CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Media

S. mutans serotype c strains Ingbritt, LT11, 3209, UA130, UA159, ATCC 25175 and GS-5 and *S. sobrinus* serotype d strains B13, 6715 and SL1 were used to study the effect of Mn on planktonic growth; otherwise, *S. mutans* strain UA159 was used throughout the study. The bacteria were stored in brain heart infusion broth containing 15% glycerol at -80°C. Bacterial cultures were prepared in a modified chemically defined medium (semi-chemically defined medium, SCDM) originally developed by Terleckyj *et al* [131]. Ingredients in the original unmodified Terleckyj's medium that were not necessary for growth of *S. mutans* were eliminated [132] including acetate, ammonium sulfate, and citrate. Amino acids were replaced by casein hydrolysate (2 g/1). Based on the percentage of individual amino acids in the casein hydrolysate (reference guide of 2001 product catalogue for Microbiology of Difco Laboratories, Detroit, MI), as well as requirements for *S. mutans* (serotype c) for individual amino acids [133,134], cysteine, glutamic acid, and leucine were added to the medium to a concentration of 200 mg/1, 30 mg/1, and 10 mg/1, respectively. Further modifications included a decrease in glucose concentration to 0.8% (Table 3.1). High-quality water (18.2 $M\Omega$ cm^{-1} at 25°C), was used for preparation of the medium. The medium was treated with Chelex® 100 (Sigma), a metal-chelating resin, to reduce trace metal contamination and then supplemented with high-purity trace metal salts to provide the optimal concentrations required for maximal growth of the microorganism. Calcium, iron, and magnesium were added to final concentrations of 50 μM, 3.6 μM, and 126 μM, respectively [132,135,136]. For planktonic growth, when desired, $MnSO_4$ was added at varying concentrations of 50 μM, 100 μM, 200 μM and 300 μM; otherwise the optimal concentration determined from the growth study was used throughout the remaining experiments. All glassware was cleaned with 70% nitric acid and rinsed three times with distilled water and three times with deionized water.

Culture Conditions

Inocula were prepared by serial subculture in manganese-depleted medium. Two hundred milliliters of freshly prepared growth media were placed aseptically into Erlenmeyer flasks and then inoculated with 1% of the inocula from the cells growing in mid exponential phase. Cultures were incubated at 37°C in 1) a 5% CO_2 incubator (Forma Scientific), 2) a O_2-enriched 5% CO_2 atmosphere with shaking at 60 rpm for 10 seconds every 1 h, or 3) an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2) (Forma Scientific). Growth of bacteria was monitored by measuring turbidity with a spectrophotometer (Spectronic Genesis 20, Thermo Electron Corp.) at wavelength 540 nm. Brain Heart Infusion broth (BHI)(BBL; Becton, Dickinson and Company) was used as the control medium, SCDM as the test medium without manganese or with $MnSO_4$ added at a final concentration of 50 μM, 100 μM, 200 μM or 300 μM for each growth condition. All growth curves were performed in triplicate.

For the subsequent study, inocula were prepared by serial subculture as described above, then inoculated with 1% of the inocula from the cells growing in mid exponential phase. Cultures were incubated at 37°C in an anaerobic chamber (Forma Scientific) throughout the study. For isolation of RNA and protein extraction from planktonic cultures, the bacteria were grown to an optical density of 0.2 (early exponential phase) at wavelength 540 nm and then split into two cultures of 100 ml each. Fifty micromolar (μM) manganese was added to one of these. The cultures were further incubated for 2 hours before collecting.

When growing the biofilm bacteria, 70 μl of inocula were added to 1.5 ml of SCDM, plus or minus 5% sucrose, with or without Mn, in a 24-well polystyrene dish or a 2-well Lab-Teck chamber slide (Lab-Tek Chambered #1.0 borosilicate coverglass system, Nalgene Nunc Intl.). For sucrose independent biofilms, the wells were first coated with saliva as previously described [137,138]. Briefly, 1 g Lab Lemco (Oxoid), 2 g yeast extract, 2.5 g mucin (Sigma), were mixed well and treated with Chelex 100 at 4°C for 1 h before 0.35 g NaCl, 0.2 g KCl, and 0.2 g $CaCl_2$ were added, filter sterilized and stored at 4°C. When used, the artificial saliva was pipetted into wells, allowed to dry, and UV sterilized. For both sucrose and non-sucrose biofilms, pre-warmed media,

with or without Mn was then added along with the bacterial inocula. The biofilms were incubated overnight in an anaerobic chamber at 37°C on a slow rotating platform (approximately 5 rpm). (Note: the sucrose biofilms were used in the image and protein study). For confocal scanning laser microscopy, the mature biofilms were washed and then stained. For RNA isolation, after 24 hour the spent medium was aspirated and pre-warmed new medium was added and incubated for another 1 hour. The biofilm bacteria were dislodged by sonication (sonic dismembrater 60, Fisher Scientific) and the cells collected by centrifugation at 4,000 rpm for 20 min at 4°C. The bacteria were then washed in PBS and resuspended in RNase-free H_2O and stored at -80°C until used.

Table 3.1

Modification of Terleckyj's medium

Composition	MW	Concentration	Per 1 liter	Unit
Amino acids:				
Caseine hydrolysate [a, ‡]		0.20%	2.00	g
L-cystine *	240.30	0.83 mM	0.20	g
L- Glutamic acid [↓, a]	147.13	2.04 mM	30.00	mg
L-Leucine [↓, a]	131.18	0.76 mM	30.00	mg
Nucleotide bases :				
Adenine *	135.10	0.26 μM	35.00	mg
Guanidine *	151.10	0.18 μM	27.00	mg
Uracil *	112.10	0.27 μM	30.00	mg
Phosphates:				
KH_2PO_4 *	136.10	1.23 mM	0.44	g
$K_2HPO_4.3H_2O$ *	228.20	1.31 mM	0.30	g
$NaH_2PO_4H_2O$ *	138.00	14.86 mM	20.50	g
Na_2HPO_4 *	141.96	22.19 mM	3.15	g
Carbohydrate :				
Glucose [↓, b]	180.20	44.40 mM	8.00	g

Table 3.1 (continued)

Modification of Terleckyj's medium

Composition	MW	Concentration	Per 1 liter	Unit
Vitamins :				
Biotin *	244.30	0.04 μM	0.01	mg
Folic acid.$2H_2O$ *	441.40	0.23 μM	0.10	mg
Niacinamide *	122.10	16.38 μM	2.00	mg
Pantothenic acid *	476.54	1.68 μM	0.80	mg
Para-aminobenzoic acid *	137.10	0.73 μM	0.10	mg
Pyridoxine HCl *	205.60	3.89 μM	0.80	mg
Riboflavin *	376.40	1.06 μM	0.40	mg
Thiamine HCl *	337.30	1.19 μM	0.40	mg
Minerals:				
$CaCl_2.2H_2O$ ↑, c	147.02	0.68 μM	0.10	mg
$FeSO_4.7H_2O$ ↓, b	278.02	3.60 μM	1.00	mg
$MgSO_4.7H_2O$ ↓, b	246.48	126.00 μM	31.06	mg
$MnSO_4.H_2O$ ↑, a, +	169.01	100.00 μM	16.91	mg

* Same concentrations as in original medium (FMC)

↓ Lower concentrations than in original medium (FMC)

↑ Higher concentrations than in original medium (FMC)

[a] Modified in this study

[b] Modified according to Aranha et al. (1982)

[c] Modified according to Aranha et al. (1986)

‡ Used instead of other amino acids in original medium (FMC)

\+ Added to manganese-supplemented medium

Confocal Scanning Laser Microscopy (CSLM) and Image Processing

For the study of both sucrose-dependent and sucrose-independent *S. mutans* biofilms, three independent biofilm experiments were performed as described above. The mature biofilms were rinsed twice with PBS buffer. LIVE *Ba*clight Bacterial Gram stain fluorescent dye mixture (5 mM SYTO9, 7 mM hexidium iodide, and 0.3% dimethyl sulfoxide in PBS buffer (Molecular Probes, Eugene, Oregon) at a dilution of 1:1,000 was added onto the biofilm and incubated for 1 hour in the dark, followed by washing twice with 1xPBS. The three dimensional biofilm images were acquired by confocal scanning laser microscopy (Carl Zeiss, LSM 510 META-NLO). At least 5 image stacks of each sample were acquired from random positions. Images were acquired at 1.0 μm intervals down through the entire depth of the biofilm. Image information and quantification were processed by the COMSTAT program analyzed in MATLAB 5.3 (The MathWorks Inc., Natick, MA) [139]. The bio-volume (biomass volume divided by substratum area), substratum area occupied by bacteria, thickness distribution, area distribution of micro-colonies at the substratum, roughness coefficient, surface area of biomass, and surface to bio-volume ratio, including the maximum thickness of the biofilm, were recorded [139]. Statistical differences between biofilm characteristics in the presence or absence of manganese were determined using paired t tests.

Protein Extraction

Cell pellets were collected by centrifuging the bacterial cultures at 6,000 rpm at 4°C for 10 min. The supernatant was collected and saved for further protein extraction. The pellets were then resuspended in 1XPBS, transferred to 1.5 ml microtubes, and centrifuged at 14,000 rpm for 5 minutes at 4°C. The resulting supernatant was discarded, and the pellet resuspended in 75 μl 4X cracking buffer [1.5 ml 0.5 M Tris HCl pH 6.8, 1 ml SDS, 0.5 ml β-mercaptoethanol, 3 ml 100% glycerol, 4 ml H_2O, bromphenol blue] and 75 μl dH_2O. The mixture was incubated at room temperature for 2 hours, periodically vortexed, then centrifuged at 10,000 rpm at 4°C for 5 minutes, and

the supernatant transferred to a new 1.5 ml microtube for storage at -20°C until all samples were ready for SDS-PAGE and western immunoblot.

The original culture supernatant was further clarified by centrifugation at 12,000 X g for 20 min at 4°C. Protein precipitation was initiated by adding 10 ml 100% trichloroacetic acid, thoroughly mixing with the supernatant, and letting stand for at least 3 h at 4°C. The mixture was centrifuged at 18,000 x g for 30 minutes at 4°C to collect the protein precipitate. The pellets were washed twice in ice-cold acetone and spun down at 14,000 x g, dried at 70°C for 5 min, and then resuspended in 250 µl of lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Triton-X-100, 0.5% Na-deoxycholate]. Samples were used immediately or stored at -80°C.

Western Immunoblot

Equal amounts of cell-associated and secreted proteins from planktonic cultures were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membrane (Bio-Rad) using the semi-dry electroblotter (Model HEP-3, Owl Separation Systems) at 80 mA for 1½ hours. The membranes were blocked using 5% skim milk in phosphate-buffered saline (pH 6.4) with 2% Tween 20 (PBST) for 1 hour at room temperature, then washed twice in PBST for 10 min. per wash. Rabbit polyclonal antibody against the GbpC or glucan-binding domain of GbpA was added at a dilution of 1:5,000 in PBST and incubated overnight. The membranes were then washed 4 times for 5 min each in PBST. Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G diluted 1: 10,000 in PBST for 30 minutes at room temperature, and then washed 4 times for 5 min each in PBST. Signals were developed using Supersignal West Pico Chemiluminescent Substrate (Pierce). Working solutions of the substrates were prepared according to the manufacturers' instructions and added to the membranes for 1 minute. Each membrane was exposed to CL-XPosure Film (Pierces) for 30 sec.

Gtf and Ftf Gel Activity Assay

Relative amounts of Gtfs proteins were determined by performing the enzyme activity as previously described [74]. Positive control was performed with cell grown in BHI. The extraction of protein was followed according to the protocol previously stated. Determination of protein concentrations was performed based on the method of Bradford (Bio-Rad) [140]. Briefly, cell wall-associated proteins from biofilm cultures were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then washed in 0.05 M Tris-Cl pH 7.5 to remove SDS followed by incubation in 0.05 M potassium phosphate buffer, pH 6.5, 1% sucrose, and 1%Triton X-100 (Fisher Scientific) overnight at 37°C. After 24 h water-insoluble glucan bands were evident. The gel was then fixed with 75% ethanol for 30 min, followed by a 30 minute incubation in 0.7% periodic acid, 5% acetic acid at RT. The gel was washed in 0.2% metasulfite and 5% acetic acid for 20 min x 3 and incubated for 1 h in Schiff's reagent (Sigma). The intensity of the stained bands reflects the relative amounts and activities of the Gtf and Ftf proteins. Quantification of the enzyme activity was determined by the scanning densitometry.

Extraction of Total RNA

RNA samples were extracted by a hot-phenol method [141] with some modifications. Briefly, frozen bacterial pellets were thawed on ice and centrifuged. Equal amounts (500 µl) of acid phenol-chloroform (pH 4.7) (Ambion, Inc.) and NAES buffer (50 mM sodium acetate pH 5, 10 mM EDTA, 10% SDS in diethyl pyrocarbonate H_2O) were used to resuspend and lyse the cell pellet. The lysate was transferred into an ice-cold screw-capped cryovial tube stored at -20°C containing 500 µl of 0.1 mm-diameter RNase-free zirconia beads (Biospec Products, Inc., Bartlesville, OK). Cell disruption was performed by a Mini-Beadbeater-8 cell disruptor (Biospec Products, Bartlesville, OK). The lysate was extracted twice with phenol:chloroform (pH 4.7). After centrifugation, the aqueous phase was collected and precipitated with isopropanol and 3M sodium acetate, pH 5.5 (Ambion, Inc.). The nucleic acid was collected by centrifugation and washed in 70% ice-cold ethanol, and suspended in diethyl

pyrocarbonate (DEPC; MP Biomedicals, LLC, OH) treated water. The RNA was further treated with RNase-free DNAse I (Ambion, Inc.); a second treatment was done when necessary as recommended by the manufacturer. RNA was further purified by means of an RNeasy Minelute cleanup column (QIAGEN). Purified RNA was eluted from the column with a final volume of 26 µL of RNase-free water and stored at -80°C until use. RNA quality and quantity were assayed by agarose gel electrophoresis, Bioanalyzer 2100 using RNA LabChips (Agilent Technologies) and by a Biophotometer 6131 (Eppendorf) (A_{260} nm/A_{280} nm 1.9-2.1). The absence of DNA was verified by PCR using primers specific for the *S. mutans* gene.

Microarrays

A custom Affymetrix array representing antisense oligonucleotides of 17 perfect match and 17 mismatch probes for each gene was manufactured based on the sequence for *S. mutans* strain UA159 [Ajdić et al. 2002]. The antisense probes were 25 mers; the mismatch control probes were identical to the perfect match probes with the exception of a single base difference in the central position. The presence of the mismatched oligonucleotide allowed cross-hybridization and local background to be estimated and subtracted from the perfect match signal. A probe pair was called positive when the intensity of the perfect match probe cell was significantly greater than the corresponding mismatch probe cell. A probe pair was called negative if the situation were reversed. Each probe was tiled in approximately one million copies per spot, targeting 1,963 ORFs. To assure minimal cross-hybridization of the probes, genomic repetitive sequences (rRNA, IS elements, transposases and gene duplications) were used for elimination of the non-desired probes. These probes were designed using Affymetrix probe-selection software.

RNA samples from three independent experiments were processed by the array facility at the New York State Department of Health Microarray Core Facility using hybridization, washing, and scanning protocols described by Affymetrix (GeneChip Expression Analysis, 2004). Briefly, biotin-labeled cDNA was prepared from purified

RNA samples using GeneChip DNA Labeling Reagent (Affymetrix, 900542) following fragmentation. The nucleic acid was fluorescently labeled by incubating with 10 μg/ml streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and 2 mg/ml BSA in 1X MES (100 mM MES, 1 M NaCl, 20mM EDTA, 0.01% Tween 20, pH 6.6). After the streptavidin solution was removed, an antibody mix was added as the second stain containing 0.1 mg/ml goat-IgG, 5 μg/ml anti-streptavidin antibody and 2 mg/ml BSA in 1X MES.

The arrays were scanned according to manufacturer protocols and analyzed with GeneChip Operating Software (Affymetrix) and GeneSpring GX v7.3 (Agilent Technologies). Data were scaled using the proportional variance RMA method (http://discover.nci.nih.gov/microarrayAnalysis/Affymetrix.Preprocessing.jsp), then normalized to the 50th percentile using GeneSpring per chip normalization, followed by per gene normalization to specific samples. The values for the Mn-depleted samples were set to 1 and the fold change calculated for the Mn-supplemented samples.

Northern Blot Analysis

Glucan binding protein A (*gbpA*), glucan binding protein C (*gbpC*) and *gyrA* probes were generated using polymerase chain reactions obtained with primers shown in Table 3.2. The probes were labeled by Digoxigenin-11-uridine-triphosphate (DIG-11dUTP) (Roche Diagnostics GmbH). Probe concentrations were determined by immunological detection of dot blotted dilutions of the probe versus control DNA,

Northern blot analysis of *gbpC* and *gbpA* gene transcription was carried out with 10-μg aliquots of total RNA isolated from *S. mutans* UA159 strains collected under the conditions described above. Samples were performed in triplicate. The RNA was separated on a 1.2% agarose-formaldehyde denaturing gel, [RNA ladder, 0.24-9.5 kb (Invitrogen)]. The gel was rinsed in 20x SSC (3 M NaCl, 0.3 M sodium acetate, pH 7.0) for 15 min x 2 to remove formaldehyde from the gel. RNA was transferred to a positively charged nylon membrane (Roche Applied Science, Germany), using 20x SSC overnight. After transfer, the membrane was fixed by UV cross-linking using a Spectrolinker XL-

1000 (Spectronics Corporation). Hybridization was carried out at 50°C using Dig Easy Hyb (Roche Applied Science) in a hybridization incubator (Barnstead International, Melrose Park, IL). Following 18 h of hybridization, the membrane was rinsed twice for 30 min at room temperature with pre-warm 37°C of 2xSSC (pH 7.0), 0.5% SDS, twice for 30 min in 2xSSC (pH 7.0), 0.1% SDS, and for 20 minutes in 0.1x SSC, 0.1% SDS. Next the membrane was washed in washing buffer consisting of maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and 0.3% Tween 20 for 2 min. The membrane was then incubated in blocking solution (10% maleic acid buffer) for 60 min at room temperature. Anti-Digoxigenin antibody tagged with alkaline phosphatase was added at the concentration of 1:10,000 and incubated at room temperature for 30 min. The membrane was washed twice in washing buffer, and transferred to equilibration buffer. CSPD (Roche Applied Science) was added as the substrate and the membrane was exposed to X-OMAT film (Eastman Kodak, Rochester, NY). The films were analyzed with the Image J image-processing program (NIH: rsb.info.**nih**.gov/ij/)

Reverse Transcriptase Polymerase Chain Reaction

RNA was isolated and cleaned as described above. Duplicate and triplicate samples were prepared from planktonic and sucrose-independent biofilm growth, respectively. The cDNA was synthesized and amplified according to the manufacturer's protocol (cMaster RTplus PCR system, Eppendorf, USA). Briefly, template RNA was serially diluted from 1 μg to 250 pg. Initial reverse transcription was at 50°C for 30 min followed by heating to 94°C for 2 min. Following this step, the subsequent PCR reaction was performed in a total reaction volume of 20 μl. The amplification program was 25 cycles of denaturation at 94°C for 15 sec, annealing (54 - 58°C; see Table 3.2) for 20 sec, and extension at 68°C for 1 min. The final elongation was at 68°C for 7 min. The PCR products were run on a 3% agarose gel. Data analysis was performed with the with Image J image processing program (NIH: rsb.info.nih.gov/ij/) based on comparison between RNA extracted from the Mn-supplemented and Mn-depleted media, using the 16S rRNA as the control.

Table 3.2 PCR Primers used in this study.

Gene	Primer	Sequence	Amplicon (bp)	Annealing Temp (°C)
gbpA	F	5'-CGCCAATAGTTCTCCAGCCGAT-3'	410	55
gbpA	R	5'-CGAACCAGCGACTGCTGCA-3'		
gbpC	F	5'-GCCATTATGAGTCTCTCATCG-3'	478	55
gbpC	R	5'-GTCACTGGAGGAACTTCCT-3'		
gbpD	F	5'-CATGCTGGTGCAATGGTAAC-3'	401	54
gbpD	R	5'-TTCTTCTCACCGCCAATAGC-3'		
gtfB	F	5'-CAGTTGACAAAACTTCTGAAGC-3'	347	54
gtfB	R	5'-TCAACATGCTCAAAGCTCTG-3'		
gtfC	F	5'-GCTTCTGGGTTCCAAGCTAA-3'	379	55
gtfC	R	5'-GGCGCTGTCCATTAACAACT-3'		
spaP	F	5'-TCAGGCTGAACTGAAACGTG-3'	386	55
spaP	R	5'-TAGCATTCTCATTGCGTTGC-3'		
wapA	F	5'-TCCAGGATCCAGTAACAACG-3'	375	55
wapA	R	5'-GTTGTCGGAACATTCGTTTGA-3'		
rgg	F	5'-TGCTGCCAATGATTTCCAT-3'	383	54
rgg	R	5'-GACGTCGATTTCGAGGTATTTC-3'		
16S rRNA	F	5'-GGGCTTAGTGCCGGAGCTA-3'	60	58
16S rRNA	R	5'-TTTCAACCTTGCGGTCGTACT-3'		
gyrA	F	5'-CAACCATTAATTCTGTTCGGC-3'	455	55
gyrA	R	5'-CTATTGAGAAGGGTGTCCC-3'		

Adherence Assay

Planktonic bacteria from early exponential phase (Optical Density [O.D.] equal to 0.2) were divided equally into Mn-supplemented or Mn-depleted cultures and incubated 2 h at 37°C. The cells were pelleted, washed, and resuspended in KCl buffer, pH 6.8. The cells were resuspended to an O.D. of 0.8 at wavelength 540 nm. One milliliter of each cell suspension was added into saliva-coated wells prepared by modification of the protocol of Vickerman & Jones [142]. Briefly, freshly collected saliva from a male subject was centrifuged at 15,000 x g at 4°C for 15 min. The supernatant was transferred into a new 50 ml tube and incubated at 60°C for 60 min, followed by centrifugation at 15,000 x g at 4°C for 15 min. The supernatant was collected and stirred with Chelex 100 at 4°C for 1 h, filter sterilized and stored at -80°C. When used, the saliva stock was diluted with KCl buffer 1:4, pipetted into 24 microtiter wells, allowed to dry and UV sterilized. The culture was incubated anaerobically at 37°C for 1 h, then aspirated and washed in KCl buffer solution. After the second aspiration, the cells attached to the wells were resuspended in equal volume of KCl buffer and sonicated. Bacteria were serially diluted and plated on brain heart infusion agar. Colony forming units were counted and expressed as the percentage of input cells.