IDENTIFICATION AND CHARACTERIZATION OF A PUTATIVE POTASSIUM TRANSPORT SYSTEM IN *STREPTOCOCCUS MUTANS*

By

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Identification and Characterization of a Putative Potassium Transport System in *STREPTOCOCCUS MUTANS*

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ABSTRACT

Streptococcus mutans demonstrates a remarkable resistance to acid stress via constitutive and pH-inducible mechanisms that activate the expression of acid survival proteins and pathways. Previous studies identified putative potassium transport genes that were linked to this response and designated the *trkB/trk/pacL* system. To further investigate this system, deletions of these genes resulted in acid sensitive mutant strains characterized by decreased bacterial yield and elevated terminal pH values in growth media at pH 5.5 and decreased survival rates at a killing pH of 3.0 when compared to the wildtype UA159 strain. Furthermore, operon deletion resulted in an inability to retain maximal intracellular potassium levels when transport assays were conducted at pH 5.5. Mutants also demonstrated defects in biofilm integrity relative to the parent strain. This work links the *trkB/trk/pacL* system to both long-term acid tolerance mechanisms of *S. mutans* and biofilm development. Supported by NIH Grant R01DE0132320 and CIHR Grant MT-15431.

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AP	**apurinic/apyrimidinic**
ATR	**acid tolerance response**
ATP	**adenosine triphosphate**
BLASTp	**Basic Local Alignment Search Tool for proteins**
CIRCE	**controlling inverted repeat of chaperone expression**
CSP	**competence stimulating peptide**
Ct	**fluorescence threshold value**
DCCD	**dicyclohexylcarbodiimide**
DNA	**deoxyribonucleic acid**
Erm	**erythromycin**
GTF	**glucosyltransferase**
LDH	**lactate dehydrogenase**
LGL	**lactoylglutathione lyase**
mRNA	**messenger ribonucleic acid**
MGT	**mean generation time**
Msm	**multiple sugar metabolism**
NADH	**nicotinamide adenine dinucleotide**
NCBI	**National Center for Biotechnology Information**
NER	**nucleotide excision repair**
ORF	**open reading frame**
PDH	**pyruvate dehydrogenase complex**
PEP	**phosphoenolpyruvate**
Pfam	**protein family database**
pH_i	**intracellular pH**
pH_o	**extracellular pH**
PMF	**proton motive force**
PTS	**phosphotransferase system**
rtPCR	**real-time polymerase chain reaction**
SMART	**Simple Architecture Research Tool**
STRING	**Search Tool for the Retrieval of Interacting Genes/Proteins**
TCSTS	**two-component signal transduction system**
THYE	**Todd Hewitt yeast extract broth**
TYEG	**tryptone yeast extract broth with 0.5% glucose (wt/vl)**
UV	**ultraviolet**

CHAPTER 1: LITERATURE REVIEW

1.1: The Impact of Dental Caries

Dental caries has been recognized as one of the most common types of bacterial infections in humans; an infection that most individuals have to contend with throughout the duration of their life (Heloe *et al.*, 1981). As such, carious lesions have received much attention due to the social and economic impact that they have on a population (Do *et al.*, 2008). Along with periodontal disease, dental caries is considered the most significant global oral health burden, consequentially forcing industrialized countries to spend 5-10% of public health expenditures on related care (Petersen *et al.*, 2005). Furthermore, caries affects a vast majority of adults and is diagnosed in 60-90% of school-aged children in industrialized nations (Petersen *et al.*, 2005). This equates to billions of dollars spent yearly by affected individuals in the treatment of tooth decay alone (Baelum *et al.*, 2007; Pennisi, 2005) and this amount is predicted to increase in response to an aging geriatric demographic (Chalmers *et al.*, 2008). That said, it has been of great interest to the scientific community to examine the ecological basis of dental caries with concern to the dynamic changes in the composition of the oral flora and how these activities can transition from health to disease.

1.2: Colonization of the Oral Cavity

The oral cavity is home to an astounding assortment of microbial life, with over 700 different bacterial phylotypes identified in recent years, including members of the Gram-positive, Gram-negative and archea taxonomic classifications (Aas *et al.*, 2005; Paster *et al.*, 2001; Keijser *et al.*, 2008). This incredible community was first encountered by the acclaimed "Father of Microbiolgy," Antony van Leeuwenhoek, whose drawings of

the "animalcules" residing in samples from his own mouth, introduced the world to the existence of this diverse microenvironment (Cohn, 1883). The human dental biofilm, more commonly known as dental plaque, is a mixed community of microorganisms that is embedded in a polymer derived polysaccharide matrix of microbial and salivary origin (Marsh, 2004). Development of the biofilm occurs in a sequential manner that begins with the formation of the acquired pellicle (Marsh *et al.*, 1995); a proteinaceous film composed of host salivary components that surround the tooth surface (Yao *et al.*, 2003). Once the pellicle has been established, bacterial colonization follows, though the actual process is a challenging task. In order to attach to the tooth, microbes must overcome physical clearance mechanisms that include chewing, speaking and the flushing of saliva (Li *et al.*, 2004), as well as chemical components, such as secretory IgA (sIgA) which causes agglutination of bacteria, that are ultimately removed through swallowing by the host (Williams and Gibbons, 1972). In an effort to evade and combat these defences, early colonizing bacteria have evolved tools of their own, such as the production of protease IgA1, which function to destroy sIgA (Genco *et al.*, 1975) and deteriorate its effects.

In addition to eluding host clearance mechanisms, the success of colonization is also determined by an organism's ability to adhere to the tooth surface, the mucosal epithelium or to the nascent bacterial layer that has been established by pioneer species (Burne, 1998; Kuramitsu *et al.*, 2007; Rickard *et al.*, 2003). Therefore, adhesion has been thoroughly studied in a multitude of oral bacteria (Whittaker *et al.*, 1996) and is demonstrated through the interaction of specific stereo-chemical molecules and their respective receptors (Marsh, 2000). Prominent early colonizers include members of the

streptococci and actinomyces genera (Dige *et al.*, 2009; Li *et al.*, 2004) that effectively use salivary constituents, such as proline-rich macromolecules (e.g. protein, peptides and glycoproteins) to initiate the interaction between specific adhesion-receptor cognate pairs (Gibbons, 1989; Gillece-Castro *et al.*, 1991; Whittaker *et al.*, 1996). Examples of these interactions include; *Streptococcus gordonii* and *Streptococcus mitis* binding α-amylase (Douglas *et al.*, 1990; Scannapieco *et al.*, 1993), as well as *Actinomyces naeslundii*, whose ability to remove terminal sialic acid residues from mucous glycoprotein 1 through the synthesis of neuraminidases, allows them to bind the resulting galactosyl residues (Ellen *et al.*, 1980) made available in the enamel pellicle (Li *et al.*, 2003). These initial events also promote the subsequent attachment of other oral microbes, deemed late colonizers, and demonstrate the types of the interspecies interactions that exist in the oral cavity (Kuramitsu *et al.*, 2007) via cell-cell adhesion (Gibbons and Nygaard, 1970); a process called co-aggregation (Kolenbrander, 1995). Of particular interest in this process, are members of the *Fusobacteria* genus that possess the ability to co-aggregate with a wide range of bacterial species, and are considered biological mediators because of their ability to adhere to both early and late colonizing bacteria (Kolenbrander, 2000).

Establishment of microbial colonization through the aforementioned adhesion processes is a prerequisite that determines the association of a particular species to the dental biofilm. The ability of these individuals to persist and thrive in this environment however, is dictated by their capacity to metabolize available nutrients (Burne, 1998), the concentrations of which fluctuate dramatically from times of famine between meals or following ingestion by the host (Carlsson, 1997). Although salivary components can provide nitrogen sources and limited carbohydrates through the breakdown of

glycoproteins for certain streptococci and actinomyces species (de Jong *et al.*, 1984), nutrient availability, especially limitations or excesses of carbohydrate in the diet, can greatly influence the cariogenic potential of the oral biofilm (Bowden and Li., 1997). In true Darwinian fashion (Darwin, 1859; Reznick and Ricklefs, 2009), these influences and resulting metabolic end-products act as selective pressures favouring the development of individuals that can best adapt to the current condition of their ecological niche (Bowden and Li, 1997).

1.3: Caries Aetiology

Relevant to the discussion on the aetiology of caries, is the effect of varying sucrose concentrations on the metabolism of oral bacteria and the changes in environmental conditions that result, specifically pH (Frostell, 1969). Studies conducted by R.M. Stephan in the 1940's provided definitive evidence linking bacterial metabolism to the lowering of plaque pH (Stephan, 1940; Stephan, 1944), which could drop to values as low as pH 3.0 following continuous sugar exposure (Jensen *et al.*, 1982). These observations supported earlier findings in the late 19th century where W. Miller had linked microbial acid production by dental plaque bacteria to the presence of dietary substrates (Miller, 1890). While he could not identify the specific species responsible for the acid produced, his ideas and findings helped researchers later determine that oral bacteria metabolize dietary carbohydrates and produce acid (mainly lactate) as an end product (Loesche, 1986). Essentially, the production of lactate by the acidogenic oral microflora causes demineralization of calcium and phosphate presented in the crystal form of hydoxyapatite that comprises the enamel of the teeth (Weatherell, 1975). When

the frequency and rate of acid production exceeds the natural remineralizing activity, demineralization occurs and results in the subsequent progression of cavitations, provided the pH remains below a "critical" value of ~5.5-5.3 for a sufficient amount of time (Hamilton, 2000; Takahashi and Nyvad, 2008).

1.4: *Streptococcus mutans*; A Role in Dental Decay

Many of the organisms responsible for acid production in plaque primarily belong to the streptococci and members of the genus *Lactobacillus* (Loesche, 1986). Of the streptococci, particular interest is attributed towards the mutans streptococci (MS) which consist of the species *Streptococcus sobrinus*, *Streptococcus rattus*, *Streptococcus cricetus* and *Streptococcus mutans*, each identified on the basis of their genetic and antigenic properties (Coykendall, 1974). Primary focus was given to *S. mutans* in the mid 1980's, when microbiologist Walter Loesche provided compelling results that clearly identified this species as the principal causative agent of tooth decay, partly because of its presence in 70-100% of human caries cases (Loesche, 1986). Historically, *S. mutans* was first seen in relation to human carious lesions in 1924, by J. Clark and was named accordingly based on its "mutated" cell morphology (Loesche, 1986). However, it was not until the late 1950's that concentrated studies on this pathogen began when R. Fitzgerald and P. Keyes demonstrated a definite link between *S. mutans* and caries in gnotobiotic rats and hamsters, thereby proving that it was an infectious, transmissible disease (Fitzgerald and Keyes, 1960; Fitzgerald, 1963).

1.5: Virulence Properties of *S. mutans*

Streptococcus mutans is a Gram-positive bacterium that is first acquired by infants soon after their first tooth emerges, with the mother being identified as the major source (Caufield *et al.*, 1993) since similar or identical genotypic profiles of the isolated strains are shared with their child. Once *S. mutans* has been acquired, selection for a cariogenic flora has been attributed to alterations in the dental plaque ecology, whereby a drastic drop in pH is observed (Jensen *et al.*, 1982) due to the exogenous consumption of fermentable dietary carbohydrates and the acidic metabolic end-products that result (Loesche, 1986). Examination and characterization of the virulence properties expressed by *S. mutans* reveals how this change in the environment can favour its propagation and explain its ability to thrive in a surrounding that many competing oral bacteria find lethal (Svensäter *et al.*, 1997). In general, the three major cariogenic factors associated with *S. mutans* are adhesion, acidogenicity (the ability to produce acid) and aciduricity (the ability to tolerate acid) (Banas, 2004). The discussion concerning adhesion and acidogenicity will be described briefly, while acid tolerance mechanisms will be described in detail serving as a major focus of this thesis.

1.6: Adhesion

Adhesion of *S. mutans* is mediated by sucrose-dependent means in the form of glucan synthesis and sucrose-independent mechanisms which are profoundly dictated by antigen I/II (Banas, 2004). Glucans produced by *Streptococcus mutans* have been under heavy scientific scrutiny due to the essential part they play in promoting colonization on the tooth surface (Gibbons and Houte, 1975). In the 1960's, Gibbons noticed that when

cariogenic bacteria were incubated in sucrose broth they accumulated on the surface of culture vessels forming large amounts of an unknown extracellular polysaccharide (Ellen *et al.*, 2005; Gibbons *et al.*, 1966). These unidentified polymers would later be revealed as glucans that undergo sucrose-dependent, glucan-mediated adhesion that is facilitated by the concerted actions of the glucosyltransferases (GTFs) and their respective glucan products (Cerning, 1990; Rolla, 1989). The GTFs are enzymes that synthesize glucans via the transfer of glucosyl residues to a growing glucan polymer chain with the energy derived from the catalytic cleavage of the glucosidic bond of sucrose (Islam *et al.*, 2007; Monchois *et al.*, 1999). Sucrose is the only natural substrate of the GTFs and interaction with this sugar molecule may induce a conformational change that results in activation of the catalytic site (Monchois *et al.*, 1999). Furthermore, the GTFs can be secreted into the surrounding environment and have the potential to bind saliva-coated hydroxyapatite (Vacca-Smith *et al.*, 1996; Vacca-Smith and Bowen, 1998), the acquired salivary pellicle (Schilling and Bowen, 1992) and other bacteria (Vacca-Smith and Bowen, 1998) through hydrogen bonding (Banas, 2004). All bound forms of the GTF remain active, allowing them to synthesize soluble and insoluble glucans (Colby and Russell, 1997) which in turn promotes the selective colonization of cariogenic mutans streptococci via cell-cell and cell-surface interactions (Schilling and Bowen, 1992).

S. mutans can also bind glucan polymers by way of non-enzymatic glucan binding proteins (GBPs) (Russell, 1979) which function to compliment the activity of the GTFs (Banas and Vickerman, 2003). These proteins have evolved high affinities for glucan, thereby providing a colonization advantage by means of enhancing cell adherence in the presence of sucrose (Banas and Vickerman, 2003).

Lastly, *S. mutans* can adhere to salivary components independent of sucrose via the cell surface fibrillar protein antigen I/II (Hajishengallis *et al.*, 1994) also designated P1 (Forester *et al.*, 1983) and SpaP (Bowen *et al.*, 1991). Shared homologies with surface proteins of other oral streptococci are observed (Ma *et al.*, 1991) and conservation of the 185 kDa protein has been implicated in the adherence of this cariogenic pathogen to the constituents of salivary pellicle (Burne, 1998; Hajishengallis *et al.*, 1994).

Collectively, these adhesion mechanisms provide favourable strategies that promote colonization of the tooth. Once these initial steps have been taken, propagation of *S. mutans* in the dental biofilm is dependent upon the ability to produce energy and grow, described next.

1.7 Acidogenicity

S. mutans is a facultative anaerobe, whose ability to ferment carbohydrates is the principal source of energy production (Jacobson *et al.*, 1989). As such, *S. mutans* can utilize a wide variety of carbohydrates by activating specific transport systems and metabolic pathways (Jacobson *et al.*, 1989) that accommodate the varying availability of said macromolecules in the oral cavity (Bowden and Li, 1997). Based on genomic data and current knowledge, *S. mutans* is the most metabolically capable Gram-positive organism at converting different sugars into usable energy (Ajdic *et al.*, 2002). Central to this designation, is the ability of *S. mutans* to uptake sugars via the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphostransferase system (PTS), the multiple sugar

metabolism (Msm) transport system and ATP-dependent glucose-specific permeases (Colby and Russell, 1997; Marsh and Martin, 1998).

Under low sugar concentrations, *S. mutans* can express the PEP-PTS; a high affinity scavenging system comprised of a sugar-specific membrane-bound permease (designated Enzyme II) that is essentially energized by PEP through the sequential phosphorylation of the soluble, non-sugar-specific, cytoplasmic intermediate proteins Enzyme I and HPr (Carlsson, 1997; Jacobson *et al.*, 1989). Sugar molecules such as glucose, fructose and lactose are thus phosphorylated during transport and can be introduced to the complete glycolytic pathway of *S. mutans* for ATP production (Ajdic *et al.*, 2002; Carlsson, 1997). Uptake of the sugars melibiose, raffinose, and isomaltotriose are conducted by the Msm transport system (Russell *et al.*, 1992), which is suggested to function between meals, utilizing the products of extracellular polysaccharide degradation (Marsh and Martin, 1998). When environmental sugar concentrations are high and the pH is lowered, the PEP-PTS system is repressed and an ATP-dependent glucose permease system becomes active (Buckley and Hamilton, 1994; Cvitkovitch *et al.*, 1995).

Once sugars have been transported into the cell, glycolysis occurs and the fate of the resulting pyruvate molecules is again dictated by the concentration of sugars in the environment (Marsh and Martin, 1998). Under low sugar concentrations and anaerobic conditions, pyruvate enters the pyruvate-formate lyase pathway which produces ATP, acetate, ethanol and formate (Abbe *et al.*, 1982). High sugar concentrations yield ATP and lactate via the enzyme lactate dehydrogenase (LDH) (Marsh and Martin, 1998; Yamada and Carlsson, 1975). Interestingly, LDH deficiency reduced the cariogenicity of

S. mutans (Fitzgerald *et al.*, 1989) and the *ldh* gene was found to be essential for growth at high glucose concentrations, though issues regarding the lethality of these mutations could not be resolved (Hillman *et al.*, 1996). Essentially, the production of lactate lowers the plaque pH, leading to decalcification of the tooth enamel as previously described. Lactate efflux and its contribution to acid tolerance will be further described below (refer to section 1.8: v) Intracellular pH Homeostasis).

1.8: Acid Tolerance

The capacity of *S. mutans* to initiate caries via acid production from the metabolism of dietary carbohydrates (Loesche, 1986) would be suicidal if not for its remarkable ability to tolerate acid; signifying a crucial aspect to its virulence (Hamilton and Buckley, 1991). Inhabitants of dental plaque experience rapid, dynamic pH fluctuations that are greatly influenced by carbohydrate intake resulting in pH levels that can drop from neutral pH 7.0 to acidic values below pH 3.0 in less than 20 min (Jensen *et al.*, 1982; Jensen and Wefel, 1989). In order to withstand these continual cycles of acid shock, *S. mutans* has evolved a repertoire of mechanisms that fall under two distinct categories; constitutive mechanisms and acid induced mechanisms (Burne, 1998), also referred to as the acid tolerance response (ATR) (Svensäter *et al.*, 1997). More specifically, the ATR of this microorganism is defined as the ability to adapt to acid stress by prior exposure to a low, sub-lethal pH of ~5.5, resulting in the induction of a mechanism (expression of certain genes) that enhances survival at a pH as low as 3.0 (Hamilton and Buckley, 1991). Rather than describing the constitutive and inducible mechanisms separately, both will be described together in an attempt to highlight the

collective benefits of these systems, focussing primarily on their contributions towards: i) the Protection and Repair of Macromolecules; ii) Alterations of Metabolic Pathways; iii) Secondary Metabolism; iv) Cell Density, Biofilm Formation and Regulatory Systems; and v) Intracellular pH Homeostasis (Cotter and Hill, 2003).

i) Protection and Repair of Macromolecules

A major consequence of bacterial life in an acidic environment is the potential of these surroundings to acidify the intracellular cytoplasm. Negative consequences of this include loss of glycolytic enzyme activity and structural damage to the cell membrane, proteins and DNA (Cotter and Hill, 2003). With concern to the latter, acid resistance is optimal when an organism has the ability to repair DNA damage or protect it from the harmful effects of intracellular acidification (Cotter and Hill, 2003). To date, multiple proteins support these functions, including RecA; a recombinase protein that is conserved throughout all kingdoms of life (Brendel *et al.*, 1997; Cox, 2007). Furthermore, RecA operates as a moderator of homologous recombination and serves a housekeeping role that repairs and restarts stalled DNA replication forks linked to genome instability (Cox, 2007). Recombination as a form of DNA repair has been extensively studied in *E. coli* and involves the mechanisms outlined by broken fork repair, double-stranded break repair and recombinational gap-filling repair; all of which are particularly important during DNA replication (Persky and Lovett, 2008).

With regard to *S. mutans,* initial studies with RecA-deficient mutants demonstrated increased susceptibility to a killing pH of 2.5 relative to the parent strain (Quivey *et al.*, 1995). Incredibly, this deficiency could be abolished when the mutant was

permitted to elicit an acid-adaptive response, directly suggesting the presence of a DNA repair system independent of RecA (Quivey *et al.*, 1995). This notion was further supported by increased resistance to hydrogen peroxide and UV radiation following acid adaptation (Quivey *et al.*, 1995). Concentrated studies in response to these phenomena would reveal that *S. mutans* does in fact induce a RecA-independent DNA repair system, which occurs through the expression of an AP endonuclease (Hahn *et al.*, 1999) and *uvr*A under acid stress (Hanna *et al.*, 2001).

At the molecular level, DNA damage is observed through the loss of purines and pyrimidines (Lindahl and Nyberg, 1972) which results in the formation of an abasic or AP (apurinic-apyrimidinic) site (Sancar, 1996). This event occurs more rapidly at an acidic pH where protonation of the nitrogenous base consequentially leads to cleavage of the glycosyl bond (Lindahl and Nyberg, 1972). Recognition of the AP site in duplex DNA and initiation of its repair is attributed to AP endonucleases that effectively cleave the phosphodiester bond located directly 5' or 3' to the damaged site, depending on the class of endonuclease involved (Hahn *et al.*, 1999; Lindahl and Nyberg, 1982). This form of base excision repair (Sancar, 1996) is a multistep process that involves different classes of endonucleases, including exonuclease III, which accounts for 90% of abasic site repair and is induced as a stress-response mechanism (Lindahl and Nyberg, 1982). Studies conducted by Hahn *et al.* (1999) demonstrated that under acid conditions, *S. mutans* upregulates the expression of an AP endonuclease that is similar to the activity and expression of exonuclease III of *E. coli*, therefore implicating its involvement in acid adaptation.

Further research in the area of DNA protection and repair in *S. mutans* includes the relatively recent characterization of *uvr*A by Hanna *et al.*(2001) who used differential display PCR to identify genes with increased expression at pH 5.0 compared to cells grown under neutral conditions (pH 7.5). Data from these experiments showed that the isogenic *uvrA* mutant in *S. mutans* demonstrated severe sensitivity to UV irradiation and was also unable to survive a killing pH of 3.0, despite previous acid exposure at a sub-lethal pH of 5.0 (Hanna *et al.*, 2001). In *Bacillus subtilis*, the *uvr*A-UV excinuclease gene is involved in the nucleotide excision repair (NER) pathway that functions to locate and excise bulky DNA lesions (Hanna *et al.*, 2001; Sancar, 1996). Moreover, the mechanism of NER is characterized by dual incisions on either side of the damaged DNA region, followed by the replacement of bound repair enzymes with replication proteins that fill the excised site and are ligated upon completion (Sancar, 1996). The *uvr*A gene of *S. mutans* shares 67% identity with *uvr*A-UV of *B. subtilis*, which suggests that *S. mutans* can successfully adapt to low pH through the contributions of the NER pathway and its ability to repair acid-induced DNA damage (Hanna *et al.*, 2001).

With respect to DNA repair mechanisms, *S. mutans* is well equipped to deal with genetic corruption via acid stress, which can be attributed to the collective induction of the AP endonuclease in concert with *uvr*A to further enhance its acid tolerant phenotype. Additionally, these mechanisms have been suggested to enable *S. mutans* to respond to minor DNA damage through base excision repair (via AP endonuclease activity), as well as larger DNA lesions through the *uvr*A pathway (Cotter and Hill, 2003; Hanna *et al.*, 2001).

My discussion will now shift to the relationship between chaperonins and their impact on the acid tolerance of *S. mutans* through protein interactions. The biological role of these molecules has been recognized in a variety of different stress responses including protein folding, protection of denatured proteins and removal of damaged proteins (Cotter and Hill, 2003). More specifically, *S. mutans* can upregulate the expression of DnaK and GroEL under acid shock, but only elevated levels of DnaK are maintained during adaptation to acid (Jayaraman *et al.*, 1997; Lemos *et al.*, 2001). Changes in levels of these proteins profoundly affected acid tolerance and also decreased the ability of the mutant strain (which expressed elevated levels of GroEL and reduced levels of DnaK) to lower the external pH relative to the parent (Lemos *et al.*, 2001).

DnaK and GroEL are part of the CIRCE (controlling inverted repeat of chaperone expression) regulon (Cotter and Hill, 2003) which is negatively regulated by HrcA and constitutes the HrcA-CIRCE system (Lemos *et al.*, 2001). Furthermore, DnaK and GroEL function as molecular chaperonins that assist in the folding process of newly synthesized or denatured proteins and aid in the transport, assembly and degradation of other proteins (Craig *et al.*, 1993). Though the precise role of these gene products and their involvement in acid adaptation in *S. mutans* is poorly understood, it has been postulated that DnaK assists in the biogenesis of the F-ATPase, which would enhance the cell's ability to maintain a functional intracellular pH through removal of protons from the cytoplasm (Lemos *et al.*, 2001). Interestingly, studies in *E. coli* have demonstrated that DnaK can increase the stability of *uvr*A, therefore suggesting a secondary target which can further promote the acid tolerance of organisms expressing this protein by ensuring the proper function of DNA repair systems (Cotter and Hill, 2003). Recent

attempts to further characterize DnaK and GroEL were achieved by the development of knockdown strains with lowered levels of these chaperonins to 95% and 80%, respectively (Lemos *et al.*, 2007). These strains exhibited multiple phenotypic changes including slower growth rate, impaired biofilm formation in glucose and major proteomic changes when compared with the wildtype (Lemos *et al.*, 2007). Notably, the DnaK knockdown strain was acid sensitive (Lemos *et al.*, 2007), again demonstrating the involvement of this protein in acid adaptation as well as the expression of other key virulence properties of *S. mutans*.

Additionally, proteomic analysis of *S. mutans* grown in continuous culture under neutral and acidic conditions identified 30 different proteins with altered levels of expression at pH 5.0 relative to pH 7.0, representing cellular and extracellular gene products associated with stress-response pathways (Len *et al.*, 2004b). Of these, 25 proteins were up-regulated or uniquely expressed at pH 5.0 and were involved in DNA replication, transcription, translation, protein folding and proteolysis (Len *et al.*, 2004b). More notably, five of these proteins (Ssb, GreA, PnpA, ClpL and PepD) were isolated based on the fact that they have never been associated with acid tolerance and/or have never been studied in oral streptococci in detail (Len *et al.*, 2004b). Although further studies are required to examine the specific roles that each of these proteins play in acid adaptation, these results demonstrate increased diversity in the molecules available for combating acid stress by *S. mutans*. Len *et al.* (2004b) conducted another proteomic analysis with *S. mutans* using steady state cells in a glucose-limited anaerobic continuous culture and examined the differential expression of proteins involved in metabolic

pathways in pH 7.0 and 5.0 growth media. Results from these studies will be described below.

In summary, this growing arsenal of macromolecule protection and repair mechanisms exhibited by *S. mutans* provides a strong advantage in an acidic environment over competitors not harbouring these crucial systems which facilitates the proper function of the essential machinery involved in transcription, translation and enzymatic activity in an acidic environment.

ii) Alteration of Metabolism

Central to caries formation is the prevalence of organic acids in the dental plaque and the decalcification of enamel that results. *S. mutans* is extremely competent at metabolising different sugars into lactic acid even when the external pH of its environment is lowered (Hamilton and Buckley, 1991). Glycolytic enzymes involved in these pathways express a wide functional pH range, demonstrating activity at pH values as low as 4.0 (Bender *et al.*, 1985; Hamilton and Buckley, 1991), which is well below the critical pH value for caries initiation. Furthermore, it has been demonstrated that in most instances, the velocity of acid production tested in a pH range of 7.0-5.0 by *S. mutans* surpasses the rates of acid production by other oral streptococci (de Soet *et al.*, 2000). This helps explain the rapid pH drop of dental plaque harbouring *S. mutans* in the presence of carbohydrates (Jensen *et al.*, 1982).

Continuous culture studies involving *S. mutans* cells grown at pH 5.5 in complex media, as compared to cells grown at pH 6.5, demonstrated a 3-fold increase in glycolytic activity of the lower pH adapted cells (Hamilton and Ellwood, 1978). Since no difference

in overall cell yield was observed in the lower pH adapted cultures, these results demonstrated the ability of *S. mutans* to alter its metabolism in response to a changing external pH (Hamilton and Ellwood, 1978). In addition, it was shown that both the pH optimum of glucose uptake and glycolysis would shift to lower levels in accordance with lower extracellular pH values, further supporting the ability of *S. mutans* to alter its metabolic activities as an acid-adaptive response (Hamilton and Buckley, 1991).

In 1992, Daspher and Reynolds determined that the extracellular pH (pH_o) optimum of glycolysis exhibited a much broader profile than the intracellular pH (pH_i) optimum, which decreased to zero as the pH_i dropped from pH 7.0 to pH 5.0. In both studies conducted by Hamilton *et al.* (1991) and Dashper and Reynolds (1992), *S. mutans* was able to maintain a transmembrane pH gradient that was inside alkaline relative to pH_o. Specific mechanisms involved with the regulation of cytopalsmic pH values will be described later (refer to v) Intracellular pH Homeostasis).

In 2004, Len *et al.* conducted a second proteome analysis of *S. mutans* that observed the metabolic phenotype associated with acid tolerance during steady-state continuous-culture. This enabled cells to be sampled under strictly defined conditions (Len *et al.*, 2004a). Interestingly, 70 of the 155 upregulated protein spots detected at pH 5.0 relative to pH 7.0 were involved in metabolism, the majority of them being associated with glycolysis, alternative acid production and branched-chain amino acid synthesis (Len *et al.*, 2004a). With regard to the latter, it has been postulated that *S. mutans* may be able to utilize amino acid biosynthesis to reduce H^+ accumulation through the buffering capacity of NH_3, which is formed by glutamine synthetase as a result of this pathway (Cvitkovitch *et al.*, 1997; Len *et al.*, 2004a). Furthermore, synthesis of branched amino

acids could also reduce H^+ concentrations in the cytoplasm through the consumption of NADPH (an intermediate step in this anabolic pathway) and by removing reducing equivalents in the form of pyruvate and 2-oxobutanoate (Len *et al.*, 2004a).

Acid tolerance via alkali production has been understudied in *S. mutans* due to the absence of genes encoding urease and arginine deiminase, although particular enzymes involved in the latter pathway have been identified (Ajdic *et al.*, 2002). Both of these systems have been characterized as alkali producing mechanisms linked to acid tolerance (Cotter an Hill, 2003). *S. mutans* does, however, possess an agmatine deiminase system, which closely resembles the arginine deiminase system, and is encoded by the *aguBDAC* operon (Griswold *et al.*, 2004). Agmatine is a decarboxylated derivative of arginine and can be acquired by the cell via an agmatine-putrescine aniporter (Griswold *et al.*, 2006). Agmatine is then hydrolized by agmatine deiminase (AgD) to produce ammonia (NH_3) and *N*-carbamoylputrescine, which can be further catabolised to CO_2, NH_3 and putrescine through a series of intermediate steps that also yields an ATP (Griswold *et al.*, 2006). The putrescine formed via this pathway can then be exchanged for agmatine which effectively restarts the cycle. Notably, this system is thought to be induced under low pH and contributes to the ATR of *S. mutans* by providing energy in the form of ATP and buffering capacity through ammonia production (Griswold *et al.*, 2006). Research conducted by Griswold *et al.* examining the particular role of this system, has demonstrated the induction of AgD under low pH and heat, which suggests the involvement of this pathway in response to certain environmental stresses (Griswold *et al.*, 2006). Further studies are required to concretely implicate the agmitine deiminase system as an acid tolerance mechanism of *S. mutans*.

The ability of *S. mutans* to alter its metabolic activity in response to external pH fluctuations reveals another acid tolerance mechanism that promotes its selection over competing species in an acidic environment. This ability, however, is not restricted to the metabolic pathways described above. Recent studies from our lab have demonstrated the involvement of secondary metabolism in the acid tolerance phenomena of this cariogenic pathogen and this topic will be the focus of the next section.

iii) Secondary Metabolism

Citric acid is ubiquitous in nature and is present in fruit juices and teeth, which are comprised of 0.3% citric acid by weight (Aranha *et al.* 2005; Pierre and Gautier-Luneau, 2000). In lactic acid bacteria, citrate uptake via the citrate/lactate antiport system (CitP), leads to increased acidurance through the generation of a proton motive force (Garcia-Quintans *et al.*, 1998). Although *S. mutans* is unable to survive when citrate is the only carbon source, it has evolved a citrate lyase pathway similar to the oxaloacetate decarboxylase pathway of *Klebsiella pneumoniae* (Dimroth *et al.*, 2001; Korithoski *et al.*, 2005). In this pathway, citrate uptake can contribute to acid tolerance via the conversion of citrate to oxaloacetate, which is then converted to pyruvate by oxaloacetate decarboxylase; a process that consumes an intracellular proton (Dimroth *et al.*, 2001). Genomic analysis of the *S. mutans* strain UA159 chromosome suggests that multiple genes are involved in citrate transport (*citM*) and metabolism (citrate lyase: *citCDEFGX* and oxaloacetate decarboxylase: *bbc*, *oad* and *pycB*) (Ajdic *et al.*, 2002; Korithoski *et al.*, 2005). When citrate is present in the growth medium at neutral and acidic pH, *S. mutans* exhibits increased acid tolerance (Korithoski *et al.*, 2005). Although the precise

relationship between the observed phenotype and citrate transport remains unclear, it has been suggested that *S. mutans* may exploit citrate uptake to acquire ferric iron into the cell as ferric citrate, as it is the preferred substrate of the system (Korithoski *et al.*, 2005). Further studies regarding this phenomenon are warranted to conclusively determine the involvement of citrate transport in acid tolerance.

The previously described metabolic proteomic analysis by Len *et al.*(2004) identified lactoylglutathione lyase (LGL) as one of the upregulated genes in *S. mutans* under pH 5.0, suggesting its involvement in acid tolerance. LGL is essential for cell survival, due to its detoxification of methyglyoxal, a glycolytic by-product that inactivates cytoplasmic macromolecules such as proteins and nucleic acids (Korithoski *et al.*, 2007). Methylglyoxal is formed via enzymatic production from the fragmentation of triose-phosphates during glycolysis (Cooper, 1984), and can accumulate under conditions of uncontrolled carbohydrate metabolism (Freedberg *et al.*, 1971). In general, LGL activity contributes to the acid tolerance of an organism via detoxification of increased methylglyoxal concentrations that result from highly glycolytically active cells (Freedberg *et al.*, 1971). In *S. mutans*, quantitative real-time PCR was used to examine the expression of LGL under acidic growth and acid adaptation, revealing increased expression in response to low pH in both parameters tested (Korithoski *et al.*, 2007). These findings were further supported by delayed generation times of the LGL-deficient mutant compared to the wildtype UA159 strain under low pH and decreased survival rates at pH 3.0 (Korithoski *et al.*, 2007). These studies confirmed the ability of LGL in *S. mutans* to detoxify methylglyoxal and support its role in acid tolerance mechanisms.

The aforementioned fate of pyruvate via homofermentation (lactic acid production) and heterofermentation (via the pyruvate-formate lyase pathway) is dictated by the extracellular concentration of glucose (Colby and Russell, 1997). Alternatively, pyruvate catabolism can occur via a third heterofermentive pathway designated the pyruvate dehydrogenase complex (PDH) pathway (Carlsson *et al.*, 1985). This pathway oxidizes pyruvate into CO_2 and acetyl-CoA, which can be further broken down into acetate or ethanol via acetate kinase or alcohol dehydrogenase, respectively. In *S. mutans*, the E1α subunit of the PDH complex is encoded by the gene *pdh*A and has been shown to be strongly upregulated in response to acid stress and acid-adaptation (Korithoski *et al.*, 2008). Moreover, loss of *pdhA* via mutagenesis, demonstrated an acid sensitive phenotype, and expression of this protein resembled metabolic activity consistent with heterofermentive growth; i.e decreased activity when glucose was in excess (Korithoski *et al.*, 2008). These observations link *pdhA* to the acid tolerant phenotype and add to the repertoire of mechanisms employed by *S. mutans* in response to acid challenges.

iv) Cell Density, Regulatory Systems and Biofilm Formation

Cell density has been shown to modulate the ability of *S .mutans* to adapt to acid challenge and its effect is dictated by whether the samples have been derived from log-phase grown planktonic or biofilm cultures (Cotter and Hill, 2003). Preadapted biofilm cells at low cell density exhibited decreased resistance to the killing pH (3.0) when compared to cells extracted at high cell density (Li *et al.*, 2001a). In *S. mutans*, cell density can be monitored via a quorum sensing system encoded by the *comCDE* operon, which is similar to the Com system in *Streptococcus pneumoniae* (Li *et al.*, 2001b). This

system is involved in competence development; a process that introduces novel genes to the chromosome through the acquisition and integration of exogenous DNA (Li *et al.*, 2001b). In addition to this role in genetic competence, the *comCDE* operon has also been directly linked to biofilm formation in *S. mutans* (Li *et al.*, 2002a). Mutants deficient in any of the genes comprising the *comCDE* operon demonstrated an abnormal biofilm, further demonstrating the critical role that this system plays on this process.

Briefly, *comC* encodes a competence-stimulating peptide (CSP), while *comD* and *comE* encode a histidine kinase and response regulator, respectively (Li *et al.*, 2001b). The histidine kinase and response regulator comprise a two component signal transduction system (TCSTS) that detects the external CSP. Also relevant to the *comC* system is the *comAB* operon which encodes an ABC-type transporter responsible for the expulsion of CSP out of the cell (Petersen and Scheie, 2000). Once a density dependent threshold has been achieved, CSP binds to ComD and results in the autophosphorylation of ComE. This leads to the transcription and ultimate expression of competence genes and consequent regulation of physiological activities (Li *et al.*, 2002a). In addition to being deficient in genetic competence, strains deficient in the *comC, -D* or *-E* genes exhibited a diminished log-phase ATR (Li *et al.*, 2001a). Notably, addition of exogenous CSP to the *comC* mutant restored the reduced ATR and demonstrated the importance of cell-density and cell-cell communication in acid adaptive mechanisms (Li *et al.*, 2001a). Moreover, the genome of *S. mutans* contains multiple TCSTS, including 14 histidine kinases and 15 response regulators (Ajdic *et al.*, 2002; Kawada-Matsuo *et al.*, 2009), some of which have already been characterized.

In attempt to understand the physiological role of these two-component systems and the genes they regulate, knockout mutations of the various response regulators and histidine kinases have been constructed and the mutants' phenotypes studied. In particular, the *hk11/rr11* TCSTS in *S. mutans* NG8 was shown to be important in stress response mechanisms and deletion of the *hk11* gene, resulted in a mutant with increased sensitivity to low pH (Li *et al.*, 2002b). The same effect was not observed in the *rr11*-deficient strain, suggesting that the histidine kinase may function as a pH sensor with the ability to interact with response regulators from different systems (Li *et al.*, 2002b). Other deletion mutants in regulatory genes demonstrating acid sensitivity and links to the ATR of *S. mutans* include GcR; a response regulator of *ffh* and *atpA/E* (genes that encode subunits of the F_1-F_0-ATPase) (Dunning *et al.*, 2008) and BrpA; a transcriptional regulator also involved in biofilm formation, autolysis and cell division (Wen *et al.*, 2006). Recent studies conducted by Kawada-Matsuo *et al.* (2009) analyzed a mutant library of histidine kinase and response regulator deficient strains and their respective double-mutants for their ability to grow under neutral and acidic conditions. In this study, the LytR, CiaH, ComC and an uncharacterized putative TCSTS had decreased doubling times and a diminished ATR (Kawada-Matsuo *et al.*, 2009).

Experimentation conducted by Li *et al.* (2001) established the differences in acid resistance expressed by *S. mutans* biofilm cells compared to planktonic cells. Further research in this area has shown that biofilm cells, specifically those that have been newly adhered to a surface, have increased acid resistance compared to their planktonic counterparts (Welin-Neilands and Svensäter, 2007). Similar results were obtained for the ATR of biofilm cells grown in a chemostat for 2 and 5 days expressing significantly

higher resistances to a killing pH of 3.5 than planktonic cells (McNeill and Hamilton, 2003). Additionally, fasting biofilm cells were more resistant to acid shock by lactic acid at pH 3.8 compared to planktonic cells and both cultures exhibited increased acid resistance when they were starved compared to sugar metabolising cells (Zhu *et al.*, 2001). Protein analysis of planktonic and biofilm cells of *S. mutans* grown under neutral conditions, via extraction and purification by two-dimensional (2D) polyacrylamide electorphoresis (2DE) and mass spectrometry identified 57 proteins that were upregulated 1.3-fold in biofilm cultures compared to planktonic cells (Svensäter *et al.*, 2001). Many of these proteins had unknown function, but the results confirm the existence of a biofilm phenotype that results in the expression of certain genes not induced in the planktonic phase of existence (Svensäter, 2001).

v) Intracellular pH Homeostasis

Discussion of the acid tolerance capabilities of *S. mutans* would be incomplete without commentary on the ability of this pathogen to maintain an intracellular pH that is more alkaline that it's surrounding medium (Booth, 1985a; Dashper and Reynolds, 1992). This is accomplished by *S. mutans* through the prevention of proton influx by altering its membrane composition and by increasing proton extrusion via end-product efflux and F_1-F_0-ATPase activity. These mechanisms allow *S. mutans* to cope with the constantly changing pH levels in plaque and reduce the denaturing effects of an acidic cytoplasm as previously described. Furthermore, these functions allow sustained growth and permit the proper functioning of enzymes and other cellular processes that would otherwise be inhibited by an acidic intracellular compartment (Kashket, 1987).

Generally, studies have shown that acid tolerance exhibited by oral lactic acid bacteria corresponds to their relative permeabilties to protons (Cotter and Hill, 2003). Studies involving increased proton permeability in *S. mutans* via the antibiotic gramicidin, affected glycolytic activity and cell viability at pH 5.5, demonstrating the effect of a compromised membrane and an acidified cytoplasm (Thibodeau and Marquis, 1983). In order to prevent the passive inflow of H^+ ions, *S. mutans* can alter the fatty acid composition of its membrane as an acid-adaptive mechanism (Quivey *et al.*, 2000). More specifically, increased levels of mono-unsaturated and longer chain fatty acids ($C_{18:1}$ and $C_{20:1}$) were observed in membranes from cells grown at pH 5, when compared to cells grown at pH 7 (Quivey *et al.*, 2000). Furthermore, the unsaturated to saturated fatty acid ratios were 4 times higher in pH 5 cells when compared to pH 7 cells, indicating the importance of these ratios in acid tolerance, which was supported by the lack of such a response in the less aciduric *S. sobrinus* (Quivey *et al.*, 2000). Fozo *et al.*(2004) demonstrated that *fabM* is the sole gene responsible for the production of monounsaturated fatty acids in *S. mutans* and deletion of this gene resulted in a mutant that exhibited distinct acid-sensitive attributes when compared to the wildtype strain. These differences included reduced acidogenicity (1.5 log units less), sensitivity to extreme acid (3.5 log units more sensitive) and an inability to maintain a transmembrane ΔpH that was equal to the parent (reduced by half) (Fozo *et al.*, 2004). These observations encouraged this group to assess the ability of the *fabM*-deficient strain to cause caries in a rodent model, and determined the cariogenicity and transmission efficacy from host to host *in vivo* (Fozo *et al.*, 2007). The *fabM* mutant strain was poorly transmissible compared to the wild-type and produced fewer and less severe cavitations

in animals that were directly infected, linking the virulence of this pathogen to a single gene product in this example (Fozo *et al.*, 2007).

In terms of acid tolerance, it has been proposed that membrane composition can affect proton permeability either directly or indirectly (Quivey *et al.*, 2000). Direct effects involve the base permeability of the lipid bilayer to H^+ ions (Quivey *et al.*, 2000), whereas indirect effects have been described in studies that demonstrated that changes in membrane lipid composition affect the optimal activity of F_1-F_0-ATPase proton pumps (Sturr and Marquis, 1992). The relationship of these ATPases to pH homeostasis will be described shortly.

From a genetic standpoint, disruptions in the genes responsible for the biogenesis, assembly and maintenance of the cell membrane, resulted in acid sensitive phenotypes compared to parent strains of *S. mutans*, further implicating the importance of membrane architecture and composition to acid adaptation (Cotter and Hill, 2003). These acid sensitive phenotypes were seen with: 1) the inactivation of *dltC,* which encodes Dcp (a $_D$-alanyl carrier protein involved in the synthesis of $_D$-alanyl-lipoteichoic acid)(Boyd *et al.*, 2000); 2) deletion of Ffh (a 54 kDa subunit homologue of the signal recognition particle of *E. coli* involved in membrane biogenesis) (Gutierrez *et al.*, 1999; Kremer, 2001); 3) a deficiency of Dgk (a diacylglycerol kinase linked to phosolipid metabolism (Shibata *et al.* 2009).

More specifically, Shibata *et al.* (2009) have recently implicated the importance of Dgk in the virulence of *S. mutans*, showing that the degree of C-terminal deletion of this protein is proportional to the acid tolerance properties of the mutants. Furthermore, when three or more amino acid residue deletions were made, acidurance was diminished

in an increasing fashion up until the eighth deletion, at which point no growth was observed at pH 5.5 (Shibata *et al.*, 2009). Dgk generates phosphatidic acid and catalyses the ATP-dependent phosphorylation of *sn*-1,2-diaylglycerols, both of which are second messengers in eukaryotic cellular signal transduction pathways (Shibata *et al.*, 2009). Infection of gnotobiotic rats with the *dg*k-deficient strain in *S. mutans* resulted in reduced levels of smooth surface caries compared to the wildtype. These results, in conjunction with similar findings with the *fabM*-deficient strain (previously described), demonstrate the importance of maintaining membrane integrity in *S. mutan's* ability to cause carious lesions *in* vivo.

As sugar levels in the oral cavity increase due to ingestion by the host, fermentation products change from mixed acid-end products to lactic acid (Yamada and Carlsson, 1975). Thus, continued carboyhydrate metabolism and fermentation through lactate dehydrogenase causes accumulation of this end-product within the cytoplasm (Dashper and Reynolds, 2000). Studies have demonstrated that lactate build-up adversely affects the cells ability to maintain an elevated intracellular pH relative to the extracellular environment, resulting in the inhibition of glycolysis at pH 5.0 (Dashper and Reynolds, 2000). In order to prevent this inhibitory effect, *S. mutans* can excrete lactate in an electroneutral process in the form of lactic acid, which dissociates in aqueous solution (pKa value of 3.86) and significantly contributes to the acidification of the external environment (Dashper and Reynolds, 1996; Dashper and Reynolds, 2000). This event is independent of metabolic energy and suggests the presence of a membrane carrier specific for lactic acid extrusion (Dashper and Reynolds, 1996). Therefore, *S. mutans* possesses the ability to remove potentially harmful metabolic end products

concomitantly with H^+ ions (Dashper and Reynolds, 1996), contributing to pH homeostasis and providing yet another advantage over competing species under acid conditions.

Central to the ability of *S. mutans* to maintain pH homeostasis is a constitutive F_1-F_0-ATPase proton pumps that become further induced at low pH (Kuhnert *et al.*, 2004). Induction of F_1-F_0-ATPases under acidic environments and consequent expulsion of protons from the cell helps maintain an elevated cytplasmic pH in relation to its surroundings (Bender *et al.*, 1986; Kuhnert *et al.*, 2004). The activity of these enzymes is paramount to acid tolerance in a variety of species, so much so that the pH optimum of the F_1-F_0-ATPases has been directly linked to an organism's ability to survive in acidic conditions (Bender *et al.*, 1986; Sturr and Marquis, 1992).

In addition to the F_1-F_0-ATPases, recent studies have shown that other enzymatic mechanisms can help maintain a neutral cytosolic pH during growth (Magalhaes *et al.*, 2003). Specifically, these results identified a 100kDa membrane protein that could maintain an intracellular pH that was one pH unit above that of the external media despite inhibition of the F_1-F_0-ATPases, with the uncoupler DCCD (dicyclohexylcarbodiimide). This group postulated that this protein functions as a P-type ATPase due to its sensitivity to orthovanadate and lanzoprazole, with H^+-ATPase or H^+, ion ATPase activity (Magalhaes *et al.*, 2003). Furthermore, these studies demonstrated that *S. mutans* is not solely dependent on the activity of the F_1-F_0-ATPases for intracellular pH regulation. Sequence analysis of the *S. mutans* UA159 genome supports these findings, identifying multiple diverse transport mechanisms with putative function for inorganic ions and other essential nutrient transport (Ajdic *et al.*, 2002). Further studies in these areas may reveal

H^+ antiport activity, which would further enhance the acid response capabilities of *S. mutans*.

A major consequence of proton efflux is the establishment of a proton motive force (PMF) across the plasma membrane, which can be defined as the sum of the pH difference (ΔpH) and the transmembrane electrical potential (Δψ , internally negative) (Bakker and Mangerich, 1981; Kashket, 1987). The PMF acts a major driving force that ultimately synthesizes ATP and facilitates transport of other solutes into the cell depending on their extracellular availability (Hamilton, 1990). As such, the PMF plays an important role in dictating the intracellular conditions of the cell and it may seem intuitive to assume that this force can determine the degree to which energy production can successfully occur in acidic conditions. However, studies have shown that this is not always the case and the observed PMF under low pH values (5.0) is insufficient for ATP production in *S. mutans* (Dashper and Reynolds, 1992; Hamilton, 1990). This implies that the PMF established from the extrusion of protons from the cytoplasm is primarily used to raise the internal pH rather than to manufacture energy (Dashper and Reynolds, 1992).

The connection between the PMF and its involvement in the acid tolerance strategies of bacteria can be seen more clearly when observing the relationship that exists between the PMF and cation uptake. More specifically, regulation of the PMF can be manipulated accordingly by the fine balancing of its components Δψ and ΔpH (Booth, 1985; Meyer-Rosberg *et al.*, 1996). Accumulating evidence has demonstrated that bacteria can adjust the PMF to their advantage under low pH conditions through the interconversion of Δψ via the uptake of cations, specifically potassium (Bakker and Mangerish, 1981; Booth, 1985; Cotter and Hill, 2003; Kashket, 1987).

1.9: pH Regulation via Potassium Uptake

Potassium is the major cation in the bacterial cytoplasm and is an essential element involved with many physiological mechanisms that dictate the proper functioning and survival of the cell (Booth, 1985). As such, the maintenance of intracellular concentrations and consequent uptake of potassium is paramount to cell viability. K^+ functions as a primary mechanism in combating dehydration (Kuo, 2005) and also serves regulatory roles in transcription, translation and protein activation by acting as a secondary messenger (Epstein, 2003). In addition to these activities, K^+ has also been linked to a variety of virulence factors, including biofilm formation via potassium leakage in *Bacillus subtilis* (Lopez *et al.*, 2009) and regulation of cytoplasmic pH in *Enterococcus faecalis* (Booth, 1985; Kashket, 1987; Kobayashi *et al.*, 1982) and *Corynebacterium glutamicum* during growth in acidic environments (Follmann *et al.*, 2009). This form of regulation has been observed in a variety of different microorganisms including *Rhodobacter sphaeroides* (Abee *et al.*, 1988), *Bradyrhizobium* sp. (Gober and Kashket, 1985), *Enterococcus faecalis* (Bakker and Mangerich, 1981; Kobayashi *et al.*, 1982) and *Helicobacter pylori* (Meyer-Rosberg, 1996), all of which have proposed similar mechanisms of action. Of the bacterial species studied, *Enterococcus faecalis* and *Escherichia coli* have been researched extensively and have provided much insight into the current understanding of the affect that potassium has on the PMF (Bakker and Mangerish, 1981; Kobayashi *et al.*, 1982).

Under relatively neutral pH, the initial generation of the pH gradient can be attributed to the constitutive proton pumps (i.e. F_1-F_0-ATPase) that eject H^+ outside of the cell, thereby acidifying the external environment and further developing a transmembrane

potential that is inside negative (Booth, 1985). When the cell is introduced to an acidic environment, the pH is further decreased, effectively increasing the magnitude of ΔpH which would promote the re-entry of H^+ back into the cell (Booth, 1985). In order to prevent this influx of protons from occurring, bacteria can compensate by increasing their uptake of potassium (Booth, 1985). The uptake of potassium results in the depolarization of the membrane, and if potassium uptake continues in this manner, dissipation of $\Delta\psi$ occurs and results in the transition from a membrane potential that was initially negative to one that is now positive (Booth, 1985; Kashket, 1987). Essentially, the uptake of potassium at low pH serves to invert and maintain a positive membrane potential inside the cell and ultimately functions to prevent the influx of protons and thus contributes to the acid tolerance capabilities of the organism (Booth, 1985).

One of the fundamental questions that continues to persist however, is the mechanism of energy coupling that occurs with potassium uptake (Booth, 1985; Rhoads *et al.*, 1977, Schlosser *et al.* 1993). Some researchers have suggested K^+/H^+ antiport activity (Booth, 1985), while more recent studies have suggested the role of NADH, a coenzyme that may interact with specific potassium uptake systems whose oxidation may provide the necessary energy requirements to facilitate potassium transport (Schlosser *et al.*, 1993).

i) Potassium Uptake in *Escherichia coli*

The aforementioned role of potassium and its affect on the PMF is an area that has not yet been unequivocally linked to the acid tolerance response (ATR) of *S. mutans*. However, it is reasonable to suggest that potassium may be intimately involved in the

ATR of *S. mutans* because of the effect uptake of this cation has on intracellular pH regulation, described later (refer to next section).

Due to the importance of potassium in cellular processes, it is generally accepted that microorganisms have multiple potassium transport systems, consisting of both ion channels and transporters (Kuo, 2005). In the Gram negative bacterium *E. coli*, potassium transport has been extensively studied (Rhoads *et al.*, 1976) and over the years, multiple potassium systems in this microorganism have been characterized consisting primarily of the Trk, Kdp and Kup systems (Dosch *et al.*, 1991). Each system is expressed under unique environmental conditions and varies in its requirements for a direct energy source (in the form of ATP), and/or the presence of a proton motive force (PMF) (Dosch *et al.*, 1991).

In *E. coli*, Trk is the major constitutive K^+ transport system and is composed of four different Trk gene products encoded by the genes *trkA*, *trkE*, *trkG* and *trkH* (Bossemeyer *et al.*, 1989; Dosch *et al.*, 1991; Epstein and Kim, 1971) Notably, the genes encoding these respective Trk proteins are located on separate regions of the chromosome and are not transcribed contiguously (Schlosser *et al.*, 1993; Schlosser *et al.*, 1995). The *trkA* protein in particular is an essential peripheral membrane component for the systems proper function (Bossemeyer *et al.*, 1989). Furthermore, it contains a KTN (K^+ transport and NAD-binding) domain that binds NAD^+ which has been postulated to be engaged in energy coupling with potassium uptake (Booth *et al.*, 2005; Schlosser *et al.*, 1993) although studies have confirmed that Trk requires both ATP and the establishment of a PMF for transport. With concern to TrkH, this gene product is a membrane associated protein that works in conjunction with TrkA and has been shown to be involved in K^+

transport (Schlosser *et al.*, 1995). In summary, the Trk system is a low affinity, high capacity potassium uptake system that helps maintain cell turgor (Schlosser *et al.*, 1993).

When extracellular potassium concentrations are low, or the turgor pressure of the cell lowers, a high affinity Kdp system is expressed and activity of the aforementioned Trk system is reduced significantly (Ballal *et al.*, 2007; Gowrishankar, 1987; Roe *et al.*, 2000; Siebers and Altendorf, 1988).The Kdp system is comprised of a four gene operon consisting of *kdpFABC* and is regulated by a two-component signal transduction system encoded by *kdpD* and *kdpE* located 5' proximal (Ballal *et al.*, 2007). The *kdpD* and *kdpE* gene products function as a histidine kinase and a response regulator, respectively (Bramkamp et al, 2007; Siebers and Altendorf, 1988). The *kdpA, B* and *C* genes each encode different subunits that combine to form a P-type ATPase, which is specifically involved in the transport of metal cations (Bramkamp *et al.*, 2007; Greie and Altendorf, 2007). Similar to the Trk system, the Kdp system requires both ATP and PMF (Greie and Altendorf, 2007), although the direct relationship between the energy requirements for these transport systems remains unclear (Bramkamp *et al.*, 2007).

When *E. coli* cells are exposed to acidic environments, the constitutive Trk system is severely inhibited and consequently, the Kup system is expressed in order to compensate under hyper-osmotic stress at low pH (Trchounian and Kobayashi, 1999; Zakharyan and Trchounian, 2001). Potassium uptake systems such as these have been identified in a number of different species, although many organisms have developed specific adaptations that are particularly fitted for the specific environment they inhabit (Follmann *et al.*, 2009; Su *et al.*, 2009). This demonstrates not only the importance of

these transport systems in the survival strategies utilized by various organisms, but also the physiological relevance that potassium has as the major cation of the cytoplasm.

ii) Effect of Potassium on *S. mutans*

Research focussing on the effects of potassium on *S. mutans* is rather limited and therefore, information regarding the uptake of this cation is almost non-existent. Nonetheless, studies have demonstrated that at least 50 μg/ml of K^+ (as KCl) are required in the growth medium by multiple strains of *S. mutans* for adequate levels of growth (Cowman and Fitzgerald, 1976). In another study, it was shown that the morphology of the cell depends on the bicarbonate/K^+ ratio in the growth media in *S. mutans* strain NCTC 10449S (Tao *et al.*, 1987). More specifically, high bicarbonate/K^+ ratios resulted in coccoid cellular forms, whereas low ratios were associated with bacillary morphology (Tao *et al.*, 1987). Although the specific role of potassium in growth and morphology remains unclear, these findings revealed the importance of this cation as an essential component for propagation and suggest that its presence, absence and acquisition have drastic consequences on *S. mutans*.

Much of what is known regarding the relationship of potassium and its effects on *S. mutans* physiology can be attributed to studies focussing on acid production and glycolytic activity. In *Streptococcus mutans* Ingbritt, addition of potassium to washed cells resulted in faster rates of acid production as well as lower terminal pH values when growth was initiated at an initial pH of 7.0 (Marsh *et al.*, 1982). Similar results were also obtained prior to these findings, where *Streptococcus mutans* K-1 exhibited increased

acid production and potassium accumulation at pH 5.8 when KCl was added to the medium (Luoma, 1971).

Further investigation into this phenomenon by S. Daspher and E. Reynolds yielded similar results. Described in two separate publications, they demonstrated that glycolyzing cells of *S. mutans* Ingbritt rapidly take up potassium under neutral (pH 7.0) conditions (Daspher and Reynolds,1992) and cells were also able to increase their glycolytic activity with the addition of KCl under all the concentrations tested in an acidic (pH 5.0) environment (Daspher and Reynolds, 2000). More importantly, their work in 1992 showed that glycolysis was optimal only when the cell was able to maintain a transmembrane pH gradient that was more alkaline than the external environment under low pH (Daspher and Reynolds, 1992). Previous work by Sato *et al.* (1987) regarding this relationship demonstrated that the development of a large ΔpH was dependent on the concentration of potassium in the media. The results of their studies will be further described below. Since, intracellular pH was directly influenced by the concentration of potassium in the extracellular medium, Daspher and Reynolds postulated that pH homeostasis near neutrality could be established through the exchange of K^+ ions for H^+ ions. More specifically, the magnitude of the $\Delta\psi$ established via F_1-F_0-ATPase proton extrusion could be dissipated by the electrogenic uptake of potassium while still maintaining a large ΔpH across the cell membrane (Dashper and Reynolds, 1992). Notably, these observations were dependent on the existence of a proton motive force and the presence of ATP (Dashper and Reynolds, 1992). These critical observations provide the bulk of knowledge concerning the impact of K^+ uptake on intracellular pH regulation in *S. mutans*.

Despite the importance of potassium to *S. mutans* as an essential component of growth and optimal glycolytic activity, little research has been conducted on the precise mechanisms involved with K^+ transport and acquisition. Studies by Noji and Sato *et al.* (1987, 1988, 1989) provide some insight into this field with concern to their work involving glutamate transport as a means of accumulating external K^+. In addition to demonstrating that supplementation of K^+ to the external environment could effectively raise the intracellular pH (Sato *et al.*, 1987; Noji *et al.*, 1988), they also showed that potassium stimulated glutamate transport in a concentration-dependent manner throughout an entire pH range from 5.5 to 8.5 (Sato *et al.*, 1989). From these results, they proposed a dual mechanism for the stimulation of glutamate transport by potassium ions, the first being regulation of the cytoplasmic pH (Sato *et al.*, 1989). The second mechanism is concerned with cancellation of the membrane potential that would be established via uptake of the anionic form of glutamate. This group suggested that the potassium cation may be co-transported with the glutamate anion in order to maintain electroneutrality and as a means of acquiring this essential element (Sato *et al.* 1989).

To date, the particular system(s) involved in potassium uptake in *S. mutans* have yet to be determined. That said, investigation into this area can provide further insight into the diverse mechanisms of acid tolerance employed by this dental pathogen in order to thrive in the dynamic and often hostile environment of the oral cavity. More specifically, our group has focussed our efforts on potassium uptake and its particular involvement in the aciduric characteristics of *S. mutans.*

1.10: Hypothesis

In *S. mutans*, studies have shown that potassium is required for adequate levels of growth (Cowman and Fitsgerald, 1976) and its presence in the external medium can directly influence glycolytic activity (Marsh *et al.*, 1982) as well as cytoplasmic pH (Daspher and Reynolds, 1992). As such, genes encoding proteins similar to those of the *E. coli* Trk system were identified by a recent microarray study conducted by our lab, which examined the global gene expression of *S. mutans* UA159 under neutral (pH 7.5) and acidic pH (pH 5.5). Analysis of the *S. mutans* UA159 genome revealed four different gene products, which were identified as putative Trk proteins and designated *trkB*, *trk*, *trkA* and *trkH* as indicated by the NCBI database (http://www.ncbi.nlm.nih.gov/). Thus far, the presence of a Trk system has not yet been verified or characterized in *S. mutans* and remains a novel area of research that can shed new light on potassium transport and its specific contribution to the acid tolerance of this organism. Since research obtained from various bacterial species, including *Rhodobacter sphaeroides* (Abee *et al.*, 1988), *Bradyrhizobium* sp (Gober and Kashket, 1986), *Enterococcus faecalis* (Bakker and Mangerich, 1981; Kobayashi *et al.*, 1982) and *Helicobacter pylori* (Meyer-Rosberg *et al.*, 1996) has suggested a direct link between potassium uptake and the regulation of intracellular pH, we hypothesize the following:

The *S. mutans* Trk proteins comprise a potassium transport system that contributes to its acid tolerance.

Objective:

To construct Trk-deficient mutants in each of the putative Trk genes identified in *S. mutans* UA159 and investigate their role in potassium transport, aciduricity and other phenotypes that are conducive to its pathogenicity.

CHAPTER 2: MATERIALS AND METHODS

2.1: Bioinformatic Analyses

Bioinformatics tools were used to identify homologues, conserved domains and potential transmembrane helices for the putative genes designated *trkB*, *trk, pacL, trkA* and *trkH*. The FASTA protein sequence of each gene was analyzed and the results are presented in Tables 3.1, 3.2 and 3.3 (CHAPTER 3: RESULTS). More specifically, homologues were identified using the BLASTp (http://www.ncbi. nlm.nih.gov/) search tool and proteins with the highest identities to the designated "Trk" proteins of *S. mutans* were reported. In addition, proteins with significant homology ($\geq$30% identity) to *trkB*, *trk, pacL, trkA* and *trkH* from known acid tolerant microorganisms were included (refer to Table 3.1). Furthermore, a protein BLAST search using known potassium transporters and channels (i.e. Trk, Kdp and Kch of *E .coli* and KcsA of *Streptomyces lividans*) as the query sequence against the genome of *S. mutans* UA159 was also conducted.

In order to identify the conserved domains harboured by the *trkB*, *trk, pacL, trkA* and *trkH* genes, the SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam. .ac.uk/) protein databases were consulted. Possible protein interactions were suggested by the STRING (http://string.embl.de/) database which predicts both direct (physical) and indirect (functional) associations based on experimentation, genomic context, coexpression and published literature.

Lastly, membrane topology of the genes of interest was determined using the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), HMMTOP (http://www.enzim. hu/hmmtop/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) software programs. These systems identify predicted transmembrane domains and

describe which portions of the amino-acid sequence exist in the cytoplasm and which segments of the protein lie on the exterior of the cell.

2.2: Quantitative rtPCR Analysis of Acid Induced Gene Expression

To study the expression of *trkB*, *trk*, *pacL*, *trkA* and *trkH* under acid stress and to confirm the initial microarray results, quantitative real-time PCR (qrt-PCR) was conducted using cDNAs derived from UA159 strains. Briefly, cells from overnight cultures (described below) were collected via centrifugation and resuspended in TYEG (Tryptone Yeast Extract + 0.5% Glucose (wt/v)) at pH 7.5 and grown to mid-log phase (OD_{600nm} of ~ 0.3-0.4). Cell suspensions were then divided into two equal aliquots; one culture was resuspended in TYEG at pH 7.5 (control) and the other in TYEG at pH 5.5 and then pelleted via centrifugation. Both cell cultures were incubated at 37°C with 5% CO_2 for 1 h, collected via centrifugation, snap frozen in liquid nitrogen and stored at -80°C until further use. When ready, Trizol reagent (Invitrogen, Carlsbad, CA) was added to the frozen pellets and total RNA was isolated using the FastPrep system (Bio 101 Savant, Holbrook, NY) according to the supplier's instructions. Following DNase treatment, mRNAs were reverse transcribed using a First-Strand cDNA (complimentary DNA) Synthesis kit (MBI Fermentas) as detailed in Senadheera *et al* (2005). Real-time PCR (rt-PCR) was conducted using cDNAs, a Quantitect SYBR-Green PCR kit (QIAGEN) and an Mx3000p Thermal cycler (Stratagene, La Jolla, CA) as previously described (Senadheera *et al.*, 2007).

Primer efficiencies were determined by using known concentrations of genomic DNA (50, 25, 5, 0.5 and 0.05 ng/µl) which was used to generate Ct values for specific primer sets (specific sequences are outlined in Table 2. "Real-time Primers"). Negative controls containing no reverse transcriptase were used to verify that no genomic contaminations were present in the cDNA synthesis reactions. Gene expression of the *trkB*, *trk*, *pacL*, *trkA* and *trkH* genes were normalized to the expression of 16S rRNA that showed invariable expression under the test conditions applied (data not shown). Expression analysis was conducted for at least 3 biological replicates, each derived from 3 technical replicates. Fold-expression was calculated using the following equation according to Pfaffl *et al.* (2002):

$$\text{Fold expression} = \frac{(\text{Efficiency of test primer})^{(Ct_A - Ct_B)}}{(\text{Efficiency of 16S primer})^{(Ct_A - Ct_B)}}$$

Where A = condition 1 (pH 7.5) and B = condition 2 (pH 5.5)

2.3: Bacterial strains and growth conditions

The genome of *Streptococcus mutans* strain UA159 (Wt: Erm^s) is publicly available (http://www.genome.ou.edu/smutans.html) and was therefore chosen as the parental strain for the construction of the deletion mutants outlined in Table 2.1. Access to the genome allowed for bioinformatic analysis to be conducted and primer design to be achieved. The strains were subcultured and maintained routinely on Todd Hewitt broth plates supplemented with 0.3% yeast extract (THYE) (Becton, Dickinson and Company, Le Pont de Claix, France) and 1.8% agar. All *S. mutans* UA159 strains were grown as

standing cultures at 37°C, with 5% CO_2 (v/v air mixture). Isogenic mutants (refer to Table 2.1 for relevant characterisitics) were grown in THYE broth or plates, both containing 10μg/ml erythromycin (Erm; Sigma-Aldrich, St. Louis, MO). Erm was added to the mutant cultures when needed in order to maintain a selective pressure and avoid loss of the Erm marker.

***S. mutans* strain**	**Relevant Characteristics**	**Source of reference**
UA159	Wild type, Erm^s	J. Ferretti, University of Oklahoma
SMUTRKB	UA159 *Smu.1561::ermAM*, Erm^r	This study
SMUTRK	UA159 *Smu.1562::ermAM*, Erm^r	This study
SMUPACL	UA159 *Smu.1563c::ermAM*, Erm^r	This study
SMUTRKA	UA159 *Smu.1708::ermAM*, Erm^r	This study
SMUTRKH	UA159 *Smu.1709::ermAM*, Erm^r	This study
SMUTKB	UA159 *Smu.1561-1562::ermAM*, Erm^r	This study
SMUTOP	UA159 *Smu.1561-1563::ermAM*, Erm^r	This study

Table 2.1. Bacterial strains used in this study. Erm^r indicates Erm resistant strains and Erm^s indicates Erm susceptible strains.

2.4: Construction of *S. mutans trkB-, trk-, pacL-, trkA-* and *trkH-* Deficient Mutants

A rapid PCR-based allelic replacement strategy (Figure 2.1.) was used to construct a mutant deficient in the *trkB, trk, pacL, trkA* and *trkH* coding regions utilizing *S. mutans* UA159 as previously described (Lau *et al.*, 2002). Briefly, PCR primers were designed using MacVector software (Carey, NC) and were used to amplify DNA fragments located on the 5' and 3' flanking regions of the genes of interest. All primers are listed in Table 2.2. PCR products were then ligated to an erythromycin resistance cassette (Nikawa and Kawabata, 1998) overnight at room temperature. Ligation constructs were then introduced into *S. mutans* UA159 via CSP-induced transformation

(CSP obtained from the Hospital for Sick Children, Toronto), which occurred at 37°C, with 5% CO_2 for three hours. Transformants harbouring the erythromycin antibiotic cassette were selected for sequence verification. Colony PCR was used to confirm successful mutagenesis of the *trkB*, *trk*, *pacL*, *trkA* and *trkH* loci using a combination of primers as described in Lau *et al.*, (2002) (Table 2). PCR products that correlated with the expected bands sizes were further verified using nucleotide sequence analysis, courtesy of ACGT Corporation (Toronto, ON). Following confirmation, the resulting mutants were designated SMUTRKB, SMUTRK, SMUPACL, SMUTRKA, SMUTRKH, SMUTKB and SMUTOP and used for further experimentation. Construction of the SMUTKB and SMUTOP mutants in particular, was achieved by using a particular combination of the designed primers used for the individual deletions. For example, SMUTOP used Δ*pacL* -P1 and P2 to amplify the 5' flanking region of the Trk operon and Δ*trkB* -P3 and P4 to amplify the 3' flanking region. Similarly, SMUTKB used Δ*trk*-P1 and P2 and Δ*trkB* -P3 and P4 primer pairs to amplify the upstream and downstream flanks, respectively.

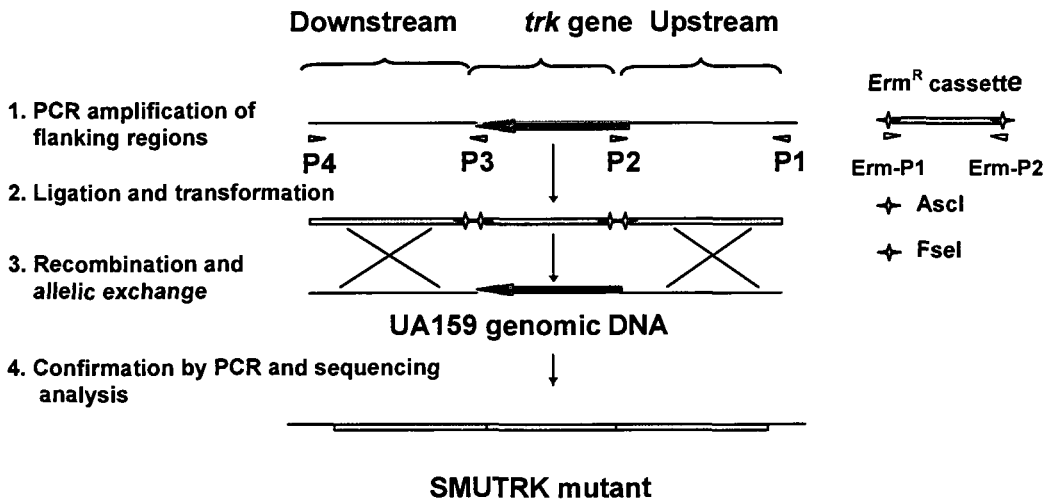

Figure 2.1. Schematic diagram of PCR ligation mutagenesis used for creating deletion mutants. (Modified from Lau *et al.* 2002).

Primer	Nucleotide sequence (5'→3')[a]	Annealing Temp. (°C)	Amplicon (bp)
Mutagenesis Primers			
Erm-P1	**GGCGCGCC**CCGGGCCCAAAATTTGTTTGAT	54	
Erm-P2	GGCCGGCCAGTCGGCAGCGACTCATAGAAT		860
Δ*trkB* -P1	GAAGTTGTCTTTGCCCGTGTAG	54	
Δ*trkB* -P2	**GGCGCGCC**ACCTAAACGACCACATCCGAC		874
Δ*trkB* -P3	GGCCGGCCCGTTCATTCCTAATAGAG		
Δ*trkB* -P4	ACTGGGTAAGGCATCAGAG		820
Δ*trk* - P1	GCTGAAGATGGGATTATGGAACG	54	
Δ*trk* -P2	**GGCGCGCC**AGCCAAACCAGAACCTAAACGAC		987
Δ*trk* -P3	GGCCGGCCTTAGCTGCATCTTCTGATACGG		
Δ*trk* - P4	GGTTTAGCAACAGGTGCCTC		1018
Δ*pacL* - P1	GAGATTGCTGAACTTGTCG	54	
Δ*pacL* - P2	**GGCGCGCC**CCATTGAACTGGTTTGTG		762
Δ*pacL* - P3	GGCCGGCCTGGCTATTTGATTCTCTG		
Δ*pacL* - P4	ATAAGCAACGGCATTCGCTTG		1005
Δ*trkA* - P1	CTGGGAGTCCTTTATCTTCACGTC	52.5	
Δ*trkA* - P2	**GGCGCGCC**TTCTTCCACCAGTGAGCGACAGAG		904
Δ*trkA* - P3	GGCCGGCCCTGGTGATGATATTCTTGAAGTAGG		
Δ*trkA* - P4	GAGACCACTTTACCAAAAACAGG		622
Δ*trkH* - P1	GGTGATGCTGTCATTCAGTCAG	52.5	
Δ*trkH* - P2	**GGCGCGCC**AAAGCAACAATCAAGGGGAC		869
Δ*trkH* - P3	GGCCGGCCCATGTTGTTGCTATTTATTCC		
Δ*trkH* - P4	TGCCCGCTATCGTATTGTC		609
Real-time Primers			
Δ*trkB* - For.	ACAATCGCATTTTCATCGTC	46.9	113
Δ*trkB* - Rev.	GTGGACAGTCGTGTTAGTCAGG		
Δ*trk* - For.	AGGTCAGCAGGTTCTTGGTTCG	50	125
Δ*trk* - Rev.	CAAAGTTCCTAAGGTCATTGCCAG		
Δ*pacL* - For.	TGGAACAAAGTGCTGCTGCTC	50	110
Δ*pacL* – Rev.	AGGTCAAGGTTATGCTTCTGATGG		
Δ*trkA* - For.	GCAAGCGTATGGAAATGATTC	46.9	131
Δ*trkA* - Rev.	AGATGACGATTGGTAGTCTTGAG		
Δ*trkH* - For.	TACTGTGGGGTGTTGGTATGC	46.9	116
Δ*trkH* – Rev.	GAGCGAACTGTGATAATGAGCAATC		
Intergenic Primers			
1560-Δ*trkB* For.	CAAACTCAGCGTTGTGATTACCC	52	297
1560-Δ*trkB* Rev.	AAGGAGAGACTGTGTTATTGGAAGG		
Δ*trkB*-Δ*trk* For.	TGTTACTTGATGATGCGAATCC	52	335
Δ*trkB*-Δ*trk* Rev.	TTTGAGTCTGGGGAATGGAC		
Δ*trk*-Δ*pacL* For.	TGCTCAATACCTGCTTTCAC	52	349
Δ*trk*-Δ*pacL* Rev.	ATGGTCTCTTCAACACGGC		
Δ*pacL*-1564 For.	CCATTTGAACTGGTTTGTG	52	424
Δ*pacL*-1564 Rev.	TGGTTTCTTCTCATCAGACAG		

Table 2.2. Primers used in this study. [a] *Asc*I sites are in boldface; *Fse*I sites are underlined.

2.5: Co-transcription Assay

To investigate whether the *trk / trkB/ pacL* genes comprised a tricistronic operon, primers were designed (Table 2.2) within the intergenic regions of these genes (Figure 2.2). Next, cDNAs obtained at pH 7.5 and pH 5.5 in TYEG broth derived from *S. mutans* UA159 (described above) were amplified using these primers to determine the presence of translated segments of DNA via PCR. The PCR product and their respective controls were added to DNA-binding dye and run on a 1% agarose (wt./vl.) gel with ~ 0.14% ethidium bromide (EtBr). Gel electrophoresis was conducted and the corresponding band sizes of the products were compared to their expected sizes for verification.

In addition, MacVector software was used to screen for transcriptional stop sites in the form of hairpin loops in the intergenic region located between SMU.1560 (hypothetical protein) and *trkB* (refer to APPENDIX: A, Figure S2).

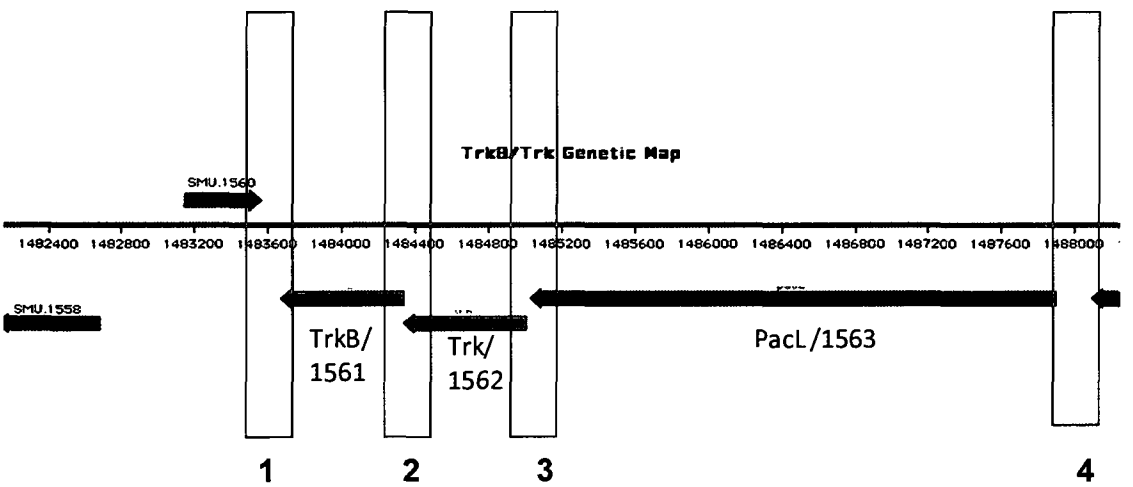

Figure 2.2. Diagram representing the intergenic primer design for the *trkB/trk/pacL* locus used in the co-transcription assay. Numbers correspond to the primer pairs used: 1) 1560-Δ*trkB* 2) Δ*trkB*-Δ*trk* 3) Δ*trk*-Δ*pacL* and 4)Δ*pacL*-1564

2.6: Growth Assays

Briefly, overnight cultures were diluted in fresh TYEG (pH 7.5) medium, incubated at 37 °C with 5% CO_2 and grown to mid-log phase growth (O.D. 600_{nm} 0.3-0.4). Consequently, 20µl of cell culture was added to 350µl of growth medium in a 96-well microtitre plate and placed in a Bioscreen C automated growth monitor (Bioscreen C Labsystems, Finland) for over 18h in an aerobic environment. Plates were incubated at 37 °C and O.D. 600_{nm} measurements were taken every 20 min after shaking for 30 seconds. Acid susceptibility of the *S. mutans* UA159 wildtype compared to SMUTRKB, SMUTRK, SMUPACL, SMUTRKA, SMUTRKH, SMUTKB and SMUTOP was tested in TYEG at pH 7.5 and pH 5.5 acidified by HCl. TYEG was chosen as the background medium because this medium was used to identify the genes of interest in our initial microarray study. In addition; 0.4M sodium chloride; $3.0x10^{-3}$% (wt/vol) sodium dodecyl sulfate; 2.5% (vol/vol) ethanol, 0.03% H_2O_2 (vol/vol) was also tested.

2.7: Terminal pH Assay

To further investigate the acid sensitivity of SMUTRKB, SMUTRK, SMUPACL, SMUTRKA, SMUTRKH, SMUTKB and SMUTOP, a terminal pH assay was conducted in TYEG at pH 7.5 and pH 5.5 acidified by HCl. Overnight cultures were pelleted and resuspended in TYEG pH 7.5 and grown to mid-log phase. Cultures were normalized to 1:40 dilutions and placed in an incubator at 37 °C with 5% CO_2 for 18 h without agitation, after which terminal pH values were documented. The pH of each culture was recorded using a pHC2001 electrode (Radiometer-Analytical, Lyon, France) which is sensitive to 3

decimal places and provides an accurate measure of pH values. Statistical analysis was conducted using a single factor ANOVA using Microsoft Excel Software.

2.8: Glycolytic Rate Assays

The effect of the deletions exhibited in the SMUTRKB, SMUTRK, and SMUTKB mutants on glycolytic activity were also determined. Overnight cultures were pelleted and resuspended in TYEG pH 7.5 and grown to mid-log phase at 37 °C with 5% CO_2. Cells were washed three times and resuspended in chilled 1% KCl, 1% peptone solution and concentrated to an O.D. 600_{nm} of ~ 1.0. Cell suspensions were divided into equal aliquots and kept in a water bath at 37 °C until glycolytic rates were measured. When ready, cultures were placed in a sample cup and the pH was adjusted to either pH 7.0 or pH 5.0. When residual glycolytic activity was depleted, glucose was added to a final concentration of 200mM. Glycolytic rates were measured for 15 minutes and the rate of acid production was determined by the addition of 10 mM NaOH expressed as (mmol of NaOH/min)/(g/mL) of cells via an ABU901 autoburrette (Radiometer, Copenhagen). Samples were analyzed in triplicates and a control was performed with sterile water added to the cell suspension instead of glucose, where the pH was recorded overtime. Cell weights were determined by drying 1.5ml Eppendorf tubes overnight in a 65 °C oven. After glycolytic rates were taken, 1ml of the final cell suspension was placed in each tube, centrifuged, supernatant removed and allowed to dry overnight. The following day the tubes were re-weighed and the appropriate calculations were carried out.

2.9: Acid Tolerance Response Assays

The role of potassium uptake and its ability to interconvert the transmembrane potential is an area that is potentially rich in information concerning the involvement of this cation in general acid tolerance mechanisms as well as the acid tolerance response (ATR) of *S. mutans*. To study the effects of the inactivation of the putative potassium transport genes on the acid tolerance response in *S. mutans*, we performed ATR assays using UA159 and SMUTRKB, SMUTRK and SMUTOP mutant strains.

Cell suspensions were diluted 10-fold and grown to mid-log growth phase in an incubator at 37 °C with 5% CO_2. Cultures were split into two equal halves: one half was pelleted and resuspended in TYEG at pH 3.0 which constituted the 'unadapted' cells. The other half was resuspended in TYEG at pH 5.5 for 2 h followed by resuspension in TYEG at a killing of pH 3.0, thereby examining the ATR and comprising the "adapted" cells. At 2-, and 3-h time points, 3ml of culture were removed, pelleted and washed 2x with 0.85% NaCl solution. Aliquots (100µl) of washed cells were placed in a 96-well microtitre plate in triplicates. Consequently, a LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, OR) was utilized to assess the viability of the bacterial strains according to the suppliers specifications. Stained cells were subjected to fluorescence readings using a FL600 Microplate Fluorescence Reader (BIO-Tek Instruments Inc., VT) at excitation/emission maxima at 480/500nm for the SYT09 'live' stain and at 490/635nm for the propidium iodide 'dead' stain.

Results were expressed as a live/dead ratio for both mutant and progenitor strains (Fig. 3). In order to determine the live/dead ratios, first, the average of the fluorescence readings from 3 biological replicates was calculated for both live (SYT09) and dead

(propidium iodide) stains. Live/dead ratios were then calculated by dividing the average live fluorescence reading by the average dead fluorescence reading for each time point in both adapted and unadapted conditions. Statistical analysis was conducted by using the Student's T-test.

2.10: Potassium Transport Assays (Neutral and Acid pH)

To assess the functionality of the putative Trk operon in potassium uptake, potassium transport assays were conducted at neutral (pH 7.0) and acidic (pH 5.5) pH values with SMUTOP and the wildtype strain. Briefly, overnight cultures were grown in THYE, pelleted and resuspended in TYEG at pH 7.5 the following day. Cells were grown to mid-log phase in an incubator at 37 °C with 5% CO_2, washed three times with 0.85% NaCl and resuspended in 1/10 the initial volume (O.D. 600 $_{nm}$ ~ 2.0). Cell suspensions were divided into 1ml aliquots and kept in a water bath at 37 °C until they were used for transport. Potassium uptake was facilitated at 37 °C and initiated with the addition of 1ml of 2x concentrated TYEG at pH 7.5 and pH 5.5. Samples were run for 0, 1, 2, 5, 8, and 10 min time points, which upon completion were aspirated through 0.22 μm filters (Millipore, Billerica, MA) followed by a wash with an equal volume of distilled water. Cells collected on the filters were allowed to dry overnight at 37 °C and final weights were taken the next day on the filter itself. Dry cells weights in mg/ml of solution were determined by subtracting the dried cells from the initial weight of the filters which were dried and pre-weighed prior to commencing the transport assays. Next, cells were removed from the filter with the addition of 1 ml of 1-Butanol, followed by vortexing for

3min. The liquid phase was removed, placed in 1.5 ml Eppendorf tubes and incubated at 37 °C for 30 min. for extraction of intracellular potassium (Daspher and Reynolds, 1992). Cell debris was removed via centrifugation and the appropriate dilutions (1/100) were made. An equal volume of 0.2% $LaCl_3$ was added to prevent ionization (Sanui and Pace, 1968). Intracellular potassium content was determined via atomic emission spectroscopy (AAnalyst 100, Perkin Elmer) set at wavelength 766.5 nm courtesy of ANALEST (University of Toronto, Toronto, ON). Concentration was plotted on a standard curve which was established by 2, 1, 0.5, 0.1 and 0.01 μg/ml standards. Samples were run in 3 biological replicates and the averages were calculated.

2.11: Potassium Accumulation Profile

The aforementioned transport assays examined potassium uptake as an immediate response to neutral and acidic pH exposure in the first 10 min. In order to determine the extent of potassium uptake over an increased exposure time in acidic conditions (pH 5.5), a potassium accumulation profile was conducted. Briefly, overnight cultures were grown and washed in identical fashion as described above. Cells were resuspended in 0.85% NaCl solution and 2ml of this solution was aspirated through a filter to determine the initial potassium content of the cells, defined as time 0. Potassium uptake was facilitated at 37 °C and began with the addition of 30ml of TYEG at pH 5.5. 2ml of sample was extracted from each culture at 2, 9, 16, 25, 34, 43, 53, 60, 75, and 120 min time points. Cells were filtered through 0.22μm filters and the filtrate was collected. Cells were then washed with an equal volume of distilled water and intracellular potassium content and

dry weights were determined as previously described. In addition, the pH of the filtrate was measured with using a pHC2001 electrode and potassium content was also determined via atomic emission spectroscopy for these samples. Prior to potassium analysis, filtrate samples were diluted 1/1000 in order to stay within the linear range of the standard curve.

2.12: Potassium Content of Growth Medium

Prior to the initiation of this project, the potassium content of various growth media utilized by our lab was unknown. As such THYE, TYEG and MMG (minimal media with 1% glucose: see APPENDIX: B) were analyzed to determine their potassium concentrations via atomic emission spectroscopy. Prior to analysis, each medium was filtered through 0.22 μm filters and the appropriate dilutions were made. An equal volume of 0.2% $LaCl_3$ was added to prevent ionization. Results were reported in μg/ml and represent the average of three independent experiments. A fourth growth medium designated MMGK- (Minimal Medium with 1% Glucose, Potassium Deficient) was also analyzed and is identical to MMG with the exception that any potassium components involved were substituted with their sodium equivalents. Use of this medium permitted the potassium content of our growth conditions to be regulated.

2.13: Growth Assays in MMGK-

To determine the ability of *S. mutans* UA159 and the knockouts mutants to grow in MMGK-, simple growth assays were conducted. Overnight cultures were resuspended in TYEG at pH 7.5. Once mid-log phase was reached, cells were washed 3x and resuspended in 0.85% NaCl. In triplicates, 20 μl of culture was inoculated into 300 μl of media in a 96-well microtitre plate and placed in an incubator for 18 hrs at 37 °C with 5% CO_2. Before readings were taken, each well was sonicated for 10 sec. to disperse both biofilm and planktonic phase cells which collectively contributed to the overall growth recorded. Plates were analyzed using an FL600 Microplate Fluorescence Reader and the O.D. at 490_{nm} of each well was recorded. Growth in terms of optical density was calculated by subtracting blank medium values from the final readings and averaging three biological replicates.

2.14: Potassium Transport Assays in MMGK-

Transport assays were also conducted in MMGK- to determine the ability of the parental strain and SMUTOP to uptake potassium under regulated levels. Growth conditions were similar to those described above in previous transport assays. Once mid-log growth phase was reached, intracellular potassium levels were diminished by allowing the cells to incubate in MMGK- for 1.5 h at 37 °C with 5% CO_2. At this point, the supernatant was removed and pelleted cells were resuspended in fresh MMGK- for 1.5 h to establish a new K^+ concentration gradient, thereby promoting K^+ levels to diminish. Potassium depletion was confirmed by analysing intracellular levels of the

samples at the 1.5 h and 3 h time points. Potassium uptake was facilitated in a water bath at 37 °C and initiated by the addition of 5 mM and 25 mM (final concentration). Samples were analyzed at 0, 15, 30, 45, 60, 80, 100 and 120 s time points. Intracellular potassium was extracted in the same manner as previously described. Potassium content at each time point was averaged from three biological replicates and plotted as $\mu g\ K^+$/mg cell vs. time.

2.15: Biofilm Integrity

Biofilm formation of *S. mutans* UA159 and the SMUTKB, SMUPACL and SMUTOP strains was assessed using different growth media with varying potassium concentrations. Briefly, overnight cultures grown in THYE were washed and resuspended in TYEG at pH 7.5 and grown to an $O.D._{600nm} > 1.0$. At this point, cultures were washed three times with 0.85% NaCl and 50 μl of cells in triplicates were added to 1.5ml of media in a 24-well microtitre plate. Plates were incubated at 37 °C with 5% CO_2 for 16.5 h. Following incubation, the supernatant and planktonic cells were removed by aspiration and biofilms were washed with 1.5 ml of distilled water. Each well was immersed in 500 μl of 0.1% Crystal Violet Dye for 10 min. Plates were washed twice and allowed to air dry overnight. The ability to form biofilms was evaluated in THYE, MMG, MMGK- and MMGK- supplemented with 0.5, 1, 1.5, 5 and 150mM KCl (final concentration).

CHAPTER 3: RESULTS

3.1: Bioinformatic Analyses

The search for potassium transporter genes in the *S. mutans* UA159 genome identified five different gene products, designated TrkB, Trk, PacL, TrkA and TrkH as indicated by NCBI (Table 3) with putative potassium uptake functionality. Of these, *trkB, trk* and *pacL* are located contiguously on the UA159 chromosome, whereas *trkA* and *trkH* are positioned adjacent to one another but are downstream from the *trkB, trk* and *pacL* genes (Figure 3.1.). A BLASTp search using the FASTA protein sequences of each query gene was used to identify closely related homologues based on % identity. The results are displayed in Table 3.1. In addition, genes from other bacteria of interest that are homologous to the *trkB, trk, pacL, trkA* and *trkH* gene products in *S. mutans* are also displayed in Table 3.1. These organisms were selected based on the acidic environments they inhabit, thereby demonstrating their need for acid tolerant properties in order to survive.

Furthermore, a BLASTp search was conducted against the *S. mutans* UA159 genome using potassium transport protein sequences that have already been characterized and studied in other organisms in attempt to identify the types of potential potassium transport systems that may exist within *S. mutans* (data not shown). These searches included the KcsA potassium channel protein sequence of *Streptomyces lividans*, the sequences representing the Trk, Kdp and Kup K^+ uptake systems as well as the KefC K^+ efflux system (Ness and Booth, 1999) of *E. coli*. Interestingly, bioinformatic results from these searches revealed no significant homology (< 30 identity) amongst all the systems tested against the UA159 genome with the exception of the *trkH* gene of *E. coli* which had 34% identity to *S. mutans' trkH* gene product.

Protein analysis was conducted for the *trkB, trk, pacL, trkA* and *trkH* translated gene products using the NCBI database as well as the SMART, Pfam and STRING bioinformatic search tools. Conserved domains and predicted functional partners were identified and are displayed in Table 3.2. More specifically, a protein search using SMART and Pfam, revealed that all the aforementioned Trk ORFs (open reading frames) contained conserved Trk domains, which are characterized by the presence of a KTN (K^+ transport and NAD-binding) binding motif that is specific for NAD^+. Notably, the *S. mutans' pacL* has 28% identity with the KdpB gene in *E.coli* (data not shown). This gene is responsible for the catalytic subunit of the Kdp system and thus comprises the functional portion of this P-type ATPase (Bramkamp and Altendorf, 2004). Predicted protein interactions were identified using the SMART database, which implicated that each of the *trkB, trk, pacL, trkA* and *trkH* genes may interact with one another as a functional unit. Each protein was also analyzed for the presence of transmembrane domains, the results of which are displayed in Table. 3.3.

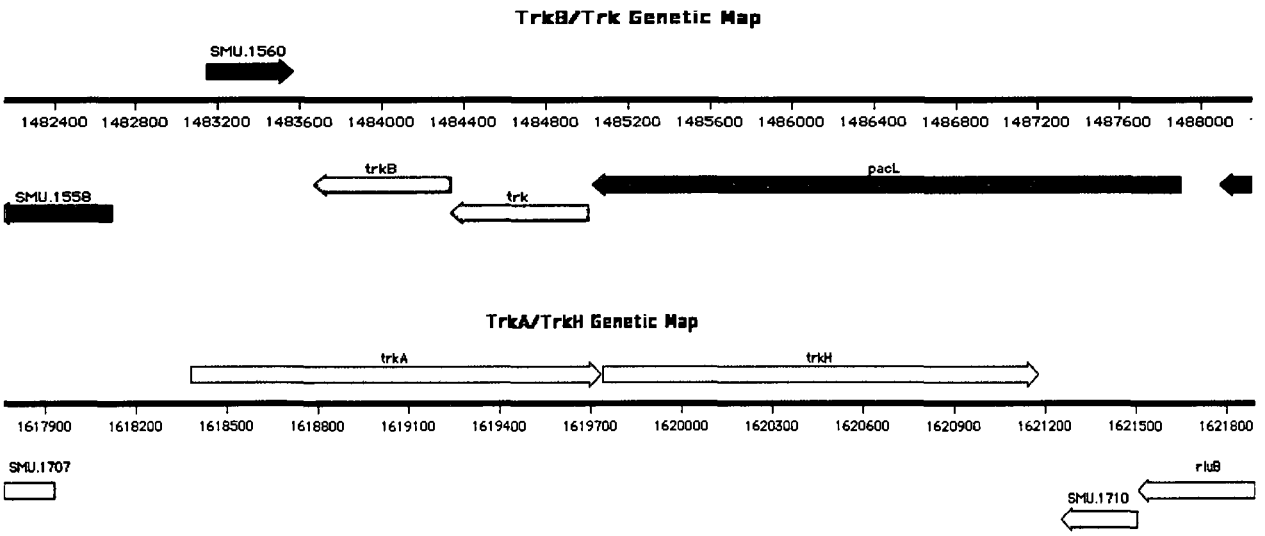

Figure 3.1. Genetic map of the *trk, trkB* and *pacL* locus (top) and the *trkA, trkH* locus (bottom). Numbers represent the specific location occupied by the base pair in the genome. Arrows represent 5' and 3'orientation of the ORFs displayed.

Protein/ Locus ID	NCBI Closest homolog (%identity)	Organisms of Interest (% identity)
TrkB / SMU.1561	TrkA domain-containing protein, *Roseiflexus sp.* RS-1 (38%)	TrkA domain-containing protein, *Acidothermus cellulolyticus* 11B (33%)
Trk / SMU.1562	TrkA domain-containing protein, *Roseiflexus castenholzii* DSM 13941 (39%)	Hypothetical protein, *Clostridium spiroforme* (37%)
PacL / SMU. 1563	Hypothetical protein, *Streptococcus infantarius* subsp. infantarius ATCC BAA-102 (67%)	H^+-K^+ exchanging ATPase, *Lactobacillus casei* BL23 (53%)
TrkA / SMU.1708	Hypothetical protein, *Streptococcus infantarius* subsp. infantarius ATCC BAA-102 (80%)	Potassium transporter peripheral membrane component, *Streptococcus salivarius* SK126 (71%)
TrkH / SMU.1709	K^+ Transporter (Trk), *Streptococcus thermophilus* strain LMG 18311 (73%)	Trk-type K^+ transport system, *Streptococcus salivarius* SK126 (72%)

Table 3.1. BLASTp results of the *trkB, trk, pacL, trkA,* and *trkH* genetic loci against the NCBI protein database. Closest homologues based on % identity are presented in column 2. Column 3 represents organisms that inhabit acidic environments and harbour genes demonstrating ≥33% identity to the genes of interest represented in column 1.

Protein/ Locus ID	Putative Function NCBI	Conserved Domain Pfam/ SMART Search	STRING Predicted Functional Partners
TrkB / SMU.1561	Putative potassium uptake system protein TrkB	N-terminal - Pfam domain Trk_N , Trk_C	TrkA, TrkH, PacL
Trk / SMU.1562	Putative potassium uptake protein TrkA	N-terminal - Pfam domain Trk_N , Trk_C	TrkB, TrkH, PacL,
PacL / SMU. 1563	Putative cation-transporting P-type ATPase	N-terminal - Pfam domain Cation ATPase_N , E1-E2 ATPase, Cation ATPase_C	Trk, TrkB
TrkA / SMU.1708	Potassium transporter peripheral membrane component	N-terminal - Pfam domain Trk_N , Trk_C, Trk, Trk_C	TrkH, Smt, SMU.482
TrkH / SMU.1709	Putative potassium uptake protein TrkH	N-terminal signal peptide, 3 transmembrane domains, Pfam domain TrkH	TrkA, TrkB, SMU.497c

Table 3.2. FASTA protein sequence analyses. Determination of putative function, conserved domains and predicted functional partners were determined using a combination of the NCBI, SMART, Pfam and STRING protein databases.

Protein/ Locus ID	Predicted Transmembrane Domains (HMMTOP, TMHMM, TMpred)
TrkB / SMU.1561	No predicted Transmembrane domain
Trk / SMU.1562	No predicted Transmembrane domain
PacL / SMU. 1563	N-terminus in; 8 predicted transmembrane helices: a.a.'s (70-92), (253-272), (279-301), (744-766), (794-816), (831-850), (870-892), (897-919)
TrkA / SMU.1708	No predicted Transmembrane domain
TrkH / SMU.1709	N-terminus in; 11 predicted transmembrane helices: a.a.'s (8-30), (35-58), (71-93), (127-150), (184-206), (237-260), (275-297), (331-350), (393-413), (418-438), (451-472)

Table 3.3. Identified transmembrane domains of the TrkB, Trk, PacL, TrkA and TrkH proteins. HMMTOP, THMM and TMpred programs were consulted for consistency.

3.2: Quantitative rtPCR Analysis of Acid Induced Gene Expression

Results from the quantitative rtPCR experiments demonstrated induction of *trkB, trk, pacL, trkA* and *trkH* in *S. mutans* UA159 upon incubation in TYEG media at pH 5.5 when compared to incubation of cells at pH 7.5 (Figure 3.2). More specifically, each of these genes were up-regulated at least 2-fold at low pH, confirming the initial microarray results that led us to investigate these genes. Interestingly, pH induction of TrkH was confirmed in the real-time PCR experiment but was not identified in the less sensitive microarray, even though RNA was isolated under identical conditions. It should be noted that expression levels were determined after 1hr of exposure to an acidic pH of 5.5 and therefore these results cannot be considered as an immediate response to combating or adapting to acid stress. These results do however suggest the involvement of these genes in acid tolerance to extended acid exposure in *S. mutans* and as such, deletion mutations of the genes described (refer to Table 2.1) were constructed to further investigate their role in the acid resistant phenotype.

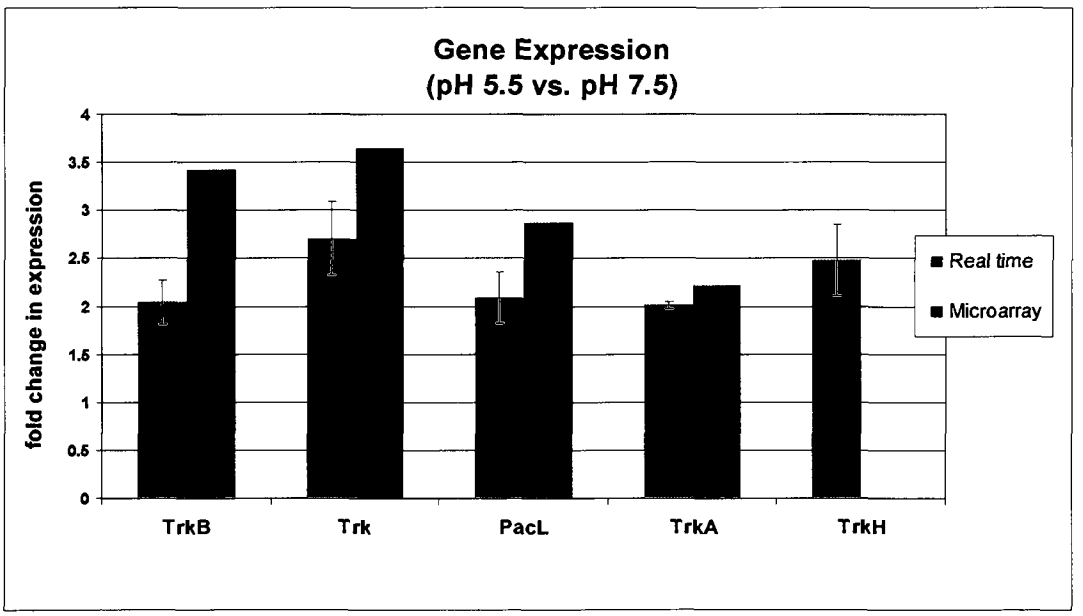

Figure 3.2 Quantitative real-time PCR for the *trk, trkB, pacL, trkA* and *trkH* genes. Results shown are the average of three independent experiments ± standard error.

3.3: Co-transcription Assay

To investigate whether the *trkB/ trk/ pacL* genes comprised a tricistronic operon, cDNAs derived from *S. mutans* UA159 at pH 5.5 were amplified using primers designed within the predicted intergenic regions of the *trkB, trk* and *pacL* genes (Table 2.2.). Amplicons were obtained only in the intergenic regions spanning the *trkB/ trk* genes and the *trk/pacL* genes shown in Figure 3.3 lane S, #2 and #3. Positive controls utilized genomic DNA to verify the functionality of the primers sets and –RT controls were included to rule out DNA contamination. Although the resolution of the picture presented (Figure 3.3.) reveals very faint bands representing the PCR products, it should be noted that the visibility of these products was far more evident on the monitor screen from which the figure was obtained. The band sizes of these products corresponded to the expected sizes dictated by their primer design (Table 2.2). More specifically, amplification of a 335 bp size band was predicted and observed for the Δ*trkB*-Δ*trk* primer set and a 349 bp size band was predicted and observed for the Δ*trk*-Δ*pacL* primer set. To further verify the co-transcription of *trkB/ trk/ pacL* as a single operon, the MacVector software program was used to scan the downstream intergenic region between *trkB* and SMU.1560 for possible termination sequences. Furthermore, this region is comprised of 98 base pairs spanning the specific location that exists between base pair 1483565 and 1483663 of the *S. mutans* UA159 genome. These results (APPENDIX A; Figure S2) identified two different possible termination sequences in the form of two adjacent hairpin loop structures. Collectively, these results confirm that transcription of *trkB, trk* and *pacL* occurs as a single mRNA and that these genes comprise a tricistronic operon.

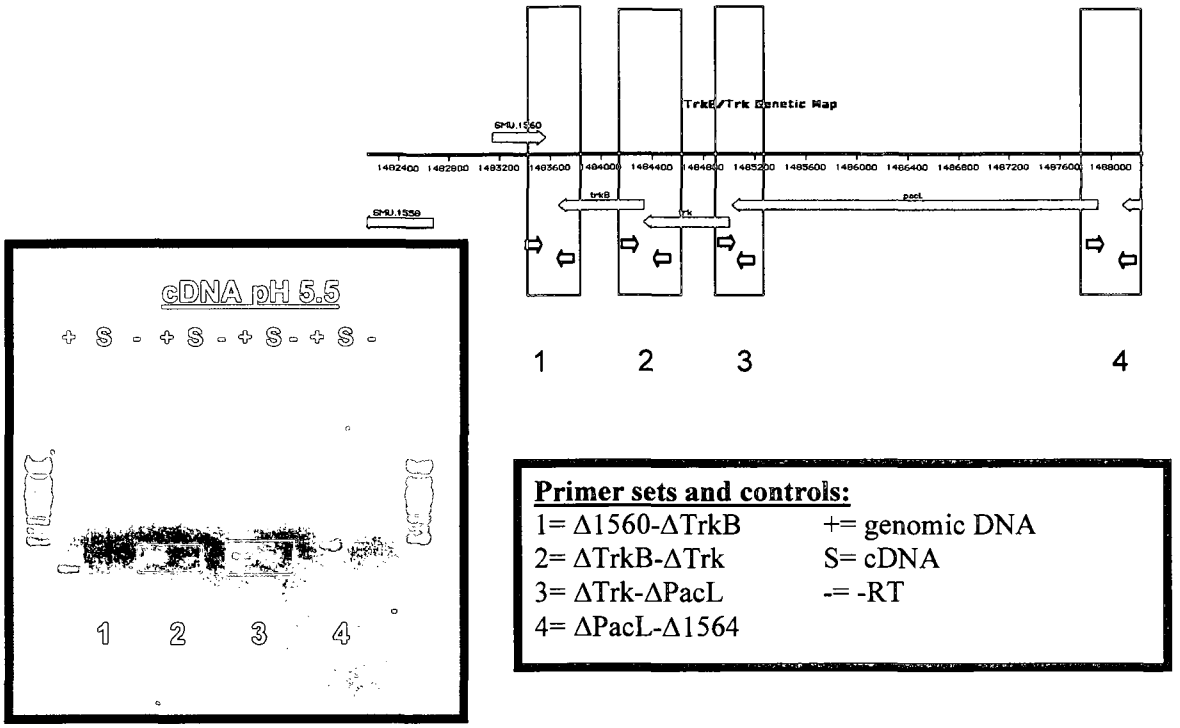

Figure 3.3. Primer design and co-transcription assay. Numbers represent the primer sets used to amplify the intergenic regions shown in the diagram and their corresponding products displayed in the gel.

3.4: Growth Assays

After successful construction of the mutants outlined in Table 2.2, growth parameters of each of these strains were examined under neutral and acid stress and compared to the wildtype UA159 strain. Results revealed that SMUTRKB, SMUTRK, SMUPACL, SMUTKB and SMUTOP had substantial growth impairment under acid stress exhibiting a lower bacterial yield in TYEG medium as compared to the wildtype strain (Figure 3.4-3.7). These deficiencies in total biomass were statistically significant in each of the mutant strains ($p<0.01$) and are expressed as the final yield after 18 hrs of growth in pH 5.5 (Figures 3.5 and 3.7). Interestingly, single and double gene deletions in strains SMUTRKB, SMUTRK, SMUPACL and SMUTKB seemed to exhibit nearly

identical acid-sensitive phenotypes as the entire operon deletion expressed by strain SMUTOP. This suggests that the optimal activity of this system is only achieved when all the genes that comprise this genetic locus are transcribed, further supporting the notion that these proteins are co-transcribed as a functional unit. These results suggest that the genes comprising the *trkB/ trk/ pacL* operon are involved in the acid tolerance mechanisms of the cell. Under neutral pH, statistical significance in total biomass was observed only in SMUPACL and SMUTOP. SMUTRKA and SMUTRKH were also evaluated for their ability to grow under acid and neutral conditions compared to the wildtype (data not shown). Results from these experiments demonstrated identical growth to the parent under both conditions.

In addition, SMUTKB and SMUTOP had differences in the time taken to enter the logarithmic phase of growth (log-phase) when compared to the UA159 parent strain. More specifically, log-phase growth of SMUTKB was delayed by approximately 40 minutes post inoculation relative to UA159, whereas log-phase growth in SMUTOP was reached nearly 1 hr ahead of the wildtype. Upon initial analysis, these results suggest that *pacL* may be involved in growth initiation under the conditions tested because the difference between SMUTKB and SMUTOP is the presence and absence of *pacL,* respectively. However, SMUPACL had no significant difference in the time taken to enter the logarithmic phase of growth compared to strain UA159, suggesting that the interaction of these particular proteins and their effects on growth initiation may be a complex process. The doubling time of each strain was also calculated (data not shown), however no statistical difference was observed between the mutant strains and the UA159 parent; only lag-phase effects were observed.

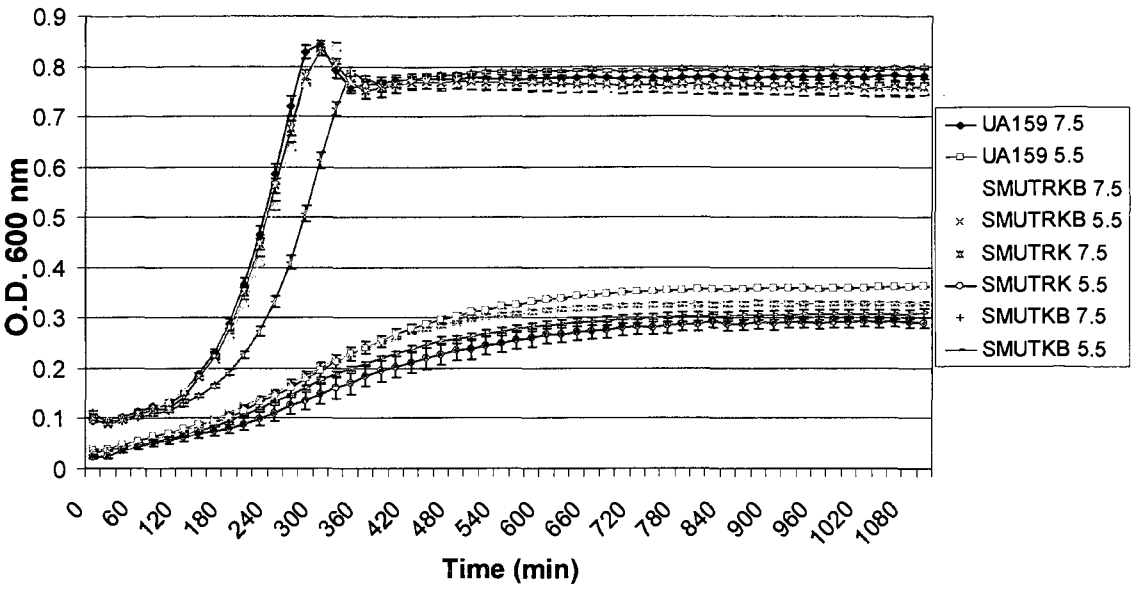

Figure 3.4. Growth curves of *S. mutans* UA159 wildtype, SMUTRKB, SMUTRK and SMUTKB at neutral (7.5) and acidic pH (5.5) in TYEG. Results shown represent the average of 4 independent experiments ± standard error.

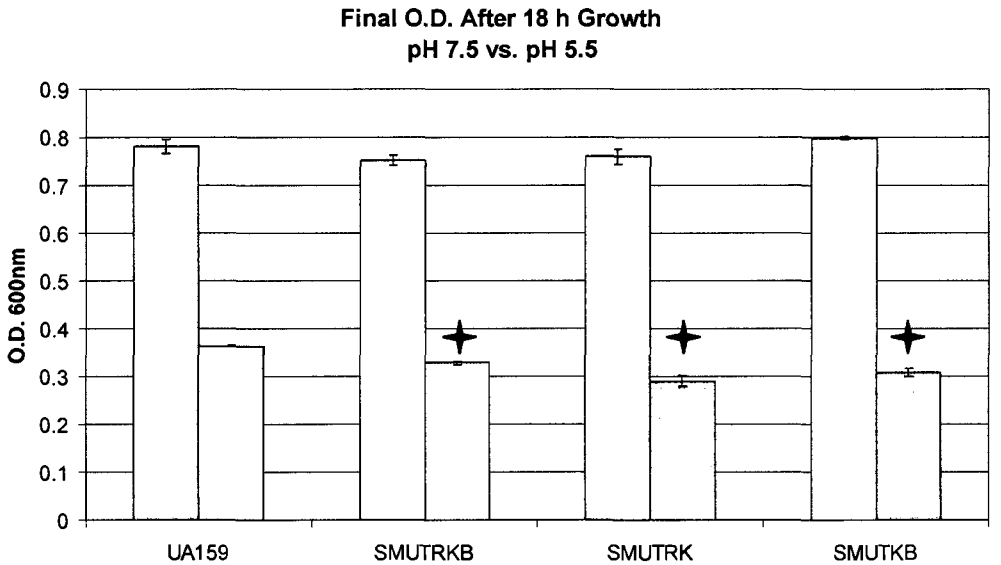

Figure 3.5. Final O.D. (600_{nm}) of *S. mutans* UA159 wildtype, SMUTRKB, SMUTRK and SMUTKB grown at neutral (7.5) and acidic pH (5.5) in TYEG for 18 h. Results shown represent the average of 4 independent experiments ± standard error. Colours correspond to the matching strain presented in the growth curves above. Stars indicate statistically significant differences relative to wildtype (p-value<0.01).

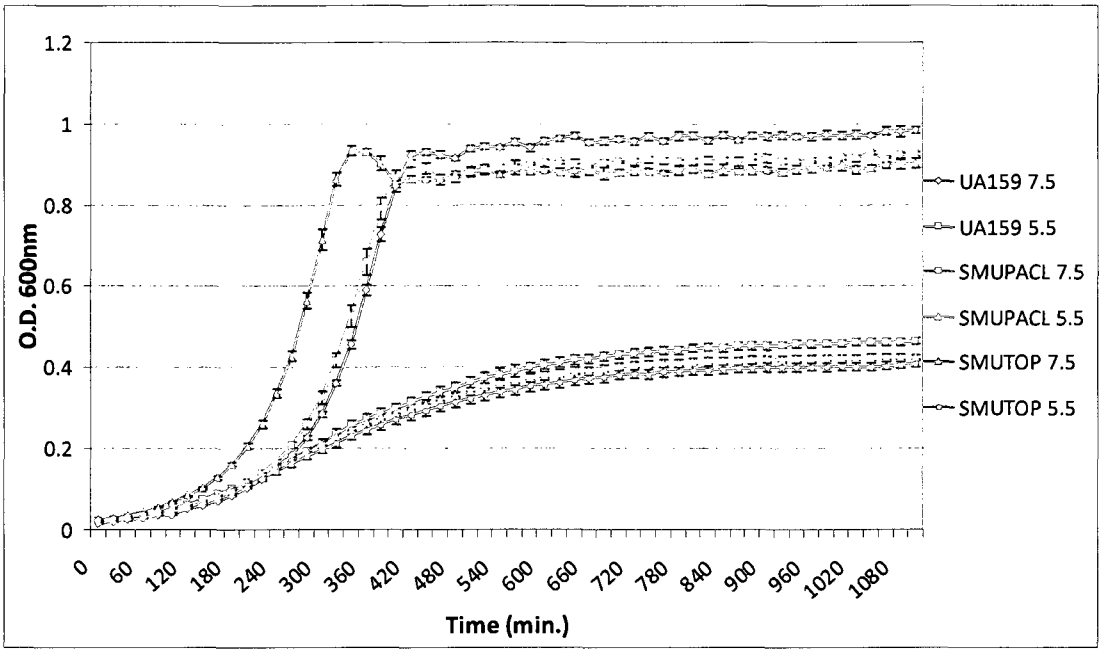

Figure 3.6. Growth curves of *S. mutans* UA159 wildtype, SMUPACL and SMUTOP at neutral (7.5) and acidic pH (5.5) in TYEG. Results shown represent the average of 4 independent experiments ± standard error.

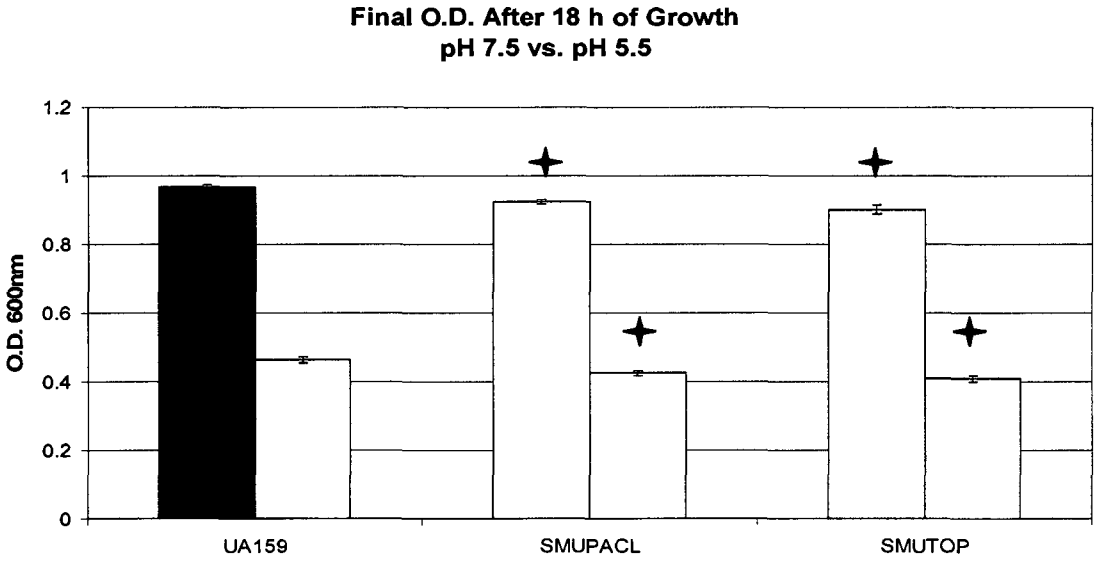

Figure 3.7. Final O.D. (600_{nm}) of *S. mutans* UA159 wildtype, SMUPACL and SMUTOP grown at neutral (7.5) and acidic pH (5.5) in TYEG for 18 h. Results shown represent the average of 4 independent experiments ± standard error. Colours correspond to the matching strain presented in the growth curves above. Stars indicate statistically significant differences relative to wildtype (p-value<0.01).

3.5: Terminal pH Assays

To further investigate the ability of the deletion mutants to withstand acid stress, terminal pH values were measured after 18 hr of growth in TYEG at pH 7.5 and pH 5.5. These terminal values are indicative of the strain's capacity to produce acid under low pH which is directly dependent on their ability to tolerate an acidic environment and metabolize nutrients under these conditions. In order to normalize the initial amount of cells inoculated into the culture media, mid-log phase cells were diluted to equal O.D. starting values. Under neutral conditions, all of the mutant strains demonstrated slightly elevated pH values when compared to the wild-type strain (Table 3.4). Of these, statistical significance (p-value<0.03) was observed in the SMUTRK, SMUPACL, and SMUTKB mutants using a one-way ANOVA analysis. Similar results were obtained with growth initiated under acidic pH, whereby each mutant tested demonstrated small but elevated terminal pH values when compared to the parent UA159 strain (Table 3.4). Statistical significance was observed between the parent strain UA159 and the SMUTRKB, SMUTRK, SMUTKB and SMUTOP strains. Collectively, these results indirectly suggest increased acid sensitivity of the mutants because a higher terminal pH value can result from an inability to produce acid due to metabolic arrest or decreased overall biomass. The latter explanation is consistent with the results obtained from the growth rate experiments previously described.

Another factor that must be considered when analyzing these results however, is the effect that the deletion mutations may have on the glycolytic activity of each individual strain. Moreover, an elevated or diminished terminal pH could be observed if the mutation results in a phenotype with decreased or increased ability to produce acid,

respectively. To test this possibility, glycolytic rate assays were performed and results of these experiments will be described next.

Initial pH of Media	Strain					
	UA159	SMUTRKB	SMUTRK	SMUPACL	SMUTKB	SMUTOP
TYEG 7.5	4.302 ± 0.0024	4.302 ± 0.0029	*4.344 ± 0.0035	*4.316 ± 0.0011	*4.327 ± 0.0012	4.309 ± 0.0013
TYEG 5.5	4.428 ± 0.0013	*4.451 ± 0.0037	*4.448 ± 0.0033	4.442 ± 0.0064	*4.453 ± 0.0013	*4.469 ± 0.0017

Table 3.4. Terminal pH values recorded after 18 h in TYEG media at pH 7.5 and pH 5.5. Results shown are the average of four independent experiments ± the standard error. * Indicate statistically significant differences relative to wildtype (p-value<0.01).

3.6: Glycolytic Rate Assays

In order to determine the effects of mutagenesis on the ability of SMUTRKB, SMUTRK and SMUTKB to produce acid at neutral and acidic pH, glycolytic rates were determined by the equimolar-pH-regulated addition of 10 mM NaOH to glycolyticaly active cells. Under both conditions, each mutant strain had similar glycolytic rates when compared to the UA159 wildtype (Figure 3.8.). No statistical difference was observed at pH 7.5 or pH 5.5 thereby suggesting that the mutations had no effect on the ability of the cell to undergo normal glycolytic activity under the test conditions. Notably, in all strains including the wildtype, glycolytic rates were 7-fold higher at pH 7.5 than pH 5.5. SMUPACL and SMUTOP were not investigated in these experiments because of complications that arose in their construction at the time these assays were being conducted. An alternative method to measuring glycolytic activity in SMUTOP was utilized and will be discussed in a subsequent section (refer to section 3.9).

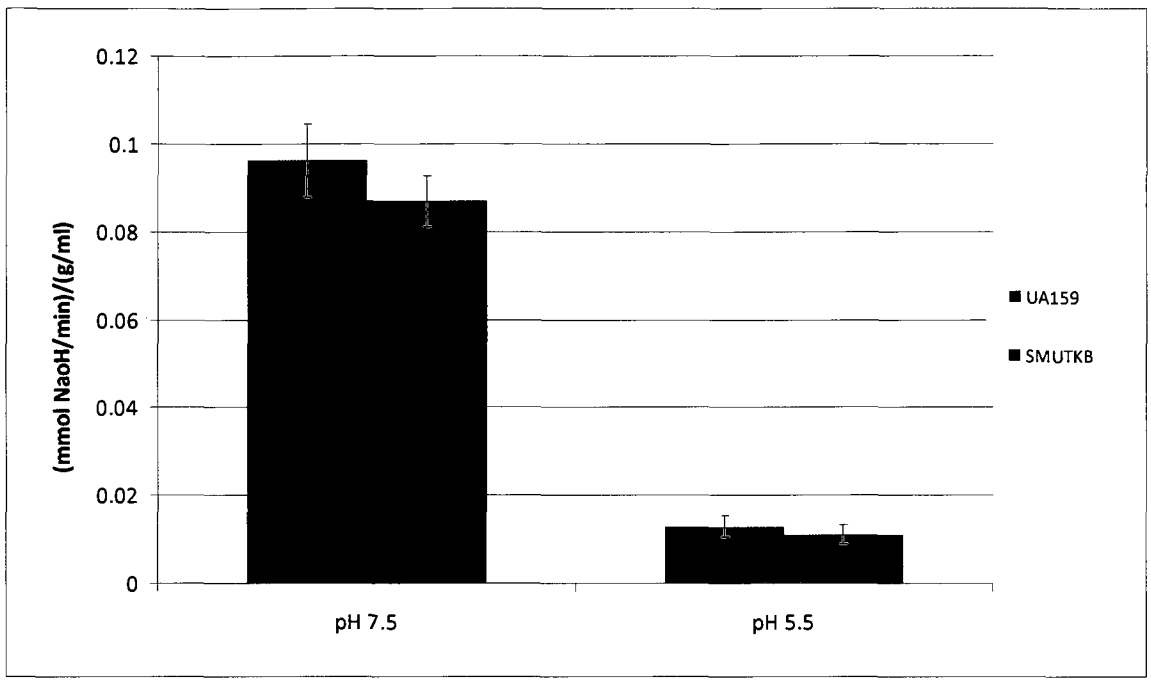

Figure 3.8. Glycolytic rate assays determined via the addition of 1mM NaOH. Results are the average of at least 5 independent experiments ± standard error. Similar results were obtained in SMUTRKB and SMUTRK (data not shown).

3.7: Acid Tolerance Response Assays

Thus far, experimental results regarding the ability of the mutant strains to grow in acidic conditions (pH 5.5) and their ability to produce acid with respect to the wildtype strain have been shown. In order to examine the role of the gene deletions on acid tolerance, ATR assays were conducted that measured survival at a killing pH of 3.0. Results from the ATR assays were consistent among the SMUTRKB, SMUTRK and SMUTKB strains in that exposure to a lethal pH of 3.0 for 2 or 3 hrs resulted in increased acid killing when compared with the wildtype strain. Statistical significance was determined via Student's T-test expressed as a two-tailed distribution with equal variance

among samples. Acid sensitivity was observed as a lower live/dead cell ratio which was statistically significant ($p<0.01$) for SMUTRKB at both time points (Figure 3.9). SMUTRK also had decreased live/dead ratios at 2 and 3 h exposure to pH 3.0 compared to UA159 (Figure 3.10), however the magnitude of this difference was not as drastic ($p<0.05$).

Interestingly, SMUTKB, which is characterized by deletions in *trkB* and *trk*, had a similar acid sensitive phenotype as its single mutation counterparts. However, statistical significance was not observed between SMUTKB and the UA159 parent.

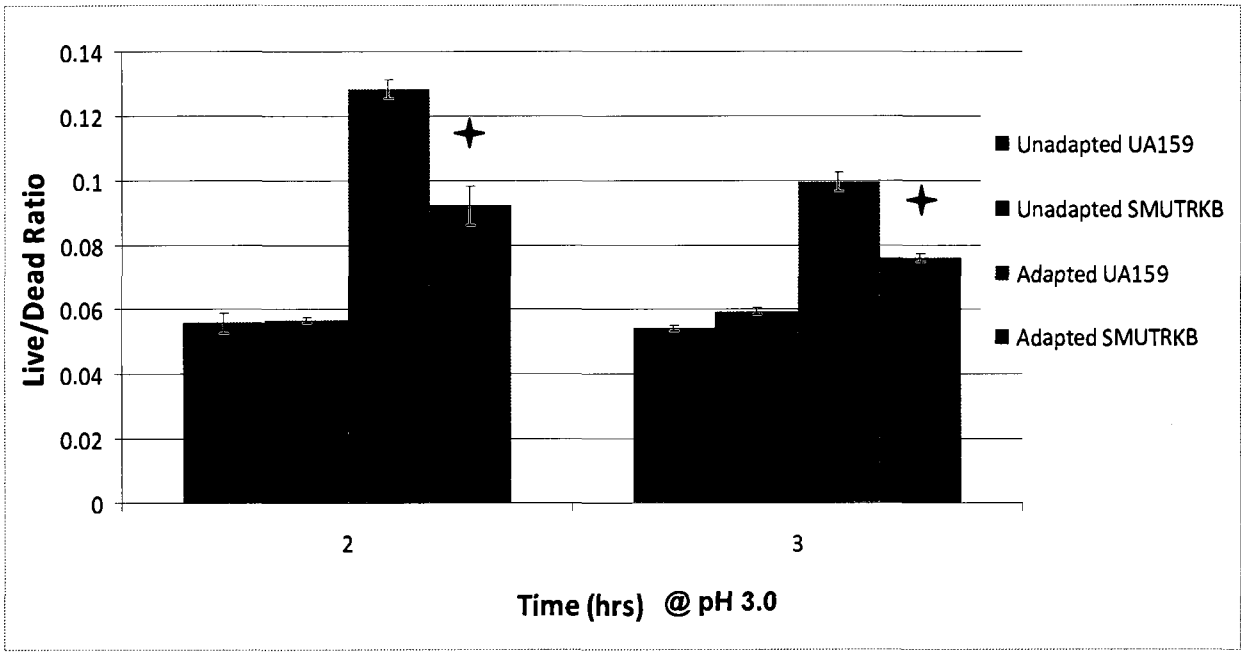

Figure 3.9. Acid tolerance response of SMUTRKB compared to wildtype UA159 in TYEG at a killing pH of 3.0. Stars represent statistically significant differences between parent and mutant strains were observed at 2 and 3 hrs (p-value <0.01) for adapted cells. Results represent the averages of 3 independent experiments ± standard error.

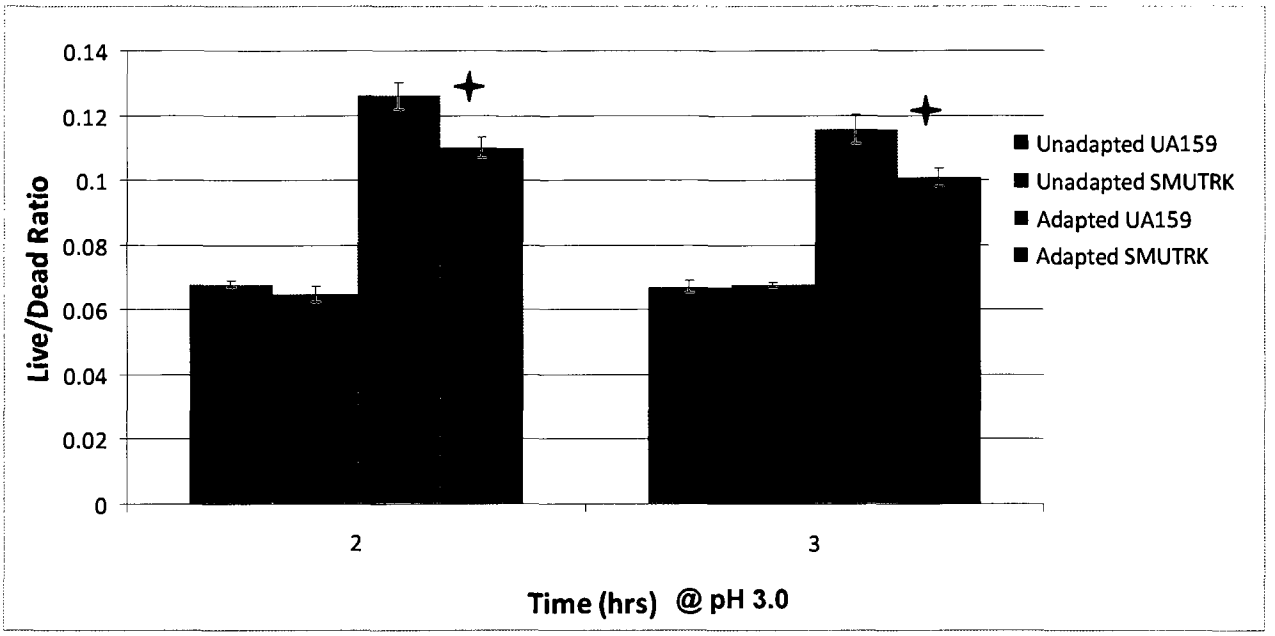

Figure 3.10. Acid tolerance response of SMUTRK compared to wildtype UA159 in TYEG at a killing pH of 3.0. Stars represent statistically significant differences between parent and mutant strains were observed at 2 and 3 hrs (p-value <0.05) for adapted cells. Results represent the averages of 3 independent experiments ± standard error.

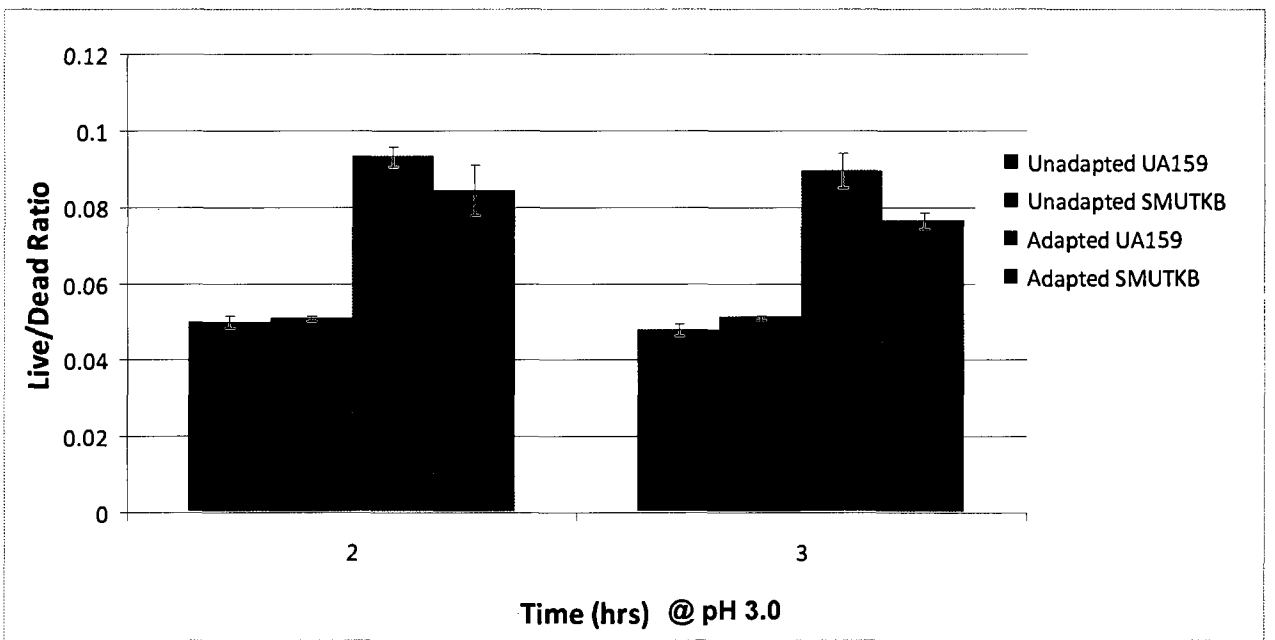

Figure 3.11. Acid tolerance response of SMUTKB compared to wildtype UA159 in TYEG at a killing pH of 3.0. Statistical significance was not observed for unadapted or adapted cells. Results represent the averages of 3 independent experiments ± standard error.

3.8: Potassium Transport Assays (Neutral and Acidic pH)

In order to demonstrate the link between potassium uptake and acid tolerance in *S. mutans* UA159, potassium transport assays were conducted at pH 5.5 and pH 7.0 in TYEG to maintain consistency with the other assays performed. For this study, *S. mutans* UA159 was evaluated along with SMUTOP, which was chosen because it contains an entire deletion of the *trkB/ trk/ pacL* operon. Transport was recorded for the first 10 min upon exposure to acidic and neutral pH and uptake was expressed as μmoles of K^+/mg dry cell vs. time (Figures 3.12. and 3.13.). Under acidic conditions, SMUTOP demonstrated lower levels of potassium at all time points tested. These values were statistically significant ($p<0.05$) at the 0, 1, 2 and 10 minute time points (Figure 3.12.). This suggests that loss of this operon prevents maximum uptake of potassium upon exposure to acidic conditions when compared with the wildtype strain. At neutral pH, no difference in the intracellular K^+ levels was observed between the UA159 strain and SMUTOP strains (Figure 3.13.).

It should be noted that the rates of K^+ uptake seemed unaffected under both neutral and acidic pH. This was demonstrated by similar K^+ transport rates (no statistical difference) observed in both UA159 and SMUTOP at pH 5.5 relative to pH 7.0 (Figure 3.14). This strongly suggests the presence of multiple K^+ transport systems encoded by the UA159 genome, since uptake was observed in both strains tested under both neutral and acidic conditons. Moreover, further analysis of the the transport profiles at pH 5.5 reveals that K^+ levels for UA159 and SMUTOP began to diminish after 10 min (Figure 3.12.). Under neutral conditions, this was not observed as the K^+ values began to plateau instead (Figure 3.13.). To determine whether this trend towards potassium loss in acidic

conditions would continue, a potassium accumulation profile was recorded over an extended time period and will be described next.

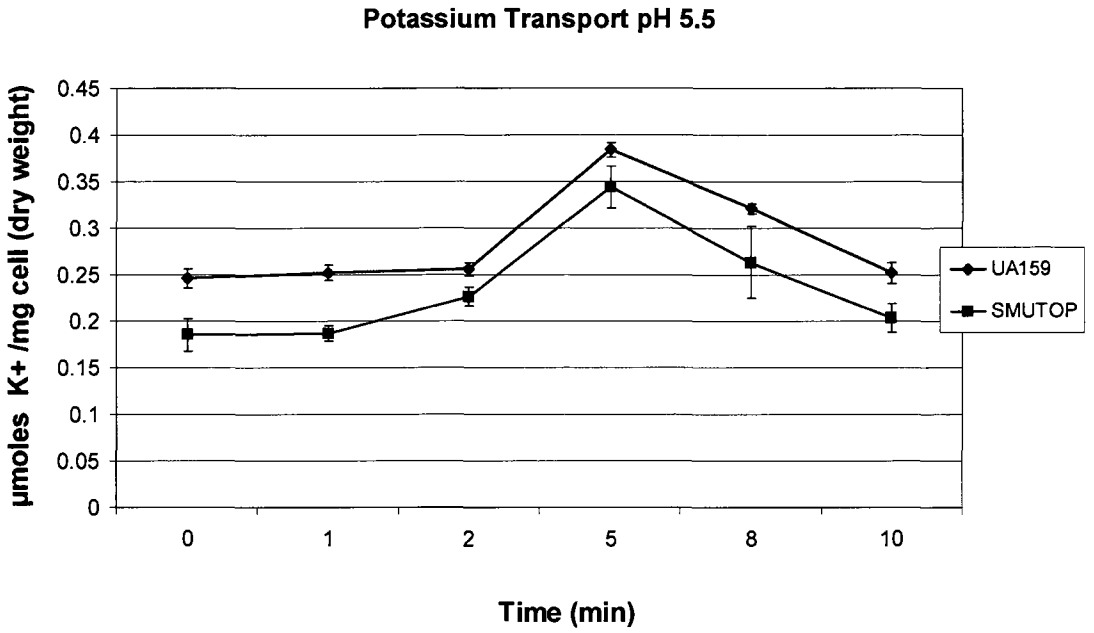

Figure 3.12. Potassium transport of UA159 vs. SMUTOP in TYEG at pH 5.5. Results are the averages of 3 independent experiments ± standard error.

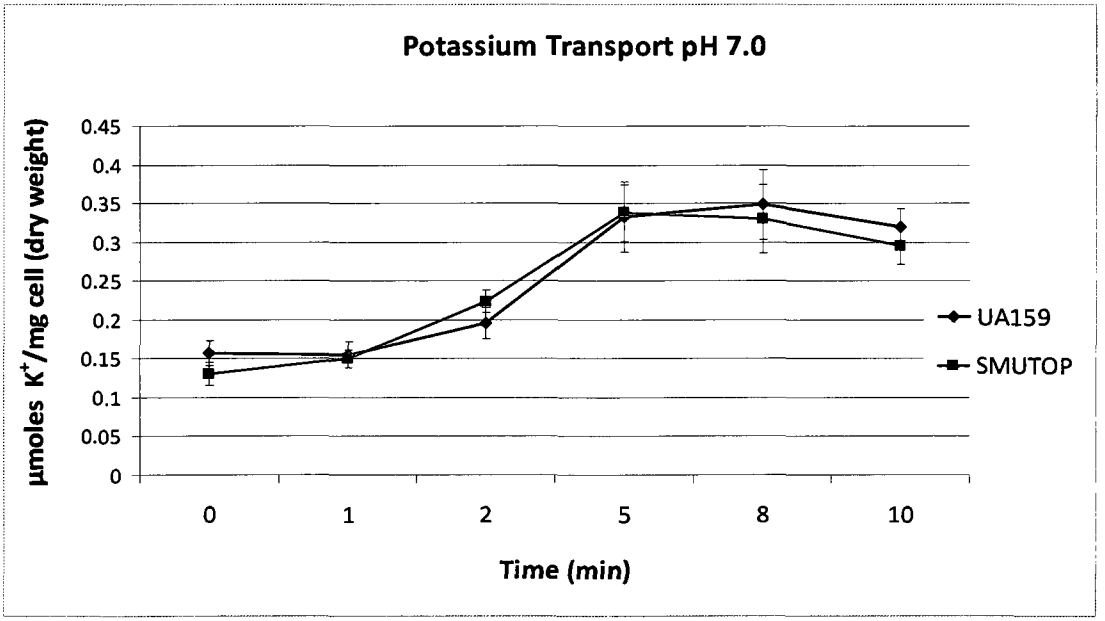

Figure 3.13. Potassium transport of UA159 vs. SMUTOP in TYEG at pH 7.0. Results are the averages of 3 independent experiments ± standard error.

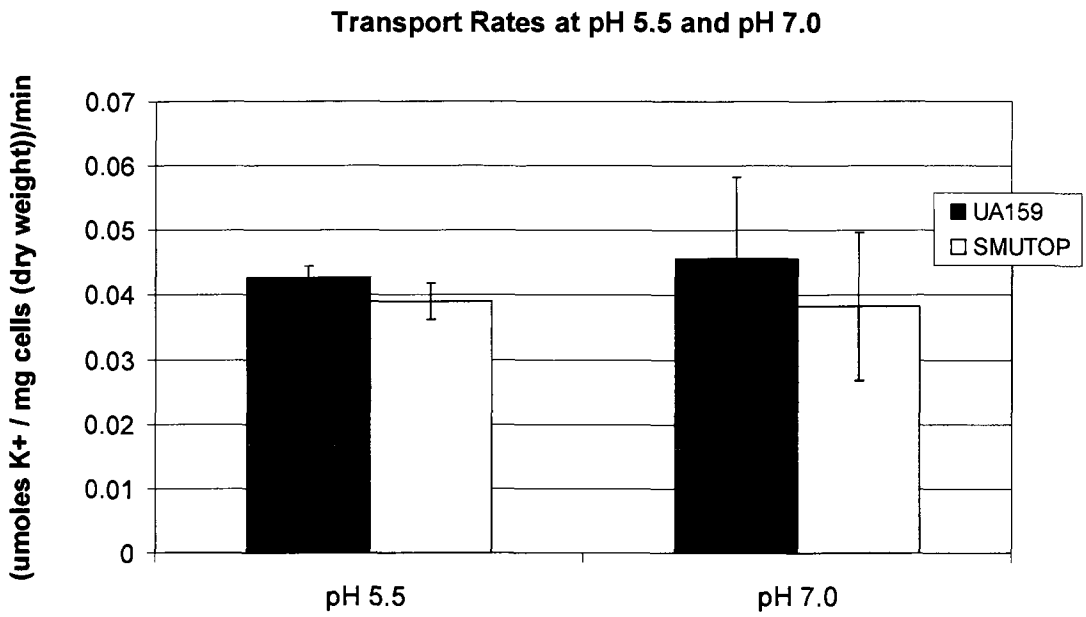

Figure 3.14. K^+ uptake rates of *S. mutans* UA159 compared to SMUTOP at pH 5.5 and pH 7.0 in TYEG medium. Values were determined between the 2 and 5 min time points for both strains at both neutral and acidic conditions, where maximum potassium levels were observed (Figure 3.12. and 3.13.) Results are the average of at least 3 independent experiments ± standard error.

3.9: Potassium Accumulation Profile

Thus far, our efforts to observe potassium transport as a means to combat acid stress were focussed on the first 10 min of exposure to TYEG medium at pH 5.5 (Figure 3.12). As described, results from these assays demonstrated uptake of potassium in the first 5 minutes after exposure to pH 5.5, followed by an apparent reduction of K^+. In order to investigate this observation, a potassium accumulation assay was conducted over a two hour time period to determine the extent of K^+ loss in both the wildtype and SMUTOP strain in response to prolonged acid exposure (Figure 3.15). As expected, similar results to those demonstrated in Figure 3.12. were observed in both strains in the initial 10 minutes of acid induced stress; SMUTOP exhibited decreased levels of intracellular K^+ content compared to UA159, although these differences were only

statistically significant at 9 min (p-value<0.05). Furthermore, UA159 again demonstrated K^+ loss after initial uptake was observed during the first 10 minutes of transport (Figure 3.15.). Interestingly, K^+ loss in UA159 stopped at 25 min., at which point elevated potassium levels seemed to be maintained for the next 27 min. This was followed by a surge in potassium uptake at 1 hr. These trends were consistent amongst the UA159 parent and SMUTOP strains.

Notably, beyond the 1 hr time point, decreased levels of intracellular potassium were once again observed in SMUTOP compared to the wildtype strain implicating the involvement of the *trkB/trl/pacL* system in long-term potassium accumulation.

In addition to evaluating the K^+ content of the cells, potassium and pH levels in the filtrate were also recorded in order to provide a more accurate depiction of the biological activity of SMUTOP and UA159. Figure 3.16 shows decreasing potassium levels over time in the culture medium of both strains therefore confirming that cellular potassium transport is occurring as extracellular K^+ is internalized. Moreover, glycolytic activity was confirmed by measuring the change in pH over time (Figure 3.17). These results confirm that both the UA159 strain and SMUTOP are metabolically active and that acid stress gradually increases as glucose is metabolized and acidic end-products released. Lastly, no difference was observed between SMUTOP and the wildtype strain in the K^+ content of the filtrates analyzed (Figure 3.16.) or in their ability to produce acid (Figure 3.17.), further supporting the observation that deletion of all or some of the genes comprising the *trkB/trk/pacL* locus does not have an effect on glycolytic activity.

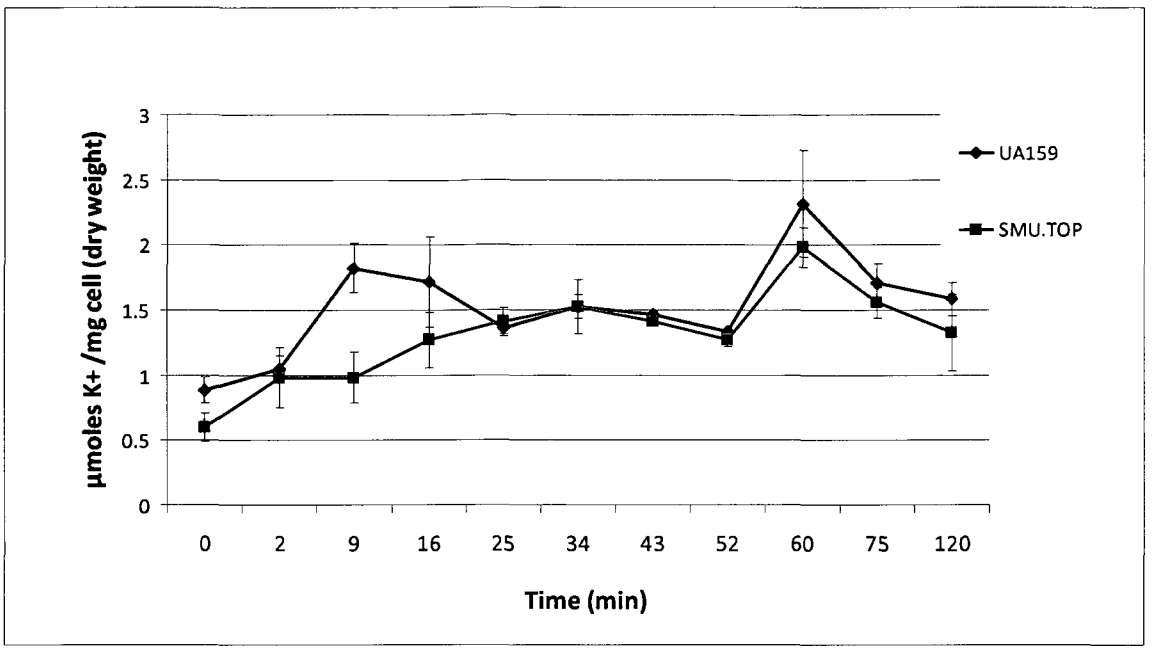

Figure 3.15. K^+ accumulation profiles of UA159 and SMUTOP over 2 h at pH 5.5 in TYEG. Results presented are the average of 3 independent experiments ± standard error.

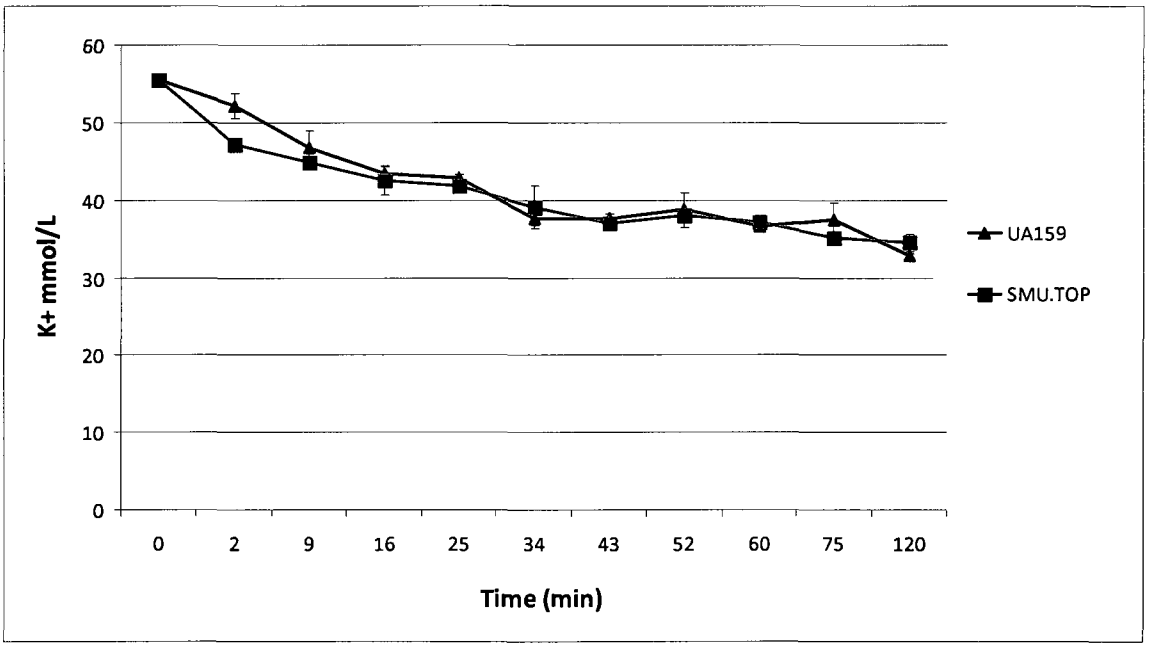

Figure 3.16. K^+ content of the filtrates of UA159 and SMUTOP over 2h at pH 5.5 in TYEG. Results presented are the average of 3 independent experiments ± standard error.

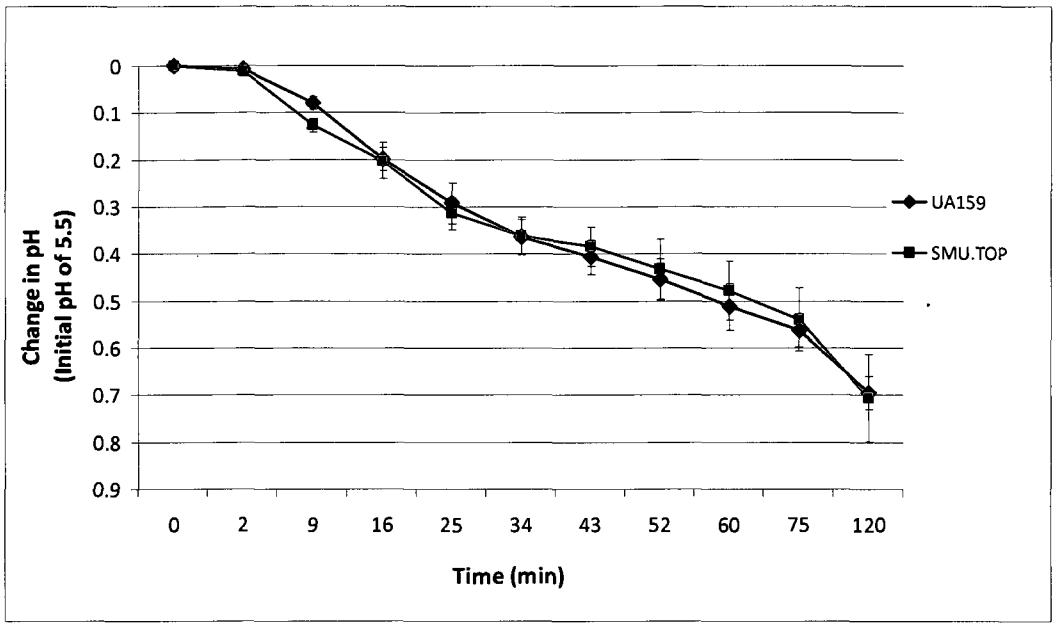

Figure 3.17. Change in pH profiles of the filtrates of UA159 and SMUTOP over 2 h at pH 5.5 in TYEG. Results presented are the average of 3 independent experiments ± standard error.

3.10: Potassium Content of Growth Medium

In order to further characterize the potassium uptake system encoded by the *trkB/ trk/ pacL* operon, a growth medium with defined potassium values was required in order to establish a means by which K^+ could be controlled. As such, various growth media utilized by our lab were subjected to K^+ analysis via atomic emission spectroscopy. Results indicated that the medium with the highest potassium concentration was minimal medium (MMG; refer to APPENDIX B), followed by TYEG (independent of pH) and THYE (Figure 3.18). Since potassium levels cannot be easily controlled in these media, a defined minimal medium (designated MMGK-) was established. More particularly MMGK- is comprised of the same components as MMG with the exception of the potassium-containing compounds which were substituted with their sodium equivalents.

This medium contained only trace amounts of potassium, so therefore, the K^+ concentration could be manipulated by supplementing with 1mM KCl.

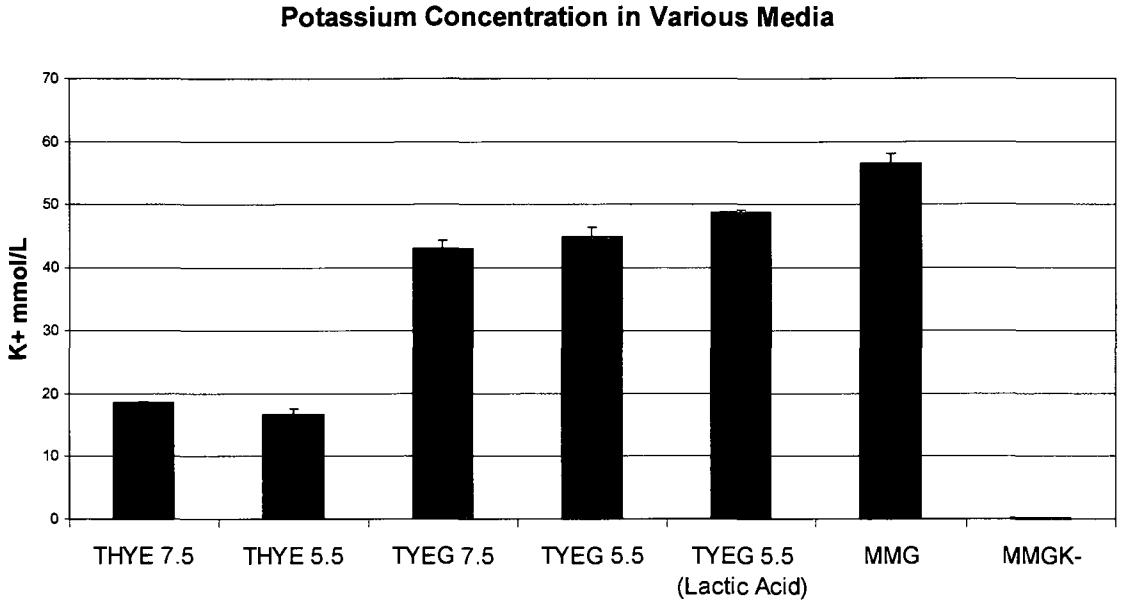

Figure 3.18. Potassium concentration (mmol/L) in THYE, TYEG, MMG and MMGK- media measured using atomic emission spectroscopy. Results are the average of a least three independent experiments ± standard error.

3.11: Growth Assays in MMGK-

Once MMGK- was defined and the potassium content was determined, growth assays were conducted in order to evaluate the ability of *S. mutans* UA159, SMUTKB, SMUPACL and SMUTOP to grow under growth limiting concentrations of potassium. As described previously, *pacL* shares significant homology with the KdpB subunit of *E. coli*, a scavenging system with high affinity for potassium. To further investigate whether the *trkB/ trk/ pacL* operon of *S. mutans* UA159 shared similar capabilities, growth was evaluated in MMGK- supplemented with 1M KCl to final concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9 and 1mM. These values were chosen based on previous data demonstrating no growth difference observed in the mutant strains and UA159 at 5 mM,

10 mM, 25 mM and 150 mM concentrations (APPENDIX A: Figure S1).Results from this assay are shown in Figures 3.19 and 3.20 and clearly demonstrate the necessity for potassium required by the cells. No growth was observed in the absence of potassium or at the 0.1 or 0.2 mM KCl concentrations. Growth was initiated in MMGK- at a final concentration of 0.3 mM KCl. Interestingly, this value equates to 11.73 μg/ml which is significantly lower than the value suggested by Cowman and Fitzgerald (1976). This group determined that at least 50 μg/ml of potassium was required for adequate levels of growth of *S. mutans*. Results also demonstrate that SMUTKB, SMUPACL and SMUTOP grew to higher final O.D. values than the parent strain, although none of the values for SMUPACL and SMUTOP were statistically significant. SMUTKB, however demonstrated statistically significant increased bacterial yield at 0.3, 0.7 and 0.9 mM KCl (p-value<0.03). Collectively, these results do not provide proof that the *trkB/ trk/ pacL* operon encodes a high affinity potassium uptake system. In fact, we observed that deletion of these genes may enhance growth under certain potassium limiting conditions.

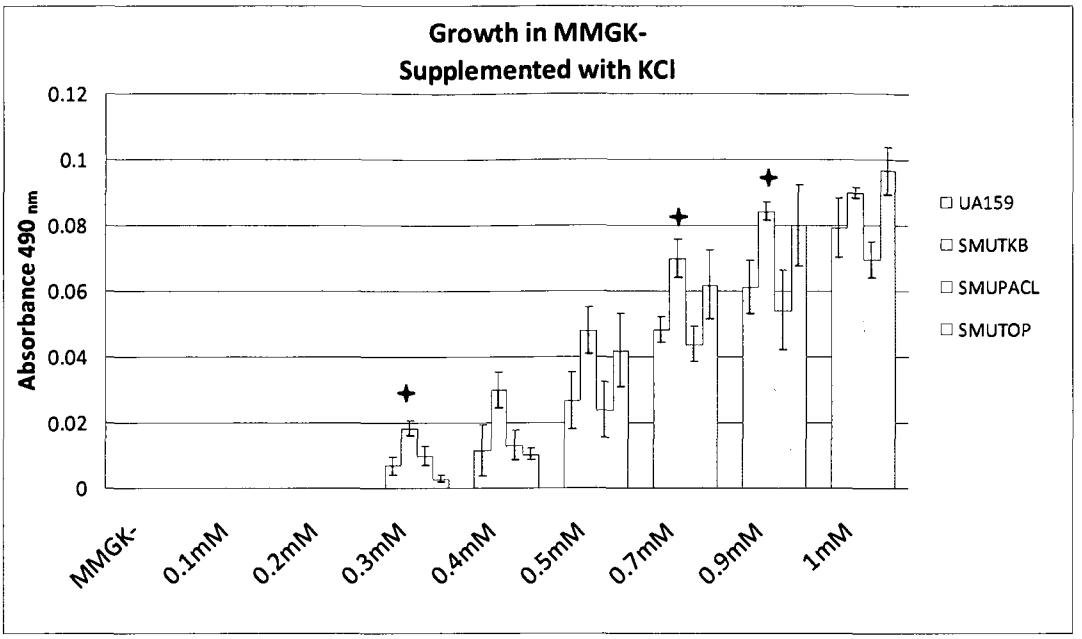

Figure 3.19. Growth of *S. mutans* UA19, SMUTKB, SMUPACL and SMUTOP in MMGK- supplemented with varying concentrations of KCl. Stars indicate statistical significance (p-value<0.03) Results shown are the average are 3 independent experiments ± standard error.

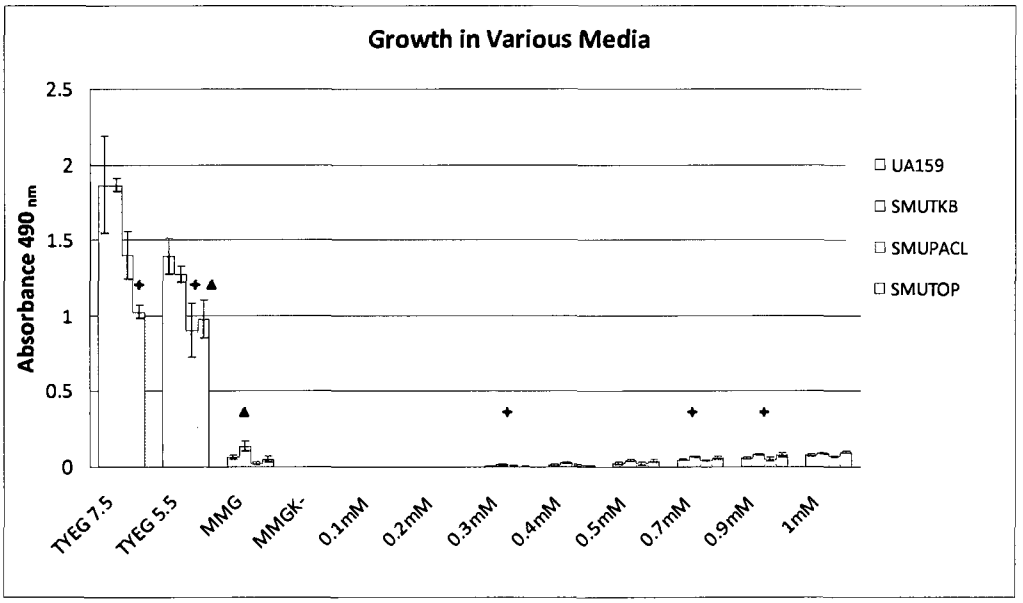

Figure 3.20. Growth of *S. mutans* UA19, SMUTKB, SMUPACL and SMUTOP in TYEG at pH 7.5 and 5.5, MMG and MMGK- supplemented with varying concentrations of KCl. Results shown are the average are 3 independent experiments ± standard error. Stars and triangles indicate statistical significance (p-values<0.03 and <0.05, respectively)

3.12: Potassium Transport in MMGK-

Although only mild growth could be attained in MMGK- supplemented with KCl, transport assays were conducted in this media to evaluate the ability of *S. mutans* UA159 and SMUTOP to transport K^+ under controlled concentrations of this required cation. Essentially, these assays followed the same procedure as the previously described transport assays, with the exception that prior to transport, cells were partially depleted of their potassium content in the hope of promoting potassium uptake (Figure 3.21.). Results from the depletion steps indicated significantly decreased potassium levels in SMUTOP at 3 h compared to UA159, further supporting the inability of the mutant to retain intracellular K^+ levels equal to that of the wildtype. Transport was evaluated in MMGK- in the first 120 sec after supplementation with 5mM and 25mM KCl, the results of which are shown in Figures 3.22 and 3.23 respectively.

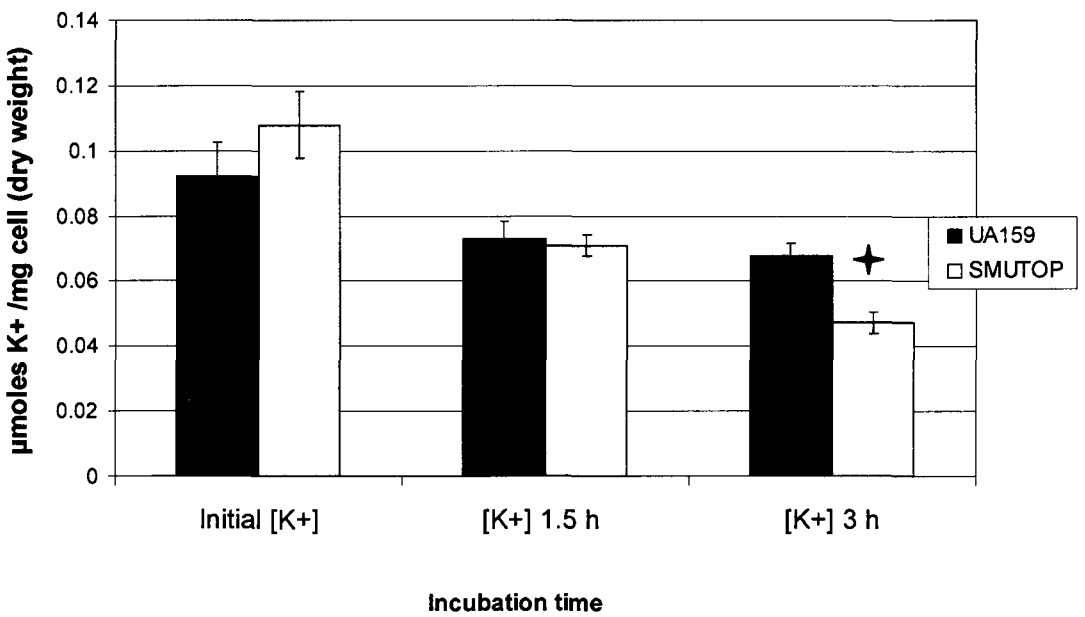

Figure 3.21. Potassium depletion of *S. mutans* UA159 and SMUTOP over a 3h incubation period in MMGK- at 37°C. Statistical significance (p-value<0.01) was observed after the 3 hr time point in SMUTOP compared to UA159. Results represent the average of three independent experiment ± standard error.

Interestingly, uptake at 5 mM was not observed under these conditions in either the wildtype strain or SMUTOP as indicated by consistent levels of potassium throughout the entire time period evaluated (Figure 3.22). SMUTOP did however have measurably lower levels of potassium at each time point, again suggesting that SMUTOP is incapable of achieving the same maximal K^+ content levels as the parent strain. Transport may not have been observed under these conditions due to limitations of other nutrients in the growth media itself. Unlike TYEG, growth in MMGK- is very limited and suggests that certain nutrients are required to initiate transport that are not present in MMGK-.

Transport in MMGK- supplemented with 25 mM KCl displayed slightly different results, whereby potassium uptake was only observed in the first 15 seconds. Upon continued incubation in the medium, UA159 and SMUTOP demonstrated gradually decreasing levels of potassium over time.

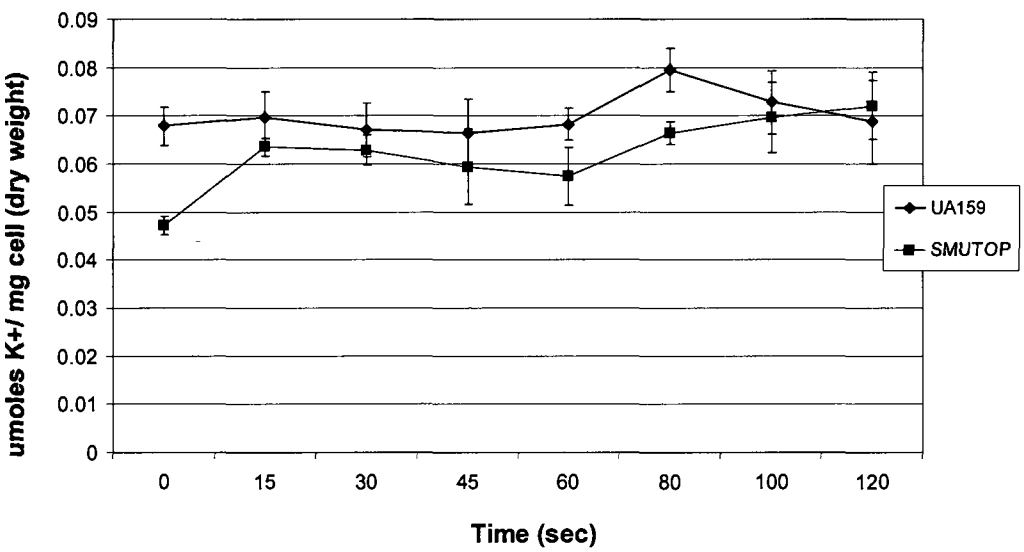

Figure 3.22. Potassium transport of *S. mutans* UA159 and SMUTOP in MMGK- supplemented with 5mM KCl. Results represent the average of 3 indepenedent experiments ± standard error.

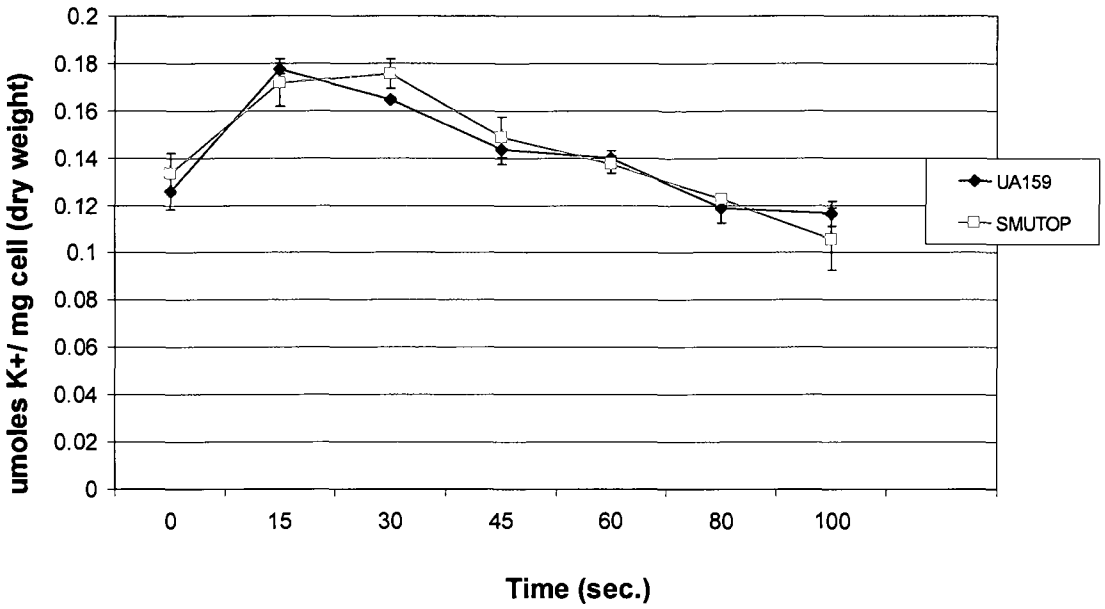

Figure 3.23. Potassium transport of *S. mutans* UA159 and SMUTOP in MMGK- supplemented with 25mM KCl. Results represent the average of 3 independent experiments ± standard error.

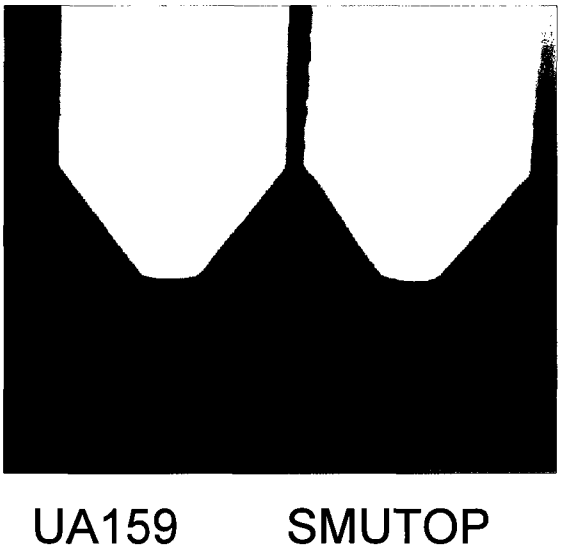

Figure 3.24. Standing cultures of *S. mutans* UA159 and SMUTOP incubated at room temperature overnight following transport assays at 25 mM in MMGK-. UA159 demonstrated a turbid culture vessel whereas SMUTOP exhibited opacity, with a majority of the cells collected at the bottom of the tube. These results are indicative of phenotypic effects involved in biofilm formation.

3.13: Biofilm Integrity

Since we observed that overnight culture of *S. mutans* UA159 and SMUTOP adhered to the culture tubes differently as depicted in Figure 3.24, we evaluated the involvement of the *trkB/ trk/ pacL* operon in biofilm formation during growth in MMGK-. This observation was further evaluated through simple biofilm assays. Results from these experiments clearly demonstrated a significant difference in biofilm formation between the SMUPACL and SMUTOP strains compared to the wildtype in their ability to form stable biofilms (Figure 3.25). Although biofilm growth was evident in wildtype and mutant strains prior to washing and processing, it became evident that the mutant biofilms (SMUPACL and SMUTOP) were severely compromised in their ability to adhere to the culture plate. Furthermore, the washing step included in the biofilm protocol, completely dissipated the biofilms formed by the SMUPACL and SMUTOP deficient mutants. This was observed in MMG media and in MMGK- at all concentrations of KCl. More specifically, compared to UA159, SMUPACL and SMUTOP demonstrated severely compromised biofilms at 0.5 mM and 150 mM KCl concentrations and in MMG. Biofilm integrity was slightly improved at the 1.0, 1.5 and 5.0 mM concentrations as indicated by the minimal presence of biofilm cells attached to the bottom of the microtitre plate (Figure 3.25).

Results also revealed that biofilms could not be established by any strain tested in the absence of potassium in the growth medium. Biofilm formation was induced when KCl was added to a final concentration of 0.5 mM in MMGK- as observed with the UA159 and SMUTKB strains. All mutant strains produced biofilms in THYE, which acted as the control to verify inoculum viability.

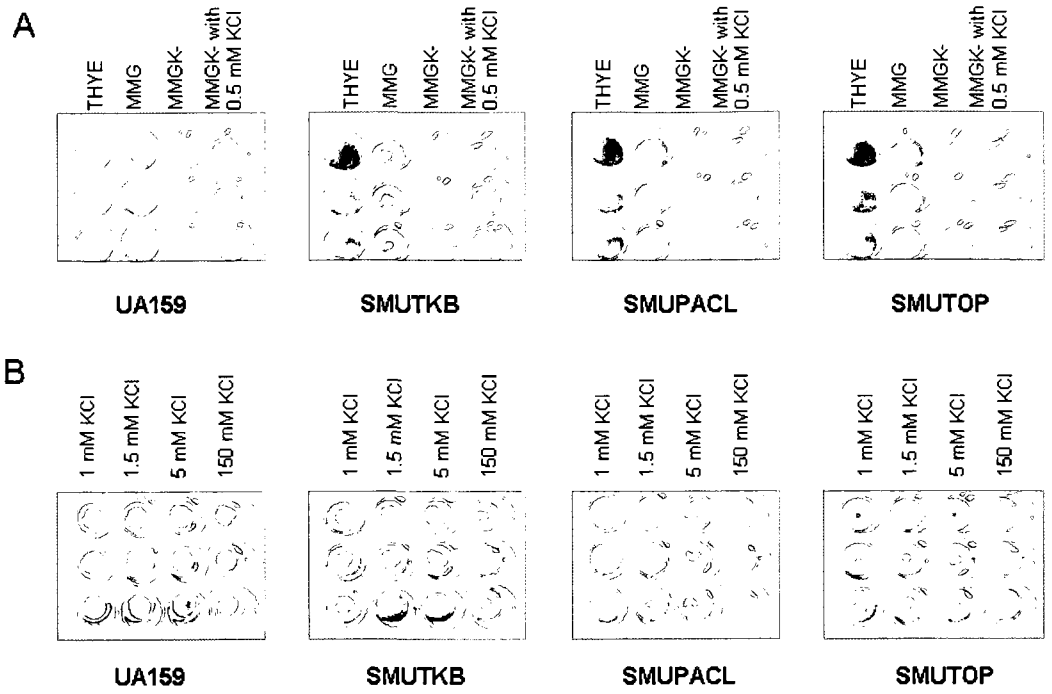

Figure 3.25. Effect of various growth media (A) and potassium concentrations in MMGK- (B) on biofilm formation using *S. mutans* UA159, SMUTKB, SMUPACL and SMUTOP. Biofilms were formed for 16h in 96-well microtitre plates, washed and stained with 0.1% crystal violet for 10 min.

CHAPTER 4: DISCUSSION

The remarkable ability of *S. mutans* to tolerate acid has been linked to a multitude of different mechanisms (refer to CHAPTER 1:LITERATURE REVIEW) that enable it to survive the pH fluctuations present in the oral cavity. The work presented here examined the involvement of the *trkB/trk/pacL* system in such mechanisms. DNA microarray analysis of *S. mutans* UA159 initially identified the *trkB/trk/pacL* system as being greater than two-fold upregulated at an acidic pH (5.5) in comparison to cells at a neutral pH (7.5). These results were corroborated by quantitative real-time PCR, confirming the genes designated *trkB, trk,* and *pacL* along with *trkA* and *trkH*, as up-regulated at pH 5.5 (Figure 3.2.). Collectively, the induction of these genes under low pH suggests their involvement in the acid tolerance strategies of *S. mutans.*

4.1 Bioinformatic Analysis Supports Potassium Uptake Functionality

Prior to the onset of bench experiments, bioinfomatic analysis of the FASTA protein sequence of each aforementioned gene was conducted in order to provide some insight into the possible functions of these gene products. The results of these searches are presented in Table 3.1, 3.2 and 3.3. Since the potassium systems in a multitude of microorganisms have been largely uncharacterized (Kuo 2005), including those presented in Table 3.1, homologues to the genes of interest were also noted in organisms that inhabit the oral cavity or in other environments that require acid tolerance mechanisms for survival. Interestingly, *trkB* shared 33% homology to Trk-A of *Acidothermus cellulolyticus*, a bacterium that resides in the acidic hot springs of Yellowstone National Park (Tucker *et al.*, 1989). Trk shared 37% identity to a hypothetical protein in *Clostridium spiroforme,* an organism that inhabits the human gastrointestinal tract. PacL

demonstrated 67% homology to H^+ /K^+ exchanging ATPase of *Lactobacillus casei*. TrkA and trkH demonstrated 80% and 71% identity with uncharacterized putative potassium transport proteins present in the chromosome of *Strepococcus salivarius,* a moderately acid tolerant species of the oral cavity (Table 3.1).

Collectively, these results suggest that transport proteins similar to *trkB, trk, pacL, trkH* and *trkA* of *S. mutans* UA159 are present in other acid tolerant bacteria, further implicating these genes in the acid survival strategies of this dental pathogen.

4.2 Multiple Potassium Systems Encoded by the *S. mutans* UA159 genome

Due to the importance of potassium as an essential cation, organisms such as *E. coli* possess 3 systems for potassium uptake that can accommodate the changing K^+ concentrations in the environment (Kuo, 2005). Therefore, the existence of multiple systems dedicated to the acquisition of this element would most likely be beneficial to an organism's survival and succession. Although K^+ transport in *S. mutans* has been largely understudied, analysis of the UA159 genome identified 3 putative transport systems. Adjic *et al.* (2002) proposed that a possible P-type ATPases (specific to Ca^+ or K^+) as well as two K^+ specific transporters are encoded in the chromosome. In addition to the genetic data, studies by Sato *et al.* (1989) proposed concomitant K^+ entry with glutamate via co-symport, thereby attributing potassium uptake to multiple distinct systems as represented in Figure 4.1. Evidence supporting the existence of multiple potassium transport systems is presented in this work, the results of which will be discussed in more detail below.

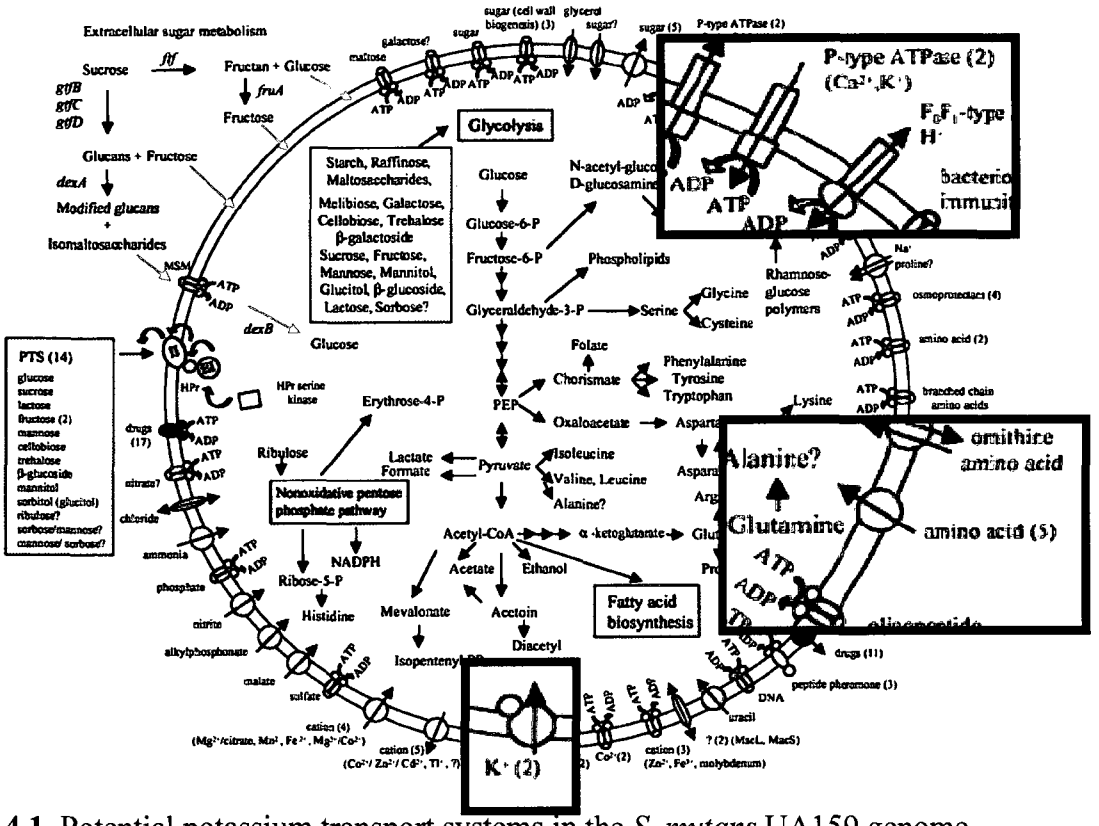

Figure 4.1. Potential potassium transport systems in the *S. mutans* UA159 genome. Modified from Adjic *et al.* (2002).

4.3: Mutations in the *trkB/trk/pacL* Locus Result in an Acid Sensitive Phenotype

In order to assess the role of the *trkB, trk* and *pacL* genes in *S. mutans'* aciduricity, deletion mutants were constructed in the UA159 wildtype strain and are shown in Table 2.1. After successful construction of these strains, mutant and UA159 cultures were subjected to three acid sensitivity experiments consisting of ATR, growth rate and terminal pH assays.

The ATR assay was used to determine the survival of SMUTRKB, SMUTRK and SMUTKB compared to parent strain UA159. In this assay, long-term (2 and 3 hrs) live/dead ratios established by exposure to a killing pH of 3.0 after a 1 hr adaptation period to pH 5.5 were examined. Results from these experiments demonstrated statistically significant decreased survival ratios in SMUTRKB and SMUTRK at the 2

and 3 hr time points when compared with the wildtype strain (Figure 3.9 and 3.10, respectively). A similar trend was noted for SMUTKB, although the result was not statistically significant (Figure 3.11). This suggested that genes comprising the *trkB/trk/pacL* locus are expressed as an acid adaptive response whose activity is required for maximum resistance to killing at pH 3.0. Moreover, this implies that under long-term exposure (2 and 3-h) to acidic conditions, potassium uptake is necessary to maintain elevated K^+ levels that likely confer maximum acidurance if in fact a K^+ uptake complex is encoded.

These conclusions are supported by the potassium accumulation profile assay, whereby the intracellular K^+ content of SMUTOP and UA159 was analyzed over a 2 hour time period at pH 5.5 (Figure 3.15). More specifically, these experiments were conducted in order to determine the extent of K^+ uptake in glycolysing cells at low pH. Notably, both UA159 and SMUTOP demonstrated elevated potassium levels at all time points following acid exposure when compared with the initial K^+ levels at time 0 (Figure 3.15). This suggests that potassium uptake occurs as a response to acid stress at pH 5.5.

When comparing UA159 and SMUTOP directly, SMUTOP had decreased levels of potassium at the initial time points (0-16) and final time points (60-120)(see Figure 3.15). Further discussion regarding initial potassium uptake will be provided below (refer to section 4.4). Statistically significant decreased levels of potassium were also observed during K^+ depletion prior to the transport assays conducted in 5 mM KCl (Figure 3.21), and during the transport assay itself conducted at this KCl concentration (Figure 3.22).Transport assays conducted at pH 5.5 with SMUTOP and UA159 also demonstrated statistically significant reduced levels of potassium content in the mutant

compared to the parent strains at all time points tested (Figure 3.12). Collectively, these findings further support the inability of SMUTOP to maintain maximum intracellular K^+ concentrations when compared to the wildtype strain.

Potassium uptake has been linked to the maintenance of intracellular pH through the adjustment of the transmembrane potential. It is believed that intracellular levels of potassium can essentially replace H^+, thereby interconverting the $\Delta\psi$ and diminishing the harmful effects of an acidified cytoplasm (Booth, 1985a; Trchunian *et al.*, 1986). Recently, it was shown that potassium uptake in *Corynebacterium glutamicum* via CglK was relevant to the activity of the respiratory chain and necessary for internal pH homeostasis under acidic conditions (Follmann *et al.*, 2009). In *S. mutans* Ingbritt, studies have determined that potassium must be present in the media in order for a large ΔpH to be established (Sato *et al.,* 1987). Furthermore, glycolysing cells of *S. mutans* at pH 5.0 could maintain an internal pH 1.37 ± 0.09 units more alkaline than the extracellular medium, only in the presence of K^+ (Daspher and Reynolds, 1992).

In light of these findings, we propose that the decreased levels of intracellular potassium exhibited by SMUTOP results in a compromised ability to combat acid stress due to the inability to interconvert $\Delta\psi$. This effect is illustrated in Figure 3.7 (further described below), whereby SMUTOP had statistically significant decreases in total biomass at pH 5.5 when compared to the wildtype strain. The rationale behind the inability of the gene deletions in the *trkB/trk/pacL* system to maintain maximum elevated intracellular K^+ levels can also be applied to the acid sensitive phenotypes observed with SMUTRKB, SMUTRK and SMUTKB in the ATR assays mentioned above. Extended exposure to increasing acidic conditions (consistent with the assay conducted at pH 3.0)

decreased the survival of adapted cells of SMUTRKB, SMUTRK and SMUTKB compared to the parent strain due to inactivation of the *trkB/trk/pacL* system. Essentially, this leads to diminished levels of cytoplasmic K^+, as K^+ uptake via the remaining potassium systems (evidence provided below) cannot accumulate potassium to the same degree as K^+ efflux is occurring. This effectively reduces the ability of the cell to maintain an alkalized intracellular compartment, ultimately leading to cell death. This mechanism seems to be relevant to the long-term acid tolerance survival strategies utilized by *S. mutans*, because elevated intracellular K^+ levels were recorded for the entire 2 h period following exposure to pH 5.5 for both UA159 and SMUTOP (Figure 3.15). Since the environmental conditions in this assay became increasingly more acidic, elevated K^+ levels may be required by the cell in order maintain an interconverted $\Delta\psi$, thus compensating for increases in the magnitude of the ΔpH and effectively preventing H^+ re-entry. Cells in this assay were metabolically active as illustrated by the decreased pH of the filtrate as displayed in Figure 3.17.

Further evidence for these conclusions is provided by the growth rate experiments which examined growth under neutral and acid pH in TYEG medium. These assays demonstrated impaired bacterial yield compared to the parent strain in all mutants tested with the exception of SMUTRKA and SMUTRKH. Since SMUTRKA and SMUTRKH lacked an acid-sensitive phenotype, further experiments focused on the acid-sensitive mutants, SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP. Acid sensitivity of these mutants can again be explained by their inability to acquire potassium with the same efficiency as the wildtype strain. As mentioned, this leads to a decrease in cytosolic pH which can negatively affect a number of physiological processes, including

transcription and translation of essential proteins and the proper functioning of metabolic enzymes, ultimately leading to the decreased yield observed with SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP.

Acid sensitivity of the mutant strains was also demonstrated by the terminal pH assays. Overnight cultures of SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP had small but statistically significant elevated pH values compared to UA159 when growth was initiated under pH 5.5. These results can be interpreted in a number of ways. First, elevated terminal pH values could result from a diminished ability of the mutants to produce metabolic acid end-products (i.e. lactate) that would acidify the medium, thus affecting glycolytic rates. Alternatively, elevated pH levels may simply result from metabolic arrest, whereby acid production is halted at a higher pH value in the mutant than in the wildtype strain due to increased sensitivity to the acid. Lastly, a decrease in overall bacterial yield would generate elevated pH levels due to lower amounts of acid produced by fewer cells. With concern to the latter, this explanation is strongly supported by the statistically significant decreases in total biomass observed in the SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP strains at pH 5.5 (Figure 3.5 and 3.7). These results provide further support for the involvement of *trkB, trk* and *pacL* in acid tolerance.

To address the possibility that the elevated terminal pH's observed in the mutant strains were the result of an effect on acid production, glycolytic assays were performed. These experiments found no differences in the glycolytic rates of SMUTRKB, SMUTRK and SMUTKB compared to the UA159 parent strain. This demonstrates that a deficiency in *trkB* and *trk* does not affect the ability of the cells to produce acid via sugar

metabolism. Similar results were obtained with an entire deletion of the *trkB/trk/pacL* operon represented by SMUTOP, whereby acid production was consistent with wildtype cells at pH 5.5 (see Figure 3.17). These results confirm that the elevated pH levels observed under acidic conditions are the result of a decreased overall biomass as observed in SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP (Figures 3.5 and 3.7).

Collectively, the data obtained from the ATR, potassium accumulation, growth curve and terminal pH assays demonstrate that expression of the *trkB/trk/pacL* system under acid results in maximum bacterial yield, decreased terminal pH and increased survival rates upon exposure to pH 3.0. These systems complement other pre-existing mechanisms of acid tolerance present in *S. mutans* (refer to CHAPTER 1: DISCUSSION) and likely contribute to its succession in the acidified plaque environment.

With respect to the roles played by the individual genes comprising the *trkB/trk/pacL* locus in the acid tolerance strategies of *S. mutans*, results from the previously discussed ATR, growth curves and terminal pH assays provide little insight and remain to be elucidated. This was demonstrated by the absence of statistical differences in the total biomass and terminal pH values amongst the SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP mutant strains when compared to each other. From this, one can conclude that each respective gene is required for optimal activity of the system. The co-transcription assay demonstrated that genes comprising the *trkB/trk/pacL* locus are transcribed together as a single tricistronic transcript (Figure 3.3). This suggests that these gene products operate as a functional unit. Further evidence in this regard is supported by bioinformatic analysis by the STRING function. This program

predicted functional partners for the genes encoded within the *trkB/trk/pacL* system and also implicated members of the *trkA/trkH* locus as encoding possible interacting protein candidates (Table 3.2).

Analysis of the *S. mutans* UA159 genome reveals an interesting but not unusual arrangement of the *trkB, trk, pacL, trkA* and *trkH* genes in *S. mutans* in relation to the *trkA, trkE, trkG* and *trkH* genes of *E. coli* (Figure 3.1). In the genetic arrangement of both organisms, the genes encoding these potassium transport proteins are either partly or entirely separated on the chromosome (refer to Figure 3.1 for genetic arrangement in *S. mutans*). For proper function, the Trk system of *E. coli* requires the interaction of at least three components including *trkA, trkE* and either *trkG* or *trkH* (Epstein, 2003). These proteins are transcribed separately from one another and are inter-dependent for attachment to the cytoplasmic membrane (Bossmeyer *et al.*, 1989). If similar interactions in *S. mutans* between *trkB, trk, pacL, trkA* and *trkH* exist, as suggested by STRING (Table 3.2), these genes may in fact compliment each other during activity. This may explain the small but statistically significant acid sensitive phenotypes and elevated terminal pH values observed with SMJUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP. Alternatively, the genetic separation of these genes and phenotypes observed may also be explained due to the existence of two or more potassium uptake systems. This will be discussed further in section 4.5.

4.4: The *trkB/trk/pacL* system Has Limited Effect on Glycolytic Activity and Growth Rates

The glycolytic rate assays showed that deletion of the *trkB* and *trk* genes had limited effects on the glycolytic rates of strain SMUTKB (Figure 3.8). Similarly, entire

operon deletion represented by SMUTOP had little effect on its ability to reduce the pH when comparedwith the wildtype strain (Figure 3.17). Upon initial analysis, these findings seem to contradict the observations of Daspher and Reynolds (1992). In addition to demonstrating that potassium was required to maintain an alkaline intracellular compartment, Daspher and Reynolds (1992) also determined that this established pH gradient dictated optimal glycolytic activity. We have already demonstrated that potassium levels are reduced in SMUTOP compared with UA159 and therefore with deficiencies in *trkB, trk*, and/or *pacL* expression, one might expect to observe decreased glycolytic activity as well. This would follow from an inability to interconvert $\pm\psi$, resulting in a compromised internal pH. Interestingly, these results were not observed in the mutant strains, as SMUTKB and SMUTOP had minor effects on the glycolytic rates when compared with the wildtype (Figures 3.8 and 3.17, respectively). This suggests that although deletion of any or all of the genes comprising the *trkB/trk/pacL* system resulted in decreased survival rates, decreased biomass, elevated terminal pH and reduced K^+ levels, these genes are not a contributing factor to glycolytic activity.

Further investigation into these results reveals that the K^+ transport rates of UA159 and SMUTOP were not only similar to each other, but were also similar between neutral and acidic conditions (Figure 3.14). These rates were calculated between 2 and 5 minutes for both UA159 and SMUTOP at pH 5.5 and pH 7.0 because K^+ uptake was maximal for both strains and conditions at this time point (Figures 3.12 and 3.13). If potassium uptake was an initial response to acid shock, it would follow that uptake rates of this cation would be increased under acidic conditions when compared to a neutral environment in order to provide maximum resistance. Since similar rates of potassium

uptake were observed at pH 7.0 and pH 5.5, initial potassium acquisition may be required by the cells for reasons other than acid tolerance and is therefore not a direct response to acid shock.

In a recent study conducted by Lee *et al.* (2007), enzyme IIA^{Ntr} of the PTS in *E. coli* was shown to regulate activity of the TrkA protein. This suggests a specific relationship between general metabolic processes and K^+ transport, which was shown to act as a signal, having regulatory roles in the transcription and activity of certain proteins involved with nitrogen catabolism (Lee *et al.*, 2007). In *Bacillus subtilis*, the presence of potassium in the medium directly affected protein synthesis and caused markedly affected growth rates in the *B. subtilis* mutant strain, which had lost its normal capacity to acquire K^+ (Willis and Ennis, 1968). Similar to the results presented here by SMUTOP, the mutant strain in *B. subtilis* had similar uptake rates as the parent, however its ability to retain and accumulate potassium was diminished (Willis and Ennis, 1961). That said, initial potassium uptake by *S. mutans* may also be required for similar functions since potassium was shown to be rapidly taken up by glycolysing cells of *S. mutans* UA159 at either pH 5.5 or pH 7.0 (Figures 3.12 and 3.13). *S. mutans* must therefore possess other transport systems that contribute to this purpose (section 4.5).

The induction of the *trkB/trk/pacL* system as a long term acid tolerance strategy is further supported by examining the relationship between potassium uptake and growth. Previous studies by Cowman and Fitzgerald (1975) have demonstrated that potassium is essential in the media for adequate growth of *S. mutans*. These ideas are further supported in this work by growth assays conducted in the defined medium designated MMGK- supplemented with various concentrations of potassium. We observed that overall growth

(both planktonic and biofilm cells) in the SMUTKB, SMUPACL, SMUTOP and UA159 strains was dependent on the presence of potassium in the medium and was similar in each of the K^+ concentrations tested (Figure 3.19). If the *trkB/trk/pacL* system is essential for potassium acquisition involved in general growth initiation mechanisms, then gene deletions in this system would result in severe growth deficiencies, regardless of the medium used. In contrast to this, mutant strains exhibited equal, if not more substantial growth than the wildtype strain in MMGK- supplemented with varying concentrations of 1 M KCl (Figure 3.19).

Similar observations were observed in the growth rates observed in TYEG at pH 7.5 and 5.5, where adequate growth was observed for all strains tested: SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP (Figures 3.4 and 3.6). Furthermore, all mutants had similar growth rates to those of the parent under both neutral and acid conditions calculated as the mean generation time of each strain (data not shown). Since the only differences observed amongst the conditions tested was the decrease in overall bacterial yield at pH 5.5 in each of the mutant strains relative to wildtype, we can conclude that the *trkB/trk/pacL* system functions as a long term acid tolerance mechanism. The absence of this system results in an inability to retain elevated potassium levels, thus leading to the acid sensitive phenotypes observed. The absence of this system however, has no effect on growth rate or glycolytic activity, therefore suggesting that other systems can compensate for its deletion, at least with regard to these processes.

Some insight into the specific characteristics of the *trkB/trk/pacL* system was also provided by our growth assays. Since equal or greater growth in MMGK- supplemented with KCl was observed in SMUTKB, SMUPACL and SMUTOP compared to the parent

UA159, we can conclude from these results that the *trkB/trk/pacL* system does not function as a high affinity K^+ system. Bioinformatic analysis determined that the *trkB, trk* and *pacL* genes contain conserved domains that are consistent with components of the Trk and Kdp systems of *E. coli* (Table 3.2). In *E. coli*, studies have shown that the Kdp system can scavenge for potassium even when concentrations exist in the nM range (Bramkamp *et al.*, 2007). If in fact a high affinity system similar to that of Kdp was encoded by the *trk, trkB* and *pacL* genes, then strains deficient in these gene products should demonstrate a decreased ability to grow when potassium concentrations are low. However, these results were not observed, suggesting that this system does not likely function as a sole high affinity K^+ transport system. Such a system in *S. mutans* may prove to be unimportant with concern to biological relevance, since potassium levels in the oral cavity exceed 570 ug/ml (Cowman and Fitzgerald, 1975).

Collectively, this work implicates various roles for general K^+ uptake in *S. mutans* physiology. First, potassium is essential for growth. In addition, initial uptake of K^+ may be utilized for metabolic and physiological functions. This work demonstrates that the *trkB/trk/pacL* system is induced at pH 5.5 and is linked to long term acid resistance via elevated potassium retention. This ultimately contributes to maximum bacterial yield and increased survival at killing pH values (~3.0), thereby providing maximum protection under acidic conditions.

4.5: Evidence for Multiple Potassium Transporters in *S. mutans*

Transport assays conducted under neutral pH (7.0) and acidic (pH 5.5) conditions with the SMUTOP and the UA159 parent strains demonstrate the existence of potassium

transport systems other than the *trkB/trk/pacL* system in *S. mutans* UA159. As previously discussed, intracellular K^+ levels were diminished in the mutant at pH 5.5 compared to the wildtype. These results can be attributed to an inability of the mutant to retain potassium, but not to an inability of the mutant strain to transport potassium, since K^+ uptake was still observed over the 10 minute time period in SMUTOP (Figure 3.12). Potassium uptake rates were similar at pH 7.0 and pH 5.5 (Figure 3.14) demonstrating that the *trkB/trk/pacL* locus is not the sole active potassium uptake system in the UA159 genome.

The data presented in this study further support the existence of multiple potassium systems encoded by the *S. mutans* UA159 genome when revisiting the potassium accumulation profile (Figure 3.15). SMUTOP, characterized by an entire deletion of the *trkB/trk/pacL*, still demonstrated an ability to take up potassium despite the absence of this system. Although initial (0-16) and (60-120) and final intracellular K^+ levels were lower than those expressed by UA159 (Figure 3.15), potassium levels in SMUTOP could return to near normal levels to those exhibited by the wildtype during the 25-53 minute time period. These results once again confirm that other K^+ uptake systems are present that can at least partially compensate for the loss of the *trkB/trk/pacL* system. The interaction between the *trkB/trk/pacL* system and the other predicted potassium transporters (section 4.2) suggests the following explanation for the reduced K^+ levels observed. Any deficiencies in intracellular K^+ content observed with SMUTOP relative to UA159, is likely due to changes in the overall rate of K^+ uptake compared with potassium efflux. Deletion of the *trkB/trk/pacL* system therefore alters this dynamic equilibrium and results in the diminished levels of potassium observed with SMUTOP. This may also

explain the small but statistically significant decreases observed in total bacterial yield and increases in terminal pH values obtained for SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP compared to UA159. Again, the presence of multiple K^+ uptake systems can be attributed to these small differences as *S. mutans* UA159 has the ability to compensate for the deficiencies in *trkB*, *trk* and *pacL* and therefore does not result in more drastic phenotypes under acid stress. Another explanation to consider is the expression of *trkA* and *trkH*, which were also induced under acidic pH (Figure 3.2). This system may complement the function of the *trkB/trk/pacL* locus or operate independently.

4.6: Transport Assays in MMGK-

Transport assays conducted in MMGK- were also performed in order to determine if the *trkB/trk/pacL* system functions at 5 mM and 25 mM K^+ (Figures 3.22 and 3.23 respectively) after the cells were partially depleted of intracellular potassium (Figure 3.21). These concentrations were chosen, because at least 25 mM of potassium (final concentration) was required for optimal glycolytic activity in the studies conducted by Daspher and Reynolds (2000). Results from our transport assays were inconclusive, as potassium transport levels remained somewhat consistant in the 5 mM transport profile (Figure 3.22) and K^+ loss was actually observed in the 25 mM assay (Figure 3.23). The lack of transport may be attributed to the particular constituents of the media and the absence of certain components that stimulate uptake or to other unbeknownst physiological factors.

4.7: Effects of the *trkB/trk/pacL* system in Biofilm Formation

In addition to the role in acid tolerance, preliminary results indicated effects of the *trkB/trk/pacL* system on biofilm formation and integrity. SMUPACL and SMUTOP were unable to form stable biofilms when compared with *S. mutans* UA159 in MMGK-supplemented with various concentrations of KCl. These results suggested that the PacL gene product is a major contributor to the phenotype observed, since SMUTKB did not produce similar results and the genetic difference between this strain and SMUPACL and SMUTOP is the absence of *pacL* (Figure 3.25). Furthermore, this implies that PacL is required for maximum biofilm integrity and adherence.

A recent study involving *Bacillus subtilis*, demonstrated that potassium leakage could induce biofilm formation (López *et al.*, 2009). Furthermore, it was proposed that lowered intracellular K^+ levels acted as a signal that was received by a membrane protein kinase designated KinC; a regulatory system involved in the expression of genes that govern biofilm formation (López *et al.*, 2009). Notably, a BLASTp search using KinC of *Bacillus subtilis* against the UA159 genome identified the histidine kinase VicK as its closest homologue (30% identity). Interestingly, a BLASTp search using KdpD and KdpE, the respective histidine kinase and response regulator responsible for regulation of the Kdp K^+ uptake system in *E. coli* was also conducted (Ballal *et al.*, 2007). These results also identified *S. mutans'* VicK as the closest homologue to KdpD, exhibiting 29% identity. VicR shared 37% identitiy with KdpE. These findings in combination with the homology expressed by PacL with KdpB (28% identity), suggest that a similar regulatory system may be present in *S. mutans*. The signal sensed by the KdpD/E was

believed to be a reduction in turgor pressure of the cell, however the precise effector for this regulatory system is still unknown (Hamaan *et al.*, 2008).

In *S. mutans*, studies conducted by our lab have characterized the VicK/R TCSTS which affects multiple virulence properties, including adhesion and biofilm formation (Senaheera *et al.*, 2005, 2007). Relevant to this study, is the effect that the VicK deletion mutant had on biofilm formation, where aberrant and easily detachable biofilms were produced when compared to the wildtype strain. Since similar phenotypes were observed with the SMUPACL and SMUTOP mutants in addition to the aforementioned bioinformatic search results, it can be suggested that the VicK/R system may have a regulatory role in expression of the *trkB/trk/pacL* system during biofilm formation. Furthermore, it is interesting to note that in the *S. mutans'* UA159 genome the VicK/R system is located adjacently to *glnQHMP* (Figure 4.2), genes that encode a glutamate transporter. This genetic arrangement may suggest a possible relationship between these two systems and can link VicK/R to potassium acquisition. Future studies with regard to the implications will be described in CHAPTER 5.

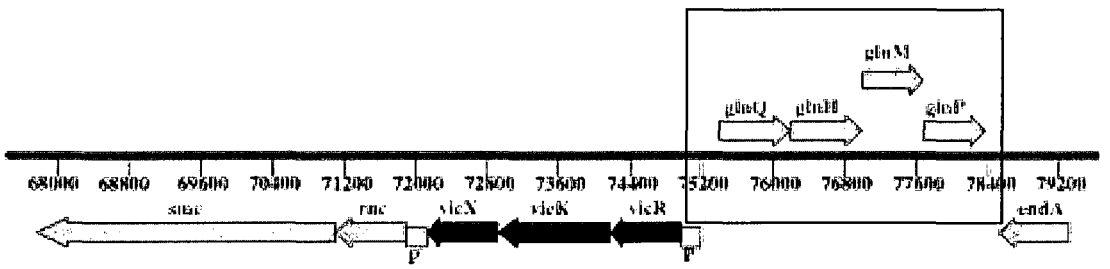

Figure 4.2. Structural organization of *vicRKX* and the glutamate transporter encoded by *glnQHMP*. Modified from Senadheera *et al.* (2005).

In accordance with the results presented by López *et al* (2009), the absence of the *trkB/trk/pacL* potassium uptake system may be involved in biofilm formation because K^+

uptake is diminished in SMUPACL and SMUTOP, as previously described. Therefore, cells of these strains would exhibit decreased amounts of intracellular K^+ and thus leakage of this cation would not be as significant as the wildtype cells. The mechanisms involved in this process are probably different than those involved in the acid induction of the *trkB/trk/pacL* system, which may explain the biofilm phenotype exhibited by SMUTKB, which was similar to the parent strain. Studies in these areas regarding the individual function of the genes encoded by the *trkB/trk/pacL* locus would need to be conducted in order to address this issue. Regardless, these results provide a foundation for further research in this area and can potentially contribute to a wealth of information that associates potassium uptake with biofilm formation and integrity.

In conclusion, this work links the *trkB/trk/pacL* system to the long-term acid tolerance of *S. mutans* UA159. Furthermore, acid tolerance is conferred by the ability of the cell to maintain elevated levels of intracellular K^+, which can function to interconvert the transmembrane potential, thus maintaining an intracellular pH that is more alkaline than the external media. Lastly, the physiological role of potassium accumulation has been extended to biofilm formation, with defects in biofilm integrity observed in the SMUPACL and SMUTOP strains.

CHAPTER 5: FUTURE WORK

To further investigate the role of the *trkB/trk/pacL* system and conclusively determine whether potassium uptake is, in fact, a long term acid tolerance mechanism, the intracellular pH of SMUTOP and UA159 in relation to the intracellular K^+ concentration should be determined over time. More specifically, the sustained levels of elevated K^+ observed in the potassium accumulation profiles (Figure 3.15) should be related to changes in pH_i and pH_o experienced by the cells. This can be accomplished through the distribution of the weak acid, [^{14}C] benzoic acid which can be delivered across the membrane with the use of the antibiotic gramicidin (Daspher and Reynolds, 1992). Gramicidin, which functions as a monovalent cation selective ionophore that causes transmembrane equilibrium between the intracellular and extracellular pH levels, should also be added to cells taking up K^+. These results would demonstrate the effects of potassium uptake on the ability of the cell to maintain an alkalized intracellular compartment, effectively contributing to the acid tolerance of the organism.

Central to the investigation of potassium uptake as a means to combat acid stress is the identification of the various systems responsible for potassium uptake present in the *S. mutans* UA159 genome. This work has suggested the possibility of three of these systems represented by the *trkB/trk/pacL* and *trkA/trkH* loci as well as possible concomitant entry with glutamate. The construction of knockout mutations utilizing this information can therefore be extremely beneficial for future studies in terms of isolating certain systems for investigation. For example, to truly examine the functionality of the *trkB/trk/pacL* system, a double knockout mutant should be constructed that is characterized by the absence of the *trkA/trkH* system and the gluatamate transport, considering that these systems are involved in potassium transport. These results may

even identify other yet to be characterized potassium systems that have not been detected by genetic or experimental data.

Although preliminary acid stress experiments conducted with single deletion mutants of the *trkA* or *trkH* genes yielded similar phenotypes as the wildtype strain, it would be interesting to determine the effects of a double knockout mutant in both the *trkB/trk/pacL* and *trkA/trkH* loci on acid tolerance. As previously mentioned, the genetically separated genes that encode the Trk system in *E. coli* have been shown to interact with each other as a functional unit (Bossemeyer *et al.*, 1989). It is therefore reasonable to suggest that similar interactions between the separated *trkB/trk/pacL* and *trkA/trkH* loci may also hold true in *S. mutans*. To further support these inquiries, physical protein-protein interaction software such as GARDEN (Global and Restrained Docking Exploration Nexus), which can effectively use conformational refinement within amino acid residues to model protein-protein complexes should be consulted. Alternatively, if the *trkB/trk/pacL* and *trkA/trkH* systems encode two separate potassium transporters, then deletion of these systems may lead to more drastic acid-sensitive phenotypes than those observed in the experiments presented in this work involving the SMUTRKB, SMUTRK, SMUTKB, SMUPACL and SMUTOP strains.

The characterization of the *trkB/trk/pacL* system can be further realized with competitive assays, using ions of the same elemental group; particularly Rb^+ and Cs^+. If this system can be isolated through knockout mutations as previously discussed, these assays can determine the affinity and specificity of the *trkB/trk/pacL* system for K^+ ions and can be conducted in a buffer described by Noji *et al.* (1988) where the constituents of the solution can be carefully regulated. Of the K^+ transport systems in *E. coli*, Kdp

expresses the highest affinity for K^+ followed by Trk (Epstein and Kim, 1971). Kup is the least specific, possessing the ability to transport Cs^+ as well as potassium (Epstein and Kim, 1971).

With concern to biofilm formation, gene expression should be quantified via rtPCR to determine the expression of the *trkB, trk* and *pacL* genes in biofilm development. From these results, biofilm assays can be conducted with the antibiotics gramicidin and surfactin, the use of which can effectively release intracellular cations into the media, particularly potassium. In *Bacillus subtilis*, these antibiotics induced biofilm formation through the proposed involvement of KinC, a membrane kinase that is involved in the expression of genes that can dictate biofilm formation as a response to decreased intracellular potassium levels (López *et al.*, 2009). If potassium uptake is involved in this process, the use of the aforementioned antibiotics in the UA159 parent strain harbouring the *trkB/trk/pacL* should demonstrate similar phenotypes to the SMUPACL and SMUTOP strains observed in the work presented. These assays should also include the VicK mutant strain to examine the relationship between the VicK system and the *trkB/trk/pacL* system. Furthermore, real-time PCR can effectively determine gene expression between the wildtype and VicK- deletion strains using real-time primers sets specific for the *trkB, trk*, and *pacL* genes. Comparing the expression of these genes from cDNAs isolated from parent and VicK strains, could produce very interesting results.

Overall, further study of potassium uptake and accumulation via the *trkB/trk/pacL* and *trkA/trkH* in *S. mutans* UA159, may provide valuable information regarding acid tolerance mechanisms and other virulence properties. Increasing knowledge in this field may prove valuable not only in the general knowledge regarding the physiological

processes engaged by *S. mutans* but also in the development of therapeutic techniques that can ultimately limit the capacity of this dental pathogen to induce caries.

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APPENDIX A: Supplemental Material

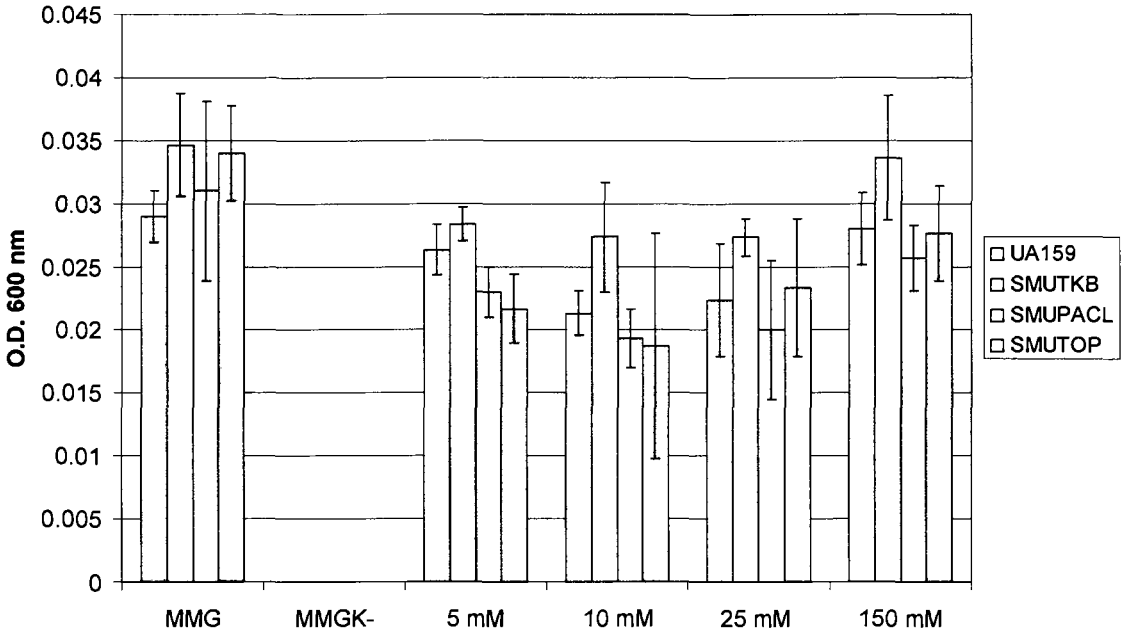


Figure S1. Growth of *S .mutans* UA19, SMUTKB, SMUPACL and SMUTOP in MMG and MMGK- supplemented with varying concentrations of KCl. Results shown are the average are 3 independent experiments ± standard error.

Hairpin Loop Structures Identified Using MacVector

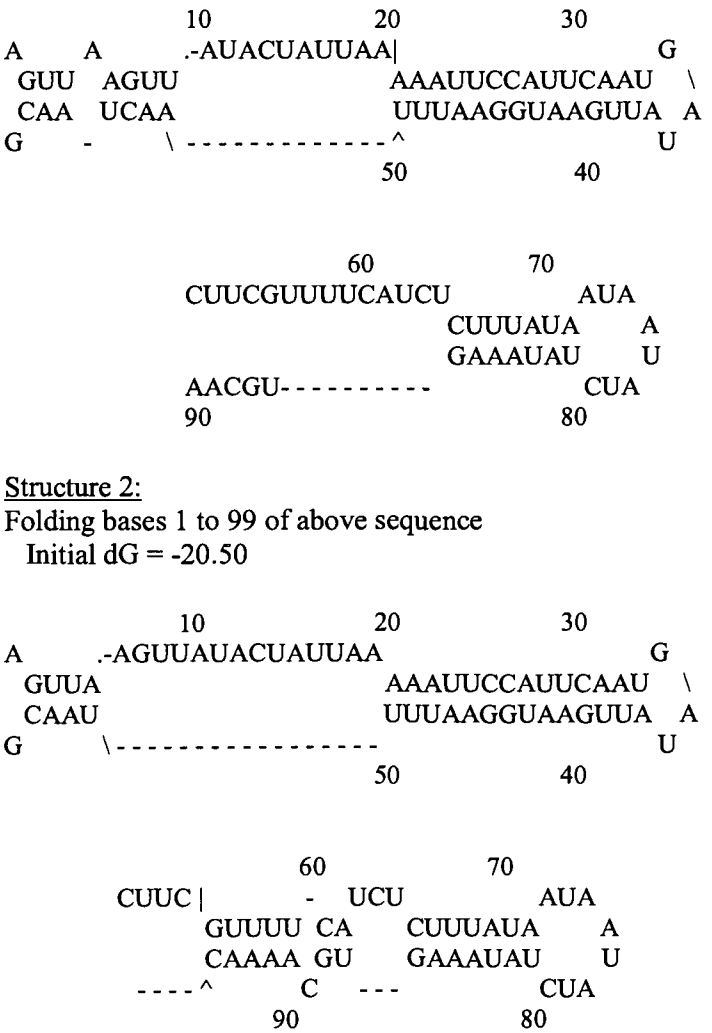
Intergenic Region Scanned (between SMU.1560 and TrkB (SMU.1561)):
1483565-1483663

DNA Sequence:
AGTTAAGTTATACTATTAAAAATTCCATTCAATGATATTGAATGGAATTTCTTCGTTT
TCATCTCTTTATAATAATATCTATAAAGTGCAAAACTAACG

RNA Sequence:
AGUUAAGUUAUACUAUUAAAAAUUCCAUUCAAUGAUAUUGAAUGGAAUUUCUUC
GUUUUCAUCUCUUUAUAAUAAUAUCUAUAAAGUGCAAAACUAACG

UAA denotes stop codon

Structure 1:
Folding bases 1 to 99 of above sequence
Initial dG = -20.60



Structure 2:
Folding bases 1 to 99 of above sequence
Initial dG = -20.50

Figure S2: Hairpin Loop Structures Identified by MacVector

APPENDIX B: Minimal Medium (MMGK-)

(modified from Fujikawa *et al.* (1978))

L-Glutamic acid	2.0 g
Glucose	10.0 g
Trizma maleate	11.9 g
L-Cysteine-HCl	0.2 g
L-Leucine	0.9 g
NH_4Cl	1.0 g
Na_2HPO_4 (dibasic)	3.5 g
NaH_2PO_4 (monobasic)	1.5 g
$NaHCO_3$	4.2 g
Sodium pyruvate	0.6 g
1000x vitamins*	1.0 ml
1000x $MnCl_2$	1.0 ml
1000x $FeSO_4$	1.0 ml
500x $MgSO_4$	2.0 ml
	1L

***1000x vitamin stock:**

Riboflavin	20 mg
Thiamine-HCl	10 mg
Bitoin	2 mg
Nicotinic Acid	20 mg
p-Aminobenzoic acid	2 mg
Calclium pantothenate	10 mg
Pyridoxal-HCl	20 mg
	20 ml