Genome-wide antisense transcription drives mRNA processing in bacteria

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RNA deep sequencing technologies are revealing unexpected levels of complexity in bacterial transcriptomes with the discovery of abundant non-coding RNAs, antisense RNAs, long 5' and 3' untranslated regions and alternative operon structures. Here, by applying deep RNA sequencing to both the long and short RNA fractions (<50 nucleotides) obtained from the major human pathogen *Staphylococcus aureus*, we have detected a collection of short RNAs that is generated genome-wide through the digestion of overlapping sense/antisense transcripts by RNase III endoribonuclease. At least 75% of sense RNAs from annotated genes is subject to this mechanism of antisense processing. Removal of RNase III activity reduces the amount of short RNAs and is accompanied by the accumulation of discrete antisense transcripts. These results suggests the production of pervasive but hidden antisense transcription used to process sense transcripts by means of creating double stranded substrates. This process of RNase III-mediated digestion of overlapping transcripts can be observed in several evolutionarily diverse Gram-positive bacteria and is capable of providing a novel genome-wide posttranscriptional mechanism to adjust mRNA levels.

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Introduction

For many years, the catalogue of transcripts (transcriptome) produced by bacterial cells was limited to the transcription products of known annotated genes (mRNA), ribosomal RNAs (rRNA) and transfer RNAs (tRNA). In the past ten years, the development of new approaches based on high-resolution tiling arrays and RNA deep-sequencing (RNA-seq) has uncovered that a significant proportion (depending on the study varies between 3% to more than half) of protein-coding genes are also transcribed from the reverse complementary strand (1-17). In most of the cases, overlapping transcription generates a non-coding antisense transcript whose size can vary between various tens of nucleotides (*cis*-encoded small RNAs) to thousands of nucleotides (antisense RNAs). The antisense transcript can cover the 5'-end, 3'-end, the middle, the entire gene or even various contiguous genes. Alternatively, overlapping transcription can also be due to the overlap between long 5'- or 3'- UTRs of mRNAs transcribed in opposite direction. Independent of the mechanism by which it is generated, it has been proposed that overlapping transcription can impact the expression of the target gene at different levels (for review see Thomasson and Storz (18)). These mechanisms include: (i) the overlapped transcript affects the stability of the target RNA by either promoting (RNA degradation) or blocking (RNA stabilization) cleavage by endoribonucleases or exoribonucleases; (ii) the overlapped transcript induces a change in the structure of the mRNA that affects transcription termination (transcription attenuation); (iii) the overlapped transcript prevents RNA polymerase from binding or extending the transcript encoded in the opposite strand (transcription interference); and (iv) the overlapping transcript affects protein synthesis either blocking or promoting ribosome binding (translational regulation). While all these regulatory mechanisms have been proposed based on studies with specific sense-antisense partners, the presence of massive amounts

1 of overlapping transcription strongly suggest that it might serve for a general purpose on
2 bacterial gene expression (5, 18-24).

3 In this work, we used RNA sequencing to analyze both the long and short RNA
4 fractions of the major human pathogen *Staphylococcus aureus*. *S. aureus* is a common
5 asymptomatic colonizer of the skin, nasopharynx and other mucosal surfaces of around a
6 quarter of healthy human population. However, when *S. aureus* traverse the epithelial
7 barrier, it becomes a leading cause of many diverse pathological syndromes such as
8 abscesses, bacteremia, endocarditis, osteomyelitis and pneumoniae (25). *S. aureus* has
9 emerged as a model organism for the study of bacterial regulatory RNAs, because key
10 discoveries in bacterial regulatory RNAs have been achieved in this bacterium. In 1993,
11 Novick and co-workers (26) identified the first example of a regulatory RNA (RNAIII) that
12 controls the expression of virulence factors by pairing with the target mRNAs followed by
13 degradation of the RNAIII–mRNA complex by the double-stranded specific RNase III (27).
14 More recently, several studies using computational analysis of intergenic regions,
15 microarray technology and deep sequencing have allowed the identification of more than
16 140 small RNAs including both *trans*-encoded and *cis*-encoded antisense RNAs (10, 28-32).
17 In this current study we uncover the existence of a genome-wide overlapping
18 transcription process covering in a genome-wide extent the expressed protein coding
19 genes. Base pairing between overlapping RNAs can create double stranded substrates for
20 RNase III endoribonuclease activity. Such duplex regions promote the cleavage of the
21 double stranded RNA and the generation of short RNAs (average size of 20nt). Thus, a
22 collection of stable small RNA molecules that symmetrically map both strands of every
23 region with overlapping transcription is generated. The presence of an identical collection
24 of short RNA molecules that symmetrically mapped both strands of annotated ORFs in
25 *Enterococcus faecalis*, *Listeria monocytogenes* and *Bacillus subtilis* indicated that this
26 process is evolutionary conserved in Gram-positive bacteria.

27 **Results**

Pervasive antisense transcription in *S. aureus*

A systematic and hierarchical strategy (Fig. S1) to characterize both long and short RNAs (< 50nt) fractions from log phase-growing *S. aureus* cells was developed. Long RNA sequencing was performed using a cDNA synthesis procedure that preserves information about transcript's direction based on the incorporation of deoxi-UTP during the second strand synthesis and subsequent destruction of the uridine-containing strand (33). The resulting 76-bp paired-end reads were mapped to the *S. aureus* NCTC 8325 reference genome. A total of 9.7 million uniquely mapped read pairs were identified (Fig. S1). 49.2%, 40.4% and 10.4% of the genome was covered by uniquely mapped reads on both strands, one of the strands and showed no coverage, respectively (Fig. 1A). Of the 2,653 annotated open reading frames (ORFs) of the *S. aureus* genome, which covers approximately 84% of the genome, we detected expression of 2,181 ORFs (coverage higher than 90%), of which 1,387 ORFs displayed 50% coverage on the antisense strand (Fig. 1B).

Naturally occurring short RNAs were also sequenced in a strand aware fashion using a two-step adaptor ligation procedure to the 3'- and 5'-ends of the RNA molecules (34). The reads were aligned by algorithmically clipping off the 3' adapter and the remaining sequences of each reads mapped to the genome using STAR (http://gingeraslab.cshl.edu/STAR/). For alignments of 10-19 bases long, up to 1 mismatch was allowed, for alignments longer than 20 bases up to 2 mismatches were allowed. Alignments shorter than 10 bases were discarded and spliced alignments were prohibited. This yielded a total of 7,778,726 million reads mapped to the genome (Fig. S1). The average length of short RNA molecules was 20 nucleotides (Fig. S1). The uniquely mapped short RNA sequences covered, in at least 50% of their length, 2268 and 1981 ORF regions on the sense and antisense strands, respectively (Fig. 1C). Thus, the percentage of ORFs covered in at least 50% of their length by reads in the antisense strand was higher in the case of short RNA (75%) than in the case of long RNA (56%), suggesting that short RNA libraries may prove to be a more sensitive way to detect antisense transcripts. Overall,

1 and given that long and short RNA libraries were generated independently, that is to say
2 from two fractions coming from the same RNA sample, these results provide the first
3 evidence of the existence of antisense transcription not seen in the long RNA sequence
4 analysis.

5 **Symmetric distribution of short RNA reads in both strands of ORF regions**

6 We next sought to determine whether the distribution of short and long reads for
7 a given ORF were linked. For that, we visualized normalized Log_2 values representing the
8 number of mapped reads per nucleotide using the Integrated Genome Browser (IGB) (35).
9 Figure 2 illustrates a randomly selected 30Kb region of the genome of *S. aureus*, which
10 represents 1% of the genome, and depicts the uniquely mapped long and short RNAs. The
11 results revealed that short RNA sequences were symmetrically distributed in both strands
12 of the ORFs, whereas long RNA transcripts follow the expected biased distribution
13 towards the sense strand. Intriguingly, the regions with detectable overlapped
14 transcription between long RNA transcripts, such as those regions corresponding to
15 antisense transcripts to ORFs (00056, 00061, *sirABC* operon), were covered with higher
16 numbers of short RNA reads in both strands. Similar symmetrical accumulation of high
17 levels of short RNAs was detected in every region of the genome where noticeable
18 overlapping transcription occurs, such as 5' and 3' overlapping UTRs, overlapping operons
19 (ORFs that being located in the middle of an operon are transcribed in opposite direction
20 to the other genes of the operon) and antisense transcripts (Fig. 3 and Fig. S2-S4 for
21 additional examples). To most accurately demonstrate that the distribution of short RNA
22 reads was symmetric genome-wide, the number of long and short RNAs mapping to the
23 sense and antisense strands of each ORF was quantified. In accordance with the images
24 observed with IGB browser, the results revealed very similar numbers of short RNA reads
25 genome-wide in both strands of ORF regions and the expected biased of long RNA reads
26 in the sense strand (Fig. 4A, B). In summary, these results show that the *S. aureus*

1 transcriptome contains both long and very short RNA molecules. The amount of long
2 RNAs is, as expected, higher in the sense strand of each ORFs. In contrast, short RNAs are
3 equally distributed in both strands of each ORF and specially enriched in those regions
4 with detectable overlapped transcription between long RNAs.

5 **RNase III is responsible for the production of symmetrically distributed short RNA**
6 **populations**

7 The fact that short RNAs display a symmetrical distribution in sense/antisense
8 strands and accumulate in higher numbers in regions with noticeable overlapping
9 transcription raised the possibility that short RNA molecules were derived from the
10 cleavage of overlapping long sense/antisense primary transcripts. *S. aureus* genome has
11 been reported to encode at least eight putative endoribonucleases and three
12 exoribonucleases (32). Among them, the RNase III endoribonuclease is the only enzyme
13 known to be able to degrade double stranded RNA. Thus, we tested the possibility that
14 RNase III might be responsible for processing the overlapping transcripts into
15 symmetrically distributed sense and antisense short RNA populations. A RNase III mutant
16 in the *S. aureus* 15981 background (*S. aureus* 15981 Δ*rnc*) was constructed using a
17 previously described approach (36).

18 Analysis of the uniquely mapped reads from long RNA seq of the RNase III mutant
19 revealed that the percentage of the genome covered by reads on both strands increased
20 up to 74.2% compared to wild type strain (49.2%) (Fig. 1A). This increase was mainly due
21 to a significant higher coverage of the antisense strand (82% ORFs displayed 50%
22 coverage on the antisense strand) (Fig. 1B). In contrast, the number of the short RNA
23 reads was drastically reduced especially in the antisense strand (Fig. 1C), reducing the
24 percentage of the genome that was covered on both strands by short RNAs to only 6%. Of
25 note, the median length of the short RNA molecules in RNase III mutant was 15 nt
26 suggesting the possibility that short RNAs detected in the RNase III mutant were produced

by another RNA processing pathway (Fig. S1). Visualization of the distribution of mapped reads using IGB confirmed that short RNA sequences had lost their symmetric distribution, whereas long RNA transcripts follow the expected biased distribution towards the sense strand (Fig. 2 and Fig. S2-S4). Accordingly, the correlation first observed in the wild-type strain between the numbers of short RNAs reads in sense and antisense strands per annotated ORF-regions disappeared in the analysis of the RNase III mutant (Fig. 4C, D). Together, these results indicate that a large majority of short RNA molecules present in the transcriptome of *S. aureus* are produced by the cleavage activity of double stranded RNase III enzyme.

As the pattern and cleavage frequency by RNase III is unknown, the short RNA molecules might be direct products of RNase III activity or processed products of larger RNA fragments generated by RNase III. Pnp is the most important 3’-5’ exoribonuclease activity in bacteria. *S. aureus* contains a gene (SAOUHSC_01251) encoding a protein that shares 66% identity with Pnp of *Bacillus subtilis*. We produced libraries from short RNA fraction of *S. aureus* 15981 *Δpnp*. Analysis of the mapped reads from *S. aureus* 15981 *Δpnp* mutant revealed that the distribution and size of the short RNAs follow a pattern indistinguishable from that of the wild type strain (Fig. S1), suggesting that Pnp activity is not required for subsequent processing of the short RNA molecules generated by RNase III activity.

The abundance of short RNAs correlates with the levels of double stranded sense /antisense transcripts

One prediction of the model that short RNAs are produced from the processing of genome-wide overlapping regions of transcription is that the abundance of short RNAs detected should be proportional to the abundance of either the sense/antisense transcripts, depending upon which transcribed strand is less abundant and available for processing. To explore this prediction, we analyzed the short and long RNA complements from a sigma B (*sigB*) mutant (*S. aureus* Δ*sigB*). The transcription factor Sigma B drives the

transcription activity of genes under specific environmental conditions. We analyzed ORF regions for which the long antisense transcripts contained a consensus SigB promoter box (Fig. S5) and their expression was significantly suppressed in the *sigB* mutant (more than 50% reduction in the Δ*sigB*/wt antisense transcript ratio based on the long RNA libraries). Consistent with the hypothesis that the abundance of short RNAs depends on the levels of double stranded RNA, knockdown of the antisense transcripts levels in *sigB* mutant correlates with a decrease in the amount of short RNAs produced from both strands (Fig. S5). These results indicated that the short RNA abundances at ORF regions are strongly correlated with the less abundant levels of overlapping long RNA capable of forming double-stranded RNA.

Detection of occurrence and abundance of antisense transcripts in RNase III mutant

Detection of antisense transcripts has been difficult in bacteria, and only the presence of few antisense transcripts has been confirmed by northern-blot techniques. The observation that RNAse III appears to be responsible for the cleavage of overlapping RNA transcripts into short RNA molecules suggests that at least one reason why antisense transcripts are difficult to detect is because the levels of detectable antisense transcript is kept extremely low within cells due to RNAse III activity. To explore this hypothesis we performed northern-hybridizations with strand specific probes to interrogate sense and antisense transcripts of several individual genes in wild-type and RNase III mutant strains. The candidate genes were selected based on their relevance to different aspects of *S. aureus* virulence (*sarA, agrBCDA, saePQRS, clpP*) or biology (*lexA, recF, yhcSR*) (Fig. S6). The results of the Northern analyses indicated a specific absence and presumed degradation of most full-length antisense transcripts in the steady state condition of the wild-type strain (Fig. 5), while the presence of discrete size antisense transcripts was clearly detectable in the RNase III mutant for all genes tested. It is worth noting that these results confirm the existence of antisense transcripts for genes that have been thoroughly

studied due to their impact on *S. aureus* virulence and antibiotic resistance. For some genes, these hybridizations showed that the RNA levels of the sense strand (*lexA*, *clpP*, *saePQRS*) increased in the RNase III mutant suggesting that the absence of RNase III cleavage can slightly modulate the expression levels of sense transcripts (Fig. 5). To confirm that the presence of antisense RNA was restricted to those regions where short RNAs were detected, we selected two genes (SAOUHSC_00086 and SAOUHSC_00410) for which very few short RNAs were detectable in the wild-type strain (Fig. S7). In both cases, no transcript antisense to these genes was detectable in the RNase III mutant. Overall, these results uncover the existence of long antisense RNAs transcripts for most ORFs of *S. aureus* genome. These long antisense transcripts are under represented in the wild type strain due to the double stranded RNase activity of RNase III.

Analysis of short RNA complement present in diverse bacterial species

To investigate whether this genome-wide sense/antisense overlapping transcript processing mechanism is specific to *S. aureus* or is active in other bacterial species, we characterized the short RNA complement present in three representative Gram-positive (*Enterococcus faecalis, Listeria monocytogenes* and *Bacillus subtilis*) and one Gram-negative (*Salmonella enterica* serovar Enteritidis) bacteria (Fig. 4E). Short RNAs libraries were produced, sequenced and mapped using the protocol previously described (Fig. S1). Analysis of the distribution of short RNAs in sense and antisense strands of ORF regions revealed a highly significant correlation between quantities of short RNAs in sense/antisense strands for the three low-GC content Gram-positive bacteria, mirroring the observations in *S. aureus*. In contrast, the results obtained from the analysis of *Salmonella* demonstrated the absence of such a correlation indicating the existence of a different processing pattern of overlapping RNA pairs in Gram-negative bacteria. Previous transcriptome analysis has allowed the identification of antisense transcripts in *Listeria monocytogenes* (5), *Bacillus subtilis* (17) and *Enterococcus faecalis* (37). Analysis of the

1 distribution of short RNAs in those regions with recognized antisense transcription
2 confirmed the accumulation of high amounts of short RNA in every region with
3 overlapping transcription indicating that genome-wide digestion of overlapping
4 sense/antisense transcripts is conserved at least in Gram-positive bacteria (Fig. S8).

5 **Discussion**

6 Development of RNA-seq technology is allowing the characterization of the multiple types
7 of RNA molecules present in a living cell. The application of this technology in bacteria has
8 primarily been restricted to the analysis of long RNA molecules due to the difficulty for
9 removing highly abundant small size ribosomal and transfer RNA molecules. Here, we
10 have used two methods developed for microRNAs analysis in eukaryotic cells to analyze
11 the RNA fraction shorter than 50 nt of the human pathogen *S. aureus*. The short RNA
12 fraction was purified by size fractionation electrophoresis and libraries for RNA-seq were
13 generated following a protocol that preserves the information about a transcript's
14 direction developed for the direct cloning of microRNA in Drosophila (34).

15 The analysis of the distribution of short RNA molecules revealed several
16 unexpected results. First, the sense strand of 2268 ORFs and the antisense strand of 1981
17 ORFs were covered with unique short RNA reads in at least 50% of their length, indicating
18 the existence of antisense transcription from both strands of most ORFs in *S. aureus*
19 genome. Second, similar numbers of short RNAs were mapping to sense and antisense
20 strands of each ORF irrespectively of the transcription levels of the sense strand. Third,
21 short RNA reads accumulated in higher numbers in regions with noticeable overlapping
22 transcription between long RNA transcripts. The simplest interpretation for these
23 observations was that short RNAs were products of the processing activity of a RNase on
24 the double stranded overlapping RNA transcripts. In support of this explanation, knockout
25 of the *rnc* gene, which encodes for the only known double stranded RNase (RNase III)

contained in the *S. aureus* genome, caused a significant decrease in the number of short RNAs, the loss of the symmetric distribution of short RNAs in sense/antisense strands of each ORF, and the accumulation of long RNA molecules (see Fig. 1) that, in the case of antisense transcripts, emerge as define visible bands in northern hybridizations. Other evidence supporting the hypothesis that short RNA molecules are generated by cleavage of overlapping RNA transcripts was obtained by the analysis of the short and long RNA fractions of the *S. aureus* Δ*sigB* mutant. As the expression of some antisense transcripts requires the presence of the SigB transcription factor, the expression of these antisense transcripts decreases in the *sigB* mutant strain. The analysis of the distribution of short RNAs for those ORFs in which the expression of the antisense transcript is SigB dependent revealed a decrease in the number of short RNAs that specifically mapped with the sense and antisense strands of these ORFs indicating that the levels of shorts RNAs is limited by the amount of double stranded RNA. Notably in most of these ORF regions, the decrease expression of the antisense RNA correlates with an increase in the expression level of the sense transcript, suggesting that RNase III-dependent sense/antisense cleavage process might serve to modulate the levels of the sense RNA.

Current RNA sequencing techniques needs microgram amounts of total RNA for analysis, which corresponds to millions of bacterial cells. The discovery of the existence of overlapping transcripts in a bacterial population does not mean that sense/antisense transcripts are simultaneously present in the same bacteria. Indeed, the transcriptome map will be identical if a subgroup in the bacterial population will synthesize the sense transcript and another subgroup will synthesize the antisense transcript. Our results imply that overlapping transcription occurs extensively in the same cell because RNase III can only produce short RNAs when both transcripts are present and emphasize that overlapping transcription plays a role in posttranscriptional regulation of RNA.

One question that emerges from these results concerns the biological role of the pervasive overlapping transcription and RNase III mediated processing. At least two important biological consequences for this process are suggested from these results. First, antisense transcription and RNase III activity could provide a means for the removal of transcriptional noise. In this circumstance RNase III would digest low levels expression of sense RNA transcription whose expression if left unchecked could unnecessarily compete with the processing and translation of required transcripts. When the transcription of the gene is increased in a regulated fashion, the sense RNA levels would exceed that of the antisense expression, leaving the unpaired sense transcripts impervious to RNase III activity and possible productive translation. Such a model predicts that the levels of expression of sense and antisense transcripts would be coordinated to achieve this threshold effect. Second, this mechanism would also permit the fine-tuning of the sense transcript levels by adjusting the levels of antisense transcription to levels that allow for more or less final sense transcripts. By controlling which regions within multiple gene operons are subject to overlapping transcription, selection of which genes will be ultimately expressed at the protein level could be regulated. It is worth noting that implementation of this mechanism could be used to avoid simultaneous expression of 5' or 3' overlapping transcripts in the same cell.

Another interesting aspect raised by these results is the fate and functional role(s) of the stable short RNAs derived from the processing of overlapping long RNA transcripts. While the answer to this question clearly requires additional studies, it is important to recall the processing of long RNA precursors into short micro-RNAs and into short interfering (si-) RNA molecules as guided by the eukaryotic RNAse III related orthologue enzymes and consider whether there are similar or related genome-wide regulatory mechanisms ongoing in eukaryotic cells.

Materials and Methods

The strains and oligonucleotides used in this study are listed in Table S1. Methods for bacterial growth, chromosomal gene deletion, RNA extraction, riboprobe synthesis, northern blots assays, read mapping and statistics analysis are described in detail in SI Methods. Short RNA libraries were prepared from an RNA fraction containing RNAs shorter than 50-nt by adapting a previously described method (34). This fraction was obtained from total RNA with the flashPAGE fractionator (Ambion). Long RNA libraries were constructed by adapting the previously described dUTP second strand method (33). Detailed protocols for short and long RNA libraries construction are presented in SI Methods. Short and long RNA libraries were sequenced using Illumina Genome Analyzer II at the CSHL facilities.

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Figure legends

Fig. 1. Genome-wide analysis of mapped reads from long and short RNA-seq libraries. **(A)** Percentage of the genome of *S. aureus* NCTC 8325 covered by uniquely mapped reads on both strands, one of the strands and showed no coverage, respectively. The long RNA-seq libraries were prepared from *S. aureus* 15981 wildtype strain (WT) and its corresponding Δ*rnc* (RNase III) mutant (Δ*rnc*). Comparison of the cumulative distribution of ORF coverage by long **(B)** and short **(C)** RNA reads. The plot represents the number of ORFs (x-axis) found above the ORF coverage value (y-axis). The coverage was computed from the collapsed reads uniquely mapped in the sense and antisense orientation to the ORFs. The dashed line represents 50% coverage.

Fig. 2. Long and short mapped reads distribution in *S. aureus* genome. The drawing is an IGB software image showing the uniquely mapped long and short RNAs in a 30Kb region (1%) of the genome of *S. aureus* NCTC 8325. Transcripts are represented as dashed red arrows. Genomic coordinates denote the position in Kb of the *S. aureus* NCTC 8325 genome. Annotated open reading frames (ORFs) are shown as blue lines. The number on the ORF indicates the gene identification. Long and short RNAs show the distribution of uniquely mapped reads of long and short RNA libraries. *S. aureus* 15981 (Black) and *S. aureus* 15981 Δ*rnc* (RNase III mutant) (Green). The scale ($\log_2$) indicates the number of mapped reads per nucleotide position.

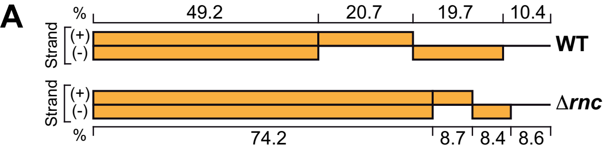
Fig. 3. Examples of mapped reads distribution in regions with overlapping transcription of *S. aureus*. Drawings are images from IGB software showing different regions of the genome of *S. aureus* NCTC 8325. Examples of **(A)** overlapping 5'-UTRs, **(B)** overlapping 3'-UTRs, **(C)** overlapping operons, and **(D)** antisense RNA are shown. Transcripts are represented as dashed red arrows. Genomic coordinates denote the position in Kb of the *S. aureus* NCTC 8325 genome. Annotated open reading frames (ORFs) are shown as blue lines. The number on the ORF indicates the gene identification. Long and short RNAs show

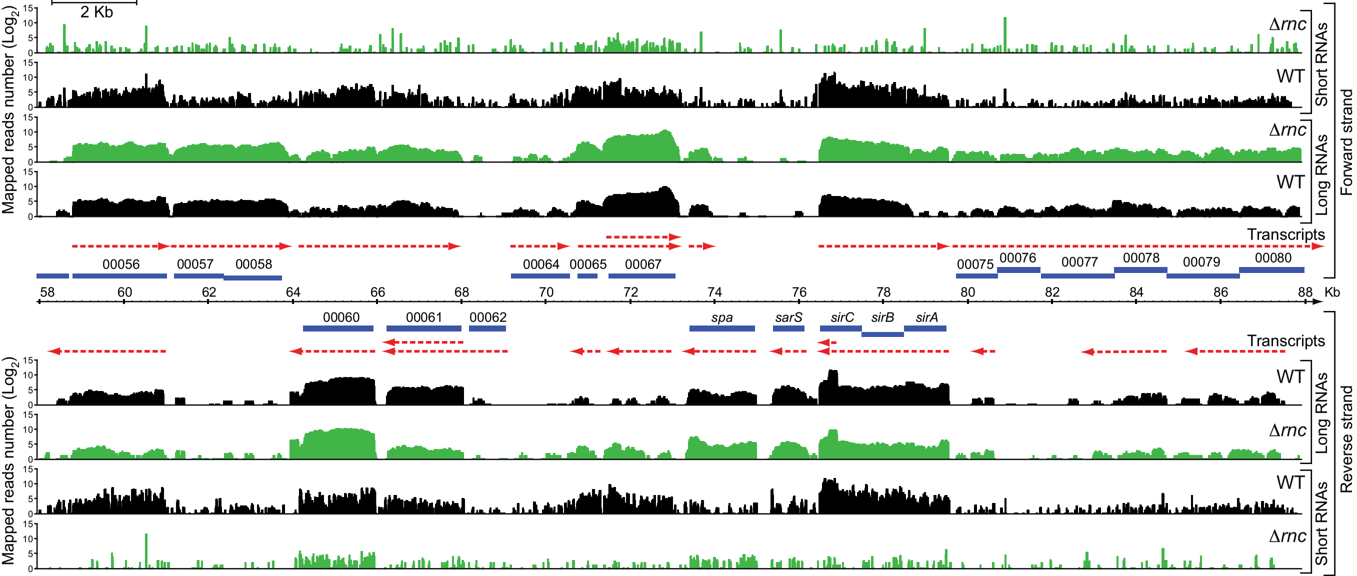
1 the distribution of uniquely mapped reads of long and short RNA libraries in *S. aureus*
2 15981. The scale (log_2) indicates the number of mapped reads per nucleotide position.
3 Dashed rectangles highlight increased accumulation of short mapped reads in regions
4 with overlapping transcription, according to long RNA reads.

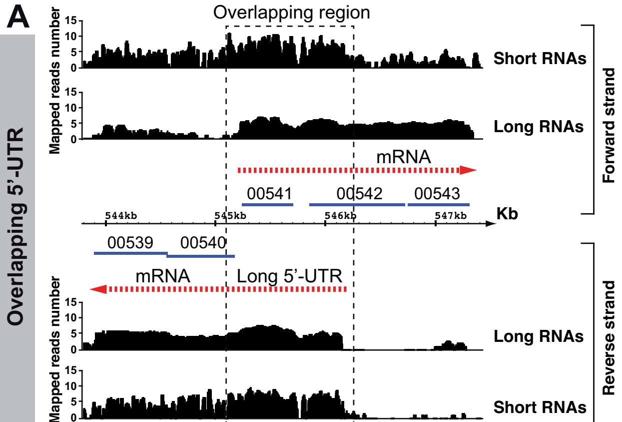
5 **Fig. 4. Expression levels of sense/antisense transcripts.** (**A** and **C**) The plot shows the
6 dependence of the antisense vs. sense ORF-averaged signal in long RNA reads. Each dot
7 corresponds to one ORF annotated in the *S. aureus* NCTC 8325 genome. **(A)** *S. aureus*
8 15981 wild type and (**C**) Δ*rnc*, *S. aureus* 15981 Δ*rnc* (RNase III mutant). (**B** and **D**) The plot
9 shows the dependence of the number of uniquely mapped reads per ORF for the
10 antisense strand vs. sense strand in the short RNA reads. (**B**) *S. aureus* 15981 wild type
11 and (**D**) Δ*rnc*, *S. aureus* 15981 Δ*rnc* (RNase III mutant). (**E**) Genome-wide analysis
12 distribution of mapped reads from short RNAseq libraries in different bacterial species.
13 The plot shows the dependence of the number of uniquely mapped reads per ORF for the
14 antisense strand vs. sense strand in the short RNA-seq libraries of *Enterococcus faecalis*,
15 *Listeria monocytogenes*, *Bacillus subtilis* and *Salmonella* Enteritidis. The colour scale
16 represents the number of points within a +/- 20% window of each point. The number in
17 the bottom right corner is the Spearman correlation coefficient R^2.

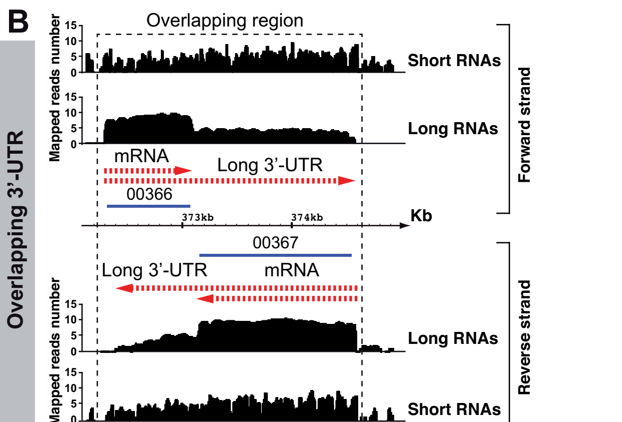
18 **Fig. 5. Expression levels of sense/antisense transcripts**. Northern blot analysis of RNA
19 harvested from *S. aureus* 15981 wild-type and its corresponding *S. aureus* 15981 Δ*rnc*. The
20 blot was probed with a riboprobe specific for sense and antisense transcripts. The
21 positions of RNA standards in Kb are indicated. The time of exposure of the
22 autoradiographies are indicated in hours (h) or days (d).

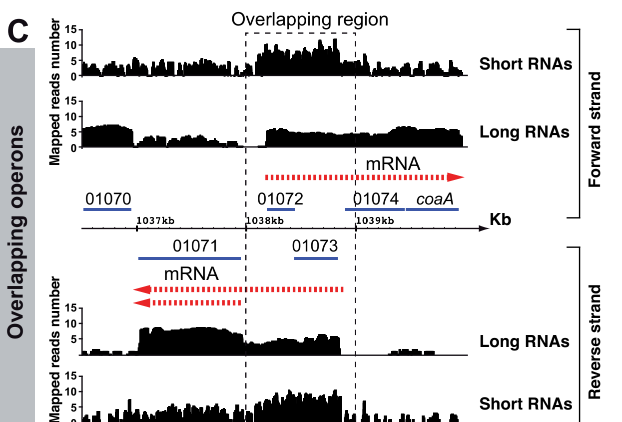
23

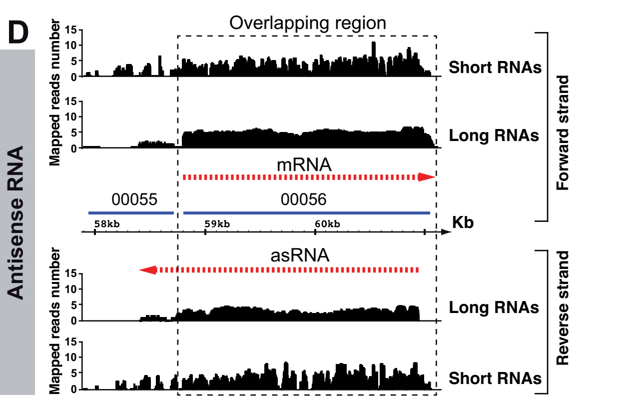

A
%
49.2
20.7
19.7
10.4
Strand
(+)
(-)
WT
Strand
(+)
(-)
Δrnc
%
74.2
8.7
8.4
8.6
B
ORF covergae (%)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Number of ORFs
S. aureus WT - Sense
S. aureus WT - Antisense
Δrnc (RNase III) - Sense
Δrnc (RNase III) - Antisense
C
ORF covergae (%)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Number of ORFs

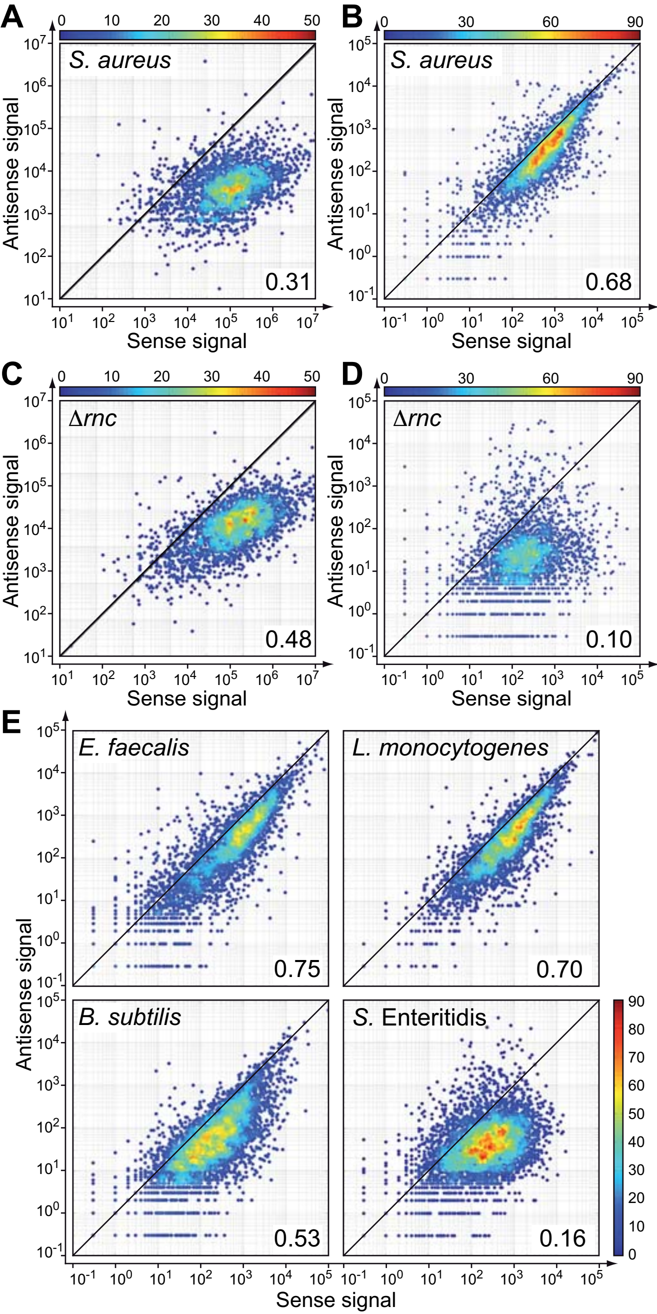

2 Kb
Mapped reads number (Log2)
Δrnc
WT
Short RNAs
Long RNAs
Forward strand
Transcripts
00056
00057
00058
00064
00065
00067
00075
00076
00077
00078
00079
00080
58
60
62
64
66
68
70
72
74
76
78
80
82
84
86
88
Kb
00060
00061
00062
spa
sarS
sirC
sirB
sirA
Reverse strand


A
Overlapping 5'-UTR
Overlapping region
Mapped reads number
Short RNAs
Long RNAs
mRNA
00541
00542
00543
544kb
545kb
546kb
547kb
Kb
Forward strand
00539
00540
mRNA
Long 5'-UTR
Reverse strand


B
Overlapping 3'-UTR
Overlapping region
Mapped reads number
Short RNAs
Long RNAs
mRNA
Long 3'-UTR
00366
373kb
374kb
Kb
Forward strand
00367
Long 3'-UTR
mRNA
Reverse strand


C
Overlapping operons
Overlapping region
Mapped reads number
Short RNAs
Long RNAs
mRNA
01070
01072
01074
coaA
1037kb
1038kb
1039kb
Kb
Forward strand
01071
01073
mRNA
Reverse strand


D
Antisense RNA
Overlapping region
Mapped reads number
Short RNAs
Long RNAs
mRNA
00055
00056
58kb
59kb
60kb
Kb
Forward strand
asRNA
Reverse strand


A
B
C
D
E
S. aureus
S. aureus
Δrnc
Δrnc
E. faecalis
L. monocytogenes
B. subtilis
S. Enteritidis
0.31
0.68
0.48
0.10
0.75
0.70
0.53
0.16
Antisense signal
Sense signal

Sense

LexA	SarA	ClpP	RecF	YhcSR	AgrBDCA	SaePQRS
WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*
7h	2h	0.3h	1h	17h	15h	2d

9.0
6.0
5.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5

16S

Antisense

LexA	SarA	ClpP	RecF	YhcSR	AgrBDCA	SaePQRS
WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*	WT Δ*rnc*
15h	17h	17h	2h	17h	45h	5d

9.0
6.0
5.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5

16S