Characterization of a *Streptococcus mutans* Intergenic Region Containing a Small Toxic Peptide and Its cis-Encoded Antisense Small RNA Antitoxin

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**Abstract**

Toxin-antitoxin (TA) modules consist of a pair of genes that encode two components: a protein toxin and an antitoxin, which may be in the form of either a labile protein or an antisense small RNA. Here we describe, to the best of our knowledge, the first functional chromosomal type I TA system in streptococci. Our model organism is the oral pathogen *Streptococcus mutans*. Our results showed that the genome of *S. mutans* UA159 reference strain harbors a previously unannotated Fst-like toxin (Fst-Sm) and its cis-encoded small RNA antitoxin (srSm) converging towards the end of the toxin gene in IGR176, a small intergenic region of 318 nt. Fst-Sm is a small hydrophobic peptide of 32 amino acid residues with homology to the Fst toxin family. Transcripts of ~200 nt and ~70 nt specific to fst-Sm mRNA and srSm RNA, respectively, were detected by Northern blot analysis throughout *S. mutans* growth. The toxin mRNA was considerably more stable than its cognate antitoxin. The half-life of srSm RNA was determined to be ~30 min, while fst-Sm mRNA had a half-life of ~90 min. Both fst-Sm and srSm RNAs were transcribed across direct tandem repeats providing a region of complementarity for inhibition of toxin translation. Overproduction of Fst-Sm had a toxic effect on *E. coli* and *S. mutans* cells which can be neutralized by coexpression of srSm RNA. Deletion of fst-Sm/srSm locus or overexpression of Fst-Sm/srSm had no effect on *S. mutans* cell growth in liquid medium and no differences in the total biofilm biomass were noted. In contrast, mild-overproduction of Fst-Sm/srSm type I TA system decreases the levels of persister cells tolerant to bacterial cell wall synthesis inhibitors.

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**Introduction**

Prokaryotic chromosomes contain small genetic elements encoding two components: a stable toxin and its less stable cognate antitoxin. These modules are called toxin-antitoxin (TA) systems [1]. Typically, the toxin inhibits an essential microbial cellular function. TA pairs form a stabilized complex in the cell preventing toxicity under normal growth conditions. However, when the balance between the toxin and antitoxin is perturbed, usually following cellular damage or stressful conditions, the toxin is released from the TA complex leading to cell growth arrest and/or cell death. The physiological significance and importance of TA modules in microbial physiology is reflected by the fact that they are ubiquitously found on the chromosome of bacteria [2]. Five different types of bacterial TA systems have been described thus far, depending on the nature and mode of action of the antitoxin component. While toxins are always proteins, antitoxins are either RNAs (type I, type III) or proteins (type II, type IV, type V). RNA antitoxins suppress toxin expression (type I) [3–5] or interfere with the toxin activity instead of preventing its expression (type III) [6,7]. Protein antitoxins neutralize the toxicity of the cognate toxin by forming a stable complex (type II) [8–10], by functioning as antagonists for the toxin activity (type IV) [11,12], or by inhibiting the toxin by cleaving specifically its mRNA (type V) [13]. Current hypotheses propose that TAs are stress-response elements that help bacteria cope with environmental stress either by promoting altruistic death of a subpopulation or by inducing formation of dormant persister cells [14–16]. The dormant state allows bacteria to survive even high doses of the antibiotic. It has been suggested that persister cells may play a significant role in the recalcitrance of chronic biofilm infections to antimicrobials [17]. In *E. coli*, multiple type II TA systems have been linked to the formation of persister cells [18,19].

The oral cavity environment is inarguably a dynamic and complex ecosystem with a wide range of environmental challenges. Our model organism is the oral pathogen *Streptococcus mutans*. *S. mutans* depends on a biofilm lifestyle for its survival and persistence in its natural habitat [20]. In presence of fermentable dietary carbohydrates, this acid-producing bacterium can cause damage (cavities) to the tooth’s hard tissues [21,22]. In fact, *S. mutans* is a major human pathogen that infects more than half of the world’s human population. We recently characterized a locus encoding a functional type II TA system, the *S. mutans* chromosomal MazEF module. We demonstrated that MazF protein was a toxic nuclease arresting cell growth through the mechanism of RNA cleavage, and that MazE antitoxin protein inhibited the ribonuclease activity of MazF. In this report, we describe a previously unannotated type I TA system that is present in the oral pathogen *S. mutans*.
activity of MazF by forming a protein complex [23]. Our most recent data showed that ectopic mild overexpression of chromosomal MazEF and RelBE type II TA systems induced formation of multidrug tolerant persister cells in S. mutans [24]. In this study, we are pursuing our investigation of the chromosomal TA systems in S. mutans. We were interested in discovering type I TA systems in the chromosome of S. mutans. In type I TA systems, antitoxins are small untranslated RNAs acting as antisense RNAs. RNA antitoxins can be encoded directly opposite the coding sequence of the toxin, opposite the 5’ UTR, or opposite the 3’ UTR of the toxin mRNA, or even divergent to the toxin gene but with long stretches of complementarity to the toxin mRNA [3]. The RNA antitoxins are acting as antisense RNAs that anneal with the corresponding toxin mRNA, thereby inhibiting its translation or promoting its degradation. All type I toxins are small, hydrophobic peptides of 19–38 amino acid residues. Overexpression of these hydrophobic toxins has been shown to lead to membrane depolarization or membrane disruption resulting in a loss of cell viability [3]. Type I TA modules were first discovered on plasmids where they were found to stabilize various plasmids in Gram-negative bacteria (post-segregational killing activity) [25]. The RNAI-RNAII encoded by the par locus of the Enterococcus faecalis plasmid pAD1 was the first type I TA system identified in Gram-positive bacteria. RNAI

Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristic(s)* | Source or reference |
|-------------------|-----------------------------|---------------------|
| **Strains**        |                             |                     |
| S. mutans         |                             |                     |
| UA159             | Wild-type S. mutans reference strain | Lab stock           |
| ΔIGR176 mutant    | In-frame IGR176 deletion mutant derived from S. mutans UA159; Em' | This study          |
| UA159(pIB166)     | UA159 harboring pIB166; Cm' | [23]                |
| ΔIGR176(pIB166)   | ΔIGR176 harboring pIB166; Em', Cm' | This study          |
| ΔIGR176(pSK10)    | ΔIGR176 harboring pSK10; Em', Cm' | This study          |
| **E. coli**       |                             |                     |
| DH10B             | Host for cloning and plasmid production | Invitrogen          |
| TOP10             | Host for cloning and plasmid production | Invitrogen          |
| LMG194            | Host strain for pBAD expression | Invitrogen          |
| LMG194(pSK1)      | LMG194 harboring pSK1; Km' | This study          |
| LMG194(pSK2)      | LMG194 harboring pSK2; Km' | This study          |
| LMG194(pSK8)      | LMG194 harboring pSK8; Km' | This study          |
| DH10B(pSK3)       | DH10B harboring pSK3; Km' | This study          |
| DH10B(pSK7)       | DH10B harboring pSK7; Km' | This study          |
| DH10B(pHSG299)(pSK3) | DH10B harboring pHSG299, pSK3; Cm' | This study          |
| DH10B(pHSG299)(pSK4) | DH10B harboring pHSG299, pSK4; Km' | This study          |
| DH10B(pHSG299)(pSK5) | DH10B harboring pHSG299, pSK5; Km' | This study          |
| DH10B(pSK3)(pSK6) | DH10B harboring pSK3, pSK6; Cm' Km' | This study          |
| DH10B(pSK4)(pSK6) | DH10B harboring pSK4, pSK6; Cm' Km' | This study          |
| DH10B(pSK5)(pSK6) | DH10B harboring pSK5, pSK6; Cm' Km' | This study          |
| **Plasmids**      |                             |                     |
| pBAD202/D-TOPO    | Expression vector linearized and topoisomerase-activated; Km' | Invitrogen          |
| pHSG299           | High-copy-number cloning vector; Km' | Takara Bio USA     |
| pPROBE-NT'        | Promoterless GFP vector; Km' | [32]                |
| pIB166            | Streptococcus-E. coli shuttle plasmid; Cm' | [33]                |
| pSK1              | Fst-Sm cloned under the control of araBAD promoter into pBAD202/D-TOPO; Km' | This study          |
| pSK2              | Fst-Sm/srSm cloned under the control of araBAD promoter into pBAD202/D-TOPO; Km' | This study          |
| pSK3              | GFP cassette cloned into pIB166; Cm' | This study          |
| pSK4              | PnαDRI-II transcriptionally fused to gfp into pSK3; Cm' | This study          |
| pSK5              | Pnα transcriptionally fused to gfp into pSK3; Cm' | This study          |
| pSK6              | srSm cloned into pHSG299; Km' | This study          |
| pSK7              | Fst-Sm G16A mutation into pSK1; Km' | This study          |
| pSK8              | Fst-Sm A11G/G16A mutation into pSK1; Km' | This study          |
| pSK9              | Fst-Sm cloned under the control of its own promoter into pIB166; Cm' | This study          |
| pSK10             | Fst-Sm and srSm cloned under the control of their own promoter into pIB166; Cm' | This study          |

* Em', erythromycin resistance; Cm', chloramphenicol resistance; Km', kanamycin resistance.
doi:10.1371/journal.pone.0054291.t001
encodes a 33-aa peptide toxin (Fs\(\text{t}\)), while RNAII codes for the ~70-nt regulatory antisense RNA [26,27]. The par addiction module kills plasmid-free cells by affecting membrane permeability and cell division [28,29]. Recently, Weaver et al. (2009) [30] identified pAD1-like TA systems on the chromosomes and plasmids of Enterococcus, Lactobacillus, and Staphylococcus species suggesting that Fs\(\text{t}\)-like toxin may be prevalent in Gram-positive bacteria.

Our results showed that the genome of \(S.\) \textit{mutans} UA159 reference strain harbors an unannotated Fs\(\text{t}\)-like toxin based on the presence of a conserved open reading frame. Examination of the genetic context revealed conserved features similar to the Fs\(\text{t}\) type I TA system. The aim of this study was to investigate the functionality of this putative \(S.\) \textit{mutans} type I TA system; we proposed the name Fs\(\text{t}\)-Sm/srSm for \(S.\) \textit{mutans} functionality of this putative type I TA system. The aim of this study was to investigate the possibility of cloning Fs\(\text{t}\)-Sm/srSm in-frame upstream from the His\(\text{6}\) sequence under control of the araBAD promoter for expression in \(E.\) \textit{coli} DH10B or TOP10. The recombinant plasmid pSK3 was confirmed by restriction digestion. To construct the fs\(\text{t}\)-Sm promoter reporter fusion constructs (P\(\text{fs\(\text{t}\)}\text{-DRI/II and P\(\text{fs\(\text{t}\)}\)}\text{-DRII}), the promoter region of fs\(\text{t}\)-Sm with and without the direct repeats (DRI+DRII) was PCR amplified using UA159 gDNA as template and the primer pairs CMT-596/CMT-597 and CMT-598/CMT-599, respectively. The PCR products were double digested with EcoRI/ KpnI and cloned into pSK3 precut with the same enzymes to generate the recombinant plasmids pSK4 (P\(\text{fs\(\text{t}\)}\text{-DRI/II}) and pSK5 (P\(\text{fs\(\text{t}\)}\text{-DRII}). To construct the srSm RNA expression vector, the srSm locus was PCR amplified using UA159 gDNA and the primer pair CMT-599/CMT-600. The PCR product was double digested with BamHI/EcoRI, purified, and cloned into pHGS299 precut by the same enzymes. The recombinant plasmid pSK6 was confirmed by sequencing.

\textbf{Materials and Methods}  

\textbf{Bacterial Growth Conditions}  
\(S.\) \textit{mutans} strains were grown in Todd-Hewitt broth supplemented with 0.5\% yeast extract (THYE) and incubated statically at 37\(^{\circ}\)C in a 5\% CO\(_2\) atmosphere. \(E.\) \textit{coli} strains were cultivated aerobically in Luria-Bertani (LB) medium at 37\(^{\circ}\)C. When needed, antibiotics were used as follows: chloramphenicol (20 \mu g/ml) or kanamycin (50 \mu g/ml) for \(E.\) \textit{coli}, and erythromycin (10 \mu g/ml), or chloramphenicol (10 \mu g/ml) for \(S.\) \textit{mutans}. Cell growth was monitored through optical density at 600 nm (OD\(_{600}\)). Cell viability was assessed by counting CFU on replica agar plates.

\textbf{Plasmid and Strain Construction}  
A summary of bacterial strains and plasmids is provided in Table 1. Primers used for the generation of PCR products indicated below are listed in Table 2. A non-polar insertion-deletion IGR176 mutant (\textit{AgIR176}) was constructed in \(S.\) \textit{mutans} UA159 wild-type (WT) strain by PCR ligation mutagenesis using the primer pairs CMT-576/CMT-577 and CMT-578/CMT-579. All plasmids were constructed in \(E.\) \textit{coli} strain DH10B or TOP10. Plasmids were introduced into \(E.\) \textit{coli} by transformation using electroporation or chemical transformation. Plasmids were transferred to \(S.\) \textit{mutans} by natural transformation as described previously [31].

\textbf{(i) Construction of expression vectors for induction in \(E.\) \textit{coli}.}  
To generate inducible expression constructs for induction of fs\(\text{t}\)-Sm and fs\(\text{t}\)-Sm/srSm in \(E.\) \textit{coli}, fragments containing the open reading frame of fs\(\text{t}\)-Sm and fs\(\text{t}\)-Sm/srSm were cloned into pBAD202/D-TOPO vector. The PCR products were double digested with BamHI/EcoRI, purified, and cloned into pBAD202/D-TOPO vector (Invitrogen). To confirm transcription of the putative Fs\(\text{t}\)-Sm toxin, reverse transcription-PCR (RT-PCR) was performed using the primers CMT-497 and CMT-498 (Table 2). Total RNA was isolated from UA159 WT strain cultures at early log (OD\(_{600}\) ~ 0.1), mid-log (OD\(_{600}\) ~ 0.5), and early stationary (OD\(_{600}\) ~ 1.5) phases using TRIzol reagent (Invitrogen), DNase treated with RQ1 DNase and stored at -80\(^{\circ}\)C. 1\% RNA was reverse transcribed using M-MLV reverse transcriptase (Promega) and subjected to PCR amplification using the primers CMT-599/CMT-600. The PCR products were double digested with EcoRI/KpnI and cloned into pSK6 precut with the same enzymes to generate the recombinant plasmid pSK7. The clone pSK7 was then electrotransformed into LMG194 cells for induction by arabinose.

\textbf{(ii) Construction of a non-toxic Fs\(\text{t}\) peptide.}  
A non-toxic Fs\(\text{t}\) peptide was generated by site-directed mutagenesis using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer’s recommendations. Briefly, plasmid pSK1 carrying the wild-type copy of fs\(\text{t}\)-Sm gene was used as a template for the replacement of G16A using the two mutagenic PCR primers CMT-621 and CMT-622. The clone used to produce Fs\(\text{t}\)-Sm G16A mutant was designated pSK7 and was confirmed by sequencing. The clone pSK7 was next used as a template with the two mutagenic PCR primers CMT-627 and CMT-628 for the replacement of A11G. The clone used for production of Fs\(\text{t}\)-Sm G16A A11G mutant was designated pSK8 and was confirmed by sequencing. The clone pSK8 was then electrotransformed into LMG194 cells for induction by arabinose to produce the recombinant non-toxic Fs\(\text{t}\) peptide (rNT-Fs\(\text{t}\)).

\textbf{(iii) Construction of GFP reporter constructs.}  
First, a promoterless GFP vector was constructed by PCR amplifying the promoterless gfp cassette flanked by its upstream and downstream terminator sequences using pPROBE-NT\footnote[32]{32} as a template and the primer pair CMT-449/CMT-450. The PCR product was purified, double digested with XhoI/SacII, and then cloned into the shuttle vector pB166\footnote[33]{33} pre-cut by the same enzymes. The recombinant plasmid pHGS299 was confirmed by sequencing. The recombinant plasmid pHGS299 was used to produce Fst-Sm G16A mutant was designated pHGS299.

\textbf{(iv) Construction of vectors for expression in \(S.\) \textit{mutans}.}  
The full-length coding region and promoter region of fs\(\text{t}\)-Sm and the fs\(\text{t}\)-Sm/srSm locus were PCR amplified using UA159 gDNA as a template and the primer pairs CMT-596/CMT-597 and CMT-598/CMT-599, respectively. The PCR products were double digested with EcoRI/HpaI and cloned into pSK3 precut with the same enzymes to generate the recombinant plasmids pSK4 (P\(\text{fs\(\text{t}\)}\text{-DRI/II}) and pSK5 (P\(\text{fs\(\text{t}\)}\text{-DRII}). To construct the srSm RNA expression vector, the srSm locus was PCR amplified using UA159 gDNA and the primer pair CMT-599/CMT-600. The PCR product was double digested with BamHI/EcoRI, purified, and cloned into pB166 precut with the same enzymes. The recombinant plasmids pSK9 (fs\(\text{t}\)-Sm in pB166) and pSK10 (fs\(\text{t}\)-Sm/srSm in pB166) were sequenced on both strands for verification.
was reverse transcribed by using the RACE outer primers CMT-583 and CMT-585 for \( \text{fst-Sm} \) gene and \( \text{srSm} \) RNA, respectively, and RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to supplier’s instructions. RNase H and RNase T1 (Ambion) were then added, followed by incubation at 37°C for 30 min. The cDNAs were purified by using a GeneJET PCR Purification Kit (Fermentas) according to the manufacturer’s instructions. Tailing of purified cDNA was performed using the terminal deoxynucleotidyl transferase (Invitrogen) and dGTP/dTTP according to the manufacturer’s instructions. Tailed cDNAs were then PCR amplified by using the RACE universal primers CMT-180 (Poly-G tail) and CMT-181 (Poly-T tail), and the RACE inner primers CMT-584 (\( \text{fst-Sm} \) gene) and CMT-586 (\( \text{srSm} \) RNA). The amplicons were analyzed by agarose gel electrophoresis, and sequenced using the RACE inner primers CMT-584 and CMT-586.

### Northern Blot Analysis

Total RNA was isolated from UA159 WT strain cultures at early log (OD\(_{600}\) ~ 0.1), mid-log (OD\(_{600}\) ~ 0.5), and early stationary phase using RNAzol B isolation reagent (Biogenesis) according to the manufacturer’s instructions.

### Table 2. Primers used in this study.

| Primer | Gene       | Sequence (5’→3’)\(^{a,b}\) | Purpose                  |
|--------|------------|-----------------------------|--------------------------|
| CMT-576| \( \text{fst-Sm/srSm} \) | GCACATTGGGAATCTGGAGA | Gene deletion              |
| CMT-577| \( \text{fst-Sm/srSm} \) | GCCGCCCAAGCTGCCGTTTATCCGCA | Gene deletion              |
| CMT-578| \( \text{fst-Sm/srSm} \) | GGCCGCCATATCTATATGTTGCC | Gene deletion              |
| CMT-579| \( \text{fst-Sm/srSm} \) | GCTATTATGTTGCC | Gene deletion              |
| CMT-19 | \( \text{erm} \) | GCCGCCGCGCGCCGAAAAATTTGGTAT | Gene deletion              |
| CMT-20 | \( \text{erm} \) | GCCGCCGCGCGCCGAAAAATTTGGTAT | Gene deletion              |
| CMT-499| \( \text{fst-Sm} \) | CACCATGTCGATTTTGATCATAATTA | pBAD cloning              |
| CMT-500| \( \text{fst-Sm} \) | ATCGTCGTCTTTCTTATCCA | pBAD cloning              |
| CMT-581| \( \text{fst-Sm/srSm} \) | CACCAGTTGCCAAATGTGGCATAAT | pBAD cloning              |
| CMT-582| \( \text{fst-Sm/srSm} \) | TTGACAAAACAATAAAAGA | pBAD cloning              |
| CMT-621| \( \text{fst-Sm} \) | CGCACCACATTTTTGCCGATAGGCTGTTCGCTAT | Mutagenesis              |
| CMT-622| \( \text{fst-Sm} \) | AYAGCACCACATTTTTGCCGATAGGCTGTTCGCTAT | Mutagenesis              |
| CMT-627| \( \text{srSm} \) | CATAATCTCTTTATATATCTGCGACCACATATTGTCGATA | Mutagenesis              |
| CMT-628| \( \text{srSm} \) | CACATTGCGAACAAATTTGGTCCGACATATATA | Mutagenesis              |
| CMT-449| \( \text{gfp} \) | CTCCGGGTCTCAGAGCTTTGGAACGCC | GFP reporter              |
| CMT-450| \( \text{gfp} \) | GCCGGGACCTGCTACATATGATTTAGCCG | GFP reporter              |
| CMT-596| \( \text{fst-Sm} \) | GAAATTCGACTGAAATTTATGAAAC | GFP reporter; \( \text{fst-Sm, fst-Sm/srSm} \) expression |
| CMT-597| \( \text{fst-Sm} \) | GGTACCATATATATAAGGAAATTTATG | GFP reporter              |
| CMT-598| \( \text{fst-Sm} \) | GTACCATATATATGATGTCATGAGGC | GFP reporter              |
| CMT-599| \( \text{srSm} \) | GCATTTATGCGACAAACAAAAATTTGG | GFP reporter; \( \text{srSm} \) expression |
| CMT-600| \( \text{srSm} \) | GAATTCGACATATATGATGTCATGAGGC | GFP reporter; \( \text{srSm} \) expression |
| CMT-597| \( \text{fst-Sm} \) | GGTACCATATATATAAGGAAATTTATG | GFP reporter              |
| CMT-598| \( \text{fst-Sm} \) | GTACCATATATATGATGTCATGAGGC | GFP reporter              |
| CMT-599| \( \text{srSm} \) | GCATTTATGCGACAAACAAAAATTTGG | GFP reporter; \( \text{srSm} \) expression |
| CMT-600| \( \text{srSm} \) | GAATTCGACATATATGATGTCATGAGGC | GFP reporter; \( \text{srSm} \) expression |
| CMT-597| \( \text{fst-Sm} \) | GGTACCATATATATAAGGAAATTTATG | GFP reporter              |
| CMT-598| \( \text{fst-Sm} \) | GTACCATATATATGATGTCATGAGGC | GFP reporter              |
| CMT-599| \( \text{srSm} \) | GCATTTATGCGACAAACAAAAATTTGG | GFP reporter; \( \text{srSm} \) expression |
| CMT-600| \( \text{srSm} \) | GAATTCGACATATATGATGTCATGAGGC | GFP reporter; \( \text{srSm} \) expression |
| CMT-449| \( \text{gfp} \) | ATGTGGCATAATTTCTTTATATA | RT-PCR; Northern blot |
| CMT-627| \( \text{srSm} \) | CATAATCTCTTTATATATCTGCGACCACATATTGTCGATA | Mutagenesis              |
| CMT-449| \( \text{gfp} \) | ATGTGGCATAATTTCTTTATATA | RT-PCR; Northern blot |
| CMT-596| \( \text{fst-Sm} \) | GAAATTCGACTGAAATTTATGAAAC | GFP reporter; \( \text{fst-Sm, fst-Sm/srSm} \) expression |
| CMT-597| \( \text{fst-Sm} \) | GGTACCATATATATAAGGAAATTTATG | GFP reporter              |
| CMT-598| \( \text{fst-Sm} \) | GTACCATATATATGATGTCATGAGGC | GFP reporter              |
| CMT-599| \( \text{srSm} \) | GCATTTATGCGACAAACAAAAATTTGG | GFP reporter; \( \text{srSm} \) expression |
| CMT-600| \( \text{srSm} \) | GAATTCGACATATATGATGTCATGAGGC | GFP reporter; \( \text{srSm} \) expression |
| CMT-597| \( \text{fst-Sm} \) | GGTACCATATATATAAGGAAATTTATG | GFP reporter              |
| CMT-598| \( \text{fst-Sm} \) | GTACCATATATATGATGTCATGAGGC | GFP reporter              |
| CMT-599| \( \text{srSm} \) | GCATTTATGCGACAAACAAAAATTTGG | GFP reporter; \( \text{srSm} \) expression |
| CMT-600| \( \text{srSm} \) | GAATTCGACATATATGATGTCATGAGGC | GFP reporter; \( \text{srSm} \) expression |
| CMT-449| \( \text{gfp} \) | ATGTGGCATAATTTCTTTATATA | RT-PCR; Northern blot |
| CMT-627| \( \text{srSm} \) | CATAATCTCTTTATATATCTGCGACCACATATTGTCGATA | Mutagenesis              |
| CMT-449| \( \text{gfp} \) | ATGTGGCATAATTTCTTTATATA | RT-PCR; Northern blot |
| CMT-596| \( \text{fst-Sm} \) | GAAATTCGACTGAAATTTATGAAAC | GFP reporter; \( \text{fst-Sm, fst-Sm/srSm} \) expression |
| CMT-597| \( \text{fst-Sm} \) | GGTACCATATATATAAGGAAATTTATG | GFP reporter              |
| CMT-598| \( \text{fst-Sm} \) | GTACCATATATATGATGTCATGAGGC | GFP reporter              |
| CMT-599| \( \text{srSm} \) | GCATTTATGCGACAAACAAAAATTTGG | GFP reporter; \( \text{srSm} \) expression |
| CMT-600| \( \text{srSm} \) | GAATTCGACATATATGATGTCATGAGGC | GFP reporter; \( \text{srSm} \) expression |

\(^{a}\)Restriction sites are underlined.

\(^{b}\)Modified residues are shown in bold and underlined.

*doi:10.1371/journal.pone.0054291.t002*
(OD$_{600}$ ~ 1.5), and late stationary (OD$_{600}$ ~ 1.1) phases using TRIzol reagent (Invitrogen). Ten micrograms of total RNA was loaded by lane and resolved on a 12% (wt/vol) polyacrylamide denaturing gel containing 8 M urea. Size-fractionated RNA was transferred to a positively charged nylon membrane (Roche) using a Bio-Rad Mini Trans-Blot Cell and subjected to UV cross-linking. Membranes were pre-hybridized for 30 min at 42°C and followed by hybridization with biotin-labeled DNA probes (1 ng/ml for PCR probe and 10 ng/ml for oligoprobes) in ULTARAbase hybridization buffer (Ambion) at 42°C overnight. The fis-Sm probe (99 bp DNA fragment) was PCR amplified from UA159 gDNA using CMT-497 and CMT-498 primers (Table 2) and labeled with Psoralen-biotin using a BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion) according to supplier’s instructions. The biotin-labeled DNA oligoprobes CMT-558 and CMT-572 (Table 2) directed against the 5’ and 3’ UTR of srSm RNA, respectively, were purchased from Integrated DNA Technologies (Coralville, IA). The BrightStar BioDetect Kit (Ambion) was used for the detection of fis-Sm mRNA and srSm RNA following the manufacturer’s instructions.

RNA Half-life Determination

An overnight culture of S. mutans UA159 WT strain was diluted (1:20) into fresh THYE broth and incubated at 37°C until mid-log phase (OD$_{600}$ ~ 0.5) was reached. Rifampicin was added to a final concentration of 300 μg/ml and total RNA was isolated from 20-mI aliquots of culture taken at 0, 2, 5, 10, 15, 20, 40, 60, 90, 120, and 150 min after the addition of rifampicin. Northern blotting was performed as described above. For the detection of fis-Sm mRNA and 5S rRNA, probes were PCR amplified from UA159 gDNA using the primer pairs CMT-497/CMT-498 and CMT-672/CMT-673, respectively, and labeled with Psoralen-biotin using a BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion) according to supplier’s instructions. For the detection of srSm RNA, the biotin-labeled DNA oligoprobe CMT-558 purchased from Integrated DNA Technologies (Coralville, IA) was used. Quantification of the signal was performed using the ChemiDoc XRS System and Quantity One software provided by Bio-Rad.

Toxicity Assays in E. coli

Overnight cultures of LMG194(pSK1), LMG194(pSK2), and LMG194(pSK8) were diluted (1:100) into fresh LB medium supplemented with kanamycin at 50 μg/ml and grown aerobi-cally at 37°C. At an OD$_{600}$ of ~0.5, 0.2% (wt/vol) glucose (uninduced control) or 0.002% (wt/vol) arabinose was added to induce the fimbria fimbriae. Overnight cultures of E. coli strains harboring the GFP reporter constructs were diluted (1:100) into fresh LB broth supplemented with chloramphenicol and kanamycin. Cells were grown aerobi-cally at 37°C until an OD$_{600}$ of ~0.4 was reached (~6×10^7 CFU/ml). Cells (2-ml aliquots) were harvested by centrifu-gation, washed once with sterile phosphate-buffered saline (PBS), and resuspended in 1 ml of sterile PBS. Green protein fluores-cence was detected in terms of relative fluorescence units (RFU) with a fluorescence spectrophotometer. The excitation and emission filters were 485 nm and 511 nm. All experiments were performed in triplicate from two independent experiments.

Persistence Assay

Overnight cultures of S. mutans (WT vs. ΔIGR176; WT[pIB166] vs. ΔIGR176[pSK10]) were diluted (1:20) into fresh THYE broth containing oxacillin (2 μg/ml), cefotaxime (2 μg/ml), or vanco-mycin (20 μg/ml) and incubated for 24 h at 37°C. Aliquots were removed at the indicated times, serially diluted, and plated on THYE agar plates. The colonies were counted after 48 h of incubation. All assays were performed in triplicate from three independent experiments.

Results

Detection of RNAs from the Intergenic Region IGR176

A study by Weaver et al. (2009) [30] previously identified pAD1-like TA systems on the chromosomes and plasmids of Enterococcus, Lactobacillus, and Staphylococcus species suggesting that Fst-like toxins may be prevalent in Gram-positive bacteria. Recently, an exhaustive PSI-BLAST and TBLAST searches across 774 bacterial genomes identified several homologs of type I toxins [4,5]. Analysis of the genome of S. mutans UA159 reference strain predicted a putative type I toxin belonging to the Fst family in the intergenic region IGR176. This small intergenic region (318 nt) is located between the genes SMU.219 and SMU.220 (Fig. 1A) encoding a Zn-dependent protease of the COG2836 family [8] and a hypothetical protein of unknown function, respectively. SMU.219 is mostly co-transcribed with an upstream gene, SMU.218, encoding an HTH domain-containing protein of the Xre family. The SMU.218/SMU.219 gene pair is predicted to function as bona fide type II TA system (TADB database: http://bioinfo.mml.sjtu.edu.cn/TADB/) [34].

Bioinformatic analysis revealed that the intergenic region IGR176 contains an unannotated open reading frame of 99 bp, which we named ORF176 (Fig. 1B). ORF176 encodes a putative peptide of 32 amino acid residues with a predicted MW of 3613.3 Da. The putative peptide showed homology to the Fst toxin family [4,5]. The APUU(A/V)GUU motif (where U equals C, A, G, or T) found in the hydrophobic region of the Fst peptide of 32 amino acid residues with a predicted MW of 3613.3 Da. The putative peptide showed homology to the Fst toxin family [4,5]. The APUU(A/V)GUU motif (where U equals C, A, G, or T) found in the hydrophobic region of the Fst toxin family [4,5].
sequencing (data not shown). To map the 5' end of ORF176, we performed 5'RACE-PCR. The apparent start site (+1) was located 7 nucleotides 3' proximal to a canonical –10 sequence (TATAAT). An obvious –35 sequence (TTGTTT) is also present spaced by 21 nucleotides from the –10 box (Fig. 1B). Taken together, these results provide genetic evidence for the presence of a previously unannotated \textit{fst}-like toxin gene in the intergenic region IGR176 of \textit{S. mutans}. We proposed the name \textit{fst-Sm} to designate the gene encoding a putative Fst-like toxin in \textit{S. mutans}, a predicted type I toxin.

We next hypothesized that the putative Fst-Sm toxin could be regulated post-transcriptionally by small RNA base pairing. Therefore, we performed a visual inspection of the DNA sequence immediately surrounding the \textit{fst-Sm} toxin gene and searched for a sequence that would generate an RNA that could potentially bind the \textit{fst-Sm} mRNA. A putative small RNA, which we named \textit{srSm} (\textit{small RNA} \textit{S. mutans}), was found directly opposite the coding sequence of \textit{fst-Sm} (Fig. 1B). The sequence was predicted to encode a small untranslated RNA as it contains potential –35 and –10 promoter elements but no open reading frame (ATG start codon) or ribosome binding site (RBS). To test further whether \textit{srSm} is an untranslated RNA, we searched for open reading frames preceded with an alternative translational start codon (GTG or TTG). The fact that the longest open reading frame was only 10 codons in length (using TTG as putative initiation codon) and no putative RBS could be identified, suggest that \textit{srSm} RNA is not translated.

Using 5'RACE-PCR, we next determined the transcriptional start site of \textit{srSm}. The \textit{srSm} RNA starts at the G position, 108 bp downstream from the +1 transcriptional start site of \textit{fst-Sm}. The regions encoding the DRI and DRII repeats are boxed. The primers CMT-497 and CMT-498 used in the RT-PCR experiments are underlined.

\textbf{Figure 1. Analysis of \textit{S. mutans} IGR176 region.} (A) Schematic representation of the location of the \textit{fst-Sm/srSm} locus on the \textit{S. mutans} chromosome. Arrows indicate the direction of transcription. The \textit{fst-Sm} and \textit{srSm} promoter sequences are indicated by \textit{P\textsubscript{fst-Sm}} and \textit{P\textsubscript{srSm}}, respectively. A predicted stem-loop bidirectional terminator is indicated between \textit{srSm} and \textit{fst-Sm}. Shown at the bottom are the boundaries of the intergenic region IGR176. (B) Nucleotide and amino acid sequences of the \textit{S. mutans} \textit{fst-Sm/srSm} type I TA locus located in the intergenic region IGR176 (from 211452 to 211769) of UA159 genome. The conserved APUU(A/V)GUU motif present in Fst-Sm peptide is boxed. Putative promoter sites of \textit{fst-Sm} toxin (–35, –10) and \textit{srSm} antitoxin (–10), ribosome binding site (RBS) of Fst-Sm toxin, and a factor-independent bidirectional terminator (double underlined) are indicated. The transcriptional start site (+1) of \textit{fst-Sm} and \textit{srSm} identified by 5’RACE-PCR are indicated below the sequence. The regions encoding the DRI and DRII repeats are boxed. The primers CMT-497 and CMT-498 used in the RT-PCR experiments are underlined. (C) Proposed RNA:RNA interactions (in shaded regions) between \textit{fst-Sm} mRNA and \textit{srSm} RNA.

doi:10.1371/journal.pone.0054291.g001
intergenic region IGR176 expresses the Sm and unannotated open reading frame, and a from the experimentally mapped are in agreement with the predicted size of the RNAs inferred detected by Northern blot analysis (Fig. 2B). The signals obtained specific to fst-Sm shown in Fig. 2A, transcripts specific to fst-Sm both detections. Interestingly, a smaller transcript was also observed for sizes of the transcripts produced from the fst-Sm of fst-Sm of S. mutans fst-Sm Consequently, the srSm transcript (Fig. 3A) suggesting that also indicated the presence of another RNA size in addition to the chromosomes. Northern blot analyses of were reported for regulatory sRNAs encoded by bacterial srSm systems [3,35]. In order to test whether consequently it has to be constantly produced to inhibit the toxin srSm life of fst-Sm was extracted and subjected to Northern blot analysis. The half- 90 min under the conditions tested, whereas long half-lives (20–60 min) were reported for regulatory sRNAs encoded by bacterial chromosomes [36,37]. Northern blot analyses of fst-Sm transcript also indicated the presence of another RNA size in addition to the full-length transcript (Fig. 3A) suggesting that fst-Sm mRNA undergoes specific processing, most likely affecting its RNA stability. These data indicate that the fst-Sm toxin mRNA is much more stable than its srSm antitoxin RNA. These results are consistent with the findings of previous studies on other type I TA systems [3,35].

Stability of the fst-Sm and srSm RNAs

The relative stabilities of both the toxin mRNA and antitoxin small RNA are an important aspect of TA regulation. In TA systems, the antitoxin is less stable than the toxin in the cell, and consequently it has to be constantly produced to inhibit the toxin [3,35]. In order to test whether srSm RNA is less stable and might therefore represent an antisense RNA antitoxin, we determined the half-life of fst-Sm mRNA and srSm RNA by Northern blotting. WT strain was cultivated in THYE medium until mid-log growth phase was reached. Rifampicin was then added to prevent the initiation of RNA synthesis, time samples were taken, and RNA was extracted and subjected to Northern blot analysis. The half-life of srSm RNA was determined to be ~30 min, while fst-Sm mRNA had a half-life of ~90 min under the conditions tested (Fig. 3). Antisense RNAs of bacterial plasmids generally exhibit very short half-lives (<2 min), whereas long half-lives (20–60 min) were reported for regulatory sRNAs encoded by bacterial chromosomes [36,37]. Northern blot analyses of fst-Sm transcript also indicated the presence of another RNA size in addition to the full-length transcript (Fig. 3A) suggesting that fst-Sm mRNA undergoes specific processing, most likely affecting its RNA stability. These data indicate that the fst-Sm toxin mRNA is much more stable than its srSm antitoxin RNA. These results are consistent with the findings of previous studies on other type I TA systems [3,35].

srSm RNA Expressed in Trans Represses GFP Expression

Our results suggest that fst-Sm/srSm locus encodes a type I TA system, in which srSm RNA antitoxin functions through an antisense base-pairing mechanism that results in inhibition of toxin translation. As both fst-Sm and srSm RNAs are transcribed across the direct tandem repeats DRI and DRII (5’ end mapping results, Fig. 1B), we hypothesized that srSm RNA (antisense) could bind to the DRI/DRII region in fst-Sm mRNA (sense) thereby controlling its expression (Fig. 1C). To test this hypothesis, we designed a two-plasmid gene reporter system to investigate whether the srSm RNA acts on the fst-Sm mRNA. The promoter of fst-Sm and its 5’ mRNA coding region with the DRI and DRII repeats were transcriptionally fused to gfp gene into the promoterless-GFP plasmid pSK3. The resulting recombinant plasmid, pSK4, was then introduced into an E. coli strain carrying the pHGS299 vector expressing srSm RNA (pSK6). Cells were grown until mid-log phase and fluorescence was measured. We reasoned that, if srSm
repressed GFP expression in this *E. coli* strain, it would indicate that srSm RNA acts directly on the fst-Sm mRNA. Indeed, when srSm was introduced in *E. coli* (pSK4), approximately two-fold repression of GFP occurred (Fig. 4). We next cloned the promoter of fst-Sm and its 5’ mRNA coding region without the DRI/II repeats in frame with gfp. The recombinant plasmid, pSK5, was then introduced into *E. coli* (pSK6) and fluorescence was measured. No repression was observed compared with an *E. coli* strain containing the empty vector (Fig. 4). Based on the above data, we can predict that srSm directly binds to the DRI/II repeats to inhibit Fst-Sm expression.

**Figure 3. RNA half-life determination.** Stability of fst-Sm mRNA (A) and srSm RNA (B) by Northern blot analysis. Total RNA was extracted from WT mid-log cells at the indicated times after addition of 300 μg/ml rifampicin. Time points of sampling are indicated above each lane. Biotin-labeled DNA probes were used for RNA detection. The probing for 5S RNA confirmed equal loading. Control RNA extraction represents total RNA extracted from cells cultivated without rifampicin at time-point 150-min (fst-Sm mRNA detection) and 60-min (srSm RNA detection). Blots shown represent results from three experiments. doi:10.1371/journal.pone.0054291.g003

Induced fst-Sm Expression Confers Toxicity in *E. coli*

The *S. mutans* fst-Sm/srSm locus was next investigated to determine whether it constitutes a functional type I TA system. The fst-Sm gene was cloned into the pBAD expression system for induction of gene expression using araBAD promoter dose-dependent regulation. The recombinant plasmid designated pSK1 was used to transform *E. coli* LMG194 strain. Overexpression of Fst-Sm resulted in inhibition of colony formation (data not shown). In cell viability assays, growth of *E. coli* was markedly affected when Fst-Sm was overexpressed from the araBAD promoter (Fig. 5). Overexpression of Fst-Sm resulted in a reduction of cell viability of ~ 90% and ~ 99% by 30 min and 45 min post-induction, respectively. By contrast, LMG194(pSK1) cultivated in presence of glucose (uninduced control) did not show any growth defects (Fig. 5). This indicated that Fst-Sm is a toxin. Furthermore, we constructed a plasmid for arabinose-inducible overexpression of a mutated Fst-Sm toxin. Single-site mutations of amino acid residues were introduced by two rounds of PCR in the conserved hydrophobic sequence of Fst toxin since this region of Fst has been shown to be important for Fst toxicity [30]. First, the conserved glycine at position 16 was changed to alanine. The alanine mutant (G16A) was then used to mutate alanine to glycine at position 11. The resulting pSK8 construction (G16A, A11G) was finally transferred to LMG194 strain and tested using our cell viability assay. As shown in Fig. 5, LMG194(pSK8) cultivated in liquid LB medium supplemented with arabinose did not present any growth defects and behaved similarly to the control curve (uninduced glucose control). These results highlight the importance of this conserved hydrophobic region in Fst-Sm toxicity.

**Figure 4. The srSm RNA represses GFP expression.** Fluorescence from plasmid-encoded DRI/II-gfp (pSK4) or ∆DRI/II-gfp (pSK5) transcriptional fusions was measured in *E. coli* DH10B carrying pHS5299 vector expressing srSm (pSK6) or the empty vector. The promoterless GFP vector (pSK3) was used as negative control. All experiments were performed in triplicate from two independent experiments. The means ± SDs are shown. doi:10.1371/journal.pone.0054291.g004

**Figure 5. Characterization of the Fst-Sm/srSm TA system in *E. coli*.** Cells of LMG194 containing pSK1 (Fst-Sm), pSK2 (Fst-Sm/srSm), and pSK8 (NT-Fst) were grown to mid-log phase, at which time arabinose (induced) and glucose (uninduced control) were added. After induction, appropriate dilutions were plated on LB agar for determination of the number of CFU per ml. The curves presented are the averages and standard deviations of results from three independent cultures. doi:10.1371/journal.pone.0054291.g005
We next measured the toxicity of Fst-Sm peptide when overexpressed in pBAD plasmid with its cis-encoded srSm RNA under the control of its own promoter. The effect of overexpressing Fst-Sm/srSm on growth was significantly less (~25% reduced cell viability by 30 min post-induction), but Fst-Sm still affected growth (~50% reduced cell viability by 3 h post-induction) most probably due to insufficient levels of srSm RNA transcribed from its own promoter (Fig. 5). Based on these results, we can conclude that Fst-Sm confers toxicity to E. coli and expression of its cis-encoded srSm is able to curb Fst toxicity.

**Overexpression of fst-sm is Lethal to S. mutans Cells**

We next measured the toxicity of Fst-Sm toxin in S. mutans. We designed our toxicity assay based on the natural transformation ability of S. mutans [23]. The individual fst-Sm gene and the fst-Sm/srSm locus were placed under the control of the constitutive P23 promoter into the plasmid pIB166. Overnight cultures of S. mutans WT strain and its ΔIGR176 mutant were diluted in fresh medium and grown to early log phase prior to addition of pIB166 (empty plasmid), pSK9 (fst-Sm in pIB166), or pSK10 (fst-Sm/srSm in pIB166). Cells were grown for a further 2.5 h before differential plating. Our results showed that WT and ΔIGR176 mutant cells were readily transformed with pIB166 and pIB166 bearing fst-Sm/srSm in the srSm locus. Indeed, the transformation efficiencies of WT and ΔIGR176 mutant for the empty vector were similar to the efficiencies obtained with the pSK10 construct (Table 3). This was not the case when the pSK9 construct was transferred to WT strain and ΔIGR176 mutant. The transformation efficiency of the WT strain was reduced by more than 1,000-fold for pSK9 bearing the fst-Sm toxin gene only, corresponding to less than 0.07% of the pIB166 control value. For ΔIGR176 mutant, less than 5 colonies were obtained when undiluted cells were plated (Table 3). The fact that the transformation efficiency was much higher for WT strain compared with the ΔIGR176 mutant for pSK9 (almost no colonies could be detected for ΔIGR176 after 48 h of incubation) suggested that the chromosomal copy of the srSm RNA (trans-encoded) in WT strain might confer some protective effects. These results clearly showed a growth-inhibitory effect of Fst-Sm on S. mutans cells and that the toxic effect elicited by Fst-Sm can be neutralized by coexpression of its srSm RNA. Hence, fst-Sm/srSm encodes a functional type I TA system in S. mutans.

**Deletion and Mild-overexpression of IGR176 Region has no Effect on S. mutans Cell Growth**

To study the cellular function of Fst-Sm/srSm system, we first investigated microbial growth kinetics of WT, ΔIGR176 mutant, and IGR176 complemented strains. The absence of Fst-Sm/srSm TA system or its mild-overexpression did not affect cell growth in liquid medium under the conditions tested (data not shown). No effect on cell growth was observed when S. mutans cells were exposed to extracellular recombinant Fst-Sm peptide at concentration up to 160 μg/ml. We next conducted a long-term survival assay using monocultures of WT and its ΔIGR176 mutant cultivated in a nutrient-rich medium (THYE) or a chemically defined medium (CDM). After 14 days, there was no difference in survival between WT and mutant strains under both nutrient growth conditions (data not shown).

**Ectopic Expression of Fst-Sm/srSm Type I TA Module Decreases Persister Cell Formation in S. mutans**

Persisters are phenotypic variants that are extremely tolerant to high concentrations of antibiotics. They make up a small part of the bacterial population [30]. In S. mutans, type II TA systems have been linked to persister formation. Indeed, mild-overexpression of the MazEF and RelBE type II TA modules resulted in an increase in multidrug-tolerant persister levels [24]. To examine whether the Fst-Sm/srSm type I TA system was also involved in persister cell formation, we first tested an IGR176 knockout mutant. As expected, deletion of Fst-Sm/srSm system did not affect the persister levels after challenge with a high dose of the cell wall synthesis inhibitors oxacillin, vancomycin, and cefotaxime (data not shown) due most likely to redundant pathways for bacterial persister formation. Similar results were obtained using the ΔmazEF and ΔrelBE deletion mutants [24], reinforcing the hypothesis that more than one single mechanism is responsible for persister formation. In contrast, the ectopic expression of Fst-Sm/srSm module caused a dramatic decrease in the number of persister cells. Our data showed that overexpression of Fst-Sm/srSm system decreased the number of oxacillin-tolerant (~145-fold decrease at 24 h), cefotaxime-tolerant (~240-fold decrease at 24 h), and vancomycin-tolerant (~50-fold decrease at 24 h) persisters (Fig. 6). The fact that the MIC values of oxacillin, cefotaxime, and vancomycin against ΔIGR176(pSK10) mutant were not different from those for the WT strain confirmed that the complemented strain was a true persister mutant.

**Discussion**

TA systems are small genetic modules that are widespread in the prokaryotic kingdom [4,8,9]. There are of five different types depending of the nature and mode of action of the antitoxin component. The type III, IV, and V were discovered very recently and are represented so far by unique examples [6,7,11–13]. While type II TAs are highly represented in bacterial chromosomes, little is known about the distribution of type I TA loci. One possible reason could be that all the toxins of the type I TAs consist of very small hydrophobic peptides [3] and gene annotation software frequently fails to identify short protein-coding genes in microbial genomes [39]. Moreover, the development of computational approaches to identify novel type I TA systems is challenging owing to the short, hydrophobic character of the toxins, and the

### Table 3. S. mutans toxicity assay based on natural competence.

| Construct Description | Transformation Efficiency ± SD* |
|-----------------------|---------------------------------|
| WT pIB166 Empty vector | (1.1 ± 0.6) × 10^9 ± (2.2 ± 0.6) × 10^2 |
| ΔIGR176 pIB166 | (2.2 ± 0.6) × 10^2 |
| pSK9 fst-Sm gene | (8.5 ± 1.5) × 10^3 |
| ΔIGR176(pSK9) vs. ΔIGR176(pIB166) | ~5 colonies |
| pSK10 fst-Sm/srSm locus | (7.0 ± 0.4) × 10^1 |

*The transformation efficiency was expressed as the percentage of chloramphenical-resistant transformants divided by the total number of recipient cells. All experiments were performed in triplicate from three independent experiments. Statistical significance: * WT(pSK9) vs. WT(pIB166); ** ΔIGR176(pSK9) vs. ΔIGR176(pIB166); SD, standard deviation.
of results from three independent cultures. The curves presented are the averages and standard deviations
plates, and the number of CFU per ml was determined from plate
counts. The curves presented are the averages and standard deviations
at the indicated time points, serially diluted, spot plated onto THYE agar
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\textit{ S. mutans
B
Cc
d), and vancomycin-treated (\textit{C}) cells were removed
Oxacillin-treated (\textit{C}) cells were removed
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investigate whether Fst-Sm toxicity is not known. Our data
discussion, we reported to our knowledge the first functional type I TA
module in the oral pathogen \textit{S. mutans}. These results are: i) \textit{fst-Sm}
encodes a small hydrophobic peptide belonging to the Fst toxin
family; ii) a cis-encoded small RNA was found converging towards the
effect on growth of \textit{S. mutans} suggests that \textit{fst-Sm} functions intracellularly since extracellular
mechanism of action of Fst-Sm toxicity is not known. Our data
discuss the antitoxin small RNAs [4]. In the present
study, we reported to our knowledge the first functional type I TA
system in streptococci. TAs are commonly described as constituents of the prokaryotic mobilome [4]. The chromosomal \textit{fst-Sm}
locus is located in a transposon-related genomic island of \textit{ S. mutans
UA159} strain suggesting that this type I TA module may have moved through horizontal gene transfer. IGR176 could not
be found in other sequenced \textit{S. mutans} genomes by searching public genomic sequence. This result was not surprising since transposon
element movement is one of the major causes for intraspecies sequence variations in the intergenic regions. Collectively our
results support that Fst-Sm/srSm forms a functional type I TA pair in the oral pathogen \textit{ S. mutans}. These results are: i) \textit{fst-Sm}
encodes a small hydrophobic peptide belonging to the Fst toxin family; ii) a cis-encoded small RNA was found converging towards the
end of the toxin gene; iii) \textit{srSm} antitoxin RNA is less stable than \textit{fst-Sm} mRNA; iv) Fst-Sm has a toxic effect on \textit{ S. mutans} which can be
neutralized by expression of \textit{srSm} antitoxin; v) both \textit{fst-Sm} mRNA and \textit{srSm} RNA are transcribed across direct tandem repeats
providing a region of complementarity between the \textit{srSm} antitoxin RNA and the translation initiation region of \textit{fst-Sm} mRNA;
v) \textit{srSm} RNA most likely binds to the direct tandem repeats in the \textit{fst-Sm} mRNA occluding Fst-Sm start codon. Although our results using the GFP constructs suggest that pairing of \textit{srSm} RNA with the 5’ end of \textit{fst-Sm} mRNA suppresses toxin translation, additional experiments will be required to determine if this pairing leads to translational silencing or mRNA degradation. It is also possible that degradation is secondary to translation inhibition [40,41]. Indeed, the \textit{srSm}/\textit{fst-Sm} duplex is a good target for cleavage by RNase III, an endoribonuclease specific for double stranded RNA. Attempts to construct a \textit{S. mutans} RNase III mutant were unsuccessful, suggesting that this may be an essential gene in \textit{S. mutans}.

The toxic phenotype of many chromosomal type I toxins have been reported upon overexpression from a multicopy plasmid. So far all type I toxins characterized from \textit{E. coli} are predicted to function through the same mechanism as phage holins, forming a pore to destroy the membrane potential and to inhibit ATP synthesis [42–45]. In \textit{E. faecalis}, the intracellular overproduction of the plasmid-encoded Fst toxin compromised the integrity of the cell membrane and specific defects in chromosome partitioning and cell division were also observed [28,29]. At present, the mechanism of action of Fst-Sm toxicity is not known. Our data suggest that Fst-Sm functions intracellularly since extracellular addition of recombinant Fst-Sm toxin to cells in cultures had no effect on growth of \textit{S. mutans} and \textit{E. coli}. Preliminary examination of Fst-induced \textit{E. coli} cells by scanning electron microscopy showed no evidence of ghost cell formation or any other abnormal cellular morphology compared with the non-toxic Fst-induced cells (data not shown). Further experiments will be required to identify the primary target of Fts-Sm toxin in its native host. For instance, construction of a tightly regulated inducible expression system for \textit{S. mutans} will be necessary to study the effects of Fst-Sm induction on \textit{S. mutans} cellular morphology. It would also be interesting to investigate whether Fst-Sm toxin could affect cell division by inhibiting the polymerization of \textit{S. mutans} cytoskeleton proteins (e.g., FtsQ, FtsA, FtsZ) in future studies.

The physiological function of chromosomal TA systems remains unclear. In fact, the functions of chromosomal TAs are diverse and may depend on the type of TA, its genomic location, and host species. Several roles have been discovered for type II TA systems, including stress survival, growth control, programmed cell death, persister formation, stabilization of the genome, biofilm formation, phage abortive infection, and anti-addiction system [1,2,13,46]. We previously demonstrated that ectopic mild-overexpression of type II TA systems generated greater number of persister cells in \textit{S. mutans}. The picture that emerged from our work and from several other studies is clearly pointing out to a multi-gene function and suggests that \textit{S. mutans} did not evolve a dedicated mechanism allowing it to adopt a persistence phenotype [24]. Not surprisingly, the deletion of \textit{fst-sm}/\textit{srSm} locus did not affect the persister levels.
reservoir of viable bacteria that can acquire resistance by random mutation or horizontal gene transfer [17], the results from this study reinforce the idea that TA systems represent an attractive target for designing new drugs that could kill persister cells that have woken up. The clinical implications of TA systems and persister cell formation in the context of chronic infections are thus highly significant.

Acknowledgments
We thank Delphine Dufour and Vincent Leung for careful reading of the manuscript. CML is a recipient of a Canada Research Chair.

Author Contributions
Conceived and designed the experiments: SK CML. Performed the experiments: SK. Analyzed the data: SK CML. Contributed reagents/materials/analysis tools: CML. Wrote the paper: SK CML.

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