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Children with severe early childhood caries: streptococci genetic strains within carious and white spot lesions

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Background and objectives: Mutans streptococci (MS) are one of the major microbiological determinants of dental caries. The objectives of this study are to identify distinct MS and non-MS streptococci strains that are located at carious sites and non-carious enamel surfaces in children with severe early childhood caries (S-ECC), and assess if cariogenic MS and non-cariogenic streptococci might independently exist as primary bacterial strains on distinct sites within the dentition of individual children.

Design: Dental plaque from children ($N = 20$; aged 3–6) with S-ECC was collected from carious lesions (CLs), white spot lesions (WSLs) and non-carious enamel surfaces. Streptococcal isolates ($N = 10$–20) from each site were subjected to polymerase chain reaction (PCR) to identify MS, and arbitrarily primed-PCR for assignment of genetic strains. Primary strains were identified as $\geq 50\%$ of the total isolates surveyed at any site. In several cases, strains were characterized for acidurity using ATP-driven bioluminescence and subjected to PCR-determination of potential MS virulence products. Identification of non-MS was determined by 16S rRNA gene sequencing.

Results: Sixty-four independent MS or non-MS streptococcal strains were identified. All children contained 1–6 strains. In many patients ($N = 11$), single primary MS strains were identified throughout the dentition. In other patients ($N = 4$), primary MS strains were identified within CLs that were distinct from primary strains found on enamel. *Streptococcus gordonii* strains were identified as primary strains on enamel or WSLs in four children, and in general were less aciduric than MS strains.

Conclusions: Many children with S-ECC contained only a single primary MS strain that was present in both carious and non-carious sites. In some cases, MS and non-cariogenic *S. gordonii* strains were found to independently exist as dominant strains at different locations within the dentition of individual children, and the aciduric potential of these strains may influence susceptibility in the development of CLs.

Keywords: *oral streptococci*; *distribution of genotypic mutans*; *streptococcal strains*; Streptococcus mutans; Streptococcus gordonii; *severe early childhood caries*

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Over the past decades, considerable progress has been made in the understanding and treatment of dental caries. However, dental caries still remains as one of the most prevalent diseases in young children, and continues to have significant medical, social, and economic consequences (1, 2). Dental caries, and especially severe early childhood caries (S-ECC), severely impacts the quality-of-life of affected children and their families, and has been found to directly correspond with several social-demographic variables, including lower education, sucrose-rich dietary habits, and poor oral hygiene (3). The American Academy of Pediatric Dentistry

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(AAPD) defines S-ECC in children aged 3–5 years as: one or more cavitated, missing (due to caries) or smooth filled surfaces in primary maxillary anterior teeth, or decayed, missing, and filled surfaces (dmfs) scores of >4 (age 3), >5 (age 4), or >6 (age 5) (4). S-ECC can be linked to a greater financial burden for families and often leads parents/caregivers to experience feelings of guilt (4).

The onset and progression of dental caries is determined by a wide array of risk factors, including genetic, dietary, environmental, and behavioral determinants (5). Changes in the acidity and metabolic status of the oral cavity may induce alterations in the microbial population of the oral biofilm (5). The aciduric mutans streptococci (MS) group, including *Streptococcus mutans* and *S. sobrinus*, are highly cariogenic, and represent microorganisms most closely associated with dental caries. The oral cavity contains at least 52 genetic strains of *S. mutans* (6, 7). Some MS strains may have enhanced abilities to adhere and propagate in specific oral environments (6–8), including selective colonization of hard-tissue sites (9, 10). In addition, several MS strains have been known to concurrently colonize a single tooth, and single genotypes have been identified to colonize multiple sites within the dentition of individual patients (9–11).

The pioneer colonizers *S. gordonii* and *S. sanguinis*, when present in high numbers, can limit *S. mutans* growth (12, 13). Oxygen availability and production of hydrogen peroxide by *S. gordonii and S. sanguinis*, allow these microorganisms to effectively compete against other streptococcal species, including *S. mutans* (12–14). Conversely, *S. mutans* can antagonize the growth of other oral streptococci by generating and releasing bacteriocins (12, 13). Interspecies antagonism, as well as other ecological factors within the oral cavity, helps determine the outcome of competition between the pioneering streptococcal colonizers and *S. mutans*, ultimately shaping the oral health status of the patient, or leading to dental caries and disease.

The cariogenic potential of *S. mutans* may be attributed to a variety of virulence factors (14–16). These virulence factors include the ability to: 1) metabolize carbohydrates with the concomitant production of lactic acid (acidogenesis), 2) tolerate and survive within acidic environments (acidurity), 3) facilitate binding to hydroxyapatite and promote cell-to-cell adhesion (adhesion), 4) form multibacterial aggregate structures in dental plaque (biofilm formation), and 5) successfully eliminate other strains of bacteria through the production of bacteriocins (14–16). When these virulence factors are phenotypically expressed by strains of *S. mutans* and other oral microorganisms and are working coordinately, the dental plaque biofilm becomes altered into a state of progressive cariogenic potential (14–16).

Individuals with high to low caries status may contain MS strains exhibiting differences in virulence and the ability to induce dental caries (15). In addition, variability in the composition of plaque bacteria has been observed in children with distinct socio-economic backgrounds, and implicates microbial diversity with onset of S-ECC (17, 18). Furthermore, the coexistence of multiple MS genotypes in individuals with dental caries has been implicated with increased caries incidence, and may serve as determinants in therapeutic success or failure (5–7, 19, 20).

In our prior work, we examined the profiles of MS genetic strains from children who had been diagnosed with S-ECC, using isolates collected both before and after full-mouth dental rehabilitation (21, 22), and found that caries restorative therapy dramatically reduced the number of MS strains to 1–2 strains within 6–12 months post-rehabilitation. In the current study, using a new cohort of 20 children with S-ECC, we now identify and describe MS and other non-MS streptococci strains within carious sites, including both carious lesions (CLs) and white spot lesions (WSLs), and non-carious sites within the dentition. In most children ($N = 11$), we find that single MS strains, including *S. mutans*, were present in all sites, including carious and non-carious sites, within each individual patient. Furthermore, within the oral cavities of four individual children, we now report the presence of MS strains and non-cariogenic *S. gordonii* as streptococcal strain pairs, independently placed on distinct sites within the dentition, and implicate the aciduric potential of these strains as an important factor in the development of CLs within specific sites of the dentition.

Methods

Patient selection and treatment

Participants were selected from children visiting the Oregon Health & Science University (OHSU) Pediatric Dentistry clinic. We obtained approval for the use of study participants from the OHSU Institutional Review Board (IRB), and written informed consent was obtained from parents or guardians. The inclusion parameters for recruitment were young children (3–6 years of age) in good general health (preferably American Society of Anesthesiologists [ASA] physical status I). The exclusion parameters included children who had been subjected within the previous 3 months to antibiotic treatment, topical fluoride application, and/or antiseptic mouth rinses, or children undergoing orthodontic therapies. We selected participants who were scheduled for full-mouth dental rehabilitation therapy, typically conducted under general anesthesia, because this permitted specimen collection under sedative and controlled conditions. Table 1 contains demographics for the 20-patient study, as well as scores for decayed, missing, and filled teeth (dmft) and dmfs.

Sampling procedure and processing of specimens

Plaque samples from each patient were collected from the buccal and lingual surfaces of the anterior teeth, prior

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Table 1. Primers for amplification of putative *S. mutans* virulence genes

Target	Forward primer	Sequence 5′-3′	Reverse primer	Sequence 5′-3′	Amplicon size (bp)
gtfB	gtfB-F2	ATGGAGCAGTGCTTTACGA	gtfB-R2	GAGACAAACGCAGCTTATTG	458
gtfC	gtfC-F2	AAGGGGCATTGCTTTACAG	gtfC-R2	GCAAGGAAAGACGTAACCTG	461
gbpA	gbpA-F	TAATTTGACGGCCCTTTGTC	gbpA-R	CTGCCGAGCGTATCAGTACA	387
gbpB	gbpB-F	CGGCAGCTGATAACACTCAA	gbpB-R	TTGTCCACCATTACCCCAGT	248
gbpC	gbpC-F	CGCATTTCTCCTTTTCTTGC	gbpC-R	CGGCTTATTATGGTGCAGGT	358
gbpD	gbpD-F	TGATTGTCTGGTTGCATGGT	gbpD-R	CGCCACCATTAGAAACACCT	282
glgA	glgA-F	TCCTGCAAGGCTGCTTTATT	glgA-R	CACCGCCATGATTCCTTACT	444
atpD	atpD-F	CACCTGCTTGCGTTTCAATA	atpD-R	TTATCGGAGACCGTCAAACC	474
comD	comD-F	AGCGCTATTCCTGCAAACTC	comD-R	TGCTGATCAAAGGAGAGCAA	416
comE	comE-F	GCAAAAGGGACCTGAAACTG	comE-R	TGACGCTATCCCTGAAAAGG	339
comX	comX1-F	CCTTCCCAAAAAGTGCTCTC	comX1-R	TGTGGACTCGTGAAGATTGG	350
ciaH	ciaH-F	ACCCTTTTGTCGTGTTCTGG	ciaH-R	GCGAAGTGGAGTCAAAAACC	588
luxS	luxS-F	TGTGGCTATTTGGGTTGTTG	luxS-R	AAAGCCCCTTATGTCCGTCT	252
S. mutans[a]	mutansF	TCGCGAAAAAGTAAACAAACA[b]	mutansR	GCCCCTTCACAGTTGGTTAG[c]	479
OPA 3		AGT CAG CAC			

[a]Accession Number NC 0043.
[b]Forward primer sequence was derived from *htrA* gene; position 2029599–2029620 in *S. mutans* genome.
[c]Reverse primer sequence was derived from downstream non-coding region of *htrA* gene; position 2030077–2030058 in *S. mutans* genome.

to full-mouth dental rehabilitation, by one of two clinicians, who were trained with the use of standardized and calibrated procedures. Sterile spoon excavators were used to isolate specimens from CLs, and disposable sterile swabs were used for isolation of specimens from WSLs and sound enamel surfaces.

Streptococcal strains and growth of MS isolates

Control streptococcal laboratory strains include *S. mutans* ATCC strains 25175 and 35668. As described previously (21, 22), for selection and growth of MS and other non-MS streptococci, plaque specimens were plated on Mitis Salivarius Agar (MSA; Difco, Becton, Dickinson and Company, Sparks, MD), supplemented with 1% sodium tellurite and the antibiotic bacitracin (0.2 U/ml). Specimens were plated on MSA plates for 48–72 hours (37°C, 5% CO_2), and then individual colonies ($N = 10$–20 for each collection site) were picked and amplified in brain heart infusion (BHI) broth for 48–72 hours (37°C, 5% CO_2). Cultures were then analyzed by Gram stain to verify isolates as bacitracin-resistant, gram-positive cocci, which includes both MS and non-MS streptococci. All isolates were then amplified and stored as frozen bacterial stocks at −80°C. All subsequent analyses were conducted using cultures initiated from frozen stocks.

Genomic DNA isolation, conventional polymerase chain reaction [PCR] and arbitrarily-primed PCR (AP-PCR)

Genomic DNA was prepared from liquid cultures using the PureLink Genomic DNA Kit (Invitrogen) and *S. mutans* isolates were verified using conventional PCR. Primers for identification of *S. mutans* isolates, as well as the OPA-3 random primer used in AP-PCR, have been utilized in previous studies (21, 22) and are defined in Table 1. Conventional PCR was also conducted using potential *S. mutans* virulence gene primers, identifying representative members of the adhesion, acidogenicity, aciduric and biofilm formation gene groups. Virulence gene primer sequences were obtained from Argimon and Caufield (16), and also defined in Table 1. The amplification parameters for PCR-determination of *S. mutans* and virulence gene products have been described previously (16, 21, 22). The amplification parameters for AP-PCR were identical to conventional PCR, with the exception that annealing was conducted at 35°C for 30 seconds. PCR products were subjected to electrophoresis in 0.8% agarose gels. Ethidium bromide was used to stain fragments that were subsequently visualized by UV transillumination or with the use of a gel imager. Electrophoretic images were optimized using Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA 94547).

16S Ribosomal RNA gene sequencing, acidurity assays, and statistical analyses

PCR and sequencing of 16S rRNA gene products were conducted with the assistance of the Human Oral Microbe Identification Microarray (HOMIM) Laboratory (Forsyth Institute, Cambridge, MA) and GENEWIZ (South Plainfield, NJ), respectively, using isolates that were not successfully amplified with *S. mutans*-specific primers and thus classified as non-MS. Alignment of sequences and identification of non-MS oral streptococci were conducted by Dr. Bruce Paster (HOMIM Laboratory, Forsyth

Institute, Cambridge, MA). Acidurity or acid tolerance (AT) was determined using the protocol of Belli and Marquis (23), with slight modifications using ATP-driven bioluminescence assays, measuring viable cells and survival at pH 2.8 versus pH 7.0. Specifically, cell pellets were resuspended in 0.1 M glycine buffer, previously adjusted to either pH 2.8 or pH 7.0, and incubated at 37°C for 5 min. Viable cells were measured by ATP-driven bioluminescence, using methods described previously (24). The AT index represents the number of cells (or ATP equivalent) surviving treatment at pH 2.8 divided by the number of cells (or ATP equivalent) surviving treatment at pH 7.0. Culture tubes for each pH were conducted in triplicate or quadruplicate ($N = 3$ or 4), and then bioluminescence values averaged, prior to dividing the mean numbers of surviving cells at pH 2.8 with the mean numbers of surviving cells at pH 7.0. Using this model, a theoretical AT index of 1 (AT index = 1) indicates equivalent growth in acidic and neutral pH environments and thus extremely high acidurity or AT for that specific isolate. Conversely, an AT index of 0 (AT index = 0) indicates no survivors at pH 2.8 and thus complete acid sensitivity for the isolate.

Results

Description of study participants

Twenty pediatric dentistry patients were enrolled in this study. The study participants, 12 males and 8 females, were between the age 3.25 and 6 years and were in good health (ASA I). The patients were all diagnosed with S-ECC, with dmft and dmfs scores ranging from 7–20 to 13–60, respectively. The mean dmft and dmfs scores were 14 and 39, respectively. Some patients were missing primary teeth, while other patients had permanent teeth. The range of teeth present for the entire patient group was 17–21 teeth. Concerning previous restorations in these patients, six children had previous failed restorations, 13 children had no previous restorations, and one child had previous extracted teeth with no previous restoration. All study participants underwent full-mouth dental rehabilitation therapy following collection of the specimens. WSLs were observed in all patients, and may have represented active or passive lesions at the time of therapy.

Identification of MS and other oral streptococcal strains

Plaque specimens from CLs, WSLs, and sound enamel surfaces of each child were collected and independently plated on MSA supplemented with bacitracin (0.2 U/ml). 10–20 isolates were obtained from each plated culture, and were used to assess the distribution of streptococcal strains within each collection site. Based on growth on MSA and Gram stain analysis, all isolates were verified as bacitracin-resistant, gram-positive oral streptococci. Using specific primers for *S. mutans* in conventional PCR, as well as using random primers (arbitrary primer OPA 3) for AP-PCR, and testing genomic DNA from isolates obtained from all 20 patients, we identified 57 genotypic strains of *S. mutans*, and seven non-*S. mutans* strains, for a total of 64 streptococcal strains. Isolates that contained identical banding patterns using the OPA-3 primer were considered to belong to the same genetic strain. Genomic DNAs from several isolates were not amplified using *S. mutans*-specific primers, or were weakly amplified; these isolates comprised the seven additional genotypic strains of non-*S. mutans* oral streptococci. Representative isolates from these non-*S. mutans* strains were subjected to 16S ribosomal RNA gene sequencing, and were identified as *S. sobrinus, S. gordonii*, and *S. constellatus.*

Primary strains by location in the oral cavity

Five patients (EE, FF, HH, NN, and XX) out of 20 exhibited primary strains that were distinct within CLs/WSLs versus sound enamel surfaces (Table 2 and Fig. 1). Primary strains were defined as constituting ≥ 5 out of the 10 isolates examined for each site, or $\geq 50\%$ of the total isolates at each site. Patient EE contained three strains, with EE1 (*S. mutans*) and EE2 (*S. gordonii*) being primary strains within CL versus WSLs/enamel surfaces, respectively (Fig. 1). EE1 was identified in all 10 isolates obtained from the CL site. All three EE strains were present on enamel surfaces (Fig. 1). Patient FF contained three strains, all verified as *S. mutans* by conventional PCR, with FF1 being a primary strain in both CLs and WSLs (present in 10 out of 10 isolates examined in both cases) and FF2 being a primary strain on enamel surfaces (present in nine out of 10 isolates examined). Patient NN also contained three strains, with NN1 (*S. mutans*) being a primary strain in CLs (present in all 10 isolates examined) and white spots lesions (present in eight out of 10 isolates examined), while NN2 (*S. gordonii*) was a primary strain in enamel surfaces (present in seven out 10 isolates examined). Patient XX had three strains, with XX2 and XX1 being a primary strain in CLs (present in 10 out 10 isolates examined) and WSLs (present in seven out 10 isolates examined), respectively; all three XX strains (XX1, XX2 and XX3) were present on enamel surfaces. Minor strain XX3 was identified as *S. constellatus.* Patient HH contained six streptococcal strains, with the HH1 strain, identified as *S. sobrinus* by 16S rRNA gene sequencing, being a primary strain in both CLs and WSLs, and HH2 being a primary strain on sound enamel surfaces.

In 10 patients (BB, CC, DD, GG, QQ, RR, VV, WW, YY, and ZZ), the primary strains, all identified as *S. mutans* by conventional PCR, were the same between collection sites within each individual patient (Table 2). In four patients (BB, DD, GG, and QQ), only one MS strain was identified for *all* the isolates surveyed for any given patient (Table 3). For example, strain GG1 within patient GG was identified in all 30 isolates obtained from CLs,

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Table 2. Primary mutans streptococci (MS) and other oral streptococci strains

Patient identifier	Number of strains found	Identification of primary strains[a] found in the oral cavity			Species identified by 16S rRNA sequencing
		Carious lesion	White spot lesion	Enamel surface	
AA	2	AA1 (5/10); AA2 (5/10)	AA1 (10/10)	AA1 (10/10)	AA1: *S. mutans* AA2: *S. mutans*
BB	1	BB1 (10/10)	BB1 (10/10)	BB1 (10/10)	*S. mutans*[b]
CC	2	CC1 (9/10)	CC1 (10/10)	CC1 (10/10)	*S. mutans*[b]
DD	1	DD1 (10/10)	DD1 (10/10)	DD1 (10/10)	*S. mutans*[b]
EE	3	EE1 (10/10)	EE2 (8/10)	EE2 (5/10)	EE1: *S. mutans* EE2: *S. gordonii*
FF	3	FF1 (10/10)	FF1 (10/10)	FF2 (9/10)	*S. mutans*[b]
GG	1	GG1 (10/10)	GG1 (10/10)	GG1 (10/10)	GG1: *S. mutans*
HH	6	HH1 (7/10)	HH1 (6/10)	HH2 (6/10)	HH1: *S. sobrinus*
JJ	5	JJ2 (9/10)	–[c]	–[c]	*S. mutans*[b]
KK	4	KK1 (6/10)	KK2 (6/10)	–[c]	*S. mutans*[b]
NN	3	NN1 (10/10)	NN1 (8/10)	NN2 (7/10)	NN1: *S. mutans*; NN2: *S. gordonii*
QQ	1	QQ2 (10/10)	QQ2 (10/10)	QQ2 (10/10)	*S. mutans*[b]
RR	2	RR1 (10/10)	RR1 (9/10)	RR1 (10/10)	*S. mutans*[b]
TT	5	TT1 (5/9)	–[c]	TT1 (6/9)	*S. mutans*[b]
UU	6	UU1 (8/10)	UU3 (7/9)[c]	UU1 (7/10)	UU1: *S. gordonii* UU3: *S. gordonii*
VV	2	VV1 (10/10)	VV1 (8/10)	VV1 (10/10)	*S. mutans*[b]
WW	2	WW1 (7/9)	WW1 (8/8)	WW1 (9/10)	*S. mutans*[b]
XX	3	XX2 (10/10)	XX1 (7/10)	–[c]	XX1: *S. gordonii*; XX2: *S. mutans*; XX3: *S. constellatus*
YY	2	YY1 (9/10)	YY1 (10/10)	YY1 (10/10)	*S. mutans*[b]
ZZ	3	ZZ1 (10/10)	ZZ1 (10/10)	ZZ1 (5/10)	*S. mutans*[b]

[a]Specimens were plated on MSA plus bacitracin. Colony isolates were identified, picked, and amplified, prior to DNA extraction and AP-PCR determination of streptococcal genetic strains. Primary strains were identified as constituting ≥50% of total isolates screened for each location in the oral cavity.
[b]Primary strains for this patient were successfully amplified by conventional PCR using *S. mutans*-specific primers, and have been identified as *S. mutans*. 16S rRNA gene sequencing was not conducted with these primary strains.
[c]No primary strain was identified for this specimen.

WSLs, and enamel surfaces (10 isolates from each site), and was the primary strain throughout all surveyed sites (Fig. 1). In addition, in several other cases, the primary strains identified within any individual site constituted a clear majority of the total isolates surveyed (eight or nine out of 10 isolates surveyed; examples: CC1, FF2, RR1, VV1 and YY1 in Table 2).

MS strains and S. gordonii *exist as primary strains within individual patients*

Using 16S rRNA gene sequencing, *S. gordonii* was identified as the microorganism for primary strains EE2, NN2 and UU1 found on sound enamel surfaces, and primary strains XX1 and UU3 found in WSLs. In all four patients (EE, NN, UU, and XX) containing the *S. gordonii* primary strains, *S. mutans* was also found to exist as the corresponding primary strain in CLs. The acidurity or AT index for these five *S. gordonii* strains ranged from 0.562 (moderate) to 0.116 (low) (Table 3), with two strains possessing very low AT indices of 0.12 (EE2) and 0.116 (UU3). As expected, the acidurity for *S. gordonii* is generally much lower than the acidurity for cariogenic MS, including *S. mutans* and *S. sobrinus* (e.g.: top five aciduric MS strains EE1, NN1, HH1, KK1, and RR1 with AT indices range = 0.855–0.58; Table 3).

The mean acidurity value (or mean AT index) for primary strains in CLs (AT index = 0.431 ± 0.16; $N = 15$) is higher than the mean acidurity values for primary strains in WSLs (AT index = 0.35 ± 0.16; $N = 15$) or on enamel surfaces (AT index = 0.354 ± 0.165; $N = 11$). This slight difference in mean acidurity values between the CL vs. WSL/enamel groups, while not statistically-significant, may be accounted for by the lower aciduric values for

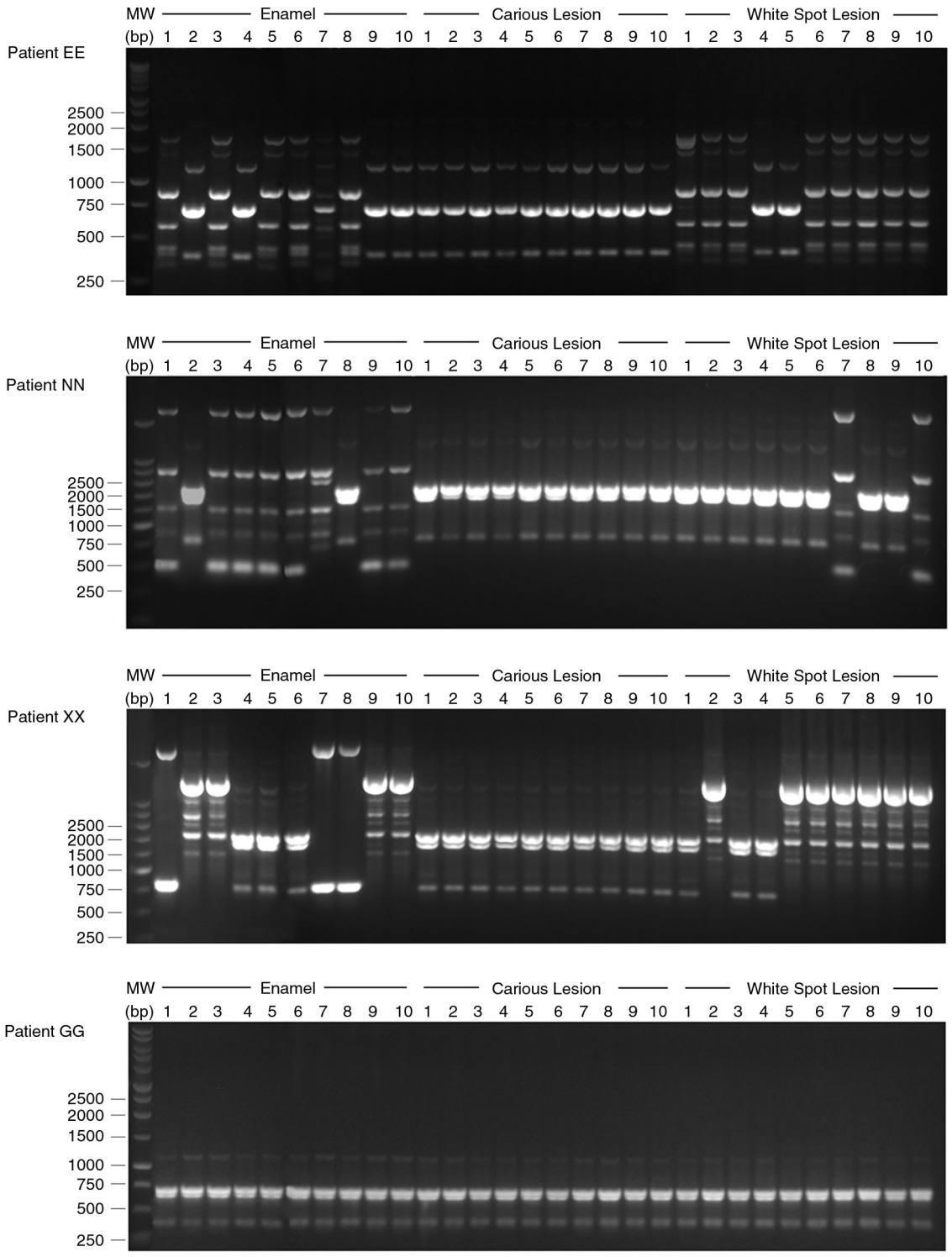

Fig. 1. AP-PCR fingerprints of isolates obtained from carious lesions, white spot lesions, and enamel surfaces from patients EE, NN, XX, and GG. Ten isolates from each collection site (carious lesions, white spot lesions, and enamel surfaces) were subjected to AP-PCR and agarose gel electrophoresis. OPA 3 primer (5′-AGTCAGCAC-3′) was used in all experiments. Note in patients EE, NN, and XX, the primary strains identified in carious lesions (EE1 in patient EE; NN1 in patient NN; and XX2 in patient XX) are distinct from the primary strains identified in enamel or WSLs (EE2 in patient EE; NN2 in patient NN, and XX1 in patient XX) for each individual patient. Patient GG is representative of many patients that contained a single MS genetic strain (GG1) in all surveyed isolates from all collections, including both carious and non-carious sites. MW = molecular weight markers in base pairs (bp).

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Table 3. Acid tolerance (AT) indices

Streptococcal strain[a]	Species identification	AT index
EE1	*S. mutans*	0.855
NN1	*S. mutans*	0.657
HH1	*S. sobrinus*	0.644
KK1	*S. mutans*	0.603
RR1	*S. mutans*	0.580
NN2	*S. gordonii*	0.562
UU1	*S. gordonii*	0.558
YY1	*S. mutans*	0.515
FF1	*S. mutans*	0.405
JJ2	*S. mutans*	0.365
DD1	*S. mutans*	0.361
WW1	*S. mutans*	0.355
AA2	*S. mutans*	0.343
XX1	*S. gordonii*	0.343
CC1	*S. mutans*	0.266
GG1	*S. mutans*	0.249
AA1	*S. mutans*	0.247
ZZ1	*S. mutans*	0.221
BB1	*S. mutans*	0.200
FF2	*S. mutans*	0.156
KK2	*S. mutans*	0.129
EE2	*S. gordonii*	0.12
UU3	*S. gordonii*	0.116
TT1	*S. mutans*	0.112
VV2	*S. mutans*	0.089
HH2	*S. mutans*	0.066
Mean acid tolerance		0.351
Standard deviation		0.213

[a]Strains may include MS and non-MS strains, both primary strains as well as minor strains present at $<50\%$ of the total isolates surveyed at any site.

S. gordonii, which has been found to be the primary strain on some non-carious enamel surfaces (example: EE2, NN2 and UU1 primary strains in non-carious enamel collections; Table 3).

PCR determination for presence or absence of S. mutans *virulence genes in primary strains*

PCR was used to confirm the presence or absence of *S. mutans* virulence genes, specifically representative members of the adhesion, acidurity, acidogenesis, and biofilm formation gene groups, in primary MS and non-MS strains. As expected, primary strains AA1, AA2, EE1, NN1, XX2 and GG1, all determined and confirmed to be *S. mutans* by PCR-amplification using *S. mutans*-specific primers, contained in most cases, the complete complement of surveyed MS virulence genes (Table 4). Primary strains EE2, NN2, UU1 and UU3, all determined to be *S. gordonii* by 16S rRNA gene sequencing, lacked many, if not all of the surveyed *S. mutans* virulence genes, consistent with the placement of these strains on non-carious enamel surfaces. Primary strain HH1, identified to be *S. sobrinus*, contained eight out of the 13 *S. mutans*-specific virulence genes surveyed in this study.

The *atpA* gene, which encodes the F_1F_0-translocating ATPase, a major determinant of AT in *S. mutans* (25, 26), was present in intact form in MS primary strains AA1, EE1, GG1, NN1 and XX2, and absent from *S. gordonii* primary strains EE2, NN2 and UU1 (Table 4). Interestingly, *S. gordonii* strain UU3, primary in WSLs, contained an intact *atpA* gene, and may represent a variant strain. In addition, the AA1-AA2 co-primary strains found in equivalent distributions within CLs in patient AA, both contain all surveyed *S. mutans* virulence genes, with the exception of *atpD* present only in AA1 (Table 4).

Discussion

S. mutans retains high cariogenic potential, because of its acid generation and AT properties, and its ability to form biofilms and produce extracellular glycans. *S. mutans* strains produce varying levels of glucosyltransferases (27), and individuals with dental caries have been identified to contain increased MS strain diversity necessary to provide the additional capacity for synthesis of water-insoluble glycans (6, 28, 29). Thus, the genetic diversity of *S. mutans* strains and divergence in cariogenic potential are now considered as important virulence factors of dental caries.

MS genotypic diversity in children with S-ECC

Mitchell et al. (30) proposed that there is a continuous and progressive re-distribution of MS strains occurring over time within the oral cavity of patients with S-ECC. The onset and progression of S-ECC are associated with several risk determinants, including caregivers containing high MS bacterial numbers or untreated CLs, frequent ingestion of sucrose-containing food or drink, and poor oral hygiene practices. In totality, these determinants can lead to MS colonization at an earlier age, and increased MS strain diversity at higher numerical levels in children with S-ECC compared to caries-free children (6, 9, 28–31).

In the current study, patients with S-ECC exhibited 1–6 streptococcal genotypic strains. Single primary MS genetic strains have been identified to concurrently exist at multiple sites within 11 of the 20 children. This is consistent with our previous reports (21, 22), where we observed MS strain diversity in children with S-ECC and demonstrated that dental rehabilitation therapy can lead to the appearance of single primary MS strains. The strain diversity observed in the current study is also consistent with findings from other investigators (32, 33), who reported that caries-active individuals contain diverse MS genotypes and hypothesized that changes in MS strain diversity may be influenced by increased

Table 4. Distribution of putative virulence genes in select primary strains

		Presence of PCR amplicon of expected size												
		Adhesion[a]					Acidogencity[b]	Acidurity[c]	Biofilm formation[d]					
Strain	Species identification	*gtfB*	*gtfC*	*gbpA*	*gbpB*	*gbpC*	*gbpA*	*glgA*	*atpA*	*comD*	*comE*	*comX*	*ciaH*	*luxS*
AA1	*S. mutans*	+	+	+	+	+	+	+	+	+	+	+	+	+
AA2	*S. mutans*	+	+	+	+	+	+	+	−	+	+	+	+	+
EE1	*S. mutans*	+	+	+	+	+	+	+	+	+	+	+	+	+
EE2	*S. gordonii*	+	−	−	+	+[e]	−	+	−	+	+	−	−	+
GG1	*S. mutans*	+	+	+	+	+	+	+	+	+	+	+	+	+
HH1	*S. sobrinus*	+	−	+	+	+	−	+	−	+	+	−	−	+
NN1	*S. mutans*	+	−	−	+	+	+	+	+	+	+	+	+	+
NN2	*S. gordonii*	−	−	+	−	−	−	−	−	−	−	−	−	−
UU1	*S. gordonii*	−	−	−	−	−	−	−	−	−	−	−	−	−
UU3	*S. gordonii*	−	−	−	−	−	−	−	+	−	+	−	−	−
XX2	*S. mutans*	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]Adhesion genes
gtfB – glucosyltransferase GTF-I
gtfC – glucosyltransferase GTF-SI
gbpA – glucan-binding protein A
gbpB – glucan-binding protein B
gbpC – glucan-binding protein C
gbpD – glucan-binding protein D
[b]Acidogenicity gene
glgA – glycogen synthase
[c]Acidurity gene
atpA – F-ATPase proton pump
[d]Biofilm formation genes
comD – competence histidine kinase
comE – competence response regulator
comX – competence alternate sigma
ciaH – competence histidine kinase factor
luxS – enzyme involved in synthesis of AI-2
[e]PCR amplicon of variant size.

acidity in the oral environment. In addition, Napimoga et al. (6) proposed that MS strain diversity may be influenced by immunological exposure to bacterial antigens, and that immune response and generation of salivary IgA antibodies would result in the elimination of specific MS strains. We are also unable to rule out the possibility that species and strain diversity, both MS and non-MS streptococci strains, within individual patients, may be due to structural irregularities in dental anatomy at the site of plaque collection, specifically on the buccal or lingual surfaces of the anterior teeth. These structural irregularities may create environmental micro-niches that allow the growth of different oral microorganisms.

There is also a possibility that our patients may have received previous caries restorative therapies at other dental clinics, and thus may have undergone reductions in MS strain diversity or MS numbers prior to their visit in our clinic. In addition, we do not know if our patients conducted plaque control at home, and therefore we are uncertain if the use of toothpastes or rinses had impacted the distribution or diversity of the streptococcal primary strains.

Caries-free children were not recruited for the current study because we believed that the study focus, to compare MS strain diversity at carious and non-carious sites, was internally controlled within the dentition of single individuals. But we acknowledge that this may represent a valid experimental group in examining the preferred localization of primary non-MS strains, such as *S. gordonii*, on sound enamel surfaces. We also conducted conventional PCR using template DNA obtained from several primary strains, with primers directed against specific *S. mutans* virulence gene sequences. The objectives of conducting conventional PCR were to help identify non-*S. mutans* isolates, later verified by 16S rRNA gene sequencing, and/or to begin identifying isolates with variations in *S. mutans*

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virulence gene sequence. To a large degree, this was a successful strategy in distinguishing *S. mutans* from non-MS streptococci. For example, EE1 and NN1, which contained a majority of the surveyed *S. mutans* virulence genes detected by PCR, were readily distinguished from EE2 and NN2, which lacked many of the *S. mutans* virulence genes and were later identified as *S. gordonii* by 16S rRNA gene sequencing (see Table 4). We also understand that the use of conventional PCR to identify the presence of *S. mutans* virulence genes may not have the ultimate precision of next-generation sequencing, and may not be able to detect small variations in sequence, or may undergo primer mis-annealing if the sequence variation occurred in the primer binding region.

We understand that the current study examined limiting numbers of patients, but believe that the study findings are statistically validated by Cheon et al. (34), who used probabilities to assess the number of MS isolates from single collections that are required to successfully evaluate strain diversity. Cheon et al. (34) proposed that screening seven MS isolates from any one specimen collection was sufficient for detecting up to four MS genetic strains with a 78% probability of success, and screening 10 isolates, as we have conducted in our study, would have a 95% probability of success for the detection of 3–4 genetic strains.

Streptococcal antagonism in oral biofilms

The oral biofilm contains a diverse polymicrobial population consisting of more than 500 identified species (12–14, 35). Streptococcal species constitute approximately 20% of the total oral bacteria, and undergo dynamic re-distributions within the oral cavity that are highly dependent on the biochemical and metabolic activities of the individual microorganisms (36, 37). The oral streptococcal species as a group retains similar metabolic activities, including the ability to metabolize assorted carbohydrates, produce antagonistic antimicrobial compounds, and compete for the same adherence sites on tooth surfaces (12–14). When present in high numbers, the pioneer colonizer *S. gordonii* can antagonize *S. mutans* with noxious hydrogen peroxide, while the latter cariogenic microorganism can produce bacteriocidins that can effectively eliminate *S. gordonii* and other oral streptococci, allowing unencumbered growth and formation of a highly cariogenic biofilm (12–14). In this study, we describe the first report of the presence of *S. mutans* and *S. gordonii* as primary streptococcal strain pairs, independently placed on distinct sites within the dentition, with *S. mutans* found primarily in CLs and *S. gordonii* on sound enamel surfaces. Interestingly, weakly acid-tolerant (or more acid-sensitive) *S. gordonii* stains, such as EE2 and UU3, are capable of establishing independent sites within patients EE and UU, who contain cariogenic MS strains at other carious sites. The non-cariogenic EE2 and UU3 strains may be located within environmental micro-niches containing reduced acidity. Even though the purpose of this study was to focus on MS and non-MS streptococci strain diversity, we also understand that several other bacterial species, including lactobacilli, *Actinomyces*, *Prevotella*, and *Scardovia*, have been implicated in dental caries, and may be complementing the cariogenic activity of MS or be present in independent micro-niches on the tooth surface leading to the development of CLs. In addition, non-cultivable bacterial species, which were not analyzed in our study, are known to exist in the oral biofilm, and may also play currently undefined roles in the development of dental caries.

There is increasing evidence that *S. gordonii* can diminish the virulence properties of *S. mutans*, and can influence bacteriocin production, genetic transformation, and generation of biofilm (38, 39). *S. gordonii* (Challis strain) secretes a protease (challisin) that can degrade competence-stimulating peptide (CSP), which serves as a quorum-sensing regulator required for bacteriocidin production in *S. mutans*. In addition, *S. mutans* requires CSP for maximal transformation, which is used in the expansion of virulence potential and permits scavenging of essential purine and pyrimidine metabolites and enhanced survival. Thus, under conditions of limiting nucleotide metabolites, the ability of *S. gordonii* to inhibit the genetic transformation of *S. mutans* may provide the former microorganism with a competitive survival advantage. Furthermore, *S. gordonii* may inhibit sucrose-dependent biofilm generation, which is an important determinant in the adhesion of *S. mutans* to hydroxyapatite tooth surfaces. Kuramitsu and Wang (38) also hypothesized that CSP may play a role in acidurity and could potentially be an important factor in the ability of *S. mutans* to survive in highly acidic environments (38). All of these factors may have influenced the ability of *S. gordonii* to become primary strains in patients examined in our study, and may have applicability in the control of human dental caries and S-ECC.

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Conflict of interest

There is no conflict of interest in the present study for any of the authors.

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