Genetic Regulation of Streptococci by Small RNAs

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Abstract

Streptococcal species constitute a large group of commensal and pathogenic Gram-positive bacteria that exist in a wide variety of habitats. The family of small RNAs is typically ranged in size from 50 to 300 nucleotides, and acts as regulators in bacteria. The last decade has witnessed the increasing findings of small RNAs (sRNAs), which play important regulatory roles in the variety of biological processes in streptococci. In this review, we summarized the recent achievements in the identification of streptococcal sRNAs, mainly in *Streptococcus pyogenes* and *Streptococcus pneumoniae*. In addition, we particularly focused on the functions that sRNAs exert in the regulatory networks of both phenotypical traits and pathogenicity. The fact that sRNAs act as a critical fine-tuning regulator of streptococci may not only reveal in-depth mechanisms of bacterial post-transcriptional regulations in response to environmental perturbance, but also provide promising approaches to the better management of streptococcal infections.

Keywords

Streptococci, small RNA, gene expression, pathogenicity, virulence

Highlights of Contents

1) sRNAs are recognized as an important regulatory component in both physiological phenotype and pathogenicity of streptococci.

2) Apart from best studied *S. pyogenes* and *S. pneumoniae,* sRNAs have also been detected in other streptococcal species including *S. suis*, *S. mutans*, *S. sanguinis, S. thermophiles*, *S. mitis* and *S. orais*.

3) sRNAs are involved in the physiological phenotypes of streptococci, and mostly in stress response and drug resistance.

4) sRNAs regulate bacterial invasion and cytotoxin secretion of streptococci.

1. Introduction

Streptococcal species are Gram-positive bacteria that possess the ability to colonize and invade into human and animals, whose interactions with host organisms can vary from commensal to life-threatening. The bacterial pathogen *Streptococcus pyogenes*, also designated as group A streptococcus (GAS), is the etiological agent of several human diseases, including pharyngitis, impetigo, acute rheumatic fever, streptococcal toxic-shock-like syndrome, and necrotizing fasciitis (Cunningham, 2000), leading to a substantial mortality and morbidity (Carapetis et al., 2005). *Streptococcus agalactiae*, commonly referred to as group B streptococcus (GBS) (Lancefield, 1933), colonizes the vaginal and gastrointestinal tracts of healthy adults asymptomatically, and it can cause fatal invasive disease such as neonatal meningitis in susceptible host (Edwards et al., 1985; Phares et al., 2008). *Streptococcus pneumoniae* is another human pathogen responsible for many human diseases, including pneumonia bacteremia and endocarditis in both children and adults (Picazo, 2009). Besides, there are large group of commensal streptococcal species including *Streptococcus mutans, Streptococcus thermophilus*, *Streptococcus sanguinis* and so on (Zorgani et al., 2016).

The polymicrobial nature of streptococcal infections in the most cases necessitates biofilm, a highly-structured community of microbial cells and extracellular matrix adhering to abiotic or biological surface (Costerton et al., 1999 Galante et al., 2015; Krzysciak et al., 2014; Rosini and Margarit, 2015) and inter-species/inter-kingdom interactions, which are mediated by complex signaling such as Quorum Sensing systems (QS) in GAS, *S. pneumoniae* as well as *S. mutans* (Bassler, 1999; Galante et al., 2015; Jimenez and Federle, 2014; Shanker and Federle, 2017). In addition, the bacterial two-component signal transduction systems (TCSTSs) including the CovRS/CsrRS system (Dalton and Scott, 2004; Heath et al., 1999; Kreikemeyer et al., 2003; Lamy et al., 2004; Levin and Wessels 1998; Pan et al., 2009; Park et al., 2012; Roberts and Scott, 2007; Trevino et al., 2009), the CiaHR system (Guenzi et al., 1994; Halfmann et al., 2007; Levesque et al., 2007; Liu and Burne, 2009; Patenge et al., 2013; Qi et al., 2004; Quach et al., 2009), the Ihk/Irr system (Han et al., 2012; Hertzen et al., 2012; Shelburne et al., 2005; Voyich et al., 2003), and the VicRK system (Li et al., 2010; Liu et al., 2006; Winkler and Hoch, 2008) have been identified to contribute to environmental fitness as well as host colonization and invasion of the streptococci Besides, eukaryotic-type serine/threonine kinases (Echenique et al., 2004; Hanks et al., 1988; Jin and Pancholi, 2006; Novakova et al., 2005; Rajagopal et al., 2003) is known to being related with biofilm formation. Surface proteins such as protein F(PrtF/Sfbl), Fba and SfbX (Roberts and Scott, 2007) have been identified as the major determinant of microbial binding to the mucosal receptor fibronection. The antiphagocytic M protein (Lancefield, 1962; Swanson et al., 1969), the hyaluronic acid capsule (Wessels et al., 1991), C5a peptidase (ScpA) (Ji et al., 1997; Lynskey et al., 2017) and streptococcal inhibitor of complement (Agarwal et al., 2015; Blom et al., 2014) function to help the streptococci escaping the host immunity (Roberts and Scott, 2007). Soluble cytolysins or secreted virulence factors, such as streptolysin S (SLS) (Datta et al., 2005; Nizet, 2002), streptococcal pyrogenic exotoxin B (SpeB) (Biswas et al., 2001; Chen et al., 2003; Chiang-Ni and Wu, 2008; Tsai et al., 1999), and streptokinase (Christensen, 1945 Steiner and Malke, 2002; Wang et al., 1998) are critical for the adherence to and internalization into host epithelial cells. Stand-alone transcriptional regulators,

such as Mga (Caparon and Scott, 1987; Hemsley et al., 2003; Hondorp and McIver, 2007; Kreikemeyer et al., 2003; Ribardo and McIver, 2006; Solano-Collado et al., 2012; Vahling and McIver, 2006), Lux/AI-2 (Blehert et al., 2003; Chao and Vogel, 2010; Federle and Bassler, 2003; Joyce et al., 2004; Lyon et al., 2001; Marouni and Sela, 2003; Petersen et al., 2006; Schauder et al., 2001; Siller et al., 2008; Vendeville et al., 2005; Vidal et al., 2011; Wang et al., 2011; Yoshida et al., 2005), metabolic regulators like CcpA (Almengor et al., 2007; Giammarinaro and Paton, 2002; Iyer et al., 2005; Saier et al., 1996; Shelburne et al., 2008; Willenborg et al., 2011), CodY (Guedon et al., 2005; Hendriksen et al., 2008; Kreth et al., 2011; Malke and Ferretti, 2007; Malke et al., 2006; Sonenshein, 2005), and CRISPR/Cas system(Barrangou et al., 2007; Deveau et al., 2008; Deveau et al., 2010; Ishino et al., 1987; Marraffini and Sontheimer, 2010) has also been identified as strategy for the pathogen to modulate its fitness and virulence in response to the microenvironment perturbations, contributing to the pathogenicity and ultimate clinical outcome of streptococcal infections.

Recently, sRNAs, whose complex regulatory roles in bacterial virulence have been reported in multiple pathogens, are recognized as an important regulatory component of streptococci (Quereda and Cossart, 2017; Waters and Storz, 2009) Despite the limited number of sRNAs discovered in streptococci compare to other bacteria to date, their critical regulatory roles streptococcal species have drawn increasing attentions of both scientists and clinical professionals.

The family of small RNAs (sRNAs) is typically composed of 50 to 300 nucleotide (nt) long transcripts in bacteria (Le Rhun et al., 2016). The first sRNA, 6S RNA, was identified in 1967 in the fractionation of crude preparations of *E.coli* tRNA by disc electrophoresis on polyacrylamide gel (Hindley, 1967). In 1984, the first chromosomally encoded sRNAs, MicF RNA was reported, which inhibits translation of the OmpF mRNA of *E.coli* (Mizuno et al., 1984). Accredited to the technological advances, sRNAs have been so far found in almost all the bacterial

species such as *Escherichia coli* (Gottesman, 2004), Vibrio cholera (Bardill and Hammer, 2012), *Listeria monocytogenes* (Mellin and Cossart, 2012), *Pseudomonas aeruginosa* (Sonnleitner et al., 2012), *Staphylococcus aureus* (Romilly et al., 2012), *Clostridium perfringens* (Mraheil et al., 2010). sRNAs regulate gene expression to allow bacteria responding more quickly to environmental cues, and consequently contribute to the fitness and microbial invasion to the host (Quereda and Cossart, 2017; Sharma and Heidrich, 2012). Both genome-wide computational and experimental approaches have been applied to predict or discover sRNAs (Le Rhun and Charpentier, 2012), and multiple regulatory mechanisms of sRNAs have been proposed (Figure 1). (Waters and Storz, 2009).

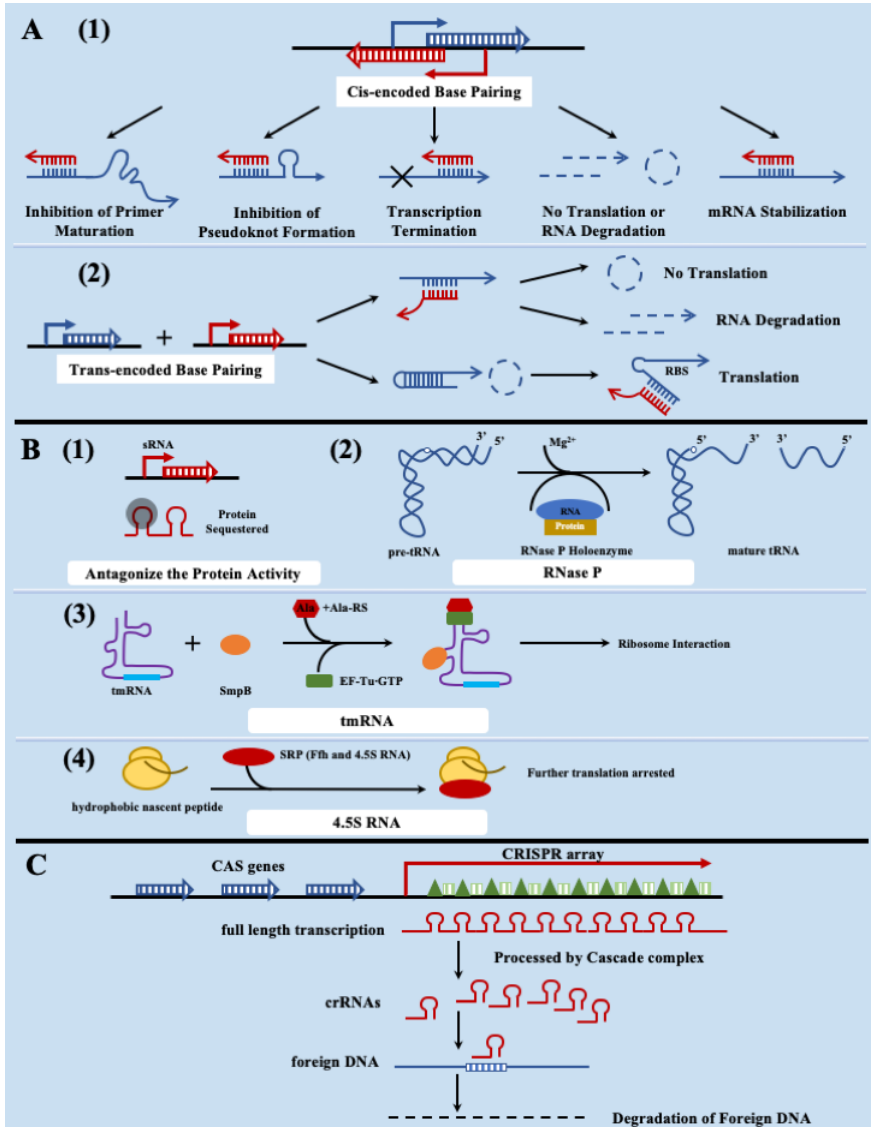

Figure 1. Regulatory Mechanism Exerted by sRNAs. A. Base-pairing Regulatory sRNAs. (1) cis-encoded base pairing sRNAs. cis-encoded base pairing sRNAs are encoded in cis on the same DNA locus and share extended regions of complete complementarity with their target mRNA, which result to the inhibition of primer maturation, inhibition of RNA pseudoknot formation, transcriptional attenuation, inhibition of translation, and promotion of RNA degradation or cleavage. (2) tran-encoded base pairing sRNAs. trans-encoded sRNAs share only limited complementarity with their target mRNAs. These sRNAs have been confirmed regulating the translation and/or stability of

target mRNAs. B. Protein-binding sRNAs. (1) sRNAs that antagonize the protein activity. Protein-binding sRNAs antagonize the activities of the cognate proteins by mimicking the structures of other nucleic acid. (2) RNase P. (3) tmRNA. (4) 4.5S RNA. C. CRISPR RNAs (crRNAs). crRNAs are a unique class of regulatory RNAs that provide resistance to bacteriophage and prevent plasmid conjugation.

There are several kinds of sRNAs. Firstly, cis-acting elements located on untranslated regions (UTRs) of a translated mRNA can control the expression of the enclosed gene(s) via modulation of the secondary structure or stability(Garst et al., 2011; Johansson et al., 2002). Another class of sRNAs is short noncoding RNA(ncRNA) which belongs to antisense RNAs (asRNAs) (Wahlestedt, 2013), and its regulatory activity occurs through base-pairing to the mRNA encoded by the opposite DNA strand molecule (Thomason and Storz, 2010). Beside, RNAs that target other RNAs, foreign DNA, or proteins is defined as trans-acting sRNAs (Gottesman and Storz, 2011). Sharing limited complementarity with their target, trans-acting sRNAs can bind to multiple mRNAs. However, this type of interaction requires the RNA chaperone protein Hfq for stabilization, and Hfq is absent in the Streptococcus species (Nielsen et al., 2010). Recently, the simplified nomenclatures and the classification of bacterial sRNAs were proposed and reemphasized (Zorgani et al., 2016), dividing sRNAs into two major categories. The first category is caRNAs (cis-acting RNAs) that act or are located at the on the untranslated regions (UTPs) of mRNA molecules. caRNAs usually refer to UTRs, which can be divided into 5’ UTRs and 3’ UTRs. The second category are the traRNAs (trans-acting RNAs), comprising the asRNAs (antisense RNAs also known as cis-encoded sRNAs) and treRNAs (trans-encoded sRNAs). asRNAs can be divided into 5’ asRNAs and 3’ asRNAs, and treRNAs consist of csRNAs (cia-dependent small RNAs), msRNAs (microRNA-size sRNAs), crRNAs (CRISPR RNAs) as well as tracrRNAs (trans-activating CRISPR RNAs).

2. sRNAs in Streptococci

In gram-positive bacterial pathogens, almost all reported biological functions of sRNAs are part of complex regulatory processes involved in adaptation or virulence processes. Most of the characterized sRNA genes are found in the intergenic regions (IGRs) of the core genome, while a few sRNAs are transcribed from the complementary strand of genes located in pathogenicity islands (PAI) (Pichon et al., 2012). Encoded on the genome, sRNAs of streptococci are associated with loci expressing TCSTSs (e.g, GAS fasX, rivX, *S. pneumoniae* csRNA) or transcriptional regulators (e.g, GAS rivX) (Romby and Charpentier, 2010). A large proportion of sRNAs act at the mRNA level, whereas a few sRNAs, such as Pel of GAS, possess the ability in encoding proteins with virulent functions (Biswas et al., 2001). The sRNAs that have been investigated to date are mainly expressed in a growth phase-dependent manner, and some of them have been discovered to be controlled by a few transcriptional regulators or TCSTSs (Romby and Charpentier, 2010).

2.1 sRNAs in S. pyogenes

GAS is the best studied streptococcal strain with sRNA. Several sRNAs including FasX, Pel, RivX, 4.5S RNA, tracrRNA and crRNA have been identified to regulate bacterial virulence in GAS (Deltcheva et al., 2011; Garneau et al., 2010; Kihlberg et al., 1995; Kreikemeyer et al., 2001; Perez et al., 2009; Roberts and Scott, 2007; Trevino et al., 2010). Pel (pleioteropic effect locus) was the first sRNA discovered in GAS in 1995 (Kihlberg et al., 1995), working as a bifunctional sRNA encoding protein and meanwhile regulating virulence factor expression. The region of *pel* gene locates at the site of Tn917 insertion and surrounding sequence on the chromosome (Li et al., 1999). *pel* has been demonstrated being identical to *sagA*, and the *sag/pel* locus is the structural gene for the key virulence factor of GAS, Streptolysin S (Li et al., 1999). Being regulated in a growth-phase-dependent manner (Mangold et al., 2004), Pel has been demonstrated exerting its effects on virulence gene expression at both transcriptional level (*emm* gene encoding M

protein and *sic* gene encoding a streptococcal inhibitor of the complement system and post-transcriptional level (cysteine protease SpeB) (Biswas et al., 2001; Li et al., 1999; Mangold et al., 2004; Perez et al., 2009). Multiple regulators are coordinated with the expression of the *sag/pel* operon, among which CodY, Irr, SLS and SptS seem regulating positively, while CcpA, CovR, FasX, LuxS, Mga, RopB and members of the Ralp regulator family have repressing effects on it. In addition, the cell nutritional state also regulates *sag/pel* expression (Le Rhun and Charpentier, 2012). Although the *pel* locus is conserved in GAS, the *pel* deletion phenotypes vary among isolates, suggesting that other regulatory factors are involved in the network of Pel-mediated pleiotropic phenotype on GAS virulence (Biswas et al., 2001; Le Rhun and Charpentier, 2012; Li et al., 1999; Perez et al., 2009). *fasBCAX* operon is a polycystronic message containing genes encoding two histidine protein kinases (HPKs) (FasB and FasC) and one response regulator (RR) (FasA) (Kreikemeyer et al., 2001). FasX is a small 300 nucleotide monocistronic transcript, whose encoded gene (*fasX*) has been identified downstream of *fasBCA* operon(Kreikemeyer et al., 2001). Deletion of *fasX* results in a similar phenotype of *fasBCA* or *fasA* mutants, while a complementary expression in trans from plasmid restores the wild-type fasBCA regulation pattern, further suggesting the main role of *fasX* in the *fas* regulon (Kreikemeyer et al., 2001). FasX sRNA has been found conserved in GAS (Ramirez-Pena et al., 2010 while at the same time presents differently in several clinical isolates. In an M49 serotype (CS101), luciferase promoter fusion revealed a growth phase-associated transcription of *fasX* with peak activities during the late exponential phase, depending on the RR FasA (Kreikemeyer et al., 2001). In M49 serotype (NZ131), expression of FasX was influenced by amino acid starvation (Steiner and Malke, 2001), while enhanced by the luxS/AI2 signaling system in an M19 serotype (RDN02) (Siller et al., 2008). RivX (RofA like proteinIV regulator X) is a processed sRNA originating from a *rivRX* transcript that encoding the regulatory protein RivR (Ralp4 family of regulators) (Roberts and Scott, 2007). Being conserved in all 12 sequenced strains of GAS, *rivX* is mapped immediately down-stream of the *rivR* gene and contains a small ORF (Roberts and Scott, 2007). By analyzing the *rivX*-harbouring nonsense mutation, the activity of RivX sRNA was

demonstrated being regulated by *rivX* transcript itself rather than the peptide encoded within it (Roberts and Scott, 2007). RivX sRNA promotes several mRNAs that encoding the virulence factors including C5a peptidase, cysteine protease SpeB and M protein, in the company of RivR (Roberts and Scott, 2007). *rivR* gene has been found directly repressed by CovR regulator (Roberts et al., 2007), and the products of the *rivRX* locus exert positive control over the transcription of Mga regulon (Roberts and Scott, 2007), indicating that RivR and RivX may link the CovRS system to the Mga regulon. 4.5S RNA (or scRNA for small cytoplasmic RNA) acts as a regulator in virulence factor expression and secretion. 4.5S RNA together with fifty-four homologue protein (Ffh) constitute SRP (signal recognition particle) from GAS, a ribonucleoprotein complex that targets polypeptides to the secretory apparatus in a co-translational manner (Trevino et al., 2010). Although not being essential, mutants affected in 4.5S RNA gene are attenuated in bacterial virulence (Trevino et al., 2010). In the SRP from *S. pneumoniae*, 4.5S RNA was demonstrated binding to the M-domain of Ffh, however the modulating ability to bacterial virulence has not been investigated yet (Zheng et al., 2002). crRNAs and tracrRNA play important roles in the CRISPR/Cas system of GAS. The precursor crRNA (pre-crRNA) is expressed in a repeat-spacer array of CRISPR/Cas system, and will be further processed into mature crRNAs. Mature crRNAs are regarded as unique RNAs conferring immunity against invading phages and plasmids by exerting as traRNAs targeting DNA and guiding Cas protein to the invading cognate nucleic sequences for Cas-executed cleavage (Deltcheva et al., 2011; Le Rhun and Charpentier, 2012; Sapranauskas et al., 2011). Discovered via dRNA-seq analysis, tracrRNA harbors 24 nucleotide complementarity to the repeat regions of the pre-crRNA transcript. By the activities of the widely conserved endogenous RNase III and the CRISPR-associated Csn1 protein，tracrRNA acts in trans to activate the maturation of pre-crRNA into crRNAs in the CRISPR/Cas system of Type II (Deltcheva et al., 2011). The presence of tracrRNA was also revealed by northern blot profiling in *S. thermophilus* LMD-9 genome (Karvelis et al., 2013). A genome-wide study using tiling microarray predicted 14 new sRNAs of GAS, which were further confirmed by Northern blot analysis (Perez et al., 2009). Recently, by using high-throughput

sRNA sequencing in GAS SF370 clinical isolate, a novel asRNA has been discovered for the first in this pathogen (Le Rhun et al., 2016).

2.2 sRNAs in S. pneumoniae

Other than three highly conserved sRNAs (RNase P, tmRNA and scRNA) or previously known families of caRNAs like riboswitches or leader regions (TPP riboswitch, T-box), several so-called functional sRNAs have been identified in the genomes of *S. pneumoniae* (Tsui et al., 2010; Wilton et al., 2015). csRNAs, the first sRNAs described in *S. pneumoniae*, were initially discovered by mapping the genome of *S. pneumoniae* for promoters regulated by CiaR regulator (Halfmann et al., 2007). Being transcribed from the five strongest promoters of the pneumococcal CiaR regulon, csRNA1-5 (or CcnA-E) share a high degree of sequence similarity especially in the characteristic unpaired region between the two stem-loop structures (Halfmann et al., 2007; Marx et al., 2010). The complementarity to the Shine-Dalgarno (SD) sequence and the start codon AUG within this unpaired region suggest that csRNA could control translation initiation of mRNAs (Marx et al., 2010). An additional conserved sequence CCUC(6N)CAU located in the middle of the unpaired region was identified in all crRNA, resulting into the potential to base pair with RBS (Halfmann et al., 2007). In *S. pneumoniae* strain R6, csRNA4 and csRNA5 are involved in stationary-phase autolysis (Halfmann et al., 2007). In strain D39, the only expression of csRNA1 is induced by competence stimulatory peptide (Tsui et al., 2010). An insertion/deletion mutant exerts strong cis-acting effects on the transcription of the adjacent gene, indicating that this sRNA region is co-transcribed in an operon (Tsui et al., 2010). Apart from 5 pneumococcal csRNA1-5, other 61 csRNA candidates (51 to 202 nt) were revealed from CiaR-activated promoters, being classified into 40 different types. Since CiaRH is highly conserved among streptococci, csRNAs have also been detected in other streptococcal genomes including *S. suis*, *S. mitis*, *S. orais*, *S. mutans*, *S. sanguinis* and *S. thermophiles*, further suggesting that csRNAs are part of the regulon of CiaRH in streptococci (Marx et al., 2010). Interestingly, *S. thermophilus* strain ST0 possesses a csRNA gene on a plasmid. It is the only one

of the identified csRNA genes that is not located in the genome and represents a new type with only one stem-loop structure (Marx et al., 2010). Nine new sRNA were later detected in *S. pneumoniae* serotype 2 strain D39, among which Spd-sr17 and Spd-sr37 were functionally analyzed. Spd-sr17 was characterized as a 144 nt sequence that decreased in stationary phase, likely folding into a structure that contains single-stranded regions between hairpin structure, while the 80 nt Spd-sr37 showed strong expression in all growth phases, forming a base-paired structure (Tsui et al., 2010). However, deletion or overexpression of Spd-sr17 and Spd-sr37 failed to cause drastic changes in bacterial growth, specific stress response, global transcription, or virulence in this strain (Tsui et al., 2010). Kumar et al further identified a total of 50 sRNA in *S. pneumoniae* TIGR4 by using a tiling array approach. However, 36 of them had no predicted function (Kumar et al., 2010). Remarkably, no asRNAs or CRISPR RNAs have been identified so far in *S. pneumoniae* (Wilton et al., 2015).

2.3 sRNAs in Other Streptococci Species

Although sRNAs can play an important role in virulence, the current knowledge on sRNAs encoded by the GBS chromosome remains limited. Some in silico studies have been conducted on the sequenced genome of GBS NEM316. Four putative csRNAs (csRNA10, csRNA11, csRNA12, and csRNA13) have been identified by searching for a CiaR-activated promoter and the transcriptional terminator sequences within the intergenic regions (Marx et al., 2010). The expression of csRNA10, csRNA11, and csRNA12 have been confirmed in a further study, being up-regulated in acidic conditions (Rosinski-Chupin et al., 2015). In another in silico study, 197 sRNAs have been detected, among which 10 sRNAs were validated by RT-PCR and northern blot hybridization. SQ18 asRNA and SQ893 sRNA act as negative regulators to their target mRNAs *gbs0031* and *gbs1263* respectively, while SQ485 asRNA positively regulates the expression of *gbs1588/gbs1589* (Pichon et al., 2012). In *S. suis* serotype 2 isolates 05ZYH33 and 98HAH33 that caused severe human infections in China, 6 sRNA candidates related to bacterial virulence have been discovered, including

cspA and rli38, which are specific to Chinese isolates(Zhang et al., 2014).

Being specific for particular species, a small number of sRNAs have been discovered in other streptococci. A novel member of the treRNA category, designated as “miRNA-size small RNAs” or “msRNAs” has been described in *S. mutans* (Lee and Hong, 2012). Before that, there was no publication regarding msRNAs in bacteria with comparable size to miRNAs, which exist in eukaryotes with 19-23 nt length transcript. A deep sequencing approach has revealed putative msRNAs by detecting reads corresponding to 15 and 26 nt (Lee and Hong, 2012). These msRNAs may result from processed RNAs and have been confirmed by northern blot as well as qRT-PCR. In a later study, three putative msRNAs targeting vicRKX have been predicted in *S. mutans* by using deep sequencing and bioinformatics analysis, and have been indicated being related to the biofilm forimg(Mao et al., 2016). In *Mycobacteria Marinum*, an msRNA that has been discovered being derived from an RNA stem-loop with the characteristics expected for a pre-microRNA(Furuse et al., 2014). Furthermore, msRNAs (20nt long) molecule named 24B_1 has been identified in *Escherichia coli* after induction of Shiga toxin-converting bacteriophage Φ24B, and it has been predicted impairing expression of the *d_ant* gene coding for an anti-repressor(Nejman-Falenczyk et al., 2015).

2.4 Highly conserved sRNAs in Streptococci

Genome-wide searches in streptococcal genome have identified several highly conserved sRNAs with housekeeping or stress-related functions, among which contain sRNA component of RNase P, tmRNA (transfer-messenger RNA), 4.5S RNA (as mentioned above), riboswitches and T-box. RNases P is the

endoribonuclease that generates mature 5'-ends of tRNA by removal of the 5'-leader elements of precursor-tRNA (Frank and Pace, 1998; Silvaggi et al., 2006). A single catalytic RNA component constitutes RNase P with a protein subunit, while the RNA subunit is catalytically active in vitro in the absence of the protein subunit (Frank and Pace, 1998). tmRNA, also designated as ssrA RNA, harbors properties of both a tRNA and an mRNA (Keiler, 2008). tmRNA together with the small protein SmpB, mediates peptide tagging of the nascent protein and release of the stalled ribosome, ensuring the protein synthesis with high fidelity in trans-translation (Keiler, 2007, 2008). In most bacteria, the inactivation of tmRNA is related to a decreased ability to response to or recover from stress conditions (Brito et al., 2016). Riboswitches are structures located in 5' region of mRNAs that have the ability to regulate the transcription and/or translation of the downstream mRNA, via directly binding intracellular metabolite (Vitreschak et al., 2004). The T-box family commonly control the expression of genes involved in tRNA aminoacylation, amino acid transport and biosynthesis in gram-positive bacteria (Gutierrez-Preciado et al., 2009).

2.5 Protein co-factors in sRNA-mediated Activities

Although protein interaction with streptococcal sRNAs is rarely reported, protein co-factors are demonstrated playing roles in sRNA activities. It is widely recognized that the RNA chaperone protein Hfq is required in sRNA targeting mRNA for most Gram-negative bacteria, however *hfq* homologues have also been found in the genome of some Gram-positive bacteria (Storz et al., 2011; Vogel and Luisi, 2011). Thus, the presence and function of Hfq have been questioned in streptococci (Chao and Vogel, 2010; Storz et al., 2011). Substituting for Hfq, proteins such as RNA-binding protein with chaperone or helicase may associate with the streptococcal traRNAs. Furthermore, interactions of streptococcal sRNAs do not require protein co-factor or require another type of co-factors (Le Rhun and Charpentier, 2012). Ribonucleases (RNase) exert critical effects on the maturation of sRNAs. Seeming to be universal, endogenous RNase III has been demonstrated playing roles in tracrRNA-directed crRNA

maturation(Deltcheva et al., 2011). In addition, RNase is also involved in the interaction between sRNAs and mRNAs. The 5'-3' exo-ribonucleases J1 and J2 was predicted to be the candidate RNase in ska mRNA decay process, and FasX enhanced the stability of ska mRNA and protects target mRNA from degradation via base-pairing to ska mRNA (Ramirez-Pena et al., 2010). Cas proteins of CRISPR/Cas system were discovered functioning in RNA binding, RNA nuclease and crRNA-guided DNA cleavage (Deveau et al., 2010; Makarova et al., 2011).

3. sRNAs regulate the physiological phenotypes of Streptococci

sRNAs are commonly involved in the stress response and drug resistance of streptococci. In GBS, 10 sRNAs have been identified differentially expressed in response to acidic stress (Pichon et al., 2012). An acidic condition of pH5.2 (natural pH of the genitourinary tract) induced the expression of Srn015 (csRNA10), Srn024 (csRNA11), Srn070 (csRNA12), Srn085 (csRNA13), Srn071 and Srn082, but downregulated the expression of Srn046, Srn056, Srn057 and Srn073, underlining the potential of these sRNAs controlled by CiaRH in acidic stress adaptation during bacterial colonization to the urogenital tract (Rosinski-Chupin et al., 2015). The ability to survive at low pH is intimately links to the carbohydrate metabolism and tooth surface colonization of *S. mutans*. L10-leader sRNA, which belongs to treRNA, has been identified as a pH-dependent sRNA and exhibits an elevated expression in the *S. mutans* strain possessing a stronger adherence capacity and acidogenicity, suggesting that L10-Leader may regulate gene expression of *S. mutans* related to cell adhesion and acid production (Xia et al., 2012)..

In *S. pneumoniae*, the phenotype of enhanced β-lactan resistance was demonstrated being dependent on the csRNAs (Schnorpfeil et al., 2013). One of the controlling csRNAs, spr2043, was confirmed negatively regulating the comC gene that codes the precursor of the competence stimulating pheromone (CSP), indicating that a hyperactive CiaRH system prevents competence development

by csRNA-dependent post-transcriptional repression of CSP production (Schnorpfeil et al., 2013). Furthermore, tmRNA also plays roles in drug resistance of *S. pneumonia*e. Fluoroquinolones, such as levofloxacin and moxifloxacin, are currently used for the treatment of pneumococcal pneumonia. This cell-killing process requires ongoing protein synthesis and is contributed by reactive oxygen species (ROS). The tmRNA deletion mutant was found significantly more resistant to fluoroquinolones compared to its wild type strain. A decreased accumulation of intracellular ROS was found in the deletion mutant, and the tmRNA deficiency was shown to prevent the levofloxacin-induced chromosome fragmentation, mainly by inhibiting protein synthesis (Brito et al., 2016).

Some sRNAs have also been identified to regulate other physiological traits of streptococci. In *S. pneumoniae*, competence in S. pneumoniae is controlled by 5 csRNAs via post-transcriptional regulation of comC, encoding the precursor of the competence stimulating peptide, which is essential to initiate the regulatory cascade leading to competence. It has been demonstrated that combinations of three csRNAs, csRNA1,2,3, or csRNA1,2,4 were sufficient to stop competence gene expression while two csRNAs were not enough to prevent competence(Laux et al., 2015). Sip is named for surface immunogenic protein that elicits protective immunity against GBS. A translational fusion system revealed that the asRNA SQ18 influences the expression of *sip* gene by acting as a negative post-transcriptional regulator (Pichon et al., 2012). In *S. pneumoniae*, stationary-phase autolysis was affected by csRNA4 and csRNA5 (Halfmann et al., 2007). In additional, the competence-inducing (XIP) peptide is predicted controling bacteriocin production via sRNAs in *S. mutans.* It has been discovered that five XIP-responsive intergenic regions express putative sRNAs. Of thses 5 regions, SMU.153-154 region is located at 3′ of bacteriocin synthetic

genes, and is believed being involved in bacteriocin induction (Wenderska et al., 2017)

4 sRNAs regulate the pathogenicity of Streptococci

4.1 sRNAs Regulate Bacterial Invasion

4.1.1 Colonization, Adherence and Internalizations

In GAS serotype M49, a significantly decreased adherence and internalization efficiency in fasX deletion mutant was observed in a comparison of HEp-2 cell infected with its wild type strain, indicating the importance of FasX in the interaction with epithelial host cells and tissue colonization (Kreikemeyer et al., 2001). Furthermore, FasX was shown to positively control the cytokine gene transcription, host cell apoptosis and cytotoxicity (Klenk et al., 2005). 4.5S RNA also exerts its effect on modulating the bacterial virulence. Using growth in human saliva as an ex vivo model of upper respiratory tract infection, a 10-fold reduction in colony-forming units were identified in 4.5S RNA mutation of GAS, consistent with the fact that 4.5S RNA contributes to GAS growth and persistence during upper respiratory tract infections (Trevino et al., 2010). In *S. pneumoniae*, by conducting a random transposon insertion, sRNA F20 was demonstrated contributing to adhesion and invasion of host nasopharyngeal (Mann et al., 2012). In addition, the srn157 and tmRNA deletion mutants of *S. pneumoniae* showed decreased adhesion/invasion to nasopharyngeal cells and endothelial cells respectively, along with compromised fitness and competitive index in the nasopharynx and lungs (Wilton et al., 2015).

Pili and capsule are recognized to play pivotal roles in adhesion of streptococci to host tissue. By base-pairing to the mRNA of the pilus biosynthesis operon, which results in the mRNA destabilization, FasX exerts negative effects on GAS

pilus (Liu et al., 2012). In addition, FasX has been found inhibiting the translation of *cpa* mRNA that coding a minor pilin protein (Liu et al., 2012). Afimbrial adhesins can also be negatively modulated by FasX, with the evidence in two matrix protein-binding adhesins (fibronectin-binding protein FBP54 and fibrinogen-binding protein MRP) in GAS (Klenk et al., 2005). In *S. sanguinis*, csRNA1-1 has been found negatively regulated the *pilT*, a constituent of the type IV pilus operon, via binding directly to *pilT* mRNA(Ota et al., 2018). In addition, csRNA1-1 and csRNA1-2 were predicted negatively regulated *S. sanguinis* biofilm formation(Ota et al., 2018). sRNA rss04 of *S. suis*, an important pathogen for pigs, has been observed facilitating *S. suis* invasion of mouse brain and lung in vivo, by repressing capsular polysaccharide (Xiao et al., 2017). In *S. mutan*s, the ability of exopolysaccharides synthesizing can affect the biofilm formation which involved in dental caries. It has been reported that *rnc* gene positively regulated exopolysaccharide synthesis significantly, meanwhile repress the downstream *vic* locus. By using deep sequencing and bioinformatics analysis, three msRNAs (msRNAs 1701, 3405 and 1657) that negatively correlated with *vicRKX* but positively correlated with *rnc* have been discovered. It has been indicated that *rnc* repressed *vicRKX* expression at the post-transcriptional level via msRNAs (Mao et al., 2016).

4.1.2 Host Defense Evasion

Appearing as hair-like projections on the cell surface, M protein is considered as the major virulence factor of GAS (Swanson et al., 1969) and protects the bacteria from phagocytosis via conferring resistance to complement-mediated killing by polymorphonuclear leukocytes and macrophages (Lancefield, 1962). In addition, M protein is required for attachment of GAS to keratinocytes, playing an important

role in skin surface infection. In previous research, an insertion mutant in the *sagA/pel* promoter region led to a decreased transcription of M protein-coding gene *emm* in the GAS M49 serotype, indicating that the locus is needed for the production of M protein (Li et al., 1999). Enhanced *sagA/pel* expression leads to an increased expression for *emm* gene in a wild-type organism (Li et al., 1999). In a subsequent study, although not affecting the transcription of *emm* gene, insertion-deletion mutation in the *sagA/pel* gene of M6 serotype (IRS470) resulted in a truncated M protein, which influenced the anchorage of M protein into the cell wall meanwhile caused a dramatic reduction in cell-surface associated M protein (Biswas et al., 2001). The production of M protein remained the same in the *sagA/pel* transposon insertion mutation of M1 and M18 serotype compared to the wild-type strain, however, the virulence ability was found impaired in a mouse model of subcutaneous infection (Betschel et al., 1998). To sum up, these observations suggest that the Pel regulator act differently on similar genes in different strains. In addition, RivX is also involved in the production of M protein, for the transcript level of the *emm* gene increased in the GAS strain that overexpresses *rivR/X* (Roberts and Scott, 2007).

Encoded by the *scpA* gene, C5a peptidase (ScpA) is a widely conserved enzyme among Streptococcal strains. The substrate for ScpA is the human anaphylatoxin C5a, which plays a critical role in neutrophil activation and recruitment to the infection site (Ji et al., 1997). In GAS, expression of *scpA* gene was lower in the absence of *rivR/X*, while overexpression of either *rivR* or *rivX* alone increased the *scpA* expression, suggesting a positive effect exerted by RivX on the production of C5a peptide (Roberts and Scott, 2007).

The complement system is a major aim of the innate immune system that functions as the first line of defense against invading pathogens by initiating a proteolytic cascade that results in the opsonization of bacteria with C3b for phagocytosis, generation of anaphylatoxins C3a and C5a, and formation of the

cytolytic membrane attack complex (MAC) or C5b-9 for direct bacterial killing (Agarwal et al., 2015). In M1 serotype d, Pel was reported to stimulate the expression of the *sic* gene that encodes the streptococcal inhibitor of complement (Mangold et al., 2004).

4.1.3 Pathogen Invasiveness

Streptokinase(SK), originally designated as streptococcal fibrinolysin (Christensen, 1945), binding plasminogen with high affinity, can hydrolytically activate plasminogen molecules to plasmin, a fibrin-degrading protease (Wang et al., 1998). Though being used in treating blood-clotting disorders, streptokinase is regarded as a virulence factor for the association with absence of fibrin in spreading streptococcal disease, contributing to the pathogen invasiveness (Malke et al., 1994). The presence of streptokinase coding gene is a stable characteristic in GAS and human isolates of group C and G streptococci (GCS and GGS) (Steiner and Malke, 2002). In GAS, the production of SKA is mostly stimulated by FasX by trans-acting to *ska* mRNA. It was observed that FasX exerted a positive influencing on SKA production in GAS M49 serotype (CS101) (Klenk et al., 2005; Kreikemeyer et al., 2001) and M1 serotype (MGAS2221) (Ramirez-Pena et al., 2010). In the strain MGAS2221, by base-pairing a short stretch of 9 nt with the very 5'end of *ska* mRNA, FasX enhanced the stability of *ska* transcript, resulting in a 10-fold increase in SKA activity compare to an isogenic FasX mutant (Ramirez-Pena et al., 2010). Further experiment demonstrated that a double-stranded structure at the 5'end of *ska* mRNA was formed by annealing of FasX to *ska* mRNA, and the structure prevented single-stranded protrusion meanwhile the mimicking of a hairpin helped to stabilize the *ska* mRNA (Ramirez-Pena et al., 2010). What's more, binding less than 30 nt upstream of the start codon AUG, which is relatively close to the RBS (ribosome binding site), the FasX positively affects the steady state level of SKA expression via protecting *ska* mRNA from degradation mediated by ribonucleases (Ramirez-Pena et al., 2010). The FasX-*ska* mRNA base-pairing also promotes a structural change within the 5'UTR. The alteration of 5'UTR structural facilitated the

ribosome binding to mRNA and subsequently activated translation initiation (Ramirez-Pena et al., 2010). Combined with aforementioned negative effect on pili, FasX seems exerting an intricate effect on GAS virulence. Other than FasX, an insertion mutation to *pel* region in M49 serotype was found decreasing the expression of SKA, suggesting a positive modulatory function exerted by Pel (Li et al., 1999). Known as the strongest SK producer yet, GCS strain H46A, whose *fasCBAX* operon is preserved at high level of primary structure identity between the GAS and GCS gene, FasX regulated SKC expression via affecting *skc* transcript levels (Steiner and Malke, 2002).

4.2 sRNAs Regulate Cytotoxin Secretion

4.2.1 Secretion of Streptolysin

Streptolysin S (SLS) can be synthesized continuously by stationary-phase cell in the presence of a minimal energy source, and primarily exists in a cell-bound form linking to the streptococcal surface by lipoteichoic acid (Nizet, 2002). As mentioned above, the chromosomal locus for SLS production is mapped to a unique ORF encoding 53 amino acid that is named *sagA* for 'streptolysin-associated gene A' (Datta et al., 2005), and is known being identical with pel locus Downstream of *sagA/pel* locus lies eight additional contiguous genes (*sagB-sagI*) that are required for effective SLS production as well (Mangold et al., 2004; Nizet et al., 2000). The relationship between SLS production and the *sagA/pel* locus has been investigated in several different serotypes of GAS, and the role of this locus in modulating the SLS function is demonstrated at the RNA level. The activity of SLS has been observed totally inhibited when Tn917 insertion disrupted the transcription of *pel* region in GAS M49 serotype (Li et al., 1999). Moreover, a reduction SLO secretion was observed in a 4.5S RNA isogenic mutant strain of GAS, which is consistent with the reduced level of transcripts for the Streptolysin O (SLO) gene, compared to the parent strain MGAS2221 (Trevino et al., 2010).

4.2.2 Secretion of Cysteine Protease (SpeB)

Cysteine protease SpeB is one of the most important virulence factors that GAS harbors. It is secreted as a 42-kDa zymogen and auto-catalyzes into an active 28-kDa cysteine protease (Chen et al., 2003). Apart from promoting epithelial cell apoptosis (Tsai et al., 1999), SpeB cleaves or degrades host serum proteins such as human extracellular matrix, immunoglobulins, complement components, and even GAS surface and secreted proteins (Chiang-Ni and Wu, 2008). In an M49 serotype, the loss of *pel* region transcript resulted in the decreased transcription of *speB* gene (Li et al., 1999). In an M1 serotype, Pel exerted its negative effect on processing and maturation of SpeB at post-transcriptional level(Mangold et al., 2004). In the *pel*-deficient mutant, the transcription level of *speB* was not affected, however the onset of SpeB activity was found clearly delayed compared to the wild type strain. The zymogen of SpeB was effeciently secreted but its processing to the active mature form appeared to be delayed (Mangold et al., 2004). Besides, the transcript of *speB* gene dramatically decreased in the *rivRX* deleted mutant compared with parent strain, while the overexpression of *rivR/X*, *rivR* or *rivX* alone led to the overproduction of *speB* transcript (Roberts and Scott, 2007). Furthermore, a 4.5S RNA isogenic mutant strain of GAS also exhibited the reduced secretion of the SpeB protease (Trevino et al., 2010).

5 Concluding Remarks and Future Prospects

Genetic regulation is critical for bacteria adaptation to environmental changes, and strategies to control genetic networks in response to extracellular stimuli has been developed in bacteria. Accredited to the advancement in genome-wide computational and experimental techniques, the sRNAs-mediated regulatory pathways in streptococci have been reported recently. Studies on streptococcal sRNAs are mainly focused on GAS and *S. pneumoniae,* however only a small number of sRNAs have been predicted bioinformatically in other streptococci like GBS, *S. mutans*, *S. thermophiles* and *S.suis*. Most investigated sRNAs are involved in bacterial physiology and pathogenesis, regulating the biological

phenotypes and infection process. Particularly, the integration of FasX, Pel, RivX and csRNAs into regulatory networks have been delineated. However, the understanding of sRNAs in streptococci remains limited. What's more, knowledges on the roles of sRNAs during streptococcal infection are still limited. More efforts are still needed to bridge the gap between sRNAs identification and functional validation, and ultimately translate the critical regulatory mechanisms of sRNAs to the better management of streptococcal infections.

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