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Loss of NADH Oxidase Activity in *Streptococcus mutans* Leads to Rex-Mediated Overcompensation in NAD^+ Regeneration by Lactate Dehydrogenase

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ABSTRACT

Previous studies of the oral pathogen *Streptococcus mutans* have determined that this Gram-positive facultative anaerobe mounts robust responses to both acid and oxidative stresses. The water-forming NADH oxidase (Nox; encoded by *nox*) is thought to be critical for the regeneration of NAD^+, for use in glycolysis, and for the reduction of oxygen, thereby preventing the formation of damaging reactive oxygen species. In this study, the free NAD^+/NADH ratio in a *nox* deletion strain (Δ*nox*) was discovered to be remarkably higher than that in the parent strain, UA159, when the strains were grown in continuous culture. This unanticipated result was explained by significantly elevated lactate dehydrogenase (Ldh; encoded by *ldh*) activity and *ldh* transcription in the Δ*nox* strain, which was mediated in part by the redox-sensing regulator Rex. cDNA microarray analysis of *S. mutans* cultures exposed to simultaneous acid stress (growth at a low pH) and oxidative stress (generated through the deletion of *nox* or the addition of exogenous oxygen) revealed a stress response synergistically heightened over that with either stress alone. In the Δ*nox* strain, this elevated stress response included increased glucose phosphoenolpyruvate phosphotransferase system (PTS) activity, which appeared to be due to elevated *manL* transcription, mediated in part, like elevated *ldh* transcription, by Rex. While the Δ*nox* strain does possess a membrane composition different from that of the parent strain, it did not appear to have defects in either membrane permeability or ATPase activity. However, the altered transcriptome and metabolome of the Δ*nox* strain were sufficient to impair its ability to compete with commensal peroxigenic oral streptococci during growth under aerobic conditions.

IMPORTANCE

***Streptococcus mutans* is an oral pathogen whose ability to outcompete commensal oral streptococci is strongly linked to the formation of dental caries. Previous work has demonstrated that the *S. mutans* water-forming NADH oxidase is critical for both carbon metabolism and the prevention of oxidative stress. The results of this study show that upregulation of lactate dehydrogenase, mediated through the redox sensor Rex, overcompensates for the loss of *nox*. Additionally, *nox* deletion led to the upregulation of mannose and glucose transport, also mediated through Rex. Importantly, the loss of *nox* rendered *S. mutans* defective in its ability to compete directly with two species of commensal streptococci, suggesting a role for *nox* in the pathogenic potential of this organism.**

Streptococcus mutans, the major etiologic agent of dental caries, is an opportunistic oral pathogen that resides in the multispecies biofilm known as dental plaque. Microorganisms that live in the oral cavity must be equipped to survive feast-or-famine cycles of nutrient availability, rapid changes in pH and temperature, and a dynamic range of oxygen concentrations (1–3). Dental plaque is somewhat porous, allowing the movement of fluids and nutrients, the establishment of chemical gradients, and the development of several environmental and ecological niches (4–6). *S. mutans* has complex stress response pathways that allow it to survive this rapidly changing environment and to thrive in dental plaque in the face of both acid and oxidative stresses (2, 7).

Oxidative stress poses a major threat to the persistence of *S. mutans*. Oxygen gas (O_2) is abundant in the habitat of *S. mutans*, particularly in the outer levels of dental plaque (8, 9). The peroxigenic oral streptococci, including *Streptococcus sanguinis*, *S. gordonii*, and *S. oligofermentans*, are major competitors of *S. mutans*. These early colonizers of dental plaque generate bactericidal levels of H_2O_2 through enzymes such as pyruvate oxidase and lactate oxidase (10–14). Both O_2 and H_2O_2 promote the formation of more-damaging reactive oxygen species (ROS), such as the hydroxyl radical and the superoxide anion radical (15). In order to survive this onslaught and establish itself in the biofilm, *S. mutans* must be able both to prevent the formation of harmful ROS and to safely eliminate the ROS that do form. *S. mutans* encodes several

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enzymes that accomplish these tasks, including two NADH oxidases (encoded by *nox* [SMU.1117] and *ahpCF* [SMU.764 and SMU.765]), superoxide dismutase (encoded by *sod* [SMU.629]), the peroxide resistance protein (encoded by *dpr* [SMU.540]), and glutathione oxidoreductase (encoded by *gor* [SMU.838]) (16). A previous study by our group revealed the oxidative stress stimulon of *S. mutans* in the context of oxidative stress on a steady-state culture, generated through elevation of the dissolved-oxygen (DO) concentration or deletion of the *nox* gene (encoding Nox, the water-forming NADH oxidase) (17).

As *S. mutans* and other colonizers of the tooth surface become established, and the plaque biofilm matures, vast amounts of organic acids are produced by the oral streptococci after the consumption of carbohydrates by the host (3). It is these acids that induce the demineralization of host tooth enamel and ultimately cause dental caries. As these acids diffuse through the dental plaque matrix, organisms in the niche are exposed to acidic pHs. *S. mutans* is more aciduric than the peroxigenic oral streptococci; thus, it can generally outcompete them at acidic pHs (3, 12). The ability of *S. mutans* to thrive in acidic environments is due in large part to the maintenance of a cytosolic pH more neutral than the pH of the extracellular milieu. This is accomplished by several mechanisms, collectively known as the acid tolerance response (ATR), which includes the upregulation of the F_1F_o ATPase (in this case catalyzing ATP hydrolysis to extrude protons from the cytosol), the agmatine deiminase system, malolactic acid fermentation, branched-chain amino acid (BCAA) biosynthesis, the downregulation of the phosphotransferase system (PTS), and the alteration of membrane fatty acid composition (2, 18–24). We have recently reported the shifts that occur in the *S. mutans* transcriptome following glucose shock and the resulting acidification of a continuous culture (25).

Several studies have indicated overlap and synergy between the oxidative stress response and the ATR. Deletion of *nox* or growth in a medium supplemented with oxygen resulted in a large increase in the percentage of unsaturated fatty acids (UFAs) in the *S. mutans* plasma membrane, reminiscent of the changes seen in the membrane upon induction of the ATR (~60% UFAs compared to ~35% seen following growth at pH 7 without oxidative stress) (23, 26). Concurrent acid and oxidative stresses were associated with even further elevation of UFA abundance, which approached 75% (26). Likewise, when cultures were grown at pH 5, ROS-metabolizing enzymes were upregulated relative to their expression during growth at pH 7, indicating induction of the oxidative stress response (25). Nox has been shown to be critical for the regeneration of NAD^+ for use in carbon metabolism in addition to its role in the reduction of oxygen, and its transcription was induced under both acidic and oxidative conditions (17, 25). Cultures of *S. mutans* UA159 subjected to simultaneous acid and oxidative stresses led to an even higher level of *nox* transcription than that under either condition alone, suggesting that imposing both stresses on the organism at the same time may lead to exacerbation of the stress response (17). In the same study, it was revealed that *nox* transcription was modulated by SpxA1 (formerly known as SpxA; encoded by *spxA1* [SMU.1044]) and by Rex (encoded by *rex* [SMU.1053]), two proteins that sense the redox state and influence their regulons accordingly (17). An attractive hypothesis was that in addition to the elevated DO concentration experienced by the Δ*nox* strain, altered NAD^+/NADH ratios caused by the loss of NADH oxidase activity may be the cause of the transcriptional changes seen solely in the Δ*nox* strain and that these changes could be mediated by redox-sensing transcription factors such as Rex (17).

TABLE 1 Strains used in this study

Strain	Description	Source or reference(s)
S. mutans UA159	Genomic type strain	16, 27
Δ*nox* strain	*nox* deletion mutant (Δ*nox*::Erm)	28
Δ*nox*-Kan strain	*nox* deletion mutant (Δ*nox*::Kan)	This study
Δ*rex* strain	*rex* deletion mutant (Δ*rex*::Erm)	28
Δ*rex* Δ*nox* strain	*rex nox* double deletion mutant (Δ*rex*::Erm Δ*nox*::Kan)	This study

In the present study, we continued the characterization of the Δ*nox* strain and determined that, contrary to expectation, the NAD^+/NADH ratio in cultures of the Δ*nox* strain was greatly elevated, not reduced, relative to the NAD^+/NADH ratio in UA159 cultures during steady-state growth. The major source of the elevated NAD^+ level was identified as an increase in lactate dehydrogenase (Ldh; encoded by *ldh* [SMU.1115]) activity, which was mediated in part by Rex activation of *ldh* transcription. Here we expanded our investigation of the effects of acid and oxidative stresses on the transcriptome of continuous cultures of *S. mutans* by observing the global response to both stresses simultaneously. As expected, exposure to concurrent acid and oxidative stresses led to a stress response synergistically heightened over that with either stress alone. Elevated glucose PTS activity was observed in the Δ*nox* strain during steady-state growth at pH 5, apparently via Rex-mediated induction of *manL* (SMU.1877) transcription. Finally, it was determined that the metabolic and transcriptomic defects of the Δ*nox* strain were sufficient to impair its ability to compete with peroxigenic oral streptococci under aerobic conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All the strains used in this study are listed in Table 1. The *Streptococcus mutans* genomic type strain UA159 has been described previously (16, 27). The *S. mutans* Δ*nox* and Δ*rex* mutant strains are derivatives of UA159 where the respective open reading frame has been replaced with an erythromycin resistance cassette (Δ*nox*::Erm or Δ*rex*::Erm). The method for the construction of the Δ*nox* and Δ*rex* mutants has been described in detail previously (17, 28). It should also be noted that Δ*nox* and Δ*rex* complement strains have been described previously and that the complement strains faithfully alleviated the phenotypes produced by their cognate deletion strains (26, 29, 30). *S. mutans* was maintained on brain heart infusion (BHI) agar plates (BD/Difco, Franklin Lakes, NJ) at 37°C in a 5% (vol/vol) CO_2–95% air environment. Where applicable, antibiotics were added to a final concentration of 5 μg ml^{-1} for erythromycin and 1 mg ml^{-1} for kanamycin. Organisms were cultured in TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH, 1 mM H_3PO_4) plus 1% (wt/vol) glucose and were grown in liquid culture or in continuous culture in a BioFlo 2000 fermentor (New Brunswick Scientific, Edison, NJ) as described previously (23, 31). Continuous cultures were grown at a dilution rate of 0.24 h^{-1} under glucose-limiting conditions (2.3 mM), with a continuous impeller speed of 200 rpm, unless stated otherwise. Steady-state growth at a pH level of 7.0 or 5.0 was maintained by the addition of 2 N KOH. Where applicable, the oxygen concentration was controlled by the addition of air to the fermentor vessel at a rate of 1 vessel volume min^{-1} (VVM), and the impeller speed was automatically adjusted to regulate oxygen diffusion on demand. The culture pH and dissolved oxygen (DO) concentration were monitored continuously throughout the experiment by using an indwelling pH probe and an

TABLE 2 Primers used in this study

Primer name	Sequence (5′–3′)	Primer application
ahpC RTF	ATGGTTTAGCACAACGTGGAAC	qRT-PCR for *ahpC*
ahpC RTR	TTGGCAGGGCAAACTTCTCC	
gor RTF	ACCTGTGTTAATGTTGGCTGTG	qRT-PCR for *gor*
gor RTR	CCTGACGATTTTGCTTCAAGAC	
tpx RTF	CTCCATCTGCTTGGACGTGCTG	qRT-PCR for *tpx*
tpx RTR	GCAAGGGCAGCGTCATAGTTG	
sod RTF	AGCACTTGATGTCTGGGAACAC	qRT-PCR for *sod*
sod RTR	CGGCATAAAGACGAGCAACAG	
ldh RTF	CGTCAAGCACTTGCTGAAAA	qRT-PCR for *ldh*
ldh RTR	CCAGCTACATTGGCATGAGA	
manL RTF	GCATCTGACACAGTTGCTAAGG	qRT-PCR for *manL*
manL RTR	CATTAGCTTTAACACCGCCAGG	
noxA-F1	CCTTGGATTGCTGCTCTTG	Construction and verification of Δ*nox*-Kan mutant
noxA-R1KpnI	GATTTTACTCATGGTACCATCCTCTTTTC	Construction of Δ*nox*-Kan mutant
noxA-F2KpnI	GCTAAATGAGGTACCATGAAACAGTG	
noxA-R2	CTACTTGACTGTCACTAC	Construction and verification of Δ*nox*-Kan mutant
Δ*rex*F	ACTCCAGATGAGGTTAAGGC	Verification of Δ*rex* Δ*nox* double mutant
Δ*rex*R	CAAGCGAGAATGACCTGCG	Construction of Δ*rex* Δ*nox* double mutant
5PmanLSacI	CGCTTTCGCAGAGCTCATTAGGTAATCTC	*manL* EMSA
3PmanLBamHI	CCGATAGCCATGGATCCTCTTCCTCCTT	

InPro 6000 oxygen sensor electrode (both from Mettler Toledo, Columbus, OH), respectively. After continuous cultures had been maintained for a minimum of 10 generations under a given condition, aliquots of the culture were removed, and cells were collected by centrifugation. Cell pellets were washed and were stored frozen at −80°C.

DNA manipulations. Chromosomal DNA was isolated from *S. mutans* as described previously (32). PCR was carried out with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Amplicons were isolated and purified via gel electrophoresis as described previously (18). *S. mutans* UA159 was transformed by methods described previously (27, 33).

Generation of the recombinant Δ*rex* Δ*nox* strain. A UA159 derivative with the *nox* open reading frame replaced by a kanamycin resistance gene (Δ*nox*-Kan) was constructed using a PCR-based, ligation-independent cloning method (LIC mutagenesis) described previously (34, 35). Briefly, the regions approximately 1,000 bp upstream and downstream of *nox* were amplified using primer pairs *noxA*-F1/*noxA*-R1KpnI and *noxA*-F2KpnI/*noxA*-R2, respectively (Table 2). The amplicons were gel purified and were digested with KpnI. The nonpolar kanamycin resistance cassette from pALH124 (a gift from José Lemos) was released by KpnI digestion and was gel purified. Equimolar amounts of each fragment were ligated and were transformed into UA159. Transformants were selected for Kan^r, and one isolate was designated the Δ*nox*-Kan strain.

The DNA construct used to create the Δ*rex* strain, described previously (28), was used to transform the Δ*nox*-Kan strain. Transformants were selected for Erm^r and were screened for Kan^r. An isolate was designated the Δ*rex* Δ*nox* strain.

The sequences of the recombinant strains were verified by using the specific primers listed in Table 2.

NAD$^+$/NADH cycling assay. The ratio of NAD$^+$ to NADH was determined using a cycling assay described previously, with modifications (36, 37). Briefly, frozen pellets of *S. mutans* were resuspended in a NAD$^+$/NADH extraction buffer containing 20 mM sodium bicarbonate, 100 mM sodium carbonate, 10 mM nicotinamide, and 0.05% Triton X-100 (pH 10.3), as described previously (38). Cells were lysed using 0.1-mm glass beads in a Mini-BeadBeater (BioSpec Products, Bartlesville, OK). Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. The samples were then deproteinated using 3,000-molecular-weight-cutoff (MWCO) spin filters (Millipore Corporation, Bedford, MA) and were split into two reaction mixtures: one to determine the relative level of NAD_{total} (both NAD$^+$ and NADH) and one to determine the relative level of NADH only. NAD$^+$ was removed from the NADH-only samples by incubation at 60°C for 30 min. The extracts were subjected (in triplicate) to the cycling assay of Bernofsky and Swan (36) with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) precipitation in NaCl as described by Gibon and Larher (37). Each reaction mixture contained the following: 100 μl 16.6 mM phenazine ethosulfate (PES), 100 μl 4.2 mM MTT, 100 μl 1 M Tricine-NaOH (pH 8), 100 μl 40 mM EDTA, 400 μl 0.1 M NaCl, and 100 μl of the sample (reagents from Sigma Chemical Co., St. Louis, MO). The mixture was preincubated for 5 min at 37°C. Ten units of alcohol dehydrogenase derived from yeast (ADH) was added in 100 μl 0.1 M Tricine-NaOH (pH 8), and the samples were mixed thoroughly and were incubated for 40 min at 37°C. The reactions were then stopped, and reduced MTT was concomitantly precipitated by the addition of 500 μl 6 M NaCl, followed by vortexing and centrifugation at 10,000 × *g* for 5 min at 4°C. The supernatants were decanted, and the collected pellets of reduced MTT were resuspended in 99% ethanol. The absorbance of each sample was determined at 570 nm. Relative amounts of NAD_{total} or NADH were determined by comparing the absorbances of the samples to a standard curve generated by the cycling assay performed on 0 to 200 pmol of pure NAD$^+$. The NAD$^+$/NADH ratio was determined using the formula (NAD_{total} − NADH)/NADH. Due to the light sensitivity of the MTT and PES, the assay, up to the spectrophotometric reading, was performed under low-light conditions.

cDNA microarray analysis. *S. mutans* UA159 microarray slides were provided by the J. Craig Venter Institute (JCVI) through a cooperative agreement with the National Institute of Allergy and Infectious Diseases (NIAID) and the National Institute of Dental and Craniofacial Research (NIDCR). Reference RNA was isolated from UA159 cells grown in BHI medium to an optical density at 600 nm (OD_{600}) of 0.5 and was purified as described previously (20). *S. mutans* UA159, grown with or without 8.4% exogenous oxygen, or the Δ*nox* strain was grown to a steady-state pH value of 5 with four replicate cultures of each. Total RNA was isolated and purified as described previously (20, 39). cDNA was synthesized and was labeled using Cy3-dUTP or Cy5-dUTP (GE Healthcare, Piscataway, NJ) as outlined previously (20). Cy3-dUTP-labeled test cDNA and Cy5-dUTP-labeled reference cDNA were hybridized overnight at 42°C. cDNA was hybridized using a MAUI hybridization system (BioMicro Systems, Inc., Salt Lake City, UT), washed according to protocols provided by the JCVI, and scanned using a GenePix 4000B microarray scanner (Molecular Devices, Inc., Sunnyvale, CA). After scanning, the images were analyzed

using TIGR Spotfinder and were normalized as described previously (20). Statistical analysis was carried out using BRB-Array Tools (linus.nci.nih.gov/BRB-ArrayTools.html).

qRT-PCR. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (20, 39) using a StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA). Gene-specific primers were designed using Beacon Designer software (version 4.0; Premier Biosoft International, Palo Alto, CA) and are listed in Table 2. RNA was isolated from UA159 grown to steady state in the chemostat at pH values of 7 and 5. The mRNA copy number was quantified based on a standard curve of the PCR product as described previously (40).

Ldh activity assay. *S. mutans* strains were grown to steady-state pH values of 7 or 5, and cells were harvested. Spent medium was recovered from 50 ml of cell pellets, and the dry weight of cells was determined from the pellets. Lactate dehydrogenase (Ldh) assays were performed on spent medium by using an L-lactic acid assay kit according to the manufacturer's instructions (R-Biopharm, Darmstadt, Germany). One unit of Ldh activity was recorded as millimoles of Ldh activity per milligram (dry weight) of cells.

Glucose phosphotransferase system assay. Glucose-specific phosphoenolpyruvate (PEP)-dependent PTS assays were performed as described previously (41). Briefly, 50 ml of cell pellets was harvested from steady-state cultures grown to pH 7 or pH 5, washed twice with 0.1 M sodium-potassium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$, and suspended in 5 ml of the same buffer. The cell suspension was permeabilized with 250 μl toluene-acetone (1:9), and 50 μl of permeabilized cells was used in the assay. The assay mixture contained 0.1 mM NADH, 10 mM NaF, 10 mM glucose, and 10 U lactic acid dehydrogenase in 0.1 M sodium-potassium phosphate buffer, pH 7.2. The assay was catalyzed by the addition of 0.5 M PEP, and the OD_{340} was monitored every 10 s for 2 min at 37°C. The OD_{340} decreased due to the oxidation of NADH by Ldh; thus, the change in absorbance reflected the amount of pyruvate produced and, therefore, the amount of glucose phosphorylated. The data are presented as millimoles of pyruvate produced per minute per milligram of protein (as determined by a bicinchoninic acid assay [Sigma Chemical Company]). Assays were performed in triplicate.

EMSA. Electrophoretic mobility shift assays (EMSA) were performed as described previously (30). Purified *S. mutans* Rex protein was a gift generously provided by Zezhang Wen (30). Briefly, a DNA fragment containing the *manL* promoter region was amplified by PCR using the primers shown in Table 2. DNA products were gel purified and were end labeled with T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP (Perkin-Elmer, Waltham, MA) for 10 min at 37°C. Labeled DNA was quantitated by scintillation counting. A representative binding reaction mixture (20 μl) consisted of the following: binding buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 75 mM KCl, 0.5 mM dithiothreitol [DTT], 10% glycerol), a radiolabeled DNA probe (5,000 cpm), and purified Rex. The reaction mixture was incubated at room temperature for 10 min. When 10 mM NAD^+ or NADH was included in the reaction, the samples were incubated for an additional 15 min upon the addition of NAD^+ or NADH. Rex-DNA binding was assessed using a 5% nondenaturing polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer. The gels were exposed to a phosphorimager screen, and binding was detected with a Molecular Imager FX phosphorimager and Bio-Rad Quantity One software (Bio-Rad, Hercules, CA). Rex-DNA binding was also assessed using a 1% agarose gel (0.5× TBE) in 0.5× TBE buffer and was visualized using SYBR Safe stain (Life Technologies, Carlsbad, CA), where indicated.

Interspecies competition assay. The interspecies competition assays with *S. mutans*, *S. gordonii*, and *S. sanguinis* were performed as described previously, with some modifications (42). Overnight cultures of *S. gordonii* and *S. sanguinis* were diluted 1:20 in fresh BHI medium and were incubated at 37°C under a 5% (vol/vol) CO_2–95% air atmosphere or in an anaerobic chamber to an OD_{600} of ~0.6. Aliquots (8 μl) of cultures were inoculated onto BHI agar. After a 24-h incubation period, *S. mutans* strains, grown to similar OD_{600} values, were inoculated next to *S. gordonii* and *S. sanguinis*, and the plates were incubated for an additional 48 h and were photographed. Where applicable, 8 μg ml^{-1} of catalase (from bovine liver; Sigma, St. Louis, MO) was added to *S. mutans* strains immediately after plating.

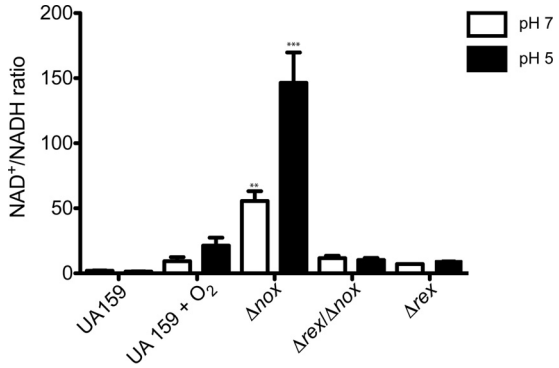

FIG 1 Free intracellular NAD^+/NADH ratios observed in steady-state cultures. A cycling assay was used to determine the free intracellular NAD^+/NADH ratio from chemostat-grown cultures of the indicated strains during steady state at either pH 7 or pH 5. Asterisks indicate statistically significant differences from the ratio for UA159 at pH 7 by Tukey's honestly significant difference test following one-way analysis of variance (**, $P < 0.01$; ***, $P < 0.001$; $n = 9$).

Microarray data accession number. The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE56118.

RESULTS

Deletion of *nox* greatly increases the intracellular free NAD^+/NADH ratio. The regulatory model proposed by Baker et al. (17) postulates that in the absence of Nox activity, the pool of NAD^+, relative to NADH, would be depleted, such that the Rex regulator would be bound to NADH and therefore would be in an inactive conformation (30, 43, 44). Consequently, downstream target genes would not be subject to Rex-mediated transcriptional enhancement or repression. To test whether the loss of *nox* altered the NAD^+/NADH ratio, continuous cultures of UA159, UA159 plus 8.4% DO, and the Δ*nox* strain were grown in chemostats, and samples were harvested at both steady-state pH 7 and steady-state pH 5. An NAD^+/NADH cycling assay was used to determine the intracellular free NAD^+/NADH ratio. In the continuous culture of UA159 without exogenous oxygen (therefore a nearly anaerobic culture [26]), the NAD^+/NADH ratio was 1.9 at pH 7 and 1.3 at pH 5 (Fig. 1). When an 8.4% concentration of DO was maintained in a continuous culture of UA159, the NAD^+/NADH ratio was elevated to 9.3 at pH 7 and 21.3 at pH 5 (Fig. 1), in agreement with the idea that in the presence of oxygen, Nox is able to generate an appreciable amount of NAD^+. Surprisingly, our hypothesis that *nox* deletion would deplete the relative NAD^+ pool was disproven, since the NAD^+/NADH ratio was remarkably elevated in the Δ*nox* strain, with values of 55.6 and 146.4 at pH 7 and 5, respectively (Fig. 1).

In the absence of *nox*, lactate dehydrogenase activity is elevated. The increase in the NAD^+/NADH ratio in the absence of *nox* was unexpected. Since the vast majority of NAD^+ regeneration in *S. mutans* during aerobic growth is thought to occur through Ldh, it was possible that Ldh activity was compensating for the loss of *nox*. Ldh activity assays were performed on all of the

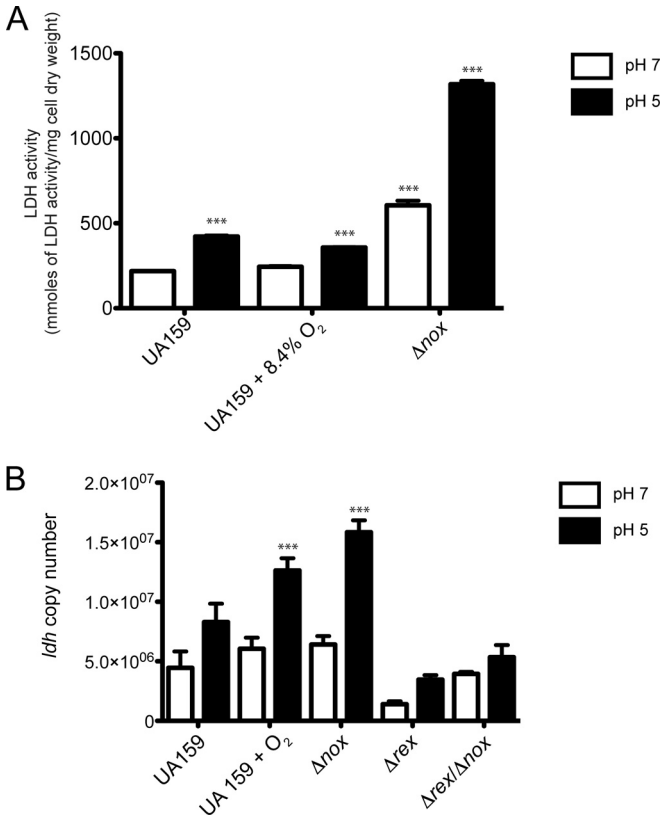

FIG 2 Deletion of *nox* increases Ldh activity and *ldh* transcription. (A) An Ldh activity assay was performed on samples of chemostat-grown cultures of UA159, UA159 plus 8.4% DO, and the Δ*nox* mutant during steady-state growth at either pH 7 or pH 5. Asterisks indicate activity levels statistically significantly different from those of *S. mutans* UA159 cultures at pH 7 by Tukey's honestly significant difference test following one-way analysis of variance (***, $P < 0.001$; $n = 3$). (B) qRT-PCR enumerating *ldh* transcripts in RNA extracted from the indicated strains during steady-state growth at either pH 7 or pH 5. Asterisks indicate expression levels statistically significantly different from those of *S. mutans* UA159 cultures at pH 7 by Tukey's honestly significant difference test following one-way analysis of variance (***, $P < 0.001$; $n = 9$).

steady-state samples described above. The addition of exogenous oxygen (8.4% DO) to UA159 had no significant effect on Ldh activity at either pH 7 or pH 5 relative to the Ldh activity for UA159 grown without exogenous oxygen, although in both cases Ldh activity at pH 5 was elevated over that at pH 7 (Fig. 2A). This is consistent with previous reports that *S. mutans* expresses larger amounts of Ldh during growth at acidic pHs (45). Deletion of *nox* resulted in a significant increase in Ldh activity, approximately 3-fold, over that for UA159 with or without exogenous oxygen (Fig. 2A). These results indicate that the highly elevated NAD^+/NADH ratio observed in the Δ*nox* strain was due, at least in part, to increased Ldh activity.

Elevated *ldh* transcription in the Δ*nox* strain is attributable, in part, to Rex-mediated transcriptional activation. To ascertain whether elevated *ldh* transcription correlated with the increased Ldh activity observed in the Δ*nox* strain, mRNA was harvested from samples grown to the steady-state conditions described above, and qRT-PCR was performed to quantify *ldh* transcripts. Generally, the qRT-PCR results agreed with the Ldh enzyme activity assay data, such that *ldh* transcript copy numbers were higher in the Δ*nox* strain than in UA159 grown either with or without exogenous oxygen (Fig. 2B). The number of *ldh* copies was slightly higher when UA159 was grown with exogenous oxygen than when cultures were grown without oxygen (Fig. 2B). The increase in the level of *ldh* transcription in UA159 plus 8.4% DO apparently had no effect on Ldh activity, since the enzymatic assay showed no significant difference between Ldh activity in UA159 alone and that in UA159 plus 8.4% DO (Fig. 2A).

It has been reported previously that Rex binds to the *ldh* promoter in *S. mutans* and that a Δ*rex* mutant had lower Ldh activity than the parent strain, although the authors did not see an effect of the presence of Rex on *ldh* transcription under the conditions tested (30). We wanted to determine whether the elevated Ldh activity in the Δ*nox* strain might be related to transcriptional regulation of *ldh* by Rex when cultures were grown under steady-state conditions. The Δ*rex* strain was grown in a chemostat to steady-state pH 7 and steady-state pH 5, as described in Materials and Methods. mRNA was purified, and qRT-PCR was performed to quantify the *ldh* transcript level in the Δ*rex* strain. While the number of *ldh* transcripts in the Δ*rex* strain appeared to be slightly decreased, this difference was not statistically significant (Fig. 2B). To determine if Rex was affecting *ldh* transcription in the absence of *nox*, a Δ*rex* Δ*nox* double mutant was constructed. The double mutant did not show significant impairment in its growth rate or its ability to withstand an acid or peroxide challenge relative to the Δ*nox* single mutant (data not shown). Like the Δ*nox* strain, the Δ*rex* Δ*nox* strain could not metabolize the dissolved oxygen in a continuous culture to the same extent as the parent strain (26) (data not shown). Samples of Δ*rex* Δ*nox* RNA were harvested during steady-state growth at pH 7 and pH 5 as described for the other strains, and qRT-PCR was performed to quantify *ldh* transcripts. The level of *ldh* transcription in the Δ*rex* Δ*nox* double mutant was similar to the levels observed in UA159 and the Δ*rex* strain, indicating that in the absence of *nox*, Rex acts to effect an increase in *ldh* transcription (Fig. 2B). Importantly, both the Δ*rex* mutant and the Δ*rex* Δ*nox* double mutant had NAD^+/NADH ratios similar to that of UA159, not elevated as in the Δ*nox* strain, further confirming that Rex-mediated *ldh* transcription was responsible for the increased NAD^+/NADH ratio in the Δ*nox* strain (Fig. 1).

Global changes in transcription due to simultaneous acid and oxidative stresses. To identify genes other than *ldh* that may be differentially expressed in the absence of *nox* during growth at pH 5, we expanded upon our previous examinations of stress responses in *S. mutans* by monitoring changes in gene expression during simultaneous acid and oxidative stresses. cDNA microarray analysis was performed on steady-state cultures of UA159, UA159 plus 8.4% DO, and the Δ*nox* strain grown to pH 5. Comparisons of transcript levels using these samples allowed exploration of the effects of concurrent acid and oxidative stresses on the *S. mutans* transcriptome. Previous efforts have shown extensive changes across the *S. mutans* transcriptome in response to both oxidative and acid stresses (17, 25, 46, 47). It is likely that *S. mutans* frequently experiences both of these stresses concomitantly: acid stress following host consumption of carbohydrates and the constitutive presence of oxidative stress in the outer levels of dental plaque exposed to oxygen gas and in microenvironments of dental plaque containing high numbers of peroxigenic oral streptococci. The microarray data ($P \leq 0.05$) are displayed in Tables S1 and S2 in the supplemental material, and the complete raw microarray

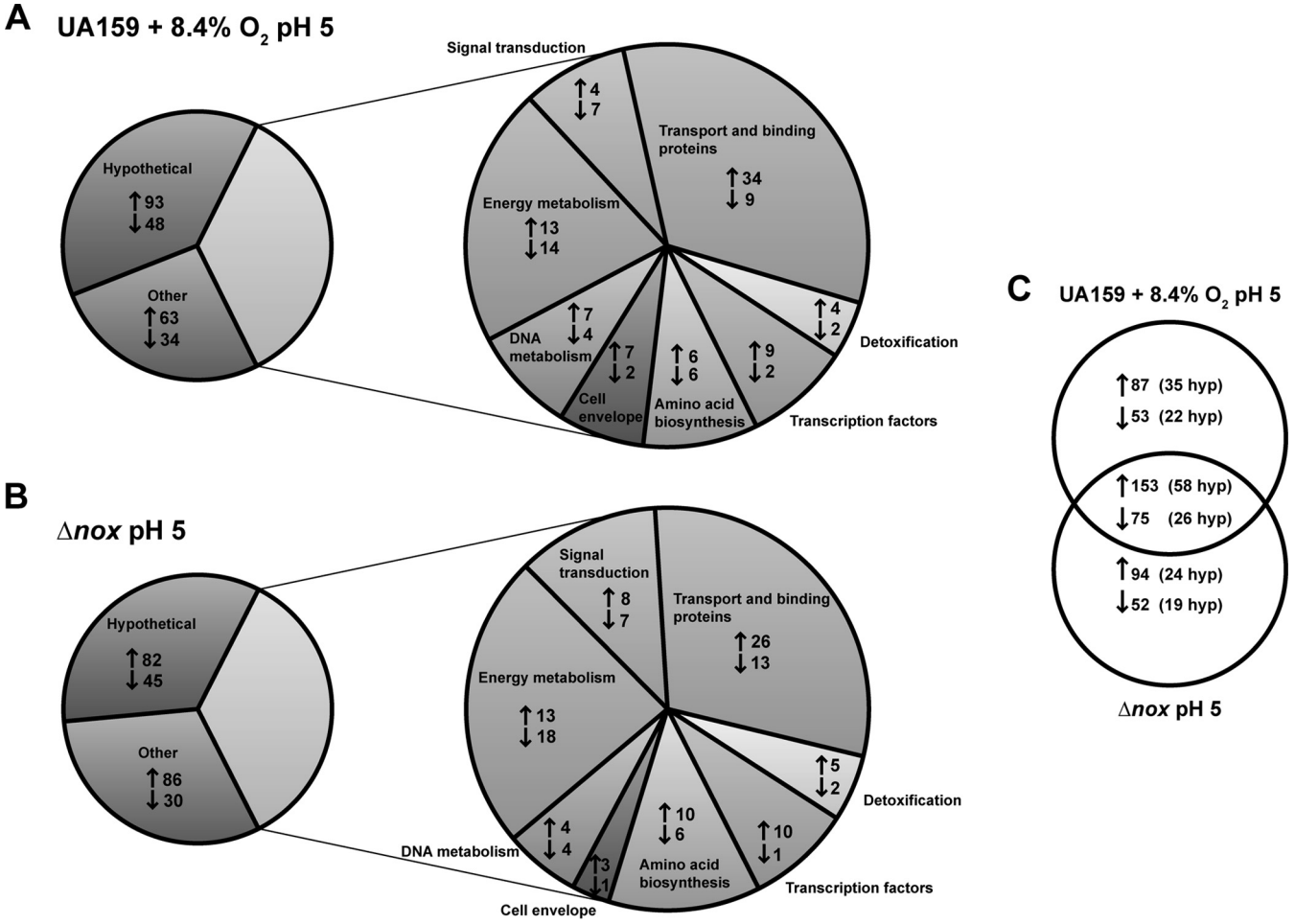

FIG 3 Overview of microarray analysis. (A and B) Pie charts of overrepresented functional categories with altered expression during simultaneous acid and oxidative stresses. *S. mutans* UA159 plus 8.4% DO (A) or the Δ*nox* mutant (B) was compared to UA159 grown without added O_2 (both strains were grown to steady state at pH 5). The numbers of genes upregulated are given next to the up arrows, and the numbers of genes downregulated are given next to the down arrows. Genes encoding hypothetical proteins are separated out in the smaller pie charts to allow for better visualization of genes with known functions. (C) The circles indicate the total numbers of up- and downregulated genes in either comparison, or in both comparisons (overlap between UA159 plus 8.4% DO and the Δ*nox* mutant). The numbers of genes encoding hypothetical proteins (hyp) in each case are given in parentheses.

data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE56118. At an assigned *P* value of ≤0.05 and a 1.5-fold cutoff, 240 genes were upregulated, and 128 genes were downregulated, in UA159 plus 8.4% DO at steady-state pH 5, relative to expression in UA159 grown without exogenous oxygen to steady-state pH 5. The Δ*nox* strain grown to steady-state pH 5 revealed 247 genes upregulated, and 127 genes downregulated, relative to expression in UA159 grown to steady-state pH 5. In accordance with the significant knowledge gap remaining in the *S. mutans* annotated genome (16), the most represented functional classes of genes differentially regulated in both data sets were those encoding hypothetical or unknown proteins (Fig. 3). Genes involved in energy metabolism and in transport and binding made up the next two functional groups with the highest numbers of differentially regulated genes in both data sets (Fig. 3). As shown in Fig. 3C, there was a large amount of overlap between the genes differentially regulated in UA159 plus 8.4% DO and those differentially regulated in the Δ*nox* strain, with more genes appearing in both data sets (153 genes upregulated and 75 genes downregulated) than in either data set alone.

A list of genes of interest appearing in the microarrays is provided in Table 3 along with the corresponding data for each particular gene from our previously published set of microarray data derived from cells grown at steady-state pH 7 under oxidative stress (17). As expected, during oxidative stress, ROS-metabolizing enzymes were upregulated in both UA159 plus 8.4% DO and the Δ*nox* strain grown at pH 5. Like microarrays of samples of UA159 plus 8.4% DO and the Δ*nox* strain grown at pH 7 (17), microarrays from cultures grown to pH 5 demonstrate that *rex* was upregulated in the Δ*nox* strain but not in UA159 plus 8.4% DO. In the Δ*nox* strain grown at pH 5, several genes encoding divisome proteins and genes involved in peptidoglycan biosynthesis were upregulated. The signal recognition particle (SRP; encoded by *ffh*), *ftsQ*, and the SRP receptor *ftsY* all showed reductions in expression in both UA159 plus 8.4% DO and Δ*nox* cultures grown to pH 5. The response regulator *vicR*, implicated in biofilm formation (48, 49), bacteriocin production, and cell

TABLE 3 Relative expression levels of genes of interest identified by microarray analysis in steady-state pH 5 cultures

Gene name	Locus	Function[a]	Fold expression in the indicated strain under the following condition[b]:			
			pH 7		pH 5	
			UA159 + 8.4% O_2	Δ*nox* strain	UA159 + 8.4% O_2	Δ*nox* strain
comD	SMU.1916	HK of competence regulon	ND	3.0	3.0	ND
comE	SMU.1917	RR of competence regulon	5.0	3.2	2.7	2.2
comYA	SMU.1987	Competence ABC transporter	ND	2.5	0.33	0.24
rex	SMU.1053	Redox-sensing repressor	ND	2.4	ND	2.7
gtfB	SMU.1004	Glucosyltransferase	ND	4.6	4.6	ND
gtfC	SMU.1005	Glucosyltransferase	ND	ND	2.5	2.7
covR	SMU.1517	VicR-like response regulator	ND	ND	0.48	0.42
adhA	SMU.127	Acetoin dehydrogenase	2.8	1.4	ND	ND
adhB	SMU.128	Acetoin dehydrogenase	3.6	ND	1.8	0.53
adhC	SMU.129	Dihydrolipoamide acetyltransferase	5.0	1.5	1.8	0.49
adhD	SMU.130	Dihydrolipoamide dehydrogenase	3.3	ND	2.2	ND
sod	SMU.629	Superoxide dismutase	ND	2.1	1.8	1.7
gor	SMU.838	Glutathione reductase	1.9	1.6	ND	1.3
ahpC	SMU.764	Alkyl hydroperoxide reductase	ND	3.1	2.3	2.7
ahpF	SMU.765	Peroxide-forming NADH oxidase	1.7	2.5	ND	2.2
ftsH	SMU.15	Cell division protein	ND	ND	ND	3.1
ftsL	SMU.454	Cell division protein	ND	ND	ND	2.8
ftsQ	SMU.550	Cell division protein	ND	ND	0.44	0.41
ftsY	SMU.774	Cell division protein	ND	ND	0.58	0.47
ffh	SMU.1060	SRP	ND	ND	0.51	0.44
mreC	SMU.20	Cell shape-determining protein	ND	2.8	6.2	4.2
mreD	SMU.21	Cell shape-determining protein	ND	ND	3.0	ND
purC	SMU.29	SAICAR synthase	6.6	ND	4.3	5.1
purL	SMU.30	FGAM synthase	10.4	ND	4.0	4.1
furR	SMU.539	Iron uptake regulator	ND	2.8	2.6	ND
manL	SMU.1877	Mannose/glucose PTS EIIAB	ND	3.1	ND	8.4
manM	SMU.1878	Mannose/glucose PTS EIIC	ND	ND	ND	7.5
manN	SMU.1879	Mannose/glucose PTS EIID	ND	2.1	0.60	5.0
vicR	SMU.1517	Response regulator	ND	ND	0.48	0.43

[a] HK, histidine kinase; RR, response regulator; SRP, signal recognition particle; SAICAR, phosphoribosylaminoimidazole-succinocarboxamide; FGAM, phosphoribosylformylglycinamidine.
[b] Relative to expression in *S. mutans* UA159. The pH 7 values are published in reference 17. ND, no significant differences in expression were detected by microarray analysis using a *P* value of ≤0.05 and a 1.5-fold cutoff.

death, whether directly or through its modulation of the ComDE system (50), was downregulated in UA159 plus 8.4% oxygen and the Δ*nox* strain during growth at pH 5.

To confirm the microarray data, and to further illustrate the additive effects of concurrent acid and oxidative stresses on transcription, qRT-PCR was performed to measure the levels of *ahpC*, *gor*, *tpx*, and *sod* transcripts in UA159, UA159 plus 8.4% DO, and the Δ*nox* strain. As expected, for all four genes, the lowest transcript level was seen in UA159 grown at pH 7 without oxidative stress (Fig. 4). Growth at either pH 5 or pH 7 with oxidative stress produced a modest increase in transcription (Fig. 4). In findings consistent with synergy between the two stress responses, growth at pH 5 with oxidative stress produced by far the greatest amount of transcript for the test genes in UA159 cultures (Fig. 4). At pH 5, the generation of oxidative stress through *nox* deletion resulted in the highest transcript levels for *ahpC*, *gor*, *tpx*, and *sod*, relative to those in UA159, with or without the addition of exogenous oxygen (Fig. 4).

The differences in expression patterns seen between UA159 plus 8.4% DO and the Δ*nox* strain from cultures grown to steady-state pH 5 are likely due to the greatly increased NAD^+/NADH ratio experienced by the Δ*nox* strain at pH 5, via the effect of redox sensors such as Rex (Fig. 1). In support of this hypothesis, the levels of transcription of *adhB* and *adhC* were higher in cultures of UA159 plus 8.4% DO than in UA159 without exogenous oxygen but were lower in the Δ*nox* strain than in UA159 without exogenous oxygen. This finding was consistent with previous reports that *S. mutans* Rex represses the *adh* operon (29, 30): expression is upregulated during oxidative stress, but in the absence of *nox*, the highly elevated NAD^+/NADH ratio leads to increased Rex activity, such that Rex-mediated repression of the *adh* operon results in reduced transcription of *adhB* and *adhC*, to levels lower than those observed in UA159. Likewise, in the mannose/glucose PTS operon, *manM* and *manN* were downregulated in UA159 plus 8.4% DO relative to expression in UA159 but were highly upregulated in the Δ*nox* strain relative to expression in UA159. This suggested that the *man* operon might be regulated by Rex, like *ldh*.

The glucose phosphotransferase system displays elevated activity under conditions of oxidative stress. In *S. mutans*, the activity of the glucose PTS is lower during growth at pH 5 than during growth at pH 7. It is thought that during growth under acidic conditions, carbohydrates can be imported via a proton symport permease and that PTS activity is downregulated in order to conserve ATP for use in proton extrusion (51, 52). According to the microarray data from this study, at pH 5, the *manL*, *manM*,

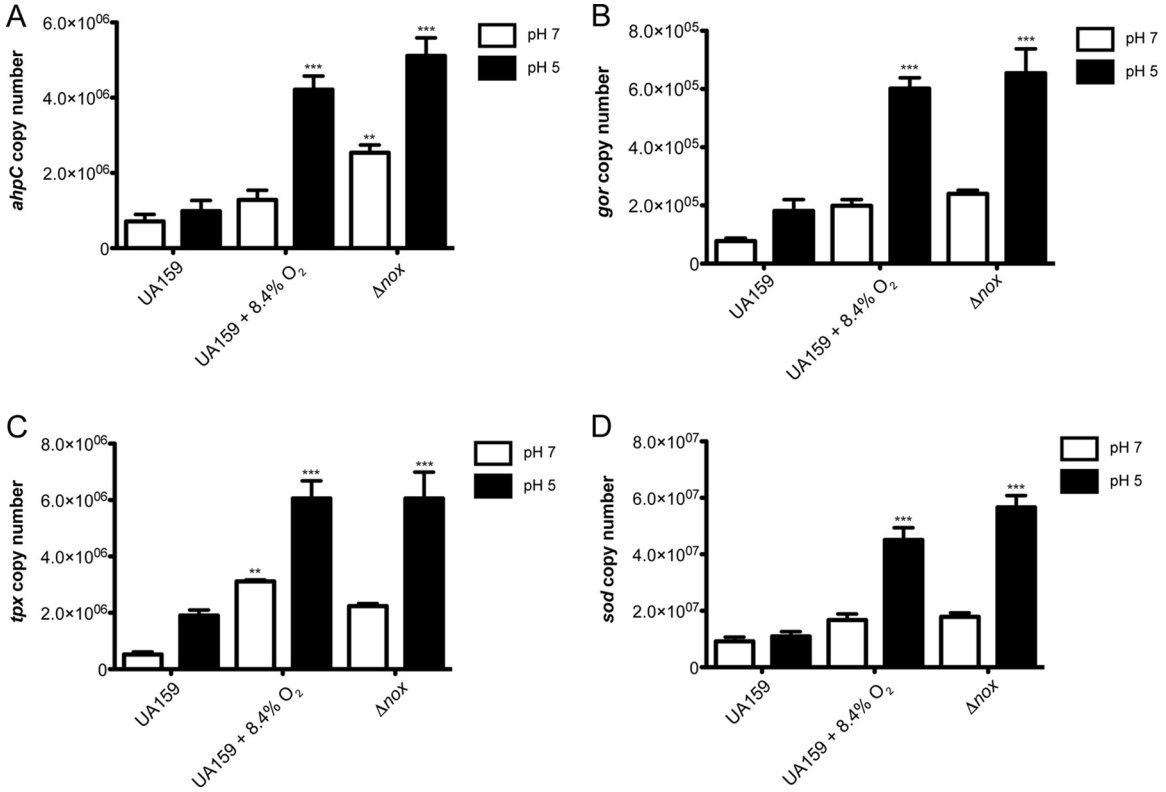

FIG 4 Genes encoding ROS-metabolizing enzymes are further upregulated during simultaneous acid and oxidative stresses. Shown are results of qRT-PCR enumerating *ahpC* (A), *gor* (B), *tpx* (C), and *sod* (D) transcripts in RNA extracted from the indicated strains under steady-state growth conditions. Asterisks indicate expression levels statistically significantly different from those for RNA from *S. mutans* UA159 cultures at pH 7 by Tukey's honestly significant difference test following one-way analysis of variance (**, $P < 0.01$; ***, $P < 0.001$; $n = 9$).

and *manN* genes were all significantly upregulated in the Δ*nox* strain relative to expression levels in UA159 grown without exogenous oxygen but were not upregulated in cultures of UA159 plus 8.4% DO. This apparent upregulation of *manL* in the Δ*nox* background was verified using qRT-PCR, and in fact, expression increased 5.6-fold at pH 7, and 15.3-fold at pH 5, in the Δ*nox* strain over that in UA159 (Fig. 5A). qRT-PCR also indicated that *manL* transcription was not significantly altered by the addition of exogenous oxygen to the continuous culture of UA159 (Fig. 5A). To confirm that the increase in *manL* transcription had a physiological effect, cultures of UA159, UA159 plus 8.4% DO, and the Δ*nox* strain were assayed for glucose PTS activity. At pH 7, glucose PTS activity did not corroborate the microarray data: both UA159 plus 8.4% DO and the Δ*nox* strain exhibited glucose/mannose PTS activity more than 2-fold higher than that of UA159 grown without the addition of oxygen (Fig. 5B). It is likely that the elevated activity seen here is due to the increased activity of another PTS enzyme, since EII^{Man} is not the only PTS enzyme capable of transporting glucose. At pH 5, UA159 plus 8.4% DO did not display glucose/mannose PTS activity significantly higher than that of UA159 grown without exogenous oxygen, but the Δ*nox* strain exhibited glucose/mannose PTS activity 2.1-fold higher than that of UA159 without oxygen, in agreement with the microarray and qRT-PCR data (Fig. 5A and B).

In the absence of *nox*, *manL* is upregulated by Rex. Predictive motif analysis using a fuzznuc algorithm (53) identified multiple putative Rex-binding sites in the promoter of *manL* (data not shown), suggesting that, like that of *ldh*, the upregulation of *manL* in the Δ*nox* strain might be mediated by Rex. qRT-PCR was used to quantify *manL* transcripts in mRNA harvested from steady-state cultures of the Δ*rex* and Δ*rex* Δ*nox* strains grown at pH 7 or pH 5. At pH 7, the number of *manL* transcripts in the Δ*rex* strain was slightly lower than that observed in UA159 and much lower than that detected in the Δ*nox* strain under the same growth conditions (Fig. 5A). At pH 5, the level of *manL* transcription was slightly higher in the Δ*rex* strain than in UA159 but, again, significantly lower than that in the Δ*nox* strain grown under the same conditions (Fig. 5A). At either pH 7 or pH 5, the level of *manL* transcription in the Δ*rex* Δ*nox* strain was similar to that in UA159, confirming that the increase in *manL* transcription seen in the Δ*nox* strain was indeed due to Rex (Fig. 5A). To determine if the Rex protein was capable of specifically binding to the *manL* promoter, EMSA was performed. When the *manL* promoter was incubated with Rex, a shift in the mobility of the DNA-protein complex was observed (Fig. 5C). This shift occurred regardless of the presence or absence of NAD^+ and still occurred even after Rex protein was preincubated with a vast excess of nonspecific DNA [poly(dG-dC)] (Fig. 5C). This shift was not seen in the absence of Rex, when Rex was preincubated with an excess of unlabeled ("cold") *manL* promoter (Fig. 5C), or when Rex was replaced with bovine serum albumin (BSA) (see Fig. S1A in the supplemental material). In agreement with the previously reported behavior of *S. mutans* Rex, the binding of Rex to the *manL* promoter appeared to be moderately inhibited in the presence of NADH relative to

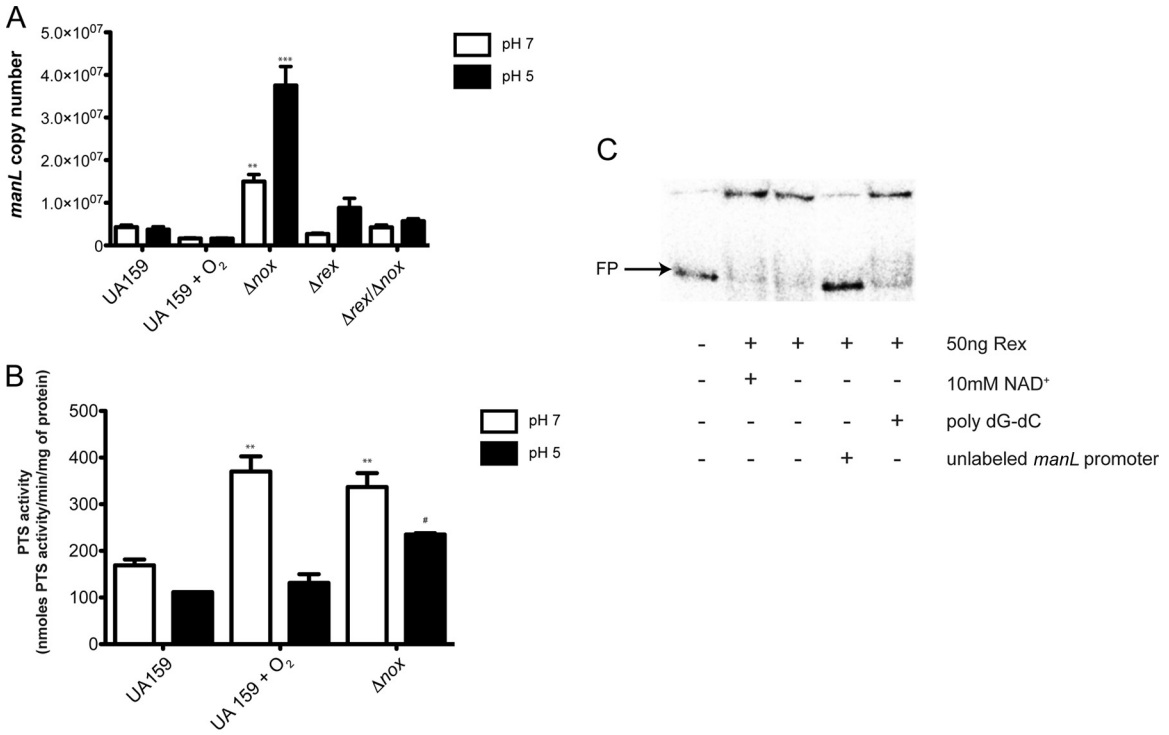

FIG 5 (A) Rex upregulates *manL* in the absence of *nox*. Shown are results of qRT-PCR enumerating *manL* transcripts in RNA extracted from the indicated strains under steady-state growth condition. Asterisks indicate expression levels statistically significantly different from those of *S. mutans* UA159 cultures at pH 7 by Tukey's honestly significant difference test following one-way analysis of variance (**, $P < 0.01$; ***, $P < 0.001$; $n = 9$). (B) Glucose PTS activity levels in samples of the indicated strains during steady-state growth at either pH 7 or pH 5. Asterisks indicate levels statistically significantly different from those for *S. mutans* UA159 cultures at pH 7 by Tukey's honestly significant difference test following one-way analysis of variance (**, $P < 0.01$; $n = 3$). The octothorpe indicates levels statistically significantly different from those for *S. mutans* UA159 cultures at pH 5 by Tukey's honestly significant difference test following one-way analysis of variance ($P < 0.05$; $n = 3$). (C) Electrophoretic mobility shift assay demonstrating the binding of Rex protein to the *manL* promoter. Free probe (FP) was incubated with 50 ng Rex in the presence or absence of either 10 mM NAD^+, an unlabeled cognate competitor (80 ng, or 200 times the amount of the labeled probe), or 2 ng poly(dG-dC).

that in the presence of NAD^+ (see Fig. S1B in the supplemental material) (30, 43). Taken together, these results indicate that in the absence of *nox*, Rex upregulates *manL* transcription.

The elevated proportion of unsaturated fatty acids incorporated into the cell membrane in the Δ*nox* strain has no apparent effect on F_1F_o ATPase activity or membrane permeability to protons. Previous work has demonstrated that both membrane composition and F_1F_o ATPase activity are factors in the aciduricity of *S. mutans* (22, 54). Since growth either in 8.4% dissolved oxygen or in the absence of *nox* resulted in an elevated acid-adaptive response, it was possible that membrane F_1F_o ATPase activity was altered, in addition to the previously observed alterations in the composition of the membrane itself, under conditions of oxidative stress. The microarray data presented in this report show modest upregulation of several ATPase subunits during oxidative stress at pH 5, relative to expression upon growth at pH 5 alone. To confirm elevated ATPase activity, in addition to transcription, an ATPase activity assay was performed on samples from the chemostat cultures grown under the conditions discussed above. Surprisingly, ATPase activity did not differ significantly between UA159, UA159 plus 8.4% DO, and the Δ*nox* strain when they were grown at either pH 7 or pH 5 (although in all three cases, activity was increased at pH 5, as expected) (data not shown) (55, 56).

In addition to F_1F_o ATPase activity, the ability of *S. mutans* to keep its cytosol more alkaline than the extracellular environment is dependent on the permeability of the cell membrane to protons (54). If the membrane is too "leaky," a transmembrane pH gradient (ΔpH) cannot be maintained, regardless of elevated ATPase activity. Our group has described other mutant strains that exhibited a reduction in the percentage of membrane UFAs and also experienced a reduced ΔpH (Δ*fabM* [57] and Δ*pgmB* [58] strains); therefore, it was of interest to learn whether the Δ*nox* strain has a greater ΔpH than the parent strain, since it incorporates a greater number of UFAs into the plasma membrane. As determined by the proton permeability assay described by Marquis and coworkers (54), the Δ*nox* strain had a ΔpH nearly identical to that of UA159 (data not shown). This observation indicated that in the case of the Δ*nox* strain, proton permeability is not related to the UFA/saturated fatty acid (SFA) ratio in the membrane.

The ability of the Δ*nox* strain to compete with *S. sanguinis* and *S. gordonii* is moderately impaired. Since the Δ*nox* strain exhibits a reduced ability to metabolize oxygen yet exhibits upregulation of genes encoding ROS-metabolizing enzymes, it was unclear whether the Δ*nox* strain would differ from the parent strain in its ability to compete with *S. sanguinis* and *S. gordonii*, both commensal organisms that produce H_2O_2 and several toxins that inhibit the growth of *S. mutans*. An interspecies competition assay was performed, placing UA159, the Δ*rex* strain, the Δ*nox*

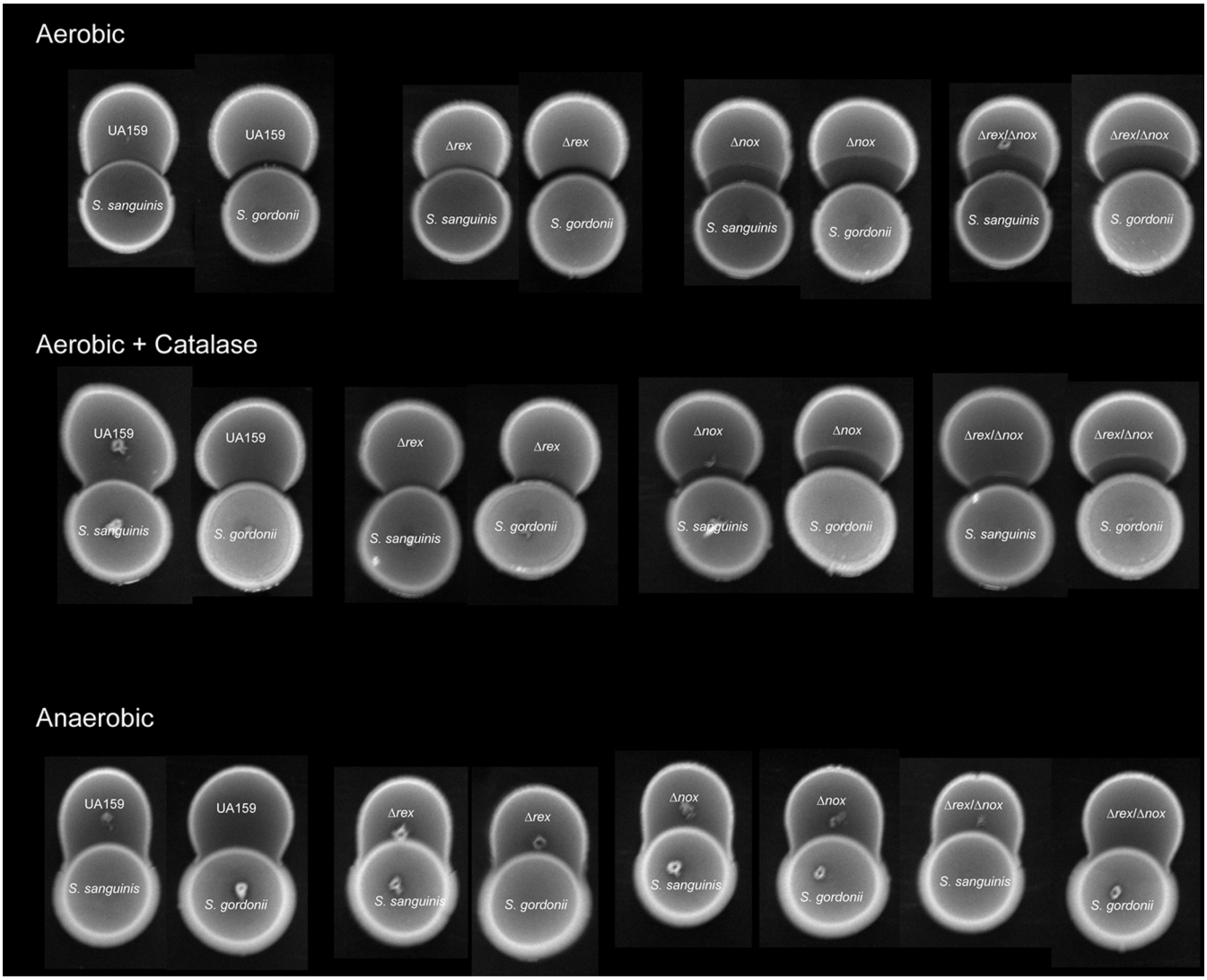

FIG 6 Interspecies competition assay. Mid-log-phase cultures of *S. mutans* were plated next to the initial colonizer (*S. sanguinis* or *S. gordonii*) in either the presence or the absence of 8 μg ml^{-1} catalase in a 5% (vol/vol) CO_2–95% air environment or an anaerobic environment, as indicated.

strain, or the Δ*rex* Δ*nox* strain, as the secondary colonizer, adjacent to either *S. sanguinis* or *S. gordonii* as the primary colonizer. While the ability of the Δ*rex* strain to compete with the peroxigenic streptococci was not significantly impaired, both the Δ*nox* and Δ*rex* Δ*nox* strains showed moderate impairment in the ability to grow in close proximity to *S. sanguinis* and *S. gordonii* under aerobic conditions (Fig. 6). During growth under anaerobic conditions, the Δ*nox* and Δ*rex* Δ*nox* strains were not inhibited by the presence of *S. sanguinis* and *S. gordonii*, indicating that Nox may be important for competition with the peroxigenic oral streptococci only during growth in aerobic environments. Additionally, when catalase was present, the abilities of all *S. mutans* strains, including the Δ*nox* and Δ*rex* Δ*nox* strains, to grow in the presence of *S. sanguinis* and *S. gordonii* were less inhibited than when catalase was absent, indicating that H_2O_2 sensitivity may play a role in the competitive defect exhibited by the Δ*nox* and Δ*rex* Δ*nox* strains.

DISCUSSION

The water-forming NADH oxidase in *S. mutans* is a key enzyme for both the regeneration of NAD^+ following glycolysis and the reduction of diatomic oxygen for the prevention of ROS formation (59). It has been established that *nox* is required for the growth of *S. mutans* on sugar alcohols under aerobic conditions, where NAD^+ cannot be regenerated by the pyruvate-formate lyase (Pfl) pathway (60). *nox* deletion also rendered *S. mutans* unable to metabolize DO in a continuous culture to the level equivalent to that metabolized by UA159 (26). The quantity of DO remaining in a continuous culture of the Δ*nox* strain was sufficient to trigger an oxidative stress response in UA159 grown in continuous culture (17). The redox-sensing regulator Rex is a transcription factor that is in an active, DNA-binding conformation when bound to NAD^+ and is inactive when bound to NADH (30, 61). Thus, this regulator, widely conserved across Gram-positive bacteria, senses the NAD^+/NADH ratio in the cell and exerts

effects on its regulon accordingly (30, 43, 44, 61, 62). Previously, we had proposed that under aerobic conditions, *nox* would contribute to the elevation of the NAD^+/NADH ratio in a continuous culture and that, in comparison, a continuous culture of the Δ*nox* strain would have a reduced NAD^+/NADH ratio due to the absence of NADH oxidase activity (17). We also proposed that it was this reduced NAD^+/NADH ratio that allowed the observed upregulation of *rex*, since Rex would be in the NADH-bound inactive conformation and therefore would be unable to repress its own transcription, as reported previously (17).

When the NAD^+/NADH ratio of UA159 was measured, it was higher in a continuous culture to which exogenous oxygen was added than in a continuous culture without the addition of oxygen, in agreement with the notion that under aerobic conditions, Nox is able to regenerate additional NAD^+ (Fig. 1). It was therefore very surprising that a continuous culture of the Δ*nox* strain exhibited an NAD^+/NADH ratio that was even further elevated (Fig. 1). The data indicated that in the Δ*nox* strain, either the NADH was being depleted or an additional source of NAD^+ regeneration was being recruited. Ldh was a logical target for investigation, since it is the primary regenerator of NAD^+ in *S. mutans*, and a *nox*-deficient strain of *S. mutans* GS-5 was previously reported to generate more lactate than its parent strain (60). Indeed, *ldh* appeared to be the source of the elevated NAD^+ regeneration in the Δ*nox* strain, since both its transcription and the activity of the encoded enzyme were significantly elevated (Fig. 2). Rex has been shown to bind to the *ldh* promoter region (30), and it appears that under these experimental conditions, Rex, which would be more active when levels of NAD^+ are high, effected an increase in the level of *ldh* transcription in a positive-feedback mechanism (Fig. 2). Although it may be difficult to rationalize the hypothesis that a <2-fold increase in expression and a ~3-fold increase in activity give rise to a >100-fold increase in the NAD^+/NADH ratio, it should be kept in mind that *in vivo*, Ldh is allosterically regulated by fructose-1,6-bisphosphate and that activity levels in the closed system of the cell may be much higher. This discrepancy is not unprecedented; a previous report describes a 1.5-fold upregulation of Ldh levels at pH 5 relative to expression at pH 7, which translated to a 127-fold increase in the amount of L-lactic acid produced at pH 5 over that at pH 7 (52).

Although the data presented above strongly support the notion that Rex-mediated activation of *ldh* transcription causes the rise in the NAD^+/NADH ratio in the Δ*nox* strain, it remains unclear how this apparent Rex/Ldh positive-feedback loop is initiated. A likely scenario is that a high level of DO present in the culture of the Δ*nox* strain provokes a change in the regulation of Ldh, either transcriptionally or allosterically, and that this initial spike in NAD^+ is sufficient to begin Rex-mediated activation of *ldh* transcription. Further studies are under way to determine if this hypothesis is borne out. Traditionally, Rex was characterized as a repressor of metabolic and fermentative genes, such as *ldh*, in several other Gram-positive bacteria. Although recent work has shown that Rex can serve as an activator of transcription in *S. mutans* (17, 29, 30), the mechanistic differences between Rex-mediated repression and Rex-mediated activation are unknown (63). The distance of the Rex binding motif from the transcriptional start site of the gene of interest may play a role, and it is noteworthy that many genes subject to Rex regulation have multiple putative Rex binding motifs in their promoters, although the effect of this positioning on Rex regulation remains unclear (17, 30, 63).

In the absence of *nox*, glucose PTS activity appeared to increase in a manner similar to that of Ldh activity. Because the use of the PTS removes phosphate from PEP (which could otherwise be used to generate ATP), the PTS is typically downregulated at pH 5 relative to expression at pH 7 (25, 52). The PTS activity data generated in this study support this hypothesis, since glucose PTS activity was lower at pH 5 than at pH 7 in all 3 strains/conditions tested (UA159, UA159 plus 8.4% DO, and the Δ*nox* strain) (Fig. 5). At pH 7, upregulation of glucose PTS activity was observed in UA159 plus 8.4% DO and the Δ*nox* strain relative to expression in UA159, suggesting that elevated glucose PTS activity is part of the oxidative stress response. At pH 5, glucose PTS activity is still upregulated in the Δ*nox* strain (Fig. 5). Our data suggest that Rex upregulates *manL*, and thus likely the entire operon encoding EII^{Man}, the primary glucose/mannose PTS (Fig. 5). The elevated NAD^+/NADH ratio of the Δ*nox* strain would ensure that more Rex is in the active conformation and promotes *manL* transcription. It remains unclear why *manL* transcription is not elevated to some extent in UA159 plus 8.4% DO, which does have a moderately increased NAD^+/NADH ratio; however, in addition to regulation by Rex, *manL* is also regulated by HPr, CcpA, and SpxA1. Therefore, the level of Rex activity may not be high enough under this condition to override repression by another regulator (39, 64). The oxidizing environment created by elevated DO concentrations in both UA159 plus 8.4% DO and the Δ*nox* strain would also lead to SpxA1 activation, which may contribute to increased *manL* transcription.

An increase in the percentage of unsaturated fatty acids incorporated into the *S. mutans* plasma membrane is an established response to both acid and oxidative stresses and is a critical component of the ATR (23, 26); however, the mechanism of the protection that this shift confers has remained elusive. A *fabM* deletion mutant is unable to synthesize unsaturated fatty acids, and its ability to withstand acid stress was severely inhibited (57). Since the Δ*fabM* strain also has a significantly decreased ΔpH across its plasma membrane and exhibits higher ATPase activity than UA159, one hypothesis is that the unsaturated fatty acids incorporated into the plasma membrane contribute to the permeability of the membrane to protons, which explains the acid sensitivity of the Δ*fabM* strain (57). Since the Δ*nox* strain has a higher percentage of unsaturated fatty acids than UA159 at both pH 7 and pH 5, it was possible that the Δ*nox* strain would have decreased permeability to protons and therefore a higher ΔpH across the membrane. However, this was not the case; a proton permeability assay showed no significant differences between the Δ*nox* strain and UA159. Oxidative stress also appeared to have no effect on ATPase activity, since there was no significant difference between UA159, UA159 plus 8.4% DO, and the Δ*nox* strain at either pH 7 or pH 5.

Survival in the face of oxidative and acid stresses is critical to the ability of *S. mutans* to cause disease. The ability of the Δ*nox* strain to compete with *S. gordonii* and *S. sanguinis* during growth under aerobic conditions was moderately inhibited, strongly suggesting that *nox* is required for the full competitive fitness of the organism. *S. mutans* must be able to withstand the oxidative stress generated by the peroxigenic oral streptococci, as well as to avoid falling victim to its own means of chemical warfare: the production of organic acids. The microarray data in this study and two previous studies reveal differences in the transcriptome of *S. mu-*

tans during acid stress, oxidative stress, and both stresses experienced concurrently (17, 25). This study clearly demonstrates that the simultaneous occurrence of acidic and oxidizing conditions exacerbates the stress response relative to that with either condition alone (17, 25). Promoter analysis of the differentially regulated genes from the collective stress response microarray studies will provide further insight into the control of the ATR and oxidative stress.

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