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Identification of genetic differences between two clinical isolates of *Streptococcus mutans* by suppression subtractive hybridization

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Streptococcus mutans is generally considered to be the principal aetiological agent for dental caries. Phenotypic variation in strains is often associated with differences in gene content, so the isolation of DNA fragments from these genes or associated regions is illuminating. The *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. Genomic DNA of strain 9-1 was used as the tester, and that of 9-2 was used as the driver. Suppression subtractive hybridization (SSH) was applied between the tester and the driver DNAs. The subtractive products were cloned into a pCR2.1 vector. Clone libraries representing sequence differences were obtained. The subtractive fragments that were found specifically in strain 9-1 but not in strain 9-2 were identified by dot blotting and then sequenced. BLASTn and BLASTx sequence homology analyses were subsequently performed. Twenty-seven sequences were found in the genome of strain 9-1 that were not in 9-2. Among them, three revealed no homology to published nucleotide sequences while the remaining sequences showed 81–100% homology to known genes of *S. mutans* strain UA159. These sequences are involved in competence development, signal transduction and transcriptional regulation, repairing stress damage, transport, carbohydrate catabolism, biochemical synthesis, or unknown functions. Differences exist in the genomes of different *S. mutans* isolates. SSH is effective in screening for *S. mutans* strain specific DNA sequences.

Key words: genome; *Streptococcus mutans*; suppression subtractive hybridization; virulence factor

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Streptococcus mutans is generally considered to be the principal aetiological agent for dental caries (21, 32). Clinical isolates of *S. mutans* show enormous variations in their genomes or genes (12). Although arbitrarily primed polymerase chain reaction (AP-PCR) (31, 41) and restriction endonuclease analysis (28) provide sensitive methods for defining the genetic relationship between *S. mutans* isolates, they provide no information on genomic differences. Such information can be obtained by *in vitro* genome comparison (13).

Phenotypic variation in strains is often associated with differences in gene content, so the isolation of DNA fragments from these genes or associated regions is illuminating (16). The characterization of such regions can lead to a greater understanding of the mechanism of cariogenesis by *S. mutans*. However, identification of the regions has primarily used time-consuming functional techniques (20, 24),

such as chemical or transposon mutagenesis followed by complement analyses, or virulence enhancement approaches using random DNA libraries. Recently, a PCR-based subtraction method called suppression subtractive hybridization (SSH) has been introduced for the rapid identification of genomic differences between bacterial pathogens (2). Its application has led to the identification of variations in the expression of virulence genes in a range of bacterial pathogens. It was employed to compare the *Escherichia coli* with *Salmonella enterica* serovar Typhimurium (7), the virulent strain of the opportunistic aquatic pathogen *Aeromonas hydrophila* with the avirulent strain (51), the uropathogenic *E. coli* strain 536 with the non-pathogenic *E. coli* strain MG1655 (25), and to identify genetic differences between two strains of *Xylella fastidiosa* (23) that differ in pathogenicity and host range. Recently, for the first time, it was applied to identify genetic elements specific to a strain of *S. mutans* that was isolated from a child with severe early childhood caries (43). This technique has the potential to identify previously unknown pathogenicity determinants rapidly and efficiently.

The purpose of this present study was to apply SSH to identify genetic differences between two isolates of *S. mutans* that colonized the same oral cavity of a caries-active young adult and that differed in suspected virulence traits.

Materials and methods

Bacterial strains and growth conditions

The clinical samples were isolated from dental plaque collected from 20 young adults aged between 18 and 29 years (22.6 ± 3.74) caries-active subjects [defined as having number of decayed, missing and filled surfaces (DMFS) = 10.0 ± 3.01]. Before the plaque samples were collected, formal written consent was obtained from each subject and the protocol was approved by the Medical Ethics Committee of the Fourth Military Medical University.

Samples of dental plaque were taken using sterile dental probes from several surfaces of the anterior and posterior teeth and from carious surfaces and were pooled for each individual. Ten-microlitre undiluted samples and 1 : 10, 1 : 100 and 1 : 1000 dilutions were cultured on mitis salivarius agar (Difco, Laboratories, Detroit, MI) supplemented with 20% sucrose and 0.2 U bacitracin/ml (MSB). Plates were incubated at 37°C for 48 h in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. After growth on MSB agar plates, 128 isolates representing morphological types from each sample were collected and inoculated in brain–heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and incubated under the same conditions as described above. All isolates were biotyped and serotyped.

E. coli strains were maintained on L agar or in Luria broth (Difco) at 37°C. As necessary, media were supplemented with kanamycin at 50 μg/ml.

Bacteria were stored as frozen cultures at −80°C in either BHI or Luria broth containing 50% (v/v) glycerol.

AP-PCR typing

Isolates identified as *S. mutans* were genotyped. Their genomic DNA was extracted with 5% chelex solution [0.25 g chelex 100 (Sigma, St. Louis, MO) in 5 ml 10 mmol/L Tris-HCL, 1 mmol/L EDTA, pH 8.0] (18). AP-PCR was performed according to the method of Li and Caufield (29) with a random primer, OPA-05 (5′-AGGGGTCTTG, Takara Biotech, Dalian, China). AP-PCR was processed in 25 μl mixtures containing 3.5 mmol $MgCl_2$, 0.2 mmol dNTPs, 0.4 mmol primer, 2.5 U *Taq* DNA polymerase and 2 μl DNA sample. The temperature profile of the reaction in a thermocycler (Perkin-Elmer systems 2400, Perkin-Elmer, Wieterstadt, Germany) was 35 cycles at 94°C for 1 min, 36°C for 2 min and 72°C for 2 min, with an initial denaturation at 94°C for 5 min and a final extension at 72°C for 5 min. Amplification products were analysed electrophoretically in 1.5% agarose gels. Ethidium bromide-stained gel images were captured with a high-resolution imaging system (Image Master VDS: Liscap Image Capture, version 1.0, Pharmacia Biotech, Cambridge, UK). Fingerprints were regarded as similar when the major bands were identical. To assure reproducibility, PCR were carried out in at least two independent amplifications, using different DNA preparations. In every AP-PCR, *S. mutans* ATCC 25175 chromosomal DNA was used as the positive control.

Individual isolates were grouped according to the pattern of amplicons generated by AP-PCR.

Screening for virulence traits: testing for the possession of suspected virulence traits

One strain representative of each genotype identified was examined for the possession of suspected virulence traits. The suspected virulence factors associated with *S. mutans* include adhesion, acidogenicity and acid tolerance (35). In this study, water-insoluble glucan (WIG) synthesis, adhesion, acid production and tolerance were used to screen for virulence.

Water-insoluble glucan (WIG) synthesis

The *S. mutans* isolates from frozen stocks were grown in defined medium (17) at 37°C for 20 h. The supernatants obtained after centrifugation were passed through a 0.45-μm pore-diameter filter. The crude glucosyltransferase (GTase) was prepared as the precipitate of 40% ethanol from culture supernatants. The crude GTase was suspended in 5 mmol triethanolamine, dialysed against the same solution and stored at −80°C until use. GTase activity was assayed using the method of Neta et al. (37). The reaction mixture consisted of 0.3 mol acetate buffer (pH 5.5), 0.15 mol sucrose and crude GTase preparation. The mixture was incubated at 37°C for 20 min and then heat-inactivated. The turbid materials were precipitated by centrifugation (15,000 **g** for 5 min) and washed three times with distilled water. The total amounts of WIG were measured by the phenol sulphuric acid method (15) and expressed as glucose equivalent (μmol glucose/min).

Adherence to saliva-coated hydroxylapatite beads

A modified method based on the report by Russell and Mansson-Rahemtulla (40) was used. Bacterial cells were radiolabelled with methyl-[^{3}H]thymidine (10 μCi/ml). Cells were harvested by centrifugation, washed three times, and re-suspended in KCl buffer (1 mmol KH_2PO_4, 50 mmol KCl, 0.1 mmol $MgCl_2$ and 1 mmol $CaCl_2$, pH 6.2) at $OD_{550} = 0.52$. Four milligrams of hydroxylapatite beads were allowed to equilibrate in 0.2 ml KCl buffer overnight. They were then incubated with 0.1 ml of clarified whole saliva or with KCl buffer for 1 hr at room temperature in an apparatus that continuously inverted at 6 r.p.m. The beads were washed twice with KCl buffer and incubated with 0.1 ml of ^{3}H-labelled bacterial suspensions. The mixtures were continuously inverted at room temperature for 90 min. The beads were allowed to settle, and the unbound bacteria were aspirated. Finally the beads were washed three times with KCl buffer, transferred to scintillation vials and counted for radioactivity.

Final pH analysis of S. mutans isolates

The lowest pH at which acidogenesis by genotypes of *S. mutans* was completely inhibited was tested (47). For this purpose, 100-μl aliquots of 20-h cultures were transferred to test tubes containing 5 ml phenol red dextrose broth (Difco) with a final concentration of 1% glucose and incubated at 37°C in an atmosphere of 10% CO_2 for 3 days. The final pH was measured with a standardized pH meter. Uninoculated control tubes were also incubated under the same conditions. The experiment was performed in triplicate.

Adaptation to acid

Adaptation to acid was measured by the ability of cultures to survive 3-h exposure to a pH that killed cells grown at pH 7.5; this killing pH was established by a previously described method (45). To test for the ability of *S. mutans* isolates to adapt to low pH, cells were grown in tryptone–yeast extract medium with 20 mmol glucose (TYEG) to mid-log phase at pH 7.5. Aliquots of the culture were then transferred to the same medium buffered in 0.5-unit increments from pH 3.0 to pH 7.5. After 2 h of incubation, the cultures were rapidly acidified to pH 3.0, either with HCl or by resuspending the cells in TYEG buffered at that pH, and incubated for a further 3 h, when the survivors were determined by plating on BHI agar. The survival rate was calculated from the control cells incubated at pH 7.5 during the 2-h adaptation period. The data are shown as the means of three determinations.

According to these suspected virulence traits, strains 9-1 and 9-2 that colonized the same oral cavity were selected. Detection of plasmid DNA was performed to identify whether strains 9-1 and 9-2 contained cryptic plasmid by the method described by Caufield et al. (11).

Generation of a subtracted DNA library by SSH

S. mutans genomic DNA was extracted according to the method described by Caufield and Walker (12). The subtracted DNA library was generated following the instructions from the PCR-select bacterial genome subtraction kit (Clontech Laboratories, Palo Alto, CA) with the method of Akopyants et al. (2). Genomic DNA of the strain 9-1 was used as the tester, and that of strain 9-2 was used as the driver. The sequences of the adaptors and primers are listed in Table 1. After comparing the genomic DNA digestion efficiency of *Hae*III, *Rsa*I and *Alu*I, *Alu*I was selected to digest the tester and driver genomic DNAs. The tester DNA was subdivided into two portions, each of which was ligated with a different adaptor provided by the subtraction kit. Two hybridizations were performed. The hybridization temperature was 61°C instead of 63°C. In the first hybridization, the driver DNA fragments were added to each adaptor-ligated tester DNA (50 : 1 ratio). Then the DNA fragments were denatured at 98°C for 1.5 min, and allowed to anneal at 61°C for 1.5 h. After the first hybridization, the two samples (one with adaptor 1, the other with adaptor 2R, Table 1) were mixed together and a further 300 ng of heat-denatured driver DNA was added. The mixture was allowed to hybridize at 61°C overnight. Because of the second-order kinetics of hybridization, the concentration of high- and low-abundance sequences was equalized among the single-stranded tester molecules. At the same time single-stranded tester molecules were significantly enriched for differential sequences. The entire population of molecules was then subjected to PCR to amplify the tester-specific sequences. Only the remaining equalized and subtracted single-stranded tester DNAs can re-associate, forming double-stranded tester molecules with different adaptors. Two sequential PCR were carried out. PCR primer 1 was adopted in the first PCR, and the nest PCR primers were used in the second PCR (Table 1).

Table 1. Sequences of adaptors and polymerase chain reaction primers

Adaptor or primer	Sequence
Adaptor 1	5′- CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ 3′-GGCCCGTCCA-5′
Adaptor 2R	5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′ 3′-GCCGGCTCCA-5′
PCR primer 1	5′-CTAATACGACTCACTATAGGGC-3′
Nested primer 1	5′-TCGAGCGGCCGCCCGGGCAGGT-3′
Nested primer 2R	5′-AGCGTGGTCGCGGCCGAGGT-3′
23S RNA forward primer	5′-CTACCTTAGGACCGTTATAGTTAC-3′

The unpurified PCR products from the second PCR were cloned into the pCR2.1 vector (Invitrogen Corporation, Carlsbad, CA), and transformed into *E. coli* TOP10F′ competent cells, which were then cultured overnight on the selective agar plates containing kanamycin (50 μg/ml), X-Gal (20 μg/cm^2), and isopropyl-thiogalactoside (12.1 μg/cm^2). White colonies were randomly picked, transferred into 100 μl LB-kanamycin medium in 96-well microtitre plates and grown at 37°C overnight. One microlitre of the cell suspension was used as a template and the inserts were colony PCR amplified under the same conditions used in the second PCR but with 25 cycles. The sizes of the inserts and the presence of one distinct PCR product were confirmed by agarose gel electrophoresis.

Evaluation of subtraction efficiency

The subtracted and unsubtracted secondary PCR products were diluted 10-fold in water. They were amplified using 23S rRNA forward primer and PCR primer 1 (Table 1) instead of 23S RNA reverse primers because of the presence of *Alu*I incision sites in the 23S rRNA gene sequence of *S. mutans*. Cycling parameters were as follows: 94°C for 10 s, 18 cycles of 94°C for 10 s, 60°C for 30 s, 68°C for 2 min. Then, 5 μl of the products was transferred to a fresh tube and the remainder of each reaction was returned to the thermal cycles and three more cycles were performed. This step was repeated twice. Each of the 5-μl samples removed after 18, 21, 24 and 27 cycles was examined on a 2% agarose gel.

Strain-specific insert screening

DNA clones were subjected to a differential screening procedure (PCR-Select differential screening kit, Clontech) to identify those that preferentially hybridized to the tester DNAs. Briefly, 5 μl of each colony PCR product was dotted on the hybond N^+ membrane (Amersham, Pharmacia Biotech, Piscataway, NJ) in duplicate. DNA fixation was carried out by irradiation under a UV transilluminator (Vilber Lourmat, Marn-la-Valléé, France) for 2 min. The *Alu*I-digested DNA fragments of the tester and driver were used as probes (the DIG DNA labelling and detection kit, Roche, Mannheim, Germany). The blots were prehybridized in the hybridization oven (Robbins Scientific Corporation, Sunnyvale, CA) at 72°C for 1 h, and then hybridized at 72°C for 16 h with 40 ng/ml of the probe. After the

Hybond N^+ membrane was washed four times for 20 min in low stringency washing solution [2× saline sodium citrate (SSC), 0.5% sodium dodecyl sulphate (SDS)] and twice for 20 min in high-stringency washing solution (0.2× SSC, 0.5% SDS), all at 68°C, the colour was developed and detected. The clones showing positive results to the tester probes and negative results to the driver probes were selected.

Table 2. WIG synthesis, adhering to saliva-coated hydroxylapatite, acid production and adaptation to acid of *S. mutans* strains 9-1 and 9-2

Virulence trait	Strain 9-1	Strain 9-2	Statistical significance *P*
WIG (μmol glucose/min), $\times 10^{-2}$	8.41 ± 1.33	5.11 ± 0.12	<0.01
Adherence (counts/min)	3659.00 ± 360.40	469.50 ± 98.50	<0.05
Final pH	4.09 ± 0.09	4.21 ± 0.15	>0.05
Adaptation to acid (maximum survivors at pH 5.5, log/million)	4.90 ± 2.06	4.57 ± 1.21	<0.05

WIG synthesis was measured by the phenol sulphuric acid method and expressed as glucose equivalent (μmol glucose/min, $\times 10^{-2}$). Adherence is the binding of radiolabelled bacteria to saliva-coated hydroxylapatite beads and was measured as counts/min. Adaptation to acid was measured as the maximum number of survivors following a 3-h exposure at a pH which kills cells grown at pH 7.5. Values are means ± SD. $n = 3$.

DNA sequencing and analysis

The subtractive hybridization fragments that were found only in strain 9-1 and not in strain 9-2 were sequenced. DNA sequencing was carried out on an Applied Biosystems PRISM 377 automated DNA sequencer by the dye termination method. Sequence assembly and further editing were performed with DNASIS DNA analysis software. BLASTn and BLASTx sequence homology analyses (4) were performed by using the BLAST network service of the National Center for Biotechnology Information (NCBI). Both the non-redundant and the unfinished microbial databases were used for comparison.

Nucleotide sequence accession number

Three nucleotide sequences have been deposited in the GenBank database. Their accession numbers are CC156476 (SFD6), CC156477 (SFD7) and CC156478 (SFD9).

Statistics

Student's *t*-test was used to analyse the *S. mutans* isolates colonizing the same oral cavity for differences in WIG synthesis, adherence to saliva-coated hydroxylapatite beads, final pH and adaptation to acid using SIGMASTAT 2.0 software (SPSS); $P < 0.05$ was considered significant.

Results

Isolation and identification of *S. mutans* strains

Eighty-seven isolates of *S. mutans* were identified as serotype c. In a comparative analysis of the fingerprints generated by AP-PCR, only high-intensity bands were used to discriminate strains. *S. mutans* from unrelated individuals displayed distinctive DNA fingerprints; 73.3% of caries-active individuals possessed more than one genotype of *S. mutans*. One strain representative of each genotype identified was examined for its WIG synthesis, adherence to saliva-coated hydroxylapatite beads, final pH and adaptation to acid. Statistically significant differences in the three traits were found between strains 9-1 and 9-2, which colonized the same oral cavity (that of subject no. 9). The results were shown in Table 2. Therefore, *S. mutans* strains 9-1 and 9-2 from the same oral cavity differed in their possession of suspected virulence traits. The AP-PCR pattern of strains isolated from subject no. 9 is shown in Fig. 1. The two strains were found to be plasmid free.

Strain 9-1-specific DNA sequencing and homologous analysis

Strain 9-1 was used as the tester, and strain 9-2, which was isolated from the same oral cavity of a caries-active young adult, also in serotype c, was used as the driver.

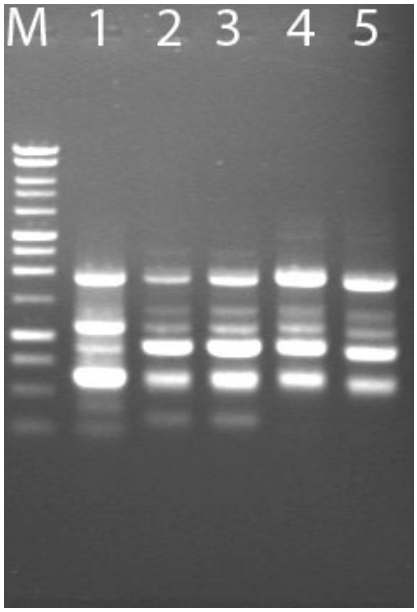

Fig. 1. AP-PCR pattern of *S. mutans* strains isolated from subject no. 9 obtained with primer OPA-05. Lane M, 1-kb DNA ladder (Promega, Madison, WI); lane 1, *S. mutans* ATCC25175; lanes 2–5, *S. mutans* isolates of serotype c from subject no. 9.

As shown in Fig. 2, expression of a 23S rRNA gene fragment existing in both tester and driver was drastically reduced. More than 300 clones were obtained from the SSH. Ninety-six white colonies were randomly chosen and the inserts were amplified (Fig. 3). The sizes of the inserts ranged from 113 to 776 bp, in agreement with the fact that shorter segments were optimal for subtraction. By dotting and fixing equal amounts of each insert in replicates on the membrane, dot blotting was carried out using the *Alu*I-digested DNA fragments as probes (Fig. 4). More than half of the clones recovered here were determined to be *S. mutans* strain 9-1-specific by hybridization. Thirty-one tester-specific clones were randomly selected for sequencing. Two had an *Alu*I cleavage site in their sequences. This observation suggested that two separate blunt-ended *Alu*I fragments were cloned into the same plasmid. They were artefacts of the cloning procedure and did not reflect gene organization in strain 9-1. There was no internal *Alu*I site present in other sequences. Twenty-five were unique and two others were each represented twice, which indicated that 27 different inserts had been obtained and the potential library of *S. mutans* strain 9-1-specific DNA was not redundant. The sequences of the 27 clone inserts were used to perform BLASTn and BLASTx searches with the completed genome of *S. mutans* UA159 (http://www.ncbi.nlm.nih.gov, GenBank accession no. AE014133). Blast analysis is shown in Table 3.

Among the 27 strain 9-1-specific DNA segments, three revealed no homology to published nucleotide sequences, five were 81–85% identical and 19 were 90–100% identical to the predicted proteins in *S. mutans* strain UA159. These segments are involved in: (i) competence development, (ii) signal transduction and transcriptional regulation, (iii) repairing stress damage, (iv) transport, (v) carbohydrate catabolism, (vi) biochemical synthesis,

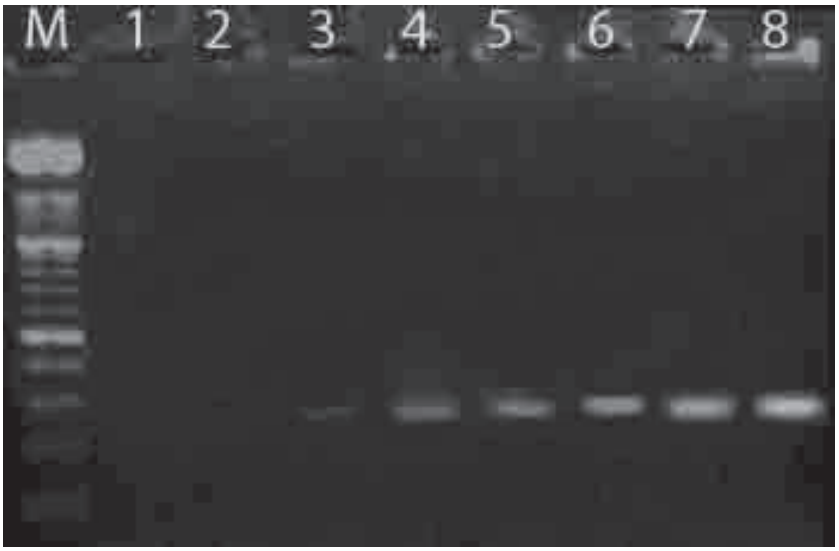

Fig. 2. Subtraction efficiency of 23S rRNA gene between subtracted (lanes 1–4) and unsubtracted (lanes 5–8) products detected by PCR. Lane M, 100-bp DNA ladder (Promega); lanes 1 and 5, 18 cycles; lanes 2 and 6, 21 cycles; lanes 3 and 7, 24 cycles; lanes 4 and 8, 27 cycles.

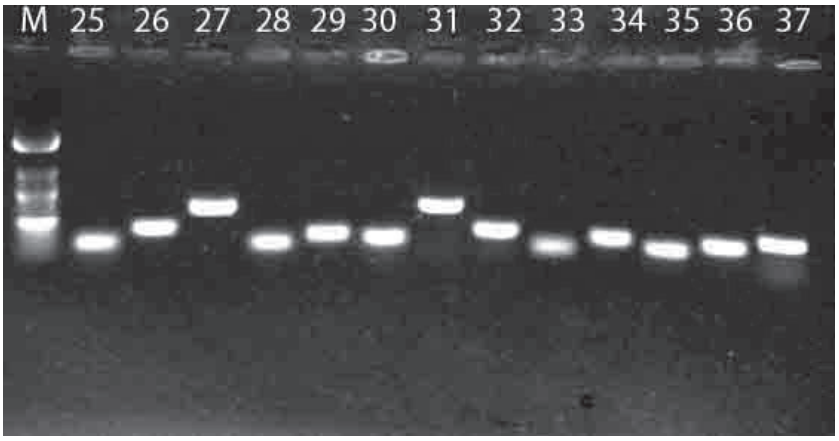

Fig. 3. Screening of *S. mutans* strain 9-1-specific minilibrary through colony PCR. Clone inserts were amplified by PCR using primers in the second PCR and were run in a 5% agarose gel. Inserts from clones 25 to 37 are shown. Lane M, 100-bp DNA ladder.

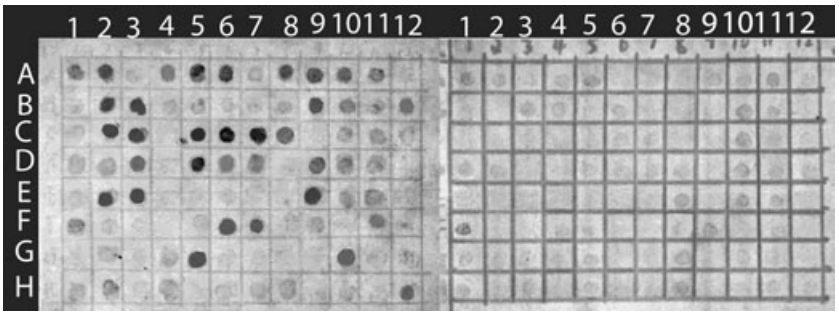

Fig. 4. Dot-blot hybridization of PCR products from the *S. mutans* strain 9-1-specific minilibrary against digoxigenin-labelled strain 9-1 DNA (left membrane) and strain 9-2 (right membrane).

(vii) unknown or unidentified function at present. The GC contents of individual fragments ranged from 31.75% to 47.31% and had an average difference of 1.79% from the GC content of UA159 sequences, which was 36.8%. Nine fragments had GC contents of greater than 40%. Four fragments had GC contents of less than 35%.

Discussion

The suspected virulence factors associated with *S. mutans* include adhesion, acidogenicity and acid tolerance (35). In our study, strains of *S. mutans* were screened for possession of these virulence traits. The adhesion of *S. mutans* within dental plaque can be mediated via sucrose-independent and sucrose-dependent means. Sucrose-dependent adhesion is mediated mainly by WIGs, which are synthesized by the action of extracellular glucosyltransferases (GTF) on sucrose (44). The ability of *S. mutans* to produce WIG is believed to be its main virulence trait (34, 36). It has been demonstrated that WIG modifies the physico-chemical properties of dental plaque and makes it more cariogenic (14). A statistically significant positive association between the level of WIG synthesis by *S. mutans* clinical isolates and caries incidence was observed (34). This suggests that WIG synthesis is associated with degrees of virulence of *S. mutans* isolates. In addition, sucrose-independent adhesion, acidogenicity and acid tolerance were important measures for the determination of the virulence of *S. mutans* strains. It is generally believed that sucrose-independent adhesion to salivary components within the acquired enamel pellicle may initiate the attachment process (8). The acidogenicity of *S. mutans* may lead to ecological changes in the plaque flora that include an elevation in the proportion of *S. mutans* and other acidogenic and acid-tolerant species (32). The acid tolerance of the *S. mutans* further enhances its accumulation as well as the accumulation of other acid-tolerant species such as the lactobacilli (22). In this present study, comprehensive assays were performed to determine adhesion, acid production and tolerance.

All of the clinical isolates of *S. mutans* from 20 caries-active young adults were examined for the possession of these suspected virulence factors. We found that the plaque of caries-active individual young adults was colonized with more than one genotype of *S. mutans*. This finding is similar to earlier reports (3, 36, 38). In a study of young adults, Redmo Emanuelsson et al. (38) found a maximum

Table 3. Summary of BLAST search of sequences that were found in *S. mutans* strain 9-1 and not strain 9-2

Fragment	Insert size (bp)	GC content (%)	GenBank accession no. (Locus tag)	Homologies to predicted encoded protein	E value[1]	Similarity (% amino acid)
SFA4	577	39.68	AAN58855 (SMU. 1164c)	Putative ABC transporter, ATP-binding protein of *S. mutans* UA159	2e-37	80/80 (100%)
			AAN58854 (SMU. 1163c)	Putative ABC transporter, ATP-binding protein of *S. mutans* UA159	1e-44	92/113 (81%)
SFA5, SFC6	348	42.52	AAN58243 (SMU. 498)	Putative late competence protein of *S. mutans* UA159	1e-60	112/115 (97%)
SFA6	243	41.15	AAN58864 (SMU. 1173)	Putative *O*-acetylhomoserine sulfhydrylase of *S. mutans* UA159	7e-40	80/80 (100%)
SFA8	340	37.05	AAN59155 (SMU. 1504c)	Hypothetical protein of *S. mutans* UA159	2e-48	90/92 (97%)
SFA9	392	38.51	AAN59090 (SMU. 1426c)	Putative phospho-sugar mutase of *S. mutans* UA159	2e-67	127/130 (97%)
SFA10	207	42.02	AAN58764 (SMU. 1066)	Putative GMP synthase of *S. mutans* UA159	2e-32	69/69 (100%)
SFB3	415	39.27	AAN58169 (SMU. 415)	Conserved hypothetical protein of *S. mutans* UA159	2e-75	138/138 (100%)
SFB9	336	47.31	AAN58519 (SMU. 801)	Putative GTP-binding protein of *S. mutans* UA159	7e-57	110/111 (99%)
SFB12	337	31.75	AAN59366 (SMU. 1733c)	Putative SNF helicase of *S. mutans* UA159	2e-58	112/112 (100%)
SFC2	159	42.76	AAN58187 (SMU. 435)	Putative *N*-acetylglucosamine-6-phosphate deacetylase of *S. mutans* UA159	6e-23	51/52 (98%)
SFC3	196	40.80	AAN58452 (SMU. 723)	Putative calcium-transporting ATPase; P-type ATPase of *S. mutans* UA159	1e-28	65/65 (100%)
SFC5, SFF11	310	36.44	AAN59234 (SMU. 1591)	Catabolite control protein A, CcpA of *S. mutans* UA159	2e-35	77/90 (85%)
			AAC46294	RegM of *S. mutans* LT11	1e-34	76/90 (84%)
			AAB58798	Catabolite control protein A of *S. mutans* GS-5	3e-34	74/90 (82%)
SFC7	633	40.59	AAN59229 (SMU. 1586)	Putative threonyl-tRNA synthetase of *S. mutans* UA159	e-123	210/210 (100%)
SFC8	356	37.63	AAN59539 (SMU.1928)	Putative ABC transporter, permease of *S. mutans* UA159	5e-49	99/118 (83%)
			AAL04089	PsaB of *S. mutans* LT11	5e-49	99/118 (83%)
SFD3	363	39.94	AAN58123 (SMU.365)	Glutamate synthase (large subunit) of *S. mutans* UA159	1e-63	119/120 (99%)
SFD5	325	37.53	AAN59089 (SMU.1425)	Putative Clp proteinase, ATP-binding subunit ClpB of *S. mutans* UA159	2e-54	107/107 (100%)
SFD6	401	36.15	AAN59070 (SMU. 1405c)	Conserved hypothetical protein of *S. mutans* UA159	6e-57	110/136 (80%)
SFD7	401	31.91	–	None[2]		
SFD9	266	36.46	–	None[2]		
SFE2	343	35.26	AAN58370 (SMU.636)	Putative *N*-acetylglucosamine -6-phosphate isomerase of *S. mutans* UA159	3e-60	114/114 (100%)
SFE3	369	36.31	AAN59308 (SMU.1669)	Putative ABC transporter, branched chain amino acid-binding protein of *S. mutans* UA159	8e-55	104/122 (85%)
SFE9	285	42.45	AAN58803 (SMU.1109c)	Putative integral membrane protein; possible permease of *S. mutans* UA159	1e-38	79/94 (84%)
SFF6	334	34.13	AAN59487 (SMU.1870)	Putative DNA mismatch repair protein MutS2 of *S. mutans* UA159	6e-58	109/111 (98%)
SFF7	571	35.19	AAN59303 (SMU.1664c)	Putative acetoin utilization protein, acetoin dehydrogenase of *S. mutans* UA159	5e-75	145/146 (99%)
SFG5	505	41.18	AAN57912 (SMU.133c)	Putative MDR permease; transmembrane efflux protein of *S. mutans* UA159	1e-72	149/165 (90%)
SFG10	240	34.58	AAN58072 (SMU. 309)	Regulator of sorbitol operon of *S. mutans* UA159	e-104	191/195 (97%)
SFH12	655	38.77	AAN59662 (SMU. 2065)	Putative UDP-glucose 4-epimerase of *S. mutans* UA159	e-119	216/218 (99%)

[1]The E value indicates the probability of the match. A match with an E value of 1e-5 and below was taken to be significant (i.e. the match was not due to chance).
[2]<30 bp identical are listed as 'None'.

of seven genotypes in subjects who had previous experience of caries. Heavy colonization and growth of multiple genotypes in the same oral cavity are likely to be consequences of frequent consumption of fermentable carbohydrates. Different clonal types of *S. mutans* detected within the oral cavity of one subject can have different phenotypic and genetic properties. The high clonal diversity of *S. mutans* is likely to result in colonization by clones of different virulence (10). Our results revealed that there was variability in WIG synthesis, adherence to saliva-coated hydroxylapatite, acid production, and adaptation to acid among the isolates that colonized the same host oral cavity. In some subjects with high incidence of caries, all genotypes possessed the suspected virulence traits. In some other subjects, strains were different in their suspected virulence traits. For example, in WIG production strain 8-3 was stronger than strains 8-1 and 8-2, while in adherence to saliva-coated hydroxylapatite and acidogenicity strain 8-2 was stronger than strains 8-1 and 8-3 and strain 8-1 was the most aciduric strain among them. Also, in subject no. 9, there were statistically significant differences in the possession of these suspected virulence traits between *S. mutans* strains 9-1 and 9-2. These findings further revealed the complexity of *S. mutans* colonization in the same oral cavity.

The saliva-coated hydroxylapatite technique is a simple and efficient way to identify the genetic differences responsible for pathogenicity-related phenotypes within bacterial strains (2, 49). The recovery rate of coding genes using saliva-coated hydroxylapatite and different restriction enzymes can reach 96% (1). The genomic sequence of *S. mutans* UA159 was recently determined, but little is known about the overall genetic diversity of this species. Saliva-coated hydroxylapatite examination of virulent strains should yield additional genetic loci associated with caries if, indeed, strains differ in virulence by virtue of specifically acquired genetic loci. In previous research (43), the PCR-based SSH was applied to identify genetic elements specific to a *S. mutans* strain associated with severe early childhood caries (S-ECC). The virulent strain was isolated from the dental plaque of a 7-year-old child from Central Africa with S-ECC (DMFT ≥14); the driver strain was isolated from a caries-free child who lived in the same village. The genomic DNA was digested with *Rsa*I. DNA fragments were obtained with similarity to genes encoding putative translation elongation factor, malolactic enzyme, putative transcriptional regulator, putative haemolysin, and function is unknown or indefinite at present. In our study, *S. mutans* strains 9-1 and 9-2 were isolated from the same oral cavity of a young adult with DMFS = 12. Compared with strain 9-2, strain 9-1 possesses suspected virulence traits. Since the two strains colonized the same oral cavity, the host environment was controlled. To generate fragments in the optimal size range (200–1000 bp), three four-base, blunt-cutting restriction enzymes were chosen to digest *S. mutans* genomic DNA. After a comparison of their digestion efficiencies, *Alu*I was selected to digest the tester and driver genomic DNAs. None of the strain variation observed by Saxena et al. was the same as that seen in this study.

The tester-strain-specific DNA sequences mean that, compared with the driver strain, these sequences are specific to the tester strain, others strains of *S. mutans* may share or may not share these sequences. This was also found in other bacterial pathogen studies in which genomic variations between two strains were identified using SSH (19). Among 27 tester-strain-specific DNA sequences, 70.4% of the tester-specific sequences showed high similarity (90–100%) to the sequences of strains UA159, LT11 and GS-5, suggesting that they are universally distributed genes in *S. mutans*. SFA5 had 97% homology to part of the putative late competence protein (ComF), which is involved in *S. mutans* DNA uptake. SFB12 contained 112 internal amino acids of the SNF helicase. SFC3 contained a portion of the P-type ATPase. SFA6, SFA10 and SFC7 contained part of putative *O*-acetylhomoserine sulphydrylase for methionine biosynthesis, putative GMP synthase, and threonyl-tRNA synthetase. SFD3 had 99% homology to the large subunit of glutamate synthase, which is involved in glutamic acid synthesis. SFA8 and SFB3 showed high homology to a hypothetical protein.

There were statistically significant differences in WIG synthesis, adhering to saliva-coated hydroxylapatite and acid tolerance between strains 9-1 and 9-2. The genetic differences between the two strains with their differences in the suspected virulence traits were correlated. Among 27 strain 9-1-specific fragments, some encode proteins putatively contributing to the phenotypic diversity. SFG5 had 90% similarity to a multidrug resistance (MDR) transporter, which participates in sucrose-dependent adhesion of *S. mutans* (46). SFH12 had 99% homology to UDP-glucose 4-epimerase, which is a key enzyme in exopolysaccharide formation and may play a role in WIG synthesis in *S. mutans*. The acquisition of SFG5 and SFH12 in the chromosome of strain 9-1 is particularly intriguing because their encoding proteins may be involved in sucrose-dependent adhesion. SFA9 had 97% homology to the phosphosugar mutases, which catalyse the first step in the biosynthetic pathway leading to the essential peptidoglycan precursor UDP-*N*-acetylglucosamine and which take part in biofilm formation (33). This protein may be involved in the sucrose-independent adhesion of strain 9-1 to the tooth surface. SFG10 shared 97% homology with the internal region of the transcriptional regulator for sorbitol P-enolpyruvate phosphotransferase transport system (PTS). This genomic variation showed that strains 9-1 and 9-2 differed in their utilization of sorbitol. SFF7 had 99% homology to acetoin dehydrogenase, which is involved in carbohydrate catabolism and will influence the final acid production. SFB9 had 99% homology to the streptococcus GTP-binding protein (SGP), which may play a role in the stress response and biofilm formation (5, 50). SFD5 encoded ClpB proteinase, which is implicated in the regulation of the stress response (30). SFF6 had 98% homology to the DNA mismatch repair protein MutS2, which recognizes mismatched base pairs and initiates repairing. The proteins encoded by SFB9, SFD5 and SFF6 may reactivate and remodel degenerated protein and make strain 9-1 acid tolerant. SFC2 was 98% homologous to *N*-acetylglucosamine-6-phosphate deacetylase, and SFE2 encoded the beginning of *N*-acetylglucosamine-6-phosphate isomerase. Both of these proteins are involved in the decomposition of *N*-acetylglucosamine (GlcNAc), and are associated with the virulence and morphogenesis of bacteria (9). The loss of SFC2 and SFE2 in chromosome may make strain 9-2 differ in its utilization of GlcNAc. We did not find a loss of the *gtf* gene in the driver genome. The reason may be that in the dot blots, only clones that showed positive results to the tester probes and negative results to the driver probes were selected. The sequenced DNA fragments were all found in the tester strain and were absent from the driver strain. Those clones that showed different intensities of positive results to both tester and driver probes were not selected for sequencing.

In summary, the strain 9-1-specific DNA fragments identified here are involved in competence development, signal transduction, stress response, transport, carbohydrate catabolism and biochemical synthesis. They are physically located throughout the strain 9-1 genome. This indicates that genetic differences between strains 9-1 and 9-2 exist over several regions of the chromosome. It had been shown that there was large genomic diversity existing among different *S. mutans* strains. The melibiose-negative *S. mutans* strains were examined and the loss of a group of adjacent genes in their genomes was identified (39). This phenomenon was observed in the present study. The sequences of SFD5 and SFA9 are adjacent in the chromosome of strain UA159. They were lost by strain 9-2.

The SSH is not limited to the discovery of known genes. It also enables the identification of low homology and new sequences and is an expansion of the phenotypic-trait-to-gene approach (29). In our study, eight of the 27 tester-specific sequences displayed either only low homology (≤85%) of the complete genomes of UA159 or no homology to the known sequences according to BLAST searches, indicating heterogeneity among different strains of *S. mutans*. SFC5 was 82–85% homologous to catabolite control protein A (CcpA), which is related to the biofilm formation of *S. mutans* (48). SFA4 contained the coding sequences of SMU.1163c and SMU.1164c, which are adjacent in the chromosome of strain UA159. Strain 9-2 lost the two adjacent genes in its genome. SFC8 has 83% homology to PsaB, which may contribute to sucrose-dependent adhesion (46). This sequence was deleted from the chromosome of strain 9-2. SFE3 shared 85% homology to a branched chain amino acid-binding protein and SFE9 had 84% homology to a possible permease. Three of the 27 strain 9-1-specific DNA fragments (SFD7, SFD9, SFD6) had no database match with known sequences in GenBank and are new gene segments. SFD7 and SFD9 did not exhibit significant protein homology to entries in current databases. The first half of the sequence of SFD6 was 80% identical to the hypothetical protein, and the second half of the fragment displayed no homology to the sequences in the public nucleotide databases. The GC contents of these fragments differed from the usual GC content of the *S. mutans* chromosome, indicating a possible acquisition by horizontal gene transfer. *S. mutans* strains may differ in their propensity to cause disease or virulence as a function of the acquisition and expression of other genetic elements. This diversity may have resulted from extensive horizontal gene transfer. In a recent study by Kreth et al. it was shown that *S. mutans* may utilize the competence-induced bacteriocin production to acquire transforming DNA from other species living in the same ecological niche (27). For opportunistic pathogens, such gene transfer can often be more potent than *de novo* mutation as an adaptive mechanism. For *S. mutans*, such recombination may speed adaptation to different human oral environments and help the bacteria cope with the changes in the tooth surface that decalcification elicits.

Investigating the genetic variability among strains is a promising approach to understanding their evolution and pathogenesis. To characterize the genetic diversity of more and less virulent strains, Salama et al. (42) examined the genomic content of 15 *Helicobacter pylori* clinical isolates by using a whole genome *H. pylori* DNA microarray. They found that a full 22% of *H. pylori* genes are dispensable in one or more strains. They also observed the strain-specific gene distributed along the chromosome, which may result from different mechanisms of gene acquisition and loss. Kato-Maeda et al. (26) detected small-scale genomic deletions among 19 clinical isolates of *Mycobacterium tuberculosis.* They found that the pattern of deletions detected differed between different strains. On average, each clinical isolate was missing 0.3% of the genome. The genomic size of clinical isolates of *E. coli* has been estimated to vary by as much as 14% (6). With the SSH approach it is possible to identify this type of genome variation between *S. mutans* strains. In this study, the 27 sequences that were lost from the genome of strain 9-2 existed in strains 9-1 and UA159. Deletions are likely to contain ancestral genes the functions of which are no longer essential for the organism's survival, whereas genes that are never deleted constitute the minimal bacterial genome. These strain 9-1-specific genes may represent those with redundant functions required for specific niches and phenotypes.

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