Genes involved in the repression of mutacin I production in *Streptococcus mutans*

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Streptococcus mutans is considered a primary pathogen for human dental caries. Its ability to produce a variety of peptide antibiotics called mutacins may play an important role in its invasion and establishment in the dental biofilm. *S. mutans* strain UA140 produces two types of mutacins, the lantibiotic mutacin I and the non-lantibiotic mutacin IV. In a previous study, we constructed a random insertional-mutation library to screen for genes involved in regulating mutacin I production, and found 25 genes/operons that have a positive effect on mutacin I production. In this study, we continued our previous work to identify genes that are negatively involved in mutacin I production. By using a high-phosphate brain heart infusion agar medium that inhibited mutacin I production of the wild-type, we isolated 77 clones that consistently produced mutacin I under repressive conditions. From the 34 clones for which we were able to obtain a sequence, 17 unique genes were identified. These genes encompass a variety of functional groups, including central metabolism, surface binding and sugar transport, and unknown functions. Some of the 17 mutations were further characterized and shown to increase mutacin gene expression during growth when the gene is usually not expressed in the wild-type. These results further demonstrate an intimate and intricate connection between mutacin production and the overall cellular homeostasis.

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INTRODUCTION

Dental caries is one of the most common infectious diseases afflicting humans. *Streptococcus mutans* is considered a primary agent in cariogenesis (Loesche, 1986). Its abilities to adhere to and form biofilms on the tooth surface, to produce acids from metabolizable carbohydrates, and to survive low pH and other environmental insults are believed to be critical in its persistence and eventual dominance in the dental plaque. The dental plaque consists of a complex bacterial community of >700 different species living in an environment with constant cycles of feast and famine (Socransky *et al.*, 1998). In addition to the previously mentioned virulence properties, *S. mutans* also possesses a tremendous ability to kill other competing species in the dental plaque by producing peptide antibiotics called mutacins (Hamada & Ooshima, 1975; Parrot *et al.*, 1989, 1990). Mutacin production may allow *S. mutans* to invade and establish itself within the

dental biofilm community and to persist when nutrients become limited in the dental plaque.

S. mutans strain UA140 produces two types of mutacins, the lantibiotic mutacin I and the non-lantibiotic mutacin IV (Qi *et al.*, 2001). The lantibiotics are extensively modified peptide antibiotics, containing dehydrated threonine and serine residues and thioether bridges (Sahl & Bierbaum, 1998), while the non-lantibiotics are unmodified peptides. Mutacin production is controlled by many genetic as well as environmental factors. While the non-lantibiotic mutacin IV is controlled by quorum sensing via the three-component system *comCDE* (Kreth *et al.*, 2005, 2006), regulation of the lantibiotic mutacin I is much more complex and less understood. Mutacin I can only be produced under high cell densities such as in a pellet or in colonies in plate culture (Qi *et al.*, 2000; Tsang *et al.*, 2005, 2006). In order to obtain a global view of how mutacin I production is regulated, we previously constructed a random insertional mutagenesis library in strain UA140. Using this library, we identified 25 genes that are positive regulators of mutacin I production (Tsang *et al.*, 2005). In the present study, we used the same library to identify genes that are negative regulators of mutacin production. We identified 17 unique genes and several of these were further characterized.

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Abbreviations: PTS, phosphotransferase system; Spc, spectinomycin.

A supplementary table of primers and two supplementary figures illustrating double- and single-crossover mutagenesis strategies are available with the online version of this paper.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain DH5 α was used for cloning and plasmid amplifications. *E. coli* cells were grown in Luria-Bertani (LB, Fisher) medium aerobically at 37 °C. *E. coli* strains carrying plasmids were grown in LB medium containing spectinomycin (Spc, 150 $\mu\text{g ml}^{-1}$). *S. mutans* wild-type strain UA140 was cultured in brain heart infusion (BHI, Difco) medium or on BHI agar plates. For selection of antibiotic-resistant colonies, BHI medium was supplemented with Spc (800 $\mu\text{g ml}^{-1}$) or erythromycin (15 $\mu\text{g ml}^{-1}$). BHI medium supplemented with potassium phosphate buffer (5 mM) was used to screen for mutants with increased mutacin production.

Library screening. A previously constructed random insertional mutation library was used (Tsang *et al.*, 2005). The library was constructed by randomly cleaving the genomic DNA of strain UA159 with *Cvi*II restriction enzyme, and ligating the 300–500 bp gel-purified fragments into the pZero-2 vector at the *Eco*RV site. After transforming into *E. coli*, the plasmid was isolated from 11 000 pooled *E. coli* clones and transformed into *S. mutans* UA140. Eleven thousand transformants were randomly selected, which were then grown on 96-well plates, and stored at –80 °C as the library. To screen for mutants that produce mutacin I in the presence of inhibitory amounts of phosphate, the library clones were grown in 96-well plates overnight and 5 μl of the culture was spotted onto BHI plates supplemented with 5 mM potassium phosphate and grown anaerobically for 24 h. Each plate was then overlaid with a thin layer of soft agar mixed with overnight cultures of *Streptococcus sobrinus* OMZ176, which was only sensitive to mutacin I. The zone of inhibition (halo) was inspected after an overnight incubation under anaerobic conditions. Since this condition inhibits mutacin I production in the wild-type, the presence of a halo around any of the mutant colonies was considered to be positive. Positive clones from the first round of screening were subjected to a second and third round of screening using the same procedures as described above. Clones that produced mutacin after all three rounds of screening were used for gene identification.

Identification of mutated genes. Chromosomal DNA from each selected mutant was prepared from 10 ml of overnight cell culture as follows. Cell cultures were centrifuged, and the cell pellet was suspended in 1 ml TE buffer with 15 mg ml^{-1} freshly prepared lysozyme and incubated at 37 °C for 2–3 h. Then 0.1 vol. 10% SDS and 0.7 vol. phenol/chloroform (pH 6.7) were added to the mixture and mixed by vortexing for 1 min. The mixture was centrifuged at 16 000 g for 5 min and the supernatant (~0.9 ml) was removed. DNA was precipitated from the supernatant by 0.7 vol. 2-propanol in the presence of 0.15 M NaCl, and washed with 70% ethanol. The DNA pellet was suspended in 50 μl TE buffer containing 0.1 mg RNase ml^{-1} .

To identify the gene mutation, chromosomal DNA was digested with *Cvi*II (CHIMERx), the enzyme that was used to generate near-random DNA fragments for construction of the insertional library used in this study (Tsang *et al.*, 2005). The digestion reaction (30 μl) was performed in a 96-well plate and incubated at room temperature overnight. Two microlitres of the digested DNA was ligated overnight at room temperature with 1 μl of plasmid pKS(+) digested with *Bam*HI and *Eco*RV, in a total volume of 11 μl , also in a 96-well plate. One microlitre of the ligation mixture was used as template in a 25 μl PCR using primers M13-reverse and Zero-F (5'-GTGTGCTGGAATTCTGCAG-3'). The PCR product was purified with the PCR Purification kit (Qiagen) and used directly for sequencing by the DNA Sequencing Facility of the Oklahoma Medical Research Foundation. Since *Eco*RV was used for cloning the original library fragments into pZero, confirmed mutations were

expected to contain *S. mutans* sequence terminating at an *Eco*RV half-site followed by pZero plasmid sequence (Tsang *et al.*, 2005). The sequences obtained were compared to the genomic sequences of *S. mutans* UA159 available at the Los Alamos Oral Pathogen Sequence Databases (<http://www.oralgen.lanl.gov>) via BLAST. The insertion point was defined as the junction with the *Eco*RV cleavage site.

Mutacin reporter assay in mutant backgrounds. Chromosomal DNA from each mutant was isolated and transformed into the *mutA-luc* reporter strain, UA140mutA-luc (He *et al.*, 2008). Transformants were selected on BHI + 800 $\mu\text{g Spc ml}^{-1}$ plates, and the right clones were further confirmed by PCR. To measure *mutA* gene expression, wild-type and mutant strains were grown overnight in BHI and 10 μl from each was spotted on BHI plates with or without supplementation with 5 mM sodium phosphate. Cells were grown anaerobically for 16 h, then scraped from the plates, and resuspended in BHI. Luciferase activity was measured immediately after resuspension using the methods described previously (Kreth *et al.*, 2004). The same cell suspensions were also measured for OD₆₀₀ and the luciferase activities were normalized against OD₆₀₀.

RESULTS

Identification of genes involved in repression of mutacin I production

Previous studies demonstrated that mutacin I gene expression is repressed during growth and activated under mild stress conditions, such as those encountered during limited nutrient availability. In an effort to identify environmental factors that repress mutacin I gene expression, we previously found that buffering BHI with 5 mM phosphate buffer inhibited mutacin I production (F. Qi, unpublished). Further studies revealed that the inhibition is temporal; mutacin I was produced after about 24 h on low-phosphate plates (BHI), whereas high-phosphate plates (BHI + 5 mM phosphate) required 48 h for mutacin production (data not shown). Based on this observation, we assumed that phosphate availability may be one of the environmental factors that inhibit mutacin I gene expression during growth. Thus, we reasoned that by selecting mutants able to produce mutacin within 24 h under high-phosphate conditions (i.e. nutrient excess), we would be able to identify genes involved in the repression of mutacin I gene expression during growth.

To this end, we screened a previously constructed random insertional mutagenesis library of 11 000 clones (Tsang *et al.*, 2005) to look for mutacin production after 24 h growth on phosphate-supplemented BHI plates. Positive clones from the first round of screening were retested; after three rounds of testing, 77 mutants were confirmed and stored at –80 °C.

To identify the mutated gene, genomic DNA (gDNA) was isolated from all 77 clones. A high-throughput protocol was developed to sequence all clones simultaneously. In this protocol, we took advantage of the near randomness of the *Cvi*II restriction enzyme. *Cvi*II recognizes 5'-PuGCPy-3' and cuts between G and C, generating blunt-ended fragments. Chromosomal DNA from each clone was

digested with *Cvi*II and ligated with pKS vector digested with *Eco*RV. To specifically amplify the chromosome region where the insertion took place, a forward primer was designed, which is immediately upstream of the *Eco*RV site in pZero (the restriction site in the vector used to construct the original library) (Tsang *et al.*, 2005). The reverse primer was the M13 reverse primer, which is located ~100 bp downstream of the *Eco*RV site in the pKS vector. After PCR amplification, most clones generated a ~300–400 bp fragment. After purification, these PCR products were directly used for sequencing with the M13 reverse primer. We were able to obtain unambiguous sequences from 34 of these clones, from which 17 unique genes were identified (Table 1). To further confirm that these genes are truly responsible for the observed phenotype, each gene was individually deleted by using a single-crossover mutagenesis strategy (see Supplementary Table S1 and Supplementary Fig. S1, available with the online version of this paper). These mutants were tested for mutacin I production on BHI plates supplemented with 5 mM phosphate. All transformants displayed the same phenotype as the original clones, indicating that the observed phenotype in these clones is indeed due to insertions in the identified genes.

Since two of the genes (SMU.478, SMU.947) reside in an operon, the downstream genes were individually deleted by a double-crossover strategy (see Supplementary Fig. S2 and Supplementary Table S1). Overall, nine mutagenesis attempts were made, and eight mutants were obtained. No mutant could be obtained for SMU.481, possibly because this gene encodes methionyl-tRNA formyltransferase, which may be essential. All eight mutants were tested for their effect on mutacin production on BHI + phosphate plates. All displayed a wild-type phenotype, except for *clpX* (SMU.949), which could not produce mutacin even on regular BHI plates, where the wild-type produced mutacin (data not shown). These results suggested that the observed phenotype for SMU.478 and SMU.947 is not due to a polar effect on the downstream genes.

Preliminary characterization of selected mutants

To further characterize the effects of these gene mutations on mutacin I production, all 17 mutants were further tested on BHI plates containing different concentrations of phosphate. Since the Difco BHI formulation contains 9.5 mM phosphate, we diluted BHI 1:1 with water and supplemented the 50 % BHI with phosphate buffer to make

Table 1. Genes associated with repression of mutacin I production

Mutant class	Oralgen ID/GenBank tag (no. of hits)	Annotation	Functional class
Class 1			
1	SMu0557/SMU.611 (1)	DeaD, ATP-dependent RNA helicase	Transcription, degradation of RNA
2	SMu0797/SMU.874 (1)	<i>methH</i> , 5-methyltetrahydrofolate-homocysteine methyltransferase	Amino acid biosynthesis, central intermediary metabolism, one-carbon metabolism
3	SMu859/SMU.947 (1)	Dfr, <i>dhfr</i> , <i>dyr</i> , dihydrofolate reductase	Biosynthesis of cofactors, prosthetic groups and carriers, folic acid
4	SMu1034/SMU.1132 (8)	<i>pepN</i> , lysyl-aminopeptidase	Degradation of proteins, peptides
5	SMu1849/SMU.2035 (2)	<i>mccF</i> , possible bacteriocin immunity protein	Toxin production and resistance
6	SMu1850/SMU.2036 (2)	<i>pepO</i> , endopeptidase O	Degradation of proteins
7	SMu1885/SMU.2077 (2)	Conserved hypothetical protein	Unknown
Class 2			
8	SMu0432/SMU.478 (1)	<i>kguA</i> , guanylate kinase	Purine metabolism
9	SMu0617/SMU.675 (1)	<i>ptsI</i> , phosphoenolpyruvate–protein phosphotransferase (enzyme I)	Signal transduction, PTS
10	SMu0646/SMU.707 (2)	Endolysin	Biosynthesis and degradation of murein sacculus and peptidoglycan
11	SMu0649/SMU.712 (1)	Ppc/ <i>pepC</i> , phosphoenolpyruvate carboxylase	Central intermediary metabolism
Class 3			
12	SMu0538/SMU.591 (1)	HTP, upstream is a transcription regulator sharing IGS with <i>furR</i>	Unknown
13	SMu0727/SMU.801 (1)	Obg, GTP-binding protein, GTP1/Obg family	Unassigned
14	SMu1319/SMU.1449 (1)	<i>fbp</i> , <i>pavA</i> , fibrinogen-binding protein A	Cellular process, pathogenesis, transport and binding proteins
15	SMu1708/SMU.1878 (1)	<i>manM</i> , mannose PTS system component IIC, a membrane protein	Signal transduction, PTS
16	SMu1709/SMU.1879 (7)	<i>manN</i> , mannose PTS system component IID, a membrane protein	Signal transduction, PTS
17	SMu1848/SMU.2033 (1)	Conserved hypothetical protein	Unknown

the final concentrations of phosphate 4.75 mM, 9.5 mM and 14.5 mM, respectively. Mutants were spotted on the plates and the zone of inhibition was determined at 9 h, 12 h and 24 h after inoculation. From the timing of mutacin production under different phosphate concentrations, three classes of mutants can be identified (Fig. 1a, Table 1). Class 1 produced mutacin I at 9 h on the 4.75 mM phosphate plate, at 12 h on the 9.5 mM phosphate plate, and at >12 h on the 14.5 mM phosphate plate. At 24 h, these mutants produced the largest halo on all plates. Class 2 produced similar sized haloes to the wild-type on the low-phosphate plate (4.75 mM), but unlike the wild-type, it produced a strong halo on medium phosphate (9.5 mM) by 24 h. These mutants also produced haloes on the highest phosphate plate, albeit smaller than the ones produced by the type 1 mutants. Class 3 produced more mutacin than the wild-type on the intermediate phosphate plate, but not on the highest phosphate plate.

To investigate further whether the mutations affected mutacin I production at the transcriptional level, three

mutations (*pepN*, *pepO* and SMU.2033) were transformed into a *mutA-luc* reporter strain (He *et al.*, 2008). The *mutA-luc* reporter activity was measured in cells grown on BHI plates as well as on BHI supplemented with 5 mM phosphate. As shown in Fig. 1(b), all mutants displayed >100-fold increased *mutA-luc* reporter activity on both plates, indicating that all three genes affected mutacin production at the transcriptional level.

DISCUSSION

This study is a continuation of our previous efforts to identify genes involved in regulating mutacin I production (Tsang *et al.*, 2005). Using BHI plates supplemented with 5 mM phosphate to suppress mutacin I production in the wild-type, 77 mutant clones were originally identified, which produced mutacin in spite of abundant available phosphate. Of these, 34 sequences were obtained, and 17 unique genes were identified. We were not able to obtain clear sequence for the remaining clones, despite using

Fig. 1. Preliminary characterization of selected mutants. (a) Representative phenotypes of the three mutant classes on BHI plates supplemented with different concentrations of phosphate. Mutants and the wild-type (wt) were grown overnight in BHI broth, and 5 μ l of the overnight culture was spotted onto 50% BHI plates containing 4.75 mM, 9.5 mM or 14.5 mM phosphate. The plates were incubated anaerobically for 9 h, 12 h or 24 h, and overlaid with the indicator strain *S. sobrinus*. Clear zones indicate the production of mutacin I. (b) Mutacin production and *mutA-luc* gene expression in SMU.2033, *pepN* and *pepO* mutants. Wild-type (WT) and mutant strains were grown overnight in BHI and 10 μ l from each was spotted on BHI plates with or without the supplementation of 5 mM sodium phosphate. Plates were incubated anaerobically for 16 h. One set was overlaid with the indicator strain for mutacin production as described above, and another set was used for luciferase assay. For luciferase assay, cells were scraped from the plates and resuspended in BHI. Luciferase activity was measured immediately after resuspension. The same cell suspensions were also measured for OD₆₀₀ and the luciferase activities were normalized against OD₆₀₀. The luciferase activity of the wild-type was taken as 1 (100%), and the level in the mutants was expressed as a ratio to the wild-type. The experiments were repeated at least three times with triplicate samples at each time point. Representative results are shown.

additional approaches such as those used in our previous studies (Tsang *et al.*, 2005). This suggests that these genes may have sequences with the *Cvi*I site too close to the cloning point (the original clone may have been generated from partial digestion), or they lack the restriction sites used in retrieving the inserted plasmid.

The effect of these genes on mutacin production was further analysed at different phosphate concentrations. Based on their temporal pattern of mutacin production, these mutants were classified into three classes. Class 1 includes SMU.611, SMU.797, SMU.859, *pepN*, SMU.2035, *pepO* and SMU.2077. These mutants exhibited the highest mutacin production on all plates tested. Although genes in this class are diverse, the two most prominent genes are *pepN* (SMU.1132) and *pepO* (SMU.2036). *pepN* was hit eight times in the library screening, and *pepO* was hit twice (Table 1). Little has been reported regarding the function of *pepN* in streptococci; however, studies of *pepN* in other bacterial species may offer some indications. *PepN* belongs to a family of ATP-independent aminopeptidases that are involved in protein turnover in the cytoplasm. In *E. coli*, *pepN* is a major aminopeptidase, and its mutation conferred resistance to sodium salicylate, indicating that it is a negative regulator for the sodium-salicylate-induced stress response (Chandu *et al.*, 2003; Chandu & Nandi, 2003). In addition, *pepN* gene expression was found to be induced by phosphate starvation in *E. coli* (Gharbi *et al.*, 1985). Conspicuously, in *S. mutans*, *pepN* is located as the last gene in a six-gene operon encoding proteins involved in phosphate uptake. Thus, based upon *pepN* gene expression in *E. coli* and its operon location in *S. mutans*, we speculate that the *S. mutans pepN* gene may be required for phosphate uptake or metabolism and its deletion may create a phosphate limitation signal responsible for inducing mutacin I production, even in the presence of an adequate supply of phosphate in the environment. *PepO* is a metalloendopeptidase, which belongs to the peptidase M13 family (Rawlings & Barrett, 1995). These peptidases are found in a wide range of organisms including mammals and bacteria. *PepO* is highly conserved among bacterial species, and among streptococci the similarity is ~90 % at the amino acid level. However, the role of this peptidase in bacteria is not defined. A recent report by the Lamont laboratory suggested that *pepO* may play a role in the invasion of *Porphyromonas gingivalis* into host epithelial cells (Park *et al.*, 2004). Here we demonstrate that both *pepN* and *pepO* mutations not only enhanced mutacin production on high-phosphate plates, but also during growth (Fig. 1b). Furthermore, both gene mutations affected mutacin I production at the transcription level, increasing *mutA* gene expression >100-fold (Fig. 1b). The mechanism of how *pepN* and *pepO* mutations trigger mutacin production awaits further investigation.

Class 2 mutations include SMU.478, 675, 707 and 712. These mutants produced intermediate levels of mutacin on the highest phosphate plate tested. All genes in this class are

annotated as genes of central metabolism. The two interesting genes are *ptsI* (SMU.675) and *pepC* (SMU.712). *ptsI* encodes phosphoenolpyruvate–protein phosphotransferase (enzyme I), which was first characterized in *S. mutans* by Boyd *et al.* (1994) as Enzyme I of the phosphoenolpyruvate-dependent phosphotransferase sugar transport system. Subsequent studies by Cvitkovitch *et al.* (1995) demonstrated growth defects of a *ptsI* mutant on glucose and raffinose. In *Streptococcus salivarius*, a *ptsI* mutant strain was found to grow slower than the wild-type on glucose as well as possessing a much longer lag time. *ptsI* was also found to play a role in urease regulation (Weaver *et al.*, 2000). It is likely that inactivation of *ptsI* reduced sugar transport, thus creating carbohydrate limitation in the cell, which triggered the stress response and mutacin I production. SMU.712 encodes a putative phosphoenolpyruvate carboxylase (PEPCase). PEPCase is an enzyme found in all multicellular plants, catalysing the formation of oxaloacetate from phosphoenolpyruvate (PEP) and a hydrocarbonate ion (Sugimoto *et al.*, 1992; Vazquez-Tello *et al.*, 1993). In bacteria, PEPCase probably supplies oxaloacetate to the TCA cycle. Since *S. mutans* does not have a complete TCA cycle, it is likely that inactivation of the gene affected pyruvate metabolism, which is a downstream intermediate in oxaloacetate metabolism. Pyruvate is a central intermediate in energy metabolism as well as in amino acid biosynthesis. We speculate that disturbance in the intracellular metabolic balance may create a metabolic stress triggering mutacin I production.

The class 3 mutations include SMU.591, 801, 1449, 1878, 1879 and 2033. These mutants produced mutacin on 9.5 mM phosphate, but could not produce mutacin on 14.5 mM phosphate. The two interesting genes in this class are *manM* (SMU.1878) and *manN* (SMU.1879), encoding mannose phosphotransferase system (PTS) IIC and IID, respectively. Both genes are located in the same operon, and *manN* was hit seven times in our library screening (Table 1). Although functions for *manM* and *manN* have not been studied, Abranches *et al.* (2006) demonstrated that inactivation of *manL*, the first gene of the *manLMN* operon in *S. mutans*, resulted in impaired biofilm formation, decreased transformation efficiency, and altered expression of 62 genes. Based on these results, it was suggested that EIIB^{Man} (*manL*) plays a central regulatory role in the physiology and virulence of *S. mutans* by sensing the energy levels of the cell. The current finding that the inactivation of the EIIC^{Man} and EIID^{Man} components resulted in increased mutacin I production under repressive conditions is consistent with this notion.

In summary, we have identified 17 genes whose mutation increased mutacin I production under repressive conditions. These genes are involved in a variety of cellular functions such as sugar transport, protein/peptide hydrolysis, amino acid and nucleotide synthesis, cell-wall metabolism, and surface binding. The involvement of these genes in mutacin I production suggests an intimate

and intricate connection between mutacin production and the overall cellular homeostasis.

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