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INVESTIGATION OF BILE STRESS TOLERANCE AND IMMUNOMODULATORY PROPERTIES OF PROBIOTIC *LACTOBACILLUS REUTERI*

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INVESTIGATION OF BILE STRESS TOLERANCE AND IMMUNOMODULATORY PROPERTIES OF PROBIOTIC *LACTOBACILLUS REUTERI*

By

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ABSTRACT

INVESTIGATION OF BILE STRESS TOLERANCE AND IMMUNOMODULATORY PROPERTIES OF PROBIOTIC *LACTOBACILLUS REUTERI*

BY

Kristi James Whitehead

This thesis focuses on characterization of different aspects of *L. reuteri* physiology that may contribute to the probiotic potential of the species. This research includes an investigation of the bile stress response of *L. reuteri* ATCC 55730 (Chapter 2), analysis of the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475 (Chapter 3), and the investigation of the potential immunomodulatory role of lactobacillic acid, a cyclopropyl fatty acid that is specific to certain strains of *L. reuteri* at particular stages of growth (Chapter 4).

The research contained within Chapter 2 includes an investigation of the physiological response of *L. reuteri* ATCC 55730 to the bile stress that would be encountered in the host small intestine, including an analysis of genes that are important for survival and growth in the presence of bile. Three genes were identified as being important for the survival of *L. reuteri* ATCC 55730 in bile: a putative esterase, a clp chaperone, and a gene of unknown function that is conserved within different *L. reuteri* isolates. One gene, encoding a multidrug resistance protein in the major facilitator superfamily, was found to contribute to the ability of the strain to grow in the presence of bile.

Chapter 3 examines the ability of *L. reuteri* ATCC PTA 6475 to deconjugate bile acids. There is a strong correlation between strains isolated from the gastrointestinal tract and the ability to deconjugate bile acids, thus it is often suggested that bile salt hydrolase activity confers a selective advantage for colonization of the gastrointestinal tract. *L. reuteri* ATCC 55730, the strain investigated in the microarray and mutational analysis of the bile stress response, exhibits a very low level of bile salt hydrolase activity. *L. reuteri* ATCC PTA 6475, on the other hand, exhibits strong bile salt hydrolase activity against all six major human bile acids. The research contained in Chapter 3 suggests that this activity confers a growth advantage in the presence of bile acids at slightly acidic pH levels.

Several strains of *L. reuteri* have been suggested to be able to modulate the host immune response through the production of compounds that inhibit production of the pro-inflammatory cytokine, TNF. The research in Chapter 4 identifies a possible novel immunomodulatory compound, lactobacillic acid, which may be responsible for the observed suppression. Production of this compound was demonstrated to be specific to *L. reuteri* strains with the TNF suppressive activity, and importantly, the appearance of lactobacillic acid in cells isolated from early stationary and late stationary phase cultures correlates with the growth stages where the immunomodulatory activity is observed. Current research is focused on identifying the role of lactobacillic acid in TNF suppression.

This work is dedicated to Daniel, who loves me unconditionally, makes me laugh, and believes in me even when I don't believe in myself.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

THE PROBIOTIC POTENTIAL OF *LACTOBACILLUS REUTERI*

Introduction

Probiotics, which are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (19), have become increasingly popular over the past several decades as reports of antibiotic resistant infections and the prevalence of gastrointestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) continues to rise. Despite the increase in usage, not much is known about the mechanisms through which these microorganisms may be able to cause their beneficial effects. Production of antimicrobial compounds, alterations of the host microbiota, and modulation of the immune response of the host are among the proposed mechanisms (18, 54, 62). In part because the mechanisms through which these microbes cause their beneficial effects are not known, the characteristics that make an effective probiotic are also speculative. Some general characteristics that are proposed to be important include that the microbe be of host origin, is nonpathogenic, is able to resist the stresses encountered during passage through the host, and is able to confer a beneficial effect on the host (18). Lactobacilli are among the most commonly used bacteria in probiotic products, in part due to their long history of safe use in the food industry. Among

the lactobacilli, many different species are currently being investigated as effective probiotics, including various strains of *L. acidophilius*, *L. rhamnosus*, and *L. reuteri* (17). *L. reuteri* is a heterofermentive species of lactic acid bacteria that is found natively in the gastrointestinal tracts of humans (52).

This review will summarize the recent literature regarding the potential of various strains of *L. reuteri* to be used as effective probiotics; both in vitro experiments and clinical trials will be discussed.

Prevalence

Although there is a lack of knowledge regarding what characteristics make an effective probiotic, it is generally considered to be important for the bacteria to have been isolated from the host in which it will be used. *Lactobacillus reuteri* is one of the few species of lactic acid bacteria that is naturally found in humans as well as in a variety of animals as part of the normal microbiota. Using culture-based isolation techniques, *L. reuteri* has been found to be one of the dominant species of *Lactobacillus* isolated from chickens, mice, dogs, and pigs (2, 39, 41, 48, 79, 89). Within the human gastrointestinal tract, only a handful of lactic acid bacteria are considered to be autochthonous; these include *L. reuteri*, *L. gasseri*, and possibly *L. ruminus* and *L. salivarius* (52). Although bacterial samples are often taken from the intestines or fecal samples for analysis, there are several reports that suggest that *L. reuteri* may also inhabit the stomach of humans (52,

57, 78), a location typically considered to be fairly uninhabitable for bacteria due to the extremely low pH. In a recent 28-day clinical trial, Valeur et al (78) administered *L. reuteri* ATCC 55730, a probiotic strain of human-origin, to participants. They were able to demonstrate that the strain survived in the stomach, duodenum, and ileum of the study participants. This supports the argument that strains isolated from the host are most likely already adapted for survival in the host, and therefore, may be strains worth investigating for probiotic usage.

Factors that aid in colonization.

Although being of host origin is generally considered to be an important trait, many of the potential probiotic strains currently in use originated from the food industry. As the definition of a probiotic requires the organism to survive passage through the gastrointestinal tract, there are several characteristics that are generally investigated through in vitro assays before the strains are used in animal studies or clinical trials. These traits include resistance to stresses encountered in the gastrointestinal tract (such as low pH in the stomach and bile in the small intestine), as well as the ability to attach to host surfaces for colonization. It is also generally desirable to understand how strains are able to survive these harsh conditions. Knowledge of the mechanisms could allow researchers to quickly screen for strains that can survive passage through the gastrointestinal tract based on a particular trait or allow for improvement of the

survival rate of a strain with intriguing beneficial properties that may have poor survival or colonization abilities. As multiple strains of *L. reuteri* have been shown to survive in the gastrointestinal tract in a wide variety of animals, this species may be ideal for investigating these mechanisms.

Acid stress. As previously mentioned, several studies have shown that *L. reuteri* can survive in the human stomach (52, 57, 78), an environment generally considered to be inhospitable to bacteria due to its extremely low pH. The pH in the human stomach can reach as low as 1.5 during the fasting stage, with values ranging between 3.0 to 5.0 after consumption of a meal (16). One of the most commonly used delivery mechanisms for probiotics, yogurt and other fermented dairy products, are also typically acidic (16). Therefore, an understanding of the ways in which various strains of *L. reuteri* can survive low pH exposure is beneficial for host, as well as industrial, applications.

Lee et al (37) exposed *L. reuteri* ATCC 23272 to pHs of 5.0, 4.5, and 4.0 for one hour and compared the protein expression under these conditions to cultures grown at pH 6.8. They identified 40 proteins involved in a wide range of cellular functions that were significantly differentially expressed during incubation at all three lower pH values, suggesting that *L. reuteri* has a complex response to pH stress. Proteins over-expressed during growth at lower pHs included those involved in transport and binding, transcription and translation, metabolism, stress, and pH homeostasis.

In a more focused study, microarray and mutational analyses of the acid shock response of *L. reuteri* ATCC 55730 were performed by Wall et al (81). The gene expression changes of cells that were exposed to pH 2.7 for either 5 or 15 minutes were identified based on comparison with the gene expression profiles of cells grown at pH 5.1. Overall, 72 genes were found to be differentially expressed when *L. reuteri* ATCC 55730 was exposed to acid shock conditions. Viability studies at pH 2.7 revealed that for this strain, more than 80% of the cells survived exposure for one hour, although no growth appeared to take place. This suggests that although the strain is able to survive passage through the stomach and even possibly colonize the stomach (78), time may be needed to adapt to the harsh acidic conditions before growth can resume. Such an adaptation period lasting approximately two hours was demonstrated when *L. reuteri* ATCC 55730 was exposed to bile in vitro (87). Based on the microarray analysis, two genes were chosen for disruption; lr1864 (*clpL*, a putative ATPase that may act as a chaperone) and lr1516 (a putative esterase in the penicillin binding protein family). Both of these mutants were found to have significantly lower survival rates compared to the wild-type strain when incubated in a synthetic gastric juice at pH 2.0 for 50 minutes. The mutational analysis suggests that the ClpL chaperone may be needed to aid the cells in degrading or refolding proteins denatured by the low pH exposure; it also suggests that the putative esterase may be involved in altering the cell wall in such a way that allows the cells to survive exposure to low pH (81). Further study is needed to

fully understand how *L. reuteri* responds to and survives exposure to the low pH found in the stomach. The importance of ClpL and the putative esterase should be investigated in an in vivo model to determine if they truly contribute to survival in the gastrointestinal tract.

Bile stress. After exposure to the low pH in the stomach, probiotic bacteria then move into the small intestine where they are exposed to bile, a complex mixture that typically aids in fat digestion and absorption (27). Bile acids, the main component in bile, are known to have potent antimicrobial effects (25), yet as previously mentioned, some strains of *L. reuteri* have been shown to colonize portions of the small intestine (78). The response of these strains to bile is important for understanding what physiological changes the bacteria undergo during passage through the gastrointestinal tract.

Bile acids are amphipathic molecules that function to solubilize fats. Their amphipathic nature allows them to form mixed micelles with fats and cholesterol in the body and also allows them to act as detergents. One of the main ways through which bile is thought to cause damage to bacterial cells is through disruption of the cell membrane and wall. Taranto et al (71) demonstrated that growth in the presence of bile induced changes in the cell membrane of *L. reuteri* CRL 1098. Exposure to bile decreased the amount of phospholipids and also decreased the ratio of saturated to unsaturated fatty acids in the cell membrane.

These changes may be important for survival during passage through the gastrointestinal tract.

L. reuteri ATCC 55730 was shown to survive in physiologically relevant concentrations of bile in vitro (87). Growth studies revealed that there was an initial pause in growth when mid-log phase cells were exposed to bile. This pause in growth is believed to be a period of adaptation, during which the cells make physiological changes that allow them to then grow in the presence of bile. In order to determine how bile exposure affects the gene expression of *L. reuteri* and to understand what changes allow the cells to resume growth in the presence of bile, microarray analyses were performed comparing the gene expression profiles of cells before and after bile exposure. Based on the microarray analysis, several genes were chosen for mutational analysis. Disruptions in three genes were shown to cause significantly lower survival rates during the first 30 minutes of bile exposure. These genes included *lr1864* (*clpL*), *lr0085* (a gene of unknown function that is conserved among *L. reuteri* only), and *lr1516* (a putative esterase). In addition, a disruption in another gene, *lr1584* (a multidrug resistance protein in the major facilitator superfamily), appeared to affect the ability of the cells to grow in the presence of bile, although the initial survival rate was not altered (87). The growth defect observed in the *lr1584* mutant is in accordance with known mechanisms of bile resistance for gram-negative organisms, where the utilization of efflux pumps to expel bile acids has been demonstrated (25).

Interestingly, there is overlap in the gene expression changes of *L. reuteri* exposed to low pH and bile based on microarray analysis (81, 87). There is also overlap in the mutational analysis; ClpL (*lr1864*) and the putative esterase (*lr1516*) were both shown to be important for survival in low pH and bile. This overlap suggests that exposure to acid in the stomach may prepare *L. reuteri* for exposure to bile in the small intestine. Further research is required to determine if the period of adaptation observed in vitro during bile exposure may be decreased by a prior exposure to low pH.

One other possibility of how *L. reuteri* may deal with the stress of bile acids is through the activity of a bile salt hydrolase (BSH) enzyme. When bile is secreted into the small intestine from the gall bladder, the majority of bile acids are conjugated to either a glycine or taurine molecule; however, by the time the bile reaches the end of the small intestine, almost all of the bile acids are unconjugated (i.e. the glycine or taurine has been removed) (47). This deconjugation activity is due to bacterial bile salt hydrolase enzymes and plays a role in bile acid metabolism of the host (7). There is much debate as to the role of the bacterial BSH enzymes. Some possible roles include lowering the cholesterol of the host, providing a nutritional benefit to the bacteria, or detoxification of the bile acids to protect the bacteria (7). Taranto et al (74) demonstrated that *L. reuteri* CRL 1098 contained an intracellular bile salt hydrolase enzyme that was expressed when the cells were in stationary phase.

Whether or not this enzyme plays a role in bile resistance of *L. reuteri* is yet to be determined. Research in our lab suggests that there is substantial strain variation in BSH enzyme specificity and activity among various *L. reuteri* (unpublished observation).

Attachment. It has been suggested that probiotic bacteria should persist in the gastrointestinal tract in order to cause their beneficial effects; the ability to attach to intestinal surfaces such as epithelial cells or mucus is often proposed to aid in this persistence. Several studies have examined the ability of *L. reuteri* to adhere to mucosal surfaces using cell lines, mucus components, or animal models.

Wang et al (85) tested nine different strains of *Lactobacillus* for their ability to attach to human enterocyte-like HT-29 cells, as well as to porcine gastric mucin, one of the main components of intestinal mucus. Of the nine strains, they found that *L. reuteri* JCM1081, a chicken isolate, was best able to adhere. Further analysis identified a 29 kDa protein, a putative ATP-binding cassette transporter protein, that was able to bind to both the cell line and the mucin in vitro, suggesting that this protein may be responsible for the adherence observed. The authors propose that based on these experiments mucin may actually act as a receptor for the binding of probiotic bacteria in the gastrointestinal tract. Roos et al (55) also previously identified a 29 kDa collagen binding protein from *L. reuteri* NCIP 11951 similar to ABC-type transport proteins that was suggested to aid in

attachment of the bacterium to epithelial surfaces. Sequence comparison revealed these two proteins to be similar (85), suggesting a conserved binding protein between the two strains.

Although there may be conserved traits identified, strain specificity is something that should be taken into careful consideration when investigating *L. reuteri* for probiotic usage. Characteristics that may be important for causing beneficial effects, such as binding to intestinal surfaces, may vary substantially between strains. For example, Miyoshi et al (42) tested *L. reuteri* strains from various sources (human, pig, mouse, and chicken) for their ability to adhere to Caco-2 cells in vitro. They identified three strains that had high levels of adherence (*L. reuteri* DSMZ 20016, 104R, and LEM83) and two strains that had low levels of adherence (*L. reuteri* ML1 and LB54). Upon further investigation, they identified a protein, MapA (mucus adhesion promoting protein) that also bound to Caco-2 cells and had the ability to inhibit the binding of *L. reuteri* to Caco-2 cells in a concentration-dependent manner. Although MapA appears to be a protein that is involved in binding to eukaryotic cells, all strains utilized in this study were found to contain the gene for MapA, despite the different adherence levels. Also, although MapA was able to compete with *L. reuteri* for binding, approximately 50% of the bacterial cells were able to bind to Caco-2 cells, even under conditions using saturating amounts of MapA. These observations suggest that binding to Caco-2 cells involves more than one factor, and that binding activity most likely is controlled by different factors in different strains of *L. reuteri*.

Although some studies have investigated binding to both cells and mucus or mucus components, other studies have focused singularly on the ability of *L. reuteri* to bind to mucus, since the epithelial surface in the gastrointestinal tract is covered with a mucus layer in healthy individuals. Jonsson et al (31) investigated the ability of seven different strains of *L. reuteri* to bind to pig gastric mucin. They observed varying levels of attachment between the strains. When the strains were grown in the presence of mucin, an increase in adherence was observed for strains with previously low levels of binding, whereas an inhibition of binding was observed for strains with naturally high levels of adherence. Proteinase K treatment was found to abolish binding, suggesting that binding is due to a protein. The effects observed by growth of the strains in the presence of mucin could also be explained by the dependence of binding on a protein or proteins. Growth in mucin could induce expression of a protein necessary for binding in the strains with naturally low levels of binding, but the presence of mucin could compete with a binding protein already present in the strains with naturally high levels of binding. This binding is not believed to be due to the previously identified 358 kDa cell surface protein, Mub, which was identified from *L. reuteri* 1063 and shown to bind to mucus and mucus components in vitro (56), as only one of the seven strains included in this study is known to produce Mub (31).

Although in vitro adherence tests provide a rapid means of examining the potential of certain strains to persist in the intestine, little is known about how host factors and physiological changes that the bacteria undergo during passage through the host may affect persistence. Initial studies in the host have identified a wide range of factors that can affect colonization success, including various cell surface proteins, exopolysaccharide production, and D-alanylation of teichoic acids in the cell membrane and wall.

Walter et al (82) examined the effect of disruption of four genes on the survival of *L. reuteri* 100-23 in the mouse. They identified one mutant with a disruption in Lsp (a high-molecular-mass surface protein) that was delayed in its colonization rate and which had impaired adherence to the forestomach epithelium of the mouse. This mutant was also found exhibit lower colonization levels when inoculated in competition with the wild-type strain. These experiments suggest that cell surface proteins are important for adherence and colonization in vivo, supporting the previously described in vitro data. The study also demonstrated somewhat reduced performance in an MsrB mutant (methionine sulfoxide reductase), although there was large inter-animal variability for this mutant.

Exopolysaccharide (EPS) production may also play a role in persistence for some strains. In *L. reuteri* TMW1.106, two proteins involved in EPS production were found to affect colonization of the strain in the gastrointestinal tract of mice. GtfA (glucosyltranferase) and Inu (inulosucrase) mutants were both found to be

defective in colonization of the mouse. The *inu* mutant was defective in colonization when put into competition with the wild-type strain; the *gtfA* mutant was defective in colonization when it was inoculated in competition with *L. johnsonii* #21, but not when it was inoculated in competition with the wild-type *L. reuteri*, suggesting that the wild-type strain was somehow able to complement the observed defect in the mutant (84).

Finally, the effect of D-alanylation of teichoic acids in the bacterial cell membrane and wall has been investigated in *L. reuteri*. The incorporation of D-alanyl esters into teichoic acids has been shown to be important for bacterial virulence when studied in various pathogens (1, 15, 51). In order to investigate whether there is overlap between the methods that pathogens and beneficial bacteria use to colonize the host, Walter et al (83) disrupted the D-alanylation operon of *L. reuteri* 100-23 by insertional disruption of *dltA*, the second gene in this operon. The *dltA* mutant was defective for colonization of the mouse when inoculated in competition with the wild-type strain, as well as when it was inoculated alone. The mutant also was demonstrated to be defective in the ability to form a biofilm on the murine forestomach epithelium. In vitro studies showed that the mutant had impaired growth under acidic conditions, which may play a role in the observed in vivo defects.

Overall, attachment to mucosal surfaces is a complex phenomenon that appears to be dependent on multiple bacterial surface characteristics such as EPS

production and cell surface proteins. Although there may be some conservation of characteristics important for attachment and persistence, there also appears to be considerable strain variation with *L. reuteri* that should be taken into consideration.

Proposed Mechanisms of Action

Although the popularity of probiotics has increased over the last few decades and they are being investigated as possible treatments for a wide variety of ailments, the mechanisms of action for the beneficial effects observed with probiotic treatment are still unknown. Some of the proposed mechanisms of action include pathogen inhibition, primarily through the production of antimicrobial compounds, and modulation of the host immune response (54, 62). Various strains of *L. reuteri* have been investigated for these traits.

Pathogen inhibition – production of antimicrobial compounds. Although production of hydrogen peroxide and lactic acid are commonly thought to contribute to pathogen inhibition by lactic acid bacteria (34, 35), most of the research regarding pathogen inhibition by *L. reuteri* has focused on other antimicrobial factors. *L. reuteri* is known to produce at least three other separate antimicrobial compounds: reuterin, a by-product of glycerol metabolism, reutericylcin, a novel tetramic acid derivative, and reutericin, a bacteriocin.

Reuterin. One of the most widely studied antimicrobial compounds produced by *L. reuteri*, and the reason commonly attributed for the beneficial effects observed with administration of the species, is reuterin, a by-product of glycerol metabolism. Reuterin has been identified as an equilibrium mixture of 3-hydroxypropionaldehyde (3-HPA) in the monomer form, the hydrate form, and the cyclical dimer form (70). Reuterin has been found to have a wide range of antimicrobial activity, including antibacterial, antimycotic, and antiprotozoal activities (3, 13). Lactobacilli are generally more resistant to reuterin than other microbes; one study found that at least two times the reuterin was needed to kill lactic acid bacteria as compared to other bacteria (13, 14). Although other organisms have been shown to produce 3-HPA as a by-product of glycerol metabolism (5, 20, 21, 40, 60), *L. reuteri* appears to be fairly unique in the ability to accumulate large amounts of reuterin (although this trait is possibly shared with certain strains of *L. coryniformis* (40)). A recent study demonstrated that *L. reuteri* had the highest reuterin MICs (minimum inhibitory concentrations) when compared to other intestinal bacteria (14).

Recent work has focused on the possible mechanism of action for reuterin's antimicrobial effects, as well as on attempts at determining whether reuterin is a relevant antimicrobial compound in vivo. Morita et al (44) were able to show that reuterin can be produced in detectable amounts in vivo using gnotobiotic BALB/c mice monoassociated with *L. reuteri* JCM 1112 if glycerol is present. Their research also demonstrated that 7 to 10 mM glycerol was found in the fecal

material of untreated mice, suggesting that reuterin may be produced in concentrations relevant for antimicrobial activity in vivo.

Schaefer et al (63) have suggested that reuterin causes its broad-spectrum antimicrobial effects by triggering oxidative stress through modification of thiol groups. This mechanism is supported by an overlap in the gene expression profile of *Escherichia coli* cells treated with reuterin and the known OxyR regulon, a group of genes known to be involved in responding to various oxidative stresses. The modification of thiol groups suggests that the aldehyde form of 3-HPA is most likely the active portion of the equilibrium mixture (63).

One intriguing characteristic about reuterin production is that larger amounts of reuterin are accumulated in the media when other bacteria are present. Various bacteria have been shown to cause this increased accumulation of reuterin (13, 63). Schaefer et al recently observed that live bacteria and UV–killed bacteria but not heat–killed bacteria, triggered this increased accumulation (63). It has not been determined whether the excess accumulation is due to increased reuterin production or increased secretion of reuterin.

Although reuterin is commonly attributed as one of the main reasons for the observed beneficial effects with *L. reuteri*, care should be taken to confirm this as the active trait for individual strains and in specific instances. Despite the evidence that reuterin is produced by various strains of *L. reuteri*, accumulation

levels can differ (69), and some strains have been shown to not contain the necessary enzymes for reuterin production (8). Some research also suggests that the genes necessary for reuterin production may have been horizontally acquired (44), possibly explaining the lack of these enzymes in certain strains. *L. reuteri* RC-14, a strain demonstrated to have many beneficial effects in urogenital health, does not make reuterin and does not contain the glycerol dehydratase enzyme necessary for converting glycerol into 3-HPA. Therefore, any probiotic effects observed with this strain are not due to reuterin production (8), and other possible mechanisms of action should be explored.

Reutericyclin. Reutericyclin was first identified as a low-molecular weight antimicrobial compound that is produced by *L. reuteri* LTH2584, a strain isolated from sourdough bread fermentations (22), although more recent research has demonstrated that it is produced by several different strains isolated from sourdough fermentations (23). Holtzel et al (29) identified reutericyclin as a novel tetramic acid derivative, 3-acetyl-1-(2-trans-decenoyl)-2-hydroxy-(5R)-isobutyl-Δ^2-pyrroline-4-one. Reutericyclin has been shown to have a broad-spectrum antimicrobial activity against various gram-positive organisms, including *Lactobacillus* spp., *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Listeria innocua* (22). Gänzle et al (24) demonstrated that the antimicrobial activity observed for reutericyclin was due to its ability to dissipate the transmembrane proton potential of sensitive cells. The main role of reutericyclin has been proposed as contributing to the persistence of

L. reuteri in sourdough fermentations. This proposed role is due to exhibited production of active amounts of reutericyclin in sourdough fermentations by *L. reuteri* (23), although due to the wide-spectrum inhibitory activity of the compound against gram-positive bacteria, further investigation of this compound is warranted.

Reutericin. Bacteriocins are antimicrobial proteinaceous compounds that have activity against closely related bacteria (53). At least one strain of *L. reuteri*, *L. reuteri* LA6, is known to produce a bacteriocin, reutericin 6. Reutericin 6 is a 2.7 kDa protein (32) that has been shown to have bacteriocidal activity against other lactobacilli, including *L. acidophilus* and *L. delbruckii.* The role of reutericin 6 has been proposed as a way for *L. reuteri* to remain the dominant species of heterofermentive lactobacilli in the gut (75). Reutericin 6 and gassericin A (a bacteriocin produced by *Lactobacillus gasseri* LA39) have been demonstrated to be identical in their molecular weight and primary amino acid structure, yet vary in the spectrum of their antimicrobial activity. The proposed reason for this difference in activity is a difference in the D-amino acid content of the two proteins (33). Investigation into the specificities of these two compounds is needed to determine the role of these similar compounds that are both produced by bacteria isolated from the same location, the feces of a human infant (33). Further research into whether these two organisms use the compounds to compete or to cooperate could indicate whether a mixture of these two strains might be advantageous for probiotic usage.

Pathogen inhibition – displacement. Apart from actually inhibiting the growth of or killing various pathogens, it is also possible that probiotics such as *L. reuteri* exert their beneficial effects through competition for binding sites or nutrients. Various studies have investigated the ability of *L. reuteri* to either inhibit the attachment of pathogens or to displace pathogens that have already adhered.

Strain specificity appears to have a large effect on the ability of *L. reuteri* to inhibit binding of pathogens to epithelial cells. Vesterlund et al (80) found that *L. reuteri* ING1 not only had significantly lower adherence levels to mucus than *Staphylococcus aureus*, but the strain also was not able to displace adherent *S. aureus*, unlike other lactic acid bacteria tested in the study. They were, however, able to demonstrate a significant reduction in the viability of *S. aureus* when incubated with *L. reuteri* ING1 in the presence of 1% glycerol, suggesting that this strain may still be effective in preventing infection by *S. aureus*.

It is also important to consider the effects that certain probiotic strains may have on one another, since many probiotic companies are marketing products containing mixtures of various strains. Larsen et al (35) tested multiple strains of *Lactobacillus*, including three strains of *L. reuteri* for the ability to bind to porcine epithelial cells and to either inhibit binding of or promote displacement of other lactobacilli or pathogenic *E. coli* O138 using in vitro assays. The authors of this study not only demonstrated a wide range in the adherence capabilities of the

three *L. reuteri* strains (ranging from 3.5 to 38% of bacteria), but also found that the most strongly adherent strain, *L. reuteri* DSM 12246, could actually displace other lactobacilli. Interestingly, in this study, there appeared to be no correlation between the ability of the lactobacilli to adhere and the inhibition of the *E. coli* O138, as all tested lactobacilli reduced adherence of the *E. coli* (35).

One possibility for inhibition of binding or displacement of pathogens is through competition for binding sites. Todoriki et al (76) tested various strains of lactobacilli for the ability to adhere to Caco-2 cells in vitro. They demonstrated that a strain of *L. reuteri*, JCM 1081, had the highest level of adhesion, while *L. crispatus* JCM 8779 had the second highest level of adhesion. Both of these strains were able to lower adherence levels of *E. coli, Salmonella typhimurium*, and *Enterococcus faecalis*. Although the adherence inhibition was suggested to be due to antimicrobial activity in the *L. crispatus* culture, no such activity was observed with the *L. reuteri* culture, suggesting either competition for binding sites or steric hindrance was the cause of the observed adherence inhibition. Using a more specific example, out of nine *L. reuteri* strains tested, two were shown to not only bind to both putative glycolipid receptor molecules of *Helicobacter pylori*, but also to inhibit binding of *H. pylori* to both, suggesting a competition for receptor binding (45). Another interesting connection regarding competition for binding sites is that Heinemann et al (26) identified a 29 kDa protein (as part of a biosurfactant mixture) from *L. reuteri* RC-14 that has strong anti-adhesive properties against *Enterococcus faecalis* 1131. The authors

suggest that this protein is the same one identified by Roos et al (55) that is believed to play a role in adherence of *L. reuteri* NCIB 11951. The identification of the same protein during studies investigating adhesion of a probiotic strain to host tissues and protection of host tissues by a probiotic strain suggest there may be similarities in the adherence mechanisms of probiotic and pathogenic strains which could lead to competition for binding sites.

One study has shown that *L. reuteri* may not only be able to displace individually bound bacteria, but also can have an effect on bacterial communities such as biofilms. *L. reuteri* RC-14, a strain demonstrated to have beneficial effects on urogenital health, was tested to determine its effect on biofilms formed by *Gardnerella vaginalis*, a pathogen commonly associated with bacterial vaginosis. *L. reuteri* RC-14 was found to disrupt and invade *G. vaginalis* biofilms in vitro, as well as cause a significant reduction in viability of the pathogen after co-incubation for 24 hours (59), suggesting that this strain can have a detrimental effect on an already well-established pathogen.

Pathogen inhibition – alteration of virulence factor expression. One other possibility for probiotic benefit through direct inhibition of pathogens is the possibility that the probiotic bacteria are able to alter either gene or protein expression of the pathogen's virulence factors. There are several in vitro studies that suggest that this may be yet another possible mechanism of action for *L. reuteri*.

Several studies have investigated the response of *E. coli* O157:H7 to incubation with *L. reuteri*. Carey et al (11) demonstrated that various strains of *Bifidobacterium*, *Pediococcus*, and *Lactobacillus*, including *L. reuteri* were able to decrease expression of Stx2A, the gene that encodes the A subunit of Shiga toxin 2. A different study demonstrated that culture supernatants from a certain strain of *L. reuteri*, *L. reuteri* ATCC 55730, were able to repress *E. coli* O157:H7 *ler* expression. The main virulence factors for *E. coli* O157:H7 are found in a pathogenicity island termed the locus of enterocyte effacement or LEE. The regulator of expression for this pathogenicity island is *ler* (or LEE regulator). The ability of *L. reuteri* culture supernatants to suppress expression of *ler* was found to be strain dependent, as supernatants from *L. reuteri* ATCC 55730 repressed expression, but supernatants from *L. reuteri* 100-23 actually induced expression of *ler* (30).

Protein expression from pathogens can also be affected, as evidenced by co-incubation of *Staphylococcus aureus* with *L. reuteri* RC-14 (36). Research demonstrated that secreted molecules of *L. reuteri* RC-14 were able to trigger a decrease in expression of SSL11 (staphylococcal superantigen-like protein 11) by *S. aureus*. SSL11 is a putative exotoxin that is proposed to be a virulence factor for *S. aureus*. The authors also demonstrated that the secreted molecules responsible for the suppression were not sensitive to protease treatment, suggesting that proteins are not responsible for the observed effects. The

responsible secreted molecules were shown to repress the SSL11 and P3 promoters of *S. aureus*, further suggesting that *L. reuteri* RC-14 may be able to alter the virulence of this common pathogen (36).

Immunomodulation. Apart from the potential for probiotic strains to cause their beneficial effects by either altering the microbial community of the host through production of antimicrobial compounds or through the prevention of establishment of various pathogens, one other popular proposed mechanism of action is the ability of probiotic bacteria to modulate the immune response of the host. Various studies have been performed to investigate the effect that administration of *L. reuteri* may have on dendritic cell maturation and cytokine production.

Dendritic cells are antigen-presenting cells that have the capability to direct the immune response of the host through the stimulation of B and T lymphocytes (4); probiotic strains that have the capability to affect the maturation of dendritic cells could therefore provide a potent modulation of the immune response. Christensen et al (12) investigated the ability of six different strains of lactobacilli to affect dendritic cell maturation by measuring cytokine production and surface marker expression. They were able to demonstrate that the irradiated bacteria had varying effects, with *L. reuteri* DSM 12246 exhibiting the weakest effect on most surface markers and cytokines measured. *L. casei* CHCC 3139, on the other hand, was shown to have the strongest effects on surface markers and IL-

12 of the strains measured. Interestingly, when *L. reuteri* was added along with *L. casei*, *L. reuteri* was able to inhibit the increase in pro-inflammatory cytokines IL-12, IL-6, TNF, and the surface marker CD86 observed with *L. casei* alone.

Several other studies have also investigated the response of dendritic cells to certain strains of *L. reuteri* and found significant differences based on the strain tested. Mohamadzadeh et al (43) determined that *L. reuteri* ATCC 23272 was able to activate and induce maturation of dendritic cells and trigger an increase in pro-inflammatory IL-12 production, suggesting that the *L. reuteri* strain was shifting the dendritic cells towards polarization of a Th1 response. The authors observed similar effects with both live and irradiated cells, suggesting that bacterial viability was not essential for this effect. On the other hand, Smits et al (67) found no effect on cytokine production by dendritic cells when they were co-incubated with *L. reuteri* ASM 20016; however, they were able to determine that the dendritic cells had been "primed" to drive the development of T regulatory cells that then led to an increase in IL-10 production by T helper cells. IL-10 is typically considered to be involved in anti-inflammatory responses. The authors of this study also demonstrated differing effects based on the ratio of bacteria to dendritic cells. They showed that a ratio of bacteria to dendritic cells that was higher than 1:1 actually resulted in a decreased priming effect, suggesting that care should be taken when choosing the dosage amount of probiotics. These studies emphasize the need for thoroughly testing probiotic strains to determine their immunomodulatory effects before using them individually or in combination

as treatments, as some strains of *L. reuteri* promoted production of IL-12 (pro-inflammatory) and others promoted production of IL-10 (anti-inflammatory).

Several labs have investigated the effect of probiotic administration on the production of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF). Soria et al (68) showed that *L. reuteri* CRL 1098 was able to decrease TNF production, while *L. acidophilus* CRL 1014 and *L. rhamnosus* CRL 1036 increased production of TNF by human peripheral blood mononuclear cells, demonstrating again that some probiotic strains may be considered immunosuppressive, while others may be considered immunostimulatory. The effects on TNF production were only observed with live bacterial cells, and these effects were suggested to be somehow involved with lipid rafts. Lipid rafts are microdomains found in eukaryotic plasma membranes that have been shown to be involved in interactions with pathogenic bacteria. This study again suggests an overlap in the mechanisms through which probiotics and pathogens interact with eukaryotic cells, despite the fact that the outcomes of these interactions are often very different.

Although the previous study demonstrated a difference in the effects of probiotics of different species of *Lactobacillus*, variation in the effect of different strains of *L. reuteri* has also been observed. A study comparing three strains of *L. reuteri*, ATCC PTA 6475, ATCC 55730, and CF48-3A, found that only *L. reuteri* ATCC PTA 6475 was able to suppress TNF production from LPS-activated monocytes

and macrophages (38). The suppression was shown to be due to secreted compounds produced by the bacteria, and the effect was demonstrated for both human cell lines and monocyte-derived macrophages isolated from Crohn's disease patients. Lin et al (38) offer evidence that suggests that the TNF suppression observed with cell-free supernatant from *L. reuteri* ATCC PTA 6475 may be caused by transcriptional regulation of TNF. The authors also note that there were differences in the responses of the isolated macrophages from different individuals, suggesting that the genetic background of the host may also play an important role in the interaction. A particularly intriguing observation is that cell-free conditioned medium from *L. reuteri* ATCC PTA 6475 was able to suppress TNF to similar levels as the current Crohn's disease treatment, infliximab (a TNF antibody), suggesting that probiotics may have potential as treatments for IBD (38).

Animal studies and clinical trials.

The most common studies performed on potential probiotic strains are in vitro tests to investigate whether the strains demonstrate the "necessary" characteristics to provide probiotic benefit, including survival to stresses in the gastrointestinal tract (acid and bile), the ability to attach to mucus or epithelial surfaces, the ability to inhibit or displace pathogens, and the ability to modify the host immune response (18, 54, 62). Although these characteristics are often considered to be important for a bacterial strain to be able to cause beneficial

effects, the mechanism of action for most of the beneficial effects attributed to probiotic strains is unknown, and therefore, what characteristics make a strain "effective" are also unknown. For example, Van Coillie et al (79) carefully screened strains of *Lactobacillus* isolated from hens for various characteristics such as production of antimicrobial acids and adherence to epithelial cells. Although they were able to identify a strain of *L. reuteri* that did decrease counts of *Salmonella enterica* from inoculated chicks, they reached the conclusion that the in vitro trait selection did not equal in vivo effectiveness. The authors suggest that this difference may be due to host-associated factors that are not included during *in vitro* testing (79). Therefore, the ultimate test for a potential probiotic strain comes in the form of *in vivo* studies, including animal models of disease, and ultimately, clinical trials. There are many animal studies and clinical trials involving *L. reuteri*; a selected few will be discussed here.

Diarrhea. *L. reuteri* has been shown to be effective against diarrhea of various causes in children. Shornikova et al (65, 66) demonstrated that *L. reuteri* could actually be used as a treatment to reduce the duration of diarrhea (primarily from rotavirus) in children. In this study, the probiotic was administered after children were brought to the hospital for treatment for the diarrhea. In another study, it was found that children in a daycare that received *L. reuteri* ATCC 55730 had significantly less diarrhea episodes and the duration of these episodes was shorter than in the control group of children (86).

Inflammatory bowel disease (IBD). Inflammatory bowel disease is a set of gastrointestinal disorders marked by chronic inflammation that affects a smaller portion of the population and is more severe than IBS. IBD can be divided into Crohn's disease or ulcerative colitis, depending on the prevalence and location of inflammation. The prevalence of IBD has increased over the last few decades, particularly in regions with historically low occurrence (88). The proposed causes of IBD include: an overly aggressive immune response to commensal bacteria due to genetic factors or irregular immune response of the host, the presence or absence of particular bacteria, or environmental factors (58, 64). Probiotics, which are proposed to act through alteration of host immune responses or alteration of host microbial communities, are proposed as possible therapies for IBD (58, 64).

Several studies have shown that IL-10 deficient mice, which are prone to spontaneous colitis unless raised in germ-free conditions, have a difference in their lactic acid bacteria population than wild-type control mice. Both Madsen (39) and Peña (48) found that the dominant *Lactobacillus* in wild-type control mice was *L. reuteri*, while the dominant type in IL-10 deficient mice was *L. johnsonii*. These studies not only suggest again that host genetic background can affect the microbial community, but also suggest that the presence of certain lactobacilli, including *L. reuteri*, may prevent spontaneous colitis. In fact, Madsen (39) was able to show that by repopulating IL-10 deficient mice with *L. reuteri*, the development of colitis was prevented, although the study also showed that

increasing the amount of lactic acid bacteria in the gut by lactulose treatment also prevented colitis, so the effect of *L. reuteri* therapy observed in that particular study may not be specific to *L. reuteri*.

Probiotic *L. reuteri* has also been demonstrated to have some level of effectiveness against various rodent models of induced colitis, including *Helicobacter hepaticus*-induced (49), acetic acid-induced (28), and TNBS (trinitrobenzenesulfonic acid)-induced (50). Peña (49) showed that inflammation levels associated with *H. hepaticus*-induced colitis in IL-10 deficient mice were decreased by pre-treatment with *L. reuteri* 6798, a strain that had previously been shown to reduce TNF levels in vitro (48). Interestingly, the colonization levels of *H. hepaticus* were not affected, suggesting that immunomodulation may be the main mechanism of action observed in this study. These animal studies demonstrating a prevention or reduction of colitis with administration of *L. reuteri* indicates that certain strains may be able to prevent or alleviate colonic inflammation associated with inflammatory bowel disease (IBD).

A one-month clinical trial investigated the effect of administration of a combination of *L. reuteri* RC-14 and *L. rhamnosus* GR-1 to individuals with IBD (either Crohn's or ulcerative colitis) or healthy control subjects. Different effects of the probiotics were observed in both groups of individuals. One effect observed was an increase in $CD4^+$ $CD25^{high}$ T cells in the IBD patients; this putatively immunosuppressive shift could help play a role in promoting and

maintaining remission in these patients (6). It is intriguing to note that probiotic effects may be altered in response to the health of the host.

Hypercholesterolemia. Probiotics are also currently being investigated for the ability to lower serum cholesterol levels in the host. *L. reuteri* CRL 1098 has been shown to not only decrease cholesterol levels in mice, but also to prevent the development of hypercholesterolemia (72, 73). In the first study, hypercholesterolemic mice were fed *L. reuteri* CRL 1098 for seven days. After this treatment, a significant reduction in total cholesterol, along with a reduction in triglyceride levels and an increased ratio of HDL (high density lipoprotein) to LDL cholesterol was observed. The authors suggest that these observed effects may be due to either lower levels of intestinal absorption of cholesterol or higher catabolism of lipids (73). One other consideration would be the possibility that bile salt hydrolase activity of the bacteria resulted in the lower cholesterol levels (7), as deconjugated bile acids are more likely to be excreted, requiring the host to pull from serum cholesterol levels to synthesize new bile acids.

Colic. *L. reuteri* ATCC 55730 has even been investigated as a probiotic for the alleviation of colic, an infantile disorder marked by excessive crying and irritability of unknown cause. When compared in a clinical trial to simethicone (a common colic treatment typically found to not be very effective), a reduction in crying time was reported for the probiotic-treated group over the 28-day course of the study. Crying time were reported at 51 minutes per day in the probiotic-treated group

and 145 minutes per day in the simethicone-treated group at the 28 day point, suggesting that probiotics may alleviate colicky symptoms (61).

General health. Weizman et al (86) demonstrated that children in a daycare setting that received *L. reuteri* not only had significantly fewer episodes of fever and diarrhea than a control group, but also had significantly less clinical visits, daycare absences, and antibiotic prescriptions. In another study where *L. reuteri* ATCC 55730 was given to Swedish workers, improved health was found in the probiotic-treated group. The probiotic-treated group had less sick leave due to respiratory or gastrointestinal infections, and also had a lower frequency of sick days (measured as the number of sick days relative to the number of work days) than the control group (77).

Oral health. *L. reuteri* has also been included in studies involving oral health, in part because of the potent antimicrobial activities of many of the strains. Many of the studies have involved determining the effects of probiotic administration on the levels of *Streptococcus mutans*, one of the main contributors to tooth decay. *L. reuteri* ATCC 55730 has been shown to have in vitro inhibitory activity against *S. mutans*; this antagonistic effect was also observed in human volunteers fed a yogurt product containing the strain. In these volunteers, a significantly lower population of *S. mutans* was isolated from the oral cavity, suggesting a decreased risk of caries (46). Çaglar has also demonstrated the effectiveness of this strain by showing a significant reduction in salivary mutans streptococci

levels in volunteers that consumed *L. reuteri* ATCC 55370 in straw, tablet, or gum form (9, 10).

Although the research in the probiotic field has increased exponentially over the past twenty years or so, there is still much that needs to be investigated. The research at this point includes many in vitro studies investigating survival of stresses that the bacteria will be exposed to in the gastrointestinal tract, the ability to inhibit pathogens, and the ability to modulate the host immune response. In vivo studies are investigating a wide range of disorders including bacterial infections, allergies, and gastrointestinal disorders. Although various strains of *L. reuteri* have demonstrated effectiveness in various in vitro and in vivo studies as discussed in this review, there are still large areas of research that need to be conducted. For example, there appears to be wide ranging differences between the physiology and effects of particular strains within the species, and very little has been determined about the actual interactions between *L. reuteri* and the host.

This thesis focuses on characterization of different aspects of *L. reuteri* physiology that may contribute to the probiotic potential of the species. Micorarray and mutational analysis of the bile stress response of *L. reuteri* ATCC 55730 identified several genes that play significant roles in either survival or growth of this strain in the presence of bile (Chapter 2). These genes may contribute to survival of the strain during passage through the host gastrointestinal tract. The study also identified a putative matrix metalloprotease

gene that is up-regulated in the presence of bile, but does not play a role in survival or growth of the strain in bile. The function of this gene is of particular interest, as it shows significant homology to eukaryotic matrix metalloproteases which are known to play important roles in the homeostasis of the gastrointestinal tract (Appendix C).

The bile salt hydrolase activity in *L. reuteri* ATCC PTA 6475 was also investigated. The bacterial function of this activity has yet to be elucidated, although the research presented in this thesis suggests that it may be involved in resistance to bile under acidic conditions (Chapter 3). An understanding of the role this activity plays in survival of the bacteria during passage through the gastrointestinal tract is important, as the activity has been proposed to also affect the host. The proposed effects on the host range from a beneficial lowering of serum cholesterol levels to putatively harmful activation of carcinogens or malabsorption of fats and nutrients.

Finally an investigation of the potential immunomodulatory role of lactobacillic acid, a cyclopropyl fatty acid that is specific to certain strains of *L. reuteri* at particular stages of growth is included. Certain strains of *L. reuteri* have been demonstrated to suppress TNF production from LPS-activated macrophages in vitro. This activity is proposed to be due to secreted molecules produced by these strains at early stationary to late stationary phases of growth. The production of lactobacillic acid is shown to be specific to strains with this

immunomodulatory activity, and the presence of this compound is shown to correlate with the growth stages during which the immunomodulatory compound is produced (Chapter 4).

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CHAPTER 2

GENOMIC AND GENETIC CHARACTERIZATION OF THE BILE STRESS RESPONSE OF PROBIOTIC *LACTOBACILLUS REUTERI* ATCC 55730

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INTRODUCTION

The idea that bacteria could benefit human health was postulated almost one hundred years ago by Elie Metchnikoff (23). Recently the use of probiotics, which are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (10), has become increasingly popular. Probiotic microorganisms are currently being investigated for many possible health benefits in many different ailments including inflammatory bowel disease, diarrhea and hypercholesterolemia (9, 33, 40, 46). The mechanisms through which probiotics confer their beneficial effects are mostly unknown; examples of current theories include immunomodulation of the host by the synthesis of immunomodulatory compounds, the production of antimicrobial

compounds that inhibit pathogen growth, and large-scale alterations of the microbiota (28, 31).

Probiotic bacteria encounter a variety of stresses that need to be overcome to remain viable. For example, many bacteria are packaged into food products such as yogurt and fermented milk, which exposes them to temperature and osmotic stress. After ingestion, probiotics must be able to survive the extreme acidic conditions in the stomach and the detergent properties of bile acids in the small intestine. Bile acids are amphipathic molecules that are synthesized from cholesterol and play an important role in the digestion of fats and absorption of fat-soluble vitamins. The concentration of bile acids ranges from 0.2 to 2% in the human small intestine and fluctuates based on the amount of fat intake in the diet (14). Bile acids have potent antimicrobial activity against many microbes and are known to cause damage to cells that are considered to be bile resistant, most likely via disruption of the membrane and cell wall. The resistance mechanisms of gram-negative bacteria are fairly well-characterized; these mechanisms include protection by the hydrophobic outer membrane and utilization of efflux pumps to expel bile salts that do enter the cell (13). The resistance mechanisms of gram-positive organisms, which in general are less bile-resistant than gram-negative bacteria, are less well understood.

Lactic acid bacteria, particularly lactobacilli, are the genus most commonly used as probiotics, in part because of their safe usage in food production. Potential

new probiotic strains should include several important characteristics; they should be of human origin, non-pathogenic, able to remain viable in the gastrointestinal tract for at least short periods of time, and be resistant to various stresses (9). In the GI tract the main sources of antimicrobial stress are the low pH encountered in the stomach and the detergent like properties of bile acids found in the small intestine. Although the precise mechanisms by which bile acids cause cell death are not understood, their chemical nature indicates they will be able to solubilize membranes and cause significant membrane damage. This is supported by genetic and genomic studies in a variety of different species that show the main response of gram-positive organisms to bile exposure appears to be alteration of the cellular envelope. Isolation of bile-sensitive mutants of *Enterococcus faecalis* and *Listeria monocytogenes* identified genes mainly involved in maintenance and synthesis of the cell membrane and wall, as well as genes involved in general stress responses (2, 16). Microarray analysis of *Lactobacillus plantarum* and *Lactobacillus acidophilus* identified expression changes in genes whose product is found in the cell envelope (6, 25). In addition there is also microscopic evidence supporting the role of bile in alteration of the cellular envelope. Bron *et al.* demonstrated that cultures of *L. plantarum* cells exposed to bile contained some shrunken cells and cells that tended to clump together and had rough surfaces (5), while bile exposure also caused the appearance of shrunken and empty cells in cultures of *Propionibacterium freudenreichii* (17).

Lactobacillus reuteri is a species with a broad host range, with isolates originating from many different species including humans, pigs, chickens, dogs, mice, and hamsters (7). *L. reuteri* is also considered to be indigenous to the human GI tract (27). *L. reuteri* ATCC 55730, a strain currently marketed for probiotic usage, has been demonstrated in clinical trials to be effective against diarrhea in children, as well as to alleviate colic in infants (26, 30, 45). In addition, consumption of *L. reuteri* ATCC 55730 reduced the number of sick days taken by workers in a large trial in Sweden (38). How these benefits are achieved at the molecular level is still unknown. *L. reuteri* ATCC 55730 is known to produce a broad-spectrum antimicrobial compound, reuterin, by metabolism of glycerol under anaerobic conditions (36). Based on the observation that this strain is able to survive in the human duodenum and ileum (41), it is an appropriate organism to use in the investigation of bile resistance mechanisms.

This research investigated the gene expression response of *L. reuteri* ATCC 55730 to bile exposure and has begun to uncover the mechanisms this strain uses to survive and grow in the presence of bile. Microarray experiments were conducted to determine the gene expression profiles of cells upon initial bile exposure (bile shock) and cells that had resumed growth in the presence of bile (bile adaptation). Based on the microarray results, nine genes were chosen for mutational analysis. These results indicate some of the mechanisms important in the bile shock and adaptation responses of *L. reuteri* ATCC 55730 *in vitro* and

may provide a further understanding of characteristics important for survival in the gastrointestinal tract.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2.1. All liquid cultures of lactobacilli were grown under microaerobic conditions (2% O_2, 5% CO_2, balanced with N_2) in MRS broth (BD Difco) at 37°C, unless otherwise specified. All plate cultures of lactobacilli were grown under anaerobic conditions using the GasPack EZ Anaerobe Container system (BD Difco) at 37°C, unless otherwise specified. *Lactobacillus reuteri* strains containing pVE6007 were grown at 35°C. All *E. coli* was grown under aerobic conditions at 37°C in LB broth (BD Difco). When specified, drugs were added to the following concentrations: 10 μg/ml (*L. reuteri*) or 400 μg/ml (*E. coli*) erythromycin, 10 μg/ml chloramphenicol, and 40 μg/ml kanamycin. *L. reuteri* mutants (containing the pORI28 disruption) were always grown in the presence of 10 μg/ml erythromycin. Dehydrated bovine bile/ox gall (Sigma) was resuspended in MRS broth to make a 50% weight/volume solution. This mixture was sterilized by autoclaving and stored at 37°C for up to four weeks.

Table 2.1. Bacterial strains and plasmids used for this study.

Strain or plasmid	Description	Reference or source
Strains		
EC1000	*E. coli* strain containing a chromosomal copy of the pWV01 *repA* gene; Kan^r	(15)
ATCC 55730	*L. reuteri* strain isolated from human breast milk	Biogaia, AB, Sweden
PRB190	*L. reuteri* ATCC 55730 *clpL* (*lr1864*) mutant, Em^r	(42)
PRB188	*L. reuteri* ATCC 55730 putative esterase (*lr1516*) mutant, Em^r	(42)
PRB167	*lr0085*::pKW01 in an ATCC 55730 background, Em^r	This study
PRB186	*L. reuteri* ATCC 55730 *clpE* (*lr0004*) mutant, Em^r	Stefan Roos
PRB126	*lr1265*::pKW02 in an ATCC 55730 background, Em^r	This study
PRB130	*lr1584*::pKW03 in an ATCC 55730 background, Em^r	This study
PRB163	*lr1291*::pKW04 in an ATCC 55730 background, Em^r	This study
PRB125	*lr1351*::pKW05 in an ATCC 55730 background, Em^r	This study
PRB114	*lr1706*::pKW06 in an ATCC 55730 background, Em^r	This study
Plasmids		
pVE6007	Cm^r *repA*-positive temperature-sensitive derivative of pWV01	(19)
pORI28	Em^r *repA*-negative derivative of pWV01	(15)
pKW01	pORI28 + 203 bp insert from *lr0085*	This study
pKW02	pORI28 + 303 bp insert from *lr1265*	This study
pKW03	pORI28 + 331 bp insert from *lr1584*	This study
pKW04	pORI28 + 310 bp insert from *lr1291*	This study
pKW05	pORI28 + 152 bp insert from *lr1351*	This study
pKW06	pORI28 + 240 bp insert from *lr1706*	This study

RNA isolation. For each of five biological replicate experiments, a culture of *L. reuteri* ATCC 55730 was grown in MRS broth to an optical density at 600 nm ($O.D._{600}$) approximately equal to 0.5. Upon reaching this stage in growth, 0.5% oxgall was added to the culture. At the correct time points, 5 ml samples were collected from the culture and immediately mixed with an equal part of ice-cold methanol. Cell pellets were collected by centrifugation, washed with STE buffer (6.7% sucrose; 50 mM Tris-Cl, pH 8.0; 1 mM EDTA), and resuspended in STE buffer containing 0.25 units/μl mutanolysin (Sigma). Cells pellets were then incubated at 37°C for 20 minutes. RNA was then isolated using the Qiagen RNeasy Kit according to manufacturer's instructions.

Microarray experiments. Long oligonucleotides (60-mers) were designed and synthesized for 1864 open reading frames from a draft genome sequence of *L. reuteri* ATCC 55730 (1) and 15 open reading frames encoding known extracellular proteins from *L. reuteri* DSM 20016 (43) using OligoArray 1.0 Software. Six control 60-mer oligonucleotides were also included. These controls are identical to DNA sequences from *E. coli* genes (*yacF*, *ybaS*, *yciC*, *yfiF*, *ygjU*, and *yjcG*) and have no sequence similarity to the *L. reuteri* genome. Once synthesized the oligonucleotide concentrations were normalized to a concentration of 25 μM and spotted onto Corning UltraGAPS-II slides using an OmniGrid Robot (GeneMachines). Each gene was represented once on the microarray. All six of the control spots were represented 8 times on the array, once in each subgrid. Oligonucleotide design, synthesis, and array construction

were performed at the Research Technology Support Facility at Michigan State University, East Lansing, MI, USA. RNA isolation, labeling, and hybridization were carried out essentially as previously described (39, 42). (Information regarding the microarray platform can be found at NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under GEO platform number GPL6366).

Five biological replicates were performed for each of the two sets of microarray experiments. In addition, technical replication was achieved by switching the dyes used for labeling each biological replicate. Therefore, each RNA sample was subjected to two hybridizations and values used for subsequent data analysis were averages of the dye swap values. The first set of experiments, referred to as the bile shock experiments, compared the gene expression profiles of cells before exposure to 0.5% bile to those that had been exposed for 15 minutes. The second set of experiments, referred to as the bile adaptation experiments, compared the gene expression profiles of cells before exposure to 0.5% bile to those that had begun growing again after exposure (Figure 2.1).

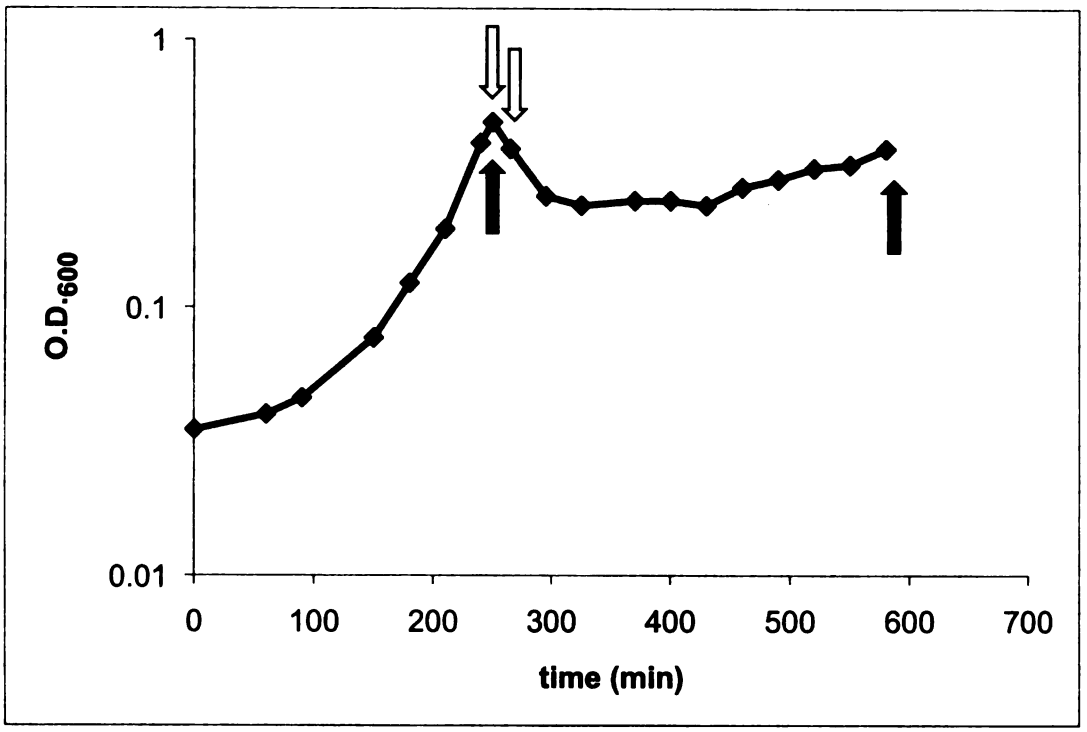

Figure 2.1. Representative growth curve of *L. reuteri* ATCC 55730 used for microarray experiments (arrows represent time points where samples were taken for RNA isolation). Open arrows represent samples for bile shock experiments; filled arrows represent samples for bile adaptation experiments. 0.5% oxgall was added at 250 minutes.

Microarray data was analyzed using iterative outlier analysis with three iterations as previously described (4, 39). Briefly, iterative outlier analysis calculates the geometric mean and standard deviation of the entire dataset. Differentially expressed genes (outliers) were selected as being more than 2.5 standard deviations away from the mean of the population. To identify additional differentially expressed genes in the dataset, the outliers were removed and the geometric mean and standard deviations were recalculated and any genes that

were more than 2.5 standard deviations from the mean were identified as differentially expressed.

Mutant construction. Mutants were created using the system developed by Russell and Klaenhammer (29) and modified for use in *L. reuteri* by Walter *et al* (44). Briefly, 200 – 300 bp from the gene of interest were PCR-amplified from *L. reuteri* ATCC 55730 and cloned into pORI28. The plasmid with the insertion was then transformed into *E. coli* EC1000, a carrier strain that contains the RepA protein needed for pORI28 to replicate; the transformed cells were grown in the presence of erythromycin and kanamycin (EC1000). pORI28 with the insertion was then extracted and transformed into *L. reuteri* ATCC 55730 cells containing pVE6007. pVE6007 is a helper plasmid that provides RepA (also allowing pORI28 to replicate). *L. reuteri* cells containing both plasmids were grown aerobically without shaking at the permissive temperature of 35°C in the presence of chloramphenicol and erythromycin for 18 hours. This culture was then diluted 1:200 and grown aerobically in the presence of erythromycin without shaking at 45°C for 8-24 hours. The 45°C culture was then plated onto MRS + erythromycin plates and incubated at 45°C for 24 hours. Isolated colonies were then obtained by streaking onto fresh MRS + erythromycin plates and incubated at 45°C for another 24 hours to ensure loss of pVE6007. Individual colonies were then selected for integration of pORI28 based on erythromycin resistance and screened for chloramphenicol sensitivity to confirm loss of pVE6007 at 37°C. Colonies that had lost pVE6007 were then screened for the correct insertion by

PCR-amplification of both flanking regions (one primer annealing to the chromosome outside of the region cloned into pORI28 and one primer annealing to pORI28) and confirmation of the absence of the correctly sized wild-type gene (also through PCR). PCR primers are available by request.

Bile stress assays. To determine levels of bile resistance for the mutants, the percent survival of wild-type and mutant cultures was determined after 30 minutes of exposure to 0.3% bile. In short, cultures were grown under microaerobic conditions in MRS at 37°C to an $O.D._{600}$ = 0.5. Samples were taken and colony counts were determined by dilution plating. 0.3% oxgall was then added to each culture, and after 30 minutes, colony counts were again determined. The before bile and after bile colony counts were used to determine percent survival for each strain. All growth curves and viability plating were performed three times for each strain, with the exception of the wild-type experiments, which were repeated eight times.

RESULTS

***Lactobacillus reuteri* ATCC 55730 is able to grow in physiologically relevant concentrations of bile.** One important characteristic for probiotic bacterial strains is the ability to remain viable during passage through the gastrointestinal tract, including the ability to overcome exposure to bile stress in the small intestine. Growth experiments were conducted to determine the response of *L.*

reuteri ATCC 55730 to physiological concentrations of bovine bile. In general, when 0.05 to 0.1% bile was added to an early or mid-log culture ($O.D._{600}$ = 0.2 or 0.5), the culture continued growing, although at a slightly reduced rate. The doubling time of the culture would slow from 38 minutes before the addition of bile to 50 minutes after the addition of bile. When concentrations of bile ranging from 0.3% to 5% were added, we observed a period of growth arrest followed by a resumption of growth. However the doubling time in the presence of these higher bile concentrations was 3-4 times slower than prior to treatment with bile.

The growth-phase of *L. reuteri* cells also influenced the ability of bile to affect cell growth and viability. Early log-phase cells (OD_{600} = 0.2) were the most resistant to the effects of bile treatment. Addition of bile at later stages of log-phase growth and early stationary phase indicated that cells become more susceptible to bile as the culture density increases (as measured by a decrease in the optical density and cell viability of the culture after the addition of bile). Active growth appears to be required for this effect as late stationary phase cultures were completely resistant to bile stress, even at concentrations of 5%.

Microarray analysis of genes involved in bile shock and adaptation. We used DNA microarrays to characterize both the bile shock response and bile adaptation response of *L. reuteri*. When cells encounter stress they often respond by altering their gene expression program to effectively counteract stress-induced damage. Because *L. reuteri* exhibits a biphasic response to bile

exposure we measured the global RNA profiles of cells that were paused for growth (which we denote as bile shock) and cells that had resumed growth in the presence of bile (bile adaptation).

i. Bile shock. In order to determine the genes involved in the bile shock response, microarray experiments were carried out to compare the gene expression profiles of mid-log cells that had not been exposed to bile to cells that had been exposed to 0.5% bile for 15 minutes. Eighty-eight genes were found to have significant expression changes, with 45 genes over-expressed and 43 genes under-expressed after 15 minutes of bile exposure. The majority of under-expressed genes are classified as being involved in substrate transport and metabolism, which is expected due to the lack of growth observed upon exposure to bile (Table 2.2). The over-expressed genes are found in a wide variety of classes and several have known roles in adaptation to other types of stresses (see below and discussion).

Table 2.2. Classes[a] of genes differentially expressed during the first 15 minutes of exposure to 0.5% bile.

Gene classification	Number of genes over-expressed during bile shock	Number of genes under-expressed during bile shock
Energy production and conversion	0	4
Cell division and envelope biogenesis[b]	1	3
Substrate transport and metabolism[c]	4	21
Translation, ribosomal structure and biogenesis	2	2
Transcription	8	1
Replication, recombination and repair	3	3
Posttranslational modification, protein turnover, chaperones	6	2
Defense mechanisms	3	0
Unknown functions	18	7
Total	45	43

[a]Genes were classified based on COG domains found in the protein sequence through a search of the JGI Integrated Microbial Genomes database.
[b]This class represents two COG categories: cell cycle control, cell division, and chromosome partitioning and cell wall/membrane/envelope biogenesis.
[c]This class represents multiple COG categories that include transport and metabolism of carbohydrates, amino acids, nucleotides, coenzymes, lipids, inorganic ions, and secondary metabolites.

ii. Bile adaptation. The expression profiles of mid-log cells that had not been exposed to bile were also compared to the profiles of cells that had resumed growth in the presence of 0.5% bile. After analysis of this set of array experiments, 84 genes were found to have significant expression changes, with

17 being over-expressed during the adaptation stage and 67 being under-expressed. Again, the majority of under-expressed genes during growth in bile are classified as being involved in substrate transport and metabolism; in addition, genes involved in energy production, translation and ribosome structure and biogenesis, and genes of unknown function are also under-expressed. Most of these changes are likely due to the dramatic reduction in growth rate of *L. reuteri* cells grown in the presence of bile. There were 17 genes that were significantly over-expressed during growth in bile; over half of these genes are annotated as having unknown functions (Table 2.3). The expression patterns of a subset of genes that were differentially expressed were found to overlap between the two sets of microarray experiments, with eight genes being over-expressed and 12 genes being under-expressed during both bile shock and adaptation. (A full description of the differentially expressed genes discovered in all of the microarray experiments can be found in Supplementary Tables 2.7 to 2.10 (located at the end of this chapter). A complete dataset of microarray data can be found at NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE10155).

Table 2.3. Classes[a] of genes differentially expressed during growth in the presence of 0.5% bile.

Gene classification	Number of genes over-expressed during bile adaptation	Number of genes under-expressed during bile adaptation
Energy production and conversion	0	11
Cell division and envelope biogenesis[b]	0	4
Substrate transport and metabolism[c]	5	23
Translation, ribosomal structure and biogenesis	0	11
Transcription	2	1
Replication, recombination and repair	0	4
Intracellular trafficking, secretion, and vesicular transport	0	2
Signal transduction mechanisms	0	2
Defense mechanisms	1	0
Unknown functions	9	9
Total	17	67

[a]Genes were classified based on COG domains found in the protein sequence through a search of the JGI Integrated Microbial Genomes database.
[b]This class represents two COG categories: cell cycle control, cell division, and chromosome partitioning and cell wall/membrane/envelope biogenesis.
[c]This class represents multiple COG categories that include transport and metabolism of carbohydrates, amino acids, nucleotides, coenzymes, lipids, inorganic ions, and secondary metabolites.

Mutations in three genes, *lr0085*, *lr1516*, and *lr1864*, decrease the ability of cells to survive bile shock. Bile salts have been proposed to cause a wide-range of cellular effects, including cell wall or membrane damage, DNA damage,

protein denaturation, oxidative stress, and low intracellular pH (3). Several genes were chosen for mutation based on their proposed functions in adapting to a variety of stresses. Disruptions were created using the pVE6007/pORI28 system in nine genes that were found to be significantly over-expressed during the bile exposure of *L. reuteri* ATCC 55730 (Table 2.4). Two Clp chaperones (*lr0004* [*clpE*]) and *lr1864* [*clpL*]) were disrupted; Clp chaperones have been implicated in the heat shock response of *Bacillus subtilis*, as well as other gram-positive organisms (8, 11). The *dps* gene (*lr1706*) and a putative esterase (*lr1516*) were also disrupted to investigate the proposed oxidative stress and cell wall damage effects of bile. The putative esterase (*lr1516*) belongs to a cluster of orthologous genes (COG) that includes β-lactamase class C and other various penicillin-binding proteins (20). Three other genes of unknown function were also chosen for disruption: *lr1291*, a putative metalloproteinase, *lr1351*, a conserved membrane protein, and *lr0085*, a gene of unknown function that appears to be specific to the species *L. reuteri*. Finally, two multidrug resistance transporters were disrupted (*lr1265* and *lr1584*). *E. coli* has the ability to actively pump bile salts out of the cell, and efflux pumps, similar to drug resistance transporters, have been found to have a major role in this activity (37).

Table 2.4. Fold gene expression changes in the presence of bile for genes chosen for disruption.

Gene	Accession no.[a]	Annotation	Fold-change[b] during bile shock	Fold-change[b] during bile adaptation
lr0004	EF421856	Clp chaperone (ClpE)	**5**	1.4
lr0085	DQ233699	Hypothetical protein	**3.3**	**2.5**
lr1265	EU038268	Multidrug resistance protein (ABC transporter family)	**3.9**	1.5
lr1291	AY970991	Metalloproteinase	**2.8**	1.6
lr1351	DQ233687	Conserved membrane protein of unknown function	**3**	**4.1**
lr1516	DQ219970	Putative esterase	**7.3**	**2.8**
lr1584	EU038252	Multidrug resistance protein (Major facilitator superfamily)	1.6	**2.2**
lr1706	EU038255	Dps	**2.6**	**2.1**
lr1864	DQ219976	Clp chaperone (ClpL)	**3.2**	1.4

[a]GenBank accession numbers are provided.

[b]Fold-changes in **bold** were found to be significantly different based on the outlier analysis.

Each of the nine mutants was subjected to treatment with various concentrations of bile to determine which mutants are defective in surviving bile shock. Based on these preliminary experiments, the survival of six of these mutant strains were quantitated and compared to the viability of wild-type cells after 30 minutes of 0.3% bile exposure; the other three mutants (*lr0004*, *lr1291*, and *lr1351*) showed no defect in bile shock and were not further tested. Using a Student's t-test, it was determined that the survival rate after 30 minutes of bile exposure for three of the mutants (*lr1864*, *lr1516*, and *lr0085*) was significantly different from that of

wild-type ($p<0.001$). Mutations in *dps* and the two multidrug resistance transporters (*lr1706*, *lr1584*, and *lr1265*) did not have a significantly different survival rate from wild-type cells (Table 2.5; Figure 2.2).

Table 2.5. Effects of 0.3% bile exposure on cell viability and final culture density.

Disrupted gene	Accession no.[a]	Annotation	Percent survival[b]	O.D.$_{600}$[c]
lr0085	DQ233699	Unknown	16 ± 3%*	1.7±0.0
lr1265	EU038268	Multidrug resistance protein (ABC transporter family)	66 ± 23%	1.0±0.1*
lr1516	DQ219970	Putative esterase	8 ± 2%*	2.1±0.1
lr1584	EU038252	Multidrug resistance protein (Major facilitator superfamily)	69 ± 32%	0.6±0.0*
lr1706	EU038255	Dps	55 ± 21%	1.3±0.3
lr1864	DQ219976	Clp chaperone (ClpL)	3 ± 1%*	1.6±0.2
		Wild-type cells	54 ± 16%	1.6±0.2

[a]GenBank accession numbers are provided.

[b](Percent survival 30 minutes after exposure. Data is presented as percent survival ± standard deviation.

[c]Data is presented as O.D.$_{600}$ ± standard deviation after growth had ceased.

*$p<0.001$ compared with wild-type.

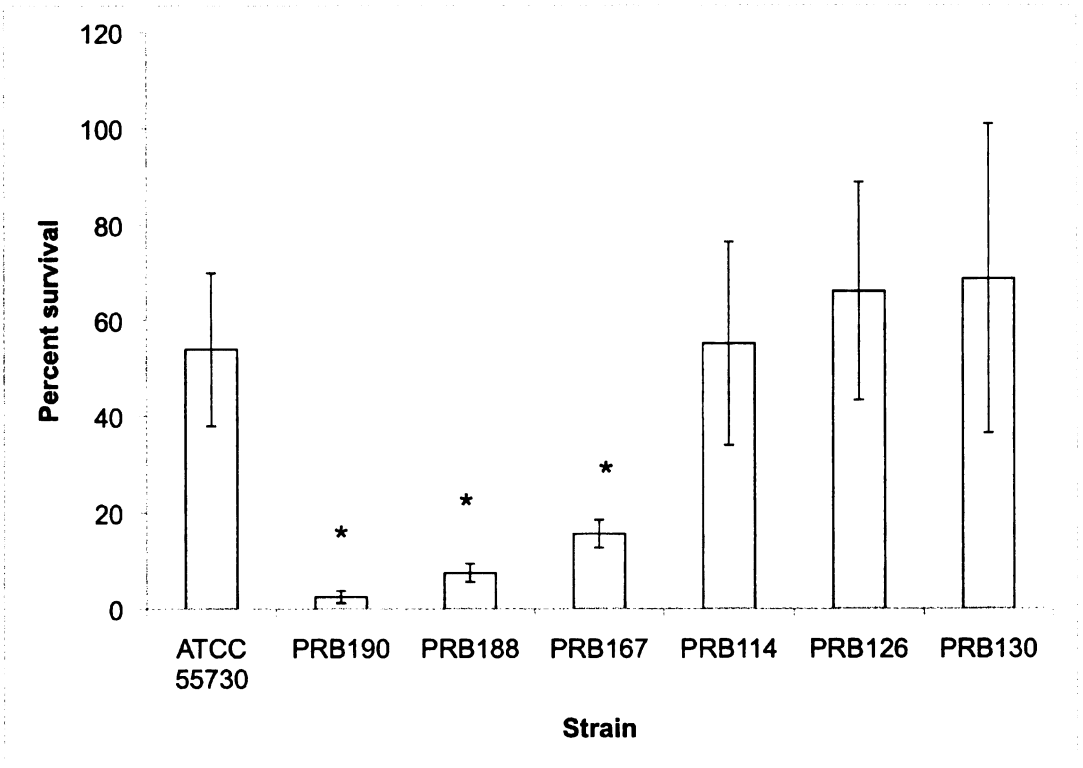

Figure 2.2. Comparison of survival after 30 minutes of exposure to 0.3% oxgall for *L. reuteri* ATCC 55730 wild-type and PRB190 (*lr1864* - ClpL), PRB188 (*lr1516* - putative esterase), PRB167 (*lr0085* - unknown), PRB114 (*lr1706* - Dps), PRB126 (*lr1265* - multidrug resistance protein in the ABC transporter family), and PRB130 (*lr1584* - multidrug resistance protein in the major facilitator superfamily) mutant strains. Cultures were plated onto MRS plates after bile exposure to determine the number of viable cells. Error bars represent standard deviation. *$p < 0.001$ compared with wild-type.

Mutations in a putative operon encoding a multidrug resistance protein and a hypothetical protein decrease the ability of *L. reuteri* to adapt in the presence of bile. When testing the mutant strains for survival in the presence of bile, it was observed that the *lr1584* mutant did not adapt to grow in the presence of bile, even after extended incubations (24 hours). The final culture density

obtained for the *lr1584* mutant was found to be three-fold lower than that of the wild-type strain. Additional experiments revealed that most of the other mutants were not affected in their ability to adapt and grow in the presence of bile. The exceptions were the *lr1265* mutant, which also obtains a lower culture density than wild-type, and *lr1516* mutant, which obtains a slightly higher culture density (Table 2.5). The decreased ability of the *lr1584* and *lr1265* mutant strains to adapt to the presence of bile suggests that multidrug resistance efflux pumps play a role in this strain's bile response. Efflux pumps have already been shown to play important roles in the bile response of other bacteria (18, 34, 37).

The *lr1584* gene is found in a putative operon with a gene encoding a conserved hypothetical protein, *lr1582*. *lr1582* is also found to be significantly over-expressed in the presence of bile; therefore, we were concerned about the possible polar effects the disruption in *lr1584* would have on the downstream gene, *lr1582*. To distinguish between the effects of the *lr1584* mutation and the possible polar effects on *lr1582*, a separate mutant strain containing a disruption in *lr1582* was created and tested for its ability to adapt in the presence of bile. This strain also showed an adaptation defect; the final culture density of the *lr1582* mutant was two-fold lower than that of wild-type cells. This demonstrates that the adaptation defect seen in the *lr1584* mutant cannot be fully explained by polar effects on the downstream gene, *lr1582*, and suggests that both genes in this operon play a role in *L. reuteri*'s adaptation to bile.

DISCUSSION

The ability of a bacterium to resist bile stress is one of the criteria often used in the selection of a potential probiotic. Bile is a complex mixture of bile acids, phospholipids, proteins, ions, and pigments that has potent antimicrobial properties, particularly against gram-positive bacteria. In this study we have identified several genes that participate in the ability of *Lactobacillus reuteri* ATCC 55730 to tolerate bile shock and to resume growth in the presence of bile (bile adaptation).

Stress responses activated in *L. reuteri* based on gene expression data. The gene expression data indicate that membrane/cell wall stress, oxidative stress, DNA damage, and protein denaturation occur when *L. reuteri* is exposed to bile. Several of these pathways have been previously shown to be involved in dealing with various forms of stress in other bacteria. First, the Clp chaperones ClpE and ClpL are induced 15 minutes after bile addition as is their known transcriptional regulator in other gram-positive organisms, CtsR. CtsR is a repressor of multiple clp chaperones in *Listeria monocytogenes* and *Bacillus subtilis* and also represses its own expression. Previous work has shown that the induction of the CtsR stress regulon is transient with an initial peak of expression under heat or salt stress that then is reduced after a period of time (32). Consistent with this mode of regulation in other bacteria is the fact that we observe that *ctsR* and the *clp* chaperones are overexpressed only during bile

shock and not during bile adaptation. *clpL* was specifically required for *L. reuteri* to resist bile shock while the *clpE* mutant did not survive significantly different than wild-type cells. Repeated attempts to construct a mutation in the *ctsR* gene were unsuccessful. Second, we also observed increased expression of *dps*, a protein involved in several types of stress adaptation in *Escherichia coli* including oxidative stress, irradiation, metal toxicity, heat stress, and pH stress (22, 24). However, disruption of *dps* in *L. reuteri* did not significantly affect their ability to survive bile shock or adapt to the presence of bile. Finally, two additional stress response genes and one additional pathway were also induced. A homolog of the gene *gls24* (*lr2108*) was induced; Gls24 was previously identified as a bile-induced protein in *Enterococcus faecalis*. Subsequent genetic analysis indicated it was required for the ability of *E. faecalis* to survive bile exposure, however no molecular function for Gls24 is known (12). The gene *lr1346* encodes a homolog of the phage shock transcriptional regulator PspC, which is proposed to be involved in sensing membrane stress during phage infection. Given that bile likely induces membrane stress it is possible that *lr1346* plays a role in bile stress survival. Unfortunately we were unable to disrupt *lr1346* due to limitations of our gene knockout technology. Lastly, genes of the arginine deiminase pathway were specifically induced during bile adaptation. This pathway has been implicated in the ability to resist mild pH shock in bacteria (21).

The identification of *lr1516*, a putative esterase of the serine β-lactamase-like superfamily, as a key enzyme in responding to bile stress suggests these cells

are experiencing cell envelope damage upon exposure to bile. Lr1516 contains the signature SxxK active site motif associated with these enzymes, which also include the D-alanyl-D-alanine carboxypeptidases. These enzymes are involved in the breakdown and reorganization of peptidoglycan, and thus we expect that *lr1516* may play a similar role when adapting to bile and acid stress (42).

Bile adaptation. Because *L. reuteri* has been shown to colonize, at least temporarily, the small intestine, we were interested in determining if *L. reuteri* can thrive in the presence of bile. Our results demonstrate that *L. reuteri* can sustain growth in the presence of bile concentrations as high as 5%. Interestingly the data suggest a multidrug resistance transporter (*lr1584*) is required for this ability to grow in the presence of bile, suggesting that removal of bile or another toxic metabolite from the cytoplasm is required for growth. Lr1584 is a member for the EmrB/QacA subfamily of the major facilitator superfamily of multidrug resistance transporters. EmrB has previously been shown to play a role in bile resistance and efflux of bile in *Escherichia coli*. (37).

Interestingly, *lr1584* is found in an operon upstream of a conserved hypothetical protein, *lr1582*; this operon is conserved in many lactic acid bacteria and is over-expressed during exposure of *L. reuteri*, *L. acidophilus*, and *E. faecalis* to bile (25, 35). Due to the limited genetic tools available for use in *L. reuteri*, we were not completely able to distinguish between the effects of disrupting *lr1584* and possible polar effects this disruption may have on the downstream *lr1582*. A

separate mutant strain with a disruption in *lr1582* was created and tested for bile adaptation. The *lr1582* mutant does result in an adaptation defect, although it is not as severe as the defect found in the *lr1584* mutant strain. The final culture density of the *lr1584* mutant is approximately 3-fold lower than that of the wild-type cells, while the final culture density of the *lr1582* mutant is approximately 2-fold lower than wild-type cells. This suggests that both genes may contribute to the adaptation defect that we have observed. Further investigation is required to elucidate the specific role of each gene in bile adaptation.

Genes that provide protection in bile stress also protect against acid stress. We identified three genes in *L. reuteri* that were induced by bile stress and that significantly reduced their ability to survive bile shock when disrupted. Two of these proteins have recently been shown to be induced by a strong reduction in pH and are necessary for increased survival at low pH (42). Both *lr1864* (*clpL*) and *lr1516* (putative esterase) play a role in surviving the initial shock of acid and bile stress.

Indeed nearly one-third of the genes Wall et al. found to be differentially regulated in acid stress conditions were also altered under bile stress conditions (Table 2.6) (42). This indicates that once cells experience acid stress in the stomach many of the important pathways for dealing with bile stress in the small intestine will already be activated.

Table 2.6. Genes over-expressed or under-expressed during both 15 minutes of bile exposure (0.5% oxgall) and 15 minutes of acid stress (pH 2.7) (42).

Gene	Accession no.[a]	Annotation
Over-expressed		
lr0597	DQ219952	Thioredoxin domain-containing protein
lr0922	DQ074860	Extracellular hydrolase
lr1139	AY970988	Conserved intracellular protein of unknown function
lr1191	DQ219999	Conserved membrane protein of unknown function
lr1468	DQ219968	Putative transcriptional regulator
lr1515	DQ074905	Unknown extracellular protein
lr1516	DQ219970	Putative esterase
lr1797	DQ219975	Phosphatidylglycerolphosphatase A and related proteins
lr1864	DQ219976	ClpL ATPase with chaperone activity
lr1937	DQ219979	Conserved intracellular protein of unknown function
lr1993	DQ219980	Putative transcriptional regulator
lr2045	DQ219981	Phage-associated protein
Under-expressed		
lr0190	DQ219995	Transcriptional regulator
lr0195	DQ219996	Putative 5-formyltetrahydrofolate cyclo-ligase
lr0382	AY971000	Putative branched-chain amino acid transport protein
lr0733	DQ219954	Conserved intracellular protein of unknown function
lr0862	DQ219957	Asp-tRNAAsn/Glu-tRNAGln amidotransferase C subunit
lr1240	DQ219962	Recombinational DNA repair ATPase
lr1297	DQ219963	Thymidine kinase
lr1432	DQ220005	Ribosomal protein S1
lr1434	DQ074902	Unknown extracellular protein
lr1628	DQ219972	Conserved intracellular protein, MarZ, of unknown function

[a]GeneBank accession numbers are provided.

Comparison of multiple genomic studies of bile stress in lactic acid bacteria. Multiple genomic studies have now been completed that identified genes important for bile tolerance in different species of *Lactobacillus* and *Enterococcus* (5, 6, 25, 35). Although different culture conditions, types of bile, and species were used for these studies, which resulted in a limited overlap in the genes identified in these studies, there are some common themes that have

emerged. In both *Lactobacillus plantarum* and *Lactobacillus acidophilus* several genes involved in the reorganization of the cell envelope were induced. Thus dealing with membrane stress is a common theme that has emerged from these three studies. In addition, the operon containing *lr1584* (multidrug resistance transporter) and *lr1582* (unknown function) is also conserved as an operon in *L. acidophilus* and *Enterococcus faecalis*. Interestingly, both homologs in *E. faecalis* and *L. acidophilus* are also overexpressed when cells were exposed to bile, indicating the function of this operon in bile adaptation may be conserved in other lactic acid bacteria (25, 35). Lastly, *clp* proteases were identified in *L. acidophilus* as being upregulated by bile stress as we found with *clpL* in our study, further supporting that protein denaturation is one stress being encountered by bile treated cells.

One common finding that is not easily explained is the reduction in gene expression of *recF*, which encodes a protein that participates in the repair of DNA damage during active DNA replication. Since bile has been implicated in generating DNA damage, on the surface it seems that a reduction in the expression of RecF would not be productive. However, recent evidence indicates that RecF is predominantly utilized in DNA repair at replication forks during active growth. The reduction in *recF* expression may simply indicate a reduction in growth rate in the presence of bile.

Although there were significant similarities in the transcriptional profiles between the *E. faecalis*, *L. acidophilus*, *L. plantarum*, and *L. reuteri* responses to bile treatment; overall there was much more discordance in the data than similarities. The lack of concordance may be due to the different physiological strategies utilized by these organisms to adapt to bile stress. In addition, the differences in experimental strategy (the type of bile used, the way bile was administered, and for how long) likely also played a significant role in the differences that were noted. Bron et al. exposed *L. plantarum* to 0.1% porcine bile on plates and looked at the response to three days of exposure, while Pfeiler et al. conducted their *L. acidophilus* experiments in liquid media containing 0.5% oxgall with 30 minutes of exposure (6, 25). The use of purified bile acids or different sources of bile will also have different effects on cell physiology (3).

Bile has been implicated as a potential signaling molecule that would indicate to a bacterium that it had entered the small intestine. Such a signal could serve to stimulate the organism to adapt its physiology to optimize growth and survival in the GI tract. Several candidates from these experiments have now been identified *in vitro*. Future work in relevant animal models will determine if the strategies uncovered here are important for survival *in vivo*.

SUPPLEMENTAL INFORMATION

Table 2.7. Genes over-expressed after 15 minutes of exposure to 0.5% oxgall.

Gene name	Accession no.[a]	Functional Classification[b]	Fold-change
Cell division and envelope biogenesis[c]			
lr1816	DQ074937	ABC-type transport system involved in lipoprotein release permease component	3.4
Defense mechanisms			
lr1265	EU038268	Multidrug resistance ABC transporter ATP-binding and permease protein	3.9
lr1516	DQ219970	Putative esterase	7.3
lr1817	EU038262	ABC transporter, ATP-binding protein	2.3
Unknown function			
lr0085	DQ233699	Hypothetical protein	3.3
lr0540	EU038232	Conserved hypothetical protein	2.9
lr0542	EU038234	Conserved hypothetical protein	3.1
lr0543	EU038235	CvpA family protein	2.3
lr0922	DQ074860	Extracellular hydrolase	2.2
lr1191	DQ219999	Conserved membrane protein of unknown function	3.2
lr1139	AY970988	Conserved intracellular protein of unknown function	2.5
lr1350	DQ233686	Intracellular protein of unknown function	5.3
lr1351	DQ233687	Conserved membrane protein of unknown function	3.1
lr1515	DQ074905	Unknown extracellular protein	13.6
lr1684	DQ857799	Conserved hypothetical protein	2.5
lr1740	EU038256	Acetyltransferase (EC 2.3.1.-)	3.5
lr1755	EU038257	Conserved hypothetical protein, putative pseudogene	2.5

Table 2.7 (cont'd)

lr1937	DQ219979	Conserved intracellular protein of unknown function	2.3
lr1990	DQ074949	Unknown extracellular protein	2.5
lr1994	EF421933	Conserved hypothetical protein	3.1
lr2045	DQ219981	Phage-associated protein	2.3
lr2108	DQ233697	Putative stress response protein	2.6
Posttranslational modification, protein turnover, and chaperones			
lr0004	EF421856	ATP-dependent clp protease ATP-binding subunit clpE	4.9
lr0597	DQ219952	Thioredoxin domain-containing protein	2.2
lr1268	DQ857779	Hypothetical protein	2.4
lr1291	AY970991	Putative metalloproteinase gene	2.8
lr1788	EU038260	Thioredoxin	2.2
lr1864	DQ219976	ClpL ATPase with chaperone activity	3.2
Replication, recombination, and repair			
lr0541	EU038233	Holliday junction resolvase-like protein	3.2
lr0685	EU421874	Transposase	3.3
lr2132	EU038269	DNA mismatch repair protein MutS2	2.5
Transcription			
lr0783	EF421875	Transcriptional regulator ctsR	2.3
lr1264	EU038242	Transcriptional regulator, MarR family	3.8
lr1346	EU038243	Stress-responsive transcriptional regulator PspC	2.7
lr1347	EU038244	Transcriptional regulator, TetR family	4.3
lr1468	DQ219968	Putative transcriptional regulator	3.1
lr1573	E7038251	Transcription regulator, AsnC-type	2.3
lr1815	EU038261	Transcriptional regulator	2.5
lr1993	DQ219980	Putative transcriptional regulator	2.2

Table 2.7 (cont'd)

Translation, ribosomal structure and biogenesis			
lr1067	EF421891	Ribosome-associated protein Y (PSrp-1)	2.4
lr1786	E7038259	Ribosomal large subunit pseudouridine synthase, RluD subfamily	3.1
Substrate transport and metabolism[d]			
lr0621	EU038237	Transcriptional regulator, GntR family / aminotransferase	2.2
lr1117	DQ857776	Thiamine pyrophosphate-requiring enzymes	2.3
lr1706	EU038255	DPS (DNA protection during starvation) protein	2.6
lr1797	DQ219975	Phosphatidylglycerolphosphatase A and related proteins	2.5

a GenBank accesion numbers are provided.

b Genes were classified based on COG domains found in the protein sequence through a search of the JGI Integrated Microbial Genomes database.

c This class represents two COG categories: cell cycle control, cell division, and chromosome partitioning and cell wall/membrane/envelope biogenesis.

d This class represents multiple COG categories that include transport and metabolism of carbohydrates, amino acids, nucleotides, coenzymes, lipids, inorganic ions, and secondary metabolites.

Table 2.8. Genes over-expressed during growth in the presence of 0.5% oxgall.

Gene name	Accession no.[a]	Functional Classification[b]	Fold-change
Defense mechanisms			
lr1516	DQ219970	Putative esterase	2.8
Unknown function			
lr0085	DQ233699	Hypothetical protein	2.5
lr0890	DQ233682	Putative acetyltransferase	2.4
lr1035	DQ857774	Conserved hypothetical protein	2.2
lr1348	DQ074898	predicted membrane protein	2.1
lr1350	DQ233686	Intracellular protein of unknown function	5.4
lr1351	DQ233687	Conserved membrane protein of unknown function	4.1
lr1515	DQ074905	Unknown extracellular protein	3.7
lr1582	EF421918	Conserved hypothetical protein	2.8
lr2108	DQ233697	Putative stress response protein	2.3
Transcription			
lr1347	EU038244	Transcriptional regulator, TetR family	2.3
lr1518	DQ233704	ArgR Arginine repressor	2.2
Substrate transport and metabolism[c]			
lr1019	DQ857773	ArcC Carbamate kinase	2.0
lr1020	DQ233707	ArgF Ornithine carbamoyltransferase	2.7
lr1517	DQ233695	ArcA Arginine deiminase	2.6
lr1584	EU038252	Permease of the major facilitator superfamily	2.2
lr1706	EU038255	DPS (DNA protection during starvation) protein	2.1

a GenBank accesion numbers are provided.

b Genes were classified based on COG domains found in the protein sequence through a search of the JGI Integrated Microbial Genomes database.

c This class represents multiple COG categories that include transport and metabolism of carbohydrates, amino acids, nucleotides, coenzymes, lipids, inorganic ions, and secondary metabolites

Table 2.9. Genes under-expressed after 15 minutes of exposure to 0.5% oxgall.

Gene name	Accession no.[a]	Functional Classification[b]	Fold-change
Cell division and envelope biogenesis[c]			
lr0548	EU038236	Cell division initiation protein	3.2
lr0957	DQ857880	Capsular polysaccharide biosynthesis protein	1.9
lr1629	EU038253	S-adenosyl-methyltransferase MraW	2.4
Energy production and conversion			
lr0599	DQ233700	CitC Citrate lyase synthetase partial CDS	1.9
lr0600	DQ240820	SfcA malic enzyme	2.1
lr1073	EF534266	Fumarase	2.0
lr1882	DQ233726	PduA propanediol utilization protein: putative microcompartment protein	2.9
Unknown function			
lr0196	EU038226	Rhomboid family protein	1.9
lr0733	DQ219954	Conserved intracellular protein of unknown function	2.7
lr1241	EF421898	Conserved hypothetical protein	3.0
lr1267	DQ074894	Unknown extracellular protein	2.1
lr1434	DQ074902	Unknown extracellular protein	2.0
lr1628	DQ219972	Conserved intracellular protein, MarZ, of unknown function	2.2
lr2134	EU038270	ComG operon protein 1	2.4
Posttranslational modification, protein turnover, and chaperones			
lr1373	EU038246	FeS assembly ATPase SufC	1.9
lr1774	EU038258	Glutaredoxin-like protein NrdH	3.0
Replication, recombination, and repair			
lr0044	EF421958	ATPase related to the helicase subunit of the Holliday junction resolvase	2.3
lr0311	DQ857869	Nuclease subunit of the excinuclease complex	2.0
lr1240	DQ219962	Recombinational DNA repair ATPase	3.4

Table 2.9 (cont'd)

Transcription			
lr0190	DQ219995	predicted transcriptional regulator	2.5
Translation, ribosomal structure and biogenesis			
lr0862	DQ219957	Asp-tRNAAsn/Glu-tRNAGln amidotransferase C subunit	2.6
lr1432	DQ220005	Ribosomal protein S1	2.4
Substrate transport and metabolism[d]			
lr0137	EF537897	Spermidine/putrescine ABC transporter, permase protein	3.4
lr0160	EF547651	Sugar kinase, ribokinase family	4.3
lr0187	EU038223	Dihydroneopterin aldolase	2.3
lr0188	EU038224	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	2.0
lr0189	EU038225	GTP cyclohydrolase I	1.9
lr0195	DQ219996	Putative 5-formyltetrahydrofolate cyclo-ligase	2.2
lr0324	EF421866	Cystathionine beta-lyase (EC 4.4.1.8) / Cystathionine gamma-lyase (EC 4.4.1.1)	2.9
lr0382	AY971000	Putative branched-chain amino acid transport protein	2.1
lr0848	EU038267	Permease of the major facilitator superfamily	2.1
lr1297	DQ219963	Thymidine kinase	2.1
lr1880	DQ233724	PduC propanediol dehydratase large subunit	2.0
lr1881	DQ233725	PduB propanediol utilization protein	3.1
lr1959	DQ857828	HemA, Glutamyl-tRNA reductase	2.2
lr1960	DQ857829	Putative siroheme synthase	2.7
lr1961	DQ857830	CbiP, cobyric acid synthase	2.3
lr1963	DQ857832	CbiQ, cobalt transport protein	2.2
lr1977	DQ857846	CbiB, cobalamin biosynthesis protein	2.0
lr1978	DQ857847	CbiA, cobyrinic acid a,c-diamide synthase	2.2
lr1979	DQ857848	CobD, L-threonine-0-3-phosphate decarboxylase	3.2

Table 2.9 (cont'd)

lr2131	EU038263	Diacylglycerol kinase family protein	2.4
lr2133	EU038271	ABC-type ribose transport system, auxiliary component	2.3

a GenBank accession numbers are provided.

b Genes were classified based on COG domains found in the protein sequence through a search of the JGI Integrated Microbial Genomes database.

c This class represents two COG categories: cell cycle control, cell division, and chromosome partitioning and cell wall/membrane/envelope biogenesis.

d This class represents multiple COG categories that include transport and metabolism of carbohydrates, amino acids, nucleotides, coenzymes, lipids, inorganic ions, and secondary metabolites.

Table 2.10. Genes under-expressed during growth in the presence of 0.5% oxgall.

Gene name	Accession no.[a]	Functional Classification[b]	Fold-change
Cell division and envelope biogenesis[c]			
lr0145	DQ219992	GidB predicted S-adenosylmethionine-dependent methyltransferase partial CDS	1.7
lr0740	DQ233678	WcaA Glycosyltransferases involved in cell wall biogenesis	1.6
lr0957	DQ857880	Capsular polysaccharide biosynthesis protein	1.7
lr1645	EU038254	UDP-MurNAc-pentapeptide synthetase	1.8
Energy production and conversion			
lr0599	DQ233700	CitC Citrate lyase synthetase partial CDS	2.1
lr0600	DQ240820	SfcA malic enzyme	1.7
lr1074	EF534267	Fumarate reductase flavoprotein subunit (EC 1.3.99.1)	2.5
lr1075	EF421892	L-lactate dehydrogenase (EC 1.1.1.27)	2.8
lr1236	EF534271	Cytochrome bd-type quinol oxidase, subunit 1	1.9
lr1402	EF421914	ATP synthase subunit A	1.7
lr1403	EF421915	ATP synthase subunit C	1.6
lr1404	EU038247	ATP synthase F0, B subunit	1.8
lr1405	EU038248	ATP synthase F1, delta subunit	1.7
lr1406	EU038249	ATP synthase F1, alpha subunit	1.7
lr1418	DQ233691	COG3051 CitF Citrate lyase alpha subunit	1.9
Intracellular trafficking, secretion, and vesicular transport			
lr0252	DQ074813	COG0706 preprotein translocase subunit YidC	1.6
lr0469	DQ074824	COG0201 preprotein translocase subunit SecY	1.8

Table 2.10 (cont'd)

Unknown function			
lr1054	DQ466579	Beta-phosphoglucomutase (EC 5.4.2.6) / Glucose-1-phosphate phosphodismutase (EC 2.7.1.41)	1.6
lr1487	DQ074904	Unknown extracellular protein	1.7
lr2054	DQ219984	Phage head maturation protease	2.0
lr2057	DQ219986	Putative phage protein	1.7
lr2058	DQ219987	Putative phage protein	1.6
lr2134	EU038270	ComG operon protein 1	2.5
lr2135	EU038264	Membrane protein, putative	1.5
lr2136	EU038265	Hypothetical protein	1.6
lr2137	EU038266	Amino acid transporter	1.6
Replication, recombination, and repair			
lr0138	EU038222	Chromosomal replication initiator protein DnaA	1.6
lr0311	DQ857869	Nuclease subunit of the excinuclease complex	1.7
lr1238	EU038240	DNA gyrase, A subunit	1.8
lr1239	EU038241	DNA gyrase, B subunit	1.6
Signal transduction mechanisms			
lr0279	EF421950	Stress response membrane GTPase TypA	1.6
lr1012	EF421886	Transcriptional regulator, MarR family	1.8
Transcription			
lr1358	EU038245	DNA-directed RNA polymerase, beta subunit	1.6
Translation, ribosomal structure and biogenesis			
lr0050	EU038217	Ribosomal protein L14	2.3
lr0051	EU038218	Ribosomal protein S17	2.2
lr0062	EF421960	Ribosomal protein S8	2.1
lr0063	EU038219	Ribosomal protein L6	2.0
lr0251	DQ219950	Rnase P protein component	1.7
lr0286	EU038227	Ribosomal protein S13p	1.9
lr0287	EU038228	Ribosomal protein S11	2.0
lr0470	EF421870	Ribosomal protein L15	1.9
lr0471	EU038230	Ribosomal protein L30	1.9
lr0472	EU038231	Ribosomal protein S5	1.9
lr1996	EF421935	TetW, Tetracycline resistance protein	1.8

Table 2.10 (cont'd)

Substrate transport and metabolism[d]			
lr0092	EF421961	Deoxyribose-phosphate aldolase (EC 4.1.2.4)	3.1
lr0101	EF421948	Phosphopentomutase (EC 5.4.2.7)	2.9
lr0136	EU038221	Spermidine/putrescine ABC transporter, permease protein	1.6
lr0137	EF537897	Spermidine/putrescine ABC transporter, permase protein	1.8
lr0160	EF547651	Sugar kinase, ribokinase family	2.3
lr0324	EF421866	Cystathionine beta-lyase (EC 4.4.1.8) / Cystathionine gamma-lyase (EC 4.4.1.1)	2.1
lr0467	EU038229	Adenylate kinase (ATP-AMP transphosphorylase)	1.8
lr0673	EU038238	Purine nucleoside phosphorylase	1.7
lr0674	EU038239	Pyrimidine-nucleoside phosphorylase	2.9
lr1009	EF421883	(acyl-carrier-protein) S-malonyltransferase, FabD	1.6
lr1013	EF421887	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase, FabA	1.8
lr1198	DQ074887	HisJ ABC-type amino acid transport/signal transduction systems periplasmic component/HisM ABC-type amino acid transport system permease component	1.9
lr1287	EF421909	3-oxoacyl-(acyl-carrier-protein) synthase, FabB	1.7
lr1417	DQ233690	Triphosphoribosyl-dephospho-CoA synthetase	1.9
lr1419	DQ233692	Citrate lyase beta subunit	1.8
lr1508	EU038250	Permease, GntP family	1.7
lr1961	DQ857830	CbiP, cobyric acid synthase	1.7
lr1963	DQ857832	CbiQ, cobalt transport protein	1.6
lr1964	DQ857833	CbiN, cobalt transport protein	1.5
lr1967	DQ857836	CbiK, cobalt chelatase	1.8

Table 2.10 (cont'd)

lr1970	DQ857839	CbiH, precorrin-3B C17-methyltransferase	1.6
lr2131	EU038263	Transcription regulator	2.0
lr2133	EU038271	Ribose transport protein	2.8

a GenBank accesion numbers are provided.

b Genes were classified based on COG domains found in the protein sequence through a search of the JGI Integrated Microbial Genomes database.

c This class represents two COG categories: cell cycle control, cell division, and chromosome partitioning and cell wall/membrane/envelope biogenesis.

d This class represents multiple COG categories that include transport and metabolism of carbohydrates, amino acids, nucleotides, coenzymes, lipids, inorganic ions, and secondary metabolites.

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CHAPTER 3

CHARACTERIZATION OF THE BILE SALT HYDROLASE ACTIVITY OF *LACTOBACILLUS REUTERI* ATCC PTA 6475

INTRODUCTION

The gastrointestinal microbiota plays an important role in the normal circulation and excretion of human bile. The primary bile acids, cholate and chenodeoxycholate, are synthesized in the liver from cholesterol. Before secretion into the small intestine, these bile acids are conjugated to either a glycine or taurine molecule, which improves the solubility and detergent properties of these molecules (18). As bile passes through the small intestine into the colon, the bile acids that are not reabsorbed in the ileum are modified by gastrointestinal bacteria. The first step in this modification is removal of the taurine or glycine molecule through the activity of bile salt hydrolase enzymes. This modification then allows for further alterations such as those carried out by bacteria with 7α-dehydroxylation activity. This removal of a hydroxyl group converts cholate and chenodeoxycholate into the secondary bile acids, deoxycholate and lithocholate, respectively (2).

Bile salt hydrolase activity has been identified in a wide range of commensal gastrointestinal microorganisms, including both bacteria and archaea (19), and it

is often suggested that bile salt hydrolase activity contributes to successful persistence in and colonization of the host gastrointestinal tract. Tanaka et al (27) examined 300 strains of lactic acid bacteria and found a strong correlation between the environment from which the organisms were isolated and the presence of bile salt hydrolase activity. The presence of bile salt hydrolase activity in most intestinal or fecal isolates, and the absence of the activity from strains isolated from milk or vegetable products suggests that there may be a selective advantage in the gastrointestinal tract for strains with deconjugation activity. Indeed, several studies have suggested that bile salt hydrolase activity can contribute to persistence in or colonization of the gastrointestinal tract (4, 11, 13, 19). It is important to note that other studies have not demonstrated this effect. Bateup et al (1) investigated five different strains of lactobacilli in regards to their ability to deconjugate bile acids and colonize the murine gastrointestinal tract. Although they demonstrated a range of bile salt hydrolase activity between the strains (from high levels of activity to no activity), all five strains colonized different locations in the gastrointestinal tract equally well. A separate study in *Lactobacillus johnsonii* showed that even disrupting all three bile salt hydrolase genes in this organism had no effect on gastrointestinal persistence as measured by fecal culturing (12).

Although the activity has been primarily studied in commensal or probiotic organisms such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. (3), recent work has suggested that this activity may act as a novel virulence

factor for some pathogens. Research in the pathogenic *Listeria monocytogenes* has revealed that the ability to deconjugate bile acids is not only controlled by PrfA, a transcriptional regulator known to control other virulence factors, but that the activity also plays an important role in the infection capabilities of this pathogen (4, 13).

Despite the fact that bile salt hydrolase activity is commonly found in gastrointestinal microorganisms and is believed to contribute to persistence of commensals and some pathogens in the gastrointestinal tract, the actual purpose of this activity is yet to be determined. Possibilities include: detoxification of conjugated bile acids, contribution to gastrointestinal persistence, nutritional benefit through utilization of the cleaved amino acid moiety, or induction of alterations in the cell membrane that may make that bacteria more resistant to other stresses encountered in the gastrointestinal tract such as defensins (3). An understanding of the function of bile salt hydrolase activity in regards to the bacteria is necessary as the activity also has important consequences on the host. Many probiotic strains exhibit the ability to deconjugate bile acids, and several of these strains are being investigated for the ability to lower serum cholesterol levels in the host. It has been proposed that administration of bacteria with bile salt hydrolase activity may lower cholesterol levels in two ways. The first proposed mechanism is that cholesterol may co-precipitate with deconjugated bile acids, thus causing a higher excretion of cholesterol. The second is that administration of bacteria with this activity may increase the

amount of deconjugated bile acids that are excreted, thus increasing the amount of bile acids that need to be synthesized from cholesterol (22, 31, 32). Despite this proposed beneficial effect, various studies have demonstrated that an excess of bacteria with bile salt hydrolase activity in the gastrointestinal tract may lead to abnormal digestive functions, gallstone formation, and possibly a higher tendency towards colon cancer (3). Therefore, a thorough understanding of the role that bile salt hydrolase activity plays regarding both the bacteria and the host is needed.

MATERIALS AND METHODS

Strains and media. The strains and vectors used in this study are listed in Table 3.1. *L. reuteri* strains were grown in MRS medium (Difco). PRB240 was grown in MRS containing 10 µg/mL erythromycin. Individual bile acids, as well as ox gall/bovine bile, were ordered from Sigma.

Table 3.1. Bacterial strains and plasmids used for this study.

Strains or plasmids	Description	Reference or source
Strains		
E. coli EC1000	Strain containing a chromosomal copy of the pWV01 *repA* gene; Kan^r	(21)
L. reuteri ATCC 55730	Human breast milk isolate	Biogaia, AB, Sweden
L. reuteri ATCC PTA 6475	Human breast milk isolate	Biogaia, AB, Sweden
PRB240	*L. reuteri* ATCC PTA 6475 bile salt hydrolase (NT01LR0487) mutant	This study
Plasmids		
pVE6007	Cm^r *repA*-positive temperature-sensitive derivative of pWV01	(23)
pORI28	Em^r *repA*-negative derivative of pWV01	(21)
pKW08	pORI28 + 289 bp insert from NT01LR0487	This study

Phenotypic plate test to determine bile salt hydrolase activity. The initial method used to determine the presence or absence of bile salt hydrolase activity for a particular strain was the plate method developed by Dashkevicz and Feighner (8). For this assay, MRS agar (Difco) was combined with 0.05 to 0.5% (w/v) of individual bile acids. The mixture was autoclaved for 20 minutes before dividing into plates. After the plates solidified, bacterial strains were struck from MRS plates onto the MRS plus 0.05 to 0.5% bile acid plates. Plates were incubated anaerobically for 72 hours at 37°C. Strains were considered to be

positive for bile salt hydrolase activity based on either precipitation of the bile acids around bacterial colonies or a change in colony morphology to white opaque matte colonies as previously described (8). Strains were assayed for the ability to deconjugate six conjugated bile acids: taurocholate (TCA), glycocholate (GCA), taurodeoxycholate (TDCA), glycodeoxycholate (GDCA), taurochenodeoxycholate (TCDCA), and glycochenodeoxycholate (GCDCA).

TLC to determine bile salt hydrolase activity. In addition to the phenotypic plate assay, thin-layer chromatography (TLC) was used to further investigate the ability of the strains to deconjugate the six bile acids mentioned above. For these assays, MRS broth was combined with 1 mM of the various conjugated bile acids listed above. The mixture was autoclaved, allowed to cool, and left in an anaerobic chamber overnight to equilibrate before inoculation with bacterial cultures. For inoculation, 100 µl of an overnight culture of *L. reuteri* grown in MRS broth was added to 10 mL of MRS plus 1 mM bile acid. These cultures were then incubated anaerobically at 37°C for up to 72 hours. Cultures were assayed for bile salt hydrolase activity at either 24 or 72 hours after inoculation. For these assays, PRB240 was grown in the presence of 10 µg/mL erythromycin.

To test for bile salt hydrolase activity, 5 mL of each culture were removed. The mixture was acidified by adding an equal volume of 1 N HCl. Bile acids were then extracted with 10 mL of ethyl acetate, and the phases were allowed to separate (separation of the two phases was aided by the addition of

approximately one mL of a saturated brine solution). The water layer was removed, and the organic layer was washed with 10 mL of a saturated brine solution. After the phases separated, the organic layer (containing the bile salts) was collected and dried over sodium sulfate. The samples were then concentrated to dryness by rotary evaporation.

For TLC, the extracted bile acids were resuspended in approximately 0.5 mL of methanol and spotted onto silica gel 60 F_{254} aluminum-backed plates, along with control bile acids (dilute control bile acid solutions contained purified bile acids dissolved in methanol). The TLC plates were run using the following solvent system: cyclohexane:ethyl acetate:acetic acid (7:23:3 v/v) as previously described (20). Using this solvent system, deoxycholate and chenodeoxycholate migrate at the same rate. A solvent system that allowed for sufficient separation of these two compounds was not identified. Plates were stained using 33 mM phosphomolybdic acid dissolved in 95% ethanol and heated to visualize spots. Cultures were considered positive for bile salt hydrolase activity based on the appearance of spots representing deconjugated bile acids on the TLC plate.

Construction of PRB240 (*L. reuteri* ATCC PTA 6475 with disruption in NT01LR0487). The following concentrations of antibiotics were used: 40 ug/mL kanamycin, 10 ug/mL chloramphenicol, 10 ug/mL erythromycin (*L. reuteri*) or 400 ug/mL erythromycin (*E. coli*). The NT01LR0487 (bile salt hydrolase) mutant was created using the system developed by Russell and Klaenhammer (26) with

modifications made for use in *Lactobacillus reuteri* (33). In short, 289 bp from NT01LR0487 from *L. reuteri* ATCC PTA 6475 was cloned into pORI28. This construct was then transformed into *E. coli* EC1000, and the transformed cells were grown in LB broth in the presence of erythromycin and kanamycin. pORI28 containing the NT01LR0487 insert was then extracted and transformed into *L. reuteri* ATCC PTA 6475 containing pVE6007. *L. reuteri* cells containing both plasmids were grown in MRS broth containing chloramphenicol and erythromycin at the permissive temperature of 35°C for 18 hours. The cultures were then shifted to the non-permissive temperature of 45°C and grown in the presence of erythromycin only. After several passages at 45°C to ensure loss of pVE6007, individual colonies were screened for integration of pORI28 at the desired location by PCR. Colonies were screened for the presence of both flanking regions (using one primer that anneals to pORI28 and one primer that anneals to the chromosome outside of the region cloned into pORI28) and the absence of a correctly-sized wild-type gene.

Growth studies to determine the effect of loss of BSH activity. Growth studies of *L. reuteri* ATCC PTA 6475 and PRB240 in the presence a synthetic "human" bile acid mixture (SHB) were conducted. SHB was made to mimic the bile acid concentrations in the small intestine (5, 15, 24) and contained the following components: 0.46 mM taurocholate, 0.93 mM glycocholate, 0.46 mM taurochenodeoxycholate, 0.93 mM glycochenodeoxycholate, 0.32 mM

taurodeoxycholate, and 0.64 mM glycodeoxycholate dissolved in MRS broth. This mixture was autoclaved, cooled, and then injected into air-tight bottles containing an anaerobic atmosphere. Overnight cultures of *L. reuteri* were used to inoculate the bottles at a starting $O.D._{600}$ = 0.02. Growth was carried out at 37° C with slow shaking. Growth curves were performed in duplicate. Samples for TLC analysis were collected at 1, 4, 7, 10, and 23 hours of growth.

In order to investigate the effect of pH on the growth of *L. reuteri* ATCC PTA 6475 and PRB240 in the presence of bile, growth experiments were performed as described above, but with the synthetic human bile mixture dissolved into a buffered version of MRS broth. For this buffered MRS, the medium was made from the various components: 10 g/L proteose peptone no. 3, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L dextrose, 1 g/L Tween 80, 2 g/L ammonium citrate, dibasic, 0.1 g/L magnesium sulfate, 0.05 g/L manganese sulfate, and 2 g/L dipotassium phosphate (sodium acetate was removed). The medium was then buffered with 200 mM MES, and the pH was adjusted to 7.0. For control experiments, MRS without sodium acetate was made, and the pH was adjusted to 7.0. Both types of medium were autoclaved for 20 minutes before use.

Samples for TLC analysis were collected from growth curves at 7 hours and 24 hours of growth and frozen at -20° C until use. pH was also monitored throughout the experiments with the use of pH strips. Growth curves were performed in triplicate.

RESULTS

***L. reuteri* ATCC PTA 6475 contains a single bile salt hydrolase that has the ability to deconjugate the six major human bile acids.** Previous work in a variety of bacteria has demonstrated that deconjugation of bile acids is due to the activity of one or more enzymes classified as choloylglycine hydrolases (EC 3.5.1.24). These bile salt hydrolase enzymes are responsible for the removal of the glycine or taurine moiety from the conjugated bile acids. A search of the *L. reuteri* ATCC PTA 6475 genome revealed a single gene, NT01LR0487, with significant similarity to known bile salt hydrolase enzymes. A disruption in the gene was created by insertion of pORI28 as described in the Materials and Methods section. The resulting strain was titled PRB240. The ability of this strain to deconjugate an assortment of bile acids was compared to that of the wild-type strain, *L. reuteri* ATCC PTA 6475, to determine the effect of this gene disruption.

Initial testing to determine the scope of activity for *L. reuteri* ATCC PTA 6475 was performed using the plate test developed by Dashkevicz and Feighner (1989). *L. reuteri* ATCC PTA 6475 was plated onto MRS plates containing between 0.05 to 0.5% taurocholate (TCA), glycocholate (GCA), taurodeoxycholate (TDCA), glycodeoxycholate (GDCA), taurochenodeoxycholate (TCDCA), or glycochenodeoxycholate (GCDCA) and incubated anaerobically for up to 72

hours. Bile salt hydrolase activity was observed by the precipitation of bile acids surrounding individual colonies of bacteria or an opaque granular white appearance of the colonies as shown in Figure 3.1 and as reported previously (8). Based on these phenotypes, the plate tests revealed that the strain could deconjugate all six of the major bile acids found in human bile.

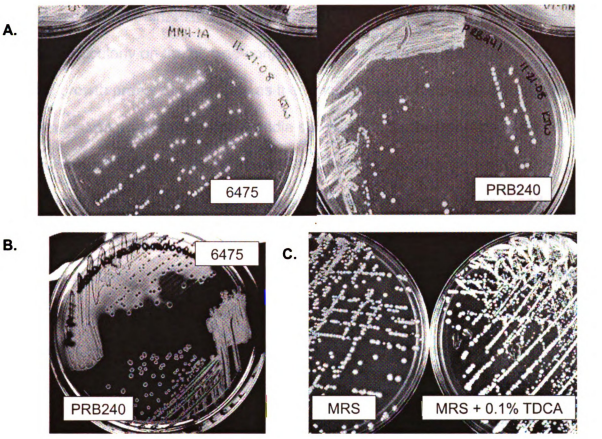

Figure 3.1. Different phenotypes observed using the plate test to determine bile salt hydrolase activity. Strains of *L. reuteri* were plated onto MRS plates with or without bile acids to determine bile salt hydrolase ability. **A.** *L. reuteri* ATCC PTA 6475 and the bile salt hydrolase mutant strain, PRB240, plated onto MRS plates containing 0.5% oxgall. Precipitation of bile is observed from ATCC PTA 6475 as halos around the colonies; no activity is observed for PRB240. This phenotype was also observed for plates containing glycodeoxycholate (GDCA) and glycochenodeoxycholate (GCDCA) (data not shown). **B.** The two strains (ATCC PTA 6475 and PRB240) plated onto MRS plates containing 0.01% taurochenodeoxycholate (TCDCA). Bile salt hydrolase activity for this bile acid was demonstrated by a cloudiness surrounding the colonies that could be observed when the plates were held against a light. No activity is observed for PRB240. **C.** ATCC PTA 6475 plated onto MRS or MRS plus 0.5% taurodeoxycholate (TDCA). Deconjugation of TDCA was demonstrated by a matte opaque granular appearance of the colonies. This phenotype was not observed for PRB240 (data not shown).

Problems with the assay arose when the screen was used for testing the bile salt hydrolase mutant strain, PRB240. This mutant demonstrated some growth defects, particularly on the glycine-conjugated bile acids (data not shown). The reduced growth presents a problem as the precipitation of deconjugated bile salts is dependent on acidification of the media surrounding the bacterial colonies (8), and this acidification depends on sufficient growth of the strain. In order to counteract this reduced growth, lower concentrations of bile acids were used. Under these conditions, the phenotypes for the wild-type strain were not as well defined, particularly the matte opaque granular colony phenotype noted on plates containing the taurine conjugated bile acids. Due to this discrepancy and to confirm that the various phenotypes shown in Figure 3.1 did represent deconjugation of the various bile acids, a different method, thin layer chromatography (TLC), was used for investigating the deconjugation activity of *L. reuteri* ATCC PTA 6475 and PRB240.

For these studies, both *L. reuteri* ATCC PTA 6475 and PRB240 were grown in MRS plus 1 mM (approximately 0.05%) of each of the six major human bile acids separately. The strains were grown for 18 hours or 72 hours and tested for deconjugation activity by TLC. The TLC results confirmed that *L. reuteri* ATCC PTA 6475 is able to deconjugate all six major human bile acids by 18 hours; no activity was observed for PRB240, even after 72 hours of incubation (data not shown). Thus, *L. reuteri* ATCC PTA 6475 contains one bile salt hydrolase gene,

NT01LR0487, which is able to deconjugate both the taurine and glycine-conjugated forms of cholate, deoxycholate, and chenodeoxycholate.

Disruption of NT01LR0487 leads to a growth defect in the presence of a synthetic human bile mixture. When *L. reuteri* ATCC PTA 6475 and PRB240 were grown in the presence of a synthetic human bile acid mixture (SHB), growth defects were observed for PRB240. The doubling time for *L. reuteri* ATCC PTA 6475 in MRS broth was 39 minutes; this time slowed to 48 $\pm$ 1 minutes when the strain was grown in the presence of SHB. PRB240 had a doubling time of 42 $\pm$ 4 minutes in the absence of bile; this time slowed to 63 $\pm$ 1 minutes in the presence of bile. Bile inhibited the growth of both strains, although the inhibition observed for the bile-salt hydrolase mutant was more severe (a 23% increase in doubling time was observed for the wild-type strain compared to a 50% increase for PRB240).

Growth in the presence of bile also decreased the final culture density that the strains were able to obtain. In the absence of bile, both strains reached a final $O.D._{600}$ approximately equal to 4.7 after 24 hours, revealing that there is not inherent growth defect due to the disruption of the bile salt hydrolase gene, NT01LR0487. In the presence of bile, *L. reuteri* ATCC PTA 6475 reached a culture density of 1.91 $\pm$ 0.24 (40% of that obtained without bile), whereas PRB240 reached a culture density of 0.8 $\pm$ 0.01 (17% of that obtained without bile).

TLC confirmed that *L. reuteri* ATCC PTA 6475 was able to deconjugate a mixture of bile acids; the deconjugation products begin accumulating after 4 hours of growth (Figure 3.2), particularly for deoxycholate and/or chenodeoxycholate. No activity above the background level was observed for PRB240, even after 23 hours of incubation.

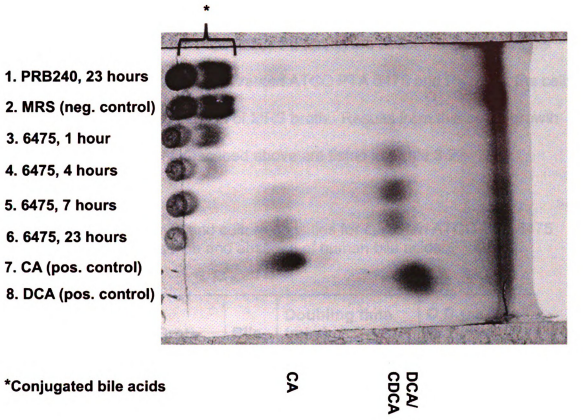

Figure 3.2. Deconjugated bile acids begin to accumulate after 4 hours in a culture of *L. reuteri* ATCC PTA 6475. Representative TLC plate showing the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475 against a synthetic "human" bile acid mixture (SHB). Samples were taken a 1, 4, 7, and 23 hours. Control solutions of cholate (CA) and deoxycholate (DCA) are included for comparison [deoxycholate and chenodeoxycholate (CDCA) migrate at the same rate in this solvent system]. The bile salt hydrolase mutant strain, PRB240, is shown to have no activity at the 23-hour time point.

Controlling the pH of the growth medium alters the growth characteristics of the wild-type and mutant strains in the presence of a synthetic human bile mixture. Various reports in the literature have suggested that pH of the

environment may play an important role in the way that bacteria respond to bile acids. Previous work in the lab has demonstrated that *L. reuteri* is capable of lowering the pH of the growth medium from 6.5 down to 4.0. In order to determine whether this shift in pH played a role in the observed growth effects and/or deconjugation activity of *L. reuteri* ATCC PTA 6475 and PRB240, the cells were grown in a buffered version of MRS broth. Results from this set of growth curves, as well as the set described above are listed in Table 3.2.

Table 3.2. Doubling times and culture densities for *L. reuteri* ATCC PTA 6475 and PRB240 in the presence and absence of human bile acids.

Strain	Broth	Bile	Doubling time (min.)	O.D.$_{600}$ (23-24 hours)
L. reuteri ATCC PTA 6475	MRS	-	39	4.69 ± 0.01
		+	48 ± 1	1.91 ± 0.24
PRB240		-	42 ± 4	4.71 ± 0.01
		+	63 ± 1	0.80 ± 0.01
L. reuteri ATCC PTA 6475	MRS - acetate	-	39 ± 3	4.54 ± 0.13
		+	47 ± 3	1.52 ± 0.40
PRB240		-	48 ± 9	4.73 ± 0.08
		+	66 ± 11	0.70 ± 0.07
L. reuteri ATCC PTA 6475	Buffered MRS - acetate	-	37 ± 5	2.41 ± 0.09
		+	44 ± 4	2.31 ± 0.22
PRB240		-	37 ± 4	1.86 ± 0.23
		+	46 ± 3	2.54 ± 0.12

Interestingly, in the buffered medium, the differences between the wild-type strain and the bile salt hydrolase mutant regarding the lower final culture density and the slower doubling time are no longer observed. The lack of differences between the two strains are not due to a difference in deconjugation activity in this medium, as confirmed by TLC (Figure 3.3). These results demonstrate that pH does not affect the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475. The growth advantage provided by bile salt hydrolase activity, however, does appear to be pH-dependent.

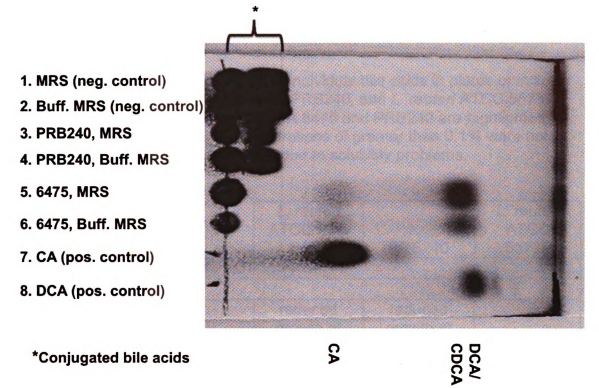

Figure 3.3. Controlling the pH of the culture does not affect the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475. Representative TLC plate showing the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475 against a synthetic "human" bile acid mixture (SHB) in MRS and buffered MRS. Samples were taken at 7 hours. Control solutions of cholate (CA) and deoxycholate (DCA) are included for comparison [deoxycholate and chenodeoxycholate (CDCA) migrate at the same rate in this solvent system]. No difference in activity is observed between cultures grown in MRS and cultures grown in buffered MRS.

Summary of the effects of various bile acids on growth of *L. reuteri*.

Throughout the course of this research, various growth effects of particular bile acids were noted for *L. reuteri* ATCC PTA 6475 and PRB240. *L. reuteri* ATCC 55730 was also included in several of these studies for comparison. Although

these experiments were carried out using different concentrations of bile acids, and the strains were grown on MRS plates or in MRS broth, these trends remained consistent. These qualitative observations are summarized in Table 3.3.

Table 3.3. Observed growth effects of individual bile acids in plates or in liquid medium on *L. reuteri* ATCC PTA 6475, PRB240, and *L. reuteri* ATCC 55730. Notable differences between ATCC PTA 6475 and PRB240 are highlighted in bold. ND = not determined. *Concentrations of greater than 0.1% were not tested for the deconjugated bile acids due to solubility problems.

	L. reuteri ATCC PTA 6475	PRB240	*L. reuteri* ATCC 55730
Cholate (CA)			
	reduced growth at 0.1%	reduced growth at 0.1%	ND
Glycocholate (GCA)			
	good growth at 0.1%	good growth at 0.1%	ND
Taurocholate (TCA)			
	good growth at 0.1%	good growth at 0.1%	ND
Deoxycholate (DCA)			
	good growth at 0.1%	good growth at 0.1%	ND
Glycodeoxycholate (GDCA)			
	good growth at 0.2%	**reduced growth at 0.05%**	**reduced growth at 0.05%**
Taurodeoxycholate (TDCA)			
	good growth at 0.5% and below	good growth at 0.5% and below	good growth at 0.5% and below

Table 3.3 (cont'd)

Chenodeoxycholate (CDCA)			
	reduced growth at 0.05%	reduced growth at 0.05%	reduced growth at 0.05%
Glycochenodeoxycholate (GCDCA)			
	reduced growth at 0.05%	**severely reduced growth at 0.05%**	**reduced growth for 0.05%**
Taurochenodeoxycholate (TCDCA)			
	good growth at 0.5%	good growth at 0.5%	good growth at 0.5%

In general, these growth studies suggest that the glycine-conjugated bile acids cause higher growth inhibition than the taurine-conjugated bile acids, and the deconjugated bile acids cause higher growth inhibition than either of the conjugated forms. PRB240, the bile salt hydrolase mutant, appears to be more sensitive to glycodeoxycholate and glycochenodeoxycholate, suggesting that the bile salt hydrolase activity may play a role in resistance to glyco-conjugated bile acids.

DISCUSSION

The ability of bacteria to deconjugate bile acids is a trait that has been investigated both in an attempt to understand what benefit this enzyme may provide for the bacteria, as well as what effect the activity may have on the host. Several large-scale studies have demonstrated a strong correlation between the isolation of bacteria from the gastrointestinal tract and the ability of these bacteria to deconjugate bile acids (19, 27). This correlation suggests that this activity most likely confers some competitive advantage to strains, although the exact function has not been elucidated.

Bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475. These studies revealed that *L. reuteri* ATCC PTA 6475 contains one bile salt hydrolase enzyme, NT01LR0487. This enzyme is capable of deconjugating all six major bile acids found in human bile: taurocholate (TCA), taurodeoxycholate (TDCA), taurochenodeoxycholate (TCDCA), glycocholate (GCA), glycodeoxycholate (GDCA), and glycochenodeoxycholate (GCDCA). PRB240, the strain containing a disruption in NT01LR0487, was shown to have lost the ability to deconjugate these bile acids. The substrate specificity of bile salt hydrolase enzymes appears to be variable, as some enzymes exhibit specificity based on the steroid nucleus of the bile acid (cholate, deoxycholate, or chenodeoxycholate) and others exhibit specificity depending on the amino acid conjugate (taurine or glycine). The occurrence of bile salt hydrolase enzymes capable of

deconjugating all six human bile acids is unknown, as many studies have demonstrated deconjugation activities using only one or two bile acids. At least two other studies have demonstrated bile salt hydrolase enzymes capable of deconjugating the full range of bile acids (28, 30).

Effect of bile salt hydrolase activity during growth in the presence of bile. Although there is a strong correlation connecting gastrointestinal strains of bacteria with the ability to deconjugate bile acids, in vitro and in vivo data demonstrating a direct relationship between the activity and persistence is conflicting. One of the main proposed functions of bile salt hydrolase activity is the detoxification of bile acids, although in vitro data regarding this is also variable. This study demonstrated that when the pH of the growth medium was not controlled (i.e., acidification due to metabolic products produced by the bacteria was allowed), bile salt hydrolase activity appeared to confer an advantage. This advantage was observed both as a decrease in doubling time and as an increase in the final culture density achieved in the presence of bile. This advantage was not present, however, when the pH of the medium was controlled (not allowed to drop below 6.5). In buffered medium, the doubling times and the final culture densities achieved by the wild-type strain and the bile-salt hydrolase mutant were virtually identical. The pH of the culture medium in the two situations was quite different, as in the unbuffered medium, the pH of the culture started at 6.5 and dropped down to 3.5 in as little as six hours (depending on the growth of the strain). In the buffered medium, the pH of the culture began

at 7.0 and only dropped down to 6.5, even after 24 hours of growth (data not shown).

As most studies have demonstrated an acidic pH is necessary for optimum activity of bile salt hydrolase enzymes (7, 17, 28, 30), and indeed one study even observed full activity at a pH of 5.2 and a complete lack of activity at a pH of 6.8 (31), TLC was performed to determine what effect the varying pHs may have had on the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475. The TLC data demonstrated that there was no observable difference in the activity levels of the enzyme in the two media types (Figure 3.3). This data strongly suggests that the growth differences observed in the two types of media are due to an advantage of bile salt hydrolase activity in acidic medium.

This phenomenon of differing effects of bile salt hydrolase activity at different pH values has been reported in the literature for other bacteria, primarily in regards to the glycine-conjugated bile acids, which are generally found to be more toxic than the taurine-conjugated forms. De Smet et al (10) demonstrated that at a low pH, glycodeoxycholate exhibited higher toxicity levels against strains with little to no bile salt hydrolase activity. Grill et al (16) also demonstrated that the toxic effect of glycodeoxycholate increased at pH values of less than 6.5 and showed that this effect was more pronounced for strains without bile salt hydrolase activity. Begley et al (4) also demonstrated that glycodeoxycholate exhibited higher levels of toxicity at pH 5.5 as compared to a pH of 6.5 for *Listeria*

monocytogenes and showed that this effect was even more pronounced in a bile salt hydrolase mutant. Interestingly, their data also demonstrated that the bile salt hydrolase mutant had a survival advantage over the wild-type strain when exposed to glycodeoxycholate at a pH above 5.5. These studies, along with the experiments performed herein, suggest that there is an advantage to bile salt deconjugation at low pH levels. As the advantage seems to be observed somewhere between a pH of 5.5 and 6.5, and the pH of the small intestine ranges from 5.7 to 7.7 (25), it is difficult to surmise whether this activity contributes a significant advantage in vivo.

Proposed model of bile salt toxicity through intracellular acidification. One proposed function for bacterial bile salt hydrolase activity is to detoxify the conjugated bile acids, which are proposed to inhibit cells through intracellular acidification in the same manner as organic acids. De Smet et al (10) outlined a model for this particular mechanism, including an explanation for the difference in toxicity observed for taurine versus glycine-conjugated bile acids. The authors propose that the protonated form of the conjugated bile acids is able to passively diffuse into the cell, where it promptly becomes deprotonated due to the alkaline intracellular environment. In a cell capable of bile salt hydrolase activity, the bile acid is then deconjugated, producing a compound with a higher pKa (around 5.0) whose conjugate base will pick up the extra proton before being transported outside of the cell, thus relieving the potential intracellular acidification. In a cell that is not capable of bile salt hydrolase activity, the cell must expend energy to

pump out the excess protons. The authors note that due to the lower pKa value of taurine-conjugated bile acids (pKa of around 0.9) versus glycine-conjugated bile acids (pKa of around 4.3), the concentration of protonated taurine-conjugated bile acids that could passively diffuse into the cell is very low at pH values achieved during bacterial growth. This reduced concentration is proposed to account for the reduced toxicity observed with these bile acids.

The research presented here does not fully support this model. Although a higher level of growth inhibition is observed for the wild-type and mutant strains when grown in the presence of GDCA and GCDCA (compared to the taurine-conjugated equivalents), and the defect is more severe for the mutant than the wild-type strain, no such defect is observed when either strain is grown in the presence of GCA (Table 3.3). As all three glycine-conjugated bile acids have very similar pKa values, ranging from 4.27 to 4.34 (6), it is unlikely that the slight variations in the concentration of protonated bile acids would cause this difference in toxicity. Also, none of the taurine-conjugated bile acids exhibited an inhibitory effect on any of the *L. reuteri* strains tested. Despite the fact that taurine-conjugated bile acids are much stronger acids, and therefore the concentration of the protonated form that is proposed to enter the cell is very low at the pH values observed during the various culture conditions, deconjugation of all three forms of taurine-conjugated bile acids by *L. reuteri* ATCC PTA 6475 was observed. As most of the bile salt hydrolase enzymes described to date are intracellular enzymes (3, 30), the taurine-conjugated forms must enter the cell in

order to become deconjugated. The lack of toxicity observed for these bile acids against PRB240 (the mutant strain without any bile salt hydrolase activity) and for *L. reuteri* ATCC 55730 (which exhibits weak bile salt hydrolase activity specific to glycine-conjugated bile acids) suggests a different mechanism of toxicity besides passive diffusion leading to intracellular acidification.

As many studies, including the research presented here, have demonstrated that the deconjugated bile acids are **more inhibitory** to bacteria than the conjugated bile acids (9, 14, 27, 29), the question of why bacteria would exhibit bile salt hydrolase activity still remains to be answered. One of the current theories specifies that the bacteria obtain some nutritional benefit from the activity, particularly through the use of the amino acid moieties (3). Nutritional benefit for *L. reuteri* appears to be an unlikely reason based on the results of this study, as both *L. reuteri* ATCC PTA 6475 and PRB240 exhibited equal doubling times and final culture densities during growth in the buffered medium, despite the presence and absence of bile acid deconjugation activity. Preliminary experiments performed to determine if the addition of taurine to the growth medium conferred a growth advantage also suggest that bile salt hydrolase activity does not confer a nutritional benefit (data not shown).

This research demonstrates that bile salt hydrolase activity provides a growth advantage to *L. reuteri* ATCC PTA 6475 during growth at acidic pH values. The mechanism providing this growth advantage has not yet been elucidated. The

lack of an effect observed in buffered MRS medium suggests that the advantage is pH-dependent, although most of the data does not support the previous model of prevention of intracellular acidification due to passive diffusion of the conjugated bile acids into the cell. Further studies, including examination of the intracellular pH values of the cells under the various growth conditions, investigation of the potential role of a conjugated bile acid transporter, and closer analysis of the specific rates of deconjugation for individual bile acids may lead to an understanding of the mechanism through which this advantage is provided to *L. reuteri* ATCC PTA 6475.

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CHAPTER 4

IDENTIFICATION OF A PUTATIVE NOVEL IMMUNOMODULATORY COMPOUND FROM *LACTOBACILLUS REUTERI*

INTRODUCTION

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts (6, 8). Many probiotics are now marketed to consumers and include organisms such as lactobacilli, streptococci, bifidobacteria, *E. coli* Nissle 1917, and the yeast *Saccharomyces boulardii* (12). In general, probiotic lactobacilli are considered safe for human consumption based on previous clinical trials, epidemiological studies, and historical usage in fermented foods (1, 23). However, the mechanisms by which probiotics promote good health and combat diseases are poorly understood. A better understanding of how probiotics influence the health of the host is critical to utilizing these organisms to their fullest potential.

One emerging area of probiotic research is the ability of these bacteria to alter the immune system. Several probiotics secrete immunomodulins that modulate the host inflammatory response, but the bacterial products responsible for the effects on inflammation are still undefined (7, 17, 19). The inflammatory cytokine TNF, which is produced primarily by monocytes and macrophages, is a key

mediator of intestinal inflammation. Immunoprobiotics, or probiotics producing key immunomodulatory factors, are potential therapies for various immune-mediated disorders such as Crohn's disease (14). Indeed, select *L. reuteri* strains inhibit TNF production by monocytoid THP-1 cells and monocytes isolated from patients with Crohn's disease (15, 19). Strains of *L. reuteri* capable of inhibiting TNF were able to reduce inflammation in a *H. hepaticus*-induced murine model of inflammatory bowel disease (19). The mechanism(s) behind this down regulation of inflammation are not understood.

Bacterial species can be identified based on their fatty acid composition. In 1950, the first cyclopropane fatty acid (CFA) was identified as cis-11,12-methylene octadecanoic acid, or lactobacillic acid, in *L. arabinosus* (9). Additional CFAs have been identified in gram-negative and gram-positive bacteria, including dihydrosterculic acid (4). As shown in Figure 4.1, lactobacillic and dihydrosterculic acids are produced from the precursors, vaccenic acid and oleic acid respectively, by the enzyme cyclopropane fatty acid synthase (Cfa). Lactobacillic and dihydrosterculic acids are 19 carbon CFAs that differ only in the placement of the propane ring. During synthesis, Cfa adds a methylene carbon across the double bond forming a three-carbon ring using S-adenosylmethionine as a methyl donor. While our knowledge of how CFAs are produced has greatly increased in the past decade, the physiological role(s) of CFAs are poorly understood. CFAs may stabilize the cellular membrane, allowing bacteria to

survive exposure to adverse conditions such as extreme acid shocks and repeated freeze/thaw cycles (4).

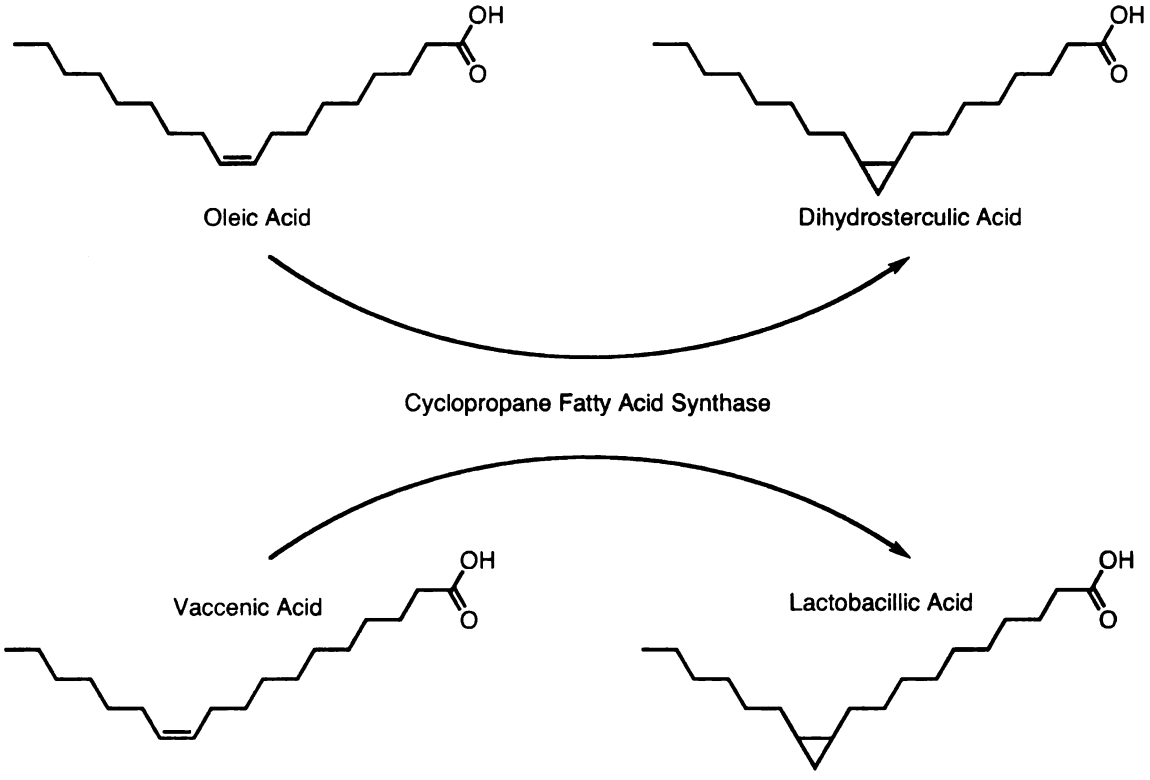

Figure 4.1. Synthesis of cyclopropane fatty acids. Cyclopropane fatty acid synthase converts oleic acid and vaccenic acid into dihydrosterculic acid and lactobacillic acid, respectively (4).

Microbial fatty acids and lipids have been previously shown to have immunomodulatory properties. Lipid A is the active component of bacterial LPS, a known agonist of TLR2 and TLR4. The length and degree of saturation of the fatty acid chains of Lipid A are important determinants of LPS activity (18). *Plasmodium falciparum* produced a mixture of fatty acids that was more effective at inhibiting TNF production than any sole fatty acid tested (5). A *Mycobacterium tuberculosis* mutant unable to synthesize trans-cyclopropyl mycolic acids was

more virulent and stimulated murine TNF production by bone marrow-derived macrophages and RAW 264.7 macrophages more than the wild type strain (21). However, the effects of cyclopropane fatty acids produced by probiotic bacteria on the host inflammatory responses are poorly understood.

This report demonstrates that synthesis of the specific CFA, lactobacillic acid, is restricted to TNF-inhibitory *L. reuteri* strains. The production of lactobacillic acid correlates with the emergence of the ability to inhibit TNF production. *L. reuteri* strains incapable of producing lactobacillic acid have an insertion of DNA at the 3' end of the *cfa* gene, which may affect their ability to synthesize lactobacillic acid (Figure 4.7). We have constructed a *L. reuteri cfa* mutant and shown that it is unable to synthesize lactobacillic acid. The *cfa* mutant has partially lost the ability to inhibit TNF production in an LPS-activated macrophage model. Possible direct and indirect effects of lactobacillic acid on immunomodulation are discussed.

MATERIALS AND METHODS

Key reagents, bacterial strains and mammalian cell lines. All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Polystyrene plates were obtained from Corning (Corning, NY). Polyvinylidene fluoride membrane filters of 0.22 μm pore size (Millipore, Bedford, MA) were used to yield *L. reuteri*-derived cell-free supernatants. All bacterial

strains and plasmids are described in Table 4.1. *L. reuteri* strains were cultured in deMan, Rogosa, Sharpe (Difco, Franklin Lakes, NJ) or LDMIIIG (supplemental material) media unless otherwise state. PRB173 was grown in the presence of 10 μg/mL erythromycin. Cis-vaccenic acid was ordered from MP Biomedicals (Ohio). For anaerobic culturing, an anaerobic chamber (1025 Anaerobic System, Forma Scientific, Waltham, MA) supplied with a mixture of 10% CO_2, 10% H_2, and 80% N_2 was used. THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2.

Table 4.1 – Bacterial strains and plasmids used in this study.

Bacterial Strains and Plasmids	**Description**	**Source**
Bacterial strains		
L. reuteri ATCC 55730	Isolate from Peruvian mother's milk	Biogaia AB (Raleigh, NC)
L. reuteri ATCC PTA 6475	Isolate from Finnish mother's milk	Biogaia AB (Raleigh, NC)
L. reuteri ATCC PTA 5289	Oral isolate from Japanese female	Biogaia AB (Raleigh, NC)
L. reuteri CF48-3A	Fecal isolate from Peruvian child	Biogaia AB (Raleigh, NC)
L. reuteri PRB173	*L. reuteri* ATCC PTA 6475 pPBR173 inserted into *cfa*	This study
E. coli EC1000	chromosomal copy of the pWV01 *repA* gene; Kan^r	(13)
Plasmids		
pVE6007	Cm^r *repA*-positive temperature-sensitive derivative of pWV01	(16)
pORI28	Em^r *repA*-negative derivative of pWV01	(13)
pPRB173	pORI28 + 239 bp insert from NT01LR1143	This study

Creation of PRB173 (*L. reuteri* ATCC PTA 6475 with a mutation in NT01LR1143). The following concentrations of antibiotics were used: 40 ug/mL kanamycin, 10 ug/mL chloramphenicol, 10 ug/mL erythromycin (*L. reuteri*) or 400 ug/mL erythromycin (*E. coli*). The NT01LR1143 (cyclopropyl fatty acid synthase) mutant was created using the system developed by Russell and Klaenhammer (22) with modifications made for use in *Lactobacillus reuteri* (26). In short, 239 bp from NT01LR1143 from *L. reuteri* ATCC PTA 6475 was cloned into pORI28. This construct was then transformed into *E. coli* EC1000, and the transformed cells were grown in LB broth in the presence of erythromycin and kanamycin. pORI28 containing the NT01LR1143 insert was then extracted and transformed into *L. reuteri* ATCC PTA 6475 containing pVE6007. *L. reuteri* cells containing both plasmids were grown in MRS broth containing chloramphenicol and erythromycin at the permissive temperature of 35°C for 18 hours. The cultures were then shifted to the non-permissive temperature of 45°C and grown in the presence of erythromycin only. After several passages at 45°C to ensure loss of pVE6007, individual colonies were screened for integration of pORI28 at the desired location by PCR. Colonies were screened for the presence of both flanking regions (using one primer that anneals to pORI28 and one primer that anneals to the chromosome outside of the region cloned into pORI28) and the absence of a correctly-sized wild-type gene.

Samples for fatty acid methyl ester (FAME) analysis of overnight cultures (strain comparison and mutant analysis). Cultures for analysis were grown aerobically without shaking for 18 hours in 10 mL of MRS (Difco) at 37°C (*L. reuteri* CF48-3A was grown at 35°C by S. E. Jones). PRB173 was grown in the presence of 10 µg/mL erythromycin. Cultures were spun down at 4000 rpm for 10 minutes at room temperature. Supernatant was removed. Pellets were resuspended in one ml of fresh MRS and spun at 13,400 rpm for 2 minutes. Supernatant was removed and pellets were frozen at -80°C until analysis. Experiments for strain comparisons were performed in quadruplicate. For mutant versus wild-type comparison, six wild-type samples were analyzed and four mutant samples were analyzed.

Samples for FAME analysis at various stages of growth. Cultures of *L. reuteri* ATCC PTA 6475 for analysis were grown in the presence of 2% oxygen with slow shaking in 110 mL of MRS (Difco) at 37°C. Culture density was measured at an optical density (O.D.) of 600 nm. 50 mL samples were collected from the cultures at mid-log (O.D. = 0.4); 10 mL samples were collected from the cultures at early stationary (O.D. = 3.1), late stationary (O.D. = 4.2), and after overnight incubation (approximately 18 hours). A representative growth curve is shown in Figure 4.2. After collection, samples were processed as described above, and pellets were frozen at -80°C until analysis. Experiments were performed in triplicate.

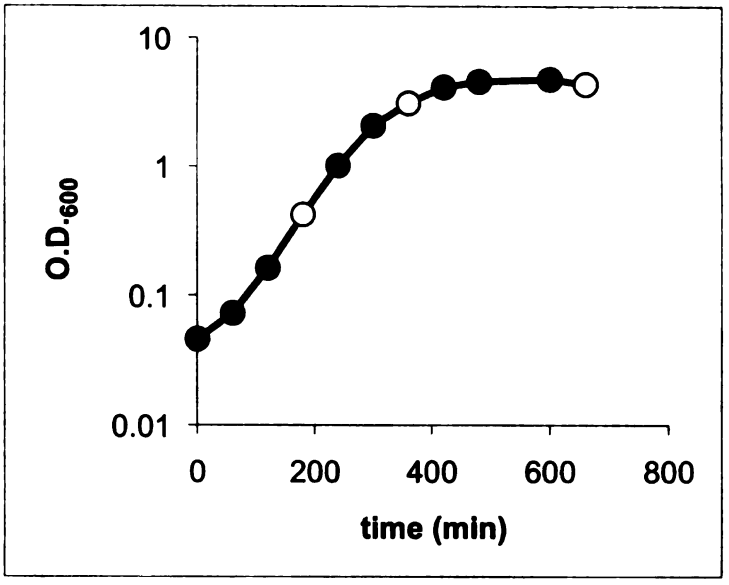

Figure 4.2. A representative growth curve for *L. reuteri* ATCC PTA 6475. Samples for fatty acid analysis (mid-log, early stationary, and late stationary phase) were collected at the time points represented by open circles.

Samples for FAME analysis in different variations of MRS media. Three variations of MRS media were used to determine the effects of various media components on the fatty acid composition of *L. reuteri* ATCC PTA 6475 and PRB173. Cultures were grown aerobically in 10 mL of MRS or modified MRS for 18 hours without shaking at 37°C. PRB173 was grown in the presence of 10 µg/mL erythromycin. Complete MRS (MRS A to distinguish it from the Difco MRS) was made from the following components: 10 g/L proteose peptone no. 3, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L dextrose, 1 g/L Tween 80, 2 g/L ammonium citrate, dibasic, 5 g/L sodium acetate, 0.1 g/L magnesium sulfate, 0.05 g/L manganese sulfate, and 2 g/L dipotassium phosphate. Two variations of MRS media were also made. The first, MRS B, is MRS media made without

beef extract or Tween 80. The second, MRS C, is MRS media made without beef extract or Tween 80 with the addition of 0.01% cis-vaccenic acid (w/v). Samples were processed as listed above, and pellets were frozen at -80°C until analysis. Experiments were performed in triplicate.

Fatty acid analysis. All fatty acid analysis was performed by Microbial ID (Newark, DE; http://www.microbialid.com).

Preparation of cell-free supernatants for immunomodulation studies. For planktonic cells, 10 mL of LDMIIIG was inoculated at a starting OD_{600} = 0.1 (~7 x 10^7 CFU) using 16-18 hr *L. reuteri* cultures. Bacteria were incubated for 24 hr at 35°C in anaerobic conditions. Serial dilutions were plated onto MRS medium to determine cell counts. Cells were pelleted (4000 x g, RT, 10 minutes) and discarded. Supernatants were filter-sterilized. Aliquots were vacuum-dried and re-suspended to the original volume using RPMI. For biofilms, *L. reuteri* cultures (16-18 hrs of incubation) were diluted 1:100 in MRS and aliquots were placed into polystyrene 24-well plates. Plates were incubated anaerobically for 24 hr at 35°C. Supernatants and planktonic cells were removed by aspiration, and biofilms were washed with 50 mM sodium phosphate buffer (37°C, 100 rpm, 10 minutes). One mL of LDMIIIG was added to each well, and the plates were incubated for 2 hours at 35°C in anaerobic conditions. The supernatants were filtered through 0.22 μm filters, vacuum-dried and resuspended in RPMI to the

starting volume. Biofilms were removed by sonication (5 min, 20°C), and serial dilutions were plated to determine cell counts.

TNF inhibition experiments. TNF assays were previously described (15). Briefly, cell-free supernatants from *L. reuteri* planktonic or biofilm cultures (5% v/v) or serial dilutions of lactobacillic acid (2% v/v) were added to human THP-1 cells (approximately 5 x 10^4 cells) activated with *E. coli* O127:B8 LPS (100 ng/mL). After the addition of *L. reuteri* supernatants and LPS, plates were incubated at 37°C with 5% CO_2 for 3.5 hours. Trypan blue (Invitrogen, Carlsbad, CA) was employed to ascertain cell viability. THP-1 cells were pelleted (1500 x g, 5 min, 4°C), and quantitative ELISAs (R&D Systems, Minneapolis, MN) were used to determine the amount of TNF present in monocytoid cell supernatants.

Statistical analyses for TNF bioassays and fatty acid profiles. For the TNF bioassays, a minimum of three biological replicates were performed and analyzed by ANOVA. Differences were considered statistically significant if $p < 0.05$. Fatty acid profiles were determined a minimum of three times. All error bars in the figures represent standard deviations.

RESULTS

Immunomodulatory activity of TNF inhibitory *L. reuteri* strains is produced upon entry into stationary phase. Some probiotic *L. reuteri* strains have the

ability to produce secreted factors that suppress lipopolysaccharide (LPS) induced TNF production in primary monocytes and macrophages (15). This activity is strain specific. We have identified two strains of *L. reuteri* (ATCC PTA 6475 and ATCC PTA 5289) that secrete one or more compounds that exhibit potent immunomodulatory activity against TNF production in activated macrophages. Secreted compounds from a third strain included in this study, *L. reuteri* ATCC PTA 4659, have also been shown to inhibit TNF production (3). Two additional strains (*L. reuteri* ATCC 55730 and CF-48/3A) were incapable of reducing TNF production (11). The inhibitory effect on TNF production is found in culture supernatants of cells grown into stationary phase and is absent from culture supernatants of exponentially growing *L. reuteri*. The goal of this study was to identify the immunomodulatory compounds produced upon entry into stationary phase using a combined genomics and genetic approach.

The cyclopropyl fatty acid, lactobacillic acid, is specifically produced in immunomodulatory strains of *L. reuteri*. Fatty acids have been shown to play important roles in the regulation of the immune system. To identify potential membrane fatty acids that are produced specifically by immunomodulatory *L. reuteri* strains we compared the membrane fatty acid profiles of the three strains capable of inhibiting TNF production with the two strains that are incapable of downregulating TNF production. These five strains were grown to stationary phase and fatty acid methyl ester analysis was performed to determine the membrane fatty acid content. The results for the major fatty acids are shown in

Figure 4.3. Briefly, the immunomodulatory strains appeared to be very similar to one another; they consisted of approximately 41% palmitic acid (16:00), 11% oleic acid (18:1 ω9c), 11% vaccenic acid (18:1 ω7c), 5% stearic acid (18:00), 18% dihydrosterculic acid (19:0 cyclo ω9c), and 10% lactobacillic acid (19:0 cyclo ω8c). The immunoneutral strains, *L. reuteri* ATCC 55730 and *L. reuteri* CF48-3A, consisted of approximately 26% palmitic acid, 21% oleic acid, 17% vaccenic acid, 4% stearic acid, and 24% dihydrosterculic acid. Interestingly, the immunomodulatory strains contained a novel cyclopropyl fatty acid, lactobacillic acid (phytomonic acid) that was absent from the immunoneutral strains. Thus lactobacillic acid is a possible candidate immunomodulin produced by *L. reuteri* strains ATCC PTA 6475, ATCC PTA 5289, and ATCC PTA 4659. A second cyclopropyl fatty acid, dihydrosterculic acid, was present in both immunoneutral and immunomodulatory strains in similar amounts. This indicated that immunoneutral strains were capable of producing fatty acids containing cyclopropane rings.

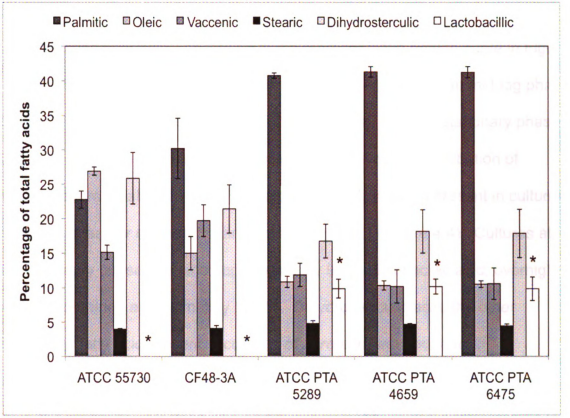

Figure 4.3 - TNF-inhibitory strains produce lactobacillic acid. Cultures of five strains of *L. reuteri* were grown in MRS broth for 18 hours at 37° C. Fatty acid profiles were determined by gas chromatography. ATCC 55730 and CF48-3A (TNF non-inhibitory strains) do not produce the CFA, lactobacillic acid. In contrast TNF-inhibitory strains (ATCC PTA 6475, ATCC PTA 5289, and ATCC PTA 4659) produce lactobacillic acid. Experiments were performed in triplicate; error bars represent standard deviation. * Lactobacillic acid.

The appearance of lactobacillic acid correlates with the appearance of the immunomodulatory activity. Previous work has demonstrated that the TNF inhibitory activity is only found in cell-free supernatant from *L. reuteri* cultures that have entered stationary phase. In order to investigate whether there was a correlation between the presence of lactobacillic acid in the cell membranes and the presence of the immunomodulatory compound in the cell-free supernatants,

fatty acid profiles were compared from *L. reuteri* ATCC PTA 6475 cultures at various stages of growth. According to the representative growth curve in Figure 4.2, samples for fatty acid analysis were collected from cultures at mid-log phase ($O.D._{600}$ = 0.4), early stationary phase ($O.D._{600}$ = 3.0), and late stationary phase ($O.D._{600}$ = 4.1). Samples were also collected after overnight incubation of cultures (approximately 18 hours). Lactobacillic acid was not present in cultures in mid-log phase or early stationary phases of growth (Figure 4.4). Cultures at late stationary phase contained approximately 9.6% lactobacillic acid; overnight cultures contained approximately 10.3%. Dihydrosterculic acid, the other cyclopropyl fatty acid present in cultures of *L. reuteri*, was found at concentrations of approximately 20% for all four time points tested (Figure 4.4). Thus, the presence of lactobacillic acid in cultures of *L. reuteri* ATCC PTA 6475 correlates with the appearance of the TNF inhibitory compound(s) in cell-free supernatant from the immunomodulatory strains.

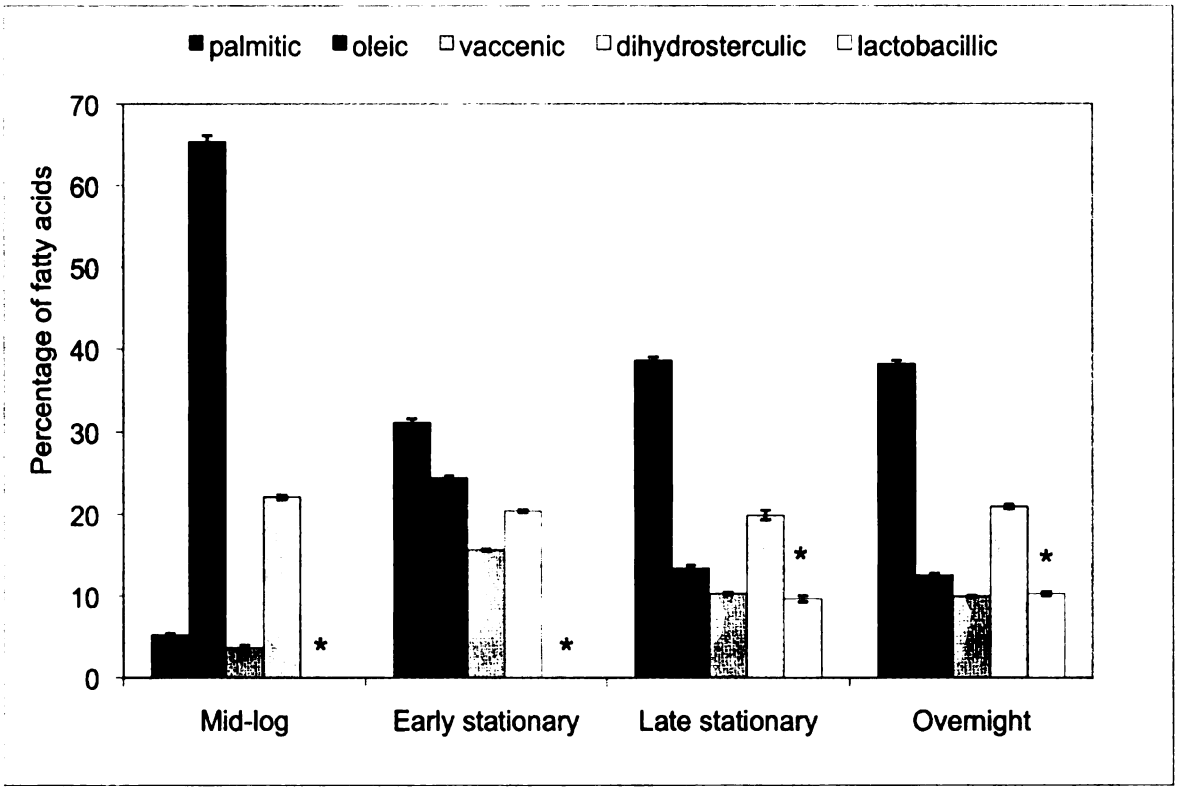

Figure 4.4. Lactobacillic acid appears in late stationary phase cultures of *L. reuteri* ATCC PTA 6475. Cultures of *L. reuteri* ATCC PTA 6475 were grown in MRS broth at 37°C. Samples were collected for FAME analysis at different stages of growth. Experiments were performed in triplicate; error bars represent standard deviation. *Lactobacillic acid.

The production of lactobacillic acid is due to the activity of NT01LR1143, a cyclopropyl fatty acid synthase. Previous work in *E. coli* has shown that the biosynthesis of bacterial cyclopropyl fatty acids is due to one enzyme, cyclopropyl fatty acid synthase. This cyclopropyl fatty acid synthase (Cfa) is responsible for the conversion of oleic acid into dihydrosterculic acid and the conversion of vaccenic acid into lactobacillic acid (Figure 4.1). A search of the *L. reuteri* ATCC PTA 6475 genome revealed a single gene, NT01LR1143, with significant similarity to known cyclopropyl fatty acid synthases. A disruption in

the gene was created by insertion of pORI28 as described in the Materials and Methods section. The resulting strain was titled PRB173. In order to confirm the effect of the disruption of NT01LR1143, cultures of *L. reuteri* ATCC PTA 6475 and PRB173 were grown overnight in MRS or MRS plus 10 µg/mL erythromycin. Samples were collected, and fatty acid analysis was performed. The results, shown in Figure 4.5, confirmed that the mutant strain, PRB173, was devoid of lactobacillic acid and contained a minor amount of dihydrosterculic acid (1.6% compared to 17.8% in wild-type). The mutant strain also contained a higher amount of oleic acid (52.5%) compared to the wild-type strain (10.7%).

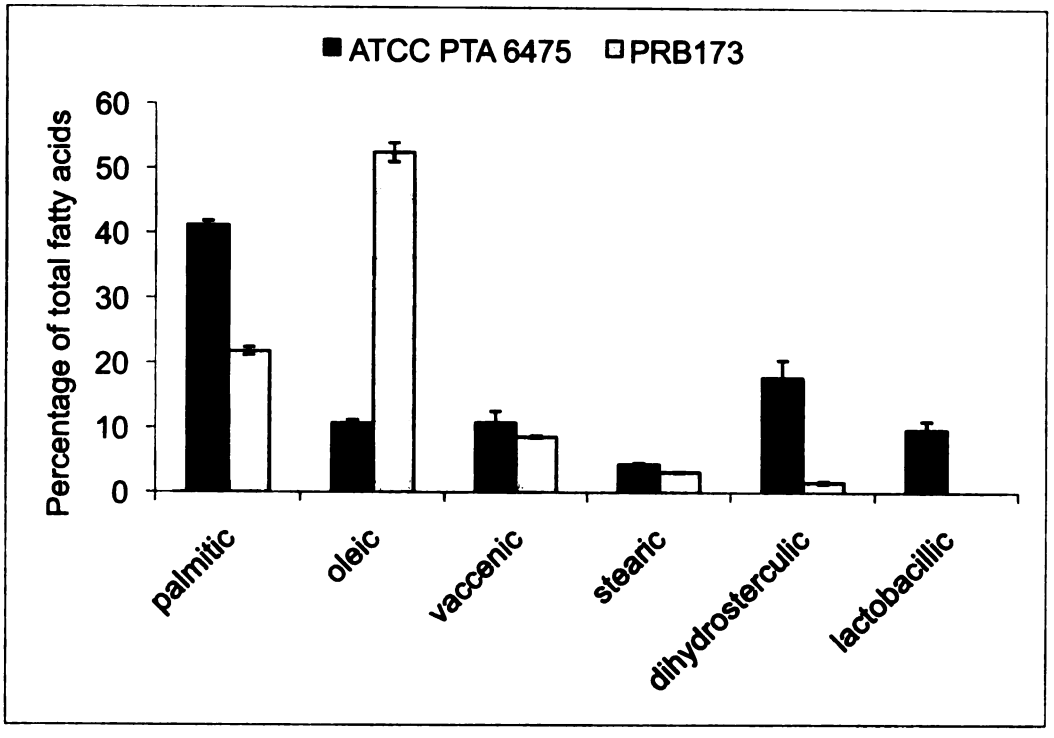

Figure 4.5. Disruption of NT01LR1143 (CFA synthase) eliminates production of lactobacillic acid by *L. reuteri* ATCC PTA 6475. L. *reuteri* ATCC PTA 6475 and PRB173 were grown overnight in MRS broth at 37° C. Samples were collected for fatty acid analysis. Experiments were performed in triplicate; error bars represent standard deviation.

Removal of Tween 80 from the growth medium increases the amount of lactobacillic acid found in *L. reuteri* ATCC PTA 6475. By disrupting the cyclopropyl fatty acid synthase gene (NT01LR1143) of *L. reuteri* ATCC PTA 6475, a strain was created that does not contain lactobacillic acid (PRB173). This strain will serve as an important control for future experiments directed at determining the possible role of lactobacillic acid in suppression of TNF by stimulated macrophages. A strain with an increased concentration of lactobacillic

acid would also be useful for these studies. To this aim, attempts were made to increase the percentage of lactobacillic acid in *L. reuteri* ATCC PTA 6475 cultures through alteration of specific media components.

L. reuteri cultures are commonly grown in MRS broth, a rich undefined media, in the laboratory. One component of MRS broth is Tween 80, which contains approximately 70% oleic acid with a balance of palmitic acid, stearic acid, and linoleic acid. Dihydrosterculic acid, the only other cyclopropyl fatty acid present in cultures of *L. reuteri* is made from oleic acid, and previous work in other lactobacilli has demonstrated that removal of oleic acid from the medium can increase the production of lactobacillic acid (10). In addition, the effect of adding 0.01% cis-vaccenic acid (the precursor for lactobacillic acid) in the absence of Tween 80 was also determined. *L. reuteri* ATCC PTA 6475 was grown in one of three variations of MRS broth for 18 hours, and samples were collected for fatty acid analysis. The three variations of broth used were: MRS A (complete MRS made from the various components), MRS B (MRS media without Tween 80 or beef extract), and MRS C (MRS B plus 0.01% cis-vaccenic acid). A summary of the results is shown in Figure 4.6. Briefly, in the absence of Tween 80 (MRS B), the lactobacillic acid concentration increased from approximately 8.7% of the total fatty acids to 29.9%, whereas the amount of dihydrosterculic acid decreased from 15.9% to 0% in the absence of its precursor. The addition of cis-vaccenic acid (MRS C) lead to a small increase in the lactobacillic acid, making it a total of 33.5% of the fatty acids found in the samples. Thus, growth of *L. reuteri* ATCC

PTA 6475 in either MRS B or MRS C would provide another sample for future studies aimed at investigating the role of lactobacillic acid in immunomodulation. PRB173, the CFA synthase mutant, was confirmed to be completely devoid of lactobacillic acid in MRS B (data not shown).

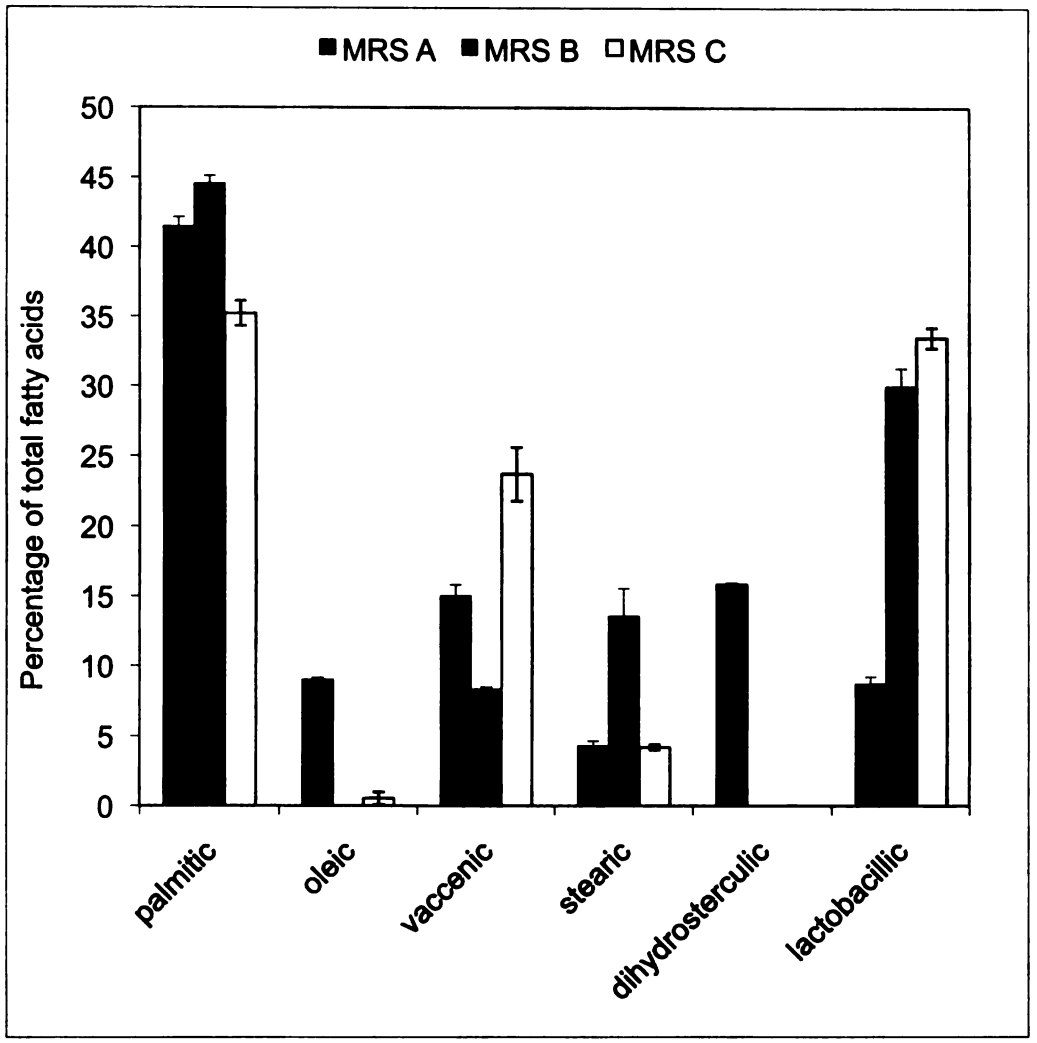

Figure 4.6. Lactobacillic acid production by *L. reuteri* ATCC PTA 6475 can be increased by removal of Tween 80 from the growth medium. Overnight cultures of *L. reuteri* ATCC PTA 6475 were grown in MRS A (complete MRS broth), MRS B (MRS broth with Tween 80 and beef extract removed), or MRS C (MRS B plus 0.01% cis-vaccenic acid); samples were collected for fatty acid analysis. Experiments were performed in triplicate; error bars represent standard deviation.

The cyclopropyl fatty acid synthase gene is truncated in strains of *L. reuteri* incapable of down-regulating TNF production. Membrane cyclopropane fatty acids (CFA) have been shown to increase as cells enter stationary phase. CFA formation is performed by the enzyme cyclopropane fatty acid synthase that is encoded by the *cfa* gene. We therefore scanned the genomes of *L. reuteri* PTA ATCC 6475 and ATCC 55730 and identified a single *cfa* gene in each genome. Interestingly, the reading frame of the *cfa* gene was truncated at the 3' end by the insertion of a DNA element in the *L. reuteri* ATCC 55730 but not the ATCC PTA 6475 strains (Figure 4.7). The insertion in *L. reuteri* ATCC 55730 results in a C-terminal deletion of 12 amino acids in the Cfa protein. To determine if this insertion correlated with the loss of immunomodulatory activity we PCR amplified the *cfa* gene and flanking sequences. The insertion was identified in both immunoneutral strains tested but was absent in both strains capable of suppressing TNF. Thus the absence of the insertion correlated with the ability to reduce TNF production.

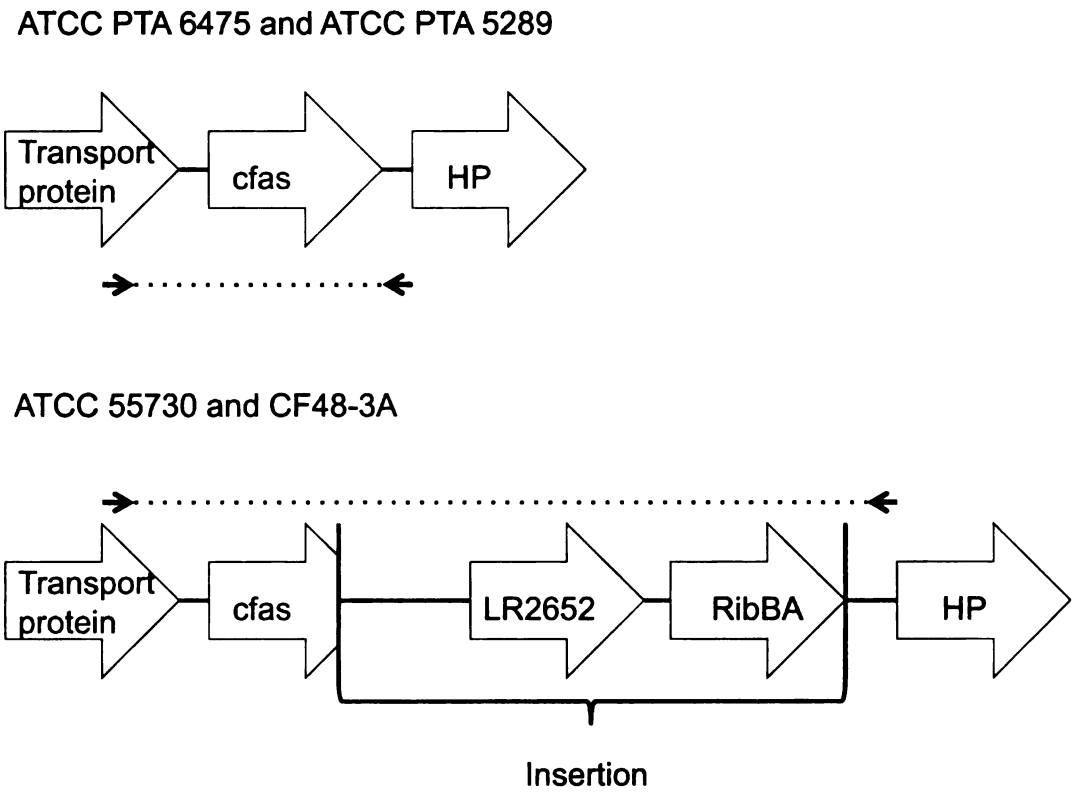

Figure 4.7. An insertion in the CFA synthase gene of *L. reuteri* ATCC 55730 and CF48-3A may prevent production of lactobacillic acid. PCR analysis revealed an insertion in the region of the genome containing the *cfa* gene for both immunomodulatory strains. This insertion results in the truncation of the cfa gene. Small arrows represent the primers used for amplification of the region surrounding the *cfa* gene.

Conditioned medium from a mutant defective in cyclopropyl fatty acid production is impaired in inhibiting TNF production. To address what possible role lactobacillic acid production has on the ability to inhibit TNF production we compared the effects of conditioned media from wild-type cells and the *cfa* mutant (PRB173) on the ability of activated monocytes to produce TNF. Human derived THP-1 monocytes were activated by the addition of LPS and the production of TNF was monitored by quantitative ELISA. Cell-free

supernatants from *L. reuteri* deficient in cyclopropane fatty acid synthase (PBR173) partially lost the ability to suppress TNF when compared to the immunomodulatory effects of wild type ATCC PTA 6475 in both planktonic cultures and biofilms (Figure 4.8).

For planktonic cultures, TNF production was efficiently suppressed by cell-free supernatant derived from *L. reuteri* ATCC PTA 6475 compared to the LDMIIIG medium control. The *cfa* mutant stain PRB173 was deficient in its ability to reduce TNF production compared to the wild-type strain. TNF production was approximately 2-fold higher in bioassays with PBR173 compared to bioassays with wild type *L. reuteri* ATCC PTA 6475 (239 pg/mL and 134 pg/mL, respectively) ($p<0.001$) (Figure 4.8 A). In addition, serial dilutions of probiotic-derived supernatants were tested. At a dilution of 1:20, wild-type supernatants still inhibited TNF by 45% compared to the media control. In contrast, at a 1:20 dilution, PRB173 supernatants had completely lost their TNF-inhibitory ability (data not shown). We also analyzed the production of immunomodulatory activity from biofilms and found similar trends with respect to TNF modulation. When supernatants from biofilm cultures were tested, ATCC PTA 6475 and PRB173 inhibited TNF production by 60% and 21%, respectively, compared to the medium alone (Figure 4.8 B). In the presence of supernatants derived from the Cfa-deficient strain, the quantities of human TNF produced by LPS-activated THP-1 cells were approximately 2-fold higher than quantities observed in the wild type strain (560 pg/mL and 282 pg/mL, respectively) ($p < 0.01$). Planktonic and

biofilm cultures of the immunoneutral *L. reuteri* ATCC 55730 did not suppress TNF when compared to the media control (Fig. 4.8). In addition, mutations in NT01LR0396 (multi-drug resistance transporter in the major facilitator superfamily) and NT01LR1106 (putative esterase) had no effect on the ability of *L. reuteri* ATCC PTA 6475 to suppress TNF, demonstrating the mutagenesis system had no effect (data not shown). These results demonstrate that mutants defective in lactobacillic acid production are unable to fully suppress TNF suppression.

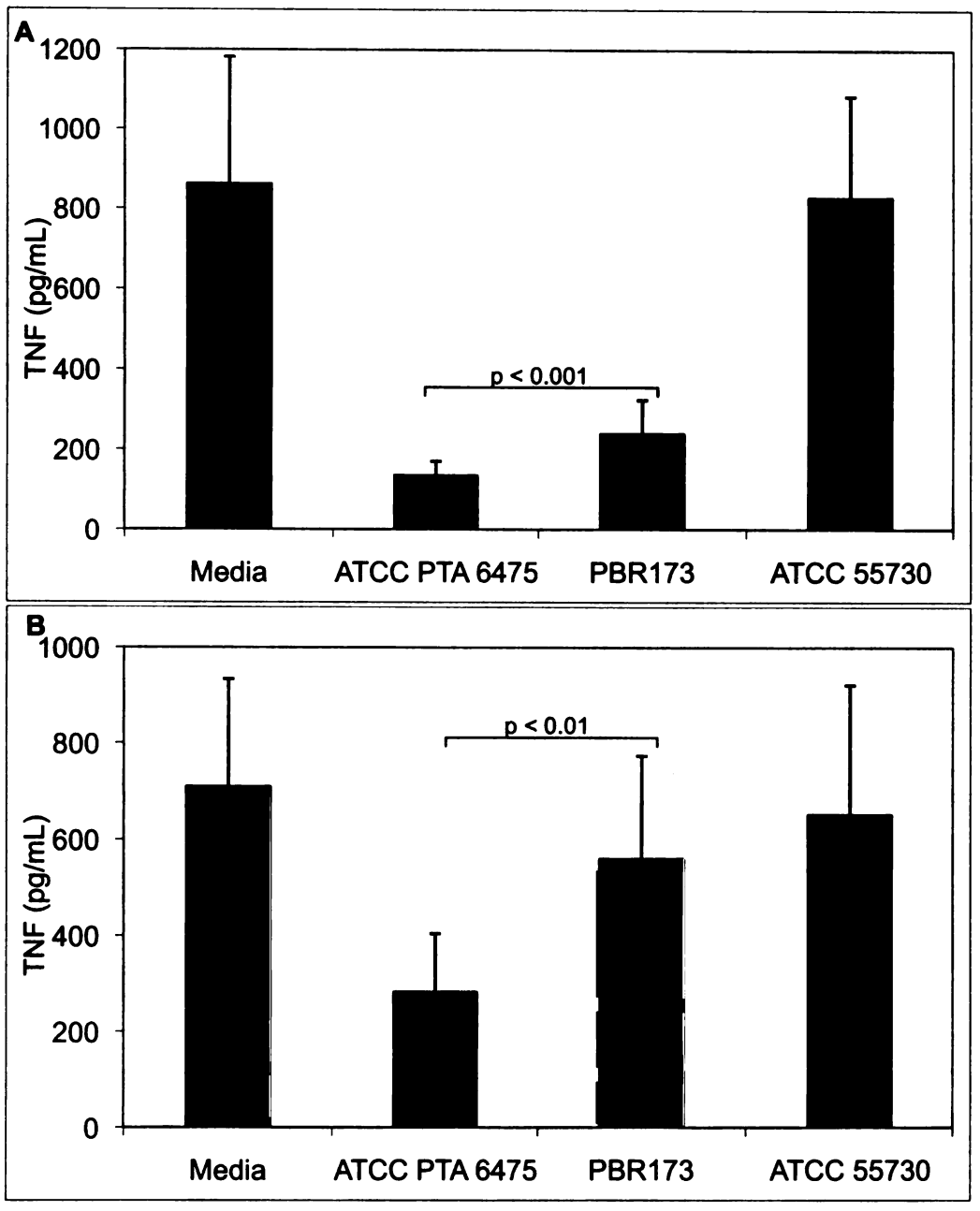

Figure 4.8 - *L. reuteri* deficient in Cfa had diminished ability to suppress TNF. Cell-free supernatants from *L. reuteri* cultured as planktonic cells or biofilms were tested for the ability to inhibit LPS-activated THP-1 cells from producing TNF. When cultured as planktonic cells (**A**) or biofilms (**B**), PBR173, which does not produce lactobacillic acid, had diminished ability to suppress TNF production compared to wild-type cells.

DISCUSSION

The ability of probiotics to affect the immune system of the host is likely to be an important mechanism for how these types of bacteria benefit human health. Several gastrointestinal disorders, including Crohn's disease, are partly caused by increased inflammation in the intestine, and therapies that target pro-inflammatory cytokines such as TNF have been effective in providing symptomatic relief is some cases. Thus candidate probiotics that can downregulate pro-inflammatory cytokines in the intestinal tract offer a means of localized delivery of an immunomodulatory agent. Several studies have identified potent immunomodulatory activities expressed by candidate probiotic bacteria, however in most cases the bacterial factors mediating these activities are not understood.

In this study we have identified the cyclopropane fatty acid lactobacillic acid as a candidate immunomodulin involved in the inhibition of TNF produced by activated macrophages. Several lines of evidence support that *L. reuteri* strains that produce lactobacillic acid have increased capabilities of inhibiting TNF. First, the presence of lactobacillic acid was only found in strains capable of down-regulating TNF production. Second, the appearance of lactobacillic acid in stationary phase correlates with the appearance of immunomodulatory activity. Third, mutation of *cfa*, which eliminates the production of lactobacillic acid,

results in a *L. reuteri* strain with reduced capacity to inhibit TNF production from both planktonic cultures and biofilms. These results indicate that lactobacillic acid is either an immunomodulatory compound itself or loss of lactobacillic acid indirectly effects the production of another immunomodulin.

We have had lactobacillic acid synthesized and have not detected TNF inhibition by lactobacillic acid at non-cytotoxic concentrations (data not shown). This either indicates that lactobacillic acid does not directly play a role in suppression of TNF expression or that we have not identified the proper conditions for delivering lactobacillic acid to macrophages. This could be due to either biological or technical reasons. We found that lactobacillic acid was very insoluble as a free fatty acid and therefore it is unclear that we were successful in delivering lactobacillic acid properly to the macrophages. In other situations fatty acids delivered to cells in phospholipids are able to mediate effects not observed with free fatty acids (7). We are currently attempting to use petroleum ether extracted lipids from the immunomodulatory *L. reuteri* strains to determine if we can reconstitute immunomodulatory activity.

The effects of cyclopropanation in lipids on the immune response has recently been studied in *Mycobacterium tuberculosis*. The disruption of cyclopropyl ring formation in mycolic acids has a profound effect on how this pathogen interacts with the immune system. Deletion of the *cma2* gene, which encodes a cyclopropane synthase enzyme which adds trans-cyclopropyl rings to mycolic

acids, produces a hypervirulent strain in a mouse model of tuberculosis. Induction of pro-inflammatory cytokines in murine-derived macrophages was more pronounced with the mutant strain and this could be mimicked with phenyl ether extracted lipids. This supports the claim that trans-cyclopropanation of mycolic acids attenuates the host response to *M. tuberculosis* (21). Interestingly, deletion of the *pcaA* gene, which encodes another cyclopropyl mycolic acid synthase in *M. tuberculosis* and is responsible for the synthesis of cis-cyclopropyl rings in mycolic acids, had the opposite effect on virulence and in vitro inflammatory responses (20). This work indicates that cyclopropane ring fatty acids can influence pathogenicity and the host immune response. Whether or not lactobacillic acid has a similar direct effect remains to be determined.

Although we currently favor a direct effect of lactobacillic acid on the inflammatory response of LPS-stimulated macrophages, it is equally possible that the observed effects of deletion of *cfa* are indirect. We have identified 36 genes that are significantly reduced in expression when *cfa* is disrupted, each of these are candidates for being involved in immunomodulation (Dephine Saulnier and Jim Versalovic, personal communication). The most interesting gene from this group is a putative cyclase (NT01LR1833) that is found only in some strains of *L. reuteri* but not other lactobacilli. Interestingly, *L. reuteri* ATCC 55730 does not contain this gene, again correlating the absence of this gene with a lack of immunomodulatory activity. The function of this cyclase is unknown. NT01LR1833 is located in a putative operon with a cobalamin-independent

methionine synthase and near an enzyme that catalyzes the final step in menaquinone (vitamin K) biosynthesis. Vitamin K, which is an essential vitamin for humans, has been linked to the regulation of the immune response. We are currently investigating whether or not this putative cyclase has any role in immunomodulation.

Further work is needed to determine if lactobacillic acid plays a direct or indirect role in modulation of the host immune response. The creation of a mutant strain of *L. reuteri* ATCC PTA 6475, PRB173, that is incapable of producing lactobacillic acid, as well as the identification of growth conditions that triple the lactobacillic acid content of the cell membranes will aid in these pursuits.

Acknowledgements. This project was carried out in collaboration with researchers at Baylor College of Medicine. Sara E. Jones grew the overnight cultures of *L. reuteri* CF48-3A (a second immunoneutral strain) for fatty acid analysis and performed the TNF inhibition experiments (including growth of bacterial and THP-1 cells, preparation of cell-free conditioned medium, and TNF assays). Delphine Saulnier identified the truncation in the *cfa* synthase gene of the immunoneutral *L. reuteri* strains. The author would also like to acknowledge Daniel Whitehead for production of Figure 4.1.

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CHAPTER 5

SUMMARY AND SIGNIFICANCE

Although research in the field of probiotics has increased exponentially over the past twenty years, the mechanisms by which these organisms exert a beneficial effect on the host are not well understood. Much research has focused on in vitro studies investigating survival of stresses that the bacteria will be exposed to in the gastrointestinal tract, the ability to inhibit pathogens, and the ability to modulate the host immune response. In vivo studies are investigating a wide range of disorders including bacterial infections, allergies, and gastrointestinal disorders. Although various strains of *L. reuteri* have demonstrated effectiveness in various in vitro and in vivo studies as discussed in Chapter 1, the mechanisms by which this effectiveness is achieved has remained elusive. For example, there appears to be wide ranging differences between the physiology and effects of particular strains within the species, and very little has been determined regarding the actual interactions between *L. reuteri* and the host.

Strain specificities. Differences between strains of *L. reuteri* need to be thoroughly investigated. Initial studies have demonstrated that there are differences in the stress resistance, production of antimicrobial compounds, immune response modulation, and effectiveness in animal trials. For example, one of the common characteristics of *L. reuteri* that is often attributed for the

ability of the species to cause beneficial effects is the production of the wide spectrum antimicrobial compound, reuterin. Research has shown that at least one strain, *L. reuteri* RC-14 does not produce reuterin, nor does it contain the necessary enzyme needed to convert glycerol into reuterin (4). Thus, any probiotic effects observed with this strain are not due to reuterin production, although this observation does not rule out reuterin's importance for other beneficial effects.

These investigations have identified some of the factors involved in the survival of *L. reuteri* in the host during exposure to bile in the small intestine, as well identified a putative novel immunomodulatory compound, lactobacillic acid. These studies have also highlighted some of the strain specificities that may play important roles in interactions with the host. Although all *L. reuteri* strains included in this work were of human origin, differences were observed in the mechanisms used for survival in the presence of bile. *L. reuteri* ATCC PTA 6475 appears to obtain a benefit from bile salt hydrolase activity during growth in the presence of bile. Preliminary research suggests that this strain's bile salt hydrolase enzyme is active against a wider range of bile acids and acts more quickly than the enzyme from *L. reuteri* ATCC 55730. Interestingly, several of the genes identified as playing an important role in the survival and growth of *L. reuteri* ATCC 55730 in the presence of bile, the putative esterase (Lr1516) and the multidrug resistance protein (Lr1584), did not appear to have overlapping roles in *L. reuteri* ATCC PTA 6475 (data not shown). Whether or not this

difference is due to the bile salt hydrolase activity of *L. reuteri* ATCC PTA 6475 has yet to be determined.

Another important strain difference is represented by the ability of some of the strains to suppress TNF, while others do not have an effect on TNF production by macrophages as described in Chapter 4. This difference suggests that some strains of *L. reuteri* may be effective in suppressing host inflammatory responses, while other strains may have no effect or may even exacerbate inflammation. Fatty acid analysis of these strains revealed that all five strains investigated had the ability to synthesize cyclopropyl fatty acids (all strains produced dihydrosterculic acid, a 19-carbon cyclopropyl fatty acid); however, only the immunomodulatory strains contained a second 19-carbon cyclopropyl fatty acid, lactobacillic acid. Although the prevalence of lactobacillic acid among the various strains, as well as the occurrence of lactobacillic acid at particular stages of growth, suggest a role for the compound in TNF suppression, it is possible that other strain differences are responsible.

Bacterial-host interactions. While strain specificity is important to investigate, ultimately the main area lacking in probiotic research, despite much effort from the field, is an understanding of the mechanisms through which these microorganisms, like *L. reuteri*, are able to cause their beneficial effects. It is unlikely that one common mechanism of action will be identified, thus careful research investigating cautiously chosen strains in a range of host genetic backgrounds should be carried out. If one strain does not alleviate a particular

disease, another strain, with different traits, should be considered. Unfortunately, the situation could be even more complicated, as at least one study involving *L. reuteri* has demonstrated that the health status of the host can alter the response to probiotic administration (3). Ouwehand et al (9) also demonstrated that the health status of the host tissue could alter adherence levels of probiotic bacteria. *L. reuteri* ING1 exhibited better adherence to colonic tissue isolated from IBD patients than healthy patients, as well as better adherence to mucus from diseased tissue versus healthy tissue. Things such as inflammatory markers, other bacteria, or mucus composition could alter this interaction (9).

One area to investigate potentially novel mechanisms of action or bacterial-host interactions would be genes that are up-regulated during stresses found in the host gastrointestinal system that do not appear to play a role in bacterial survival of this stress. The putative matrix metalloprotease discussed in Appendix C is an example of such a gene. Microarray analysis revealed that *lr1291* was up-regulated in *L. reuteri* ATCC 55730 during bile shock exposure, yet mutational analysis revealed that this gene does not appear to play a role in either survival or growth in the presence of bile. As bile is known to act as a signal for some pathogens to up-regulate virulence factors (5), it is possible that it may also act as a signal for commensals or probiotic strains to up-regulate factors involved in interaction with the host. The sequence similarity between *lr1291* and eukaryotic matrix metalloproteases, along with the important roles of matrix

metalloproteases within the gastrointestinal tract (7, 8, 14), make this a particularly intriguing gene to investigate.

Despite all of the unknown aspects of probiotics, there is substantial clinical and experimental evidence of their beneficial effects. *L. reuteri* appears to have great promise as probiotic species, exhibiting many of the in vitro characteristics classically considered to be "necessary" for a strain to cause beneficial effects, such as being of host origin, surviving stresses present in the gastrointestinal tract, and producing antimicrobial compounds. Importantly there are also multiple *L. reuteri* strains exhibiting beneficial effects in clinical trials, including RC-14's effects on urogenital health (1, 2, 6) and ATCC 55730's effects on general health and diarrhea in children (10-13). A better understanding of how *L. reuteri* responds to situations within the host and possibly modifies the responses of the host may lead to further applications for this species.

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APPENDIX A

MICROARRAY ANALYSIS OF *LACTOBACILLUS REUTERI* ATCC 55730 DURING MID-LOG AND EARLY STATIONARY PHASES OF GROWTH

PURPOSE

The purpose of this investigation was to compare the gene expression profiles of a commonly used probiotic strain, *L. reuteri* ATCC 55730, during mid-log and early stationary phases of growth. Collaborators at Baylor College of Medicine identified that certain strains of *L. reuteri* produce compounds that are able to suppress TNF production by activated macrophages (as described in Chapter 4). This activity is found in cell-free conditioned medium isolated from cells at the stationary phase of growth. The microarray studies described herein were performed in an attempt to identify potential targets to investigate as the possible immunomodulatory compounds. *L. reuteri* ATCC 55730 is not one of the strains that exhibits the TNF-suppressive activity, yet it was the only sequenced strain at the time of the experiments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. reuteri* ATCC 55370 was grown in MRS broth (BD Difco) at 37°C under microaerobic conditions (2% O_2, and 5%

CO_2, balanced with N_2) with slow shaking, or grown on MRS agar plates incubated anaerobically using the GasPack EZ Anaerobe Container system (BD Difco) at 37°C unless otherwise specified.

RNA isolation. *L. reuteri* ATCC 55730 from an 18-hour old culture was inoculated into 40 ml of MRS broth to a starting $O.D._{600}$ approximately equal to 0.03. Cultures were grown to late stationary phase. For each of the five biological replicate experiments, 5 ml samples were collected at mid-log phase ($O.D._{600}$ approximately equal to 0.3) and early stationary phase ($O.D._{600}$ approximately equal to 2.4) and immediately mixed with an equal portion of ice-cold methanol to halt transcription. Cells were pelleted by centrifugation at 4000 rpm for 10 minutes and washed with STE buffer (6.7% sucrose, 50 mM Tris-Cl, pH 8.0, 1 mM EDTA). The washed cells were resuspended in 0.25 units/μl mutanolysin (Sigma) dissolved in STE buffer and incubated at 37°C for 20 minutes. RNA was then isolated from lysed cells using the Qiagen RNeasy Kit with optional on column DNase treatment according to manufacturer's instructions.

Microarray experiments. Microarray experiments were performed as previously described (5). In short, 60-mer oligonucleotides for 1864 open reading frames were synthesized for the *L. reuteri* ATCC 55730 genome (1); 15 open reading frames from *L. reuteri* DSM20016 encoding extracellular proteins were also incuded. Various *E. coli* genes with no known similarity to the *L. reuteri* genome

were included as controls. The synthesized oligonucleotides were spotted onto Corning UltraGAPS-II slides; with each gene represented once on the microarray, while the control spots were spotted eight times, once in each subgrid. All work, from the design of the oligonucleotides to the array construction were performed at the Research Technology Support Facility at Michigan State University, East Lansing, MI, USA. RNA isolation, labeling, and hybridization were performed as previously described (3, 4). (Information regarding the microarray platform can be found at NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under GEO platform number GPL6366).

Five biological replicates, along with technical replication through dye-swapping, were performed for the microarray experiments. In other words, each RNA sample was subjected to two hybridizations and values used for subsequent analysis were averaged. Microarray data was analyzed using iterative outlier analysis with three iterations as previously described (2, 3).

RESULTS

Microarray experiments were performed to investigate the differences in the gene expression profiles of *L. reuteri* ATCC 55730 cultures at mid-log and early stationary phases of growth. The points of samples collection are shown in Figure A.1, which shows a representative growth curve for the strain. Overall, 85

genes were found to be significantly over-expressed as the cultures entered stationary phase, and 41 genes were found to be significantly under-expressed based on the iterative outlier analysis. A list of genes with significantly different expression patterns can be found in Tables A.1 to A.2.

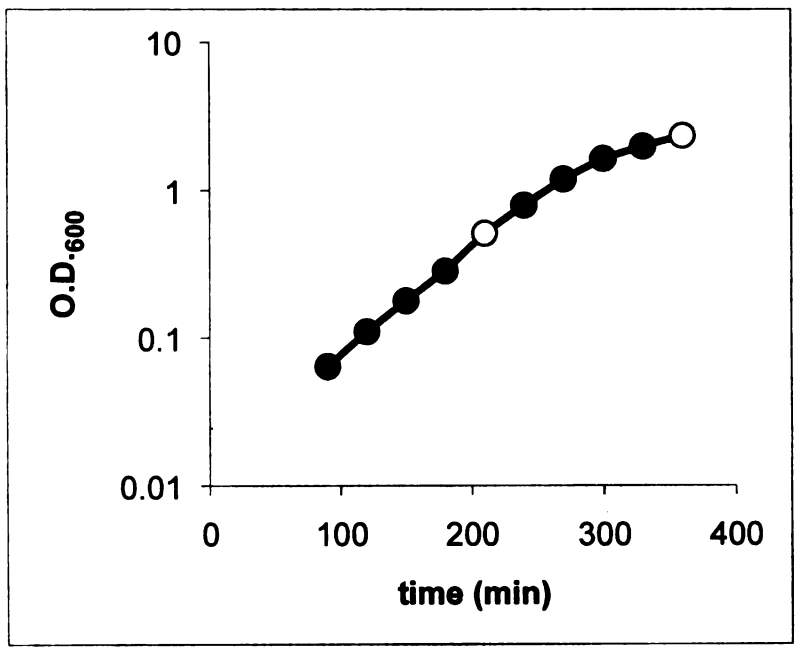

Figure A.1. A representative growth curve of *L. reuteri* ATCC 55730. Samples for microarray analysis (mid-log and early stationary phase) were taken at the time point represented by the open circles.

Table A.1. Genes significantly over-expressed by *L. reuteri* ATCC 55730 during early stationary phase.

Gene name	Accession no.[a]	Functional classification[b]	Fold-change
Energy production and conversion			
lr0904	NS	Malate dehydrogenase (EC 1.1.1.37)	5.1
lr1475	NS	Malolactic enzyme	2.6
lr1744	NS	L-2-hydroxyisocaproate dehydrogenase	4.9
lr1777	EF421920	bifunctional protein: alcohol dehydrogenase; acetaldehyde dehydrogenase	3.5
lr1793	EF421923	1,3-propanediol dehydrogenase	3.2
lr1866	DQ233710	PduW putative acetate kinase	7.6
lr1867	DQ233711	COG1454 EutG Alcohol dehydrogenase class IV	11.6
lr1868	DQ233712	PduP putative NAD-dependent aldehyde dehydrogenase	10.3
lr1882	DQ233726	PduA propanediol utilization protein: putative microcompartment protein	4.9
lr1919	NS	Malate dehydrogenase (EC 1.1.1.37)	2.9
Amino acid transport and metabolism			
lr0054	NS	Homoserine kinase (EC 2.7.1.39)	2.9
lr0124	NS	Amino acid permease	4.6
lr0125	EF537904	aminotransferase	6.1
lr0213	NS	Aminotransferase	6.3
lr0324	EF421866	Cystathionine beta-lyase/cystathionine gamma-synthase	6.0
lr0529	NS	Arginine/ornithine antiporter	5.6
lr0530	NS	Arginine/ornithine antiporter	4.0
lr0560	NS	Cysteine synthase (EC 4.2.99.8)	8.5
lr0621	NS	Transcriptional regulator, GntR family / TYROSINE AMINOTRANSFERASE (EC 2.6.1.5)	2.7
lr0650	NS	4-aminobutyrate aminotransferase (EC 2.6.1.19)	3.8
lr0701	NS	Aspartokinase (EC 2.7.2.4)	4.2
lr1019	DQ857773	ArcC, Carbamate kinase	4.7
lr1020	DQ233707	COG0078 ArgF Ornithine carbamoyltransferase partial CDS	7.2
lr1113	EF534269	Threonine synthase	2.7

Table A.1 (cont'd)

lr1162	NS	Aspartate aminotransferase (EC 2.6.1.1)	5.1
lr1258	EF421900	Xaa-Pro aminopeptidase, PepP	3.8
lr1517	DQ233695	COG2235 ArcA Arginine deiminase	6.6
lr1731	DQ233708	COG0624 ArgE Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases partial CDS	7.9
lr1784	DQ857803	Peptidase U34	3.1
lr1865	DQ233709	PduU putative ethanolamine utilisation protein	9.8
lr1881	DQ233725	PduB propanediol utilization protein	8.0
lr2088	DQ857861	Carbamoylphosphate synthase small subunit	2.6
Nucleotide transport and metabolism			
lr0302	DQ857868	Dihydroorotase and related cyclic amidohydrolases	4.6
lr0521	DQ857762	Dihydroorotate dehydrogenase	3.0
Carbohydrate transport and metabolism			
lr1525	EF534272	Arabinose efflux permease	3.1
lr1985	DQ857854	CobC, Alpha-ribazole-5'-phosphate phosphatase	3.2
Coenzyme transport and metabolism			
lr0969	NS	2-dehydropantoate 2-reductase (EC 1.1.1.169)	2.8
Lipid transport and metabolism			
lr0149	NS	Putative lipase/esterase	2.7
lr1009	EF421883	(acyl-carrier-protein) S-malonyltransferase, FabD	24.4
lr1011	EF421885	3-oxoacyl-[acyl-carrier-protein] synthase III, FabH	5.9
lr1013	EF421887	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase, FabA	23.7
lr1279	EF421902	Enoyl-[acyl-carrier protein] reductase (NADH), FabI	19.1
lr1280	EF421903	Acetyl-CoA carboxylase alpha subunit, AccA	16.3
lr1281	EF421904	Acetyl-CoA carboxylase beta subunit, AccD	14.3
lr1282	EF421905	Biotin carboxylase, AccC	20.7
lr1283	EF421906	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein), FabA	16.1

Table A.1 (cont'd)

lr1284	EF421907	Biotin carboxyl carrier protein, AccB	18.1
lr1286	EF421908	3-oxoacyl-(acyl-carrier-protein) synthase, FabB	21.4
lr1287	EF421909	3-oxoacyl-(acyl-carrier-protein) reductase, FabG	19.5
Transcription			
lr1012	EF421886	Transcriptional regulator, MarR family	20.7
lr1310	EF421911	hypothetical protein	4.2
lr1518	DQ233704	COG1438 ArgR Arginine repressor partial CDS	5.2
lr1573	NS	Leucine-responsive regulatory protein	3.2
Replication, recombination and repair			
lr0044	EF421958	ATPase related to the helicase subunit of the Holliday junction resolvase	4.7
Posttranslational modification, protein turnover, chaperones			
lr0107	NS	Thioredoxin	6.1
Inorganic ion transport and metabolism			
lr0629	DQ074834	COG1464 NlpA ABC-type metal ion transport system periplasmic component/surface antigen	3.6
lr1151	DQ074882	COG1464 NlpA ABC-type metal ion transport system periplasmic component/surface antigen	3.7
lr2077	NS	Copper-exporting ATPase (EC 3.6.3.4)	2.6
Secondary metabolites biosynthesis, transport and catabolism			
lr1745	NS	2-oxo-hepta-3-ene-1,7-dioic acid hydratase	3.3
lr1873	DQ233717	PduL propanediol utilisation protein	9.6
lr1878	DQ233722	PduE propanediol dehydratase small subunit	4.6
lr1880	DQ233724	PduC propanediol dehydratase large subunit	8.7
lr1871	DQ233715	PduN propanediol utilization protein: putative microcompartment protein	7.2
lr1874	DQ233718	PduJ propanediol utilization protein: putative microcompartment protein	4.5
lr1875	DQ233719	PduK propanediol utilization protein: putative microcompartment protein	11.3
General function prediction only			
lr0079	EF471975	D-lactate dehydrogenase	6.1
lr0272	EF537899	flavodoxin	5.6
lr0313	EF421952	HAD-superfamily hydrolase	2.6

Table A.1 (cont'd)

lr1115	NS	NAD-dependent oxidoreductase	3.4
lr1163	NS	Nitrilase (EC 3.5.5.1)	4.0
lr1834	NS	Nitrilase (EC 3.5.5.1)	4.8
lr1869	DQ233713	PduObis propanediol utilization protein	9.2
Signal transduction mechanisms			
lr0628	DQ233673	LuxS protein	3.7
lr0793	DQ074847	COG0834 HisJ ABC-type amino acid transport/signal transduction systems periplasmic component/domain	4.2
lr2123	DQ857864	Protein-tyrosine phosphatase	8.7
Unknown function			
lr1010	EF421884	Acyl carrier protein, AcpP	12.4
lr1047	EF421890	Uncharacterized conserved protein, DegV	3.7
lr1319	DQ074896	COG4716 Myosin-crossreactive antigen	3.7
lr1515	DQ074905	Unknown extracellular protein	2.9
lr1870	DQ233714	PduO putative propanediol utilization B12 related protein	3.8
lr1872	DQ233716	PduM propanediol utilization protein	10.9
lr1876	DQ233720	PduH putative diol dehydratase reactivation protein	6.1
lr1877	DQ233721	PduG putative diol dehydratase reactivation protein	7.2
lr1879	DQ233723	PduD propanediol dehydratase medium subunit	8.1
lr2078	NS	unknown protein	3.9

a GenBank accesion numbers are provided (NS = not submitted)
b Genes were classified based on COG domains found in the protein sequence through a
search of the JGI Integrated Microbial Genomes database.

Table A.2. Genes significantly under-expressed by *L. reuteri* ATCC 55730 during early stationary phase.

Gene name	Accession no.[a]	Functional classification[b]	Fold-change
Energy production and conversion			
lr0599	DQ233700	[Citrate [pro-3S]-lyase] ligase (EC 6.2.1.22)	5.0
lr0600	DQ240820	NAD-dependent malic enzyme (EC 1.1.1.38)	5.6
lr1418	DQ233691	COG3051 CitF Citrate lyase alpha subunit	4.3
Amino acid transport and metabolism			
lr0137	EF537897	spermidine/putrescine ABC transporter, permease protein	5.3
lr1197	NS	Glutamine transport ATP-binding protein glnQ	5.1
lr1198	DQ074887	COG0834 HisJ ABC-type amino acid transport/signal transduction systems periplasmic component/domain COG0765 HisM ABC-type amino acid transport system permease component	7.0
lr1508	NS	Gluconate permease	2.9
lr1664	NS	Histidine transport ATP-binding protein hisP	3.2
NS86:1	NS	amino acid permease-associated region	3.0
Nucleotide transport and metabolism			
lr0092	EF421961	Deoxyribose-phosphate aldolase	3.2
lr0115	DQ219946	COG1428 Deoxynucleoside kinase	4.1
lr0674	NS	Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	3.3
Carbohydrate transport and metabolism			
lr0101	EF421948	Phosphopentomutase (EC 5.4.2.7)	2.9
lr0160	EF547651	Sugar kinase, ribokinase family	13.7
lr0590	NS	Deoxyribose transporter	3.9
lr0675	DQ857878	Phosphopentomutase	2.9
lr1419	DQ233692	COG2301 CitE Citrate lyase beta subunit	5.8
NS359:2	NS	Ribose transport protein	12.6

Table A.2 (cont'd)

Coenzyme transport and metabolism			
lr1417	DQ233690	COG1767 CitG Triphosphoribosyl-dephospho-CoA synthetase	5.4
Lipid transport and metabolism			
NS207:1	NS	Transcription regulator	5.8
Translation, ribosomal structure and biogenesis			
lr0050	NS	LSU ribosomal protein L14P	2.9
lr0251	DQ219950	COG0594 RnpA RNase P protein component	3.2
lr1551	NS	tRNA (Guanine-N(1)-)-methyltransferase (EC 2.1.1.31)	3.5
Transcription			
lr0647	NS	Catabolite control protein A	3.2
Replication, recombination and repair			
lr1237	NS	DNA gyrase subunit A (EC 5.99.1.3)	3.6
lr1238	NS	DNA gyrase subunit A (EC 5.99.1.3)	3.4
lr1239	NS	DNA gyrase subunit B (EC 5.99.1.3)	3.6
lr1240	DQ219962	COG1195 RecF Recombinational DNA repair ATPase	4.1
Cell motility			
NS376:1	NS	putative type II secretion system protein	7.0
General function prediction only			
lr0517	NS	Zinc protease (EC 3.4.99.-)	3.2
lr1416	DQ233689	COG0596 MhpC Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily)	3.2
lr1666	NS	putative zinc-dependent peptidase	4.2
NS337:1	NS	Conserved hypothetical protein	4.3
Signal transduction mechanisms			
lr0279	EF421950	Stress response membrane GTPase TypA	3.0
lr1665	DQ074920	COG0834 HisJ ABC-type amino acid transport/signal transduction systems periplasmic component/domain	3.5
Intracellular trafficking, secretion, and vesicular transport			
lr0252	DQ074813	COG0706 Preprotein translocase subunit YidC	3.0
Unknown function			
lr0646	DQ233675	Hypothetical membrane protein	3.0
lr0982	DQ857874	Hypothetical protein	3.4

Table A.2 (cont'd)

lr1434	DQ074902	Unknown extracellular protein	3.1
lr1487	DQ074904	Unknown extracellular protein	4.1
NS104:1	NS	unknown protein	3.4

a GenBank accesion numbers are provided (NS = not submitted)
b Genes were classified based on COG domains found in the protein sequence through a
search of the JGI Integrated Microbial Genomes database.

The microarray experiments described were performed to identify potential immunomodulatory compounds based on gene expression changes as *L. reuteri* transitions from mid-log to early stationary phases of growth. Although *L. reuteri* ATCC 55730 is not one of the strains capable of producing compounds that suppress TNF production, it was the only strain for which sequence information and therefore, microarrays, were available at the time of the study. The microarray studies revealed two main pathways that were up-regulated as the bacteria transitioned from mid-log to early stationary phase, propanediol utilization and fatty acid biosynthesis. The interest in fatty acid biosynthesis led to the identification of fatty acid differences between the immunomodulatory and immunoneutral strains as described in Chapter 4.

This data is not included in Chapter 4 because preliminary experiments carried out by the lab at Baylor College of Medicine using microarrays designed for one of the immunomodulatory strains, *L. reuteri* ATCC PTA 6475, did not confirm similar gene expression changes for this strain. This may be due to genuine differences in gene expression between the two strains or due to the collection of

samples at different time points (and possibly different stages of growth) between the two labs.

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APPENDIX B

IDENTIFICATION OF A PUTATIVE BILE-INDUCIBLE TRANSCRIPTIONAL ELEMENT FROM *LACTOBACILLUS REUTERI*

PURPOSE

Bile has been demonstrated to serve as a signal for particular bacteria regarding their location in the gastrointestinal tract. For example, certain pathogens, including *Vibrio*, *Shigella*, *Salmonella*, and *Campylobacter*, are known to alter expression of virulence factors such as toxin production, motility, and type III secretion systems in response to bile (2). Various bile acids have also been shown to affect the germination of *Clostridium difficile* spores (3, 4). As there is known to be overlap in the interaction of pathogenic and commensal bacteria with the host, it is possible that bile also acts as a signal for probiotic bacteria. Using the microarray analysis of the bile shock response of *L. reuteri* ATCC 55730 (5), the purpose of this study was to use a bioinformatic approach to identify a bile-inducible transcriptional element associated with promoters induced by bile.

RESULTS

The upstream regions (300 bp) of all genes over-expressed during the bile shock response of *L. reuteri* ATCC 55730 were analyzed for over-represented motifs using the freely-available internet program MEME (1). As it is unlikely that all of the genes over-expressed during bile shock will be regulated by the same element, multiple MEME runs were performed comparing the upstream regions of smaller subsets of genes. One statistically significant 13 base pair motif was identified when the upstream regions of 13 genes over-expressed during bile shock were compared. The upstream regions included in this particular run were from the following genes: *lr0359*, *lr0540*, *lr0685*, *lr0783*, *lr0922*, *lr1067*, *lr1139*, *lr1351*, *lr1468*, *lr1684*, *lr1786*, *lr1864*, and *lr1937*. The sequence of the motif at each of these locations, as well as the consensus sequence is shown in Figure B.1.

Gene number	Motif sequence												
lr0359	C	A	A	T	T	G	C	G	T	C	A	T	C
lr1067	C	A	A	T	T	G	C	T	T	G	A	T	C
lr1786	C	A	A	T	T	G	C	A	T	A	A	T	C
lr0685	C	A	A	T	T	G	G	G	T	T	A	T	C
lr1351	C	A	G	T	T	G	C	C	T	T	A	T	C
lr0922	C	A	T	T	T	G	C	T	T	C	A	T	C
lr1468	G	A	A	T	T	G	C	A	T	C	A	T	C
lr0783	C	A	A	T	T	G	G	G	T	G	A	A	C
lr1139	C	A	A	T	T	G	C	A	T	A	A	T	T
lr1937	C	A	A	T	T	C	C	T	T	C	A	T	G
lr1684	C	C	G	T	C	G	C	G	T	T	A	T	C
lr0540	C	A	A	T	T	G	C	C	A	A	A	T	G
lr1864	T	A	A	A	T	G	C	G	T	T	A	A	C

Consensus	C	A	A	T	T	G	C	G	T	C	A	T	C
								A		T			
								T		A			

Figure B.1. The sequences of the 13 occurrences of the possible bile-inducible transcriptional element in *L. reuteri* ATCC 55730 identified by MEME.

In collaboration with C. Titus Brown at Michigan State University, genome-wide analysis of this putative bile-inducible transcriptional element was performed. Dr. Brown identified that this particular 13 base pair sequence was found a total of 70 times throughout the *L. reuteri* ATCC 55730 genome and 54 times throughout the genome of another probiotic strain, *L. reuteri* ATCC PTA 6475. If this were a random sequence element it would be expected to be found only 11 times in a genome with the size (approximately 2.3 mega-bases) and GC content of *L. reuteri* ATCC 55730.

Further analysis revealed that of the 70 occurrences of this particular 13 base pair element in *L. reuteri* ATCC 55730, 66 of them are within the coding regions of other genes. It was also demonstrated that none of the additional 57 elements (beyond the original 13 identified) are located upstream of genes that are significantly over-expressed or under-expressed during the bile shock response of this strain.

FUTURE DIRECTIONS

Current work in the lab is focused on developing a reporter gene system for use in *L. reuteri*. This system will ultimately allow us to identify whether this putative 13 base pair transcriptional element is a bile-inducible promoter. A bile-inducible promoter could have great potential for application in the probiotic industry, as it would allow for targeted expression of a gene product in the host small intestine.

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APPENDIX C

INVESTIGATION OF A PUTATIVE MATRIX METALLOPROTEASE FROM *LACTOBACILLUS REUTERI*

PURPOSE

The purpose of this study was to investigate the role of a putative metalloprotease identified in *Lactobacillus reuteri*. This gene, *lr1291*, was identified as being significantly over-expressed during the bile shock response of *L. reuteri* ATCC 55730. Mutational analysis did not reveal a role for this gene in survival or growth in the presence of bile (14). Sequence analysis revealed that the protein has significant similarity to eukaryotic matrix metalloproteases (MMPs), a class of proteins known to be involved in a wide range of important functions such as recruitment of immune cells, cytokine and chemokine activation, and degradation of the extracellular matrix. As very little is currently understood regarding how probiotic bacteria interact with the host to cause their beneficial effects, the presence of a gene with significant similarity to eukaryotic proteins known to play important roles in the gastrointestinal tract is of interest.

Note: Although the gene was originally identified in *L. reuteri* ATCC 55730 (*lr1291*), the homolog found in *L. reuteri* ATCC PTA 6475 (NT01LR0595) was used for all bioinformatic and over-expression studies discussed herein. There is

96% similarity between Lr1291 and NT01LR0595 at the amino acid level, and 93% similarity at the nucleic acid level; both proteins are referred to as Lr1291 for simplicity.

BIOINFORMATICS

A BLAST of the protein sequence of Lr1291 against the non-redundant protein sequences in GenBank revealed that the closest matches were peptidases in the M10A or M12B family (including matrixins and adamalysins), putative metalloproteinases, or zinc-dependent proteases in either *Lactobacillus* spp. or *Streptococcus* spp (1). Lactic acid bacteria with similar proteins included primarily host-associated strains such as lactobacilli commonly used as probiotics, including *L. reuteri*, *L. acidophilus*, *L. gasseri*, *L. johnsonii*, and *L. plantarum*, as well as pathogenic streptococci including *Streptococcus pneumoniae* and *S. mutans*. The significant hits also included several human matrix metalloproteases (MMPs) that aligned with the second half of Lr1291. A search of the conserved domain database (5) revealed that the last 130 amino acids of Lr1291 contains cd04268 (ZnMc_MMP_like subfamily). This group includes matrix metalloproteases, serralysins, and astacin-like family of proteases.

To further investigate the sequence of Lr1291, the protein sequence was compared to the MEROPS database (10). This search revealed that Lr1291 fits

into the M10 family, although it was classified along with other unassigned peptidases. The active site for the M10 family is HExxHxxgxxH, including the histidine residues that act as zinc ligands and the catalytic glutamate. This family shares the same active site as several other families of metalloproteases (M12, M43, and M57). The M10 family peptidases are classified as "metzincins" because of the conserved methionine residue found C-terminal to the active site. The M10 family peptidases often are also found to function outside of the cell. In line with this, Lr1291is predicted to be secreted, due to the presence of an N-terminal signal peptide without any discernable cell envelope anchor (2). Figure C.1 shows the protein sequence of Lr1291with several characteristics of interest marked.

>NT01LR0595
VNEINFIFRLLKRIFIIGLLVGGGWLYFNDARVQA //
TANQTAWNVRDRIAKLIGRDDTNSNDNSNLHLNDANNGSSKNEQGPTTEQQT
STQTSIPSTGRWATNQATVYVNTNNAQLDAATNTAIQNWNQTGAFTFKPVNNQ
SKADIVVTTMNRSDSNAAGLTKTSSNSLTRRFMHATVYLNTYYLTDPSYGYSQE
RIVNTAE **HELGHAIGLDH** TNAVSV **M** QPAGSFYTIQPDDVQAVQKLYANNK

Figure C.1. The protein sequence of *L. reuteri* ATCC PTA 6475 NT01LR0595 (Lr1291 homolog). The putative cleavage site is marked with two forward slashes (//); this site was predicted by SignalP 3.0 (3, 8). The putative active site is underlined, with the conserved residues in bold. The conserved methionine residue ("met-turn") is also in bold.

These similarities and sequence comparisons suggest that Lr1291 is part of a novel series of bacterial zinc-dependent metalloproteases of unknown function. Although there are other known bacterial zinc-dependent metalloproteases, none

of these proteins of known function were found to have significant sequence similarity.

EXPERIMENTS AND RESULTS

This section will briefly outline some of the experimental attempts to determine the function of Lr1291.

Expression vectors and protein forms. Various expression vectors were used in attempts to over-express Lr1291. Original cloning attempts were made into traditional pET vectors, including pET-21b (C-terminal his-tag expression vector) and pET-19b (N-terminal his-tag expression vector). Due to the difficulty in cloning (owing to low transformation efficiencies and high background levels, a pET-21b construct was never constructed; one pET-19b construct was constructed after multiple attempts), the Gateway system (Invitrogen) was employed. Several constructs were made successfully using this system, albeit at much lower efficiencies than suggested by the Invitrogen product manual. The vectors successfully used for constructs included: pET-DEST42 (C-terminal his-tag expression vector), pDEST-17 (N-terminal his-tag expression vector), and pDEST-15 (N-terminal GST-tag expression vector). Various forms of the gene were also used: the complete Lr1291 sequence, the Lr1291 sequence beginning with the putative cleavage site, and the complete Lr1291 sequence with the

active site mutated from HExxHxxGxxH to AAxxHxxGxxH. All constructs were confirmed by sequencing.

Induction strains and conditions. Multiple strains were tested for induction with the various expression vectors. These included: *E. coli* BL21, *E. coli* Tuner, and *E. coli* Rosetta (DE3) pLysS cells (Novagen). Induction for these strains with the various vectors was attempted at 16° C, 25° C, 30° C, and 37° C in the presence and absence of 100 uM zinc acetate. Cell lysates from all cultures were prepared by sonication and loaded onto SDS-PAGE gels. No difference was detected between proteins from uninduced and induced cultures under any conditions tested. Lysis of the induced cultures was observed on multiple instances, suggesting a toxic effect of the protein.

Codon usage. Lr1291 contains multiple codons that are rarely used in *E. coli* (including four occurrences of AUA encoding isoleucine and six instances of GGA encoding glycine). In order to determine if codon usage played a role in the lack of high expression levels in *E. coli* BL21, Rosetta cells were employed for expression. *E. coli* Rosetta (DE3) pLysS cells from Novagen contain the pRARE plasmid, which supplies the tRNAs that recognize the following rare codons: AUA, AGG, AGA, CUA, CCC, and GGA (9). Use of Rosetta cells did not result in detectable expression levels of Lr1291.

Other systems for expression. When multiple attempts were made to express the protein in *E. coli* using various vectors, expression strains, and induction conditions, other systems were employed. These included an in vitro translation system (Qiagen EasyXPress Protein Synthesis Kit, catalog number 32501), over-expression in *Bacillus subtilis* (using the integrative pDR111 plasmid), and over-expression in *Kluyveromyces lactis* (New England Biolabs yeast expression kit, catalog number E1000S). None of these systems resulted in detectable over-expression of Lr1291. When a construct containing Lr1291 was included in the in vitro translation reaction with the control vector, over-expression of the control protein (elongation factor EF-Ts) was completely shut down. This result suggests that production of Lr1291 may interfere with translation.

Isolation from conditioned medium. After attempts at over-expression failed, experiments were carried out to try to isolate the protein from conditioned medium of *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 6475. For these experiments, both wild-type strains and mutants for each strain containing a disruption in Lr1291 were used. Cell-free conditioned medium from cells grown in deMan, Rogosa, Sharpe (MRS) broth (Difco) were used. Samples were collected from mid-log phase cultures, before and 15 minutes after exposure to bile, and from overnight cultures. The cell-free medium was concentrated under nitrogen, as some component of MRS broth clogged all types of centrifugation concentration devices tried. Despite using the same conditions where Lr1291

was shown to be over-expressed (15 minutes after bile exposure, Chapter 2) no consistent protease activity was identified in these samples.

Because of the inconsistencies observed with concentrated conditioned medium from *L. reuteri* cultures, attempts were made to precipitate proteins from the cultures using 20% trichloroacetic acid. The samples were loaded onto SDS-PAGE gels and onto casein and gelatin zymograms. No difference in the secreted proteins was detected between the wild-type *L. reuteri* and the Lr1291 mutant strains. Importantly, no protease activity was detected in any of the samples tested. The protocol for precipitation of proteins from *L. reuteri* conditioned medium is included at the end of this Appendix.

Detection of activity. In all cases (protein expression and isolation from conditioned medium), samples were applied to either casein or gelatin zymograms to determine if any protease activity was detected. In several cases, protease activity was observed. Further investigation revealed that this protease activity was present in any *E. coli* lysate and was not specific to the production of the protein of interest.

Note: Although some companies sell pre-made zymogram gels, the use of these products is not recommended. The results obtained with these products were highly variable. Due to the difficulty and inconsistencies in various zymogram

protocols, the protocol listed at the end of this Appendix obtained from Dr. Jenifer Fenton at Michigan State University is recommended for use.

DISCUSSION

The role of Lr1291, a protease of unknown function with homologs in various strains of host-associated lactic acid bacteria, should be investigated. The protein sequence shows intriguing similarity to eukaryotic matrix metalloproteases, a class of proteins known to play wide-ranging roles in normal development, inflammation, and the immune response. The MMPs have been implicated in important roles in the gastrointestinal tract, specifically in the intestinal immune response, the activation of defensins (antimicrobial peptides produced in the small intestine) and the development of inflammatory bowel disease (6, 7, 15). It is possible that proteases like Lr1291 may play an important role in the interaction of bacteria with the host.

It is difficult to determine why there was a lack of experimental success for this project. The problems with the protein expression systems (low success rate in cloning, lysis of induced cultures, etc) suggest a level of protein toxicity, although the lack of success in over-expression of the mutant protein (active site changed from HExxHxxGxxH to AAxxHxxGxxH to inhibit activity) suggests that factors other than the protein activity are involved. A similar protein also found in *L. reuteri*, Lr1017, did not cause lysis of the expression strains and was shown to

be over-expressed using a pDEST-17 construct in the *E. coli* BL21 expression strain, suggesting that the problem is specific to Lr1291 and not a simple technical issue.

The lack of activity observed with cell-free culture supernatant and precipitated secreted proteins from *L. reuteri* ATCC 55730 and ATCC PTA 6475 could be due to a low level of protein production under the growth conditions used or the use of an inappropriate substrate for this particular protease. It is also possible that the protein in question, Lr1291, is not actually secreted or may be secreted in an inactive form.

Although casein and gelatin zymography are commonly used to detect general protease activity, it is possible that this particular protease has a more specific substrate. Several eukaryotic MMPs have either shown little or no activity against gelatin in vitro, thus the use of other substrates such as elastin, collagen, and various synthetic peptides should be considered for future use (4, 11, 12). Detection of activity from matrilysin (MMP-7) has been particularly difficult, and sensitive assays using synthetic fluorogenic peptides as substrates have been employed (13). A screen of several of these synthetic peptides may be a more sensitive and possibly useful way of identifying protease activity from Lr1291.

Other difficulties have been encountered in attempts to determine the role of various eukaryotic proteases. For example, MMP-23 did not demonstrate any

protease activity against multiple substrates in vitro, suggesting either unique substrate specificity or a mis-folding of the protein during purification. Weak protease activity was finally achieved with a chimeric protein, combining the propeptide domain of MMP-19 (a metalloprotease known to autoactivate) with the catalytic domain of MMP-23, suggesting that activation of the protein (cleavage of the prodomain) may have been an issue in vitro (12). Despite the multiple attempts in this study at detecting activity from cultures of *E. coli* induced to express the protein and from conditioned medium of *L. reuteri*, mis-folding or a lack of proper activation of Lr1291 are possibilities that should also be investigated.

Current work in the lab by another researcher suggests that some success in over-expressing the cleaved form of Lr1291 may be found using a *Lactococcus lactis* expression system. If this finding is confirmed, careful selection of a wide-range of substrates and sensitive assays should be employed for detection of protease activity. The value of the mutant strains of *L. reuteri* containing disrupted Lr1291 genes should also not be ignored. If attempts to purify the protein are not successful, these strains could be employed in various assays, such as activation of defensins or production of cytokines and chemokines by eukaryotic cells, in attempts to determine the role of this protease.

PROTOCOLS OF INTEREST

Protein precipitation from *L. reuteri* conditioned medium.

1. Grow *L. reuteri* in LDM-III broth containing Tween 80 and glucose (MRS broth contains too many proteins; the background levels of precipitated proteins will interfere with results). At desired time, collect cells by centrifugation at room temperature for 10 minutes at 4000 rpm. Filter supernatant through a 0.22 μM filter into a 50 mL conical tube.

2. Add ice-cold 100% trichloroacetic acid (TCA) to a final concentration of 20%. Incubate on ice at 4° C (cold-room) for 48 hours.

3. Transfer mixture to Sorvall tubes and spin at 12,000 x g for 30 minutes at 4° C. Remove supernatant.

4. Resuspend pelled in 2 mL of ice-cold acetone (if starting with original culture volume of approximately 20 mL). Incubate on ice for 30 minutes. Transfer mixture to two 1.5 mL eppendorf tubes.

5. Spin at maximum speed in a microcentrifuge kept at 4° C for 30 minutes. Remove supernatant and air-dry pellets for 2 to 3 minutes. Resuspend pellet in desired sample buffer and proceed. **Note:** For zymograms, do not resuspend

pellets in the LDS sample buffer provided for use with the pre-cast Invitrogen protein gels. Although this buffer works well for visualization of the proteins, it will destroy any protease activity present in the samples.

Zymograms (SDS-PAGE Substrate Gel Method)

Stock Solutions

- 30% Acrylamide/bis-Acrylamide (29:1) solution (purchase from Bio-rad, catalog #161-0156). Store in refrigerator.

- 2.0 M Tris, pH 8.8. Store in refrigerator

- 0.5 M Tris, pH 6.8. Store in refrigerator.

- 0.625 M Tris, pH 6.8. Store in refrigerator.

- 10% SDS stock. Store at room temperature.

- 10% ammonium persulfate. Weigh 0.1 g ammonium persulfate in a microcentrifuge tube, add 1 mL of ddH_20 the day of use, throw away when done.

- Substrate - Gelatin: 2.0 mg/mL Swine skin gelatin (Sigma, #G2500). Weigh 100 mg gelatin in a 50 mL conical tube, add 50 mL of ddH_20, warm slightly to dissolve gelatin completely in water. Store in refrigerator.

- Substrate - Casein: 2.0mg/mL casein in 5mM Tris (Sigma, #C7078). Dissolve 100mg of casein in 20 mL of 10mM NaOH and heat to get in solution while stirring. Neutralize by slowly dripping in 20mL of 10mM HCl while stirring on ice (sep funnel). Add 500 mM Tris, pH 7.5 (.5mL). Add 9.5 mL ddH2O. Store in refrigerator for no more than a month.

*may not have to add the entire 20 mL of 10 mM HCl. Add slowly, stopping as soon as the solution becomes slightly cloudy (usually somewhere between 14 to 20 mL in my experience). Can make up any needed volume by

the addition of more ddH2O. The addition of excess HCl will cause the casein to crash out of solution.

- 4X Sample buffer (40% glycerol, 4% SDS, and 0.25 M Tris; pH 6.8). For 10 mL total volume, add in this order to a 15 mL conical tube: 0.4 g SDS, 4 mL of 0.625 M Tris stock, 2 mL ddH_20. Vortex. Add 4 mL glycerol and a small amount of powdered bromophenol blue. Vortex.

- Running buffer (total volume of 4 mL). Add 1 L ddH_20 and a stir bar to a 2 L graduated cylinder, weigh out 57.6 g glycine, 12.1 g Tris, and 4.0 g SDS, add the three chemicals to the 2 L graduated cylinder, fill the graduated cylinder to the 2 L mark and stir until clear. Pour into a 4 L amber bottle. Add an addition 2 L of ddH_20 to the amber bottle, cap, and shake.

- Equilibration buffer - 2.5% Triton-X100 (total volume of 4 mL). Add 1.9 L ddH_20 and a stir bar to a 2 L graduated cylinder place on a warm plate (medium setting) and stir, add 100 mL of Triton-X100 very slowly. Stir until clear. Pour into a 4 L amber bottle. Add an additional 2 L of ddH_20 to the amber bottle, cap, and shake.

- Incubation Buffer. 50 mM Tris HCl pH 7.5, 200 mM NaCl, 10 mM CaCl2, 10 uM $ZnCl_2$, 0.02% Brij 35 (only good for one week once the calcium and zinc are added).

- 4X Stain. Add 1 g Coomassie blue R250 to 1L methanol, stir and gravity filter.

- 1X Stain. Add to a 500 mL glass bottle, 100 mL 4X stain, 40 mL glacial acetic acid, and 260 mL ddH_20, shake.

Procedure (Mini gels = 7.5 mL)

Day 1

1. Substrate gel

a. Mix the following in a conical vial for **one gel**:

2.0 mL 30% acrylamide stock

150 μL 10% SDS

1.4 mL 2.0 M Tris, pH 8.8

2.13 mL ddH_20

1.86 mL gelatin stock or casein stock

b. Add together simultaneously to mixture above:

83.6 μl ammonium persulfate

10.7 μl TEMED

c. Mix pour immediately by slowly adding liquid gel to chamber with a transfer pipette. Slowly overlay gel with approximately 320 μL ddH_20 (160 μL twice).

d. Let stand at room temperature for 1 hour (remove water overlay before pouring stacker).

2. 4 % Stacking gel

a. Mix the following in a conical vial for **two stackers**:

0.65 mL acrylamide

1.25 mL 0.5 M Tris, pH 6.8

50 mL 10% SDS

3.05 mL ddH_20

b. Add together simultaneously to mixture above:

25 mL ammonium persulfate

6 mL TEMED

c. Center comb in chamber and add liquid stacking gel with a transfer pipette, be careful not to create air bubbles. Let sit at room temperature for 30 minutes.

3. Loading the gel

a. Enzyme samples are mixed with 4X sample buffer (32 mL sample: 8 mL sample buffer); load 40 μL into well.

b. For molecular weight ladders follow the protocol for each.

c. Load samples with pipette tips while gels are completely submersed in running buffer, use a new tip for each sample. Check that the inner area is completely filled with running buffer and that the outer area is at least covered by buffer to the top black knob with running buffer. You can use a

transfer pipette to fill the inner area of the apparatus. This allows good current flow.

4. Running the gel

Be sure that the red electrode is connected to the red outlet and the black to the black. Run the gel at 65 V until the samples are thru the stacking gel (approximately 30 minutes); then at 120 V (or 100 V if separation looks odd) until the blue line is below the gray seal at the bottom of the glass plates. Be sure that the protein does not run off the plates. While running the gel watch that the samples run at the same speed; watch for "smile" or "frown" sample lines. If they start to form, either increase or decrease the voltage to eliminate the "smile" or "frown".

5. Preparing the gel for staining

a. Once the samples are done, remove the gel apparatus from the buffer chamber set-up. Remove the gel from between the glass plates. Carefully remove the plates from their position on the gel apparatus. Gently lift one spacer (to release the vacuum that forms while running the gel between the plates) to remove the top plate (shorter plate). The gel will remain attached to the top (shorter) plate.

b. Remove the stacker from the gel by pressing a piece of paper towel along the stacker portion of the stacker-gel junction. Next, gently pull up

on the paper towel to remove the stacker from the gel. Cut the upper right hand corner so that the orientation of the gel is known. By cutting the right top corner you are cutting the area where the marker is loaded or lane 1of the gel. If more than one gel is run, cut two (same side) or three corners so that the gels can be distinguished from each other.

c. Place each gel in a container, submerse each gel in equilibration buffer and cover container. Shake for 30 minutes in rocking water bath. Pour off old buffer in sink (be sure to retain gels in the container). Add new equilibration buffer to submerse each gel and cover container. Shake for 30 minutes in rocking water bath. Pour off buffer in sink (be sure to retain gels in the container). Add incubation buffer to submerse gel, cover container and incubate at 37C overnight (or for up to 48 hours).

Day 2

6. Staining the gel

Stain gel for 1 hour with 1X stain (completely submerse gel) at room temperature. Rock on rocker in covered container.

7. Destain

Destain the gel in ddH_20.

8. Storage of the gel

Store gel in 10% glycerol or ddH_20.

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