

The *copYAZ* Operon Functions in Copper Efflux, Biofilm Formation, Genetic Transformation, and Stress Tolerance in *Streptococcus mutans*

Kamna Singh, Dilani B. Senadheera, Céline M. Lévesque, Dennis G. Cvitkovitch

Dental Research Institute, Faculty of Dentistry, University of Toronto, Toronto, Canada

ABSTRACT

In bacteria, copper homeostasis is closely monitored to ensure proper cellular functions while avoiding cell damage. Most Gram-positive bacteria utilize the *copYABZ* operon for copper homeostasis, where *copA* and *copB* encode copper-transporting P-type ATPases, whereas *copY* and *copZ* regulate the expression of the *cop* operon. *Streptococcus mutans* is a biofilm-forming oral pathogen that harbors a putative copper-transporting *copYAZ* operon. Here, we characterized the role of *copYAZ* operon in the physiology of *S. mutans* and delineated the mechanisms of copper-induced toxicity in this bacterium. We observed that copper induced toxicity in *S. mutans* cells by generating oxidative stress and disrupting their membrane potential. Deletion of the *copYAZ* operon in *S. mutans* strain UA159 resulted in reduced cell viability under copper, acid, and oxidative stress relative to the viability of the wild type under these conditions. Furthermore, the ability of *S. mutans* to form biofilms and develop genetic competence was impaired under copper stress. Briefly, copper stress significantly reduced cell adherence and total biofilm biomass, concomitantly repressing the transcription of the *gtfB*, *gtfC*, *gtfD*, *gpbB*, and *gpbC* genes, whose products have roles in maintaining the structural and/or functional integrity of the *S. mutans* biofilm. Furthermore, supplementation with copper or loss of *copYAZ* resulted in significant reductions in transformability and in the transcription of competence-associated genes. Copper transport assays revealed that the Δ *copYAZ* strain accrued significantly large amounts of intracellular copper compared with the amount of copper accumulation in the wild-type strain, thereby demonstrating a role for CopYAZ in the copper efflux of *S. mutans*. The complementation of the CopYAZ system restored copper expulsion, membrane potential, and stress tolerance in the *copYAZ*-null mutant. Taking these results collectively, we have established the function of the *S. mutans* CopYAZ system in copper export and have further expanded knowledge on the importance of copper homeostasis and the CopYAZ system in modulating streptococcal physiology, including stress tolerance, membrane potential, genetic competence, and biofilm formation.

IMPORTANCE

S. mutans is best known for its role in the initiation and progression of human dental caries, one of the most common chronic diseases worldwide. *S. mutans* is also implicated in bacterial endocarditis, a life-threatening inflammation of the heart valve. The core virulence factors of *S. mutans* include its ability to produce and sustain acidic conditions and to form a polysaccharide-encased biofilm that provides protection against environmental insults. Here, we demonstrate that the addition of copper and/or deletion of *copYAZ* (the copper homeostasis system) have serious implications in modulating biofilm formation, stress tolerance, and genetic transformation in *S. mutans*. Manipulating the pathways affected by copper and the *copYAZ* system may help to develop potential therapeutics to prevent *S. mutans* infection in and beyond the oral cavity.

Many bacteria utilize copper as an essential cofactor for enzymes involved in electron transfer reactions, including superoxide dismutase, cytochrome *c* oxidase, and NADH dehydrogenase (1, 2). Although it is required in low concentrations, copper at higher levels can pose a threat because of its high reactivity. Copper can exist in cuprous (Cu^+) and cupric (Cu^{2+}) oxidative forms; the interchange between these states via Fenton reactions can lead to the generation of reactive oxygen species (ROS), especially superoxide radicals. The ROS are believed to cause cellular damage by reacting with a number of cellular macromolecules, such as lipids, proteins, and nucleic acids (3–5). However, there is little or no direct evidence that shows that copper-induced ROS generation is the main mode of copper toxicity in bacteria (6–8). In *Escherichia coli*, copper was demonstrated to target and replace iron from the iron-sulfur clusters of dehydratases, thereby rendering them inactive and perturbing the biosynthesis of branched-chain amino acids (6–9). Supplementation of *E. coli* cultures with branched-chain amino acids was shown to reverse the copper-induced growth inhibition (7). However, a similar mechanism was not observed in *Salmonella enterica* sero-

var Typhimurium, where the addition of exogenous branched-chain amino acids did not revert copper-mediated growth inhibition, thereby suggesting the involvement of a different cellular target for copper toxicity (10). Copper-mediated membrane depolarization has also been reported in *E. coli* (11). The challenges

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Address correspondence to Dennis G. Cvitkovitch, d.cvitkovitch@dentistry.utoronto.ca.

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posed by copper necessitate the involvement of complex regulatory machinery to maintain copper homeostasis in the cell. To contend with copper toxicity, bacteria utilize at least one of three principal mechanisms, including (i) copper export across the plasma membrane into the periplasmic space or the extracellular environment, (ii) extracellular and/or intracellular copper sequestration via copper binding proteins, and (iii) copper oxidation to a less toxic Cu^{2+} state (12). The mechanisms involved in copper stress tolerance can vary among bacterial species, and exploring these pathways is an important step to implement the use of such metal cations as antimicrobial therapeutics.

Streptococcus mutans is considered the primary etiological agent of dental caries, one of the most widespread infectious diseases worldwide (13, 14). Numerous studies have indicated the inhibitory effects of copper on *S. mutans* growth and caries formation (15–22). The copper concentrations in saliva and dental plaque vary between individuals and depend on the age, sex, nutrient intake, etc. (23–26). Copper concentrations in saliva can range from 0.05 to 61.7 μM , whereas the concentrations fluctuate between 26 and 1,520 ppm within the plaque biofilm. By the administration of copper-containing mouth rinses, copper has been shown to dramatically influence the growth and pathogenicity of *S. mutans* (25). In *S. mutans* strain GS-5, copper irreversibly inhibited the activity of F-ATPases, thereby compromising the cell's ability to carry out glycolysis in acidic environments (27). Furthermore, this cation was shown to modulate the expression of the glucosyltransferase D gene (*gtfD*), whose products are associated with soluble glucan production and biofilm formation in *S. mutans* (28). Like the genomes of many Gram-positive bacteria, the genome sequence of *S. mutans* does not appear to encode proteins or enzymes that require copper for their functional activity (29). Even though copper is not required for its cellular processes, *S. mutans* must still possess a functional copper-exporting machinery to avoid cell damage under excessive copper concentrations.

Unlike Gram-negative bacteria, which traffic copper through different cellular compartments from the cytosol to the periplasm and from the periplasm to the extracellular environment, Gram-positive bacteria possess rather simple copper homeostatic systems dedicated to extrusion of excess copper cations from the cytosol to the extracellular milieu (30). The CopYABZ copper homeostasis system of *Enterococcus hirae* is the best understood copper homeostasis model in Gram-positive bacteria (30–33). This operon encodes four proteins that include two copper P-type ATPases, CopA and CopB, a copper-responsive repressor, CopY, and a copper chaperone, CopZ (31–33). The CopZ family of metallochaperones is conserved within various bacterial and eukaryotic systems (30, 33). A homologous copper transport and resistance system, encoded by *copYAZ*, has been partially characterized in *S. mutans* strain JH1005 (34). The sensitivity of the *copYAZ* knockout mutant was shown to be specific to copper, and CopY was demonstrated to act as a negative regulator of the operon (34). Although the idea was not tested conclusively, it was speculated that CopZ derepressed the *cop* operon activity, and a deficiency of *copYAZ* was shown to result in enhanced sensitivity to cell killing under copper stress (34).

Here, we investigated the effects of copper on the physiology of *S. mutans* and the role of CopYAZ in copper homeostasis. While previous studies have implicated metal cations in biofilm formation and the development of genetic competence (35–37), the role of copper and the CopYAZ system in regulating these phenotypes

in *S. mutans* is poorly understood. The ability of this organism to adhere to hard surfaces and form a biofilm is an important virulence factor that is critical for its survival and persistence in the oral cavity. Within the plaque biofilm, *S. mutans* is capable of natural transformation that is made possible when it accomplishes a transient physiological state referred to as genetic competence (38, 39). Genetic transformation is important for acquiring novel, heritable functions that can enhance fitness and drive evolution (40). In *S. mutans*, genetic competence is induced by two signaling peptides, designated the competence-stimulating peptide (CSP; encoded by *comC*) and the *comX*-inducing peptide (XIP; encoded by *comS*) (41–44). Under specific competence-inducing conditions, both CSP and XIP can activate the transcription of the master competence regulator that is encoded by the *comX* (or *sigX*) alternate sigma factor and is required for the transcription of genes involved in DNA uptake and recombination (41–44). Notably, the *S. mutans* competence development pathway activated by CSP and XIP is closely intertwined with its biofilm pathway (38, 41, 45–47). However, it remains to be studied how and whether copper exerts an influence on the regulation of these phenotypes via the putative CopYAZ copper transport system in *S. mutans*.

Here, we show that copper instigates a compromised state in *S. mutans* by dissipating membrane potential and decreasing the organism's ability to endure environmental oxidative and pH stress, produce biofilm, and develop genetic competence. Utilizing a *copYAZ* deletion mutant, we validated the function of *S. mutans* CopYAZ in copper efflux. We also report that the addition of copper or the absence of the *copYAZ* system reduces the transcription of genes involved in biofilm matrix production and the development of genetic competence; loss of *copYAZ* leads to impairment of stress tolerance, transformability, and membrane potential in *S. mutans*.

MATERIALS AND METHODS

Strains and growth conditions. All *S. mutans* strains were grown in Todd-Hewitt yeast extract (THYE) (Becton Dickinson, Sparks, MD) broth as standing cultures or on THYE medium with 1.5% (wt/vol) agar (Bioshop; Burlington, Ontario, Canada) at 37°C in air with 5% (vol/vol) CO_2 . Tryptone yeast extract (TYE) medium (10% tryptone [Bioshop, Burlington, Ontario, Canada], 5% yeast extract, 17.2 mM K_2HPO_4) was utilized for the acid tolerance response (ATR) assays. For the ATR assays, NaOH or HCl was added to TYE medium to adjust the pH to 7.5 or 5.5 and 3.5, respectively. *E. coli* strains were cultivated aerobically in Luria-Bertani (LB) medium at 37°C. Chemically defined medium (CDM) (43, 48) was used for transformation frequency assays. Antibiotics were added whenever required at the recommended concentrations as follows: erythromycin (10 $\mu\text{g}/\text{ml}$), chloramphenicol (10 $\mu\text{g}/\text{ml}$), or spectinomycin (1,000 $\mu\text{g}/\text{ml}$) for *S. mutans* and ampicillin (100 $\mu\text{g}/\text{ml}$) or chloramphenicol (20 $\mu\text{g}/\text{ml}$) for *E. coli*.

MIC assays. MIC assays were conducted as previously described (49). Briefly, 100 μl of mid-log-phase bacterial cells adjusted to an optical density at 600 nm (OD_{600}) of ~ 0.01 were added to a 96-well microtiter plate containing THYE medium supplemented with 2-fold serial dilutions of CuSO_4 or CuCl_2 (Sigma-Aldrich) solution (concentrations ranging from 0 to 25 mM) and AgNO_3 (1 to 100 μM), CdSO_4 (0 to 1.5 μM), HgNO_3 (0 to 10 μM), ZnCl_2 (0 to 25 mM), MnCl_2 (0 to 100 mM), or CaCl_2 (0 to 100 mM). After incubation at 37°C with 5% (vol/vol) CO_2 for 24 h, bacterial growth was spectrophotometrically measured by using a microtiter plate reader at an absorbance of 600 nm. The MIC was determined as the lowest concentration that inhibited visible cell growth relative to the growth in the no-copper control.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>S. mutans</i> strains		
UA159	Wild type; Erm ^s	J. Ferretti, University of Oklahoma
$\Delta copYAZ$ mutant	In-frame <i>copYAZ</i> deletion mutant derived from UA159; Erm ^r	This study
Comp $\Delta copYAZ$	$\Delta copYAZ$ strain transformed with pIB-YAZ; Cm ^r Erm ^r	This study
<i>E. coli</i> strains		
W3110	Wild type	Keio Collection
DW3110	W3110 $\Delta copA$	Keio Collection
DW3110compYAZ	DW3110 transformed with pJET- <i>copYAZ</i> ; Amp ^r Em ^r	This study
Plasmids		
pIB166	Shuttle plasmid containing the <i>P23</i> lactococcal promoter; Cm ^r	51
pIB-YAZ	<i>S. mutans copYAZ</i> fragment cloned into pIB166; Cm ^r Em ^r	This study
pJET	Plasmid containing T7 promoter; Amp ^r	Thermo Scientific CloneJET PCR cloning kit
pJET- <i>copYAZ</i>	<i>S. mutans copYAZ</i> fragment cloned into pJET; Amp ^r Em ^r	This study
pDL277	<i>E. coli-Streptococcus</i> shuttle vector; Sp ^r	109

Mutant and complemented-strain construction. A mutant with deletion of the *copYAZ* operon (NCBI database gene annotations, SMU_424, SMU_426, and SMU_427) in the *S. mutans* UA159 wild-type background was constructed for this study. Briefly, a knockout mutant was constructed using the PCR-ligation mutagenesis strategy as described by Lau et al. (50) by deleting the operon and inserting an erythromycin resistance cassette at the locus in the UA159 background. The deletion of the *copYAZ* operon in the $\Delta copYAZ$ strain was confirmed by PCR amplification, DNA sequencing, and quantitative real-time PCR (qRT-PCR) analyses. The complementation analysis was done using an *E. coli copA*-deficient mutant (strain DW3110 from the Keio collection). Complemented strains of *E. coli copA* were constructed using the Thermo Scientific CloneJET PCR cloning kit according to the manufacturer's instructions. Briefly, *S. mutans copYAZ* was PCR amplified using UA159 genomic DNA as the template and ligated into the pJET vector. The vector was first transferred into *E. coli* DH5 α chemically competent cells (Subcloning Efficiency DH5 α competent cells; Invitrogen). The clones were selected on LB agar plates supplemented with ampicillin (100 μ g/ml) and confirmed using nucleotide sequencing. The resulting plasmid was then transferred into chemically competent cells of the *E. coli* DW3110 mutant to obtain the complemented strain DW3110compYAZ. Similarly, to generate a complemented strain in *S. mutans*, similar PCR products were utilized and ligated into plasmid pIB166 (51), which harbors a chloramphenicol resistance marker and a constitutive promoter upstream from a multiple cloning site. The cloned vector with the entire *copYAZ* operon was transferred into the $\Delta copYAZ$ mutant, and the resulting strain was designated Comp $\Delta copYAZ$. Complementation with the empty vector was utilized as a control [DW3110(pJET) or $\Delta copYAZ$ (pIB) strain] (data not shown). Bacterial strains utilized in this study are summarized in Table 1, and primers are in Table S1 in the supplemental material.

Biofilm assays. Biofilms were cultivated in 96-well polystyrene microtiter plates using one-quarter-THYE medium supplemented with 10 mM sucrose and various concentrations of CuSO₄ or CuCl₂ (0, 100 μ M, 250 μ M, 500 μ M, 1 mM, 2 mM, or 5 mM). All wells were inoculated with overnight cell suspensions of *S. mutans* wild-type and mutant strains, and the plates were incubated for 24 h at 37°C with 5% (vol/vol) CO₂. Following incubation, the broth was carefully removed and the biofilms were stained with 0.01% (wt/vol) safranin to determine the relative biomass, as previously described (52). For biofilm initial adhesion studies, cells (20 μ l of mid-logarithmic cells) were incubated in 12-well polystyrene microtiter plates containing 2 ml one-quarter-THYE medium supplemented with 10 mM sucrose and various concentrations of CuSO₄ (0, 500, or 1,000 μ M). The plates were incubated for different time intervals, and the planktonic cells were removed. Attached cells were washed twice with 1×

phosphate-buffered saline (PBS) before being scraped from the plates and resuspended in 200 μ l of PBS. Cells were briefly sonicated and serially diluted in 10-fold decrements in 1× PBS, and 20- μ l amounts of each dilution were spotted in triplicates on THYE agar plates. After 48 h of incubation, colonies were counted for the dilutions showing individual colonies (30 to 300 in number), and the percentages of cells that were attached/able to grow in the presence and absence of copper were calculated.

ATR assays. Acid tolerance response (ATR) assays were conducted as previously described (53). Briefly, overnight cultures grown in THYE medium were diluted 1:20 using sterile prewarmed tryptone-yeast extract medium at pH 7.5 and supplemented with 1% (wt/vol) glucose (TYEG medium). Cultures were then grown to mid-logarithmic phase (OD₆₀₀ of ~0.4), divided into two equal aliquots, and pelleted via centrifugation. For nonadapted cells, one aliquot was resuspended in TYEG medium at a lethal pH of 3.5, and for adapted cells, the other aliquot was first resuspended in TYEG medium at pH 5.5 and incubated at 37°C with 5% CO₂ for 2 h before being exposed to the lethal pH of 3.5. Following incubation at 37°C with 5% CO₂, cell fractions were removed from cultures at time zero and after 2 h. Cells were gently sonicated, serially diluted in 10 mM potassium phosphate buffer (pH 7.2), and spotted in triplicate (20 μ l of each dilution) onto THYE agar plates. After incubation for 48 h, CFU were counted and ATR was calculated as the percentage of CFU obtained at lethal pH after 2 h relative to the number of CFU present at time zero.

Growth kinetics analysis. For growth kinetics assays, *S. mutans* strains were grown in THYE medium with various concentrations of CuSO₄ or CuCl₂, paraquat (0, 5 mM, 10 mM, 25 mM, or 50 mM), or hydrogen peroxide (H₂O₂) (0, 0.0015, 0.003, 0.0045, or 0.006% [vol/vol]). Overnight cultures of *S. mutans* strains were diluted 1:20 in fresh THYE medium and grown until mid-logarithmic phase (OD₆₀₀ of ~0.4). Amounts of 20 μ l of cell inocula were added to 350- μ l volumes of THYE medium containing various concentrations of test reagents, in quadruplicate. Cells were incubated at 37°C for 24 h; uninoculated wells and wells containing THYE medium alone, with or without reagents, were used as controls. No antibiotics were used in growth assays to avoid additional stress. Optical density readings were taken every 20 min using an automated growth reader workstation (Bioscreen C; Growth Curves USA) and were plotted over time to obtain growth curves.

Copper transport assays. Overnight cultures grown in THYE medium were diluted 1:20 and suspended in fresh medium supplemented with or without 1 mM CuSO₄ or CuCl₂ (suspended in MilliQ water). Samples were incubated at 37°C with 5% CO₂ until they reached mid-logarithmic phase (OD₆₀₀ of 0.4 to 0.6). Following incubation, cells were harvested by centrifugation at 3,800 \times g. To minimize the probability of measuring membrane-associated copper cations, cells were washed twice

with 0.01 M NaClO₄, an experimental electrolyte previously shown to remove residual metal from medium and bacterial surfaces (54). Cells were resuspended in ice-cold 1× PBS and filtered through a 0.22-μm-pore-size nitrocellulose filter. The filter was washed twice with 1 ml of ice-cold 1× Tris-EDTA buffer and once with 1 ml of ice-cold MilliQ water. The filter was digested with 4% HNO₃, followed by inductively coupled plasma-atomic emission spectroscopic (ICP-AES) analysis using the ANALEST facilities (Analytical Laboratory for Environmental Science Research and Training) at the University of Toronto. A 100 mg/ml CuSO₄ or CuCl₂ (suspended in 4% HNO₃ in MilliQ water) stock solution was used to prepare standards ranging from 3 μg/ml to 100 μg/ml for ICP-AES calibration. A standard curve was plotted and utilized to analyze the intracellular copper concentrations. No detectable amounts of copper were observed in THYE or LB medium alone. The measured intracellular copper concentrations were normalized per mg dry weight of cells.

Quantitative real-time PCR assays. Total RNA was isolated from 18-h-old biofilms grown in the presence or absence of 500 μM CuSO₄ in one-quarter-THYE medium supplemented with 10 mM sucrose. Due to its poor biomass production, higher numbers of wells (three times the number for the wild type) were inoculated to isolate RNA from 18-h-old biofilms of the *ΔcopYAZ* strain grown in the presence of copper. The planktonic cells were removed, and the biofilm cells were washed with 1× PBS. The biofilm cells were scraped from the polystyrene surface and suspended in ice-cold 1× PBS. The cells were sonicated and collected after centrifugation at 3,800 × g. For expression analysis of the genes associated with the competence regulon, cells were cultivated in CDM (43, 48) at 37°C with 5% CO₂ until they reached mid-logarithmic growth phase. Cells were then treated with 100 μM CuSO₄ for 30 min. For expression analysis of the genes associated with acid stress, mid-logarithmic cells cultivated in THYE medium were treated with 500 μM CuSO₄ for 30 min. Total RNA was harvested from the cell pellets as previously described (55). After DNase treatment, RNA was subjected to reverse transcription using a first-strand cDNA synthesis kit (Fermentas). Quantitative real-time PCR analyses were conducted using the QuantiTect SYBR green PCR kit (Qiagen, Mississauga, Ontario, Canada). The fold change in expression was calculated according to the method of Pfaffl et al. (56, 57) using the following formula: fold change = $[E^{\text{target gene (control CT)} - \text{experimental gene CT}}] / [E^{16S \text{ rRNA (control CT)} - \text{experimental gene CT}}]$, where $E^{(10^{-1/\text{slope}})}$ represents the efficiency of gene amplification and C_T is threshold cycle (49, 55). The results were normalized against *S. mutans* 16S rRNA or *gyrA* expression; the expression of these housekeeping genes was found to be stable under the test conditions (data not shown). Statistical analysis was conducted using Student's *t* test, and a *P* value of <0.05 was considered significant. The primers utilized are listed in Table S1 in the supplemental material.

Membrane potential assays. Overnight *S. mutans* cultures were diluted 1:20 in fresh THYE medium, and cells were grown to mid-logarithmic phase before being washed and resuspended in minimal medium (58) or in 1× PBS supplemented with 25 mM glucose. The membrane potential assays were conducted by measuring the fluorescence intensity of an anionic dye, DiSBAC1(3) [bis-(1,3-diethylthiobarbituric acid)trimethine oxonol, 5,5'-(prop-1-en-1-yl-3-ylidene)bis(2-tiobarbituric acid)], using the manufacturer's protocol for the FIVEphoton kit. As bis-oxonol is a lipophilic anionic molecule, the dye accumulates in the cell upon membrane depolarization, where it binds to intracellular components and results in an increase in cytosolic bis-oxonol fluorescence intensity. After incubating the cells with dye for 10 min, fluorescence was measured using the TECAN fluorescence plate reader (excitation wavelength of 530 nm and emission wavelength of 560 nm), and the fluorescence intensities were expressed as arbitrary units (AU). After dye stabilization, 1 mM CuSO₄ was added. The plate conditions were set at 37°C with shaking for 5 s before every read, and fluorescence intensity measurements were taken every 15 min for at least 6 h. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a known membrane depolarizer, was used as a positive control, wherein the addition of 10 μM CCCP resulted in an immediate increase in fluorescence intensity. The change in membrane potential ($\Delta\Psi$, ex-

pressed as AU) was taken as the difference between the fluorescence value at a specific time point (every 15 min) of incubation (Ψ_t) and the initial stabilization value (Ψ_i). To control for the dye's intensity change, the value for the culture sample (Ψ_c) was normalized to the value for a blank with dye (Ψ_b). Statistical analysis was conducted using Student's *t* test, with a *P* value of <0.05 considered significant.

Genetic transformation assays. Overnight cultures of *S. mutans* grown in THYE medium were pelleted, washed, diluted (1:20), and resuspended in chemically defined medium (43, 48). Cultures were allowed to grow at 37°C with 5% CO₂ to mid-logarithmic growth phase (OD₆₀₀ of ~0.4). Cultures were then divided into three aliquots and treated with (i) no copper, (ii) 100 μM CuSO₄, or (iii) 250 μM CuSO₄. The copper concentrations used were based on the MICs calculated from assays in CDM: the MIC for *S. mutans* UA159 was 1,000 μM, and the MIC for the *ΔcopYAZ* strain was 500 μM CuSO₄. Next, 1 μg of plasmid DNA (pDL277, containing a spectinomycin resistance marker) was added to 500-μl aliquots of cell suspensions and incubated in 5% CO₂ at 37°C for 90 min. Following incubation, samples were briefly sonicated, serially diluted, and plated onto THYE agar with or without spectinomycin, to determine the number of transformants and the total number of possible viable cells, respectively. Transformation frequency was calculated by dividing the number of transformant CFU by the total number of viable CFU, times 100. Statistical analysis was conducted using Student's *t* test, with a *P* value of <0.05 considered significant.

RESULTS

Copper is toxic to *S. mutans*, and *copYAZ* is required for copper resistance in *S. mutans*. To investigate the physiological function of the *copYAZ* operon in *S. mutans*, an isogenic *ΔcopYAZ* mutant was constructed and characterized *in vitro*. Growth kinetics and MICs were assessed using the wild-type and mutant strains under various concentrations of CuSO₄ solutions. A 2-fold decrease in the MIC was observed in the *ΔcopYAZ* strain (2 mM CuSO₄) compared with the MIC for the UA159 strain (4 mM CuSO₄) under copper stress (*P* < 0.05). At higher concentrations (≥ 6 mM for the *ΔcopYAZ* strain and ≥ 12 mM for UA159), copper had a bactericidal effect on the viability of *S. mutans*. Complementation studies conducted with the Comp*ΔcopYAZ* (*ΔcopYAZ* harboring pIB-YAZ) strain showed restoration of copper-dependent growth inhibition to levels observed with the UA159 wild-type strain (MIC of 4 mM CuSO₄; *P* < 0.05). Growth assays conducted with the wild-type, *ΔcopYAZ*, and Comp*ΔcopYAZ* strains revealed that exposure to added copper resulted in growth impairment of all strains and that loss of the *copYAZ* operon resulted in enhanced toxicity of copper relative to its toxicity for the wild-type and complemented strains (Fig. 1; see also Fig. S1 in the supplemental material). To validate that the effects observed in the growth assays were due to copper cations, the experiments were repeated with CuCl₂, which showed an influence on the growth of *S. mutans* similar to that observed with CuSO₄. In addition, we performed growth-inhibitory assays for the wild-type and *ΔcopYAZ* strains in the presence of various concentrations of other metal ions, including Ag, Cd, Zn, Mn, Mg, Hg, and Ca; the MICs from these assays did not reveal statistically relevant differences (data not shown). Hence, these results highlighted the importance and specificity of the CopYAZ system in copper-induced toxicity in *S. mutans*.

CopYAZ has a role in copper export in *S. mutans*. In several Gram-positive bacteria, CopA functions in copper export. Copper transport studies using ICP-AES analysis revealed that *S. mutans* UA159 cells grown in the presence of 1 mM CuSO₄ had a significantly large amount of intracellular copper (Fig. 2) compared to the amount in those grown without copper supplementation (no

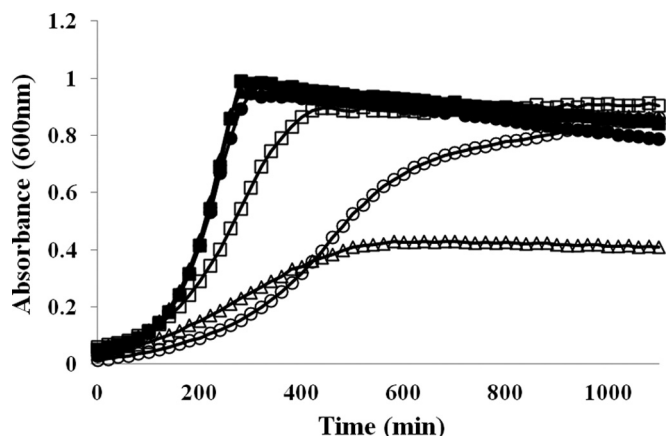


FIG 1 Growth analysis of *S. mutans* strains cultivated in the presence (open symbols) or absence (filled symbols) of 2 mM CuSO_4 . The results shown are representative of six independent experiments conducted with duplicates for each strain. Squares, *S. mutans* wild-type strain; triangles, ΔcopYAZ strain; circles, $\text{Comp}\Delta\text{copYAZ}$ strain.

detectable intracellular copper). A 2-fold increase in the amount of intracellular copper was observed in the ΔcopYAZ strain compared with the amount in the wild-type strain ($P < 0.05$) (Fig. 2), thus suggesting the involvement of *copYAZ* in copper efflux in *S. mutans*. In the wild-type and ΔcopYAZ strains, transport studies conducted with Ag and Mn cations (using 50 μM AgNO_3 or 1 mM MnCl_2) showed no statistically significant differences in the intracellular levels of these cations (data not shown). The latter results support the specificity of the CopYAZ transport system for copper cations, which was further validated using the *CopYAZ*-complemented $\text{Comp}\Delta\text{copYAZ}$ strain, which had an ability to efflux copper at levels comparable to the ability of the wild-type parent (Fig. 2). In the complementation experiments, we employed an *E. coli* strain deficient in *copA* (DW3110), which exhibits a defect in copper efflux and detoxification (59). The recombinant *S. mutans copYAZ* operon successfully complemented the deficiency in *E. coli* DW3110, as observed by a significantly decreased sensitivity to copper-induced killing and reduced intracellular copper ion accumulation (see Fig. S2A and B in the supplemental material), thus supporting a specific function for the *S. mutans* CopYAZ in copper transport and resistance.

Copper inhibits biofilm formation, and *copYAZ* is required to tolerate copper stress under biofilm growth. The wild-type and ΔcopYAZ strains produced comparable amounts of biomass in 18-h biofilms grown in the absence of copper. A significant reduction of biofilm biomass was observed in ΔcopYAZ strain biofilms cultivated with 500 μM CuSO_4 relative to the biofilm biomass formed by the wild type (Fig. 3A). Biofilm biomass was drastically reduced at 1 mM CuSO_4 in both wild-type and mutant strains (Fig. 3A). Initial cell attachment assays were conducted to evaluate the percentage of cells attached to a surface in the presence or absence of copper for different time intervals. At 1 h of incubation, the percentage of cells adhered in the presence of 500 μM CuSO_4 was significantly reduced in both the wild-type (~50% decrease) and the ΔcopYAZ strain (~80% decrease) relative to the percentage in the no-copper control (Fig. 3B). At 1 mM CuSO_4 , a further reduction in the percentage of cells adhered was observed in both wild-type and the mutant strain. After prolonged

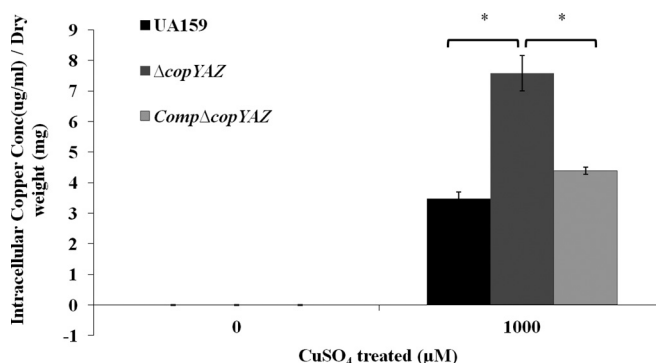


FIG 2 Role of CopYAZ in copper export. Cells of *S. mutans* wild-type strain UA159, the ΔcopYAZ strain, and the $\text{Comp}\Delta\text{copYAZ}$ strain were grown in the presence or absence of 1 mM CuSO_4 until the mid-logarithmic growth phase. Cells were lysed, and the intracellular copper concentrations, normalized to the cell dry weight, were determined. The results shown are the mean values obtained from three independent experiments \pm standard errors. *, $P < 0.05$.

incubation of 2 h or 4 h, no significant decrease in the number of cells adhering to the surface was observed in the wild-type strain at 500 μM CuSO_4 . However, the ΔcopYAZ strain cultivated in the presence of copper showed significant reductions in the numbers of cells attached relative to the numbers for the no-copper control at all time periods (see Fig. S3 in the supplemental material). The results from these experiments showed that the addition of copper and deletion of the *copYAZ* operon reduced the ability of cells to adhere, likely leading to defective biofilms.

Copper affects the transcription of *gtf* and *gpb* genes. To assess the underlying molecular mechanism associated with copper-mediated reduced cell adherence and reduced biofilm biomass of *S. mutans*, we examined the effects of copper on the transcription of *gtf* genes (*gtfB*, *gtfC*, and *gtfD*) and glucan binding protein (*gpb*) genes (*gpbB* and *gpbC*), whose products are needed to maintain the structural and functional integrity of the *S. mutans* biofilm (60–65). Gene expression analysis using qRT-PCR of biofilm-derived cDNAs with or without 500 μM CuSO_4 demonstrated a significant, ≥ 2 -fold downregulation of all five genes ($P < 0.05$) in the presence of copper relative to their transcription levels in the no-copper control (Fig. 3C). In the absence of copper, the transcription of *gtf* and *gpb* genes was not significantly affected in ΔcopYAZ and wild-type strain biofilms (data not shown). Furthermore, in the presence of copper, further transcriptional repression of *gtfC*, *gtfD*, *gpbB*, and *gpbC* was observed in ΔcopYAZ strain biofilms relative to their levels of transcription in the wild-type strain biofilms. In *S. mutans*, the negative regulatory role of copper in the transcription of the *gpb* genes, whose products facilitate biofilm cell adherence, as well as the transcription of the *gtf* genes, whose products are responsible for glucan production that maintains the integrity of the biofilm matrix, might explain the impaired biofilms observed under copper stress.

Copper induces oxidative stress, and CopYAZ modulates oxidative stress tolerance. The copper-induced growth inhibition was assessed in the presence of the antioxidants glutathione and thiourea. The addition of 1.5 mM glutathione dramatically improved the growth of *S. mutans* UA159, ΔcopYAZ , and $\text{Comp}\Delta\text{copYAZ}$ strains cultivated in the presence of 2 mM CuSO_4 compared to the growth of the no-added-glutathione control (Fig. 4A). Treatment with glutathione in the absence of copper did not

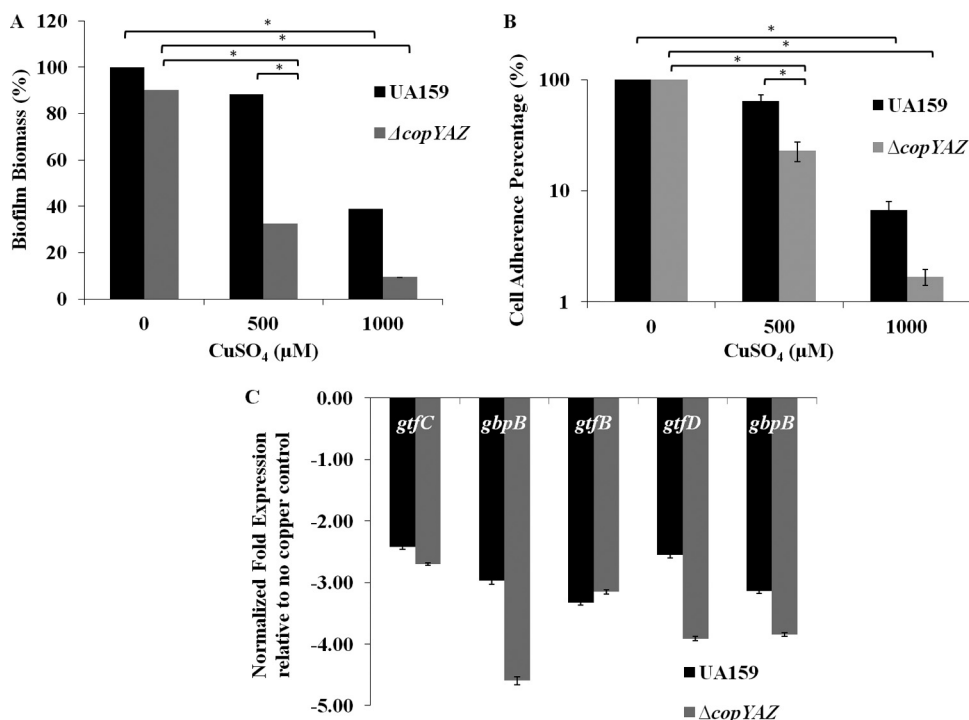


FIG 3 Effects of copper on biofilm formation. (A) Biomass of 18-h biofilms derived from wild-type UA159 and the $\Delta copYAZ$ strain grown in the absence or presence of $CuSO_4$. The results shown represent the mean values obtained from three independent experiments with three replicates for each strain \pm standard errors. *, $P < 0.05$. (B) UA159 and the $\Delta copYAZ$ strain were incubated in the presence of various concentrations of $CuSO_4$, and the percentages of cells adhered to the surface after 1 h of incubation relative to the adherence in the no-copper control were determined. The results shown represent the mean values obtained from three independent experiments with three replicates for each strain \pm standard errors. *, $P < 0.05$. (C) Analysis of the expression of different biofilm matrix-related genes in UA159 and the $\Delta copYAZ$ strain in the presence of 500 μM $CuSO_4$ relative to their expression in the no-copper control. The results shown represent the mean values obtained from four independent experiments with three replicates for each strain \pm standard errors. The P value was < 0.05 for all the genes shown in the graph.

alter the growth of *S. mutans* strains used in this study (data not shown). The reversion of copper-induced growth defects was also observed in the presence of the antioxidant thiourea (data not shown). These results emphasize that copper-induced toxicity in *S. mutans* is likely dependent on copper-dependent generation of oxidative stress in *S. mutans*. Biofilms were also assessed to study the effect of glutathione (or thiourea) in reversal of the copper-induced biofilm defect by cultivating 18-h biofilms in the presence or absence of copper supplemented with or without 1.5 mM glutathione. Glutathione alone did not have any effect on the biofilm biomass of any of the three strains tested. The addition of 1.5 mM glutathione did not improve the biomass production of the biofilms cultivated in the presence of 500 μM or 1 mM copper in *S. mutans* UA159, $\Delta copYAZ$, and $Comp\Delta copYAZ$ strains. These results suggest that *S. mutans* can utilize an alternate route to contend with copper stress during biofilm growth.

We next investigated the role of *copYAZ* in protecting the cells against oxidative stress. Growth kinetics analyses were performed using wild-type UA159, $\Delta copYAZ$, and $Comp\Delta copYAZ$ strains in the presence of the oxidizers methyl viologen dichloride (paraquat) and hydrogen peroxide. Though all strains cultivated in the presence of either paraquat or hydrogen peroxide reached a comparable final optical density after 24 h (see Fig. S4 in the supplemental material), the lag phase of $\Delta copYAZ$ was drastically prolonged relative to that of the wild type (Fig. 4B), thereby suggesting a role of the efflux system in protecting cells under oxidative stress. In the complemented strain $Comp\Delta copYAZ$, the

lag phase in the presence of oxidative stressors was restored to a length similar to that of the wild type.

Copper inhibits growth under acid stress, and CopYAZ modulates the acid tolerance response of *S. mutans*. A previous report indicates that cuprous ions added to *S. mutans* strain GS-5 cultures can irreversibly inhibit the activity of F-ATPases, thereby compromising the ability to carry out glycolysis in acidic environments (27). Here, growth kinetic analyses were performed in the presence and absence of copper in neutral and acidic pH using wild-type UA159, $\Delta copYAZ$, and $Comp\Delta copYAZ$ strains. Although the $\Delta copYAZ$ strain displayed doubling times similar to those of the wild type under acid (pH 5.5) or copper (1 mM $CuSO_4$) stress, the doubling times and the yield of the $\Delta copYAZ$ strain were markedly impaired in THYE medium at pH 5.5 supplemented with 1 mM $CuSO_4$ (Fig. 5). The combined effect of acid and copper stress caused approximately 2- and 5-fold increases in the doubling times of *S. mutans* UA159 and $\Delta copYAZ$ strains, respectively, compared to their growth in the presence of either of these stressors alone (Fig. 5). These results emphasize the synergistic effects of copper and acid against the growth of *S. mutans*. The increased susceptibility of the $\Delta copYAZ$ strain under acid and copper stress could be due to (i) surplus accumulation of copper ions or (ii) lack of cellular protection offered by the *CopYAZ* copper efflux system. To examine the importance of *copYAZ* in the acid adaptation process, we conducted acid tolerance response (ATR) assays. Not surprisingly, for all three strains tested, the percentage of adapted cells (previously subjected to pH 5.5) that

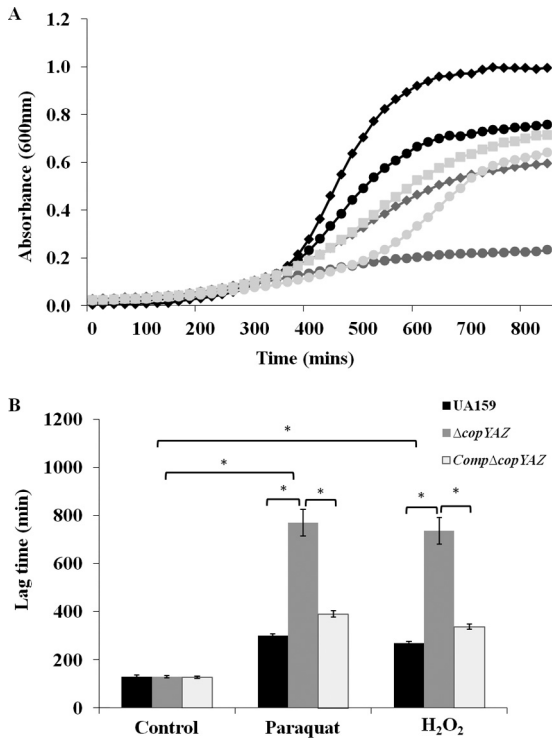


FIG 4 Copper-induced oxidative stress and involvement of CopYAZ in protection against oxidative stress. (A) Growth curves of wild-type UA159 (black), $\Delta copYAZ$ (medium gray), and $Comp\Delta copYAZ$ (light gray) strains in the presence of 2 mM $CuSO_4$ copper with (diamonds and squares) or without (circles) 1.5 mM glutathione. The results shown are representative of six independent experiments conducted using duplicate samples for each strain. (B) Lag times for UA159, $\Delta copYAZ$, and $Comp\Delta copYAZ$ strains to reach an OD_{600} of nearly 0.1 in the presence and absence of the oxidative stressors paraquat (25 mM) and H_2O_2 (0.0045%). The top two brackets indicate the results for statistical significance within strains under different stressors. The results are the means from three independent experiments conducted with three replicates for each strain. *, $P < 0.001$.

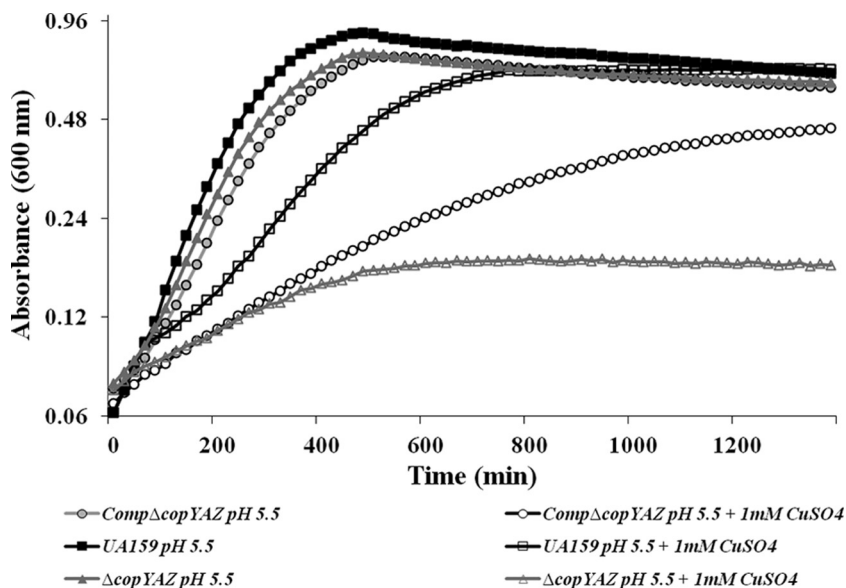


FIG 5 Growth of *S. mutans* under acid stress with or without copper. Growth curves of *S. mutans* UA159, $\Delta copYAZ$, and $Comp\Delta copYAZ$ strains in the presence of acid (pH 5.5) and/or copper (1 mM $CuSO_4$). The results shown are representative of six independent experiments with duplicate samples for each strain.

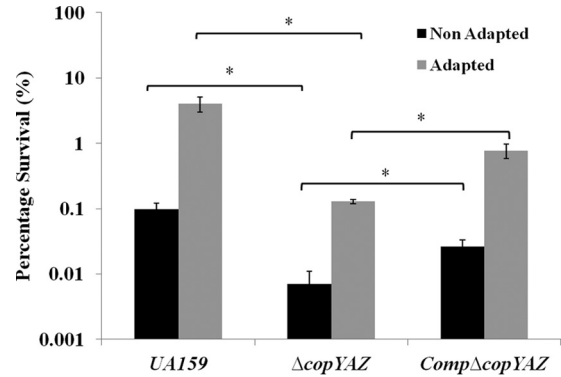


FIG 6 Acid tolerance response assays. Percentages of surviving *S. mutans* cells (in log scale) after 2 h of incubation at lethal pH of 3.5. Cells were either exposed (Adapted) or not exposed (Non Adapted) for preadaptation at the signal pH of 5.5. The results shown are the mean values obtained from three independent experiments \pm standard errors. *, $P < 0.05$.

survived was higher than that of nonadapted cells (maintained at pH 7.5). The loss of *copYAZ* decreased the ability of *S. mutans* to survive the pH 3.5 challenge relative to the survivability of the wild type, regardless of pre-acid adaptation, (Fig. 6). No significant difference in the percentages of surviving cells of the $Comp\Delta copYAZ$ and wild-type strains at pH 3.5 was observed under either nonadapted or preadapted conditions, thereby demonstrating that *copYAZ* contributes to the ATR response in *S. mutans*.

Previously, copper has been shown to reduce the activity of F-ATPase in *S. mutans* (27). Therefore, we examined the role of copper in the transcription of acid stress-related genes that include components of the *S. mutans* F_0F_1 ATPase (SMU.1530 [*atpD*], SMU.1531 [*atpE*], SMU.1532 [*atpF*], and SMU.1534 [*atpH*]) and *uvrA*, a DNA damage repair gene that is induced under acid stress (66). The expression levels of these genes in the wild-type strain cultivated in the presence and absence of copper

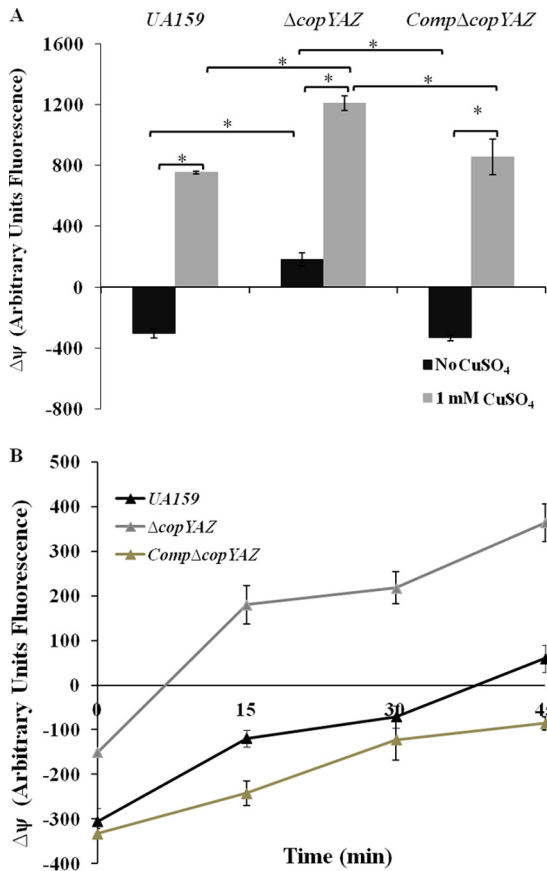


FIG 7 Membrane potential in cells exposed to copper. The changes in membrane potential ($\Delta\Psi$) of *S. mutans* strains over 45 min were measured using a bis-oxonol probe. (A) Fluorescence intensities of cells after 15 min of incubation in the absence or presence of 1 mM CuSO_4 . The statistical significance of the results at each time point was calculated using Student's *t* test. *, $P < 0.05$. (B) Fluorescence intensities of wild-type UA159, ΔcopYAZ , and $\text{Comp}\Delta\text{copYAZ}$ strains in the absence of copper over a period of 45 min. Data shown are the mean results from four independent experiments \pm standard errors. Statistically significant differences between the results for the ΔcopYAZ strain and the other two strains were observed at each time point. $P < 0.05$.

were not significantly different. However, in the ΔcopYAZ strain, the transcription of *uvrA* was downregulated significantly, by more than 2-fold ($P < 0.05$) and 3.6-fold ($P < 0.05$) for incubation with and without copper, respectively, relative to the transcription levels in the wild-type strain. A significant, 2.2-fold reduction ($P < 0.05$) in the expression of *atpH* was observed in the ΔcopYAZ strain in the presence of copper relative to its expression in the control without copper. Studies are under way to analyze the effects of copper and the *copYAZ* operon on the acid-inducible regulon of *S. mutans* that is responsible for the observed acid-sensitive phenotype in this oral pathogen.

Copper induces membrane depolarization, and CopYAZ helps maintain membrane potential. In *E. coli* and *Salmonella*, contact killing on copper surfaces involves immediate membrane depolarization, leading to compromised cell viability (11). To test whether copper and CopYAZ modulate the membrane potential of *S. mutans*, we conducted fluorometric assays with bis-oxonol dyes, which can enter depolarized cells and bind intracellular proteins or membranes. Higher fluorescence intensities, as a result of

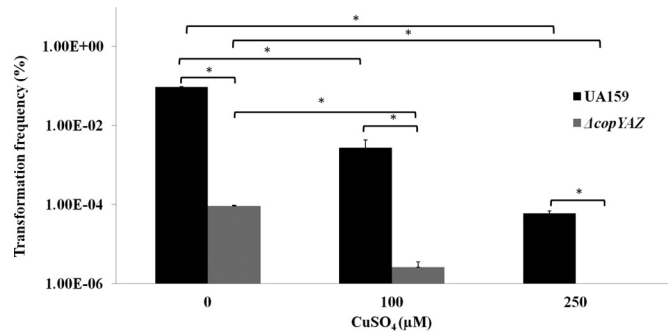


FIG 8 Transformation frequencies (in log scale) for *S. mutans* UA159 and the ΔcopYAZ strain in the presence of various concentrations of copper. Frequencies were calculated as the number of transformant CFU divided by the total number of viable CFU, times 100. The results are the means from three independent experiments conducted in triplicates. *, $P < 0.05$.

elevated influx of the dye, indicate increased membrane depolarization. In *S. mutans*, the addition of copper initiated a gradual increase in the fluorescence intensity, which was maximal relative to that in the no-copper control after 15 min of incubation (Fig. 7A). The increase in fluorescence intensity suggested that copper influx induced a dissipation of the membrane potential. The *S. mutans* ΔcopYAZ cells exhibited a depolarized membrane phenotype where the cells cultivated even in the absence of copper showed a gradual and sustained increase in the DiSBAC1(3)-dependent fluorescence intensity compared with the fluorescence intensity in the wild type (Fig. 7B). Furthermore, the restoration of the fluorescence intensity in the complemented strain $\text{Comp}\Delta\text{copYAZ}$ to the wild-type level provided additional evidence for the involvement of this operon in the membrane depolarization of *S. mutans*. In membrane potential assays, CCCP was used as a positive control; the addition of 10 μM CCCP resulted in an immediate increase in fluorescence intensity as a result of instant membrane depolarization (data not shown).

The addition of copper and loss of *copYAZ* alter the transformability of *S. mutans*. Genetic transformation assays were conducted to investigate the effect of copper on the genetic competence development of *S. mutans*. These assays were conducted in two different growth media, THYE medium and CDM, which activate separate competence induction pathways via the CSP-activated ComDE and XIP-activated ComRS signaling pathway, respectively (43, 67). When UA159 and the ΔcopYAZ strain were grown in the peptone-abundant, nutrient-rich THYE medium supplemented with 10 μM , 100 μM , 250 μM , 500 μM , or 1 mM CuSO_4 , a 5-fold decrease in transformation frequency relative to the frequency in the controls without copper was observed in the wild-type strain only at the 1 mM concentration of CuSO_4 ($P < 0.05$; data not shown). In the ΔcopYAZ mutant, transformability was significantly reduced, by over 10-fold relative to that of the wild type, irrespective of copper supplementation (data not shown). The effects of copper on transformability were stronger when cells were grown in CDM. For instance, in CDM, a 20-fold reduction in transformation frequency was observed in both the wild-type and ΔcopYAZ strains in the presence of 100 μM CuSO_4 relative to the frequency in the no-copper control ($P < 0.05$) (Fig. 8). Furthermore, in the presence of copper, we noted that the transformability of wild-type and mutant strains was significantly reduced in a dose-dependent manner (Fig. 8); our comparison of

the numbers of viable recipient cells in these strains showed that total cell viability in UA159 and $\Delta copYAZ$ strains was not affected at these copper concentrations (see Fig. S6 in the supplemental material). Also of interest, loss of the copper efflux system in the $\Delta copYAZ$ strain impaired its transformability by over 30-fold relative to that of the wild type ($P < 0.05$), even in the absence of copper stress (Fig. 8). To argue against the possibility of this transformation defect arising due to copper-mediated plasmid DNA damage, we conducted transformation assays using cells preincubated with copper. These cells were washed to remove exogenous or cell-associated copper and then supplemented with fresh medium containing plasmid DNA. A similar decrease in genetic transformation was observed in the presence of copper relative to the transformation frequency of the no-copper control, thereby confirming that the defect in genetic transformation was indeed due to copper present within the cells (data not shown).

Copper represses the expression of genes associated with genetic competence. To determine the pathways by which copper and CopYAZ modulate the transformability of *S. mutans*, we compared the expression levels of the competence-related genes *comC*, *comD*, *comE*, *comR*, *comS*, and *comX* in the isogenic strains treated with or without copper. In *S. mutans* UA159, supplementation with 100 μ M $CuSO_4$ reduced the expression of *comX* and *comS* by 2-fold and 2.8-fold, respectively, compared with the expression levels in the no-copper control ($P < 0.001$). Regardless of the presence of copper, the expression of *comX*, which is critical for competence development, was reduced by 2.5-fold in the $\Delta copYAZ$ strain compared with its expression in the wild-type strain ($P < 0.001$). Moreover, relative to the expression in the wild type, the expression of *comS* in the copper transporter mutant was repressed more than 8-fold and 12.5-fold in the absence and presence of copper, respectively ($P < 0.001$).

DISCUSSION

Fluctuations in intracellular levels of copper can have severe implications for the physiology of all prokaryotes. The mechanisms involved in copper-induced killing vary among bacterial species. Bacteria usually initiate a global adaptive genetic response to copper, depending on their physiological copper requirements and the presence of copper in their environmental niches (68–73). While in some bacteria, such as *Enterococcus faecalis* and *Pseudomonas aeruginosa*, a large portion of the genome (approximately 300 genes) is differentially expressed under copper stress, in others, such as *Lactococcus lactis*, only 11 genes are copper responsive (70, 72, 73). While we did not analyze changes in the entire copper regulon of *S. mutans*, targeted gene expression analysis in this work revealed that copper-responsive genes included the *gtfBCD* and *gpbBC* genes, which are associated with biofilm formation, the *comS* and *comX* genes, which are critical for competence development, and the *uvrA* and *atpH* genes, which are involved in acid stress tolerance. An important aspect of this work was also to validate the role of the *S. mutans* CopYAZ system in copper efflux, which influenced the bacterium's ability to tolerate environmental stressors and maintain its membrane potential. The link between copper homeostasis via the CopYAZ system and its effects on *comS* and *comX* transcription for competence development is novel. Since the competence pathway is closely linked with the biofilm and stress tolerance pathways of *S. mutans* (38, 46, 47, 74), this work adds to the current knowledge as to how these pathways can be modulated by environmental copper stress.

The results of growth kinetic assays and MIC testing in this work indicated that higher concentrations of copper are toxic to *S. mutans* and that copper toxicity is counteracted through CopYAZ activity. In most bacteria, CopA has been speculated or proven to remove copper cations from the cell (30, 31, 59), which we validated using copper transport assays. Since we observed that cells of both the wild-type and the $\Delta copYAZ$ strain were able to accumulate copper cations, we suspect that in *S. mutans*, copper import/influx occurs passively, in a nonspecific manner, as demonstrated in other bacteria, such as *Helicobacter pylori* and *E. coli* (75–77).

In the oral cavity, since *S. mutans* is constantly exposed to fluctuating levels of oxidative stress, its ability to adapt to such conditions is important for its survival. Here, we demonstrate that the addition of glutathione dramatically reversed the copper-induced growth defect in *S. mutans* cultures, thus suggesting that the toxicity of copper is likely a result of copper-dependent generation of oxidative stress in *S. mutans*. Copper can generate oxidative stress either by depleting glutathione (due to its capability of forming a complex with glutathione) (78) or by generating reactive oxygen species (ROS), especially superoxide radicals, which are toxic to cells (3, 30). Copper, due to its high reactivity, is capable of replacing Fe or Mn in the active site of proteins, thereby rendering them inactive (79). In *S. mutans*, the proteins encoded by *perR* (peroxide regulator), *sod* (superoxide dismutase), and *dpr* (dps-like peroxidase resistance protein) are associated with resistance to oxidative stress (80–83). The *dpr*-encoded protein requires Fe for its activity, the *sod*-encoded protein requires Mn/Fe, and PerR has been speculated to require Mn or Fe (80, 82–84). Hence, it can be speculated that copper replaces Fe/Mn in the active sites of the products of *dpr*, *sod*, and/or *perR*, thereby inactivating these proteins and compromising the cell viability under oxidative stress. However, further research is warranted to dissect the specific underlying mechanisms involved in this process. Our results also demonstrated the importance of the *S. mutans* *copYAZ* operon in resistance against oxidative stress, thereby suggesting its involvement in the stress adaptation process of this oral pathogen.

Copper and acidic pH exerted a synergistic effect against the growth of *S. mutans*. Growth in THYE medium at pH 5.5 or in THYE medium with 1 mM $CuSO_4$ did not produce discernibly different phenotypes in the $\Delta copYAZ$ and UA159 strains. However, significant increases in the doubling times were noted when these strains were cultivated in THYE medium at pH 5.5 with 1 mM $CuSO_4$, thus suggesting a synergistic effect of acid and copper stress against the growth of *S. mutans*. Since cuprous ions in *S. mutans* have previously been associated with impaired glycolysis under acid stress (27), our study supports this finding by demonstrating a notable growth defect in the presence of copper and acid stress. The impaired growth observed for the $\Delta copYAZ$ strain in the presence of copper and acid stress could be due to the accumulation of surplus copper in the cells or to the involvement of the *copYAZ* operon in protection under acid stress. *S. mutans* produces lactic acid as a metabolic end product of dietary sugars and mounts an ATR that affords it growth and survival under pH values as low as 3.5 (53, 85). During the ATR, *S. mutans* undergoes a number of physiological changes, such as increased synthesis of stress-responsive proteins, membrane fatty acid changes, and increased activity of proton-translocating ATPases (53, 85). Such adaptation requires preexposure to a sublethal signal of pH 5.5 to activate the processes to protect cells against killing pH values (pH

2.0 to 3.0) (53, 85). Severe decreases in the viability of both adapted and nonadapted cells of the $\Delta copYAZ$ strain compared with those of the wild type at pH 3.5 implicated the contribution of *copYAZ* to the ATR of *S. mutans*. In the presence of copper, the transcriptional repression of the acid stress-related genes *uvrA* and *atpH* likely contributed to the observed acid-sensitive phenotype of *S. mutans*. The repression of the expression of *uvrA* in the $\Delta copYAZ$ strain also supported the association of the *copYAZ* operon with the acid stress response. Although we did not analyze the effect of copper in influencing the acid-inducible regulon of *S. mutans*, the results of this study offer a foundation for understanding the copper-mediated acid tolerance response in this oral pathogen. Further studies are under way to elucidate the effect of copper in modulating the proteome of *S. mutans*, with the specific aim of determining the proteins involved in the acid adaptation process under copper stress.

Under normal growth conditions, bacteria maintain their membrane potential by establishing multiple ion gradients across their intact cytoplasmic membrane. The maintenance of intact cell membranes is crucial to maintain ATP hydrolysis and proton motive force (86, 87). A disturbance in the amount of ions can result in hyperpolarization (higher negative intracellular electrical potential) or depolarization (higher positive intracellular electrical potential) of the membrane potential. A rational target for copper-induced toxicity in bacteria is their cell wall or cell membrane, as these are the parts of bacteria that are exposed to external stress. Reports have indicated that, in Gram-positive bacteria, the metal binding sites usually lie within the peptidoglycan layer (88, 89). Copper also tends to accumulate on the inner side of the cell membrane, making the inner side more positive and, thus, causing membrane depolarization (11, 90). Here, we demonstrated the effect of copper on initiating an immediate membrane potential dissipation, which was significantly higher in the $\Delta copYAZ$ strain than in the wild-type strain. The *S. mutans* $\Delta copYAZ$ cells exhibited a depolarized membrane phenotype, even in the absence of copper, compared with the membrane phenotype of wild-type cells. The gradual increase in membrane depolarization observed in cells of the $\Delta copYAZ$ strain could be due to membrane perturbation caused by the loss of the CopA transmembrane protein. Our findings are in agreement with those of another study, where the loss of the arsenic and antimony efflux system in *E. coli* was shown to be linked with cell membrane depolarization (91). Disturbances in the bacterial membrane potential can alter certain cellular processes, such as cell division and differentiation, maintenance of the integrity of cellular membranes, electron transport across the membranes, localization of specific proteins and protein complexes, and regulation of the levels of cellular energy (86, 92–94). In *S. mutans*, copper acting as a membrane potential dissipater might contribute to the generation of copper-dependent oxidative and acid stress, where both these stresses are likely to be affected by variations in the bacterial membrane integrity and/or changes in electron transport across the bacterial membrane (87, 95–97).

Copper has an inhibitory effect on biofilm formation in *S. mutans*. This mechanism relies on the ability of copper to inhibit early attachment of cells to the surface and to significantly reduce biomass production in *S. mutans*. The reduction in cell adherence and biomass production was more prominent in the $\Delta copYAZ$ strain, likely due to surplus accumulation of copper within the cells. In the presence of sucrose, cell wall-associated Gtf proteins mediate the synthesis of D-glucose polysaccharides, which are glu-

cans that promote *S. mutans* cell adherence to the tooth surface and to other adhered bacteria (62, 65). *S. mutans* produces three different Gtf proteins: GtfB, which produces water-insoluble glucans composed predominantly of α -1,3 linkages; GtfD, which mostly produces soluble α -1,6-linked glucans; and GtfC, which synthesizes both α -1,3 and α -1,6 glucans (98–100). In our experiments, the expression levels of *gtfB*, *gtfC*, and *gtfD* were significantly repressed by copper. The transcriptional repression of these *gtf* genes validated the effects of copper in influencing genes critical for glucan production and biofilm matrix formation; their downregulation under conditions of excess copper explains the poor biofilm biomass that we observed. In addition to Gtf proteins, surface-associated Gbp proteins in *S. mutans* promote cell-cell aggregation by mediating the binding of *S. mutans* to glucans (61, 101, 102). The addition of copper significantly reduced the transcription of *gbpB* and *gbpC*, further expanding the knowledge of components affected by environmental copper. The downregulation of these *gbp* genes distinctly emphasized the effect of copper in the initial sucrose-dependent biofilm formation, where expression of these genes is essential for the transition from planktonic to biofilm growth (102, 103). In contrast to our *gtf* transcription results, others showed previously that supplementation with 1 mM copper induced the transcription and translation of *gtfD* but not *gtfBC* (28). In another study, RNAs isolated from planktonic cells were used for expression analysis (31), whereas this study utilized RNAs from 18-h biofilms cultivated with or without added copper. In the absence of copper, we did not note any significant differences in the transcription levels of *gtfBCD* and *gbpBC* between the $\Delta copYAZ$ and wild-type strains; hence, it is not surprising that we obtained comparable amounts of biofilm biomass for these strains. In addition to the repressive effects of copper on *gtfBCD* and *gbpBC* transcription, loss of copper efflux in the $\Delta copYAZ$ mutant led to reduced expression of the *gtf* and *gbp* genes in *cop* mutant biofilms with added copper in the medium. These results provide strong evidence that copper is extremely effective in inhibiting biofilm formation, one of the most vital virulence attributes of this oral pathogen.

The influence of copper and the CopYAZ system on the development of genetic competence and the expression of *comX* and *comS* in *S. mutans* is novel. The ComDE and ComRS signaling pathways involved in *S. mutans* competence development differ in their mechanisms of action (42–45). The CSP-ComDE pathway is activated by CSP, whose precursor peptide is encoded by *comC* (38, 44, 104). Upon activation of ComD and subsequent phosphorylation, transfer to ComE leads to the activation of ComX, which is a critical switch required for the competent state of *S. mutans* (44, 47). Recently, the proximal regulator of ComX was determined to be ComR, an Rgg-like transcriptional regulator in the cytosol that is able, in conjunction with internalized XIP, to activate ComX for the transcription of late competence genes required for DNA uptake and recombination (43). It has been shown that the nutrients and peptones present in the growth medium of *S. mutans* can differentially affect the CSP and XIP activities needed for competence activation (42, 43, 45, 67, 105). CDM was shown to be optimal only for XIP-mediated competence induction, whereas the peptone-rich THYE medium was optimal for transformation in the presence of CSP (42, 43, 45, 67). In our assays, since the effect of copper on *S. mutans* transformability is dramatically pronounced in CDM relative to the effect in THYE medium, we speculate that its influence on genetic competence

occurs primarily via the XIP-induced signaling pathway. Moreover, the observation that only *comS* and *comX* out of all competence-related genes tested (i.e., *comCDE*, *comRS*, and *comX*) had significantly changed expression under our test conditions provides additional evidence that the effects of copper on transformation are modulated by the XIP-ComRS pathway. The fact that copper affects the expression of *comS* but not *comR* suggests that copper represses the activity of ComX by affecting the function or secretion of ComS and/or XIP, thus causing a dramatic reduction in transformation frequency. In the absence of copper, a significant reduction in the transformation frequency of the $\Delta copYAZ$ strain relative to that of the wild type implies the importance of this operon in genetic competence development in *S. mutans*. Previously, in *Streptococcus pneumoniae*, inactivation of a putative metal-transporting operon (*AdcCBA*) was shown to confer a competence-deficient phenotype (106, 107). The operon was speculated to be involved in zinc transport, and the addition of zinc improved the transformability of the *adc* null mutants (106, 107). In our study, although the mechanism involved in modulation of competence by the *copYAZ* system is not fully understood, two suggestions can be made based on the observed transcriptional repression of *comS*: (i) the *CopYAZ* may have a role in modulating the function, processing, or export of XIP, and/or (ii) the membrane perturbation that results from the loss of *CopA* may hinder the process of DNA acquisition, resulting in reduced transformation frequency.

In conclusion, the results of the present study enhance our understanding about the effects of copper on *S. mutans* survival under planktonic and biofilm growth conditions. We also demonstrate the role of the *copYAZ* system in maintaining *S. mutans* physiology under stress conditions. Insight into the mechanism of copper toxicity and its connection with biofilm formation and genetic transformation is instrumental in understanding and devising new strategies to utilize copper as an effective antibiofilm agent to combat *S. mutans* infections. Since *S. mutans* can be considered an ideal model organism to study the genetics and physiology of pathogenic Gram-positive bacteria (108), our study holds relevance in suggesting the importance of copper acquisition and homeostasis in closely related pathogens.

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