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Analysis of a DNA Polymorphic Region in the *gtfB* and *gtfC* Genes of *Streptococcus mutans*

JEAN-SAN CHIA,[1,2] SHU-WHA LIN,[3] TSUEY-YING HSU,[1] JEN-YANG CHEN,[1]*
HSUEH-WAN KWAN,[2] AND CZAU-SIUNG YANG[1]

Graduate Institute of Microbiology,[1] School of Dentistry,[2] and Graduate Institute of Medical Technology,[3] College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China*

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We have previously demonstrated the existence of DNA polymorphisms at the 5′ coding regions of the *gtfB* and *gtfC* genes specifying the streptococcal glucosyltransferases (J. S. Chia, T. Y. Hsu, L. J. Teng, J. Y. Chen, L. J. Hahn, and C. S. Yang, Infect. Immun. 59:1656–1660, 1991). DNA sequence analysis by polymerase chain reaction and direct sequencing revealed that while several nucleotide changes were identified, accounting for the polymorphisms, the amino acids which they code for remain unchanged. The polymorphic region is located in a highly conserved amino terminus of the glucosyltransferases. A peptide of 19 amino acids from this region reversed the inhibiting activity of an antiserum raised against the proteins coded for by the *gtfB* and *gtfC* genes. The results suggest that the polymorphic region, varying in DNA but not in amino acid sequences, might specify some biological function.

Glucosyltransferases (GTFs) are enzymes responsible for the synthesis of water-soluble and -insoluble glucans with sucrose as a substrate. GTFs are important in the colonization of cariogenic streptococci and the development of dental caries (9). Genetic studies of the serotype c strains of *Streptococcus mutans* have identified three distinct GTF genes, *gtfB*, *gtfC*, and *gtfD* (2, 10–12, 20, 22). The *gtfB* and *gtfC* genes share extensive sequence identity and both encode proteins (162 and 149 kDa, respectively) which synthesize primarily insoluble glucan in a primer-independent manner (2, 10). The *gtfD* gene specifies a protein synthesizing water-soluble glucan exclusively in a primer-dependent manner (11). Using restriction fragment length polymorphism, we have previously demonstrated genetic variations in the *gtfB* and *gtfC* genes among serotype c and e strains of *S. mutans* isolated in Taiwan (3). One of the restriction fragment length polymorphisms relates to an *Eco*RI restriction site polymorphism located in the coding region near the amino termini of *gtfB* and *gtfC* (Fig. 1). Computer analysis revealed that the amino acid sequence of this polymorphic region is highly conserved in several *S. mutans* GTFs (1, 6, 7, 12, 20, 22). Our initial goal was to determine the DNA sequence of the polymorphic region and the nucleotide changes that might affect important amino acid residues.

Both the *gtfB* and *gtfC* genes from a total of seven *S. mutans* isolates, six serotype c and one serotype e, were analyzed (NTU-2s, -2p, -3s, -4s, -16, and -45 and MT8148) (3, 4). Previous results of Southern blotting indicated that NTU-3s, -4s, and -16 exhibited a polymorphic *Eco*RI site in *gtfB*. The other three isolates, NTU-2s, -2p, and -45 (a serotype e strain), and MT-8148 (a laboratory strain) contained the *Eco*RI polymorphism in *gtfC* (4). By using the polymerase chain reaction (PCR) combined with direct sequencing, a region of about 200 nucleotides which extends from the region corresponding to nucleotides 1866 to 1959 of the *gtfB* gene (20) and nucleotides 1435 to 1527 of the *gtfC* gene was sequenced (22) (Fig. 1). PCR was performed by the method of Saiki et al. (18) as previously described (14). Two pairs of appropriate primers (Clonetech, Palo Alto, Calif.) were synthesized to allow amplification of specific regions corresponding to the individual *gtfB* and *gtfC* genes. Asymmetric PCR was carried out by adding primers at a molar ratio of 1:20 to obtain single-stranded DNA for sequencing by the dideoxy-chain termination method (Sequenase version 2; U.S. Biochemical Corp., Cleveland, Ohio) (15). The results of the DNA sequences of six clinical isolates are summarized in Fig. 1. An A-to-G alteration at residue 1923 which abolished the *Eco*RI site and resulted in the *gtfB* polymorphism for NTU-4s and NTU-16 was found, while an intact *Eco*RI site was present at the relative position of *gtfC*. In the case of *gtfC* polymorphism, an A-to-G alteration at residue 1491 was found to be responsible for the *Eco*RI polymorphism in strains NTU-2s, -2p, and -45. Other DNA variations not related to the *Eco*RI polymorphism were also found, one of which was at the 399th codon of the gene coding for the GtfB protein. The sequences of NTU-4s, -16, and -45 revealed a CGC triplet coding for arginine at amino acid residue 399 of GtfB instead of the common AAC (specifying asparagine) for the other GtfB proteins. The importance of this amino acid alteration in GtfB awaits determination. All of the other DNA variations identified in these strains were silent without affecting the amino acid sequence. In addition, most of the nucleotide changes were shared by the clinical isolates and a laboratory strain, MT-8148, originally from the National Institute of Health in Japan (17). The sequencing results confirmed our previous Southern blot analysis and identified DNA alterations none other than point mutations in the 5′ coding regions of the *gtfB* and *gtfC* genes.

DNA sequence alignment of *gtfB* and *gtfC* from these clinical isolates and the GS-5 strain, from which both genes were originally identified, revealed that the polymorphic sequences are in a highly conserved peptide region. The fact that most nucleotide substitutions are of a synonymous nature without affecting their amino acid sequences suggested that this region might be structurally and/or functionally important. To investigate this possibility, a 19-amino-acid peptide from this region (ANDVDNSNPVVQAEQL

* Corresponding author.

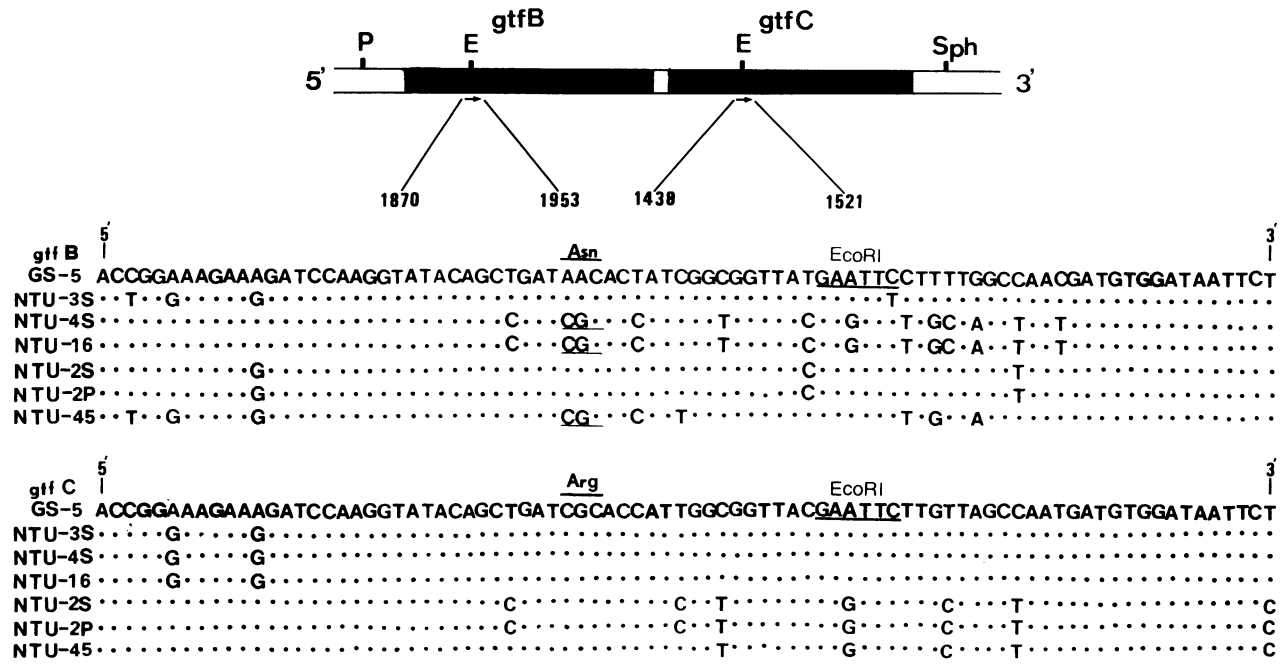

FIG. 1. Restriction map (top panel) of a portion of the GS-5 chromosome showing the coding region of *gtfB* and *gtfC* (filled area), the polymorphic *Eco*RI sites (E) (3), and the DNA fragments sequenced (arrows) and summary of the DNA sequences analyzed by PCR and direct sequencing (bottom panel). The sequences of GS-5 and the six clinical isolates were aligned. Identical nucleotides are dotted. In the GS-5 strain, the amino acid sequences of GtfB and GtfC in these regions are identical, except at residue 399 of GtfB (Asn) and residue 425 of GtfC (Arg). Note that in three clinical isolates (NTU-4s, -16, and -45) the 399th residue of GtfB is arginine. The polymorphic *Eco*RI sites (underlined) were found in the *gtfB* of NTU-3s, -4s, and -16 and in the *gtfC* of NTU-2s, -2p, and -45. The nucleotide sequence of MT-8148 was identical to those of NTU-2s and -2p.

NWL), corresponding to residues 409 to 427 of GtfB (435 to 453 of GtfC), was chemically synthesized and used in competition assays. Peptide synthesis was performed with the Applied Biosystems model 430A peptide synthesizer with Fmoc methodology. The peptide was verified by amino acids analysis with the High-Performance Analyzer System 6300 (Beckman) and automated sequence analysis with Edman degradation by the Applied Biosystems sequenator (model 477A). The possible function of this 19-amino-acid peptide was tested by incubation with partially purified proteins coded for by *gtfB* and *gtfC* and a polyclonal antibody recognizing both enzymes. Because of their similarity in molecular size and enzymatic activities, both GtfB and GtfC proteins were isolated from a genetically constructed mutant, GS-5DD, whose *gtfD* gene has been insertionally inactivated (11). These two proteins are cell associated and therefore were extracted from the cell surface with 8 M urea (8). After DEAE-Sephacel and hydroxylapatite chromatography, the proteins coded for by the *gtfB* and *gtfC* genes were separated from the other proteins and coeluted into the same fractions. This preparation (abbreviated as GtfB/C) was used for generation of polyclonal antibodies in rabbits and for subsequent competition assays (see below).

A polyclonal antibody generated against GtfB/C, PJS-3, was purified (ImmunoPure [immunoglobulin G (IgG)] purification kit; Pierce) and its IgG fraction recognized specifically GtfB/C but not GtfD by Western blot (immunoblot) analysis (Fig. 2). Incubation of GtfB/C with PJS-3 IgG resulted in specific inhibition of the total and insoluble glucan synthesis of GtfB/C in a dose-dependent manner, while the antibody had no effect on GtfD's enzyme activity. Table 1 summarizes the inhibition of the total glucan synthesis. Total and insoluble glucan syntheses were measured as follows. The GTFs' enzyme activities were determined by the [^{14}C]glucose-sucrose (New England Nuclear Corp., Boston, Mass.) incorporation assay as described previously (13). The reaction

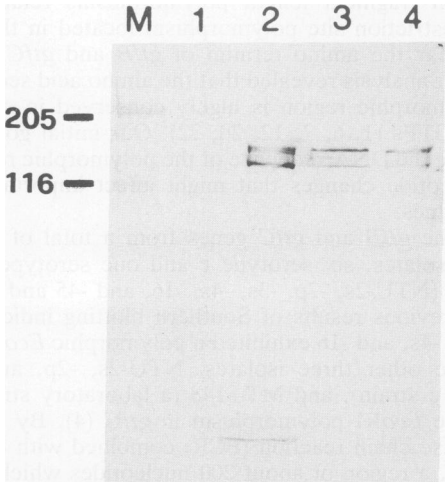

FIG. 2. Western blot analysis of the GTFs. Purified GtfD and partially purified GtfB/C preparations were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose paper (21) and then were reacted with affinity column-purified IgG from a polyclonal PJS-3 antibody recognizing GtfB/C. Lanes: 1, GtfD (4 μg); 2, 3, and 4, GtfB/C (4, 2, and 0.6 μg, respectively); M, prestained molecular mass marker (in kilodaltons).

TABLE 1. Competitive inhibition of GTFs by polyclonal antibody PJS-3 with various concentrations of the synthetic peptide

GTF	PJS-3/IgG (1 mg/ml)[a]	Concn of peptide (mg/ml)	Total glucan synthesis (cpm/h)[b]	% Inhibition[c]
GtfB/C (75 μg/ml)[d]	−	0	8,270 ± 240	0
	+	0.01	4,023 ± 320	51
	+	0.1	4,992 ± 562	40
	+	0.4	6,023 ± 488	27
	+	1.0	5,989 ± 370	27
	e	0	9,235 ± 142	0
GtfC[f]	−	0	7,352 ± 160	0
	+	0.01	3,769 ± 243	48
	+	0.1	4,130 ± 365	43
	+	0.4	5,238 ± 412	29
	+	1.0	5,682 ± 396	22
	e	0	7,892 ± 120	0
GtfD (50 μg/ml)[g]	−	0	9,345 ± 245	0
	+	0	10,996 ± 367	0
	−	2.0	10,667 ± 253	0
	e	0	9,987 ± 164	0

[a] −, absent; + present.
[b] The GTFs' enzyme activities were determined by the [^{14}C]glucose-sucrose incorporation assay as described previously (13) (see text for details). Values are means ± standard deviations from triplicate assays for total glucan synthesis.
[c] Determined as 100 − percentage of activity in controls, which was normalized to 100%.
[d] Partially purified GtfB/C preparation from *S. mutans* GS-5DD (see text for details).
[e] PJS-2/IgG (1 mg/ml), a control antibody for inhibition assays, was used instead of PJS-3/IgG.
[f] Crude enzyme from 10-ml broth culture of *E. coli* pNH3 containing the cloned *gtfC* gene.
[g] Purified from culture supernatant of *S. mutans* NHS1 (see text for details).

mixture consisted of enzyme, 2.9 mM labeled sucrose (0.017 μCi/μmol), without dextran T10 for GtfB/C and GtfC or with dextran T10 for GtfD, and 0.10 M potassium phosphate buffer (pH 6.0) in a total volume of 0.5 ml. The reaction mixtures were incubated at 37°C for 1 h, and the synthesis was terminated by the addition of 5 ml of methanol for total glucan synthetic activity or by heating at 100°C for 5 min for insoluble glucan synthesis. For the inhibition assay, the enzyme preparation was incubated in an equal volume of PJS-3/IgG and peptide (or phosphate-buffered saline for control) for 15 min at 37°C before substrate was added. PJS-2/IgG, a control antibody for inhibition assays, was added. Percent inhibition was determined as 100 − percentage of activity in the controls, which was normalized to 100%.

For assay of GtfC, crude enzyme from 10-ml broth culture of *Escherichia coli* pNH3 containing the cloned *gtfC* gene (10) was used. The *E. coli* cells were disrupted by sonication in extraction buffer (20 mM Tris hydrochloride buffer [pH 8.3] containing 1.0 mM phenylmethylsulfonyl fluoride and 2.5 mM EDTA) followed by centrifugation at 12,000 × *g* for 30 min, and the supernatant was collected, dialyzed against sodium phosphate buffer (50 mM, pH 6.5), and used as the crude enzyme preparation.

The GtfD protein was purified from the culture supernatant of *S. mutans* NHS1, containing deletions in both *gtfB* and *gtfC* (11), first by 50% (saturation) ammonium sulfate precipitation and then by chromatofocusing on a Polybuffer exchanger PBE94 (Pharmacia) followed by hydroxylapatite chromatography (19). In order to further verify the inhibitory effects of PJS-3, we have obtained a single protein coded for by *gtfC* devoid of GtfB from an *Escherichia coli* (pNH3) strain which expresses recombinant GtfC (10). As shown in Table 1, PJS-3 was found to inhibit total glucan synthesis of recombinant GtfC. An antibody against GtfD (PJS-2) showed no inhibitory activity against GtfB/C or recombinant GtfC (Table 1), although it recognized GtfD and GtfB/C by Western blot analysis (data not shown).

When GtfB/C or recombinant GtfC proteins were incubated with purified PJS-3 IgG in the presence of the synthetic 19-amino-acid peptide and the residual enzymatic activities of total glucan synthesis were measured, the peptide was found to compete with the proteins for binding to PJS-3 and, as a result, the inhibitory effect of PJS-3 was reduced. As shown in Table 1, the peptide could reverse the inhibition of GtfB/C or GtfC by PJS-3 in a dose-dependent manner. When incubated with the GTF enzymes in the absence of PJS-3, the peptide had no effect on the enzymatic activities. The ability of the peptide to reverse the inhibitory effect of PJS-3 antibody when the single protein coded for by *gtfC* was tested suggests that one of the binding regions of PJS-3 specified by this peptide is important for the enzyme activities of GtfC. The active site(s) of the streptococcal GTFs is still unknown, but a reaction intermediate composed of the active site of the GtfI protein of *Streptococcus sobrinus* has already been identified (16). A recent finding by Dertzbaugh and Macrina (5) indicated that an antiserum recognizing a sequence of 15 amino acids (342 to 356 of GtfB) was able to inhibit the water-insoluble glucan synthesis of the GTFs. Our peptide extends from residues 404 to 427 of *gtfB* and is within a highly conserved region, as is the 15-amino-acid peptide identified by the previous investigators (5). Taken together, this information suggests that this amino-terminal conserved region of the GTFs, initially identified by DNA polymorphisms, might be important for some enzyme activity which needs further investigation.

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