Distribution of Putative Virulence Genes in Streptococcus mutans Strains Does Not Correlate with Caries Experience

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Streptococcus mutans is a member of the human oral flora, is a widely recognized etiological agent of dental caries. The cariogenic potential of S. mutans is related to its ability to metabolize a wide variety of sugars, form a robust biofilm, produce copious amounts of lactic acid, and thrive in the acid environment that it generates. The remarkable genetic variability present within the species is reflected at the phenotypic level, notably in the differences in the cariogenic potential between strains. However, the genetic basis of these differences is yet to be elucidated. In this study, we surveyed by PCR and DNA hybridization the distribution of putative virulence genes, genomic islands, and insertion sequences across a collection of 33 strains isolated from either children with severe early childhood caries (S-ECC) or those who were caries free (CF). We found this genetically diverse group of isolates to be remarkably homogeneous with regard to the distribution of the putative virulence genes and genetic elements analyzed. Our findings point to the role of other factors in the pathogenesis of S-ECC, such as uncharacterized virulence genes, differences in gene expression and/or enzymatic activity, cooperation between S. mutans strains or with other members of the oral biota, and host factors.

S. mutans is a member of the human indigenous oral microbiome commonly associated with the etiology of dental caries (43, 77). The propensity of S. mutans to initiate caries (i.e., its virulence) likely results from a combination of attributes and not from a single virulence factor (4, 34). More specifically, S. mutans utilizes dietary sucrose to produce glucans that facilitate cell-to-cell adhesion and capsule formation, thus promoting attachment to the tooth surface and the formation of a resilient biofilm. In addition, the bacterium can uptake and metabolize various carbohydrates, with the concomitant production of lactic acid (acidogenicity), which erodes dentin and enamel. S. mutans thrives in this acidic environment (acidurance), which may confer a selective advantage over other members of the dental biofilm (for a comprehensive review, see reference 8).

Several genes associated with these virulence attributes have been identified (4, 34) and, in some cases, exhaustively studied with the expectation that they might be key to the cariogenic potential of S. mutans. For some of these genetic loci, a more direct connection to virulence has been implied by studies relying on gene inactivation followed by in vitro assays (37, 41, 49, 70) or by virulence testing in animal models (23, 69, 87). Remarkable intraspecies genetic variability of S. mutans was first revealed by restriction enzyme fingerprinting (12, 33) and, more recently, by multilocus sequence type (MLST) (17, 52), comparative genome hybridization (89), and the comparison of two sequenced genomes (1, 47). In addition, several studies have documented S. mutans genetic variability in individual genes (54, 56, 82). As a result of the differences in their genetic repertoire, S. mutans strains also exhibit phenotypic variability (3, 16, 20, 36, 53). Notably, the evidence suggests that not all the strains are equally virulent in promoting dental caries (18, 30). It seemed reasonable to expect that at least some of the virulence-related genes would be unequally distributed in strains according to caries status; i.e., strains associated with caries might possess a greater number of virulence attributes than strains isolated from caries-free (CF) individuals.

A previous study failed to identify genes that defined caries status by comparing isolates from children with high and low levels of caries to reference strain UA159 (89). The association between S. mutans virulence and caries may be less ambiguous with one form of caries called rampant or severe early childhood caries (S-ECC). Most studies support the role of S. mutans (5, 21, 80), with some reporting S. mutans levels as high as 40% or more of the total cultivable biota (80). We reasoned that if the distribution of virulence traits did differ, the difference would be most apparent in a comparison between strains from children with rampant caries and caries-free children. In this respect, our group previously identified by suppressive subtractive hybridization a set of five genetic biomarkers that could classify a collection of strains from children with S-ECC or caries-free children according to their caries status with a 92% accuracy (66). However, when these biomarkers were restested on a new set of isolates, they failed to properly classify them (unpublished results).

The aim of this study was to detect distinct differences in the distribution of putative virulence genes from S. mutans described in the literature between two disparate caries groups, young children with rampant caries (S-ECC) and a caries-free matched control group (CF). Pronounced differences in the distributions of putative virulence genes between the two groups would support the hypothesis that strains of S. mutans differ both in gene content and in propensity to initiate dental caries.
RESULTS

Genetic diversity. Thirty-three S. mutans isolates representing unique genotypes were collected, 19 from S-ECC children and 14 from CF children, all of Hispanic origin. CDF profiles generated with HaeIII averaged 22.5 ± 5.7 discrete bands above a 3.5-kb cutoff (Fig. 1). All isolates displayed a 14-kb band common to all serotype c strains (13). The highest similarity between two genotypes was 90%, and the lowest similarity was 58%, confirming that our isolates were genotypically diverse. Similarity comparisons of banding profiles showed no evidence of clustering according to caries status. A cryptic plasmid was not detected in any of the 33 isolates, neither by the CDF profiles nor by PCR amplification of the plasmid hypervariable region (HVR) (11).

Distribution of S. mutans putative virulence genes. We assayed the distribution of 31 chromosomally encoded putative S. mutans virulence genes by PCR. Genes spanning different virulence attributes, e.g., adhesion, acidogenicity, acidurty, biofilm formation, and bacteriocin production, were selected from the literature (Table 1). We also included genes representing different mechanisms/pathways, e.g., sucrose-dependent and sucrose-independent adhesion, phosphotransferase system (PTS) and non-PTS sugar transport systems, and different acid tolerance mechanisms. Primers internal to each gene were designed to yield amplicons of between 200 and 600 bp (see Table S1 in the supplemental material). Twenty-five of these genes were present in 100% of the isolates (Table 1). In contrast, we did not detect the cnm gene, encoding collagen-binding protein, in any of the 33 isolates. This was confirmed by dot blot hybridization with a cnm probe amplified from strain AF199 (67) (data not shown).

The presence of structural genes for either mutacin I or (Ambion) according to the manufacturer's instructions and after exposure to BioMax X-ray films (Kodak, Carestream Health, Rochester, NY).

Southern blot hybridizations were employed to determine the number and distribution of insertion sequences (IS) across the bacterial isolates. The chromosomal DNA of the isolates (1 µg each) was digested for 4 h at 37°C with 2.5 U of the NsiI restriction enzyme (New England Biolabs, Ipswich, MA), in a final volume of 20 µL. The digested DNA was separated by electrophoresis on a 0.8% agarose gel and blotted onto a BrightStar-Plus positively charged nylon membrane (Ambion) using a vacuum blotted, model 785 (Bio-Rad). The membranes were then cross-linked, prehybridized, hybridized with the probe, and detected in the same way as that described for the dot blot.

Bioinformatic and statistical analyses. AP-PCR and CDF profiles were normalized and analyzed using BioNumerics version 6.0 (Applied Maths, Belgium).

Bands were predicted from standardized density curves, with minimum profiling set to 4% for both AP-PCR and CDF profiles, and used uniformly across samples and gels. Similarity matrices of pairwise comparisons for AP-PCR and CDF were evaluated using the Dice coefficient algorithm with optimization set to 1% and tolerance to 0.5%. Clustering of the isolates was based on the unweighted pair group method with arithmetic mean (UPGMA).

Evolutionary relatedness that might support clonal clustering among strains based on disease status was inferred from maximum likelihood (ML) modeling as implemented in BioNumerics. Top-score tree and bootstrap values were calculated by bootstrap resampling of 100 replicates. Independent analyses with different evolutionary models were carried out on the individual nucleotide and amino acid data sets and also on the concatenated amino acid sequences.

S. mutans genes homologous to well-characterized group A streptococcal virulence genes (50) were identified by BLASTP (2) of the group A streptococcus (GAS) protein sequences to a nonredundant protein database of S. mutans coding sequences (CDS). Ten hits with E values of 2 × 10⁻⁶ or smaller were selected. The corresponding genes were then identified in the genome sequence of S. mutans strain UA159.

MATERIALS AND METHODS

Study population. Thirty-three children of Hispanic origin (age 3 to 8 years) were included in this study. Nineteen children were diagnosed by pediatric dental specialists with rampant caries or severe early childhood caries (S-ECC) and scheduled for extensive caries restorative treatment under general anesthesia in the operating room at the Bellevue Hospital, New York, NY. Fourteen children were diagnosed as being free from detectable caries (caries-free [CF]) and constituted the control group. The study protocol for human subjects was approved by the Institutional Review Board of New York University School of Medicine and Bellevue Hospital.

Bacterial sample collection and processing. Bacterial samples from saliva and pooled plaque of the S-ECC and CF children were collected as previously described (11, 40). Additionally, for S-ECC children, four individual plaque samples were obtained from caries lesions of four posterior quadrants of the dentition with a sterile round bur (29). S. mutans isolates were selected from mitis salivarius-bacitracin agar medium (MSB) based upon colony morphology (22) and confirmed by their fermentation profiles (39) and by PCR with species-specific primers (13). Approximately 10 isolates of S. mutans per individual were pure cultured onto Todd-Hewitt agar and preserved at −70°C for later genetic characterization. The genomic DNA of single isolates was obtained by using a genomic DNA purification kit (Qiagen, Hilden, Germany). Genotyping of isolates was carried out by arbitrarily primed PCR (AP-PCR) with primer OPA-02 (38) and chromosomal DNA fingerprinting (CDF) with restriction endonuclease HaeIII, as previously reported (12). Fifty-one genotypically unique S. mutans isolates were initially identified. Both S-ECC and CF subjects presented 1 to 3 unique genotypes, with the majority of the subjects harboring a single genotype.

We limited our study to a single isolate from each patient to ensure both parity and independence among subjects and samples, yielding a final sample size of 33 isolates.

PCR amplification and sequencing. To screen for the presence or absence of putative virulence genes across our collection of S. mutans isolates, PCR primers internal to each of the target coding sequences were designed with the Primer3 software (62) based on the UA159 genome sequence. Primers are shown in Table S1 in the supplemental material. PCR amplification was carried out in a Mas-tercyler (Eppendorf, Hamburg, Germany) in a 25-μL reaction mixture containing 1× PCR buffer, 1.5 mM MgCl₂, 200 nM each 2 ng each forward and reverse primer, 1.25 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 25 ng of genomic DNA. PCR conditions were typically as follows: one initial denaturation at 94°C for 3 min and then 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were resolved by electrophoresis on 1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer and stained with SybrSafe (Invitrogen). Results were captured with an Alpha IS-1000 digital imaging system (Alpha Inno- tech Corp., San Leandro, CA).

The gfb/gfc/c locus was amplified with the Expand Long Template PCR system (Roche, Mannheim, Germany) by following the manufacturer's instructions and the following PCR conditions: an initial denaturation at 94°C for 3 min and then 10 cycles of 94°C for 30 s, 55°C for 90 s, and 68°C for 8 min, followed by 20 cycles of 94°C for 30 s, 55°C for 90 s, and 68°C for 9 min, with a final extension at 68°C for 7 min. Amplicons were resolved in 0.7% agarose-TAE gels.

PCR products targeted for sequencing were purified with a PCR purification kit (QIAquick, Qiagen, Hilden, Germany) and resuspended in distilled water (UltraPure, Invitrogen). Amplicons were sequenced in both directions at Ge-newiz (South Plainfield, NJ).

DNA hybridization procedures. Dot blot hybridizations were used to validate PCR results for select genes. The chromosomal DNA of the isolates (150 ng each) was heat denatured for 10 min at 99°C in a solution of 0.4 M NaOH, 1 M NaCl, and 200 mM EDTA (pH 8) and quickly chilled in ice/water slush. Subsequently, the DNA was spotted onto a BrightStar-Plus positively charged nylon membrane (Ambion, Applied Biosystems, Austin, TX), using a Bio-Dot microfiltration apparatus and a vacuum pump (both from Bio-Rad, Hercules, CA). The DNA was UV cross-linked to the membrane with a Stratalinker (Stratagene, Santa Clara, CA). Membranes were air dried before prehybridization with 7 ml of prewarmed ULTRAhyb buffer (Ambion) for 30 min at 42°C in a rotating hybridization oven. Probes were PCR amplified as described above. The amplicons were purified with the QIAquick PCR purification kit (Qiagen) and biotin labeled with the BrightStar psoralen-biotin nonisotopic labeling kit (Ambion) by following the manufacturer’s indications. Hybridization with the probe was carried out at 42°C for 16 h. The biotinylated probe was detected with the BrightStar BioDetect nonisotopic detection kit (Ambion) according to the manufacturer's instructions and after exposure to BioMax X-ray films (Kodak, Carestream Health, Rochester, NY).

Southern blot hybridizations were employed to determine the number and distribution of insertion sequences (IS) across the bacterial isolates. The chromosomal DNA of the isolates (1 µg each) was digested for 4 h at 37°C with 2.5 U of the NsiI restriction enzyme (New England Biolabs, Ipswich, MA), in a final volume of 20 µL. The digested DNA was separated by electrophoresis on a 0.8% agarose gel and blotted onto a BrightStar-Plus positively charged nylon membrane (Ambion) using a vacuum blotted, model 785 (Bio-Rad). The membranes were then cross-linked, prehybridized, hybridized with the probe, and detected in the same way as that described for the dot blot.

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mutacin II was rare across these isolates (less than 10%) (Table 1), while mutA for mutacin III was not detected in any of the strains tested. An amplicon spanning the *nlmA* and *nlmB* genes encoding the nonlantibiotic mutacin IV was detected for 63.2% of the S-ECC isolates and 57.1% of the CF isolates, showing no remarkable differences in the distribution between the two groups (Table 1).

**Analysis of gtfB and gtfC genes.** The glucosyltransferases (GTFs) encoded by the *gtfB* and *gtfC* genes are thought to play a key role in the cariogenicity of *S. mutans* (34, 87). A previous study suggested that *S. mutans* strain UA101 underwent a homologous recombination event between the tandemly arranged *gtfB* and *gtfC* genes that resulted in a fusion *gtfBC* gene (88). Notably, strain UA101 also exhibited low levels of smooth caries activity in rats fed on a high-sucrose diet, likely as a result of the reduced synthesis of insoluble glucan exhibited by cells carrying the single *gtfBC* gene (88). We thus investigated the presence of the fusion *gtfBC* gene in our collection by long-range PCR (25) and found that both S-ECC and CF isolates yielded an amplicon consistent with the presence of both *gtfB* and *gtfC* genes (see Fig. S1 in the supplemental material). We independently confirmed the PCR results by Southern hybridization of PstI-digested chromosomal DNA with a probe derived from a conserved region between *gtfB* and *gtfC* (data not shown). Taken together, our results suggested that the presence of the tandemly arranged *gtfB* and *gtfC* genes or the *gtfBC* fusion is not correlated with caries status.

In addition to the variability in the number of *gtf* genes, a DNA region rich in sequence polymorphisms has been previously identified for both the *gtfB* and *gtfC* genes, and it was
suggested that the corresponding protein region might be important for the enzymatic activity of the GTFs (14). We investigated whether there was a clonal underpinning in either of the contiguous \textit{gtfB} and \textit{gtfC} genes that clustered our population of isolates according to caries status. A 132-nucleotide (nt) region, spanning from positions 1144 to 1275 of the \textit{gtfB} gene and from positions 1222 to 1352 of the \textit{gtfC} gene, was sequenced for 10 S-ECC isolates and 10 CF isolates. Twenty-four and 25 polymorphic sites were identified for \textit{gtfB} and \textit{gtfC}, respectively (not shown), the vast majority representing silent mutations. The only nonsynonymous nucleotide substitution resulted in an amino acid change at position 399, as previously described by Chia and coworkers (14). Using both distance (neighbor-joining and minimum evolution) and optimality criteria (maximum parsimony and maximum likelihood) modeling on both the nucleotide and amino acid data sets, we found no evidence of a population structure that might suggest a correlation between caries status and the evolutionary history of the \textit{gtfB} or \textit{gtfC} gene, at least with the fragments we analyzed (see Fig. S2 in the supplemental material).

\textbf{Distribution of other potential virulence genes.} The difference in cariogenicity between \textit{S. mutans} isolates could also be connected to gene products that have not been evaluated to date. For this reason, we searched the \textit{S. mutans} UA159 genome for other putative virulence genes and found the following distribution of putative virulence genes across \textit{S. mutans} isolates:

\begin{table}[h]
\centering
\caption{Distribution of putative virulence genes across \textit{S. mutans} isolates}
\begin{tabular}{llll}
\hline
\textbf{Locus} & \textbf{Gene product} & \textbf{\% of isolates that yielded a PCR amplicon of the expected size} & \textbf{Reference(s)} \\
\hline
\textbf{S. mutans putative virulence genes} & & & \\
\textbf{Adhesion} & \textit{gtfB}, \textit{gtfC} & Glucosyltransferases GTF-I and GTF-SI & 100 100 87 \\
& \textit{gpbA} & Glucan-binding protein A & 100 100 25 \\
& \textit{gpbB} & Glucan-binding protein B & 100 100 48 \\
& \textit{gpbC} & Glucan-binding protein C & 100 100 48 \\
& \textit{gpbD} & Glucan-binding protein D & 100 100 71 \\
& \textit{wapA} & Wall-associated protein A & 100 100 90 \\
& \textit{spa} & Antigen I/II & 100 100 15 \\
& \textit{cmm} & Collagen-binding protein & 0 0 64 \\
& \textit{eno} & Enolase & 100 100 26 \\
\textbf{Acidogenicity} & \textit{glgA} & Glycogen synthase & 100 100 10 \\
& \textit{dltC} & D-Alanyl carrier protein & 100 100 7, 75 \\
& \textit{scrA} & Enzyme II(sc) & 100 100 81 \\
& \textit{scrB} & Sucrose-6-phosphate hydrolase & 100 100 81 \\
& \textit{msmF} & Integral membrane protein, transport & 100 100 78 \\
& \textit{fruA} & Fructanase & 100 100 9 \\
& \textit{fft} & Fructosyltransferase & 100 100 69 \\
\textbf{Acidity} & \textit{atpA} & F-ATPase proton pump & 100 100 32 \\
& \textit{fabM} & \textit{trans}-2, \textit{cis}-3-Decenoyl-ACP isomerase & 100 100 19 \\
& \textit{sloR} & Metallorregulatory protein & 100 100 61 \\
& \textit{vicK} & Histidine kinase & 100 100 76 \\
& \textit{hisA} & Serine protease/chaperone & 100 100 28 \\
\textbf{Biofilm formation} & \textit{comD} & Competence histidine kinase & 100 100 41 \\
& \textit{comE} & Competence response regulator & 100 100 41 \\
& \textit{comX} & Competence alternate sigma factor & 100 100 41 \\
& \textit{ciaH} & Competence histidine kinase & 100 100 59 \\
& \textit{luxS} & Enzyme involved in synthesis of AI-2 & 100 100 84 \\
\textbf{Mutacin production} & \textit{mua} (I) & Mutacin I & 10.5 7.1 57 \\
& \textit{mua} (II) & Mutacin II & 5.3 0 86 \\
& \textit{mua} (III) & Mutacin III & 0 0 58 \\
& \textit{nlmAB} (IV) & Mutacin IV & 63.2 57.1 56 \\
\textbf{Other putative virulence genes} & \textit{pgk} & Putative phosphoglycerate kinase & 100 100 50 \\
& \textit{adcA} & Putative surface adhesin/Zn-binding lipoprotein & 100 100 50 \\
& \textit{lytC} & Putative hemolysin & 100 100 50 \\
& \textit{gdpC} & Putative glyceraldehyde-3-phosphate dehydrogenase & 100 100 50 \\
& \textit{pulA} & Putative pullulanase & 100 100 50 \\
& \textit{Smu.399} & Putative C3-degrading proteinase & 100 100 50 \\
& \textit{Smu.583} & Putative hemolysin & 100 100 50 \\
& \textit{Smu.761} & Putative collagenase & 100 100 50 \\
& \textit{Smu.940c} & Putative hemolysin III & 100 100 50 \\
& \textit{Smu.1449} & Putative fibronectin/fibrinogen-binding protein & 100 100 50 \\
& \textit{Smu.2130} & Putative exfoliative toxin & 100 100 50 \\
\hline
\end{tabular}
\end{table}
neme for homologues to well-characterized GAS virulence attributes (50) through a BLASTP search. We selected 10 genes with homology to GAS adhesins, fibronectin-binding proteins, proteases, and toxins and evaluated their distribution across the clinical isolates. We also included an S. mutans gene coding for a putative collagenase (Smu.761) (85). All 11 genes were present in 100% of the isolates (Table 1), showing no clear association with caries status.

**Distribution of S. mutans genomic islands.** Genomic islands (GI) are large but discrete DNA segments, usually acquired by horizontal gene transfer, which become integrated in the bacterial chromosome (27). Their presence varies between closely related strains and often confers a selective advantage to the host. Eleven potential GI have been identified in the genome sequence Database [http://www.oralgen.lanl.gov/], many of which are related to virulence or fitness. For this reason, it was of interest to analyze the occurrence of these GI in the collection of S-ECC and CF isolates.

Primers specific for one or more genes in each of nine GI were designed based on the UA159 sequence information (see Table S1 in the supplemental material). All of the isolates yielded amplicons of the expected size for genes representative of GI 1, 3, 9, 10, and 15 (Table 2), which suggests that these GI are ubiquitous across our collection. Amplicons representative of GI 4, 6, and 7 were obtained for 57%, 21%, and 88% of the isolates, respectively, and their distribution was not strongly associated with caries status (Table 2). The analysis of the distribution of four different genes within the large GI 12 (TnSmu2) (1) revealed the existence of variability in gene content across the isolates; i.e., some isolates yielded the expected amplicon for all four genes, others showed various combinations of 1 to 3 genes, and a few isolates did not yield an amplicon for any of them (not shown). This suggests that, while most isolates contained at least parts of GI 12, there was substantial sequence variation. However, the distribution of these four genes did not appear to be correlated with caries status (Table 2). In summary, we found no evidence of an association between the distribution of nine S. mutans GI and caries status.

**Distribution of S. mutans insertion sequences.** Insertion sequences (IS) are short, genetically compact sequences that usually encode only the functions required for their own mobility (46). Importantly, IS can affect the expression of genes by insertion or by promoting deletions and rearrangements (46). Importantly, IS can affect the expression of genes by insertion or by promoting deletions and rearrangements (46). Importantly, IS can affect the expression of genes by insertion or by promoting deletions and rearrangements (46). Importantly, IS can affect the expression of genes by insertion or by promoting deletions and rearrangements (46). Importantly, IS can affect the expression of genes by insertion or by promoting deletions and rearrangements (46).

### Table 2. Distribution of S. mutans GI across S. mutans isolates

| GI ID | Descriptiona | ORF tested | % of isolates that yielded a PCR amplicon of the expected size | S-ECC (n = 19) | CF (n = 14) |
|-------|--------------|------------|---------------------------------------------------------------|---------------|------------|
| 1     | Cell division| Smu.12     | 100 100                                                       | + (2)         | + (1)      |
| 3     | Purine metabolism | Smu.51 | 100 100                                                       | -             | -          |
| 4     | Sorbose PTS   | Smu.105    | 47.4 71.4                                                     | -             | -          |
| 6     | Transposon    | Smu.208    | 21.1 21.4                                                     | -             | -          |
| 7     | Sugar metabolism/transport | Smu.267c | 89.5 85.7                                                     | + (1)         | + (1)      |
| 9     | Virulence or fitness | Smu.591c | 100 100                                                       | -             | -          |
| 10    | Acid tolerance| Smu.1060   | 100 100                                                       | -             | -          |
| 12    | Bacitracin synthesis | Smu.1334 | 57.9 71.4                                                     | -             | -          |
| 15    | Bacteriocin related | Smu.1913 | 100 100                                                       | -             | -          |

* Based on annotation available at http://www.oralgen.lanl.gov/.

### Table 3. Distribution of IS across S. mutans isolates

| Strain ID | Presence (+) or absence (-) (no. of copies)* of: | S-ECC (n = 19) | CF (n = 14) |
|-----------|--------------------------------------------------|---------------|------------|
|           | ISSmu1 ISSmu2 ISSmu5 IS199                        |               |            |
| S-ECC     | 8 + (2) + (1) - + (2)                             |               |            |
|           | 10 - + (1) - + (1)                                |               |            |
|           | 11 - + (1) - + (1)                                |               |            |
|           | 13 - + (1) - -                                    |               |            |
|           | 14 - - -                                         |               |            |
|           | 15 - - -                                         |               |            |
|           | 17 - - -                                         |               |            |
|           | 19 + (1) + (1) - + (2)                            |               |            |
|           | 20 - - -                                         |               |            |
|           | 21 - - -                                         |               |            |
|           | 22 - + (2) -                                     |               |            |
|           | 23 + (1) -                                       |               |            |
|           | 24 + (23) + (1) -                                |               |            |
| Total S-ECC (%) | 31.6 52.6 5.3 31.6                              |               |            |
| CF        | 1 - + (1) - + (1) -                              |               |            |
|           | 3 - + (2) -                                       |               |            |
|           | 4 - - -                                          |               |            |
|           | 6 - + (1) -                                       |               |            |
|           | 7 - + (1) + (2) + (7)                             |               |            |
|           | 25 - - -                                         |               |            |
|           | 26 - + (1) -                                     |               |            |
|           | 27 - + (1) -                                     |               |            |
|           | 28 - - -                                         |               |            |
|           | 29 + (1) + (1) + (10) + (23)                     |               |            |
|           | 30 - - -                                         |               |            |
|           | 31 - + (2) -                                     |               |            |
|           | 32 - - -                                         |               |            |
|           | 33 - - -                                         |               |            |
|           | 34 - - -                                         |               |            |
|           | 35 + (1) + (1) + (10) + (23)                     |               |            |
|           | 37 + (2) + (1) -                                 |               |            |
|           | 42 + (23) + (1) -                                |               |            |
| Total CF (%) | 0 57.1 7.1 35.7                                 |               |            |
| Total (%) (n = 33) | 18.2 54.5 6.1 33.3                              |               |            |
| UA159     | + (6) + (1) -                                   |               |            |

* Detected by Southern hybridization.
and 3% of the isolates, respectively (Table 3). More importantly, even though ISSm1 seemed to be overrepresented in the S-ECC strains, the distributions of ISSm1, ISSm2, ISSm5, and IS199 did not appear strongly associated with the caries status of the isolates.

We also analyzed the location of ISSm1 and ISSm2 in our collection of isolates by PCR with primers designed on the basis of the sequences flanking the insertion sites of the six ISSm1 copies and the single ISSm2 copy in the UA159 genome (55) (see Table S1 in the supplemental material). All isolates but one yielded an amplicon of the expected size if no IS was present, in contrast to the bigger amplicon size observed for UA159 and one clinical isolate, which was consistent with the presence of ISSm1 or ISSm2 (not shown). Therefore, the locations of ISSm1 and ISSm2 in the genome of most clinical isolates are different from those in the genome of UA159.

**DISCUSSION**

In this study, we surveyed the distribution of a battery of putative virulence genes and other genetic elements selected from the literature across a collection of 33 strains isolated from children with rampant caries or free from detectable caries, with the aim of identifying genetic elements associated with disease. The distributions of the genetic elements tested did not support an association with caries status. Although *S. mutans* displays intraspecies genetic diversity (Fig. 1) (12, 17, 52), we observed remarkable homogeneity with regard to the distribution of the putative virulence genes (Table 1). We did find differences in the distribution of the structural genes encoding mutacins I, II, and IV, as well as of foreign DNA elements, such as genomic islands 4, 6, 7, and 12 (Table 2) and insertion sequences ISSm1, ISSm2, ISSm5, and IS199 (Table 3), but our results did not suggest a strong association with caries status.

Intraspecies genetic variability has been commonly found in bacteria, either as genes that are shared by some but not all isolates or as strain-specific genes that are unique to each isolate (51). They constitute the dispensable genome, while those genes shared by all strains within a species are regarded as the core genome. In *S. mutans*, comparison of the genome sequences of strains UA159 and NN2025 revealed that these two strains differ in 10% of the genes (47), while 20% of the open reading frames (ORFs) in strain UA159 have been estimated to belong to the dispensable genome by a DNA hybridization-based comparison with nine other strains (83). In addition, comparative genome hybridization of 11 strains showed that 16.6% of the ORFs included in the microarray were missing in at least one of the genomes (89). Likewise, comparisons between three strains of either *Streptococcus pyogenes*, *Streptococcus thermophilus*, or *Streptococcus agalactiae* showed that approximately 25% of their genes belonged to the dispensable genome (35). Our finding that most of the genes tested are present in all 33 isolates suggests that they may be part of the core genome. This is not surprising in the case of genes involved in carbon metabolism (Table 1, acidogenicity genes), since housekeeping genes are usually overrepresented in the core genome (79). Virulence-related genes, on the other hand, would be expected to reside in the dispensable genome if differences in the virulences between strains stemmed from differences in the genetic repertoires.

Strain-to-strain variability in gene content has been previously reported for various *S. mutans* genes. For example, the *gbpA* gene encoding a glucan-binding protein was previously found in all but five isolates from a collection of 39 laboratory and clinical strains (82). In our study, however, the *gbpA* gene was present in all the isolates tested (Table 1), in concordance with results from another study with 11 strains (89). We found the genes for mutacin I, II, and IV to be present in 9%, 3%, and 60% of the isolates, respectively, while the gene for mutacin III was not detected in any of them (Table 1). This is in agreement with previous studies, which found the mutacin IV *nlmA* and *nlmB* genes in 50% of a population of 70 clinical isolates (31), while a study with 19 clinical isolates found that none harbored the genes for mutacin I and III and only one isolate presented the gene for mutacin II (44). On the other hand, the *cmr* gene encoding the collagen-binding protein was not detected in our collection of 33 clinical isolates (Table 1), but two recent studies reported its presence in 9.8% (54) and 21.6% (52) of the strains analyzed, including serotype c strains. Nevertheless, we found the *cmr* gene to be present in strain AF199 (67) by PCR amplification followed by sequencing. These genes are therefore part of the dispensable genome of *S. mutans*, but we found no association with the pathogenesis of S-ECC.

Many of the genes associated with the dispensable genome are encoded in syntenic blocks called genomic islands with different G+C contents than the average of the genome (27). In bacterial pathogens, virulence genes are often clustered into GI termed pathogenicity islands (68). We therefore investigated the distribution of the GI reported for *S. mutans* strain UA159 (http://www.oralgen.lanl.gov/) in relation to the pathogenicity of S-ECC. We detected the presence of genes within GI 1, 3, 9, 10, and 15 in all 33 isolates (Table 2), which suggests that they may confer a selective advantage by increasing fitness more than virulence. Moreover, GI 1, 3, and 10 showed the same map location to that in UA159 (not shown), likely due to the fact that they do not contain mobile elements. On the other hand, GI 4, 6, 7, and 12 were not ubiquitous, but they did not appear to be overrepresented in S-ECC isolates (Table 2). GI 4, which includes genes for sorbose and fructose phosphotransferase systems, was also found to be lacking in some strains in a previous study (89). GI 12 (TnSmu2), a large GI encoding various gramicidin and bacitracin synthetases (1), displayed sequence variation across our collection of isolates, as previously shown by Waterhouse and Russell (82). This reflects a mosaic-like structure, likely related to the presence of multiple insertion sequence transposases. All together, the distributions and locations of the different GI analyzed in this study did not correlate with caries status, and no associations could be made with the pathogenicity of S-ECC.

Our results showed that 64% of the isolates carried at least one IS, suggesting that IS are frequently found in the genome of *S. mutans*. The most commonly found IS was ISSm2 (54.5%), followed by IS199 (33.3%), ISSm1 (18.2%), and ISSm5 (6.1%) (Table 3). These observations are in agreement with those of Old and Russell (55), except that they found ISSm1 to be more abundant than IS199. Importantly, the distribution of ISSm1, ISSm2, ISSm5, and IS199 did
not appear strongly associated with the caries status. ISSmu1 was not detected in any of the CF strains included in this study and thus seemed to be overrepresented in the S-ECC strains (Table 3). However, we tested a few additional strains that had been randomly excluded in this study to ensure independence of the samples and detected ISSmu1 in three CF strains (not shown), which indicated that ISSmu1 is not exclusively found in S-ECC strains.

Insertion sequences may modify the expression of genes by gene inactivation (due to insertion or deletion) or by modulation of gene expression (46, 72). This has been shown to be the case in S. mutans, where an incomplete insertion sequence (ISSmu3) replaces the msn and gal operons in melibiose-negative and galactose-negative strains (60). Additionally, sucrose-resistant revertants from the scrB mutant strain were obtained by sucrose-induced transposition of IS199 into the scrA gene (45). In group B Streptococcus agalactiae, several insertion sequences were found to be associated with virulence (42), e.g., an insertion sequence of the ISJ family that was reported to be present in a hypervirulent strain near genes involved in capsule synthesis (63). Furthermore, in the oral pathogen Porphyromonas gingivalis, the insertion sequence ISJ598 was found interrupting the ORF encoding a 67-kDa outer membrane protein in abscess-forming strains but not in avirulent strains (65). In UA159, ISSmu1 and ISSmu2 are found at intergenic regions, but comparison of the locations of ISSmu1 and ISSmu2 in our clinical isolates to those in strain UA159 showed that in all cases but one the IS are located at different genomic sites (not shown). The precise genomic locations of the insertion sequences remain to be determined.

We found no clear association between any of the genes or genetic elements tested and S-ECC. This is in agreement with a comparative genomic hybridization study of reference strain UA159 and 11 S. mutans isolates from children with high levels of caries and low levels of caries (89), as well as with studies on both group A streptococci (GAS) (50) and group B S. agalactiae (24, 74), which found no significant association between the presence of virulence genes and disease. Both S-ECC and CF isolates inhabit the oral cavity; therefore, at least some of the genes we found to be widespread across our collection may be more related to the fitness of the bacterium, i.e., its growth in the oral niche, than to the propensity of one strain to cause disease (virulence) compared to that of another strain.

Although we cannot exclude the possibility that the pathogenicity of S-ECC relies on one or more genes not included in this study, we provide substantial evidence that the putative virulence genes prevalent in the literature are not distinctly distributed according to caries status. A small sample size could conceal potential differences in the distribution of genetic elements between S-ECC and CF isolates. However, this is an unlikely scenario in view of the high equality in the distributions of loci between the two groups (Table 1).

Other factors that could potentially contribute to the pathogenesis of caries are differences in gene expression (up- or downregulation) of caries-promoting attributes or allelic differences resulting in enzymes with different activities or proteins with different structural properties. We found no such pattern of allelic similarities according to caries group in sequences of two genes linked to S. mutans virulence (34), gtfB and gtfC (see Fig. S2 in the supplemental material). Likewise, none of the isolates harbored the gffBC gene fusion (Table 1; see also Fig. S1 in the supplemental material), so differences in adhesion that may result from carrying either the two tandemly arranged genes or the gene fusion (76) are not fundamental to the development of caries. Finally, S. mutans is a member of the oral biota, and as such it is in constant interaction with many other microorganisms, as well as with host factors, all likely to play a role in caries pathogenesis. The genetic differences that may contribute to differences in virulence of S. mutans strains remain the subject of our research.

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