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Abstract

Staphylococcus aureus is a versatile opportunistic pathogen that adapts readily to a variety of different growth conditions. This adaptation requires a rapid regulation of gene expression including the control of mRNA abundance. The CshA DEAD-box RNA helicase was previously shown to be required for efficient turnover of the agr quorum sensing mRNA. Here we show by transcriptome-wide RNA sequencing and microarray analyses that CshA is required for the degradation of bulk mRNA. Moreover a subset of mRNAs is significantly stabilised in absence of CshA. Deletion of the C-terminal extension affects RNA turnover similar to the full deletion of the cshA gene. In accordance with RNA decay data, the C-terminal region of CshA is required for an RNA-independent interaction with components of the RNA degradation machinery. The C-terminal truncation of CshA reduces its ATPase activity and this reduction cannot be compensated at high RNA concentrations. Finally, the deletion of the C-terminal extension does affect growth at low temperatures, but to a significantly lesser degree than the full deletion, indicating that the core of the [...]
The C-terminal region of the RNA helicase CshA is required for the interaction with the degradosome and turnover of bulk RNA in the opportunistic pathogen *Staphylococcus aureus*

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**Introduction**

*Staphylococcus aureus* is a versatile opportunistic pathogen that adapts readily to a variety of different growth conditions. This adaptation requires a rapid regulation of gene expression including the control of mRNA abundance. The CshA DEAD-box RNA helicase was previously shown to be required for efficient turnover of the *agr* quorum sensing mRNA. Here we show by transcriptome-wide RNA sequencing and microarray analyses that CshA is required for the degradation of bulk mRNA. Moreover a subset of mRNAs is significantly stabilised in absence of CshA. Deletion of the C-terminal extension affects RNA turnover similar to the full deletion of the *cshA* gene. In accordance with RNA decay data, the C-terminal region of CshA is required for an RNA-independent interaction with components of the RNA degradation machinery. The C-terminal truncation of CshA reduces its ATPase activity and this reduction cannot be compensated at high RNA concentrations. Finally, the deletion of the C-terminal extension does affect growth at low temperatures, but to a significantly lesser degree than the full deletion, indicating that the core of the helicase can assume a partial function and opening the possibility that CshA is involved in different cellular processes.
In *E. coli*, the DEAD box helicase RhlB is associated with the degradosome, a protein complex responsible for RNA degradation. The RNase E endonuclease consists of a N-terminal domain that possesses the catalytic activity and a C-terminal domain that serves as scaffold for the other degradosome proteins. In addition to RhlB, a 3’→5’ exonuclease polynucleotide phosphorylase (PNPase) and the glycolytic enzyme enolase, whose role in the complex is not understood, are part of the core complex. Other minor components, such as RraA, RraB, Hfq, L4, polyphosphate kinase (PPK), DnaK, and GroEL, were also found to be associated with the degradosome. Moreover, it has been shown that the *E. coli* degradosome associates with translating ribosomes. During the mRNA degradation process, RNase E cleaves 5’ monophosphorylated RNAs that are subsequently degraded by the PNPase and other 3’→5’ exoribonucleases that are not associated with the degradosome (for review see ref. 19). RNase E and the PNPase are single-strand specific enzymes and RhlB activity is required to facilitate the RNA degradation in a context where the RNAs can form double-stranded structures.

Moreover, it has been shown that a C-terminal region of RNase E stimulates the RNA-dependent ATPase activity of RhlB.

Bacterial two hybrid analyses have shown that the DEAD box helicase CshA of *S. aureus* is also part of a putative degradosome, similar to its *Bacillus subtilis* counterpart. The interaction network includes the endonuclease RNase Y, the endo and 5’→3’ exoribonucleases RNase J1 and RNase J2 and the 3’→5’ exonuclease PNPase. The complex also contains the RNaseP component RnpA, the glycolytic enzymes enolase and phosphofructokinase. Nevertheless, a complex containing several of these proteins has not yet been purified as such. Mutations in *cshA* revealed in addition to a cold sensitive phenotype a reduced biofilm formation and increased hemolysis, the latter due to a stabilization of *agr* mRNA. In this context, CshA could be needed to allow the degradosome enzymes to degrade the RNA, as it was shown for RhlB. In *H. pylori* it has been shown that, *in vitro*, the DEAD box RNA helicase RhpA allows RNase J to degrade double-stranded RNA. The ribonucleases RNase Y, RNase J1, RNase J2 and PNPase are specific for single-stranded RNA and a stable duplex on the RNA can form a protective structure to prevent degradation. The unwinding of such structures by CshA would allow the degradosome to degrade this RNA.

In this work we show that the C-terminal domain of CshA is necessary for its function *in vivo*, confirming the importance of the DEAD box accessory domains. We show that CshA is implicated in the stability of several mRNAs, including many that encode regulators or virulence factors. Finally we show that CshA interacts *in vivo* with degradosome components and that the C-terminal domain of CshA is important for these interactions.

**Results**

The C-terminal region of CshA plays a crucial role

DEAD-box proteins contain 2 domains with a RecA fold, constituting together the core or motor domain involved in ATP dependent RNA binding and RNA-dependent ATP hydrolysis. Accessory N- or C-terminal extensions of the DEAD box helicases can play a regulatory or/and a specificity role. We therefore investigated whether the C-terminal extension of CshA is required for its function and deleted the region corresponding to amino acids 383 to 506, creating a protein similar in length to eIF4A, the godfather of DEAD-box proteins (Fig. 1A, Fig. S1). The region after amino acid 434 appears to be unstructured by contrast to the globular catalytic core of the protein (GlobPlot 2.3, 28). To avoid changes in the expression level, we deleted the corresponding region of the gene directly on the chromosome using the *pyrEF5-FOA selection/antiselection system* in a derivative of the clinical strain SA564, resulting in strain PR01*chA*ΔCter. We checked by western blot that the truncated protein was still correctly expressed (Fig. 1B). To assess the functionality of the truncated protein, we followed 2 readouts for CshA activity, the cold sensitive phenotype and the *agr* mRNA decay. The PR01*chA*ΔCter strain showed a cold sensitive phenotype at 16°C similar to the full deletion of the *chA* gene. Importantly, at 24°C only the PR01*chA* strain showed a cold sensitivity phenotype whereas the PR01*chA*ΔCter strain was still able to grow. Importantly, growth at 16°C can be restored in PR01*chA*ΔCter by expressing CshA from a plasmid. At 42°C, 37°C and 30°C both strains have a growth similar to the parental PR01 strain (Fig. 1C). Since we have previously shown that a *chA* mutant strain is affected in the decay of the *agr* quorum sensing mRNA, we also measured the decay of this mRNA in the PR01*chA*ΔCter strain by qRT-PCR. A time course after rifampicin treatment showed that the *agr* mRNA decay was comparable to the PR01*chA* strain, showing that the C-terminal extension of CshA is of great importance *in vivo* for the RNA turnover function of the protein (Fig. 1D).

The truncated version of CshA retains ATPase activity

The reduced *in vivo* functionality of the C-terminal truncated RNA helicase could be due to lack of interactions with partner proteins or simply be due to the absence of enzymatic activity. We therefore expressed and purified the full length and the truncated proteins from *E. coli* and checked for their ATPase activity *in vitro* in presence or absence of rRNA.

Purification of the CshA-His protein was achieved by adsorption to nickel-agarose and elution with 250 mM imidazole. The nickel-agarose preparation was highly enriched with respect to the globular catalytic core of the protein, as indicated by SDS-PAGE (Fig. 2A). In parallel we purified a mutated version of CshA in which Lys52 was replaced by alanine (K52A; Fig. 2A). An analogous mutation in eIF4A was shown to abrogate nucleotide binding. Recombinant wild-type CshA catalyzed the release of Pi from *[^gamma-32P]*ATP in the presence of *E. coli* rRNA (rRNA) and the extent of ATP hydrolysis was proportional to enzyme concentration (Fig. 2B). The ATPase activity of the K52A mutant was <1% of the activity of wild-type enzyme. We therefore conclude that the observed phosphohydrolyase activity is intrinsic to CshA. Moreover, the ATPase activity measured after a glycerol gradient followed exactly the position of the protein as determined by Western analysis (see below). As it was reported previously and characteristic for most DEAD-box
Figure 1. Functional analysis of CshA and CshAΔCter. (A) Schematic representation of the CshA protein. Gray boxes and the letters above represent the conserved domains characteristic of the DEAD-box family. The numbers below indicate the amino acid position. For CshAΔCter, the protein was truncated after amino acid 382. (B) Presence of CshA in the total cell extracts of PR01, PR01ΔcshA and PR01cshAΔCter. The CshA protein was detected in a western blot performed with an anti-CshA polyclonal antibody. Equal amounts of lysed cells were loaded and verified by Ponceau staining. (C) The strains PR01, PR01ΔcshA and PR01cshAΔCter, containing the plasmids pEB01 (vector) or pEB07 (containing CshA) were spotted in serial dilutions (indicated above) on rich medium containing 15 μg/ml chloramphenicol. All plates were incubated at 42°C (24 h), 37°C (24 h), 30°C (48 h), 24°C (3 days) and 16°C (6 days). Only representative 10⁻³ and 10⁻⁴ dilutions are shown. (D) Cultures of PR01 (black diamonds), PR01ΔcshA (gray squares) and the PR01cshAΔCter (white triangles) were rifampicin treated to block de novo RNA synthesis. Samples were taken for RNA isolation at 0, 2.5, 5, 10 and 15 min after treatment, and qRT-PCR was performed using primers and probes specific for agrA, and using HU mRNA as an internal reference. The quantity of agr, relative to HU, was normalized to 1 at time zero, and plotted in the graph. Error bars represent the 99% confidence level.
proteins, only background ATPase activity could be detected in absence of RNA (data not shown).

The truncated CshAΔCter and its K52A version were also expressed in E. coli and purified in parallel. The mobility of the CshAΔCter proteins during SDS-PAGE was fairly consistent with their calculated sizes of 43 kDa (Fig. 2A). At saturating RNA concentrations, the CshAΔCter (1–382) protein retained 30% specific activity of wild type whereas the CshAΔCter-K52A was <1% of the wild-type enzyme. We concluded that the C-terminal 124 amino acids of CshA are involved in, but not essential for, RNA dependent ATP hydrolysis (Fig. 2B).

To test the possible effects of the C-terminal domain deletion on the RNA interactions of CshA, we measured ATP hydrolysis as a function of the concentration of the rRNA (Fig. 2C). Wild-type CshA and CshAΔCter displayed a typical hyperbolic dependence of ATP hydrolysis on input rRNA (Fig. 2B). From the titration curve we estimate that the deletion of the C-terminal domain elicited about a 3-fold decrement in the activation of the CshA ATPase. From this experiment we concluded that the C-terminal domain of CshA is not the major RNA interaction domain.

Sedimentation Analysis of CshA

The recombinant protein was subjected to zonal velocity sedimentation in a 15–30% glycerol gradient. Marker proteins catalase (native size 248 kDa), bovine serum albumin (66 kDa), and cytochrome c (12 kDa) were included as internal standards. CshA-His$_6$ (calculated to be a 57-kDa polypeptide) sedimented cytochrome c (12 kDa) were included as internal standards.

CshA is required for the decay of a subset of mRNAs in S. aureus

CshA was suggested to be part of the Firmicute degradosome, and we have shown that CshA is involved in agr mRNA decay. We therefore tested if the decay of other RNAs was also affected in absence of CshA or in absence of the C-terminal extension of CshA. The mutant and parental strains were grown to early exponential growth phase and transcription was stopped by the addition of rifampicin. Total bacterial RNA was purified from aliquots of cells before transcriptional arrest and at 2.5, 5, and 10 minutes post-transcriptional arrest. After depletion of rRNA, the purified RNA was then analyzed by RNA deep sequencing and the analysis of genes was fitted with the highly similar genome of N315. Genes that showed less than 100 reads on average over the 4 time points were eliminated, resulting in 1961 ORFs and tRNAs from strain SA564 out of the 2662 found in the N315 genome. The read counts were then normalized to the mRNA of HU, which was previously shown to be well expressed and relatively stable. Similar to published studies, these results clearly showed that most RNAs are present at background levels at >10 minutes after rifampicin treatment. Half-lives were computed by fitting a linear model on log$_2$ transformed gene expressions. Genes whose RNA levels had the largest divergence with the fitted model are excluded to finally retain 1448 genes. Comparing the half-lives of each strain in the 2 experiments showed a good correlation of the 2 independent experiments (Fig. S2).
Interestingly, the comparison of the full deletion and the truncated cshA mutant strains revealed a large similarity in the RNA stabilization in respect to the wild type (Fig. 4A). From the cumulative distribution of RNA stabilization in the 2 mutant strains it is clear that in both the full deletion and the truncated CshAΔCter mutant strains over 80% of the RNAs are stabilised, but only a relatively small portion is significantly (≥2 fold) stabilised in the absence of the helicase or in absence of the C-terminal extension of CshA (Fig. 4B).

To identify genes that had RNAs stabilised at least 2 fold we compared the data of the full deletion and the CshAΔCter mutants with the wild type data. Since the 2 mutant types behaved similarly we retained genes that showed in at least 3 out of the 4 experiments (2 times the full deletion and 2 times the C-terminus truncation) a 2 fold or larger increase of the half-life. The eluted fractions were analyzed on a SDS-PAGE and the protein partners were identified by mass spectrometry. A total of 113 RNAs that were stabilised 2 fold or more (Table 1). For many RNAs, the stabilization also leads to an increase of the steady state level, as judged from the number of reads at time zero of the rifampicin treatment (Fig. 5).

Similar results were obtained by microarray analysis for RNA preparations carried out at 0, 2.5, 5, 10, and 30 minutes after rifampicin treatment (data not shown). Among 118 identified genes in the micro array experiment, 51 are present in the list obtained by the sequencing approach. The genes that are not present in the final list of the sequencing approach were eliminated due to quality restrictions, low expression levels, or are slightly below the cut-off (≥2×) in the sequencing data set. Nevertheless the overall concordance of the 2 methods validates the sequencing approach. It should be noted that due to our RNA purification, we did not quantitatively purify small RNAs (<200 nt), and we therefore concentrate here on the analysis of coding RNAs. Interestingly, a few mRNAs seemed to be destabilised in absence of a fully functional CshA protein. At present we have no explanation for this.

Altogether these results clearly indicate that CshA is involved at least to some degree in the degradation of many RNAs. Moreover, CshA seems to play a significant role in the turnover of a subset of mRNAs. Moreover, we show that the C-terminal extension of this DEAD-box protein is important for this function within RNA decay.

Identification of CshA interaction partners
The C-terminal truncation of CshA in the CshAΔCter mutant strain showed reduced growth at 16°C but not at higher temperatures. However the analysis of the RNA-decay patterns showed virtually identical profiles for the full deletion and the C-terminal truncation. Thus it is likely that the C-terminal extension is important for interactions with the RNA-decay machinery and we set out to identify interacting proteins using a tandem affinity purification (TAP) approach. We used a PR01ΔcshA strain producing a full-length CshA protein or the C-terminal truncated CshAΔCter, containing a Strep/FLAG-tag at the C-terminus. We verified that the tagged version of full length CshA was able to restore the cold sensitive phenotype of the PR01ΔcshA strain, showing that the protein is functional (data not shown). We also checked the production of the proteins by western blot, using a polyclonal antibody raised against CshA. We noted a slight overproduction in comparison with the wild type level of CshA, probably due to the fact that the constructs are expressed from a plasmid on its native promoter (data not shown). Since some interactions could be mediated through RNA, we performed the lysis steps both in absence or presence of RNase A. The Strep/FLAG tagged versions of CshA and their potential interaction partners were purified using first a Strep-Tactin column. The corresponding elution fractions were pooled and subjected to a second round of purification on an α-FLAG resin. In order to be able to discriminate protein interactions from column background, we performed in parallel the same purifications with a lysate from a PR01 strain with the empty vector. The eluted fractions were analyzed on a SDS-PAGE and the protein partners were identified by mass spectrometry. A total of 106 proteins were identified in the elution fractions coming from the lysates containing the tagged version of CshA, with or without RNase A treatment (Table S2, Table S3 for the full data set). Among these proteins, we found proteins involved in each step of the RNA metabolism from transcription to decay, enzymes in DNA metabolism and others. Indeed, we found interactions with the β and β’ subunits of the RNA polymerase, various ribosomal proteins, translation initiation and elongation factors. Most importantly, we also identified RNase J1, RNase J2, RNase Y and enolase, components of the putative degradosome. Interestingly, the PNPase was only detected without RNase A treatment, suggesting a lose association, if any, with the degradosome. To identify the interactions mediated through the C-terminal extension, we also identified proteins that copurified with the truncated CshAΔCter. The analysis showed that out of the 106 proteins from the CshA purification, 68 proteins were
absent in the CshAΔCTer preparation after RNase A treatment (Table 2). Importantly, RNase J1, RNase J2, RNase Y and enolase were no longer detected, as well as many ribosomal proteins, indicating an association mediated through the C-terminal extension. We therefore also purified RNase J1 to see whether the reciprocal co-purification could be observed. Interestingly, relatively few proteins copurified with RNase J1, with the exception of ribosomal proteins and

Figure 4. Stabilization of mRNAs. (A) Scatterplot showing half-life change between mutants and the parental strain. Each point represents a gene and is positioned on the x-axis according to the mean half-life estimated in both replicates of the decay experiment; similarly, the point on the y-axis is positioned according to the mean half-life estimated in both replicates of the decay experiment of the parental strain. Kendall tau correlation coefficient is given for each case. A diagonal line marks the limit between genes whose half-life is increased in the mutants (below the line), and genes whose half-life is decreased in the mutants (above the line). Only genes satisfying minimum quality criteria are reported. (B) Distribution of the half-life fold change between the mutants and the parental strain. The areas under the curves sums to 1.0, and reflects the proportion of genes affected with a given stabilization factor. The vertical line at x = 1 marks the limits between stabilization (on the right) and destabilization (on the left). The shift of the curves on the right side of the vertical line reflects the longer half-life measured in the mutants compared to the ancestor strain.
Table 1. Stabilized RNAs. The half-life of the RNAs in the 2 mutant and the parental strains in 2 independent experiments were compared. The ratio of the half-life of the mutant compared to the parental strain is indicated. Genes that showed a stabilization of the RNA at least 2-fold in 3 out of the 4 mutant strains were retained and are listed in the table.

| gene ID | operon | Description | Gene Name | First data set | Second data set |
|---------|--------|-------------|-----------|---------------|----------------|
| SA0013  | SA0012-SA0016 | Cyclic di-AMP phosphodiesterase | GdpP | 2.1 | 2.1 |
| SA0014  | SA0012-SA0016 | Ribosomal protein L9 | rplI | 2.1 | 2.0 |
| SA0127  | SA0124-SA0127 | Uncharacterized protein, similar to E. coli Putative O-antigen transporter [rfbX] | SA0127 | 3.4 | 2.7 |
| SA0129  | Uncharacterized protein | SA0129 | 2.9 | 2.8 |
| SA0132  | SA0131-SA0134 | Uncharacterized protein, weak similarity to Bacillus Tetracycline resistance protein [tetB] | SA0132 | 2.6 | 2.4 |
| SA0173  | SA0173-SA0174 | Uncharacterized protein, similar to amino acid adenylases, | SA0173 | 3.4 | 3.1 |
| SA0174  | SA0173-SA0174 | Uncharacterized protein, homolog to Bacitracin synthase 3 | SA0174 | 3.0 | 3.5 |
| SA0198  | Uncharacterized protein | SA0198 | 1.9 | 2.0 |
| SA0285  | Uncharacterized protein | SA0285 | 2.4 | 2.1 |
| SA0301  | SA0300-SA0302 | Pseudouridine-5'-phosphate glycosidase | psuG | 2.1 | 1.8 |
| SA0309  | Lipase 2 | geh | 2.0 | 2.7 |
| SA0318  | SA0318-SA0321 | Uncharacterized protein, similar to Ascorbate-specific permease IIC component UlaA | ulaA | 2.1 | 2.1 |
| SA0319  | Uncharacterized protein | SA0319 | 2.5 | 2.1 |
| SA0340  | SA0430-SA0431 | Glutamate synthase large subunit | gatB | 2.7 | 2.7 |
| SA0345  | SA0453-SA0456 | 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase | ipk | 2.1 | 2.1 |
| SA0454  | SA0453-SA0456 | purR transcription regulator | purR | 2.0 | 2.0 |
| SA0475  | Lysine-tRNA synthetase | ulAA | 2.4 | 2.3 |
| SA0484  | SA0484-SA0485 | DNA repair protein radA | radA | 2.5 | 2.8 |
| SA0485  | Uncharacterized protein, similar to putative Bacillus RNase YacL (PIN and TRAM-domain) | SA0485 | 2.4 | 2.9 |
| SA0507  | Uncharacterized protein, similar to putative amidohydrolase YhaA | SA0507 | 2.0 | 2.2 |
| SA0578  | SA0577 - SA0584 | Putative antipporter subunit mnhA2 | SA0578 | 2.3 | 2.7 |
| SA0587  | SA0587-SA0589 | Uncharacterized protein, similar to Manganese ABC transport system membrane protein MntC | SA0587 | 1.8 | 2.0 |
| SA0588  | SA0587-SA0589 | Uncharacterized protein, similar to Manganese transport system membrane protein MntB | SA0588 | 1.7 | 2.0 |
| SA0589  | SA0587-SA0589 | Uncharacterized protein, similar to Manganese transport system ATP-binding protein MntB | SA0589 | 1.7 | 2.0 |
| SA0682  | Uncharacterized protein, similar to D/L-tripeptide transporter [dtpT] | SA0682 | 2.2 | 2.1 |
| SA0701  | Uncharacterized membrane protein with similarity to diguanylate cyclase DgcC | SA0701 | 3.2 | 2.9 |
| SA0868  | SA0863-SA0868 | Uncharacterized protein, similar to Putative Na(+)H (+) antipporter YjbQ [yjbQ] | SA0868 | 2.5 | 2.4 |
| SA0927  | SA0927-SA0929 | Uncharacterized protein, weakly similar to Putative HMP/thiamine permease protein YkoC | SA0927 | 2.3 | 2.1 |
| SA0950  | SA0949-SA0954 | Spermidine/putrescine import ATP-binding protein | potA | 2.3 | 2.3 |
| SA0951  | SA0949-SA0954 | Spermidine/putrescine transport system permease protein | potB | 2.3 | 2.5 |
| SA0952  | SA0949-SA0954 | Spermidine/putrescine transport system permease protein | potC | 2.3 | 2.4 |
| SA0964  | Heme A synthase | ctaA | 2.0 | 2.1 |
| SA0975  | Uncharacterized protein | SA0975 | 2.4 | 2.0 |
| SA0987  | Ribonuclease Hill | mhc | 2.7 | 2.7 |
| SA1007  | Alpha-Hemolysin | hlaY | 3.6 | 3.8 |
| SA1073  | SA1071-SA1074 | Malonyl CoA-acyl carrier protein transacylase | fabD | 2.4 | 2.2 |
| SA1074  | SA1071-SA1074 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | fabG | 2.3 | 2.1 |
| SA1139  | SA1137-SA1139 | Glycerol uptake operon antiterminator regulatory protein | glpP | 2.2 | 2.0 |
| SA1187  | Glycerol-3-phosphate acyltransferase | phlY | 2.8 | 2.4 |

(continued on next page)
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| gene ID | operon | Description | Gene Name | First data set | Second data set |
|---------|--------|-------------|-----------|---------------|----------------|
|         |        |             |           | ∆cshA/wt | CshA/Cter/wt | ∆cshA/wt | CshA/Cter/wt |
| SA1193  |        | oxacillin resistance-related FmtC protein (fmtC) | fmtC      | 2.4     | 2.3      | 1.8    | 2.4    |
| SA1224  |        | Uncharacterized protein, similar to uncharacterized ABC transporter ATP-binding protein YkpA | SA1224    | 2.8     | 2.8      | 2.0    | 2.9    |
| SA1267  | SA1267-SA1268 | Extracellular matrix-binding protein EbhA | ebhA      | 1.6     | 2.0      | 7.7    | 6.1    |
| SA1268  | SA1267-SA1268 | Extracellular matrix-binding protein EbhB | ebhB      | 2.7     | 2.6      | 5.5    | 5.4    |
| SA1281  | SA1279-SA1281 | Uncharacterized protein | SA1281    | 2.7     | 2.3      | 2.0    | 2.8    |
| SA1282  | SA1282-SA1283 | Holliday junction resolvase RecU | recU      | 3.6     | 3.0      | 2.5    | 2.4    |
| SA1283  | SA1282-SA1283 | penicillin binding protein 2 | pbp2      | 2.6     | 2.5      | 2.2    | 1.5    |
| SA1337  |        | Uncharacterized protein, weakly similar to transcriptional regulator | SA1337    | 2.3     | 2.2      | 1.9    | 2.0    |
| SA1375  | SA1375-SA1380 | Uncharacterized protein, similar to Probable metallo-hydrolase YqgX | SA1375    | 2.0     | 2.0      | 2.1    | 2.1    |
| SA1376  | SA1375-SA1380 | Uncharacterized protein, Similar to YggV from B. subtilis | SA1376    | 2.3     | 2.2      | 2.2    | 2.4    |
| SA1377  | SA1375-SA1380 | Glucokinase | glcK      | 2.5     | 2.3      | 2.8    | 3.1    |
| SA1474  | SA1474-SA1475 | Uncharacterized protein | SA1474    | 2.3     | 2.0      | 1.7    | 2.0    |
| SA1490  |        | Uncharacterized protein | SA1490    | 2.8     | 2.7      | 2.1    | 6.3    |
| SA1536  | SA1535-SA1538 | Uncharacterized protein, weakly similar to UPF0721 transmembrane protein | SA1536    | 2.2     | 2.2      | 2.1    | 2.5    |
| SA1579  | SA1578-SA1579 | Leucine-tRNA synthetase | leuS      | 1.9     | 2.0      | 2.1    | 2.4    |
| SA1580  |        | Uncharacterized protein, similar to Uncharacterized MFS-type transporter YttB | SA1580    | 2.3     | 2.3      | 2.1    | 1.9    |
| SA1587  | SA1586-SA1589 | Riboflavin biosynthesis protein RibA | ribA      | 2.1     | 1.6      | 2.1    | 2.0    |
| SA1588  | SA1586-SA1589 | Riboflavin synthase α chain | ribB      | 2.1     | 1.6      | 2.3    | 2.0    |
| SA1589  | SA1586-SA1589 | Riboflavin biosynthesis protein RibD | ribD      | 2.1     | 1.5      | 2.4    | 2.1    |
| SA1606  |        | Uncharacterized protein, similar to Glyoxal reductase | SA1606    | 2.8     | 3.6      | 2.4    | 3.2    |
| SA1617  | SA1617-SA1618 | Uncharacterized protein | SA1617    | 2.8     | 1.8      | 3.1    | 7.0    |
| SA1618  | SA1617-SA1618 | Uncharacterized protein | SA1618    | 4.2     | 2.3      | 4.1    | 4.4    |
| SA5053  |        | Uncharacterized protein | SA5053    | 2.4     | 2.3      | 1.1    | 2.1    |
| SA1679  | SA1678-SA1680 | Uncharacterized protein | SA1679    | 2.8     | 2.6      | 2.0    | 3.2    |
| SA1680  | SA1679-SA1680 | Uncharacterized protein | SA1680    | 3.1     | 2.9      | 1.7    | 6.4    |
| SA1755  |        | Chemotaxis inhibitory protein, extracellular | SA1755    | 7.0     | 2.9      | 2.0    | 19.7   |
| SA5066  | SA1843-SA1844 | AgrD protein | agrD      | 2.7     | 2.4      | 3.3    | 16.8   |
| SA1843  | SA1843-SA1844 | Accessory gene regulator C | agrC      | 4.2     | 3.7      | 2.0    | 19.3   |
| SA1844  | SA1843-SA1844 | Accessory gene regulator A | agrA      | 3.3     | 3.5      | 2.1    | 16.9   |
| SA1851  |        | Redox-sensing transcriptional repressor Rex | rex       | 2.3     | 2.3      | 2.3    | 2.0    |
| SA1879  | SA1879-SA1881 | Potassium-transporting ATPase C chain 1 | kdpC      | 3.0     | 2.9      | 4.5    | 6.4    |
| SA1880  | SA1879-SA1881 | Potassium-transporting ATPase B chain 2 | kdpB      | 3.0     | 2.9      | 3.8    | 6.2    |
| SA1881  | SA1879-SA1881 | Potassium-transporting ATPase A chain 1 | kdpA      | 4.0     | 3.4      | 2.8    | 6.5    |
| SA1883  | SA1882-SA1883 | KDP operon transcriptional regulatory protein KdpE | kdpE      | 2.2     | 2.3      | 2.5    | 2.7    |
| SA1897  | SA1893-SA1897 | Putative thiaminase-2 | SA1897    | 2.7     | 2.4      | 1.1    | 4.1    |
| SA1923  |        | Transcription termination factor Rho | rho       | 4.1     | 3.4      | 3.8    | 2.6    |
| SA1970  |        | Uncharacterized protein, similar to Uncharacterized MFS-type (major-facilitator-superfamily) transporter YcnB | SA1970    | 2.1     | 2.2      | 2.2    | 2.2    |
| SA1993  | SA1991-SA1997 | Lactose-specific phosphotransferase enzyme IIA component | lacF      | 2.3     | 1.9      | 2.5    | 5.4    |
| SA1994  | SA1991-SA1997 | Tagatose 1,6-diphosphate aldolase | lacD      | 3.4     | 2.1      | 2.7    | 3.3    |
| SA1996  | SA1991-SA1997 | Tagatose-6-phosphate kinase | lacC      | 2.3     | 1.6      | 2.4    | 3.8    |
| SA1997  | SA1991-SA1997 | Galactose-6-phosphate isomerase subunit LacB | lacB      | 2.6     | 1.6      | 2.3    | 3.3    |
| SA2050  |        | Uncharacterized protein, similar to Guanine/ hypoxanthine permease PbuG | SA2050    | 3.5     | 3.2      | 3.3    | 2.2    |
| SA2069  |        | Cyclic pyranopterin monophosphate synthase accessory protein | moaC      | 2.3     | 2.7      | 2.5    | 2.3    |
| SA2119  | SA2119-SA2120 | Cyclic pyranopterin monophosphate synthase accessory protein | SA2119    | 2.9     | 2.5      | 2.1    | 3.8    |

(continued on next page)
Importantly, purification of RNase J1 allowed the copurification of CshA, further confirming an interaction of CshA and RNase J1. To check for specificity of CshA, we also purified CshB by the same procedure. In contrast to CshA, relatively few proteins copurified with CshB. However, both helicases purified with a considerable number of ribosomal proteins (Table S2), which may reflect an association with the ribosome or a role in ribosome biogenesis.35

Intriguingly we were so far unable to demonstrate an in vitro interaction of individually prepared recombinant N-terminal Flag-Streptavidin tagged RNase J1 with CshA. This could be explained either by cotranslational folding or by interaction through a third partner. However, if both proteins were co-expressed together in E. coli, purification of Flag-Streptavidin tagged RNase J1 in presence of RNase A, allowed co-purification of low amounts of CshA, visible on Commassie blue stained gels and confirmed by western blot analysis (data not shown).

Table 1. Stabilized RNAs. The half-life of the RNAs in the 2 mutant and the parental strains in 2 independent experiments were compared. The ratio of the half-life of the mutant compared to the parental strain is indicated. Genes that showed a stabilization of the RNA at least 2-fold in 3 out of the 4 mutant strains were retained and are listed in the table (Continued)

| gene ID | operon | Description | Gene Name | First data set | Second data set |
|---------|--------|-------------|-----------|----------------|----------------|
| S.2A147 | | Uncharacterized protein, similar to TcaR transcription regulator | tcaR | 2.5 | 2.4 | 1.9 | 17.5 |
| S.2A167 | | Uncharacterized protein, similar to PTS system, sucrose-specific IIBC component | scrA | 3.5 | 2.5 | 3.6 | 2.5 |
| S.2A172 | | Uncharacterized protein, similar to Proton/sodium-glutamate symport protein | gltT | 3.6 | 3.6 | 2.9 | 2.5 |
| S.2A174 | | Uncharacterized protein, similar to HTH-type transcriptional regulator SarZ | SarZ | 2.5 | 2.3 | 1.9 | 2.6 |
| S.2A206 | | Uncharacterized protein, similar to Immunoglobulin-binding protein sib | sib | 2.9 | 3.3 | 3.4 | 6.1 |
| S.2A208 | | Probable glycine betaine/carnitine/choline ABC transporter opuCD | opuCD | 1.8 | 2.0 | 2.0 | 2.0 |
| S.2A224 | | Uncharacterized protein | S.2A224 | 2.9 | 2.7 | 2.9 | 2.2 |
| S.2A226 | | Uncharacterized protein, similar to p-aminobenzoyl-glutamate transport protein | S.2A226 | 3.1 | 4.5 | 2.0 | 2.0 |
| S.2A237 | | Uncharacterized protein, similar to p-aminobenzoyl-glutamate transport protein | S.2A237 | 4.6 | 3.9 | 3.7 | 4.3 |
| S.2A238 | | Holin-like protein CidB | CidB | 4.2 | 4.2 | 4.0 | 4.7 |
| S.2A236 | | Uncharacterized protein, similar to ATP-dependent Clp protease ATP-binding subunit ClpL | clpL | 3.6 | 2.8 | 1.3 | 2.9 |
| S.2A254 | | O-acetyltransferase OatA, cell membrane | S.2A254 | 2.1 | 2.0 | 2.1 | 1.9 |
| S.2A261 | | Uncharacterized protein | S.2A261 | 2.1 | 1.8 | 2.0 | 2.2 |
| S.2A262 | | Uncharacterized protein | S.2A262 | 2.2 | 1.9 | 2.2 | 2.2 |
| S.2A266 | | Uncharacterized protein | S.2A266 | 2.0 | 2.0 | 1.4 | 2.0 |
| S.2A268 | | Uncharacterized protein | S.2A268 | 2.1 | 1.9 | 2.5 | 2.3 |
| S.2A291 | | Pantothenate synthetase, pantoate-β-alanine ligase | panC | 2.3 | 2.0 | 1.8 | 2.0 |
| S.2A292 | | 3-methyl-2-oxobutanoate hydroxymethyltransferase | panB | 2.4 | 2.2 | 2.0 | 1.7 |
| S.2A240 | | Oxygen-dependent choline dehydrogenase | betA | 2.0 | 2.2 | 2.2 | 2.0 |
| S.2A241 | | Sensor histidine kinase | BraS | 3.1 | 2.5 | 2.9 | 3.4 |
| S.2A248 | | Uncharacterized protein, similar to transcriptional regulatory protein | BraR | 4.0 | 2.7 | 2.5 | 3.6 |
| S.2A249 | | Uncharacterized protein | S.2A249 | 3.5 | 2.5 | 3.0 | 3.1 |
| S.2A2431 | | Uncharacterized protein, similar to Immunodominant staphylococcal antigen B, extracellular | isaB | 2.2 | 2.4 | 1.8 | 2.3 |
| S.2A2440 | | Glycosyltransferase stabilizing protein Gtf2, part of part of the accessory SecA2/SecY2 system | gtf2 | 2.1 | 1.9 | 2.4 | 2.8 |
| S.2A2441 | | Glycosyltransferase Gtf1 | gtf1 | 2.0 | 1.9 | 2.9 | 2.9 |
| S.2A2442 | | Protein translocase subunit SecA2 | S.2A2442 | 2.5 | 2.1 | 2.7 | 2.7 |
| S.2A2487 | | Uncharacterized protein, similar to transporter | S.2A2487 | 2.1 | 2.1 | 2.1 | 2.1 |
| S.2A2491 | | Uncharacterized protein, similar to CidB | S.2A2491 | 2.4 | 2.1 | 1.3 | 9.4 |
| S.2A2495 | | Uncharacterized protein, similar to Cro/CI family transcriptional regulator | S.2A2495 | 2.6 | 2.5 | 1.7 | 2.0 |
| S.2A0156 | S.2A0144-S.2A0159 | Capsular polysaccharide synthesis enzyme Cap5M | cap5M | 0.2 | 0.4 | 0.4 | 1.0 |
| S.2A194 | | Uncharacterized protein | S.2A194 | 0.3 | 0.3 | 0.5 | 1 |
| S.2A1310 | | Probable L-asparaginase | ansA | 0.5 | 0.7 | 0.5 | 0.5 |
| S.2A1532 | | Putative universal stress protein SA1532 | SA1532 | 0.4 | 0.5 | 0.4 | 0.4 |
| S.2A1989 | | Uncharacterized protein similar to Putative NADP-dependent oxidoreductase YfmJ | SA1989 | 0.2 | 0.2 | 0.3 | 0.4 |
| S.2A1990 | | Uncharacterized protein | SA1990 | 0.3 | 0.4 | 0.5 | 0.6 |

RNase J2 (Table S2).
Interestingly the H. pylori RNase J and RNA helicase interaction was also observed only by co-expression. 26

Figure 5. Correlation of half life and steady-state levels. Scatterplot showing correlations between the changes in steady state level of RNAs a time point 0 and in the half-life from the ΔcshA and cshAΔCter mutants as compared to the parent. Each point represents a gene and is positioned on the x-axis according to log2 change in half-life (stabilization), while it is positioned on the y-axis according to log2 of the expression of the gene at time point 0 (steady-state level). Each change is computed in a conservative way making use of all replicates information in a worst case scenario strategy: to estimate the change in steady state level, the normalized gene expression of the 2 parent replicates at time point 0 are compared to the normalized gene expression of the 4 mutants at time point 0; only the combination yielding the smallest change is considered. Half-life change on the y-axis is computed with the same strategy.

Discussion

We have previously shown that the S. aureus CshA DEAD-box RNA helicase is required for efficient turnover of the agr mRNA and that inactivation of cshA results in decreased biofilm formation and increased hemolysis. Here we showed that CshA is more generally involved in RNA turnover and that the C-terminal extension is required for this activity, as well as for the interaction with components of a Gram-positive degradosome. Interestingly, the cold sensitive phenotype at 24°C was almost absent with the truncated protein, but very pronounced with the full deletion. We also showed that in absence of the C-terminal extension the RNA-dependent ATPase activity was reduced.

Using the strains PR01ΔcshA and PR01cshAΔCter, we were able to show that the bulk of the mRNAs is slightly stabilised in absence of a fully functional CshA protein and that a defined subset of the mRNAs is significantly stabilised in absence of a wild-type helicase. As expected from our previous observations, the agr quorum sensing system is among the identified operons. In addition, 2 other 2-component systems, BraS/BraR and Kpd/KpdD (KpdD was eliminated because of quality limitations) and 6 putative or established transcription factors (purR, SA1337, Rex, tcaR, SA2174, SA2495), as well as genes encoding proteins involved in intracellular signaling, such as the cyclic-di-AMP phosphodiesterase GdpP,36,37 were among the genes with stabilised mRNAs. Interestingly, many genes encoding membrane-associated proteins (36) were also in the list of significantly stabilised mRNAs. At present we do not know if this observation has a biological significance, or if this is due to a potential localization of the degradosome to the membrane through the RNase Y membrane anchor. The stabilization of RNA could be due to a direct effect of a deficient degradosome, or due to an indirect effect. As an example, studies from the Dunman laboratory have shown that certain mRNAs require SarA for stabilization, which was suggested to be due its RNA binding activity.34,38 Several RNAs that were identified in the study by38 also are present in our list of 2 fold or more stabilised RNAs (radA, ctaA, rpmF, glnP fmtC, agrA, kdpA, lacA, moaC, pyruvate oxidase, opuCD, cidB, betA). The sarA mRNA is slightly stabilised in the ΔcshA strain (1.6-fold) and the cshAΔCter strain (2.2-fold). Moreover, DEAD-box proteins were shown to be able to remove RNA-binding proteins from their substrate,39 and therefore SarA stabilised mRNAs might be stabilised in absence of CshA either by increased SarA or due to the absence of a putative RNase activity of the RNA helicase. In a similar vein, certain genes, affected in absence of a fully functional CshA RNA helicase, comprise also genes like the one encoding the holin-like protein CidB is also largely stabilized in our experimental conditions. Altogether, this suggests that the helicase plays an important role in adaptation to changing environments and thereby influence the outcome of infections by this feared pathogen.

From the studies with the E. coli degradosome, it was strongly suggested that the helicase is required for RNAs that contain secondary structures. It is reasonable to expect that mRNAs, coding for proteins that are required only under certain growth conditions or that are involved in adaptation to stress conditions, need to be rapidly turned over when no longer necessary. Therefore one possibility could be that the CshA-dependent decay of RNAs is regulated by the RNA helicase. In this scenario the CshA protein plays a regulatory role, eventually assisted by other proteins. How the helicase itself would be regulated is not known, but in E. coli regulatory proteins that inhibit the RhlB helicase have been described.85 Another more direct explanation for the requirement of a DEAD-box RNA helicase in turnover would be the presence of secondary structures that perform a biological function, such as regulating the accessibility of the ribosome-binding site. For the degradation of such RNAs, the degradosome needs to be assisted by a helicase since it was shown
Table 2. Summary of proteins identified for CshA. Proteins identified in the purifications of CshA, CshAΔCter, RNase J1, or CshB. Numbers represent the peptides identified in presence and absence (within brackets) of RNase A during the purification. The table was established by selecting, for each purification, all proteins that were represented by 2 or more peptides. Proteins were retained for the table if they were present in the CshA purification and after RNase A treatment, but not in the purification of the truncated CshAΔCter. The full data set is given in Supplementary Table 3 Proteins identified in the mock sample (Elongation factor Tu, ASIQ2 (14 peptides) and Pyruvate dehydrogenase E1 P0A0A1 (5 peptides)) are not indicated.

| gene name    | Identified Proteins                  | MW       | CshA                    | CshAΔCter                | RNase J1                | CshB                    |
|--------------|------------------------------------|----------|-------------------------|--------------------------|-------------------------|-------------------------|
| CshA         | CshA (used as bait for the purification in first column) | 29 (27)  | 31 (32)                 | 18 (17)                  | 4 (12)                  |
| rmj1         | Ribonuclease J 1                    | 63 kDa   | 11 (13)                 | 56 (54)                  |                         |
| rmj2         | Ribonuclease J 2                    | 63 kDa   | 4 (8)                   | 37 (31)                  |                         |
| rny          | Ribonuclease Y                      | 59 kDa   | 4 (4)                   |                          |                         |
| eno          | Enolase                             | 47 kDa   | 12 (11)                 |                          |                         |
| rpsD         | 30S ribosomal protein S4            | 23 kDa   | 7 (10)                  |                          | 10 (12)                 | 12 (10)                 |
| rpsF         | 30S ribosomal protein S6            | 12 kDa   | 3 (6)                   |                          | 4 (5)                   | 6 (6)                   |
| rpsG         | 30S ribosomal protein S7            | 18 kDa   | 4 (5)                   |                          | 4 (8)                   | 7 (7)                   |
| rpsL         | 30S ribosomal protein S9            | 15 kDa   | 2 (3)                   |                          | 4 (4)                   | 4 (4)                   |
| rpsL         | 30S ribosomal protein S12           | 15 kDa   | 2 (3)                   |                          | 2                       |                         |
| rpsM         | 30S ribosomal protein S13           | 14 kDa   | 4 (5)                   |                          | 3 (5)                   | 4 (4)                   |
| rpsR         | 30S ribosomal protein S18           | 9 kDa    | 2 (4)                   |                          | 3 (2)                   | 3 (4)                   |
| rpsS         | 30S ribosomal protein S19           | 11 kDa   | 2 (2)                   |                          |                         |                         |
| rplB         | 50S ribosomal protein L2            | 30 kDa   | 12 (13)                 |                          | 11 (13)                 | 10 (13)                 |
| rplC         | 50S ribosomal protein L3            | 24 kDa   | 3 (8)                   |                          | 7 (7)                   | 4 (8)                   |
| rplD         | 50S ribosomal protein L4            | 22 kDa   | 6 (7)                   |                          | 3 (5)                   | 5 (5)                   |
| rplO         | 50S ribosomal protein L15           | 16 kDa   | 7 (10)                  |                          | 6 (7)                   | 8 (15)                  |
| rplR         | 50S ribosomal protein L18           | 13 kDa   | 2 (2)                   |                          | 2 (3)                   | 4 (7)                   |
| rplS         | 50S ribosomal protein L19           | 13 kDa   | 4 (4)                   |                          | 6 (5)                   | 5 (7)                   |
| rplT         | 50S ribosomal protein L20           | 14 kDa   | 2 (5)                   |                          | 2 (2)                   | 2 (3)                   |
| rplU         | 50S ribosomal protein L21           | 11 kDa   | 6 (7)                   |                          | 6 (5)                   | 8 (8)                   |
| rplV         | 50S ribosomal protein L22           | 13 kDa   | 4 (3)                   |                          | 3 (2)                   | 3 (4)                   |
| rplW         | 50S ribosomal protein L23           | 11 kDa   | 2 (5)                   |                          | 2 (3)                   | 2 (5)                   |
| rpmC         | 50S ribosomal protein L29           | 8 kDa    | 3 (4)                   |                          | 2                       | 3 (3)                   |
| rpmE2        | 50S ribosomal protein L31           | 10 kDa   | 2                       |                          |                         | 3 (4)                   |
| rsmA         | rRNA small subunit methyltransferase A | 34 kDa   | 3                       |                          |                         |                         |
| infB         | Translation initiation factor IF-2  | 78 kDa   | 3 (3)                   |                          |                         |                         |
| serS         | Serine–rRNA ligase                  | 49 kDa   | 2                       |                          |                         |                         |
| METABOLISM   |                                    |          |                        |                          |                         |                         |
| ald1         | Alanine dehydrogenase 1, SA1272     | 40 kDa   | 3                       |                          |                         |                         |
| adh          | Alcohol dehydrogenase               | 36 kDa   | 10 (3)                  |                          |                         |                         |
| atpF         | ATP synthase subunit                | 20 kDa   | 2                       |                          |                         |                         |
| qoxB         | Probable quinol oxidase subunit 1   | 75 kDa   | 3                       |                          |                         |                         |
| qoxA         | Probable quinol oxidase subunit 2   | 42 kDa   | 3                       |                          |                         |                         |
| ubiE         | Demethylmenaquinone methyltransferase, menaquinone biosynthesis | 27 kDa | 3 | |
| pyrR         | Bifunctional protein PyrR, RNA binding | 20 kDa | 3 (2) | |
| ftnA         | Ferritin                            | 20 kDa   | 5 (4)                   |                          | 5 (4)                   |                         |
| glmS         | Glutamine–fructose-6-phosphate aminotransferase | 66 kDa | 4 | |
| relA         | GTP pyrophosphokinase, ppGpp formation | 85 kDa | 4 (6) | |
| pgk          | Phosphoglycerate kinase             | 43 kDa   | 2                       |                          |                         |                         |
| lacF         | Lactose-specific phosphotransferase enzyme II A component | 11 kDa | 2 | |
| lacE         | PTS system lactose-specific EIICB component | 62 kDa | 3 | |
| ptsG         | PTS system glucose-specific EIICIA component | 74 kDa | 4 | |
| glcB         | PTS system glucoside-specific EIICBA component | 74 kDa | 6 | |
| murA1        | UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1 | 45 kDa | 10 | |
| kdpB         | Potassium-transporting ATPase B chain | 73 kDa | 2 | |
| ugpT         | Procesive diacylglycerol glucosyltransferase | 45 kDa | 2 | |
| gtaB         | UTP–glucose-1-phosphate uridylyltransferase | 32 kDa | 4 (3) | |
| SA0216       | Uncharacterized TCS response regulatory protein | 30 kDa | 2 | |
| saeS         | Histidine protein kinase SaeS, TCS  | 40 kDa   | 4                       |                          |                         |                         |
| hsiU         | ATP-dependent protease ATPase subunit HsiU, protein degradation | 52 kDa | 2 | |
| ftsH         | ATP-dependent zinc metalloprotease FtsH, protein degradation | 81 kDa | 3 | |
| OTHER FUNCTIONS |                                  |          |                        |                          |                         |                         |
| esaA         | Protein EsaA, protein secretion     | 115 kDa  | 5                       |                          |                         |                         |

(continued on next page)
Previously both the 5'–3' RNase J and the 3'–5' PNPase are sensitive to secondary structures.\textsuperscript{40,41} However, it remains to be shown, whether CshA is a permanent member of the decay machinery, or if it is only recruited on demand. A detailed analysis of some of the target RNAs requiring CshA will certainly help to further distinguish between these possibilities.

RNA turnover in \textit{S. aureus} has been analyzed previously in exponential phase, heat shock, cold shock, stringent and SOS response conditions.\textsuperscript{42} Under heat shock, cold shock and stringent response conditions, the stability of most mRNAs increased, but so far the components required for the turnover have not been identified. Using tandem affinity purifications we were able to determine the interaction partners of CshA, CshA\textDelta Cter, RNase J1 and CshB. Importantly, CshA copurifies with the predicted degradosome proteins RNase J1, RNase J2, RNase Y and enolase. This is consistent with bacterial 2-hybrid analyses\textsuperscript{23} and the finding that the minimal \textit{H. pylori} degradosome is formed by RNase J and RhpA.\textsuperscript{26} In addition we noted that CshA interacts with 28 ribosomal proteins and various other proteins. Most of these interactions are lost when the purification is performed with CshA\textDelta Cter, indicating that the C-terminal region drives these interactions. However, it should be noted that many of these interactions might be indirect. Nevertheless it was shown that both the \textit{H. pylori} and the \textit{E. coli} degradosomes associate with poly-ribosomes\textsuperscript{18,26} and that RNase J1 and RNase J2 from \textit{B. subtilis} are tightly ribosome associated. Moreover, most ribosomal proteins that copurified with CshA were also found in the purification of RNase J1. Finally, CshA was reported to be involved in ribosome biogenesis in the same bacterial species.\textsuperscript{15}

To distinguish between an interaction with RNase J or the ribosome, we intended to co-purify recombinant RNase J1 and CshA. However, so far we were not able to demonstrate co-purification by mixing the purified proteins, but found a weak interaction if co-expressed together in \textit{E. coli} (data not shown). Further experiments will be required to confirm or reject such a putative interaction between CshA and RNase J1, or an indirect interaction through the ribosome. We observed that CshA copurifies with a higher number of proteins than RNase J1 and CshB and that many more proteins unrelated to RNA metabolism co-purify with CshA. It is not clear to what extent CshA could be associated with translating ribosomes or could be anchored to the membrane through its interaction with RNase Y\textsuperscript{24,43} and thereby indirectly co-purify with a large number of proteins.

It has been shown for several DEAD-box proteins that N- and C-terminal extensions are important for interactions with either their substrate or partner proteins, and thereby confer specificity to these RNA-dependent ATPases. Here we showed that the deletion of the C-terminal 124 amino acids results in reduced growth at low temperature (16°C) and stabilization of the \textit{agr} mRNA similar to the full deletion. The slightly better growth at 24°C indicates that the truncated helicase, despite reduced enzymatic activity (see below) and the absence of many interacting proteins, retains some functionality which may be provided by interactions through the core domain, as it is observed for the minimal RNA helicases elf4A or elf4AIII, interacting with elf4G and the exon-junction complex components, respectively.\textsuperscript{44,45} It may also support the hypothesis that CshA is involved in another process, such as ribosome biogenesis. Indeed, in \textit{E. coli} the deletion of RhlB does not confer a cold sensitive phenotype, whereas the deletion of CsdA/DeaD or SrmB results in cold-sensitivity, probably due to erroneous 23S rRNA translation into 5S RNA METABOLISM

| gene name     | Identified Proteins                                                                 | MW    | RNase J1 | CshA | CshA\textDelta Cter | RNase J1 | CshB |
|---------------|-----------------------------------------------------------------------------------|-------|----------|------|----------------------|----------|------|
| ezrA          | Septation ring formation regulator EzrA                                             | 66 kDa| 14       | 14   | + (−) RNase          | + (−) RNase| 2 (2) |
| map           | Protein map, 4 microtubule associated protein motifs                               | 53 kDa| 3 (3)    | 3 (3)|                     | + (−) RNase| 2 (2) |
| SA1813        | Uncharacterized leukocidin-like protein 2                                          | 40 kDa| 4 (5)    | 4 (5)|                     | + (−) RNase| 2 (2) |
| uvrA          | UvrABC system protein A                                                            | 105 kDa| 2 (8)    | 2 (8)|                     | + (−) RNase| 2 (2) |
| gyrB          | DNA gyrase subunit B                                                              | 73 kDa| 2        | 2    |                     | + (−) RNase| 2 (2) |
| ftsK          | DNA translocase FtsK                                                               | 88 kDa| 3 (3)    | 3 (3)|                     | + (−) RNase| 2 (2) |
| fmtA          | ProteinFmtA, unknown function,                                                     | 46 kDa| 3 (5)    | 3 (5)|                     | + (−) RNase| 2 (2) |
| lip2          | Lipase 2                                                                          | 76 kDa| 2        | 2    |                     | + (−) RNase| 2 (2) |
| clfB          | Clumping factor B                                                                 | 97 kDa| 4        | 4    |                     | + (−) RNase| 2 (2) |
| spa           | Immunoglobulin G-binding protein A                                                | 56 kDa| 4 (6)    | 4 (6)|                     | + (−) RNase| 2 (2) |
| sbi           | Immunoglobulin-binding protein sbi                                                | 50 kDa| 5        | 5    |                     | + (−) RNase| 2 (2) |
| spsB          | Signal peptidase IB                                                               | 22 kDa| 3        | 3    |                     | + (−) RNase| 2 (2) |
| ssaB          | Staphylococcal secretory antigen ssaA2                                             | 29 kDa| 4 (3)    | 4 (3)|                     | + (−) RNase| 2 (2) |
| femX          | Lipid II glycine glycytransferase                                                   | 49 kDa| 8 (9)    | 8 (9)|                     | + (−) RNase| 2 (2) |
| SA0778        | UPF0051 protein                                                                   | 53 kDa| 2        | 2    |                     | + (−) RNase| 2 (2) |
| SA1727        | UPF0316 protein                                                                   | 23 kDa| 2        | 2    |                     | + (−) RNase| 2 (2) |
| SA1560        | UPF0478 protein                                                                   | 19 kDa| 2 (2)    | 2 (2)|                     | + (−) RNase| 2 (2) |
rRNA hybridization, as elegantly shown with a srmB suppressor analysis.\(^\text{46}\) Such a ribosome biogenesis function was also suggested for the \textit{B. subtilis} CshA\(^\text{35}\) and would be in accordance with the co-purification of a large number of ribosomal proteins.

Altogether, our results show that CshA is required for efficient turnover of the bulk of mRNAs and that a selected subset of RNAs is significantly stabilised in absence of the RNA helicase. For efficient degradation, the RNA helicase interacts through its C-terminal extension with the degradosome components. The molecular function of the RNA helicase could be the destabilisation of secondary structures or the removal of hindering RNA binding proteins. Further analysis of individual RNAs and different mutants in \textit{cshA} or the other components of the degradosome will help to further elucidate this exciting and important aspect of gene expression.

### Materials and Methods

**Bacterial strains**

Bacteria (Table 3) were grown under standard laboratory conditions. The deletion mutants were constructed using the \textit{pyrFE}/FOA counter selection system.\(^\text{30}\) Standard molecular biology methods for plasmid and strain constructions were employed according to either the manufacturer’s instructions or Sambrook and Russell.\(^\text{47}\) Plasmids used in this study are listed in Table 4.

**RNA isolation, GeneChip analysis and RNA-sequencing**

Overnight cultures were diluted in fresh Mueller-Hinton media to a final OD\(_{600}\) of 0.05. Cells were grown until mid-exponential phase (OD 0.3 – 0.4). A 1 ml sample was harvested and rifampicin was immediately added to arrest the transcription (final concentration 200 \(\mu\)g/ml). 1 ml samples were harvested at 0, 2.5, 5, and 10 minutes after rifampicin treatment. After centrifugation, the supernatant was removed and 0.5 ml of ice-cold acetone-ethanol (1:1) was added and the samples were stored at –80°C until the lysis step. For the lysis, the pellet was resuspended in 200 \(\mu\)l of TE containing 0.05 \(\mu\)g/ml lysostaphin and was incubated 10 minutes at 37°C. The RNA isolation was done immediately after, using the QIAshredder and RNasy mini column, according to the manufacturer’s recommendations (Qiagen). 1 \(\mu\)g of total RNA were ribo-depleted using the ribo-zero magnetic kit for Bacteria from epicenter. Libraries were then prepared using the Illumina TruSeq stranded mRNA kit according to manufacturer’s recommendations. Libraries were validated on the Bioanalyzer 2100 (Agilent) and the Qubit fluorimeter (Invitrogen). Samples were multiplexed by 8 and loaded at 8 pM on one lane of a Illumina HiSeq 2500 according to a single read – 50 cycles protocol. Alternatively, RNA was then reverse transcribed, and cDNA was fragmented, 3’-biotinylated, mixed with exogenous labeled “spike-in” transcripts, and hybridized to \textit{S. aureus} GeneChips by following the manufacturer’s recommendations for antisense prokaryotic arrays (Affymetrix, Santa Clara, CA).

**Assembly of the Staphylococcus aureus SA564 genome**

Total DNA was sequenced in a HiSeq 2500 machine using Illumina technology to obtain paired 100 nt reads, which were assembled into 13 contigs with the aid of the complete genome sequence from the closely related \textit{S. aureus} N315.\(^\text{48}\) One contig was a circular plasmid (pSA564), and the relative order of the remaining 12 contigs was inferred from the \textit{S. aureus} N315 chromosome, whereupon unique primers were designed to PCR-amplify across the gaps. All 12 gap-spanning PCR products could be obtained, thus confirming the contig-order, and the PCR products were sequenced individually by primer-walking with Sanger-sequencing to completely fill the gaps and generate a closed circular assembly. Finally, the original Illumina reads were re-mapped onto the SA564 assembly, and regions of uncertainty were PCR-amplified and confirmed/corrected with Sanger-sequencing. The final assembly was submitted to the NCBI database with accession number CP010890 and CP010891.

**Bioinformatic analysis**

The stranded mRNA-sequencing reads were mapped to the genome of \textit{S. aureus} N315 using the software BWA.\(^\text{49}\) Reads that did align to multiple positions on the genome are dropped. The number of reads overlapping a gene was counted taking into consideration the agreement between the mapped read strand and the gene strand. This was done under the R programming language environment (www.R-project.org), making use of Bioconductor packages (www.bioconductor.org). Obtained counts were normalized according to the count obtained for gene \textit{HU}, and scaled to reflect the average expression level of \textit{HU} in the 2 WT samples.

To include an RNA, we defined a threshold of 100 reads on average across the 4 time points to avoid too much noise in correctly estimating the half-life of a gene. The threshold of 100 was chosen arbitrary and roughly corresponds to a 5% error on the half-life estimate for a difference of 1 in the count. As reference we used the \textit{hu} mRNA which was shown to be highly expressed.

### Table 3. Strains

| Strains          | Description                                                        | Reference |
|------------------|-------------------------------------------------------------------|-----------|
| \textit{E. coli} |                                                                  |           |
| DH5a             | Standard laboratory cloning strain                                 | Invitrogen |
| PR01             | \textit{SA564} disrupted for 2 restriction systems and deleted \textit{pyrFE} | 30        |
| PR01\textDelta \textit{cshA} | PR01 with \textit{cshA} deleted                                       | 25        |
| PR01\textit{cshA}A\textit{Cter} | PR01 with \textit{cshA} region corresponding to amino acids 386 to 506 deleted | This study |
| PR01\textDelta \textit{cshB} | PR01 with \textit{cshB} deleted                                        | 30        |
| PR01\textDelta J | PR01 with \textit{RNaseJ} deleted                                    | 30        |

\textbf{S. aureus strains}

PR01 with \textit{cshA} deleted

PR01 with \textit{cshB} deleted

PR01 with \textit{RNaseJ} deleted

\begin{figure} [h]
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\caption{Figure caption goes here.}
\end{figure}
Table 4. Plasmids

| Name     | Description                                      | Reference |
|----------|--------------------------------------------------|-----------|
| pEB01    | pCN47 with the cat194 cassette from pCN38 replacing the Erm cassette | 25        |
| pEB07    | pEB01 plasmid with cshA gene from SA564 and 542 bp upstream | 25        |
| pCG      | pEB01 plasmid for expression of proteins with a C-terminal Strep/Flag tag | This study |
| pCGcshA  | pCG vector with cshA gene from SA564 and 542 bp upstream | This study |
| pCG cshAΔCter | pCG vector with cshA gene region from SA564 corresponding to CshA aminoacids 1–385 and 542 bp upstream | This study |
| pCGJ1    | pCG vector with RNaseJ1 gene from SA564 and 493 bp upstream | This study |
| pGCcshB  | pCG vector with cshB gene from SA564 and 589 bp upstream | This study |

and stable, and has been used previously. A qRT-PCR analysis of 16S rRNA and hup mRNA showed very similar stability over a time range of 10 minutes.

Using log2 transformed expression levels, measured at the 4 time points (0, 2.5', 5', 10'), a linear model was fitted to estimate the half-life. Indeed, the level of expression \( e_t \) at time \( t \), for a gene characterized by a half-life \( h \), and an expression level \( e_0 \) at time 0 is expected to be \( e_t = e_0 (1/2)^{t/h} \). The Log2 transformation of this formula results in a linear expression in \( t \): \( \log_2 (e_t) = \log_2 (e_0) - t/h \); so that the half-life is given by: \( h = -1/a \) where \( a \) is the linear coefficient of the model fitted on the log2 transformed expressions. For simplicity, half-life estimates > 30 minutes, or negative half-lives (as stable as HU or negative half-lives) were set to 30 minutes.

Model fitting was effectively performed by minimizing a weighted sum of square errors. Since most of the RNAs have a very short half-life ranging in background levels at time point 10 minutes or even earlier, weights were set accordingly (256, 64, 16, and 1 fold, for the 0, 2.5, 5, 10 minutes time points). We empirically determined that fitted models resulting in a weighted error sum higher than 20 are not satisfying and this score was therefore used as cut-off for quality control.

Tandem affinity purification

CshA, CshAΔCter, CshB and RNase J1 were cloned in the pCG vector, resulting in the expression of proteins containing a Strep/Flag tag at their C-terminal extremity. PR01ΔcshA, PR01ΔcshB and PR01ΔJ1 strains replicating respectively the pCGcshA or the pCGGshAΔCter vector, the pCGcshB, and the pCGJ1 were grown until OD = 1. One liter of bacteria was harvested and resuspended in lysis buffer (Tris 50 mM pH 7.5; CaCl₂ 1 mM; Triton 0.1%; DNaseI; lysozyme) and subjected to 3 freeze/thaw cycles. The lysate was cleared by centrifugation and loaded on a Streptactin column (IBA). The elution fractions were then loaded on a α-Flag column (Sigma). The purifications were done according to the manufacturer recommendations and the yield varied between 55 and 158 μg per preparation. Proteins were identified by LC/MS starting with 10 μg of material. Proteins were considered as being present in the sample if at least 2 different exclusive peptides were detected.

Bacterial expression vectors for recombinant CshA and CshAΔCter

The pET22b-CshA plasmids were transformed into E. coli Rosetta (DE3). Cultures (500 ml) derived from single transformants were grown at 37 °C in LB medium containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol until the OD₆₀₀ reached 0.6. The cultures were adjusted to 0.2 mM IPTG and 2% (v/v) ethanol and incubation was continued for 20 h at 17 °C. Cells were harvested by centrifugation and stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacteria were resuspended in 25 ml of buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM MgCl₂) and supplemented with one tablet of protease inhibitor cocktail (Roche). The suspension was adjusted to 0.1 mg/ml lysozyme and incubated on ice for 30 min. Imidazole was added to a final concentration of 5 mM and the lysate was sonicated to reduce viscosity. Insoluble material was removed by centrifugation. The soluble extracts were mixed for 30 min with 1.6 ml of Ni²⁺-NTA-agarose (Qiagen) that had been equilibrated with buffer A containing 5 mM imidazole. The resins were recovered by centrifugation, resuspended in buffer A with 5 mM imidazole, and poured into columns. The columns were washed with 8 ml aliquots of 10 and 20 mM imidazole in buffer A and then eluted step-wise with 2.5 ml aliquots of buffer A containing 50, 100, 250, and 500 mM imidazole. The elution profiles were monitored by SDS-PAGE. The 250 mM imidazole eluates containing the CshA polypeptides were stored at −80 °C. The protein concentrations were determined using the Bio-Rad dye reagent with BSA as the standard.

Glycerol gradient sedimentation

An aliquot (50 μg) of the nickel-agarose preparation of CshA was mixed with catalase (50 μg), bovine serum albumin (50 μg), and cytochrome c (50 μg). The mixture was applied to a 4.8-ml 15–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 1 mM EDTA, and 2 mM DTT. The gradient was centrifuged for 20 h at 4 °C in a Beckman SW50 rotor at 48,000 rpm. Fractions (0.17 ml) were collected from the bottom of the tube.

Triphosphatase assay

Reaction mixtures (15 μl) containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl₂, 1 mM [γ-³²P]ATP, 400 ng/μl rRNA and CshA as specified were incubated for 15 min at 37 °C. The reactions were quenched by adding 3.8 μl of 5 M formic acid. An aliquot (2 μl) of the mixture was applied to a polyethyleneimine cellulose TLC plate, which was developed using 0.5 M LiCl, 1 M formic acid. The radiolabeled material was visualized by autoradiography, and ³²P formation was quantified.
by scanning the TLC plate with laser Scanner Typhoon FLA 7000 (General Electric).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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