Japanese Dental Science Review 57 (2021) 209–216

Contents lists available at ScienceDirect

Japanese Dental Science Review

journal homepage: www.elsevier.com/locate/jdsr

Review Article

Small regulatory RNAs of oral streptococci and periodontal bacteria

Yuichi Oogai, Masanobu Nakata *

Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, 890-8544, Japan

ARTICLE INFO

Article history:
Received 29 June 2021
Received in revised form
20 September 2021
Accepted 24 September 2021

Keywords:
Small regulatory RNA
Non-coding RNA
Antisense RNA
Oral bacteria
Streptococcus
Periodontal pathogens

ABSTRACT

Small regulatory RNAs (sRNAs) belong to a family of non-coding RNAs, and many of which regulate expression of genes via interaction with mRNA. The recent popularity of high-throughput next generation sequencers have presented abundant sRNA-related data, including sRNAs of several different oral bacterial species. Some sRNA candidates have been validated in terms of their expression and interaction with target mRNAs. Since the oral cavity is an environment constantly exposed to various stimuli, such as fluctuations in temperature and pH, and osmotic pressure, as well as changes in nutrient availability, oral bacteria require rapid control of gene expression for adaptation to such diverse conditions, while regulation via interactions of sRNAs with mRNA provides advantages for rapid adaptation. This review summarizes methods effective for identification and validation of sRNAs, as well as sRNAs identified to be associated with oral bacterial species, including cariogenic and periodontal pathogens, together with their confirmed and putative target genes.

1. Bacterial sRNA introduction

Small regulatory RNA (sRNA) is a member of a family of non-coding RNAs, some of which have regulatory functions [1,2]. As compared with eukaryotic miRNA with sizes ranging from 20 to 30 nucleotides (nt), bacterial sRNAs are structurally heterogenous with a wider range of length from approximately 20–500 nt [1–3], and can be classified into intergenic region sRNAs (IGR sRNA) and antisense sRNAs (asRNAs), depending on their genomic location and orientation [1].

IGR sRNAs are encoded in intergenic regions, which generally contain no protein-coding sequence, and control translation or mRNA stability of target genes situated in various genomic regions (*trans*-acting regulation). In many instances, IGR sRNAs form a stem-loop structure and regulate expression of target mRNAs in a manner that involves imperfect base-pairing. For example, McaS [4] of *Escherichia coli* and RNAIII [5] of *Staphylococcus aureus* mediate translational activation of the target genes *flhD* (flagellar transcriptional regulator) and *hla* (α-Hemolysin), respectively. These target mRNAs form a hairpin structure on the 5′ untranslated region (UTR) containing the ribosome binding site (RBS), which halts translation due to low accessibility of the ribosome to mRNA. Binding of the sRNAs to target mRNA resolves the hairpin structure and relieves the restricted accessibility (Fig. 1A). The *Listeria monocytogenes* sRNA LhrA binds to the 5′ UTR region containing the RBS of target mRNAs and functions as a translational repressor by preventing ribosome access (Fig. 1B) [6]. Furthermore, mRNA levels of target genes are modulated by binding of IGR sRNA. Also, the sRNA-mRNA complex enhances RNA degradation by endogenous RNases, resulting in decreased expression levels of target genes (Fig. 1C). The *Escherichia coli* sRNA RyhB regulates the level of *sodB* encoding superoxide dismutase by RNase E cleavage [7], while the *Salmonella typhimurium* sRNA MicA binds *ompA* mRNA encoding an outer membrane protein, and the complex of MicA and target mRNA is cleaved by RNase III [8]. In contrast, some sRNAs are known to enhance target mRNA stability (Fig. 1D), including the *Streptococcus pyogenes* sRNA FasX that interacts with the 5′ UTR of *ska* mRNA encoding streptokinase [9].

asRNAs are encoded on the opposite strand of the gene. In many instances, they bind to mRNAs expressed from the antisense strand and mediate regulation (*cis*-acting regulation) by various mechanisms, such as inhibition/activation of translation as well as alteration of RNA stability [10]. Gene regulation of several different bacterial species, especially Gram-negative bacteria, via sRNAs often requires RNA chaperons, such as Hfq [11], ProQ [12], or CsrA [13]. These chaperons mediate interactions between sRNA and mRNA, which effects the regulation mechanisms of sRNAs [14].

This review summarizes methods used for identification and validation of sRNAs, and also presents findings by which the functions of thus far reported sRNAs and asRNAs of oral bacteria

* Corresponding author.
E-mail address: nakata@dent.kagoshima-u.ac.jp (M. Nakata).

https://doi.org/10.1016/j.jdsr.2021.09.004

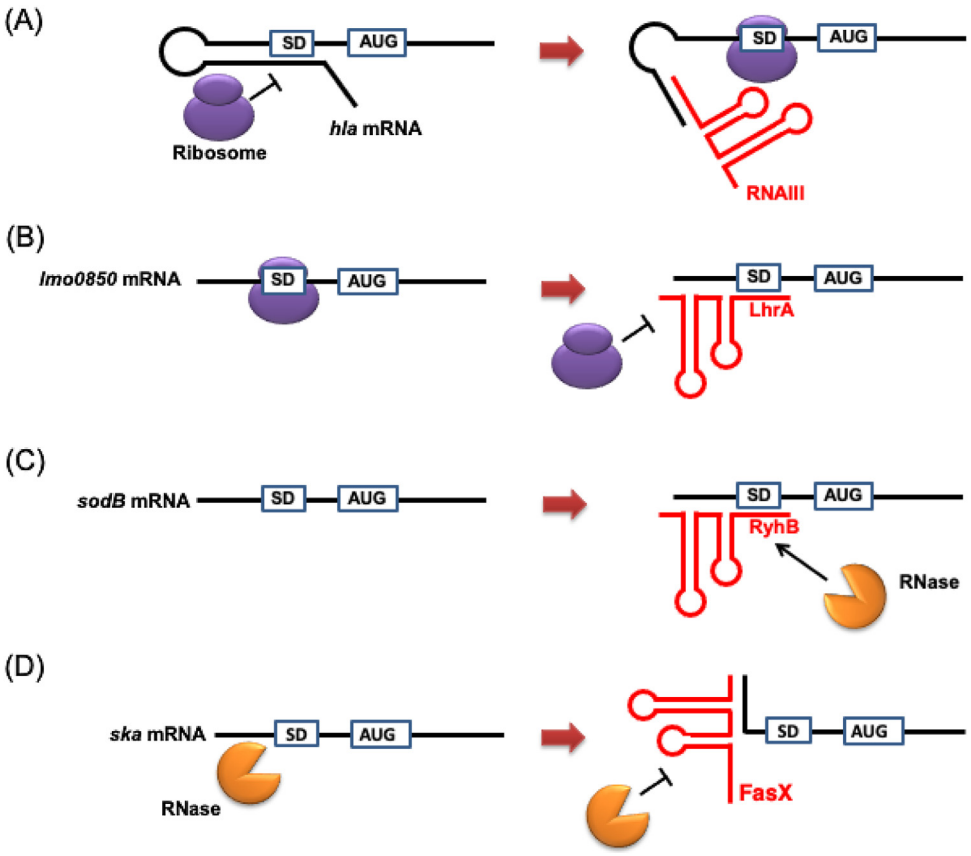

Fig. 1. Regulatory functions of sRNA.
(A) Translational activation by sRNA. Base-pairing of RNA III, a multifunctional sRNA of *Staphylococcus aureus*, with the α-hemolysin-encoding *hla* mRNA modifies the mRNA secondary structure and exposes the RBS to the ribosome. (B) Translational suppression by sRNA. Binding of the *lmo0850* mRNA encoding a hypothetical protein to the ribosome is inhibited by base-pairing of the LhrA sRNA in *Listeria monocytogenes*. (C) mRNA degradation induced by sRNA. In *Escherichia coli*, base-pairing of the RyhB sRNA and the superoxide dismutase-encoding *sodB* mRNA recruits endogenous RNase, which cleaves the complex. (D) RNA stabilization by sRNA. Binding of the sRNA FasX to the streptokinase-encoding *ska* mRNA enhances the mRNA stability in *Streptococcus pyogenes*.

can be confirmed or deduced, including the cariogenic bacterium *Streptococcus mutans*, and periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Additionally, various sRNA and asRNA functions are introduced in the context of virulence-related regulation.

2. sRNA identification

The complete genome sequences of many bacterial species have been deposited in databases. However, rather than identification of open reading frames, experimental analysis is basically required for identification of IGR sRNAs and asRNAs. Prior to development of the next-generation sequencing technology, software-based *in silico* analyses and microarrays covering the entire genome were utilized to predict and identify sRNAs [15,16], while more recently RNA-seq analysis has become the main method for sRNA identification [17–19]. Furthermore, oral bacteria (genus *Streptococcus* and periodontal pathogens) sRNAs have also been detected by use of this method (Table 1) [20–30].

Techniques used for RNA purification and cDNA library construction have effects on the number and class of identifiable sRNAs. Those similar to the method for sequencing eukaryotic miRNA have been used for bacterial sRNA identification, including (1) Small RNA fractions extracted with a column specialized for miRNA and (2) a cDNA library constructed from fractions extracted from a polyacrylamide gel after electrophoresis (Fig. 2A). sRNAs have been identified with these sampling methods by some research groups [20–26] that focused on short sRNAs with a length less than 50 nt, termed miRNA-Size sRNA (msRNAs) (Table 1), as they are not suitable for detection of relatively large sRNA molecules. Representative sRNAs with a large size (>200 nt) that have been well studied include RNAIII of *S. aureus* [5], VR-RNA of *Clostridium perfringens* [31], and FasX [32] of *Streptococcus pyogenes*.

Jorth et al. sequenced all transcripts of strain 624 of the periodontal pathogen *A. actinomycetemcomitans*, using a total RNA-seq method (Fig. 2B), which provided identification of 202 sRNAs containing long molecules (>200 nt) and defined the transcriptional start sites of all sRNAs [28]. RNA-seq analysis targeting total RNA is appropriate for revealing sRNA repertories, though the data obtained will also contain a large amount of unnecessary sequences, including those related to protein-coding mRNA and ribosomal RNA. In addition, the cost for such a sequencing procedure is higher than that for sequencing only small molecules. A library of fragmented RNA is generally used for RNA-seq analysis (Fig. 2B), because the sequence platform limits the size (for example, <600 nt with an Illumina sequencer).

Previously, one of the present authors reported sRNAs of *A. actinomycetemcomitans* strain HK1651 [29]. In that study, total RNA was intentionally not fragmented and long transcripts were excluded in the following step of cDNA library construction by PCR (Fig. 2C). Sequencing was performed using an illumina MiSeq platform and a total of 120 sRNAs with lengths of 35–386 nt were identified. Most of the obtained reads were 5s rRNA, thus elimination of 5s rRNA from a sequence library may be useful for efficient sRNA identification. Several rRNA depletion kits are commercially available, including MICROBExpress Bacterial mRNA

Table 1
sRNAs of oral bacteria identified by RNA-seq.

Species	Strain	Source of sequence library	Number of sRNA	References
Streptococcus mutans	ATCC 25175	Small molecules of cDNA were extracted after PAGE[a]	922	20
	UA159	Small molecules of cDNA were extracted after PAGE	1879	21
	UA159	Small RNA fraction was extracted using miRNA column	2125	23
	UA159	Small molecules of cDNA were extracted after PAGE	736	25
	UA159	Small RNA fraction was extracted using miRNA column	2749	26
Streptococcus sanguinis	SK36	Small RNA fraction was extracted using miRNA column	219	24
Aggregatibacter actinomycetemcomitans	624	Total RNA-seq without rRNA	202	28
	ATCC 33384	Small RNA fraction was extracted using miRNA column	59	22
	HK1651	Total RNA was converted to cDNA without fragmentation	120	29
Porphyromonas gingivalis	ATCC 33277	Total RNA-seq without rRNA	2480	30
	W83	Small RNA fraction was extracted using miRNA column	30	27
	ATCC 33277	Small RNA fraction was extracted using miRNA column	40	22
Treponema denticola	ATCC 35405	Small RNA fraction was extracted using miRNA column	11	22

[a] Polyacrylamide gel electrophoresis.

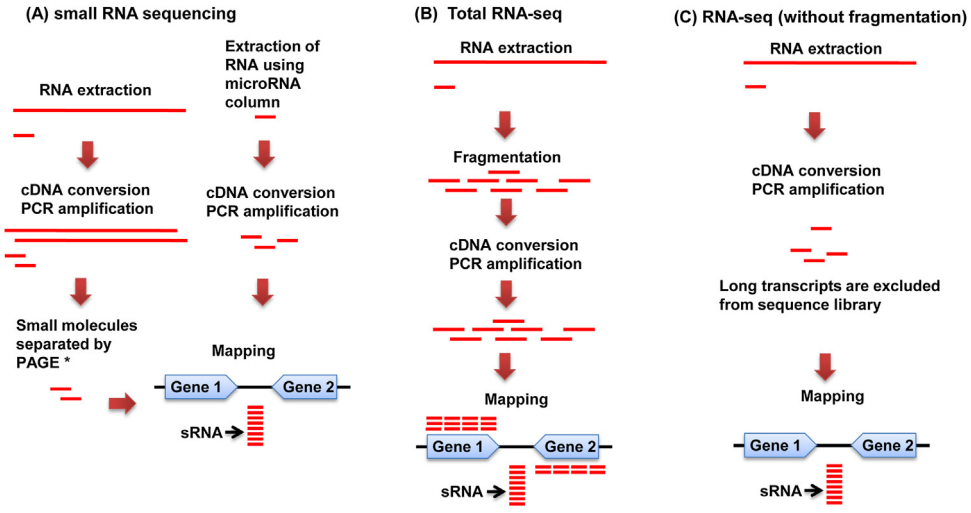

Fig. 2. Flowchart illustrating bacterial sRNA identification.
(A) Sequence samples of small RNA-seq were prepared from gel extracts after PAGE or from eluates produced by an miRNA specialized column. (B) Total RNA-seq allows for sequences of all transcripts containing sRNA. (C) RNA-seq analysis with non-fragmented RNA allows for effective sequencing of small molecules as well as sRNAs with a relatively large size (>200 nt). *Polyacrylamide gel electrophoresis.

Enrichment Kit (Thermo Fisher Scientific, Waltham, MA, USA), RiboMinus Transcriptome Isolation Kit, bacteria (Thermo Fisher Scientific), and Ribo-Zero rRNA Depletion Kit (Illumina, San Diego, CA, USA), though only the Ribo-Zero kit contains 5S rRNA-specific probes that can be used to eliminate 5S rRNA by RNA hybridization. Alternatively, terminator 5′ monophosphate-dependent exonuclease (TEX) can be utilized to remove rRNA from total RNA. TEX is a processive 5′ → 3′ exonuclease and specifically digests RNA with 5′ monophosphate, while it does not degrade RNA with the 5′-triphosphate, 5′ -cap, or 5′-hydroxyl group. The primary bacterial transcripts contain a triphosphate at the 5′ terminus. On the other hand, rRNA and RNA degradant have a mono-phosphate at the 5′ terminus, which can be digested by TEX. Unlike eukaryotic miRNA, several bacterial sRNAs contain a triphosphate at the 5′ terminus, and TEX treatment can be used to eliminate rRNA and RNA degradants.

Co-immunoprecipitation with RNA chaperones has been applied for identification of RNA molecules bound to those chaperones [33,34]. Since several functional sRNAs form the mRNA-sRNA-protein complex, sequencing of RNAs co-precipitated with chaperones can be used to identify such sRNAs [11–13]. In particular, Hfq, a bacterial member of the Sm family of RNA-binding proteins, has been used for identification of RNA, including sRNAs and their target mRNA [35,36].

3. sRNA validation

The detected expression levels of sRNAs vary, depending on the strain and culture conditions. Furthermore, previously identified sRNAs were listed by use of criteria determined by individual researchers, which were mainly based on expression levels and genomic locations. Therefore, the numbers of sRNAs identified differ considerably among studies, even of the same species (Table 1). In addition, there is a possibility of false-positive results that might have been caused by sequencing RNA degradation products even though expression level was high. Thus, sRNA validation in additional experiments is important to reconfirm expression and orientation, as well as the encoded chromosomal locations. Northern blot analysis has been frequently used for validation of sRNA candidates [37,38] as well as reverse transcription PCR (RT-PCR) [37], though use of Taq-man probes is necessary to distinguish transcription orientations. Additionally, it is also difficult to design primers for short IGR sRNA, thus rapid amplification of cDNA ends (RACE) analysis is suitable for validation. In a previous study, 70 of 120 sRNA candidates were validated by use of 3′ RACE analysis [29], as that is useful for determination of the transcriptional start site and 3′ terminal end of sRNA.

4. Oral *Streptococcus* sRNAs

The genus *Streptococcus* is comprised of Gram-positive cocci and dominant in the oral cavity. RNA-dependent regulation of virulence, competence, and bacterial immunity has been examined in studies of pathogenic streptococci [39–41]. Organisms belonging to a subset of oral *Streptococcus* species, such as mitis group *Streptococcus*, are known to be primary colonizers on tooth surfaces and promote dental biofilm formation. Mutans group *Streptococcus* is involved in dental caries development by their activities to metabolize carbohydrates and produce acids. These oral commensals also occasionally cause systemic infections such as endocarditis by gaining access to the bloodstream. *Streptococcus mutans* and *Streptococcus sanguinis* sRNAs have been investigated, with a brief discussion of those findings following. Table 2 summarizes information about their notable sRNAs.

4.1. *Streptococcus mutans*

S. mutans is the major pathogen responsible for dental caries, which arises from decalcification of the tooth tissues under an acidic condition (less than pH 5.5) [42]. This bacterium can metabolize a variety of carbohydrates by fermentation and produces acid as a metabolic byproduct, leading to decalcification. The acid tolerance of this bacterium has been noted as a crucial factor related with its cariogenic activity and ability to survive within acidic carious lesions [43].

Liu et al. identified small-sized sRNAs (18−50 nt) in *S. mutans* strain UA159 cultured under an acidic condition and further examined two sRNAs abundantly expressed (referred to as srn884837 and srn133480, with length of 29 and 27 nt, respectively) [21]. Using RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) [44], target mRNAs were predicted to encode factors involved in acid tolerance, including the glutamate transporter components GlnQ and GlnM [45], Ffh, homologous to the signal recognition particle of *E. coli* [46], the surface-associated protein BrpA, homologous to LytR-CpsA-Psr family proteins [47], and RelA responsible for synthesis and hydrolysis of guanosine tetraphosphate/pentaphosphate [48] (*glnQ*, *glnM*, *brpA*, and *relA* for srn884837; *ffh*, *brpA*, and *relA* for srn133480). The expression levels of the two sRNAs were decreased with lowered pH (pH 6.5, 5.5, 4.5), while those of the corresponding putative target genes were increased, implying that these sRNAs have negative effects on expression of acid tolerance related genes.

Expression of sRNA molecules containing both the srn133480 sequence and 6 additional bases at the 5′ end (referred to as sRNA133474) was also shown to be decreased along with a decrease in pH in ten tested clinical isolates [49]. Among the examined strains, the expression levels of sRNA133474 varied, while highly acid-tolerant strains showed lower expression levels of srn133480 as compared with low acid-tolerant strains. Furthermore, target mRNAs predicted with use of the RNApredator system (http://rna.tbi.univie.ac.at/RNApredator) [50] included mRNAs of two-component system (TCS) genes, including *liaS*, *liaR*, *comE*, *covR*, and *ciaR* [51–54], with the expression levels of *liaR*, *covR*, and *ciaR* shown to be negatively correlated with that of sRNA133474. The authors suggested that sRNA133474 could be utilized for adaptation to acidic conditions via TCS modulation.

Other TCS genes are also under the control of an asRNA and sRNAs. The VicRK TCS is highly conserved in Gram-positive bacteria and is related to virulence-associated traits, such as biofilm formation, genetic competence, bacteriocin production, and exopolysaccharide synthesis [55–57]. Mao et al. reported that three putative sRNAs (referred to as msRNA 1701, msRNA 3405, and msRNA 1657) potentially target *vicRK* mRNA, and their expression levels were negatively correlated with that of *vicRK* and positively correlated with that of *rnc* encoding RNase III [58]. Consequently, the authors inferred that RNase III post-transcriptionally reduced *vicRK* mRNA levels through sRNAs. Furthermore, VicR protein level was shown to be negatively regulated by asRNA of *vicR* (ASvicR), which was confirmed by Northern blot analysis findings [59]. Overexpression of ASvicR was reported to delay bacterial growth, and compromised the ability of the organisms to synthesize exopolysaccharide and form carious lesions on teeth in a rat infection model [59]. Furthermore, transcriptome analysis indicated that ASvicR inhibits galactose and glucose metabolism. Thus, the elaborate interplay between VicRK TCS genes, ASvicR, putative sRNAs, and RNase III could be related to the cariogenic properties of *S. mutans*.

Adhesion to tooth surfaces and synthesis of water-insoluble glucan are also determinants for *S. mutans* cariogenicity [60]. Liu et al. reported that sRNAs participate in regulation of bacterial adherence and glucan synthesis in response to extracellular sucrose [23]. Of 2125 sRNA candidates expressed under a 1% or 5% sucrose condition, 22 were differentially expressed depending on the sucrose concentration, six of which were validated by quantitative RT-PCR analysis. Target mRNAs predicted with an RNAhybrid algorithm were found to include *spaP*, *gtfB*, and *gtfC*, and shown to encode antigen I/II family surface proteins, protein antigen C (PAc) [61], and two types of glucosyltransferases (GTFs) responsible for synthesis of extracellular water-insoluble glucan [62,63]. However, details regarding direct interaction of sRNAs with mRNAs of these putative targets and the potential consequences remain unknown. A subset of sRNAs were also reported to be induced in response to extracellular glucose [26]. In addition, expression of two sRNAs (referred to as sRNA0187 and sRNA0593) were correlated with bacterial adherence to a Petri dish surface in the presence of 1% sucrose [25].

The 5′ UTR of the *irvA* gene encoding a putative repressor [64] was shown to interact with glucan-binding protein C (GbpC)-encoding mRNA [65], also to modulate mRNA stability as well as GbpC production [66]. Furthermore, the protein encoding *irvA* mRNA has an ability to function as a trans-RNA, while sRNAs have also been reported to be involved in bacterial toxin-antitoxin (TA) systems [67]. This system consists of two components, a peptide toxin and cognate antitoxin. Of six classes of bacterial TA systems, *S. mutans* strain UA159 possesses the type I TA system, where the untranslated antisense small RNA (~70 nt) serves as the antitoxin [68]. Base-pairing of the toxin mRNA and *cis*-encoded small antitoxin sRNA may suppress the toxin translation.

Although several *S. mutans* sRNAs have been reported, the exact mechanisms related to their functions remain largely elusive. Elucidation of the detailed functions and critical roles of those sRNAs in regulatory networks may provide a molecular basis for therapeutic approaches for treatment of dental caries.

4.2. *Streptococcus sanguinis*

S. sanguinis, a commensal oral bacterium, is a member of mitis group *Streptococcus* and known as an early colonizer on tooth surfaces, thus is involved in development of dental plaque [69]. In addition, this bacterium has frequently been isolated from endocarditis lesions [70]. Using RNA-seq analysis, Choi et al. identified 219 msRNAs and then focused on an asRNA (referred to as S.S-1964) expressed from the antisense strand corresponding to the 3′ UTR of the gene encoding ATP:cob(I)alamin adenosyltransferase (SSA0513), an enzyme involved in conversion of cobalamin (vitamin B12) to its coenzyme form. Expression of SSA0513 was increased following addition of skim milk to the culture, while the expression of asRNA was decreased. The authors also showed that sRNAs including S.S-1964 were secreted by extracellular mem-

Table 2
Notable sRNAs found in oral streptococci.

sRNA name	Species	Strain	Expression	Targets	Putative phenotype	References
srn884837	*Streptococcus mutans*	UA159	Decreased in low pH	*glnQ*, *glnM*, *brpA*, *relA*	Acid tolerance	20
srn133480	*Streptococcus mutans*	UA159	Decreased in low pH	*ffh*, *brpA*, *relA*	Acid tolerance	20
sRNA133474	*Streptococcus mutans*	UA159 and clinical strains	Decreased in low pH	*liaR*, *ciaR*, *covR*	Acid tolerance	49
msRNA 1701	*Streptococcus mutans*	UA159	Decreased in *rnc* mutant	*vicK*	Biofilm formation	58
msRNA 3405	*Streptococcus mutans*	UA159	Decreased in *rnc* mutant	*vicR*	Biofilm formation	58
msRNA 1657	*Streptococcus mutans*	UA159	Decreased in *rnc* mutant	*vicK*	Biofilm formation	58
ASvicR	*Streptococcus mutans*	UA159	Decreased expression in biofilm	*vicR*	Biofilm formation	59
sRNA0187	*Streptococcus mutans*	UA159 and clinical strains	Increased in high sucrose		Biofilm formation	25
sRNA0593	*Streptococcus mutans*	UA159 and clinical strains	Decreased in high sucrose		Biofilm formation	25
irvA 5′ UTR	*Streptococcus mutans*	UA159		*gpbC*	Dextran-dependent aggregation	66
srSm	*Streptococcus mutans*	UA159		*fst-Sm*	Toxin-antitoxin system	68
S.S-1964	*Streptococcus sanguinis*	SK36	Decreased in skim milk	SSA0513	Conversion of cobalamin	24
csRNA1-1	*Streptococcus sanguinis*	SK36	Decreased in *ciaRH* mutant	*pilT*	Biofilm formation	40
csRNA1-2	*Streptococcus sanguinis*	SK36	Decreased in *ciaRH* mutant	*pilT*	Biofilm formation	40

Table 3
Notable sRNAs found in periodontal bacteria.

sRNA name	Species	Strain	Expression	Targets	Putative phenotype	References
sRNA PG_RS02100	*Porphyromonas gingivalis*	W83	Decreased in stationary phase cell		Oxidative stress resistance and heme accumulation	81
sRNA JA03	*Aggregatibacter actinomycetemcomitans*	HK1651	Increased in iron-chelated medium	*hitC*	Iron acquisition	83
AaHKsRNA042	*Aggregatibacter actinomycetemcomitans*	HK1651		*ltxD*	Cytolysin production	29
AaHKsRNA093	*Aggregatibacter actinomycetemcomitans*	HK1651		*cdtA*	Cytolethal distending toxin production	29
AaHKsRNA051	*Aggregatibacter actinomycetemcomitans*	HK1651		*flp-1*	Adhesin to host cell	29

brane vesicles. However, the interaction of asRNA and *ssa*0513 mRNA was not elucidated in that study [24].

Streptococcus pneumoniae, a member of the mitis group, was shown to produce five *cia*-dependent small RNAs (csRNA), of which expression is regulated by CiaRH TCS [71]. Thereafter, homology searches revealed six csRNA genes present in *S. sanguinis*, including csRNA1-1, csRNA1-2, csRNA1-3, csRNA2, csRNA7, and csRNA8 [72]. Ota et al. performed target prediction of those csRNAs using TargetRNA2, which showed the *pilT* gene encoding a component of the type IV pilus to be a target of csRNA1-1 [40,73]. While *S. sanguinis* strains, such as strain SK36, produce cell wall anchored pili that bind to salivary amylase [74,75], type IV pili are produced in only a few of those strains [76]. Type IV pili mediate a variety of cellular processes, such as twitching motility, genetic competence, and biofilm formation [77]. Furthermore, Ota et al. demonstrated that csRNA1-1 directly binds to *pilT* mRNA using RNA-RNA electrophoretic mobility shift assay, while mutation of both csRNA1-1 and csRNA1-2 compromised the ability to form biofilm [40].

5. Periodontal pathogen sRNAs

Periodontal disease is a polymicrobial disease caused by a variety of bacterial virulence factors in concert with the immune response [78,79]. Currently, periodontal pathogens are recognized as pathobionts related with the onset of systemic diseases [80]. In the following section, reported sRNAs of *P. gingivalis*, *A. actinomycetemcomitans*, and *Treponema denticola* are introduced. Table 3 summarizes information about the notable sRNAs.

5.1. P. gingivalis

P. gingivalis is a Gram-negative anaerobe that belongs to the phylum Bacteroidetes. Hirano et al. performed RNA-seq analysis of *P. gingivalis* strain ATCC 33277 and confirmed 11 sRNAs based on 5′ RACE analysis results [30], while Philips et al. identified 30 sRNAs in *P. gingivalis* strain W83 using RNA-seq and microarray analyses [27]. Thereafter, the latter group constructed an inactivated mutant strain of sRNA PG_RS02100 and demonstrated that the mutation rendered bacteria more resistant to oxidative stress. The color of the mutant strain colonies grown on blood agar plates was darker than that of those of the wild type. The authors suggested that sRNA regulates genes involved in oxidative stress resistance and heme accumulation [81].

5.2. A. actinomycetemcomitans

The major periodontal pathogen *A. actinomycetemcomitans* is a Gram-negative facultative anaerobe that belongs to the phylum Proteobacteria. Jorth et al. validated nine sRNAs in strain VT1169 from sRNA candidates predicted by bioinformatics [82]. Additionally, the authors also performed a more systematic screening of sRNA in strain 624 using RNA-seq and identified 202 sRNAs [28]. Utilizing bioinformatic analysis and strain HK1651, Amarasinghe et al. identified four sRNAs expressed under iron-limiting conditions and found that overexpression of the sRNA JA03 significantly repressed expression of *hitC* encoding an ATP-binding protein, a component of the Fe(III) ABC transporter [83]. Oogai et al. examined strain HK1561 and reported a total of 120 sRNAs (90 IGR-sRNAs, 30 asRNAs) [29]. Distribution of the identified sRNAs among five *A. actinomycetemcomitans* strains was analyzed by RT-PCR and intraspecies sRNA variations were noted. Target mRNA prediction of identified sRNAs using intaRNA [84] showed interactions of sRNAs and mRNAs encoding major virulence factors (*cdtA*, *lktD*, and *flp-1* encoding cytolethal distending toxin subunit A, leukotoxin export protein LtxD, and fimbrial protein Flp 1, respectively),

suggesting that *A. actinomycetemcmoitans* virulence is controlled by sRNAs.

5.3. T. denticola *and outer membrane vesicles of periodontal pathogens*

T. denticola is an oral spirochete associated with chronic periodontitis. Choi et al. identified 11 msRNAs from the *T. denticola* strain ATCC 35405, three of which (T.D2161, T.D15612, and T.D16563) were detected in secreted outer membrane vesicles (OMVs) by Northern blotting [22]. Additionally, they also found 40 msRNAs from *P. gingivalis* strain ATCC 33277 and 59 msRNAs from *A. actinomycetemcomitans* strain ATCC 33384. Furthermore, msRNAs were also detected in OMVs of these species, including an msRNA (A.A_20050) from *A. actinomycetemcomitans* and three msRNAs (P.G_45033, P.G_4378, P.G_122) from *P. gingivalis*. When OMVs from the three species were purified and separately added to NIH3T3 fibroblastic cells, penetration of all tested msRNAs into cytoplasm of those cells was detected. Also, transfection of a synthetic msRNA oligonucleotide, such as A.A_20050, P.G_45033, or T.D_2161, into Jurkat T cells decreased expression levels of interleukin (IL) -5, IL-13, and IL-15. Together, these findings indicated that msRNAs delivered to mammalian cells by OMVs potentially modulate host immune responses through RNA interference.

6. Conclusion

Over the recent decade, numerous sRNAs of oral bacteria have been identified. As shown in the above sections, the sRNA repertoire obtained from RNA-seq analyses of the same bacterial species (even the same strain) among various conducted studies have revealed great diversity. The differences can be mainly attributed to strain-specific expression due to genomic diversity as well as the criteria used for analysis. To better understand the relationship of sRNAs with virulence-related phenotypes of oral bacteria, detailed experimental analyses together with the computational validations [85,86] are anticipated. It will be necessary to confirm the expression mode, precise chromosomal locations encoded, target mRNA, and mechanisms of action. sRNAs demonstrate post-transcriptional regulation of genes important for cellular events, including adaptation to environmental cues, biofilm formation, and virulence [87]. Although several studies thus far presented have predicted that sRNAs of oral pathogens interact with virulence gene mRNAs, functional information regarding sRNAs is very limited. Additional studies of oral bacteria sRNAs together with metatranscriptome data of the oral microbiome [88,89] should provide additional insight into the regulatory networks and exact roles of sRNAs related to the pathogenesis of oral diseases. Since the sRNA-mediated artificial regulation has been utilized for modulating gene expression and engineering metabolic status in bacteria [90,91], application of sRNAs, such as delivering effective synthetic sRNAs into distinct oral bacteria, may provide a new strategy to combat oral diseases.

Conflicts of interest

The authors have no conflicts of interest to declare in regard to this study.

References

[1] Waters LS, Storz G. Regulatory RNAs in bacteria. Cell 2009;136:615–28.
[2] Fröhlich KS, Vogel J. Activation of gene expression by small RNA. Curr Opin Microbiol 2009;12:674–82.
[3] Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642–55.
[4] Thomason MK, Fontaine F, De Lay N, Storz G. A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli*. Mol Microbiol 2012;84:17–35.
[5] Morfeldt E, Taylor D, von Gabain A, Arvidson S. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO J 1995;14:4569–77.
[6] Nielsen JS, Lei LK, Ebersbach T, Olsen AS, Klitgaard JK, Valentin-Hansen P, et al. Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*. Nucleic Acids Res 2010;38:907–19.
[7] Afonyushkin T, Vecerek B, Moll I, Bläsi U, Kaberdin VR. Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. Nucleic Acids Res 2005;33:1678–89.
[8] Viegas SC, Silva IJ, Saramago M, Domingues S, Arraiano CM. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. Nucleic Acids Res 2010;39:2918–30.
[9] Ramirez-Peña E, Treviño J, Liu Z, Perez N, Sumby P. The group A *Streptococcus* small regulatory RNA FasX enhances streptokinase activity by increasing the stability of the *ska* mRNA transcript. Mol Microbiol 2010;78:1332–47.
[10] Saberi F, Kamali M, Najafi A, Yazdanparast A, Moghaddam MM. Natural antisense RNAs as mRNA regulatory elements in bacteria: a review on function and applications. Cell Mol Biol Lett 2016;21:6.
[11] Geissmann TA, Touati D. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. EMBO J 2004;23:396–405.
[12] Attaiech L, Boughammoura A, Brochier-Armanet C, Allatif O, Peillard-Fiorente F, Edwards RA, et al. Silencing of natural transformation by an RNA chaperone and a multitarget small RNA. Proc Natl Acad Sci USA 2016;113:8813.
[13] Vakulskas CA, Potts AH, Babitzke P, Ahmer BMM, Romeo T. Regulation of bacterial virulence by Csr (Rsm) systems. Microbiol Mol Biol Rev 2015;79:193–224.
[14] Quendera AP, Seixas AF, dos Santos RF, Santos I, Silva JPN, Arraiano CM, et al. RNA-binding proteins driving the regulatory activity of small non-coding RNAs in bacteria. Front Mol Biosci 2020;7.
[15] Pichon C, Felden B. Small RNA gene identification and mRNA target predictions in bacteria. Bioinformatics 2008;24:2807–13.
[16] Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, et al. The *Listeria* transcriptional landscape from saprophytism to virulence. Nature 2009;459:950–6.
[17] Shinhara A, Matsui M, Hiraoka K, Nomura W, Hirano R, Nakahigashi K, et al. Deep sequencing reveals as-yet-undiscovered small RNAs in *Escherichia coli*. BMC Genomics 2011;12:428.
[18] Gómez-Lozano M, Marvig RL, Molin S, Long KS. Genome-wide identification of novel small RNAs in *Pseudomonas aeruginosa*. Environ Microbiol 2012;14:2006–16.
[19] Carroll RK, Weiss A, Broach WH, Wiemels RE, Mogen AB, Rice KC, et al. Genome-wide annotation, identification, and global transcriptomic analysis of regulatory or small RNA gene expression in *Staphylococcus aureus*. mBio 2016;7:e01990-15.
[20] Lee H-J, Hong S-H. Analysis of microRNA-size, small RNAs in *Streptococcus mutans* by deep sequencing. FEMS Microbiol Lett 2012;326:131–6.
[21] Liu S, Tao Y, Yu L, Zhuang P, Zhi Q, Zhou Y, et al. Analysis of small RNAs in *Streptococcus mutans* under acid stress—a new insight for caries research. Int J Mol Sci 2016;17:1529.
[22] Choi JW, Kim SC, Hong SH, Lee HJ. Secretable small RNAs via outer membrane vesicles in periodontal pathogens. J Dent Res 2017;96:458–66.
[23] Liu SS, Zhu WH, Zhi QH, Liu J, Wang Y, Lin HC. Analysis of sucrose-induced small RNAs in *Streptococcus mutans* in the presence of different sucrose concentrations. Appl Microbiol Biotech 2017;101:5739–48.
[24] Choi J-W, Kwon T-Y, Hong S-H, Lee H-J. Isolation and characterization of a microRNA-size secretable small RNA in *Streptococcus sanguinis*. Cell Biochem Biophy 2018;76:293–301.
[25] Zhu W, Liu S, Liu J, Zhou Y, Lin H. High-throughput sequencing identification and characterization of potentially adhesion-related small RNAs in *Streptococcus mutans*. J Med Microbiol 2018;67:641–51.
[26] Liu S, Zhou Y, Tao Y, Zhuang P, Pang L, Zhi Q, et al. Effect of different glucose concentrations on small RNA levels and adherence of *Streptococcus mutans*. Curr Microbiol 2019;76:1238–46.
[27] Phillips P, Progulske-Fox A, Grieshaber S, Grieshaber N. Expression of *Porphyromonas gingivalis* small RNA in response to hemin availability identified using microarray and RNA-seq analysis. FEMS Microbiol Lett 2014;351:202–8.
[28] Jorth P, Trivedi U, Rumbaugh K, Whiteley M. Probing bacterial metabolism during Infection using high-resolution transcriptomics. J Bacteriol 2013;195:4991–8.
[29] Oogai Y, Gotoh Y, Ogura Y, Kawada-Matsuo M, Hayashi T, Komatsuzawa H. Small RNA repertoires and their intraspecies variation in *Aggregatibacter actinomycetemcomitans*. DNA Res 2017;25:207–15.
[30] Hirano T, Beck D, Demuth D, Hackett M, Lamont R. Deep sequencing *of Porphyromonas gingivalis* and comparative transcriptome analysis of a LuxS mutant. Front Cell Infect Microbiol 2012;2.
[31] Shimizu T, Yaguchi H, Ohtani K, Banu S, Hayashi H. Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. Mol Microbiol 2002;43:257–65.
[32] Kreikemeyer B, Boyle MD, Buttaro BA, Heinemann M, Podbielski A. Group A streptococcal growth phase-associated virulence factor regulation by a novel

operon (Fas) with homologies to two-component-type regulators requires a small RNA molecule. Mol Microbiol 2001;39:392–406.
[33] Chao Y, Papenfort K, Reinhardt R, Sharma CM, Vogel J. An atlas of Hfq-bound transcripts reveals 3′ UTRs as a genomic reservoir of regulatory small RNAs. EMBO J 2012;31:4005–19.
[34] Dugar G, Svensson SL, Bischler T, Wäldchen S, Reinhardt R, Sauer M, et al. The CsrA-FliW network controls polar localization of the dual-function flagellin mRNA in *Campylobacter jejuni*. Nat Commun 2016;7:11667.
[35] Saadeh B, Caswell CC, Chao Y, Berta P, Wattam AR, Roop 2nd RM, et al. Transcriptome-wide identification of Hfq-associated RNAs in *Brucella suis* by Deep Sequencing. J Bacteriol 2016;198:427–35.
[36] Sittka A, Sharma CM, Rolle K, Vogel J. Deep sequencing of Salmonella RNA associated with heterologous Hfq proteins in vivo reveals small RNAs as a major target class and identifies RNA processing phenotypes. RNA Biol 2009;6:266–75.
[37] Grüll MP, Peña-Castillo L, Mulligan ME, Lang AS. Genome-wide identification and characterization of small RNAs in *Rhodobacter capsulatus* and identification of small RNAs affected by loss of the response regulator CtrA. RNA Biol 2017;14:914–25.
[38] Ryan D, Jenniches L, Reichardt S, Barquist L, Westermann AJ. A high-resolution transcriptome map identifies small RNA regulation of metabolism in the gut microbe *Bacteroides thetaiotaomicron*. Nat Commun 2020;11:3557.
[39] Nakata M, Sumitomo T, Patenge N, Kreikemeyer B, Kawabata S. Thermosensitive pilus production by FCT type 3 *Streptococcus pyogenes* controlled by Nra regulator translational efficiency. Mol Microbiol 2020;113:173–89.
[40] Ota C, Morisaki H, Nakata M, Arimoto T, Fukamachi H, Kataoka H, et al. *Streptococcus sanguinis* noncoding *cia*-dependent small RNAs negatively regulate expression of type IV pilus retraction ATPase PilT and biofilm formation. Infect Immun 2018;86:e00894-17.
[41] Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 2011;471:602–7.
[42] Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980;44:331–84.
[43] Welin-Neilands J, Svensäter G. Acid tolerance of biofilm cells of *Streptococcus mutans*. Appl Environ Microbiol 2007;73:5633–8.
[44] Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. RNA 2004;10:1507–17.
[45] Krastel K, Senadheera DB, Mair R, Downey JS, Goodman SD, Cvitkovitch DG. Characterization of a glutamate transporter operon, *glnQHMP*, in *Streptococcus mutans* and its role in acid tolerance. J Bacteriol 2010;192:984–93.
[46] Kremer BH, van der Kraan M, Crowley PJ, Hamilton IR, Brady LJ, Bleiweis AS. Characterization of the *sat* operon in *Streptococcus mutans*: evidence for a role of Ffh in acid tolerance. J Bacteriol 2001;183:2543–52.
[47] Wen ZT, Baker HV, Burne RA. Influence of BrpA on critical virulence attributes of *Streptococcus mutans*. J Bacteriol 2006;188:2983–92.
[48] Lemos JA, Brown Jr TA, Burne RA. Effects of RelA on key virulence properties of planktonic and biofilm populations of *Streptococcus mutans*. Infect Immun 2004;72:1431–40.
[49] Zhu W, Liu S, Zhuang P, Liu J, Wang Y, Lin H. Characterization of acid-tolerance-associated small RNAs in clinical isolates of *Streptococcus mutans*: potential biomarkers for caries prevention. Mol Med Rep 2017;16:9242–50.
[50] Eggenhofer F, Tafer H, Stadler PF, Hofacker IL. RNApredator: fast accessibility-based prediction of sRNA targets. Nucleic Acids Res 2011;39:W149–54.
[51] Suntharalingam P, Senadheera MD, Mair RW, Lévesque CM, Cvitkovitch DG. The LiaFSR system regulates the cell envelope stress response in *Streptococcus mutans*. J Bacteriol 2009;191:2973–84.
[52] Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 2001;183:897–908.
[53] Alves LA, Nomura R, Mariano FS, Harth-Chu EN, Stipp RN, Nakano K, et al. CovR regulates *Streptococcus mutans* susceptibility to complement immunity and survival in blood. Infect Immun 2016;84:3206–19.
[54] Ahn SJ, Wen ZT, Burne RA. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect Immun 2006;74:1631–42.
[55] Fabret C, Feher VA, Hoch JA. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J Bacteriol 1999;181:1975–83.
[56] Senadheera MD, Guggenheim B, Spatafora GA, Huang Y-CC, Choi J, Hung DCI, et al. A VicRK signal transduction system in *Streptococcus mutans* affects *gtfBCD*, *gbpB*, and *ftf* expression, biofilm formation, and genetic competence development. J Bacteriol 2005;187:4064–76.
[57] Senadheera DB, Cordova M, Ayala EA, Chávez de Paz LE, Singh K, Downey JS, et al. Regulation of bacteriocin production and cell death by the VicRK signaling system in *Streptococcus mutans*. J Bacteriol 2012;194:1307–16.
[58] Mao M-Y, Yang Y-M, Li K-Z, Lei L, Li M, Yang Y, et al. The *rnc* gene promotes exopolysaccharide synthesis and represses the *vicRKX* gene expressions via microRNA-size small RNAs in *Streptococcus mutans*. Front Microbiol 2016;7.
[59] Lei L, Stipp RN, Chen T, Wu SZ, Hu T, Duncan MJ. Activity of *Streptococcus mutans* VicR is modulated by antisense RNA. J Dent Res 2018;97:1477–84.
[60] Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK. Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. Infect Immun 1993;61:3811–7.
[61] Okahashi N, Sasakawa C, Yoshikawa M, Hamada S, Koga T. Molecular characterization of a surface protein antigen gene from serotype c *Streptococcus mutans*, implicated in dental caries. Mol Microbiol 1989;3:673–8.
[62] Aoki H, Shiroza T, Hayakawa M, Sato S, Kuramitsu HK. Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. Infect Immun 1986;53:587–94.
[63] Hanada N, Kuramitsu HK. Isolation and characterization of the *Streptococcus mutans gtfC* gene, coding for synthesis of both soluble and insoluble glucans. Infect Immun 1988;56:1999–2005.
[64] Merritt J, Kreth J, Shi W, Qi F. LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. Mol Microbiol 2005;57:960–9.
[65] Sato Y, Yamamoto Y, Kizaki H. Cloning and sequence analysis of the *gbpC* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. Infect Immun 1997;65:668–75.
[66] Liu N, Niu G, Xie Z, Chen Z, Itzek A, Kreth J, et al. The *Streptococcus mutans irvA* gene encodes a trans-acting riboregulatory mRNA. Mol Cell 2015;57:179–90.
[67] Sarpong DD, Murphy ER. RNA regulated toxin-antitoxin systems in pathogenic bacteria. Front Cell Infect Microbiol 2021;11.
[68] Koyanagi S, Lévesque CM. Characterization of a *Streptococcus mutans* intergenic region containing a small toxic peptide and its *cis*-encoded antisense small RNA antitoxin. PLoS One 2013;8:e54291.
[69] Kawamura Y, Hou XG, Sultana F, Miura H, Ezaki T. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol 1995;45:406–8.
[70] Dyson C, Barnes RA, Harrison GA. Infective endocarditis: an epidemiological review of 128 episodes. J Infect 1999;38:87–93.
[71] Halfmann A, Kovács M, Hakenbeck R, Brückner R. Identification of the genes directly controlled by the response regulator CiaR in *Streptococcus pneumoniae*: five out of 15 promoters drive expression of small non-coding RNAs. Mol Microbiol 2007;66:110–26.
[72] Marx P, Nuhn M, Kovács M, Hakenbeck R, Brückner R. Identification of genes for small non-coding RNAs that belong to the regulon of the two-component regulatory system CiaRH in *Streptococcus*. BMC Genomics 2010;11:661.
[73] Kery MB, Feldman M, Livny J, Tjaden B. TargetRNA2: identifying targets of small regulatory RNAs in bacteria. Nucleic Acids Res 2014;42:W124–9.
[74] Okahashi N, Nakata M, Sakurai A, Terao Y, Hoshino T, Yamaguchi M, et al. Pili of oral *Streptococcus sanguinis* bind to fibronectin and contribute to cell adhesion. Biochem Biophys Res Commun 2010;391:1192–6.
[75] Okahashi N, Nakata M, Terao Y, Isoda R, Sakurai A, Sumitomo T, et al. Pili of oral *Streptococcus sanguinis* bind to salivary amylase and promote the biofilm formation. Microb Pathog 2011;50:148–54.
[76] Gurung I, Spielman I, Davies MR, Lala R, Gaustad P, Biais N, et al. Functional analysis of an unusual type IV pilus in the Gram-positive *Streptococcus sanguinis*. Mol Microbiol 2016;99:380–92.
[77] Melville S, Craig L. Type IV pili in Gram-positive bacteria. Microbiol Mol Biol Rev 2013;77:323–41.
[78] Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000 2005;38:72–122.
[79] Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrzadeh V, Kafil HS. Oral pathogenesis of *Aggregatibacter actinomycetemcomitans*. Microb Pathog 2017;113:303–11.
[80] Carrizales-Sepúlveda EF, Ordaz-Farías A, Vera-Pineda R, Flores-Ramírez R. Periodontal disease, systemic inflammation and the risk of cardiovascular disease. Heart Lung Circ 2018;27:1327–34.
[81] Phillips PL, Reyes L, Sampson EM, Murrell EA, Whitlock JA, Progulske-Fox A. Deletion of a conserved transcript PG_RS02100 expressed during logarithmic growth in *Porphyromonas gingivalis* results in hyperpigmentation and increased tolerance to oxidative stress. PLoS One 2018;13:e0207295.
[82] Jorth P, Whiteley M. Characterization of a novel riboswitch-regulated lysine transporter in *Aggregatibacter actinomycetemcomitans*. J Bacteriol 2010;192:6240–50.
[83] Amarasinghe JJ, Connell TD, Scannapieco FA, Haase EM. Novel iron-regulated and Fur-regulated small regulatory RNAs in *Aggregatibacter actinomycetemcomitans*. Mol Oral Microbiol 2012;27:327–49.
[84] Busch A, Richter AS, Backofen R. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics 2008;24:2849–56.
[85] Yu SH, Vogel J, Förstner KU. ANNOgesic: a Swiss army knife for the RNA-seq based annotation of bacterial/archaeal genomes. Gigascience 2018;7.
[86] Leonard S, Meyer S, Lacour S, Nasser W, Hommais F, Reverchon S. APERO: a genome-wide approach for identifying bacterial small RNAs from RNA-Seq data. Nucleic Acids Res 2019;47:e88.
[87] Chakravarty S, Massé E. RNA-dependent regulation of virulence in pathogenic bacteria. Front Cell Infect Microbiol 2019;9:337.

[88] Duran-Pinedo AE, Yost S, Frias-Lopez J. Small RNA transcriptome of the oral microbiome during periodontitis progression. Appl Environ Microbiol 2015;81:6688–99.
[89] Ram-Mohan N, Meyer MM. Comparative metatranscriptomics of periodontitis supports a common polymicrobial shift in metabolic function and identifies novel putative disease-associated ncRNAs. Front Microbiol 2020; 11:482.
[90] Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY. Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. Nat Biotechnol 2013;31:170–4.
[91] Villa JK, Su Y, Contreras LM, Hammond MC. Synthetic biology of small RNAs and riboswitches. Microbiol Spectr 2018;6.