Functional Characterization of the Chromosomal MazEF Toxin-Antitoxin Addiction System in *Streptococcus mutans*

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Graduate Department of Dentistry
University of Toronto

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ABSTRACT

Chromosomal type II toxin-antitoxin (TA) modules consist of a pair of genes that encode two components: a stable toxin and a labile antitoxin interfering with the lethal action of the toxin through protein complex formation. Typically, the toxin inhibits an essential cell function such as DNA replication or protein synthesis. Chromosomal type II TAs have been proposed to function as regulators of cell growth in response to environmental stress or as a mechanism for growth control by phenotypic variation (bacterial persistence). *Streptococcus mutans*, a major causative agent in the formation of dental caries, encodes four predicted chromosomal type II TA modules. The objective of this study was to characterize the MazEF TA system of *S. mutans*. *S. mutans* mazEF genes form a bicistronic operon that is cotranscribed from a σ70-like promoter. MazF toxin had a toxic effect on *S. mutans* and this effect can be neutralized by coexpression of its cognate antitoxin MazE. Although MazF expression inhibited cell growth, no cell lysis of *S. mutans* cultures was observed under the conditions tested. The *S. mutans* MazEF TA is also functional in *E. coli*, where the expression of MazF leads to growth arrest and MazF-stasis can be prevented by MazE coexpression. Furthermore, we demonstrated that *S. mutans* MazE and MazF proteins interact with each other in vivo, confirming the nature of this TA as a type II addiction system. Our data indicate that MazF is a toxic nuclease arresting cell growth through the mechanism of RNA cleavage and that MazE inhibits the RNase activity of MazF by forming a protein complex. Our results suggest that the chromosomal MazEF TA module might represent a cell growth modulator facilitating the persistence of *S. mutans* in the oral cavity.
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CSP</td>
<td>competence-stimulating peptide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HK</td>
<td>histidine kinase</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-turn-helix</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>Mbp</td>
<td>mega base pair</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MU</td>
<td>Miller unit</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMF</td>
<td>proton motive force</td>
</tr>
<tr>
<td>PNPG</td>
<td>para-nitrophenyl-β-D-glucuronide</td>
</tr>
<tr>
<td>PSK</td>
<td>post-segregational killing</td>
</tr>
<tr>
<td>RHH</td>
<td>ribbon-helix-helix</td>
</tr>
<tr>
<td>R/M</td>
<td>restriction-modification</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RR</td>
<td>response regulator</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>VNBC</td>
<td>viable but non-culturable</td>
</tr>
<tr>
<td>β-Gal</td>
<td>β-galactosidase</td>
</tr>
</tbody>
</table>
"Most people live - whether physically, intellectually or morally - in a very restricted circle of their potential being. We all have reservoirs of life to draw upon of which we do not dream"

William James
CHAPTER 1

LITERATURE REVIEW

1. Introduction

Prokaryotic chromosomes contain genetic modules encoding two components: a stable toxin and its cognate labile antitoxin. These modules are called toxin-antitoxin (TA) systems (Gerdes et al., 2005). Typically, the toxin inhibits an essential microbial cell function such as translation or DNA replication. Importantly, chromosomal TAs induce a reversible bacteriostatic state as no toxin has been reported to be directly bacteriolytic (Van Melderen, 2010). TAs are ubiquitous in chromosomes of archaea and bacteria, often found in multiple copies (Van Melderen, 2010). Because TAs are thought to invade prokaryotic genomes through horizontal gene transfer and intragenomic recombination, they are commonly described as mobile genetic elements (Makarova et al., 2009).

Bacterial TAs are of three different types depending on the nature and mode of action of the antitoxin component (Blower et al., 2011). While toxins are always proteins, antitoxins are either antisense RNAs (type I and type III) or proteins (type II) (Van Melderen, 2010). Antisense RNA antitoxins suppress toxin expression (type I) while labile protein antitoxins bind to and inactivate the activity of its cognate toxin (type II) (Blower et al., 2011). The antisense RNA antitoxin can also interfere with the toxin activity instead of preventing its expression (type III), although the precise molecular mechanism is still unknown (Fineran et al., 2009). In all three TA systems, the cells are “addicted” to the antitoxin as its production is essential for cell survival. For this reason, these systems are called addiction modules. Although restriction-modification (R/M) systems are also addictives, they are not considered TA systems. R/M systems are composed of a restriction endonuclease and a methylase. By degrading unmethylated native DNA, bacterial R/M systems prevent invasion by foreign DNA (Rocha et al., 2001).
2. Discovery of bacterial TA systems

Type I and type II TA systems were first described in the 1980s as addiction modules that contribute to plasmid maintenance, a mechanism called post-segregational killing (Gerdes et al., 1988). When a plasmid copy is not transmitted to daughter cells, the antitoxin and toxin pool is not replenished. Since the antitoxin is less stable and rapidly degraded, the toxin is released from inhibition leading to the killing of plasmid-free cells. Type III was discovered very recently and is represented so far by a unique example (Fineran et al., 2009). The hok-sok, ccdAB, and toxIN loci encoding TAs of type I, type II, and type III, respectively, will be briefly described below.

2.1 Type I TA: hok-sok system

In 1986, a two-gene set was shown to be involved in the stable inheritance of the naturally occurring R1 plasmid in Escherichia coli (Gerdes et al., 1986). The genes were called hok and sok for host killing and suppressor of host killing, respectively. The first experiments demonstrated that when hok expression was induced, it resulted in the loss of bacterial membrane potential and prevented the consumption of oxygen by the bacteria leading to cell killing. When sok was co-induced, the killing was suppressed. Upon analysis it was found that hok gene encoded a stable protein, while sok was a non-coding unstable antisense RNA that was complementary to hok mRNA (Gerdes et al., 1988). Hok/sok TA mediates plasmid stabilization by promoting post-segregational killing of cells that have lost TA-encoding plasmids. When cells undergo division and a plasmid copy is not transmitted to daughter bacteria, the sok antisense RNA is rapidly degraded by the host RNAses and the hok mRNA is released from inhibition, leading to killing of plasmid-free cells (Gerdes et al., 1988).

2.2 Type II TA: ccdAB system

During the same period, another two-gene set named ccdAB_{F} for ability to control cell death (subscript denotes where the TA was found: plasmid designation if plasmidic or bacterial genus/species initials if chromosomal) was shown to enhance the stable inheritance of the F
plasmid in *E. coli* (Jaffe *et al.*, 1985). Later studies revealed that CcdB$_F$ was a toxin that inhibits DNA replication by damaging the DNA gyrase enzyme, while CcdA$_F$ was a protein antitoxin that prevents CcdB$_F$ toxicity (Bernard & Couturier, 1992). CcdAB$_F$ is classified as a type II TA as both toxin and antitoxin are proteins, where the labile antitoxin neutralizes the toxicity of the cognate toxin by forming a harmless protein complex (Bernard & Couturier, 1992). Recently, genes homologous to the plasmidic genes were found during the bioinformatic analysis of complete genome sequence of clinical *E. coli* isolates. The functions of the chromosomal *ccdAB$_{0157}$* genes corresponded to the function of the *ccdAB$_F$* genes located on plasmid F (Wilbaux *et al.*, 2007). Interestingly, cross-expression experiments showed that while CcdB$_{0157}$ toxin could be neutralized by CcdA$_F$ antitoxin, CcdA$_{0157}$ antitoxin could not interfere with the toxic activity of CcdB$_F$ toxin (Wilbaux *et al.*, 2007).

### 2.3 Type III TA: toxIN system

Recently, a novel class of TA systems was discovered on a cryptic plasmid of the Gram-negative phytopathogen *Erwinia carotovora* subspecies *atroseptica*. ToxIN is a type III TA system composed of a toxin-antitoxin pair, with ToxN toxin inhibiting bacterial growth and ToxI antitoxin counteracting the toxicity of ToxN (Fineran *et al.*, 2009). The RNA nature of ToxI (*toxI* is transcribed but not translated) suggested that the ToxIN system was an antisense TA module. However, when *toxI* RNA was overexpressed, the ToxN protein was detectable, suggesting a new class of TAs in which the RNA antitoxin interferes with the biochemical activity of the protein toxin (Fineran *et al.*, 2009). Interestingly, ToxN toxin has sequence identity (31%) to AbiQ protein of *Lactococcus lactis* (Emond *et al.*, 1998). AbiQ is involved in protection against multiple phages through abortive infection (Abi) and provides population protection by promoting altruistic suicide of a phage-infected cell. ToxIN TA module also functions as an Abi system by directing abortive infection of multiple phages (Fineran *et al.*, 2009).
3. TA distribution

Although TAs were initially described on plasmids, it has since been shown that TAs are also ubiquitously found on the chromosome of archae and bacteria (Pandey & Gerdes, 2005). Type I TAs appear to be less represented in microbial chromosomes relative to the predominantly studied and characterized type II TAs (Fozo et al., 2010). In addition, exhaustive PSI-BLAST and TBLAST searches across 774 bacterial genomes to identify homologs of known toxins, suggested that type I TA loci were not horizontally transferred, while type II TAs moved through lateral gene transfer (Fozo et al., 2010). Type III TAs were discovered only recently and are still pending an exhaustive bioinformatics analysis.

Little is known about the distribution of type I TA loci. The development of computational approaches to identify novel type I TAs is challenging due to the short, hydrophobic character of the toxin proteins and the difficulty in predicting the antitoxin small RNAs. Until now, type I TA loci have only been observed in Firmicutes and γ-Proteobacteria (Fozo et al., 2010).

In contrast, type II TAs are widely distributed throughout the chromosomes of almost all free-living bacteria and archae. Upon analysis of 750 sequenced prokaryotic genomes, Makarova et al. (2009) predicted that individual chromosome of bacteria and archae may contain over 50 TA loci, with the vast majority encoding at least one. The maximum distribution was shown for the vapBC and relBE TA systems (42% and 23% of the number of all found TA loci). No correlation between the number of TAs, the lifestyle, the phylum or growth rate could be drawn. One of the trends observed was that archae generally possess more TAs relative to their genome size than bacteria (Pandey & Gerdes, 2005). Also, thermophiles have more TAs compared to mesophiles and psychrophiles (Pandey & Gerdes, 2005). Moreover, terrestrial and multi-environmental microbes possess fewer TAs, while microorganisms living in aquatic, host-associated and specialized environments contain a greater number of TAs (Pandey & Gerdes, 2005). A certain tendency was also revealed in the location of TA modules in the chromosome. If they are numerous, members of the same TA family can be found in genomic islands, mobile genetic elements or superintegrons (Makarova et al., 2009). Consistent with the focus of our study, all further references within this thesis to TA will refer solely to type II TA systems.
4. Toxin families

The toxin-antitoxin database (TADB) is an integrated database that provides comprehensive information about type II TA systems in bacteria and archae (http://bioinfomml.sjtu.edu.cn/TADB). Using the web-interface, users can retrieve the full list of 14 TA families that encompass the full set of current TADB entries that have been to date mapped to a specific family. Recently, Makarova et al. (2009) reported a comprehensive comparative-genomic analysis of TA systems in prokaryotes and predicted 12 new families of toxins and 13 new families of antitoxins. Table 1 lists the currently known type II toxins and their characteristics.

**TABLE 1. Current toxin families**

<table>
<thead>
<tr>
<th>Toxin family</th>
<th>Target</th>
<th>Mode of action</th>
<th>Cellular process</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcdB</td>
<td>DNA gyrase</td>
<td>Generates DS breaks</td>
<td>Replication</td>
</tr>
<tr>
<td>ParE</td>
<td>DNA gyrase</td>
<td>Generates DS breaks</td>
<td>Replication</td>
</tr>
<tr>
<td>RelE</td>
<td>Translating ribosome</td>
<td>Induces mRNA cleavage</td>
<td>Translation</td>
</tr>
<tr>
<td>Doc</td>
<td>Translating ribosome</td>
<td>Induces mRNA cleavage</td>
<td>Translation</td>
</tr>
<tr>
<td>HigB</td>
<td>Translating ribosome</td>
<td>Induces mRNA cleavage</td>
<td>Translation</td>
</tr>
<tr>
<td>HipA</td>
<td>EF-Tu</td>
<td>Protein kinase</td>
<td>Translation</td>
</tr>
<tr>
<td>MazF</td>
<td>RNA</td>
<td>Endoribonuclease</td>
<td>Translation</td>
</tr>
<tr>
<td>VapC</td>
<td>RNA</td>
<td>Endoribonuclease</td>
<td>Translation</td>
</tr>
<tr>
<td>HicA</td>
<td>RNA</td>
<td>Induces mRNA cleavage</td>
<td>Translation</td>
</tr>
<tr>
<td>Zeta</td>
<td>nd</td>
<td>Phosphotransferase</td>
<td>nd</td>
</tr>
</tbody>
</table>

*aAdapted from Van Melderen, 2010.*
4.1 CcdB toxin family

The CcdB toxin family has been found so far only in Gram-negative bacteria. The most extensively studied CcdB toxin is located on the F sex factor plasmid of *E. coli*. Plasmid F of *E. coli* contains a pair of genes, *ccdA* and *ccdB*, whose protein gene products are involved in plasmid maintenance (Bernard & Couturier, 1992). Recently, it has been shown that the foodborne pathogen *E. coli* O157:H7 (Wilbaux et al., 2007), the plant pathogen *Erwinia chrysanthemi* (Saavedra De Bast et al., 2008) and the marine bacterium *Vibrio fischeri* (De Jonge et al., 2007) also contained *ccd* homologues, named *ccdAB*$_{0157}$, *ccdAB*$_{Ech}$, and *ccdAB*$_{Vf}$, respectively, in their chromosomes.

In *E. coli*, the *ccdAB*$_{F}$ locus is located adjacent to the origin of replication of F plasmid (Ogura & Hiraga, 1983). The two genes are organized in an operon in which the antitoxin *ccdA*$_{F}$ gene is upstream of the toxin *ccdB*$_{F}$ gene. CcdA$_{F}$ antitoxin prevents CcdB$_{F}$ toxicity by forming a tight CcdA$_{F}$–CcdB$_{F}$ complex (Afif et al., 2001). CcdB$_{F}$ toxin inhibits DNA replication by DNA gyrase poisoning (Bernard & Couturier, 1992). More specifically, CcdB$_{F}$ prevents DNA gyrase from disentangling DNA for replication resulting in double-stranded breaks in the DNA (Doa-Thi et al., 2005). CcdA$_{F}$ and CcdB$_{F}$ are able to form complexes of different stoichiometry depending of the CcdA$_{F}$–CcdB$_{F}$ molar ratio (Van Melderen et al., 1996). CcdA$_{F}$ antitoxin also possesses a ribbon-helix-helix (RHH) domain which has been implicated in the negative autoregulation of the *ccdAB*$_{F}$ TA system by binding directly to the operator region of the operon. When CcdB$_{F}$ toxin is present, the affinity of CcdA$_{F}$ antitoxin for the operator region is enhanced (Van Melderen et al., 1996). CcdA$_{F}$ antitoxin is an unstable protein mainly due to its partially unfolded C-terminal end that is readily degraded by the host protease Lon (Van Melderen, et al., 1994). When CcdB$_{F}$ toxin is present in excess, a hexameric complex, $\{\text{CcdA}_F\}_2 \cdot \{\text{CcdB}_F\}_4$, unable to bind to the operator region of *ccdAB*$_{F}$ operon is formed, leading to de-repression of *ccdAB*$_{F}$ transcription (Van Melderen et al., 1996). Therefore, the molar ratio of the CcdAB$_{F}$ complex determines whether the *ccdAB*$_{F}$ operon is negatively autoregulated at the level of transcription.
4.1.1 Anti-addiction model

The discovery of chromosomal ccd homologues has led some research groups to suggest that chromosomal TA systems can selectively advantage their host in post-segregational killing conditions. By having a homologous ccdA antitoxin gene encoded on the chromosome, the plasmid-borne CcdA antitoxin would be replenished preventing CcdB-induced DNA cleavage (Van Melderen & Saavedra De Bast, 2009). This phenomenon has been observed in *E. chrysanthemi* with respect to its F plasmid-encoded ccd homolog (Saavedra De Bast et al., 2008). In an *E. coli* strain containing the ccdAB_Ech genes inserted into its chromosome, no post-segregational killing was observed upon the loss of a plasmid carrying CcdAB_F TA system (Wilbaux et al., 2007). Consequently, chromosomal TA systems were hypothesized to act as anti-addiction modules.

4.2 ParE toxin family

The ParE toxin family is found in both Gram-positive and Gram-negative bacteria, and has been discovered on the broad-host-range plasmid RK2 (Roberts & Helinski, 1992). Three homologues of the parDE_RK2 TA system, parDE1_Vc, parDE2_Vc, and parDE3_Vc, are also present in the genome of *Vibrio cholerae* within the superintegron located on chrII (*V. cholerae* possesses two circular chromosomes, named chrI and chrII; 13 TA loci have been found in chrII) (Yuan et al., 2011). The ParE toxins inhibit bacterial cell division and reduce viability, presumably due to their capacity to damage DNA (Jiang et al., 2002). Recent studies have shown that ParE1_Vc, ParE2_Vc, and ParE3_Vc toxins act as DNA gyrase inhibitors (Yuan et al., 2010). The mechanism by which the ParE2_Vc toxin poisons the DNA gyrase was studied in more details. Results showed that ParE2_Vc, like unrelated F-encoded CcdB toxins, targets the gyrase enzyme and stalled the DNA–gyrase complex. However, in contrast to other DNA gyrase inhibitors, ParE2_Vc interferes with gyrase-dependent supercoiling but not DNA relaxation (Yuan et al., 2010).
4.2.1 Genome stabilization model

The plasmid-borne ParDE\textsubscript{RK2} TA system has been implicated in plasmid stabilization. In the same way, it has been suggested that chromosomal par TA systems may be stabilizers of genomic regions to prevent their loss during evolution. Recent studies suggested that the ParAB\textsubscript{2} TA system located on chrII led to post-segregational killing of \textit{V. cholerae} cells missing chrII in a manner similar to post-segregational killing mediated by plasmid-encoded par homologs (Yuan \textit{et al}., 2011). In a \textit{parAB2} deletion mutant of \textit{V. cholerae}, chrII is mislocalized and frequently fails to segregate, yielding a high frequency of cells that lack chrII (Yuan \textit{et al}., 2011). Cells containing only chrI divide once and then become CHUB (condensed nucleoid, hypertrophic, undividing bacteria) cells (Yuan \textit{et al}., 2011). The authors concluded that the chromosomal ParAB\textsubscript{2} TA system could function in a chromosome-specific manner to promote the accurate subcellular localization and maintenance of \textit{V. cholerae} chrII but not chrI (Yuan \textit{et al}., 2011).

4.3 RelE toxin family

The RelE toxin of the RelBE TA system is one of the best-studied chromosomal toxins. The RelBE systems are widely distributed in archae and bacteria and have been mostly studied in \textit{E. coli}, \textit{Mycobacterium tuberculosis}, \textit{Caulobacter crescentus}, \textit{Streptococcus pneumoniae}, \textit{Staphylococcus aureus}, and \textit{Methanococcus jannaschii} (Flebig \textit{et al}., 2010; Francuski \& Saenger, 2009; Gerdes \textit{et al}., 2005; Korch \textit{et al}., 2009; Yoshizumi \textit{et al}., 2009).

The RelBE TA modules usually constitute a two-component toxin-antitoxin system, a stable toxin and a labile antitoxin. The RelE toxin forms a stable complex with the RelB antitoxin (Overgaard \textit{et al}., 2009). The RelB–RelE complex (and to a lesser degree the antitoxin alone) regulates its own transcription. In \textit{E. coli}, the RelBE system is composed of three genes (\textit{relB}, \textit{relE}, and \textit{relF}) and thus belongs to a new TA gene family (Gotfredsen \& Gerdes, 1998). The \textit{relE} gene encodes a cytotoxin lethal or inhibitory to host cells, while \textit{relB} gene encodes its cognate antitoxin. The \textit{relF} gene (also named \textit{hokD}) encodes a \textit{hok} homolog (Gotfredsen \& Gerdes, 1998). When overexpressed \textit{in vitro}, RelF causes rapid cessation of cell growth, arrest of
respiration, and collapse of the cell membrane potential (Gotfredsen & Gerdes, 1998). The physiological significance of RelF is still unknown but translation of relF is most probably inhibited since analysis of the relF gene revealed that it was missing a key sequence element upstream called the translation activation element (tac) essential for the hok-homologue to be translated in vivo (Gotfredsen & Gerdes, 1998). RelF probably constitutes an evolutionary relic of an ancient hok-homolog (Gotfredsen & Gerdes, 1998).

Although TA systems do not generally cross-talk with other homologous TAs present on the same chromosome, cross-talking has been observed amongst a set of RelE toxins in M. tuberculosis. One study reported cross-complexation between three chromosomal RelBE-like systems (RelBE, RelFG, and RelJK) in M. tuberculosis H37Rv strain (Yang et al., 2010). Results showed that RelB antitoxin was able to form a harmless complex with RelG toxin and prevent growth inhibition. However, when RelF antitoxin complexed with RelE toxin, RelF enhanced RelE toxicity. Similarly, replacing RelJ antitoxin with RelB antitoxin increased the toxicity of RelK toxin. Consequently, interactions between different pairs of TA modules is possible and cross-regulation can cause either an enhanced protective or toxic effect on bacterial growth.

Similarly to CcdB and ParE toxin families, RelE toxin family autoregulates their own transcription. In E. coli, RelB antitoxin binds specifically to palindromic repeats (PR) located in the promoter region of the operon (Li et al., 2008). By complexing with RelB antitoxin, RelE toxin enhances the binding affinity of the antitoxin to the PR region. It was suggested that RelE toxin could modify the secondary structure of the antitoxin to make its DNA-binding motif more accessible to the PR region resulting in a stronger bond with DNA sequence (Li et al., 2008). It was also hypothesized that the large RelB–RelE complex can act as a roadblock preventing the RNA polymerase from transcribing the operon (Li et al., 2008). Interestingly, RelE toxin can also stimulate TA operon transcription. Using in vitro transcription studies, Gerdes and coworkers demonstrated how RelE toxin can function both as co-repressor and de-repressor of relBE transcription in a process called conditional cooperativity (Overgaard et al., 2008). When RelB antitoxin is in excess to RelE toxin, the {RelB}$_2$–{RelE} complex binds cooperatively to two adjacent operator sites in the promoter region and repressed transcription. In contrast, RelE
in excess stimulates transcription of the operon by releasing the \{RelB\}_2–\{RelE\} complex from the promoter region (Overgaard \textit{et al.}, 2008).

The RelE toxin family is composed of translation inhibitors. RelE is an endoribonuclease that inhibits protein synthesis by cleaving mRNA through its association with the ribosome (Pedersen \textit{et al.}, 2003). Further analysis showed that RelE cleaved mRNAs between the second and third base of specific codons at the A-site to halt and thus prevent termination of the translation process. The highest cleavage rate was observed for the UAG, UAA and UGA stop codons as well as CAG and UCG sense codons (Pedersen \textit{et al.}, 2003). RelE toxins are commonly referred to as mRNA interferases.

\textbf{4.3.1 Growth modulation model}

The RelBE chromosomal TA system is hypothesized to modulate cell growth to cope with stress and survive adverse environmental conditions. By inhibiting global protein synthesis, RelE toxin induces a dormant state (stasis). When favorable conditions return, production of RelB antitoxin resumes resulting in a reversal of RelE-induced translation blockade. Upon amino acid starvation, \textit{relBE} transcription is enhanced through the Lon-dependent degradation of RelB antitoxin in a ppGpp-independent manner (Christensen \textit{et al.}, 2001). As a consequence, RelE toxin inhibits translation by cleaving mRNA present in the ribosome (Christensen and Gerdes, 2003). Such mRNAs lack their natural stop-codons and tmRNA (transfer-messenger RNA) is needed to release ribosomes locked at their termini (Karzai \textit{et al.}, 1999). When favorable growth conditions return, the ribosomes are rescued by tmRNA and RelB antitoxin in a reaction called trans-translation that simultaneously mediates ribosome recycling and tagging of incomplete proteins for degradation by cellular proteases. Once the ribosome is released, the translation cycle resumes, and the cells become capable of normal growth (Christensen & Gerdes, 2003).
4.4 Doc toxin family

The *phd-doc* TA system is responsible for the stable inheritance of the plasmid prophage of bacteriophage P1 in *E. coli* and represents the archetype of a very small family of TA modules (Gerdes *et al.*, 2005). The *phd-doc* operon encodes the Doc toxin (death on curing) and Phd antitoxin (prevent host death) (Lehnherr & Yarmolinsky, 1995). The Phd antitoxin possesses a DNA-binding motif in its N-terminal region allowing for the negative autoregulation of the operon by binding to two palindromic sequences in the promoter region of *phd-doc* operon (Zhao & Maguson, 2005). Doc toxin also interacts with Phd antitoxin in the repressive complex to mediate cooperative interactions between the two palindromic regions and thereby enhances transcriptional repression (Zhao & Magnuson, 2005). When the plasmid prophage is lost, the unstable Phd antitoxin is rapidly degraded by the host-encoded ClpXP protease (Lehnherr & Yarmolinsky, 1995). Doc toxin targets the translation process through the inhibition of the elongation step by associating with the 30S ribosomal subunit causing cell growth arrest on curing (Liu *et al.*, 2008).

4.5 HigB toxin family

The HigB toxin family has been found in both Gram-positive and Gram-negative bacteria. Interestingly, one or more chromosomal *higB* homologs have been reported for several pathogens, including *V. cholerae*, *S. pneumoniae*, and *E. coli* CFT073 (uropathogenic) and O157:H7 (enterohemorrhagic) strains (Pandey & Gerdes, 2005). Some characterization of the two *V. cholerae* HigBA TA modules has been performed. In contrast to other TA systems, the toxin gene (*higB*) is found upstream the antitoxin gene (*higA*) (Budde *et al.*, 2007). Overexpression of the toxin gene (*higB1, higB2*) inhibits the growth of *V. cholerae* and decreases its viability. The antitoxin (HigA1, HigA2) is able to counteract the toxic activity of its cognate toxin only. Similarly to RelBE TA system, HigB antitoxin is degraded by the host-encoded Lon protease and HigB toxin leads to inhibition of protein synthesis through translation-dependent mRNA cleavage, though its target cleavage sequences have not yet been identified (Christensen-Dalsgaard *et al.*, 2010). The function of the *V. cholerae* HigBA systems remains to be determined. One possibility is that HigB toxin inhibits cell growth under stress or unfavorable
growth conditions (growth-modulation model) (Budde et al., 2007). Another possibility is that HigBA systems play a role in stabilization of *V. cholerae* chromosomes (genome stabilization model) (Christensen-Dalsgaard & Gerdes, 2006).

### 4.6 HipA toxin family

The *hipA* gene, encoding the toxic factor, together with *hipB*, encoding a putative antitoxin, constitutes one of the several TA modules present in bacteria. The HipBA TA system has been mainly studied in *E. coli*. Similar to typical TA operons, *hipB* and *hipA* are organized in an operon with the gene encoding the antitoxin, *hipB*, located upstream of *hipA*, the toxin gene (Schumacher et al., 2009). Overexpression of the HipA toxin causes cell stasis and ectopic induction of HipB antitoxin rescues cells overexpressing HipA (Schumacher et al., 2009). HipB is a Cro/CI-like DNA binding protein that negatively regulates the *hipBA* operon. Interestingly, the *hipA* (high persister) gene was the first gene linked to persisters (Moyed & Bertrand, 1983). Persisters are cells that neither grow nor die in presence of bactericidal antibiotics (Lewis, 2010). These cells are not mutants but phenotypic variants of regular cells that form stochastically in microbial populations (Lewis, 2010). HipA toxin is a protein kinase. The crystal structure of HipA in complex with its antitoxin HipB was recently resolved, and a pull-down experiment showed that the substrate of HipA is the elongation factor EF-Tu that catalyses the transfer of aminoacyl-transfer RNA to the ribosome during translation. Phosphorylated EF-Tu is inactive, which leads to a block in translation and bacterial persistence (Schumacher et al., 2009).

#### 4.6.1 Persistence model

The persistence phenomenon was first discovered by Joseph Bigger in 1944 (Bigger, 1944). Bigger treated *S. aureus* with high concentrations of penicillin and observed a small fraction of surviving cells. These survivors were able to re-grow after the removal of penicillin and were still as susceptible to penicillin as the original population. Thus, Bigger termed this phenomenon as ‘persistence’. Moreover, he postulated that these persisters were in a state of
dormancy, which allowed them to withstand the bactericidal effects of penicillin. This is similar to the viable but non-culturable (VBNC) state in which bacteria do not divide and have a very low metabolic activity (Keep et al., 2006). Although bacteria in a VBNC state have altered peptidoglycan (Signoretto et al., 2002), little is known about changes in the peptidoglycan structure of persister cells. Despite the fact that persisters are highly important in antibiotic therapy, the mechanism for persistence is still not thoroughly understood. Persisters are not genetic mutants. They are phenotypic variants of regular cells that form stochastically in microbial populations (Lewis, 2010). It has been suggested that persisters may play a significant role in the recalcitrance of chronic biofilm infections to antimicrobials (Lewis, 2001). Indeed, biofilms show a surprising ability to resist killing by antibiotics without having any obvious drug resistance mechanisms (Davies, 2003). Persisters are an inherent part of all microbial populations (Jayaraman, 2008). One of the main questions about the phenomenon of persistence is whether bacteria have evolved a dedicated mechanism that allows them to survive lethal stresses (e.g. antibiotic exposure). In E. coli multiple TA systems have been linked to the formation of persisters. It has been shown that ectopic mild-overexpression of TA systems induced bacterial persistence (Lewis, 2010). The stochastic expression of toxic proteins from TA modules in a subpopulation of cells maintains persisters in a dormant non-growing state. By shutting down antibiotic targets, toxins can thus produce tolerant cells.

4.7 MazF toxin family

Among chromosomal TA modules other than RelBE, only MazEF has been studied extensively. In E. coli, the mazEF system consists of two genes, mazE and mazF, located downstream of the relA gene encoding a (p)ppGpp synthetase (Aizenman et al., 1996). The MazE and MazF pair forms a stable complex under normal growth conditions, while under stress or unfavorable growth conditions (e.g., amino acid starvation, DNA damage, oxidative stress, antibiotics), degradation of MazE antitoxin by the host-encoded ATP-dependent ClpPA serine protease results in immediate MazF toxin release, allowing MazF to exert its effect to the cell. MazF toxin is an endoribonuclease which cleaves mRNAs at ACA sequences in a ribosome-independent manner (Zhang et al., 2005). Although MazE antitoxin autoregulates mazEF
operon, it does not possess a classical HTH or RHH motif as found in other antitoxins (Loris et al., 2003). The MazE monomer has no hydrophobic core on its own and represents only one-half of a typical protein domain. A complete domain structure is formed by the association of two chains, creating a hydrophobic core between two four-stranded β-sheets. The folded part of MazE antitoxin constitutes a new DNA binding motif (Loris et al., 2003).

The *E. coli* chromosomal MazEF system can be expressed from two promoters, P2 and P3, which are located 13 bp apart (Aizenman et al., 1996; Marianovsky et al., 2001). Expression from both promoters is weakly repressed by MazE and efficiently autoregulated by MazE–MazF complex. Under conditions of extreme amino acid starvation, *mazEF* expression is regulated at the P2 promoter by the cellular level of ppGpp, the product of the RelA enzyme.

### 4.7.1 Programmed cell death model

MazEF TA has been implicated in programmed cell death (PCD). PCD can be described as any death mediated by a genetically regulated intracellular death program (Lewis, 2000). In more developmentally complex multicellular organisms, PCD is required for homeostasis, development of tissues, and elimination of defective cells (Skulachev, 2002). In prokaryotes, it has been hypothesized that PCD could be an active process that results in altruistic suicide occurring either during the developmental life cycle or to remove damaged cells from a population in response to a wide variety of stresses (Lewis, 2000).

The MazEF TA module of *E. coli* was the first to be described as responsible for bacterial PCD in response to a variety of environmental stresses (Aizenman et al., 1996). MazEF-directed bacterial PCD is a population phenomenon that depends on the density of the bacterial culture; death occurs in a dense culture, while not in a diluted culture (Kolodkin-Gal et al., 2007). The phenomenon requires an extracellular quorum sensing molecule, called EDF (extracellular death factor) (Kolodkin-Gal et al., 2007). EDF is a linear pentapeptide (Asn-Asn-Trp-Asn-Asn) sensitive to extreme pH, high temperatures, and proteinase K. Each of the five amino acids is important for the MazEF-directed PCD, and the terminal Asn residues are the most important. EDF production involves the glucose-6-phosphate 1-dehydrogenase and the ClpXP protease.
Based on preliminary results obtained by Engelberg-Kulka’s group, glucose-6-phosphate 1-dehydrogenase, carrying the amino acid sequence Asn-Asn-Trp-Asn-Asn, may be the precursor of EDF, and ClpXP is most probably the protease involved in the generation of EDF (Kolodkin-Gal et al., 2007). Until now, only one group presented evidence that MazEF TA was involved in bacterial PCD. Indeed, Engelberg-Kulka and coworkers demonstrated that *E. coli* MazEF is a stress-induced suicidal TA module, and yet MazEF-induced PCD is still subject to debate since it has not been reproduced by other groups.

### 4.7.2 Development model

MazEF TA has also been implicated in the development model. This model was proposed for the fruiting body formation in *Myxococcus xanthus*, a Gram-negative soil-dweller that undergoes a complex developmental cycle in response to nutrient limitation conditions (Nariya & Inouye, 2008). Interestingly, *M. xanthus* possesses a solitary chromosomal *mazF* gene that lacks a co-transcribed *mazE* antitoxin gene (Nariya & Inouye, 2008). Instead, the *mazF* gene is integrated in a regulatory cascade controlled by the key developmental regulator MrpC (Sogaard-Andersen & Yang, 2008). During the process of fruiting body, 80% of the whole myxococcal population dies by cell lysis while 20% develops into myxospores (Nariya & Inouye, 2008). It has been shown that MazF toxin plays a critical role in fruiting body formation as a *mazF* deficient mutant showed a dramatic reduction of spore formation (Nariya & Inouye, 2008). The global regulator MrpC functions as an antidote and transcriptional activator (Sogaard-Andersen & Yang, 2008). During vegetative growth, the bifunctional transcription factor/antitoxin is phosphorylated through a Ser/Thr protein kinase and negatively regulates *mrpC* and *mazF* expression. When *M. xanthus* engages in fruiting body formation, MrpC transcription activity is activated most likely by a Lon-dependent cleavage. MazF toxin, an mRNA interferase, is then produced and cleaves cellular mRNAs to efficiently block protein synthesis and induces cell death (Sogaard-Andersen & Yang, 2008). This example can be seen as an evolution of the TA modules and how effectively they can be integrated into the regulatory network of the bacteria.
### 4.8 VapC toxin family

The first VapBC TA module has been identified in pathogenic strains of the Gram-negative strict anaerobe *Dichelobacter nodosus*. The TA has been named *vap* for virulence associated protein (Katz *et al.*, 1992; 1994). The VapC toxin family is now widely disseminated and has been identified in archae, Gram-positive and Gram-negative bacteria. For example, *M. tuberculosis* genome encodes 45 *vapBC* loci (Miallau *et al.*, 2009) while the nitrogen-fixing symbiont of legumes, *Sinorhizobium meliloti* has 21 *vapBC* loci (Bodogai *et al.*, 2006). The chromosomal *vapBC* loci encode the VapC toxin preceded by the VapB antitoxin which contains a transcription factor domain (Robson *et al.*, 2009). VapC toxins possess PIN domains containing a motif thought to be associated with ribonuclease activity and have been shown to block translation via RNA cleavage (Daines *et al.*, 2007; Miallau *et al.*, 2009).

### 4.9 HicA toxin family

The HicAB TA family has many members in bacteria and archae. The *hicAB* locus has been described first as an insertion into the major pilus gene cluster in several strains of *Haemophilus influenza* (Syed & Gilsdorf, 2007). Indeed, a bicistronic locus closely linked to the pilus gene cluster of *H. influenzae* (*hif*) was called *hic* for *hif* contiguous. Predicted HicA toxins are small and have a double-stranded RNA-binding fold, while predicted HicB antitoxins have a DNA binding domain fused to a degraded RNase H fold (Makarova *et al.*, 2006). Work done in *E. coli* has shown that HicA inhibited translation and induced mRNA cleavages in a ribosome-independent manner, while induction of *hicB* counteracted the effect of the toxin (Jorgensen *et al.*, 2009). Transcription of *hicAB* locus was induced by amino acid and carbon starvation by a Lon-dependent mechanism (Jorgensen *et al.*, 2009).

### 4.10 Zeta toxin family

The zeta toxin family has been found only in Gram-positive bacteria. The first omega-epsilon-zeta TA locus has been identified in pSM19035 of *Streptococcus pyogenes*, a low-copy-
number plasmid carrying erythromycin resistance (Zielenkiewicz & Ceglowski, 2005). The omega-epsilon-zeta operon constitutes a novel plasmid addiction system in which the epsilon and zeta genes encode an antitoxin and toxin, respectively, while omega plays an autoregulatory function (Zielenkiewicz & Ceglowski, 2005). The toxic protein zeta contains a highly conserved Walker-A motif for ATP/GTP binding, as well as a phosphoryltransferase active site. Epsilon antitoxin neutralizes the toxicity of zeta by blocking access to the phosphoryltransferase active site through formation of a tight complex with zeta. Expression of toxin zeta is bactericidal for Bacillus subtilis and bacteriostatic for E. coli. However, the mode of action of the zeta toxin remained elusive.

5. *Streptococcus mutans* as a model organism

The human oral cavity contains a complex microbial flora (Aas *et al*., 2005). Recent estimates of the number of bacterial species using massively parallel DNA sequencing have suggested that the oral cavity may contain as many as 19,000 bacterial phylotypes, while each individual will only have a proportion of these microbes (Keijser *et al*., 2008). These bacterial species maintain themselves in the mouth by attaching to different surfaces on which they form oral biofilms (Kuramitsu *et al*., 2007). Several factors may contribute to the microbial diversity of the oral biofilm such as abundance of nutrients, moisture, hospitable temperature, and the availability of different surfaces (tongue, epithelial cells, enamel). The community of microorganisms that form on the tooth surface is called the dental plaque biofilm (Marsh, 2005). Dental plaque is perhaps the best-studied biofilm and serves as a good model for the study of other microbial biofilms. Dental plaque forms within minutes or few hours after a professional cleaning, and is composed of more than 700 different bacterial species (Aas *et al*., 2005). Extensive clinical studies have indicated that the oral microbial flora is responsible for two major biofilm-related diseases: dental caries (tooth decay) and periodontal diseases (Kuramitsu *et al*., 2007).

Of all the bacterial species found in dental plaque biofilm, only the mutants streptococci have been shown to have a clear association with the initiation of dental caries (Loesche, 1986). *Streptococcus mutans* is a low G+C Gram-positive, non-haemolytic, non-spore forming, non-
motile bacteria and is considered the leading etiological agent of dental caries, which is perhaps the most common bacterial infection in humans (Hamada & Slade, 1980). Caries is the dissolution of tooth enamel caused by the prolonged exposure to bacterially derived organic acids. *S. mutans* has a central role in the etiology of dental caries because it can adhere to the enamel salivary pellicle and to other plaque bacteria (Mitchell, 2003). *S. mutans* is also a strong acid producer (acidogenicity) and hence causes an acidic environment creating the risks for cavities (Banas, 2004). *S. mutans* can also survive at pH values that are lethal to most of the other bacterial species present in the oral biofilm (aciduricity) (Lemos & Burne, 2008). Indeed, the acidogenicity and aciduricity of *S. mutans* lead to ecological changes in the plaque flora that includes an elevation in the proportion of *S. mutans* and other acidogenic and acid-tolerant species (Takahashi & Nyvad, 2008). Although *S. mutans* is the predominant species, it is also recognized that caries can occur in its absence. Other oral bacteria potentially responsible for dental caries include *Actinomyces odontolyticus* and *Lactobacilli* species (Loesche, 1986).

*S. mutans* has also been isolated from cases of infective endocarditis (Nomura et al., 2006; Schelenz et al., 2005). Various dental procedures (e.g., dental extractions, brushing of teeth) can lead to a transient bacteremia with oral microorganisms. About 20% of the infective endocarditis cases attributed to mutans streptococci are caused by *S. mutans*.

### 5.1 *S. mutans* in the oral biofilm

The bacteria comprising the dental plaque biofilm are not randomly distributed. Indeed, dental plaque shows a high degree of organization and is formed by sequential and ordered colonization of multiple species of bacteria (Kolenbrander et al., 2006). During the process of dental plaque formation, some oral bacterial species, known as early colonizers, adhere to the acquired pellicle coating the tooth surface, which is composed of salivary proteins (sialyted mucins, α-amylase, prolin-rich proteins, agglutinin, statherin) and bacterial cell fragments. Late colonizers then bind to early colonizers that are already attached (Fig. 1). Under normal circumstances, the dental plaque biofilm remains relatively stable contributing to dental health (Marsh, 2006; Sbordone & Bortolaia, 2003).
The ecological plaque hypothesis proposed that potentially pathogenic organisms can be present in healthy individuals but at levels that are not clinically relevant (Sbordone & Bortolaia, 2003). The disease is a result of a shift in the balance of the resident microflora due to a response to a change in local environmental conditions (e.g., sugar-rich diet, low saliva flow, immune suppression) leading to a major ecological pressure. These conditions disrupt the natural homeostasis present in plaque during health, leading to the environment of organisms that can cause disease (Takahashi & Nyvad, 2008). For example, a sucrose-rich diet produces prolonged conditions of acidic pH. This environmental change in dental plaque favors the growth of acid-tolerating bacteria, which are capable of demineralizing enamel, at the expense of healthy-associated species (Takahashi & Nyvad, 2008).

*S. mutans* has evolved a biofilm lifestyle for survival and persistence in the dental plaque. *S. mutans* colonizes the teeth via a two-stage process: initial attachment (both sucrose-dependent and sucrose-independent mechanisms) followed by sucrose-dependent accumulation (Nobbs et al., 2009). The action of glucosyltransferases in the synthesis of glucans is the major mechanism behind sucrose-dependent attachment. During the sucrose-independent process, *S. mutans* expresses several adhesins including P1 (also known as SpaP, antigen I/II, PAc, Sr), a cell surface protein of 185 kDa (Navarre & Schneewind, 1999). P1 contains multiple binding specificities. The alanine-rich and proline-rich domains are thought to be primarily responsible for the interaction between antigen I/II and salivary components coating the tooth surface. Table 2 provides a list of all *S. mutans* cell surface-associated adhesins and their putative role.
FIG. 1. Formation of dental plaque biofilm. Although streptococci make up 47-82% of the microbiota colonizing the tooth surface, studies by Socransky and coll. (1998, 2002) identified six specific microbial groups or bacterial ‘complexes’ within dental plaque biofilm (early colonizers: yellow, blue, green, and purple complexes; late colonizers: orange and red complexes).
<table>
<thead>
<tr>
<th>Protein family</th>
<th>Protein</th>
<th>Function and/or substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva-binding proteins</strong></td>
<td>Agl/II</td>
<td>Salivary components</td>
</tr>
<tr>
<td></td>
<td>SmFnB</td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>FBP-130</td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>PavA-like</td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>Agl/II</td>
<td>Fibronectin</td>
</tr>
<tr>
<td><strong>Fn-binding proteins</strong></td>
<td>GbpABCD</td>
<td>Sucrose-dependent adhesion, glucan</td>
</tr>
<tr>
<td><strong>Glucan-binding proteins</strong></td>
<td>WapA</td>
<td>Collagen</td>
</tr>
<tr>
<td></td>
<td>Cnm</td>
<td>Collagen</td>
</tr>
<tr>
<td></td>
<td>Agl/II</td>
<td>Collagen</td>
</tr>
<tr>
<td><strong>Collagen-binding proteins</strong></td>
<td>Enolase</td>
<td>Plasminogen, plasmin</td>
</tr>
<tr>
<td><strong>Plasminogen-binding proteins</strong></td>
<td>Agl/II</td>
<td>Laminin</td>
</tr>
<tr>
<td></td>
<td>Cnm</td>
<td>Laminin</td>
</tr>
<tr>
<td><strong>Laminin-binding proteins</strong></td>
<td>Agl/II</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td></td>
<td>Cnm</td>
<td>Fibrinogen</td>
</tr>
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<td><strong>Fibrinogen-binding proteins</strong></td>
<td>FBP-130</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td><strong>Host cell-binding proteins</strong></td>
<td>Agl/II</td>
<td>Epithelial/endothelial cells</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td>HtrA</td>
<td>Serine protease</td>
</tr>
<tr>
<td></td>
<td>FruA</td>
<td>ß-D-fructosidase</td>
</tr>
<tr>
<td></td>
<td>DexA</td>
<td>Dextranase</td>
</tr>
</tbody>
</table>

*a Adapted from Nobbs et al., 2009.*
5.2 S. mutans TA modules

By sequence homology, a total of 45 putative toxin and antitoxin genes have been identified in the chromosome of S. mutans UA159 reference strain on the basis of protein sequence and comparative genomic analysis. A number of orphan type II toxin genes lacking the adjacent antitoxin gene have also been found in searches for type II loci; it is possible that the synthesis of these toxins is repressed by antisense small RNAs. Of these loci, only four TA pairs possessed all of the features and could be predicted to function as bona fide type II TA systems (Table 3).

<table>
<thead>
<tr>
<th>Toxin family</th>
<th>Toxin</th>
<th>Antitoxin</th>
<th>TA pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>MazF</td>
<td>SMU.173</td>
<td>SMU.172</td>
<td>AbrB/MazE – MazF</td>
</tr>
<tr>
<td>COG2856(^a)</td>
<td>SMU.219</td>
<td>SMU.218</td>
<td>Xre – COG2856</td>
</tr>
<tr>
<td>RelE</td>
<td>SMU.896</td>
<td>SMU.895</td>
<td>RHH – RelE</td>
</tr>
<tr>
<td>VapC</td>
<td>SMU.1767</td>
<td>SMU.1768</td>
<td>AbrB – VapC</td>
</tr>
</tbody>
</table>

\(^a\)TA pairs identified by using the PostgreSQL database of type II TA systems (Shao et al., 2011) and the web-based RASTA-bacteria tool (Sevin & Barloy-Hubler, 2007).

\(^b\)New toxin family. Proteins of COG2856 contain a conserved HEXXH motif that is the sequence signature of numerous families of Zn-dependent proteases.
6. Statement of the problem

Mutants lacking mazF, relE, or both putative toxin-encoding genes were previously constructed and tested for their impact on the capacity of *S. mutans* to form biofilm and withstand acid stress. When both toxin genes were deleted, growth properties of *S. mutans* were affected suggesting that chromosomal TAs of *S. mutans* could function as regulators of cell growth in response to environmental stresses.

6.1 General Hypothesis & Specific Objectives

*S. mutans* TAs provide a control mechanism to help cells cope with environmental stress and thereby enable survival of the species in the event of sudden unfavorable environmental change. My overall goal is to answer the following question: Does the *S. mutans* MazEF module constitute a functional type II TA system? There are three specific objectives:

**Objective 1**: Genetic characterization of the SMU.172-SMU.173 locus

**Objective 2**: Biochemical characterization of MazE and MazF proteins

**Objective 3**: Functional characterization of MazEF type II TA module
The Chromosomal \textit{mazEF} Locus of \textit{Streptococcus mutans} Encodes a Functional Type II Toxin-Antitoxin Addiction System

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2.1 ABSTRACT

Type II chromosomal toxin-antitoxin (TA) modules consist of a pair of genes that encode two components: a stable toxin and a labile antitoxin interfering with the lethal action of the toxin through protein complex formation. Bioinformatic analysis of Streptococcus mutans UA159 genome identified a pair of linked genes encoding a MazEF-like TA. Our results show that S. mutans mazEF genes form a bisictronic operon that is cotranscribed from a σ70-like promoter. Overproduction of S. mutans MazF toxin had a toxic effect on S. mutans which can be neutralized by coexpression of its cognate antitoxin, S. mutans MazE. Although mazF expression inhibited cell growth, no cell lysis of S. mutans cultures was observed under the conditions tested. The MazEF TA is also functional in E. coli, where S. mutans MazF did not kill the cells but rather caused reversible cell growth arrest. Recombinant S. mutans MazE and MazF proteins were purified and were shown to interact with each other in vivo, confirming the nature of this TA as a type II addiction system. Our data indicate that MazF is a toxic nuclease arresting cell growth through the mechanism of RNA cleavage, and that MazE inhibits the ribonuclease activity of MazF by forming a complex. Our results suggest that the MazEF TA module might represent a cell growth modulator facilitating persistence of S. mutans under the harsh conditions of the oral cavity.
2.2 INTRODUCTION

Most prokaryotic chromosomes contain a number of toxic or suicidal genes that induce reversible cell growth arrest or death (18). These chromosomal toxin-antitoxin (TA) modules consist of a pair of genes that encode two components: a protein toxin and an antitoxin (labile protein or untranslated antisense RNA) that interferes with the lethal action of the toxin (15). Typically, the toxin inhibits an essential cell function such as translation or DNA replication (18). The TA modules are classified into two major types based on the nature of the antitoxin, the toxin always being a protein. Type I TA systems consist of an RNA antitoxin and a protein toxin, in which the RNA antitoxin inhibits translation of the toxin mRNA (13, 14). In type II TA systems, both toxins and antitoxins are proteins, where the unstable antitoxin neutralizes the toxicity of the cognate toxin by forming a harmless complex (for a recent review, see reference 32). In all studied type II TA systems, the protein antitoxin has a much shorter \textit{in vivo} half-life than the toxin and is degraded by a specific intracellular protease such as Lon or Clp (6). TA systems are also known as addiction modules as cells become ‘addicted’ to the short-lived antitoxin product (the unstable antitoxin is degraded faster than the more stable toxin) since its \textit{de novo} synthesis is essential for their survival (9). All reported type II TA operons are autoregulated at the level of transcription by the antitoxin or the concerted action of toxin and antitoxin binding to their promoter (32). The type II toxins characterized so far include protein kinases, phosphotransferases, DNA gyrase poisons, and ribosome-dependent and ribosome-independent ribonucleases (45). The vast majority of type II toxins are mRNA-specific endonucleases, also called mRNA interferases. Importantly, chromosomal type II TAs induce a
reversible bacteriostatic state since no toxin has been reported to be directly bacteriolytic (7, 32, 38, 43).

TA modules are ubiquitous in the chromosomes of archaea and bacteria, often found in multiple copies (32, 37). Because TA systems are thought to invade prokaryotic genomes through horizontal gene transfer and intragenomic recombination, they are commonly described as mobile genetic elements (32). Comprehensive genome analyses have revealed the diversity in the distribution of TA systems. Among bacteria, *Bacteroidetes*/*Chlorobi*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Cyanobacteria* possess the greatest variety of TAs, while *Firmicutes* are characterized by a particularly low TA diversity (32, 37). Recently, Makarova et al. (32) reported the most detailed and comprehensive comparative analysis of type II TAs in 750 complete genomes of archaea and bacteria. This analysis resulted in the prediction of 12 new families of putative toxins and 13 new families of putative antitoxins. Since these small modules are highly diverse and broadly distributed in prokaryotes, one can postulate that they might have multiple biological roles. For example, the fact that almost all obligate intracellular microorganisms (endosymbiotic species) are devoid of TA loci, whereas free-living prokaryotes have several TA loci, has led some researchers to propose the hypothesis that TAs are stress-response elements that help free-living prokaryotes to cope with environmental stress (6, 15). Indeed, obligate intracellular microorganisms thrive in constant environments and are thus expected to encounter minimal stress or environmental fluctuations. Nevertheless, the physiological role of chromosomal TAs still remains the subject of debate. Different hypotheses have been proposed to explain the role of TAs in bacterial physiology, including stress survival, growth control, programmed cell death, persister formation, stabilization of genome, and anti-addiction system (7, 8, 28, 31, 43, 45).
*Streptococcus mutans* is a member of the division *Firmicutes*. In the presence of fermentable dietary carbohydrates, this acid-producing bacterium present in the oral biofilm can cause damage (cavities) to the tooth’s hard tissues (4, 12, 34). Although tooth decay is largely preventable, it remains the most common and costly disease worldwide. By sequence homology, at least four type II TA systems have been identified in the *S. mutans* UA159 reference strain (3, 32, 40). In an attempt to investigate the physiological roles of *S. mutans* TA modules, mutants lacking MazF (SMU.173), RelE (SMU.896), or both putative toxins were constructed and tested for their impact on the capacity of *S. mutans* to form biofilm and withstand acid killing (26). Interestingly, when both putative toxin-encoding genes were inactivated, acid tolerance and growth properties of *S. mutans* were affected. These results led Burne and coworkers (26) to propose that chromosomal TA modules of *S. mutans* could function as regulators of cell growth in response to environmental stresses. We provide here definitive evidence that the SMU.172–SMU.173 locus of *S. mutans* encodes a functional type II TA addiction system, the first characterized TA module in an oral pathogen.
2.3 MATERIALS AND METHODS

**Bacterial strains and growth conditions.** A summary of bacterial strains and plasmids used in this study is provided in Table 1. *S. mutans* UA159 wild-type strain and its mutants were grown in Todd-Hewitt yeast extract (THYE) broth and incubated statically at 37°C in air with 5% CO₂. Mutants were constructed in UA159 wild-type as described previously (25). *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium at 37°C. When needed, antibiotics were added as follows: ampicillin (100 µg/ml), chloramphenicol (20 µg/ml), or kanamycin (50 µg/ml) for *E. coli* and chloramphenicol (10 µg/ml), kanamycin (300 µg/ml), erythromycin (10 µg/ml), or spectinomycin (1000 µg/ml) for *S. mutans*. Cell growth was monitored based on the optical density at 600 nm (OD₆₀₀). Cell viability was assessed by counting CFU on replica agar plates.

**RT-PCR.** To confirm the genetic organization and transcriptional coupling between *S. mutans mazE* and *mazF* genes, reverse transcription-PCR (RT-PCR) was performed with the primers CMT-264 (5’-ACTGTAGAAGATCTGTTTAAAG-3’) and CMT-265 (5’-AAAATCGATTGTAACCAGTTGG-3’) spanning the 3’ end and 5’ end of antitoxin- and toxin-encoding genes, respectively. Total RNA was isolated from mid-log-phase UA159 cultures (OD₆₀₀ ~ 0.4) using TRIzol reagent (Invitrogen), DNase treated, and converted to cDNA by using a First-Strand cDNA synthesis kit (MBI Fermentas). PCR was performed with 800 ng of cDNA. Controls without reverse transcriptase enzyme were included in all experiments. As a positive control, the same RT-PCR primers were used to directly amplify the genomic DNA (gDNA) of UA159. PCR products of 323 bp were resolved on a 1.0% (wt/vol) agarose gel.
5’ RACE-PCR. A 5’ rapid amplification of cDNA ends (5’ RACE)-PCR technique was used to define the transcriptional start site of the mazEF operon. Total RNA was isolated from mid-log-phase UA159 cultures using TRIzol reagent (Invitrogen). DNA-free RNA (10 µg) was reverse transcribed by using the RACE outer primer (5’-TTACCATTGTTCTTTCCACA-3’) and Superscript II reverse transcriptase (Invitrogen) according to the supplier’s instructions. RNase H and RNase T1 (Ambion) were then added, followed by incubation at 37°C for 30 min. The cDNA was purified by using a StrataPrep PCR purification kit (Stratagene) according to the manufacturer’s instructions. Tailing of purified cDNA using the terminal deoxynucleotidyltransferase (Sigma) and dGTP/dTTP was performed according to the manufacturer’s instructions. Tailed cDNAs were amplified by PCR using RACE universal primers (5’-GAATTCGAATTCCCCCCCCCCC-3’ and 5’-GAATTCGAATTCAAAAAAAAAAAA-3’) and a RACE inner primer (5’-CTTTAAACAGATCTTCTACAGT-3’). The amplicons were analyzed by agarose gel electrophoresis and sequenced using the RACE inner primer.

Expression vectors for induction in S. mutans. The full-length coding region of the S. mutans mazEF and mazF genes were first PCR amplified using UA159 gDNA as a template and the primers: CMT-392 (5’-GAATTCGAATAGGAGGTGGCATTATG-3’) and CMT-388 (5’-AAGCTTAGATTTCAGTAGAAGTTTAAC-3’) for the mazEF operon and the primers CMT-387 (5’-GAATTCGAATGTTGGAATGAACAATG-3’) and CMT-388 for the mazF gene. The purified PCR products were double digested with EcoRI/HindIII and cloned under the control of the lactococcal promoter P23 in the shuttle plasmid pIB166 (5) precut by the same enzymes. The recombinant plasmids pMAS2 (mazEF in pIB166) and pMAS3 (mazF in pIB166) were
transferred into electrocompetent DH10B and DH10B(pMAS1) cells, respectively. The recombinant plasmids were sequenced on both strands for verification. The plasmids pIB166, pMAS2, and pMAS3 were then used for the toxicity assays.

**Toxicity assays: *S. mutans* transformation.** *S. mutans* UA159 overnight cells were diluted (1:20) with fresh THYE, grown to OD$_{600}$ of ~ 0.1 and divided into three 0.5-ml aliquots of (i) pIB166 (control), (ii) pMAS2 (*mazEF* in pIB166), (iii) pMAS3 (*mazF* in pIB166). Portions (10 µg) of plasmids were added to the cultures, which were grown for a further 2.5 h at 37°C with 5% CO$_2$ in the presence of *S. mutans* synthetic competence-stimulating peptide at a final concentration of 0.5 µg/ml. Cultures were serially diluted and plated on THYE agar. Transformation efficiency (TE) was calculated as the percentage of chloramphenicol-resistant transformants divided by the total number of recipient cells, which was determined by the number of CFU on antibiotic-free THYE agar plates. All assays were performed in triplicate from three independent experiments. Statistical significance was determined by using a Student $t$ test and a $P$ value of <0.01.

**Cell lysis.** The β-glucuronidase (GUS) enzyme was chosen as the marker enzyme for the cell lysis assay since it is known to be rather stable against proteolytic degradation and there has been no indication that the expression of this protein is toxic (35). Cell lysis was assessed by harvesting the supernatant of cultures expressing the *gusA* reporter gene cloned into pIB187 as described previously (39). Briefly, supernatants were combined in equal parts with 2× GUS buffer (100 mM Na$_2$HPO$_4$ [pH 7.0], 20 mM β-mercaptoethanol, 2 mM EDTA, 0.2% Triton X-100, 2 mM *para*-nitrophenyl-β-D-glucuronide [PNPG] substrate; Sigma). The reaction was
incubated at 37°C for 180 min and stopped by addition of 100 mM Na₂CO₃. The absorbance at 420 nm (A₄₂₀) was measured after 180 min of color development. The GUS activity was expressed as \((1000 \times A_{420})/(\text{time in min} \times \text{OD}_{600})\) in Miller units (MU).

**Production and purification of recombinant fusion proteins in *E. coli.*** The full-length coding region of the *S. mutans* mazF gene and mazEF operon were first PCR amplified using UA159 gDNA as a template and the primers CMT-445 (5’-CACCATGGTAAACCATAAGCAAGG-3’) and CMT-451 (5’-TCCTTTTTCAATAGCACCTTG-3’) for the mazF gene and CMT-444 (5’-CACCATGCAACTAGTCATCAATAA-3’) and CMT-451 for the mazEF operon. The PCR products were purified, cloned in-frame upstream from the His₆ sequence into pBAD202/D-TOPO vector (Invitrogen), and transferred into TOP10 chemically competent *E. coli* cells. The recombinant plasmids were sequenced on both strands for confirmation. The plasmids designated pMAS4 and pMAS5 were then transferred into electrocompetent *E. coli* LMG194 (Invitrogen) cells to express the His₆-tagged MazF (rMazF-His₆) and His₆-tagged MazEF (rMazEF-His₆) recombinant proteins, respectively. Overnight, LMG194(pMAS4) and LMG194(pMAS5) cells were diluted (1:100) into fresh LB medium supplemented with kanamycin at 50 µg/ml and grown aerobically at 37°C until an OD₆₀₀ of ~0.5 was reached. Arabinose was then added at a final concentration of 0.02% to induce the expression of recombinant fusion proteins and the incubation was continued for another 4 h at 37°C with agitation.

The cells were collected by centrifugation, resuspended in 1× binding buffer (Novagen), and disrupted on ice by sonication. The soluble fractions of the disrupted cells were recovered by centrifugation, and the rMazF-His₆ and rMazEF-His₆ proteins were then purified by affinity chromatography on Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin (Novagen) as described by the
manufacturer. The Bradford protein assay was used to determine the protein concentration of the purified samples.

**Protein electrophoresis.** SDS-PAGE was performed using the buffer system of Laemmli at a constant voltage (200 V) with gels containing 12% polyacrylamide in the separating gel and 4.5% polyacrylamide in the stacking gel. The protein bands were visualized by staining with Coomassie brilliant blue. For native PAGE, the Laemmli’s gel system was used without SDS and β-mercaptoethanol. The proteins were transferred to a nitrocellulose membrane as described previously (27), and the recombinant proteins were detected by Western blotting with an anti-His$_6$ monoclonal antibody (Sigma).

**In-gel digestion and mass spectrometry.** Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry was performed at the Proteomics Platform, Quebec Genomics Center (Québec city, Québec, Canada). Briefly, protein bands of interest were extracted from the gels, placed in 96-well plates, and then washed with water. Tryptic digestion was performed on a MassPrep liquid handling robot (Waters, Milford, CT) according to the manufacturer’s specifications and the protocol of Shevchenko et al. (41) with the modifications suggested by Havlis et al. (17). Peptide samples were separated by using online reversed-phase nanoscale capillary liquid chromatography and analyzed by electrospray-tandem mass spectrometry. Database searches were conducted using Mascot software (version 2.2.0; Matrix Science, London, United Kingdom) on an *S. mutans*-specific database containing protein sequences deduced from the genome of UA159 reference strain (GenBank accession number AE014133).
**RNase activity.** Total RNA was extracted from *S. mutans* UA159 as described above. Total RNA was extracted from *E. coli* LMG194 cultures (OD$_{600}$ ~ 0.5) using the RNeasy Minikit (Qiagen), and isolated RNA preparations were treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer’s protocol. DNase-treated RNA substrates were incubated with rMazF at 37°C for 15 min. The purified recombinant β-galactosidase (rLacZ) protein was used in the control experiments. Each reaction mixture contained 4 µg of DNase-treated RNA substrate, 20 pmol rMazF, and 1× nuclease buffer (20 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM dithiothreitol). The reaction mixture was then subjected to 1.2% agarose gel electrophoresis. Inhibition of cleavage with the addition of rMazE antitoxin was also tested. Briefly, 4 µg of DNase-treated RNA substrate was incubated in 1× nuclease buffer at 37°C for 15 min with rMazE and rMazF in different ratio combinations.
2.4 RESULTS

**Sequence analysis of the chromosomal mazEF locus.** DNA sequence analysis of the *S. mutans* UA159 genome revealed that the SMU.172–SMU.173 locus encodes a putative TA pair (3). BLASTP analysis revealed that SMU.172 encodes a putative protein of 81 amino acids. PSI-BLAST search revealed a statistically significant similarity (E-value, 7.9e⁻⁶) with the AbrB/MazE superfamily of DNA-binding proteins. AbrB proteins have been identified as antitoxins in well-characterized type II TA systems such as MazEF, Kis-Kid, and PemIK (32). Apparently arranged in a bicistronic operon, the SMU.173 open reading frame overlaps with SMU.172 by seven nucleotides (Fig. 1A). Bioinformatic analysis of SMU.173 revealed that the putative protein of 110 amino acids belongs to the PemK/MazF interferase superfamily of toxins (E-value, 4.84e⁻¹²). This family consists of growth inhibitor proteins such as PemK, ChpA, ChpB, Kid, and MazF (32). Thus, the *S. mutans* SMU.172–SMU.173 locus was investigated to determine whether the two genes encoded for a functional type II TA system.

To confirm the genetic organization and transcriptional coupling between *mazE* (SMU.172) and *mazF* (SMU.173) genes, RT-PCR was performed using primers spanning the 3’ end and 5’ end of antitoxin- and toxin-encoding genes, respectively. A single PCR product of 323 bp was amplified and sequenced, confirming that the *mazEF* mRNA was bicistronic (Fig. 1B). Using 5’ RACE-PCR, we determined the transcriptional start site of the *mazEF* operon to be located seven nucleotides 3’ proximal to the inferred Pribnow box (TATAAT), suggesting that *mazEF* comprises a transcriptionally discrete locus in the *S. mutans* UA159 genome.
The *mazEF* locus encodes a functional TA module. The lack of a tightly regulated inducible expression system for *S. mutans* prompted us to exploit the ability of *S. mutans* to become naturally competent to design our toxicity assay. Naturally competent bacteria have the ability to take free DNA from the surrounding medium and incorporate this DNA into their genomes by homologous recombination, a phenomenon first discovered in *Streptococcus pneumoniae* (16). *S. mutans* strains behave like pneumococci since they are naturally competent for transformation (29). The toxicity of the predicted MazF toxin in its native host was tested by natural transformation of the Δ*mazEF* and Δ*mazF* mutants (UA159 background) with the empty plasmid (pIB166), the plasmid pIB166 containing *mazEF* operon driven by the constitutive P$_{23}$ promoter (pMAS2), and the plasmid pIB166 containing *mazF* gene under the control of P$_{23}$ (pMAS3). It is worth mentioning that despite several attempts, the *mazF* gene could be cloned into pIB166 plasmid and expressed in *E. coli* background only when the MazE antitoxin was preliminary induced. Consequently, the *mazE* gene was cloned under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter into pHGS299 vector (a plasmid unable to replicate in streptococci). The *E. coli* cells were made electrocompetent and used as recipient cells to propagate pMAS3 plasmid necessary for the toxicity assay. The transformation efficiencies of the Δ*mazEF* mutant were $(7.2 \pm 1.0) \times 10^{-2}$ and $(8.0 \pm 0.3) \times 10^{-2}$ for pIB166 (empty vector) and pMAS2 (MazEF), respectively, whereas no colonies were recovered when Δ*mazEF* cells were transformed with pMAS3 expressing the MazF toxin alone (Table 2). In contrast, the Δ*mazF* mutant benefited from the presence of the wild-type *mazE* copy into its genome to protect the cells since plasmid pMAS3 was able to replicate. Indeed, the transformation efficiency of the Δ*mazF* mutant for pMAS3 was similar to those obtained with pIB166 vector and pMAS2 construct (Table 2). These results clearly showed a growth-inhibitory effect of MazF toxin on
S. mutans cells and that the toxic effect elicited by MazF can be neutralized by co-expression of its cognate MazE antitoxin. Hence, mazEF operon encodes a functional TA system in S. mutans.

Effect of MazF mutations on toxicity. As previously mentioned, all our attempts at cloning the S. mutans mazF gene without its cognate antitoxin gene in E. coli gave rise to mutated S. mutans mazF clones, and most of these amino acid changes occurred at conserved positions (see Fig. S1 in the supplemental material). In order to test the importance of these amino acid mutations in the functionality of the MazF protein, we tested randomly selected clones (Table 3) in our toxicity assay (natural transformation). Dramatic effects were observed with the substitutions I29F, L61Q, E21K, and F45L since all four point mutations restored transformation efficiency to the wild-type level (Fig. 2). Although clones S193, S195, and S197 did not restore transformation efficiency to full wild-type levels, the decrease in transforming ability was not considered statistically significant. We can hypothesize that these point mutations substantially reduced or completely abolished the toxic activity of MazF toxin since cells of the naturally transformable ΔmazEF mutant were able to replicate the MazF recombinant plasmids.

MazF toxicity does not result in S. mutans autolysis during natural transformation. In order to investigate whether the growth-inhibitory effect of MazF toxin during natural transformation leads to lysis of S. mutans, we used the β-glucuronidase (GUS) enzyme as the marker enzyme; the release of the cytoplasmic GUS enzyme into the supernatant of transformed cells was quantified as a measure of cell lysis. The ΔmazEF mutant was thus transformed with pIB187 plasmid harboring a GUS reporter gene under the control of the P23 lactococcal promoter for constitutive expression. The new S. mutans ΔmazEF(pIB187) background strain was then used in
our toxicity assay (natural transformation) as described previously. As expected, the transformation efficiency was similar for pIB166 (empty plasmid) and pMAS2 (expressing both the antitoxin and the toxin genes), whereas no transformant could be detected with the pMAS3 construct containing the mazF toxin gene alone (Table 4). Interestingly, GUS activity was not significantly altered in the supernatants of cultures tested with pMAS3, suggesting that MazF toxic activity does not lead to cell lysis of S. mutans under the experimental conditions tested. Moreover, the fact that the transformation assays with pMAS3 construct did not alter the total number of recipient cells, since MazF expression had no effect on the proportion of dying cells, further support our conclusion.

Expression of MazF toxin leads to E. coli cell growth arrest and inhibits colony formation. E. coli cells have been successfully used previously as a host for verification of the function of various heterologously expressed TA loci (1, 11, 21, 36, 46). The S. mutans mazF gene was thus cloned into the pBAD expression system for induction of gene expression using araBAD promoter dose-dependent regulation. The recombinant plasmid designated pMAS4 harboring the streptococcal toxin gene was used to transform E. coli LMG194, and transformants were selected on LB-kanamycin agar plates supplemented with glucose to repress the basal expression level. The growth of LMG194(pMAS4) transformants in liquid medium supplemented with 0.02% arabinose as an inducer showed significant growth differences (Fig. 3A). Strain LMG194(pMAS5), in which both MazE and MazF proteins were expressed, behaves similarly to the LMG194(pBAD202/D/lacZ) control strain. In cell viability assays, overexpression of MazF resulted in growth inhibition with a reduction of ~10^2 in cell viability by 4 h postinduction compared to LMG194(pMAS5) and LMG194(pBAD202/D/lacZ) (Fig. 3B). When tested on
solid media, LMG194 cells harboring pMAS4 could not grow in the presence of arabinose at 0.2%, whereas LMG194(pBAD202/D//lacZ) and LMG194(pMAS5) were able to grow (data not shown). The lack of colony formation indicates either that MazF kills the cells or that MazF induces a state wherein most cells remained viable but in which colony formation is inhibited. Nevertheless, these results demonstrated that MazF is also toxic to *E. coli* cells, and this toxicity is abolished by coexpression of MazE antitoxin.

**Recovery of *E. coli* cell growth arrest after the induction of *S. mutans* MazE antitoxin in cultures treated with *S. mutans* MazF toxin.** To determine whether MazE antitoxin can rescue MazF-induced cell growth arrest, we tested the effect of MazE on *E. coli* cells in which MazF was previously overexpressed. The *mazE* gene was cloned into pET15TV-L under the IPTG-inducible T7 promoter. The recombinant plasmid pMAS6 was transferred into BL21(DE3) containing plasmid pMAS4 for MazF expression in the presence of arabinose. Cells of BL21(DE3) cotransformed with plasmids pMAS4 and pMAS6 were grown to mid-log phase, at which time arabinose was added to induce MazF expression. At time 2, 3, and 4 h after arabinose induction, IPTG was added to induce MazE expression for 1 h; the cells were then spread onto LB plates containing glucose to repress the pBAD promoter. As seen in Fig. 4, overexpression of MazF toxin led to a rapid decrease of CFU counts. When IPTG was added to the culture medium to induce MazE, the CFU counts increased (Fig. 4), indicating that the expression of MazE antitoxin reversed the toxicity of MazF when the *mazE* reading frame was provided *in trans*. These results demonstrated that MazF toxin did not kill the cells but rather caused reversible cell growth arrest.
**MazE and MazF form a complex.** To show direct interaction between MazE antitoxin and MazF toxin, we used plasmid pMAS5, in which both MazE and MazF proteins were expressed and which was constructed so that only MazF protein was expressed as a His\textsubscript{6}-tagged fusion protein. Recombinant proteins were expressed in LMG194 and purified by affinity chromatography. When the mixture was subjected to native PAGE, a single band was observed and detected by Western blotting using an anti-His\textsubscript{6} monoclonal antibody (Fig. 5A). The protein band was cut out and analyzed by mass spectrometry to identify the protein components. The results showed that the complex was composed of MazE and MazF proteins, and the molar ratio of MazE to MazF was 1:2.

When the mixture was subjected to SDS-PAGE, two distinct bands were observed that corresponded to the expected mobilities of rMazE (22.8 kDa) and rMazF-His\textsubscript{6} (16 kDa), thus confirming that under native conditions both rMazE and rMazF proteins were copurified (Fig. 5B). Mass spectrometry also demonstrated that both rMazE and rMazF proteins had the expected molecular mass.

**MazF has RNase activity.** The PemK/MazF toxin superfamily is composed of endoribonucleases that interfere with protein synthesis by cleaving cellular mRNA. To determine whether MazF toxin has RNase activity, DNAse-treated total RNA from *S. mutans* UA159 was incubated with rMazF in a dose-dependent manner. As shown in Fig. 6, *S. mutans* RNA was cleaved into small fragments with 20 pmol of rMazF. The β-galactosidase fusion protein was also purified by affinity chromatography and used in the nuclease assay to confirm that the observed RNase activity was due to rMazF alone and not to contaminating *E. coli* RNases (Fig. 6). The RNase activity of rMazF was inhibited when the molar ratio of rMazE to rMazF
was increased to 1:2, supporting the mass spectrometry results that the molar ratio of MazE–MazF complex is approximately 1:2. Similar results were obtained when the DNase-treated RNA from *E. coli* was used as substrate (data not shown). These results indicated that MazF is a toxic RNase causing bacterial cell growth arrest through the mechanism of RNA cleavage and that MazE antitoxin most probably inhibits the RNase activity by forming a stable MazE–MazF complex.
2.5 DISCUSSION

Identification of the signaling mechanisms that regulate bacterial cell growth is critical to understanding how microorganisms colonize and persist in specific niches. The possible role of chromosomal TAs in growth control is relevant to a number of important aspects of bacterial physiology, including antibiotic tolerance, maintenance of pathogen reservoirs, and chronic infections. In the present study, we reported a functional chromosomal type II TA, MazEF, in the pathogenic organism *S. mutans*. The *mazEF* locus possesses the characteristic organization of type II TAs in which the gene for the antitoxin component precedes the toxin gene and where the two genes share one or several nucleotides; overlaps have been demonstrated to be potentially important in transcriptional and translational regulators of gene expression and to influence gene evolution (20). Although most type II TAs conform to this arrangement, there are examples in which the gene order is reversed or where the product of a third gene is involved (32, 45). A number of orphan toxin genes lacking the adjacent antitoxin gene have also been found in searches for type II loci; it is possible that the synthesis of these toxins is repressed by antisense small RNAs (9, 30). For instance, a total of 45 putative toxins and antitoxins have been identified in the genome of UA159 on the basis of protein sequence and comparative genomic analysis (32), and yet only four TA pairs (AbrB/MazF, Xre/COG2856, RHH/RelE, and PIN/AbrB) possessed all of the features and could be predicted to function as *bona fide* type II TAs (32).

We focused our study on the functional characterization of the MazE(AbrB)/MazF system. Our results confirmed that the chromosomal *mazF* homologue in *S. mutans* encoded a toxic protein with the adjacent *mazE* gene encoding its cognate protein antitoxin. In *S. mutans* as well as in most cases, the type II toxin is a RNase that cleaves mRNA, leading to inhibition of protein
synthesis and cell growth arrest. We therefore propose that the *S. mutans* chromosomal MazEF module represents a growth modulator system that induces under particular stress conditions a reversible dormancy state to enhance fitness and competitiveness. Indeed, the oral cavity is a complex ecological system in which bacteria must overcome a wide range of conditions in order to survive (22, 33, 42). Previous work done by Lemos et al. (26) showed that the deletion of both *mazEF* and *relBE* in *S. mutans* increased resistance to low pHs when the cells were grown in biofilm. Although the molecular mechanisms underlying this process are still not known, these data are consistent with the growth modulator model. Recently, preliminary work done in our lab suggested that the stochastic expression of the MazF toxin in a small fraction of *S. mutans* population could lead to dormancy, allowing dormant cells to survive lethal stresses, including exposure to bactericidal antibiotics. These preliminary data are in accordance with the present study demonstrating that MazF does not cause cell lysis but inhibits cell growth by cleaving single-stranded RNA. By stopping bacterial growth, MazF could allow stressed cells to remain in a dormant or nongrowing stress-tolerant state until more favorable environmental conditions return.

One group presented evidence that MazEF of *E. coli* was involved in bacterial programmed cell death, a form of apoptosis (10). Indeed, Engelberg-Kulka and coworkers demonstrated that in *E. coli*, MazEF is a stress-induced suicidal TA module (19), and yet MazEF-induced PCD is still subject to debate since it has not been reproduced by other groups (44). Recently, Kolodkin-Gal et al. (23, 24) demonstrated that *E. coli* MazF-induced death was dependent on the bacterial cell density and required an extracellular death factor (EDF) in order for the stresses to be effective. The role of the EDF peptide resembles to some extent the function of the peptide pheromone CSP involved in *S. mutans* quorum sensing. Our lab
previously demonstrated that CSP was involved in stress response and induced a dose-dependent growth inhibition of *S. mutans* cultures. Interestingly, cell lysis could be observed in a small fraction of the population at high concentrations of CSP (39). We sought to determine whether *S. mutans* could integrate its stress response with the MazEF TA via the CSP pheromone. To test the impact of MazEF on the stress response directly, Δ*mazEF* mutant cells were exposed to a protein synthesis-inhibitor antibiotic and acid stress (conditions known to induce expression of CSP). When exposed to sub-MICs of spectinomycin or to pH 5.0, Δ*mazEF* cells behave like the wild-type since no significant increase or decrease of lag phase before recovering from the stress was observed between the two strains. Although these results could not allow us to make an intimate connection between the CSP-induced stress regulon and MazEF, we cannot rule out the contribution of CSP in MazF-induced stasis under different stresses. Further experiments will be required to explore this possibility.

Assuming that chromosomal TAs are functioning as regulators of bacterial growth in response to environmental stress, we can speculate that TAs could limit bacterial growth upon encountering eukaryotic host cells (hostile environment) and allow pathogens to persist into specific niches. As a result, pathogens can persist several months (or sometimes years) and give rise to recurrent infections exceedingly difficult to eradicate. Consequently, the understanding of bacterial growth regulation with particular emphasis on the mechanism of action of TA-induced growth arrest represents a promising avenue for the development of novel and effective antimicrobial strategies.
2.6 ACKNOWLEDGMENTS

We are grateful to Indranil Biswas for gifts of expression vectors. We thank Greg Brown and Robert Flick for helpful technical advice with protein purification. We thank Delphine Dufour for careful reading of the manuscript and Elena Voronejskaia for technical assistance.

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2.7 REFERENCES


2.8 TABLES & FIGURES

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)(^a)</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>S. mutans</td>
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<tr>
<td>UA159</td>
<td>Wild-type reference strain</td>
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<td>In-frame (mazEF) deletion mutants derived from UA159; Em(^r) or Sp(^r)</td>
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<td>Host strain for pBAD expression</td>
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<td>Host strain for pET15TV-L expression</td>
<td>Novagen</td>
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<td><strong>Plasmids</strong></td>
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<td>pIB166</td>
<td>Shuttle plasmid containing the (P_{23}) lactococcal promoter; Cm(^r)</td>
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<tr>
<td>pIB187</td>
<td>Shuttle plasmid containing the (gusA) gene encoding the (\beta)-glucuronidase enzyme under the control of (P_{23}); Em(^r)</td>
<td>5</td>
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<td>pHSG299</td>
<td>High-copy-number cloning vector; Km(^r)</td>
<td>Takara Bio USA</td>
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<tr>
<td>pBAD202/D-TOPO</td>
<td>Expression vector linearized and topoisomerase-activated; Km(^r)</td>
<td>Invitrogen</td>
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pBAD202/D/lacZ  Control plasmid encoding the β-galactosidase LacZ; Km
pET15TV-L  Expression vector derived from pET15b; Ap Lab stock
pMAS1  mazE cloned under the control of the IPTG inducible lac promoter into pHSG299; Km
pMAS2  mazEF cloned into pIB166; Cm This study
pMAS3  mazF gene cloned into pIB166; Cm This study
pMAS4  mazF cloned under the control of araBAD promoter into pBAD202/D-TOPO vector; Km
pMAS5  mazEF\textsuperscript{Smu} cloned under the control of araBAD promoter into pBAD202/D-TOPO vector; Km
pMAS6  mazE cloned under the control of T7 promoter into pET15TV-L vector; Ap

\textsuperscript{a}Ap, ampicillin resistance; Em, erythromycin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Sp, spectinomycin resistance.
TABLE 2. *S. mutans* toxicity assay based on natural competence

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>TE ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(\Delta mazEF) mutant</th>
<th>(\Delta mazF) mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIB166</td>
<td>Empty vector</td>
<td>((7.2 \pm 1.0) \times 10^{-2})</td>
<td>((12.2 \pm 3.1) \times 10^{-2})</td>
<td></td>
</tr>
<tr>
<td>pMAS2</td>
<td>(mazEF) genes</td>
<td>((8.0 \pm 0.3) \times 10^{-2})</td>
<td>((11.1 \pm 3.7) \times 10^{-2})</td>
<td></td>
</tr>
<tr>
<td>pMAS3</td>
<td>(mazF) gene</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>((7.5 \pm 1.0) \times 10^{-2})</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The transformation efficiency (TE) was calculated as the percentage of transformants over the total number of recipient cells.

<sup>b</sup><em>P < 0.01</em>, as determined using a Student t test comparing results for the \(\Delta mazEF\) mutant for pMAS3 to those for the \(\Delta mazEF\) mutant for pIB166.
TABLE 3. Amino acid modifications of randomly selected MazF clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Amino acid modification(s)</th>
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<tbody>
<tr>
<td>S193</td>
<td>R53H, R102W</td>
</tr>
<tr>
<td>S194</td>
<td>I29F</td>
</tr>
<tr>
<td>S195</td>
<td>K11E</td>
</tr>
<tr>
<td>S196</td>
<td>L61Q</td>
</tr>
<tr>
<td>S197</td>
<td>Q74R</td>
</tr>
<tr>
<td>S198</td>
<td>E21K</td>
</tr>
<tr>
<td>S199</td>
<td>F45L</td>
</tr>
<tr>
<td>S200</td>
<td>N41I, Q74L</td>
</tr>
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</table>
TABLE 4. Effect of MazF on *S. mutans* lysis during the transformation experiments

<table>
<thead>
<tr>
<th>Construct</th>
<th>TE ± SD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GUS activity&lt;sup&gt;c&lt;/sup&gt; (MU ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIB166 (empty vector)</td>
<td>$(1.2 ± 0.2) \times 10^{-2}$</td>
<td>$8.02 ± 0.57$</td>
</tr>
<tr>
<td>pMAS2 (<em>mazEF</em>)</td>
<td>$(1.8 ± 0.7) \times 10^{-2}$</td>
<td>$7.37 ± 0.77$</td>
</tr>
<tr>
<td>pMAS3 (<em>mazF</em>)</td>
<td>$0^d$</td>
<td>$7.23 ± 0.92$</td>
</tr>
</tbody>
</table>

<sup>a</sup> All assays were performed in triplicate from three independent experiments.

<sup>b</sup> TE, transformation efficiency.

<sup>c</sup> GUS activity was expressed in Miller units (MU). See Materials and Methods for details.

<sup>d</sup> $P < 0.01$, as determined using a Student *t* test comparing results of the *ΔmazEF*(pIB187) strain for pMAS3 to those of the *ΔmazEF*(pIB187) strain for pIB166.
FIGURE LEGENDS

FIG. 1. (A) Nucleotide and deduced amino acid sequences of the mazEF (SMU.172–SMU.173) locus. Putative –35 and –10 promoter sites and putative ribosome-binding sites (RBS) are indicated. A factor-independent terminator-like structure is underlined with broken lines. The transcriptional start site (+1) identified by 5′ RACE-PCR is indicated below the sequence. (B) Agarose gel electrophoresis of the RT-PCR amplification product. Lanes: L, 1-kb Plus DNA Ladder (Invitrogen); 1, positive control (UA159 gDNA); 2, negative control (no RT); 3, RT-PCR (UA159 cDNA).

FIG. 2. Effect of MazF mutations on S. mutans toxicity based on natural competence. Portions (10 µg) of the plasmids were added to S. mutans ΔmazEF cultures grown to early log phase. After 2.5 h at 37°C, the cultures were serially diluted and plated. The transformation efficiency was expressed as the percentage of chloramphenicol-resistant transformants divided by the total number of recipient cells. Although clones S193, S195, and S197 did not restore transformation efficiency to full wild-type levels (C, control), the decrease in transforming ability was not considered statistically significant. All assays were performed in triplicate from three independent experiments and statistical significance was determined by using a Student t test and a P value of <0.01.

FIG. 3. Characterization of the MazEF TA in E. coli. (A) The growth characteristics of LMG194 cells expressing LacZ (pBAD202/D/lacZ), MazF (pMAS4), and MazEF (pMAS5) were analyzed by measuring the absorbance (OD₆₀₀) after every hour. (B) After arabinose induction, appropriate dilutions were plated on LB agar for determination of the number of CFU per ml.
FIG. 4. Recovery of MazF-induced cell growth arrest by MazE antitoxin. Cells of BL21(DE3) cotransformed with pBAD-MazF (pMAS4) and pET15-MazE (pMAS6) were grown to mid-log phase, at which time 0.02% arabinose was added (MazF induced). At indicated time points (after arabinose induction), 1 mM IPTG was added for 1 h (MazE coinduced), after which the cells were spread on LB plates containing 0.2% glucose, 50 µg of kanamycin/ml, and 100 µg of ampicillin/ml.

FIG. 5. Copurification and interaction of rMazE antitoxin and rMazF-6His toxin. (A) Native PAGE and Western blot analysis using the anti-His₆ monoclonal antibody. The protein composition of the immunoreactive band was identified by MS. Lanes: 1, LMG194(pMAS5) uninduced culture; 2, LMG194(pMAS5) arabinose-induced culture. (B) SDS–12% PAGE was performed to analyze the protein composition of the immunoreactive band. Lanes: L, PageRuler Prestained protein ladder (Fermentas); 1, total cell lysate from LMG194(pMAS5) uninduced culture; 2, total cell lysate from LMG194(pMAS5) arabinose-induced culture; 3, elution of absorbed lysate from LMG194(pMAS5) uninduced culture; 4, elution of absorbed lysate from LMG194(pMAS5) arabinose-induced culture. The identity of the two distinct protein bands was confirmed by mass spectrometry. Both rMazE and rMazF-6His fusion proteins had the expected molecular mass: ~23 kDa for rMazE, including the His-patch thioredoxin on the N-terminal end, and ~16 kDa for rMazF-6His including the polyhistidine region on the C-terminal end.

FIG. 6. RNase activity of MazF. Each reaction was performed for 15 min at 37°C with 4 µg of S. mutans DNase-treated RNA. Lanes: L, GeneRuler 1-kb Plus DNA ladder (Fermentas); No P, no protein, RNA alone; LacZ, RNA incubated with rLacZ (20 pmol); MazE, RNA incubated
with rMazE (20 pmol); MazF, RNA incubated with rMazF (20 pmol); 2E:1F, RNA incubated with rMazE (40 pmol) and rMazF (20 pmol); 1E:1F, RNA incubated with rMazE (20 pmol) and rMazF (20 pmol); 1E:2F, RNA incubated with rMazE (20 pmol) and rMazF (40 pmol); 1E:4F, RNA incubated with rMazE (20 pmol) and rMazF (80 pmol).
FIG. 1A

170881 aatattatgctaataccaaagatttacagagaatgattatatata_35
tataataagtcacagaaaaaggaattgctgttttcgaagttcaagtcat
-10 _ +1 RBS
Maze
MQLVIN
171001 aatgtgggataatgtctccccctttccttgacttaacatatatata
KWNSSAIRLPKQLYQELQL
171061 caaacaataatgtctctagacatteggtaaacgaattatatctctagaaaaagtg
QTNVDVLDYKVSNGIKIKILEKV
171121 aataatatccctggaatccggtgaacctgtgtctcctgtagagatctgttttaag
RT-PCR Forward Primer
NNINPELTVEDELFDYQGEPV
171181 aatgtttacccccatgtctatttgaaatgttgtgggaatgaaac__ATGgtaaccatcaagcaag
NVTPALFESVGVQNWSTOP
MazF
MVTIKQ
171241 ggtcaatattcaaaaatcaattgacattcctaaaccaaaagagaagctcat
GSIIKINLDPKQHGHEQKGYR
171301 cgtacatctggcataacattataaggataccag acctatataggttcttttgccccgc
P YICLNSHSIVTKYSNIGIFA
171361 caatttagcattcagagctttgatatacttcctgtatgtggcaacctaggaacaagaatccca
PISNTKRDYPFYVSLEGTES
171421 cagggaaagtattattagaccaactgtggtataaatcgtggtttttatatattaggdggtta
RT-PCR Reverse Primer
TGYKLDDLQIDTFNARQYD Y
171481 atgtggaggaatctcagagagaccttttagatgaaccttttagcgtggcttaaagtctat
YVEDIQEDDLDELLARVKL
171541 tttgaaaggttagcattcaggttaaagatgggtctttttttctctcactgaatatatatatatatatCGSTOP
FIG. 1B
FIG. 2

![Graph showing transformation efficiency (%) for C, S193, S194, S195, S196, S197, S198, S199, and S200.](image-url)
FIG. 3A
FIG. 3B

![Graph showing CFU/ml vs. Time post-induction (h)]

- pBAD202/D/lacZ
- pMAS5
- pMAS4

CFU/ml scales:
- 1.0E+05
- 1.0E+06
- 1.0E+07
- 1.0E+08
FIG. 4

[Bar graph showing CFU/ml as a function of time post MazF induction (h).]

- MazF co-induced
- MazF induced
FIG. 5B

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<tr>
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<th>1</th>
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<tr>
<td>B)</td>
<td></td>
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</tbody>
</table>

- ~35 kDa
- ~25 kDa
- ~15 kDa

rMazE

rMazF-His$_6$
FIG. S1

Multiple sequence alignment of MazF toxin. The amino acid sequence of MazF from *S. mutans*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, and *E. coli* were aligned. Residues highlighted in black are identical for all sequences, dark and light grey for conserved and semi-conserved residues, respectively. The secondary structure of MazF from *E. coli* (PDB ID: 1UB4) is shown under the alignment. Mutation sites of *S. mutans* MazF are designated with an arrow above the alignment.
CHAPTER 3

DISCUSSION

3.1 Conclusion

Prokaryotic type II TA systems are widespread, mobile two-gene modules that can be considered as more than selfish genetic entities because they also possess cellular functions in various forms of stress response (Van Melederen, 2010). They also belong to the prokaryotic mobilome as they are extensively, if not preferentially, spread via horizontal gene transfer (Makarova et al., 2009). In this study, we reported a functional chromosomal type II TA module, the MazEF system, in the pathogenic organism \textit{S. mutans}. This bacterium colonizes the tooth surface and has a central role in the etiology of dental caries, one of the most common chronic biofilm infections in the world. The genetic organization of the \textit{S. mutans} MazEF chromosomal TA locus and the functional gene products identified are similar to the emergent prokaryotic stress-inducible suicide modules (Gerdes et al., 2005). The fact that almost all obligate endosymbiotic species are devoid of TA loci, whereas free-living prokaryotes have several TA loci further supports the hypothesis that TA modules provide a control mechanism to cope with environmental stresses (Pandey & Gerdes, 2005).

The \textit{S. mutans} mazEF bicistronic operon encodes a functional type II TA module, where \textit{mazF} encodes a toxin and \textit{mazE} its cognate antitoxin. We showed that overexpression of MazF toxin was toxic to \textit{S. mutans} and \textit{E. coli} (\textit{E. coli} cells have been successfully used previously as a host for verification of the function of various heterologically expressed TA loci) cells leading to severe inhibition of cell growth; MazF-stasis could be prevented by MazE antitoxin coexpression. We also demonstrated that the purified streptococcal MazE antitoxin and MazF toxin interacted with each other \textit{in vivo} confirming the nature of this module as type II addiction system, which refers to neutralization of toxin by avid binding of protein antitoxin. In \textit{S. mutans} as well as in most cases, the type II toxin is a ribonuclease that cleaves mRNA, leading to inhibition of protein synthesis and cell growth arrest (Makarova et al., 2009).

The function of the chromosomal type II TA systems is still not elucidated and continues to be the subject of debate. Preliminary work done in our lab showed that after exposure to high
temperature stress, $\Delta mazEF$ mutant had a slower growth rate compared with wild-type strain and that $\Delta mazEF$ mutant was unable to survive an exposure of 90 min at 50°C. In contrast, $\Delta mazEF$ cells were more tolerant to mitomycin C. Gene expression analysis demonstrated that $S. mutans mazEF$ locus was increased (~25-fold) after exposure to high temperature stress and repressed (~3-fold) when cells were exposed to DNA damaging conditions (unpublished data). We therefore propose that the $S. mutans$ chromosomal MazEF module represents a growth modulator system that induces under particular stressful environmental conditions a reversible dormancy state to enhance fitness and competitiveness. Indeed, the oral cavity is a complex ecological system in which bacteria must overcome a wide range of conditions in order to survive (Marsh, 2005; 2006). The natural flow of saliva and mastication will detach bacteria not firmly attached to a surface. The pH levels are highly variable and frequently shift from neutral pH to as low as 3.0 during the ingestion of dietary carbohydrates by the host. Despite the fact that the mouth is highly aerobic, oxygen gradients exist in the oral biofilm. Solutes can also accumulate from different sources, including exogenous nutrients from the diet and salts from tooth demineralization.

One research group presented evidence that the MazEF system of $E. coli$ was involved in bacterial programmed cell death, a form of apoptosis. Indeed, Engelberg-Kulka and colleagues demonstrated that in $E. coli$, the MazEF system is a stress-induced suicidal TA module (Hazan et al., 2004). Stressful conditions that prevent the expression of $mazEF$ operon (e.g., inhibition of transcription and translation by antibiotics) lead to a decrease in MazE protein levels – a consequence of MazE degradation by the host cellular protease ClpAP – leaving MazF toxin free to exert its killing effect. The altruistic death of a portion of the bacterial population is proposed to leave more resources in the environment for the siblings and therefore improve long-time survival of the bacterial population as a whole. $S. mutans$ genome encodes several proteases, including the ATP-dependent ClpP protease. ClpP has been shown to be essential for $S. mutans$ biofilm formation and for the ability of $S. mutans$ to respond to various environmental stresses, such as temperature and oxidative stresses (Kajfasz et al., 2009). Furthermore, $S. mutans clpP$ gene expression was induced by acidic conditions (Lemos & Burne, 2002; Chattoraj et al., 2010). Whether $S. mutans$ ClpP degrades the MazE antitoxin, and if there is a relationship
between *S. mutans* aciduricity and regulation of the MazEF TA system will require further investigation.

### 3.2 Future direction

To test the impact of type II TA systems on the stress response directly, TA-deficient mutants can be constructed in each of the putative TAs identified in the UA159 reference strain and the growth kinetics tested following exposure to different stressors. Additionally, the putative toxin genes could be cloned into an *E. coli*-Streptococcus shuttle expression plasmid for constitutive expression. The knockout mutants and overexpressing clones could be easily tested for multidrug tolerance. Multiple knockouts mutants can also be created to assess whether only MazEF system is involved in forming *S. mutans* dormant persister cells, or if other type II TA systems provide redundancy in developing persistence. Indeed, work currently under progress in our lab shows that overexpression of MazEF system in *S. mutans* caused a ten-fold increase in multidrug tolerant persister cells (Leung and Lévesque, unpublished data). Lastly, as cells within biofilms do not behave uniformly but are in different physiological states and appear to be functionally distinct, we could construct transcriptional fusions to analyze the spatial location of *mazEF* promoter activity in multi-species oral biofilms exposed to different environmental stresses.


