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Identification of protein differences between two clinical isolates of *Streptococcus mutans* by proteomic analysis

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Introduction: *Streptococcus mutans* is generally considered to be the principal etiological agent for dental caries. Different strains of *S. mutans* may display different virulence mechanisms, so the isolation of the differential proteins is illuminating.
Methods: *S. mutans* strains 9-1 and 9-2, which both colonized the same oral cavity, were selected after screening for the possession of suspected virulence traits. The soluble cellular proteins were extracted from steady-state planktonic cells of strains 9-1 and 9-2 and were analyzed using high-resolution two-dimensional gel electrophoresis. Then, replicate maps of proteins from the two strains were generated. Proteins expressed only in strain 9-1 or 9-2 were excised and digested with trypsin by using an in-gel protocol. Tryptic digests were analyzed using matrix-assisted laser desorption/ionization time of flight mass spectrometry, by which peptide mass fingerprints were generated, and these were used to assign putative functions according to their homology with the translated sequences in the *S. mutans* genomic database.
Results: There were 12 proteins only expressed in strain 9-1 and three proteins only expressed in strain 9-2. They were involved in protein biosynthesis, protein folding, cell wall biosynthesis, fatty acid biosynthesis, nucleotide biosynthesis, repair of DNA damage, carbohydrate metabolism, signal transduction, and translation.
Conclusion: The identification of proteins differentially expressed between strains 9-1 and 9-2 provides new information concerning the mechanisms of cariogenesis.

Key words: matrix-assisted laser desorption/ionization–time of flight mass spectrometry, proteome; *Streptococcus mutans;* two-dimensional electrophoresis; virulence factor

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Streptococcus mutans is generally considered to be the principal etiological agent for dental caries (4, 44). The virulence determinants of this organism have received much attention over decades and have been investigated using a range of biochemical, physiological and genetic techniques. Because proteins are the executors of cell functions, it is necessary to find out and identify the proteins related to virulence traits. A number of recent reports have made use of the more holistic strategy made possible by proteome analysis to re-evaluate the cariogenesis of *S. mutans*. Comparative proteomic analysis can reveal alterations in protein expression and hence cellular function, enabling researchers to identify and focus on proteins of interest without the need to re-identify each and every protein.

Today, comparative proteomic analysis of *S. mutans* is more frequently used to establish changes in protein expression when one strain is under a different mode of growth (biofilm or planktonic) (34, 42, 46) or under different physiological adaptations including acid shock (23, 24, 47, 49), oxidative stress, starvation, or salt and heat stress (41). Researchers have paid more attention to alterations in protein expression when one strain of *S. mutans* was occurring in different situations. Only one strain of *S. mutans* was used however, which could mean that much physiological and cariogenic information has been missed. No single strain is likely ever to be entirely representative of its species, so it is necessary to look at the differences in protein expression between strains. The

reason for this is that there is a general truth to the recently proposed 'distributed genome hypothesis', which supposes that the majority of genes of a bacterial species are not found in all members of that species. For example, in *Helicobacter pylori*, *Mycobacterium tuberculosis*, and *Escherichia coli* the genomic size of clinical isolates has been estimated to vary by as much as 22%, 0.3%, and 14% respectively (3, 20, 37). In *S. mutans*, the genomic size was also found to vary in different isolates (38). Waterhouse et al. (45) found that 20% of the UA159 open reading frames were absent from other *S. mutans* strains. Differences in the genome of *S. mutans* strains may also cause changes in corresponding protein expression profiles. In a recent study, Luppens and ten Cate (25) found that there 21–28% of proteins were differentially expressed between strains C180-2, GS5, and UA159. For each of the strains they found a different set of proteins, and none of the proteins were differentially expressed in all three strains. It seems that each strain of *S. mutans* has its own protein pattern and possibly its own physiology under similar growth conditions. Therefore, a study of changes in protein expression between *S. mutans* strains differing in virulence may find some new virulence-associated proteins and will provide new information concerning the mechanisms of cariogenesis.

Many researchers have proved that caries-active individuals are colonized with more than one genotype of *S. mutans* in plaque (1, 31, 35). Different genotypes of *S. mutans* detected within the oral cavity of one subject can have different phenotypic properties. The high genotype diversity of *S. mutans* is likely to result in the colonization by genotypes of different virulence (6).

In our previous work (13), clinical strains of *S. mutans* were isolated from dental plaque collected from 20 young adults aged between 18 and 29 years (22.6 ± 3.74 [mean ± SD]), who were caries-active [the number of decayed, missing, and filled surfaces (DMFS) = 10.0 ± 3.01]. All isolates were biotyped, serotyped, and genotyped. Different genotypes of *S. mutans* that colonized the same oral cavity of caries-active individuals have been screened for possession of virulence traits. The suspected virulence factors associated with *S. mutans* include adhesion, acidogenicity, and acid tolerance (30). In our previous work, water-insoluble glucan synthesis, adhesion, acid production, and tolerance were used to screen for virulence. According to the possession of suspected virulence traits, *S. mutans* strains 9-1 and 9-2 that colonized the same oral cavity were selected. Statistically significant differences in the water-insoluble glucan synthesis, adhesion, and acid tolerance were found between them.

Since the two strains were isolated from the same oral cavity, their growth conditions could be assumed to be very similar. Hence, they were suitable candidates for a comparative proteomic analysis study. On the other hand, because the clinically isolated strains had survived in the severe conditions of the oral cavity, they were considered superior for testing compared to laboratory strains and were thought to express important proteins for cariogenesis. Therefore, the purpose of this present study was to identify protein expression differences between *S. mutans* strains 9-1 and 9-2.

Materials and methods

Bacterial strains and growth conditions

Details of the subjects, plaque sample collection, and bacterial strain separation and isolation are given elsewhere (13).

Strains 9-1 and 9-2 of *S. mutans* that had been isolated from the same oral cavity and were different in their possession of suspected virulence traits were stored as frozen cultures at −80°C in brain–heart infusion (BHI) broth containing 50% (v/v) glycerol. For this work, the strains were grown statically at 37°C in BHI broth or on BHI-agar in an anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2).

Preparation of cellular proteins

Stationary-phase planktonic cells of strains 9-1 and 9-2 were harvested after incubation at pH 7.0 and 37°C for 2 days and washed in the presence of 2.5 μg/ml Sigma proteinase inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany), and the cell pellet was lyophilized. The frozen cell pellets were then thawed in warm water, frozen and thawed again. Cells were resuspended in 350 μl lysis buffer containing 8 M urea, 2% [volume/volume (V/V)] 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS), 62 mM dithiothreitol (DTT), and 4% (V/V) pharmalyte (IPG buffer 4-7, Amersham Biosciences, Uppsala, Sweden) and subjected to ultrasonication in the presence of 0.2-mm glass beads for 4×5 min with cooling between. Intact cells were sedimented by centrifugation at 16,000 ***g*** for 5 min at 4°C and the resultant cell extracts were treated with nuclease mix (Amersham Biosciences, Piscataway, NJ). The extracts were further cleaned up with a 2D Clean-Up kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendation. The protein concentration was determined by the method of Bradford (5) with the appropriate concentration of lysis buffer in the standards, as described by Fey et al. (11). Individual sets of cells from strains 9-1 and 9-2 were processed simultaneously to ensure consistency. Each set of cells was prepared separately to monitor reproducibility of the preparation method.

Two-dimensional electrophoresis

Proteins in the cell extracts were separated by two-dimensional electrophoresis (2DE) essentially as described by Svensäter et al. (41). Before first-dimension separation of the proteins, the IPG strips (18 cm, pH 4–7; Amersham Biosciences, Sweden) were rehydrated overnight at room temperature. One milligram of the protein samples was then loaded on to the corresponding IPG strips using the paper bridge method (36). Proteins were focused on a Multiphor II (Amersham Biosciences, Sydney, Australia). The focusing was initiated by 30 V for 6 h, 500 V for 1 h, and 1000 V for 1 h. The voltage was then increased to 8000 V and maintained at this level for 16 h. Before the second-dimension separation, the IPG gel strips were equilibrated in 50 mM Tris–HCl (pH 8·8), 6 M urea, 2% [weight (W)/V] sodium dodceyl sulfate (SDS), 30% (V/V) glycerol, 2.5% DTT and 5 mM tributylphosphine for 15 min and then for a further 15 min in the same solution except that DTT was replaced with iodoacetamide to alkylate any free DTT. The equilibrated IPG strips were embedded on top of 12.5% polyacrylamide gradient gels using 0.5% (W/V) molten agarose. The SDS–polyacrylamide gel electrophoresis (PAGE) was performed at a constant current of 20 mA per gel overnight at 4°C using an Ettan DALTsix electrophoresis unit (Amersham Biosciences, Australia) according to the manufacturer's instructions. Gels were then fixed and stained with silver before being 'double-stained' with Coomassie brilliant blue G (7, 22). At least three protein preparations were analyzed by 2DE, and proteins that were subjected to further analysis were visible in at least two gels.

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS)

Specific expressed proteins were excised from gels stained with colloidal Coomassie

brilliant blue and digested with trypsin using an in-gel protocol (39, 48). Peptides were purified and desalted using ZipTips (C18 reverse-phase tips; Millipore Ltd., Watford, UK) according to the manufacturer's instructions and eluted in 1 : 1 acetonitrile/0.1% trifluoroacetic acid. Peptide solutions (0.5 μl) were applied to a stainless steel target plate and mixed in equal volumes with a saturated solution of K-cyano-4-hydroxycinnamic acid (99% purity; Sigma-Aldrich, Gillingham, Dorset, UK) in 70% acetonitrile and 0.033% trifluoroacetic acid (high-pressure liquid chromatography grade; Perbio Science UK Ltd., Chester, UK). Samples were allowed to air dry before the acquisition of spectra on a Voyager Elite MALDI-TOF spectrometer (Applied Biosystems, Warringhton, Cheshire, UK) operating with delayed extraction in reflector mode. Peptides were irradiated with a nitrogen laser giving a 337-nm output with 3-ns pulse width and molecular ions accelerated at a potential of 20 kV. The laser intensity used was maintained at 1000 units. Peptide mass fingerprints were calibrated using a mixture of des-Arg1-bradykinin, angiotensin 1 and Glu1-fibrinopeptide with close external calibration, and DATA EXPLORER software (Applied Biosystems) was used to label monoisotopic peaks.

Protein identification

All searches were performed against the completed genomic database for *S. mutans* (http://www.genome.ou.edu). Mass spectroscopic parameters for protein identification included a mass tolerance of 150 p.p.m. and a maximum of one missed cleavage per peptide while taking into consideration methionine sulfoxide and cysteine acrylamide modifications. Matches were defined on the basis of the number of matching peptide masses and the total percentage sequence covered by the peptides.

Protein isoforms

The term 'isoform' is used here to describe the multiple charged forms of a protein that exist on a given 2DE gel, where the mean observed M_r for each form calculated from the second (SDS–PAGE) dimension deviated by approximately 10% or less, and where there was no evidence from peptide-mass mapping for some form of truncation or degradation.

Results

Cellular proteins extracted from stationary-phase planktonic cells of strains 9-1 and 9-2 were resolved by 2DE and visualized following colloidal Coomassie brilliant blue staining. The total numbers of spots visible on the 2DE gels were approximately 475 and 490 in the cell extracts of strains 9-1 and 9-2 respectively.

As shown in Fig. 1 and Table 1, 96 protein spots were enhanced 1.3-fold or more in strain 9-1 with 12 proteins expressed only in stain 9-1. In contrast, 112 protein spots were diminished 1.3-fold or more in strain 9-1 with three proteins expressed only in strain 9-2, but not in strain 9-1. The differentially expressed proteins for strains 9-1 and 9-2 comprised approximately 20.2–22.9% of the total protein spots observed on 2DE gels.

To focus on the most significant protein changes, further MALDI-TOF MS analysis was restricted to those specific expressed proteins. These strain-specific proteins (Table 2) are involved in protein biosynthesis, protein folding, cell wall biosynthesis, fatty acid biosynthesis, nucleotide biosynthesis, repair of DNA damage, carbohydrate metabolism, and signal transduction and translation.

Discussion

For 2DE gels that were prepared for peptide mass fingerprinting, colloidal Coomassie brilliant blue staining was used because this yielded better MALDI-TOF mass spectra than silver staining. Examination of colloidal Coomassie brilliant blue-stained 2DE gels demonstrated that 475 and 490 protein spots were detected when strains 9-1 and 9-2 were cultured at pH 7.0 in planktonic phase for 2 days. The total number of spots visible on the 2DE gels in our study is similar to the findings of other proteomic analyses of *S. mutans* in which the same staining method was used. In a previous study, about 347 protein spots were identified on colloidal Coomassie brilliant blue-stained 2DE gels over the pH range 4–7 (33). Other research displayed about 200 proteins on the 2DE gels (49). In our study, 475–490 proteins were visualized following the same staining over the same pH ranges. Therefore, the sensitivity of our results is comparable with that of other studies. However, the sensitivity of colloidal Coomassie brilliant blue staining is lower than fluorescent staining and radioactive labeling of protein samples (25, 42).

In our study, almost 500 protein spots were detected in the cell extract, although this is a lower number than might be expected from the 1963 open reading frames of *S. mutans*. However, bacteria are known to express only part of their genome to be a functional unit of their environment. A limitation of the present

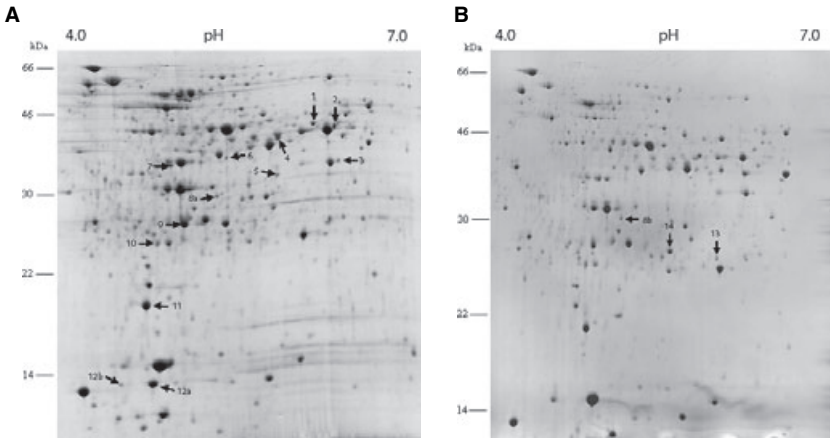

Fig. 1. Coomassie brilliant blue G-stained 2DE protein profiles of *Streptococcus mutans* growing at pH 7.0 in stationary state and planktonic phase. (A) Protein expression in strain 9-1; (B) protein expression in strain 9-2. The proteins indicated with arrows were only expressed in one strain or the other. Several protein spots marked with the same number represent isoforms of the same protein. The gels are representatives of three independent experiments.

Table 1. Changes in protein expression of strains 9-1 and 9-2 1.3-fold or greater after incubation at pH 7.0 and 37°C for 2 days

Cells	Total number of changes	1.3- to 2-fold change	2- to 3-fold change	>3-fold change	Specific proteins
Strain 9-1	96	60	19	5	12
Strain 9-2	112	91	13	5	3

Table 2. Identification of the proteins expressed in only strain 9-1 or 9-2 (spot number refers to 2DE gels in Fig. 1)

Spot no.	M_r (Da)	pI	Gene ID	Protein, EC no. and Gene	Protein function	Identified in other comparative proteomic analysis of *S. mutans* (reference)			
						Upregulated in biofilm cells	Downregulated in biofilm cells	Upregulated in acid pH	Downregulated in acid pH
Only expressed in strain 9-1									
1	42230	6.03	SMU.1653	D-3-phosphoglycerate dehydrogenase, EC 1.1.1.95, *serA*	Amino acid biosynthesis		(34, 42)		
2	41595	6.16	SMU.1457	dTDP-glucose-4,6-dehydratase, EC 4.2.1.46, *rmlB*	Cell wall biosynthesis		(33)	(29)	
3	35325	6.19	SMU.1649	exodeoxyribonuclease III, EC 3.1.11, *exoA*	Repair of DNA damage			(10)	
4	39750	5.81	SMU.65	Low molecular protein tyrosine phosphatase, EC 3.1.3.48	Signal transduction				
5	32950	5.80	SMU.1741	Malonyl-CoA-acyl carrier protein transacylase, EC 2.3.1.39, *fabD*	Fatty acid biosynthesis		(33)		
6	35809	5.48	SMU.116	Tagatose-1,6-bisphosphate aldolase, EC 4.1.2.40, *lacD2*	Carbohydrate metabolism				
7	34532	5.12	SMU.1043c	Phosphotransacetylase, EC 2.3.1.8, *pta*	Carbohydrate metabolism		(33, 34)	(29)	
8a	29787	5.41	SMU.900	Dihydrodipicolinate reductase, EC 1.3.1.26, *dapB*	Cell wall biosynthesis				
9	26276	5.20	SMU.715	Triose phosphate isomerase, EC 5.3.1.1, *tpi*	Carbohydrate metabolism		(31, 42)	(29)	(47)
10	23917	5.03	SMU.714	Elongation factor-Tu	Translation	(42)	(34)	(24)	
11	18498	4.95	SMU.957	50S ribosomal protein L10	Translation				
12a	13602	4.99	SMU.1955	GroES, *groES*	Protein folding	(42)		(23)	
12b	13501	4.79	SMU.1955	GroES, *groES*	Protein folding	(42)		(23)	
Only expressed in strain 9-2									
8b	30776	5.20	SMU.900	Dihydrodipicolinate reductase, EC 1.3.1.26, *dapB*	Cell wall biosynthesis				
13	26187	5.87	SMU.143c	Peptide deformylase, EC 3.5.1.88, *pdf*	Protein synthesis				
14	26925	5.55	SMU.2005	Adenylate kinase, EC 2.7.4.3, *adk*	Nucleotide biosynthesis	(42)	(34)		

study is that the data represent only a partial picture of the total protein fraction. Only those proteins within the pH range 4–7 and with molecular masses from 10 to 100 kDa were taken into account. Another limitation was indicated by the 2DE technology. Proteins with extreme pH values, especially very basic proteins, as well as very low and high M_r proteins, are not readily resolved by current 2DE technology (16). In practice, many low-abundance proteins and hydrophobic proteins, particularly intrinsic cytoplasmic membrane proteins, although representing 15–30% of the proteome of a bacterium, are either not detected or represent less than 1.0% of the proteins displayed on 2DE gels (22).

In the present study, only some strain-specific proteins were taken into account. There were 12 proteins only expressed in strain 9-1 and three proteins only expressed in strain 9-2. These strain-specific proteins were not expressed in the other strain and may represent those with redundant functions required for specific phenotypes. As shown in Table 2, the proteins identified as specific to strain 9-1 are involved in amino acid biosynthesis, protein folding, cell wall biosynthesis, fatty acid biosynthesis, repair of DNA damage, carbohydrate metabolism, and signal transduction and translation. Specific expression of glucosyltransferase was not found in strain 9-2. The reason may be that only specific protein spots were selected. Spots that showed different intensities of expression for both strains were not selected for protein identification.

S. mutans is well adapted to tolerate rapid changes in dental plaque pH, as well as fluctuations in carbohydrate availability and multiple environmental stresses. Central to the tolerance of environmental insults by this microorganism is the production of a variety of stress proteins, such as the molecular chaperone GroES (10). This protein assists in the folding of newly synthesized or denatured proteins. The level of GroES in *S. mutans* has been found to be elevated at acid pH (23) and highly expressed by biofilm grown cells (42). In this study, two isoforms of GroES (chaperonin 10) were only expressed in strain 9-1. The isoforms most likely arise as a result of post-translational modifications, including phosphorylation, glycosylation, or acylation.

The acid environmental conditions will damage DNA and lead directly or indirectly to the formation of apurinic/apyrimidinic (AP) sites and strand breaks (40). Damaged sites are most efficiently repaired by exonuclease III (ExoA; EC

3.1.11). ExoA has been found previously to be upregulated in cells grown at low pH (12, 14). In this study, it was only expressed in strain 9-1 and may make the strain more acid tolerant. In our study, samples of dental plaque were taken from several surfaces of the anterior and posterior teeth and from carious surfaces and were pooled for each individual. Therefore, we cannot ascertain whether strains 9-1 and 9-2 colonized the same ecological habitat or not, such as different teeth and different tooth surfaces. In our previous work (13), a statistically significant difference in adaptation to acid was found between strains 9-1 and 9-2. Strain 9-1 was more acid tolerant than strain 9-2. The strain 9-1 might colonize the plaque on the demineralized or decayed surface where the pH is rather low. ExoA might enable strain 9-1 to compete successfully at low pH. Strain 9-2 might colonize the caries-free surface where the pH is neutral. It is also possible that strain 9-2 colonized the decayed surface and harbored other proteins involved in acid tolerance response (ATR) and repair of DNA damage.

EF-Tu, a protein involved in the polypeptide elongation cycle, was found to be only expressed in strain 9-1. Besides specific expression in strain 9-1, an increase in the expression of EF-Tu was also observed by growing *S. mutans* at pH 5.0 (23). The increase in EF-Tu is of interest from another standpoint, because it has reported that EF-Tu could recognize the same hydrophobic-binding motifs in proteins as the chaperone DnaK (26).

Two proteins that have been found to be enhanced at acid pH (29) and diminished in biofilm grown cells (33, 37) in previous *S. mutans* proteomic analyses were identified as being only expressed in strain 9-1 in this study. One is dTDP-D-glucose 4,6-dehydratase (RmlB; EC 4.2.1.46), which catalyzes L-rhamnose biosynthesis (43). The serotype-specific rhamnose-containing polysaccharide antigens of *S. mutans* have been proposed as putative mediators for the colonization of cells on tooth surfaces (27). Our previous work has identified that strain 9-1 has stronger adherence to saliva-coated hydroxylapatite beads than strain 9-2 (13). In strain 9-1 RmlB might play a role in adhesion to the tooth surface. However, in another proteomic analysis, RmlB was downregulated in biofilm-grown cells (33). *S. mutans* cells will alter their protein expression profile under different modes of growth. In the present study, the two strains were incubated in planktonic phase. Therefore we postulate that RmlB may participate in the adhesion of planktonic cells, but not biofilm cells. Another strain 9-1-specific protein is phosphotransacetylase (Pta, EC 2.3.1.8), which participates in acetate formation in the pyruvate–formate–lyase pathway. The *pta* gene of *S. mutans* contains putative competence-induced (cin)-boxes in its promoter regions and was predicted to be under the control of the alternative sigma factor ComX. The previous studies have found that Pta could be upregulated as a response to the maintenance of competence (32). A *pta* mutant of *Salmonella enterica* serovar *typhimurium* was attenuated in mice (21) and in *Campylobacter jejuni*, pellicle formation was found to be reduced in a *pta* mutant (19). In *S. mutans*, mutagenesis studies are needed to further assess the physiological significance of *pta*.

Another three proteins specific to strain 9-1 also diminished in *S. mutans* biofilm-grown cells compared to their planktonic counterparts (33, 34, 42). One of them, D-3-phosphoglycerate dehydrogenase (SerA; EC 1.1.1.95), catalyzes the first step in the serine biosynthetic pathway. Malonyl-coenzyme A-acyl carrier protein transacylase (FabD; EC 2.3.1.39), which is involved in polymerizing acetyl-coenzyme A into fatty acids, has also been found failure to be detected in biofilm-grown cells (33), compared to their planktonic counterparts. The fatty-acid synthetic pathway is the principal route for the production of membrane phospholipid acyl chains in bacteria (28) and another specific protein is triose phosphate isomerase (Tpi; EC 5.3.1.1), which catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Here we postulate that the coexistence of the two strains has allowed them to evolve such that redundant functions can be eliminated from one strain as long as the other strain is still competent for that activity.

Adenylate kinase (Adk; EC 2.7.4.3) was only expressed in strain 9-2. This enzyme catalyzes the synthesis of adenosine diphosphate from adenosine triphosphate and adenosine monophosphate and plays a major role in adenine nucleotide homeostasis. In *S. mutans*, Rathsam et al. (34) found that Adk was downregulated in biofilm-grown cells. However, in other research, this enzyme was found to be enhanced in biofilm-grown *S. mutans* cells (42). The cause could be differences in physiology between cells grown in different models. Luppens and ten Cate (25) have found that there was 0.3–7.8% differential protein expression when different biofilm models were used.

There are several strain-specific proteins in our study that have not been identified in previous comparative proteomic analyses. One protein, the low-molecular-weight protein tyrosine phosphatase, was only expressed in strain 9-1. Protein tyrosine phosphatases (PTPs; EC 3.1.3.48) constitute a large family of signaling enzymes that are important for the regulation of cell proliferation, differentiation, metabolism, migration, and survival (18). A new concept has emerged suggesting the existence of a biological link between protein tyrosine phosphorylation and bacterial pathogenicity (9). Another differentially expressed protein is dihydrodipicolinate reductase (DapB, EC 1.3.1.26), an enzyme within the diaminopimelate pathway involved in the formation of cell wall cross-linking peptides (2). In this study, two isoforms of DapB were found to be strain specific. Most notable, however, was the finding that one was only expressed in strain 9-1 and another was only expressed in strain 9-2, i.e. a different isoform of the same protein is present in the two strains. A similar observation was made with elongation factor Tu (EF-Tu) in a previous study (23). At pH 5.0, some charged isogenic forms of EF-Tu were upregulated and other truncated forms were downregulated. One of the explanations for the observed differences in isoforms among *S. mutans* strains may be that the post-translational modification among the strains differs. Tagatose-1,6-bisphosphate aldolase (LacD2; EC 4.1.2.40) was not detected in strain 9-2. It is situated at the branching of the tagatose-6-phosphate and Embden–Meyerhof–Parnas (glycolysis) metabolic pathways, where it catalyzes the reversible cleavage of tagatose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (15).

In our previous work (13), genomic comparison was performed between strains 9-1 and 9-2. Twenty-seven sequences were found in the genome of strain 9-1 but not in 9-2. These sequences are involved in competence development, signal transduction, and transcriptional regulation, repairing stress damage, transport, carbohydrate catabolism, biochemical synthesis, or unknown functions. In the present study, no proteins identified were correlated with genome subtractions. The reasons may include the following. First, whether or not the twenty-seven strain-9-1-specific sequences are transcribed and translated, which is unknown, bacteria are known to express only part of their genome. Although 95% of a bacterial

genome is expressed as protein products (17), the theoretical resolving power of 2DE is still estimated to be approximately 75% of the proteome (8). In the comprehensive proteomic reference map for the protein expression of *S. mutans*, approximately 11% of the total open reading frames of the bacterium were expressed (22). Second, in the research of comparative genomics between strains 9-1 and 9-2, the strain-9-1-specific clones were randomly selected for sequencing and identification. Therefore, they may not overlap with the 15 proteins identified in this study. On the other hand, the data in this study represent only a partial picture of the total protein fraction. The failure of other proteins to be detected emphasizes an important aspect of current proteomics involving incomplete 2DE displays, because any undetected changes may be of equal importance in an understanding of the true nature of phenotypic change.

In our research we could not determine whether the failure to express these proteins in one of the two strains was cause or effect of dental caries. Many important questions are raised concerning the mechanism for the protein expression variation and their ecological advantage or disadvantage. In this study, we pooled the plaque at the time of collection. In future work, investigation of site-specific plaque may provide more information.

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