Abstract

BUCK, BROCK LOGAN. Functional analysis of adhesion factors and signaling mechanisms in *Lactobacillus acidophilus* NCFM. (Under the direction of Professor Todd R. Klaenhammer).

The lactobacilli are traditionally used for the fermentation of food products, but have recently been investigated for their potential health promoting factors. One important property of these probiotic lactobacilli is their ability to adhere to the intestinal mucosa. *Lactobacillus acidophilus* NCFM is a probiotic organism which has the ability to adhere to intestinal cells *in vitro*, although the mechanisms for this adhesion are not clear. A functional genomic approach was utilized to identify specific genes related to the adhesion process of *L. acidophilus*. The Caco-2 cell model was employed for analysis of the adhesive properties of multiple isogenic mutant strains of *L. acidophilus*. Mutation of genes encoding a fibronectin-binding protein (FbpA, LBA1148), mucus-binding protein (Mub, LBA1392), and a surface layer protein (SlpA, LBA0169) resulted in a significant decrease in adhesion to Caco-2 cells compared to the control strain. It appears that multiple factors work together to contribute to the adhesive ability of *L. acidophilus* NCFM. When then environmental conditions of *L. acidophilus* were modified immediately before adhesion, an explosive adhesive state was induced, termed the Adhesion Adaptive Response (AAR). Microarray analysis was used to study the transcriptional response of the *L. acidophilus* population to AAR conditions. Interestingly, multiple stress-related genes were overexpressed under AAR conditions along with the quorum sensing related gene, *luxS*. LuxS acts to synthesize autoinducer-2 (AI-2) which acts as an interspecies signaling molecule. A mutant strain of *L. acidophilus* deficient in LuxS activity was constructed and analyzed for its adhesive

ability. The LuxS$^-$ mutant strain exhibited a decrease in adhesion compared to the control when harvested directly from logarithmic phase, but still presented an explosive adhesive response following exposure to AAR conditions.

Further analysis of the effect of LuxS on *L. acidophilus* was performed using microarray analysis and phenotypic confirmation studies. *L. acidophilus* was found to produce AI-2 throughout the logarithmic growth phase. As such, three points were selected for transcriptional analysis of both the wild type and LuxS- mutant strain. Following a mixed model statistical analysis we discovered that the majority of differential expression related to LuxS occurs in the early-log stages of growth. Multiple genes related to the stress response, host-microbe interactions, and general growth and metabolism were positively differentially expressed in the presence of AI-2.

Multiple surface proteins of were identified that contributed to the ability of *L. acidophilus* NCFM to adhere to intestinal epithelial cells, in vitro. An explosive adhesive response was induced by altering the environmental conditions in a process called the Adhesion Adaptive Response. Finally, the impact of the autoinducer AI-2 was studied using a novel microarray loop design. We reported that AI-2 influenced the transcriptional response of *L. acidophilus* at the early stages of growth, suggesting that AI-2 acts as a unique quorum sensing molecule.

FUNCTIONAL ANALYSIS OF ADHESION FACTORS AND SIGNALING MECHANISMS IN *LACTOBACILLUS ACIDOPHILUS* NCFM

By

B. Logan Buck

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APPROVED BY:

_____	_____
Dr. Todd R. Klaenhammer Chairman of Advisory Committee	Dr. Robert M. Kelly
_____	_____
Dr. Amy M. Grunden	Dr. Hosni Hassan

Biography

Brock Logan Buck was born 9 April, 1979 in Raleigh, NC. He was raised in North Carolina where he graduated from Monroe High School in 1997. Logan was awarded the Park Scholarship to attended North Carolina State University where both his mother, Jamie R. Fish and father, E. Gale Buck, graduated. Upon his own graduation in 2001 from the Department of Food Science and the Department of Microbiology, he began graduate school under the direction of Dr. Todd R. Klaenhammer in the Department of Microbiology. Logan was awarded the NIH Biotechnology Training Program fellowship in 2001. During his time at NC State University, Logan was the founding member of an internationally recognized student exchange organization called IAESTE and served as the drumming instructor for the NC State University Pipes and Drums for four years.

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CHAPTER I

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1.1 Introduction

Higher life forms would have never arisen and could not now be sustained without microorganisms. Diversity and functions of microorganisms have been shaped over millions of years and continue to change as the organisms themselves adapt to new habitats. Microorganisms, bacteria especially, exist in a wide variety of environmental niches as members of complex communities, rather than single planktonic cells. The diversity present in each niche requires bacterial populations to continually modulate their own behavior in order to adapt and survive. Although examination and identification of all bacterial species in a given niche are not yet possible, advances in biotechnology have provided researchers with the tools necessary to investigate the genetic control and evolution of bacterial behavior.

Human epithelial surfaces are inhabited by bacterial populations throughout the life of the host. The presence of these populations in the respiratory epithelium, vaginal epithelium, and gastrointestinal epithelium serves various functions. At birth the human gastrointestinal tract (GIT) is sterile, though it is quickly colonized by anaerobes and facultative anaerobes via oral inoculation. The intestinal microflora is subsequently established and after two years the fecal flora is fully constituted (54). The analysis of prokaryotic ribosomal RNA gene sequences isolated from the human colon and feces suggested that approximately 80% of the bacterial phylotypes detected corresponded to uncultivated species and novel microorganisms. Most of the inferred organisms were either *Firmicutes* or *Bacteroidetes* (20). Accurate definition of the GIT-associated microbiota is problematic due to limitations in sampling procedures and heterogeneity

between subjects. Although the actual composition is unique between subjects, the intestinal microbiota performs similar functions in each person.

Immediately following inoculation, the intestinal flora begins to provide benefits to the host that include nutritional contributions, protection from infection, maturation of the immune system, and maturation of the mucosa (69). For example, B-group vitamins necessary for normal homeostasis and vitamin K required for proper blood coagulation are both produced by the normal intestinal microflora (34). Short-chain fatty acids produced by the microbiota offer additional energy sources and support the healthy growth of intestinal epithelial cells (96). The high numbers of commensal flora in the intestine are antagonistic to the activity of pathogens by numerous mechanisms, including competition for essential substrates, competition for bacterial receptor sites, creation of a restrictive physiological environment, and production of antimicrobial substances (21). Maturation of both the mucosal immunity and preimmune antibody (Ab) repertoire are initiated by antigens presented by commensal bacteria (89). Finally, intestinal permeability and epithelial cell function are shaped by the exposure to bacteria in the early stages of life and weaning (35). It is clear that the human immune system and GIT requires the presence of a diverse and high population of microorganisms for proper maturation and function throughout the life of the host.

The commensal flora populates and maintains homeostasis in the human intestine, and any alteration of this flora could potentially affect the health of the host. For example, when antibiotics decrease the commensal population, pathogens are given a selective environment for growth and possible infection. The prospect that human health can be positively influenced by manipulation of the commensal flora is an exciting

possibility. Recently defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (86), probiotic species have been found useful in the maintenance of gastrointestinal health and prevention and treatment of disease. Many of these probiotic species are members of the lactic acid bacteria (LAB), a family of microorganisms that ferment various substrates primarily into lactic acid. The majority of LAB are anaerobic, Gram-positive, non-sporulating, and acid tolerant. Early in the 20th century Russian scientist, Eli Metchnikoff, proposed that sugar-fermenting LAB found in fermented milk products could prolong human life by preventing or reducing the toxic effects of putrefactive organisms in the gut (63). Since that time, research on probiotic LAB has focused on identifying strains with functionally desirable characteristics, collecting data to confirm health benefits offered by probiotics, and determining the molecular basis for these modes of action. Recently, certain *Lactobacillus* strains have been investigated for their potential as live vaccine delivery vehicles (31).

1.2 The intestinal mucosa

The intestinal mucosa can be separated into three distinct layers: the intestinal crypt, intestinal villus, and mucus bilayer. Stem cells in the intestinal crypts divide to produce new enterocytes (intestinal epithelial cells) that serve to constantly replace expiring enterocytes at the intestinal villus surface. Adjacent to the stem cells in the intestinal crypt are Paneth cells which secrete a number of antimicrobial substances including defensins and lysozyme. Consequently, the environment of the crypt is generally kept sterile to protect the stem cells, ensuring adequate numbers of new

enterocytes at the villus surface. Along with the mature absorptive enterocytes at the intestinal villus surface are Peyer's patches containing M-cells used for antigen sampling and presentation at their basolateral surface. A polymeric mucus gel bilayer is directly exposed to the apical surface of the intestinal villus and forms the first physical layer of intestinal defense against pathogens. The top layer of free flowing mucosal gel has the special ability to reanneal if fractured during normal contraction of the underlying smooth muscle. It is advantageous for the host to trap microorganisms in this top mucus layer because it is continually removed by passing intestinal contents and subsequently replaced. The bottom mucus layer is tethered to the apical surface of the intestinal epithelial cells and forms the primary mucus barrier. This mucosal gel bilayer is composed of high-molecular-weight glycoproteins called mucins, secreted by goblet cells dispersed between the mature enterocytes of the villus layer. Currently, 20 human mucin genes (*MUC*) have been identified, and at least 14 of these *MUC* are expressed in the GI tract (78). Together the three layers of the intestinal mucosa inhibit bacterial adhesion to the epithelial cells, and sample the intestinal contents for new antigens.

Before ingested bacteria are exposed to intestinal epithelial surfaces, they must survive passage through the dynamic and harsh environment of the stomach and upper intestinal tract. Acidity in the stomach combined with bile salts and pancreatic secretions in the intestine serve as barriers to intestinal persistence and colonization. However, some bacteria possess genetic mechanisms to overcome these barriers and survive passage to the mucosal surfaces in the intestine. Once in the intestinal environment, not all bacteria have the ability to establish colonization and are only transient, or allochthonous members of the intestinal microflora (101). Microorganisms that can be

detected in the feces over long periods of time are thus referred to as being autochthonous to the gut (101). Although the specific genetic determinants that confer autochthony have not yet been identified, it is likely that adhesion to the intestinal mucosa plays an important role in this process. While lasting colonization is desirable for certain probiotic effects such as competitive exclusion of pathogens, transient interaction appears to be sufficient for some probiotic results, such as immune stimulation. The body of knowledge surrounding the adhesion requirements for probiotic activity is not extensive enough to draw definitive conclusions, primarily due to the lack of understanding of the adhesion process itself. Although studied extensively in enteric pathogenic bacteria, the mechanisms of adhesion of probiotic lactic acid bacteria with the intestinal mucosa are not fully understood. Presented here is a review of selected studies describing the molecular mechanisms of adhesion to the intestinal mucosa by lactobacilli, focusing on strains with reputed probiotic activities.

1.3 Model Systems

The interactive nature between the microflora and intestinal mucosa requires that methods exist to study this relationship. Unfortunately, the ecosystem of the human GIT presents numerous obstacles for the study of associated microflora and the interaction between the microflora and mucosa. Efforts to avoid these obstacles have led to the development and utilization of numerous *in vitro* cell culture models, *in vivo* animal systems, and *in silico* mathematical models. An accurate replica of the human gastrointestinal environment has not been developed for adhesion studies, but through the

conscientious use of model systems, experiments can be performed that provide insight to specific intestinal relationships of interest.

In vitro model systems of the human GIT allow researchers control over specific conditions to examine a single parameter in detail. Most *in vitro* studies of the relationship between bacteria and intestinal mucosal surfaces use cultures of enterocytes or explanted sections of intestinal mucosa. These model systems allow the study of both the bacterial and host factors involved with bacterial association with the mucosal surface of interest. One of the more common enterocyte cell lines used for bacterial adherence studies is the Caco-2 model. Originally studied as a gastrointestinal tumor cell line for performing studies on cancer mechanisms, Caco-2 cells were unique in their ability to spontaneously differentiate in culture (82). Although colonic in origin, Caco-2 cells express several morphological and enzymatic features of small intestinal enterocytes upon differentiation. These cells grow with a cylindrical polarized morphology, in a monolayer, expressing brush border microvilli and small intestinal hydrolase activity on the apical surface with tight junctions between cells (93). For intestinal transport and bacterial invasion studies, Caco-2 cells are grown on permeable filter supports that allow access of ions and nutrients to both sides of the monolayer. Bacterial adhesion studies generally use cells grown on multi-well plates specially treated for cell culture. In culture, the Caco-2 is a heterogeneous population of cells with different morphologies. Clonal cell lines have been created from the parental Caco-2 culture (ATCC HTB-37) to improve the homogeneity of the population or isolate a specific desired phenotype from a subpopulation. For example, the C2BBe cell line was subcultured for more homogeneous brush border expression comparable to *in vivo* human intestinal enterocytes

(81). Although Caco-2 cells are widely used for bacterial adhesion studies because of their morphological and functional similarity to human small intestinal enterocytes, the inherent heterogeneity of the Caco-2 line, combined with different culture conditions makes the comparison of results between laboratories difficult.

While Caco-2 cells are appropriate as an intestinal enterocyte model, they are not able to accurately replicate the mucus layer of the intestinal mucosa. Another colonic carcinoma cell line, HT29, has been subcultured into distinct populations with varied morphologies and mucin expression. Following exposure to either 5-fluorouracil (HT29-FU) or methotrexate (HT29-MTX), subpopulations were isolated that showed varied morphology and mucus secretions. The original HT29-FU and HT29-MTX cultures were found to contain mucus-secreting cells and enterocyte-like cells. Enterocyte-like cells isolated following FU and MTX treatment were resistant to FU and MTX, respectively, and did not produce mucins. The mucins of the secretory HT29-FU goblet-shaped cells (HT29-5F12) were similar to mucins of the human colon and were resistant to 5-FU but not to MTX. Conversely, columnar mucus-secreting cells of HT29-MTX origin (HT29-M21) produced gastric immunoreactive mucins and were sensitive to 5-FU but not to MTX (50). The availability of mucin-specific subclones allows researchers to study the adhesion of bacteria to either colonic or intestinal mucins. The availability of immunoassays for specific mucin types provides a mechanism for studying mucin induction by intestinal bacteria. For example, certain adhesive *Lactobacillus* species have the ability to induce the expression MUC3. The MUC3 gene product is a secreted small intestinal mucin with the ability to inhibit enteric pathogen adherence (55). Mack *et al*. (56) reported the induction of the intestinal mucins MUC2 and MUC3 from HT29

cells, but not from the non-intestinal HEp-2 cells. MUC2 and MUC3 were highly induced in HT29 cells following exposure to both *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG, and both mucins were able to inhibit the adherence of pathogenic *E. coli* species (56).

Other cell lines have been used as models to study the adhesion of commensal and probiotic bacteria to the human intestinal mucosa. HeLa cells, originally isolated as malignant cervical cells, are a very aggressive cell line that can easily overwhelm other cell lines. As such, much research thought to be performed on other cell lines was actually performed on HeLa cells and care should be taken to maintain homogeneity of cells grown in proximity to HeLa cells. Due to their cervical origin, HeLa do not necessarily model the structure and receptors of the human intestinal epithelium, but their ease of maintenance makes them a popular choice for adhesion experiments (22, 62). Intestinal 407 (Int-407) cells were found to have a similar DNA fingerprinting profile to HeLa cells, although Int-407 cells were isolated from the small intestine of a human embryo, and likely show a greater similarity to the healthy human intestine. Although not a common choice for bacterial adhesion experiments, Int-407 cells have been used occasionally (5, 38, 44). HEp-2 cells, a human larynx epithelioma cell line, are also used for bacterial adhesion studies, but do not necessarily present an accurate model of the human intestinal mucosa. Selection and proper maintenance of *in vitro* cell lines is of premier importance when surveying the adhesive properties of pathogenic, commensal, and potentially probiotic bacteria. When conclusions concerning the adhesion ability of a bacterial strain or collection of strains are desired, care should be taken to select a cell line that models the *in vivo* environment as accurately as possible.

In vitro cell lines are commonly used due to their relatively low cost, their ability to be controlled for targeted experimentation, and their ease of access and manipulation. However, the lack of realism of these cell lines makes animal models more illustrative of the human gastrointestinal environment. Although ethically restrictive, animal models offer a more accurate representation of the complexity present in the human GIT. For example, animal models more closely replicate the integration of mucosal and luminal surfaces along with the immune response naturally established in the GIT of humans (10). Conventional animal models present the highest degree of realism and fewest ethical restrictions, but the complexity of factors in, the gastrointestinal tract limit their use in bacterial adhesion studies. Germ free and gnotobiotic animals are not as representative of the human GIT as conventional animals, but experimental conditions can be more easily controlled. The lack of a complex flora makes the interpretation of data obtained from bacteria-host interactions in gnotobiotic animals more straightforward. Use of germ-free animals is essential for determining the effect of a single bacterial population on normal development, establishment and maintenance of the mucosa-associated immune system, and epithelial-cell functions. Experiments in germ-free animals illustrate the importance of indigenous microflora in protecting against intestinal colonization by exogenous bacteria (10).

More recently, *in silico* analyses utilizing mathematical models of bacterial adhesion to epithelial cells and mucosal surfaces are being employed. Lee et al. (48) created a mathematical model of the adhesion of *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, and *Escherichia coli* TG1 to Caco-2 cells. The model was based on predetermined numbers of bacterial receptors on the Caco-2 cells and bacterial

efficiency of binding to those receptors. Although the mathematical model was used to estimate the competitive exclusion properties of the lactobacilli against *E. coli*, it was clear that "wet lab" experimentation is necessary for quantitation of the adhesive abilities of these strains. These and other mathematical models allow researchers to study the principles of known interactions within a specified model system. The effects of specific probiotics, prebiotics, or other compounds can not be accurately estimated. However, with the integration of data from a growing body of knowledge on bacterial adhesion mechanisms, *in silico* analyses could move closer to representing *in vivo* relationships.

Animal models offer insight into the accuracy of hypotheses in a controlled, yet complex system. *In vitro* cell culture models use a reductionist approach to study specific relationships in a manageable and reproducible environment. Integration of data from other models into mathematical models allows for the relatively rapid estimation of general relationships between bacteria and the intestinal environment (10). Human models are the final, integrated step in assessing theoretical predictions. Unfortunately, ethical restrictions and lack of environmental control make human trials both expensive and difficult to perform.

1.4 Mucus layer and ECM binding

The surface of gram-positive bacteria is studded with strain-specific proteins either bound to the cell wall, or extending through the cell wall and linked to the underlying cytoplasmic membrane. A bacterial proteinaceous component likely participates in the association of bacteria, and specifically lactobacilli, with the intestinal mucosa. Bacterial proteins involved with adhesion could either be secreted or bound to

the cell surface. Proteins translocated across the cytoplasmic membrane generally have an N-terminal signal peptide sequence proteolytically cleaved by signal peptidases upon translocation (70). Some proteins are targeted to the cell surface by covalent linkage following sortase-dependent cleavage (103). Although these proteins are easily identified by the C-terminal LPXTG motif, few have been characterized. Some cell-wall associated proteins contain tandem repeat regions varying in size from a few to several hundred amino acids (70). While these tandem repeats are a common feature in surface proteins, the sequence and number of repeats is frequently strain-specific. Certain common features of surface-associated proteins provide a starting point for the identification of adhesion proteins, but to date, no common feature has been identified that differentiates adhesion proteins from proteins of other function. However, with the recent availability of genome sequence data for a variety of bacterial species, more surface proteins can be identified and characterized, providing insight into features influencing surface presentation and binding to host components.

Some lactic acid bacteria have the ability to associate with the mucus in the small intestine as a means of initiating transient or extended residence. Specifically, several lactobacilli used in probiotic products were reported to bind human mucus isolated from feces of newborns, infants, and adults (45). The reported binding was strain-dependent and varied depending on the source of the mucus. Roos and Jonsson (91) characterized a large cell-surface protein, Mub, from *Lactobacillus reuteri* 1063 that contributes to adhesion to pig and hen mucus and mucus components. Binding of Mub to mucus and mucus components was higher at pH 3-6 than at pH 7.4, with maximum binding occurring between pH 4-5. The 358 kDa Mub contains two sets of different tandem

repeat regions and a predicted N-terminal signal sequence. The C-terminal end was predicted to contain both cell-wall and membrane spanning regions, along with the LPXTG cell-wall anchoring motif (91). Multiple copies of *mub* are frequently found in the genomes of lactobacilli. At least one homolog of *mub* can be found in *Lactobacillus acidophilus* (3), *Lactobacillus gasseri*, *Lactobacillus johnsonii* (84), *Lactobacillus plantarum* (46), *Pediococcus pentosaceus*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus fermentum* (http://genome.jgi-psf.org/mic_home.html). One *mub* homolog in *L. acidophilus* NCFM (LBA1392) was disrupted by insertional inactivation and the resulting mutant was analyzed for its ability to adhere to Caco-2 cells. When compared to the control, the isogenic Mub mutant of *L. acidophilus* NCFM exhibited a 65% decrease in adhesion to Caco-2 cells (11). Although Caco-2 cells do not produce a mucus layer, the contribution of Mub to intestinal cell binding is evident.

Although *mub* homologs are present in a number of lactic acid bacteria, very few reports characterize the influence of Mub on adhesion to the intestinal mucosa. However, an unknown proteinaceous component has been linked with adhesion to mucus in *Lactobacillus rhamnosus* GG, and *L. johnsonii* LC1 (104). At the time of that report, genomic sequence data was not available to identify Mub in *L. johnsonii*, so the influence on mucin adhesion of the *L. johnsonii* Mub (LJ_0574) remains unclear. In the same study, proteolytic treatment of *L. rhamnosus* LC-705 and *Lactobacillus casei* strain Shirota did not significantly influence adhesion to mucus components (104). A mucin adhesion promoting protein (MAPP) was identified in *Lactobacillus fermentum* 104R which is much smaller (29 kDa) than Mub, but was able to bind porcine small intestinal mucus and gastric mucin (90). The ability of *L. reuteri* to adhere to mucin was

stimulated by the addition of mucin to the growth medium (43). The same study reported that proteinase treatment of the bacteria prior to binding reduced their mucin-binding capacity, suggesting the involvement of surface proteins. It could be assumed from these results that the growth environment can influence the adhesion properties of bacteria to the intestinal mucosa. The adhesion of *Lactobacillus crispatus* BG2FO4 (formally classified as *L. acidophilus* BG2FO4) to mucus producing HT29-MTX cells occurred in dense clusters suggesting mucus could be involved with aggregation of this strain (17). A strictly bioinformatic analysis identified a putative mucus-binding domain, called MUB (8). In contrast to the pfam MucBP domain, complete MUB domains were only found in lactic acid bacteria and ranged in size from 100 to more than 200 amino acids per domain. The authors postulate that the high variability in the number of MUB domains in putative mucus-binding proteins suggests that the MUB domain is often duplicated or deleted in evolution. A number of lactobacilli were predicted to contain multiple proteins with the MUB domain including: *L. gasseri* ATCC33323 (13 predicted proteins), *L. acidophilus* NCFM (12 predicted proteins), *L. johnsonii* NCC533 (9 predicted proteins), *L. plantarum* WCFS1 (4 predicted proteins), *L. reuteri* 1063 (2 predicted proteins), *L. brevis* ATCC367 (2 predicted proteins), and *L. fermentum* BR11 (2 predicted proteins). The exclusivity of MUB to lactic acid bacteria and high frequency in intestinal lactobacilli, suggests that the MUB domain could play an important role in host-microbe relationships (8).

Beneath the mucus layer lays the host cell extracellular matrix (ECM) composed of various secreted proteins including laminin, fibrin, heparin, collagen, and fibronectin. Also found in blood plasma, fibronectin is an adhesive dimeric glycoprotein (~440 kDa)

that plays a key roll in cell adhesion to the ECM of vertebrates. The group of bacterial surface adhesins that bind to ECM molecules are collectively referred to as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (77). One of these MSCRAMMs, the fibronectin-binding protein, has been identified and characterized in various species of pathogenic *Staphylococcus* and *Streptococcus* (41, 42, 97). Fibronectin was implicated in the adhesion of lactobacilli to intestinal 407 cells in vitro (44). Using immuno-fluorescent microscopy, adherent lactobacilli seemed to localize in areas of the cell surface where fibronectin was detected. Additionally, when the lactobacilli were treated with fibronectin, blocking any fibronectin-binding sites, the level of adhesion to intestinal 407 cells was decreased (44). The MSCRAMM on lactobacilli responsible for the binding of fibronectin has not been identified, but trypsin and proteinase K decreased the ability of *L. acidophilus* CRL 639 to bind to immobilized fibronectin (53). This binding inhibition was more dramatic in exponentially growing cells compared to stationary phase cells. A fibronectin-binding protein (LBA1148) was identified in *L. acidophilus* NCFM containing a pfam domain for fibronectin binding and showing homology to characterized fibronectin-binding proteins in *Streptococcus mutans* and *Streptococcus gordonii*. An isogenic mutant containing a functionally inactivated fibronectin-binding protein (FbpA) was reported to have a 76% decrease in adhesion to Caco-2 cells when compared to the control (11). While these results suggest that fibronectin is important to the binding of lactobacilli to epithelial surfaces, it is clear that other mechanisms are also involved.

Other ECM molecules likely participate in the binding of lactic acid bacteria to the intestinal mucosa. Some industrial lactobacilli strains used for their probiotic

properties were shown to bind various components of the ECM including fibrinogen, fibronectin, lactoferrin, transferrin, and fetuin, although binding ability varied between strains (100). Lorca *et al*. (53) reported the binding of various strains of *L. acidophilus*, *Lactobacillus helveticus*, *L. reuteri*, *L. gasseri*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *L. fermentum*, *L. casei*, *L. rhamnosus*, and *Lactobacillus paracasei* to both collagen and fibronectin. Although a molecular determinant for binding was not identified, the lactobacilli tested generally bound collagen more readily than fibronectin. Collageneous molecules are the major constituents of the ECM and represent a popular target for pathogenic bacteria (53). *Bifidobacterium bifidum* and *L. reuteri* were also reported to bind intestinal glycolipids found on the surface of epithelial cells (68). Periodate treatment of glycolipids extracted from Caco-2 cells prevented any binding of either *B. bifidum* or *L. reuteri*, suggesting that bacterial cells bind the carbohydrate moieties of glycolipids. The same study reported that actinase treatment of cell-surface fractions from *B. bifidum* prevented binding to glycolipids, also suggesting the involvement of a proteinaceous component (68). Binding of lactobacilli to these ECM components could potentially block the adhesion of pathogens and help maintain intestinal homeostasis.

The ability to bind ECM components could represent an important criterion for selection of probiotic strains. In an effort to confer epithelial cell-binding ability to a non-adherent *Lactobacillus*, the gene (*cbsA*) encoding a collagen-binding surface-layer protein from *L. cripatus* JCM5810 was expressed in *L. casei* (61). CbsA was successfully presented at the surface of *L. casei*, conferring collagen-binding properties to the transformed strain. Further characterization of CbsA identified two collagen-binding

domains and a crystalline structure with sheet morphology, which proved important for optimal binding (95).

1.5 Surface layers

Surface-layers (S-layers) are formed by a paracrystalline monomolecular assembly of proteins or glycoproteins on the outside of bacteria and archea. Due to the lattice structure of S-layers, a majority of the surface area is composed of pores of identical size and morphology (98). Protein subunits of the S-layer are non-covalently linked to each other as well as the bacterial cell wall adding rigidity to the cellular structure. S-layer proteins in Gram-positive bacteria generally contain an N-terminal secretion signal, common for the Sec pathway (94), and the resulting signal peptide in lactobacilli is usually 25-30 amino acids (6). Although limited knowledge is currently available on the biological roles of S-layers, proposed functions include cell shape determinants, protective coats, molecule traps, exoenzyme adhesion sites, cell adhesion, and surface recognition [(6), reviewed in (98)].

Genes responsible for the production of S-layers have been identified by either functional or sequence analysis in several species of the genus *Lactobacillus*. In lactobacilli that contain multiple S-layer genes, their genetic arrangement is strain-dependent, and no general consensus structure of their genetic organization has been identified (6). S-layer protein encoding genes from *Lactobacillus brevis* (39), *L. acidophilus* (9), *L. helveticus* (13), and *L. crispatus* (95) have been cloned and sequenced to provide insight to the function of the S-layer in lactobacilli. When the S-layer was removed from the cell surface of *L. brevis* with guanidine hydrochloride (guanidine HCl),

adhesion to Intestinal 407, urinary bladder T24, and endothelial EA-hy926 cells was almost completely eliminated, as well as binding to immobilized fibronectin (37). However, adhesion to Caco-2 cells was only partially decreased by guanidine HCl treatment in the same study. Some lactobacilli that do not present an S-layer also have the ability to bind to Caco-2 cells, suggesting that the S-layer is not the only adhesin responsible for Caco-2 adhesion, but is essential for adhesion to other cell types (37). For example, *L. gasseri* and *L. johnsonii* do not appear to have an S-layer, but adhere readily to Caco-2 cells. An S-layer protein encoded by the aforementioned *cbsA* from *L. crispatus* was shown to bind both solubilized and immobilized collagens. Hybrid CbsA molecules which failed to crystallize into an S-layer were unable to bind collagens, suggesting that the regular crystal structure of S-layers is important to binding ECM components (95). A mutant strain of *L. acidophilus* NCFM containing an insertional inactivation of the S-layer gene, *slpA*, was analyzed for tolerance to environmental stress and genetic stability (2). The knockout strain was more sensitive to 2.5% NaCl and 15% ethanol, but more tolerant to 2.0 % Oxgall (fresh dehydrated bile). Interestingly, long-term evaluation of the mutant revealed the reappearance of a functional S-layer due to chromosomal inversion at the *slpA-slpB* locus and subsequent expression of *slpB* (2). Prior to the chromosomal inversion, the S-layer mutant strain demonstrated an 84% decrease in adhesion to Caco-2 cells, likely due to the absence of the S-layer and coinciding disruption of multiple surface-associated molecules (11).

The location of the adhesive domain(s) of S-layer proteins has been studied in both *L. brevis* and *L. crispatus*. Using *E. coli* flagellum display of chimeric SlpA fragments, the N-terminal part of the S-layer protein was identified as an epithelial cell-

binding region (37). Specifically, an 81 amino acid peptide from position 96-176 of the unprocessed *L. brevis* S-layer protein was the shortest fragment with binding activity. Likewise, fragments of the S-layer protein, CbsA, from *L. crispatus* were expressed in a non-adhesive *L. casei* strain to determine the binding activities of the individual peptides (4). In accordance with SlpA, N-terminal CbsA peptides bound immobilized laminin and collagen. C-terminal CbsA peptides bound teichoic and lipoteichoic acids. Additionally, the C-terminal peptides only bound *L. crispatus* cells following S-layer removal (4) , suggesting cell wall anchoring activity at the C-terminus of CbsA.

1.6 Other adhesion proteins

Although mucin-binding, collagen and fibronectin-binding, and surface layer proteins have been studied in multiple *Lactobacillus* species, a few adhesion proteins remain poorly characterized. Some proteins identified in lactobacilli show similarity to the α–like family of streptococcal proteins (Alp), extensively studied for their pathogenic and immunoreactive properties. The Alp family includes the α protein, Rib protein, R28 protein and Alp2 protein noted by their highly repetitive sequence and ability to elicit protective immunity (51). Proteins from the Alp family contain general surface protein features including an N-terminal signal sequence followed by a nonrepeated N-terminal region, an LPXTG cell wall-anchoring motif, and a short hydrophobic region that could extend into the cellular membrane. R28, initially characterized in *Streptococcus pyogenes*, was shown to promote adhesion to ME180 cervical epithelial cells using a R-28 negative mutant (99). The same study reported that antibodies against purified R28 conferred protection against infection by two different mouse-virulent strains of *S.*

pyogenes. However, conclusive evidence that Alp family proteins participate in streptococcal virulence is lacking and their major biological function remains unclear (51).

Two tandem genes (LBA1633 and LBA1634) in *L. acidophilus* NCFM show homology to the Alp family, and specifically the R28 protein of *S. pyogenes*, although they lack the extensive repeated domains present in R28 and other Alp family proteins. Each of these genes was disrupted in separate strains and the resulting mutants were analyzed for their ability to adhere to Caco-2 cells (11). Neither of the strains showed a consistent difference in adhesion, suggesting that, individually, these R28 homologs do not affect the adhesion phenotype of *L. acidophilus*. However, the disruption of both genes in a single strain would be useful in determining if these proteins work together to participate in either adhesion or immunoreactivity. *Lactobacillus fermentum* BR11 also has two tandem genes, *rlp* and *mlp*, that could participate in adhesion (105). Rlp is similar to the Alp family proteins R28 from *S. pyogenes*, Rib from *Streptococcus agalactiae*, and Alp3 also from *S. agalactiae*, as well as an uncharacterized protein from *L. johnsonii* encoded by LJ0621, and previously mentioned proteins from *L. acidophilus* NCFM encoded by LBA1633 and LBA1634. Rlp contains the LPQTG C-terminal cell wall-anchoring motif as well as multiple imperfect amino acid repeats. Directly downstream of *rlp*, *mlp* also contains the LPQTG motif and is similar to *mub* from *L. reuteri* (105). The authors also report multiple homologs of *mlp* exist in *L. fermentum* BR11, although the binding of that strain to mucin has not been demonstrated. Both Rlp and Mlp were successfully used for the heterologous expression and surface display of small peptides (105). Other uncharacterized genes whose products show similarity to

Alp family proteins include the previously mentioned LJ0621 from *L. johnsonii* and lr1694 from *L. reuteri*, both of which have predicted N-terminal signal peptides and C-terminal LPQTG cell wall-anchoring motifs. Although the Alp family of proteins has been primarily examined in group B streptococci, a number of proteins in lactobacilli show similarity to the Alp family and could also participate in host-microbe interactions.

Some proteins contribute to the adhesion of lactobacilli to the human intestinal mucosa, but do not belong to the major family of adhesins discussed above. A cell surface-associated elongation factor mediates the association of *L. johnsonii* NCC533 to Caco-2 cells and mucins (25). EF-Tu, also found in *L. acidophilus* NCFM (LBA0845) (3), *Lactobacillus sakei* (LSA1063), and *L. plantarum* WCFS1 (lp_2119) (46), does not contain an identifiable signal sequence, an LPXTG motif, or transmembrane domain to suggest localization at the cell surface. Nevertheless, EF-Tu was shown to localize at the cell surface of *L. johnsonii*, as well as exist unbound in the cytoplasm (25). Although EF-Tu isolated from *L. johnsonii* was not able to bind fibronectin or collagen, it successfully bound both gastric and intestinal mucin preparations, suggesting possible involvement in binding to the mucus layer, but not the underlying ECM. Three genes of *L. reuteri* 100-23 specifically induced in the murine gut were identified using in vivo expression technology (IVET) (110). One of those genes encoding a methionine sulfoxide reductase (MsrB) and a separately identified high-molecular-mass surface protein (Lsp) were shown to be involved with the maintenance and ecological performance of *L. reuteri* in the murine GIT (109). Insertional inactivation of *lsp* impaired the adherence of the bacteria to the mouse forestomach epithelium and extended the time required for the bacteria to reach maximum population size in vivo. Three of the

previously mentioned Alp family homologs, *L. acidophilus* LBA1633 and LBA1634 and *L. johnsonii* LJ0621 all have sequence similarity to Lsp from *L. reuteri*. A mannose-specific adhesin identified in *L. plantarum* contributed to the binding ability of that strain to HT29 cells (1). Periodate treatment of the HT29 cells eliminated adhesion, along with proteinase treatment of the bacteria, suggesting a carbohydrate receptor on the epithelial cells and a proteinaceous component on the bacterial cell surface both contribute to adhesion. The authors report that HT29 binding of *L. plantarum* correlated with binding to D-mannose-coated agarose beads and the ability to agglutinate *Saccharomyces cerevisiae* in a mannose-sensitive manner. Further investigation revealed that upon deletion of one gene (lp_1229), yeast agglutination ability was lost, while overexpression of lp_1229 quantitatively enhanced the yeast agglutination phenotype (83). This gene, designated *msa*, is directed to the cell surface in a sortase dependent manner and contains known carbohydrate-binding domains along with perfect repeat regions. Two regions at the C-terminal end of Msa show homology to a mucus-binding domain present in the *L. reuteri* Mub (83). In some cases, proteins previously considered to be involved with other cellular functions have been linked with adhesive ability. An *L. johnsonii* NCC533 protein, GroEL, was recently shown to bind intestinal mucin and undifferentiated HT29 cells (7). GroEL contains no signal sequence or membrane binding domain, and is customarily associated with the mediation of protein folding during stressful conditions, such as heat stress. However, recombinant GroEL (rGroEL) from *L. johnsonii* NCC533 not only binds to HT29 cells, but when incubated in low concentration on HT29 cells, blocks the binding of *L. johnsonii* (7).

Clearly, a variety of proteins may participate in the interactions between the intestinal mucosa and commensal or probiotic lactobacilli. It is likely that these proteins are strain-dependant and their expression could vary depending on environmental conditions. *L. acidophilus* NCFM, *L. crispatus* BG2FO4 (previously classified *L. acidophilus* BG2FO4), and *L. gasseri* ADH were subjected to a number of treatments and subsequently analyzed for their adherence ability to Caco-2 cells (28). Adherence of both *L. acidophilus* NCFM and *L. crispatus* BG2FO4 was decreased by protease treatment, while the adherence of *L. gasseri* ADH was not affected. Periodate oxidation of cell-surface carbohydrates significantly reduced adherence of *L. gasseri* ADH, moderately reduced the adherence of *L. crispatus* BG2FO4, but did not affect the adherence of *L. acidophilus* NCFM (28). Not only are adhesion proteins likely expressed in a strain-dependant manner, but bacterial surface molecules other than proteins also contriubute to adhesion.

1.7 Non-protein mediated adhesion

Certain surface-associated and environmental factors, other than proteins, could participate in the association of lactobacilli with the intestinal mucosa. For example, lipteichoic acids are key components of the Gram-positive bacterial cell walls and could contribute to the adhesion of bacteria to the intestinal mucosa. Anchored to the underlying cell membrane, lipoteichoic acid (LTA) is an amphipathic molecule that extends through the peptidoglycan layer to present at the cell surface. LTA binds target eukaryotic cells, membrane phospholips, and CD14 and Toll-like receptors. As an immune stimulator, LTA has been shown to activate the complement cascade and trigger

the release of reactive oxygen and nitrogen species from macrophages and neutrophils (23). Although LTA is found in a wide variety of Gram-positive bacteria, there exists a great diversity in lipoteichoic acids between species. LTA present at the cell surface was shown to participate in the adhesion of *L. johnsnoii* NCC533 to Caco-2 cells (26). Purified LTA from the cell surface was incubated on Caco-2 cells prior to bacterial adherence, resulting in a 60% decrease in adhesion. Additionally, LTA from the spent culture supernatant inhibited the adhesion of *L. johnsonii* to Caco-2 cells in a concentration-dependent manner (26). The C-terminal domain of the S-layer protein CbsA of *L. crispatus* (discussed previously) was shown to bind LTA isolated from several bacterial species (4). Teichoic acids were also implicated in the adhesion of *Staphlyococcus epidermidis* to immobilized fibronectin (36). Although much of the research on the adhesion of LTA has been performed on Gram-positive pathogens, it is clear that LTA from lactobacilli is likely to play a role in adhesion and is an important activator of the immune system (27, 47, 107). In addition to LTA, the production of exopolysaccharides (EPS) by lactobacilli could influence their adhesion to the intestinal mucosa. EPS are long-chain polysaccharides composed of branched, repeating units of sugars or sugar derivatives loosely attached to the cell surface or secreted into the environment. Lorca *et al*. (53) postulated that elevated binding of *L. acidophilus* CRL639 to ECM components during exponential phase compared to stationary phase could be attributed to the production of different types of EPS. *L. acidophilus* CRL639 produces slime polysaccharide (EPSs) during exponential phase and capsular polysaccharide (EPSc) during stationary phase. When stationary phase *L. acidophilus*

CRL639 was treated with periodate, binding to immobilized collagen and fibronectin was improved, suggesting the interference of EPSc with adhesion to ECM components.

The environment of the human GIT could also affect the adhesion ability of lactobacilli and other bacteria. Acid and bile-salt stress increased the adhesion of enteropathogenic *E. coli* (EPEC) to HEp-2 epithelial cells (19). This increase in adhesion was observed with or without acid adaptation prior to adhesion. When the *E. coli* cells were pretreated with erythromycin, the adhesion enhancement from acid or bile-salt exposure was eliminated, further implicating the involvement of proteins with adhesion. Interestingly, the surface expression of a heat-shock protein 70 (Hsp70) was increased on acid-shocked EPEC, and pretreatment with anti-Hsp70 eliminated the adhesion increase observed following acid stress. The adhesion of the gastrointestinal pathogen *Clostridium difficile* was increased following exposure to a number of stress-related conditions, including high sodium concentration, calcium-rich medium, an acidic pH, and iron starvation (108). Although *C. difficile* is a gastrointestinal pathogen, Vero cells isolated from the kidney of an African green monkey were the cell line used for in vitro adhesion experiments and may not accurately model the gastrointestinal mucosa. When the environmental conditions were altered prior to adherence of *L. acidophilus* NCFM to Caco-2 cells, the adhesion ability significantly increased in a response termed Adhesion Adaptive Response (AAR) (12). The AAR was achieved by the one hour incubation of a concentrated exponential phase culture immediately prior to adherence. The pH of the AAR conditions was pH 4.5 prior to adhesion, thus also exposing the bacterial cells to an acid-stress condition. The adhesion of lactobacilli to Caco-2 cells was also previously reported to be higher at lower pH values (28). *L. acidophilus* NCFM, *L. crispatus*

BG2FO4, *L. gasseri* ADH, and *L. delbrueckii* subsp. *bulgaricus* 1489 all adhered to Caco-2 at least ten-fold greater at pH 3 than at pH 7, although the influence of low pH on Caco-2 cells was not clear. The same study reported that high adhesion of *L. crispatus* BG2FO4 occurred in spent culture supernatant at pH 4.2, but when fresh growth medium was acidified to pH 4.2 the same level of adhesion could not be reached, suggesting a factor in the supernatant contributed to the adhesion increase in addition to low pH. These results, combined with the influence of GroEL on adhesion (7) mentioned previously, implicate the possible involvement of a stress response with induction of factors that promote bacterial adhesion to the intestinal mucosa.

The diverse nature of the bacterial population in the human GIT allows for the possibility of communication between microbial species, as well as host-microbe communication. One method of interaction used in bacterial communities is mediated through the density dependant response to small molecules called autoinducers present in the environment. This form of interaction is termed quorum sensing, and has been reported in a wide variety of microorganisms. Genetic analysis and genome sequencing has revealed numerous bacteriocin operons in intestinal lactobacilli (3, 46), many of which are autoinducible (71). The structure of autoinducers produced by Gram-negative bacteria is generally a homoserine lactone while Gram-positive bacteria frequently produce small peptides that act as autoinducers. One quorum sensing system used for communication both among and between species is based on a furanosyl borate diester known as autoinducer-2 (AI-2). The production of AI-2 has been shown to regulate the expression of various phenotypes including virulence factors, DNA processing, cell morphology, motility, biofilm formation, toxin production, light production, and cell

division in numerous species (111). The formation of biofilms is one important phenotype regulated by AI-2 in both Gram-negative and Gram-positive bacteria (24, 112). Pathogenic oral streptococci utilize biofilms as a virulence factor in the early stages of dental plaque formation. However, when the AI-2 synthase gene, *luxS*, was inactivated, the biofilm formation ability of *Streptococcus mutans* was eliminated (112). Although biofilms are important for adhesion to some substrates, the influence of biofilms on epithelial cell adhesion of lactobacilli remains unclear. In contrast to results presented in streptococci, a LuxS deficient strain of *L. reuteri* formed thicker biofilms than the wild type strain on both plastic surfaces in a bioreactor and epithelial surfaces of a murine forestomach (102). The same study reported a reduction in the ecological competitiveness of the LuxS deficient strain of *L. reuteri* in the cecum, but no difference in the stomach or jejunum. The influence of LuxS on bacterial association with the intestinal mucosa has not been extensively studied, but could represent an important mechanism of communication within the GIT and host-microbe interactions. The *luxS* gene of *S. pyogenes* was shown to influence internalization by epithelial cells (60). A LuxS mutant was constructed in an invasive serotype of *S. pyogenes* and was subsequently internalized by HEp-2 cells with higher efficiency than the wild type strain. The mutant strain also showed altered expression of genes previously reported to have involvement with epithelial cell internalization. When the *luxS* gene of *L. acidophilus* NCFM was inactivated, the resulting mutant strain did not produce AI-2 and showed a 58% decrease in adhesion to Caco-2 cells (12). The complete pathway for the production of AI-2 from methionine has been identified in *L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L.*

plantarum, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus*, but the general influence of AI-2 on the behavior of lactobacilli has not been fully characterized.

Given the bacterial diversity present in the human GIT, physical interactions and communication within single species and between species could occur. Although it has proven difficult to determine the influence of these interactions on association with the intestinal mucosa *in vivo*, the interactive nature of bacteria associated with the intestinal environment can be examined *in vitro*. Another mechanism of interaction between organisms traditionally studied in oral pathogens is bacterial aggregation. In contrast, little is known about the aggregation of lactobacilli and the influence of aggregation on intestinal maintenance. A 56 kDa cell-surface protein that mediates autoaggregation of *L. reuteri* was cloned and sequenced (92). Overexpression of the corresponding gene, *aggH*, enhanced aggregation and inactivation of *aggH* resulted in an autoaggregation-deficient phenotype, although the influence of AggH on association with the intestinal mucosa was not examined. An aggregating strain of *L. crispatus*, however, was shown to adhere to components of the intestinal mucosa better than a non-aggregating spontaneous *L. crispatus* mutant (15). The wild-type strain adhered at a higher efficiency to both mucus and Caco-2 cells than did the non-aggregating mutant. When both strains were administered in human colonization trials, the wild-type strain was recovered from fecal and biopsy samples following an 8 day oral administration while the non-aggregating mutant strain could not be identified. Interestingly, the populations of the wild-type strain recovered from the trials were often mixed, and different enterococci and *Lactobacillus* strains coaggregating with *L. crispatus* were isolated (15). The same wild-type strain of *L. crispatus* reduced the severity of DSS (dextran sodium sulfate)

induced colitis in a mouse model, but neither heat killed *L. crispatus* nor the non-aggregating mutant were able to present the same therapeutic effect (14). Unfortunately, a cell-surface related factor was not identified that was responsible for the observed aggregation and ecological performance. In contrast, a secreted 32 kDa aggregation-promoting factor (APF) was identified in *L. gasseri* 4B2 (87). Following washing, *L. gasseri* 4B2 was unable to aggregate, but upon addition of filtered supernatant or purified APF, the washed cells again reaggregated. APF was also shown to participate in cell-shape maintenance of *L. gasseri* 4B2 (40). Genes ecoding APF were identified and sequenced from six different strains of *L. johnsonii* and *L. gasseri* (106). Two copies of *apf* genes, *apf*1 and *apf*2, were identified in both species and showed strong sequence conservation except in their central region. The amino acid composition, physical properties, and genetic organization of the *apf* gene products were found to be similar to those of S-layer proteins. However, *apf* genes were identical in both the aggregating and non-aggregating strains of *L. crispatus*, and APF was detected from the supernatant of both strains (58). These results suggest that APF influences the aggregation of lactobacilli in a strain-dependant manner and could also participate in the association of some *Lactobacillus* strains with the intestinal mucosa.

1.8 Influence of adhesion on immune response

As discussed previously, the microflora of the intestinal environment is very diverse and delicate, yet important in maintaining a balanced and functional immune response. A very significant property of the immune system is the ability to discriminate between pathogenic and non-pathogenic bacteria within this microflora. While many

pathogens possess mechanisms to evade and defeat the immune system, some bacteria can positively modulate the immune system to successfully avoid and fight pathogens. The ability of bacteria to exist in the intestinal environment, and associate with the epithelial layers therein, likely provides the opportunity for immune stimulation. Certain bacteria, including some lactobacilli, are noted for use in anti-tumor and anti-allergy immunotherapy, and also for stimulation of a protective immune response to enhance resistance to microbial pathogens. This immunomodulation affects both the innate and adaptive immune response. Lactic acid bacteria have been shown to enhance macrophage and lymphocyte activation (59), T- and B-cell proliferation (80), antibody production (74), and natural killer-cell function (29). The ability of some lactobacilli to competitively exclude pathogens from adhesion sites in the intestinal mucosa represents another means of defense against pathogens provided by probiotic or commensal microorganisms (49, 72).

Healthy immune function, both systemic and local, can be influenced by signals provided by lactic acid bacteria in the intestinal tract. Dendritic cells open the tight junction between epithelial cells, send dendrites outside the epithelium, and directly sample the intestinal environment for antigens, including those present on the surface of bacteria (88). This process of sampling allows bacteria that are unable to translocate across the intestinal epithelium to signal the immune system. Dendritic cells can promote the development of naïve T cells toward Th1, Th2, or unpolarized T-cell responses. A balance of dendritic cell stimulation and tolerance following stimulation by lactobacilli in the GIT may be important to maintain the homeostasis necessary for commensal bacteria to carry out their functions in host nutrition, intestinal permeability, and pathogen

protection. Human myeloid dendritic cells secreted high levels of pro-inflammatory IL-12 and IL-18 following exposure to lactobacilli, but not anti-inflammatory IL-10. In the presence of lipopolysaccharide (LPS) from *E. coli* combined with lactobacilli, IL-12 was still released at high levels while LPS-induced IL-10 was substantially inhibited (67). The same study reported that dendritic cells activated with lactobacilli skewed $CD4^+$ and $CD8^+$ T-cells to Th1 polarization. When sampled, lactic acid bacteria can also transduce nuclear factor-κB (NF- κB) and STAT-mediated signals in macrophages (64). Once activated, these macrophages play a key role in initiating the innate immune response, leading to activation of the adaptive response to the antigen. Specifically, lipoteichoic acid on the surface of gram-positive bacteria induces IL-12 production by macrophages, leading to enhanced activity of natural killer (NK) cells, cytolytic T-cells, and Th1 cells (16, 33). The composition of lipoteichoic acid on the surface of lactobacilli can modulate proinflammatory or anti-inflammatory immune responses (27). A mutant strain of *L. plantarum*, Dlt^-, synthesizing modified teichoic acids dramatically reduced the secretion of proinflammatory cytokines by peripheral blood mononuclear cells (PBMCs) when compared to the wild type strain. An increase was also observed in IL-10 production by PBMCs exposed to the Dlt^- mutant compared to the wild type. In accordance with the in vitro results, the Dlt^- mutant was significantly more protective in a murine colitis model when compared to the wild type (27). *Bifidobacterium*, another member of the lactic acid bacteria, has been shown to stimulate hydrogen peroxide and nitric oxide production by macrophages (75). Production of these oxidative compounds mediates killing or growth inhibition of tumor cells and bacteria. Along with IL-12 production by macrophages, lactobacilli also induce secretion of IFN-γ by T-cells and NK cells (65).

IL-12 stimulates T and NK cells to secrete IFN-γ, so the secretion of this cytokine could be seen as an indirect stimulation of the host immune system by non-pathogenic bacteria. In addition to the pro-inflammatory cytokines such as TNF-α, IL-12 and IFN-γ, lactic acid bacteria also induce anti-inflammatory or regulatory cytokines such as IL-10 and transforming growth factor-B. However, the production of these anti-inflammatory cytokines, especially IL-10, varied significantly between strains (66). Therefore, these results indicate that the presence of lactobacilli in the intestinal mucosa could stimulate the immune system to activate macrophages and initiate both innate and acquired immune response through the production and release of various cytokines.

In addition to stimulating the immune system through induced cytokine production, many lactic acid bacteria can act directly on the immune system and influence systemic events. The gut associated lymphoid tissue (GALT) is a well-developed immune network involved both in protecting the host from pathogens and preventing the host from reacting to ingested protein. Antigen uptake can occur through M-cells overlying Peyer's patches or across normal epithelial cells over the lamina propria. When an intact or partially degraded antigen is absorbed and immune response induced, secretory IgA is the primary mediator. The IgA binds the antigen surface, preventing microbial adherence and translocation of antigens. Additionally, IgA migrates to the other mucosal sites, indicating that intestinal immune stimulation can induce immunity in mucosal sites removed from the GIT. In a murine model, yogurt supplemented with LAB greatly increased the numbers of IgA secreting B-cells and T-lymphocytes (80). The increased levels of specific antibody would provide a faster immune response to pathogens displaying any of the same surface antigens as the yogurt

cultures. When rats were administered *L. plantarum* and *E. coli* they had significantly higher total serum IgA and marginally higher IgM and IgA antibody levels against *E. coli* than those colonized with *E. coli* alone (32). These results indicate that the presence of certain lactobacilli could compete with *E. coli* for intestinal colonization and may influence intestinal and systemic immunity. Another mechanism by which lactobacilli mediate immune enhancement might be through an adjuvant effect. Adjuvants enhance the immune response for a specific antigen when administered along with the antigen. For example, *L. casei* GG increased IgA specific for β-lactalbumin and casein in Crohn's patients (57). The same strain also increases serum IgA titers to rotavirus in humans, although a similar response was not observed when fed heat killed bacteria (30). Lactobacilli that promote cell-mediated immunity have been shown to induce IL-12 and IFN-γ expression, to activate NK cell tumoricidal activity, and to limit tumor growth in certain forms of non-metastatic carcinoma (85). Strains that increase the expression of regulatory cytokines (IL-10) have shown promise as both prophylactic and therapeutic agents for the prevention of immune hypersensitivity and the alleviation of inflammatory bowel disease (73).

Complete characterization of the immunomodulation properties of *Lactobacillus* strains in humans will require well-designed human feeding trials. Several studies have been conducted using rodent infection/challenge models, but these do not represent true human enteropathogen infection. A combination of *L. acidophilus* and *L. casei* prior to oral challenge with *S. typhimurium* elevated both serum and GIT anti-Salmonella antibody titres, suggesting that *Lactobacillus*-mediated enhancement of the acquired immune response likely contributed to the protective effects (79). In addition to

enhanced pathogen-specific antibody, probiotic feeding increased non-specific phagocytic activity of macrophages and blood-born neutrophils, suggesting that enhanced cell-mediated immunity may also contribute to increased protection (18). While pathogen studies cannot be conducted in humans, some studies have been completed using attenuated or non-virulent strains of enteric pathogens. One such study used the attenuated Ty21A strain of *Salmonella typhi* as a live oral vaccine. In this study, 21 days of prefeeding a mixture of potential probiotic strains significantly increased the serum IgA pathogen-specific antibody (52). While it is likely that lactobacilli are able to illicit an immune response, the actual features of each strain that are responsible for the immune activation have not been characterized. The previously discussed influence of bacterial surface molecules on the association of lactobacilli with the intestinal mucosa and maintenance in the GIT may indirectly implicate their interaction with the immune system. The identification of bacterial factors involved with adhesion, therefore, could provide critical insight into the specific molecules that influence the host immune system and allow for a more efficient selection of probiotic strains.

1.9 Conclusions

Currently, interest in food products and dietary supplements containing beneficial "probiotic" bacteria is increasing. Some of the reported beneficial effects of probiotic lactobacilli require that the organisms survive passage through the GIT and associate with the intestinal mucosa. However, despite recent improvements in genetic manipulation and detection strategies, the molecular mechanisms responsible for transient or extended association with the mucus and epithelial layers in the intestinal tract have not been fully

characterized. The relatively high complexity of the gastrointestinal microflora, combined with the strain-specific nature of many adhesion molecules makes identification and characterization of adhesive determinants difficult. In addition, possible involvement of host environmental conditions and communication between bacteria makes it difficult to study the in vivo behavior of individual adhesion molecules. High-throughput genomic sequencing offers the first critical insights into the unique and shared features of lactobacilli that might participate with adhesion and interaction with the host. As more genome sequence data becomes available, selection of putative adhesive determinants, followed by functional and adhesion analysis should provide more insight into possible mechanisms used by lactobacilli that support probiotic roles. Already, the surface features of probiotics are being altered to selectively bind pathogenic bacteria in an effort to prevent bacterial infections (76). By mimicking host cell receptors for toxins and adhesins, these "designer probiotics" can prevent disease by inhibiting pathogenic interaction with host cells. Increasing knowledge of bacterial adherence mechanisms and interaction with host tissues will aid in the selection and engineering of lactobacilli for formulations designed to elicit maximum benefits to human health.

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Table 1. Cell lines used to study bacterial adhesion

Strain	Origin	Relevant phenotype
Caco-2	Colonic carcinoma	Intestinal cell morphology, microvilli
C2BBe	Caco-2 subclone	Homogeneous brush border expression
HT29	Colonic carcinoma	Mucin production
HT29-FU	HT29 subclone	Colonic mucin production
HT29-MTX	HT29 subclone	Gastric mucin production
HeLa	Cervical carcinoma	Low maintenance
Int-407	Embryonic intestine	Similar to HeLa, viral studies
HEp-2	Larynx epithelioma	Epithelial-like, primarily used for viral studies

Table 2. *Lactobacillus* proteins affecting adhesion to the intestinal mucosa

Protein	Bacterial species	Target	Reference
CbsA	*L. crispatus*	Collagen	(61)
EF-Tu	*L. johnsonii*	Mucins, Caco-2	(25)
FbpA	*L. acidophilus*	Caco-2	(11)
GroEL	*L. johnsonii*	HT29	(7)
Lsp	*L. reuteri*	Mouse forestomach epithelium	(109)
LuxS	*L. acidophilus*	Caco-2	(12)
MAPP	*L. fermentum*	Mucus	(90)
Msa	*L. plantarum*	Mannose	(83)
Mub	*L. reuteri,*	Mucus	(11, 91)
	L. acidophilus	Caco-2	(11)
SlpA	*L. brevis*	Int-407, T24 (bladder), EA-hy926	(37)
	L. acidophilus	Caco-2	(11)

CHAPTER II

Functional Analysis of Putative Adhesion Factors in *Lactobacillus acidophilus* NCFM

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2.1 Abstract

Lactobacilli are major inhabitants of the normal microflora of the gastrointestinal tract, and some select species have been used extensively as probiotic cultures. One potentially important property of these organisms is their ability to interact with epithelial cells in the intestinal tract which may promote retention and host-bacterial communication. However, the mechanisms by which they attach to intestinal epithelial cells are unknown. The objective of this study was to investigate cell surface proteins in *Lactobacillus acidophilus* that may promote attachment to intestinal tissues. Using genome sequence data, predicted open reading frames were searched against known protein and protein motif databases to identify four proteins potentially involved in adhesion to epithelial cells. Homologous recombination was used to construct isogenic mutations in genes encoding a mucin-binding protein, a fibronectin-binding protein, a surface layer protein, and two Streptococcal R28 homologs. The adhesive abilities of the mutants to intestinal epithelial cells were then evaluated, in *vitro*. Each strain was screened on Caco-2 cells, which differentiate and express markers characteristic of normal small intestinal cells. A significant decrease in adhesion was observed in the fibronectin-binding protein mutant (76%) and the mucin-binding protein mutant (65%). A surface layer protein mutant also showed reduction in adhesion ability (84%), but the effect of this mutant is likely due to the loss of multiple surface proteins that may be embedded in the S-layer. This study demonstrated that multiple cell surface proteins in *L. acidophilus* NCFM can individually contribute to the organism's ability to attach to intestinal cells, in *vitro*.

2.2 Introduction

Probiotics are commonly included in dairy products, especially fermented milks. One major selection criterion considered for selection of probiotic bacteria has been their capacity to adhere to the human intestinal epithelial cells. Adhesion is believed to be a requirement for the realization of certain probiotic effects such as immunomodulation (37, 44) and pathogen exclusion (6, 28). However, the mechanisms of attachment are not understood. Association with the intestinal mucosa can initiate and extend transient associations which afford these bacteria a distinct advantage when in the gastrointestinal tract. *Lactobacillus acidophilus* NCFM is an industrial bacterial strain used widely in dietary supplements and cultured yogurts (36). Recent studies have implicated the involvement of some surface proteins from lactobacilli with adhesion to epithelial cells (16), mucin (32) and various extracellular matrix (ECM) proteins (40). The surface layer protein, SlpA, from other lactobacilli has also been shown to bind epithelial cells and ECM components (4, 21). However, no studies have demonstrated unequivocally the function and significance of S-layers in either adherence or improved retention of probiotic cultures in the gastrointestinal tract.

Lactobacilli are normal components of the intestinal microbiota and appear to be a key factor in the processes of competitive exclusion (13) and immunomodulation (44, 45) exerted by commensal organisms. Although extensive genetic characterization has been performed on the adhesive abilities of enteropathogenic bacteria, the genetic systems responsible for intestinal adhesion of probiotic bacteria are not fully understood. The difficulties in performing human trials, the complex and kinetic nature of the intestinal environment, and the absence of mutant strains for isogenic comparisons have made it

difficult to study these processes. Development of appropriate intestinal model systems has allowed us to study bacterial adhesion to epithelial and mucosal surfaces, in *vitro*, particularly in pathogen models. In *vitro* model systems together with high-throughput sequencing of *Lactobacillus* genomes now provides a platform for functional analysis of genes that may contribute to adherence and aggregation processes.

In order to identify genes potentially involved with adhesion, the complete genome sequence of *L. acidophilus* NCFM (2) was analyzed and open reading frames (ORFs) similar to genes implicated previously in adhesion were targeted for insertional inactivation including two streptococcal R28 homologs (LBA1633 and LBA1634), a fibronectin-binding protein (FpbA), a mucin-binding protein (Mub), and a surface layer protein (SlpA). In this study, we provide evidence that the genes encoding fibronectin-binding protein (FbpA), mucin-binding protein (Mub) and the surface layer protein (SlpA) contribute to the adhesive abilities of NCFM to intestinal cells, in *vitro*.

2.3 Materials and Methods

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are identified in Table 1. *Lactobacillus* strains were cultivated anaerobically at 37°C or 42°C in MRS broth (Difco Laboratories Inc., Detroit MI) or, when appropriate, in MRS supplemented with 1.5% agar. *Escherichia coli* was propagated aerobically in Luria-Bertani (LB, Difco) medium or on LB medium supplemented with 1.5 % agar at 37°C. Brain Heart Infusion (BHI, Difco) medium supplemented with 1.5 % agar and 150 μg/ml erythromycin (Em) was used for selection of *E. coli* transformants. Chloramphenicol (Cm, 5.0 μg/ml) and Em

(5.0 μg/ml or 150 μg/ml) were used for selection when appropriate. Colony forming units (CFU) per ml were determined using a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, West Yorkshire, England).

Computational Analysis

Conserved protein domains were detected using Pfam (http://pfam.wustl.edu) and ClustalX (41) was used to align sequences. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) was used to identify signal sequences and THMM v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) predicted transmembrane domains in selected protein sequences. Putative functions were assigned to target genes manually by sequence comparison to an existing protein database using the BLAST-P algorithm (3). Phylogenetic trees of related protein sequences were constructed using ClustalX (41) and MEGA2 (25).

DNA manipulation techniques

Total *Lactobacillus* genomic DNA was isolated according to the method of Walker and Klaenhammer (47). Standard protocols were used for endonuclease restriction, ligation, DNA modification and transformation (35). Plasmid preparations for the purpose of screening *E. coli* transformants followed the method of Zhou *et al*. (48). Large scale plasmid preparations were performed with the QIAprep Spin kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). PCRs were carried out according to manufacturer's recommendations using a *Taq* DNA polymerase PCR system (Roche Molecular Biochemicals). PCR primers (Table 2) were synthesized by Integrated

DNA Technologies (Coralville, IA) and, when appropriate, restriction sites were designed into the 5' end of the primers to facilitate future cloning steps. DNA fragments were extracted from 1.0 % agarose gels using the Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA). Electrocompetent *Lactobacillus* cells were prepared as described by Walker et al. (46). Southern hybridization of genomic DNA was carried out using Magnacharge nylon transfer membranes (MSI, Westboro, MA) according to the manufacturer's instructions.

Site-specific integration into *L. acidophilus* NCFM

Using *L. acidophilus* NCFM chromosomal DNA as a template, an internal fragment of each target ORF was amplified using PCR with primers listed in Table 2. The internal fragment was cloned onto the integrative vector pORI28 (26) and subsequently transformed by electroporation into *L. acidophilus* NCFM containing the temperature sensitive helper plasmid pTRK669 (34). Steps were then carried out according to Russell and Klaenhammer (34) for selection of integrants. Successful integration of the plasmid was confirmed by PCR and Southern hybridization analysis of junction fragments.

Tissue culture

The Caco-2 (ATCC HTB-37, Rockville, MD) cells were only used between the 40^{th} and 60^{th} passage. All reagents used in maintenance of Caco-2 cells were obtained from Gibco (Invitrogen Corp., Carlsbad, CA). The cells were routinely grown in 95% air-5% CO_2 atmosphere in Minimum Essential Medium (MEM) supplemented with 20%

(v/v) inactivated (56°C, 30min) fetal bovine serum (FBS), 0.10 mM non-essential amino acids and 1.0 mM sodium pyruvate . Monolayers were trypsinized for 10 min, counted using a hemocytometer, and seeded at $1.3x10^5$ cells/well in 2.0 ml of cell culture medium. The medium was replaced every two days and all adherence assays were performed after 14 days of incubation. Cells were grown on 15 mm Thermanox plastic coverslips (Nalge Nunc International, Rochester, NY) in Costar 12-well tissue culture treated plates (Corning Inc., Acton, MA).

Adherence Assay

Adhesion of *Lactobacillus* strains to Caco-2 cells was examined according to the method described previously by Chauviere *et al*. (8), with the following modifications. Briefly, middle log-phase bacterial cells (OD_{600} 6.0) were prepared in MRS with 3.0 μg/ml Em to maintain selective pressure on integrants. Cells were removed to eliminate any effect of low pH or extracellular proteins in culture supernatants by centrifugation for 10 min at 4000 x g, and washed twice with phosphate-buffered saline (PBS). Bacterial pellets were resuspended in 5 mL of fresh MRS prior to adherence. Fifteen-day Caco-2 monolayers were washed twice with PBS and treated with a bacterial suspension at a concentration of 4×10^8 CFU/ml. Bacteria were incubated on the monolayer for 1.5 hr at 37°C in a mixture (1:2 v/v) of MRS and cell line culture medium. Following incubation, the monolayers were washed five times with PBS, fixed in methanol and Gram stained. Adherent bacterial cells were then enumerated microscopically. For statistical purposes, 17 fields were examined in a pre-determined fixed grid for each coverslip (Fig. 1). The pattern of the grid was selected by choosing the pattern of fields that most ideally

represented the average count per field obtained by counting all fields on the coverslip. Duplicate coverslips were counted for each experiment. The final data presented collectively represented at least 5 experiments. Total counts for each coverslip were used and adhesion was expressed as percent (%) of the control strain NCK1398 (34) which carries an insert in the *lacL* (β-galactosidase) gene. Using this control, all mutant cultures and the parental control could be prepared with Em to maintain selective pressure on the integrant.

2.4 Results

2.4.1 Sequence analysis of putative adhesion ORFs

Three regions of the *L. acidophilus* NCFM genome were identified as harboring genes predicted to encode proteins that may participate in adhesion to the intestinal epithelium and mucosa. The first region encoded two adjacent ORFs (LBA1633 and LBA1634) showing homology to R28, a protein involved in adhesion of *Streptococcus pyogenes* to the vaginal epithelium (39). The second and third regions contained a putative fibronectin-binding protein (LBA1148) and a mucin-binding protein (LBA1392). Figure 2 shows the sizes of the predicted proteins, and surrounding features in the genome.

Fibronectin-binding protein (LBA1148) was identified containing the N-terminal PFam domain (pfam05833) for fibronectin-binding protein A (FbpA); it was the only ORF in the NCFM genome containing that domain. LBA1148 is transcribed on the forward strand with its own promoter, ribosomal binding site (RBS) and terminator (Fig 2). LBA1148 showed 41% amino acid identity to a predicted adherence protein

(NP_785358) in *Lactobacillus plantarum* WCFS1, a putative fibronectin-binding protein (NP_814975) in *Enterococcus faecalis* V583, and exhibited > 65% amino acid identity to an uncharacterized putative proteins in *Lactobacillus gasseri* (Lgas1024) and *Lactobacillus johnsonii* (LJ1182). Interestingly, the fibronectin-binding proteins identified in *L. acidophilus* NCFM, *L. gasseri*, and *L. johnsonii* are clustered away from other fibronectin-binding proteins when compared using amino acid sequence alignments (Fig 3). Previously studied fibronectin-binding proteins in *Streptococcus mutans* and *Streptococcus gordonii* also contain the FbpA PFam domain and showed homology to LBA1148 (38% and 39% amino acid identity respectively). Due to the similarities to other binding proteins, LBA1148 (FbpA) was inactivated by site specific integration via a 734 bp internal region of homology. Confirmation of the integration event was performed by PCR analysis of junction fragments and by Southern hybridization (Fig. 4).

LBA1633 and LBA1634 both show similarity to R28, a characterized adhesion protein in *S. pyogenes*. R28 contains 10 identical 79-residue repeats and was shown to promote the adhesion of *S. pyogenes* to cervical epithelial cells *in vitro* (39). *Lactobacillus fermentum* BR11 also has two tandem genes, *rlp* and *mlp* proposed to be involved in adhesion (43); Rlp exhibits homology to R28, LBA1633 and LBA1634. Interestingly, Mlp is also similar to the mucus binding protein (LBA1392) discussed below. Both LBA1633 and LBA1634 have exhibited LPxTG sortase target signal sequences for cell-wall anchoring. A gene encoding a sortase (LBA1244) was identified and twelve other ORFs in the *L. acidophilus* NCFM genome harbored the sortase target sequence. LBA1634 also contains a gram-positive cell-wall anchor motif (PF00746) and a SIRK-type signal peptide sequence, suggesting that this protein is secreted and

anchored to the cell wall. C-terminal transmembrane domains were found in both proteins, while only LBA1634 contains both C- and N-terminal transmembrane domains. These two ORFs contain both perfect and non-perfect internal repeats and show 39% amino acid identity to each other. LBA1633 and LBA1634 are transcribed on the complement strand, translated in different frames and each has a terminator immediately downstream. Ribosomal binding sites along with -10 and -35 promoter regions were predicted upstream of each ORF. Upstream of LBA1634 is an ORF of unknown function (LBA1636), and one predicted membrane transporter (LBA1637). Downstream of LBA1633 is a predicted aldehyde dehydrogenase transcribed in the opposite direction. The similarity of LBA1633 and LBA1634 to each other and to other adhesion factors made them a logical target for characterization. Site specific integrations (data not shown) were individually made into LBA1633 and LBA1634 and the mutants were evaluated for their adhesive ability to Caco-2 intestinal cells, *in vitro*.

Mucin-binding proteins are common in various forms in the genome of *L. acidophilus* NCFM. Of the thirteen ORFs that are annotated as mucus-binding proteins, five have BlastP e-values smaller than $1e^{-29}$ (non-redundant database), and two of those contain the LPxTG sortase targeting signal for cell wall anchoring. One of these two, LBA1392, is of similar size and shows homology (25% amino acid identity) to a previously characterized mucus-binding protein in *L. reuteri* (33). However, the amino acid repeat pattern in the *L. reuteri* protein is not conserved in LBA1392. Instead, LBA1392 contains two sets of three repeats each, ranging in size from 70-87 amino acids and showing 61-100% sequence identity between the repeats. The three repeats in each set are adjacent and the two sets of repeats are located next to each other covering an

overall stretch of 1586 amino acids. Transmembrane domains were identified at both the amino and carboxy terminus of LBA1392 and a SIRK signal sequence and cleavage site was identified at amino acid position 50. Collectively, these predicted features strengthen the functional classification of LBA1392 as a candidate for mucin association and epithelial cell adhesion. Mucus-binding protein homologs have been identified in several different lactobacilli (24, 31, 33), but are not generally found among the lactic acid bacteria. Although these proteins have a similar predicted function and are found in similar probiotic lactobacilli, the lack of amino acid identity indicates substantial divergence for these proteins with a presumed similar function (Fig. 5). LBA1392 (Mub) was also insertionally inactivated and the integration event confirmed by both Southern hybridization and PCR (data not shown).

2.4.2 Adhesion of Mutants to epithelial cells

The effect of insertional inactivation of LBA1633, LBA1634, FbpA (LBA1148), Mub (LBA1392) and the surface layer gene, slpA (LBA1377) (1), was determined by screening the mutants ability to adhere to Caco-2 cells, in *vitro*. Caco-2 is a colonic carcinoma cell line, commonly used to study bacterial adherence, which expresses many of the markers associated with normal small intestine villus cells (30). Log-phase bacterial cells and a 1.5 hr adherence time have demonstrated the most consistent levels of adherence to Caco-2 cells, in our experiments (data not shown). A derivative of *L. acidophilus* NCFM (NCK1398) harboring a plasmid integration into the *lacL* gene, encoding β-galactosidase, was used as the control so antibiotic pressure could be maintained on all strains used in adherence comparisons. There was no significant

difference in adhesion between NCK1398 and wild type *L. acidophilus* NCFM (data not shown).

The cell morphologies of individual mutant strains were not altered under the growth conditions used in the study with the exception of the SlpA$^-$ mutant which grew as small curved bacilli (1). Each inactivated gene contained a predicted downstream terminator so any effects observed were not considered likely to result from downstream polar effects. Insertional inactivation of 3 of the 5 genes targeted in this study resulted in a significant reduction ($p < 0.05$) in adhesion to Caco-2 intestinal cells, when compared to the wild type control (Fig. 6). No reproducible effects on adhesion were observed with the two R28 homolog mutants, NCK1662 and NCK1720. Inactivation of a predicted fibronectin-binding protein resulted in a 76 % decrease in adhesion. Mucin-binding proteins were predicted to be abundant in the genome of *L. acidophilus* NCFM. Inactivation of a single putative mucin-binding protein decreased the ability of the resulting mutant strain to adhere by 65 %. The SlpA$^-$ mutant demonstrated the largest decrease in adhesion ability at 84 %, although it is likely that multiple surface-associated proteins could be disrupted with the removal of the S-layer.

2.5 Discussion

Adhesion of lactic acid bacteria to epithelial and mucosal surfaces is a complex process involving many different factors. The ability of these microorganisms to adhere to intestinal surfaces is potentially a major distinguishing feature for selection of bacteria as probiotic strains. Close interaction with host tissues may provide probiotics with a distinct advantage when establishing residence in the gastrointestinal tract or interacting

with cells of the intestinal mucosa. This study exploited genome sequence data to identify four genes that may be involved in adhesion of NCFM to intestinal epithelial cells. A targeted approach for identifying adhesion factors and creating isogenic chromosomal mutations allows us to systematically evaluate cell surface proteins for their contribution to adherence or attachment capability. Previous studies have concentrated on identifying the location of adhesive determinants (16, 20), determining the nature of adhesive determinants (8, 10, 17-19), comparing the adhesiveness of different strains (8, 42), or identifying adhesion molecules using binding assays (17, 22, 33). In contrast, this reports the first study that relied on genome sequence data to identify multiple cell surface proteins potentially involved with adhesion and specifically investigated their individual contributions via isogenic strain comparisons. In this study a fibronectin-binding protein, mucin-binding protein, and surface layer protein each contributed, individually, to the ability of NCFM to adhere to Caco-2 cells, in *vitro*.

The human Caco-2 cell line was originally isolated from a human adenocarinoma of the colon. In culture, Caco-2 cells spontaneously differentiate post-confluence to display functional apical brush border microvilli and epithelial cell polarization. This cell line has traditionally been employed to study the adherence of lactobacilli and select for adhesive probiotics strains (5, 7, 8, 16, 18, 21). Additionally, the Caco-2 model is commonly used to demonstrate the competitive exclusion properties of probiotic bacteria against enteropathogenic strains such as various *Salmonella* and *E. coli* species (6, 11, 15). When examining the adhesive properties of probiotic bacteria to Caco-2 cells, many different protocols have emerged, each with its own benefits and shortcomings. After evaluating different protocols, we selected and adapted one based on its reproducibility

and applicability to the cell-surface knockout mutants constructed. Blum *et al*. (1999) noted that the adhesion percentage of a probiotic strain under given assay conditions does not represent an absolute value (7). Instead, adhesion should be reported as a value relative to a type strain. This study uses a β-galactosidase mutant as the control to ensure that conditions for propagation, specifically under antibiotic selection to maintain an integrated plasmid, are identical between the control strain and mutants. If conditions of the adhesion assay are closely controlled, the effect of isogenic mutations in cell surface proteins can be accurately evaluated and compared.

We observed a significant decrease in adhesion to Caco-2 cells with inactivation of the surface layer gene, *slp*A. The surface layer is a self-assembled paracrystalline monolayer of proteins that coats the entire surface of some bacteria and archea. The function of surface layers can include cell-shape determination, protection, and epitope display. Additionally, S-layers have been reported to be involved with epithelial cell attachment (14, 38). However, these studies used chemical treatments such as LiCl to remove existing S-layers from the organism. This approach does not account for the reappearance of the S-layer after chemical removal. Use of an isogenic S-layer mutant allows for a more definitive study of the effect of the S-layer on adhesion. The absence of an S-layer was confirmed in NCK1377 at the time of experimentation; however, after approximately 6 months of storage a chromosomal inversion resulted in the reappearance of an alternate S-layer (1). It is not surprising that such a dramatic effect on adhesion was observed in the current study when the gene encoding the surface layer protein was inactivated. Removal or alteration of a surface layer can have many dramatic cellular

affects, not the least of which may be surface charge, architecture, and the presence or conformation of various surface proteins involved with attachment.

Surface proteins involved with adhesion could either adhere directly to the epithelium, or to specific components of the epithelial environment. The extracellular matrix (ECM) surrounding mammalian epithelial cells is composed of various secreted proteins including laminin, collagen, and fibronectin. Fibronectin is a dimeric glycoprotein reported to be involved with adhesion of *Lactobacillus* cells to intestinal cells, in *vitro* (22). Recent studies have reported fibronectin binding by lactobacilli (27, 40) and implicated involvement of fibronectin with epithelial cell adhesion (22). Other studies have proposed involvement of bacterial-associated proteinaceous components with the adhesion of lactic acid bacteria to intestinal cells, in *vitro* (18, 19). Multiple fibronectin-binding proteins have been examined in various *Streptococcus* species, one of which is the surface associated protein FbpA in *S. gordonii* (9). FbpA appears to act as a bridging protein between the bacterial surface and the ECM of the host, although the precise mechanism by which this interaction occurs is not understood. By inactivating the predicted bridging protein, FbpA in *L. acidophilus* NCFM, we expected to eliminate any adhesive properties due to fibronectin binding. Accordingly, we observed an approximate 76% reduction in adherence capacity, indicating that FbpA is an important, but not the only factor contributing to the ability of lactobacilli to adhere to epithelial cells.

In addition to the ECM, epithelial cells in the mammalian gastrointestinal tract are covered with a thick layer of mucus composed mainly of glycoproteins called mucins. Mucin expression and composition are dynamic, balanced between production by goblet

cells and degradation by proteases and physical erosion in the gut due to transit functions. Bacteria able to adhere to mucus, but unable to associate with the epithelium, may be washed away with degraded mucins, partially accounting for the transient nature of intestinal colonization observed for most probiotic bacteria. Studies have reported the binding ability of many lactobacilli to human intestinal mucus (23). The ability of lactobacilli to colonize this mucus layer could be vital in the realization of certain probiotic properties. A mucin-binding protein in *L. reuteri*, Mub, was not only shown to be associated with the cell surface via immunofluorescence, but also to bind intestinal mucins (33). The repeat regions of the *L. reuteri* Mub were shown to bind mucus components when examined as a part of a fusion protein. Therefore, the region targeted for integration of pTRK834 (the *mub* integration vector) was the repeat region of *mub* in *L. acidophilus* NCFM. Although the vector was designed to integrate into the repeat region, care was taken to avoid multiple regions of homology between the vector and *mub*. The resulting mutant showed a consistent 65% decrease in adhesion to Caco-2 cells. Although the Caco-2 cells in this study do not produce mucins, the contribution of the mucin-binding protein to intestinal cell binding is evident.

Both the Mub$^-$ and FbpA$^-$ mutants exhibited significant and reproducible decreases in adhesion to Caco-2 cells. Follow-up experiments will focus on the specific ability of those mutant strains to bind mucin and fibronectin. The mutants with integrations in the contiguous R28 homologs (LBA1663 and LBA1664) did not produce convincing changes in adhesive properties. LBA1663 and LBA1664 both showed substantial homology to adhesins R28 in *S. pyogenes* and Rlp in *L. fermentum* BR11. The participation of R28 in the adhesion of *S. pyogenes* to vaginal epithelial cells might

suggest that an in *vitro* model of the vaginal epithelium would be useful in characterizing these mutant strains, but is beyond the scope of this study. Although these two ORFs in NCFM encode proteins similar to those in the Rib family, they do not contain the identical repeats present in most other Rib proteins, particularly Rib proteins in pathogenic organisms. Neighboring binding proteins with notable identity were identified in *S. gordonii* at two separate chromosomal loci (12, 29). Although there are terminators downstream of both genes in NCFM, the fact that these genes are adjacent and very similar could account for the substantial variation observed in attachment levels. Perhaps the cell relies upon both proteins for a similar function in adhesion and disruption of only one leads to a variable functional attachment phenotype. A mutant strain deficient in both of these proteins would be useful in determining their effect on adhesiveness, and is planned as a follow-up to this study.

A significant involvement of SlpA, FbpA and Mub with adherence of *L. acidophilus* NCFM to Caco-2 cells was demonstrated. Given that the inactivation of LBA1633 and LBA1634 produced neither dramatic changes nor reproducible results, the affects of $SlpA^-$, $FbpA^-$ and Mub^- mutations are even more striking in comparison. To date, the genetic mechanisms and components involved with the binding of *Lactobacillus* to the human intestinal epithelium have yet to be fully characterized. However, with the increasing availability of genomic data and the molecular tools necessary to construct functional isogenic mutants, cell surface components can be identified and tested in *vitro*. No single gene inactivated in this study was able to completely eliminate adhesion of NCFM to Caco-2 cells, thereby strengthening the hypothesis that adhesion is achieved through an intimate interplay of multiple factors. Elucidation of the molecular

components, and their interactions, on adhesion of lactobacilli to human epithelial cells is expected to progress rapidly through the use of functional genomics to identify and confirm those key factors.

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TABLE 1. Bacterial strains and plasmids used in this study

Strains	Origin or relevant characteristics	Source or reference
L. acidophilus		
NCFM	Human intestinal isolate	(36)
NCK 1377	NCFM::pTRK826 (*slpA* integrant)	(1)
NCK 1392	NCFM containing pTRK669	(34)
NCK 1398	NCFM::pTRK685 (*lacL* integrant)	(34)
NCK 1660	NCFM::pTRK834 (*mub* integrant)	This study
NCK 1661	NCFM::pTRK833 (*fbpA* integrant)	This study
NCK 1662	NCFM::pTRK835 (*R28* integrant)	This study
NCK 1720	NCFM::pTRK832 (*R28* integrant)	This study
E.coli		
EC1000	$RepA^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA*; host for pOR128-based plasmids	(26)
Plasmids		
pORI28	Em^r, ori (pWV01), replicates only with *repA* provided in *trans*	(26)
pTRK669	ori (pWV01), Cm^r, $RepA^+$	(34)
pTRK832	835bp internal region of *r28* (LBA1634) cloned into BglII-XbaI sites of pORI28	This study
pTRK833	734bp internal region of *fbpA* (LBA1148) cloned into BglII-XbaI sites of pORI28	This study
pTRK834	955bp internal region of *mub* (LBA1392) cloned into NcoI-XbaI sites of pORI28	This study
pTRK835	867bp internal region of *R28* (LBA1633) cloned into BglII-XbaI sites of pORI28	This study

TABLE 2. Primer sets used

ORF		Internal[a]	External[b]
LBA1148 (*fpbA*)	Forward	GATCTCTAGA-TGGAAGGTTCAGTCTTAC (a)	CAACGCTCAATATCCTAGAA
	Reverse	GATCAGATCT-TACGATAGCCCTCAGAAT (b)	CTTGTGGATCAGCATTATCA
LBA1392 (*mub*)	Forward	CATGCCATGG-TAAGGTCACAGGTGTAACAA (c)	AGTGCTAGTGACCAACACTT
	Reverse	GATCTCTAGA-AGCCTACTTCACTAGGAGTC (a)	ATTGCTTAGGTGCGTTAGTA
LBA1633 (*R28*)	Forward	GATCAGATCT-CAGGTTCTACAGCATCAA (b)	TAGGCAATACTGGTAATGAA
	Reverse	GATCTCTAGA-CCAACGCCTTCACTATAA (a)	TTAACCGGAATTACAACATC
LBA1634 (*R28*)	Forward	GATCAGATCT-CCAGCAGTTGTTGTCGTA (b)	CCAGCAAGTTCAATCAAGGT
	Reverse	GATCTCTAGA-ACTGTGGCCGGCTTGTTA (a)	ACGTGGATAGTTACTGAGAC

[a] Primer sets used to amplify an internal region of the specified ORF for cloning onto pORI28. Restriction enzyme digestion sites for XbaI (a), BglII (b) and EcoRI (c) are underlined.
[b] Primer sets used to detect junction fragments in integrants.

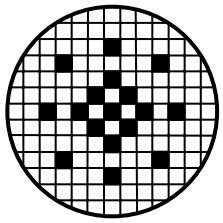

Fig. 1. Grid pattern used for microscopic enumeration of bacterial cells adhered to Caco-2 monolayers on coverslips. Each square represents one microscopic field. Shaded squares indicate fields on the coverslips (represented by complete circle) selected for enumeration.

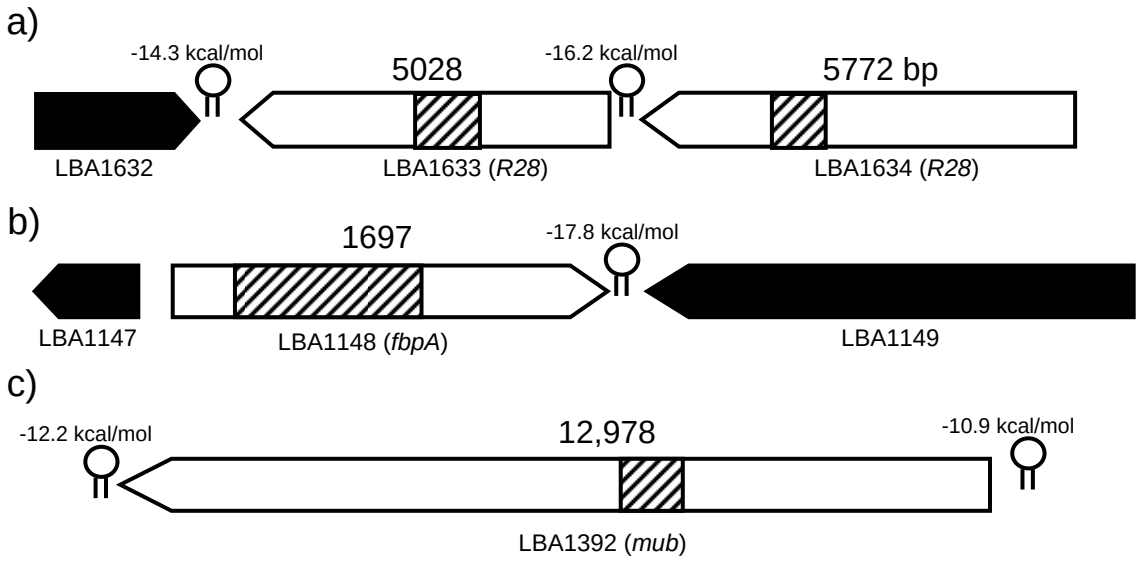

Fig. 2. Putative adhesion ORFs in the *L. acidophilus* NCFM genome targeted for insertional inactivation are represented by white arrows. The shaded region in the white arrrows designates the region cloned onto pORI28 for homologous recombination and the subsequent region of integration. Shaded regions are drawn to scale with each gene, but the relative size between genes is not to scale. Predicted rho-independent terminator free energies are represented by hairpin structures. Potential functions of target genes are listed next to the ORF number.

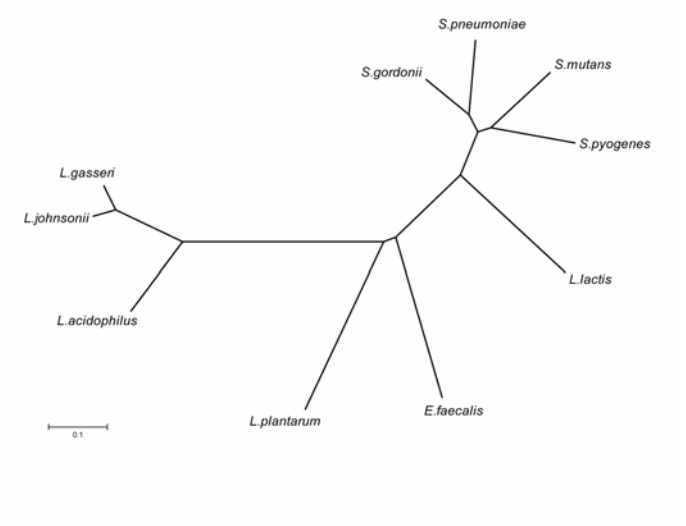

Fig. 3. Phylogenetic tree of selected fibronectin-binding proteins in Gram-positive bacteria. Protein alignment and tree construction performed using ClustalX and MEGA2. Sequences for alignment of fibronectin-binding proteins were obtained from GenBank and were selected based on similarity to FbpA (LBA1148) in *L. acidophilus* NCFM.

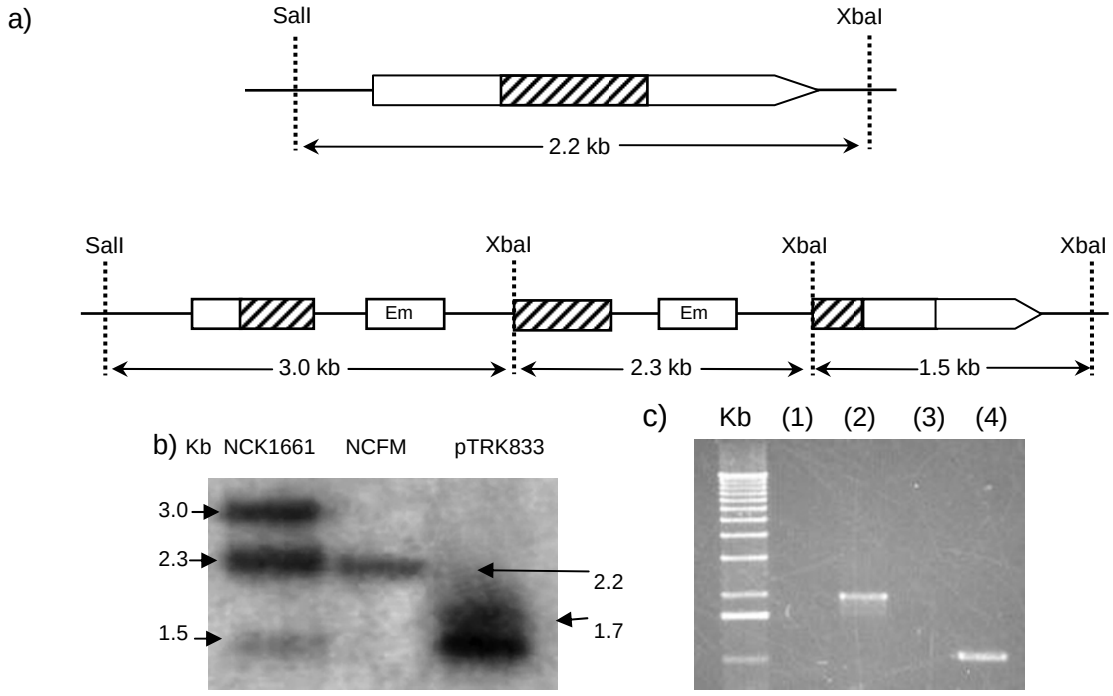

Fig. 4. Confirmation of integration event in the gene putatively encoding the fibronectin-binding protein, LBA1148. Panel A: Chromosomal insertion of pTRK833 into the target region of LBA1148. The internal target region is represented by a shaded box. Restriction sites SalI and XbaI are indicated. Panel B: Southern hybridization analysis of genomes of NCFM and the fibronectin binding protein mutant, NCK1661. Chromosomal DNA was digested with SalI and XbaI and the internal fragment (shaded region) was used as a probe. Lane 1, NCK1661; Lane 2, NCFM; Lane 3, plasmid pTRK833 (double band represents supercoiled and nicked plasmid). Panel C: PCR amplification of junction fragments in the fibronectin-binding protein mutant using primers outside of the region of homology paired with primers in the Em resistance gene of the integration plasmid. Lane 1 and lane 3, NCFM controls; Lane 2, upstream junction fragment; Lane 4, downstream junction fragment. The absence of a band in Lanes 1 and 3 indicates that no plasmid was integrated into this region in the control.

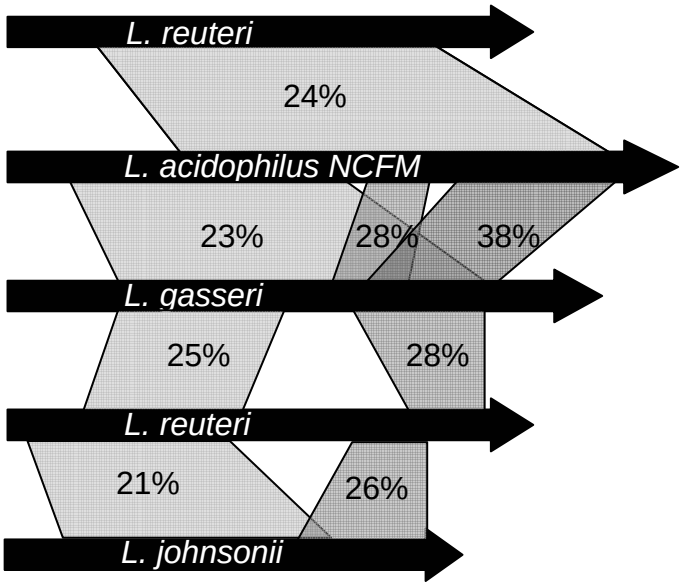

Fig. 5. Amino acid identities compared for predicted mucin-binding proteins in four *Lactobacillus* species. Amino acid alignment was performed with BLAST-P and identity represented by shaded regions between genes. Sequences for alignment were obtained from GenBank. Protein lengths are drawn approximately to scale; lengths in amino acids are, *L. reuteri* Mub, 3269; *L. acidophilus* Mub, 4326; *L. gasseri*, 3692; *L. johnsonii*, 2139.

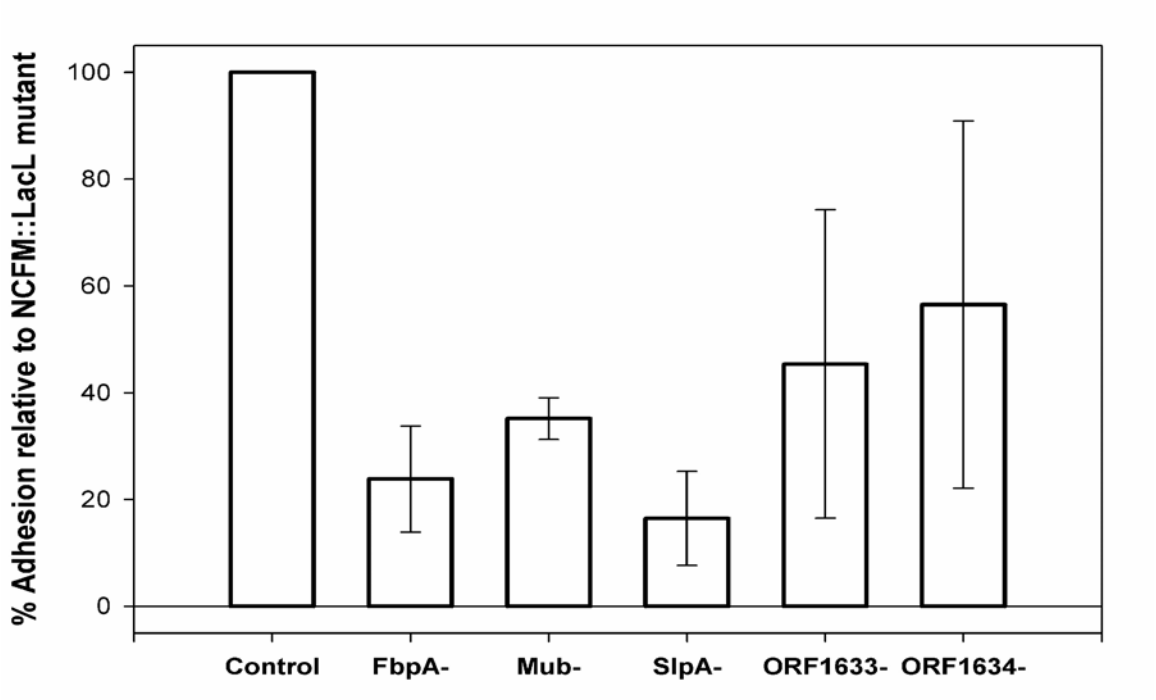

Fig. 6. Adhesion properties of *L. acidophilus* NCFM mutants expressed as a percent of the NCFM::*lacL* control, enumerated microscopically. Error bars represent one standard deviation.

CHAPTER III

Influence of environmental conditions and autoinducer-2 on the adhesion adaptive response of *Lactobacillus acidophilus* NCFM

3.1 Abstract

It is suspected that lactobacilli adhere to the intestinal epithelium for retention in the gastrointestinal tract and communication with the immune system, although the mechanisms by which these bacteria attach are largely unknown. Environmental conditions, including the presence of other microorganisms and signals present in the gastrointestinal tract, are likely to influence the adherence of bacteria to epithelial cells. This study reports an explosive increase in the adhesive ability of *Lactobacillus acidophilus* NCFM to Caco-2 cells following a 1 hour incubation of cells that were concentrated ten-fold, immediately prior to adhesion. Microarray analysis of the global transcriptional response with and without exposure to the adhesion adaptive conditions revealed several genes potentially involved with adhesion to the intestinal epithelial cells and a classic stress response. Interestingly, putative genes linked to the synthesis of an interspecies signaling molecule, autoinducer-2 (AI-2), were overexpressed. Examination of the *L. acidophilus* NCFM genome revealed the complete pathway for AI-2 synthesis. AI-2 activity was detected in *L. acidophilus* NCFM during stationary growth phase using the *Vibrio harveyi* BB170 assay system. Using site-specific integration, an isogenic mutation was created in *luxS* and the resulting derivative of *L. acidophilus* NCFM did not produce AI-2. A 58 % decrease in adherence to Caco-2 cells was also observed in the LuxS$^-$ mutant when the cells were used for adhesion directly from logarithmic phase cultures. However, the LuxS$^-$ mutant strain still responded to adhesion adaptive conditions with significantly increased adherence indicating that additional factors contribute to the explosive adhesion response. The decrease in adhesion detected represents one of the first phenotypes attributed to LuxS in lactobacilli.

3.2 Introduction

Lactic acid bacteria (LAB) are naturally found in a variety of environmental niches where they exist as members of complex microbial communities. Survival in each niche depends on the ability of the organism to sense and respond to varying conditions such as temperature, pH, nutrient availability, and cell population density. One major niche that LAB frequently occupy is that of the mammalian gastrointestinal tract (GIT). Multiple signal transduction pathways likely control the expression of adhesion factors, stress response genes, and other genetic determinants that promote survival and competition of LAB within the various compartments of the GIT. Some intestinal lactobacilli, along with Gram-positive and Gram-negative pathogenic bacteria, have the ability to associate with the intestinal epithelium and mucosal layers therein. This association is thought to be important for the realization of certain probiotic properties including competitive exclusion, immunomodulation, and the delivery of biotherapeutics, among others. The difficulties and cost of human trials, along with the dynamic and variable environment of the intestine have led to the development of in vitro model systems for the study of bacterial adherence to the intestinal epithelium, such as Caco-2 cells. Traditionally used for the study of pathogens (13, 33, 39), probiotics (4, 10, 17, 18), and the inhibition of pathogens by probiotics (8, 12, 16), Caco-2 monolayers are also employed as models of the intestinal epithelium for the selection of adhesive probiotic strains. Although the molecular mechanisms involved with this association are not understood, it is clear that the process is complex, involving host-specific, bacterial-specific, and environmental factors. Lactobacilli must maintain a balance between meeting their own growth requirements and surviving the hostile conditions, including

gastric-acid shock, presented by the host defenses and competing microflora (36). However, little is known about how these stressors influence the ability of lactobacilli to associate with the varied substrates in the intestinal environment.

The microflora of the healthy human intestine contains an estimated 500 to 1,000 different species that combine to represent a uniquely vast genetic diversity. It would, therefore, not be surprising if the members of the intestinal community communicate among themselves to coordinate processes such as maintenance of the commensal microflora or disease resistance (21). Bacterial cells frequently communicate via quorum sensing mechanisms, which involve the density-dependant recognition of an autoinducer followed by changes in gene expression. One important quorum sensing system that can be used to communicate among and between species is based on a furanosyl borate diester called autoinducer-2 (AI-2), produced in four enzymatic steps from methionine. The gene encoding the AI-2 synthase, LuxS, has recently been identified in many different Gram-positive and Gram-negative species. AI-2 regulates the expression of various phenotypes including virulence factors, DNA processing, cell morphology, motility, biofilm formation, toxin production, light production, and cell division in numerous species (47). For example, a $LuxS^-$ mutant strain of *Streptococcus mutans* was more resistant to antibiotics and showed altered virulence and biofilm formation (26). However, the influence of AI-2 on bacterial signaling in the GIT has not been characterized. Production of AI-2 in *Lactobacillus rhamnosus* and *Lactobacillus casei* has been confirmed (15), and in *Lactobacillus reuteri*, AI-2 production was reported to be involved with biofilm formation (37).

This study reports a significant increase in the adhesive ability of *L. acidophilus* to Caco-2 cells following a preconditioning treatment. A whole genome microarray was employed to identify genes potentially involved with the adhesion adaptive response. Among those induced was *luxS*, one gene in the biosynthetic pathway for the signaling molecule, AI-2. This study investigated the role of LuxS, and AI-2, on the adhesive capability of *L. acidophilus*.

3.3 Materials and Methods

Bacterial strains and growth conditions. *Lactobacillus* strains were cultivated anaerobically at 37°C or 42°C in MRS broth (Difco Laboratories Inc., Detroit MI) or, when appropriate, in MRS supplemented with 1.5% agar. *Escherichia coli* was propagated aerobically in Luria-Bertani (LB, Difco) medium or on LB medium supplemented with 1.5 % agar at 37°C. Brain Heart Infusion (BHI, Difco) medium supplemented with 1.5 % agar and 150 μg/ml erythromycin (Em) was used for selection of *E. coli* transformants. Autoinducer Bioassay (AB) media (5) was used for the propagation of all *Vibrio harveyi* strains. When appropriate, chloramphenicol (Cm, 5.0 μg/ml) and Em (5.0 μg/ml or 150 μg/ml) were used for selection. Colony forming units (CFU) per ml were determined with appropriate dilutions using a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, West Yorkshire, England).

DNA manipulation techniques. Total *Lactobacillus* genomic DNA was isolated according to the method of Walker and Klaenhammer (42). Standard protocols were

used for endonuclease restriction, ligation, DNA modification and transformation (30). Plasmid preparations for the purpose of screening *E. coli* transformants followed the method of Zhou *et al*. (50). Large scale plasmid preparations were performed with the QIAprep Spin kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). PCRs were carried out according to manufacturer's recommendations using a *Taq* DNA polymerase PCR system (Roche Applied Science, Indianapolis, IN). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA) and, when appropriate, restriction sites were designed into the 5' end of the primers to facilitate future cloning steps. DNA fragments were extracted from 1.0 % agarose gels using the Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA). Electrocompetent *Lactobacillus* cells were prepared as described by Walker et al. (41). Southern hybridization of genomic DNA was carried out using standard protocols.

Tissue culture. The Caco-2 (ATCC HTB-37, Rockville, MD) cells were only used between the 40^{th} and 50^{th} passage. All reagents used in maintenance of Caco-2 cells were obtained from Gibco (Invitrogen Corp., Carlsbad, CA). The cells were routinely grown in 95% air-5% CO_2 atmosphere in Minimum Essential Medium (MEM) supplemented with 20% (v/v) inactivated (56°C, 30 min) fetal bovine serum (FBS), 0.10 mM non-essential amino acids and 1.0 mM sodium pyruvate. Monolayers were trypsinized for 10 min, counted using a hemocytometer, and seeded at $1.3x10^5$ cells/well in 2.0 ml of cell culture medium. Cells were grown on 15 mm Thermanox plastic coverslips (Nalge Nunc International, Rochester, NY) in Costar 12-well tissue culture

treated plates (Corning Inc., Acton, MA). The medium was replaced every two days and all adherence assays were performed after 14 days of incubation.

Adherence Assay. Adhesion of *Lactobacillus* strains to Caco-2 cells was examined according to the method described previously (9). Briefly, mid-log phase bacterial cells (OD_{600} 0.6) were prepared in 10 ml MRS with 3.0 μg/ml Em to maintain selective pressure on integrants. Cells were removed by centrifugation for 10 min at 4000 x g, and washed twice with phosphate-buffered saline (PBS). Bacterial pellets were resuspended in 5 ml of fresh MRS prior to adherence. For adhesion adaptive conditions, cell pellets from mid-log phase cultures were resuspended in 1 ml of fresh MRS at ~1.0 x 10^9 cfu/ml and incubated for 1 hr at 37°C. Cells were centrifuged again and resuspended in 5 ml fresh MRS prior to addition to the monolayers. Fifteen-day Caco-2 monolayers were washed twice with PBS and treated with a bacterial suspension at a concentration of 4 x 10^8 CFU/ml. Bacteria were incubated on the monolayer for 1.5 hr at 37°C in a mixture (1:2 v/v) of MRS and cell-line culture medium. Following incubation, the monolayers were washed five times with PBS, fixed in methanol and Gram stained. Adherent bacterial cells were then enumerated microscopically. Duplicate coverslips were counted for each experiment. The final data presented collectively represents at least 3 independent experiments in duplicate. Total counts for each coverslip were used and adhesion was expressed as percent (%) of the control strain NCK1398 (29) which carries an insert in the *lacL* (β-galactosidase) gene. Using this control, all mutant cultures and the parental control could be prepared with Em to maintain selective pressure on the $LuxS^-$ integrant.

RNA Isolation. A single 20 mL culture of *L. acidophilus* was grown in MRS to OD_{600} of 0.6, divided into two 10 ml aliquots, and harvested by centrifugation for 8 min at 3,150 x g. One aliquot was resuspended in 10 ml of fresh MRS, the other resuspended in 1 ml of fresh MRS. Both cultures were then incubated for 1 hr at 37°C. Following incubation, cells were harvested by centrifugation and frozen immediately in a dry ice-ethanol bath. RNA isolation was conducted using TRIzol (Life Technologies, Rockville, MD) according to the protocol described previously (3). RNA purity and concentration were determined by electrophoresis on agarose gels and standard spectrophotometer measurements.

Microarray hybridization and data analysis. Gene expression analysis was performed using a whole-genome DNA microarray based on PCR products of predicted ORFs from the *L. acidophilus* NCFM genome (3). Briefly, identical amounts (25 µg) of total RNA were aminoallyl-labeled by reverse transcription with random hexamers in the presence of amino-allyl dUTP (Sigma Chemical Co.), using Superscript II reverse transcriptase (Life Technologies) at 42°C overnight, followed by fluorescence-labeling of aminoallylated cDNA with *N*-hydroxysuccinimide-activated Cy3 or Cy5 esters (Amersham Pharmacia Biotech). Labeled cDNA probes were purified using the PCR Purification Kit (Qiagen). Coupling of the Cy3 and Cy5 dyes to the AA-dUTP labeled cDNA and hybridization of samples to microarrays were performed according to the protocols by TIGR (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). Fluorescence intensities were acquired using a General Scanning ScanArray 4000

Microarray Scanner (Packard Biochip BioScience, Biochip Technologies LLC, Mas.) and processed as TIFF images. Signal intensities were quantified using the QuantArray 3.0 software package (Packard BioScience). Two slides, each containing the complete genome of *L. acidophilus* NCFM spotted in triplicate, were hybridized reciprocally to Cy3- and Cy5-labeled probes per experiment (dye swap) for each of two biological replicates. Spots were analyzed by adaptive quantitation. The local background was subsequently subtracted from the recorded spot intensities. Data was median normalized. The median of the six ratios per gene was recorded. The ratio between the average absolute pixel values for the replicated spots of each gene with and without treatment represented the fold change in gene expression. Confidence intervals and *P* values on the fold change were also calculated with the use of a two-sample *t* test. *P* values of 0.05 or less were considered significant (23).

Site-specific integration into *L. acidophilus* NCFM. Using *L. acidophilus* NCFM chromosomal DNA as a template, a 471 bp internal fragment of LBA1081 was amplified using PCR with primers 1081-IF (5'-GATCA GATCT AAGTT AAGGC ACCTT ACG-3') and 1081-IR (5'-GATCT CTAGA TTTCG AATGG GTCAT CAC-3'). The internal fragment was cloned onto the integrative vector pORI28 (25) and subsequently transformed by electroporation into *L. acidophilus* NCFM containing the temperature sensitive helper plasmid pTRK669 (29). Steps were then carried out according to Russell and Klaenhammer (29) for selection of integrants. Successful integration of the plasmid was confirmed by PCR and Southern hybridization analysis of junction fragments using standard protocols.

AI-2 Detection. The detection of autoinducer-2 from the supernatant of selected bacterial strains was performed as described previously (15) with modifications as follows. The detection of AI-2 produced by lactic acid bacteria can be problematic due to the decreased pH of the spent culture supernatants, and catabolite repression by glucose of the *lux* operon in the reporter strain *V. harveyi* BB170 (15). Accordingly, all *Lactobacillus* populations used for detection of autoinducer-2 were grown in modified MRS containing 1% galactose rather than glucose. At specified time points, 4 ml aliquots were collected, OD_{600} measured, and cell-free supernatants isolated by centrifugation at 4,000 x g for 10 min. The pH of the supernatant was neutralized to pH 6.5 with 2N NaOH and filtered through a 0.2 μm membrane. Prepared supernatants were stored at 4°C until the end of the time course experiments and all samples from each culture were assayed together in separate 96-well microtiter plates. The reporter strain *V. harveyi* BB170 (3) was grown overnight in autoinducer bioassay (AB) media, washed with and resuspended in fresh AB to OD_{600} 0.5. Ten μl of sterile supernatant was mixed with 90 μl of a 1/1000 dilution of the reporter strain BB170 in each well of a 96-well plate. Luminescence was measured at 30°C every 10 min for 6 h in a fluorescent microtiter plater reader (FLOUStar Optima, BMG Technologies, Durham, NC). Fold induction was calculated by averaging at least 6 standard time points and dividing the average value obtained from the wild type by the average value obtained from the $LuxS^-$ mutant. Each sample was measured in at least 3 independent wells and each data point represents 3 independent samples.

3.4 Results

3.4.1 Adhesion Adaptive Response. Standard adhesion protocols generally harvest bacterial populations at a predetermined point in the growth phase and immediately process the cells for adhesion. Previously, the wild type and mutants strains of *L. acidophilus* cells were selected at mid-log phase for evaluation of adherence to Caco-2 cells (9). In this study, we compared the adherence of mid-log phase cells against cells that were further treated by concentration to 1 x 10^9 CFU/ml and incubated for 1 hr at 37°C, just prior to addition to Caco-2 cells. The concentrated and incubated cells exhibited a significant increase in adhesion (Figure 1). The level of adherence for the concentrated and incubated cells was at least 20 fold greater than the non-concentrated and incubated control cells (Figure 2), even though the same number of bacterial cells was added initially to each well. In each experiment, cells were resuspended in 5 ml of fresh MRS and enumerated to determine the absolute concentration of cells added to the Caco-2 monolayer. Only adherence data resulting from the addition of 3.8 x 10^8 – 4.2 x 10^8 CFU/ml was reported. Similar increases in bacterial adherence were not obtained by either 10 X concentration without incubation, or incubation (37°C, 1 hr) without concentration of the mid-log cells prior to adherence, or by incubation (37°C, 1 hr) without concentration in MRS adjusted to pH 4.5 with lactic acid (data not shown). After the one hour incubation, the pH of the unconcentrated cells was 5.5, whereas the concentrated cells lowered the pH to 4.5. Therefore, a combination of cell concentration and decreased pH resulted in an adaptation of the *L. acidophilus* cells to a state

significantly more amenable to adherence. We designated this phenomenon an Adhesion Adaptive Response (AAR).

3.4.2 Microarray Analysis of AAR. Utilizing an existing microarray platform (3), a whole genome transcriptional analysis was performed on a mid-log phase cell population of *L. acidophilus* either exposed to AAR conditions (test) or cells that were not concentrated but still incubated for 1 h at 37°C (control). ORF's showing significant differences in expression, as determined by the Student's t-test ($P < 0.02$), are listed in table 2. Notably, multiple stress response genes were over expressed, including *radA*, *groES*, *groEL*, *clp*-type proteases, *bshA*, and *dnaK*. A putative amino acid ABC transporter operon (LBA1042 – LBA1046) correlated with acid tolerance and amino acid transport was differentially expressed (2.67 – 4.87 fold higher) following exposure to AAR conditions. ORFs shown in bold in table 2 exhibit a similar expression pattern to a previous microarray analysis of *L. acidophilus* NCFM exposed to MRS acidified with lactic acid to pH 4.5 (3). These include many of the putative stress response genes, along with a putative aggregation promoting protein, myosin cross-reactive antigen, and multiple genes encoding membrane associated proteins. Interestingly, AAR conditions significantly upregulated expression of a region encoding genes related to lactose metabolism (LBA1457-LBA1469), within which was embedded a predicted surface protein (LBA1460). The operon predicted to encode an amino acid ABC transporter (LBA1042 – LBA1046) was not significantly differentially expressed at pH 4.5 with the exception of LBA1045, a putative ATP-binding protein, which was repressed (mean ratio 0.28). In contrast, this ABC transporter operon was significantly differentially expressed

under AAR conditions. Notably, one mucus binding protein (LBA1460) and the predicted bacteriocin helveticin J (LBA1566) were induced at 2.5 fold. Moreover, two tandem ORFs (LBA1080 and LBA1081) putatively encoding proteins involved in the synthesis pathway for autoinducer-2 (AI-2) were induced 1.8 – 2.1 fold in response to AAR conditions.

3.4.3 AI-2 production. Further analysis of the genome of *L. acidophilus* NCFM revealed four ORFs (LBA1622, LBA0931, LBA0820, LBA1081) showing homology to each gene in the biosynthetic pathway for the production of AI-2 from methionine (Fig. 3). MetK converts methionine to S-adenosylmethionine (SAM) and is putatively coded by LBA1622. A methyl group is removed from SAM by an SAM-dependent methyltransferase (encoded by LBA931) forming S-adenosylhomocysteine (SAH) which is subsequently detoxified by an MTA/SAH nucleosidase, Pfs (encoded by LBA820), forming S-ribosylhomocysteine (SRH) and adenine. LuxS (encoded by LBA1081) converts SRH to homocysteine and 4,5-dihydroxy-2,3-pentanedione which circularizes, incorporating boron, to form AI-2 (45). Homocysteine is finally methylated back to methionine by MetE (encoded by LBA1080) located directly upstream of the *luxS* homolog, LBA1081. A terminator between the two ORFs with a free energy of -16.2 kcal suggests that LBA1080 and LBA1081 are expressed separately. Table 3 lists other lactobacilli, for which genomic sequence data is available, with the complete pathway for the production of AI-2 from methionine. Interestingly, based on predicted protein sequence, ORFs from the *Lactobacillus* genomes (2, 22, 28) annotated as *metE* (methionine synthase or cobalamin independent homocysteine methyltransferase)

grouped away from other available MetE proteins by neighbor-joining bootstrap analysis (Fig. 4).

In order to determine when AI-2 was produced during the growth phase of *L. acidophilus* NCFM, supernatants were harvested at 0, 3, 6, 9, 12, 16, 20, 24, 36, and 48 hrs from triplicate cultures. AI-2 production during the growth of *L. acidophilus* is shown in figure 5. Coinciding with a rapid drop in pH, a noticeable increase in AI-2 production occurs throughout the logarithmic phase of growth. Upon entrance into stationary phase the AI-2 activity in the supernatant maintains a constant level up to 48 hrs.

3.4.4 Inactivation of *luxS* and adherence assays. To investigate the potential effect of AI-2 on the adherence phenotype of *L. acidophilus*, an isogenic chromosomal mutation was introduced into the *luxS* gene, LBA1081. For insertional inactivation, a 471 bp internal fragment of LBA1081 was PCR amplified and cloned into pORI28. The resulting plasmid, pTRK854, was then transformed by electroporation into *L. acidophilus* NCFM harboring the temperature sensitive helper plasmid pTRK669. Successful integrants of pTRK854 into LBA1081 were selected according to the protocol described by Russell and Klaenhammer (29) to generate NCK1765. The integration event and location was subsequently confirmed by PCR amplification and Southern hybridization of junction fragments (data not shown). No AI-2 activity was detected from the supernatant of NCK1765, at any time point tested over 48 hours of growth.

The LuxS$^-$ mutant strain, NCK 1765, was tested for the ability to adhere to Caco-2 cells using bacterial cells from mid-log phase of growth and cells exposed to AAR

conditions. When the mid-log phase cells were added directly to the Caco-2 cells from mid-log phase, a 58% decrease in adhesion (Student's t-test, $P < 0.001$) was observed for the LuxS$^-$ mutant strain (Fig. 6) compared to the control. A derivative of *L. acidophilus* NCFM (NCK1398) harboring an integration in a β–galactosidase was used as the control so that antibiotic pressure could be maintained on the mutant and control. No difference in adherence was observed between NCK1398 and the wild type *L. acidophilus* NCFM (data not shown). Therefore, elimination of AI-2 production by the inactivation of *luxS* significantly lowered the adherence ability of *L. acidophilus* NCFM. Following exposure to AAR conditions, the LuxS$^-$ mutant showed a dramatic increase in adhesion that was, unexpectedly, comparable to the LuxS$^+$ control. Similar to the FbpA, SlpA, and Mub mutants (Fig. 2), the LuxS$^-$ mutant exhibited a dramatic increase in adherence capability following treatment with the AAR conditions (data not shown).

3.5 Discussion

The ability of lactobacilli to associate with the intestinal epithelium and mucosal layers is an important property for bacteria that are part of the microbiota of the GI tract, or in some cases, when used for potential probiotic properties. This study reports a significant increase in adhesion when *Lactobacillus acidophilus* NCFM cells were concentrated for 1 hr prior to adherence measurements. This adhesion adaptive response (AAR) overcame the effects of three separate isogenic mutations to surface proteins, shown to negatively impact adhesion to Caco-2 cells, in vitro (9). The reported AAR resulted from incubation of 10X concentrated cells for 1 hr prior to adhesion to Caco-2

cells. A whole genome microarray analysis of cultures exposed to AAR conditions suggested the potential involvement of AI-2 via the LuxS mediated quorum sensing system. All 4 genes responsible for the biosynthesis of AI-2 were identified in the genome of *L. acidophilus*. While the production of AI-2 was important to the adherence ability of *L. acidophilus*, it does not appear to be the signaling pathway responsible for expression of other factors that promote the AAR.

Probiotic bacteria must survive multiple stressors following ingestion in order to maintain transient or extended association with the epithelial layers in the gastrointestinal tract. It is possible that these stressors prepare the bacteria for adhesion to the intestinal epithelial layers by promoting the expression of adhesion factors. The harvested bacterial cells were originally suspended in fresh MRS (pH 6.25), but the pH following exposure to AAR conditions dropped to pH 4.5 in the concentrated sample, and only pH 5.5 in the non-concentrated sample. A two-component regulatory system involved with acid tolerance was previously inactivated in *L. acidophilus* NCFM and the resulting mutant demonstrated increased expression of both *metE* (LBA1080) and *luxS* (LBA1081) in acidified media compared to the wild type (3). These results suggest that regulation of acid tolerance might participate in the control of *luxS* expression. Previous research reported an increase in the adhesion of *Clostridium difficile* when pre-exposed to pH 4 (40). Both low-pH stress and bile-salt stress have been shown to increase enteropathogenic *E. coli* adhesion to HEp-2 cells (14). An increase in adhesion was also observed for various lactobacilli at pH 3 and pH 4 (18), however *Lactobacillus fermentum* 104 was shown not to have an adhesion increase in pH 2 buffer when compared to the same buffer at pH 7 (20). Greene and Klaenhammer (18) observed that

the increased adhesion at a lower pH could not completely be attributed to the pH differences. They reported that high adhesion of *Lactobacillus crispatus* BG2FO4 (formerly classified as *L. acidophilus* BG2FO4) occurred in spent culture supernatant at pH 4.2, but when fresh MRS was acidified to pH 4.2 the same level of adhesion could not be reached, suggesting a factor in the spent culture supernatant increased adhesion in addition to pH (18). Other studies have implicated the involvement of a factor secreted in stationary phase with adhesion to epithelial cells, though this factor has not been identified (7, 11, 17).

Expression results from a whole genome microarray were used to identify genes that may participate in the adhesion adaptive response. Gene expression from log-phase cells exposed to AAR conditions was compared to that of log-phase cells incubated for 1 hr at 37°C. Peptide methionine sulfoxide reductases (Msr) have been previously implicated in epithelial cell adhesion in both lactobacilli (43) and pathogens (46). During adhesion adaptive conditions, a putative Msr (LBA1208) was over expressed providing further evidence that associates methionine metabolism with adhesion. The increased expression of the heat shock related groELS operon agrees with previous results in *C. difficile* suggesting the involvement of heat shock proteins with adhesion (19). Multiple putative *clp* family proteases (LBA638, LBA694, LBA1910) were over expressed under adhesion adaptive conditions with the exception of the regulatory ATPase component, *clpX* (LBA847), which was repressed. Zhang et al. (49) reported that *clpP* (proteolytic component) is not only upregulated in response to epithelial cell exposure, but is also required for optimal invasive activity of the oral pathogen *Porphyromonas gingivalis*. These results, along with the over expression of putative bile salt hydrolases (LBA892

and LBA1078), suggest that *L. acidophilus* NCFM responds to AAR conditions similarly to that which might also be expected from the general stressors of the gastrointestinal environment. Due to the relative abundance of over expressed genes related to stress responses identified by microarray analysis, it is possible that the increase in adhesion following adhesion adaptive conditions is a non-specific stress response. No specific component of the adhesion adaptive conditions tested alone was able to induce the adhesion adaptive response including incubation in MRS acidified to pH 4.5 with lactic acid, non-concentrated incubation, or concentration without incubation prior to adherence. However, two ORFs (LBA1080, LBA1081) putatively associated with the AI-2 production cycle were over expressed under AAR conditions, but not in response to pH 4.5. The predicted product of LBA1080 shows amino acid similarity to various vitamin B-12 independent methionine synthases (MetE). MetE recycles methionine by converting homocysteine also produced by LuxS (LBA1081), and other predicted MetE proteins from certain lactobacilli group away from the main family of MetE proteins (Fig 4), possibly suggesting a functional divergence.

LBA1081 putatively encodes a protein homologous to the AI-2 synthase, LuxS. LuxS is found in a wide variety of bacterial species and has been functionally linked to the expression of virulence factors in *E. coli* (32, 34), *Vibrio cholerae* (27), and *Streptococcus mutans* (26, 44), among others. The production of LuxS is required for synthesis of AI-2 from S-ribosylhomocysteine, and insertional inactivation of LBA1081 resulted in a loss of AI-2 production by *L. acidophilus* NCFM as determined by the *V. harveyi* BB170 reporter assay (15, 35). The complete pathway for the production of AI-2 was identified in other lactobacilli including *L. gasseri*, *L. johnsonii*, and *L. plantarum*, *L.*

casei, and *L. delbrueckii* subsp. *bulgaricus* (Table 3), but no phenotype has been attributed to the production of AI-2 in these organisms. Examination of gene synteny revealed that an ORF putatively coding for MetE is found adjacent upstream of *luxS* in *L. acidophilus*, *L. johnsonii*, *L. gasseri*, and *L. casei*, but not in *L. delbrueckii* subsp. *bulgaricus* or *L. plantarum*. Biofilm thickness was increased in a strain of *L. reuteri* containing a mutation in *luxS* compared to the wild type strain (37). Although *L. reuteri* was reported to have over 2000 fold AI-2 activity, it should be noted that catabolite repression by glucose of the control strain grown in MRS could have resulted in overestimation of the actual AI-2 activity. As reported previously (15), when AI-2 is measured from the supernatant using *V. harveyi* as the reporter, modified MRS, not containing glucose, should be used for the propagation of *Lactobacillus* strains. Interestingly, in *Salmonella*, the production of AI-2 seems to be transcriptionally regulated at the SAH-nucleosidase and not at LuxS (6). Beeston and Surette (6) also suggest that AI-2 production is not linked to LuxS availability. The over expression of LuxS during AAR conditions, therefore, may not necessarily correlate to an increase in AI-2 production. However, various studies have reported an altered stress response of LuxS mutant strains during mid-log phase (44, 48). When mid-log phase cultures of *L. acidophilus* NCK1765 ($LuxS^-$) were analyzed, without exposure to AAR conditions a 58 % decrease in adhesion was observed. During mid-log phase, the pH of the growth media rapidly decreased and AI-2 was also rapidly produced. It is possible that AI-2 influences the expression of adhesion factors during normal growth, but accumulation of the signal is not necessarily required for a response related to adhesion. Therefore, the

increased expression of both LBA1081 and LBA1080 might be part of a metabolic response by the cell, recycling important metabolites, to the stress of AAR conditions.

A significant increase in the adhesion of *L. acidophilus* to Caco-2 cells was observed following AAR conditions. AI-2 was detected from the supernatant of *L. acidophilus* and implicated in promoting adherence of log-phase bacterial cells to Caco-2 cells in vitro. However, the inactivation of *luxS* did not alter the adhesion adaptive response. At this point, it is not apparent what gene system or systems are being orchestrated to promote the elevated adherence ability of *L. acidophilus* NCFM. Although only limited research has been reported, AI-2 mediated signaling likely represents an important means of interspecies communication within the intestinal environment and should be investigated further in fermenting lactic acid bacteria, and commensal and probiotic intestinal bacteria.

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TABLE 1. Bacterial strains and plasmids used in this study

Strains	Origin or relevant characteristics	Source or reference
L. acidophilus		
NCFM	Human intestinal isolate	(31)
NCK 1377	NCFM::pTRK826 (*slpA* integrant)	(1)
NCK 1392	NCFM containing pTRK669	(29)
NCK 1398	NCFM::pTRK685 (*lacL* integrant)	(29)
NCK 1661	NCFM::pTRK833 (*fbpA* integrant)	(9)
NCK 1765	NCFM::pTRK854 (*luxS* integrant)	This study
V. harveyi		
BB170	*luxN*::Tn5 AI-1 sensor$^-$ AI-2 sensor$^+$	(35)
E.coli		
EC1000	RepA$^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA*; host for pOR128-based plasmids	(25)
Plasmids		
pORI28	Em^r, ori (pWV01), replicates only with *repA* provided in *trans*	(25)
pTRK669	ori (pWV01), Cm^r, RepA$^+$	(29)
pTRK854	471 bp internal region of *luxS* (LBA1081) cloned into BglII-XbaI sites of pORI28	This study

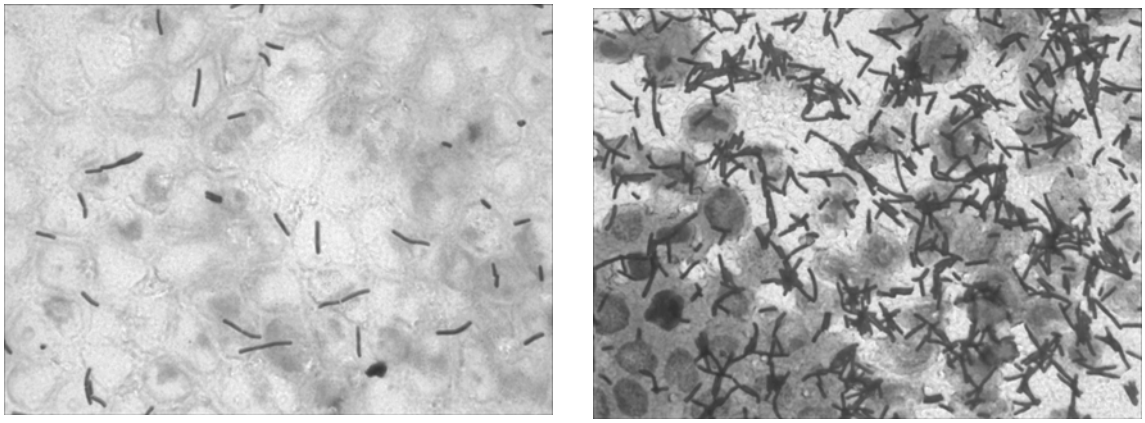
TABLE 2. Selected differentially expressed ORFs under adhesion adaptive conditions[a]

ORF#	Gene Annotation	Ratio[1]	ORF#	Gene Annotation	Ratio[1]
41	ribonucleoside triphosphate reductase	2.767	1046	ABC transporter substrate-binding protein	4.241
55	**D-lactate dehydrogenase**	**3.225**	1078	bile salt hydrolase	2.538
132	**Putative transcr. reg. tetR family**	**0.157**	1080	MetE	2.125
144	n-acetylglucosamine-6-P deacetylase	2.385	1081	autoinducer-2 production protein LuxS	1.852
154	phosphonate ABC trans. (permease)	0.489	1115	amino acid permease	4.056
160	anaer. NTP reductase	2.383	1119	Putative inner membrane prot.	0.444
161	anaer. NTP reductase or activator	2.507	**1122**	**DNA topoisomerase IV subunitB**	**0.328**
165	neutral endopeptidase	2.348	**1140**	**lysin**	**0.221**
166	**(K+) uptake protein**	**0.327**	**1177**	**iron-sulfur cofactor synthesis protein YrvO**	**0.430**
199	inosine-monophosph. dehydrogen.	3.000	**1178**	**[nucleolar protein]**	**0.393**
204	aminopeptidase e	2.881	**1197**	**DNA primase**	**0.353**
205	**heat shock low mol. weight**	**2.496**	1208	peptide methionine sulfoxide reductase msrA	2.357
234	**carboxyvinyltransferase**	**0.487**	**1243**	**DNA-specific exonuclease RecJ**	**0.457**
262	aa transporter	0.459	1247	heat shock protein DnaK	3.532
266	**d-ala-d-ala adding enzyme**	**0.321**	1248	cochaperonin GrpE, Hsp70 cofactor	3.266
267	**ATP-dep. RNA helicase ydbR**	**0.225**	1249	Heat inducible transcription repressor HrcA	3.831
281	**lys-trna synthetase lysrs**	**0.406**	**1269**	**translation elongation factor Ts**	**0.430**
346	**dna repair RadA**	**0.500**	**1270**	**30s ribosomal proteinS2**	**0.446**
358	transcr. antiterm. nusG	0.485	1300	oligopeptide ABC substrate binding prot oppA	3.007
359	50S rib. prot. L11	0.397	1341	branched-chain amino acid aminotrans. ILVE	3.354
395	Putative dehydratase	5.362	**1376**	**transmembrane prot.**	**0.192**
396	oxalyl-coa decarboxylase	5.525	**1401**	**Nadh Peroxidase (Npx)**	**6.032**
405	**cochaperonin GroES**	**8.045**	**1433**	**dihydroxyacetone kinase**	**2.208**
406	**chaperonin GroEL**	**9.273**	**1457**	**galactose-1-epimerase (mutarotase)**	**3.434**
493	**aggregation promoting protein**	**0.295**	**1458**	**galactose-1-phosphate uridylyltransferase**	**3.315**
523	P-type ATPase	2.750	**1460**	**Mucus binding protein precursor**	**2.470**
555	**myosin-crossreactive antigen**	**6.833**	**1462**	**beta-galactosidase**	**3.765**
617	**ATP-dep. RNA helicase**	**0.498**	**1467**	**beta-galactosidase large subunit (lactase)**	**3.868**
638	ATP-dep.t Clp protease, ClpE	6.577	**1469**	**udp-glucose 4-epimerase**	**3.024**
652	>>H+/K+ ATPase to 657<<	3.583	1551	phosphoribosylamine-glycine ligase PUR2	0.414
655	Phosphotrans. system II pthA	2.187	**1552**	**Phosphoribosylaminoimidazole. PUR9**	**0.207**
672	Phos. starvation induced prot YvyD	2.345	**1553**	**phosphoribosyl glycinamide PUR3**	**0.259**
680	glucan branching enzyme glgB	3.643	**1554**	**Phosphoribosyl. cyclo-ligase PUR5**	**0.219**
682	adenylyltransferase glgD	3.489	**1555**	**Phosphoribosylpyroph. amidotrans. PUR1**	**0.203**
694	ATP-dependent Clp protease P	2.945	**1556**	**Phosphoribosylformylglyc. synthase PURL**	**0.187**
789	**aminotranfserase - NifS family**	**0.480**	**1557**	**Phosphoribosylformylglyc. synthase PURQ**	**0.387**
791	**16s pseudouridylate synthase**	**0.448**	**1559**	**Phosphoribosylaminoimid. synthase PUR7**	**0.243**
822	**tRNA-methyltransferase**	**0.453**	1564	Putative membrane prot.	0.318
847	**clpX (stress related protease)**	**0.465**	1566	bacteriocin helveticin J	2.762
851	diaminopimelate decarboxylase	3.351	1595	glycerol uptake facilitator protein glpF	0.132
852	tetrahydrodipicolinate succinylase	3.348	**1632**	**ssdh**	**5.685**
853	amino acid amidohydrolase	2.961	1665	oligopeptide ABC trans. substrate binding	2.936
854	dihydrodipicolinate synthase	3.637	**1699**	**exodeoxyribonuclease**	**7.479**
855	dihydrodipicolinate reductase	2.390	**1743**	**Cell wall-associated hydrolase**	**0.300**
877	**PTS system IIa**	**2.562**	**1744**	**Putative glycosidase**	**0.232**
882	transc. regulator (GntR family)	2.163	**1768**	**lctP lactate premease**	**0.288**
892	bile salt hydrolase	1.815	1812	alpha-glucosidase II	2.952
911	**aminopeptidase**	**3.671**	**1821**	**ABC transporter, ATPase component**	**0.362**
913	peroxidase	1.988	**1822**	**ABC transporter, ATPase component**	**0.399**
925	**transcr. regulator**	**2.036**	**1848**	**di-/tripeptide transporter**	**0.272**
927	Putative membrane prot.	0.363	**1869**	**beta-phosphoglucomutase**	**6.740**
953	**nucleotide phosphodiesterase**	**0.430**	**1870**	**maltose phosphorylase**	**10.17**
986	**Galactose mutarotase related**	**3.146**	1879	phosphomethylpyrimidine kinase	2.192
994	**aminoacyl-histidine dipeptidase**	**0.326**	1883	probable NLP/P60 family secreted protein	0.388
995	**amino acid permease**	**0.257**	**1893**	**GMP reductase**	**0.478**
999	**Na+-transporting ATP synthase**	**2.293**	**1910**	**ATP-dependent protease ClpE**	**2.571**
1027	oxidoreductase	2.381	**1943**	**Putative lipoprotein A/antigen precursor**	**0.444**
1042	ABC transporter permease	3.010	1945	sugar ABC transporter permease	0.426
1044	abc transporter	2.670	**1974**	**pyruvate oxidase**	**4.304**
1045	ABC transporter ATP-binding	4.866	**1999**	**glycyl-tRNA synthetase alpha chain**	**0.444**

[a]ORFs shown in bold in table 2 exhibit a similar expression pattern to a previous microarray analysis of *L. acidophilus* NCFM exposed to MRS acidified to pH 4.5 (3).

TABLE 3. Complete pathway for production of AI-2 from methionine

Organism	***metK***	**Methyltransferase**	**MTA nucleosidase**	***luxS***
L. acidophilus NCFM	1	2	1	2
L. gasseri	1	5	1	1
L. johnsonii NCC533	1	6	1	1
L. plantarum WCFS1	1	2	1	1
L. casei ATCC334	1	7	1	1
L. delbrueckii subsp. *bulgaricus* ATCC BAA-365	1	3	1	1



(a) (b)

Fig. 1. Adhesion of *L. acidophilus* NCFM to Caco-2 cells in vitro observed microscopically. Bacterial cells were either (a) incubated for one hour at 37 °C in non-concentrated condition (1 x 10^8 CFU/ml) or (b) pelleted and incubated in a 10 X concentrated condition (1 x 10^9 CFU/ml) (AAR conditions) for 1 hour at 37°C. Cells from both conditions were pelleted and then resuspended in 5ml and enumerated to 4 x 10^8 CFU/ml, prior to the adhesion assay.

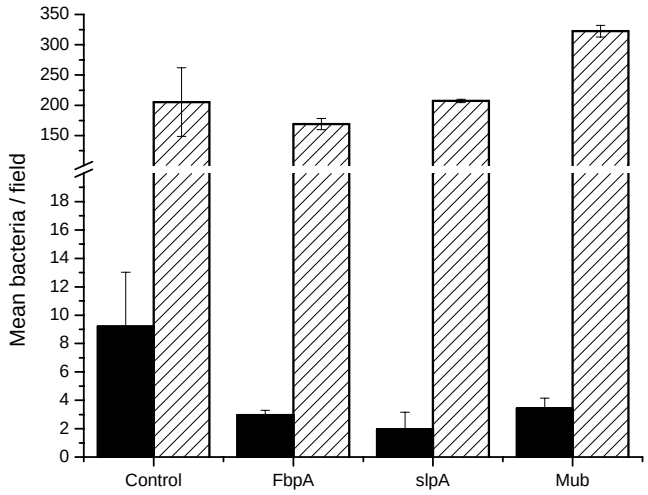


Fig. 2. Adhesion properties of mutant strains and NCFM::*lacL* control strain to Caco-2 cells in standard incubation conditions (solid bars) and adhesion adaptive conditions (striped bars). Counts are expressed as mean bacteria adhering per microscopic field and error bars represent one standard deviation from the mean.

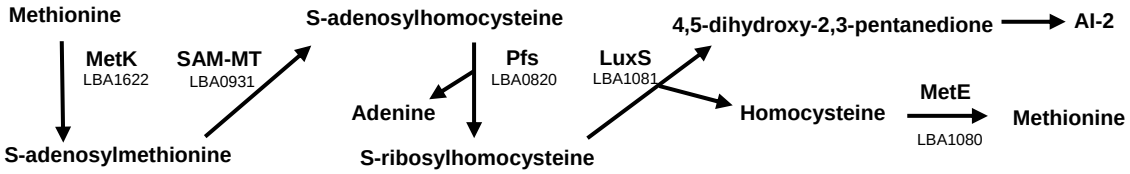


Fig. 3. Pathway for the production of AI-2 from methionine in *L. acidophilus* NCFM. Essential intermediates for the production of AI-2 are represented in bold, and ORFs encoding each enzyme are listed under the enzyme name. The SAM-dependent methyltransferase is abbreviated SAM-MT. The final step involves the nonenzymatic circularization of 4,5-dihydroxy-2,3-pentanedione into AI-2.

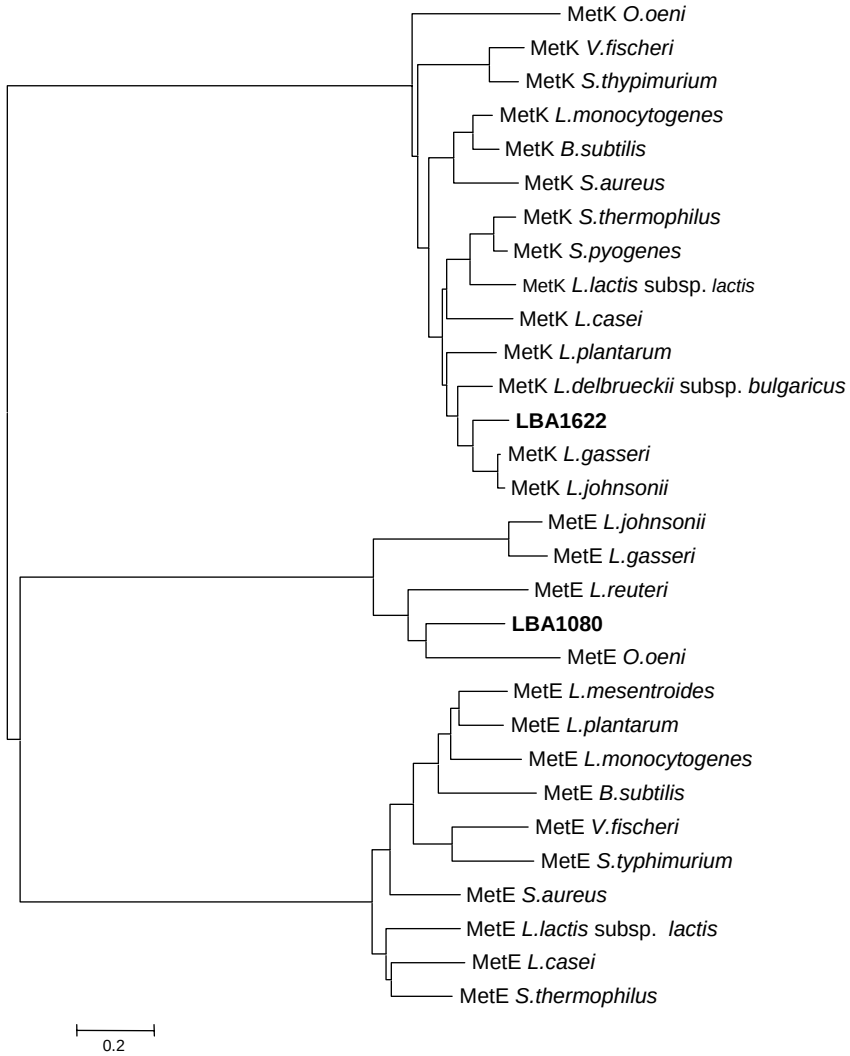


Fig. 4. Phylogenetic tree of MetK and MetE protein sequences from selected bacterial species. Protein sequence alignment and tree construction was performed using ClustalX (38) and Mega2 (24). MetK and MetE sequences were obtained from GenBank and represent a cross-section of available sequences with focus on the lactic acid bacteria. ORFs from *L. acidophilus* NCFM showing similarity to selected sequences are shown in bold.

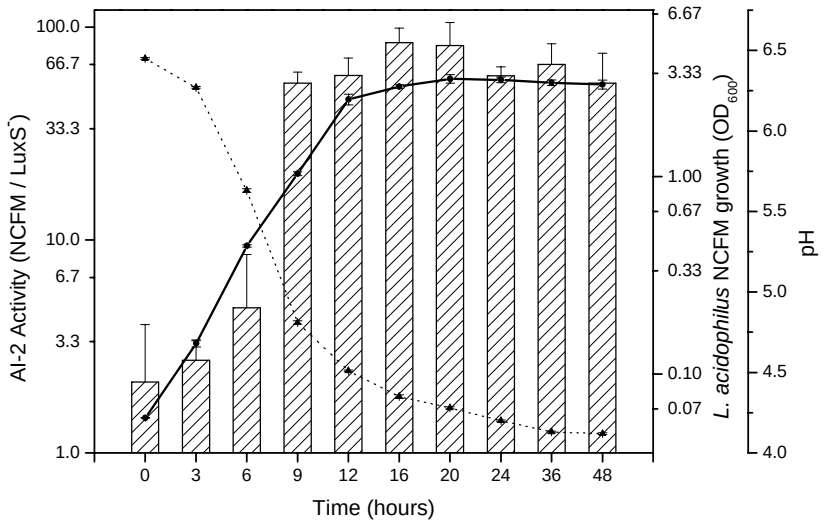


Fig. 5. AI-2 produced during the growth phase of *L. acidophilus* NCFM, as detected from sterile neutralized supernatant (striped bars) and expressed as mean luminescence of the parent strain / mean luminescence of the LuxS$^-$ mutant strain. Growth of *L. acidophilus* NCFM, represented by OD_{600} (–●–) and the drop in pH (-▲-), coincides with rapid AI-2 production during logarithmic phase of growth. Each value represents the mean of triplicate experiments and error bars are one standard deviation from the mean.

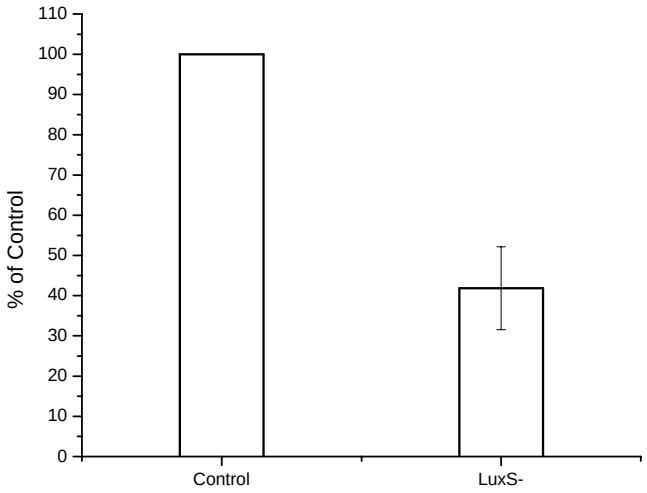


Fig. 6. Adhesion ability of a LuxS$^-$ mutant strain of *L. acidophilus* NCFM to Caco-2 cells compared to the NCFM::*lacL* control strain.

CHAPTER IV

Growth phase microarray analysis of genes influenced by LuxS in *Lactobacillus acidophilus* NCFM

4.1 Abstract

The bacterium *Lactobacillus acidophilus* NCFM employs a variety of mechanisms to survive passage and compete within the gastrointestinal tract. One important mechanism is likely cell-to-cell communication, mediated by autoinducer-2 (AI-2), to signal and adapt to changing environmental conditions. AI-2 is produced by *L. acidophilus* NCFM and accumulates throughout logarithmic growth phase through expression of the AI-2 synthase, LuxS. A whole genome microarray analysis of *L. acidophilus* NCFM and a LuxS$^-$ mutant strain was performed at early, middle, and late-logarithmic phase to investigate global gene expression influenced by AI-2 during growth. Hybridizations were carried out in a round-robin design and data were analyzed using a two-stage mixed model ANOVA. The highest number of genes was differentially expressed between the mutant and wild-type at early-log phase (OD_{600} 0.2), and included genes related to growth, metabolism, and stress response. Few genes showed differential expression at mid-log phase (OD_{600} 0.7), but included a transporter (LBA1796) and response regulator (LBA1798) of a bacteriocin production and transport operon. No differentially expressed genes were detected at late-log phase (OD_{600} 1.2) under the conditions tested. Of the differentially expressed genes, most were positively influenced by LuxS. Examination of the stress response of *L. acidophilus* NCFM revealed that upon exposure to heat and bile stress, the LuxS$^-$ mutant was more sensitive than the wild type when populations were harvested from early or middle-log phase. These results agreed with the overexpression of heat and bile-stress related genes in the wild type compared to the mutant strain during the early growth stages. AI-2 signaling during the early exponential phase appears to prepare the developing cell population for stress and interactive conditions naturally encountered during planktonic growth. This study reports the first microarray analysis of the impact of a *luxS* mutation throughout the bacterial growth phase.

4.2 Introduction

The human gastrointestinal tract (GIT) is the site of the largest and most complex environment in the mammalian host (15). Beginning in the oral cavity, and extending through the stomach, small intestine, and colon, the GIT is composed of multiple macro environments each with its own diverse microbial population. Given the immense number and diversity of bacteria that permanently or transiently inhabit the gastrointestinal environment, it is likely that the members of this population communicate to manage host-microbe relationships and ensure maintenance of the intestinal homeostasis. This interaction depends on the ability of microorganisms to sense the environment and adapt to changing conditions by regulating the expression of specific genes (29). Consequently, bacteria have developed mechanisms to gather, process, and transduce environmental information such as temperature, pH, nutrient availability, and cell density. One of these mechanisms centers on the production of autoinducer-2 (AI-2) by LuxS, and the subsequent response to exogenous AI-2. Although LuxS acts as an AI-2 synthase (27), it also functions as an integral component of the activated methyl cycle, recycling *S*-adenosylhomocysteine (SAH) to homocysteine (29). The activated methyl cycle is important in providing methyl groups for use in the methylation of DNA, RNA, proteins, and certain metabolites.

Phenotypes related to the synthesis of an active LuxS have been described in numerous bacteria associated with the gastrointestinal tract. LuxS$^-$ mutant strains of periodontal pathogens have demonstrated altered ability to form biofilms (19, 20, 33). Enteropathogenic *Escherichia coli* (EPEC) strains showed a decreased adhesion to epithelial cells when the *luxS* gene was inactivated (25). Similarly, the expression of a

type III secretion system participating with the virulence of enterohaemorragic *E. coli* was decreased in a LuxS$^-$ mutant. Microarray analysis of gene expression affected by LuxS and AI-2 has been performed on a number of different microbial species in order to determine the function of LuxS on cellular processes. The influence of AI-2 on *E. coli* K-12 grown in the presence and absence of glucose revealed altered gene expression of genes related to the *lsr* operon, methionine metabolism, and carbon utilization (12, 32). Another study linked LuxS production with genes involved in cellular growth and division using *E. coli* microarrays (9). Transcriptional microarray analysis of a LuxS$^-$ mutant of *Porphyromonas gingivalis* and its wild type implicated the participation of LuxS with the stress response of that organism (36). In addition to pathogenic bacterial strains, several non-pathogenic strains have demonstrated a phenotype associated with AI-2 production. For example, the ecological performance of *Lactobacillus reuteri* in the murine gastrointestinal tract was altered in a LuxS$^-$ mutant (28). In a previous study we observed that when the *luxS* gene of *Lactobacillus acidophilus* NCFM was inactivated, the resulting mutant demonstrated decreased adherence ability to Caco-2 epithelial cells (8).

L. acidophilus NCFM is a Gram-positive, homofermentive, non-sporulating bacilli originally isolated from human feces (12). Commercially available in fermented dairy products, *L. acidophilus* NCFM has been characterized in vitro, in animal studies, and human clinical trials for its probiotic functionality (24). Analysis of the sequenced genome of *L. acidophilus* NCFM revealed genes encoding the complete pathway for the production of AI-2 from methionine. Detection of AI-2 activity from culture supernatants was recently reported (8). A whole genome microarray platform was

developed for this organism and has been used for the analysis of acid resistance (3) and carbohydrate utilization (4).

This study utilized a whole genome microarray to identify gene expression influenced by LuxS in *L. acidophilus* NCFM. Multiple genes involved with the stress response, growth, and metabolism were differentially expressed in a LuxS$^-$ mutant strain. Phenotypic analysis of a LuxS$^-$ mutant strain examined the influence of a *luxS* mutation on the heat and bile tolerance of *L. acidophilus* NCFM.

4.3 Materials and Methods

Bacterial strains and growth conditions. *Lactobacillus* strains were cultivated anaerobically at 37°C or 42°C in MRS broth (Difco Laboratories Inc., Detroit MI) or, when appropriate, in MRS supplemented with 1.5% agar. For detection of AI-2, lactobacilli were grown in modified MRS, following the ingredients for MRS except substituting 1 % w/v galactose in place of glucose. *E. coli* was grown aerobically in Luria-Bertani (LB, Difco) medium or on LB medium supplemented with 1.5 % agar at 37°C. Brain Heart Infusion (BHI, Difco) medium supplemented with 1.5 % agar and 150 μg/ml erythromycin (Em) was used for selection of *E. coli* transformants. Autoinducer Bioassay (AB) media (5) was used for the propagation of all *Vibrio harveyi* strains. When appropriate, chloramphenicol (Cm, 5.0 μg/ml) and Em (5.0 μg/ml or 150 μg/ml) were used for selection. Colony forming units (CFU) per ml were determined with appropriate dilutions in 10 % MRS broth using a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, West Yorkshire, England).

DNA manipulation techniques. Total *Lactobacillus* genomic DNA was isolated according to the method of Walker and Klaenhammer (31). Standard protocols were used for endonuclease restriction, ligation, DNA modification and transformation (23). Plasmid preparations for the purpose of screening *E. coli* transformants followed the method of Zhou *et al*. (37). Large scale plasmid preparations were performed with the QIAprep Spin kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). PCRs were carried out according to manufacturer's recommendations using a *Taq* DNA polymerase PCR system (Roche Applied Science, Indianapolis, IN). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA) and, when appropriate, restriction sites were designed into the 5' end of the primers to facilitate future cloning steps. DNA fragments were extracted from 1.0 % agarose gels using the Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA). Electrocompetent *Lactobacillus* cells were prepared as described by Walker et al. (30). Southern hybridization of genomic DNA was carried out using standard protocols.

Marker free inactivation of *LuxS*. A LuxS$^-$ mutant strain of *L. acidophilus* NCFM was constructed by deletion of 97 bp within *luxS* (LBA1081) according to the protocols described previously (2, 6). Briefly, a 1,243 bp fragment containing luxS was amplified using primers LuxS-EF and LuxS-ER (Table 2). That fragment was subsequently ligated to pORI28, at the XbaI and BglII sites. Transformants were sleected in *E. coli* EC1000 and pORI::LuxSbig was isolated. Inverse PCR (Expand High Fidelity PCR system, Roche Applied Science, Indianapolis, IN) was performed on that plasmid

using primers LuxS-delF and LuxS-delR (Table 2) each containing EcoRI enzyme recognition sites at the 5' end. The resulting 2.7 kbp PCR product was cleaned using the Qiagen PCR purification kit and digested for 18 hrs at 37°C with EcoRI. Following gel purification with the Zymoclean Gel DNA Recovery Kit (Zymogen) the product was ligated to itself for 16 hrs at 16 °C. The resulting plasmid, pTRK884, was then transformed into *E. coli* EC1000, and plasmid construction confirmed with PCR amplification. *L. acidophilus* NCFM containing the temperature sensitive helper plasmid, pTRK669, was then electrotransformed with pTRK884 and integrants selected according to the method previously described by Russell and Klaenhammer (22). Selected integrants were passed six times in non-selective media, to allow the second cross-over event to occur, and then plated on MRS devoid of antibiotics. Colonies were screened for Em sensitivity and colony PCR conducted using primers LuxS-delNF and LuxS-delNR, flanking the deleted region. PCR amplification of the deletion region and Southern hybridization assays using an internal fragment of luxS as the probe confirmed the deletion via gene replacement in NCK1818 (data not shown).

AI-2 detection. Detection of autoinducer-2 from the supernatant of selected bacterial strains was performed as described previously (8). Briefly, the supernatants from bacterial populations harvested at each specified time point were adjusted to pH 6.5 with 2N NaOH and filtered through a 0.2 µm membrane. The reporter strain *Vibrio harveyi* BB170 (3) was grown overnight in autoinducer bioassay (AB) media, washed with and resuspended in fresh AB to OD_{600} 0.5. Ten µl of sterile supernatant from the test culture was mixed with 90 µl of a 1/1000 dilution of the reporter strain BB170 in

each well of a 96-well plate. Luminescence was measured at 30°C every 10 min for 6 h in a fluorescent microtiter plate reader (FLOUStar Optima, BMG Technologies, Durham, NC). Fold induction was calculated by averaging at least 6 standard time points from the *V. harveyi* luminescence curve and dividing the average value obtained from the wild type by the average value obtained from the LuxS$^-$ mutant. Time points were selected when luminescence was at a constant level before the reporter strain responded to the production of its own AI-2. Each sample was measured in at least 3 independent wells and each data point represents 2 independent experiments.

RNA Isolation. Both *L. acidophilus* NCFM and NCK1818 were transferred two times from frozen stock cultures in semi-defined media using galactose as the primary carbon source to prevent catabolite repression by glucose of the *lux* operon in the *V. harveyi* reporter strain. A 400 ml culture of both strains was inoculated at 1 % from a stationary phase (16 hr) culture and allowed to grow at 37°C. At OD_{600} 0.2, a 5 ml sample was obtained for CFU determination and pH measurement. Six 10 ml samples of each strain were then quick cooled on dry ice, and cell pellets harvested at 4°C by centrifugation for 10 min at 3,150 x g. The supernatant from each strain following centrifugation at every time point was retained for AI-2 detection. At the final two sampling points (OD_{600} 0.7 and OD_{600}1.2) 5 ml samples were collected from each culture for plating and pH determination; two 10 ml samples were then harvested from each culture and treated as described above. RNA isolation from cell pellets was conducted as described previously (3) using TRIzol (Invitrogen Life Technologies, Rockville, MD). RNA purity and concentration were determined by electrophoresis on 1.0 % agarose gels

and standard spectrophotometer measurements. RNA was isolated from two biological replicates of both strains.

cDNA Production and Microarray Hybridization. Gene expression analysis of the LuxS$^-$ mutant strain and the wild type strain at each of the three selected sampling points was performed using a whole-genome DNA microarray (3). PCR products of 1,889 predicted ORFs from the *L. acidophilus* NCFM genome (1) were spotted in triplicate on ULTRA GAPS glass slides (Corning Inc., Acton, MA). First strand cDNA synthesis and labeling were performed with the SuperScript Indirect cDNA Labeling System (Invitrogen). Briefly, equal amounts (20 μg) of RNA were aminoallyl-labeled by reverse transcription with random hexamers in the presence of amino-modified nucleotides, using SuperScript III reverse transcriptase at 42°C for 3 hrs. First-strand cDNA was purified with S.N.A.P columns, precipitated, labeled with *N*-hydroxysuccinimide-activated Cy3 or Cy5 esters (GE Healthcare Life Sciences, Piscataway, NJ), and purified again with S.N.A.P columns. The resulting labeled cDNA was hybridized onto microarray slides according to the protocols outlined by TIGR (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). Hybridizations were performed according to a single round-robin pattern, so that all possible direct pair-wise comparisons were conducted in an unbalanced design. A total of fifteen different hybridizations were performed at three different sampling points (OD_{600} 0.2, 0.7, and 1.2) and two different strains (*L. acidophilus* NCFM and NCK1818).

Microarray Data Analysis. Fluorescence intensities were acquired using a General Scanning ScanArray 4000 Microarray Scanner (Packard Biochip BioScience, Biochip Technologies LLC, Mass.) and processed as TIFF images. Signal intensities were quantified, including background subtraction, and spot replicates averaged using the QuantArray 3.0 software package (Packard BioScience). The resulting raw intensity data was imported into SAS (SAS Institute, Cary, NC), $\log_2$ transformed and fit to the normalization mixed model analysis of variance (ANOVA) in order to center the data to the mean intensity. A gene-specific mixed model ANOVA was then performed on the normalized data in which dye, strain, and time were considered fixed effects and the array effect was considered a random effect (34). The resulting difference between least-square estimates for two different treatments is analogous to $\log_2$-transformed ratio of gene expression between those two treatments. Differences were calculated for dye effect, strain effect, time effect, and the combined effect of strain and time, strain*time. A *t* test was performed using these differences and their standard errors, with $P < 0.05$ considered significant. Volcano plots were constructed for each comparison using the estimate and $-\text{Log}_{10}$(P value) with JMP 5.0 (SAS Institute) in order to visualize contrast between treatments and statistical significance of the results.

Stress experiments. Both *L. acidophilus* NCFM and NCK1818 were grown in MRS broth at 37°C until the population reached OD_{600} 0.2, 0.7 and 1.2, at which point cells were collected for stress tolerance experiments.

(i) Bile tolerance. At the predetermined sampling points, each culture was diluted and plated in duplicate on MRS agar supplemented with 0.75, 1.0, or 2.0 % w/w

Oxgall (BD Biosciences, San Jose, CA). The plates were incubated anaerobically for 48 hrs and CFU/ml and percent survival were calculated. Each assay was performed in triplicate.

(ii) Heat tolerance. Each strain was grown in 250 ml of MRS broth and at each sampling point, 10 ml of cells were harvested from each population by centrifugation at 3,150 x g for 10 min at 21°C. Following centrifugation, the supernatant was discarded and cell pellets resuspended in 10 ml fresh MRS, preincubated to 55°C. Samples were taken at 0, 10, 20, 30, and 45 min, diluted, and plated in duplicate. Each assay was performed in triplicate.

(iii) Statistical analysis. Data obtained from the above experiments were analyzed using the Student's *t* test with $P < 0.05$ considered significant.

Bacteriocin production. Five µl of both *L. acidophilus* NCFM and NCK1818 were spotted onto MRS agar and incubated at 37°C overnight in an anaerobic chamber. The following day, 100 µl of the indicator strain *Lactobacillus delbrueckii* (NCK235) was added to 10 ml of molten MRS overlay agar (0.75% w/v) and poured evenly onto the surface of the agar plate. After 24 hours of incubation, zones of inhibition indicating antagonistic activity of lactacin B, were evaluated (10).

Growth curves. *L. acidophilus* NCFM and NCK1818 were both transferred three times from frozen stock cultures in MRS and modified MRS (mMRS). The mMRS media used in this study followed the ingredients for commercially available MRS (Becton, Dickinson and Company, Sparks, MD), replacing dextrose with 1 % w/v of

either glucose, lactose, trehalose, or sucrose. Growth curves were performed at 37°C in 96 well plates (Corning) containing 200 µl of each supplemented semi-defined medium or MRS broth. Cultures were allowed to grow for 16 hours and OD_{600} was measured every 15 min in triplicate wells. Plates were incubated at 37°C, and growth was automatically monitored by determining the changes in A_{600} as a function of time using a FLUOStar OPTIMA microtiter plate reader (BMG Labtech). The maximum specific growth rate was calculated from the slope of a linear regression line during exponential growth with a correlation coefficient (r^2) of 0.99. Each point represented the mean of three independent cultures.

4.4 Results

4.4.1 Construction of the LuxS$^-$ mutant. Until recently, site-specific gene inactivation in lactobacilli has been performed using plasmid integration strategies. Maintenance of plasmid integration requires growth in selective media. However, both plasmid integration and selective growth may influence gene expression results obtained by microarray analysis. As such, a marker-free strategy was used to inactivate *luxS* (LBA1081) in *L. acidophilus* NCFM using a double-crossover homologous recombination approach. The *luxS* gene of the resulting mutant strain, NCK1818, lacked a 97 bp internal region, and contained an additional EcoRI restriction site. The deletion region was selected to disrupt the reading frame and result in a non-functional gene product. Gene replacement was confirmed by PCR analysis of the deleted region and Southern hybridization using an internal fragment of *luxS* as the probe (data not shown).

NCK1818 was Em sensitive and did not produce AI-2 as determined by the *V. harveyi* reporter assay (5). Previously, the pattern of AI-2 expression by *L. acidophilus* NCFM was determined using a *luxS* integration mutant as the negative control (8). The same pattern of AI-2 production during the growth of *L. acidophilus* NCFM was observed using NCK1818 as the negative control.

4.4.2 Microarray analysis of the LuxS regulon. In order to study the genes differentially expressed in response to LuxS production, a microarray design was developed that examined the differences in expression between the wild type and $LuxS^-$ mutant in the early, middle, or late-exponential growth phase. AI-2 production during growth was previously determined for *L. acidophilus* NCFM (8). From that data, three growth points were selected for RNA isolation: OD_{600} 0.2, before AI-2 has accumulated; OD_{600} 0.7, during the rapid production of AI-2; and, OD_{600} 1.2, when production of AI-2 slows and the level of AI-2 in the supernatant remains elevated but constant. RNA was isolated from both strains at each time point, in duplicate, and AI-2 activity determined using the *V. harveyi* reporter assay. In total, six points were compared against each other in every possible pair of comparisons (15 hybridizations). Analysis of expression data was performed using a two-stage mixed model ANOVA and least square mean ratios considered significant at a fold change of 1.8 and $P < 0.05$.

Each differentially expressed ORF at the first two time points were grouped according to their COG classification (Fig. 1). The two COG classifications with the highest number of overexpressed genes in NCFM, accounting for 42% of the total, were translation ribosomal structure and biogenesis (J), and replication, recombination, and repair (L). In contrast, 40% of the overexpressed genes in the $LuxS^-$ mutant were

categorized as function unknown (S). The increased expression of genes related to normal growth and metabolism in NCFM compared to the LuxS$^-$ mutant suggests that LuxS positively influences the expression of many of these genes either directly or indirectly. It is not clear from these data the function of the genes whose expression was negatively influenced by LuxS. There were no significantly differentially expressed genes under the conditions tested in either NCFM or the LuxS$^-$ mutant at late-log phase (OD_{600} 1.2), when the level of AI-2 in the media is no longer increasing.

A round robin microarray design combined with mixed model analysis was used to compare the effect of strain, time and the combined effect of both strain and time (strain*time) on the gene expression of *L. acidophilus* NCFM. A strain to strain comparison revealed that, before AI-2 began to appreciably accumulate in the media (OD_{600} 0.2), 84 ORFs were significantly overexpressed in NCFM (Table 4a) while only 13 ORFs were significantly overexpressed in the LuxS$^-$ mutant (Table 4b). The most highly induced genes in NCFM compared to the mutant at early-log phase (OD_{600} 0.2) were *relA* (3.14 fold), putatively related to signal transduction, and IF-2 (3.14 fold), a putative translation initiation factor. At mid-log phase (OD_{600} 0.7), as AI-2 was rapidly accumulating, only 3 ORFs were significantly overexpressed in NCFM (LBA1497, LBA1796, LBA1798) and 4 ORFs (LBA0026, LBA0089, LBA0568, and LBA1596) overexpressed in the LuxS- mutant. Interestingly, the only ORF that was significantly differentially expressed at both of the first two time points was *labT* (LBA1796), an ABC-transporter previously implicated with bacteriocin export (10). At early-log phase, *labT* was overexpressed in NCFM 2.53 fold, and at mid-log it was overexpressed 2.10 fold compared to the LuxS$^-$ mutant. A response regulator (LBA1798), of a neighboring

two component regulatory system, was also overexpressed in NCFM compared to the mutant at mid-log phase. In this regard, the LuxS$^-$ strain was analyzed for bacteriocin production and found to elicit the same level of bacteriocin production as the wild type. Additionally, an isogenic *labT* mutant strain was tested for AI-2 production and found to produce AI-2 at the same level as the wild-type.

The trehalose hydrolase, *treC* (LBA1014), was overexpressed 2.56 fold in NCFM compared to the LuxS$^-$ mutant strain at early-log phase. Previous research reported deficient growth on trehalose of a *L. acidophilus* TreC$^-$ mutant (11). Therefore, the maximum growth rate of *L. acidophilus* NCFM and the LuxS$^-$ mutant on mMRS media containing various sugars was measured in order to determine the possible influence of a *luxS* mutation on sugar utilization (Table 5). Growth was not affected by the *luxS* mutation when the strains were grown on MRS or mMRS containing glucose or trehalose. However, the LuxS$^-$ mutant exhibited a lower maximum growth rate compared to NCFM on both lactose and sucrose.

The transcriptional profile of each strain from early to late-log phase was also examined (Fig. 2). A similar number of genes, 27 in NCFM and 41 in the LuxS$^-$ mutant, increased in expression from early to mid-log phase in both strains. The expression of 12 genes increased in both strains and included sugar metabolism genes (LBA0874, LBA1012, LBA1812, and LBA1974), and a putative myosin-crossreactive antigen (LBA555). Only 24 genes in the wild type strain, compared to 97 genes in the mutant strain, decreased in expression from early to mid-log phase. Again, from middle to late-log phase, the expression of a similar number of genes increased in both strains, but 45 genes decreased in expression during this growth phase in the wild type, and only 15 in

the LuxS$^-$ mutant. These results suggest that AI-2 could be responsible for increasing or maintaining the expression of genes during the early stages of growth. When AI-2 is deficient in the media, the expression of a significant number of genes appears to be decreased during the early log phase.

4.4.3 Stress tolerance. The involvement of LuxS with various stress responses of *L. acidophilus* NCFM was tested. Growth on MRS agar supplemented with 2.0 % Oxgall (dehydrated fresh bile) significantly decreased the growth of the LuxS$^-$ mutant compared to NCFM when bacterial populations were plated at OD_{600} 0.2. When cells were plated from OD_{600} 0.7 populations, a significant decrease in growth was observed in the LuxS$^-$ strain on MRS supplemented with 1.0 % Oxgall. Cells harvested from the final time point, OD_{600} 1.2, showed no difference in growth on Oxgall (Fig 3). These results indicate that AI-2 production correlates with bile tolerance through mid-log phase, but as the LuxS$^-$ population reaches stationary phase, sensitivity to bile due to the absence of AI-2 is not present.

Additionally, when populations harvested from OD_{600} 0.2 and OD_{600} 0.7 were exposed to 55°C heat stress, the LuxS$^-$ mutant was more sensitive (Fig 4). Cell populations of the LuxS$^-$ mutant harvested from the final time point, OD_{600} 1.2, did not show a significant decrease when compared to NCFM. This heat-stress survival pattern further implicates the involvement of AI-2 with stress response during the early and middle-log phases of growth, but not as the population approaches stationary growth phase where the cells were inherently more heat tolerant.

4.5 Discussion

The human gastrointestinal tract is populated by a diverse number of microorganisms whose combined activity establishes protective immunity and maintains intestinal homeostasis. It is likely that these microorganisms communicate between populations to coordinate gene expression for maintenance of the integrity and function of the microbiota (15). LuxS, produced by a variety of microbial species, is responsible for the synthesis of the interspecies bacterial signal AI-2 which could participate in communication within the intestinal environment as well as during pure culture fermentations. Using microarray analysis, this study reports the identification of genes whose expression was influenced by LuxS over the growth phase of *L. acidophilus* NCFM. The highest level of differential expression from the wild type strain compared to a LuxS$^-$ mutant strain occurred, surprisingly, at early-log phase of growth. At the late stages of logarithmic growth phase, no differentially expressed genes were identified under the conditions tested. The overexpression of certain stress-associated genes in the wild type strain suggested the involvement of AI-2 with stress response. Analysis of the mutant strain indicated that AI-2 participates in the survival of *L. acidophilus* NCFM following heat and bile stress.

This study is the first to report a transcriptional analysis of the LuxS regulon at different stages of growth. Previous microarray studies of the gene expression modulated by AI-2 have either studied the response of a LuxS$^-$ mutant strain to exogenous AI-2 (9) or identified a single growth point and analyzed the transcriptional difference between an insertional mutant and the wild type (20, 32, 36). While these approaches can successfully identify genes regulated by AI-2 at single points, the expression profile only

represents a snapshot of the LuxS regulon. In order to gain a more complete understanding of gene expression influenced by LuxS, we selected three time points throughout the growth phase of *L. acidophilus* NCFM for transcriptional analysis. At the first sampling point, OD_{600} 0.2, AI-2 activity detected in the growth media was minimal as the populations were in the early stages of logarithmic growth phase. The second sampling point, during mid-log growth (OD_{600} 0.7), was taken during the rapid accumulation of AI-2 in the media. Cells were harvested at OD_{600} 1.2, late-log phase, for the final sampling point, which was approximately 30 minutes after AI-2 levels reached their peak in the media. These points were chosen to represent the gene expression before, during, and after the production of AI-2 as determined previously (8). The $LuxS^-$ strain used in this study was a gene deletion mutant and therefore was expected to be free of any pleiotropic effects caused by integrations or addition of selected exogenous components to the media.

The largest number of differentially expressed genes was identified during the early-log phase of growth. A relatively high number of genes were induced in the wild type compared to the $LuxS^-$ strain, indicating that AI-2 facilitates the expression of certain genes during the early phases of growth. The majority of these genes were related to transcription, translation, and replication. Three genes (LBA772, LBA773, and LBA775) of the operon encoding the F_1F_0-ATPase (17) (LBA772-LBA779) were overexpressed in the wild type strain. Synthesis of ATP by the F_1F_0-ATPase could associate AI-2 with the generation of energy needed for logarithmic growth phase or prepare the cell of deacidification of the cytoplasm as the pH falls during growth. The trehalose hydrolase (*treC*, LBA1014) also showed increased expression in the wild type,

although no trehalose was present in the media. The absence of AI-2 did not affect the growth of NCFM on trehalose (Table 5), while neither a TreB$^-$ (transporter) or TreC$^-$ mutant strain were able to grow on trehalose (11). The upregulation of the TreC hydrolase could be another effort to generate energy needed for growth through the hydrolysis of more complex sugars that may accumulate in the cytoplasm. The differential expression of these putative energy production systems further associates LuxS with growth and metabolism. Similar results obtained by deLisa *et al*. (9) implicated AI-2 with regulation of cell division, DNA processing, and morphological processes. Both the production of AI-2 and phenotypic differences detected in the LuxS$^-$ strain suggest that AI-2 can be sensed and affect gene expression. However, *L. acidophilus* NCFM does not contain a gene homolog to the Lsr transporters characterized as AI-2 ABC transporters in *E. coli* (35). An ABC transporter (LBA1783) was differentially expressed in the presence of AI-2 in early-log phase and perhaps could be involved with the internalization of AI-2.

In addition to normal growth and metabolic processes, LuxS affected the expression of various cell-surface factors. Two genes (LBA1735 and LBA1736) of a putative exopolysaccharide (EPS) operon were also differentially expressed in the wild type in early-log phase. Although EPS production by *L. acidophilus* NCFM has not been demonstrated, these genes could potentially encode proteins for assembly of polysaccharides into adhesive structures on the surface of the bacterium. LuxS has similarly been shown to reduce cell-to-cell adhesion by regulating the expression of biofilm exopolysaccharides in staphylococci (16). We previously reported that a LuxS$^-$ strain of *L. acidophilus* NCFM exhibited decreased adhesion ability when tested on

Caco-2 cells (8). In the same study, the LuxS$^-$ mutant was only demonstrated to influence adhesion of NCFM in log phase, but when the mutant strain was harvested for adhesion after being in a concentrated cell suspension, adhesion significantly increased, but no difference in adhesion was observed between NCFM and the LuxS$^-$ mutant. These observations correlate with the lack of differentially expressed genes in this study between the mutant and wild type strain during late-log phase. A putative fibronectin-binding protein, previously shown to participate with adhesion to Caco-2 cells (7), was also induced during early-log phase by AI-2. Fibronectin is a component of the human intestinal extracellular matrix (ECM) and could be a target for the adhesion of bacterial cells in the intestinal environment (14). A LuxS$^-$ mutant strain of *Lactobacillus reuteri* exhibited decreased ecological performance in the murine gastrointestinal tract (28). Regulating the expression of cell-surface factors early in the growth phase may pre-adapt the bacterium for interaction with a diverse microbial community or ecological performance in the intestinal tract.

Cell-surface associated molecules of intestinal microorganisms are recognized by the host and can be important in both the inflammatory response and maintenance of intestinal homeostatsis (21). One of these bacterial surface molecules, lipoteichoic acid, is bound to the cellular membrane and extends through the cell wall to present itself in the environment of the bacteria. The *dltD* gene (LBA1923), a member of the *dlt* operon responsible for proper D-alanation of lipoteichoic acids in lactobacilli, was induced in the presence of LuxS at early-log growth phase. When a Dlt$^-$ mutant of *Lactobacillus plantarum* was exposed to peripheral blood mononuclear cells (PBMCs), the secretion of proinflammatory cytokines TNFα and IL-12 was decreased (13). The expression of IL-

10 by the PBMCs was increased following exposure to the Dlt$^-$ mutant, skewing the cytokine profile to an anti-inflammatory response. Regulation by AI-2 of the structure of these lipoteichoic acids could play a role in host-microbe interactions in the intestinal tract.

AI-2 is also thought to participate in the regulation of stress responses in bacteria (33, 36). Accordingly, the expression of multiple genes putatively related with stress responses were influenced by AI-2, including *dnaK* (LBA1247), *grpE* (LBA1248), and *clpX* (LBA847) involved with the removal of misfolded proteins and premature polypeptides produced during heat stress. Analysis of the influence of AI-2 on heat stress survival revealed that when compared to the wild type strain, the LuxS$^-$ mutant strain of *L. acidophilus* NCFM was more sensitive to 55°C heat stress (Fig 4). This sensitivity was only observed when the bacterial populations were stressed from early or mid-log growth phase. These results are consistent with the increased expression of the heat-shock response genes at the early stages of growth. In a recent study of *Porphyromonoas gingivalis*, a LuxS$^-$ mutant strain was more resistant than the wild type to heat stress (36), suggesting strain-dependent alteration of stress response by AI-2

Intestinal bacteria must survive passage through the harsh conditions of the gastrointestinal tract in order to persist in the intestine. One of the hurdles that must be overcome is the exposure to bile in the gastric region. A putative bile salt hydrolase (LBA0892) was induced by LuxS at early-log phase. The LuxS$^-$ mutant strain was tested for its tolerance to bile and found to be more sensitive when cultures were harvested from either early or mid-log growth phase. Cultures harvested from late log phase did not exhibit any differences in bile tolerance from the wild type. These results indicate that

AI-2 could act to prepare the cell for stressful conditions by positively influencing the expression of genes that have a function with growth and survival in different environmental conditions.

The mixed model analysis of microarray data used in this study allows for significant differences in gene expression to be detected based on strain, time, and the combined effect of strain and time. Strain-dependent differential expression indicates a gene is differentially expressed between the two strains where all experiments are analyzed together, independent of growth phase. Only two genes from the genome of *L. acidophilus* NCFM showed strain-dependent regulation. A helicase, *dnaB* (LBA1545), was overexpressed in the wild type, indicating activation by AI-2 and further associating AI-2 with the regulation of cell growth and division. The other strain dependent gene, also overexpressed in the wild type, was *labT* (LBA1796), an ABC transporter associated with the export of the bacteriocin, lactacin B. At mid-log growth phase, a response regulator (LBA1798) in the lactacin B operon was also activated by LuxS. The induction of genes related to lactacin B production and transport by AI-2 provides some insight into the regulation of lactacin B activity, albeit that the transporter does not appear to be involved directly with AI-2 transport. Mutacin I production by *Streptococcus mutans* was eliminated by mutation of the LuxS gene (20). Mutacin I is a class I lantibiotic while lactacin B is a class II small peptide and any regulation of different types of bacteriocins by AI-2, therefore, varies. The results from both *S. mutans* and *L. acidophilus*, however, suggest that AI-2 signaling and expression of some bacteriocin components are connected in these two Gram-positive bacteria.

AI-2 was reported to be a quorum sensing factor that accumulates as the environment as cell density increases, although the growth phase in which AI-2 affects gene expression has not been reported. Our results suggest that AI-2 acts on cell populations in the early stages of logarithmic growth, before appreciable accumulation of AI-2. The high number of genes that decreased in expression from early to mid-log growth phase in the LuxS$^-$ mutant strain compared to the wild type (Fig. 2) supports the hypothesis that LuxS positively influences gene expression early in the growth phase. When gene expression was examined throughout the growth phase, LuxS seems to prepare the population for the stresses and interactive conditions naturally encountered during planktonic growth. *In vivo*, *L. acidophilus* is not provided with the ideal nutrients and an optimum environment presented by laboratory conditions. AI-2 could be part of a signaling mechanism that optimizes the performance of bacteria throughout rapid growth. Therefore, regulation by AI-2 of growth and stress responses is likely condition and strain dependant. It is apparent, however, that the behavior of AI-2 as a quorum sensing autoinducer occurs in a low cell density dependant manner, rather than at high cell density. The results presented here, along with previous results, implicate AI-2 production with the expression of genes related to growth and metabolism. Therefore, for a more complete understanding of the gene expression related to AI-2 production, transcriptional analysis should not only be considered at one growth condition, but rather at multiple conditions that combine to provide insight into the overall response of bacteria to AI-2. It is clear that AI-2 impacts the expression of genes related to rapid growth and stress response of *L. acidophilus* NCFM, as well as possibly influencing host-microbe interactions.

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Table 1. Bacterial strains and plasmids used in this study

Strains	Origin or relevant characteristics	Source or reference
L. acidophilus		
NCFM	Human intestinal isolate	(24)
NCK 1392	NCFM containing pTRK669	(22)
NCK 1818	NCFM containing luxS deletion	This study
NCK 1758	NCFM with insertionally inactivated *labT*	(10)
NCK 235	*Lactobacillus delbruekii* used as indicator strain in bacteriocin assay	ATCC 4797
V. harveyi		
BB170	*luxN*::Tn5 AI-1 sensor$^-$ AI-2 sensor$^+$	(26)
E.coli		
EC1000	RepA$^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA*; host for pOR128-based plasmids	(18)
Plasmids		
pORI28	Em^r, ori (pWV01), replicates only with *repA* provided in *trans*	(18)
pTRK669	ori (pWV01), Cm^r, RepA$^+$	(22)
pTRK884	pORI28 ligated to *luxS* containing a 97 bp deletion and introduced EcoRI restriction site	This study

Table 2. Primers used in this study

Primer	Sequence[a]
LuxS-EF	5'- GATCTCTAGATGACAGAAGACGATGAATG -3'
LuxS-ER	5'- GATCAGATCTATTGCGACTAAGTTCAGAC -3'
LuxS-delF	5'- GATCGAATTCTCGTTCGGTTGAACTAAACGTAAGTC -3'
LuxS-delR	5'- GATCGAATTCCGAACAGGATTCCACCTAATCGTTTG -3'
LuxS-XXF	5'- GCCAACTTAGCCTTAAGCACTC -3'
LuxS-XXR	5'- TTGTTCCTGCTCCTCAGCCTTC -3'
LuxS-delNF	5'- TGCTTTAGCAACTTCAGTAG -3'
LuxS-delNR	5'- TAAAGTTAAGGCACCTTACG -3'

[a]Restriction enzyme sites used for cloning are underlined.

Table 3. Culture conditions of cell populations harvested for microarray analysis[a]

OD_{600}	CFU/ml (± SD)	AI-2 activity (± SD)	pH (± SD)
0.2	3.26×10^6 ($\pm 4.27 \times 10^5$)	2.27 (±0.09)	6.08 (±0.04)
0.7	2.67×10^7 ($\pm 3.13 \times 10^6$)	10.78 (±0.60)	5.35 (±0.13)
1.2	7.81×10^7 ($\pm 1.22 \times 10^7$)	30.13 (±0.25)	4.89 (±0.12)

[a]All values represent the mean and one standard deviation calculated from combined data obtained from both *L. acidophilus* NCFM and NCK1818 in three independent experiments.

Table 4a. COG classification of differentially expressed genes

Genes induced in *L. acidophilus* NCFM at early-log phase (OD_{600} 0.2)					
Gene Annotation[a]	ORF	Ratio[b]	Gene Annotation	ORF	Ratio
Energy production and conversion					
F1F0-ATPase subunit a	LBA 0772	1.92	F1F0-ATPase subunit d	LBA 0775	1.98
F1F0-ATPase subunit c	LBA 0773	1.99	*lctP*	LBA 1768	1.97
Cell cycle control, cell division, chromosome partitioning					
spoIIIe	LBA 0659	1.95	*epsC*	LBA 1735	1.85
ftsZ	LBA 0812	2.18			
Amino acid transport and metabolism					
asd2	LBA 0857	1.81	*pepP*	LBA 1336	2.50
pepT	LBA 1190	2.28			
Nucleotide transport and metabolism					
deoxyribose-p aldolase	LBA 0391	2.24	UMP kinase	LBA 1268	2.46
Carbohydrate transport and metabolism					
phosphoglucomutase	LBA 0687	1.91	*treC*	LBA 1014	2.56
Lipid transport and metabolism					
thil	LBA 0626	2.07	*fabG*	LBA 0662	2.28
hmdH	LBA 0627	2.15			
Translation, ribosomal structure and biogenesis					
trprs	LBA 0209	1.94	*hemK*	LBA 0768	2.42
meth-trna synthetase	LBA 0213	2.15	pseudouridylate synthase	LBA 0791	1.85
ribosomal protein S17	LBA 0300	2.24	trna synthetase	LBA 0817	1.93
ribosomal protein L14	LBA 0301	2.34	*gidA*	LBA 0982	2.00
ribosomal protein L24	LBA 0302	2.53	tRNA ligase	LBA 1198	1.82
ribosomal protein L36	LBA 0314	2.24	*rrf*	LBA 1267	1.89
ribosomal protein S13	LBA 0315	2.02	*miaA*	LBA 1503	1.94
tRNA pseudouridine synthetase	LBA 0322	2.12	*pheS*	LBA 1518	2.31
ampM	LBA 0623	1.93	*leuS*	LBA 1617	1.80
pcrf I	LBA 0767	2.75	GTP-binding protein	LBA 1824	2.20
Transcription					
*gntR_*	LBA 0393	2.09	*nusA*	LBA 1259	1.88
fibronectin-binding prot.	LBA 1148	1.84	*parB*	LBA 1828	2.52
Replication, recombination and repair					
recF	LBA 0004	1.88	DNA primase	LBA 1197	2.66
gyrA	LBA 0006	1.89	transposase	LBA 1464	2.33
DNA polymer. III	LBA 0376	1.89	transposase	LBA 1487	1.92
dnlJ	LBA 0529	1.89	*dnaB*	LBA 1545	2.97
uvrB	LBA 0688	2.35	*mutM*	LBA 1549	1.90
radC	LBA 0797	2.02	transposase	LBA 1570	2.11
uvrC	LBA 0946	1.96	RNA helicase	LBA 0416	2.06
DNA topoisomerase	LBA 0981	1.87	*mutT* family protein	LBA 0819	2.89
DNA topo.sub.B	LBA 1122	2.89			
Cell wall, membrane, and envelope biogenesis					
mraW	LBA 0803	2.04	*epsB*	LBA 1736	1.98
murG	LBA 0809	2.16	*gidB*	LBA 1829	1.90
bshA	LBA 0892	1.94	*dltD*	LBA 1923	2.29
eep	LBA 1263	2.23			
Posttranslational modification, protein turnover, chaperones					
clpC	LBA 0283	2.05	*hslV*	LBA 0984	1.88
radA	LBA 0346	2.04	*dnaK*	LBA 1247	1.95
clpX	LBA 0847	2.49	*grpE*	LBA 1248	2.97
Signal transduction mechanisms					
luxS	LBA 1081		*relA*	LBA 0932	
phoH	LBA 1203				
Defense mechanisms					
labT	LBA 1796	2.53	permease	LBA 1839	2.36
General Function or Function Unknown					
putative transport protein	LBA 0635	2.07	dihydroacetone kinase	LBA 1310	1.81
putative hydrolase	LBA 0796	1.97	helicase	LBA 1676	2.39
GTP binding protein	LBA 0947	2.46	ABC transporter	LBA 1783	2.25
oxidoreductase	LBA 0950	2.39	serine/threonine prot. kinase	LBA 1317	1.85
phosphoglycerate dehydrogenase	LBA 0969	2.05	hypothetical	LBA 1191	2.04
O-GlcNAc transferase	LBA 0971	1.85	conserved hypothetical protein	LBA 1204	2.10
conserved hypothetical protein	LBA1202	2.70	hypothetical	LBA 1823	1.88

[a] Putative identification by manual annotation for that gene or ORF

[b] Least square means ratio of $\log_2$ estimates between wild type *L. acidophilus* NCFM and the LuxS$^-$ mutant strain. The cutoff ratio for the fold difference was 1.8 and $P < 0.05$.

Table 4b. COG classification of differentially expressed genes

Genes induced in *L. acidophilus* NCFM compared to NCK1818 at mid-log phase (OD_{600} 0.7)		
Gene Annotation[a]	ORF	Ratio[b]
Cell cycle control, cell division, chromosome partitioning		
unknown	LBA 1497	1.94
Transcription		
response regulator	LBA 1798	1.92
Defense mechanisms		
labT	LBA 1796	2.11

Genes repressed in *L. acidophilus* NCFM compared to NCK1818 at early-log phase (OD_{600} 0.2)		
Cell cycle control, cell division, chromosome partitioning		
hypothetical	LBA 1156	0.53
Amino acid transport and metabolism		
ansA	LBA 1687	0.51
Translation, ribosomal structure and biogenesis		
rib. prot. L11	LBA 0359	0.52
seryl-trna synthetase	LBA 1626	0.52
kanamycin kinase	LBA 1348	0.54
Posttranslational modification, protein turnover, chaperones		
thioredoxin reductase	LBA 0422	0.51
Inorganic ion transport and metabolism		
K+ uptake protein	LBA 0166	0.47
ABC transporter	LBA 0154	0.54
Secondary metabolites biosynthesis, transport and catabolism		
hypothetical	LBA 0644	0.53
Intracellular trafficking, secretion, and vesicular transport		
hypothetical	LBA 0448	0.52
General function or function unknown		
hypothetical	LBA 0031	0.50
cons. hypothetical	LBA 0217	0.50
hypothetical	LBA 0690	0.53
unknown	LBA 1127	0.53
lysM	LBA 1850	0.51
cons. hypothetical	LBA 0100	0.55

Genes repressed in *L. acidophilus* NCFM compared to NCK1818 at mid-log phase (OD_{600} 0.7)		
Transcription		
cadX	LBA 0022	0.55
dinG	LBA 1164	0.55
General function or function unknown		
malate permease	LBA 0568	0.49
hypothetical	LBA 0026	0.53
cons. hypothetical	LBA 0089	0.50
unknown	LBA 0883	0.55
unknown	LBA 1596	0.51

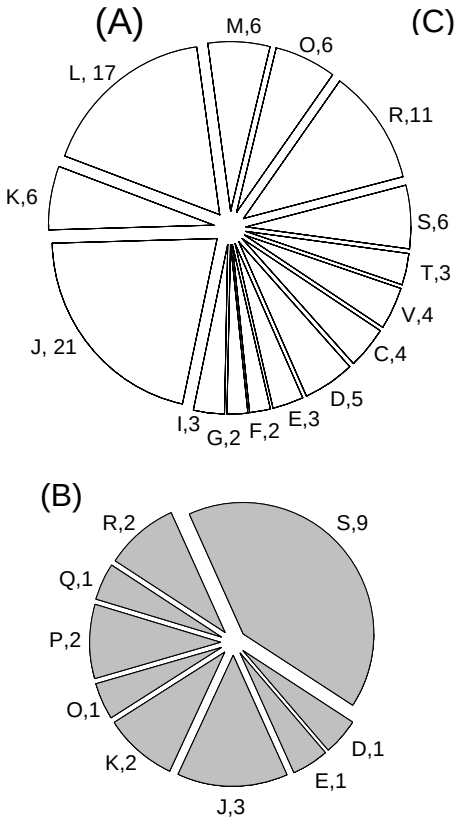
[a] Putative identification by manual annotation for that gene or ORF
[b] Least square means ratio of $\log_2$ estimates between wild type *L. acidophilus* NCFM and the LuxS$^-$ mutant strain. The cutoff ratio for the fold difference was 1.8 and $P < 0.05$.

Table 5. Maximum specific growth rate (μ hr^{-1}) on various carbohydrate sources

Medium		NCFM ($\mu \pm$ SD)		LuxS$^-$ ($\mu \pm$ SD)
MRS		0.45 (± 0.005)		0.42 (± 0.006)
mMRS[a]	Glucose	0.34 (± 0.04)		0.32 (± 0.05)
mMRS[a]	Trehalose	0.28 (± 0.003)		0.29 (± 0.02)
mMRS[a]	Lactose	0.28 (± 0.004)	*	0.19 (± 0.006)
mMRS[a]	Sucrose	0.33 (± 0.003)	*	0.23 (± 0.003)

* Identifies significantly different growth rates as determined by the Student's *t* test ($P < 0.01$)

[a] Refer to Materials and Methods section for medium composition



(C)

Group	COG Classification
C	Energy production and conversion
D	Cell cycle control, cell division,
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
G	Carbohydrate transport and metabolism
I	Lipid transport and metabolism
J	Translation, ribosomal structure and biogenesis
K	Transcription
L	Replication, recombination and repair
M	Cell wall, membrane, envelope biogenesis
O	Posttranslational modification, protein turnover, chaperones
P	Inorganic ion transport and metabolism
Q	Secondary metabolites biosynthesis,
R	General function prediction only
S	Function unknown
T	Signal transduction mechanisms
V	Defense mechanisms

Figure 1. COG classification of total ORFs overexpressed in the wild-type (Panel A) or LuxS^{-} mutant strain (Panel B). COG groups (Panel C) are listed on pie charts with the number of overexpressed ORFs in that group (COG, # of ORFs).

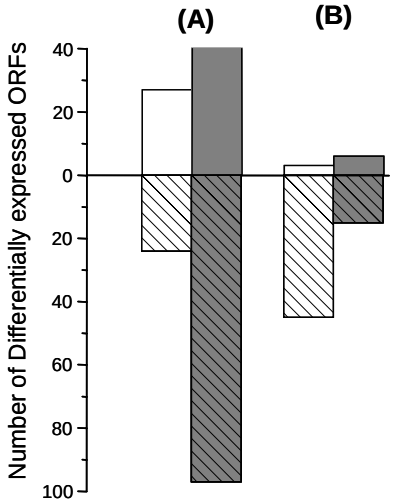


Figure 2. Number of ORFs whose expression increased (no slashes) or decreased (slashes) from (A) early to middle-log phase or (B) from middle to late-log phase. The wild type is represented by white bars (□) and the LuxS$^-$ mutant strain represented by gray bars (■).

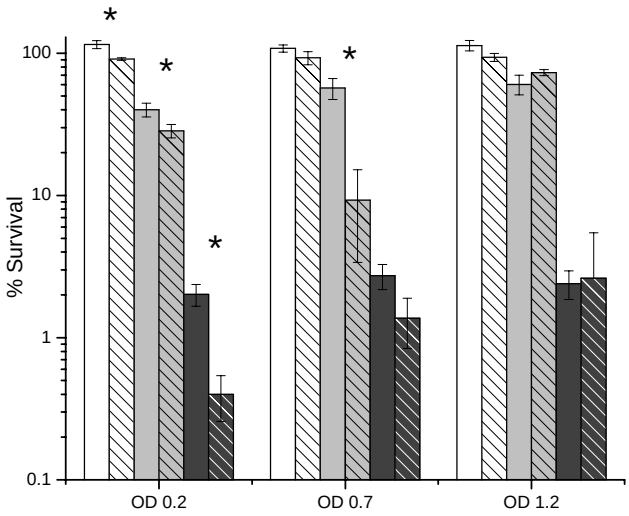


Figure 3. Bile tolerance of *L. acidophilus* NCFM (no slashes) and the LuxS$^-$ mutant strain (slashes) harvested at early-log (OD_{600} 0.2), middle-log (OD_{600} 0.7), and late-log (OD_{600} 1.2) growth phase. Bacterial populations were diluted and plated on MRS supplemented with either 0.75 % (□), 1.0 % (▨), or 2.0 % (■) Oxgall. Percent survival was calculated by comparison to CFU/ml grown on non-supplemented MRS. Significant differences detected by the Student's *t* test ($P < 0.05$) are represented by an asterisk (*). Error bars represent one standard deviation from the mean.

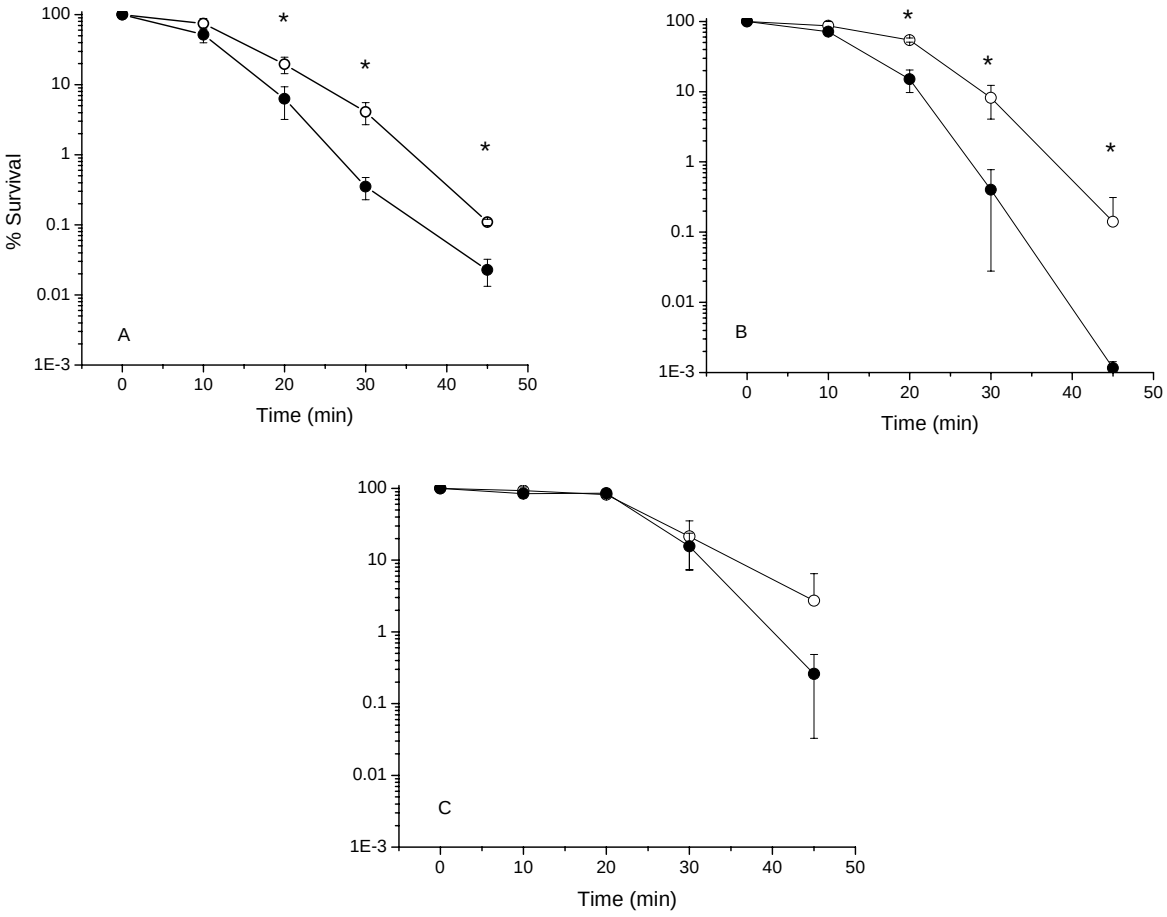


Figure 4. Survival of wild type (open circles) and LuxS^{-} (closed circles) populations harvested at (Panel A) early-log, OD_{600} 0.2, (Panel B) middle-log, OD_{600} 0.7, or (Panel C) late-log, OD_{600} 1.2, growth phase. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) has been detected for that time point. Error bars represent one standard deviation from the mean.