ACCESS MICROBIOLOGY

RESEARCH ARTICLE
Tian *et al.*, *Access Microbiology* 2019;1
DOI 10.1099/acmi.0.000054

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ClpP is required for proteolytic regulation of type II toxin–antitoxin systems and persister cell formation in *Streptococcus mutans*

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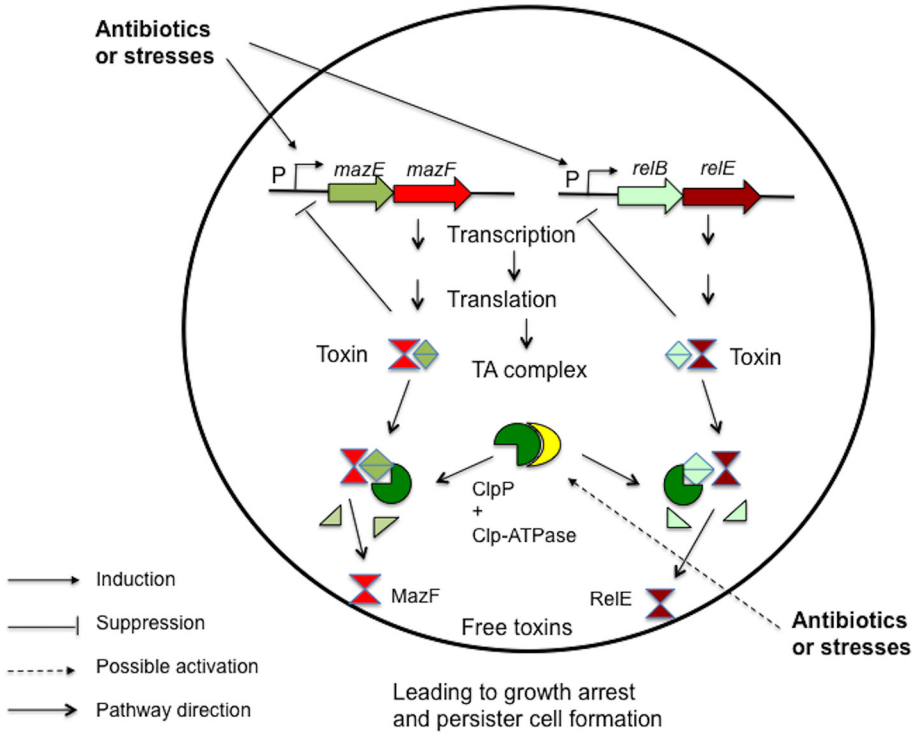

Graphical abstract

Transcriptional and proteolytic regulation of the type II toxin–antitoxin systems, MazEF and RelBE, and persister cell formation of *Streptococcus mutans* in response to antibiotics or stresses.

Abstract

The type II toxin–antitoxin (TA) modules, mazEF and relBE, in *Streptococcus mutans* have been implicated in stress response, antibiotic tolerance and persister cell formation. However, how *S. mutans* regulates these systems to prevent unwanted toxin activation and persister cell formation is unclear. In this study, we provide evidence that ClpP is required for the proteolytic regulation of these TA systems and persister cell formation in *S. mutans* following antibiotic challenge. A persister viability assay showed that *S. mutans* UA159 (WT) formed a larger quantity of persister cells than its isogenic mutant ΔclpP following antibiotic challenge. However, the *lux* reporter assay revealed that clpP deletion did not affect the transcriptional levels of mazEF and relBE, since no significant differences (P>0.05) in the reporter activities were detected between the wild-type and ΔclpP background. Instead, all antibiotics tested at a sub-minimum inhibitory concentration (sub-MIC) induced transcriptional levels of mazEF and relBE operons. We then examined the protein profiles of His-tagged MazE and RelB proteins in the UA159 and ΔclpP backgrounds by Western blotting analysis. The results showed that *S. mutans* strains grown under non-stress conditions expressed very low but detectable levels of MazE and RelB antitoxin proteins. Antibiotics at sub-MICs induced the levels of

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the MazE and RelB proteins, but the protein levels decreased rapidly in the wild-type background. In contrast, a stable level of MazE and RelB proteins could be detected in the ΔclpP mutant background, suggesting that both proteins accumulated in the ΔclpP mutant. We conclude that ClpP is required for the proteolytic regulation of cellular levels of the MazE and RelB antitoxins in *S. mutans*, which may play a critical role in modulating the TA activities and persister cell formation of this organism following antibiotic challenge.

INTRODUCTION

Many bacteria are able to survive antibiotic treatments not only by acquiring resistance through genetic mutations but also through the presence of phenotypically distinct, antibiotic-tolerant cells or persister cells [1–3]. Unlike resistant cells that grow in the presence of antibiotics, persister cells are phenotypic variants that are transiently tolerant to antibiotics by restraining their growth [4]. Upon removal of the antibiotics, persister cells can resume their growth and repopulate in the culture. The formation of persister cells is a reversible and low-frequency phenomenon allowing a small fraction of bacterial populations to survive antibiotic treatments [3, 5]. Importantly, persister cells can be the cause of relapsing infections, which are a major public health concern [6], although the mechanism of persister cell formation is not fully understood. It is generally accepted that bacterial toxin–antitoxin (TA) systems function as 'regulatory switches' to play critical roles in regulating stress response and antibiotic tolerance via persister cell formation [7]. Among six types of TA systems, bacterial type II TA systems are the largest group and the best studied so far [8]. Type II TA modules typically consist of two co-transcripted genes (bicistronic operon), one encoding a stable toxin that can disrupt cellular processes and another encoding a labile antitoxin that inactivates the toxin by forming a TA complex [8]. Under normal conditions, bacterial cells produce low levels of such toxins that can be effectively inactivated by the cognate antitoxins, so that the cells remain protected from the toxins [7]. However, antibiotics or stresses may disrupt this balance in the regulation of these TA systems, resulting in the activation of the toxins and triggering growth arrest and persister cell formation [5–8]. Despite considerable advances, the question of how these TA systems are regulated to prevent the activation of these toxins remains controversial.

Streptococcus mutans is a Gram-positive bacterium that depends on a biofilm lifestyle for survival and persistence in its natural ecosystem, dental biofilms [9, 10]. Under appropriate conditions, *S. mutans* can rapidly produce acids from dietary fermentable carbohydrates and lower the biofilm pH, initiating demineralization of the tooth surface or dental caries [9]. *S. mutans* is therefore considered to be a primary aetiological agent of dental caries worldwide [10]. In addition, *S. mutans* can be a cause of subacute infective endocarditis, with ≈14% of viridans streptococcus-induced endocarditis triggered by *S. mutans* [11]. In dental biofilms, *S. mutans* is frequently exposed to various antimicrobial molecules produced by competing species and by the host or to challenge by daily mouth rinses of antimicrobials [12–14]. To cope with such life-threatening insults, *S. mutans* has developed a number of strategies that allow it to survive and persist under harsh conditions [12, 15]. For example, *S. mutans* can transiently induce the expression of subsets of stress proteins, such as Clp superfamily chaperones, in response to these threats, depending on the type, extent and duration of a stress [16, 17]. Recent studies have reported that *S. mutans* is able to form persister cells following antibiotic challenge, and this has been linked to the activation of two type II TA systems, MazEF and RelBE, in *S. mutans* [18, 19]. Although they are implicated in antibiotic tolerance and persister cell formation, it is unclear how *S. mutans* regulates these TA systems to prevent unwanted toxin activation and persister cell formation. While screening a transposon mutant library in our laboratory [15], we identified a ClpP-deficient mutant that was defective in long-term survival and more sensitive to antibiotics. In this study, we further investigated the effects of *clpP* deletion on the persister cell formation and transcriptional and proteolytic regulation of these TA systems in *S. mutans* following antibiotic challenge.

METHODS

Bacterial strains, media and growth conditions

The bacterial strains and plasmids, along with their characteristics, are presented in Table 1. *S. mutans* UA159 was grown in brain heart infusion (BHI) medium, whereas all other strains derived from *S. mutans* UA159 were maintained

Received 24 July 2019; Accepted 25 July 2019; Published 25 September 2019
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Keywords: toxin–antitoxin systems; antibiotic tolerance; persister cell formation; proteolytic regulation; *Streptococcus mutans*.
Abbreviations: AP, alkaline phosphatase; BHI, brain heart infusion; c.f.u., colony-forming units; LB, Luria-Bertani; Lux reporter assay, luciferase reporter assay; $MDK_{99.99}$, minimum duration for killing 99.99% of bacterial cells; MIC, minimum inhibitory concentration; OD_{590}, optical density at 590 nm; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RIDV, relative integrated density values; RLU, relative luminescent unit; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sub-MIC, sub-minimum or sub-inhibitory concentration; TA, toxin-antitoxin.
One supplementary figure and one supplementary table are available with the online version of this article.

Table 1. Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Reference/source
S. mutans		
UA159	WT, the genome sequence reference strain	[10]
Sm-ΔclpP	UA159 △*clpP*::*em*, Em^r	This study
Sm-pCclpP	UA159 △*clpP*::*erm* harbouring pCclpP, Em^r, Spec^r	This study
UA-pPmazEF	UA159 carrying pGF-PmazEF (P*mazEF*::*luxAB*), Kan^r	This study
UA-pPrelBE	UA159 carrying pGF-PrelBE (P*relBE*::*luxAB*), Kan^r	This study
ΔclpP-pPmazEF	ΔclpP carrying pGF-PmazEF (P*mazEF*::*luxAB*), Em^r, Kan^r	This study
ΔclpP-pPrelBE	ΔclpP carrying pGF-PrelBE (P*relBE*::*luxAB*), Em^r, Kan^r	This study
UA-pMazE-His	UA159 carrying pMazE-His, Spec^r	This study
ΔclpP-pMazE-His	ΔclpP carrying pMaxE-His, Em^r, Spec^r	This study
UA-pRelB-His	UA159 carrying pRelB-His, Spec^r	This study
ΔclpP-pRelB-His	ΔclpP carrying pRelB-His, Em^r, Spec^r	This study
E. coli		
DH5α	Cloning host	Invitrogen
NovaBlue	Cloning host	Novagen
BL21(DE3)pLysS	Host for protein expression	Novagen
Plasmid		
pDL278	*E. coli–Streptococcus* shuttle vector, Spec^r	[22]
pCclpP	pDL278::clpP, Spec^r	This study
pGF-kan	pWAR303 but the *erm* replaced by the *kan*, Kan^r	[24]
pGF-PmazEF	pGF with a fusion of P*mazEF*::*luxAB*, Kan^r	This study
pGF-PrelBE	pGF with a fusion of P*relBE*::*luxAB*, Kan^r	This study
pET-20b(+)	*E. coli* expression vector (6His-tag), Amp^r	Novagen
pET-20b(+)mazE	pET-20b::*mazE,* His-tag fusion, Amp^r	This study
pET-20b(+)relB	pET-20b::*relB,* His-tag fusion, Amp^r	This study
pMazE-His	pDL278::mazE-His, Spec^r	This study
pRelB-His	pDL278::relB-His, Spec^r	This study

in BHI medium supplemented with an appropriate antibiotic (or antibiotics). *Escherichia coli* host strains were grown in Luria–Bertani (LB) medium supplemented with an appropriate antibiotic whenever needed.

Construction of the *clpP* deletion mutant

To determine the effect of *clpP* deletion on *mazEF* and *relBE* modules, a *clpP* deletion mutant was constructed by a PCR ligation mutagenesis method [20]. Briefly, the flanking regions from ≈100 bp internal to the start and stop codons of *clpP* gene were amplified by PCR using primers clpP-P1 and clpP-P2 (AscI) and clpP-P3 (FseI) and clpP-P4 (Table 2). Both PCR products were digested, purified and ligated to the AscI and FseI sites of an 876 bp PCR fragment, which was derived from PCR amplification of an ermAM cassette containing an erythromycin resistance gene (erm^R) and its own promoter [20, 21]. The ligation product, clpP-up::Erm::clpP-dw, was transformed into *S. mutans* UA159. Following double-crossover recombination, the internal region of *clpP* was completely replaced by the erm^R cassette. The constructed mutant was verified by PCR using four combinations of the primers against genomic DNAs of the mutant and parent, as shown in detail in Fig. S1 (available in the online version of this article). The presence of the predicted sizes of the erm^R marker and its flanking regions in the mutant but not in the parent indicated successful deletion of the target gene by replacement of the erm^R cassette. The confirmed mutant together with other strains was then grown in BHI medium for analyses of their phenotype and growth kinetics.

Construction of *clpP* complementation strain

To confirm the phenotype of the *clpP* deletion, we also constructed a complementary strain that carried a low-copy-number *E. coli–Streptococcus* shuttle vector, pDL278 [22], which was engineered to harbour a wild-type copy of *clpP* and its promoter region. Briefly, the entire *clpP* gene and its promoter region were amplified by PCR using primers CclpP-F and CclpP-B (Table 2). The amplicon was digested with Nco1 and EcoR1 and ligated to the same restriction sites at the multiple cloning site of pDL278. The ligation product was transformed into *E. coli* host DH5α (Invitrogen). Positive transformants were selected from LB plates plus spectinomycin (50 μg ml^{-1}) for genetic confirmation. A confirmed plasmid, designated pCclpP, was then transformed into the ΔclpP mutant. The constructed strain conferred resistance to both spectinomycin (800 μg ml^{-1}) and erythromycin (10 μg ml^{-1}). This strain was designated Sm-pCclpP and used for complementation analysis *in trans*.

Table 2. Primers used to construct bacterial strains this study

Name	Nucleotide sequence (5′ --->3′)*	Purpose
clpP-P1	CTAAATGAAAAGGGCTATATTGTTC	
clpP-P2	TAGGCGCGCCCGATCTTTTAATAGACGTGA	
clpP-P3	GCTGGCCGGCCGGATGGATGCTAAGGAAACA	*clpP* deletion
clpP-P4	TTATTGCCATATCCTTTTTTGTGA	
erm-P1	TAGGCGCGCCCCGGGCCCAAAATTTGTTTGAT	Erm marker
erm-P2	GCTGGCCGGCCAGTCGGCAGCGACTCATAGAAT	
CclpP-F	CCATGGGCGTTCCCAAAAGAAACAAGCTTGTT	Complement
CclpP-B	GGAATTCGCGCGAAGCTTTTATTTTAATTCATTAT	
PmazE-F	GGGGTACCCTACATTACAGCATTTTCAAACAC	*lux* reporter
PmazE-B	AACTGCAGAATGCCACCTCCTATTTCTGTG	
PrelB-F	GGGGTACCACTTATTGCGTAACTTGTTAG	*lux* reporter
PrelB-B	AACTGCAGATTATCAACTCCTTTTATATCTAT	
mazE-F1	CGGAATTCGAACAGCTACATTACAGCATTTTC	MazE-His
mazE-B1	AAGCGGCCGCCCATTGTTCATTTCCCACACT	
mazE-B2	ACATGCATGCCAAAAAACCCCTCAAGAC	
relB-F1	CGGAATTCTAGAATAATAAAACAAAAGCAAGA	RelB-His
relB-B1	AAGCGGCCGCATCTTTAGCATAGGCACTCAT	
relB-B2	ACATGCATGCCAAAAAACCCCTCAAGAC	

*The restriction sites are indicated in bold: GGCGCGCC (AscI); GGCCGGCC (FseI); CCATGG (Nco1); GAATTC (EcoR1), GCGGCCGC (Not1); GCATGC (Sph1).

Construction of transcriptional reporter strains and luciferase activity assay

To analyse the transcriptional levels of the *mazEF* and *relBE* modules, we constructed several transcriptional reporter strains that carried a shuttle vector pGF-kan harbouring *luxAB* fused to the promoter region of each of these operons. Briefly, DNA fragments containing the promoter region (≈250–350 bp) of these operons were generated by PCR, purified and cloned into pGF-kan to generate two *luxAB* fusion plasmids (Table 1). The constructed plasmids were generically confirmed by PCR and sequencing. The confirmed plasmids were then transformed into *S. mutans* UA159 and the ΔclpP mutant to generate four reporter strains. All of the strains were grown under tested conditions for the *lux* reporter assay by reading luciferase activity and related cell density at 590 nm using a microtitre plate reader (Synergy HT, Biotek, USA). Since *luxAB*-catalyzed luciferase activity requires the presence of nonanal as a substrate, 1 % nonanal (Sigma-Aldrich) was used for the reaction with a previously described protocol [23]. Aliquots (300 µl) of cell suspension (log growth phase) were transferred to each well of a prewarmed (37 °C) microtitre plate. Aliquots (50 µl) of the nonanal solution in a volatile form were placed in the spaces between the wells of the microplate, which was covered with the lip. The microtitre plate was set at 37 °C for incubation and the readings were taken at time intervals of 15 min for 5 h. The results were expressed as relative luminescence units (RLU) divided by the cell densities (OD_{590}) of these strains grown under the same conditions.

Construction of plasmids pMazE-His and pRelB-His

To determine whether *clpP* deletion affected the protolytic regulation of MazE and RelB proteins, we constructed two plasmids by a two-step cloning strategy as described previously [24, 25]. In the first step, we amplified the entire target gene (except the stop codon) of *mazE* or *relB* from the *S. mutans* genome using primers with two restriction sites, EcoR1 and NotI (Table 2). The PCR fragments were cloned into an expression vector pET-20b(+) (Novagen), generating two plasmids of pET-20b(+)mazE and pET-20b(+)relB, respectively. In the second step, we used a new backward primer with a restriction site Sph1-B2 and the same forward primer to amplify inserted mazE or relB along with the His-tag from these plasmids and cloned into the shuttle vector pDL278 [22]. The constructed plasmids were confirmed by PCR and restriction analysis. The confirmed plasmids were designated pMazE-His and pRelB-His and then transformed into *S. mutans* UA159 and the ΔclpP mutant to generate four strains (Table 1) for Western blot analysis of His-tagged antitoxin proteins.

Protein extraction and Western blot analysis

Next, these strains were grown in BHI medium with or without the addition of bacitracin (1 unit ml^{-1}) to examine cellular levels of His-tagged MazE and RelB proteins. Aliquots (10 ml) of samples were taken at different times to prepare crude protein lysates after the adjustment of cell densities to 0.8 at OD_{600} in BHI medium. The cellular levels of His-tagged proteins were analysed by Western blotting using an anti-His antibody (GeneScript, NJ, USA). All crude protein samples were resolved on SDS-PAGE gels with a Bio-Rad Mini-Protein II gel apparatus. The proteins were then transferred to two polyvinylidene difluoride (PVDF) membranes: one was used for the detection of His-tagged proteins and the other was used as a protein loading control with an anti-*S. mutans* (serotype C) antibody (Abcam, MA, USA). The membranes were blocked with 5% fat-free milk in TBS-T buffer at 4 °C overnight and added to the first antibody (1:2500 dilution) and incubated for 90 min. After three washes, the membranes were added to the second antibody (1:5000 diluted anti-rabbit IgG conjugated to alkaline phosphatase or AP). The membranes were incubated for 1 h, washed and detected with AP detection reagents (Novagen). The protein profiles were then examined using FluorChem SP imaging system (Alpha Innotech). To quantify the intensities, the protein bands were scanned using UN-SCAN-IT software 6.1 (Silk Sci. Inc.) and the data were converted to relative integrated density values (RIDVs), in comparison with the maximum density value (100 %) of each sample at time zero [24].

Persister viability assay

A persister assay was used to determine the numbers of survivor or persister cells by examining bacterial strains following antibiotic challenge [26]. Briefly, *S. mutans* UA159, Sm-Δ*clpP* and its complementary strain Sm-pCclpP were first grown under normal conditions (BHI broth) at 37 °C until they reached the late stationary phase (20 h). The cultures were diluted to ≈0.1 at OD_{600} in fresh BHI medium containing one of following antibiotics (at 10-fold MIC)L a cell wall biosynthesis inhibitor, bacitracin (40 unit ml^{-1}); a cell membrane-disrupting agent, chlorhexidine (20 μg ml^{-1}); a DNA-damaging agent, mitomycin (0.5 μg ml^{-1}); or a protein synthesis inhibitor, chloramphenicol (10 μg ml^{-1}). The 10-fold MIC concentrations were chosen based on MIC tests (Table S1), because this increased the effectiveness of the antibiotics with little possibility of spontaneous resistance mutation [15]. The cultures continued to be incubated at 37 °C and aliquots (200 μl) of samples were taken at different time points of T_0, T_1, T_2, T_4, T_8 and T_{16} to perform viable cell counts. Following anaerobic incubation at 37 °C for 2 days, the agar plates were examined for colony-forming units (c.f.u.). The percentages of persister cells were calculated based on the number of survival cells (c.f.u. ml^{-1}) in relation to he total viable cell counts at T_0. The data were plotted against time (hour) by indicating $MDK_{99.99}$, which was defined as the minimum duration required to kill 99.99% of bacterial cells [4]. All assays were performed in triplicate in two independent experiments. The possibility of spontaneous resistance mutation was evaluated by plating aliquots of the cell suspension onto BHI agar plates containing each of the antibiotics after the last sampling time.

Statistical analysis

Whenever needed, the results were analysed by Student's *t*-test and *P* values of ≤0.05 were considered statistically significant.

RESULTS

Growth of the Δ*clpP* mutant under the normal and stress conditions

Previously, we identified a ClpP-deficient mutant that was defective in long-term survival and more sensitive to antibiotics than wild-type *S. mutans* [15, 17]. However, it was unknown whether the observed phenotypes genuinely resulted from *clpP* inactivation or from a polar effect of the transposon insertion within the *clpP* locus. To answer this question, we constructed a new *clpP* deletion mutant using a PCR ligation mutagenesis method that enabled *clpP* to be deleted without interrupting downstream reading frames, because the PCR primers were designed in such a way that the original downstream reading frames were preserved when the erm^R cassette was inserted into the deletion site [20]. We ensured that the erm^R gene and its promoter had the same reading frame as the target gene. No specific sequence within or downstream of the erm^R determinant could affect the transcription and translation of the downstream chromosome genes [20, 21]. The newly constructed *clpP* deletion mutant, Sm-ΔclpP, along with its parent, UA159 (WT), and the complementary strain, Sm-pCclpP, were examined for their growth kinetics under both normal (non-stress) conditions (37 °C, pH 7.5) and under stress conditions (either at 42 °C or at pH 5.5). The growth curves showed that the Sm-Δ*clpP* mutant grew slightly more slowly than the parent under normal conditions, but the final cell density of the mutant nearly reached that of UA159 (Fig. 1a). At a temperature of 42 °C (Fig. 1b), however, the Sm-Δ*clpP* mutant not only grew more slowly, but it also showed lower cell density than the parent. Similarly, the Sm-Δ*clpP* mutant grew more slowly and had a lower cell density than the parent at pH 5.5 (Fig. 1c), suggesting that *clpP* deletion affected the growth of *S. mutans* to some extent in terms of its growth rate and growth yield, especially under stress conditions (higher temperature or lower pH). The introduction of a wild copy of *clpP* into the mutant *in trans* (Sm-pCclpP) restored the growth of Sm-Δ*clpP* to a level that was close to that of the parent under the tested conditions. The results confirm that the newly constructed mutant Sm-Δ*clpP* showed the expected phenotype, as described previously [15–17]. This mutant was then used for further experiments.

Effect of *clpP* deletion on persister cell formation of *S. mutans* following antibiotic challenge

Next, we performed a persister viability assay to determine whether *clpP* deletion affected the persister cell formation of *S. mutans* following antibiotic challenge. *S. mutans* UA159 (WT), Sm-Δ*clpP* and Sm-pCclpP were examined for their ability to form persister cells following antibiotic challenges,

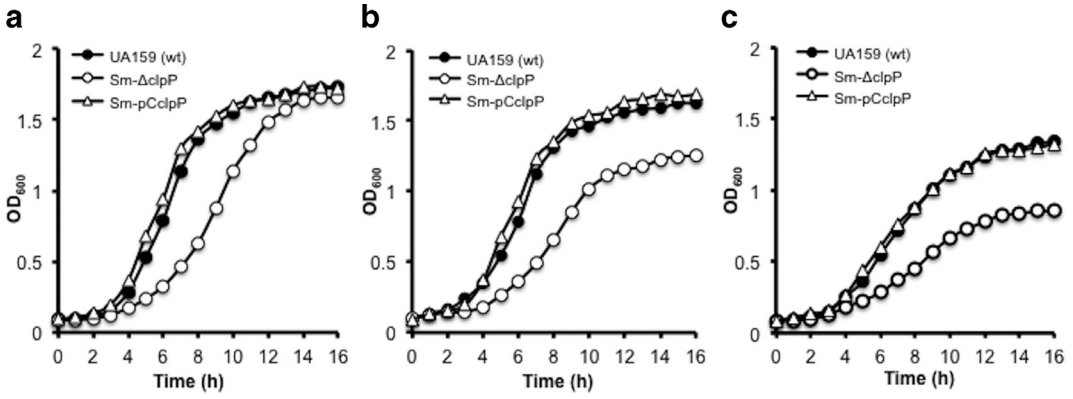

Fig. 1. Growth curves for *S. mutans* UA159 (WT), Sm-Δ*clpP* and its complementary strain Sm-pCclpP under standard (non-stress) conditions (BHI medium, pH 7.5, 37 °C) (a) or under stress conditions [BHI, pH 7.5, 42 °C (b) or BHI, pH 5.5, 37 °C (c)].

including 10-fold MIC treatments with bacitracin (40 unit ml^{-1}), chlorhexidine (20 μg ml^{-1}), mitomycin (0.5 μg ml^{-1}) or chloramphenicol (10 μg ml^{-1}). The results showed that the minimum duration for killing 99.99% ($MDK_{99.99}$) of *S. mutans* UA159 (WT) with bacitracin was approximately 10 h, while the $MDK_{99.99}$ for Sm-ΔclpP was approximately 4 h (Fig. 2a). The $MDK_{99.99}$ for *S. mutans* UA159 (WT) with chlorhexidine was approximately 8 h, while the $MDK_{99.99}$ for Sm-ΔclpP was approximately 4 h (Fig. 2b). Similarly, the $MDK_{99.99}$ for *S. mutans* UA159 (WT) with mitomycin and chloramphenicol was approximately 12 h, while the $MDK_{99.99}$ for Sm-ΔclpP was 4–6 h (Fig. 2c, d). For all antibiotics tested, strain Sm-CclpP showed nearly the same $MDK_{99.99}$ as the wild-type strain, suggesting that complementation of *clpP* deletion restored

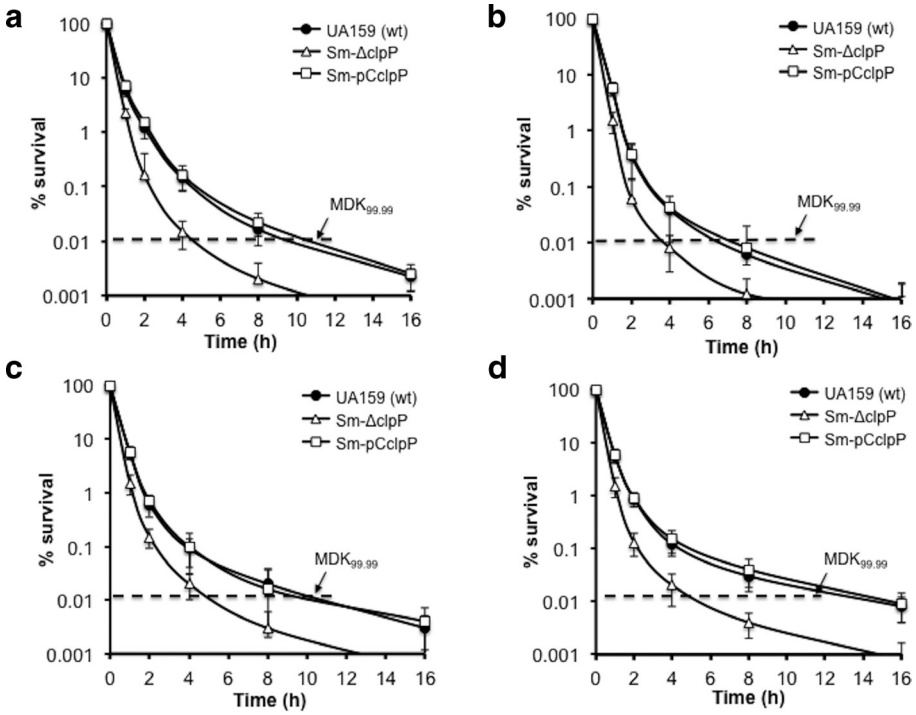

Fig. 2. Effects of *clpP* deletion on persister cell formation by *S. mutans* following antibiotic treatments. *S. mutans* UA159 (WT), Sm-Δ*clpP* and Sm-pCclpP were grown in BHI medium for 16 h. The cultures were diluted to 0.1 at $O.D_{600}$ in fresh BHI medium containing one of four antibiotics, (a) bacitracin (40 unit ml^{-1}), (b) chlorhexidine (20 μg ml^{-1}), (c) mitomycin (0.5 μg ml^{-1}) or (d) chloramphenicol (10 μg ml^{-1}). The cultures continued to be incubated. Aliquots (200 μl) were taken from the samples at the indicated time points, serially diluted and spotted onto BHI agar plates for viable cell counts. The log percentages of survivor or persister cells were plotted against time (hours). $MDK_{99.99}$ is defined as the minimum duration required to kill 99.99% of bacterial cells, as indicated by the dashed lines.

the tolerance of the ΔclpP mutant to these antibiotics. To rule out the possibility of spontaneous antibiotic resistance, we also took aliquots of the cell suspension from the cultures (after 16 h) and plated them onto BHI agar plates containing the same concentrations of each of the four antibiotics used. The results showed no visible colonies (antibiotic resistance) on the antibiotic-containing agar plates after incubation for 2 days, suggesting that no spontaneous antibiotic resistance had developed during the experiments.

Effects of *clpP* deletion on transcriptional regulation of *mazEF* and *RelBE*

The *mazEF* and *relBE* are located at two separate loci in the *S. mutans* genome and both are organized as bicistronic operons [27]. This suggests that two genes within the *mazEF* or *relBE* operons are co-transcripted at the genetic level. However, it was unknown whether *clpP* deletion affected the transcriptional regulation of the *mazEF* and *relBE* operons. To answer this question, we constructed two *luxAB* reporter plasmids, which contained a promoterless *luxAB* fused to the promoter region of either the *mazEF* or the *relBE* operon, and transformed these plasmids into the UA159 and the Δ*clpP* mutant. We then assayed the *lux* reporter activities of these strains grown under culture conditions with or without an antibiotic. For ease of comparison, only the maximal *lux* reporter activities of these strains grown under each condition are presented in Fig. 3. The results showed that under normal conditions (without an antibiotic) all the reporter strains displayed a low level of *lux* reporter activity. No significant differences in the *lux* reporter activities were observed between the wild-type and Δ*clpP* backgrounds ($P>0.05$). The results suggested that the *clpP* deletion did not affect the transcriptional levels of these TA modules. Upon exposure to a sub-MIC of an antibiotic, however, all the strains showed significantly increased levels of *lux* reporter activity ($P<0.01$) compared to the same reporter strains grown without antibiotics. The results suggested that antibiotics at a sub-MIC induced transcriptional levels of *mazEF* and *relBE* in *S. mutans*, but the *clpP* deletion did not appear to affect the transcription of these operons.

To further determine whether other stresses affected the transcription of the *mazEF* and *relBE* operons, we also examined the *lux* reporter activities of these strains grown at a higher temperature 42 °C or at a lower pH of 5.5. The results showed that the higher temperature (42 vs 37 °C) slightly increased the transcriptional levels of the *mazEF* and *relBE* operons, but the differences in the reporter activities between 42 and

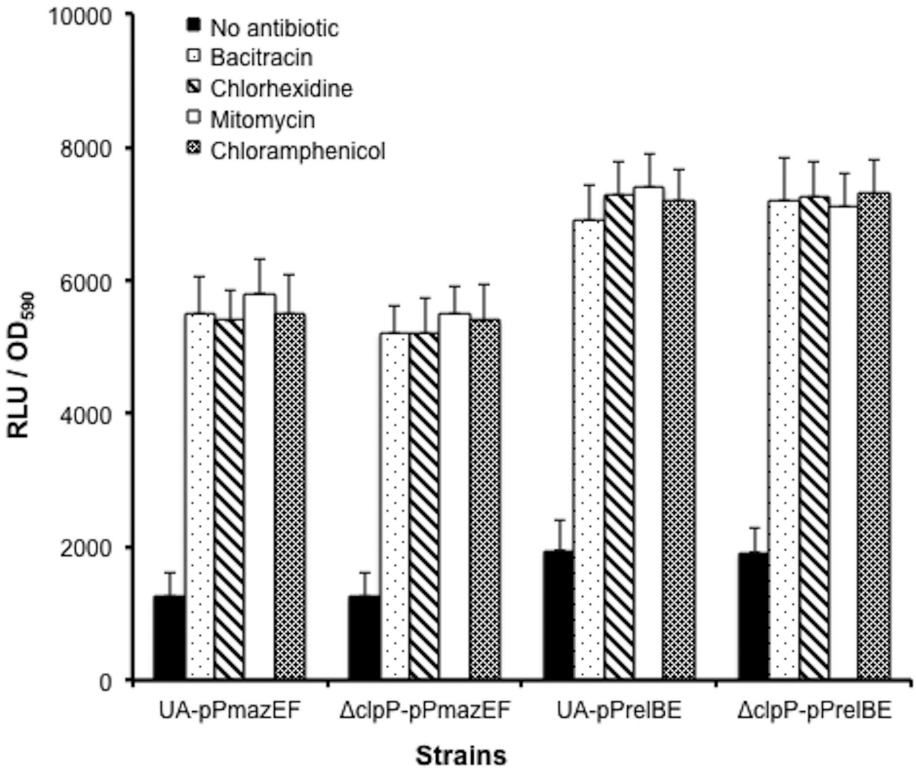

Fig. 3. The luciferase reporter activities of the *S. mutans* strains, UA-pPmazEF, ΔclpP-pPmazEF, UA-pPrelBE and ΔclpP-pPrelBE, were assayed in response to sub-MICs of antibiotics (bacitracin, 1.0 unit ml^{-1}; chlorhexidine, 2 μg ml^{-1}; mitomycin, 0.01 μg ml^{-1}; chloramphenicol, 0.25 μg ml^{-1}). The results are expressed as relative luminescence units divided by the cell densities (RLU/OD_{590}) of these strains grown under the same conditions. Only the maximal reporter activities (one-point values) of these strains have been plotted based on the data obtained during a 5 h reading period with the microtitre plate reader.

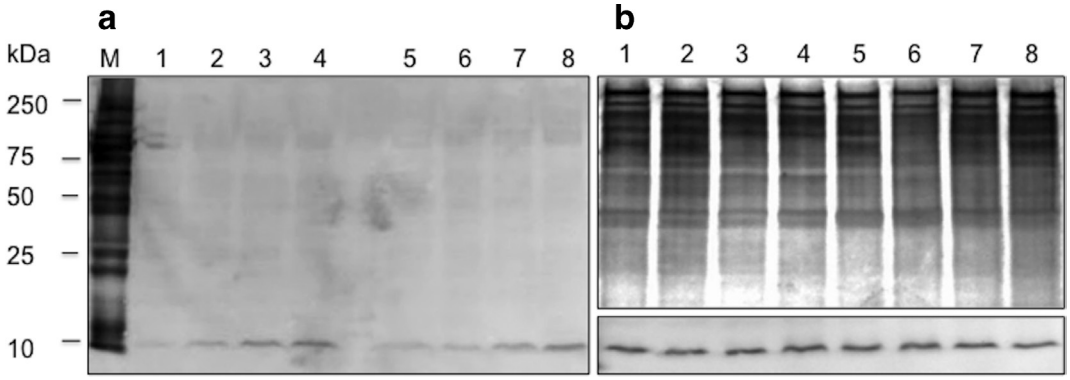

Fig. 4. Western blot analysis of His-tagged MazE and RelB antitoxin proteins in the crude cell lysates from four *S. mutans* strains. (a) Lanes 1–2, UA-pMazE-His; lanes 3–4, ΔclpP-pMazE-His; lanes 5–6, UA-pRelB-His; lanes 7–8, ΔclpP-pRelB-His, grown under normal conditions. A 10 kDa band was detected by an anti-His antibody in all the strains. (b) An example of the loading controls from these strains following exposure to bacitracin (1 unit ml^{-1}), although the blotting membrane was cut into two: one detected by an anti-*S. mutans* antibody (up) and another detected by the anti-His antibody (down). All of the presented samples were taken at the mid-log phase (0.6 at OD_{600}) and at the late-log phase (1.0 at OD_{600}), respectively, but the same quantity of proteins was loaded into each well based on the BioRad protein assay. **M:** Molecular weight of precision plus protein standards (Bio-Rad).

37 °C were not significant (data not shown). Interestingly, all the reporter strains grown at pH 5.5 showed lower levels of reporter activity than those grown at pH 7.5, suggesting that an acidic pH appears to repress the transcription of these operons (data not shown).

Effects of *clpP* deletion on the intracellular levels of MazE and RelB proteins

In addition to transcriptional regulation, the intracellular levels of antitoxins of *E. coli* TA systems are proteolytically regulated by the ATP-dependent proteases Lon and ClpP [27]. However, it remains unclear whether a similar regulatory mechanism exists in *S. mutans*, since *S. mutans* only has ClpP but lacks a Lon homologue [10, 17]. To answer this question, we constructed two plasmids, pMazE-His and pRelB-His, and transformed them into both UA159 (WT) and the Δ*clpP* mutant. We then examined the cellular levels of MazE and RelB antitoxin proteins by Western blotting. Based on the genome sequence of *S. mutans*, *mazE* encodes a 9.2 kDa protein and *relB* encodes a 10 kDa protein [10, 27]. Both are small proteins of a similar size, indicating a challenge to gain sufficient proteins. Despite this challenge, we successfully extracted and detected both proteins in the crude cell lysates from the *S. mutans* strains, which expressed one of the His-tagged MazE and RelB proteins, by Western blotting. As shown in Fig. 4, S. *mutans* wild-type background grown under non-stress conditions produced very low but detectable levels of the MazE and RelB proteins (Fig. 4a, lanes 1–2 and 5–6). Under the same conditions, the Δ*clpP* background strains showed slightly higher levels of these proteins (Fig. 4b, lanes 3–4 and 7–8). These results suggested that *clpP* deletion might result in the cellular accumulation of both MazE and RelB proteins. To confirm this observation, we further examined the stability of these proteins in both the wild-type and the Δ*clpP* backgrounds in response to a sub-MIC of bacitracin (Fig. 5). The results revealed that bacitracin increased the cellular levels of both the MazE and RelB proteins in all the strains, which appeared to be consistent with the transcriptional levels of both the *mazEF* and *relBE* operons based on the *lux* reporter assay. However, both MazE and RelB antitoxin proteins appeared to be degraded in the wild-type background, with a half-life of 80 min for MazE (Fig. 5a) and one of 70 min for RelB (Fig. 5c), but not in the Δ*clpP* mutant background, which showed relatively stable levels of MazE and RelB (Fig. 5b, d). The results suggest that *clpP* deletion results in the cellular accumulation of MazE and RelB proteins following antibiotic challenge (sub-MIC). These results confirm that ClpP is required for proteolytic regulation of both the MazE and RelB antitoxin proteins of *S. mutans* grown under antibiotic stress.

DISCUSSION

The type II toxin–antitoxin (TA) systems, MazEF and RelBE, which are linked to persister cell formation and antibiotic tolerance, have been identified in the low-GC Gram-positive *S. mutans,* and their roles in persister cell formation have been partially characterized [18, 27]. Previous studies have shown that ecotopic expression of MazEF or RelBE TA systems or overproduction of MazF or RelE toxin results in cell growth arrest and an increase in the number of *S. mutans* persister cells following antibiotic challenge [18]. These studies have suggested that both MazEF and RelBE TA systems contribute to antibiotic tolerance through persister cell formation in *S. mutans*. However, relatively little is known regarding how these TA systems are regulated to prevent unwanted activation of these toxins and their effects on the cellular processes in this organism. In this study, we have explored this question by investigating the effects of *clpP* deletion on persister cell formation and the transcriptional and proteolytic regulation

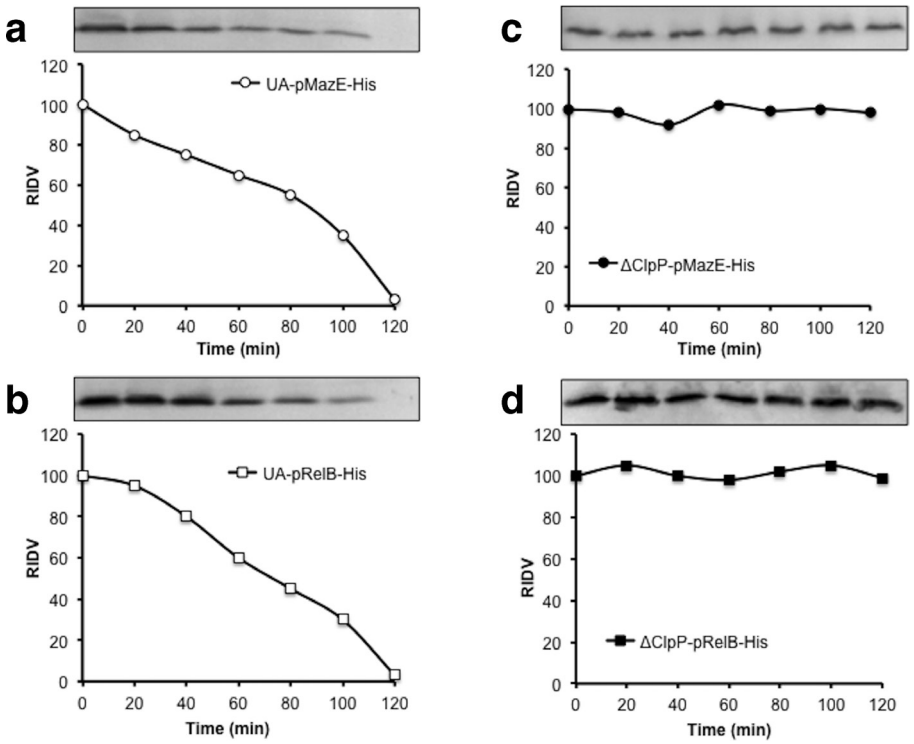

Fig. 5. Western blot analysis of the effects of *clpP* deletion on the cellular levels and stability of MazE-His and RelB-His proteins in the wild-type background (a and c) and the ΔclpP mutant background (b and d) following exposure to bacitracin (1 unit ml^{-1}). The protein bands representing the cellular levels of these proteins were scanned and the intensities of the protein bands were converted to relative integrated density values (RIDVs), which were normalized to a maximum value of 100% for each sample.

of MazEF and RelBE type II TA systems of *S. mutans* in response to antibiotic challenge. The data from this study confirm that after antibiotic challenge (regardless of the type of antibiotics), *S. mutans* UA159 (WT) is able to form a small quantity of antibiotic-tolerant or persister cells, ranging from 0.01–0.001% (or below) of the population. However, *clpP* deletion results in significantly reduced persister cells under the same antibiotic challenge. In addition, the Δ*clpP* mutant is more sensitive to all four tested antibiotics than the parent and complementary strain. The results strongly suggest that ClpP is required for persister cell formation and antibiotic tolerance in *S. mutans*. Our initial work confirms that the small quantity of survivor cells following antibiotic challenge are not antibiotic-resistant cells, because they can only grow on agar plates without antibiotics, but cannot grow on the plates containing the same concentrations of these antibiotics. Interestingly, *clpP* deletion did not significantly affect the transcriptional levels of the *mazEF* and *relBE* operons, which only responded to antibiotic challenge. Since the transcriptional levels of these TA modules are very weak under non-stress conditions, the data suggest that *S. mutans* may only switch to TA-mediated growth arrest and persister cell formation when it encounters a life-threatening challenge, such as antibiotics. Thus, the transcriptional data appear to be insufficient to explain how antibiotic stress triggers the activation of MazF and RelE toxins and subsequent cell growth arrest, because increased toxins could be inactivated by an equal amount of the cognate antitoxins. This suggests that it must be another level of regulatory mechanism that allows these toxins to become free from interaction with the cognate antitoxins or activated following antibiotic challenge.

Another important finding from this study is that in addition to transcriptional regulation, *S. mutans* is able to respond to antibiotic challenge by proteolytic regulation of the cellular levels of the MazE and RelB antitoxins. In particular, ClpP protease is confirmed to play a critical role in the regulatory proteolysis of the MazE and RelB antitoxins in *S. mutans* following antibiotic challenge. Three lines of evidence support this conclusion. First, *clpP* deletion significantly reduces the formation of *S. mutans* persister cells following antibiotic challenge. Second, *clpP* deletion increases the sensitivity of *S. mutans* to all four types of antibiotics tested. Third, *clpP* deletion results in the cellular accumulation of both MazE and RelB antitoxins following antibiotic challenge, suggesting that ClpP protease is required for regulatory proteolysis of these antitoxins under antibiotic stress. The data support the idea that the cellular levels of TA toxins and their cognate antitoxins can be maintained both by transcriptional autoregulation and regulatory proteolysis when *S. mutans* is grown under normal conditions. Upon antibiotic challenge, however,

S. mutans may upregulate the transcription of *mazEF* and *relBE*, increasing the synthesis of the gene products, MazEF and RelBE. Antibiotic challenge may also increase the activity of the ClpP proteolytic complex, leading to degradation of the more labile antitoxins, MazE and RelB, under such a stress condition [28–30]. This might allow the MazF and RelE toxins to become free from interaction with the cognate antitoxins, resulting in the dysregulation of cellular processes, such as growth arrest and persister cell formation in a subpopulation of *S. mutans* grown under the stress conditions, as described in the Graphical Abstract. However, it seems debatable whether the effect of ClpP on persister cell formation is the direct cause of its regulation of the TA systems, since ClpP deficiency may have impacts on multiple cellular processes, including the TA systems, which could affect antibiotic tolerance and persister cell formation. This question could be further explored by examining the effect of *mazF* or/and *relE* inactivation on persister cell formation. However, a work by Syed *et al*. revealed that inactivation of *mazF* or *relE* or both had little effect on the persister cell phenotype in *S. mutans*, but rather, overexpression of these genes could cause cell growth arrest and persister cell formation [18, 19]. These works suggest the possibility that more than two TA systems might exist in the *S. mutans* genome or that a more complicated regulatory mechanism may be involved in the TA-mediated persister cell formation.

It has been well established in *E. coli* that both Lon and ClpP are engaged in the proteolytic regulation of TA antitoxins, such as MazE, RelB and other antitoxins [26–28]. Lon represents a major class of ATP-dependent protease *in E. coli* and is a homo-oligomer with an ATPase domain and a proteolytic domain [28]. However, many bacteria, including *S. mutans,* do not have a Lon homologue in their genome, so that ClpP, the second well-characterized protease, is considered to be the core protease that is essential for the proteolytic regulation of various proteins in these bacteria [28–30]. ClpP is a cylinder-like protease that requires interaction with a Clp-ATPase for its proteolytic activity. In the *S. mutans* genome, all of the genes that encode one ClpP and five Clp-ATPase chaperons, ClpB, ClpC, ClpE, ClpL and ClpX, have been identified [10, 16]. In *S. mutans*, however, only ClpC, ClpE and ClpX have the tripeptide sequence that is needed to interact with ClpP [17]. In this study, we have focused on the *clpP* locus by examining the effects of ClpP deficiency on persister cell formation and the regulation of the MazEF and RelBE TA systems in *S. mutans*. Despite the evidence that strongly suggests that ClpP has a crucial role in persister cell formation and the proteolytic regulation of the MazEF and RelBE systems, it may be necessary to include the three Clp-ATPase chaperons, ClpC, ClpE and ClpX, in future studies to determine their roles in the proteolytic regulation of the MazE and RelB antitoxins in *S. mutans* following antibiotic challenge.

In summary, this study demonstrates that the ClpP core protease is required for the proteolytic regulation of cellular levels of the MazE and RelB antitoxins in *S. mutans* following antibiotic challenge. To the best of our knowledge, this is the first report to provide direct evidence that *S. mutans* is able to tolerate antibiotics through ClpP-mediated proteolytic regulation of the MazEF and RelBE TA systems in *S. mutans*.

Funding information
This work was supported in part by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant RGPIN 311682–07 and the Canadian Institutes of Health Research (CIHR) Operating Grant MOP-115007. M. L. was the recipient of a Visiting Scholarship Award from China. Z. S. and H. R. were the recipients of the Faculty Summer Research Studentship Award.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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