Oral Microbiology Immunology 2004: 19: 395–402
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Role of a *Streptococcus gordonii* copper-transport operon, *copYAZ*, in biofilm detachment

K. Mitrakul, C. Y. Loo, C. V. Hughes, N. Ganeshkumar
Department of Pediatric Dentistry, Goldman School of Dental Medicine, Boston University, Boston, MA, USA

Mitrakul K, Loo CY, Hughes CV, Ganeshkumar N. Role of a Streptococcus gordonii *copper-transport operon,* copYAZ, *in biofilm detachment.*
Oral Microbiol Immunol 2004: 19: 395–402. © Blackwell Munksgaard, 2004.

Streptococcus gordonii is a pioneer oral bacterium that is associated with the initiation of dental plaque development. Located downstream of the *S. gordonii adc* operon, which is involved in competence and biofilm formation, were three open reading frames, designated *copY, copA* and *copZ*. These open reading frames were homologous to the *copYAZ* genes in *Streptococcus mutans* that are involved in copper homeostasis and biofilm detachment. This study examined whether *copYAZ* genes play any role in the biofilm formation and detachment of *S. gordonii*. The *copY* gene encodes a 143-amino acid protein homologous to the negative transcriptional regulator of a copper-transport operon, *copA* encodes a 748-amino acid copper-transporting P-type ATPase, and *copZ* encodes a 69-amino acid putative metallochaperone protein in *S. mutans*. Each open reading frame in the *copYAZ* operon in *S. gordonii* was inactivated by insertional mutation and the growth, biofilm formation and detachment of each mutant were examined. *S. gordonii* copY::*spec*^{R}, *copA*::*spec*^{R}, and *copZ*::*spec*^{R} mutants were able to form biofilms on both polystyrene and glass surfaces. However, inactivation of *copZ* and to a lesser extent *copY* resulted in phenotypes that were defective in biofilm detachment, which is consistent with previous observations in *S. mutans* and suggests that the trace element copper might influence biofilm detachment of bacterial biofilms.

Key words: biofilm; cop operon; copper; dental plaque; streptococci; *Streptococcus gordonii*

N. Ganeshkumar, Department of Pediatric Dentistry, Goldman School of Dental Medicine, Boston University, 801 Albany Street, Room 215, Boston, MA 02118, USA
Tel. +1 617 638 4773;
fax: +1 617 638 5033;
e-mail: nganesh@bu.edu
Accepted for publication July 25, 2004

Tooth surfaces are usually colonized by structurally complex bacterial consortia termed dental plaque, which are microbial biofilms that survive by adapting to environmental fluctuations within the oral cavity such as changes in pH, oxygen tension and cyclic variations in nutrient availability. Biofilms develop in several stages, beginning with cells attaching to a surface, accumulating subsequent to bacterial growth and colonization by other bacteria, and eventually forming complex climax communities interspersed with fluid-filled channels. These biofilms display increased resistance to antibiotics and host defense and are associated with several chronic persistent infections (7).

Recent studies have demonstrated that metal ions might play an important role in biofilm formation and pathogenesis. In *Vibrio cholerae*, calcium ions are essential for biofilm development and dissolution in seawater, and biofilm dissolution during times of estuarine instability may contribute to cholera epidemics (18). In *Streptococcus pneumoniae,* manganese is transported by PsaA, the lipoprotein component of an ABC transporter which has been shown to be a virulence factor (24). A screen for *in vivo* expressed genes in *Staphylococcus aureus* identified a virulence gene *ivi44*, which encodes a protein that has 50% identity to CopA of *Enterococcus hirae* (22). In *Listeria monocytogenes*, mutants of *ctpA* (copper transport protein), which encodes a P-type adenosine 5′ triphosphatase (ATPase), are involved in copper transport and have reduced virulence (15). A reduction of colonization by *Pseudomonas aeruginosa* biofilms on copper-coated butyl rubber was observed when compared with the control material over 14 h using a modified Robbins device (26). In *Streptococcus mutans,* a mutant that is defective in biofilm detachment had a Tn*917* insertion adjacent to the promoter region of the *cop* operon (37, 38). Intracellular copper is tightly regulated in bacteria as it is critical to cell viability. As large concentrations are toxic, the maintenance of intracellular copper at

trace levels requires homeostatic mechanisms (35). Bacterial integral membrane P-type ATPases play a critical role in metal ion homeostasis, including copper (36).

Previous *in vivo* and *in vitro* studies have examined the potential cariostatic effect of a number of trace elements including copper (12, 14). Copper ions have been shown to be cariostatic in rats (2, 3), to reduce plaque (33), to inhibit the ability of plaque to generate acids after exposure to sucrose in rats (4, 39), and to inhibit demineralization of human enamel *in vitro* (5).

Streptococcus gordonii, which is a pioneer oral bacterial species that initiates the formation of biofilm on tooth surfaces, belongs to the viridans group of oral streptococci (16). This group constitutes a large proportion of the commensal bacteria that colonize oral surfaces and when introduced into the bloodstream can cause transient bacteremia-initiated infections in susceptible individuals.

The oral cavity in which *S. gordonii* dwells is an environment of highly fluctuating levels of nutrients and trace elements. Metal ions such as those of copper and silver can come from consumed foods and dental restorative materials such as amalgam, which is still a widely used metal alloy in dentistry (27). A *cop* operon in *S. gordonii* was identified downstream of the *adc* (*ad*hesion *c*ompetence) operon (*adcRCBA*) (20). This was homologous to the *cop* operon in *S. mutans*, which was previously shown to be associated with biofilm detachment and copper transport (37, 38). This study examined the possible role of *S. gordonii copYAZ* genes in biofilm formation and detachment.

Material and methods

Bacteria, media, and chemicals

S. gordonii Challis 2, the rifamycin resistant (500 μg/ml) strain of *S. gordonii* Challis (19), was used as the parent strain. Bacteria were subcultured and maintained routinely on Brain Heart Infusion (BHI) agar (BBL, Becton Dickinson, Cockeysville, MD) or Todd Hewitt Broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% yeast extract (THBYE) at 37°C under anaerobic conditions (CO_2 : H_2 : NO_2; 5 : 5 : 90%; VWRbrand anaerobic chamber, VWR, Plainfield, NJ).

Mutagenesis of the *cop* operon in *S. gordonii*

Polymerase chain reaction (PCR) ligation mutagenesis with a spectinomycin resistance gene, $spec^R$ (20), was used to construct deletion mutants in each of the three open reading frames (ORFs) present in the putative *S. gordonii cop* operon. PCR amplifications of the two flanking regions and the antibiotic marker insert were performed with the appropriate primers that incorporated *Mlu*I and *Xba*I, respectively, and subsequent purification of PCR products and ligation were performed as previously described (20). Four μg of the ligated DNA was used for transformation of *S. gordonii* Challis 2 by the method described previously (19). The transformation mixture was then plated on BHI agar containing spectinomycin (1000 μg/ml) and incubated at 37°C anaerobically for 2–5 days.

The *copY::*$spec^R$ was constructed by deleting residues 7–110 of 143 amino acids, the *copA::*$spec^R$ was constructed by deleting residues 10–720 of 749 amino acids and the *copZ::*$spec^R$ was constructed by deleting residues 11–53 of 69 amino acids.

Reverse Transcription-PCR (RT-PCR)

RT-PCR was performed to confirm that the mutants generated were nonpolar. Total RNA was extracted from *S. gordonii* Challis 2, *copY*::$spec^R$ and *copA*:: $spec^R$ strains grown to mid-log phase (A_{600}nm of 0.3–0.4) using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA). RT-PCR was performed using RNA isolated from *copY*::$spec^R$ with primers copA for 5 (5′-ATAGATGGACAGCTGGCAGGTAT TTTAG-3′) and copZ rev 5 (5′-GTGAC-GTGT TTG ACACAGTTTTGA CAAG-3′) spanning from *copA* to *copZ*, RNA from *copA*::$spec^R$ with primers copZ for (5′-CTTGTCAAAACTGTGTCAAACA-CG TCAC-3′) and copZ rev (5′-AATC-GTTTTAGCTAAAGCCGCTTCATAG-3′), which span within *copZ*. RNA from Challis 2 strain was used as a control with identical primers pairs. The location of the primers used are shown in Fig. 1A.

Growth assays

The growth rates of *S. gordonii* Challis 2, *cop*Y::$spec^R$, *copA*::$spec^R$ and *copZ*::$spec^R$ mutants were assessed by inoculating the strains from an overnight THBYE culture into fresh 10 ml THBYE and growing them at 37°C under anaerobic conditions. The effects of copper on the growth of *S. gordonii* Challis 2, *cop*Y::$spec^R$, *copA*::$spec^R$, and *copZ*::$spec^R$ mutants were assessed by inoculating the strains from an overnight THBYE culture into fresh 100 μl biofilm medium (BM) supplemented with various concentrations of $CuSO_4$ (0–800 μM) in a microtiter plate and growing them at 37°C under anaerobic conditions (21). Growth was quantified by recording the absorbance at 575 nm over 24 h. The growth of each strain in 400 μM $CuSO_4$ was compared to growth of the same strain in BM without $CuSO_4$ (considered as 100% growth) after 24 h and expressed as relative growth.

Biofilm detachment assay

A microtiter plate biofilm detachment assay described by Kaplan et al. (17) was used to measure the biofilm detachment of *S. gordonii* Challis 2 and the *cop* mutants. Polystyrene rods were attached to the lid of a 96-well polystyrene microtiter plate so that the rods were suspended approximately 2 mm above the bottom of the wells. The modified lid was sterilized with 70% ethanol. After the microtiter plate containing BM with appropriate antibiotics (100 μl per well) was inoculated, the modified lid was placed on the inoculated plate so that the polystyrene rods remain immersed in the inoculated medium. The plate was then incubated for 24 h to allow bacteria to form biofilms on the immersed rods. The lid was then transferred to a new microtiter plate containing 100 μl BM per well with the appropriate antibiotics and

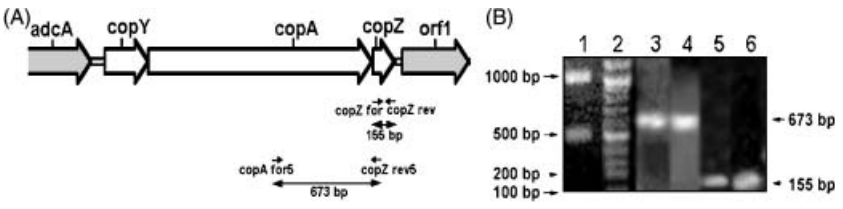

Fig. 1. A) Organization of *cop* and the adjacent genes, and the location of primers used are shown. Positions of primers that successfully produced an amplicon and their predicted sizes are shown. B) RT-PCR of RNA extracted from *S. gordonii* Challis 2 and *cop* operon mutants. Lane 1: 1 kb DNA marker. Lane 2: 100 bp DNA marker. Lane 3: primers copA for 5 and copZ rev 5 using RNA extracted from Challis 2. Lane 4: primers copA for 5 and copZ rev 5 using RNA extracted from *copY*::$spec^R$. Lane 5: primers copZ for and copZ rev using RNA extracted from Challis 2. Lane 6: primers copZ for and copZ rev using RNA extracted from *copA*::$spec^R$.

incubated for an additional 24 h to allow biofilm cells to detach from the rods. Cells that detached from the rods fell to the bottom of the well, where they are likely to attach to the surface and form new biofilms. The amount of biofilm growth on the well was proportional to the number of cells that detached from biofilms that formed on the rods, and was measured by staining with crystal violet (CV). The lid was removed and the plate was stained by adding 1% (wt/vol) CV solution to each well. After 15 min, wells were rinsed three times with 200 μl of distilled H_2O and air-dried. Bacterial growth and biofilm formation were quantified by measuring the absorbance at 575 nm of the bacterial culture and CV-stained biofilm, respectively. Each assay was performed in triplicate.

Biofilm formation assay

The *in vitro* biofilm formation assay was performed in BM as previously described (19). Various concentrations of $CuSO_4$, $CaCl_2$, KCl, $NiCl_2$, $CdCl_2$, $ZnSO_4$, $MgSO_4$, $FeSO_4$, $CoCl_2$, and $AgCl_2$ (0.05–1 mM) were added to the BM to assess the effect of metal ions on biofilm formation. In addition to the microtiter plate biofilm assay, biofilm formation on borosilicate glass coverslips was visualized directly using phase-contrast microscopy as described previously (21).

Statistical analyses

Mutant strains were compared to the Challis 2 strain and the significance of differences in growth, biofilm formation and biofilm detachment was assessed by Student's *t*-test.

Results

Genetic organization of the *cop* operon

Downstream of the *adc* operon were three ORFs, *copY*, *copA* and *copZ*, which are homologous to *copYAZ* of *S. mutans* and *copYZAB* of *E. hirae* (Fig. 2A). Inactivation of the *cop* operon inhibited *S. mutans* growth in the presence of higher levels of copper and reduced detachment of adherent *S. mutans* biofilm (37, 38). This study examined the role of *copYAZ* genes on the growth, biofilm formation and biofilm detachment of *S. gordonii*.

The genetic organization of *copYAZ* of *S. gordonii* and the alignment of the deduced amino acid sequences were examined by identifying homologous sequences from streptococci in the DNA databases (http://www.tigr.org). The predicted amino acid sequences of the proteins encoded by the three ORFs in *S. gordonii* showed strong homology to the *S. mutans* CopY, CopA and CopZ, respectively. Upstream of the *copY* start site, a putative ribosomal binding site, putative −10 and −35 sites were identified (Fig. 2B). Two imperfect inverted repeats present in the promoter regions of the *cop* operon in *S. mutans* (38) and *E. hirae* (30) were also found in the *S. gordonii cop* promoter region (Fig. 2B).

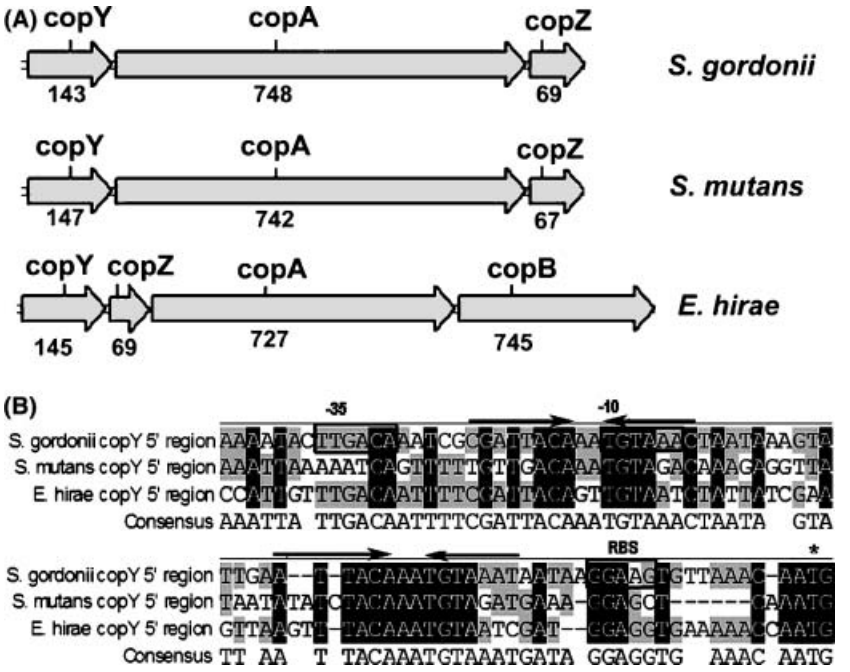

Fig. 2. A) The genetic organization in the *cop* genes in *S. gordonii* Challis, *S. mutans* and *E. hirae.* The number of amino acid residues encoded by each gene is shown below. Unlike *S. gordonii*, the *cop* operon in *S. mutans* is not downstream of the *adc* operon. B) Alignment of the promoter regions of *S. gordonii* Challis, *S. mutans* and *E. hirae.* Nucleotides that are identical and conserved are highlighted in black and gray, respectively. Arrows indicate two potential imperfect inverted repeat regions, putative rbs, potential −10 and −35 regions, and the start codon are indicated by an asterisk (*).

The first gene in the *cop* operon of *S. gordonii* is the *copY* gene, which starts 148 bp 3′ of the *adcA* stop codon and encodes CopY, a deduced 143-amino acid protein with a predicted molecular mass of 16.7 kDa. The CopY in *S. gordonii* contains a consensus heavy-metal-binding motif ($CXCX_4CXC$) at the C-terminus and two stretches of conserved sequences ($IX_3EXXVMX_3W$ and $WX_3TX_2TX_3RLX_2K$) at the N-terminus (Fig. 3A), which are also present in *S. mutans* CopY (38) and *E. hirae* CopY (41). In *S. mutans* and *E. hirae*, CopY is a negative transcriptional regulator of the *cop* operon and shares extensive homology with other bacterial negative transcriptional regulators (31). Amino acid sequence identities between CopY of *S. gordonii* and the CopY homologs from *S. pneumoniae*, *Streptococcus agalactiae, S. mutans, Streptococcus pyogenes*, *E. hirae*, *Streptococcus equi*, and *Streptococcus sobrinus* are approximately 30, 32, 34, 30, 35, 28, and 32%, respectively (Table 1).

copA is 2246 bp in length, starting 7 bp 3′ of the *copY* stop codon and predicted to encode CopA, a deduced 749-amino acid protein with a predicted molecular mass of 80.3 kDa. CopA shares extensive homology with P-type ATPases from other bacteria (36). P-type ATPases are involved in heavy metal transport and contain a number of conserved structural and functional domains. They include an N-terminal heavy-metal binding motif (GMXCXXC), eight transmembrane helices, a CPC ion-transduction motif, an ATP-binding site, and a DKTGT phosphorylation domain (1, 38, 42), all of which are also present in CopA of *S. gordonii* (data not shown). Amino acid identities between the CopA of *S. gordonii* and the CopA homologs from *S. pneumoniae*, *S. agalactaciae, S. mutans, S. pyogenes*, *E. hirae*, *S. equi,* and *S. sobrinus* are approximately 39, 50, 52, 51, 44, 50 and 54%, respectively (Table 1).

The *copZ* gene is 209 bp in length, starting 20 bp 5′ of the *copA* stop codon. It

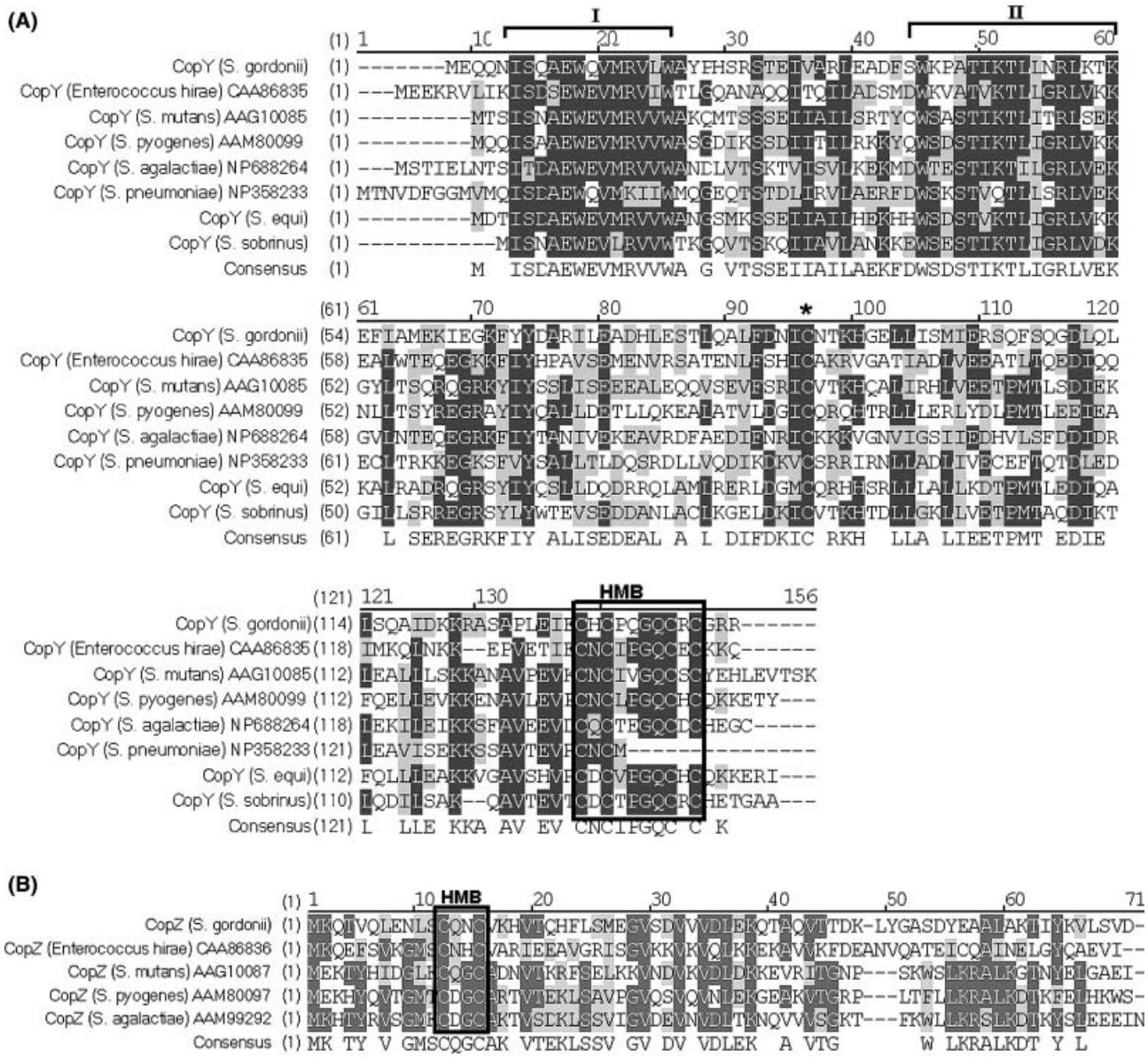

Fig. 3. A) Multiple alignments of *S. gordonii* CopY deduced amino acid sequence with CopY homologs from various streptococci. *S. gordonii* CopY was aligned with CopY from *S. pneumoniae* (NP358283), *S. agalactiae* (NP688264), *S. mutans* (AAG10085), *S. pyogenes* (AAM80089), and with CopY homologs from *S. equi* and *S. sobrinus* (identified from their respective unfinished genome sequences) using the AlignX program of Vector NTI (Informax). Conserved sequences I; $IX_3EXXVMX_3W$, and II; $WX_3TX_2TX_3RLX_2K$, are shown. The putative metal binding motif $CXCX_4CXC$), HMB is boxed. Amino acids that are identical and conserved are highlighted in dark gray and light gray, respectively. A conserved cysteine residue is indicated by an asterisk (*). B) Multiple alignments of *S. gordonii* CopZ deduced amino acid sequence with CopZ homologs from various streptococci. *S. gordonii* CopZ was aligned with CopZ from *S. mutans* (AAG10087), *S. agalactiae* (AAM99292), and *S. pyogenes* (AAM80097 using the AlignX program of Vector NTI (Informax). The putative metal binding motif (CXXC), HMB is boxed. Amino acids that are identical and conserved are highlighted in dark gray and light gray, respectively.

encodes a deduced 69-amino acid protein with a predicted molecular mass of 7.8 kDa that contains a consensus heavy-metal-binding motif (CXXC) at the N-terminus (Fig. 3B) that is also present in CopZ of *S. mutans* (38), *E. hirae* (41) and CopP of *Pseudomonas putida* (1). Amino acid identities between the CopZ of *S. gordonii* and the CopZ homologs from *S. agalactiae, S. mutans, S. pyogenes*, *E. hirae* and *S. sobrinus* are approximately 26, 31, 29, 26, and 41%, respectively (Table 1).

There are significant differences in the arrangement of the *cop* operons in a variety of streptococci, as determined by sequences from their completed and unfinished microbial genome databases (http://www.ncbi.nih.gov). The organization of the *cop* operons in *S. gordonii* and *S. mutans* are similar (*copYAZ*), which are different from *E. hirae* where the *cop* operon contains four genes (*copYZAB*) with the additional gene *copB* encoding a copper efflux protein. No *copB* homolog was found in the unfinished, nonannotated *S. gordonii* Challis genome (http://www.tigr.org.) Among other streptococci, *S. agalactiae* contained a *copB* homolog, which was noncontiguous with the *cop* operon, and a partial *copB* homolog was identified in the incomplete *S. sobrinus* sequence. In *S. pneumoniae*, a hypothetical ORF was

Table 1. Homology of the proteins encoded by *cop* genes of *S. gordonii, E. hirae* and those of other oral streptococci

S. gordonii	CopY %Identity (%Similarity)	CopA %Identity (%Similarity)	CopZ %Identity (%Similarity)
Streptococcus pneumoniae	29.8 (43.0)	38.5 (54.0)	NP
Streptococcus agalactiae	32.4 (48.0)	49.5 (66.2)	25.7 (34.3)
Streptococcus mutans	34.2 (51.0)	51.5 (66.9)	31.4 (40.0)
Streptococcus pyogenes	30.1 (47.9)	51.2 (66.4)	28.6 (35.7)
Enterococcus hirae	34.5 (46.6)	43.6 (59.8)	25.7 (34.3)
Streptococcus equi	28.1 (47.9)	49.8 (64.8)	NA
Streptococcus sobrinus	31.5 (45.2)	53.7 (69.2)	41.4 (32.9)

NP, not present. NA, genome sequence incomplete.

located between *cptY* (*copY* homolog) and *cptA* (*copA* homolog); however, no *copZ* homolog was present in the genome.

The genes encoded by the *cop* operon in *S. gordonii* were inactivated by constructing c*opY, copA* and *copZ* deletion mutants using PCR ligation mutagenesis (21) and inactivation was confirmed by PCR. The location of the primers used are shown in Fig. 1A. RT-PCR performed using RNA isolated from *S. gordonii copY*::*spec*^{R} with primers copA for 5 and copZ rev 5 produced a 673 bp amplicon, with a span from *copA* to *copZ*. RNA isolated from *S. gordonii copA*::*spec*^{R} with primers copZ for and copZ rev produced a 155 bp amplicon that spans within *copZ* (Fig. 1B). RT-PCR using RNA from *S. gordonii* Challis 2 as a control confirmed that amplicons of similar size were produced when identical pair primers were used. These results confirmed that the insertional inactivation of *copY*::*spec*^{R} and *copA*::*spec*^{R} mutants were nonpolar in that the transcription of downstream genes was not affected. The genes were replaced with a spectinomycin cassette designed to ensure a read through so that downstream genes will be nonpolar. A negative control without reverse transcriptase was included to detect any DNA contamination in the RNA sample.

Growth assays

The growth rates and final yields of the *copY*::*spec*^{R}, *copA*::*spec*^{R}, and *copZ*::*spec*^{R} mutants were similar to the growth rate and the final yield of *S. gordonii* Challis 2 in THBYE medium (Fig. 4A). The sequence similarities between *S. gordonii copYAZ* operon and *S. mutans copYAZ* operon indicated that the *S. gordonii copYAZ* might be involved in copper transport and resistance. Hence the effect of copper on growth of *S. gordonii* Challis 2, *copY*::*spec*^{R}, *copA*::*spec*^{R} and *copZ*::*spec*^{R} mutants was examined. *S. gordonii* Challis 2 grew normally up to a final $CuSO_4$ concentration of 200 μM, above which bacterial growth was reduced. The growth of each strain in 400 μM $CuSO_4$ was compared with growth of the same strain in BM without $CuSO_4$ (considered as 100% growth) and expressed as relative growth (Fig. 4B). In the presence of 400 μM $CuSO_4$, the relative growth of the Challis 2 strain was 67%, of the *copY* mutant 100%, of the *copA* mutant 74%, and of the *copZ* mutant 89%. Results indicate that with the exception of the *copY*::*spec*^{R} mutant, growth of all other strains was reduced in the presence of 400 μM $CuSO_4$. The relative growth of *copY* and *copZ* mutants was significantly higher when compared to Challis 2, indicating that a mutation in *copY* or *copZ* increases the resistance to copper, whereas the resistance after a mutation in *copA* was the same as

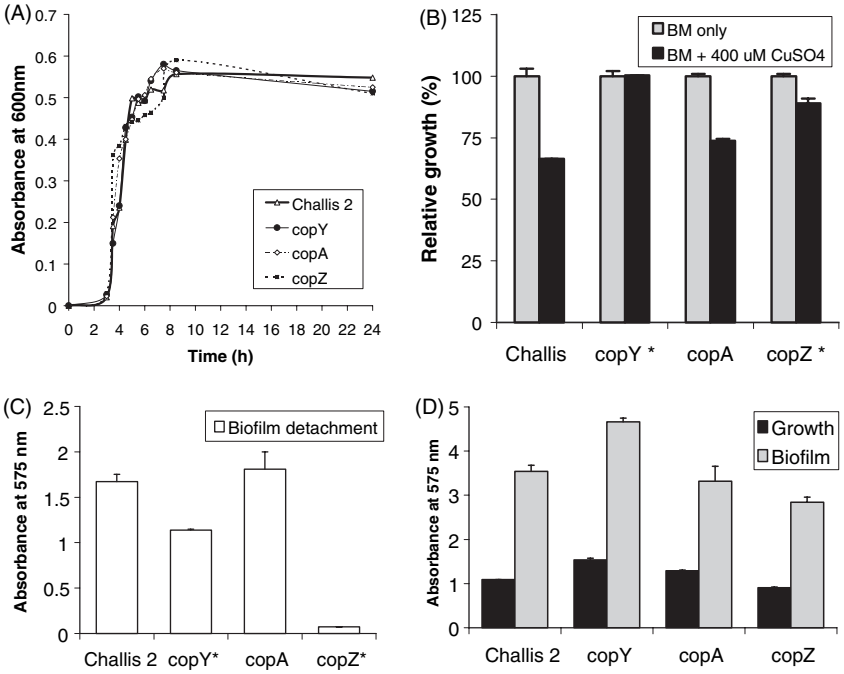

Fig. 4. A) Growth of *S. gordonii* Challis 2 and *cop* mutants in THBYE under anaerobic conditions 37°C for 24 h. All assays were performed in triplicate, and mean values and standard deviations are given. B) Effect of copper on growth of *S. gordonii* Challis 2 and *cop* mutants. Bacteria were grown under anaerobic conditions at 37°C in BM and BM supplemented with 400 μM of $CuSO_4$. The growth of each strain in 400 μM $CuSO_4$ was compared with growth of the same strain in BM only and expressed as relative growth. Growth in BM only was considered 100% growth. All assays were performed in triplicate, and standard deviations are given. Mutant strains that have a statistically significant difference when compared to Challis 2 are marked with an asterisk (*****). C) Biofilm detachment of *S. gordonii* Challis 2 and *cop* mutants. Following the detachment assay described by Kaplan et al. (17), bacteria were grown in BM in a microtiter plate with a modified lid with attached polystyrene rods immersed in the BM. After 24 h, bacteria have formed biofilms on the immersed rods, and the lid was transferred to a new microtiter plate containing BM and incubated for an additional 24 h to allow biofilm cells to detach from the rods. The amount of cells that detached was proportional to the amount of biofilm formation on the second plate, measured by staining with crystal violet (CV). Bacterial growth and biofilm formation were quantified by measuring the absorbance at 575 nm of the bacterial culture and CV-stained biofilm, respectively. All assays were performed in triplicate, and mean values and standard deviations are given. Mutant strains that have a statistically significant difference when compared to Challis 2 are marked with an asterisk (*****). D) Biofilm formation of *S. gordonii* Challis 2 and the *cop* mutants. Bacteria were grown in BM in polystyrene microtiter plates under anaerobic conditions at 37°C for 24 h. Bacterial growth was quantified by measuring the absorbance at 575 nm of the bacterial culture and biofilm formation was quantified by measuring the absorbance of the CV-stained biofilm. All assays were performed in triplicate, and mean values and standard deviations are given.

the parent strain. Therefore it appears a functional *copY* and *copZ* decreases the tolerance of the parent strain to copper, probably by negatively regulating the copper transport operon.

Biofilm assays

Previous studies that isolated a biofilm detachment defective mutant in *S. mutans* showed that a Tn*917* insertion had occurred adjacent to the promoter region (69 bases upstream of the start codon) of the *cop* operon (38). Therefore the detachment phenotype of *S. gordonii* Challis 2, *copY*::*spec*^{R}, *copA*::*spec*^{R}, and *copZ*::*spec*^{R} strains was examined using a biofilm detachment assay described by Kaplan et al. (17). Results from this assay demonstrated that the *copZ* mutant was defective in biofilm detachment. When compared to the parent strain, the *copZ* mutant displayed >95% reduction in biofilm detachment. The *copY* mutant exhibited a smaller reduction (>31%) in the ability to detach when compared with the parent strain (Fig. 4C). The growth was equivalent in *S. gordonii* Challis 2 and the *cop* mutants. This suggests that copper homeostasis in *S. gordonii* is associated with the maintenance of biofilms and is consistent with previous observations of reduced detachment in a *S. mutans* mutant with a transposon insertion adjacent to the promoter region of the *cop* operon (37, 38).

The biofilm-formation phenotype of *copY*::*spec*^{R}, *copA*::*spec*^{R} and *copZ*::*spec*^{R} mutants were also examined in an *in vitro* biofilm assay using polystyrene microtiter plates in BM (Fig. 4D). Biofilm formation of *copY*::*spec*^{R} (A_{575nm} ± standard deviation, 4.12 ± 0.42), *copA*::*spec*^{R} (A_{575nm} ± standard deviation, 4.19 ± 0.19) and *copZ*::*spec*^{R} mutant (A_{575nm} ± standard deviation, 3.103 ± 0.22) were not significantly different from *S. gordonii* Challis 2 (A_{575nm} ± standard deviation, 3.54 ± 0.14).

In order to observe the presence of any microscopic differences in biofilm formation of *S. gordonii* Challis 2 and each mutant biofilms formed on glass coverslips suspended in BM were visualized directly using phase-contrast microscopy (21). Phase-contrast microscopy was used to follow biofilm development of *S. gordonii* Challis 2, *copY*::*spec*^{R}, *copA*::*spec*^{R} and *copZ*::*spec*^{R} mutants on glass coverslips 1, 3, 24, 48, 72, and 96 h after inoculation. Results indicate that biofilm formation of Challis 2 and the mutants were not significantly different (data not shown). No significant growth differences between the four strains were observed. These data demonstrate that there were no differences in biofilm formation between *S. gordonii* Challis 2 and the *cop* mutants.

The effect of extracellular copper on biofilm formation on *S. gordonii* Challis 2 was also examined in a microtiter plate. *S. gordonii* Challis 2 was inoculated into BM containing various concentrations of $CuSO_4$ (0.05–1 mM). Addition of extracellular copper as a supplement to BM did not affect either the growth or biofilm formation (data not shown). None of the other metal ions examined had any effect on *S. gordonii* Challis 2 biofilm formation.

Discussion

The isolation and characterization of a biofilm-defective mutant that had a Tn*917-lac* insertion within an ORF that was homologous to the *adcR* of *S. pneumoniae* (9, 10) demonstrated that the *adc* operon plays a role in zinc/manganese homeostasis, biofilm formation and competence in *S. gordonii* (20). Immediately downstream of the *adc* operon were three ORFs designated *copYAZ*, based on their homologies to the *copYAZ* operon in *S. mutans*. In addition to copper homeostasis, the *copYAZ* operon is involved in biofilm detachment in *S. mutans*, as a transposon insertion adjacent to the promoter region of the *cop* operon in *S. mutans* displayed a biofilm detachment-defective phenotype (38). This study was initiated to investigate the role of *S. gordonii copYAZ* in biofilm detachment, and the influence of extracellular copper on growth and biofilm formation in *S. gordonii*.

None of the mutations generated in *S. gordonii copY*, *copA* and *copZ* by insertional inactivation using a *spec*^{R} cassette affected the biofilm formation of *S. gordonii*. *S. gordonii copZ*::*spec*^{R} mutant and, to a lesser extent, *S. gordonii copY*::*spec*^{R} were defective in biofilm detachment. This is consistent with the previous report of a biofilm detachment-defective mutant in *S. mutans* (38). Our observations from the *S. gordonii copZ*::*spec*^{R} mutant suggest that the detachment-defective phenotype may be specifically associated with *copZ*. Based on studies with the *E. hirae cop* operon, the CopZ protein is now recognized as a metallochaperone that is involved in the trafficking of intracellular copper to CopY and CopA, fulfilling the vital cellular function of copper transport to copper-dependent enzymes while protecting the intracellular milieu from copper toxicity (35). In the current model depicting the function of the *cop* operon in *E. hirae*, it is proposed that CopA transports copper intracellularly, resulting in copper loading of apo-CopZ. Subsequently, CopZ delivers copper to CopY in cases of high intracellular levels of copper (6). However, a high concentration of copper-loaded CopZ is toxic to cells and its levels appear to be regulated by copper-induced proteolysis (23).

In the gram-positive bacterium *E. hirae*, the *cop* operon consists of four genes that encode a metalloregulated repressor (CopY), a copper metallochaperone (CopZ), and two Cpx-type copper ATPases (CopA and CopB). *E. hirae* CopA is responsible for copper uptake under copper-limiting conditions and CopB for copper efflux when copper reaches toxic levels (29). However, examination of the unfinished *S. gordonii* DNA database did not identify a *copB* gene elsewhere in the genome. Likewise, no CopB homolog was present in the completed genomes of *S. mutans* and *S. pneumoniae*. Thus the *cop* operon in *S. gordonii* may be mainly responsible for copper uptake. It is possible that a CopB homolog is present in *S. gordonii* and may be identified when the genome is completely sequenced. Alternatively, another metal efflux protein could be responsible for excreting excess copper. For example, the CopB in *E. hirae* can also modulate silver transport, illustrating the presence of redundancy within metal transport systems in bacteria (34). Also, it is possible that as *S. gordonii* exists in environments that are relatively low in copper, a copper efflux system may be unnecessary. In contrast to the observations in *S. mutans*, *S. gordonii copY*::*spec*^{R}, and *copZ*::*spec*^{R} mutants grew well in the presence of higher levels of copper in the growth medium (>400 μM), whereas the growth of *S. gordonii* Challis 2 was inhibited by increased copper in the growth medium (>200 μM). The growth studies with *S. mutans* used mutants where the whole *cop* operon was disrupted, whereas this study used *S. gordonii* mutants where individual *cop* genes were disrupted. This could have resulted in the differences observed between the two growth studies. *S. gordonii copY::spec*^{R} and *copZ::spec*^{R} mutants are able to tolerate higher levels of extracellular copper than the parent strain, probably by affecting the transport of extracellular copper ions into the cell. Transport studies with copper are required to verify these observations.

Although various trace elements are of interest in dentistry as potential modulators of dental caries, copper and fluoride have a

consistent, inverse relationship with the caries experience in children. The concentration of copper in saliva ranges from 0.2 to 7.05 mg/l (13), and several studies have suggested that copper may have a potent cariostatic effect due to antibacterial activity. The action of copper on dental plaque may be mediated by inhibiting bacterial growth through their antiglycolytic and anti-ureolytic properties (33). Metal ions such as copper can also inactivate glucosyltransferase produced by mutans streptococci via a Fenton type reaction (8). Several *in vitro* and *in vivo* studies have demonstrated the following:

- topical copper application in combination with fluoride reduced caries incidence in hamsters and rats (3, 25);
- copper provided in the drinking water reduced experimental caries in rats infected with *S. mutans* (2);
- copper and chlorhexidine showed synergistic growth inhibition of *S. mutans* and *Actinomyces* spp. (11);
- glucose containing $CuSO_4$ and zinc acetate oral rinses reduced acid production in plaque (4).

A recent study found that phosphate-based glass containing 5–10% copper reduced the viability of *in vitro Streptococcus sanguis* biofilms grown over 24 h in a constant-depth film fermentor. The reduction of viability was attributed to the antibacterial effect of copper (28). However, the antibacterial effect on *S. sanguis* that was observed may also be attributed to the ability of copper to modulate *S. sanguis* biofilms as suggested by the biofilm detachment-defective phenotype of the *copZ* mutant of *S. gordonii*. Although further work is required on the specific action of copper on acidogenic bacteria, this study suggests biofilm detachment may also contribute to the disruption of dental plaque and perhaps to the reduction in caries experience previously reported (13). The long-term durability and success of amalgam (copper silver alloy) restorations in dentistry, where copper ions are known to leach out (27, 40), could have been partially due to the effect of copper ions on acidogenesis and biofilm detachment of oral streptococci. These findings support the hypothesis that metalloregulation of bacterial oral biofilms may be a common phenomenon and needs to be further investigated.

Acknowledgment

This work was supported by PHS grant RO1-DE13328 from the National Institute of Dental and Craniofacial Research.

References

1. Adaikkalam V, Swarup S. Molecular characterization of an operon, *cueAR*, encoding a putative P1-type ATPase and MerR-type regulatory protein involved in copper homeostasis in *Pseudomonas putida*. Microbiology 2002: **148**: 2857–2867.
2. Afseth J, Amsbaugh SM, Monell-Torrens E, Brown WH, Rolla G, Brunelle J, et al. Effect of copper applied topically or in drinking water on experimental caries in rats. Caries Res 1984: **18**: 434–439.
3. Afseth J, Amsbaugh SM, Monell-Torrens E, Brown WH, Rolla G, Brunelle J, et al. Effect of topical application of copper in combination with fluoride in drinking water on experimental caries in rats. Caries Res 1984: **18**: 134–140.
4. Afseth J, Opperman RV, Rolla G. The *in vivo* effect of glucose solutions containing Cu^{2+} and Zn^{2+} on the acidogenicity of dental plaque. Acta Odontol Scand 1980: **38**: 229–233.
5. Brooks SJ, Shore RC, Robinson C, Wood SR, Kirkham J. Copper ions inhibit the demineralization of human enamel. Arch Oral Biol 2003: **48**: 25–30.
6. Cobine P, Wickramasinghe WA, Harrosin MD, Weber T, Solioz M, Dameron CT. The *Enterococcus hirae* copper chaperone CopZ delivers copper (I) to the CopY repressor. FEBS Lett 1999: **445**: 27–30.
7. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999: **284**: 1318–1322.
8. Devulapalle KS, Mooser G. Glucosyltransferase inactivation reduces dental caries. J Dent Res 2001: **80**: 466–469.
9. Dintilhac A, Alloing G, Granadel C, Claverys JP. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and manganese resulting from inactivation of putative ABC metal permeases. Mol Microbiol 1997: **25**: 727–739.
10. Dintilhac A, Claverys JP. The *adc* locus, which affects competence for genetic transformation in *Streptococcus pneumoniae*, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. Res Microbiol 1997: **148**: 119–131.
11. Drake DR, Grigsby W, Cardenzana A, Dunkerson D. Synergistic, growth-inhibitory effects of chlorhexidine and copper combinations on *Streptococcus mutans*, *Actinomyces viscosus*, and *Actinomyces naeslundii*. J Dent Res 1993: **72**: 524–528.
12. Driezen S, Spies HA, Spies BS. Copper and cobalt levels in human saliva and dental caries. J Dent Res 1952: **31**: 137–142.
13. Duggal MS, Chawla HS, Curzon MEJ. A study of the relationship between trace elements in saliva and dental caries in children. Arch Oral Biol 1991: **36**: 881–884.
14. Forbes JC, Smith SD. Studies on the effect of metallic salts on acid production in saliva. J Dent Res 1952: **3**: 129–136.
15. Francis MS, Thomas CJ. The mutants in the *ctpA* copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. Microb Pathog 1997: **22**: 67–78.
16. Frandsen EVG, Pedrazzoli V, Kilian M. Ecology of viridans streptococci in the oral cavity and pharynx. Oral Microbiol Immunol 1991: **6**: 129–133.
17. Kaplan JB, Meyenhofer MF, Fine DH. Biofilm growth and detachment of *Actinobacillus actinomycetemcomitans*. J Bacteriol 2003: **185**: 1399–1404.
18. Kierek K, Watnick PI. The *Vibrio cholerae* 0139 O-antigen polysaccharide is essential for Ca^{2+}-dependent biofilm development in sea water. PNAS 2003: **100**: 14357–14362.
19. Loo CY, Corliss DA, Ganeshkumar N. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. J Bacteriol 2000: **182**: 1374–1382.
20. Loo CY, Mitrakul K, Voss IB, Hughes CV, Ganeshkumar N. Involvement of the *adc* operon and manganese homeostasis in *Streptococcus gordonii* biofilm formation. J Bacteriol 2003: **185**: 2887–2990.
21. Loo CY, Mitrakul K, Voss IB, Hughes CV, Ganeshkumar N. Involvement of an inducible fructose phosphotransferase operon in *Streptococcus gordonii* biofilm formation. J Bacteriol 2003: **185**: 6241–6254.
22. Lowe AM, Beattie DT, Deresiewicz RL. Identification of novel staphylococcal virulence genes by *in vivo* expression technology. Mol Microbiol 1998: **27**: 967–976.
23. Lu ZH, Solioz M. Copper-induced proteolysis of the *copZ* copper chaperone of *Enterococcus hirae*. J Biol Chem 2001: **276**: 47822–47827.
24. Marra A, Lawson S, Asundi JS, Brigham D, Hromockyj AE. *In vivo* characterization of the *psa* genes from *Streptococcus pneumoniae* in multiples models of infection. Microbiology 2002: **148**: 1483–1491.
25. Matz M, Emilson CG. Effect of copper fluoride and copper sulphate on dental plaque, *Streptococcus mutans* and caries in hamsters. Scand J Dent Res 1988: **96**: 390–392.
26. McLean RJ, AA. Hussain AA, M. Sayer M, Vincent PJ, Hughes DJ, Smith TJ. Antibacterial activity of multiplayer silver-copper surface films on catheter material. Can J Microbiol 1993: **39**: 895–899.
27. Morrier JJ, Suchett-Kaye G, Nguyen D, Rocca JP, Blanc-Benon J, Barsotti O. Antimicrobial activity of amalgams, alloys and their elements and phases. Dent Mater 1998: **14**: 150–157.
28. Mulligan AM, Wilson M, Knowles JC. The effect of increasing copper content in phosphate-based glasses on biofilms of *Streptococcus sanguis*. Biomaterials 2003: **24**: 1797–1807.
29. Odermatt A, Solioz M. Primary structure of two P-type ATPases invloved in copper homeostasis in *Enterococcus hirae*. J Biol Chem 1993: **268**: 12775–12779.
30. Odermatt A, Solioz M. Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of *Enterococcus hirae*. J Biol Chem 1995: **270**: 4349–4354.

31. Odermatt A, Solioz M. Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. J Biol Chem 1995: **270**: 9217–9221.
32. Opperman RV, Johansen JR. Effect of fluoride and non-fluoride salts of copper, silver and tin on the acidogenecity of dental plaque *in vivo*. J Dent Res 1980: **88**: 476–480.
33. Opperman RV, Rolla G, Johansen JR, Assev S. Thiol groups and reduced acidogenecity of dental plaque in the presence of metal ions *in vivo*. Scand J Dent Res 1980: **88**: 389–396.
34. Solioz M, Odermatt A. Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. J Biol Chem 1995: **270**: 9217–0221.
35. Solioz M, Stoyanov JV. Copper homeostasis in *Enterococcus hirae*. FEMS Microbiol Rev 2003: **27**: 183–195.
36. Solioz M, Vulpe C. CPx-type ATPases. a class of P-type ATPases that pump heavy metals. Trends Biochem Sci 1996: **21**: 237241.
37. Vats N, Lee SF. Active detachment of *Streptococcus mutans* cells adhered to epon-hydroxylapatite surfaces coated with salivary proteins *in vitro*.: Arch Oral Biol 2000: **45**: 305–314.
38. Vats N, Lee SF. Characterization of a copper-transport operon, *copYAZ*, from *Streptococcus mutans*. Microbiology 2001: **147**: 653–662.
39. Waler SM, Rolla G. Comparison between plaque inhibiting effect of chlorhexidine and aqueous solutions of copper and silver ions. Scand J Dent Res 1982 1982: **90**: 131–133.
40. Wataha JC, Lockwood PE. Release of elements from dental casting alloys into cell-culture medium over 10 months. Dent Mater 1998: **14**: 158–163.
41. Wunderli YeH, Solioz M. Copper homeostasis in *Enterococcus hirae*. Adv Exp Medical Biol 1999: **448**: 255–264.
42. Wunderli YeH, Solioz M. Purification and functional analysis of the copper ATPase CopA of *Enterococcus hirae*. Biochem Biophys Res Commun 2001: **280**: 713–719.