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Effects of Oxygen on Virulence Traits of *Streptococcus mutans*▽

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Oxygen profoundly affects the composition of oral biofilms. Recently, we showed that exposure of *Streptococcus mutans* to oxygen strongly inhibits biofilm formation and alters cell surface biogenesis. To begin to dissect the underlying mechanisms by which oxygen affects known virulence traits of *S. mutans*, transcription profiling was used to show that roughly 5% of the genes of this organism are differentially expressed in response to aeration. Among the most profoundly upregulated genes were autolysis-related genes and those that encode bacteriocins, the ClpB protease chaperone subunit, pyruvate dehydrogenase, the tricarboxylic acid cycle enzymes, NADH oxidase enzymes, and certain carbohydrate transporters and catabolic pathways. Consistent with our observation that the ability of *S. mutans* to form biofilms was severely impaired by oxygen exposure, transcription of the *gtfB* gene, which encodes one of the primary enzymes involved in the production of water-insoluble, adhesive glucan exopolysaccharides, was down-regulated in cells growing aerobically. Further investigation revealed that transcription of *gtfB*, but not *gtfC*, was responsive to oxygen and that aeration causes major changes in the amount and degree of cell association of the Gtf enzymes. Moreover, inactivation of the VicK sensor kinase affected the expression and localization the GtfB and GtfC enzymes. This study provides novel insights into the complex transcriptional and posttranscriptional regulatory networks used by *S. mutans* to modulate virulence gene expression and exopolysaccharide production in response to changes in oxygen availability.

Of the hundreds of bacterial species that colonize and persist in the mouth, *Streptococcus mutans* is the organism that is most effective at causing dental caries. The abilities of this organism to form biofilms, to generate acid, and to tolerate environmental stresses are critical to its virulence (10, 11, 24, 25). All organisms in oral biofilms are exposed to rapid variations in the amount and type of metabolizable energy sources, to rapid changes in environmental pH, and to a considerable spectrum of local oxidation-reduction potentials. All of these environmental variables are known to have a profound impact on bacterial gene expression and have been unequivocally shown to be the factors that have the greatest impact on the microbial composition and biological activities of oral biofilms (10, 11, 24, 25). It is becoming generally accepted that the ability of *S. mutans* to adapt to comparatively hostile environments is a primary mechanism by which this organism emerges as a dominant member of cariogenic dental biofilms (11, 17, 40, 41). While the molecular mechanisms underlying the control of carbohydrate acquisition, acid production, and adaptation to low pH by *S. mutans* have been the focus of a substantial number of studies, the role of oxygen in fundamental aspects of gene regulation and physiologic homeostasis is poorly understood.

Oxygen is required by many oral organisms for respiration and energy generation. Organisms that initially colonize the surfaces of the mouth are exposed to levels of oxygen approaching those found in air or air-saturated water (30). Mature oral biofilms, however, support a wide range of aerobes, facultative anaerobes, and obligately anaerobic bacteria, indicating that limited diffusion and rapid metabolism of oxygen in mature biofilms combine to substantially reduce oxygen tension and lower the redox potential of dental plaque (22). In fact, oxygen tension in the oral cavity has been estimated to range from 5 to 27 mm Hg (34), while estimates of the redox potential (E_h) of early biofilms (+294 mV) is much higher than that (−141 mV) measured in mature biofilms (22). Although oral streptococci do not possess a full electron transport chain and cannot carry out oxidative phosphorylation, these organisms maintain a high capacity to metabolize oxygen, primarily through NADH oxidase enzymes (19, 30).

We have recently demonstrated that the ability of *S. mutans* to form biofilms, an essential virulence attribute of this organism, was dramatically reduced when cells were cultivated in the presence of oxygen (2). This finding alone has substantial implications, since it reveals that *S. mutans* cells that initially colonize a surface in the oral cavity may display much different behaviors than when the organisms are growing in a mature biofilm with reduced oxygen availability or redox potential. We also demonstrated that the behavior of cells in the presence of oxygen is largely influenced by the VicK sensor kinase of a CovRS-like two-component system (TCS) and by the AtlA autolysin pathway, which plays a major role in modulating cell surface composition (2). It was particularly interesting that inactivation of the gene for AtlA or VicK restored the capacity of *S. mutans* to form biofilms in the presence of oxygen (2). Collectively, these observations indicate that oxygen is a key environmental factor that strongly influences cell envelope composition and biofilm formation and that *S. mutans* has evolved specialized pathways to regulate gene expression, protein secretion, and cell surface biogenesis in response to redox (2). The purpose of this study was to identify genes differentially expressed in response to oxygen availability to define

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further the network of genes involved in virulence expression, particularly biofilm formation, by *S. mutans*. On the basis of previous work (2) and the gene expression profiling data described herein, we subsequently reveal important aspects of posttranscriptional control of the exopolysaccharide machinery that may have substantial implications for how *S. mutans* regulates biofilm maturation in vivo in response to oxygen.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Escherichia coli* DH10B was grown in Luria broth, and *S. mutans* UA159 and its derivatives were grown in brain heart infusion (BHI) broth (Difco). For selection of antibiotic-resistant colonies after genetic transformation, ampicillin (100 μg ml^{-1} for *E. coli*) and kanamycin (50 μg ml^{-1} for *E. coli* or 1 mg ml^{-1} for *S. mutans*) were added to the media as needed. For aerobic growth, an overnight culture of *S. mutans* UA159 was diluted 1:50 into a 250-ml conical flask containing 50 ml of BHI broth (Difco) and cultures were grown on a rotary shaker (150 rpm) at 37°C until the absorbance at 600 nm reached 0.4 (mid-exponential phase). For anaerobic growth, cultures were similarly diluted and incubated without agitation in a BBL GasPak Plus anaerobic system (BD, Franklin Lakes, NJ) to the same optical density. Under these conditions, no significant differences in growth rate were noted between aerated cells and cells cultures anaerobically (2).

Microarray experiments. Total RNA was isolated from 10 ml of exponential-phase (optical density at 600 nm = 0.4) cultures as described previously (4). All RNA samples were DNase I treated and purified with the RNeasy mini kit (QIAGEN). RNA concentration was estimated spectrophotometrically in triplicate. Reverse transcription (RT) and microarray reactions were performed with 5.0 μg of total bacterial RNA as described elsewhere (1, 49). *S. mutans* UA159 microarrays were provided by The Institute for Genomic Research (TIGR), and each slide contained four copies of 1960 corresponding to open reading frames in the genome. For each microarray slide, a separate RNA preparation from a separate culture was used, so a total of eight slides (four for each condition) were used in this study. Hybridizations were performed in a Maui hybridization chamber (BioMicro Systems, Salt Lake City, UT). Additional details regarding the array protocols used are available at http://pfgrc.tigr.org/protocols/protocols.shtml.

Data analysis. After the slides were scanned, the resulting images were analyzed by TIGR Spotfinder software (http://www.tigr.org/software/) and further normalized with LOWESS and iterative log mean centering with default settings, followed by in-slide replicate analysis with the TIGR microarray data analysis system (MIDAS; http://www.tigr.org/software/). Statistical analysis was carried out with BRB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html/) with a cutoff *P* value of 0.001.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR was performed on a subset of the genes to validate the microarray data as described previously (4, 5) and to measure the levels of the *gtfB* and *gtfC* mRNAs in the wild-type and vicK-NP strains (Table 1). The gene-specific primers (see Table 2) used in all real-time PCR experiments were designed with Beacon Designer 4.0 software (Premier Biosoft International, Palo Alto, CA). Standard curves for each gene were prepared as described elsewhere (55).

Preparation of protein fractions from *S. mutans*. Various protein fractions were prepared from *S. mutans* cells that were grown under anaerobic or aerobic conditions. In all cases, cells were harvested from BHI broth cultures at an optical density at 600 nm of 0.5, centrifuged, and washed twice with Tris-buffered saline (10 mM Tris, 0.9% NaCl, pH 7.4) (3). Culture supernatant proteins were obtained by passing the supernatant fluid through a 0.45-μm-pore-size filter and concentrating the proteins 80-fold by precipitation with 10% trichloroacetic acid. Whole-cell lysates for protein analysis were obtained by homogenization with a Bead Beater (Biospec, Bartlesville, OK) in sodium dodecyl sulfate (SDS) boiling buffer (60 mM Tris [pH 6.8], 10% glycerol, 5% SDS) in the presence of glass beads. SDS extraction of surface-associated proteins was done by incubating the cells in 4% SDS for 30 min at room temperature (3). For isolation of the cellular soluble fraction, the supernatant fluid was recovered from cells that had been homogenized in the presence of glass beads (0.1 mm, 2 min) in 20 mM Tris buffer (pH 7.4) and centrifuged at 10,000 × *g* for 15 min at 4°C. The remaining pellet was resuspended in SDS boiling buffer and designated the insoluble fraction.

Protein electrophoresis and Western blotting. Protein preparations were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) through a 3 to 8% Tris-acetate gradient gel (Invitrogen), and the proteins were transferred to Immobilon P membranes (Millipore). Both GtfB and GtfC were detected with a polyclonal rabbit antiserum raised against purified GtfB (1:500 dilution) (52), which shares 75% amino acid identity with GtfC. Peroxidase-labeled goat anti-rabbit immunoglobulin G (KPL) and Sigma FAST (3,3′-diaminobenzidine tablets) were used to disclose antibody reactivity. The anti-GtfB antiserum was a kind gift from William H. Bowen, University of Rochester. The protein concentration of samples was determined by a bicinchoninic acid assay (Sigma).

TABLE 1. The *S. mutans* strains used in this study

Strain	Relevant characteristic(s)	Source or reference
UA159	Wild type	
TW54	UA159::P_{gtfB}-*cat*	This study
TW55	UA159::P_{gtfC}-*cat*	This study
vicK-NP	Δ*vicK*::NPKm^r	2

Construction of reporter gene fusions and CAT assays. To construct a reporter gene fusion for measuring transcription from the promoters of the *gtfB* and *gtfC* genes, a 0.2-kbp fragment containing the putative promoter regions and cognate ribosome binding sites (RBS) of each gene was generated by PCR with the primers listed in Table 2. The products were then cloned into BamHI- and PstI-digested plasmid pU1 so as to drive the transcription and translation of a chloramphenicol acetyltransferase (CAT) gene (*cat*) derived from *Staphylococcus aureus* plasmid pC194 (12). Accurate amplification of PCR products was always confirmed by sequencing. The transcriptional fusions were then released by partial SmaI and HindIII digestions and inserted into EcoRV- and HindIII-digested pBluescript KS(+). The resulting plasmids were then digested with SmaI and HincII, and the DNA fragments with the gene fusions were gel purified and ligated into the integration vector pBGK (50) at the unique SmaI site. The resulting *cat* fusion was integrated into the *gtfA* locus of the wild-type strain in single copy to create strains TW54 and TW55, respectively (Table 1). Double-crossover recombination of the reporter gene fusions into the *S. mutans* chromosome was confirmed by PCR amplification with primers internal to *gtfA*.

Microarray data accession number. Microarray data have been deposited at NCBI-GEO (GSE8544).

RESULTS AND DISCUSSION

Overview of the effects of oxygen on the transcriptome of *S. mutans*. To gain further insights into the molecular basis for the effects of oxygen on biofilm formation by *S. mutans* (2), we used DNA microarrays to analyze gene expression profiles of cells cultured under aerobic or anaerobic conditions. Analysis of microarray data revealed that about 5% of the *S. mutans* genome displayed altered expression with a *P* value of ≤0.001 in response to aeration (Fig. 1; Table 3). Substantially more genes were upregulated ($n = 83$) than downregulated ($n = 23$) in response to growth in air. Real-time quantitative RT-PCR was performed on a subset of the genes ($n = 11$) to validate the microarray data (4, 5), and all of the genes displayed the same trend observed in the microarrays (Table 4).

Up-regulated genes. The majority of the upregulated genes were in five functional groups, i.e., (i) hypothetical, unassigned, and unknown proteins ($n = 28$); (ii) energy metabolism proteins ($n = 25$); (iii) signal transduction proteins ($n = 9$); (iv) transport and binding proteins ($n = 8$); and (v) cellular-process proteins ($n = 4$) (Fig. 1; Table 3). Notably, 16 genes were strongly induced by aeration (10- to 42-fold) and most of these were either established or predicted bacteriocin-encoding genes (SMU.150 [*nlmA*], SMU.151 [*nlmB*], SMU.423, SMU.1905c, SMU.1906c, and SMU.1914c [*bip*]) (16) that are localized in two gene clusters, SMU.148 to SMU.152 and SMU.1903c to SMU.1914c. Because mutacins (*S. mutans* bacteriocins) are known to kill or inhibit the growth of closely related bacteria (16, 20), their biogenesis in an aerated environment may reflect a defense mechanism by *S. mutans* to

TABLE 2. Primers used for amplification of putative promoters for *cat* fusions and for real-time PCR in this study

Application and name	Sequence	Promoter or amplicon	Primer name	Sequence
Amplification of putative promoters				
PgtfB-5	ATGGATCCGACAATTGTGGTGGGTAC	P_{gtfB}	PgtfB-3	CGCTGCAGCTTGTCCATTAGGAACCTCC
PgtfC-5	GTGAATTCGATGCTAACTCTGGAGAACG	P_{gtfC}	PgtfC-3	GAGGATCCAAAAATAGTTAGAGTTAGTG
Real-time RT-PCR				
gtfB-sense	AGCAATGCAGCCATCTACAAAT	*gtfB*	gtfB-antisense	ACGAACTTTGCCGTTATTGTCA
gtfC-sense	GGTTTAACGTCAAAATTAGCTGTATTAGC	*gtfC*	gtfC-antisense	CTCAACCAACCGCCACTGTT
SMU.150-sense	GGAAGGTATCGGGTGGAGAAGC	SMU.150	SMU.150-antisense	GAGTCGCACCTGCCAGTCC
SMU.299-sense	GGAGCTAATGGCTATGCTTACCG	SMU.299	SMU.299-antisense	GCACCTGAAGCCCAGCTATTTAC
SMU.423-sense	CAACTGTTGAGGGTGGTGGTATG	SMU.423	SMU.423-antisense	AAGCCGCTCCAGATACTGTACC
SMU.575-sense	TTGCCTAAAGCCTTACCGATTCC	SMU.575	SMU.575-antisense	GCCTGATGGGACAAACATAAAGC
SMU.577-sense	TCCATCTACTTCGCAGGTCATTG	SMU.577	SMU.577-antisense	AATCCCAGCCAGTAAAGAAACCG
SMU.879-sense	TTCAGAGCCTGGTTCTTCTTTCC	SMU.879	SMU.879-antisense	TTCCCAAAGCACTGCCAACTG
SMU.1175-sense	GAGAAGTCTCTGGCGGACCTATG	SMU.1175	SMU.1175-antisense	GCCACTAAGATACCTGCCAATGC
SMU.1508-sense	AGGAGAGATGTCTGTTGAGAAGC	SMU.1508	SMU.1508-antisense	AACGGTTCACCTCCAGTAACAC
SMU.1609-sense	TGCAGCCTCAAAAGAATCCTAGC	SMU.1609	SMU.1609-antisense	GCAATCGCAAGCCAAAAGAAGAC
SMU.1913-sense	TCACAAGAAAGTTCGGCTGCTAC	SMU.1913	SMU.1913-antisense	CTGCTGGCAAATTCGCTTACTTG
SMU.1960-sense	ACTGTAACACGTTGGGCGAAAG	SMU.1960	SMU.1960-antisense	AGCAGCAAAGGCTTCTTTAGACC

inhibit the colonization of competitor organisms during early biofilm formation. A putative bacteriocin immunity protein gene, *bip*, that is located immediately upstream from *comC* was also was found to be up-regulated by about 18-fold. Interestingly, Bip has been reported to affect sensitivity to a variety of antimicrobial agents, as well as to be upregulated in the presence of low concentrations of antibiotics and during biofilm formation (31). It was postulated that Bip may have broad specificity and could provide protection against a variety of inhibitory substances (i.e., antibiotics, bacteriocins, metals, etc.), which is likely important for the resistance of *S. mutans* to host defenses and antagonistic interactions in nascent and mature biofilms. Consistent with the activation of bacteriocin-related genes, *comD* (SMU.1916c), which encodes the histidine kinase of a TCS and coordinates multiple environmental signals for the induction of *com* genes and development of

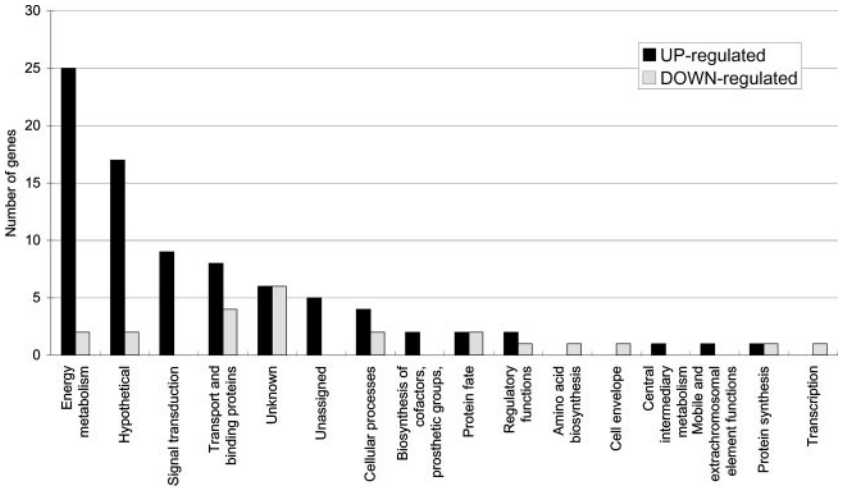

FIG. 1. Distribution of functions of genes affected by oxygen availability. The 106 genes differentially expressed at $P \leq 0.001$ were grouped by functional classification according to the Los Alamos *S. mutans* genome database (http://www.oralgen.lanl.gov/).

TABLE 3. Genes differentially expressed under aerobic conditions

Unique identification no.	Description	Gene(s)	*n*-Fold difference between aerobic/anaerobic geometric means	*P* value
SMU.423	Possible bacteriocin		41.969	<1e−07
SMU.1912c	Hypothetical protein		33.572	<1e−07
SMU.1904c	Hypothetical protein		32.951	<1e−07
SMU.1913c	Hypothetical protein; immunity protein, BlpL like		33.875	0.0000001
SMU.1903c	Hypothetical protein		29.797	<1e−07
SMU.1425	ATP-dependent Clp protease, ATP-binding subunit ClpB	*clpB*	29.838	0.0000006
SMU.150	Nonlantibiotic mutacin IVA	*nlmA*	28.789	<1e−07
SMU.151	Nonlantibiotic mutacin IVB	*nlmB*	28.011	<1e−07
SMU.1905c	Hypothetical protein		26.629	<1e−07
SMU.152	Hypothetical protein		24.281	<1e−07
SMU.1906c	Bacteriocin-related protein		24.048	<1e−07
SMU.1914c	Bacteriocin immunity protein, BlpO like	*bip*	17.848	0.0000002
SMU.1909c	Hypothetical protein		14.212	0.0000001
SMU.148	Alcohol-acetaldehyde dehydrogenase	*adhE*	14.326	0.0003066
SMU.1424	Dihydrolipoamide dehydrogenase	*acoL adhD*	13.986	0.0000364
SMU.575c	Murein hydrolase regulator	*lrgA*	11.656	0.0000492
SMU.2057c	Cadmium efflux ATPase, E1-E2 (heavy-metal-transporting ATPase)	*cadA cadD*	9.516	0.0000005
SMU.78	Fructan hydrolase; exo-β-D -fructosidase	*fruA*	8.624	0.0000002
SMU.1539	1,4-α-Glucan branching enzyme	*glgB*	8.222	0.0000007
SMU.79	Fructan hydrolase; exo-β-D -fructosidase	*fruB*	7.871	0.0000032
SMU.1421	Dihydrolipoamide acetyltransferase (acetoin dehydrogenase E2 component)	*acoC yugF*	7.696	0.0000193
SMU.997	Inorganic ion ABC transporter, ATP-binding protein; possible ferrichrome transport system	*fecE yclP*	7.5	0.0000001
SMU.2127	Succinic semialdehyde dehydrogenase (NAD-dependent aldehyde dehydrogenase)	*gabD*	7.463	0.0000001
SMU.180	Oxidoreductase, possible fumarate reductase		7.063	0.00001
SMU.998	ABC transporter, ferrichrome-binding protein	*fatB yclQ*	6.683	0.0000001
SMU.925	Hypothetical protein		6.386	0.0001306
SMU.179	Conserved hypothetical protein (possible oxidoreductase)		6.287	0.0000006
SMU.1423	Acetoin dehydrogenase E1 component	*acoA*	6.193	0.0003586
SMU.402	Pyruvate formate-lyase	*pfl*	6.077	0.0000062
SMU.995	Ferrichrome ABC transporter (permease)	*yclN*	5.876	0.0000006
SMU.252	Hypothetical protein		5.833	0.0000004
SMU.1536	Glycogen synthase	*glgA*	5.513	0.0000081
SMU.1117	H_2O-forming NADH oxidase	*naoX nox nox-2*	5.493	0.0000507
SMU.996	ABC transporter, permease protein; possible ferrichrome transport system	*yclN*	5.35	0.0000091
SMU.577	Sensor histidine kinase	*lytS*	4.969	0.0000003
SMU.1535	Glycogen phosphorylase	*glgP*	4.957	0.0000031
SMU.1077	Phosphoglucomutase	*pgmA*	4.922	0.0000133
SMU.799c	Conserved hypothetical protein		4.806	0.0000024
SMU.149	Transposase fragment (IS*605*/IS*200* like)	*tpn*	4.794	0.000023
SMU.1596	PTS system, cellobiose-specific IIC component	*celB lacE*	4.414	0.0000996
SMU.1116c	Hypothetical protein		4.362	0.0001648
SMU.1957	Fructose-specific enzyme IID component	*levG ptnD*	3.766	0.0000435
SMU.1410	Fumarate reductase	*frdC*	3.677	0.0000493
SMU.113	Fructose-1-phosphate kinase	*pfk*	3.643	0.0009101
SMU.1591	Catabolite control protein A	*ccpA regM*	3.635	0.0001455
SMU.1843	Sucrose-6-phosphate hydrolase	*scrB*	3.432	0.000015
SMU.500	Ribosome-associated protein	*yfiA*	3.274	0.0000558
SMU.1960c	Fructose-specific enzyme IIB component	*levE*	3.101	0.0000133
SMU.1088	Thiamine biosynthesis lipoprotein	*apbE*	3.098	0.0000072
SMU.101	Sorbose PTS system, IIC component	*sorC*	3.048	0.0003228
SMU.1956c	Conserved hypothetical protein		2.985	0.0000254
SMU.883	Glucan 1,6-α-glucosidase	*dexB*	2.983	0.0004012
SMU.1411	Conserved hypothetical protein		2.956	0.0001324
SMU.1958c	Fructose-specific enzyme IIC component	*levF*	2.949	0.0000213
SMU.1090	Conserved hypothetical protein		2.783	0.0000132
SMU.1495	Galactose-6-phosphate isomerase	*lacB rpiB*	2.61	0.0003498
SMU.629	Superoxide dismutase	*sod sodA*	2.601	0.0000356
SMU.878	ABC transporter, sugar-binding protein	*msmE*	2.585	0.0002202

Continued on facing page

TABLE 3—*Continued*

Unique identification no.	Description	Gene(s)	*n*-Fold difference between aerobic/anaerobic geometric means	*P* value
SMU.765	Alkyl hydroperoxide reductase, subunit F	*ahpF nox1*	2.566	0.0000345
SMU.674	Phosphotransferase system phosphohistidine-containing protein	*pthP*	2.523	0.0000325
SMU.882	Multiple sugar-binding transport ATP-binding protein MsmK	*msmK*	2.527	0.0002513
SMU.1758c	Conserved hypothetical protein		2.471	0.0004122
SMU.1490	Phospho-β-D -galactosidase	*lacG*	2.448	0.0002415
SMU.130	Dihydrolipoamide dehydrogenase	*acoL adhD*	2.401	0.0003251
SMU.879	ABC transporter, sugar permease protein	*msmF*	2.379	0.0000512
SMU.1844	Sucrose operon repressor	*scrR*	2.365	0.0000449
SMU.131	Lipoate-protein ligase	*lplA*	2.286	0.0000561
SMU.1916	Histidine kinase	*comD hk08 iH*	2.267	0.0000234
SMU.1590	Intracellular α-amylase	*amy*	2.205	0.0002606
SMU.880	Multiple-sugar-binding transport system permease protein MsmG	*msmG*	2.178	0.000069
SMU.924	Thiol peroxidase	*tpx*	2.127	0.0009348
SMU.1754c	Conserved hypothetical protein		2.122	0.0002191
SMU.633	Thioesterase	*tesA*	2.117	0.0001955
SMU.1126	Pantothenate kinase	*coaA*	2.116	0.0007698
SMU.1183	Phosphotransferase system enzyme II	*mtlA2*	2.113	0.000973
SMU.1563	Cation-transporting P-ATPase	*pacL*	2.093	0.0007923
SMU.404c	Hypothetical protein		2.046	0.0003166
SMU.1978	Acetate kinase	*ackA comYI*	1.964	0.0003371
SMU.881	Sucrose phosphorylase	*gftA*	1.825	0.0009275
SMU.251	ABC transporter permease		1.789	0.0009034
SMU.204c	Hypothetical protein		1.752	0.0007489
SMU.926	GTP pyrophosphokinase family protein		1.703	0.0008682
SMU.273	Hexulose-6-phosphate synthase	*rmpD*	1.635	0.0009137
SMU.1609c	Preprotein translocase, SecG subunit	*secG*	0.575	0.0002803
SMU.2129c	Conserved hypothetical protein		0.566	0.000596
SMU.363	Transcriptional regulator; glutamine synthetase repressor	*glnR*	0.564	0.0005633
SMU.1519	Glutamine ABC transport, ATP-binding protein	*glnQ*	0.537	0.0002578
SMU.672	Isocitrate dehydrogenase	*citC icd*	0.513	0.0009986
SMU.2071	Anaerobic ribonucleoside triphosphate reductase-activating protein	*nrdG*	0.488	0.0005869
SMU.697	Translation initiation factor IF-3	*infC*	0.486	0.00014
SMU.299c	Bacteriocin peptide precursor	*ip*	0.481	0.0004078
SMU.2073c	Conserved hypothetical protein		0.445	0.0000822
SMU.1897	ABC transporter, ATP-binding protein; similar to BlpA		0.435	0.0004475
SMU.1554c	Conserved hypothetical protein		0.434	0.0008506
SMU.1997	Competence-specific sigma factor	*comX*	0.434	0.0000765
SMU.1505c	Conserved hypothetical protein (phenylalanyl-tRNA synthetase fragment)	*pheT*	0.432	0.0006737
SMU.611	ATP-dependent RNA helicase/DEAD family	*deaD rheA*	0.432	0.0004757
SMU.40	Conserved hypothetical protein		0.422	0.0009784
SMU.396	Glycerol uptake facilitator protein	*glpF*	0.416	0.0005311
SMU.1701c	Conserved hypothetical protein		0.396	0.0004313
SMU.1502c	Conserved hypothetical protein		0.386	0.0000699
SMU.1657c	Nitrogen regulatory protein PII	*glnB*	0.354	0.0000544
SMU.758c	Conserved hypothetical protein		0.303	0.0001941
SMU.539c	Prepilin peptidase type IV	*comC hopD*	0.278	0.00063
SMU.1004	Glucosyltransferase I	*gtfB*	0.262	0.0000241
SMU.1175	Sodium:alanine (or glycine) symporter	*dagA*	0.117	0.0004863

competence (5, 27), was upregulated about 2.3-fold in the presence of oxygen. Of note, the *comC* quorum-sensing system (ComCDE) of *S. mutans* has been shown to regulate the expression of antimicrobial peptides (23, 46), including the two-peptide nonlantibiotic bacteriocin encoded by *nlmAB* and *bip* (46).

It is known that cultivation of lactic acid bacteria in the presence of air can affect glycolytic rates and elicit a shift to heterofermentative metabolism (21, 35, 45, 53, 57). Consistent with this knowledge, growth in oxygen markedly increased the expression of genes that encode the partial tricarboxylic acid cycle of *S. mutans*, upregulated pyruvate formate lyase, and increased expression of the water- and peroxide-forming NADH oxidases (Table 3). In addition to these changes, which

TABLE 4. Comparison of real-time PCR and microarray differences between selected genes

Unique GenBank identification no.	Description	*n*-Fold difference between aerobic/anaerobic geometric means	
		Microarray	Real-time PCR
SMU.423	Possible bacteriocin	41.969	20.619
SMU.1913	Hypothetical protein, putative immunity protein, BlpL like	32.951	11.852
SMU.150	Nonlantibiotic mutacin IVA (*nlmA*)	28.789	21.951
SMU.575	Murein hydrolase regulator (*lrgA*)	11.656	16.139
SMU.577	Sensor histidine kinase (*lytS*)	4.969	3.201
SMU.1960	Fructose-specific enzyme IIB component (*levE*)	3.101	4.681
SMU.879	ABC transporter, sugar permease protein (*msmF*)	2.365	1.882
SMU.1609	Preprotein translocase, SecG subunit	0.575	0.473
SMU.299	Bacteriocin peptide precursor (*ip*)	0.481	0.425
SMU.1004	Glucosyltransferase I (*gtfB*)	0.262	0.451
SMU.1175	Sodium:alanine (glycine) symporter (*dagA*)	0.117	0.071

can modify the way carbohydrate flows through the cell, it is noteworthy that the *ccpA* (*c*arbon *c*atabolite *p*rotein *A*) gene was upregulated 3.6-fold in response to growth in air. CcpA is a DNA binding protein that functions as part of a global regulatory system that controls transcription, in response to levels of glycolytic intermediates, of a variety of genes that encode products involved in the transport and catabolism of carbohydrates (38, 51). Recently, CcpA in *S. mutans* has been shown to serve as a major regulator of the expression of glycolytic and tricarboxylic acid cycle enzymes, carbohydrate transporters, and catabolic pathways (Burne et al., unpublished data), as well as to have a central role in the control of biofilm formation (51) and the expression of the *gtfBC* and *ftf* genes (9), which encode the major exopolysaccharide-producing enzymes of *S. mutans* (38, 51). Consistent with the differential regulation of CcpA, a variety of carbohydrate uptake systems, including PTS (phosphoenolpyruvate:sugar transferase system) and ABC (ATP-binding cassette) transporters, were substantially upregulated (Table 3) in cells exposed to oxygen. It is noteworthy that oxygen is a major factor in the *ccpA*-mediated control of metabolism in *Lactococcus lactis* (14). Collectively, these data indicate that the machinery for the uptake and catabolism of carbohydrates is extensively regulated by oxygen at the transcriptional level for efficient carbohydrate utilization, maintenance of NAD-NADH balances, and active oxygen metabolism during growth in air. The relationship of these changes to alterations in the expression levels of CcpA remains to be explored, especially in light of the fact that the DNA binding activity of CcpA is tightly controlled by binding to serine-phosphorylated HPr and particular glycolytic intermediates (13, 14, 28, 48).

There may be considerable significance to the observation that gene products that are predicted to influence autolysis, including *lrgA* (SMU575c), *lytT* (SMU.576c), *lytS* (SMU.577c), SMU.1700c, and SMU.1701c, were strongly up- or down-regulated during growth with aeration (Table 3). It has been established that autolysins participate in a variety of biological processes, including cell wall turnover, cell growth, antibiotic resistance, cell-to-surface adhesion, genetic competence, and protein secretion (7, 15, 18, 33, 39). In *S. mutans*, the AtlA autolysin is required for efficient biofilm maturation and profoundly influences the composition of the cell envelope (3, 8). In *S. aureus*, the *lrgAB* gene products are under the control of the LytSR TCS and control autolytic behavior by modulating autolysin activity and penicillin tolerance (15). Given the demonstrated importance of autolytic activity and oxygen for *S. mutans* biofilm maturation (2), further investigation of the roles of the LrgAB and LytST proteins in the control of virulence-related phenotypes is warranted. It is also of note that the staphylococcal *lrgAB* and *cidABC* genes, which encode a second group of gene products that participate in autolysin regulation, are strongly influenced by glucose and acetic acid (36). Thus, the alterations in the expression of the apparent homologues of *lrg* and *lytS* in *S. mutans* may be induced secondarily by changes in acid end products caused by growth in air or through alterations in the levels or binding activity of CcpA. In fact, the expression of the genes in the two regions, SMU.574c to SMU.577c and SMU.1700c to SMU.1701c, was significantly influenced by loss of CcpA and affected by growth under conditions that alleviate catabolite repression in *S. mutans* (Burne et al., unpublished data). Finally, the large number ($n = 28$) of hypothetical proteins that display increased expression in cells cultivated in air are of considerable interest, as they may be important in factors in initial adherence or biofilm formation by *S. mutans*.

Down-regulated genes. The 23 down-regulated genes encode predominantly hypothetical or unknown proteins ($n = 8$), products involved in transport and binding processes ($n = 4$), cellular processes ($n = 2$), energy metabolism ($n = 2$), and protein fate ($n = 2$). It is of note that regulators and transporters associated with amino acid metabolism, such as *glnR* (SMU.363), *dagA* (SMU.1175), *glnQ* (SMU.1519), and *glnB* (SMU.1657c), were downregulated, possibly implicating the engagement of stringent control of gene expression mediated by the nutritional alarmones (p)ppGpp. This idea is supported by the observation that *relP* (SMU.926), which has recently been shown to catalyze (p)ppGpp synthesis in *S. mutans* (26), was up-regulated about 70% in the presence of oxygen. It has been proposed that (p)ppGpp adjusts cellular metabolism by favoring the transcription of genes involved in amino acid biosynthesis and stress tolerance at the expense of those essential for growth (29). On the other hand, the down-regulation of genes involved in amino acid biosynthesis is confined to a relatively small subset of genes, and the observations could be due to other factors, including alterations in carbohydrate metabolism, in CcpA levels, or in other regulatory proteins, for example, SMU.1657c, which encodes a putative nitrogen-dependent regulator of gene expression.

Oxygen and exopolysaccharide metabolism. One particularly interesting finding was that the *gtfB* gene was down-regulated 3.3-fold under aerobic conditions. Since GtfB is a major enzyme involved in the production of adhesive, water-insoluble glucan exopolymers, down-regulation of this gene could contribute to the diminished capacity to form biofilms in air (2). It

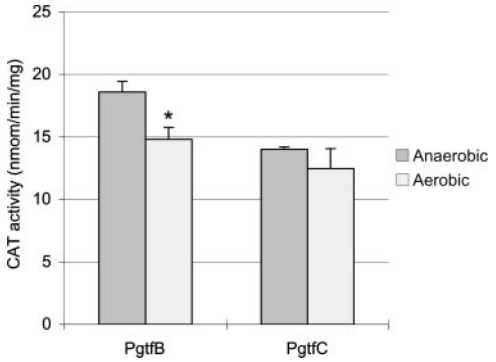

FIG. 2. CAT assay. The *gtfB* and *gtfC* promoter fusions with the *cat* gene were inserted in a single copy into the chromosome of the wild type, and the resulting strains were designated TW54 (PgtfB) and TW55 (PgtfC), respectively. The strains were grown under aerobic or anaerobic conditions. See the text for more details. The data presented are means ± standard deviations (error bars) of at least three independent experiments. *, $P < 0.01$ (Student's *t* test).

is also of interest that the expression of the *gtfC* gene was not altered, even at $P < 0.01$, considering that *gtfB* and *gtfC* are tandemly arranged and have been reported to be cotranscribed (44, 56). Given the critical roles that GtfB and GtfC play in establishing the extracellular polysaccharide matrix (glucans) that is crucial for the organisms' adherence to and accumulation on tooth surfaces (6, 32, 54), we investigated in more detail the regulation of *gtfBC* in response to oxygen. To measure the expression of *gtfB* and *gtfC*, transcriptional fusions to the promoters of these genes were constructed as described in Materials and Methods, with the primers shown in Table 2 and the strains described in Table 1. Consistent with the microarray data, the expression of *gtfB* (TW54) alone was reduced by about 20% in response to growth in oxygen (Fig. 2). However, there was no significant difference in the expression of *gtfC* (TW55) under these same conditions. This result confirms the microarray data showing that *gtfB* expression is sensitive to aeration but also reveals that the two genes can be independently and differentially regulated in response to an environmental signal.

Recently, we observed that a protein band of approximately 150 kDa was present in much greater quantity in cell-associated fractions of *S. mutans* UA159 grown under aerobic conditions compared with anaerobic conditions (data not shown). To identify the band, it was excised from the gel and trypsin-digested protein was analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry. The protein was identified as GtfC, suggesting that that the amount of GtfC enzyme associated with the surface of *S. mutans* increased dramatically in aerobically cultivated cells. Taking into account this finding, we investigated further the posttranscriptional regulation of the Gtf proteins by oxygen.

Localization of GtfB and GtfC is significantly affected by oxygen. SDS-PAGE and Western blot analysis were used to compare GtfB and GtfC levels in various protein fractions from *S. mutans* cells grown under anaerobic or aerobic conditions (3). The proteins were separated by SDS-PAGE through a 3 to 8% gradient gel and transferred to Immobilon P membranes. Both GtfB and GtfC were detected with a polyclonal antiserum raised against purified GtfB (1:500 dilution) (52), which shares 75% amino acid identity with GtfC. In the Western blot profile of whole-cell lysates prepared by homogenizing cells in the presence SDS, both GtfB (top) and GtfC (bottom) were readily detected (Fig. 3A). Interestingly, in cells cultured aerobically, the intensity of the GtfC band, as a proportion of the reactivity with GtfB, in the 4% SDS extracts of cells (Fig. 3B), the soluble fraction prepared by bead beating in Tris buffer without SDS (Fig. 3C), and the insoluble fraction consisting of the remaining pellet that had been treated with SDS boiling buffer (Fig. 3D), was always greater than that of the GtfC band in the same fraction prepared from anaerobically grown cells. However, the relative amounts of GtfC were equivalent in the supernatant fractions, regardless of the atmosphere in which the cells were grown (Fig. 3E). These observations contrast with the transcriptional data and demonstrate that not only the localization but probably also the stability of the GtfC enzyme is affected by oxygen. On the basis of our previous observations, we believe that alterations in the localization of GtfC arise from changes in the envelope induced by growth in oxygen (2). The basis for the increase in the absolute amount of GtfC in aerobically grown cells may be due to enhanced translation or secretion of GtfC or to the downregulation or inhibition of an activity that degrades GtfC. However, there was no evidence of increased amounts of Gtf breakdown products in Western blot assays of anaerobically grown cells (data not shown). Importantly, the increased production of the GtfC enzyme in aerated cells and its strong association with the cell surface may reflect an important role of this enzyme in the early stages of biofilm formation by this organism in vivo. Specifically, strong association of the enzyme with the cells in newly forming biofilms, where O_2 concentrations may be high, may enhance the synthesis of, and adherence to, water-insoluble glucans by the organism. Along these lines, the kinetic properties and stability of GtfC are known to be enhanced by binding of the enzyme to a solid surface (42, 43, 47), so cell association may increase the efficiency of this enzyme in

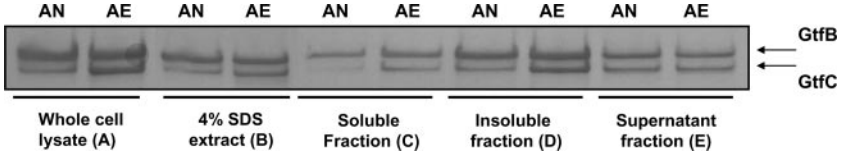

FIG. 3. Western blot analysis of different cell extracts from wild-type *S. mutans* grown under anaerobic (AN) or aerobic (AE) conditions, i.e., bead-beaten SDS boiling extract (whole-cell lysates, A), 4% SDS extracts (B), bead-beaten Tris extracts (soluble fraction, C), the insoluble fraction (D), and the supernatant fraction (E). See Materials and Methods for more details about the preparation of the cellular fractions. Following SDS-PAGE, proteins were blotted onto a nitrocellulose membrane and subjected to Western blotting with an anti-GtfB polyclonal antiserum at a 1:500 dilution.

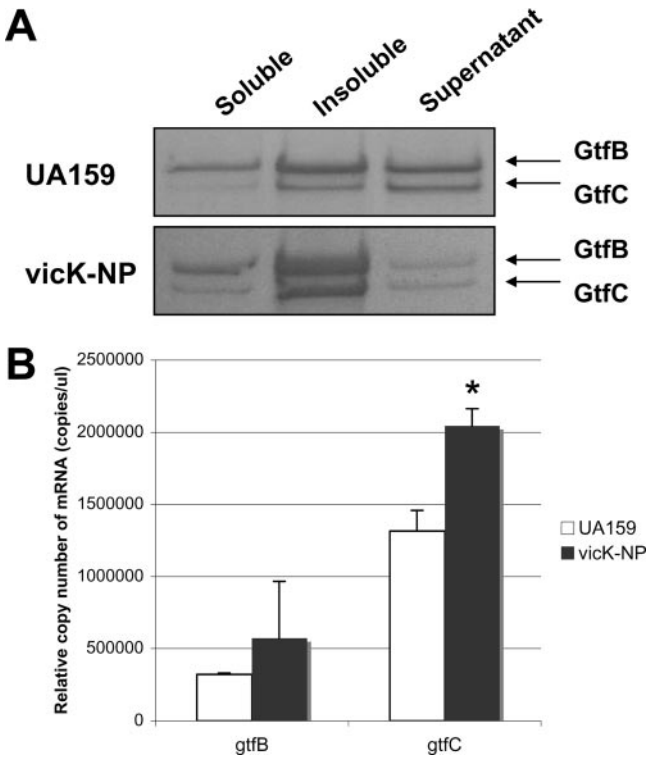

FIG. 4. The behaviors of GtfB and GtfC in the *vicK*-deficient mutant (vicK-NP). (A) Western blot analysis of different cell extracts from wild-type (UA159) and vicK-NP strains of *S. mutans*, i.e., bead-beaten Tris extract (soluble fraction), the insoluble fraction, and the supernatant fraction. Following SDS-PAGE, proteins were blotted onto a nitrocellulose membrane and subjected to Western blotting with an anti-GtfB polyclonal antiserum at a dilution of 1:500. See the text for more details. (B) Differential expression of *gtfB* and *gtfC* in the wild-type and *vicK* mutant strains by real-time PCR. The data shown are means ± standard deviations (error bars) from at least three independent experiments. *, $P < 0.001$ (Student's *t* test).

vivo and alter the nature of the polysaccharides produced in early biofilms. Future studies will be oriented toward understanding the basis for the enhanced cell association of GtfC, which may arise, for example, from changes in the physicochemical properties of the wall, from altered expression or localization of proteins that interact with Gtf proteins, or through the synthesis of carbohydrate bridging molecules that are bound by wall-associated proteins and the Gtf proteins.

The VicRK TCS affects the localization of GtfB or GtfC. In our recent study (2), elevated levels of cell-associated GtfC were observed in Coomassie-stained gels of whole-cell lysates and 4% SDS extracts of a VicK-deficient strain (vicK-NP, Table 1). The VicK sensor kinase protein, as a component of the VicRKX TCS, was recently reported to harbor a PAS domain, which can function as a sensor of redox potential (37). When various fractions of proteins from wild-type and vicK-NP cells were separated by SDS-PAGE, GtfB and GtfC levels were significantly elevated in both the soluble and insoluble fractions. In contrast, there was a reduction in the amount of both proteins in the supernatant fractions (Fig. 4A). Consistent with the SDS-PAGE data, *gtfC* mRNA levels were 20 to 30% higher in the *vicK* mutant ($P = 0.002$) during growth in the presence oxygen (Fig. 4B). Expression of the *gtfB* gene also showed a trend for increased transcription, but the increase was not statistically significant ($P = 0.38$). Collectively, the data are most supportive of the idea that while some increase in the transcription of the genes occurs in the VicK-deficient strain, alterations in the composition and biochemical properties of the surface of the cells occur (2) that allow a stronger association of the Gtf proteins with the cell. A central player in Vic-dependent modification of the cell surface appears to be the AtlA autolysin complex (2), but the array data support the idea that other autolytic pathways may play a role in modulation of the properties of the cell surface. Studies are under way to understand why loss of VicK causes substantial changes in surface protein profiles and to elucidate the nature of the changes to the cell surface or Gtf proteins that affect not only the localization of GtfB and GtfC but also biofilm formation and virulence gene expression by *S. mutans*.

Concluding remarks. To our knowledge, this is the first report that shows a global gene expression profile of *S. mutans* in the presence of oxygen. The results presented herein indicate that oxygen is a key environmental signal that significantly alters the transcriptome and that these alterations strongly influence bacteriocin production, carbohydrate metabolism, and the expression of known virulence attributes. As shown here and in our previous work (2), oxygen also has profound effects on the biogenesis of a normal cell surface, exerted in large part through the Vic signal transduction pathway and the AtlA autolytic circuit. The dramatic changes in the localization of enzymes that produce the polysaccharide matrix that is characteristic of *S. mutans* biofilms indicates that the organisms have evolved a sophisticated sensing pathway to alter their capacity to form biofilms in response to the redox environment. Continued analysis of the effects of O_2, Vic, and AtlA on the expression and localization of specific gene products is under way to provide a comprehensive picture of the way in which *S. mutans* integrates environmental signals during biofilm maturation.

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