CHAPTER V

DISCUSSION

When bacteria are grown in an oxygenated environment they need mechanisms to defend against the damage caused by reactive oxygen species such as superoxide radicals (O_2^-), H_2O_2, and hydroxyl radicals ($^{\bullet}OH$). They use enzymes such as superoxide dismutases (SODs), catalases and peroxidases for protection. The role of manganese as a co-factor for superoxide dismutase (SOD) has been well-established. The lower plateau O.D. of the Mn-SCDM cultures compared with BHI cultures was due to the more limited supply of nutrients within the defined medium. The relatively long lag phase observed in SCDM cultures implied that the bacteria needed to adapt to the less nutrient rich environment. However, once adapted, they were able to multiply at a rate comparable to bacteria in nutrient rich environments. The very low population densities measured in most cultures grown in Mn-depleted media under aerobic conditions (except UA130) suggested that the culture were greatly impacted by the absence of manganese and strongly supports a role for manganese in enzymatic activity against oxygen stress.

Strain UA130 showed only a 34.4% decrease in growth in Mn-depleted media under O_2-enriched 5% CO_2. Under condition 1 (5% CO_2) UA130 could utilize manganese at any of the concentrations tested with no negative effect on growth, probably due to the ability of this strain to take up more manganese than the others resulting in the highest yields among all strains tested. Strains UA159 and 3209 also grew under these conditions though not as richly. We can only speculate on why these strains could grow under aerobic conditions in Mn-depleted media. It is probable that other enzymes, including peroxidases and oxidases, can contribute to the capacity for aerobic growth [144,145]. The full consequences of prolonged O_2 stress were not explored. However, it was noted that strains inhibited from growth in Mn-depleted media were able to resume growth when transferred into an enriched medium (data not shown). UA130 and UA159 were the least sensitive to Mn deprivation which may partly explain differences in virulence between strains.

Our study indicated that the optimal concentrations of manganese for growth were 50 and 100 μM. The manganese concentration of 50 μM gave the best yield among all concentrations tested for each strain, except for UA130 which grew best with 100 μM manganese in condition 1 and 3. This is consistent with the fact that concentrations of dissolved manganese in natural waters can range from 10 to >10,000 μg/litre (0.18 – 182 μM) [146]. The metal chelating resin, Chelex® 100, used to prepare our media lowered the background manganese concentration to less than 0.05 μM. In addition to testing the requirement for manganese we explored manganese toxicity. Although a trend of lower yields of growth was observed when higher concentrations of manganese were added, significant drops in O.D. for strains 3209, Ingbritt and ATCC 25175 suggested a toxic effect from manganese. Strains 3209 and Ingbritt exhibited profound growth inhibition at manganese concentrations in excess of 200 μM when oxygen was present (condition 1 and 2) which is considered a relatively low concentration. Interestingly, ATCC 25175 was affected by a Mn concentration of 300 μM in all conditions. Since a role for manganese in getting rid of reactive oxygen species is widely accepted [132,147], the toxic effect observed at 200 and 300 μM in the oxygenated atmosphere was extremely interesting. We would naturally assume that a Mn toxic effect would be present in an anaerobic atmosphere where it would tend to be needed less. We hypothesize that excessive manganese may negatively affect particular genes involved in growth within oxygenated atmospheres while not affecting the repressed state of those genes under anaerobic conditions. This idea is supported by studies that showed repression of two glycolytic enzymes, enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), when *Lactobacillus plantarum* was grown in manganese-rich medium (100 mM) [5,148]. Another possible explanation is that excess manganese may interfere with iron (or other essential divalent cations) uptake which could result in starvation for other trace metals.

Microarrays were performed with RNA recovered from bacteria incubated in an anaerobic atmosphere. Growth was not evident since we collected cultures only 2 hours after they reached an O.D. of 0.2 which is rather low but enough to see the effect of manganese on gene transcription. The expression of Mn SOD (SMU_629) showed an insignificant difference between the absence and presence of Mn in the anaerobic

environment (Table 4.4, +1.15 fold change with p-value of 0.217). Muramidase (SMU_76) and Hit-like protein (SMU_412c) mRNAs were a few examples of what may happen in the process of the growth differences between the two conditions. Muramidase functions in cell wall activity. The histidine triad (HIT) protein is extremely well conserved not only among prokaryotes but in higher eukaryotes like humans where it is known to be a tumor suppressor gene [149,150]. When Mn was plentiful, the muramidase activity was higher than when Mn was depleted, whereas, we can speculate that the putative function of the Hit-like protein in *S. mutans* might depress cell multiplication if its prokaryotic function is similar to that in eukaryotes. We propose that deprivation of Mn may somehow affect the transcription of this putative hit-like protein and repress the cell cycle activity of *S. mutans*.

The observation that *S. mutans* ATCC 25175 and LT11 displayed poor growth in the absence of manganese even under anaerobic conditions strongly suggested another role for this trace metal beyond oxygen intermediate detoxification. Iron has a well established role in promoting bacterial pathogenicity, yet other metal ions have not been as extensively studied. An increasing body of evidence has highlighted roles for manganese. For example, two different DNA binding transcriptional regulators in *Salmonella enterica typhimurium* are known to be co-factored by manganese, Fur and MntR [118-124]. Fur was also found to regulate some genes in response to acid pH [125]. In *Bacillus subtilis*, MntR, the manganese–dependent repressor, was demonstrated to repress *mntH*, a gene whose product belongs to the natural resistance associated macrophage protein (NRAMP) family. In contrast, the MntR acts as a transcriptional activator of the *mntABCD* operon, encoding an ABC manganese transporter under low manganese conditions [14,127,128,151]. Upregulation of an ABC-type Mn transporter under manganese limiting conditions was also observed in the dental plaque colonizer *Streptococcus gordonii* [13]. The *gpmA* gene (glycolytic enzyme, phosphoglycerate mutase) in *Treponema pallidum* is regulated by a Mn-binding transcription factor, TroR [12]. Recently, the *S. mutans* SloR adhesin in the LraI family (SMU_184) was proposed as a manganese importer due to homology with TroR and DtxR [116]. Furthermore, manganese has recently been shown to play a role in regulating morphological changes in *Mycobacterium tuberculosis* during cell division

through a eukaryotic-type serine/threonine kinase [129,130]. Clearly, manganese can make several contributions to bacterial metabolism beyond an association with superoxide dismutase.

In the planktonic state under Mn depleted conditions, our microarray data revealed high expression of Mn transporter gene-SMU_770c (three-fold increase), and increases in a few genes that are putatively involved in Mn transport system: the Mn/Zn ABC transporter and Mn or Zn ATP binding protein (SMU_183 and SMU_182) had 26- and 22-fold increases, respectively. This could be explained by the bacteria recognizing a critical need to acquire Mn for cellular functions. They kept producing more Mn transporter in an effort to bring in more Mn into the cells. In our study, the effect of Mn deprivation on these genes indicated that they are likely involved in the acquisition of essential cationic trace elements, especially Mn. We cannot conclude that manganese is the sole cation involved since its function is based only on our microarray results. However, we conclude here that they may be involved in Mn transport or in some way, respond to the absence of manganese. Direct testing will be necessary in order to make a firm conclusion.

Another gene of potential interest was SMU_184 (Table 4.4) defined as a putative metal binding protein similar to Tro in *Treponema pallidum* [12], or to the surface adhesion precursor and saliva binding protein in the LraI family (lipoprotein receptor antigen I). The LraI family of polypeptides are surface-associated lipoproteins (32–33 kDa) involved in adherence of streptococci to the salivary glycoprotein pellicle and to oral *Actinomyces* [152]. The mRNA was expressed 20-fold more under conditions of Mn deprivation than when grown in Mn-supplemented culture. Though the environments accompanying the microarray and adherence experiments may be somewhat different, the product of SMU_184 may contribute to the adherence in our saliva-coated biofilm along with WapA and SpaP.

The effects of manganese on biofilm formation were interesting and may indicate how *S. mutans* responds when nutrient-deprived. Clearly, the lack of manganese induced bacterial clumping. Ordinarily, aggregation of *S. mutans* is associated with the synthesis of glucans from sucrose. In this instance, however, the bacterial aggregation

was more pronounced in the absence of sucrose. Although the Mn-supplemented and Mn-depleted biofilms appeared qualitatively different from one another both in the presence and absence of sucrose, the presence of sucrose suppressed most quantitative differences as measured using COMSTAT software. The bacterial clumping may be a means whereby the bacteria come together to pool their nutritional resources, perhaps by parasitizing some members of the aggregate. At the same time the formation of large aggregates may preclude efficient biofilm formation allowing the bacteria to more easily move to new sites that might be more nutritionally abundant. Determining the basis for the clumping may have to further transcriptional analysis of *S. mutans* under Mn-supplemented and Mn-depleted conditions.

Biofilm formation is initiated by interaction between planktonic cells, and a surface in response to appropriate environmental signal. It requires many metabolic and physiological changes to go from the planktonic state into a well differentiated structure [153]. Various gene regulation systems, including quorum sensing have been found to be involved in this process [154]. Bacteria use quorum sensing as means of cell-cell communication and instrument for establishing a specific niche. In Gram positive bacteria, the quorum sensing system generally consists of three components, a signal peptide, and a two-component signal transduction system involving a histidine-kinase membrane associated receptor, and an intracellular response regulator [155]. When activated, the receptor autophosphorylates itself on a histidine residue that is usually part of the intracellular domain, and then transfers that phosphate to an aspartate on the response regulator, which most often acts as a transcriptional regulator. The data from our microarray analysis of planktonic cells suggested that *S. mutans* responds to the presence/absence of manganese. The response included higher expression of the competence response genes involved in quorum sensing (SMU_1915, SMU_1916c, SMU_1917c, SMU_1983c, SMU_1984c, SMU_1985c, SMU_1987c — Table 4.4) [156]. This may suggest that the bacteria recognize a nutritionally deprived state where some bacteria may turn themselves into static phase while the others are primed to acquire advantageous DNA sequences. Many Gram-positive and Gram-negative bacteria also possess the *luxS* gene. This highly conserved gene is present in *S mutans* and its 990 bp encode a protein of 160 amino acids similar in size to other reported LuxS proteins

[157]. The LuxS enzyme is primarily responsible for the production of autoinducer-2 [158]. A previous study demonstrated that *S. mutans* biofilms formed by a strain mutated in *luxS* displayed a more granular appearance than the typically smooth, confluent layer normally seen with the wild type [157]. Our study has shown that a more aggregated, granular appearing biofilm was formed under Mn depleted SCDM. Our microarray data showed upregulation of the *luxS* gene (SMU_474c) under those conditions. When these observations are considered along with the finding that several highly conserved amino acids in the LuxS protein are reported to coordinate binding of the Zn^{2+} ion and form the catalytic center of the protein [159], we can speculate that the more granular appearance of the Mn-depleted biofilm was due to a defect in the function of LuxS due to a requirement for another trace metal such as Mn. Furthermore, *luxS* gene was suggested to regulate the *gtfB* and *gtfC* expression with mechanism is yet to explore [160].

Some studies have suggested that manganese availability can influence caries potential [2-4,103]. Although the effects of manganese likely can be manifested in multiple ways, one plausible manner is to influence the expression of *S. mutans* genes encoding virulence factors. We have demonstrated here that manganese affects the expression of several *S. mutans* virulence genes that promote sucrose-independent and -dependent adhesion, as well as biofilm development. The changes in gene expression were consistent between the different methodologies employed for analyzing planktonic cultures, but differences were noted when comparing virulence gene expression between planktonic and biofilm cultures.

In the planktonic state the *spaP* gene product, believed to promote sucrose-independent adhesion, was slightly up regulated despite the fact that an adherence assay demonstrated decreased sucrose-independent adherence of bacteria grown under manganese-supplemented conditions. It is tempting to conclude that the down regulation of *wapA* was responsible for the decreased adhesion though an effect by other genes cannot be ruled out. In biofilm cultures the expression of *spaP* reversed and showed a slight decrease in the presence of manganese. It can be speculated that down regulation of sucrose-independent adhesins within the biofilm may indicate they

are not needed to maintain biofilm integrity. Alternatively, the relatively higher expression of *wapA* and *spaP* in the absence of manganese may promote the likelihood of adhesion to nutritionally rich sites.

The results from the gel activity assay suggested that glucosyltransferase enzyme activity did not significantly fluctuate between Mn-supplemented and –depleted growth conditions. This differed from the results at the transcriptional level. This may mean the time frame of our experiment was sensitive enough to detect changes in transcription that may not yet have been notable at the protein level. Alternatively, it may indicate that the transcriptional differences were not indicative of protein activity. The activity assay showed prominent differences for the fructosyltransferase enzyme. This may have been due to less cellular activity under Mn-depleted conditions, so that energy was conserved rather than used to synthesize energy stores.

Sucrose-related virulence genes generally showed higher or steady expression when grown in the presence of manganese either in a biofilm or in a planktonic state. The exceptions were *gbpA* and *gbpD* which showed decreased expression within a biofilm. The GbpA and GbpD have been linked to biofilm elevation [Hazlett et al., 1999 and unpublished data]. Decreasing levels of these two extracellular Gbps might provide protection against building the height of the biofilm beyond its limits of cohesion. Conversely, considering it from the perspective of manganese deprivation, the relatively higher levels of GbpA and GbpD might represent an attempt to remodel the biofilm architecture in order to improve diffusion of exogenous nutrients. The concomitant down regulation of *gbpC* in the absence of manganese would at first appear contradictory. However, at the population level it might also be beneficial to down regulate genes that promote biofilm cohesiveness so that some organisms can detach and find more nutritionally beneficial sites for colonization. The relatively lower expression of the glucan synthesizing genes *gtfB* and *gtfC* would fit this scenario. The *rgg*, is upregulated in the Mn-depleted media in both planktonic and biofilm grown cultures. It is a positive regulator of *gtfG* in *S. oralis* and *S. gordonii* [161]. Unlike these two, *S. mutans rgg* is not located upstream of the *gtf* gene [162]. However, based on the result in this study, we propose that *rgg* may directly or indirectly regulate the *gtfB*, since *gtfB* was down

regulated in both planktonic bacteria and biofilm cultures under Mn-depleted conditions. These data may indicate that the Rgg regulatory protein functions in a metal dependent fashion. Since the location in the genome of *gtfC* is downstream of *gtfB*, it is surprising that *gtfC* is not similarly downregulated to *gtfB*. Rather *gtfC* expression was not significantly different in the biofilm culture. We speculate that there the greater water-solubility of the glucan synthesized by the *gtfC* gene product may be more advantageous to *S. mutans* under this nutritionally deprive state. The upregulation of *wapA* and *spaP* within the manganese-depleted biofilms could mean that *S. mutans* is primed for sucrose-independent attachment elsewhere if dissociated from the biofilm.

CONCLUSION

Dental plaque is a complex community comprised of many bacteria residing in the oral cavity plus various organic materials in saliva. The manganese content in saliva and dental plaque can vary over a wide range. The strain-specific responses documented in this study to the absence of manganese, as well as to its abundance, suggest that the relative availability of manganese may help shape the ecology of dental plaque by influencing colonization with particular strains of *S. mutans*. These strains might harbor differences in cariogenic potential or be susceptible to manganese influences on virulence gene expression that may explain earlier reports linking manganese levels with dental caries.

The data clearly suggest that manganese availability has an impact on the expression of *S. mutans* virulence factors. However, the mechanism behind the effect may be indirect. The microarray data revealed a manganese effect on an assortment of genes including the *S. mutans* homologue encoding the Rgg transcriptional regulator. It is conceivable that there are several layers of regulation, some of which may be gene-specific and others global. The altered expression of virulence and biofilm-related genes as a function of manganese availability may suggest that *S. mutans* has evolved means of responding to the nutritional state of its environment. Further investigation will

be necessary to determine if host variations in salivary levels of manganese influence the epidemiology and virulence potential of select strains of *S. mutans*.

FUTURE STUDY

Microarray is a very powerful tool in that it generates a handful of data from the whole genome in one experiment. The results from this study have suggested numerous possibilities for further exploring the effects of Mn on *S. mutans* behavior as well as for understanding the mechanisms that accompany changes in gene expression. There must be certain mechanisms that control how the cells sense and regulate in response to Mn deprivation whose specific identity awaits discovery. Comparative microarrays of biofilm cultures may also help us understand how Mn can affect the transition from a planktonic state in oral cavity into the biofilm that forms on the surfaces of teeth. Clearly, cells growing in biofilms have distinct behavior from their planktonic counterparts. Our speculation related to SMU_184 may open a new aspect of research into *S. mutans* adhesion via a novel sucrose-independent pathway. Real-time biofilm formation could be monitored with the help of reporter genes engineered downstream of promoters from our gene of interest under the same sets of conditions as analyzed in this study. Further study will also focus on the strains that were insensitive to Mn-deprivation or strains that displayed a toxic effect to high Mn concentrations. The knowledge derived from these studies will hopefully allow us to manipulate strain colonization in humans by adjusting exposure to this essential trace element.