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RNA-mediated regulation in Gram-positive pathogens: an overview punctuated with examples from the group A *Streptococcus*

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Abstract

RNA-based mechanisms of regulation represent a ubiquitous class of regulators that are associated with diverse processes including nutrient sensing, stress response, modulation of horizontal gene transfer, and virulence factor expression. While better studied in Gram-negative bacteria, the literature is replete with examples of the importance of RNA-mediated regulatory mechanisms to the virulence and fitness of Gram-positives. Regulatory RNAs are classified as *cis*-acting, e.g. riboswitches, which modulate the transcription, translation, or stability of co-transcribed RNA, or *trans*-acting, e.g. small regulatory RNAs, which target separate mRNAs or proteins. The group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is a Gram-positive bacterial pathogen from which several regulatory RNA mechanisms have been characterized. The study of RNA-mediated regulation in GAS has uncovered novel concepts with respect to how small regulatory RNAs may positively regulate target mRNA stability, and to how CRISPR RNAs are processed from longer precursors. This review provides an overview of RNA-mediated regulation in Gram-positive bacteria, and is highlighted with specific examples from GAS research. The key roles that these systems play in regulating bacterial virulence are discussed and future perspectives outlined.

Keywords

control; sRNA; CRISPR; S. pyogenes

Introduction

The idea that bacterial RNA molecules could serve a regulatory role was proposed more than 50 years ago (Jacob & Monod, 1961), although the multiple mechanisms by which this can occur have only recently begun to be appreciated. For example, there have been bacterial RNA molecules described that (i) regulate target mRNAs at the levels of transcription, mRNA stability, and translation (Storz *et al.*, 2004, Cavanagh & Wassarman,

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2014), (ii) regulate the activity of target proteins through binding and sequestration (Babitzke & Romeo, 2007), and (iii) regulate the ability of horizontally transferred nucleic acids (e.g. bacteriophage and plasmids) to be maintained within the cell (Brouns *et al.*, 2008). A common method by which regulatory RNAs are classified is based upon whether the regulatory activity is targeted to the same RNA molecule (*cis*-acting RNAs) or to different RNAs, DNA, or proteins (*trans*-acting RNAs). The best described class of *cis*-acting RNAs are riboswitches, RNA elements typically located in the 5′-untranslated regions (5′-UTRs) of select mRNAs that respond to effector molecules (e.g. cyclic di-GMP) (Sudarsan *et al.*, 2008) or a physical parameter (e.g. temperature) (Johansson *et al.*, 2002), and modulate transcription, translation, or cleavage of the associated mRNA (Henkin, 2008, Serganov & Nudler, 2013). The best described class of *trans*-acting RNAs are the small regulatory RNAs (sRNAs, also known as non-coding RNAs [ncRNAs]) that function by binding to target mRNAs and/or proteins to modify their expression or activity (Storz *et al.*, 2011). In general, research into the RNA-mediated regulation of Gram-positive bacterial species lags behind that of Gram-negative species. The importance of RNA-mediated regulatory mechanisms in the virulence of Gram-positive pathogens (Brantl & Bruckner, 2014, Johansson et al., 2002, Mann *et al.*, 2012), as well as apparent differences between Gram-positive and -negative bacteria, such as many Gram-positive genomes lacking a gene encoding the RNA chaperone protein Hfq (Nielsen *et al.*, 2010, Sun *et al.*, 2002), warrant the study of RNA-mediated regulation in these organisms.

The group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a Gram-positive pathogen that causes a range of human infections, from mild, self-limiting infections such as pharyngitis (a.k.a. strep throat), to severe invasive infections such as necrotizing fasciitis (a.k.a. the flesh-eating disease) (Cunningham, 2000). GAS has a large repertoire of virulence factors that promote colonization, immune evasion, and the disruption of host tissue barriers (Reglinski & Sriskandan, 2014, Thomas & Lee, 2012). The coordinated expression of these virulence factors is believed to be critical to the ability of GAS to cause distinct human infections. RNA-mediated regulatory mechanisms are an important component of the ability of GAS to regulate gene expression (Liu *et al.*, 2012, Ramirez-Pena *et al.*, 2010, Fuchs *et al.*, 2006). Throughout this review, we will use examples from GAS research to highlight the mechanisms and activity of different categories of regulatory RNAs. Please note that due to space constraints we will not discuss house-keeping small RNA molecules that provide critical cell functions such as the 4.5S and 6S RNAs (Steuten *et al.*, 2014, Trevino *et al.*, 2010).

Trans-acting RNAs

The *trans*-acting RNAs include (a) sRNAs, which modify the expression or function of target molecules, (b) CRISPR RNAs, which contribute to a form of immunity that reduces acquisition of horizontally transferred elements, and (c) plasmid-encoded RNAs that contribute to plasmid maintenance and stability.

Highlights of sRNA-mediated regulation in Gram-positive bacteria—Similar to their Gram-negative counterparts, most sRNAs from Gram-positive species range from 50 to 250 nucleotides in length and, with notable exceptions (Balaban & Novick, 1995, Gimpel *et*

al., 2010), do not encode for any proteins; rather, the RNA molecules themselves have intrinsic regulatory activity. For the most part, given the absence of the regulator-protein sequestering CsrB-like sRNAs from Gram-positive genomes (Babitzke & Romeo, 2007), sRNAs from Gram-positive organisms fulfill their regulatory activity by base-pairing to one or more target mRNAs, leading to an alteration in the stability and/or translation of the hybridized mRNAs.

The stability of an sRNA-targeted mRNA can be increased or decreased depending upon multiple factors, in particular the location of the sRNA:mRNA interaction relative to the 5′ end, the Shine-Dalgarno ribosome binding site (RBS), and/or RNase cleavage sites, of the mRNA molecule. For example, the *Clostridium perfringens* sRNA VR-RNA increases the stability of *colA* mRNA, encoding the toxin collagenase, after binding to the mRNA 5′-UTR between the 5′ end and RBS (Obana *et al.*, 2010). VR-RNA:*colA* mRNA duplex formation leads to a single cleavage event within the *colA* mRNA 5′-UTR, which generates a new 5′ end (Figure 1A). Crucially, the nucleotides of the processed *colA* mRNA generate a stem-loop structure at the 5′ end, unlike the situation with the full-length transcript (Obana et al., 2010). Secondary structure at the 5′ end of an mRNA molecule promotes stability through the inhibition of RNA pyrophosphorylase binding as this enzyme converts 5′ tri-phosphate ends into mono-phosphate ends; the preferred substrates of several ribonucleases (Condon & Bechhofer, 2011, Lehnik-Habrink *et al.*, 2012). An example of an mRNA with decreased stability following sRNA binding is the *lmo0850* mRNA following LhrA sRNA binding in *Listeria monocytogenes* (Nielsen et al., 2010). While the exact mechanism by which *lmo0850* mRNA stability is reduced has not been investigated, it is believed to be a consequence of reduced ribosome activity on the mRNA (Figure 1B). Translocating ribosomes enhance the stability of mRNAs by inhibiting ribonuclease access (Deana & Belasco, 2005).

The translation of an sRNA-targeted mRNA can also be increased or decreased depending upon the location and nature of the sRNA:mRNA interaction. For example, the best described sRNA from a Gram-positive pathogen, the 514 nt RNAIII from *Staphylococcus aureus* (Novick *et al.*, 1993), positively regulates translation of *hla* mRNA encoding α-hemolysin and negatively regulates translation of *rot* mRNA encoding the repressor-of-toxins protein Rot. RNAIII positively regulates *hla* mRNA translation by disrupting intramolecular base-pairing in the *hla* mRNA that ordinarily blocks ribosome access to the RBS (Morfeldt *et al.*, 1995) (Figure 1C). Conversely, RNAIII negatively regulates *rot* mRNA translation by binding to the RBS and occluding ribosome access (Geisinger *et al.*, 2006) (Figure 1D).

Candidate sRNAs are encoded throughout the GAS genome—Multiple distinct approaches have been used to identify sRNAs expressed by bacterial pathogens. In GAS, these include the use of tiling microarrays (Perez *et al.*, 2009, Patenge *et al.*, 2012), RNAseq analysis (Deltcheva *et al.*, 2011), and bioinformatics (Livny *et al.*, 2006, Tesorero *et al.*, 2013, Raasch *et al.*, 2010). In combination, over 100 candidate sRNAs have been predicted to be encoded within the GAS genome. Importantly, multiple of the candidate sRNAs are differentially expressed in serotype, growth phase, and/or growth media-specific fashion. Thus, it has been proposed that GAS differentially expresses sRNAs during infection and

that this influences GAS virulence. While there are many candidate sRNAs within the GAS genome the only one thus far characterized is FasX.

The GAS sRNA FasX differentially regulates virulence factor expression—The fibronectin/fibrinogen-binding/hemolytic activity/streptokinase-regulator (*fas*) locus was discovered in GAS, based upon modest homology to the *S. aureus* Agr and *Streptococcus pneumoniae* Com systems (Kreikemeyer *et al.*, 2001). The *fas* locus consists of the *fasBCAX* genes which encode for two putative membrane-spanning histidine kinases (FasB and FasC), one putative DNA-binding response regulator (FasA), and a 205 nt sRNA (FasX). While the FasBCA proteins have not been studied in detail, it is hypothesized that they function similar to classical two-component systems. FasX abundance increases during the exponential phase of growth and is significantly decreased in stationary phase, similar to the abundance of RNAIII from the *agr* quorum sensing system of *S. aureus* (Boisset *et al.*, 2007). However, while auto-inducing peptides have been detected in GAS culture supernatants none function with the FasBCA system (Chang *et al.*, 2011, Belotserkovsky *et al.*, 2009), reducing the possibility that the Fas locus functions as part of a quorum sensing mechanism (Kreikemeyer et al., 2001).

Two virulence factor-encoding mRNAs have been confirmed as targets of FasX and the molecular mechanisms behind the regulation delineated. The first confirmed FasX-regulated GAS virulence factor was the thrombolytic agent streptokinase (Ramirez-Pena et al., 2010, Kreikemeyer et al., 2001). Streptokinase is a secreted virulence factor that promotes the conversion of the human protein plasminogen into the protease plasmin (McArthur *et al.*, 2012). Plasmin has multiple activities, including degrading blood clots (directly by degrading the fibrin fibers within a blood clot) and tissue barriers (directly by degrading extracellular matrix components and indirectly by activating collagenases and metalloproteases) (Syrovets *et al.*, 2012). FasX base-pairs to the first nine nucleotides of the streptokinase-encoding (*ska*) mRNA, an interaction that increases the stability of the mRNA leading to a ten-fold increase in *ska* mRNA abundance and streptokinase protein expression (Figures 2A and 2B) (Ramirez-Pena et al., 2010). The increase in *ska* mRNA stability following FasX binding is a consequence of the creation of secondary structure at the 5′ end of *ska* mRNA, a heretofore unrecognized mechanism of positive regulation by an sRNA (Podkaminski & Vogel, 2010). Thus, although the mechanism differs between the FasX-mediated regulation of *ska* mRNA stability in GAS and the VR-RNA-mediated regulation of *colA* mRNA stability in *C. perfringens*, both ultimately promote stability through formation of 5′ end secondary structure (Obana et al., 2010, Ramirez-Pena et al., 2010). Differences between the two mechanisms include that FasX must remain bound to its mRNA target to enhance stability while VR-RNA does not. The reversible nature of the FasX:*ska* mRNA interaction implies that the positive regulation afforded by FasX could be removed by decreasing FasX transcription, or increasing FasX turnover.

It has previously been proposed that the hybridization of an sRNA to an mRNA between nucleotides −35 to +15, relative to the A of the ATG start codon, leads to inhibition of mRNA translation (Frohlich & Vogel, 2009, Sharma *et al.*, 2007, Storz et al., 2004). However, this does not appear to be the case for the FasX:*ska* mRNA interaction, which occurs at *ska* mRNA nucleotides −32 to −24. This is supported by the fact that the increase

in streptokinase expression mirrors the increase in *ska* mRNA abundance (Ramirez-Pena et al., 2010). Therefore, the consequences to translation with respect to sRNA:mRNA interactions must be determined on a case-by-case basis.

The second confirmed FasX-regulated GAS virulence factor is the pilus (Liu et al., 2012). GAS pili bind to collagen and promote the ability of this pathogen to adhere to host cells, as well as to form biofilms (Abbot *et al.*, 2007, Lizano *et al.*, 2007, Manetti *et al.*, 2007). FasX base pairs to 16 of the first 17 nucleotides of *cpa* mRNA, the first gene in the pilus biosynthesis operon that encodes the collagen-binding minor pilus protein located at the pilus tip (Quigley *et al.*, 2009). In contrast to the FasX:*ska* mRNA interaction, the FasX:*cpa* mRNA interaction negatively regulates expression (Figures 2A and 2C). The main mechanism by which FasX negatively regulates pilus expression is through the inhibition of *cpa* mRNA translation, reducing access of ribosomes to the *cpa* mRNA RBS and consequently, translation of the pilus biosynthesis genes (Figure 2A) (Liu et al., 2012). By reducing expression of adhesins and enhancing expression of streptokinase, which aids GAS spread, FasX is proposed to be a key regulator in the transition of GAS from the colonization to the dissemination stages of infection.

The FasX sequences complementary to *ska* and *cpa* mRNAs are UCAAUCCCC and CUCUCUCUCUUUUGUU, respectively. The concentrations of U and C nucleotides in these regions are reminiscent of the conserved UCCC sequence motif recently identified in 11 previously uncharacterized *S. aureus* sRNAs (Geissmann *et al.*, 2009). Similarly, three UCCC sequences present in the *S. aureus* RNAIII sRNA are known to interact with target mRNA sequences (Boisset et al., 2007). It has been proposed that the UCCC motif highlights a novel subset of *S. aureus* sRNAs that function by inhibiting translation of target mRNAs through sRNA:mRNA interactions (Geissmann et al., 2009), and we propose that similarly functioning sRNAs are also present in other low GC% Gram-positive pathogens such as GAS.

Highlights of CRISPR/Cas systems in Gram-positive bacteria—CRISPR/Cas (clustered, regularly interspaced short palindromic repeat/CRISPR-associated proteins) together constitute an adaptive immune system which reduces the ability of bacteriophage and plasmids to be maintained in a recipient cell following transfer (Brouns et al., 2008). Thus, this activity, first described in *Streptococcus thermophilus* (Barrangou *et al.*, 2007), represents a major impediment to horizontal gene transfer. CRISPRs consist of repeat sequences 24–28 bp in size that are separated by unique ‘spacer’ regions which vary in size (26 to 72 bp), with the majority showing homology to foreign DNA, such as bacteriophages and plasmids (Bolotin *et al.*, 2005, Jansen *et al.*, 2002, Mojica *et al.*, 2005). CRISPR/Cas systems enable the bacterial cell to develop an ‘immune response’ against horizontally transferred DNA that is complementary to one or more spacer sequences.

CRISPR elements are transcribed as a single precursor transcript (pre-CRISPR RNA [pre-crRNA]). Subsequently, the pre-crRNA undergoes a maturation process whereby each repeat-spacer region is cleaved into a separate crRNA. How the pre-crRNA is processed into mature crRNAs provides the basis of a classification system (Makarova *et al.*, 2011). In Type I CRISPR/Cas systems, the pre-crRNA is cleaved by a Cas endonuclease that is part of

a larger complex termed Cascade (CRISPR-associated complex for antiviral defense) (Sinkunas *et al.*, 2013, Brouns et al., 2008). In Type II CRISPR/Cas systems, the generation of crRNAs requires a small RNA molecule termed tracrRNA (trans-activating CRISPR RNA) which base-pairs to the repeat units in pre-crRNA and promotes their cleavage via the double-stranded RNA-cleaving enzyme RNase III (Deltcheva et al., 2011) (Figure 3). In Type III CRISPR/Cas systems, the pre-crRNA is cleaved by a single Cas endonuclease prior to being transferred to a Cas protein complex for additional processing (Carte *et al.*, 2008).

A key factor in the functionality of CRISPR/Cas systems is their ability to integrate new information in the form of additional spacer sequences, thus enabling the constant monitoring of horizontally transferred elements. Typically, only a single new spacer is inserted following plasmid or bacteriophage transfer, with the integration also resulting in the duplication of a repeat sequence to create a novel spacer-repeat unit (Figure 3). The region of bacteriophage or plasmid DNA that forms the spacer sequences is not randomly selected, rather the selection of spacer precursors (proto-spacers) is determined by adjacent sequences termed proto-spacer-adjacent motifs (PAMs) (Shah *et al.*, 2013, Heler *et al.*, 2014). While the exact mechanism by which new spacers are acquired is not known, the Cas1 and Cas2 proteins appear to play key roles (Nunez *et al.*, 2014).

The “immune” activity of generated crRNAs occurs through the ability of crRNA/Cas ribonucleoprotein complexes to target and cleave bacteriophage or plasmid DNA at sites complementary to the crRNA sequences (Figure 3). While all Type I and II CRISPR/Cas systems target DNA, Type III systems can be subdivided into Type III-A or III-B, where Type III-A systems also target DNA, while Type III-B systems target RNA (Staals *et al.*, 2013). The site-specific targeting achieved by CRISPR/Cas systems has led to their exploitation as a tool for genome engineering, with a Type II system from GAS being the most extensively used, in part due to this being the first Type II system characterized (Cong *et al.*, 2013, Wang *et al.*, 2013, Hwang *et al.*, 2013, Bassett *et al.*, 2013, Deltcheva et al., 2011).

Do CRISPR-Cas systems influence GAS virulence?—GAS strains are poly-lysogenic such that between ~5 to 10% of any one genome is attributable to integrated bacteriophage, most of which encode one or more virulence factors (Banks *et al.*, 2002). The assortment of bacteriophage present within GAS strains is highly variable and, given the importance of phage-encoded virulence factors to GAS pathogenicity (e.g. the superantigen SpeA and the immune modulating DNase SdaD2) (Sumby *et al.*, 2005, Kasper *et al.*, 2014), mechanisms that influence bacteriophage acquisition such as CRISPR systems are believed to have an indirect effect on GAS virulence (Nozawa *et al.*, 2011). Given that antibiotic resistance genes are commonly associated with plasmids (e.g. *erm(T)*) (DiPersio *et al.*, 2011, Woodbury *et al.*, 2008), CRISPR systems may also impact the ability of GAS to gain antibiotic resistances. Finally, at least one CRISPR spacer identified in GAS shares homology not with mobile genetic elements, but with a chromosomally-encoded gene (Nozawa et al., 2011). This, plus similar observations in other pathogens (Westra *et al.*, 2014), has led to the hypothesis that CRISPR/Cas systems may be able to influence the expression of targeted chromosomal genes, and hence would represent a novel regulatory mechanism.

RNA-based regulation of plasmid maintenance and stability—The regulatory RNA RNAI is encoded on the *E. coli* plasmid pColEI and is involved in modulating plasmid replication (Dooley *et al.*, 1985). Multiple plasmids from Gram-positive bacteria also have a regulatory RNA component controlling plasmid replication. For example, the GAS plasmid pSM19035 has at least three genes that modulate replication, the protein-encoding genes *copS* and *repS*, and the regulatory RNA-encoding RNAIII (Brantl *et al.*, 1993, Lioy *et al.*, 2010) (not to be confused with the RNAIII sRNA from *S. aureus*) (Figure S1A). RepS mediates plasmid synthesis and is regulated by both the transcription factor CopS and RNAIII. RNAIII and *repS* are divergently transcribed from one another and are arranged such that the 5′ ends of the two RNA molecules are complementary. As a consequence of the sequence complementarity between RNAIII and *repS* mRNA, there is base-pairing between the two 5′ ends which sequesters the *repS* mRNA RBS and inhibits translation. Deletion of either CopS or RNAIII results in a 10–20 fold increase in plasmid copy number. This tight regulation of RepS controls the rate of plasmid replication, maintaining a low plasmid count per cell (Lioy et al., 2010).

In some cases, the maintenance and propagation of low-copy plasmids are also controlled through the action of an RNA molecule. Toxin-antitoxin (TA) systems contain a stable, protein-encoded toxin which is repressed by either an unstable RNA antitoxin (Type I TA systems) or by an unstable protein antitoxin (Type II TA systems) (Van Melderen, 2010). As the cells replicate and segregate, the stable toxin molecule remains present in the daughter cells. If the daughter cell loses the plasmid, it loses the ability to produce the antitoxin and is killed by the toxin. The *Enterococcus faecalis* plasmid pAD1 has a well characterized Type I TA system termed the *par* cassette (Figure S1B). The *par* region harbors two convergently transcribed RNA molecules, RNA I (which encodes the 33 amino acid toxin) and RNA II (the RNA antitoxin) (Weaver, 2012). Due to complementarity between RNA I/II molecules they are able to hybridize to one another, resulting in the inhibition of RNA I translation (Greenfield *et al.*, 2000). While the GAS plasmid pSM19035 harbors a Type II TA system no Type I systems have thus far been described in this pathogen (Brzozowska *et al.*, 2012, Lioy et al., 2010). A chromosomally-encoded homolog of the Type I TA system *par* cassette has been identified in *Streptococcus pneumoniae* (Fozo et al., 2010). Chromosomally-encoded TA systems appear, in part, to function in the general stress response (Durand *et al.*, 2012).

Cis-acting RNAs

Cis-acting RNAs are sequences that are co-transcribed with their respective mRNA regulatory targets, the best described class of which are riboswitches.

Riboswitches trigger structural rearrangement of mRNA—Riboswitches are RNA motifs located almost exclusively in the 5′-UTR of select mRNAs that, either through binding effector molecules or sensing a physical parameter, modulate the transcription, translation, or stability of the associated mRNA (Serganov & Nudler, 2013, Breaker, 2012). Regulation occurs as a consequence of conformational changes in the secondary RNA structure of the riboswitch following activation. This leads to the formation of transcriptional terminators or anti-terminators, the sequestrating or unmasking of the RBS

altering mRNA translation, or the exposing or masking of RNase cleavage sites altering mRNA stability (Caron *et al.*, 2012, Hollands *et al.*, 2012) (Figure 4A). Recently, several S-box riboswitches, which bind the metabolite S-adenosylmethionine (SAM), have been identified that not only regulate *in cis* but also regulate *in trans*. For example, two S-box riboswitches (SreA/B) from *Listeria monocytogenes* regulate *in trans* by binding the 5′-UTR of the mRNA encoding the major virulence factor regulator PrfA (Loh *et al.*, 2009) (Figure 4B). Thus, while regulatory RNAs are commonly classified into different groups they should be viewed as fluid classifications, not rigid, as new information is gathered and new functions discovered.

The investigation of riboswitches in GAS has thus far been limited to a SAM-binding riboswitch termed the S_{MK}-box riboswitch (Fuchs *et al.*, 2007), which shares little sequence homology with the classic S-box riboswitch. The S_{MK}-box riboswitch is found in the 5′-UTR of *metK* genes, encoding SAM synthetase, in lactic acid bacteria, including Streptococcal and Enterococcal species (Grundy & Henkin, 1998). The S_{MK}-box regulates *metK* mRNA translation by sequestering the RBS and inhibiting ribosome binding in the presence of SAM (Fuchs et al., 2006, Fuchs et al., 2007) (Figure 4C). Following SAM binding, the S_{MK}-box also regulates *metK* transcription by stabilizing a 5′ terminator and disrupting RNA polymerase activity.

Putative regulatory roles for the 5′-UTRs of the C5a peptidase and CovR-encoding mRNAs—While no GAS riboswitches have been confirmed to regulate virulence factors or their regulators, there are three 5′-UTRs that have been proposed to have regulatory activity, with riboswitch-like activity being one of several possibilities. The three 5′-UTRs are located in the *scpA* mRNA encoding the C5a peptidase immune evasion protein (Pritchard & Cleary, 1996), the *rivR* mRNA encoding the virulence factor regulatory protein RivR (Trevino *et al.*, 2013), and the *covR* mRNA encoding the response regulator component of the major virulence factor regulatory two component system CovR/S (Sumby *et al.*, 2006). A characteristic shared by the *scpA* and *rivR* mRNAs is that their transcription can terminate prematurely within the 5′-UTR sequence, leading to 170 nt and 140 nt RNA truncation products, respectively (Pritchard & Cleary, 1996, Trevino et al., 2013). In both instances, the transcriptional termination occurs at the site of an inverted repeat, which is hypothesized to form a hairpin and terminate transcription in a Rho-independent manner (Figures S2A and S2B). Removal of the inverted repeat region upstream of the *scpA* and *rivR* genes enhances transcript abundance four and three-fold, respectively (Pritchard & Cleary, 1996, Trevino et al., 2013). Whether the rate of transcriptional termination within the 5′-UTRs is regulated is hypothesized but remains untested.

Bioinformatic analysis of the 196 nt *covR* 5′-UTR is consistent with this region possessing a high degree of secondary structure that includes the RBS (Figure S2C). If correct, then the sequestering of the RBS in a secondary structure would be expected to reduce ribosome binding and therefore, inhibit *covR* mRNA translation. Additional support for a regulatory role for the *covR* 5′-UTR comes from related pathogens. For example, the *covR* 5′-UTR from *Streptococcus mutans* shares only 45% nucleotide identity with that of GAS (Chong *et al.*, 2008), but is predicted to form a similar secondary structure (data not shown). Given the critical contribution of the CovR/S regulatory system to GAS pathogenicity (Sumby et al.,

2006, Federle *et al.*, 1999, Hollands *et al.*, 2010, Trevino *et al.*, 2009), the investigation of how this system is regulated is currently under investigation.

Conclusions and Future Prospects

In general, tools for the genetic manipulation of Gram-positive bacteria trail those of Gram-negatives, and is likely a contributing factor into why mechanisms of RNA-mediated regulation have been better studied in Gram-negative bacteria. Another contributing factor is that most Gram-positive bacteria lack a homologue of the RNA chaperone protein Hfq, a protein which binds sRNAs and has been exploited by Gram-negative researchers to identify candidate sRNAs via pull-down assays (Pfeiffer *et al.*, 2007, Sittka *et al.*, 2009, Sonnleitner *et al.*, 2008, Zhang *et al.*, 2003). While RNA-mediated regulation may be better studied in Gram-negatives, this does not mean that this large class of regulators are any less important in Gram-positives. Indeed, multiple studies, from the RNAIII sRNA in *S. aureus* (Novick et al., 1993), to the SreA/B riboswitches in *L. monocytogenes* (Loh et al., 2009), to our own work with the FasX sRNA in GAS (Ramirez-Pena et al., 2010), all highlight the critical importance of RNA-mediated regulation to the virulence of Gram-positive pathogens. Furthermore, Gram-positive regulatory RNA research has uncovered novel aspects of this class of regulators. For example, GAS regulatory RNA research has led to the discovery of a novel positive regulatory mechanism by an sRNA (FasX regulating streptokinase by formation of 5′ end secondary structure) (Ramirez-Pena et al., 2010), and the discovery of a novel mechanism to generate crRNAs from pre-crRNA precursors (via tracRNA and RNase III; thus describing the prototypical Type II CRISPR/Cas system) (Deltcheva et al., 2011). These and other findings highlight the importance of continuing the investigation of RNA-mediated regulatory mechanisms in Gram-positive pathogens. Giving credence to the term “be careful what you wish for”, the current bottleneck to characterizing novel mechanisms of RNA-mediated regulation comes not from identifying candidate regulatory RNAs, but rather from the testing of the many candidates identified from recent next-generation sequencing, tiling microarray, and bioinformatic approaches. Improvements in the accuracy of bioinformatic-based analyses and the speed of lab-based confirmation of candidate regulatory RNA activities are crucial to future efforts in delineating the overall contribution of this fundamental class of regulatory system in Gram-positives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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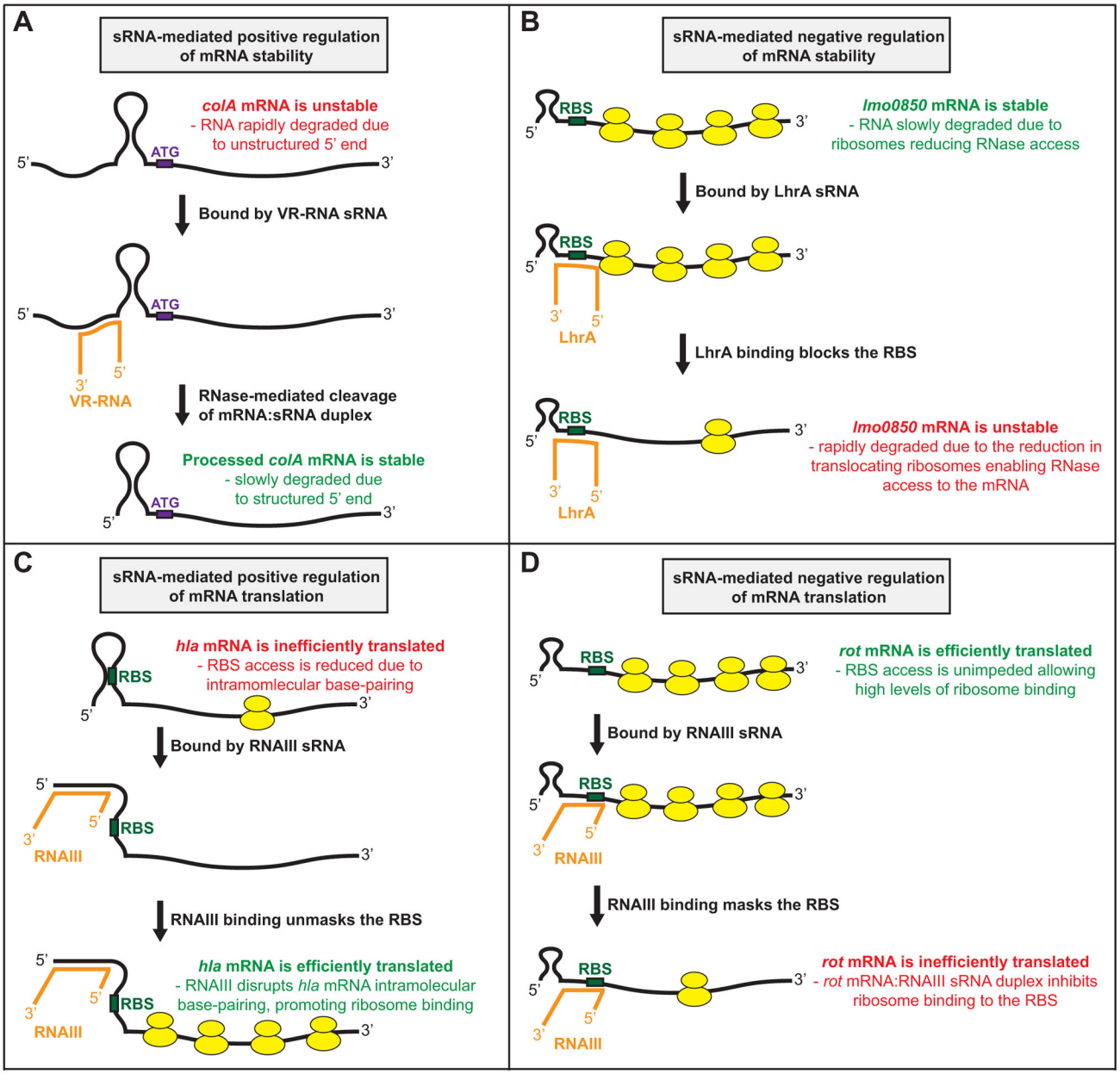

Figure 1. Mechanisms by which sRNAs from Gram-positive pathogens regulate the stability or translation of target mRNAs

(**A**) The *Clostridium perfringens* sRNA VR-RNA positively regulates the stability of *colA* mRNA (Obana et al., 2010). The *colA* mRNA (black), *colA* mRNA start codon (ATG; purple), and VR-RNA sRNA (orange) are shown. (**B**) The *Listeria monocytogenes* sRNA LhrA negatively regulates the stability of *lmo0850* mRNA (Nielsen et al., 2010). The *lmo0850* mRNA (black), *lmo0850* mRNA RBS (green), LhrA sRNA (orange), and ribosomes (yellow) are shown. (**C**) The *Staphylococcus aureus* sRNA RNAIII positively regulates the translation of *hla* mRNA (Morfeldt et al., 1995). The *hla* mRNA (black), *hla* mRNA RBS (green), RNAIII sRNA (orange), and ribosomes (yellow) are shown. (**D**) The

Staphylococcus aureus sRNA RNAIII negatively regulates the translation of *rot* mRNA (Boisset et al., 2007). The *rot* mRNA (black), *rot* mRNA RBS (green), RNAIII sRNA (orange), and ribosomes (yellow) are shown.

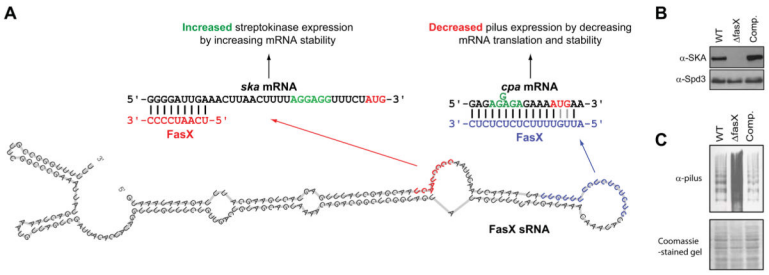

Figure 2. The FasX sRNA positively and negatively regulates GAS virulence factor expression

(**A**) Schematic showing the putative FasX secondary structure with nucleotides involved in the positive regulation of *ska* mRNA (red) and the negative regulation of *cpa* mRNA (blue) highlighted. Only the 5′ ends of the *ska* and *cpa* mRNAs are shown, with the Shine-Dalgarno ribosome binding sites (green) and AUG start codons (red) highlighted. This panel is a modified version of a previously published figure (Liu et al., 2012). (**B** + **C**) Western blot analyses of secreted (B) or cell wall (C) protein fractions from a parental GAS strain (WT), an isogenic *fasX* mutant (ΔfasX), and a complemented mutant derivative (Comp). The secreted protein Westerns used an anti-SKA antibody as the test antibody and an anti-Spd3 antibody as a loading control. The cell wall protein Westerns used an anti-pilus antibody as the test antibody, with the gel coomassie-stained prior to performing the Western as a loading control. Note the characteristic laddering pattern of pili.

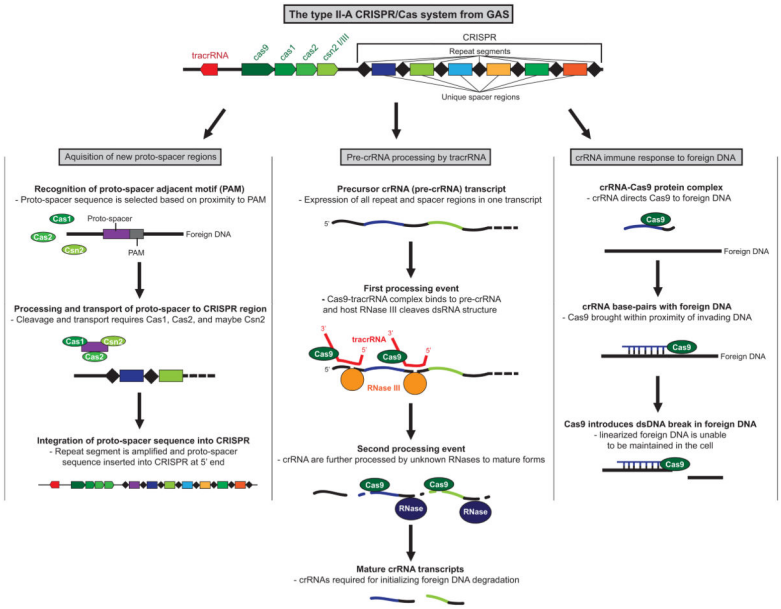

Figure 3. Schematic of how a type II-A CRISPR/Cas system generates new spacer sequences, processes pre-crRNA into mature crRNA transcripts, and targets foreign DNA for cleavage

The three functions associated with CRISPR/Cas systems are shown. The conserved repeat segments of the CRISPR region are represented by black diamonds, while unique spacers are represented by colored rectangles.

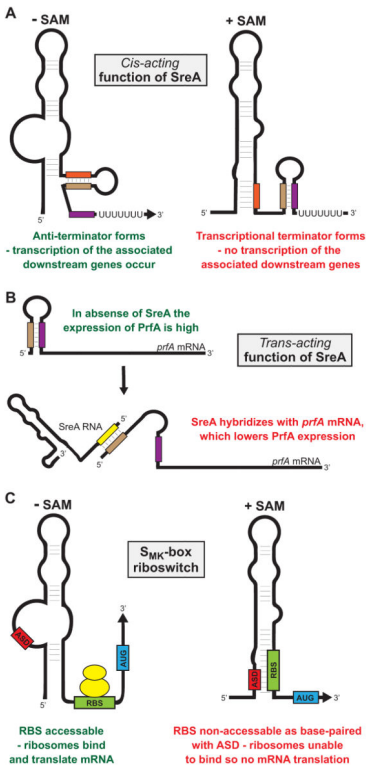

Figure 4. Examples of regulatory mechanisms by SAM-binding riboswitches from Gram-positive bacteria

(**A**) The *Listeria monocytogenes* S-box riboswitch SreA functions *in cis* to terminate transcription of the downstream genes *lmo2417–2419* in the presence, but not in the absence, of SAM (Loh et al., 2009). In the presence of SAM the rearrangement of secondary structure results in the formation of the terminator hairpin through nucleotides highlighted as brown and purple rectangles. In the absence of SAM the anti-terminator forms through nucleotides highlighted as red and brown rectangles. (**B**) The *Listeria monocytogenes* S-box riboswitch SreA can also function *in trans* to reduce expression of the virulence factor regulatory protein PrfA (Loh et al., 2009). SreA RNA can bind near the 5′ end of *prfA* mRNA. Through unknown mechanisms the interaction between SreA RNA and *prfA* mRNA lowers PrfA expression levels. (**C**) The S_{MK}-box riboswitch from lactic acid bacteria negatively regulates the translation of its associated gene in the presence, but not in the absence, of SAM (Fuchs et al., 2006). The anti-RBS sequence (ASD), the RBS (green) and start codon (AUG; blue) of the associated gene, and ribosomes (yellow) are shown. In the absence of SAM the ASD nucleotides do not base-pair the RBS, enabling ribosome binding and translation of the gene. In the presence of SAM the rearrangement of secondary structure results in base-pairing between the ASD and RBS sequences, occluding the RBS and preventing mRNA translation.