Comparative Analysis of Gtf Isozyme Production and Diversity in Isolates of *Streptococcus mutans* with Different Biofilm Growth Phenotypes

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Streptococcus mutans **is the main pathogenic agent of dental caries. Glucosyltransferases (Gtfs) produced by these bacteria are important virulence factors because they catalyze the extracellular synthesis of glucans that are necessary for bacterial accumulation in the dental biofilm. The diversity of GtfB and GtfC isozymes was analyzed in 44 genotypes of *S. mutans* that showed a range of abilities to form biofilms in vitro. Several approaches were used to characterize these isozymes, including restriction fragment length polymorphism analysis of the *gtfB* and *gtfC* genes, zymographic analysis of the identified GtfB and GtfC genotypes, and quantitation of isozyme production in immunoblot experiments with specific monoclonal antibodies. A high diversity of *gtf* genes, patterns of enzymatic activity, and isozyme production was identified among the isolates tested. GtfC and, to a lesser extent, GtfB were produced in significantly higher amounts by strains that had high biofilm-forming ability than by strains with low biofilm-forming ability. Biofilm formation was independent of the GtfB and GtfC genotype. Atypical strains that showed an apparent single Gtf isozyme of intermediate size between GtfB and GtfC were also identified. The results indicate that various expression levels of GtfB and GtfC isozymes are associated with the ability of distinct *S. mutans* genotypes to grow as biofilms, strengthening the results of previous genetic and biochemical studies performed with laboratory strains. These studies also emphasize the need to identify factors that control *gtf* gene expression.**

Streptococcus mutans is the major pathogen of dental caries. One of its most intensively studied virulence factors is the ability to synthesize extracellular water-insoluble glucan from sucrose through the activity of secreted glucosyltransferases (Gtf), leading to bacterial accumulation in the dental biofilm. Three distinct Gtfs (GtfB, GtfC, and GtfD) were demonstrated to participate in the sucrose-dependent adherence process (16, 23, 26). Antibodies raised against Gtfs have been shown to protect against dental caries in animal models (18, 21). Gtfs have also been incorporated into vaccines used in human clinical trials (3, 19, 20). GtfB and GtfC enzymes are encoded by highly homologous genes that are organized in a sequential operon-like fashion (17, 24). These two Gtfs are involved in the synthesis of water-insoluble α(1-3)-rich glucans (17, 24). A third isotype, GtfD, shows less homology with GtfB and GtfC and is encoded by a gene located elsewhere in the chromosome. This enzyme catalyzes the synthesis of water-soluble α(1-6)-rich glucans (8).

Genes coding for each Gtf isozyme have been inactivated, and the resulting mutant strains were analyzed for sucrose-dependent adherence in vitro and cariogenicity in animal models. These studies indicated that GtfB and GtfC have the highest association with virulence (16, 26). We have shown that there is significant variability in the ability to form biofilms in vitro among a large group of clinical isolates containing distinct *S. mutans* genotypes and have suggested that these variations may be associated with differential expression of proteins involved in the accumulation phase of biofilm growth (12).

A study of distinct genotypes that vary in biofilm growth may help us to understand how virulence factor expression is related to the ability of *S. mutans* to accumulate in the dental biofilm and ultimately help to explain the differences in caries activity observed among *S. mutans*-infected children (11). Toward these goals, in this study, we characterized Gtf isozymes with respect to *gtf* gene polymorphisms and enzyme production and activity patterns in a large number of clinical isolates which differ in their abilities to grow as biofilms. The results of our study indicate that *S. mutans* biofilm formation is influenced more by the amounts of GtfC and GtfB produced than by the inherent *gtf* gene polymorphisms, suggesting that factors that control expression of GtfB and GtfC may account for differences in virulence among infecting *S. mutans* strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 76 *S. mutans* isolates included in this study were obtained from 35 young children (11) and included 44 distinct *S. mutans* amplitypes, as previously determined by AP-PCR (13). Laboratory strains were *S. mutans* SJ32, UA130 (ATCC 700611), and GS5 (kindly provided by H. K. Kuramitsu, State University of New York). *S. mutans* strains were grown in either Todd-Hewitt broth (THB; Difco), brain heart infusion

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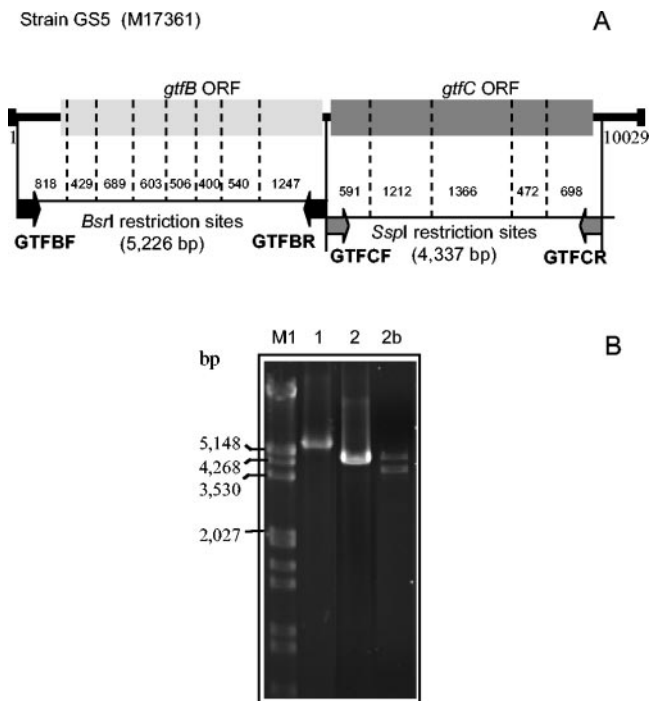
FIG. 1. Chromosome locus of the GS5 strain containing the *gtfB* and *gtfC* genes (GenBank accession number M17361). (A) Primer pairs used for specific amplification of *gtfB* and *gtfC* genes are represented as black and shaded arrows, respectively. The restriction sites of the 5.2-kb *gtfB* and 4.3-kb *gtfC* amplicons are represented by the dashed lines; within them, the size of fragments are indicated. (B) PCR products obtained with the GTFBF-GTFBR (lane 1) and GTFCF-GTFCR (lane 2) primer pairs. In 18 isolates, efficient amplification of the *gtfC* genes was not obtained. Instead, PCRs yielded two weaker products as shown in lane 2b. Thus, RFLP analysis of *gtfC* could not be performed with these 18 strains. ORF, open reading frame.

(Difco), or chemically defined medium (CDM) under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) or in candle jars.

DNA isolation. *S. mutans* chromosomal DNA was isolated by using a Master-Pure DNA purification kit from Epicentre Technologies (Madison, Wis.) as described by the manufacturer.

PCR-RFLP analysis of *gtfB* and *gtfC*. Polymorphisms of *gtfB* and *gtfC* genes were identified by PCR-restriction fragment length polymorphism (RFLP) in the 44 *S. mutans* amplitypes and in strains SJ32, UA130, and GS5. Seven additional isolates of duplicate amplitypes were also included to check agreement between RFLP patterns of *gtf* genes and *S. mutans* AP-PCR identity, giving a total of 51 isolates tested. Since *gtfB* and *gtfC* sequences are sequentially arranged in the chromosome and are highly homologous (76% amino acid sequence identity), primer sets used for independent amplification of *gtfB* and *gtfC* included a nonconserved region located between the GtfB and GtfC open reading frames. The sequence used for primer design was obtained from *S. mutans* strain GS5 (GenBank accession number M17361). Figure 1 shows primer locations and restriction maps of the *gtfB* and *gtfC* amplicons. Amplification of *gtfB* with the specific primers GTFBF (5'-CAAAGATGAAGAAGCAGTAAT-3') and GTFBR (5'-TTCTATAACAAAAGCACAATG-3') yielded amplicons of 5.2 kb. Amplicons of 4.3 kb, corresponding to *gtfC*, were obtained with primers GTFCF (5'-TTGGAGGAAATATGGAAAAG-3') and GTFCR (5'-GTCTAAAACGA ACAGCACT-3'). Amplification reactions were performed with a polymerase enzyme system specific for the generation of long PCR products (Advantage2; Clontech Labs, Inc.). The PCR included 23 cycles of denaturing at 95°C (30 s), annealing at 59°C (1 min), and extension at 70°C (2 min). The restriction enzymes BsrI and SspI were selected for RFLP analysis of the *gtfB* and *gtfC* amplicons, respectively, since restriction maps indicated that these enzymes give the highest number of cleavage sites and yielded fragments suitable for electrophoretic separation. Fragments of digested *gtfB* and *gtfC* amplicons were separated by electrophoresis at 5 V/cm for 3 h in 3 and 2% low-melting-point agarose gels (GTG; Novagen), respectively, in 0.5× Tris-borate-EDTA. Gels were then stained with ethidium bromide.

Measurement of GtfB, GtfC, and GtfD produced in culture fluids. After overnight growth in CDM, bacterial suspensions were adjusted to the same optical density (A_{550}) so that the same size inocula were added to fresh CDM (4 ml) containing 10 μM hydrochloride 4-(2-aminoethyl)-benzolsulfonylfluoride (AEBSF) (Roche Diagnostics, Indianapolis, Ind.) (12). After growth for 18 h, final absorbances of cultures (A_{550}) were measured and cells were pelleted from 2 ml of culture. The supernatant was filtered through a 0.22-μm-pore-size filter membrane (Spin-X; Costar, New York, N.Y.) and immediately frozen at −70°C. Fifty microliters of sample was diluted twofold in 0.02 M sodium phosphate buffer (pH 6.8) and applied to nitrocellulose membranes (Bio-Rad, Foster City, Calif.), as described elsewhere, by using a dot blot apparatus (Bio-Rad) (12). Membranes were blocked overnight in 100 mM Tris-HCl, 2.5 mM NaCl (pH 7.5), and 5% nonfat milk, followed by incubation with monoclonal antibodies (MAbs) P72 (anti-GtfB), P32 (anti-GtfC), and P4 (anti-GtfD) (5) at dilutions of 1:60, 1:30, and 1:60, respectively, in the blocking buffer. Following 2 h of incubation at room temperature with MAbs or the blocking buffer (negative control), membranes were washed four times with 100 mM Tris buffer containing 0.25% Tween, pH 7.5. Incubation with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Amersham Biosciences, Piscataway, N.J.) was then performed at room temperature for 2 h. Membranes were washed four times with 100 mM Tris buffer containing 0.25% Tween and drained, and immunological reactions were developed by using the ECL-plus system (Amersham). Intensities of dot blot reactions were quantified with ImageQuant software (Molecular Dynamics) from digital images of membranes exposed to X-ray films. Samples of all strains were blotted to the same membrane for direct comparison, and the immunoassay was repeated at least twice. The intensities of dot blots were normalized according the final absorbance (A_{550}) of the respective culture to minimize the effect of varying growth rates. The A_{550} values ranged from 1.30 to 1.69 (mean ± standard deviation [SD], 1.49 ± 0.11).

Analysis of GtfB and GtfC activity patterns in zymographic assays. To compare the patterns of GtfB and GtfC enzyme activity among the distinct RFLP classes of *gtfB* and *gtfC*, 3 or more strains representative of each *gtfB* and *gtfC* genotype identified were randomly selected from the 51 strains analyzed. These strains represented a subset of 34 clinical isolates. Briefly, the samples of culture fluids were obtained from 18-h cultures grown in 4 ml of brain heart infusion during incubation in candle jars. These cultures had been inoculated with an adjusted inoculum size of 18-h cultures from frozen stocks prepared under the same conditions. The final absorbance (A_{550}) of cultures used for enzyme analysis was measured again to check for variations in growth (mean ± SD, 0.89 ± 0.10; range, 0.71 to 1.05). Bacterial cells were removed from 3 ml of culture by centrifuging twice at 4°C (1,385 × g, 3 min). Culture supernatants were dialyzed at 4°C against 0.02 M sodium phosphate buffer, pH 6.8, containing 10 μM phenylmethylsulfonyl fluoride, followed by dialysis against 0.2 mM sodium phosphate containing 10 μM phenylmethylsulfonyl fluoride, a protease inhibitor. After dialysis, samples were concentrated 100-fold by lyophilization. To prepare for electrophoresis, 7.5 μl of each concentrated culture fluid was resuspended in 2× Laemmli buffer (1:1), boiled for 1 min, and applied to sodium dodecyl sulfate (SDS)–6% polyacrylamide gels by using the mini-Protean II system (Bio-Rad).

GtfB and GtfC enzyme activities were analyzed in simultaneously run quadruplicate SDS–6% polyacrylamide gel electrophoresis (PAGE) gels. The first gel was used for protein detection by staining with Coomassie blue. The second gel was used for the zymogram assay. Proteins separated in the other two gels were transferred to nitrocellulose membranes for detection of the corresponding GtfB and GtfC isozymes in Western blot assays performed with MAbs P72 (anti-GtfB) and P32 (anti-GtfC), respectively.

For zymogram analysis, following electrophoretic separation, gels were washed twice for 15 min each with renaturing buffer containing 2.5% Triton X-100. Gels were then incubated for 18 h at 37°C with 0.2 M sodium phosphate buffer (pH 6.5) containing 0.2% dextran T70 and 5% sucrose. The reactions were stopped by washing gels with distilled water at 4°C for 10 min, and digital images of the resulting white opaque glucan bands, synthesized by Gtf within the gels, were captured by using a black background to enhance visibility.

Western blot analyses of GtfB and GtfC from electrophoretic separation of culture supernatants were performed as described above for the dot blot assay, except that MAbs P72 and P32 were used at 1:200 dilutions. The antibody-reactive GtfB and GtfC bands were detected by using the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G reagent followed by the ECL system (Amersham). The relationships of antibody-stained Gtf bands to the corresponding glucan bands observed in zymograms were confirmed by measuring the distances of antibody-recognized bands from prestained molecular size markers (Gibco BRL, Carlsbad, Calif.), which were included in separate lanes of

all gels. Intensities of the zymographic and Western blot bands were obtained from digital images captured by a scanning densitometer (Bio-Rad GS-700 imaging densitometer). To control for interassay variability, intensities of the principal glucan and protein bands of all isolates tested were divided by the intensity of a control strain (3VF2) used in all the experiments.

Biofilm formation in microtiter plates. Biofilm growth in microtiter plates was initiated from 1:100 dilutions of 18-h THB cultures transferred into fresh medium. THB contains trace amounts of sucrose, thus providing the substrate for Gtf activities (7). Briefly, after incubation, planktonic cells were removed by washing and the biofilms were stained with a 1% aqueous solution of crystal violet. The absorbances of crystal violet dissolved from ethanol treatment of the stained biofilms (A_{575}) and of planktonic cultures grown under the same conditions (A_{550}) were measured.

Statistical analysis. Pearson correlation analyses were used to evaluate associations between production of GtfB, GtfC, and GtfD (normalized according to the A_{550} of the tested cultures) and biofilm or planktonic growth among *S. mutans* amplitypes. Means of glucan band intensities were compared among the PCR-RFLP patterns identified for their respective Gtf isozyme by the Kruskal-Wallis U test.

RESULTS

Polymorphisms of *gtfB* and *gtfC* genes and zymographic patterns. Significant genetic diversity was identified in both *gtfB* and *gtfC* genes. Figure 2 illustrates the distinct representative classes of *gtfB* (Fig. 2A) and *gtfC* (Fig. 2B) genes identified in the RFLP analysis. The frequencies of each RFLP class among the 44 *S. mutans* amplitypes tested are also shown (Fig. 2). The *gtfB* RFLP class b was, as predicted, the pattern identified in strain GS5. This same *gtfB* genotype was also identified in strain SJ32 and in 8 clinical amplitypes (18.2%). The *gtfB* RFLP class c was the most frequently detected genotype, observed in 40.9% of the clinical amplitypes ($n = 18$) and in UA130. Only strains 3SN1, 2SN3, and 4SM1 reflected *gtfB* RFLP class f. PCR products corresponding to the 4.3-kb *gtfC* gene were not efficiently generated in 18 strains, although GtfC was recognized by the appropriate MAb (MAb P32) in the immunological analysis. Thus, the *gtfC* RFLP patterns were analyzed in 26 clinical amplitypes. Class k was the most frequently identified *gtfC* genotype ($n = 12$) and was also observed in strain GS5. The *gtfC* RFLP class n genotype was detected in strain SJ32 and 10 other distinct *S. mutans* amplitypes.

Figure 3 shows representative protein, zymogram, and Western blot patterns of Gtf isozyme activities identified in the 34 strains that demonstrated distinct *gtfB* and *gtfC* RFLP classes. Strains 3SN1 and 4SM1 showed atypical patterns of Gtf activity, with an apparent single glucan-synthesized band of intermediate size between the commonly detected GtfB (165.8 kDa) and GtfC (153 kDa) correspondent glucan bands. Three other strains, 17A2, 2SN3, and 12JP3 (data not shown), also showed this single band pattern. As in strain 3SN1 (Fig. 3), strain 2SN3 did not produce detectable levels of GtfB in the immunological assays (see below). Similar to 4SM1 (Fig. 3), other strains (17A2 and 12JP3) showed detectable levels of GtfB, although the anti-GtfB MAb P72 reactions in Western blot analysis of these strains were with a protein that migrated more rapidly than typically seen for GtfB.

Strains that did not demonstrate GtfB reactivity by Western blot analyses (e.g., 5ST1, 11SSST2, and 3SN1) (Fig. 3) did not contain cell-associated protein, as observed in Western blot assays with cell extracts (data not shown). There was general agreement between *gtf* polymorphisms and Gtf production patterns when strains representing the same amplitype (AP-PCR
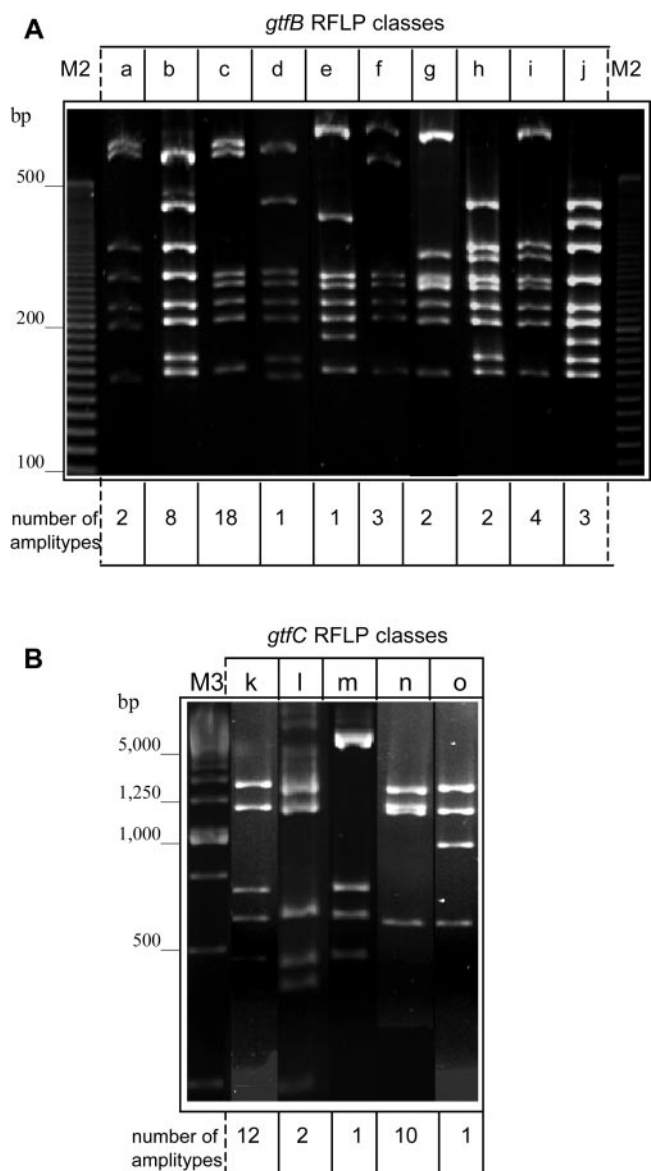

FIG. 2. (A) Ten distinct RFLP classes of the *gtfB* amplicon (a to j) were detected by BsrI digestion among the clinical strains analyzed. (B) Patterns of RFLP identified by SspI digestion of *gtfC* amplicons (k to o).

profile) were compared, indicating that the genetic and phenotypic traits of the Gtf expressed were related to the genetic background of the isolates tested. Intensities of Gtf-related glucan bands, adjusted by the A_{550} of the applied cultures ranged from 0.44 to 2.30 for GtfB (mean ± SD, 1.09 ± 0.49) and from 0.71 to 9.64 for GtfC (mean ± SD, 2.00 ± 1.83). No significant differences in activity were detected when glucan band intensities were compared among distinct *gtfB* (Kruskal-Wallis, $P = 0.651$) and *gtfC* (Kruskal-Wallis, $P = 0.091$) RFLP classes.

Comparisons of Gtf production and *gtf* gene polymorphisms with biofilm growth. Relative amounts of secreted Gtf isozymes quantified by immuno dot blot analysis were quite variable among the 44 *S. mutans* amplitypes tested. Relative
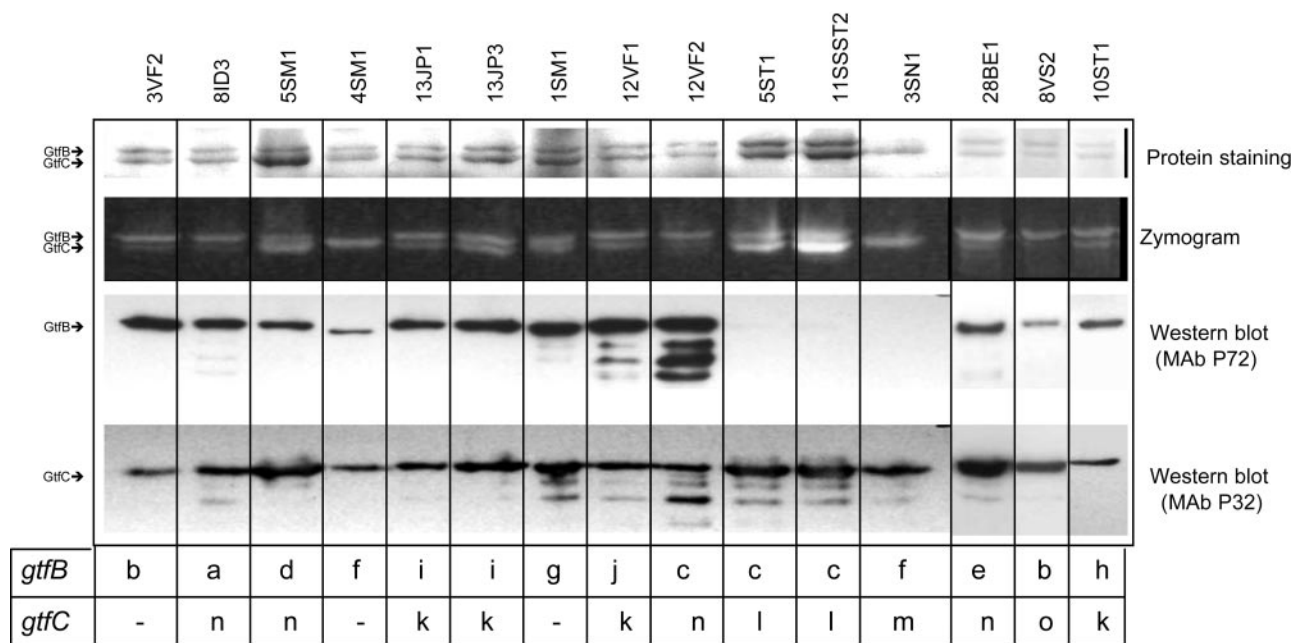
FIG. 3. Representative patterns of Gtf isozyme production and activities analyzed in clinical isolates representative of the *gtfB* and *gtfC* genotypes identified by RFLP. GtfB and GtfC were electrophoretically separated on quadruplicate SDS–6% PAGE gels and then analyzed for protein by Coomassie staining, for glucan production after incubation of gels with sucrose, and immunologically for GtfB and GtfC in Western blots developed with MAb P72 or MAb P32, respectively. Strains are indicated above each lane. RFLP classes of *gtfB* and *gtfC* amplicons are shown beneath each lane. Note that strains 4SM1 and 3SN1 presented a glucan-synthesized band of intermediate size between the typical GtfB- and GtfC-related glucan bands. Strains 5ST1 and 11SSST2, representative of a single *S. mutans* amplitype, did not display detectable levels of GtfB.

amounts of GtfB, expressed as the intensities of dot blot reactivities, normalized by the A_{550} of the cultures tested, ranged from 0 to 131 arbitrary units (U) (mean ± SD, 54.8 ± 44.1 U). Relative amounts of GtfC ranged from 19.2 to 80.7 U for GtfC (mean ± SD, 50.2 ± 16.8 U). For GtfD, relative amounts ranged from 4.5 to 72.7 U (mean ± SD, 20.7 ± 15.2 U). Relative amounts of GtfB produced were associated with amounts of GtfC in a high proportion of amplitypes studied (Pearson correlation, $r = 0.49$, $P < 0.001$) but not with amounts of GtfD ($r = 0.01$; $P = 0.95$). Amounts of GtfC also were not associated with amounts of GtfD ($r = 0.07$; $P = 0.62$).

To investigate the influence of varying production of Gtf isozymes from clinical isolates on their ability to grow as biofilms, the relative amounts of GtfB, GtfC, and GtfD produced were compared to biofilm growth measurements (Fig. 4). Relative amounts of GtfC produced by the 44 amplitypes were significantly associated with the ability of these strains to grow as biofilms (Pearson correlation, $r = 0.36$, $P < 0.02$). Amounts of GtfB expressed were also associated with biofilm formation, although to a lower extent than those of GtfC (Pearson correlation, $r = 0.31$, $P < 0.05$). An inverse relationship was observed between amounts of GtfD produced and biofilm growth (Pearson correlation, $r = -0.33$, $P < 0.05$). Planktonic growth was not related to levels of Gtf isozyme production (GtfB, $r = -0.39$, $P = 0.01$; GtfC, $r = -0.48$, $P = 0.76$; GtfD, $r = 0.20$, $P = 0.20$).

Comparisons of *gtf* polymorphisms with biofilm growth showed no clear differences in means of biofilm intensities between amplitypes carrying distinct *gtfB* and *gtfC* genotypes. The five *S. mutans* amplitypes expressing an apparent single

Gtf synthesizing glucan band of intermediate size between GtfB and GtfC showed low biofilm formation, with crystal violet intensities (A_{575}) ranging from 0.16 to 0.35 (mean, 0.26; SD, 0.07).

DISCUSSION

The production and activity of *S. mutans* Gtfs and their association with virulence has been studied extensively in laboratory strains (16, 18, 23, 26). Mutants of *gtfB*, *gtfC*, or *gtfD* from strain UA130 showed a significantly lower potential to induce caries on smooth tooth surfaces in animal models when compared to the wild type, suggesting that all Gtf isozymes are important for colonization and caries induction (26). However, analyses of GS5 mutants carrying different combinations of *gtf* gene deletions indicated that GtfB and GtfC were important in sucrose-dependent adhesion in vitro while deletion of GtfD had only a modest effect (23). The *gtfC* mutations in GS5 caused the largest decrease in adherence in vitro (23). Since these studies suggested that differential expression of *gtf* genes may be related to phenotypic expressions of virulence, we explored the potential variability in the expression and activity of Gtf isotypes in a large group of clinical *S. mutans* isolates that had successfully colonized young children but differed in in vitro biofilm growth.

Previously, we compared both sucrose-dependent adherence on glass surfaces and Gtf activities among clinical strains isolated from young children who exhibited large differences in caries activity and established that children with high caries activity were often infected by strains showing significantly
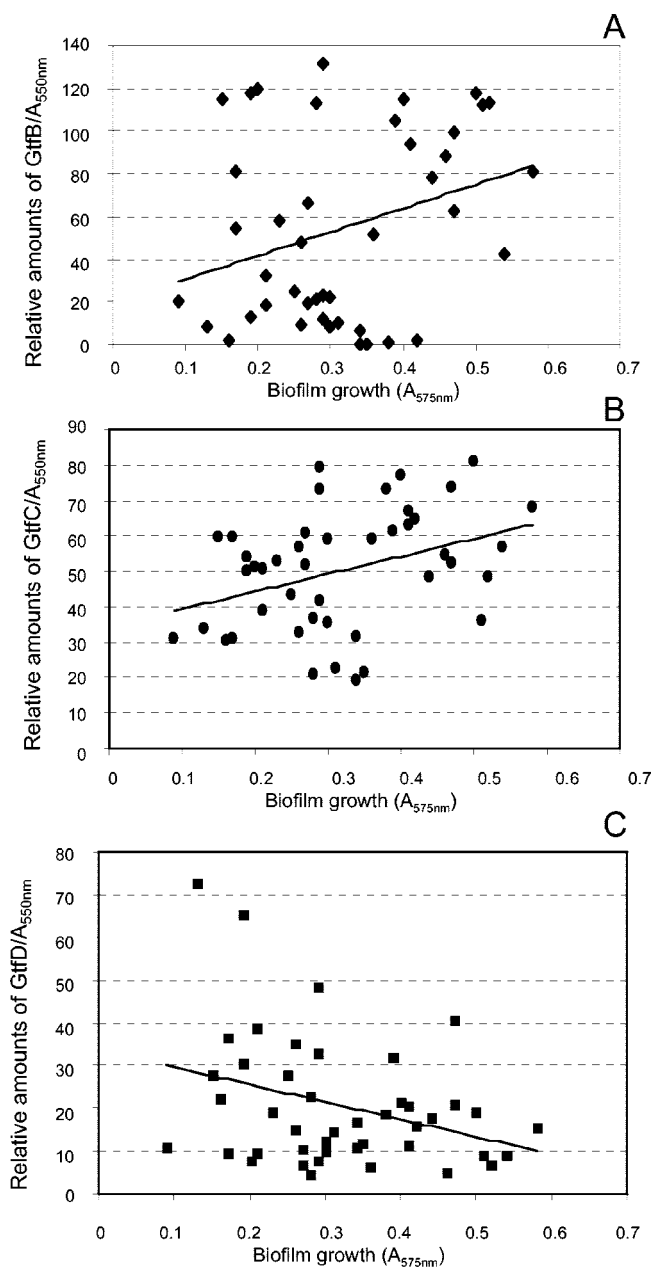
FIG. 4. Distribution of Gtf isozyme production and biofilm growth in 44 *S. mutans* amplitypes. The relative amounts of Gtf produced were expressed as the intensities of the immunological dot blot reactions adjusted for the A_{550} of the cultures tested. Biofilm growth was expressed as the A_{575} of ethanol-eluted crystal violet of the stained biofilms. A significant positive association was observed between amounts of GtfB (A) and GtfC (B) produced and biofilm formation. No positive association was detected between amounts of GtfD and biofilm growth (C).

higher amounts of glucan synthesis compared to strains infecting caries-free children (14). The results suggested that diversity in Gtf expression or specific activity may be associated with the level of cariogenicity. However, analyses of specific Gtf isozymes were not performed (14).

The expression patterns of GtfB, GtfC, and GtfD were characterized using isozyme-specific MAbs (5, 22). This is the first

study that characterized Gtf isozymes in a large number of clinical isolates, and the results support the notion that differences in expression of GtfB and GtfC are associated with the ability of the organism to form biofilms. The relationships between amounts of each Gtf isotype secreted with amounts of biofilm is consistent with the phenotypic traits observed in *gtfB*, *gtfC*, and *gtfD* mutants, supporting a pronounced role for GtfC in adherence (23). Contrary results have been observed with respect to the individual participation of GtfD in the virulence phenotype (23, 26). We found the amplitypes with the highest biofilm growth showed the lowest amounts of GtfD production, which may explain the lack of association observed between amounts of total secreted Gtfs and biofilm growth previously reported (12).

In the present study, no relationship was detected between specific *gtfC* genotypes and amounts of GtfC produced, which concurred with the assumption that differences in GtfC amounts were indeed due to variation in expression levels rather than to *gtfC* polymorphisms. Five atypical strains (3SN1, 2SN3, 4SM1, 17A2, and 12JP3) showed a GtfB/C intermediate size glucan band and a low level of biofilm growth (A_{575} from 0.16 to 0.35). The Western blot patterns of these strains were similar to those previously observed with the strain UA101 (27). The intermediate size Gtf of UA101 had been suggested to be a product of homologous recombination of *gtfB* with *gtfC* in the UA101 ancestor (27).

Although an important role for GtfB and GtfC in the virulence of *S. mutans* was defined, the mechanisms that control expression of these proteins are poorly understood. For most genotypes analyzed in this study, the amounts of GtfB were positively associated with the amounts of GtfC expressed (Pearson correlation, $r = 0.49$, $P < 0.001$), supporting the notion that *gtfB* and *gtfC* genes are part of an operon (29). However, several high GtfC producers showed no detectable GtfB (3SN1, 2SN3, 5ST1, and 11SSST1), a finding which is consistent with independent transcription of these genes, as proposed in other studies (6). Isolates 5ST1 and 11SSST3 showed no detectable levels of GtfB in immunological assays, although a glucan band of migration similar to that synthesized by the GtfB isozyme was detected (Fig. 3). These apparently GtfB-related glucan bands could, however, be the consequence of GtfD activities because this latter isozyme has a molecular mass of approximately 160 kDa and may not have adequately separated from GtfB on the SDS–6% PAGE gels (Fig. 2). Another possible explanation for GtfB-like glucan bands in strains with no detectable GtfB in Western blots is that the GtfB isozymes produced by these strains were not recognized by the MAb P72, although their *gtfB* genes were of RFLP class c, the same as identified in high GtfB producers (Fig. 3). Sequence analysis of the whole *gtf* locus in these atypical *S. mutans* amplitypes may help to resolve these anomalies.

Measurement of glucan band intensities should be an indirect measurement of Gtf expression. However, zymograph variations were not always related to Western blot intensities (Fig. 3). The similar sizes of the Gtf isotypes made it difficult to determine the precise location of glucan-related bands, even when parallel Western blot assays were probed with specific MAbs. Despite this limitation, the relative amounts of glucans synthesized, which corresponded to the migration position of either GtfB and GtfC, were positively associated with biofilm

growth in a subset of 29 strains (data not shown). The five atypical strains that synthesized single glucan bands were not included in these analyses. Variations in migration patterns, enzymatic kinetics, or protein degradation could influence zymographic results. For example, we cannot exclude the possibility that some glucan bands, which were associated with GtfC because of their migration position, could result from degraded GtfB that retained activity but migrated faster.

Given the high number of polymorphisms identified in the *gtf* genes, we presumed that different Gtfs may vary in their enzymatic kinetics, although no specific *gtf* genotypes were related to low or high enzymatic activity measured in zymograms. Polymorphisms in *gtf* genes have been described among isolates from other populations (1, 2, 4), including several that encode silent mutations that have no effect on isozyme activity (2). The *gtfB* RFLP class c was the most common genotype, followed by class b, which included the laboratory GS5 strain. The majority of clinical isolates also demonstrated *gtfC* of pattern k, the same as identified in GS5. Previous sequence comparison of the *gtfB* and *gtfC* genes between five clinical isolates and the GS5 laboratory strain have indicated several frameshift mutations in the *gtfB* and *gtfC* genes in GS5 which accounted for changes in the protein sequence (4). Besides the five atypical strains observed in the present study, no other clear influence of *gtf* gene polymorphism on isozyme production, activity pattern, or biofilm growth were observed. Thus, the results indicate that the amount of Gtf isozyme produced is more important to biofilm growth than *gtf* gene polymorphism. The positive associations of GtfB/C production with biofilm formation in vitro, determined from the large number of clinical isolates tested, suggests that further studies are required to elucidate mechanisms that regulate the expression of these isozymes. It is noteworthy that other factors could contribute to the variability in the amounts of Gtfs produced. For example, strains could differ in the affinities of their expressed Gtfs for the cell surface. We have only quantified Gtfs in culture fluids, since it has been shown that essentially all Gtf is released into the supernatant when strains are grown in non-sucrose-containing medium (7). Also, differences in the turnover of extracellular proteins cannot be excluded.

Experiments with strains GS5 and UA130 with reporter genes fused to the promoter sequences of *gtfB*/*C* indicated that the expression of these *gtf* genes is enhanced in bacteria growing as biofilms compared to planktonic growth (9, 25, 29). The amounts of Gtf that we quantified were from culture supernatants of planktonic cultures, and its relationship with biofilm growth suggests an intrinsic diversity in expression. Chloramphenicol acetyltransferase gene fusions performed with two *S. mutans* variant strains that diverged in the production of several proteins involved in virulence indicated that differences in the production of GtfC and GtfB were due to transcriptional activities of their respective *gtf* genes. Since no relationships to *gtfB*/*C* promoter region polymorphisms were identified, other factors controlling multiple virulence gene expression were suggested (28). In the present study, sequencing of promoter regions of the strains with divergent expression of GtfB and GtfC may shed new light on this question. A peak of expression of *gtfB* and *gtfC* genes was observed at the beginning of the log phase in planktonic cultures, suggesting that a cell density-dependent intercell communication system may play a role in regulating expression of *gtfB*/*C* genes (6). Since the biofilm growth phenotype was also shown to be regulated by such cell density-dependent systems (10, 15), factors important for biofilm growth which affect the expression of *gtf* genes were hypothesized (9). Alternatively, independent factors controlling *gtf* expression may increase the ability of distinct genotypes to grow and accumulate in the biofilm phase. Comparative transcriptional profiling or differential display analysis between the clinical strains described herein which exhibit highly divergent patterns of Gtf isozyme production may reveal factors involved in the regulation of *gtf* genes.

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