ACID TOLERANCE AND THE INVOLVEMENT OF SECONDARY METABOLISM IN *STREPTOCOCCUS MUTANS*

By

Bryan M Korithoski

A thesis submitted in conformity with the requirements for the

Degree of Doctor of Philosophy

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ABSTRACT

Dental caries is the dissolution of tooth enamel caused by the acidic end-products produced from bacterial metabolism of dietary carbohydrates. *Streptococcus mutans*, a ubiquitous constituent of human dental plaque, is an etiological agent of dental caries. Two of *S. mutans*' cariogenic attributes are acidogenicity and aciduricity. Acidogenicity is the ability to produce acids from carbohydrate catabolism. Furthermore, *S. mutans* has developed a complex response network allowing it the ability to survive in this acidic environment, termed aciduricity. Studies have shown regulation at the genomic and proteomic levels is paramount in aciduricity for *S. mutans* (29, 91, 92, 153, 168, 170). Among the pathways showing up-regulation to acid were the citrate metabolism pathway (29), the glyoxalase pathway (91, 170), and the pyruvate dehydrogenase pathway (168).

The goal of this work was to investigate the mode of acid tolerance for three secondary metabolic pathways; citrate transport and metabolism, the glyoxalase pathway (focused on LGL) and pyruvate dehydrogenase (focused on PdhA) in *S. mutans*.

S. mutans is capable of citrate transport, via the CitM transporter. Transport is most efficient when coupled with Fe^{3+}. The end-product of citrate metabolism is aspartate. The citrate metabolic pathway includes citrate lyase as citrate accumulates in a citrate lyase

knockout mutant. Citrate was found to be toxic to *S. mutans*, however, pre-exposure to citrate resulted in increased acid tolerance.

LGL is involved in the detoxification of methylglyoxal, illustrated by the absence of enzyme activity in an *lgl* mutant (LGLKO), hypersensitivity of LGLKO to methylglyoxal, and increased transcription of *lgl* in the presence of exogenous methylglyoxal.

Transcriptional analysis revealed *pdhA* responded to conditions favouring heterofermentation; decreased expression in response to excess glucose, and increased expression during stationary growth phase compared with mid-logarithmic phase growth.

The isogenic mutants, LGLKO and PDHAKO, were acid-sensitive. Both mutants had reduced growth capacity at pH 5.0, decreased survival at pH 3.2, and decreased survival in an acidic bio-fermenter compared with wild-type. Both genes were up-regulated during growth at pH 5.0 compared with growth at pH 7.5 and during the process of acid adaptation.

This work highlighted the importance of these three secondary metabolic pathways in the aciduricity of *S. mutans*.

PREFACE

Dissertation Format

This Ph.D. dissertation is written in a publication style format. Chapter 1 is a general introduction providing context for the experimental work presented in this dissertation based on past publications and the most current knowledge in the field. Chapters 2 and 3 describe experimental data published as full length, peer reviewed manuscripts in the Journal of Bacteriology. Chapter 4 is a manuscript submitted for publication to FEMS Microbiology Letters. Chapters 2 - 4 have been modified slightly from the versions published to improve the readability of this dissertation. Information presented in the Supplemental Data sections of Chapters 2 - 4 has not been submitted for publication. Chapter 5 contains the general conclusions that can be drawn from the experimental data, as well as a general discussion, and possible future work. Publications cited in each chapter appear at the end of each chapter.

Publications from the Dissertation Chapters

1. **Korithoski, B.**, Krastel, K., Cvitkovitch, D.G., (2005). Transport and metabolism of citrate by *Streptococcus mutans*. J. Bacteriol. 187:4451-4456

2. **Korithoski, B.,** Lévesque, C.M., Cvitkovitch, D.G. Involvement of the Detoxifying Enzyme Lactoylglutathione Lyase in *Streptococcus mutans* Aciduricity. J. Bacteriol. Aug. 24, 2007. [Epub ahead of print].

3. **Korithoski, B.,** Lévesque, C.M., Cvitkovitch, D.G. The Involvement of the Pyruvate Dehydrogenase E1α subunit in *Streptococcus mutans* Acid Tolerance. Submitted to FEMS Microbiol. Lett.

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Awards

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LIST OF ABBREVIATIONS

AGEs	Advanced Glycation End-products
ATP	Adenosine Triphosphate
ATR	Acid Tolerance Response
BB	Berman's Broth
cDNA	Complementary DNA
CO_2	Carbon Dioxide
COHN	An anaerobic atmosphere of 10% CO_2, 10% H_2, and 80% N_2
CSP	Competence Stimulating Peptide
Ct	Cycle Threshold
D	Dilutions per hour
EDTA	Ethylenediaminetetraacetate
Ext_{coeff}	Extinction Coefficient
Ftf	Frucosyltransferase
g	The force of Gravity
Gbp	Glucan Binding Protein
Gtf	Glucosyltransferase
h	Hour
IPS	Intracellular Polysaccharide
kDa	Kilo-Dalton
KOH	Potassium Hydroxide
LAB	Lactic Acid Bacteria
LGL	Lactoylglutathione Lyase
MG	Methylglyoxal
MIC	Minimum Inhibitory Concentration
MM4	Minimal Media 4
mRNA	Messenger RNA
MS	Mutans Streptococci
Msm	Multiple Sugar Metabolism
OAD	Oxaloacetate decarboxylase
OD	Optical Density
PCR	Polymerase Chain Reaction
PDH	Pyruvate Dehydrogenase
PEP:PTS	Phosphoenolpyruvate Phosphotransferase Transport System
PFL	Pyruvate-Formate Lyase
PIPES	piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)
PK solution	1% Peptone, 1% Potassium Chloride
qRT-PCR	Quantitative Real-Time PCR
RNA	Ribonucleic acid
TCA	Trichloroacetic Acid
THYE	Todd Hewitt/Yeast Extract
TLC	Thin Layer Chromatography

Main Body of Dissertation

CHAPTER I: LITERATURE REVIEW

I. *S. MUTANS* AND THE DENTAL PLAQUE ENVIRONMENT

A). Oral Microbiology and Plaque:

The oral cavity is an unusual human microbial habitat because it contains hard, permanent surfaces (ie. teeth) and is easily accessed by external environmental sources. The teeth allow for the build up of large quantities of bacteria, and their extracellular products, especially at stagnant or retentive sites. The accumulation of bacteria along with their associated extracellular products is collectively referred to as dental plaque. The dental plaque is a hostile ecosystem in which bacteria must withstand inhospitable environmental insults including variable nutrient availability, oxidative stress, pH fluctuations, and physical sheer forces.

Dental plaque is a typical biofilm that consists predominantly of bacteria encapsulated in a polysaccharide matrix. This matrix is comprised of polymers that are of both salivary and microbial in origin. The bacterial composition of plaque is both highly structured and variable, and is know to contain over 400 different bacterial species (128). The microflora of plaque also varies in composition from site to site. Subgingival plaque (below the gum line) is mostly supported by nutrients provided by the gingival crevicular fluid and is dominated by gram-negative, anaerobic bacteria, including members of the genera, *Propionibacterium, 'Bacteroides', Fusobacterium, Prevotella, Selenomonas* and *Veillonella* as well as the gram-positive *Actinomyces* and *Peptostreptococcus* (145, 164). Supragingival plaque (above the gum line) is characteristically inhabited by gram-positive bacteria of the genera *Streptococci* and *Actinomyces* (125). Supragingival plaque can be further divided into smooth surface plaque and fissure plaque. Due to its inaccessibility

fissure plaque is enriched, compared with smooth surface plaque, with anaerobic, acid-tolerant bacteria chiefly *streptococci* (157).

Marsh and Bradshaw (108) arbitrarily divided the maturation and formation of dental plaque into the following six-step process: 1) the formation of the acquired pellicle on the tooth surface; 2) a non-specific reversible phase involving physicochemical interactions between salivary bacteria and the pellicle; 3) short-range specific stereo-chemical molecular interactions between primary bacterial colonizers and host receptor molecules in the acquired pellicle; 4) co-aggregation of secondary colonizers to already attached primary colonizers; 5) development of horizontal and vertical stratification within the developing biofilm, and increased bacterial succession; and 6) growth and the formation of a climax community (108).

Central to plaque formation is the ability of the bacteria to attach to the tooth surface or the polysaccharide matrix. Oral bacteria do not bind to the teeth directly but adhere to the acquired pellicle (60). The pellicle is a thin coating covering the teeth and is composed primarily of host derived salivary glycoproteins, mucins, amylase, statherin, immunoglubulins, proline rich proteins, as well as other proteins (3). The constituents of the pellicle act as specific receptors for bacterial adhesion, and therefore selectively influence the dental plaque development. Most oral bacteria are nonmotile, and therefore the early colonizers are transported passively to the tooth surface via salivary flow. As a bacterium approaches the pellicle, long-range physico-chemical forces provide for weak, non-specific, reversible attachment (23, 136). The attachment can be reinforced by shorter range stereo-chemical interaction between the pellicle and bacterial surface structures such as fimbriae (31).

It has been shown that specific pellicle proteins bind to specific bacteria. For example α-amylase binds *Streptococcus gordonii* (49, 137), PRP-1, a proline-rich protein, binds to *Streptococcus gordonii* and *Actinomyces naeslundii* (61, 62), and statherin binds *A. naeslundii* and *Fusobacterium nucleatum* (62, 87). Several bacterial species, including *S. oralis* and *S. sanguis* produce lectin-like binding proteins capable of binding to pellicle-associated glycoproteins (113, 122, 142).

This ability of bacteria to interact with other bacterial species, especially with the early colonizers already attached to the tooth surface, is an important mechanism in the development of a complex, mature dental plaque. This cell-cell adhesion, termed co-aggregation, is a process by which almost all plaque bacteria have been found to co-aggregate with at least one other bacterial species (88). *Fusobacteria* co-aggregate with the widest range of bacterial genera, and it has been proposed that *Fusobacteria* act as a bridge between early- and late-colonizing plaque bacteria (86, 87).

Once the early-colonizing bacteria have adhered to the tooth enamel, via the salivary pellicle, they grow and produce exo-polymers, forming a confluent plaque biofilm (109). The metabolism by early colonizers, feeding on the constituents of saliva, results in changes in the plaque micro-environment, such as decreased oxygen concentration and lowered redox potential. These changes allow for obligate anaerobes such as *Porphyromonas gingivalis* and *Prevotella intermedia* to colonize the plaque (109). Further maturation results in a dynamic climax community within dental plaque. Once the climax community has been established, the composition of the microbial constituents remains remarkably unchanged (107). Like other biofilms, a mature dental plaque has its own architecture containing open channels facilitating the passage, and creating gradients, of nutrients, water, and enzymes (171). The

close proximity within the plaque allows for increased and novel nutrient utilization, cell-cell interactions, such as quorum sensing, and genetic transfer (40, 42). It is believed a healthy mature dental plaque can help prevent colonization by other foreign, more pathogenic bacteria. Additionally it has been proposed that oral diseases, such as periodontitis and dental caries, are the result of imbalances within this community (110).

Phenotypic and genotypic studies have shown that the mother or major care-giver is the primary source of *S. mutans* acquisition for children infected with dental caries (63, 84, 85, 95), with the transfer mainly occurring via saliva (83, 85). Also, *S. mutans* colonization seems to have a window of infectivity, with establishment within the plaque occurring shortly after the eruption of the primary dentition (median age of 26 months) (28). *S. mutans* can under favourable conditions, such as a high carbohydrate diet, dominate this ecological niche. The domination of this niche by *S. mutans* and its corresponding acid production often leads to the dissolution of the tooth enamel hydroxyapatite, and subsequent progression of dental caries. The epidemiology and microbiological nature of dental caries is discussed in depth in the following section.

II. PATHOGENICITY OF *S. MUTANS*

A). *S. mutans* and Dental Caries

Simply stated, dental caries is an infectious disease typified by the dissolution of tooth enamel, resulting from the acidic metabolic end-products of oral bacteria. The development of dental caries involves a triad of essential factors, including cariogenic plaque bacteria, dietary carbohydrates, time, and a susceptible host (81). This dissertation is focused on the bacteriological factors associated with caries development.

Several cross sectional epidemiological studies surveying dental decay have indicated that caries is a disease of youth occurring in teeth shortly after eruption. Cavitation does not occur uniformly on all teeth or tooth surfaces (99), the prevalence of decay is highest on the occlusal surfaces of first and second molars and lowest on the lingual surfaces of mandibular anterior teeth.

S. mutans, was first isolated and associated with dental caries by Clarke in 1924 (32). Clarke, noted a variation in morphology in response to the pH of the medium, and thus named this 'mutant' streptococci *Streptococcus mutans* (32). Due to the complex multi-factorial nature of dental caries, *S. mutans* did not become a major subject of caries research for another forty years. *S. mutans* is a normal inhabitant of dental plaque and is found in subjects with active caries and those deemed caries free (17). Additionally, caries has been reported in the absence of *S. mutans* (154). This led some to the conclusion that dental infections were the result of general bacterial overgrowth on the tooth surfaces and that therapy should be directed toward the prevention of this overgrowth. This theory is known as the 'nonspecific plaque hypothesis', and the recommended therapy consists of tooth brushing and professional cleanings, which are known to be effective in reducing caries (105).

A part of the problem in identifying the causative caries agent was often the mis-identification of *S. mutans*. Upon closer study, and when *S. mutans* strains were collected from different sources, it was revealed that there was serological (16, 129) and genetic heterogeneity in organisms previously named as *S. mutans* (35, 51). Eight serotypes could be recognized on the basis of carbohydrate antigens (129) and DNA studies revealed four separate species [based on genetic composition (37)]. These species are *S. mutans*, *S.*

sobrinus, *S. rattus*, and *S. cricetus*, and combine to make up what is commonly referred as mutans streptococci. *S. mutans* contains strains that possess the c, e, or f antigens, and the c serotype accounts for about 70 to 100% of the human isolates [reviewed by Loeshe (99)]. Most of the remaining human isolates are *S. sobrinus* possessing d, g, and h carbohydrate antigens.

B). Epidemiology of Caries

The process of identifying mutans streptococci (MS) to the species level has been performed in relatively few epidemiological studies. As mentioned previously, the majority of human cases of caries can be attributed to *S. mutans*. The following section identifies the relationship of MS with the epidemiology of caries, and extrapolates the findings to *S. mutans*.

Buccal and lingual smooth surfaces normally are not highly colonized by MS, however the ease of access to these sites has proved to be invaluable in the research of caries. Studies by de Stoppelaar et. al. were first to demonstrate significantly higher proportions of MS in white spots (an early sign of enamel destruction) or decayed sites compared with those sites deemed to be free of any decay (46). Additionally the development of decay on buccal surfaces was associated with a significant increase in the proportions of MS in the plaque. These studies unequivocally demonstrated the relationship between MS and human tooth decay.

Fissure surfaces are the most caries-prone sites on the teeth (9, 24). Interestingly, MS have the strongest association with fissure-caries, with every study showing a strong correlation between caries and an increase in MS within saliva or plaque (22, 74, 98, 100,

102, 114). Some longitudinal studies have shown increased proportions of MS from 0 to 24 months prior to cavitation at the fissure site (22, 101). Loesche *et. al.* saw an increase in the proportion of MS exactly at time of early cavitation as detected by an explorer (101).

The caries rate on approximal surfaces is highly variable and depends largely on the tooth type and the age of the patient (9, 24). The decay on the approximal surfaces occurs at the contact point between adjacent teeth and is therefore difficult to observe. This location also makes sampling of the bacteria present at the actual lesion site difficult, and these samples may contain a mixture of plaque from both caries-active and caries-inactive sites. These difficulties have made the association between MS and approximal caries in clinical research quite variable, most studies do however show a correlation between the presence or increase in MS and approximal caries (9, 24).

Decay at the root surface occurs when the gingival tissue of the teeth recedes. This recession is often secondary to periodontal disease and occurs mainly in older people. Ellen et. al. showed a strong association between the presence of *S. mutans* and root caries in a longitudinal study (52). An even stronger risk predictor was established when both *S. mutans* and lactobacilli were detected (52). Additionally, 90% of sites that were found to be *S. mutans* and lactobacilli free were found in caries-free subjects (52). These findings were further supported by a cross-sectional survey conducted by van Houte et. al. (163). This study found a greater proportion of MS in the plaque and saliva of subjects with root-caries compared with root-caries free patients. Moreover, plaque obtained from carious root sites in patients with active root-caries had a higher portions of MS compared with plaque from sites free of root-caries in the same individual. Another study found the number of *S. mutans* to be greatest at the time of the initiation of root-caries, over that of caries-free root sites and

advanced lesions. These results indicated *S. mutans* is probably involved in the initiation but not likely the progression of lesions affecting root-sites (18).

Associations between MS and rampant caries and nursing/bottle caries have also been well established (8, 117, 162).

C). *S. mutans* and Endocarditis:

Oral streptococci frequently cause bacteraemia and sometimes leading to infective endocarditis. Infective endocarditis is an inflammation of the inner layer of the heart, the endothelium, caused by a microorganism. A recent retrospective study found that in nearly 40% of infective endocarditis cases oral streptococci were detected (124). *S. mutans*, in particular, has also been identified as causing endocarditis and is presented in several case reports (59, 161, 166). Virulence factors of *S. mutans* known to be associated with endocarditis are the glucosyltransferases (GtfB, GtfC, and GtfD), and protein antigen I/II. The Gtfs have been shown to induce the production of cytokines from monocytes (143). Subjects with endocarditis had higher levels of antigen I/II titres than healthy subjects (135). Additionally, *S. mutans* antigen I/II defective mutants survived in greater numbers and for a greater period of time than the parent strain in a rat model (123).

III. CARIOGENIC PROPERTIES OF *S. MUTANS*

A) Acidogenicity

In 1890, Miller was the first to recognize that carbohydrate metabolism by oral bacteria resulted in the generation of metabolic acid end-products known to have a central role in the process of dental caries. Since that time *S. mutans* has been implicated as the major etiological agent of caries. As *S. mutans* is highly acidogenic, the exogenous application of fermentable dietary carbohydrates results in a dramatic drop in plaque pH to as low as pH 3 (78). Frequent carbohydrate intake leads to a sustained drop in local pH leading to decalcification of tooth enamel and subsequently the progression of caries.

Studies in *S. mutans* utilizing two-dimensional gel electrophoresis coupled to mass spectroscopy have revealed that glycolytic enzymes are highly regulated, showing differential expression between growth in pH 7 and pH 5, most enzymes were up-regulated by low pH (91, 170). *S. mutans* biofilms have also been investigated using two-dimensional gel electrophoresis and have revealed the increased expression of glycolytic enzymes during the first two hours of biofilm formation versus planktonic growth (167). These studies have also revealed the increased expression of enzymes associated with secondary metabolic processes.

Central metabolism of *S. mutans* is well documented and is shown in Figure 1.2. Under anaerobic conditions and high sugar concentration for every one glucose molecule, 2 lactate, and 2 ATP molecules are produced. As the sugar concentration drops in an anaerobic environment, the metabolic pathway is shifted to the more energy efficient pyruvate:formate lyase system. This results in the production of 3 ATP, 1 acetate, 1 ethanol, and 2 formate molecules per glucose molecule. Pyruvate formate lyase is an extremely oxygen sensitive

enzyme and is almost completely inactive under aerobic conditions. Therefore glucose metabolism under aerobic conditions is mostly homofermentive and results in 2 lactate and 2 ATP molecules produced per glucose molecule consumed. However, under these aerobic conditions *S. mutans* is still capable of heterofermentation via pyruvate dehydrogenase. The robust metabolic capacity of *S. mutans* allows it not only to survive but to out compete other oral bacteria under fluctuating environmental conditions, an example includes carbohydrate concentrations in the oral cavity that can increase 10,000-fold following ingestion of food (26).

S. mutans is capable of producing acid from fermentable carbohydrates at a higher rate and over a broader pH range than most other streptococci (45). In fact many virulence/cariogenic factors of *S. mutans* involve aspects of carbohydrate metabolism, these include: 1) high acidogenicity with the ability to rapidly metabolize a wide variety of carbohydrates into lactic, acetic, and formic acid; 2) high aciduricity, that is the ability to survive, grow, and metabolize carbohydrates at low pH values; 3) Intracellular glycogen synthesis in the presence of a high sugar concentration which serves as a carbohydrate reserve during periods of low exogenous carbohydrate; 4) Extracellular synthesis of glucans and fructan from sucrose that aid in adhesion and colonization, and also serve as an endogenous food source (68). *S. mutans'* exceedingly high glycolytic rate can also be attributed partly to its robust ability to transport sugars from the environment to inside the cell. Sugars can enter the cell through three general transport mechanisms including a glucose-specific permease, the multiple sugar metabolism (Msm) transport system, and the high affinity phosphoenolpyruvate phosphotransferase transport system (PEP:PTS).

B). Aciduricity and the Acid Tolerance Response

The robust metabolic capacity of *S. mutans* can lead to rapid drops in plaque pH from resting pH 7 to as low as pH 3 (78). As such, *S. mutans* has evolved highly elaborate systems allowing it to tolerate these large and frequent fluctuations in pH. *S. mutans* has both constitutive and acid inducible mechanisms for acid tolerance. One of these constitutive mechanisms is the F_1F_0-ATPase proton pump. The F_1F_0-ATPase proton pump helps maintain intercellular pH by the extrusion of protons (7, 69). Another continuously functioning pH mediating mechanism is the functional pH range of *S. mutans'* glycolytic enzymes. Studies have revealed that glycolysis can function at pH as low as pH 4 (7, 69). Also involved in constitutive pH homeostasis is the rapid efflux of acidic metabolic end-products such as lactic acid (25, 43).

S. mutans has also evolved acid-inducible mechanisms to tolerate acid, these collectively being referred to as the acid-tolerance response (ATR). The ATR is typified by increased survival at apparent sub-lethal pH upon pre-exposure to a moderately acidic 'adaptive' environment (pH 5.5) (153). Several proteins that have shown to be important in ATR have been characterized. Among these proteins are the molecular chaperones GroEL and DnaK (76, 90). Molecular chaperones are important in assisting the folding of newly synthesized or denatured proteins, as well as in the assembly, transport, and degradation of cellular proteins (90). Also showing genetic up-regulation during acid challenge are the DNA repair proteins UvrA and RecA (70, 133). The cell membrane's fatty acid composition is also modified by acid challenge, where the fatty acid profile at pH 7 consists of short-chained, saturated fatty acids compared to that of the long-chained, monounsaturated profile at pH 5 (54).

Work by Svensater et. al. showed that protein synthesis is an essential component of the acid tolerance response in *S. mutans* in which at least 30 proteins were up-regulated during acid stress (153). Another study using an *S. mutans* clinical isolate revealed the up-regulation of 64 proteins and the down-regulation of 49 proteins during the first 30 minutes of acid adaptation at pH 5.5 (152). Recent work utilizing two dimensional gel-electrophoresis and mass spectroscopic analysis has identified 123 proteins that were up-regulated at least 1.5-fold during chemostatic growth at pH 5 compared to pH 7. Of these proteins, 53 were associated with stress responsive and/or regulatory pathways (92) and the remaining 70 were associated with metabolism (91). This proteomic work collectively highlights the coordination and intricacies of the *S. mutans* response to an acidic environment.

C). Adhesion

As mentioned previously in this dissertation, attachment to the salivary pellicle and to other bacteria within plaque is an essential step in order for *S. mutans* to colonize dental plaque. *S. mutans* mediates its attachment through three main mechanisms, and these factors, to some extent, have all been linked to the cariogenic potential of *S. mutans*.

i) Antigen I/II

Of the main adhesion mechanisms only the antigen I/II protein is completely sucrose-independent. Antigen I/II is a 185 kDa surface protein with homologues found is almost all other oral streptococci; these homologues are designated PAc, P1, SpaP, and Sr (104). The alanine-rich and proline-rich domains within all these homologues are believed to responsible for binding to various salivary components (15, 38, 66, 130). Several *in vitro*

studies have shown mutant strains lacking antigen I/II have reduced adhesion to saliva-coated hydroxyapatite (13, 50, 89, 126). Crowley et. al. reported an antigen I/II mutant was less virulent in rats fed a modest 5% sucrose diet (39). Additionally antigen I/II has been shown to mediate dentinal tubule invasion (103).

ii) Gtfs

Also involved in *S. mutans* adhesion are glucosyltransferases, which enzymatically convert sucrose to glucans. These enzymes utilize the glucose moiety of sucrose to generate glucan polymers. Glucans form an important component of the extracellular plaque matrix that adheres to the acquired pellicle (121). Glucosyltransferases not only produce glucans but are also believed to be responsible for anchoring *S. mutans* cells within the milieu of glucans. *S. mutans* has three Gtfs encoded by *gtfB*, *gtfC*, and *gtfD*. GtfB and GtfC are responsible for producing water-insoluble α(1-3) glucans, mainly mutan. GtfD is responsible for the synthesis of α(1-6) glycosidically linked-dextran like glucans (119). Several groups have shown strains of *S. mutans* defective in one or more *gtf* genes have diminished virulence in rodent models (120, 155, 174).

iii) Gbps

Acting as complements to the glucosyltransferases, glucan-binding proteins produced by *S. mutans* are responsible for non-enzymatically adhering *S. mutans* to glucan (6). Four separate Gbp's have been isolated from *S. mutans*, and are encoded by *gbpA*, *gbpB*, *gbpC*, and *gbpD* (6). Both *gbpA* and *gbpC* have been linked to cariogenicity, as isogenic mutants in each individual gene had reduced cariogenicity in a rat model (112).

D). Carbohydrate storage

i) Ftf

In addition to producing glucans, *S. mutans* produces extracellular fructans from the fructose moiety of sucrose. *S. mutans* produces β(2,1) and β(2,6)-linked fructans (inulin and levan respectively) via the fructosyltransferase (Ftf) enzyme. Both levan and inulin (and also sucrose and raffinose) can be degraded by the exo-β-D fructosidase enzyme FruA. FruA results in the release of fructose from these polymers, (19). Expression of *fruA* in *S. mutans* is very sensitive to carbohydrate catabolite repression and is optimal when the cells are grown in the presence of fructans (175). These fructan polymers have been shown to serve as a carbohydrate reservoir in situations of low food supply. In a rat model, a fruA knockout mutant had reduced virulence compared with the parent strain (20). There is some additional evidence that fructans can also aid in the adhesion of *S. mutans* to a glucan matrix (134).

ii) Glycogen

Studies have revealed that *S. mutans* produces a glycogen-like intracellular polysaccharide (IPS). Research by Spatafora et. al. has shown there is a relationship between IPS and virulence in *S. mutans* using a germ free rat model. The first study showed transposon knockouts in the *dlt* operon, resulted in loss of IPS synthesis and reduced virulence (150). A corroborating study showed that a hyper-producing-IPS mutant had increased virulence in the same rodent model (148). Interestingly, the dlt operon (*dltABCD*), is responsible for the synthesis of D-alanyl-lipoteichoic acid, a surface component of gram-positive bacteria (14). The exact relationship between this operon and IPS synthesis has yet to be determined.

E). Biofilm Formation

Human dental plaque is one of the most studied, naturally occurring biofilms. As such, the ability to form biofilms is considered a major virulence property of *S. mutans*. The adhesion mechanisms previously mentioned in this dissertation have been shown to be important in biofilm formation. The biofilm 'lifestyle' is also characterized by modulation of gene expression and cellular physiology compared to planktonic growth. These changes allow *S. mutans* to withstand various environmental stressors. Growth in biofilms may also facilitate novel nutrient availability, toxin removal, and horizontal gene transfer via genetic transformation. A report identified 33 proteins modulated by biofilm growth compared to planktonic growth (167). Other reports have shown quorum sensing through various two-component signal transduction systems may act as global regulators, affecting things such as *gtf* and *ftf* expression (141).

F). Quorum Sensing

S. mutans regulates many of its physiological processes via quorum sensing. Like other gram-positive bacteria, the *S. mutans* quorum sensing system contains three principle constituents. These components being a signal peptide (CSP), a histidine kinase, and a response regulator. The latter two elements comprise a two-component signal transduction system. The major components of quorum sensing in *S. mutans* are reviewed in Figure 1.1. In *S. mutans* the signal peptide is encoded by *comC* (94). The *S. mutans* quorum sensing *comCDE* locus has been extensively studied and is believed to control many physiological processes (93, 94, 96, 97). Quorum sensing has also been linked to aciduricity displayed by *S. mutans*. Knockout mutants of the genes *comC*, *comD*, and *comE* each displayed a

diminished log-phase acid tolerance response (96). The addition of exogenous synthetic CSP to the *comC*- strain resulted in the partial recovery of acid tolerance (96). Interestingly, with the addition of cell free culture supernatant from acidic grown cells in conjunction with CSP resulted in the compete restoration of acid tolerance to wild-type levels (96). This finding indicates the presence of at least one other signal molecular, in addition to CSP, is required for a full and robust acid tolerance response in *S. mutans*. This work by Li et. al. clearly shows the link between quorum sensing and the acid tolerance of *S. mutans*.

Additionally, *S. mutans* quorum sensing influences biofilm formation and the biofilm 'lifestyle'. Studies utilizing individual knockouts in *comC, comD* and *comE* revealed these mutants had altered biofilms. The *comC* knockout mutant produced biofilms with abhorrent structure, in which cells formed large aggregates of long chains, whereas wild-type biofilms consisted of confluent microcolonies composed of shorter cell chains (97). The addition of synthetic CSP to the *comC* knockout partially restored wild-type biofilm architecture (97). Interestingly, *comD* and *comE* mutants had differing biofilm architecture from that of *comC* mutant. Mutations in these genes resulted in biofilms with substantially reduced biomass (97). The addition of CSP to both the *comD* and *comE* mutants did not result in the restoration of biofilm biomass, indicating these genes are part of a two-component signal transduction system responsible for detecting CSP (97).

The varying biofilm phenotypes of the *com* locus mutants alluded to the existence of a second receptor. *S. mutans* genome analysis revealed 13 separate two-component signal transduction systems. One pair consisting of the histadine-kinase *hk11* and the response-regulator *rr11* has been previously indepthly investigated. Deletion of either *hk11* or *rr11* resulted in biofilms with reduced biomass and a sponge-like architecture, that differed from

wild-type and the *comC*, *comD*, and *comE* mutant biofilms (94, 97). The addition of synthetic CSP to the *hk11* and *rr11* mutants did not restore wild-type architecture to the biofilms (94). Results show that the *hk11/rr11* system is important for proper biofilms formation in a manner that is completely separate from that of *comDE*. The *hk11* knockout strain also had significant decreases in both the ability to grow at low pH and the ability to mount an acid tolerance response. Interestingly, the cognate *rr11* knockout mutant did not display these phenotypic insufficiencies (94). These data suggest that Hk11 acts as a pH sensor mediating acid-tolerance pathway and 'cross talk' exists between Hk11, Rr11, and another non-cognate response regulator. The presence of cross talk within two-component system has been previously described in *E. coli* (165).

Additionally, *S. mutans* has a *luxS*/autoinducer-II (AI-2) system homologous to that of *Vibrio harveyi*. As opposed to the peptide based signal molecule of CSP, the *luxS* signal molecule (AI-2) is a furanosyl borate diester. The absence of AI-2 resulted in the diminishment of virulence properties, including reduced acid tolerance (169).

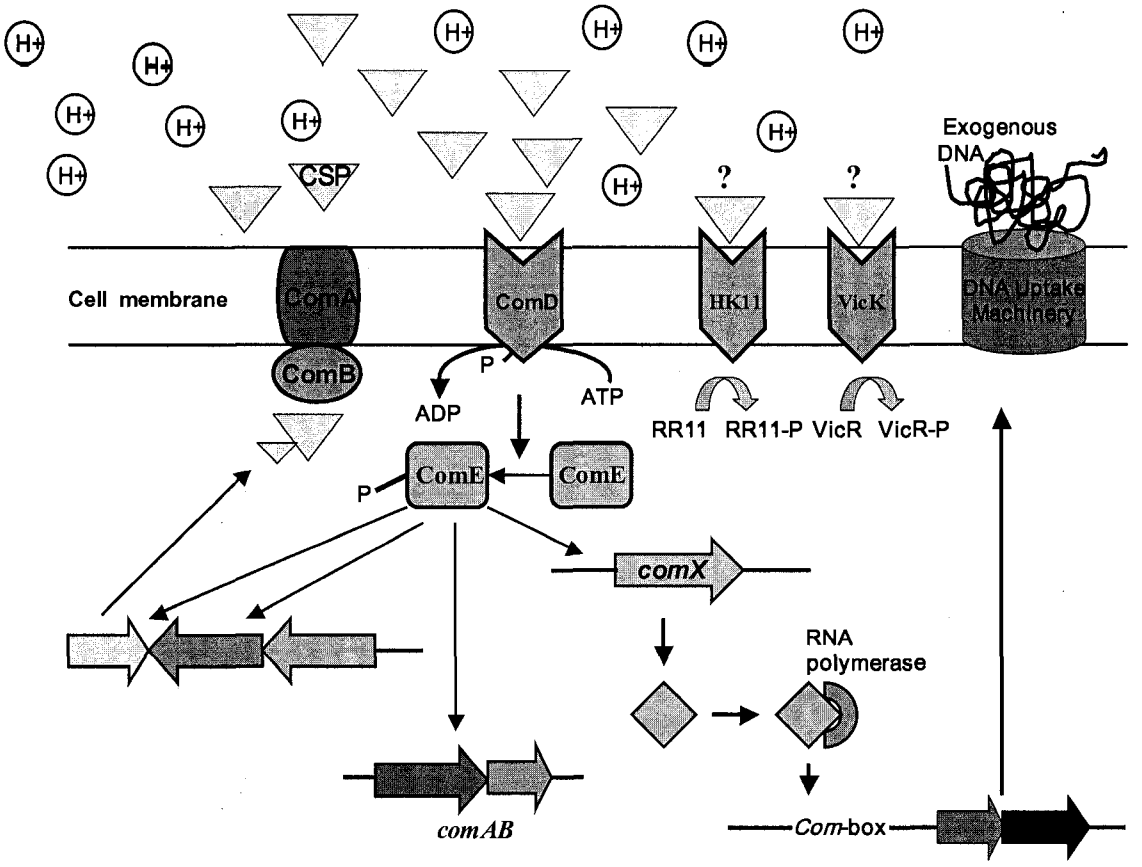

Figure 1.1. Quorum Sensing: The two-component signal transduction systems of *S. mutans.*

IV. *S. mutans* Secondary Metabolism and Virulence

S. mutans has developed many adaptive mechanisms in order to survive in the highly dynamic dental plaque environment. A component of this ever-changing environment is the rapid and drastic pH fluctuations. Many studies have illuminated the importance of regulation in response to acid challenge both at the RNA and protein levels. These studies in *S. mutans* have suggested that regulation of diverse secondary metabolic pathways is important for the survival of *S. mutans* in an acidic environment (91, 168). Transcriptional analysis of genes responding to acid shock revealed modulated expression of 9 secondary metabolic genes, including the 3 components of the citrate/α-ketoglutarate pathway and the β subunit of oxaloacetate decarboxylase (29). Utilizing 2D-GEL electrophoresis, Wilkins et. al. showed the up regulation of 18 proteins when *S. mutans* was grown in an acidic environment, with 4 of these considered secondary metabolic enzymes (170). Of particular interest was the 51-fold increase in lactoylglutathione lyase (170). Proteomic analysis by Len et. al. (91) showed increased synthesis of 70 proteins during chemostatic growth at pH 5.0 relative to growth at pH 7.0. Of these 70 proteins, 7 proteins could be considered secondary metabolic enzymes, including a 2.5-fold increase in lactoylglutathione lyase expression (91). Additional proteomic studies have also shown another secondary metabolic enzyme, pyruvate dehydrogenase, to be increased in expression under continuous acidic culture conditions versus cells grown continuously in a neutral environment (168). These data help form the basis of the research presented within this document. The goal of this work was to investigate the 3 secondary metabolic pathways and their relationship to acid tolerance of *S. mutans*. We specifically investigated citrate transport and metabolism, the

glyoxalase pathway, and pyruvate dehydrogenase A, and their relationship in the acid tolerance of *S. mutans*.

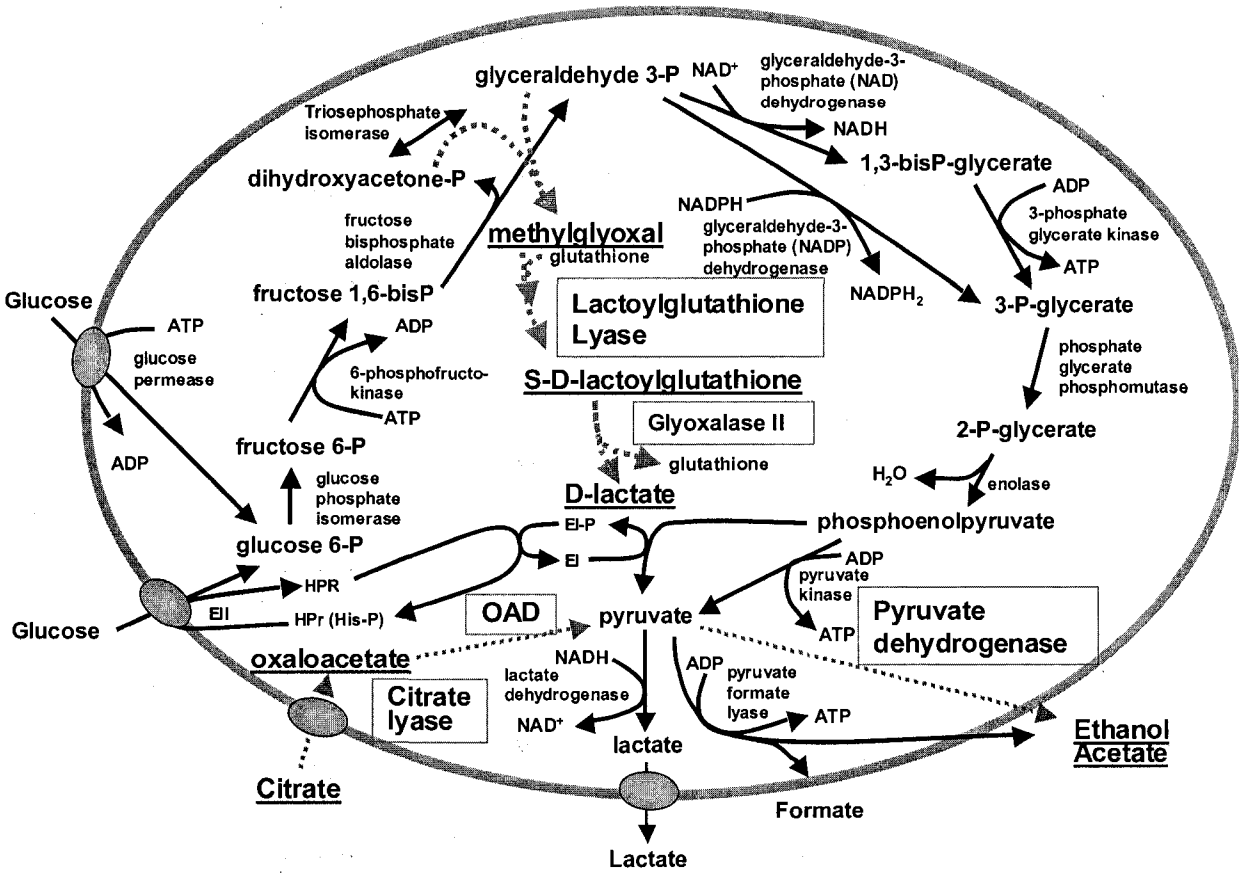

Figure 1.2. Central Metabolism of *S. mutans*, including the secondary metabolic pathways under investigation (boxed areas).

A). Citrate Transport, Metabolism and Iron Acquisition

Many LAB have been shown to metabolize citrate. *S. mutans* is unable to survive when citrate is the sole source of carbon as observed by no growth on Simmons citrate agar. *S. mutans* also does not evolve CO_2 gas from citrate, which is a common by-product of citrate metabolism in other lactic acid bacteria (LAB). These observations have left the relationship between *S. mutans* and citrate metabolism widely understudied.

The transport of citrate in some LAB generates a proton motive force in which citrate is exchanged for lactate via the CitP anti-porter (58). The co-metabolism of citrate with glucose results in the activation of heterofermentation and the end-products of CO_2, diacetyl, acetoin, and butanediol. A growth advantage at low pH is conferred to *L. lactis* by this citrate co-metabolism (58). The genetic expression of citrate metabolism genes is also up-regulated by an acidic environment (58).

Previous investigations in *S. mutans* have revealed a link between acid tolerance and citrate metabolism. A transposon insertion into the aconitase gene (*citB*) resulted in an acid sensitive phenotype (65). Further work investigating the pathway involving CitB as well as citrate synthase (CitZ), and isocitrate dehydrogenase (Icd) was found to be essential for nitrogen assimilation and glutamate biosynthesis (41)

A review of the *S. mutans* genome reveals many putative genes that could be associated with citrate metabolism as displayed in Figure 1.3 (2). Based on citrate metabolism of LAB (11, 73) and *Klebsiella pneumoniae* (12, 140), and *S. mutans* (2, 41) Figure 1.4 represents the putative citrate metabolic network within *S. mutans*. The citrate transporter of *S. mutans* has homology to the citrate transporter of *Bacillus subtilis*, CitM. Transport studies in *B. subtilis* have revealed CitM is responsible for the uptake of citrate in complex with divalent metal ions, mainly Mg^{2+} (5).

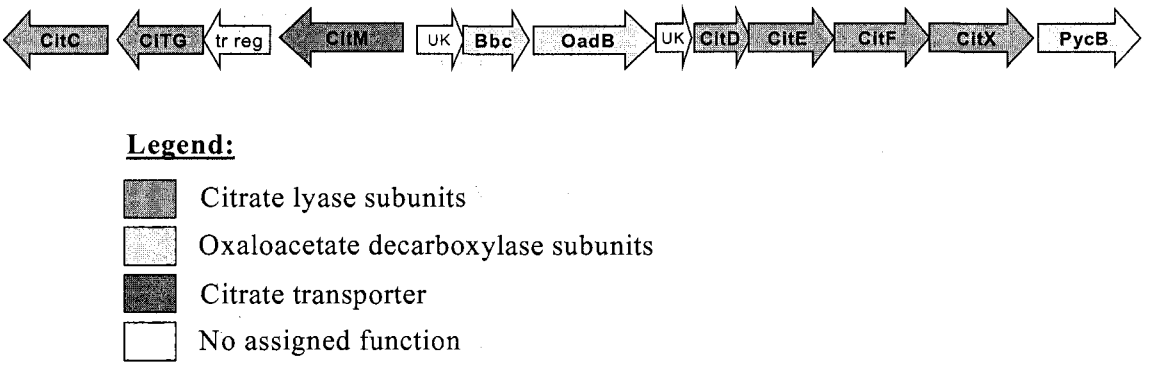

Figure 1.3: Citrate transport and metabolism genetic locus.

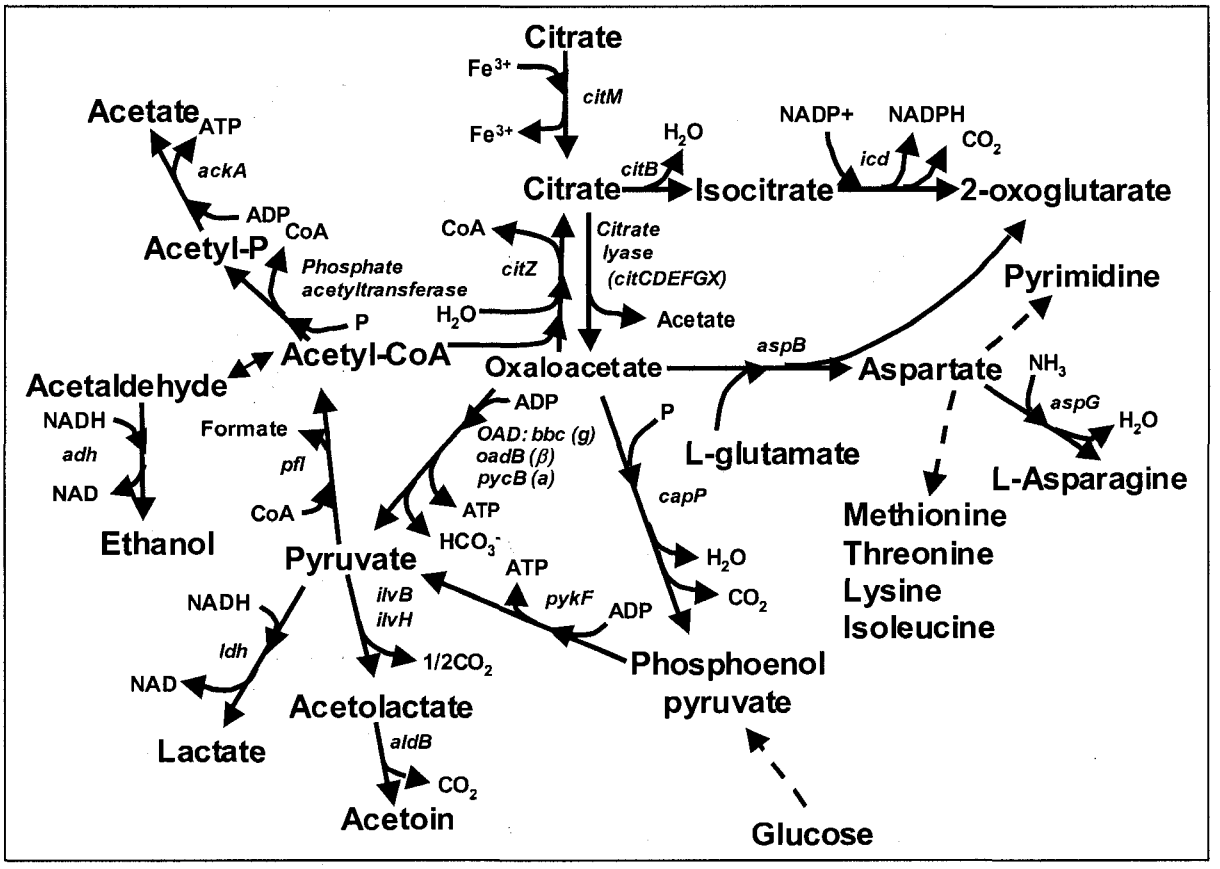

Figure. 1.4. Putative citrate utilization pathway of *S. mutans.*

Work investigating the citrate lyase complex (CitCDEFGX) in *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, and *Klebsiella pneumoniae* has revealed complex functional and regulatory mechanisms for this enzyme. The functional core of the enzyme is composed of α, β, and γ subunits (encoded by *citFED* respectively) in a 6:6:6 stoichiometry (schematically shown by Figure 1.5) (144, 151). The *citC* gene encodes for citrate lyase ligase. CitX subunit controls the enzyme structure between the holo- and apo-comfomation (139, 140), and CitG has a function yet to be determined. Citrate lyase is active only when the thoiester residue of the prosthetic group is linked to the γ subunit (as known as the acyl carrier protein) (138). Acetylating the γ subunit, via CitC, and consuming an ATP molecule accomplishes this activation. The α subunit catalyses the exchange of the acyl group with the citryl moiety. Lastly, the β subunit cleaves off oxaloacetate regenerating an active acyl-γ subunit (48).

In *Klebsiella*, and other microbes containing an operative tricarboxylic acid cycle, citrate lyase is negatively controlled by L-glutamate concentrations (4). In *L. mesenteroides* citrate lyase activity is regulated positively by citrate concentration (72).

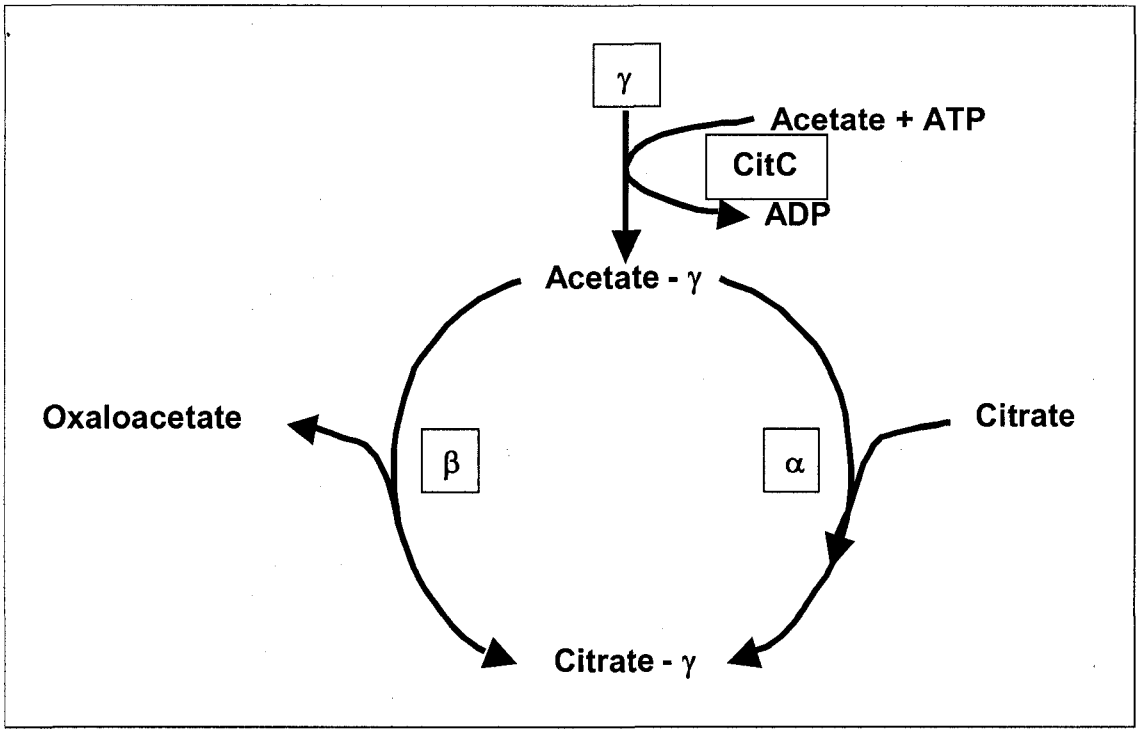

Figure 1.5. Putative Citrate Lyase Pathway for *S. mutans*

Along this putative citrate metabolism pathway, citrate is converted to oxaloacetate and oxaloacetate converted to pyruvate. Oxaloacetate decarboxylase is responsible for the second step, the conversion of oxaloacetate to pyruvate. Oxaloacetate decarboxylase (OAD) is a trimeric enzyme composed of α, β, and γ subunits, encoded in the *S. mutans* genome by *pycB*, *oadB*, and *bbc* respectively (47). The decarboxylation of oxaloacetate into pyruvate and carbon dioxide consumes an intracellular proton resulting in an increase in cytoplasmic pH. OAD has been extensively studied in the gram-negative anaerobe *Klebsiella pneumoniae*, where it has been show to extrude 2 Na^+ ions in conjunction with its decarboxylation activity (47). This Na^+ efflux has not been shown in gram-positive bacteria.

Additionally, *Leuconostoc mesenteroides*, has been shown to convert citrate into the amino acid aspartate (111). This conversion would be mediated by the conversion of oxaloacetate into aspartate via aspartate transaminase.

Preliminary work in our laboratory revealed that citrate, when added to rich culture media, conferred increased acid tolerance and a decreased growth rate to *S. mutans*. The goal of this research into citrate transport and metabolism is two fold: Firstly to determine how much, if any, of this metabolic network actually functions in *S. mutans*, and secondly if the catabolism of citrate promotes *S. mutans* aciduricity.

Iron has an essential role in cellular metabolism, but also has the potential to cause oxidative damage and eventual cell death. *S. mutans* has a nutritional requirement for trace metals including magnesium, manganese, and iron (5). *S. mutans* optimally requires iron at a concentration of 3.6 μM (5), which is far greater that the 0.01 to 1.0 μM present in human saliva (Spatafora, personal communication). *S. mutans*, therefore, must have a mechanism by which to capture iron. A review of the *S. mutans* genome sequence reveals multiple putative mechanisms for iron transport. This redundancy suggests that these pathways are extremely important. The processes involving iron transport in gram-negative bacteria are well studied. However, the mechanisms of iron transport are poorly characterized in gram-positive bacteria. The most well understood iron regulatory system in gram-positive bacteria is that of *Corynebacterium diptheriae*. At high concentrations, iron binds to the diptheria toxin regulator protein, DtxR. Upon binding of iron DtxR binds specifically to the diptheria *tox* operator and represses toxin production. When the concentration of iron is low, as in the human host, derepression occurs and toxin genes are expressed (156). The DtxR homologue in *S. mutans* is called Dlg. DtxR homologues have been associated with proteins of the

lipoprotein receptor antigen I (LraI) family (34, 71, 77) and have been shown to be involved in bacterial adhesion (10, 21, 57). An *S. mutans* homologue, *sloC*, has been identified as a Fe^{2+} transporter (149). Experiments have shown that iron increases the dental plaque pH *in vivo*, however the mechanisms are not known (116, 127). It is proposed that iron displaces essential Mg^{2+}, inhibits glucose metabolism, or prevents proton efflux. Iron also has toxic properties in the presence of oxygen. Iron ions stimulate the generation of highly reactive oxygen species, such as hydroxyl radicals, via the Fenton reaction (67). *S. mutans* is catalase negative, and therefore requires a different mechanism of oxygen tolerance. One portion of this oygen tolerance mechanism involves Dpr, an iron-binding protein, which protects cells from peroxides by sequestering iron (172, 173). Iron has an important, yet not fully understood role in the pathogenesis of dental caries.

Citrate readily chelates numerous metal ions, including both Fe^{2+} and Fe^{3+}. The uptake of iron coupled with citrate, and transported into the cell via CitM, may prove to be a mechanism by which *S. mutans* obtains iron.

B). Methylglyoxal and Lactoylglutathione Lyase

Lactoylglutathione Lyase (LGL) is a highly evolutionary conserved enzyme, with homologues found in all living cells types. LGL is the first enzyme of the glyoxalase pathway (Figure 1.6) responsible for the detoxification of methylglyoxal (MG), a highly reactive electrophile. As a result, LGL and MG have been extensively studied in eurkaryotic systems as chemotherapy targets for the treatment of cancers and diabetes mellitus. Only recently have investigations been undertaken in prokaryotes, but mostly in enteric gram-negative organisms.

MG has also shown to be clinically important in the oral cavity, with dramatically increased concentrations in the gingival crevicular fluid of patients with periodontitis when compared with healthy subjects (80). The periodontal pathogen *Bacteroides forsythus* produces high levels of MG when cultured in the presence of glucose (106). Additionally, MG modified the arginine residues of collagen, resulting in the disruption of collagen phagocytosis (30). Matrix remodeling, via collagen phagocytosis is essential to maintain the integrity of sites such as the periodontium. These observations have lead to a novel theory; the connective tissue destruction associated with periodontitis, may be the result of altered collagen homeostasis caused by the elevated levels of MG present in the periodontal pocket produced by periodontal pathogens such as *B. forsythus*.

LGL neutralizes methylglyoxal toxicity via the formation of *S*-D-lactoylglutathione from the hemimercaptal adduct that is formed nonenzymatically between glutathione and the 2-oxoaldehyde methylglyoxal (56). Glyoxalase II then converts *S*-D-lactoylglutathione into reduced glutathione and D-lactate (56). An examination of the *S. mutans* UA159 genome reveals the presence of Lgl, however, a glyoxalase II homologue does not appear to be present.

The formation of methylglyoxal occurs via enzymatic production during glycolysis from the fragmentation of triose-phosphates (36, 79). In *E. coli*, methylglyoxal was accumulated under physiological conditions of uncontrolled carbohydrate metabolism (1, 55, 82). The concentration of methylglyoxal was seen to increase from approximately 0.1% of triphosphates metabolized to 1% in highly metabolically active human red blood cells where *lgl* expression appeared to be regulated by the rate of glycolysis (132, 159).

Methylglyoxal is a natural by-product of glycolysis and reacts with and inactivates intracellular macromolecules, including both proteins and nucleic acids forming advanced glycation end-products (AGEs) (75, 79). Its rapid degradation is therefore vital for cell survival, as even low concentrations can cause mutagenicity and protein degradation. Methylglyoxal reacts irreversibly with proteins at arginine, lysine, and cysteine residues (158). Reactions between MG and arginine residues result in the formation of the hydroimidazolone, 5-methylimidazol-4-one. MG glycates lysine to form N^{ε}-carboxylethyl-lysine, pyrraline, and a lysine dimer 1,3-di(N^{ε}-lysino)-4-methyl-imidazolium. MG also reacts with cysteine residues reducing the product of a hemithioacetal adduct. Research has shown MG to have the greatest affinity for arginine residues resulting in functional impairment of the glycated protein (158).

Deoxyguanosine is the nucleotide most susceptible to attack by MG under physiological conditions. The major nucleotide derived AGEs are N2-(1-carboxylethyl)-deoxyguanosine, and the imidazopurinone derivatives 3-(2'-deoxyribosyl)-6,7dihydro-6,7-dihydroxyimidazo-[2,3-b]purin-9(8)one and 6,7dihydro-6,7-dihydroxy-6-methylimidazo-[2,3-b]purin9(8)one (158). The formation of nucleotide AGEs is associated with increased mutation frequency, DNA strand breaks and cytotoxicity.

Methylglyoxal also produces DNA-protein cross links, and is capable of cross-linking the Klenow fragment of DNA polymerase to the DNA template, effectively inactivating both molecules (160). Maintenance and elimination of MG is obviously beneficial to the health of a multitude of biological systems.

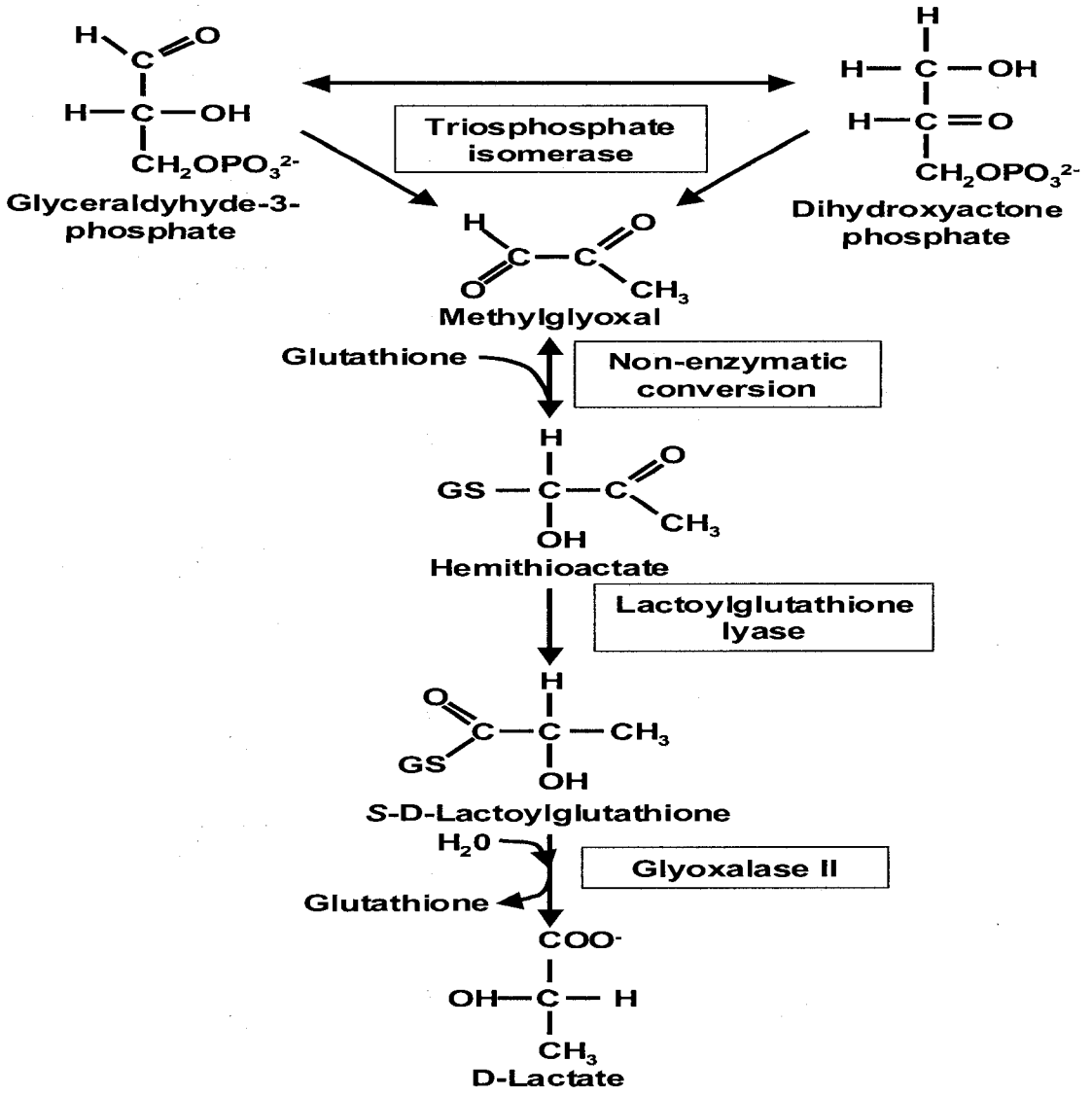

Figure 1.6. Methylglyoxal detoxification pathway.

Reactions involved in the lactoylglutathione lyase pathway. Toxic methylglyoxal is formed as a by-product of the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate catalyzed by triose-phosphate isomerase. Methylglyoxal non-enzymatically combines with reduced glutathione to produce hemithioacetate. Lactoylglutathione lyase catalyses the conversion of hemithioacetate to S-D-lactoylglutathione which is subsequently hydrolyzed by glyoxalase II into D-lactate and glutathione.

Salmonella enterica expressed elevated levels of *lgl* mRNA when engulfed in the acidic environment of the macrophage (53). Separate two-dimensional gel electrophoresis

studies also demonstrated that LGL was up-regulated in an acidic environment (91, 170). These findings suggest LGL may be linked to *S. mutans* acid tolerance (91, 170). The goal of this line of study was to investigate the role LGL may play in the aciduricity and acidogenicity of *S. mutans*.

C) Pyruvate Dehydrogenase

When *S. mutans* is grown in excess glucose, lactate is the major fermentation product. However, under conditions of glucose limitation, ethanol, acetate, and formate, in conjunction with lactate are the resulting end-products of metabolism (26). This divergence in metabolism occurs at pyruvate, which has been shown to be a key metabolic control node in many bacteria (33, 64, 118). Pyruvate can be converted to lactate via lactate dehydrogenase (homofermentation), or to formate, ethanol, and acetate via pyruvate-formate lyase (heterofermentation) (26). A third alternative pathway of pyruvate catabolism is via the pyruvate dehydrogenase complex in which pyruvate is oxidized into acetyl-CoA and CO_2 (27). Acetyl-CoA is further catabolized into ethanol, via alcohol dehydrogenase, or acetate, via acetate kinase.

In gram-positive bacteria the pyruvate dehydrogenase complex (PDH) is composed of four distinct polypeptides E1α, E1β, E2 and E3 (146). These proteins combine to from an icosahedral complex consisting of pyruvate dehydrogenase (E1 subunits), dihydrolipoyl transacetylase (E2) and dihydroipoamide dehydrogenase (E3) (118).

Pyruvate decarboxylase (E1$\alpha\beta$) uses thiamin diphosphate for the oxidative decarboxylation of pyruvate resulting in the formation of acetyl lipoic acid. The acetyl moiety is next covalently attached to the acetyltransferase E2-component. The E2-component

catalyses the transfer of the acetyl group to coenzyme A. The resulting dihydrolipoyl group is reoxidized by NAD^+ with this reaction catalyzed by dihydrolipoamide dehydrogenase (E3-component) (44). The net reaction is the production of 1 CO_2, 1 Acetyl-CoA and 1 NADH from 1 pyruvate, 1 NAD^+, and CoASH.

The activity of PDH in gram-negative bacteria depends largely on allosteric regulation, however in gram-positive bacteria PDH seems to be regulated by redox potential (146). Such is the case in *Lactococcus lactis,* due to pyruvate formate lyase (PFL) being extremely oxygen sensitive, PDH is responsible for pyruvate metabolism an aerobic environment (146). In *Enterococcus faecalis*, however, both PFL and PDH are active under anaerobic conditions, analogous to pyruvate oxidation in gram-negative *Klebsiella pneumoniae* (115, 147). PFL is also extremely oxygen sensitive in *S. mutans*. A previous report in *S. mutans* described increased PDH activity in response to an aerobic environment (27).

The human dental plaque routinely goes through cycles of carbohydrate excess and limitation, and therefore metabolic regulation between homofermentation and heterofermentation is paramount to the survival of *S. mutans*. Heterofermentation is thought to play an important role in the acid tolerance of *S. mutans* as the metabolic end-products are weaker organic acids and heterofermentation yields an additional ATP molecule per pyruvate molecule (26). During acid challenge, in *S. mutans*, the protein expression levels of the E1α subunit of PDH (encoded by *pdhA*) were shown to increase (168). PdhA is the α component of the heterodimeteric E1 subunit responsible for the decarboxylation of pyruvate, and this liberated carboxyl group could help increase intracellular pH, via the formation of carbonate. These data suggest that PdhA may play an important role in the acid

tolerance of *S. mutans*. The present study looks to address the involvement of PDH, specifically *pdhA*, in the metabolism and acid tolerance of *S. mutans*.

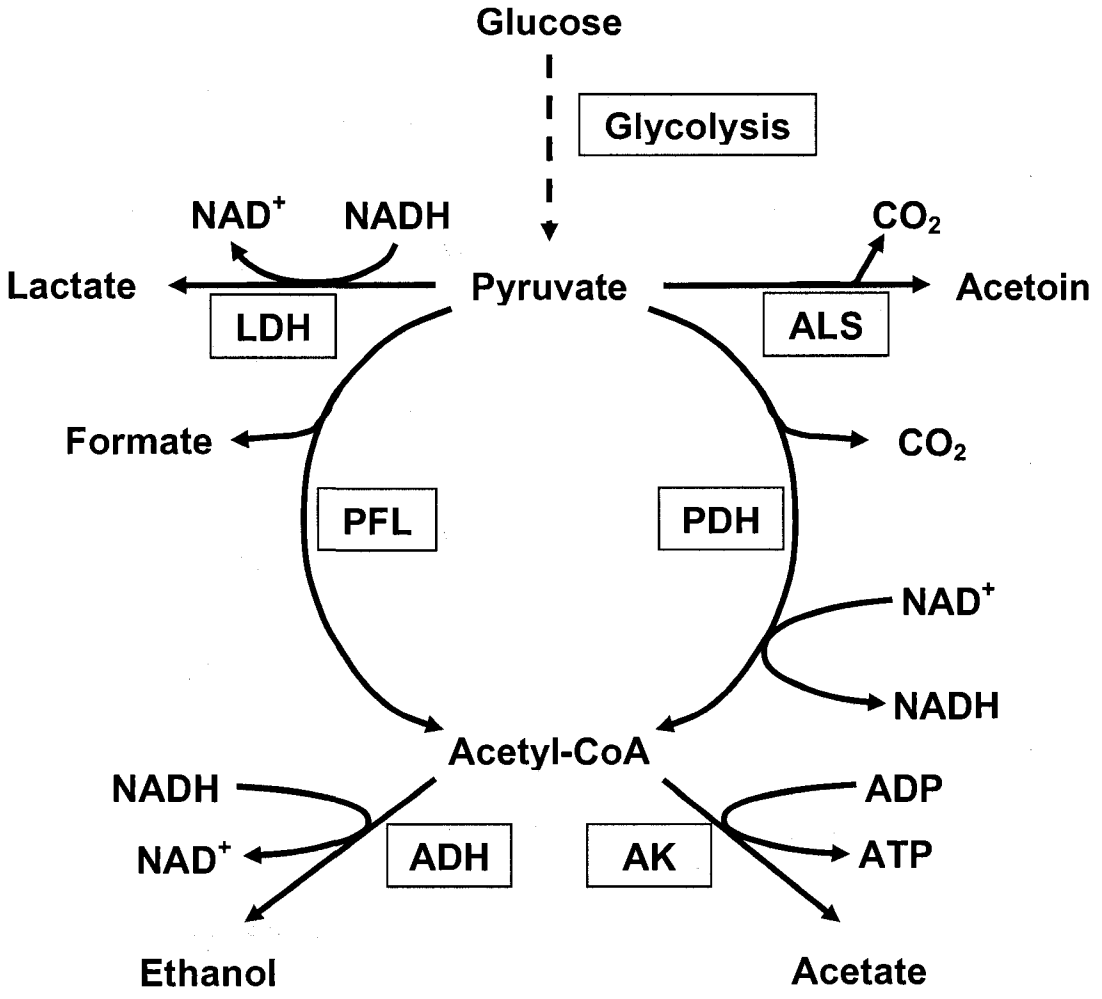

Figure 1.7. Reactions involved in the metabolism of pyruvate.

Lactate dehydrogenase (LDH), α-acetolactate synthase (ALS), pyruvate-formate lyase (PFL), pyruvate dehydrogenase (PDH), acetate kinase (AK), and alcohol dehydrogenase (ADH).

Statement of the Problem

Streptococcus mutans is a normal inhabitant of dental plaque and is considered a major etiological agent of dental caries, one of the most prevalent dental diseases. Two virulence determinants of *S. mutans* linked to its cariogenicity are acidogenicity and aciduricity. These are the ability to produce acid and the ability to survive and grow at low pH respectively. Acidogenicity and aciduricity allow *S. mutans* to out compete other organisms within the dental plaque, which can lead to the progression of carious lesions. Previous studies have shown the secondary metabolic pathways of citrate metabolism, methylglyoxal detoxification, and pyruvate dehydrogenase to be putatively important to the acid tolerance of *S. mutans*. The general aim of my research plan is to gain insight into the genetics and biochemistry of these specific secondary metabolic pathways in relation to the acidogenicity and aciduricity in *S. mutans*. Furthermore, disruption of these secondary metabolic processes may prove to be a novel and valuable strategy to reduce the cariogenic potential of *S. mutans*.

General Hypothesis: Secondary metabolism plays an important role in acid tolerance of *S. mutans*.

Primary objective: To investigate the three previously mentioned secondary metabolic pathways and determine the methods by which they contribute to the acid tolerance of *S. mutans*.

Rationale: Recent studies in *S. mutans* have suggested that modulation of secondary metabolic processes is important in acid tolerance (91, 152, 168, 170). In other gram-positive cocci, namely *Leuconostoc* and *Lactococcus* species, the metabolism of citrate is known to confer growth advantages and increased survival at low pH (35, 58). Citrate may also confer these same advantages to *S. mutans*. Previous reports have yet to show *S. mutans*

as being capable of catabolic breakdown of citrate, while the *S. mutans* genome contains all the necessary homologues.

Two-dimensional gel electrophoresis studies have identified the modulated expression of *S. mutans* proteins under acidic conditions (91, 168, 170). Two enzymes that were shown to be up-regulated by these studies were pyruvate dehydrogenase A (PdhA) and lactoylglutathione lyase (Lgl), therefore suggesting these enzymes might be important to acid tolerance by *S. mutans*.

Homologous proteins to Lgl have been found in all living organisms. Lgl is part of the glyoxalase pathway responsible for the detoxification of methylglyoxal. Methyglyoxal, a natural by-product of glycolysis, modifies both proteins and nucleic acids and therefore its removal is vital for cell survival.

PdhA is the α subunit of the four-enzyme pyruvate dehydrogenase complex, responsible for the conversion of pyruvate to acetyl-CoA. Acetyl-CoA can be further degraded into either, ethanol or acetate. This pathway may afford a greater degree of acid tolerance due the additional production of ATP over the production of solely lactate of the Emden-Meyerhof pathway.

Based on the knowledge gleaned from the previous research, the goal of this proposal is to investigate the following specific aims and their relationship to acid tolerance of *S. mutans*

Specific Aim I: Elucidate the role of citrate transport and metabolism in acid tolerance by *S. mutans*.

Specific Aim II: Examine the mode of acid tolerance for the secondary metabolic glyoxalase pathway enzyme lactoylglutathione lyase (Lgl),

Specific Aim III: Investigate the relationship between the heterofermentative enzyme pyruvate dehydrogenase A (PdhA) and acid tolerance in *S. mutans*.

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CHAPTER II: TRANSPORT AND METABOLISM OF CITRATE BY *STREPTOCOCCUS MUTANS*

Korithoski, B., Krastel, K., Cvitkovitch, D.G., (2005). Transport and metabolism of citrate by *Streptococcus mutans*. J. Bacteriol. **187**:4451-4456

Abstract

Streptococcus mutans, a normal inhabitant of dental plaque, is considered a primary etiological agent of dental caries. Two virulence determinants of *S. mutans* are its acidogenicity and aciduricity, the ability to produce acid and the ability to survive and grow at low pH respectively. Citric acid is ubiquitous in nature; a component of fruit juices, bones and teeth (3, 16). In lactic acid bacteria citrate transport has also been linked to increased survival in acidic conditions (5). We have identified putative citrate transport and metabolism genes in *S. mutans*, which lead us to investigate citrate transport and metabolism. This study proposed to determine the mechanisms of citrate transport and metabolism in *S. mutans*, and to examine if citrate modulates *S. mutans* aciduricity. Radiolabeled citrate was used during citrate transport to identify citrate metal ion cofactors and thin layer chromatography (TLC) was used to identify metabolic end-products of citrate metabolism. *S. mutans* was grown in MM4 with varying citrate concentrations and pH and the effects on growth rate and cell survival monitored. Intracellular citrate was growth inhibitory to these bacteria, especially at low pH. The most effective cofactor for citrate uptake by *S. mutans* was Fe^{3+}. The metabolic end-product of citrate metabolism was aspartate and a citrate transporter mutant was more citrate tolerant than the parent.

Introduction

Streptococcus mutans is a normal inhabitant of dental plaque and is considered a major etiological agent of dental caries, one of the most prevalent dental diseases. Two virulence determinants of *S. mutans* linked to cariogenicity are acidogenicity and aciduricity. These are the ability to produce acid and the ability to survive and grow at low pH respectively. Acidogenicity and aciduricity allow *S. mutans* to out compete other organisms within dental plaque, which can lead to the progression of carious lesions.

Citric acid is ubiquitous in nature; for example fruit juices contain between 5 and 8% citric acid and teeth are composed of 0.3% citric acid by weight (3), (16). Citric acid is also a cause of tooth enamel erosion (3). Citrate is widely used as a food preservative and also has bactericidal activity against coagulase-negative staphylococci and *S. aureus* (11).

Most bacteria have transport systems that allow for the uptake of citrate. In the genera *Lactococcus* and *Leuconostoc* citrate transport is mediated by homologous citrate/lactate anti-porters named CitP (5), (6). This transport leads to the generation of a proton motive force and to increased cell survival in acidic conditions (5), (6). In these bacteria the co-metabolism of citrate with glucose results in heterofermentation and the production of the end-products CO_2, diacetyl, acetoin, and butanediol. Some bacteria including *Klebsiella pneumoniae* even have the ability to grow in an anaerobic environment utilizing citrate as the sole carbon source [reviewed in (4)]. *K. pneumoniae* possesses two distinct citrate transporters, under aerobic conditions a proton dependent CitH citrate transporter is expressed, whereas under anaerobic conditions a sodium-ion dependent CitS is expressed (12).

Unlike *K. pneumoniae, S. mutans* is unable to survive when citrate is the sole source of carbon as observed by no growth on Simmons citrate agar. *S. mutans* also does not evolve CO_2 gas when citrate is available, which is a common by-product of citrate metabolism in many other lactic acid bacteria (LAB). These observations have left the relationship between *S. mutans* and citrate widely unstudied. Interestingly, the *S. mutans* genome contains orthologs of many citrate metabolic genes from bacterial species known to utilize citrate (3). These genes encode the subunits of citrate lyase (citCDEFGX) and oxaloacetate decarboxylase (*bbc* (α), *oadB* (β), & *pycB* (γ)) (2). The *S. mutans* genome also harbors a putative Mg^{2+}-dependent citrate transporter which we have designated *citM*. This citrate metabolism locus is illustrated in Figure 1.3 and the possible metabolic outcomes for citrate in Figure 1.4. This study, the first of its kind in *S. mutans*, focuses on the mechanisms of citrate uptake and metabolism by *S. mutans*, and the effect of citrate on cell survival at low pH.

Materials and Methods

Bacterial Strains and Growth Conditions.

The *S. mutans* strains used in this study are listed in Table 2.1. For transport assays *S. mutans* UA159 cells were grown in Berman's Broth (BB) (2% trypticase peptone, 0.1% yeast extract, 25 mM potassium phosphate, 34 mM NaCl, 0.5% thioglycolic acid, 1 mM $MgSO_4$, 0.1 mM $MnSO_4$)(BB) (14). For growth curve analysis cells were grown in a defined minimal media MM4 (7). When needed erythromycin was added at a final concentration of 5 μg/ml.

TABLE 2.1. Bacterial Strains Used in Chapter II

Strain	Relevant Characteristics	Source
UA159	Wild type	2
SMCitM	UA159::CitM-, Em^r (putative Mg^{2+} citrate transporter)	This Study
SMOAD	UA159::Oad-, Em^r (oxaloacetate decarboxylase B)	This Study
SMCLY	UA159::Cly-, Em^r (citrate lyase a)	This Study
SMASPB	UA159::AspB-, Em^r (aspartate transaminase)	This Study

Construction of Isogenic Mutants.

Knockouts in the *S. mutans citM*, *citF*, *oadB*, and *aspB* were generated using PCR ligation mutagenesis (10). Briefly, from the nucleotide sequence of each gene 2 sets of primers were designed. The first set of primers were directed towards the 5' flanking region of the gene of interest and the second set directed towards the 3' flanking region of the gene. Using UA159 genomic DNA as a template these two fragments were amplified via PCR. The two amplicons were subsequently ligated to an erythromycin resistance cassette. This entire fragment was then transformed into genetically competent *S. mutans* UA159 cells previously exposed to synthetic *S. mutans* competence stimulating peptide (CSP) (10). The fragment was integrated into the *S. mutans* chromosome via a double crossover event. The resulting

erythromycin resistant transformants were evaluated by PCR to confirm integration at the desired loci. Primers used for mutagenesis are listed in Table 2.2.

TABLE 2.2. Oligonucleotides Used in Chapter II

Primer	Primer Sequence (5' to 3')	Description
Erm 1	GGCGCGCCCGGGCCCAAAATTTGTTTGAT	*erm*AM cassette
Erm2	GGCCGGCCAGTCGGCAGCGACTCATAGAAT	"
CitM1	TCTGTTGTAGGAGTGATGGGGG	*citM* mutagenesis
CitM2[a]	GGCGCGCCCTCAGTGCCGTAAAAGGCGAC	"
CitM3[b]	GGCCGGCCCTTAACGGGCTTAGACATGGGC	"
CitM4	TCGGTAACGATCTCTCGTAGGC	"
OAD1	CAATCTGAATGGGAGCCTATTTCG	*oad* mutagenesis
OAD2[a]	GGCGCGCCCCGCCGTTAATAACCTGAGTTAGGAC	"
OAD3[b]	GGCCGGCCCGCAGTAGGTGCTAATGTTTCAGGAC	"
OAD4	TGACCATCAGGATAACGGCG	"
CLY1	TTCTGTTATCGCAGGTGGCTTAC	*cly* mutagenesis
CLY2[a]	GGCGCGCCCAAGGAGCATCTCGCAGCATAGC	"
CLY3[b]	GGCCGGCCCCGCTATTGTTGGTAATCCTCAACC	"
CLY4	TGTCCGCCAGCTTCCTTAGTAG	"
ASPB1	TGCTGGATGAAGCGAAACTTACTC	*aspB* mutagenesis
ASPB2[a]	GGCGCGCCAGGTGTTACAAAATCAGGCTGTCC	"
ASPB3[b]	GGCCGGCCCAGGTGTCGCTTTGGTAACAGG	"
ASPB4	GCATTGCCCGATAAGATTGGAC	"
CitMRT1	AGTTGTTATCCTTGTCTTCGCTGC	*citM* Rt-PCR
CitMRT2	CGATGACTAAGCCCCAGAAACC	"

[a.] Underlined based indicate *Asc I* restriction sequence
[b.] Underlined based indicate *Fse I* restriction sequence

Growth Curves.

Single colonies of the appropriate strain were inoculated into THYE and incubated overnight at 37°C in a 5% CO_2 environment. Overnight cultures were subsequently inoculated 1:10

into anaerobic MM4 at pH 7 and incubated overnight at 37°C under an atmosphere of 10% CO_2, 10% H_2, and 80% N_2 (COHN). The overnight subcultures were then inoculated into a microtitre plate 1:10 in quadruplicate into anaerobic MM4, pH 7 containing appropriate levels of citrate (0 mM, 2 mM, 4 mM, 6 mM or 8 mM citrate). Plates were then sealed and placed into a Bioscreen C automated growth monitor (Lab-Systems). The bacteria were incubated for 3 days at 37°C. Data points were taken every 20 minutes with 20 seconds of shaking prior to each optical density measurement at 600 nm. From these data growth curves were obtained and doubling times were calculated.

Citrate Killing Curves.

Single colonies of the appropriate strain were inoculated into THYE and incubated overnight at 37°C in a 5% CO_2 atmosphere. Overnight cultures were subsequently inoculated 1:100 in anaerobic MM4 at pH 7 COHN. Two aliquots of the overnight subcultures were then centrifuged at 4000 x g for 10 minutes. The pellets were then suspended to 10-fold their original culture volume in MM4 at pH 5 containing either no citrate or 8 mM citrate and incubated at 37°C in COHN. An aliquot was removed and serially diluted in 10 mM potassium phosphate buffer, pH 7.2. Twenty μL of each dilution was spotted in triplicate onto THYE 1% agar plates that were incubated at 37°C in COHN for 2 days. This first plate was considered time zero. Subsequent aliquots were removed, at 5 hrs, 1 day, 2 days, and 3 days, serial diluted and plated.

Citrate Adaptation.

Single colonies of the appropriate strain were inoculated into THYE and incubated overnight at 37°C in a 5% CO_2 atmosphere. Overnight cultures were subsequently inoculated 1:10 in anaerobic MM4 at pH 7 and incubated overnight at 37°C under an atmosphere of COHN. Two aliquots of the overnight subcultures were then centrifuged at 4000 x g for 10 minutes. The pellets were then suspended to 10-fold their original volume in MM4 at pH 7 containing either no citrate or 8 mM citrate and incubated at 37°C in COHN for 18 hrs. Following incubation, two aliquots from each subculture were then centrifuged at 16000 x g for 5 minutes. The pellets were then suspended to 10-fold their original volume in MM4 at pH 5 containing either no citrate or 8 mM citrate and incubated at 37°C in a COHN. An aliquot was removed and serially diluted in 10 mM potassium phosphate buffer, pH 7.2. Twenty μL of each dilution was spotted in triplicate onto THYE 1% agar plates that were incubated at 37°C in COHN for 2 days. This first plate was considered time zero. Subsequent aliquots were removed, serial diluted and plated at 24 hrs, and 48 hrs.

Citrate Transport Assay.

Citrate transport assays were preformed as described by Krom et. al. (9) with minor modifications. Single colonies of *S. mutans* UA159 were inoculated into 10 ml of modified BB at pH 6.5 supplemented with 0.1% raffinose and 10 mM citrate and incubated overnight at 37°C in a 5% CO_2 atmosphere. Overnight cultures were subsequently diluted 5-fold in fresh BB at pH 6.5 supplemented with 0.35% raffinose and 10 mM citrate and incubated at 37°C until cells reached mid-logarithmic phase (OD_{600} ~ 0.4 - 0.6). The cells were then harvested by centrifugation and washed twice with cold 50 mM PIPES [piperazine-*N*,*N*'-bis

(2-ethanesulfonic acid)], pH 6.5. PIPES buffer was previously treated with 15 g/L chelex 100 (Sigma Chemicals) for 18 h to remove residual metal ions. Cells were then resuspended in 10 ml of the same buffer supplemented with the appropriate amount of metal ions. One hundred µl aliquots were incubated at 37°C and allowed to equilibrate for 15 minutes. At time zero [1,5-^{14}C] citrate (4.5 µM final concentration at 112 mCi/mmol) was added. Uptake was stopped by the addition of 2 ml of ice cold 0.1 M LiCl and cells were immediately filtered through a 0.2 µm pore-size nitrocellulose filter. The filtered cells were washed once with 2 ml of ice cold 0.1 M LiCl and submerged into scintillation fluid and the internalized radioactivity counted in a liquid scintillation counter. To obtain dry weights for standardization, 1 ml aliquots of resuspended cells were taken in quadruplicate and filtered through dried pre-weighed filters, dried for 24 hrs and re-weighed.

Quantitative Real-time PCR analysis of *citM* expression.

To determine the effects of citrate presence or pH on *S. mutans citM* transcription levels, quantitative Real-time PCR (qRT-PCR) was performed. *S. mutans* UA159 cells were grown in Berman's Broth (pH 5.0, pH 6.0 and pH 7.0) to mid-log phase. Total RNA was extracted as described by Hanna et. al. (8). A First Strand cDNA synthesis kit (MBI Fermentas) was used to manufacturer's specifications to generate cDNA from 1 µg of DNAse treated RNA. The resulting single stranded cDNA was diluted to 50 ng/µl for RtPCR analysis. To ensure the absence of contaminating DNA two negative control reaction mixtures were setup, one without template RNA and another lacking reverse transcriptase.

For amplification and detection of RtPCR products, we will use the Quantitect™ SYBR-Green® PCR kit (Qiagen). RtPCR reactions contained 100 ng of template DNA, 250

nM of each primer and 2X SYBR-Green mix (contains SYBR-Green, dNTPs, $MgCl_2$ and Hotstar Taq polymerase). RtPCR was carried out in a Cepheid Smart Cycler (Sunnyvale, CA) using *citM* specific primers (Table 2). For each reaction the Cycle Threshold (Ct) was measured, this value reflects the starting amount of cDNA in each sample. Initial DNA concentrations were determined by comparing experimental Ct values to a standard curve for *citM* which was generated by plotting these Ct values against the absorbance of serially diluted known quantities of amplicons. These data were normalized using *gyrA* expression results which were invariant for the conditions examined.

Citrate Metabolism Assay.

S. mutans cells were grown in Berman's Broth containing 10 mM citrate to mid log phase (~OD_{600} 0.4). Cells were subsequently washed and re-suspended in 10 mM potassium phosphate, 10 mM $MgCl_2$ buffer at appropriate pH. This mixture was then incubated at 37°C for 10 minutes to allow for equilibration after which 2.5 mM [1,5-^{14}C] citrate (1 Ci mol^{-1}) was added to the reaction. The cells were allowed to metabolize the citrate for 1 hr, followed by centrifugation to separate the cells from the extracellular metabolites. Cells were then lysed using glass beads and a Fast Prep machine to release the intracellular metabolic end-products. The intracellular extract was treated with trichloroacetic acid (TCA) to precipitate proteins and nucleic acids, followed by an ethylenediaminetetraacetate (EDTA) treatment to chelate metal ions. The extract was then neutralized with potassium hydroxide (KOH). The extracellular metabolites were also treated in the same fashion as the intracellular extract; with additions of TCA, EDTA, KOH. Both extracts were spotted onto 60 angstrom Silica gel TLC plates. Standards of radiolabeled citrate, aspartate, glutamate, lysine, and histidine

(Amersham Biosciences) were also spotted onto the TLC plates. The plates were then placed into a developing chamber, previously charged with a 4:1:1 n-butanol, acetic acid and water eluent, and allowed to resolve. Following migration of 4-6 hours, plates were air-dried and the spots visualized by autoradiography. This was repeated for the SMCitM, SMCLY, SMOAD, and SMASPB strains. Non-radiolabeled standards of citrate, oxaloacetate, and aspartate were also spotted onto the TLC plates, and subsequent to resolution the standards were detected colourimetrically using a 10% solution of bromocresol green in ethanol.

Results

The Effect of Citrate on Growth.

In lactic acid bacteria citrate provides increased growth yield and acid tolerance (5,6). To determine if similar benefits could be realized in *S. mutans*, UA159 cells and putative citrate metabolic knockout strains SMCitM, SMCLY, SMOAD, and SMASPB were grown in a defined minimal media MM4 in the presence of citrate, as described previously, results for which are shown in Table 2.3. These genes were selected for their putative role in citrate catabolism as displayed in Figure 1.4. Citrate at 4 mM caused no statistically significant growth rate changes to any of the strains expect for SMASPB. Increasing the citrate concentration to 8 mM caused a major increase in generation time and completely inhibited the growth of SMAPSB. Of the strains that could grow in the presence of 8 mM citrate, interestingly, the UA159 wild-type strain was most affected by the citrate as displayed by a 5.5-fold increase in doubling time versus a 3-fold increase in doubling time for the isogenic mutants SMCitM, SMOAD, and SMCLY.

TABLE 2.3. Growth Rates of Citrate Metabolic Mutant Strains

	Doubling Time (min)		
Strain	0 mM Citrate	4 mM Citrate	8 mM Citrate
UA159	135.4 ± 4.7	130.1 ± 2.7	748.8*[1] ± 130.7
SMCitM	141.6 ± 5.4	131.3 ± 2.6	402.8* ± 69.7
SMOAD	138.4 ±5.9	127.5 ± 2.2	458.1* ±107.5
SMCLY	131 ± 2.9	139.1 ± 3.6	411.9* ± 40.0
SMASPB	132.5 ± 1.5	155.3 ± 6.5*[2]	No Growth

* Significant difference when grown without citrate $P<0.01$
[1] Significant difference from the other strains $P<0.01$
[2] Significant difference from the other strains $P<0.05$

Citrate enhances killing of *S. mutans* at low pH.

A growth advantage at low pH is conferred to *Lactococcus lactis* by citrate co-metabolism with glucose (6). Therefore the role of citrate in survival under acidic conditions was investigated for *S. mutans*. No growth was observed at pH 5.0 in MM4 broth, with or without citrate, using the previously described protocol. The rate of cell death was then investigated in MM4 media with and without citrate to determine what effect citrate would have at low pH. It was observed that citrate markedly increased the rate at which *S. mutans* was killed at pH 5.0 for both UA159 and CitM- (Figure 2.1). The CitM- cells were more resistant to citrate killing than UA159 cells when 8 mM citrate was added to the medium (Figure 2.1).

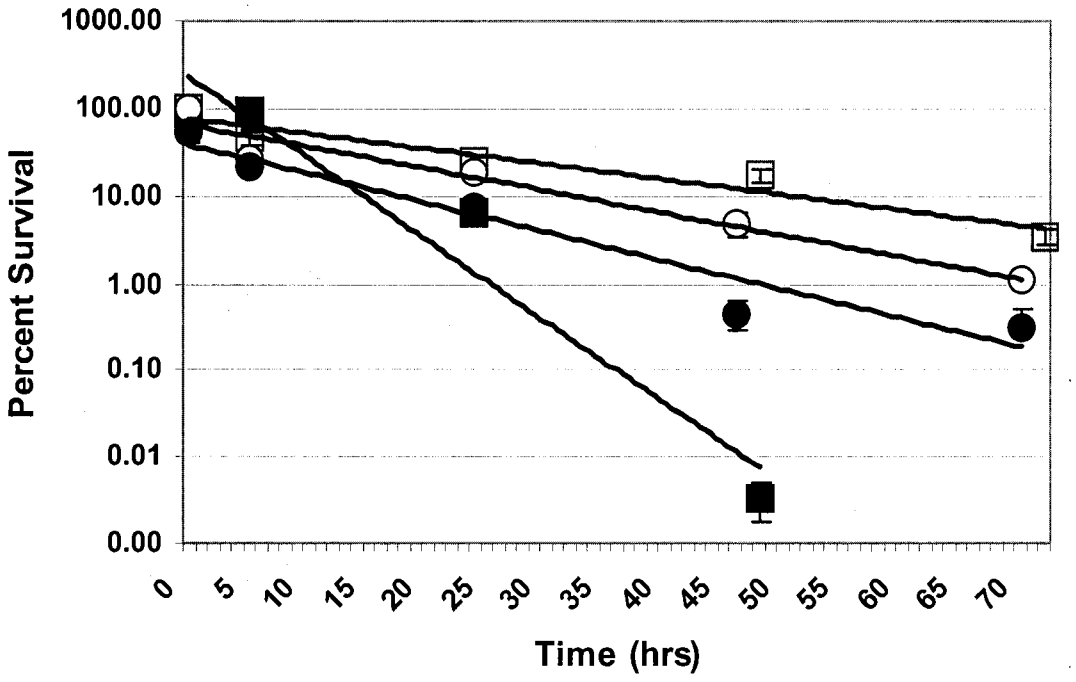

Figure 2.1. Relative survival of *S. mutans* at pH 5 in MM4 with or without 8 mM citrate. Cells were grown in MM4 medium and then subjected to MM4 pH 5 containing 8 mM citrate (UA159 (■), SMCitM (●)) or without citrate (UA159 (□), SMCitM (○)) and subsequently serial diluted and plated on THYE agar plates. Survival-fold, relative to growth without citrate, for each strain was determined by counting the colonies present at each time point and dividing that by the colonies present at time zero h without citrate.

Adaptation to citrate enhances survival at low pH.

As seen in Figure 2.1, citrate enhancess killing of *S. mutans* at low pH. Investigations into whether *S. mutans* could adapt to citrate where subsequently undertaken. *S. mutans* cells where subcultured at neutral pH in the presence or absence of citrate prior to being subjected to killing at pH 5 with or without citrate. Cells that were previously exposed to citrate (citrate adapted) displayed greater survival at pH 5 than those that were not exposed to citrate (unadapted) (Figure 2.2). Citrate enhanced killing of both the adapted and unadapted *S.*

mutans cells, the adapted cells survived to a much greater extent than the unadapted cells (Figure 2.2).

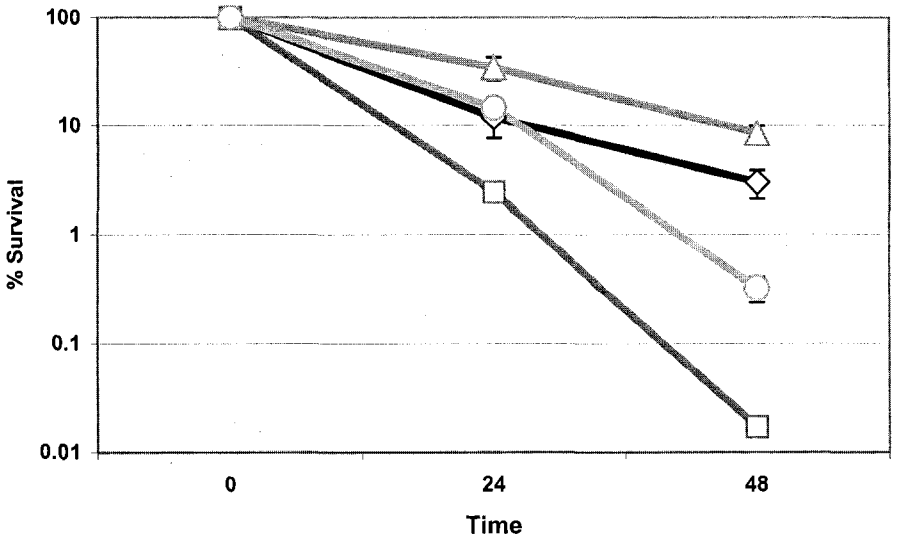

Figure 2.2. Effect of pre-incubating *S. mutans* UA159 with citrate cells prior to pH 5 exposure.

Cells were grown in medium MM4 at pH 7 in the absence or presence of 8 mM citrate and subsequently exposed to medium MM4 at pH 5 with 8 mM citrate. After this the cells were serially diluted and plated on THYE agar plates. The level of survival for each strain was determined by dividing the number of colonies present at each time by the number of colonies present at time zero. Symbols: (◊), cells grown at pH 7 without citrate and then incubated at pH 5 without citrate; (□), cells grown at pH 7 without citrate and then incubated at pH 5 with 8 mM citrate; (Δ), cells grown at pH 7 with 8 mM citrate and then incubated at pH 5 without citrate; (○), cells grown at pH 7 with 8 mM citrate and then incubated at pH 5 with 8 mM citrate.

S. mutans transports citrate with divalent metal ion cofactors.

The *citM* gene was designated as a putative Mg^{2+}-citrate transporter but only has limited homology (36% identity at the amino acid level) to the *B. subtilis* Mg^{2+}-citrate transporter CitM, known to catalyze the uptake of citrate in complex with Mg^{2+} (2,9). With all divalent metal ions depleted from the reaction mixture, very little citrate was transported by *S. mutans*

(Figure 2.3). When 1 μM $FeCl_3$ or 5 mM $MnCl_2$ was added to the reaction citrate uptake was increased (Figure 2.3). With 5 mM $CaCl_2$ or 5 mM $NiCl_2$ added to the reaction the rate of uptake had no increase over that without metal cations (results displayed in Supplemental Data section). Negligible amounts of citrate entered SMCitM cells in which the citrate transport gene was mutated (Figure 2.3). These observations indicated that Fe^{3+} was the best cofactor tested to facilitate citrate transport via CitM.

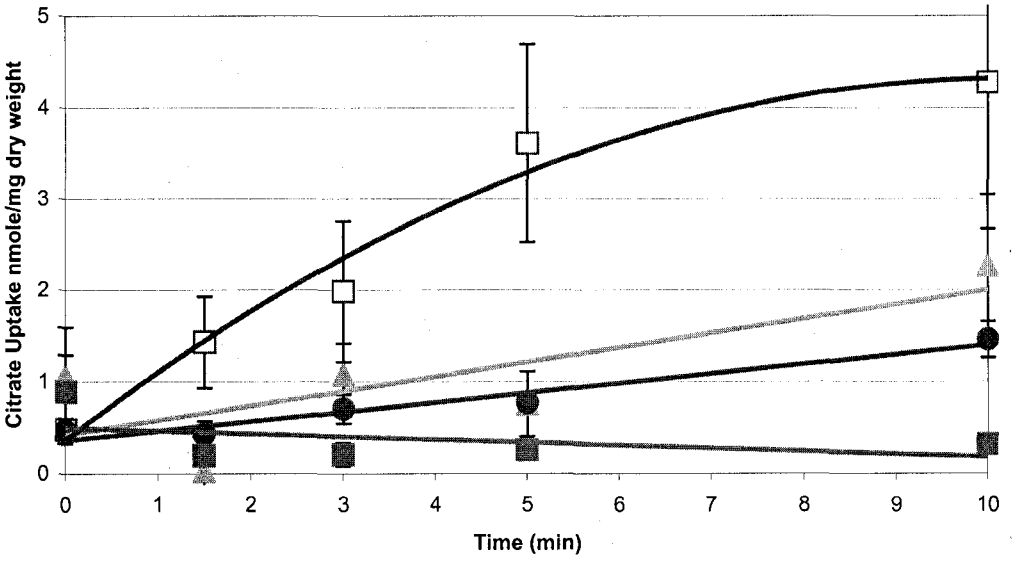

Figure 2.3. Uptake of [1,5-^{14}C]citrate by *S. mutans* in the presence of different metal cations. The uptake of [^{14}C]citrate was measured in 50 mM PIPES, pH 6.5, in the presence of 1 μM Fe^{3+} (□) or 5 mM Mn^{2+} (▲) or without added metal ions (●). The uptake of [1,5-^{14}C]citrate by SMCitM was measured in the presence of 1 μM Fe^{3+} (■). [1,5-^{14}C]citrate uptake was not detected with 5 mM Mg^{2+}, Ni^{2+}, or Ca^{2+} as a cofactor (data shown in Supplemental Figure 2.7).

***citM* expression is increased by citrate.**

Our transport data indicated that citrate was transported by *S. mutans* in complex with ferric ions, and this process was mediated by the CitM transporter. To determine if *citM* expression was modulated by exposure to citrate or varying pH qRT-PCR expression analysis was performed. The expression of *citM* was up-regulated 25-fold at pH 7.0 upon exposure to citrate as seen in Fig. 2.4. In following the hypothesis that citrate uptake and metabolism may be involved in acid tolerance, citM expression levels were measured at decreasing pH (pH 6.0 and pH 5.0). Our data indicated that the mean expression-fold increased as the pH was reduced displayed in Fig. 2.4. However, the differences in *citM* expression levels at pH 6.0 and pH 5.0 were not significantly from the expression levels seen at pH 7.0.

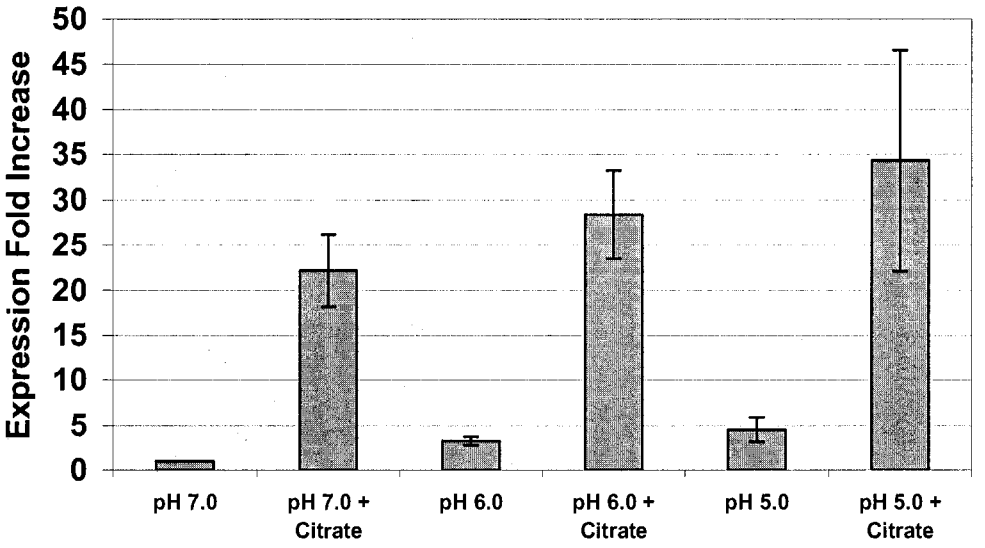

Figure 2.4. Expression of *citM* in response to pH and citrate.
The data indicate the increased expression compared to the expression at pH 7 without citrate, normalized to *gyrA* expression levels. The values are means of five replicates.

***S. mutans* metabolizes citrate.**

Although many lactic acid bacteria are capable of citrate metabolism, no previous studies to our knowledge examined the ability of *S. mutans* to metabolize citrate. To determine if *S. mutans* was capable of citrate metabolism wild type UA159 cells were incubated in the presence of [1,5-^{14}C] citrate and metabolites were then separated on silica TLC plates. Our results indicated that *S. mutans* converts internalized citrate into the amino acid aspartate (as visualized for Wild type in Figure 2.5). However, using nonradiolabeled standards, and colourimetric detection, aspartate resolved the same as oxaloacetate. This suggested the spot identified in Figure 2.5 as aspartate could also be oxaloacetate, or more likely a combination of the two metabolites (colourimetric detection not shown). Lowering the external pH or oxygen concentration had no effect on the metabolic end-product composition (data not shown).

The knockout strains SMCitM, SMOAD, SMCLY and SMASPB were used to determine if these genes encoded enzymes involved in citrate metabolism. As with UA159, these strains were incubated in the presence [1,5-^{14}C] citrate and the metabolites separated via TLC. The metabolites of the oxaloacetate decarboxylase mutant, SMOAD, separated in the same fashion as those of wild type, shown in Figure 2.5, indicating noninvolvement of the enzyme in citrate metabolism. The metabolites of the aspartate mutant, SMASP also separated in the same fashion as those of wild type, but since oxaloacetate could not be resolved from aspartate using the techniques employed a firm conclusion cannot be drawn. Citrate was seen to accumulate inside the citrate lyase mutant, SMCLY, as shown in Figure 2.5, and therefore it can be concluded that citrate lyase is responsible for the conversion of

citrate to oxaloacetate. The SMCitM mutant was unable to transport citrate as no spot was visualized corresponding to citrate with TLC as shown in Figure 2.5.

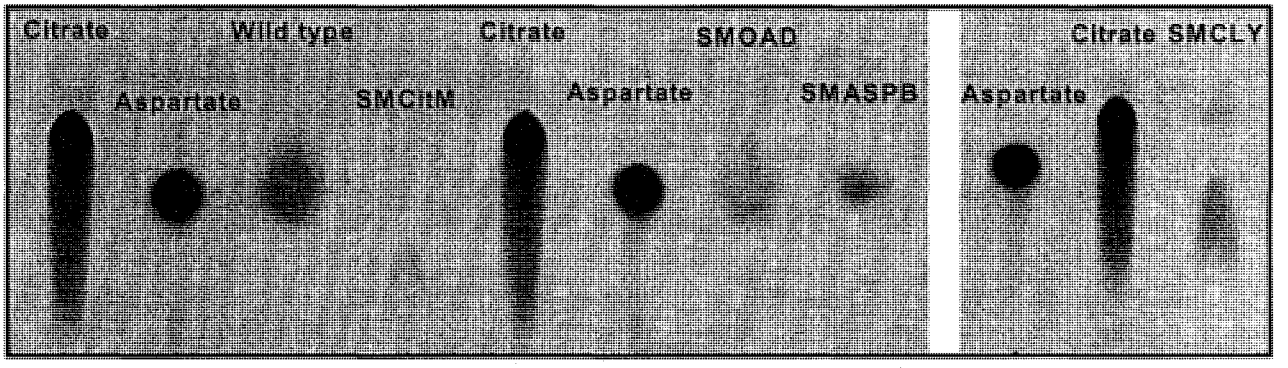

Figure 2.5. Autoradiograph of intracellular metabolites separated by TLC from *S. mutans* strains.

The cells were resuspended in 50 mM PIPES, pH 6.5, and allowed to take up 2.5 mM [1,5-^{14}C] citrate for 1 h. Radiolabeled [1,5-^{14}C]citrate and [^{14}C]aspartate standards were also spotted on the plate. The strains examined were *S. mutans* UA159 (Wild type), SMCitM, SMCLY, SMOAD, and SMASPB.

Discussion

This is the first known description of citrate transport and metabolism by *S. mutans*. Other bacterial species, including *B. subtilis*, *K. pneumoniae*, and *Lactococcus lactis*, have been shown to both transport and metabolize citrate (9,12,6). This study demonstrated that *S. mutans* does, in fact, transport and metabolize citrate. In contrast to the advantages citrate provides to other LAB, in *S. mutans* citrate appears to affect growth and survival negatively at concentrations that are physiologically plausible. The addition of citrate increased killing of the SMCitM transport knockout mutant at pH 5 to a much lesser extent than the killing observed by the addition of citrate to UA159. The results from these killing curves (Figure 2.1) indicated that citrate killing works via both intracellular and extracellular processes. Citrate could therefore potentially be used as an *S. mutans* growth inhibitor. In fact, this may explain the basis behind the observed effectiveness of citrate as an additive in toothpaste (1).

Ferric citrate transport has been well documented in gram-negative bacteria. We believe this is the first known report of a Gram-positive citrate transporter preferentially utilizing ferric ions as a cofactor. Citrate chelates ferric ions and readily precipitates them out of solution, therefore transport analyses were performed using 1 μM $FeCl_3$ compared to 5 mM of other metal ions. *S. mutans* is known to have a nutritional requirement for trace metals including magnesium, manganese, and iron (3). *S. mutans* optimally requires iron at a concentration of 3.6 μM (3), which is far greater that the 0.01 to 1.0 μM present in human saliva (18). An *S. mutans* ABC transporter, encoded by the *sloABCR* operon, has previously been identified as a Fe^{2+} transporter (17). Experiments have shown that iron increases the dental plaque pH *in vivo*, however the mechanisms are not known (13,15). Iron has an important role in the cariogenesis of *S. mutans,* and CitM may play a part in iron acquisition.

This work is also the first to describe citrate metabolism by *S. mutans*. This metabolism results in the conversion of citrate to aspartate. Aspartate production in the SMOAD mutant can be explained since the Oad enzyme is not part of the citrate to aspartate pathway. The precise metabolic end-product of SMASPB was not determined, however it can be inferred that AspB is involved in citrate metabolism as its corresponding mutant was more sensitive to growth in the presence of citrate than the parent strain. Citrate lyase is responsible for the conversion of citrate to oxaloacetate as citrate was seen to accumulate within the SMCLY strain. Citrate does appear to enter central metabolism and does not seem to provide any growth or survival benefit under acidic conditions. Interestingly, however, the presence of citrate during growth at neutral pH affords *S. mutans* enhanced acid tolerance as well as tolerance to citrate at low pH. *S. mutans* may, in fact, exploit citrate's ability to chelate essential ferric iron to transport it into the cell.

Supplemental Data

Previous investigations into citrate metabolism in other LAB have shown citrate metabolism to vary in response to environmental changes including pH and the concentration of oxygen. Using the TLC protocol described above, we examined the resulting end-products of citrate metabolism in *S. mutans* in the absence of oxygen and in response to low pH. Data from these experiments are shown on Figure 2.6. These data indicate that *S. mutans* can metabolize citrate under aerobic and anaerobic conditions at both pH 6.5 and 5.5. Additionally, extracellular pH and oxygen environment have no apparent effect on the composition of citrate metabolic end-products, since the intracellular end-products of metabolism all displayed the same R_f value.

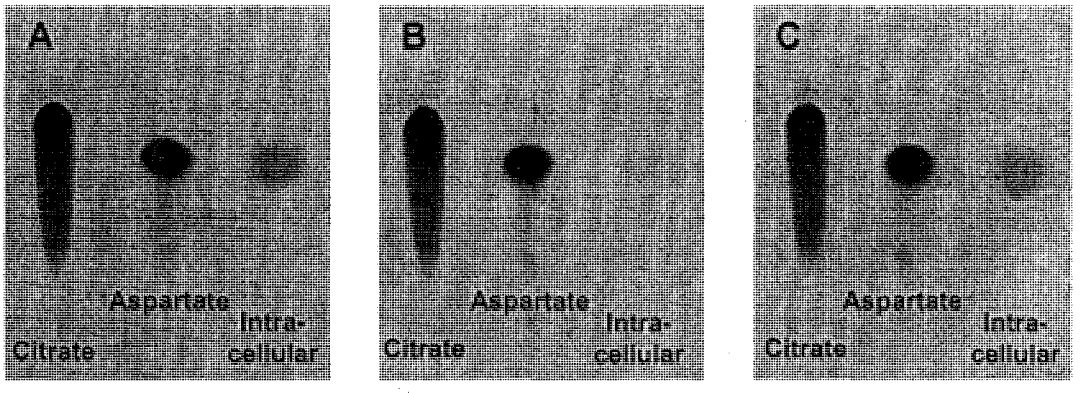

Figure 2.6. Autoradiograph of TLC plates in which *S. mutans* was cultured under various environmental conditions.

All cells were grown in BB at 37°C. Aerobic = 5% CO_2; Anaerobic = 10% CO_2, 10% H_2, and balanced N_2. Only intracellular components are shown. **A:** Anaerobic growth at pH 6.5, **B:** Aerobic growth at pH 5.5, and **C:** Anaerobic growth at pH 5.5.

To further compliment the above citrate transport data, citrate transport by *S. mutans* was investigated in the presence of additional metal cations including magnesium, calcium, and nickel. Using these metal cations as co-factors resulted in almost negligible amounts of

citrate to by taken up by the cells, shown in Figure 2.7. These data further strengthen our previously reported findings stating ferric irons induce citrate uptake to the greatest extent.

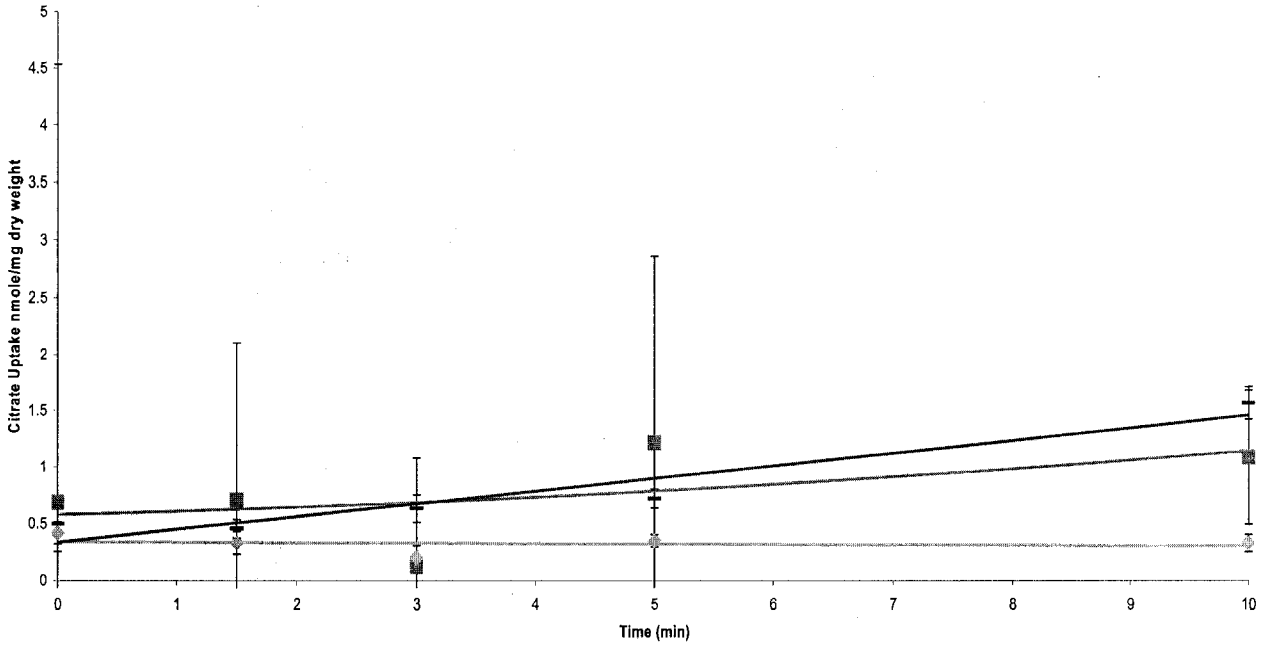

Figure 2.7 Uptake of [1,5-^{14}C] citrate by *S. mutans* in the presence of different metal cations. The uptake of [^{14}C] citrate was measured in 50 mM PIPES, pH 6.5, in the presence of 1 mM Mg^{2+} (squares) or 5 mM Ca^{2+} (dashes) or Ni^{2+} (diamonds).

Acknowledgements

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CHAPTER III: INVOLVEMENT OF THE DETOXIFYING ENZYME LACTOYLGLUTATHIONE LYASE IN *STREPTOCOCCUS MUTANS* ACIDURICITY

Korithoski, B., Lévesque, C.M., Cvitkovitch, D.G. Involvement of the Detoxifying Enzyme Lactoylglutathione Lyase in *Streptococcus mutans* Aciduricity. J. Bacteriol. Aug. 24, 2007. [Epub ahead of print].

Abstract

Streptococcus mutans, a normal inhabitant of dental plaque, is considered a primary etiological agent of dental caries. Its main virulence factors are acidogenicity and aciduricity, the ability to produce acid and survive and grow at low pH, respectively. Metabolic processes are finely regulated following acid exposure in *S. mutans*. Proteome analysis of *S. mutans* demonstrated that lactoylglutathione lyase (LGL) was up-regulated during acid challenge. The LGL enzyme catalyzes the conversion of toxic methylglyoxal, derived from glycolysis, to S-D-lactoylglutathione. Methylglyoxal inhibits the growth of cells in all types of organisms. The current study aimed to investigate the relationship between LGL and both aciduricity and acidogenicity in *S. mutans*. An *S. mutans* isogenic mutant defective in *lgl* (LGLKO) was created and its growth kinetics characterized. Insertional inactivation of *lgl* resulted in an acid sensitive phenotype in relation to UA159 wild-type. However, the glycolytic rate at pH 5.0 was greater for LGLKO than UA159 wild-type cells. LGL was involved in the detoxification of methylglyoxal, illustrated by the absence of enzyme activity in LGLKO and hypersensitivity of LGLKO to methylglyoxal compared with UA159 (MIC of 3.9 and 15.6 mM, respectively). Transcriptional analysis of *lgl* conducted by quantitative Real-Time PCR revealed that *lgl* was up-regulated (~ 7-fold) during the exponential growth phase compared with the stationary growth phase. Gene expression studies conducted at low pH, demonstrated *lgl* was induced during acidic growth (~ 3.5-fold) and following acid adaptation (~ 2-fold). This study demonstrates that in *S. mutans,* Lgl functions in the detoxification of methylglyoxal, resulting in increased aciduricity.

Introduction

Dental caries is one of the most prevalent infectious dental diseases afflicting humans. The microorganism most strongly associated with human dental caries is *Streptococcus mutans*, a normal inhabitant of dental plaque. Two of the foremost cariogenic determinants of *S. mutans* are its acidogenicity and aciduricity (reviewed by Banas (3)). These properties are the ability to produce acid end products from the metabolism of dietary carbohydrates and the ability to survive and grow at low pH respectively. Acidogenicity and aciduricity allow *S. mutans* to dramatically drop the pH within the dental plaque biofilm, which can promote the development and progression of carious lesions via its sustained dominance and acid production via glycolysis. The feast and famine conditions generated by the host diet can result in rapid increases in sugar that must be quickly removed from the environment. Likewise the glycolytic end-products must be quickly removed from the cell to avoid toxicity.

LGL is an enzyme involved in the detoxification of methylglyoxal, a highly toxic electrophilic glycolytic by-product that reacts with and inactivates intracellular macromolecules, including both proteins and nucleic acids (9, 10). Therefore its rapid degradation is vital for cell survival. The formation of methylglyoxal occurs via enzymatic production during glycolysis from the fragmentation of triose-phosphates (4, 10). LGL is involved in methylglyoxal detoxification via the formation of *S*-D-lactoylglutathione from the hemimercaptal adduct that is formed nonenzymatically between glutathione and the 2-oxoaldehyde methylglyoxal (7). Glyoxalase II then converts *S*-D-lactoylglutathione into reduced glutathione and D-lactate (7). An examination of the *S. mutans* UA159 genome does not reveal the presence of a glyoxalase II homologue, suggesting an alternate pathway by

which *S*-D-lactoylglutathione is neutralized. In *E. coli*, methylglyoxal was accumulated under physiological conditions of uncontrolled carbohydrate metabolism (1, 6, 12) and the concentration of methylglyoxal was seen to be greater in highly metabolically active human red blood cells where *lgl* expression appeared to be regulated by the rate of glycolysis (20, 24).

Salmonella enterica expressed elevated levels of *lgl* mRNA when engulfed in the acidic environment of the macrophage (5). Recent studies in *S. mutans* have suggested that regulation of metabolic pathways is important for the survival of *S. mutans* in an acidic environment (15, 25, 26). Proteomic analysis by Len et. al. (15) showed increased synthesis of 70 proteins during chemostat growth at pH 5.0 relative to growth at pH 7.0. Separate two-dimensional gel electrophoresis studies also demonstrated that LGL was up-regulated in an acidic environment (15, 26). These findings suggest LGL may be linked to *S. mutans* acid tolerance (15, 26). The goal of this study was to investigate the role LGL may play in the aciduricity and acidogenicity of *S. mutans*.

Materials and Methods

Bacterial strains and growth conditions.

S. mutans wild-type strain UA159 was used in this study. The *S. mutans lgl* isogenic knockout mutant (LGLKO) was generated using PCR ligation mutagenesis (14). Primers used for mutagenesis are shown in Table 3.1. *S. mutans* UA159 cells were grown in Todd Hewitt broth supplemented with 0.3% yeast extract (THYE). Erythromycin was added to a final concentration of 10 μg/ml when needed.

TABLE 3.1. Primers Used in Chapter III

Primer	Primer Sequence (5' to 3')[a]	Description
Erm 1	GGCGCGCCCGGGCCCAAAATTTGTTTGAT	Erythromycine cassette (13)
Erm 2	GGCCGGCCAGTCGGCAGCGACTCATAGAAT	"
LGL 1	GGACAATCAAAATCAACCTC	*lgl* mutagenesis
LGL 2	GGCGCGCCTAACTCATAGTCGGGTCG	"
LGL 3	GGCCGGCCACTACTTCATCACAGACCCC	"
LGL 4	ATGCCGACACACATAGCAAC	"
LGL RT 1	CCTTGGCAATGGCTATGGTC	*lgl* qRT-PCR
LGL RT 2	TCGGGGTCTGTGATGAAGTAG	"
16S RT 1	CTTACCAGGTCTTGACATCCCG	*16S* qRT-PCR
16S RT 2	ACCCAACATCTCACGACACGAG	"

[a]Restriction sites are underlined: *Asc*I GGCGCGCC; *Fse*I GGCCGGCC.

Growth kinetics.

Overnight cultures of *S. mutans* UA159 and LGLKO strains were diluted (1:20) into pre-warmed THYE and incubated at 37°C under an atmosphere of 5% CO_2 until mid-log phase (optical density at 600 nm (OD_{600}) ~ 0.4). The subcultures were then inoculated (1:20) into microtitre plates in quadruplicate containing THYE broth at pH 7.5 and pH 5.0. Growth was

followed for 24 h using an automated growth monitor (Bioscreen C, Labsystems, Finland). From these data, growth curves were generated and doubling times calculated (11).

Acid tolerance response (ATR) assay.

Overnight cultures of *S. mutans* UA159 and LGLKO strains were diluted (1:20) into TYE (10% Tryptone, 5% Yeast Extract, 17.2 mM K_2HPO_4) supplemented with 5 mM glucose at pH 7.5 and incubated at 37°C, in a 5% CO_2 enhanced environment until mid-log phase (OD_{600nm} ~ 0.4). Cultures were then divided into two equal aliquots, (termed 'adapted' and 'non-adapted'), and pelleted via centrifugation. Non-adapted cells were resuspended in TYE at the lethal pH value of 3.2. An aliquot was immediately removed (t = 0), serially diluted in 10mM potassium phosphate buffer (pH 7.2) and the incubation if the culture was continued for 3 h at 37°C in an atmosphere of 5 % CO_2. Twenty μL of each dilution was spotted in triplicate onto THYE agar plates and incubated at 37°C, in a 5% CO_2 supplemented atmosphere for 2 days. Adapted cells were first resuspended in TYE at pH 5.5 for 2 h prior to being subjected to TYE at pH 3.2. The ATR was expressed as the percentage of cells to survive the lethal pH for 1 h, 2 h, and 3 h compared to the number of cells present at time zero.

Terminal pH determination.

Overnight cultures of *S. mutans* UA159 and LGLKO strains (8 independent cultures of each strain) were diluted (1:40) in fresh THYE at pH 7.5, pH 6.0 and pH 5.0 and incubated at 37°C in an atmosphere of 5% CO_2 for 20 h prior to the terminal pH measurement.

Continuous culture acidic competition assay.

Overnight cultures of UA159 and LGLKO were simultaneously inoculated (a dilution of 1:80 for each in ¼ THYE at pH 6.0) into a chemostat biofermentor with glass rods for biofilm accumulation. Cells were continuously cultured as described previously (17) with some modification. The culture was established initially at pH 6.0 at a fresh media flow rate of 0.1 dilutions/h (D) for 18 h. After culture establishment, culture pH was reduced to pH 5.0 and flow rate increased to D = 0.5. Aliquots of planktonic cells were removed, serially diluted and plated onto THYE agar and THYE + 10 μg/ml erythromycin agar. Biofilm cells were quantified by the removal of glass rods. Biofilm cells were released by vortex and cells serially diluted and plated as previously described.

Minimum inhibitory concentration of methylglyoxal

Commercial methylglyoxal (Sigma) was diluted in THYE and serially diluted (1:1) into 96-well microtitre plates. Microtitre plates were inoculated with 2 μl of *S. mutans* UA159 and LGLKO cells (at a concentration of 0.5 McFarland Standard), and subsequently incubated for 24 h at 37°C in an atmosphere of 5% CO_2. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of methylglyoxal that inhibited visible bacterial growth. To determine the minimum bactericidal concentration bacteria from each microtitre well were streaked out onto THYE agar plates that were incubated for 48h to test for cell viability.

Measurement of glycolytic rates.

Overnight cultures of *S. mutans* UA159 and LGLKO strains were diluted 1:10 into THYE at pH 7.5 and incubated at 37°C in air with 5% CO_2 until cultures reached mid-log phase (OD_{600nm} ~ 0.4). The cells were then harvested by centrifugation and washed twice with cold PK solution (1% peptone, 1% KCl) at either pH 7.0 or pH 5.0. Cells were next resuspended in PK solution at the appropriate pH to a final OD_{600nm} ~ 1.0. Aliquots (18 ml) of cell suspension were equilibrated in the reaction vessel at 37°C until residual glycolytic activity had diminished. Following equilibration, glucose was added to a final concentration of 200 mM and glycolysis followed by the rate of addition of potassium hydroxide (10 mM KOH at pH 7.0 and 2 mM KOH at pH 5.0) required to keep the pH constant, utilizing a Radiometer ABU901 autoburrette in conjunction with a PHM290 pH controller (Radiometer, Denmark). The glycolytic rate was defined as μmoles of acid neutralized per milligram (dry weight) of cells per minute. Additional experiments were conducted in the presence or absence of exogenously added 5 mM methylglyoxal.

Cell preparation for gene expression analysis and LGL enzyme activity.

S. mutans UA159 cells were grown under the following conditions prior to RNA isolation. **Acid growth:** Overnight cultures of *S. mutans* UA159 were diluted (1:40) in THYE at pH 7.5 and pH 5.0, and incubated at 37°C in an atmosphere of 5% CO_2 until mid-log growth phase (OD_{600} ~ 0.4). **Acid adaptation:** Overnight cultures of *S. mutans* UA159 were diluted (1:20) in TYE pH 7.5 and incubated at 37°C, in a 5% CO_2 atmosphere until mid-log growth phase was reached. Cultures were then divided into 2 aliquots, cells harvested via centrifugation, and subsequently resuspended in TYE pH 5.0. The first aliquot (unadapted)

was processed immediately, and the second aliquot (adapted) was incubated at 37°C in a 5% CO_2 supplemented atmosphere for 2 h prior to further manipulation. **Growth phase:** Overnight cultures of *S. mutans* UA159 were diluted (1:20) into TYE at pH 7.5 and incubated at 37°C in air with 5% CO_2 supplementation. An aliquot was removed when the cultures reached mid-log phase (OD_{600nm} ~ 0.4) and cells were harvested by centrifugation. The remaining cells were harvested after reaching stationary phase (20 h). **Glucose response:** Cell suspensions of *S. mutans* UA159 were prepared at both pH 7.0 and pH 5.0 as described for measurement of glycolytic rates. Cells were equilibrated for 20 min at 37°C to eliminate residual glycolytic activity. Subsequent to equilibration, 200 mM glucose was added to the suspensions for 15 min prior to cell harvesting. **Methylglyoxal response:** *S. mutans* UA159 cells were grown as described for glucose response, however cells were exposed to 10 mM methylglyoxal for 15 min before being harvested.

Quantitative Real-Time PCR (qRT-PCR) analysis of *lgl* expression.

Total RNA was isolated, processed and treated with RQ1 RNAse-free DNAse (Promega, USA) as described previously (8). From this RNA, cDNA was generated via reverse transcription using a First Strand cDNA synthesis kit (MBI Fermentas) according to the manufacturer's instructions. RNA samples lacking reverse transcriptase were included as controls to assure results were not the product of residual DNA contamination. The single stranded cDNA template qRT-PCR reactions were carried out using the QuantiTect SYBRGreen PCR kit (Qiagen, USA) in a Mx3005P QPCR system (Stratagene, USA). Specific primer sequences used (Table 2) in the reactions were designed to yield 100 – 150 bp products. For each reaction the Cycle Threshold (Ct) was measured; this value was

inversely proportional to the starting amount of DNA in each sample. All data was normalized against the expression of an internal standard, 16S rRNA. The expression fold-change was determined using the $2^{-\Delta\Delta}$ CT method (18).

Assay for glyoxalase enzyme activity.

To prepare soluble cellular proteins, *S. mutans* UA159 and the LGLKO strains were subjected to conditions as described previously. Cells were harvested via centrifugation after which cell pellets were resuspended in 10 mM potassium phosphate buffer (pH 7.2) and stored at –20°C. To release the intracellular soluble proteins cells were lysed using glass beads and a FastPrep homogenizer (Savant, USA), Glass beads and cell debris were sedimented by centrifugation at 16,000 × g for 5 min. The protein supernatant was removed and subsequently dialyzed overnight in 10 mM potassium phosphate buffer, pH 7.2, and then snap frozen with liquid nitrogen. Protein concentrations were determined following the manufacturer's protocol using the Bio-Rad Protein Dye micro-assay (Bio-Rad).

Enzyme activity assays for LGL were performed similar to the protocol of Frickel et. al. (7). Briefly, the reaction substrate, hemi-thioacetal, was prepared by incubating 4 mM each of reduced glutathione and methylglyoxal in 50 mM sodium phosphate buffer (pH 6.6) for 10 min at 37°C and its concentration determined spectrophotometrically (Ext_{coeff} 0.44 mM^{-1} cm^{-1} at 240 nm). The activity of Lgl was analyzed by measuring the initial rate of formation of S-D-lactoylglutathione from hemi-thioacetal in the presence of soluble cellular protein, followed spectrophotometrically by the increase in absorbance at 240 nm (S-D-lactoylglutathione Ext_{coeff} 2.86 mM^{-1} cm^{-1} at 240 nm). Rates for each reaction are expressed in μmol/min/μg of protein. Assays for glyoxalase II activity were performed as described by

Allen et. al. with some modifications (2). The initial rates of hydrolysis of 400 μM exogenously added D-lactoylglutathione by soluble cellular protein were followed spectrophotometrically by determining the decrease in absorbance at 240 nm.

Results

Lgl catalyzed the metabolism of methylglyoxal.

In order to determine if LGL detoxifies methyglyoxal, enzyme activity assays were carried out using soluble protein extracts isolated from both *S. mutans* UA159 and LGLKO strains. The UA159 protein extracts were able to efficiently convert the hemithioacetal (formed non-enzymatically between methylglyoxal and glutathione) to *S*-D glutathione; in contrast, the LGLKO protein extracts had no detectable enzyme activity (Fig. 3.1). These data, confirmed that Lgl was responsible for the detoxification of methyglyoxal in *S. mutans*. Involvement of LGL in methylglyoxal detoxification was further confirmed by determination of bactericidal MICs for methylglyoxal. The LGLKO strain was unable to survive at 3.9 mM methylglyoxal, as opposed to 15.6 mM for UA159 wild-type strain.

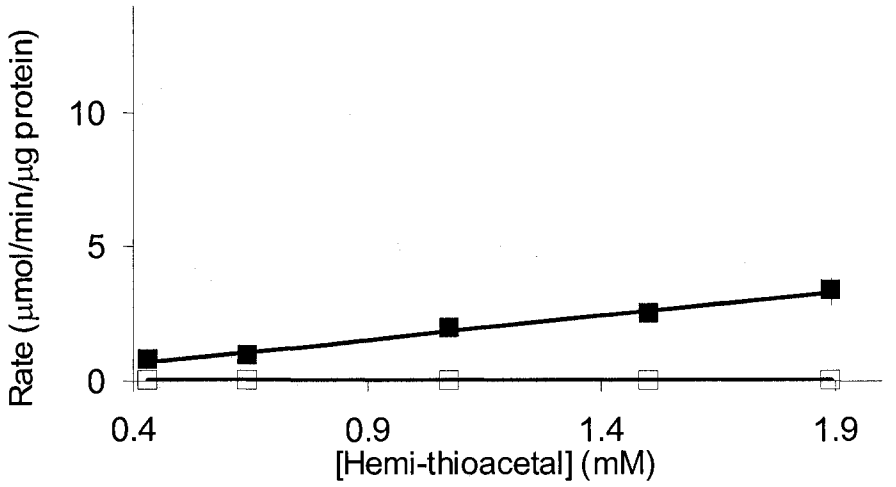


Figure 3.1. Lgl enzyme activity in *S. mutans*.

Soluble cell extracts from UA159 (■) and LGLKO (□). Initial rates were measured in triplicate and monitored by the increase in absorbance at 240 nm due to the conversion of hemi-thioacetal to S-D-lactoylglutathione.

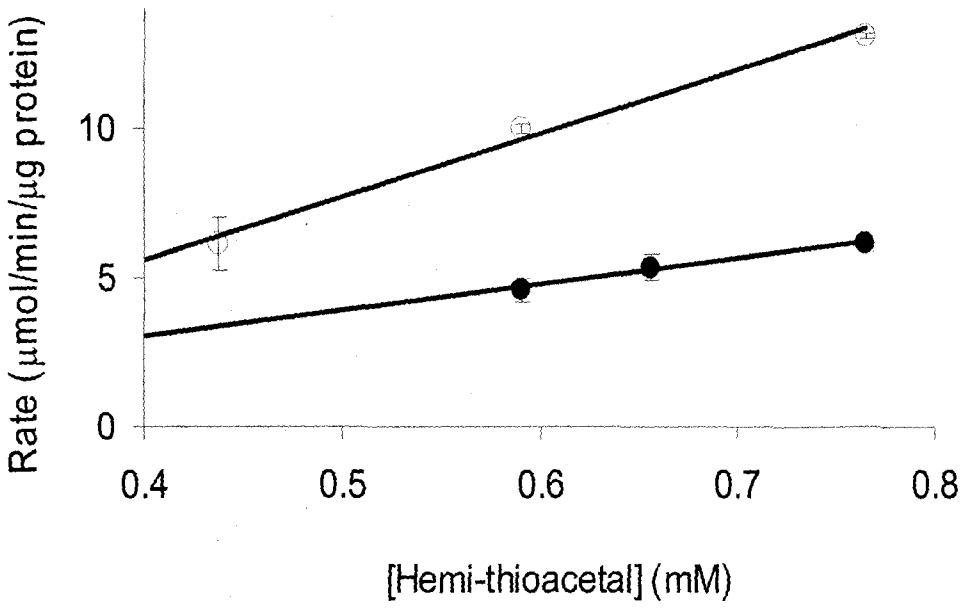
S. mutans displays glyoxalase II activity.

A search of the *S. mutans* genome reveals no obvious homologue to glyoxalase II. However, *S. mutans* does have glyoxalase II activity as displayed by the ability of cellular protein extracts to hydrolyze S-lactoylglutathione. In vitro analysis revealed that approximately 50 mg of *S. mutans* cellular protein extracts hydrolyzed S-lactoylglutathione at a rate of 4.50 ± 1.77 μmol/min.

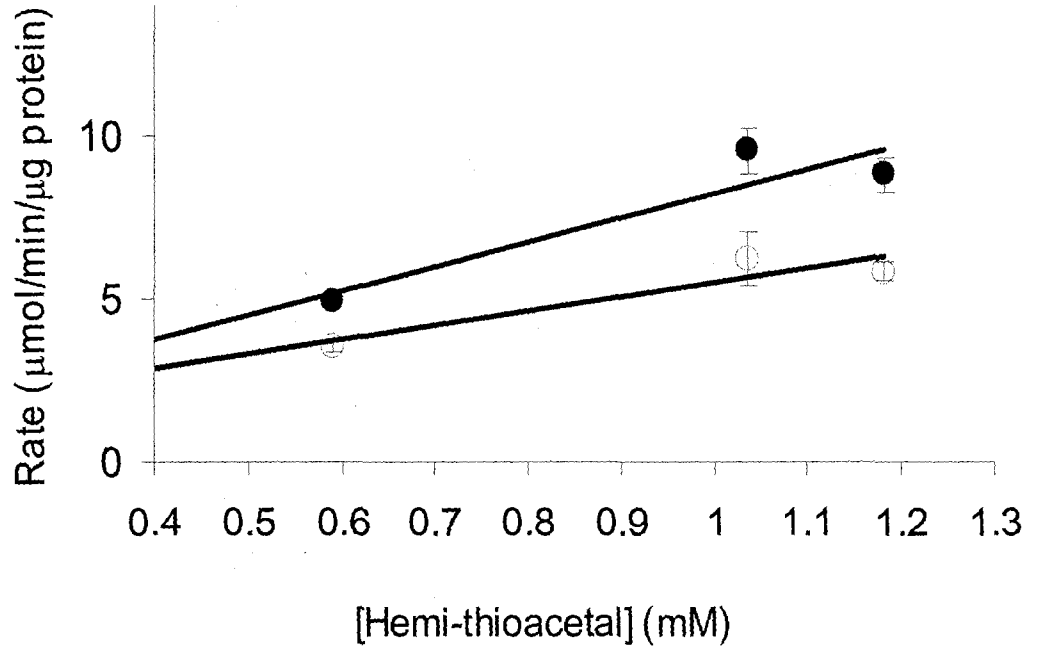
Lgl enzyme activity profile.

To corroborate qRT-PCR analysis, LGL enzyme activity was measured under the same environmental conditions used for gene expression studies. There was higher LGL specific activity in the *S. mutans* protein extracts when cells were exposed to an acidic environment; when grown at low pH compared with neutral pH (Fig. 3.2A) or adapted to pH 5.5 for 2 h (Fig. 3.2B). Growth phase also influenced LGL specific activity as seen by the increased activity in stationary growth phase cells compared with mid-logarithmic phase cells (Fig. 3.2C). The presence or absence of glucose had no effect on LGL activity in a dense *S. mutans* cell suspension at neither pH 7.0 nor pH 5.0 (Fig. 3.2D-E).

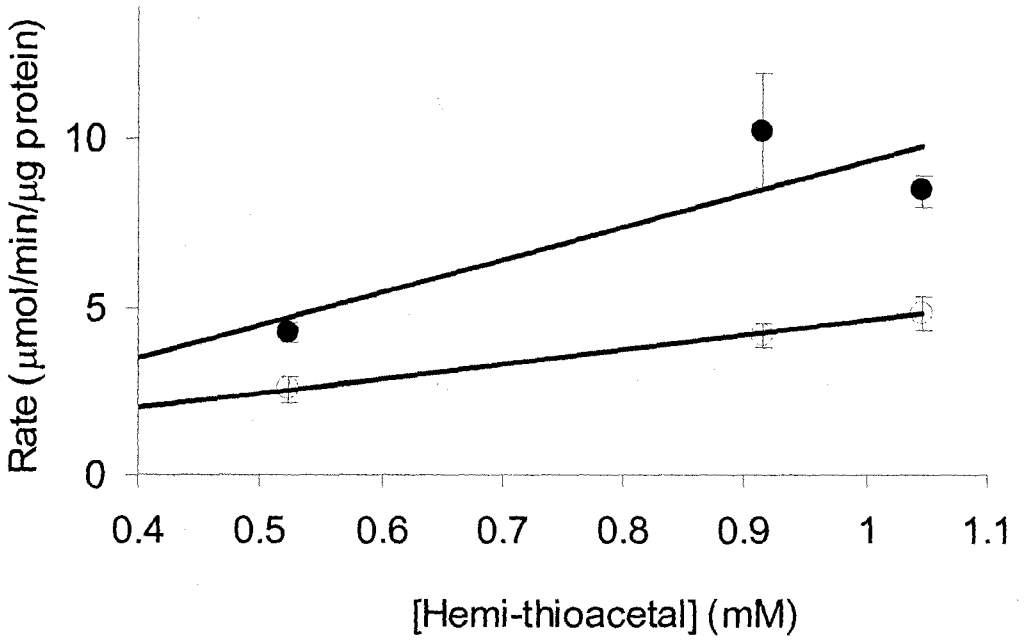
A.


Rate (µmol/min/µg protein)
10
5
0
0.4
0.5
0.6
0.7
0.8
[Hemi-thioacetal] (mM)

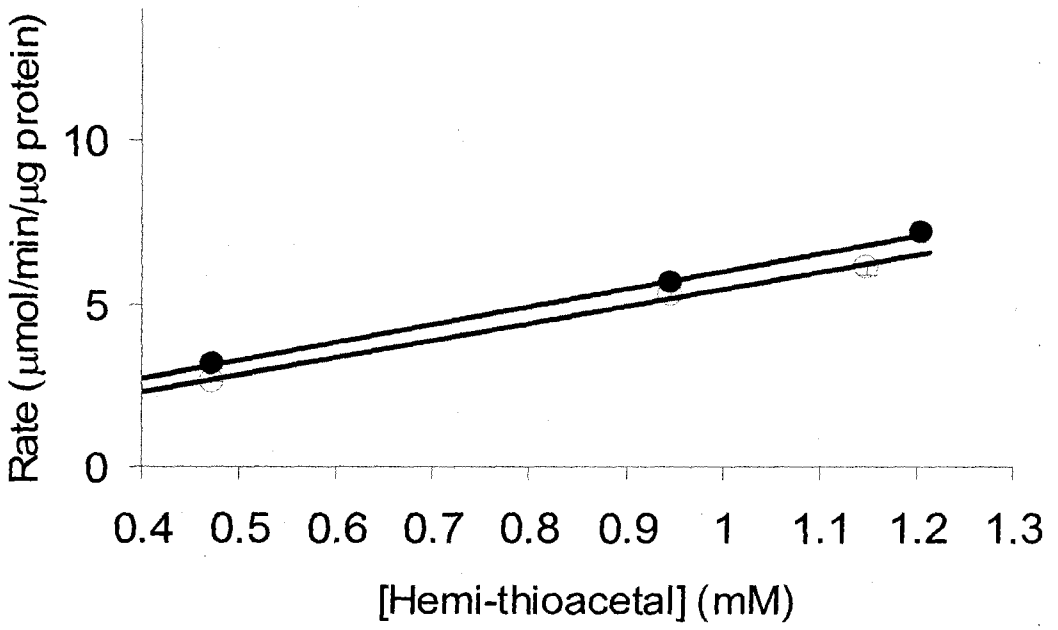
B.


Rate (µmol/min/µg protein)
10
5
0
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
1.3
[Hemi-thioacetal] (mM)

C.


Rate (µmol/min/µg protein)
10
5
0
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
[Hemi-thioacetal] (mM)

D.


Rate (µmol/min/µg protein)
10
5
0
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
1.3
[Hemi-thioacetal] (mM)

E.

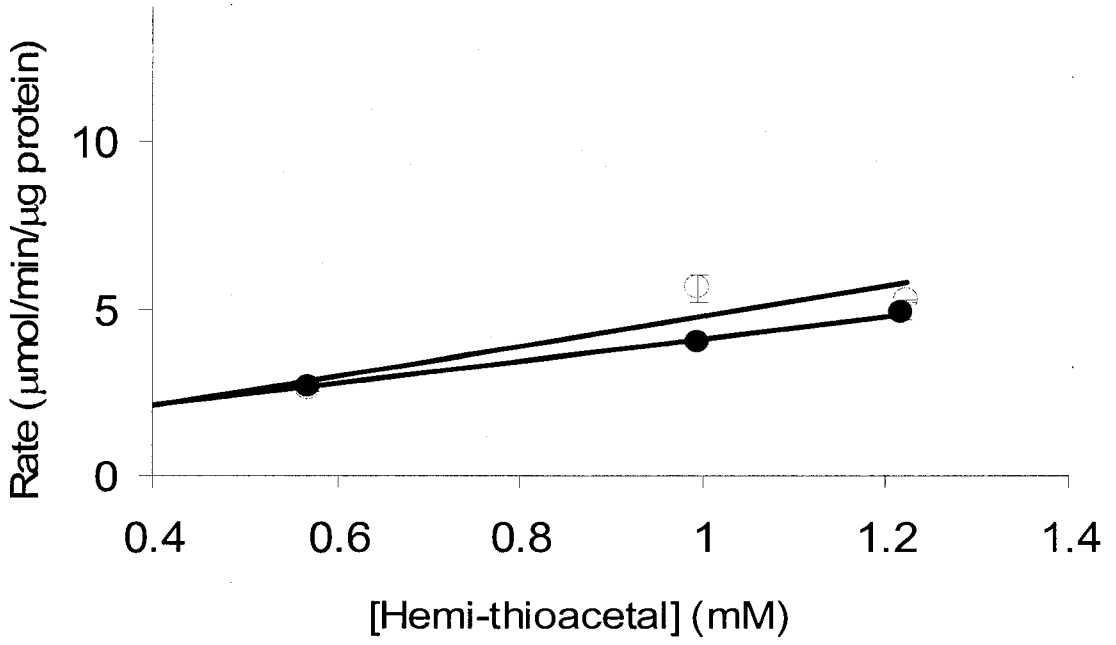


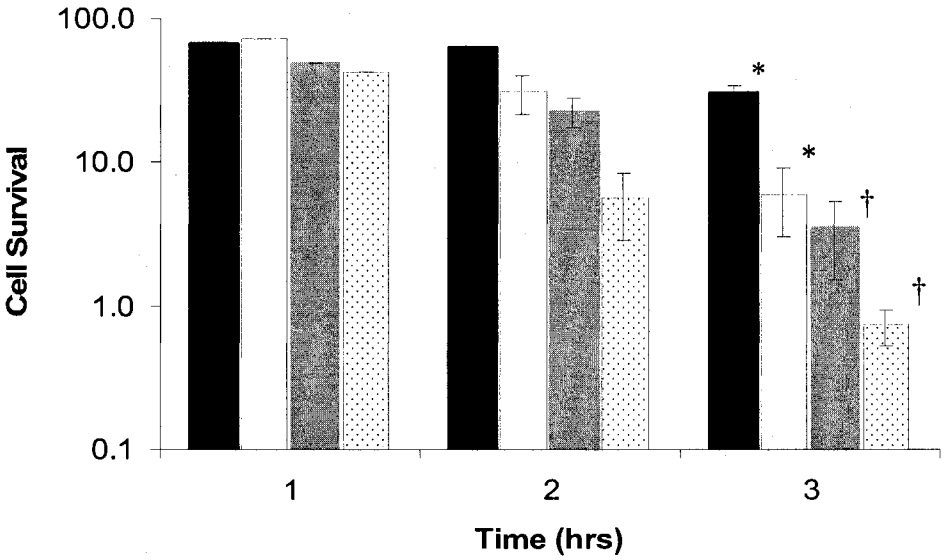
Figure 3.2. Lgl enzyme activity in *S. mutans* UA159 subjected to various environmental factors.

Initial rates were measured in triplicate and monitored by the increase in absorbance at 240 nm due to the conversion of hemi-thioacetal to S-D-lactoylglutathione. **A.** *S. mutans* UA159 cells grown to mid-log phase at pH 7.5 (○) and pH 5.0 (●), **B.** *S. mutans* UA159 acid adapted cells (●) and non-adapted cells (○), **C.** *S. mutans* UA159 cells grown to mid-logarithmic growth phase (○) and stationary growth phase cells (●), **D.** *S. mutans* UA159 cells at pH 7.0 subjected to glucose starvation (○) and 200 mM glucose (●), **E.** *S. mutans* UA159 cells at pH 5.0 subjected to glucose starvation (○) and 200 mM glucose (●).

The involvement of Lgl in aciduricity.

The phenotypic effect of the *lgl* mutation on *S. mutans* acid tolerance was first quantified by measurements of growth rates at acidic pH. Growth kinetics showed that the LGLKO mutant and wild-type UA159 grew similarly in THYE pH 7.5 (mean doubling times of 70.2 ± 1.1 min and 70.4 ± 0.2 min respectively). However, during growth in THYE at pH 5.0, LGLKO displayed a significantly slower doubling time compared to UA159 (mean doubling times of 179.2 ± 5.4 min and 164.3 ± 5.2 min respectively, p value $\leq$0.5). The final growth yield of LGLKO after 18 h of growth at either pH 7.5 or pH 5.0 growth conditions were the same as the UA159 wild-type strain.

To further investigate the involvement of *lgl* in acid tolerance, an ATR assay was employed again comparing *S. mutans* UA159 wild-type and LGLKO mutant strains (Fig. 3.3). The non-adapted LGLKO cells were more sensitive to acid compared with non-adapted UA159 wild-type cells. LGLKO cells that were adapted to acid at pH 5.5 media prior to exposure to pH 3.2 medium, also had diminished survival ability compared with UA159. Interestingly, the 3 h adaptation period caused similar increases in survival for both strains; an 8.2-fold increase in survival for LGLKO and a 9.0-fold increase in survival for UA159.



*Statistical significance comparing non-adapted cells (P<0.05)

†Statistical significance comparing adapted cells (P<0.05)

Figure 3.3. Acid tolerance response of *S. mutans* UA159 and LGLKO mutant strains. Cells were grown in TYE supplemented with glucose at pH 7.5 to mid-log phase and subjected to TYE pH 3.2 (unadapted UA159 (▪), LGLKO (▨) or incubated in TYE pH 5.5 for 2 h and then subjected to TYE pH 3.2 (adapted UA159 (■) and LGLKO (□)). Percentage of cell survival was calculated as the CFU/ml at a given time divided by the CFU/ml at time zero × 100. The results are expressed as the mean ± standard error of 3 independent experiments.

As another indication of acid tolerance, terminal culture pH was measured for UA159 and LGLKO for which a small, but statistically significant difference was observed. With the initial culture at pH 7.5, the LGLKO strain was unable to acidify cultures to the same extent as UA159. In addition, this trend became more pronounced as the initial culture pH was reduced to pH 5.0 (mean terminal pH's of 4.22 ± 0.0017 and 4.26 ± 0.0042 respectively; Statistical significance determined with single factor ANOVA, $P = 7.35 \times 10^{-7}$).

Acidic continuous culture competition.

To further strengthen the argument for LGL being an important component in the aciduricity of *S. mutans*, the parent and mutant strain were grown simultaneously in competition with each other in an acidic environment in continuous culture Fig. 3.4). The LGLKO cells were less fit in an acidic environment compared with the UA159 wild-type cells as demonstrated by the significant decrease in their proportional contribution to the total culture for both planktonic and biofilm fractions. This decrease, for both fractions, increased from 48 h to 96 h. Interestingly, LGLKO cells, when grown in a biofilm, were better able to maintain themselves within the competitive culture, compared to planktonic cells.

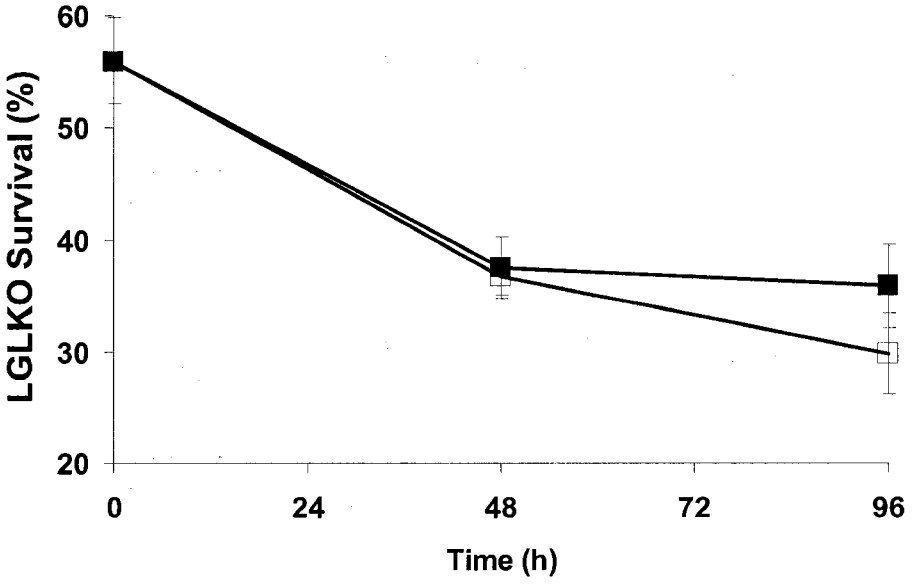
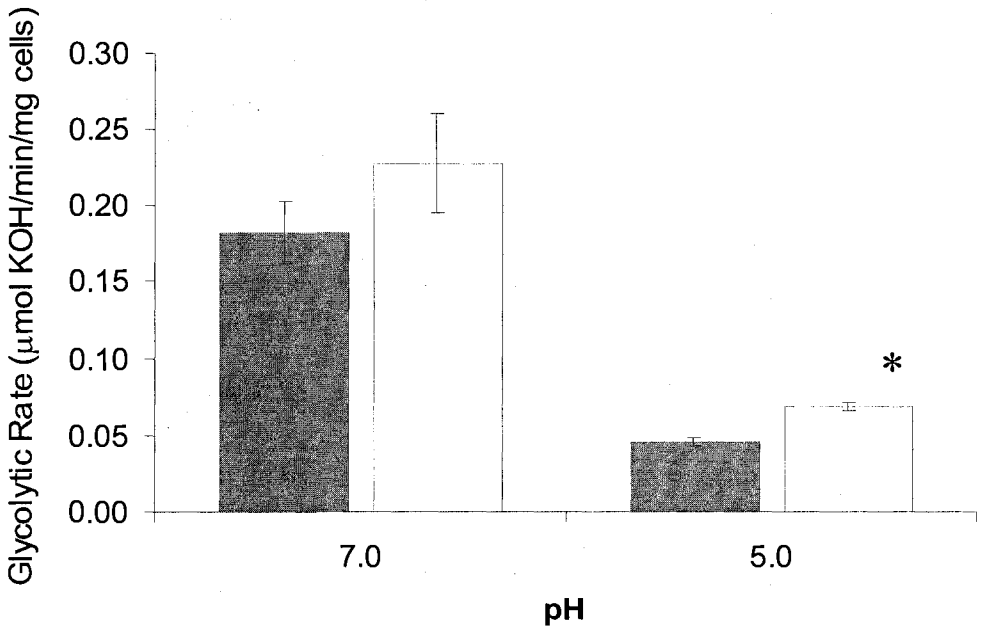


Figure 3.4: Biofermentor growth competition between UA159 and LGLKO strains. Cultures were initiated at pH 6.0 prior to exposure to pH 5.0. Percentage of LGLKO survival in both biofilm (■) and planktonic (□) culture fractions was calculated as the CFU/ml present on THYE + 10 μg/ml erythromycin agar plates divided by the CFU/ml present on THYE agar. The results are expressed as the mean ± standard error of 3 independent experiments.

The effect of exogenous methylglyoxal and LGL deletion on glycolytic rates.

The effect of *lgl* deletion on the glycolytic rates of *S. mutans* was investigated. The absence of *lgl* caused no statistically significant change in glycolytic rates, at pH 7.0. Interestingly, when the pH of the reaction was decreased to pH 5.0 LGLKO had increased glycolytic rates, with LGLKO able to produce acid at a rate of 68.7 ± 0.2 μmole·min^{-1}·mg^{-1} compared to UA159 of 46.2 ± 0.2 μmole·min^{-1}·mg^{-1} (data shown in Fig. 3.5). To determine whether these results were caused by increased methylglyoxal concentrations, exogenous methylglyoxal was added and glycolytic rates measured. Interestingly, the addition of methylglyoxal resulted in a modest 28% decrease in glycolytic rate at pH 7.0 and conversely a significant 98% increase in rates at pH 5.0.



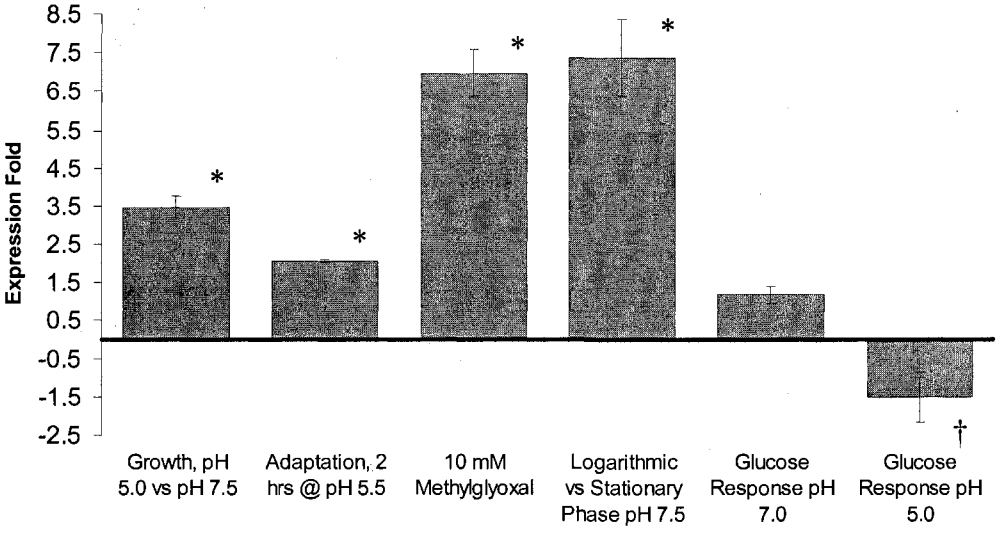
*Statistical significance comparing with UA159 pH 5.0 (P<0.05)

Figure 3.5. Glycolytic rates of *S. mutans* UA159 and LGLKO.

The strains are labeled as UA159 (■) and LGLKO (□). Glycolytic rates were monitored by measuring the addition rate of 10 mM KOH to cell suspension following the addition of 200 mM glucose at pH 7.0 and pH 5.0. Results are expressed as the mean ± standard error of 3 independent experiments.

The expression profile of *lgl*.

In order to compare differential expression of *lgl*, a series of experiments with *S. mutans* UA159 cells grown under specific environmental conditions related to acidogenicity and aciduricity was performed. In batch cultures, the mRNA levels of *lgl* were markedly higher, 3.5-fold, in cells grown at pH 5.0 compared with cells grown at pH 7.5 (Fig. 3.6). Levels of *lgl* transcript were also increased 2.1-fold in cells exposed to pH 5.5 for 2 hr. The exogenous addition of 10 mM methylglyoxal resulted in a 7.0 fold increase in *lgl* mRNA. At pH 7.0, glucose addition induced no significant change in *lgl* expression, whereas at pH 5.0 *lgl* levels were modestly reduced 1.5-fold upon addition of 200 mM glucose. Levels of *lgl* mRNA were markedly increased, 7.4-fold, during logarithmic phase of growth as compared with stationary phase growth (Fig. 6).



*Statistical significance, expression fold > 1 ($P<0.01$)

†Statistical significance, expression fold < -1 ($P<0.01$)

Figure 3.6. Differential gene expression of *lgl* during various environmental conditions. Expression fold was standardized to 16S rRNA expression. Results are expressed as the mean ± standard error of at least 4 independent experiments.

Discussion

When living within the complex, multi-species environment of dental plaque, *S. mutans* rapidly metabolizes carbohydrates into organic acids. This can result in a dramatic pH drop leading to the initiation and progression of carious lesions via dissolution of tooth enamel. *S. mutans* has evolved elaborate regulatory systems affording it the abilities of rapid carbohydrate metabolism and acid survival. This investigation focused on one enzyme, LGL, it's function, and it's involvement in the aciduricity and acidogenicity of *S. mutans*.

Methylglyoxal is a strong electrophile that occurs as a natural by-product of glycolysis, therefore cells, both eurkaryotic and prokaryotic, have developed methods to neutralize its toxic effects (4, 24). One system known to detoxify methylglyoxal is the glyoxalase system in which toxic methylglyoxal is converted to D-lactate in a process involving two enzymes; the first of these enzymes being LGL. Utilizing total soluble protein obtained from *S. mutans* UA159 and its *lgl* defective mutant, we confirmed that LGL was responsible for the conversion of methylgloxal to *S*-D-lactoylglutathione in *S. mutans*. This conclusion was further supported by the increased sensitivity of the LGLKO strain to methylglyoxal. Isogenic inactivation of *lgl* proved not to be lethal, indicating *S. mutans* may have at least one other system capable of detoxifying methylglyoxal or the intracellular concentrations of methylglyoxal under the conditions tested were sub-lethal. A possible alternative detoxification system may be comprised of aldose and aldehyde reductases as reported in *E. coli* and *Saccharomyces cerevisiae* (13, 19, 21).

Previous investigations into the proteome-wide response of *S. mutans* to acid challenge have revealed a vast and diverse list of proteins with altered expression profiles (15, 16, 25, 26). Of these proteins LGL has been shown to be up-regulated 2.6- and 51-fold

under acidic conditions (15, 26). As opposed to measuring protein abundance, we investigated the 'real-time' regulation of *lgl* mRNA expression in *S. mutans*, under various environmental queues relevant to acidogenicity and aciduricity. Our data clearly showed pronounced increases in *lgl* mRNA levels during both acidic growth and acid adaptation. The gene expression data were additionally validated by the increase in Lgl specific enzymatic activity in cell extracts prepared from acidic grown cells and acid adapted cells. This increased *lgl* expression in response to low pH at the mRNA level and increased enzymatic activity in response to an acidic environment, strongly implicate LGL as important in acid tolerance.

The involvement of LGL in *S. mutans* acid tolerance was further supported by our findings that the LGLKO strain had statistically significant longer generation times at pH 5.0 compared to wild-type UA159. Furthermore, LGLKO showed even greater acid sensitivity than UA159 under the conditions of the ATR assay. Although adapted LGLKO cells did not survive to the same extent as UA159 wild-type cells, interestingly, this adaptation caused a nearly identical fold increase in survival for both strains, indicating LGL is likely involved in intrinsic acid survival. The mutant, LGLKO, was also unable to acidify liquid culture to the same extent as UA159. These data were further supported by the inability of LGLKO to compete with UA159 when grown competitively in the bio-fermentor. These results definitively show the compromised competitiveness of the acid sensitive phenotype resulting from *lgl* inactivation in *S. mutans*. We can, therefore, conclude that LGL is involved in acid tolerance in *S. mutans*.

Generally the concentration of methylglyoxal increases during unregulated growth and in highly glycolytically active prokaryotic and eukaryotic cells (1, 6, 12, 20, 23). Also,

the formation of methylglyoxal was seen to increase following the addition of metabolites that stimulate the flux of triosephosphates, such as glucose (12). We therefore set out to determine the relationship between glucose metabolism and LGL activity in *S. mutans*. Remarkably, and what we believe to be a novel observation, the rate of acid production at pH 5.0 increased in response to the addition of exogenous methylglyoxal and in the absence of LGL. This suggests that excess intracellular methyglyoxal acts as a glycolytic regulator in an acidic environment. One possible mechanism by which glycolysis could be regulated is through the stimulation of pyruvate kinase. This would lead to a coordinate drop in the concentration of intracellular phosphenolpyruvate, which is known to activate LDH activity (22). This increase in homofermentation would rob *S. mutans* of the additional ATP generation by heterofermentation required for robust acid tolerance, especially under conditions of limiting glucose

Further investigation into the connection between *lgl* expression, glycolysis and growth phase were undertaken by measuring *lgl* expression in response to glucose under neutral and acidic conditions and at different growth phases. The expression of *lgl* was maximal during the mid-log phase of cellular growth, whereas enzymatic activity of LGL was greater in stationary phase cells. These somewhat contradictory results likely allude to LGL being highly stable and persisting in an active form long after being synthesized. At pH 5.0, the modestly decreased *lgl* expression in the presence of excess glucose was inversely proportional to the increase in glycolytic rates observed under the same conditions. Also at pH 7.0, in the presence of excess glucose, *lgl* expression remained stable. Enzymatic analysis under these same conditions yielded similar results. These data suggest that *lgl*

expression is tightly regulated, and this regulation is dependent upon more factors than the acidity or exogenous glucose concentration.

This report examined the role of the detoxifying enzyme, LGL, in the acidogenicity and aciduricity of *S. mutans*. The data clearly demonstrated LGL is an enzyme responsible for the detoxification of methylglyoxal and suggests a link between LGL and the acid tolerance afforded to *S. mutans*. The ability to regulate LGL may prove to be a valuable strategy to modulate *S. mutans* acid tolerance and hence virulence.

Supplemental Data

As mentioned above the *S. mutans* genome contains no obvious homologue of the glyoxalase II gene. Glyoxalase II is the second and final enzyme of the glyoxalase pathway required to convert *S*-D-glutathione into D-lactate. Investigations to determine whether *S. mutans* possesses glyoxalase II activity were undertaken as described above (Chapter 3: Materials and Methods), in which initial rates were measured in triplicate and monitored by the decrease in absorbance at 240 nm due to the conversion of D-lactoylglutathione to D-lactate. In presence of D-lactoylglutathione, total cell extracts from *S. mutans* were able to convert the substrate to lactate. The enzymatic activity was found to be $86 \pm 33 \times 10^{-3}$ μmol/min/mg protein. These data clearly indicate that *S. mutans* does have glyoxalase II enzyme.

To further investigate the acid sensitive phenotype of LGLKO terminal culture pH values were measured as described above (Chapter 3: Materials and Methods). Results from these experiments are shown Table 3.2 and show a small but statistically significant elevated pH values for the LGLKO cultures compared with UA159.

TABLE 3.2. Terminal pH of *S. mutans* cultures

Initial pH	Strain	
	UA159	LGLKO
7.5	5.39 ± 0.0008	5.40 ± 0.0017
6.0	4.33 ± 0.0006	4.35 ± 0.0016
5.0	4.22 ± 0.0017	4.26 ± 0.0042

Results displayed are the means ± standard error from 8 independent experiments
Statistical significance comparing UA159 with LGLKO at pH 7.5, 6.0, & 5.0 using single factor ANOVA: 3.3×10^{-4}, 1.0×10^{-7}, and 7.4×10^{-7} respectively.

The *lgl* gene was genetically complemented in trans to further validate the link between LGL and the observed acid sensitive phenotype of LGLKO. Primers were designed to amplify the full-length coding region of *lgl* with its putative promoter. These primers, designated LGLCOMPF and LGLCOMPR contained *Sac*I and *BamH*I restrictions sites added at the 5' end of the primers respectively (underlined) for cloning into the *E. coli/Streptococci* shuttle vector pDL277. Primer sequences are as follows:

LGLCOMPF: 5'-GAGCTCGAGCTCCATGGGCAATATGATGACTGC-3'

LGLCOMR: 5'-GGATCCGGATCCAAAGGTCGGAACGCTTTCG-3'

The resulting plasmid, pDLGLCOM, was isolated from *E. coli* DH5α chemically competent cells, and cloned into LGLKO, yielding the LGLCOMP strain. LGLCOMP cells were subjected to growth in acidic media and compared with UA159. The LGLCOMP strain performed equal to the wild-type UA159 under these conditions. Double times in THYE pH 5.0 of 154.7 ± 1.4 minutes and 154.4 ± 3.7 minutes for UA159 and LGLCOMP respectively were observed (Using single factor ANOVA analyzing 6 independent cultures, P value = 0.94). These data indicate that *lgl* was successfully complimented in trans and the phenotypes observed for LGLKO are the result of the absence of *lgl*.

Acknowledgements

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CHAPTER IV: THE INVOLVEMENT OF THE PYRUVATE DEHYDROGENASE E1α SUBUNIT, IN *STREPTOCOCCUS MUTANS* ACID TOLERANCE.

Korithoski, B., Lévesque, C.M., Cvitkovitch, D.G. The Involvement of the Pyruvate Dehydrogenase E1α subunit in *Streptococcus mutans* Acid Tolerance. Submitted to FEMS Microbiol. Lett.

Abstract

Streptococcus mutans, considered the primary etiological agent of dental caries, is a normal inhabitant of dental plaque. Two main virulence factors of *S. mutans* are acidogenicity and aciduricity, the ability to produce acid and survive and grow at low pH, respectively. Metabolic processes, including the catabolism of pyruvate, are finely regulated following acid exposure in *S. mutans*. Proteome analysis of the *S. mutans* response to acid has shown a subunit of pyruvate dehydrogenase, PdhA, to be up-regulated. PdhA is the E1α subunit of the four-enzyme pyruvate dehydrogenase complex (PDH) responsible for the heterofermentative catalysis of pyruvate into Acetyl-CoA. Acetyl Co-A is subsequently broken down into ethanol and acetate yielding additional ATP. This investigation examined the relationship between PdhA and aciduricity and metabolism in *S. mutans*. An *S. mutans pdhA* knockout (PDHAKO) was created revealing an acid sensitive phenotype. Quantitative Real-Time PCR studies revealed *pdhA* increased dramatically in expression during growth in acid and acid adaptation (increases of 15-fold for both). Additionally *pdhA* seems to respond to heterofermentative conditions; decreased expression in the presence of excess glucose, and more abundant during stationary phase compared with mid-log phase growth. This study demonstrates that, in *S. mutans, pdhA* expression responds to conditions conducive to heterofermentation and deletion of *pdhA* results in decreased aciduricity.

Introduction

According to the World Health Organization, dental caries is still a major oral health problem in industrialized countries affecting up to 90% of children (14). Dental caries is the dissolution of tooth enamel caused by the acidic end-products resulting from bacterial metabolism of dietary carbohydrates. *Streptococcus mutans*, a ubiquitous constituent of human dental plaque, is the main bacterial species associated with the initiation and progression of dental caries. *S. mutans* has the ability to rapidly metabolism a variety of carbohydrates into lactate; a highly acidic organic acid end product of glycolysis. Furthermore, *S. mutans* has developed a complex response network allowing it the ability to survive in the acidic environment created by its own acid production. These two previously mentioned characteristics, termed acidogenicity and aciduricity respectively, are vital cariogenic determinants of this bacterial species (reviewed by Banas (1)). During periods of high carbohydrate concentration and low pH, acidogenicity and aciduricity allow *S. mutans* to dominate over other organisms within the dental plaque biofilm, leading to the development and progression of carious lesions.

When *S. mutans* is grown in excess glucose, lactate is the major fermentation product. However, under conditions of glucose limitation, ethanol, acetate, and formate, in conjunction with lactate are the resulting end-products of metabolism (2). This divergence in metabolism occurs at pyruvate, which has been shown to be a key metabolic control node in many bacterial species (4, 5, 13). Pyruvate can be converted to lactate via lactate dehydrogenase (homofermentation), or to formate, ethanol, and acetate via pyruvate-formate lyase (heterofermentation) (2). A third pathway of pyruvate catabolism is via the pyruvate dehydrogenase complex in which pyruvate is oxidized into acetyl-CoA and CO_2 (3). Acetyl-

CoA is further catabolized into ethanol, via alcohol dehydrogenase, or acetate, via acetate kinase.

In gram positive bacteria the pyruvate dehydrogenase complex (PDH) is composed of four distinct polypeptides E1α, E1β, E2 and E3 (15). These proteins combine to from an icosahedral complex consisting of pyruvate dehydrogenase (E1 subunits), dihydrolipoyl transacetylase (E2) and dihydroipoamide dehydrogenase (E3) (13).

The activity of PDH in gram negative bacteria depends largely on allosteric regulations, however in Gram positive bacteria PDH seems to be regulated by redox potential (15). Such is the case in *Lactococcus lactis,* due to pyruvate formate lyase (PFL) being extremely oxygen sensitive, PDH is responsible for pyruvate metabolism an aerobic environment (15). In *Enterococcus faecalis*, however, both PFL and PDH are active under anaerobic conditions, analogous to pyruvate oxidation in the gram-negative bacterium *Klebsiella pneumoniae* (12, 16). A previous report in *S. mutans* described increased PDH activity in response to an aerobic environment (3).

Human dental plaque routinely goes through cycles of carbohydrate excess and limitation, and therefore metabolic regulation between homofermentation and heterofermentation is paramount to the survival of *S. mutans.* Heterofermentation is thought to play an important role in the acid tolerance of *S. mutans* as the metabolic end-products are weaker organic acids and heterofermentation can yield an additional ATP molecule per pyruvate molecule (2). During acid challenge, in *S. mutans*, the protein expression levels of the E1α subunit (termed PdhA) were shown to increase (19). These data suggest that PDH may play an important role in the acid tolerance of *S. mutans.* The present study looks to

address the involvement of PDH, specifically *pdhA*, in the metabolism and acid tolerance of *S. mutans*.

Materials and Methods

Bacterial strains and growth conditions.

S. mutans wild-type UA159 strain was used in this study. The *pdhA* isogenic insertional inactivation mutant (PDHAKO) was generated using a PCR ligation mutagenesis methodology (7). Primers used for mutagenesis are shown in Table 4.1. *S. mutans* UA159 cells were grown in Todd Hewitt broth supplemented with 0.3% yeast extract (THYE). Erythromycin was added to a final concentration of 10 μg/ml as needed.

TABLE 4.1. Primers Used in Chapter IV

Primer	Primer Sequence (5' to 3')[a]	Description
Erm 1	GGCGCGCCCGGGCCCAAAATTTGTTTGAT	Erythromycine cassette (6)
Erm 2	GGCCGGCCAGTCGGCAGCGACTCATAGAAT	"
PDHA 1	CGACTGGTAGCCATCCTTTTATTC	*pdhA* mutagenesis
PDHA 2	GGCGCGCCGCTTGCTCTTTGGAAACTTC	"
PDHA 3	GGCCGGCCGATGTATTTGCTGAGTAAAG	"
PDHA 4	CTGTCGCTGTATTATTATGTGGG	"
PDHA RT 1	TGGAGATGGTGCCGCTAATG	*pdhA* qRT-PCR
PDHA RT 2	TGTGGTGTTGATTCAGCAAC	"
16S RT 1	CTTACCAGGTCTTGACATCCCG	16S rRNA qRT-PCR
16S RT 2	ACCCAACATCTCACGACACGAG	"
GYRA RT 1	ATTGTTGCTCGGGCTCTTCCAG	*gyrA* qRT-PCR
GYRA RT 2	ATGCGGCTTGTCAGGAGTAACC	"

[a]Restriction sites are underlined: *AscI* GGCGCGCC; *FseI* GGCCGGCC.

Growth kinetics.

Overnight cultures of UA159 and PDHAKO were diluted (1:20) into fresh THYE and incubated at 37°C under an atmosphere of 5% CO_2 until cultures reached mid-logarithmic

growth phase (Optical Density at 600 nm (OD_{600nm}) ~ 0.4). These subcultures were subsequently inoculated 1:20 into microtitre plates in quadruplicate containing THYE broth at pH 7.5 and pH 5.0. Cultures were incubated at 37°C for 24 h and growth followed using an automated growth reader (Bioscreen C, Labsystems, Finland). Growth curves were generated and doubling times (T_d) calculated from the data.

Acid tolerance response (ATR) Assay.

Overnight cultures of *S. mutans* UA159 and PDHAKO strains were diluted (1:20) into TYE (10% Tryptone, 5% Yeast Extract, 17.2 mM K_2HPO_4) with additional 5 mM glucose at pH 7.5 and incubated at 37°C, in a 5% CO_2 enhanced environment until mid-log growth phase was achieved (OD_{600nm} ~ 0.4). Cultures were divided into two aliquots, termed 'adapted' and 'nonadapted', and pelleted by centrifugation. Nonadapted cells were immediately resuspended in TYE at the killing pH of pH 3.2. An aliquot was immediately removed (t = 0) and serially diluted in 10 mM potassium phosphate buffer (pH 7.2) and incubation continued for 3 h at 37°C in an atmosphere of 5% CO_2. Each dilution was spotted in triplicate (20 μL per spot) onto THYE agar plates and incubated at 37°C, in a 5% CO_2 supplemented atmosphere for 2 days. Adapted cells were resuspended in TYE at pH 5.5 for 2 h prior to being subjected to the killing pH (pH 3.2). The ATR was expressed as the percentage of cells to survive the lethal pH at 1 h, 2 h, and 3 h compared with the number of cells present at time 0.

Terminal pH.

Overnight cultures of *S. mutans* UA159 and PDHKO strains (8 independent cultures of each strain) were diluted (1:40) in THYE at pH 7.5, pH 6.0 and pH 5.0 and incubated at 37°C in an atmosphere of 5% CO_2 for 20 h prior to the terminal pH being recorded.

Measurement of glycolytic rates.

Overnight cultures were diluted (1:10) into THYE at pH 7.5 and incubated at 37°C until mid-log growth phase was reached (OD_{600nm} ~ 0.4). The cells were subsequently pelleted via centrifugation and washed twice with ice-cold PK solution (1% Peptone, 1% KCL) at either pH 7.0 or pH 5.0. Cells were then resuspended in PK solution at the appropriate pH to yield an OD_{600nm} ~ 1.0. Aliquots (18 ml) of cell suspension were equilibrated in the reaction vessel at 37°C until residual glycolytic activity had diminished. After equilibration, the reaction was initiated with the addition of 200 mM glucose. Glycolytic rates were monitored by the rate of addition of potassium hydroxide (10 mM KOH at pH 7.0 and 2 mM KOH at pH 5.0) required to keep the pH constant utilizing a Radiometer ABU901 autoburrette in conjunction with a PHM290 pH controller (Radiometer, Denmark). The glycolytic rates are defined as μmoles of acid neutralized per milligram (dry wt.) of cells per minute.

Continuous culture acidic competition assay.

Cultures of UA159 and LGLKO were simultaneously inoculated (1:80) into a chemostat biofermentor previously charged with ¼ THYE + 5 mM glucose at pH 6.0 and containing glass rods for biofilm accumulation. Cells were continuously cultured as described previously with some modification (10). Bacterial cultures were initially established at pH

6.0 and a fresh media flow rate of 0.1 dilutions/h (D) for 18hrs. After culture establishment, culture pH was reduced to pH 5.0 and flow rate increased to D = 0.5. Aliquots of planktonic cells were removed, serially diluted and plated onto THYE agar and THYE + 10 μg/ml erythromycin agar. Biofilm cells were quantified by the removal of glass rods. Biofilm cells were released by vortex and cells serially diluted and plated.

Cell preparation for gene expression analysis.

S. mutans UA159 wild-type cells were grown under the following conditions prior to RNA isolation. **Acid Growth:** Overnight cultures of *S. mutans* cells were diluted (1:40) in THYE (pH 7.5 and pH 5.0), and incubated at 37°C in an atmosphere of 5% CO_2 until mid-log growth phase was reached (OD_{600nm} ~ 0.4). **Acid Adaptation:** Overnight cultures of *S. mutans* UA159 cells were diluted (1:20) in TYE pH 7.5 and incubated until mid-log growth phase in a 37°C, 5% CO_2 environment. Cells were divided into 2 aliquots, centrifuged and subsequently resuspended to their original concentration in TYE pH 5.0. RNA from one aliquot was processed immediately, and the second aliquot was incubated at 37°C supplemented with 5% CO_2 for 2 h prior to RNA extraction. **Oxygen Environment:** Overnight cultures were diluted (1:20) into TYE pH 7.5 supplemented with 5 mM glucose that had been pre-equilibrated in an anaerobic chamber (10% CO_2, 10% H_2, and balanced N_2) as well as into TYE pH 7.5 with supplemented 5 mM glucose pre-incubated at 37°C under standard atmospheric conditions. RNA was harvested from the cultures after cells reached mid-log phase (OD_{600nm} ~ 0.4). **Biofilm Growth:** Overnight cultures were inoculated 1:100 into 24-well polypropylene microtitre plates containing ½× concentrated THYE pH 5.0 supplemented with 5 mM glucose. Cells were allowed to grow at 37°C with 5% CO_2 for 18

h. After incubation planktonic cells were separated from biofilm cells and RNA extract from each. **Growth Phase:** Overnight cultures of *S. mutans* UA159 cells were diluted (1:20) into TYE at pH 7.5 and incubated at 37°C with 5% CO_2 supplementation. RNA was harvested from the cultures when the cells reached mid-log growth phase (OD_{600nm} ~ 0.4) as well as after reaching stationary phase (20 h). **Glucose Response:** Cell suspensions of UA159 were prepared at both pH 7.0 and pH 5.0 as described for measurement of glycolytic rates. Cells were equilibrated for 20 min at 37°C to eliminate residual glycolytic activity. Subsequent to equilibration 200 mM glucose was added to the suspensions for 15 min prior to RNA isolation.

Quantitative Real-Time PCR (qRT-PCR) analysis of *pdhA* RNA expression.

Total RNA was harvested and treated with RQ1 RNAse-free DNAse (Promega, USA) as described previously (6). From this RNA, cDNA was generated via reverse transcription using a First Strand cDNA synthesis kit (MBI Fermentas) according to the manufacturer's instructions. RNA samples lacking reverse transcriptase were incorporated as controls to assure results were not the product of residual DNA contamination. These single stranded cDNA templates were used for qRT-PCR reactions that were carried out using the QuantiTect SYBRGreen PCR kit (Qiagen, USA) in a Mx3005P QPCR system (Stratagene, USA). Specific primer sequences used (Table 2) for the reactions were designed to yield 100 – 150 bp products. For each reaction the Cycle Threshold (Ct) was measured; this value was inversely proportional to the starting amount of target DNA in each sample. All data was normalized against the expression of an internal standard. For all experiments 16S rRNA was used, except *gyrA* was the internal standard used for biofilm experiments, as the expression

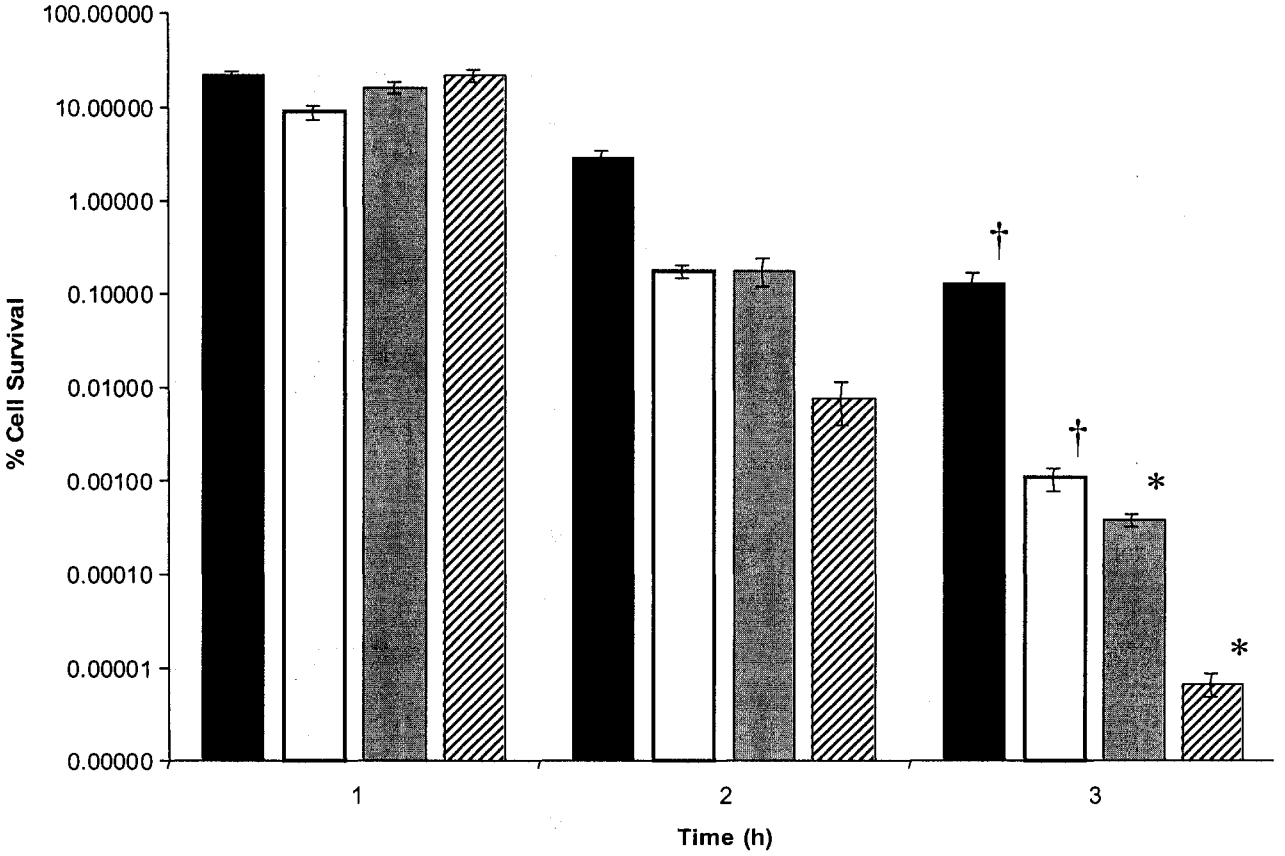
of these genes was shown to be stable under experimental conditions. The expression fold change was determined using the $2^{-\Delta\Delta}$ CT method (11).

Results

The involvement of PdhA in aciduricity:

The phenotypic effect of the *pdhA* knockout on *S. mutans* was first determined by measurements of growth rates at both initial pH 7.5 and pH 5.0. The PDHAKO mutant and wild-type UA159 displayed similar growth kinetic when grown at pH 7.5 (mean T_d's of 68.2 ± 1.1 min and 70.4 ± 0.2 min respectively). Under both pH growth conditions the final growth yield was the same for both UA159 and the mutant PDHAKO. However, the PDHA strain displayed significantly slower growth than wild-type UA159 when grown at pH 5.0 (mean T_d of 190.4 ± 6.3 min and 164.3 ± 5.2 min respectively).

The involvement of *pdhA* in the aciduricity of *S. mutans* was further investigated by employing ATR assays comparing the PDHAKO and UA159 strains (Figure 4.1). The PDHAKO strain displayed reduced innate survival capacity compared with UA159 under nonadapted conditions (0.00001% and 0.0015% survival for PDHAKO and UA159 respectively). PDHAKO cells that were acid adapted, by exposure to pH 5.5 media prior to killing pH 3.2 media, also had significantly reduced survival ability compared with UA159 (0.00038% and 0.131% survival for PDHAKO and UA159 respectively).



*Statistical significance comparing nonadapted cells (P<0.05)

†Statistical significance comparing adapted cells (P<0.05)

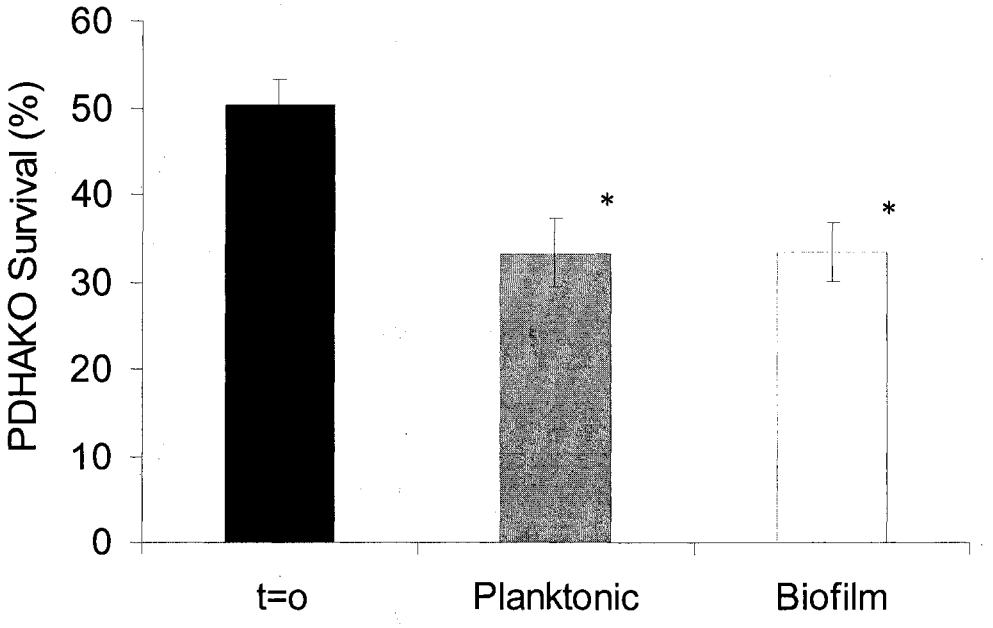
Figure 4.1: Acid tolerance response of *S. mutans* UA159 and PDHAKO strains. Cells were grown in TYE supplemented with glucose at pH 7.5 to mid-log phase and subjected to TYE pH 3.2 (Nonadapted UA159 (▩), PDHAKO (▨)) or incubated in TYE pH 5.5 for 2 h and then subjected to TYE pH 3.2 (adapted UA159 (■) and PDHAKO (□)). Percentage of cell survival was calculated as the CFU/ml at a given time divided by the CFU/ml at time zero.

Terminal culture pH was measured for UA159 and PDHAKO. PDHAKO was unable to acidify cultures to the same degree when compared with UA159. This phenomenon became more pronounced as the initial culture pH was reduced. With initial pH of the media reduced to pH 5.0, the mean terminal pH for UA159 and PDHAKO were 4.118 ± 0.0027 and

4.149 ± 0.0025 (Statistical significance determined using single factor ANOVA, P = 1.47 × 10^{-6}).

Simultaneous biofermentor competition.

S. mutans UA159 and PDHAKO were grown in simultaneous competition in an acidic environment in a continuous culture biofermentor (Fig. 4.2). In only 48 h of growth competition, a significant reduction in PDHAKO cells present in both planktonic and biofilm fractions was observed.



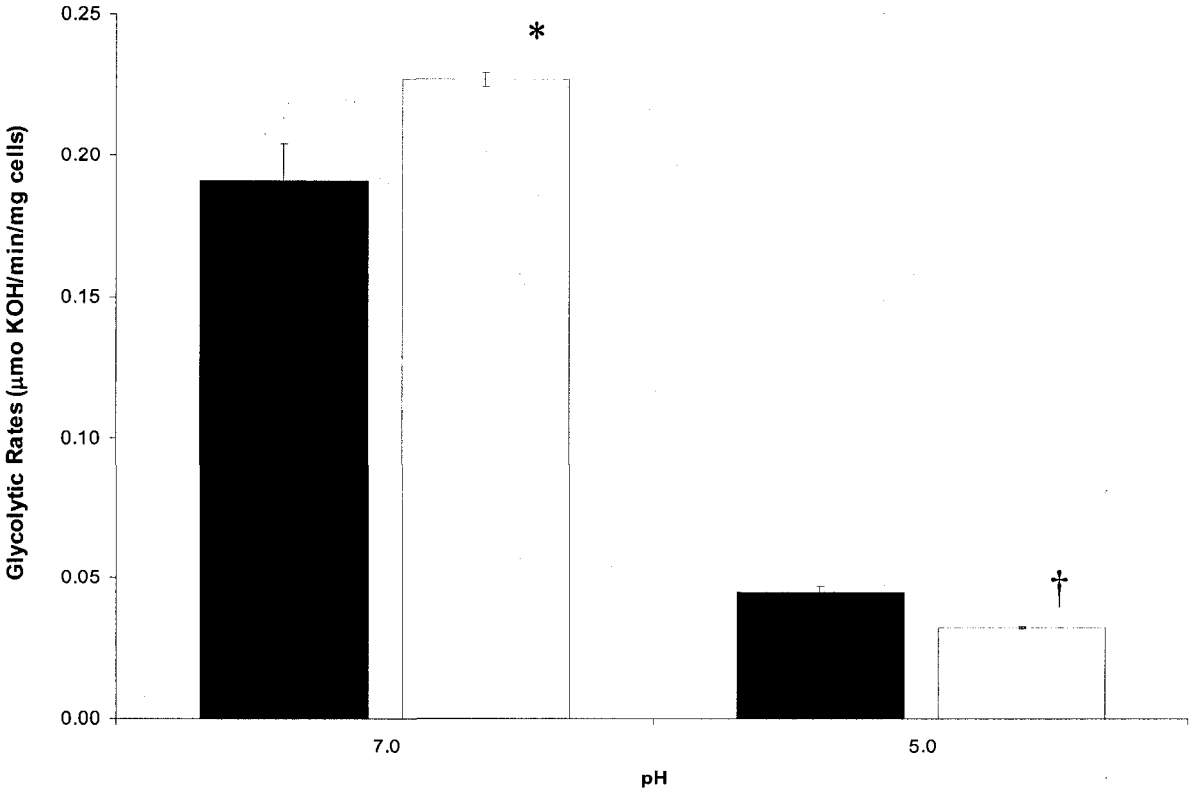
*Statistical significance compared with t=0 (P<0.05)

Figure 4.2. Bio-fermentor growth competition between UA159 and PDHAKO.

Cultures were initiated at pH 6.0 prior to exposure to pH 5.0. Percentage of PDHAO survival in both biofilm (□) and planktonic (■) culture fractions was calculated as the CFU/ml present on THYE + 10 μg/ml erythromycin agar plates divided by the CFU/ml present on THYE agar. The results are expressed as the mean ± standard error of 3 independent experiments.

The effect of PdhA on glycolytic rates.

The effect of knocking out *pdha*, thought to play a major role in pyruvate metabolism, on glycolytic rates of *S. mutans* was investigated at both pH 7.0 and pH 5.0, (Figure 4.3). At pH 7.0, the absence of *pdhA* resulted in an increased glycolytic rate for PDHAKO over that of UA159 (0.19 $\mu mole \cdot min^{-1} \cdot mg^{-1}$ & 0.23 $\mu mole \cdot min^{-1} \cdot mg^{-1}$ respectively). An inverse trend was observed when the reaction pH of was decreased to pH 5.0, when the deletion of *pdhA* resulted in a decreased glycolytic rate for PDHAKO (32.2 $nmole \cdot min^{-1} \cdot mg^{-1}$) compared with UA159 (44.9 $nmole \cdot min^{-1} \cdot mg^{-1}$).



*Statistical significance relative to UA159 pH 7.0 ($P<0.05$)

Figure 4.3. Glycolytic rates of *S. mutans* UA159 and PDHAKO.

Glycolytic rates for UA159 (■) and PDHAKO (□) were monitored by measuring the addition rate of 10 mM KOH to cell suspension following the addition of 200 mM glucose at pH 7.0 and pH 5.0. Results are expressed as the mean ± standard error of 3 independent experiments.

The expression profile of *pdhA*.

The differences in expression of *S. mutans pdhA*, at the mRNA level, were quantified using qRT-PCR under various environmental conditions relating to aciduricity, growth phase, and exogenous glucose. Results from these experiments are presented in Figure 4.4. In liquid culture *pdhA* levels escalated 14.7-fold in cells grown at pH 5.0 compared with those grown at pH 7.5. A similar trend was observed when cells were exposed to pH 5.5 for 2 h; *pdhA* RNA increased 14.6-fold during this period. Transcription of *pdhA* was dramatically higher at stationary grown phase compared with logarithmic growth phase increasing 10.4-fold. The transcriptional response of *S. mutans pdhA* levels in reaction to excess glucose was assessed via qRT-PCR. Transcription of *pdhA* mRNA decreased significantly in the presence of excess glucose both at pH 7.5 and pH 5.0, diminishing 18.4-fold and 4.3-fold respectively. Additionally, *pdhA* had a 2.0-fold increase in expression in acidic biofilm cells over planktonic cells.

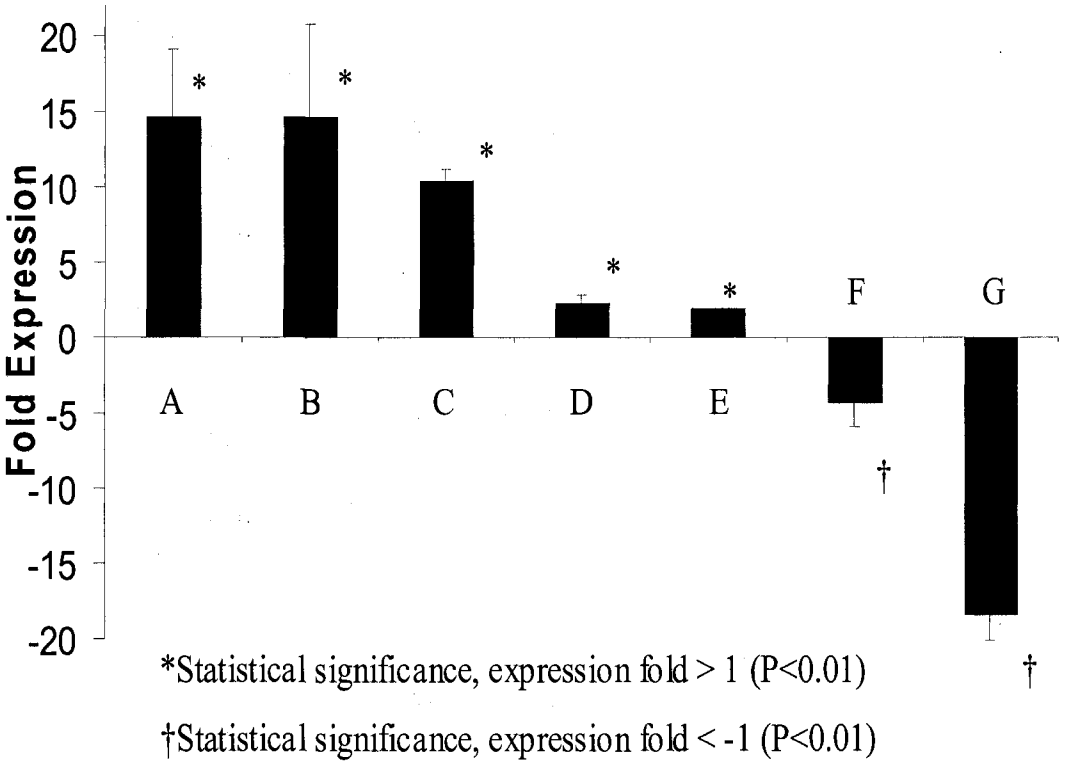


Figure 4.4. Differential gene expression of *pdhA* during various environmental stresses.

Expression fold was standardized to 16S rRNA expression. Conditions investigated: Column A, Growth at pH 5.0 vs pH 7.5; Column B, 2 h acid adaptation at pH 5.5; Column C, Stationary vs. mid-log growth phase; Column D, Anaerobic vs. Aerobic growth; Column E, Biofilm vs. planktonic growth at pH 5.0; Column F, Response to the presence of 200 mM glucose at pH 5.0; Column G, Response to the presence of 200 mM glucose at pH 7.0. Results are expressed as the mean ± standard error of at least 4 independent experiments.

Discussion

S. mutans is a frequent inhabitant in human dental plaque, and is the species mostly commonly associated with dental caries. Carious lesions are the end result of dissolution of tooth enamel by the carboxylic acid end products of dietary carbohydrate metabolism by oral bacteria, especially *S. mutans*. In order to survive this dramatic pH drop, *S. mutans* has developed a complex network of stringently regulated, coordinated responses affording it the abilities of varied carbohydrate metabolism and acid survival (1). The focus of this investigation was to discern the role of pyruvate dehydrogenase, specifically PdhA, in the acid tolerance of *S. mutans* and its expression relating to heterofermentation.

In *S. mutans*, the switch to mixed acid metabolism results in increased concentrations of ethanol and acetate as end products (2). We propose PDH to be pivotal to the regulation of acid production in *S. mutans*, therefore investigations into the effects of a *pdhA* knockout on glycolysis were undertaken by measuring glycolytic rates and utilizing qRT-PCR. Our results revealed drastic decreases in *pdhA* transcription in the presence of excess glucose at both pH 7.0 and pH 5.0 indicating *pdhA* is almost entirely repressed during conditions conducive to homofermentation. Additionally *pdhA* expression was monitored in response to growth phase, with results displaying dramatically increased expression at stationary growth phase over logarithmic phase, further supporting *pdhA* involvement in homofermentative pyruvate metabolism in *S. mutans*. In the absence of *pdhA*, the rates of glycolysis increased at pH 7.0 and decreased at pH 5.0 in PDHAKO relative to wild-type UA159. These results indicate PdhA aids in *S. mutans* metabolic regulation over a broad pH range.

The PDH complex is involved in the switch from homofermentation to heterofermentation in many bacterial species. Pyruvate formate lyase, in *S. mutans* has been

shown to be extremely sensitive to oxygen (17, 18). It has been shown in other lactic acid bacteria that the PDH complex is the mechanism by which heterofermentation is accomplished in an oxygen rich environment. As such, we hypothesized the PDH complex would be important for *S. mutans* heterofermentation, and this importance would be paramount in the presence of oxygen (15). This hypothesis was tested by monitoring the level of *pdhA* transcription under various environmental conditions via qRT-PCR. Interestingly, expression of *pdhA* was elevated in an anaerobic environment compared with an aerobic environment, contrary to results seen in *L. lactis*. These data suggest that PDH and pyruvate formate lyase are both responsible for pyruvate heterofermentation under anaerobic conditions, while PDH is solely responsible in an aerobic environment.

Previous investigations into the proteome-wide response of *S. mutans* to acid challenge have revealed a vast and diverse list of proteins with altered expression profiles (8, 9, 19, 20). Work conducted by Welin *et. al.* (19) investigating the translation response of *S. mutans* to acid challenge via 2D gel electrophoresis revealed a number of proteins with altered expression profiles. This work showed PdhA to be up-regulated by acid challenge 3.5- and 2.5-fold in planktonic and biofilm cells respectively. To investigate mRNA expression of *pdhA* under acidic conditions we implemented qRT-PCR for this study. Our data clearly showed dramatic increases in *pdhA* mRNA levels during both acidic growth and acid adaptation.

The involvement of PdhA in the acid tolerance of *S. mutans* is further supported by our growth kinetics findings that the PDHAKO mutant strain had statistically significant slower doubling times at pH 5.0 compared to UA159. The PDHAKO mutant was also unable to acidify culture media to same extent as UA159. Additionally, the PDHAKO strain

was far more acid sensitive compared with wild-type UA159 under the conditions of the ATR assay. These data illustrate the acid sensitive phenotype resulting from *pdhA* deletion in *S. mutans*. These findings, together with the qRT-PCR results under acid challenge suggest PdhA is an important component of aciduricity in *S. mutans*.

This article examined the involvement of the PdhA in heterofermentation, and the aciduricity of *S. mutans*. Our data clearly indicate *pdhA* expression dramatically increases under conditions conducive to mixed acid production and decreases during conditions favouring homofermentative lactic acid production Additionally the deletion of *pdhA* results in an acid sensitive phenotype suggesting a link between PdhA and the acid tolerance afforded to *S. mutans*. The ability to inhibited or down regulate *pdhA* may lead to novel strategies to reduce the acid tolerance of *S. mutans* and hence reduce its associated cariogenicity. This work adds to our further understanding of *S. mutans*' dynamic metabolic flexibility that has evolved to facilitate its domination in the low pH environment of carious dental plaque.

Suppplemental Data

As mentioned above terminal culture pH values were measured to add further weight to our claim for a role for PdhA in the acid-tolerance of *S. mutans*. Results from these experiments are shown in Table 4.2 and show small, but statistically significant elevated pH values for the PDHAKO cultures compared with UA159.

TABLE 4.2. Terminal pH of *S. mutans* cultures

Initial pH	Strain	
	UA159	LGLKO
7.5	5.24 ± 0.0024	5.26 ± 0.0026
6.0	4.31 ± 0.0019	4.33 ± 0.0016
5.0	4.12 ± 0027	4.15 ± 0.0025

Results displayed are the means ± standard error from 8 independent experiments
Statistical significance comparing UA159 with LGLKO at pH 7.5, 6.0, & 5.0 using single factor ANOVA: 2.7×10^{-5}, 6.8×10^{-6}, and 1.5×10^{-6} respectively.

Attempts were made to gain kinetics data for PdhA from total soluble cell protein extracts (isolation described in Chapter 3, Material and Methods), using the method set out by Menzel et. al. (11). Unfortunately, using these crude extracts, no discernable enzyme activity could be observed. This is probably the result of the relatively low abundance PdhA in the extracts. However, this does allow one to suggest that this enzyme even in low abundance, has a great effect on S. *mutans*.

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CHAPTER V: DISCUSSIONS AND FUTURE DIRECTIONS

I. Summary, Conclusions, and Discussions

The ability of *S. mutans* to sense and respond to its environment is paramount to its survival. Of critical importance is the ability to sense and become more resistant to acid. *S. mutans*, like other gram-positive bacteria, possesses a myriad of acid resistance systems to help it overcome the numerous challenges posed by the oral cavity and, in particular, human dental plaque. Previous investigations have examined the phenomenon of acid tolerance, in terms of RNA expression patterns, proteomic expression, and the production of acid fermentation by-products (2, 3, 5, 12, 13, 16, 20-22). These numerous reports solidify the belief that *S. mutans* is capable of a complex, multileveled response to environmental stimuli such as a reduction in extracellular pH. These reports have taken a holistic approach to unraveling the enigma of aciduricity of *S. mutans*. The goal of the research presented in this dissertation was to investigate three secondary metabolic pathways previously suggested to have importance to acid tolerance of *S. mutans*. Specifically, I investigated citrate transport and metabolism (Chapter 2), the glyoxalase pathway (Chapter 3), and pyruvate dehydrogenase A (Chapter 4), and their relationship to the acid tolerance of *S. mutans*.

Citrate is a relatively abundant organic acid that readily chelates a variety of metal cations. *S. mutans* cannot survive when citrate is the sole carbon, and as such, relatively little research has been directed toward citrate transport and metabolism in *S. mutans* prior to the investigation set forth in this work. Extensive research into citrate utilization by other lactic acid bacteria has shown a significant role for citrate in acid tolerance (6, 8-10, 14).

Citrate uptake assays, using radiolabelled citrate, conclusively identified CitM as the major citrate transporter of *S. mutans*. Additionally, in a novel finding, and contrary to its

closest homologues, CitM transported citrate most efficiently when the organic acid was coupled with Fe^{3+} ions.

Using radiolabeled citrate, we were able to show, via thin layer chromatography, that *S. mutans* is capable of citrate catabolism, with the major end-product being the amino acid aspartate. The conversion of citrate to oxaloacetate was mediated by the citrate lyase enzyme. Furthermore, no activity for oxaloacetate decarboxylase could be identified.

The addition of citrate to a chemically defined minimal medium resulted in decreased growth rates at pH 7.5 and accelerated death in the same medium at pH 5.0. However, cells previously grown in the presence of citrate in a neutral pH environment had significantly higher subsequent survival in an acidic environment. These data indicate that citrate can elicit an acid tolerance response at neutral pH in *S. mutans* prior to exposure at low pH.

The most novel and major finding of this work was the identification of ferric ions as the major cofactor for citrate transport. Although there is some evidence that citrate transport and catabolism may provide a small degree of increased acid tolerance to *S. mutans*, my hypothesis is that the primary function of citrate transport is for the acquisition of metal ions, mainly ferric ions. Iron is an element, essential in trace amounts for the survival of many bacterial species including *S. mutans*. Further supporting this hypothesis is the up-regulation of the citrate synthase-aconatase-isocitrate dehydrogenase operon in the presence of iron, compared with an environment completely devoid of metal ions (Spatafora, personal communication). In conclusion citrate transport and metabolism are beneficial to *S. mutans* by 'priming the pump' of the acid tolerance response, providing a molecular backbone for amino acid synthesis, and providing a novel means by which iron can be acquired.

My second line of investigation, research into the *S. mutans* glyoxalase pathway, responsible for the detoxification of methylglyoxal, was conducted because of its known association with acid tolerance (12, 22). Methylglyoxal (MG) is a highly toxic electrophile, resulting from aberrant conversion of glycolytic intermediates during glycolysis. The removal of this toxic compound is important to all living cells, as MG attacks and inhibits both proteins and nucleic acids (17-19). The glyoxalase pathway consists of two enzymes [lactoylglutathione lyase (LGL) and glyoxalase II (GlyII)] responsible for the conversion of MG into D-lactate. Much work has been conducted regarding this pathway with eukaryotic cells, however very little work has focused on prokaryotes and, to date none in gram-positive bacteria. Previous research conducted by 2 separate groups showed the up-regulation of LGL in *S. mutans* upon exposure to an acidic environment, leading to the hypothesis that the glyoxalase pathway, and in particular LGL, is important in the acid tolerance of *S. mutans*.

I demonstrated that, in *S. mutans*, LGL is responsible for the conversion of MG to the intermediary S-D-lactoylglutathione. I was also able to prove *S. mutans* has GlyII activity, although preliminary homology searches found no obvious *glyII* homologue in the *S. mutans* genome (homology searches performed using pBlast searching against, the human *glyII*, and *E. coli glyII*). This suggests the existence of a novel, yet to be identified mechanism.

I was able to demonstrate a solid link between LGL and *S. mutans* acid tolerance. Using qRT-PCR, *lgl* was shown to be up-regulated by acidic environments (both growth in acid medium and response to acid). An isogenic deletion mutant in *lgl* was created, named LGLKO. LGLKO had an acid sensitive phenotype compared with wild-type UA159, showing increased generation times at pH 5.0, diminished survival via ATR, increased

terminal culture pH, and a decreased ability to compete with UA159 during simultaneous growth in an acidified biofermentor.

The influence of MG and LGL inactivation on glycolytic rates generated a quite remarkable discovery. In what I believe to be a unique finding, at pH 5.0 the rate of glycolysis increased in response to exogenous methylglyoxal and in the absence of LGL, suggesting excess intracellular methyglyoxal acts as a glycolytic regulator in an acidic environment.

The strong association between the requirement for LGL and a robust ability to survive acid challenge, as uncovered by this work, suggests LGL may be a new and exciting target to potentially modulate the cariogenicity of dental caries. One strategy would be to incorporate powerful specific inhibitors of LGL into consumer products such as chewing gum, or toothpaste, similar to the addition of xylitol and fluoride to these products respectively. This would inhibit the growth of all plaque bacteria, however it is very likely that fast growing bacteria, such as *S. mutans*, would be most affected under caries conducive conditions. S-(N-Aryl-N-hydroxycarbamoyl) glutathione derivatives are powerful competitive inhibitors of LGL, and have been investigated as antitumor agents (15).

Pyruvate dehydrogenase A (PdhA) is a component of 4-enzyme pyruvate dehydrogenase complex (PDH). It has been shown that PDH of *S. mutans* is partly responsible for the heterofermentative conversion of pyruvate to acetyl-CoA, which can be subsequently catabolized to ethanol and/or acetate (4). This is in contrast to homofermentation, typified as the production of lactate from pyruvate. PDH has been proposed to be of importance in the aciduricity of *S. mutans* as PDH produces additional ATP, and end-products of weaker acidicity compared with homofermentation mediated by

lactate dehydrogenase. *S. mutans* doesn't evolve CO_2 during fermentation, and as it has been proposed that intracellular aqueous CO_2 is rapidly converted to basic carbonate, increasing cytosolic pH. Of particular interest is *pdhA* as it encodes for the E1α-subunit of PDH. Together with the E1β, the E1 subunit of PDH is responsible for the decarboxylation of pyruvate, and therefore may contribute to *S. mutans* acid tolerance to a greater extent than the other subunits. Additionally PdhA was the only PDH subunit to show increased expression in response to acid using 2D-GEL electrophoresis (21).

A knockout of *pdhA* proved to confer an acid sensitive phenotype to the resulting strain, PDHAKO. This strain exhibited increased doubling time during growth at pH 5.0, decreased ATR assay survival, increased terminal culture pH, and reduced simultaneous survival in an acidic biofermentor. This acid sensitivity was further supported by qRT-PCR, in which pdhA dramatically increased in expression in acidic environments (growth in pH 5.0 media, and acid adaptation for 2 h at pH 5.5).

Additional qRT-PCR experiments showed *pdhA* responded in accordance with it being involved in heterofermentation. These experiments showed *pdhA* to be up-regulated at stationary growth phase compared with mid-log growth phase, and down regulated in the presence of excess glucose.

As PDH is involved in acidic end-product production, analysis of glycolytic rates was carried out. These analyses showed the deletion of *pdhA* resulted in glycolysis increasing at pH 7.0 and decreased at pH 5.0. From these data, I hypothesize that at pH 7.0 the PDHAKO strain is producing solely lactate (homofermentation) and as such the glycolytic rates are greater compared with wild-type. Conversely, at pH 5.0 heterofermentation, through PDH, would be favoured and as such the glycolytic rates are lower in PDHAKO compared with

wild-type. The glycolytic rate experiments were conducted in an aerobic environment, and therefore, no contributions to overall glycolysis by pyruvate formate lyase would be observed, as pyruvate formate lyase is exceedingly oxygen sensitive (23).

All these data combined show the association between PdhA and the acid tolerance of *S. mutans*. In addition the qRT-PCR and the glycolytic rate data provide good, albeit circumstantial, evidence that PdhA is important for heterofermentation, and furthermore this switch in metabolism is important to acid tolerance. I believe PDH, of which PdhA is an integral component, supplies the additional ATP required for a fully robust acid tolerance response in *S. mutans*. The end-products of PDH are less acidic, allowing a more gradual reduction in extracellular pH.

Neither the uptake/utilization of citrate nor the glyoxalase pathway had been previously investigated in *S. mutans*. Through this work it has been determined both these pathways are indeed functional in *S. mutans*. Only one previous paper investigating PDH in *S. mutans* has been published prior to this work (4).

The work contained in this dissertation represents significant strides in understanding the secondary metabolic networks of *S. mutans* and their importance to aciduricity. We determined PdhA and LGL are important enzymes in the acid tolerance of *S. mutans*. We also determined that the organic acid citrate can stimulate an acid tolerance response. The most notable findings of this work are that the CitM transporter has a preference for ferric iron ions as cofactors for citrate uptake, and LGL and/or intracellular MG concentrations regulate glycolysis at low pH.

II. Future Directions

The study of three secondary metabolic pathways in *S. mutans*, has yielded much information regarding their function and involvement in acid tolerance. However this is only a small snapshot of the total biochemical networks in *S. mutans* that respond to decreasing pH. Further investigations are currently being conducted and future experiments are being planned including work in our research group as well as others. As a complement to the proteomic work our research group, thanks to the efforts of Richard Mair, has begun to use RNA microarray analysis of *S. mutans* to investigate, among other things, the transcriptome of the acid tolerance response.

The previous proteomic work suggested that many other secondary metabolic genes are up-regulated by low pH challenge (12, 20, 22), and therefore likely involved in *S. mutans* acid tolerance. One such pathway is the diacetyl-producing pathway. Diacetyl is a common by-product of fermentation, giving wines and beers a buttery flavour (1). Two enzymes in this pathway, acetolacetate synthase (12) and acetoin reductase (22) have shown up-regulation in response to an acidic environment in *S. mutans*. It is unknown what advantage may be gained from diacetyl production, however acetoin reductase does produce CO_2 (1). However diacetyl production may be a general response to the increased protein synthesis required for an acid tolerance response, as diacetyl accumulates during brewing processes using yeast in environments low in valine concentrations.

Our investigations into citrate transport and metabolism focused on the transporter CitM, its affinity for cofactors, its genetic response to citrate and acid, and the phenotype of the knockout. More in-depth investigations into the other genes in the pathway could be accomplished. Using capillary electrophoresis the concentrations of organic acids in a

sample could be detected, following a similar procedure used by Izco et. al. (11), who measured the concentrations of organic acid products of LAB dairy fermentation. Exogenous citrate could be supplied to wild-type UA159 cells to metabolize, and the resulting organic acids identified. Additionally, our mutant strains could be subjected to similar conditions and the resulting organic acid profile identified.

Further citrate metabolism investigations could be conducted using qRT-PCR. The transcriptional response of the network of citrate metabolism genes could be monitored in response to acid, citrate, and iron species. These experiments would further the understanding of how citrate metabolic genes respond to various environmental stimuli.

One interesting observation from my work is the lack of any detectable activity for oxaloacetate decarboxylase. This is despite its β subunit showing increased transcription in previous work (5). This enzyme complex may not have displayed any activity under the conditions we tested or alternatively its activity may be minimal compared with aspartate transaminase, responsible for the conversion of oxaloacetate to aspartate.

Additionally the hypothesis of citrate transport functioning in the acquisition of iron is currently being conducted in collaboration with Dr. Grace Spatafora at Middlebury College. These experiments will investigate the transport of iron through CitM as well as following the intracellular fate of the iron. Dr. Spatafora has already investigated the transcriptional response of *S. mutans* to iron using microarrays. Results from these experiments have shown the genes within the citrate pathway are up-regulated in response to iron (personal communication). Dr. Spatafora also intends to determine whether iron remains in complex with citrate once it enters the cell, or if and when it becomes a free ion using modified siderophore assays (7).

Research presented in Chapter 3, showed the importance of LGL in the acid tolerance of *S. mutans*. The work mainly focused on LGL, as this was previously shown to be up regulated in response to acid (12, 22). However, LGL is only the first enzyme in the glyoxalase pathway, the second enzyme being GlyII. Activity of the enzyme was found in my total soluble protein extracts of *S. mutans*. This preliminary GlyII work could be furthered, by identifying the gene or genes encoding the enzyme. With the aid of our bio-informatics specialist Richard Mair, novel approaches to locating GlyII could be performed by comparing putative protein structures, and functional groups, as opposed to mere amino acid and nucleotide sequence alignments conducted by blast searches. Once GlyII has been identified, factors governing the expression and activity of *glyII* could be uncovered in a similar fashion as for LGL.

My work clearly demonstrated that the regulation of *pdhA* is important in *S. mutans* acid tolerance. This work also indicated that *pdhA* is regulated by growth phase, glucose concentration and low pH. This work could be further supported by measuring the end-products of metabolism of PDHAKO compared with wild-type UA159 under conditions known to favour heterofermentation. This would require sophisticated techniques as PdhA activity is considerably small, and it has been shown that lactate always remains the dominant organic acid end-product (3). Work in other laboratories has shown that a powerful technique, capillary electrophoresis, is capable of detecting small variations and concentration of organic acids (11). The organic acids produced by wild-type UA159 and PDHAKO under environmental conditions such as varying pH, glucose concentration, and oxygen would be monitored. A decrease in proportion of lactate compared with acetate would be an indication of increased heterofermentation, and would likely occur at low pH,

low glucose concentration and during stationary growth phase. These are the conditions that I found increased *pdhA* expression.

Its is obvious that more work is needed to gain a better, more in depth understanding of the intricacies involved in the ability of *S. mutans* to respond to and thrive in an acidic environment. This report represents a systematic approach to understanding how secondary metabolism plays an important role in acid tolerance. Additionally, this work represents a valuable starting point for future investigations not only into citrate metabolism, methyglyoxal detoxification, and pyruvate oxidation, but other secondary metabolic networks as well.

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