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Small regulatory RNAs from low-GC Gram-positive bacteria

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Small regulatory RNAs (sRNAs) that act by base-pairing were first discovered in so-called accessory DNA elements—plasmids, phages, and transposons—where they control replication, maintenance, and transposition. Since 2001, a huge body of work has been performed to predict and identify sRNAs in a multitude of bacterial genomes. The majority of chromosome-encoded sRNAs have been investigated in *E. coli* and other Gram-negative bacteria. However, during the past five years an increasing number of sRNAs were found in Gram-positive bacteria. Here, we outline our current knowledge on chromosome-encoded sRNAs from low-GC Gram-positive species that act by base-pairing, i.e., an antisense mechanism. We will focus on sRNAs with known targets and defined regulatory mechanisms with special emphasis on *Bacillus subtilis*.

Introduction

Bacterial small regulatory RNAs (sRNAs) are the most abundant class of post-transcriptional regulators and had first been discovered in plasmids, phages, and transposons, where they control replication, maintenance, and transposition.[1] Whereas until 2001, only a dozen riboregulators were known from bacterial chromosomes, since then, various systematic approaches have been performed to predict and identify chromosome-encoded sRNAs in prokaryotes. The majority of them have been investigated in *E. coli* and *Salmonella*, whereas only a few well-studied examples are known from Gram-positive bacteria.

In the past five years, a combination of computational predictions, transcriptome analyses, and RNA sequencing approaches has been applied to identify chromosome-encoded riboregulators in low GC Gram-positive bacteria (see **Table 1**). Five searches have been performed in *B. subtilis.* Rasmussen et al. found 84 putative non-coding trans-encoded sRNAs in the *B. subtilis* genome,[2] and Irnov et al. increased the total number to 108.[3] A recent RNA-Seq in *B. licheniformis* identified 461 independently transcribed sRNAs in addition to 855 RNAs transcribed in antisense to known protein and RNA encoding genes.[4] Six searches were performed in *S. aureus* and five in *Listeria monocytogenes,* three in *Streptococcus pyogenes*, and four in *Streptococcus pneumoniae* (see below). Two searches in *Enterococcus faecalis* identified 29 novel sRNAs, among them an antisense RNA to 6S RNA.[5,6] In *Clostridium*, three searches have been performed (*C. difficile*,[7] *C. ljungdahlii*,[8] and *C. acetobutylicum*[9]).

sRNAs either regulate translation or RNA stability. The majority of them inhibit translation (**Fig. 1A and B**), whereas only a few of them activate translation (**Fig. 1C**). Translational inhibition can principally occur in three different ways, (1) by direct blocking of the ribosome-binding site (RBS) (**Fig. 1A**), by induction of structural alterations downstream of the RBS (see **Fig. 1B** or iii) by blocking of a ribosome standby site required for efficient translation (reviewed in ref. 10). So far, the latter case has been only found in *E. coli*.[11] In some cases, translational inhibition is accompanied by mRNA degradation (see below, *S. aureus* sRNAs). Both cis-and trans-encoded sRNAs can inhibit translation (**Fig. 1A**) or promote RNA degradation (**Fig. 1D**). Some sRNAs can stabilize their target RNAs (**Fig. 1E**), whereas others process an unstable mRNA into a stable, translationally active RNA (**Fig. 1F**). In 2011, the first sRNA required for the maturation of long RNAs—the CRISPR RNAs—was discovered[12] (**Fig. 1G**). A mechanism of action that can be exclusively used by cis-encoded sRNAs is transcriptional interference (**Fig. 1H**, see below). In this report, we summarize (**Fig. 1**) and discuss all currently known mechanisms employed by chromosome-encoded sRNAs from low-GC Gram-positive bacteria. Thereby, mechanisms discovered for cis-encoded and for trans-encoded sRNAs were assembled. **Tables 2 and 3** list all currently known sRNAs with their targets, mechanisms of action, and regulators. In a recent report that also includes plasmid-encoded antisense RNAs that control replication or maintenance of these accessory DNA elements, two additional control mechanisms were considered: transcription and translation attenuation.[13] Although both mechanisms are employed by riboswitches, they have not yet been observed for chromosome-encoded bona fide sRNAs.

Chromosome-Encoded sRNAs in Low GC Gram-Positive Bacteria and Their Biological Functions

In the past years, a variety of articles have been published on the identification of sRNAs in Gram-positive bacteria (reviewed in ref. 13). On average, ≈100–200 sRNAs have been discovered in a single genome. Despite newly available methods, it is still a

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Table 1. Systematic searches for sRNAs in low GC Gram-positive bacteria

Species	sRNAs predicted/ confirmed*	References
Bacillus subtilis	108	2, 3, 97-99
Bacillus licheniformis	461 indepently encoded	4
Staphyloccus aureus	100 trans-,100 cis-encoded	50, 54, 99, 100-103
Listeria monocytogenes/ Listeria innocua	113 trans-, 70 cis-encoded	72, 104-107
Enterococcus faecalis	69	5, 6
Clostridium difficile	94 trans-, 91 cis-encoded	7
Clostridium acetobutylicum	159	9
Clostridium ljungdahlii	36	8
Streptococcus pyogenes	75	84, 108, 109
Streptococcus pneumoniae	179	78, 80, 82, 110

*The total number of predicted or confirmed sRNAs for the corresponding organisms from all published searches is indicated.

challenging task to identify the targets of these novel riboregulators. In the following, we focus on base-pairing sRNAs for which targets have been verified experimentally. For an overview that also includes data on riboswitches and protein-binding sRNAs from Gram-positive bacteria, see reference 14.

Chromosome-encoded sRNAs are involved in a wide variety of biological functions. Mostly, they fine-tune metabolic processes and regulate stress adaptation or virulence. Fine-tuning functions are reflected by the lack of severe phenotypes upon deletion or overexpression of such RNAs. Examples for metabolic regulation include arginine catabolism (*B. subtilis* SR1[15]) and iron-transport and storage (*B. subtilis* FsrA[16]) or central metabolism (*S. aureus* RsaE[17]).

A few trans-encoded sRNAs contain additionally small open reading frames (ORFs) that are translated. Such RNAs were designated dual-function sRNAs. Examples for small ORFs are the 26 codon δ-hemolysin ORF of *S. aureus* RNAIII,[18] the 22 codon psmα-ORF in *S. aureus P*sm-mec,[19] the streptolysin SLS-ORF of *Streptococcus* Pel RNA,[20] and the 39 codon ORF *sr1p* on *B. subtilis* SR1 RNA (see below[21]). The translation products of the small ORFs can either operate in the same pathway as the base-pairing sRNA (in RNAIII and Pel RNA) or in another pathway: *B. subtilis* SR1 acts as a base-pairing sRNA in arginine catabolism, whereas SR1P acts in sugar metabolism.[21] To date, neither for the 72 codon *hyp7* ORF of *Clostridium perfringens* VR[22] nor the 32 codon ORF of *Streptococcus pyogenes* RivX[23] data are available on translation or possible biological function(s).

In different approaches, genome-wide overlapping (antisense) transcription was found (reviewed in ref. 24). Antisense transcription in the same bacterial cells yields a collection of short RNA fragments that result from RNase III processing, which appears most prominently in Gram-positive bacteria. Examples are provided below for *B. subtilis* and *S. aureus*. The mechanisms through which overlapping transcription can affect sense RNA expression are diverse. Primarily direct base-pairing interactions between sense and antisense transcripts, which result in RNase III cleavage of the complexes, can be imagined. An alternative is transcriptional interference that has, so far, been documented only in one case (see below).

In the following, sRNAs discovered in *Bacillus subtilis, Staphylococcus aureus, Listeria monocytogenes*, as well as *Clostridium* and *Streptococcus* species, and for which targets have been identified, are discussed in detail with regard to their biological function, expression conditions, and mechanism of action.

sRNAs from *Bacillus subtilis*

Trans-encoded sRNAs

The first trans-encoded sRNA in *B. subtilis,* FsrA (84 nt) was discovered in 2008[16] and resembles *E. coli* RyhB as it is transcriptionally repressed by Fur and regulates target mRNAs involved in iron metabolism and storage, e.g., *sdhCAB* (succinate dehydrogenase) and *citB* (aconitase). Using transcriptomics, additional FsrA targets were identified, among them, *gltAB* encoding the iron-sulfur-containing enzyme glutamate synthase and *lutABC, dctP, resA*, and *qcrA*.[25] Consequently, FsrA is a global regulator in *B. subtilis.* In contrast to *E. coli* RyhB, which requires the RNA chaperone Hfq, FsrA cooperates with one, two, or three Fur-regulated small basic proteins, FbpA, FbpB, and FbpC, suggested to be RNA chaperones to repress translation of its different targets.[16] Under iron-limited growth conditions, both FsrA and FbpB (48 aa) inhibit translation of *lutABC* encoding iron-sulfur-containing oxidases, which allows iron to be directed to higher-priority target proteins.[26] FsrA is predicted to target the region upstream of and including the RBS, while FbpB might facilitate FsrA/l*utABC* RNA pairing or recruit the RNA degradation machinery.[26] Thereby, FsrA is the main regulator, as its modest overexpression can bypass the need for FbpB.[26] The authors suggest that FsrA uses a C-rich single-stranded region to interact with the ribosome binding sites of its different target mRNAs.[25]

In 2009, it has been found that the expression of BsrF (115 nt) is controlled by the global regulator CodY that responds to branched chain amino acids and GTP.[27] However, no BsrF targets have been identified so far. In 2011, CsfG, a sporulation-specific, non-coding sRNA highly conserved in endospore formers was found.[28] Its target has not yet been determined. The same holds true for a number of non-coding sRNAs that are under sporulation control, which were discovered in 2006[29] and further analyzed later on.[30] Among them, SurC is transcribed under control of σ^K and is conserved in the distantly related *B. anthracis*.[29]

SR1—The first dual-function sRNA in *Bacillus subtilis*

The small RNA SR1 was discovered using a bioinformatics approach for sRNAs in intergenic regions of the *B. subtilis* chromosome combined with northern blotting.[15] SR1 comprises 205 nt and is encoded between *pdhD* and *speA*. Knockout and overexpression of *sr1* were not detrimental for *B. subtilis* growth. Using 2D gel electrophoresis and northern blotting with a wild-type and an *sr1*-knockout strain, two arginine catabolic operons—*rocABC* and *rocDEDF*—were identified as secondary targets whose expression is upregulated in an *sr1*-knockout strain. The mRNA encoding

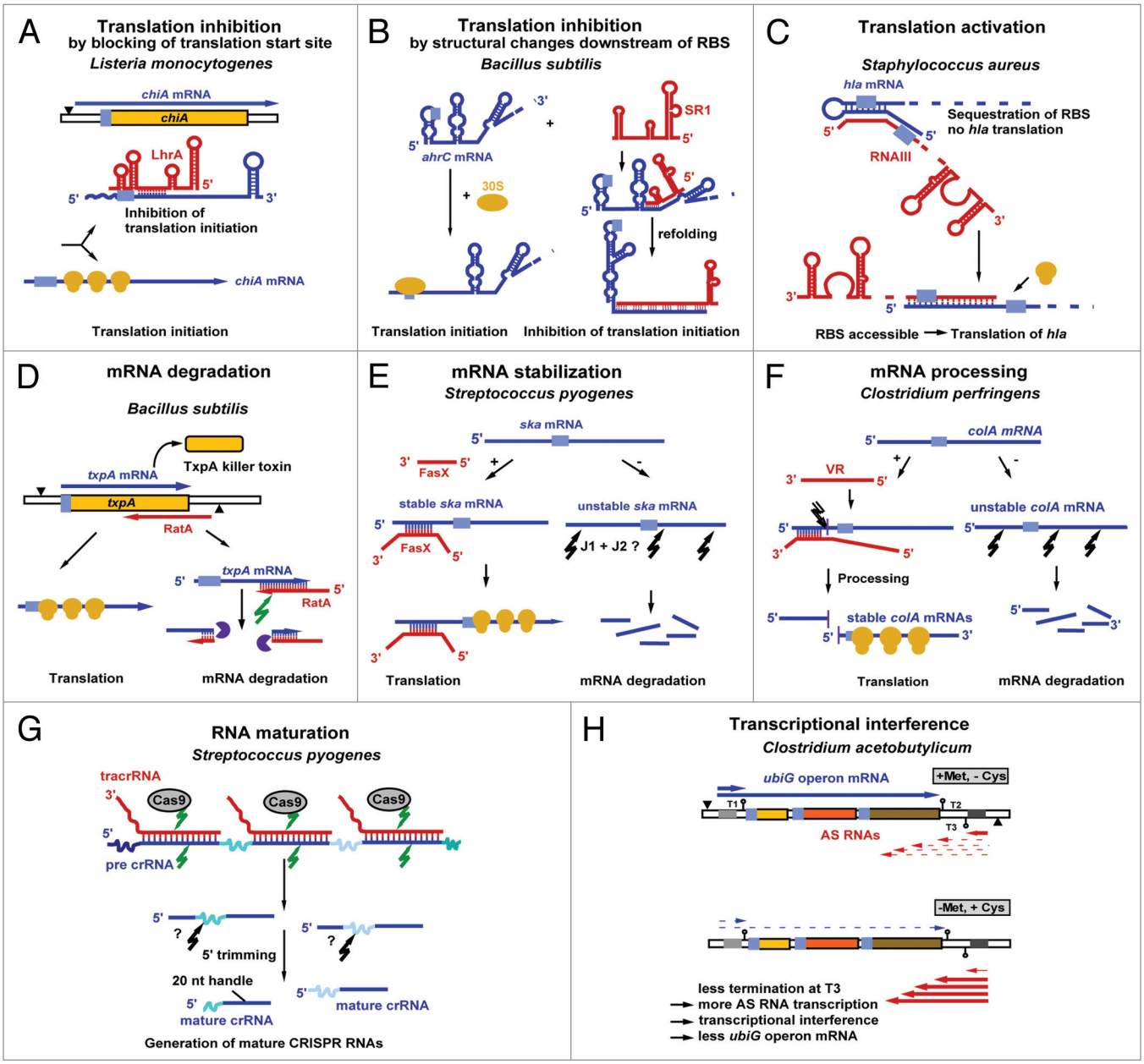

Figure 1. Mechanisms employed by sRNAs encoded from low-GC Gram-positive bacteria. All currently known mechanisms for sRNAs encoded from chromosomes are summarized. For additional mechanisms employed by plasmid-encoded sRNAs, see reference 13. Antisense RNAs are drawn in red, sense RNAs in blue. Black triangles denote promoters. Light blue, ribosome binding sites (RBS). Yellow symbols indicate ribosomes. Green arrows denote RNase III cleavage; black arrows indicate unknown RNase action. The violet symbol represents RNase R. For details, see text. B, C, E, F, and H are based on reference 13.

AhrC, the transcription activator of these operons, is the primary target of SR1.[31] Both RNAs share seven regions of complementarity in the central part of *ahrC* and the 3′ half of SR1 (**Fig. 2A**). SR1 does not affect the stability or amount of *ahrC* mRNA, but inhibits its translation by a novel mechanism:[32] Although it binds ≈100 nt downstream of the *ahrC* RBS, it induces structural changes 20–40 nt downstream of the *ahrC* RBS that inhibit translation initiation (**Fig. 1B**). This was shown by secondary structure probing of the *ahrC*/SR1 complex and toe-printing studies.[32] SR1 is only expressed under gluconeogenic conditions and 20–30-fold repressed by CcpN, and, to a minor (3-fold) extent, CcpA, under glycolytic conditions.[15] CcpN binds upstream of and overlapping the *sr1* promoter,[33] whereas CcpA binds 250 upstream of the transcription start site (TSS) at a *cre* site (**Fig. 2A**). CcpN represses *sr1* transcription in the presence of ATP and slightly acidic pH (6.5)[34] by interacting with the α-subunit of the RNA polymerase, thereby inhibiting promoter escape.[35] Transcriptomics and northern blotting suggested a second target for SR1, the *gapA* operon. In the presence of SR1, *gapA* mRNA is stabilized, whereas it is barely detectable in the absence of SR1 under gluconeogenic conditions.[21] We

Table 2. Overview of cis-encoded sRNAs from low-GC Gram-positive bacteria

Antisense RNA/ Target-RNA	Length of sRNA (nt)	Species	Biological function	Mechanism	Specific characteristic
RatA/*txpA*	222	*B. subtilis*	antitoxin/toxin	RD	
SR4/*bsrG*	180	*B. subtilis*	antitoxin/toxin	RD + TI	*bsrG* temperature dependent
SR5/*bsrE*	163[b]	*B. subtilis*	antitoxin/toxin	?	
antibsrH/*bsrH*[c]	≈202	*B. subtilis*	antitoxin/toxin[c]	?	
antiYonT/*yonT*[c]	≈80	*B. subtilis*	antitoxin/toxin[c]	?	
ECF/*yab*	750	*B. subtilis*	autolysin	RD*	σ^X, σ^M
SprA1AS/SprA1	60	*S. aureus*	antitoxin/toxin	RD	SprA1 is additionally a cytolisin and acts on human erythrocytes
RsaOX/*sa0062**	129	*S. aureus*	transposase?	RD*	
RsaOW/*is1181*		*S. aureus*	transposase?	?	8 RsaOW copies
p3 RNA/*glnA*	43	*C. acetobutylicum*	N metabolism	?	N-induced (p3)
Antisense/*ubiG*	264/424/730/1000	*C. acetobutylicum*	S metabolism	TINF	S-box riboswitch

[a]Renamed (Jahn and Brantl, unpublished). [b]Length determined (Maiwald, Jahn, Brantl, unpublished). [c]Not yet demonstrated in Bacillus that indeed TA system. RD, RNA degradation; TI, translation inhibition; TINF, transcriptional interference; *, mechanism proposed but not experimentally substantiated; ?, no mechanism proposed.

demonstrated that the 39 aa peptide encoded by SR1, SR1P, is responsible for this effect: SR1P binds to GapA (glyceraldehyde-3P dehydrogenase A), thereby stabilizing *gapA* operon mRNA by a hitherto unknown mechanism.[21] Consequently, SR1 is a dual-function sRNA: It acts as a base-pairing regulatory RNA on *ahrC* RNA, and as a peptide-encoding mRNA on the *gapA* operon.

Recently, a computer prediction revealed SR1 homologs in 23 other species belonging to the *Bacillales*.[36] The expression of the SR1 homologs in seven of these species was experimentally verified. Furthermore, the ability of SR1P homologs to bind *B. subtilis* GapA was investigated in northern blots and co-elution experiments, and the interaction between SR1 homologs and the corresponding *ahrC* homologs studied in vitro.[36] The results demonstrated that both functions of SR1, the base-pairing and the peptide-encoding function, are remarkably conserved over ≈1 billion of years of evolution. **Figure 2A** represents the two functions of SR1.

To elucidate the interaction surface of SR1P and GapA, a series of peptide mutants were constructed and analyzed in northern blotting and co-elution experiments. To verify the predicted binding regions, both protein-coding genes have to be mutated and expressed simultaneously and independently in *B. subtilis*. For this purpose, the pMG vector family, a series of modular plasmids suitable for chromosomal integration and gene expression under single copy conditions in *B. subtilis,* was constructed.[37] Preliminary data show that for the stabilizing effect of GapA/SR1P, only part of the *gapA* operon mRNA must be present, and this sequence must contain sequences adjacent to the *gapA* ORF (Gimpel, unpublished).

Recently, we observed that the amount of SR1 is significantly reduced after an 18 °C cold-shock. This was due to altered transcription initiation and not RNA degradation. Upon investigation of the cold-shock effect, we discovered a novel trigger enzyme (Preis et al., unpublished).

Future work will be aimed at the investigation of the molecular mechanism behind the SR1P–GapA interaction, in particular, the determination of the SR1P binding pocket and properties as K_D-value or stoichiometry of the interaction. Moreover, the biological role of SR1P-GapA interaction will be elucidated.

Cis-encoded sRNAs

With the exception of ECF RNA (see below), only cis-encoded sRNAs that act as type I antitoxins have been identified so far in *B. subtilis*. Currently, five types of toxin-antitoxin systems (TA systems) are known (reviewed in ref. 38). In type I TA systems, the antitoxin is a small RNA, and the toxin mRNA encodes a hydrophobic peptide. RNA antitoxins are cis-encoded regulatory RNAs that interact with their target RNAs either at their 5′ or 3′ end by a base-pairing mechanism. In *Bacillus subtilis*, 14 type I TA systems have been postulated, and three of them verified experimentally: *txpA*/RatA,[39] *bsrG*/SR4,[40] and *yonT*/as-yonT.[41,42] The majority of them are located on prophage elements or phage remnants in the chromosome.

RatA—The first identified RNA antitoxin from B. subtilis

In 2005, *txpA*/RatA was identified as the first type I TA system on the *skin* element of the *B. subtilis* chromosome.[39] In the absence of RatA, TxpA causes cell lysis on agar plates. The RatA RNA is 220 nt long and overlaps the 3′ end of *txpA* mRNA by ≈120 nt.[39] The interaction between *txpA* mRNA and RatA promotes the degradation of *txpA* mRNA by an RNase III-dependent mechanism.[42] Degradation of *txpA* mRNA by RNase III is essential for viability of *B. subtilis*.[42] The secondary structures of RatA and *txpA* RNA, as well as their complex, have been determined.[42] The ribosome binding site (RBS) of *txpA* is located in a 5 bp ds region. This sequestration is not altered upon RatA binding.

SR4—An RNA antitoxin with two functions

The *bsrG/sr4* module is located on the SPβ prophage of the *B. subtilis* chromosome. We corroborated experimentally that

Table 3. Overview of trans-encoded antisense RNAs from low GC Gram-positive bacteria

sRNA (nt)	target RNA(s)	Biological function	Mechanism of action	Control of expression/ specific characteristic
Bacillus subtilis				
SR1 (205)	*ahrC*	arginine catabolism	TI	CcpN, CcpA
	gapA operon*	sugar metabolism	SR1P/GapA*	
FsrA (84)	*sdhCAB, citB*	succinate dehydrogenase	TI	Fur, iron;; some targets need FbpA, B or C
	gltA	glutamate synthase (iron-sulfur)		
	lutABC	iron-sulfur oxidase		
	dctP	dicarboxylate permease		
	yvfW, leuCD			
RsaE (114)	*cstA*[a], *sucC*[a], *galK*[a] central metabolism[a]		TI	exponential phase
Staphylococcus aureus				
RNAIII (514)	*hla*	α hemolysin	TA	AgrC/AgrA, stationary phase
	rot	repressor of toxins Host-pathogen interactions	TI[b] and RD	
	sa1000/sa2261	fibrinogen BP, ABC transporter		
	spa	protein A		
	coa	coagulase		
	lytM	peptidoglycan hydrolase		
	map	MCH class II analogous protein	TA?	
RsaE (96)	*opp-3B/3A, sucC, sA0873*	central metabolism	TI	pre-stationary phase, heat-shock
SprA (202)	*sa2216*[#]	ABC transporter?	Post-translational?	strain-specific
SprD (142)	*sbi*	immune response	TI, RD	growth phase
Psm-mec (157)	*agrA*	virulence regulator	TI, RD independent of TI	
Art A (345)	*sarT*	transcriptional regulator of *hla*	RD	ArgA
Streptococcus pyogenes				
Pel RNA (459)	*speB,*	cysteine protease	post-transcriptional	multiple transcription regulators and conditioned media
	emm, sic, nga	M- and -related proteins	transcriptional control	
FasX (205)	*ska*	streptokinase	RNA stabilization	FasBCA, *luxS*
	sagS	streptolysin	?	aa starvation
	fbp54	fibronectin binding protein	?	
	mrp	fibrogen binding protein	?	
RivX (289,237,189)	*mga*	virulence	TA?	CovR/CovS
tracrRNA (171, 89)	*crRNA* precursor	CRISPR maturation	RNA processing	Csn1[b]

Only sRNAs are listed for which target genes have been identified or #proposed. [a]In analogy to *S. aureus* RsaE, which interacts via C-rich loops with SD sequences, targets have been predicted. [b]Required for processing of the sRNA/target RNA duplex by RNase III *SR1 acts on *gapA* as a peptide encoding mRNA, i.e., SR1P interacts with GapA, thereby stabilizing *gapA* mRNA. TI, translation inhibition; TA, translation activation; RD, mRNA degradation. Control of expression: All proteins and growth conditions known to regulate sRNA expression are listed. It is not indicated whether these factors promote or inhibit sRNA expression. For more details, see see text. RS, riboswitch.

this module acts as a type I toxin/antitoxin system:[40] The *bsrG* RNA is 294 nt long and codes for a 38 aa toxic peptide with a predicted central transmembrane domain and a charged C terminus (see ref. 41). The antisense RNA SR4 is 180 nucleotides long and overlaps the 3′ end of *bsrG* by 123 nucleotides. The interaction of both RNAs at their 3′ ends promotes degradation of *bsrG* RNA

Table 3 (continued). Overview of trans-encoded antisense RNAs from low GC Gram-positive bacteria

sRNA (nt)	target RNA(s)	Biological function	Mechanism of action	Control of expression/ specific characteristic
S. pneumoniae				
srn206 (120)	*comD*	histidine kinase, competence	TI?	
csRNA1–5 (87–151)	*comC*	competence	TI?	All 5 homologous sRNAs are regulated by CiaRH
	spr0081	ABC transporter		
	spr0159	DNA binding protein		
	spr0551	branched chain aa transport		
	spr1097	formate/nitrite transporter		
Clostridium perfringens				
VR (386)	*pfoA, vrr, virT, virU, ccp*	collagenase, toxin genes	RNA processing	VirR/VirS
	CE1446/CE1447		TA?	
VirX(?)	*pfoA, plc, colA*	α, κ, τ enterotoxin production	TA?	Independent of VirR/VirS
	spo0A σ^E, σ^F, σ^K	sporulation		Encodes 51 aa peptide VirX
Listeria monocytogenes				
LhrA (268)	*lmo0850*	protein of unknown function	TI and RD	Hfq required for stability and target binding
	chiA	two chitinases	TI	
	lmo0302	protein of unknown function	TI	
SreA (228)	*prfA*	virulence master regulator	TI	SAM RS, blood
	lmo0049 = agrD	quorum sensing molecule	TA?	
SreB (179)	*prfA, lmo2230*	arsenate reductase homol.	?	
RilB	*lmo2104/2105*#	iron transport proteins	?	
RilE	*comEA/FA, lmo0945*#	competence factors	?	
RliI	*lmo1035*#	phosphotransferase system	?	
Rli23, 25, 35	*lmo0172*#	transposases		
Rli45	*rli46*#		?	
Rli29	*lmo9471*#		?	
Rli30	*lmo0506*#		?	

Only sRNAs are listed for which target genes have been identified or #proposed. [a]In analogy to *S. aureus* RsaE, which interacts via C-rich loops with SD sequences, targets have been predicted. [b]Required for processing of the sRNA/target RNA duplex by RNase III *SR1 acts on *gapA* as a peptide encoding mRNA, i.e., SR1P interacts with GapA, thereby stabilizing *gapA* mRNA. TI, translation inhibition; TA, translation activation; RD, mRNA degradation. Control of expression: All proteins and growth conditions known to regulate sRNA expression are listed. It is not indicated whether these factors promote or inhibit sRNA expression. For more details, see text. RS, riboswitch.

(**Fig. 2B**). RNase III cleaves the *bsrG* RNA/SR4 duplex at position 185 of *bsrG* RNA, 8 nt downstream from the stop codon, but is not involved in the degradation of either *bsrG* RNA or SR4 alone.[40] Endonuclease Y and the 3′-5′ exoribonuclease R are responsible for further degradation of both RNAs. PNPase processes SR4 precursors into the mature RNA. As a Δrnc suppressor strain, neither lysed on agar plates nor had mutations in the *bsrG* ORF, RNase III is not essential to the function of the *bsrG*/SR4 system.[40] Later it was found that the Δ*rnc* suppressor strain has a deletion of the *skin* prophage and a tendency to loose SPβ in order to reduce TxpA and YonT toxicity.[41] Hfq is not required for the function of the *bsrG*/SR4 system,[40] since a Δ*hfq* strain does neither show lysis nor altered half-lives of *bsrG* RNA or SR4.

Recently, the secondary structures of SR4 and *bsrG*-RNA, as well as the SR4/*bsrG* RNA complex, were experimentally determined. The results demonstrated that SR4 induces structural alterations around the RBS of *bsrG*[43] (**Fig. 2B**): A 4 bp region that sequesters the *bsrG* RBS is extended to 8 bp in the presence of SR4. It was shown experimentally that this extended double-stranded region inhibits translation.[43] Consequently, SR4 is the first type I antitoxin with two clearly separable functions: it promotes degradation of *bsrG* mRNA and impedes ribosome access to the *bsrG* SD, thereby preventing translation. Complex formation assays with wild-type *bsrG* RNA/SR4 yielded an apparent binding constant k_{app} of 6.5×10^5 M^{-1} s^{-1},[43] which is in the same order of magnitude as those of other *cis*-encoded sense/antisense RNA pairs.[44] The binding pathway of *bsrG* mRNA and SR4 was elucidated: Binding starts with a single loop–loop contact between loop 3 of *bsrG* RNA and loop 4 of the SR4 terminator stem-loop. Intermolecular base-pairing progresses via

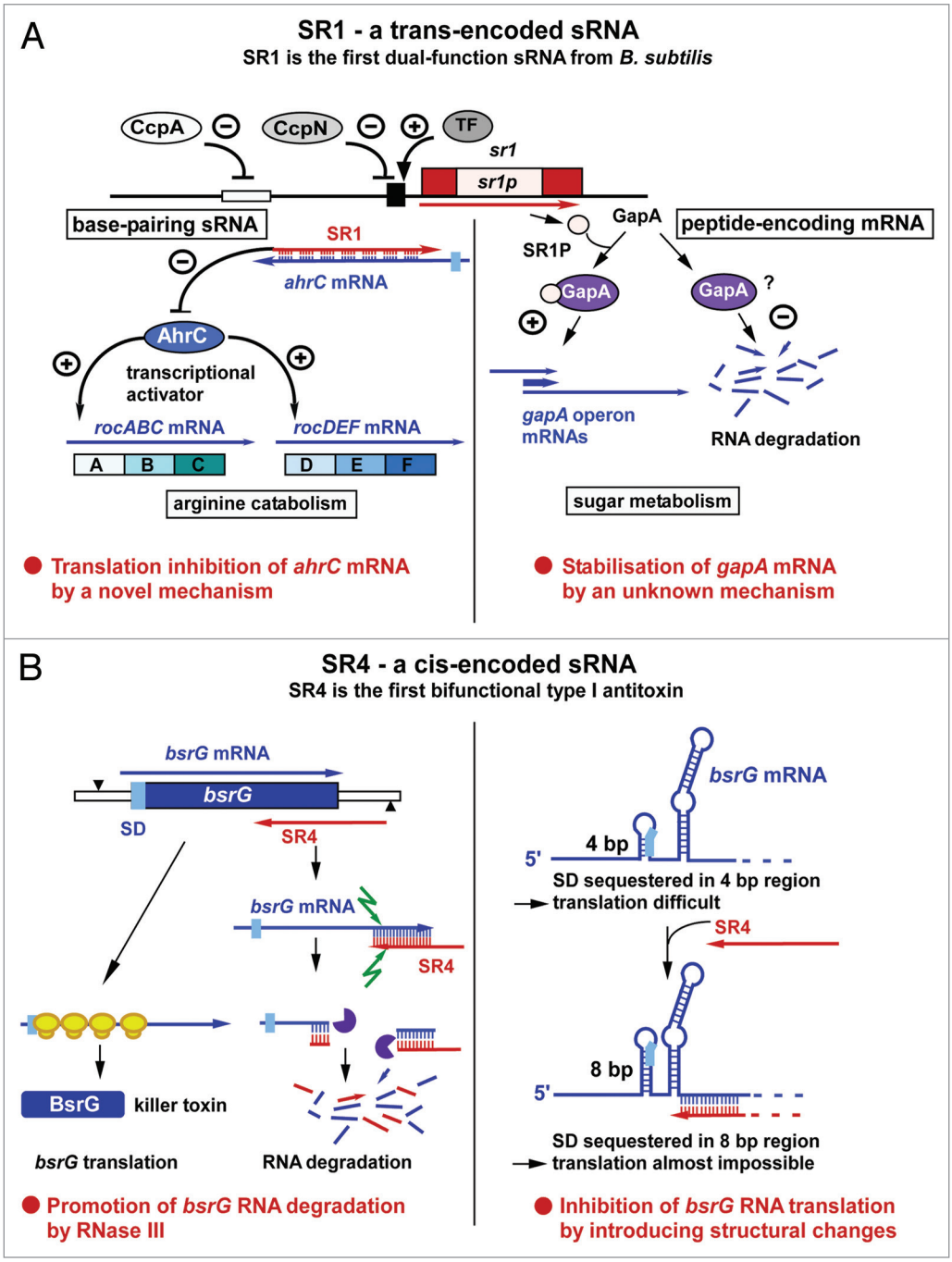

Figure 2. SR1 and SR4, a trans- and a cis-encoded sRNA from *B. subtilis*. As in **Figure 1**, the antisense RNAs are indicated in red, the sense RNAs in blue, RBS in light blue, ribosomes are in yellow, RNase III in green, and RNase R in violet. (**A**) SR1, a trans-encoded sRNA, is the first identified dual-function sRNA from *B. subtilis*. +, activation; -, repression. CcpA and CcpN repress *sr1* transcription under glycolytic conditions. TF is a novel transcription factor that activates *sr1* transcription at cold-shock. (**B**) SR4, a cis-encoded sRNA, is the antitoxin of the type I TA system *bsrG*/SR4. It is the first antitoxin for which two independent functions have been found. For details, see text.

the single-stranded region between L4 and L3 toward loop 3 of SR4, and, finally to loop 2, which pairs with the *bsrG* terminator-stem-loop. However, the latter interaction was not required for efficient binding.[43] Loop 3 of *bsrG* RNA contains a 5′ YUNR motif, which apparently forms a U-turn that provides a scaffold for the efficient initial interaction between both RNAs.[43] The *sr4* promoter is about 6- to 10-fold stronger than the *bsrG* promoter,[40] which should result in an excess of the antitoxin over the toxin, as was shown for *txpA*/RatA.[42]

Other cis-encoded sRNAs in *B. subtilis*

A recent review summarizes the current knowledge about other cis-encoded sRNAs that act as type I antitoxins in *B. subtilis*.[41] Among them is *yonT80*, which is regulated by as-yonT, and was recently confirmed to be a toxin in *E. coli*, and indirectly in

B. subtilis.[42] Furthermore, BsrE, regulated by as-BsrE (renamed SR5) acts as a toxin in *B. subtilis* (Maiwald, Jahn, and Brantl, unpublished).

The only other cis-encoded sRNA known to date is the long (750 nt) ECF RNA, which is expressed under control of extracytoplasmic sigma factors σ^X and σ^M and was found to regulate an autolysin encoded by the *yabE* gene.[45]

A transcriptome analysis indicated a widespread antisense transcription in bacterial chromosomes: Using micorarrays, for 2.9% of all *B. subtilis* genes, antisense transcripts were detected,[2] and a dRNA Seq approach found in total 29 cis-encoded antisense RNAs.[3] To date, the biological role of these antisense RNAs is unknown.

In *Staphylococcus, Streptococcus, Listeria*, and *Clostridium* species, mostly sRNAs implicated in the regulation of pathogenesis and virulence, but also a few metabolic riboregulators, have been discovered and investigated in more detail.

sRNAs from *Staphylococcus aureus*

Meanwhile, six different approaches have identified about 100 trans- and 100 cis-encoded sRNAs in the human pathogen *S. aureus* (**Table 1**). The majority of the hitherto characterized sRNAs are involved in pathogenesis. The most prominent and best-characterized of them, which was already discovered in 1995, is the unusually long RNAIII (514 nt).[18] Transcription of RNAIII is induced by AgrA, the response regulator of the quorum-sensing *agr* (accessory gene regulation) system, and RNAIII is the effector of this system. Its secondary structure was mapped in vitro and in vivo and revealed 14 hairpin structures, out of which the conserved hairpin 13 was involved in repression of protein A expression.[46] RNAIII was the first antisense RNA for which an activating function has been found[18] (**Fig. 1C**). However, it does not only activate translation of the α-hemolysin mRNA, but also inhibits translation of a variety of targets:[47] *spa* (main surface adhesin protein), SA1000 (novel fibrinogen-binding protein), *sa2261* (ABC transporter), *rot* (pleiotropic transcriptional factor Rot[48]), *lytM* (peptidoglycan hydrolase), and coagulase mRNA.[49] To exert its inhibitory effect, RNAIII employs a combination of translation inhibition by a base-pairing interaction of its two redundant 3′ hairpin loops with the target mRNA and recruitment of RNase III for target degradation.[50] As shown for *spa*, RNA degradation was required for permanent translational arrest. Specificity for RNAIII on all translationally inhibited targets is obtained by either propagating the first loop–loop contact at the RBS into the stem regions (*sa1000* and *sa2353* mRNAs) or by addition of a second loop–loop interaction (*rot* and *coa* mRNAs). The RNAIII/*coa* mRNA duplex comprises an imperfect duplex masking the SD sequence and a loop–loop interaction in the *coa* ORF. The imperfect duplex is sufficient to prevent translation initiation. RNase III cleaves the two regions of the *coa* mRNA bound to RNAIII that may contribute to the degradation of the repressed mRNA.[49] Interestingly, RNAIII represents the first identified dual-function sRNA as it is on the one hand a regulatory sRNA that acts by base-pairing and on the other hand a protein-encoding mRNA with an ORF for the 26 aa δ-hemolysin.[18]

The second dual-function sRNA in *S. aureus*, Psm-mec (157 nt), was discovered only in 2013.[19] The *psm-mec* gene is located on the mobile genetic element SCCmec that confers—via *mecA*—methicillin resistance to MRSA strains. Psm-mec encodes the 22 aa-secreted cytolytic toxin PSMα (α phenol-soluble modulin). Additionally, it inhibits translation of *agrA* mRNA by base-pairing of its 5′ nt 21–50 with the *agrA* coding sequence (most important are nt 199 to nt 267). Furthermore, it causes a ≈2-fold RNase III-dependent decrease in *agrA* half-life, which was—in contrast to the inhibitory effect of RNAIII on *rot* mRNA—independent of the translation inhibition effect. Psm-mec RNA itself is stable (half-life 20 min) and not affected by *agrA* mRNA. AgrA activates transcription of RNAIII and of the *psm*α 3 operon. Twenty-five percent of 325 analyzed clinical isolates (HA strains) have a promoter mutation that causes attenuated *psm-mec* transcription, and 9% have no *psm-mec*, which results in high virulence. By contrast, community-acquired MRSA strains (CA strains) have no Psm-mec. Kaito et al. proposed that Psm-mec sRNA attenuates virulence in HA comparison to CA strains.[19]

Recently, a second AgrA-regulated sRNA, ArtA, was discovered that controls α-toxin expression by targeting the 5′ UTR of *sarT* mRNA.[51]

In 2005, a search in the clinical *agr* negative *S. aureus* strain N315 resulted in seven experimentally confirmed small pathogenicity islands RNAs = Spr (SprA to SprG), among which SprA, E, F, and G were present in multiple copies, partly also on the core genome.[52] For SprA (202 nt), in vitro data suggested an interaction with three putative mRNA targets, among them a 3.5 kb ABC transporter mRNA.[52] However, in 2012 it was found instead that SprA1 encodes a 30 aa peptide toxin, which inserts into the cell membrane and kills *S. aureus* cells, and is regulated by a cis-encoded antisense RNA (SprA1AS) that facilitates degradation of SprA1 mRNA. Therefore, SprA1/SprA1AS constitutes a type I toxin-antitoxin system.[53] SprA1AS combines—similar to RNAI from *E. faecalis* plasmid pAD1 (reviewed in ref. 13)—features of a cis- and a trans-encoded sRNA.[54] Surprisingly, the SprA1 peptide was previously also found to act as cytolysin on human erythrocytes.[54] In 2010, SprD (142 nt), another sRNA of the first search, was shown to inhibit translation initiation of the abundant secreted immune-evasion protein Sbi by an interaction between its central region and the 5′ 41 nt of *sbi* mRNA, including RBS and start codon.[55] In contrast to RNAIII and Psm-mec, SprD did not affect the *sbi* mRNA levels. In an animal septicaemia model, SprD impaired both adaptive and innate host immune responses.[54]

The first staphylococcal sRNA involved in metabolic regulation, RsaE, was discovered in 2009[17] among 11 novel Hfq-independent sRNAs (RsaA–RsaK). RsaE (96 nt) is highly conserved in four differential *S. aureus* species and was also found in *Macrococcus* and *Bacillus*.[17] It co-regulates several metabolic pathways involved in amino acid and peptide transport, cofactor synthesis, carbohydrate and folate metabolism, and TCA cycle by inhibiting translation of two cistrons of an oligopeptide transporter operon, *opp-3A*[56] and *opp-3B*,[17] and *sucC/sucD*-encoding succinyl-CoA synthetase subunits α and β, and by targeting

sA0873.[56] Expression of *rsaE* is AgrA-dependent and very low in pre-stationary phase. Similar to *B. subtilis* FsrA and *S. aureus* RNAIII, RsaE seems to recognize its target mRNAs at the RBS via a conserved C-rich loop (UCCC motif). In another screen for sRNAs, RsaOX (129 nt) and RsaOW were proposed to target the transposase genes *sa0062* and *is1181*, respectively, possibly by promoting RNA degradation.[56]

Recent tiling arrays revealed long antisense RNAs that are rapidly degraded by RNase III.[57] It is suggested that such long sRNAs might play an important role in staphylococcal gene regulation, in particular, of genes involved in pathogenesis and virulence. One example is SSR42 (891 nt) that affects erythrocyte lysis and pathogenesis in a murine infection model.[58] SSR42 is stabilized in stationary phase, upregulates genes involved in capsule biosynthesis, and downregulates ≈80 genes, among them, virulence factors. However, its role seems to be indirect as no SSR42 binding to the corresponding mRNAs was found.[58] A summary of all kinds of regulatory RNAs hitherto found and investigated in *Staphylococcus areus* and their biological functions has been published recently.[59]

sRNAs from *Clostridium*

In 2013, RNA-seq approaches in three *Clostridium* species discovered between 36 and 182 sRNAs (**Table 1**). So far, for only a few of them, targets are known. Already > 20 y ago, a cis-encoded antisense RNA was found in the biotechnologically important *Clostridium acetobutylicum*, which is involved in control of nitrogen metabolism by interacting with the 5′ UTR of the glutamin synthetase gene *glnA*.[60,61] However, no further reports were published on this issue. In the same species, four antisense RNAs were discovered in 2008 that are encoded downstream of the *ubiG* operon and act in concert with an S-box riboswitch to regulate sulfur metabolism,[62] **Figure 1H**. These long antisense RNAs (between 200–1000 nt) represent the so-far-unique example for transcriptional interference as mechanism of action of base-pairing sRNAs. In the human pathogen *Clostridium difficile*, a genome-wide association study identified 94 trans-encoded sRNAs and 91 cis-encoded sRNAs, and confirmed 35 of them experimentally.[7]

In the food-born pathogen *Clostridium perfringens*, two sRNAs have been characterized in more detail: The sRNA VR is part of the VirR/S regulon that controls toxin production and induces collagenase (K-toxin) and b2-toxin synthesis,[63] **Figure 1F**. VR has been shown to regulate five genes by direct binding to their mRNAs: *pfoA, vrr, virT, ccp*, and *virU*. Additionally, VR positively affects synthesis of CPE1447 and CPE1446, which form a protein heterodimer that controls toxin gene expression.[64] The function of the small ORF encoded on VR is still unclear. In 2013, another sRNA, VirX, which had been shown before[65] to regulate *pfoA, plc*, and *colA* mRNAs independent of the VirR/VirS system has been found to repress genes encoding positive sporulation regulators like Spo0A and sigma factors E, F, and K.[66] Inactivation of *virX* led to higher levels of sporulation and enterotoxin production. Data on sRNAs in Clostridium species available until 2011 have been summarized.[67]

sRNAs from *Listeria*

Although five different approaches have discovered sRNAs in *Listeria monocytogenes* and *L. innocua* (**Table 1**), only a few targets have been identified so far. One screen for Hfq-binding sRNAs in *L. monocytogenes* identified LhrA, LhrB, and LhrC.[68] LhrA is stabilized by Hfq and targets at least three mRNAs directly, *chiA* mRNA encoding two chitinases and two genes of unknown function (*lmo0850* and *lmo0302*).[69,70] In a global screen, 300 genes were found to be affected by LhrA. In the presence of Hfq, LhrA inhibits translation of *chiA* mRNA (**Fig. 1A**).[70] LhrA is, so far, the only example from Gram-positive bacteria for which an effect of the RNA chaperone Hfq on target binding was found[69] (see below). However, the majority of the sRNAs from Listeria do not seem to need Hfq for stabilization or interaction with their targets (reviewed in ref. 69). In the case of *lmo0850*, LhrA both inhibits translation and promotes RNA degradation. For a few recently discovered sRNAs, targets were predicted, but their mechanism of action on them is still elusive:[71] RliB is proposed to target *lmo2104/5* involved in iron transport, RliE the competence factors *comEA/FA,* and *RliL* a phosphotransferase system (*lmo1035*). The absence of 15 of the 29 sRNAs recently found in *L. monocytogenes*[72] in the non-pathogenic *L. innocua* underlines the importance of riboregulators for pathogenesis and virulence. Rli38 from *L. monocytogenes* is 25-fold higher expressed in blood and in the presence of H_2O_2, i.e., under oxygen stress.

In RNaseq approaches, unusually long antisense RNAs (las RNA) complementary to more than one ORF or operon were found in *Listeria* species. The authors designated such a genomic locus excludon.[72,73] Thereby, the 5′ or 3′ non-coding part of a lasRNA negatively affects the expression of one or several gene(s) on the complementary strand, whereas the remaining (major) part functions as mRNA for the downstream or upstream genes. The first reported excludon in *L. monocytogenes* controls flagellum biosynthesis at the motility gene repressor locus. MogR is the transcriptional repressor of flagellum and motility genes in *Listeria* species, and *mogR* is transcribed from a promoter 45 nt upstream of the AUG. In opposite direction, the flagellum operon with *lmo0675* (unknown), *lmo0676* (fli), *lmo0677* (fliQ), and *lmo0678* (fliR) is transcribed. FliP and FliQ form the flagellum export apparatus. Additionally, transcription initiated at a third promoter upstream of p_{mogR} results in an excludon transcript, Anti0677, whose 5′ region is antisense to *lmo0675-lmo0677*, and whose 3′ part contains the *mogR* ORF. Anti0677 is under control of stress-activated σ^B located within the *lmo0677* ORF. Other recently summarized examples[73] comprise two putative permease-efflux pump excludons and a putative carbon source utilization excludon. However, the detailed mechanism of action of the proposed lasRNAs has not yet been elucidated.

In 2009, two trans-acting S-adenosylmethionine riboswitches (SreA and SreB) that can function as trans-encoded sRNAs were discovered in *L. monocytogenes*.[74] SreA upregulates *argD* and represses translation of *prfA* encoding the virulence master regulator by base-pairing upstream of the RBS. SreB (179 nt) regulates *prfA* and *lmo2230* (arsenate reductase homolog).[74] By targeting PrfA, SreA and SreB link virulence to nutrient availability.

sRNAs from *Streptococcus*

Streptococci include species that cause severe human diseases. In the group A (GAS) *Streptococcus pyogenes*, 75 sRNAs were identified (**Table 1**), and three of them were implicated in virulence control: Pel, FasX and RivX. The 450 nt long bifunctional sRNA Pel regulates M- and M-related proteins and codes for streptolysin S (SLS).[20] It exerts pleiotropic effects on virulence. Pel expression is repressed both by a multitude of transcription factors, in fact CcpA, CovRS/CsRS, LuxS, Mga, Nra, and RopB/Rgg, and, additionally, by FasX RNA, but activated at amino acid starvation by CodY, Irr, and SLS itself.

FasX (≈200 nt) is encoded in the *fasBCAX* operon and transcriptionally activated by response regulator FasA. FasX stabilizes the *ska* mRNA encoding the secreted plasminogen activator streptokinase[75] (**Fig. 1E**). It also controls *sagS* mRNA encoding streptolysin S (see above) and, by unknown mechanisms, transcription of *fbp54* mRNA and *mrp* mRNA encoding two fibronectin-binding proteins. In *S. dysgalactiae ssp. equisimilis* (group C streptococci GCS), FasX also affects *ska* and streptolysin.[76]

The third recently found sRNA, RivX, is processed from a longer mRNA-encoding transcription regulator RivR and has, thus, three alternative 5′ ends resulting in 189, 237, 289 nt-long species. Together with transcription factor RivR, RivX upregulates the global virulence gene regulator *mgA* by enhancing its translation.[23] Mga itself controls *emm*, C5a peptidase, and cysteine protease *speB*. As mentioned above, RivX contains a 23 codon ORF of unknown function.

In 2011, tracrRNA (89 nt), a trans-encoded sRNA involved in maturation of CRISPR RNA was discovered.[12] CRISPR protects its host against prophage-derived DNA. In type II CRISPR systems, tracrRNA induces—together with Cas9 and RNase III—cleavage of pre-crRNA to yield mature crRNA (**Fig. 1G**), which, upon phage infection, can target phage DNA. By probing selected loci, functional tracrRNA homologs were also found in *Streptococcus mutans*, *S. thermophiles,* and *Listeria innocua*, and even the Gram-negative *Neisseria meningitidis*.[12]

Details about expression regulation and the involvement of streptococcal sRNAs in global networks controlling virulence and pathogenesis have been summarized recently.[77]

sRNAs from *Streptococcus pneumoniae*

In *Streptococcus pneumoniae,* far more than one hundred sRNAs have been identified mainly by high-throughput approaches (**Table 1**). One sRNA, *srn206*, has been implicated in competence control[78] and the target was suggested to be *comD*, the gene encoding the histidine kinase of the two-component regulatory system ComDE, which is essential for initiation of competence development.[79] Several sRNAs were shown to be involved in the control of various aspects of virulence and a number of differentially expressed proteins were detected in sRNA mutants.[80] However, no direct regulatory link was established between sRNAs and putative targets. In addition, two-component regulatory systems appeared to be involved in sRNA expression control, but the underlying mechanism was not determined.[80] Despite the identification of numerous sRNAs in whole genome approaches, no clearly defined targets or regulatory mechanisms were determined so far.

More information is available for the first sRNAs described in *S. pneumoniae,* which have been detected in an analysis to define the regulon of the two-component regulatory system CiaRH.[81] The five strongest promoters in the CiaRH regulon were found to drive expression of sRNAs between 87–151 nt in size. These non-coding sRNAs, designated csRNAs (cia-dependent small RNA), show a high degree of similarity to each other. They are predicted to adopt a secondary structure with two stem-loops at the 5′- and 3′-ends, respectively. Sequences complementary to the SD sequence and the start codon AUG are present in the unpaired region between the stem-loops. The csRNAs appear to affect pneumococcal physiology pleiotropically. Stationary phase autolysis was affected by csRNA4 and csRNA5,[81] and a csRNA5 mutant was defective in lung infection.[80] Furthermore, csRNA1 was shown to act negatively on competence development.[82] The csRNAs were originally detected in *S. pneumoniae* R6,[81] but are found in all *S. pneumoniae* genome sequences available to date. Curiously, Hungarian *S. pneumoniae* serotype 19A isolates carry and express longer versions of csRNA5, which apparently arose by internal sequence duplication (R. Brückner, unpublished observations). Expression of CiaR-controlled csRNAs was also confirmed in *Streptococcus mitis*, *Streptococcus oralis, Streptococcus sanguinis,*[83] and *Streptococcus pyogenes*.[84] The presence of multiple csRNAs genes could be predicted in all streptococcal genomes[83] suggesting that they serve an important function in this group of organisms.

In a recent study, csRNA target predictions in *S. pneumoniae* R6 were evaluated by analyzing translational fusions of candidate genes.[85] Six targets could be identified, which were all downregulated by the csRNAs. At least for the three genes tested, each of the csRNAs could act upon the targets reflecting the high degree of csRNA similarity. Regulation by the csRNAs was additive, no single csRNA was as effective as all csRNAs together. Four of the regulated genes, *spr0081, spr0371, spr0551, spr1097*, encode transport proteins of various protein families, but their physiological roles in *S. pneumoniae* are currently unknown. A putative transcriptional regulator *spr0159* and *comC*, the gene encoding the precursor of the competence stimulating peptide CSP[86] were the remaining csRNA targets. Especially the identification of the latter was intriguing, since CiaRH was known to act negatively on competence development.[87,88] Mutation of *comC* between the SD sequence and the start codon partially disrupting complementarity to the csRNAs greatly diminished csRNA-mediated repression of a translational fusion. Replacing wild-type *comC* by the mutated version in the genome of *S. pneumoniae* R6 relieved competence from CiaRH-dependent control.[85] Therefore, the csRNAs block CSP precursor production thereby interfering with pheromone signaling that initiates competence.

Interestingly, CiaRH controls production of the serine protease HtrA, which is also able to act negatively on competence by degradation of CSP.[89] Which negative CiaRH-dependent control mechanism prevails, csRNA- or HtrA-mediated, depends strongly on the growth conditions.[85]

In addition to competence control, csRNAs are involved in another CiaRH-dependent phenotype. Mutations in the histidine kinase gene *ciaH* leading to a hyperactive CiaRH system[87,88] have been shown to increase β-lactam resistance. Without csRNAs, these CiaRH hyperactive strains are no longer resistant, but the target(s) involved in this phenotype has not yet been identified. Thus, the csRNAs certainly control at least one more target in *S. pneumoniae*.

Role of RNA Chaperones in sRNA-Mediated Gene Regulation in Low GC-Gram-Positive Bacteria

An important characteristic of many trans-encoded antisense RNAs from *E. coli* is their ability to bind the RNA chaperone Hfq (reviewed in ref. 90). Hfq is present in 50% of all sequenced bacterial species, and a few species like *Bacillus anthracis* encode even two Hfq proteins. Hfq is a homohexamer that is very similar to the eukaryotic Sm proteins involved in splicing.[90] It binds to AU-rich sequences in single-stranded regions flanked by one or two stem-loops. Among others, Hfq is involved in mRNA stability, polyadenylation, and translation.[90] In Gram-negative bacteria, the majority of trans-encoded sRNAs need Hfq either for their stability or for sRNA/target interaction (reviewed in ref. 10). In 2010, it was shown that sRNAs can displace each other on Hfq on a short time scale by RNA concentration-driven cycling.[91]

Currently, the only example for Hfq-dependent antisense regulation in Gram-positive bacteria is LhrA from *L. monocytogenes* (see above).[68,69] For two trans-encoded sRNAs in Gram-positive bacteria, *B. subtilis* SR1[32] and *S. aureus* RNAIII,[92] Hfq does not impact sRNA/target interaction. In the latter case, this was even tested in three virulent genetic backgrounds.[92] However, as *hfq* expression levels differ between strains, the role of Hfq in *S. aureus* is discussed controversially (e.g. ref. 93). By contrast, for the trans-encoded sRNA FsrA from *B. subtilis*, a role of other small putative RNA binding proteins that might act as RNA chaperones—in particular FbpB—has been proposed.[16,26] However, it has not yet been demonstrated experimentally that FbpA, B, or C indeed bind RNA. Interestingly, *Streptococcus* species do not encode Hfq. A study on the interchangeability of Hfq-like proteins between Gram-negative and Gram-positive bacteria demonstrated that neither *S. aureus* nor *Borrelia* Hfq expressed chromosomally in *S. enterica* Typhimurium from the location of endogenous Hfq could functionally substitute *Salmonella* Hfq in sRNA-mediated regulation and protection from degradation.[94] Future research will show whether the LhrA case is an exception and whether other, yet-unidentified RNA binding proteins function as additional RNA chaperones in Gram-positive bacteria. In addition to the Fbp proteins, the SMc01113 protein could be a possible candidate, as it alters sRNA/target mRNA accumulation in *Sinorhizobius meliloti*,[95] but is highly conserved and present in almost all bacteria, also those that lack Hfq.

Future Perspectives

In the near future, the multitude of newly discovered sRNAs in low-GC Gram-positive bacteria will be investigated in detail to identify their targets, to analyze their biological role, and to elucidate their mechanisms of action. It can be expected that novel mechanisms or such known so far only from plasmid-encoded antisense RNAs or from sRNAs in Gram-negative bacteria, will be found for these sRNAs. Likewise, one sRNA might act in cis on one target and in trans on one or several others, thereby employing different modes of action.

As only in one case, LhrA from *Listeria monocytogenes*, a role for Hfq has been established, it might well be that other chaperones will be detected that play equivalent roles in Gram-positive bacteria. First possible candidates are the small basic proteins FbpA, FbpB, and FbpC, which were linked to the function of *B. subtilis* FsrA (see above).

Like in *E. coli* or *Salmonella*, target mRNAs will be found that are regulated by different sRNAs. In this context, global regulatory networks will be uncovered that implicate both sRNAs and transcriptional repressors and activators.

Furthermore, the small number of dual-function sRNAs will increase considerably, and new unprecedented functions for small peptides encoded by sRNAs will be detected.

The excludon concept recently established for long antisense RNAs (lasRNAs) from *Listeria* might be confirmed for novel lasRNAs from other low-GC-Gram-positive bacteria. The modes of action used by the lasRNAs may not only comprise classical antisense RNA concepts or RNA interference, but so far unanticipated mechanisms. Additionally, sRNAs might be found that act directly on the genome like e.g., the siRNAs from *Schizosaccharomyces pombe* that are involved in chromatin silencing (reviewed in ref. 96).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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