ORIGINAL ARTICLE

Morphological and proteomic analyses of the biofilms generated by *Streptococcus mutans* isolated from caries-active and caries-free adults

Shan Jiang [a], Xiaojing Huang [a*], Chengfei Zhang [b], Zhiyu Cai [c], Ting Zou [b]

[a] *Department of Endodontics and Operative Dentistry I, School and Hospital of Stomatology, Fujian Medical University, Fuzhou, Fujian, China*
[b] *Department of Endodontics, Comprehensive Dental Care, Faculty of Dentistry, The University of Hong Kong, Hong Kong Special Administrative Region, China*
[c] *Department of Oral and Maxillofacial Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian, China*

Abstract *Background/purpose:* Biofilm formation by *Streptococcus mutans* is a prerequisite for the development of caries. Different strains of *S. mutans* may differ in their capacity in biofilm formation and protein expression. The objective of this study was to investigate the morphological features and proteomes of biofilms of *S. mutans* clinical isolates.
Materials and methods: Clinical strains isolated from caries-active (SM 593) and caries-free (SM 18) adults were cultured on polystyrene sheets in tryptone—polypeptone—yeast extract medium. Biofilm formation and structure were assessed by confocal laser scanning microscopy and scanning electron microscopy. Proteins were extracted from SM 593 and SM 18 presented in biofilms and separated with two-dimensional gel electrophoresis, followed by peptide mass fingerprinting using matrix-assisted laser desorption time-of-flight mass spectrometry analysis.
Results: Initially detected 2 hours after incubation, biofilm formation reached its maximum level at 20 hours. The biofilm formed by SM 593 was thicker with a higher percentage of viable bacteria compared with that formed by SM 18. Hydrolase and pantothenate kinase were detected in the SM 593 biofilm only, whereas 6-pyruvoyl tetrahydropterin synthase and phosphoribosylglycinamide formyltransferase were expressed exclusively in the SM 18 biofilm. Expressions of D-alanyl-D-alanine carboxypeptidase and response regulator homolog of RumR were most greatly enhanced in the SM 593 and SM 18 biofilms, respectively.

* Corresponding author. Department of Endodontics and Operative Dentistry I, School and Hospital of Stomatology, Fujian Medical University, 246 Yangqiao Zhong Road, Fuzhou, Fujian 350002, PR China.
    E-mail address: hxiaoj@163.com (X. Huang).

Conclusion: SM 593 exhibited greater biofilm-forming capacity compared to SM 18. SM 593 and SM 18 biofilms expressed specific proteins involved in nucleic acid metabolism and intermediary metabolism, respectively, which may account for the differences in their biofilm-forming abilities.

Introduction

One of the greatest challenges facing any pathogen attempting to cause dental caries is simply surviving the complex environment of the oral cavity. Considerable research has shown that the cells growing in biofilms are more antibiotic resistant and acid tolerant than their planktonic counterparts.[1-3] Dental biofilm formation is the prerequisite for bacteria to adhere and grow, and to withstand dynamic changes in oral cavity environment as well.[4] Polysaccharides produced by exoenzymes from *Streptococcus mutans* are the main constituents of the matrix of cariogenic plaque biofilms and are recognized as essential virulence factors associated with dental caries.[5] Among important virulence factors of this pathogen, the ability of *S. mutans* to form and sustain a polysaccharide-encased biofilm is vital not only to its survival and persistence in the oral cavity, but also for its pathogenicity.[6]

Although *S. mutans* are generally considered to be the principal etiological agent of dental caries,[7,8] they are widely distributed in both caries-active populations[9,10] and populations having no or low caries experience.[11,12] In order to find the possible explanation for their presence in caries-free individuals, several studies have been carried out to investigate the genetic heterogeneity among *S. mutans* strains.[13,14] However, the relationship between caries activity and the genetic diversity of *S. mutans* still remains controversial.[15,16]

Protein is the product of gene expression and is the final executor of function of gene. Surface-associated proteins play an important role in cariogenicity. Research has shown that biofilm regulatory protein A and glucan-binding proteins expressed by *S. mutans* are essential for survival of bacteria and biofilm formation within the host, and play a fundamental role in the interaction between the bacterial cell and its environment.[17,18] Our previous study demonstrated that, compared with those in planktonic status, clinical isolations of *S. mutans* in biofilms have higher expression of certain surface-associated proteins that are presumed to be essential for formation of biofilms.[19]

Based on the importance of biofilm-forming capability and surface-associated protein expression by *S. mutans* in the initiation and progression of caries, we hypothesized that differences exist between biofilm-forming capability and surface-associated protein expression by strains of *S. mutans* isolated from caries-active and caries-free individuals. To testify this, the process of biofilm formation by *S. mutans* strain was monitored with confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM); expression of surface-associated proteins in bacteria was detected using two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Materials and methods

Bacterial strains and culture conditions

SM 593 was isolated from caries-active adults [the number of decayed and filled teeth (DFT) = 10, no missing tooth, 3 cavitated lesions]. SM 18 was isolated from caries-free adults [the number of decayed, missing, and filled teeth (DMFT) = 0] (both SM 593 and SM 18 were isolated in our previous study). The strains were stored at $-80°C$ in basic growth medium containing 15% (v/v) glycerol. Bacteria were cultured anaerobically on tryptone–polypeptone–yeast extract (TPY) (Oxoid, Hampshire, England) at 37°C as described previously.[20] Cultivation of bacteria was performed in an anaerobic environment (80% N_2, 10% CO_2, and 10% H_2). Pure cultures of each test strain were obtained and suspended in fresh TPY to an optical density (OD) of 1.0 at 630 nm (approximately 10^8 cells/mL) for the following experiments.

Biofilm formation on polystyrene sheets and petri dishes

S. mutans biofilms were formed on sterile plastic sheets (1×1 mm^2) and plastic petri dishes (Dow Corning, Wiesbaden, Germany). In one group, the plastic sheets were immersed in plastic petri dishes containing 19 mL of TPY and 1 mL of bacterial suspension, as mentioned above. The plastic sheets were then incubated in an anaerobic chamber at 37°C for 2 hours, 4 hours, 6 hours, 12 hours, 20 hours, and 24 hours. For the other group, 19 mL of TPY and 1 mL of bacterial suspension were placed directly in plastic petri dishes without the plastic sheets and incubated in an anaerobic chamber at 37°C for 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, and 20 hours.

Colony forming units assay

Biofilms were washed twice with 0.01M phosphate buffered saline (PBS, pH 7.4) at 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 20 hours, and 24 hours to remove excess medium and unattached cells. Then, 100 μL of PBS was added to each dish, and biofilm cells were scraped off the surface of the polystyrene petri dishes using a sterile cell scraper. The detached biofilm cells were serially diluted 10-fold and inoculated on TPY agar at 37°C for 24 hours in triplicate.

Colony-forming units (CFUs) were calculated by the method described by Miles et al.[21] Briefly, plates with >30 but <300 colonies were chosen for counting, and the CFU/mL was calculated as follows: CFU/mL = (number of colonies × dilution factor)/volume of culture plate. Meanwhile, the population doubling time was calculated on the basis of the following formula: $\ln Z - \ln Z_0 = k(t - t_0)$.[22] Each independent assay was performed in triplicate.

CLSM analysis of biofilms

CLSM observation was performed as follows: First, the biofilms were stained with the L-7012 Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. The bacterial viability kit contains SYTO 9 and propidium iodide, each of which was thoroughly mixed with distilled water at a ratio of 1.5:1000. Viable bacterial cells were stained with green fluorescence (SYTO 9), while bacterial cells with damaged membranes were stained with red fluorescence (propidium iodide). The biofilms were washed twice with 0.01M PBS at 2 hours, 4 hours, 6 hours, 12 hours, and 20 hours to remove excess medium and unattached cells. Each biofilm was immersed in 200 μL of the above staining solutions, incubated in the dark at room temperature for 15 minutes, and rinsed with 200 μL PBS. Second, P-phenylenediamine and PBS were added on top of the biofilm in succession and covered with a coverslip. Third, biofilms were examined using a CLSM (Zeiss LSM 510; Carl Zeiss Microscopy, Jena, Germany) equipped with argon-ion and helium–neon lasers set to emit at 488 nm and 543 nm, respectively. In each experiment, the same settings of exciting laser intensity, background level, contrast, and electronic zoom size were maintained. A series of optical cross-section images were acquired at 1-μm intervals from the surface of the biofilm to the polystyrene surface using a computer-controlled motor drive. The total average area covered by bacterial cells on the surface was obtained from at least four different images of the same sample. Each biofilm was scanned from the outside (the side in direct contact with the planktonic bacteria) to the inside (the side in contact with the polystyrene sheets). Three series were generated following optical sectioning at each of these positions. Finally, image stacks were analyzed using the Java-based image analysis program Image J (version 1.38; National Institutes of Health, Bethesda, MD, USA). Image J was used to count the green and red areas of outer (the topmost surface of the biofilm that is in direct contact with planktonic bacteria), medium (the midpoint of all scanned layers), and inner (the side in direct contact with the polystyrene sheets) layers of the biofilm. The area occupied by live and dead bacteria in each layer indicates the fraction (percentage) of the area occupied by either component in each image of a stack and provides the vertical distribution of each of the biofilm components. The three-dimensional architecture of the biofilm was visualized using Zeiss LSM software. Further, the thickness and architecture of the biofilm were determined from the vertical (xz) sections. The percentage of viable microorganisms was calculated using the following formula: green areas/(red areas + green areas) × 100%. The assay was performed in triplicate on three separate occasions.

SEM analysis of biofilms

The biofilms were removed from the plates after 2 hours, 4 hours, 6 hours, 12 hours, and 20 hours; washed twice with PBS; and placed in 1% osmium tetroxide for 1 hour. Samples were then washed in distilled water, dehydrated in increasing concentrations of ethanol, dried in a desiccator, sputter-coated with gold, and examined using SEM (XL30ESM; FEI Co., Amsterdam, the Netherlands). Each specimen was divided into quadrants. One field of vision was randomly selected from each quadrant. Each independent assay was carried out in triplicate.

Extraction and analysis of biofilm surface-associated proteins

Biofilm growth was ceased at 20 hours for protein extraction.[23] Cells that did not adhere to the inner surface of polystyrene petri dishes were removed by immersing polystyrene petri dish surfaces in 25 mL of wash medium. The rinsing procedure was repeated three times with fresh medium to remove all nonadherent cells. After washing the inner surfaces of polystyrene petri dishes, a cell scraper was used to gently scrape the adherent cells (biofilm cells) off the bottom of the polystyrene petri dishes into a microcentrifuge tube. The cells were centrifuged at 4000g (20°C, 5 minutes), then suspended in fresh TPY to an approximate OD_{630} of 1.0, and centrifuged again at 4000g (20°C, 5 minutes); the supernatant was discarded, and the surface-associated proteins were extracted from the pelleted cells according to the method of Wilkins et al.[24] Cell pellets were washed twice in PBS and suspended in PBS with 0.2% (w/v) N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent; Sigma-Aldrich, Chicago, IL, USA). The suspensions were incubated at 25°C with shaking at 50 rpm for 1 hour, and the cells were pelleted at 13,000g (20°C, 10 minutes). The supernatant containing the extracted cell surface proteins was diluted 1:4 with 50mM Tris-HCl (pH 7.5), passed through a filter (0.22 μm pore-size Acrodisc syringe filter) to remove remaining cells, dialyzed until the liquid in the dialysis bag became clear, and then four volumes of ice-cold acetone were added. Samples were stored overnight at −20°C. The cell surface-associated proteins were collected by centrifugation at 12,000g (4°C, 20 minutes) and subjected to 2-DE, and then some spots in 2-DE gel, corresponding to proteins of interest, were identified by MALDI-TOF-MS. Image analysis and protein identification were performed following the method described by Wilkins et al.[24]

Statistical analysis

Data were analyzed using repeated-measures analysis of variance (ANOVA) (time-dependent data) and two-sample t test. All analyses were conducted using the SPSS software (SPSS 13.0 for Windows; SPSS, Chicago, IL, USA). The level of significance was defined as P = 0.05. There were six samples in each experiment on three separate occasions.

Results

Growth of SM 593 and SM 18 in planktonic bacteria

The mean OD values of SM 593 and SM 18 were similar and followed the same trend (Fig. 1). Specifically, there was a steady increase in the mean OD value (from 0 to 0.2) from 0 hour to 2 hours, followed by a dramatic rise in the mean OD value (from 0.3 to 0.8) between 3 hours and 5 hours. The OD values plateaued at approximately 1.0 (approximately 10^8 cells) at 6 hours. Therefore, the 6 hours of cultivation of SM 593 and SM 18 were chosen to conduct further experiments to assess biofilm formation. There were no significant differences between the mean OD values between SM 593 and SM 18 at the various times (P > 0.05).

Growth of SM 593 and SM 18 biofilms

The mean CFUs in biofilms of SM 593 and SM 18 between 2 hours and 24 hours are shown in Fig. 2. The mean CFUs of SM 593 and SM 18 increased sharply from 2 hours to 12 hours, followed by slow growth until 20 hours, and plateaued between 20 hours and 24 hours. Moreover, the mean CFUs of biofilms formed by SM 593 were always greater than those of SM 18 at each time point (P < 0.05). In addition, the SM 593 biofilm doubling time (122 ± 6 minutes) was significantly lower than that of the SM 18 biofilm (151 ± 11 minutes) (P < 0.05).

Thickness of biofilms

Thickness of the biofilms formed by SM 593 and SM 18 increased with time. After a rapid growth from 2 hours to 12 hours, their thickness increased slightly until 16 hours and was then stabilized up to 20 hours. More important, a significant difference in thickness was observed between the biofilms formed by SM 593 and SM 18 at each time (P < 0.05) (Fig. 3).

CLSM analysis of biofilms

SM 593 and SM 18 biofilms were mainly composed of viable bacteria from 2 hours to 20 hours (Fig. 4). Within the initial 2 hours, only a few scattered bacteria were observed,
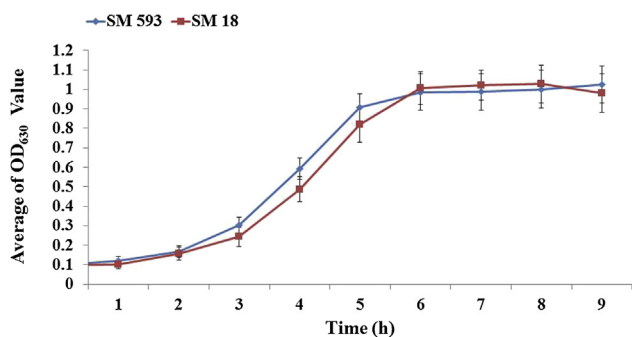

Figure 1 Growth rates (OD values) of planktonic SM 593 and SM 18 incubated at pH 7.0 and 37°C. Bars indicate standard deviations. The data represent the average of triplicate independent essays. OD = optical density.
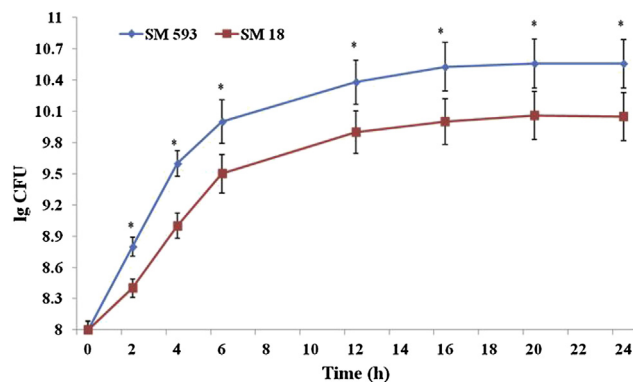

Figure 2 Growth rates of SM 593 and SM 18 in biofilms. The graph shows the mean CFU counts of SM 593 and SM 18 biofilm cells from 2 hours to 24 hours. Bars indicate standard deviations. The data represent the average of triplicate independent essays. * Significant difference between SM 593 and SM 18 at P < 0.05. CFU = colony-forming unit.

whereas by 6 hours, the bacteria fused to form a tangle of flocculation agglomerates. From 12 hours to 20 hours, numerous viable bacteria emerged, forming a network-like structure. However, compared with those in the SM 18 biofilm, the number of living bacterial cells was higher and the structure was denser in the SM 593 biofilm at each time between 2 hours and 20 hours.

Three-dimensional spatial structure of biofilms at 20 hours

A three-dimensional reconstruction was generated by biofilms at 20 hours. Both the SM 593 and the SM 18 biofilm had open and heterogeneous structures. Nevertheless, the SM 593 biofilm was thicker and gully shaped, compared with the flat SM 18 biofilm (Fig. 5).

Analysis of viable bacteria in biofilms at 20 hours

The biofilms contained an uneven distribution of viable and dead bacteria, with the ratios of viable to dead
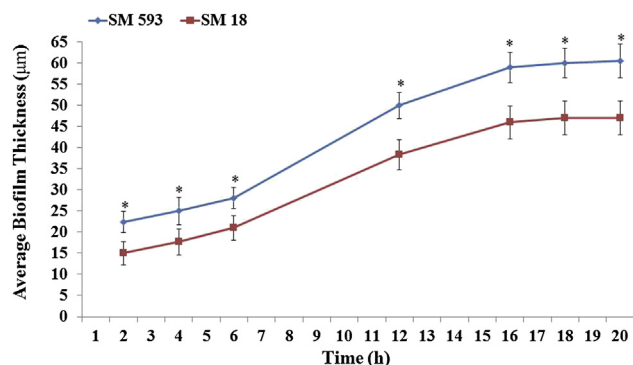

Figure 3 Average thickness of SM 593 and SM 18 biofilms from 2 hours to 20 hours, determined using CLSM. Bars indicate standard deviations. The data represent the average of triplicate independent essays. * Significant difference between SM 593 and SM 18 at P < 0.05. CLSM = confocal laser scanning microscopy.
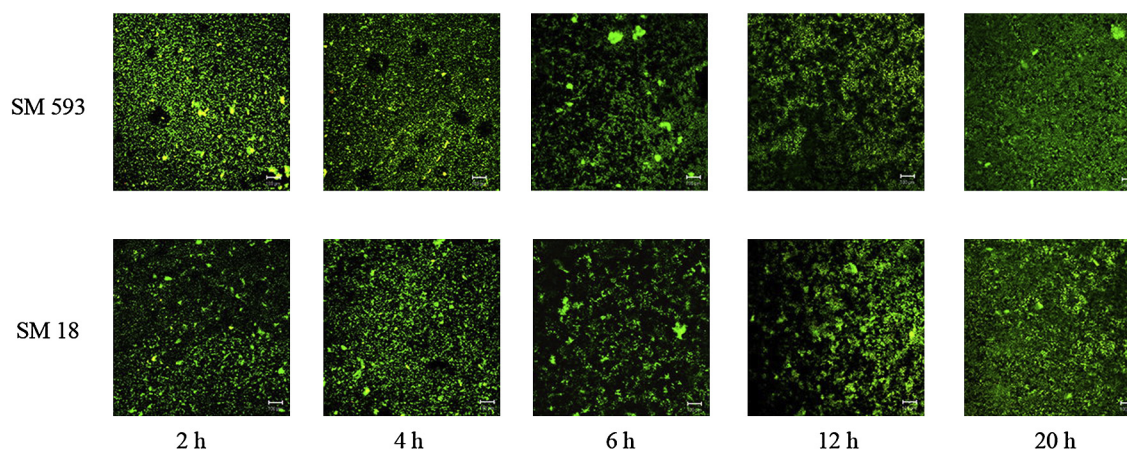
Figure 4 CLSM analysis of SM 593 and SM 18 biofilms. Time series of CLSM images of development of SM 593 and SM 18 biofilms stained with live/dead BacLight fluorescent stains (scale bars, 2 μm; original magnification 100×). The images are representative of three independent experiments. CLSM = confocal laser scanning microscopy.

bacteria changing within the different layers. At 20 hours, the percentage of viable microorganisms in SM 593 and SM 18 biofilms increased gradually from the inner to the outer layer. Further, the differences among several layers were statistically significant (P < 0.05). The number of viable bacteria in the outer layer was greater than that in the inner layer, and the percentage of viable bacteria in the SM 593 biofilm was significantly higher than that in the SM 18 biofilm at each corresponding layer (P < 0.05) (Fig. 6).

SEM analysis of biofilms

After 2 hours of culture, the bacteria that attached to the polystyrene sheets formed long chains with little overlap. At 6 hours, the bacteria aggregated and began forming a biofilm, which was completed by 20 hours. However, compared with SM 18, the number of long chains formed by SM 593 increased at 2 hours with more overlap. By 20 hours, the structure of the SM 593 biofilm was denser than that of the SM 18 biofilm. Further, increased extracellular polysaccharides were observed surrounding the bacteria in the SM 593 biofilm (Fig. 7).

Two-dimensional gel electrophoresis analysis of surface-associated proteins of SM 593 and SM 18 biofilms

The surface-associated proteins extracted from biofilms formed by SM 593 and SM 18 were separated using 2-DE. There were approximately 727 and 575 distinct spots on the 2-DE gels of SM 593 and SM 18, respectively, which were mainly distributed in the isoelectric point (p*I*) range of 4.0−7.0 with relative molecular masses ranging from 10 kDa to 100 kDa (Fig. 8).

Differences between surface-associated proteins present in biofilms formed by SM 593 and SM 18

Two hundred and twenty-nine proteins in the mass and p*I* ranges described above were differentially expressed

(increases or decreases of a factor of 1.3) when the biofilms formed by SM 593 to SM 18 were compared. Nine and five proteins were specifically detected in SM 593 and SM 18 biofilms, respectively. Using MALDI-TOF, we determined the identities of the unique proteins and proteins of interest expressed at levels that differed between the two biofilms by a factor of at least 3. A hydrolase of the MutT family, purine-nucleoside phosphorylase, DNA repair protein RecO, pantothenate kinase, and heat shock protein DnaJ (HSP40) were present only in the SM 593 biofilm, and 6-pyruvoyl tetrahydropterin synthase, phosphoribosylglycinamide formyltransferase (GART), ribose 5-phosphate isomerase A, and ABC transporter (ATP-binding protein, MsmK-like protein) were present only in the SM 18 biofilm (Table 1). The expression of D-alanyl-D-alanine carboxypeptidase, glycerol-3-phosphate dehydrogenase, and histidyl-tRNA synthetase (histidine-tRNA ligase) was greatly increased in the SM 593 biofilms, and the expression of the transcriptional regulator, response regulator homolog of RumR and ScnR, tagatose 1,6-aldolase, glycerol-3-phosphate dehydrogenase, oxidoreductase, and isopentenyl pyrophosphate isomerase was greatly increased in the SM 18 biofilms (Table 2).

Discussion

In nature, most bacteria form biofilms that adhere to the surfaces of living organisms and inanimate objects.[25] Microbial biofilms can be created by several techniques *in vitro*.[26] The multispecies biofilm model offers opportunities to explore the inter-relationship among the various species of oral bacterial communities, while analysis of the monospecies model contributes significantly to the accurate identification of genes and specific proteins associated with biofilm formation. In the present study, we established an *S. mutans* monospecies biofilm model *in vitro*. Consequently, biofilm formation and surface-associated protein expression of *S. mutants* strains SM 593 and SM 18 were compared.

CLSM and SEM observations revealed that biofilm formation by SM 593 and SM 18 was dynamic and could be
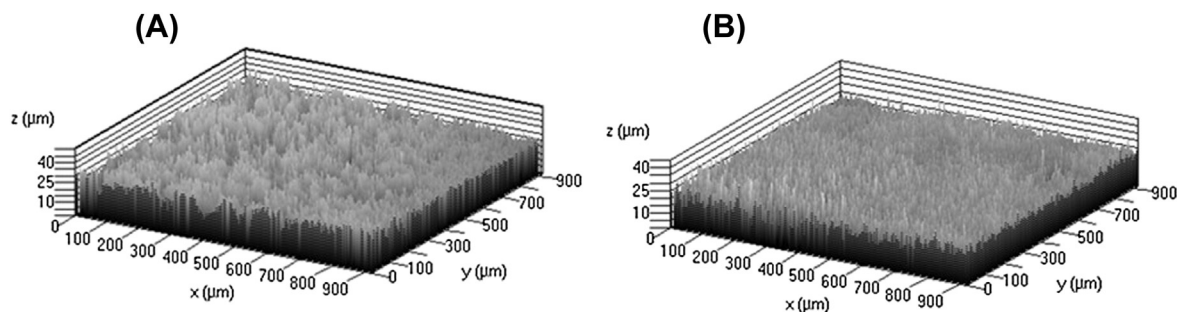
Figure 5   Three-dimensional structures of 20-hour (A) SM 593 and (B) SM 18 biofilms, reconstructed with Zeiss LSM software.

classified into three stages as follows: In the first stage, a few single bacteria adhere to the surface (0—6 hours). In the second stage, the primary colonizers proliferate (6—12 hours). The biofilm matures during the final stage (12—20 hours). The structure of the biofilms formed by SM 593 and SM 18 were typically three dimensional, heterogeneous, and dispersed, and vary in morphology.

Image analyses of biofilms demonstrate that bacterial aggregates are separated by fluid-filled channels that facilitate oxygen transport throughout the biofilm.[27,28] During investigation of the distribution of dead and viable bacteria during biofilm formation, CLSM and fluorescent staining techniques revealed an uneven spatial distribution, with an escalating percentage of viable cells located toward the outer layer, which is consistent with the results of another study.[29] The following biofilm features were observed in both SM 593 and SM 18: In the outer layer of the biofilm, bacteria enjoy ready access to nutrients, and toxic metabolic products are removed more efficiently. Conversely, bacteria in the inner layer live under conditions of inadequate nutrition, which inhibits their proliferation. Further, relatively more metabolites are accumulated, leading to cytotoxicity, within the inner layer of the biofilm. In contrast, bacteria residing on the top layers of dental plaque are directly exposed to adverse stimuli from the oral environment. Therefore, the present study conducted *in vitro* may not completely represent the clinical situation.

Although biofilm formation by SM 593 and SM 18 was similar as described above, there were still the following significant differences: First, the mean number of CFUs of the SM 593 biofilms was always higher compared with those of the SM 18 biofilms. This may be because of the stronger adherence of SM 593 to the matrix.[30] Second, SM 593 biofilms contained more cells and were denser, compared with SM 18 biofilms, from 2 hours to 20 hours. These properties may enhance the adaptation of SM 593 to the environment. Third, in all three stages of biofilm formation, SM 593 biofilms grew faster, which may be due to their lower population doubling time. Moreover, the percentage of viable cells in the biofilms was significantly greater at each corresponding layer. Finally, the biofilms of SM 593 were always thicker, as the SM 593 biofilm contained more living and dead bacteria, as well as higher amounts of exopolysaccharides,[31] which are a key component of the biofilm matrix.[32]

The behavior of bacteria in biofilms depends on the number of viable cells, as well as on the structure and thickness of the biofilm.[32,33] The thickness of a mature biofilm plays a key role in bacterial survival and is of pivotal importance during immune evasion and induction of resistance to antibacterial agents.[28] The superior ability of SM 593 to form biofilms is consistent with the significant differences in cariogenicity between planktonic SM 593 and SM 18 in our previous work.[19,30,34]

To further define the differences between *S. mutans* biofilms, surface-associated proteins that may influence biofilm formation were extracted from 20-hour biofilm cells. Approximately 727 and 575 protein spots were detected in 20-hour extracts of SM 593 and SM 18 biofilms, respectively. We identified proteins uniquely expressed by either SM 593 or SM 18, and proteins expressed by SM 593 or SM 18 whose levels differed by at least a factor of 3. We found that hydrolase (MutT family), purine-nucleoside phosphorylase, DNA repair protein RecO, and putative pantothenate kinase were expressed only by SM 593, and histidyl-tRNA synthetase (histidine-tRNA ligase) was expressed at higher levels by SM 593 (20-hour biofilm cells). Phosphoribosylglycinamide synthetase, formyltransferase (GART) and ribose 5-phosphate isomerase A were expressed
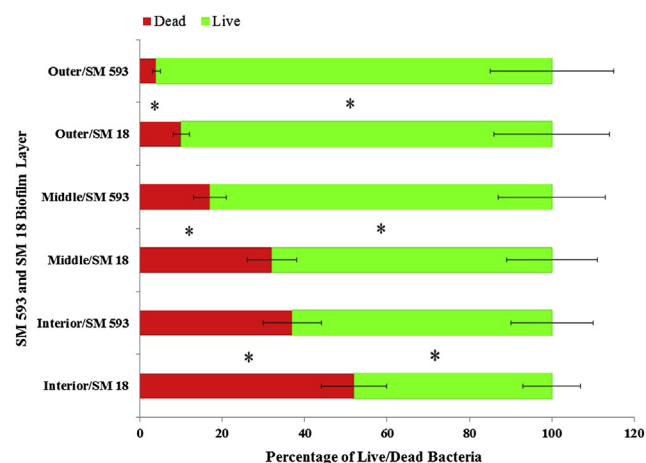

Figure 6   Percentage of viable (green) and dead (red) bacteria in different (interior, middle, and outer) layers of 20-hour SM 593 and SM 18 biofilms. The images of biofilms were analyzed using Image J to count the red and green areas of interior, middle, and outer layers of the biofilms. Bars indicate standard deviations. The data represent the average of triplicate independent experiments. * Significant difference between SM 593 and SM 18 at P < 0.05.
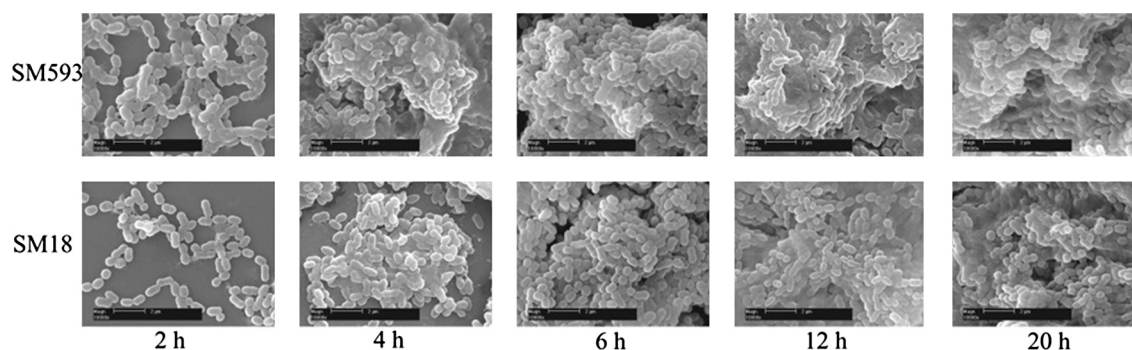
Figure 7   Time series SEM images of SM 593 and SM 18 biofilms formed on surfaces of polystyrene sheets (scale bars, 2 μm; original magnification 10,000×). The data represent three independent experiments. SEM = scanning electron microscopy.

only by SM 18 (20 hours). These proteins are all involved in nucleic acid metabolism, DNA and RNA synthesis.[35,36] The findings are partially consistent with the results of Rathsam et al[37,38] that competence-related proteins, such as DNA-processing protein, in *S. mutans* biofilms were upregulated.

Proteins always exist as an unfolded polypeptide or random coil when translated.[39] Therefore, heat shock protein DnaJ (HSP40), which was expressed only in SM 593 biofilm cells and is involved in protein fate (folding, modification, and destination), could effectively fold into its characteristic and functional three-dimensional structure from a random coil to function as a DnaK-dependent chaperone, which is critical for biofilm formation by providing adequate levels of the required proteins.[40] Moreover, lack of expression of this protein by SM 18 may adversely affect biofilm development.

D-alanyl-D-alanine carboxypeptidase is required during the final steps of peptidoglycan synthesis and for biofilm formation by *S. mutans*.[41] This enzyme was expressed at 6.5 times higher levels by SM 593, indicating that it enhanced biofilm formation. Downregulation of D-alanyl-D-alanine carboxypeptidase by SM 18 would slow biofilm formation. The 6.2-fold enhanced expression of glycerol-3-phosphate dehydrogenase in the SM 593 biofilms might impart greater stability to the biofilm by maintaining the cells in the stationary phase of growth.[42]

Interestingly, oxidoreductase and isopentenyl pyrophosphate isomerase, which function in intermediary metabolism, were upregulated in SM 18 biofilm cells by at least four-fold in contrast to SM 593. This may explain the enhanced formation of biofilms by SM 593. The decreased levels of intermediary metabolism in SM 593 biofilm cells
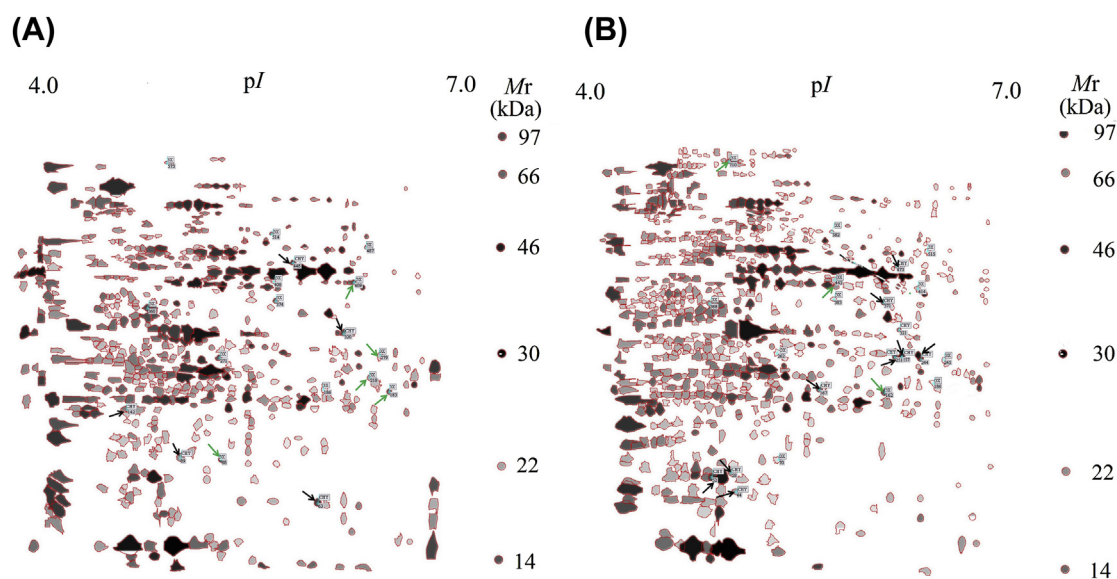

Figure 8   Two-dimensional gel electrophoresis protein profiles of 20-hour SM 593 and SM 18 biofilm cells. Extracted proteins were separated by isoelectric focusing in the pH range of 4—7 in the first dimension and a gradient (12—14%) SDS-PAGE in the second dimension. (A) Protein expression by SM 593. (B) Protein expression by SM 18. Proteins indicated by "CHY" were expressed by either SM 593 biofilm cells or SM 18 biofilm cells. The proteins indicated with "3X" were enhanced or diminished by more than three-fold in SM 18 biofilm cells or SM 593 compared with each other. The gels represent three independent experiments. "CHY" spots of interest: black arrow label, "3X" spots of interest: green arrow label. SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1 Identification of proteins expressed only in 20-hour SM 593 or SM 18 biofilm cells.

Protein function	Spot no.	Mr (Da)	pI	Gene ID	Protein
Only expressed in SM 593 biofilm cells					
Nucleic acid metabolism	58	19,850	4.92	SMU.0561	Putative hydrolase (MutT family)
	167	26,976	5.63	SMU.2126	Putative purine-nucleoside phosphorylase
	251	29,354	6.20	SMU.0025	Putative DNA repair protein RecO
	371	35,738	6.12	SMU.1126	Putative pantothenate kinase
Protein fate	473	40,858	6.28	SMU.0083	Heat shock protein DnaJ (HSP40)
Unknown	44	18,911	5.05	SMU.0984	Hypothetical protein
	52	19,746	4.85	SMU.0393	Hypothetical protein
	244	29,098	6.42	SMU.0725	Hypothetical protein
	257	31,512	6.25	SMU.1428	Hypothetical protein
Only expressed in SM 18 biofilm cells					
Transport proteins	63	17,711	6.16	SMU.0917	6-Pyruvoyl tetrahydropterin synthase
	448	42,325	6.05	SMU.1571	ABC transporter, ATP-binding protein, MsmK-like protein
Nucleic acid metabolism	89	20,640	5.22	SMU.0035	Phosphoribosylglycinamide formyltransferase (GART)
	142	24,568	4.74	SMU.1234	Ribose 5-phosphate isomerase A
Unknown	320	32,859.81	6.43	SMU.2099	Conserved hypothetical protein

Spot number refers to 2-DE gels in Fig. 8.
Mr = relative molecular mass; 2-DE = two-dimensional gel electrophoresis.

might allow SM 593 to readily adapt to an experimental environment that limits bacterial growth.[43]

The ABC transporter (ATP-binding protein, MsmK-like protein) and 6-pyruvoyl-tetrahydropterin synthase were detected only in SM 18 biofilm cells. ABC transporters mediate the flux of essential substances such as carbohydrate, amino acids, proteins, lipids, inorganic ions, and complex molecules required for biofilm formation by SM 18.[44] Sugar transport is also mediated by 6-pyruvoyl-tetrahydropterin synthase.[45] These two proteins may likely compensate for protein functions absent in the SM 18 biofilms. In contrast, other enzymes involved in intermediary metabolism, including hydrolase (MutT family), purine-nucleoside phosphorylase, DNA repair protein RecO, putative pantothenate kinase, glycerol-3-phosphate dehydrogenase, and histidyl-tRNA synthetase (histidine-tRNA ligase) were expressed uniquely or at enhanced levels by SM 593.[36,42] This may explain why SM 593 formed thicker biofilms more rapidly. Thus, it was speculated that SM 593 has the intrinsic ability to produce substances required for biofilm formation, whereas biofilm formation by SM 18 requires exogenous supplementation.

Response regulator homolog of RumR and ScnR was elevated 5.3-fold in the SM 18 biofilm than in the SM 593 biofilm. However, biofilm formation capacity of SM 18 was much lower than that of SM 593, suggesting that response regulator homolog of RumR and ScnR was not a key regulator of *S. mutans* biofilm formation. Similarly, it was reported in a previous study that inactivation of scnR in *S. mutans* caused no change in biofilm formation.[46]

Transcription factors are a set of sophisticated proteins that could promote and regulate diverse metabolic

Table 2 Identification of proteins enhanced in 20-hour SM 593 or SM 18 biofilm cells.

Protein function	Spot no.	Mr (Da)	pI	Gene ID	Protein	Enhanced (fold)
Enhanced in SM 593 biofilm cells						
Cellular processes	162	27,845	6.10	SMU.0075	D-alanyl-D-alanine carboxypeptidase	6.5
Lipid metabolism	443	37,312	5.74	SMU.0323	Glycerol-3-phosphate dehydrogenase	6.2
Nucleic acid metabolism	710	48,896	5.70	SMU.2102	Histidyl-tRNA synthetase (histidine-tRNA ligase)	3.0
Enhanced in SM 18 biofilm cells						
Regulatory functions	70	21,853	5.35	SMU.1282	Transcriptional regulator	5.9
	183	26,244	6.65	SMU.1146	Response regulator homolog of RumR and ScnR	5.3
Intermediary metabolism	218	26,752	6.31	SMU.0374	Oxidoreductase	4.7
	409	37,309	6.37	SMU.0939	Isopentenyl pyrophosphate isomerase	4.1
Unknown	279	30,537	6.54	SMU.1108	Conserved hypothetical protein	3.3

Spot number refers to 2-DE gels in Fig. 8.
Mr = relative molecular mass; 2-DE = two-dimensional gel electrophoresis.

processes.[47,48] In addition, different transcriptional regulators play different roles in biofilm formation.[49] Therefore, the function of the transcriptional regulator (spot #70), 5.9-fold upregulated expression in SM 18, in the biofilm formation process is still unclear, and more investigations are needed to analyze the subgroup of this protein spot #70.

Furthermore, apart from the above-identified proteins, in accordance with the previous studies,[38,50] several conserved hypothetical proteins with unknown functions and biological roles were altered or uniquely expressed in the present study. Therefore, there is abundant room for further progress in S. mutans biofilm proteome study.

Surprisingly, in contrast to previous studies reporting that glucosyltransferase and glucan-binding proteins were upregulated by S. mutans biofilm,[38,51,52] the expression of these two proteins were not elevated in this comparative proteome analysis study. This discrepancy may attribute to these two proteins being essential for S. mutans biofilm formation,[53,54] playing the same role in both SM 593 and SM 18 biofilms.

In conclusion, two clinical isolates of S. mutans, SM 593 and SM 18, from caries-active and caries-free individuals, respectively, exhibited significant differences in their abilities to form biofilms and in their profiles of protein expression. These findings may indicate that S. mutans strains from caries-active individuals are able to form more robust biofilms compared with those from caries-free individuals, which contributes to their higher cariogenicity. Proteins expressed differently by SM 593 and SM 18 present in biofilms are likely to be associated with biofilm development. Undoubtedly, the limitations of this study focus on S. mutans single species biofilm is obvious. Further research is required for understanding the physiology of multispecies biofilms formed by many other caries-related microbial species.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

References

1. Lewis K. Riddle of biofilm resistance. *Antimicrob Agents Chemother* 2001;45:999—1007.
2. Costerton JW, Cheng KJ, Geesey GG, et al. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 1987;41:435—64.
3. Fletcher M. The physiological activity of bacteria attached to solid surfaces. *Adv Microb Physiol* 1991;32:53—85.
4. Bowden GH, Hamilton IR. Survival of oral bacteria. *Crit Rev Oral Biol Med* 1998;9:54—85.
5. Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res* 2011;45:69—86.
6. Senadheera D, Cvitkovitch DG. Quorum sensing and biofilm formation by *Streptococcus mutans*. *Adv Exp Med Biol* 2008;631:178—88.
7. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 1980;44:331—84.
8. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986;50:353—80.
9. Alaluusua S, Kleemola-Kujala E, Nystrom M, Evalahti M, Gronroos L. Caries in the primary teeth and salivary *Streptococcus mutans* and lactobacillus levels as indicators of caries in permanent teeth. *Pediatr Dent* 1987;9:126—30.
10. Beighton D, Rippon HR, Thomas HE. The distribution of *Streptococcus mutans* serotypes and dental caries in a group of 5- to 8-year-old Hampshire schoolchildren. *Br Dent J* 1987;162:103—6.
11. Carlsson P, Olsson B, Bratthall D. The relationship between the bacterium *Streptococcus mutans* in the saliva and dental caries in children in Mozambique. *Arch Oral Biol* 1985;30:265—8.
12. Matee MI, Mikx FH, de Soet JS, Maselle SY, de Graaff J, van Palenstein Helderman WH. Mutans streptococci in caries-active and caries-free infants in Tanzania. *Oral Microbiol Immunol* 1993;8:322—4.
13. Saarela M, Hannula J, Mättö J, Asikainen S, Alaluusua S. Typing of mutans streptococci by arbitrarily primed polymerase chain reaction. *Arch Oral Biol* 1996;41:821—6.
14. Li Y, Caufield PW. Arbitrarily primed polymerase chain reaction fingerprinting for the genotypic identification of mutans streptococci from humans. *Oral Microbiol Immunol* 1998;13:17—22.
15. Alaluusua S, Mättö J, Grönroos L. Oral colonization by more than one clonal type of mutans streptococcus in children with nursing-bottle dental caries. *Arch Oral Biol* 1996;41:167—73.
16. Kreulen CM, de Soet HJ, Hogeveen R, Veerkamp JS. *Streptococcus mutans* in children using nursing bottles. *ASDC J Dent Child* 1997;64:107—11.
17. Wen ZT, Baker HV, Burne RA. Influence of BrpA on critical virulence attributes of *Streptococcus mutans*. *J Bacteriol* 2006;188:2983—92.
18. Lynch DJ, Fountain TL, Mazurkiewicz JE, Banas JA. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. *FEMS Microbiol Lett* 2007;268:158—65.
19. Huang XJ, Jiang S, Qiu M, Jiang YP. Protein expression by planktonic and biofilm cells of clinical isolations of *Streptococcus mutans*. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2009;44:739—44.
20. Burne RA, Chen YY, Penders JE. Analysis of gene expression in *Streptococcus mutans* in biofilms *in vitro*. *Adv Dent Res* 1997;11:100—9.
21. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. *J Hyg (Lond)* 1938;38:732—49.
22. Qi F, Merritt J, Lux R, Shi W. Inactivation of the ciaH Gene in *Streptococcus mutans* diminishes mutacin production and competence development, alters sucrose-dependent biofilm formation, and reduces stress tolerance. *Infect Immun* 2004;72:4895—9.
23. Huang XJ, Qiu M, Jiang YP. Preliminary analysis of differences in protein expression at early mature stages of *Streptococcus mutans* biofilm formation. *Chin J Stomatol Res (Electron Ver)* 2008;2:13—6.
24. Wilkins JC, Beighton D, Homer KA. Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*. *Appl Environ Microbiol* 2003;69:5290—6.

25. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol* 1995;49:711—45.

26. Coenye T, Nelis HJ. *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J Microbiol Methods* 2010;83:89—105.

27. de Beer D, Stoodley P, Roe F, Lewandowski Z. Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnol Bioeng* 1994;43:1131—8.

28. Wood SR, Kirkham J, Marsh PD, Shore RC, Nattress B, Robinson C. Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. *J Dent Res* 2000;79:21—7.

29. Hope CK, Clements D, Wilson M. Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling. *J Appl Microbiol* 2002;93:448—55.

30. Huang XJ, Liu TJ, Chen Z, Zhan L, Yang JB. Evaluation of cariogenic potential of *Streptococcus mutans* isolated from caries-free and -active persons: adherence properties to saliva-coated hydroxyapatite. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2000;18:416—8.

31. Huang XJ, Jiang S, Cai ZY, Yan FH, Zhong S. The difference of the ability to synthesize exopolysaccharides of clinical strains of biofilm *Streptococcus mutans*. *J Chongqing Med Univ* 2011;36:281—4.

32. Schue M, Fekete A, Ortet P, Brutesco C. Modulation of metabolism and switching to biofilm prevail over exopolysaccharide production in the response of *Rhizobium alamii* to cadmium. *PLoS One* 2011;6:e26771.

33. Thenmozhi R, Balaji K, Kumar R, Rao TS, Pandian SK. Characterization of biofilms in different clinical M serotypes of *Streptococcus pyogenes*. *J Basic Microbiol* 2011;51:196—204.

34. Huang XJ, Liu TJ, Yang JB, Chen Z, Liu JG. Evaluation of cariogenic potential of *Streptococcus mutans* isolated from caries-free and -active persons: abilities to synthesize water-soluble and -insoluble glucans. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2000;18:419—21.

35. Wolff E. The contribution of experimental embryology to medicine and surgery. *Bull Acad Natl Med* 1990;174:1337—42.

36. Montanaro L, Poggi A, Visai L, et al. Extracellular DNA in biofilms. *Int J Artif Organs* 2011;34:824—31.

37. Rathsam C, Eaton R, Simpson C, et al. Up-regulation of competence but not stress-responsive proteins accompanies an altered metabolic phenotype in *Streptococcus mutans* biofilms. *Microbiology* 2005;151:1823—37.

38. Rathsam C, Eaton RE, Simpson CL, et al. Two-dimensional fluorescence difference gel electrophoretic analysis of *Streptococcus mutans* biofilms. *J Proteome Res* 2005;4:2161—73.

39. Raghunathan G, Rein R. Structural requirements for a primitive adaptor molecule. *J Biomol Struct Dyn* 1987;4:1041—50.

40. Oli MW, Otoo HN, Crowley PJ, et al. Functional amyloid formation by *Streptococcus mutans*. *Microbiology* 2012;158:2903—16.

41. Craig EA, Gambill BD, Nelson RJ. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol Rev* 1993;57:402—14.

42. Spoering AL, Vulic M, Lewis K. GlpD and PlsB participate in persister cell formation in *Escherichia coli*. *J Bacteriol* 2006;188:5136—44.

43. Shemesh M, Tam A, Steinberg D. Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. *Microbiology* 2007;153:1307—17.

44. Zhu X, Long F, Chen Y, Knøchel S, She Q, Shi X. A putative ABC transporter is involved in negative regulation of biofilm formation by *Listeria monocytogenes*. *Appl Environ Microbiol* 2008;74:7675—83.

45. Ajdic D, Chen Z. A novel phosphotransferase system of *Streptococcus mutans* is responsible for transport of carbohydrates with α-1,3 linkage. *Mol Oral Microbiol* 2013;28:114—28.

46. Kim JN, Stanhope MJ, Burne RA. Core-gene-encoded peptide regulating virulence-associated traits in *Streptococcus mutans*. *J Bacteriol* 2013;195:2912—20.

47. Ajdić D, McShan WM, McLaughlin RE, et al. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A* 2002;99:14434—9.

48. Tropel D, van der Meer JR. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol Mol Biol Rev* 2004;68:474—500.

49. Wen ZT, Burne RA. Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. *Appl Environ Microbiol* ;68:1196—203.

50. Khan AU, Islam B, Khan SN, Akram M. A proteomic approach for exploring biofilm in *Streptococcus mutans*. *Bioinformation* 2011;5:440—5.

51. Lévesque CM, Voronejskaia E, Huang YC, Mair RW, Ellen RP, Cvitkovitch DG. Involvement of sortase anchoring of cell wall proteins in biofilm formation by *Streptococcus mutans*. *Infect Immun* 2005;73:3773—7.

52. Liao S, Klein MI, Heim KP, et al. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. *J Bacteriol* 2014;196:2355—66.

53. Tsumori H, Kuramitsu H. The role of *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the GtfC enzyme. *Oral Microbiol Immunol* 1997;12:274—80.

54. Sato Y, Senpuku H, Okamoto K, Hanada N, Kizaki H. *Streptococcus mutans* binding to solid phase dextran mediated by the glucan-binding protein C. *Oral Microbiol Immunol* 2002;17:252—6.