Chromosomal inversion between *rrn* operons among *Streptococcus mutans* serotype *c* oral and blood isolates

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Streptococcus mutans causes dental caries and infective endocarditis. The aim of this study was to determine genomic diversity among serotype *c* *S. mutans* laboratory and clinical strains and to characterize the genetic events involved. A genome-based approach using PFGE coupled with Southern hybridization was employed to examine a total of 58 serotype *c* oral and blood isolates and seven laboratory strains and to compare them with *S. mutans* UA159. No significant differences were found in the phenotypic characteristics of the strains tested, except that some of the strains exhibited smooth rather than rough colony morphology. In contrast, PFGE profiles of clinical isolates, from either diseased or healthy subjects, exhibited diverse patterns, suggesting that recombination or point mutations occurred frequently *in vivo*. Diverse PFGE patterns, with various lengths of insertions and deletions, could be detected even within a localized chromosomal region between rRNA operons. Comparative analysis using Southern hybridization with specific markers revealed that a large chromosomal inversion had also occurred between *rrn* operons in 25 strains.

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INTRODUCTION

Streptococcus mutans together with members of mutans streptococci are the primary aetiological agents of dental caries in humans (Loesche, 1986). *S. mutans*, and other viridans streptococci, such as *Streptococcus sanguinis* and *Streptococcus oralis*, are also common causes of infective endocarditis (Mylonakis & Calderwood, 2001). Based on the serological properties of cell-wall polysaccharide antigens, nine serotypes (Nomura *et al.*, 2005) and seven distinct species have been described in mutans streptococci, indicating a substantial heterogeneity among mutans streptococci. Of all serotypes, serotype *c* *S. mutans* is the most prevalent isolate found in humans, irrespective of age or race of subjects studied (Loesche, 1986). The whole genomic sequence of a serotype *c* *S. mutans* strain (UA159) was completed recently (Ajdic *et al.*, 2002). The unique pathogenic potential of *S. mutans* in distinct tissue compartments, oral cavity or heart valves, is attributed in part to its ability to establish biofilms on teeth or damaged tissue matrix. The natural competence of *S. mutans* and the

close contact with other bacteria in the dental biofilm have laid a perfect foundation for the promotion of horizontal gene transfer for the benefit of adaptation or survival in distinct environments (Davey & O'Toole, 2000).

Although serotyping is used broadly for identification of *S. mutans*, phenotypic or biochemical diversity is often observed within the same serotype. Certain serotype *c* clinical isolates are unable to ferment melibiose (Beighton *et al.*, 1991), and both rough and smooth colony formers on Mitis Salivarius (MS) agar are found within the serotype *c* isolates (Okahashi *et al.*, 1984). Clonal diversity of *S. mutans* depending on colonization niches has also been suggested (Gronroos & Alaluusua, 2000). Although it has been postulated that, in addition to interspecies differences among mutans streptococci, heterogeneity exists among closely related *S. mutans* isolates belonging to the same serotype *c*, genetic evidence is still lacking. In this study, a genome-based approach using PFGE coupled with Southern hybridization was conducted on a total of 58 serotype *c* oral and blood isolates as well as seven

laboratory strains. We have confirmed that genome diversity exists among serotype *c* clinical isolates and have also provided evidence to indicate that insertion, deletion and inversion between *rrn* operons contribute to the diversity.

METHODS

Bacterial strains and growth conditions. The seven serotype *c* laboratory strains included in this study were UA159 (ATCC 700610), GS-5 (Kuramitsu, 1975), MT8148 (Okahashi *et al.*, 1984), JH1005 (Hillman *et al.*, 1984), PC3370 (Crowley *et al.*, 1999), Xc (Koga, *et al.*, 1989) and NTU-5R. Twenty-five of the 50 clinical strains studied were isolated from the oral cavities of healthy individuals (caries-free) and the other 25 strains were from diseased (caries-active) individuals. In the caries-active group, samples were collected from dental plaque overlying untreated carious lesions from different sites (pooled plaque samples), whereas stimulated saliva samples were collected from caries-free individuals. *S. mutans* was isolated from the samples following serial dilution and plating on Mitis Salivarius (MS) agar plates (Difco). Selected colony-forming units from each donor were subsequently confirmed by biochemical reactions as well as by the serotyping analysis described below. Eight additional serotype *c* *S. mutans* strains that had been isolated previously from blood cultures of patients with infective endocarditis, were also included in the study. Approval for collection and investigation of human specimens was obtained from the Research Ethics Committee of the National Taiwan University Hospital.

All *S. mutans* strains used were grown routinely in Brain Heart Infusion (BHI) broth (Difco) and on MS agar at 37 °C with 5% CO₂.

Identification and morphology of the *S. mutans* clinical isolates. The identities of the *S. mutans* strains were confirmed by using an API Rapid ID 32 Strep kit (bioMérieux). The Rapid ID 32 Strep system serotype *c* *S. mutans* generally forms small (0.5–1 mm in diameter), raised and rough colonies on MS agar. Among all the clinical isolates, 51 strains formed rough colonies and seven strains formed smooth colonies (Okahashi *et al.*, 1984) on MS agar. In the seven laboratory strains examined, only strains JH1005 and GS-5 showed smooth colonies on MS plates.

Serotyping of *S. mutans* clinical isolates. The serotype of each isolate was determined by using an immunodiffusion test with rabbit serotype-specific antibodies. The serotype-specific polysaccharide antigens were extracted from *S. mutans* UA159 (serotype *c*), *S. mutans* MT73OR (serotype *e*), *S. mutans* OMZ175 (serotype *f*), *Streptococcus sobrinus* B13 (serotype *d*) and *S. sobrinus* 6715 (serotype *g*) and serotype-specific antisera were prepared and confirmed as described by Hamada *et al.* (1976). The serotypes of all the clinical isolates were determined by specific reaction with antisera for serotype *c*, and a lack of response to antisera for serotypes *d*, *e*, *f* and *g*. Over 90% of the isolates belonged to serotype *c* and a total of 58 serotype *c* isolates were selected for further analysis.

Biofilm formation and transformation of *S. mutans*. The ability to form biofilms is an important virulence trait of *S. mutans*. The degree of biofilm formation was evaluated by using the method of O'Toole & Kolter (1998), except that the bacteria were grown in 3 ml BHI medium overnight at 37 °C. All of the strains tested in this study produced biofilm, albeit at different degrees. The density of the biofilm was quantified from triplicate assays by measuring optical density at 570 nm after staining with crystal violet. Statistical analysis was conducted by using analysis of variance (ANOVA) and

differences were considered significant when the error probability was less than 0.05. Most of the isolates formed biofilm at a density comparable to that observed for strain UA159.

Preparation of competent *S. mutans* cells and transformation were performed as described by Perry & Kuramitsu (1981). A shuttle-plasmid, pDL278 (Aspiras *et al.*, 2000), was used to determine the transformation efficiency for each strain. All test strains were transformable, with variable transformation efficiencies ranging from 10 to 10⁶ c.f.u. (µg plasmid DNA)⁻¹.

DNA manipulation and PCR. Genomic DNA from *S. mutans* was isolated as described previously (Chia *et al.*, 1991). The PCR primers used in this study are listed in Table 1. For PCR products greater than 15 kbp, 400 µM dNTPs and 2.5 U LA *Taq* polymerase (Takara Bio Inc.) were used in each reaction. All reactions were carried out according to the manufacturer's instructions.

PFGE and Southern blots. Genomic DNA from all of the *S. mutans* isolates was characterized by using macrorestriction analysis with *NotI* or *I-CeuI* (New England Biolabs). DNA agarose plugs for PFGE were prepared according to Martin *et al.* (1998) with minor modifications. The agarose plugs containing the genomic DNA were cut to about 1 mm-thick slices and digested overnight with 20 U *NotI* or *I-CeuI*. The digested DNA fragments were separated in a 1.0% agarose gel in TBE buffer, and lambda DNA concatemers (size range, 50–1000 kbp; New England Biolabs) were used as molecular mass markers. PFGE was carried out at 14 °C in a CHEF Mapper XA system (Bio-Rad Laboratories). Electrophoresis was performed at 6 V cm⁻¹, and various switch-times and run-times were used, depending on the resolution ranges. Following electrophoresis, the gels were visualized under UV illumination and photographed using Analysis System 120 (Scientific Imaging Systems, Eastman Kodak Co.). Digital images of ethidium bromide-stained gels were saved as TIFF files and analysed visually with GelCompar (Applied Maths), which involved the Dice coefficient and UPGMA with 1% tolerance and 0.5% optimization settings.

Southern hybridization of DNA fragments from PFGE agarose gels was carried out at high stringency, as described previously (Chia *et al.*, 1991). The hybridization probes were generated by PCR and were based on the genome sequence of strain UA159. All probes were labelled with [α -³²P]dATP by random priming (NEBlot kit; New England Biolabs).

Statistical analysis. Analysis of the characteristics of the laboratory and clinical strains was performed using the chi-square test. *P* values of <0.0001 were regarded as significant. ANOVA was used to analyse the variance of biofilm production. *P* values of <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Heterogeneity of serotype *c* *S. mutans* strains

The serotypes of all the clinical isolates were determined using antisera specific for serotypes *c*, *d*, *e*, *f* and *g*, as detailed in Methods, and 58 serotype *c* isolates were selected for further analysis. Phenotypic characterization showed that all test strains exhibited identical biochemical characteristics, with the exception of strains 16-1 and 16-7, which were negative for α -galactosidase. In addition, all but five strains (16-1, 16-7, 37, 41-2 and 8585) were able to ferment melibiose. Regardless of the minor differences in biochemical profiles and variable transformation

Table 1. PCR primers used in this study

Name	Forward*	Reverse*	Length of PCR product (bp)
Probe 1 (<i>NotI</i> fragment H-specific)	719893–719910	721316–721333	1440
Probe 2 (<i>NotI</i> fragment G-specific)	563789–563806	564568–564585	796
Probe 3 (<i>NotI</i> fragment A-specific)	514905–514922	516013–516030	1125
Probe 4 (<i>NotI</i> fragment A-specific)	183750–183767	185197–185214	1464
Probe 5 (<i>NotI</i> fragment F-specific)	1974287–1974304	1975250–1975267	980
Probe 6 (<i>NotI</i> fragment E-specific)	1938418–1938435	1940330–1940347	1929
Probe 7 (<i>NotI</i> fragment D-specific)	1701070–1701087	1702453–1702470	1400
Probe 8 (<i>NotI</i> fragment B-specific)	1324473–1324490	1325547–1325564	1091
Probe 9 (<i>NotI</i> fragment C-specific)	956792–956809	957478–957495	703
1st (for amplification of region 1)	1874458–1874482	1889401–1889425	14967
2nd (for amplification of region 2)	1889401–1889425	1900355–1900379	10978
3rd (for amplification of region 3)	1900355–1900372	1920323–1920340	19985
4th (for amplification of region 4)	1920323–1920340	1939427–1939444	19121
5th (for amplification of region 5)	1940323–1940347	1955510–1955534	15211
6th (for amplification of region 6)	1955510–1955534	1970848–1970872	15362
7th (for amplification of region 7)	1970855–1970872	1990885–1990902	20047
8th (for amplification of region 8)	1990885–1990902	2010919–2010936	20051
a (for amplification of region a)	1970855–1970872	1974870–1974887	4029
b (for amplification of region b)	1974791–1974808	1975893–1975910	1119
c (for amplification of region c)	1975893–1975910	1976884–1976901	1008
d (for amplification of region d)	1976884–1976901	1977809–1977826	942
e (for amplification of region e)	1977826–1977845	1978819–1978836	1010
f (for amplification of region f)	1982766–1982783	1983764–1983781	1015
g (for amplification of region g)	1983781–1983798	1984796–1984813	1032
h (for amplification of region h)	1984796–1984813	1985801–1985818	1022
i (for amplification of region i)	1985801–1985818	1986998–1987015	1214
j (for amplification of region j)	1986922–1986939	1990885–1990902	3980
<i>gfpA</i> (accession no. M30945) amplification	1981872–1981888	1980004–1980030	1884

*The positions on the chromosome of *S. mutans* UA159 (GenBank accession no. AE014133) are listed.

frequency, biofilm formation was quite compatible in all strains examined. To test whether genomic diversity is a general phenomenon within serotype *c* *S. mutans* strains, chromosomal DNA from all 58 clinical isolates and the seven laboratory strains was subjected to PFGE analysis. Total chromosomal DNA from all strains was digested initially with *NotI* or *I-CeuI* to completion and separated using PFGE. *I-CeuI* is an intron-encoded endonuclease that recognizes a unique 19 bp sequence present in the *rrn* locus for 23S rRNA. The reproducibility of PFGE was confirmed by using UA159 as the reference strain in each run. The PFGE profiles of all strains were confirmed in at least triplicate gels. The PFGE profiles of either *NotI* or *I-CeuI* digests revealed a substantial polymorphism, encompassing the whole genome present among all isolates, including the laboratory strains, and it was too complicated to clearly categorize the isolates into simple groups (data not shown). For strain UA159, complete digestion with *I-CeuI* constantly revealed three consecutive bands of 189, 177 and 169 kbp. Albeit a substantial polymorphism was present in terms of the size of the *I-CeuI* fragments, all isolates, including the laboratory strains, exhibited the same number of *I-CeuI* fragments.

Characterization of the large chromosomal rearrangement in laboratory strains

Using PFGE analysis, Cappiello *et al.* (1999) constructed a genetic map of *S. mutans* GS-5. Based on the complete genome of strain UA159, we constructed a genetic map of UA159 using the Vector NTI Advance software package (data not shown). Comparative analysis of genetic maps of *I-CeuI* and *NotI* fragments from both *S. mutans* UA159 and GS-5 revealed major rearrangements within the C and D *I-CeuI* fragments (i.e. between *rrnA* and *rrnC*; Fig. 1a). Previously, we characterized a *S. mutans* laboratory strain (NTU-5R) that did not contain the *gfpA* locus, and found that the deletion was located between *rrnB* and *rrnC* (Chia and others, unpublished data). Therefore, strains GS-5 and NTU-5R were selected for further genetic characterization, with an attempt to explore the genetic elements or events that contribute to the macrorestriction polymorphism observed among *S. mutans* strains.

Based on a computer-aided BLASTN search, a series of probes (Table 1) specific for each *NotI* fragment of *S. mutans* UA159 were constructed and were used to locate corresponding regions on the chromosomes of *S. mutans*

Fig. 1. Physical maps and hybridization patterns of PFGE-separated DNA of *S. mutans* strains with various probes. (a) Comparison of the physical maps of *NotI* and *I-CeuI* fragments from *S. mutans* UA159, GS-5 and NTU-5R. The fragments are named according to Cappiello *et al.* (1999). The estimated size of each fragment in kbp is shown. The locations and orientations of *rrn* loci are indicated by open triangles. All hybridization probes are numbered and the locations are indicated by solid lines. (b) Hybridization patterns of *NotI*-digested, PFGE-separated chromosomal DNA from *S. mutans* UA159 (U), GS-5 (S) and NTU-5R (R). The probe used in each hybridization reaction is indicated at the top. *The relative locations of probes 4, 5 and 6 on NTU-5R are not clear. (c) Hybridization patterns of *I-CeuI*-digested, PFGE-separated, *S. mutans* chromosomes with various probes. R, S and U are the same as in (b). The sizes of the restriction fragments in strain UA159 were used as a reference.

GS-5 and NTU-5R. When chromosomal DNA was hybridized with probes specific for the B, C, G and H fragments (probes 8, 9, 2 and 1), signals with sizes predicted by the maps were observed in both GS-5 and NTU-5R (data not shown). These results indicate that the arrangement between *NotI* -B, -C, -H, and -G fragments is conserved among all three *S. mutans* strains. When chromosomal DNA was hybridized with two A-specific probes (probes 3 and 4 – one to each end of the A fragment), two signals, approximately 430 and 280 kbp, corresponding to GS-5 *NotI*-A and -D fragments, respectively, were observed in strain GS-5, but not in NTU-5R (Fig. 1b, probes 3 and 4). Furthermore, the same 430 kbp *NotI* fragment of strain GS-5 also hybridized to probe 6 (specific for fragment E), confirming inversion of *S. mutans* UA159 *NotI*-A and -E fragments in *S. mutans* GS-5. In contrast, with the NTU-5R chromosome, all probes (probes 3, 4, and 6) hybridized to a single *NotI* fragment of approximately 800 kbp (the size was confirmed in separate PFGE experiments under running conditions for resolution between 100 and 870 kbp) (Fig. 1b). Similarly, when the same blot was hybridized

with probe 5, whose location was close to the juncture of fragments E and F on the UA159 chromosome, a signal of approximately 140 kbp was detected in GS-5, but not in NTU-5R (Fig. 1b, probe 5). This probe hybridized to the same *NotI* fragment that also hybridized with probes 3, 4 and 6 in GS-5, indicating that sequence alterations resulting in the loss of *NotI* recognition sequences had occurred in NTU-5R.

To further confirm the genomic arrangements in strains UA159 and GS-5, the hybridization patterns of *I-CeuI*-digested chromosomal DNA with probes 4, 6 and 7 were also determined (Fig. 1c). In agreement with the hybridization pattern of *NotI*-digested chromosomal DNA, signals with predicted sizes as found in UA159 were also observed in GS-5 with all three probes, indicating the presence of a genomic inversion that might occur through the combination of neighbouring *rrn* operons. In contrast, different hybridization patterns of *I-CeuI* fragments were observed in NTU-5R (Fig. 1c), suggesting that insertions within the *I-CeuI*-B and -C fragments (probes 4 and 6) and deletions within the *I-CeuI*-D fragment (probe 7) may have

occurred in the NTU-5R chromosome. Together, these data indicate that, compared with strain UA159, a large chromosomal inversion had occurred between *rrn* operons in *S. mutans* GS-5 and that deletion (other than *gfpA*) could also occur in these regions, as found in NTU-5R.

Chromosomal inversion in clinical isolates

Based on the hybridization pattern of strain GS-5, we also investigated the *NotI*-digested PFGE patterns of all isolates to predict whether recombination between *rrnA* and *rrnC* might have occurred. If inversion had occurred, probes 3 and 6 would hybridize to the same signals, whereas probe 4 would not. As shown in Fig. 2, in isolates NTU-1020, -3432 and -3930, the hybridization patterns obtained using probes 3 and 6 were identical, and probe 4 also hybridized to a signal (260 kbp) similar to that found in GS-5. These results indicated that chromosomal inversion might also

occur in these three clinical isolates. Similar approaches indicated that a total of 24 clinical isolates (41.3%) and one laboratory strain (14.3%) exhibited *NotI*-digested PFGE patterns similar to those of GS-5, suggesting that recombination might have occurred in both clinical and laboratory isolates.

Characterization of the large chromosomal shuffling in strain NTU-5R

Genomic analysis of gene positions in closely related micro-organisms suggests that replication forks are preferred sites for DNA recombination (Tillier & Collins, 2000). We noticed that the *I-CeuI*-C fragments of strain UA159 contained the replication origin (*ori*) and that an inversion had occurred in this region between strains UA159 and GS-5. Our analysis of the NTU-5R genome also indicated that a major deletion and/or sequence shuffling

Fig. 2. PFGE and hybridization patterns of *NotI*-digested chromosomes of *S. mutans* strains. (a) Digested chromosomal DNA from *S. mutans* NTU-1020, 1037, 3432, 3368 and 3930 were hybridized with various probes as indicated in Fig. 1(a). (b) PFGE of *NotI*-digested DNA from laboratory *S. mutans* strains. S, R and U indicate chromosomes from strains GS-5, NTU-5R and UA159, respectively. 1020, 1037, 3432, 3368 and 3930 are the same as in (a). The sizes of restriction fragments of UA159 were used as a reference.

Fig. 3. Analysis of the region between *rrnB* and *rrnC* using long-PCR. (a) Physical maps of *S. mutans* UA159 chromosomes digested with *I-CeuI*. The estimated size of each fragment is shown in kbp. Diagrammatic representations of the relative positions of the primers are indicated by arrows. The *rrns* are indicated by open triangles. The relative location of *gbpA* is indicated. (b) Agarose gel electrophoresis of PCR products generated from *S. mutans* strains. Lanes 1, 2 and 3 show chromosomes from GS-5, NTU-5R and UA159, respectively.

had occurred within this region (Fig. 1). To further characterize the differences within this region between UA159, GS-5 and NTU-5R, an extensive series of PCR primers was designed based on the UA159 genome, and was used to amplify long stretches of DNA fragments within the *I-CeuI* C-fragment (Fig. 3a). Amplicons of the predicted sizes were observed only in UA159 and GS-5 with all pairs of primers used (Fig. 3b). PCRs with NTU-5R chromosomal DNA yielded a larger amplicon in the 5th region and a smaller one in the 7th region. The estimated insertion and deletion are 8 and 10 kbp, respectively. In addition, the deletion observed in the 7th region contained the *gbpA* locus (Fig. 3b). To further analyse the deleted region, chromosomal walking by PCR was performed and the nucleotide sequences of the PCR-amplified fragments were determined by direct sequencing. A series of 10 pairs of primers was included to encompass a total length of 16 kb flanking the 7th region of *I-CeuI*-C. The overlapping

and contiguous PCR fragments obtained were about 1–4 kb, named segments a, b, c, d, e, f, g, h, i and j, as indicated in Fig. 3(a). Surprisingly, sequencing results confirmed that all PCR fragments, from a to j, derived from NTU-5R exhibited entities that were almost identical to that from UA159 (Fig. 4b). When PCR amplification of larger fragments was conducted by combining primers from segments c and h, a correct amplicon with a size of around 10 kbp was obtained (Fig. 4a, lane U). However, PCR amplicons obtained from NTU-5R consisted of two shorter fragments of 5 and 2.5 kbp (Fig. 4a, lane R). Further analysis using primers derived from segments e and g revealed similar PCR fragments (e–g) around 8 kbp from DNA from either NTU-5R or UA159. Therefore, a positive identity of fragments e–g found in NTU-5R suggested that this region was not deleted but might have located elsewhere in the chromosome. Taken together, these results suggest that sequence shuffling might have

Fig. 4. Analysis of a 10 kbp chromosomal fragment of strain NTU-5R by PCR. The position and primers used to generate internal fragments of the 7th region were those depicted in Fig. 3(a). (a) Agarose gel electrophoresis of long-PCR products generated from *S. mutans* UA159 (U) and NTU-5R (R). M, 1 kb DNA ladder; N, negative control of PCR reaction. Bands of predicted sizes were found for UA159 with a 'c-h' PCR product, about 10 kbp, and an 'e-g' PCR product of about 8 kbp. In contrast, a shorter fragment of 'c-h' but not 'e-g' was obtained in NTU-5R. (b) Agarose gel electrophoresis of PCR products generated from subfragments in the 7th region from UA159 (U) or NTU-5R (R). 'c, d, e, f, g and h' PCR products amplified from either UA159 or NTU-5R are of identical length and with DNA sequence homology around 97% (data not shown).

occurred in the 7th region at the I-*CeuI*-C region of NTU-5R. Whether deletion in addition to *gbpA* or the underlying mechanism of chromosomal shuffling is involved awaits further investigation.

Deletion of the virulence gene *gbpA* among clinical isolates

The *gbpA* gene encodes one of the glucan-binding proteins (GBPA) and contributes to the adherence and biofilm formation of *S. mutans* on the surfaces of teeth (Banas & Vickerman, 2003). Using primers specific for *gbpA* of *S. mutans* UA159, we were able to detect a PCR product with the predicted size in chromosomal DNA from *S. mutans* GS-5 but not from NTU-5R (data not shown), confirming a deletion of *gbpA* in NTU-5R. The *gbpA*-deficient genotype was also observed in isolates from caries-free and caries-active subjects, and patients with endocarditis, at frequencies of 16, 4 and 12.5%, respectively (Table 2). Based on this small-scale survey, deletion of *gbpA* was observed at a significantly higher frequency in isolates from caries-active than caries-free or endocarditis individuals ($P < 0.0001$; chi-square test).

In conclusion, we have demonstrated that genomic diversity exists among *S. mutans* serotype *c* laboratory and clinical isolates from different colonizing niches in the volunteers tested. The most interesting findings were that genomic insertion, deletion and inversion occur frequently in *S. mutans* even within rather localized and defined regions between *rrn* operons. A large genomic rearrangement across the replication axis or recombination between *rrn* regions has been shown to occur in M3 and M5 strains (Manfredo)

of *Streptococcus pyogenes*, respectively (Nakagawa *et al.*, 2003; Holden *et al.*, 2007). It is clear that intrachromosomal recombination is an important mechanism that contributed to the evolution of the genomes of the two *S. pyogenes* strains (Holden *et al.*, 2007). Polymorphisms in *rrn* operons have also been identified between *Streptococcus thermophilus* strains (Pebay *et al.*, 1992; Roussel *et al.*, 1997). Different serovars of *Salmonella* (general or host-specific) undergo rearrangements at *rrn* operons at similar frequencies *in vitro*, indicating that the observed difference in genomic stability between general and host-specific serovars is a consequence of their distinct lifestyles, and not intrinsic differences in recombination frequencies (Helm *et al.*, 2003). In *S. mutans*, PFGE analysis following *NotI* digestion has been carried out previously in strain MT8148 (Okahashi *et al.*, 1990). The distribution of a number of insertions and deletions has been identified between the closely related laboratory strain UA159 and its derivative LT11 (Tao *et al.*, 1993) or among a collection of clinical isolates of *S. mutans* from different areas of the world. It has been suggested that *S. mutans* had a core genome and a dispensable genome, and that dispensable genes have become widely distributed through horizontal transfer (Waterhouse & Russell, 2006). Interestingly, in that report, the deletion of *gbpA* was also identified in several strains and the deletion was replaced by insertion of other genes as well as IS elements. The most recent microarray analysis indicated that a total of 385 (20%) of the UA159 open-reading frames were absent from one or more of the nine test *S. mutans* strains (Waterhouse *et al.*, 2007). We found that IS199 (Macrina *et al.*, 1996) was present in NTU-5R, but was absent from both GS-5 and UA159, by using PCR analysis (data not shown). However, chromosomal walking and sequence analysis of the 10 kb

Table 2. Characteristics of laboratory and clinical strains

Strain (no.)	Colony morphology		<i>gbpA</i> deletion	Recombination between <i>rrn</i> loci
	Smooth	Rough		
Caries-active				
Laboratory (7)	2 (28.6 %)	5 (71.4 %)	2 (28.6 %)	1 (14.3 %)
Clinical (25) (DMFS=13.08 ± 4.19)*	2 (8 %)	23 (92 %)	1 (4 %) [†]	10 (40 %)
Caries-free				
Clinical (25) (DMFS=0)	3 (11.5 %)	22 (88 %)	4 (16 %)	10 (40 %)
Endocarditis (8)	2 (25 %)	6 (75 %)	1 (12.5 %)	4 (50 %)
Total (65)	9 (13.8 %)	56 (86.2 %)	8 (12.3 %)	25 (38.5 %)

*Mean DMFS (decayed–missing–filled surfaces) of caries-active clinical strains.

[†]*P* < 0.0001, chi-square test (statistically significant).

fragment in the 7th region at the I-*CeuI*-C region of NTU-5R (Fig. 3) did not reveal elements belonging to IS199 or others. Therefore, IS elements might not be involved in the gene shuffling observed in NTU-5R.

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