Identification of genes associated with mutacin I production in *Streptococcus mutans* using random insertional mutagenesis

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Streptococcus mutans is a major pathogen implicated in dental caries. Its virulence is enhanced by its ability to produce bacteriocins, called mutacins, which inhibit the growth of other Gram-positive bacteria. The goal of this study is to use a random insertional mutagenesis approach to search for genes that are associated with mutacin I production in the virulent strain UA140. A random insertional mutagenesis library consisting of 11 000 clones was constructed and screened for a mutacin-defective phenotype. Mutacin-defective clones were isolated, and their insertion sites were determined by PCR amplification or plasmid rescue followed by sequencing. A total of twenty-five unique genes were identified. These genes can be categorized into the following functional classes: two-component sensory systems, stress responses, energy metabolism and central cellular processes. Several conserved hypothetical proteins with unknown functions were also identified. These results suggest that mutacin I production is stringently controlled by diverse and complex regulatory pathways.

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INTRODUCTION

Dental caries is a chronic condition affecting the teeth. Bacteria adhering to tooth surfaces produce acids from carbohydrate fermentation, which can demineralize tooth structures, resulting in cavitation on tooth surfaces. One of the major pathogens implicated in dental caries is *Streptococcus mutans* (Loesche, 1986). In addition to its various virulence attributes, such as glucan synthesis, acid production and acid tolerance, the production of bacteriocins, called mutacins, may also play an important role in the persistence of *S. mutans* in the dental-plaque community (Gronroos *et al.*, 1998; Hillman, 2002).

Bacteriocins produced by Gram-positive bacteria are peptide antibiotics classified as class I (lantibiotics) or class II (non-lantibiotics), based on their post-translational modifications (Guder *et al.*, 2000; Riley & Wertz, 2002). In *S. mutans*, both classes of bacteriocins are produced. The lantibiotic mutacins have a wide spectrum of activity against Gram-positive bacteria, including multiple-drug-resistant pathogens (Novak *et al.*, 1994; Qi *et al.*, 1999, 2000), while the non-lantibiotic mutacins are more specific to closely related streptococcal species such as the mitis group streptococci and group A streptococci (Qi *et al.*, 2001). Mutacin I, the focus of this study, is a 24 aa lantibiotic with a

molecular mass of 2364 Da. The mutacin I biosynthesis gene locus consists of 14 genes in the order of *mutR*, *mutA*, *mutA'*, *mutB*, *mutC*, *mutD*, *mutP*, *mutT*, *mutF*, *mutE*, *mutG*, *orfX*, *orfY*, *orfZ* (Qi *et al.*, 2000). MutR is thought to be the positive regulator for the expression of the mutacin I operon. MutA and MutA' show strong similarity to each other. While *mutA* is the structural gene for prepromutacin I, *mutA'* is not required for mutacin I activity. MutB, MutC and MutD constitute the modification apparatus for the premature peptide, and MutT and MutP are the ABC transporter and protease, respectively, for the transporting and processing of premature mutacin. MutF, MutE and MutG are probable immunity proteins for mutacin I. The functions of OrfX, OrfY and OrfZ proteins in mutacin I production are unknown (Qi *et al.*, 2000).

Previous studies indicate that the proteins encoded by *ciaH* and *luxS* are required for mutacin I production (Merritt *et al.*, 2005; Qi *et al.*, 2004). CiaH is the histidine kinase of a two-component signal-transduction system, CiaH/R (Giammarinaro *et al.*, 1999; Zähner *et al.*, 2002), while LuxS is responsible for the synthesis of the interspecies cell signalling molecule autoinducer 2 (AI-2) (Schauder *et al.*, 2001; Xavier & Bassler, 2003). In addition to regulating mutacin I production, both CiaH and LuxS were also found to be involved in regulating other cellular functions in *S. mutans* such as biofilm formation and stress tolerance (Len *et al.*, 2004; Merritt *et al.*, 2003; Qi *et al.*, 2004). As a preliminary attempt to understand the control of mutacin I

Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; TCS, two-component sensory system.

production and its relationships with these pleiotropic regulators, we employed a random insertional mutagenesis approach to obtain a global view of genes involved in mutacin I production. Our results indicate that mutacin I production seems to be affected by numerous global regulatory systems.

METHODS

Bacterial strains and culture conditions. All *S. mutans* strains were grown in brain–heart infusion (BHI) medium (Difco) or on BHI agar plates. *S. mutans* mutant strains were selected using the same medium with 800 µg spectinomycin ml⁻¹. All *S. mutans* strains were grown anaerobically (80% N₂, 10% CO₂, 10% H₂) at 37 °C. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium with aeration or on LB agar plates at 37 °C. *E. coli* strains carrying p0SC plasmids were grown in LB medium supplemented with 250 µg spectinomycin ml⁻¹.

Construction of a random mutagenesis library. Genomic DNA extracted from *S. mutans* strain UA159 was randomly cleaved by *Cvi*JI restriction enzyme digestion. Digested DNA fragments of 300–500 bp were purified using the QIAquick gel extraction kit (Qiagen).

The pZero-2 cloning vector (Invitrogen) was used to facilitate library construction in *E. coli*, and as a suicide vector in *S. mutans*. To adapt the use of this plasmid for the selection of positively transformed *S. mutans*, the kanamycin-resistance gene from the pZero-2 vector was excised by *Ava*I and *Bgl*II restriction enzymes and the pZero-2 backbone was ligated with a spectinomycin-resistance gene (*aad9*) (Podbielski *et al.*, 1996). The modified vector, p0SC, was linearized by *Eco*RV digestion and purified by gel extraction. For ligation, the reaction contained *S. mutans* DNA fragments combined with the linearized p0SC vector in a 5:1 ratio and 2 U T4 DNA ligase (Promega). Competent *E. coli* DH5α cells were transformed with the ligation mixture by electroporation. Plasmids harvested from positive *E. coli* transformants were used to transform *S. mutans* strain UA140 for mutagenesis, using protocols described by Perry *et al.* (1983). Transformants were selected on BHI plates containing 800 µg spectinomycin ml⁻¹. The overall strategy for the construction of this library is summarized in Fig. 1. Confirmation of DNA integration was performed by PCR and Southern blotting. PCRs were performed using primers specific to the spectinomycin-resistance gene: Scaad9Up (5'-ACTGCATTTCGCAATATC-3') and Scaad9Dn (5'-GGATCAGGAGTTGAGAGTGG-3'). Southern blotting was performed with digoxigenin (DIG)-labelled *aad9* PCR product as a probe, synthesized and detected by the DIG DNA labelling and detection kit (Roche Diagnosis).

Fig. 1. Construction of the random mutagenesis library. *S. mutans* (strain UA159) genomic DNA was randomly cleaved by *Cvi*JI into 300–500 bp fragments and ligated with the modified pZero-2 vector, p0SC. These plasmids were used to transform *S. mutans* strain UA140 for single-cross-over mutagenesis. DNA from the mutants was digested by *Xmn*I, *Sca*I, *Acl*I or *Bst*BI, and recircularized by ligation for sequence identification.

Screening for the mutacin-defective phenotype. *S. mutans* mutants were inoculated on Todd-Hewitt (TH) agar plates and incubated anaerobically (80 % N₂, 10 % CO₂, 10 % H₂) at 37 °C for 48 h. The indicator strain *Streptococcus sobrinus* OMZ 176 was grown in BHI anaerobically at 37 °C overnight. A mixture of the *S. sobrinus* culture and melted BHI agar was overlaid on top of the *S. mutans* mutant plates. The zone of *S. sobrinus* inhibition in the overlay agar was inspected after overnight incubation at 37 °C under anaerobic conditions.

Identification of mutated genes. Chromosomal DNA from each mutacin-defective mutant was prepared from 10 ml cell culture at OD₆₀₀ 0.8 by a standard DNA extraction protocol (Sambrook *et al.*, 1989). Chromosomal DNA from each mutant was digested with one of the following restriction enzymes: *Xmn*I, *Sca*I, *Acl*I or *Bst*BI. All of these restriction enzymes do not cut within the pOSC plasmid sequence but excise the genome into fragments amenable to further manipulation. DNA fragments were circularized by self-ligation with T4 DNA ligase (Promega). The recircularization of the DNA fragments allowed the targeting inserts to be directly amplified by PCR using M13 primers flanking the multiple cloning site of the pOSC plasmid: M13F (5'-GTAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3'). The PCR was performed with initial denaturation of DNA at 95 °C for 4 min, followed by 40 cycles of amplification consisting of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 5 min and 1 cycle of final extension of 72 °C for 10 min. The recircularized DNA fragments were also used to transform *E. coli* DH5 α cells. The rescued plasmids or PCR products were analysed by gel electrophoresis, purified with a Qiagen gel extraction kit and sequenced by the UCLA core DNA sequencing facility. 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.

RESULTS AND DISCUSSION

Construction of the mutagenesis library

Random insertional mutagenesis has been previously used to identify genes involved in biofilm formation in *S. mutans* (Yoshida & Kuramitsu, 2002). For this assay, suicide plasmids were inserted into the *S. mutans* genome via single-crossover homologous recombination. The integration of the inserted plasmid may inactivate either a single gene or an operon by polar mutation. Previous studies have shown that the size of the targeting fragment affects the chance of mutagenesis. For instance, to ensure 95 % of the genes are mutated with 99 % probability in the *Streptococcus pneumoniae* genome, roughly 9000 clones are required with randomly chosen 100 bp inserts, while 11 000 clones are required with 300 bp inserts (Lee *et al.*, 1999). In contrast, the recombination rate for insertion mutagenesis is found to be dependent on the targeting fragments size. As the insert size decreases, recombination efficiency drops rapidly and greatly reduces the number of mutants obtained per μ g of DNA (Lee *et al.*, 1998). In order to balance the probability of saturating the genome by mutagenesis with a practical library size, the DNA fragment was chosen to be between 300 and 500 bp. Various methods for generating genomic DNA fragment of the target size, including sonication, DNase treatment and restriction enzyme digestion, were compared. Based on preliminary experimentation, *Cvi*II restriction

enzyme digestion was selected for this study. The star activity of restriction enzyme *Cvi*II creates nearly random cleavages throughout the genome. Furthermore, *Cvi*II generates blunt-ended DNA fragments and, therefore, bypasses the need for Klenow treatment. Genomic DNA from *S. mutans* strain UA159 was used to generate the random fragments for mutagenesis. Since UA159 does not contain the mutacin I structural operon, it was expected that none of these genes would be affected in the mutagenized *S. mutans* strain UA140. This arrangement improved the efficiency of screening for mutacin I negative phenotypes since each mutation was independent of the mutacin I structural operon. However, there is a possibility that some mutacin I regulators in UA140 may not be mutated due to genomic differences between the two strains.

To allow the use of pZero-2 in *S. mutans* the plasmid was adapted by replacing the kanamycin resistance gene with a spectinomycin-resistance gene (*aad9*) (Podbielski *et al.*, 1996). The replacement of the antibiotic-resistant gene did not affect the lethal selection property of the plasmid. Analysis by PCR amplification using universal primers flanking the multiple cloning sites demonstrated that nearly 100 % of *E. coli* clones carried an insert (data not shown).

Overall, the utilization of the restriction enzyme *Cvi*II and the pOSC plasmid greatly streamlined the preparation of the *S. mutans* random insertional library. A total of 11 000 *E. coli* clones were obtained to represent approximately 6 times the total number of ORFs (1963) in the *S. mutans* genome (Ajdić *et al.*, 2002). Plasmids extracted from these clones were used to transform competent *S. mutans* UA140. Approximately 11 000 *S. mutans* transformants were subjected to phenotypic screening.

Screening for mutacin-defective mutants and identification of the disrupted genes

All 11 000 clones were subjected to a mutacin-activity assay. Clones that did not inhibit the growth of an indicator strain were selected and subjected to a total of three rounds of screening. To exclude those mutants whose mutacin-defective phenotype might be a result of severe growth defects, only clones with apparent normal growth rates (overnight culture with OD₆₀₀ ~0.8) were chosen for the second round of screening. Clones with a mutacin-negative phenotype in all three rounds of screening were selected for gene identification.

Digested chromosomal DNA fragments were recircularized and used to transform competent *E. coli*, or used as PCR templates (see Methods). The PCR products or the rescued plasmids were directly sequenced, and 25 unique genes were identified after a BLAST search of the UA159 genome sequence database (Tables 1 and 2). The protein sequences were also compared with other known sequences available in the NCBI database. The genes proposed functions and their frequencies of disruption in the mutagenesis library are

Table 1. Putative genes associated with mutacin I expression

Gene ID*	Assigned name	Functional class	Frequency of disruption
Smu0072	HrcA – transcriptional repressor of class I heat-shock protein	Regulation of stress response	3
Smu0131	AdhE – alcohol-acetaldehyde dehydrogenase	Energy metabolism, fermentation	10
Smu0434	PriA – primosomal replication factor Y	DNA replication	1
Smu0440	Hk03 – two-component sensory kinase	TCS	1
Smu1008	Pgm – phosphoglycerate mutase protein	Energy metabolism: glycolysis	1
Smu1271	LepA – GTP-binding proteins	Cell division	2
Smu1364	RexB – ATP-dependent exonuclease subunit B	Replication/repair	1
Smu1378	CovS – two-component sensory kinase	TCS	2
Smu1389	AtpD – ATPase beta subunit	Energy metabolism: ATP-proton motive force interconversion	1
Smu1403	LigA – DNA ligase A	Replication/recombination	1
Smu1852	PttB – phosphotransferase system trehalose-specific IIBC component	PTS transport	1
–	23S RNA	rRNA	4
–	tRNA	tRNA	2
–	16S RNA	rRNA	1

*Gene ID from Los Alamos oral pathogen sequences database.

summarized in Table 1. These genes are classified by their functional classes as described in the Los Alamos National Laboratory – oral pathogen sequence database: (1) two-component sensory systems (TCSs), (2) stress responses, (3) energy metabolism, (4) central cellular processes and (5) genes with unknown function.

Two-component sensory systems

TCSs, composed of membrane-bound sensors and cytoplasmic response regulators, are important mechanisms for bacteria to sense and respond to environmental stimuli. In this study, we identified two TCSs encoded by genes whose disruptions caused a mutacin I defective phenotype: *covS/R* (Smu1378) and *hk03/rr03* (Smu440). CovS is a predicted transmembrane histidine kinase of the CovS/R TCS. It shares 84 % protein similarity with VicK in *Streptococcus pyogenes* and 80 % similarity with VicK in *S. pneumoniae*. Hk03 is also a predicted transmembrane histidine kinase, which shares 81 and 69 % protein similarity to putative TCS histidine kinases in *S. pyogenes* and *S. pneumoniae*, respectively. Previous studies have illustrated the role of TCSs in the control of bacteriocin production (Engelke *et al.*, 1994; Klein *et al.*, 1993; Qi *et al.*, 2004). In group A streptococci, CovS/R have been implicated as global virulence regulators (Federle *et al.*, 1999; Levin & Wessels, 1998), influencing transcription of 15 % of all chromosomal genes and many mediators of host-pathogen interactions (Graham *et al.*, 2002). In *S. mutans*, CovS/R has been shown to negatively regulate the expression of fructosyltransferase and the synthesis of a novel extracellular carbohydrate (Lee *et al.*, 2004). Li *et al.* (2002) reported that mutations in the Hk03/Rr03 TCS caused alterations in the biofilm architecture

and acid tolerance of *S. mutans* NG8. Our findings suggest that the disruption of the CovS/R and Hk03/Rr03 TCSs abolished mutacin I production, which implies that *S. mutans* may regulate mutacin production based on external environmental stimuli. However, currently the input signals of these two component sensors are unknown.

Stress-response genes

The ability of bacteria to adapt to stresses, such as pH, temperature and oxidative changes, is essential for their survival. Lactic acid producing bacteria like *S. mutans* are constantly bombarded with dissociated protons from acids passively diffusing through the cell membrane into the cytoplasm. The resulting decrease in intracellular pH drastically alters the proton motive force required for numerous transmembrane transport processes (Presser *et al.*, 1997). The internal acidification also reduces the activity of acid-labile enzymes and damages proteins and DNA (van de Guchte *et al.*, 2002). To stabilize the transmembrane pH during an acidic challenge, the multimeric F₀F₁-ATPase is utilized to expel protons out of the cell (Kuhnert *et al.*, 2004; Suzuki *et al.*, 2000). Mutations in the β (AtpD) and γ (UncG) subunits of the F₀F₁-ATPase resulted in mutacin I defective phenotypes. BLASTP analysis of AtpD and UncG in *S. mutans* revealed high sequence conservation with orthologues in other streptococci, with AtpD and UncG exhibiting approximately 80–90 % similarity to other ATPase subunits. It is speculated that mutacin I production may be abolished due to protein denaturation resulting from intracellular pH change. Alternatively, a stress signal secondary to the disruption of the ATPase may affect mutacin I production. A stress-modulated mutacin I regulation pathway is also

supported by the evidence that another stress-response regulatory gene, *hrcA*, was also detected in our assay. BLASTP analysis of HrcA in *S. mutans* shows approximately 80 % similarity with HrcA in other streptococci. HrcA is a transcriptional regulator of class I heat-shock proteins, GroEL and DnaK, which assist the folding of newly synthesized proteins or proteins denatured during environmental insults (Jayaraman *et al.*, 1997; Schulz & Schumann, 1996). HrcA represses the stress-related proteins by binding to a DNA element called CIRCE (controlling inverted repeat of chaperone expression) located in the regulatory region of the stress genes (Zuber & Schumann, 1994). Mutation of the HrcA/CIRCE system in *S. mutans* has been demonstrated to derepress *groESL* and *dnaK* gene expression, and render the mutant sensitive to low pH (Lemos *et al.*, 2001). It is unlikely that HrcA directly controls the production of mutacin I since no CIRCE element has been found in the mutacin I operon sequence. Further investigation is needed to decipher the relationship between HrcA and mutacin I production.

Genes involved in energy metabolism

In *S. mutans*, pyruvate generated from glycolysis can be converted into a variety of acidic end products (Carlsson *et al.*, 1985). Under anaerobic conditions and limited glucose, pyruvate is converted into formate and acetate or ethanol through the pyruvate formate-lyase pathway. In one branch of this pathway, pyruvate becomes acetyl-CoA, which is in turn converted to ethanol by a bifunctional enzyme, alcohol-acetaldehyde dehydrogenase (AdhE), in a two-step process (Carlsson & Griffith, 1974; Yamada & Carlsson, 1975). Interestingly, among all of the mutacin-defective mutants selected from our library, *adhE* had the highest frequency of disruption. A BLASTP search revealed numerous matches to AdhE, with the best match to the enzyme from *Lactococcus lactis* (82 % similarity). The dysfunction of AdhE may cause shunting of pyruvate towards the production of formate. Formic acid is a strong acid, which may contribute to a greater production of intracellular H⁺ (Len *et al.*, 2004). This possible internal acidification may create a severe stress signal leading to the abrogation of mutacin production. Another gene involving metabolism, *pgm*, encodes a phosphoglycerate mutase-like protein. Pgm participates in the final conversion of 3-phosphoglycerate to pyruvate in the glycolysis pathway. Pgm matches phosphoglycerate mutases in other species, with the best match exhibiting 76 % similarity to a predicted enzyme from *Listeria innocua*. Pgm mutation may alter the metabolic balance and affect mutacin I production similarly to AdhE mutation.

Another disruption was located in *pttB*, which encodes a transmembrane trehalose-specific IIBC component in the phosphoenolpyruvate:sugar phosphotransferase systems (PTS). PttB appears to be conserved among both Gram-positive and Gram-negative species. The *S. mutans* PttB is 85 % similar to *S. pneumoniae* EIIBC-tre and 66 % similar to *E. coli* EIIBC-tre. *S. mutans* contains numerous PTS systems (Ajdić *et al.*, 2002), and is remarkably adaptable in the types of carbohydrates it can utilize as an energy source (Gauthier

et al., 1984; Schachtele, 1975; Vadeboncoeur & Pelletier, 1997). Besides their roles in sugar transportation, PTS components have been demonstrated to influence urease expression (Weaver *et al.*, 2000), haemolysin production (Lun & Willson, 2005) and biofilm formation (Abranches *et al.*, 2003) in various streptococci. Furthermore, reports have shown that sensitivity to bacteriocin is related to the presence of the mannose-specific phosphotransferase enzyme IIAB in *Enterococcus faecalis* and *Listeria monocytogenes* (Dalet *et al.*, 2001; Héchard *et al.*, 2001; Ramnath *et al.*, 2000). However, there is currently no data, to our knowledge, that supports regulation of bacteriocin production by the PTS.

Genes involved in central cellular processes

Mutations in *rexB*, *priA*, *ligA*, 23S/16S rRNA and tRNA sequences were all predicted to affect central cellular processes such as DNA replication, repair and recombination as well as translation. RexB is 78 % similar to RexB in *Streptococcus agalactiae*, and 72 % similar to the B subunit of a putative ATP-dependent exonuclease in *S. pyogenes*. PriA is approximately 80 % similar to the putative primosomal replication factor Y in *S. pyogenes* and *S. pneumoniae*. LigA is approximately 85 % similar to putative DNA ligases in other streptococci.

Recently, emerging evidence has suggested alternative mechanisms to those of classical transcriptional regulators, such as those involving termination/antitermination factors or regulatory RNA elements participating in gene expression (Anderson & Schneewind, 1997; Henkin, 2000; Johansson & Cossart, 2003; Ma *et al.*, 2001; Novick *et al.*, 1993; Shimizu *et al.*, 2002). It is interesting to find that a possible polar mutation in the *nusA-infB* operon, and alterations to rRNA and tRNA sequences in our mutants, have eliminated the production of mutacin I. It is arguable that mutacin I reduction in these mutants is not a specific phenomenon, but a part of the global physiologic stress due to major disruptions in cellular pathways. Nonetheless, the possible role of transcription termination and regulatory RNA elements in mutacin I regulation warrants further exploration. In *Pseudomonas*, mutation of the DNA repair pathway has been shown to be associated with a reduced production of bacteriocin LlpA (de Los Santos *et al.*, 2005). Similarly, mutations in *rexB*, *priA* and *ligA* in our study represent major insults to the cells DNA replication, repair and recombination systems, and may create a negative effect on mutacin I gene expression through stress-response pathways.

Genes with unknown functions

Conserved hypothetical proteins and their homologies with other proteins are outlined in Table 2. Smu0274 is a protein that is 80 % similar to hypothetical proteins in *S. pyogenes* and *S. agalactiae*. Protein-domain analysis assigned this protein to the metallo- β -lactamase superfamily. Smu0378 is a conserved hypothetical protein immediately upstream of

Table 2. Conserved hypothetical proteins associated with mutacin I expression

Gene ID* (gene size)	Homology (identity/similarity)	Frequency of disruption
Smu0274 (744 bp)	Homologous to hypothetical protein in <i>S. pyogenes</i> (80/89 %) and <i>S. agalactiae</i> (75/85 %)	1
Smu0378 (489 bp)	Homologous to other unknown proteins in <i>S. agalactiae</i> (77/89 %), <i>S. pyogenes</i> (71/82 %) and <i>S. pneumoniae</i> (70/83 %)	6
Smu0530 (825 bp)	Homologous to haemolysins in <i>S. agalactiae</i> (79/88 %) and <i>S. pyogenes</i> (77/86 %)	1
Smu0755 (2538 bp)	Homologous to hypothetical protein in <i>S. pyogenes</i> (48/67 %)	1
Smu1019 (1134 bp)	Homologous to unknown protein in <i>S. agalactiae</i> (33/49 %)	1
Smu1272 (126 bp)	No significant match	1
Smu1281 (4035 bp)	Homologous to unknown protein in <i>S. agalactiae</i> (71/82 %) and <i>S. pyogenes</i> (63/78 %)	1
Smu1292 (726 bp)	Homologous to putative acyl-ACP thioesterase in <i>S. pneumoniae</i> (50/76 %)	1
Smu1616 & 1617 (177 & 243 bp)	Smu1616 – no match with other proteins Smu1617 – homologous to putative DNA damage inducible protein J in <i>S. agalactiae</i> (73/91 %)	1
Smu1713 (1071 bp)	Homologous to putative membrane protein in <i>S.</i> <i>agalactiae</i> (52/69 %)	1

*Gene ID from Los Alamos oral pathogen sequences database.

the putative transcription termination/antitermination factor *nusA*. This protein is 80 % similar to unknown proteins in other streptococci. Smu0530 is 85 % similar to putative haemolysins in *S. pyogenes* and *S. pneumoniae*. Smu0755 is a conserved hypothetical protein that has 11 transmembrane domains, and is linked to the cell-wall localization and side-chain formation of rhamnose-glucose polysaccharides in *S. mutans* (Yamashita *et al.*, 1998). It shares 48 % identity with a hypothetical protein in *S. pyogenes* from residues 53–882. Smu1019 is predicted to have one transmembrane domain and is 49 % similar to another hypothetical protein in *S. agalactiae*. Smu1272 does not have any significant match with proteins in other species. It is unlikely that the mutacin I negative phenotype was a result of insertional mutation of Smu1272 due to its small size (42 aa). The phenotype could be a result of promoter-sequence interruption to the downstream gene, *lepA*. Smu1281 is a large protein (1345 aa) with unknown function, and is also found in the species of *Streptococcus*, *Listeria*, *Neisseria* and *Fusobacterium*. It does not have any significant paralogue in the *S. mutans* genome and its protein structure does not match with any known protein domain. Smu1292 shares 50 % identity with a putative acyl-acyl carrier protein (ACP) thioesterase in *S. pneumoniae*, and is predicted to belong to the fatty acid biosynthesis pathway. Smu1616 (59 aa) and Smu1617 (81 aa) were identified in the same mutant. Due to the small sizes of the genes, the phenotype observed in this mutant was probably

due to duplication of these genes rather than insertional inactivation. Residues 1–77 of Smu1617 are assigned to the RelB family, which is a component of the RelE/RelB toxin–antitoxin system. RelE represses translation, probably through binding ribosomes, whereas RelB stably binds RelE, presumably deactivating it (Christensen *et al.*, 2001; Galvani *et al.*, 2001; Pedersen *et al.*, 2002). Smu1713 is predicted to be a transmembrane protein with 70 % similarity to a putative membranous protein in *S. agalactiae*.

Limitations of this study

As an alternative to insertional inactivation, mutacin I defective phenotypes could have resulted from polar mutations to genes downstream of the disruptions. Among the 25 mutants, 18 were probably a consequence of polar mutations, which are outlined in Table 3. The random mutagenesis approach effectively identified the potential loci involved in mutacin I regulation. Nonetheless, in order to pinpoint the candidates responsible for mutacin regulation, experiments are in progress to distinguish between polar and non-polar mutational effects of these mutations.

It is worth noting that among the 25 genes identified in the mutacin-defective mutants, the previously characterized mutacin I regulatory genes, *ciaH* and *lux S*, were not isolated. The exclusion could be due to the growth retardation of the *ciaH* mutant, which would have likely been eliminated during the initial screening process. The small size of the

Table 3. Possible polar mutations associated with insertional inactivation

Gene ID*/name	Possible polar mutations (functional class)
Smu0072 <i>hrcA</i>	GrpE – heat-shock protein, possible activation of DnaK (stress response)
Smu0274	Cdd – cytidine/deoxycytidylate deaminase (nucleotide and nucleoside interconversions)
	Smu0276 – conserved hypothetical protein
Smu0378	NusA – transcription termination/antitermination factor (transcription termination)
	InfB – translation initiation factor 2 (translation: protein modification)
Smu0434 <i>priA</i>	Fmt – methionyl-tRNA formyltransferase (translation: amino acyl tRNA synthetases and tRNA modification)
	SunL – possible rRNA methylase
	PppL – phosphoprotein phosphatase (translation: protein modification)
	PknB – serine/threonine protein kinase (translation: protein modification and translation factors)
Smu0440 <i>hk03</i>	Smu0441 – response regulator (TCS)
	Smu0442 – possible hydrolase
	PNPase – polyribonucleotide nucleotidyltransferase, general stress protein (salvage of nucleosides and nucleotides)
Smu0530	ArgR – arginine repressor (general regulation)
	RecN – DNA repair and genetic recombination protein (replication/recombination)
Smu0755	Smu0756 – conserved hypothetical protein (unknown)
	GtrB – glycosyltransferase (unknown)
Smu1271 <i>lepA</i>	Smu1270, Smu1269, Smu1268 – conserved hypothetical protein (unknown)
Smu1272	LepA – GTP-binding protein (cell division)
	Smu1270, Smu1269, Smu1268 – conserved hypothetical protein (unknown)
Smu1281	Smu1280, Smu1279, Smu1278 – conserved hypothetical protein (unknown)
Smu1292	MutT – mutator protein T (replication/repair)
	NagD – N-acetylglucosamine catabolic protein (sugars, energy metabolism)
	Smu1289 – conserved hypothetical protein (unknown)
Smu1364 <i>rexB</i>	RexA – ATP dependent exonuclease subunit A (replication/repair)
Smu1378 <i>covS</i>	CovX – unknown function (TCS)
Smu1390 <i>uncG</i>	AtpD – ATPase β subunit (energy metabolism: ATP-proton motive force interconversion)
Smu1403 <i>ligA</i>	Smu1402 – conserved hypothetical protein (replication/recombination)
Smu1852 <i>pttB</i>	DexS – dextran glucosidase; probable trehalose-6-phosphate hydrolase (sugars, energy metabolism)

*Gene ID from Los Alamos oral pathogen sequences database.

luxS gene (430 bp) would have made it less likely to be disrupted by the single-crossover recombination approach in this study. It is likely that additional screenings, including of those mutants with growth defects, would yield more genes involved in mutacin I production, though more experiments will be needed to distinguish between general growth defects and specific mutacin I regulatory defects amongst these mutants. Additionally, the inability to disrupt small ORF by the current method could be overcome by targeted gene disruption via double-crossover recombination in future studies.

The data presented in this study indicate that mutacin I production is stringently controlled by complex regulatory pathways. *S. mutans* may utilize the TCS or PTS to survey

environmental changes and fine tune mutacin I expression accordingly. During stressful situations, such as extreme nutrient deprivation, temperature changes or pH changes, *S. mutans* may minimize energy expenditure by exerting a negative feedback on mutacin I production via stress-regulatory networks. Further experiments are under way to explore the mechanisms involving the proteins affected in these mutants of mutacin I production.

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