The Many Facets of the Small Non-coding RNA RsaE (RoxS) in Metabolic Niche Adaptation of Gram-Positive Bacteria

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Abstract

Small regulatory RNAs (sRNAs) are increasingly recognized as players in the complex regulatory networks governing bacterial gene expression. RsaE (synonym RoxS) is an sRNA that is highly conserved in bacteria of the Bacillales order. Recent analyses in *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis* identified RsaE/RoxS as a potent riboregulator of central carbon metabolism and energy balance with many molecular RsaE/RoxS functions and targets being shared across species. Similarities and species-specific differences in cellular processes modulated by RsaE/RoxS suggest that this sRNA plays a prominent role in the adaptation of Gram-positive bacteria to niches with varying nutrient availabilities and environmental cues. This review summarizes recent findings on the molecular function of RsaE/RoxS and its interaction with mRNA targets. Special emphasis will be on the integration of RsaE/RoxS into metabolic regulatory circuits and, derived from this, the role of RsaE/RoxS as a putative driver to generate phenotypic heterogeneity in bacterial populations. In this respect, we will particularly discuss heterogeneous RsaE expression in *S. epidermidis* biofilms and its possible contribution to metabolic niche diversification, programmed bacterial lysis and biofilm matrix production.

Introduction

RNA-mediated control of bacterial gene expression is an emerging and dynamic field of research. Among the many RNA-based mechanisms studied, particularly the discovery and investigation of non-coding regulatory RNAs (ncRNAs) greatly influenced novel conceptual views on the physiology of microorganisms [1,2]. ncRNAs are poststranscriptional regulators that act by base-pairing interactions with mRNA targets. They may be encoded on the opposite strand of the regulated RNA (*cis*-encoded) or are transcribed distantly from their RNA targets on which they act in *trans* [1]. According to their size between 40 and 400 nucleotides, *trans*-acting ncRNAs are often referred to as non-coding small RNAs (sRNAs). Occasionally, sRNAs may encode short peptide sequences and exert functions both as *trans*-acting sRNAs and protein-coding regions [3]. Prime examples for such dual-function sRNA are RNAIII, the effector of the staphylococcal Agr quorum-sensing system [4,5] and SR1, an sRNA from *Bacillus subtilis* modulating glyceraldehyde-3-P-dehydrogenase (GapA) functions [6,7]. Based on their expression as autonomous transcription units and their operation in *trans*, sRNAs are considered as *bona fide* regulatory RNAs in bacteria [8]. Many sRNAs are transcribed in response to distinct growth or stress conditions, and riboregulation through sRNAs can influence gene expression at all levels, ranging from transcription and translation control to RNA stability and modulation of protein activity [1,9]. sRNAs add enormous complexity to bacterial gene regulatory networks both by acting as fine tuners of gene expression and through their local integration into mixed regulatory circuits, including feed-forward and feedback loops [2]. Many sRNAs were shown to be involved in the control of important cellular processes such as virulence [10–16], antibiotic resistance [16–21], biofilm formation [22–28] and, last but not least, in the control of metabolism [29–36]. With the advent of next-generation sequencing techniques, the number of sRNAs discovered in bacteria is constantly increasing [37]. On the

(a)

(b)

Phylum	Class	Order	Family	Genus	RsaE
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Anoxybacillus*	+
				Bacillus	+
				Geobacillus	+
				Parageobacillus	+
			Staphylococcaceae	*Macrococcus*	+
				Salinococcus	+
				Staphylococcus	+
			Planococcaceae	*Jeotgalibacillus*	+
		Lactobacillales	Enterococcaceae		-
			Lactobacillaceae		
			Streptococcaceae		
			etc.		

Fig. 1. RsaE/RoxS sequence conservation and detection in eubacteria. (a) An alignment of RsaE/RoxS sequences is shown from four *S. epidermidis* strains, four *S. aureus* strains and one *B. subtilis* strain. The putative binding sites of different regulators as well as the −35 and −10 sequences in the upstream regions are boxed in gray. The C-rich motifs (CCRs) are marked by rectangles. The transcriptional start site of RsaE/RoxS is indicated by +1. Nucleotides varying between *S. aureus* and *S. epidermidis* RsaE are highlighted in bold. Abbreviations are as follows: Se, *S. epidermidis*; Sa, *S. aureus*; Bsu, *B. subtilis*. (b) Occurrence of RsaE orthologs among Firmicutes. The figure was generated using the BLASTN megablast algorithm for identifying highly similar nucleotide sequences at the National Center for Biotechnology Information (NCBI) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [60]. The RsaE nucleotide sequence from *S. aureus* 8325-4 was used as input sequence to query the non-redundant nucleotide collection at NCBI, setting the maximum number of aligned sequences to display to 20,000.

species level, a number of sRNAs exist which are specific for a distinct species or even strain, while others are more broadly distributed and conserved throughout phylogenetic clades [38]. A prime example for a highly conserved sRNA is RsaE, which can be found in numerous members of the Bacillales order [8,39–44]. This unusual conservation across families and genera suggests selective pressure to preserve the sequence and structure integrity of RsaE and points to a prominent function of this riboregulator in the physiology of low-GC Gram-positive bacteria [8,45]. RsaE was mainly studied in staphylococci [8,16,39,40,42,44] and the Gram-positive model organism *B. subtilis* [43,46,47], demonstrating a multitude of functions of RsaE in these bacteria. A common theme revealed in these studies is the role of RsaE in the control of important metabolic pathways such as central carbon flux and amino acid metabolism, with regulatory links existing toward control of programmed bacterial cell death and biofilm formation. A general hallmark of biofilms is the existence of various metabolic niches, which are determined by different nutrient, water and oxygen availabilities within the biofilm architecture [48–50]. Organization of planktonic bacteria as biofilm communities requires heterogeneity of gene expression and division of labor within a bacterial population to meet these varying metabolic challenges [51–53]. Recent studies indicate that RsaE is heterogeneously expressed within populations of the prototype biofilm-forming bacterium *Staphylococcus epidermidis* [16]. In this species, RsaE promotes extracellular matrix production and seems to support the spatiotemporal organization of the biofilm *via* influencing metabolic pathways and localized bacterial lysis [16]. This review summarizes recent knowledge on the molecular functions of RsaE as a riboregulator of metabolic functions in *B. subtilis* and staphylococci, with a particular focus on the supposed role of RsaE in staphylococcal biofilm formation and the generation of phenotypic population heterogeneity.

Table 1. Selected cellular functions influenced by RsaE/RoxS

	Pathway	Genes influenced by RsaE	Species	References
Carbon and energy metabolism	TCA cycle	*sucA*, *sucB*, *sucC*, *sucD*, *citB*, *citC*, *fumC*, *mqo1*, *pycA*	*S. aureus*	[39,40,44]
		citZ, *sucC*	*B. subtilis*	[47]
	Oxidoreductases and redox-related functions	*hmp*, *nrdG*, *SA0084*, *ndh2*	*S. aureus*	[39,40,44]
		ahpC, *catD*, *catE*, *dhbA*, *msrA*, *nfrA*, *resA*, *ycgT*, *ydbP*, *ygaF*, *ykuP*, *yoxD*, *ypjG*, *yrdP*, *yrkL*, *yvaA*, *cydA*, *dps*, *cccB*, *etfA*, *ppnKB*, *tpx*, *rocA*, *rocG*	*B. subtilis*	[47]
	Pyruvate metabolism and overflow	*ald2*, *pdhB*, *accB*	*S. aureus*	[39,40,44]
		yqfL, *ldh*, *pta*	*B. subtilis*	[47]
	Central carbon flux and aminosugar metabolism	*fdh*, SA2307, SAOUHSC_01138, *pgk*	*S. aureus*	[39]
		sacA, *yrdC*, *yvgN*, *nagBB*, *nagP*, *glpK*, *glpF*	*B. subtilis*	[47]
	Sugar transport and regulators of carbon metabolism	*lldp2* (lactate), SAOUHSC_0698 (malate), SA0237 (sorbitol), SA0186	*S. aureus*	[39,40,44]
		sacP (sucrose), *yflS* (malate), *manP* (mannose), *manR*, *iolR*	*B. subtilis*	[46,47]
	Folate biosynthesis	*fhs*, *folD*, *gcvP*, *gcvT*	*S. aureus*	[39,40,44]
	Nucleotide metabolism	*pyrR*, *pyrB*, *pyrC*, *guaA*, *purK*, *purF*, *purN*, *purH*	*S. aureus*	[39,40,44]
		pyrE, *pyrF*, *pyrK*	*B. subtilis*	[46,47]
Amino acid transport and metabolism	Arginine and proline	*arcA*, *arcB*, *arcC*, *arcD*, *arg*, *rocF*, *rocD*, *gudB*	*S. aureus*	[39,40,44]
		rocA, *rocG*	*B. subtilis*	[46,47]
	Branched-chain amino acids	*ilvB*, *ilvC*, *ilvD*, *ilvH*, *ilvN*, *leuA*, *leuB*, *leuD*	*S. aureus*	[39,40]
	Glycine, serine and threonine	*betA*, *betB*, *thrB*, *thrC*, *gcvP*, *gcvT*, *gcvPB*, *asd*, SAOUHSC_02723	*S. aureus*	[39,40,44]
		gcvH	*B. subtilis*	[47]
	Oligopeptide transport	*oppA*, *oppB*, *oppC*, *oppD*, *oppF*	*S. aureus*	[39,40]
Others	Biofilm formation	*icaR*	*S. aureus*	[44]
			S. epidermidis	[16]
	CcpA regulated genes	*ald2*, *citB*, *citC*, *citZ*, *fhs*, *gudB*, *rocA*, *rocD*, *rocF*, *rsaOG (rsaI)*	*S. aureus*	[44]
		etfA, *sucCD*, *rbsD*, *sacP*, *pta*, *yqgX*, *citZ*	*B. subtilis*	[47]
	SigB regulated genes	*ydpB*, *ytkL*, *dps*, *sigB*	*B. subtilis*	[47]

RsaE Discovery and Conservation among Bacillales

The discovery of sRNAs as players in the control of bacterial physiology prompted extensive experimental work to identify such elements in the important human pathogens *Staphylococcus aureus* [8,39–41,54–56] and *S. epidermidis* [57]. Most of these studies used a combination of computational methods and experimental validations in their quest of novel *bona fide* sRNAs. Thus, a systematic search in *S. aureus* using this global approach discovered 10 novel sRNA which were named RsaA-J [39]. RsaA-J shared common features such as accumulation in late-exponential growth stage, long half-life and structural similarities, the latter comprising the

Table 2. Validated direct mRNA targets of RsaE/RoxS

Pathway	mRNA	Function	Organism	RsaE effect on mRNA translation	References
TCA cycle and carbon metabolism	*sucCD*	Succinyl-CoA synthase	*B. subtilis*	Negative	[47]
			S. aureus		[39]
			S. epidermidis		[16]
	yflS	Malate transporter	*B. subtilis*	Positive	[46]
Amino acid metabolism and transport	*rocF*	Arginase	*S. aureus*	Negative	[44]
	opp3A	Oligopeptide transporter	*S. aureus*	Negative	[40]
	opp3B	Oligopeptide transporter	*S. aureus*	Negative	[39]
Folate biosynthesis	*fhs*	Formate tetrahydrofolate (THF) ligase	*S. aureus*	Negative	[39]
	folD	Methylene-THF enzyme	*S. aureus*	Negative	[39]
Nicotinamide metabolism	*ppnKB*	NAD^+/NADH kinase	*B. subtilis*	Negative	[47]
Programmed cell death and eDNA release	*lrgA*	Anti-holin protein	*S. epidermidis*	Negative	[16]
Biofilm formation	*icaR*	Repressor of biofilm genes	*S. epidermidis*	Negative	[16]
Others	SA0873	Hypothetical protein	*S. aureus*	Negative	[39]

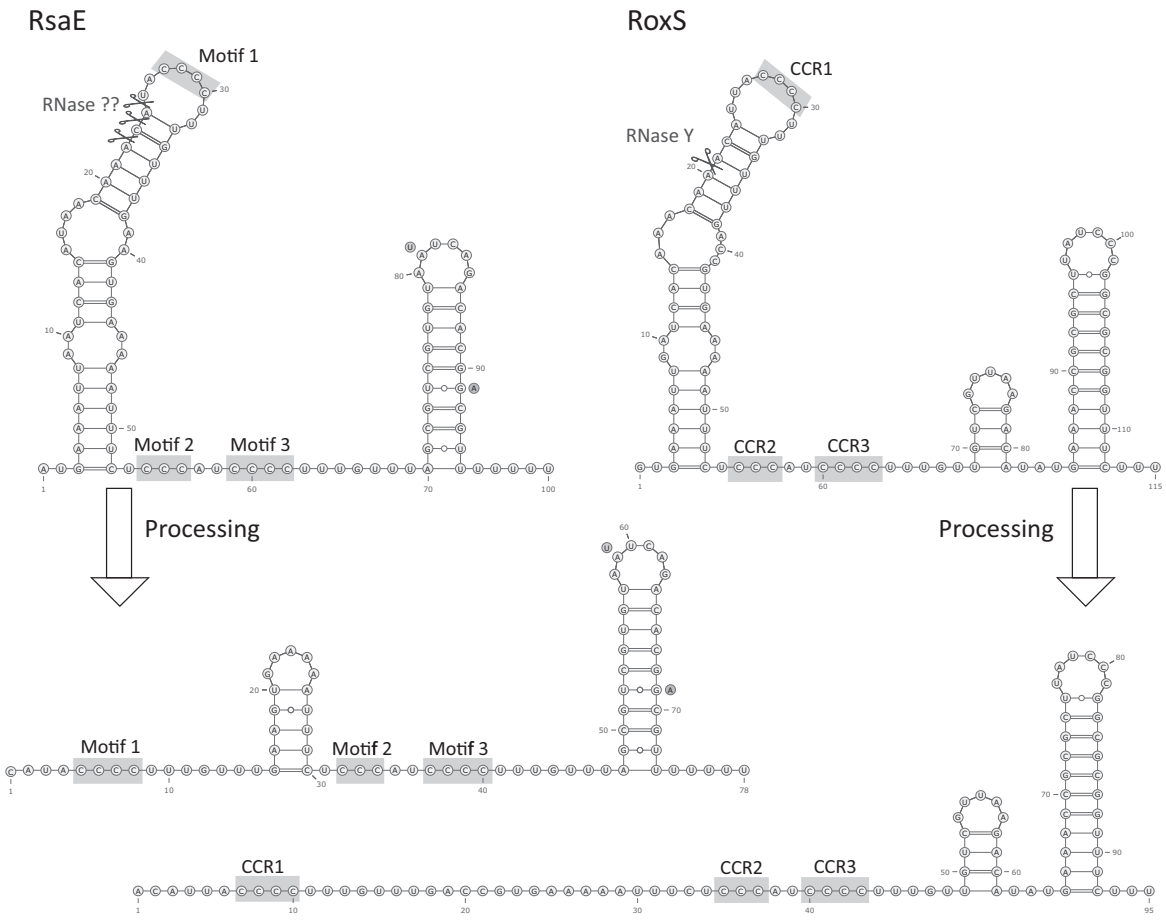

Fig. 2. RsaE/RoxS structure and processing. Secondary structure of RsaE from *S. epidermidis* (left) and RoxS from *B. subtilis* (right). Varying nucleotides present in *S. aureus* RsaE are indicated next to the corresponding positions in *S. epidermidis* RsaE. C-rich motifs are boxed in gray, and processing sites are depicted by scissors. The respective processed forms of RsaE and RoxS are shown at the bottom of the figure. Note that in *S. aureus*, RsaE is not processed.

exhibition of stem–loops linked by long unpaired RNA regions harboring a typical C-rich motif [39] (Fig. 1a). Interestingly, RsaC, RsaE and RsaG carried multiple copies of this sequence motif, which was later recognized to be important for target mRNA interaction involving ribosomal binding sites (RBS) [39,40]. RsaE was also detected in another global study in which 14 novel *S. aureus* sRNAs (RsaOH-OX) were described [40]. In both reports, RsaE was revealed to influence a number of genes associated with central metabolic functions such as tricarboxylic acid (TCA) cycle activity, amino acid metabolism and folate-dependent one-carbon metabolism [39,40] (Tables 1 and 2). Furthermore, the transcript (tentatively named ncr22/nr626) emerged in early *B. subtilis* transcriptome studies employing tiling arrays or RNA-sequencing approaches [58,59]. Interestingly, RsaE was recognized as highly conserved among Firmicutes, which arouse interest of researchers in the first place and prompted the initial functional studies listed above. Orthologs of the sRNA were found across the genera *Staphylococcus* and *Bacillus*, but also in *Macrococcus*, *Geobacillus* and other bacteria of the Bacillales order [39,44,47,58]. When searching for RsaE using the BLASTN megablast algorithm (for identifying highly similar nucleotide sequences) [60], RsaE orthologs can be identified in many, but not all genera of the Bacillaceae and Staphylococcaceae families (Fig. 1b). In contrast, RsaE is lacking in bacteria of the Lactobacillales order which also comprises the Streptococcaceae and Enterococcaceae families, harboring many common pathogens (Fig. 1b). The BLASTN algorithm is based on pairwise comparisons of nucleotide sequences [60], and the RsaE orthologs identified by this approach share three C-rich motifs, which are characteristic for RsaE and its function (see also below). When employing for the database query the GLASSgo algorithm [61], the same RsaE orthologs are found as with BLASTN (own unpublished data). However, in addition RsaE is detected in Listeria and a number of other bacteria

by this approach ([44] and own unpublished data). GLASSgo is a dedicated program for sRNA homolog detection that also considers RNA secondary structure information [61]. Of note, the additional RsaE-like sequences identified by GLASSgo are poorly conserved on the nucleotide sequence level, and most of them only retain one or two of the three C-rich motifs (data not shown). It is therefore currently not clear whether or not these sRNAs indeed represent functional RsaE molecules. So far, the molecular function of RsaE was addressed in *B. subtilis*, *S. aureus* and *S. epidermidis*, demonstrating similarities regarding TCA cycle control and mRNA target recognition *via* the C-rich motifs [16,44,46,47]. In *S. epidermidis*, RsaE was additionally found to facilitate biofilm matrix and extracellular (e)DNA production, and in *B. subtilis*, a number of mRNA targets were identified which were involved in oxidative stress response, leading to renaming of RsaE as RoxS (for *r*elated to *ox*idative *s*tress) in this organism [16,47].

Molecular Basis of mRNA Target Interaction and Processing of RsaE/RoxS

Figure 1a shows a nucleotide sequence alignment of RsaE/RoxS in *B. subtilis* and various *S. aureus* and *S. epidermidis* strains highlighting the positions of the three conserved C-rich motifs (Fig. 1a). Resolution of the RsaE structure was achieved through computational methods and enzymatic probing [39]. The sRNA was shown to form two main stem–loops (hairpins) that are interconnected by a long unpaired RNA stretch (Fig. 2). Despite minor differences, this overall structure is well preserved between RsaE from staphylococci and RoxS from *B. subtilis* (Fig. 2). In both sRNA species, the C-rich motifs are located in single-stranded RNA regions which would allow for interactions with target mRNAs. Thus, motif/CCR 1 is exposed in the outmost loop of the long hairpin at the 5′-end, while motifs/CCR 2 and 3 are part of the unpaired region linking the stem–loops (Fig. 2). All three motifs are *in silico* predicted to be able to interact with the G-rich regions of bacterial RBS [39]. Repetition of identical binding motifs on the same sRNA molecule is an unusual feature. *In vitro* studies gave evidence that RsaE/RoxS indeed binds to the 5'-untranslated regions of mRNA targets and prevents the formation of translation initiation complexes both in *S. aureus* and in *B. subtilis* [39,40,47]. Later, importance of the RBS and the C-rich motifs for this interaction was shown *in vitro* and *in vivo* for mRNA targets from *S. aureus* and *S. epidermidis* [16,44]. Interestingly, in *S. aureus*, motifs 1 and 2 were shown to contribute independently to the activity of RsaE on mRNA targets. Thus, RsaE species carrying mutations of either motif 1 or motif 2 were still able to complement RsaE functions in an *rsaE* deletion mutant. In contrast, simultaneous disintegration of both motifs abolished the activity of RsaE in *S. aureus* [44]. Similarly, in *S. epidermidis*, mutation of all three C-rich motifs was required to abrogate the effect of RsaE on biofilm matrix production and eDNA release ([16] and own unpublished observations). Most of the direct mRNA targets identified for RsaE/RoxS so far are negatively affected by the sRNA through binding of RsaE/RoxS to RBS regions, thereby generating double-stranded RNA molecules that are most likely subject to rapid RNase III-driven decay [62]. Recently, an additional mode of action was described for *B. subtilis* RoxS, leading to enhancement of mRNA target stability. Thus, RoxS was found to bind to the extreme 5′-end of *yflS* mRNA (encoding a malate transporter) *via* the CRR3 motif. This interaction prevents RNase J1-dependent exonucleolytic degradation of *yflS* mRNA and also enhances translation of the mRNA [46]. In the light of the many cellular functions influenced by RsaE/RoxS (see below), it is very likely that other direct targets will undergo a similar positive regulation by RsaE/RoxS as well.

Interestingly, in *B. subtilis* and *S. epidermidis*, RoxS and RsaE were found to undergo processing at the 5′-end, which generates sRNA species that are shortened by approximately 20 nucleotides and which coexist with full-length RsaE/RoxS [16,47]. Processing is predicted to result in a conformational change of RsaE/RoxS that disintegrates the 5′-stem–loop and moves motif 1/CCR1 to a long unpaired RNA stretch (Fig. 2). Together with the two other motifs, this structure would offer more options for interactions of processed RsaE/RoxS with G-rich regions in mRNA targets. Indeed, in *B. subtilis* and *S. epidermidis* processing results in expansion of the RsaE/RoxS target spectrum [16,47]. In both species, opening of the RoxS/RsaE structure enabled motif 1/CCR1 to take part in additional base pairing interactions involving the early coding regions of *sucC* mRNA, while motifs/CRRs 2 and 3 covered the RBS [16,47]. A similar mechanism was found for *sucD* mRNA binding as well as for the interaction with *icaR* mRNA, encoding a repressor of *S. epidermidis* biofilm formation [16]. In *B. subtilis*, RNase Y was identified as the endoribonuclease that mediates RoxS processing, and cleavage by this enzyme was identified as a prerequisite for subsequent degradation of processed RoxS by RNase J1 [47]. The cleavage site for RNase Y resides in a double-stranded region of RoxS (scissors in Fig. 2), which is unusual for the enzyme that normally prefers single-stranded RNAs for cleavage [63–65]. The RNase that mediates RsaE processing in *S. epidermidis* has not been identified yet [16]. Surprisingly, in contrast to *S. epidermidis* and *B. subtilis*, RsaE seems not to undergo processing in *S. aureus*. Indeed, no

significant RsaE target mRNA variation was observed in a *S. aureus* strain lacking RNase Y [44,64]. Moreover, transcriptome comparisons of a *S. aureus* wild-type and RNase Y mutant by differential RNA sequencing confirmed the absence of (major) processed forms of RsaE in *S. aureus*, suggesting distinct species-specific differences in RsaE/RoxS turnover (Marincola G., unpublished data). Interestingly, variations in RNase functions between species are also reflected by varying consequences in bacterial physiology. Thus, an RNase Y mutant in *B. subtilis* grows very slowly, exhibits major defects in sporulation and competence and shows striking alteration in cell morphology [66]. Because of these dramatic effects, RNase Y was previously even regarded as being essential in *B. subtilis* [65,67]. In contrast, in *S. aureus*, RNase Y is non-essential and a mutant grows comparable to the wild type [64,68]. It is an interesting open question whether or not the observed differences in sRNA processing might be

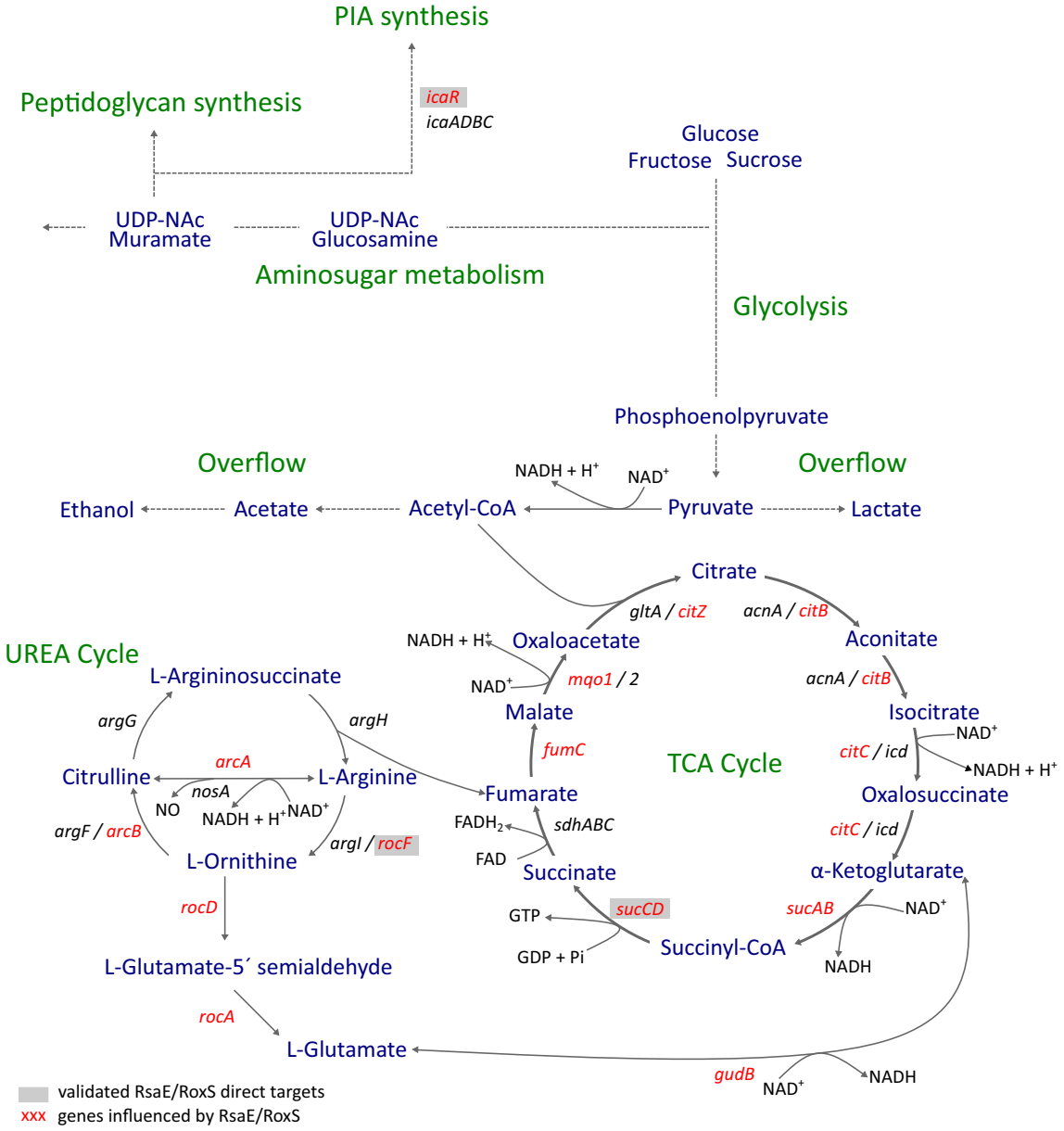

Fig. 3. Overview on selected central carbon flux pathways influenced by RsaE/RoxS (adapted from Ref. [44]). In staphylococci and *B. subtilis*, RsaE/RoxS influences numerous steps of the TCA and Urea cycles. In *S. epidermidis*, this results in the redirection of carbon sources from energy gain into GlcNac and eventually PIA synthesis. Genes known to be influenced by RsaE/RoxS in *S. aureus*, *S. epidermidis* and/or *B. subtilis* are in red color (for references, see Table 1). Verified RoxS/RsaE targets are boxed in gray (for references, see Table 2).

associated with the species-specific RNase functions and effects on bacterial physiology.

Metabolic Targets of RsaE/RoxS

RsaE was specified as a riboregulator of central metabolism that originates in the striking influence of this sRNA particularly on carbon flux genes [39,40,44,47]. Table 1 summarizes (selected) cellular functions affected upon overexpression or deletion of *rsaE*/*roxS* in global transcriptome and proteome analyses. Regardless of which method was used (microarray, qRT-PCR, RNAseq, proteome) or which approach was pursued (*rsaE*/*roxS* deletion or overexpression), an impact on TCA cycle-associated genes and proteins was a common finding in these studies (Table 1). Except for succinate/fumarate conversion, all TCA cycle steps were found to be negatively influenced by RsaE/RoxS, and the *sucC* and *sucD* mRNAs were shown to represent direct targets for RsaE/RoxS binding in *S. aureus*, in *B. subtilis* and in *S. epidermidis* (Table 2, Fig. 3). Phenotypic growth inhibition observed in *S. aureus* upon RsaE overproduction is in line with these molecular findings and speaks indeed in favor for a negative effect of RsaE/RoxS on TCA cycle functions [40]. Other RsaE/RoxS-influenced genes of carbon and energy metabolism comprise pyruvate and overflow metabolism, sugar transport, aminosugar and nucleotide synthesis as well as the biosynthesis of folate (Table 1). In a growing bacterial culture, the TCA cycle is usually repressed in early growth stages when sufficient glucose is available, and activated during stationary growth when glucose is depleted. Glucose is catabolized to pyruvate through glycolysis during exponential growth. In the presence of oxygen, excess glucose/pyruvate is directed to overflow metabolism to produce acetate, lactate, ethanol or acetoin, which are excreted into the medium. In stationary growth stage, when glucose is depleted, these compounds are re-imported and used as alternate carbon sources in the then activated TCA cycle. Main purpose of the TCA cycle is to generate reducing power through oxidation of substrates and the transfer of electrons to NAD^+ and FAD to yield NADH and $FADH_2$, which are used to drive ATP synthesis during oxidative phosphorylation (Fig. 3). In addition, NADH and $FADH_2$ serve as reducing agents in a multitude of cellular redox reactions. In *B. subtilis*, RsaE/RoxS was found to negatively influence a number of oxidoreductases, and many of these downregulated proteins use NAD and FAD as prosthetic groups to accomplish their oxido-reduction/electron transfer reactions [47]. In this respect, the inorganic polyphosphate/ATP-NAD kinase PpnKB, which converts NAD^+ to $NADP^+$, was shown to represent a direct target of RoxS in *B. subtilis* [47] (Table 2). Although this enzyme does not represent an oxidoreductase itself, it contributes to the reducing power of the cell by influencing NADPH levels. Thus, balancing cellular NAD^+/NADH (and $NADP^+$/NADPH) ratios in response to nutrient availability is now considered to represent the most likely primary function of RsaE/RoxS in Gram-positive bacteria [47]. The demonstrated control of RsaE/RoxS transcription by redox-sensitive regulators (see below) and its influence on amino acid transport and metabolism further support this hypothesis. Thus, in *S. aureus*, RsaE was recently identified to repress arginine catabolism by directly targeting *rocF* mRNA, encoding arginase, which mediates the conversion of arginine into ornithine and plays a role in staphylococcal arginine synthesis from proline *via* the urea cycle [44,69] (Fig. 3). Other pathways involved in amino acid biosynthesis and oligopeptide transport are negatively influenced by RsaE/RoxS as well (Tables 1 and 2). Downregulated genes comprise, for example, genes of the glycine cleavage pathway, yielding either pyruvate or building blocks for methionine biosynthesis (e.g., methyl groups). Furthermore, RsaE/RoxS directly targets *fhs* and *folD* mRNAs, involved in the synthesis of 10-formyl-tetrahydrofolate, which is required for the formylation of initiator methionyl-tRNA, pointing to a negative role of RsaE/RoxS in protein translation initiation and, more globally, to an inhibitory function in amino acid degradation. Generally, amino acids can be used as alternate substrates in the TCA cycle once sugar sources are depleted. As RsaE/RoxS reduces amino acid catabolism, this may limit feeding of the TCA cycle and consequently reduce NADH production. Utilization of alternate substrates for energy gain in bacteria is hierarchically coordinated with glucose representing the preferred carbon source, followed by other sugars as well as fatty and amino acids, whose catabolized intermediates can enter the TCA cycle at different checkpoints. In bacteria, the metabolic switch from a preferred to an alternate carbon source is mainly controlled by the catabolite control protein A (CcpA), which is a transcription repressor that binds to specific palindromic sequences called catabolite responsive elements (*cre*) in the promoter regions of regulated genes [70,71]. Interestingly, RsaE/RoxS influences a number of genes of the CcpA regulon of both *S. aureus* and *B. subtilis*, assigning RsaE/RoxS a function in the metabolic adaptation of Gram-positive bacteria to alternate substrates [44,47] (Table 1). Moreover, RsaE/RoxS seems to influence other cellular processes such as stress-response *via* the alternative sigma factor SigB and staphylococcal biofilm formation, which are discussed below.

Regulation of RsaE/RoxS

Given the role of RsaE as a regulator of metabolic functions, the question arises how control of this versatile switch is achieved. Interestingly, the RsaE/RoxS sequence conservation in staphylococci and

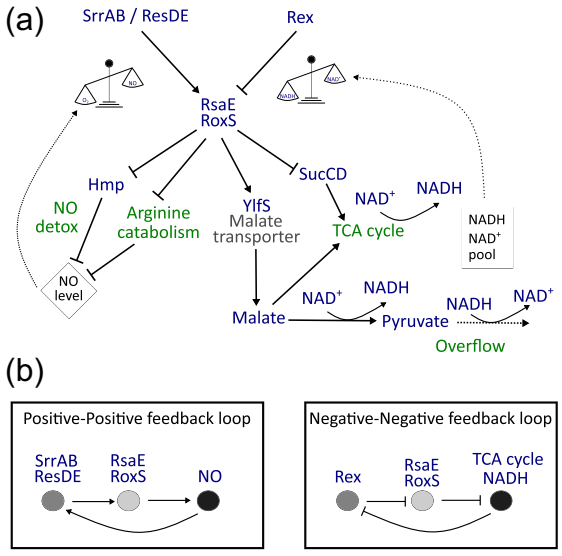

Fig. 4. RsaE/RoxS regulation. (a) The SrrB/ResE histidine kinase senses decreasing oxygen concentrations and increasing NO levels in the environment, which leads to phosphorylation of the SrrA/ResD response regulator, which mediates transcription of RsaE/RoxS. Rex is a repressor protein that senses the NAD^+/NADH ratio of the cell. Presence of NAD^+ enhances binding of Rex to the RsaE/RoxS promoter region and represses RsaE/RoxS transcription. By acting on the TCA cycle and on malate transport, RsaE/RoxS regulates the NADH/NAD^+ pool, which in turn regulates RsaE/RoxS transcription. Furthermore, RsaE/RoxS controls NO levels *via* arginine catabolism and NO detoxification pathways, thus indirectly influencing SrrAB/ResDE activity. (b) Breakdown of the SrrAB- and Rex-mediated regulatory circuits into positive and negative feedback loops to govern RsaE/RoxS transcription.

bacilli extends into the region upstream of the *rsaE* gene. Here, binding sites for at least two transcription factors (i.e., SrrA/ResD and Rex) are present whose activities are known to be responsive to distinct metabolic and stress signals [46,47]. Thus, the *rsaE* promoter region harbors a binding site for the SrrA protein, which represents the response regulator of the *S. aureus* two-component system (TCS) SrrAB [72] (Fig. 1). SrrAB, which stands for *s*taphylococcal *r*espiratory *r*esponse AB, responds to low oxygen levels and nitric oxide (NO) stress [73]. The system is a homolog of the *B. subtilis* ResDE TCS, which is involved in the global regulation of aerobic and anaerobic respiratory metabolism [72,74,75]. Thus, the membrane-associated SrrB/ResE histidine kinase senses decreasing oxygen concentrations as well as increasing NO levels in the environment, which leads to phosphorylation of the SrrA/ResD response regulator and mediates binding of SrrA/ResD to target DNAs [76]. Both in *S. aureus* and in *B. subtilis*, SrrA/ResD was recently shown to act as an activator of RsaE/RoxS transcription in response to increasing NO levels in the growth medium [47] (Fig. 4a). In the same study, RoxS was revealed to negatively influence a number of mRNA targets in *B. subtilis* with functions related to oxidative stress and oxidoreduction reactions, suggesting feedback control of RsaE expression in response to the redox status of the cell [47]. Importance of the redox equilibrium for RsaE control is further highlighted by presence of a Rex binding site immediately downstream of the *rsaE*/*roxS* transcription start (Fig. 1). Rex is a repressor protein that senses the NAD^+/NADH ratio of the cell [77,78]. The two compounds compete for Rex binding with NADH having a 20,000-fold higher affinity than NAD^+ [46,79]. Presence of NAD^+ enhances binding of Rex to target promoters and represses expression of downstream genes, while NADH mediates a conformational change of the protein and alleviates Rex-mediated repression [77,79]. In *B. subtilis*, Rex was recently shown to bind to the *roxS* promoter region, leading to effective repression of RsaE/RoxS transcription [46]. Addition of malate (and/or glucose) to the growth medium released Rex-mediated repression and increased RoxS transcription [46]. Interestingly, RoxS was also found to facilitate malate uptake by stabilizing *yflS* mRNA, encoding a malate transporter [46] (Fig. 4a). In *B. subtilis*, malate is a common carbon source that is metabolized through the TCA cycle or is used for gluconeogenesis, or (when present in excess) is directed to overflow/fermentation metabolism [46]. TCA cycle and overflow metabolism are important pathways to generate NADH and NAD^+, respectively. Thus, malate-induced RoxS expression was suggested to be linked to the production of NADH during malate metabolism either through the TCA cycle or by conversion of the compound into pyruvate, leading to alleviated Rex repression and *roxS* transcription. In contrast, (excess) malate fermentation through the overflow metabolism will allow for the regeneration of NAD^+ whose binding to Rex will result in restoration of Rex-mediated *roxS* repression (Fig. 4a). As RoxS/RsaE downregulates a number of mRNA targets involved in TCA cycle and cofactor synthesis, carbohydrate-induced RoxS/RsaE expression is likely to represent an effective feedback loop to control transcription of this sRNA (Fig. 4b). The alleged role of RsaE/RoxS in adjusting the cellular NAD^+/NADH equilibrium in response to metabolic stimuli [46] is in good agreement with early studies on RsaE function in *S. aureus*, demonstrating growth phase-dependent RsaE transcription with pre-stationary RsaE accumulation and a drop in stationary phase, suggesting indeed a role of RsaE in adaptation to nutrient availability and growth phase changes [39,40]. Downregulation of RsaE in the stationary growth phase (when an active TCA cycle is required) was recently also confirmed for *S. epidermidis* [16]. During early-, mid- and late-exponential growth, however, striking variations in

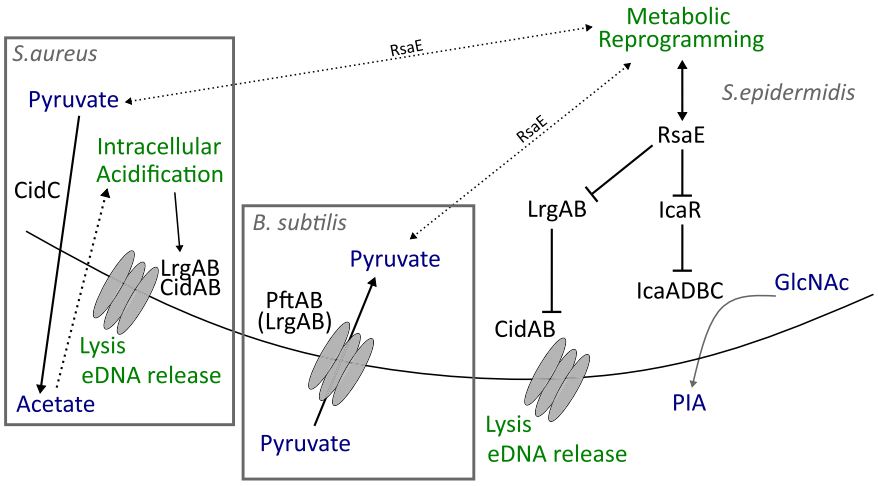

Fig. 5. RsaE influence on programmed cell death and biofilm matrix production. In *S. epidermidis*, RsaE targets the mRNA of IcaR, thereby diminishing its translation and thus enabling transcription of the *icaADBC* genes and PIA production. RsaE also targets the antiholin LrgAB of *S. epidermidis*, thus inducing lysis and eDNA release through activation of the CidAB holin complex [16]. In *S. aureus*, intracellular pyruvate is metabolized to acetate by the pyruvate oxidase CidC, encoded by the *cidABC* operon. Intracellular acidification by acetate decreases cellular respiration, which in turn triggers CidAB/LrgAB-mediated lysis [107]. In *B. subtilis*, the LrgAB homologs PftAB exhibit a (second) function as a pyruvate import/export transporter [102]. In both species, pyruvate is the crucial compound to be fueled into TCA cycle and overflow metabolism, thereby indirectly influencing RsaE/RoxS transcription and metabolic reprogramming (symbolized by dotted lines).

RsaE transcription patterns were observed within different *S. epidermidis* strains and isolates, indicating heterogeneous expression of the sRNA, particularly within *S. epidermidis* biofilm populations [16]. Also in *S. aureus* clinical isolates, distinct and variable RsaE transcription patterns were recorded with major differences existing between colonizing and infection-associated strains [80]. These findings suggest that staphylococci have a broad flexibility regarding RsaE expression, and some conflicting results in *S. aureus* concerning involvement of other global regulators in control of RsaE might be explained by strain-dependent differences. Thus, in an initial study in *S. aureus*, RsaE transcription was found to be low in strains expressing the alternative sigma factor B (SigB), and increased when an active Agr quorum sensing system was present [39]. In another study, SigB and Agr dependency was not observed when using isogenic mutants of these global regulators in an *S. aureus* 8325-4 background [40]. Although a systematic analysis is still lacking, involvement of SigB and Agr in RsaE control of staphylococci would be plausible. The Agr quorum sensing system is the major player to control gene expression in response to cell density and growth stage in staphylococci [81]. In *S. aureus*, the Agr system was shown be repressed by SrrAB under low-oxygen conditions [72,75], and it is therefore tempting to speculate that Agr and SrrAB might act together to control RsaE in response to oxygen availability. However, such a scenario still requires experimental validation. Likewise, an influence of SigB on RsaE/RoxS is conceivable as some *bona fide* sRNAs (i.e., RsaA, RsaD and RsaF) were proposed to undergo SigB-dependent control [8,39,42]. SigB is an alternative sigma factor of the RNA polymerase that recognizes dedicated promoter elements to coordinate numerous stress-induced and metabolic genes in *B. subtilis* and staphylococci [82,83]. In *B. subtilis*, RoxS was recently found to repress the production of proteins belonging to the SigB regulon, including the SigB factor itself [47]. Conversely, however, it is unlikely that SigB directly influences RsaE/RoxS transcription as no *sigB* consensus exists within the *rsaE*/*roxS* promoter regions. Hence, any effect of SigB on RsaE/RoxS is likely to be indirect and may reflect integration of the SigB-mediated oxidative stress response into the RsaE/RoxS regulatory circuit.

RsaE as a Player in *S. epidermidis* Biofilm Formation and Control of Programmed Cell Death

Many microorganisms are capable to produce extracellular matrix substances, which are excreted and prompt the bacteria to organize themselves as biofilm populations [84]. Biofilms are regarded as a kind of multicellular lifestyle that bacteria have evolved to protect from unfavorable external conditions [51,52]. Within the multilayered structure of a biofilm, access to nutrients, water and oxygen may vary significantly, and accordingly, single bacteria

will be exposed to very different conditions for growth [49]. To meet these challenges, life in biofilms requires heterogeneous gene expression patterns as well as division of labor and communication between consortium members, features that are very similar to multicellular tissues and organisms [52,53,85,86]. Diversification into cell types with varying phenotypes may involve regulatory pathways, reversible and non-reversible genetic events as well as stochastic variations during transcription and translation, resulting in fluctuations of transcript and protein levels [87–90]. Recently, RsaE was found to be heterogeneously expressed in populations of the prototype biofilm-forming bacterium *S. epidermidis*, and RsaE was suggested to contribute to *S. epidermidis* biofilm organization by influencing biofilm matrix production and eDNA release in this organism [16]. In *S. epidermidis*, the biofilm matrix may contain surface-associated proteins, eDNA and the polysaccharide intercellular adhesin PIA [91,92]. PIA synthesis is accomplished through enzymes encoded by the *icaADBC* operon, which is under control of its cognate repressor IcaR whose translation in turn is influenced by a novel, recently discovered ncRNA [93,94]. RsaE directly targets mRNAs involved in PIA synthesis control (i.e., *icaR*), metabolic reprogramming (i.e., *sucCD*) and eDNA release (i.e., *lrgA*) [16]. Thus, processed RsaE covers the RBS of *icaR* mRNA and diminished translation of the repressor will enable transcription of *icaADBC* genes (Fig. 5). However, Ica enzyme production alone would not be sufficient to synthesize the PIA biofilm matrix. PIA is a homopolymer consisting of beta-1,6 linked *N*-acetylglucosamines (GlcNac), and production of the polysaccharide requires ample sugar and energy resources to be fueled into GlcNac synthesis as PIA building blocks. Accordingly, synthesis of PIA exclusively takes place during exponential growth when carbon flow through the TCA cycle is low. Indeed, mutational or chemical blockage of the pathway was shown to result in the redirection of carbon sources from energy gain into GlcNac and eventually PIA synthesis [95,96]. Downregulation of the TCA cycle by RsaE in *S. epidermidis* may therefore trigger metabolic reprogramming in favor of biofilm matrix production (Figs. 3 and 5). Finally, in *S. epidermidis*, RsaE facilitates the release of eDNA, which is a stabilizing matrix component of many bacterial biofilms and whose release is thought to be mainly due to bacterial lysis [16,97–99]. In staphylococci, (auto)lysis is regarded to represent a form of programmed bacterial cell death, which is mediated and controlled by the *cidABC* and *lrgAB* operons [100]. These gene clusters encode membrane-associated protein complexes in which the CidA protein acts as lysis-triggering holin, while the LrgA antiholin counteracts the lytic function of CidA [101]. RsaE favors lysis by targeting *lrgA* mRNA through binding to the RBS, thereby diminishing the LrgA-mediated antiholin activity [16]. Of note, this interaction is specific for *S. epidermidis* and does not occur with *S. aureus lrgA*, which is due to sequence variations in the RBS [16]. The *cidABC* and *lrgAB* system is conserved in staphylococci as well as in *B. subtilis* where *lrgAB* is named *pftAB* [102]. Interestingly, *cidABC* and *lrgAB* genes are differentially expressed within *S. aureus* and *S. epidermidis* biofilm populations, and the system is suggested to play a major role in the generation of metabolically distinct niches during maturation of *S. aureus* biofilms [16,101,103–105]. Recent findings on the function(s) of *cidABC*/*lrgAB* in *B. subtilis* and *S. aureus* suggest that the system itself is capable to modulate central carbon flux [102,106–109], which would offer opportunities to (indirectly) influence RsaE/RoxS expression and biofilm matrix production (Fig. 5). Thus, the *cidABC* and *lrgAB* operons are under the control of their respective cognate regulators CidR and LytSR, which are responsive to oxygen and carbon flow as metabolic triggers [100,107,110]. In *S. aureus*, CidAB/LrgAB-mediated lysis was found to be linked to overflow metabolism *via* a CidR controlled cascade, in which intracellular pyruvate is either metabolized to acetate by the pyruvate oxidase CidC or converted into acetoin by AlsSD (acetolactate synthase/decarboxylase) [107]. Intracellular acidification by acetate decreased cellular respiration, which in turn triggered CidAB/LrgAB-mediated lysis, while neutral acetoin production through AlsSD efficiently counteracted the process [107]. Thus, CidAB/LrgA-mediated cell death is obviously modulated by carbon flux through the pyruvate node [107]. In this regard, it is interesting to note that in *B. subtilis* the LrgAB homologs PftAB were recently revealed to exhibit a second function as a pyruvate import/export system whose expression is influenced by varying extra- and intracellular pyruvate levels [102]. *pftAB* is activated by extracellular pyruvate *via* phosphorelay through the LytSR-like LytST TCS and repressed by CcpA-dependent catabolite repression in the presence of glucose or malate. Also, the circuit comprises CcpA-independent *pftAB* retroinhibition by intracellular pyruvate levels [102]. Impaired pyruvate utilization in a *S. epidermidis lytSR* mutant together with increased biofilm formation and diminished cell death points to a putative similar function of LrgAB in *S. epidermidis* [111]. Together, the Cid/Lrg systems exhibit unexpected second functions as checkpoints to control levels and the metabolic fate of intracellular pyruvate, the crucial compound that feeds both overflow metabolism and the TCA cycle, offering plenty of opportunity to indirectly influence RsaE/RoxS transcription (Fig. 5).

RsaE/RoxS as a Putative Factor to Generate Population Heterogeneity

In the light of the many inhibitory effects of RsaE/RoxS on a broad range of vital cellular functions,

homogeneous expression of RsaE/RoxS within a bacterial community would represent a risky strategy that might potentially kill the population. However, RsaE/RoxS also greatly benefits the community by contributing to growth stage adaptation and utilization of alternate carbon sources, which ensures survival under nutrient limited conditions. Hence, strict condition-dependent control of RsaE/RoxS levels is required to exploit the beneficial properties of RsaE/RoxS. Another possibility to balance the potential negative and positive effects of RsaE/RoxS would be stochastic or bistable expression of this potent switch within a population. In fact, heterogeneous RsaE expression was recorded in *S. epidermidis* biofilm populations, and it is reasonable to suggest that this might be the case in other species as well [16]. The genuine regulatory function of RsaE/RoxS seems to be the balancing of cellular NAD^+/NADH ratios (as a proxy for the energy status of the cell), and in this regard, RsaE/RoxS is subject to feedback control involving transcription factors and its own target mRNAs. Figure 4b shows a breakdown of the two main RsaE/RoxS regulatory circuits governed by SrrAB/ResDE and Rex, with activation by SrrAB/ResDE forming a positive–positive feedback loop and Rex-mediated repression organized as negative–negative feedback loop (Fig. 4b). Such regulatory relays employing (mixed) feedback and feedforward loops are typical for the integration of sRNAs into the complex gene expression networks of bacterial cells [2,112,113]. In mathematical modeling approaches, particularly negative–negative feedback loops were identified as prone to induce bistability of gene expression, resulting in two stable states that can coexist, with one dominated by the sRNA and the other governed by the transcription factor controlling the sRNA [2,87,112]. According to this model, the Rex-driven negative–negative feedback loop would induce RsaE/RoxS transcription in one part of the population, while in the other, RsaE/RoxS would be switched off through Rex-mediated repression (Fig. 4b). This scenario is in good agreement with the experimental findings in *S. epidermidis*, and due to the deep impact of RsaE on a multitude of metabolic functions, heterogeneous RsaE expression is likely to have consequences for the diversification of a population into cells with varying phenotypes [16]. In case of *S. epidermidis* biofilms, RsaE might trigger metabolic diversity and division of labor resulting in spatiotemporal differentiation of cells with only a part of the biofilm population undergoing costly matrix synthesis in RsaE-expressing microniches. Furthermore, the direct and indirect influence that RsaE exerts on bacterial cell death can be understood as a form of bacterial altruism, not only by releasing eDNA as important biofilm matrix component and common good, but also through providing nutrients to non-lysing and surviving cells in the immediate neighborhood. In this respect, expression of RsaE by only a part of the population makes sense as it might function as a mean to prevent total lysis of the population. Collectively, RsaE/RoxS seems to be a good candidate to drive phenotypic heterogeneity of bacterial communities, which might in the end serve long-term survival of the population as a whole. However, more research is required to further experimentally underpin this hypothesis.

Concluding Remarks

The combined data highlight that RsaE/RoxS is an extremely versatile player in the gene regulatory networks of many Gram-positive bacteria. The prominent role of RsaE/RoxS in energy balance and carbon flux control can explain its conservation throughout the Bacillales order with many molecular functions and targets being shared across species. In addition, interesting species-specific differences in RsaE/RoxS functions do exist which are likely to reflect adaptation to distinct habitats. Together, dissecting similarities and differences in RsaE functions as well as investigating its role in the population dynamic of Gram-positive bacteria make RsaE/RoxS a fascinating subject of (future) research.

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Abbreviations used:
ncRNA, non-coding regulatory RNA; sRNA, small RNA; RBS, ribosomal binding site; TCA, tricarboxylic acid; CcpA, catabolite control protein A; NO, nitric oxide; PIA, polysaccharide intercellular adhesin; TCS, two component system.

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