The Tea Catechin Epigallocatechin Gallate Suppresses Cariogenic Virulence Factors of *Streptococcus mutans*[▽]

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Streptococcus mutans, the primary etiologic agent of dental caries, possesses a series of virulence factors associated with its cariogenicity. Alternatives to traditional antimicrobial treatment, agents selectively inhibiting the virulence factors without necessarily suppressing the resident oral species, are promising. The anticariogenic properties of tea have been suggested in experimental animals and humans. Tea polyphenols, especially epigallocatechin gallate (EGCg), have been shown to inhibit the growth and glucosyltransferases activity of *S. mutans*. However, their effects on biofilm and cariogenic virulence factors of oral streptococci other than glucosyltransferases have not been well documented. In this study, we investigated the biological effect of EGCg on the virulence factors of *S. mutans* associated with its acidogenicity and acidurity. The antimicrobial effects of EGCg on *S. mutans* biofilm grown in chemically defined medium were also examined. EGCg inhibited growth of *S. mutans* planktonic cells at an MIC of 31.25 μg/ml and a minimal bactericidal concentration (MBC) of 62.5 μg/ml. EGCg also inhibited *S. mutans* biofilm formation at 15.6 μg/ml (minimum concentration that showed at least 90% inhibition of biofilm formation) and reduced viability of the preformed biofilm at 625 μg/ml (sessile MIC_{80}). EGCg at sub-MIC levels inhibited acidogenicity and acidurity of *S. mutans* cells. Analysis of the data obtained from real-time PCR showed that EGCg significantly suppressed the *ldh*, *eno*, *atpD*, and *aguD* genes of *S. mutans* UA159. Inhibition of the enzymatic activity of F_1F_o-ATPase and lactate dehydrogenase was also noted (50% inhibitory concentration between 15.6 and 31.25 μg/ml). These findings suggest that EGCg is a natural anticariogenic agent in that it exhibits antimicrobial activity against *S. mutans* and suppresses the specific virulence factors associated with its cariogenicity.

Dental caries is a multifactorial infectious disease dependent on diet, nutrition, resident oral flora, and host response. Unlike most infectious diseases exhibiting classic virulence factors such as endotoxin (lipopolysaccharide), the etiology of dental caries is unique, being associated with the bacterial metabolism of carbohydrates, leading to prolonged periods of plaque acidification and demineralization of the tooth enamel. Bacterial virulence factors contributing to dental caries result in stable biofilm formation, stress tolerance (e.g., acid tolerance), and efficient acid production from carbohydrates (7, 36, 37, 53). Despite the complexity of oral flora, oral streptococci, including *Streptococcus mutans* and *Streptococcus sobrinus*, have generally been considered the primary etiologic agents of dental caries (1, 38). One of the most often documented characteristics of the virulence of *S. mutans* is its ability to produce glucosyltransferases (GTFs), which synthesize intracellular polysaccharides (IPS) and extracellular polysaccharides (EPS). The EPS, especially water-insoluble glucans, mediate the adherence of *S. mutans* and other oral bacterial species to tooth surfaces, contributing to the formation of dental plaque biofilms (21, 60). Other crucial virulence factors of *S. mutans* include the membrane-bound F_1F_o-ATPase system, the agmatine deiminase system, enolase, and lactate dehydrogenase. The membrane-bound F_1F_o-ATPase system pumps protons

from cells, while maintaining the internal pH value. This is considered the primary determinant of acid tolerance in *S. mutans* (5, 14, 33, 34). Another inducible pathway for alkali production is through the agmatine deiminase system (AgDS), which increases the competitive fitness of *S. mutans*, contributing to its persistence and pathogenesis (19, 20). Enolase is responsible for the production of phosphoenolpyruvate (PEP), which functions as the key component of the PEP:carbohydrate phosphotransferase system (PTS). Lactate dehydrogenase (LDH), responsible for lactate production, further contributes to the virulence of *S. mutans* (36). Mutants of *S. mutans*, deficient in specific virulence factors, were more sensitive to environmental stress and less cariogenic than their parent strains (11, 27, 36, 39, 71). These findings suggest that suppression of virulence-associated genes and enzymes could be appealing for the prevention of dental caries.

Fluoride has long been proven to be an effective cariostatic agent (15, 17, 44) via the inhibition of demineralization and the enhancement of remineralization at the crystal surface, and the inhibition of bacterial activity (16). However, in the case of high bacterial challenge and/or xerostomia, fluoride alone is insufficient to prevent the progression of caries. In addition, adverse effects such as fluorosis have led to the limited use of fluoride for public health in many countries (16, 66). Therefore, the development of an alternative cariostatic agent with minimal side effects has been promising.

Tea, an aqueous infusion of dried leaves of the plant *Camellia sinensis* (family: Theaceae), is the most popular beverage consumed by humans worldwide (18). Numerous *in vitro* and animal studies have demonstrated that polyphenols from

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tea possess antioxidant, antimutagenic, antidiabetic, hypocholesterolemic, antibacterial, anti-inflammatory, and cancer-preventive properties (23, 41, 70). Although tea is a source of fluoride and many other dietary trace elements, studies have demonstrated that tea polyphenols, rather than the fluoride content, contribute to its anticariogenic potential (31, 55, 72). Analysis of data from our previous studies and from other researchers has shown that tea catechins, the main antimicrobial polyphenol components of green tea, possess antimicrobial effects against oral streptococci (22, 23, 54, 57, 65, 69, 70). Animal studies have demonstrated that various tea extracts prevented or reduced caries formation (46, 48, 49, 55). Clinical trials have also shown that drinking tea (without added sugar) is associated with lower caries levels in humans (30, 47, 50). Our previous study in adult humans found that rinsing with black tea extract resulted in a significantly less-pronounced plaque pH fall and a lower plaque index than the water-rinse group (70). Frequent short-term rinses with black tea also inhibited subsequent regrowth and glycolysis of human supragingival plaque bacteria compared with the water-rinse group (69). Several mechanisms proposed for the observed anticariogenic properties of tea polyphenols, especially the galloylated catechins, include the inhibition of bacterial growth, adherence, acid production and metabolism, and enzymatic activities such as those of glucosyltransferases (GTFs) and amylase (22, 25, 40, 43, 73).

Despite the aforementioned anticariogenic potential of tea and tea polyphenols, their mechanisms of action on dental plaque and caries remain to be elucidated. At present, most of the antimicrobial studies have been performed in planktonic cultures of oral streptococci, while limited data are available regarding their biofilm counterpart. The use of different culture media and growth conditions has also contributed to the variable antimicrobial effects of tea polyphenols. The *in vitro* investigations into the effects of tea polyphenols on cariogenic virulence factors have focused on GTFs activity, EPS production, and cell adherence (26, 41, 45, 51). We hypothesized that the major antimicrobial monomeric component of tea polyphenols, epigallocatechin gallate (EGCg), contributes to the suppression of virulence factors of *S. mutans* other than GTFs. We thus investigated the effects of EGCg on F_1F_o-ATPase, agmatine deiminase, enolase, and lactate dehydrogenase of *S. mutans* UA159 cells at the transcriptional and enzymatic levels. We also examined the antimicrobial effects of EGCg against planktonic culture and biofilm of *S. mutans* UA159 grown in chemically defined and complex media.

MATERIALS AND METHODS

Chemicals, test bacterium, and growth conditions. Epigallocatechin gallate from green tea (EGCg, 95% [wt/wt] by high-performance liquid chromatography), and all chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The test bacterium *S. mutans* UA159 was grown in chemically defined medium (CDM) (62) at 37°C in an anaerobic chamber (37°C, 10% H_2, 5% CO_2, and 85% N_2; Forma Scientific, Inc., Marietta, OH). Brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI) was also used for antimicrobial assays. Tryptone-yeast extract medium containing 20 mM glucose (TYEG) was used for acid tolerance experiments (63). For the agmatine deiminase (AgD) activity assay, *S. mutans* UA159 was grown anaerobically in a low-carbohydrate tryptone-vitamin base medium (TV medium) containing 25 mM galactose and 10 mM agmatine (9), to avoid the carbon catabolite repression (CCR) caused by the glucose in the CDM and BHI.

Bacterial susceptibility assays. The MIC and minimum bactericidal concentration (MBC) of EGCg against *S. mutans* UA159 were determined by a microdilution method in both CDM and BHI, as described previously (45).

The kinetics of the bacterial killing effect of EGCg against *S. mutans* UA159 was determined with the method modified from that of Koo et al. (35). Tubes containing *S. mutans* UA159 (1×10^6 CFU/ml) and EGCg (31.25 to 125 µg/ml) in CDM were incubated at 37°C. Cell samples were removed at 0, 0.5, 1, 2, 4, and 8 h, serially diluted in phosphate-buffered saline (PBS, 50 mM, pH 6.8), and plated onto BHI agar with a spiral plater (Autoplate model 4000; Spiral Biotech, Inc., Norwood, MA). All plates were incubated anaerobically at 37°C for 48 h, and the number of colonies was then determined with a colony counter (Synbiosis Acolyte 7510; Microbiology International, Frederick, MD). Control contained *S. mutans* UA159 grown in the absence of EGCg. We constructed the killing curves by plotting the $\log_{10}$ CFU/ml versus time over 8 h. A bactericidal effect was defined as a $\geq$ 3-$\log_{10}$ CFU/ml decrease from the original inoculum.

Biofilm susceptibility assay. The effect of EGCg on *S. mutans* biofilm formation was examined by the microdilution method described previously (68). *S. mutans* UA159 (1×10^6 CFU/ml) was grown in CDM supplemented with 1% (wt/vol) sucrose and EGCg (0 to 1,000 µg/ml) at 37°C for 24 h. The culture supernatant from each well was then decanted, and the adherent biofilm was washed three times with PBS, fixed with methanol for 15 min, and stained with 0.1% (wt/vol) crystal violet (Sigma) for 5 min. Subsequently, the wells were rinsed with deionized water until the blank wells appeared colorless; 200 µl of 95% ethanol was added. The plates were shaken at room temperature for 30 min, and the absorbance at 595 nm was recorded. The minimum biofilm inhibition concentration (MBIC) was defined as the lowest EGCg concentration that resulted in at least 50% ($MBIC_{50}$) or 90% ($MBIC_{90}$) inhibition of the formation of biofilms compared with that in the untreated control. A parallel study was also performed with BHI medium supplemented with 1% (wt/vol) sucrose.

The effect of EGCg on the viability of *in vitro* *S. mutans* biofilm was determined by the microdilution method modified from that of Pierce et al. (51). A 200-µl quantity of an *S. mutans* UA159 cell suspension (1×10^6 CFU/ml) in CDM containing 1% (wt/vol) sucrose was added to the wells of a 96-well microtiter plate for biofilm formation. After anaerobic incubation at 37°C for 24 h, the growth medium was removed without disrupting the integrity of the biofilms. The formed biofilms were then washed three times with PBS to remove nonadherent cells. CDM supplemented with EGCg (9.77 to 1,250 µg/ml) was added to wells containing biofilm and incubated at 37°C for 24 h. The control wells contained CDM without EGCg. After incubation, the culture media were removed, and the treated biofilms were washed three times with PBS. The metabolic activity of the biofilms was determined by the addition of 120 µl of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)–menadione solution (0.2 mg/ml XTT and 16 µM menadione). All plates were further incubated in the dark at 37°C for 2 h. A 100-µl quantity of the colored cell-free supernatant in each well was then transferred to another blank microtiter plate, and absorbance at 490 nm was determined. The sessile MIC ($SMIC_{50}$ and $SMIC_{80}$) was defined as the lowest concentration resulting in at least 50% or 80% reduction compared with that of the untreated control. A parallel study was also performed with BHI medium supplemented with 1% (wt/vol) sucrose.

Glycolytic pH drop and acid tolerance assay. The effect of EGCg on *S. mutans* glycolysis was measured by the method modified from that of Song et al. (63). *S. mutans* UA159 was harvested at mid-logarithmic phase, washed with 0.5 mM potassium phosphate buffer containing 37.5 mM KCl and 1.25 mM $MgCl_2$ (pH 6.5), and resuspended (optical density at 600 nm [OD_{600}] = 0.5) in the same solution containing sub-MIC levels of EGCg (3.9 to 15.6 µg/ml). The control mixture contained no EGCg. Glucose was added in the mixture to give a final concentration of 1% (wt/vol). The decrease in pH, as a result of glycolytic activity of *S. mutans* UA159 cells, was monitored at 15-min intervals over a period of 75 min (Corning pH meter 240; Corning Inc., New York, NY).

The effect of EGCg on the acid tolerance of *S. mutans* UA159 was determined by measurement of the viability of bacteria after 2 h of exposure at pH 5.0 (63, 64). *S. mutans* UA159 was grown in TYEG medium until the cells reached the mid-logarithmic phase (OD_{600} = 0.5). The cells were collected by centrifugation and resuspended (OD_{600} = 0.2) in TYEG medium buffered with 40 mM phosphate-citrate buffer (pH 5.0) containing sub-MIC levels of EGCg, and incubated at 37°C for 2 h. The control mixture contained no EGCg. Samples were removed before and after incubation at pH 5.0 for viable counts as described above.

Cell permeabilization. *S. mutans* UA159 cells were permeabilized according to the method modified from Hoelscher and Hudson (29). *S. mutans* UA159 was grown in BHI broth (for F_1F_o-ATPase and enolase assays) or TV medium (for AgD assay) until late exponential phase; 30-ml aliquots were harvested by centrifugation (4,000 $\times$ g, 5 min, 4°C). Cells were resuspended in 2.5 ml of 75 mM Tris-HCl–10 mM $MgSO_4$ (pH 7.0). Toluene (Fluka, Steinheim, Germany) was

TABLE 1. Specific primers used for real-time PCR

Primers	Sequences (L and R)
16S rRNA	AGCGTTGTCCGGATTTATTG CTACGCATTTCACCGCTACA
atpD	TGTTGATGGTCTGGGTGAAA TTTGACGGTCTCCGATAACC
aguD	TGGTGCTGCTCTTGCTAATG TAAAAGGACGCGGTGTATCC
ldh	GGCGACGCTCTTGATCTTAG GGTTAGCAGCAACGAGGAAG
eno	CAGCGTCTTCAGTTCCATCA TCACTCAGATGCTCCAATCG

added to a final concentration of 10% (vol/vol), and the cell suspensions were vigorously vortexed and incubated for 5 min at 37°C. The cell suspensions were further frozen in liquid nitrogen and thawed twice at 37°C. The permeabilized cells were harvested by centrifugation (4,000 × *g*, 5 min, 4°C), resuspended in 75 mM Tris-HCl–10 mM MgSO₄ at pH 7, and stored at −80°C until use.

F₁Fₒ-ATPase assay. We determined F₁Fₒ-ATPase activity by using permeabilized cells of *S. mutans* UA159 according to the method described by Belli *et al.* (2). Permeabilized cells were pretreated by EGCg (7.8 to 62.5 μg/ml) at room temperature for 15 min. The F₁Fₒ-ATPase activity was measured in terms of the release of inorganic phosphate in the following reaction mixture: 100 mM Tris-maleate buffer (pH 7.0) containing 5 mM adenosine-5′-triphosphate (ATP), 10 mM MgCl₂, and permeabilized cells. The released phosphate was then determined (4), and results were expressed as enzymatic activity relative to that of the untreated control.

Enolase assay. Enolase activity was determined by the use of permeabilized cells of *S. mutans* UA159 according to the method described by van Loveren et al. (67). Permeabilized cells were pretreated with EGCg (7.8 to 62.5 μg/ml) at room temperature for 15 min, and we measured enolase activity directly by monitoring the formation of PEP at 240 nm. The reaction mixture (200 μl) contained 180 μl of 20 mM KPO₄ buffer (pH 6.5) with 2 mM MgSO₄, 10 μl of D-(+)-2-phosphoglycerate (final concentration of 17.6 mM), and 10 μl of pretreated permeabilized cells. Results were expressed as enzymatic activity relative to that of the untreated control.

Agmatine deiminase (AgD) assay. Permeabilized *S. mutans* UA159 cells were pretreated with EGCg (7.8 to 62.5 μg/ml) at room temperature for 15 min, and AgD activity was measured by colorimetric determination of *N*-carbamoylputrescine production from agmatine as previously described by Griswold et al. (19). Results were expressed as enzymatic activity relative to that of the untreated control.

Lactate dehydrogenase (LDH) assay. *S. mutans* UA159 cells were collected at late exponential phase and incubated in Tris-HCl buffer (pH 7.0) containing 0.5 mg/ml of lysozyme at 37°C for 1 h. The lysate was further sonicated by means of a cup horn (Thermo Fisher Scientific Inc., Pittsburgh, PA) on ice for 2 cycles of 60 s, and the cell-free supernatant was collected by centrifugation (14,000 × *g*, 4°C, 10 min). The crude extract was further dialyzed against 10 mM phosphate buffer (pH 6.9) overnight at 4°C. The dialyzed preparation was designated the crude LDH, and its total protein content was determined by the Bradford method (8).

For the LDH assay, crude LDH was pretreated with EGCg (7.8 to 62.5 μg/ml) at room temperature for 15 min, and the LDH activity was estimated by measurement of the rate of NADH oxidation at 340 nm (13). The reaction mixture (200 μl) contained 180 μl of 50 mM phosphate-buffered saline (pH 6.9) with 0.167 mM NADH and 10 mM sodium pyruvate; 10 μl of fructose 1,6-diphosphate (final concentration of 1 mM); and 10 μl of pretreated LDH. Results were expressed as enzymatic activity relative to that of the untreated control.

RNA isolation, purification, and reverse transcription. For determination of the effect of EGCg on selected virulence factors at the transcriptional level, *S. mutans* UA159 was grown in either CDM or BHI medium supplemented with sub-MIC levels of EGCg (15.6 μg/ml and 62.5 μg/ml in CDM and in BHI, respectively). Cells were collected during the late exponential phase by centrifugation, and RNA was immediately stabilized with an RNAprotect bacterial reagent (Qiagen, Valencia, CA). Cells were then pelleted, resuspended in 100 μl of lysis buffer (20 mM Tris-HCl, 3 mM EDTA, 20 mg/ml lysozyme, 60 mAU/ml proteinase K, 1,000 U/ml mutanolysin [pH 8.0]), and incubated at 37°C with gentle agitation for 45 min. The lysate was further sonicated by means of a cup horn (Thermo Fisher Scientific Inc.) on ice for 2 cycles of ultrasonication for 60 s

TABLE 2. Antimicrobial effect of EGCg against *S. mutans* planktonic culture and biofilms in chemically defined medium (CDM) and BHI medium

Medium	Planktonic cells		Biofilm			
			Formation		Viability	
	MIC (μg/ml)	MBC (μg/ml)	MBIC₅₀ (μg/ml)	MBIC₉₀ (μg/ml)	SMIC₅₀ (μg/ml)	SMIC₈₀ (μg/ml)
CDM	31.25	62.5	7.8	15.6	156.25	625
BHI	625	>1,250	312.5	625	1,250	>1,250

and then purified in an RNeasy minikit (Qiagen). Reverse transcription was performed by use of a 1st Strand cDNA synthesis kit with random hexamer primers (Invitrogen, Madison, WI).

Quantitative real-time PCR. Real-time PCR was used for quantification of *atpD* (part of the F₁Fₒ-ATPase operon), *aguD* (part of the agmatine deiminase system operon), *ldh* (LDH), and *eno* (enolase) mRNA expression, with 16S rRNA as an internal control. All primers for real-time PCR were designed with Primer3 (56) and obtained commercially from Sigma-Aldrich Corp. (Table 1). Real-time PCR amplification was performed on the iCycler iQ detection system (Applied Biosystems, Foster City, CA). The reaction mixture (25 μl) contained 1× SYBR green PCR Master Mix (Applied Biosystems), 1 μl of template cDNA, and forward and reverse primers (10 μM each). Thermal cycling conditions were designated as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. An additional step, from 95°C for 15 s to 60°C for 1 min (0.05°C s⁻¹), was performed to establish a melting curve. Threshold cycle values (C_T) were determined, and the data were analyzed by StepOne software v2.0 (Applied Biosystems) according to the $2^{-\Delta\Delta CT}$ method.

Statistical analysis. All experiments were performed in triplicate and reproduced at least three separate times. Differences between the experimental group and the untreated control group were analyzed by SPSS (version 15.0 for Windows). One-way analysis of variance (ANOVA) was performed, and a post hoc Tukey test was used for the comparison of multiple means. Significance was set at a *P* value of <0.05.

RESULTS

Antimicrobial activity of EGCg against *S. mutans* UA159 planktonic culture and biofilm *in vitro*. EGCg inhibited the *in vitro* growth of *S. mutans* UA159 (MIC = 31.25 μg/ml) in the chemically defined medium and had an MBC of 62.5 μg/ml (Table 2). The kinetics of the antimicrobial effect of EGCg against *S. mutans* UA159 is shown in Fig. 1. At 62.5 μg/ml, EGCg was bactericidal against *S. mutans* UA159, with a more
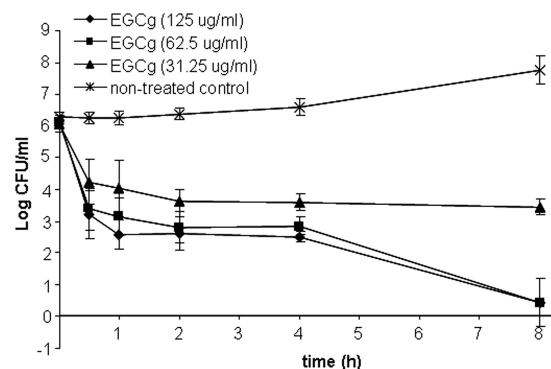

FIG. 1. Kinetic killing effect of EGCg on *S. mutans* UA159 planktonic cell cultures. The concentrations of EGCg ranged from 31.25 μg/ml to 125 μg/ml. The untreated control was *S. mutans* UA159 cells grown in chemically defined medium in the absence of EGCg. Data are means ± standard deviations (SD) (*n* = 3).
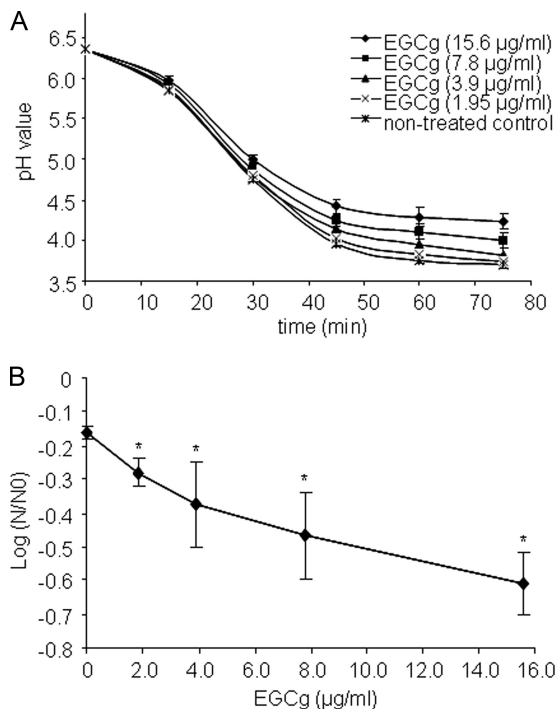
FIG. 2. Effect of EGCg on glycolytic acid production and acid tolerance of *S. mutans* UA159 cells at sub-MIC levels. (A) Glycolytic acid production was determined by monitoring the pH decrease in sucrose solution (1%, wt/vol) over a period of 75 min. Values (means ± SD, $n = 4$) for all EGCg groups were significantly different from those of untreated controls at 30, 45, 60, and 75 min (Tukey test, $P < 0.01$). (B) Acid tolerance was determined by measuring the survival rate of *S. mutans* UA159 cells at pH 5.0. *, EGCg at test concentrations (1.95 to 15.6 μg/ml) significantly inhibited the survival rate (means ± SD, $n = 4$) of *S. mutans* UA159 cells compared with the untreated control ($P < 0.05$).
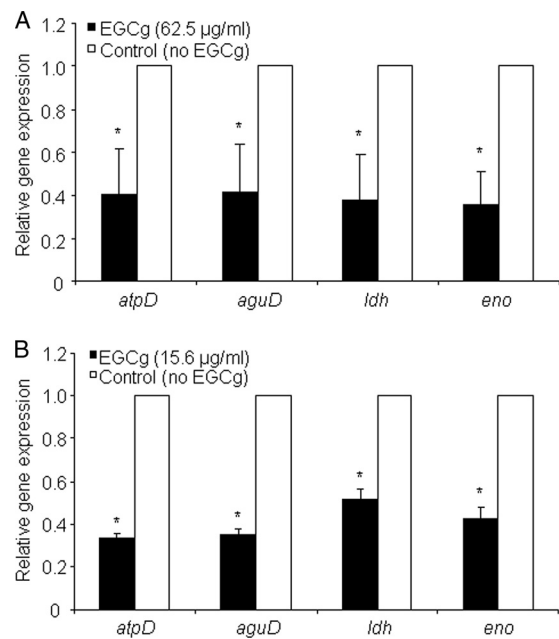

FIG. 3. Effects of sub-MIC levels of EGCg on the gene expression of specific virulence factors associated with the acidogenicity and acidurity of *S. mutans* UA159 at sub-MIC levels. (A) Relative gene expression compared with untreated control in BHI medium supplemented with 62.5 μg/ml of EGCg. (B) Relative gene expression compared with untreated control in chemically defined medium supplemented with 15.6 μg/ml of EGCg. Data are means ± SD ($n = 3$). *, Significant difference compared with the untreated control ($P < 0.05$).

than 5-$\log_{10}$ CFU/ml decrease (>99.99%) over the 8 h of incubation compared with the original inoculum. This bactericidal effect was found to be dosage dependent. However, when *S. mutans* UA159 cells were grown in the complex medium, i.e., BHI, a higher MIC, 625 μg/ml, was noted, and no bactericidal concentration was detected up to 1,250 μg/ml.

In addition to growth inhibition on *S. mutans* UA159 planktonic culture, EGCg inhibited the formation of *S. mutans* UA159 biofilm in CDM ($MBIC_{50} = 7.8$ μg/ml; $MBIC_{90} = 15.6$ μg/ml). EGCg was also found to inhibit the viability of biofilm, with an $SMIC_{50}$ of 156.25 μg/ml and an $SMIC_{80}$ of 625 μg/ml. When parallel experiments were performed in BHI medium, EGCg was less effective against *S. mutans* UA159 biofilm formation with higher MIBCs ($MBIC_{50} = 312.5$ μg/ml; $MBIC_{90} = 625$ μg/ml). Reduced efficacy of EGCg in BHI medium against *S. mutans* UA159 biofilm viability was also observed with an $SMIC_{50}$ of 1,250 μg/ml (Table 2).

EGCg inhibits the acid production and acid tolerance of *S. mutans* UA159 *in vitro*. We determined the effect of EGCg on acid production by *S. mutans* UA159 by monitoring the glycolytic pH drop of *S. mutans* culture. As shown in Fig. 2A, the acid production by *S. mutans* UA159 cells was significantly inhibited by EGCg at sub-MIC levels, 7.8 μg/ml and 15.6 μg/ml ($P < 0.05$).

The acidurity of *S. mutans* UA159 cells was also suppressed by EGCg at sub-MIC levels. As shown in Fig. 2B, the survival rate of *S. mutans* UA159 cells at pH of 5.0 was significantly reduced in the presence of EGCg (1.95 to 15.6 μg/ml) compared with the untreated control. Dosage-dependent inhibition was observed, and the survival rate was significantly reduced by EGCg at concentrations as low as 1.95 μg/ml ($P < 0.05$).

EGCg inhibits the virulence factors of *S. mutans* UA159 *in vitro*. The virulence factors of *S. mutans* UA159 examined in this study included those encoded by *atpD*, *eno*, *ldh*, and *aguD*. In CDM, EGCg at 15.6 μg/ml significantly suppressed expression of *atpD*, *eno*, *ldh*, and *aguD* by >50% compared with expression in the untreated control ($P < 0.05$). A similar result was observed in BHI medium supplemented with 62.5 μg/ml of EGCg (Fig. 3). In addition to the suppression of virulence factors at the transcriptional level, EGCg inhibited F_1F_o-ATPase activity, with half-maximal inhibitory concentrations (IC_{50}) between 15.6 μg/ml and 31.25 μg/ml (Fig. 4A) ($P < 0.05$). A similar IC_{50} was noted for LDH (Fig. 4B). However, less than 10% reduction of enolase or AgD activity was observed up to the highest concentration of EGCg tested (Fig. 4C and D).

DISCUSSION

Tea catechins, especially the galloylated derivatives such as EGCg, have been documented to possess antimicrobial effects against oral streptococci (22, 23, 32, 40, 65, 70). The antimicrobial mechanism is mainly attributable to irreversible dam-
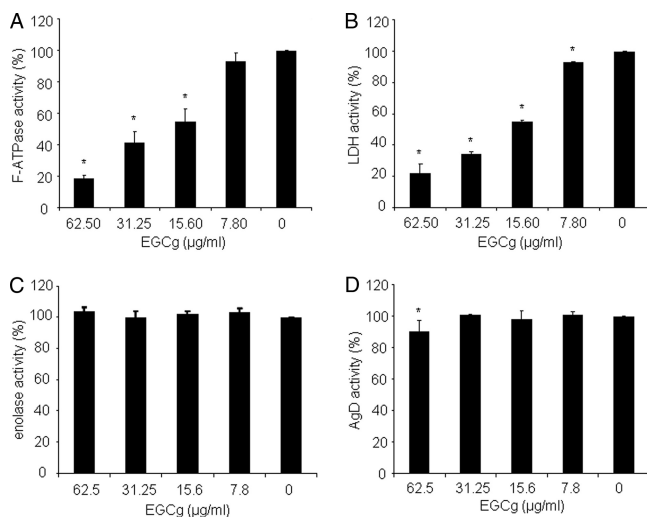
FIG. 4. Effect of increasing concentrations of EGCg on the activity of enzymes associated with the acidogenicity and acidurity of *S. mutans* UA159. Data are means ± SD ($n = 3$). *, Significant difference compared with the untreated control ($P < 0.05$).

age of the microbial cytoplasmic membrane (65). However, great variability has been reported from different studies. Muroi and Kubo (42) found no significant antimicrobial activity against oral streptococci at 500 μg/ml, while others reported MICs between 50 and 500 μg/ml (32, 57). In this study, we compared the susceptibility of *S. mutans* UA159 cells in CDM and BHI medium. We found that the MIC of EGCg in BHI medium was 20 times higher than that in CDM, and the bactericidal effect was observed only in CDM (MBC = 62.5 μg/ml). These results are in agreement with previous findings that the antibacterial activity of Oolong tea extract (OTE) was significantly inhibited in BHI medium, and that pretreatment of OTE with bovine serum albumin also reduced its antibacterial activity against *S. mutans* (58). Despite the different methods used for the determination of bacterial susceptibility in various independent studies, proteins present in the undefined medium such as BHI may bind to or even precipitate tea catechins. The binding may compromise their antimicrobial efficacy, resulting in the discrepancies reported in previous studies.

Microbial biofilm commonly exhibits increasing levels of resistance to most antibiotics or therapeutic agents (12). Stable biofilm formation is also considered one of the key factors of caries pathogenesis. To the best of our knowledge, the effect of EGCg on *S. mutans* biofilm formation and viability has not yet been well documented. In this study, we found that the *in vitro* pre-established *S. mutans* biofilm was susceptible to EGCg (SMIC$_{50}$ = 156.25 μg/ml; SMIC$_{80}$ = 625 μg/ml), suggesting that EGCg may represent a novel natural agent against dental biofilms. Moreover, EGCg in this study was also found to disrupt *S. mutans* biofilm formation at concentrations below the MIC (MBIC$_{90}$ = 15.6 μg/ml). This indicates that, in addition to its known bacteriostatic effect, EGCg may be capable of inhibiting the adherence of cells to the host surface, thus disrupting the initial step in the formation of mature biofilms.

Given the difficulties of maintaining effective levels of therapeutic agents to achieve antimicrobial efficacy in the oral

cavity, especially in the dental plaque, approaches selectively inhibiting the virulence factors of caries-associated bacteria without necessarily suppressing the resident oral flora are promising. Previous studies have demonstrated that tea catechins, especially EGCg, could act as promising cariostatic agents, due to their antimicrobial activity and various biological effects on key virulence factors of oral streptococci (65, 70). In addition to its bacteriostatic and bactericidal effects, EGCg is able to inhibit GTF activity, thus disrupting the formation and integrity of oral biofilm (25, 49). EGCg and other catechins from green tea have also been reported to suppress salivary and bacterial amylase activities, leading to the inefficiency of carbohydrate metabolism (26). A previous study showed that EGCg was able to suppress the *in vitro* acid production of *S. mutans*, and rinsing with EGCg solution also inhibited the *in situ* acidity of dental plaque (28). Those authors suggested that the antimicrobial activity of EGCg against oral streptococci may contribute to its inhibitory effect on acid production. However, in the same study, the viability of *S. mutans* in sucrose-containing culture or adherent to hydroxyapatite was not affected by the test concentrations of EGCg, indicating that other mechanisms may contribute to the observed inhibition. In this study, we found that the *in vitro* acid production by *S. mutans* UA159 was inhibited by sub-MIC levels of EGCg, which confirmed that other biomolecular mechanisms may also be involved in inhibition of the acid production of *S. mutans*. The previously reported inhibitory effect of EGCg on LDH activity may explain its suppression of the acid production of *S. mutans*, despite the fact that LDH used in that study was commercially obtained from *Leuconostoc mesenteroides* (28). The *in vitro* suppression of the LDH activity of *S. mutans* UA159 observed in our study, in concert with the inhibition of *ldh* gene expression obtained from real-time PCR, suggests that the inhibitory effect of EGCg on acid production is attributable not only to its antimicrobial activity but also to the suppression of LDH at both the transcriptional and enzymatic levels.

Moreover, the inhibitory effect on acid production may result from the biological effect of EGCg on the bacterial glycolytic pathways. Not only may the suppression of enolase by EGCg at the transcriptional level exert direct inhibition on glycolysis, but also the reduction of downstream phosphoenolpyruvate (PEP) produced by enolase may suppress the PEP:carbohydrate phosphotransferase system (PTS), the major system for internalizing sugar into the cell during a depleted sugar situation (52). The net result would be a decrease in sugar internalization and suppressed glycolysis, leading to the reduced acid production by *S. mutans* cells, as observed in this study.

Since the pathology of dental caries is associated almost exclusively with bacterial metabolism of carbohydrates, the metabolites, especially acids produced after carbohydrate consumption, in turn impose physiological stress on the oral microflora. Due to its acidogenic and aciduric properties, *S. mutans* gains a competitive advantage over less-acid-tolerant species and emerges as the numerically predominant resident in the cariogenic plaque when environmental conditions are less favorable. Thus, stress tolerance, especially the acidurity of *S. mutans*, is closely involved in its virulence. The acid tolerance of *S. mutans* is mainly attributed to the activity of F_1F_o-
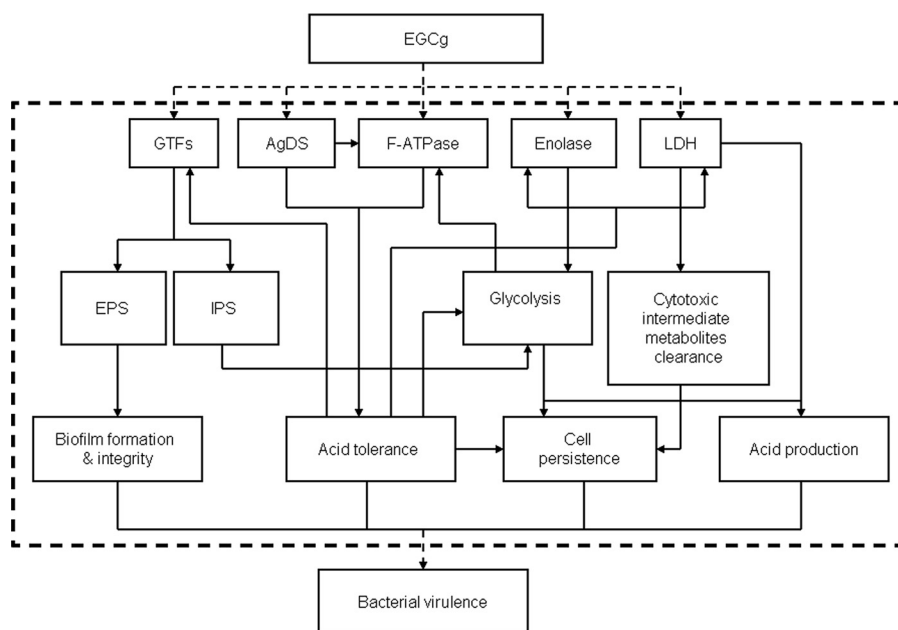
FIG. 5. Proposed physiological effects of EGCg on *S. mutans* UA159 cells. Dashed arrows represent the inhibitory effect of EGCg. The portion inside the dashed box represents the interaction among individual virulence factors.

ATPase (3, 24). It has been demonstrated that increased F_1F_o-ATPase activity correlates with increased transcription of the F_1F_o-ATPase operon (53), and the operon of *S. mutans* can be regulated at the transcriptional level in response to growth pH (61). The inhibition of F_1F_o-ATPase enzymatic activity and *atpD* expression (α subunit of the proton translocator) observed in this study may lead to increased cytoplasmic acidity and decreased acid adaptation. Cytoplasmic acidification may impair the normal function of the serial enzymes involved in glycolysis and EPS production, compromising the competitive fitness of, or having a potential lethal effect on, *S. mutans*.

In addition to the F_1F_o-ATPase system, the agmatine deiminase system (AgDS) is also able to increase the competitive fitness of *S. mutans*, contributing to the persistence and pathogenesis of this organism (19, 20). The AgDS system converts agmatine to putrescine, ammonia, and CO_2. The production of ammonia from agmatine increases the cytoplasmic pH, and the ATP generated in this process can also be used for growth or to extrude protons (20). The inhibition of *aguD* (antiporter of AgDS) expression observed in our study may further exacerbate the starvation stress of *S. mutans* cells due to reduced ATP production by AgDS.

Maintaining ΔpH across the cell membrane is the key factor allowing aciduric bacteria such as *S. mutans* to carry out glycolysis at low pH values within the biofilm, because glycolytic enzymes are not acid tolerant (2). Suppression of F_1F_o-ATPase and AgDS by EGCg may lead not only to energy starvation but also to disruption of ΔpH across the cell membrane (36), which in turn may trigger a series of physiological effects in the cell. As summarized in Fig. 5, we propose that cytoplasmic acidification and impaired acid tolerance as the result of suppression of AgDS and F_1F_o-ATPase may inhibit the normal function of various acid-intolerant enzymes. The optimum pH range of GTFs in *S. mutans* was reported to be

5.5 to 6.0 (59). The malfunction of GTFs at the lower pH value may lead to reduced production of EPS and IPS. The latter can be metabolized when exogenous fermentable substrate is depleted in the oral cavity (10). Therefore, the malfunction of GTFs may disrupt both bacterial adherence to the tooth surface and biofilm integrity and may augment the starvation stress of *S. mutans* cells due to the reduced preservation of IPS. Cytoplasmic acidity, in concert with the inhibition of enolase by EGCg, may also inhibit the normal process of glycolysis, as described above. This in turn will diminish the ATP pool and further suppress the activity of the proton translocator (F_1F_o-ATPase), aggravating cytoplasmic acidification. The inhibition of LDH at both transcriptional and enzymatic levels may also increase the levels of NADH and decrease the redox potential of the cell, leading to the NAD^+/NADH imbalance and/or accumulation of glycolytic intermediates in the cell, which is toxic for *S. mutans* (11, 27). The net result would be cytoplasmic acidification and disrupted glycolytic processes with diminished ATP pools, thereby triggering a series of cascaded biological effects at molecular levels, leading to compromised competence to environmental stress and impaired cellular functions, even cell death.

The interaction of EGCg with proteins, leading to the distortion of their tertiary structure, may account for the observed *in vitro* inhibition of EGCg in certain enzyme activity, e.g., F_1F_o-ATPase and LDH. However, the affinity for salivary proteins may limit its oral application (6). The reduced antimicrobial activity of EGCg in BHI medium, compared with that in CDM observed in our study, confirmed this point of view, which makes the inhibition of certain virulence factors at sub-MIC levels promising. The observed suppression of the virulence factors at the transcriptional level was comparable in both BHI and CDM, indicating that the interaction of EGCg with proteins may be unable to diminish the biological effect of

EGCg on *S. mutans* cells. However, further studies are needed to confirm this observation.

In summary, based on our data, EGCg represents a natural and alternative anticariogenic agent because (i) EGCg inhibits growth of both *S. mutans* planktonic and biofilm cultures, and (ii) EGCg inhibits various cariogenic virulence factors of *S. mutans* at the transcriptional and enzymatic levels, leading to reduced acidogenicity and compromised stress tolerance (especially acid tolerance). Considering the complexity of the acid stress and starvation stress elicited by EGCg, further studies with transcriptomic and proteomic approaches are warranted.

REFERENCES

1. **Beighton, D.** 2005. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent. Oral Epidemiol. **33:**248–255.
2. **Belli, W. A., D. H. Buckley, and R. E. Marquis.** 1995. Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5. Can. J. Microbiol. **41:**785–791.
3. **Belli, W. A., and R. E. Marquis.** 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. Appl. Environ. Microbiol. **57:**1134–1138.
4. **Bencini, D. A., M. S. Shanley, J. R. Wild, and G. A. O'Donovan.** 1983. New assay for enzymatic phosphate release: application to aspartate transcarbamylase and other enzymes. Anal. Biochem. **132:**259–264.
5. **Bender, G. R., S. V. Sutton, and R. E. Marquis.** 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. Infect. Immun. **53:**331–338.
6. **Bennick, A.** 2002. Interaction of plant polyphenols with salivary proteins. Crit. Rev. Oral Biol. Med. **13:**184–196.
7. **Bowen, W. H.** 2002. Do we need to be concerned about dental caries in the coming millennium? Crit. Rev. Oral Biol. Med. **13:**126–131.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
9. **Burne, R. A., Z. T. Wen, Y. Y. Chen, and J. E. Penders.** 1999. Regulation of expression of the fructan hydrolase gene of *Streptococcus mutans* GS-5 by induction and carbon catabolite repression. J. Bacteriol. **181:**2863–2871.
10. **Busuioc, M., K. Mackiewicz, B. A. Buttaro, and P. J. Piggot.** 2009. Role of intracellular polysaccharide in persistence of *Streptococcus mutans*. J. Bacteriol. **191:**7315–7322.
11. **Chen, A., J. D. Hillman, and M. Duncan.** 1994. L-(+)-lactate dehydrogenase deficiency is lethal in *Streptococcus mutans*. J. Bacteriol. **176:**1542–1545.
12. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
13. **Crow, V. L., and G. G. Pritchard.** 1977. Fructose 1,6-diphosphate-activated L-lactate dehydrogenase from *Streptococcus lactis*: kinetic properties and factors affecting activation. J. Bacteriol. **131:**82–91.
14. **Dashper, S. G., and E. C. Reynolds.** 1992. pH regulation by *Streptococcus mutans*. J. Dent. Res. **71:**1159–1165.
15. **DePaola, P. F., et al.** 1993. The relative anticaries effectiveness of sodium monofluorophosphate and sodium fluoride as contained in currently available dentifrice formulations. Am. J. Dent. **6**(Spec. No.)**:**S7–S12.
16. **Featherstone, J. D.** 1999. Prevention and reversal of dental caries: role of low level fluoride. Community Dent. Oral Epidemiol. **27:**31–40.
17. **Featherstone, J. D.** 2009. Remineralization, the natural caries repair process—the need for new approaches. Adv. Dent. Res. **21:**4–7.
18. **Graham, H. N.** 1992. Green tea composition, consumption, and polyphenol chemistry. Prev. Med. **21:**334–350.
19. **Griswold, A. R., Y. Y. Chen, and R. A. Burne.** 2004. Analysis of an agmatine deiminase gene cluster in *Streptococcus mutans* UA159. J. Bacteriol. **186:**1902–1904.
20. **Griswold, A. R., M. Jameson-Lee, and R. A. Burne.** 2006. Regulation and physiologic significance of the agmatine deiminase system of *Streptococcus mutans* UA159. J. Bacteriol. **188:**834–841.
21. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44:**331–384.
22. **Hamilton-Miller, J. M.** 2001. Anti-cariogenic properties of tea (*Camellia sinensis*). J. Med. Microbiol. **50:**299–302.
23. **Hamilton-Miller, J. M.** 1995. Antimicrobial properties of tea (*Camellia sinensis L.*). Antimicrob. Agents Chemother. **39:**2375–2377.
24. **Hamilton, I. R., and N. D. Buckley.** 1991. Adaptation by *Streptococcus mutans* to acid tolerance. Oral Microbiol. Immunol. **6:**65–71.
25. **Hattori, M., I. T. Kusumoto, T. Namba, T. Ishigami, and Y. Hara.** 1990. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from *Streptococcus mutans*. Chem. Pharm. Bull. (Tokyo) **38:**717–720.
26. **He, Q., Y. Lv, and K. Yaoa.** 2007. Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase. Food Chem. **101:**1178–1182.
27. **Hillman, J. D., A. Chen, and J. L. Snoep.** 1996. Genetic and physiological analysis of the lethal effect of L-(+)-lactate dehydrogenase deficiency in *Streptococcus mutans*: complementation by alcohol dehydrogenase from *Zymomonas mobilis*. Infect. Immun. **64:**4319–4323.
28. **Hirasawa, M., K. Takada, and S. Otake.** 2006. Inhibition of acid production in dental plaque bacteria by green tea catechins. Caries Res. **40:**265–270.
29. **Hoelscher, G. L., and M. C. Hudson.** 1996. Characterization of an unusual fluoride-resistant *Streptococcus mutans* isolate. Curr. Microbiol. **32:**156–161.
30. **Jones, C., K. Woods, G. Whittle, H. Worthington, and G. Taylor.** 1999. Sugar, drinks, deprivation and dental caries in 14-year-old children in the north west of England in 1995. Community Dent. Health **16:**68–71.
31. **Kashket, S., V. J. Paolino, D. A. Lewis, and J. van Houte.** 1985. In-vitro inhibition of glucosyltransferase from the dental plaque bacterium *Streptococcus mutans* by common beverages and food extracts. Arch. Oral Biol. **30:**821–826.
32. **Kawamura, J., and T. Takeo.** 1989. Antibacterial activity of tea catechin to *Streptococcus mutans*. J. Jpn. Soc. Food Sci. Technol. **36:**463–467.
33. **Kobayashi, H.** 1985. A proton-translocating ATPase regulates pH of the bacterial cytoplasm. J. Biol. Chem. **260:**72–76.
34. **Kobayashi, H., T. Suzuki, and T. Unemoto.** 1986. Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. J. Biol. Chem. **261:**627–630.
35. **Koo, H., P. L. Rosalen, J. A. Cury, Y. K. Park, and W. H. Bowen.** 2002. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. Antimicrob. Agents Chemother. **46:**1302–1309.
36. **Lemos, J. A., J. Abranches, and R. A. Burne.** 2005. Responses of cariogenic streptococci to environmental stresses. Curr. Issues Mol. Biol. **7:**95–107.
37. **Lemos, J. A., and R. A. Burne.** 2008. A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology **154:**3247–3255.
38. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
39. **Matsumoto-Nakano, M., K. Fujita, and T. Ooshima.** 2007. Comparison of glucan-binding proteins in cariogenicity of *Streptococcus mutans*. Oral Microbiol. Immunol. **22:**30–35.
40. **Matsumoto, M., et al.** 1999. Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. Caries Res. **33:**441–445.
41. **Mitscher, L. A., et al.** 1997. Chemoprotection: a review of the potential therapeutic antioxidant properties of green tea (*Camellia sinensis*) and certain of its constituents. Med. Res. Rev. **17:**327–365.
42. **Muroi, H., and I. Kubo.** 1993. Combination effects of antibacterial compounds in green tea flavor against *Streptococcus mutans*. J. Agric. Food Chem. **41:**4.
43. **Nakahara, K., et al.** 1993. Inhibitory effect of oolong tea polyphenols on glycosyltransferases of mutans streptococci. Appl. Environ. Microbiol. **59:**968–973.
44. **Newbrun, E.** 1989. Effectiveness of water fluoridation. J. Public Health Dent. **49:**279–289.
45. **Nudera, W. J., et al.** 2007. Antimicrobial effect of triclosan and triclosan with Gantrez on five common endodontic pathogens. J. Endod. **33:**1239–1242.
46. **Ooshima, T., et al.** 1993. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. Caries Res. **27:**124–129.
47. **Ooshima, T., T. Minami, W. Aono, Y. Tamura, and S. Hamada.** 1994. Reduction of dental plaque deposition in humans by oolong tea extract. Caries Res. **28:**146–149.
48. **Ooshima, T., et al.** 1998. Comparison of the cariostatic effects between regimens to administer oolong tea polyphenols in SPF rats. Caries Res. **32:**75–80.
49. **Otake, S., M. Makimura, T. Kuroki, Y. Nishihara, and M. Hirasawa.** 1991. Anticaries effects of polyphenolic compounds from Japanese green tea. Caries Res. **25:**438–443.
50. **Parajas, I. L.** 1995. Caries preventive effect of wild tea (tsaang-gubat) among school children. J. Philipp. Dent. Assoc. **47:**3–13.
51. **Pierce, C. G., et al.** 2008. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat. Protoc. **3:**1494–1500.
52. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.

53. Quivey, R. G., Jr., W. L. Kuhnert, and K. Hahn. 2000. Adaptation of oral streptococci to low pH. Adv. Microb. Physiol. 42:239–274.

54. Rasheed, A., and M. Haider. 1998. Antibacterial activity of Camellia sinensis extracts against dental caries. Arch. Pharm. Res. 21:348–352.

55. Rosen, S., M. Elvin-Lewis, F. M. Beck, and E. X. Beck. 1984. Anticariogenic effects of tea in rats. J. Dent. Res. 63:658–660.

56. Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132:365–386.

57. Sakanaka, S., M. Kim, M. Taniguchi, and T. Yamamoto. 1989. Antibacterial substances in Japanese green tea extract against Streptococcus mutans, a cariogenic bacterium. Agric. Biol. Chem. 53:2307–2311.

58. Sasaki, H., et al. 2004. Antibacterial activity of polyphenol components in oolong tea extract against Streptococcus mutans. Caries Res. 38:2–8.

59. Satoa, S., T. Koga, and M. Inoue. 1984. Isolation and some properties of extracellular d-glucosyltransferases and d-fructosyltransferases from Streptococcus mutans serotypes c, e, and f. Carbohydrate Res. 134:293–304.

60. Schilling, K. M., and W. H. Bowen. 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for Streptococcus mutans. Infect. Immun. 60:284–295.

61. Smith, A. J., R. G. Quivey, Jr., and R. C. Faustoferri. 1996. Cloning and nucleotide sequence analysis of the Streptococcus mutans membrane-bound, proton-translocating ATPase operon. Gene 183:87–96.

62. Socransky, S. S., J. L. Dzink, and C. M. Smith. 1985. Chemically defined medium for oral microorganisms. J. Clin. Microbiol. 22:303–305.

63. Song, J. H., et al. 2006. In vitro inhibitory effects of Polygonum cuspidatum on bacterial viability and virulence factors of Streptococcus mutans and Streptococcus sobrinus. Arch. Oral Biol. 51:1131–1140.

64. Svensater, G., U. B. Larsson, E. C. Greif, D. G. Cvitkovitch, and I. R. Hamilton. 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol. Immunol. 12:266–273.

65. Taylor, P. W., J. M. Hamilton-Miller, and P. D. Stapleton. 2005. Antimicrobial properties of green tea catechins. Food Sci. Technol. Bull. 2:71–81.

66. ten Cate, J. M. 2009. The need for antibacterial approaches to improve caries control. Adv. Dent. Res. 21:8–12.

67. van Loveren, C., M. A. Hoogenkamp, D. M. Deng, and J. M. ten Cate. 2008. Effects of different kinds of fluorides on enolase and ATPase activity of a fluoride-sensitive and fluoride-resistant Streptococcus mutans strain. Caries Res. 42:429–434.

68. Wei, G. X., A. N. Campagna, and L. A. Bobek. 2006. Effect of MUC7 peptides on the growth of bacteria and on Streptococcus mutans biofilm. J. Antimicrob. Chemother. 57:1100–1109.

69. Wu, C. D., and G. Wei. 2009. Tea as a functional food for oral health, p. 396–417. In M. Wilson (ed.), Food constituents and oral health: current status and future prospect. Woodhead Publishing, Cambridge, United Kingdom.

70. Wu, C. D., and G. X. Wei. 2002. Tea as a functional food for oral health. Nutrition 18:443–444.

71. Yamashita, Y., W. H. Bowen, R. A. Burne, and H. K. Kuramitsu. 1993. Role of the Streptococcus mutans gtf genes in caries induction in the specific-pathogen-free rat model. Infect. Immun. 61:3811–3817.

72. Yu, H., T. Oho, and L. X. Xu. 1995. Effects of several tea components on acid resistance of human tooth enamel. J. Dent. 23:101–105.

73. Zhang, J., and S. Kashket. 1998. Inhibition of salivary amylase by black and green teas and their effects on the intraoral hydrolysis of starch. Caries Res. 32:233–238.