Characterization of a Copper Resistance and Transport System in *Streptococcus Mutans*

By

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A thesis submitted in conformity with the requirements for the degree of

Doctor of Philosophy

School of Graduate Studies

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I. Abstract

Proteins and enzymes require metal ions as enzymatic cofactors for optimal biological activities. Copper metal ion is utilized by various bacterial systems in trace amounts; however, it can be extremely toxic at higher concentrations. The \textit{copYABZ} operon, involved in copper homeostasis, has been extensively studied and characterized in several Gram positive bacteria. CopA and CopB encode for P-type ATPases, involved in copper translocation, whereas, CopY and CopZ regulate the expression of the \textit{cop} operon. In \textit{Streptococcus mutans}, the primary etiological agent of dental caries, \textit{copYAZ} has been partially investigated for its role in copper transport and resistance. In this study, we demonstrated the probable mechanisms by which copper induces toxicity in \textit{S. mutans}; and elucidated the role of \textit{copYAZ} operon in modulating copper transport and resistance, as well as other physiological processes of this oral pathogen. Copper induces toxicity by generating oxidative stress and dissipating membrane potential in \textit{S. mutans}. During biofilm growth, copper impaired \textit{S. mutans} ability to adhere to a surface and produce biofilm biomass; while significantly repressing the expression of genes involved in
maintaining the functional and structural integrity of biofilm matrix. Results from copper transport studies validated the role of copYZ in copper efflux in *S. mutans*. The knock-out strain in *copYZ* (Δ*copYZ*) was more sensitive to copper, oxidative and acid stress relative to its wild type. Loss of *copYZ* resulted in prolonged membrane depolarization in *S. mutans*. The presence of copper and/or the absence of *copYZ* significantly impaired *S. mutans* ability to acquire foreign DNA from the surrounding environments; while repressing the transcription of genes involved in competence development. The *copYZ*-associated phenotypes were further validated via genetic complementation studies. Taken collectively, we illustrated the implications of copper-induced toxicity in *S. mutans*; and have provided evidence describing the importance of *copYZ* operon in copper resistance and transport, biofilm formation, acid and oxidative stress tolerance, maintenance of membrane potential, and genetic transformation in *S. mutans*. 
II. Acknowledgments

First and foremost, I would like to express my deepest gratitude to my supervisor Dr. Dennis Cvitkovitch for his support, expert guidance, understanding, patience, and encouragement over the course of my graduate program. Very special thanks to my co-supervisor Dr. Celine Levesque, for providing me with the motivation and encouragement to pursue this research project. I am very grateful to my advisory committee members Dr. Trevor Moraes, Dr. Anil Kishen and Dr. Dilani Senadheera for their aspiring guidance and invaluably constructive criticism.

I am thankful to Ms. Gursonika Binepal for her help and support. I would like to extend my thanks to other members of Cvitovitch Lab, particularly, Mr. Andrew Latos, Ms. Kirsten Krastel, Ms. Marie-Christine Kean and Ms. Iwona Wenderska.

Finally, I thank my family for their unconditional love and support. To Mom, Papa, Ravi, Sahil, Jai and Tanu, I am ever grateful to you all for always being there for me. Thank you.
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<tr>
<th>Abbreviation</th>
<th>Meaning</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>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide 3-chlorophenylhydrazone</td>
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<tr>
<td>CDM</td>
<td>chemically-defined medium</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CFUs</td>
<td>colony forming units</td>
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<tr>
<td>CSP</td>
<td>competence-stimulating peptide</td>
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<tr>
<td>Cu</td>
<td>copper</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<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>
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<tr>
<td>Ftf</td>
<td>fructosyltransferase</td>
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<tr>
<td>Gbp</td>
<td>glucan-binding protein</td>
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<tr>
<td>Gtf</td>
<td>glucosyltransferase</td>
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<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>GCF</td>
<td>gingival crevicular fluid</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<td>min</td>
<td>minute</td>
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<tr>
<td>Mg</td>
<td>magnesium</td>
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<tr>
<td>Mn</td>
<td>manganese</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>Spec</td>
<td>spectinomycin</td>
</tr>
<tr>
<td>spp</td>
<td>species</td>
</tr>
<tr>
<td>TCSTS</td>
<td>two-component signal transduction system</td>
</tr>
<tr>
<td>THYE</td>
<td>Todd Hewitt yeast extract</td>
</tr>
<tr>
<td>TYE</td>
<td>Tryptone yeast extract</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
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<tr>
<td>XIP</td>
<td>$\text{sig}X$-inducing peptide</td>
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VII. Preface

Format of the dissertation

The presented dissertation is written in the "Publishable Style". Chapter 1 provides introduction to the subject and presents the background for the following chapters. Chapter 2, the review article published in Future Microbiology, describes the link between bacterial two component systems and their metal transporters. Chapter 3 states the purpose and rationale of the study. Chapter 4 describes the experimental data that have been accepted for publication in Journal of Bacteriology. Chapter 5 provides a brief summary and conclusions drawn from this study and chapter 6 presents the future directions and significance of this study. The thesis is presented in a format to improve readability and avoid repetition between the chapters.
1 Literature Review

1.1 The oral cavity

1.1.1 Microbial habitats of the oral cavity

The oral cavity represents one of the most diverse and dynamic ecosystems in the human body known to be colonized with over 700 identified microbial species [1-2]. It is distinct from other body sites due to the presence of 1) specialized mucosal surfaces such as lips, tongue, cheek, and palate; 2) hard non-shedding tooth surfaces; 3) saliva; and 4) gingival crevicular fluid (GCF), that sustain a diverse array of microbes adapted to a biofilm lifestyle, commonly referred to as plaque [3-4]. The mucosal and tooth surfaces provide the substratum for microbial colonization, whereas, host oral fluids such as saliva and gingival crevicular fluid provide nutrients and assist in bacterial adherence for community development on these surfaces [3-4]. Chronologically, in the oral cavity of a new born baby, the only site available for microbial colonization is the infant's oral mucosa [5]. The oral mucosa is predominately comprised of stratified squamous epithelium, and contains specialized mucosal surfaces containing either keratinized (hard palate, attached gingiva and dorsal surface of tongue) or non-keratinized (all other mucosa of oral cavity) epithelial cells [3, 6]. The biomass of microbial communities in the oral mucosa in an infant is restricted by the continuous desquamation of the epithelial cells [7]. On the mucosal surfaces a variety of distinct habitats exists, each of which provides unique ecological and biochemical conditions to selectively support the growth and co-existence of specific microbial species [4, 8]. A major ecological perturbation in the infant’s oral cavity occurs following tooth eruption around a few months after birth that provides new hard non-shedding surfaces for microbial colonization [9-11]. While the process of primary dentition is completed by the age of 3 years, the permanent teeth start to erupt around 6 years and completed by the age of 12 years (Source: American Academy of Pediatric Dentistry). The loss and eruption of teeth causes perturbations in the local environment, resulting in alterations in the composition of microbial community [3, 12]. Like the oral mucosa, teeth also provide a variety of sites for colonization and growth of different microbial communities [9, 13-14]. Variations in the biochemical properties depending on the anatomy of the tooth can sustain diverse microbial populations [15-
For instance, the plaque formed in the hidden area between the teeth (approximal plaque) and in the gingival crevice (gingival crevice plaque) are favorable habitats for facultative and obligate anaerobes such as Streptococci and Actinomyces [17-18]. Whereas, the tooth fissures (fissure plaque) is predominated by species such as certain aerobes and facultative anaerobes, for e.g. Streptococci [15, 19]. Another important component of oral cavity is the saliva, which plays an important role in maintaining the oral ecosystem and is critical for oral health by providing antimicrobial, buffering and conditioning functions in the mouth [20]. Saliva aids the clearing of food and buffers the oral cavity following the ingestion and metabolism of dietary carbohydrates by the acidogenic consortia [7, 21]. Salivary components (e.g., mucins, histatins and cystatins) can adsorb on the tooth surface to form a conditioning film known as the acquired pellicle, which facilitates bacterial adherence on the surface [22-24]. Saliva is the primary nutrient source for the resident oral microbes, and also contains antimicrobial factors (e.g. lysozyme and lactoferrin) to prevent the growth of unwanted exogenous microorganisms [22, 25]. The last major component of oral cavity is the GCF that flows through the junctional epitelium of gingiva. It is a serum-like fluid that can act as a source of nutrients and contains components of host defence that include lysozyme, lactoferrin, peroxidases and Immunoglobulin G (IgG) antibodies [26-29].

1.1.2 Acquisition of oral microflora

The oral cavity of an infant is inoculated with microbes that are acquired from mother's milk; these microbes can then initialize the process of acquisition of resident oral microflora. The mode of birth delivery also appears to influence the development of oral microflora. On comparing the oral microbiota of 3-month old infants delivered vaginally and by caesarean section, a higher prevalence of Streptococci and Lactobacilli was found in the oral microbiota of vaginally delivered infants [30-31]. In addition to microbial acquisition from the mother, the infant can also acquire microbes from family members in close proximity, or external food source such as milk or water [32-34]. This microbial acquisition is controlled by host factors including saliva. The detailed roles of saliva in microbial acquisition and homeostasis have been discussed under the section 1.1.3. The first colonizers are referred to as pioneer species and are mainly streptococci belonging to the mitis-group that includes: Streptococcus salivarius,
Streptococcus mitis, and Streptococcus oralis [5, 35-36]. The pioneer microbial community continue to grow and colonize until it encounters an environmental resistance, such as: shedding of epithelial cells, shear force due to salivary flow, nutritional limitations and alterations in redox potential or pH [15, 36]. Many of the pioneer species secrete proteases against secretory immunoglobulin A (IgA), one of the major host immune defense factors present in the infant’s saliva [21, 37-38]. The inhibitory activity against secretory IgA allows these pioneer organisms to colonize the oral mucosal surfaces by overcoming key host-defenses in the early stages of colonization [38]. Over time, the physiological activity of pioneer microbial community modifies the surrounding environment making it conducive for the colonization and succession of other bacterial populations [16, 39]. Some of these modifications include: exposure of new receptors necessary for bacterial adherence to the surface or to the pre-adhered bacteria (co-aggregation), generation of metabolic end-products by pioneer species that can be utilized by subsequent populations, and other environmental changes [40]. The progressive development of pioneer community also prevents the colonization of unwanted exogenous bacterial species by: 1) competing for essential nutrients and co-factors; 2) competing for the receptors for adhesion; 3) creating an environment not favorable for the growth of exogenous species and; 4) generating inhibitory substances such as bacteriocins, hydrogen peroxide, etc [16, 41].

As the process of dentition progresses, new microenvironments are created that increases the diversity of the microbial community, with the introduction of members of genera Neisseria, Lactobacillus, Veillonella, and Actinomyces [35]. The progressive diversity in the microbial community results to the formation of a complex dental biofilm, commonly called dental plaque. The dental plaque is highly organized with microbes embedded in a matrix of self-composed extracellular carbohydrate and/or nucleotide polymers [42-43]. The microbial homeostasis in the oral cavity is constantly challenged by physical and non-physical changes, such as tooth extractions, dental treatment, antibiotic use, variation in salivary flow or saliva pH, food habits, and the presence of host-derived antimicrobial factors [8, 16, 44].

The microbial successions typically fall under two defined categories, namely: 1) Allogenic succession resulting due to the environmental changes generated by the host, or 2) Autogenic succession that is a result of the microbial interrelations [45]. During allogenic succession, the community development is influenced by the non-microbial factors such as tooth eruption,
insertion or removal of dentures, and introduction of antimicrobial agents. Whereas, during autogenic succession, factors derived from pioneer species such as growth factors, alteration in the environmental pH and the redox potential regulate the progression of microbial community [45]. The pioneer community continues to develop through series of microbial successions until equilibrium is attained which results in the formation of the climax community. The climax community exhibits a highly dynamic relationship between the host, the environment and the resident microflora in the oral cavity. The oral microflora continues to acquire new bacterial species over the age of the host. In adults, the 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 [15, 46-47].

1.1.3 Oral biofilms

In natural environments bacteria primarily exist as surface-attached complex microbial communities known as biofilms [48]. A biofilm is a surface associated four-dimensional structure that contains mono- or multi-species bacteria co-adhered and embedded into a matrix of extracellular polymeric substance(s) (EPS) derived from host and/or bacterial factors [43]. Biofilm formation includes three distinct steps: attachment of cells to a surface, growth of the cells into a sessile biofilm colony, and dispersal of cells from the mature biofilm to colonize into the surrounding environment. Environmental signals, chemical and physical forces, bacterial communication factors, and effectors of bacterial or host origin play a significant role in each distinct stage of biofilm formation [49-50]. Adapting to a biofilm mode of growth provides bacteria with multiple advantages such as protection from antimicrobial factors, dynamic host and environmental conditions, and the ability to communicate among themselves [51-52].

The formation of dental plaque occurs via specific steps, each of which determines the successful attachment and colonization of microbes on the tooth surface. The distinct stages of plaque formation are described below:

a) Acquired pellicle formation: Once a clean tooth surface is exposed to the oral environment, adsorption of salivary proteins and glycoproteins to the dental enamel creates an acquired enamel
pellicle (AEP) [22]. The AEP is composed of bacterial components such as glucans (explained in detail in Section 1.2.2), and host components including salivary proteins such as sialyted mucins, α-amylase, proline-rich proteins, agglutinin, and statherin [24, 53]. An equilibrium attained between the continuous adsorption and desorption of the salivary molecules on the tooth surface determines the thickness of pellicle layer [7, 54]. The thickness of the pellicle layer also depends on the site of formation and the presence of salivary shear forces [54]. The molecular composition and the physiochemical properties of the pellicle layer play a critical role in determining the composition and the pattern of dental plaque formation.

b) Attachment and colonization of bacteria on the tooth surface: Microbes usually are transported passively to the tooth surface via the salivary flow; however few of them possess specialized structures such as fimbrae or pili that assist them to attach to the surface [32, 39]. The components of the acquired pellicle play an important role in bacterial adherence to the surface. Initially, as the microbes approach the pellicle-coated surface, weak and long-range physiochemical forces are generated by the negatively charged bacterial cells and hydrophobic tooth surface, that allows reversible attachment of bacteria to the surface [42, 53]. Within a short period of time, irreversible attachment of bacteria is mediated by strong and specific but short-range interactions between the microbial adhesins (components of microbial cell surface) and the complementary receptors on the pellicle layer [42, 53]. After successful attachment, the pioneer species constituting mainly of the members of the mitis group of streptococci start to divide and propagate on the surface. The cell walls of several oral streptococci possesses a number of adhesins such as antigen I/II, glucosyltransferases and lipoproteins that bind to their cognate receptors on the pellicle layer [10]. For instance, antigen I/II binds to the human salivary glycoproteins (agglutinins) and other microbial cells; whereas, glucosyltransferases interacts with blood-related proteins or the adsorbed dextrans and glucans in the pellicle layer [10]. In some cases, the salivary molecules in the acquired pellicle can undergo conformational changes leading to exposure of new bacterial receptors known as cryptitopes [20, 22]. These cryptitopes are recognized by specific bacteria that possess cognate adhesins on their cell surfaces for plaque formation [20, 22].

The pioneer species continue to grow and propagate in the dental plaque environment. These species are equipped with special features that enable them to initiate plaque formation on tooth
These species secrete IgA protease that allows them to overcome the host defenses; and they also possess a range of glycosidase activity that enables them to use salivary glycoproteins as nutrient substrates [16, 21]. On reaching an equilibrium in their population, these pioneer species start to make conditions conducive for the growth of secondary colonizers. The pioneer species provides additional adhesins for the secondary colonizers that were previously unable to adhere to the adhesins of pellicle layer [39]. Additionally, the metabolic activity of the pioneer communities alters the local environment, thereby, generating an improved atmosphere for the new bacteria to colonize the dental plaque [16, 50, 55]. Over time, the diversity in the plaque microflora increases with the introduction and expansion of Actinomyces and other Gram positive bacilli. A series of complex interactions between the microbes in the plaque community changes the composition of plaque microflora in due course of time [55-56]. The conditions continue to progressively change to eventually become favourable for obligate anaerobes. The graphical demonstration of early and late colonizers of the plaque formation has been presented in Figure 1-1, with the initial colonizers constituting the yellow (Streptococcus gordonii, Streptococcus intermedius, S. mitis, S. oralis, and Streptococcus sanguinis), blue (Actinomyces spp), green (Capnocytophaga plus Campylobacter concisus, Eikenella corrodens, and Aggregatibacter actinomycetemcomitans serotype a) and purple (Veillonella parvula and Actinomyces odontolyticus) complexes, whereas the late colonizers forming the orange (Fusobacterium nucleatum, Prevotella intermedia, Peptostreptococcus micros, Eubacterium nodatum, and other species) and red complexes (Treponema denticola, Porphyromonas gingivalis and Tannerella forsythia) [3, 57-59]. 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 [50, 60]. In oral health, the microbial composition of dental plaque is diverse and remains relatively stable over time [14-15].

c) Biofilm maturation and detachment: On attaining maturation, the growth rate of individual bacteria within the plaque community decreases, significantly increasing the doubling rate from 1-2 hours during early growth to 12-15 hours after 1-3 days of biofilm development [12, 61]. The microbial diversity reaches its maximum in the mature plaque. Biofilms are embedded in the EPS matrix composed of different types of biopolymers such as, polysaccharides (glucan,
fructans and hetero-polymers), proteins, deoxyribonucleic acid (DNA) and lipids, most of which are self-produced by the microbes [43, 62]. The adhered bacteria simultaneously propagate and synthesize EPS to facilitate their adherence together in a mass and firmly to the surface. The constituents of the EPS matrix assist biofilms in providing protection against desiccation and in facilitating physical interactions between bacterial cells or between bacteria and the adhered surface, thereby, encouraging biofilm stabilization [43, 62]. The matrix critically maintains the structural integrity of the plaque and accounts for biofilm's ability to tolerate environmental insults. The matrix also assists in retaining water, nutrients and growth factors, while restricting the penetration of detrimental antimicrobials and host-defence factors [43]. In addition, the EPS immobilize the cells in the matrix, allowing them to be in close proximity for complex interactions and cell-to-cell communication, thereby, enabling the bacteria to communicate and to co-ordinate their metabolic and physiological activities [43, 63].

The final stage in biofilm development is the detachment of cells from the mature biofilms and their dispersal into the surrounding environment to colonize new sites, thus, playing an important role in transmission of bacterial infection [41, 64]. The biofilm life-style impacts the genomics and proteomics of the plaque bacteria during distinct stages of plaque formation. For instance, in the initial stage of plaque formation, bacterial adhesins and enzymes involved in carbohydrate catabolism are induced; during plaque colonization and propagation, proteins involved in biochemical functions are expressed; whereas, at the stage of biofilm maturation, enzymes that cleave bacterial adhesins are activated, thereby, facilitating cell dispersal from one location to another [37, 65-66]. Additionally, cell-cell signalling molecules of the plaque bacteria assist them to communicate and coordinate their gene expression, resulting in formation of complex, interactive, multi-species, spatially and functionally organized dental plaque [40, 50-51, 55, 67].
**Figure 1-1** Diagram representing microbial composition of dental plaque

The base of the pyramid is comprised of species thought to colonize the tooth surface and proliferate at an early stage. The orange complex becomes numerically more dominant later and is thought to bridge the early colonizers and the red complex species that become numerically more dominant at late stages in plaque development. Adapted from Socransky and Haffajee, 2000 [68].

### 1.1.4 Oral diseases

The healthy oral cavity is predominantly colonized with health-associated commensals, and some opportunistic pathogens at levels insufficient to cause a disease [47]. In the state of ecological stability, the microbial homeostasis is achieved by numerous host-microbial interactions in the oral cavity [47]. Perturbation in the microbial homeostasis can shift the composition and properties of the resident microflora [19, 52]. This shift in microflora may lead to progression of oral diseases such as gingivitis, oral candidiasis, endodontic (root canal)
infections, alveolar osteitis (dry socket), tonsillitis, dental caries and periodontitis [52, 69]. Oral bacteria have also been implicated in causing systemic conditions such as infective endocarditis, coronary heart disease, and increased risk of preterm low-birth-weight babies [70-72]. Additionally, plaque-mediated periodontal disease has been associated with other chronic diseases, such as chronic kidney disease, diabetes and hypertension [28, 73-76].

Dental caries, one of the most common chronic biofilm infections, is considered as a major oral health problem due to its high prevalence [77]. In most industrialized countries, dental caries affects 60-90% of school-aged children and vast majority of adults; whereas, the global prevalence of dental caries among adults can be nearly 100% of the population [77]. It is a multifactorial disease that depends on the availability of susceptible tooth surface, cariogenic pathogens and preferred substrates (in form of dietary sugars) for sufficient period of time to initiate the dissolution of tooth enamel [52].

Unlike most medical infections, where a specific pathogen is associated with the disease, in plaque-mediated diseases particularly dental caries and periodontitis, the disease-associated traits are usually not restricted to a single species. Therefore, to explain the etiology of plaque-mediated diseases, three hypotheses have been proposed: Specific, Non-Specific and Ecological plaque hypothesis. According to the "Specific Plaque Hypothesis", out of the diverse microbial community present in the plaque microflora, only a few specific species are actively involved in causing a disease [78-79]. On the contrary, the "Non-Specific Plaque Hypothesis" states that a disease is an outcome of the overall activity of the total plaque microflora [80]. The "Ecological Plaque Hypothesis" reconciled the key elements of the earlier two hypotheses and explained the dynamic relationship between the resident microflora and host ecology in health and disease [15] [14]. The hypothesis states that changes in the host environment such as: a) shifts in the overall community metabolism, b) subsequent modification of local environment, c) host factors, and d) the balance between potential pathogens and species associated with oral health, determines the conditions conducive for oral health or disease [14-15]. 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 [14-15]. For example, for the treatment of periodontal disease caused by oral pathogens such as P. gingivalis, T. forsythia, and A. actinomycetemcomitans, special measures,
such as reducing the severity of inflammation and altering the redox potential of periodontal pockets, should be considered to restrict the growth of associated microbes [14-15]. Similarly, low pH environments encourage the growth of acid-producing, acid-tolerating bacteria such as mutans streptococci and lactobacilli, commonly known for their involvement in dental caries. The prevention measures against dental caries should target both the associated pathogens and the environmental factors causing the ecological shifts in the plaque population [14-15, 81]. The role of oral streptococci in the etiology of dental caries is described under sections 1 and 0.

1.1.5 Oral streptococci

A large population of resident oral microflora is comprised of streptococci, which have been isolated from various sites in the mouth. Most oral streptococci are usually commensal bacteria, while some are involved in causing dental caries, periodontitis and occasional extra-oral infections such as infective endocarditis or brain abscesses [61, 82-84]. The most prevalent oral streptococci are clustered within four phylogenetic groups: mutans group (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 and S. oralis) [85-86]. The pioneer colonizers on 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 sections 1.1.2 and 1.1.3). Streptococci from the mutans group are commonly associated with the etiology of dental caries. The mutans streptococci along with Lactobacillus are capable of rapidly metabolizing carbohydrates into acid end-products and generating acidic environment, while activating their adaptive response under low pH [87]. The microbial succession during the caries formation implies the association of mutans streptococci with the caries initiation, whereas lactobacilli are associated with caries progression [9, 21]. In mutans streptococci, nine serotypes (a-h and k) have been recognized based on the specificity of carbohydrates present in their cell walls [84, 88-89]. 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). The isolates of S. cricetus serotype a, and S. sobrinus serotype d and g have also been recovered from the human oral
cavity [84, 88-90]. *Streptococcus downei* serotype h and *Streptococcus ratti* serotype b are rarely isolated from macaques and rats, respectively[86, 90]. The members of salivarius-group colonize preferably on mucosal surfaces and are not considered as significant pathogens of oral diseases [14, 91]. Species from the anginosus-group are colonizers of dental plaque and mucosal surfaces, and are recognized as the main causative agents of maxillo-facial infections [92-93]. 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 [3, 12, 40].

### 1.2 *Streptococcus mutans*

*S. mutans* was first isolated from a caries lesion by J Clarke in 1924 [94]. However, it was decades later that *S. mutans* was associated with dental caries [87, 95]. In rare cases, *S. mutans* has also been shown to cause infective endocarditis, a life threatening heart valves inflammation [82, 96-97]. In both conditions, *S. mutans* adapts a biofilm mode of growth to colonize specific host sites. *S. mutans* can be transmitted to an infant directly from the mother, or from different members of the family, indicating both vertical and horizontal mode of transmission [98-99]. During the course of life, most of the initially acquired *S. mutans* strains persist, some are lost and other new strains are introduced [98-99]. Following the advent of complete *S. mutans* genome sequence, various genomic and proteomic approaches have facilitated our understanding on the genetics, biochemistry and physiology of this dental pathogen. The genomes of four different strains of *S. mutans* have been sequenced with *S. mutans* UA159 being the first to be sequenced, followed by *S. mutans* NN2025, *S. mutans* LJ23 and *S. mutans* GS-5 [100-103].

*S. mutans* harbors several virulence factors that provide the bacterium an ecological advantage over other oral streptococci. The majority of the genome of *S. mutans* is dedicated towards the metabolism of dietary sugars and their associated transport systems. The main virulence features of *S. mutans* include: a) the ability to produce large quantities of organic acids (acidogenicity) from metabolized carbohydrates; b) the ability to sustain under low pH conditions (aciduricity); and c) the ability to synthesize extracellular polymer matrix from dietary sugars for initial
attachment, colonization and accumulation of biofilms on tooth surfaces [87]. The factors influencing the survival and virulence of S. mutans in the oral biofilm are described below.

### 1.2.1 Acidogenicity and aciduricity

S. mutans has the ability to transport and metabolize several sugars, such as glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β-glucosides, trehalose, maltose, raffinose, ribulose, mellibiose, iso-maltosaccharides, and possibly sorbose [101]. S. mutans utilizes these dietary sugars and generates acid end products such as lactate, formate, acetate and ethanol [104]. During periods of heavy sugar intake, excessive acid production by acidogenic bacteria lowers the pH of the dental plaque [105]. S. mutans are capable of maintaining their glycolytic activity under the acidified milieu, which provides them a competitive advantage over other oral streptococci and assists them and other acidogenic/aciduric bacteria to dominate in the dental plaque [105-107]. These acid-induced adaptation and selection processes can perturb the balance between the demineralization and remineralization of tooth enamel, resulting in initiation and progression of dental caries.

The ability of S. mutans to tolerate acid stress depends on the implementation of several constitutive and inductive mechanisms, which assist in their adaptation under acidic environments [105-106, 108]. The central constitutive mechanism involves the activity of the F₁F₀ ATPase proton pump, which is functional under low pH and plays a role in: 1) expelling protons out of the cell to normalize the intracellular pH, and 2) simultaneously generating ATP to provide energy for the cellular functions [105-106, 108-109]. The inductive mechanisms are collectively referred to as the Acid Tolerance Response (ATR), whereby bacteria adjust several catabolic pathways to maintain their viability [105-106, 108]. Some of these inducible mechanisms include: 1) alteration in the integrity and composition of the cell envelope resulting in impaired proton permeability; 2) induction of genes encoding for molecular chaperones, proteases, and DNA repair enzymes to maintain stability of macromolecules and to prevent accumulation of misfolded proteins; 3) activation of metabolic pathways involved in recycling of the carbon acids produced during sugar fermentation, thereby, assisting in alkalization of cytoplasm; 4) and, synthesis of insoluble glucans, leading to adaptation to a biofilm mode of
growth, which have increased acid resistance compared with their planktonic counterparts [105-106, 108].

### 1.2.2 Biofilm formation

*S. mutans* adapts a biofilm-dependent lifestyle to colonize the host body [95]. *S. mutans* utilizes certain mechanisms to adhere to the tooth surface and under favorable conditions becomes significantly predominant in the dental plaque community. The fermentable dietary carbohydrates, especially sucrose, act as one of the key environmental factors in the initiation and development of dental caries. In the absence of sucrose, the multifunctional adhesin SpaP (antigen I/II, P1, PAc) is considered to be the primary factor required for initial adherence of *S. mutans* to the tooth surface [53, 110]. *S. mutans* metabolizes dietary sugars to produce extracellular polysaccharides that act as major constituent of the EPS in the biofilm matrix [105-106]. EPS promotes bacterial adherence to the tooth surface or to adhered bacteria, thereby enhancing the structural integrity of the dental plaque [105-106]. Sucrose, a disaccharide of fructose and glucose, is a major component of the human diet and is strongly associated with the initiation and progression of dental caries. In the presence of sucrose, multiple glucosyltransferases exoenzymes mediate the adherence of *S. mutans* to the tooth surface, by synthesizing D-glucose polysaccharides, known as glucans. *S. mutans* produces three distinct glucosyltransferases, namely: the glucosyltransferase enzyme GtfB, which acts on sucrose to produce water-insoluble glucans composed predominantly of α-1,3-linkages, GtfD that converts sucrose to mostly soluble α-1,6-linked glucans and GtfC makes both α-1,3 and α-1,6 glucans [111-113]. The high molecular weight polysaccharides produced by the Gtf enzymes have been implicated in attachment and biofilm formation on the smooth surfaces of the tooth in *S. mutans* [63]. In addition, a fructosyltransferase (FTF) enzyme acts on sucrose to produce a fructose homopolymer that is primarily composed of β-2, 1-linked fructose (inulin) [114]. Glucans are required for initiating and facilitating *S. mutans* adherence and accumulation on the tooth surface and acting as a source of metabolizable polysaccharides outside the cell; whereas fructans are believed to function exclusively as extracellular storage polysaccharides [10, 115-118].
The binding of *S. mutans* to glucans is mediated by cell associated Gtf enzymes and glucan binding proteins (Gbps) [119]. *S. mutans* produces four different Gbps that include GbpA, GbpB, GbpC and GbpD [120-124]. Immunologically distinct from other Gbps, GbpB affects the initial steps of sucrose-dependent biofilm formation by modulating cell division and other physiological processes required for planktonic to biofilm transition [125]. GbpA, GbpC and GbpD are each transported across membrane, where only GbpA and GbpD are released; whereas, GbpC is cell-wall bound [119, 126]. GbpA contributes to biofilm architecture by linking glucans molecules, more or less independent of the bacteria [124-125]. GbpC is essential in the early stages of biofilm formation and is involved in glucan-dependent aggregation of bacteria; the loss of GbpC results in impairment in virulence, biofilm biomass, and bacterial aggregates formation [122, 126-127]. GbpD contribute to the scaffolding of the biofilm and is required to provide cohesiveness between bacteria and glucans in the biofilm matrix; loss of GbpD results in extremely fragile biofilms [119].

### 1.2.3 Regulatory systems in *S. mutans*

Both Gram positive and Gram negative bacteria utilize quorum sensing signaling system to communicate under an array of diverse physiological conditions. Quorum sensing in Gram positive bacteria regulates a number of physiological activities, such as competence development in *Streptococcus gordonii, S. pneumoniae,* and *S. mutans,* sporulation in *Bacillus subtilis,* antibiotic synthesis in *L. lactis,* and induction of virulence factors in *Staphylococcus aureus.* The bacterial communication via quorum sensing involves the production, release and detection of small signaling molecules namely: the auto-inducers (e.g. acylated homoserine lactones) or by processed oligo-peptides (e.g. competence stimulating peptide) [40, 128]. In the oral cavity auto-inducer 2 (AI-2), produced by pioneer species, have a tendency to alter the structure and composition of the developing dental plaque [129]. A recent research has proposed the involvement of bacterial signaling molecules in eliciting specific responses from host cells, thereby mounting the possibility of cross-communication between prokaryotic and eukaryotic cells [49]. The concentration of signaling molecules increases with cell growth and on reaching a
threshold density; a signaling cascade is induced leading to alteration in the expression of target genes thereby shifting the dynamics of bacterial population [40, 128].

Quorum signaling in Gram-positive bacteria can involve the activity of auto-inducers or processed oligopeptides, also known as pheromones. The pheromones, which are initially synthesized as inactive pro-peptides in the ribosome, are exported from the cell either by the general secretion system or by utilizing dedicated ABC transporters [130]. During the export event, the pro-peptides undergo proteolytic and maturation processes to generate the active and mature pheromone. Pheromones can be imported into the cytoplasm via peptide transporter complexes, where they can bind and directly modulate the activity of their cognate transcriptional regulators [130-132]. Alternatively, on reaching a threshold concentrations in the extracellular milieu, pheromones can be detected by transmembrane receptors of the two-component system signal transduction family (TCSTS) [128, 130]. TCSTS are typically composed of a membrane-bound histidine kinase and cytoplasmic response regulator [133-134]. On responding to a specific signal, the histidine kinase undergoes auto-phosphorylation at a conserved histidine-serine residue and then transfers the phosphoryl group to a conserved aspartate in the response regulator [135]. The resulting phosphorylation of the response regulator triggers a conformational change in its domain, prompting its ability to bind specific DNA promoter regions, thereby rendering transcriptional regulation of the target genes [135]. In S. mutans, 14 TCSTSs and an orphan response regulator have been identified until now [101]. Cross-talk between certain histidine kinases and their non-cognate response regulators have also been identified in this bacterium [136]. In S. mutans, the most extensively studied TCSTSs and their associated affected phenotypes are as follows: VicKR, which is involved in biofilm formation, sucrose-dependent adhesion, competence development, acid tolerance, oxidative and cell envelope stress, and bacteriocin production; CiaHR, which is involved in biofilm formation, sucrose-dependent adhesion, competence development, acid tolerance, calcium signaling, and mutacin production; LiaSR, which is involved in cell envelope stress, acid tolerance, osmotic stress, biofilm formation, and mutacin production; LevSR, which is involved in fructose metabolism and sugar transport; ScnKR, which is involved in acid tolerance and oxidative stress; orphan RR GcrR, which is involved in sucrose-dependent adhesion and acid tolerance; and ComDE, which is involved in genetic competence, biofilm formation, bacteriocin production,
and quorum sensing [109, 137-140]. An extensive literature review describing the individual properties of these TCSTTs can be found in Spatafora et al [139].

Although S. mutans produces and responds to AI-2, its significant role in quorum sensing in not been ascribed [141]. In S. mutans, two density dependent quorum sensing systems have so far been identified: one comprising the peptide pheromone competence stimulating peptide (CSP) and the ComDE TCSTS; and the other one consisting the double tryptophan containing signal peptide XIP (SigX-inducing peptide), regulated by an Rgg-like transcriptional regulator ComR [132, 142]. Both quorum sensing pathways convey their respective signals to the activation of the only alternative sigma factor SigX (also known as ComX) of S. mutans, which is therefore a master regulator of quorum sensing [143-145]. The two signaling pathways differ in many ways such as: the CSP peptide pheromone is sensed outside the cell by ComD HK, whereas XIP is sensed inside the cell after its internalization [146-147]; CSP is active only in peptide-rich media whereas XIP is active only in peptide-free chemically defined media (CDM) [148]; and CSP induces the expression of sigX only in a fraction of population while the majority remains un-induced and a small fraction undergoes cell death, while, stimulation via XIP confers 100% compliance in sigX activation [142, 149-150]. Despite of all these differences, the ultimate activation of sigX by either of the pathways modulates genetic competence in S. mutans [143-144].

1.2.4 Genetic competence

Natural genetic transformation is one of the processes of horizontal gene transfer observed in several bacteria, including streptococci. The genetic transformation has been reported to play a role in modulating genetic diversity, cell survival, biofilm formation, bacteriocin production and general stress response in several bacteria [151-152]. During the process of genetic transformation, the bacteria enter into a physiological state of genetic competence facilitating the acquisition of foreign DNA from the external environment. In some bacterial species, the occurrence of a competent state is constitutive, whereas, in others competence is tightly regulated and depends on specific factors or conditions, such as pheromones, nutrient availability and stress [151]. On sensing the specific inducible conditions and/or factors, the expression of
master regulator(s) is stimulated which leads to activation of the genes involved in DNA uptake and recombination [151].

All streptococcal species harbor the master regulator SigX and SigX-dependent effector genes, usually required for natural genetic transformation [153-154]. However, not all streptococcal species have been shown to be naturally competent [153-154]. *S. mutans* is a naturally competent bacterium, which utilizes at least two quorum signaling pathways in transmitting information from the secreted peptides CSP and XIP to the SigX, involved in development of competence for genetic transformation (Figure 1-2) [142, 147]. As described previously, the two quorum sensing pathways differ both in basic architecture and in mechanism of signaling. CSP, encoded by ComC as a 46-amino-acid pro-peptide, contains a leader sequence with a double-glycine motif [147]. During secretion through a dedicated ABC transporter, ComAB, the N-terminal leader peptide is cleaved off by the proteolytic activity of the transporter to generate a peptide that is 21 residues long (21-CSP) [155]. 21-CSP can either be degraded by HtrA proteases in a process affected by misfolded proteins or can undergo productive processing by SepM extracellular protease to generate a mature 18-CSP that binds to ComD leading to phosphorylation of ComE [156]. As described previously, on reaching a threshold density processed CSP is sensed by the ComD HK, leading to its auto-phosphorylation and thus activation of the response regulator ComE RR [143, 153]. In contrast, the peptide encoded by ComS is involved in *comR-comS* quorum sensing [132]. ComS encodes for a 17-amino-acid polypeptide, which is secreted and processed to mature XIP [132, 146]. Though the steps involved in XIP secretion and processing remain entirely unknown, a predicted mature form of ComS is proposed to be of 7 amino acids in length. Additionally, synthetic XIP consisting of the C-terminal seven amino acids of ComS induce a significant increase in *sigX* transcription when added to cells grown in CDM. XIP interacts with the Rgg-like transcriptional regulator ComR and collectively, ComR and XIP activate the expression of *sigX* and *comS*, thus creating an auto-induction loop in the signaling system [132]. The absence of *comR* or *comS* shuts down the competence machinery even when cells are stimulated by CSP, whereas the absence of *comE* does not have any effect on XIP induced *comX* activation [132, 148]. Therefore, the *comRS* pathway is not only necessary but sufficient in inducing the expression of SigX, involved in competence development.
Figure 1-2 Model of CSP- and XIP-induced quorum sensing in *S. mutans*.

The ComCDE quorum signaling is mostly devoted towards the activation of bacteriocins (antimicrobial peptides), whereas ComRS signaling system have a prominent role in competence development. The two signalling pathways converge on transcription of *sigX* transcriptional factor, where the ComRS complex acts as the proximal regulator of the *sigX* transcription. Figure is reproduced from Federle *et al*, 2012 with permission of the copyright owner [142].

The activation of *sigX* by either CSP-ComDE or ComRS sensing systems also depends on the components of the growth media: XIP is only active in CDM, devoid of any exogenous peptides, whereas CSP is active only in nutrient-rich media such as brain heart infusion (BHI) or typtone.
yeast extract (THYE) [148, 150]. Moreover, CDM medium facilitates a high frequency of natural transformation without exogenous supplementation of CSP or XIP, whereas genetic transformation in nutrient-rich media is strongly dependent on the addition of exogenous CSP. Another difference in the pathways of sigX induction via the two quorum sensing system lies in their mode of induction: the XIP heptapeptide induces sigX expression broadly across the population in a unimodal way, on the other hand CSP induces in a bimodal way where only a fraction of the cells acquires competence state [149-150, 157]. S. mutans growing in mixed biofilms with Candida albicans shows interesting patterns of regulation of the competence pathway [158], where, comS is not activated in single culture biofilms of S. mutans whereas in mixed biofilms the dramatic induction of comS triggers the activation of quorum sensing signaling cascade resulting in induced expression of comR and comX [158]. The study provides a novel mechanism of competence induction in mixed biofilms and holds relevance as C. albicans have been isolated from the dental caries lesions associated with S. mutans [158].

1.2.5 Metal homeostasis

Metals play an important role in modulating various cellular processes, thereby, ensuring optimal cell viability. The unique properties of metals allow them to perform a multitude of tasks that include: as structural component of bio-molecules, as signaling molecules, and as catalytic co-factors for proper functioning of various essential metabolic enzymes [159-160]. Metal requirement is closely monitored within the cell, as in the absence/deficiency of a particular metal ion a stress response is induced that can lead to altered cellular metabolism [161]. Similarly, at higher concentrations metals can be extremely destructive by affecting bacterial physiology such as: induction of oxidative stress, impairment of protein stability and function, structural damage of bio-molecules, etc [161-162]. In order to sustain cell viability, extensive regulatory and protein-coding machinery is devoted to maintain the metal homeostasis. Metal homeostasis is achieved by maintaining the intracellular metal concentration at an optimal bio-available concentration and is mediated by balancing efflux and intracellular trafficking/storage of metal ions (Figure 1-3).
Figure 1-3 Metal homeostasis model

Metals play an important role in modulating various cellular processes in all living systems. The unique properties of metals allow them to perform a multitude of tasks that include: as structural component of bio-molecules, as signaling molecules, and as catalytic co-factors for proper functioning of various essential metabolic enzymes. Required in trace amounts, metals can be extremely toxic at higher concentrations, therefore there trafficking is closely monitored across the cell membrane and within the cytoplasm.

In *S. mutans* divalent metal ions such as iron (Fe), manganese (Mn) and calcium (Ca) are known be required for its growth and survival [163-165]. Of these divalent cations, role of Mn in *S. mutans* physiology and its homeostasis has been well characterized. In bacteria, Mn functions as the co-factor for superoxide dismutase involved in dis-mutation of the toxic superoxide radicals[166], and for the enzymes required in lactic acid fermentation. *S. mutans* contains the *sloABCR* operon, which encodes an ATP-binding protein, an integral membrane protein, a solute-binding lipoprotein, and a metal-dependent regulator [164]. Together SloABC forms a transport system for the transport of both Fe and Mn, whereas the SloR metallo-regulator
represses the operon only in response to intracellular Mn [164]. SloR homologs are found in other bacterial species such as MntR in B. subtilis, IdeR in Mycobacterium tuberculosis, ScaR in S. gordonii. In S. mutans, SloR has been associated with several physiological activities such as cell adherence, biofilm formation, genetic competence, metal ion homeostasis, and oxidative stress tolerance [167]. Moreover, SloR-Mn regulates the expression of the orphan response regulator GcrR, which acts downstream of SloR to control ATR in S. mutans [140, 168]. GcrR also modulates sucrose-dependent adherence and aggregation by repressing the transcription of gbpC, gtfB and gtfC, genes critical for biofilm formation and virulence in S. mutans [122, 140]. Loss of GcrR results in reduced virulence and cariogenicity in S. mutans [140].

Another metal cation, calcium, has been shown to activate CiaHR TCSTS system involved in ATR in S. mutans [165]. In S. mutans, CiaHR is known to modulate acidogenesis, biofilm formation, and genetic transformation. The ciaHR is part of the ciaRHX operon, where ciaX encodes a Ca-sensing secreted peptide, which responds to Ca levels and allows the auto-regulation of CiaHRX system [79]. With these examples, it is clear that there is a close link between metal homeostasis and TCSTSs in S. mutans; detailed literature review on discussing the link between bacterial metal homeostasis and their cognate TCSTSs is presented in Chapter 2.

In this study we investigated the role of copper (Cu) on the physiology and virulence in S. mutans. Copper homeostasis has been extensively studied in Enterococcus hirae, L. lactis, and B. subtilis [169-172]. In all these bacterial systems, the genes involved in copper transport are usually located on the chromosome as copper transporting operon (cop operon). The knock-out mutants in cop operon become more prone to killing under copper induced stress, confirming their importance in copper resistance [169-172]. The best-understood copper transport and resistance system is that of E. hirae [173]. The copYABZ operon of E. hirae encodes two P-type ATPases, CopA, and CopB involved in copper translocation, and CopY and CopZ, which regulate the expression of the cop operon in response to both copper starvation and copper excess (Figure 1-4) [169]. Under normal conditions, zinc forms complex with CopY and binds to the inverted-repeat sequence, upstream of the copY gene and negatively regulates the expression of the co-transcribed genes in the cop operon. At high intracellular concentrations of copper ions, CopZ binds to copper and form Cu⁺- CopZ complex [174]. For the de-repression of the operon,
Cu\(^+\)-CopZ donates copper to CopY, thereby displacing the bound Zn\(^{2+}\) and releasing CopY from the DNA [169, 173, 175].

**Figure 1-4 The copYABZ operon of E. hirae**

Two P-type ATPases, CopA and CopB transport copper across the membrane and CopY and CopZ, regulate the expression of the cop operon in response to both copper starvation and copper excess. CopB was shown to catalyze the accumulation of copper and silver ions in native membrane vesicles of *E. hirae* in an energy dependent manner. These vesicles accumulate copper in ATP-dependent manner and extrude copper ions in whole cells. These ATPases are induced at high extracellular concentration of copper and silver. The regulation of these ATPases is accomplished by a negative repressor CopY and copper chaperone CopZ. Under normal conditions, CopY binds as a Zn(II)-CopY complex to the inverted repeat sequence upstream of the *copY* gene and thereby, negatively regulates the expression of the co-transcribed genes on the cop operon. At excessive concentrations of copper ions into the cell, CopZ binds to copper and form Cu(I)-CopZ complex. For the induction of the operon, Cu(I)-CopZ donates copper to CopY, thereby displacing the bound Zn(II) and releasing CopY from the DNA, resulting in the de-repression of the operon. Adapted from Solioz *et al* (2003) [169]
In dental plaque, copper alone or in combination with other antimicrobials have been linked with reduction in occurrence of dental caries [176-177]. Copper has an inhibitory effect on the growth of *S. mutans* [176-177]. In *S. mutans*, the absence of any putative or known cupro-enzyme signifies non-essentiality of copper in bacterial metabolism; however the presence of an entire copper resistance and transport operon, *copYZ*, suggests the primary function of the system in defending against Cu stress.

![Figure 1-5 Genetic organization of *S. mutans* *copYZ* operon](image)

The *copYZ* operon has been partially investigated in *S. mutans* (Figure 1-5) [178]. The *copYZ* is co-transcribed as a polycistronic operon, where the knock-out mutant in the entire operon becomes more sensitive to killing specifically under copper stress relative to the wild type strain [178]. Moreover, *copYZ* operon is specifically induced in the presence of copper and is negatively regulated by CopY [178]. Also, *copZ* has been shown to de-repress the activity of *cop* operon. Despite of all these findings, the function of this operon in copper transport (import and/or export of copper) and its contribution to other virulence features in *S. mutans* has not yet been established. Copper is utilized to maintain optimal cellular metabolism by several bacteria, however at higher concentrations it can be extremely toxic. Copper induces toxicity by targeting different processes or pathways in different bacteria. In our study we focused on investigating the effects of copper on different physiological activities of *S. mutans*. Copper was demonstrated
to induce oxidative stress, affect the survival of the cells under copper and acid stress, inhibit biofilm formation, disrupt membrane potential and reduce genetic transformation frequency in *S. mutans*. Our results also indicate that the *copYAZ* operon is not only involved in copper transport but also in protecting the cells under acid and oxidative stress, assisting in maintenance of cell membrane potential, and in modulation of biofilm formation and genetic transformation under copper stress.
An intimate link: two component signal transduction systems and metal transport systems in bacteria

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Future Microbiol 9(11), 1283-1293 (2014)
2.1 Abstract

Bacteria have evolved various strategies to contend with high concentrations of environmental heavy-metal ions for rapid, adaptive responses to maintain cell viability. Evidence gathered in the past two decades suggests that bacterial two component signal transduction systems (TCSTTs) are intimately involved in monitoring cation accumulation, and can regulate the expression of related metabolic and virulence genes to elicit adaptive responses to changes in the concentration of these ions. Using examples garnered from recent studies, we summarize the cross-regulatory relationships between metal ions and TCSTTs. We present evidence of how bacterial TCSTTs modulate metal ion homeostasis and also how metal ions, in turn, function to control the activities of these signaling systems linked with bacterial survival and virulence.

Keywords: Transition metal ion homoeostasis; two component signal transduction systems; gene regulation.

2.2 Introduction

Bacterial interactions with transition metal ions present a dual challenge: while many metal ions are biologically necessary at low levels, they can also be toxic at high concentrations. Bacteria use metal ions as co-factors for the function of several critical enzymes involved in electron transport and/or cell metabolism [179-182]. Accumulation of metal ions can impose deleterious effects on metabolic and cellular pathways thus compromising cell survival: different metal ions in the cytoplasm can tend to displace the metal co-factors at the active site(s) of enzymes ultimately leading to their inactivation [180, 183]. For instance, when cellular metal homeostasis is disrupted, metals at the upper end of the Irving-Williams series - Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II) - have the potential to displace enzymatic metal co-factors at the lower end, thus rendering the proteins inactive [183-184]. In other cases, metal ions can also affect cell growth and viability by disrupting the structure of nucleic acids, phospholipid membranes, and enzyme function [185-186]. Therefore, bacteria have developed complex
mechanisms to monitor cellular metal ion levels and simultaneously maintain the homeostasis of multiple cations within a cell [187-188]

Bacteria use various strategies to regulate heavy-metal homeostasis, which include the use of metal efflux pumps, channels, cation-specific metallo-regulatory proteins, small non-coding RNAs, and two component signal transduction systems (TCSTs) [180]. The intracellular import of metal ions is often facilitated by ATP-binding cassette transporters and Nramp transporters, whereas their export is usually carried out by cation-diffusion facilitators (CDFs) [189], P-type ATPases [190-191], and tripartite resistance-nodulation-cell division (RND) transporters [192]. The regulation of metal trafficking proteins or their encoding genes is usually modulated by TCSTs or metallo-regulatory proteins. Bacterial TCSTs are comprised of a membrane-bound histidine kinase (HK) and an intracellular cognate response regulator (RR) protein [133]. Upon reaching an appropriate threshold signal, the bacterial HK undergoes auto-phosphorylation, which in turn, transfers the phosphate to its cognate RR protein. Once phosphorylated, the RR undergoes a conformational change which alters its binding affinity to specific sequences in the promoter/operator regions of its target genes [135]. As a result, the RR can activate or inhibit the transcription of target genes required for an adaptive response. Another group of regulatory proteins include cytoplasmic metalloregulators, which unlike TCSTs are comprised of a single protein that can perform dual functions of sensing and responding to metal ions [18-20]. In fact, these proteins are specialized allosteric proteins that can directly bind to a specific or a small number of cognate metal ions [193-195]. Upon binding, the protein undergoes a conformational change in the regulatory region allowing it to control the transcription of target genes [195]. The products encoded by these genes can have multiple functions and may include proteins involved in growth, stress tolerance, virulence and metal trafficking within or between cellular compartments [18-20]. The function, mechanism and ion specificity of metallo-regulators has been previously reviewed by others [193-195]. TCSTs or cytoplasmic metallo-regulators can exist together in bacteria. While they are capable of independent activation and regulation [18-20], intracellular cross-talk between these signaling systems has been noted in some species [36-37].

Of transition metal ions, the homeostasis of iron (Fe) has been widely investigated and extensively reviewed [196-201]. In this review, we will focus on the relationship between the
activity of bacterial TCSTs and homeostasis of Copper (Cu), Manganese (Mn), Zinc (Zn), Nickel (Ni), Silver (Ag), Cobalt (Co) and Cadmium (Cd).

### 2.3 Copper homeostasis

Cu is an important transition metal required for the growth of most living organisms. Owing to its redox properties, it has a vital role in maintaining biological processes important for cell viability. Cu exists in two oxidation states, Cu\(^+\) and Cu\(^{2+}\), and is utilized by an array of metallo-enzymes to catalyze electron transfer reactions. In bacteria, Cu functions as a cofactor for over 30 enzymes, such as superoxide dismutase, cytochrome c oxidase or lysyl oxidase [171, 202]. Although required at lower concentrations, accumulation of excess Cu within the cell under aerobic conditions can catalyze the production of hydroxyl radicals via Fenton and Haber-Weiss reactions. The production of reactive oxygen species (ROS) in the presence of Cu is believed to cause cellular damage by reacting with a number of cellular macromolecules such as lipids, proteins, and nucleic acids [203]. However, there is little or no direct evidence that shows that Cu-induced ROS generation is the major cause of Cu toxicity. Alternative mechanisms of Cu toxicity in *E. coli* have been suggested where Cu, instead of being involved in oxidative DNA damage, was shown to suppress Fe-mediated oxidative damage [204]. In *E. coli* growth inhibition due to Cu could be reversed by the addition of branched chain amino acids [204]. The authors proposed that Cu can block the synthesis of branched chain amino acids by targeting the Fe-S cluster containing dehydratases, where Cu replaces the Fe, rendering them inactive and blocking their activity [204]. However, a similar mechanism was not observed in *Salmonella*, where the addition of exogenous branched chain amino acids didn't revert the Cu-mediated growth inhibition [30]. It was suggested that Cu might have different targets than Fe-S clusters or the bacterium might have evolved multi-factorial mechanisms to contend with Cu-induced toxicity [205]. In another study, it was shown that the *E. coli* Cu efflux system CusCFBA (described below) was induced during anaerobic amino acid limiting conditions to protect Fe-S cluster enzymes from endogenous Cu toxicity [206]. Challenges posed by Cu necessitate the activity of complex regulatory networks to maintain Cu homeostasis in the cell. The evolution of
To contend with Cu toxicity, bacteria utilize at least one of three principal mechanisms. These include, a) Cu export across the plasma membrane into the periplasmic space or the extracellular environment, b) extracellular and/or intracellular Cu sequestration via Cu binding proteins, c) Cu oxidation to a less toxic Cu$^{2+}$ state [207]. The mechanisms to maintain Cu homeostasis have been extensively studied in several bacteria including *E. coli*, *Pseudomonas*, *Xanthomonas*, *Enterococcus* and *Bacillus* [208]. In Gram negative bacteria, excess Cu is either accumulated in the periplasm or is exported out of the cells. In these organisms, the genes contributing to Cu homeostasis are either located on the chromosome or are plasmid born. In many Enterobacteriaceae, such as *E. coli*, Cu stress tolerance is modulated by chromosomal regulons designated *cue* (Cu efflux) and *cus* (Cu sensing), as well as by plasmid-born machinery such as the *pco* system [209]. *E. coli* utilizes cytoplasmic MerR-type regulator CueR, which in concert with the CusRS TCSTS, regulate the expression of target genes involved in Cu homeostasis [209-211]. Under aerobic conditions, CueR is activated by elevated intracellular Cu concentrations, which can then directly bind the CueR box in the promoter region of *copA* and *cueO* encoding a P-type ATPase and an oxygen-dependent multi-copper oxidase, respectively [209, 211]. The CopA protein helps export excess Cu$^{+}$ from the cytoplasm into the periplasm, where it is oxidized to the less toxic Cu$^{2+}$ form with the aid of CueO [212]. Under anaerobic conditions, the CusRS TCSTS maintains Cu homeostasis, wherein the CusS sensor kinase is activated by a threshold periplasmic Cu concentration, which then transfers this signal via a phopho-relay event to its cognate responder protein CusR [211]. As a result, CusR regulates transcription of the *cusRS* operon, as well as the adjacent, but divergently oriented *cusCFBA* operon by directly binding to the CusR box (AAAATGACAANNTTGTCATTTT) between the *cusC* and *cusR* promoters [213]. The CusCBA (a proton-cation antiporter) and CusF (a Cu chaperone) proteins have also been demonstrated to aid in Cu stress tolerance [214]. Interestingly, no other sequence in the entire genome of *E. coli* was found to contain a CusR box suggesting it binds specifically and only to the intergenic region between the *cusRS* and *cusCFBA* operons [213]. In *Coryn bacterium glutamicum*, the *E. coli* homologue of CusRS was identified and characterized as *copRS* TCSTS. CopRS senses extra-cytoplasmic levels of Cu$^{+}$
concentrations, and induces the set of genes involved in Cu homeostasis and resistance [215]. Like in *E. coli*, CopR specifically binds and regulates the expression of two divergently oriented operons cg3286-cg3289 and *copR*-cg3281, which encodes for Cu resistance proteins (e.g. putative multicopper oxidase CopO and the putative copper export ATPase CopB) and CopRS TCSTS respectively [215].

In cyanobacterium *Synechocystis* sp. PCC 6803, CopRS two-component system is known to be essential for copper resistance [216]. CopS was shown to have a high affinity to bind Cu in vitro, moreover its localization both in the plasma and thylakoid membrane suggested that CopS can possibly bind and respond to Cu both in the periplasmic and thylakoid lumen in *Synechocystis* [216]. However, further studies are warranted to completely understand the mechanism and conditions involved in Cu binding to CopS under natural conditions. In the presence of Cu, CopR directly binds and regulates the expression of both *copBAC*, the putative heavy-metal efflux-RND copper efflux system and its own locus (*copMRS* operon) [216].

In *E. coli* two other TCSTSs, CpxAR and YedVW, were shown to be induced in the presence of Cu [213]. CpxAR is involved in membrane stress tolerance; it activates the transcription of CpxP protease for degradation of denatured proteins. Furthermore, a CpxR binding-site, a conserved tandem repeat pentanucleotide sequence GTAAA(N)(4-8)GTAAA, was identified in the promoter region of several copper-inducible genes [217]. Knockout mutants of CpxAR were more sensitive to killing under Cu stress suggesting the role of this TCSTS in copper homeostasis in *E. coli* [217]. The function of YedVW is not well characterized; however, YedV kinase has been shown to transfer phosphate not only to YedW but also CusR. The YedVW was not induced by Cu in a CusR-deficient mutant suggesting the induction of YedVW system in a CusR-dependent manner [213]. Although the function of YedVW in Cu homeostasis is not yet characterized, its induction likely results from cross-talk between the CusRS and YedVW systems in the presence of Cu [213].

In *Helicobacter pylori*, a major colonizer of the human gastric mucosa, Cu is imported in a non-specific manner [218]. Cu ions accumulated in the periplasm are detoxified via the metal export system, *crdAB* and *czcBA*, orthologous to that of the *E. coli* four-component Cu export system CusCFBA and is directly controlled by the CrdRS TCSTS [219]. The expression of *crdA*,
encoding the protein most highly expressed under copper stress is directly controlled by the CrdRS TCSTS [219], where CrdR was shown to bind the operator region of the crdA promoter. Despite their speculations, the authors did not demonstrate that CrdS can directly sense Cu from the surrounding environment. *H. pylori* mutants' deficient in CrdS and CrdR cannot colonize the mouse stomach, indicating a crucial role for Cu homeostasis in the adaptation to the gastric environment [219].

In addition to the utility of chromosomal Cu resistance genes, *E. coli* also utilizes a conjugative plasmid harboring the pco gene cluster comprised of *pcoABCDRSE* to contend with Cu stress [220]. These encode the multi-Cu oxidase, PcoA, the outer membrane protein, PcoB, the periplasmic proteins, PcoC and PcoE, and the inner membrane protein PcoD. The *pco* operon is Cu-inducible and is regulated by a specific *pCoRS* TCSTS encoded by the same operon [220]. Although *cusRS* and *pcoRS* encode homologous Cu-responsive regulatory systems, they do not substitute for one another. The CusRS and PcoRS have different sensitivities or induction profiles to varying Cu concentrations and are comprise of independent copper-responsive regulatory systems in this bacterium [220].

Similar plasmid-encoded regulation of Cu resistance operon, *copABCDRS*, is observed in *Pseudomonas syringae* and is regulated by the corresponding *copRS* TCSTS [221]. CopABCD functions in the binding of copper, while CopR and CopS form a TCSTS system that regulates the expression of *copABCD* [221-223]. In *P. syringae* multi copper oxidase *copA* and outer membrane protein *copB* are essential to fundamental copper resistance whereas periplasmic proteins, *copC* and *copD* are required for full Cu resistance [221-223]. *In vitro* studies suggested the direct binding of CopR to the cop box (a conserved CopR binding region) in the promoter region of the Cu resistance operon [224]. In *Pseudomonas fluorescens* strain TSS, homologous to plasmid encoded cop operon however chromosomally located, Cu resistance genes *copRSCD* but not *copAB* have been identified [225]. The expression of *copCD* is directly regulated by the two component responder protein CopR, which auto-regulates its expression in response to Cu [225-226]. In addition to Cu resistance, CopRS modulates bacterial growth, biofilm formation, and tissue dissemination, thereby suggesting its involvement in the regulation of bacterial virulence determinants in *P. fluorescens* [225].
2.4 A link between Copper and Silver homeostasis

In the periodic table Cu and Ag both belong to Group 11, of the transition metal elements. Similar to Cu, Ag has an antimicrobial effect on bacteria and is widely incorporated into products in medical equipment and public environments [227]. Silver cations (Ag\(^{+}\)) are bactericidal at low concentrations and are used to treat burns, wounds and ulcers. In addition, silver catheters and silver-coated catheters are used for their inhibitory role in microbial growth and biofilm formation [227]. Silver nanoparticles are effective not only against bacterial infections, but also are active against several types of viruses including the human immunodeficiency virus, hepatitis B virus, herpes simplex virus, respiratory syncytial virus, and monkey pox virus [228]. Because of similar chemical properties of Cu and Ag, bacteria utilize a similar set of genes to cope with their homeostasis and resistance. In *E. coli*, the central role of CusS has been associated with modulating transcription of genes involved in Cu and Ag efflux [210]. CusS is activated in a metal-dependent manner and up-regulates the expression of *cusCFBA* that is required for Cu and Ag resistance. In a clinically isolated Ag resistant strain of *Salmonella*, metal ion resistance was conferred by the plasmid pMGH100, which has nine genes required for Ag resistance that are arranged as three transcriptional units [227]. Genes present within these units encode the SilRS TCSTS, a periplasmic Ag\(^{+}\)-binding protein (SilE), an Ag\(^{+}\) efflux pump (a P-type ATPase SilP), and a membrane potential-dependent three-polypeptide cation/proton antiporter (SilCBA). The function of SilP in transporting cytoplasmic Ag directly into the periplasm or across the outer membrane is yet not investigated. However, SilABC forms the three polypeptide RND protein which removes Ag from the cytoplasmic region directly to the outside of the cell without its release into the periplasmic space. The silver dependent activation of the SilE, SilP and SilCBA is regulated by the SilRS system [227].

2.5 Manganese homeostasis

Mn has a vital function in regulation of bacterial metabolism, since it acts as a cofactor for metabolic enzymes such as superoxide dismutase, catalase, pyruvate carboxylyase, and phosphoenolpyruvate carboxykinase[229-230]. Mn is essential for detoxification of ROS thereby
protecting the cells against oxidative stress, also recently, low molecular weight Mn complexes have been shown to reduce oxidative tissue injury and ROS in \textit{in vitro} and \textit{in vivo} biological systems [166, 231]. \textit{B. subtilis} utilizes Mn during different stages of its developmental cycle and particularly for efficient sporulation [232].

In \textit{Pseudomonas putida}, a Mn$^{2+}$-oxidizing bacterium [233], the MnxSR two-component regulatory system is comprised of two sensor kinases, MnxS1 and MnxS2, as well as one responder protein, MnxR. Together MnxS1, MnxS2 and MnxR have a central role in regulating Mn$^{2+}$ oxidation [234]. The oxidation of Mn$^{2+}$ to Mn$^{3+}$ and Mn$^{4+}$ is assumed thermodynamically favorable as bacteria may derive energy from this reaction. The MnxR regulates Mn$^{2+}$ oxidation; however, in order to be activated, MnxR requires signaling from both cognate sensor kinases since deletion of MnxS1 or MnxS2 results in complete loss of its ability to oxidize Mn$^{2+}$ [234].

Mn together with Ca$^{2+}$ and Cl$^{-}$ act as a catalytic center for the oxygen-evolving photosynthetic machinery in higher plants and cyanobacteria [235]. Cyanobacterial cells take up Mn$^{2+}$ ions using an ABC-type transporter encoded by the mntCAB operon: mntA encodes an ATP binding subunit, mntB encodes a gene for its hydrophobic subunit, and the mntC encodes a Mn$^{2+}$ binding subunit [236]. Two different studies in \textit{Synechocystis} identified a TCSTS comprised of ManS and ManR involved in sensing and regulating the mntCAB operon, respectively [237-238]. At high Mn$^{2+}$ concentrations, cell membrane localized ManS is speculated to bind Mn$^{2+}$ and convey the signal to its cognate responder protein, ManR. Once activated, ManR can repress the transcription of the mntCAB operon thus inhibiting Mn uptake. On the other hand, under low Mn$^{2+}$ concentrations, ManS is speculated not to bind Mn$^{2+}$ ions and thus, ManR remains inactive resulting in de-repression of the mntCAB operon, facilitating uptake of Mn [237-238].

The effect of Mn in activating and regulating the expression of two component signaling systems has been observed in several organisms [239-242]. In \textit{Streptomyces reticuli}, a soil microbe, the SenS/SenR system modulates the expression \textit{cpeB}, which encodes a Mn-dependent peroxidase involved in protecting the cells under oxidative stress [240]. In \textit{M. tuberculosis}, Mn and Ca are known to activate the TrcRS TCSTS [243]. The TrcRS TCSTS suppresses the expression of \textit{rv1057}, which encodes the only seven-bladed \(\beta\)-propeller protein required for the structural integrity of the cell envelope of \textit{M. tuberculosis} and might be a component of the mycobacterial
envelope [242]. Though not much has been published regarding \textit{rv1057}, studies suggest that the Rv1057 protein may function similar to TolB, a β-propeller protein that interacts with peptidoglycan-associated proteins and maintains envelope integrity in Gram negative bacteria [244]. In \textit{E. coli}, Mn-containing serine/threonine protein phosphatases, PrpA and PrpB regulate the CpxAB TCSTS and the periplasmic protease HtrA/DegP, which help contend with environmental stress tolerance [239, 241].

In \textit{Streptococcus mutans}, which is one of the primary causative agents of human dental caries [245], the \textit{sloABCR} operon encodes components of a putative metal uptake system. These genes encode an ATP-binding protein, an integral membrane protein, a solute-binding lipoprotein, and a metal-dependent regulator, respectively [164]. The SloABC transport both Fe and Mn, although the SloR represses this system only in response to Mn [164]. The SloR in \textit{S. mutans} is a one component cytoplasmic metal dependent regulator, which modulates several physiological functions that include cell adherence, biofilm formation, genetic competence, metal ion homeostasis, oxidative stress tolerance, and antibiotic gene regulation [167]. In this organism, SloR-Mn regulates the expression of an orphan responder protein designated GcrR, which acts downstream to tolerate acid stress, an important virulence factor of this cariogenic pathogen [140, 168]. The interaction between the metalloregulatory protein SloR and RR GcrR provides an excellent example of cross-talk between one component and two component metal regulatory systems in bacteria. In \textit{S. mutans}, acid tolerance is known to be mediated by several TCSTSs including the CiaHR system that is activated by Ca\textsuperscript{2+} [165]. This system also regulates multiple virulence phenotypes including the ability to produce acid, form biofilm and develop genetic competence [165]. The \textit{ciaRH} is part of the \textit{ciaRHX} operon, where \textit{ciaX} encodes a calcium sensing signaling peptide that allows the CiaRH system to modulate its operon own expression in response to this cation [165].

\section{2.6 Cadmium Zinc Cobalt Homeostasis}

Bacteria utilize Zn as an enzymatic co-factor and for the integrity of structural elements of the cell. Like other heavy metals, at high concentrations Zn can be highly toxic to the cell.
Therefore, intracellular levels of Zn are often tightly regulated by an extensive network of transporters, ligands and transcription factors. In *E. coli*, Zn detoxification is primarily achieved by exporters such as ZntA, a P-type ATPase, ZntB, a cation diffusion facilitator and periplasmic proteins such as Spy or ZraP [246].

In *E. coli*, the BaeSR TCSTS is one of three extra-cytoplasmic stress response (ESR) systems that helps elicit adaptive responses to changing environmental conditions [247]. On sensing transient environments (e.g., stresses posed by indole, tannin, flavonoids, sodium tungstate, or high levels of metal cations), the BaeS is activated and transduces its signal by catalyzing phosphorylation of the transcription factor BaeR [247]. In *E. coli* and *Salmonella* BaeR activates the expression of its own operon as well as genes involved in responses to contend with envelope stress, drug resistance, and metal resistance [248-250]. In *E. coli*, the BaeSR TCSTS can sense Fe and Zn in the surrounding environment, and in turn, regulate the expression of genes involved in the formation and modification of membrane structure and function [251]. The BaeSR together with the CpxAR TCSTS co-regulate the ZraP and Spy periplasmic chaperones required for envelope stress sensing [252]. Though the function of the periplasmic chaperone, Spy, in Zn detoxification is not known, its induction in the presence of Zn and impaired growth of the knock out mutant in *spy* under Zn stress suggest that it might play a role in Zn detoxification probably by facilitating the folding of the transmembrane or periplasmic transporters involved in Zn export [248]. In *S. typhimurium*, BaeSR was shown to be involved in regulating the expression of multidrug efflux pumps and providing resistance against Cu and Zn toxicity [253].

In *E. coli* another Zn-responsive TCSTS, HydHG TCSTS system, when under excess Zn concentrations, modulates the expression of the periplasmic Zn-binding protein ZraP to maintain Zn homeostasis [254]. Similarly in *S. typhimurium*, ZraSR (HydHG), have been shown to modulate the expression of periplasmic protein, ZraP required for regulation of the ZntA transporter of Zn [255]. The periplasmic protein and Zn-dependent chaperone ZraP has an important role in maintaining envelope homeostasis and contributes to the regulation of response regulator *zraR* of the ZraSR TCSTS [255]. In another study, it was shown that while ZraP and ZraSR can be induced by indole. In the *zraP* knock out mutant a significant reduction in *zraR* expression was observed even in the presence of indole [255]. Although it was suggested that ZraP regulated the expression of the ZraSR TCSTS, the fact that both ZraP and ZraSR are
induced by indole is noteworthy; since ZraP is a periplasmic chaperone, there is a possibility that it might act as a signal transducer for indole, wherein the absence of this protein, the signal for ZraR activation is not conveyed resulting in its low expression levels [255]. The same group further showed that regulatory cross-talk occurs between the ZraPSR and BaeSR systems, wherein the loss of BaeR led to the induction of ZraPSR [255].

In *P. aeruginosa*, the CzcRS TCSTS regulates the expression of CzcCBA efflux system, which confers specific resistance against Zn, Cd, and Co cations [256]. CzcA functions as a cation-proton antiporter, and CzcB acts as a cation binding subunit. Both CzcA and CzcB proteins form a membrane bound protein complex which can catalyze an energy-dependent efflux of Zn, Co, and Cd ions [257]. The CzcC provides substrate specificity towards Co and Cd [257]. In the presence of Zn, Cd or Co, the metal-inducible TCSTS, CzcRS is activated, and in turn, induces the expression of *czcCBA* encoding a metal efflux pump [256]. Further, the CzcRS is involved in cross-talk with the CopRS TCSTS, wherein the CopR regulator required for Cu homeostasis, links Cu resistance to Zn tolerance by activating the *czcRS* operon [258]. In *P. aeruginosa*, a putative *cop* box was identified between *czcC* and the *czcR* regulatory gene suggesting that CopR can bind to this region and regulate the transcription of the *czcRS* and *czcCBA* operons [259].

## 2.7 Nickel Homeostasis

Ni is required as a structural component of metallo-enzymes [260-261]. It is also needed to form a part of the active site of a number of enzymes including peptide deformylase, reductase and ureases, as well as some superoxide dismutases and hydrogenases [260-261]. Like other transition metal ions, at higher concentrations, Ni cations can confer harmful effects such as the generation of free radicals, inhibition of enzyme activity, contribution to DNA damage, developmental defects and cancer [262-263]. *E. coli* utilize Ni to survive under anaerobic growth. In fact, high levels of Ni are used for the optimal activity of Ni/Fe hydrogenases, enzymes involved in H\textsubscript{2} oxidation [264-265]. The NikR, transcription factor has been characterized in various bacteria, and is known to repress or activate specific genes in response to Ni availability [266].
Microbial Ni uptake is either accomplished by non-specific transport systems for divalent cations or by high affinity Ni-specific systems [267-268]. In *E. coli*, an ATP-binding cassette transporter, designated as NikABCDE, serves as the main importer for Ni ions. NikABCDE is comprised of NikA, a periplasmic Ni-binding and Ni-sensing protein; NikB and NikC integral inner membrane proteins; as well as NikD and NikE membrane-associated ATPase proteins [266, 269]. The nikABCDE operon responds to the presence of intracellular Ni, oxygen tension and nitrate availability. The transcription of the nikABCDE operon is positively regulated by the fumarate nitrate regulatory protein (FNR) transcription factor in the absence of oxygen [270] and is negatively regulated by the NikR repressor in the presence of high concentrations of Ni ions [271-272]. Another major transcriptional regulator identified for the nikABCDE operon is the nitrate responsive NarLX two-component system [273]. In the presence of nitrate, NarX HK phosphorylates the cognate RR NarL, which represses the expression of the nikABCDE operon by binding to a distinct operator site from that of NikR [273]. The expression of the operon is tightly regulated by the mechanisms described depending on the presence of stressors involved [266].

In *Synechocystis* species, the nrsBACD operon encodes proteins required for Ni resistance [274]. NrsB and NrsA are homologues of the *P. aeruginosa* CzcB and CzcA proteins, respectively [274]. Together the NrsA and NrsB, form a membrane-bound protein complex that can catalyze Ni efflux by a proton/cation antiport system. The role of NrsC in Ni export is unknown. NrsD, a membrane protein, confers resistance to Ni and also acts as a Ni exporter [274]. Upstream of this operon and in reverse orientation, the NrsRS TCSTS can sense and respond to Ni. Once activated, it modulates expression of the nrsBACD operon, whose products regulate Ni homeostasis [274]. When Ni accumulates in the periplasm, the nrsBACD operon is activated under the control of NrsSR TCSTS; the accumulation of Ni in the cytosol is sensed by InsR (internal nickel-responsive sensor) [275]. InsR, a CsoR/RcnR like metallo-regulator, binds directly at the cryptic transcription start sites within the nrs operon which enables independent repression of nrsD (of other nrs genes) in response to cytosolic Ni ions [275]. In the mutant deficient in inrS, as a result of Ni-dependent derepression of nrsD, an increase in export of Ni from the cytosol to periplasm was observed resulting in enhanced Ni resistance and reduced cytosolic Ni accumulation [275]. The regulation of the nrsBACD operon either by cytoplasmic
metallo regulatory protein or membrane bound TCSTS occurs depending on the surplus accumulation of Ni within the cytosol or periplasm respectively [275]. However, whether the regulatory pathways involve any crosstalk for efficient homeostasis remains a question [275].

2.8 Future perspectives

Despite our enhanced understanding of the role of TCSTSSs in bacterial physiology, more studies are required to dissect the direct mechanisms underlying these processes. For instance, the nature of cation-induced activation (i.e. direct or indirect stimulation of the sensor kinases) remains an area of research that deserves greater attention. As discussed earlier, in *Synechocystis*, in vitro studies have shown direct binding and activation of the HK by Cu cations [216]. Such studies provide groundwork for more extensive research to understand the bio-physical interactions between the TCSTS and metal cations. Emerging research in the field of bacterial metallo-regulation reveals the conservation and pairing of TCSTSSs with corresponding cation uptake/efflux systems. Recently, upon analyzing the phylogeny of responder proteins belonging to TCSTSSs, patterns of orthology/paralogy between Cu, Cd, Zn, and Co efflux proteins, as well as among their regulatory proteins (e.g., CopR, CzcR, CopS, and CzcS) was discovered [276]. Further, comparative analyses of three-dimensional structures has confirmed a common evolutionary origin for these regulatory proteins [276]. Hence, the shared mechanism of activation or function of these systems can be exploited to develop novel therapies against bacterial infections. Bacteria deprived of the genes involved in metal homeostasis or those tied to their regulation tend to have diminished virulence relative to their parent strains. Since metal-ion homeostasis and TCSTSSs are closely coupled to bacterial survival and virulence mechanisms, understanding the molecular mechanisms underlying these processes can have significant clinical implications in curbing bacterial virulence.
2.9 **Executive summary**

- Transition metal-ion homeostasis is important for bacterial survival. While these cations can be detrimental at high concentrations, they can sometimes serve essential functions in maintaining optimal physiology of these organisms.

- Of cellular components that are involved in metal homeostasis, bacterial TCSTSs have intimate and complex roles in responding, as well as in eliciting cellular responses to metal cation stress.

- Cytoplasmic metallo-regulators differ from the TCSTSs, as the former are comprised of a single protein that can perform dual functions of sensing and responding to metal ions whereas latter utilizes two different proteins to perform these functions.

- Bacterial TCSTSs accomplish metal-ion homeostasis by not only regulating metal export, but also by modulating the production of proteins associated with metal detoxification.

- Many RRs can directly bind to the promoter regions of their target genes whose products modulate metal trafficking and homeostasis (summarized in Table 1); however sensing and activation of their cognate HKs by metal cations is still an area which demands more investigative work.

- Bacterial metallo-regulation reveals the conservation and pairing of TCSTSs with their corresponding cation uptake/efflux systems.
<table>
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<th>TCSTS</th>
<th>Organism</th>
<th>Operon regulated</th>
<th>RR directly binding to operon</th>
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</table>
3 Statement of the problem

It is established that metal ions play an important role in bacterial cellular processes to ensure optimal cell viability at all times. The unique properties of metals allow them to function as structural component of bio-molecules, signalling molecules, and catalytic co-factors of various essential metabolic enzymes [159-160]. Bacteria are known to have specialized mechanisms to counter the perturbations in the intracellular metal homeostasis. The unwanted metal imbalance can have deleterious effects on bacterial physiology such as: induction of oxidative stress, impairment of protein stability and function, or structural damage of bio-molecules, etc [161-162].

Most bacteria require copper as an essential co-factor for metabolic enzymes, although there are others where copper is not required for any of their metabolic activity [277]. Copper exists in two oxidation states: cuprous (Cu⁺) and cupric (Cu²⁺) forms; the interchange between these states via Fenton reactions can lead to the generation of reactive oxygen species (ROS), especially superoxide radicals which are toxic to the cells [208]. The production of ROS causes major damage to nucleic acids, proteins or lipids, thereby compromising cell viability [203]. However, some reports indicate the involvement of non-redox mechanisms of copper toxicity [204, 206, 278]. One such example is copper displacing the iron cation from Fe-S clusters of the dehydratases enzymes thereby, rendering them inactive [206, 278]. Therefore, mechanisms involved in copper homeostasis can have different cellular targets among different bacterial species.

Copper has been linked to the inhibition of biofilm formation and detachment in S. gordonii [279]. Previous studies have shown that in S. mutans the addition of copper modulates the expression of gtfD both at the transcriptional and translational levels [280]. In S. mutans, in two different microarray studies conducted in our lab showed that the expression of the copYAZ genes was modulated in the presence of acid or CSP [109, 157]. In S. mutans strain GS-5, copper was shown to irreversibly inhibit the activity of F-ATPases, thereby compromising the cell's ability to carry out glycolysis in acidic environments [281]. The mechanism by which copper renders toxicity in S. mutans has yet not been fully discovered.
3.1 Rationale and objectives

*S. mutans* is known to contribute in initiation and progression of dental caries- a chronic transmissible bacterial infection. Since its post-genome sequence era, the research on and understanding *S. mutans*' physiology and identifying its virulence attributes has vastly expanded, accredited to the recent advances in the field of genomics, proteomics and transcriptomics. The three established virulence attributes of *S. mutans* include: acidogenicity, aciduricity and biofilm formation.

*S. mutans* genome encodes the *copYAZ* operon, a copper transport and resistance system. The structure analysis for the *S. mutans* CopYAZ was performed using Phyre-2 structure prediction site (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The *S. mutans* *copA* encodes for a 742 amino acids (a.a) long predicted protein belonging to the P-type ATPases class of metal exporters. The CopA product is predicted to contain 7 trans-membrane domains with an intracellular N- and an extracellular C-terminal. The *copY* encodes translated product of 147 a.a residues and is predicted to be a negative transcriptional regulator of *copA*, harboring winged helix DNA-binding domain. The *copZ* encodes for a 67 a.a residues putative copper chaperone and harbors a metal binding domain. Using BLASTP, the percentage sequence similarity analysis of *S. mutans* *copA* encoded protein with other bacterial Cop proteins showed high sequence similarity with: *E. hirae* CopA (96%), *E. hirae* CopB (86%), *Staphylococcus aureus* CopB (91%), *S. aureus* CopA (81%), *Enterococcus faecalis* CopA (99%), *S. thermophilus* copper P-type ATPase (100%), *S. salivarius* CopA (100%). Moreover, the gene expression analysis revealed an induction of the *copYAZ* operon under copper stress in *S. mutans*. With these preliminary results we postulated the presence of a functional *copYAZ* operon in *S. mutans*, which resembles other functional bacterial copper transporters in copper efflux, and in protecting the cell under copper and/or other cellular stressors.

Copper is toxic to *S. mutans*, however, the pathways affected by copper warrants further investigation. *S. mutans* genome does not appear to have any intracellular copper requirement, either in cupro-proteins (copper-containing proteins) or as enzymatic co-factors; therefore implying the major function of copper homeostatic systems is protecting the cells against copper stress [170, 277]. Although copper resistance and the transport system *copYAZ* have been
partially characterized in *S. mutans*, the role of this operon in bacterial virulence is not yet characterized. Based on above observations we presented the following hypothesis.

### 3.2 General hypothesis

In *S. mutans*, an imbalance in copper homeostasis induces toxic effects on its main features such as biofilm formation, competence and stress tolerance. The CopYAZ system is required for copper homeostasis and for the modulation of virulence attributes in *S. mutans*.

### 3.3 Objectives and aims

Given that biofilm formation and stress tolerance are vital to the virulence of *S. mutans*, understanding the contribution of copper to these processes remains the question warranting further research. In an attempt to elucidate the physiological and molecular mechanisms underlying copper response in *S. mutans*, the goal of this dissertation is to investigate the following specific aims:

**Specific Aim 1:** Determine the effects of copper on biofilm formation, genetic competence and stress response in *S. mutans*.

**Specific Aim 2:** Understand the role of the *copYAZ* operon in copper transport and stress tolerance in *S. mutans*.

**Specific Aim 3:** Determine the underlying molecular mechanism by which copper modulates genetic competence and biofilm formation in *S. mutans*. 
4 The copYAZ operon functions in copper efflux, biofilm formation, genetic transformation and stress tolerance in *Streptococcus mutans*

Running Title: *Streptococcus mutans* copper transport system

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Epub 2015 May 26
4.1 Abstract

In bacteria, copper homeostasis is closely monitored to ensure proper cellular functions, while avoiding cell damage. Most Gram positive bacteria utilize the copYABZ operon for copper homeostasis, where copA and copB encode for copper transporting P-type ATPases, whereas copY and copZ regulate the expression of the cop operon. Streptococcus mutans is a biofilm forming oral pathogen that harbours a putative copper transporting copYAZ operon. Here, we characterized the role of copYAZ operon in the physiology of S. mutans and delineated the mechanisms of copper-induced toxicity in this bacterium. We observed that copper induced toxicity in S. mutans by generating oxidative stress and disrupting its membrane potential. The deletion mutant of the copYAZ operon in S. mutans UA159 strain, designated ΔcopYAZ, resulted in reduced cell viability under copper-, acid- and oxidative-stress relative to the wild type. Furthermore, the ability of S. mutans to form biofilms and develop genetic competence was impaired under copper stress. Briefly, copper stress significantly reduced cell adherence and total biofilm biomass, concomitantly repressing the transcription of the gtfB, gtfC, gtfD, ghpB, and ghpC genes, whose products have a role in maintaining the structural and/or functional integrity of the S. mutans biofilm. Further, supplementation with copper or loss of the copYAZ resulted in a significant reduction in transformability and transcription of competence- associated genes. Copper transport assays revealed that the ΔcopYAZ strain accrued significantly high amounts of intracellular copper compared with the wild-type strain, thereby, demonstrating a role for CopYAZ in copper efflux of S. mutans. The complementation of CopYAZ system restored copper expulsion, membrane potential, and stress tolerance in copYAZ-null mutant. Taken collectively, we have established the function of the S. mutans CopYAZ system in copper export, and have further expanded the importance of copper homeostasis and the CopYAZ system in modulating streptococcal physiology including the stress tolerance, membrane potential, genetic competence and biofilm formation.
4.2 Importance

*S. mutans* is best known for its role in initiation and progression of human dental caries, one of the most common chronic diseases worldwide. *S. mutans* is also implicated in bacterial endocarditis, a life-threatening inflammation of heart valves. *S. mutans* core virulence factors include its ability to produce and sustain acidic conditions, and to form a polysaccharide-encased biofilm that provides protection against environmental insults. Herein, we demonstrated that the addition of copper and/or deletion of *copYAZ* (the copper-homeostasis system) have serious implications in modulating biofilm formation, stress tolerance and genetic transformation in *S. mutans*. Manipulating the pathways affected by copper and *copYAZ* system may help to develop potential therapeutics to prevent *S. mutans* infection in, and beyond, the oral cavity.

4.3 Introduction

Many bacteria utilize copper as an essential co-factor for enzymes involved in electron transfer reactions that include, superoxide dismutase, cytochrome c oxidase or NADH-dehydrogenase [171, 202]. Although required in low concentrations, copper at higher levels can pose a threat owing to its high reactivity. Copper can exist in cuprous (*Cu*⁺) and cupric (*Cu*²⁺) oxidative forms; the interchange between these states via Fenton reactions can lead to the generation of reactive oxygen species (ROS), especially superoxide radicals. The ROS are believed to cause cellular damage by reacting with a number of cellular macromolecules such as lipids, proteins, and nucleic acids [203, 208, 282]. However, there is little or no direct evidence that shows that copper-induced ROS generation is the main mode of copper-toxicity in bacteria [204, 206, 278]. In *Escherichia coli*, copper was demonstrated to target and replace iron from the iron-sulphur clusters of dehydratases, thereby, rendering them inactive and perturbing the biosynthesis of branched chain amino acids [172, 204, 206, 278]. Supplementation of *E. coli* cultures with branched chain amino acids was shown to reverse the copper-induced growth inhibition [278]. However, a similar mechanism was not observed in *Salmonella*, where the addition of exogenous branched chain amino acids did not revert copper-mediated growth inhibition, thereby suggesting the involvement of a different cellular target for copper-toxicity [205]. Copper-mediated
membrane depolarization has also been reported in *E. coli* [283]. Challenges posed by copper necessitate the involvement of complex regulatory machinery to maintain copper homeostasis in the cell. To contend with copper toxicity, bacteria utilize at least one of three principal mechanisms that include, a) copper export across the plasma membrane into the periplasmic space or the extracellular environment, b) extracellular and/or intracellular copper sequestration via copper binding proteins and, c) copper oxidation to a less toxic Cu$^{2+}$ state [207]. The mechanisms involved in copper stress tolerance can vary among bacterial species and exploring these pathways is an important step to implement the use of such metal cations as antimicrobial therapeutics.

*Streptococcus mutans* is considered as the primary etiological agent of dental caries, one of the most widespread infectious diseases worldwide [19, 77]. Numerous studies have indicated the inhibitory effects of copper on *S. mutans* growth and caries formation [87, 95, 177, 284-288]. The copper concentration in saliva and dental plaque fluctuate between individuals and depend on the age, sex, nutrient intake, etc. [289-292]. Copper concentrations in saliva can range from 0.05-61.7 µM, whereas the numbers fluctuate between 26 and 1520 ppm within the plaque biofilm. By administering copper-containing mouth rinses, copper has been shown to dramatically influence the growth and pathogenicity of *S. mutans* [291]. In *S. mutans* strain GS-5, copper irreversibly inhibited the activity of F-ATPases, thereby compromising the cell's ability to carry out glycolysis in acidic environments [281]. Further, this cation was shown to modulate *S. mutans* gtfD expression whose products are associated with soluble glucan production and biofilm formation of *S. mutans* [280]. Like many Gram positive bacteria, *S. mutans* genome sequence does not appear to encode proteins or enzymes that require copper for their functional activity [277]. Even though copper is not required for its cellular processes, *S. mutans* must still possess a functional copper-exporting machinery to avoid cell damage under excessive copper concentrations.

Unlike Gram negative bacteria that traffic copper through different cellular compartments from the cytosol to the periplasm and from the periplasm to the extracellular environment, Gram positive bacteria possess rather simple copper homeostatic systems dedicated towards extrusion of excess copper cations from the cytosol to the extracellular milieu [170]. The CopYABZ copper homeostasis system of *Enterococcus hirae* is the best understood copper homeostasis
model in Gram positive bacteria [169-170, 293-294]. This operon encodes four proteins that include two copper P-type ATPases, CopA and CopB, a copper responsive repressor, CopY, and a copper-chaperone, CopZ [169, 293-294]. The CopZ family of metallo-chaperones is conserved within various bacterial and eukaryotic systems [169-170]. A homologous copper transport and resistance system, encoded by \textit{copYAZ}, has been partially characterized in \textit{S. mutans} strain JH1005 [178]. The sensitivity of the \textit{copYAZ} knockout mutant was shown to be specific to copper and CopY was demonstrated to act as a negative regulator of the operon [178]. Although not tested conclusively, CopZ was speculated to de-repress the \textit{cop} operon activity and the deficiency of \textit{copYAZ} was shown to result in enhanced sensitivity to cell-killing under copper stress [178].

Here we investigated the effects of copper on the physiology of \textit{S. mutans} and the role of CopYAZ in copper homeostasis. While previous studies have implicated metal cations in biofilm formation and genetic competence development [295-297], the role of copper and the CopYAZ in regulating these phenotypes is poorly understood in \textit{S. mutans}. The ability of this organism to adhere to hard surfaces and form a biofilm is an important virulence factor that is critical for its survival and persistence in the oral cavity. Within the plaque biofilm, \textit{S. mutans} is capable of natural transformation that is made possible when it accomplishes a transient physiological state referred to as genetic competence [147, 153]. Genetic transformation is important for acquiring novel, heritable functions that can enhance fitness and drive evolution [151]. In \textit{S. mutans}, genetic competence is induced by two signaling peptides designated the competence-stimulating peptide (CSP encoded by \textit{comC}) and the \textit{comX}-inducing peptide (XIP encoded by \textit{comS}) [132, 142-143, 146]. Under specific competence-inducing conditions, both CSP and XIP can activate the transcription of the master competence regulator encoded by the \textit{comX} (or \textit{sigX}) alternate sigma factor required for the transcription of genes involved in DNA uptake and recombination [132, 142-143, 146]. Notably, the \textit{S. mutans} competence development pathway activated by CSP and XIP is closely intertwined with its biofilm pathway [142, 145, 147, 298-299]. However, it remains to be studied how and whether copper exerts its influence to regulate these phenotypes via the putative CopYAZ copper transport system in \textit{S. mutans}.

Herein, we showed that copper instigates a compromised state in \textit{S. mutans}, by dissipating membrane potential and decreasing its ability to endure environmental oxidative and pH stress,
produce biofilm, and develop genetic competence. Utilizing a *copYAZ* deletion mutant, designated as Δ*copYAZ*, we validated the function of *S. mutans* CopYAZ in copper efflux. We also report that the addition of copper or the absence of *copYAZ* system reduces the transcription of genes involved in biofilm matrix production and genetic competence development; loss of *copYAZ* leads to impairment of stress tolerance, transformability, and membrane potential in *S. mutans*.

### 4.4 Material and methods

#### 4.4.1 Strains and growth conditions

All *S. mutans* strains were grown in Todd-Hewitt yeast extract (THYE) (Becton Dickinson, Sparks, MD) broth as standing cultures or on THYE with 1.5% (wt/vol) agar (Bioshop, Burlington, Ontario, Canada) at 37°C in air with 5% (vol/vol) CO₂. Tryptone yeast extract medium (TYE) (10% Tryptone, 5% yeast extract, 17.2 mM K₂HPO₄) was utilized for the acid tolerance response (ATR) assays (Tryptone was obtained from Bioshop, Burlington, Ontario, Canada). For the ATR assays, NaOH or HCl was added to TYE to adjust the pH to 7.5 or 5.5 and 3.5, respectively. *E. coli* strains were cultivated aerobically in Luria Bertani (LB) medium at 37°C. Chemically defined media [132, 300] was used for transformation frequency assays. Antibiotics were added whenever required in recommended concentrations: erythromycin (10 µg/ml), chloramphenicol (10 µg/ml), spectinomycin (1000 µg/ml) for *S. mutans* and ampicillin (100 µg/ml), chloramphenicol (20 µg/ml) for *E. coli*.

#### 4.4.2 Minimum inhibitory concentration (MIC) assays

MIC assays were conducted as previously described [301]. Briefly, 100 µl of mid-log phase bacterial cells adjusted to an OD₆₀₀ of ~0.01 were added to a 96-well microtiter plate containing THYE medium supplemented with two-fold serial dilutions of CuSO₄ or CuCl₂ (Sigma-Aldrich) solutions (concentrations ranging from 0 to 25 mM), AgNO₃ (1 to 100 µM), CdSO₄ (0 to 1.5 µM), HgNO₃ (0 to 10 µM), ZnCl₂ (0 to 25 mM), MnCl₂ (0 to 100 mM), or CaCl₂ (0 to 100 mM). After incubation at 37°C with 5% (vol/vol) CO₂ for 24 h, bacterial growth was
spectrophotometrically measured by using a micro-titer plate reader at an absorbance of 600 nm. The MIC was determined as the lowest concentration that inhibited visible cell growth relative to no copper control.

### 4.4.3 Mutant and complemented strain construction

A deletion mutant (designated as ΔcopYAZ) of the copYAZ (NCBI database gene annotation: SMU_424, SMU_426, SMU_427) in *S. mutans* UA159 wild type background was constructed for this study. Briefly, a knockout mutant was constructed using the PCR-ligation mutagenesis strategy as described by Lau *et al.* [302], by deleting the operon and inserting an erythromycin resistance cassette at the locus in the UA159 background. The deletion of *copYAZ* operon in the ΔcopYAZ strain was confirmed by PCR amplification, DNA sequencing and quantitative Real Time PCR (qRT-PCR) analyses. The complementation analysis was done using *E. coli* copA deficient mutant (designated as DW3110, obtained from the Keio collection). Complemented strains of *E. coli* copA were constructed using the Thermo Scientific CloneJET PCR Cloning Kit as per the manufacturer's instructions. Briefly, the *S. mutans* copYAZ was PCR amplified using UA159 genomic DNA as the template, and ligated into the pJET vector. The vector was first transferred into *E. coli* DH5α chemically competent cells (Invitrogen Subcloning Efficiency™ DH5α™ Competent Cells). The clones were selected on LB agar plates supplemented with ampicillin (100 µg/ml) and confirmed using nucleotide sequencing. The resulting plasmid was then transferred into chemically competent cells of the *E. coli* DW3110 mutant to obtain the complemented strain DW3110compYAZ. Similarly, to generate a complemented strain in *S. mutans*, similar PCR products were utilized and ligated into plasmid pIB166 [303] harbouring a chloramphenicol resistance marker and a constitutive promoter upstream of a multiple cloning site. The cloned vector with the entire *copYAZ* was transferred into the ΔcopYAZ and the resulting strain was designated as CompΔcopYAZ. Complementation with empty vector was utilized as control (DW3110pJET or ΔcopYAZpIB) (data not shown). Bacterial strains and primers utilized in this study are summarized in Table 4-1 and Table 4-2, respectively.
Table 4-1 Bacterial strains and plasmids used in this study

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<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td><em>S. mutans</em> Wild type Erm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J. Ferretti, University of Oklahoma</td>
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<td>ΔcopYAZ</td>
<td>In-frame <em>copYAZ</em> deletion mutants derived from UA159; Erm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>CompΔcopYAZ</td>
<td>ΔcopYAZ transformed with pIB-YAZ Cm&lt;sup&gt;+&lt;/sup&gt;, Erm&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>DW3110</td>
<td>W3110 ΔcopA</td>
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<td>DW3110 transformed with pJET-<em>copYAZ</em> Amp&lt;sup&gt;+&lt;/sup&gt;, Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pIB166</td>
<td>Shuttle plasmid containing the <em>P23</em> lactococcal promoter; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[303]</td>
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<td>This study</td>
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4.4.4 Biofilm assays

Biofilms were cultivated in 96-well polystyrene microtiter plates using ¼THYE media supplemented with 10 mM sucrose and varying concentrations of CuSO₄ or CuCl₂ (0, 100 µM, 250 µM, 500 µM, 1 mM, 2 mM, 5 mM). All wells were inoculated with overnight cell suspensions of S. mutans wild-type and mutant strains and the plates were incubated for 24 h at 37°C with 5% (vol/vol) CO₂. Following incubation, broth was carefully removed and the biofilms were stained with 0.01% (wt/vol) safranin to determine the relative biomass, as previously described [305]. For biofilm initial adhesion studies, cells (20µl of mid-logarithmic cells) were incubated in 12-well polystyrene microtiter plates containing 2 ml ¼-THYE media supplemented with 10 mM sucrose and varying concentrations of CuSO₄ (0, 500, 1000 µM). The plates were incubated for different time intervals, and the planktonic cells were removed. Attached cells were washed twice with 1× phosphate buffered saline (PBS) solution before being scraped from plates and re-suspended in 200 µl of PBS. Cells were briefly sonicated, serially diluted in 10-fold decrements in 1× PBS, and 20 µl of each dilution was spotted in triplicates on THYE agar plates. After 48 h of incubation, colonies were counted for the dilutions showing individual colonies (30-300 in number), and the percentage of cells attached/able to grow were calculated between the presence and absence of copper.

4.4.5 Acid tolerance response (ATR) assays

ATR assays were conducted as previously described [109]. Briefly, overnight cultures grown in THYE media were diluted 1:20 using sterile pre-warmed tryptone-yeast extract media at pH 7.5 supplemented with 1% (wt/vol) glucose (TYEG). Cultures were then grown until mid-logarithmic phase (optical density at 600 nm [OD₆₀₀], ~0.4), divided into two equal aliquots and pelleted via centrifugation. For non-adapted cells, one aliquot was re-suspended in TYEG at a lethal pH value of 3.5, and for adapted cells, the other aliquot was first re-suspended in TYEG at pH 5.5 and incubated at 37°C with 5% CO₂ for 2h before exposing them to lethal pH 3.5. Following incubation at 37°C with 5% CO₂, cell fractions were removed from cultures at time zero and after 2h. Cells were gently sonicated, serially-diluted in 10 mM potassium phosphate
buffer (pH 7.2) and spotted in triplicate (20 μl of each dilution) onto THYE agar plates. After incubation for 48 h, colony forming units (CFUs) were counted and ATR was calculated as the percentage of CFU obtained at lethal pH after 2 h relative to the number of CFU present at time zero.

### 4.4.6 Growth kinetic analysis

For growth kinetic assays, *S. mutans* strains were grown in THYE with varying concentrations of CuSO₄ or CuCl₂, paraquat (0, 5 mM, 10 mM, 25 mM, 50 mM) or hydrogen peroxide (H₂O₂) (0, 0.0015%, 0.003%, 0.0045%, 0.006% (v/v)). Overnight cultures of *S. mutans* strains were diluted 1:20 in fresh THYE medium, and grown till mid-logarithmic phase (O.D ~ 0.4). 20 μl of cell inocula was added to 350 μl THYE containing varying concentrations of test reagents in quadruplicate. Cells were incubated at 37°C for 24 h; non-inoculated wells and wells containing THYE medium with or without reagents alone were used as controls. No antibiotics were used in growth assays to avoid additional stress. The optical density readings were taken every 20 min using an automated growth reader workstation (Bioscreen C, Growth Curves USA) and were plotted over time to obtain growth curves.

### 4.4.7 Copper transport assays

Overnight cultures grown in THYE were diluted 1:20 and suspended in fresh media supplemented with or without 1 mM CuSO₄ or CuCl₂ (suspended in milliQ water). Samples were incubated at 37°C with 5% CO₂ till they reach mid-logarithmic phase (OD₆₀₀ 0.4-0.6). Following incubation, cells were harvested by centrifugation at 3800 g. To minimize the probability of measuring membrane-associated copper cations, cells were washed twice with 0.01 M NaClO₄, an experimental electrolyte previously shown to remove residual metal from media and bacterial surfaces [306]. Cells were re-suspended in ice cold 1× PBS and filtered through a 0.22-µm-pore-size nitrocellulose filter. The filter was washed twice with 1 ml of ice-cold 1× Tris-EDTA buffer and once with 1 ml of ice-cold milliQ water. The filter was digested with 4% HNO₃ followed by
inductively coupled plasma atomic emission spectroscopic (ICP-AES) analysis using the ANALEST facilities (Analytical Lab for Environmental Science Research and Training) at University of Toronto. A 100 mg/ml CuSO₄ or CuCl₂ (suspended in 4% HNO₃ in milliQ water) stock solution was used to prepare standards ranging from 3 µg/ml to 100 µg/ml for ICP-AES calibration. A standard curve was plotted and utilized to analyse the intracellular copper concentrations. No detectable amounts of copper were observed in THYE or LB media alone. The measured intracellular copper concentrations were normalized per mg dry weight of cells.

4.4.8 Quantitative real time PCR Assays

Total RNA was isolated from 18-h-old biofilms grown in the presence or absence of 500 µM CuSO₄ in ¼ THYE media supplemented with 10 mM sucrose. Due to its poor biomass production, higher numbers of wells (three-times the number of wild type) were inoculated to isolate RNA from 18-h-old biofilms of the ΔcopYAZ grown in the presence of copper. The planktonic cells were removed and the biofilm cells were washed with 1× PBS. The biofilm cells were scraped from the polystyrene surface and suspended in ice-cold 1× PBS. Cells were sonicated and collected after centrifugation at 3800×g. For expression analysis of the genes associated with the competence regulon, cells were cultivated in chemically defined media (CDM) [132, 300] at 37°C with 5% CO₂, until they reached mid-logarithmic growth phase. Cells were then treated with 100 µM CuSO₄ for 30 min. For expression analysis of the genes associated with acid stress, mid-logarithmic cells cultivated in THYE were treated with 500 µM CuSO₄ for 30 min. Total RNA was harvested from the cell pellets as previously described [307]. After DNase treatment, RNA was subjected to reverse transcription using the First Strand cDNA Synthesis Kit (Fermentas). Quantitative Real Time PCR analyses were conducted using the Quantitect SYBR-Green PCR kit (Qiagen, Mississauga, Ontario, Canada). The fold expression change was calculated according to the method of Pfaffl et al. [308-309] using the following formula: Fold change = \[ \frac{[\text{Eff}]_{\text{target gene}} \times (\text{Ct-control} - \text{Ct-experimental})}{[\text{Eff}]_{16S \text{ rRNA}} \times (\text{Ct-control} - \text{Ct-experimental})} \]. Where E = (10⁻¹/slope) represents the efficiency of gene amplification and Ct values are the threshold cycle values of the target gene [301, 307]. Results were normalized against S. mutans 16S rRNA or gyrA expression; expression of these house-keeping genes was found stable under
the test conditions (data not shown). Statistical analysis was conducted using a Student’s t-test and a P value of <0.05 was considered significant. Primers utilized are listed in Table S1.

4.4.9 Membrane potential assays

Overnight S. mutans cultures were diluted 1:20 in fresh THYE and cells were grown to mid-logarithmic phase before washing and re-suspending them in minimal media [310] or in 1× PBS supplemented with 25 mM glucose. The membrane potential assays were conducted by measuring the fluorescence intensity of an anionic dye DiSBAC$_3^-$ using the Five Photon kit manufacturer’s protocol. As bis-oxonol is a lipophilic anionic molecule, the dye accumulates in the cell upon membrane depolarization, where it binds to intracellular components and results in an increase in cytosolic bis-oxonol fluorescence intensity. After incubating the cells with dye for 10 min, fluorescence was measured using the TECAN fluorescence plate reader (excitation wavelength of 530 nm, emission wavelength of 560 nm) and the fluorescence intensity was expressed as Arbitrary Units (AU). After dye stabilization 1 mM CuSO$_4$ was added. The plate conditions were set at 37°C with shaking for 5 sec before every read and fluorescence intensity measurements were taken every 15 min for at least 6 h. carbonyl cyanidem-chlorophenylhydrazone (CCCP), a known membrane depolarizer, was used as a positive control, wherein the addition of 10 µM CCCP resulted in an immediate increase in fluorescence intensity. Change in membrane potential (ΔΨ, expressed as AU) was taken as the difference between the fluorescence value at a specific time point (every 15 min) of incubation (Ψ$_f$) and the initial stabilization value (Ψ$_i$). To control for the dye’s intensity change, Ψ$_c$ (culture sample) was normalized with Ψ$_b$ (blank with dye). Statistical analysis was conducted using a Student’s t-test with a P value of <0.05 considered as significant.

4.4.10 Genetic transformation assays

Overnight cultures of S. mutans grown in THYE were pelleted, washed, diluted (1:20) and re-suspended in chemically defined media [132, 300]. Cultures were allowed to grow at 37°C with
5% CO$_2$ to mid-logarithmic growth phase (OD600 of ~ 0.4). Cultures were then divided into three aliquots and treated with: 1) no copper; 2) 100 µM CuSO$_4$ and 3) 250 µM CuSO$_4$. The copper concentrations used were based on the MICs calculated in the CDM media: *S. mutans* UA159: 1000 µM and Δ*copYAZ*: 500 µM CuSO$_4$. Next, 1 µg of plasmid DNA (pDL277, containing a spectinomycin resistance marker) was added to 500 µl aliquots of cell suspensions and incubated in 5% CO$_2$ at 37°C for 90 min. Following incubation, samples were briefly sonicated, serially diluted, and plated onto THYE agar, with and without spectinomycin, to determine the number of transformants and the total number of possible viable cells, respectively. Transformation frequency was calculated as the percentage between transformant CFUs and total number of viable CFUs, times 100. Statistical analysis was conducted using a Student’s *t*-test with a *P* value of <0.05 considered as significant.
4.5 Results

4.5.1 Copper is toxic to S. mutans and copYAZ is required for copper resistance in S. mutans

To investigate the physiological function of the copYAZ operon in S. mutans, an isogenic mutant, designated ΔcopYAZ, was constructed and characterized in vitro. Growth kinetics and minimum inhibitory concentration (MIC) were assessed using the wild type and mutant strain under varying concentrations of CuSO₄ solutions. A two-fold decrease in the MIC was observed in the ΔcopYAZ (2 mM CuSO₄) as compared with the UA159 strain (4 mM CuSO₄) under copper stress \((P < 0.05)\). At higher concentrations \((≥ 6 \text{ mM for ΔcopYAZ and } ≥ 12 \text{ mM for UA159})\), copper had a bactericidal effect on S. mutans viability. Complementation studies conducted with the CompΔcopYAZ (ΔcopYAZ harboring pIB-YAZ) strain showed restoration of copper-dependent growth inhibition to levels observed with the UA159 wild-type strain (MIC of 4 mM CuSO₄; \(P < 0.05\)). Growth assays conducted with the wild-type, ΔcopYAZ and CompΔcopYAZ strains revealed that exposure to added copper resulted in growth impairment of all strains and that loss of the copYAZ resulted in enhanced toxicity to copper, relative to wild-type and complemented strains (Figure 4-1 and Figure 4-2). To validate that effects observed in growth assays were due to copper cations the experiments were repeated with CuCl₂ which showed a similar influence on S. mutans growth as observed with CuSO₄. In addition, we performed growth inhibitory assays for wild-type and ΔcopYAZ in the presence of varying concentrations of other metal ions that included Ag, Cd and Hg; MICs for these assays did not reveal statistically relevant differences (MICs 100 µM, 1.5 µM, 6 µM respectively for Ag, Cd and Hg). Hence, these results highlighted the importance and specificity of the CopYAZ system in copper-induced toxicity in S. mutans.
Figure 4-1 Growth analysis in the presence and absence of copper

Growth analysis of *S. mutans* strains cultivated in the presence or absence of 2 mM CuSO$_4$. Results are representative of six independent experiments conducted with duplicates for each strain. ■ indicate *S. mutans* wild type strain, ▲ indicate Δ*copYAZ*, and ● indicate *compΔcopYAZ*; solid-filled indicate strains grown in the absence of CuSO$_4$, and hollow icons indicate strains grown in the presence of 2 mM CuSO$_4$. 
Figure 4-2 Growth kinetics under copper stress

*S. mutans* UA159 and ΔcopYAZ strains cultivated under varying concentrations of CuSO₄. Results are the representative of six independent experiments conducted with duplicates for each strain.
4.5.2 *CopYAZ has a role in copper export in S. mutans*

In several Gram positive bacteria CopA functions in copper export. Copper transport studies, using ICP-AES analysis, revealed that *S. mutans* UA159 grown in the presence of 1 mM CuSO$_4$ had a significantly high amount of intracellular copper (Figure 4-3) as compared to those grown without copper supplementation (no detectable amount of intracellular copper). A two-fold increase in the amount of intracellular copper was observed in the ∆*copYAZ* compared with the wild type strain (Figure 4-3; $P<0.05$), thus suggesting the involvement of *copYAZ* in copper efflux in *S. mutans*. In the wild type and ∆*copYAZ* strains, transport studies conducted with Ag and Mn cations (using 50 µM AgNO$_3$ or 1 mM MnCl$_2$) showed no statistically significant differences in the intracellular levels of these cations. This latter result support the specificity of the CopYAZ transport system to copper cations, which was further validated using the CopYAZ complemented Comp∆*copYAZ* strain whose ability to efflux copper at levels comparable to the wild type parent (Figure 4-3). In the complementation experiments, we employed an *E. coli* strain deficient in *copA* (DW3110), which exhibits a defect in copper efflux and its detoxification [212]. The recombinant *S. mutans* *copYAZ* operon successfully complemented the deficiency in DW3110, as observed by a significantly decreased sensitivity to copper-induced killing, and reduced intracellular copper ion accumulation (Figure 4-4A and Figure 4-4B); thus supporting a specific function for the *S. mutans* CopYAZ in copper transport and resistance.
Figure 4-3 Role of CopYAZ in copper export

Intracellular copper concentration normalized to dry weight of cells of *S. mutans* wild type UA159 (■ bar), ΔcopYAZ (■ bar), and compΔcopYAZ (□ bar). Cells were grown in the presence or absence of 1 mM CuSO₄ until the mid-logarithmic growth phase. Cells were lysed and intracellular copper concentration was normalized with cell dry weight. Results shown here are the mean values obtained from three independent experiments ± standard error (*P* < 0.05).
Figure 4-4 Growth kinetics and transport studies using *E. coli* strains

A) *E. coli* strains cultivated in the presence or absence of 2 mM CuSO₄. Results are representative of six independent experiments conducted with duplicates for each strain. Legend: squares indicate wild type, triangles indicate *cop* mutant, circles indicate complemented strain of *E. coli* strains, solid-filled indicate strains grown in the absence of CuSO₄, and hollow icons indicate strains grown in the presence of 2 mM CuSO₄.

B) Intracellular copper concentration normalized to dry weight of cells of *E. coli* wild type W3110 (black bar), *DW3110* (grey bar), and *DW3110compYAZ* (white bar) was quantified using ICP-AES. No copper was detected in cells with no copper treatment. Results shown here are the mean values obtained from three independent experiments ± standard error (*P <0.05*).
4.5.3 Copper inhibits biofilm formation and copYAZ is required to tolerate copper stress under biofilm growth.

Both the wild type and ΔcopYAZ strains produced comparable biomass in 18-h biofilms grown in the absence of copper. A significant reduction of biofilm biomass was observed in ΔcopYAZ biofilms cultivated with 500 µM CuSO$_4$ relative to the wild type (Figure 4-5A). Biofilm biomass was drastically reduced at 1 mM CuSO$_4$ in both wild type and mutant strains (Figure 4-5A).

Figure 4-5 Effect of copper on biofilm formation

A) Biomass of 18-h biofilms derived from wild type UA159 (■ bars) and ΔcopYAZ (■ bars) strains grown in the absence or presence of CuSO$_4$. Results shown represent the mean values obtained from three independent experiments with three replicates for each strain ± standard error (*$P$ <0.05). B) Percentage of the cells adhered to the surface after 1 h incubation using UA159 (■ bars) and ΔcopYAZ (■ bars) in the presence of varying concentrations of CuSO$_4$ relative to no copper. Results shown represent the mean values obtained from three independent experiments with three replicates for each strain ± standard error (*$P$ <0.05).
Initial cell attachment assays were conducted to evaluate the percentage of cells attached to a surface in the presence or absence of copper for different time intervals. At 1 h incubation, the percentage of cells adhered in the presence of 500 μM CuSO$_4$ was significantly reduced in both wild type (~50 % decrease) and ΔcopYAZ strains (~80 % decrease) relative to the no copper control (Figure 4-5B). At 1 mM CuSO$_4$, further reduction in the percentage of cells adhered was observed in both wild type and mutant strains. After prolonged incubation of 2 h or 4 h, no significant decrease in the number of cells adhering to surface was observed in wild type strain at 500 μM CuSO$_4$. However, the ΔcopYAZ cultivated in the presence of copper showed a significant reduction in the number of cells attached relative to the no copper control at all time periods (Figure 4-6). Results from these experiments showed that the addition of copper and deletion of the copYAZ operon reduced the ability of cells to adhere likely leading to defective biofilms.
Figure 4-6 Cell adherence assays

Percentage of the cells (log scale) adhered to the polystyrene surface after A) 2h and B) 4h incubation using UA159 (black bars) and ΔcopYAZ (grey bars) in the presence of varying concentrations of CuSO₄ relative to no copper. Results shown represent the mean values obtained from three independent experiments with three replicates for each strain ± standard error (*P <0.05).
4.5.4 *Copper affects the transcription of gfts and glucan binding protein (gbp) genes.*

To assess the underlying molecular mechanism associated with copper-mediated reduced cell adherence and reduced biofilm biomass of *S. mutans*, we examined the effects of copper on the transcription of *gfts* (*gtfB, gtfC, gtfD*) and *gbps* (*gbpB* and *gbpC*) whose products are needed to maintain the structural and functional integrity of the *S. mutans* biofilm [63, 116-117, 119, 124, 127]. Gene expression analysis using qRTPCR of biofilm-derived cDNAs with or without of 500 µM CuSO$_4$ demonstrated a significant $\geq 2$-fold down-regulation of all five genes ($P < 0.05$) in the presence of copper relative to the no copper control (Figure 4-7). In the absence of copper, the transcription of *gtf* and *gbp* genes was not significantly affected between ∆copYAZ and wild type biofilms (data not shown). Furthermore, in the presence of copper an enhanced transcriptional repression of *gtfC, gtfD, gbpB* and *gbpC* was observed in ∆copYAZ biofilms relative to those of the wild-type strain. In *S. mutans*, the negative regulatory role of copper on the transcription of the *gbp* genes- whose products facilitate biofilm cell adherence, as well as the *gfts*- whose products are responsible for glucan production that maintain the integrity of the biofilm matrix, might explain the impaired biofilms observed under copper stress.
Figure 4-7 Gene expression analysis of different biofilm matrix-related genes

*S. mutans* UA159 (■ bars) and Δ*copYAZ* (■ bars) treated with 500 µM CuSO₄ relative to no copper control. Results shown represent the mean values obtained from four independent experiments with three replicates for each strain ± standard error (*P* <0.05 for all the genes shown in the graph).

4.5.5 *Copper-induces oxidative stress and CopYAZ modulates oxidative stress tolerance.*

The copper-induced growth inhibition was assessed in the presence of the antioxidants, glutathione and thiourea. Addition of 1.5 mM glutathione dramatically improved the growth of *S. mutans* UA159, Δ*copYAZ*, and *compΔcopYAZ* strains cultivated in the presence of 2 mM CuSO₄ compared to no added glutathione control (Figure 4-9A). Treatment with glutathione in the absence of copper did not alter the growth of *S. mutans* strains used in this study (Figure 4-8). The reversion of copper-induced growth defects was also observed in the presence of the antioxidant thiourea. These results emphasize that copper-induced toxicity in *S. mutans* is likely dependent on copper-dependent generation of oxidative stress in *S. mutans*. Biofilms were also assessed to study the effect of glutathione (or thiourea) in reversal of the copper-induced biofilm defect by cultivating 18 h biofilms in the presence or absence of copper supplemented with or
without 1.5 mM glutathione. Glutathione alone did not have any effect on the biofilm biomass of all three strains tested. Addition of 1.5 mM glutathione did not improve the biomass production of the biofilms cultivated in the presence of 500 μM or 1 mM copper in S. mutans UA159, ΔcopYAZ, and compΔcopYAZ strains. These results suggest that S. mutans can utilize an alternate route to contend with copper stress during biofilm growth.

![Growth Kinetics in the presence and absence of glutathione](image)

**Figure 4-8 Growth Kinetics in the presence and absence of glutathione**

S. mutans UA159, ΔcopYAZ and compΔcopYAZ grown in the presence of the 1.5mM glutathione. Results are the representative of six independent experiments conducted with duplicates for each strain.
Figure 4-9 Copper-induced oxidative stress, and involvement of CopYAZ in protection against oxidative stress

A) Growth curves of wild type UA159 (■), ΔcopYAZ (■), and compΔcopYAZ (□) strains in the presence of 2mM CuSO₄ copper with (○) or without (▲) 1.5mM glutathione. Results are representative of six independent experiments conducted using duplicate samples for each strain. B) Lag times of UA159 (■ bars), ΔcopYAZ (■ bars), and compΔcopYAZ (□ bars) to reach an OD₆₀₀ of nearly 0.1 in the presence and absence of the oxidative stressors paraquat (25 mM) paraquat and H₂O₂ (0.0045%). The top two lines indicate the statistical significant results within the strains under different stressors. Results are the mean of three independent experiments conducted with three replicates for each strain (* P <0.001).
We next investigated the role of *copYAZ* in protecting the cells against oxidative stress. Growth kinetics analyses were performed using wild type UA159, Δ*copYAZ* and *compΔ*copYAZ strains in the presence of the oxidizers methyl viologen dichloride (paraquat) or hydrogen peroxide. Though all strains cultivated in the presence of either paraquat or hydrogen peroxide reached a comparable final optical density after 24 h (Figure 4-10A and Figure 4-10B), the lag growth phase of Δ*copYAZ* was drastically prolonged relative to wild type (Figure 4-9B), thereby suggesting a role of the efflux system in protecting cells under oxidative stress. In the complementary *CompΔcopYAZ* strain, the lag phase in the presence of oxidative stressors was restored to that of wild type levels.
Figure 4-10 Growth kinetics under oxidative stressors

*S. mutans* UA159, Δ*copYAZ* and *compΔcopYAZ* grown in the presence of the oxidative stressors A) paraquat (25 mM) and B) H$_2$O$_2$ (0.0045%). Results are the representative of six independent experiments conducted with duplicates for each strain.
4.5.6 Copper inhibits growth under acid stress and CopYAZ modulates the acid tolerance response of S. mutans

Previous reports indicate that cuprous ions added to S. mutans strain GS-5 cultures can irreversibly inhibit the activity of F-ATPases, thereby compromising its ability to carry out glycolysis in acidic environments [281]. Here, growth kinetic analyses were performed in the presence and absence of copper in neutral and acidic pH using wild type UA159, ΔcopYAZ, and CompΔcopYAZ strains. Although the ΔcopYAZ strain displayed similar doubling times relative to wild type under acid (pH 5.5) or copper (1 mM CuSO₄) stress, the doubling times and the yield of the ΔcopYAZ was markedly impaired in THYE at pH 5.5 supplemented with 1 mM CuSO₄ (Figure 4-11 and Figure 4-12). The combined effect of acid and copper stress caused an approximate two- and five-fold increase in the doubling times of S. mutans UA159 and ΔcopYAZ respectively, compared to their growth in the presence of either of these stressors alone (Figure 4-12).
Figure 4-11 Growth of *S. mutans* under acid stress with or without copper

Growth curves of *S. mutans* UA159, ∆copYAZ, and comp∆copYAZ strains in the presence of acid (pH 5.5) and/or copper (1 mM CuSO₄) stress. Results are representative of six independent experiments with duplicate samples for each strain.
Figure 4-12 Doubling times under acid stress with or without copper

Evaluation of *S. mutans* doubling time in the presence of various stressors using wild type UA159 (black bars), ∆*copYAZ* (grey bars), and *comp∆copYAZ* (light grey bars). Results are the mean of three independent experiments with duplicate samples of each strain ± standard error (*P < 0.05).
These results emphasize the synergistic effects of copper and acid against *S. mutans* growth. The increased susceptibility of the ∆*copYAZ* strain under acid and copper stress can be due to 1) surplus accumulation of copper ions, or 2) lack of cellular protection offered by the *CopYAZ* copper efflux system. To examine the importance of *copYAZ* in the acid adaptation process, we conducted acid tolerance response (ATR) assays. Not surprisingly, for all the three tested strains the percentage survival of adapted cells (which were previously subjected to pH 5.5) was higher than that for non-adapted cells (which were maintained at pH 7.5). The loss of *copYAZ* decreased the ability of *S. mutans* to survive the pH 3.5 challenge regardless of pre-acid adaptation, relative to wild type (Figure 4-13). No significant difference in the percentage survival at pH 3.5 was observed between the Comp∆*copYAZ* and wild type strains under either non-adapted or pre-adapted conditions, thereby demonstrating that *copYAZ* contributed to ATR response in *S. mutans*.

![Figure 4-13 Acid tolerance response assays](image)

Percentage survival (in log scale) of *S. mutans* cells after 2 h incubation at lethal pH 3.5. Cell were either exposed (adapted cells- ■ bars) or not (non-adapted cells- ■ bars) for pre-adaptation at signal pH of 5.5. Results shown are the mean values obtained from three independent experiments ± standard error (*P* <0.05).
Previously, copper has been shown to reduce the activity of F-ATPase in S. mutans [281]. Hence we examined the role of copper on the transcription of acid stress-related genes that include components of the S. mutans F$_0$F$_1$ ATPase (SMU.1530 (atpD), SMU.1531 (atpE), SMU.1532 (atpF), SMU. 1534 (atpH)), and uvrA, a DNA damage repair gene, which is induced under acid stress [311]. Expression of genes was not significantly affected between the wild type strain cultivated in the presence and absence of copper. However, in ΔcopYAZ, the transcription of uvrA was significantly down-regulated by more than 2-fold ($P < 0.05$) and 3.6-fold ($P < 0.05$) incubated with and without copper respectively, relative to the wild type strain. A significant 2.2-fold reduction ($P < 0.05$) in the expression of atpH was observed in ΔcopYAZ in the presence of copper relative to control without copper. Studies are currently underway to analyze the impact of copper and copYAZ operon on the acid-inducible regulon of S. mutans that is responsible for the observed acid-sensitive phenotype in this oral pathogen.

**4.5.7 Copper induces membrane depolarization and CopYAZ helps maintain membrane potential.**

In *E. coli* and *Salmonella sp.*, contact-killing on copper surfaces involves immediate membrane depolarization, subsequently leading to compromised cell viability [283]. To test whether copper and CopYAZ modulate S. mutans membrane potential, we conducted fluorometric assays with bis-oxonols dyes, which can enter depolarized cells and bind intracellular proteins or membranes. Higher fluorescence intensity, as a result of elevated influx of the dye, indicates increased membrane depolarization. In *S. mutans*, addition of copper initiated a gradual increase in the fluorescence intensity, which was maximal after 15 min of incubation relative to the no copper control (Figure 4-14A). The increase in fluorescence intensity suggested that copper influx induced a dissipation of the membrane potential. The *S. mutans* ΔcopYAZ cells exhibited a depolarized membrane phenotype, where the cells cultivated even in the absence of copper, showed a gradual and sustained increase in the DiSBAC$_{3}$.dependent fluorescence intensity, compared with wild type (Figure 4-14B). Furthermore, restoration of the fluorescence intensity in the complemented CompΔcopYAZ strain to the wild type levels provided additional evidence for the involvement of this operon in *S. mutans* membrane depolarization. In membrane potential
assays, CCCP was used as a positive control; the addition of 10 µM CCCP resulted in an immediate increase in fluorescence intensity, as a result of instant membrane depolarization ($\Delta\psi$ units exceeding over 2-fold compared to no CCCP control).
Figure 4-14 Membrane potential in cells exposed to copper

Changes in membrane potential of *S. mutans* strain measured using a bis-oxonol probe over 45 min. A) Fluorescence intensity of cells with 15 min incubation in the absence (■ bars) or presence (■ bars) of 1 mM CuSO₄. Statistical significance was calculated using a Student's *t*-test at each time point (* *P* < 0.05). B) Fluorescence intensity of wild type UA159 (●), ΔcopYAZ (—), and compΔcopYAZ (—) in the absence of copper over a period of 45 min. Data shown are the mean of four independent experiments ± standard error. Statistical significance was observed at each time point between the ΔcopYAZ and other two strains (*P* < 0.05).
4.5.8 Addition of copper and loss of copYAZ alters transformability of 
*S. mutans*

Genetic transformation assays were conducted to investigate the effect of copper on genetic 
competence development of *S. mutans*. These assays were conducted in two different growth 
media, namely THYE and CDM, which activate separate competence induction pathways via the 
CSP-activated ComDE and XIP-activated ComRS signaling pathways, respectively ([132, 148]. 
When supplemented with 10µM, 100 µM, 250 µM, 500 µM or 1 mM CuSO₄, UA159 and 
ΔcopYAZ strains grown in THYE, which is a peptone-abundant nutrient rich medium, a five-fold 
decrease in transformation frequency was observed in the wild type strain only at the 1 mM 
CuSO₄ relative to controls without copper (P <0.05; data not shown). In the ΔcopYAZ mutant 
transformability was significantly reduced by over 10-fold relative to wild type, irrespective of 
copper supplementation (data not shown). The effects of copper on transformability were 
stronger when cells were grown in CDM medium. For instance, in CDM medium a 20-fold 
decreduction in transformation frequency was observed in both the wild type and ΔcopYAZ strains in 
the presence of 100 µM CuSO₄ relative to the no copper control (P <0.05; Figure 4-15). 
Furthermore, in the presence of copper, we noted that transformability of wild type and mutant 
strains were significantly reduced in a dose-dependent manner (Figure 4-15); our comparison of 
viable recipient cells between these strains, showed that total cell viability was not affected under 
these copper concentrations for UA159 and ΔcopYAZ strains (Figure 4-16). Also interestingly, 
loss of the copper efflux system in the ΔcopYAZ strain impaired its transformability by over 30-
fold relative to wild type (P<0.05), even in the absence of the copper stress (Figure 4-15). To 
argue against the possibility of this transformation defect arising due to copper-mediated plasmid 
DNA damage, we conducted transformation assays by utilizing cells pre-incubated with copper. 
These cells were washed to remove exogenous or cell-associated copper and then supplemented 
with fresh medium containing plasmid DNA. A similar decrease in genetic transformation was 
observed in the presence of copper relative to the no copper control, thereby confirming that the 
defect in genetic transformation was indeed due to copper present within the cells (data not 
shown).
Figure 4-15 Transformation frequency assays

Transformation frequency assays were conducted using *S. mutans* UA159 (■ bar) and ΔcopYAZ (■ bar) strains in the presence of varying concentrations of copper. The transformation frequency was calculated as the percentage between transformants and recipients cells (log scale). Results are mean of three independent experiments conducted in triplicates. (* P <0.05)*
Figure 4-16 Colony forming units of viable cells at different copper concentrations

*S. mutans* colony forming unit of the total viable cells (during the transformation frequency assays) in the presence of varying copper concentrations using wild type UA159 (black line), ΔcopYAZ (grey line). Results are the mean of three independent experiments with duplicate samples of each strain ± standard error (*P* <0.05).
4.5.9 Copper represses the expression of genes associated with genetic competence.

To determine the pathways by which copper and CopYAZ modulate transformability of *S. mutans*, we compared the expression of *comC*, *comD*, *comE*, *comR*, *comS*, and *comX* competence-related genes between the isogenic strains treated with or without copper. In *S. mutans* UA159, compared with the no copper control, supplementation with 100 μM CuSO$_4$ reduced the expression of *comX* and *comS* by 2-fold and 2.8-fold, respectively (*P<0.001*). Regardless of the presence of copper, expression of the *comX*, which is critical for competence development, was reduced by 2.5-fold in the ΔcopYAZ, compared with the wild type strain (*P<0.001*). Moreover, relative to the wild type, the expression of *comS* was repressed over 8-fold and 12.5- fold in the copper transporter mutant in the absence and presence of copper, respectively (*P<0.001*).
4.6 Discussion

Fluctuations in intracellular levels of copper can have severe implications on the physiology of all prokaryotes. The mechanisms involved in copper-induced killing vary amongst bacterial species. Bacteria usually initiate a global adaptive genetic response to copper, depending on their physiological copper requirements and presence of copper in their environmental niches [312-317]. While in some bacteria, such as Enterococcus faecalis and Pseudomonas aeruginosa, a large portion of the genome (approximately 300 genes) is differentially expressed under copper stress; in other bacteria such as Lactococcus lactis, only 11 genes were copper-responsive [314, 316-317]. While we did not analyze changes in entire copper-regulon of S. mutans, targeted gene expression analysis in this work revealed that copper-responsive genes included the gtfBCD and gbpBC associated with biofilm formation, the comS and comX genes critical for competence development, and the uvrA and atpH genes involved in acid-stress tolerance. An important aspect of this work was also to validate the role of the S. mutans CopYAZ in copper efflux, which influenced its ability to tolerate environmental stressors and maintain its membrane potential. The link between copper homeostasis via the CopYAZ and its effects on comS and comX transcription for competence development is novel. Since the competence pathway is closely linked with the biofilm and stress tolerance pathways of S. mutans [144-145, 147, 299], this work adds to the current knowledge as to how these pathways can be modulated by environmental copper stress.

The growth kinetic assays and MICs in this work indicated that higher concentrations of copper are toxic to S. mutans and its toxicity is counteracted through CopYAZ activity. In most bacteria, CopA has been speculated or proven to remove copper cations from the cell [170, 212, 293], which we validated using copper transport assays. Since we observed that both the wild type and ∆copYAZ cells were able to accumulate copper cations, we suspect that in S. mutans copper import/influx occurs passively, in a non-specific manner, as demonstrated in other bacteria such as Helicobacter pylori and E. coli [219, 318-319].

In the oral cavity, since S. mutans is constantly exposed to fluctuating oxidative stress, its ability to adapt to such conditions is important for its survival. Here, we demonstrated that the addition of the glutathione dramatically reversed the copper-induced growth defect in S. mutans cultures,
thus suggesting that toxicity to copper is likely a result of copper-dependent generation of oxidative stress in *S. mutans*. Copper can generate oxidative stress either by depleting glutathione (due to its capability of forming a complex with glutathione) [320] or by generating reactive oxygen species (ROS), especially superoxide radicals which are toxic to the cells [170, 208]. Copper, due to its high reactivity, is capable of replacing Fe or Mn from the active site of proteins, thereby rendering them inactive [184]. In *S. mutans*, *perR* (peroxide regulator), *sod* (superoxide dismutase) and *dpr* (dps-like peroxidase resistance protein) gene products are associated with resistance to oxidative stress [163, 321-323]. The *dpr*-encoded protein requires Fe, *sod*-encoded protein requires Mn/Fe and PerR has been speculated to require Mn or Fe for their activity [163, 321, 323-324]. Hence, copper can be speculated to replace Fe/Mn from the active sites of *dpr*, *sod*, and/or *perR* encoded products, thereby inactivating these proteins and compromising the cell viability under oxidative stress. However, further research is warranted to dissect the specific underlying mechanisms involved in this process. Our results also demonstrated the importance of *S. mutans* *copYAZ* operon in resistance against oxidative stress, thereby suggesting its involvement in stress adaptation process of this oral pathogen.

Copper and acidic pH exerted a synergistic effect against growth of *S. mutans*. Growth in THYE at pH 5.5 or in THYE with 1 mM CuSO₄ did not produce a discernible phenotype between the Δ*copYAZ* and UA159 strains. However, a significant increase in the doubling times were noted when these strains when cultivated in THYE at pH 5.5 with 1 mM CuSO₄, thus suggesting a synergistic effect of acid and copper stress against *S. mutans* growth. Since cuprous ions in *S. mutans* have been previously associated with impaired glycolysis under acid stress [281], our study supports this finding by demonstrating a notable growth defect in the presence of copper and acid stress. The impaired growth observed for the Δ*copYAZ* strain in the presence of copper and acid stress can be due to accumulation of surplus copper in the cells or due to the involvement of the *copYAZ* operon in protection under acid stress. *S. mutans* produces lactic acid as a metabolic end-product of dietary sugars and mounts an ATR that affords it growth and survival under pH values as low as 3.5 [105, 109]. During the ATR, *S. mutans* undergoes a number of physiological changes such as increased synthesis of stress responsive proteins, membrane fatty acid changes, and increased activity of proton translocating ATPases [105, 109]. Such adaptation requires a pre-exposure to a sub-lethal signal of pH 5.5 to activate the processes
to protect cells against killing pH values (pH 2.0-3.0) [105, 109]. A severe decrease in the viability of both the adapted and non-adapted ΔcopYAZ compared with the wild type at pH 3.5, implicated the contribution of copYAZ to the ATR of S. mutans. In the presence of copper, the transcriptional repression of the acid stress related genes, uvrA and atpH, likely contributed to the observed acid sensitive phenotype of S. mutans. Repression in the expression of uvrA in ΔcopYAZ also supported the association of the copYAZ operon in acid stress response. Although, we did not analysed the impact of copper on influencing the acid-inducible regulon of S. mutans; results from this study offer the foundation towards understanding the copper-mediated acid-tolerance response in this oral pathogen. Further studies are currently underway to elucidate the effect of copper on modulating S. mutans proteome; hence, aiming to determine the proteins involved in acid adaptation process under copper stress.

Under normal growth conditions, bacteria maintain their membrane potential by establishing multiple ion gradients across their intact cytoplasmic membrane. The maintenance of intact cell membranes is crucial to maintain ATP hydrolysis and proton motive force [325-326]. A disturbance in the amount of ions can result in hyper-polarization (higher negative intracellular electrical potential) or depolarization (higher positive intracellular electrical potential) of the membrane potential. A rational target for copper-induced toxicity in bacteria is their cell wall or cell membrane, as these are the exposed parts of bacteria to external stress. Reports have indicated that in Gram positive bacteria, the metal binding sites usually lie within the peptidoglycan layer [327-328]. Copper also tends to accumulate on the inner side of the cell membrane, making the inner side more positive and thus causing membrane depolarization [283, 329]. Here, we demonstrated the effect of copper on initiating an immediate membrane potential dissipation, which was significantly higher in the ΔcopYAZ strain relative to the wild type strain. The S. mutans ΔcopYAZ cells exhibited a depolarized membrane phenotype, even in the absence of copper, compared with wild type cells. The gradual increase in membrane depolarization observed in ΔcopYAZ can be due to membrane perturbation caused by the loss of the CopA trans-membrane protein. Our findings are in agreement with another study, where the loss of E. coli arsenic and antimony efflux system was shown to be linked with cell membrane depolarization [330]. Disturbances in the bacterial membrane potential can alter certain cellular processes such as cell division and differentiation, integrity of cellular membranes, electron
transport across the membranes, localization of specific proteins and protein complexes, and levels of cellular energy [325, 331-333]. In S. mutans, copper acting as a membrane potential dissipater might contribute to the generation of copper-dependent oxidative and acid stress, where both these stresses are likely to be affected by variations in the bacterial membrane integrity and/or changes in the electron transport across the bacterial membrane [326, 334-336].

Copper has an inhibitory effect on biofilm formation in S. mutans. This mechanism relies on the ability of copper to inhibit early attachment of cells to the surface and to significantly reduce biomass production in S. mutans. The reduction in cell adherence and biomass production was more prominent in the ΔcopYAZ strain, likely due to surplus accumulation of copper within the cells. In the presence of sucrose, cell-wall associated Gtfs mediate the synthesis of D-glucose polysaccharides, which are glucans that promote S. mutans adherence to tooth surface and other adhered bacteria [63, 116]. S. mutans produces three different Gtfs that include: GtfB, which produces water-insoluble glucans composed predominantly of α-1,3-linkages; GtfD that mostly produces soluble α-1,6-linked glucans; and GtfC that synthesizes both α-1,3 and α-1,6 glucans [111-113]. In our work expression of gtfB, gtfC, and gtfD were significantly repressed by copper. The transcriptional repression of these gtf genes validated the effects of copper in influencing genes critical for glucan production and biofilm matrix formation; their downregualtion under copper explains poor biofilm biomass observed. In addition to Gtfs, surface associated Gbps in S. mutans promote cell-cell aggregation by mediating the binding of S. mutans to glucans [119, 125, 337]. Addition of copper significantly reduced the transcription of gbpB and gbpC further expanding on components affected by environmental copper. The down-regulation of these gbps distinctly emphasized the effect of copper in initial sucrose-dependent biofilm formation, where expression of these genes is essential for the transition from planktonic to biofilm growth [37, 125]. In contrast to our gtf transcription results, previously others showed that supplementation with 1 mM copper induced the transcription and translation of gtfD but not gtfBC [280]. In their study, RNAs isolated from planktonic cells were used for expression analysis [31], whereas this study utilized RNAs from 18-h biofilms cultivated with or without added copper. In the absence of copper, we did not note a significant change in the transcriptional levels of gtfBCD, and gbpBC between the ΔcopYAZ and wild type strains; hence, it is not surprising that we obtained comparable amounts of biofilm biomass between these strains. In addition to the repressive
effects of copper on \textit{gtfBCD} and \textit{gbpBC} transcription, loss of copper efflux in the \textit{ΔcopYAZ} mutant lead to reduced \textit{gtfs} and \textit{gbps} expression in \textit{cop} mutant biofilms with added copper in the medium. These results provide strong evidence that copper is extremely effective in inhibiting biofilm formation, one of the most vital virulence attributes of this oral pathogen.

The influence of copper and the CopYAZ system on genetic competence development and \textit{comX} and \textit{comS} expression in \textit{S. mutans} is novel. The ComDE and ComRS signaling pathways involved in \textit{S. mutans} competence development differ in their mechanism of action [132, 143, 146, 298]. The CSP-ComDE pathway is activated by CSP whose precursor peptide is encoded by \textit{comC} gene [143, 147, 149]. Upon activation of the ComD and subsequent phosho-transfer to ComE leads to activation ComX that is a critical switch required for the competent state of \textit{S. mutans} [143, 145]. Recently, the proximal regulator of ComX was described as the ComR, an Rgg-like transcriptional regulator in the cytosol, which in conjunction with internalized XIP is able to activate ComX for the transcription of late competence genes required for DNA uptake and recombination [132]. It has been shown that nutrients and peptones present in the growth medium of \textit{S. mutans} can differentially affect CSP and XIP activity needed for competence activation [132, 146, 148, 150, 298]. CDM medium was shown to be optimal only for XIP-mediated competence induction, whereas the peptone-rich THYE medium was optimal for transformation in the presence of CSP [132, 146, 148, 298]. In our assays, since the effect of copper on \textit{S. mutans} transformability is dramatically pronounced in CDM relative to that under THYE, we speculate that its influence on genetic competence primarily occurs via the XIP-induced signaling pathway. Moreover, the observation that of all competence-related genes tested (e.g., \textit{comCDE}, \textit{comRS}, and \textit{comX}), only \textit{comS} and \textit{comX} expression was significantly affected under our test conditions provides additional evidence that the effects of copper on transformation is modulated by the XIP-ComRS pathway. The effect of copper on the expression of \textit{comS} but not \textit{comR} suggests that copper represses the activity of ComX by affecting the function or secretion of ComS and/or XIP, thus causing a dramatic reduction in transformation frequency. In the absence of copper, a significant reduction in transformation frequency of the \textit{ΔcopYAZ} relative to wild type implies the importance of this operon in genetic competence development in \textit{S. mutans}. Previously, in \textit{Streptococcus pneumoniae}, inactivation of a putative metal transporting operon (AdcCBA) was shown to have a competence-deficient phenotype.
[338-339]. The operon was speculated to be involved in zinc transport and the addition of zinc improved the transformability of the \textit{adc} null mutants [338-339]. In our study, although the mechanism involved in modulation of competence by the \textit{copYAZ} is not fully understood, two suggestions can be proposed based on the observed transcriptional repression of \textit{comS}: 1) the CopYAZ may have a role in modulating the function, processing, or export of XIP, and/or 2) the membrane perturbation resulted due to loss of the CopA may hinder the process of DNA acquisition resulting in defective transformation frequency.

Conclusively, results from the present study enhance our understanding about the effects of copper on \textit{S. mutans} survival under planktonic and biofilm growth conditions. We also demonstrated the role of the \textit{copYAZ} system in maintaining \textit{S. mutans} physiology under stress conditions. Insights into the mechanism of copper toxicity and its connection with biofilm formation and genetic transformation is instrumental in understanding and devising new strategies to utilize copper as an effective anti-biofilm agent to combat \textit{S. mutans} infections. Since, \textit{S. mutans} can be considered as an ideal model organism to study the genetics and physiology in pathogenic Gram positive bacteria [340], our study holds relevance in suggesting the importance of copper acquisition and homeostasis in closely-related pathogens.

### 4.7 Acknowledgements

We acknowledge the technical assistance provided by Ms. Kirsten Krastel in performing the MIC assays and DNA cloning for this study. This research was supported by NIH RO1DE013230-03 and CIHR-MT15431 to DGC. CML is a recipient of a Canada Research Chair. KS is a recipient of Cell Signaling Fellowship CIHR-STP-53877.
5 Summary and Conclusions

This dissertation characterizes the importance of copYAZ-mediated copper homeostasis in modulation of stress tolerance and biofilm formation, two important virulence factors of S. mutans. Our results show that copper-induces toxicity in S. mutans UA159 by generating oxidative stress and dissipating its membrane potential. Furthermore, we established that copper exposure impaired various important physiological attributes of S. mutans such as: 1) acid tolerance, 2) genetic transformation and 3) surface adherence to initiate biofilm formation. Using gene expression analysis we have shown that copper had a repressing effect on the transcription of S. mutans genes involved in biofilm matrix formation, genetic competence and acid tolerance. We have also demonstrated the importance of copYAZ in copper homeostasis by facilitating copper expulsion from S. mutans, which otherwise can exert a detrimental effect on the survival and stress tolerance (towards copper, acid and oxidative stress) of this bacterium. In S. mutans, the loss of copYAZ resulted in cells having a dissipated membrane potential compared to wild type in spite of controlled ionic environment. In conclusion, the inhibitory effects of copper, and importance of copYAZ in affecting several virulence features in S. mutans, provides strong evidence in associating copper homeostasis with the bacterium’s ability to adapt and survive under fluctuating environments.

5.1 Effects of copper on bacterial physiology

The medicinal use of copper dates back to the 19th century, where a variety of copper preparations -including metallic surfaces and coatings- were utilized to treat bacterial and viral infections [341]. However, the application of copper was reduced with the advent of new and efficient antibiotics in antimicrobial therapeutics. In the last few decades, the acquisition of antibiotic resistance genes by the pathogenic bacteria via the process of natural selection has challenged their eradication, thereby, posing a huge threat against clearance of bacterial infections. The use of metals as antimicrobials have gained special interest in recent years due to their ability to disrupt antibiotic-resistant biofilms, to exert synergistic bactericidal activity with other biocides, and to inhibit metabolic pathways in a selective manner [162, 342]. The
conventional antimicrobial activity of metal ions allows them to participate in targeting specific or discrete metabolic reactions, depending upon the physical and chemical properties of both the metal atoms and the accessible donor ligands within the intracellular biomolecules [342].

One such transition metal ion, copper, induces killing of the antibiotic resistant bacterial populations, either via direct contact killing or by copper in solutions. The direct contact with copper surface exhibits a 7-8 log higher rate of killing than when exposed to copper in solutions [343-345]. There are various proposed models to understand the mechanisms by which copper renders toxicity in different bacterial species. Bacteria usually initiate a global adaptive genetic response to copper insult, which involves induction of stress regulons [314, 316-317]. However, the range of copper induced toxic effects can be determined from the stretch of regulatory response it generates in particular species. The difference in the copper-responsive regulons between bacterial species depends on their physiological copper requirements and the presence of copper in their respective niches [172, 346-347]. In this study, we made a significant progress in establishing the effect of copper in modulating the transcription of the vital genes involved in stress tolerance, genetic competence development, and biofilm formation in *S. mutans*.

We demonstrated a novel inhibitory effect of copper on *S. mutans* biofilm formation. Addition of copper reduced the ability of *S. mutans* to adhere to a surface, thus leading to poor biomass production. Moreover, with copper supplementation, there was an over 2-fold repression in the transcription of genes encoding GTFs and GBP. We know that GTFs and GBP are required for sucrose-dependent bacterial adherence to the tooth surface and to pre-adhered bacteria, which provides a link for the copper-mediated biofilm impairment in *S. mutans* [63, 117, 119, 127].

Herein, we demonstrated that copper induced an oxidative stress in *S. mutans* and instigated the depolarization of membrane potential. The disturbance in the ion gradient across the cell membrane can result in hyper-polarization (higher negative intracellular electrical potential) or depolarization (higher positive intracellular electrical potential) of the membrane potential. Metal cations, like copper, tend to accumulate on the inner side of the cell membrane making it more positive and thus causing membrane depolarization. Addition of copper induces oxidative stress and causes severe growth defects in *S. mutans* cells growing under acid stress, while suppressing the expression of related acid stress genes. Since, both the oxidative and acid stresses can be
affected by variations in the bacterial membrane integrity and/or changes in the electron transport across the bacterial membrane as shown in retrograde studies, we suggest that these defects are the consequences from the copper affecting membrane potential in *S. mutans* [326, 334-335, 348]. Furthermore, it can be postulated that in *S. mutans* as a consequence of copper-induced membrane depolarization, other cellular processes such as cell division, integrity of cellular membranes and dissipation of cellular energy might also be affected, where these processes vastly rely on maintenance of membrane potential [325, 336]. Future studies are warranted to study the specific targets involved in copper induced toxicity in this oral pathogen.

The presence of copper in dental amalgams and drinking water has been reported to inhibit *S. mutans* growth and reduce caries formation [287]. In this study, we demonstrated copper-dependent inhibition of *S. mutans* biofilm formation and its effect on attenuating cell survival under acid-stress. Residing in the highly fluctuating oral environment, the integral ability to tolerate the stresses and proliferate and sustain life as a biofilm are considered as the two major virulence attributes in *S. mutans* [349-350]. Our study provides strong evidence by which copper affects these virulence features on physiological and molecular levels, thereby, holding strong relevance in offering the background knowledge of copper utilization as an effective antimicrobial against dental caries. More detailed discussion is presented in section 4.6.

### 5.2 Characterization of the copYAZ operon

The ability of bacteria to survive under copper stress depends on the expression of the genes involved in copper resistance and tolerance. As described previously, the principle mechanisms of copper tolerance include: trans-membrane copper expulsion from the cytoplasm to periplasm or to extracellular milieu; sequestration of copper by metallo-proteins; and oxidation of toxic cuprous ions to less toxic cupric ions by multi-copper oxidases. Bacterial copper transporting P$_{1B}$-type ATPases (a sub-class of P-type ATPases specialized for heavy metal ion transport) are highly specific for copper (in some cases they also transport silver cations); disruption in their function results in intracellular accumulation of copper cations (and/or silver cations) and modulation of bacterial virulence [190, 212, 293, 351-352]. All bacteria possess at least one
copper exporting P1B-type ATPases to expel excess cytoplasmic copper. Mutations in the genes encoding for copper transporters results in increased sensitivity to stresses and decreased virulence in many bacteria such as *P. aeruginosa* and *S. pneumoniae* [315, 353]. *E. coli* utilizes copper P1B-ATPases to cope with host phagosomal oxidative burst during infection [354]. More examples are reported in the literature describing the role of the copper exporting ATPases in modulating certain physiological features required for bacterial colonization and pathogenesis [191, 312, 347].

*S. mutans* have a *copYAZ* system, and in this study we have demonstrated its specific role as a copper exporter. The strain lacking *copYAZ* accumulated significantly higher amounts of copper within the cell as compared with the wild type UA159 strain. The intracellular copper accumulation in our study coincides with various published reports, which suggest the involvement of an unidentified copper-specific uptake system, or an unknown passive non-specific copper uptake mechanism in *S. mutans* [208, 347, 355]. We tested two putative cadmium transporter ATPases, SMU_723 and SMU_2057 with high sequence similarity to the *S. mutans* *copA* gene, which did not show difference in copper sensitivity compared with the wild type, thereby, we dismissed the role of these putative transporters in *S. mutans* copper transport. The role of *copYAZ* in copper efflux, was substantiated by utilizing a *E. coli* strain lacking *copA* that was complemented with the *S. mutans* *copYAZ* operon. The presence of *S. mutans* *copYAZ* significantly reduced the intracellular copper ion accumulation in the test *E. coli* mutant, thereby, reinforcing the role of *S. mutans* CopYAZ in copper efflux. The *S. mutans* ∆*copYAZ* strain was complemented with *copYAZ* operon, which restored copper efflux and further validated the function of this system in *S. mutans* copper export. We have confirmed that the *copYAZ* affords a resistance against copper-induced toxicity by specifically expelling copper; while other test metal stressors such as silver, cadmium, mercury did not result to an increased killing in *copYAZ*-null mutant.

Amongst other stress-related phenotypes of *S. mutans*, we observed that ∆*copYAZ* cells exhibited a depolarized membrane phenotype suggesting a state of compromised cell membrane integrity or a significantly increased accumulation of intracellular cations. The gradual increase in membrane depolarization observed in ∆*copYAZ* can be due to membrane perturbation caused by the loss of the CopA trans-membrane protein. This is in consonance with the previous reports
showing that the loss of the metal efflux system have a membrane depolarization phenotype [283].

Figure 5-1 Summary of role of copper and copYAZ operon in S. mutans

The loss of copYAZ also resulted in sensitivity to killing under acid or oxidative stress, thereby, emphasizing the involvement of this operon in general adaptive response of S. mutans. As described previously, the involvement of copYAZ in modulating membrane potential partially explains the sensitivity of ΔcopYAZ to acid and oxidative stress. Conclusively, this is the first study to identify and characterize the role of copYAZ as the specific copper efflux system (
Figure 5-1), which has significant implications on stress induced physiological adaptation in *S. mutans*.

### 5.3 Effect of copper and CopYAZ in genetic competence

Association of metal ions and genetic competence has been previously reported in Gram positive bacteria; for instance, calcium in *S. pneumonia* [296] and either calcium or magnesium in *Azotobacter vinelandii* [295, 297] are considered as important factors in competence development. Previously, in *S. mutans* the development of genetic competence is shown to be associated with the bacterium's ability to form biofilms and to tolerate stress [143-144, 147, 153]. In this study, we also established the effect of copper in modulating *S. mutans* stress tolerance response and its ability to acquire foreign DNA. We demonstrated that copper supplementation resulted in a 20-fold repression of the natural genetic transformation frequency in *S. mutans* and a simultaneous 2-fold repression in the expression of *comS* and *comX*, (involved in XIP-mediated competence development). The deletion of *copYAZ* also resulted in a significant 30-fold reduction in the transformation frequency compared with UA159 strain and repression of *comX* and *comS* relative to wild type strain, by 2.5 fold and 8.5 fold respectively. These results clearly signify the importance of the *copYAZ* system in modulation of genetic competence in *S. mutans*. Taken collectively, we have demonstrated the novel activity of copper and copper transport system in modulating genetic competence development in *S. mutans* (Figure 5-2). These results imply the importance of copper and *copYAZ* system in circumventing the ability of *S. mutans* to uptake foreign DNA that may assist bacteria in acquiring resistance against certain antimicrobials and/or host factors. Moreover, copper and *copYAZ* dependent impairment in genetic competence development might also result in compromising *S. mutans* ability to cause infection in a changing environment [151]. More detailed description is provided in section 4.6.
Figure 5-2 Effect of copper and copYAZ on the competence regulon of S. mutans
6 Future directions and significance

6.1 Future directions

In this study, our specific objectives were to delineate the mechanism involved in copper-mediated cell toxicity, to define the systems involved in copper transport, and to enhance our understanding about copper homeostasis. We made significant progress towards understanding the phenotypes associated with copper in *S. mutans*, which lays the foundation to several important questions. It is predicted that CopZ functions as a copper-chaperone to translocate copper within the cytoplasm. However, the protein machinery involved in copper exchange between the cytoplasm and extracellular milieu still needs further exploration. Moreover, the chemistry of copper's affinity to associate with these proteins, and their detailed role in homeostasis awaits further exploration. Future research is also warranted to investigate the importance of the copper transport and resistance machinery in modulating *S. mutans* pathogenesis and virulence in the host. The homeostasis of other metal ions, except manganese and iron, has not yet been defined in *S. mutans*, further investigation studying these homeostatic systems and the possible cross talk between their pathways involved in homeostasis also remains an interesting area to be explored.

Metal ions with high reactivity in the Irving–Williams series can replace less reactive metal ions from metalloprotein complex [184]. Considering the fact that copper is a highly reactive, it can be postulated that copper can affect the functioning of various metalloproteins in *S. mutans*. Emerging research have also shown that metal sensors and transporters participate in allowing the cell to overcome inadequate protein:metal affinities; thereby, assisting the metalloproteins to acquire the right metal ion [356-357]. Future experiments need to be directed towards understanding the possible role of *S. mutans* CopYZ system in modulating the association of copper with specific metalloproteins. Understanding the mechanisms of how *S. mutans'* physiology is regulated by cellular copper holds a strong relevance in devising new therapeutic measures, where metal transporters and sensors proteins can be targeted to circumvent its pathogenicity.
One of the most significant contributions of this study was to provide evidence relevant to association between copper/CopYAZ system and genetic transformation in *S. mutans*. While the impact of addition of copper or absence of *copYAZ* on repressing the transcription of *sigma-X* and *comS* provide an explanation for the observed genetic transformation defect, the mechanism by which copper and *copYAZ* regulate the expression of these genes remains unclear. Copper can be postulated to modulate the processing, sensing and/or uptake machinery of precursor peptide; thereby affecting the XIP mediated genetic transformation. The impairment in the genetic transformation can also be a result of altered membrane potential as observed in ∆*copYAZ* strain relative to wild type. Future quantification of secreted XIP in presence and absence of copper stress can be done to provide an explanation if the peptide secretion or uptake through specific system is affected. Further, a proteome analysis in presence or absence of copper stress is required to understand the mechanisms underlying copper related phenotypes observed in this study.

In the past few decades, instrumental progress has been made in understanding the metal utilization pathways and identifying metalloproteins [179, 195]. However, the computational and systems biology analyses of the total content metallomes (entirety of metal and metalloid species within a cell or tissue type [358]) and metalloproteome (proteome associated with the metallome [358]) are limited. There is a direct need to understand the metal transport systems in terms of their origin (which would identify the functional environment), how these systems were first acquired (categorically linking the genetic exchange), and if these systems are involved only in response to a signal or can behave as a source of a signal. Although, an advanced computational and comparative genomics study suggested a common evolutionary origins of metal transporters and their regulatory signal transduction in bacteria [276], further studies are warranted to understand the functional diversification of these systems across the bacterial kingdom. Moreover, in oral cavity, studies should also be conducted utilizing comparative genomic approaches to decipher the importance of copper and other metal ions in the physiology and metabolism of oral microbes. Results from such studies can help to design novel therapeutic measures to reduce oral infections; for instance, manipulation of the metal-dependent physiological processes in accordance to inhibit disease-associated but encourage health-associated microbial colonization in the oral cavity.
6.2 Significance

Significant advances have been made over the past few decades on illustrating the importance and homeostasis of essential transition metal ions in bacteria. These efforts have led to fruitful information regarding the mechanisms that bacteria employ on regulation, utilization, transportation and detoxification of metal ions. Here we described the mechanisms by which copper renders toxicity in \textit{S. mutans}. An emphasis was made on demonstrating these mechanisms on physiological and molecular levels. \textit{S. mutans} ability to form biofilms and tolerate fluctuating environments provides it with a competitive advantage over many commensal organisms in the oral cavity. A number of studies have reported the importance of other metal ions in \textit{S. mutans}, for instance, manganese and iron acts as a co-factor of superoxide dismutase; while zinc have inhibitory effect on the GTF activity, acid production, and glycolysis [359-361]. The transport and homeostasis of iron and manganese in \textit{S. mutans} has been attributed to SloABC system, and is associated with cell adherence, biofilm formation, genetic competence, and oxidative stress tolerance [164, 167]. Another metal cation thoroughly studied for its importance in metabolism of \textit{S. mutans} is calcium. In \textit{S. mutans}, calcium ions activate CiaRH TCS, which is involved in acid tolerance and production, biofilm formation, and development of genetic competence [165]. These studies establish the foundation towards understanding on the importance of transition metal ions and their regulatory role in modulating the various physiological processes in \textit{S. mutans}. In our study we focused on understanding copper-induced toxicity and the effect of copper on several virulence factors in this oral pathogen. Copper has an inhibitory effect under both planktonic and biofilm growth conditions in \textit{S. mutans}. Interestingly, during planktonic growth copper induces toxicity by the generation of oxidative stress and disruption of membrane potential; whereas during biofilm formation copper represses the transcription of genes associated with matrix production resulting in poor biofilm biomass production. Copper's effect on biofilm formation and its involvement in stress response makes it an ideal antimicrobial candidate against \textit{S. mutans} infections. In several bacteria, metal transporters are known to play a crucial role in bacterial virulence [171, 179, 182, 338] and with our study we demonstrated the significance of \textit{copYAZ} operon in copper transport, genetic transformation, and stress response in \textit{S. mutans}. While further genetic and biochemical studies are warranted to decipher the underlying genetic pathways that modulate these phenotypes,
results from our study have set a platform to understand the important role of \textit{copYAZ} system in modulating the growth and survival of \textit{S. mutans} under stress and normal conditions. Understanding the mechanisms by which copper exerts its toxicity in \textit{S. mutans} can have implications in devising novel clinical applications of copper in anti-caries therapeutics. Since \textit{S. mutans} is considered as a Gram positive paradigm, our study also provides a basic background required to study other copper homeostatic systems in other closely related bacterial pathogens.
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