Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism

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ABSTRACT

Using an experimental approach, we investigated the RNome of the pathogen *Staphylococcus aureus* to identify 30 small RNAs (sRNAs) including 14 that are newly confirmed. Among the latter, 10 are encoded in intergenic regions, three are generated by premature transcription termination associated with riboswitch activities, and one is expressed from the complementary strand of a transposase gene. The expression of four sRNAs increases during the transition from exponential to stationary phase. We focused our study on RsaE, an sRNA that is highly conserved in the bacillales order and is deleterious when over-expressed. We show that RsaE interacts *in vitro* with the 5′ region of *opp3A* mRNA, encoding an ABC transporter component, to prevent formation of the ribosomal initiation complex. A previous report showed that RsaE targets *opp3B* which is co-transcribed with *opp3A*. Thus, our results identify an unusual case of riboregulation where the same sRNA controls an operon mRNA by targeting two of its cistrons. A combination of biocomputational and transcriptional analyses revealed a remarkably coordinated RsaE-dependent downregulation of numerous metabolic enzymes involved in the citrate cycle and the folate-dependent one-carbon metabolism. As we observed that RsaE accumulates transiently in late exponential growth, we propose that RsaE functions to ensure a coordinate downregulation of the central metabolism when carbon sources become scarce.

INTRODUCTION

Regulatory RNAs in prokaryotes are frequently referred to as small RNA (sRNAs). The sRNAs are typically between 50 and 300 nts in length. Most sRNAs with identified regulatory functions pair with mRNAs to modulate their translation, stability or transcription (1). The sRNAs encoded opposite to mRNA genes are referred to as 'cis-encoded sRNAs', since they often regulate the associated mRNA expressed from the complementary coding strand by extended perfect base-pairing (2). In contrast, sRNAs expressed from intergenic regions (IGRs) often act on target mRNAs transcribed from genetically unlinked regions. Base-pairing between these *trans*-encoded sRNAs and mRNA targets is usually imperfect and limited to short sequences. In many bacteria, including the Gram-negative bacterium *Escherichia coli*, the activity of *trans*-encoded sRNAs requires the RNA-chaperone Hfq (3). However, the Gram-positive bacterium *Staphylococcus aureus* might not require Hfq for sRNA activity since an *hfq* deletion mutant of this

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organism has no discernable phenotype (4). A third class of sRNAs can exert a regulatory function by directly interacting with proteins (5–7). Lastly, some mRNAs possess structured 5′ untranslated regions (UTR) that, under specific growth conditions, can adopt alternative conformations to affect gene expression of the downstream genes in *cis*. Binding of molecules such as metabolites or tRNAs to these so-called riboswitches induces RNA structure changes of the transcribed UTR, causing premature transcriptional termination or translational inhibition (8). Some riboswitches are known to produce sRNAs (9), which might independently function on *trans*-encoded target mRNAs by base-pairing mechanisms (10).

Several methods have been used to identify sRNAs, including computational predictions, direct labeling, DNA microarrays and shotgun sequencing (11,12). *Escherichia coli* and *Salmonella* each encode an estimated 100 sRNAs (11). These respond to diverse environmental factors such as glucose availability, iron limitation, envelope stress, or transition to anaerobiosis, to facilitate adjustments, respectively, in carbon metabolism (13,14), of levels of iron-requiring enzymes (15–17), amounts of metabolic transporters (18,19) or quantities of enzymes required for anaerobic growth (20,21). Other regulatory functions of sRNAs include quorum sensing and virulence (22–26).

Staphylococcus aureus is a human commensal of skin and nares and an opportunistic pathogen responsible for a wide variety of human infections including superficial skin and wound infections to deep abscesses (endocarditis and meningitis), septicemia or toxin-associated syndromes (27). It is a leading cause of nosocomial and community acquired diseases and many strains acquire antibiotic resistance (28). *Staphylococcus aureus* virulence gene expression is regulated by the coordinated action of numerous regulators. One of the best characterized regulators is an sRNA called RNAIII (514 nt), which modulates virulence gene expression (29). Its 3′ domains can mediate translational repression of *S. aureus* virulence genes directly by interacting with *spa* mRNA, which encodes the surface-anchored Protein A, or indirectly, by interacting with *rot* mRNA, which encodes Rot, a transcriptional regulator of several exoproteins (30–32).

Staphylococcus aureus has recently emerged as a model organism for the study of bacterial sRNAs (33). A pioneering study based on a computational analysis of IGRs led to the identification of 12 sRNAs that were experimentally confirmed, among which seven were expressed from pathogenicity islands (34). Thirty-four new sRNAs, including some that likely originate from putative riboswitches were recently identified (35–37). Finally, one deep sequencing and two global transcriptome studies on *S. aureus*, performed using different growth conditions suggested the existence of numerous non-coding small stable RNAs; however, most of them were not confirmed by alternative methods (37–39).

As sRNAs are potent regulators that can potentially affect viability, adaptability to different growth conditions, or virulence of this deadly pathogen, we undertook a search for new sRNAs in *S. aureus*. The characterization of one of these new sRNAs indicates that it coordinately downregulates multiple enzymes of two major metabolic pathways, likely to help *S. aureus* prepare for the entry into stationary phase.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The RNomics approach was performed on strain N315 (27). Transcriptome analysis was performed on strain RN6390 (40), a laboratory derivative of the NCTC8325 clinical strain (41) that was shown to be virulent in different animal models (42,43). Note that for simplicity, we only used the N315 gene name nomenclature. Engineered plasmids were constructed in *E. coli* TG1, and transferred to RN4220 (*rsbU*, *agr*), which is transformable with exogenous DNA (44), and subsequently to RN6390.

Staphylococcus aureus strains were routinely grown in BHI medium with aeration at 37°C. *Escherichia coli* TG1 used for cloning experiments was grown in LB broth at 37°C. Chloramphenicol was added to media as needed at the following concentrations: 5 μg/ml for *S. aureus* and 10 μg/ml for *E. coli*. To determine the effect of sRNA induction, staphylococcal cultures were prepared as follows: 10-fold serial dilutions of exponential cultures were spotted on BHI plates containing or not 1 μM anhydrotetracycline (aTc) and incubated at 37°C for 24 h.

pAT12 contains the *repA*, *repC*, *tetR* genes and the *xyl/tetO* promoter (45). The pAT12 derivative containing *rsaA*, *rsaH*, *rsaE* or expressing RNA sequences antisense of RsaA, RsaH and RsaE (named pAT12-RsaA, pAT12-RsaH, pAT12-RsaE, pAT12-ASRsaA, pAT12-ASRsaH and pAT12-ASRsaE, respectively) were constructed as follows: DNA sequences of interest were amplified from N315 genomic DNA by PCR, using primers indicated in Supplementary Data S1. The resulting products were digested by EcoRI and NotI, ligated to EcoRI-NotI-treated pAT12. Expression of the cloned sequences on constructed plasmids was induced by adding anhydrotetracycline (aTc) to the media, and confirmed by northern blot analysis.

DNA preparation

Lysis of *S. aureus* cell suspensions was achieved by treatment with 100 μg/ml of lysostaphin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C, followed by standard methods for DNA preparation. DNA transformation of *E. coli* and for *S. aureus* was performed as described (46,47).

RNA manipulation

For northern blot analysis and 454 pyrosequencing, overnight cultures of staphylococcal strains grown in BHI medium were diluted 1000-fold in the same medium and grown at 37°C; samples were withdrawn at mid-or post-exponential or stationary phase cultures. To study the effect of stress conditions, cultures at $OD_{600} = 0.2$ were either shifted to 45°C, or supplemented with NaCl 1.5 M or H_2O_2 10 mM for 2 h. Total RNAs were extracted

as described (48) or using NucleoSpin RNAII (Macherey Nagel, Düren, Germany).

Northern blots were performed as described (35,46,49). Samples were separated by either PAGE or agarose gels and probed with γ^{32}-P 5′ labeled oligonucleotides (described in Supplementary Data S1).

Rapid amplification of cDNA ends (RACE) mapping was performed as described (50,51).

Synthetic RNAs were generated using the T7 MEGAshortscript kit (Ambion, Foster City, CA, USA) according to manufacturer's instructions.

RNA gel mobility shift assays were performed as described (52). [α^{32}-P]UTP was added to the transcription mix to produce radioactive transcripts. The transcripts were denatured in 50 mM HEPES (pH 6.9), 50 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$ for 3 min at 85°C, followed by refolding for 10 min at 30°C. Labeled RsaE (0.04 pmol) was incubated with increasing amounts of un-labeled synthetic RNAs or yeast tRNAs in 10 mM Tris–HCl (pH 7.5), 6 mM NaCl, 10 mM EDTA and 5 mM dithiothreitol for 10 min at 30°C. Samples were supplemented with 10% glycerol (final concentration) and loaded on a native 5% polyacrylamide gel containing 5% glycerol.

Toeprint experiments were performed as described (53,54) with the following modifications. Annealing mixtures contained 0.2 pmol unlabeled *opp3A* mRNA and 1 pmol labeled SA0849 mRNA (primer 404) in a buffer containing 10 mM Tris–acetate (pH 7.5), 60 mM NH_4Cl and 1 mM DTT. The effect of RsaE was tested by adding various quantity of RsaE (25–300 pmol) to the annealing mixtures prior to the addition of purified *E. coli* 70S ribosomes. Ribosomes were activated for 15 min at 37°C and diluted in the reaction buffer in the presence of 1 mM $MgCl_2$. For each sample, 4 pmol of 70S were added, followed by 5 min incubation and adjustment of $MgCl_2$ to 10 mM. Ten picomoles of uncharged tRNA^{fMet} were then added and samples were incubated 15 min. cDNA was synthesized using two units of AMV RT (New England Biolabs, Ipswich, MA, USA) for 15 min at 37°C. Reactions were stopped by addition of 10 μl loading buffer II (Ambion, Foster City, CA, USA). The cDNAs were loaded and separated by Urea 8% PAGE. The toeprint position on *opp3A* mRNA was determined by DNA sequencing.

cDNA library from sRNAs, 454 pyrosequencing and analysis of data

RNA preparations (cf. above) were treated twice with DNase (DNA-free, Ambion, Foster City, CA, USA) according to manufacturer's instructions. For cDNA library construction, *S. aureus* RNAs from six different growth conditions (see 'Results' section) were mixed in a equimolar ratio. In addition, we used a new treatment protocol to enrich for primary bacterial transcripts (most mRNAs and sRNAs) (73). For this purpose, the pooled RNA was treated with Terminator 5′-phosphate dependent exonuclease (Epicentre, Madison, WI, USA). From this RNA, a cDNA library was prepared and analyzed on a Roche GS20 sequencer (F. Hoffmann-La

Roche, Basel, Switzerland) as described (55). The cDNA library was constructed by Vertis Biotechnology AG (http://www.vertis-biotech.com/). In brief, the Terminator exonuclease-treated RNA was first treated with tobacco acid pyrophosphatase (TAP). The RNA samples were subsequently poly(A)-tailed using poly(A) polymerase followed by ligation of an RNA adapter to the 5′-monophosphate of the sRNAs. First-strand cDNA synthesis was then performed using an oligo(dT)-adapter primer and MMLV H- reverse transcriptase. The resulting cDNAs were then PCR-amplified and the specific barcode sequence CAGC was attached to the 5′ ends of the cDNAs.

DNA sequences obtained from a multiplex 454 run on a Roche GS20 sequencer that contained the specific barcode tag CAGC were extracted. The 5′ end linker and polyA-tails were clipped, yielding 38 052 insert sequences (reads). 543 reads with lengths <14 nt were excluded from the study. The remaining 37 507 reads ($\geq$14 nt) were blasted with WU-blast (http://blast.wustl.edu/, chosen parameters were $E = 0.0001$, $m = 1$, $n = -3$, $Q = 3$, $R = 3$) against the *S. aureus* N315 genome sequence (NC_002745.fna), and 36 021 reads that could be mapped were kept for further analysis. Those with blast hits that corresponded to rRNA, tRNA, tmRNA, RNaseP, SRP RNA and known mRNA were removed. 202 reads (Supplementary Data S2) corresponded to sequences putatively issued from IGRs, and were assembled with cd-hit-est (similarity threshold $c = 0.92$) into 160 clusters (56). Further blast analysis of these 160 remaining clusters revealed additional tRNA or mRNA sequences that were excluded. The assembled sequences were manually analyzed to identify the sRNAs presented in this study. For example, at this last step, reads issued from sequences adjacent to open reading frames were considered as likely mRNA UTRs and discarded.

Transcriptome analysis

Overnight cultures grown in BHI supplemented with chloramphenicol were diluted 1000-fold in the same medium at 37°C. At $OD_{600} = 0.6$, aTc (1 μM) was added to the medium and 5 min later, the cultures were sampled. Total RNAs were extracted and treated twice with DNase I. RNA quality was controlled with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Two conditions were tested, RN6390 containing pAT12-RsaE and pAT12-AS-RsaE. Trancriptome experiments were performed using *S. aureus* Affymetrix DNA chips (Santa Clara, CA, USA) by Cogenics (Newton, MA, USA) according to the standard manufacturer's instructions.

Quantitative real-time PCR (qRT–PCR)

Overnight cultures grown in BHI supplemented with chloramphenicol were diluted 1000-fold in the same medium at 37°C. At $OD_{600} = 0.3$, aTc (1 μM) was added to the medium and cultures were sampled at 0, 5, 10, 20 and 30 min. RNAs were prepared as described (48). Genes for normalization were selected, qRT–PCR experiments were performed and data were analyzed as described (57). The geometric mean of 3 genes (*recA*, *gyrA* and *ftsZ*) chosen among 13 was used to normalize the samples.

RESULTS

Screening for new *S. aureus* sRNAs

As compared to enteric bacteria, comparatively few sRNAs have been experimentally confirmed to date in *S. aureus* (34–37,58). Our goal was to identify new sRNAs without any *a priori* restrictions in the selection; we therefore used an RNomics approach based on pyrosequencing of cDNA libraries (12). The study was performed on N315, a methicillin-resistant *S. aureus* strain that was isolated in a case of a hospital-acquired infection in 1982 (27). As sRNAs are often expressed in response to specific signals, total RNA was extracted from six different conditions. N315 was inoculated in rich medium (BHI) under aeration at 37°C and samples were withdrawn in (i) exponential phase, (ii) post-exponential phase, (iii) stationary phase and after 2 h of exposure to (iv) osmotic, (v) thermal or (vi) oxidative stress (cf. experimental procedures). For cDNA library preparation the RNAs extracted from these six growth conditions were pooled in an equimolar ratio, treated with Terminator 5′-phosphate dependent exonuclease to deplete processed sRNAs, thus enriching primary transcripts, and converted to cDNA libraries to be analyzed by 454 pyrosequencing as described (55,59,73).

A total of 36 021 cDNA sequences (referred to as reads) > 14 nt matched to the N315 *S. aureus* reference DNA sequence. The vast majority of these reads corresponded to rRNAs (33 094, mainly 5S) or tRNAs (2548), and were not analyzed further. Likewise, 173 reads matching small mRNAs or probable mRNA degradation products were discarded. A total of 126 reads that did not correspond to the above-described RNAs were retained for further analysis (Supplementary Data S2). Of these, 50 were likely issued from mRNA UTR regions, rather than sRNAs *per se*, and 73 were putatively derived from IGRs. In addition, seven sequences mapped to the complementary strand of coding sequences (Supplementary Data S2), suggesting the expression of *cis*-encoded RNAs. The 71 sequences of interest were clustered in 32 IGRs. None of the sequences matched RNAIII, as expected since the N315 strain expresses little if any RNAIII. 4.5S RNA, tmRNA, RNAse P RNA and 6S RNA were found in *S. aureus via* a computational screening (34); we identified all of these (corresponding to 54 reads, Table 1). In addition, six of the seven previously reported sRNAs of *S. aureus* pathogenicity islands, named SprA through SprG (34), were recovered by our RNomics approach (Table 1); the exception was SprE, which has turned out to be an rRNA fragment and not an sRNA (S.C. and B.F., unpublished results). In addition to already known sRNAs, our global RNomics search suggested the existence of new *trans*-encoded sRNAs [of which two were suggested, but not confirmed, by previous studies (34,38,39)]. We confirmed 16 of these putative sRNAs by northern blot (two other tested regions could not be confirmed). We named them RsaOH to RsaOW [for RNA *S. aureus* Orsay (35)] and the corresponding genes *rsaOH* to *rsaOW* (Table 1). While the present work was being completed, the sRNAs RsaOJ, RsaOK and RsaON were independently reported; we will refer to them using the recently published names, RsaA, RsaH and RsaE, respectively (36).

Expression of *S. aureus* sRNA genes encoded in IGRs

Expression of the 15 identified RNAs was followed as a function of the growth phase. An overnight culture of N315 was diluted into a fresh rich medium and grown aerobically at 37°C; samples were withdrawn at different stages of growth and sRNA amounts were evaluated by northern blot analysis (Figure 1). We also examined the expression of six sRNAs in the six growth and stress conditions used for the RNomics approach (Supplementary Data S4). The studied sRNAs presented below are ordered according to their position on the N315 genomic map. Conservation of the identified sRNAs in other species is indicated in Table 1.

RsaOH is about 100-nt long. Its gene, *rsaOH*, is located between SA0065 and *kdpE* genes encoding a putative C4-dicarboxylate transporter and a putative transcriptional regulator belonging to the OmpR family of two-component response regulators, respectively. The *rsaOH* detected sequence is adjacent to the 3′-end of *kdpE* (Supplementary Data S3). One may speculate that RsaOH interacts with the 3′-end of the *kdpD-kdpE* mRNA and therefore could regulate its stability. RsaOH increases with the growth phase but strongly decreases in the stationary phase (Figure 1).

RsaOI is about 240-nt long; it is the longest sRNA that we identified, and contains no expected ORF. Its gene, *rsaOI*, is located between *proP*, encoding a putative transporter and partially (38 nt) overlaps in antisense orientation with the 5′ end of SA0532 encoding a *Staphylococcus*-specific hypothetical protein (Supplementary Data S3). It is therefore possible that RsaOI affects transcription or translation of SA0532. In contrast to RsaOH, RsaOI expression drastically increases in stationary phase (Figure 1).

RsaA (RsaOJ) is 142-nt long (36). Its gene, *rsaA*, is located between SA0543 encoding a conserved putative membrane protein and SA0544 encoding a putative heme peroxidase. Like RsaOI, RsaA, is detected in all growth phases and drastically increases in stationary phase (Figure 1). RsaA did not accumulate upon thermal, salt or oxidative stress (Supplementary Data S4).

RsaH (RsaOK) is 128-nt long (36). Its gene is located between SA0724, encoding a hypothetical protein similar to a cell-division inhibitor, and SA0725, encoding a protein of unknown function. RsaH could encode a putative 23 amino-acid polypeptide (MCKTLHDTNEGKNLTPFSSGPVR). In the corresponding IGR of *S. aureus* strain RF122 the SAB0725 ORF of a putative 52 amino-acid protein with no significant similarity to proteins from other organisms has been annotated. However, in the majority of *S. aureus* strains, including N315, the coding sequence is interrupted by a stop codon leading to the putative 23 amino-acid peptide. RsaH was previously reported to be induced in stationary phase (36). We found that in N315, RsaH is barely detectable in early exponential phase, but accumulates during

Table 1. sRNAs identified in this RNomics study

RNA name[a]	IGR	Flanking genes	Strand Orientation	Nb of reads[b]	Alternative names	Conservation[c]
RsaOH	72446/73508	SA0065/*kdpE*	< > <	1	IGR59 (34)	*S. aureus*, *S. epidermidis*, *S. haemolyticus*
4.5S RNA (34)	501358/502001	SA0434/SA0435	> > >	23	SRP RNA	Conserved
RsaOI	626797/627130	*proP*/SA0532	> < >	4	Sau-6477 (37)	*S. aureus*
RsaA (36)	636758/637419	SA0543/SA0544	< > <	2	RsaOJ (this work), Sau-64 (37)	*S. aureus*, *S. epidermidis*
RsaC (36)	679620/680738	SA0586/SA0587	< < <	1		*S. aureus*
RsaH (36)	829363/830064	SA0724/SA0725	< > >	2	RsaOK (this work)	Staphylococcal strains
tmRNA (34)	843706/844543	smpB/SA0737	> > >	27	SsrA	Conserved
RsaOL	856992/857482	*cspC*/SA0748	> < <	1	Sau-07 (37)	*S. aureus*, *S. epidermidis*
RsaOM	875349/875597	SAS023/SA0769	> > >	1	Predicted SAM-I riboswitch	Conserved
RsaE (36)	975283/975742	SA0859/SA0860	> > <	3	RsaON (this work) Sau-20 (37)	Bacillales order
Sau-02 (37)	1006365/1007020	SAS028/SAS029	> < >	1		*S. aureus*, *S. epidermidis*, phages, plasmids
RsaOO	1012811/1013050	SA0891/SA0892	> < <	1		*S. aureus*
RsaOP	1089149/1089566	SA0961/SA0962	> > >	1		Staphylococcal strains
RsaOQ	1372074/1372564	SA1198/SA1199	> > >	1	Predicted T-box	Conserved
RNaseP RNA (34)	1483578–1484012	SA1277/SA1278	< < <	2		*S. aureus*, phages
6S RNA (34)	1660651–1660795	SAS050/*aspS*	< < <	2	Srr80 (38,39)	Conserved
SprA (34)	1856223/1856978	SA1623/tnp IS232	< > <	1		Staphylococcal strains
SprB (34)	1866662/1867133	SA1633/SA1634	> < <	3		*S. aureus*
SprC (34)	1871168–1872530	*luckE*/SA1639	< < >	1		*S. aureus*, *S. epidermidis*, *S. haemolyticus*
SprD (34)	2006879–2007560	SA1754/SA1755	< > <	3		*S. aureus*
RsaOR	2008572–2009085	SA1756–57/*sak*	< < <	1	Srr6 (38,39, SprX)	*S. aureus*, phages
SprF3	2010790–2011000	SA1956/SAS069	> > >	1	RsaOS (this work)	Staphylococcal strains
Sau-30 (37)	2294189/2294725	cbiO/rplQ	< < <	2		Staphylococcal strains
RsaOG (35)	2367825/2368208	SA2104/SA2105	< < >	2	RsaI (36)	Staphylococcal strains
SprA2 (34)	2490680–2491385	SA2217/SA2218	< < >	1	WAN014FZW (38,39)	Staphylococcal strains
RsaOT	2544336–2544594	SA2267/SA2268	> < <	7	Srr43 (38,39)	*S. aureus*
RsaOU	2611619/2611918	*ptsG*/SA2327	< < <	2	Rat sequence	
RsaOV	2632123/2632361	SA2343/*copA*	> > >	1	Sau-40 (37)	*S. aureus*
RsaOW	Eight possible positions	Tnp for IS1181	< > –	4		
RsaOX		SA0062	*Cis*-encoded	1		

[a]Gene names corresponding to sRNAs confirmed by northern blot previously or in this study (cf. Figure 2 and Supplementary Data S4).
[b]Number of reads obtained by 454 sequencing matching the corresponding indicated IGR in given orientations (cf. Supplementary Data S2).
[c]Conservation determined by Blast analysis (default parameters) using the identified or predicted sRNA gene sequence. Conserved indicates that the sequence can be found in phylogenetically distantly related bacteria.

pre-stationary phase (Figure 1), and then strongly decreases in stationary phase.

RsaOL is estimated to be about 100-nt long, according to our northern blot probing. It is transcribed from a gene located in the IGR between *cspC* (encoding a cold shock protein from the CspA family) and SA0748 (encoding a poorly conserved putative protein of unknown function). RsaOL is expressed in all growth phases tested. Its quantity increases with the growth phase, except in stationary phase, where levels are slightly decreased (Figure 1).

RsaE (RsaON) is 93-nt long (36). The *rsaE* gene is located between SA0859, encoding a predicted dithiol-disulfide isomerase involved in polyketide biosynthesis, and SA0860 encoding an oligoendopeptidase. The RsaE sequence is present in the bacillales order and the synteny is conserved within the Firmicute phylum with the noticeable exception of the Listeriaceae family. The *rsaE* gene is identical in all *S. aureus* strains sequenced to date. RsaE is thus likely under a strong selective pressure for the conservation of its primary sequence: one possibility is that

RsaE interacts with a specific protein requiring a defined nucleotide sequence for interaction and subsequent function. However, experiments based on the incubation of synthetic RsaE with protein extracts did not identify an RsaE-specific binding protein (data not shown). RsaE was also reported as induced in stationary phase (36). However, a detailed analysis reveals that in N315, although RsaE is detectable during all growth phases, it accumulates specifically during pre-stationary phase ($OD_{600} = 6$) and almost disappears in stationary phase (Figure 1). Its expression profile is similar to that of RsaH. In several strains, RNAIII is also induced in late exponential phase. Transition to stationary phase and possibly virulence activate the expression of several sRNAs, which make them candidates for being key regulators of transition adaptation processes. Furthermore, RsaE is reduced under heat-shock (Supplementary Figure S4).

RsaOO is about 180-nt long. Its gene, *rsaOO*, is located between SA0891 and SA0892, both of which encode membrane proteins. A second product, about 140-nt
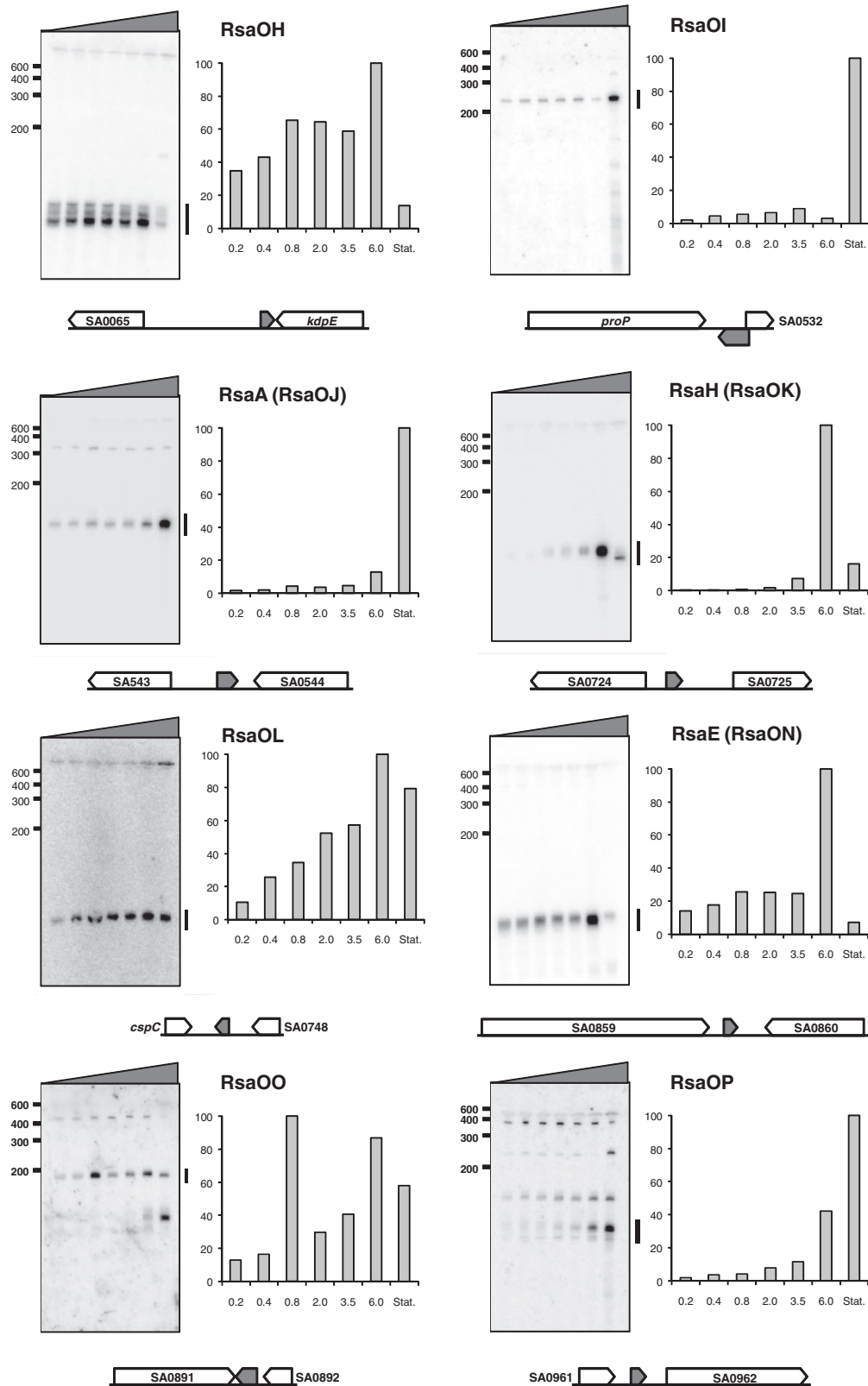
Figure 1. Identification and characterization of new N315 *S. aureus* sRNAs derived from the RNomics approach. Northern blots showing the expression of various sRNA candidates in nutrient-rich medium are presented. Total RNAs were prepared from cultures harvested at OD_{600}: 0.2, 0.4, 0.8, 2, 3.5, 6 and 10. Black wedges indicate the increasing OD_{600} values from left to right. All blots presented were performed using the same RNA extracts. Marker sizes are shown to the left of each blot. RNAs were detected using $\gamma^{32}P$-labeled oligonucleotides designed to pair with the predicted RNAs (cf. Supplementary Data S1). 5S rRNA was used as a loading control. Histograms indicate the relative expression of each sRNA in different growth phases (the intensity of each band was divided by the intensity of the 5S band from the same RNA extraction; and the maximum value for each histogram was set to 100). A bar on the right side of the blot indicates the band(s) selected for quantification. Flanking or overlapping genes (open arrows) of each sRNA (grey arrow) is presented to indicate genetic localization. Arrows indicate the direction of transcription and are proportional to known or expected transcript sizes.
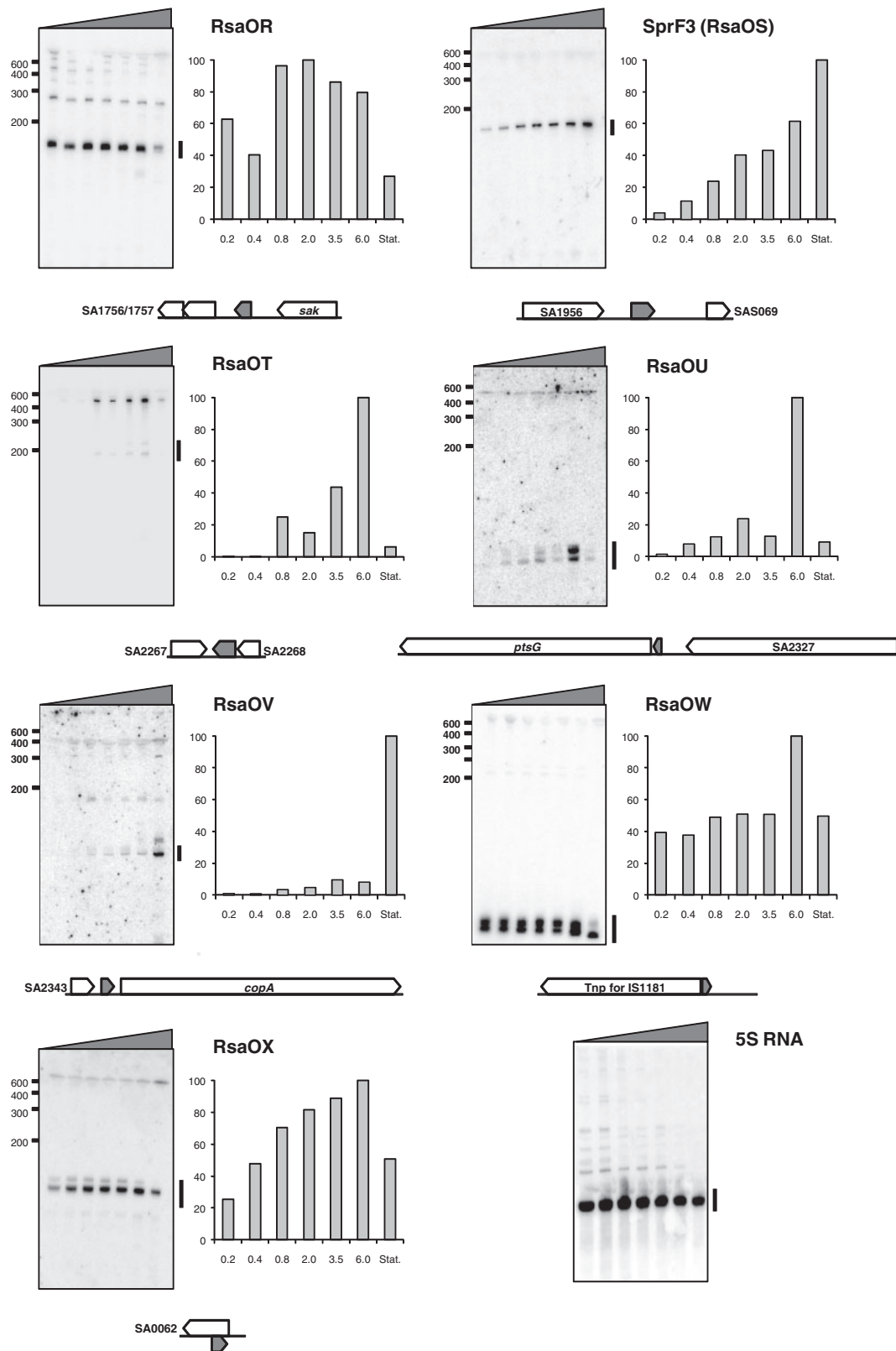
Figure 1. Continued.

long, accumulates in stationary phase, possibly issued from the processing of RsaOO (Figure 1).

The *rsaOP* gene is located between SA0961 and SA0962, which encode a protein of unknown function and an integral membrane protein (FtsW) involved in cell division, respectively. Several products were detected when probing for RsaOP; the major one is about 100-nt long and accumulates in stationary phase (Figure 1).

RsaOR (alias SprX) is about 160-nt long with a possible longer form. Its gene is located between SA1757, encoding a putative truncated amidase, and *sak*, encoding staphylokinase. The sequence comprising this region, including adjacent gene SA1757 is also found in several phages (tp310-3, 52A, phi12, tp310-2, 80apha, phiNM4, etc.). RsaOR is thus likely acquired via horizontal transfer and expressed by phages. RsaOR is expressed throughout growth, but amounts are reduced in stationary phase (Figure 1). It also accumulates upon salt stress (Supplementary Data S4). N315 RsaOR may encode a putative polypeptide of 40 amino acids (MHQLFTSLFT QACHWVFFLM IESIVFILLP RSIYDFSIPV). However, the initiation codon is absent in most *S. aureus* sequenced genomes and there is no obvious ribosome binding site sequence. Therefore, if expressed, this peptide would be expressed only in few *S. aureus* strains. We therefore favor the hypothesis that RsaOR is a regulatory sRNA.

The intergenic SA1760/SAS059 region contains *sprF* and on its complementary strand *sprG*. In N315, the corresponding RNAs SprF and SprG were proposed to have paralogs expressed from two other separated regions located on pathogenicity islands (34). A read obtained by 454 (Supplementary Data S3) matched with the SA1956/SAS069 IGR containing the putative *sprF3* (*rsaOS*) gene. Northern blot experiments show that the expression of *sprF3* increases gradually with the growth phase (Figure 1). 5' RACE mapping experiments (Supplementary Data S3) indicate that the sequence of the first 47 nt of *sprF3* does not match *sprF* and *sprF2*, suggesting that targets of SprF3 could differ from those of SprF and SprF2.

RsaOT is about 180-nt long. It is poorly expressed and we could not identify its extremities. *rsaOT* expression is at a maximum during pre-stationary phase (Figure 1) and is also stimulated by the presence of H_2O_2 in the medium (Supplementary Data S4). As one of the RsaOT cDNA sequences obtained via our RNomic study is only at 6 nt downstream from the SA2268 stop codon, it is possible that the sRNA detected by northern blot is issued from 3' end processing of SA2268.

RsaOV is about 100-nt long. Its gene, *rsaOV*, is located between SA2343 and *copA*, which encode a putative poorly conserved protein and a copper translocating ATPase, respectively. RsaOV accumulates in stationary phase (Figure 1).

The *rsaOW* gene is present in eight copies (*rsaOW1* to *rsa0W8*) on the N315 genome; it is located immediately upstream of the ORF encoding the IS1181 transposase (Supplementary Data S3). RsaOW is therefore perfectly complementary to the transposase 5' UTR region (including its ribosome binding site). As RsaOW is

detected in all growth conditions (Figure 1), one may hypothesize that its role is to control, likely lowering, the expression of the IS1181 transposase. RsaOW would therefore affect the *S. aureus* genome evolution.

Our results confirm the existence of several *trans*-encoded *S. aureus* sRNAs and show that their expression is, for the most part, dependent on growth conditions.

Expression of *cis*-encoded *S. aureus* sRNA genes

Our RNomics approach predicted seven candidates of *cis*-encoded sRNAs; we were able to detect one of them, RsaOX, by northern blot analysis (Figure 1). The others gave either no hybridization signal or signals corresponding to high-molecular weight RNAs (data not shown). RsaOX is about 120-nt long and is transcribed from the complementary strand of SA0062; it was expressed in all of the tested growth phases. As SA0062 encodes a putative transposase, the existence of RsaOX is reminiscent of the system described for the *E. coli* IS10 transposase, whose mRNA (called RNAin) is controlled by RNAout, its *cis*-encoded sRNA (60,61). SA0062 mRNA was recently proposed to be the target of MazF$_{SA}$, a sequence-specific endoribonuclease (62). One can postulate that in addition to MazF$_{SA}$, RsaOX could prevent the synthesis of the likely deleterious SA0062-encoded transposase and therefore might limit transpositions.

Identification and expression of sRNA for putative *S. aureus* riboswitches

The RNomics analysis allowed us to identify transcripts that likely result from premature transcriptional termination associated with riboswitch activities. RsaOM corresponds to the sequence upstream of SA0769 encoding a D-methionine transport system ATP-binding protein. Probing for this region revealed several small transcripts more expressed in the high salt stress condition (Supplementary Data S4). The SAS023-SA0769 IGR is predicted to contain a SAM riboswitch [also known as S-box leader or SAM-I riboswitch; (63)], which is found upstream of genes involved in methionine or cysteine biosynthesis (64). The effector, S-adenosyl-methionine, specifically binds to the nascent S-box RNA leading to a premature transcription arrest (65,66), which would explain the observed short transcripts (Supplementary Data S4). Surprisingly, the N315 predicted SAM riboswitch may encode a putative protein of 60 amino acids conserved in *S. aureus* genomes. However, this ORF does not exist in other species, suggesting that if expressed in *S. aureus*, its activity might not be related to the SAM riboswitch control.

RsaOQ corresponds to the upstream sequence of SA1199, which encodes component I of the anthranilate synthase involved in phenylalanine, tyrosine and tryptophan biosynthesis. Probing for this sequence revealed several bands with a different profile in stationary phase as compared to the exponential phase or stress conditions (Supplementary Figure S4). The SA1198-SA1199 IGR is predicted to contain two tandem T-boxes (63,67). These leader elements sense uncharged tRNA to regulate the expression of aminoacyl–tRNA synthetase genes and

amino-acid biosynthetic genes such as anthranilate synthase (68).

RsaOU is a tiny sRNA (estimated length is <70 nt) originating immediately upstream of *ptsG*, which encodes a component of the glucose-specific phosphotransferase system. RsaOU is highly expressed in pre-stationary phase (Figure 1). It is likely that *rsaOU* acts as a *cis*-element on *ptsG* expression. Indeed, the *ptsG* operon of *Staphylococcus carnosus* is regulated by a termination/antitermination mechanism associated with a sequence called RAT for ribonucleic antiterminator (69,70). HPr-dependent phosphorylation of GlcT facilitates antitermination (69,70). A similar regulation is likely to exist in *S. aureus*; in addition to *ptsG*, *glcA*, which encodes another enzyme from the PTS system, is preceded by a RAT sequence that generates an sRNA (36).

RsaE downregulates the one carbon pool by folate and central metabolic pathways

The *rsaA* (*rsaOJ*), *rsaE* (*rsaON*) and *rsaH* (*rsaOK*) genes (corresponding to RNAs highly expressed during specific growth phases) were cloned under the regulatory control of the $P_{xyl/tetO}$ promoter of pAT12, giving rise to pAT12-RsaA, pAT12-RsaE and pAT12-RsaH, respectively. These plasmids express the Tet repressor that binds to the *tetO* operator; sRNA expression was induced by addition of anhydrotetracycline (aTc) to the media. We also constructed derivatives, pAT12-ASRsaA, pAT12-ASRsaH and pAT12-ASRsaE expressing antisense RNAs of RsaA, RsaH and RsaE, respectively. RN6390 containing pAT12-RsaE, but not the other above described plasmids, was sensitive to aTc (100 nM) in a solid rich media at 30, 37 or 42°C (Figure 2 and data

not shown). Accumulation of RsaE may cause toxicity by affecting essential functions; we therefore focused our interest on the functional characterization of RsaE.

Many sRNAs act *via* base-pairing to target mRNAs to affect their stability and/or translation. We hypothesized that RNA targets of RsaE would vary upon RsaE accumulation, and could thus be identified by transcriptome analysis. We therefore performed an mRNA analysis of strains expressing a plasmid-encoded RsaE (pAT12-RsaE) or RsaE-antisense (pAT12-ASRsaE), sampling RNA 5 min after induction of RsaE or its antisense sequence; this approach was designed to identify those mRNAs with which RsaE directly interacts, and is similar to the pulse-expression approaches that facilitated sRNA target discovery in *E. coli* and *Salmonella* (16,19). Preliminary northern blot analysis showed that 5 min of induction was sufficient for strong accumulation of either RsaE or its antisense RNA (data not shown). Induction was performed at $OD_{600} = 0.6$, when the expression of the chromosomally encoded RsaE is low (Figure 1). RsaE accumulation led to the downregulation of 25 genes; surprisingly, it also upregulated 39 genes at least 2-fold (Table 2 and Supplementary Data S5). To consolidate our results, the expression of 16-selected genes was studied *via* a kinetic of RsaE induction at time 0, 5, 10, 20 and 30 min by qRT–PCR (Table 2 and Supplementary Data S6). With the exception of one gene (*opp3A*, cf. Table 2 legend), the qRT–PCR results fully confirmed the transcriptome data. The mRNAs modulated either directly or indirectly by RsaE mainly encode membrane proteins or metabolic enzymes.

Strikingly, mRNAs that are downregulated by RsaE can be clustered around two essential pathways (Figure 3): (i) SA1366-SA1367 (*gcvP-gcvT*) and SA1553 (*fhs*) mRNAs are associated with production of methenyl tetrahydrofolate (THF) required for the

Figure 2. Accumulation of RsaE induces a growth defect that is partly alleviated by acetate. Overnight cultures were prepared in BHI from the strains listed below. Five-microliter spots of ten-fold serial dilutions of cultures were deposited from top (most concentrated) to bottom on BHI plates supplemented or not with aTc and acetate (100 mM) as indicated, and incubated at 37°C. Strains are RN6390 derivatives containing the indicated plasmids pAT12, pAT12-RsaE and pAT12-ASRsaE. pAT12-RsaE and pAT12-ASRsaE contain the *rsaE* gene and its antisense, respectively, both of which are under the transcriptional control of the P_{tet} promoter (inducible by aTc). Experiments were also performed at 30 and 42°C, and in LB medium, and gave similar results (data not shown).

Table 2. Variation of gene expressions upon 5 min of RsaE induction

Gene name[a]	Affim.[b]	qRT–PCR[c]	Δg[d]	Function
SA0845 (*opp3B*)	1.10	0.79	−25.47	Oligopeptide transport system
SA0849 (*opp3A*)[e]	2.18	0.76	−24.31	Oligopeptide transport system
SA0850 (*opp4A*)	4.38	6.06		Peptide/nickel transport system
SA0851 (*opp4D*)	3.15	4.59	−14.70	Peptide/nickel transport system
SA0873	0.48[f]		−21.13	Hypothetical protein
SA1088 (*sucC*)	0.54[f]		−18.24	Succinyl–CoA synthetase subunit beta
SA1089 (*sucD*)	0.47[f]		−15.51	Succinyl–CoA synthetase subunit alpha
SA1184 (*citB*)	0.47		−15.61	Aconitate hydratase
SA1365 (*gcvPB*)	0.48			Glycine dehydrogenase subunit 2
SA1366 (*gcvPA*)	0.36	0.48	−21.88	Glycine dehydrogenase subunit 1
SA1367 (*gcvT*)	0.32	0.36		Aminomethyltransferase
SA1432	0.43	0.46	−18.04	Hypothetical Mn^{2+} and Fe^{2+} transporter
SA1434	0.43			Acetyl–CoA carboxylase
SA1435	0.45			Acetyl–CoA carboxylase
SA1517 (*citC*)	0.40			Isocitrate dehydrogenase
SA1518 (*citZ*)	0.37	0.48	−14.80	Methylcitrate synthase
SA1553 (*fhs*)	0.30[f]		−14.08	Formate–tetrahydrofolate ligase
SA1858 (*ilvD*)	3.45	1.90		Dihydroxy-acid dehydratase
SA1859 (*ilvB*)	2.75			Acetolactate synthase large subunit
SA1860 (*ilvH*)	3.12			Acetolactate synthase 1 regulatory subunit
SA1861 (*ilvC*)	2.17			Ketol-acid reductoisomerase
SA1862 (*leuA*)	2.77			2-Isopropylmalate synthase
SA1863 (*leuB*)	2.56			3-Isopropylmalate dehydrogenase
SA1865 (*leuD*)	3.11			Isopropylmalate isomerase small subunit
SA1968 (*arg*)	0.35	0.44	−18.81	Arginase

RN6390 strains containing either pAT12-RsaE or p12-ASRsaE were grown at 37°C in BHI to OD600: 0.6. aTc was added to the medium and after 5 min, samples were removed and total RNA were extracted.
[a]SA0845 to SA849, SA1088-SA1089, SA1367-SA1365, SA1435 to SA1432, SA1518-SA1517, SA1858 to SA1865, likely organized in six distinct operons, respectively (genes for the same operon are expected to have a RsaE-dependent coordinated regulation).
[b]Gene expression ratios between strains containing pAT12-RsaE versus p12-ASRsaE obtained by Affimetrix microarray analysis (see also Supplementary Data S6).
[c]Gene expression ratios between strains containing pAT12-RsaE versus p12-ASRsaE obtained by qRT–PCR analysis. Experiments were also performed with samples taken at 0, 10, 20 and 30 min after the aTc addition to the media (Supplementary Data S7).
[d]Free energy of pairing between RsaE and the 5′ region of corresponding mRNAs as calculated by RNAup software (71).
[e]Discrepancy was observed between transcriptome and qRT–PCR experiments in the case of *opp3A* mRNA is possibly due to cross-hybridization of opp4A cDNA with the transcriptome opp3A probes.
[f]Results supported by published transcriptome results (36).

one-carbon shuffle, and (ii) SA1088-SA1089 (*sucC-sucD*), SA1184 (*citB*) and SA1517-SA1518 (*citC-citZ*) mRNA, which produce enzymes of the Krebs cycle. The growth inhibitory effect of RsaE overproduction is consistent with the observed downregulation of these genes; furthermore, addition of acetate to the media, which should allow acetyl–CoA formation for partial bypass of the TCA cycle, partly suppressed the toxicity (Figure 2).

Genes upregulated by RsaE include the operon for valine, leucine and isoleucine biosynthesis. In addition, several genes, likely encoding membrane proteins involved in peptide transport, are upregulated. Among them, two encode subunits of an oligopeptide permease (Opp) [SA0850 (*opp4A*), SA0851 (*opp4D*)].

The RsaE accumulation appears to correlate with an adaptation response aimed at reducing central metabolic pathways and increasing the amino acid pool.

RsaE binds the *opp3A* mRNA ribosome-binding site

To identify the primary RsaE targets among the putative candidates revealed by the transcriptome analysis, we performed a computational screen using RNAup. This software determines pairing energies between sRNAs and putative mRNA targets (71). Pairing energies

between RsaE and the 5′ region (−150/+20) of all *S. aureus* CDSs were evaluated; one of the best scores was obtained with SA0849 (Δ*G*:−21 kcal/M) encoding Opp3A. The transcript of the two immediate *opp3A*-downstream genes strongly accumulates upon RsaE induction (Table 2 and Supplementary Data S5 and S6). These *opp* genes encode components of ABC transporters possibly involved in peptide/nickel transport (72). The combination of computational and transcriptome data indicates that RsaE could affect *opp* regulation by pairing with the SA0849 mRNA.

The interaction of RsaE with different mRNAs predicted to be RsaE targets (i.e. by trancriptome results and computational analyses using RNAup) was tested by gel retardation assays. The candidates correspond to (i) *opp3A* (SA0849) and *opp4D* (SA0851) encoding components of oligopeptide transport systems (72), (ii) SA1432 and SA0645 encoding putative manganese and anion transporters, respectively, (iii) *sucC* (SA1088) and *sucD* (SA1089) encoding the succinyl–CoA synthetase alpha and beta subunits, respectively, (iv) *gcvP* (SA1366) and *gcvT* (SA1367), encoding enzymes from the glycine cleavage pathway, (v) *acsA* (SA1554) encoding the acetyl–CoA synthetase and (vi) *arg* (SA1968) encoding
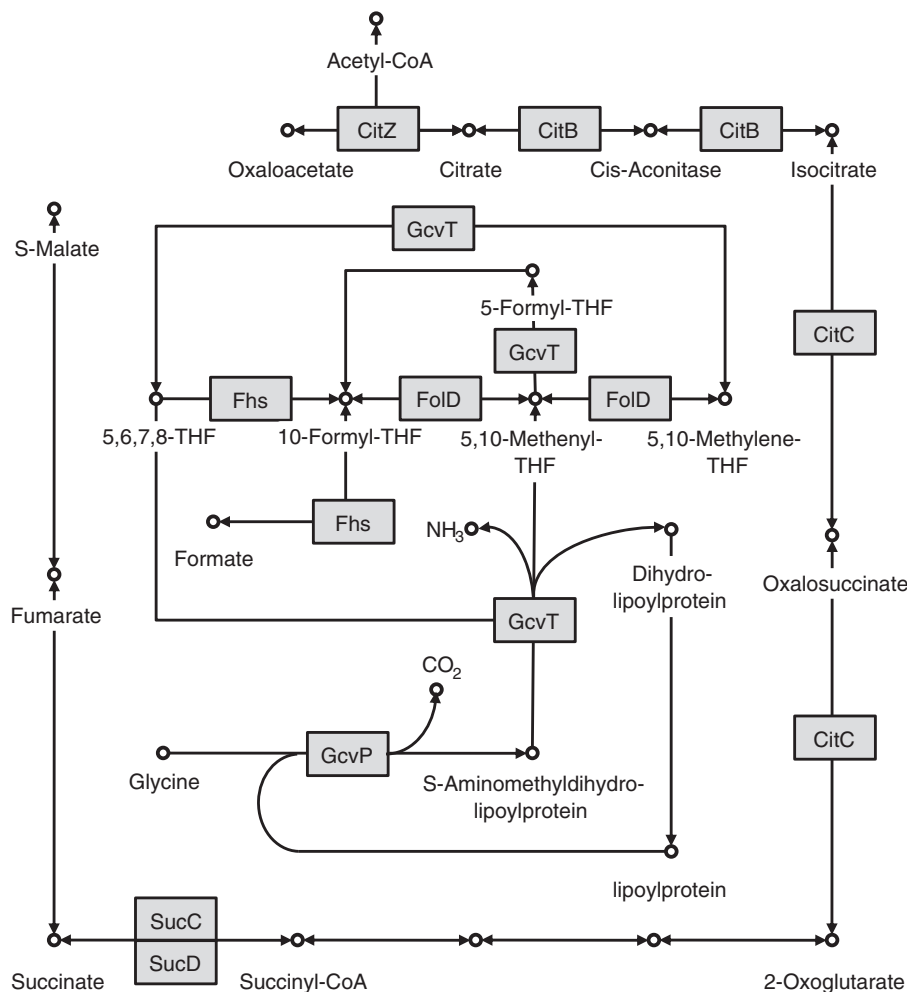
Figure 3. RsaE accumulation downregulates specific metabolic pathways. The metabolic charts are adapted from the Kegg database (90). Enzymes corresponding to downregulated genes by RsaE are indicated by square boxes highlighted in grey [FolD [SA0915] indicated on the figure was not found in this study, but was previously reported (36)]; metabolites are shown as open circles; biochemical reactions that are demonstrated or predicted in N315 by arrows, respectively. The outer pathway is the TCA cycle. The inner pathway is the THF biosynthesis pathway. In certain organisms, these two pathways are connected *via* the glyoxylate shunt, which leads to a modified version of the TCA cycle that bypasses certain steps. However, connecting enzymes in N315 are not yet known.

arginase. (Additional candidates based solely on bio-computing data were also tested: *opp3B* [SA0845], *opp4C* [SA0854], SAS045, SA0308, SA0785 and SA2118.) A synthetic full-length RsaE and truncated mRNAs (~180 nt long; for oligonucleotides used, see Supplementary Data S1) were produced from PCR-generated genes placed under the control of the T7 RNA polymerase using an *in vitro* transcription assay. The putative RsaE targets tested were about 140-nt long with about 80-nt corresponding to the Shine-Dalgarno (SD) upstream sequences. Among these, only one mRNA, corresponding to *opp3A* (SA0849), formed a complex with RsaE *in vitro* (Figure 4A and B). Complex formation between RsaE and SA0849 is specific since a thousand fold excess of tRNA did not disrupt the sRNA–mRNA duplex (Figure 4B).

Predicted pairings between RsaE and the SA0849 mRNA (Figure 6) suggest that RsaE interacts with the opp3A SD sequence, as well as part of the AUG initiation codon and therefore could prevent translation

initiation. To test this, toeprint assays were performed on ternary initiation complexes including purified 70S ribosomes, initiator tRNA[fMet] and the SA0849 mRNA. Two ribosome toeprints were detected on the mRNA, at 14 and 15-nt downstream from the initiation codon respectively (Figure 4C), supporting the location of the SA0849 mRNA start codon as drawn on Figure 6. RsaE reduced ribosome loading onto the SA0849 mRNA in a concentration-dependent manner (Figure 4C). This demonstrates the formation of a translational initiation complex on the SA0849 mRNA. We conclude that RsaE inhibits SA0849 mRNA translation by antisense pairing, thus preventing ribosome binding. The fact that RsaE (i) is predicted to efficiently pair with the *opp3A* mRNA at the SD site, (ii) forms a specific complex *in vitro* with the *opp3A* mRNA transcript and (iii) prevents *in vitro* formation of an *opp3A* mRNA translation initiation complex, together suggest that RsaE targets interacts with the *oppA3* mRNA and prevents its translation.
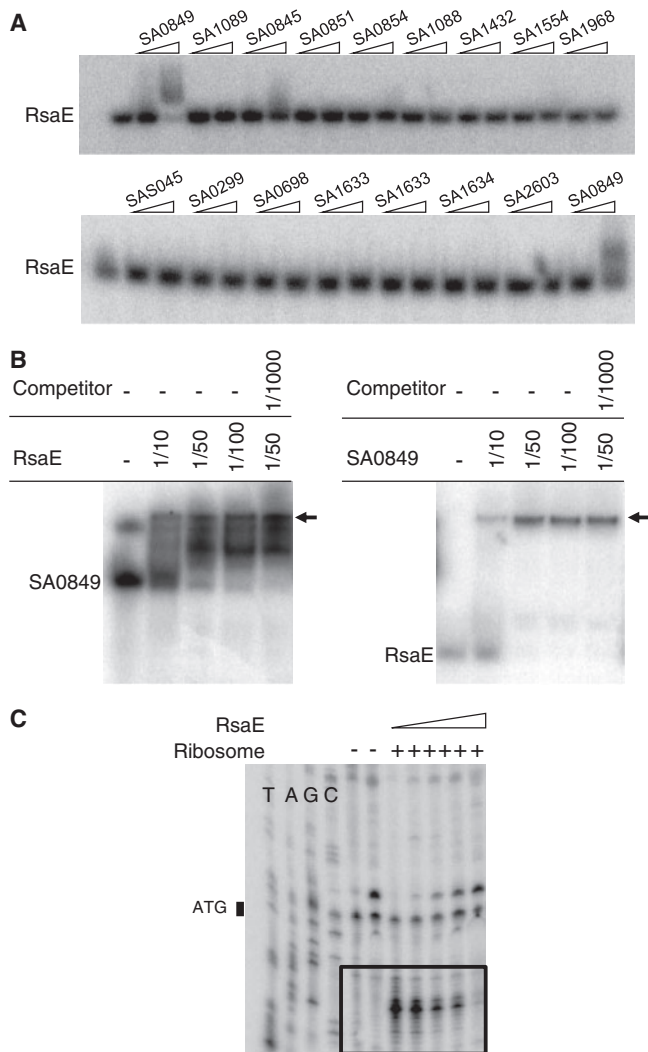
Figure 4. RsaE interacts with the 5′ end region of *opp3A* (SA0849) mRNA. (**A**) Gel retardation assay of labeled RsaE (0.4 pmol) incubated with synthetic RNAs (4 and 40 pmol), corresponding to different 5′ gene ends of the indicated genes (see Supplementary Data S1 for the sequences). The RsaE/SA0849 mRNA interaction is shown by a mobility shift of labeled RsaE in the presence of SA0849 mRNA (RsaE mobility was not affected by the other tested RNAs). (**B**) Left: Gel retardation assay of synthetic labeled SA0849 mRNA 5′ end fragment (0.4 pmol) incubated with increasing concentrations (molar ratio is indicated) of synthetic RsaE. Right: Gel retardation assay of labeled RsaE (0.4 pmol) incubated with increasing concentrations (molar ratio is indicated) of synthetic SA0849 mRNA 5′ end fragment. Addition of a large excess of unlabelled tRNAs did not alter the RsaE/SA0849 mRNA interactions in either assay. (**C**) RsaE prevents ribosome loading and translation initiation on opp3A SA0849 mRNA. Ribosome toeprints on SA0849 mRNA. '−/+' indicates the absence or presence of purified ribosomes. The wedge indicates increasing amounts of RsaE. The experimentally determined toeprints are boxed. T, A, G and C refer to the SA0849 mRNA sequencing ladders. The position of the SA0849 mRNA initiation codon is indicated (ATG).

DISCUSSION

We used an RNomics approach based on deep sequencing of RNA enriched for primary transcripts (73) to search for novel staphylococcal sRNAs. Our analysis of 36 000 reads identified 30 known or putative sRNAs encoded within

IGRs (Table 1 and Figure 5). Fourteen sRNAs confirmed by northern blots were categorized as: (i) sRNAs that could target genetically unlinked mRNAs (eight new instances) or (ii) sRNAs that pair with the UTRs of mRNAs expressed from the adjacent gene (two new instances). We also detected small transcripts likely associated with three riboswitch activities. Seven sRNAs were expressed from the opposite strand of ORFs; one is a bona fide *cis*-encoded RNA. We recently predicted 50 regions as encoding sRNAs; out of 24 tested, seven were confirmed to express sRNAs (35). Additionally, five of the newly reported sRNAs (RsaE, RsaOM, RsaOP, RsaOQ and RsaOW) are among the untested previously predicted regions. This observation brings further support for this computational screen based on phylogenetic profiling, which is particularly efficient at detecting novel bacterial sRNAs (35).

Most *S. aureus* RNAs are differentially expressed in a growth phase-dependent fashion (34,36,58). The detailed analysis of sRNA expression performed in this study reveals that some sRNAs accumulate in exponential (RsaOH, RsaOR), pre-stationary (RsaH, RsaE, RsaOT, RsaOU) or stationary (RsaOI, RsaA, RsaOP, RsaOV) phases. We observed abrupt changes in the expression levels of several sRNAs between $OD_{600} = 6.0$ and stationary phase ($OD_{600} = 10$). It is striking that several sRNAs such as RsaH and RsaE accumulate specifically in pre-stationary phase, and disappear in stationary phase, suggesting a role in adaptation to growth phase changes. Numerous staphylococcal regulatory functions are coordinated with environmental conditions or growth phase. Virulence factor expression is correlated with growth phase: surface adhesins are generally expressed in exponential phase, whereas exoproteins are expressed in stationary phase. The newly identified sRNAs conceivably play role(s) in this regulation, in addition to RNAIII (29). We tested the effect of several regulators on the expression of *rsaE*. RsaE quantities were compared in isogenic 8325-4 strains deficient for regulators SigB (74), SarA (75), Agr (76), PerR (77), Fur (78) or Spa (79), or proficient for RsbU (80); no significant differences were observed in any of those genetic contexts, suggesting that RsaE expression is probably regulated independently of these regulators (data not shown). A σ^A-consensus binding site is located just upstream of the *rsaE* start site, indicating expression driven by the *S. aureus* vegetative σ factor.

The primary RsaE nucleotide sequence is surprisingly well conserved among the sequenced bacillales, and RsaE over-expression is toxic. Altogether, these observations suggest important functional role(s) for RsaE. A variation in the quantity of RsaE induced a significant change in the transcriptional pattern. Among the up or downregulated genes, many express mRNAs that are predicted to pair with RsaE. Structure probing (36) and computational analyses suggest that RsaE folds as two stem loops separated by a 17-nt-long RNA single strand. RsaE has a duplicated, CCCCUUUGUUU accessible sequence with one copy in the loop of the first hairpin and a second within the central single stranded domain. The predicted pairings between RsaE and the targeted mRNAs
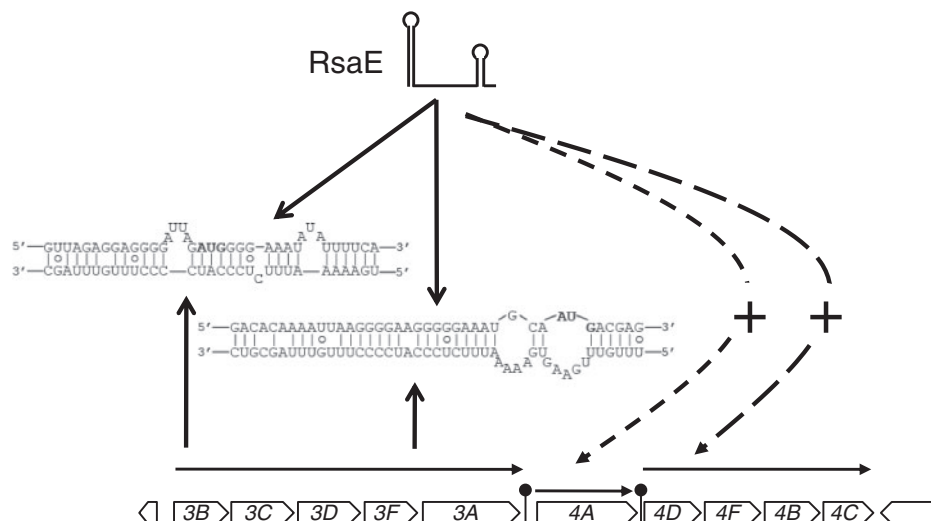
Figure 5. Genomic map showing the distribution of *S. aureus* sRNAs. Flags indicate the names of sRNAs that were predicted and confirmed by northern blot analysis; sRNAs uncovered by the present work are highlighted in grey (34–37,58).

involve the duplicated sequence and the mRNA SD sequences (Supplementary Data S7). Repetition of that interacting sequence on the sRNA suggests several pairing combinations with its mRNA substrates, involving either the RsaE apical loop 1 or its unstructured central domain.

The *opp* operons encode ABC transporters involved in the transport of small peptides. *Staphylococcus aureus* has several putative *opp* operons, three of which (*opp-1*, *opp-2* and *opp-3*) are conserved in all strains (72). The *opp-3* operon is dedicated to nitrogen nutrition by peptide import and is also involved in the regulation of extracellular proteases (SspA and Aur) via an unknown mechanism (81). In several Enterobacteriales, expression of dipeptide, oligopeptide and microcin ABC transport systems are partly controlled by sRNAs such as GcvB and RydC (52,82–84). RsaE targets the *opp* operons and is thus a possible GcvB/RydC functional analog. However, we detected no sequence similarities between RsaE and these sRNAs. In N315, the *opp-3* operon comprises *opp3B*, *opp3C*, *opp3D*, *opp3F* and *opp3A*; it is followed by a second *opp* operon, *opp-4*, comprising *opp4A* and *opp4D* (72). The function of the *opp-4* operon is at present unknown. Toeprint experiments show that RsaE interacts with the *opp-3* mRNA (Figure 4). Surprisingly, RsaE induction had no significant effects on the amounts of *opp-3* mRNAs, but strongly

upregulated the downstream operon, *opp-4* (quantified by qRT–PCR, cf. Table 2). Several interpretations can be considered for these results: (i) the observed RsaE mediated inhibition of ribosome loading on *opp3A* mRNA (Figure 4) could in turn, upregulate the *opp-4* operon (i.e. Opp proteins could mediate this regulation) (ii) interactions between RsaE and *opp-3* mRNA generate an anti-termination mechanism that leads to increased *opp-4* operon transcription or (iii) RsaE interacts directly with the *opp-4* mRNA preventing its degradation; however, we did not observe RsaE gel retardation by the *opp4D* 5′ mRNA. Recently, RsaE was reported to target the SD sequence of the *opp3B* mRNA, the first gene of the *opp-3* operon (36). Here, we provide evidence that RsaE targets *opp3A*, the last gene of the *opp3* operon. These observations suggest that RsaE operates simultaneously on two genes of the same mRNA, revealing an unusual mode of sRNA action (Figure 6).

Our results point to a regulatory role of RsaE in primary metabolism. It is astonishing that four enzymes converging directly to the 5, 10 methenyl-THF have their mRNA coordinately downregulated by the same sRNA, RsaE (Figure 3). They correspond to glycine decarboxylase (GcvP) and aminomethyltransferase (GcvT) which belong to the glycine cleavage pathway that provides the one-carbon units to methenyl-THF, and the formate-tetrahydrofolate ligase (Fhs) and methylene-THF

Figure 6. RsaE targets two SD sequences within the same mRNA. The transcriptional organization of the *S. aureus* N315 *opp-3* and *opp-4* operons is presented [based on (72)]. RsaE prevents ribosome loading onto *opp-3* mRNA in front of *opp3B* (36) and *opp3A* ORF (this work). Predicted base pairing between RsaE and the *opp-3* operon is indicated. RsaE positively regulates *opp-3* downstream genes. Dashed lines indicate RsaE-mediated activation, as deduced from microarray and qRT–PCR results.

enzyme (FolD) (36) which convert THF into methenyl-THF. This latter compound is the supplier of one-carbon units for the synthesis of purines and the formyl group of *N*-formylmethionyl-tRNA(f). It is also striking that RsaE downregulates four key enzymes of the tricarboxylic acid (TCA) cycle: the succinyl–CoA synthetase (SucC-SucD) [also in ref (36)], the aconitase (CitB), the isocitrate dehydrogenase (CitC) and the citrate synthase (CitZ) (Table 2, Figure 3). The TCA cycle is involved in the synthesis of usable energy. General downregulation of these different pathways might induce the observed toxicity of *rsaE* over-expression via an ectopic copy. The partial toxicity alleviation by acetate could be due to the utilization of glyoxylate pathway that shunts some RsaE-repressed steps of the TCA cycle. As endogenous RsaE is induced in a pre-stationary phase, an expected role for this sRNA would be to coordinate downregulation of energy metabolism (TCA cycle), and of cofactors, vitamins, translation, and purine biosynthesis (folate-dependent one-carbon metabolism) to facilitate adaptation to the entry into stationary phase.

CcpA is a master regulator for carbon catabolite regulation in numerous Gram-positive bacteria. Many genes that are also modulated by RsaE (e.g. *sucC*, *sucD*, *arg*, *citC*, *citZ* and *ilvB*) show CcpA-dependent regulation (85–87). This suggested that RsaE could affect a CcpA. However, accumulation of RsaE (by means of pAT12-RsaE) did not affect the quantity of the *ccpA* mRNA (Supplementary Data S5 and S6). In addition, we did not observe any band shift of the *ccpA* UTR mRNA sequence by RsaE (data not shown) suggesting that the RsaE effect is likely not *via* CcpA.

Computer predictions indicate the possibility that RsaE-dependent downregulation is mediated via pairing of RsaE with mRNA 5′ UTRs. However, *in vitro* experiments performed between RsaE and synthetic transcripts of the candidate UTR sequences displayed gel retardation

with only one mRNA sequence. It is possible that the sRNA/RNA target binding in *S. aureus* is more labile *in vitro* than with sRNAs from other species such as *E. coli* (possibly because sequences are A/U-rich). For example, the *sucD* mRNA was previously identified and validated as an RsaE target using *in vitro* toeprinting (36); however, the RsaE-*sucD* interaction seemed too labile to be detected in our gel retardation assays (Figure 3A). In *E. coli*, the activity of *trans*-encoded RNA usually requires the RNA chaperone protein Hfq, but it is not the case for known sRNA–mRNA interactions in *S. aureus* (4,30,32,36); thus an unknown factor might be required to promote such regulatory interactions in *S. aureus*. Further evidence for RsaE-dependent gene downregulation via a direct interaction of RsaE with 5′ mRNA UTRs was obtained using a statistical method: Indeed, the probability that the seven of the 22 most downregulated genes (among a total of 2790 *S. aureus* genes) are found by random among the 139 best RsaE/mRNA matching scores (determined using the RNAup software) is 0.000054 (data from Table 2 and Supplementary Table S5). This result rules out the possibility of the random appearance of so many genes matching RsaE among the RsaE-dependent downregulated genes. Strikingly, among these 22 genes that are the most downregulated, 11 are directly involved in Krebs/one carbon by folate metabolism, providing further indication that a pairing with RsaE is required for the observed downregulation of metabolic pathways.

Since RsaE is induced in late exponential phase when the growth medium is depleted, we speculate that RsaE would contribute to downregulating bacterial metabolism (one carbon by folate and TCA cycle), and at the same time activate Opp transporters, as a last-ditch effort to adapt to limited nutrient conditions as cells approach stationary phase.

sRNAs are often expressed during specific growth conditions and have numerous targets. As such, they constitute key regulators of an intricate network of metabolic pathways and nutrient uptake systems. So far, most of the evidence for these roles comes from studies on enteric bacteria, while little is known concerning Gram-positive bacteria (36,88,89); results on RsaE make it obvious that the concept of coordinated control of metabolic enzymes by sRNAs should be extended to the Gram-positive pathogen, *S. aureus*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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