RESEARCH LETTER

The serotype-specific glucose side chain of rhamnose–glucose polysaccharides is essential for adsorption of bacteriophage M102 to *Streptococcus mutans*

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Abstract

Bacteriophage M102 is a virulent siphophage that propagates in some serotype c *Streptococcus mutans* strains, but not in *S. mutans* of serotype e, f or k. The serotype of *S. mutans* is determined by the glucose side chain of rhamnose–glucose polysaccharide (RGP). Because the first step in the bacteriophage infection process is adsorption of the phage, it was investigated whether the serotype specificity of phage M102 was determined by adsorption. M102 adsorbed to all tested serotype c strains, but not to strains of different serotypes. *Streptococcus mutans* serotype c mutants defective in the synthesis of the glucose side chain of RGP failed to adsorb phage M102. These results suggest that the glucose side chain of RGP acts as a receptor for phage M102.

Introduction

Mutans streptococci are thought to play an important role in caries development in humans and in some animals (Loesche, 1986). The group of mutans streptococci consists of seven different species: *Streptococcus mutans*, *Streptococcus rattus*, *Streptococcus sobrinus*, *Streptococcus cricetus*, *Streptococcus ferus*, *Streptococcus macacae* and *Streptococcus downei*. *Streptococcus mutans* and *S. sobrinus* are frequently found in the human oral cavity (Loesche, 1986). Apart from the classification at the species level, mutans streptococci have also been classified into nine different serotypes, based on differences in their cell wall polysaccharides. *Streptococcus mutans* is divided into four different serotypes: c, e, f and k. They produce cell wall rhamnose–glucose polysaccharides (RGPs) consisting of a backbone structure of α1,2- and α1,3-linked rhamnosyl units with glucose side chains linked to alternate molecules on the cell wall (Pritchard *et al.*, 1986). Each serotype RGP has unique linkages of its glucose side chains (Fig. 1; Pritchard *et al.*, 1986).

RGP synthesis in *S. mutans* is initiated by transfer of *N*-acetylglucosamine-1-phosphate to a lipid carrier, most likely undecaprenyl phosphate (Yamashita *et al.*, 1999). Synthesis of the rhamnan backbone requires dTDP-L-rhamnose, which is produced from D-glucose-1-phosphate by the products of the *rmlABCD* genes (Tsukioka *et al.*, 1997a, b). The activated rhamnose units are linked to *N*-acetylglucosamine-lipid through RgpA, RgpB and RgpF to form the rhamnan backbone (Yamashita *et al.*, 1998a; Shibata *et al.*, 2002). Glucose side chain formation requires *gluA*, encoding glucose-1-phosphate uridyltransferase, for the synthesis of UDP-D-glucose (Yamashita *et al.*, 1998b). This precursor is attached to the rhamnan backbone by RgpE, RgpH and RgpI (Ozaki *et al.*, 2002). RgpE and RgpH are glucosyltransferases required for glucose side chain formation of RGP, and RgpI regulates the branching frequency of glucose side chain on RGP in serotype c strain Xc (Yamashita *et al.*, 1998a; Ozaki *et al.*, 2002). The differences in the glucose side chain formation of RGP among serotype c, e and f strains were found to depend on a serotype determinant locus downstream from *rgpF* (Fig. 2) (Shibata *et al.*, 2003). In

variants of serotype c, named serotype k, the glucose side chain of RGP is lacking, probably because *rgpE* is not expressed (Nomura *et al.*, 2005).

Bacteriophage M102 is a virulent siphophage specific for *S. mutans* of serotype c (Delisle & Rostkowski, 1993; van der Ploeg, 2007). M102 produces clear plaques on only some of the serotype c strains of *S. mutans*, but cannot infect serotype e and f strains (Delisle & Rostkowski, 1993). Because the first step in the bacteriophage infection process is the adsorption of the phage, the specificity of phage M102 for serotype c strains might be determined by the receptor. Here, we investigated whether serotype-specific RGP functions as a receptor of bacteriophage M102.

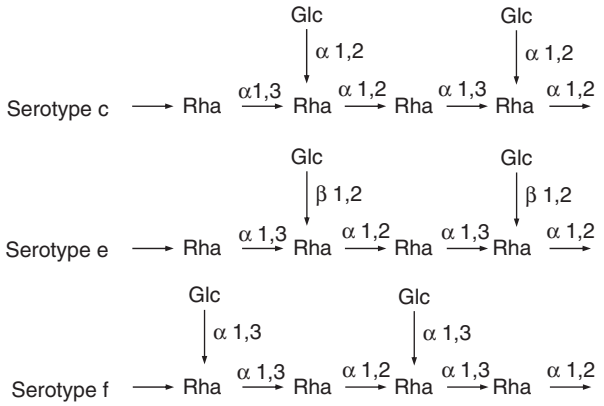

Fig. 1. Structure of cell wall-linked RGPs in *Streptococcus mutans* serotypes c, e and f.

Materials and methods

Strains and growth conditions

Mutans streptococci (Table 1) were routinely grown in Todd–Hewitt broth (THY, Becton-Dickinson) with 0.3% yeast extract (Oxoid), at 37 °C in an environment of 10% CO_2 and 90% air. *Streptococcus mutans* OMZ 381S is a spontaneous streptomycin-resistant mutant of OMZ 381 (van der Ploeg, 2007). THY plates contained 1.5% agar. THY soft agar contained 0.7% agar. Bacteriophage M102 and its propagation have been described before (van der Ploeg, 2007). The antibiotics used for selection of *S. mutans* were spectinomycin (1000 μg mL^{-1}), erythromycin (15 μg mL^{-1}) and streptomycin (1000 μg mL^{-1}).

Construction of mutants

For the construction of plasmids pSTN and pSTC, a DNA fragment containing the genes from *rgpF* to ORF12 (Fig. 2) from *S. mutans* strain Xc was inserted into the multicloning site of pBluescript SK II (+) to yield plasmid pST. For pSTN, the region containing ORF7 through ORF11 was replaced with a spectinomycin resistance gene. For construction of pSTC, the region containing ORF7 in pST was first replaced with the erythromycin resistance gene; subsequently, the region harbouring ORF10 was deleted.

Strain OMZ 381SN was constructed by transformation of OMZ 381S with BssHII-digested plasmid pSTN to spectinomycin resistance. OMZ 381SC was generated by transformation of OMZ 381SN with BssHII-digested plasmid pSTC to erythromycin resistance.

Replacement of serotype f-specific genes in strain OMZ 175 was as described previously (Shibata *et al.*, 2003).

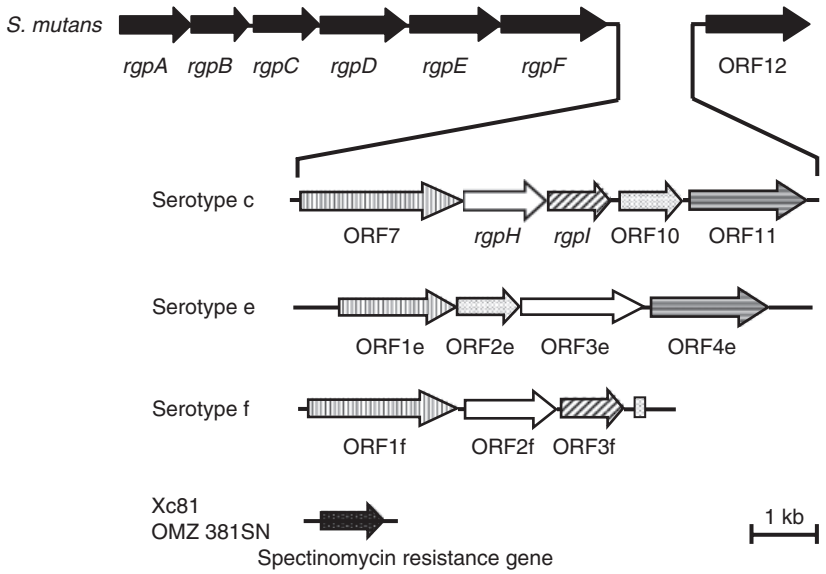

Fig. 2. Organization of the *rgp* loci in *Streptococcus mutans* serotype c, e and f strains. The lower part of the diagram indicates the regions responsible for glucose side chain formation during RGP synthesis. Genes with a high sequence similarity are indicated by identical shading. The *rgpA* through *rgpF* genes and ORF12 are common to the three serotypes. In strains Xc81 and OMZ 381SN, the serotype-specific genes were replaced by a spectinomycin resistance gene.

Table 1. Strains used in this study

Strains	Serotypes	Original designation/characteristics	Sources or references
S. mutans OMZ 381	c	P42, host strain for M102	van der Ploeg (2007)
S. cricetus OMZ 62	a	AHT	Laboratory collection
S. cricetus OMZ 165	a	HS6	Laboratory collection
S. mutans OMZ 7	c	NCTC 10449	Laboratory collection
S. rattus OMZ 63	b	FA1	Laboratory collection
S. mutans OMZ 64	c	IB1600	Laboratory collection
S. mutans OMZ 67	c	GS5	Laboratory collection
S. mutans OMZ 918	c	UA159	ATCC
S. mutans OMZ 1040	c	PK1	Laboratory collection
S. mutans Xc	c		Laboratory collection
S. sobrinus OMZ 176	d		Laboratory collection
S. mutans LM7	e		Laboratory collection
S. mutans MT4653	e		Laboratory collection
S. mutans OMZ 175	f		Laboratory collection
S. mutans MT6219	f		Laboratory collection
S. sobrinus 6715	g		Laboratory collection
S. downei MFe28	h		Laboratory collection
S. mutans OM98x	k		Yutaka Sato, Tokyo Dental College
S. mutans OMZ 381S	c	Spontaneous streptomycin-resistant mutant of OMZ 381	This study
S. mutans OMZ 381SN	None	OMZ 381S, but serotype-specific genes replaced with spectinomycin resistance gene	This study
S. mutans OMZ 381SC	c	OMZ 381SN, but harbouring *rgpH*, *rgpI* and ORF11	This study
S. mutans Xc81	None	Xc, but serotype-specific genes replaced with spectinomycin resistance gene	Shibata *et al*. (2003)
S. mutans LM7-81C	c	LM7, but serotype exchanged from e to c	Shibata *et al*. (2003)
S. mutans OMZ 175C	c	OMZ 175, but serotype exchanged from f to c	This study
S. mutans MT6219-81C	c	MT6219, but serotype exchanged from f to c	Shibata *et al*. (2003)
S. mutans Xc41	None	Xc, but *rgpA*::*erm*	Yamashita *et al*. (1998a)
S. mutans Xc42	None	Xc, but *rgpB*::*erm*	Yamashita *et al*. (1998a)
S. mutans Xc52	None	Xc, but *rgpG*::*erm*	Yamashita *et al*. (1999)
S. mutans OMZ 381S-45	None	OMZ 381S, but *rgpE*::*erm*	This study
S. mutans OMZ 381S-48	None	OMZ 381S, but *rgpH*::*erm*	This study

Briefly, OMZ 175 was first transformed with BssHII-digested plasmid pSTN to spectinomycin resistance. The resulting strain was then transformed with BssHII-digested plasmid pSTC to erythromycin resistance.

Streptococcus mutans strains Xc41, Xc42 and Xc52 have been described previously (Yamashita *et al*., 1998a, 1999). *rgpE* and *rgpH* mutations in strain OMZ 381S were obtained by transformation with genomic DNA from *S. mutans* strains Xc45 (Yamashita *et al*., 1998a) and Xc48 (Ozaki *et al*., 2002), respectively, with selection for erythromycin resistance. Correct gene replacement was verified by PCR analysis.

Adsorption assays

Adsorption of phage M102 to mutans streptococci was investigated as follows: 100 μL of phage M102 (*c.* 10^4 PFU) was mixed with 100 μL of an exponentially growing culture ($OD_{550\,nm}$ of about 0.4) of the strain and incubated for 30 min at 37 °C. Phages adsorbed to bacteria were pelleted by centrifugation for 5 min at 16 000 ***g***. The amount of nonadsorbed phages remaining in the supernatant was then determined by a standard plaque assay. For this, 100 μL of an appropriate dilution was mixed with 100 μL of an overnight grown culture of OMZ 381S and incubated for 30 min at 37 °C. After addition of 4 mL of molten THY soft agar (THY with 0.7% agar), the mixture was poured over an THY agar plate, which was incubated overnight at 37 °C in 10% CO_2 and 90% air. The number of plaques was counted.

Results

It was first examined whether the serotype specificity of phage infection was determined by adsorption (Fig. 3). M102 adsorbed to all seven serotype c strains tested, whereas it did not adsorb to strains of different serotypes. On the other hand, whereas M102 adsorbed to all tested serotype c *S. mutans* strains, these strains were not infected. Because

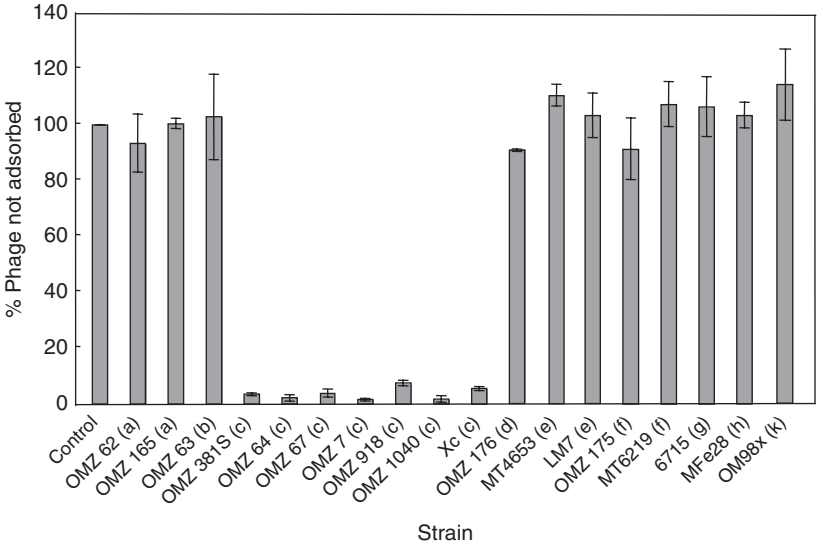

Fig. 3. Adsorption of phage M102 to mutans streptococci. Phage adsorption is expressed as the percentage of phage remaining in the supernatant after incubation with the strain of interest relative to the negative control with medium only. Results are the mean of at least three independent experiments. The serotype of the strains is indicated in parentheses.

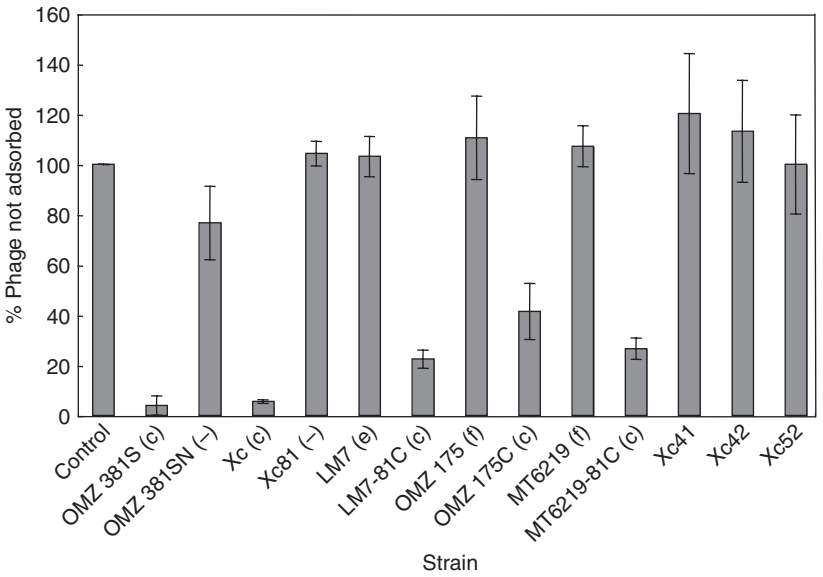

Fig. 4. Adsorption of phage M102 to *Streptococcus mutans rgp* mutants and to *S. mutans* with a converted serotype. Results are the mean of at least three independent experiments. Control, medium without addition of *S. mutans*. The serotypes of the strains are indicated in parentheses; (–), no serotype. Strains Xc41, Xc42 and Xc52 are mutants of the serotype c strain Xc (see text).

S. mutans serotype c, e, f and k strains have a different RGP structure, but are very similar otherwise, further investigations focused on these serotypes.

Three mutants of *rgpA* (Xc41), *rgpB* (Xc42) or *rgpG* (Xc52), which did not produce a rhamnan backbone and hence no RGP at all (Yamashita *et al.*, 1998a, 1999; Shibata *et al.*, 2002), were examined for adsorption by phage M102. M102 did not adsorb to these mutants (Fig. 4), which suggests that RGPs function as the phage receptor. We then constructed strains from which the serotype-specific genes had been removed or exchanged, and determined their ability to adsorb M102 (Fig. 4). The phage adsorbed to serotype c wild-type strains OMZ 381S and Xc, but not to the respective strains OMZ 381SN and Xc81, in which the locus downstream from *rgpF* and upstream from ORF12 had been deleted (Fig. 2).

As mentioned above, phage M102 did not adsorb to serotype e strain LM7 or serotype f strains MT6219 and OMZ 175, but replacing the serotype-specific loci of these strains by the serotype c-specific locus from strain Xc led to recombinant strains showing drastically increased adsorption of M102 (Fig. 4). Nevertheless, M102 could not infect these mutants.

The results demonstrate that the presence of the serotype c-specific locus involved in the glucose side chain formation of RGP is essential for the adsorption of M102, thus strongly suggesting that the serotype-specific glucose side-chain acts as the receptor for M102. To confirm our hypothesis, it was

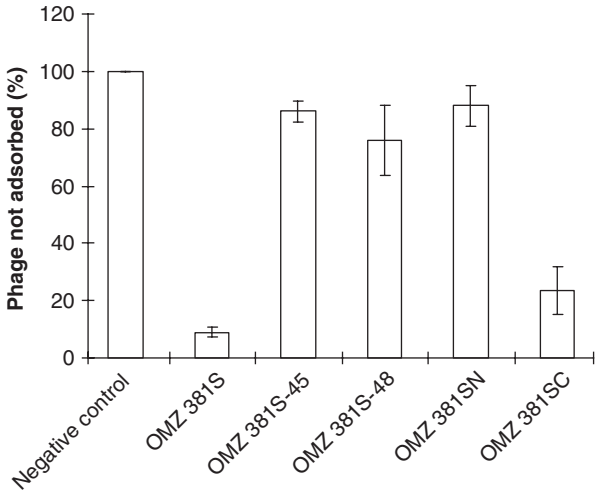

Fig. 5. Adsorption of phage M102 to *Streptococcus mutans rgp* mutants of OMZ 381S. Results are the mean of at least three independent experiments.

investigated whether bacteriophage M102 could be propagated in OMZ 381S mutants impaired in the formation of the glucose side chain of RGP. Strains OMZ 381SN, OMZ 381S-45 and OMZ 381S-48 did not adsorb bacteriophage M102 (Fig. 5) and were resistant to infection (relative efficiency of plating $< 1 \times 10^{-9}$). The serotype-specific *rgpH*, *rgpI* and ORF11 (Fig. 2) from strain Xc were then introduced into OMZ 381SN as described in Materials and methods. The resulting strain, OMZ 381SC, adsorbed phage M102 (Fig. 5) and was sensitive to infection (relative efficiency of plating 0.14), which demonstrates that the serotype-specific genes from strain Xc are sufficient for phage infection.

Discussion

Bacteriophage infection requires irreversible adsorption to a specific receptor. Bacteriophages infecting gram-positive bacteria first encounter the cell wall, which is decorated with polysaccharides and teichoic acids, either linked to the cytoplasmic membrane or linked to the peptidoglycan. Not surprisingly, these structures have previously been shown to function as a bacteriophage receptor (Vinga *et al.*, 2006). Here, we have demonstrated that the glucose side chain of RGP is indispensable for adsorption and infection of *S. mutans* bacteriophage M102.

Cell wall carbohydrates frequently serve as a phage receptor in lactococci or thermophilic streptococci, but the exact molecular nature of the receptor has not yet been elucidated for any of the bacteriophages investigated. Studies to identify the receptor of *Lactococcus lactis* bacteriophages bIL170 and ϕ645 by random mutagenesis revealed a gene cluster likely involved in the synthesis of rhamnose-containing cell wall polysaccharides (Dupont *et al.*, 2004). Interestingly, one of the genes identified showed similarity to *S. mutans rgpE*, which exists in all serotypes of *S. mutans* and whose gene product acts as a glucosyltransferase (Fig. 2).

The requirement for glucose substitution of a phage receptor is not unprecedented. Glycosylation of cell wall-linked teichoic acid was found to be essential for phage adsorption and susceptibility of *Listeria monocytogenes* (Cheng *et al.*, 2008). Cell wall-bound teichoic acid consisting of glycosylated poly(glycerol-phosphate) is the primary receptor for *Bacillus subtilis* bacteriophage SPP1. In this case, reversible adsorption of phage to teichoic acid accelerates binding to the membrane receptor YueB, which is irreversible (Baptista *et al.*, 2008). Finally, *Lactobacillus delbrueckii* phage LL-H adsorption to lipoteichoic acid depends on α-glucose substitution (Räisänen *et al.*, 2007).

Although M102 adsorbed to all tested serotype c *S. mutans* strains, only strain OMZ 381S was sensitive to phage infection. The mutants that were converted from serotype e or f to serotype c showed drastically increased phage adsorption, but were not infected by M102. Thus, adsorption to the host is an essential, but not the sole determinant for successful phage infection. Several different mechanisms of phage resistance have been described (Forde & Fitzgerald, 1999; Barrangou *et al.*, 2007), but at present it is unknown what mechanism causes resistance to M102 in serotype c strains.

The potential use of bacteriophage to remove cariogenic mutans streptococci from dental plaque is thus limited by serotype specifity, but also by other, unknown, factors. For these reasons, it might be better to use phage-encoded endolysins for selective elimination of *S. mutans* from dental plaque.

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