Biological functions of UDP-glucose synthesis in *Streptococcus mutans*

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A gene encoding glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) was isolated from *Streptococcus mutans*. A cell extract of *Escherichia coli* expressing the cloned gene exhibited glucose-1-phosphate uridylyltransferase activity. The enzyme catalyses the conversion of D-glucose 1-phosphate and UTP into UDP-D-glucose. Rabbit antiserum against the serotype-c-specific antigen did not react with autoclaved extracts from mutant cells in which the cloned gene was insertionally inactivated. The glucose content of the cell-wall preparation purified from the mutant was very much lowered, whereas there was no observable decrease in the content of rhamnose. When the mutant strain was grown in an acidic environment, its cell viability was much lower than that of the wild-type. These results suggest that UDP-D-glucose functions not only as an immediate precursor of the serotype-c-specific antigen of *S. mutans* (as a glucose donor for side-chain formation), but is also important for the organism's viability in environmental conditions of low pH.

Keywords: *Streptococcus mutans*, UDP-glucose, glucose-1-phosphate uridylyltransferase, polysaccharide antigen, acid tolerance

INTRODUCTION

Serotype-specific polysaccharide antigens of *Streptococcus mutans* are composed of a backbone structure of 1,2- and 1,3-linked rhamnosyl polymers with glucose side-chains (Wetherell & Bleiweis, 1975; Linzer *et al.*, 1986; Pritchard *et al.*, 1986) and their serological detection has been used extensively in classification of cariogenic streptococci (Loesche *et al.*, 1986). These polysaccharide antigens have received increasing attention because *in vitro* stimulation of human monocytes with the serotype-f-specific polysaccharide antigen of *S. mutans* was reported to induce the release of inflammatory cytokines such as tumour necrosis factor alpha and interleukin-1 β (Soell *et al.*, 1995), and shown to provoke nitric oxide production in the rat aorta (Martin *et al.*, 1997). However, the biosynthetic pathway of these polysaccharide antigens has not been elucidated. We have recently cloned two loci related to the dTDP-L-rhamnose synthesis pathway and demonstrated that four genes (*rmlA*, *rmlB*, *rmlC* and *rmlD*) are involved in dTDP-L-rhamnose synthesis from glucose 1-

phosphate in *S. mutans* (Tsukioka *et al.*, 1997a, b). Rhamnose is not detected in cell-wall preparations from mutants in which any one of the four *rml* genes is insertionally inactivated. Furthermore, rabbit antiserum against *S. mutans* serotype-c-specific antigen does not react with autoclaved extracts from these mutants. These findings suggest that dTDP-rhamnose is used as an immediate precursor for the synthesis of the backbone of the serotype-c-specific antigen of *S. mutans*.

Glucose-1-phosphate uridylyltransferase (EC 2.7.7.9), which catalyses a reaction converting D-glucose 1-phosphate and UTP to UDP-D-glucose, is widely distributed in animals, plants and micro-organisms. The UDP-D-glucose produced is then used as a glucosyl donor in the biosynthesis of various carbohydrates (Ginsburg, 1964; Markovitz, 1977; Schnaitman & Klena, 1993; Sundararajan *et al.*, 1962; Weissborn *et al.*, 1994). It is possible that UDP-D-glucose is a nucleotide sugar used as a glucosyl donor for side-chain formation of the serotype-specific antigen in *S. mutans*. There is, however, no information concerning the mechanism of glucose side-chain formation of the antigen. Several reports have suggested that bacterial UDP-D-glucose synthesis is involved in osmotolerance and thermotolerance (Böhringer *et al.*, 1995; Giæver *et al.*, 1988;

The EMBL/GenBank/DDBJ accession number for the 3313 bp nucleotide sequence described in this paper is AB001562.

Hengge-Aronis *et al.*, 1991). Acid tolerance of *S. mutans* plays a key role in demineralization of human dental enamel. We have previously shown that a mutation in the gene encoding a diacylglycerol kinase homologue leads not only to the loss of acid tolerance but also to the loss of osmotolerance and thermotolerance (Yamashita *et al.*, 1993). Little is known, however, about the role that UDP-D-glucose synthesis may play in the organism's tolerance of environmental stresses.

In the present study, we cloned the gene from *S. mutans* which encodes a glucose-1-phosphate uridylyl-transferase, and analysed the function of the protein encoded by this gene.

METHODS

Bacterial strains and culture conditions. *S. mutans* strains, *Escherichia coli* strains and plasmids used in this study are listed in Tables 1 and 2. *Streptococcus pneumoniae* WU2 was kindly provided by J. Yother, Department of Microbiology, University of Alabama at Birmingham, AL, USA. The other bacteria used in this study were obtained as previously described (Tsukioka *et al.*, 1997a). Strains of *S. mutans* and *E. coli* were maintained and grown routinely as described previously (Yamashita *et al.*, 1993). Antibiotics were used at the following concentrations: 150 mg erythromycin l⁻¹ or 50 mg ampicillin l⁻¹ for *E. coli*; 10 mg erythromycin l⁻¹ for *S. mutans*. *Bacillus subtilis* was grown aerobically in Luria-Bertani medium at 37 °C. *Clostridium bifermentans* was grown anaerobically in GAM broth (Nissui Medical, Tokyo, Japan) at 37 °C. *S. pneumoniae* was grown in Todd-Hewitt broth (Difco) supplemented with 1% (w/v) yeast extract and 5% horse serum at 37 °C in a 5% CO₂ atmosphere. *Streptococcus* species (except for *S. pneumoniae*), *Enterococcus faecalis* and *Eubacterium limosum* were grown anaerobically in brain heart infusion broth (Difco) at 37 °C. *Staphylococcus aureus*, *Micrococcus luteus*, *Lactobacillus casei* and *Mycobacterium smegmatis* were grown aerobically in brain heart infusion broth at 37 °C.

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DNA manipulations and sequence analyses. Standard DNA recombinant procedures such as DNA isolation, endonuclease restriction and ligation were carried out as described by Sambrook *et al.* (1989). Transformation of *S. mutans* and *E. coli* was carried out as previously described (Yamashita *et al.*, 1993). Chromosomal DNA of all the bacteria used in this study except for *Mycobacterium smegmatis* was prepared as described by Perry *et al.* (1983). Chromosomal DNA of *Mycobacterium smegmatis* was prepared by the method of Imai *et al.* (1994). The nucleotide sequence of the insert fragment was determined using a 373 STRETCH automated sequencer (Applied Biosystems) as described previously (Yamashita *et al.*, 1993). The *galU* gene with the Tn5 insertion was transferred from *E. coli* strain JC7623-16 to *E. coli* strain Sφ874 by P1 transduction (Miller, 1992). The nucleotide sequence was assembled using the DNASIS sequence analysis program (Hitachi Software Engineering). Database searching was performed using the FASTA program (Lipman & Pearson, 1985) of the DDBJ electronic-mail server in the National Institute of Genetics, Mishima, Japan. Multiple alignments of the amino acid sequences were generated with the CLUSTAL V program (Higgins *et al.*, 1992).

Southern blot analysis and colony hybridization. Southern blot analysis and colony hybridization were performed with digoxigenin-labelled PCR probes using a nonradioactive digoxigenin DNA labelling and detection kit (DIG-ELISA) (Boehringer Mannheim) according to the instructions of the supplier. To detect the *S. mutans* gene homologous to *cps3U* encoding a glucose-1-phosphate uridylyltransferase of *S. pneumoniae* WU2, a set of primers (5'-TACGTTCCGGTA-

Table 1. *E. coli* and *S. mutans* strains used

Strain	Genotype or relevant characteristic	Reference
<i>E. coli</i>		
JM109	F ⁻ <i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i>	Yanisch-Perron <i>et al.</i> (1985)
Sφ874	<i>lacZ2286 trp-49 Δ(sbcB-rfb)86 upp-12 relA1 rpsL150 λ⁻</i>	Jiang <i>et al.</i> (1991)
JC7623-16	<i>recBrecCsbcd galU::Tn5-16</i>	Weissborn <i>et al.</i> (1994)
Sφ874-16	As Sφ874 but with <i>galU::Tn5-16</i>	This study
KD300	Sφ874-16 carrying pBluescriptII KS ⁺	This study
KD301	Sφ874-16 carrying pMG1	This study
<i>S. mutans</i>		
Xc	Serotype c wild-type strain	Koga <i>et al.</i> (1989)
Xc22	Em ^r ; strain Xc carrying P15A replicon and Em ^r gene inserted into ORF3, immediately upstream of <i>rmlA</i>	Tsukioka <i>et al.</i> (1997a)
Xc24	Em ^r ; strain Xc carrying P15A replicon and Em ^r gene inserted into <i>rmlB</i>	Tsukioka <i>et al.</i> (1997a)
Xc31	Em ^r ; strain Xc carrying P15A replicon and Em ^r gene inserted into <i>gluA</i>	This study
MT8148	Serotype c strain	Ohta <i>et al.</i> (1989)
MT703	Serotype e strain	Hamada & Slade (1976)
OMZ175	Serotype f strain	Bratthall (1970)

Table 2. Plasmids used

Plasmid	Relevant characteristic	Source or reference
pBluescriptII KS ⁺	Ap ^r ; phagemid cloning vector	Stratagene
pYT6	pBluescriptII KS ⁺ containing 3.3 kb <i>Xba</i> I fragment of Xc chromosomal DNA	This study (Fig. 1)
pMG1	pBluescriptII KS ⁺ containing <i>gluA</i>	This study (Fig. 1)
pResEmNot	Em ^r ; P15A replicon	Shiroza & Kuramitsu (1993)
pYT31	pMG1 containing pResEmNot ligated into <i>Hinc</i> II site of <i>gluA</i>	This study
pJD366	pJY4164 containing 3.2 kb <i>Hind</i> III fragment of <i>S. pneumoniae</i> WU2 chromosomal DNA	Dillard & Yother (1994)

TTGAAG-3' and 5'-AGGTGTCCACTTGACGAACA-3') and pJD366, which carries *cps3U* (Dillard & Yother, 1994), were used for amplification of a 0.8 kb digoxigenin-labelled PCR probe (probe 1). Hybridization with probe 1 was carried out overnight at 25 °C in the presence of 50% formamide. To confirm appropriate insertional inactivation of the *gluA* gene and to detect *gluA*-specific hybridization signals in the chromosomal DNA of various bacterial species, a set of primers (5'-AGCTGTCATCCCTGCGGC-3' and 5'-CCGAGTTCTTTTGTAGTCG-3') was designed and a digoxigenin-labelled PCR probe (probe 2) was amplified using the primers and pMG1.

Insertional inactivation of the *gluA* gene. pResEmNot, containing an erythromycin resistance cassette (Shiroza & Kuramitsu, 1993), was digested with *Not*I and blunt-ended with T4 DNA polymerase. The linearized 1.6 kb pResEmNot was ligated into the *Hinc*II site in the *gluA* gene of pMG1. The resultant plasmid, designated pYT31, was digested with *Bss*HII and the digested plasmid DNA was introduced into *S. mutans* Xc by homologous recombination. The transformants were screened on a mitis-salivarius agar plate containing 10 µg erythromycin ml⁻¹ and three independent transformants which appeared on the agar plate were isolated. The appropriate insertion of pResEmNot in the *gluA* gene was confirmed by Southern blot analysis of *Xba*I digests with probe 2. Hybridization with probe 2 was carried out overnight at 42 °C in the presence of 50% formamide. The *Xba*I-digested chromosomal DNA from all three transformants showed a 4.9 kb fragment which hybridized with probe 2, while that of Xc hybridized to a 3.3 kb fragment (data not shown). One of these three transformants was designated Xc31 and tested for further characterization. The phenotype of the other transformants was very similar to that of Xc31 (data not shown).

SDS-PAGE. Expression of the *gluA* gene in *E. coli* Sφ874-16 was confirmed by SDS-PAGE (Laemmli, 1970). *E. coli* strain KD301 or KD300 was grown at 37 °C in 5 ml Luria-Bertani broth containing 50 mg ampicillin l⁻¹. Gene expression was induced with 0.3 mM IPTG for 3 h. The cells were harvested and suspended in SDS sample buffer containing 10 mM Tris/HCl (pH 6.8), 1% (w/v) SDS, 1% (v/v) mercapto-ethanol and 20% (v/v) glycerol, and heated for 5 min at 100 °C. The cell extract was then clarified by centrifugation. The samples (10 µl) were electrophoresed for 60 min at room temperature at 25 mA per gel in a 12% (w/v) resolving gel and a 4.8% (w/v) stacking gel, and the gels were stained with Coomassie brilliant blue.

Preparation of crude enzyme extracts. *E. coli* KD301 was grown at 37 °C in 200 ml Luria-Bertani broth containing 50 mg ampicillin l⁻¹ and gene expression was induced with 0.3 mM IPTG to OD₅₅₀ approx. 1.0 (within 2 h after addition of IPTG). The cells were harvested by centrifugation at 10000 g for 20 min at 4 °C, washed twice with ice-cold 50 mM Tris/HCl buffer, pH 7.0, containing 10 mM MgCl₂ and 1 mM EDTA (buffer A), and resuspended in the same buffer (1 g, wet wt, ml⁻¹). 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). The cell extract was centrifuged at 18000 g for 30 min at 4 °C and the supernatant was passed through a Sephadex G-25 prepacked PD10 column (Pharmacia LKB Biotechnology) using buffer A containing 20% (v/v) glycerol as the eluent. Control extracts from the cells of KD300 were prepared in the same manner as the cell extracts from KD301. The protein-containing fraction was collected and glycerol was added to a final concentration of 50% (v/v). The resultant crude enzyme extract was stored at -20 °C until used.

Enzyme analysis. Glucose-1-phosphate uridylyltransferase activity was determined by measuring the conversion of UTP to UDP-D-glucose by HPLC essentially as described by Marolda & Valvano (1996). The reaction mixture (300 µl) of 50 mM Tris/HCl buffer, pH 8.0, containing 12 mM MgCl₂, 24 mM α-D-glucose 1-phosphate, 6 mM UTP, 1.8 U inorganic pyrophosphatase (Boehringer) and an appropriate amount of the crude enzyme extract, was incubated at 37 °C. Aliquots (30 µl) of the reaction mixture were withdrawn at timed intervals of up to 10 min. The aliquot was immediately mixed with 1.0 ml 50 mM potassium phosphate buffer, pH 3.0, to stop the reaction, and the diluted solutions were stored at 4 °C until HPLC analysis. Samples (100 µl) were analysed by HPLC using a TSKgel QAE-2SW column (0.46 × 25 cm; Tosoh). The chromatogram was developed with a linear gradient of 50–600 mM potassium phosphate (pH 4.0, 20 ml) delivered at a flow rate of 1.0 ml min⁻¹ at 30 °C and the A₂₅₄ of the effluent was monitored. From the integrated peak areas of the HPLC chromatogram, the molar amount of UDP-glucose formed was calculated. One unit of enzyme activity is defined as the amount of the enzyme catalysing the synthesis of 1 µmol UDP-D-glucose min⁻¹. To calculate specific activity, the protein concentration in the crude enzyme extract was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Immunological methods. Lyophilized cell suspensions (20 g l⁻¹) of *S. mutans* strains were suspended in phosphate-buffered

saline (10 mM, pH 6.8) and autoclaved at 121 °C for 15 min. After being autoclaved, the suspensions were centrifuged at 10000 *g* for 20 min and the supernatants were collected and used as Rantz–Randall extracts (Rantz & Randall, 1955). The serotype c antigen was extracted with 5% (w/v) trichloroacetic acid from a cell wall preparation of *S. mutans* MT8148 and then the extracted serotype antigen was purified by chromatography on DEAE-Sephadex 25A and Sephacryl S-300 columns as described by Linzer *et al.* (1976). Rabbit antisera against whole cells of strain MT8148 (serotype c) were kindly provided by T. Ooshima, Osaka University Faculty of Dentistry, Japan, and S. Alaluusua, University of Helsinki, Finland. These antisera were absorbed with whole cells of *S. mutans* MT703 (serotype e). Immunodiffusion was performed in 1% (w/v) Noble agar in saline (Ouchterlony, 1958).

Analysis of cell-wall sugar components. Bacteria were grown overnight at 37 °C in 40 ml brain heart infusion broth (Difco), collected by centrifugation and washed three times with distilled water. The cells were resuspended in 1 ml cold distilled water, transferred to a chilled 2 ml microcentrifuge tube containing 0.1 mm diameter glass beads (GMB-10; Nippon Rikagaku Kikai) and were disrupted with a Mini-Bead Beater homogenizer (Biospec Products) for 20 min. The glass beads were removed by decantation and the undisrupted cells were separated by centrifugation at 450 *g* for 20 min. The crude cell walls were obtained by centrifugation at 8000 *g* for 30 min and washed once with distilled water. The cell walls were treated with RNase (10 mg l⁻¹) and DNase (10 mg l⁻¹) at 37 °C for 2 h and with Pronase E (100 mg l⁻¹) at 37 °C for 24 h. After being washed twice with distilled water, the cell wall preparations were treated with trypsin (100 mg l⁻¹) at 37 °C for 2 h. The purified cell walls were collected by centrifugation at 8000 *g* for 30 min, washed twice with distilled water and lyophilized.

Component sugars in the purified cell wall preparations were analysed by HPLC with fluorescence labelling. The lyophilized cell wall preparation (1 mg) was resuspended in 0.5 ml distilled water, and 40 µl 5 M trifluoroacetic acid was added to 10 µl suspension. The mixture was heated at 100 °C for 3 h and dried at 50 °C. Free amino groups were acetylated by adding 50 µl of a mixture of pyridine/methanol/water (3:6:2, by vol.) and 2 µl acetic anhydride. The solution was left standing for 30 min at room temperature. After the hydrolysed solution had been dried at 50 °C, 10 µl coupling reagent (670 g 2-aminopyridine l⁻¹ in acetic acid) was added. The suspension was heated at 90 °C for 20 min, the excess reagents were removed by evaporating, and then 10 µl reducing reagent (60 g borane–dimethylamine complex l⁻¹ in acetic acid) was added. The mixture was reduced at 90 °C for 35 min and dried under a stream of nitrogen gas at 50 °C for 10 min. The dried sample was dissolved in 1 ml distilled water, and 5 µl of the solution was analysed by HPLC with a PALPAK type A column (Takara Shuzo). D-Glucose, L-rhamnose, N-acetylglucosamine and N-acetylmuramic acid were also coupled with 2-aminopyridine as described above and the resultant pyridylamino D-glucose, pyridylamino L-rhamnose, pyridylamino N-acetylglucosamine and pyridylamino N-acetylmuramic acid were used as standards. 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⁻¹ 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. Pyridylaminated D-fucose (10 µl, 1 µmol l⁻¹) was used as a standard for quantification of the pyridylamino sugars. The total hexose content in the purified cell-wall preparation was

quantified by the anthrone/sulfuric acid method (Morris, 1948). The hexosamine content in the purified cell-wall preparation was determined by the colorimetric method of Strominger *et al.* (1959).

Determination of UDP-D-glucose concentration in cells. The intracellular UDP-D-glucose concentrations of strain Xc and its derivatives were determined by using UDP-D-glucose dehydrogenase and NAD. The cells were grown in 45 ml Todd–Hewitt broth to OD₅₅₀ 0.7, harvested by centrifugation at 7000 *g* for 10 min at 4 °C and washed twice with distilled water. The cells were resuspended in 400 µl distilled water and transferred to 0.5 ml microcentrifuge tubes containing 0.5 g 0.1 mm diameter glass beads. The bacterial cells were disrupted with a Mini-Bead Beater homogenizer for 5 min. The supernatant which was obtained by centrifugation at 20000 *g* for 30 min at 4 °C was separated into two equal portions. One of the portions was boiled for 90 s, cleared by centrifugation at 20000 *g* for 15 min and used to determine the UDP-D-glucose concentration as described by Keppler & Decker (1974). The other portion was used for determination of the protein concentration by the method of Bradford (1976).

Evaluation of tolerance of the mutant to environmental stress. Tolerance of *S. mutans* Xc31 toward environmental stresses was evaluated by monitoring growth in Todd–Hewitt broth containing 50 mM sodium acetate buffer at appropriate pH values or various concentrations (0–0.9 M) of NaCl. Temperature sensitivity was determined from 37 to 45 °C. Strain Xc24 (Tsukioka *et al.*, 1997a), which was totally defective in the serotype antigen due to the insertion of an erythromycin resistance gene into the *rmlB* gene, was also examined to determine the role of the serotype antigen in environmental stress tolerance of *S. mutans*. In addition to the wild-type strain Xc, strain Xc22 (Tsukioka *et al.*, 1997a), in which the erythromycin resistance gene was inserted into the gene immediately upstream of *rmlA* and in which serotype-specific polysaccharide antigen was not affected, was used as an erythromycin-resistant control strain. To initiate the growth experiments, overnight cultures of each strain were inoculated into fresh Todd–Hewitt broth and the growth was monitored by measuring OD₅₅₀. Except for Xc24, the culture was grown at 37 °C to OD₅₅₀ 0.7 and 150 µl culture broth was then inoculated into 3 ml Todd–Hewitt broth with the indicated modification to evaluate cell viability in conditions of low pH and high osmotic pressure. When thermotolerance was evaluated, 300 µl culture was used as inoculum. The growth was evaluated by measuring OD₅₅₀. Inocula of Xc24 were taken from cultures grown to OD₅₅₀ 0.4 because Xc24 grown in the broth exhibited aggregation and about 70% increase in OD₅₅₀ was obtained by sonication. When OD₅₅₀ increase had ceased in the experimental cultures, the culture broth was sonicated at 20% pulsed power three times for 5 s with a sonicator (Cell Disrupter model W-225R) and final OD₅₅₀ values for Xc24 were measured.

RESULTS

Cloning and nucleotide sequencing of the *S. mutans* *gluA* gene

Southern blot analysis revealed that the 3.3 kb *Xba*I fragment of the *S. mutans* Xc chromosomal DNA hybridized to probe 1. An *S. mutans* clone bank constructed with *Xba*I-digested *S. mutans* Xc chromosomal DNA and pBluescriptII KS⁺ was screened for the gene by colony hybridization. A plasmid designated

Fig. 1. Restriction map of the *gluA* gene of *S. mutans* Xc and its flanking regions. Locations of the four ORFs are indicated by the arrows. The pResEmNot insertion site (*HincII* site) for insertional inactivation of the *gluA* gene is indicated by the inverted open triangle. The inverted repeating unit is denoted by an omega loop.

pYT6 containing the target 3.3 kb *XbaI* fragment was obtained (Fig. 1).

Nucleotide sequence analysis of the fragment revealed the presence of four ORFs. Their locations, extents and directions are shown in Fig. 1. The amino acid sequence deduced from the nucleotide sequence of the second ORF demonstrated significant homology to glucose-1-phosphate uridylyltransferase encoded by the *cps3U* gene (Dillard *et al.*, 1995) (Table 3) and therefore ORF2 was designated *gluA* (Fig. 1). The first ORF encoded a putative amino acid sequence showing 55.9% identity with the carboxyl-terminal end of an NAD(P)H-dependent dihydroxyacetone phosphate reductase of *B. subtilis*, which is required for phospholipid synthesis (Morbidoni *et al.*, 1995) and was designated *gpsA*. Two other ORFs, ORF3 and ORF4, on the opposite strand were also identified downstream of the *gluA* gene. No proteins homologous to the predicted gene products of these two ORFs were identified in a search of the PIR and SWISS-PROT protein databases.

The region downstream of the *gluA* gene contained an inverted repeat structure (positions 1764–1783) followed by a poly(T) sequence, which may act as a transcription terminator for the *gluA* gene. On the opposite strand,

this inverted repeat structure was also followed by a poly(T) sequence possibly acting as a transcription terminator for ORF3. The *gluA* gene was preceded by a putative 5' GGAG 3' ribosome-binding site located 8 bp upstream of the initiation codon found at nucleotide position 855. The predicted translational product of the *gluA* gene is a protein of 306 amino acids with a molecular mass of 33903 Da. The ATG translational initiation codon of the *gluA* gene was identified at 1 bp behind the TAA stop codon of the *gpsA* gene and a putative promoter sequence for the *gluA* gene was not identified in the upstream region of the *gluA* gene, suggesting polycistronic transcription of *gpsA* and *gluA*.

Expression of the *gluA* gene in *E. coli*

To characterize the product of the *gluA* gene, the fragment containing the gene was subcloned into pBluescriptII KS⁺ (Fig. 1). The resultant plasmid (pMG1) was introduced into *E. coli* strain Sφ874-16. SDS-PAGE followed by Coomassie brilliant blue staining (Fig. 2) demonstrated distinct expression of the *gluA* gene in *E. coli* Sφ874-16 harbouring pMG1 (lane 1) in the presence of IPTG as compared with the same transformant grown in the absence of IPTG (lane 2) and with KD300 (lane 3) as a control strain. The apparent molecular mass estimated by SDS-PAGE was around 41 kDa.

Enzyme activity of the *gluA* gene product

The *gluA* gene product was prepared from KD301 (*E. coli* Sφ874-16 harbouring pMG1). The activity of glucose-1-phosphate uridylyltransferase was assayed by HPLC. The enzyme preparation from KD301 showed a specific activity of 1.01 ± 0.04 U (mg protein)⁻¹ (mean $\pm$ SD, $n = 3$). The activity was significantly higher than that of the cell extract from KD300 used as a control: 0.011 ± 0.002 U (mg protein)⁻¹.

Characterization of the function of the *gluA* gene in *S. mutans*

To analyse the function of the *gluA* gene in *S. mutans*, the gene was insertionally inactivated by homologous recombination. The gene was interrupted by pRes-

Table 3. Amino acid sequence identity among uridylyltransferase-related gene products

Protein	Percentage identity*				Species	Reference
	Cps3U	HasC	GalU	GalF		
GluA	76.8	85.2	40.1	33.8	<i>S. mutans</i> Xc	This study
Cps3U		76.3	38.7	32.5	<i>S. pneumoniae</i> WU2	Dillard <i>et al.</i> (1995)
HasC			38.1	35.1	<i>S. pyogenes</i> WF51	Crater <i>et al.</i> (1995)
GalU				53.0	<i>E. coli</i> K-12	Weissborn <i>et al.</i> (1994)
GalF					<i>E. coli</i> K-12	Yao & Valvano (1994)

* Percentage of identical amino acids over length of the protein sequence based on CLUSTAL V alignment.

Fig. 2. SDS-PAGE analysis of *gluA* gene expression in *E. coli*. Lane 1, cell lysate of *S*Ø874-16(pMG1) grown in the presence of 0.3 mM IPTG; lane 2, cell lysate of *S*Ø874-16(pMG1) grown in the absence of IPTG; lane 3, cell lysate of *S*Ø874-16(pBluescriptII KS⁺) grown in the presence of 5 mM IPTG. The positions of standard molecular mass markers are indicated.

EmNot (Shiroza & Kuramitsu, 1993) at the *Hinc*II site and one of the resultant mutants was designated Xc31. The intracellular concentration of UDP-D-glucose in Xc31 grown in Todd-Hewitt broth was lower than 0.1 nmol (mg protein)⁻¹, whereas that of UDP-D-glucose in Xc grown in the broth was 11 ± 3 nmol (mg protein)⁻¹ (mean $\pm$ SD, $n=3$). The concentrations of UDP-D-glucose in Xc22 and Xc24 were not significantly different from that in the parental strain Xc. The Rantz-Randall extracts from strains Xc and Xc31 were analysed by immunodiffusion with serotype antiserum (Fig. 3). Serotype antiserum reacted with the serotype c antigen purified from *S. mutans* MT8148 and with Rantz-Randall extract from strain MT8148, but not with Rantz-Randall extracts from MT703 (serotype e) and OMZ175 (serotype f), indicating that the serotype antiserum used in the present study does indeed possess serotype c specificity (Fig. 3). The serotype-c-specific antiserum reacted with the extract from strain Xc but not with that from Xc31 (Fig. 3). The sugar compositions of the cell wall preparations isolated from strain Xc and the mutant strain were analysed by HPLC (Fig. 4). The ratio of rhamnose to glucose in the cell wall

Fig. 3. Immunodiffusion analysis of Rantz-Randall extracts with anti-serotype-c serum. The centre well contains rabbit anti-serotype-c serum. The outer wells contain the Rantz-Randall extracts (20 µl) from strains Xc (well 1), Xc31 (well 2), MT703 (well 3), OMZ175 (well 4) and MT8148 (well 5). Well 6 contains the purified serotype c antigen (1 µg) from strain MT8148.

preparations from Xc was nearly 2. In contrast, glucose was barely detectable in the cell-wall preparation from the mutant strain Xc31. No significant difference in hexosamine content of the purified cell-wall preparations was observed between Xc and Xc31 (data not shown).

Environmental stress tolerance of the mutant

The mutant strain Xc31 barely grew in medium of pH below 5.6 and showed apparent deficiency of aciduricity as compared with the control strains Xc22 and Xc when the culture in exponential phase was inoculated (Table 4). Strain Xc24 showed an overall reduction in growth compared with Xc and Xc22 in an acidic medium, but it grew better than Xc31. Xc31 also showed obvious growth inhibition but grew better than Xc24 at temperatures at or above 43 °C, while Xc and Xc22 grew relatively well at 44 °C (Table 5). In contrast, Xc31, Xc and Xc22 did not greatly differ from each other in their growth in the presence of various concentrations of NaCl (0–0.9 M), whereas the growth of Xc24 was drastically inhibited by NaCl (Table 6).

Southern blot analysis

Fragments reactive to probe 2 were evident in all of the streptococci tested, as well as in *L. casei*, *Enterococcus faecalis*, *B. subtilis* and *C. bifementans*, and a faint band was detected in *Staphylococcus aureus* (Fig. 5). Two distinct hybridization bands were detected in *Streptococcus pyogenes* and *S. pneumoniae* (Fig. 5, lanes

Fig. 4. HPLC pattern of monosaccharides obtained by acid hydrolysis of cell-wall preparations of *S. mutans* strains Xc31 (a) and Xc (b). The pyridylamino sugars were analysed with HPLC using 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, v/v), the flow rate was 0.3 ml min⁻¹ 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 labelled Rha, GlcNAc, Glc and MurNAc indicate the elution times of pyridylaminated L-rhamnose, pyridylaminated *N*-acetylglucosamine, pyridylaminated D-glucose and pyridylaminated *N*-acetylmuramic acid, respectively.

12–14). However, no band for *gluA* was detected in other Gram-positive bacteria such as *Eubacterium limosum*, *Micrococcus luteus* or *Mycobacterium smegmatis* (Fig. 5), nor in the Gram-negative bacteria *Porphyromonas gingivalis*, *Actinobacillus actino-*

mycetemcomitans, *E. coli*, *Salmonella typhimurium* and *Shigella flexneri* (data not shown).

DISCUSSION

In the present study, we cloned the *gluA* gene of *S. mutans* using the *cps3U* gene (Dillard *et al.*, 1995) of *S. pneumoniae* as a probe. The amino acid sequence deduced from the nucleotide sequence of the *gluA* gene has 76.8% identity with that deduced from the nucleotide sequence of the *cps3U* gene (Table 3). However, inactivation of *cps3U* does not affect the phenotype of *S. pneumoniae* (Dillard *et al.*, 1995). Furthermore, the GalF protein of *E. coli*, which has substantial amino acid sequence identity with bacterial glucose-1-phosphate uridylyltransferases such as the GalU protein of *E. coli* (Table 3), was shown to be non-catalytic but rather to act as a modulator of GalU activity by increasing the formation of UDP-D-glucose (Marolda & Valvano, 1996). Therefore, it is important to confirm enzymic activity, especially in the case of glucose-1-phosphate uridylyltransferase. We tried to evaluate the enzymic activity of the *gluA* gene product expressed in *E. coli*. Because the enzyme encoded by *rmlA*, which was previously referred to as *rfaA* (Reeves *et al.*, 1996), is known to exhibit not only glucose-1-phosphate thymidylyltransferase activity but also glucose-1-phosphate uridylyltransferase activity in wild-type *E. coli* (Weissborn *et al.*, 1994), we constructed an *E. coli* strain in which both the *galU* and *rmlA* genes were inactivated to enable evaluation of the enzymic activity of the *gluA* gene product synthesized in *E. coli*. Recently, it has been reported that the *rffH* gene encodes glucose-1-phosphate thymidylyltransferase (Marolda & Valvano, 1995). It is possible that the *rmlA*- and *galU*-defective strain Sϕ874-16 might retain residual glucose-1-phosphate uridylyltransferase activity. However, the background activity was low enough to evaluate observed increases in

Table 4. Effect of pH on growth of *S. mutans* strains

Cultures were grown in Todd-Hewitt broth containing 50 mM sodium acetate buffer. The indicated pH value was the starting pH of each Todd-Hewitt broth after addition of sodium acetate buffer. The Todd-Hewitt broth was prepared three times, and each value (mean $\pm$ SD) of the starting pH was obtained from independent triplicate samples using each preparation. Growth of each strain was determined as the increase of OD₅₅₀. The values were obtained by subtraction of the OD₅₅₀ at initiation of the culture from that at stationary phase. Each value represents the mean $\pm$ SD for triplicate cultures from three independent colonies.

pH	Growth (increase in OD ₅₅₀) of strain:			
	Xc31 (<i>gluA</i> inactive)	Xc24 (<i>rmlB</i> inactive)	Xc22 (ORF3 inactive)	Xc (wild-type)
7.59 $\pm$ 0.06	1.156 $\pm$ 0.050	0.842 $\pm$ 0.063	1.277 $\pm$ 0.024	1.283 $\pm$ 0.039
5.97 $\pm$ 0.06	0.591 $\pm$ 0.144	0.737 $\pm$ 0.052	1.166 $\pm$ 0.043	1.155 $\pm$ 0.057
5.75 $\pm$ 0.08	0.235 $\pm$ 0.113	0.678 $\pm$ 0.071	1.086 $\pm$ 0.083	1.098 $\pm$ 0.092
5.60 $\pm$ 0.08	0.129 $\pm$ 0.097	0.612 $\pm$ 0.080	1.051 $\pm$ 0.088	1.049 $\pm$ 0.076
5.41 $\pm$ 0.06	0.035 $\pm$ 0.018	0.374 $\pm$ 0.118	0.878 $\pm$ 0.142	0.912 $\pm$ 0.104
5.24 $\pm$ 0.07	0.014 $\pm$ 0.010	0.177 $\pm$ 0.038	0.490 $\pm$ 0.215	0.463 $\pm$ 0.191

Table 5. Effect of temperature on growth of *S. mutans* strains

Each strain was cultured at the indicated temperature in Todd–Hewitt broth. Growth of each strain was determined as described for Table 4. Each value represents the mean $\pm$ SD for triplicate cultures from three independent colonies.

Temp. (°C)	Growth (increase in OD ₅₅₀) of strain:			
	Xc31	Xc24	Xc22	Xc
37	1.165 $\pm$ 0.049	1.042 $\pm$ 0.055	1.298 $\pm$ 0.041	1.287 $\pm$ 0.038
43	0.569 $\pm$ 0.083	0.103 $\pm$ 0.028	1.224 $\pm$ 0.033	1.225 $\pm$ 0.057
44	0.117 $\pm$ 0.028	0.014 $\pm$ 0.005	1.040 $\pm$ 0.036	1.056 $\pm$ 0.029
45	0.024 $\pm$ 0.016	0.005 $\pm$ 0.003	0.311 $\pm$ 0.072	0.292 $\pm$ 0.064

Table 6. Effect of NaCl concentration on growth of *S. mutans* strains

Cultures were grown in Todd–Hewitt broth containing the indicated concentration of NaCl. Growth of each strain was determined as described for Table 4. Each value represents the mean $\pm$ SD for triplicate cultures from three independent colonies.

NaCl concn (M)	Growth (increase in OD ₅₅₀) of strain:			
	Xc31	Xc24	Xc22	Xc
0	1.176 $\pm$ 0.052	1.086 $\pm$ 0.047	1.310 $\pm$ 0.027	1.319 $\pm$ 0.047
0.4	0.980 $\pm$ 0.074	0.290 $\pm$ 0.158	1.082 $\pm$ 0.033	1.063 $\pm$ 0.055
0.6	0.648 $\pm$ 0.130	0.043 $\pm$ 0.022	0.814 $\pm$ 0.073	0.779 $\pm$ 0.062
0.7	0.285 $\pm$ 0.094	0.026 $\pm$ 0.013	0.478 $\pm$ 0.091	0.492 $\pm$ 0.079
0.8	0.059 $\pm$ 0.026	0.017 $\pm$ 0.017	0.073 $\pm$ 0.032	0.076 $\pm$ 0.034
0.9	0.017 $\pm$ 0.012	0.008 $\pm$ 0.007	0.028 $\pm$ 0.011	0.029 $\pm$ 0.024

Fig. 5. Southern blot hybridization of chromosomal DNA from various bacterial species using probe 2 (see Methods). Lanes: 1, *Streptococcus mutans* Xc; 2, *S. cricetus* HS1; 3, *S. rattus* FA1; 4, *S. sobrinus* 6715; 5, *S. downei* MFe28; 6, *S. salivarius* HT9R; 7, *S. milleri* NCTC 10703; 8, *S. mitior* ATCC 12396; 9, *S. oralis* ATCC 10557; 10, *S. gordonii* ATCC 10558; 11, *S. agalactiae* IID1625; 12 and 13, *S. pyogenes* T29; 14, *S. pneumoniae* WU2; 15, *Enterococcus faecalis* SS499; 16, *Eubacterium limosum* GA15456; 17, *Lactobacillus casei* ATCC 393; 18, *Bacillus subtilis* 168; 19, *Staphylococcus aureus* IFO12732; 20, *Micrococcus luteus* GIFU8717; 21, *Mycobacterium smegmatis* RIMD1332001; 22, *Clostridium bifermentans* KZ1012. Lanes M contain standard markers with their sizes indicated in kb. DNA was digested with the following enzymes: lane 1, *Xba*I; lane 12, *Hind*III; lane 14, *Bgl*II; lane 21, *Bam*HI; lanes 13, 16 and 20, *Pst*I; all other lanes, *Eco*RI. Hybridization with probe 2 was carried out overnight at 25 °C in the presence of 50% formamide.

enzyme activity. The enzyme extract from KD301 expressing the *gluA* gene exhibited significant glucose-1-phosphate uridylyltransferase activity compared with

that of KD300, suggesting that *gluA* encodes the glucose-1-phosphate uridylyltransferase of *S. mutans*. Based on SDS-PAGE analysis, the protein product of *gluA* has an

apparent molecular mass of 41 kDa. This size is larger than the size of 35.3 kDa predicted from the sequence analysis. The difference could be due to protein secondary structure causing slower than expected migration in the gel. Such a difference in molecular mass has also been noted in glucose-1-phosphate uridylyltransferases of other bacteria, including GalU of *E. coli* (Weissborn *et al.*, 1994) and HasC of *S. pyogenes* (Crater *et al.*, 1995). The HasC protein is involved in hyaluronic acid synthesis in *S. pyogenes* and the amino acid sequence of the protein has 85.2% identity with that of the *gluA* gene product (Table 3).

Serotype-specific polysaccharide antigens of *S. mutans* have been reported to have a poly-L-rhamnose backbone with D-glucose side-chains (Linzer *et al.*, 1986; Pritchard *et al.*, 1986). Insertional inactivation of the *gluA* gene of *S. mutans* resulted in loss of serotype-c-specific antigenicity of the autoclaved extract (Fig. 3) and intracellular UDP-glucose in the *gluA* mutant cells (Xc31) was scarcely detected. Furthermore, HPLC analysis of cell wall preparation from Xc31 showed a dramatic decrease in glucose content but not in rhamnose content (Fig. 4). These findings suggest that the serotype-c-specific antiserum used in this study might recognize the glucose side-chains of the polysaccharide antigen, and lack of incorporation of glucose into the branches of the rhamnose backbone might lead to loss of serotype-c-specific antigenicity of Xc31. Moreover, the inactivation of the *gluA* gene apparently has no effect on the synthesis of the core rhamnose moiety. It may be concluded that UDP-D-glucose is an immediate precursor for glucose side-chain formation of the serotype-c-specific antigen of *S. mutans*. Recently, the *S. mutans* galactose operon comprising genes for galactose metabolism (galactokinase, galactose-1-phosphate uridylyltransferase and UDP-glucose-4-epimerase) was cloned and characterized (Ajdic *et al.*, 1996). UDP-D-glucose is theoretically synthesized from galactose via this pathway. However, the extract from Xc31 grown in the presence of 0.5% (w/v) galactose did not react with the antiserum (data not shown), suggesting that the galactose metabolism pathway does not complement the *gluA* gene function in Xc31.

Southern blot analysis showed that two *gluA*-specific probe-reactive fragments were positioned at 6.2 kb and 10 kb in the *Bgl*II-digested chromosomal DNA of *S. pneumoniae* WU2 (Fig. 5, lane 14). This indicates that two copies of the homologous gene exist in the chromosomal DNA of *S. pneumoniae* WU2, since there is no restriction site for *Bgl*II within the *cps3U* gene (Dillard *et al.*, 1995). Two probe-reactive fragments were also identified in *S. pyogenes* chromosomal DNA (Fig. 5, lanes 12 and 13). The BLAST program was used to search the *S. pyogenes* genome sequencing database for homology to the *gluA* gene. The database can be found at the WorldWide Web site of the University of Oklahoma's Advanced Center for Genome Technology (URL: <http://dna1.chem.uoknor.edu/strep.html>) and the sequence data were registered in the various contigs in 'random' order. Two genes homologous to *gluA*

were found in different contigs. The arrangement of the flanking genes of the homologous gene in one contig was similar to that of the *gluA* gene, whereas the arrangement of the flanking genes found in the other contig resembled that of the flanking genes of the *hasC* and *cps3U* genes (Crater *et al.*, 1995; Dillard *et al.*, 1995). In addition to the major band, a weakly reactive fragment was observed in *S. milleri* (Fig. 5, lane 7), *S. gordonii* (Fig. 5, lane 10) and *C. bifermentans* (Fig. 5, lane 22). These minor bands might be derived from an occurrence of the restriction site within the *gluA* homologue gene in these bacteria. *Streptococcus* species other than *S. pneumoniae* and *S. pyogenes* examined in this study are likely to have a single copy of the gene encoding glucose-1-phosphate uridylyltransferase based on the results of Southern blot analysis (Fig. 5).

The observation that the lack of UDP-D-glucose in *E. coli* results in an increase in the basal level of the expression of the σ^S gene (*rpoS*) and σ^S -dependent genes induced by osmotic pressure or during entry into stationary phase suggests that UDP-D-glucose serves as an intracellular signalling molecule controlling expression of the genes required for osmotolerance and thermotolerance in stationary phase (Böhringer *et al.*, 1995; Gæver *et al.*, 1988; Hengge-Aronis *et al.*, 1991). In earlier work, we reported the isolation of a mutant defective in aciduricity, GS5Tn1, in which a gene homologous to the *E. coli* *dgk* (diacylglycerol kinase) gene was partially inactivated (Yamashita *et al.*, 1993). This mutant grew poorly in an acidic environment and was sensitive to high osmolarity and elevated temperatures, leading to speculation that the *gluA* gene product, glucose-1-phosphate uridylyltransferase, may also be involved in acid tolerance of *S. mutans*. Interestingly, we found that strain Xc31, a *gluA* null mutant, as well as GS5Tn1, showed drastically reduced aciduricity (Table 4). Aciduricity is known to be one of the most important virulence factors of the organism. In this regard, it is possible that the inability of the *gluA* mutant to synthesize the serotype-c-specific polysaccharide is directly responsible for its reduced cell viability in an acidic environment. If the serotype-c-specific polysaccharide is really required for the maintenance of aciduricity, then a mutant that is totally unable to synthesize serotype-c-specific polysaccharide should exhibit the same phenotype as the *gluA* mutant. However, Xc24, which is a mutant defective in synthesis of serotype-c-specific polysaccharide (Tsukioka *et al.*, 1997a) but has the same levels of intracellular UDP-D-glucose as the wild-type Xc, grew better than Xc31 in an acidic environment (Table 4), whereas Xc24 scarcely grew at elevated temperature and high osmolarity (Tables 5 and 6), suggesting that UDP-D-glucose synthesis may be required for the maintenance of aciduricity of *S. mutans*. It is also clear that Xc31 has a reduced cell viability at elevated temperatures, but this reduction was not as pronounced as that of Xc24. At the present stage, we do not have any data to confirm whether UDP-D-glucose or serotype-specific antigen is required for cell viability of *S. mutans* at elevated temperature. Growth

of Xc31 was slightly inhibited even in the absence of NaCl compared with that of Xc and Xc22. Inhibition of the growth of Xc31 at high osmolarity was probably due to a comprehensive growth defect, not a defect in osmotic tolerance. Indeed, Xc31 grew well in the presence of 0.6 M NaCl, despite the fact that Xc24 scarcely grew at that concentration of NaCl, suggesting that osmotolerance of *S. mutans* is not drastically affected by the null mutation of the *gluA* gene.

In the present study, we evaluated stress tolerance of *S. mutans* growing in the exponential phase and not the stationary phase. Two acid-tolerance responses induced at low pH were recognized in *Salmonella typhimurium*: an exponential-phase acid-tolerance response and a stationary-phase acid-tolerance response (Lee *et al.*, 1994). Although induction of these two responses is σ^S independent, σ^S is required for a sustained induction of the exponential-phase acid-tolerance response (Lee *et al.*, 1995). Considering that UDP-glucose is a potential intracellular signal molecule in the control of expression of the *rpoS* gene (Böhringer *et al.*, 1995), it is possible that growth inhibition of Xc31 under acidic conditions may relate to unusual expression of the *rpoS* homologue in Xc31 cells. Meanwhile, UDP-D-glucose is thought to be a precursor of other cellular components such as glycopospholipids, which are important membrane constituents, and lipoteichoic acid anchors. It is possible that defects in such components might lessen the tolerance of *S. mutans* to conditions of acidity and elevated temperature. However, little information is currently available regarding regulation of the *rpoS* homologue and UDP-D-glucose metabolism in *S. mutans*. In addition, the mechanism of acid tolerance of *S. mutans* is at present ill-defined. Further studies are required to elucidate the details of the unique biological function of UDP-D-glucose synthesis in this organism.

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