UNDERSTANDING THE ROLE OF POTASSIUM AND ITS TRANSPORT SYSTEMS IN STREPTOCOCCUS MUTANS

BY

GURSONIKA BINEPAL

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Faculty of Dentistry
University of Toronto

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I. ABSTRACT

*Streptococcus mutans* is a cariogenic bacterium found in the human dental plaque biofilm. Amongst its various attributes, the ability to metabolize a variety of dietary sugars to produce acid, resistance to pH stress by activating an acid tolerance response, ability to produce sticky glucan polymers and the ability to naturally take up genetic material are important properties of *S. mutans*. One way bacteria withstand external environmental stress is by accumulating intracellular potassium. In the genome of *S. mutans*, four putative potassium transport systems were identified, namely, the Trk1 (*trkB/trk/pacL*) system, Trk2 (*trkA/trkH*) system, GlnQHMP, and Kch (SMU_1848). The hypothesis of this study was that *S. mutans* transports potassium using specific transport systems, and relies on $K^+$ homeostasis for regulation of its multiple physiological and virulence attributes. To investigate the role of these systems, strains with deletions of each system were constructed. The most profound effects on the growth, pH tolerance, genetic transformation and biofilm formation were observed when the Trk2 system was absent compared to wild type UA159 and *trk1-, glnQHMP-* or *kch* deficient strains.
Furthermore, deletion of the Trk2 transport system resulted in reduced intracellular potassium accumulation. To confirm the role of Trk2 as a potassium uptake system, this system was cloned, expressed and functionally characterized in the *Escherichia coli* TK2420 mutant strain lacking constitutive potassium uptake systems. Collectively, this study identified Trk2 as the key potassium transport system in *S. mutans*, and demonstrated its importance and the role of potassium homeostasis with regard to biofilm formation, stress tolerance, growth and genetic transformation. This work constitutes the first characterization of the Trk systems in oral streptococci.
II. ACKNOWLEDGEMENTS

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AEP</td>
<td>acquired enamel pellicle</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>acid tolerance response</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CDM</td>
<td>chemically-defined medium</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFUs</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CSP</td>
<td>competence-stimulating peptide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
</tr>
<tr>
<td>Erm</td>
<td>erythromycin</td>
</tr>
<tr>
<td>Ftf</td>
<td>fructosyltransferase</td>
</tr>
<tr>
<td>Gbp</td>
<td>glucan-binding protein</td>
</tr>
<tr>
<td>Gtf</td>
<td>glucosyltransferase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HKLM</td>
<td>high potassium growth medium</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
</tr>
</tbody>
</table>
LKLM  low potassium growth medium
MMGK- minimal medium with glucose and no potassium
GCF gingival crevicular fluid
Ig immunoglobulin
MIC minimum inhibitory concentration
min minute
OD optical density
PBS phosphate buffered saline
PCR polymerase chain reaction
qRT-PCR quantitative real time PCR
SDS Sodium Dodecyl Sulphate
sec second
Spec spectinomycin
spp species
TCSTS two-component signal transduction system
THYE Todd Hewitt yeast extract
TYE Tryptone yeast extract
TE Tris-EDTA
v/v volume per volume
XIP sigX-inducing peptide
VI. AWARDS

2010-15 University of Toronto Fellowship
2012 CIHR Institute Community Support -IMHA travel award
2013 School of Graduate Studies Conference Grant
2013 Investigator award for Research day poster presentation
2014 Second Prize for oral presentation at OHSS Faculty of Dentistry
2014 First prize for the NCOHR poster presentation
2015 Third Prize CADR-NCOHR student research award
Chapter 1: Literature Review
1.1 The oral cavity

1.1.1 The oral cavity as a microbial habitat

The human oral cavity is a complex and fluctuating environment. The oral cavity, is a major access point for microorganisms into the human body. Intake of nutrients occurs through the mouth, where they are masticated and mixed with digestive enzymes from saliva before being passed to the stomach and intestinal tract. The nose and mouth are also the entry point for the required physiological gases, which are then passed to the trachea and lungs. A moist environment with a relatively constant temperature (34 to 36°C) and a pH close to neutrality is maintained in most areas of the oral cavity (Marcotte & Lavoie, 1998). The topology in the oral cavity includes both hard non-shedding surfaces such as teeth, and soft surfaces such as gingival sulcus, attached gingiva, tongue, cheek, lip, hard palate, and soft palate, which are available for microbial colonization. Contiguous with the mouth are additional sites for microbial colonization such as tonsils, pharynx, esophagus, trachea, lungs, nasal passages, and sinuses. These factors together with different favorable and complex conditions for microbial inhabitation of the oral cavity, which facilitates the occurrence of a highly structured and intricate human oral microbiome consisting of distinct microbial communities. The oral microbiome is an extension of the terminology “microbiome”, which was coined by Joshua Lederberg to define the community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space, and are determinants of its health and
disease (Lederberg & McCray, 2001). This is an accepted nomenclature in the Human Microbiome Project that is categorically defined as the multifaceted oral bacterial population, their genetic material and ecological interactions, which may be involved in both health and disease (Dewhirst et al., 2010). More recently, terminologies have been redefined to avoid the confusion arising due to interchangeability of ‘microbiome’ with ‘microbiota’, wherein the human microbiome is now defined as the catalog of the microbes and their genes, and the microbiota is defined as the microbial taxa associated with humans (Ursell et al., 2012, Marchesi & Ravel, 2015).

The human oral microbiota includes the microbial communities in the contiguous extensions of the oral cavity up to the distal esophagus. This means that the oral microbes from a specific site of the oral cavity have a significant probability of disseminating to adjacent epithelial surfaces. It is established that the microbial residents of an individual affect the well-being of the individual. Thus, it is imperative to understand their dynamic complexity pertaining to survival strategies, propagation and death in their natural niche or simulated environment.

The composition of the normal flora evolves with the host, adapting with simultaneous changes in the environment. Previous paradigms of microbial evolution, where babies were believed to be germ-free before birth, is now disregarded, as emerging evidence shows the presence of microbes in the placenta, the umbilical cord blood (Jimenez et al., 2005), the amniotic fluid (Bearfield et al., 2002), and the meconium (Jimenez et al., 2008). The oral colonization commences at various sites through vertical
transmission of microbes from mother to a newborn child, and also from the environment. The acquisition of microorganisms can vary depending on the mode of birth (vaginal or Caesarian), which would determine if the microbial composition that will be encountered first by the newborn is vaginal-derived or skin-derived (Dominguez-Bello et al., 2010). This is interesting because the children born through Caesarian have an ecological disadvantage and predisposition to colonization with opportunistic oral pathogens such as *Streptococcus mutans* compared with the babies born through the vaginal mode (Li et al., 2005). Further, the type of infant feeding during the early postpartum period can determine the presence (breast-fed infants) or absence (formula-fed) of bacteria such as oral lactobacilli (Holgerson et al., 2013, Vestman et al., 2013). During edentulous neonatal stage, microbial colonization occur on the moistened soft tissues such as lips, cheeks, tongue and palate. Streptococci, especially *Streptococcus salivarius* dominate and constitute a majority of the total oral microbiota until the appearance of the teeth (6 - 9 months in humans) (Carlsson et al., 1970). With the eruption of deciduous teeth from first year allows colonization by species such as *S. mutans* and *Streptococcus sanguis* (Smith et al., 1993). These bacteria require a non-desquamating surface for adherence to initiate the formation of a biofilm and usually persist as long as teeth remain. Other strains of streptococci found in the oral cavity during this period can exist on the gums and cheeks. The surface changes post-dentition result in the formation of the gingival crevice area around the tooth, which can harbor a variety of anaerobic species like *Actinomyces* (Slots, 1977). The complexity of the oral flora continues to increase with time and other confounding factors; bacteroides and spirochetes colonize around puberty (Gusberti et al., 1990).
The microbiome tends to coevolve with the host and depends on their immune tolerance, and the conditions established by primary colonizers. The primary colonizers condition the environment with the production and excretion of their metabolic products, which often potentiates the growth of other secondary species. The oral microbiota, has been predominated by the bacteriome or the study of bacterial population, which chronologically evolves with the host (Nobbs & Jenkinson, 2015). This bacterial population in infants includes six phyla: *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria*, and *Spirochaetes*. The most prevalent genera are *Streptococcus, Haemophilus, Neisseria*, and *Veillonella* (Cephas et al., 2011).

The oral microbiota of children with deciduous dentition has bacteria belonging to the class *Gammaproteobacteria*, families of *Pseudomonaceae* (genus *Pseudomonas*), *Moraxellaceae* (genera *Acinetobacter, Moraxella*, and *Enhydrobacter*), *Enterobacteriaceae* and *Pasteurellaceae* (genus *Aggregatibacter*) (Crielaard et al., 2011). As the deciduous dentition is replaced with the permanent dentition, the bacterome shifts with an increased prevalence of the *Veillonellaceae* family (genus *Veillonella* and *Selenomonas*) and the genus *Prevotella*, while the bacteria of the *Carnobacteriaceae* family (genus *Granulicatella*) decreased (Crielaard et al., 2011). Recently, the study of the microbiota has been extended beyond bacteria to fungi (Krom et al., 2014), *Archaea*, viruses and protozoa, which form a part of a normal oral microbiota (Wade, 2013).
1.1.2 Dental biofilm

In the seventeenth century, Antonie van Leeuwenhoek first reported the diversity and abundance of ‘animulcules’ or microbes in dental plaque (Leeuwenhoek, 1684). Dental plaque is one of the most important biofilms found in the human body with a microbial diversity that can enhance the biochemical repertoire of the biofilm. The co-inhabitants share the overall biochemical burden, enhance resistance to antimicrobial agents and host defenses, and elicit pathogenicity under conducive conditions. Dental plaque has been one of the most studied biofilms and forms the paradigm to understand the basic characteristics of a biofilm. Dental plaque can be sampled easily by non-invasive methods and cultured under in vitro conditions. The study of dental biofilms has led to the understanding of intercellular interactions, such as: production and utilization of adhesins by a microbial population; cell-cell interactions; structuring of nutrient hierarchy between various species; and ecological control determined by the local factors. Although, microbes attach to the mucosal surfaces, the tongue and the dentures, a well-structured three-dimensional biofilm is mostly formed on the hard non-shedding tooth surfaces. All biofilms are formed using similar mechanisms, which can be derived from understanding dental biofilms.

1.1.2.1 Mechanism of plaque acquisition

Dental plaque has a non-uniform structure (Zijnge et al., 2010) that varies between teeth and between various sites on each tooth. The process of dental biofilm
formation was reported in the classic study by Løe (Løe et al., 1965) and the shift in the microbial community with the progression of biofilm initiation to biofilm maturation was reported by Ritz (Ritz, 1967). Ritz study examined the plaque samples by culture methods over a period of nine days. This study was one of the initial studies to indicate a specific progression of species within a dental microcosm with the streptococci being the dominant ‘pioneer’ species, leading to increasing proportions of Actinomyces and eventually transforming to a ‘mature’ community with high levels of Gram-negative anaerobic filamentous organisms. The pioneer communities include streptococci species that possess IgA1 protease activity, which helps them to escape the host defense. Once established, this community alters the environment by generating metabolic end products as a nutrient source for their successors, making specific receptors available for attachment of these successors and modifying the physical attributes of the environment such as pH and redox potential to favor colonization by succeeding species (Marsh et al., 2009, Marcotte & Lavoie, 1998). The microbial succession depends on factors of microbial and non-microbial origin. Succession is influenced by microbial factors such as those generated by the pioneer community for the colonization of secondary community. This phenomenon is referred to as autogenic succession. Whereas, the ecological shift that arises due to host-dependent physical changes such as food intake, medication, or availability of new surfaces for colonization is called allogenic succession. One such phenomenon occurs when bacteria colonize the tooth surface, adapt to the fluctuating environment and induce conditions that would progress towards disease such as dental caries as represented in the Fig 1.1.
Fig 1.1 Progression of dental caries. Tooth decay as a result of shift towards a higher rate of demineralization due to increased acid production by the plaque acid producing bacteria such as *Streptococcus mutans* and Lactobacilli.

The first step to microbial colonization on the tooth surface requires conditioning of the tooth surface with a film of saliva-derived proteins, glycoproteins, lipoproteins such as statherins, amylase and other host factors (Marsh *et al.*, 2009). This film is called the Acquired Enamel Pellicle (AEP). The pellicle allows a weak physiochemical attachment between microbes and pellicle in the presence of certain ions (Marsh *et al.*, 2009). Appearance of bacterial factors, including enzymes such as glucosyltransferase and their glucan products create new receptors for bacterial attachment. This result in irreversible specific short-range interactions between adhesins on *Streptococcus gordonii* binding to α-amylase, *Fusobacterium nucleatum* interacts with statherins, and *S. mutans* with proline-rich proteins (PRPs). Streptococci harbor high molecular weight proteins in their cell walls, which function as adhesins for both surface attachment and for attachment to other bacteria. Once microbes are in favorable conditions, they activate the
adhesion process by altering the conformation of hidden receptors or cryptitopes that facilitate selective attachment, while avoiding attachment during passage period (Marsh et al., 2009, Gibbons, 1989). The growth rate of bacteria in the oral biofilm changes from rapid in the early biofilm stage to slow at the mature biofilm stage (Chandki et al., 2011, Marya, 2011). Diverse communities are found embedded in the mature oral biofilm matrix rich with extracellular polysaccharides (soluble and insoluble glucans, fructans) and nucleic acid polymers. The oral microcosm continues to acquire new bacterial species synchronized with the exposure of the host to various species and conditions. The overall composition and proportion of the resident oral microflora remains fairly stable over time, owing to the microbial homeostasis attained from various inter-bacterial and host-bacterial interactions (Marsh, 2006, Marsh, 1994a).

The early and late colonizers of the dental plaque can be grouped and represented as depicted in Fig. 1.2. The initial colonizers are coloured yellow (example of members: Streptococcus gordonii, Streptococcus intermedius, S. mitis, S. oralis, and Streptococcus sanguinis), blue (Actinomyces spp), green (Capnocytophaga species, Campylobacter concisus, Eikenella corrodens, and Aggregatibacter actinomycetemcomitans serotype a) and purple (Veillonella parvula and Actinomyces odontolyticus) complexes, whereas the late colonizers form the orange (Fusobacterium nucleatum, Prevotella intermedia, Peptostreptococcus micros, Eubacterium nodatum, and other species) and red complexes (Treponema denticola, Porphyromonas gingivalis and Tannerella forsythia) (Liljemark & Bloomquist, 1996, Paster et al., 2001, Aas et al., 2005, Moore et al., 1983, Socransky & Haffajee, 2005). F. nucleatum functions as a bridge organism during plaque formation.
owing to its capability to co-aggregate with both initial and late colonizers, while the early and late colonizers do not co-aggregate with each other (He et al., 2012, Kolenbrander, 2000).

**Fig 1.2. Microbial composition of dental plaque.** The base level is comprised of species thought to colonize the tooth surface and proliferate at an early stage. The orange complex are predominantly secondary colonizers and bridge the early colonizers with the red complex species that become more prevalent at late stages in plaque development. Adapted from Socransky and Haffajee, 2005.

The homeostasis of the plaque microbiota plays an important role in the etiology of caries and periodontal diseases. The historical hypothesis, called the ‘specific plaque hypothesis’, states that only a few species out of the diverse consortium of residing
plaque microbes are involved in causing disease (Loesche, 1992, Emilson & Krasse, 1985). This hypothesis was replaced by the ‘non-specific plaque hypothesis’, which was formulated since patients had disease even in the absence of disease-causing pathogens (Theilade, 1986). The more recent "Ecological Plaque Hypothesis" concedes the key elements of the earlier two hypotheses simultaneously addressing the dynamic relationship between the resident microflora and host ecology in health and disease (Marsh, 1994b, Marsh, 2006). The hypothesis states that changes in the host environment such as: a) shifts in the overall community metabolism, b) subsequent modification of the local environment, c) host factors, and d) the balance between potential pathogens and species associated with the oral health determines the conditions conducive for oral health or disease (Marsh, 1994b, Marsh, 2006). The hypothesis suggests that the regimens aimed for long term prevention of oral disease should control both the associated pathogens and the underlying changes in the environment that drive the deleterious shifts in the microflora (Marsh, 1994b, Marsh, 2006).

1.1.2.2 Oral Streptococci

Oral streptococci constitute the most prevalent genus of the dental microbiota and are also present at other various sites in the mouth. Oral streptococci are commensal bacteria; however, some streptococci species are opportunists and are known for causing dental caries, periodontitis and occasional systemic infections such as infective endocarditis or brain abscesses (Whitmore & Lamont, 2011, Knirsch & Nadal, 2011, Whiley & Beighton, 1998, Rosan & Lamont, 2000). The most predominant oral
streptococci are clustered into four phylogenetic groups, namely the: mutans group (MS) (prominent members are *Streptococcus mutans* and *Streptococcus sobrinus*), salivarius group (*S. salivarius*, and *Streptococcus vestibularis*), anginosus group (*Streptococcus anginosus* and *S. intermedius*), and mitis group (*S. mitis*, *S. gordonii*, *S. sanguinis*, *S. parasanguinis* and *S. oralis*) (Kawamura *et al.*, 1995, Hardie & Whiley, 1997). The pioneer colonizers of the tooth surface are members of the mitis group. These colonizers play a critical role in the initiation and development of dental plaque (as described previously in section 1.1.2.1). Streptococci from the mutans group are usually associated with cariogenesis. The mutants streptococci along with lactobacilli are capable of metabolizing different dietary carbohydrates into acid end-products that acidify the plaque environment (Colby & Russell, 1997). While this local acidification makes it uninhabitable for the commensal bacteria to survive, MS activate their specialized adaptive response to survive under low pH conditions (Loesche, 1986, Svensater *et al.*, 1997, Harper & Loesche, 1984). The microbial succession associated with the cariogenesis includes MS during the initiation process, whereas lactobacilli are associated with caries progression (Nyvad, 1993, Marcotte & Lavoie, 1998). The MS group is comprised of nine serotypes (*a*-*h* and *k*), with each serotype having specific types of carbohydrates present in their cell walls (Facklam, 2002, Whiley & Beighton, 1998, Nakano *et al.*, 2007). *S. mutans* is classified into serotypes c, e, f and k with approximately 70-80% of strains as serotype c, followed by e (∼20%), f and k (less than 5% each).
Streptococcus downei (serotype h) Streptococcus ratti (serotype b), the isolates of S. cricetus serotype a, and S. sobrinus serotype d and g can also be found in the human oral cavity (Facklam, 2002, Whiley & Beighton, 1998, Nakano et al., 2007). The members of the salivarius group preferentially colonize the mucosal surfaces and are not considered as significant pathogens of oral diseases. Species from the anginosus group are colonizers of the mucosal surfaces, and they are recognized as the main causative agents of maxillo-facial infections (Bancescu et al., 1997). The oral streptococci participate in numerous co-operative and antagonistic bacterial interactions within the plaque community that determines the health and disease of the oral cavity (Aas et al., 2005, Zijnge et al., 2010, Hojo et al., 2009).

1.2 Streptococcus mutans

Of the mutans Streptococci group, S. mutans has remained the prime focus of cariology research since Walter Loesche established its prevalence in more than 70% of dental caries cases studied (Loesche, 1986). However, S. mutans was first isolated from a caries lesion by J Clarke in 1924 and assigned a name according to variations observed in its morphology that led him to think it was a mutant (Hamada & Slade, 1980). Fitzgerald and Keyes later demonstrated the virulence of S. mutans in gnotobiotic rats and hamsters, thereby confirming its role in cariogenesis (Fitzgerald & Keyes, 1960). In addition, studies have associated S. mutans with the systemic diseases such as infective endocarditis, a life threatening inflammation of heart valves (Whitmore & Lamont, 2011, Robbins et al., 1977, Schelenz et al., 2005).
1.2.1 Virulence factors of *S. mutans*

Transmission of *S. mutans* to an infant can occur from the mother concurrent to the eruption of teeth (Caufield *et al.*, 1993), or from different members of the family (Lapirattanakul & Nakano, 2014, Berkowitz, 2006). The ecological shift to promote the predominance of cariogenic bacteria is initiated with a drop in plaque pH resulting from rapid metabolism of dietary carbohydrates and the production of acid end products by species such as *S. mutans* (Loesche, 1986). Figure 1.3 demonstrates the virulence properties of *S. mutans* in its natural environment. Examination of various virulence attributes of *S. mutans* has uncovered how pH, nutritional and redox changes in the environment allow this bacterium to thrive when similar changes have lethal effect on many competing co-inhabitants. The discussion below elaborates on the main virulence attributes and survival mechanisms of *S. mutans* including acidogenicity, aciduricity, adhesion and genetic competence.

The genomes of four different strains of *S. mutans* have been sequenced, of which *S. mutans* UA159 was the first to be annotated, followed by *S. mutans* NN2025, *S. mutans* GS-5 and *S. mutans* LJ23 (Biswas & Biswas, 2012, Ajdić *et al.*, 2002, Aikawa *et al.*, 2012, Maruyama *et al.*, 2009). It is evident from the annotated sequences that protein machinery involved in sugar transport and metabolism constitutes almost 15% of its genetic repertoire (Ajdić & Pham, 2007)
1.2.1.1 Biofilm formation by S. mutans

*S. mutans* adheres to the tooth surface and adapts to a sessile lifestyle at the most favorable site (Hamada & Slade, 1980). In the presence of common dietary sugars such as sucrose, this bacterium is able to produce gluey substances, for example glucans (constituent of exopolysaccharide matrix) (Hamada et al., 1984), and glucan binding proteins. In the absence of sucrose, surface adhesin SpaP (antigen I/II, P1, PAc) can facilitate the attachment of *S. mutans* to the tooth surface (Bowen et al., 1991, Crowley et al., 1999, Brady et al., 1993, Russell et al., 1980). Glucans are formed with the help of glucosyltransferases, which forms a polymer of D-glucose. At least three distinct glucosyltransferases are functional in *S. mutans*, namely: the glucosyltransferase enzyme GtfB, which converts sucrose to produce a polymer of α-1,3-linkages; GtfD, which converts sucrose to mostly soluble α-1,6-linked glucans; and GtfC, which makes both α-1,3 and α-1,6 glucans (Hanada & Kuramitsu, 1988, Hanada & Kuramitsu, 1989, Gilpin et al., 1985). Being a polymer of glucose, glucans can also function as a carbon source reservoir under famine conditions in addition to their primary role in cell adhesion. Another enzyme, fructosyltransferase (FTF) metabolizes sucrose to produce a fructose homopolymer composed of β-2, 1-linked fructose (inulin). These polymers are exclusively synthesized as a carbon reservoir (Koo et al., 2013, Yamashita et al., 1993, Bowen & Koo, 2011, Decker et al., 2011, Nobbs et al., 2009).

The glucan binding proteins (Gbps) are the proteins on the *S. mutans* cell surface that attach to glucan (Lynch et al., 2007, Matsumura et al., 2003a). *S. mutans* produces four
different types of Gbps namely; GbpA, GbpB, GbpC and GbpD (Smith et al., 1994b, Russell, 1979, D.S. & R.R.B., 2002). Of these, GbpB is known to participate in sucrose-dependent biofilm formation (Duque et al., 2011). GbpA, GbpC and GbpD are each transported across the membrane, where only GbpA and GbpD are released; whereas, GbpC is bound to the cell wall (Matsumura et al., 2003b, Lynch et al., 2007). GbpA contributes to the biofilm architecture by linking glucan molecules (Mattos-Graner et al., 2006, Duque et al., 2011), whereas GbpC is essential in the glucan-dependent aggregation of bacteria; the loss of GbpC results in an impaired biofilm biomass and bacterial aggregation (Biswas et al., 2007, Matsumura et al., 2003b, Nakano et al., 2002). GbpD contributes to the scaffolds and robustness of the biofilm and linkage between bacteria and glucans in the biofilm (Lynch et al., 2007). Further elaboration can be found in the model of the virulence attributes of S. mutans as shown in Figure 1.3.
Fig 1.3. Summary of S. mutans’ contributory factors to carogenicity in dental plaque. S. mutans adheres to the tooth surface and other bacteria with the help of glucan and glucan binding proteins in the presence of sucrose. In the absence of sucrose, adhesion occurs via SpaP, also known as antigen I/II, which binds to salivary agglutinin glycoprotein. S. mutans can metabolize various dietary sugars through multiple sugar transport systems and a robust glycolytic pathway, to produce acid end products, which leads to dissolution of the tooth enamel and killing of other commensal bacteria. A reduction in environmental pH can activate a specialized acid tolerance response (Bancescu et al.). During stress tolerance adaptation, S. mutans secretes antimicrobial and signaling molecules. The function of these antimicrobial molecules is to cause cell death of other competing bacteria, while S. mutans alters its community phenotype dramatically by the secretion and detection of signaling peptides. Figure is adapted from Mitchell, 2003 (Mitchell, 2003).
1.2.1.2 Mechanisms of dietary sugar metabolism

*S. mutans* is an opportunistic pathogen and one of the most proactive plaque residents in fermenting different dietary sugars as its primary source of energy (Jacobson *et al.*, 1989). It has specialized mechanisms to accommodate the transport and metabolism of various carbohydrates available in the oral cavity such as glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β-glucosides, trehalose, maltose, raffinose, ribulose, mellibiose, starch, isomaltosaccharides and sorbose (Ajdić *et al.*, 2002). Depending on the type of sugar, these aforementioned specialized systems are designated as: phosphoenolpyruvate sugar phospho-transferase system (PEP:PTS), multiple sugar metabolism (MSM) or ATP dependent glucose specific permeases (Colby & Russell, 1997, Marsh *et al.*, 2009). For example, when under famine or low sugar conditions, *S. mutans* can express the PEP:PTS, a high affinity uptake system, which has sugar-specific membrane-bound permeases (designated as Enzyme II) that is essentially energized by the sequential phosphorylation of the soluble, non-sugar-specific, cytoplasmic intermediate proteins Enzyme I and HPr (Carlsson, 1997, Jacobson *et al.*, 1989, Cvitkovitch *et al.*, 1995). Sugar molecules such as glucose, fructose and lactose are thus phosphorylated during their transport and introduced to the glycolytic pathway of *S. mutans* for energy generation (Carlsson, 1997). Alternately, sugars such as melibiose, raffinose, and isomaltotriose are taken up by the MSM transport system (Russell *et al.*, 1992), which can be utilized during famine situations between meals, by degrading the products of extracellular polysaccharides. As a result of these multiple sugar transport and metabolizing systems, *S. mutans* can acidify its microcosm with acid end-products.
such as lactate, formate, acetate and ethanol (Guo et al., 2013, Matsui & Cvitkovitch, 2010a).

1.2.1.3 Mechanisms of acid tolerance

*S. mutans* is capable of maintaining its glycolytic activity under the acidified milieu, which affords it a competitive advantage over other oral streptococci and assists it and other aciduric bacteria to dominate in the plaque environment (Matsui & Cvitkovitch, 2010a, Li et al., 2001a, Hamilton & Buckley, 1991). These acid-induced adaptation and selection processes in the presence of high sugar exposure can perturb the balance between the demineralization and remineralization of the tooth enamel, resulting in initiation and progression of dental caries. *S. mutans*’ ability to tolerate, adapt and utilize these otherwise suicidal acid stress conditions for its benefit depends on its acid tolerance as defined under two categories: i) constitutive and ii) inducible mechanism (Svensater et al., 1997, Burne, 1998). The acid tolerance cascade of this microorganism is activated by prior exposure to a low, sub-lethal pH of ~5.5, resulting in the induction of the tolerance mechanism that enhances survival at a pH as low as 3.0. Both the constitutive and inductive mechanisms work to provide a holistic benefit to the cell by: i) protection and repair of macromolecules; ii) alterations of metabolic pathways; iii) activation of secondary metabolism; iv) regulation of cell density, biofilm formation and regulatory systems; and v) intracellular pH homeostasis (Cotter & Hill, 2003, Matsui & Cvitkovitch, 2010b). An example of each adaptive process will be discussed briefly in the following paragraphs.
The function of acid tolerance mechanisms is to preserve the cellular macromolecules and keep glycolysis functional in the cell to provide it with energy. Central to this mechanism is protection of the DNA during intracellular acidification. DNA protection involves proteins like Recombinase-A that moderates homologous recombination and serves a housekeeping function to re-activate DNA replication forks (Cox, 2007). However in an *S. mutans* mutant, that lacks Recombinase-A, DNA repair is carried out by an independent repair system, which functions through the expression of an AP (apurinic-apyrimidinic) endonuclease (Hahn *et al.*, 1999) and *uvrA* under acid stress (Hanna *et al.*, 2001b). Acid inside the cell causes protonation of the nitrogenous base, which follows with cleavage of the glycosyl bond and loss of purines and pyrimidines (Lindahl & Nyberg, 1972). This process results in DNA damage via the formation of an abasic or AP site (Sancar, 1996), which is recognized by AP endonucleases to repair the phosphodiester bond (Hahn *et al.*, 1999). Previous reports have shown that a mutant lacking *uvrA* was unable to survive a killing pH of 3.0, despite previous acid exposure at a sublethal pH of 5.0 (Hanna *et al.*, 2001b), thus implicating an adaptive and protective role of *uvrA* against acid stress. Collectively, acid-induced genetic damage in *S. mutans* can be counteracted by the induction of the AP endonuclease in concert with UvrA to further enhance its acid tolerant phenotype.

While genetic material is protected and preserved under acid stress as described above, chaperones DnaK and GroEL operate to protect the integrity of cellular proteins. These chaperones are part of the controlling inverted repeat of chaperone expression (CIRCE) regulon (Cotter & Hill, 2003), which is negatively regulated by HrcA and
constitutes the HrcA-CIRCE system (Lemos et al., 2001). In response to the acid shock, initially the expression of both DnaK and GroEL is elevated, followed by a stage of adaptation where high levels of DnaK are maintained (Lemos et al., 2001, Jayaraman et al., 1997). DnaK and GroEL molecular chaperones facilitate the folding process of new or denatured proteins and the transport, assembly and degradation of other proteins (Craig et al., 1993). Proteomic analysis of acid induced S. mutans grown in continuous culture revealed a measurable change in expression of 30 different proteins upon culture acidification, of which 25 proteins were most sensitive to pH 5.0 and were connected with DNA replication, transcription, translation, protein folding and proteolysis (Len et al., 2004).

Central to the constitutive mechanism employed by S. mutans is the activity of the F1F0 ATPase proton pump (Bender et al., 1986), which expels unwanted protons out of the cell to normalize the intracellular pH relative to the external environment, and generates ATP in the process (Dashper & Reynolds, 1992). S. mutans maintain a slightly alkaline intracellular pH compared to its environment. This is achieved by modulating the composition of the cell membrane to make it impermeable to proton intrusion, while maintaining the acid end product efflux and F1F0 ATPase activity (Quivey et al., 2000). The membrane composition under these conditions had increased levels of mono-unsaturated long chain fatty acids (C18:1 and C20:1) (Quivey et al., 2000). In addition to F1F0 ATPase, there are other P-type ATPases and ion transporters that have similar function and share the ultimate goal to alkalinize the cytoplasm. Together, these ion transport systems establish a gradient required to maintain the membrane potential and
the pH difference, as they are the two main factors that drive the proton motive force (Bakker & Mangerich, 1981).

In addition to these mechanisms *S. mutans* activates the recycling of the carbon acids, which contributes to alkalization of its cytoplasm. For example, the decarboxylation of L-malic acid to L-lactic acid occurs in *S. mutans* under low pH conditions; by this process the bacterium alkalinizes its cytoplasm and also generates ATP by utilizing the F-type ATPase in the synthase mode (Sheng & Marquis, 2007).

### 1.2.2 Mechanism of sensing and responding

*S. mutans* along with other plaque bacteria, face fluctuating and stressful environments in their natural niche. The microbial residents of the biofilm can share the stressful burden of the environment and simultaneously keep a close check on their population. Quorum sensing is a term used to define the process by which bacteria orchestrate a population check and a global response to the changing environment.

In order to regulate the behavioral traits between individuals of a microbial population and to synchronize population behavior, cells require inter-cellular signaling mechanisms (Keller & Surette, 2006). After Tomasz and Hotchkiss first reported that bacterial population density coordinated processes such as DNA uptake by autogenic
factors (Tomasz & Hotchkiss, 1964), successive reports have established the existence of many cell-cell communication mechanisms in bacteria. To accomplish this, a signal can be generated by an individual or a sub-population of cells and detected by the rest of the cells in a nearby community, which leads to phenotypic or genotypic changes in the receiving cells. The signal is generated by release and detection of small molecules such as the auto-inducers (e.g. acylated homoserine lactones) in Gram negative bacteria, and processed oligo-peptides (e.g. competence stimulating peptide) in Gram positive bacteria. Moreover, reports have proposed a novel function for bacterial signaling molecules where they can affect host cells, which leads to cross-communication between bacteria and host (Cuadra-Saenz et al., 2012). The mechanism of quorum sensing and that of genetic competence partly overlaps as bacteria utilize common peptides for both these processes. Genetic competence is the ability of a cell to first bind, import and integrate exogenous DNA. Both the quorum sensing and genetic competence process will be discussed in the section 1.2.3.

*S. mutans* possesses a total of 14 two-component signal transduction system or TCSTS (Smith & Spatafora, 2012). TCSTS are typically composed of a membrane-bound kinase with a conserved histidine/serine residue, which undergo a process of auto-phosphorylation in response to environmental stimuli. The histidine kinase interacts with its cognate or a non-cognate cytoplasmic regulator, which usually gets phosphorylated at a conserved aspartate residue (West & Stock, 2001, Stipp et al., 2013). The concomitant conformational change in response regulator, enables it to bind at specific DNA promoter regions (West & Stock, 2001). From the genetic point of view, such binding between the
response regulator and the promoter of DNA sites is required for modulating genes involved in processes such as biofilm formation, resistance to environmental stresses (pH, temperature, osmolarity, antibiotics, and others); biotic stresses (host antimicrobials and other competing microbes), which are all critical factors for persistence of this bacterium in its niche (Beier & Gross, 2006).

Of the 14 two component signal transduction systems, VicKR is one of the most studied signal transduction system in S. mutans. Its role has been reported in the biofilm formation, sucrose-dependent adhesion, competence development, acid tolerance, oxidative and cell envelope stress, and bacteriocin production (Senadheera et al., 2007, Senadheera et al., 2005). Other signal transduction systems that have been characterized and have overlapping functions include CiaHR, LiaSR, ScnKR, and ComDE, which are described in figure 1.4. LevSR is another signal transduction system, which is involved in nutrient acquisition, more specifically, in fructose metabolism and sugar transport. In addition to the paired specific Histidine Kinase {Tomasz, #79} with cognate Response Regulator {RR} in S. mutans, this bacterium possesses an orphan response regulator GcrR, which functions in sucrose dependent adhesion and acid tolerance (Chen et al., 2008, Deng et al., 2007, Smith & Spatafora, 2012, Idone et al., 2003, Senadheera et al., 2009a).
Fig. 1.4. Signal transduction by the membrane-associated sensor kinases in *S. mutans*. Environmental cues from alterations in the levels of osmolytes, pH, nutrients, peptides, ions and other factors result in the activation of the membrane bound component of the TCSTS. The most commonly accepted downstream effect of this type of signal transduction is considered to be through gene expression modulation, which occurs when the activated response regulator binds to the promoter region of the specific target genes. The most well-characterized signal transduction systems are depicted in the figure. The superscript letter represent the physiological change regulated by a specific TCSTS. The function of each TCSTS has been reviewed by Smith and Spatafora (Smith & Spatafora, 2012).
1.2.3 Genetic competence and Quorum Sensing in

*S. mutans*

Genetic competence and quorum sensing are two closely related processes in *S. mutans*. Genetic competence in *S. mutans* was first established by Perry and Kuramitsu, who demonstrated the acquisition of streptomycin resistance by three strains of *S. mutans* (Perry & Kuramitsu, 1981). Genetic competence is the processes by which the cell prepares itself for horizontal gene transfer, and the process of taking up extracellular genetic material and integrating it in the host genome is called transformation. Genetic transformation regulates, and contributes to the genetic repertoire, growth and metabolism, biofilm formation and general stress response (Johnsborg *et al.*, 2007, van der Ploeg, 2005a). *S. mutans* is capable of inducing competence under the selection pressure of a stressful oral environment through self-generated competence inducing peptides, which could contribute to its overall fitness. Two peptides linked with quorum sensing and genetic competence in *S. mutans* characterized so far include, a peptide pheromone designated the Competence Stimulating Peptide (CSP) that acts via the ComDE signal transduction system (Li *et al.*, 2002c, Li *et al.*, 2001b), and a double tryptophan-containing signal peptide termed XIP (comX-inducing peptide), which acts in conjunction with an Rgg-like transcriptional regulator ComR (Federle & Morrison, 2012). The CSP mediated competence pathway includes *comCDE, comAB, comR* and *comX* genetic components (Reck *et al.*, 2015), wherein the *comC* synthesizes a precursor peptide, which is processed to CSP and exported by the ComAB transporter (Li *et al.*, 2001b). As CSP accumulates in the extracellular milieu, it reaches a critical threshold that
is sensed by the histidine kinase ComD, which transduces the information to its cognate response regulator ComE. Although the primary cellular response generated via ComE signaling is bacteriocin production (Mashburn-Warren et al., 2010a), there is a concomitant indirect activation of comX via the CSP regulon, which affects the expression of various late competence genes (Cvitkovitch, 2001). A precursor peptide of CSP consisting of 46 amino acids is processed both during export and extracellularly to an 18 amino acid mature signaling peptide (Hossain & Biswas, 2012). The XIP is a more recently identified competence peptide, which induces a competence state in S. mutans by directly activating the expression of comX and the genes required for DNA acquisition and recombination (Federle & Morrison, 2012). The XIP-induced competence regulon is partially described, and includes genetic components comRS and an opp gene (Fig 1.5). The comS encodes a precursor peptide, which is processed and secreted out of cell through an unknown mechanism. After being imported into the cell, the processed form of ComS or XIP activates ComR, a transcriptional regulator, which belongs to the Rgg family (Mashburn-Warren et al., 2010a). The ComR-XIP interaction is believed to further regulate expression of genes such as comS and comX (Reck et al., 2015). The XIP peptide is a smaller secreted 7-amino-acid peptide processed from the C terminus of its 17-amino acid long precursor peptide (Mashburn-Warren et al., 2010b, Khan et al., 2012). The process of maturation of the ComS to XIP is not well characterized but its uptake is proposed to occur via the Opp oligopeptide transporter.

It is understood that both XIP and CSP mediated pathways converge to activate a common master regulator, the alternative sigma factor ComX (also known as SigX) of S.
mutans critical for competence development in *S. mutans* (Li *et al.*, 2002b, Ahn *et al.*, 2006, Aspiras *et al.*, 2004). How *S. mutans* utilizes these two mechanisms in its natural niche is unclear, however various functional differences have been reported based on *in vitro* assays. For example, while ComD senses extracellular CSP (reported to occur only in rich growth medium), the XIP-mediated competence development (which occurs only in chemically defined medium) requires the internalization of XIP (Khan *et al.*, 2012, Li *et al.*, 2001d, Desai *et al.*, 2012, Mashburn-Warren *et al.*, 2010a). CSP also induces response in a subpopulation of a community, whereas XIP activates 100% of the population (Lemme *et al.*, 2011, Federle & Morrison, 2012, Desai *et al.*, 2012, Li *et al.*, 2001b). Nevertheless, it is known that there are two sets of genetic material in *S. mutans*; the core genetic material, which is essential for the cell viability and the dispensible genetic material, which can function in enhancing the physiological and pathological properties for optimal survival. The genomic plasticity also enables one *S. mutans* cell to differ from another in the same microcosm (Cornejo *et al.*, 2013).
Fig. 1.5. A schematic diagram depicting the synthesis and processing of the Sigma X inducing peptide (XIP) adapted from Mashburn-Warren et al (Mashburn-Warren et al., 2012). The precursor XIP is secreted from the cell by an unknown mechanism to produce the mature XIP. XIP is imported via the Opp transporter. In the cell, XIP interacts with ComR and together ComR-XIP regulate the transcription of comS and comX.

1.2.4 Bacteriocin production in S. mutans

S. mutans can secrete a range of ribosomally synthesized antimicrobial peptides to kill its competitors. The ubiquitous occurrence of antimicrobial peptides in S. mutans clinical isolates, highlights its ecological significance in this bacterium (Rogers, 1976). The antimicrobial peptides secreted by S. mutans are members of the bacteriocin family and are considered as potential replacements for conventional antibiotics. Generally, bacteriocins from Gram positive bacteria are classified under three groups: class I comprises of the post-translationally modified bacteriocins or lantibiotics, class II includes small unmodified, heat-stable peptide bacteriocins, and class III contain large,
heat-labile bacteriocins (Nes & Holo, 2000). *S. mutans* can produce bacteriocins that belong to group I lantibiotics, such as, mutacin I and mutacin 1140 depending on the growth conditions (Qi et al., 2001, Hillman et al., 1998). *S. mutans* UA140 also secretes non-lantibiotic class IIB bacteriocin, mutacin IV; preference of the type of bacteriocin being produced depends on the growth conditions (Qi et al., 2001). The production of mutacin IV, other class IIB bacteriocins and expression of other associated gene products in *S. mutans* coordinates with the quorum sensing via CSP and genetic transformation (van der Ploeg, 2005b). By this way, the bacterial community conducts a selection process, such as competent cells undergo the process of competence and biofilm development, and the damaged cells such as those irreversibly injured by environmental undergo a process of elimination (Rice & Bayles, 2008).

### 1.3 Chemistry of the oral cavity

#### 1.3.1 Saliva and its composition

Saliva has multifactorial control over the oral environment, which makes it an important component to study in oral microbiology. Humans secrete this slightly acidic mucoserous exocrine secretion via the salivary glands diversely located in the mouth at sites, which are evidently favourable for the microbial inhabitation (Humphrey & Williamson, 2001). The salivary glands include the paired parotid glands, which are located opposite the maxillary first molars, and the submandibular and sublingual glands,
which are found in the floor of the mouth. Minor glands that produce saliva are found in the lower lip, tongue, palate, cheeks, and pharynx. Amongst various roles of saliva towards host, its digestive and protective functions are the most important. Saliva aids in mechanical handling of food, its chemical degradation by amylase and lipase, dissolves the tastants, facilitating the interaction with the taste buds for a cephalic regulation of gastric secretion. The important function of saliva include lubrication of the oral structures with mucins, regulation of the pH with components including bicarbonate, phosphates, and protein, remineralization of the enamel by calcium, antimicrobial defense action by immunoglobulin A, α-defensins, and β-defensins, and wound healing by growth-stimulating factors such as epidermal growth hormone, statherines, and histatins (Ekström et al., 2012). Saliva is also composed of electrolytes, including sodium, potassium, calcium and magnesium. Together, these components have a huge impact on other oral fluids such as the plaque fluid.

The volume and electrolyte composition of saliva depends on factors such as, the individual, the age, the type of secreting gland, the properties of a stimulus, local circulatory and metabolic conditions, the nutritional and medication status, the endocrine balance and the oral health status (Martinez, 1987). According to Thaysen's hypothesis, saliva is formed isotonic in the acini, but it is modified as it flows through the glandular duct system by the removal or addition of specific ions, thus making it hypotonic (Thaysen et al., 1954). Later, this hypothesis was revised to include the newly emerged concepts about water and electrolyte secretion in the salivary glands: 1. Ions and water were initially secreted in salivary acini as an isotonic and similar to plasma secretion; 2.
external stimuli could alter the rate of formation, but not osmolarity, of this primary secretion; 3. the electrolyte content of the primary secretion was significantly altered by the type of secreting duct cells; and 4. autonomic stimuli could alter the ion transport functions of these cells; for example, the increase in duct cell membrane conductance produced by a stimulation is associated with fluxes of $K^+$, $Na^+$, and $Cl^-$ (Catalan et al., 2009). The relationships between $Na^+$, $K^+$, $Cl^-$, and $HCO_3^-$ transport in salivary ducts are complex and depend on individual factors. However, exchange of $K^+$ and $Na^+$, between the duct cells and the external environment is strongly affected by stimulation, with an increased loss of $K^+$ into the venous effluent in response to stimuli (Burgen, 1956). Subsequent studies reinforced that stimulation could enhance $K^+$ exchange between the salivary duct cell and the environment (Schneyer & Schneyer, 1962).

### 1.3.2 Composition of the Plaque fluid

The oral microbes, saliva, and the fluid in the gingival crevices exert a confluent effect on the plaque fluid composition, which can affect the health of the oral cavity. Dental biofilms consist of a rich *saliva-like* fluid phase, which was initially called the "plaque saliva" (Brudevold et al., 1964). The existence of chemical equilibrium between the tooth enamel and the "inner surface of plaque" or the "plaque fluid", rather than saliva was reported by Jenkins (Jenkins, 1966). Plaque fluid is an interface between the tooth surface and the microbial community in the plaque, and its composition is extremely important to understand the physiology of caries formation. Saliva and gingival crevicular fluid can contribute and influence the composition of the plaque fluid.
Tatevossian and Gould were the first to isolate the plaque fluid from saliva and gingival crevicular fluids, and examine the chemical composition of each (Tatevossian & Gould, 1976). In their report, the inorganic ion compositions for the rested plaque fluid (obtained one to several hours after eating) and the starved plaque fluid (obtained following overnight fasting) of human subjects contained significant quantities of Na$^+$, NH$_4^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, PO$_4^{3-}$, and Cl$^-$. The plaque fluid had appreciable concentrations of organic acid end-products derived from bacterial metabolism. Although, there was a variation in the concentration of these products, both the rested and starved plaque fluids contained acetic and propionic acids and relatively low concentrations of lactic, succinic, butyric, and formic acids (Margolis & Moreno, 1994). Concomitant to concentrations of acids and ions in rested and starved samples, pH values in the rested samples were reported to be lower (pH 5.69 to 6.54) than those found in the starved samples (Margolis & Moreno, 1994). Although, one may see a fluctuation in the concentrations of most of these components of plaque fluid, the levels of K$^+$ remains high relative to the other cations in that environment. Thus, it is important to understand the impact of essential monovalent cations such as K$^+$ on the physiology of Streptococci.

### 1.4 Importance of potassium (K$^+$) and its transport

Bacteria can respond to osmotic stresses in two ways: some halophilic species, including eubacterial and archaeal species, use K$^+$ and to some extent Na$^+$ as major cytoplasmic–osmotic solutes regardless of the extracellular osmolarity. This is also
referred as the ‘salting in’ adaptation. Some other halophilic bacteria use $K^+$ as a primary osmotic solute only during growth under low osmolarity (Epstein, 2003).

Most of the understanding about the transport of ions in streptococci is based on the extensive studies of the transport of $H^+$ and $Na^+$ in *Enterococcus hirae*, which was initiated by Harold *et al* (Harold & Papineau, 1972) and continued by Kakinuma *et al* (Kakinuma & Harold, 1985). Heefner and Harold established the presence of an ATP driven primary $Na^+$ pump in *E. hirae* that expelled $Na^+$ in exchange for $H^+$, and this transport was independent of the proton motive force (Heefner & Harold, 1982). Kakinuma’s group identified the close relationship between the electrogenic co-translocation of $Na^+$ and $K^+$ through a Ktr transporter in *E. hirae* (Kawano *et al.*, 2000). It was suggested from these studies that $Na^+$ is not only extruded out of *E. hirae* to make ionic space for $K^+$, a more compatible solute, but $K^+$ was actively and specifically accumulated to adapt with the transitory environment.

The role of $K^+$ has been studied for decades, and has been shown to play a crucial role in maintaining the cell integrity and cellular functions (Epstein, 2003). $K^+$ is a ubiquitously abundant cation. $K^+$ accumulation under stress conditions enables contention with dehydration, membrane damage, regulation of the $Na^+/H^+$-dependent cell energetics and pH homeostasis, while simultaneously minimizing interference with the structures and functions of intracellular proteins (Csonka, 1989, Christian & Waltho, 1961, Booth, 1985).
The importance of \( K^+ \) in bacterial physiology is illustrated by the fact that it utilizes different types of \( K^+ \) transporters and channels that are specific to the conditions under which they respond, and have specific energy requirements. Only a few types of specific \( K^+ \)-transport systems are found in bacteria, many are widely distributed in eubacteria, while some are found in the distantly related Archaea (Epstein, 2003).

The \( K^+ \) transport system most commonly found in bacteria is a multicomponent complex referred to as Trk. This system has a moderate affinity for \( K^+ \) in the vicinity of 1 to 5 mM and a relatively high specificity for \( K^+ \). The Trk system of \( E. coli \) is the one most extensively studied. The products of three different and unlinked genes: \( trkA; trkE; \) and \( trkH \) are necessary for Trk activity in \( E. coli \) and similar enterics (Epstein & Kim, 1971). The corresponding genes have also been studied in \( Salmonella typhimurium \) and are referred to as \( sapG, sapD, \) and \( sapJ \), respectively (Parra-Lopez et al., 1994). The \( trk \) systems are mainly of two types depending upon their energy requirement. They may have either an NADH/ NAD binding site (Bossemeyer et al., 1989) or an ATP binding component (Parra-Lopez et al., 1993). In addition, these systems have a transmembrane-path component for \( K^+ \) movement (Schlosser et al., 1995). A distant relative of \( trk \) systems are the two transport systems referred as Ktr found in bacteria like \( Vibrio alginolyticus \) and \( B. subtilis \) (Rhoads et al., 1977, Bakker, 1983). The Ktr system is energized by \( K^+/Na^+ \) symport instead of by \( H^+/K^+ \) symport inferred for the Trk system. The third type of transport system that is found in \( E. coli \) and many other bacteria is Kdp, an inducible system with high affinity and specificity for \( K^+ \) (Altendorf & Epstein, 1996). Kdp is a P-type ATPase consisting of four subunits, all membrane proteins. It is
expressed when the ion concentration in environment is too low to be efficiently taken up by the other K\textsuperscript{+}-transport systems or too high when other K\textsuperscript{+}-transport systems are abolished by mutation (Epstein, 2003).

In addition to the main systems mentioned above there are other transporters and channels, which play a role in the transport of K\textsuperscript{+}. There are two purified bacterial K\textsuperscript{+} channels that have been characterized, namely: KcsA in *Streptomyces lividans* and MthK in *Methanobacterium thermoautotrophicum* (Doyle et al., 1998, Jiang et al., 2002). Also, a gene that encodes a bacterial homolog of eukaryotic K\textsuperscript{+} channels has been identified and referred as *kch* of *E. coli* (Milkman, 1994). In terms of its role in virulence, K\textsuperscript{+} acquisition during the infectious cycle of *Francisella tularensis*, which is a Gram-negative facultative bacterium, was reported as a critical factor for it to infect and replicate inside macrophages (Alkhuder et al., 2010). When trkH was mutated, *F. tularensis* showed growth defects in blood (Alkhuder et al., 2010). In other bacteria such as *B. subtilis*, leakage of potassium ions strongly affected biofilm formation (López et al., 2009). Also, *E. coli* and *B. subtilis* accumulate potassium ions against a concentration gradient during growth. The high intracellular potassium content is required for macromolecular synthesis during growth, germination, and lytic infection by bacteriophage (Eisenstadt, 1972).

K\textsuperscript{+} efflux has been recently associated with independent events critical for infection, inflammasome activation and histone modification in the case of *Listeria*
monocytogenes (Hamon & Cossart, 2011). Thus, potassium cations can be considered critical for bacterial growth and pathogenesis.

### 1.5 K$^+$ and its role in Streptococci

Various oral streptococci require K$^+$ for optimal growth and stress tolerance. However, the requirement for K$^+$ may vary depending on different strains; for example, K$^+$ available in saliva is sufficient for *S. mutans* but not for *S. sanguis* (Cowman & Fitzgerald, 1976). The knowledge pertaining to the effect of K$^+$ on *S. mutans*, its uptake and contribution to stress tolerance are limited. Nevertheless, K$^+$ transport has been associated with the ability of *S. mutans* to take up and metabolize dietary sugars and, thus, contribute to its virulence (Luoma & Tuompo, 1975). In a previous study, it was shown that glycolyzing cells of *S. mutans* Ingbritt rapidly take up K$^+$ under neutral Huang *et al.* (2008) conditions and the glycolytic activity increased with addition of KCl under all the concentrations tested in an acidic (pH 5.0) environment, suggesting a connection between active uptake of K$^+$ and acid production in *S. mutans* (Dashper & Reynolds, 1992). The cell morphology of *S. mutans* depends on the bicarbonate/ K$^+$ ratio in the growth medium. More specifically, high bicarbonate/ K$^+$ ratios resulted in coccoid cellular forms, whereas low ratios were associated with bacillary morphology. Although the specific role of K$^+$ in growth and morphology remains unclear, there is a possibility that the cell is changing its intracellular volume to contend with stress. It has been proposed before that an optimal level of intracellular K$^+$ is required for maintaining the shape of cell by regulating the osmotic homeostasis as seen in other bacteria (Tao *et al.*, 2008).
1987). 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 for *S. mutans.*
Chapter 2: Introduction to K⁺ transport in *S. mutans*
2.1 K\textsuperscript{+} transport systems in \textit{S. mutans}

The \textit{S. mutans} UA159 genome harbors four putative K\textsuperscript{+} transport systems, annotated as Trk1, Trk2, Kch and GlnQHMP. Trk1 and Trk2 are interpreted to be the primary K\textsuperscript{+} transport systems in \textit{S. mutans}. The Trk1 system is encoded by a tricistronic operon of \textit{trkB}, \textit{trk} and \textit{pacL}. Both TrkB and Trk are predicted to be regulators of PacL mediated transport activity. The Trk2 putative K\textsuperscript{+} transport system in \textit{S. mutans} is comprised of two components, TrkA and TrkH, whose encoding genes form a bicistronic operon. The TrkA is predicted to have similar conserved domains as those observed for TrkB (of the Trk1 system) and probably gates the entry of K\textsuperscript{+} through TrkH. TrkH is believed to form a complex with the TrkA and is a transmembrane component.

In addition to Trk1 and Trk2, \textit{S. mutans} possess secondary K\textsuperscript{+} transporter GlnQHMP and Kch systems. Of these, our lab has previously characterized the GlnQHMP, an ABC-type transporter for its role in transport of glutamate, the main substrate and K\textsuperscript{+} as a co-transported ion (Krastel \textit{et al.}, 2010). This system plays an important role to modulate growth and acid tolerance of \textit{S. mutans} UA159 in K\textsuperscript{+} rich THYE medium (Krastel \textit{et al.}, 2010).

Kch is a hypothetical protein in the \textit{S. mutans} UA159 chromosome encoded by \textit{SMU_1848} (NCBI) with high homology to conserved ion channel-like domains with selectivity to K\textsuperscript{+} ions. Details about these systems are described in chapter 3.
Protein structure prediction was done for the four putative transport systems using Phyre 2 server (Protein structure prediction on the web: a case study using the Phyre server Kelley LA and Sternberg MJE. Nature Protocols 4, 363 - 371 (2009). Figures 2.1-3 represent the structural predictions for the components of the Trk1, Trk2, GlnQHMP and Kch systems. The best prediction outcome we could generate revealed that the PacL of the Trk1 system, which is a P-type ATPase, has a transmembrane domain, as shown in figure 2.1, and on its cytoplasmic side, it has structural domains that resemble Nucleotide (N) domain, Phosphorylation (P) and Actuator (A) domains (Shinoda et al., 2009).

Fig 2.1: Predicted structural models for the products of the genes—trkB, trk and pacL. The PacL protein has domains similar to those found in the structure of P-type ATPases. The Trk and TrkB are probably the PacL associated cytoplasmic proteins, which have similar structures. The model for PacL is a side view representation of the predicted structure.

The Trk2 system has two components as shown in the figure 2.2, of which TrkA harbors a probable K⁺ selective Rectifying Channel for K⁺ (RCK) motif and a cytoplasmic region, whereas, TrkH has a predicted structure, similar to the structure that
has been recently resolved with four main D domains that would sit in the transmembrane region (Cao et al., 2011).

![Diagram of TrkH and TrkA proteins]

**Fig. 2.2: Predicted structural models for the products of the genes–trkA and trkH.** TrkH is a trans-membrane protein with four helical D domains. The TrkA is predicted as an attached protein with selective RCK domains, which regulates the entry of K⁺ into the cytoplasm from the TrkH tunnel. The interaction of TrkA with TrkH probably occurs at the DD domain of TrkH.

The putative Kch predicted structure shows similarity to the chain C of KcsA K⁺ channel of *Streptomyces lividans*. KcsA was predicted to form a tetramer of four chains (Negoda et al., 2007). The GlnQHMP system has components similar to the ABC type transporters.
(Figure 2.3). All these systems show a portion of trans-membrane domains and selective motifs that determine their role in selective $K^+$ transport.

**Fig. 2.3:** Predicted structural models for the products of the operons–glnQHM and kch. The operon glnQHM encodes for four components, GlnQ, GlnH, GlnM and GlnP. GlnQHM is annotated to form an ABC-type transporter. Predicted transmembrane helices were observed in GlnM and GlnP. The GlnQ and GlnH proteins, both possess a D-loop, which could provide specificity to the charged moiety being transported. The Kch is encoded by a single gene $kch$ in the genome of *S. mutans*. Its basic structure prediction revealed the presence of a $K^+$ selective domain.
2.2 Rationale and Hypothesis

The physiological importance of K\(^+\) is well studied in both Gram negative and Gram positive bacteria *per se*, which explains why bacteria would accumulate this cation in and around itself. K\(^+\) is the most abundant cation present in the fluids of dental biofilms (Tatevossian & Gould, 1976), which are the natural niche of *S. mutans* in the oral environment. The cariogenic attributes of *S. mutans* have been studied extensively at both the genetic and metabolic levels, and most of the studies have reinforced the connection between its virulence properties and its microenvironment. Even though much of the abovementioned knowledge has existed since 1970s, this research area had been inadequately explored to understand the role of K\(^+\) and its regulation in *S. mutans*.

*S. mutans*, is believed to have a specific acid tolerance regulon that is activated in response to the dropping pH as shown by some 2-D proteomic (Gong *et al.*, 2009) and DNA microarray analyses (Senadheera *et al.*, 2009b). As a part of this ATR regulon, a set of potassium (K\(^+\)) transport proteins was strongly up regulated. Interestingly, K\(^+\) has been reported to play a vital role for glycolysis, cell integrity of *S. mutans*; however, the importance of K\(^+\) transport in biofilm formation and stress tolerance makes it an important focus point to understand the physiology of *S. mutans*. 
Hypothesis:

*S. mutans* transport $K^+$ using specific transport systems, and relies on $K^+$ homeostasis for regulation of its multiple physiological and virulence attributes.

**General Objectives:** 1) To investigate the role of Trk1, Trk2, GlnQHMP and Kch in $K^+$ acquisition and homeostasis in *S. mutans* under normal and stress conditions. 2) Examine the effect of $K^+$ perturbation on *S. mutans’* phenotypes that are conducive to its pathogenicity.

The following specific aims have been accomplished and described in Chapters two and three:

**Specific Aim 1.** To identify the role of *trk1, trk2, kch* and *glnQHMP* operon in *S. mutans* $K^+$ transport.

**Specific Aim 2.** To investigate the effect of $K^+$ on the main virulence properties of *S. mutans* including: efficient growth, biofilm formation, acid tolerance and acid production.

**Specific Aim 3.** To define the molecular mechanism that governs $K^+$ dependent growth defects in *S. mutans*.

**Specific Aim 4.** To examine the effect of the $K^+$ gradient between inside and outside the cell, on genetic transformability of *S. mutans*. 
Chapter 3: The Trk2 potassium transport system in *Streptococcus mutans* and its role in potassium homeostasis, biofilm formation and stress tolerance

Running Title: Potassium homeostasis in *Streptococcus mutans*

Authors: Gursonika Binepal¹#, Kamal Gill¹#, Paula Crowley², Martha Cordova¹, Jeannine Brady², Dilani B. Senadheera¹ and Dennis G. Cvitkovitch¹*

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Contributions: GB performed the experiments, analyzed the data, wrote the manuscript; KG contributed with the acid tolerance and acid production results; PC performed Western blot assays and contributed to the scientific language of the manuscript; MC performed the qRT-PCR assays and assisted in the confirmation assays; JB and DS contributed to the scientific content of the manuscript; DGC was in charge of the project and contributed to the scientific inputs, planning of the project, funds to support the project.
3.1 Abstract

Potassium (K⁺) is the most abundant cation in the fluids of dental biofilm. The biochemical and biophysical functions of K⁺, and a variety of K⁺ transport systems have been studied for most pathogenic bacteria but not for oral pathogens. In this study we establish the modes of K⁺ acquisition in Streptococcus mutans, and the importance of K⁺ homeostasis on its virulence attributes. S. mutans’ genome harbors four putative K⁺ transport systems that included two Trk-like transporters (designated Trk1 and Trk2), one glutamate/K⁺ co-transporter (GlnQHMP) and a channel-like K⁺ transport system (Kch). Mutants lacking Trk2 had significantly impaired growth, acidogenicity, aciduricity and biofilm formation. [K⁺] less than 5 mM effaced the biofilm formation in S. mutans. The functionality of the Trk2 system was confirmed by complementing an Escherichia coli TK2420 mutant strain, which resulted in significant K⁺ accumulation, improved growth and survival under stress. Taken together, these results suggest that Trk2 is the main facet of K⁺-dependent cellular response of S. mutans against environment stresses.

3.2 Importance

Biofilm formation and stress tolerance are important virulence properties of caries-causing Streptococcus mutans. To limit aforementioned properties of this bacterium, it is imperative to understand its survival mechanisms. Potassium is the most abundant cation in dental plaque, the natural environment of S. mutans. K⁺ is known to
function in stress tolerance and bacteria have specialized mechanisms for its uptake. However, there are no reports to identify or characterize specific K⁺ porters in *S. mutans*. We identified the most important system for K⁺ homeostasis and its role in biofilm formation, stress tolerance and growth. We also show the requirement of environmental K⁺ for the activity of biofilm-forming enzymes, which rationalizes why such high levels of K⁺ would favor biofilm formation.

### 3.3 Introduction

Bacteria utilize specialized endogenous mechanisms to survive and proliferate in transient environments. A common bacterial response to environmental perturbations such as acid stress, osmotic tension, or nutrient deprivation, is to accumulate certain solutes such as potassium (K⁺). K⁺, a naturally abundant cation, is present in all types of cells and is essential for both cell survival and physiology. K⁺ accumulation under stress conditions enables organisms to contend with dehydration, membrane damage, regulation of the Na⁺/ H⁺-dependent cell energetics and pH homeostasis while simultaneously minimizing interference with the structures and functions of intracellular proteins (Csonka, 1989, Christian & Waltho, 1961, Booth, 1985).

In prokaryotes, four main K⁺ transport systems have been identified, which include proteins Trk or Ktr, Kdp, Kup, and channel-like Keh (Epstein, 2003), all of which have different K⁺ affinities. Their variety and independent functioning have likely evolved as a cell survival strategy to ensure that adequate levels of K⁺ are present at all
times to contend with environmental changes (Corratgé-Faillie et al., 2010). These systems are responsible for maintaining a typical range of intracellular $K^+$ concentration, i.e. from 200-600 mM (Harold & Kakinuma, 1985, Poolman et al., 1987).

*Streptococcus mutans* is an opportunistic oral pathogen, which is a causative agent of dental caries (Becker et al., 2002). This bacterium can metabolize dietary sugars to produce acid end-products (acidogenicity) and produces sticky insoluble glucan polymers for the formation of dental plaque. Furthermore, *S. mutans* can rapidly mount a pH stress tolerance response that allows its survival at pH conditions as low as pH 3.2 (Matsui & Cvitkovitch, 2010b). The low pH in plaque environment is a general growth limiting factor, which is modulated and favored by certain inhabitant bacteria, and is distinct from other oral environments. It is known that the concentration of $K^+$ (80 mM) in the fluid portion of dental plaque is markedly higher relative to other cations found there such as sodium (20 mM) and calcium (7 mM) (Margolis & Moreno, 1994, Dibdin et al., 1986). Despite these findings, it is not well understood how such high $K^+$ concentrations affect physiology and virulence of oral bacteria such as *S. mutans*. It has been suggested that in response to acid stress, *S. mutans* can modulate its metabolic pathways by accumulating $K^+$ inside the cell (Matsui & Cvitkovitch, 2010b, Dashper & Reynolds, 2000). According to previous reports, $K^+$ can affect growth and glycolytic activity (Dashper & Reynolds, 1992), membrane potential and maintenance of cytoplasmic pH homeostasis (Dashper & Reynolds, 1996, Sato et al., 1989).
The *S. mutans* UA159 genome has at least four putative K\(^+\) transport systems, annotated as Trk1, Trk2, Kch and GlnQHMP (Supplementary Table 1). To gain an understanding of potassium acquisition and its role in *S. mutans* pathophysiology, isogenic mutants were used to measure K\(^+\) transport. The main emphasis was to first elucidate the roles of two Trk systems in the main virulence attributes of *S. mutans* namely: acidogenicity, aciduricity, and biofilm formation. The role of GlnQHMP in modulating growth and acid tolerance of *S. mutans UA159* has been reported (Krastel et al., 2010). In the current study, we utilized the glnQHMP-null mutant to extend our knowledge regarding its role in K\(^+\) dependent phenotypes. Beyond these systems, we also tested Kch, which is annotated as a hypothetical protein but has some sequence similarities with K\(^+\) channel proteins.

To address our limitations in characterizing the K\(^+\) uptake by putative K\(^+\) transport systems individually in *S. mutans*, we cloned and expressed each system separately in an *E. coli* mutant strain TK2420, which is deficient in its three major K\(^+\) transport systems (*kdp, trk* and *kup*) (Supplementary Table 2). In the current study we report that the distinctive K\(^+\) transport system for *S. mutans* Trk2 is required for physiological adaptation of *S. mutans* in K\(^+\)-limited stress conditions. We show that the Trk2 system is required for regulating membrane potential and sugar metabolism. Finally, the Trk2 system is critical in the ability of *S. mutans* to form biofilms under low K\(^+\) conditions by regulating levels of glucosyltransferases and glucan production.
3.4 Material and Methods:

3.4.1 Bacterial Strains and Culture Conditions

*S. mutans* strains were grown in Todd Hewitt Yeast Extract broth (THYE from BD, Sparks, MD) or minimal defined potassium-deprived medium with 1% glucose (MMGK) or with 1% sucrose (MMSK) at 37°C with aeration. The MMGK was prepared as previously reported (Fujiwara *et al.*, 1978); potassium salts were replaced by sodium salts, and final concentrations of 5 mM cystine and 5 mM glutamic acid were added immediately before use. For growth under osmotic stress induced by 0.4M NaCl, *S. mutans* strains were grown in chemically defined medium MMGK with 5mM, 25mM and 50mM KCl with or without 0.4M NaCl. The *E. coli* strains were grown in Luria Broth (Luria-Bertani Broth from BD, Sparks, MD) or potassium limiting medium (KLM) supplemented with 10g/L KCl for high K⁺ medium (HKLM), or with 10g/L NaCl for low K⁺ medium (LKLM) as described previously (Parfenova & Rothberg, 2006). Strains and primers used are listed in Table S2. To construct various K⁺ transport system null mutants, previously described PCR-ligation mutagenesis (Lau *et al.*, 2002) was used.

3.4.2 Growth and stress tolerance

To monitor sensitivity to extracellular K⁺ and determine the concentration for optimal growth, UA159 wild-type cells were grown in MMGK- supplemented with 0 to 150 mM filter-sterilized KCl. Based on the results for growth response to K⁺ of wild-type
UA159 cells, the range of K\(^+\) was then narrowed to 5-50 mM to monitor growth of various K\(^+\) transport null mutants. Growth was monitored in the Bioscreen C automated growth monitor (Lab Systems) as previously described (Li et al., 2002a). Briefly, 16 h cultures were diluted 1/20 in fresh THYE and grown to an OD\(_{600}\) of at least 0.400. Cells were washed and re-suspended in minimal media to give equivalent OD\(_{600}\)’s of ~0.040 and then plated in quadruplicate wells in a microtiter plate. Covered plates were placed in the growth monitor and maintained at 37\(^{\circ}\)C for at least 18 hours. Medium turbidity was measured at an absorbance of 600 nm every 20 minutes following 20 seconds of shaking. Static planktonic growth was assessed by diluting overnight cultures 1/20 in fresh THYE and measuring medium turbidity and medium pH from samples of a stock growth culture at select time points. Medium turbidity was measured by placing samples in a 96-well plate and reading the optical density at an absorbance of 595 nm on a Bio-rad Model 3550 microtiter plate reader. In parallel, medium pH was measured using a VWR SB20 SympHony pH meter. The method used was similar for *E. coli* strains with the following modifications; cells were grown and sub-cultured in HKLM medium and to test K\(^+\) sensitivity, cells were subjected to low K\(^+\) medium (LKLM). For osmotic stress assays, test strains were grown in MMGK supplemented with specific concentrations of KCl with or without 0.4M NaCl and growth was monitored using the above protocol.
3.4.3 Acid stress response

ATR (acid tolerance response) was assessed according to our published protocol (Li et al., 2001a). Briefly, overnight cultures were diluted 1/20 and grown to an OD$_{600}$ of 0.400 in Tryptone Yeast Extract (TYE) with 0.5% (w/v) glucose at pH 7.5. Half of the cultures were immediately placed in pH 3.5 TYE with 0.5% (w/v) glucose (‘kill’ medium) for up to 2 hours and are referred to as ‘un-adapted’ cells/cultures. To obtain ‘adapted’ cells, the other half of cultures were placed in TYE medium with 0.5% (w/v) glucose at pH 5.5 for 2 hours prior to placing in the pH 3.5 medium. Cells were pelleted via centrifugation at 4000 x g for 10 minutes at 22°C and re-suspended in the desired medium pre-warmed to 37°C. Cultures were sampled at the 0-, 1- and 2-hour time points in the pH 3.5 medium, serially diluted, and plated in triplicate on THYE agar to obtain colony counts. Survival percentage was calculated as: [(average colony count at post-zero time point for a particular treatment / average colony count at the zero time point for the same treatment) x 100].

3.4.4 Biofilm staining, scanning electron and confocal laser scanning microscopy

Biofilms were grown as described (Li et al., 2002a) with few modifications. Overnight cultures were diluted 1/50 in minimal medium and plated in 24-well Falcon tissue culture plates. Following a 20-hour incubation, cultures were dried and stained overnight with a 0.1% crystal violet dye solution. After removing excess stain by rinsing
with dH₂O, stained biofilms were imaged using Canon Power Shot SD 1200 IS digital camera. Experiments were performed in quadruplicate wells and repeated four times. In parallel, biofilms were grown as above but on glass slides and processed for scanning electron microscopy [SEM] as previously described (Li et al., 2001b).

For confocal analysis, 1 μM Texas Red-labeled dextran conjugate (molecular weight, 70 kDa; absorbance/fluorescence emission maxima of 595/615 nm; Molecular Probes, Invitrogen Corp., Carlsbad, CA) was added to the biofilm medium. All the bacterial cells in the biofilms were labeled with SYTO 9 green fluorescent nucleic acid stain (485/498 nm; Molecular Probes) using standard protocol (Xiao et al., 2012, Koo et al., 2010). The imaging was done using Zeiss LSM700 confocal microscope at the Advanced Optical Microscopy Facility, University of Toronto. The plan-Apochromat 60X/1.4 NA oil immersion (Ajdić & Pham) objective was used to obtain images. Two independent biofilm experiments were performed using the Thermo Scientific™ Nunc™ Lab-Tek™ II Chambered Coverglass with 8 wells, and 4 image Z-stacks at 1.0μm intervals (512×512 pixel for quantification or 1024×1024 pixel for visualization) were collected for each experiment. The images were analyzed with the Imaris 7.0.0 software (Bitplane, Saint Paul, MN) and quantified for the average surface thickness and average spot objects of the biofilms.
3.4.5 qRT-PCR analysis of gtfB expression

Expression of the *gtfB* gene in wtUA159 and the SMΔtrak2 mutant was assessed using quantitative real time polymerase chain reaction analysis. Briefly, planktonic cultures were grown to O.D.~0.4 in THYE. RNA was purified from both strains using method describes elsewhere (Hanna et al., 2001a) and *gtfB* quantitated following PCR amplification with primers 5′ ACACCTTTCGGGTGGCTTG 3′ and 5′ GCTTAGATGTCACTTCGGTTG 3′. Expression change was normalized with the expression of the house keeping 16S rRNA gene using primers 5′-CTTACCAGGTCTTGACATCCCG-3′ and 5′-ACCCAACATCTCACGACACGAG-3′. Using the Pfaffle method (Pfaffl, 2001) fold change in the expression in the mutant strain was calculated relative to expression of the *gtfB* gene in UA159. Fold change was calculated for at least 4 biological replicas and 3 technical replicas.

3.4.6 Western analysis of glucosyltransferase from cell-surface extracts

Wild type (UA159) and SMtrakΔ2 strains were cultured for 16 h in THYE broth at 37°C and passaged 1:20 into warm THYE. Growth was continued until O.D.~0.6 was reached, then cells were centrifuged, washed once in MMSK, resuspended in warm MMSK and diluted 1:20 dilution into duplicate 40 ml tubes of warm MMSK with KCl added at 5, 25 or 50 mM final concentrations. Cultures were grown 24 hrs at 37°C then
cells were pelleted and washed twice in 20 mM Tris-Cl, pH 8.0 and extracted for 1 hr with 1.0 ml of 4% SDS followed by boiling for 10 min, which allowed the extraction of non-covalently attached proteins from the cell surface without lysing the cells. Samples were centrifuged at 16,000 x g and the supernatant saved aside for Western analysis. The O.D. of each SDS extract was measured using the Nanodrop 1000 (ThermoScientific, Wilmington, DE) and extracts were standardized for protein concentration. Twenty microgram of extract protein from duplicate cell cultures was applied to a 4-20% TGX gel (BioRad) and proteins were separated at 150 V. Separated proteins were blotted onto PVDF membranes using the Transblot Turbo (Biorad) and membranes were blocked overnight in PBS-0.3% Tween20, 5% skim milk. Rat anti-GtfD antibody (Smith et al., 1994a) was added to the blot at a 1:500 dilution, incubated for 1 hour at room temperature, washed three times in PBS-Tween and incubated for 1 hour with 1:1000 goat-anti-rat-HRP conjugated antibody. The blots were washed in PBS-Tween and developed using ECL-Prime (GE Lifesciences, Piscataway, NJ) for 1 min prior to imaging in the Gbox-Chemi XL1.4 imager (Syngene, Frederick, MD).

3.4.7 Visualization of glucan production from agar plate-grown colonies

*S. mutans* UA159 and the trk2-null mutant colonies were grown for 48 h on TSY20B (4% trypticase soy agar, 0.5 % plain agar, 1% yeast extract, 20 % sucrose, and 0.2 U/ml of bacitracin; ThermoFisher) plates at 37°C with an additional 72 h incubation
at 25 °C. Colonies and glucan puddles produced were imaged with a Leica MZ8 dissecting microscope with DFC320 camera and Firecam v.3.4.1 software (Leica Microsystems, Buffalo Grove, IL) using incident lighting. Magnifications were 63X (smallest images) – 160X (largest images).

3.4.8 Measurement of intracellular K\(^+\) ion content

Overnight *S. mutans* cultures, diluted 1/20 in fresh THYE, were grown to an OD\(_{600}\) of 0.400. Cells were washed and incubated in MMGK without KCl for at least 2 hours to deplete the cellular K\(^+\). Cells were stimulated with 10 mM KCl and an aliquot was taken and filtered (0.22 µm) every 30 seconds. The dry weights of filters for each sample were measured and the filters were immersed in 10% HNO\(_3\) and boiled for 30 minutes. Inductively Couple Plasma Optical Emission Spectroscopy (ICP-OES) was performed on each sample using an Optima 7300 DV instrument (Perkin Elmer) housed at the ANALEST facility, Department of Chemistry, University of Toronto. Triplicate emission reads of samples were averaged and calibrated against a standard curve (minimum correlation of 99.9%) generated for different concentrations of potassium (766.49 nm) using a serially diluted QC4 standard (SCP Science, Quebec). For *E. coli* cultures, the overnight cultures grown were sub-cultured 1/20 in HKLM medium until they reached an OD\(_{600}\) of 0.4. Cells were then processed similar to *S. mutans* cultures described above.
3.4.9 Membrane potential measurement using fluorescence

The alterations in electrical membrane potential of various wild-type and K\(^+\) transport mutant cells were measured using the membrane-potential-sensitive fluorescent dye bis-oxonol \{DiSBaC\(_2\)(3) [bis-(1,3-diethylthiobarbituric acid) trimethine oxonol]\}. As bis-oxonol is an anionic, lipophilic molecule, a higher concentration of dye accumulates in the cell upon membrane depolarization, binding to intracellular components and resulting in an increase in cytosolic bis-oxonol fluorescence intensity, whereas upon hyperpolarization, less dye is located intercellularly and fluorescence intensity decreases.

Overnight *S. mutans* cultures, diluted 1/20 in fresh THYE, were grown to an OD\(_{600}\) of 0.4. Cells were then washed and re-suspended in MMGK supplemented with defined KCl, with or without stressors such as pH or osmotic stress. 100 µL of cell suspension was aliquoted in a 96-well plate and mixed with fluorescent dye reaction mixtures prepared using the FIVEphoton kit manufacturer’s protocol (FIVEphoton Biochemicals). The plate was incubated for 15 minutes and fluorescence emission intensity (emission wavelength of 560 nm) was measured using a TECAN microplate reader (excitation wavelength of 530 nm). The plate conditions were set at 37°C with shaking for 5 seconds before every read, and the fluorescence emission intensity measurement was taken every 15 minutes for at least 6 hours. For *E. coli* strains, the experimental protocol followed was the same as for *S. mutans* with the following
modifications: cultures were grown in HKLM medium and the mid-exponential phase cells were washed and re-suspended in either MMGK with defined concentrations of KCl, or cells were re-suspended in KLM medium with low and high K⁺ content and test stressors. The measurement protocol was altered to accommodate desired culture conditions at 37°C with continuous shaking and read every 2 minutes for at least 6 hours. For fluorescence intensity change analysis, data were transformed to control for bias potentially generated by experimental setting variations: fluorescence was expressed as change between time t and time 0 according to the formula: ΔFt = [(Ft-F0)/F0] \cite{Sorio et al., 2011}. To control for intensity change of the dye, ΔFt (culture sample) was normalized with ΔFt (blank with dye) and final values were plotted against time for each condition used.

### 3.4.10 Complementation assay

The *E. coli* strain TK2420 \cite{Epstein, 2003} was a generous gift from Wolfgang Epstein (University of Chicago). Open reading frames (ORFs) for each K⁺ transport system (trk1: SMU_1561-63, trk2: SMU_1708-09, glnQHMP: SMU_1519-22 and putative kch SMU_1848) were each amplified using specific primers and cloned into the pET-DUET expression vector (Novagen, Inc.). DNA sequencing confirmed proper ligation of inserts into expression vectors. Complementation was assayed as described with few modifications \cite{Buurman et al., 2004}. Briefly, competent TK2420 cells were heat-shock transformed with each expression vector of interest and grown with ampicillin selection on potassium rich (HKLM) agar. Colonies were then re-streaked on low
potassium (LKLM) agar with ampicillin selection to confirm successful complementation. Starter cultures were grown overnight by inoculating single colonies in HKLM with ampicillin. Subsequently, cultures were grown to mid-exponential phase, washed and resuspended in sterile H2O or PBS and serially-diluted before plating as 10-µL droplets on minimal medium MMGK-agar.

3.5 Results

3.5.1 K+ transport systems identified in S. mutans

The S. mutans UA159 genome harbors two orthologs of putative K+ porters, which are designated as Trk1 and Trk2 (Table S1, Fig. 3.1). The Conserved Domains Database (CDD) search was used to elaborate the predicted protein domains and families (Marchler-Bauer et al., 2015). The predicted interactive partners and proteins that share sequence identity in other bacteria are listed in Table S1. The Trk1 system is annotated to be comprised of TrkB, Trk and PacL and is encoded by a tricistronic operon. Both TrkB and Trk are predicted to contain N-terminal-conserved Rossmann-fold (+)-binding regions with a GXGXXG consensus sequence. This motif is known to confer specificity to NAD(+)-binding through its first two glycine residues. Glycine rich regions are also found and associated with selectivity to K+ ions. The C-terminal ends of these proteins have a predicted conserved permease-like domain, which is conducive to configuring its beta structure and has specificity for an unknown ligand. PacL has four predicted conserved domains that include an N-terminal cation ATPase, an E1-E2 ATPase (pfam
00122), a conserved putative hydrolase of \( \text{Na}^+/\text{K}^+ \) ATPase alpha-subunit like domain, and a C-terminal cation ATPase.

The Trk2 putative \( \text{K}^+ \) transport system in \( S. \text{mutans} \) is comprised of two components, TrkA and TrkH, whose encoding genes form a bicistronic operon. The TrkA is predicted to have similar conserved domains as those observed for TrkB (of the Trk1 system) with a Rossmann-fold NAD (+)-binding region. TrkH is believed to form a complex with TrkA and is a transmembrane component.

In addition to Trk1 and Trk2, other putative systems that could modulate the transport of \( \text{K}^+ \) in \( S. \text{mutans} \) are the GlnQHMP (Krastel et al., 2010), and Kch. Kch is a hypothetical protein in the \( S. \text{mutans} \) UA159 chromosome encoded by \( \text{SMU}_1848 \) (NCBI) with high homology to conserved ion channel-like domains of either two helices or tetrameric structure, and belongs to the family of pfam07885, which have a conserved GYG sequence shown to render glycine-rich selectivity to \( \text{K}^+ \) ions (Sansom et al., 2002).
FIG. 3.1: Genetic map of operons encoding the putative K⁺ transport systems in *S. mutans* UA159.
3.5.2 Role of K$^+$ in *S. mutans*

To understand the roles of Trk1, Trk2, GlnQHMP and Kch in *S. mutans* physiology, the isogenic mutant strains deficient in individual operons (strains SM$\Delta$trk1, SM$\Delta$trk2, SM$\Delta$glnQHMP, or SM$\Delta$kch) and knockout mutants, deficient in genetic components of these systems (strains SM$\Delta$trk, SM$\Delta$trkB, SM$\Delta$trkA, SM$\Delta$trkH or SM$\Delta$pacL), were used (Table S2). To examine the effects of both Trk systems, we constructed a double-knockout mutant strain lacking trk1 and trk2 operons, designated SM$\Delta$trk12 (Table S2).

3.5.2.1 K$^+$ dependent growth characterization

The concentrations of K$^+$ required for optimal growth of *S. mutans* and the K$^+$ concentrations present in some commonly used growth media (THYE, TYE, MMGK-, elaborated in Materials and Methods) were estimated using inductively coupled plasma optical emission spectroscopy (ICP-OES). Our results indicated that both THYE and TYE had sufficient but not optimal K$^+$ (> 10 mM) for the growth of *S. mutans* (Table S3). To test the effect of limited K$^+$, the planktonic growth of *S. mutans* UA159 wild-type strain was tested in MMGK- medium supplemented with a range of K$^+$ concentrations (5-150 mM), and growth was monitored using the Bioscreen C automated growth monitor. By comparing the final absorbance values (Fig. 3.2A) for growth, we noted that optimal growth was accomplished in MMGK supplemented with 25 mM K$^+$. At this concentration, the growth yield was significantly higher than that at \( \leq 5 \text{ mM} \) or \( \geq 50 \text{ mM} \).
K⁺ (p<0.01). For our experiments (unless otherwise stated), we used MMGK- medium supplemented with 1) 5 mM K⁺ as “low” K⁺ conditions, 2) 25 mM K⁺ as “optimal” conditions, and 3) 50 mM K⁺ as “high” K⁺ conditions.

FIG. 3.2: Effects of K⁺ and its transport systems in growth and final pH changes in *S. mutans*. A) Growth analysis of the *S. mutans* UA159 parent strain. Final absorbance measurements were taken from cell cultures grown in MMGK supplemented with varying concentrations of KCl (0 mM to 150 mM). B) Growth curves of wild-type and Trk mutants grown in K⁺-rich THYE medium (~ 19 mM). C) pH measurements of Trk1, Trk2 and the Trk1/Trk2 double mutant grown in THYE growth medium over time. A single asterisk (*) above or below the boxed symbols denotes result is statistically significant by p < 0.05 and a double asterisk (**) denotes result is statistically significant by p < 0.001 in comparison to WT (for B and C) and comparison of growth in various K⁺ concentrations to growth in 25 mM K⁺ (for A). Values for all graphs represent mean standard error (SE) for experiments repeated three times and statistical comparison was performed using a two-way ANOVA.

3.5.2.2 Role of K⁺ transport systems in growth

The roles of the Trk1- and Trk2-dependent K⁺ acquisition on the growth of *S. mutans* were examined in K⁺-rich THYE medium. Notably, loss of the *trk2* operon (SMΔtrk2), and the combined *trk1/trk2* deletion mutant (SMΔtrk12) showed significantly reduced growth yields at the 2-, 4-, 6- and 8-hour time points relative to the wild-type and
the \textit{trk1} knockout mutant (p$< 0.01$) (Fig. 3.2B). Concomitant with the growth defect, we noted that the drop in pH values was slower relative to the parent strain, reflective of significantly reduced acid production by strains SM$\Delta$trk2 and SM$\Delta$trk12 (p$<0.001$) (Fig. 3.2C). Further, the growth retardation of the \textit{trk2} deficient strain was observed to be consistent when cells were grown under low K$^+$ conditions in presence of various dietary sugars such as fructose, sucrose, mannose and glucose (S5). Unlike the Trk systems, loss of the Kch system did not have any effect on the growth phenotype of \textit{S. mutans}.

3.5.2.3 Trk2 is essential for acid stress tolerance

The acid tolerance response of \textit{S. mutans} is a major virulence attribute that facilitates its survival under low pH conditions (pH$< 3.5$) that are often detrimental to other competing oral bacteria. When supplemented with sugars, \textit{S. mutans} produces acid end-products, primarily lactic acid, which decreases its environmental pH. When pre-exposed to a “mildly acidic” signal (pH $5.5$), \textit{S. mutans} can activate protective mechanisms for survival at a killing pH (pH $3.5$) (Svensater \textit{et al.}, 1997, Svensater \textit{et al.}, 2000). To examine the role of Trk1 and Trk2 systems on the ATR of \textit{S. mutans}, we quantified survival using acid killing assays with acid-adapted or un-adapted cultures. In agreement with other studies, adaptation to acid significantly increased the survival rates of the \textit{S. mutans} UA159 wild-type strain (Fig. 3.3A, 3.3B). Although this was the case with the Trk1 deletion mutant, loss of Trk2 did not show a remarkable difference in percentage viability between adapted and un-adapted cells (Fig. 3.3A, 3.3B). As observed with the growth phenotypes, the response to acid killing of the Trk2 mutant was similar
to that of the Trk1/Trk2 double mutant, suggesting the importance of Trk2 for the acid stress response of *S. mutans*.

**FIG. 3.3: Acid stress response of *S. mutans* wild type UA159 and Trk mutants.** Acid tolerance assays were conducted with unadapted (white bars) and adapted (black bars) cultures incubated for (A) 1 hour or (B) 2 hours at a killing pH of 3.5. For pH adaptation, cells were pre-exposed to TYE medium plus 0.5% glucose that was adjusted to pH 5.5. After exposure to the killing pH, cells were plated and acid tolerance was expressed as percent survivors relative to the total number of CFUs present at time 0, just prior to exposure at pH 3.5. Number sign (#) or asterisk (*) denotes statistical significance by p<0.01 or p<0.001, respectively compared to unadapted cultures of the same strain. Values for all graphs represent mean standard error (SE) for experiments repeated three times and statistical comparison was performed using a one-way ANOVA.

### 3.5.2.4 Role of K⁺ in osmoregulation

K⁺ accumulation is an essential aspect of bacterial response to osmotic stress. Perturbations in environmental osmolality of oral biofilms can significantly affect the overall cell energetics and expression of various systems involved in survival and virulence of *S. mutans* (Abranches *et al.*, 2006b). To assess the influence of the putative
K⁺ transport systems on osmo-adaptation, we first tested the growth of mutant and wild-type S. mutans strains under osmotic stress induced by 0.4 M NaCl in MMGK- medium supplemented with low K⁺ (5 mM), optimal K⁺ (25 mM), and high K⁺ (50 mM) concentrations. The cultures were depleted of K⁺ prior to growth analysis by first incubating cells in MMGK- for 1 hour. At low K⁺ (5 mM), the growth of SMΔtrk2 was significantly reduced as compared with UA159 (Fig. 3.4). However, increasing K⁺ to 25 mM or 50 mM improved the growth of trk2 mutant strains relative to low K⁺ conditions. Under low K⁺ and osmotic stress of 0.4 M NaCl, growth retardation was observed for all test strains. Increasing the K⁺ concentration to 25 mM in MMGK improved the growth of UA159, and concentration of 50 mM improved that of SMΔtrk1 under osmotic stress, while the growth of SMΔtrk2 remained low even in presence of 50 mM K⁺. Thus we concluded that Trk2 is the most important for K⁺ mediated osmo-adaptation in S. mutans.
FIG. 3.4: Osmotic stress adaptation of wild type UA159 and SMΔtrk2 under limited K⁺. Strains were grown in MMGK with 5 mM, 25 mM, and 50 mM KCl for 24 hrs. To examine growth rate under osmotic stress, cells were supplemented with 0.4M NaCl (labeled with concentration of KCl + 0.4M NaCl) and compared to those without NaCl (labeled with concentration of KCl). Final growth yield under varying K⁺ concentration with or without 0.4M NaCl for four biological and three technical replicates are presented. Student’s t-test showed statistical significance of p<0.05 (*) when compared to wild type strain UA159.
3.5.3 Biofilm formation in limited K$^+$ requires Trk2 system

The ability of *S. mutans* to form a biofilm is critical to its survival in the oral cavity (J. Nishimura, 2012, Marsh, 2004). To date, the biofilm phenotype of *S. mutans* and its relationship with K$^+$ transport systems have not been examined. We examined the biofilm of the K$^+$ transport mutant strains attached to the micro-titre plate in MMGK supplemented with defined concentrations of K$^+$ (0-150 mM). Without K$^+$ in the growth medium (0 mM), biofilms were completely abolished for wild-type UA159 and mutant strains (Fig. 2.5A). When supplemented with 5 mM K$^+$, the SMΔtrk2 strain formed a fragile and extremely thin biofilm compared to the parent strain. Biofilms formed by the Trk1/Trk2 double mutant were also unstable and easily disrupted relative to wild-type and the Trk2 mutant. We observed a restoration in the biofilm phenotype of SMΔtrk12 at a K$^+$ concentration of 150 mM. Although the discrepancy in the biofilm phenotype between SMΔtrk2 and SMΔtrk12 mutants suggested an influence of Trk1 on biofilm formation, we did not notice an impairment of the biofilm phenotype with the loss of Trk1 alone. In contrast to the influence of Trk systems in biofilm formation, loss of GlnQHMP and Kch systems did not affect the biofilm phenotype relative to wild-type under test conditions.

Scanning electron microscopy for wild-type or Trk1-knockout strains grown in 5 mM K$^+$ (Fig. 3.5B) showed normal entangled chains of spherical cells. In contrast, SMΔtrk2 and SMΔtrk12 mutant biofilms were hypocellular in nature containing cells forming less entangled and smaller chains. These defects were more pronounced in the
double knockout, as revealed by crystal violet staining (Fig. 3.5A). Supplementation with excess K\(^+\) (>50mM) recovered the biofilm phenotypes in the mutant strains. Since we had previously noted that loss of Trk2 affected growth of \textit{S. mutans}, we tested to see if the Trk2-mediated biofilm phenotype was related to a growth deficiency or the result of compromised cell viability. Our live/dead stains of the Trk biofilms with PI and SYTO-9 showed that cell viability was not affected in the SMΔtrk2 mutant strain relative to wild-type.

**FIG. 3.5:** Evaluation of biofilm attachment to solid surfaces with scanning electron microscopy for \textit{S. mutans} wild type UA159 and mutant strains deficient in primary K\(^+\) transport systems. (A) Biofilms were grown on microtiter plates in defined medium MMGK supplemented with various concentrations of KCl (0-150 mM). After a 20-hour incubation, supernatant was removed and biofilms were dried and stained with 0.1% crystal violet dye. The experiment was repeated at least three times using triplicate wells each time. Representative photographs are shown. (B) Biofilms were grown on glass cover slips placed in microtitre plates containing MMGK growth medium supplemented with 5 mM. Following a 20-hour incubation period, biofilms were dehydrated and further processed for SEM. Representative micrographs are shown.
Preliminary qRT-PCR analysis revealed \textit{gtfB} expression of 0.5 ± 0.12 in the planktonic cultures of SMΔtrk2 mutant, which is 2-fold down-regulation relative to wt UA159 strain. Although, not statistically different than the wt UA159, this down regulation led us to hypothesize that biofilm formation is disrupted in the \textit{trk2}-null mutant under low K\textsuperscript{+} concentrations because glucosyltransferase activity is in some way affected in these mutants. To test this, we grew the biofilms for \textit{trk2}-null and UA159 strains in minimal medium with sucrose (MMSK) instead of glucose and tested the formation under low and high K\textsuperscript{+} using confocal laser scanning microscopy analyses. Sucrose was utilized as the sugar source, because the incorporation of Texas-red labeled dextran as a function of GTF activity can be best measured by keeping sucrose as the substrate. The assembly of the extracellular matrix was evaluated by incorporating Texas-Red dextran at the beginning of the biofilm assay and the cells were stained with Syto 9. The (fluorescently-labeled) dextran acts as a primer and acceptor for GtfB, and gets incorporated with the help of exoenzyme into the EPS-matrix over the course of biofilm formation (Klein \textit{et al.}, 2009, Xiao \textit{et al.}, 2012).

Biofilms matrix produced by the \textit{trk}-null mutant (Fig. 3.6) were significantly lower than those produced by the wild type at the 25 and 50 mM K\textsuperscript{+} medium concentrations while no different at the 5 mM K\textsuperscript{+} concentration. A similar result was seen for cell scores (Fig. 3.6) at all K\textsuperscript{+} concentrations. Notably, biofilm thickness and cell scores were not significantly different for the \textit{trk} mutant at all K\textsuperscript{+} concentrations where, conversely, significant differences in cell scores were observed at the 25 and 50 mM K\textsuperscript{+} concentrations and for biofilm thickness at all K\textsuperscript{+} concentrations for the wild type strain.
To normalize the results for impact of K\(^+\) levels on GTF function by total cell count, total dextran thickness measured was normalized to total number of Syto 9 stained cells and the final dextran thickness per cell is presented in the figure 3.6. There was a significant increase in the dextran thickness per cell observed in the presence of 25 mM K\(^+\) for wtUA159, while an improved dextran thickness per cell was observed for SM\(\Delta\)trk2 in the presence of 50 mM K\(^+\). We were also able to see some biofilm formation by SM\(\Delta\)trk2 mutant under low K\(^+\) conditions, which was not observed in the previous method, but SM\(\Delta\)trk2 biofilm remained significantly impaired relative to UA159 strain.

Western blot analysis was then carried out to further examine if low intracellular K\(^+\) levels in the \(trk2\)-null mutant causes a defect in Gtf levels in the SM\(\Delta\)trk2 mutant compared with UA159 under limited K\(^+\) (Fig. 3.7A). Using polyclonal anti-GtfD sera we observed a reduction in the SDS-extracted, cell-associated, GtfD levels in the mutant at all medium-K\(^+\) concentrations. Notably, the protein profiles for UA159 (WT) were different from those for the \(trk2\)-null mutant in the Gtf MW region (~155kDa), with high MW bands prominent for UA159 and lower MW bands (~30 kDa) that did not react with the antibody, prominent in the mutant. For further confirmation, colony morphology was monitored for the presence or absence of glucan puddles for both the wtUA159 and SM\(\Delta\)trk2 strains. Interestingly, large clear glucan puddles could be seen around the UA159 colonies (Fig 3.7B), where none could be seen around the SM\(\Delta\)trk2 mutant colonies, although colonies of the latter “glistened” suggesting the presence of very modest amounts of glucan. Taken together with the Western analysis data this suggests
that glucosyltransferase activity, and hence glucan production, is severely impaired in the Trk2 mutant.

FIG. 3.6: Confocal laser scanning microscopy of biofilms formed by *S. mutans* wild-type UA159 and *trk2*–null mutant strains. Biofilms were grown on the 8 well-chambered coverglass containing MMSK growth medium supplemented with 5mM, 25mM or 50mM KCl and 1 μM Texas Red-labeled dextran conjugate. Following 18 h all of the bacterial cells in the biofilms were labeled with SYTO 9 green fluorescent nucleic acid stain and processed for CLSM. The histograms below represent: A) the average surface thickness of dextran, B) average spot counts of objects in the biofilms and C) the dextran thickness normalized to the SYTO 9 stained cell count. Values for all graphs represent mean standard error (SE) for experiments repeated three times with three technical replicates and statistical comparison was performed using a student’s t-test. P-value < 0.05 was considered significant and represented with an asterisk (*).
FIG. 3.7: A) Western blot analysis for Glucosyltransferase derived from cell surface protein extracts of \textit{S. mutans} wild type and SM\textDelta trk2 mutant strains grown in MMSK with varying KCl concentrations. 20 μgs of SDS-extracted cell-surface proteins from 24 hr duplicate cultures of wild type and \textit{trk2}-null mutant strains grown in MMSK with 5, 25 and 50 mM KCl added were separated on 4-20% TGX gradient gels, blotted and stained for total protein (left panel) or probed with anti-GtfD (right panel). B) \textbf{G}lucan production by \textit{S. mutans} wild type and \textit{trk2}-null mutant strains. Strains were grown on TSY20B (20% sucrose) agar plates for 48 h at 37°C and an additional 72 h at 25°C. Colony morphology was imaged using a Leica MZ8 dissecting microscope with DFC320 camera and Firecam v.3.4.1 software (Leica Microsystems, Buffalo Grove, IL) using incident lighting. Magnifications were 63X (a, c) – 160X (b, d). Images a and b represent colony morphology for wt UA159 strain; images c and d represent the morphology of SM\textDelta trk2 strain.
2.5.4 K\(^+\) acquisition by *S. mutans*

Despite the critical role of K\(^+\) in growth, glycolysis, and stress tolerance of *S. mutans*, there are no studies to date examining how this organism acquires K\(^+\). To establish a clear role for Trk1, Trk2, GlnQHMP and Kch in K\(^+\) acquisition, we used ICP-OES for quantifying the amounts of K\(^+\) accrued by each null mutant relative to wild-type UA159 (Fig. 3.8A) over time. First we measured the temporal intracellular concentrations of K\(^+\) with respect to extracellular supplemented concentrations of KCl (5, 10, 25, 50 mM) in the growth medium, MMGK (not shown). Prior to analysis, we ensured cell-depletion of K\(^+\) by incubation in MMGK for at least 2 hours. For Trk1, GlnQHMP, and Kch deletion mutants, growth, as assessed by dry weight measure of each cellular sample was unaffected at 5 mM K\(^+\), while at this same concentration the Trk2 mutants were unable to grow leading us to conclude that K\(^+\) uptake of Trk2 mutants did not occur. When K\(^+\) in the medium was increased to 50 mM, we did not observe statistically significant differences in growth or total K\(^+\) uptake in any of the mutants relative to wild-type (Fig. S6). To compare K\(^+\) uptake in wild-type vs mutant cells, we used cells grown in MMGK supplemented with either 10 mM or 25 mM KCl, to determine initial and prolonged K\(^+\) uptake, respectively. With media supplemented with 10 mM K\(^+\), the intracellular content of K\(^+\) increased significantly over time (15 seconds to 2 minutes) for SMΔtrk1 (up to 1.22 ± 0.5 µmol/mg dry weight of cells) relative to wild-type (0.28 ± 0.05 µmol/mg dry weight of cells). However, no significant differences in intracellular K\(^+\) concentration were observed between UA159 and the mutant strains SMΔtrk12, SMAkch, and SMΔglnQHMP.
FIG 3.8A: Estimation of intracellular K⁺ content in *S. mutans* wild-type UA159 strain and K⁺ transport knockout mutants. To deplete cells of K⁺ prior to uptake assays, mid-exponential phase cells were incubated in K⁺-deficient MMGK growth medium for 2 hours. Cells were then supplemented with 10 mM KCl and aliquots were sampled over time to measure intracellular K⁺ content using ICP-OES. Values represent mean ± SD of at least four biological and two technical replicates. Repeated measure ANOVA was performed and comparisons were made between different time points within each group (*) and by comparing intracellular K⁺ levels between groups (†), *p*< 0.05 is significant.

Since we observed growth defects in SMΔtrk2 at 25 mM K⁺ that were overcome by the media being supplemented with 50 mM K⁺, we used a K⁺ concentration of 25 mM to test whether its growth retardation was due to the lack of adequate intracellular K⁺. We also assessed K⁺ accumulation in various isogenic Trk1 and Trk2 mutants relative to that of the wild-type supplemented with 25 mM K⁺ after an extended time of 30 minutes (Fig. 3.8B). At 30 minutes, K⁺ accumulation by the Trk1/Trk2-deficient strain was significantly reduced compared to that of UA159 cells (*p*<0.01). Moreover, as previously observed with similarities in biofilm and acid tolerance phenotypes, the SMΔtrk12 (0.118
± 0.029 µmol/mg dry weight of cells) had comparable levels of K⁺ uptake relative to the SMAΔtrk2 mutant (0.115 ± 0.039 µmol/mg dry weight of cells). Our results showed that UA159 had significantly increased cellular levels of K⁺ (0.295 ± 0.025 µmol/mg dry weight of cells) compared with potassium-starved UA159 cells (0.060 ± 0.021 µmol/mg dry weight of cells) (p<0.001). The amount of cellular K⁺ was not statistically different between K⁺-starved UA159 and mutant strains (0.113 ± 0.023 µmol/mg, 0.11 ± 0.011 µmol/mg, and 0.08 ± 0.009 µmol/mg dry weight of cells) for SMAΔtrk1, SMAΔtrk2 and SMAΔtrk12, respectively.

**FIG 3.8B: Intracellular K⁺ accumulation after 30 min.** Wild-type UA159 and knockout mutant strains were grown to an OD₆₀₀ ≈ 0.4 in THYE medium prior to incubation in minimal media for 10-12 h to deplete cellular K⁺. Cells were subsequently challenged with 25 mM K⁺ for 30 min followed by measurement of cellular cation content via ICP-OES. Values represent mean ± SE and experiments were repeated at least four times and statistical comparison was performed using a two-way ANOVA. Asterisk (*) indicates values are statistically different to at least p<0.01 from WT cells stimulated with 25 mM K⁺ while the number sign (#) denotes values are statistically different to at least p<0.01 from WT cells starved of K⁺.
3.5.5 Influence of K$^+$ transport systems on the membrane potential of S. mutans

The trans-membrane potential of bacterial cells is a critical feature governing growth and cell physiology by affecting cellular proton motive force, pH homeostasis, protein function and protein localization (Strahl & Hamoen, 2010). Here we examined the role of K$^+$ transport systems in maintaining the membrane potential of S. mutans in response to changes in external K$^+$ concentrations. We had observed that the loss of Trk1 and Trk2 systems had a profound effect on growth, stress tolerance, and K$^+$ acquisition of S. mutans. After washing S. mutans strains with K$^+$-deficient growth medium, we challenged cells with 0, 5, 25 and 50 mM K$^+$ in MMGK- with and without acid stress conditions. Membrane potential assays were then conducted by measuring the fluorescence intensity of a potentiometric dye, DiSBAC$_2$(3), that can only enter a depolarized cell or cell with high cation accumulation (Gášková et al., 1998). We analyzed the membrane potential of wild-type UA159, SMtrk1 and SMΔtrk2 mutants after 15 minutes, 30 minutes, and 2 hours (Fig. S1). The wild-type UA159 strain showed high depolarization at 5 mM K$^+$ at pH 5.5, which was in contrast to the mutants lacking trk1 or trk2 (Fig. S1). While the trk1 mutant showed recovery at 25 mM K$^+$ concentration, the trk2 mutant showed recovery at only 50 mM K$^+$ concentration at pH 5.5. The defect in K$^+$ gradient dependent membrane potential of SMΔtrk2 mutants under pH 5.5 stress can be linked with the defect observed in acidogenicity and aciduricity of this mutant.
3.5.6 Characterizing *S. mutans* K⁺ transport systems in *E. coli* strain TK2420

Our aim was to dissect the function of each of the *S. mutans* K⁺ transport systems individually, which we were unable to do using the parent strain under our test condition. The genes containing the four *S. mutans* K⁺ transport systems were cloned into the pET-DUET cloning and expression vector and transformed into *Escherichia coli* TK2420, which lacks its own constitutive K⁺ transport systems. The complementation of these systems was confirmed by selection on high and low K⁺ plates with ampicillin. We also included recombinant plasmids containing the individual components of the Trk systems to determine whether or not these proteins can function independently. We were able to obtain clones on low K⁺ agar plates for strains harboring plasmids that contained *pacL*, *glnQHMP*, *trk2* or *kch*. The *trk1a* component of the Trk1 system and TrkA or TrkH of the Trk2 system did not complement the *E. coli* TK2420 as deduced by a lack of colonies on low K⁺ - ampicillin plates.

3.5.6.1 *E. coli* TK2420 incorporated with *S. mutans* K⁺ transport systems recovers K⁺ acquisition and growth

Previously, it has been reported in *E. coli* TK2420 that K⁺ uptake by non-specific transport systems is nearly abolished when supplemented with ≤ 5 mM K⁺ in defined growth medium (Buurman *et al.*, 2004). We tested if K⁺ transport systems from *S. mutans* could restore growth under low K⁺ concentrations in growth medium by screening the *E.
coli TK2420 mutant derivatives with Trk1, Trk2, GlnQHMP or Kch systems that were able to grow on low K$^+$ (MMGK- agar plates containing either 1 mM or 5 mM K$^+$). The plates were incubated for up to 90 hours to accommodate the cells with slower recovery. Within an 18 hour-incubation period we recovered the TKtrk2 growth on both 1 mM and 5 mM K$^+$ plates (Fig. 3.9). Gradually, (36-90 hours) we also observed recovery of growth of TKtrk1 and TKglnQHMP with 5 mM K$^+$ supplementation. However, even after 90 hours under 1 mM K$^+$ supplementation, only TKtrk2 grew. Thus, we concluded that Trk2 is an important K$^+$ transport system that has a crucial function in cell growth under low K$^+$ conditions.
FIG. 3.9: Complementation analysis using *E. coli* TK2420 and its mutant derivatives complemented with *S. mutans* Trk1, Trk2, GlnQHMP and Kch systems. Strains were grown to mid-exponential phase in HKLM medium with a high concentration (10g/L) of KCl before washing and diluting with K⁺-deficient, MMGK medium. Cells were spotted on MMGK agar plates with 5 mM KCl (top panel) or 1 mM KCl (bottom panel), and selected for ampicillin resistance after incubation for (A) 18 hours, (B) 36 hours, and (C) 90 hours.
The intracellular K\(^{+}\) concentration was measured for various complemented strains of *E. coli* TK2420 using ICP-OES method. Mid-exponential phase *E. coli* TK2420 and complemented strains were grown in high K\(^{+}\) broth (HKLM). Cells were then washed and incubated in MMGK- for 2 hours before challenging them for K\(^{+}\) uptake. Trk1, Trk2 and GlnQHMP systems showed a significantly higher acquisition of K\(^{+}\) (1.234 ± 0.06, 1.488 ± 0.063, and 1.709 ± 0.38 µmol/mg dry weight of cells, respectively), compared with TK2420 cells harboring pET-DUET alone (0.746 ± 0.04 µmol/mg dry weight of cells) after 120 seconds of exposure to 5 mM K\(^{+}\) supplemented defined MMGK- (Fig. 3.10). TKkch harboring the Kch system showed no difference (0.78 ± 0.05 µmol/mg dry weight of cells) compared with TK-DUET, suggesting its inability to acquire K\(^{+}\) under extracellular availability of 5 mM K\(^{+}\). These results showed that Trk1, Trk2 and GlnQHMP were able to pump significant amounts of K\(^{+}\) intracellularly from a low K\(^{+}\) extracellular environment (5 mM) within 120 seconds.
FIG. 3.10: Estimation of intracellular K$^+$ content in *E. coli* strain TK2420 deficient in major K$^+$ transport systems and its complemented strains. Strains were grown to mid-exponential phase in K$^+$-rich KLM medium and incubated in MMGK for 2 hours to deplete cellular K$^+$ levels. Cells were subsequently supplemented with 5 mM KCl and aliquots sampled over time to measure intracellular K$^+$ content using ICP-OES. Data are representative of two independent experiments performed in duplicate. Error bars represent mean concentrations ± S.D. Repeated measure ANOVA was used for statistical analysis between: a) different time points compared with the initial time point (*), and b) different mutants compared with *E. coli* strain TK2420 harboring an empty vector at the same time point (#).

3.5.6.2 Trk1, Trk2 and GlnQHMP enable *E. coli* TK2420 growth under stress

The role of each of the K$^+$ transport systems that complemented *E. coli* TK2420 (TKtrk1, TKtrk2, and TKglnQHMP) was assessed in terms of planktonic growth under low K$^+$ and high osmotic stress (0.4 M NaCl) conditions. The *E. coli* mutant strain TK2420 and its complemented variants harboring Trk1, Trk2 and GlnQHMP systems initially grown in HKLM, were washed and diluted 1/100 in either LKLM or LKLM with
400 mM NaCl. A control for each strain was prepared similarly except the dilution medium contained high K$^+$ (HKLM) with 0.4 M NaCl. The growth was monitored in a Bioscreen automated growth monitoring system for at least 18 hours. All the complemented strains, TKtrk1, TKtrk2 and TKglnQHMP, showed improved growth relative to the deficient strain, TK2420, when grown in low K$^+$ medium with or without osmotic stress (Fig. S2), with the most profound improvement observed in the TKtrk2 strain. Further, we tested if these systems could accumulate sufficient K$^+$ to aid acid sensitive *E. coli* TK2420’s response to low pH. We observed both Trk1 (including individual component pacL) and Trk2 enable the TK2420 mutant’s survival at low pH (4.0) stress under high K$^+$ conditions (Fig. S2). It was concluded from these results that Trk1, Trk2 and GlnQHMP function to accumulate K$^+$ inside the cells.

### 3.5.6.3 K$^+$ transporters influence the membrane potential of *E. coli* TK2420

The alterations in the membrane potential were assayed by measuring the change in fluorescence intensity of DiSBAC$_2$(3), and normalized with fluorescence intensity decay of dye alone. Our analysis revealed that the TK-DUET strain exhibited a lower intensity change over time under low K$^+$ (5 mM) in minimal medium or LKLM medium; whereas on addition of 400 mM NaCl, we observed an initial hyperpolarized state (Fig. S3). With increasing extracellular K$^+$ concentration to $\geq$ 25 mM, a more depolarized state was observed. Amongst TKtrk1, TKtrk2 and TKglnQHMP, we did not observe a
profound overall difference, although TKtrk2 had a relatively higher depolarized state compared with both TKtrk1 and TKglnQHMP under low $K^+$ (5 mM) (Fig. S3).

3.6 Discussion

*S. mutans* has highly organized adaptive mechanisms that come into effect under physiological stress conditions. Here, we have established the mechanism and importance of $K^+$ uptake and its accumulation in the physiological adaptation to stress. We investigated how critical $K^+$ is for the growth and survival of *S. mutans*; how it acquires $K^+$ from the extracellular environment; and how $K^+$ transport systems affect its physiology and virulence attributes. We demonstrated the essentiality of $K^+$ and the roles of four putative transport systems, Trk1, Trk2, Kch, and GlnQHMP, in $K^+$ homeostasis of *S. mutans*. Of these, the function of Trk2 was critical to establish a link between extracellular $K^+$ availability and several virulence attributes of *S. mutans* growth, for instance, planktonic growth, biofilm formation and acid tolerance phenotypes were impaired in the absence of Trk2. In the absence of Trk1 and Trk2, *S. mutans* was capable of acquiring $K^+$ from its surroundings. While we had previously characterized the function of GlnQHMP in glutamate transport and acid tolerance of *S. mutans* (Krastel *et al.*, 2010), we were able to demonstrate that this system displays specificity to $K^+$ transport in the absence of both Trk1 and Trk2 systems. Loss of the Kch system did not affect $K^+$ acquisition, growth, or virulence phenotypes in *S. mutans*. Collectively, our results and the presence of at least four putative $K^+$ transport systems in the *S. mutans* UA159 genome highlight not only the significance of $K^+$ homeostasis, but also that its
tight regulation is required to maintain cellular functions. Previously, effects of K\(^+\) on the growth of \textit{S. mutans} were not investigated in detail. We showed here that \textit{S. mutans}’ growth was sensitive to extracellular K\(^+\) availability, and that low or high K\(^+\) concentrations lead to growth retardation. This result concurs with the previously reported dependency on K\(^+\) for normal growth and glycolysis in \textit{S. mutans} (Dashper & Reynolds, 1996).

K\(^+\) is an important cation in the dental plaque fluid, wherein the plaque biofilm constitutes the natural environment of \textit{S. mutans} in the oral cavity. The ability to form and maintain a biofilm is a critical virulence property of \textit{S. mutans}. Here we report that K\(^+\) is essential to initiate biofilm formation by \textit{S. mutans}. Specifically, \textit{S. mutans} is not capable of forming a biofilm without K\(^+\) in the growth medium. We observed that supplementing cells with K\(^+\) concentrations as low as 5 mM allows the formation of robust biofilms, whose biomass remains stable even under K\(^+\) concentrations as high as 150 mM. Effects of K\(^+\) leakage across the membrane, and the concentration of extracellular K\(^+\) on biofilm formation have been well-studied in \textit{Bacillus subtilis} (López \textit{et al.}, 2010, Fall \textit{et al.}, 2006). A possible relationship between K\(^+\) in the biofilm matrix and its integrity has been presented in the study by Markevics and Jacques (Markevics & Jacques, 1985), who showed that optimal K\(^+\) in the matrix enhanced the function of glucosyltransferase enzymes of \textit{Streptococcus salivaris} We show here that the K\(^+\) homeostasis mediated by Trk2 of \textit{S. mutans} affects the Gtfs function. There are reports suggesting the role of membrane potential in translocation of proteins across the membrane or their secretion into extracellular spaces (Daniels \textit{et al.}, 1981, Palmen \textit{et al.},
It is thus possible that the altered membrane potential due to the K\(^+\) gradient across the membrane in the \textit{trk2-null} mutant could cause the defects in secretion or synthesis of functional Gtfs or Gbps that result in abrogating the biofilm under low K\(^+\) conditions. Our Western blots showed that the K\(^+\) perturbation induced by the \textit{trk2} deletion affected the reactivity of cell surface-extracted GtfD to anti-GtfD antibody. We did not, however, observe a significant reduction in the expression levels of the \textit{gtfB} gene in the \textit{trk2-null} mutant as compared to its expression in the wild type strain suggesting that Gtf secretion may be impaired in the mutant or that some conformational change to the enzyme has occurred during its maturation on the cell surface that alters its detection by the antibody. Morphologically, a dramatic, almost complete absence of glucan puddling around the colonies was observed for the \textit{trk2-null} mutant as compared to its wild-type parent strain UA159, which confirmed that the absence of the Trk2 system is affecting Gtf activity. The pathway of Gtf secretion is unknown still, but the best assumption convenes on the Sec pathway (Huang \textit{et al.}, 2008). The Sec pathway includes a translocon such as SecYEG and SecA as the cytoplasmic ATPase, which undergoes an insertion and de-insertion process to translocate the protein (Economou \textit{et al.}, 1995). Since, fluctuation in the levels of K\(^+\) can affect the oligomeric to monomeric state of SecA (Wowor \textit{et al.}, 2011), it seems reasonable that translocation of proteins such as Gtfs to the cell surface is affected under K\(^+\) perturbation as seen in \textit{trk2-null} mutant. Western analysis was performed to confirm the presence or absence of other cell surface-localized proteins such as P1 and Antigen A (WapA) in the mutant and resulted in positive reactivity with each cognate antibody (data not shown) suggesting that translocation by suspected Sec pathway substrates, in general, is not affected in the mutant. Results
reported here, however, suggest that the K⁺ gradient across the membrane or K⁺ levels in the extracellular milieu affects the functioning of Gtfs.

In some bacteria, extracellular K⁺ can act as a signal cation to activate virulence mechanisms (Xue et al., 2011, Su et al., 2009, Gries et al., 2013). In S. mutans Ingbritt, the addition of K⁺ increased the rate of acid production, which is an essential virulence factor of this cariogenic organism (Marsh et al., 1982). Another study demonstrated that S. mutans K-I cells grown in K⁺-supplemented medium under low pH resulted in increased accumulation of K⁺ and increased acid production (Luoma, 1971). However, in both of these studies the authors did not provide the mode of K⁺ acquisition and its underlying mechanism of action. In addition to acid production, tolerance to low pH is an important determinant in maintaining glycolysis and surviving acidic environments such as those found in carious lesions. A study conducted by Gong et al. examined the acid-induced transcriptome of S. mutans, and found that the expression of nearly 14% of the genes encoded in its genome was altered by acid stress (Gong et al., 2009). These included trkA, trkH and trkB all of which were significantly up-regulated at low pH (Gong et al., 2009), suggesting their involvement in acid stress tolerance. Our acid tolerance assays confirmed the relation between the Trk2 system and aciduricity as the loss of Trk2 resulted in increased susceptibility of cells to acid stress. This mutant was also significantly impaired in its ability to grow and produce acid, whereas SMΔtrk2 showed slower growth relative to wild-type and SMΔtrk1. Taken together, Trk2 has a critical role in maintaining K⁺ homeostasis at different growth phases and conditions encountered by S. mutans.
Amongst various bacterial K\(^+\) transport systems, the Trk systems have gained much attention due to their role in general cell physiology (Epstein, 2003) and virulence (Alkhuder et al., 2010, Buurman et al., 2004). Here, we tested several isogenic Trk deletion mutant strains for their abilities to accumulate intracellular K\(^+\) in the presence of low (5 mM), normal (25 mM), and high (50 mM) concentrations of this cation in the growth medium. We noted that S. mutans required at least a 25 mM K\(^+\) concentration in the growth medium to maintain normal cellular activities. With the exception of the mutant lacking Trk1 system, none of our mutant strains displayed considerable alterations in their ability to accrue K\(^+\) at initial time points (0-2 minutes), relative to the wild-type strain. After 30 minutes of incubation, the SM\(\Delta\)trk2 strain and the Trk1/2 double mutants had significantly decreased levels of intracellular K\(^+\) relative to the wild-type strain. The diminished ability to acquire K\(^+\) by these mutants likely explains their growth defect (i.e. low final yield) and decreased biofilm biomass compared to the parent strain. The presence of membrane bound and intracellular gating components have been suggested for the TrkAH systems in *Halomonas elongate* (Kraegeloh et al., 2005), *Vibrio alginolyticus* (Nakamura et al., 1998), and *Vibrio parahaemolyticus* (Cao et al., 2013). Proteins similar to Trks have been also been shown to function in response to interaction with physiological messengers such as c-di-AMP (Corrigan et al., 2013, Bai et al., 2014).

The Trk2 in *S. mutans* has two components that include a membrane-bound TrkH component that presumably allows the transfer of cations through the cell membrane, and a TrkA component those likely controls the movement of K\(^+\) into the cytoplasm. In our study, the deletion of either component affected the cellular physiology of *S. mutans*.
UA159, suggesting that both TrkA and TrkH are required for optimal cellular function. Due to the drastic phenotypic effects conferred by the Trk2 $K^+$ uptake mutant relative to the SMΔtrk1 mutant, we inferred that loss of Trk2 raised $S. mutans$' net $K^+$ requirement for maintenance of normal cellular activities. Although we noted an increased ability of the Trk1 mutant to acquire $K^+$ in the first 2 minutes, the accrued $K^+$ levels in this mutant dropped to levels observed in other strains, including the wild-type, at later time points. This observation challenged our initial hypothesis that the $S. mutans$ Trk1 acts as a $K^+$ uptake system, which prompted us to perform complementation studies in an $E. coli$ mutant strain unable to transport extracellular $K^+$.

Each of the four $K^+$ transport systems, Trk1, Trk2, GlnQHMP and Kch were tested for their role in $K^+$ uptake by complementing the $E. coli$ strain TK2420 with these systems. The $E. coli$ TK2420 strain is a well-established model organism to characterize $K^+$ transporters because of its lack of constitutive transporters and inability to transport $K^+$ (Buurman et al., 2004, Sturr et al., 1997, Kitko et al., 2010, Sun et al., 2006). We observed that complementing $E. coli$ TK2420 with Trk1, Trk2 or GlnQHMP facilitated significant $K^+$ acquisition, and enabled normal growth under low $K^+$ and stress conditions posed by high salt concentrations and low pH. These results confirm the role of Trk1 in $K^+$ uptake, even though we were unable to functionally dissect these properties in $S. mutans$ under our test conditions.

In $S. mutans$, emerging data from microarray studies suggest that $K^+$ transport systems are linked with the acid stress response (Gong et al., 2009, Senadheera et al.,
2009b), sugar transport via the EII component of phospho-transferase system (Abranches et al., 2006a), and oxidative stress tolerance (Ahn et al., 2007). Klein and colleagues (Klein et al., 2010) used S. mutans cDNAs derived from biofilms to show that Trk1-component genes, trkB and pacL, were up-regulated when cells were grown in sucrose and starch compared to sucrose alone (Klein et al., 2010). These authors proposed the role for these genes in osmoregulation of S. mutans. Trks in bacteria (other than streptococci) have been shown to function in response to various environmental stressors such as hyper saline conditions, low pH, oxidative stress, etc. (Holtmann et al., 2003, Epstein, 2003). To maintain cellular functions under such stressful conditions, bacteria utilize two forms of metabolic energy obtained by either breakdown of energy-rich phosphate bonds such as ATP or electrochemical energy provided by ion gradients. We postulated that if Trks in S. mutans functioned as early stress responders, then they would have a measurable impact on the overall electrochemical membrane potential. In the absence of Trk2, the mutants were unable to grow in hyper saline conditions, and had altered electro-potential gradients in response to extracellular K\(^+\). In addition to modulating K\(^+\) import, we reasoned that the Trk2 also had a function in contending with osmotic stress. Further, by complementing E. coli TK2420 with Trk2, we concluded that the K\(^+\)-dependent growth defect under osmotic stress was a direct result of the loss of Trk2 as opposed to simply an indirect physiological response to hyperosmotic stress.

Others have shown a relationship between intracellular Na\(^+\) to K\(^+\) cationic ratios (Schultz & Solomon, 1961) and pH of the medium, age of the culture, and cell metabolism (Epstein & Kim, 1971, Ignatov et al., 2002). Since the Na\(^+\) to K\(^+\) ratios affect
the cellular proton motive force (PMF), we investigated how the membrane potential varied during stressful growth conditions (e.g. low pH, osmotic stress). We reasoned that if K$^+$ acquisition were crucial under these conditions, an efficient K$^+$ transport system such as the Trk2 would be required for cell survival and growth, as shown in our acid tolerance assays. We then hypothesized that under low extracellular K$^+$, the intracellular levels of K$^+$ could be manipulated to collapse internal pH homeostasis of cells and affect the trans-membrane potential. Cells typically maintain a negative intracellular potential with the exception of acidophilic cells and during certain phases in the cell cycle when cells undergo ‘alternate positive potential’ (Ivanov et al., 2013). The defect in membrane potential of SMΔtrk2 mutants reinforces its phenotypes’ requirement of a high K$^+$ concentration for cellular functions and pH sensitivity.

In this study, we have presented an in-depth characterization of four K$^+$ transport systems in S. mutans. In addition to relating K$^+$ acquisition to growth, we have identified an important function for this cation and its uptake systems in maintaining virulence properties of S. mutans, including acidogenicity, aciduricity and biofilm formation. Although we have outlined possible stimuli that may facilitate the exchange or import of K$^+$, and the proposed pathways involved (Fig. 3.11), the precise mechanisms of the activation and regulatory pathways require further study. Our study highlights the important role of the Trk2 system in S. mutans’ K$^+$ transport, and demonstrates the complexity of other independently functioning, and secondary, multiple K$^+$ transport systems. This work constitutes the first characterization of the Trk systems in oral streptococci.
FIG. 3.11: Representation of regulatory mechanisms in S. mutans that include K⁺ transport systems in physiological responses. Environment changes, such as pH and cations, result in alterations of the cell membrane for survival. The membrane proteins (e.g. transport systems) and membrane bound sensors (e.g. histidine kinases) can respond in a feedback-regulated manner to changes by increasing the inflow of cations (e.g. K⁺). Activation of K⁺ accumulation can affect the overall physiological responses of cells by bolstering their defense and repair mechanisms. K⁺ levels can affect various intracellular and extracellular physiological aspects of S. mutans.

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Chapter 4: Dissecting a novel link: Effects of K⁺ on stress and competence networks in Streptococcus mutans

Running title: Effect on K⁺ on genetic transformation and stress tolerance in S. mutans

Authors: Gursonika Binepal¹, Paula Crowley², Iwona Wenderska¹, Richard N. Besingi², Dilani B. Senadheera¹, L. Jeannine Brady², Dennis G. Cvitkovitch¹*

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Contributions: GB performed the experiments, analyzed the data, wrote the manuscript; PC performed Western blot assays and contributed to the scientific language of the manuscript; IW provided the synthetic XIP peptide and contributed to genetic competence assays; LJB and DBS contributed to the scientific content of the manuscript; DGC was the project in-charge and contributed to the scientific inputs, planning of the project, funds to support the project.
4.1 Abstract

The success of *Streptococcus mutans* as a cariogenic pathogen can be attributed to several features that it possesses including: i) efficient metabolism of different sugars; ii) rapid stress adaptation; iii) the ability to form a biofilm; iv) highly coordinated signaling networks and genetic competence. Potassium (K\(^+\)) is the most abundant cation in the plaque fluid, and its transport via Trk2 system affects this bacterium through several physiological pathways. Herein, we characterized and report the influence of K\(^+\) perturbations on various molecular components of *S. mutans*. Keeping 25 mM K\(^+\) as our concentration for the control cultures, we analyzed the expression of genes involved in the stress regulon, sugar metabolism, and cell adhesion, in the presence of two test K\(^+\) concentrations: low (5mM) or high (50mM) relative to the control. The gene expression of most stress response elements was up-regulated under low K\(^+\) conditions at late exponential phase. Western blot analysis confirmed that in presence of 5mM K\(^+\), the levels of membrane-associated or secreted proteins were disturbed. Since the stress regulon in *S. mutans* is closely related to genetic competence, we also examined if low K\(^+\) can affect the competence induction in *S. mutans*. In the presence of 5mM K\(^+\), the transformation frequency of *S. mutans* UA159 was reduced by almost 100-fold relative to that observed in the presence of 25mM K\(^+\). While K\(^+\) concentrations \(\geq 25\) mM reinstated endogenous competence in the UA159 strain, no transformants were obtained for trk2-null mutants even at K\(^+\) concentrations \(\geq 25\) mM. A concomitant reduction was observed in the expression of genes encoding the sigma X inducing peptide (XIP) and the alternate sigma factor SigX (\(\sigma^X\)) under low K\(^+\), or in the
absence of the Trk2 system. Thus, we conclude that the stress regulon and competence development in *S. mutans* is highly sensitive to K\(^+\) concentrations in its environment.

### 4.2 Introduction

*Streptococcus mutans* is a cariogenic pathogen that exhibits a high degree of persistence and virulence in its naturally transient environment. The oral environment provides this bacterium with a favorable niche to colonize and nourish on host factors such as salivary components and dietary sugars. In its ecological niche, the predominance of *S. mutans* is directed by its ability to form a biofilm, generate acid metabolites, and activate a cascade of stress adaptive mechanisms. This bacterium is able to exhibit its virulence properties in a well-coordinated manner, which is closely associated with the competence regulon (Seaton *et al.*, 2011, Guo *et al.*, 2014). Moreover, previous reports have shown that the regulatory networks initially believed to be involved mainly in quorum sensing and genetic competence, also impact the stress tolerance and biofilm formation in *S. mutans* (Senadheera & Cvitkovitch, 2008, Li *et al.*, 2001d).

Competence is the natural ability of bacteria such as *S. mutans* to take up extracellular DNA, which could be integrated into the gene repertoire of the cell, thus affording it the genetic plasticity to adapt to environmental stresses. Thus, environmental factors such as cations, which strongly affect the stress response of *S. mutans*, could influence its other attributes such as genetic competence. It is established that *S. mutans* develops the competence state by activation of “master regulator” of competence, ComX,
through two self-generated peptides: the Competence Stimulating Peptide (CSP) (Li et al., 2001c, Aspiras et al., 2004) and the Sigma X Inducing Peptide (XIP) (Mashburn-Warren et al., 2010a). While there are marked differences in the mechanisms adopted by their specific regulon, the paths adopted by both the regulons intersect at ComX to facilitate the induction of late-competence genes required for DNA uptake and integration. The density of CSP in the extracellular milieu is a signal sensed by the histidine kinase ComD, which transduces the information to its cognate response regulator ComE (Li et al., 2001c). Although, the primary cellular response generated via ComE signaling is bacteriocin production, there is a concomitant indirect activation of comX via the CSP regulon, which affects the expression of various late competence genes. The more recently identified competence peptide, XIP, generates a competence state in S. mutans by directly activating the expression of comX and the genes required for DNA acquisition (Mashburn-Warren et al., 2010a). The threshold signal of extracellular XIP is processed via the Opp oligopeptide transporter and a synchronous activation of ComR a transcriptional regulator belonging to the Rgg family. In Bacillus subtilis, the DNA binding and transport depends on membrane potential and pH gradient (van Nieuwenhoven et al., 1982). Thus, an acceptable understanding is that appropriate alterations in the membrane physiology and its electrophysiology are required to attain a state of genetic competence. For example, the gradient of ions across the membrane could affect the state of membrane and membrane proteins. K⁺ cations are believed to partake in the initial reversible attachment of negatively charged DNA with negatively charged cell wall (Kohoutova, 1965). This whole process depends on membrane physiology at two key steps: first, the secretion and processing of the signaling peptide; and second, the uptake of extracellular genetic material. Since, the natural environment of S. mutans is K⁺-
Bacteria have specialized mechanisms to combat environmental stress, one of which is to accumulate potassium (K\(^+\)). K\(^+\) is a monovalent cation essential for the bacterial growth (Cowman & Fitzgerald, 1976), and is the most abundant cation in the plaque fluid (Moreno & Margolis, 1988). Recently, we characterized the role of potassium (K\(^+\)) in \textit{S. mutans} growth, biofilm formation and stress tolerance (Binepal & Gill \textit{et al.}, 2015 submitted). It was evident from our results that the growth under low K\(^+\) (< 5mM KCl) resulted in an impaired glucosyltransferase activity; thereby impacting the biofilm formation, reduced the growth of \textit{S. mutans} UA159, and affected its membrane potential. These low K\(^+\) phenotypic characteristics were significantly enhanced with deletion of the Trk2 K\(^+\) transporter in \textit{S. mutans}, making it the most important K\(^+\) transport system in this bacterium.

In this report, we sought to identify the underlying mechanisms responsible for the significant impact observed on various virulence attributes of \textit{S. mutans} due to reduced K\(^+\) availability in its environment; or due to low K\(^+\) uptake resulting from \textit{trk2}-deletion. Thus, we dissected various elements of this low K\(^+\) induced stress cascade, at the genetic and proteomic levels in \textit{S. mutans} UA159. K\(^+\) can play a critical role in the maintenance of the membrane chemistry, which further affects cell metabolism, adhesion and material exchange with the environment.
4.3 Materials and Methods

4.3.1 Bacterial Strains and Culture Conditions

*S. mutans* strains were grown in Todd Hewitt Yeast Extract broth (THYE) or Minimal Defined potassium-deprived medium (with 1% glucose MMGK(-) or 1% sucrose MMSK(-)) or Chemically Defined potassium-deprived Medium (CDM (K-) at 37°C with aeration. The MMGK(-) was prepared as previously reported (Fujiwara *et al.*, 1978); potassium salts were replaced by sodium salts, and final concentrations of 5 mM cystine and 5 mM glutamic acid were added fresh immediately before use; and the CDM(K-) was prepared as previously described except the potassium containing components were omitted (Mashburn-Warren *et al.*, 2010a, van de Rijn & Kessler, 1980). Strains and primers used are listed in Supplementary Table 2. The *trk2* null mutant was previously constructed using PCR-ligation mutagenesis method (Lau *et al.*, 2002).

4.3.2 Transformation frequency assays

Overnight *S. mutans* strains grown in THYE were pelleted, washed, and re-suspended in phosphate buffered saline (1X PBS). The re-suspended culture was diluted 50-folds using pre-warmed CDM(K-) with 5 mM, 25 mM or 50 mM K⁺ and grown to an OD600 nm ~ 0.6. Next, 1 µg mL⁻¹ of the donor plasmid DNA (pDL277; specR) was added to 1-mL aliquots of the culture in the presence or absence of CSP (0.4 µM) or XIP (10 µM), and samples were incubated for 90 min. For XIP, control cultures containing
1% DMSO were utilized. After incubation, cultures were serially diluted and plated on THYE plates with and without antibiotics. Percent TF was calculated as transformant colony-forming units (CFUs) divided by the total number of viable CFUs, times 100.

**4.3.3 Quantitative real-time PCR (qRT-PCR) analyses**

For evaluating the expression change in the genes involved in genetic transformation: Overnight cultures of UA159 and its derivative strains: *trk2-null* and *SMU_63c null* were diluted 20 fold in fresh CDM (K-) supplemented with either 5 mM, 25 mM or 50 mM K⁺ and grown to an OD₆₀₀ nm of 0.6. RNA was isolated and treated with DNAse before proceeding for cDNA synthesis. The gene expression analyses were carried out using Pfaffle method (Pfaffl, 2001). Primers used for qRT-PCR are as presented in table 2. Expression was normalized to that of 16SrRNA gene, and fold change was calculated relative to expression in UA159 grown under matched O.D. and K⁺ conditions.

To evaluate the expression change of various genes in Table 2, at different growth O.D.’s under varying concentrations of K⁺: Overnight cultures of UA159 strain were washed and diluted 20 folds in MMGK- with specific K⁺ concentrations, and grown up to the desired O.D.- 0.2, 0.4 or 0.8. Cells were then pelleted and RNA was extracted and processed for cDNA synthesis as described above. The primers used are mentioned in the Table 2.
4.3.4 Western Blot analysis

Strains were started in THYE broth, grown overnight and passaged (1:20) into warm THYE the next day. Once cultures reached $O.D._{600nm} \sim 0.6-7$, cells were pelleted, washed once in MMSK and resuspended in warm MMSK. Washed cells were used to inoculate (1:20 dilution) duplicate tubes of warm MMSK with KCl added at 5, 25 or 50 mM concentration. Cultures were grown overnight at 37°C, following which cells were pelleted and washed twice in 20 mM Tris-Cl, pH 8.0 and extracted for 1 hr with 0.5-1.0 ml of 4% SDS followed by boiling for 10 min. Samples were centrifuged at 16,000 x g and the supernatant saved aside for Western analysis. $O.D._{280}$ of each SDS extract was measured using the Nanodrop 1000 and extracts were standardized for protein concentration. Using and $OD_{280}$ of 1.0 = 1 mg/ml protein, 20 ug of extract protein from duplicate cell cultures was applied to a 4-20% TGX gradient gel (BioRad) and proteins were separated at 150 Volts constant voltage. Following electrophoresis, separated proteins were blotted onto PVDF membrane for 7 min using the Transblot Turbo (Biorad) and membranes were blocked overnight in PBS-0.3%ECL Prime substrate was applied to the blot for 1 min prior to chemi imaging in the G-Box (Syngene).
4.4 Results

4.4.1 K⁺ levels regulate expression of various genes involved in the stress response

In our previous report, we showed a significant impact of K⁺ limitation on the growth of *S. mutans* UA159, and concluded that the optimal level of K⁺ in the defined growth medium was 25 mM for its planktonic growth. To understand how K⁺ concentrations higher or lower than 25 mM could affect the growth of *S. mutans*, we tested the expression of various genes involved in sugar metabolism, stress regulation, cell adhesion, and cation transport of the *S. mutans* UA159 cells, grown in the presence of 5 mM (Fig. 4.1A) or 50 mM (Fig. 4.1B) K⁺, relative to their expression in the presence of 25 mM K⁺. As shown in Fig. 4.1A, most of the metabolism genes were down regulated at the early exponential growth phase in the presence of 5 mM K⁺, which is in consensus with the growth retardation observed previously (Binepal and Gill *et al.*, 2015). However, when growth of wt UA159 cells reached the late exponential growth phase, there was an up-regulation in the expression of sugar metabolism associated genes namely; *bgIP* (~20-fold), *scrA* (~2-fold) and *ptnA* (~6-fold). Likewise, the expression was up-regulated for genes involved in cation transport, specifically proton and K⁺ transport (~5-fold), and genes encoding the adhesion proteins (>3-fold), at the late exponential growth in the medium with 5 mM K⁺ (Fig 4.1A). Concurrent to the changes in the expression of above mentioned genes at late exponential growth in 5 mM K⁺, we also observed a high fold change (> 5-fold) in the expression of genes encoding regulators involved in the stress regulon, such as *codY*, *relR*, *gerR* and *vicR*. With an
exception to an increased expression of *codY* in the presence of 50 mM K\(^+\) at late exponential growth phase, the expression of the other regulators tested were either down-regulated in 50 mM K\(^+\), or equivalent to their expression in 25 mM K\(^+\). Collectively, these results depict the global impact of an imbalance in K\(^+\) homeostasis on the transcription on various genes in *S. mutans*.

**Fig 4.1A:** Fold change in the expression of specific genes in *S. mutans* UA159, grown in the presence of 5 mM K\(^+\) as compared with their expression in wt UA159 grown in the presence of 25 mM K\(^+\). The fold change > 2 fold or < 0.5 fold was considered a significant change in the expression of a specific gene. Values are presented as mean for a total of 3X3 replicate values ± standard error of means.
Fold change in the expression of specific genes in *S. mutans* UA159, grown in the presence of 50 mM K\(^+\) as compared with their expression in wt UA159 grown in the presence of 25 mM K\(^+\). The fold change > 2 fold or < 0.5 fold was considered a significant change in the expression of a specific gene. Values are presented as mean for a total of 3X3 replicate values ± standard error of means.

4.4.2 Effect of K\(^+\) on various surface and secreted proteins

Our previous Western blot results showed a difference in the levels of glucosyltransferase derived from wt UA159 vs *trk2*-null mutant strains. In the present study, we tested the effect of K\(^+\) on the levels of some other surface and secreted proteins in the wt
UA159 and trk2-null mutant strains using Western blots (Figure 4.2). The proteins tested included surface localized adhesion protein P1, autolysin AtlA, glucan binding protein GbpB, cell wall associated WapA and an unknown secreted protein encoded by SMU_63c. Amongst these, the most profound impact of K$^+$ availability was observed on the level of reactivity of 63c with its cognate antibody (Fig. 4.2). The detection of this protein was higher at 5 mM K$^+$ concentration in the growth medium and decreased as the K$^+$ concentration increased to 50 mM. The level of 63c detection was lower in the trk2-null mutant as compared to wt UA159 at 5 mM K$^+$. We also observed an effect of K$^+$ levels on the reactivity of P1-specific monoclonal antibodies in both wt UA159 and SMtrk2 strains. While the reactivity of 4-10A (P1-stalk specific) increased with an increase in concentration of K$^+$ in the medium, the opposite effect was observed for 3-10E (base of the stalk), where a decrease in reactivity was observed with an increase in K$^+$ level. Differences in reactivity with antibody 1-6F (globular head of P1) with increasing K$^+$ concentration were negligible. The expression of WapA and AtlA proteins were also affected at the low K$^+$ conditions and in the absence of the Trk2 transporter. WapA expression was visually slightly more in the presence of 25 mM or 50 mM K$^+$ levels compared with 5 mM K$^+$, and AtlA had a modest decrease in the reactivity with its specific antibody in the absence of Trk2 transporter under 5 mM or 50 mM K$^+$ levels; however, a clear pattern for the expression profiles in response to K$^+$ levels could not be deduced.
**FIG 4.2: Western blot analysis of wt and trk mutant cell surface extracts.** SDS-extracted surface proteins of UA159 (wt) and SMtrk2 mutant (Δ) cells grown 24 hrs in MMSK were subject to SDS-PAGE and electroblotting onto PVDF membranes. Membranes were reacted with anti-P1 Mabs 4-10A, 3-10E and 1-6F specific for the stalk, an epitope formed by the interaction between the N- and C-terminal regions at the base of the stalk, and the globular head domains of the mature P1 protein, respectively (Heim *et al*., 2013).
4.4.3 Transformation frequency is reduced under K$^+$ stress

Development of a competence state in *S. mutans* is strongly affected by its microenvironment, including changes in osmotic and pH conditions. It is known that K$^+$ homeostasis plays a critical role in stress tolerance and that this cation is required to take up negatively charged molecules such as DNA. Here, we hypothesized that K$^+$ homeostasis should affect the ability of cells to attain a state of competence. It has been shown before that, compared to UA159 cells, *trk2*-null mutant cells remain K$^+$ deficient for a prolonged time ($\geq 30$ min) after they were incubated in a K$^+$ lacking medium (Binepal and Gill *et al.*, 2015). This effect was also evident from the membrane potential assays, where the *trk2*-null mutant remained less depolarized compared with the wt UA159 cells, suggesting that K$^+$ was accumulated at low levels in this mutant strain. Thus, wt UA159 and *trk2*-null mutant cells were used to test the effect of low intracellular K$^+$ levels on the transformation frequency (Fig. 4.3). In the presence of 5mM K$^+$, the wt exhibited nearly a 100-fold reduction in the natural transformation frequency compared with the transformation frequency in presence of $\geq 25$ mM K$^+$. These results indicated that low K$^+$ had a negative impact on the endogenous competence development in UA159 strain. To confirm this, wt UA159 cells were supplied with exogenous synthetic XIP (10µM) under similar K$^+$ conditions and nearly 100% transformants were obtained; thus suggesting that the supplied synthetic XIP helped to surpass the K$^+$ effect on *S. mutans* genetic transformation.
FIG 4.3: Genetic competence of wtUA159 and SMtrk2 strains in varying levels of supplied K⁺. Cells grown in chemically-defined media were supplemented with 5mM, 25 mM or 50 mM KCl, were transformed with pDL277 (Dunny et al., 1991) plasmid DNA in the presence or absence of the competence stimulating peptide, XIP. Percent transformation frequency was calculated using the number of transformants obtained over the total number of viable cells multiplied by 100. Bars shown above are the means of six replicas with standard deviation for each condition.
Natural competence (XIP treatment) was completely abolished in trk2-null mutant, while the exogenous induction of competence with 10µM XIP could be observed only in the presence of ≥ 25mM K⁺ (Figure 4.3).

To further confirm the effect of K⁺ on the ability of S. mutans to attain a state of competence, it was necessary to test the effect of another competence stimulating peptide, CSP, under similar conditions. The conditions for competence induction in the presence or absence of 0.4 µM synthetic CSP were modified to suit the use of CSP in the assay. Since, CSP induction is not functional in CDMK-, cells of wtUA159 and SMtrk2 strains were grown to 0.1 in MMGK medium for in the presence of 5, 25 or 50 mM K⁺ conditions before testing transformation in Todd Hewitt Yeast broth with or without CSP. While no significant effect of K⁺ concentration was seen on the transformation frequency of UA159 in the presence or absence of CSP, natural competence (CSP treatment) in the trk2-null mutant cells was abolished (Fig. S4). Further, as compared to the wild type, a reduction in CSP-induced competence was observed for cells grown in the presence of K⁺ concentrations ≥ 25mM for mutant strain SMtrk2 (Fig. S4). Collectively these results reinforce the fact that K⁺ homeostasis is an important determinant of competence development in S. mutans.

**4.4.4 K⁺ levels influence the SMU_63c gene**

We tested the effect of K⁺ perturbations on SMU_63c, which encodes for a hypothetical protein secreted extracellularly and is potentially involved in signaling mechanisms as this gene neighbors the comR and comS genes in S. mutans (Fig. 4.4). The
expression of this gene is believed to be regulated by quorum sensing peptides XIP and CSP and in the absence of this gene the mutant strain is hyper-transformable. Thus, we argued that disturbance in K\(^+\) homeostasis might exert similar effect on genetic transformation in the strain lacking SMU_63c and regulate the expression of SMU_63c product as for the ComCDE and ComRS pathways.

**FIG 4.4:** Genetic locus for SMU_63c. The genes comR and comS are involved in XIP-mediated genetic competence in S. mutans.

We first tested the fold change in the expression of SMU_63c at different growth phases in response to 5- or 50 mM K\(^+\) compared with expression in the presence of 25 mM K\(^+\). In the presence of 50 mM K\(^+\), the expression of SMU_63c was up-regulated at early exponential-phase; However, in the presence of 5 mM K\(^+\) the expression was 8 fold higher at near-stationary growth phase (Fig. 4.5).
FIG 4.5: Expression fold change of SMU_63c in S. mutans UA159. UA159 cells were grown in the presence of 5 mM, 25 mM or 50 mM K⁺ supplemented in MMGK media. Expression fold change was calculated by normalizing gene expression in the wtUA159 grown in the presence of 5 mM or 50 mM K⁺ to the expression obtained when UA159 strain was grown in the presence of 25 mM K⁺. The fold change > 2 fold or < 0.5 fold was considered a significant change in the expression of SMU_63c gene. Values are presented as means for three biological and three technical replicate values ± standard error of the mean.

Since, SMU_63c is adjacent to the XIP-induced comRS operon, we tested the effect of K⁺ limitation on the transformation frequency of the SMU_63c null mutant as compared to wt UA159. The natural transformation was abolished for the SMU_63c null mutant in the presence of 5 mM K⁺. Interestingly, as the K⁺ concentration was increased to 25 mM, the natural transformability of this strain remained abolished; however, in the presence of exogenous XIP the transformation frequency increased to the levels observed for UA159 strain as can be seen in the fig. 4.6.
**FIG 4.6: Genetic transformation in the SMU_63c null mutant.** Chemically-defined media grown cells supplemented with 5mM, 25 mM or 50 mM KCl\(^+\), were transformed with pDL277 (Dunny et al., 1991) plasmid DNA in the presence or absence of the competence stimulating peptide, XIP. Percent transformation frequency was calculated using the number of transformants obtained over the total number of viable cells multiplied by 100. Bars shown above are the means for a total of six replicas with standard deviation for each condition. No natural transformants were obtained for the SMU_63c null mutant strain under 5mM or 25 mM K\(^+\) condition.

### 4.4.4.1 K\(^+\) dependent growth of SMU_63c null mutant

We also tested the effect of K\(^+\) concentrations on the growth of the mutant lacking SMU_63c. While, this mutant exhibited non-significant growth retardation in the presence of 5 or 25mM K\(^+\) relative to the wt UA159, a significant improvement in its growth was observed in the presence of 50mM K\(^+\) as shown in the Fig. 4.7.
FIG 4.7: Growth curves for the *SMU_63c* mutant strain in defined K⁺ concentrations. Growth of wtUA159 and *SMU_63c*-null mutant cells in CDM supplemented with 5-, 25-, or 50mM K⁺ was monitored using the automated Bioscreen C for at least 20h. The left panel represents the logarithmic growth curves obtained for each strain; the right panel represents the terminal absorbance, all results were obtained using at least three biological and three technical replicas. Statistical relevance (*) was performed using Student’s t-test and p-value <0.05 was considered significant.
4.4.5 K\(^+\) affects the expression of competence genes

A common and critical point for CSP- or XIP-mediated competence induction is activation of the expression of the alternative sigma factor ComX (Son et al., 2015). The current model suggests that a two-way activation mechanism exist between the CSP-ComDE and XIP-ComRS pathways in \textit{S. mutans} via the ComX regulon, and it is this mutual interlink that controls bacteriocin expression and genetic competence via the ComDE and ComRS pathways, respectively (Son et al., 2015). Competence induction with exogenous XIP results in the highest transformation frequency, and the pathway include genes such as \textit{comR}, a transcription regulator; and \textit{comS}, which encodes the sigma X inducing peptide (XIP). We tested the effect of K\(^+\) levels on the expression of these genes in both wtUA159 and \textit{trk2}-null mutant strains. The expression levels of \textit{comS}, \textit{comR} and \textit{comX} were tested using cDNA obtained from cells grown in the presence of 5mM, 25mM and 50mM K\(^+\), and compared with expression in UA159 grown in the presence of CDMK supplemented with 25mM K\(^+\). Concomitant to the effect of K\(^+\) on transformation frequency, the expression of \textit{comS} and \textit{comX} was reduced (<0.5 fold) under low K\(^+\) conditions (5mM K\(^+\) and/or absence of \textit{trk2}) (Fig. 4.8). Thus, it was concluded that K\(^+\) limitation affected the ability of \textit{S. mutans} to achieve the competence state by down-regulating the expression of competence-associated genes.
FIG 4.8: Transcriptional analysis using qRT-PCR. The expression of \textit{comR}, \textit{comS} and \textit{comX} involved in XIP mediated genetic competence were examined in wtUA159 and SMtrk2 strains grown to O.D. \textsubscript{600nm} ~ 0.6 in CDMK with different K\textsuperscript+ concentrations. Values are expressed as mean of fold change in the expression of each gene for a total of three biological and three technical replicas and error bars represent standard error of the mean.
4.5 Discussion

Competence in *S. mutans* is a natural and well-regulated process that exert an advantage under stressful conditions. The role of K⁺, an essential and a compatible cation in genetic transformation has not been reported previously. This study identifies a new link between K⁺ and natural genetic transformation of *S. mutans* and highlight molecular elements involved in the response to K⁺ stress by this bacterium. Effect of imbalance in the intracellular K⁺ on natural transformation due to absence of Trk2, the main K⁺ uptake system of *S. mutans* has been examined in this study. We suggest that low K⁺ is perceived as a stress signal, which induces the expression of genes encoding for stress response regulators. These results collectively suggest that an imbalance in the K⁺ levels could affect multiple molecular pathways in *S. mutans*.

We tested the effect of K⁺ perturbations on transformation frequency of *S. mutans*. Our results show that a low level of K⁺ (≤ 5 mM) in the medium impairs competence in *S. mutans*. Genetic transformation in *S. mutans* occurs most efficiently in the presence of 25mM K⁺. This concentration of K⁺ is also in range of the physiological concentration of K⁺ in saliva. To confirm if only extracellular K⁺ or both intra- and extra- cellular K⁺ is required for genetic transformation, we performed transformation assays using K⁺ uptake deficient *trk2*-null mutant strain. A significant reduction in transformation frequency was observed for the mutants lacking Trk2 transporter, such that very low induction was observed with exogenous XIP. Thus, we concluded that K⁺ homeostasis is crucial for cell to attain competence state. A concurrent decrease in expression of *comS* and *comX* was observed under 5 mM K⁺ growth condition. To understand if the aforementioned low transformation
frequency was due to an effect of K$^+$ on the expression of competence genes; we tested the expression of three important genes involved in the XIP mediated competence, namely: \textit{comR}, \textit{comS} and \textit{comX} in wtUA159 and \textit{trk2}-null mutant strains. The strains were grown to O.D. $\approx 0.6$ in presence of 5-, or 50 mM K$^+$ and the expression of \textit{comR}, \textit{comS} and \textit{comX} under these concentrations of K$^+$ was compared to the expression of the respective genes in wt UA159 strain grown in 25 mM K$^+$. While, the expression of \textit{comR} was not affected, a remarkable reduction in the expression of \textit{comS} and \textit{comX} was observed in cells grown under 5mM K$^+$ levels and in \textit{trk2}-null mutants. To confirm if K$^+$ limitation is directly affecting the genetic competence in \textit{S. mutans}, and that the competence phenotypes observed are not mutant specific, we tested the transformation frequency of the mutant lacking \textit{SMU\textunderscore 63c} under above-mentioned K$^+$ conditions. Deletion of \textit{SMU\textunderscore 63c} in \textit{S. mutans} UA159 increased its transformation frequency through an uncharacterized pathway (Unpublished data of Richard N. Besingi). However, under our test condition, the natural transformation was abolished in presence of 5mM K$^+$, which was in consent to our observations for wt UA159 under same K$^+$ levels. The transformation frequency increased in presence of high K$^+$ levels or in presence of exogenous XIP. While molecular mechanisms underlying the \textit{SMU\textunderscore 63c} competence phenotypes are in progress, we conclude from our Western blot and transformation assay results that this gene has a strong response towards the available K$^+$ levels; which does not included an effect on its planktonic growth.

In \textit{S. mutans}, sugar metabolism is a major process that contributes significantly to its pathogenicity. Sugar metabolism can be categorized under three factions, depending on the type of sugar being metabolized namely; phosphoenolpyruvate (PEP)-dependent,
multiple sugar metabolism (msm), or ATP dependent glucose specific permeases (Colby & Russell, 1997, Marsh et al., 2009). Of these, the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (transport PTS) represents a major carbohydrate uptake system, and at least 14 types of PTS pathways are identified in the genome of S. mutans (Ajdić & Pham, 2007). The PTS for glucose, fructose, maltose and sucrose are believed to be constitutively expressed in S. mutans (Ajdić & Pham, 2007). The components of PTS also contribute to a global signaling cascade that orchestrates the carbohydrate utilization with other cellular processes including the stress response regulons. PTS is composed of two common components; the phosphotransferases enzyme I (EI), and the histidine protein (HPr), which transfers the phosphoryl groups from phosphoenol-pyruvate to the EII enzymes (EIIs). The EIIs are composed of membrane bound transport proteins and associated cytoplasmic components, which are specific to the carbohydrates. Depending on the type of carbohydrate being transported, its cognate PTS proteins will undergo a sequence of phosphorylation states (Bettenbrock et al., 2007, Hogema et al., 1998). This phosphorylation is required to further regulate other protein activities. In an analogous PTS\textsuperscript{ntr} system, which is required for nitrogen and carbon metabolism in bacteria including Escherichia coli, PTS\textsuperscript{ntr} has been related with K\textsuperscript{+} uptake, as EIIA\textsuperscript{ntr} can bind to the TrkA component of the Trk- transporter (Lee et al., 2007) and to the kinase component KdpD of the KdpDE system, which regulates the expression of Kdp transporter (Lüttmann et al., 2009). In E. coli, there is a strong indication of an association between the intracellular levels of K\textsuperscript{+} and stress response protein SspA, which further regulates the phosphorylation status of PTS\textsuperscript{ntr} (Bahr et al., 2011). Thereby, an intricate two-way regulation exists between the intracellular K\textsuperscript{+} levels and the activity of these PTS systems. Although, a canonical PTS\textsuperscript{ntr} is
absent in *S. mutans*, an analogous PTS regulatory system is present. Similar to *S. mutans* PTS, the system in *B. subtilis* was proposed to depend on the phosphorylation status of the antiterminator LicT that controls the β-glucoside utilization (Bahr et al., 2011). In this study, we examined the effect of K⁺ on expression of genes involved in sugar metabolism, which included *bglP* (β-glucoside utilization), *scrA* (sucrose uptake) and *ptnA* (mannose acquisition).

Our results revealed that the level of K⁺ in the growth medium regulates the expression of the aforementioned genes. Specifically, the expression of various genes (Table 1) was low at early growth phase in the presence of 5 mM K⁺, but increased significantly as the growth reached late logarithmic to early stationary phase. This effect of K⁺ on the expression of the aforementioned genes was negated when the growth medium had high levels of K⁺ (50 mM). We also observed a concomitant increase in the expression of *atpE* (proton transporter) and *trkH* (K⁺ transporter) at late exponential growth phase of UA159 grown in medium with low K⁺. The increment in the expression of these genes encoding components of ion transporters indicates towards the support required for cellular metabolism through regulation of membrane energetics. Previously, we tested the combined effect of various sugars and limited K⁺ on the growth of UA159 and found that in the presence of various specific sugars under K⁺ limiting conditions growth was comparable to that obtained in the presence of glucose under similar conditions (Binepal and Gill et al., 2015). Collectively, these results show that under low K⁺ conditions, sugar metabolism is affected and the genes involved are not activated until late exponential phase, which explains
the growth retardation under low K$^+$ conditions or in the absence of the Trk2 K$^+$ transporter in *S. mutans*.

Low K$^+$ in the growth medium is a stressful condition for *S. mutans*. To probe how this stress signal is perceived, we tested the effect of growth in the presence of 5 mM K$^+$ on the expression of certain stress response regulators such as *relR* (associated with pppGpp nutritional alarmone) (Lemos *et al.*, 2007); *gcrR* (stress associated orphan response regulator); *vicR* (stress associated response regulator) (Senadheera *et al.*, 2005) and *codY* (stress response regulator) (Lemos *et al.*, 2008). Individually, these regulators have been characterized and all have an overlap between their functions, especially in the stress response. In this study, we observed that K$^+$ limitation induced more than 5-fold up-regulation in the expression of all these stress response regulators. Interestingly, increase in the levels of K$^+$ in the growth medium initially normalized, then gradually repressed the expression of most of these regulators in *S. mutans* UA159; except for *codY*, which remained 5-fold high even in the presence of 50mM K$^+$. Levels of isoleucine and GTP in the cell of Gram-positive can regulate CodY (Sonenshein, 2005). Specifically in *S. mutans*, the regulation of CodY is dictated more by isoleucine levels than by GTP levels(Lemos *et al.*, 2008). *S. mutans* accumulate branched chain amino acids (BCAAs) with the help of the proton motive force (Dashper & Reynolds, 1993). Limitation in the environmental K$^+$ levels affects the cell energetics that could impact the BCAAs accumulation in the cell. Another reason could be due to reduction in biosynthesis of BCAAs such as isoleucine due to reduced levels of intracellular K$^+$. It is known that K$^+$ can impact the biosynthesis of BCAA by affecting the activity of enzymes involved in the process (Xing & Whitman, 1991).
way, the reduction in isoleucine can trickle down to up-regulation of codY, as seen under limited K⁺ growth conditions.

We had previously observed a reduction in the levels of glucosyltransferaseS (GtfD) and extracellular polysaccharide levels under low K⁺ growth conditions (Binepal and Gill et al., 2016). In this study, we examined the effect of K⁺ limitation on various genes involved in cell adhesion, namely; gbpB (glucan binding protein B)(Banas & Vickerman, 2003), gtfB (adhesin metabolizing enzyme)(Bowen & Koo, 2011), spaP (surface antigen P1 involved in sucrose independent adhesion) (Russell & Mansson-Rahemtulla, 1989). Surprisingly, at the late exponential phase of cell growth, in the presence of low K⁺, expression of all of these genes was up regulated; even though we had previously observed a reduced biofilm biomass (Binepal and Gill et al., 2016).

A plausible explanation for the inconsistency observed in Gtf activity (Binepal and Gill et al., 2016) and gene expression of these adhesion proteins could be due to an effect on the protein secretion, or a conformational change of the protein due to altered K⁺ gradient across the membrane of S. mutans. To investigate this further, we processed the UA159 and trk2-null mutant cells grown in limited K⁺ conditions (5, 25 and 50 mM KCl in MMSK-) and identified the level of surface associated or secreted proteins such as GbpB, WapA, AtlA, SpaP and SMU_63c product using specific monoclonal antibodies. Our results revealed that depending on the medium K⁺ level, the reactivity of these proteins was altered. For SpaP, which is also referred to as antigen P1, a total of three monoclonal antibodies were tested, of which 4-10A has a specificity for the helical stalk, 3-10E which recognizes an
epitope formed by the interaction between the N- and C-terminal regions at the base of the stalk, and 1-6F which recognizes an epitope within the globular apical head of the molecule. Previously, it was shown that the P region contributes to the conformation of the stalk of P1 antigen, and its presence is necessary for surface expression of the molecule on S. mutans. The structural arrangement of these domains is critical for the adhesive properties of this protein. Our Western Blots revealed that alterations in the K\(^+\) concentrations affected the level of reactivity of domain specific antibodies; thereby suggesting an effect on the conformation of this protein.

Of the proteins tested, we also observed an inverse effect of extracellular K\(^+\) concentration on SMU\(_{63c}\) encoded hypothetical protein of unknown function. Its predicted regulators are CodY and RelR based on the prediction tool RegPrecise (http://regprecise.lbl.gov/RegPrecise/regulon.jsp?regulon_id=45107) both of which are stress response regulators and we had observed that the expression of these regulators was sensitive to the K\(^+\) level during the growth of S. mutans. SMU\(_{63c}\) is adjacent to the comRS operon, which is responsible for XIP-mediated genetic competence. The expression of this gene is up-regulated in response to CSP exposure, suggesting its involvement in quorum sensing or genetic transformation. Reactivity of anti-63c mAb to the SMU\(_{63c}\) product was high under 5 mM K\(^+\) as compared to 25 or 50 mM K\(^+\) in the growth medium. Examining the expression of SMU\(_{63c}\) gene in UA159 at different growth phases in the presence of 5, 25 or 50mM K\(^+\), corroborated our protein expression results; with maximum expression of this gene being observed in late exponential phase cells grown in the medium with 5mM K\(^+\).
In the present study, we have shown that the perturbation in the K$^+$ concentrations across the membrane, due to changes in the extracellular or intracellular K$^+$ levels acts as a signal to the cell. This signal can then generate a stress response by altering the expression of genes involved in the metabolic and regulatory pathways, ion transportation to restore the gradient and cell adhesion. We have also shown that a high K$^+$ concentration (≥ 25 mM) is required during the process of genetic competence. A direct effect on the genetic transformation can be seen when K$^+$ homeostasis is disrupted, implying that the cell shifts its efforts in restoring the gradient or adapting to this change, instead of taking up genetic material. This is the first study to show a relationship between the K$^+$ and genetic transformation in *S. mutans*. 

Chapter 5: Summary of the dissertation
5.1 Summary

This dissertation characterizes the biological significance and mechanisms of K$^+$ homeostasis in the dental plaque colonizing bacterium *S. mutans*. We have shown that at least 25 mM K$^+$ is required for optimal physiological activities in *S. mutans*. The role of K$^+$ is two-fold: in the extracellular environment of *S. mutans*, levels of K$^+$ are maintained over 25 mM to allow optimal environment for biofilm formation by enzymes such as Gtfs; the intracellular K$^+$ accumulation is required for maximal growth and optimal physiology of *S. mutans*. The Gtfs are required to synthesize extracellular sticky polymers, which contribute to the integrity of the biofilm matrix. The loss of K$^+$ in the growth environment inhibits Gtf activity thus resulting in the loss of the biofilm phenotype. In the present study, we tested four putative transport systems for their role in K$^+$ uptake. Of the four systems, we concluded that the Trk2 system is critical for K$^+$ homeostasis and stress tolerance; disruption of this system impairs the growth, deregulates the membrane potential and affects the expression of various essential genes in *S. mutans* (Figure 5.1). This imbalance in the K$^+$ levels, either due to decreased K$^+$ concentration in the growth medium or due to loss of Trk2, affected growth, biofilm formation, metabolism and stress tolerance of *S. mutans*. Perturbation in the K$^+$ concentration across the membrane can be a potential signal to generate a stress response by altering the expression of genes involved in the metabolic and regulatory pathways, ion transportation to restore the gradient and cell adhesion. We have also shown that a high K$^+$ concentration is required during the process of genetic transformation in *S. mutans*. A direct effect on the genetic transformation can be seen when K$^+$ homeostasis is disrupted, implying that the cell shifts its efforts in restoring the gradient or adapting to this change, instead of
taking up genetic material. Complete loss in the ability of DNA uptake and genomic integration was observed when the Trk2 system was absent. Since, loss of Trk2 results in perturbed membrane potential, which is critical to take up charged molecules such as DNA, we could infer that the Trk2 mediates adaptation in membrane physiology during the process of genetic transformation by regulating the potential gradient across the membrane. This is the first study to show a relationship between the K⁺ and genetic transformation in *S. mutans.*

**Streptococcus mutans**

FIG 5.1: Perturbations in the level of pH and cations acts as signals to the cell membrane for activation of an adaptive response. The membrane proteins (e.g. transport systems) respond in a feedback-regulated manner to K⁺ changes by increasing its inflow. In *S. mutans,* Trk2 mediated K⁺ uptake affect its essential attributes such as stress tolerance, growth and sugar metabolism. K⁺ in the extracellular fluids affect the activity of glucosytransferases and regulate the biofilm formation.
Chapter 6: Significance, Limitations and Future Directions
6.1 Limitations of the study and future directions

This study provides novel insights to how \textit{S. mutans} accumulates K$^+$, and how K$^+$ contributes to the various virulence attributes of \textit{S. mutans}. Although, this study covers a broad understanding on the impact of K$^+$ disturbance on genetic, physiological and molecular aspects, the molecular pathway underlying these effects remains unclear. For example, even though the decrease in extracellular concentration of K$^+$ or decrease in its uptake due to Trk2 deletion affects Gtfs and other secreted or surface associated proteins, we have not yet defined if the impact on Gtf is due to an effect of low K$^+$ on a molecular mechanism like the Sec pathway or due to an effect on the physiological aspect like low metabolism and acid production.

We have utilized a K$^+$ concentration of 25 mM as our control, as it yielded in maximum growth and is in the physiological range of the K$^+$ concentrations found in the un-stimulated saliva. Testing the growth and biofilm phenotypes of \textit{trk2-null} mutant in the pooled human saliva could provide useful information towards utility of Trk2 as a potential blocking target for therapeutic purposes.

The link provided in this study, between genetic transformation and Trk2 mediated K$^+$ homeostasis is new. Future experiments monitoring the effect of K$^+$ concentration on the uptake of labeled eDNA would be required to confirm if the transformation defect is due to disruption of the membrane potential due to limiting K$^+$ or trk2 deletion, which results in defect in uptake to the negatively charged eDNA by the cell.
There are four transport systems identified, of which Trk2 and GlnQHMP have been characterized for their functions, but the roles of Trk1 and Kch remain inconclusive. It is possible that Trk1 is functional at initial stages of growth and is the cell’s way to regulate K$^+$ balance; we did observe changes in the membrane potential and initial K$^+$ uptake when Trk1 was absent in *S. mutans* UA159 strain. We were also able to retrieve its role as the K$^+$ uptake system in complemented *E. coli* TK2420 strain, but its role as the K$^+$ transporter in *S. mutans* and conditions under which this system is activated need to be characterized.

Using our complemented *E. coli* TK2420 strain, it is possible in the future to probe the molecular mechanisms underlying the activity of the Trk2 system under specific environment conditions. Future experiments including construction of a triple knockout strain from a wt UA159 parent strain, in which only one K$^+$ transport system is analyzed, could provide better understanding about why multiple K$^+$ transport systems are present in the genome of *S. mutans* and also help in characterizing conditions under which each of these systems is activated.

### 6.2 Significance of the study

Dental plaque is a distinct and a complex microbial environment in the oral cavity. The fluid retained in mature dental plaque is drastically different from other oral fluids such as saliva or gingival crevicular fluids; one of the key differences is that plaque fluid is highly saturated with potassium. Even though the importance of potassium in bacterial physiology
is an established fact, questions such as why dental plaque microbes prefer a high potassium environment, and if potassium accumulation fosters an environment conducive to cariogenesis remains underexplored. The hypothesis for our study was that potassium homeostasis is essential for the cariogenic attributes of \textit{S. mutans}. We tested five main properties of \textit{S. mutans} namely, 1) biofilm formation, 2) sugar metabolism, 3) acid and osmotic stress tolerance, 4) genetic transformation and 5) growth, in defined media with potassium concentrations ranging 0-50mM. Our results show that at least 25mM K$^+$ is required for optimal physiological functioning of \textit{S. mutans}. Interestingly, 25mM K$^+$ is the reported K$^+$ concentration found in the un-stimulated saliva. We further identified Trk2 as the key K$^+$ transport system in \textit{S. mutans} UA159 strain; absence of this system lead to disruption in all the aforementioned virulence attributes of \textit{S. mutans}. During the course of this study, we also identified the molecular components involved in biofilm formation, genetic transformation and stress tolerance of \textit{S. mutans} that are affected by disruption in K$^+$ homeostasis. Collectively, with the identification of the key K$^+$ transporter in \textit{S. mutans}, understanding the role of K$^+$ in virulence properties and defining the requirement of K$^+$ by this bacterium, we have opened new prospects in cariology research.
Figure S1: Membrane potential analysis of UA159, Trk1 null, and Trk2 null strains. Cells were grown in THYE to mid-exponential phase before washing and re-suspending in MMGK in the presence of 0, 5, 25, or 50 mM KCl. Aliquots of cell suspensions were incubated with DiSBAC<sub>2</sub> fluorescence dye. The effect of membrane stressors such as low pH (5.5) and introduction of potential dissipater carbonyl cyanide-m-chlorophenylhydrazone (CCCP) were also tested as described. Alterations in the membrane potential were calculated by measuring the changes in fluorescence intensity after (A) 15 minutes, (B) 30 minutes, and (C) 2 hours, and normalized with the fluorescence intensity decay of the dye.
Figure S2: Growth curves for *E. coli* TK2420 mutant and its complemented strains. Cells are: (A) *E. coli* TK2420 mutant, and its complemented counter strains with (B) GlnQHMP, (C) Trk1, and (D) Trk2 systems. Strains were grown in HKLM medium ( ), LKLM (Δ) and LKLM with 400 mM NaCl (✘) for 16 hours. Growth curves are representative of at least three independent experiments conducted with five replicates each. The lower panel (E) shows the effect of pH 4.0 on the growth of TK2420 *E. coli* mutant and TK2420 strains complemented with pacL or Trk2 systems as indicated.
Figure S3: Change in membrane potential for complemented TK2420 strains: TK-DUET, TKtrk1, TKtrk2 and TKglnQHMP. All strains were grown in K⁺-rich medium to OD₆₀₀ ~0.4. Aliquots of cells were then incubated with DiSBAC₂(3) fluorescent dye and change in fluorescence intensity was measured and normalized against decay of dye intensity was calculated for each sample at the following time points: (A) 2 minutes, (B) 4 minutes, and (C) 10 minutes. Asterisk (*) represents a statistical significance of p-value < 0.05.
Figure S4: Genetic transformation in the UA159 and trk2-null mutant. Cells were grown to O.D. ~0.1 in MMGK- medium supplemented with 5mM, 25 mM or 50 mM KCl. 0.5 ml of cells were then pelleted and re-suspended in equal volume of THYE and transformed with pDL277 (Dunny et al., 1991) plasmid DNA in the presence or absence of the competence stimulating peptide, CSP. Percent transformation frequency was calculated using the number of transformants obtained over the total number of viable cells multiplied by 100. Bars shown above are the means for a total of four replicas with standard deviation for each condition.
Figure S5: Growth curves for *S. mutans* UA159 and isogenic mutant strains. Cells are grown in minimal media (MMGK-) where 1% glucose was replaced with 1% fructose or 1% sucrose and grown for 16 hours. Growth curves are representative of at least three independent experiments conducted with five replicates each.
Figure S6: Estimation of intracellular K\(^+\) content in *S. mutans* wild-type UA159 strain and K\(^+\) transport knockout mutants. To deplete cells of K\(^+\) prior to uptake assays, mid-exponential phase cells were incubated in K\(^+\)-deficient MMGK growth medium for 2 hours. Cells were then supplemented with 50 mM KCl and aliquots were sampled over time to measure intracellular K\(^+\) content using ICP-OES. Repeated measure ANOVA was performed and comparisons were made between different time points within each group (*) and by comparing intracellular K\(^+\) levels between groups (\(^*\)), p<0.05 is significant.
Table S1: Putative K⁺ transport proteins and their predicted functions in *Streptococcus mutans* UA159.

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<th>Protein/ Locus ID</th>
<th>Conserved Domain SMART Search</th>
<th>NCBI Closest Homolog (%identity/%positive)</th>
<th>STRING Predicted Functional Partners</th>
<th>Function</th>
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<td>Trk, TrkA, PacL</td>
<td>Putative potassium uptake system protein TrkB</td>
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<td>Putative cation-transporting P-type ATPase</td>
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<td>Function</td>
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**Table S2A: Strains and their isogenic variants.**

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**Table S2B: Primers used in this study.** KO: knockout primers; CP: cloning primers; RT: primers used in qRT-PCR

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</tbody>
</table>

**Table S3 – Two nutrient-rich media preparations, THYE and TYE, both at pH7.5, were subjected to ICP-OES to quantify cation content.** Detected levels of cations were calibrated against standard curves generated for each analyte [wavelength listed after the element in brackets – Na (589.592 nm), K (766.49 nm), Mg (285.213 nm), Ca (317.933 nm), Fe (238.204 nm), Mn (257.61), Cu (327.393 nm), Al (396.153 nm), Ni (231.604 nm) and Zn (206.2 nm)] using a serially diluted QC4 (SCP Science, Quebec) cation standard. Each standard curve had a minimum correlation coefficient of 99.9%. Cations below the detection limits of this assay [Fe(179 µM), Mn (182 µM), Cu (157 µM), Al (371 µM), Ni (170 µM), Zn (153 µM)] are excluded. Values represent analyte concentration mean ± SE for experiments repeated three times.

<table>
<thead>
<tr>
<th>Cation</th>
<th>THYE</th>
<th>TYE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>65.62 ± 0.29 mM</td>
<td>37.46 ± 0.12 mM</td>
</tr>
<tr>
<td>Potassium</td>
<td>18.55 ± 0.047 mM</td>
<td>12.85 ± 0.043 mM</td>
</tr>
<tr>
<td>Magnesium</td>
<td>4.89 ± 0.036 mM</td>
<td>0.263 ± 0.00141 mM</td>
</tr>
</tbody>
</table>
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