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Biofilm Formation of *Streptococcus equi* ssp. *zooepidemicus* and Comparative Proteomic Analysis of Biofilm and Planktonic Cells

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Abstract *Streptococcus equi* ssp. *zooepidemicus* (SEZ) is responsible for a wide variety of infections in many species, including pigs, horses and humans. Biofilm formation is essential for pathogenesis, and the ability to resist antibiotic treatment results in difficult-to-treat and persistent infections. However, the ability of SEZ to form biofilms is unclear. Furthermore, the mechanisms underlying SEZ biofilm formation and their attributes are poorly understood. In this study, scanning electron microscopy (SEM) demonstrated that SEZ strain ATCC35246 formed biofilms comprising a thick, heterogeneous layer with clumps on the coverslips when incubated for 24 h. In addition, we used a two-dimensional gel electrophoresis (2-DE) based approach to characterize differentially expressed protein in SEZ biofilms compared with their planktonic counterparts. The results revealed the existence of 24 protein spots of varying intensities, 13 of which were upregulated and 11 were downregulated in the SEZ biofilm compared with the planktonic controls. Most of proteins expressed during biofilm formation were associated with metabolism, adhesion, and stress conditions. These observations contribute to our understanding of the SEZ biofilm lifestyle, which may lead to more effective measures to control persistent SEZ infections.

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Introduction

Streptococcus equi ssp. *zooepidemicus* (SEZ) is responsible for a wide variety of infections in many species, including pigs, horses and humans [10, 16]. In China, SEZ is the predominant disease causing pathogen in swine. The 1975 pandemic in Sichuan led to the death of 300,000 pigs, resulting in large economic losses [12]. The disease is generally sporadic and outbreaks are usually of short duration; however, in large populations the bacteria may be present for longer periods [5, 24, 27, 30]. Why this bacterium causes persistent infection and disease under certain conditions remains largely unknown.

A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface to form tower- and mushroom-shaped microcolonies [7]. Formation of biofilms allows microbial pathogens to create a protected environment in which sessile cells are more resistant to antibiotics compared to their planktonic counterparts. Many animal-infecting bacteria, such as *Streptococcus suis* (SS) [17] and *Pseudomonas aeruginosa* [1], have the ability to form biofilms, which plays a key role in causing persistent infections. Recent developments in proteomic technologies have paved the way for exploration of the molecular mechanisms of natural phenomena such as biofilm formation.

The ability of SEZ to form biofilms and the relationship between biofilm formation and pathogenesis or persistent SEZ infections is unclear. In this study, the ability of SEZ to form biofilms was investigated, and the differences in the protein expression profiles of SEZ grown either as planktonic cells or biofilms were identified using comparative proteomic analysis, with the aim of gaining a better understanding of SEZ biofilm formation. This study is the first examination of biofilm formation in SEZ.

Materials and Methods

Bacteria and Culture Condition

Bacterial strain SEZ ATCC35246 was originally isolated from an infected pig in Sichuan and was purchased from American Type Culture Center. It was grown at 37 °C in Todd–Hewitt broth (THB).

Microtiter Plate Biofilm Assay

Quantification of biofilm production was carried out by a microtiter plate assay [34] with some modifications. The wells of sterile 96-well flat-bottomed polystyrene microplates (Greiner, Germany) were filled with 100 µl of THB medium containing 1 % fibrinogen and, then, 10 µl of overnight inoculum was added to each well and incubated at 37 °C for 24 h without shaking. Medium alone served as a negative control. The sample was visualized by staining with 1 % crystal violet for 10 min after washing with PBS. After adding 200 µl of 95 % ethanol, $A_{595\ \mathrm{nm}}$ was measured with a Tecan GENios Plus microplate reader (Tecan, Austria). All assays were performed in triplicate.

Scanning Electron Microscopy

Overnight cultures of strains were diluted to a final density of 1.0×10^6 CFU ml^{-1} with fresh THB medium containing 1 % fibrinogen. Scanning electron microscopy (SEM) was performed on biofilms formed on glass coverslips by dispensing 2 ml of cell suspensions into the wells of six-well microtiter plates. Plates were statically incubated at 37 °C for 24 h. The coverslips were then washed with PBS. The cells were fixed with 25 % SEM grade glutaraldehyde in PBS. Fixed samples were then dehydrated through a graded series of ethanol concentrations, mounted, and sputter coated with gold–palladium. Dried samples were adhered to metal holders with double-sided tape and finally coated in an evaporator with gold and palladium. Observations were performed at 5 kV with a scanning electron microscope (model S800; Hitachi).

Preparation of Whole-Cell Proteins

For biofilm cultures, SEZ was grown in THB medium supplemented with 1 % fibrinogen in 100 mm polystyrene petri dishes at 37 °C for 24 h. The supernatant was then removed and the plates were washed twice with 50 mM Tris/HC1 (pH 7.5). Biofilms were detached by scraping, then resuspended in 50 mM Tris–HC1. The cells were pelleted by centrifugation at approximately $8{,}000 \times g$ for 5 min and washed with 50 mM Tris/HC1. SEZ planktonic cell was grown in 500 Erlenmeyer flasks containing 100 ml of the above culture medium at 37 °C for 24 h. Planktonic cells were pelleted and washed as described for the biofilm cultures above. Protein precipitations were performed as described previously by Wang et al. [35]. Protein samples were treated with a 2-D Clean-up Kit (GE Healthcare) to remove contaminants that interfere with electrophoresis. The protein content was determined using the PlusOne 2-D Quant Kit (GE Healthcare).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed using the immobiline/polyacrylamide system. Protein sample (200 µg) was absorbed onto a 13 cm immobilized pH gradient strips and isoelectric focusing (IEF) was performed in a Protein IEF Cell (GE Healthcare) using a stepwise increase of the voltage to 8,000 V. Before electrophoreses in the second dimension, strips were equilibrated for 2 × 15 min in equilibration buffer (6 M urea, 2 % SDS, 30 %glycerol, 0.05 M Tris–HCl, pH 8.8) containing 1 % DTT and 4 % iodoacetamide. SDS-PAGE was carried out vertically in an Ettan DALT II system (GE Healthcare) using 12 % polyacrylamide gels. Resolved proteins were routinely stained with Coomassie Brilliant Blue G-250 for protein identification purposes. Samples were run in triplicate. Gel comparison analysis was conducted using Image Master Platinum 5.0 software (GE Healthcare). An analysis set was created to find the spots with a difference (minimum 2-fold increase or decrease) in the biofilm samples compared with the planktonic samples.

Mass Spectrometry Analysis of Protein Spots and Database Searches

Spots identified as being differentially expressed were excised from the triplicate 2-D gels and sent to Nanjing Ji'ao BioTechnologies Co., Ltd. for tryptic in-gel digestion and matrix-assisted laser desorption ionization time-of-flight time-of-flight-mass spectrometry (MALDI-TOF/TOF-MS) or MALDI-TOF-MS analysis. The peptide mass fingerprinting (PMF) data were analyzed using the MASCOT server (http://www.matrixscience.com/). Protein

identification was assigned when the following criteria were met: presence of at least four matching peptides and sequence coverage was >15 %. The protein scores as well as the original PMF data, including extent of sequence coverage, number of peptides matched, and score of probability were used for acceptance of protein identifications.

Verification of Comparative Proteomics Results by Quantitative Real-Time PCR

Total RNA was isolated from SEZ grown as biofilms and planktonic cells for 24 h with using the E.Z.N.A bacterial RNA Kit (Omega, USA) according to the manufacturer's protocol. cDNAs were generated by reverse transcription using a PrimeScript RT-PCR Kit (TaKaRa).The mRNA levels were measured using a two-step relative qRT-PCR. A specific primer set was used to analyze *glmS*, *citE*, *gapdh*, *fabF*, and *16s rRNA* mRNA content (Table 1). 16s rRNA was used as internal control in qRT-PCR analysis. The relative change in gene expression was recorded as comparative CT ($2^{-\Delta\Delta CT}$) [23].

Results

Appearance of Biofilm In Vitro

The ability to form biofilms was investigated for SEZ strain ATCC35246 using a crystal violet microtiter plate assay. The results revealed that strain ATCC35246 had the ability to form biofilms in microtiter plates (Fig. 1). The strain showed a strong biofilm with a mean ± SD optical density at 595 nm (OD_{595}) of 1.19 ± 0.05.

The structure of SEZ biofilms on glass coverslips was examined by SEM. SEM observations revealed that strain ATCC35246 formed a thick, heterogeneous layer with clumps on the coverslips after incubation for 24 h. The aggregates and microcolonies of SEZ were patchy with confluent areas of growth contrasting with cell-free areas (Fig. 2).

Comparative Proteomics

For further characterization of differences in protein expression between the biofilm and the planktonic cells, representative 2-DE of whole-cell protein extracts was performed. The reproducibility of separation of the total proteins was the same in all triplicate gels (data not shown). The majority of proteins were distributed in the pH range of 4–7 (IPG linear gradient) (Fig. 3a, b).

By matching and comparing the 2-DE proteomes, 24 protein spots were identified with a minimum 2-fold change in expression level. These 24 spots corresponded to

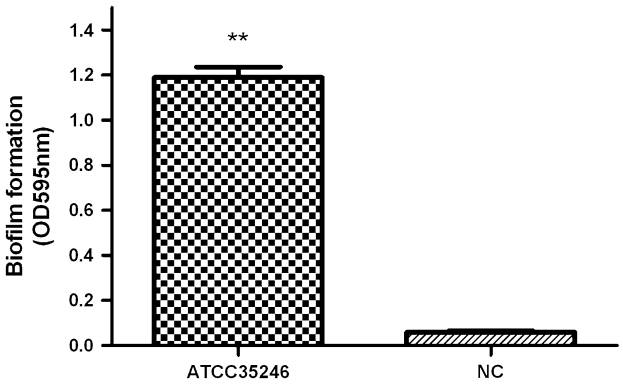

Fig. 1 Quantitative microtiter plate assay for biofilm production. SEZ strain was cultured in THB medium supplemented with 1 % fibrinogen. Negative control (NC) wells contained medium. The *asterisks* showed significant difference ($p < 0.01$)

Table 1 Primers used for qRT-PCR

Name	Primers sequence (5′-3′)	Gene	Protein
glmS-F	TACACTATGCTGCTCCATGCAGGT	glmS	Glucosamine: fructose-6-phosphate aminotransferase
glmS-R	TCCTTCTTGCCATTAGCCTCACCA		
citE-F	TGGTGCTCTGGATATTGAGGCAGT	citE	Citrate lyase subunit beta
citE-R	TCGATAGCTGCCATCATGCGTGTA		
fabF-F	AAGCGCGTTAAGCCGATGACTCTA	fabF	3-oxoacyl-[acyl-carrier-protein] synthase II
fabF-R	CCTTGGGCATTCAGTTTCATGGCA		
Gapdh-F	TCGGTCGTATCGGTCGTCTT	gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gapdh-R	TGTGCTAACATCGCTGGATCTG		
16sRNA-F	TGTAACTGACGCTGAGGCTCGAAA	16sRNA	16S rRNA
16SRNA-R	AAAGGGCCTAACACCTAGCACTCA		

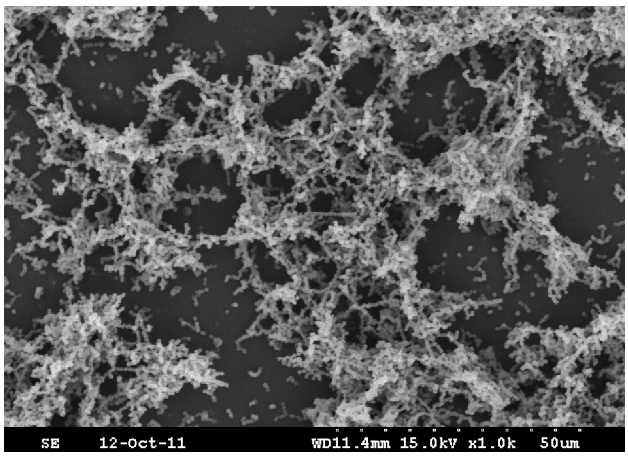

Fig. 2 Scanning electron micrographs of SEZ ATCC35246 biofilms formed on the glass coverslips after 24 h of cultivation at 37 °C

22 individual proteins, 13 of which were upregulated and 11 were downregulated in SEZ biofilms. The spots were identified by MALDI-TOF/TOF-MS. These data were compared to those in the NCBI sequence database. The probability score for the match, molecular weight (MW), isoelectric point (p*I*), number of peptide matches, and the percentage of the total translated ORF sequence covered by the peptides were used as confidence factors in protein identification (Tables 2, 3).

Quantitative Real-Time PCR Detection

For the analysis of gene expression in biofilm and planktonic cells, we followed the same procedure described for bacterial strain and culture condition. Quantitative RT-PCR analysis was performed on four selected genes to confirm the results of comparative proteomics analysis. The qRT-PCR results indicated that SEZ grown as biofilms expressed 2.1-fold higher *glmS* mRNA and *gapdh* mRNA ($P < 0.01$), 0.5-fold lower *citE* mRNA levels and 0.4-fold lower *fabF* mRNA levels ($P < 0.01$) than SEZ grown as planktonic cells (Fig. 4). The qRT-PCR results confirmed the results of comparative proteomic analysis.

Discussion

Biofilm production is considered as a marker of clinically relevant infection. SEM observations revealed very similar architectures of biofilms formed by SEZ. Such biofilm coverage has been already described for biofilms of *S. aureus* [20] and other organisms grown [8] in vitro. In this study, differences in the whole-cell protein expression of SEZ grown under either biofilm or planktonic conditions were also analyzed. Using the selective capture of transcribed sequences (SCOTS) technique, we also revealed differential gene expression in biofilms compared with planktonic cells (data not shown). Similar studies have shown differential gene expression and protein profiles in biofilm compared to planktonic cells [2, 14, 28, 35], suggesting that biofilm formation is a complex and highly regulated process. It can be speculated that marked differences in protein profiles detected under conditions of biofilm growth are responsible for the physiological changes of cells in SEZ biofilm.

The four upregulated proteins we found encode enzymes of the glycolysis or fermentation pathway. 6-phosphofructokinase (PFK) is a key enzyme in glycolysis [15]. Glutamine: fructose-6-phosphate aminotransferase (GlmS), also known as glucosamine synthase, is the rate-limiting enzyme of the hexosamine biosynthetic pathway [13]. dTDP-glucose-4,6-dehydratase RmlB (rfbB) is responsible for the conversion of dTDP-D-glucose into dTDP-4-keto-6-

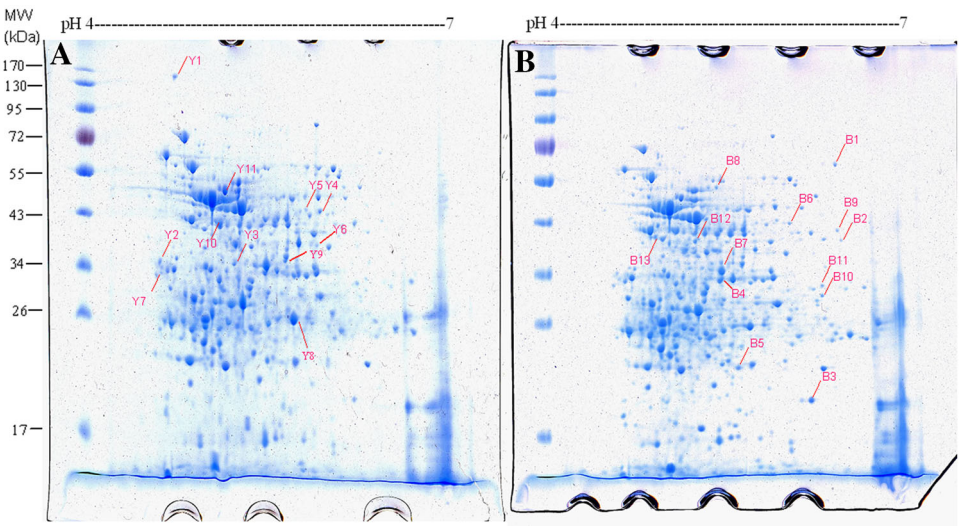

Fig. 3 2D gel electrophoresis patterns of SEZ from whole-cell lysate proteins. SEZ was grown as biofilms and planktonic conditions and the proteins separated by 2DGE. Molecular weight markers are on the *left lane* (kDa). **a** Protein pattern in the planktonic culture. **b** Protein pattern in the biofilm culture

Table 2 Proteins with increased expression levels in the SEZ biofilm, identified by MALDI-TOF/TOF-MS

Spot no.	Protein identified[a]	BLASTX similarity matched protein/ species/identity score	Theoretical MW/p*I*[b]	Experimental MW/p*I*	MASCOT score[c]	No. of peptides matched[d]	Coverage (%)[e]
B1	gil225867864	CTP synthase	60,153/5.80	59,000/5.98	97	13	28
B2	gil195977849	Transcriptional regulator	50,438/5.94	40,000/6.10	224	18	49
B3	gil338847405	Elongation factor-Tu	44,545/4.89	21,000/5.30	109	9	22
B4	gil338846290	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	35,866/5.24	35,000/5.00	207	18	51
B5	gil195978427	Elongation factor-Tu	43,841/4.88	23,800/5.10	83	10	23
B6	gil338847987	Adenylosuccinate synthetase	47,501/5.47	45,000/5.20	298	13	27
B7	gil195978008	6-phosphofructokinase	35,648/5.11	35,000/5.00	129	12	31
B8	gil338846963	Glucosamine: fructose-6-phosphate aminotransferase	63,670/5.19	54,000/5.00	230	29	50
B9	gil338846425	Seryl-tRNA synthetase	48,064/5.66	40,000/6.10	317	27	56
B10	gil338847316	dTDP-glucose 4,6-dehydratase RmlB	39,225/5.30	30,000/5.30	302	11	27
B11	gil338846831	Conserved hypothetical protein	34,333/4.91	31,000/5.30	277	18	82
B12	gil338846215	Glucose-6-phosphate isomerase	49,478/4.88	40,000/4.80	207	23	48
B13	gil338846140	Elongation factor-Ts	37,893/4.84	38,000/4.40	192	20	58

[a] gi number in NCBI

[b] Theoretical p*I* was calculated using AnTheProt (http://antheprot-pbil.ibcp.fr/)

[c] MASCOT score obtained for the peptide mass fingerprint (PMF). The significance threshold was 70

[d] Number of peptides that match the predicted protein sequence

[e] Percentage of predicted protein sequence covered by matched peptides

Table 3 Proteins with decreased expression levels in the SEZ biofilm, identified by MALDI-TOF/TOF-MS

Spot no.	Protein identified[a]	BLASTX similarity matched protein/ species/identity score	Theoretical MW/p*I*[b]	Experimental MW/p*I*	MASCOT score[c]	No. of peptides matched[d]	Coverage (%)[e]
Y1	gil338846410	Chaperone protein DnaK (heat shock protein 70)	65,045/4.64	100,000/4.30	171	19	30
Y2	gil338847448	6-phospho-beta-glucosidase	54,062/5.72	34,000/4.10	125	12	26
Y3	gil338847238	Branched-chain amino acid aminotransferase	37,078/4.90	32,000/4.90	239	14	62
Y4	gil338846419	3-oxoacyl-[acyl-carrier-protein] synthase II	43,909/5.44	45,000/5.20	514	20	56
Y5	gil338846346	Pyridine nucleotide-disulfide oxidoreductase	47,715/5.30	45,000/5.00	164	19	51
Y6	gil338847610	Oxidoreductase family protein	35,858/5.47	35,000/5.10	323	14	29
Y7	gil338847677	Conserved hypothetical protein	13,669/6.49	31,000/4.10	69	4	40
Y8	gil195977771	Phosphoglyceromutase	26,124/5.30	26,000/5.20	533	33	81
Y9	gil338847065	Citrate lyase subunit beta	31,193/5.05	34,000/5.10	220	15	53
Y10	gil195977234	DNA-directed RNA polymerase subunit alpha	34,462/4.83	38,000/4.70	93	8	25
Y11	gil338847236	30S ribosomal protein S1	43,769/4.93	45,000/4.90	172	17	50

deoxy-D-glucose (TKDG), which is a key intermediate in most deoxysugar biosynthetic pathway [36]. Glucose-6-phosphate isomerase catalyzes the reversible aldose-ketose isomerization of D-glucose-6-phosphate in glycolysis and gluconeogenesis, and in the recycling of hexose-6-phosphate in the pentose phosphate pathway, which is important in defense against oxidative stress [38]. Previous studies of protein expression in *S. mutans* [22] and *Mycoplasma mycoides* subsp. *Mycoides* [21] biofilms have shown that glycolytic enzymes analogous to the ones identified in this study are highly expressed during early biofilm formation. These results strongly indicate that glycolysis and

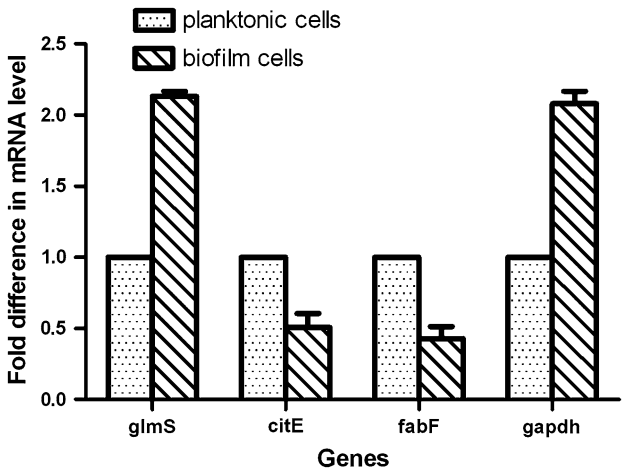

Fig. 4 *n*-Fold changes in the expression of selected SEZ genes as measured by qRT-PCR. SEZ 16S rRNA was chosen as the internal control

fermentation pathways in a SEZ biofilm are upregulated to produce the energy required for this process. CTP synthase (pyrG) may be important to metabolic enzyme by catalyzing the conversion of UTP to CTP [25]. Adenylosuccinate synthase (purA) is an important enzyme in purine biosynthesis [26]. The identification of proteins involved in metabolic processes, such as amino acid metabolism, carbon metabolism and cofactor biosynthesis, indicates that metabolism is important for the sessile mode of growth.

The protein most similar to B4 was glyceraldehyde-3-phosphate dehydrogenase GAPDH, which is a streptococcal surface protein that mediates cell adhesion and plays an important role in bacterial infection and invasion [6, 33]. GADPH has also been shown to be upregulated in biofilms of SS2 [35], *Pseudomonas aeruginosa* [18] and *Staphylococcus xylosus* [19]. The results found in our study support the overall observation that biofilm cells express more factors for binding and sessile growth, which may play a role in early biofilm development [29].

In addition to oxidative stress and oxygen limitation, the bacteria have to cope with nutrient limitation, especially in the deeper layers of the biofilm. Regulation of transcription is needed for the cell to quickly adapt to the ever-changing outer environment and stress tolerance [32]. The stress-related proteins were upregulated expressed only in the biofilm condition, suggesting that the biofilm cells specifically express proteins in different adverse environments. It is of interest that we found the upregulation of Elongation factor-Tu (EF-Tu) protein in biofilm. EF-Tu is a translation elongation factor, responsible for the binding and transport of specific tRNA on the ribosomes. It can also intervene as a molecular chaperone in *E. coli* by binding to denatured proteins [3]. This factor has also been linked to stress response in *M. pulmonis* [11]. EF-Tu also exerts other functions. In *E. coli*, it regulates both transcription and translation of proteins during nutritional deprivation [9]. It has also been reported extracellular localization of EF-Tu can also increase adhesion to human plasminogen [31]. A similar result has been found EF-Tu was upregulated during biofilm formation by *S. mutans* [4]. In conclusion, most of upregulated proteins in the biofilm growth were associated with metabolism, adhesion, and stress conditions.

Bacteria growing in biofilms are different from those growing in planktonic cells. In this study, Y1–Y11 were identified and significantly downregulated in biofilm cells (Table 3). Most of the downregulated proteins are likely to be involved in protein synthesis or encode membrane proteins/transporters. The results support the hypothesis that general metabolic functions are downregulated in response to a reduction in growth rate during biofilm formation. Similar conclusions were found in *S. mutans* [37] and SS2 [35].

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