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Identification of a Fourth Gene Involved in dTDP-Rhamnose Synthesis in *Streptococcus mutans*

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We had isolated three genes (*rmlA*, *rmlB*, and *rmlC*) involved in dTDP-rhamnose synthesis in *Streptococcus mutans* and found that three genes were insufficient for dTDP-rhamnose synthesis (Y. Tsukioka, Y. Yamashita, T. Oho, Y. Nakano, and T. Koga, J. Bacteriol. 179:1126–1134, 1997). The *rmlD* gene of *S. mutans*, encoding the enzyme which catalyzes the last step of dTDP-rhamnose synthesis, has been cloned and sequenced. The cell extract of *Escherichia coli* expressing the *rmlD* gene of *S. mutans* exhibited enzymatic activity corresponding to its counterpart in *Shigella flexneri*, a gram-negative bacterium. Rhamnose was not detected in the cell wall preparation purified from the mutant in which the cloned gene was insertionally inactivated. Rabbit antiserum against *S. mutans* serotype c-specific antigen did not react with autoclaved extracts from the mutant. The *rmlD* gene product of *S. mutans* compensated for the incompleteness of dTDP-rhamnose synthesis by the three previously isolated genes. These results indicate that the *rmlD* gene product is indispensable for the dTDP-rhamnose pathway and subsequently for the synthesis of serotype-specific antigen in *S. mutans*. Furthermore, conservation of the *rmlD* gene in *Streptococcus* species was demonstrated by Southern blot analysis.

The serotype-specific rhamnose-containing polysaccharide antigens of *Streptococcus mutans*, which are composed of backbone structures of 1,2- and 1,3-linked rhamnosyl units (3, 7), have been proposed as putative mediators for the colonization of tooth surfaces by the organism (5) and for its binding to heart, kidney, and muscle tissues (14). Furthermore, in vitro stimulation of human monocytes with the serotype f-specific polysaccharide antigen of *S. mutans* has been reported to induce the release of inflammatory cytokines such as tumor necrosis factor alpha and interleukin-1β (13). We recently cloned three genes involved in the dTDP-L-rhamnose synthesis pathway in *S. mutans* and demonstrated that the pathway is involved in the biosynthesis of the serotype c-specific polysaccharide antigen in the organism (15). In gram-negative bacteria such as *Salmonella typhimurium*, *Shigella flexneri*, and *Xanthomonas campestris*, the dTDP-L-rhamnose pathway is considered to consist of four enzymes catalyzing the reaction sequence from D-glucose-1-phosphate and dTTP to dTDP-L-rhamnose through three intermediates, as shown in Fig. 1A (1, 2, 4, 8, 9). The *rmlA*, *rmlB*, *rmlC*, and *rmlD* genes for the four enzymes have been identified in such gram-negative bacteria, and the four genes are closely located in a locus (1, 2, 4, 8, 9, 10), but the gene corresponding to the *rmlD* gene of the gram-negative bacteria was not found in the *rml* locus of *S. mutans* (15). It was considered possible that only three gene products were enough to catalyze an anabolism from D-glucose-1-phosphate and dTTP to dTDP-L-rhamnose in *S. mutans*. However, high-performance liquid chromatography (HPLC) analysis revealed that the enzyme extract from KD105, in which all three *rml* genes were expressed simultaneously, synthesized dTDP-L-rhamnose only in the presence of the enzyme extract containing the *rmlD* gene product of *S. flexneri*, suggesting that the *rmlD* gene homolog is indispensable for the production of dTDP-L-rhamnose from D-glucose-1-phosphate and dTTP in *S. mutans* (15). Therefore, speculating that the *rmlD* gene of *S. mutans* might map elsewhere, we sequenced the approximately 10-kb flanking regions both upstream and downstream of the *rmlABC* locus of *S. mutans*, but the gene showing homology with the *rmlD* genes isolated from the gram-negative bacteria described above was not identified in these areas. In addition, no signal was detected in the *S. mutans* chromosome by Southern blot analysis with the *rmlD* gene of *S. flexneri* as a probe. Finally we attempted to detect the *rmlD* gene with a probe which was designed on the basis of the result of a homology search of the recently constructed *Streptococcus pyogenes* genome sequencing database and succeeded in detecting and cloning the *rmlD* gene of *S. mutans*. Here we describe the characteristics of the cloned gene.

Bacterial strains and DNA manipulations. Bacterial strains used in this study were obtained and maintained as described previously (15). Standard recombinant DNA procedures such as DNA isolation, endonuclease restriction, and ligation were carried out as described by Sambrook et al. (11). Southern blot analysis with a digoxigenin-labeled probe, preparation of chromosomal DNA, and transformation of *S. mutans* and *Escherichia coli* were carried out as previously described (15).

The gene homologous to the *rmlD* gene of *S. typhimurium* was searched by use of the BLAST program of the *S. pyogenes* genome sequencing database, which is based on the results of the Streptococcal Genome Sequencing Project, on the World Wide Web site of the University of Oklahoma's Advanced Center for Genome Technology. In this project, over 93% of the sequence of the entire *S. pyogenes* genome had been determined up to the data release of 12 January 1997. The gene encoding the protein which showed 29% identity with the *rmlD* gene product of *S. typhimurium* was found in the DNA sequence of contig 247, which is registered in the database. A set of primers (5′-ATTACAGGAAGCAATGGTC-3′ and 5′-ATGCCTTCAAGGCTTCTTG-3′) was designed to amplify a digoxigenin-labeled PCR probe, and a PCR probe with an appropriate size (around 850 bp) was successfully amplified with the primers and the chromosome of *S. pyogenes* T29. Southern blot analysis revealed that a 4.2-kb *Eco*RI fragment

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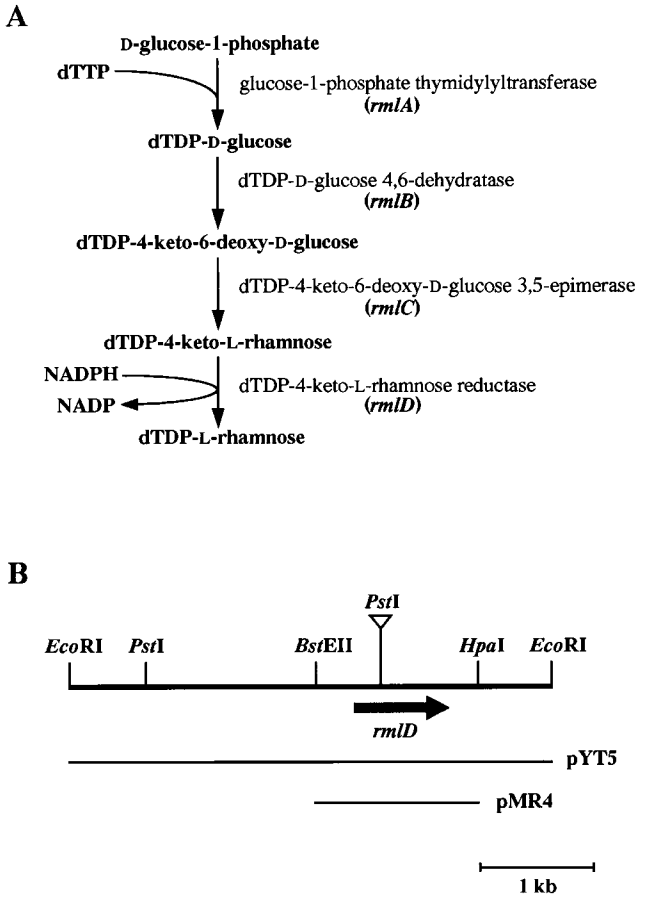

FIG. 1. (A) Biosynthesis pathway of dTDP-L-rhamnose. The names of the genes encoding the enzymes catalyzing the pathway are shown in parentheses. (B) Restriction map of the 4.2-kb *Eco*RI fragment on which the *rmlD* gene of *S. mutans* Xc is located. The location of the *rmlD* gene is indicated by the arrow. The pResEmNot insertion site for insertional inactivation of the *rmlD* gene is indicated by the inverted triangle. The regions of the insertion fragments of pYT5 and pMR4 are indicated in the lower portion of the diagram.

of the chromosome of *S. mutans* Xc hybridized with the probe (data not shown). An *S. mutans* clone bank constructed with *Eco*RI-digested *S. mutans* Xc chromosome and pBluescriptII KS^+ was screened for the gene which hybridized with the *rmlD* gene-specific digoxigenin-labeled PCR probe by colony hybridization. After screening, pYT5, which contained the target 4.2-kb *Eco*RI fragment, was obtained (Fig. 1B). The nucleotide sequence of the insert fragment was determined with a model 373 STRETCH automated sequencer (Applied Biosystems, Inc., Foster City, Calif.) as described previously (17). Nucleotide sequencing analysis revealed the presence of five open reading frames (ORFs) in the same orientation on this fragment. The fourth ORF consists of 852 bp, starting at an ATG codon (position 2521) preceded by a 5′-AGGA-3′ sequence, a typical ribosome binding site located 8 bp in front of the initiation codon. A promoter-like sequence, TTGACA-N_{19}-TATCAC, exists between positions 2353 and 2383. The predicted translational product of the ORF is a protein of 284 amino acids with a molecular weight of 32,410. The deduced amino acid sequence of this ORF showed a high degree of similarity (81% identity) to that of the *rmlD* homolog of *S. pyogenes* identified in this study and relatively less homology (27% identity) with the *rmlD* gene of *S. typhimurium* LT2 (1). This gene of *S. mutans* Xc was designated *rmlD* (Fig. 1B).

To characterize the gene product of the *rmlD* gene of *S. mutans*, the 1.4-kb *Bst*EII-*Hpa*I fragment (positions 2139 to 3582) was subcloned into pBluescriptII KS^+. The plasmid, which was designated pMR4 (Fig. 1B), was introduced into *E. coli* Sϕ874, and the resultant transformant (KD104) was obtained. KD104 was grown in Luria-Bertani broth containing 50 μg of ampicillin per ml and 5 mM isopropyl-β-D-thiogalactopyranoside at 37°C until an optical density at 550 nm of about 1.0 was attained. The cells were harvested by centrifugation and resuspended with ice-cold 50 mM Tris-HCl buffer (pH 7.0) containing 10 mM $MgCl_2$ and 1 mM EDTA (1 g [wet weight]/ml). The cell suspension was sonicated on ice at 30% pulsed power eight times for 15 s with a sonicator (Cell Disrupter model W-225R; Heat Systems, Inc., Farmingdale, N.Y.). The cell extract was centrifuged at 18,000 × *g* for 30 min at 4°C, and the supernatant was passed through a Sephadex G-25 prepacked PD10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and 22% (vol/vol) glycerol as the eluent. The protein fraction was collected, and glycerol was added to a final concentration of 50%. The solution was used as the enzyme extract containing the *rmlD* gene product. The enzyme extracts containing the *rmlA*, *rmlB*, and *rmlC* gene products and the control extract were prepared from transformants KD101, KD102, KD103, and KD100 by the procedure described above (15). Overall activity to produce dTDP-L-rhamnose from dTDP-4-keto-6-deoxy-D-glucose was assayed by a spectrophotometric method as described previously (15). The reaction mixture containing the enzyme extracts from both KD103 and KD104 showed stronger activity (6.2 ± 0.8 U/mg of protein) to convert dTDP-4-keto-6-deoxy-D-glucose into dTDP-L-rhamnose than that (<0.1 U/mg of protein) of the cell extract from KD100 (15), which is a control strain harboring pBluescriptII KS^+. On the other hand, the conversion activity of the enzyme extract from KD103 or KD104 cells alone was almost at the same level as that of the cell extract from KD100. The conversion of dTTP and D-glucose-1-phosphate to dTDP-L-rhamnose was confirmed by HPLC with a fluorescence detector as described previously (15). The reaction mixture (500 μl) containing the enzyme extracts (120 mU) from KD101 and KD102 and the enzyme extracts (50 μg of protein from each extract) from KD103 and KD104 was incubated at 37°C for 0 min, 30 min, and 60 min and hydrolyzed with 0.05 M HCl after termination of the enzymatic reaction. The sugar parts of the nucleotide sugars in the reaction mixture were coupled with 2-aminopyridine, and the pyridylamino sugars were analyzed by HPLC with a PALPAK type A column (Takara Shuzo Co., Shiga, Japan). The reaction mixture containing all of the four enzyme extracts showed distinct activity in the production of dTDP-L-rhamnose from dTTP and D-glucose-1-phosphate, whereas the reaction mixture which did not contain the enzyme extract from KD104 did not show such activity (data not shown).

To analyze the function of the *rmlD* gene in *S. mutans*, the *rmlD* gene was insertionally inactivated. pResEmNot (12), which was linearized with *Not*I, was inserted into the unique *Pst*I site on the *rmlD* gene in pMR4. The resultant plasmid was digested with *Bss*HII, and the insertion fragment was introduced into the chromosome of strain Xc by a double crossover recombination. The transformant was designated Xc26. The appropriate insertion of pResEmNot in the *rmlD* gene was confirmed by Southern blot analysis of *Eco*RI digests (data not shown). The Rantz-Randall extracts from *S. mutans* strains and purified serotype c-specific polysaccharide antigen were examined by immunodiffusion analysis (6) in 1% (wt/vol) Noble agar in saline with rabbit serotype c-specific antiserum (Fig. 2). The serotype c-specific antiserum reacted with the extract

FIG. 2. Immunodiffusion analysis of Rantz-Randall extracts with rabbit anti-serotype c serum. The center well contains rabbit anti-serotype c serum. The outer wells contain the Rantz-Randall extracts (20 μl) from strains Xc (well 1), Xc26 (well 2), OMZ175 (well 3), MT703 (well 4), and MT8148 (well 5). Well 6 contains the purified serotype c antigen (0.5 μg) from strain MT8148.

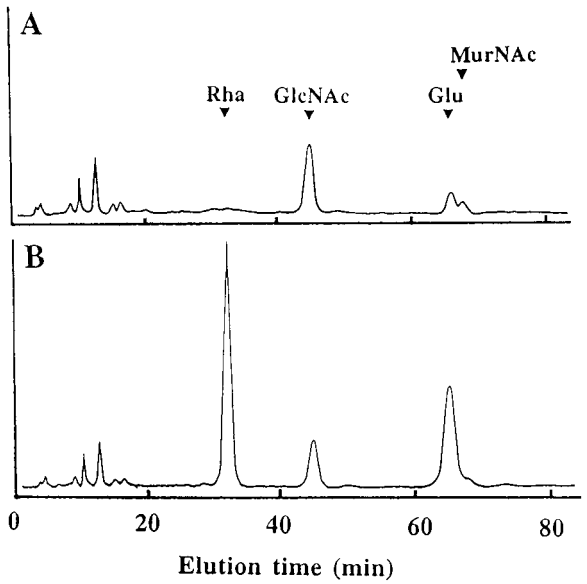

FIG. 3. HPLC pattern of monosaccharides obtained by acid hydrolysis of cell wall preparations of *S. mutans* Xc26 (A) and Xc (B). The pyridylamino sugars were analyzed by HPLC with a PALPAK type A column. The buffer used was a mixture of 0.7 M boric acid (pH 9.0) and acetonitrile (9.5:0.5). The flow rate was 0.3 ml/min, and the column was operated at 65°C. An excitation wavelength of 310 nm and an emission wavelength of 380 nm were used to detect the pyridylamino sugars. Arrowheads labeled Rha, GlcNAc, Glu, and MurNAc indicate the elution times of pyridylaminated L-rhamnose, pyridylaminated *N*-acetylglucosamine, pyridylaminated D-glucose, and pyridylaminated *N*-acetylmuramic acid, respectively.

from strain Xc, whereas the antiserum did not react with the extract from Xc26. Serotype specificity of the antiserum was confirmed with serotype c antigen purified from *S. mutans* MT8148 and Rantz-Randall extracts from strains MT8148, MT703 (serotype e), and OMZ175 (serotype f). The sugar compositions of the cell wall preparations isolated from strain Xc and mutant strain Xc26 were analyzed by HPLC with fluorescence labeling as described previously (15). The cell wall preparations (2 mg) were hydrolyzed with 4 M trifluoroacetic acid at 100°C for 3 h, and free amino groups were acetylated by adding 50 μl of a pyridine-methanol-water mixture (3:6:2) and 2 μl of acetic anhydride. The hydrolyzed sugars were coupled with 2-aminopyridine, and pyridylamino sugars were analyzed by HPLC with a PALPAK type A column. The peak of rhamnose was not detected in the cell wall preparation from strain Xc26. The amount of glucose in the cell wall preparation from strain Xc26 was markedly smaller than that in the preparation from strain Xc (Fig. 3).

The *rmlD*-specific hybridization signal was detected in the chromosomal DNA of various bacterial species by Southern blot analysis with the *rmlD* probe, as those for the *rmlA*, *rmlB*, and *rmlC* genes had been detected (15). To detect the gene homologous to the *rmlD* gene of *S. mutans*, a PCR probe was amplified with a set of primers, 5′-ATCACAGGAAGTAATGGTC-3′ (complementary to positions 2530 to 2548) and 5′-TCTTGTAAAGCTTCTTGCC-3′ (complementary to positions 3347 to 3329), and pMR4 as a template. Specific signals for the *rmlD* gene were observed in all tested streptococci, except that only a faint signal for the *rmlD* gene was detected in *Streptococcus mitior* (Fig. 4). No signal was detected in *Enterococcus faecalis* or *Lactobacillus casei* (Fig. 4), other gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Clostridium bifermentans*, and *Listeria monocytogenes*), or gram-negative bacteria (*E. coli*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *S. typhimurium*, and *S. flexneri*) (data not shown). These results suggest that well-conserved *rmlD*-like genes, as well as the homologs of the *rmlA*, *rmlB*, and *rmlC* genes (15), exist in most species of streptococci. Although the *rmlA*, *rmlB*, and *rmlC* genes each hybridized with a band of the same size in most streptococcus species (15), all bands with which the *rmlD* gene hybridized were different in size from the bands with which the *rmlA*, *rmlB*, and *rmlC* genes in each species hybridized. These findings strongly suggest that the *rmlD* gene is located apart from the locus of the *rmlA*, *rmlB*, and *rmlC* genes in streptococci. The G+C content of the *rml* gene locus consisting of four genes (*rmlA*, *rmlB*, *rmlC*, and *rmlD*) in *S. typhimurium* is lower than that of the chromosomal DNA, suggesting that the gene locus might be transferred from an ancestral species with a low G+C content (16). The genetic analysis of the separate locations of the *rml* genes in streptococci may be of considerable interest for resolving the origin of the *rml* genes.

Enzymatic and functional analyses revealed that the cloned

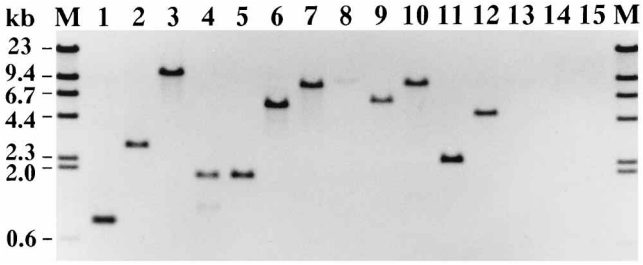

FIG. 4. Southern blot hybridization of chromosomal DNA from various bacterial species with the *rmlD* gene. Lanes: 1, *S. mutans* Xc; 2, *Streptococcus cricetus* HS1; 3, *Streptococcus rattus* FA1; 4, *Streptococcus sobrinus* 6715; 5, *Streptococcus downei* MFe28; 6, *Streptococcus salivarius* HT9R; 7, *Streptococcus milleri* NCTC 10703; 8, *S. mitior* ATCC 12396; 9, *Streptococcus oralis* ATCC 10557; 10, *Streptococcus gordonii* ATCC 10558; 11, *Streptococcus agalactiae* IID1625; 12, *S. pyogenes* T29; 13, *E. faecalis* SS499; 14, *Eubacterium limosum* GAI5456; 15, *L. casei* ATCC 393; M, *Hin*dIII-digested and digoxigenin-labeled lambda DNA. Lanes 1 and 12, *Hin*dIII digests; lane 14, *Pst*I digest; other lanes, *Eco*RI digests. The sizes of the molecular mass markers are indicated on the left. Hybridization with a probe was carried out at 25°C in the presence of 50% formamide overnight.

rmlD gene was involved in dTDP-L-rhamnose synthesis and subsequently in biosynthesis of serotype c-specific carbohydrate antigen in *S. mutans* (Fig. 2 and 3). The *rmlD* gene identified in this study, as well as the *rmlA*, *rmlB*, and *rmlC* genes of *S. mutans* (15), may be useful for identifying and cloning the genes involved in dTDP-L-rhamnose synthesis in most species of streptococci and for elucidating the mechanism of cell surface carbohydrate antigen synthesis in the organisms.

Nucleotide sequence accession number. The 4,177-bp nucleotide sequence presented in this paper has been submitted to the EMBL/GenBank/DDBJ data bank under accession no. AB000631.

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