LACTOSE/GALACTOSE METABOLISM IN STREPTOCOCCUS GORDONII

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To my mom, dad, sister, brother and aunt

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Streptococcus gordonii is a Gram-positive, facultative anaerobic commensal bacterium commonly found in dental plaque. Its genome contains two gene clusters encoding of tagatose pathway genes (*lacABCD*) and sugar phosphotransferase system (PTS) enzyme II components likely responsible for the transport of lactose (EII^{Lac}) and galactose (EII^{Gal}). Also encoded with the EII^{Gal}-containing operon is a DeoR-like transcription regulator (LacR) and with the EII^{Lac}-containing operon a putative PRD (PTS-regulated domain)-containing transcription antiterminator. In contrast, the *Streptococcus mutans* genome contains only one tagatose operon and a homologous LacR protein that are required for galactose metabolism. Our goal was to determine the contributions of these genes to lactose/galactose metabolism and to test whether *S. gordonii* has a selective advantage over *S. mutans* attributable to high-affinity galactose transport. Genetic analyses of the lactose and galactose gene clusters in *S. gordonii* were carried out using various mutants and Real-Time qRT-PCR and promoter-reporter fusions. Growth analysis showed that EII^{Lac} transports lactose and galactose. Analyses of Real-Time qRT-PCR results indicate that the expression of EII^{Gal} is upregulated in galactose and lactose. Results from promoter-*cat* (chloramphenicol acetyltransferase)

fusions implicate LacR as a negative regulator of the *lacA1* and *lacA2* promoters and show that CcpA is a negative regulator of the *lacA2* promoter. *In vitro* binding assays confirmed that LacR could bind to both the *lacA1* and *lacA2* promoter regions. A mixed-species liquid culture competition assay was performed with *S. gordonii* and *S. mutans* grown in glucose and galactose media with and without phosphate buffer. Results showed that *S. gordonii* maintained an advantage over *S. mutans* due to its galactose PTS. However, *S. mutans* was better able to persist due to its acidogenic and aciduric properties. Collectively these results support that the galactose and lactose systems are differentially regulated in *S. gordonii* and that a high-affinity galactose PTS is advantageous when *S. gordonii* is competing against the caries pathogen *S. mutans*.

CHAPTER 1
INTRODUCTORY REMARKS

General Overview Of The Oral Microbiome In Health And Disease

Genus *Streptococcus* and *Streptococcus gordonii*

The bacterial genus *Streptococcus* is comprised of Gram-positive, non-motile, facultative, anaerobic cocci. Belonging to the phylum Firmicutes, these bacteria divide along a single axis and often form chains of cells. Most streptococci are catalase- and oxidase-negative. Both pathogenic and commensal organisms are encompassed in this genus. Streptococci are broadly classified based on a hemolysis pattern, with the viridans group of streptococci displaying alpha hemolysis due to its ability to oxidize the iron in hemoglobin. When grown on a blood agar plate, a green halo forms around the streptococcal colonies and is termed partial hemolysis.

The viridans streptococci are further classified into groups based on gene sequence homology and a few defining characteristics, including the ability to ferment mannitol and sorbitol, produce glucans from sucrose and sustain growth at low pH (22). There are five viridans groups including the anginosus, mitis, salivarius, bovis and mutans streptococci (46). *Streptococcus gordonii* belongs to the mitis group, which also includes *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus oralis*, *Streptococcus sanguis* and *Streptococcus parasanguis* (46). Previously, *S. gordonii* was considered to be a part of the *S. sanguis* species until Kilian and co-workers defined it as a new species based on DNA sequence information (48). However, both species are still considered to be commensal organisms associated with oral health. *Streptococcus mutans* belongs to the mutans group and is considered the primary etiological agent of dental caries. Earlier studies have indicated an inverse relationship in plaque samples

between the presence of *S. mutans* and strains classified as *S. sanguis* (including *S. gordonii*) (31, 55).

Composition of the Normal Oral Flora

Recognized as one of the most densely populated regions of the body, the oral cavity harbors upwards of approximately 700 species of indigenous bacteria with about half of those organisms uncultivated *in vitro* thus far (47, 74). *Streptococcus* spp, *Lactobacillus* spp and other Gram-positive filamentous rods (e.g. *Actinomyces* spp) constitute the vast majority of the resident flora in the mouth; however, Gram-negative cocci (e.g. *Veillonella* spp, *Neisseria* spp) and Gram-negative rods (e.g. *Bacteroides* spp, *Fusobacterium* spp, *Vibrio* spp, *Spirillum* spp, *Prevotella* spp, *Porphyromonas gingivalis*) are also abundant. Spirochetes (*Treponema*, *Borrelia*) are commonly found in the gingival crevice and *Candida albicans* is the yeast most commonly isolated throughout the mouth (1, 39).

Oral streptococci are abundant in dental plaque found on the surface of the teeth. According to a study conducted in Pisa, Italy, *S. mutans* prevalence was measured at 14% in caries-free patients, 27% in caries-inactive patients and 67% in caries-active patients (13). Mitis group streptococci, including *S. gordonii* and *S. sanguis*, are associated with good oral health and higher proportions are present in caries-free and caries-inactive patients (14, 51, 71).

Colonization of the Oral Cavity and Biofilm Formation

There are a number of distinct microcosms in the oral cavity such as saliva, the tongue dorsum, buccal mucosa, gingival crevice and supragingival space. Each ecological niche provides a unique microenvironment with characteristics that render it capable of sustaining colonization and growth of certain microbial communities. For

example, the normal flora of the gingival crevice is dissimilar from that of the flora on the tooth surface. Colonization of these microcosms is based on ecological determinants that include preferential adherence to a particular surface, nutritional need, tolerance of environmental stresses and inhibitory substances and competition from neighboring organisms (39, 70). *S. gordonii* is most commonly isolated from the supragingival environment, which is located on the surface of the tooth enamel. Supragingival biofilms, often referred to as plaque, consists of a community of microorganisms with the capability to adhere to each other and to surfaces. Successful biofilm formation is an essential step in progression to a carious lesion.

Biofilm formation begins when the acquired pellicle, a thin film over the tooth enamel, accumulates after cleaning of the surface. This coating is comprised of salivary glyocproteins and other proteinaceous and non-proteinaceous material (10). Pioneer (early) colonizers, including *S. gordonii* and *Actinomyces* spp, possess adhesins that allows for binding via receptors to the host pellicle. In a time-dependent manner, late colonizers begin to coaggregate with previously attached early colonizers on the tooth structure, resulting in a multi-tiered cluster of cells with a surrounding matrix of bacterial and host components called the glycocalyx (60). Glucosyltransferase enzymes produced by most oral streptococcal species hydrolyze sucrose and polymerize the glucose monomers into sticky extracellular glucans, which contribute to glycocalyx synthesis and adherence to the enamel (96). Mature biofilms have a high degree of structural complexity, bacterial heterogeneity and differ metabolically, physiologically and biochemically from planktonic cells (27, 28). The levels of gene expression in an established biofilm differ from planktonic cells. In addition, a mature biofilm develops an

increased resistance to antibiotics, host defenses and environmental stresses (27, 28, 44).

Because *S. mutans* is both acidogenic (creating abundant amounts of acid) and aciduric (able to sustain growth in especially acidic conditions), biofilms with high proportions of this species (considered the disease state) are at an increased risk for producing copious amounts of acid. Prolonged acid exposure is responsible for demineralization of the tooth enamel and the primary cause for developing a carious lesion (49, 84, 85, 99).

Ecological Determinants in the Human Oral Cavity

Inside the human mouth is an ever-changing environment. There are a multitude of different bacterial species suspended in saliva due to wash-off from the many different surfaces (50). However, most bacteria cannot remain in the saliva permanently due to its constant flow and movement. It is imperative for an organism to adapt by adhering to a surface. If not properly adherent, the microorganism can easily be washed away with the current of saliva and passed into the intestinal tract via swallowing. In order to circumvent physical removal, the correct niche must be located and adherence achieved via receptors on the acquired host pellicle or neighboring microbes (66). Attachment ensures that the microorganism is able to compete in the surrounding environment; however, it does not guarantee survival.

Fermentation of carbohydrates is the main mechanism in which viridans streptococci gain a source of energy. The availability of nutritional resources depends on the host diet, food debris, metabolic products of other bacteria, and constituents in saliva (42). Of those mentioned, saliva is the main biological fluid in the oral cavity and the most constant source available to most microbes *in vivo*. Therefore, salivary

glycoconjugates (especially O- and N-linked oligosaccharides of glycoproteins and mucins) can serve as an important nutrient resource for those bacteria harboring the genetic machinery necessary to exploit it. Secreted and/or surface-associated glycosidases are enzymes that modify and cleave glycoconjugates found in the saliva. Both *S. pneumoniae* and *S. oralis* have been shown to possess glycosidases capable of sequentially breaking down α1-acid glycoprotein (AGP), first with neuraminidase to cleave off the terminal sialic acid and then with β-galactosidase to cleave off the second galactose moiety (18, 21, 86). The study on *S. oralis* also reported that almost the entire oligosaccharide was deglycosylated from the glycoprotein (21). The release of these sugar moieties served as a nutritional source and supported growth even when no free carbohydrates were present.

S. gordonii is annotated to possess two genes encoding extracellular β-galactosidase activity, bgaA and bgaC. Thus, if *S. gordonii* were capable of catabolizing glycoconjugates in saliva, this mechanism would serve as an additional nutrition source. It is important to keep in mind that the release of sugar moieties from glycoproteins would produce only low concentrations of free sugar in the environment as opposed to large spikes in sugar concentrations such as after a meal. Further conjecture would hint that a high-affinity transport system directed at the uptake of sugars embedded in glucoconjugates has the potential to confer a selective advantage over species that do not have the same genetic components. Therefore, it may be advantageous for S. gordonii to possess a high-affinity galactose-specific PTS since galactose is often the second moiety cleaved from glycoproteins after sialic acid (41).

Adaptation to environmental stresses such as pH, oxygen tension and inhibitory substances within the mouth is vital to the survival of both planktonic and biofilm-associated microbes. The ability to withstand these pressures will determine which species flourish. Most microorganisms are sensitive to the effects of acid. Any pH not in the tolerable range is likely to kill the microbe or lessen its colonization and/or virulence (65). Exceptions include the mutans streptococci and lactobacilli, which are acidogenic and aciduric (reference). These species are responsible for much of the acid production by the degradation of carbohydrates via the Embden-Meyerhof-Parnas (EMP) pathway, which creates acid as an end product (16). When acid accumulates above the acceptable limits for acid-sensitive species, the balance between commensal and cariogenic bacteria favors disease.

Oxygen concentration in the supragingival space varies greatly depending on the age, activity and type of biofilm formed. In a newly forming biofilm, oxygen flows relatively freely throughout the structure due to its thin layer of cells (65). In contrast, it is much more difficult for oxygen to diffuse through a mature biofilm because of its thickness; therefore, an oxygen gradient forms (19). Due to the heterogeneous nature of dental plaque, aerobic bacteria become positioned closer to the edge of the biofilm while anaerobic bacteria remain deeper in the biofilm (65). This is because oxygen levels tend to decrease as biofilm depth increases (65). Oral bacteria have a high oxygen metabolism and, as a result, toxic products are produced by metabolic activity that are susceptible to the limitations of diffusion (19). This can increase the exposure time to stress and is especially harmful for anaerobic bacteria.

Inhibitory substances secreted by the host and other bacteria can threaten a microbial population as well. Although mature biofilms have a lower risk of being affected by these substances (28), both planktonic and biofilm-associated cells must contend with this threat. Bacteriocins and hydrogen peroxide (H_2O_2) production by various microbial species, secretion of proteases, generation of alkaline products, and antibiotics are all examples of the various types of substances that can interfere with the growth of bacteria (54). In some cases, two species will produce opposing bacteriocins or inhibitory substances in an attempt to outcompete each other for a particular space (42, 54). The microbe with the better adaptive mechanism often wins. All of the factors mentioned in this section generate constant competition among the microbial community and the outcome determines whether the oral cavity will be one of health or disease.

Interspecies Interactions in Dental Plaque

Because dental plaque contains a multitude of bacterial species, it is logical to anticipate extensive bacterial interactions among the community. Furthermore, these interactions not only extend to the process of biofilm formation, but also to growth and survival. Interactions between cariogenic bacteria, such as *S. mutans*, and non-cariogenic bacteria, such as *S. gordonii*, can modulate the pathogenic potential of a biofilm (49). Multiple researchers have reported evidence suggesting both cooperative and antagonistic interactions among species in the oral microbiome (42, 52, 54, 59, 73).

Microorganisms established in a biofilm have evolved a host of metabolic pathways designed to more efficiently utilize the varying carbohydrate sources encountered in the respective niches. For example, although many streptococcal species possess genes for glucosyltransferase enzymes, only mutans streptococci

possess extracellular glucan-binding proteins (11). These extracellular glucan-binding proteins may increase the cariogenic potential of *S. mutans* by mediating the binding of glucan to the bacterial cell and promoting adhesion to the biofilm matrix (11). In addition, the ability to metabolize sucrose into lactic acid more rapidly than other bacteria provides another competitive advantage for *S. mutans* (54).

In contrast, synergistic interactions in a biofilm have been described as well. For example, *Actinomyces naeslundii* and *S. oralis* have been shown to poorly colonize saliva-coated surfaces when incubated separately, but form considerable biofilms on the same surfaces when incubated together (73). The combination of metabolic activities from each species was sufficient to support a mutualistic relationship and sustain growth of a biofilm.

The metabolites produced from each microbe may likely be affecting other organisms in the biofilm. For example, plaque with large proportions of *S. mutans* often presents low quantities of *S. sanguinis* (59). Production of high amounts of lactic acid by the former is detrimental to non-aciduric species such as *S. sanguinis*, thereby favoring *S. mutans*. In response, *S. sanguinis* and *S. gordonii* produce H_2O_2, which cannot be metabolized by *S. mutans* and acts as a nonspecific antimicrobial agent (53, 54). In addition, the metabolic products of one microbe may support the growth of another. For example, the lactic acid generated by *S. mutans* is metabolized into H_2O_2 and pyruvate via the enzyme lactate oxidase by *Streptococcus oligofermentans*, which is associated with caries-free patients (90, 91).

Both commensal and pathogenic bacteria produce inhibitory substances and/or enzymes in order to effectively compete against neighboring species. Kuramitsu

demonstrated that *S. gordonii* is able to degrade the competence stimulating peptide (CSP) made by *S. mutans* via the challisin protease, leading to attenuation of bacteriocin production and inhibition of sucrose-dependent biofilm formation (55). Bacteriocins are proteinaceous toxins usually affecting closely related organisms (25). *S. mutans* is able to produce at least five bacteriocins (mutacins I – V). Mutacins I, II and III are lanthionine-containing lantibiotics with a broad killing spectrum whereas mutacins IV and V are unmodified lantibiotics with mutacin IV specifically targeting the mitis group of streptococci (54).

Importance of Sugar Metabolism

In Prokaryotes

The most critical objective for a prokaryote is to persist and grow by utilizing biosynthetic pathways to produce the necessary constituents of a living cell. These pathways require energy in the form of ATP. Therefore it is of interest for the cell to obtain energy in the most efficient manner. Heterotrophic bacteria often catabolize carbohydrates to acquire energy. These carbohydrates can be taken into the cell in the form of monosaccharides or disaccharides, depending on the enzymes available to the microbe. Each species possesses genes encoding a variety of enzymes and this determines which sugars are available for uptake and catabolism. It also determines the niche occupied by the species since uptake of the correct substrates in the microenvironment is required for energy and survival.

In the Oral Cavity

The primary function of the oral cavity is to provide the host with nutrition, often in the form of carbohydrates. It therefore follows that bacteria colonizing the mouth should regard carbohydrate utilization as an important aspect of survival and growth since

there are so many different substrates to choose from. Because of the extensive variety of nutritional resources available, there is a constant struggle among different species to utilize the most efficient transport mechanisms in hopes of outcompeting neighbors and keeping energy expenditures as low as possible. This is evidenced by the fact that many species in the mouth possess multiple substrate-specific carbohydrate transport mechanisms (7, 93).

The importance of carbohydrate metabolism first became evident in 1860 when Pasteur reported that bacteria produce lactic acid via sugar fermentation (75). *In vitro* studies then demonstrated that exposure to the acidic by-products of sugar metabolism can induce dental caries (64). Finally, Miller published results indicating that bacteria from human saliva can produce lactic acid from dietary constituents and that the quantities made were sufficient enough to cause demineralization of the tooth enamel (68).

A few decades later, Stephan provided the first *in vivo* evidence linking bacterial carbohydrate metabolism with acid production (84). He attributed the findings to a causal relationship between the sharp decrease in plaque pH measured after a sugar rinse and the presence of a mixed population of bacteria (84, 85). Sugar metabolism by way of the EMP pathway (glycolysis + homolactic acid fermentation) creates lactic acid as an end product, which is responsible for the decline in pH (81). However, Stephan also noted a gradual increase in pH with an eventual plateau as acid production diminished. It was shown years later that the gradual rise in pH was due to the generation of alkaline products by acid-sensitive microbes present nearby (98)

With the information gained from the previously mentioned studies, it appears that there is a fluctuating balance between acid-tolerant and acid-sensitive bacteria within a dental biofilm. This balance stems from the fact that acid-tolerant species tend to rapidly metabolize sugar and lower the pH while acid-sensitive species tend to counteract that by producing basic products to increase the pH. Only when the composition of the microbial community begins to deviate from that of the normal healthy flora does the cariogenic potential of the biofilm increase. The normal healthy flora includes bacterial species that either benefit the host or do not benefit the host but also do not cause disease. Microbes associated with disease such as *S. mutans* and lactobacilli (dental caries) or *Porphyromonas gingivalis* (periodontal disease) are not found in high numbers, if any, of dental plaque isolated from healthy teeth (1). Therefore, a deviation from the normal healthy flora would include increased numbers of pathogenic species or the conversion of opportunistic pathogens into full-fledged pathogens.

Sugar Transport and Utilization

Lactose and Galactose Structure and Function

Lactose is a disaccharide composed of glucose and galactose fused together via a β(1→4) glycosidic linkage. This bond can be broken by hydrolysis via the β-galactosidase/lactase enzyme to produce glucose and β-galactose. Commonly found in milk and other dairy products, lactose acquired its name from *lactis*, the Latin word for milk. Its molecular formula is $C_{12}H_{22}O_{11}$. There are two forms of lactose: α-lactose and β-lactose. The difference between these isomers lies in the orientation of the hydrogen and hydroxyl groups of carbon number 1 in the glucose moiety. In reality, lactose actually exists in equilibrium as a combination of both of these forms.

Galactose is a monosaccharide composed of 6 carbon atoms and is a C-4 epimer of glucose. It is often found in dairy products, pectins, gums and mucilages as well as sugar beets and various fruits and vegetables. Its molecular formula is $C_6H_{12}O_6$. Aside from transportation across the bacterial membrane for use as energy, galactose is also often incorporated as the second moiety in glycoproteins on the bacterial cell membrane surface (41). These glycoproteins can often be cleaved via extracellular β-galactosidase enzymes made by the cell to release the galactose for energy utilization (21, 86). The free galactose is then available for uptake by the cell.

Sugar Transport Systems

Lactose and galactose are both transported through complex systems embedded in the bacterial membrane. In oral streptococci there are three transport systems known to exist for these sugars: (1) primary active transport, (2) secondary active transport and (3) group translocation (see this reference for review - (33)).

Primary and secondary active transport systems pump in the sugar without modifying it chemically. Primary transport systems are common to all bacteria and require energy from ATP for the internalization of the sugar substrate. This sugar-specific system is termed an ATP-binding cassette (ABC) transporter and usually includes four domains that are often fused together, two inserted into the membrane and two located in the cytoplasmic space. The two integral membrane proteins serve as the channel for the sugar substrate to pass through while the two cytoplasmic proteins act as ATPases, which harvest energy from ATP to power the movement across the membrane. Secondary transport systems do not use the energy from ATP to power the transfer across the membrane, but rather use a concentration gradient to force the sugar molecule into the cell. Two different types of secondary transport systems are

often employed for lactose and/or galactose uptake: (1) a proton-coupled antiporter, or (2) lactose-galactose antiporter.

The last transport system, group translocation, is the most efficient from a bioenergetics standpoint. Also known as the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS), this multi-component system transfers a phosphoryl group to inbound sugar substrates via a high-energy PEP molecule (Figure 1-1). The chemical composition of the carbohydrate is therefore altered. A signal cascade comprised of two general cytoplasmic components and one transport permease aids in the transfer of the phosphate group from PEP to the sugar. This mechanism is found in both Gram-negative and Gram-positive bacteria with minor differences. While the cytoplasmic constituents are not substrate-specific, the transport permease is. Usually each PTS only allows for one or two specific sugars to be transported, although a particular sugar can be transported by more than one PTS. For example, *S. mutans* has been shown to transport mannose, glucose and galactose through the mannose PTS (EII^{Man}).

PEP Group Translocation - Phosphotransferase System (PTS)

The process starts with phosphoenolpyruvate, a glycolytic intermediate (Figure 1-1) (see this reference for review - (93)). The high-energy bond created from detachment of the phosphoryl group is enough to power a signal cascade and allow for importation of the sugar. The phosphoryl group is transferred to a histidine residue located on the first cytoplasmic protein, Enzyme I (EI). EI subsequently transfers it to histidine residue 15 on the second cytoplasmic protein, histidine-phosphocarrier protein (HPr). HPr continues the cascade by donating the phosphate to the carbohydrate-specific Enzyme II (EII) complex. This complex consists of a hydrophobic integral membrane domain

(subunit C, EIIC) and two hydrophilic cytosolic domains (subunit A and B, EIIAB). EIIA catalyzes the phosphate transfer from HPr and passes it on to either the histidine or cysteine of EIIB. The sugar enters via the translocating EIIC domain and docks at a sugar-binding site. EIIB then provides the final phosphorylation onto the sugar at the position of carbon 6. The sugar is now free to move into the cytoplasm where it can be metabolized through the proper pathway.

Tagatose-6-Phosphate Pathway

Once lactose or galactose has been phosphorylated, the cell will direct it towards the correct pathway for catabolism (Figure 1-2). In the case of lactose-6-phosphate (Lac-6-P), a cytosolic phospho-β-galactosidase (*lacG*) will catalyze its breakdown into glucose and galactose-6-phosphate (Gal-6-P). Glucose enters the glycolytic pathway whereas Gal-6-P enters the tagatose pathway. Because galactose is already transported into the cell as Gal-6-P, there are no necessary steps to facilitate its entry into the tagatose pathway.

The first enzymes in the pathway, both galactose-6-phosphate isomerases (*lacA, lacB*), convert Gal-6-P into tagatose-6-phosphate via an aldose → ketose reaction. Then an additional phosphate is attached at the carbon 1 position of tagatose-6-phosphate to create tagatose-1,6-bisphosphate after tagatose-6-phosphate kinase (*lacC*) harvests energy from a molecule of ATP. The final enzyme in the pathway, tagatose-1,6-diphosphate aldolase (*lacD*), cleaves tagatose-1,6-bisphosphate into glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP).

Leloir Pathway

Galactose can also be brought into the cell through a second mechanism of transport. A non-PTS permease can uptake the hexose and lead it into the alternative

Leloir pathway (see this reference for review - (36)). The permease does not alter the galactose substrate by phosphorylation. Instead, the sugar is transported in its free form. Once galactose has entered the Leloir pathway, it is phosphorylated at the carbon 1 position by ATP-dependent galactokinase (GalK) to produce galactose-1-phosphate (Gal-1-P). The next enzyme in the pathway, galactose-1-phosphate uridylyltransferase (GalT), requires the co-factor UDP-glucose. Gal-1-P is then transformed into the galactose derivative UDP-galactose + glucose-1-phosphate (Glc-1-P) via GalT. Finally, UDP-glucose-4-epimerase (GalE) recycles UDP-galactose by converting it back into UDP-glucose while phosphoglucomutase converts Glc-1-P into glucose-6-phosphate.

Galactose transport can occur through both the PTS and non-PTS permeases; however, usually one mechanism is more dominant than the other. There have been conflicting reports on which mechanism plays a dominant role in the metabolism of galactose in *S. mutans*. Ajdic and Pham suggest that the Leloir pathway plays a dominant role due to abolished growth in galactose when *galK* is inactivated (7). In contrast, Zeng and co-workers argue that the lactose PTS plays a dominant role. Their findings show that a lacG deficient strain is unable to grow on galactose and over expression of the Leloir pathway in that same strain did not rectify growth on galactose (103). Therefore, it appears that in *S. mutans* the dominant transport system for galactose uptake is the lactose PTS.

Genetic Organization of the Lactose and Galactose Gene Clusters *Streptococcus mutans* UA159

The *S. mutans* UA159 genome contains two main types of sugar transporters: the PEP-dependent PTS or the ABC transporter. In a microarray analysis of the *S. mutans* genome after growth in 13 different sugars, Ajdic and Pham found that

monosaccharides, disaccharides, β-glucosides, and sugar alcohols were all transported through the PTS while the ABC transporters were mostly specialized for oligosaccharide transport (7). Lactose enters *S. mutans* through the lactose PTS and continues to the tagatose pathway. Galactose may enter through the lactose PTS or through an unidentified non-PTS permease that leads the sugar into the Leloir pathway. It is likely that galactose enters the cell through both mechanisms.

The genes for the lactose PTS, *lacRABCDFEG*, are situated adjacently in the genome (Figure 1-3). The lactose-specific transporter is annotated by *lacF* (EIIA) and *lacE* (EIIBC). A phospho-β-galactosidase (*lacG*) catabolizes Lac-6-P before it enters the tagatose-6-phosphate pathway. The enzymes in this pathway include the two subunits of the heteromeric galactose-6-phosphate isomerase (*lacAB*), a tagatose-6-phosphate kinase (*lacC*) and a tagatose-1,6-bisphosphate aldolase (*lacD*). A transcriptional regulator (*lacR*) sits just upstream of the entire operon (79). All of these genes are based on annotation and not experimentation.

***Streptococcus gordonii* DL-1 Challis**

S. gordonii DL1 harbors a similar genetic layout to *S. mutans* UA159 in terms of the lactose-specific PTS and tagatose pathway genes (Figure 1-4). However, in contrast to *S. mutans*, the *S. gordonii* genome contains a unique set of genes that appear to be part of a dedicated galactose-specific PTS, including SGO_1522 (EIIA), SGO_1521 (EIIB) and SGO_1520 (EIIC). In addition, a second set of tagatose pathway genes are situated just upstream of the EII^{Gal} genes. Another difference between the genetic organizations of the *lac* genes in *S. mutans* UA159 and *S. gordonii* DL1 lies in a regulatory transcriptional antiterminator SG0_1515 (*lacT*), which is situated between the first set of tagatose genes and the lactose-specific PTS. Although the *lacT* gene is not

found in the *S. mutans* UA159 genome, it is present in the genome of another oral microbe, *Lactobacillus casei* (33).

In addition to the lactose PTS, *S. gordonii* also contains genes for the Leloir pathway. Therefore, galactose is likely transported by both a PTS and non-PTS permease. However, because it appears from the *S. gordonii lac* genomic structure that a dedicated galactose-specific PTS exists, the main route of transport into the cell could likely be through the PTS.

For the purposes of this thesis, the tagatose pathway genes just up stream of the lactose-specific PTS have been termed the lactose cluster while the second set of tagatose pathway genes just upstream of the galactose-specific PTS have been designated as the galactose cluster.

Regulation of PTS Activity and the Tagatose-6-Phosphate Pathway

Carbon Catabolite Repression

Under normal circumstances in the mouth, bacteria are often bathed in multiple sugar sources due to the variety of carbohydrates consumed in the diet and sugar moieties contained in glycoconjugates of saliva. During these conditions, the bacteria must decide the best course of action in regards to satisfying their nutritional requirements from the environment in the most efficient manner. Therefore, many bacterial species have developed a mechanism that preferentially utilizes a particular sugar source while repressing expression of the genetic components associated with an alternative carbon source (83). This mechanism is known as carbon catabolite repression (CCR) and is executed by both Gram-negative and Gram-positive microbes. In many cases, glucose is a preferred sugar source over sugars such as lactose and galactose. The main components involved in CCR of Gram-positives include HPr, HPr

kinase/phosphatase (HPrK/P), the global transcriptional regulator catabolite control protein A (CcpA) and various carbohydrate-specific EII of the PTS (37, 83). Global control and regulation by these proteins is based on the physiological state of the cell and the available carbon sources (76).

HPr is the primary protein involved in CCR in that it relays information about the nutritional needs of the cell based on its phosphorylation state. When HPr is phosphorylated by EI at histidine residue 15 (P-His-HPr) it is responsible for signaling the transport of carbohydrates into the cell via PTS permeases. In addition, HPr is capable of being phosphorylated at serine residue 46 (P-Ser-HPr), through the enzymatic activity of HPrK/P. The fluctuating levels of P-His-HPr and P-Ser-HPr constantly change depending on signals in the cytosolic environment (76).

CcpA, a member of the LacI-GalR family of bacterial transcriptional regulators, is implicated in controlling genes related to energy, transport, metabolism and other functions (4). Although CcpA often acts as a repressor, this protein is capable of functioning as an activator of transcription as well depending on the location of the catabolite response element (*cre*) binding sequence in relation to the gene promoter (61). If the *cre* site is located downstream of the promoter then CcpA is most often a repressor whereas if the *cre* site is located upstream of the promoter then CcpA is most often an activator (37). Binding of CcpA to a *cre* sequence located proximal to promoter regions is stimulated by P-Ser-HPr and together they form a complex that co-represses genetic transcription of a gene by binding (38).

CCR in Gram-positive bacteria can be achieved via two different mechanisms: CcpA-dependent or CcpA-independent. In CcpA-dependent CCR, phosphorylation by

EI produces P-His-HPr, which is necessary for the uptake of carbohydrates through the PTS. As the carbohydrates are metabolized, glycolytic intermediates such as fructose-1,6-bisphosphate (FBP) and glucose-6-phosphate (G-6-P) are released . When these intermediates accumulate to a threshold concentration in the cell, HPrK/P is activated and an ATP-dependent phosphorylation of HPr occurs at the serine residue (P-Ser-HPr) (80). With CcpA-dependent CCR activated, P-Ser-HPr and CcpA bind to the *cre* sequence located in the promoter of the gene of interest and co-repress transcription of the operon (83). When the concentration of FBP in the cell drops too low and the concentration of inorganic phosphate (Pi) builds up, HPrK/P works in the reverse direction to dephosphorylate P-Ser-HPr and produce pyrophosphate (PPi) + free HPr, which then becomes available to again bind phosphate at the histidine residue for carbohydrate transport (76). In CcpA-independent CCR, components of the PTS are involved in regulating genetic transcription. In *S. mutans*, the carbohydrate-specific mannose permease (ManL), fructose permeases (FruI/FruCD), and the fructose/mannose permease (EII^{Lev}) have been identified as contributors in the CcpA-independent repression of *fruA* and *levDEFG* (2, 97, 101). The mechanism for this type of CCR begins similarly to that of CcpA-dependent CCR. The uptake of preferred sugar sources by the respective PTS creates FBP, which in turn activates ATP-dependent phosphorylation by HPrK/P to create P-Ser-HPr (83). At this point, P-Ser-HPr has been postulated to bind to LevR and inhibit its activation of the *fruA* and *levDEFG* promoters via an allosteric mechanism, thus repressing transcription of the genes (102). Furthermore, underphosphorylation of the EIIA and EIIB subunits of the ManL (and possibly FruI/FruCD, EII^{Lev}) transporter also represses transcription of the genes (102).

Regulation of the *lac* Genes

There is not much information available in the literature in regards to the regulation of the lactose and/or galactose PTS for *S. gordonii*; however, research studies completed on species with similar *lac* genetic organization may provide insight into how *S. gordonii* regulates these gene clusters.

Secondary regulators often exist such as repressors and antiterminators, which work in conjunction with CCR-mediated controls (37). The repressor associated with the lactose PTS is LacR. The *lacR* gene, along with the rest of the lactose PTS, is present in *S. mitis*, *S. sanguinis*, *S. pyogenes*, *S. aureus*, *S. mutans*, *S. pneumoniae* and *L. lactis* (33, 62, 87, 94, 100). Principally, *lacR* is annotated as a transcriptional repressor, which is supported by evidence showing that LacR binds to its own promoter as well as the *lacA* promoter in *L. lactis* (32, 35, 95). Comparable roles for LacR have been hypothesized for *S. aureus* and *S. mutans*; however, *L. casei* does not possess a *lacR* gene (33).

A transcriptional antiterminator (LacT) similar to that of the BglG/SacY family is putatively present in the genome of all species mentioned except *S. mutans*, *S. aureus* and *L. lactis* . The activity of LacT in *L. casei* is controlled by EII^{Lac} and the common PTS elements and is modulated by phosphorylation of two PTS regulation domains (PRD) (40). There have been no other studies published on the regulatory mechanism of LacT, indicating that this area of research is very novel.

Specific Aims

- Aim I: Characterization of contiguous lactose/galactose operons in *S. gordonii*.
- Aim II: Gene regulation in response to lactose/galactose utilization and enzymatic metabolism.

- Aim III: Impact of lactose/galactose utilization on the inter-specific competition between cariogenic *S. mutans* and commensal *S. gordonii* oral streptococci.

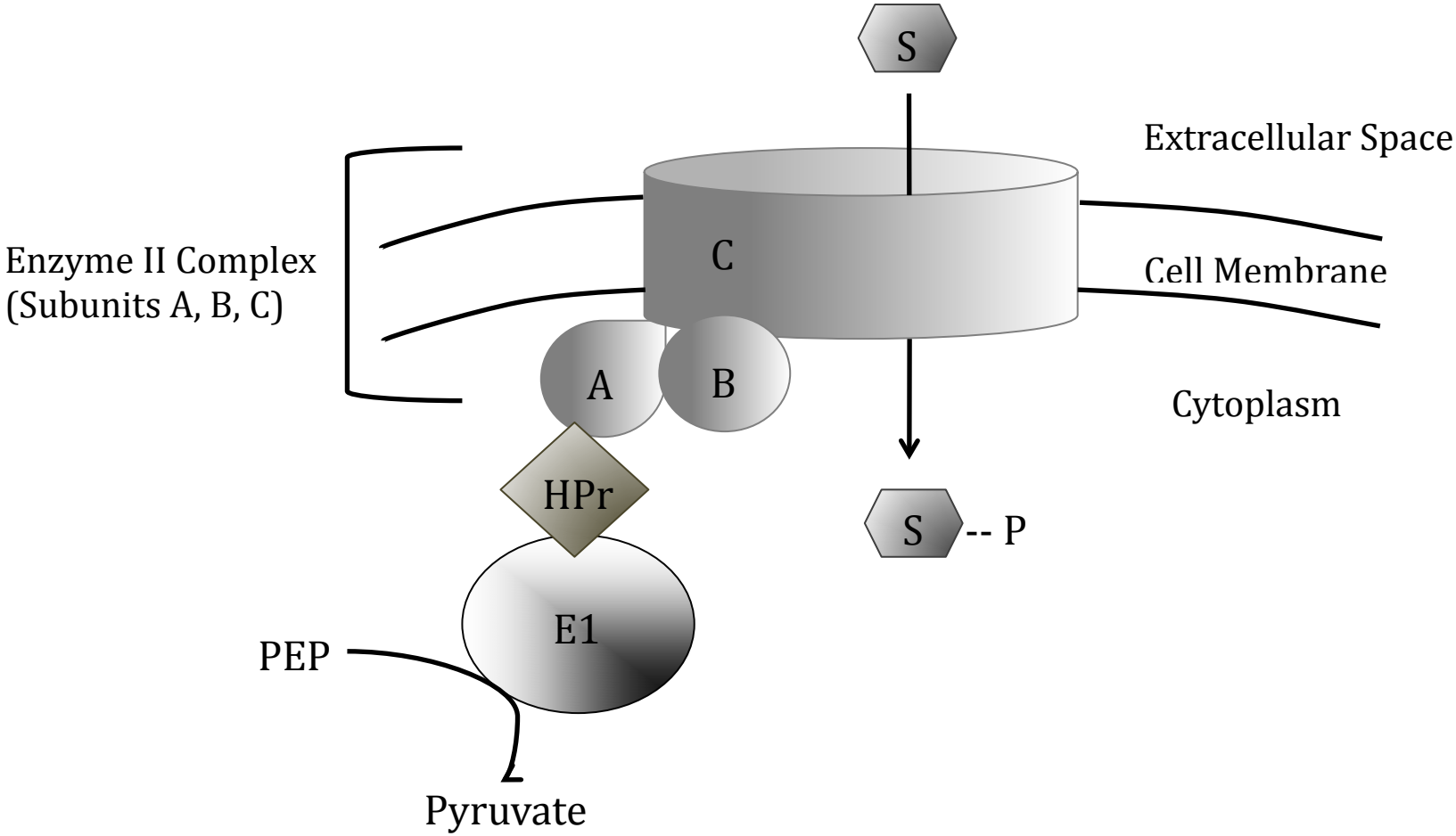


Figure 1-1. A general overview of the phosphoenolpyruvate-dependent sugar:phosphotransferase system (PEP-dependent PTS) involved in carbohydrate transport across the cell membrane. A phosphate molecule is transferred from phosphoenolpyruvate to Enzyme I (EI). EI transfers the phosphate to residue histidine 15 of the histidine-phosphocarrier protein (HPr). Subunit A of the Enzyme II (EII) complex passes the phosphate from P-His-HPr to subunit B of the EII complex. The incoming sugar is transported and concomitantly phosphorylated by the transmembrane protein subunit C of the EII complex.

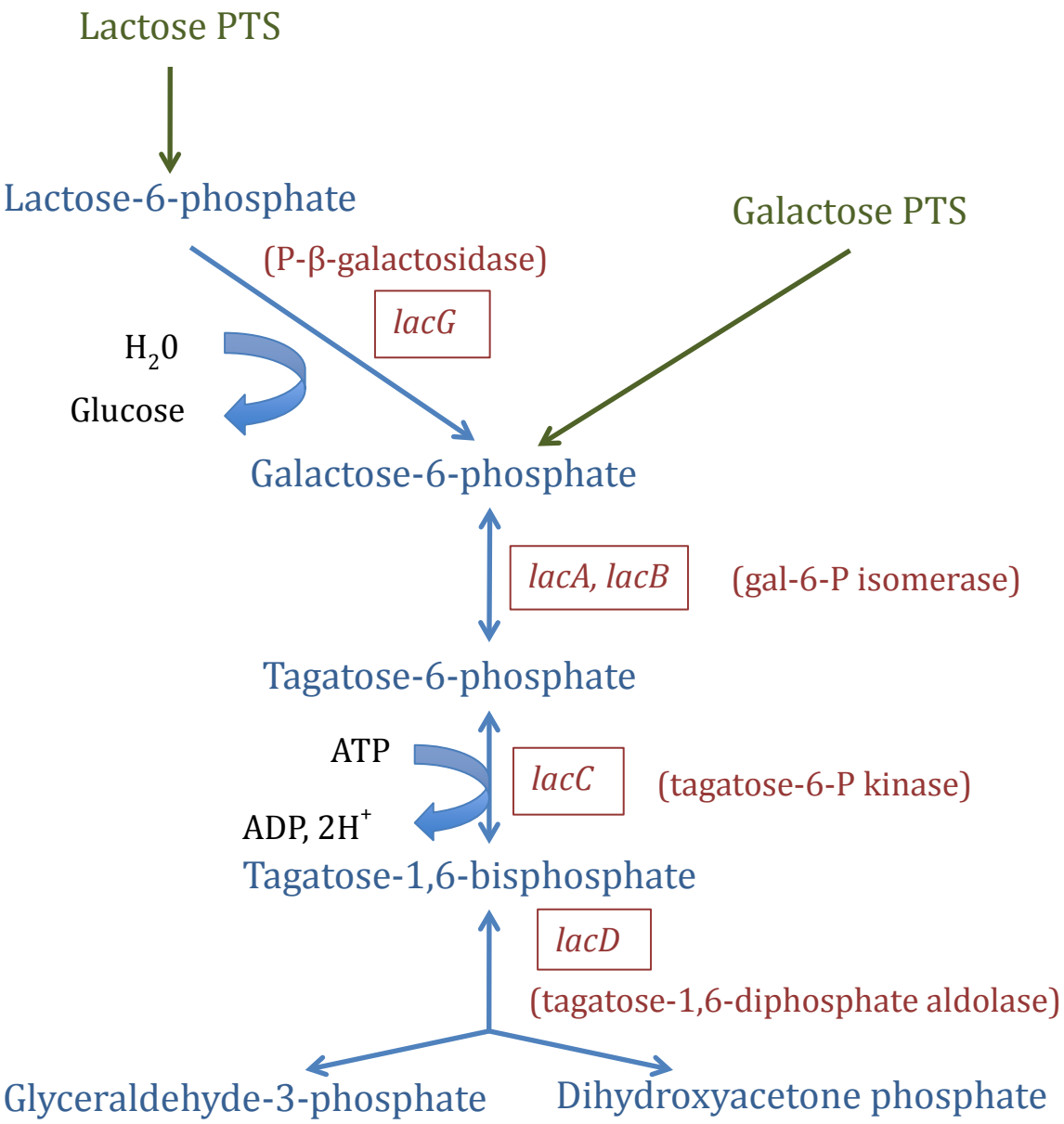


Figure 1-2. Schematic showing predicted pathways for catabolism of lactose and galactose by *S. gordonii* following transport via a sugar-specific PTS and shunted through the tagatose-6-phosphate pathway. Once the sugar enters the cell, *lacG* will catabolize Lac-6-P into glucose and Gal-6-P. Gal-6-P enters the tagatose pathway where the galatose-6-phosphate isomerases (*lacA, lacB*) convert it into tagatose-6-phosphate. Tagatose-6-phosphate kinase adds a phosphate to the carbon one position to produce tagatose-1,6-bisphosphate. Lastly, tagatose-1,6-disphosphate aldose (*lacD*) converts the sugar intermediate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

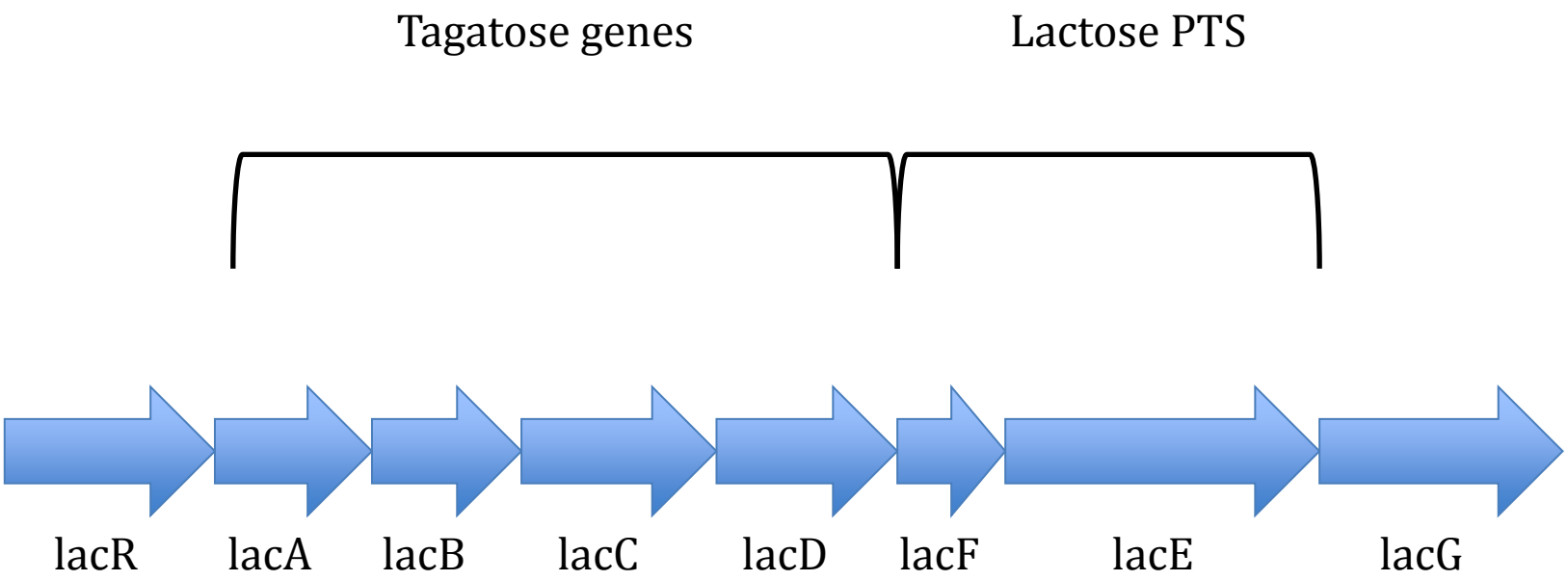


Figure 1-3. Genetic organization of the *lac* operon in *S. mutans* UA159. The *lac* operon contains the genes for a repressor (*lacR*), the tagatose-6-phosphate pathway (*lacABCD*), the lactose transporter (*lacF, lacE*) and phospho-β-galactosidase (*lacG*).

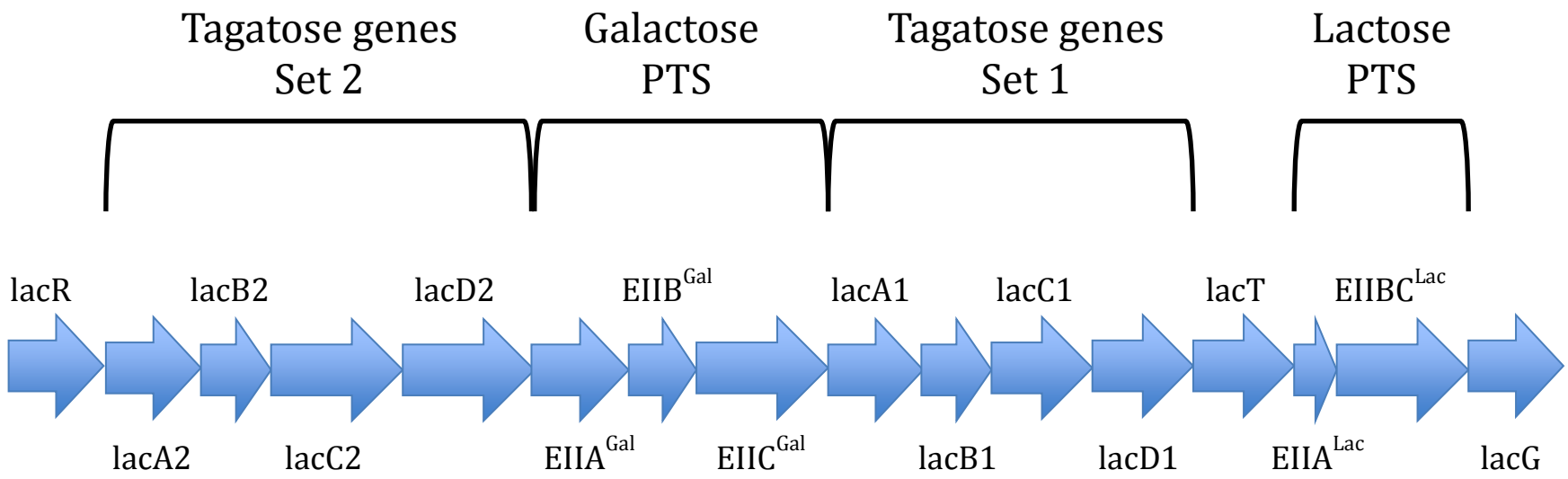


Figure 1-4. Genetic organization of the *lac* operon in *S. gordonii* DL1. The lactose cluster contains the genes for the first set of tagatose-6-phosphate pathway enzymes (*lacABCD1*), a transcriptional antiterminator (*lacT*), lactose transporter (*EIIA^{Lac}, EIIBC^{Lac}*) and phospho-β-galactosidase (*lacG*). The galactose cluster contains the genes for a repressor (*lacR*), a second set of tagatose-6-phosphate pathway enzymes (*lacABCD2*) and the galactose transporter (*EIIA^{Gal}, EIIB^{Gal}, EIIC^{Gal}*).

CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 2-1. All strains were stored in 50% glycerol at -80°C. All *S. gordonii* and *S. mutans* strains were maintained on brain heart infusion (BHI) agar plates (Difco Laboratories, Detroit, MI) and broth cultures were routinely grown in either BHI or Tryptone-Vitamin (TV) (Difco Laboratories, Detroit, MI) base medium. All cultures were incubated at 37°C in a 5% CO_2 chamber. Selection and maintenance of antibiotic resistant strains on agar was achieved by supplementing media with erythromycin (Em) (10 µg/mL), kanamycin (Km) (1 mg/mL), or spectinomycin (Sp) (1 mg/mL) as needed. BHI broth cultures supplemented with antibiotics employed half those concentrations.

E. coli strains were maintained on Luria-Bertani (LB) agar plates under aerobic conditions at 37°C. All liquid cultures were grown in LB broth in an aerobic chamber with vigorous agitation of the cells at either 30°C or 37°C depending on experimental conditions. Vigorous agitation is defined as shaking at 250 rpm in a vessel where the culture occupied less than 20% of the total vessel volume. Antibiotics utilized in the selection of relevant strains include Km (25 µg/mL) and ampicillin (Ap) (100 µg/mL) for both agar and broth conditions. Induction of the *lac* operon with isopropyl β-D-thiogalactoside (IPTG) (Sigma-Aldrich, St. Louis, MO) yielded expression of recombinant protein in an *E. coli* M15 expression system. At an OD_{600} of 0.5-0.6, IPTG was added to a final concentration of 0.005 mM and the cells were incubated another three hours before harvesting. Uninduced cultures were treated exactly the same with the exception that no IPTG was added.

DNA Manipulations

Coding sequences of the genes in the *lac* and *gal* clusters of *S. gordonii* were deleted in whole or in part and replaced with a nonpolar Em, Km, or Sp resistance cassette via allelic exchange, as described previously (56). Briefly, two DNA sequences flanking the gene of interest were amplified via PCR using gene specific primers with integrated restriction cut sites (Table 2-2). These fragments, along with the appropriate antibiotic resistance vector, were cut using restriction enzymes (New England BioLabs, Ipswich, MA; Invitrogen, Carlsbad, CA) and ligated together using T4 DNA ligase overnight at 16°C. This mutated, linear DNA was then transformed into naturally competent *S. gordonii* DL-1 cells using 10% horse serum (Sigma-Aldrich, St. Louis, MO) (63, 67). Resulting strains were subjected to PCR verification and DNA sequencing before use in experiments. Specific genes targeted for mutation include *lacR*, *lacA2B2*, EII^{Gal}, *lacA1B1*, EII^{Lac} (*lacEF*) and *lacG* (Table 2-1). Double deletion mutants were made by transformation of one strain with the chromosomal DNA of the second strain.

Plasmid PlacA2-*cat* was constructed by fusing a 308-bp sequence from the promoter region of *lacA2* with the promoterless chloramphenicol acetyltransferase (*cat*) gene (43) located on plasmid pJL84 (105). The promoter-*cat* fusion was excised from the plasmid using restriction enzymes *SacI* and *BamHI* and sub-cloned into the integration vector pMJB8. This vector integrates a single copy of the promoter fusion into the *S. gordonii* genome at a remote site (*gtfG* locus). This plasmid encodes a Km marker for selection purposes.

Plasmid PlacA1-*cat* was constructed in the same manner by fusing a 385-bp sequence from the promoter of *lacA1* with the *cat* gene from plasmid pYQ4, a plasmid derived from pMJB8. pYQ4 can be used directly to integrate into the *S. gordonii*

chromosome at the *gtfG* locus. Restriction enzymes used include *BamHI* and *KpnI*. This plasmid contains an Em marker for selection purposes.

A 4-bp mutation was created in the *cre* of the *lacA2* promoter by site-directed mutagenesis. Briefly, two pairs of primers were used to generate two separate DNA fragments via PCR: the original pair of primers used to make the PlacA2-*cat* DNA and another pair designed to incorporate the 4-bp mutation into the DNA fragments (Table 2-2). Therefore, the two separate DNA fragments each contained the mutation, one fragment at the 5' end and the other fragment at the 3' end. The two fragments were then combined in recombinant PCR along with the original pair of primers, producing a single product containing both fragments and the desired 4-bp mutation. This mutated *lacA2* promoter region was then fused to the *cat* gene in plasmid pYQ4 to create the *cre*-PlacA2-*cat* plasmid (104). The plasmid encodes Em resistance and integrates into the *S. gordonii* genome at the *gtfG* locus. All three of these plasmids were then transformed into various mutated genetic backgrounds and the impact assessed using the chloramphenicol acetyltransferase (CAT) reporter assay of Shaw (82).

A site-directed point mutation was generated in the *lacT* gene whereby the translational start codon (Met8) was replaced with a stop codon. This strain was designated *lacT*(M1stop). Briefly, a 1-2 kb DNA fragment containing the *lacT* point mutation was generated using recombinant PCR. The mutated *lacT* DNA fragment was transformed into wild-type *S. gordonii* DL-1 along with an indicator plasmid carrying the PlacA2-*cat* reporter fusion and a Km resistance cassette (101). A lower concentration of the plasmid was used (at least 1000-fold) than of the mutated DNA fragment, with integration occurring at the *gtfG* locus. Transformants were then plated on BHI + Km

and several colonies were screened for the desired mutation by using an allele-specific mismatch amplification mutation analysis (MAMA) PCR (23) and verified via sequencing. This type of mutation allows for transcription to occur while halting translation.

MAMA PCR was used to verify integration of the mutated *lacT* DNA fragment into the *S. gordonii* genome. The reaction included three primers: (1) a 5' primer (SGO1515-5'), (2) a 3' MAMA primer (SGO1515-M8stop-3') and (3) a 3' control primer (SGO1515-3') that anneals at a distal site (>0.5 kb) on the same strand (Table 2-2). The MAMA primer is designed to detect site-directed mutations and usually contains one mismatch with the wild-type allele and two mismatches with the mutant allele within the last three nucleotides of the primer sequence. Therefore, when wild-type DNA is tested via MAMA PCR, the MAMA primer binds preferentially to generate the shorter DNA fragment. This indicates that no point mutations exist in the tested DNA region. When cells containing mutated *lacT* DNA are tested, amplification of the larger control product is observed. This is a result of the MAMA primer's inability to anneal to the mutated *lacT* DNA region and indicates that the site-directed mutation is present in the template strand. *S. gordonii* colonies were screened in a 50 mL reaction that contained 0.6 mM primer 1, 0.4 mM primer 2 (MAMA primer) and 0.2 mM primer 3, treated at 95°C for 5 minutes, followed by 30 cycles as follows: 95°C for 25 seconds, 55°C for 25 s and 72°C for 2 min (102).

Growth Rate Assays

Phenotypic growth analysis was documented for all oral streptococci strains with a Bioscreen C™ reader (Oy Growth Curves Ab, Ltd., Helsinki, Finland). Individual colonies were inoculated in triplicate into BHI and grown overnight. Strains were then

sub-cultured the next morning into fresh BHI media and grown to an OD_{600} of 0.5 (mid-exponential phase). Samples were then sub-cultured into fresh TV medium containing glucose, galactose, or lactose at a concentration of 0.5%. Samples were loaded onto a 100 well plate at 300 µL each with a 50 µL mineral oil overlay to mimic anaerobic conditions. The plate was then incubated in the Bioscreen C™ at 37°C and the optical density measured at 600 nm (OD_{600}) every 30 min with 10 s of shaking before each reading.

Analysis of Promoter Gene Fusion Strains

S. gordonii cat fusion strains for the *lacA1* and *lacA2* promoters were assayed in various mutated backgrounds. Strains were grown overnight in TV medium containing glucose, galactose, lactose, glucose + galactose, or glucose + lactose at a concentration of 0.5%. In the morning, the cells were sub-cultured into 30 mL of the same medium and grown to early exponential phase (OD_{600} of 0.4-0.5). Cells were harvested and washed with 10 mM Tris-HCl pH 7.8 followed by resuspension in 750 µL of the same buffer on ice. The concentrated cell suspension was mixed with glass beads (0.1-mm diameter) equal to a volume of 500 µL. Homogenization and mechanical breakdown of the cells was achieved by subjecting the cells to a Bead Beater (Biospec Products, Inc., Bartlesville, OK) for 30 s pulses twice at 4°C with a 2 min incubation period on ice between pulses. Centrifugation produced a pellet of the cellular debris while leaving proteins suspended in the supernatant fluid. The cell-free extract was used to measure the CAT specific activity via the spectrophotometric method outlined by Shaw (82).

This kinetic assay determines the rate at which acetyl-CoA acetylates the antibiotic chloramphenicol (Cm) via the chloramphenicol acetyltransferase (*cat*) enzyme, generating a free CoA sulfhydryl group end product in the process. Addition of 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) activates a second reaction in which the free CoA sulfhydryl group and DTNB react to form a mixed disulfide of CoA and thionitrobenzoic acid + a molar equivalent of free 5-thio-2-nitrobenzoate (TNB). The second reaction is used to quantitatively measure *cat* enzymatic rates with a spectrophotometer since the reaction of DTNB with the sulfhydryl group on CoA results in an increase in absorbance at 412 nm (due to the TNB anion) (82). The reaction mixture contains the protein to be measured, 0.1 mM acetyl-CoA and 0.4 mg/mL of DTNB in 100 mM Tris-HCl, pH 7.8. The reaction mixture was adjusted to 37°C in a spectrophotometer equipped with a temperature-controlled cuvette chamber. Subsequently, Cm was added to initiate the reaction. The assay was run with a control containing no Cm and triplicates of each sample containing Cm at a final concentration of 0.1 mM. The rate of increase in absorption at 412 nm was measured and used to calculate the CAT specific activity. The BCA protein assay was used to measure total protein content of the cell lysate samples with BSA employed as the standard.

RNA Isolation and Gene Expression via quantitative Real-Time RT-PCR

RNA was isolated from cultures of wild-type and genetically modified *S. gordonii* strains to evaluate genetic expression via quantitative Real-Time RT-PCR (6). Cells were grown in 10 mL of fresh medium and harvested for RNA isolation during the mid-exponential phase. Cells were frozen in 1 mL Bacterial RNA Protectant in -80°C storage until time for extraction. Media conditions tested included TV with 0.5% of the appropriate sugar (either glucose, galactose, or lactose). Total RNA was then isolated

using protocols described elsewhere (26). Briefly, cells were pelleted, resuspended in TE buffer containing SDS and homogenized in the presence of glass beads and acidic phenol. The cell lysate was centrifuged for 15 min at top speed and cellular RNA was extracted from the supernatant fluid via the RNeasy Mini Kit™ (Qiagen, Germantown, MD). Total RNA was estimated in triplicate using a spectrophotometer. The RNA was also analyzed by electrophoresis on a 0.8% TAE gel to determine quality of the isolated nucleic acid.

High-quality RNA was then converted to cDNA using Reverse Transcriptase PCR (RT-PCR). Each RT reaction combined 50 ng random hexamer primers, 10 mM dNTP mix, 1 µg total extracted RNA and DEPC-treated water up to 10 µL. This reaction was incubated at 65°C for 5 min and then placed on ice for 1 min. The cDNA synthesis master mix combined 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNaseOUT and SuperScript III™ RT. Addition of 10 µL of cDNA synthesis master mix to each sample reaction completed the set up of the RT-PCR reaction. The mixture was incubated at 25°C for 10 min followed by 50°C for 50 min. The reaction was then terminated at 85°C for 5 min and chilled on ice. Any residual RNA was removed by adding 1 µL of RNase H to each sample and incubating for 20 min at 37°C. Each cDNA sample was diluted 10 times for later use in the quantitative Real-Time PCR reaction.

Sense and anti-sense primers (Table 2-2) were designed for a specific gene and used to create a standard curve template via standard PCR. This amplified PCR product was purified, measured and diluted into a series ranging from $0.5x10^1$ to $0.5x10^8$ copies/µL. A 96 well plate was loaded with the 8-point standard curve series (doubles), each sample condition (triplicates) and a negative control (doubles). A master

mix was prepared for each sample with 12 µL of SYBR-Green™ super mix, 100 µM sense primer, 100 µM anti-sense primer and 8.6 µL water. Each well contained 4 µL of nucleic acid plus 21 µL of master mix for a total of 25 µL. Once the plate was set up, the quantitative Real-Time PCR was run using the Biorad iCycler™ program (Hercules, CA). All data were normalized to 1 µg of total RNA.

Expression, Purification and Dialysis of Recombinant N-terminal 6X-His-tagged LacR protein

The QIAexpressionist™ system was used to create a recombinant LacR protein. This system uses the low-copy pQE-30 vector, which is based on the T5 promoter transcription-translation system. The plasmid includes an optimized promoter-operator element consisting of phage T5 promoter and two *lac* operator sequences which increase *lac* repressor binding to ensure efficient repression of the T5 promoter. It also contains a synthetic ribosomal binding site, a 6xHis-tag coding sequence located at the 5' end of the cloning region, multiple cloning site and translational stop codons in all reading frames, two strong transcriptional terminators, β-lactamase gene (*bla*) conferring ampicillin resistance, *cat* gene (no promoter – not normally expressed) and a ColE1 origin of replication.

The *lacR* region was amplified via PCR using primers lacR-5'*BamHI* (GCTAGAGAGG AGGATCCATGGGTAA) and lacR-3'*SalI* (GCCCTTTAAAGAGCTGTCGACTAACT) and subsequently PCR purified. Both the *lacR* PCR product and the pQE-30 plasmid were sequentially digested for 3 hours at 37°C with restriction enzymes *BamHI* and *SalI*. Each digestion reaction was verified on a 0.8% agarose gel and gel purified. Both the *lacR* DNA and digested plasmid DNA were vacuum dried together and set up in a ligation reaction overnight at 16°C

containing 7 µL water, 2 µL buffer with ATP and 1 µL T4 DNA ligase. The ligation product was then transformed by electroporation into competent *E. coli* M15[pREP4] cells, cultured in 1 mL LB broth for 2 hours in an aerobic shaker and plated on LB agar containing Km and Ap for selection. The resulting colonies were screened via PCR to verify the correct size of the plasmid. Primers used for this PCR were pQE30-forward (5'-CCGA AAAGTGCCACCTGACGTCTAAG-3') and pQE30-reverse (5'-AGGTCATTACTGGATCTA TCAACAGG-3'). Four colonies were chosen for sequencing and storage in -80°C. Sequencing of three colonies verified the correct *lacR* sequence.

To determine if the recombinant LacR protein was soluble, 10 mL of LB broth containing Km and Ap was inoculated overnight with the transformed *lacR* expressing *E. coli* M15 cells. This culture was diluted (1:20) into 100 mL of fresh medium and grown to OD_{600} of 0.6. An uninduced control was collected, pelleted and placed in the -80°C freezer. Adding 1 mM IPTG induced expression of the LacR protein. The culture was incubated for another 3 hours. A 1 mL induced control sample was collected, pelleted and placed in the -80°C freezer. The rest of the culture was centrifuged at 4,000 x g for 20 min and frozen at -80°C until further use. An SDS-PAGE gel was prepared to evaluate the induction of LacR in the pellet versus the supernatant samples. Three samples were prepared. The uninduced control and induced pellet were both combined with 2X SDS buffer and boiled for 10 min. The third sample included 50 mL of induced culture combined with 750 µL lysis buffer. The cells were then homogenized with 500 µL glass beads for 30 s and spun down at top speed for 10 min in 4°C. The supernatant was extracted from the sample and combined with 2X SDS buffer. All three

samples were run on the SDS-PAGE gel, stained with coomassie blue and de-stained for visualization of protein bands.

To purify a large scale (1 liter) *E. coli* expression culture, cells were grown overnight with Km and Ap. In the morning, cells were diluted into 1 L fresh media and grown aerobically in 30°C to OD_{600} of 0.6. Adding IPTG to a final concentration of 0.005 mM induced expression of LacR. The cells were harvested after 3 hours, centrifuged at 4,000 x g for 30 min and stored in -80°C.

To prepare the protein lysate under native conditions, the pellet was thawed for 15 min and suspended in 4 mL lysis buffer containing 10 mM imidazole-HCl. The mixture was then incubated for 30 min on ice along with lysozyme (1 mg/mL) and RNase A (10 µg/mL). The cells were added to 500 µL glass beads and homogenized for 30 s twice with a 2 min cooling period in between. The sample was centrifuged at top speed for 15 min and the cleared lysate collected. To the lysate, 200 µL of 50% Ni-NTA slurry was added and mixed gently on a rotary shaker at 200 rpm for 90 min in 4°C. After this incubation period, the mixture was spun down via centrifugation and the supernatant fluid containing the recombinant protein was collected. The nickel slurry was then washed 5 times with increasing concentrations of imidazole buffer. All washes were saved for later evaluation.

- Wash 1 = 10 mM
- Wash 2 = 30 mM
- Wash 3 = 30 mM
- Wash 4 = 50 mM
- Wash 5 = 50 mM

The protein was then eluted from the nickel slurry eight times using 100 µL of an elution buffer containing 250 mM imidazole. All eluates were saved for later evaluation.

A protein gel was run with samples from each step of the purification process. This was to allow for proper tracking of the protein at all times. The eluate with the purest protein sample was then dialyzed against PBS overnight.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assay (EMSA) was used to evaluate the ability of the recombinant LacR protein to bind to the *lacA1* and *lacA2* promoters of *S. gordonii*. Biotinylated and unbiotinylated DNA probes were created using standard PCR for both promoter sequences. Each binding reaction was comprised of 50 mM $MgCl_2$, 2 µg poly dI-dC and 5X binding buffer (50 mM Hepes, pH 7.9, 250 mM KCl, 5 mM EDTA, 25 mM DTT, 50% glycerol) as well as varying concentrations of LacR purified protein, biotinylated/unbiotinylated DNA probe (for either the *lacA1* or *lacA2* promoter sequence) and water up to 10 µL. The binding reactions were subsequently incubated on ice for 30 min, after which time each was loaded onto a 4% non-denaturing PAGE gel (5X TBE, 30% acrylamide, 2% bisacrylamide, 30% APS and TEMED) and run for 1 hour at 35 mA. Following electrophoresis, the DNA was transferred to a positively charged hybridization membrane via Genescreen Plus™ (Boston, MA) and UV crosslinked. The biotinylated DNA was then detected with a Chemiluminescence EMSA kit (Pierce Biotechnology, Rockford, IL) by probing with Streptavidin-Horseradish Peroxidase Conjugate and the Chemiluminescent Substrate. An autoradiograph captured the resulting image after exposure for 30 s to 2 min.

Mixed-Species Liquid Culture Competition Assay

S. gordonii DL-1 and *S. mutans* UA159 genomes were both modified by replacing the *fruA* gene in each with either Em (DL-1) or Km (UA159) resistance determinants, thus allowing for enumeration of these strains individually on selective media. Each

strain was cultured in triplicate overnight in 10 mL BHI supplemented with antibiotics. The next morning, each sample was sub-cultured into 10 mL fresh BHI in a 1:50 fold dilution. The cells were grown for 3 hours, the OD_{600} was determined and *S. gordonii* and *S. mutans* samples were mixed together in a 1:1 cell ratio (OD_{600} = 0.5) into fresh TV supplemented with either 0.5% glucose or galactose, with or without 50 mM phosphate buffer (final concentration). This was designated as t = 0 hrs. The samples were allowed to incubate for 6 hours (t = 6 hrs) at 37°C, 5% CO_2. At this time, the absorbance and pH were measured. The samples were serially diluted from 10^{-1} – 10^{-8} and plated on BHI + Em and BHI + Km. Samples were then diluted 1:50 into fresh media. Both the re-diluted (viability) and original (persistence) cultures were then incubated overnight (t = 22 hrs). At this time, both the viability and persistence cultures were measured for OD_{600} and pH. Again the samples were serially diluted and plated on BHI containing Em or Km. In the last part of the assay, the persistence culture was kept as is while the viability culture was re-diluted once more in a 1:50 fold dilution. These samples were incubated for 8 hours until t = 30 hrs (endpoint). In this last step, the samples were again serially diluted and plated on BHI containing Em or Km. All plates were incubated for 2 days in the anaerobic chamber at 37°C, 5% CO_2 and then CFUs were counted for dilution plates that contained between 30-300 colonies.

Table 2-1. Bacterial strains used in this study.

Strain	Phenotype or description	Reference or source
E. coli strains:		
DH10B	General cloning strain	Invitrogen
M15[pREP4]	General purpose protein expression host	Qiagen
LacR	M15[pREP4]/pQE-30 expressing LacR	This study
S. gordonii strains:		
DL1	Wild-type host	ATCC 49818
ΔEII^{Lac}	*EII*^{Lac}::Em	This study
ΔlacG-Em	*lacG*::Em	This study
ΔlacG-Km	*lacG*::Km	This study
ΔlacA1B1	*lacA1B1*::Km	This study
lacT(M1stop)	*lacT*::Km point mutation at amino acid Met1	This study
ΔEII^{Gal}	*EII*^{Gal}::Em	This study
ΔlacR-Em	*lacR*::Em	This study
ΔlacR-Km	*lacR*::Km	This study
ΔlacA2B1	*lacA2B1*::Em	This study
ΔlacA1B1-A2B2	*lacA1B1-A2B2*::Km-Em	This study
WT::P_{lacA1}-*cat*	*S. gordonii* P_{lacA1} integrated into gtfG locus of DL1	This study
lacT(M1stop)::P_{lacA1}-*cat*	*S. gordonii* P_{lacA1} integrated into gtfG locus of *lacT*(M1stop)	This study
ΔlacR::P_{lacA1}-*cat*	*S. gordonii* P_{lacA1} integrated into gtfG locus of *ΔlacR*	This study
ΔlacG::P_{lacA1}-*cat*	*S. gordonii* P_{lacA1} integrated into gtfG locus of *ΔlacG*	This study
WT::P_{lacA2}-*cat*	*S. gordonii* P_{lacA2} integrated into gtfG locus of DL1	This study
lacT(M1stop)::P_{lacA2}-*cat*	*S. gordonii* P_{lacA2} integrated into gtfG locus of *lacT*(M1stop)	This study
ΔlacR::P_{lacA2}-*cat*	*S. gordonii* P_{lacA2} integrated into gtfG locus of *ΔlacR*	This study
ΔlacRT::P_{lacA2}-*cat*	*S. gordonii* P_{lacA2} integrated into gtfG locus of *ΔlacRT*	This study
ΔlacG::P_{lacA2}-*cat*	*S. gordonii* P_{lacA2} integrated into gtfG locus of DL1	This study
WT::*cre*-P_{lacA2}-*cat*	*S. gordonii cre*-P_{lacA2} integrated into gtfG locus of DL1	This study
ΔlacR::*cre*-P_{lacA2}-*cat*	*S. gordonii cre*-P_{lacA2} integrated into gtfG locus of *ΔlacR*	This study
ΔlacG::*cre*-P_{lacA2}-*cat*	*S. gordonii cre*-P_{lacA2} integrated into gtfG locus of *ΔlacG*	This study
ΔfruA	*fruA*::Em	Tong *et al.* (2011)
S. mutans strains:		
UA159	Wild-type host	ATCC 700610
ΔfruA	*fruA*::Km	This study

Table 2-2. Primers used in this study.

Primer	Sequence	Application
EIIGal-1	5'-GGAACAAGGTGCAGATGCTGTTAAGTT-3'	Inactivation of EII^{Gal}
EIIGal-2HIII	5'-CTTGATTATAAGCTTGTAGACAGAAGACCAA-3'	Inactivation of EII^{Gal}
EIIGal-3	5'-CCAAGGAGACACCAGCTGTTAAACCAA-3'	Inactivation of EII^{Gal}
*lacE*1	5'-GAGAAGATTTCTCGGAATATTTATCTCC-3'	Inactivation of *lacE*
*lacE*2	5'-GCTGCATAGTCCACAAAGTGCTCAATA-3'	Inactivation of *lacE*
lacG-1	5'-GACTTTGCGGCTCTAGAAGCAGCTA-3'	Inactivation of *lacG*
lacG-2	5'-GGGTGCGCGCCTATGAAATAAGG-3'	Inactivation of *lacG*
lacG-3BamHI	5'-GAAACGCAGGATCCCTATCCAAAGAAA-3'	Inactivation of *lacG*
lacA1-1	5'-GGTAACGCAACAACCGGTGATA-3'	Inactivation of *lacA1*
lacA1-2EcoRI	5'-GCTCCAATAAGAATTCCCATGATCATTCTC-3'	Inactivation of *lacA1*
lacA1-3EcoRI	5'-CGTGATATGAATTCAGCTCTCTATGCT-3'	Inactivation of *lacA1*
lacA1-4	5'-CTTCCCGAGATACTAACTACCTCA-3'	Inactivation of *lacA1*
lacA2-1	5'-GGTCCTGGAACATCGGTCGAA-3'	Inactivation of *lacA2*
lacA2-2XbaI	5'-TCCAGCAGCATCTAGACCTATAATAATTG-3'	Inactivation of *lacA2*
lacA2-3	5'-CATCAAGCAGATGCAAACTTCTTTAC-3'	Inactivation of *lacA2*
lacA2-4	5'-GGAATATCTACCTTGTAGAAAGTATCAC-3'	Inactivation of *lacA2*
lacR-1	5'-CCGCCAAGGATCCTCTCATAACGA-3'	Inactivation of *lacR*
lacR-2BamHI	5'-GTTCCAGCTGGATCCACAAATTTAAGAA-3'	Inactivation of *lacR*
lacR-3SphI	5'-GCTTATACTCAGAAGCATGCCTTAGAAC-3'	Inactivation of *lacR*
lacR-3BamHI	5'-GCTTATACTCAGAAGGATCCCTTAGA-3'	Inactivation of *lacR*
lacR-4	5'-CGGACCAGCCCCATAAGCAT-3'	Inactivation of *lacR*
SGO1515-5'	5'-GCACCAAGCGCCTGCCAGACTGTCT-3'	*lacT* Point Mutation
SGO1515-M8stop-5'	5'-CGAATCATACATCCTTAGAACAACAATGTTGCTC-3'	*lacT* Point Mutation
SGO1515-M8stop-3'	5'-GAGCAACATTGTTGTTCTAAGGATGTATGATTCG	*lacT* Point Mutation
SGO1515-M8stop-3'MAMA	5'-GTTTGGCTAGAGCAACATTGTTGTACA-3'	*lacT* Point Mutation
SGO1515-3'	5'-TCGCCTGCCCAAGTGATTTTGC-3'	*lacT* Point Mutation
PlacA1-5'KpnI	5'-ATTGTCCCGGTACCATTTCTGTTTATCAGA-3'	P_{lacA1} Amplification
PlacA1-3'BamHI	5'-CCAATAATAATTGCCATGGATCCTCTCCTTTGTT-3'	P_{lacA1} Amplification; lacA1 and lacA2 Probes
PlacA2-5'SacI	5'-GGCAAGGAGGAGCTCACGGCTATCT-3'	P_{lacA2} Amplification
PlacA2-3'BamHI	5'-CCTATAATAATTGCCATGGATCCTCTCCTTTGTT-3'	P_{lacA2} and *cre*-P_{lacA2} Amplification
PlacA2-5'KpnI	5'-GGCAAGGAGGGTACCACGGCTATCT-3'	*cre*-P_{lacA2} Amplification
PlacA2-MCRE-3'	5'-CAGAAAGAATTATAGTAAGATACGTTCTTTTAATATATTAA-3'	*cre*-P_{lacA2} Amplification
PlacA2-MCRE-5'	5'-TTAATATATTAAAAGAACGTATCTTACTATAATTCTTTCTG-3'	*cre*-P_{lacA2} Amplification

Table 2-2. Continued

Primer	Sequence	Application
lacR-5'BamHI	5'-GCTAGAGAGGAGGATCCATGGGTAAGAATCA-3'	LacR Expression
lacR-3'SalI	5'-GCCCTTTAAAGAGCTGTCGACTAACTGTTTAT-3'	LacR Expression
PlacA1-5'Biotin	5'-/5Biosg/ATTGTCCCCCTCCCATTTCTGTTTATCAGA-3'	*lacA1* Probe (Hot)
PlacA1-5'	5'-ATTGTCCCCCTCCCATTTCTGTTTATCAGA-3'	*lacA1* Probe (Cold)
PlacA2-5'Biotin	5'-/5Biosg/GGCAAGGAGGATTTCACGGCTATCT-3'	*lacA2* Probe (Hot)
PlacA2-5'	5'-GGCAAGGAGGATTTCACGGCTATCT-3'	*lacA2* Probe (Cold)
lacG-S	5'-GACAGGCTATGGAGAGGTCAATC-3'	RT-PCR
lacG-AS	5'-TGGTGTATCAAAGTGGTGAAGGG-3'	RT-PCR
SGO1520-S	5'-GATAACAACGGAGTAAGCCAAGG-3'	RT-PCR
SGO1520-AS	5'-TTGGAGCATTTAGGAGGTCGTC-3'	RT-PCR
lacR-S	5'-TGGAACTGTAACGGTTGCTGAG-3'	RT-PCR
lacR-AS	5'-CTCTTCGCCCCACCAAATACTC-3'	RT-PCR
SGO1515-S	5'-TCTGGACCGAAGCCGTGATG-3'	RT-PCR
SGO1515-AS	5'-CCGTCTTCTGGGCAATGATGTG-3'	RT-PCR
lacA1-S	5'-GGTCAGGATTTTGTTGATGTGACCC-3'	RT-PCR
lacA1-AS	GGACCAGCCCCATAAGCATCGAT-3'	RT-PCR
lacA2-S	5'-GGTGCAGATGCTGCTGGAAAT-3'	RT-PCR
lacA2-AS	CACCTCAGCTGCAACTGCCAAT-3'	RT-PCR

CHAPTER 3
CHARACTERIZATION OF CONTIGUOUS LACTOSE/GALACTOSE OPERONS IN S. GORDONII DL-1 CHALLIS

Introduction

Unpublished data from Zeng and coworkers in the Burne laboratory indicated that *S. gordonii* is able to grow well on galactose-containing medium at a 10-fold lower concentration than *S. mutans*. Upon examining the *S. gordonii* genome, two tandem *lac* gene clusters were found, both containing a set of tagatose genes (*lacABCD*) as well as a PTS specific for either galactose or lactose. The redundant nature of these two gene clusters is unlike the organization of the *lac* genes in the *S. mutans* genome, which only contains one set of tagatose genes and a lactose-specific PTS. Of most importance, *S. mutans* lacks the transcriptional antiterminator and galactose-specific transporters found in *S. gordonii*. Thus, *S. gordonii* appears to have a selective advantage over the caries-producing pathogen. Based on the growth data and the major differences in genomic structure between the two species, it is conceivable that this advantage may be due to the specific genes that *S. gordonii* harbors dedicated to a high-affinity galactose transport system. On the other hand, *S. mutans* seems to have either failed to acquire this transport mechanism or lost it through evolution. Therefore, since *S. gordonii* is more sensitive to galactose consumption (103), this species could potentially compete successfully against the pathogen in an environment where galactose is present in small quantities. This aim was dedicated to characterizing the two gene clusters in *S. gordonii* as a comparison against other species and for further use in later aims.

In order to understand more about how *S. gordonii* utilizes galactose and lactose, a bioinformatics analysis was necessary to understand and compare homologous genes from other species. Growth assessments were important for appointing a specific

phenotype to each gene. Finally, further evidence supporting the growth data was obtained through Real-Time RT-PCR, helping to decipher which gene cluster was inducible by which sugar.

Results

Bioinformatics Review of the Lactose and Galactose Gene Clusters

An NCBI nucleotide BLAST search comparing the genome of *S. gordonii* strain DL-1 Challis to *S. mutans* strain UA159 in the online database Oralgen (http://oralgen.lanl.gov) resulted in the finding that *S. gordonii* contains two sets of genes dedicated to the tagatose-6-phosphate pathway, whereas the *S. mutans* genome only contains one set. Although both species have a lactose-specific transporter contained in the *lac* operon, *S. gordonii* also possess a second transport system designated as a putative galactose-specific PTS component. The first set of tagatose genes found in *S. gordonii* appears to be homologous (between 66-77% identity according to an NCBI BLAST search) to those in *S. mutans*; however, a second set is situated adjacent to the first set, just upstream of the galactose transporter genes (SGO1520-SGO1522; $EIIABC^{Gal}$). Interestingly, the galactose transporter genes are completely absent in the *S. mutans* UA159 genome. Based on an Oralgen database search, *S. pyogenes* also contains genes annotated for a galactose-specific PTS along with a paralogous set of the tagatose genes, excluding *lacC*. *LacD.1* of *S. pyogenes*, which is contained in the second set of tagatose genes, plays an important role as a global regulator of virulence factor expression rather than the traditional role in the tagatose-6-phosphate pathway (62). In addition, *L. casei* contains a galactose-specific PTS (24) and an NCBI Gene search suggests *S. pneumoniae* also has a potential galactose-specific PTS.

A transcriptional antiterminator designated by our lab as *lacT* is present in the *S. gordonii* DL1 genome, but is noticeably absent from the *S. mutans* UA159 genome. This transcriptional antiterminator belongs to the BglG/SacY family of proteins associated with regulation of sugar metabolism (34). It contains a Co-AntiTerminator (CAT) RNA-binding domain located at the amino terminus that facilitates binding to the ribonucleic antiterminator (RAT) sequence of nascent mRNA transcripts. This binding stabilizes the RAT structure and effectively prevents formation of a termination structure, allowing RNA Polymerase to continue transcription of the genes downstream (34). Two PTS regulatory domains (PRD) of approximately 100-aa in length constitute most of the remaining part of the gene. Each domain contains a highly conserved histidine residue at position 7 that serves as the phosphorylation site for the PRDs (92). The site is phosphorylated when there is no sugar entering the cell (9, 45). This phosphorylation inactivates *lacT* and allows termination of transcription to occur since the genetic machinery needed to metabolize the sugar is not required. Upon transport of sugar across the cell membrane, most of the phosphate in the cytosol is used by the general components of the PTS (EI and HPr) to phosphorylate the incoming substrate (29). This leaves relatively little phosphate left over for binding to histidine 7 in the PRDs of *lacT*, causing dephosphorylation. Thus, *lacT* is activated and the antiterminator protein attaches to mRNA to prevent termination of transcription. Software prediction (Softberry - BPROM) evaluating the intergenic region located just upstream of *lacT* suggested a potential promoter site (Table 3-1). A *cre* sequence has also been found upstream of *lacT*, suggesting that *lacT* is regulated by another protein. *L. casei* has a *lacT* similar to *S. gordonii*. It has been reported that in *L. casei, lacT* is transcribed along with *lacEFG*

as one single mRNA of 4.4 kb (8). Because the *S. gordonii lacTEFG* genes are similar in size to *L. casei* and *lacT* has a potential promoter with a *cre* sequence, *lacT* may have a comparable function.

A transcriptional regulator, LacR, located upstream of the second set of tagatose genes is predicted by Oralgen to have a helix-turn-helix domain of about 50-60 amino acids. This domain is located in the amino terminal region of the protein and binds DNA for effective repression of the lactose operon. The carboxy terminal end may contain either an effector-binding domain or an oligomerization domain. Effector molecules for this type of regulator are often phosphorylated and act as intermediates in the appropriate metabolic pathway. This specific gene has homology to the *deoR* gene sequence of *Escherichia coli*, which functions as a transcriptional repressor of the *deo* operon (72, 79). The LacR protein is present in multiple genera, including *Streptococcus*, *Staphylococcus* and *Bacillus* and often participates in the regulation of sugar catabolism. A protein alignment of LacR located in the *S. mutans* UA159 genome revealed 59% identity to LacR of *S. gordonii*. A BLAST search indicated that *S. sanguinis* strain SK49 has a protein that most closely relates to *S. gordonii* LacR, with 86% identity. The exact mechanism of regulation by LacR has not been elucidated for Gram-positive bacteria as of yet. Software prediction also revealed a potential promoter site in the region just upstream of *lacR* (Table 3-1).

Comparison of the two isomerases, LacA1 and LacA2, in an NCBI protein BLAST search disclosed that the two proteins are 97% identical. The species with the closest homology to LacA1 was *S. sanguinis* strain SK36 with an identity of 100%. Softberry –

BPROM also predicted potential promoter regions in front of both *lacA1* and *lacA2* (Table 3-1).

A few other streptococci, lactococci and lactobacilli retain various combinations of these genes as well (17, 24, 69, 78, 79). As mentioned earlier, the *S. pyogenes* genome most closely resembles the one found in *S. gordonii*, with two sets of the tagatose genes (excluding *lacC.1*), a lactose PTS, a separate galactose PTS and a BglG family transcriptional antiterminator (78). In contrast, the *S. mutans* genome contains only one operon involving the lactose PTS, the tagatose genes and regulatory component LacR (79). Also found through searches in the Oralgen and NCBI databases, the following species have similar transport systems as well: *Lactococcus lactis*, *Lactobacillus casei*, *S. sanguinis*, *S. mitis*, *S. pneumoniae* and *Staphylococcus aureus* (Table 3-2). *S. gordonii* is a good model organism for studying lactose and galactose utilization because of the similarity between its *lac* genes and those of other important human pathogens such as *S. pyogenes* and *S. pneumoniae* and its ease of genetic manipulation.

Growth Phenotype of the *S. gordonii* Lactose and Galactose Gene Clusters

Various mutant strains were created by deleting genes of interest from the genome and replacing each with an antibiotic resistance cassette via allelic exchange. All strains were grown in TV supplemented with 0.5% of the appropriate sugar and a mineral oil overlay to reduce oxygen tension. Incubation in a Bioscreen-C reader allowed for measurement of the optical density (OD_{600}) every 30 min, which was used to calculate doubling times (Table 3-3).

TV – 0.5% glucose

When grown in TV supplemented with 0.5% glucose (Figure 3-1), the wild-type (WT) *S. gordonii* strain grew to a final OD_{600} of 0.72 with a doubling time of 92 ± 6.2 min. Several mutant strains from the lactose gene cluster including Δ*EII*^{Lac}, Δ*lacA1B1* and *lacT*(M1stop) displayed a similar final optical density and growth rate as WT in glucose. In contrast, the Δ*lacG* mutant exhibited a much longer lag phase of about 12 hours, a slightly slower growth rate with a doubling time of 120 ± 15 min and a lower final yield of 0.53. It is normally characteristic of streptococcal growth curves to depict a slight dip in optical density just after reaching peak growth. This phenomenon is due to cell lysis, as described in experiments performed on *S. mutans* (5). However, the Δ*lacG* mutant experienced cell lysis more extensively than all of the other strains.

Results for deletion mutants from the galactose gene cluster (Figure 3-2) indicate that Δ*EII*^{Gal} *and* Δ*lacA2B2* both displayed similar growth rates and final yields to WT. A slightly slower growth rate was observed in Δ*lacR* with a doubling time of 120 ± 5.2 min. The double mutant, Δ*lacA1B1-A2B2*, grew identically to the WT (Figure 3-3).

TV – 0.5% galactose

When grown in TV supplemented with 0.5% galactose (Figure 3-4), the WT strain grew to a final OD_{600} of 0.91 with a doubling time of 190 ± 2.2 min and a lag phase of about 5 hours. Most of the deletion mutants from the lactose cluster showed signs of defective growth on galactose. The lactose-specific transporter mutant strain, Δ*EII*^{Lac}, grew to a similar yield as WT with an OD_{600} of 0.89, however, the lag phase lasted about 16 hours (vs. a lag of 5 hours in WT) and the doubling time was slower at 260 ± 7.4 min. Surprisingly, the Δ*lacG* strain did not grow in galactose. The Δ*LacA1B1* strain also had a lag phase of about 15 hours, a slightly decreased growth rate with a doubling

time of 250 ± 22 min and a yield of 0.84. The *lacT*(M1stop) strain grew slower than WT with a doubling time of 220 ± 16 min and had a comparable final yield.

Growth results for the galactose cluster deletion mutants were not as straightforward as expected (Figure 3-5). Analysis of the Δ*EII*^{Gal} strain grown in galactose was inconsistent. Final yield was always similar to WT, but the growth rate and lag phase sometimes varied between replicates. With a calculated doubling time of 170 ± 4.2 min, this mutant appears to grow faster than WT; however, in certain instances the strain actually grew slower than WT and had a lag phase of about 25 hours. It is recommended that additional experiments be completed before fully assessing the impact of this mutation on the growth of *S. gordonii* in a galactose-containing medium. The *ΔLacR* strain displayed a comparable growth rate to WT with a doubling time of 200 ± 18 min. When *lacA2B2* was deleted, the growth rate increased with a doubling time of 180 ± 19 min; however, final yield was the same as WT. When the double deletion mutant Δ*lacA1B1-A2B2* was grown on galactose, no growth was observed (Figure 3-6).

TV – 0.5% lactose

When grown in TV supplemented with 0.5% lactose (Figure 3-7), the WT strain grew to a final OD_{600} of 0.69 with a doubling time of 140 ± 32 min and a lag phase of about 4 hours. As expected, all of the mutant strains within the lactose gene cluster were severely defective when grown in lactose. When the lactose-specific transporter was removed (Δ*EII*^{Lac}), almost no growth was observed with a doubling time of 2600 ± 720 min and a final yield of 0.37. Similarly, the Δ*lacG* strain displayed very little growth with a final optical density of 0.18. With a lag phase of 15 hours and a doubling time of 360 ± 26 min, the Δ*lacA1B1* strain reached a final yield of 0.59. The mutant lacking the

transcriptional antiterminator, *lacT*(M1stop), had the highest final yield (OD_{600} of 0.62) and a doubling time of 240 ± 36 min; however, its lag phase extended to 20 hours.

As for the galactose cluster, deletion mutants did not appear to compromise bacterial growth on lactose (Figure 3-8). In fact, all three strains (*ΔEII*^{Gal}*,* Δ*LacR and* Δ*lacA2B2*) grew more rapidly than WT in lactose. The final optical density of the *ΔEII*^{Gal}*,* Δ*LacR and* Δ*lacA2B2* strains were equal to WT. As seen in Table 3-3, the doubling time of the galactose-specific transporter mutant was 93 ± 9.8 min, the Δ*LacR* strain was 120 ± 3.3 min and the Δ*lacA2B2* strain was 100 ± 2.8 min. All of these strains experienced a lag phase of 4 hours, similar to the WT strain. Conversely, when both sets of isomerase genes were deleted (Δ*lacA1B1-A2B2*), no growth was observed with a final yield of 0.11 (Figure 3-9).

Operon-specific Gene Expression via Quantitative Real-Time RT-PCR

Real-time RT-PCR was performed to evaluate the expression of a representative gene from what we believed to be two distinct operons to allow for clarification as to which cluster was induced by which carbohydrate source and hint at possible operon arrangements. The *lacG* and *EIIC*^{Gal} genes were chosen as the representative genes based on the fact that these genes are situated at the end of each gene cluster. Therefore, if the clusters were inducible by a particular sugar, RNA Polymerase would have to pass through all of the other genes before transcribing either *lacG* or *EIIC*^{Gal}. RNA was extracted from *S. gordonii* WT cells that were grown in TV supplemented with 0.5% glucose, galactose, or lactose and converted to cDNA via RT-PCR. Using Real-Time PCR, this cDNA was used to measure the expression level of *lacG* and *EIIC*^{Gal}. Standard curves were prepared to measure copy numbers of cDNA. The data were normalized to copies/(µg of total RNA).

Figure 3-10 shows the expression of *lacG* in a WT background. When compared to growth in glucose, *lacG* expression was induced 24-fold when grown in galactose ($p=0.04$). Even higher expression was noted when the cells were grown in lactose, with a 390-fold induction compared to glucose ($p=0.2$). Figure 3-10 indicates the expression of $EIIC^{Gal}$ in a WT background. Comparing growth in glucose and galactose, $EIIC^{Gal}$ expression was induced 28-fold ($p=7.6x10^{-5}$). In a comparison of glucose- and lactose-grown cells, a 2.5-fold increase in induction occurred ($p=0.04$). All comparisons were calculated as significant based on pair-wise Student's t-tests.

Discussion

Analysis of the growth curve data shows that while the ΔEII^{Lac} strain is not affected by growth in glucose, this strain is moderately affected by growth in galactose and severely affected by growth in lactose. These conclusions suggest that the lactose PTS does not transport glucose. The extensive lag phase and severely diminished growth rate displayed when grown in lactose is a strong indication that EII^{Lac} is the primary transporter of lactose through the cell membrane. However, since some growth in lactose is achieved, the cell is must be using another mechanism to bring lactose inside, such as through another transporter or it is producing secreted enzymes that break down lactose outside the cell. Interestingly, growth in galactose was also slower in the ΔEII^{Lac} strain, along with a lag phase of about 20 hours, indicating that EII^{Lac} likely transports galactose into the cell, albeit in lower quantities than it brings in lactose.

No growth was detectable for the *lacG* mutant in either lactose or galactose. While this is understandable in the case of the former sugar, it is perplexing in the case of the latter. *LacG* is required for the catabolism of Lac-6-P into glucose and Gal-6-P; therefore, a *ΔlacG* strain would be unable to fulfill that role when grown in the presence

of lactose substrate and the cells would not be able to grow. It seems though that *lacG* should not affect growth in galactose since phospho-β-galactosidase is not required for the metabolism of Gal-6-P. The *lacG* mutant also has an extended lag phase when grown in glucose. Again, the *lacG* enzyme is not necessary for the metabolism of glucose. The findings regarding growth on glucose and galactose imply that *lacG* is more important than previously thought and it is likely responsible for providing a second, equally important and possibly regulatory, function.

Similar to the *ΔlacG* strain, the *lacA1B1* mutant displayed impaired growth on galactose and lactose with a long lag phase. The *lacA1B1* enzymes are responsible for converting Gal-6-P into tagatose-6-P in the first step of the tagatose pathway. Since both galactose and lactose are converted to Gal-6-P before entering the tagatose pathway, a deletion of *lacA1B1* would prevent Gal-6-P from being metabolized by the tagatose pathway. When this occurs, Gal-6-P begins to accumulate inside the cell leading to inhibition of growth. Zeng and co-workers have proposed that this occurs in *S. mutans*, where Gal-6-P builds up to such high levels that it actually becomes inhibitory to the cell (103). Growth is not however totally abolished since there is a second set of tagatose pathway genes capable of metabolizing the Gal-6-P, apparently at a lower rate than LacA1B1.

Since the strain containing the *lacT* mutation has a reduced growth rate and long lag phase when grown in lactose, it appears that *lacT* may enhance utilization of this sugar. We propose that when lactose is being transported that LacT would be de-phosphorylated and capable of binding to nascent mRNA to prevent termination of transcription. In a *lacT* mutant, the cell is missing this crucial step and the tagatose

genes are mostly not transcribed because termination structures in the mRNA are able to form without *lacT* there to interfere. In this scenario, Lac-6-P continues to enter the cell and is catabolized into glucose and Gal-6-P, but the Gal-6-P cannot be metabolized because the LacABCD gene products have not been synthesized. Therefore, Gal-6-P again builds up to toxic levels.

As mentioned previously, it has been shown in *L. casei* that *lacT* is transcribed as part of a polycistronic mRNA with *lacEFG*. This may also be the case in *S. gordonii* since software prediction indicates a putative promoter site in the region directly upstream of *lacT*. If this were the case, then when *lacT* is deleted, *lacEFG* would not be transcribed, thereby removing production of the transporters and phospho-β-galactosidase genes. Therefore, it is proposed that *lacT* can act to regulate transcription of the tagatose genes and autoregulate so as to control expression of the genes for the lactose transporter and phospho-β-galactosidase. This explanation is supported by studies by Fujita and co-worked which showed that each antiterminator controls the expression of a cognate EII of the PTS and that EII in turn negatively regulates its antiterminator (37). The fact that the *lacT*(M1stop) strain grew only slightly slower than WT in galactose suggests that it does not play an important role in galactose metabolism.

Deletion of the putative galactose transport and metabolism genes provided unexpected results. Although the *ΔEII*^{Gal} strain grew similarly to wild-type when in glucose medium, the same strain grew much faster than wild-type in lactose. It is therefore possible that EII^{Gal} plays a role in the negative regulation of the tagatose pathway, which is still necessary for the metabolism of lactose; thus explaining the

faster growth rate in lactose by the *ΔEII*^{Gal} strain. Absence of EII^{Gal} would then de-repress the tagatose genes, leading to increased expression. Therefore, the cell would have an enhanced capacity for metabolizing lactose. In addition, extracellular β-galactosidases may be cleaving lactose into glucose and galactose before it enters the cell. This released galactose can then be taken up by the lactose- (and possibly mannose-) PTS and metabolized via the tagatose-6-phosphate pathway, thus metabolizing more galactose than a wild-type cell. Growth results were often inconsistent when the *ΔEII*^{Gal} strain was grown in galactose. More often than not the growth rate of the *EII*^{Gal} mutant was slightly faster than WT. However, at times the *EII*^{Gal} strain displayed a slower growth rate with a lag phase of about 25 hours compared to WT. The extreme difference in growth often occurred within replicates of the same sample. If the galactose transporter were specialized for high-affinity transport, then the cell would express the *EII*^{Gal} gene the most under conditions where there are only small concentrations of galactose in the environment, such as may occur in saliva. Therefore, if the cell is bombarded with high quantities of galactose, the lactose transporters are utilized instead. Since 0.5% galactose is considered a much higher concentration than the physiological levels found inside the oral cavity, this may account for the inconsistency in growth rates and lag phase. In regards to the slightly faster grow rate displayed by the *ΔEII*^{Gal} strain in galactose, it is possible that the cell sensed removal of the galactose transporter. In effect, the cell attempted to over-compensate for the loss of the galactose PTS by transcribing more copies of the *EII*^{Lac} (and possibly *EII*^{Man}) genes so that galactose could continue to be brought in through other transporters. The absence of *EII*^{Gal} may not have affected growth rate significantly because the galactose

transporter is a high-affinity system. In effect, the cell may not express the galactose PTS if the concentration of galactose in the environment is considered too high. In this scenario, both the lactose- and mannose- PTS would likely transport the galactose inside the cell.

The *lacR* mutant grew slower in glucose compared to WT. The *ΔlacR* strain also presented a clumping phenotype and tended to aggregate when grown in BHI, which is a medium that contains glucose. Further investigation revealed that the cells lacking LacR grow in much longer chains than WT (about 15-20 cells per chain compared to 2-5 in WT). Under stress-inducing conditions, *S. mutans* forms long chains and aggregates in culture, resulting from impairment of cell septation and/or autolysis (58). In addition, a *lacR* deficient strain of *S. mutans* grown in glucose was observed to have lower glucose PTS activity compared to the parental strain (3). Therefore, LacR may be involved in other processes in the cell, such as in the regulation of the glucose PTS. When grown in galactose, the *ΔlacR* strain and WT exhibited similar growth rates. Since the role of LacR is to repress the genes in the tagatose pathway, removal of this protein would result in constitutive expression of *lacABCD*. Therefore, when the *ΔlacR* strain is grown in galactose, one would expect an increased growth rate since the tagatose pathway genes are constantly being expressed and available to metabolize more Gal-6-P than a WT cell. Because the growth rate of the *lacR* mutant is not significantly affected in galactose, it seems likely that there is another layer of regulation not yet accounted for. Based on the *ΔlacR* strain growth data in galactose, the tagatose genes seem to be expressed at similar levels to the WT. Therefore, even with LacR removed, *lacABCD* expression could still be under the control of another regulatory protein.

The *ΔlacA2B2* strain displayed a faster growth rate in glucose compared to WT. As with the *lacR* deficient strain, *lacA2B2* may also serve a function relating to the regulation of the glucose PTS. The *LacA2B2* mutant also displayed a faster growth rate in galactose and lactose. If *lacA2B2* were absent, one would expect growth rate to be slower since one of the tagatose pathways has been shut off. However, similar to the Δ*EII*^{Gal} *strain*, the loss of *lacA2B2* may be sensed by the cell. To compensate for the loss of one of the tagatose pathways, the cell relays a message to increase expression of the genes in the other tagatose pathway. This way, the incoming sugar does not build up inside the cell as Gal-6-P and inhibit growth.

The double mutant *lacA1B1-A2B2* strain did not grow at all in either galactose or lactose, likely because both sets of these genes are important for metabolizing Gal-6-P. It is also clear from these data that the tagatose pathways are the only way in which either galactose or lactose is metabolized since no growth was supported.

Data generated by Real-Time PCR on *lacG* gene expression confirmed that the phospho-β-galactosidase enzyme is induced in lactose (390-fold induction), a result that was expected since the cell requires lactose to be broken down into glucose and Gal-6-P before it can be metabolized. Without the LacG enzyme, the incoming lactose never gets processed. At first glance, it was difficult to understand why there was a significant induction in *lacG* expression under galactose conditions (24-fold induction) since this enzyme is not required for galactose metabolism. However, once it was combined with the growth data, it seemed likely that *lacG* might have another role in activation of gene expression in addition to its enzymatic activity. In *S. mutans*, it has been shown that *lacG* must be catalytically active for optimal *lac* gene expression. It has been proposed

that *lacG* converts Gal-6-P into another compound that functions as the inducer for the expression of the *lac* genes (103). It is likely that *lacG* serves a similar purpose in *S. gordonii* by converting Gal-6-P into some other intermediate that acts as an inducer for LacR, thereby de-repressing transcription of the tagatose pathway genes.

Transcription of the galactose transporter gene is significantly higher (28-fold induction) in cells growing in galactose as compared to glucose, which supports a role for EII^{Gal} as the galactose transporter. The 2.5-fold induction of EII^{Gal} gene expression when grown in lactose was also statistically significant compared to glucose. One suggestion for why EII^{Gal} is transcribed when grown in lactose may be that the extracellular β-galactosidases mentioned earlier cleave lactose into glucose and galactose outside of the cell, providing an inducing signal.

Table 3-1. Potential promoter sites involved in the lac operon of *S. gordonii* based on software prediction (Softberry – BPROM).

	Sequence (-10 region)	Sequence (-35 region)
lacT	TGCTATAAT	TTTACA
lacR	GGATAAAAT	TTGAA
lacA1	GATTAAAAT	TTTTCA
lacA2	TAGTATATT	TTGACA

Table 3-2. A list of the genes involved in the lactose and galactose PTS and tagatose-6-phosphate pathway in related species.

	Lactose PTS	Galactose PTS	*lacABCD*	*lacG*	*lacR*	*lacT*
Streptococcus mutans	✓	×	✓	✓	✓	×
Streptococcus sanguinis	✓	×	✓	✓	✓	✓
Streptococcus mitis	✓	×	✓ (putative *lacA* only)	✓	✓	✓
Streptococcus pyogenes (contains 2 sets)	✓	✓	✓ (2)	✓	✓ (2)	✓
Staphylococcus aureus	✓	×	✓	✓	✓	×
Lactococcus lactis	✓	×	✓	✓	✓	×
Lactobacillus casei	✓ (plasmid)	✓ (chromosome)	? (putatively on chromosome)	✓ (plasmid)	?	✓ (plasmid)
Streptococcus pneumoniae	✓	✓	✓	✓ (2)	✓	✓

✓ is defined as present in the genome.
× is defined as not present in the genome.

Table 3-3. Calculated doubling times of the wild-type strain and mutated *lac* gene strains of *S. gordonii* based on growth curve analysis.

Strain	Glucose T_d	Glucose OD_{600}	Galactose T_d	Galactose OD_{600}	Lactose T_d	Lactose OD_{600}
Wild-type	92 ± 6.2	0.72	190 ± 2.2	0.91	140 ± 32	0.69
ΔEII^{Lac}	110 ± 2.9	0.72	260 ± 7.4 (16 h lag)	0.89	2600± 720	0.37
ΔlacG	120 ± 15 (12 h lag)	0.53	∞	0.08	∞	0.18
ΔlacA1B1	96 ± 2.3	0.76	250 ± 22 (15 h lag)	0.84	360 ± 26 (15 h lag)	0.59
lacT(M1stop)	88 ± 8.3	0.70	220 ± 16	0.93	240 ± 36 (20 h lag)	0.62
ΔEII^{Gal}	88 ± 3.3	0.79	170 ± 4.2	0.90	93 ± 9.8	0.71
ΔlacR	120 ± 5.2	0.71	200 ± 18 (12 h lag)	0.91	120 ± 3.3	0.69
ΔlacA2B2	78 ± 3.2	0.78	180 ± 19	0.93	100 ± 2.8	0.73
ΔlacA1B1-A2B2	91 ± 2.4	0.78	∞	0.10	∞	0.11

T_d is defined as the doubling time.
OD_{600} is defined as the optical density measured at a wavelength of 600 nm in a spectrophotometer.
∞ is denoted when a strain displayed minimal growth and the doubling time was too large for calculation.

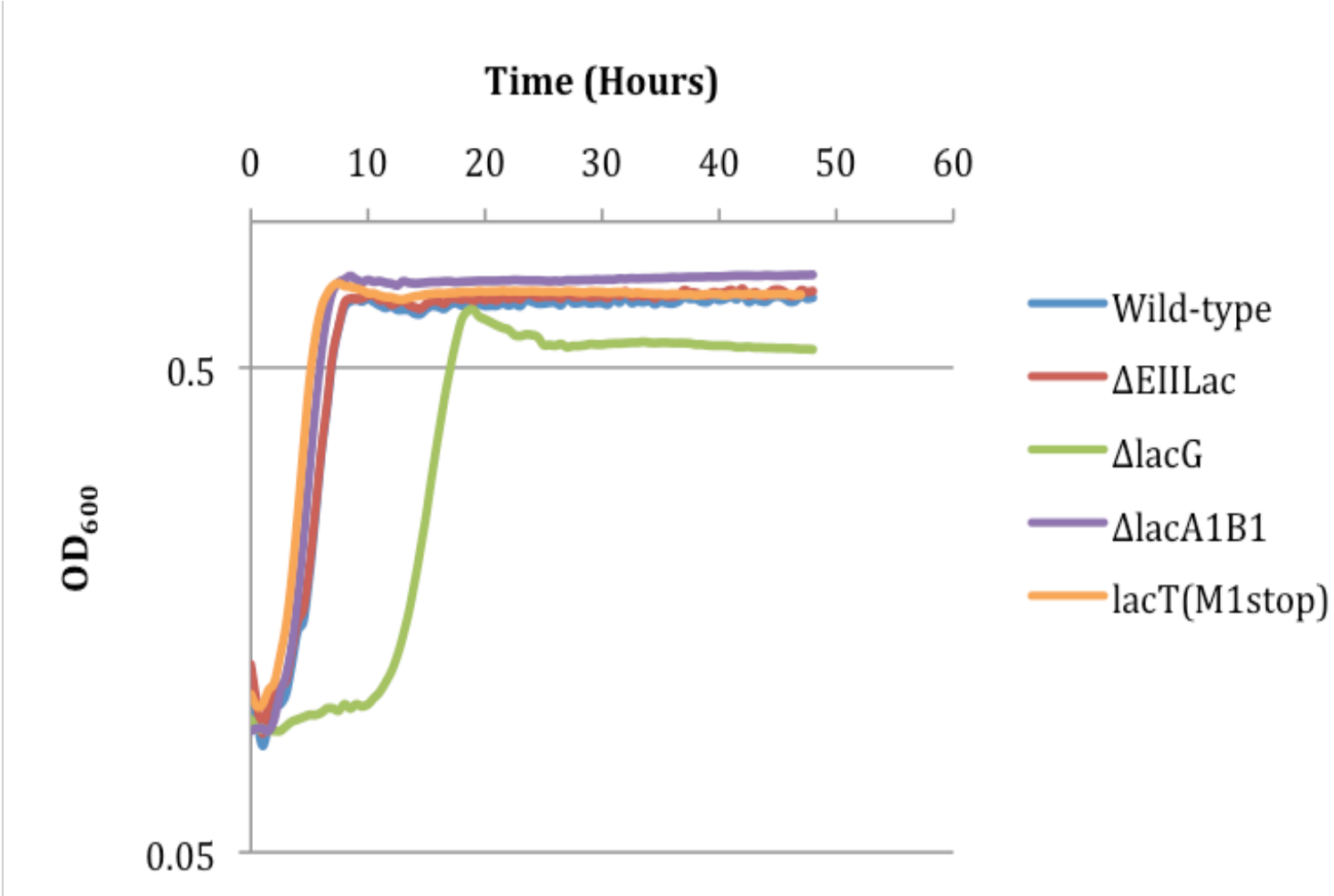


Figure 3-1. Growth of *S. gordonii* DL1 wild-type, *ΔEII*^{Lac}, *ΔlacG*, *ΔlacA1B1* and *lacT*(M1stop) strains in TV – 0.5% Glucose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

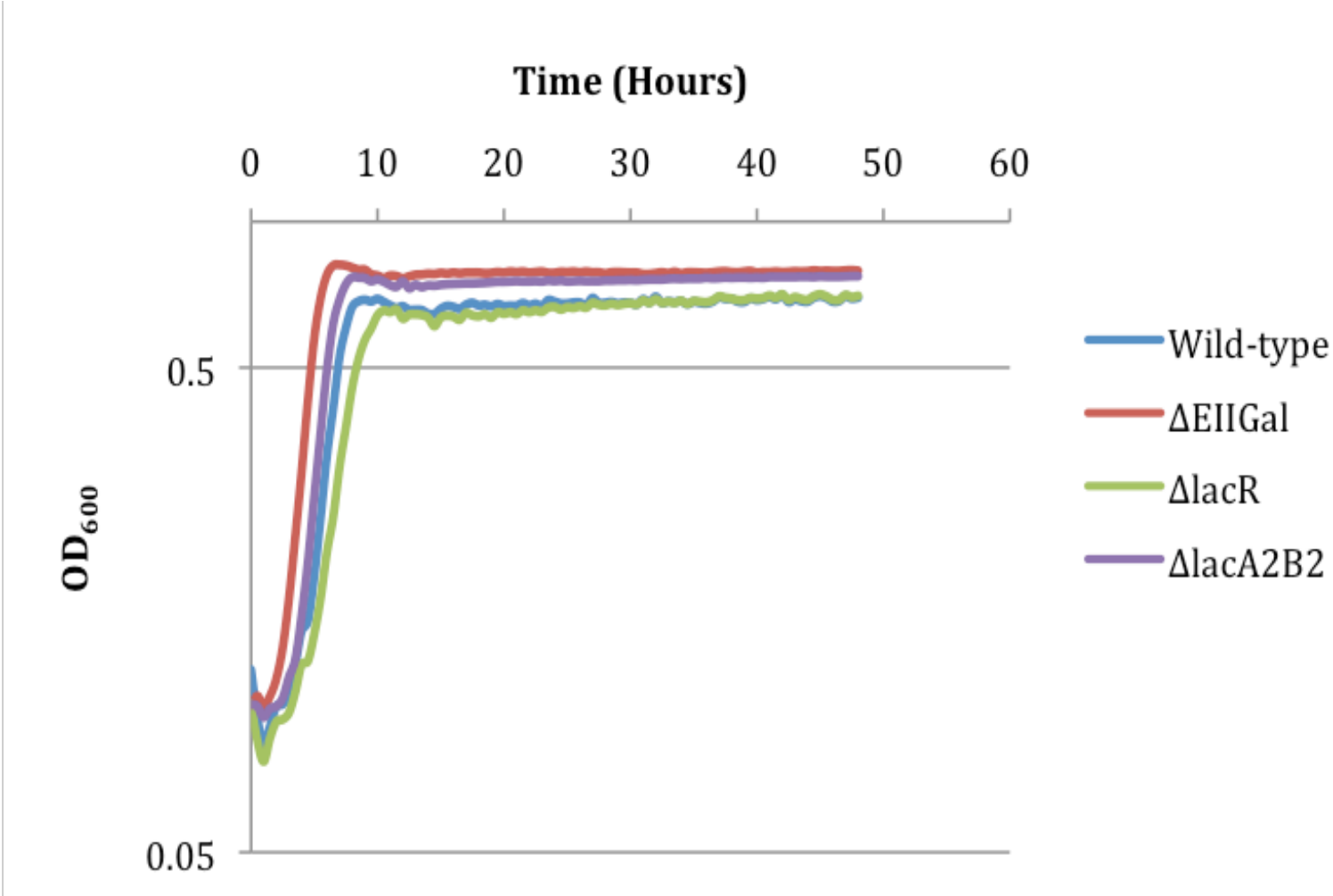


Figure 3-2. Growth of *S. gordonii* DL1 wild-type, *ΔEII*^{Gal}, *ΔlacR* and *ΔlacA2B2* strains in TV – 0.5% Glucose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

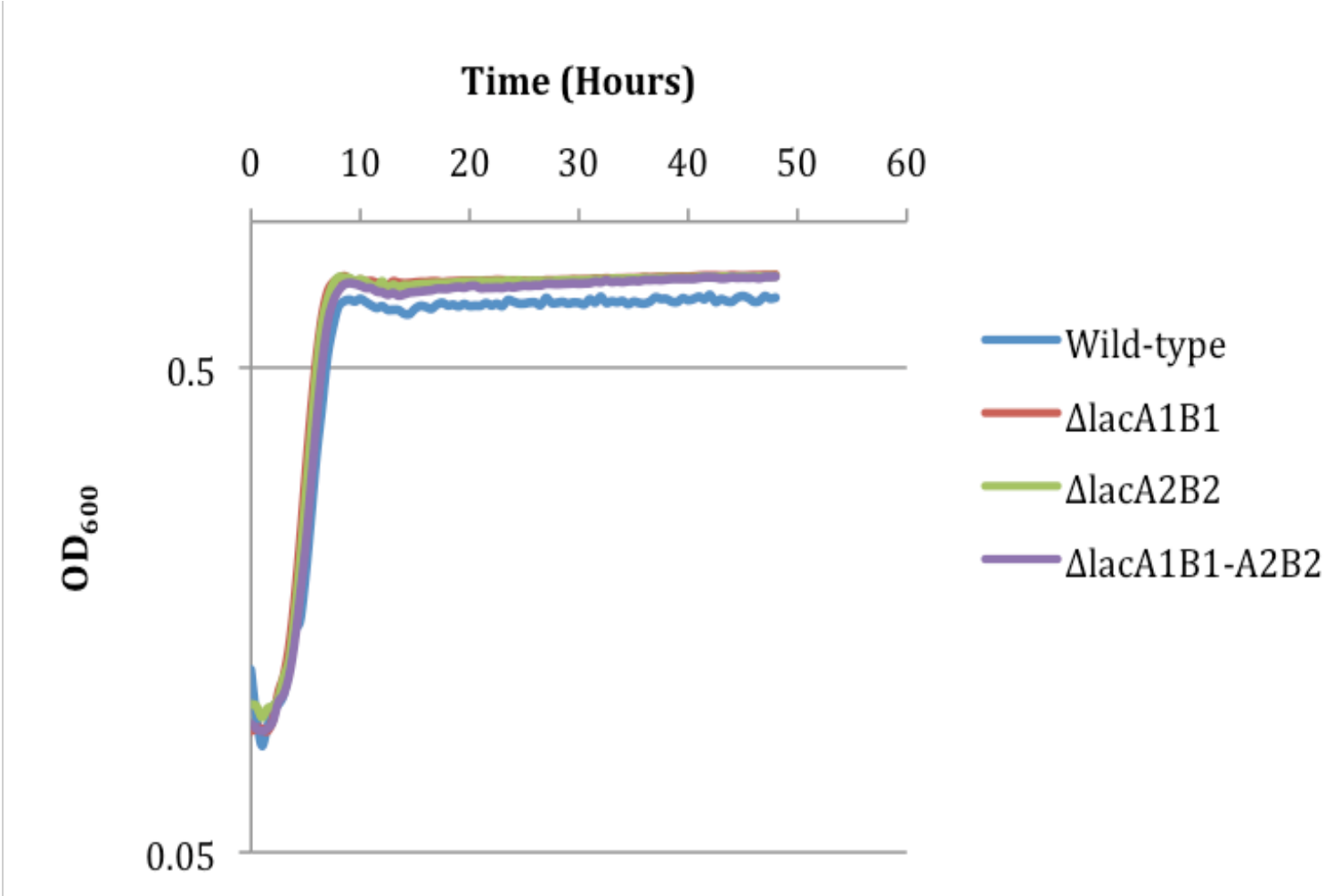


Figure 3-3. Growth of *S. gordonii* DL1 wild-type, *ΔlacA1B1*, *ΔlacA2B2* and *ΔlacA1B1-A2B2* strains in TV – 0.5% Glucose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

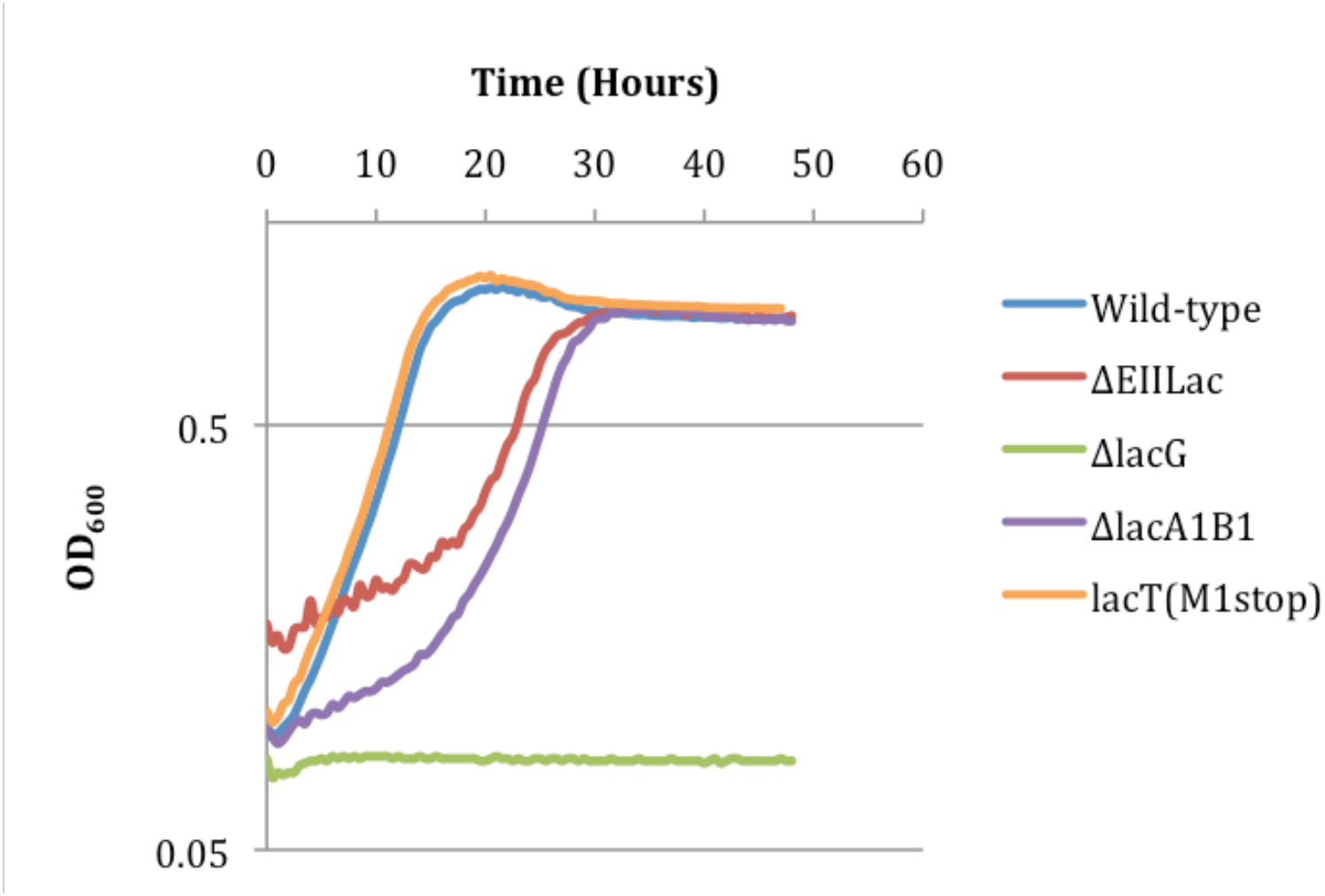


Figure 3-4. Growth of *S. gordonii* DL1 wild-type, *ΔEII*^{Lac}, *ΔlacG*, *ΔlacA1B1* and *lacT*(M1stop) strains in TV – 0.5% Galactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

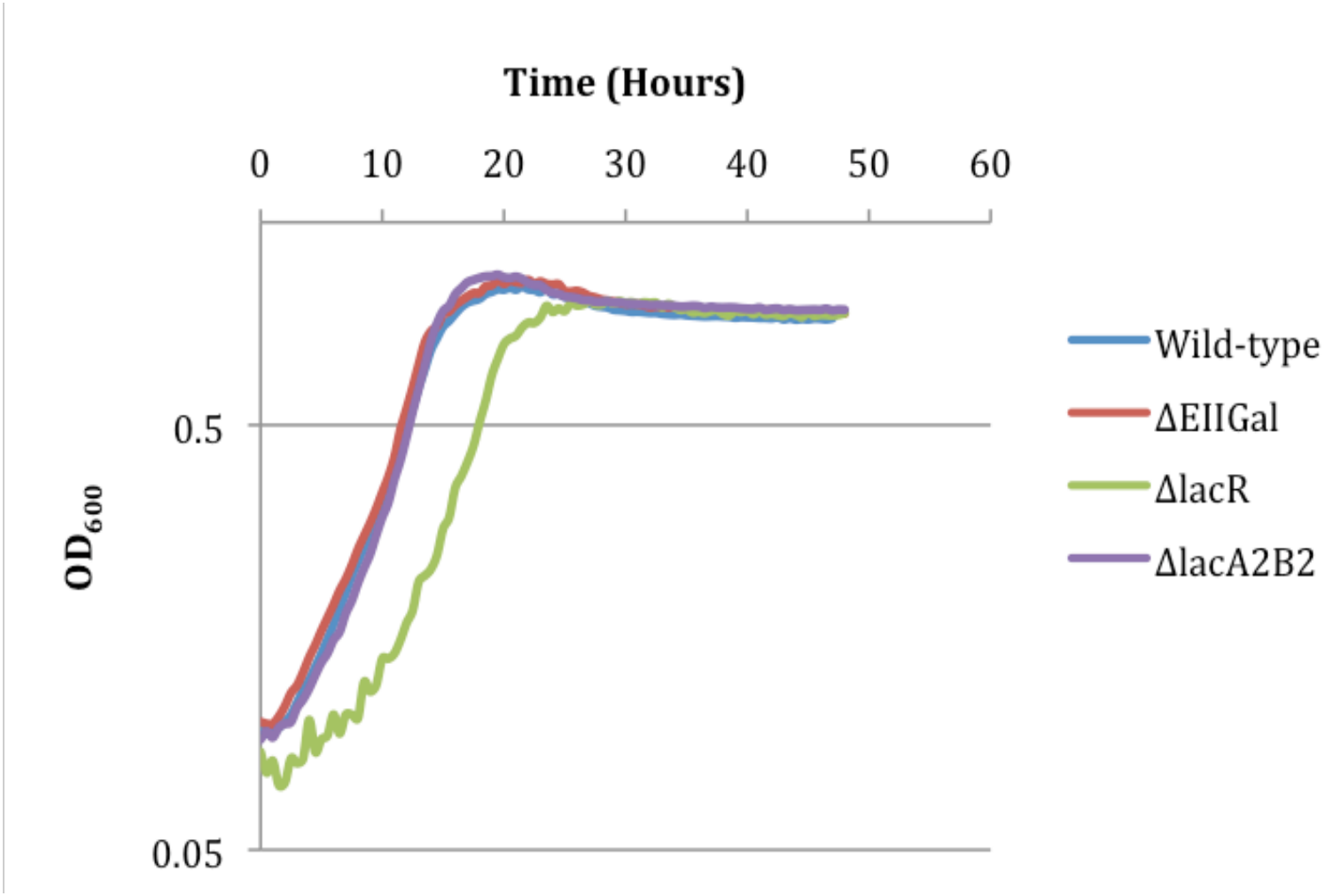


Figure 3-5. Growth of *S. gordonii* DL1 wild-type, *ΔEII*^{Gal}, *ΔlacR* and *ΔlacA2B2* strains in TV – 0.5% Galactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

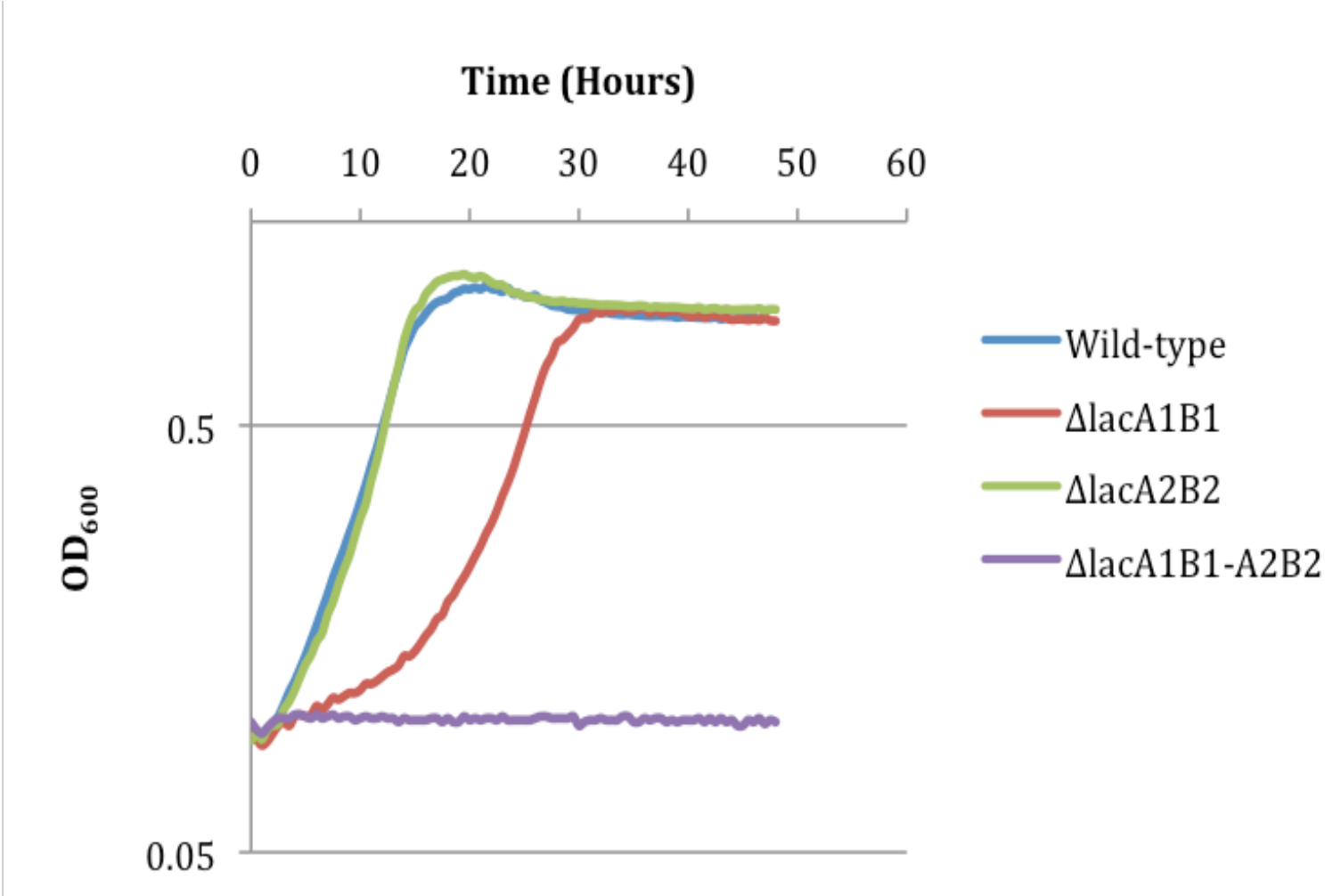


Figure 3-6. Growth of *S. gordonii* DL1 wild-type, *ΔlacA1B1*, *ΔlacA2B2* and *ΔlacA1B1-A2B2* strains in TV – 0.5% Galactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

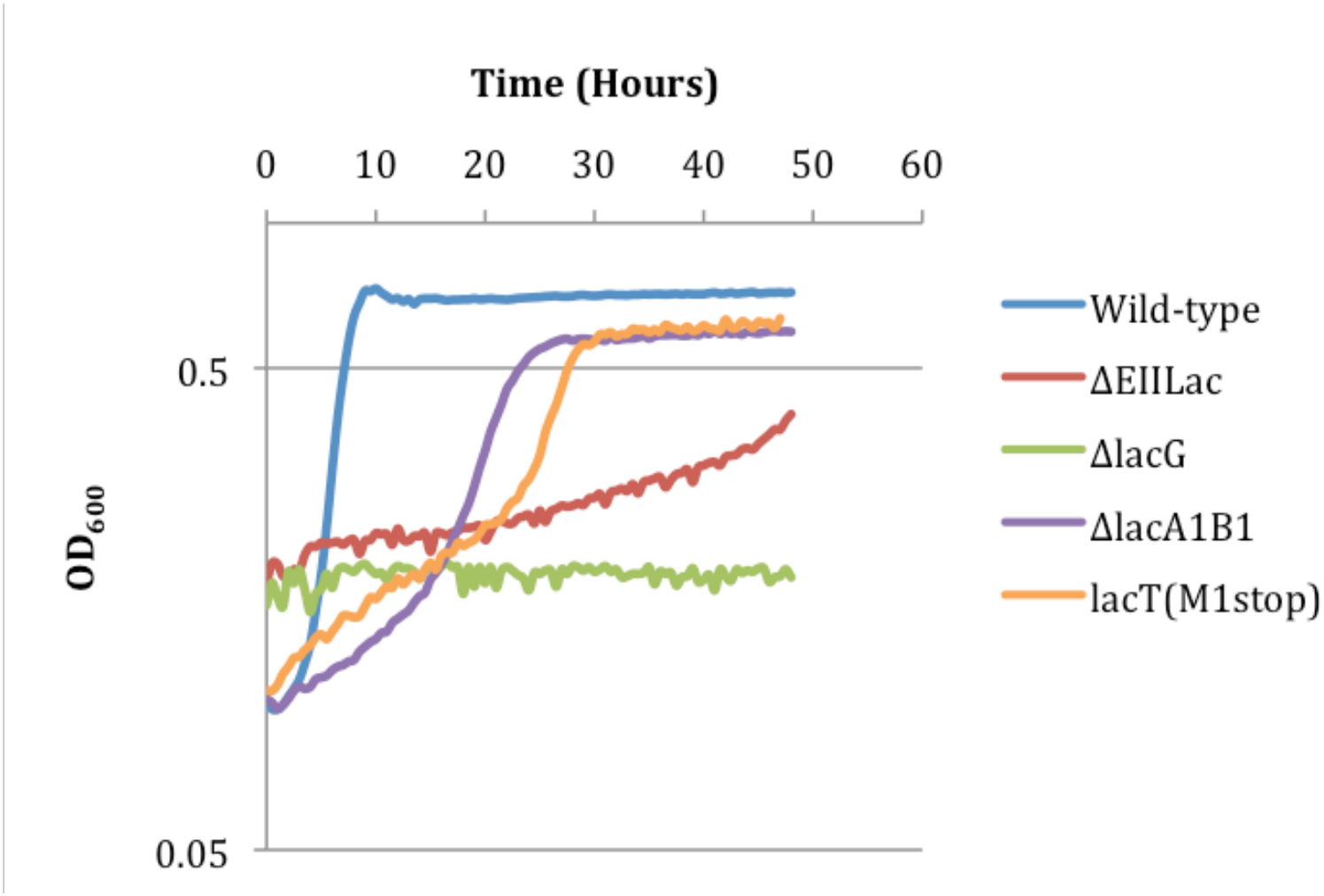


Figure 3-7. Growth of *S. gordonii* DL1 wild-type, *ΔEII*^{Lac}, *ΔlacG*, *ΔlacA1B1* and *lacT*(M1stop) strains in TV – 0.5% Lactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

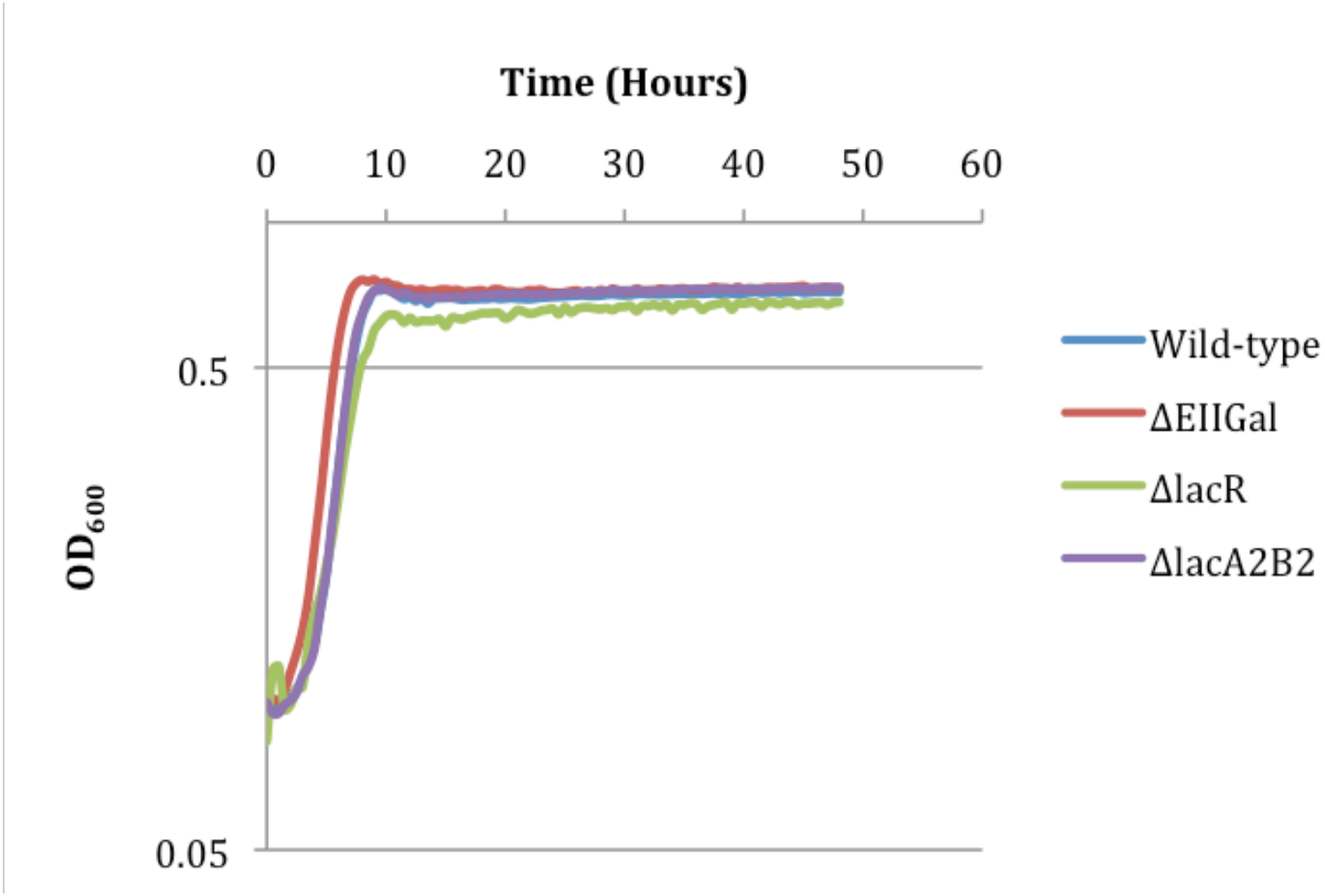


Figure 3-8. Growth of *S. gordonii* DL1 wild-type, *ΔEII*^{Gal}, *ΔlacR* and *ΔlacA2B2* strains in TV – 0.5% Lactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

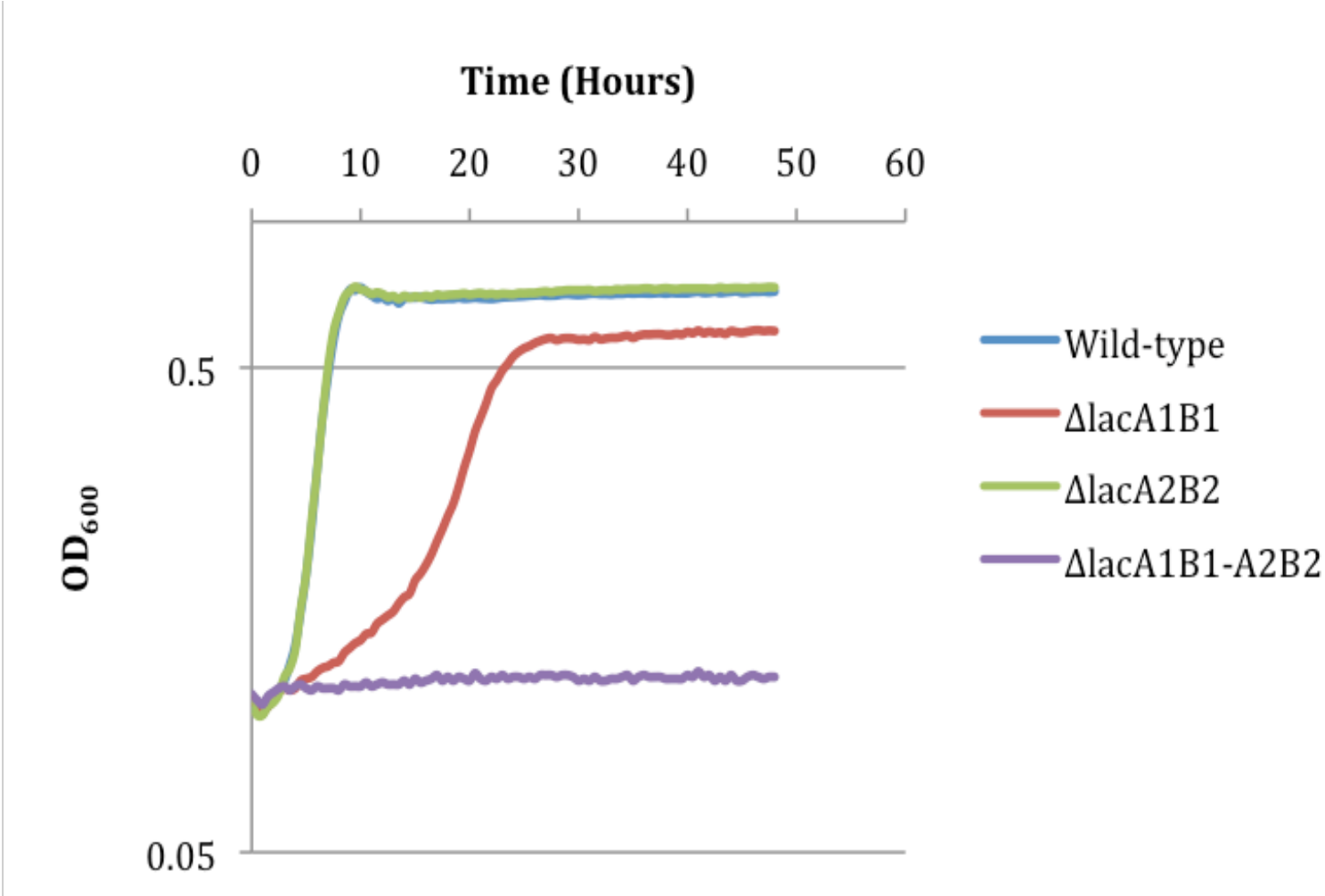


Figure 3-9. Growth of *S. gordonii* DL1 wild-type, *ΔlacA1B1*, *ΔlacA2B2* and *ΔlacA1B1-A2B2* strains in TV – 0.5% Lactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 3-3.

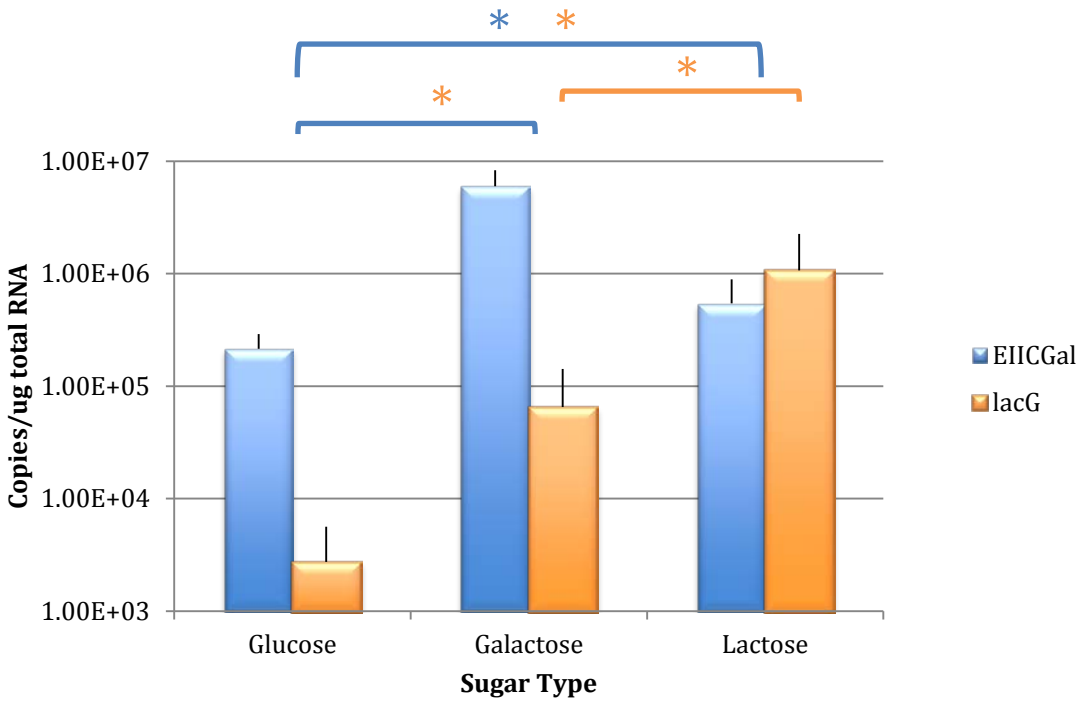


Figure 3-10. Expression levels of the *lacG* and *EIIC*^{Gal} transcripts via quantitative Real-Time RT-PCR when grown in various carbohydrate sources. Cells were grown to mid-exponential phase in TV – 0.5% glucose, galactose, or lactose at 37°C with 5% CO_2. Results shown are the mean and standard deviations of three separate cultures assayed in triplicate for each strain. Pair-wise Student t-tests were used to determine significant differences ($p < 0.05$).

CHAPTER 4
GENE REGULATION IN RESPONSE TO LACTOSE/GALACTOSE TRANSPORT AND ENZYMATIC METABOLISM

Introduction

As described in Chapter 3, there are several genes identified in the Oralgen database as putative regulatory components for the *lac* operons in *S. gordonii*. Although a lactose-specific PTS is present in many Gram-positive bacteria, the presence of two seemingly separate gene clusters dedicated to both galactose and lactose utilization is unique to only *S. pyogenes*, *S. pneumoniae*, *L. casei* and *S. gordonii (15, 103)*. To further complicate matters, those four species are not unified in the arrangement of their *lac* genes in the genome. Although all four appear to contain a double set of tagatose pathway genes, including the lactose PTS, galactose PTS, *lacR*, *lacT* and *lacG*, these genes are not always adjacent to each other in the genome. In the case of *L. casei*, some of the *lac* genes are located on a plasmid while the rest appear to be chromosomal (24, 33).

Galactose is one of the major carbohydrate components found in host glycoproteins (41), including many abundant salivary proteins. As a result, galactose is always available in low concentrations in the oral environment. In turn, species such as *S. gordonii* that are equipped with extracellular β-galactosidases capable of cleaving galactose from glycoproteins may utilize a high-affinity galactose PTS to obtain carbon sources for energy (18, 21). With the advantage of having constant access to a supply of carbohydrates, even during fasting periods by the host, *S. gordonii* would have a competitive advantage over *S. mutans* and other oral bacteria that appear to lack a mechanism for high-affinity internalization of galactose.

It is equally important to understand the regulatory mechanisms behind the *S. gordonii lac* operons. If it is accurate that commensal *S. gordonii* gains a selective advantage over pathogenic *S. mutans* with enhanced growth in galactose, then understanding the differences in regulation between the two species could help in the development of ways to exploit this process to improve oral health.

Thus, the goals of the studies described in Chapter 4 were (1) to characterize *lacA1* and *lacA2* promoter activity, (2) to use *lacA1* and *lacA2* promoter gene fusions to assess the regulatory effects of three *lac* genes in a variety of sugars, (3) to measure mRNA transcript levels of key putative *lac* regulators in a variety of sugars, and (4) to determine the ability of the putative repressor LacR to bind to both the *lacA1* and *lacA2* promoters.

Results

Analysis of *lacA1* and *lacA2* Promoter Activity Using *cat* Gene Fusions

Bioinformatics analysis revealed the existence of a promoter situated directly upstream of both *lacA1* and *lacA2* (Figure 4-1). To determine the validity of this discovery, reporter gene fusions were constructed for use in Chloramphenicol Acetyltransferase (CAT) assays. The 385-bp region directly upstream of the ATG start site of *lacA1* containing the putative promoter was cloned in front of a promoterless chloramphenicol acetyltransferase (*cat)* gene on plasmid pYQ4 and integrated into the *S. gordonii* genome at the *gtfG* locus. A similar fusion was made by cloning the 308-bp region upstream of the ATG start site of *lacA2* in front of the *cat* gene on plasmid pMJB8, with integration into *S. gordonii* also occurring at the *gtfG* locus. Once the promoter fusion was cloned into *S. gordonii* DL1 and it was evident that there was promoter activity, the promoter gene fusion was sub-cloned into various mutant strains

to assess the impact of the loss of each *lac* gene on the regulation of the promoters. For a quick reference to all CAT activity numbers in table form, see Table 4-1 and Table 4-2.

Focusing on the *lacA1* promoter, the WT::PlacA1-*cat* strain verified that growth in galactose or lactose resulted in activation of the promoter with 2300 and 1900 units of CAT activity, respectively (p=0.3, p=0.5) (Figure 4-2). When grown in the presence of the repressing sugar glucose and galactose, promoter activity decreased to 80 units of CAT activity (p=0.03). In contrast, when the cells were grown in a combination of glucose and lactose, promoter activity was reduced to approximately half of the activity level (1000 units) seen in only lactose. Mutation of *lacT* did not affect promoter activity in galactose or a glucose plus galactose mixture. However, promoter activity was abolished when the cells were grown in a mixture of glucose plus lactose as compared to wild-type. Because growth is severely limited in lactose, the *lacT* mutant was not assayed for promoter activity under those conditions. Removal of *lacR* produced drastic effects, with promoter activity increased in glucose, galactose and lactose (P_{glc}=0.02). The *lacG* mutant is not able to grow in galactose or lactose; therefore, this strain was assayed in the presence of each sugar in combination with the repressing sugar glucose. Loss of *lacG* resulted in decreased activity by about half in glucose plus galactose as compared to the wild-type strain, while there was no activity in glucose plus lactose conditions. Again, the *lacG* mutant was not assayed in galactose or lactose due to poor growth on that substrate.

Evaluation of the *lacA2* promoter proved to be more complicated than originally anticipated (Figure 4-3). Comparison between WT and mutant strains only showed

small changes in promoter activity. In the WT::PlacA2-*cat* strain, activity was much lower than in WT::PlacA1-*cat*. The *lacA2* promoter was inducible by galactose with 32 units, but not by lactose with only 4 units of CAT activity. In a *lacT* mutant background, activity was only slightly reduced to 23 units of CAT activity when the cells were grown in galactose. Removal of *lacR* caused modest de-repression in glucose (8 units) and galactose (37 units). A double deletion of *lacRT* resulted in slight increases in activity compared to the *lacR* background with 13 units in glucose and 54 units in galactose. Compared to the wild-type strain, the *lacG* deficient strain did not affect *lacA2* promoter activity in glucose plus lactose, but it did increase significantly in glucose and glucose plus galactose (Figure 4-4).

Because none of the potential gene regulators associated with this system appeared to have any major effects on promoter activity of *lacA2*, it was theorized that regulation of this promoter is CcpA-dependent. A *ΔccpA*::PlacA2-*cat* strain resulted in significant de-repression of the promoter in all carbohydrate conditions (Figure 4-3). Of note, promoter activity in lactose increased to a similar level as in galactose with 120 and 130 units of CAT activity reported, respectively. To investigate whether CcpA was masking the regulatory actions of LacR, a Δ*ccpA/lacR*::PlacA2-*cat* strain was also assayed (Figure 4-3). Surprisingly, promoter activity was decreased greatly in both galactose and lactose conditions. In order to eliminate any effects on the rest of the genome caused by a loss of CcpA, a site-specific four base pair mutation starting 49-bp upstream from the ATG start site (AGC GTT → CGT ATC) was created in the predicted CcpA binding site (catabolite response element - *cre*) of the *lacA2* promoter using recombinant PCR techniques (Figure 4-1). It was not until this strain, WT::*cre*-PlacA2-

cat, was assayed that the true promoter activity was revealed (Figure 4-3). In all three carbohydrate conditions, promoter activity increased significantly. Without an active binding site for CcpA, the *lacA2* promoter yielded 44 units of CAT activity in glucose and 230 units of CAT activity in both galactose and lactose.

Oftentimes, a promoter is under the regulation of multiple transcription factors. Although the *lacR* mutation did not dramatically affect *lacA2* promoter activity previously, mutation of the CcpA binding site combined with a *lacR* deletion caused substantial de-repression of the promoter activity in glucose, galactose and lactose conditions. When the *lacG* mutant was introduced into the strain with the *cat* fusion lacking the *cre,* promoter activity was similar to that of the WT::*cre*-PlacA2-*cat* strain in glucose and glucose plus galactose, but was lower in the mutant growing in glucose plus lactose (Figure 4-3).

Gene Expression of LacA1, LacA2, LacR and LacT

To complement the data acquired via the CAT assays, quantitative Real-Time PCR was used to measure the expression of various *lac* genes in the background of WT, *ΔlacR* and *lacT*(M1stop) strains when grown in glucose, galactose, or lactose (Figure 4-5).

Expression of the *lacR* gene was significantly upregulated in galactose in a *lacT*(M1stop) mutant compared to wild-type (p=0.03). In the *lacT* mutant strain, copy numbers of the *lacR* transcript in galactose were significantly higher in magnitude than in glucose (p=0.03). Transcript levels of *lacT* were not significantly different in the *ΔlacR* or *lacT*(M1stop) strains. Increased expression level in galactose compared to glucose of the wild-type strain was the only noteworthy distinction (p=0.04). Transcription of *lacA1* in the wild-type strain was markedly upregulated in both galactose and lactose (p=0.0.3

and p=0.03, respectively). This same trend appeared in the *lacT* mutant strain, with *lacA1* expression being upregulated in galactose ($p=2.8x10^{-3}$). Comparison of the wild-type and *lacT*(M1stop) strains also indicated a significant difference in LacA1 expression in galactose (p=0.02). Lastly, transcript levels of *lacA2* in the wild-type strain were significantly different when the cells were grown in glucose, galactose and lactose with the highest induction measured in galactose ($p_{glc-gal}=4.0x10^{-3}$, $p_{glc-lac}=7.3x10^{-3}$, $p_{gal-lac}=9.1x10^{-3}$). The last significant variance in gene expression was between the wild-type and *ΔlacR* strains growing in galactose, where the copy number of *lacA2* was significantly higher than the wild-type strain (p=0.04).

In Vitro Binding Analysis of *S. gordonii* LacR Recombinant Protein to the *lacA1* and *lacA2* Promoters

Being that LacR is the putative repressor protein for the *lac* system in *S. gordonii*, attention was focused on delving deeper into the regulatory role of this gene. As mentioned previously, LacR contains a helix-turn-helix region at its amino terminal end that is capable of binding to DNA for the purpose of repressing gene transcription. An online database called RegPrecise (http://regprecise.lbl.gov/RegPrecise/) was employed to find several consensus LacR binding sequences for the *S. gordonii* genome (Table 4-1). Using these sequences, one putative LacR binding site was found within the *lacA1* promoter as well as six putative LacR binding sites within the *lacA2* promoter (Figure 4-1).

To determine if LacR could bind directly to either promoter, a 6X-His-tagged recombinant LacR protein sequence was generated using pQE-30 in *E. coli* M15[pREP4] cells. Expression was induced with IPTG and the protein was subsequently purified via nickel affinity chromatography (Figure 4-6). The 28 KDa

protein was then dialyzed before addition into the binding reaction. After a 30-min incubation period on ice, the reactions were run on a non-denaturing polyacrylamide gel. The DNA was transferred to a hybridization membrane and UV crosslinked. The biotinylated DNA was then detected with chemiluminescence by probing with Streptavidin-Horseradish Peroxidase Conjugate and Chemiluminescent Substrate. All of the EMSA results presented here are preliminary findings and further work must be completed with the proper controls to accurately confirm these results.

Addition of 2.08, 4.16 and 6.24 pmol of the recombinant LacR protein to a biotinylated probe of the *lacA1* promoter resulted in increasing shift of the probe (Figure 4-7). When both biotinylated and unbiotinylated *lacA1* probe were mixed in the same reaction tube, competition from the unbiotinylated probe reduced the binding of LacR incrementally (based on a 1:1 and 1:10 ratio of biotinylated:unbiotinylated probe). Similar results were obtained when the biotinylated probe of the *lacA2* promoter was tested in the same conditions (Figure 4-8). Densitometry analysis confirmed that there was in fact shift for both promoters.

Discussion

Based on the results presented here, the *lacA1* and *lacA2* promoters are differentially regulated. Some of the key differences in the structure of the promoters include a CcpA binding site and six putative LacR binding sequences in the *lacA2* promoter, but only one predicted LacR binding site and no *cre* sequence in the *lacA1* promoter. CAT assay data revealed that the *lacA1* promoter is activated by both galactose and lactose, indicating a role of the first set of tagatose pathway genes (*lacABCD1*) in metabolism of both sugars. In addition, the *lacA1* promoter is likely not regulated by CcpA, at least in a direct manner. Aside from the fact that there are no *cre*

sequences in the *lacA1* promoter, promoter activity of the wild-type strain in galactose alone was very high, while promoter activity in glucose plus galactose was abolished. Had CcpA been exerting an effect on the *lacA1* promoter, some activity would still have been noted in glucose plus galactose. Also noted, cells grown in glucose plus lactose resulted in promoter activity of about half that measured in lactose alone. The most reasonable explanation for this is that lactose is preferred over galactose and therefore better transported as a sugar substrate under repressing conditions and a better inducer of the *lacA1* promoter.

The transcriptional antiterminator LacT does not appear to regulate the *lacA1* promoter since activity in the *lacT* mutant strain in galactose was comparable to that of WT. Once more, regulation of the *lacA1* promoter seems to be CcpA-independent due to the fact that promoter activity in a *lacT* mutant is again abolished in glucose plus galactose. Although a lactose culture was not assayed due to poor growth, a glucose plus lactose culture also measured no promoter activity. That result aligns with the previous line of reasoning concerning LacT's autoregulation. Because the lactose transporters are located directly downstream of LacT, autoregulation by the transcriptional antiterminator also affects the transcription of the downstream lactose transporter as well. As it follows, if LacT is removed from the cell, there will be no transcription of the lactose transporters. So normally when the wild-type strain is grown in glucose plus lactose, a small amount of lactose is able to enter the cell via the lactose transporter, resulting in some *lacA1* promoter activity. However, in the *lacT*(M1stop) strain, no promoter activity is measured due to the fact that the lactose transporters are not synthesized without LacT present.

Evaluating the *ΔlacR* strain is slightly more complicated. Promoter activity of *lacA1* is higher in the *lacR* deficient strain compared to wild-type for glucose, galactose and lactose. This was expected in glucose because, since the LacR protein is not present, the *lacA1* operon cannot be repressed and will be expressed constitutively. Conversely, when comparing WT to the *ΔlacR* strain in terms of galactose and lactose growth conditions, increased promoter activity was observed as well. If LacR is not present, the promoter activity should not change considerably from wild-type levels since these two sugars already induce the *lacA1* promoter. The most suitable explanation for why *lacA1* promoter activity is considerably higher in the *ΔlacR* strain stems from the hypothesis that LacG converts Gal-6-P into an inducer for LacR. In the WT strain, Gal-6-P is not operating as an inducer at its most efficient level since much of the carbohydrate is actually entering the tagatose pathway. Therefore, some LacR is still binding to the *lacA1* promoter and exerting a repressive effect. It is only when LacR is completely removed from the cell that the *lacA1* promoter activity increases dramatically. Therefore, LacR acts as a repressor for the *lacA1* promoter.

A *lacG* deletion decreased *lacA1* promoter activity by at least half in every sugar tested. This lends support to the theory that LacG is helping to activate the *lacA1* promoter by converting Gal-6-P into an inducer for LacR. Cells grown in glucose plus lactose actually displayed almost no *lacA1* promoter activity. Similar to the reasoning behind this in the *lacT* mutant, LacG is required for the catabolism of Lac-6-P into glucose and Gal-6-P. Without this enzyme, there is no Gal-6-P available to metabolize and the *lacABCD* genes in this pathway are not necessary.

The *lacA2* promoter is under tighter regulatory control via at least two different mechanisms uncovered here. The first mechanism involves CcpA and is based on a more global control of carbohydrate utilization and catabolite repression. The second mechanism is specific for the *lac* operon and involves LacR, LacT and LacG.

In the WT strain, the *lacA2* promoter is activated in galactose but less so in lactose, which is most likely due to CcpA-dependent effects as well as better induction by Gal-6-P. Even then, the activity is extremely low compared to the levels measured from the *lacA1* promoter. This same trend is evident in the *ΔlacR* strain, albeit a slight de-repression. Absence of *lacT* produced a small repressive effect in both glucose and galactose. The double mutant *lacR* and *lacT*(M1stop) strain caused just slightly more de-repression than the *ΔlacR* strain alone. All of this data led to the conclusion that LacR acts as a repressor and lacT as an activator of the *lacA2* promoter, but that the effect of each is not very influential in regulating the *lacA2* promoter. This was puzzling since LacR and LacT are the two transcriptional factors associated with the second set of tagatose genes.

It appeared that in glucose and glucose plus galactose, the absence of *lacG* had a negative effect on the *lacA2* promoter, but no significant effect in glucose plus lactose. Interestingly, this data agreed with the growth data in that LacG plays some kind of role in glucose metabolism. Although it appears that LacG is repressing the *lacA2* promoter in glucose plus galactose, this effect could also be due to the glucose present only and have nothing to do with the galactose. Most surprising though was the fact that a cell lacking *lacG* did not interfere with *lacA2* promoter activity in glucose plus lactose, where one would assume that *lacA2* promoter activity would decrease due to no enzymatic

cleavage of lactose for use in the tagatose pathway. This could be explained by our theory that EII^{Gal} specializes in the transport of low concentrations of galactose. With LacG absent in lactose conditions, Lac-6-P cannot be catabolized into Gal-6-P. However, extracellular β-galactosidases can still release galactose from lactose. Once EII^{Gal} transports the sugar inside, the *lacA2* promoter is stimulated.

The other possibility for regulation of the *lacA2* promoter may be related to global control of transcription by CcpA, as many other carbohydrate operons are regulated in this manner. Dr. Zeng, from the Burne lab, performed a CAT assay on a CcpA-deficient strain showing significantly increased *lacA2* promoter activity in glucose, galactose and lactose (Zeng and Burne, unpublished). This de-repression unmasked the role of CcpA as a direct repressor of the *lacA2* promoter. After this revelation, we turned to LacR to investigate if an effect of LacR on *lacA2* expression could be observed in a CcpA-deficient genetic background. Despite expectations that promoter activity would increase dramatically, the *ΔCcpA/LacR*::PlacA2-*cat* strain produced the opposite effect with activity dropping to baseline in galactose and lactose. We deduced from the *lacA1* promoter data and *ΔccpA/lacR*::PlacA2-*cat* results that LacR was acting as an activator under inducing conditions and as a repressor under repressing sugar conditions.

Upon further inquiry, this premise proved to be incorrect. Since CcpA affects hundreds of genes, its deletion could produce unintended effects across the entire genome. A point mutation in the *cre* site ensuring only site-specific effects to the *lacA2* promoter produced activity levels of the same magnitude in galactose and lactose, thus confirming that CcpA has a direct effect in negatively regulating the *lacA2* promoter.

Testing the *cre*-PlacA2-cat gene fusion in a *lacR* mutant revealed significantly increased levels of *lacA2* promoter activity in glucose, galactose and lactose. Following the same logic as in the *lacA1* promoter, when LacR is removed the operon will be constitutively expressed in glucose while activity will be enhanced in galactose and lactose. Since removal of LacR causes de-repression, it can be concluded that LacR is repressing the *lacA2* promoter. Lastly, when the *ΔlacG*::*cre*-PlacA2-*cat* strain was assayed in glucose and glucose plus galactose, *lacA2* promoter activity was not affected. However, a significant repression occurred in glucose plus lactose, supporting the role of LacG as an activator for the *lacA2* promoter.

Much of the Real-Time PCR data is in agreement with the findings from the promoter gene fusions. The *lacA1* gene was expressed significantly higher in galactose and lactose of WT, indicating that the first set of tagatose genes is important for the metabolism of both carbohydrates. It also confirms that LacR represses the *lacA1* promoter since, in glucose, *lacA1* is expressed constitutively when LacR is taken out of the equation. Expression data also verifies that LacT does not regulate the *lacA1* promoter as evidenced by no difference in expression of the *lacA1* gene between wild-type and the *lacT* mutant.

RNA transcript copy numbers for *lacA2* in the WT strain align well with the promoter activities obtained with CAT assays. It is noticeable, however, that *lacA1* is more highly expressed than *lacA2* in the WT strain. At the time of this experiment, it was not known that the *lacA2* promoter was under the control of CcpA. Now that CcpA has been implicated in the negative repression of the *lacA2* promoter, the decreased *lacA2* expression (compared to *lacA1* expression) in wild-type seems likely due to the fact that

CcpA was functional and probably repressing the *lacA2* promoter. In a strain with a mutation in the *cre* of the *lacA2* promoter, it is expected that *lacA2* expression would rise significantly in both galactose and lactose.

Real-Time PCR data also suggests a negative interaction between LacR and LacT since a *lacT* mutation caused increased expression of *lacR* in galactose. From this data, it is plausible that LacT somehow negatively regulates *lacR*, either directly or indirectly, but there is currently no evidence to support this idea. Conversely, *lacT* gene expression is not affected by the deletion of LacR, implying that the repressor does not regulate *lacT* transcription. It is more likely that *lacT* is autoregulated. The reason why there is *lacT* expression in the *lacT* mutant is because of the manner in which this strain was made. Instead of deleting the sequence from the genome, a point mutation was made at the ATG start site. In effect, transcription is not affected, but translation is blocked.

Promoter fusion assays and/or Real-Time PCR data suggest a repressive role for LacR in both the *lacA1* and *lacA2* promoters. *In vitro* binding assays using a recombinant LacR protein demonstrated a band shift when either *lacA1* or *lacA2* DNA probes were supplied. In a cold probe competition, binding of LacR to both promoters was reduced incrementally as the ratio of unbiotinylated to biotinylated probe was increased. This data provides support for the role of LacR as a repressor of both the *lacA1* and *lacA2* promoters. Although these results are preliminary, when data from the CAT assays, quantitative Real-Time PCR and *in vitro* binding assays are combined, the conclusion can be made that LacR negatively regulates the *lacA1* and *lacA2* promoters by binding to putative *lacR* consensus sequences.

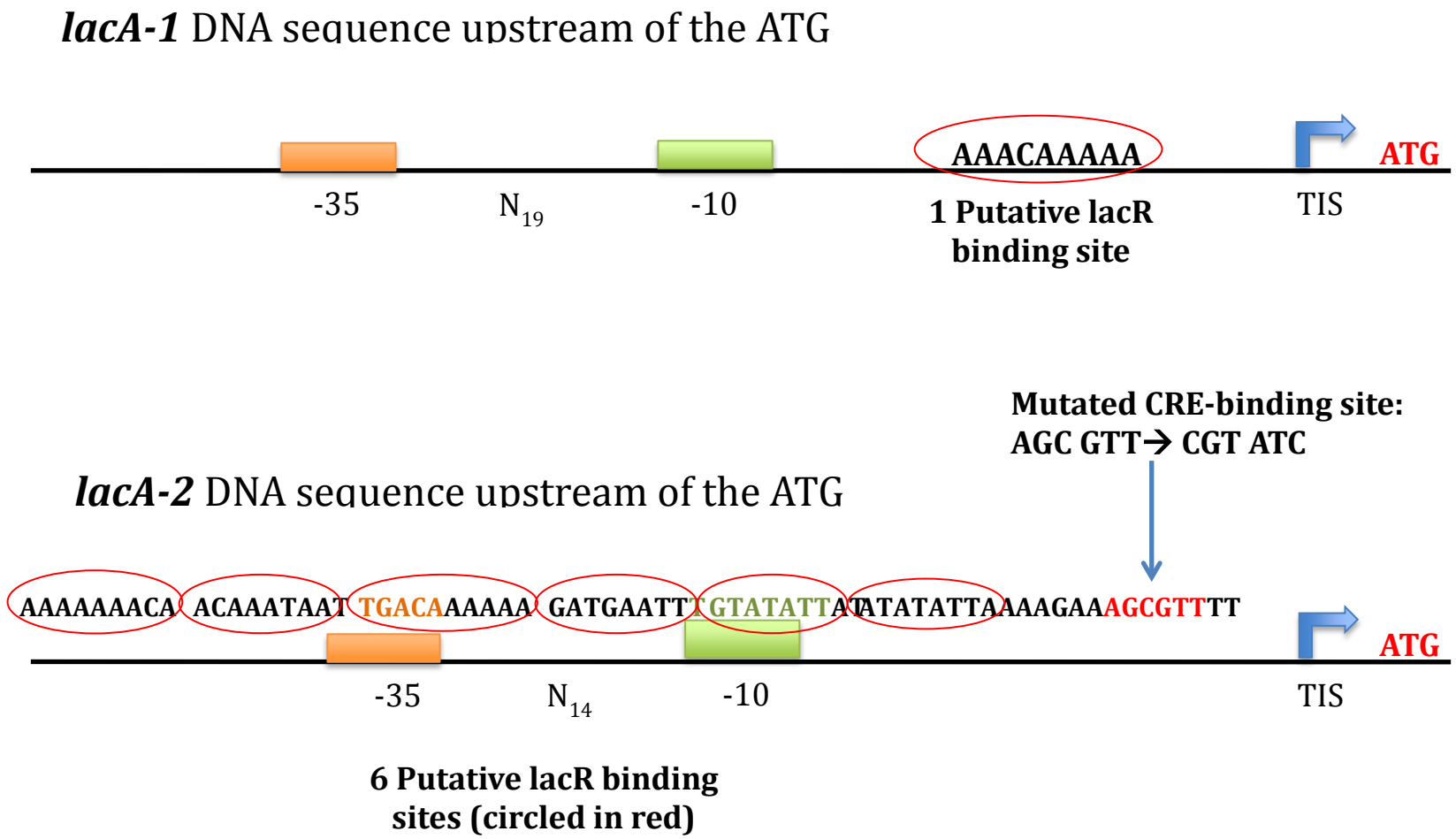


Figure 4-1. Critical elements comprising the putative promoter regions of *lacA1* and *lacA2* based on bioinformatics analysis. The *lacA1* promoter contains one LacR binding sequence 114-bp upstream of the ATG start site. The *lacA2* promoter contains six LacR binding sequences starting 116-bp upstream of the ATG start site and a *cre* sequence starting 54-bp upstream of the ATG start site. An online database named RegPrecise (http://regprecise.lbl.gov/RegPrecise/) was used to find the consensus LacR binding sequences for the *S. gordonii* genome. The promoter prediction program Softberry – BPROM was used to find the promoter regions.

Table 4-1. CAT activity of the *lacA1* promoter of *S. gordonii* DL1 in the background of the wild-type, *lacT*(M1stop), *ΔlacR* and *ΔlacG* strains.

	Glc	Glc SD	Gal	Gal SD	Lac	Lac SD	Glc/ Gal	Glc/ Gal SD	Glc/Lac	Glc/ Lac SD
Wild-type	84	15	2300	170	1900	180	80	12	1000	480
lacT(M1stop)	37	3.0	2200	150	NA	NA	37	4.3	48	6.2
ΔlacR	10000	3500	15000	7400	11000	5700	NA	NA	NA	NA
ΔlacG	45	3.7	1200	39	NA	NA	43	12	50	23

SD is defined as the standard deviation among triplicate samples.

Table 4-2. CAT activity of the *lacA2* promoter of *S. gordonii* in the background of the wild-type, *lacT*(M1stop), *ΔlacR*, *ΔlacR-lacT*(M1stop) and *ΔlacG* strains.

	Glc	Glc SD	Gal	Gal SD	Lac	Lac SD	Glc/Gal	Glc/Gal SD	Glc/Lac	Glc/Lac SD
Wild-type	0.50	0.07	32	0.91	3.8	0.93	0.33	0.13	1.2	1.0
lacT(M1stop)	0.07	0.06	23	2.6	NA	NA	NA	NA	NA	NA
ΔlacR	8.3	0.53	37	0.89	2.4	1.3	NA	NA	NA	NA
ΔlacR-lacT(M1stop)	13	0.43	54	3.0	NA	NA	NA	NA	NA	NA
ΔlacG	1.3	0.34	NA	NA	NA	NA	1.5	0.51	1.6	0.25
WT::*cre*-PlacA2-cat	44	9.3	200	6.0	230	7.5	41	1.0	120	22
ΔlacR::*cre*-PlacA2-cat	430	22	300	35	140	29	350	17	320	60
ΔlacG::*cre*-PlacA2-cat	32	17	NA	NA	NA	NA	32	23	30	9.3

SD is defined as the standard deviation among triplicate samples.

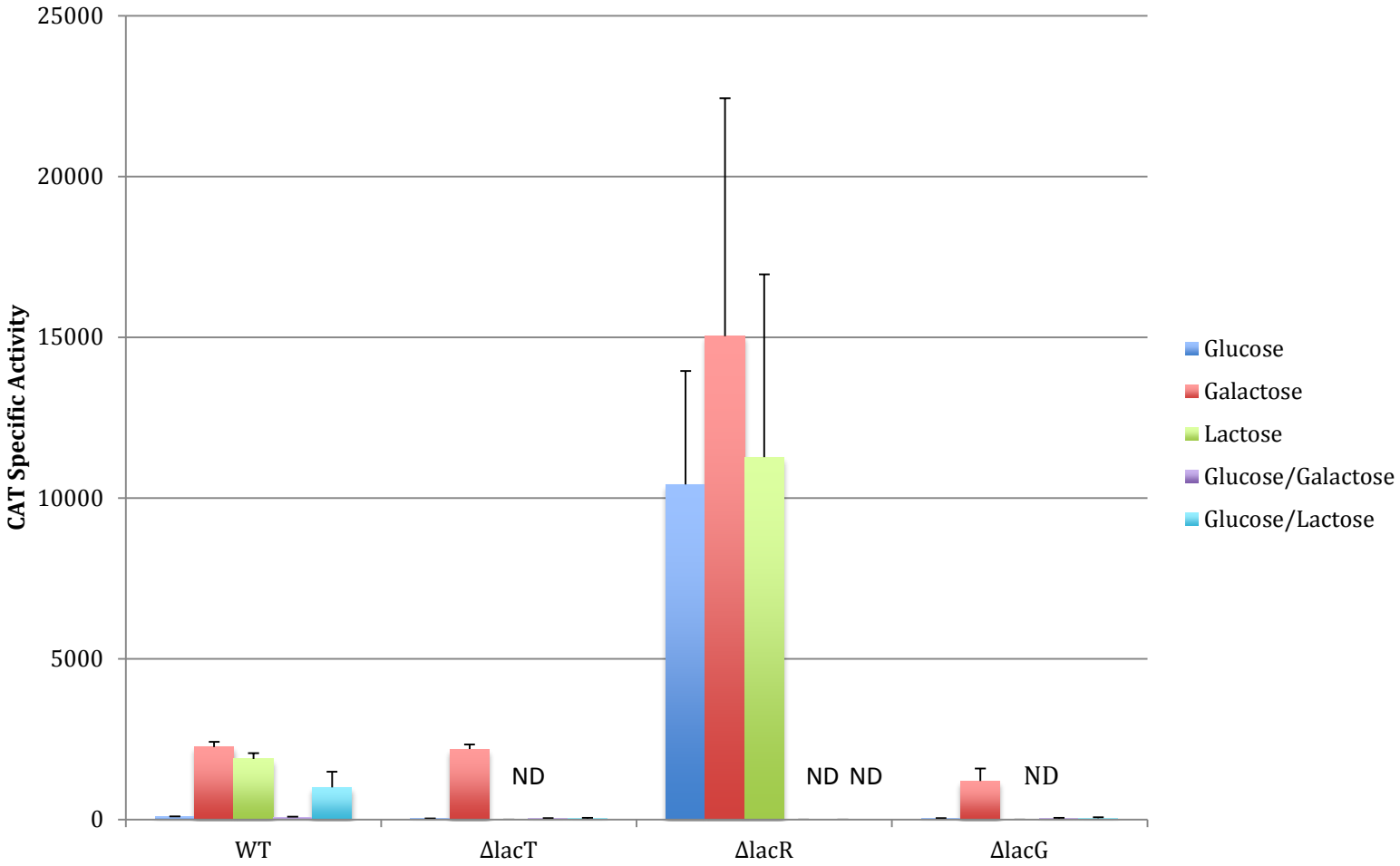


Figure 4-2. CAT activity of the 385-bp region directly upstream of the ATG start site of *lacA1*. Cells were grown to mid-exponential phase in TV – 0.5% carbohydrate at 37°C with 5% CO2, collected by centrifugation and then measured for CAT activity. Results shown are the mean and standard deviations of three separate cultures for each strain. Pair-wise Student t-tests were used to determine significant differences ($p < 0.05$). ND = Not Determined.

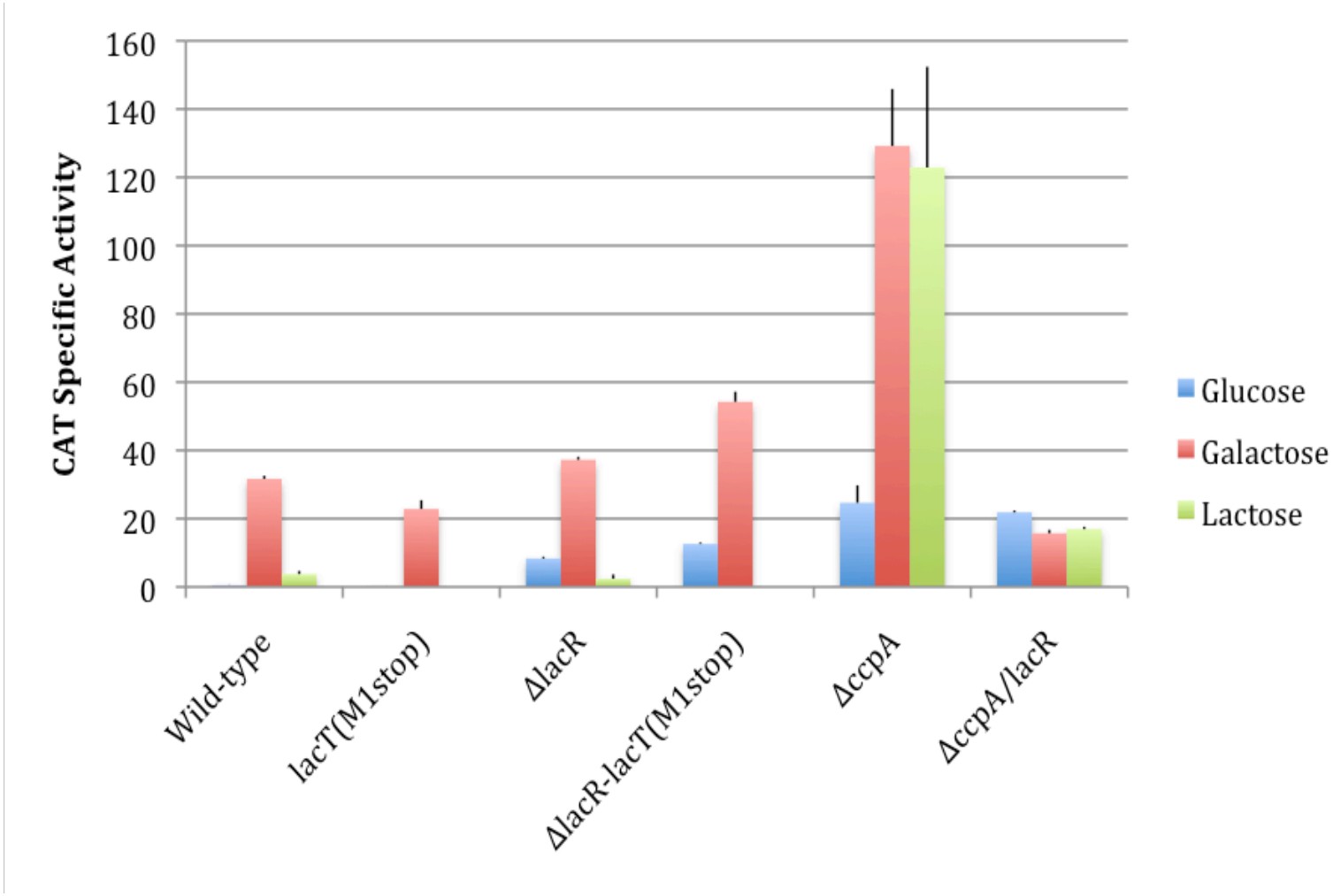


Figure 4-3. CAT activity of the 308-bp region directly upstream of the ATG start site of *lacA2*. Cells were grown to mid-exponential phase in TV – 0.5% carbohydrate at 37°C with 5% CO_2, collected by centrifugation and then measured for CAT activity. Results shown are the mean and standard deviations of three separate cultures for each strain. Pair-wise Student t-tests were used to determine significant differences ($p < 0.05$).

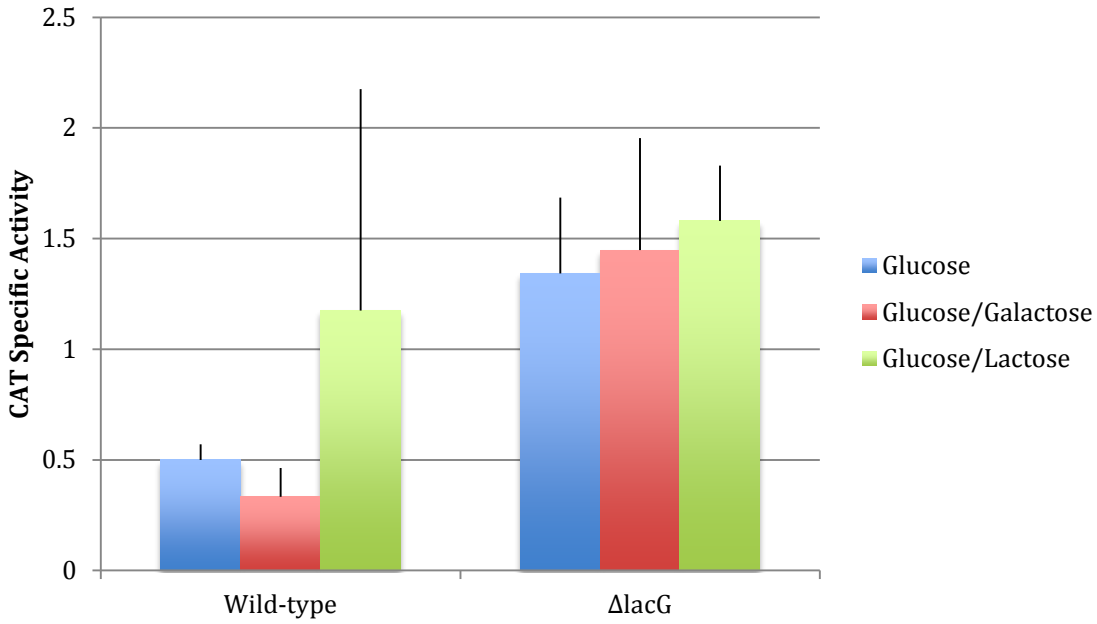


Figure 4-4. CAT activity of the 308-bp region directly upstream of the ATG start site of *lacA2*. Cells were grown to mid-exponential phase in TV – 0.5% carbohydrate at 37°C with 5% CO_2, collected by centrifugation and then measured for CAT activity. Results shown are the mean and standard deviations of three separate cultures for each strain. Pair-wise Student t-tests were used to determine significant differences ($p < 0.05$).

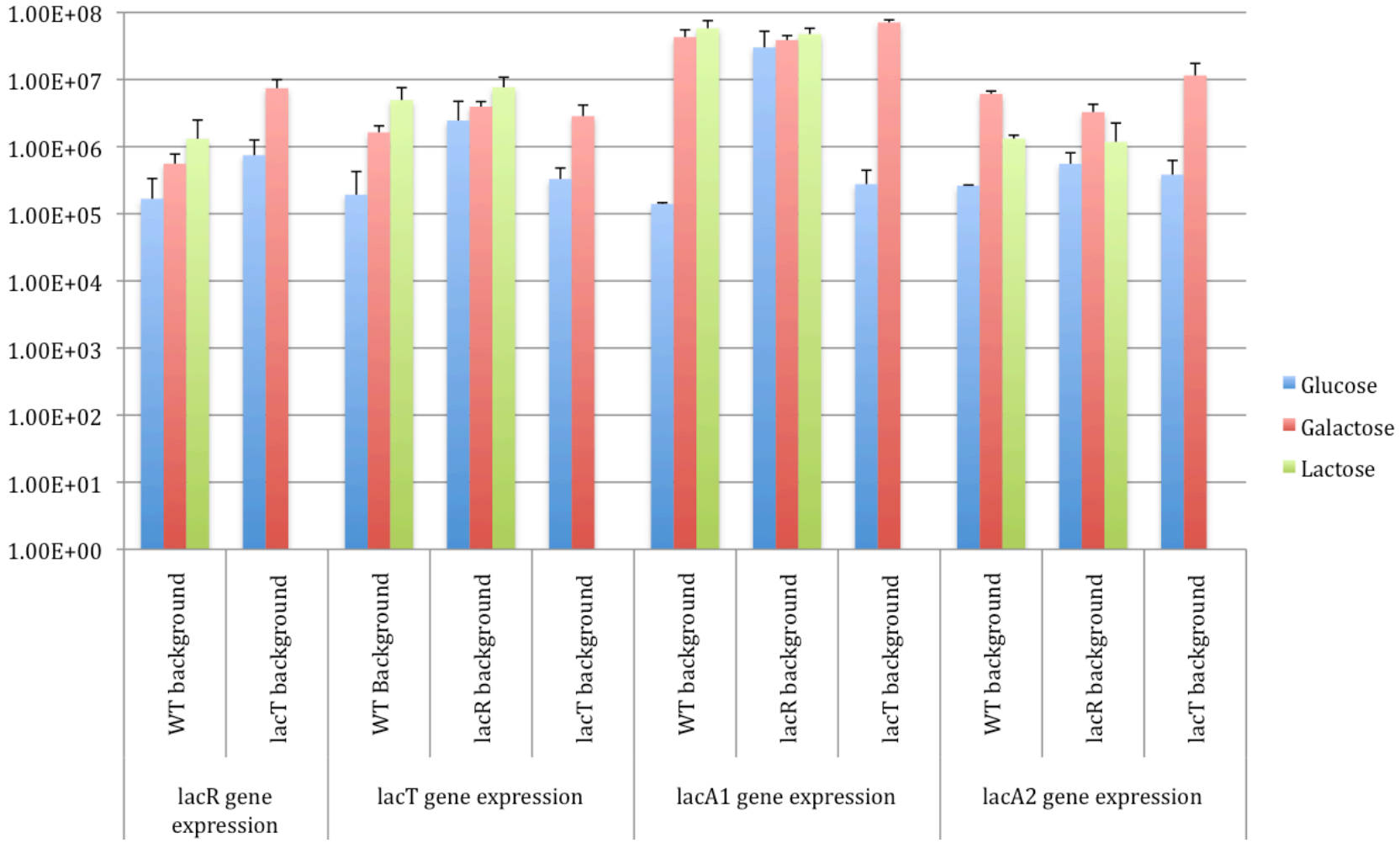


Figure 4-5. Expression levels of the *lacR*, *lacT*, *lacA1* and *lacA2* transcripts via quantitative Real-Time RT-PCR when grown in various carbohydrate sources. Cells were grown to mid-exponential phase in TV – 0.5% glucose, galactose, or lactose at 37°C with 5% CO_2. Results shown are the mean and standard deviations of three separate cultures assayed in triplicate for each strain. Pair-wise Student t-tests were used to determine any significant differences ($p < 0.05$).

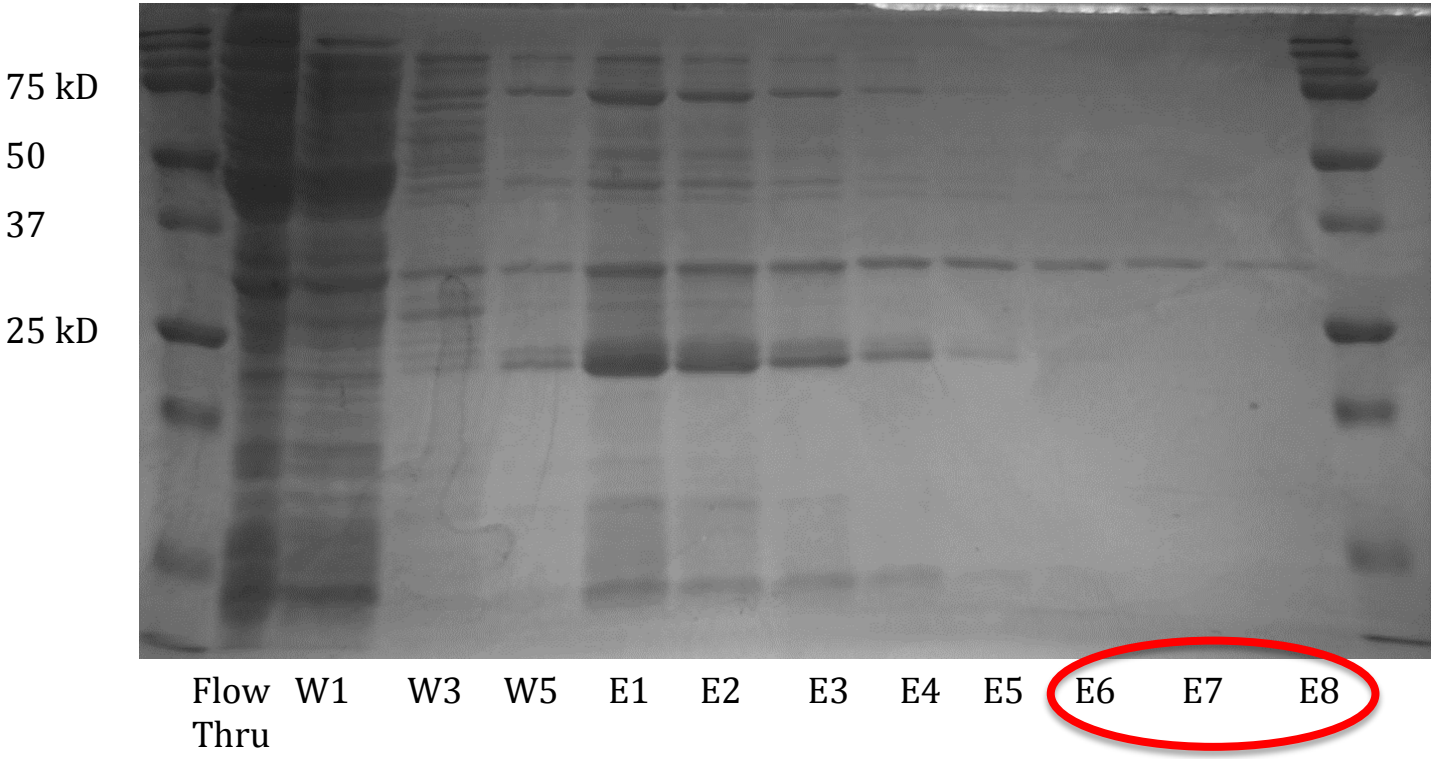
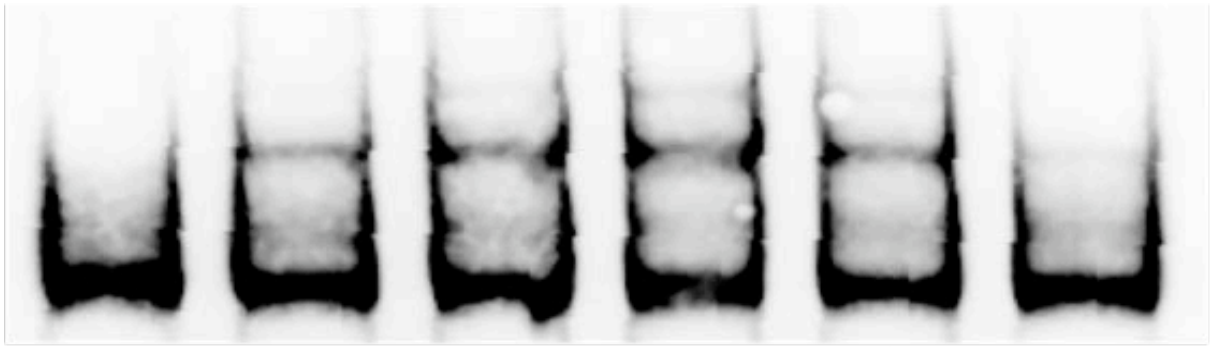


Figure 4-6. Expression and purification of the *S. gordonii* LacR recombinant protein. The 6X-His-LacR protein vector was transformed into competent *E. coli* M15[pREP4] cells. Those cells were grown aerobically in 30°C and expression of LacR was induced with 0.005 mM of IPTG. Cells were collected by centrifugation and purified using nickel-affinity chromatography. The samples were run on an SDS-PAGE gel for verification of the 28 kDa LacR protein.



Sample #	1	2	3	4	5	6
lacR protein (pmol)	0	2.08	4.16	6.24	6.24	6.24
Unbiotinylated probe (fmol)	0	0	0	0	10 fmol	100 fmol
Biotinylated P*lacA1* probe (fmol)	10	10	10	10	10	10

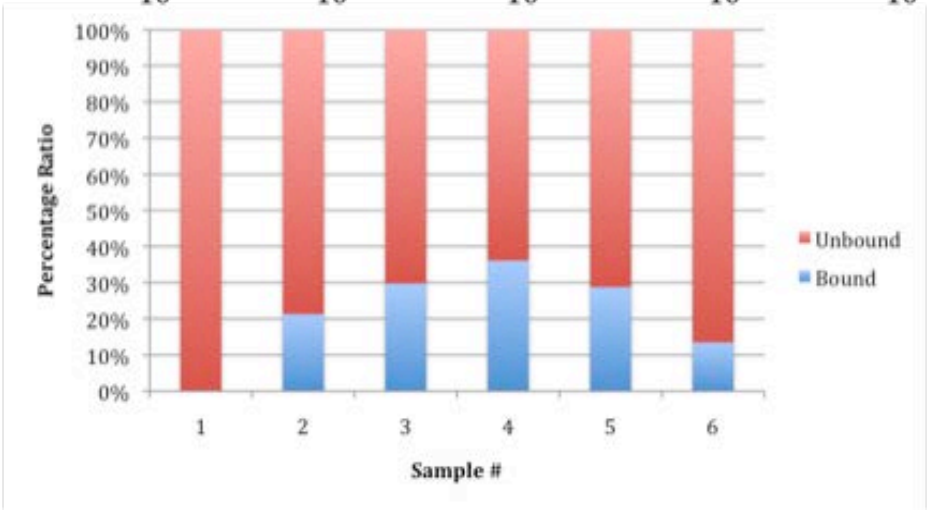
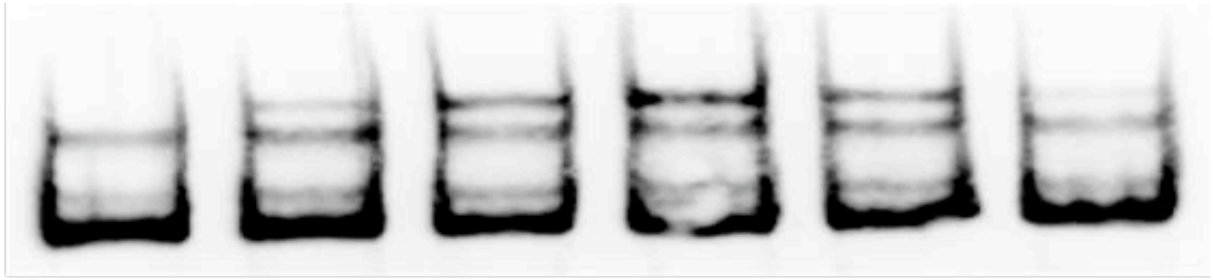


Figure 4-7. EMSA of the *S. gordonii* recombinant LacR protein and the *lacA1* promoter. The *lacA1* promoter contains one putative LacR binding sequence. All columns contain 10 fmole of biotinylated *lacA1* probe. Column 1 contains *lacA1* probe alone. Columns 2, 3 and 4 contain increasing concentrations of purified 6X-His-LacR protein of *S. gordonii* at 2.08, 4.16 and 6.24 pmole, respectively. Columns 5 and 6 contain *lacA1* probe and 6X-His-LacR in the same concentrations as column 4 plus 10 and 100 fmole of unbiotinylated *lacA1* probe, respectively. Quantitative results shown below image represent shift as a percent ratio of bound to unbound protein.



Sample #	1	2	3	4	5	6
lacR protein (pmol)	0	2.08	4.16	6.24	6.24	6.24
Unbiotinylated probe (fmol)	0	0	0	0	10 fmol	100 fmol
Biotinylated P*lacA2* probe (fmol)	10	10	10	10	10	10

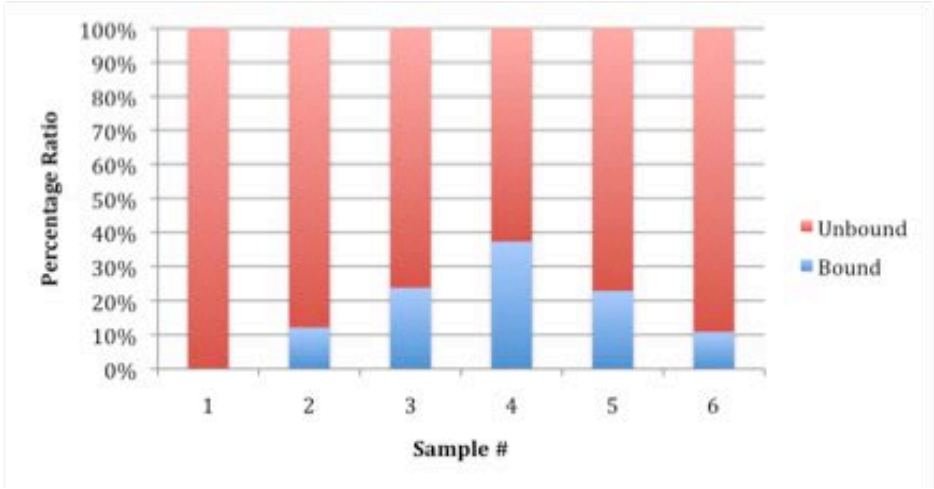


Figure 4-8. EMSA of the *S. gordonii* recombinant LacR protein and the *lacA2* promoter. The *lacA2* promoter contains one *cre* sequence and six putative LacR binding sequences. All columns contain 10 fmole of biotinylated *lacA2* probe. Column 1 contains *lacA2* probe alone. Columns 2, 3 and 4 contain increasing concentrations of purified 6X-His-LacR protein of *S. gordonii* at 2.08, 4.16 and 6.24 pmole, respectively. Columns 5 and 6 contain *lacA2* probe and 6X-His-LacR in the same concentrations as column 4 plus 10 and 100 fmole of unbiotinylated *lacA2* probe, respectively. Quantitative results shown below image represent shift as a percent ratio of bound to unbound protein.

CHAPTER 5
IMPACT OF GALACTOSE UTILIZATION ON THE INTER-SPECIFIC COMPETITION BETWEEN CARIOGENIC S. MUTANS UA159 AND COMMENSAL S. GORDONII DL-1 ORAL STREPTOCOCCI

Introduction

After initial growth experiments performed in the Burne laboratory suggested that *S. gordonii* is able to sustain growth on galactose at a concentration that is 10 times lower than what is necessary for *S. mutans* (103), we became interested in understanding the mechanism behind the results. Evaluation of the *S. gordonii* genome revealed two separate PTS, one each for lactose and galactose, along with a redundant set of tagatose genes. This genetic setup differs from *S. mutans*, which only has a lactose PTS and one set of tagatose genes. Further research into the *S. gordonii* genome confirmed the presence of two extracellular β-galactosidases. Results from Chapters 3 and 4 of this document support the hypothesis that *S. gordonii* has a galactose-specific PTS capable of high-affinity transport.

Competition for available carbon resources is a critical ecological determinant in dental biofilms, especially during periods of famine when nutrient levels are the lowest. Saliva provides a continuous source of nutrients; therefore, it is advantageous for oral microbes to acquire the genetic machinery necessary to metabolize oligosaccharides of glycoconjugates. Galactose is one of the major constituents in the oligosaccharides of many glycoconjugates (41). Therefore, a microbe with the ability to compete for galactose from salivary constituents may hold a distinct advantage over neighboring microbes that cannot utilize this carbohydrate or that cannot efficiently transport galactose at lower concentrations.

Since we are suggesting that *S. gordonii* possesses a high-affinity galactose-specific transport system, the goal of Chapter 5 was to design an *in vitro* mixed-species liquid culture competition assay assessing the viability and persistence of both *S. gordonii* and *S. mutans* when grown together in galactose or glucose media. In order to ensure that the results were pH-independent, the assay was conducted with and without phosphate buffer for both sugar conditions.

Results

Growth Comparison of *S. gordonii* DL-1 and *S. mutans* UA159

Both strains were grown in TV supplemented with either 0.5% or 2% of the desired sugar and a mineral oil overlay to reduce oxygen tension, since oxygen antagonizes the growth of *S. mutans* as does H_2O_2 production from oxygen by *S. gordonii*. Incubation in a Bioscreen-C reader allowed for measurement of the optical density every 30 min, which was used to calculate doubling times (Table 5-1). When grown in TV supplemented with 0.5% glucose (Figure 5-1), both strains grew to the same final yield, though *S. gordonii* grew at a faster rate. *S. gordonii* grew to a final OD_{600} of 0.61 with a doubling time of 88 ± 2.3 min and a lag phase of 7 hours. *S. mutans* grew to a final OD_{600} of 0.56 with a doubling time of 180 ± 17 min and a lag phase of 12 hours.

When grown in TV supplemented with 0.5% galactose (Figure 5-2), *S. gordonii* produced a higher yield with a final OD_{600} of 0.84 and a much more rapid doubling time of 170 ± 30 min. *S. mutans* grew to a final OD_{600} of 0.52 with a doubling time of 410 ± 61 min. Both strains left lag phase at 15 hours. Although the final OD_{600} for *S. gordonii* was 0.84, this strain actually reached a peak OD_{600} of 1.0, apparently due to the fact that *S. gordonii* is more prone to lysis during stationary phase than *S. mutans*.

When grown in TV supplemented with 2% galactose (Figure 5-3), the results were similar to that seen in TV supplemented with 0.5% galactose. *S. gordonii* again outcompeted *S. mutans* by growing at a faster rate (240 ± 3.1 min) and generating a higher final optical density (OD_{600} = 0.87). *S. mutans* grew to a final OD_{600} of 0.64 with a doubling time of 340 ± 22 min. As mentioned in the paragraph above, *S. gordonii* actually reached a peak OD_{600} of 1.0 before cells began to lyse in the stationary phase.

Mixed Species Liquid Culture Competition Assay

Based on the growth phenotypes displayed above when medium was supplemented with either glucose or galactose, a mixed-species liquid culture competition assay was created to assess the bacterial interaction between planktonic *S. gordonii* DL1 and *S. mutans* UA159 cells. Cells were grown to mid-exponential phase at an OD_{600} of 0.5 and mixed together in one liquid culture tube in a 1:1 ratio (t = 0 h). Serial dilutions were plated to ensure that the correct ratio was achieved. At several time points (t = 6, 22, 30 h), the OD_{600} and pH were measured, serial dilutions were plated for the purpose of CFU counting and the sample was sub-cultured in a 1:100 dilution of fresh media of the same kind. These samples measured the viability of the cells. At the same time, the original culture with a measured ratio of 1:1 was also incubated and subjected to all of the same measurements at the same time points. However, the media were never changed, nor was any buffer added. This sample measured the ability of the cells to persist over time. The *fruA* gene, which does not interfere with metabolism of glucose or galactose (20, 105), was used as a selective marker. In *S. gordonii*, *fruA* was replaced with an Em resistance cassette while in *S. mutans*, *fruA* was replaced with a Km resistance cassette. All conditions were tested in triplicate.

TV + 0.5% galactose without phosphate buffer

In TV supplemented with 0.5% galactose and no phosphate buffer, *S. gordonii* outcompeted *S. mutans* in both viability and persistence (Figure 5-4, 5-5). As far as viability is concerned, at t = 6 h both *S. gordonii* and *S. mutans* had grown in number, although the CFU count favored *S. gordonii*. After this point, *S. gordonii* growth appeared to stall as the CFU count remained the same throughout the rest of the viability assay. On the other hand, *S. mutans* consistently declined in numbers by one log at t = 22 and 30 h. The media became fairly acidic overnight at pH 4.8 but stabilized around pH 6.0 at the end of the assay (Table 5-2).

Results from the persistence assay, showed that at t = 22 h *S. gordonii* outnumbered *S. mutans* by only a small margin. At a pH of 4.7 the media became very acidic overnight (Table 5-3). By t = 30 h, *S. mutans* outnumbered *S. gordonii* by about one log difference.

TV + 0.5% galactose + 50 mM phosphate buffer

In TV supplemented with 0.5% galactose and 50 mM phosphate buffer, *S. gordonii* outcompeted *S. mutans* in both viability and persistence (Figure 5-4, 5-5). In terms of viability at t = 6 h, *S. gordonii* appeared to hold an advantage with a CFU count one log higher than *S. mutans*. The pH of the media was fairly neutral at 6.7 (Table 5-2). From this point on, *S. gordonii* continued to hold the advantage with the CFU count between the two strains widening further at each time interval. At t = 22 h, *S. gordonii* was more than two logs higher than *S. mutans*. Although the media had been buffered with fresh 50 mM phosphate buffer at t = 6 h, the culture was acidic in the morning, reaching a pH measurement of 5.4. At t = 30 h, there was again a two log difference between the two species as *S. gordonii* remained viable. The pH recorded was a neutral 6.9.

In the persistence portion of the assay, *S. gordonii* again gained the advantage and continued to grow in numbers until the final time point. However, the margin between the strains appeared to be slightly different from that of the viability portion of the assay. At t = 22 h, the *S. gordonii* CFU count was slightly higher than at t = 0 h. However, the *S. mutans* cells were severely depleted by two logs. Even though the pH measured 5.4 after overnight incubation, it was not enough to assist *S. mutans* in regaining strength. At t = 30 h, *S. gordonii* continued to persist while *S. mutans* decreased further by another two logs. The pH remained around 5.3 since no phosphate buffer had been added since t = 0 h (Table 5-3).

TV + 0.5% glucose without phosphate buffer

In TV supplemented with 0.5% glucose and no phosphate buffer, *S. gordonii* was still able to outcompete *S. mutans* at all time points in the viability test (Figure 5-4, 5-5). At t = 6 h, both strains had grown past their initial CFU counts. Despite the acidic conditions of pH 4.9, *S. gordonii* was able to sustain growth and maintain an advantage over *S. mutans* (Table 5-2). At t = 22 h, both strains had decreased similarly in number with a pH measurement of 4.6. The last time point, t = 30 h, showed no signs of change from t =22 h while the pH from this culture was less acidic at 6.6.

The persistence assay is the only assay that *S. mutans* was able to outcompete *S. gordonii*. At t = 22 h, *S. mutans* had grown slightly while *S. gordonii* cells already started to die. The pH at this point was 4.4 (Table 5-3). At t = 30 h, *S. mutans* cell counts remained similar to the previous time point while more *S. gordonii* cells continued to die. The pH did not change and was measured at 4.5.

TV + 0.5% glucose + 50 mM phosphate buffer

In TV supplemented with 0.5% glucose and 50 mM phosphate buffer, *S. gordonii* once more outcompeted *S. mutans* both in viability and persistence (Figure 5-4, 5-5). During the viability portion of the assay, *S. gordonii* began to show a slight advantage at t = 6 h. The pH of the culture at this time measured 5.8 (Table 5-2). At t = 22 h, a two log difference in cell count was recorded. Because the culture sat overnight, the pH dropped to 5.8. However, the acidic environment did not benefit *S. mutans*, which dropped two logs down in CFU count. Finally at t = 30 h, *S. gordonii* cell counts remained the same whereas *S. mutans* cell counts decreased further. A final pH of 6.2 indicated that the phosphate buffer was indeed neutralizing most of the acid in the culture tube.

Although the persistence results for this condition still indicate that *S. gordonii* outcompeted *S. mutans*, the cell count ratio is altered slightly from that seen in the persistence assay for the galactose plus phosphate condition. At t = 22 h, the *S. gordonii* CFU count climbed 4 logs higher than the original culture at t = 0 h. Despite this large increase, the *S. mutans* CFU count also rose 1.5 logs. So instead of dying or lysing, *S. mutans* was still able to persist, albeit less so than *S. gordonii*. The culture was acidic with a pH of 5.8 (Table 5-3). At t = 30 h, it appears that both strains were beginning to die or lyse, although *S. gordonii* still reigned. However, both strains still maintained CFU counts that were higher than the original culture count. The pH dropped only slightly to 5.7.

Discussion

In TV – 0.5% galactose, *S. gordonii* grew about 2.5 times faster than *S. mutans*. In addition, final yield was much higher in *S. gordonii*. As previously mentioned, our lab

discovered that *S. gordonii* is able to grow well in galactose at 10-fold lower concentrations than *S. mutans* UA159. Both of these pieces of data suggest that *S. gordonii* may be better equipped than *S. mutans* UA159 to utilize galactose, as evidenced by both a faster growth rate, higher final yield and seemingly higher affinity and specificity for this hexose. In comparison, *S. gordonii* grew about 1.5 times as fast as *S. mutans* when grown in TV – 2% galactose. *S. mutans* produced a slightly higher final yield and faster growth rate compared to its growth in 0.5% galactose. In contrast, *S. gordonii* grew slower in 2% galactose than it did in 0.5% galactose.

This substantial change in growth rate of *S. gordonii* in varying galactose concentrations could be attributed to the change in relative participation between the galactose PTS and ATP-dependent galactose permease. If the galactose PTS is indeed a high-affinity transporter, then the cells might actually grow better in lower concentrations of galactose as opposed to higher concentrations if the cells switch to a lower affinity, lower capacity transporter at high galactose concentrations. Specifically, in high concentrations of galactose, activation of the presumably lower-affinity galactose permease (and possibly lactose- and mannose-PTS) would permit growth, albeit at a slower rate. In the case of *S. mutans*, transport of galactose occurs via the lactose and mannose PTS, which appear to have about 100-fold lower affinity for galactose than for their cognate PTS substrates (103).

It has been shown in *Saccharomyces cerevisiae* that there is a reciprocal relationship between high- and low-affinity transport systems wherein cells grown in high percentages of glucose almost exclusively display activity from low-affinity transporters while cells grown in low percentages of glucose displayed activity mainly

from high-affinity transporters (77). Furthermore, multiple studies have reported that both *Streptococcus cremoris* and *Streptococcus lactis* possess a galactose PTS that is low-affinity in nature due to cell requirements for high concentrations of substrate for maximal growth rates (57, 88, 89). *S. cremoris* was also found to predominantly transport galactose through the galactose PTS as opposed to the galactose permease (88). In contrast, it has been suggested by Thomas and co-workers (88) that *S. lactis* strain ML_3 uses a high-affinity permease to transport galactose based their results indicating less dependency on galactose concentration by this strain and Thompson and co-workers (89) findings that the affinity for the galactose permease was 10-fold higher than for the galactose PTS. Therefore, the slower growth rate of *S. gordonii* in 2% galactose (compared to 0.5% galactose) could be indicative of activation of the lower-affinity galactose permease and lactose- and mannose-PTS whereas the faster growth rate of *S. gordonii* in 0.5% galactose (compared to 2% galactose) is indicative of activation of the high-affinity galactose PTS.

Results for the competition assay predominantly favored *S. gordonii* in both the viability and persistence portions of the experiment. In evaluating the viability of the cells, *S. gordonii* showed a strong ability to maintain survival and recover from stress no matter which sugar it was grown in. *S. mutans* grew faster in glucose conditions, indicating its preference for glucose over galactose substrates; however, *S. gordonii* appeared to grow equally well in both sugars, indicating its enhanced utilization of galactose. At t = 6 hrs, all testing conditions appeared to show similar growth patterns for both *S. mutans* and *S. gordonii*. This indicates that in the first 6 hours of co-habitation neither sugar-type nor acid production affected competition between *S.*

mutans and *S. gordonii*. However, as time progressed, it became evident that *S. gordonii* was able to outcompete *S. mutans* with regards to sugar type. Although *S. gordonii* grew better in both glucose and galactose, it is clear that the commensal displayed enhanced growth in galactose that likely contributed to its competitive edge. In contrast, *S. mutans* grew considerably less in galactose, especially when the media was buffered. In regards to acid production, *S. gordonii* was able to tolerate the stress despite acidic conditions in unbuffered media. This suggests that *S. gordonii* has some mechanism to combat acid production in its surroundings. In fact, it has been shown in *S. gordonii* that H_2O_2 production is insensitive to changes in pH between 5.0 and 7.5 (12). Therefore, the release of hydrogen peroxide by *S. gordonii* may likely have contributed to the decrease in *S. mutans* cells via oxidative stress. Based on the pH measurements from this assay, production of acid is decreased when the cells are grown in galactose compared to glucose. There are two plausible explanations for this: (1) because *S. gordonii* is better able to compete against *S. mutans* in galactose, the increase in the number of *S. gordonii* cells (and subsequent decrease in number of *S. mutans* cells) means that less acid overall is produced, or (2) *S. gordonii* displays enhanced H_2O_2 production when grown in galactose. In contrast, it appears that bacteriocin production by *S. mutans*, if any, did not affect the ability of *S. gordonii* to stay viable.

When evaluating the ability to persist, *S. gordonii* was only able to effectively compete with *S. mutans* when phosphate buffer was added to the media. In regards to carbohydrate-type, *S. gordonii* still maintained a competitive advantage over pathogenic *S. mutans*; however, this advantage was only limited to buffered medium, although

severity of the final outcome was decreased in unbuffered medium . This suggests that while *S. gordonii* is still able to outcompete *S. mutans* when galactose is present, the commensal cannot persist without continual removal of acid from the environment. This aligns with the acid-sensitive phenotype of *S. gordonii*. Therefore, despite the advantage that enhanced galactose metabolism confers to *S. gordonii*, this mechanism alone is not enough to overcome the effects of too much acid. It should be noted, however, that saliva flow inside the mouth contributes greatly to the diffusion and level of acid and can also be considered a constant source of carbohydrate, especially galactose. With that in mind, the viability portion of this competition assay likely emulates an *in vivo* setting more closely than the persistence portion. This data also suggests that the decreased acid production when the cells are grown in galactose may be more due to the fact that *S. gordonii* outnumbers *S. mutans* rather than enhanced H_2O_2 production by *S. gordonii* when grown in galactose. Once *S. mutans* gained a competitive advantage over *S. gordonii*, bacteriocin production likely may have also contributed to the persistence of the pathogen.

Table 5-1. Calculated doubling times of the *S. gordonii* DL1 wild-type and *S. mutans* UA159 wild-type strains based on growth curve analysis.

	0.5% Glucose T_d	0.5% Glucose OD_{600}	0.5% Galactose T_d	0.5% Galactose OD_{600}	2% Galactose T_d	2% Galactose OD_{600}
DL1	88 ± 2.3	0.61	170 ± 30	0.84	240 ± 3.1	0.87
UA159	180 ± 17	0.56	410 ± 61	0.52	340 ± 22	0.64

T_d is defined as the doubling time.
OD_{600} is defined as the optical density measured at a wavelength of 600 nm in a spectrophotometer.

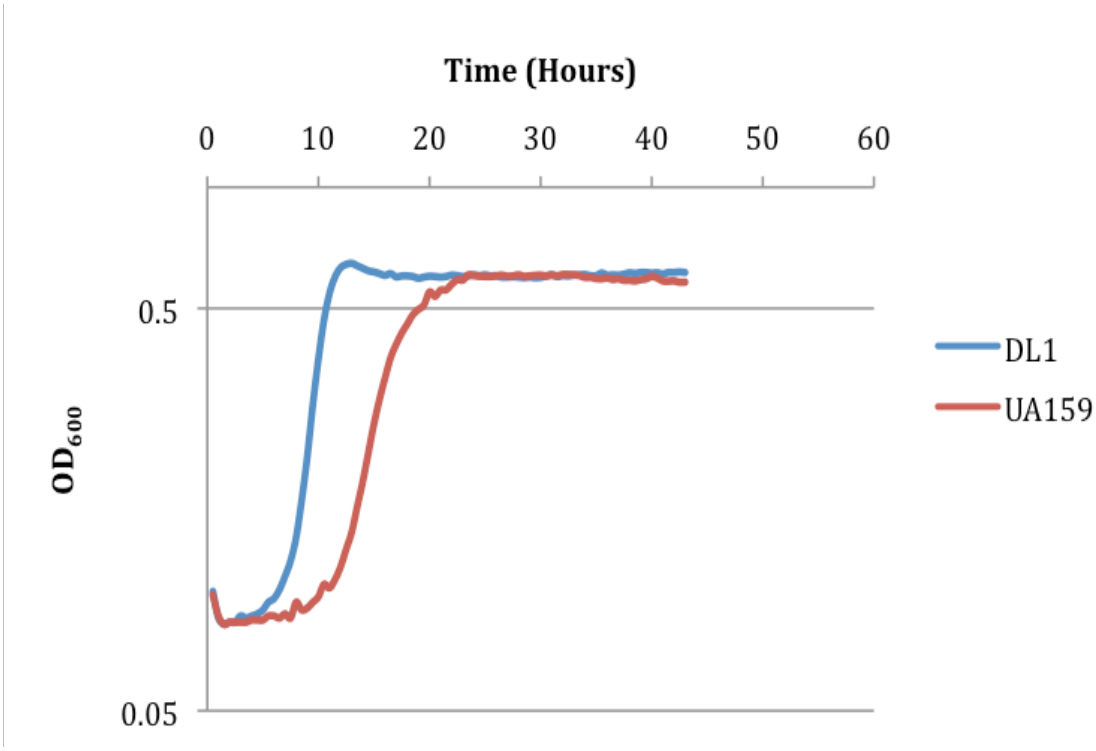


Figure 5-1. Growth of *S. gordonii* DL1 and *S. mutans* UA159 in TV – 0.5% Glucose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 5-1.

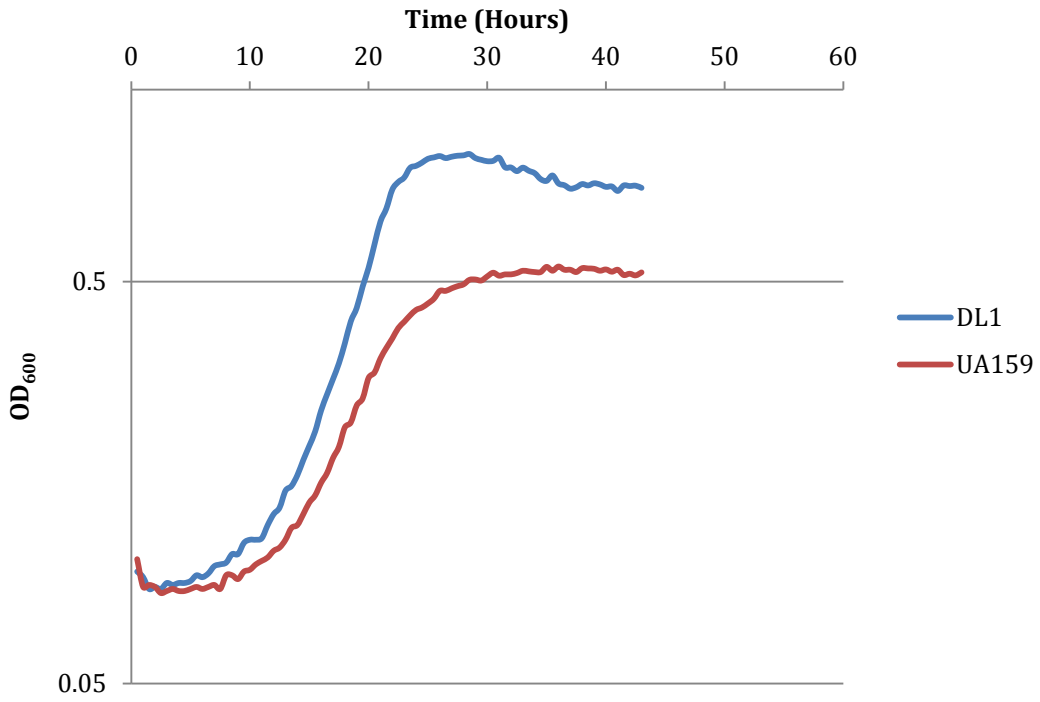


Figure 5-2. Growth of *S. gordonii* DL1 and *S. mutans* UA159 in TV – 0.5% Galactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 5-1.

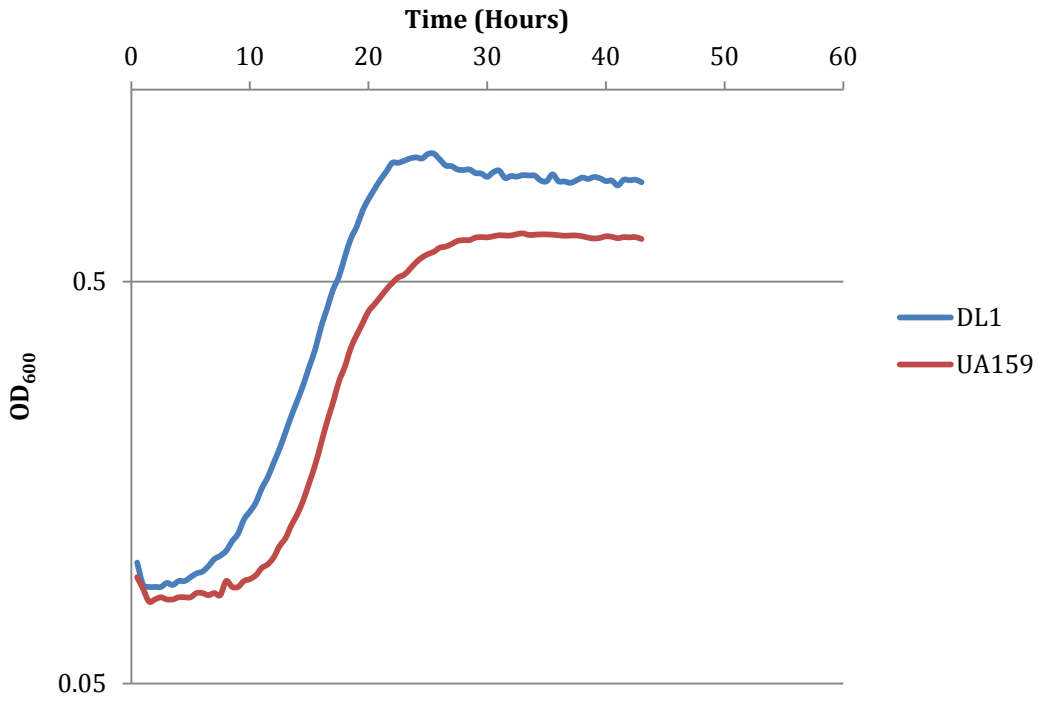


Figure 5-3. Growth of *S. gordonii* DL1 and *S. mutans* UA159 in TV – 2% Galactose with an oil overlay. Optical density at 600 nm was measured every 30 min using a Bioscreen C™. Each point represents the mean of three separate cultures in triplicate. Doubling times and standard deviations are located in Table 5-1.

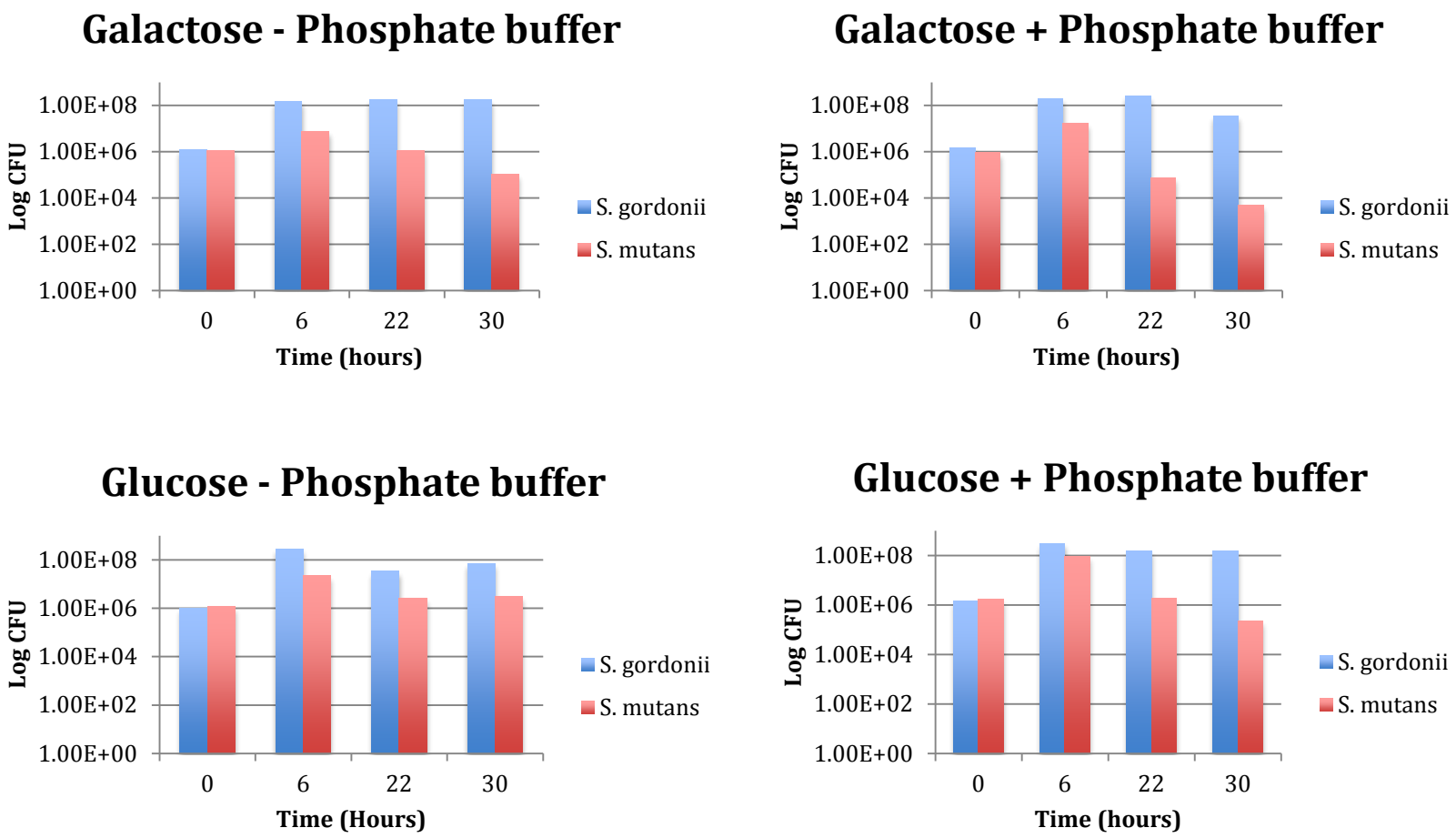


Figure 5-4. Mixed-species Competition Assay testing viability of the *S. gordonii* DL1 and *S. mutans* UA159 strains when grown in TV supplemented with 0.5% glucose or galactose, with or without phosphate buffer. *S. gordonii* and *S. mutans* cells were grown separately to mid-exponential phase in triplicate, mixed together in a 1:1 ratio and incubated at 37°C, 5% CO_2. At time points t = 6, 22, 30 h, the cultures were diluted into fresh media. In addition, at t = 0, 6, 22, 30 h, the optical density and pH of each mixed-species culture was measured and serial dilutions were plated for enumeration of the strains individually on selective media.

Table 5-2. Average measured pH values from the mixed-species competition assay testing viability.

Time	Galactose + Phosphate	Galactose - Phosphate	Glucose + Phosphate	Glucose - Phosphate
t = 6 hours	6.7	5.8	5.8	4.9
t = 22 hours	5.4	4.8	5.8	4.6
t = 30 hours	6.9	6.0	6.2	6.6

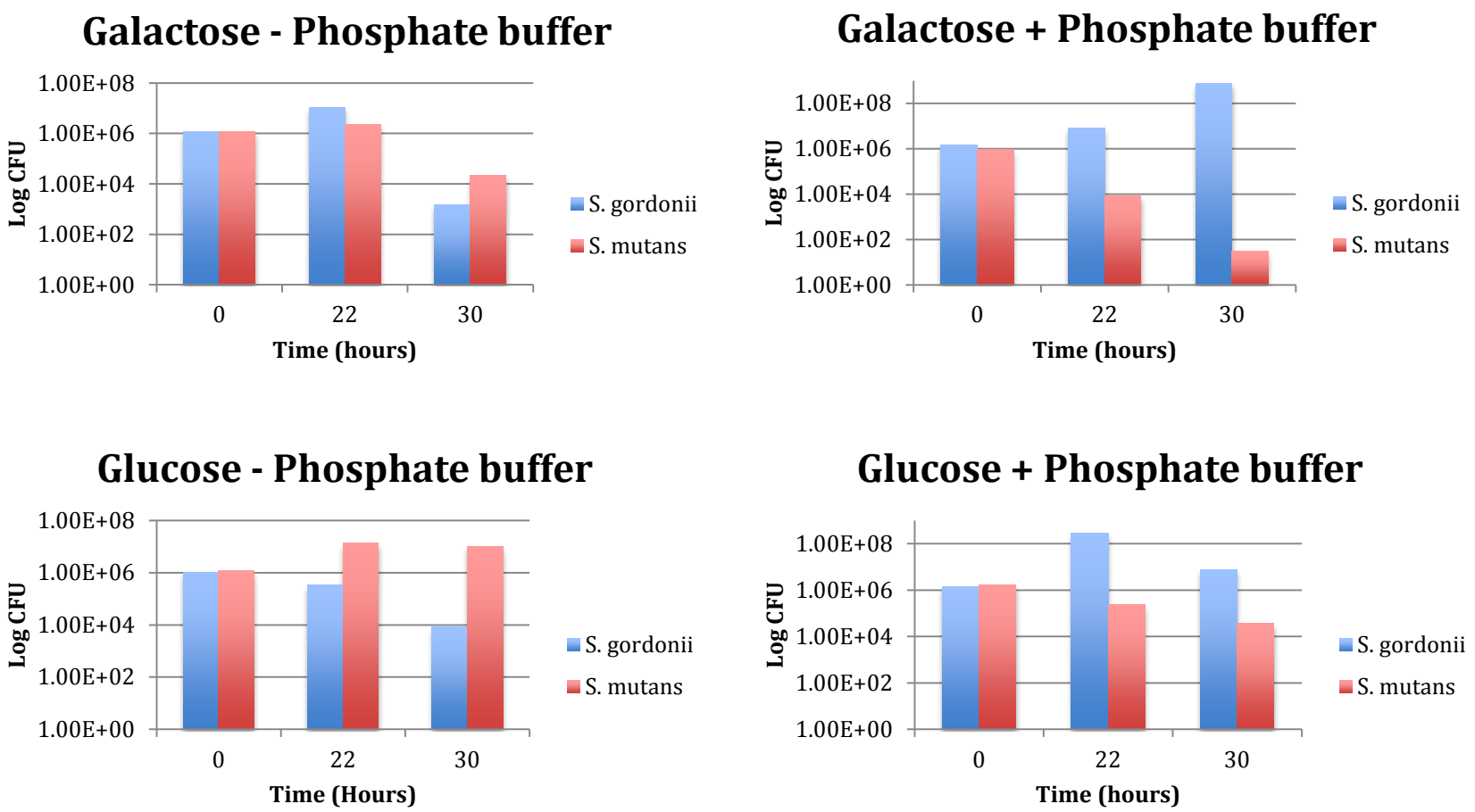


Figure 5-5. Mixed-species Competition Assay testing persistence of the *S. gordonii* DL1 and *S. mutans* UA159 strains when grown in TV supplemented with 0.5% glucose or galactose, with or without phosphate buffer. *S. gordonii* and *S. mutans* cells were grown separately to mid-exponential phase in triplicate, mixed together in a 1:1 ratio and incubated at 37°C, 5% CO_2. At time points t = 0, 6, 22, 30 h, the optical density and pH of each mixed-species culture was measured and serial dilutions were plated for enumeration of the strains individually on selective media.

Table 5-3. Average measured pH values from the mixed-species competition assay testing persistence.

Time	Galactose + Phosphate	Galactose - Phosphate	Glucose + Phosphate	Glucose - Phosphate
t = 22 hours	5.4	4.7	5.8	4.4
t = 30 hours	5.3	4.7	5.7	4.5

CHAPTER 6
SUMMARY AND FUTURE DIRECTIONS

Summary

Dental plaque is an organized and complex structure with diverse microbial communities (28). Bacterial gene expression is constantly affected by frequent changes in the oral cavity such as nutrient availability, pH, oxygen tension and bacteriocin production (28, 66). Those microbes capable of adapting and persisting in stressful environmental conditions ultimately determine which species will inhabit the biofilm. Commensal organisms are associated with good oral health. In contrast, cariogenic *S. mutans* and other lactic acid bacteria are often isolated from carious lesions (30). Therefore, it is desirable to shift the equilibrium towards commensal bacteria such as *S. gordonii*.

After learning that *S. gordonii* grows well in galactose at concentrations 10-fold less than *S. mutans*, a review of the genome revealed two tandem gene clusters containing duplicate copies of the tagatose-6-phosphate pathway genes, a lactose PTS and a galactose PTS as well as a repressor, transcriptional antiterminator and phospho-β-galactosidase. One cluster appears to be dedicated to lactose metabolism while the other may be dedicated to a high-affinity galactose transport system. If the *S. gordonii* genome does encode a galactose PTS capable of high-affinity transport, it could likely give the cell a selective advantage over pathogenic bacteria such as *S. mutans* and provide a potential mechanism for directing the microbiome towards health.

Experiments described in Chapter 3 showed the initial characterization of the genes in the lactose and galactose clusters by observing growth and measuring the transcription of two representative genes (one gene from each cluster). Major findings

included that the transport of both lactose and galactose occurs by the lactose PTS, identification of a role for LacT in the expression of the lactose transporter, a function for *lacG* in regulating induction of the operons, verification that the two sets of tagatose pathway genes are the only way that lactose and galactose can be metabolized and demonstrating that increased expression of the galactose transporter is observed when cells are grown in galactose.

Further investigation into the regulation of the *lac* gene clusters was described in Chapter 4. Two potential promoters were found, one in front of *lacA1* and the other in front of *lacA2*. Promoter gene fusion experiments showed that both *lacA1* and *lacA2* have promoter activity. Continued analysis revealed that LacR acts as a negative regulator for both promoters. However, the *lacA2* promoter is under the additional negative regulation of the global regulator of CCR, CcpA. A role for LacG was described in galactose metabolism whereby LacG is posited to convert Gal-6-P into some other form that would act as an inducer for LacR. Real-Time PCR data also suggested that LacT may negatively regulate *lacR* in some way. An *in vitro* binding assay showed that LacR is capable of binding to both the *lacA1* and *lacA2* promoters.

Chapter 5 outlined a mixed-species liquid culture competition assay between *S. gordonii* DL1 and *S. mutans* UA159 grown in galactose and glucose with and without phosphate buffer. Results indicated that *S. gordonii* is able to compete effectively against *S. mutans* under conditions testing viability. The ability of *S. gordonii* to stay viable was dependent on the type of sugar in the environment. Growth in galactose gave a competitive advantage to the commensal versus growth in glucose. When testing the ability to persist, *S. gordonii* was only able to outcompete *S. mutans* if the

media was buffered, whereas unbuffered media gave *S. mutans* a competitive advantage. Therefore, persistence appears to be dependent on the ability to survive in an acidic environment and growth in galactose alone was not effective enough for *S. gordonii* to outcompete *S. mutans* at low pH. This assay is limited in scope, as it only reflects the interactions of two oral microbial species and was performed *in vitro* as opposed to *in vivo*. It is possible that in an *in vivo* environment the cells would behave differently. For example, *S. gordonii* may gain an enhanced ability to survive at low pH when grown in galactose. In this case, the commensal could then continue to compete against *S. mutans* when the pH increased. Despite these limitations, the viability portion of the competition assay more accurately reflects the natural environment of the oral cavity due to the nature of saliva flow. In that case, *S. gordonii* would be a good candidate organism for use in shifting the ecological balance to one of oral health via its high-affinity galactose PTS.

Future Directions

The *S. gordonii lac* genes presented here introduce a novel genetic organization of a system that is widespread in both Gram-positive and Gram-negative bacteria. What appear to be multiple layers of regulation only constitute the beginning of this story. In the future, this research can be branched into multiple directions.

A more thorough characterization of the galactose transporter is necessary to confirm that EII^{Gal} is indeed a high-affinity transporter. In addition, measuring the gene expression of EII^{Lac} and EII^{Man} as well as the Leloir pathway enzymes in a *ΔEII*^{Gal} strain may provide some insight into the high-affinity nature of the galactose transporter and possible explain the growth data concerning this strain. Northern blot experiments or Reverse Transcriptase PCR will reveal whether the *lacTEFG* genes are transcribed as

an operon or separately. The long chain formation and clumping phenotype expressed by the *lacR* mutant suggests that LacR is an important protein that probably has far-reaching effects across the genome and warrants further examination.

It is also important to continue the investigation into how LacR interacts with and regulates the *lacA1* and *lacA2* promoters. The 5'RACE method would allow for mapping of the *lacA1* and *lacA2* promoters. Exploration of the deeper layers of regulation that have been suggested here can provide a role for LacT and LacG as regulators. Investigation into the putative *lacT* and *lacR* promoters should include promoter gene fusions to evaluate promoter activity in various deletion strains and Real-Time PCR to measure gene expression of suspected regulators. Since there is a cre sequence in the promoter of *lacT*, CcpA regulation over this promoter should be investigated as well.

The competition assay could be expanded further by performing the same assay between the *S. gordonii* wild-type strain and the ΔEII^{Gal} strain using varying concentrations of galactose. With this kind of setup it would be possible to evaluate high-vs low-affinity transport by adjusting the galactose concentration. In order to better imitate an *in vivo* model, the competition assay can also be repeated in a biofilm culture rather than a planktonic culture.

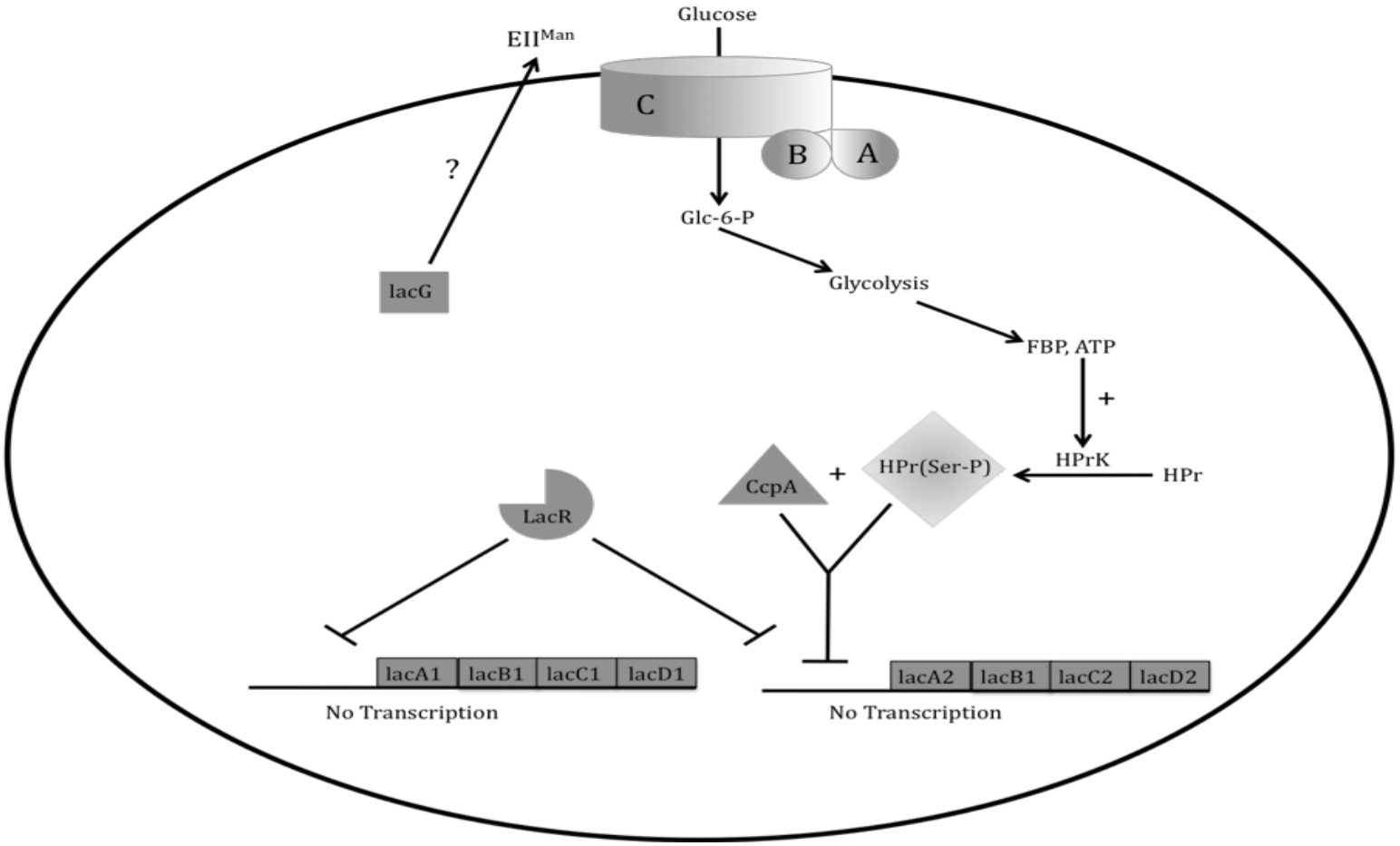


Figure 6-1. Model depicting LacR and carbohydrate catabolite repression of the *lacA1* and *lacA2* promoters in *S. gordonii* when glucose is present. Binding of the LacR repressor to the *lacA1* and *lacA2* promoters inhibits transcription of both sets of tagatose pathway genes (*lacABCD1*, *lacABCD2*). In addition, Glc-6-P is converted to FBP and ATP, both of which activate the enzyme HPrK/P to phosphorylate HPr and produce HPr(Ser-P). CcpA binds to HPr(Ser-P) and together these two proteins co-repress the *lacA2* promoter to further prevent transcription of the second set of tagatose genes.

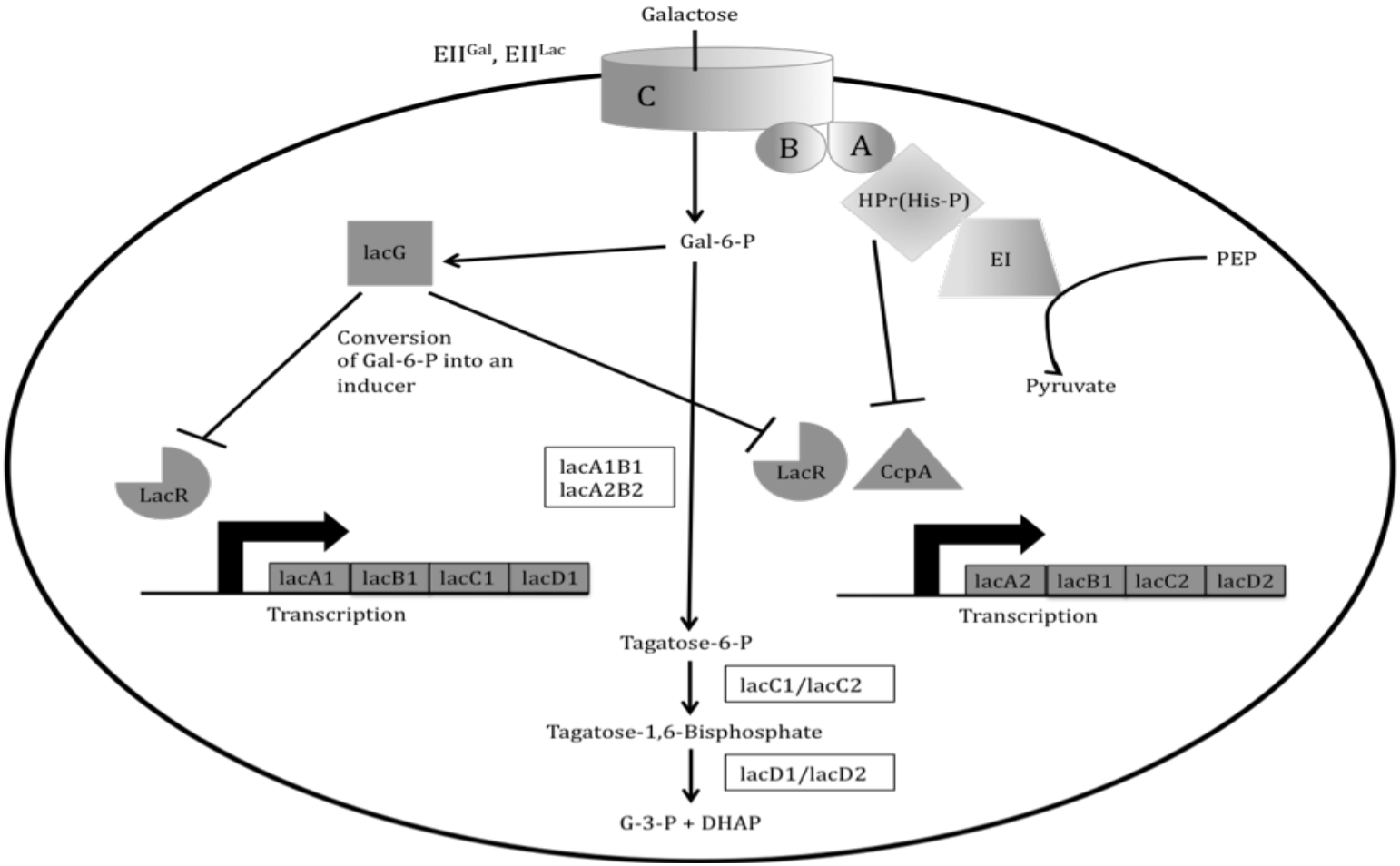


Figure 6-2. Model depicting the regulation of the *lacA1* and *lacA2* promoters in *S. gordonii* when galactose is present. Binding of an unknown inducer molecule to LacR causes an inhibition of repression of the *lacA1* and *lacA2* promoters, resulting in transcription of the tagatose genes. LacG has been proposed to convert or alter the incoming sugar, Gal-6-P, into the unknown inducer molecule. Furthermore, CcpA is inhibited from binding to the *lacA2* promoter due to low availability of the co-repressor HPr(Ser-P). With Gal-6-P present in the cell and transcription of the tagatose genes uninhibited, metabolism of galactose ensues.

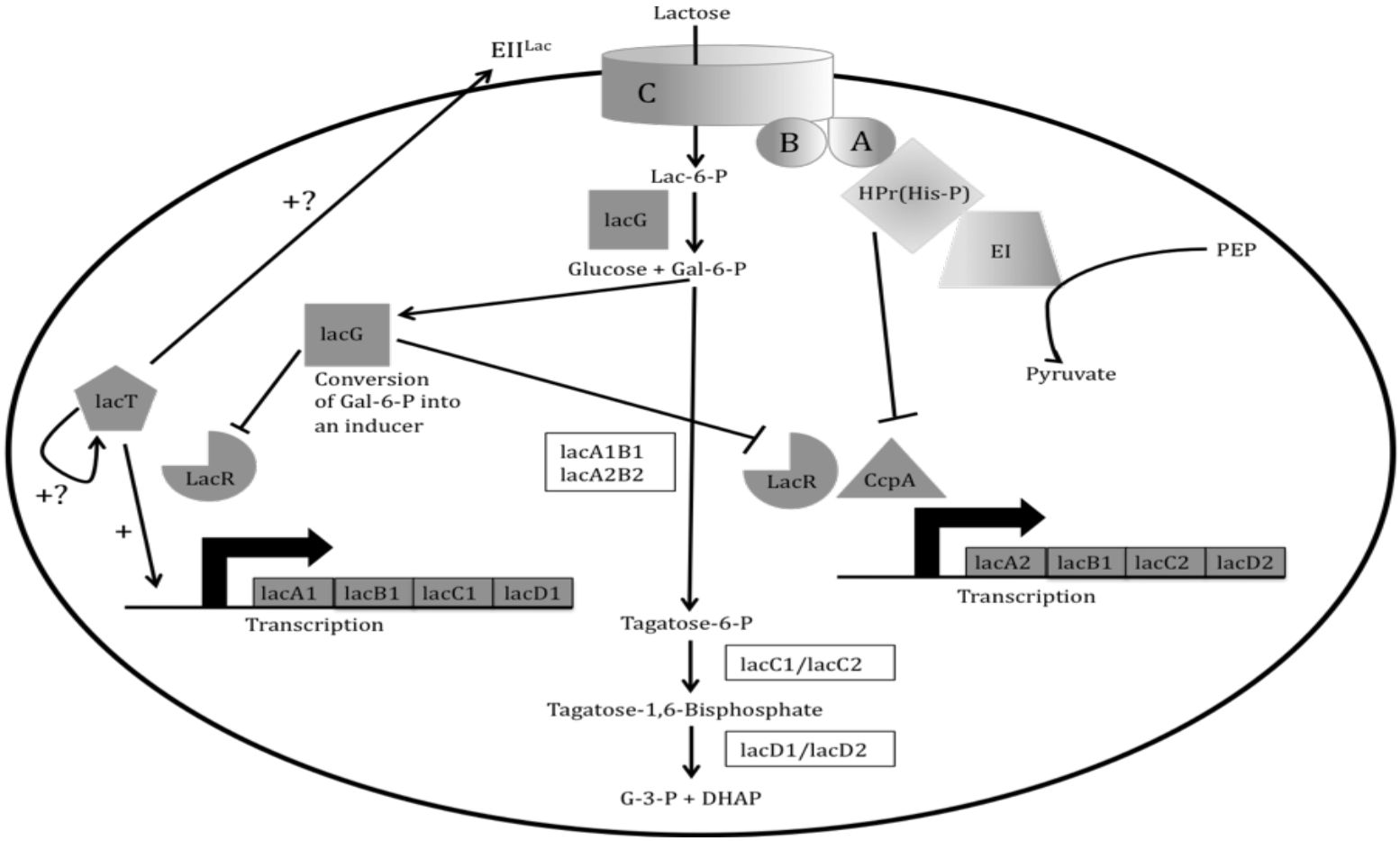


Figure 6-3. Model depicting the regulation of the *lacA1* and *lacA2* promoters in *S. gordonii* when lactose is present. Two separate mechanisms inhibit repression of the *lacA1* and *lacA2* promoters and allow transcription of the tagatose genes: (1) after Lac-6-P is catabolized into glucose and Gal-6-P, LacG converts Gal-6-P into an unknown inducer molecule that binds to LacR and inhibits binding to both promoters, (2) CcpA is inhibited from binding to the *lacA2* promoter due to low availability of the co-repressor HPr(Ser-P). In addition, LacT activates the *lacA1* promoter to increase transcription of the first set of tagatose genes. With transcription of both sets of tagatose genes occurring, the Gal-6-P present in the cell (originating from lactose uptake) can be metabolized.

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BIOGRAPHICAL SKETCH

Nicole Christine Martino was born in 1986 in Fort Lauderdale, Florida. The eldest of three children, she spent 18 years in South Florida with her family before graduating from Boyd H. Anderson High School in 2004 with a diploma from the International Baccalaureate (IB) Programme.

She earned her B.S. in Food Science and Human Nutrition with an emphasis on Human Nutrition from the University of Florida (UF) in 2008. During this time, she contributed to dental-related research on the etiology of canker sores with Dr. Lorena Baccaglini. In October 2007 and April 2008, she presented an abstract and poster of her work at the Hispanic Dental Association (HDA) annual meeting and American Association for Dental Research (AADR) annual meeting, respectively.

After taking a year off to work and travel, Nicole enrolled as a graduate student in the UF College of Medicine. Working towards a Master of Science degree, she completed research under the supervision of Dr. Robert A. Burne for two years. In May 2010, she presented her work in an abstract and poster at the American Society for Microbiology (ASM) General Meeting.

Upon completion of her M.S. program, Nicole plans to publish a journal article related to her recent work in Dr. Burne's laboratory and will be attending the UF College of Dentistry (UFCD) in Gainesville, Florida in order to earn a D.M.D. degree and enter the professional field of dentistry.