Chapter 9
Molecular Typing of *Streptococcus mutans*

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9.1 Properties of *Streptococcus mutans*

Streptococcus mutans, a Gram-positive facultative anaerobic bacterium, is generally known to be a pathogen of dental caries and also considered to be one of the oral streptococcal species that can cause infective endocarditis since it was reported to be recovered from 8 to 10% of patients with endocardial disease [1] (Fig. 9.1). *S. mutans* is one of the members of the "mutans streptococci" group, which also consists of *Streptococcus sobrinus*, *Streptococcus cricetus*, *Streptococcus rattus*, *Streptococcus ferus*, *Streptococcus macacae*, and *Streptococcus downei*. Mutans streptococci were previously classified into eight serotypes based on the chemical composition of their serotype-specific polysaccharides, among which five serotypes (*a* through *e*) were designated in 1970, followed by three additional serotypes (*f*, *g*, *h*) determined during the next decade. *S. mutans* (*c*/*e*/*f*) and *S. sobrinus* (*d*/*g*) are detected in humans, while *S. cricetus* (*a*) and *S. rattus* (*b*) strains are mainly identified in hamsters and rats, respectively. In addition, *S. ferus* (*c*) was reported to be isolated from rats, and *S. macacae* (*c*) and *S. downei* (*h*) were isolated from monkeys. Among the mutans streptococci, *S. mutans* is the most frequently identified in the

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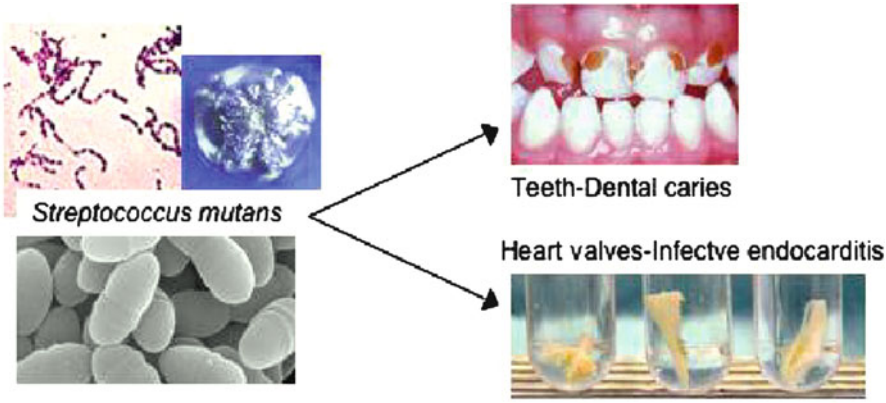

Fig. 9.1 *Streptococcus mutans* and its associated diseases

human oral cavity, followed by *S. sobrinus*. On the other hand, the presence of the mutans streptococci in the oral cavities of animals is considered to result from the ingestion of sucrose contained in feed.

The serotype-specific polysaccharides of *S. mutans* are known to be composed of rhamnose-glucose polymers, with a backbone of rhamnose and side chains of glucose polymers [2]. The chemical linkage of each rhamnose unit is common in each *c*/*e*/*f* serotype (α-1,2 and α-1,3 repeatedly), while that for glucose side chains is different; α-1,2 for serotype *c*, β-1,2 for serotype *e*, and α-1,3 for serotype *f*. The distribution frequency of serotype *c* strains in the oral cavity is the highest with a rate of 70–80%, followed by serotype *e* (approximately 20%) and *f* (less than 5%). Strains, which could not be classified into any of *c*/*e*/*f* serotypes, have been described in the literature from subjects in several countries. However, details of the chemical composition of their serotype-specific polysaccharides have not been investigated. In 2004, the 9th serotype "*k*" was designated for the non-*c*/*e*/*f* serotype *S. mutans* strains which were isolated from the blood of the patients with bacteremia after tooth extraction and infective endocarditis complicated with subarachnoid hemorrhage [3].

Analysis of the distribution of serotype *k* strains revealed that the detection frequency in the oral cavity of Japanese children was 2–5%, which was shown to be consistent with that of Thai subjects [4]. In addition, several *S. mutans* strains of non-*c*/*e*/*f* serotypes isolated in Finland and UK were classified into serotype *k* [5, 6]. There have been several reports demonstrating the presence of non-*c*/*e*/*f* strains but an estimation of the presence of serotype *k* strains is not possible due to the lack of adequate description of the chemical composition of the serotype-specific rhamnose glucose polymers. On the other hand, a recent study in Chile considered the possibility of the presence of serotype *k* for the strains of non-*c*/*e*/*f S. mutans* serotypes isolated [7]. Taken together, these studies suggest that the serotype *k* strains are prevalent worldwide.

In addition to polysaccharide antigens, cell surface protein antigens are important for the virulence of *S. mutans* in dental caries. Among the various cell surface

antigens, an approximately 190-kDa protein antigen (PA), glucosyltransferases (GTFs), and glucan-binding proteins (Gbps) are known to be major virulence factors for *S. mutans* [8–10]. PA, also referred to by other names (PAc, SpaP, antigen I/II, antigen B, SR, IF, P1, and MSL-1) is correlated with the sucrose-independent initial adhesion to tooth surfaces by the bacterium. In addition, GTFs are composed of three types (GTFB/GTFC/GTFD), and are known to be associated with sucrose-dependent adhesion. GTFB and GTFC, located on the cell surface, mainly synthesize water-insoluble glucans, which contain a high degree of branching of α-1,3-glucosidic linkages, whereas GTFD, released into the culture supernatant, produces water-soluble glucans that are predominantly linear polymers linked by α-1,6-glucosidic bonds, similar to dextran.

The complete genome of *S. mutans* strain UA159 (serotype *c*) was sequenced in 2002 by a team at the University of Oklahoma Health Sciences Center, which revealed that it was composed of 2,030,936 bp and contained 1,963 ORFs [11]. Detailed information is available in the Oral Pathogen Sequence Database provided by the Database Team at the Bioscience Division of Los Alamos National Laboratory (http://www.oralgen.lanl.gov/). Recently, the complete genome of another *S. mutans* strain NN2025 (serotype *c*) isolated from a Japanese child with severe dental caries was sequenced, which showed that it was composed of 2,013,587 bp and contained 1,869 ORFs [12]. When comparing the complete genomes of the two strains, core-genome was shown to be highly conserved, whereas a large genomic inversion between homologous ribosomal operons across the replication axis was identified. In addition, at least 25 different regions, which might be transferred following conjugation transfer or mediated by insertion elements, were identified in the two strains. At this moment, the complete genome of an additional *S. mutans* strain LJ23 (serotype *k*) is now being sequenced in order to identify the serotype-*k* specific genomic features by comparison with UA159 and NN2025.

9.2 Detection of *S. mutans*

In the early 1990s, DNA probe methods targeting *gtf*s and other genes were constructed to detect *S. mutans*. However, these are hampered by complex procedures and low sensitivity and one of the studies demonstrated that more than 300 pg of DNA and as many as 2×10^5 cells would be required for detection [13]. In the middle of the 1990s, the PCR-based approach for *S. mutans* detection was introduced, which was regarded as rapid, sensitive and relatively simple method. The *spaP* gene encoding the 190 kDa-protein antigen (PA) [14] and the *dexA* gene encoding extracellular dextranase were targeted for construction of species-specific sets of primers [15] (Table 9.1). The sensitivity was drastically increased with a lower limit of 1 pg of chromosomal DNA or 12 colony-forming units of *S. mutans* cells. Subsequently, several molecular methods for detecting *S. mutans* DNA in specimens from dental plaque and saliva have been reported. In addition, cardiovascular specimens, such as those from heart valves and atheromatous plaques, have been examined [16, 17].

Table 9.1 PCR primers for identification of *S. mutans*

Target gene	Name	Sequence (5′ to 3′)	Product Size (bp)	References
spaP	Sense	AAC GAC CGC TCT TCA GCA GAT ACC	192	[14]
	Antisense	AGA AAG AAC ATC TCT AAT TTC TTG		
dexA	SD1	TAT GCT GCT ATT GGA GGT TC	1,272	[15]
	SD2	AAG GTT GAG CAA TTG AAT CG		

Table 9.2 PCR primers for identification of *S. mutans*

Target gene	Name	Sequence (5′ to 3′)	Product Size (bp)	References
gtfB	GTFB-I	ACT ACA CTT TCG GGT GGC TTG G	517	[20]
	GTFB-R	CAG TAT AAG CGC CAG TTT CAT C		
gtfD	MKD-F	GGC ACC ACA ACA TTG GGA AGC TCA GTT	433	[21]
	MKD-R	GGA ATG GCC GCT AAG TCA ACA GGA T		
16S rRNA	8UA	AGA GTT TGA TCC TGG CTC AG	1,505	[24]
	1492R	TAC GGG TAC CTT GTT ACG ACT T		
16S rRNA	Forward	GGT CAG GAA AGT CTG GAG TAA AAG GCT A	282	[25]
	Reverse	GCG TTA GCT CCG GCA CTA AGC C		
gorESL	ES.5-29	TAA AAC TAG GFG AHC GWR TBG T	430	[29]
	EL.35-18R	CKK GCA TCT GCT GAA AAT		
ddlA	Forward	ATT GAA GGC GAG CCT TTA GAA AG	351	[30]
	Reverse	GTT GCT ATT GTC CTA G		
sodA	*d1*	CCI TAY ICI TAY GAY GCI YTI GAR CC	480	[31]
	d2	ARR TAR TAI GCR TGY TCC CAI ACR TC		

The lower limit of detection for *S. mutans* DNA was reported to be detected in heart valve specimens extirpated from infective endocarditis patients, in which multiple species were identified in each specimen [18]. Thus, it is possible to speculate that *S. mutans* is one of the possible etiological agents and/or it is incidentally detected during transient bacteremia. As for atheromatous plaque, the high detection rate of *S. mutans* DNA does not necessarily mean a direct association of *S. mutans* with atheromatous plaque formation [19]. It is advantageous that PCR methods are very sensitive, however, careful interpretation of the results is required since bacterial DNA from nonviable and/or incidentally disseminated strains can be identified as positive reactions.

Table 9.2 summarizes the commonly used primer sets for *S. mutans* detection, among which those designed based on the *gtf* genes are widely used. Many oral streptococcal species reside in dental plaque, and the glucan synthesizing by the glucosyltransferases encoded by the *gtf* genes is one of the major factors in dental plaque formation [9]. The species-specific sets of primers have been constructed based on the differences in the nucleotide alignments of the *gtf* genes among several oral streptococci. The primer sets for *S. mutans* designed based on the *gtfB* or the

gtfD genes are widely used [20, 21]. As for the methods using the *gtfD* sequence, the lower limit of detection for *S. mutans* DNA was reported to be 1.5 pg, indicating that this method is very sensitive. In addition, the methods for quantifying the numbers of *S. mutans* cells were developed using real-time PCR with the primer set SmF5 (5′-AGC CAT GCG CAA TCA ACA GGT T-3′) and SmR4 (5′-CGC AAC GCG AAC ATC TTG ATC AG-3′) targeting the *gtfB* gene [22]. It was reported that high levels of *S. mutans* in the parents is one of the important factors for vertical transmission into children [23]. Thus, this method could be one of the possible tools for identifying subjects with high risk for transmission. In addition, it could also be used to determine the number of the bacterial cells in cardiovascular specimens, which might lead to the identification of specific pathogenic bacterial species when multiple species are identified in each specimen by PCR.

The other molecular approach for detecting *S. mutans* is the restriction fragment length polymorphism (RFLP) of amplified 16S rRNA fragments, in which approximately 1,500 bp fragments are amplified with the universal primers 8UA and 1492R followed by digestion with *Hpa*II [24]. In addition, another primer set based on 16S rRNA alignments [25] has been modified to amplify approximately 1,500 bp lengths of 16S rRNA followed by nested PCR amplification of an internal 282 bp region [24]. However, it was reported that false positive could result since the 16S rRNA sequence of the mutans streptococci and neighboring group are quite similar [26]. On the other hand, the determination of the entire 16S rRNA sequence amplified by the primer sets 8UA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′) was used to confirm that the tested strain was truly a *S. mutans* [27]. This obviates misleading results although it is more time consuming and expensive than PCR methods with species-specific primer sets.

The *groESL* genes encoding 10-kDa (GroES) and 60-kDa (GroEL) heat shock proteins are reported to be ubiquitous and evolutionary highly conserved genes [28]. However, the *groESL* sequence is known to be less conserved as compared to the 16S rRNA sequence, indicating the possibility for its application in the differentiation of species with high degrees of similarity in their 16S rRNA sequences. The primer set for identification of mutans streptococci using the *groESL* genes was designed to amplify the fragment containing a region of partial *groES*, partial *groEL*, and the *groES-EL* spacer [29]. Since the nucleotide length of the *groES-groEL* spacer is varied among each species (111 bp for *S. mutans*, 218 bp for *S. sobrinus*, 200 bp for *S. cricetus*, 125 bp for *S. rattus*, and 310 bp for *S. downei*), the molecular sizes of the positive bands are different depending on the species. The other species-specific sets of primers were also constructed based on the nucleotide alignment of the *ddlA* gene encoding D-alanine:D-alanine ligase, which is known to be essential for bacterial cell wall synthesis [30]. In addition, a method for sequencing the internal fragment representing 85% of the *sodA* gene encoding manganese-dependent superoxide dismutase, was also developed, which discriminates between a large numbers of the various streptococcal strains [31].

The 16S–23S ribosomal RNA intergenic spacer (ITS) region, known to contain low levels of intraspecies variation and high levels of interspecies divergence, can also be used for speciation of *S. mutans*. PCR using universal primers 13BF (5′-GTG

Table 9.3 Primers used for broad-range PCR

Target gene	Name	Sequence (5′ to 3′)	References
16S rRNA	536f	CAG CAG CCG CGG TAA TAC	[33]
	1050r	CAC GAG CTG ACG ACA	
16S rRNA	PA	AGA GTT TGA TCC TGG CTC AG	[34]
	PD	GTA TTA CCG CGG CTG CTG	

AAT ACG TTC CCG GGC CT-3′) and 6R (5′-GGG TTY CRT TCR GAA AT-3′) was designed based on the sequence of the 3′-region of the 16S rRNA gene and the 5′-portion of the 23S rRNA gene [32]. This amplifies fragments of variable sizes depending on the species. When the specimens contain *S. mutans* DNA, the amplified fragments include the 387 bp or 388 bp regions of ITS. Determination of the nucleotide alignment is initially performed, after which the identification of *S. mutans* is made by comparing the sequence of species-specific ITS and that of the specimens.

It is possible that the conventional methods for identification of bacterial species fail to identify phenotypically aberrant strains. On the other hand, the broad-range PCR and sequencing method, in which full and partial 16S rRNA nucleotide alignments are determined, is a reliable tool. As compared to PCR with species-specific sets of primers, the broad-range PCR and sequencing method enables the identification of multiple species in the specimens. Several primers for broad-range PCR methods have been developed and the amplified fragments are then sequenced, for comparison with those in the GenBank, EMBL, and DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/) [33, 34] (Table 9.3). Identification to the species level is generally defined as a 16S rRNA sequence similarity of more than 99% with that of the prototype strain sequence in the databases. This approach is widely used for the investigation of bacterial profiles in the clinical specimens such as saliva and dental plaque as well as with cardiovascular specimens.

S. mutans strains are easily isolated from oral specimens, such as saliva and dental plaque, using selective medium and Mitis-salivarius agar plates containing bacitracin (MSB plates) [35]. On the other hand, *S. mutans* is occasionally isolated from the blood of the patients with bacteremia and infective endocarditis. However, it is difficult to isolate the strains from blood specimens once antibiotic treatments are initiated. The molecular methods enable detection of *S. mutans* using bacterial DNA extracted from even nonviable cells, which should be regarded as the most advantageous aspects in the use of molecular approaches.

9.3 Differentiation of *S. mutans* and *S. sobrinus*

S. mutans is highly prevalent in the oral cavity of humans, with a detection rate ranging from 74 to 94%, while *S. sobrinus* is known to be less prevalent [36]. In general, *S. mutans* is reported to be associated with coronal caries, whereas *S. sobrinus* is considered to be correlated to the lesions found on the smooth surfaces [36, 37].

It is generally known that subjects harboring both *S. mutans* and *S. sobrinus* have a significantly higher caries experience than those with only *S. mutans* [38, 39].

S. mutans and *S. sobrinus* are easily discriminated based on the rough and smooth colonies, respectively, on Mitis-salivarius agar plates. However, the GTF-defective strains of *S. mutans* are known to show smooth colony morphology on the agar although the distribution frequency is extremely low [40, 41]. Thus, it is possible that phenotypic variation prevents the appropriate discriminations of *S. mutans* and *S. sobrinus*. However, PCR-based approaches using species-specific primers for each species are not influenced by the phenotypic variations of *S. mutans*. The primer sets for *S. mutans* were designed based on the *gtfB* or *gtfD* genes of *S. mutans*, whereas *gtfI* was used for construction of the primer sets for *S. sobrinus* [20, 21].

One of the other molecular approaches is the PCR-RFLP method. In this method, the 4-kb region of the ribosomal RNA (*rrn*) operon, which most bacteria possess several copies for, with a high degree of homology, is amplified by PCR, followed by the comparison of the digestion patterns of the amplified fragments following *Hin*fI, *Mbo*I, or *Taq*I digestion [42]. Another approach is the chromosomal DNA fingerprint (CDF) and arbitrarily primed (AP)-PCR methods [43]. The CDF method can discriminate between two major patterns after *Hae*III digestion of the chromosomal DNA of each strain. One is designated as the CDF-1 group with restriction fragments equal to or greater than 6.6 kb in size and the other the CDF-2 group with fragments less than 6.6 kb. All of the tested *S. mutans* strains are classified as CDF-1, whereas most of the *S. sobrinus* strains were classified as CDF-2.

As for AP-PCR approach characterized by the short length of primers and low annealing temperatures, the primer OPA-02 (5′-TGC CGA GCT G-3′) is the most commonly used for the analysis of *S. mutans*, followed by OPA-13 (5′-CAG CAC CCA C-3′). This choice is primarily due to the appearance of readily identifiable electrophoretic products with the former compared to the other 40 sets of the primers [44] (Fig. 9.2). *S. mutans* and *S. sobrinus* strains showed similar patterns in each group, which are reported to consist of the major common amplified fragments of 782 bp and 1,070 bp, respectively. In addition, OPA-03 (5′-AGT CAG CCA C-3′), OPA-05 (5′-AGG GGT CTT G-3′) and OPA-18 (5′-AGG TGA CCG T-3′) were also used in several studies [44].

9.4 Classification of Serotypes

The genes involved in the biosynthesis of serotype-specific polysaccharides are estimated to be located in four different regions and those in strain UA159 (serotype *c*) are illustrated in Fig. 9.3. There are multiple enzymes required for the biosynthesis of the polysaccharides and the biochemical steps and their relevant genes have been identified [1]. The enzyme RgpG encoded by *rgpG* is proposed to be involved in the first step in the formation of the polysaccharides catalyzing the transfer of *N*-acetylglucosamine-1-phosphate to a lipid carrier [45]. On the other hand, the

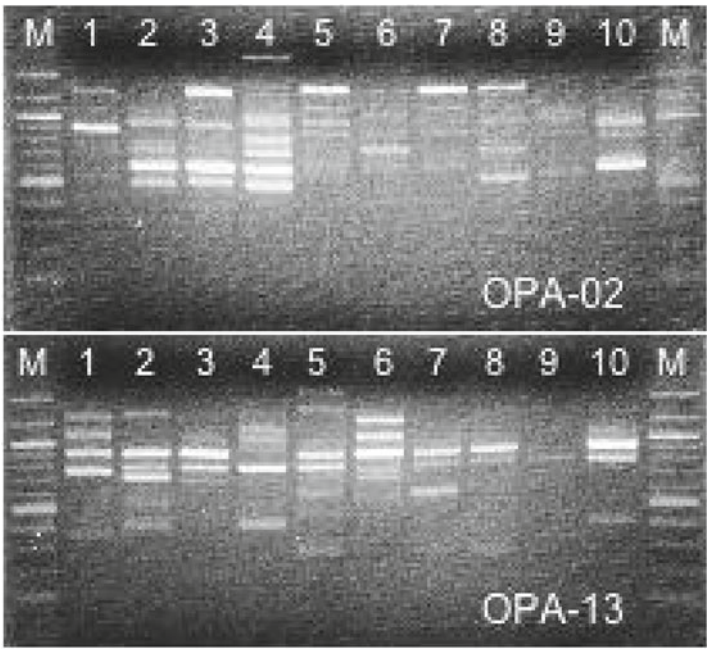

Fig. 9.2 Electrophoretic appearance of the results of arbitrarily primed (AP)-PCR for ten clinical strains (*Lane 1–10*) using OPA-02 and OPA-13 primer sets

basic units of the rhamnose polymers and glucose side chains are considered to be dTDP-L-rhamnose and UDP-D-glucose, respectively. These units are synthesized from the UDP-D-glucose-1-phosphate by the actions of multiple enzymes encoded by their respective genes [46, 47]. The *rmlA*, *rmlB*, *rmlC*, and *rmlD* genes are known to encode the enzymes that catalyze the pathway from UDP-D-glucose-1-phosphate to the rhamnose units [46, 48], and the *gluA* gene encoding GluA is known to be involved in the biosynthesis of the units of the glucose side chain [49].

The *rgpA*, *rgpB*, and *rgpF* genes encoding RgpA, RgpB, and RgpF, respectively, are reported to function in the polymerization of the rhamnose units [47]. Specifically, RgpA is proposed to be associated with the first rhamnose unit, whereas RgpB and RgpF are presumed to be correlated with the polymerization of the even and odd numbers of rhamnose units from the second unit, respectively [50]. In addition, the *rgpE* gene encoding RgpE is considered to be involved in the side-chain formation by glucose units [47]. Furthermore, RgpH was shown to encode a glucosyltransferase, while RgpI is thought to control the frequency of branching [51]. As for polysaccharide export, the *rgpC* and *rgpD* genes encoding RgpC and RgpD were demonstrated to regulate this function [47].

As compared to serotype *c/e/f* strains, the region from downstream of *rgpF* to the upstream of ORF12 was demonstrated to be highly variable among each serotype [38]. Using the differences in the nucleotide alignments in these regions, primer sets

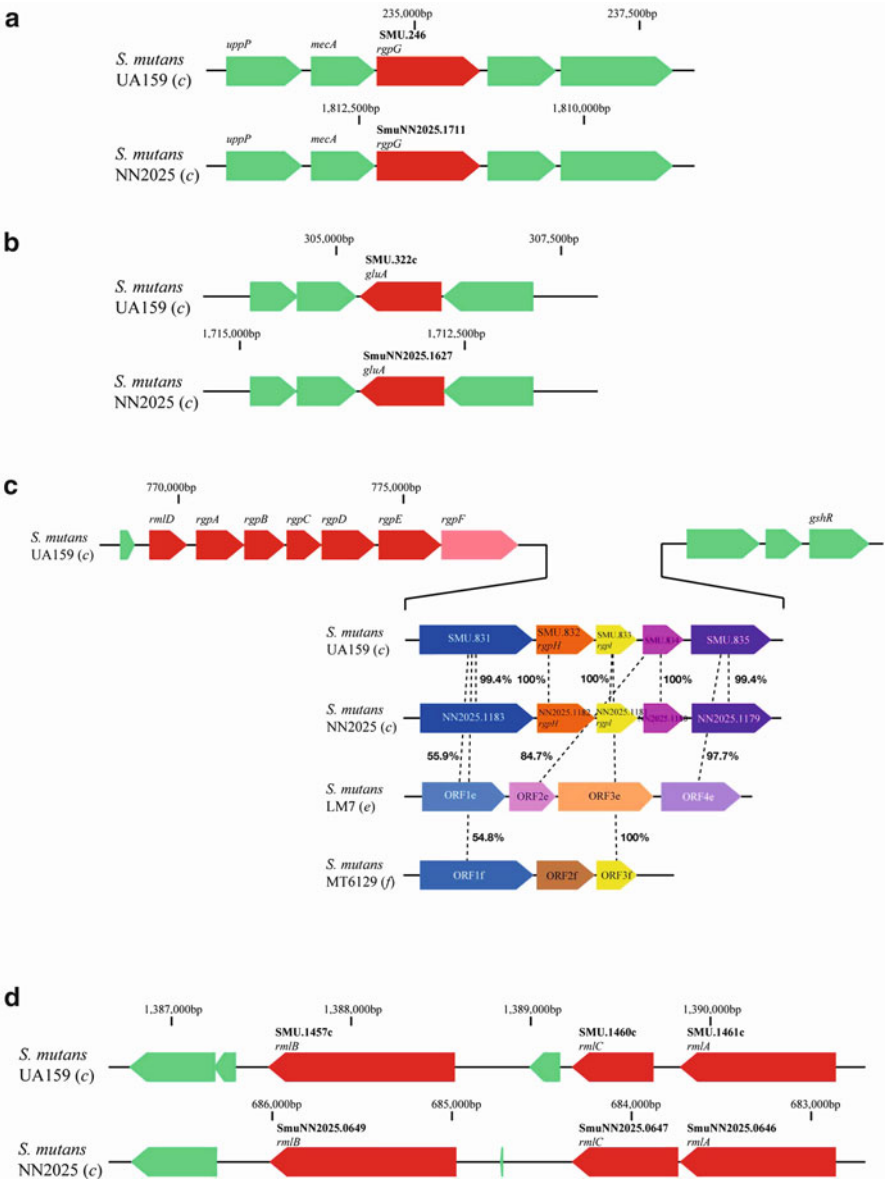

Fig. 9.3 Genes involved in the biosynthesis of the serotype-specific polysaccharide of *S. mutans*. The genes were located four different regions (**a**–**d**). *rgpG* (**a**) and *gluA* (**b**) genes are completely conserved between two sequenced serotype *c* strains (UA159 and NN2025). However, genes located downstream of *rgpF* are quite diverse (**c**). *rmlA-C* genes are also conserved between the two serotype *c* strains, but several differences are found in the intergenetic regions (**d**)

Table 9.4 PCR primers for determination of serotypes

Serotype	Name	Sequence (5′ to 3′)	Product size (bp)	References
c	SC-F	CGG AGT GCT TTT TAC AAG TGC TGG	727	[38]
	SC-R	AAC CAC GGC CAG CAA ACC CTT TAT		
e	SE-F	CCT GCT TTT CAA GTA CCT TTC GCC	517	[38]
	SE-R	CTG CTT GCC AAG CCC TAC TAG AAA		
f	SF-F	CCC ACA ATT GGC TTC AAG AGG AGA	316	[38]
	SF-R	TGC GAA ACC ATA AGC ATA GCG AGG		
k	CEFK-F	ATT CCC GCC GTT GGA CCA TTC C	296	[52]
	K-R	CCA ATG TGA TTC ATC CCA TCA C		

for identification of each *c*/*e*/*f* serotype were constructed. On the other hand, no drastic differences in the nucleotide alignments between serotype *c* and *k* strains were identified in that region [52]. However, a serotype *k*-specific alignment is present in the 5′ one-third end of the *rgpF* gene, upon which the serotype-*k* specific set of primers was constructed. It should be noted that these alterations in the nucleotide alignments in this region for the serotype *k* strains are considered to be inconsequential for the observed variety of glucose side chains in the serotype-specific polysaccharides [53]. Table 9.4 lists the primers for determination of each serotype *c*/*e*/*f*/*k* strain of *S. mutans* [38, 52]. PCR detection system using these primer sets were demonstrated to be very sensitive, with the minimum number of cells detected being 5–50 per reaction.

9.5 Identification of Virulent Strains

Considering the prevention of dental caries, the approaches used should be ideally based on the common risk factors for dental caries [54]. Thus, the identification of subjects with highly virulent strains could be beneficial for the prevention of dental caries. This should be true also when considering the pathogenesis of *S. mutans* in blood. It is generally accepted that considerable phenotypic variations exist within *S. mutans* species, which is derived from a consequence of a variety of genetic events, such as point mutations, translocations and inversions [35]. Therefore, some of the strains show strong virulence and others are regarded as weak virulent strains. In order to develop molecular methods to identify subjects who harbor the highly virulent *S. mutans* strains, several PCR approaches using the extracted bacterial DNA from the specimens have been evaluated.

One of the methods for possible clinical use in estimating the risk of dental caries in subjects is the identification of the multiple serotypes of *S. mutans* in specimens from the oral cavity by PCR with serotype-specific sets of primers [38]. The use of such methods is supported by the evidence that dental caries scores for preschool children with multiple serotypes of *S. mutans* were shown to be significantly higher than those with a single serotype or with no detectable *S. mutans*. It should be noted

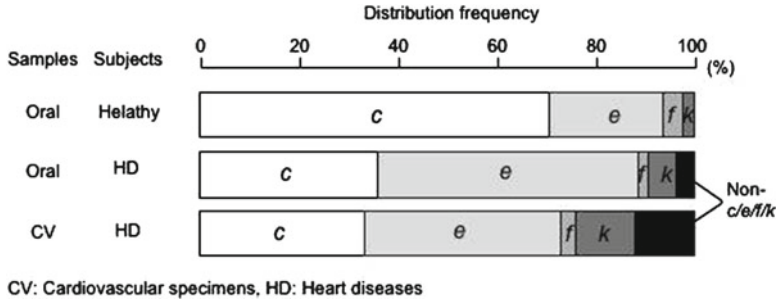

Fig. 9.4 Serotype distribution of oral or cardiovascular specimens from healthy subjects or patients with cardiovascular diseases

that the risk for subjects should be estimated based upon their clinical conditions, such as the number of lesions or fillings, in addition to the results of the molecular analyses. The other study showed the unique distribution of the serotypes in the oral and cardiovascular specimens in subjects with cardiovascular diseases (Fig. 9.4) [55]. The serotype distribution patterns in the subjects with cardiovascular diseases were demonstrated to be totally different compared with healthy subjects. Thus, it might be possible to also use serotype determinations as a means of identifying subjects at risk for developing cardiovascular diseases although additional confirmation of such a relationship is still required.

Although there are a large number of studies attempting to identify the association of the cell surface protein antigens and the pathogenesis of dental caries, the development of molecular methods to analyze for virulence genes is relatively uncommon. In this regard, one of the approaches is RFLP analysis of the *gtf* genes, which is based upon the high diversity of the *gtf* genes [56]. In that method, the 5.2-kb *gtfB* and 4.3-kb *gtfC* genes amplified by PCR are digested with *Bsr*I and *Ssp*I, respectively. Ten and five genotypes were designated based on the digestion patterns for *gtfB* and *gtfC*, respectively. However, there were no correlations found between specific genotypes and the GTF enzymatic activities.

Recently, the *cnm* gene, encoding a 120-kDa cell-surface collagen-binding adhesin of *S. mutans*, was cloned and sequenced [57], which has received attention due to the possible association of dental caries with infective endocarditis [58]. *S. mutans* strains with the *cnm* gene are estimated to be present in approximately 10–20% of individuals. The *cnm*-positive strains possess significantly higher activities for binding type I collagen than the *cnm*-negative strains (Fig. 9.5a). Thus, *S. mutans* strains with *cnm* were predicted to show high virulence for dental caries since type I collagen is also a major organic component of dentin. It was also proposed that the *cnm*-positive strains could bind with higher affinity than the *cnm*-negative strains once the dentin is exposed as caries progresses. In fact, clinical parameters indicate that dental caries in children with *cnm*-positive *S. mutans* in saliva was significantly higher than those with *cnm*-negative *S. mutans* strains as well as *S. mutans*-negative children (Fig. 9.5b).

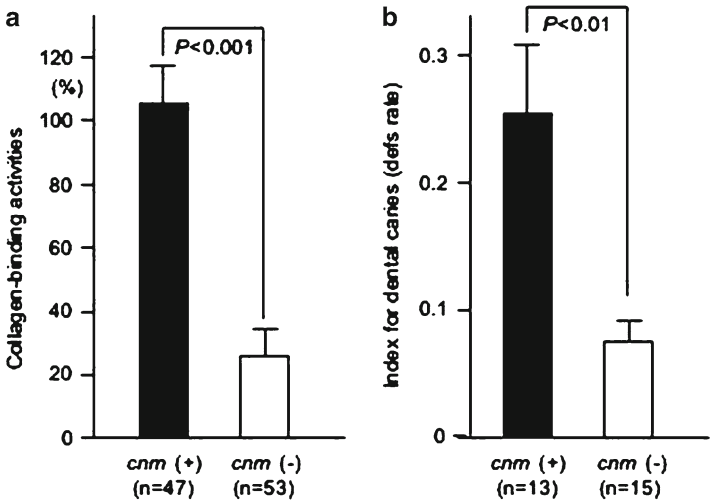

Fig. 9.5 Properties of *S. mutans* strains with *cnm* genes. (**a**) Collagen-binding activity of the strains with or without *cnm* genes when that of strain TW871 as 100%. (**b**) Dental caries index for the subjects harboring strains with or without the *cnm* gene

The *cnm* gene consists of the conserved collagen-binding domain in the 5′-region, followed by a region containing multiple B-repeats, whose length varied among the different strains. The primer set specific for the *S. mutans cnm* gene was constructed based on the nucleotide alignment of the *cnm* gene (cnm-1F 5′-GAC AAA GAA ATG AAA GAT GT-3′ and cnm-1R 5′-GCA AAG ACT CTT GTC CCT GC-3′). The size of the amplified fragments varied from approximately 1,650–1,750 bp due to the number of repeats within the amplicon. The distribution frequency of the *cnm* gene in *S. mutans* strains in the oral cavity is estimated to be approximately 10–20%, with the *cnm*-positive strains showing a predominant distribution among strains with the minor serotypes *f* and *k*.

It was reported that a *S. mutans* strain with defects in the expression of all three GTFs has been isolated [58]. This defect of caused a drastic reduction in its virulence potential for inducing dental caries [40], however, the concomitant decrease in GTF antigenicity was speculated to result in lower susceptibility to phagocytosis by polymorphonuclear leukocytes. This could result in the enhanced survival of such strains in blood compared to GTF-expressing strains. Using the specific nucleotide alignment in *gtf* regions, primer sets specific for detection of similar non-GTF expressing strains were constructed [59]. The detection rate for such strains was shown to be quite low. It has been hypothesized that alterations of cell surface structures of *S. mutans* are considered to be related to the survival in blood as well as the pathogenicity for infective endocarditis [60]. Therefore, further studies focused on the relationship between the cell surface antigens and pathogenesis as well as the development of PCR methods to identify subjects with these highly virulent strains should be considered.

9.6 Transmission Studies

Acquisition of *S. mutans* is considered to be initiated after the first primary tooth erupts, which provides a location for the bacterium to be colonized [61]. The first tooth which erupts is the generally mandibular primary central incisor, which emerges into the oral cavity at an age between 6 and 12 months. The number of erupted teeth increases as children grow, and they face a critical period for colonization at the age between 19 and 31 months called the "window of infectivity" [62], although the speed of colonization could depend on the caries activity of the bacterial population. More recent studies indicate that *S. mutans* can colonize the mouths of predentate infants [63], which indicates the possibility that the predentate children receive *S. mutans* frequently in their mouth and some are transient and some can colonize on the tongue. They are then able to attach to the surface of the first erupting tooth when circumstances are favorable such as with sugar ingestion. The original sources of *S. mutans* have been demonstrated to be mainly their mothers from a large number of studies conducted worldwide. Longitudinal studies regarding the genotypes of *S. mutans* in children demonstrate that most of the initially acquired genotypes generally transmitted from the mothers persist and some are lost and new strains are also acquired. In addition, the sharing of *S. mutans* genotypes between siblings has also been reported, which suggests the possibility of horizontal transfer of strains acquired from mothers between siblings [64]. Furthermore, the transmission of *S. mutans* strains from other family members or other care givers has also been considered.

Saliva is considered to be the major vehicle for oral bacterial transmission, and a high level of salivary *S. mutans* in mothers results in the earlier colonization of the bacterium in their children [65]. In addition, saliva specimens are thought to reflect the composition of the whole oral cavity, whereas dental plaque specimens primarily indicate localized colonization [66]. Studies regarding the transmission of *S. mutans* have been performed by comparison of the isolated strains using various subtyping strategies, such as serotyping, bacteriocin activity profiles, and molecular typings [4]. As for molecular biological methods, CDF techniques commonly employed with *Hae*III digestion, ribotyping, AP-PCR assays, or random-amplified polymorphic DNA (RAPD) analyses are generally used [43, 67]. In addition, the diversity of *S. mutans* strains from children and their mothers was investigated by RFLP of the *gtfB* gene digested by *Hae*III [68].

It should be advantageous to analyze as many strains as possible in a single subject when performing transmission studies. Since there exist time and financial limitations, the number of the estimated genotypes in the populations should be carefully considered when constructing the study design. As for Japanese, approximately 90% of the subjects are estimated to harbor one or two genotypes (average; 1.9 genotypes) [69]. Another study conducted in China demonstrated that 95% of the subjects aged 9–14 years possess one or two genotypes (average; 1.5 genotypes) [70]. Thus, 3–5 randomly selected representative strains should be sufficient for *S. mutans* to be analysis when performing transmission studies.

On the other hand, a study carried out in Sweden showed that only 60% of the subjects between the ages of 20–40 possessed fewer than two genotypes (average; 2.6 genotypes), and the maximum number of the genotypes was shown to be seven [67]. In addition, analysis of the subjects aged 18–29 years held in Brazil showed that a caries-free group possessed one to four genotypes (average; 3.0 genotypes) and that the caries-active group possessed two to eight genotypes (average; 5.5 genotypes). When analyzing these populations, the numbers of the strains are recommended for the study should be as large as possible [71]. In addition, it should be noted that the number of genotypes of *S. mutans* could be influenced by the dental caries status of each individual. AP-PCR analyses revealed that the children with severe dental caries based on the inappropriate usage of nursing bottles showed higher numbers of genotypes as compared with that of caries-free children [72]. This finding suggests that during favorable circumstances, such as sugar exposure, it is easier for new genotypes to be colonized.

The intra-familial transmission rates have been reported by a large number of groups worldwide and could be influenced by many factors such as cultural background, even within similar populations in the same country. Although most of the studies focus on the mother–child transmission of *S. mutans*, there is one study considering father–child transmission [69]. In that study, analysis of 1908 isolates from 76 subjects with 20 Japanese families including children below the age of 12 demonstrated that the transmission ratio from mothers and fathers were demonstrated to be 51.4% and 31.4%, respectively.

9.7 Multilocus Sequencing Typing Approach

Multilocus sequencing typing (MLST) is a generic typing method, employed to date principally, but not solely with bacterial pathogens, which aims to be a robust and portable method for the characterization of bacterial isolates at the molecular level. This method differs from many other approaches for characterization in that it is based explicitly on population genetic concepts [73]. MLST usually employs allele fragments of housekeeping genes approximately 400–600 bp in length and 6–10 loci were selected for sequencing because MLST provides sufficient discrimination for bacterial typing without being subject to diversifying selections which could obscure relationships among isolates. This method was first developed in 1998 in a study of *Neisseria meningitides* [74] and it has been applied for examination of approximately 40 species of microorganisms.

As mentioned above, several genotypic typing methodologies have been used to subtype *S. mutans* including multilocus enzyme electrophoresis (MLEE), ribotyping, and RAPD [64, 67, 71]. More discriminating methods for the subtyping of *S. mutans* include pulsed-field gel electrophoresis (PFGE) [75]. However, these methods differ in their discriminatory abilities power and reproducibility. Therefore, we have developed the MLST method for *S. mutans* typing (Fig. 9.6). Table 9.5 lists the eight housekeeping gene loci applied for a MLST scheme for *S. mutans* [6].

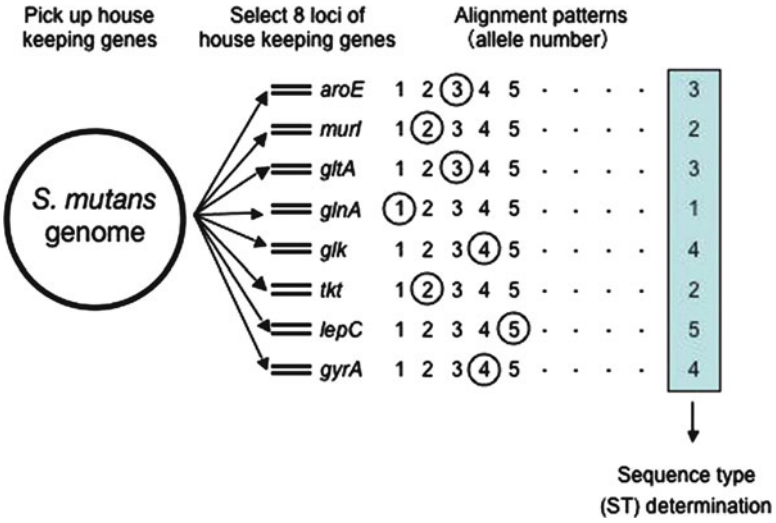

Fig. 9.6 Strategy for multilocus sequence typing (MLST) method of *S. mutans*

Table 9.5 Characteristics of housekeeping genes in *S. mutans* MLST scheme

Genes	Gene locus tags	Gene products	Number of allele identified[a]
tkt	Smu.291	Transketorase	14
glnA	Smu.364	Glutamine synthase	18
gltA	Smu.365	Glutamate synthase	26
glk	Smu.542	Glucose kinase	19
aroE	Smu.778	Shikimate 5-dehydrogenase	22
gyrA	Smu.1114	DNA gyrase subunit A	17
murI	Smu.1718	Glutamate racemase	19
lepC	Smu.1874	Signal peptidase I	25

[a]Number was determined based on the analyses of total of 238 strains from 142 subjects

Initially, the internal fragments of the housekeeping genes were amplified and their nucleotide sequences determined (GenBank accession numbers AB281702-AB282509 and AB427220-AB428307). The sequences for each allele are compared with those in nonredundant databases (http://pubmlst.org/oralstrep/) and allele numbers were assigned for each strain, which defines the allelic profile. Finally, the sequence types (STs) for each strain are assigned. Numbers of the alleles for eight kinds of housekeeping genes are between 14 and 26 and STs 1–108 are assigned for 238 strains from 142 subjects at present [4, 6].

Figure 9.7 shows a phylogenetic tree based on hierarchical clustering of MLST results with 108 STs from 238 strains isolated in Japan and Finland. This result indicated that *S. mutans* contains a diverse population. This method was proven to theoretically distinguish more than 1.2×10^{10} sequence types. The serotype *c* strains

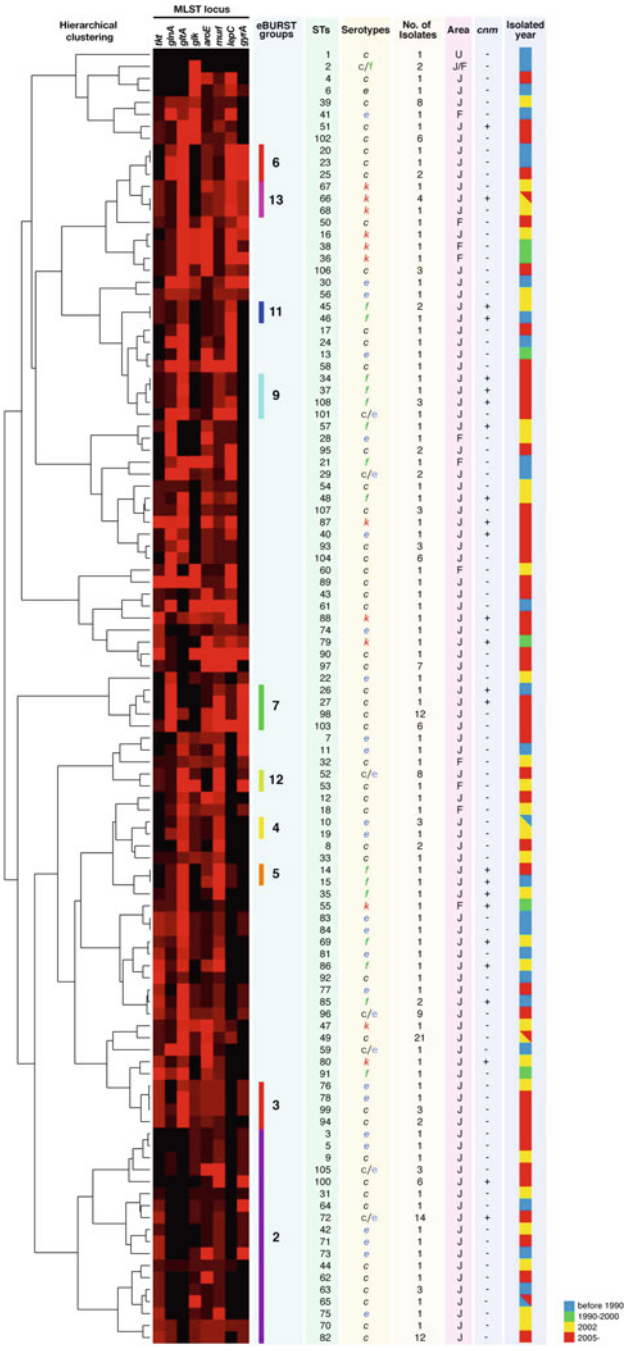

Fig. 9.7 Cluster analysis of ST profiles and the relationship among serotypes, year of isolation, and areas of isolation. Phylogenetic tree constructed based on the 164 strains isolated from Japan and Finland using CLUSTER3 software (http://bonsai.ims.u-tokyo.ac.jp/%7Emdehoon/software/cluster/software.htm) and Java TreeView (http://jtreeview.sourceforge.net/). Area—U, USA; J, Japan; F, Finland

are shown to be widely distributed in the tree, whereas the serotype *e*, *f*, and *k* strains were differentiated into clonal complexes, suggesting that the original ancestral strain of *S. mutans* was serotype *c*. Although no geographic specificity was identified, the distribution of the *cnm* gene was demonstrated to be clearly evident.

The superior discriminatory capacity of this MLST method for *S. mutans* may have important practical implications. Although various kinds of subtyping methods have been applied for transmission studies of *S. mutans*, the high discriminatory power gained by the MLST method is considered to result in greater sensitivity. Using MLST, 20 Japanese mother–child pairs whose children were between 2 and 10 years of ages showed that transmission could be observed with 70% of the pairs [4]. The MLST method could be applied for various epidemiological studies, which possibly could lead to grouping of the virulent strains of *S. mutans* into specific clusters to aid clinical assessment in the near future.

9.8 Summary

In summary, recent development of molecular biological techniques enables the detection of target bacterial species and their virulence genes without direct isolation of the strains. As for *S. mutans*, there have been a large number of such approaches developed. Identification of *S. mutans* followed by speciation of the highly virulent strains for dental caries as well as other diseases could be one of the powerful tools for clinical interventions in the future. Accumulation of data from clinical studies using molecular biological techniques might lead to the development of novel relevant systems for clinical use.

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