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Analysis of CRISPR in *Streptococcus mutans* suggests frequent occurrence of acquired immunity against infection by M102-like bacteriophages

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Clustered regularly interspaced short palindromic repeats (CRISPR) consist of highly conserved direct repeats interspersed with variable spacer sequences. They can protect bacteria against invasion by foreign DNA elements. The genome sequence of *Streptococcus mutans* strain UA159 contains two CRISPR loci, designated CRISPR1 and CRISPR2. The aims of this study were to analyse the organization of CRISPR in further *S. mutans* strains and to investigate the importance of CRISPR in acquired immunity to M102-like phages. The sequences of CRISPR1 and CRISPR2 arrays were determined for 29 *S. mutans* strains from different persons. More than half of the CRISPR1 spacers and about 35 % of the CRISPR2 spacers showed sequence similarity with the genome sequence of M102, a virulent siphophage specific for *S. mutans*. Although only a few spacers matched the phage sequence completely, most of the mismatches had no effect on the amino acid sequences of the phage-encoded proteins. The results suggest that *S. mutans* is often attacked by M102-like bacteriophages, and that its acquisition of novel phage-derived CRISPR sequences goes along with the presence of *S. mutans* phages in the environment. Analysis of CRISPR1 of M102-resistant mutants of *S. mutans* OMZ 381 showed that some of them had acquired novel spacers, and the sequences of all but one of these matched the phage M102 genome sequence. This suggests that the acquisition of the spacers contributed to the resistance against phage infection. However, since not all resistant mutants had new spacers, and since the removal of the CRISPR1 array in one of the mutants and in wild-type strains did not lead to loss of resistance to infection by M102, the acquisition of resistance must be based on further elements as well.

INTRODUCTION

The human oral cavity is inhabited by more than 500 different bacterial species (Paster *et al.*, 2001), of which only a few cause disease. Dental caries, one of the most frequent diseases in humans, is caused by micro-organisms that metabolize dietary sugars into acids. The concomitant decrease in pH results in demineralization of the hard tissues of teeth. Mutans streptococci, in particular *Streptococcus mutans*, are considered the principal aetiological agents of dental caries. Most efforts to develop strategies for prevention of this disease are therefore aimed towards reduction of *S. mutans* by interference with colonization or by passive or active immunization while leaving beneficial micro-organisms untouched. Phage therapy, the application of bacteriophages to combat bacterial infections, has regained interest during the last few years due to the increase in bacterial resistance to conventional antibiotics. In principle, phage therapy could be used to specifically remove *S. mutans* from dental plaque.

Relatively little is known about *S. mutans* bacteriophages and about the role they play in the ecology of *S. mutans* in the oral cavity. It is difficult to isolate bacteriophages specific for mutans streptococci (Bachrach *et al.*, 2003; J. R. van der Ploeg, unpublished results). Armau *et al.* (1988) isolated 16 lytic bacteriophages specific for strains of *S. mutans* and *Streptococcus sobrinus* by screening more than 1000 plaque samples. Three of these, phages M102, e1 and f2, appear to be similar, as suggested by comparable virion sizes, GC content, DNA–DNA hybridization and restric-

Abbreviation: CRISPR, clustered regularly interspaced short palindromic repeats.

A supplementary figure and six supplementary tables are available with the online version of this paper. The supplementary figure shows a comparison of part of the *cas1* gene from *Streptococcus mutans*, *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus pyogenes* strains. The supplementary tables list oligonucleotides used in this study, CRISPR1 and CRISPR2 spacer sequences, sequences of the CRISPR1 and CRISPR2 repeats, and similarity of *S. mutans* CRISPR spacers to sequences other than those of M102.

tion fragment analysis, although the phages show different serotype specificity (Delisle & Rostkowski, 1993). The genome sequence of M102, which is specific for *S. mutans* serotype c strains, has been determined (van der Ploeg, 2007). Another serotype c-specific bacteriophage, M101, appears to be closely related to M102 (van der Ploeg, 2007).

One of the weaknesses of phage therapy is the eventual development of resistance against bacteriophage infection. *S. mutans* strain OMZ 381 is one of only a few strains supporting propagation of M102 (Delisle & Rostkowski, 1993; J. R. van der Ploeg, unpublished results). The causes of this limited host range are not known. Several mechanisms that render lactic acid bacteria resistant to phage infection have been described: inhibition of phage adsorption, inhibition of DNA injection, restriction modification of phage DNA, and abortive infection (Forde & Fitzgerald, 1999). In the case of *S. mutans*, nothing is known about the last three mechanisms, but it has been shown that M102 does adsorb also to *S. mutans* serotype c strains that do not support propagation (Shibata *et al.*, 2009). This suggests that factors different from phage adsorption must determine resistance of *S. mutans* serotype c strains to infection by M102.

Recently, a novel phage resistance mechanism, which relies on clustered regularly interspaced short palindromic repeats (CRISPR) and a set of CRISPR-associated genes (*cas*), has been discovered (Barrangou *et al.*, 2007). CRISPR clusters consist of direct repeats of 24–47 bp, which are separated by unique spacers of 25–72 bp (Makarova *et al.*, 2006; Sorek *et al.*, 2008). Several CRISPR subtypes exist and each subtype has its own particular set of *cas* genes (Kunin *et al.*, 2007). CRISPR are found in about 40 % of sequenced bacterial genomes and in about 90 % of sequenced archaeal genomes (Sorek *et al.*, 2008). Frequently, a high number of the CRISPR spacer sequences show homology to bacteriophage or plasmid sequences. It has been shown that *Streptococcus thermophilus* mutants with acquired resistance to phage infection have incorporated an additional CRISPR spacer, whose sequence matches 100 % with part of the phage genome (Barrangou *et al.*, 2007). Removal of this spacer sequence leads to sensitivity to phage infection (Barrangou *et al.*, 2007). Apart from providing resistance to bacteriophages, CRISPR interference has also been shown to block plasmid DNA conjugation and transformation (Marraffini & Sontheimer, 2008).

In the present study, the incidence and the composition of CRISPR in *S. mutans* was examined and the role of CRISPR in protection against bacteriophage infection was investigated.

METHODS

Strains, plasmids and phages, and growth conditions. *S. mutans* strains used are listed in Table 1. Unless specified otherwise, they were routinely grown at 37 °C under anaerobic conditions or in an atmosphere of 10 % CO_2 and 90 % air in THY medium, which consists of Todd–Hewitt medium (Beckton-Dickinson) with 0.3 % yeast extract (Oxoid). Solid medium contained 1.5 % agar. Bacteriophages M102 and M101 were propagated in *S. mutans* strain OMZ 381 as described previously (van der Ploeg, 2007). When required, erythromycin was added to a final concentration of 10 μg ml^{-1}. *Escherichia coli* JM109 was used as the host for propagation of plasmids and was grown in LB medium at 37 °C with aeration. When required, erythromycin was added to a final concentration of 200 μg ml^{-1}.

PCR amplification and DNA sequencing. Chromosomal DNA was isolated with a GenElute bacterial genomic DNA kit (Sigma). CRISPR1 arrays were amplified by PCR with primers CRu and CRd or Cru2 and Crd2 (Supplementary Table S1). For amplification of CRISPR2 arrays, primers Cau and Cad or Cau2 and Cad2 were used. *Taq* DNA polymerase (New England Biolabs) or Jumpstart *Taq* polymerase (Sigma) were employed. The PCR program consisted of an initial denaturation for 3 min at 95 °C, followed by 30 cycles of annealing for 30 s at 55 °C, extension for 3 min at 72 °C and denaturation for 30 s at 95 °C. PCR products were purified with a Jetquick PCR purification kit (Genomed) and directly sequenced using dye-terminator technology (Microsynth) with one of the primers used for amplification. When required, a primer-walking strategy was employed for the sequencing of large CRISPR arrays.

The serotypes of *S. mutans* strains OMZ 380, OMZ 382, OMZ 737 and OM1079 were determined by PCR analysis (Shibata *et al.*, 2003). In addition, *spaP*, *atlA*, SMU.1757c and SMU.1762c from some strains were amplified by PCR with the primers listed in Supplementary Table S1 and sequenced with one of the primers used for amplification. Specific fragments of bacteriophage M101 were amplified and sequenced using primers specified in Supplementary Table S1.

Bioinformatic analyses. CRISPR spacers were identified by using the CRISPR finder tool (available at http://crispr.u-psud.fr/; Grissa *et al.*, 2007) combined with visual inspection of the sequence. Similarity of spacers to M102 was analysed by TBLASTX (using 0.1 as e-value cut off). Similarity between spacer and database sequences was searched for by BLAST analysis. WebLogo (Crooks *et al.*, 2004) was used to visualize sequence consensus motifs.

Isolation of mutants resistant to infection by phage M102. *S. mutans* strain OMZ 381 is one of a few strains which support propagation of bacteriophage M102 (Delisle & Rostkowski, 1993; Shibata *et al.*, 2009). OMZ 381 was therefore chosen for isolation of phage-resistant mutants. For this, 100 μl of serial dilutions of an exponentially growing OMZ 381 culture was mixed with $\sim 3 \times 10^8$ p.f.u. of phage M102. After incubation at 37 °C for 30 min, 4 ml M1D soft agar (van der Ploeg, 2007) was added, immediately poured on an M1D plate and incubated for 48 h at 37 °C. Colonies that appeared were recloned on M1D agar and tested for resistance to phage infection.

One further M102-resistant mutant was isolated in the course of an animal experiment. In brief, in a study approved by the 'Veterinäramt des Kantons Zürich' and conformed to the Swiss laws on animal protection, 10 specific-pathogen-free, caries-susceptible Osborne–Mendel rats (Institute of Oral Biology, University of Zurich, Zurich, Switzerland) were associated orally with *S. mutans* OMZ 381 as described elsewhere (Thurnheer *et al.*, 2008). Thereafter, phage M102 (10^5 p.f.u., 0.1 ml) was applied in the oral cavity of the rats (twice daily on days 23, 24, 25 and 26). Oral swabs were taken from all rats on days 29, 34 and 43 and suspended in 0.9 % NaCl, and various dilutions were plated on *S. mutans* selective medium. Three *S. mutans* colonies from each rat were tested for resistance to phage M102. Only two mutants were obtained, both from day 43, of which one, OMZ 432, was used for further investigation.

Table 1. Strains of *S. mutans* used in this study

Strain	Original designation	Serotype	Presence of CRISPR1*	Presence of CRISPR2*	Origin	Reference or source
OMZ 7	NCTC 10449†	c	3	7	UK	Edwardsson (1968)
OMZ 20	MT118	c	33	–‡	Japan	Hamada & Ooshima (1975)
OMZ 25	MT142	c	18	–	Japan	Hamada & Ooshima (1975)
OMZ 26	C67-1	c	–	6	The Netherlands	de Stoppelaar *et al.* (1971)
OMZ 27	C67-25	c	–	50	The Netherlands	de Stoppelaar *et al.* (1971)
OMZ 30	MT12	c	–	42	Japan	Hamada & Ooshima (1975)
OMZ 64	IB1600	c	12	–	Sweden	Krasse (1966)
OMZ 67	GS-5	c	22	2	USA	Gibbons *et al.* (1966)
OMZ 70	ATCC 33535§	c	–	–	Switzerland	Guggenheim (1968)
OMZ 186	KPSK2=JC2	c	5	–	Sweden	Carlsson (1967)
OMZ 380	P34	c	8	–	France	G. Tiraby, Université Paul Sabatier, Toulouse, France
OMZ 381	P42	c	6	–	France	G. Tiraby
OMZ 382	S61	c	31	–	France	G. Tiraby
OMZ 737	R9	c	–	–	UK	D. J. Bradshaw, CAMR, Salisbury, UK
OMZ 918	UA159	c	6	1	USA	ATCC
OMZ 949	'NG8'	c	5	–		D. Cvitkovitch, University of Toronto
OMZ 960	UA130	c	–	13	USA	G. Spatafora, Middlebury College
OMZ 961	'NG8'	c	5	–		G. Spatafora, Middlebury College
OMZ 1040	PK1	c	20	–	USA	Gibbons *et al.* (1966)
OMZ 1079		c	38	–	Switzerland	J. R. van der Ploeg, unpublished results
At10		c	6	–	Sweden	Edwardsson (1968)
L18		c	15	–	Finland	Grönroos & Alaluusua (2000)
LML5		c	–	–	UK	Waterhouse & Russell (2006)
LML7		c	9	–	UK	Waterhouse & Russell (2006)
MT4863		c	6	–	Japan	Hamada *et al.* (1980)
OMZ 125	LM7	e	26	–	USA	Gibbons *et al.* (1966)
MT4653		e	16	–	Japan	Hamada *et al.* (1980)
OMZ 175		f	–	1	Switzerland	Guggenheim (1968)
OM98x		k	25	33	Japan	T. Sato, Tokyo Dental College, Chiba, Japan

*The number of spacers is shown.
†NCTC, National Collection of Type Cultures.
‡–, None detected.
§ATCC, American Type Culture Collection.

Construction of mutations in CRISPR1 and SMU.1405c. For construction of mutants with a deletion in CRISPR1, a double-crossover replacement strategy was employed. The region 5′ to CRISPR1 was amplified by PCR with primers CR1 and CR2, using genomic DNA from *S. mutans* OMZ 1001 as template (OMZ 1001 is a derivative of UA159 with a high transformation frequency). The product was digested with *Bam*HI and *Hin*dIII and cloned into plasmid pFW15 (Podbielski *et al.*, 1996) to create plasmid pOMZ357. Subsequently, the region 3′ to CRISPR1 was amplified with primers CR3 and CR4, digested with *Eco*RI and *Nco*I, and cloned into pOMZ357 to create pOMZ358. Plasmid pOMZ358 was linearized by digestion with *Bam*HI and introduced into *S. mutans* strains by transformation of naturally competent cells (Perry & Kuramitsu, 1981). Selection for chromosomal integration was done on THY agar plates containing erythromycin. Correct integration was confirmed by PCR analysis.

A similar strategy was used for the construction of SMU.1405c knockout mutants. An approximately 500 bp fragment internal to SMU.1405c was amplified by PCR with primers 1405c1 and 1405c2. The fragment was digested with *Bam*HI and *Hin*dIII and cloned into pFW15 to give plasmid pOMZ417. A second fragment harbouring the 3′ end of SMU.1405c was amplified with primers 1405c3 and 1405c4, digested with *Pst*I and *Eco*RI, and cloned into pOMZ417 to yield plasmid pOMZ418. The *Eco*RI-linearized plasmid pOMZ418 was introduced into different strains of *S. mutans* by transformation. Selection for and confirmation of plasmid integration were done as described above.

RESULTS

Bioinformatic analysis of CRISPR arrays and *cas* genes in *S. mutans* UA159

The annotated genome of *S. mutans* UA159 (Ajdić *et al.*, 2002) was found to harbour two CRISPR loci, designated CRISPR1 and CRISPR2. Two *S. mutans* CRISPR types have also been identified recently by others (Haft *et al.*, 2005; Horvath *et al.*, 2008a). As outlined in Fig. 1(a), CRISPR1 is

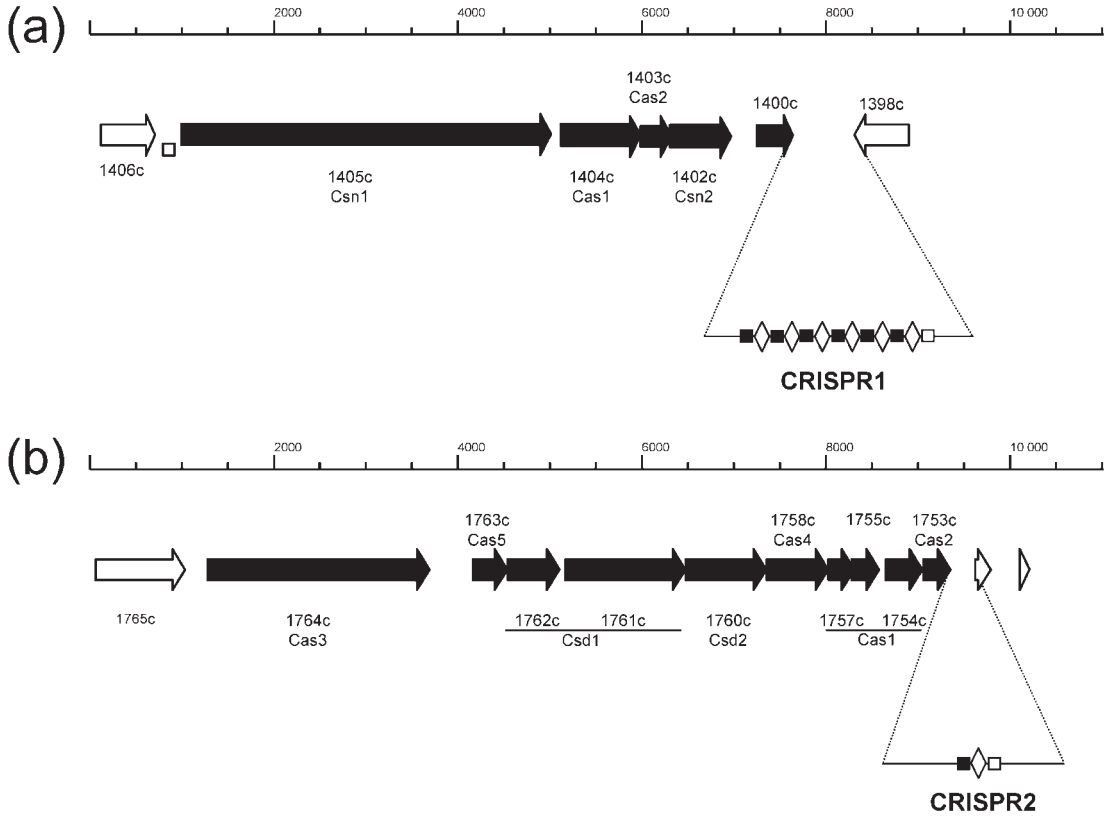


Fig. 1. Organization of CRISPR1 (a) and CRISPR2 (b) arrays and associated genes in *S. mutans* UA159. Lines below the ORF numbers show the size that the gene would have if the frame shift or stop codons (see text) were removed. CRISPR repeats are indicated by boxes (black, conserved; white, degenerated). Spacers are indicated by diamonds.

located between ORFs SMU.1400 and SMU.1398, and contains six identical copies of a partially palindromic sequence of 36 bp (5′-GTTTTAGAGCTGTGTTGTTT-CGAATGGTTCCAAAAC-3′). A seventh degenerated repeat with 67 % identity (5′-GTTTTAGAGCCATGTTAGTT-ACTGATTTACTAAAAT-3′) is located proximal to SMU.1398. All repeats are interspersed by spacers of 30 bp in size. The CRISPR1 array is preceded by four ORFs (SMU.1405c, SMU.1404c, SMU.1403c and SMU.1402c), whose amino acid sequences show similarity to those of *cas* genes of the Nmeni subtype (Haft *et al.*, 2005). This subtype is usually associated with the CRISPR repeat cluster 10 (Kunin *et al.*, 2007), to which the *S. mutans* CRISPR1 repeat also belongs. An additional partially homologous repeat (5′-TTTAACTTGCTGTGTTGTTTCGAATGATTCCAACA-C-3′) is present upstream of SMU.1405c (Fig. 1), and is a common feature of this CAS/CRISPR subtype (Haft *et al.*, 2005). Another annotated ORF, SMU.1399, overlaps with the CRISPR1 array (not shown). Its deduced amino acid sequence shows no similarity to proteins of known function.

The CRISPR2 locus of UA159 is located between SMU.1753c and SMU.1752c, and consists of two 32 bp partially homologous repeats (5′-GTCGCACCCTTCA-CGGGTGCGTGGATTGAAAT-3′ and 5′-GTCGCACCC-TTTAAAGGTTGGGTTTGCTTTTT-3′), separated by a 34 bp spacer (Fig. 1b). The first repeat is identical to a CRISPR repeat present in the genome of *Streptococcus pyogenes* M49 (McShan *et al.*, 2008). It is a member of repeat cluster 3 (Kunin *et al.*, 2007), associated with subtype Dvulg *cas* genes (Haft *et al.*, 2005). The genes from SMU.1764c to SMU.1753c located upstream of the first repeat (Fig. 1) belong to this subtype. However, two of these genes appear to be truncated by a frame shift mutation (*csd1* and *cas1*) and one by a premature stop codon (*cas1*).

Distribution of CRISPR1 and CRISPR2 in mutans streptococci

The distribution of CRISPR1 and CRISPR2 in 29 isolates of *S. mutans* (Table 1) was investigated by PCR analysis and sequencing, using primers derived from the sequences bordering the CRISPR of *S. mutans* UA159. Since it turned out that strains OMZ 186, OMZ 949 and OMZ 961 contained identical CRISPR (see below), OMZ 949 and OMZ 961 were omitted from the analysis. CRISPR1 was detected more frequently (in 19 out of 27 strains) than CRISPR2 (nine out of 27). Both CRISPR clusters were

present in only four strains (OMZ 7, UA159, OMZ 67 and OM98x). Three serotype c strains yielded neither a CRISPR1 nor a CRISPR2 PCR product. Most positive strains displayed many more repeats and spacers in both CRISPR1 and CRISPR2 than *S. mutans* UA159 (Fig. 2a, b).

A total of 305 CRISPR1 spacers were found in the 19 strains that yielded a PCR product; the mean number was 16.0 (range 3–38; Supplementary Table S2). Within CRISPR2, 155 spacers were found in nine positive strains, with a mean number of 17.2 spacers per strain (range 1–50).

Sequence analysis of CRISPR1 and CRISPR2 spacers and repeats

Spacers. More than 90 % of the CRISPR1 spacer sequences were 30 bp in size; the remaining spacers consisted of 28, 29 or 31 bp (Supplementary Table S2). Most were unique, and only the terminal spacer (preceding the terminal degenerated repeat) showed some conservation. A total of six different terminal spacers were found, of which one occurred in eight different strains. Apart from the conservation of the last spacer, there were only eight identical CRISPR1 spacer pairs, and these pairs were always located close to each other within a single strain. CRISPR1 from OM98x was unusual, with a block of three spacers in repeat. These duplications may have been selected in response to phage predation, as they could lead to an increased efficiency of inhibition of the target nucleic acid. Alternatively, identical spacers could have been generated by errors of the CRISPR machinery.

With respect to CRISPR2 spacers, sequences of 34 bp occurred most frequently, followed by 35, 33 and 36 bp (Supplementary Table S3). As for CRISPR1, identical spacers were detected only occasionally. An exception was again the terminal spacer sequence, which occurred in three variants.

Repeats. The sequences of the CRISPR1 and CRISPR2 repeats are summarized in Supplementary Tables S4 and S5, respectively. In contrast to spacer diversity, no sequence variation was found in the regular CRISPR1 repeats, except for strain MT4863, where the last residue was a T instead of C. The terminal CRISPR1 repeat was found in three variants. Regular CRISPR2 repeats occurred in two variants, with only one base pair being affected. The terminal CRISPR2 repeat was different from the regular CRISPR2 repeats and also occurred in two variants.

Inactive CRISPR2 in UA159 due to mutations

As described above, *cas1* and *csd1*, which are associated with CRISPR2, appeared to be truncated in UA159. Since this strain contains only one CRISPR2 spacer, it has been postulated that the mutations in *cas1* and *csd1* render the CRISPR2 system inactive (Horvath *et al.*, 2008a). To find evidence for this hypothesis, part of the *cas1* gene (861 bp) from strains OMZ 960 and OM98x was sequenced. The data show that these strains contain 13 and 33 CRISPR2 spacers respectively, which suggests an active CRISPR2 system. Compared with the sequence of UA159, two base pairs (GT) are inserted into both OMZ 960 and OM98x (Supplementary Figure S1). In addition, the stop codon present in the UA159 sequence is absent in OMZ 960 and OM98x.

Likewise, part of the sequence of *csd1* (660 bp) from OMZ 960 was determined. Comparison with UA159 revealed two differences: an A–G transition, which has no influence on the amino acid sequence of the encoded protein, and an insertion of 1 bp within a homopolymeric dA tract in the UA159 sequence, which results in a frame shift (data not shown). Together, these results suggest that both *cas1* and *csd1* are non-functional in UA159, implying that UA159 has lost the ability to incorporate novel CRISPR2 spacers.

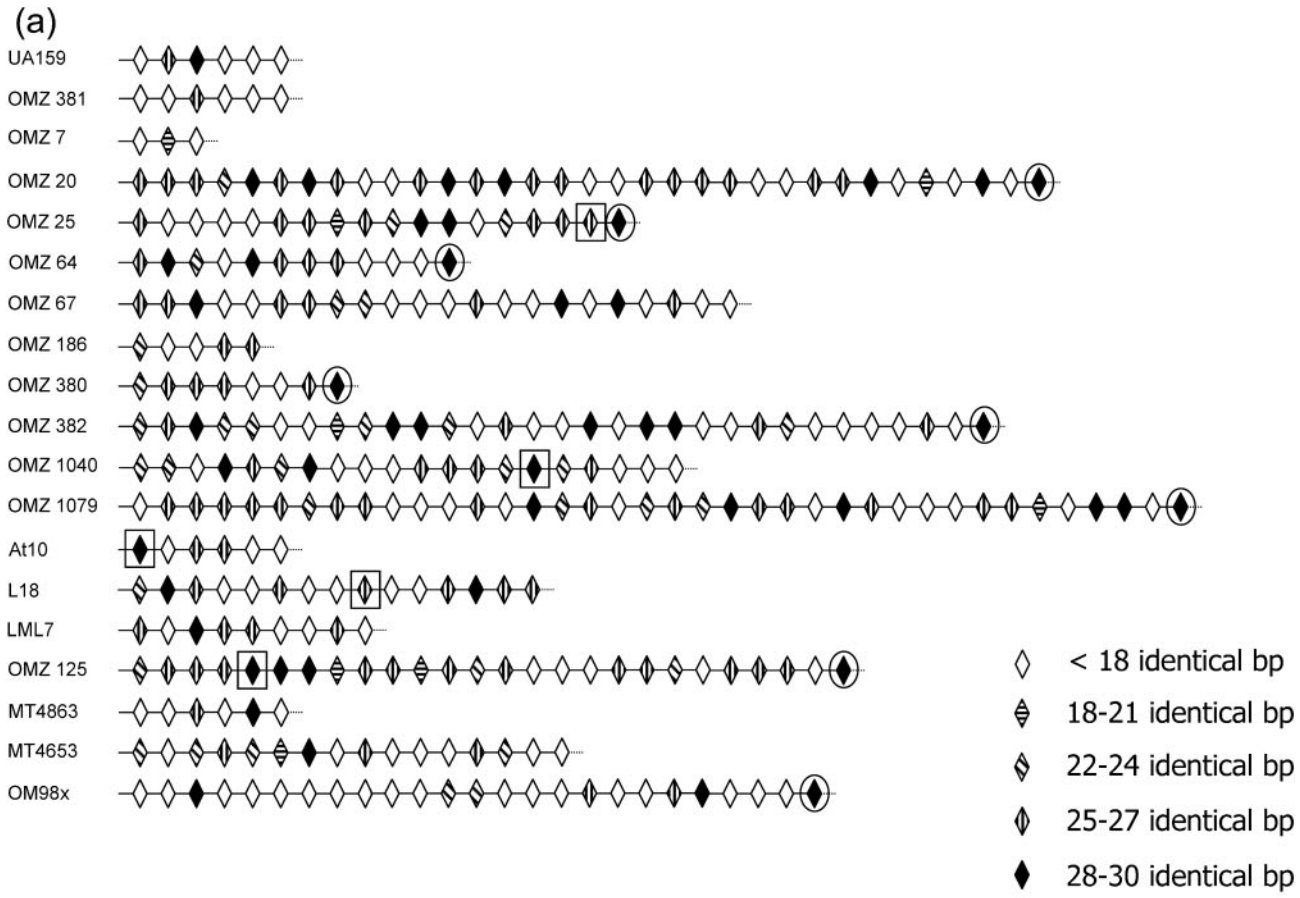
Identical CRISPR1 sequences in OMZ 186 and OMZ 961

The CRISPR1 sequences of OMZ 961 (NG8, obtained from G. Spatafora, Middlebury College) and OMZ 186 (KSPK2 or JC-2) were identical. This appeared rather unlikely, since the strains had been isolated in New Guinea and Sweden, respectively (R. Russell, personal communication). Hence, the CRISPR1 sequence of OMZ 949 (NG8, obtained from D. Cvitkovitch, University of Toronto) was determined as well and was found to be identical to that of CRISPR1 from OMZ 961 and OMZ 186. To investigate this further, a part of the sequence of the *spaP* gene of these three strains was determined, since it had been reported that *spaP* is different in KSKP2 and NG8 (Demuth *et al.*, 1990; P. Crowley, personal communication). However, in the present study, the *spaP* sequences obtained from OMZ 961 and OMZ 186 were identical to KSPK2 (data not shown), which strongly suggests that some laboratories hold the same strain under different names.

Similarity of CRISPR spacers to bacteriophage M102

Of the 305 CRISPR1 spacer sequences determined, only five matched the bacteriophage M102 genome sequence exactly. However, an additional 172 CRISPR1 spacers showed sequence similarity to M102 (more than 17 identical residues; Fig. 2a). The majority of them contained mismatches at degenerate positions, and the encoded amino acid sequence remained unchanged compared with M102. Of 201 mismatches investigated, 161 concerned the third position of the codon of the targeted M102 ORF, and were mostly silent (n=152). The remaining 49 mismatches resulted in 30 amino acid changes.

Of the 155 CRISPR2 spacers, 55 (35%) showed sequence similarity to M102 (more than 22 identical residues; Fig. 2b), but none matched perfectly. Again, the majority of the mismatches were positioned at the third base of the codon (69 out of 93 mismatches investigated). In total, only 18 amino acid changes resulted from these mismatches.



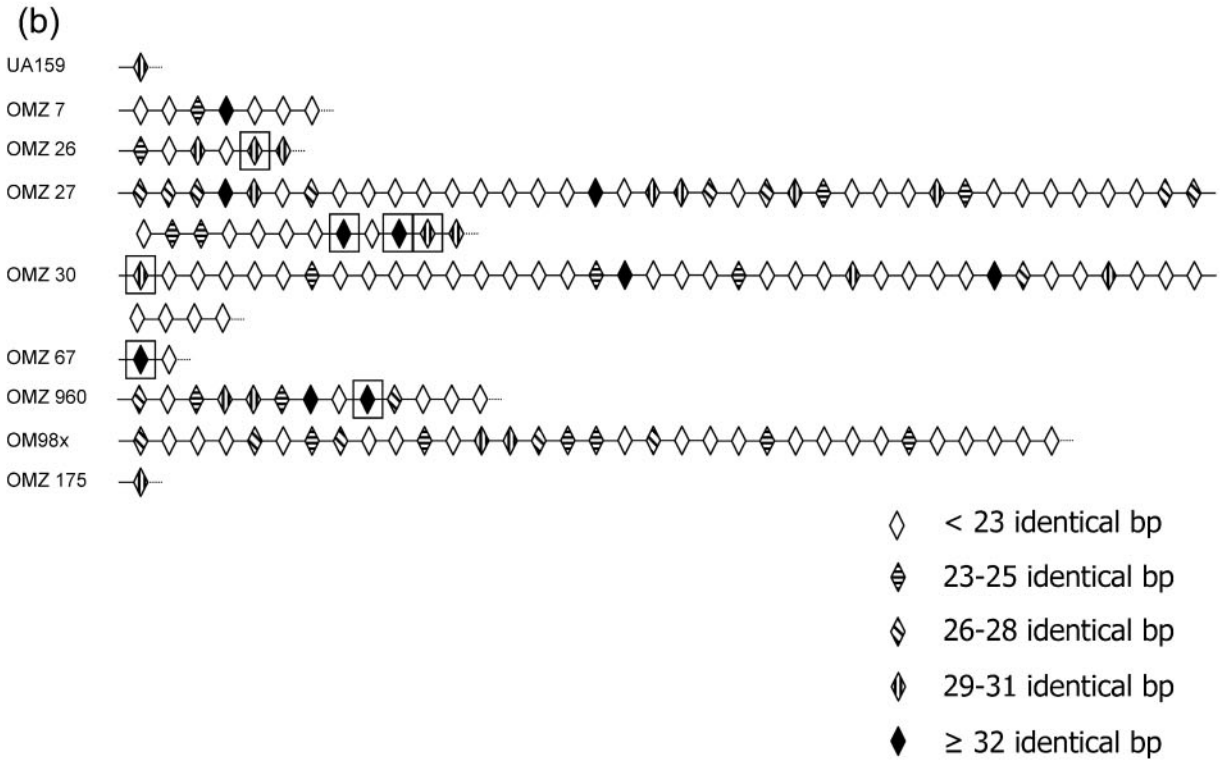


Fig. 2. Analysis of CRISPR1 (a) and CRISPR2 (b) in *S. mutans*. Repeats are shown as lines; the last non-conserved repeat is shown as a dotted line. Spacers are represented by diamonds; shading indicates similarity to known sequences. Spacers that are encircled have identical sequences; spacers that are surrounded by a rectangle show similarity to sequences different from the genome of phage M102.

It has been shown that within a CRISPR repeat family, bacteriophage-derived sequences located adjacent to the proto-spacers, the region in the phage genome corresponding with the spacer, exhibit conservation (Bolotin *et al.*, 2005; Deveau *et al.*, 2008; Horvath *et al.*, 2008b). In this study, comparison of the sequences adjacent to CRISPR1 proto-spacers showed conservation within the three-base stretch at the 3′ end (5′-WRR; Fig. 3a). Two submotifs were recognized in different strains. In OMZ 64, OMZ 382, OMZ 1040, OMZ 1079, OMZ 381 and MT4863, a 5′-WAAR-3′ motif is present (Fig. 3b), whereas the remaining strains show a 5′-WGG-3′ motif (Fig. 3c). In contrast, no conservation was observed next to the 5′ end of the proto-spacers (results not shown).

With regard to CRISPR2, the sequences 3′ to the CRISPR2 proto-spacers showed no conservation, whereas the three bases located at the 5′ site commonly exhibited the motif 5′-TTC-3′ (Fig. 3d).

Similarity of CRISPR spacers to other sequences

Apart from the evident sequence similarity to bacteriophage M102, some CRISPR1 and CRISPR2 spacers showed similarity to other invading mobile elements, namely bacteriophages from other streptococci, plasmids and transposons (Supplementary Table S6). Three CRISPR1 and CRISPR2 spacer sequences showed similarity to a 48 kb plasmid present in three copies in the genome of *Streptococcus agalactiae* pNEM316-1 (Glaser *et al.*, 2002). Some CRISPR2 spacers from OMZ 26, OMZ 27 and OMZ 960 match the sequence of the genomic island Tnsmu1, which encompasses SMU.191c to SMU.221c and is present in UA159 and other *S. mutans* strains (Waterhouse *et al.*, 2007). The clustering of spacers in OMZ 27 hints at repeated attacks of Tn1smu or at simultaneous incorporation of multiple spacers from the Tn1smu genome. Two CRISPR1 spacers matched the ORFs SMU.1705 and SMU.820 of *S. mutans* UA159.

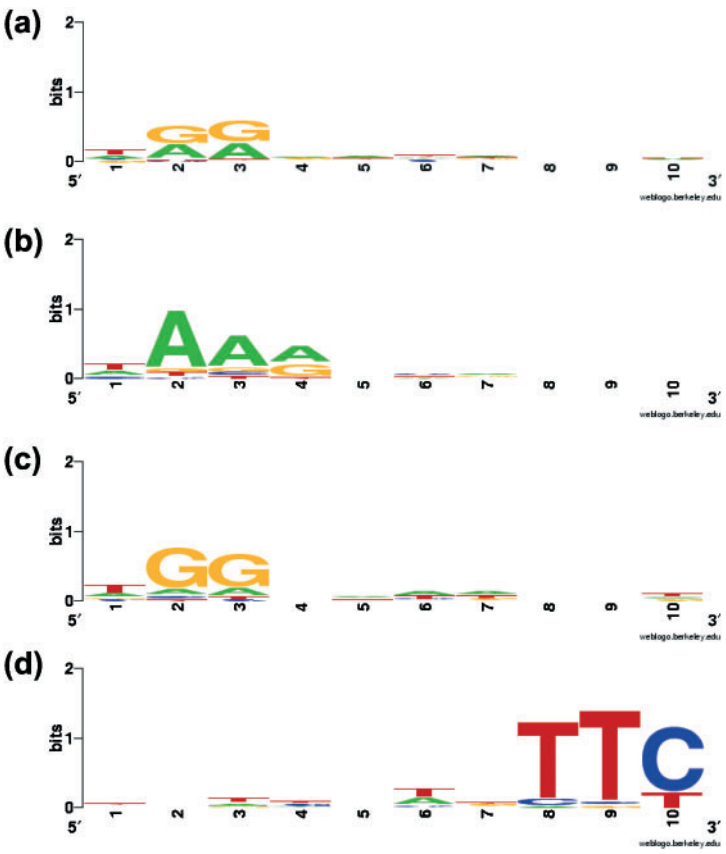


Fig. 3. WebLogo motifs of sequences 5′ and 3′ to proto-spacers. (a) Sequences located 3′ to CRISPR1 proto-spacers; (b) sequences 3′ to CRISPR1 proto-spacers targeted by spacers from strains OMZ 64, OMZ 382, OMZ 1040, OMZ 1079 and OMZ 381; (c) sequences 3′ to CRISPR1 proto-spacers targeted by spacers from strains OMZ 20, OMZ 25, OMZ 67, OMZ 186, OMZ 380, OMZ 382, UA159, At10, L18, LML7, MT4863, OMZ 125, Mt4653 and OM98x; (d) sequences 5′ to CRISPR2 proto-spacers.

Analysis of CRISPR1 in M102-resistant mutants

Spontaneous mutants of OMZ 381 resistant to infection by M102 could easily be isolated. PCR analysis showed that in 13 of 57 analysed mutants the size of the CRISPR array had increased by approximately the size of one spacer and one repeat (Table 2). The acquisition of a novel spacer and repeat in these mutants was confirmed by DNA sequencing. In OMZ 432, a strain isolated from a rat whose oral cavity had been inoculated with the wild-type strain (OMZ 381) and then challenged with phage M102, two additional CRISPR1 spacers were detected (Table 2).

With one exception (OMZ 381-M5) all newly acquired spacers and repeats were inserted proximal to the CRISPR1 leader. This corroborates results from earlier studies with phage-resistant *S. thermophilus* mutants (Barrangou *et al.*, 2007; Deveau *et al.*, 2008). In all but one case (OMZ 381-M71), the novel spacer sequences matched the genome sequence of M102 perfectly. In OMZ 381-M71, one mismatch at the ultimate 5′ base of the proto-spacer is present. In some mutants, nearly identical spacers are incorporated (Table 2).

In mutants M3, M60 and M71, the spacer matched the same phage sequence, but the length of the spacer differed. This was also found for two other mutants (OMZ 432 and M56). The region 3′ to the proto-spacer sequence exhibited a conserved motif (YAAAWY) that is similar to the CRISPR1 motif described above (Fig. 3b).

Table 2. M102-resistant mutants of OMZ 381

Mutant	Novel spacer sequence	Size (bp)	M102 co-ordinates*	5′ Sequence†	3′ Sequence‡	Sensitivity to M101§	M101 proto-spacer‖
OMZ432	AGGAAATCTCCTTAC-ACGCTTAGCAAGTGT	30	21 421–21 450	AACAGAAAAG	TAAATGCTAG	−	AGGAAATCTCCTTACA-CGCTTAGCAAGCAT
OMZ432	AAGATGGCTGAAGAG-ACCGGGGTTAACGCC	30	12 228–12 198 (r)	TACCATTTCC	TAAATTAGCA	−	AAGATGGCTGAAGAG-ACCGGGGTTAACGCC
OMZ 381-HS	TGACAGCAATTTTTA-CACTGGCACAGTAGC	30	21 301–21 330	ATGTAGGTGA	TAAATTTATG	−	TGATAGCAACCTCTAC-ACCGGAACAGTAGC
OMZ 381-M3	ATATGTAATAGGTCA-TAATGATGATGGCAC	30	17 268–17 297	TTGCCCGTGA	TAAAACAATT	−	ATATGTAATAGGTCATA-ATGATGATGGCAC
OMZ 381-M5	GCAGTCAGTAGCGCA-ACAAGAGCTACAGGC	30	16 560–16 579	TTGCAGACGC	TAAAGCAAGT	−	ACAATCAGTCGCACAAC-AAGAACTGCAAGC
OMZ 381-M19	TATGCGTTGGATTGTT-TTTGCTCTGTCTA	29	21 869–21 841 (r)	TAGCACCGAT	TAAAGCCTTC	−	TGTGCGTTGAATTGTC-TTTGCTCTGTCTA
OMZ 381-M46	TCATTTCTCCTGCATT-TTATTAGTAACTAG	30	21 687–21 658 (r)	ACCTCTATTT	TAAATTATGC	−	TCCTTTCTCCTGCATTT-TATTAGGCGCTAG
OMZ 381-M51	TAGCAAACGGAGCTTT-GCTGTATAGAGCCCTT	32	1463–1432 (r)	TTAGCAACTT	AAAGTTGAAG	−	TAGCAAACGGAGCTTT-GCTGTATAGAACCCTT
OMZ 381-M54	GATAATGCTTTTGCCA-AAGCACAGCTAATC	30	4621–4650	CGACGTTATC	CAAAACGCTT	−	GATAATGTTTTTGCTAA-AGCGCAGTTAATC
OMZ 381-M56	CAAGATGGCTGAAGAG-ACCGGGGTTAACGCC	31	12 228–12 198 (r)	TTACCATTTC	TAAATTAGCT	–	CAAGATGGCTGAAGAGA-CCGGGGTTAACGCC
OMZ 381-M60	AATATGTAATAGGTCAT-AATGATGATGGCAC	31	17 267–17 297	TTTGCCCGTG	TAAAACAATT	+	AATATGTAATAGGTCAT-AATGATGATGGCAC
OMZ 381-M62	TAAGAATTTGTTTTC-TTTGGCATTCAGCCA	30	1993–1964 (r)	CTTTCAATTC	CAAATTCAAG	−	TAAGAATTTGTTTTCTT-TGGCATTCAGCCA
OMZ 381-M63	GTGGCAAAAGTGATA-GCTAGGAACATGCCT	30	9798–9827	AATCGAAGAT	CAAATCATCA	−	ND¶
OMZ 381-M69	AAAGAGGAATACAAAG-ACCCGAATACTTGGA	31	1808–1838	CAAGATGATA	TAAAGTCTAA	−	AAAGAGGAATACAAA-GACCCGAATACTTGGA
OMZ 381-M71	CATATGTAATAGGTCA-TAATGATGATGGCAC	31	17 267–17 297	TTTGCCCGTG	TAAAACAATT	+	AATATGTAATAGGTCAT-AATGATGATGGCAC

*Co-ordinates of M102 proto-spacer; (r), reverse strand.
†Sequence 5′ to the M102 proto-spacer.
‡Sequence 3′ to the M102 proto-spacer.
§+, Sensitive; −, resistant to infection by phage M101.
‖Proto-spacer sequence of M101. Underlined residues are different from the corresponding proto-spacer of M102.
¶ND, Not determined.

Bacteriophage M101 is related to phage M102 (van der Ploeg, 2007). It was therefore investigated whether the M102-resistant mutants were also resistant to phage M101. The results showed that only mutants M60 and M71 were sensitive to M101 infection (Table 2). In these mutants, the acquired CRISPR1 spacer is homologous to a sequence of M102. However, this sequence is also present in the CRISPR1 spacer in mutant M3, which was resistant to infection by M101. To determine whether the differences in sensitivity to phage infection were related to differences in the proto-spacers, their sequences were determined. Some of the proto-spacer sequences in M101 were identical to those in M102 (M3, M60 and M62), whereas others were different. In fact, the proto-spacer in M101 differed from the novel spacer in mutant M5 by as many as seven nucleotides. Although M5 is resistant to infection by M101, it is hard to conceive that the acquired spacer in this mutant could provide protection. It has been reported that mutation of only one nucleotide in the phage proto-spacer rendered *S. thermophilus* bacteriophage 2972 unaffected by CRISPR-mediated resistance (Deveau *et al.*, 2008).

Inactivation of SMU.1405c or CRISPR1 is insufficient to render sensitivity to infection by the bacteriophage

In order to confirm that resistance to phage M102 is conferred by the CRISPR1 system, mutants in the *cas5* gene SMU.1405c were constructed in OMZ 67, OMZ 961, OMZ 1001, OMZ 381-M3 and OMZ 381-M46. However, all resulting mutants remained resistant to infection by phage M102 (data not shown). Next, the CRISPR1 clusters of OMZ 7, OMZ 67, OMZ 1001, OMZ 381-M3 and OMZ 381-HS were deleted and replaced by an erythromycin-resistance cassette. These mutants also remained resistant to infection by phage M102 (not shown).

DISCUSSION

The results of this study show that mutants of strain OMZ 381 which have become resistant to bacteriophage M102 have acquired novel CRISPR1 spacers that are most likely derived from the phage genomic DNA, since their sequences matched the genome sequence of M102. This finding is in agreement with studies of phage-resistant mutants of *S. thermophilus* (Barrangou *et al.*, 2007; Horvath *et al.*, 2008b). However, just as remarkable is the fact that in the majority of the M102-resistant OMZ 381 mutants no change in the CRISPR1 cluster had occurred. Thus, it is evident that in *S. mutans* there must exist other CRISPR1/2-independent and as yet unidentified mechanism(s) of acquired immunity to phage infection. In line with this conclusion are two further observations: first, inactivation of the CRISPR1 cluster in several different *S. mutans* strains and in a phage-resistant mutant of OMZ 381 with an acquired supplementary CRISPR1 spacer did not re-establish sensitivity to M102 infection; second, some of the novel M102-resistant mutants were also resistant to infection by M101, although there was only partial homology between the novel spacer sequence and the proto-spacer of M101. One possible explanation is that many *S. mutans* strains (other than UA159) may carry further, still unknown CRISPR cluster(s) with CRISPR-associated genes. In fact, some *S. thermophilus* strains possess three different CRISPR arrays (Horvath *et al.*, 2008b). Whole genomic sequencing of other *S. mutans* strains may reveal whether one or more additional CRISPR clusters exist.

This study demonstrates that the prevalence, the composition and the sequences of CRISPR1 and CRISPR2 in *S. mutans* are highly variable. The polymorphic nature of CRISPR makes the two clusters useful markers for epidemiological studies. CRISPR1 was found to occur more frequently than CRISPR2. Although the choice of primers and or PCR conditions might have had an influence on the results, the distribution frequency was congruent with results from a comparative genomic hybridization study (Waterhouse *et al.*, 2007). In that study, it was shown that a complete set of CRISPR1-associated genes (SMU.1405 to SMU.1400) is present in seven of nine strains investigated. Of these nine strains, seven were also part of the present work (GS5, At10, L18, LML5, LML7, MT4863 and MT4653), and in all but LML5, a CRISPR1 array was found. LML5 has been reported to lack CRISPR1-associated genes (Waterhouse *et al.*, 2007), and indeed, no CRISPR1 array could be detected. With respect to CRISPR2, however, a complete set of CRISPR2-associated genes (SMU.1764 to SMU.1753) and a CRISPR2 array were present in a single strain only, GS5 (OMZ 67). These results agree with earlier observations that particular CRISPR repeat clusters are functionally coupled with particular CAS subtypes (Horvath *et al.*, 2008b; Kunin *et al.*, 2007).

My comparison of the sequences of *cas1* and *csd1* from strain UA159 with those from other strains and species suggests that both genes are non-functional in UA159. It has been speculated that Cas1 functions to integrate novel spacers into CRISPR2 (Makarova *et al.*, 2006). Hence, UA159 should have lost the ability to do so, and this would fit with the observation described here that UA159 contains only a single CRISPR2 spacer. This hypothesis is also consistent with the observation in *E. coli* that the Cas1 protein is dispensable for resistance to infection by phage λ (Brouns *et al.*, 2008).

About half of the CRISPR1 and CRISPR2 spacers exhibited similarity to the genome of phage M102. It has been shown for *S. thermophilus* that a correlation exists between resistance to phage infection and the number of spacers with sequence similarity to phage sequences (Bolotin *et al.*, 2005). In the present study, only one pair, consisting of an infecting phage (M102) and a receptive strain (OMZ 381), was available for investigation. This limited material does not allow conclusions about the relationship between CRISPR content and phage resistance to be drawn.

However, the high prevalence of M102-homologous spacer sequences among *S. mutans* strains suggests that *S. mutans* is frequently exposed to M102-like phages. Interestingly, most mismatches between homologous spacers and the M102 phage sequence affected the third base of the codon. This indicates that many different variants of M102 exist and could evade CRISPR-mediated resistance through silent or conservative mutations without affecting the function of phage-encoded proteins. Such a mechanism has been proposed for *S. thermophilus*, in which a single base pair change in the phage genome is sufficient to escape resistance caused by the CRISPR machinery (Barrangou *et al.*, 2007; Deveau *et al.*, 2008). This could represent a major driving force for the evolution of bacteriophage mutants.

Plasmids occur in about 5 % of all *S. mutans* strains (Caufield *et al.*, 1988). However, none of the CRISPR1 or CRISPR2 spacers showed sequence similarity to the previously identified *S. mutans* plasmids pLM7 and pUA140 (Zhou *et al.*, 2001). This is surprising, since spacers with similarity to a plasmid from *S. agalactiae* were present in some strains. Equally unexpected, though not unprecedented, was the finding that two CRISPR1 spacers matched ORFs present in the *S. mutans* UA159 genome. There are no indications that these ORFs are located on genomic islands or are part of prophage sequences. Hence, it is not clear why spacers derived from these ORFs were incorporated. Speculatively, one could postulate that the CRISPR machinery occasionally confuses host DNA and host mRNA with invading nucleic acids and erroneously attacks the former. Alternatively, uptake of homologous chromosomal DNA of lysed *S. mutans* cells during natural competence might provoke capture of novel spacers.

In summary, this study demonstrates a high degree of variability in the sequences of CRISPR1 and CRISPR2 in *S. mutans*. A large fraction of CRISPR1 and CRISPR2 spacers have homology to M102, suggesting that the organism often encounters M102-like phages. Although the data presented indicate that acquired resistance to M102 infection cannot be explained by the function of the two identified CRISPR clusters alone, the emergence of phage-resistant mutants following exposure to M102, as observed experimentally *in vivo* in a rat model, seems to be a frequent event.

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