CHAPTER IV

RESULTS

The effect of manganese on planktonic growth

All strains of *S. mutans*, except GS-5 and *S. sobrinus* serotype d strains B13, 6715 and SL1, were able to grow upon subculturing in Mn-depleted media at 37°C in a 5% CO_2 atmosphere. For this reason *S. mutans* GS-5 and *S. sobrinus* serotype d strains B13, 6715 and SL1 were not included in subsequent experiments. All strains but ATCC 25175 when grown in BHI reached O.D. above 1. None of the *S. mutans* strains grew to as high a yield in Mn-SCDM as when grown in BHI (Table 4.1). This could be explained by limited amounts of glucose and carbon sources within the SCDM compared to the enriched nutrients in BHI. It is interesting to note that each strain grew at a different rate and obtained different maximal yields despite the same enriched media.

Results on growth curves of *S. mutans* under each culture condition are categorized into 2 groups based on the criteria of how well the bacteria grew in the absence of manganese. Each atmospheric condition is defined as condition 1, 2 or 3 as indicated in Table 4.2.

Table 4.1 Maximum optical densities ± standard error of mean (S.E.) for *S. mutans* cultures grown with and without manganese in different atmospheres

	UA159	UA130	3209	Ingbritt	LT11	ATCC25175
	O.D. ± S.E.†	O.D. ± S.E.	O.D. ± S.E.	O.D. ± S.E.	O.D. ± S.E.	O.D. ± S.E.
5%CO_2						
BHI	1.215 ± 0.037	1.158 ± 0.034	1.277 ± 0.022	1.271 ± 0.141	1.094 ± 0.048	0.707 ± 0.026
Mn-depleted	0.588 ± 0.030	0.557 ± 0.050	0.695 ± 0.059	0.256 ± 0.021	0.010 ± 0.006	0.079 ± 0.113
Mn 50 µM	0.869 ± 0.044 *	1.019 ± 0.039	0.997 ± 0.078 *	0.973 ± 0.157 *	0.787 ± 0.037 *	0.653 ± 0.084 *
Mn 100 µM	0.811 ± 0.022	1.100 ± 0.048 *	0.890 ± 0.025	0.860 ± 0.092	0.743 ± 0.050	0.654 ± 0.110
Mn 200 µM	0.834 ± 0.065	1.048 ± 0.025	0.200 ± 0.053↓	0.161 ± 0.045↓	0.710 ± 0.020	0.558 ± 0.118
Mn 300 µM	0.653 ± 0.094	0.927 ± 0.115	0.191 ± 0.050↓	0.169 ± 0.077↓	0.718 ± 0.070	0.048 ± 0.014↓
O_2-enriched 5% CO_2						
BHI	1.174 ± 0.015	1.184 ± 0.020	1.120 ± 0.02	1.174 ± 0.056	1.019 ± 0.143	0.659 ± 0.055
Mn-depleted	0.186 ± 0.065	0.728 ± 0.056	0.075 ± 0.012	0.223 ± 0.025	0.031 ± 0.024	0.009 ± 0.002
Mn 50 µM	0.852 ± 0.053 *	1.109 ± 0.097 *	0.906 ± 0.038 *	0.843 ± 0.017	0.729 ± 0.017 *	0.501 ± 0.061 *
Mn 100 µM	0.691 ± 0.102	1.095 ± 0.045	0.798 ± 0.023	0.857 ± 0.012 *	0.729 ± 0.094 *	0.413 ± 0.040
Mn 200 µM	0.720 ± 0.043	0.973 ± 0.181	0.141 ± 0.010↓	0.175 ± 0.058↓	0.732 ± 0.111	0.383 ± 0.039
Mn 300 µM	0.675 ± 0.05	1.033 ± 0.022	0.141 ± 0.041↓	0.159 ± 0.060↓	0.688 ± 0.078	0.038 ± 0.036↓
Anaerobic						
BHI	1.168 ± 0.032	1.245 ± 0.057	1.210 ± 0.117	1.120 ± 0.101	1.096 ± 0.078	1.023 ± 0.044
Mn-depleted	0.573 ± 0.017	0.895 ± 0.064	0.954 ± 0.056	0.625 ± 0.046	0.074 ± 0.083	0.171 ± 0.021
Mn 50 µM	0.841 ± 0.024 *	1.158 ± 0.033	1.130 ± 0.036 *	0.820 ± 0.139 *	0.833 ± 0.037 *	0.656 ± 0.017 *
Mn 100 µM	0.748 ± 0.012	1.173 ± 0.020 *	1.065 ± 0.052	0.786 ± 0.078	0.727 ± 0.098	0.641 ± 0.002
Mn 200 µM	0.724 ± 0.022	1.106 ± 0.045	1.065 ± 0.069	0.603 ± 0.079	0.713 ± 0.099	0.637 ± 0.050
Mn 300 µM	0.672 ± 0.025	0.999 ± 0.068	1.044 ± 0.040	0.617 ± 0.062	0.727 ± 0.071	0.243 ± 0.028↓

†, Optical density (O.D.) at 540 nm and standard error of the mean (S.E.)

*, Highest yield within a growth condition for each strain

↓, Significant drop in O.D. as higher concentrations of manganese were added.

Table 4.2 Categorization of strains according to conditions

Condition	Group 1	Group 2
Condition 1 5%CO_2	UA159, UA130, 3209, Ingbritt	LT11, ATCC 25175
Condition 2 O_2-enriched 5%CO_2	UA159, UA130, Ingbritt	LT11, ATCC 25175, 3209
Condition 3 Anaerobic	UA159, UA130, 3209, Ingbritt, ATCC 25175	LT11

Group 1, condition 1: UA159, UA130, 3209, Ingbritt (Fig. 4.1a, 4.1b, 4.1c and 4.1d)

This group could grow in both manganese-depleted medium and in media with manganese added up to a concentration of 300 μM. UA159 and UA130 grew to yields of O.D. = 0.588 and 0.557 respectively in Mn- depleted media. When 50, 100, 200 and 300 μM of manganese were added, the O.D.s were 0.869, 0.811, 0.834 and 0.653 for UA159 and 1.019, 1.100, 1.048, and 0.927 for UA130. Strain 3209 and Ingbritt had O.D.s of 0.695 and 0.256, respectively, in Mn-depleted media. These latter two strains showed significantly retarded growth at high concentrations of manganese. When 200 and 300 μM manganese were added, the 3209 yields were at 0.200, and 0.191; Ingbritt yields were 0.161, and 0.169, respectively (Table 4.1). The percentage of maximal growth is tabulated in Table 4.3.

Group 2, condition 1: LT11, ATCC 25175 (Fig. 4.1e and 4.1f)

LT11 and ATCC 25175 are categorized in a separate group since both LT11 and ATCC 25175 grew very poorly in Mn-depleted media (O.D. = 0.010 and 0.079). While both strains grew well at manganese concentrations of 50, 100 and 200 μM, the ATCC 25175 at 300 μM showed retarded growth with an O.D. = 0.048 (Table 4.1, Fig. 4.1e and 4.1f).

Figure 4.1a: *S. mutans* UA159

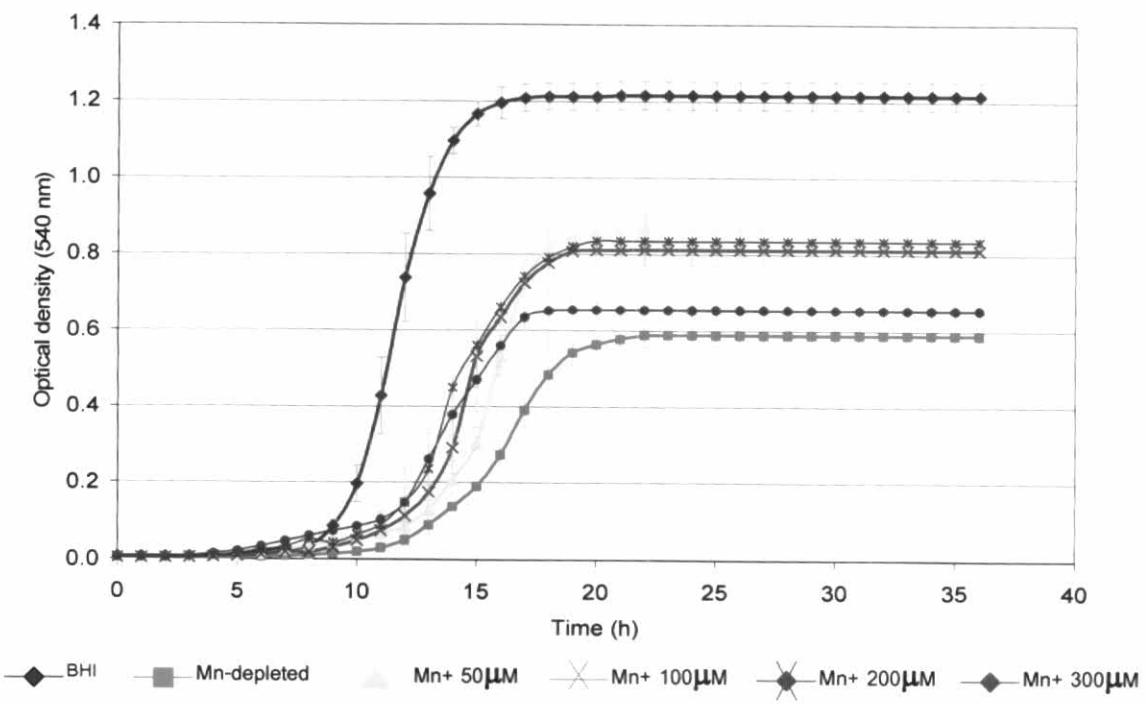


Figure 4.1b: *S. mutans* UA130

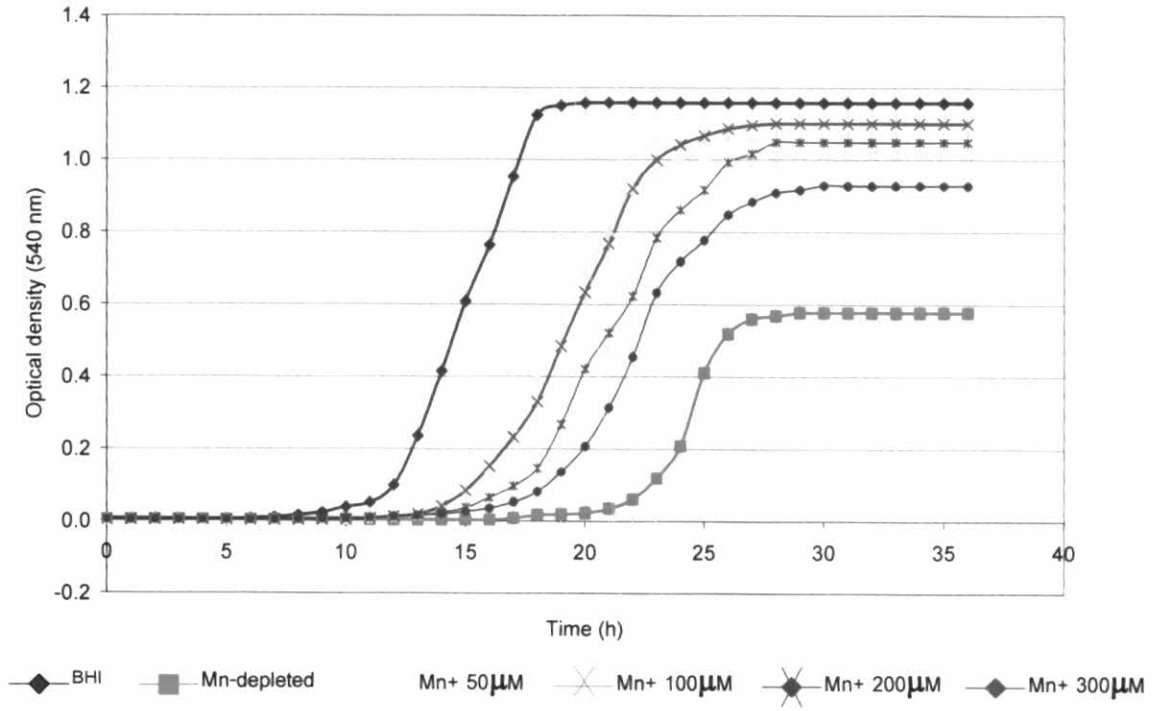


Figure 4.1c: *S. mutans* 3209

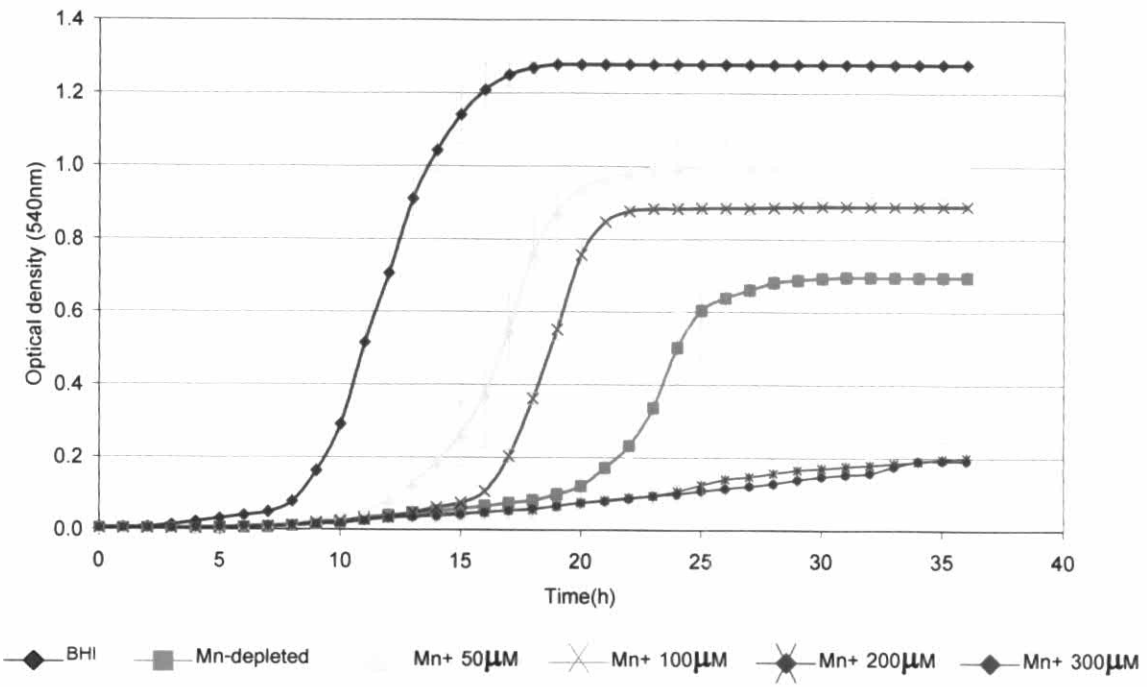


Figure 4.1d: *S. mutans* Ingbritt

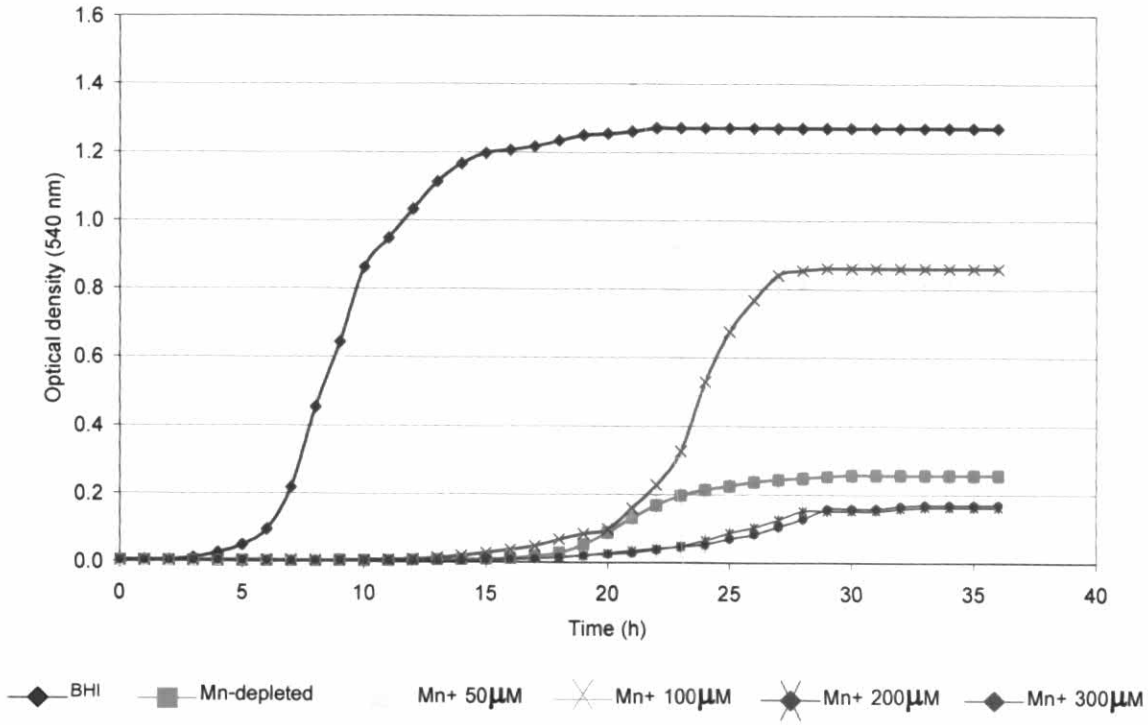


Figure 4.1e: *S. mutans* LT11

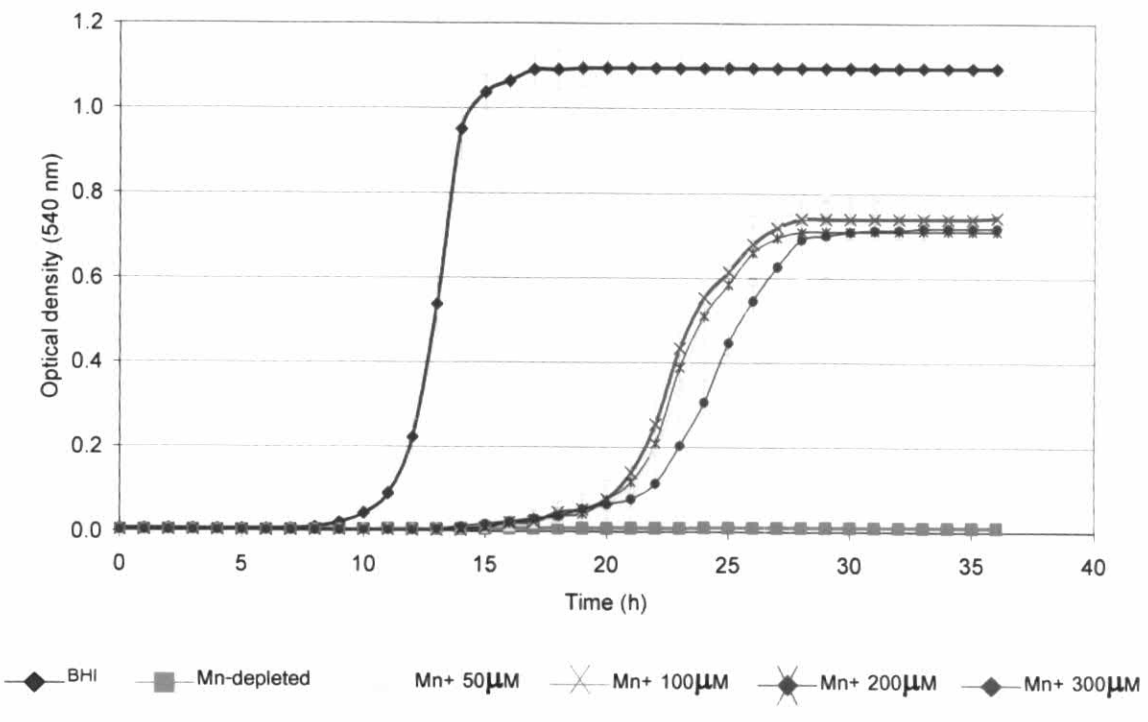


Figure 4.1f: *S. mutans* ATCC 25175

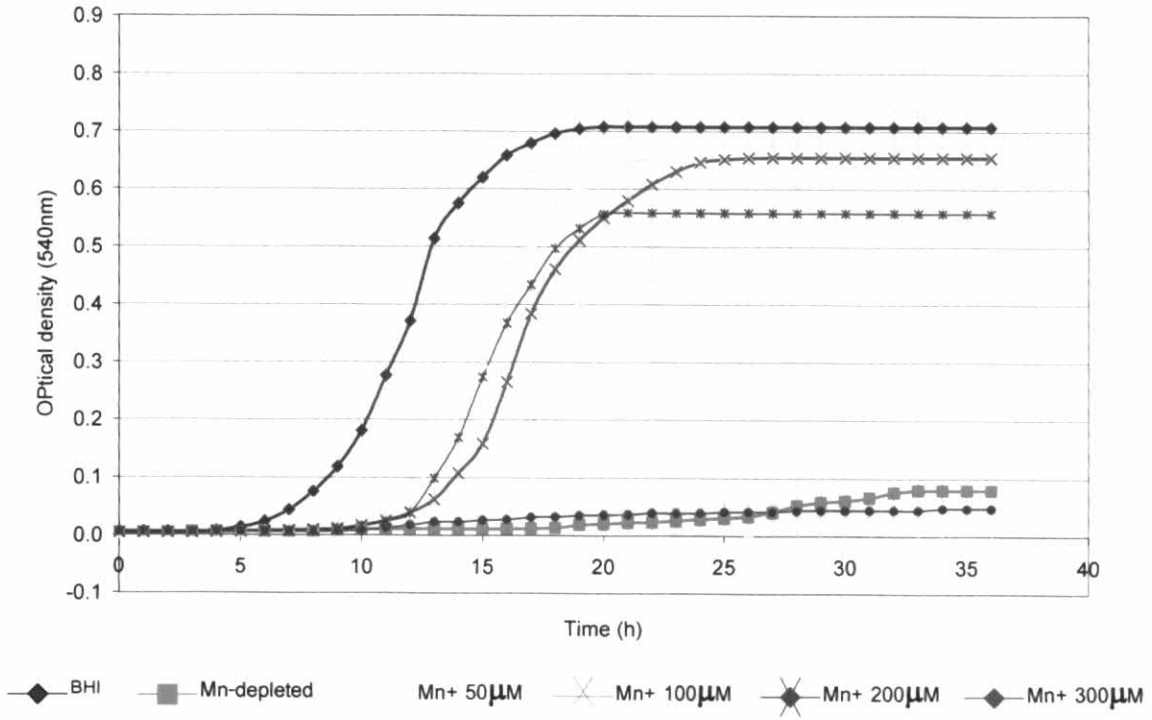


Figure 4.1. Growth curves of *S. mutans* culture in 5% CO_2 atmosphere. The bacteria were serially subcultured in Mn-depleted media; 1% inocula was added to BHI broth (positive control), Mn-depleted medium, and Mn-supplemented medium at concentrations of 50, 100, 200 and 300 µM. Growth was monitored by measuring O.D. at 540 nm. Fig.4.1a: UA159, Fig.4.1b: UA130, Fig.4.1c: 3209, Fig. 4.1d Ingbritt, Fig. 4.1e: LT11, Fig. 4.1f: ATCC 25175. Vertical bars represent the standard error of the mean.

When the cultures were grown in an O_2-enriched 5% CO_2 atmosphere (condition 2), the growth pattern was different. All strains were greatly affected when deprived of manganese, which was evident in the decrease of population density when compared to cultures grown in SCDM supplemented with manganese. Strain UA130 was affected the least while strains ATCC 25175, LT11, and 3209 could barely grow at all. In manganese supplemented SCDM, strains Ingbritt, 3209 and ATCC 25175 were sensitive to high concentrations of manganese (200 and 300 μM in 3209 and Ingbritt, 300 μM in ATCC 25175) as evidenced by the low final O.D.s of less than 0.2.

Group 1, condition 2: UA159, UA130, Ingbritt (Fig. 4.2a, 4.2b and 4.2d)

This group could grow in manganese depleted SCDM, though the population density was low (except UA130). The UA159, UA130 and Ingbritt could multiply to O.D.s of 0.186, 0.728 and 0.223, respectively. In manganese supplemented SCDM, UA159 reached O.D.s of 0.852, 0.691, 0.72 and 0.675, while UA130 was at 1.109, 1.095, 0.973 and 1.033 in 50, 100, 200 and 300 μM of manganese. Only strain Ingbritt was negatively affected by high concentrations of manganese (200 and 300 μM) (O.D. = 0.175 and 0.159).

Group 2, condition 2: strains 3209, LT11, ATCC 25175 (Fig. 4.2c, 4.2e and 4.2f)

This group could barely grow in Mn-depleted media: strains 3209, LT11 and ATCC 25175 had O.D.s = 0.075, 0.031 and 0.009, respectively. In manganese supplemented SCDM, strains 3209 and ATCC 25175 were sensitive to high concentrations of manganese (200 and 300 μM) as strain 3209 reached an O.D. = 0.141) and ATCC 25175 reached an O.D. = 0.038 with manganese at 300 μM. The LT11 grew well under all concentrations of manganese.

Figure 4.2a: *S. mutans* UA159

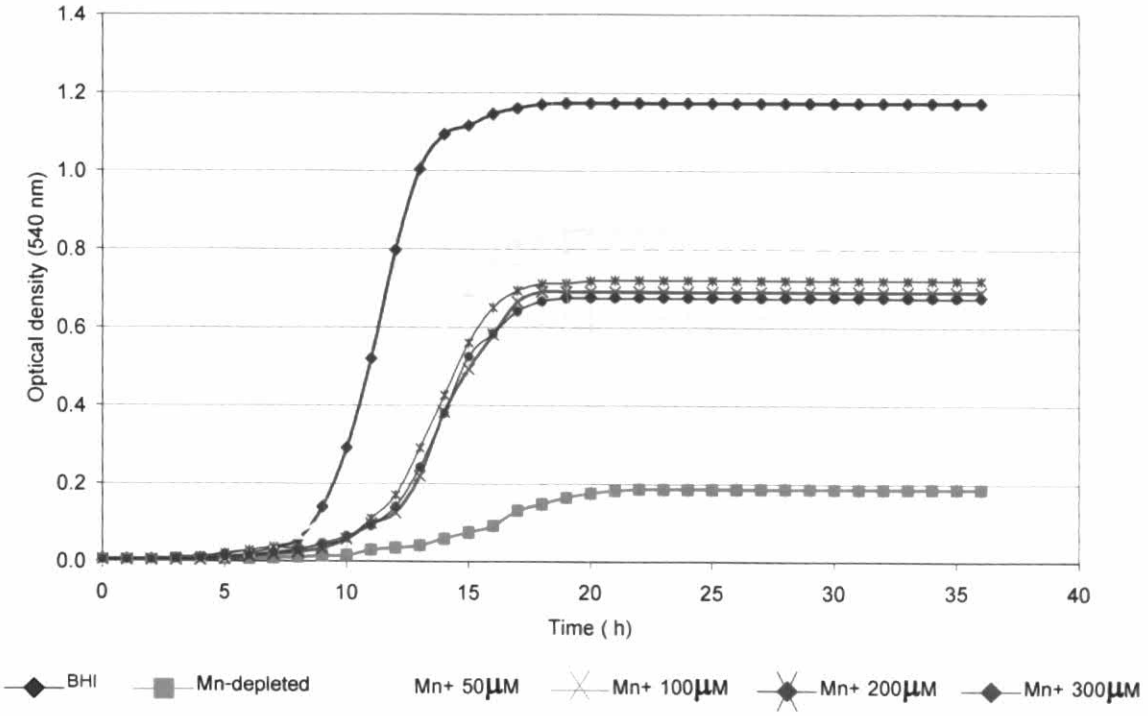


Figure 4.2b: *S. mutans* UA130

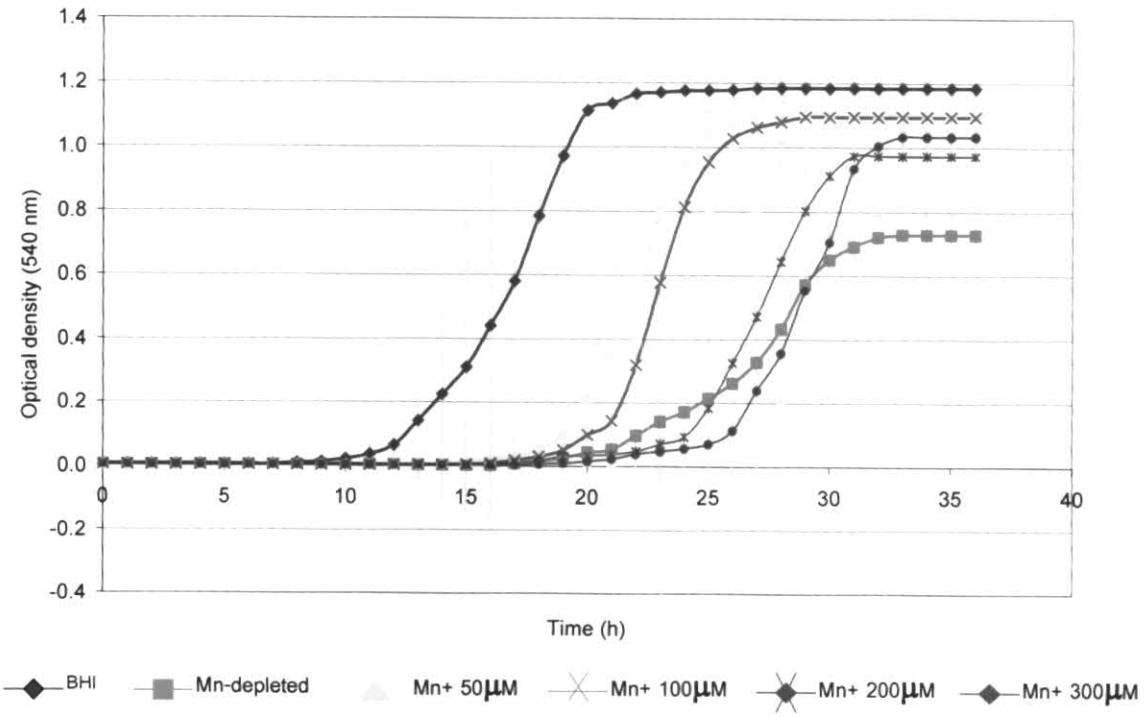


Figure 4.2c: *S. mutans* 3209

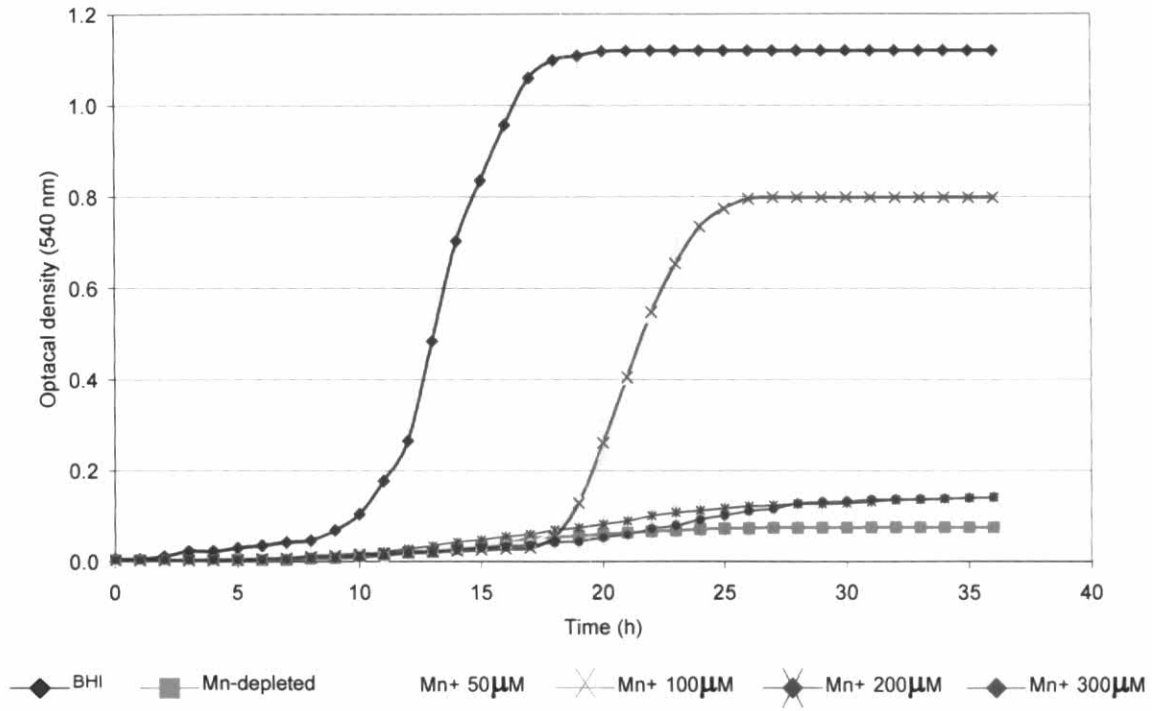


Figure 4.2d: *S. mutans* Ingbritt

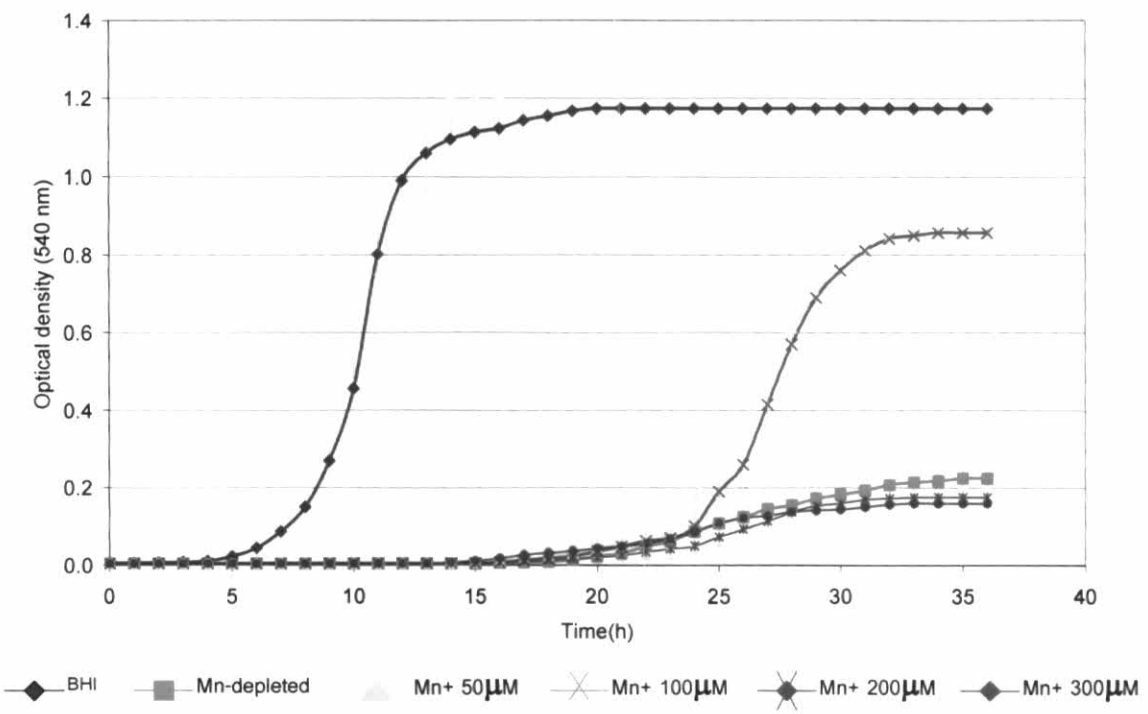


Figure 4.2e: *S. mutans* LT11

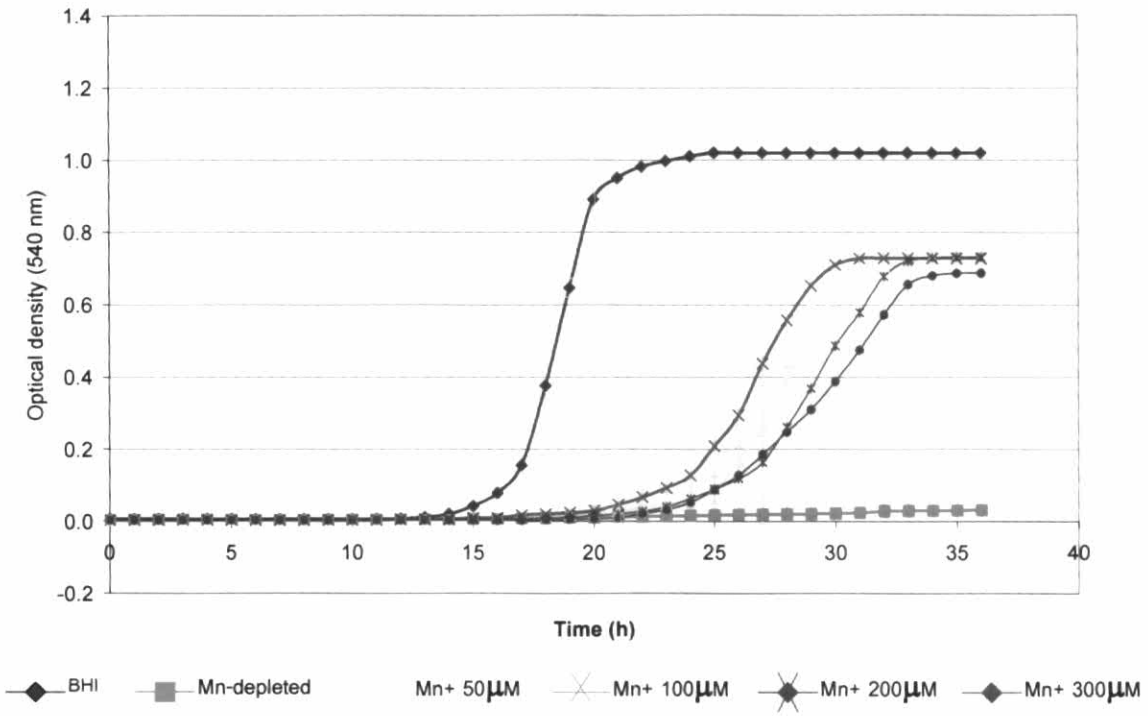


Figure 4.2f: *S. mutans* ATCC 25175

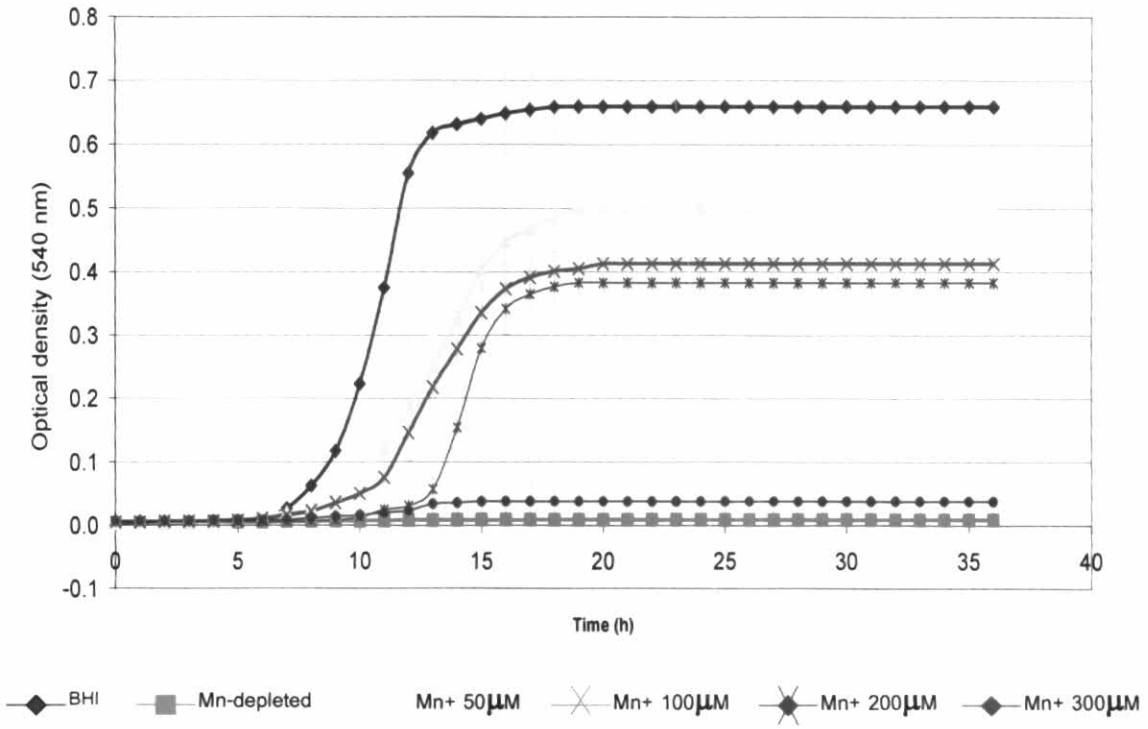


Figure 4.2. Growth curves of *S. mutans* culture in O_2-enriched 5% CO_2 atmosphere. The bacteria were serially subcultured in Mn-depleted media; 1% inocula was added to BHI broth (positive control), Mn-depleted medium, and Mn-supplemented medium at concentrations of 50, 100, 200 and 300 μM. Growth was monitored by measuring O.D. at 540 nm. Fig.4.2a: UA159, Fig.4.2b: UA130, Fig.4.2c: 3209, Fig. 4.2d Ingbritt, Fig. 4.2e: LT11, Fig. 4.2f: ATCC 25175. Vertical bars represent the standard error of the mean.

Group 1, condition 3: UA159, UA130, 3209, Ingbritt, ATCC 25175 (Fig. 4.3a, 4.3b, 4.3c, 4.3d, 4.3f)

S. mutans strains UA159, UA130, 3209, Ingbritt and ATCC 25175 showed higher density population growth in Mn-depleted SCDM (O.D. = 0.573, 0.895, 0.954, 0.625 and 0.171, respectively) than they did under aerobic conditions. The decreases in population density compared to growth in media containing 50 μM manganese-anaerobic atmosphere were more modest: 32.1%, 22.7%, 15.6%, 23.8%, and 74%, respectively. ATCC 25175 was also found to be sensitive to Mn at a concentration of 300 μM (Fig. 4.3f).

Group 2, condition 3: LT11 (Fig.4.3e)

LT11 culture grown in Mn-depleted SCDM, showed a 91% decrease in O.D. when compared to the identical condition in the presence of 50 μM manganese (Table 4.1). This group was considered to strongly require manganese for growth despite the absence of oxidative stress.

Figure 4.3a: *S. mutans* UA159

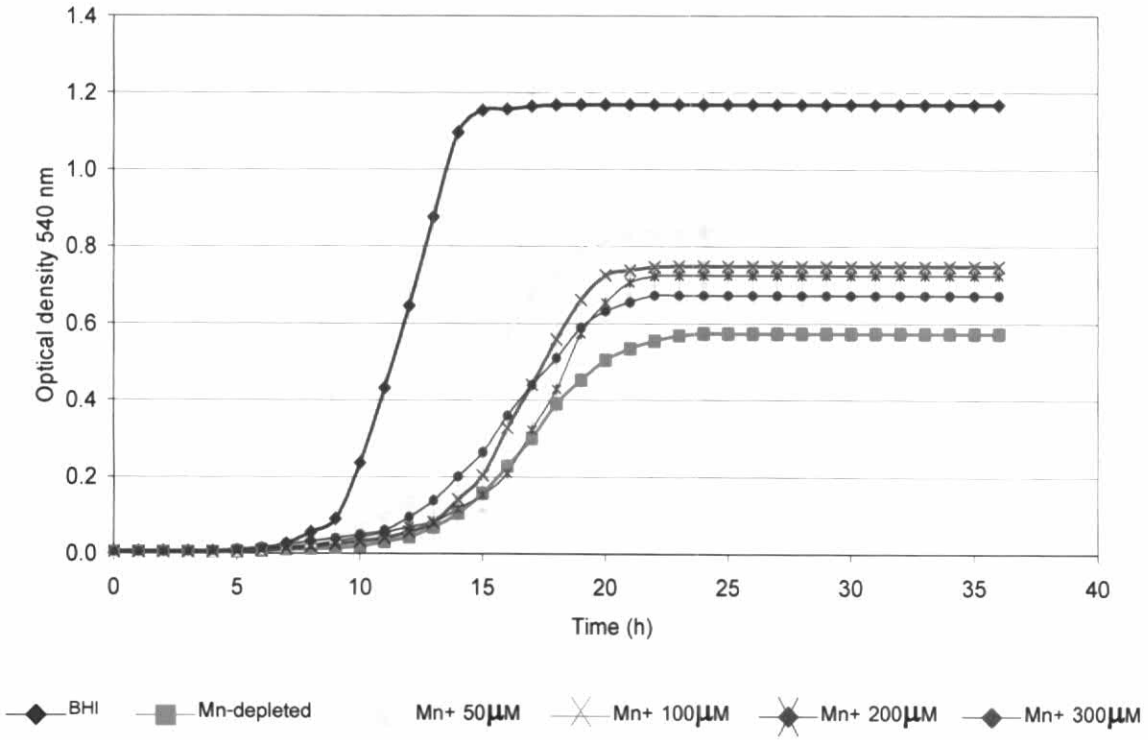


Figure 4.3b: *S. mutans* UA130

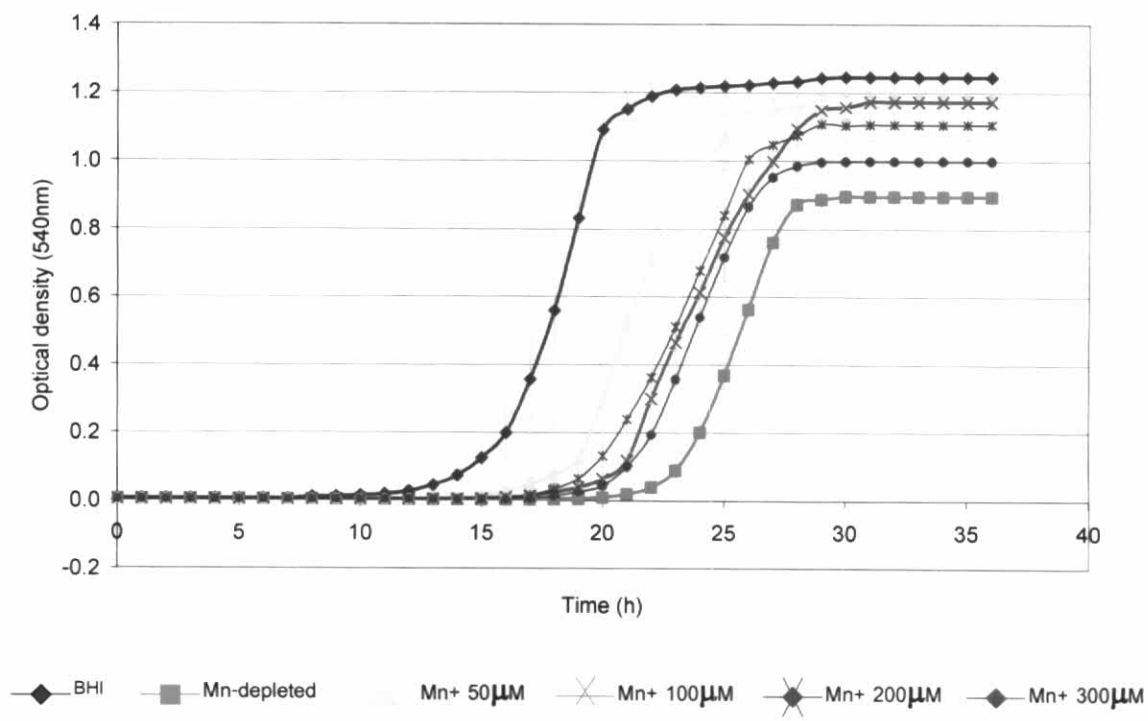


Figure 4.3c: *S. mutans* 3209

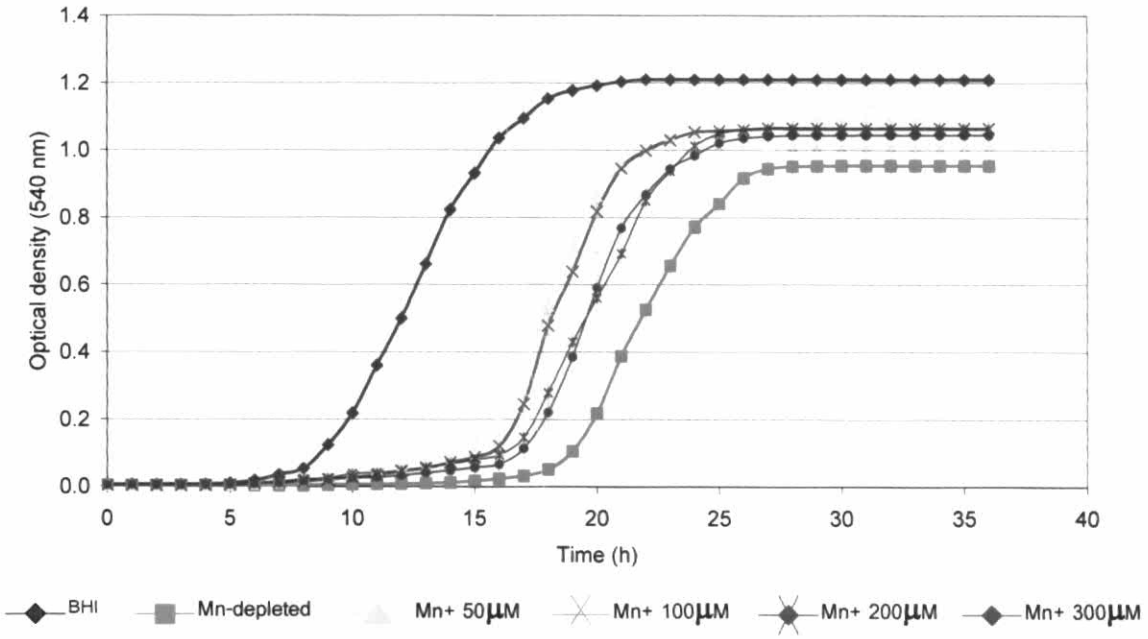


Figure 4.3d: *S. mutans* Ingbritt

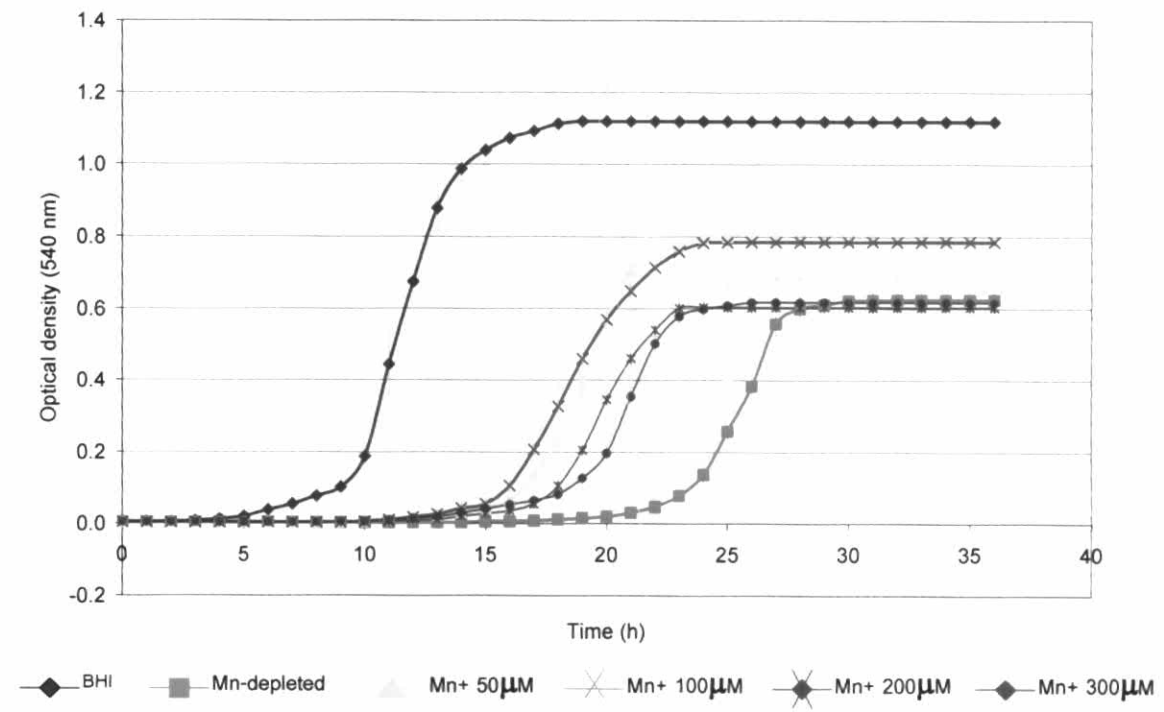


Figure 4.3e: *S. mutans* LT11

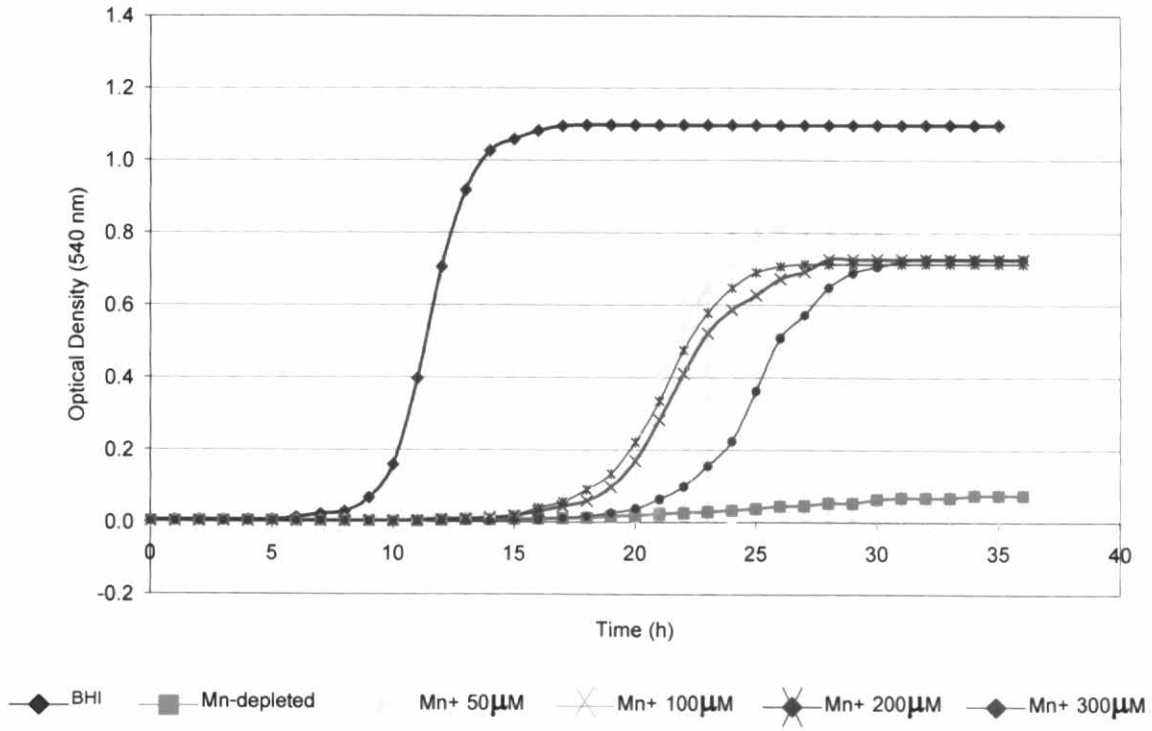


Figure 4.3f: *S. mutans* ATCC 25175

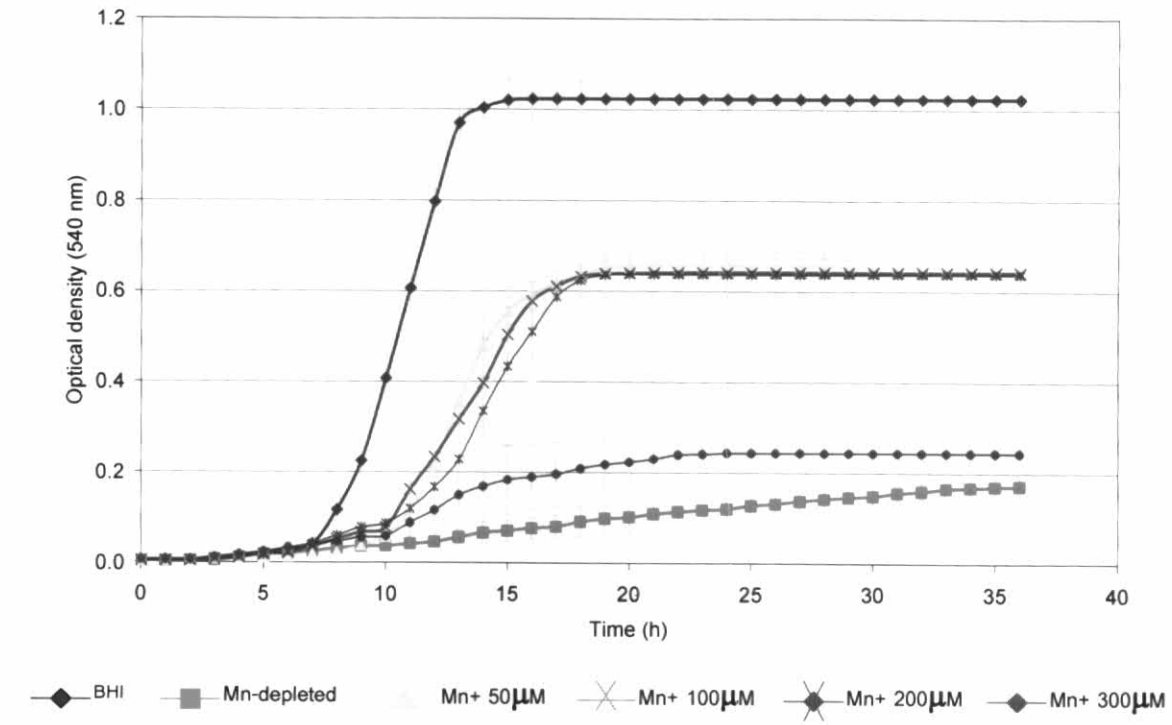


Figure 4.3. Growth curves of *S. mutans* cultures in anaerobic atmosphere. The bacteria were serially subcultured in Mn-depleted media; 1% inocula was added to BHI broth (positive control), Mn-depleted medium, and Mn-supplemented medium at concentrations of 50, 100, 200 and 300 μM. Growth was monitored by measuring O.D. at 540 nm. Fig.4.3a: UA159, Fig.4.3b: UA130, Fig.4.3c: 3209, Fig. 4.3d Ingbritt, Fig. 4.3e: LT11, Fig. 4.3f: ATCC 25175. Vertical bars represent the standard error of the mean.

Growth yield was enhanced for all strains when supplemented with any concentrations of manganese relative to manganese-depleted media, regardless of the atmosphere used for incubation (Table 4.1). The 50 and 100 μM manganese concentrations seemed to be optimal. A trend of lower yields of growth was observed when higher concentrations of manganese were added. Similar patterns were observed in all strains. When the bacterial growth in Mn-supplemented and Mn-depleted SCDM was compared, the 50 μM manganese concentration was chosen to simplify discussion, and is shown in Table 4.3.

Table 4.3 Percent growth yield in Mn-depleted medium[†]

Condition	UA159	UA130	3209	Ingbritt	LT11	ATCC 25175
5%CO_2-*Condition 1*	64.2	57.4	69.7	26.3	1.3	12.1
O_2-enriched 5% CO_2-*Condition 2*	21.8	65.6	8.3	26.5	4.3	1.8
Anaerobic-*Condition 3*	67.9	77.3	84.4	76.2	8.9	26.0

[†], = O.D. of culture grown in Mn-depleted medium / O.D. of cultures grown at 50 μM manganese concentration * 100

Group 1. (Table 4.2) under any atmosphere condition Group 1 strains are considered the least sensitive to manganese deprivation. Results indicated that UA159 and UA130 were consistently categorized in group 1 under conditions 1, 2 and 3 as they multiplied well despite the absence of Mn. Both strains could grow in Mn-depleted SCDM at approximately 64.2 and 54.7% of the Mn-supplemented population under condition 1, 21.8 and 65.6% under condition 2, and 67.9 and 77.3% under condition 3. However strains 3209 and Ingbritt seemed to be somewhat more sensitive to high concentrations of Mn (200 and 300 μM) in the presence of O_2 (conditions 1 and 2) as exemplified by drastic drops in O.D.s (Fig. 4.1c, 4.1d, 4.2c, 4.2d and Table 4.1).

Group 2. This group, consisting of strains LT11 and ATCC 25175 strongly required Mn. Even under anaerobic conditions, when deprived of Mn, LT11 did not grow (8.9% growth yield). On the other hand, too much Mn had a negative effect on the growth of ATCC 25175 as seen with a Mn concentration of 300 μM.

When oxygen was present (conditions 1 and 2), LT11 and ATCC 25175 consistently demonstrated their narrow range of optimal Mn requirement, since they did not grow either under conditions of depleted-Mn or high Mn concentrations (Table 4.1, and Fig. 4.1e, 4.1f, 4.2e, and 4.2f).

In summary: most strains could grow in Mn depleted media at 5% CO_2 atmosphere. Growth yields were decreased when O_2 enrichment was introduced as seen in all strains except UA130 and Ingbritt. It is interesting to note that the effect seen under O_2-enriched 5% CO_2 in strains 3209, Ingbritt and ATCC 25175 were consistent with the result shown with the same strains in 5% CO_2 atmosphere. In the presence of oxygen, with higher amounts of manganese (200-300 μM), ATCC 25175, 3209 and Ingbritt showed significantly retarded growth (O.D. ~ 0.048-0.2) (Fig. 4.1c, 4.1d, 4.1f, 4.2c, 4.2d, 4.2f and Table 4.1). In some instances the growth in the high concentrations of manganese was less than that in Mn-depleted media (Fig. 4.1c, 4.1d, 4.1f, 4.2d, and Table 4.1). When grown in Mn-depleted SCDM under anaerobic conditions, *S. mutans* strains UA159, UA130, 3209 and Ingbritt showed better growth (O.D. = 0.573-0.954) (Fig. 4.3a, 4.3b, 4.3c, 4.3d Table 4.1) than under aerobic conditions. The decreases in population density compared to growth in media containing manganese were more

modest: 32.1%, 22.7%, 23.8%, and 15.6%, respectively. The bacteria grew best in media supplemented with 50 μM manganese. However, Mn-depleted ATCC 25175 and LT11 showed a 74% and 91% decrease in O.D. when compared to the identical condition with 50 μM manganese, thereby consistently demonstrating a strong requirement for manganese under all atmospheric conditions.

The effect of manganese on virulence genes of *S. mutans*

Microarrays

Strain UA159 was chosen to investigate the effects of manganese for the rest of the study since this was one of the strains capable of growth in the absence of manganese, and in anticipation of subsequent investigations that would take advantage of the fact that its genome sequence was known. Microarrays were performed in triplicate using RNA isolated from planktonic cultures grown in Mn-depleted or Mn-supplemented media. The array data indicated that *gtfC* and *gbpC* were expressed more highly under Mn-supplemented conditions than Mn-depleted conditions (Table 4.4; full array results are in Appendix). The expression of *wapA* declined marginally and that of *gtfB* increased marginally. Selected virulence genes (Table 4.4) were then chosen for further study using both planktonic and biofilm cultures with or without manganese.

The strong induction of manganese transport operons under conditions of manganese deprivation confirmed the utility of the experimental design (see appended table of array results: SMU_182, SMU_183, SMU_184, SMU_186). Expression differences for genes involved in metabolism, growth, and possible two-component systems were observed. The expression of *dexB* (SMU_883), a gene involved in metabolic activity which has similar function to *gtfB* and *gtfC* are upregulated in the presence of Mn. The upregulation of SMU_78, SMU_79, SMU_883, and SMU_2028 genes, encoding fructosidase-FruA, fructosidase-FruB, dextran glucosidase and levansucrase, also supported higher metabolic activity associated with higher growth yields when Mn was present (Table 4.4).

Table 4.4 Selected microarray results of *S.mutans* genes

S. mutans UA159 ORF	Gene description	Mn^{+} fold change	p Value
Selected virulence genes			
SMU_1509	*rgg*	-4.77	**0.013**
SMU_987	*wapA*	-1.34	0.112
SMU_2112c	*gbpA*	-1.13	0.348
SMU_772	*gbpD*	-1.09	0.511
SMU_610	*spaP*	+1.03	0.798
SMU_1004	*gtfB*	+1.37	0.243
SMU_1005	*gtfC*	+1.42	**0.014**
SMU_1396c	*gbpC*	+1.72	**0.038**
Selected genes involved in metabolism of sucrose and its products			
SMU_79	Fructan hydrolase, exo beta-D-fructosidase, FruB	+1.15	0.460
SMU_883	Dextran glucosidase, DexB	+1.38	0.050
SMU_542	Putative glucose kinase	+1.47	0.008
SMU_2028c	Levansucrase, precursor beta D fructosyl transferase	+1.58	0.006
SMU_78	Fructan hydrolase, exo beta-D-fructosidase, FruA, fructanase	+1.69	0.040
Selected genes involved in metal transport			
SMU_183	Putative Mn/Zn ABC transporter	-26.43	0.013
SMU_182	Putative ABC transporter, ATP-binding protein, possible iron and/ or Mn ABC transport system	-22.75	0.014

S. mutans UA159 ORF	Gene Description	Mn^{+} fold change	p Value
SMU_184	Putative ABC transporter, metal-binding lipoprotein, surface adhesion precursor, saliva-binding protein	-19.68	0.013
SMU_186	Putative metal dependent transcriptional regulator	-9.13	0.013
SMU_770c	Mn transporter	-2.91	0.017
SMU_1695	Putative ABC transporter, ATP-binding protein, possible molybdenum transport system	-1.83	0.027
SMU_995	ABC transporter, possible ferrichrome	-1.8	0.28
SMU_996	ABC transporter, possible ferrichrome	-1.7	0.035
SMU_997	Putative inorganic ion ABC transporter, possible ferrichrome transport systems	-1.53	0.037
SMU_1993c	Putative ABC transporter, Zn permease protein	+1.62	0.029
SMU_1302c	Putative surface adhesion, AdcA protein, homolog to putative Zn-binding lipoprotein	+2.04	0.004
Other genes			
SMU_412c	Putative Hit-like protein, involved in cell cycle regulation	-2.15	0.038
SMU_1270c	Putative histidinol dehydrogenase	-2.10	0.030
SMU_1269c	Putative phosphoserine phophatase	-1.93	0.023
SMU_474c	Putative autoinducer-2 production, LuxS	-1.93	0.023
SMU_1146c	Putative response regulator, homolog of RumR, ScnR	-1.51	0.048
SMU_1145c	Putative histidine kinase, homolog of RumK, ScnK	-1.49	0.059

S. mutans UA159 ORF	Gene Description	Mn^{+} fold change	p Value
SMU_629	Putative Mn type SOD, Fe/Mn SOD	+1.15	**0.217**
SMU_287	Putative ComB, accessory factor of ComA	+1.3	0.021
SMU_1917	Putative response regulator of the competence regulon, comE	+1.36	**0.061**
SMU_1983	Putative competence protein, Com YD	+1.64	0.016
SMU_1984	Putative competence protein, Com YC	+1.72	0.012
SMU_76	Putative N-acetyl muramidase	+2.00	0.001
SMU_1915	ComC, competence signal peptide	+2.10	0.003
SMU_1985c	Putative ABC transporter, ComYB, possibly part of the DNA transport machinery	+2.51	8.20E-04
SMU_1987c	Putative ABC transporter, ATP binding protein ComYA; late competence gene	+3.90	1.59E-05

Western Immunoblot

GbpA and GbpC western immunoblots were carried out to determine if differences in protein expression as a function of manganese availability could be detected. GbpA was detected in a concentrated culture supernatant fraction since this protein was secreted extracellularly. GbpC was extracted from the cell pellet since it was anchored to the cell wall. Fig. 4.4 shows that the relative amounts of GbpA were similar whether the bacteria were grown with or without manganese. For GbpC, however, there was more protein associated with the bacteria grown in the presence of manganese.

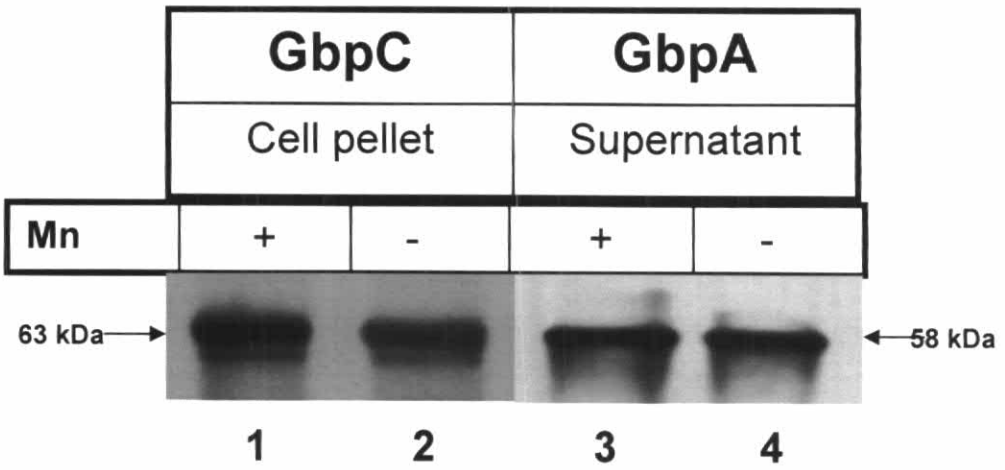


Figure 4.4 Representative western immunoblot from three independent experiments. Equal amounts of cell-associated (lanes 1-2) or secreted proteins (lanes 3-4) from planktonic cultures grown in Mn-supplemented or Mn-depleted media were resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and incubated with rabbit polyclonal antibody against GbpC or the glucan-binding domain of GbpA. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase. Signals were developed using chemiluminescent substrate and semi-quantified by densitometry.

Northern Blot Analysis

When the *gbpA* and *gbpC* were further investigated at the transcriptional level by northern blotting, the *gbpC* mRNA was significantly increased in bacteria grown in manganese-supplemented media (Fig. 4.5). The *gbpA* mRNA still demonstrated no difference between the two conditions. The *gyrA* probe was included with the intention that this gene would serve as the control for normalization. However, it appeared that manganese availability affected *gyrA* expression. Therefore the amount of loaded RNA was calibrated by running 10 μg of total RNA on a 1.2% denaturing agarose gel (Fig. 4.5).

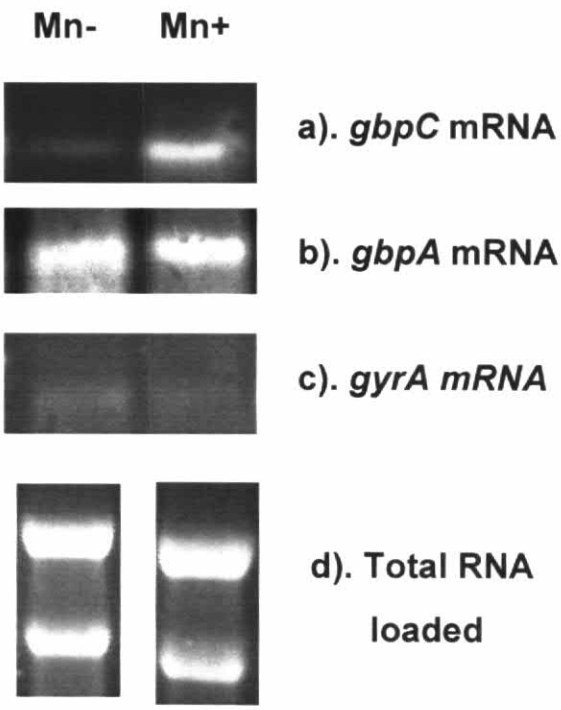


Figure 4.5

Northern blot of RNA collected from bacteria grown in Mn-supplemented or Mn-depleted media, separated by agarose gel electrophoresis, blotted onto nitrocellulose and probed for a) *gbpC*, b) *gbpA*, or c) *gyrA*. The loading control is shown in part d).

Reverse Transcriptase Polymerase Chain Reaction

These results confirmed that manganese availability could have differential effects on *S. mutans* virulence genes. To more broadly examine the effects of manganese on virulence gene expression, a semi-quantitative analysis using reverse transcriptase polymerase chain reaction (RT-PCR) was performed on *S. mutans* cultures grown under planktonic or biofilm conditions. The results from the planktonic culture analysis agreed with those from the microarray experiments. The presence of manganese resulted in increased expression of *gbpC*, *gtfB*, and *gtfC* (Figure 4.6). The expression of the *spaP* gene was slightly increased. Interestingly, however, the expression of *wapA* was decreased under manganese-supplemented growth conditions (Figure 4.6). When *S. mutans* was grown in a biofilm culture on saliva-coated polystyrene, the effects of manganese were not always similar to those observed for planktonic cultures. The presence of manganese still resulted in increased expression of *gtfB*, slight increase in *gbpC* and decreased expression of *wapA* (Figure 4.7). But *gtfC* expression appeared to be unaffected, and the expression of *gbpA*, *gbpD*, and to

a lesser extent *spaP*, all decreased under the manganese-supplemented conditions. Expression of *rgg*, the *S. mutans* homologue encoding the regulatory protein Rgg, was also decreased under manganese-supplemented conditions in both planktonic and biofilm cultures (Figure 4.8).

The decreased expression of *wapA* when grown in manganese-supplemented media led us to examine whether *S. mutans* adherence to saliva-coated polystyrene was affected by manganese availability. Data from multiple trials of an adherence assay (Table 4.5) indicated that the bacteria grown under manganese-supplemented conditions adhered in a significantly lower percentage than organisms grown without manganese.

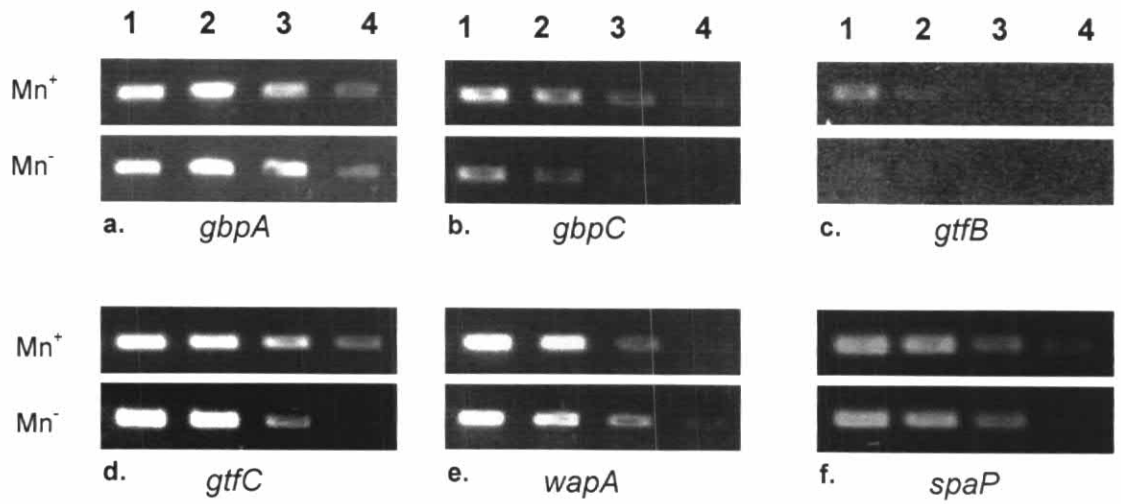


Figure 4.6 Reverse transcriptase PCR of RNA from planktonic cultures grown in Mn-supplemented or Mn-depleted media. Total RNA from the cultures were serially diluted prior to amplification with gene-specific primers (as labeled in a-f) and represented in lanes 1 (most concentrated) to 4 (least concentrated). The PCR products were separated on a 3% agarose gel. The experiments were run in duplicate and, along with the microarray data, confirmed reproducibility.

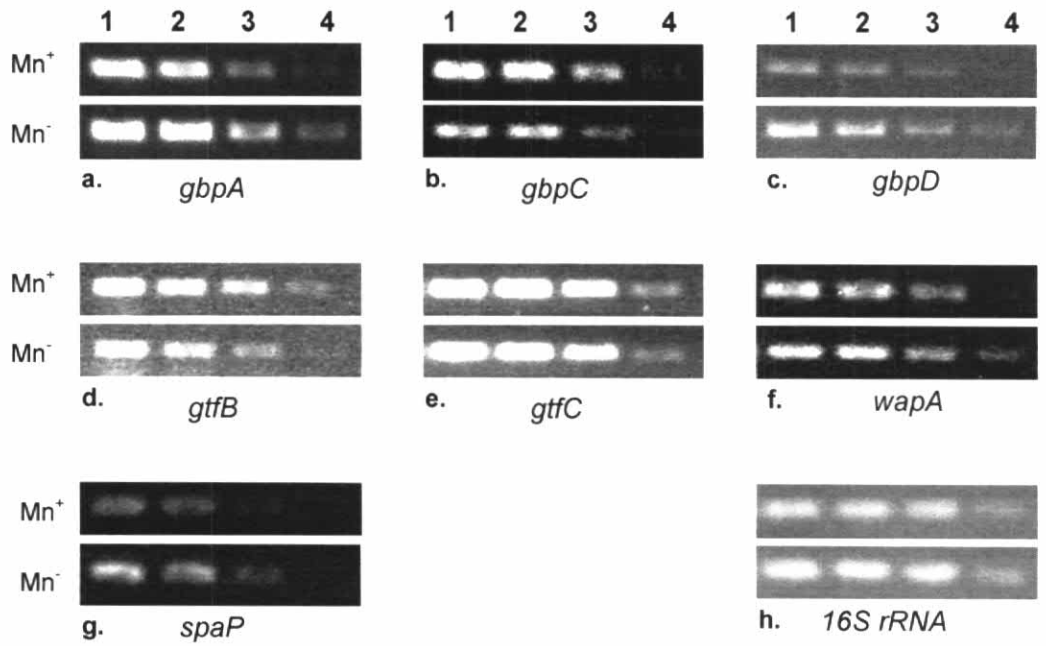


Figure 4.7 Reverse transcriptase PCR of RNA from biofilm cultures grown in Mn-supplemented or Mn-depleted media. Total RNA from biofilm bacteria were serially diluted prior to amplification with gene-specific primers (as labeled a-h) and represented in lanes 1 (most concentrated) to 4 (least concentrated). The PCR products were separated on a 3% agarose gel. The experiments were run in triplicate to confirm reproducibility. 16S RNA was run as a control.

Adherence Assay

Table 4.5 Adherence to saliva-coated polystyrene wells

Sample	**No. of samples**	**Mean % adherent cells / total cells** ±S.D.
Mn^-	9	14.54 ± 5.16
Mn^+	9	3.04 ± 1.14

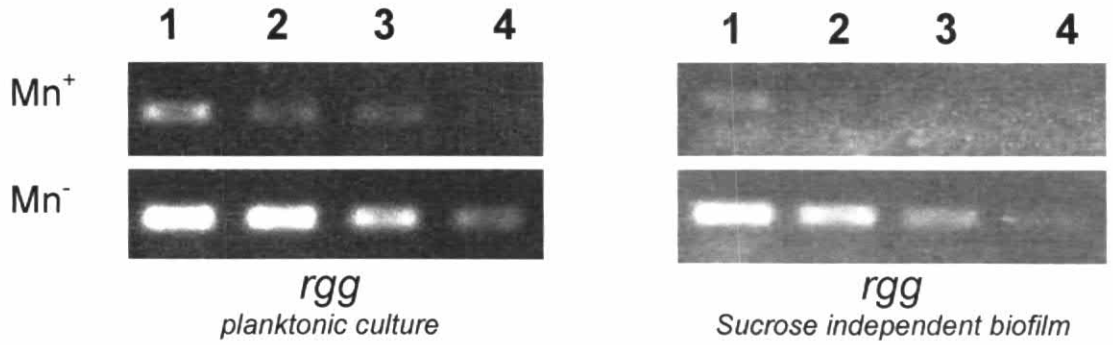


Figure 4.8 Reverse transcriptase PCR of RNA from either planktonic or biofilm cultures grown in Mn-supplemented or Mn-depleted media. Total RNA from bacteria was serially diluted prior to amplification with *rgg*-specific primers and represented in lanes 1 (most concentrated) to 4 (least concentrated). The PCR products were separated on a 3% agarose gel. The experiment was performed in triplicate to confirm reproducibility. 16S RNA was run as control.

Gtf and Ftf Gel Activity

Figure 4.9 shows the GTF activity of *S. mutans* UA159. Two glucan bands represent GtfB activity at ~166 kDa, GtfC, and GtfD at 163 kDa, respectively. Equivalent amount of protein extracted from cell grown in Mn-supplemented and Mn-depleted SCDM was loaded in lane 3 and 8, lane 4 and 9, lane 5 and 10 at 1, 1.5, and 2 folds, respectively. The enzymatic activity of GtfB (protein size of 166 kDa) produces only insoluble glucan, whereas GtfC produces both soluble and insoluble glucan and GtfD produces only soluble glucan (red arrows). The band indicated at molecular weight 163 kDa is fainter than that of GtfC at the molecular weight 166 kDa. No significant difference was observed in glucosyltransferase activity in cell grown in Mn-supplemented and Mn-depleted media. The lower two bands at molecular weight approximately 80-87 kDa of fructosyltransferase activity were observed in cell cultured in media added Mn with significant difference (black arrows).

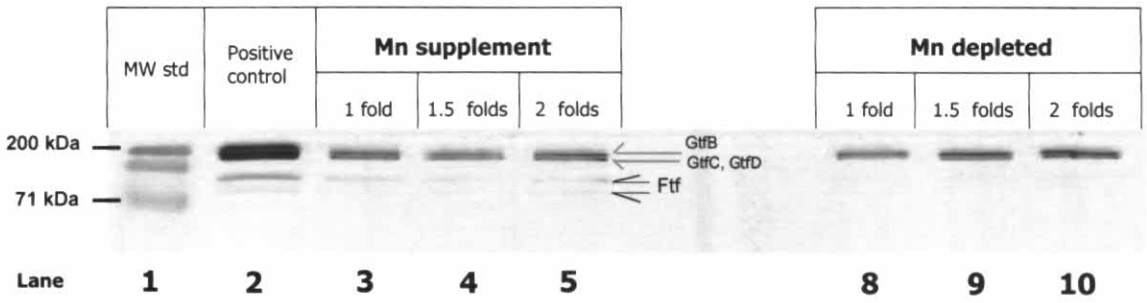


Figure 4.9 Activity gel. Red bands indicate polysaccharide formation on SDS-10%PAGE gel. Lane 1 indicated positions of protein molecular mass standards. Protein extract of cell culture grown in BHI was used as a positive control (lane 2). protein samples of culture grown in biofilm with/without manganese and quantitatively measured and loaded in lane 3 to 5 (Mn supplemented SCDM) and lane 8 to 10 (Mn depleted SCDM) at 1, 1.5 and 2 folds, respectively. Densitometric scanning of the bands at approximately 166 and 87 kDa showed that there was no difference in the Gtf activity, but the Ftf bands demonstrated significant difference between the two conditions.

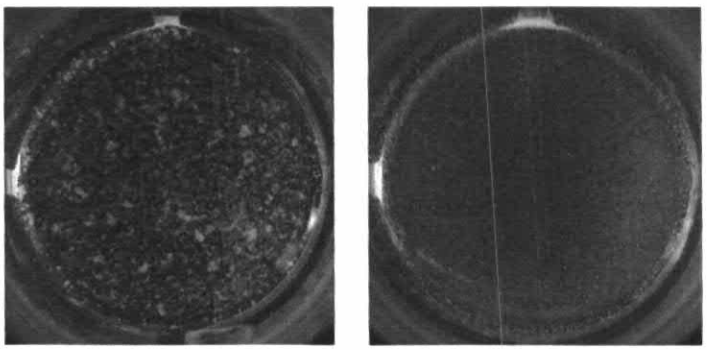
Glucosyltransferase B and C (GtfB and GtfC) produce water-insoluble extracellular glucan from sucrose consisted primarily α 1, 3 linkages. These glucans facilitate adhesion and accumulation of the bacteria and promote growth of oral biofilms. *S. mutans* also produce fructosyltransferase (Ftf), from *ftf* gene [143]. Ftf catalyses sucrose into glucose and fructose, then incorporates the fructose moiety into a fructan polymer. Although fructans usually do not facilitate the adhesion or aggregation of the bacteria, they are thought to provide a source of carbohydrate during times of limited exogenous nutrients.

The effect of manganese on biofilm architecture

Biofilm generated in the absence of manganese displayed macroscopic differences compared to those grown in the presence of manganese, whether or not sucrose was present (Fig. 4.10a, 4.10b). Mn-supplemented biofilms are uniform, with

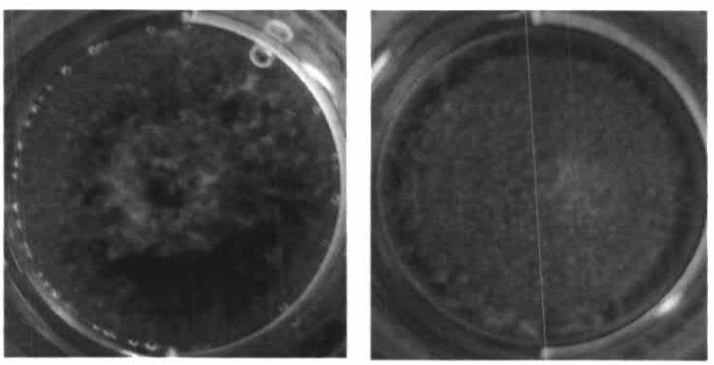
more coverage to the attached surface. They tend to have small aggregates spread fairly evenly throughout the biofilm matrix (similar to the positive control BHI grown (data not shown)). The organization of Mn-depleted biofilm is more heterogeneous with noticeable large gap in the biofilm matrix and the cell aggregates appeared much larger. The most recognizable difference was that the Mn-depleted biofilm showed extensive clumping or aggregation of the bacteria. However, many of the clumps of Mn-depleted in the absence of sucrose were not firmly adhered to the biofilm and could easily be washed away. When bacteria adhered at the substratum were viewed by confocal microscopy the pattern seemed to be the reverse. The manganese supplemented bacteria adhered in more discreet aggregates whereas the manganese-depleted bacteria adhered more evenly over the surface (Fig. 4.11a, 4.11b). Computer analysis of various biofilm properties using the COMSTAT program [139] did not detect significant differences between Mn-supplemented and Mn-depleted biofilms when grown with sucrose (Table 4.6). The lone exception was the ratio of surface to biovolume which was higher for the Mn-depleted biofilm. In contrast, the ratio of surface to biovolume was the only parameter that was not significantly different when comparing biofilms generated in the absence of sucrose (Table 4.6).

(a) 5% sucrose biofilms



Mn-depleted Mn-supplemented (50 μM)

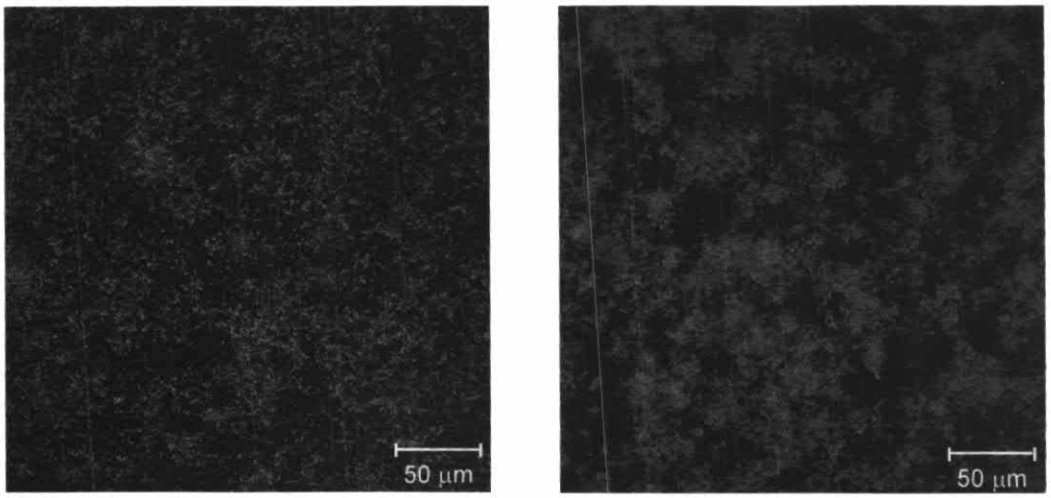
(b) Non-sucrose biofilms formed over saliva coating



Mn-depleted Mn-supplemented (50 μM)

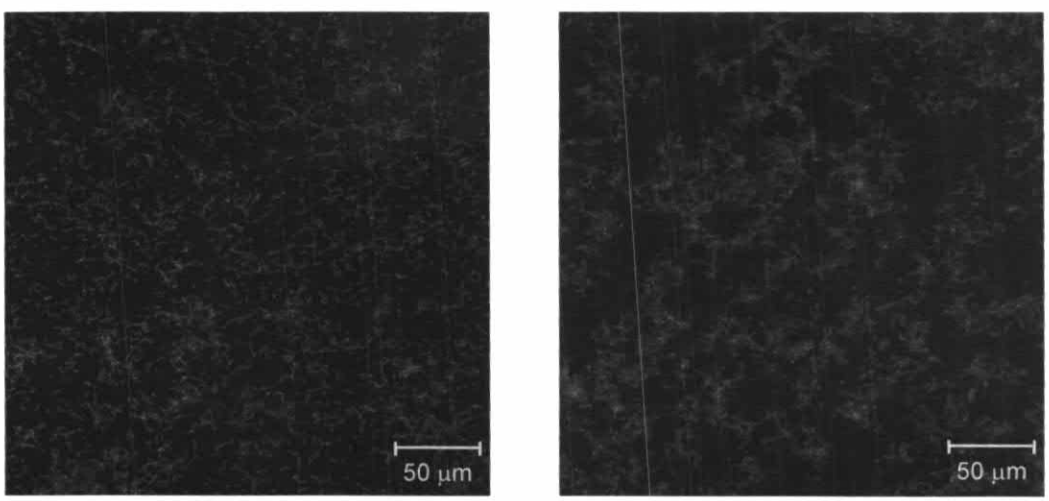
Figure 4.10 Whole-well images of biofilms formed by *S. mutans* in polystyrene dishes following overnight incubation under anaerobic conditions. (a): Biofilms grown in SCDM with 5% sucrose and without manganese (left) or with manganese (50 μM; right). (b): Biofilms grown in SCDM within saliva-coated wells without manganese (left) or with manganese (50 μM; right).

(a) 5% sucrose biofilms



Mn-depleted Mn-supplemented (50 μM)

(b) Non-sucrose biofilms formed over saliva coating



Mn-depleted Mn-supplemented (50 μM)

Figure 4.11 Confocal microscopic images of fluorescently stained bacteria adhered to the substratum. (a): Biofilm at the substratum after growth in SCDM with 5% sucrose and without manganese (left) or with manganese (50 μM; right). (b): Biofilms grown in SCDM within saliva-coated wells without manganese (left) or with manganese (50 μM; right).

Table 4.6 COMSTAT comparisons of quantitative differences in *S. mutans* UA159 biofilms formed in the presence and absence of manganese

Sucrose biofilms	Mn-depleted	50 μM Mn	t test p value
Biomass (μm^3 (μm^2)$^{-1}$)[ab]	7.37 ± 0.12	8.50 ± 1.36	0.361
% Substratum occupied[a]	30.32 ±16.09	39.27 ±16.69	0.118
Average thickness (μm)[a]	14.12 ± 2.40	14.16 ± 0.98	0.982
Roughness coefficient[c]	0.77 ± 0.07	0.70 ± 0.08	0.325
Surface to biovolume ratio (μm^2 (μm^3)$^{-1}$ [a]	1.79 ± 0.04	1.34 ± 0.12	0.004
Maximum thickness (μm)[a]	58.17 ± 28.74	56.80 ± 33.38	0.785
Non-Sucrose Biofilms	**Mn-Depleted**	**50 μM Mn**	**t test p value**
Biomass (μm^3 (μm^2)$^{-1}$)[ab]	0.73 ± 0.68	2.62 ± 1.55	0.000
% Substratum occupied[a]	5.94 ± 3.35	10.03 ± 4.78	0.001
Average thickness (μm)[a]	1.75 ± 2.09	9.91 ± 7.16	0.000
Roughness coefficient[c]	1.75 ± 0.16	1.21 ± 0.31	0.000
Surface to biovolume Ratio (μm^2 (μm^3)$^{-1}$ [a]	2.56 ± 0.49	2.77 ± 0.48	0.131
Maximum thickness (μm)[a]	43.96 ±19.67	76.44 ± 27.79	0.000

[a]Average of three independent trials ± the standard error of the mean.

[b]Estimated value based on total biovolume divided by the field area.

[c]Roughness Coefficient is a unitless measure of the variation in biofilm thickness.