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Posttranscriptional Control of Gene Expression and Role of Small RNAs in *Streptococcus mutans*

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Abstract

Streptococcus mutans is a key contributor to the formation of biofilms associated with dental caries disease. Living in the oral environment and developing of disease require tight gene regulation. In bacteria, gene expression is most commonly regulated at the level of transcription initiation. This control involved interactions of specific DNA sequences with regulatory proteins. A second mechanism of control of gene expression is mediated at the RNA level by several mechanisms and is generally called posttranscriptional regulation. These mechanisms include *cis*- and *trans*-acting small, non-coding RNAs, RNA-binding proteins, riboswitches, thermosensors, RNases, and Type I toxin-antitoxin systems, and may result in changes in RNA stability, efficiency of ribosome binding, translation initiation, and transcript secondary structures. Here I review the posttranscriptional regulation in *S. mutans*.

Keywords

Dental Carries, Gene Regulation, Antisense Therapy

1. Introduction

The general rules of life are the same for all living organisms. Everything that is true for bacteria, is true for big animals as well. That theory coined in the XIX century resulted in focusing scientific research on model organisms which were easy to cultivate (propagate), stable and amenable to experimental manipulation. Our knowledge on bacterial physiology is based mostly on two models: *Escherichia coli,* a Gram-negative rod, which is a common constituent of the human digestive system and *Bacillus subtilis,* a Gram-positive, rod shaped, sporulating,

free living organism. Due to an enormous progress in DNA sequencing technologies the last decade allowed researchers to establish and annotate hundreds of microbial genomes. Based on that data it can be noticed that many genes are specific for a specific organism, have no similarities with other genes and no function can be assigned. That leads to the conclusion that many organisms are unique and do not undergo the common paradigms. One of those organisms, *Streptococcus mutans,* has been proposed to be a new model organism for Gram-positive pathogenic bacteria [1].

It has been almost 90 years since *S. mutans* was described for the first time in 1924 and almost thirty years since its role in formation of dental caries was established [1]. The last decade, which started with the first completed genome sequence [2], revealed many interesting features of the *S. mutans* specific physiology. Developing many new molecular tools and usage of the "tip top" molecular biology techniques also contribute to understanding of those processes on molecular level [1] [3]-[10]. That knowledge leads also to developing new therapies for human carries [11] [12] [13] [14].

Regulation of gene expression is fundamental for the coordinate synthesis, assembly and localization of all macromolecular structures of cells. This is achieved by a multi-step program that is highly interconnected and regulated at diverse levels. In bacteria, gene expression is most commonly regulated at the level of transcription initiation. This control involved interactions of specific DNA sequences with regulatory proteins. A second mechanism of control of gene expression is mediated at the RNA level by several mechanisms and is generally called post-transcriptional regulation. These mechanisms include *cis*- and *trans*-acting small, non-coding RNAs, RNA-binding proteins, riboswitches, thermosensors, RNases, and Type I toxin-antitoxin systems, and may result in changes in RNA stability, efficiency of ribosome binding, translation initiation, and transcript secondary structures [15] [16] [17] [18] [19].

In *S. mutans* UA159 genome more than 100 different transcriptional regulators have not has been identified [2]. Only two RNA polymerase sigma factors have been found: σ^{70} (RpoD: SMU.822 and SMU.1803) and ComX (SMU.1997), a transcriptional regulator of competence-specific genes [2]. The major role in gene regulation and adaptation to environmental conditions in *S. mutans* is being held by the RpoE, delta subunit of RNA polymerase [20] [21] and two component signal transduction systems (TCSTS). The role of each of the 14 TCSTS as well as a single orphan regulator GcrR has been studied extensively and recently reviewed [22]. Here I am going to shed some light on a question of post-transcriptional regulation of gene expression in *S. mutans*.

2. Mechanisms of Posttranscriptional Regulation

For many bacterial operons, the mechanism of posttranscriptional control of gene expression relies on sequence signals in the 5′-untranslated region (5′-UTR) found upstream of gene coding sequences. These leader regions can fold into at

least two mutually exclusive RNA structures, like an intrinsic terminator and an anti-terminator. Switching between different conformations of this transcribed region controls the fate of the elongation complex, and thus the expression of the downstream gene. This switch is controlled by diverse regulators that include accessory proteins, small molecules, uncharged tRNAs and translating ribosomes [23] [24] [25]. Another mechanism of posttranscriptional regulation involves regulatory proteins which bind to mRNA and block its translation initiation region (negative regulation) or stabilize mRNA molecule and/or structure (positive regulation) [26]. The third mechanism involves the complementary RNA molecules (asRNA, sRNA, miRNA) that action similar to RNA binding proteins but may also target a complementary mRNA strand for degradation (RNA decay) [27] [28] [29].

3. Regulation by RNA Binding Proteins

3.1. BglG(LicT)/SacY Antiterminator Proteins

Antiterminator (AT) proteins modify the structure of the target mRNA in order to prevent formation of the termination signals and subsequent dissociation of the polymerase. However direct interaction with the transcription complex is not required in that case. The best known bacterial AT belongs to the BglG(LicT)/SacY family, which has been shown to be involved in carbohydrate transport and metabolism in many Gram-positive and Gram-negative bacteria [30]. The RNA target sequences (called RAT-ribonucleic antiterminator) for these proteins are usually 30 nucleotides long and are proposed to adopt a hairpin structure with a variable apical loop and two asymmetric internal loops interrupting a central stem [31]. Mutational analysis on different RATs has shown that base pairings in the stem are required for the antitermination function and that the non-conserved nucleotides within the internal loops are involved in the control of the specificity of the AT-RAT interaction. In contrast, RAT recognition by the AT appears largely independent of the length and nucleotide sequence of the apical loop [32].

In the genome sequence of *S. mutans* UA159 five genes encoding AT proteins and fourteen PTS systems have been identified [2] [32]. Two of those PTS are responsible for utilizing β-glucosides such as cellobiose, esculin, arbutin, and salicin which can be found in foods containing plant extracts. Cellobiose and salicin are utilized by proteins encoded in the *cel* locus (SMU.1596 to -1601). The second locus, designated *bgl* (SMU.977 to -985), consists of the genes for PTS transport (EII, SMU.980) and the metabolism of esculin. Arbutin is hydrolyzed by its own phospho-β-glucosidase (encoded by the *arb* gene, SMU.1102), but it is transported by the *cel* or *bgl* system [33]. Two out of those five identified AT genes are located within the *cel* and *bgl* operons (SMU1599-*celR* and SMU977) and one is located within *mtl* operon (mannitol operon *mtlR*-SMU1184) while the fourth is encoded by a single SMU289 gene located ~8 kb downstream of the putative ribulose utilization operon (SMU270-275). The mechanism of action between

AT and the RAT sequence have been described only for the SMU977-LicT in *S. mutans* NG8 [34] [35] [36]. Based on DNA sequence homology between NG8 and UA159 strains it was possible to identify the *bgl* RAT structure and the terminator in front of the *bglP* gene (**Figure 1(a)**). Another hypothetical RAT sequence has been described in front of a fructan hydrolase gene (*fruA*) [37]. However further experiments showed that this structure was not involved in gene regulation [38].

The role of CelR in regulation of the *cel* operon has been shown recently [39]. Although the detailed mechanism of interaction has not been determined a similarity with the MtlR protein of *Bacillus stearothermophilus* (29% identity) suggests that CelR might act as the transcriptional regulator at the DNA level [39] [40]. The latest data on *Vibrio parahemeolyticus* MtlR protein structure and function showed that it does not bind to the promoter regions directly [41]. Therefore, identification of the hypothetical RAT and terminator structures in the *celA* mRNA leader (**Figure 1(b)**) might suggest the antitermination mechanism of regulation.

The mechanism of action of three remaining AT proteins, MtlR, SMU289 and SMU787, which show limited homologies to other proteins is not known. Although the location of *S. mutans* MtlR protein suggests its role in regulation of mannitol operon, it shows even lower similarity level with MtlR *B. stearothermophilus* (23% identity) than CelB. A target sequence for the orphan regulators, SMU787 and SMU289, remains unknown but a presence of the fluoride riboswitch in front of SMU787 might suggest its role in fluoride resistance (see riboswitch section).

3.2. Ribosomal RNA Regulation—NusAB Antitermination

Transcription termination factors NusAB (N protein-Utilization Substance AB) have been described for the first time in bacteriophage lambda [42]. Further studies showed that the same system plays a key role in the regulation of ribosomal RNA biosynthesis in eubacteria by modulating the efficiency of transcriptional antitermination [43]. NusB along with other Nus factors (NusA, NusE/S10 and NusG) forms the core complex with the boxA element of the *nut* site of the rRNA operons [44] [45]. These interactions help RNA polymerase to counteract polarity during transcription of rRNA operons and allow stable antitermination. Although the Nus proteins recognize the boxA sequence in rRNA leader they act rather as transcription elongation factors modifying RNA polymerase sensitivity to the presence of *rho*-dependent transcription termination sites [46].

Five rRNA operons have been identified in the *S. mutans* UA159 genome [2]. Also all three components of the antitermination system: NusA (SMU.418), NusB (SMU.1845) and NusG (SMU.1947) have been described [2]. Although blast search revealed many sequences identical with the original boxA sequence (5'-(t/c)GCtCTTtaaca(a/t)(T/C)-3'), none of them was actually located in the vicinity of the rRNA genes. Therefore, the role of Nus antiterminators, as well as rRNA transcription regulation in *S. mutans,* remains unknown.

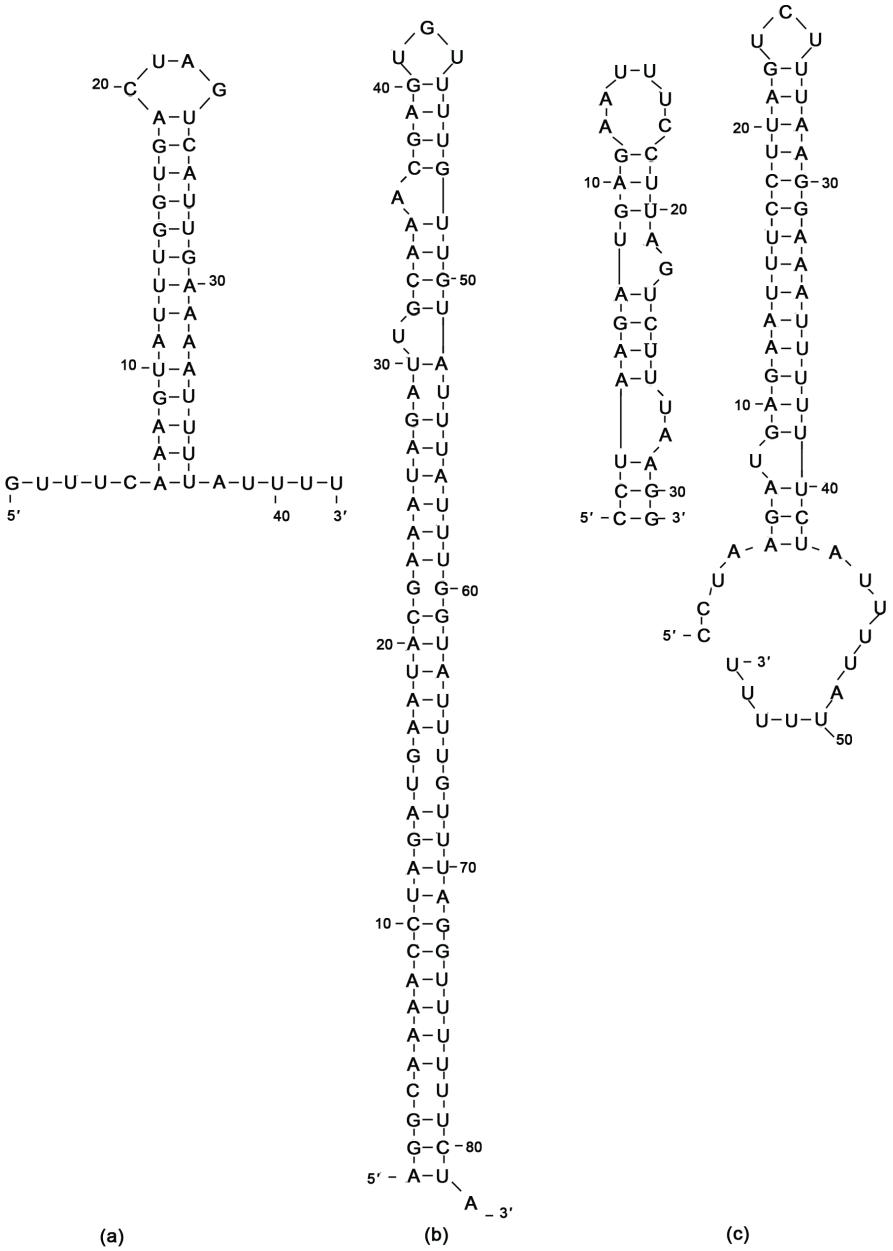

Figure 1. RAT structure and terminator of *S. mutans* UA159 *bglP* mRNA (a) and a hypothetical RAT structure and terminator in the *celA* leader (b).

3.3. Termination Protein—PyrR Regulon

The PyrR protein regulates expression of the genes of *de novo* pyrimidine nucleotide biosynthesis (*pyr* genes) in almost all Gram positive and many other bacteria by a transcription attenuation mechanism [47]. PyrR acts by binding to a segment of *pyr* mRNA with conserved sequence and secondary structure. 5'-UTR leader folds to form three alternative structures, an antiterminator, a terminator, and an anti-antiterminator that precedes the antiterminator [48]. The PyrR protein, in the presence of UMP, can bind to, and stabilize, the anti-antiterminator. This prevents formation of the antiterminator, resulting in

termination [48].

In *S. mutans pyrR* regulon contains two loci: SMU.856-860 (*pyrRPBcarAB*) and SMU.1224-1221 (*pyrKDFE*) (http://regprecise.lbl.gov/RegPrecise). PyrR regulatory elements has been identified in front of *pyrR* (−133 nt) and *pyrK* (−131 nt). Both elements contain motifs similar to the highly conserved sequence motif 5′-ARUCCNGNGAGGYU-3′in PyrR-binding loop [49] (**Figure 2**).

3.4. Leaders of Ribosomal Protein Genes

Another example of proteins which interacts with 5'-UTR RNA leader sequences are some ribosomal proteins [26] [50]. These proteins regulate not only their own expression but also expression of the co-transcribed genes via translational coupling [26] [50] [51] [52].

In *S. mutans* four ribosomal protein leader sequences have been identified: L10, L13, L20 and L21 (http://www.oralgen.org/srna_result/index.html#S.mut). L10 leader is located upstream of the SMU.957-958 operon encoding L10 and L7/L12 ribosomal proteins. L13 leader precedes the SMU169-170 operon for L13 and S9 ribosomal proteins. Both L20 and L21 leaders are located in front of the three gene operons SMU.697-699 and SMU.847-849, respectively. SMU.697 and SMU.699 encode L35 and L20 ribosomal protein, while the first gene in that operon encodes for a hypothetical translation initiation factor 3. Similarly, SMU.847 and SMU.849 encode for L21 and L27 proteins. The central gene in that operon shows homology to uncharacterized *ysxB* gene from *B. subtilis* [2].

4. Riboswitches

Riboswitches are bacterial RNA regulatory elements located at the 5′ end of mRNAs, which can adopt different conformations in response to the environmental signals. The RNA elements fit a general pattern where formation of the helix of the intrinsic terminator is, in the absence of effector, prevented by formation of a more stable competing antiterminator structure. Effector binding is proposed to stabilize a third competing structure that serves as an anti-antiterminator, which allows formation of the terminator. For some riboswitches, like temperature sensing ones, the effector binding domains are not present [53].

```
SMU.1224_pyrK_      AATAACCTTTAATTTAGTCCAGAGAGGCTGTCAAGGGAAATCAAGATTAACAG-GCAGAG 59
SMU.856_pyrR_       --ATTCCTTTAACACTGTCCCGTGAGGCAGGCAAGGAGCGACAAAGAAAAAAGTGCAG-- 56
                      :::******* : :****.*:*****:* *****....:***..::**.** ****

SMU.1224_pyrK_      GTACAATCTG-CCCACTTTTTTGTGTAACCTTGCCAGTTAGTTGGTAAGGTTTTTTTTCA 118
SMU.856_pyrR_       GAACAA-CTGTACTTATATTTTGTGAAATCT--CCTTGTATGAGG--AGATTTTTTCTTA 111
                    *:**** *** .* :.*:*******:** **  **:  **  :**  **.****** * *

SMU.1224_pyrK_      T- 119
SMU.856_pyrR_       TT 113
                    *
```

Figure 2. Sequence alignment of the PyrR binding sites in *S. mutans* UA159. PyrR-binding loop is underlined.

4.1. Metabolite-Sensing Riboswitches

In many bacteria genes involved in vitamins production and transport, as well as amino acid and nucleotides biosynthesis are regulated by metabolite-sensing riboswitches. Specific sequences responsible for binding flavin mononucleotide (FMN, rfn-box), thiamin pyrophosphate (TPP, tpp-box), adenosylcobalamin (B12, B12-box), *S*-adenosyl-l-methionine (SAM, S-box; S_{MK}-box), lysine (L-box), guanine and hypoxanthine (Purine, G-box) have been identified [54] [55] [56].

In *S. mutans* UA159 genome sequence originally four metabolite—sensing riboswitches, FMN, TPP, Glycine and *yybP*|*ykoY* have been identified (http://www.oralgen.org/srna_result/index.html#S.mut). The TPP riboswitch has been found upstream of SMU706 gene. It shows similarity to *yuaJ B. subtilis* encoding for thiamine permease [57]. FNM riboswitch is located in front of the SMU1703-1702 operon (1615112-1615325). SMU1703 shows homology to *ypaA* gene from *B. subtilis* which is a membrane protein involved in riboflavin uptake [58]. Glycine riboswitch typically resides upstream of genes that express protein components of the glycine cleavage system which catalyzes the initial reactions for use of glycine as an energy source like *B. subtilis gcvT-gcvPA-gcvPB* operon. However in *Vibrio cholerae* it is located immediately upstream of the *VC*1422 gene (a putative sodium and alanine symporter) [59]. In *S. mutans,* Glycine riboswitch is located in front of the SMU1175 gene which is similar to *VC*1422. The last riboswitch element *yybP*|*ykoY* is common and widely distributed in other bacteria. The role of that riboswitch and the mechanism of regulation have not been described. The *yybP*|*ykoY* element resides upstream of two separate monocistronic transcripts in *B. subtilis* and *E. coli* and is found mainly upstream of genes classified into four groups. The first cluster includes *E. coli ygjT* and *B. subtilis ykoY*, which are similar to an *E. coli* gene (*terC*) that encodes a membrane protein with a poorly defined function related to tellurium resistance. The second group encodes a cation-transport ATPase, whereas the final two clusters are predicted to be membrane proteins (one includes *E. coli yebN*) [60]. In *S. mutans yybP*|*ykoY* element is located upstream of SMU.723 gene encoding for a putative calcium-transporting ATPase [2]. Location of that riboswitch in front genes involved in cation transport might suggest the role of cations in its activation. Other riboswitches involved in sensing intracellular level of ions like magnesium riboswitch described in *Salmonella enterica* [61] have not been so far identified in *S. mutans*.

Additional studies revealed the presence of additional three riboswitches in *S. mutans*. Although a standard S-box has not been identified in *S. mutans* genome, Fuchs and coworkers found an alternative S_{MK}-box in front of *metK* gene (SMU1573) encoding for S-adenosylmethionine synthetase [62]. Recently, an additional riboswitch interacting with tetrahydrofoliate (THF) that controls the *folT* gene in *Streptococcus mutans* UA159 have been identified [63] [64]. Two molecules of folinic acid, THF analog, were observed to bind to that structure and prevent synthesis of genes involved in THF transport and synthesis [63].

Another newly described riboswitch, *crcB*/*eriC*^{F} senses fluoride concentrations. The *crcB* motifs are located upstream of genes encoding proteins annotated as ion transporters (for example, chloride, sodium, proton). Some others are involved in various physiological (e.g., universal stress adaptation, DNA repair) or metabolic (e.g., enolase, formate-hydrogen lyase) processes [65]. In *Streptococcus mutans* UA159 the *eriC*^{F} riboswitch has been shown to regulate SMU.787 gene [65]. The SMU.787 protein is a transcriptional regulator related to cell envelope and contains transcriptional attenuator domain [2]. As fluoride has been widely used as an additive in oral hygiene products and water since the 1950s because of its usefulness in preventing tooth decay [66], it would be important to reveal the role of that riboswitch in *S. mutans* fluoride toxicity resistance.

4.2. T-Box Regulation Mechanism

The T-box mechanism is widely used in Gram-positive bacteria to regulate expression of aminoacyl-tRNA synthetase genes and genes involved in amino acid biosynthesis and uptake. Binding of a specific uncharged tRNA to a riboswitch element in the nascent transcript causes a structural change in the transcript that promotes expression of the downstream coding sequence. In most cases, this occurs by stabilization of an antiterminator element that competes with formation of a terminator helix. Specific tRNA recognition by the nascent transcript results in increased expression of genes important for tRNA aminoacylation in response to decreased pools of charged tRNA [67] [68].

In *S. mutans* UA159 genome sequence originally four T-boxes in front of the *trp* operon as well as Ala-, Thr- and Ser-tRNA synthetases have been identified (http://www.oralgen.org/srna_result/index.html#S.mut). In other studies ten T-box sequences (8 for AA-tRNA synthetases, 1- AA synthesis and 1- unknown function) were described [69]. In total 10 AA-tRNA synthetases are regulated by the T-box mechanism (AlaS, AsnS, GlyS, HisS, AspS, IleS, PheS, SerS, ThrS and ValS). Some of those genes constitute polycistronic transcriptional units.

T-box regulates also histidine biosynthesis operon. That mechanism is specific for *Lactobacillales*, including *L. lactis* and *S. mutans* [69]. The *his* operon in *S. mutans* contains also genes for serine biosynthesis (*serB*) and *hisC* and *hisH*, which encode the enzymes that catalyze some of the last steps of the tyrosine, and phenylalanine pathways. The rarity of T-box regulation for *his* operon may be caused by the fact that the acceptor arm of $tRNA^{His}$ has only three unpaired nucleotides (CCA) at its 3′ end. Therefore, $tRNA^{His}$ lacks one of the unpaired nucleotides involved in the interaction of tRNAs with the antiterminator bulge. The absence of the fourth position of pairing could potentially result in a lower efficiency of tRNA-dependent antitermination [69].

The T-box sequence identified upstream of the *trpEGDCFBA* operon (SMU532-538) was located upstream of the small gene SMU531 with similarity to chorismate mutase (*pheA*). As that gene location is unique only to *Streptococcus thermophilus* and *S. mutans* it has been speculated that SMU531 had

been horizontally transferred and inserted in front of *trpE* gene [69].

5. Antisense RNA

Antisense RNA (asRNA) is a single-stranded RNA that is complementary to a messenger RNA (mRNA) strand. Antisense RNA reflects usually to long transcripts in contrast to small interference RNA (RNAi); however the mechanism of action is probably similar [70] [71]. The simplest example of such antisense structure is when two adjacent genes overlap and they are transcribed in the opposite direction. There are two possible conformations for those overlapping genes: "convergent" (→←) and "divergent" (←→). Analysis of 50 microbial genomes showed that while co-transcribed, directly overlapping genes represent 71.4%, the two inverted orientations have the same numbers (14.3%) of gene pairs, but the fraction of the divergent overlapping genes (←→) is lower than that of the convergent overlapping genes (→←) [72]. The lower ratio of the divergent structure is probably due to the evolutionary constraints on the 5′-end of the gene and the upstream region, which have structures that are essential for the gene expression [72]. Although these data represents only annotated ORFs which contain translation signals (RBS and start codon) while those signals are not required in asRNA, the number of divergent transcripts should not dramatically increase. However, like other regulatory genes, expression of many asRNAs depends on cell physiology and environmental conditions, advances of new microarray and whole transcriptome sequencing techniques should allow the precise identification of those molecules.

Toxin-Antitoxin System

The role of toxin-antitoxin systems in cell physiology, specifically in biofilm formation, persister cell formation and the general stress response, is becoming clearer [73] [74]. So far, five types of toxin-antitoxin (TA) systems have been described [75] [76] [77] [78] [79]. In two TA types (I, III) the RNA molecules play important roles.

In type III a 36 nt ToxI antitoxin RNA repeat sequesters ToxN protein antitoxin and prevent its cytostatic activity [80]. In type I TA systems, antitoxins are small untranslated RNAs acting as asRNAs. RNA antitoxins can be encoded directly opposite to the coding sequence of the toxin, opposite the 5′-UTR, or opposite the 3′-UTR of the toxin mRNA, or even divergent to the toxin gene but with long stretches of complementarity to the toxin mRNA [81]. First described systems were involved in stability of plasmids in *Enterobactericeae* [81].

Although in *S. mutans* plasmid encoded TA system belongs to the type II, $RelBE_{plas}$ family [82], the presence of type I TA system has been recently reported [83]. The system named Fst-Sm/srSm is encoded by a small intergenic region (318 nt) located between the genes SMU.219 and SMU.220. Although the regions of base pairing between *srSm* RNA and the 5′end of *fst-Sm* mRNA suppresses toxin translation, the mechanism of action has not been determined [83].

Both mechanisms of action, translational silencing or mRNA degradation as well as their combination are probable. The *srSm*:*fst-Sm* duplex is a good target for cleavage by RNase III, an endoribonuclease specific for double stranded RNA which is encoded by SMU.1415 gene. Attempts to construct a *S. mutans* RNase III mutant were unsuccessful, suggesting that this gene may be essential in *S. mutans* [83].

6. Small Non-Coding RNA

The bacterial non-coding RNA transcripts are usually 80 - 100 nt in length and are not generally processed. Many sRNAs like *oxyR*, *ryhB*, *dsrA*, *sgrS*, RNAIII, *pel* or *fasX* have key roles in the bacterial response to stress and regulation of factors important for virulence [84] [85] [86]. Many of those RNAs interact with small protein Hfq protein which increases their activity [87] [88]. So far neither *hfq* gene nor any of the well known small RNAs has been described in *S. mutans,* even if they are present in other *Streptococci* [89] [90] [91] [92]. Lemme and co-workers showed that the expression of 30 intergenic regions was significantly changed in a cell fraction where expression of *comX* gene was induced by CSP protein [93]. When the transcriptome of cells induced by CSP was compared to that of non-induced cells much more (380) differentially expressed intergenic regions were found [93]. Although no detailed analysis has been conducted that data suggests a potential role of small non-coding sequences in gene regulation of CSP induced pathways.

6.1. Clustered, Regularly Interspaced Short Palindromic Repeats (CRISPRs) Immunity—crRNA and TracrRNA

CRISPRs motifs, discovered in late 1980s, are involved in bacterial immunity mechanisms to protect cells against invasion of an "alien" DNA. That RNA -based adaptive defense system which targets phages or plasmids has been identified in almost 40% of currently sequenced bacterial genomes [94]. Currently eight types of CRISPR have been described [95]. The major part of the system contains a different number of 31 bp (23 to 50) long CRISPR motifs separated by an average 36 bp (17 to 84) long spacer sequences [96] The spacers show similarities to phage and plasmid sequences that can infect a particular cell. Typically, a repeat cluster is preceded by a long "leader" sequence. The second part constitutes genes encoding CRISPR associated proteins (*cas* genes) which are involved in both immunity acquisition and function. The mechanism of immunity acquisition is not known. It has been shown that new units (one or a few) are attached to the proximal (5') region and some Cas proteins are necessary in that process [94]. After activation the CRISPR region is transcribed as pre-crRNA and undergoes maturation. The final form of crRNA molecule contains an 8-nt part of the CRISPR motif on its 5' end, a spacer region and another part of CRISPR on the 3' end. For some types of CRISPR, Cas proteins alone are responsible for the maturation. Recently it has been described that maturation of

csRNAs in *S. pyogenes* type II CRISPR (CRISPR01) depends on the presence of a small -171- and 89- nt long tracrRNA as well as the RNase III and Crn1—Cas protein [97]. After maturation csRNP (csRNA/Cas) complexes are able to recognize and inactivate any foreign DNA (RNA) with homology to the spacer region however the molecular mechanism of that process still remains elusive.

The annotated genome of *S. mutans* UA159 was found to harbour two CRISPR loci, designated CRISPR1 and CRISPR2 [2] [98]. CRISPR1 is located between ORFs SMU.1400 and SMU.1398, and contains seven copies of a partially palindromic sequence of 36 bp. All repeats are interspersed by spacers of 30 bp in size [98]. Four *cas* genes have been identified upstream of CRISPR1 (SMU.1405-*csn*1, SMU.1404-*cas*1, SMU.1403-*cas*2 and SMU.1402-*csn*2). *S. mutans* CRISPR1 shows similarity with *S. pyogenes* CRISPR01 and the tracrRNA gene has been identified upstream of the SMU.1405 gene [97] [98]. The fact that, in contrast to *S. mutans*, the RNase III mutant of *S. pyogenes* has been described might suggest a different role of that enzyme in these two species [83] [97].

The CRISPR2 locus of UA159, a member of the type 3 CRISPR, is located between SMU.1753c and SMU.1752c, and consists of two 32 bp partially homologous repeats separated by a 34 bp spacer [98]. The expression of *cas* genes of the CRISPR2 was upregulated in a strain defective in *clpP* protease [99].

6.2. Cia-Dependent Small RNA (csRNAs)

These small RNAs were originally described in *Streptococcus pneumoniae*. The first csRNAs were found in the regulon of the two-component regulatory system (TCS-TCSTS) CiaRH [100] and were designated csRNAs (cia-dependent small RNA). They show a high degree of similarity to each other, especially in the unpaired region between the two stem-loop structures characteristic for these sRNAs. Complementarity to the translation initiation region (TIR)-Shine-Dalgarno (SD) sequence and the start codon AUG within this unpaired region suggested that the csRNA could control translation initiation of mRNAs.

In *Streptococcus mutans*, the CiaRH system is involved in bacteriocin production, competence regulation, biofilm formation, and tolerance to environmental stresses [22]. The search for csRNAs in *S. mutans* genome sequence revealed the presence of 3 csRNA molecules—csRNA23-1 (79nt), csRNA23-2 (81nt) and csRNA24 (144nt). CsRNA23-1 and csRNA23-2 shows a high degree of similarity on both sequence and structure levels. They form 2 stem-loop structures on both 5' and 3' ends. CsRNA24 is much longer and contains 4 stem-loop structures. Despite the size and structural differences the sequence complement to the TIR shows high degree of similarity. Therefore potential target prediction with the sTarPicker software (https://omictools.com/starpicker-tool) revealed 162 hypothetical target genes ($P > 0.5$) that were identical to CsRNA23-1, csRNA23-2 and csRNA24. It is not surprising that genes involved in processes regulated by the CiaRH system like *bacA*1 and SMU.1341 involved in bacteriocin production, *comB*, *comYB*—competence, *gbpA*—biofilm formation and *grpE*—stress re-

sponse were identified among these targets. The presence of genes belonging to some sugar PTS systems (like SMU.102, SMU.872, SMU.1598, SMU.1600, SMU1491, SMU.977) and specific regulators like MalR, LacR, GalR might suggest that csRNAs are involved in sugar metabolism. Eight more regulatory genes with *rpoD* gene encoding for housekeeping, sigmaA RNA polymerase subunit identified among csRNA targets also suggest that the role of those small RNAs is more complex.

It is worth to noting that some of the target sequences are localized within long transcriptional units. Therefore, one of the possible functions of these sRNA might be differentiation of protein translation levels within a single operon. In a single polycistronic mRNA the difference in number of proteins synthesized from the consecutive genes relies on the affinity of ribosome to the TIR regions. The presence of csRNA molecule in the TIR region might modulate that interaction and subsequently change the protein amount produced by that specific gene. It is also interesting that in several different TCSTS sRNA have been described to modulate transcription of activated genes [101] therefore that mechanism seems to be more common.

7. MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNAs with a size of 22 nt found in many living organisms and function as an important modulators of gene expression mostly in Eukaryota [102] [103] [104]. Advantages of high-throughput transcriptome sequencing methods (RNAseq) allow identifying miRNAs and analyzing their role in bacterial physiology. Recently, Lee and Hong have analyzed small transcripts in *S. mutans* and they identified 922 miRNAs [105]. Despite the possibility that some of those molecules resulted from the processing of long mRNA transcripts, the presence of some of those miRNA species in a great number might suggest that they are not simply a degradation products. As the role of miRNAs in eukaryotic cells has been established it is important to study those systems in bacterial cells to reveal their functions and possible phylogenetic relations.

8. Housekeeping Small RNAs

Some sRNAs play an important role in regulation of important physiological processes and they are widely distributed among different taxa therefore they were named housekeeping sRNAs. The most known housekeeping RNA are ribosomal RNAs, RNaseP, tmRNA, 6S RNA and SRP RNA.

8.1. tRNA Processing—RNase P

A proper structure of tRNA molecules necessary for its transfer function and T-box regulation is maintained by two RNases: exoribonuclease-RNase P and endoribonuclease-RNase Z. RNase P is a ribozyme and contains two components: an RNA chain, called M1 RNA, and a polypeptide chain, or protein, called

C5 protein [106]. Both components have been identified in *S. mutans* genome (M1 RNA pos. 439800-440179; C5, rnaP-SMU336) [2]. The major activity of RNase P is processing of the 5′leader sequence of precursor tRNA. The 3' end of tRNA, on the other hand, is processed by the RNase Z which does not contain any RNA components. RNase Z is responsible for the processing of tRNA precursors lacking an encoded CCA 3′terminus [107]. In *E. coli* all tRNA transcripts are terminated on CCA and it has been suggested that RNase Z plays a role in RNA decay [108], while in *Bacillus subtilis*, the best studied of the Gram-positive bacteria, about two-thirds of tRNAs (59 out of 86) have the CCA motif encoded by their genes and the presence of RNase Z gene is essential [107]. In *S. mutans* UA159 the RNase Z is encoded by SMU1474 gene. As only 9 out of 65 identified tRNA molecules are terminated on CCA the RNase Z plays a very important role in that strain.

8.2. Transfer-Messenger RNA—tmRNA

Transfer-messenger RNA (*ssrA*) is a bacterial RNA molecule with dual tRNA-like and messenger RNA-like properties. The tmRNA forms a ribonucleoprotein complex (tmRNP) together with Small Protein B (SmpB), Elongation Factor Tu (EF-Tu), and ribosomal protein S1. In *trans*-translation, tmRNP binds to bacterial ribosomes which have arrested on mRNA due to the presence of rare codons, lack of specific charged tRNA rare or stop codon. The tmRNA is remarkably versatile: it recycles the stalled ribosome, adds a proteolysis-inducing tag to the unfinished polypeptide, and facilitates the degradation of the aberrant messenger RNA [109] [110] [111].

Both *ssrA* (1139684-1139337) and *smpB* (SMU1606) genes have been identified on *S. mutans* UA159 chromosome [2]. However tmRNA functions mostly as a quality control and "recycling" pathway in some bacteria it can modulate gene activity acting as asRNA and binding to the RBS sites [112] and is required for full virulence, either enhancing or repressing the expression of specific genes during infection [113]. As the oral environment undergoes constant changes in nutrient availability it is likely that tmRNA plays an important role in *S. mutans* and other oral bacteria, however no experimental data are currently available. It has been shown that proteins tagged by *ssrA* are degraded by ClpP protease complex in *S. mutans* (Tao and Biswas, unpublished). As *clpP* deletion induces a striking difference in gene expression [99] and affects many physiological traits [114] it is possible that tmRNA plays also a role in gene regulation.

8.3. 6S/SsrS RNA

The 6S RNA specifically associates with RNA polymerase holoenzyme containing the sigma70 specificity factor. This interaction represses expression mostly from σ^{70}-dependent (so called σ^{70} extended promoters) but also some σ^{32} promoters during stationary phase [115] [116]. *B. subtilis* and several closely related bacteria have two 6S RNAs that are differentially expressed suggesting they might act at different stages of growth [116] [117].

In *S. mutans* only one *ssrS* gene has been identified (pos. 1927946-1928139). It is interesting that *ssrS* gene overlaps (39 nt) in antisens orientation with *rpoB* gene encoding RNA polymerase B subunit. Recently it has been shown that *E. coli* 6S RNA affects the amount of global stress alarmon ppGpp and it has been suggested that 6S RNA modulates transcription of several global regulators directly, including *relA*, to downregulate expression of key pathways in response to changing environmental conditions [118].

8.4. Signal Recognition Particle RNA—SRP RNA

SRP RNA is the RNA component of the signal recognition particle (SRP) ribonucleoprotein complex. SRP is universally conserved between many kingdoms and functions as a chaperone that directs the traffic of proteins within the cell and allows them to be secreted. The SRP RNA, together with one or more SRP proteins contributes to the binding and release of the signal peptide. SRP RNA is responsible for interaction and conformational changes of SRP and membrane receptor SM protein [119] [120].

In *S. mutans* SRP RNA have been identified in the SMU305-SMU307 (pos.293137-293237) and the SRP protein is encoded by SMU1060 (*ffh*) gene [2]. The two SM proteins, YidC1 and YidC2, are present and expressed in *S. mutans*. Deletion of YidC1 demonstrated no obvious phenotype. Elimination of YidC2 resulted in a stress-sensitive phenotype similar to SRP pathway mutants. In contrary to *E. coli* and *B. subtilis* mutations in *S. mutans* SRP RNA and/or *ffh* genes was not lethal but affected cells viability in stress conditions [121] [122]. That emphasizes the role of SRP mechanism in stress response processes and pointed out the differences between *S. mutans* and other model organisms.

9. Future Prospects

Posttranscriptional regulation of gene expression plays an important role in cell physiology of all living organisms. The most important role in that process is being held by small RNA molecules. Current technology allows identifying those small molecules; however functions of many sRNA still remain unknown. For some human pathogens like *S. mutans* which is naturally able to uptake any extracellular DNA an antisense therapy targeting sRNA might be a method of choice. That technique has been used effectively in prokaryotic systems, including *S. mutans* to inhibit gene expression [123]-[128]. Recently, more stable, chemically modified oligonucleotides (phosphorothioate oligodeoxyribonucleotides, PS-ODNs) have been used for gene silencing in *S. mutans* [129] [130]. Developing new strategies for delivery of those oligonucleotides and selecting the most appropriate targets might be one of the major goals to overcome the problem of increased multidrug-resistance in pathogenic microorganisms.

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Abbreviations

TCSTS—two component signal transduction systems
asRNA—antisense RNA
sRNA—small RNA
miRNA—micro RNA
RAT—ribonucleic antiterminator
CSP—competence-stimulating peptide
RNAi—interference RNA
TIR—translation initiation region
TA—toxin /antitoxin system
PTS—phosphotransferase systems