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Caitlin D. Goin

University of Nebraska at Lincoln, caitlin.goin@huskers.unl.edu

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Growth of Lactic Acid Bacteria: Influence of Protocooperation, Bacteriophage Infection
and Prebiotic Carbohydrates

by

Caitlin D. Goin

A THESIS

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Growth of Lactic Acid Bacteria: Influence of Protocooperation, Bacteriophage and Prebiotic Carbohydrates

Caitlin D Goin, M.S

University of Nebraska, 2010

Advisor: Robert W. Hutkins

Lactic acid bacteria are a vital part of the fermented food industry and are the subject of much interest and research. Industry is especially interested in using modern molecular approaches to maintain and improve selected strains; several industrial uses could be improved by closer investigation, namely the protocooperation of *S. thermophilus* and *L. bulgaricus*, the role of CRISPRs in phage resistance, and the utilization of prebiotic carbohydrates. Questions such as what genes and pathways are shared during milk fermentation between *S. thermophilus* and *L. bulgaricus*, when and what genes are active during bacteriophage infection, and how and where does prebiotic carbohydrate utilization occur can be answered. By using microarrays, complete snapshot of gene expression during each of these conditions are generated and detailed expression profiles can be produced. By devising a screening system, the distribution of the phenotype of GOS fermentation over a wide array of lactic acid bacteria from *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *S. thermophilus* can be explored. Once this phenotypic distribution is generated, selected strains that are able

to utilize GOS can be studied in detail to determine the mechanics of GOS fermentation. Answering these questions will add to the understanding of what factors are involved in successful fermentation and eventually be able to improve strain selection methods.

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Preface

This thesis is comprised of three chapters and a conclusion section. The first chapter is a literature review on the influence of proto-cooperation and bacteriophage infection in *Streptococcus thermophilus*, and the fermentation of prebiotic carbohydrate by lactic acid bacteria. The second chapter describes our completed *S. thermophilus* transcriptome project in publication form. In the third chapter, we report our results of the fermentation of the prebiotic galactooligosaccharide (GOS), by lactic acid bacteria, with a focus of fermentation by *Lactobacillus reuteri*. A conclusion section is included at the end to summarize the major research findings outlined within this thesis.

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Chapter 1

Literature Review

Introduction

Lactic acid bacteria, commonly referred to as LABs, are a group of commercially important organisms that are classified by their ability to ferment hexose sugars into mainly lactic acid. These bacteria are an integral part of the fermented foods industry, performing the main bioconversions in fermentations involving dairy products, meats, and vegetables [20]. The manufacture of these fermented foods is dependent on the proper and reliable function of the bacterial cultures used to produce those foods. As such, production of high cell density and active cultures are among the top priorities of the fermented food industry. Many factors can play a role in the difference between a successful fermentation and a failed one. Historically, strictly maintained culture collections and careful strain selection methods have been used to help minimize failed fermentations and to maintain product quality. In addition, much research has been done to help assess other aspects of the fermentation process. This literature review will focus on three such aspects: the influence of protocoooperation among LAB groups, the influence of bacteriophage infection, and the fermentation of the prebiotic carbohydrate galactooligosaccharides (GOS).

A Story of Protooperation: The Functional and Evolutionary Relationship of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*

In fermented dairy products such as cheese and yogurt, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are often paired together in an equally beneficial relationship, termed 'protooperation' [18]. In such a relationship, each provides something that the other needs, and both organisms grow better as a result of this association. The production of these fermented dairy products rely on this synergistic relationship for a prompt and complete fermentation. *S. thermophilus* grows first, fermenting lactose to lactic acid. This initial growth of *S. thermophilus* continues until all the free amino acids within the medium are depleted. This production of lactic acid lowers the pH to a level more favorable to *L. bulgaricus*, thereby stimulating cell growth. As *L. bulgaricus* begins to grow, it secretes an extracellular protease that hydrolyzes proteins in milk or any other medium, and secretes amino acids and other peptides that both itself and *S. thermophilus* can utilize. The result is that both bacteria grow better and faster together than apart [6].

Recent analysis of the genomes of these organisms gives several clues to the importance of this relationship. The genomes of *S. thermophilus* and *L. bulgaricus* have each undergone extensive and reductive evolution, driven in part by their high adaptation to the milk environment as well as to each other [13,14]. This is evidenced by the overall small genome size and the large number of pseudogenes, or genes that do not code for functional sequences, found within each genome.

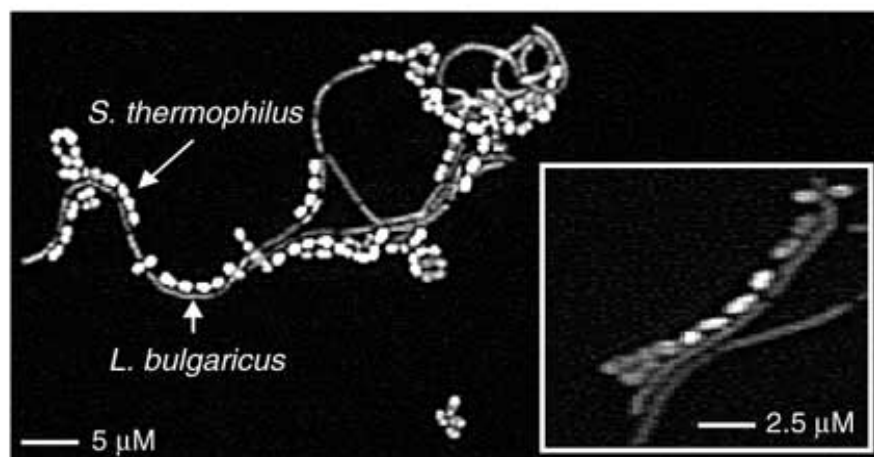
At around 1700 genes in size, the genome of *L. bulgaricus* is one of the smaller genomes of lactic acid bacteria, where the largest genome size, that of *Lactococcus lactis*, is approximately 2700 genes [20]. In addition to this small size, recent annotation of the sequenced genome has determined that almost 27 percent of the *L. bulgaricus* genome consists of either pseudogenes or fragments of non-coding regions [30]. This high percentage indicates active genome change and gives evidence for a strong environmental driving force. The genome of *S. thermophilus* has also been sequenced and its genome annotated. Very much paralleling the genome of *L. bulgaricus*, it is small, only 1800 genes in size, and 10 percent of its genome is comprised of pseudogenes [5].

Perhaps more interesting is what genes are missing, what genes have been retained, and what genes are otherwise non-functional in these two genomes. When compared to the genomes of other dairy-related members of the genus *Lactobacillus* that have been sequenced and annotated, the *L. bulgaricus* genome has lost several sugar transport and metabolism genes and amino acid biosynthetic pathways [30]. This loss of functioning metabolic systems has been suggested to be a direct result of adaptation to the milk environment [20]. The ability to transport and metabolize multiple sugars was no longer necessary in an environment where lactose availability is high, and likewise the ability to synthesize amino acids was non-essential in the nutrient-rich milk [31]. The genes missing or non-functional in the *S. thermophilus* genome also indicate a high amount of adaptation. Multiple carbohydrate uptake and

fermentation pathways are missing and four of the seven sugar phosphotransferase system transporters (PTS) are pseudogenes [5].

As for the genes that were retained, the relationship between these two organisms has also become particularly clear. The extracellular proteinase of *L. bulgaricus*, which enables it to hydrolyze proteins in the environment, is absent in *S. thermophilus*. *S. thermophilus* has retained its ability to synthesize amino acids, something that *L. bulgaricus* has lost [30]. The two organisms must therefore work together in a multi-dimensional capacity for the benefit of both.

The occurrence of lateral gene transfer between *S. thermophilus* and *L. bulgaricus* has also played a role in their evolutionary relationship. Their close association with one another within the milk environment necessitates the occurrence of cell-to-cell contact at regular intervals, thereby increasing the probability of exchange of genetic material. The limited number of cell wall-bound or other extracellular proteins enables close contact of each organism's cell surface with the other [30]. In fact, such a cell-to-cell contact has been documented (Fig 1).



Bolotin, et al. 2004. Nature Biotechnology. 12:1554-1558.

Figure 1: Cell to cell contact of *S. thermophilus* and *L. bulgaricus*.

In addition to this physical evidence, there is also genetic evidence that lateral gene transfer occurs. Methionine is an amino acid not commonly found in milk, yet the methionine biosynthesis regions of *S. thermophilus* and *L. bulgaricus* are 95 percent identical to each other [5]. Since these two organisms are relatively phylogenetically distant, such a high level of similarity indicates a lateral gene transfer event. There has also been evidence of exopolysaccharide (EPS) gene transfer between *S. thermophilus* and *L. bulgaricus* [18].

The relationship of *S. thermophilus* and *L. bulgaricus* in fermented dairy products has become so intertwined that it is difficult to examine one genome and not see evidence of the other. The highly specific milk environment and the evolutionary changes observed in each organism's genome as a result of this environment is clearly seen in many aspects of the metabolism and molecular biosynthesis capacities of these

two organisms. As the rapid adaptation and reductive evolution continues, additional changes may occur that not only affect their own genomes, but each other's as well. The importance of such a close relationship as these two share necessitates a deeper and more complete understanding than has currently been studied.

Methods and Techniques of Bacteriophage Infection Resistance and the Role of CRISPRs

One of the most common causes of bacterial culture failure is bacteriophage infection. Bacteriophage infection can result in inferior fermentations or fermentation products and loss of culture viability. To ensure product quality and avoid monetary losses as a result of bacterial infection, several factors should be considered. These factors include limiting conditions favorable for bacteriophage growth and infection, rotation of bacterial strains used as starter cultures, selection and use of naturally occurring bacteriophage resistant strains, and genetic engineering of bacterial strains to improve bacteriophage resistance.

A variety of practical strategies for limiting bacteriophage growth have long been used and established as an effective way to avoid bacteriophage infection. Functional design of the manufacturing plant, proper aseptic culture propagation, and increased sanitation are among the most important components of a bacteriophage control plan. Another practical strategy is to introduce changes into the media composition. The addition of compounds like phosphates and citrates to bind calcium that could otherwise be used by the bacteriophage to infect cells can help to limit bacteriophage growth [7]. Temperature control and maintenance is also an important factor in reducing the chance of bacteriophage infection and the reduction of bacteriophage multiplication rates, as extremely high temperatures or low temperatures slow bacteriophage multiplication [1]. Unfortunately, the optimum temperature of the starter culture is often also the optimum temperature of bacteriophage infection, so

great changes in temperature are not possible, as this would impair fermentation.

While these strategies have become accepted as necessary steps to avoid infection, they do not provide full protection.

Careful selection of starter culture composition and strain rotation has been a relatively simple, yet effective way to avoid fermentation failure due to bacteriophage infection. It is thought that by rotating the strains used in fermentation, bacteriophage that are able to infect one strain will not necessarily be able to infect another strain, and thus the fermentation will be saved. Several methods of rotation have been developed along this line. A common method is the introduction and rotation of isogenic strains that each have a diverse anti-phage system to ensure maximum bacteriophage resistance without sacrificing fermentation ability [7]. Another common method is the rotation of strains based on bacteriophage sensitivity. This method employs using a starter culture with a mix of bacteriophage sensitivities, so that no one bacteriophage can infect all strains within the culture. Strain rotation is also a desirable alternative to more complex molecular methods which may carry legal and safety ramifications.

In addition to strain rotation, the utilization of naturally occurring bacteriophage resistant strains can decrease the occurrence of bacteriophage infection. Use of such bacteriophage resistant strains is much less expensive than genetic manipulations, and the selection process is quite simple. The main approach is to generate bacteriophage insensitive mutants, or BIMs, directly from the starter species by exposing the culture to bacteriophage in successive rounds of exposure until a highly resistant strain is

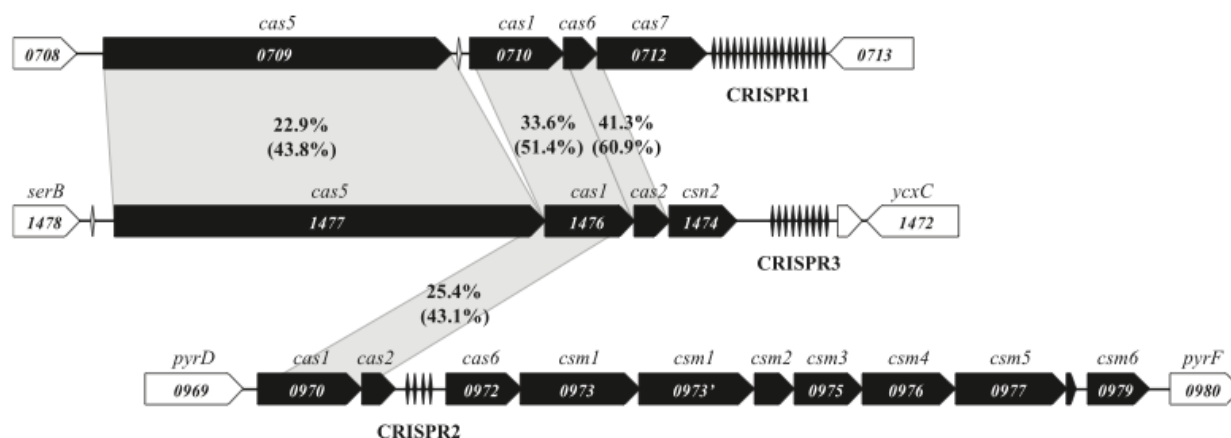
produced [21]. For this technique to be useful industrially, the bacteriophage insensitive mutants must, and perhaps most importantly, retain their desired fermentation phenotype, be generated without the use of specialized equipment, and remain stable during fermentation

Though often more costly and subject to regulatory restrictions, genetic engineering of bacteriophage resistant strains remains a useful tool. While other methods may result in resistant strains, often the range of bacteriophage resistance is narrow and does not confer total protection from infection. In addition, changes in other important phenotypic properties may also occur. Genetic engineering and recombination by use of plasmids and food-grade vectors to introduce bacteriophage-resistance mechanisms can result in a much wider range of bacteriophage resistance. Phage-resistance systems are commonly plasmid linked, which can be both helpful and a hindrance for genetic engineering. It is helpful in the sense that plasmid DNA can more easily be involved in horizontal gene transfer, and so bacteriophage-resistance genes can be transferred in this manner. It is also a hindrance in that plasmid DNA is often easily lost, and therefore bacteriophage resistance can be unstable.

In addition to plasmids, food-grade vectors are an important means to introduce or improve bacteriophage resistance mechanisms. Food-grade vectors are often difficult to engineer, and several conditions must be met before any vector can be determined as safe for use in foods. The first condition is that the vector must not contain any commonly used markers that would be unsafe in food, such as antibiotic

resistance markers. A second condition is that gene sequence and expression integrity is maintained, without risking the occurrence of horizontal gene transfer [28]. Third, in general, the vector must be comprised of DNA obtained from “food-grade” organisms; thus *E. coli*-based vectors would not be permissible. Once these conditions are met, the vector can be approved for use. While the use of food grade vectors can be costly and arduous to design, they remain one of the most powerful tools available and are often the most successful at conferring stable bacteriophage infection resistance.

Recently, the role of CRISPRs, or clustered regularly interspaced short palindromic repeats, in phage resistance was shown by Barrangou et al. [3] and van de Guchte [30], in *S. thermophilus* and *L. bulgaricus* respectively, with the potential to be found in many more bacterial genomes. The CRISPR locus consists of several noncontiguous direct repeats separated by stretches of variable regions, or spacers, and are often next to CRISPR-associated genes (*cas* genes) [3]. It is thought that these CRISPR regions confer a RNA-interference mechanism, whereby the composition of sequences within these regions corresponds to particular and specific phage-expressed mRNA. When the phage-expressed mRNA is present within a phage infected cell, it hybridizes with the complementary CRISPR sequence and no phage proteins can be expressed. *S. thermophilus* LMD-9 possess three separate CRISPR loci, and 14 *cas* genes, with possibly more as yet uncharacterized (Fig 2).



Horvath, et. al. 2008. J Bacteriology. 190:1401-1412.

Figure 2: Overview of the three *S. thermophilus* CRISPR loci in the LMD-9 genome. The *cas* genes are shown in black. Numbers within the genes indicate the genomic ORF number. Numbers on the gray shading indicate percent identity and percent similarity between homologous *cas* protein sequences.

Though the methods described above to prevent bacteriophage infection can be effective, phage-host interactions are constantly evolving and changing. As the fermented food industry continues to grow, failed batches or inferior fermentations cannot be tolerated. Multiple strategies and techniques are available and in use, but that is not likely to be enough. As time passes and the industry continues to grow, the occurrence of bacteriophage mutations and recombination will continue to increase, and novel approaches must be developed to ensure product quality remains standard and consistent.

Prebiotic Oligosaccharides, Probiotics, and Synbiotics: Their role in gut health and utilization by Lactic Acid Bacteria

The term “prebiotic” refers to non-digestible food ingredients that selectively stimulate the growth or activity of a limited number of intestinal bacteria which results in improved host health [9]. The most common commercially available prebiotics include inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS).

Galactooligosaccharides are complex carbohydrates that are formed from lactose via a transgalactosylation reaction by beta-galactosidase. The GOS molecule is comprised of a glucose subunit with several galactose subunits attached in either a 1,4 or 1,6 beta linkage (Fig 3).

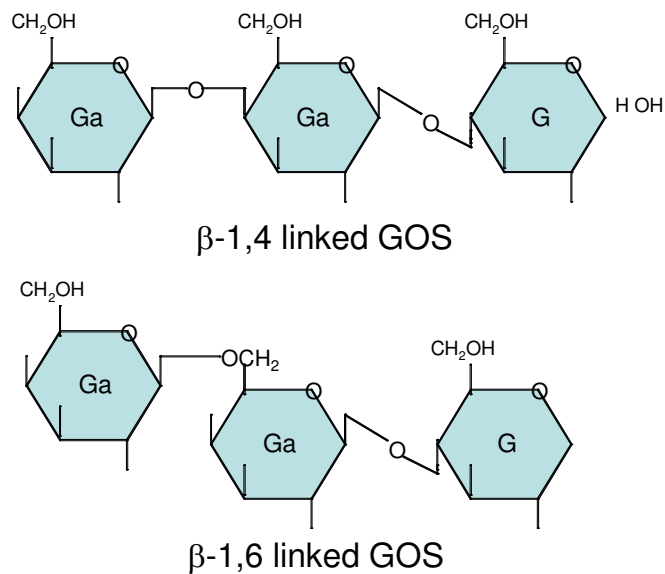


Figure 3: Structure of GOS including linkages

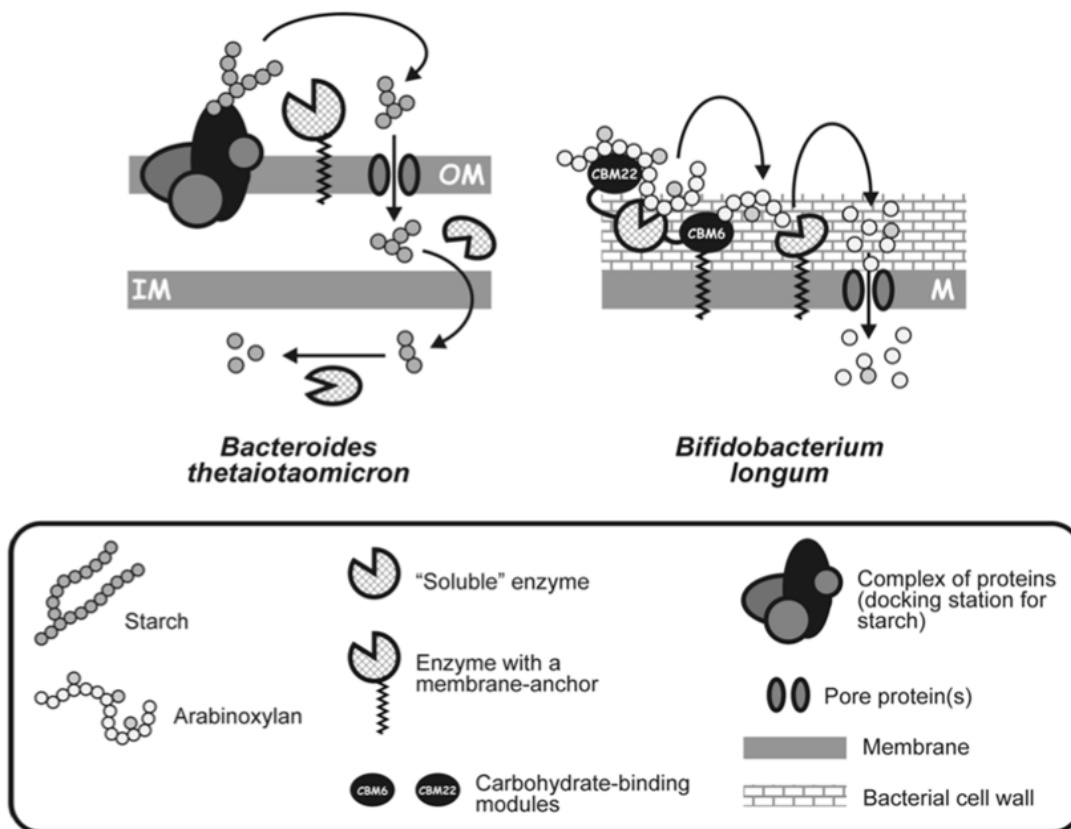
GOS qualifies as a prebiotic by virtue of its stability throughout passage through the digestive tract [19]. Few organisms inhabiting the GI tract possess the enzymes needed to hydrolyze GOS, therefore by ingesting GOS it is possible to, in essence, “enrich” for only those organisms able to utilize it as a substrate. GOS also fulfills other desirable requirements to be a successful prebiotic: temperature stability, resistant to acid and bile, and low calorific value [19]. Among the organisms capable of fermenting GOS are species and strains from the genera *Bifidobacterium* and *Lactobacillus*. *Bifidobacterium*, a gram-positive, anaerobic, non-motile, non-spore forming organism, is often the first to colonize the gut as infants, and higher numbers of bifidobacteria are found in breast-fed infants when compared to formula fed infants [8]. The levels of *Bifidobacterium* in adults varies, but is usually found in health adults at around 1% [31]. *Bifidobacterium* are attributed to be active in many aspects of host health, from immunostimulation/modulation [23,31], to the prevention of diarrhea [24].

In addition to these specific health aspects, *Bifidobacteria* and *Lactobacillus* may play an important role in colonization resistance. In other words, if “good” bacteria such as *Bifidobacteria* and *Lactobacillus* are present in the gut, then they, complimented by the normal host microflora, occupy every available niche, thereby excluding any possible pathogens or attempted colonization by undesirable bacteria.

The term “probiotic” refers to “defined viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects” [25]. For a bacteria to be considered “probiotic”, it must fulfill the following

criteria: exert positive health effects whether by colonization or during passage through the GIT, be acid and bile resistant, be safe and non-pathogenic, and selectively stimulate growth and activity of intestinal bacteria. Most of the probiotic bacteria used commercially are either lactobacilli or bifidobacteria, which as previously discussed are known to be beneficial to host health. When a specific pairing of a probiotic bacterial strain to a prebiotic carbohydrate is made within a food product, it is called a synbiotic [19]. This idea of specific pairings within food products is a particularly effective strategy when trying to exert a change in the gut microflora, as it essentially “self-feeds” the probiotic bacteria during its time in the GI tract.

Most lactic acid bacteria have abundant proteolytic systems, with the ability to take up macromolecules, such as oligosaccharides [25], and so have been suggested for use as probiotics. Probiotic strains must be able to survive passage through the GI tract, and be able to compete with the host microflora for nutrients during passage. The specific pathways used to ferment prebiotics may be strain specific and unique, or may involve “borrowing” a pathway normally used for a different carbohydrate. The carbohydrate utilization pathways of several important lactic acid bacteria, whether significant health wise in the gut or commercially, have been studied [3,10, 11,12,19, 25, 26,27, 29, 31]. The first question to answer is how does carbohydrate uptake happen? Most research completed so far indicates that carbohydrate transporters sit within cell membranes, with capture of the carbohydrate accomplished by specific receptors, while the breakdown of the carbohydrate can occur intra- or extra- cellularly (Fig 4).



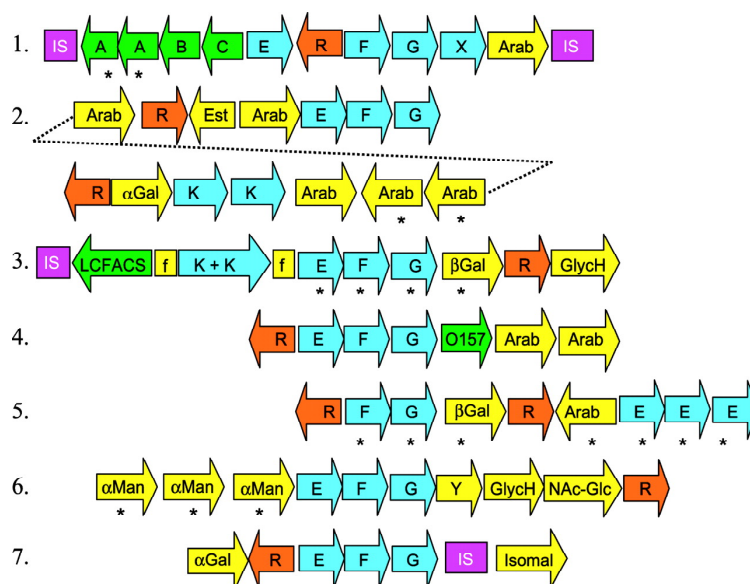
van den Broek et al. 2007. Mol. Nutr. Food Res. 52:146-163.

Figure 4: Schematic of different strategies to secure carbohydrate nutrients; IM= inner membrane, OM= outer membrane, M=membrane, *B. longum* = *B. longum* biotype *longum*

The utilization of large molecules, like prebiotic carbohydrates, presents a particular challenge. From the microbe's perspective, the important factor in where uptake and utilization occurs is how to keep the carbohydrates for themselves and not lose them to the environment or other microbes during the breakdown and utilization process. The inner and outer membrane of a gram negative organism, such as *B. thetaiotaomicron*,

allows for larger carbohydrates to enter the intermembrane space and then be further hydrolyzed within the periplasm or intracellularly by enzymes within the cell cytoplasm. Gram positive organisms, such as *Bifidobacterium longum*, have a thicker, single membrane, so greater hydrolysis must occur outside the cell via either secreted or cell-anchored enzymes, with the smaller carbohydrate subunits transported into the cell via specific transporters.

Previous studies performed have examined how prebiotic carbohydrates are metabolized in bifidobacteria and certain *Lactobacillus* species. Schell [26] sequenced the genome of *Bifidobacterium longum* and found that many of the oligosaccharide transporters are organized in seven separate clusters with conserved modular architecture, consisting of: (1) a LacI-type repressor, (2) an ABC-type oligosaccharide transporter, and (3) one to six genes encoding glycosyl hydrolases (Fig 5).



Schell M A et al. PNAS 2002;99:14422-14427

Figure 5: Oligosaccharide utilization gene clusters in *B. longum*

When the genome of *Bifidobacterium longum* ssp. *infantis* was sequence by Sela et al [27], they found that oligosaccharides were broken down into monosaccharides by glycosidases extracellularly before entering the bifid shunt, which converts D-fructose - 6-phosphate into acetyl phosphate .

Gonzalez et al [11] used microarrays to study the transcriptional response of *Bifidobacterium longum* to media with GOS. They found an upregulation of a cluster containing a β -galactosidase and an ABC sugar permease transporter, with additional upregulation of other sugar permease transport systems, including those with glycosyltransferases and genes involved in galactose transport, indicating that carbohydrate metabolism is specifically induced. In lactobacilli, utilization of prebiotic carbohydrates is usually tied to specific hydrolases and transporters. Barrangou et al [2] determined that fructooligosaccharide (FOS) utilization in *Lactobacillus acidophilus* was an adaptation by the microbe where a raffinose operon was combined with a β -fructosidase to form a four-component ABC transport system with regulation based on preferred carbohydrate availability (Fig 6).

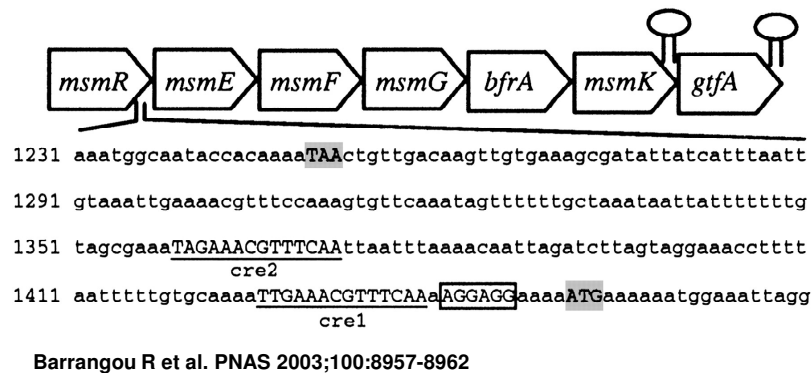


Figure 6: Operon layout of *L. acidophilus* NCFM

Goh et al [10] also examined FOS utilization, in *Lactobacillus paracasei*. In this case, it was determined that a cell surface-anchored fructosidase cleaved FOS via a phosphotransferase system, and the resulting free fructose was transported into the cell.

The ability to utilize prebiotic carbohydrates can be very strain specific, as only those microbes with the enzymes to cleave the specific linkages in complex oligosaccharides are capable of utilizing them. In general, screening methods and phenotypic test are performed based on growth curves of strains in appropriate media. Screenings of many strains from major groups of lactic acid bacteria including *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *S. thermophilus*, reveal that the ability to ferment GOS is overwhelmingly limited to the so called “good” bacteria, *Bifidobacteria* and *Lactobacillus*.

Conclusion

Lactic acid bacteria are a vital part of the fermented food industry and are the subject of much interest and research. Industry is especially interested in using modern molecular approaches to maintain and improve selected strains; several industrial uses could be improved by closer investigation, namely the proto-cooperation of *S. thermophilus* and *L. bulgaricus*, the role of CRISPRs in phage resistance, and the utilization of prebiotic carbohydrates. Questions such as what genes and pathways are shared during milk fermentation between *S. thermophilus* and *L. bulgaricus*, when and what genes are active during bacteriophage infection, and how and where does prebiotic carbohydrate utilization occur can be answered. By using microarrays, complete snapshot of gene expression during each of these conditions are generated and detailed expression profiles can be produced. By devising a screening system, the distribution of the phenotype of GOS fermentation over a wide array of lactic acid bacteria from *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *S. thermophilus* can be explored. Once this phenotypic distribution is generated, selected strains that are able to utilize GOS can be studied in more detail to determine the mechanics of GOS fermentation. Answering these questions add to the understanding of what factors are involved in a successful fermentation and eventually be able to improve strain selection methods.

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Chapter 2

**Transcriptomics of *Streptococcus thermophilus*: Bacteriophage Resistance and
Protooperation with *Lactobacillus bulgaricus* in milk**

**Transcriptomics of *Streptococcus thermophilus*: Bacteriophage Resistance and
Protocooperation with *Lactobacillus bulgaricus* in milk**

C. Goin and R. Hutkins*

Department of Biological Sciences and Department of Food Science and Technology,
University of Nebraska-Lincoln, Lincoln, NE 68583, USA

***Corresponding author:** Dr. Robert Hutkins, Department of Food Science and
Technology, 338 Food Industry Complex, University of Nebraska-Lincoln, Lincoln, NE,
68583; Tel: 402-472-2820; email: rhutkins1@unl.edu

Introduction

Streptococcus thermophilus is a Gram-positive, low GC bacterium belonging to a larger cluster of lactic acid bacteria (LAB). This organism is an integral part of the fermented foods industry, performing the main bioconversions in fermentations involving dairy products such as yogurt and Italian cheeses [18]. According to traditional classification schemes, *S. thermophilus* belongs to the viridans group streptococci, specifically, the *S. salivarius* group, whose other members include *Streptococcus salivarius* and *Streptococcus vestibularis* [25]. Despite its somewhat close phylogenetic proximity to pathogenic streptococci, *S. thermophilus* has “Generally Recognized As Safe” (GRAS) status and is widely used as a starter culture in dairy foods. In fact, this organism is now second only to *Lactococcus lactis* with respect to its use in fermented dairy products [5,6].

The genomes of most LAB are relatively small, encoding for both auxotrophic and prototrophic biosynthetic capabilities [18]. The genome of three strains of *S. thermophilus* have been sequenced, and comparative genome hybridization studies by Rasmussen et al. reveal a core genome of 1,271 genes, the majority of which are energy metabolism and transport systems. Although *S. thermophilus* is thought to have originally been a soil organism, it has undergone extensive genome evolution fueled by its adaptation to a milk environment rich in lactose and casein [10,11]. Indeed, approximately 10 percent of the genes are classified as pseudogenes [2], reflecting active gene decay consistent with a strong environmental driving force. It has been suggested that the evolution of *S. thermophilus* during the last 3,000 to 30,000 years

correlates with human dairy activities and the domestication of milk-producing animals, beginning around 7,000 years ago [23]. Indeed, several of its most important physiological properties appear to be intimately associated with its adaptation to the milk environment.

During most milk fermentations in which *S. thermophilus* is used as a starter culture, strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus* are ordinarily included. These organisms share a well-studied synergistic relationship [1,2,10,18,29,30]. It is thought that there are three major phases that occur during growth in milk. In the first phase, growth of *S. thermophilus* results in rapid formation of lactic acid, with decreases the pH of the medium. However, when the free amino acids are exhausted, growth of the weakly proteolytic *S. thermophilus* decreases [4]. The second phase consists of growth of *L. bulgaricus*, which is stimulated by the lactic acid and reduced redox potential resulting from growth of *S. thermophilus*. *L. bulgaricus* produces an extracellular protease that generates amino acids and other peptides. The formation of a pool of amino acids stimulates a final stage of growth of both *S. thermophilus* and *L. bulgaricus*. This interaction is supported by the observation that amino acid transport machinery accounts for up to 33% of all membrane transport activity in both bacteria [18]. The increased growth rate of both organisms that results from their association with each other is crucial to successful milk fermentations, not only for prompt milk coagulation, but also for generating flavor compounds [21].

The successful manufacture of yogurt and other *S. thermophilus*-fermented dairy products is also dependent on the ability of this organism to withstand bacteriophage

infections, which can otherwise result in slow or arrested fermentations. Ordinarily, most starter strains of *S. thermophilus* are sensitive to phage, and research aimed at understanding phage resistance in this organism is now actively being pursued.

Recently, it was reported that clustered regularly interspaced short palindromic repeats (CRISPRs) exist in *S. thermophilus* and provide a natural mechanism by which this organism defends itself against lytic phage. The CRISPR locus consists of several noncontiguous direct repeats separated by stretches of variable regions, or spacers, and are often next to CRISPR-associated genes (*cas* genes) [1]. It is thought that these CRISPR regions confer a RNA-interference mechanism, whereby the composition of sequences within these regions corresponds to particular and specific phage-expressed mRNA. When the phage-expressed mRNA is present within a phage infected cell, it hybridizes with the complementary CRISPR sequence and no phage proteins can be expressed. *S. thermophilus* LMD-9 possess three separate CRISPR loci, and 14 *cas* genes, with possibly more as yet uncharacterized [13].

Given the importance of *S. thermophilus* in the dairy industry, our goal was to understand how this organism responds to the milk environment. Specifically, we sought to identify genes that were transcribed during typical milk fermentations, including growth in the presence of *Lactobacillus delbrueckii* ssp *bulgaricus* and lytic bacteriophage.

Materials and Methods

Organisms and growth conditions. *Streptococcus thermophilus* LMD-9 was routinely propagated in Elliker broth (Difco, Inc. Ann Arbor, MI) statically at 42°C. *Lactobacillus delbrueckii* ssp. *bulgaricus* BAA-365 was routinely propagated in MRS broth ((Difco, Inc. Ann Arbor, MI) statically at 37°C. For phage expression experiments, *S. thermophilus* LMD-9 was grown at 42°C in M17 broth (Difco, Inc. Ann Arbor, MI) supplemented with 1% glucose. The lytic phage DT1 (provided by Moineau), was propagated in modified M17 broth containing (per liter): 19.0 g disodium β -glycerophosphate, 5.0 g beef extract, 5.0 g papiac digest of soybean meal, 2.5 g yeast extract, 0.5 g ascorbic acid, and 0.25 g MgSO₄, supplemented with 10mM calcium chloride, then purified by 0.45 μ m filter. For *S. thermophilus*/*L. bulgaricus* symbiosis expression experiments, *S. thermophilus* and *L. bulgaricus* were propagated in indicated media, then used to inoculate 10% rehydrated skim milk that had been steamed previously for 1 hour and stored at 4°C until usage.

Microarray Fabrication. Microarrays were fabricated as 60mer oligo-chip arrays generated from the *S. thermophilus* LMD-9 genome (Invitrogen). Each oligomer was contact-printed using the OminGrid robotic arrayer (GeneMachine), in triplicate, for a total of 4,866 features per microarray. Slides were pre-treated according to the manufacturer's recommendations using a UV cross-linking method to anchor the oligos to the surface of the epoxy slide. Steps include: (1) using diamond pen, mark the outside

edges of the printed area on the back side of the slide, (2) Heat water to 65°C and heat slide moat to 90°C. (3) Hold slide spotted side down over the water to steam for 10 seconds, followed by placing the slide spotted side up on the slide moat for 5 seconds, (4) Repeat step 3, (5) Place slide spotted side up on paper towel inside UV Stratalinker and set to 2400, (6) Gently wash slide in 1% SDS solution for 5 minutes, (7) dip slide quickly in water 20 times, then in ethanol 10 times, (8) place slides in glass slide holder and centrifuge at 750 rpm for 4 minutes to dry.

Cell harvesting and RNA isolation procedures. Cells were harvested for centrifugation after the treatment and incubation was completed, RNeasy Protect (Qiagen) was used to stop gene expression and stabilize the RNA (protocol modified from Monnet [20]). RNA isolation was achieved using the chaotropic agent TRI reagent (Molecular Research Center) according to manufacturer's instructions. Following the use of this reagent, 0.1 mm glass beads and a beadbeater were used to complete seven cycles of 2 minutes in the beadbeater and 2 minutes on ice in between cycles. Homogenate was incubated at room temperature for 5 minutes before chloroform extraction and purification. Steps include: (1) Add 0.2 ml chloroform to homogenate and shake vigorously for 15 seconds, (2) store mixture at room temperature for 15 minutes, (3) centrifuge at 13000 rpm for 15 minutes at 4°C, (4) transfer upper phase to new tube and add 0.5 ml isopropanol and mix by inversion, (5) store at room temperature for 8 minutes, (6) centrifuge at 13000 rpm for 8 minutes at 4°C, (7) Decant supernatant and wash pellets with 1 ml cold 75% ethanol and mix, (8) centrifuge at 12000 rpm for 3

minutes at 4°C, (9) repeat steps 7 and 8, (10) decant ethanol and air dry tubes until ethanol has evaporated, (11) add 100 uL Ambion nuclease-free water to each pellet and incubate at 55-60°C for 10 minutes. DNase treatment (Turbo DNase, Ambion), was used to treat RNA, also according to manufacturer's instructions. Steps include: (1) Add 5 uL of 10x DNase I Buffer and 1.5 uL DNase (2U/uL) to 50 uL RNA sample, (2) mix gently and incubate at 37°C for 1 hour, (3) add 5 uL DNase Inactivation Reagent and mix well, (4) incubate mixture at room temperature for 2 minutes, (5) centrifuge for 5 minutes at room temperature to pellet, (6) transfer supernatant to new tubes.

cDNA synthesis and hybridization cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) from 30 µg of extracted RNA and directly labeled with two different fluorochromes; Cy3 (Perkin Elmer) was used to label the experimental group or the group of cells that underwent the treatment and Cy5 (Perkin Elmer) was used to label the control group. Steps include: (1) Prepare reaction mixture as follows:

	<u>Cy3</u>	<u>Cy5</u>
treatment RNA	13.5 ul	-
control RNA	-	10 ul
random hexomers (1ug/ul)	1 ul	1 ul
nuclease-free H ₂ O	to 14.5 ul	to 14.5 ul

(2) incubate mixture at 65°C for 10 minutes and place on ice, (3) on ice, add to each tube:

	<u>Cy3</u>	<u>Cy5</u>
5x 1 st strand buffer	6 ul	6 ul
0.1 M DTT	3 ul	3 ul
dNTP mix (low dCTP)	0.6 ul	0.6 ul
RNase out	1.0 ul	1.0 ul
Cy3-dCTP dye	3.0 uL	-

Cy5 -dCTP dye - 3.0 ul

(4) add 3 ul Superscript III reverse transcriptase (200U/ul), (5) incubate at 42°C for 2 hours, (6) add 3 ul of 0.2 um-filtered 0.5 M EDTA and incubate for 2 minutes at room temperature. (7) add 3 ul of 0.2 um-filtered 1M and incubate at 65°C for 30 minutes, (8) cool to room temperature and add 3 ul of 0.2 um-filtered 1M HCl and incubate for 3 minutes at room temperature. The labeled probes were hybridized to the microarray surface using Hyb Low Temp/Target buffer and incubating in a HybChamber (GeneMachine) for 16-20 hours before the slide was washed in a series of three washing buffers: (1) 1x SSC, 0.03% SDS, (2) 0.2x SSC, (3) 0.05 x SSC, and scanned using the GenePix 4000B scanner (Axon Instruments) at 5 um per pixel resolution.

Response to bacteriophage. *S. thermophilus* LMD-9 was incubated in M17 + glucose at 42°C until reaching OD₆₂₅ ~ 0.4, then the culture was split. The lytic bacteriophage DT1 was added at a M.O.I of 1 to one culture as the expression treatment and 10 ml samples were taken at time points of -5 minutes before infection and 0,5,10,20,30, and 40 minutes after infection and added directly to 20 ml RNAprotect (Qiagen) to halt gene expression. The cells of the other half of the culture were immediately harvested by centrifugation as the control. RNA was collected using the RNA protect method. Steps are as follows: (1) 10 ml of sample is added to 20 ml RNA protect (Qiagen) + 0.89 ul of rifampicin (2.25 mg/ml), (2) mixture is incubated 5 minutes at room temperature, (3) centrifuge at 5,500 x g for 15 minutes at 4°C, (4) resuspend pellet in 5 ml lysozyme (20mg/ml) and 0.22 ml rifampicin (2.25 mg/ml), (5) incubate mixture for 25 min at 37°C

before centrifuging at 5,500 x g for 15 min at 4°C, (6) resuspend pellet in 5 ml Trizol reagent, (7) incubate 10 minutes at room temperature, (8) centrifuge 16,000 x g for 15 minutes at 4°C, (9) remove upper phase and mix with equal volume isopropanol, (10) shake gently on shaker for 30 minutes before pelleting RNA by centrifugation and continuing with RNA purification and DNase treatment as described above.

S. thermophilus and L. bulgaricus synergism in milk. *L. bulgaricus* BAA-365 was grown in skim milk for 7 hours at 42°C, and then added at an equal volume to *S. thermophilus* LMD-9, also in skim milk. Sterile skim milk at a similar pH was added to a parallel culture of *S. thermophilus* LMD-9 as a control. After incubating both cultures at 42°C for 4 hours or when pH 5.5 is reached, RNA was collected using the RNA protect method and purified as described above.

Statistical Analysis. The median feature pixel intensity at wavelengths of 635 and 532 nm in raw data amounts generated by the GenePix scanner were normalized between spots and between each of the three replicates performed using LimmaGui software package (<http://bioinf.wehi.edu.au/limmaGUI/>) using general loess after background correction. The least squares method was used to determine differentially expressed genes, and only those genes with a p value of ≤ 0.05 were considered significantly differentially expressed.

Results and Discussion

Response to Bacteriophage

In order to obtain an accurate representation of the activity of CRISPR genes during the phage shock time trial, gene expression levels of each of the 14 known CRISPR genes, spread among 3 separate loci, in the *S. thermophilus* LMD-9 genome were compared at each time point (Figure 1). The phage response arrays indicate a transient increase in global gene expression, including that of CRISPR genes, 5 minutes post infection, followed by decrease in gene expression. Genes significantly differentially expressed at each time point are listed in Table 1.

The increased expression of the CRISPR1 locus reflects the expected response to phage infection. CRISPR1 is known to have repeat degeneracy within the CRISPR gene sequence, with spacer size more highly conserved and in the highest number when compared to the other two loci [13]. This increase in spacer number and conservation in CRISPR1 is likely evidence of an effective mechanism to integrate novel spacers when faced with novel phage infection [19]. CRISPR3 plays a lesser role in phage response, and likewise we see a lesser degree of gene expression. CRISPR 2 has not been associated with active phage response [13,19].

Global gene expression profiles during this phage infection time course did not indicate great shifts or specific trends. At 5 minutes after infection, the most highly expressed gene is a replication and repair gene, STER_1614. Three of the seven other genes upregulated at this timepoint were membrane transport genes including two PTS

system components. Twenty minutes after infection, we see only one gene with a statistically significant change in gene expression, a P-loop containing kinase, STER_0875. At 30 minutes post infection, there is very little significant change in any upregulated genes, but there is a slight down regulation in a nucleotide metabolism gene *gmk*, a guanylate kinase (STER_1398), as well as a down regulation in an arsenate reductase (STER_1485). Finally, after 40 minutes post infection, an even steeper down regulation of *gmk* was observed. Guanylate kinase is involved in purine metabolism, so this down regulation may indicate that the cell is in distress and is no longer normally synthesizing nucleotides.

S. thermophilus and L. bulgaricus synergism in milk.

The analysis of the milk expression arrays, listed in Table 2 and shown graphically by KEGG function in Figure 2, revealed several trends in gene expression. First, there is an up regulation of amino acid metabolism and transport genes, with the highest fold change in expression coming from STER_1318, and amino acid transporter. This result was expected, as the presence of *L. bulgaricus* and its extracellular protease would increase the level of hydrolyzed proteins that would be available for use once the free amino acids from the milk media were depleted. A significant increase in replication and repair genes, seven in total, was observed indicating a stimulation of cell activity and growth rate. Interestingly, there was a down-regulation trend in membrane transport gene expression, specifically ABC-type PTS (STER_1007) and an ABC-type dipeptide transport (STER_1407). The most down-regulated gene was STER_1274, an

aspartate-semialdehyde dehydrogenase, and enzyme involved in glycine, serine and threonine metabolism and lysine biosynthesis. This was surprising as Herve-Jimenez et al. [10] showed that there was an upregulation of AA biosynthesis, particularly branched chain amino acids (BCAA) and nucleic acid metabolism in *S. thermophilus* when grown in co-culture with *L. bulgaricus*. While the change in gene expression on the global scale does not show any great shifts either up or down, based on the overall relatively few significantly expressed genes and somewhat low \log_2 ratios observed, there was a general increase in cell activity in the presence of *L. bulgaricus* compared to that of *S. thermophilus* LMD-9 alone, indicating that the protocoooperation between these two bacteria many involve a subtle give and take, rather than an overwhelming change in just one or two aspects of interaction.

Conclusion

In this study, global gene expression profiles of *Streptococcus thermophilus* were generated using DNA microarrays during specific conditions to which the microbe would be exposed during processing. This allowed us to gather large amounts of data within a single experiment, which resulted in a much clearer picture of the cell activity during these conditions. The role of CRISPR genes emerges, as the response of the genes on each of the three CRISPR loci respond to phage infection by 5 minutes after phage is introduced into the culture, and then expression shift downwards by 20 minutes after phage infection, indicating that the CRISPR response in *S. thermophilus* is likely involved in early bacteriophage defense. A subtle pattern of reciprocity between *S. thermophilus* and *L. bulgaricus* emerges, where the cell activity of *S. thermophilus* is enhanced by incubation in co-culture with *L. bulgaricus*. By increasing our understanding in this way of the transcriptomics of this important dairy organism, we can begin to more accurately relate phenotype to genotype and ultimately greatly improve strain selection methods.

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Acknowledgements

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Tables and Figures

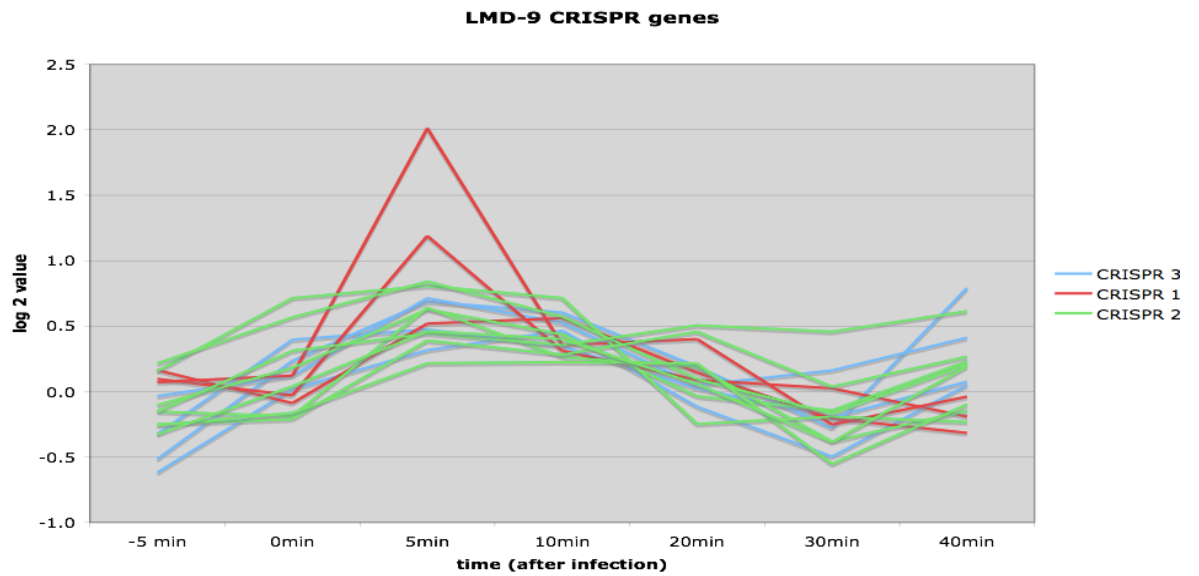
Table 1. Genes differentially expressed during phage shock by time point

Locus Tag	Function	KEGG function code	Fold change (log 2 ratio)
-5 min			
STER_1485	arsenate reductase	PS	0.542
STER_1649	response regulator LytR/AlgR	PS	-0.354
5 min			
STER_1614	N6-adenine-specific methylase	RR	2.93
STER_0100	predicted flavoprotein	PS	2.23
STER_0111	transposase	RR	2.039
STER_0239	phosphotransferase system IIA component	MT	1.96
STER_0941	alpha-acetolactate decarboxylase (<i>alsD</i>)	C	1.91
STER_1318	amino acid transporter	MT	1.760
STER_0370	mannose-specific PTS system component	MT	1.313
20 min			
STER_0875	predicted P-loop containing kinase	PS	0.319
30 min			
STER_1115	signal transduction histidine kinase (<i>vick</i>)	PS	0.739
STER_0239	phosphotransferase system IIA component	C	0.719
STER_0998	restriction endonuclease S subunit	N	0.545
STER_0977	CRISPR-system related protein (<i>Csm5</i>)	PS	0.460
STER_0786	Mn ²⁺ /Fe ²⁺ transporter of NRAMP family	PS	0.396
STER_1607	ABC-type molybdenum transport	MT	0.239
STER_0875	predicted P-loop-containing kinase	PS	0.188
STER_1584	predicted RNA-binding protein	TR	0.1309
STER_0721	uncharacterized protein	FU	0.0635
STER_0138	transposase	PS	0.0426
STER_0249	ribose/xylose/arabinose/galactoside ABC transporter	MT	-0.00433
STER_1485	arsenate reductase	PS	-0.00723
STER_1398	guanylate kinase (<i>gmk</i>)	N	-0.759

40 min			
STER_0239	phosphotransferase system IIA component	C	0.509
STER_1624	uncharacterized protein	FU	0.434
STER_1990	S4-like RNA binding protein	TS	0.321
STER_1256	DNA gyrase, A subunit (<i>gyrA</i>)	RR	0.123
STER_1398	guanylate kinase (<i>gmk</i>)	N	-1.09

KEGG (Kyoto Encyclopedia of Genes and Genomes) function codes: PS, cellular processes and signaling; RR, replication and repair; MT, membrane transport; C, carbohydrate metabolism; N, nucleotide metabolism; FU, function unknown; TR, translation. Values given are indicative of the fold change in gene expression.

Figure 1: CRISPR gene activity by time point



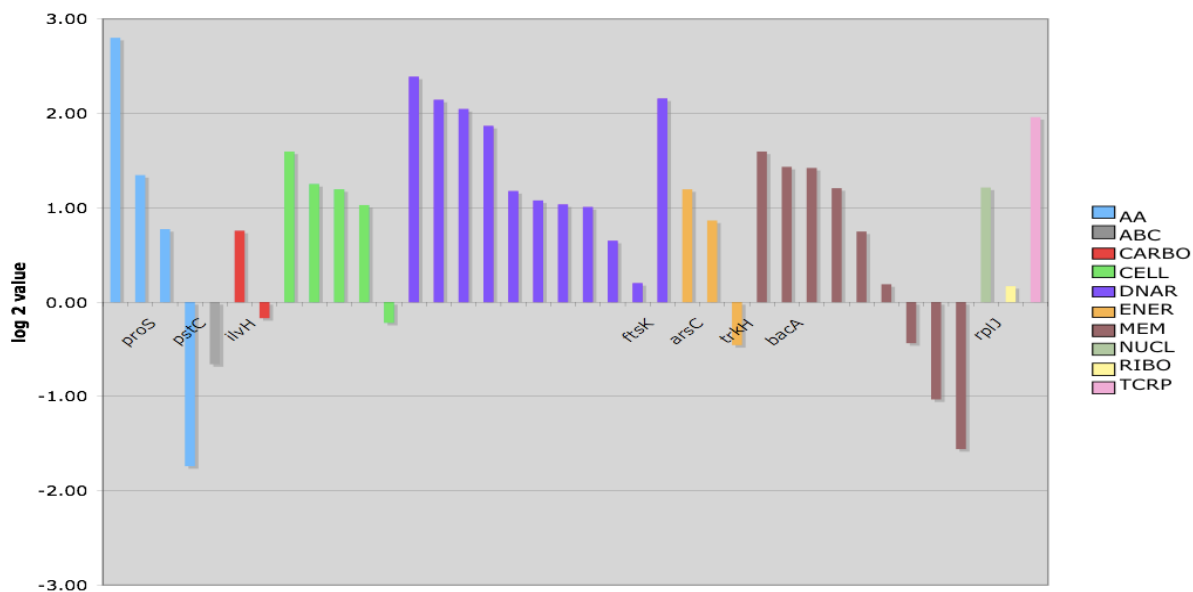
Numbers refer to each of the three CRISPR loci; red = CRISPR 1, green= CRISPR 2, blue, CRISPR 3. Each line indicates one of the 14 *cas*, or CRISPR associated genes. Log₂ values are indicative of fold change in gene expression.

Table 2. Genes differentially expressed during growth in the presence of *L. bulgaricus*

Locus Tag	Function	KEGG function code	Fold change (log 2 ratio)
STER_1318	amino acid transporter	AA	2.80
STER_0842	transposase	RR	2.39
STER_1381	DNA segregation ATPase	RR	2.16
STER_0003	transposase	RR	2.14
STER_0018	transposase	RR	2.05
STER_0300	transcriptional regulator	RR	1.96
STER_0111	transposase	RR	1.87
STER_0311	trk-type K ⁺ transport system, membrane	MT	1.59
STER_0375	xanthine/uracil/vitamin C permease	PS	1.59
STER_1169	predicted membrane protein	FU	1.43
STER_0215	undecaprenyl pyrophosphate phosphatase	C	1.42
STER_0886	cystathionine beta-lyase	AA	1.34
STER_1406	ABC-type dipeptide transport system	PS	1.25
STER_0193	CTP synthase	N	1.21
STER_0312	K ⁺ transport system	MT	1.21
STER_1454	ABC-type amino acid transport	PS	1.20
STER_1192	short-chain dehydrogenase	EM	1.20
STER_0139	transposase	RR	1.18
STER_0001	ATPase involved in DNA replication	RR	0.20
STER_0190	small-conductance mechanosensitive channel	MT	0.19
STER_1849	acetolactate synthase	C	-0.17
STER_1571	predicted ABC-type exoprotein transport system	MT	-0.22
STER_1925	ABC-type multidrug transport system	MT	-0.43
STER_1800	carbonic anhydrase	EM	-0.45
STER_1007	ABC-type PTS	MT	-0.66
STER_1407	ABC-type dipeptide transport system	MT	-1.03
STER_1980	very conserved membrane protein	MT	-1.56
STER_1274	aspartate-semialdehyde dehydrogenase	AA	-1.74

KEGG (Kyoto Encyclopedia of Genes and Genomes) function codes: AA, amino acid transport and metabolism; EM, energy metabolism; PS, cellular processes and signaling; RR, replication and repair; MT, membrane transport; C, carbohydrate metabolism; N, nucleotide metabolism; FU, function unknown; TR, translation. Values given are indicative of the fold change in gene expression.

Figure 2. Fold change in gene expression by KEGG function



Genes are separated based on KEGG function and abbreviated as follows: AA; amino acid metabolism, ABC; ABC-type transport, CARBO; carbohydrate metabolism, CELL; cellular processes, DNAR; DNA replication, ENER; energy metabolism, MEM; membrane related genes, NUCL; nucleic acid metabolism, RIBO; ribosome related genes, TCRP; transcription. Those genes with specific names are labeled as such. Log₂ values are indicative of fold change in gene expression.

Chapter 3

Fermentation of the prebiotic carbohydrate galactooligosaccharide by lactic acid bacteria

**Fermentation of the prebiotic carbohydrate galactooligosaccharide by lactic acid
bacteria**

C. Goin and R. Hutkins*

Department of Biological Sciences and Department of Food Science and Technology,
University of Nebraska-Lincoln, Lincoln, NE 68583, USA

***Corresponding author:** Dr. Robert Hutkins, Department of Food Science and
Technology, 338 Food Industry Complex, University of Nebraska-Lincoln, Lincoln, NE,
68583; Tel: 402-472-2820; email: rhutkins1@unl.edu

Introduction

The term “prebiotic” refers to non-digestible food ingredients that selectively stimulate the growth or activity of a limited number of intestinal bacteria which results in improved host health [4]. Galactooligosaccharides (GOS) are complex carbohydrates that are formed from lactose via a transgalactosylation reaction by beta-galactosidase. The GOS molecule is comprised of a glucose subunit with several galactose subunits attached in either a 1,4 or 1,6 beta linkage. GOS are of prebiotic importance, as few organisms inhabiting the GI tract possess the enzymes needed to hydrolyze GOS, therefore GOS “enriches” for only those organisms able to utilize it as a substrate. GOS fulfills other necessary requirements to be a successful prebiotic: temperature stable, resistant to acid and bile and low calorific value [8]. Among organisms in the GI tract that are able to utilize GOS are certain groups of bacteria considered beneficial to the health of the host. These include *Bifidobacteria* and *Lactobacillus*. *Bifidobacterium*, a gram-positive, anaerobic, non-motile, non-sporeforming organism, is often the first to colonize the gut as infants, and higher numbers of bifidobacterium are found in breast-fed infants when compared to formula fed infants [3]. Levels of *Bifidobacterium* are highest in infants, but steadily decrease after weaning and into adulthood. *Bifidobacterium* are attributed to be active in many aspects of host health, from immunostimulation/modulation [9,15] , to the prevention of diarrhea [10].

In addition to these specific health aspects, *Bifidobacteria* and *Lactobacillus* may play an important role in colonization resistance. In other words, if “good” bacteria

such as *Bifidobacteria* and *Lactobacillus* are present in the gut, then they, complimented by the normal host microflora, occupy every available niche, thereby excluding any possible pathogens or attempted colonization by undesirable bacteria.

The term “probiotic” refers to “defined viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects” [25]. For a bacteria to be considered “probiotic”, it must fulfill the following criteria: exert positive health effects whether by colonization or during passage through the GI tract, be acid and bile resistant, be safe and non-pathogenic, and selectively stimulate growth and activity of intestinal bacteria. Most of the probiotic bacteria used commercially are either lactobacilli or bifidobacteria, which as previously discussed are known to be beneficial to host health. When a specific pairing of a probiotic bacterial strain to a prebiotic carbohydrate is made within a food product, it is called a synbiotic [19]. This idea of specific pairings within food products is a particularly effective strategy when trying to exert a change in the gut microflora, as it essentially “self-feeds” the probiotic bacteria during its time in the GI tract.

Most lactic acid bacteria have abundant proteolytic systems, with the ability to take up macromolecules, such as oligosaccharides [25], and so have been suggested for use as probiotics. Probiotic strains must be able to survive passage through the GI tract, and be able to compete with the host microflora for nutrients during passage. The specific pathways used to ferment prebiotics may be strain specific and unique, or may involve “borrowing” a pathway normally used for a different carbohydrate. The

carbohydrate utilization pathways of several important lactic acid bacteria, have been well studied [3,10, 11,12,19, 25, 26,27, 29, 31]. Most research indicates that carbohydrate transporters sit within cell membranes, with capture of the carbohydrate accomplished by specific receptors, while the breakdown of the carbohydrate can occur intra- or extra- cellularly [1, 5, 6, 12, 13, 14]. In *Lactobacillus*, utilization of prebiotic carbohydrates is usually tied to specific hydrolases and transporters. The ability to utilize prebiotic carbohydrates can be very strain specific, as only those microbes with the enzymes to cleave the specific linkages in the complex oligosaccharides are capable of utilizing them. We designed a screening method and media to carry out phenotypic tests and growth curves of many strains from major groups of lactic acid bacteria including *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *S. thermophilus*. Once this phenotypic distribution is generated, we will be able identify strains that are able to utilize GOS and determine the mechanics of GOS fermentation in more detail.

Materials and Methods

Organisms and growth conditions. Lactic acid bacteria used in this study were propagated in the following media according to their nutritional needs: *Bifidobacteria*, MRS supplemented with 0.5% L-cysteine (Difco, Inc. Ann Arbor, MI); *Lactobacillus* and *Leuconostoc*, MRS (Difco, Inc. Ann Arbor, MI); *Lactococcus*, M17 (Difco, Inc. Ann Arbor, MI); *Streptococcus thermophilus*, Elliker (Difco, Inc. Ann Arbor, MI). All bacteria were incubated statically at 37°C, with *Bifidobacteria* incubated anaerobically in an anaerobic chamber. Media used to complete growth curve and plate screenings was modified to contain lower concentration of extraneous carbohydrates per 900 mL as follows: MRS; 5 g protease peptone #3, 5g beef extract, 2.5g yeast extract, 1g polysorbate 80, 2g ammonium citrate dibasic, 5g sodium acetate, 0.1 g MgSO₄, 0.5g MnSO₄, 2g K₂HPO₄, 0.5g L-cysteine (bifidobacteria only); Elliker; 20g pancreatic digest of casein, 5g yeast extract, 4g NaCl, 2.5g gelatin, 1.5g sodium acetate, 0.5g ascorbic acid; M17; 19.0g disodium β-glycerophosphate, 5.0g beef extract, 5.0g papiac digest of soybean meal, 2.5g yeast extract, 0.5g ascorbic acid, 0.25g MgSO₄. The remaining 100 mL was made up of a 2% galactooligosaccharide (GOS) solution added after sterilization. The commercial GOS, from GTC Nutrition, was provided in a powder form and then rehydrated into stock solutions. Composition of commercial GOS is comprised of mix of three sugar components: 92% GOS, 7% lactose, 0.75% glucose, 0.25% galactose. To account for the 8% of non-GOS sugars, from here on referred to as “contaminating sugars”, these sugars were added to media in the same concentration as would be

found in the 2% GOS solution used in the other modified media to act as a control. The final control used was the basal media without any sugars added.

Strain Screening Strains were screened with a combination of plating and liquid media. Plate screening was completed using the modified media appropriate for the nutritional needs of each type of bacteria supplemented with 2% GOS solution and bromocresol purple as a pH indicator. Liquid media screening consisted of measuring cell growth over a time course at OD₆₂₀ in three separate media: (1) basal media with no additional carbohydrate added, (2) control media with only the “contaminating sugars” added, and (3) media with 2% GOS added. These screenings were completed statically at 37°C, with only the bifidobacteria incubated anaerobically. Strains were considered positive for GOS fermentation if they produced yellow colonies on the plate screening (indicating that they were producing lactic acid which lowered the pH), and had a p-value of <0.05 in a paired t-test of the OD values between the three medias at each time point in three separate biological replicates.

Random mutagenesis of *Lactobacillus reuteri* DSM 20016T Random mutagenesis was achieved using a mariner transposon system from *Bacillus subtilis* (provided by Haldenwang). Transposons was propagated in *B. subtilis* and transformed colonies selected using LB kanamycin⁵/erythramycin¹ at 50°C, and plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen) with the following modification: 250U/ml of mutanolysin (Sigma) and 10mg/ml of lysozyme (Fisher) was added to Buffer P1.

After checking plasmid DNA quality on agarose gel, 400 μ L of pelleted and washed *L. reuteri* DSM 20016T cells were electrotransformed with 5 μ L plasmid DNA in an electroporation cuvette (Midwest Scientific) using an electroporator and a single pulse (12.5 KV/cm, 200 Ω , 25 μ FD). Cells are then transferred to 10 mL prewarmed MRS and incubated for 2.5h at 37°C before 100 μ L is plated on MRS + kan⁵/ery¹ and incubated at 30°C for 48h. The resulting transformed colonies were picked and then screened using the same plating method as described above.

Directed Mutagenesis of *Lactobacillus reuteri* DSM 20016T Directed mutagenesis was achieved via site-directed gene inactivation as described by Walter et al. [16]. Four genes and two separate clusters were chosen to be inactivated based on their likelihood to be involved in GOS hydrolysis: Cluster 1; (1) LAR_0276 beta-galactosidase large subunit, (2) LAR_0277 beta-galactosidase small subunit; Cluster 2; (3) LAR_1032 beta-galactosidase, (4) LAR_1033 PTS system transporter. Gene inactivation was confirmed using test primers (see Table 4).

GOS synthesis GOS synthesis was achieved by adding 0.25U commercial enzyme (Biolacta, provided by GTC Nutrition) to 400 μ L of 40% lactose solution and 200 μ L 5% lactose solution in AC buffer (50 ml glycerol, 10 ml 5M NaCl, 10 ml 1M Tris, pH 7.6, 1 ml 0.5M EDTA) and incubating at 60°C for 18h. The solution was then boiled at 100°C for 5 minutes to deactivate any remaining enzyme. Radiolabeled GOS was synthesized in the same manner but with the following substitutions: 100 μ L of ¹⁴C-labeled lactose (ARC

chemicals) was added in addition to 200 μ L 5% lactose in AC Buffer, 400 μ L 40% lactose solution, and 100U commercial enzyme.

Thin Layer Chromatography of Carbohydrates The products of GOS enzymatic synthesis were purified using preparative TLC. High Performance TLC glass plates coated with silica 60 at 200 μ m (Dynamic Absorbents Inc.) were used to separate carbohydrates generated during GOS synthesis or other experiments. Plates were spotted using capillary pipettes, with volumes averaging 1-2 μ l per spot. Plates were developed two times in a 22:9:9 butanol-acetic acid-water solution, then sprayed with a 1:1 (v/v) sulfuric acid-ethanol solution and baked at 275 $^{\circ}$ F for 5-10 minutes to char the carbohydrates into distinct spots. Plates were run with 2% solutions of sugar standards such as full GOS, lactose, galactose, and/or glucose.

Radiolabeled GOS uptake experiments MRS media supplemented with 2% GOS was inoculated from an overnight and incubated at 37 $^{\circ}$ C for 8-12h, then pelleted and washed twice with 0.1M PBS after taking the initial OD₆₂₀. The pellet was then resuspended in buffer so the final OD₆₂₀ is 1.0-1.2. A pulse of 0.1M glucose was added to energize cells when necessary. Radiolabeled GOS is added and 1 mL samples are taken in duplicate at time points 1, 5, 10, 20, and 30 minutes. These 1 mL samples are added to tubes containing 500 μ L mineral oil, then centrifuged for 1 minute. The cells are separated from the supernatant by the oil, leaving the cells in a pellet at the bottom of the tube. The supernatant and oil is poured off, and the tubes are inverted over

absorbent towels to dry. The intact pellet is removed from the tube using guillotine clippers, and placed in scintillation vials. Four ml of scintillation liquid (Fisher) is added to the vials and mixed well. The vials are counted in a scintillation counter and each vial is measured at 5 minute intervals within the ^{14}C spectrum. Counts are then averaged over the duplicate samples at each time point and graphed.

Intracellular and Extracellular enzyme tests To determine the location of GOS hydrolysis enzyme activity, two separate cell components were extracted from a *L. reuteri* DSM 20016T cell population grown in MRS supplemented with 2% GOS at 37°C for 12h. To measure the intracellular activity, cells were pelleted after incubation and washed twice with 1X PBS. Cells were resuspended in buffer and cells lysed using microbeads and beadbeating for 7 cycles of 1 min with 1 min on ice in between cycles. After centrifugation, the supernatant was removed and 100ul added to new tubes containing 2% GOS. The tubes were incubated at 37°C and 1 ml samples taken at 0,2,4, and 8 hours. To measure the extracellular activity, cells were incubated as before, then the 150ml cell solution was pelleted and the supernatant was filtered to remove any remaining cells. 2% GOS was added to the filtered cell-free supernatant and samples were taken at 0, 2, 4, and 8 hours during incubation at 37°C. Samples from both enzyme activity tests were run on HPTLC plates to qualify any GOS hydrolysis activity, as well as intracellular enzyme assays of the mutant knock-outs.

Microarray Fabrication. Microarrays were fabricated as 60mer oligo-chip arrays generated from two separate *L. reuteri* genomes, DSM 20016T, a human isolate, and 100-23, a rat isolate (Invitrogen). Each oligomer was contact-printed using the OminGrid robotic arrayer (GeneMachine), in duplicate, for a total of 5,026 features per microarray. Slides were pre-treated according to the manufacturer's recommendations using a UV cross-linking method to anchor the oligos to the surface of the epoxy slide. Steps include: (1) using diamond pen, mark the outside edges of the printed area on the back side of the slide. Label slide with number if necessary, (2) Heat water to 65°C and heat slide moat to 90°C. (3) Hold slide spotted side down over the water to steam for 10 seconds, followed by placing the slide spotted side up on the slide moat for 5 seconds, (4) Repeat step 3, (5) Place slide spotted side up on paper towel inside UV Stratalinker and set to 2400, (6) Gently wash slide in 1% SDS solution for 5 minutes, (7) dip slide quickly in water 20 times, then in ethanol 10 times, (8) place slides in glass slide holder and centrifuge at 750 rpm for 4 minutes to dry.

Cell harvesting and RNA isolation procedures. Cells were harvested for centrifugation after the treatment and incubation was completed, RNAprotect (Qiagen) was used to stop gene expression and stabilize the RNA (protocol modified from Monnet 2008). RNA isolation was achieved using the chaotropic agent TRI reagent (Molecular Research Center) according to manufacturer's instructions. Following the use of this reagent, 0.1 mm glass beads and a beadbeater were used to complete seven cycles of 2 minutes in the beadbeater and 2 minutes on ice in between cycles.

Homogenate was incubated at room temperature for 5 minutes before chloroform extraction and purification. Steps include: (1) Add 0.2 ml chloroform to homogenate and shake vigorously for 15 seconds, (2) store mixture at room temperature for 15 minutes, (3) centrifuge at 13000 rpm for 15 minutes at 4°C, (4) transfer upper phase to new tube and add 0.5 ml isopropanol and mix by inversion, (5) store at room temperature for 8 minutes, (6) centrifuge at 13000 rpm for 8 minutes at 4°C, (7) Decant supernatant and wash pellets with 1 ml cold 75% ethanol and mix, (8) centrifuge at 12000 rpm for 3 minutes at 4°C, (9) repeat steps 7 and 8, (10) decant ethanol and air dry tubes until ethanol has evaporated, (11) add 100 µL Ambion nuclease-free water to each pellet and incubate at 55-60°C for 10 minutes. DNase treatment (Turbo DNase, Ambion), was used to treat RNA, also according to manufacturer's instructions. Steps include: (1) Add 5 µL of 10x DNase I Buffer and 1.5 µL DNase (2U/µL) to 50 µL RNA sample, (2) mix gently and incubate at 37°C for 1 hour, (3) add 5 µL DNase Inactivation Reagent and mix well, (4) incubate mixture at room temperature for 2 minutes, (5) centrifuge for 5 minutes at room temperature to pellet, (6) transfer supernatant to new tubes.

cDNA synthesis and hybridization cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) from 30 µg of extracted RNA and directly labeled with two different fluorochromes; Cy3 (Perkin Elmer) was used to label the experimental group or the group of cells that underwent the treatment and Cy5 (Perkin Elmer) was used to label the control group. Steps include: (1) Prepare reaction mixture as follows:

	<u>Cy3</u>	<u>Cy5</u>
treatment RNA	13.5 ul	-
control RNA	-	10 ul
random hexomers (1ug/ul)	1 ul	1 ul
nuclease-free H ₂ O	to 14.5 ul	to 14.5 ul

(2) incubate mixture at 65°C for 10 minutes and place on ice, (3) on ice, add to each tube:

	<u>Cy3</u>	<u>Cy5</u>
5x 1 st strand buffer	6 ul	6 ul
0.1 M DTT	3 ul	3 ul
dNTP mix (low dCTP)	0.6 ul	0.6 ul
RNase out	1.0 ul	1.0 ul
Cy3-dCTP dye	3.0 uL	-
Cy5 -dCTP dye	-	3.0 ul

(4) add 3 ul Superscript III reverse transcriptase (200U/ul), (5) incubate at 42°C for 2 hours, (6) add 3 ul of 0.2 um-filtered 0.5 M EDTA and incubate for 2 minutes at room temperature. (7) add 3 ul of 0.2 um-filtered 1M and incubate at 65°C for 30 minutes, (8) cool to room temperature and add 3 ul of 0.2 um-filtered 1M HCl and incubate for 3 minutes at room temperature. The labeled probes were hybridized to the microarray surface using Hyb Low Temp/Target buffer and incubating in a HybChamber (GeneMachine) for 16-20 hours before the slide was washed in a series of three washing buffers: (1) 1x SSC, 0.03% SDS, (2) 0.2x SSC, (3) 0.05 x SSC, and scanned using the GenePix 4000B scanner (Axon Instruments) at 5 um per pixel resolution.

GOS expression of *L. reuteri* measured by DNA microarray *L. reuteri* DSM

20016T was used to inoculate MRS supplemented with 2% GOS as the treatment and MRS supplemented with 2% glucose as the control in parallel cultures incubated

statically at 37°C. Thirty ml samples of each treatment were taken at time points 3, 8, and 16 hours after inoculation. Cells were immediately harvested by centrifugation at 10,000 rpm for 10 min at 4°C. RNA was extracted and purified as described above.

Statistical Analysis. The median feature pixel intensity at wavelengths of 635 and 532 nm in raw data amounts generated by the GenePix scanner were normalized between spots and between each of the three replicates performed using LimmaGui software package (<http://bioinf.wehi.edu.au/limmaGUI/>) using general loess after background correction. The least squares method was used to determine differentially expressed genes, and only those genes with a p value of ≤ 0.05 were considered significantly differentially expressed.

Results and Discussion

Strain screening 238 strains were screened using plating on selective media and liquid media growth curves from *Bifidobacteria*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus thermophilus*. GOS fermentation seemed to be the most common in Bifidobacteria, where 12 of the 19 strains screened were positive (Table 1). GOS fermentation was also fairly common in lactobacillus, where 60 of the 117 strains screened were positive, or roughly 50% (Table 2). The stand out among the *Lactobacilli* was definitely *L. reuteri*, where 42 of the 46 strains tested were positive for GOS fermentation, or 91 %. The screenings of the other groups of lactic acid bacteria showed GOS fermentation was much less common, only 10 out of 102 strains (10%) tested among *Lactococcus*, *Leuconostoc*, and *S. thermophilus* were positive (Table 3). This indicates that GOS fermentation, or at least the ability to use complex oligosaccharides, is an important ability in bifidobacteria and lactobacilli. As such, we decided to focus our efforts on *L. reuteri*, specifically *L. reuteri* DSM 20016T, the sequenced type strain.

Mutagenesis of *L. reuteri* DSM 20016T Both random and site-directed mutants were generated to determine which genes might be involved in GOS hydrolysis. Random mutations were problematic to quantify, as screening is difficult and many genes may be involved in providing the GOS fermentation phenotype. As a result, we did not find a suitable random mutant over many plating screens. We therefore concentrated on site-directed mutagenesis. Four genes on two separate clusters were

chosen to knock-out (Figure 1), as they seemed the most likely to be involved in GOS fermentation. Once gene inactivation was validated using test primers and PCR (Table 4), we screened the resulting mutants in liquid media (Figure 2). Until 16 hours, growth in media with GOS is greatly reduced compared to the wild type (Figure 3). After 16 hours, the phenotype is somewhat repaired, possibly due to the presence of secondary GOS hydrolysis systems.

Radiolabeled GOS uptake experiments We first completed uptake experiments with phenotypically positive and negative *L. reuteri* strains from our screening to determine uptake patterns and relate those patterns to a known phenotype (Figure 4). Phenotypically positive strains, such as DSM 20016T, take up the radiolabeled GOS steadily until plateauing at 20 minutes after adding the radiolabeled GOS. The phenotypically negative strains, such as LMS 11-3, for the most part do not take up any GOS at any statistically significant rate. We continued these uptake assays with our knock-out mutants to determine if the phenotype was lost. When compared to the wild-type DSM 20016T, all the mutants have little or no uptake, even the mutants with presumably intact transporters (Figure 5). This may be a result of polar effects between neighboring genes among the beta-galactosidase gene clusters, or because of the relatively low uptake counts.

Intracellular and Extracellular enzyme tests Samples from both the intra- and extra-cellular tests were run on TLC to determine if any GOS hydrolysis occurred. For

the extracellular enzyme test, samples from each timepoint were spotted and run on TLC plates. There is no change at any of the time points in the spotting pattern, and the pattern that is seen is the same as the GOS standard (Figure 6). This indicates that enzymatic hydrolysis activity is not likely extracellular. For the intracellular enzyme test, samples from each timepoint were likewise spotted and run on TLC plates. In this assay, there is a gradual shift in GOS fractions over the timepoints, with spots appearing at the different fraction locations at the 4,6, and 8 hour timepoints (Figure 7). In the intracellular enzyme tests completed with the knock-out mutants, the phenotype of the is maintained. The spots remain constant throughout the timepoints in the mutants, indicating no significant hydrolysis occurs to produce separate fractions (Figure 8). This further supports that knocking out these four genes impairs *L. reuteri* DSM 20016T from normal levels of GOS hydrolysis activity.

GOS expression of *L. reuteri* measured by DNA microarray

The gene expression

profile generated by the GOS treatment were not very revealing. Only 7 genes were found to be statistically differentially expressed in the three timepoints combined (Table 5). At 3 hours after inoculation, the majority of genes upregulated were transcription (Lreu_1039, Lreu_0673) and transportation genes (Lreu_0074), with a riboflavin synthase (Lreu_0879) being slightly downregulated. At 8 hours after inoculation only 1 gene is statistically differentially expressed, *glpQ*, a glycerophosphoryl phosphodiesterase (Lreu_0065), is slightly upregulated. At 16 hours after inoculation, only 2 genes are differentially expressed: Lreu_0849, a hypothetical protein, and Lreu_1113, an

uncharacterized phage protein, are both slightly down regulated. This small number of genes and relatively moderate fold changes in gene expression indicate that expression of GOS hydrolysis related genes may be constitutive, or otherwise expressed in a manner that global expression profiles would not likely reveal.

Conclusion

Common prebiotic bacterial groups such as Bifidobacteria and Lactobacillus groups such as *L. reuteri*, are among the highest percentages of strains that are able to utilize GOS, indicating that these strains may be able to utilize many different oligosaccharides and complex carbohydrates, an important characteristic for a commensal organism in the GI tract or as a probiotic. Indeed, when genes thought to be associated with GOS hydrolysis, specifically beta-galactosidases, were knocked out of a *L. reuteri* strain, we see a loss of the ability to ferment GOS. As for the other groups of LABs screened, namely *Lactococcus*, *Leuconostoc*, and *S. thermophilus*, only 10% were positive for GOS fermentation, indicating that these strains may not be as well suited as probiotics, or may not possess the cell machinery or transport systems to utilize oligosaccharides. The overall conclusion that can be drawn is that for a particular strain to be a successful probiotic, or to be paired with GOS as a synbiotic, it must possess specific enzymes to successfully hydrolyze complex oligosaccharides such as GOS.

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Tables and Figures

Table 1: GOS fermentation by *Bifidobacterium*

Species (total number)	Number positive (%)	Number negative (%)
<i>B. adolescentis</i> (2)	1 (50%)	1 (50%)
<i>B. bifidum</i> (3)	2 (67%)	1 (33%)
<i>B. breve</i> (2)	1 (50%)	1 (50%)
<i>B. coagulans</i> (4)	2 (50%)	2 (50%)
<i>B. infantis</i> (2)	2 (100%)	0 (0%)
<i>B. lactis</i> (1)	1 (100%)	0 (0%)
<i>B. longum</i> (3)	3 (100%)	0 (0%)
Other <i>Bifidobacterium</i> spp. (2)	1 (50%)	1 (50%)
TOTAL (19)	12 (63%)	7 (37%)

Table 2: GOS fermentation by *Lactobacillus*

Species (total number)	Number positive (%)	Number negative (%)
<i>L. acidophilus</i> (9)	1 (11%)	8 (89%)
<i>L. amylophilus</i> (1)	0 (0%)	1 (100%)
<i>L. amylovorus</i> (4)	0 (0%)	4 (100%)
<i>L. brevis</i> (5)	3 (60%)	2 (40%)
<i>L. buchneri</i> (1)	0 (0%)	1 (100%)
<i>L. casei</i> (8)	3 (38%)	5 (62%)
<i>L. confusus</i> (1)	0 (0%)	1 (100%)
<i>L. delbrueckii subsp. bulgaricus</i> (10)	2 (20%)	8 (80%)
<i>L. helveticus</i> (3)	0 (0%)	3 (100%)
<i>L. hilgardii</i> (1)	0 (0%)	1 (100%)
<i>L. lactis</i> (2)	0 (0%)	2 (100%)
<i>L. paracasei</i> (3)	0 (0%)	3 (100%)
<i>L. pentosus</i> (1)	1 (100%)	0 (0%)
<i>L. plantarum</i> (5)	2 (40%)	3 (60%)
<i>L. reuteri</i> (46)	42 (91%)	4 (9%)
<i>L. rhamnosus</i> (1)	0 (0%)	1 (100%)
<i>L. xylosus</i> (1)	0 (0%)	1 (100%)
Other <i>Lactobacillus</i> spp. (16)	6 (38%)	10 (62%)
TOTAL (117)	60 (51%)	57 (49%)

Table 3: GOS fermentation by other Lactic Acid Bacteria

Species (total number)	Number positive (%)	Number negative (%)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> (16)	1 (6%)	15 (94%)
<i>Streptococcus thermophilus</i> (67)	4 (6%)	63 (94%)
<i>Leuconostoc citrovorum</i> (4)	0 (0%)	4 (100%)
<i>Leuconostoc cremoris</i> (3)	1 (33%)	2 (33%)
<i>Leuconostoc dextranicum</i> (5)	1 (20%)	4 (80%)
<i>Leuconostoc mesenteroides</i> (2)	0 (0%)	2 (100%)
<i>Leuconostoc paramesenteroides</i> (1)	0 (0%)	1 (100%)
Other <i>Leuconostoc</i> spp. (4)	3 (75%)	1 (25%)
TOTAL (102)	10 (10%)	92 (90%)

Table 4: Primers used to generate knock-out mutants

Target gene	Primer	Sequence (5'-3')	Application
LAR_0276	BLF	TTACCCCCTCTGAATTTGAC	Insert primer
	BLR	CCAGATTATTTTCGGCCATC	Insert primer
	BLFT	GGTCACTTTATTGGTTACGC	Test primer
	BLRT	TTGCTCCCATCTTCTGCC	Test primer
LAR_0277	BSF	ATGCGCTATGGTGGTGA	Insert primer
	BSR	GTAACCGTCAACATGGAA	Insert primer
	BSFT	GTTAAGGATCCGGTAAGTGGGC	Test primer
	BSRT	GGGGAACGAGATACTTAGTAACC	Test primer
LAR_1032	BGF	CTGAATTACAGGCAGTTGC	Insert primer
	BGR	GGTACC GCGGGAGTC	Insert primer
	BGFT	CGTCCGGGACAGATGGCAGCC	Test primer
	BGRT	CCAACATACCATGCTTTCCC	Test primer
LAR_1033	PF	TGGATTGTTGGGGTCATC	Insert primer
	PR	GGGCGTACAACCTTATCAC	Insert primer
	PFT	GTTATTGGTAAACCTGGTG	Test primer
	PRT	TTCTTTCATGGCGACTATCTCC	Test primer

Table 5: Genes significantly differentially expressed in *L. reuteri* DSM 20016T during growth in media supplemented with 2% GOS

Locus Tag	Function	KEGG function code	Fold change (log 2 ratio)
3 hours			
Lreu_1039	transcriptional regulator	TC	2.216
Lreu_0074	transposase	RR	2.031
Lreu_0673	phage transcriptional regulator	TC	1.585
Lreu_0879	riboflavin synthase	EM	-0.552
8 hours			
Lreu_0065	glycerophosphoryl diester phosphodiesterase	EM	1.214
16 hours			
Lreu_1113	uncharacterized phage protein	FU	-0.667
Lreu_0849	hypothetical protein	FU	-0.889

KEGG (Kyoto Encyclopedia of Genes and Genomes) function codes: AA, amino acid transport and metabolism; EM, energy metabolism; PS, cellular processes and signaling; RR, replication and repair; MT, membrane transport; C, carbohydrate metabolism; N, nucleotide metabolism; FU, function unknown; TR, translation; TC, transcription. Values given are indicative of the fold change in gene expression.

Figure 1: Beta-galactosidase gene clusters and surrounding genes in *L. reuteri*. Genes knocked-out are in grey.

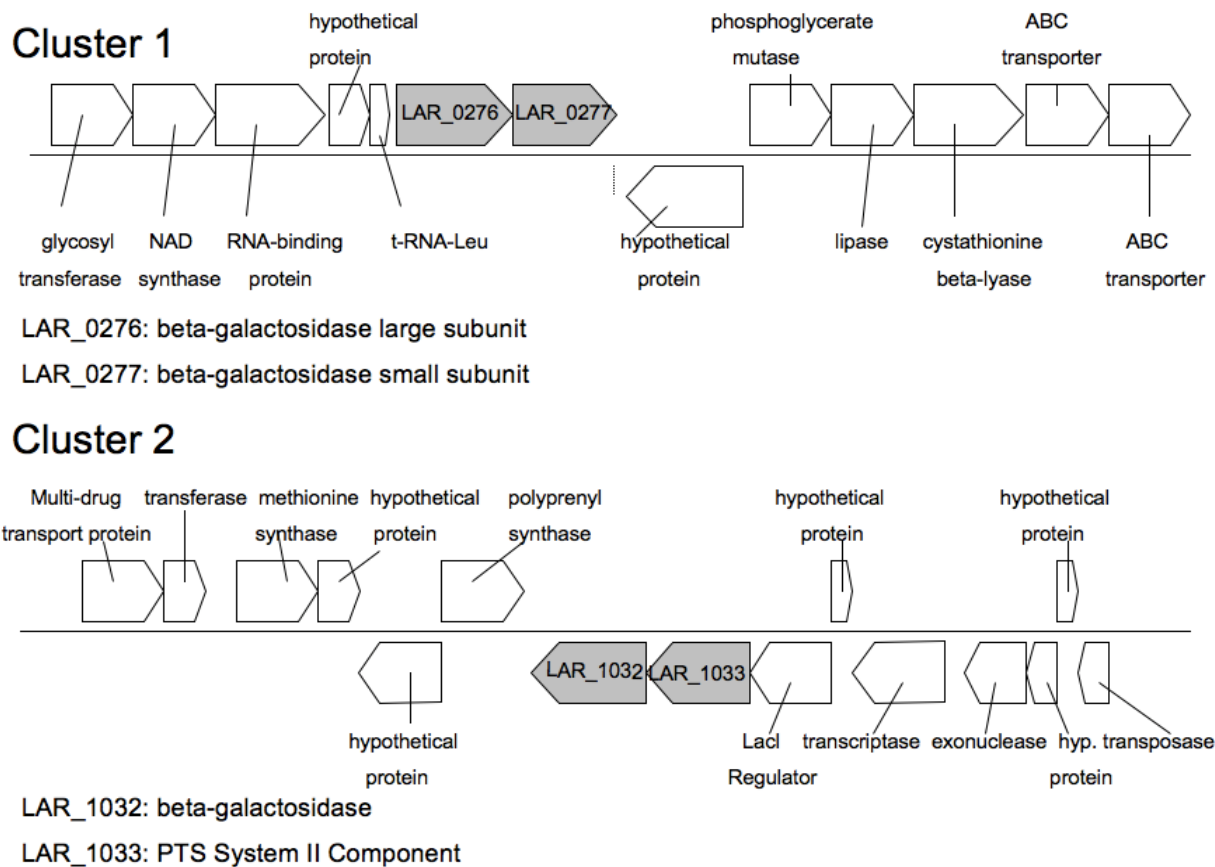


Figure 2: *L. reuteri* DSM 20016T knock-outs in liquid media. Blue = basal media, Red = control media (basal + “contaminating sugars”), Yellow = basal + GOS. Graphs are of OD over time. Top graphs are of knock-out mutants in cluster 2 (P = PTS transporter knockout mutant, BG = beta-galactosidase mutant); Bottom graphs are of knock-out mutants in cluster 1 (BS= beta-galactosidase small subunit, BL= beta-galactosidase large subunit).

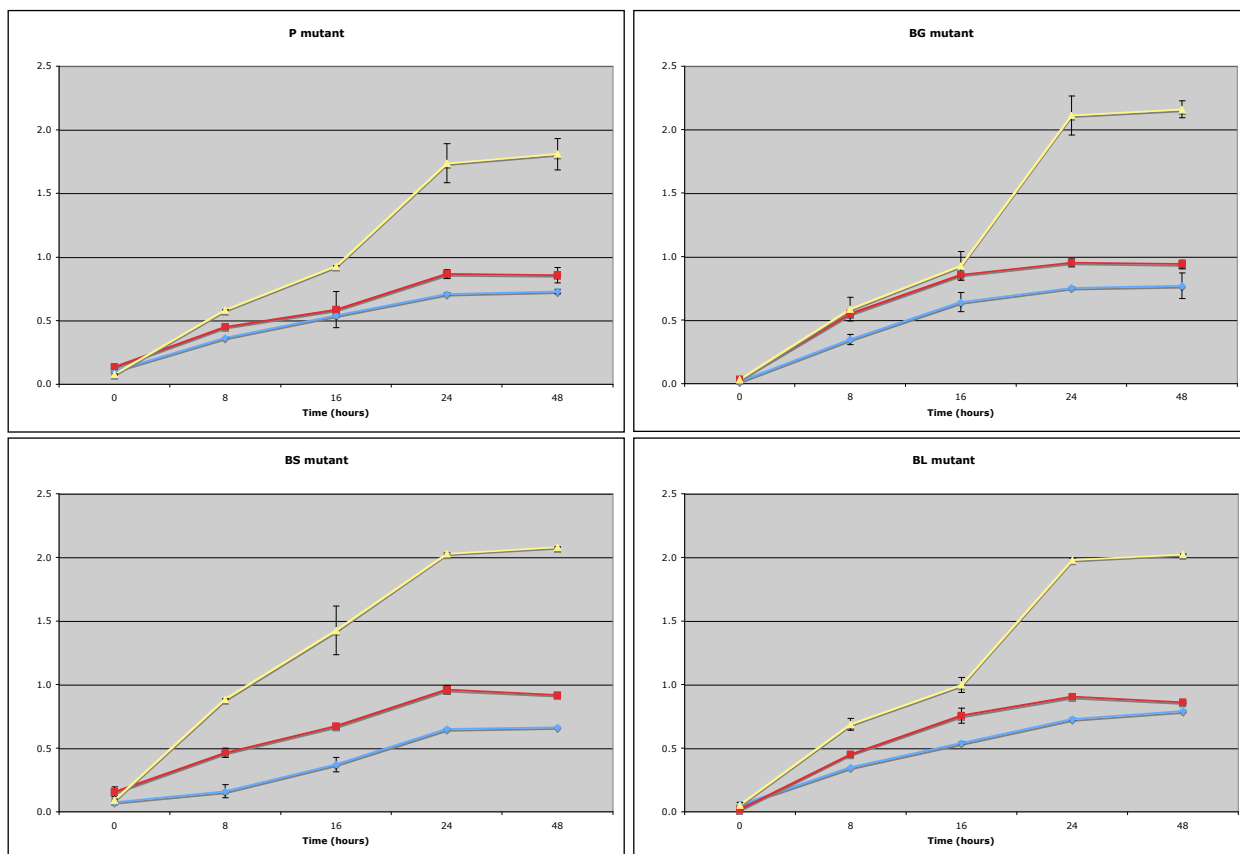


Figure 3: *L. reuteri* DSM 20016T wild type in liquid media. Blue = basal media, Red = control media (basal + “contaminating sugars”), Yellow = basal + GOS

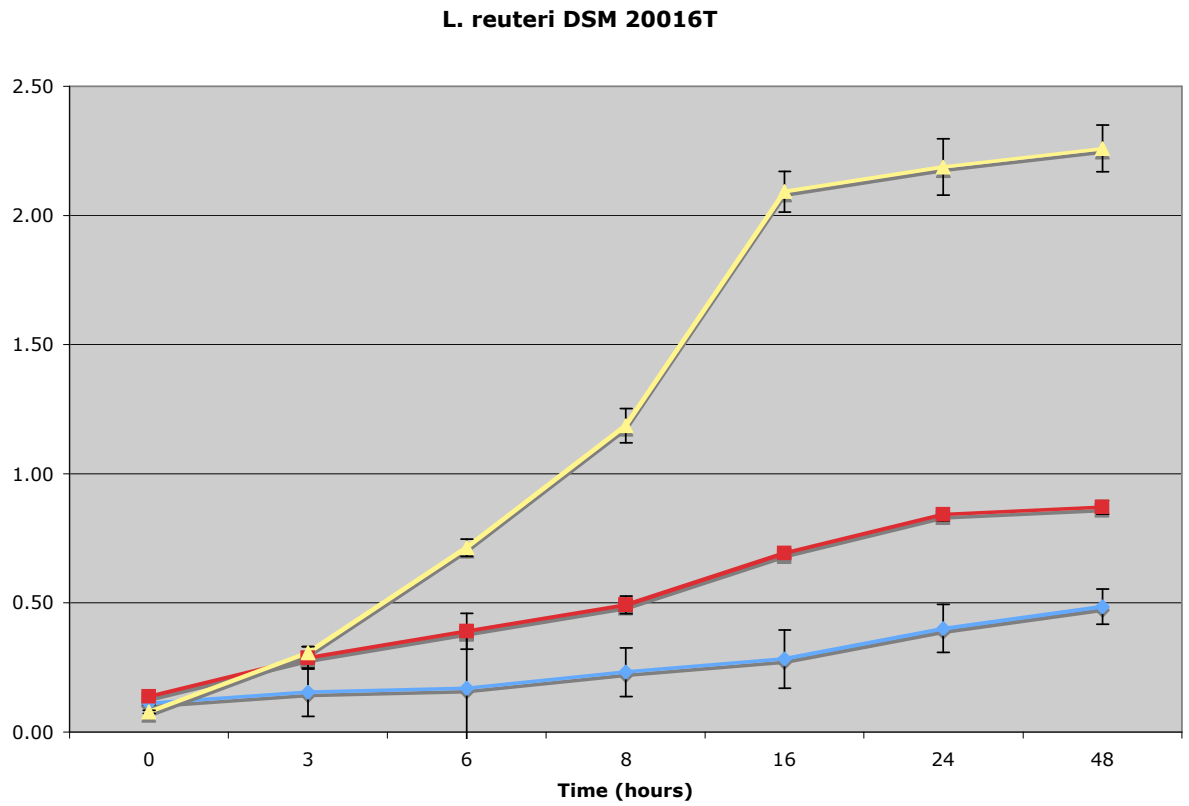


Figure 4: Radiolabeled GOS assays of *L. reuteri*. Positive strain: DSM 20016T; Negative strain: LM11-3. Graphed in radioactivity (DPM) over time. Blue = DSM, Yellow = LM11-3. Significant uptake is seen in the positive strain, DSM 20016T, and little uptake is seen in the negative strain LM11-3.

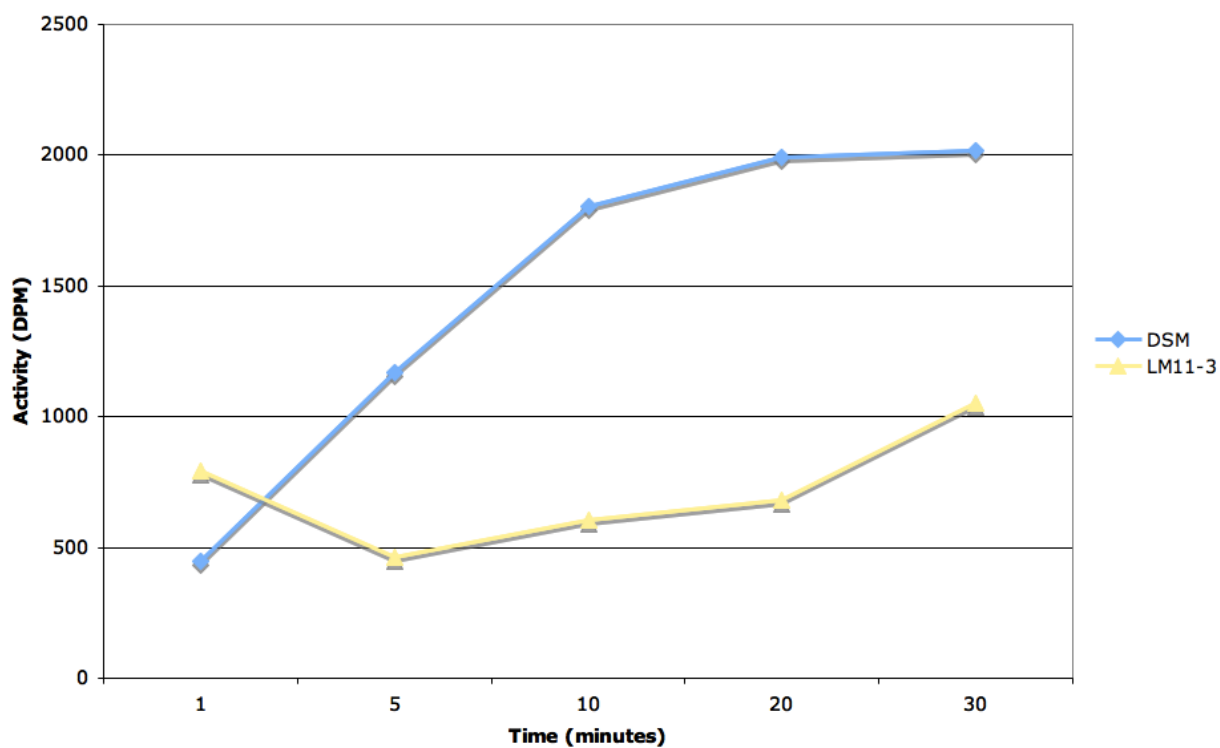


Figure 5: Radiolabeled GOS assays of *L. reuteri* knockout mutants. Graphed in radioactivity (DPM) over time. Blue = DSM wild type, Red = PTS transporter mutant, Yellow = Beta-galactosidase from cluster 2 mutant, Green = Beta-galactosidase large subunit from cluster 1 mutant. Moderate uptake is seen in DSM wild type, with no uptake activity in any of the three mutants.

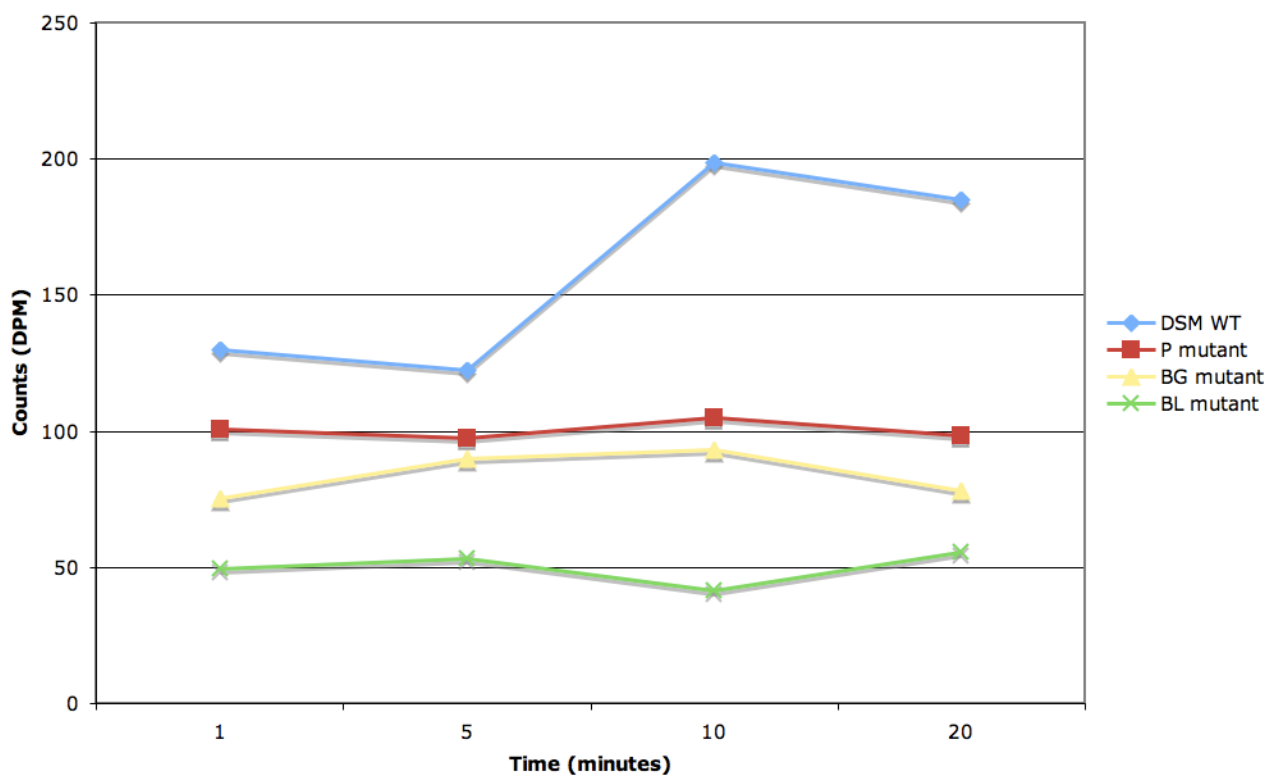


Figure 6: TLC separation of samples taken from extra-cellular enzyme assay. Samples are spotted at 10-12 ul volumes per spot. TLC plates are developed twice in developing buffer (22:9:9 butanol-acetic acid-water), sprayed with 1:1 (v/v) sulfuric acid:ethanol, and baked at 275°C until developed spots appear. Spotting pattern consistent with standards run on each end of plate, indicating that no GOS hydrolysis occurred.

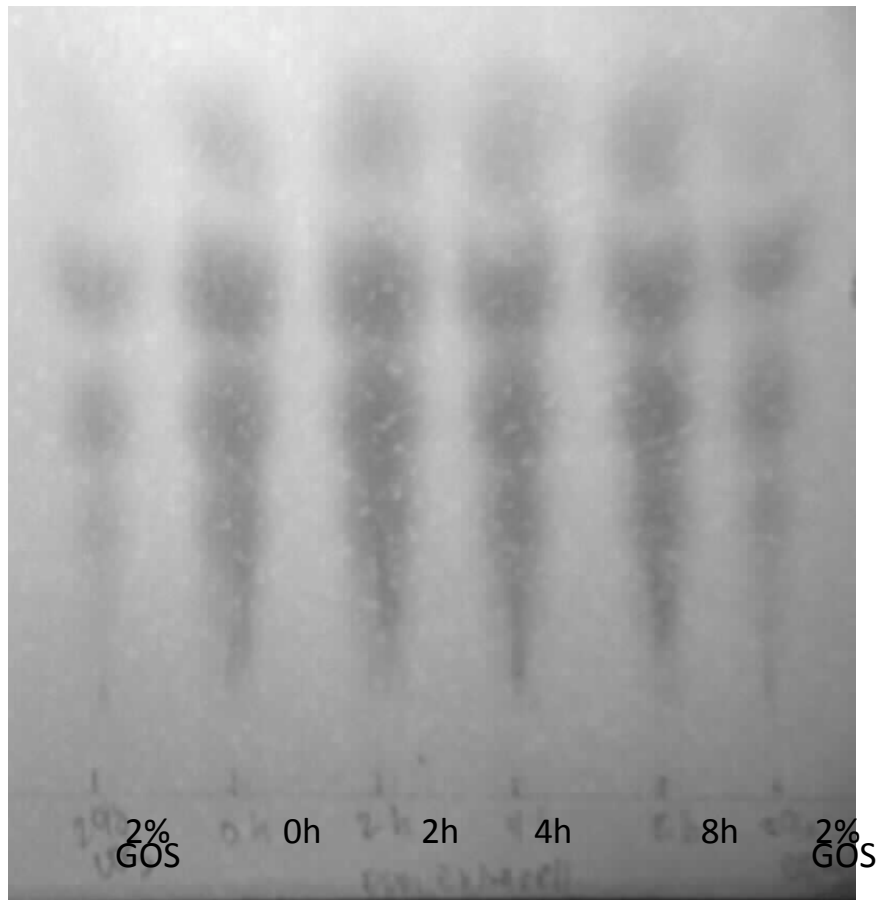
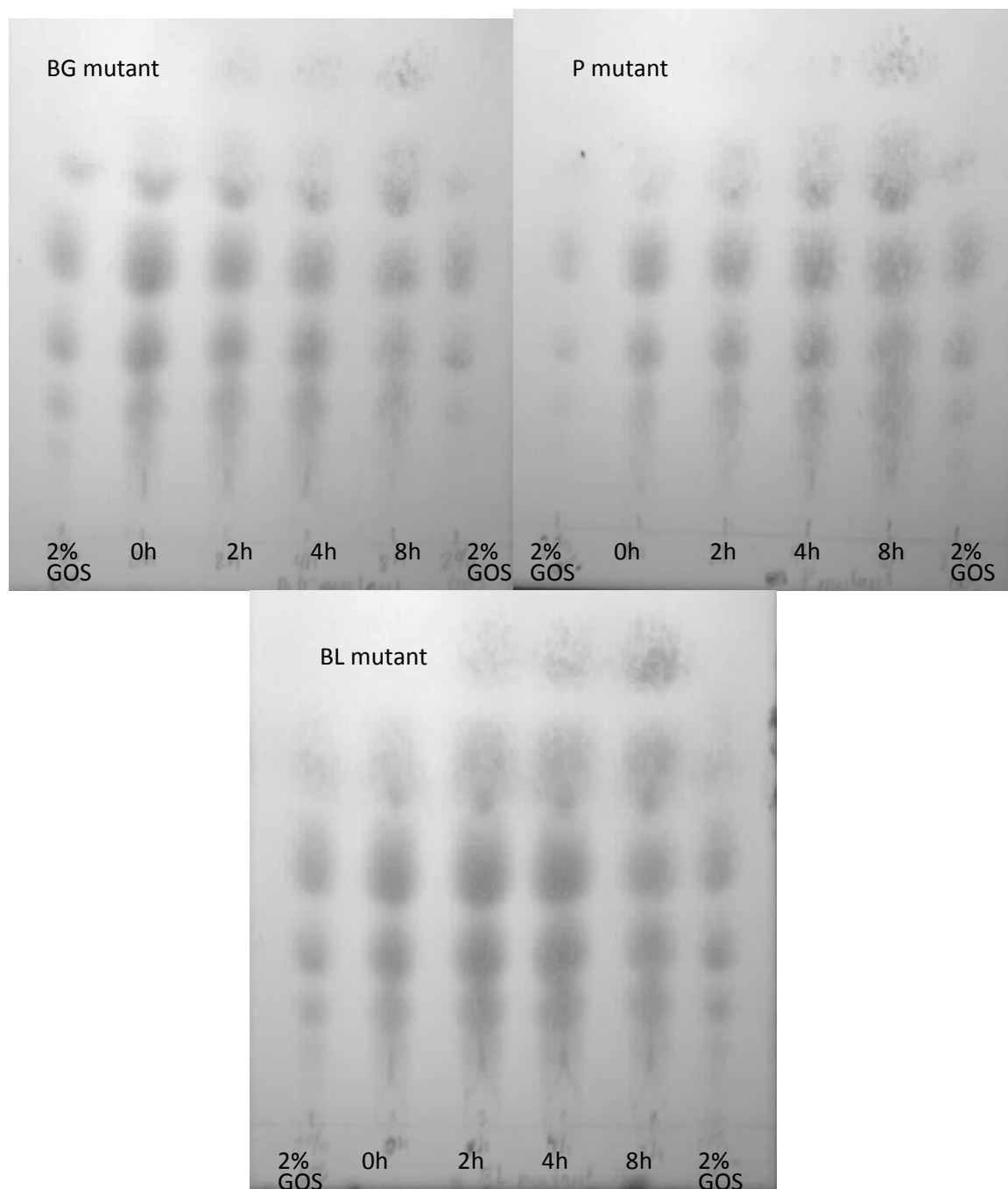


Figure 7: TLC separation of samples taken from intra-cellular enzyme assay. Samples are spotted at 3-4 ul volumes per spot. TLC plates are developed twice in developing buffer (22:9:9 butanol-acetic acid-water), sprayed with 1:1 (v/v) sulfuric acid:ethanol, and baked at 275°C until developed spots appear. The locations of spots change over time, indicating GOS hydrolysis activity.



Figure 8: TLC separation of samples taken from intra-cellular enzyme assay of three knock-out mutants. Samples are spotted at 3-4 ul volumes per spot. TLC plates are developed twice in developing buffer (22:9:9 butanol-acetic acid-water), sprayed with 1:1 (v/v) sulfuric acid:ethanol, and baked at 275°C until developed spots appear. Spotting pattern consistent with standards run on each end of plate, indicating that no GOS hydrolysis occurred.



Chapter 4

Conclusion

At the start of this thesis work, our goals were to answer the following questions: (1) what genes and pathways are involved in the protocoeoperation relationship during milk fermentation between *S. thermophilus* and *L. bulgaricus*, (2) when and what genes are active during bacteriophage infection, and (3) how and where does prebiotic carbohydrate utilization occur.

The ramifications and implications of this research are as follows:

1. Microarray experiments showed the relationship between *S. thermophilus* and *L. bulgaricus* to be a subtle give-and-take, with the sharing of amino acids and spurring overall cell activity and replication.
2. Microarray experiments showed the three CRISPR loci in *S. thermophilus* have different expression profiles during bacteriophage infection, indicating that the CRISPR genes are involved almost at the onset of infection, and within 30 minutes, they are no longer actively participating in bacteriophage infection, whether because infection has been stemmed, or because they are no longer able to provide a beneficial function to the cell.
3. Seeing the overwhelming number of phenotypically positive *L. reuteri* strains during screening, knock-out mutants were generated to uncover

which genes may be involved in GOS fermentation. It was shown that indeed there is a loss-of-function phenotype when beta-galactosidase function is impaired. By completing intracellular and extracellular enzyme test and separating carbohydrates using thin layer chromatography, it was determined that GOS is broken down intracellularly, rather than extracellularly. We can therefore propose the pairing of GOS with either a *Bifidobacterium* or *Lactobacillus* strain, specifically *L. reuteri*, to make a potential synbiotic food product.