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The CshA DEAD-box RNA helicase is important for quorum sensing control in *Staphylococcus aureus*

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DEAD-box RNA helicases are present in almost all living organisms and participate in various processes of RNA metabolism. Bacterial proteins of this large family were shown to be required for translation initiation, ribosome biogenesis and RNA decay. The latter is primordial for rapid adaptation to changing environmental conditions. In particular, the RhlB RNA helicase from *E. coli* was shown to assist the bacterial degradosome machinery. Recently, the CshA DEAD-box proteins from *Bacillus subtilis* and *Staphylococcus aureus* were shown to interact with proteins that are believed to form the degradosome. *S. aureus* can cause life-threatening disease, with particular concern focusing on biofilm formation on catheters and prosthetic devices, since in this form the bacteria are almost impossible to eradicate both by the immune system and antibiotic treatment. This persistent state relies on the expression of surface encoded proteins that allow attachment to various surfaces, and contrasts with the dispersal mode of growth that relies on the secretion of proteins such as hemolysins and proteases. The switch between these two states is mainly mediated by the Staphylococcal cell density sensing system encoded by *agr*. We show that inactivation of the *cshA* DEAD-box gene results in dysregulation of biofilm formation and hemolysis through modulation of *agr* mRNA stability. Importantly, inactivation of the *agrA* gene in the *cshA* mutant background reverses the defect, indicating that *cshA* is genetically upstream of agr and that a delicate balance of *agr* mRNA abundance mediated through stability control by CshA is critical for proper expression of virulence factors.

Introduction

Tightly regulated RNA metabolism is important for cells to adapt to changing environments and to optimally utilize available nutrient resources. DEAD-box RNA helicases play crucial roles in almost all living organisms within the complexed interplay of RNA synthesis, RNA folding, RNA-RNA interactions, RNA localization, and, not least, RNA degradation. The RNA helicases are characterized by 12 conserved sequence motifs that are involved in ATP binding, RNA binding and intramolecular interactions.[1] These enzymes are able to locally unwind double stranded RNA in an ATP-dependent manner or they can clamp protein complexes to a substrate RNA under conditions in which ATP hydrolysis or Pi release are delayed.[1-3]

The model organisms, *Escherichia coli* and *Bacillus subtilis,* encode 5 and 4 DEAD-box proteins, respectively. Among the first DEAD-box proteins identified, the *E. coli* CsdA (previously called DeaD), SrmB, and DbpA were shown to be involved in ribosome biogenesis.[4-6] The *E. coli* DbpA and the *B. subtilis* YxiN (DbpA) proteins are specifically stimulated by hairpin 92 of the 23S rRNA, and both interact with this substrate via their C-terminus.[7-10] CsdA was also found to be involved in translation initiation.[11] The *E. coli* RhlE protein was proposed to be involved in ribosome biogenesis and interacts genetically with other DEAD-box proteins.[12] The *E. coli* RhlB protein was found to be part of the degradosome, which is composed of the scaffolding protein RNase E, PNPase and enolase.[13] The ATPase activity of recombinant RhlB protein is stimulated by RNase E.[14] An attractive hypothesis for the role of the RNA helicase RhlB within the degradosome would be the local unwinding of secondary structures that are inhibitory for RNA degradation by the PNPase.[13,15] Additionally, CsdA was also found to be associated with the degradosome under cold shock conditions.[16]

Several bacterial DEAD-box proteins were identified using a variety of screens, although the molecular basis for these phenotypes remains unclear. For example, DEAD-box proteins are required for phenolic acid metabolism, bacterial aggregation, oxidative stress response, cold adaption, and for growth in the absence of prey in the case of obligatory bacteriovorous bacteria.[17-22] In *Staphylococcus aureus*, the DEAD-box protein CshA, was identified in a screen for mini-Mu insertions that reduced biofilm formation.[23] Furthermore, CshA and its homolog from

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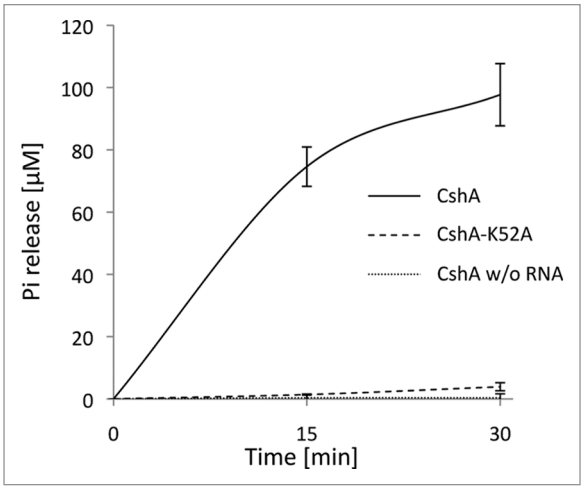

Figure 1. ATPase activity of CshA is RNA dependent. ATPase activity expressed in µM of Pi released, for wild type CshA (solid line) and the K52A mutant (dashed line) in the presence of RNA and in the absence of RNA (dotted line). Error bars show the standard deviation.

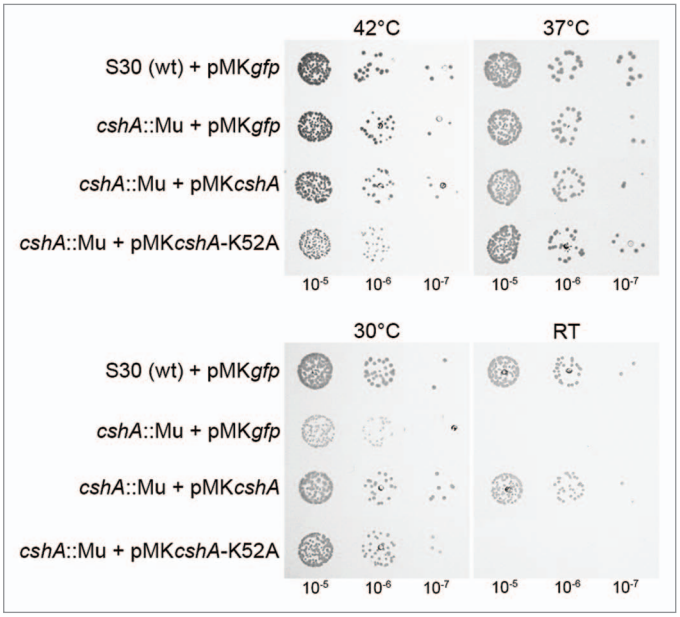

Figure 2. Inactivation of the *cshA* gene results in a cold-sensitive growth phenotype. The parental wild type strain S30, the *cshA*::mini-Mu insertion, the mutant complemented with wild type *cshA* and a Walker A motif mutant (K52A) under the control of a xylose-inducible promoter were spotted in serial dilutions (indicated underneath) on rich medium containing 1% xylose and chloramphenicol (15 µg/ml). All were incubated at 42°C, 37°C, 30°C, and room temperature for 24 h, 24 h, 48 h and 4 d, respectively. Plasmids expressing GFP instead of CshA were used as controls.

B. subtilis were recently reported to interact with proteins present within the Gram-positive degradosome.[24,25]

S. aureus is an opportunistic pathogen carried asymptomatically by approximately one third of the human population. It can cause infections ranging from furuncles to severe osteomyelitis and endocarditis.[26] These very different clinical pathologies reflect the versatility of this bacterium to adapt to changing environments and its ability to switch from a harmless sessile state to a very aggressive and dispersive growth mode.[27] Importantly, *S. aureus* can cause highly persistent infections that are difficult or even impossible to eradicate and it is generally assumed that these are due to intracellular survival,[28] the formation of small colony variants,[29] or the presence of biofilms.[30] Biofilm formation can formally be divided into several stages.[31,32] After primary adhesion via surface components, bacteria secrete proteins and exopolysaccharides to form an extracellular matrix, forming mushroom-like structures that eventually detach to seed new surfaces. This detachment is due to the presence of secreted proteases and surfactant peptides.[32]

S. aureus uses a quorum-sensing system, *agr*, to regulate the switch from adhesive to dispersal behavior.[27] The *agr* operon encodes an auto-inducing peptide (AgrD) secreted by AgrB. At high AgrD concentrations, the peptide binds to the two-component system AgrC/AgrA. The AgrA protein then stimulates transcription of the *agr* operon resulting in a positive feedback loop and promotes synthesis of RNAIII, a regulatory RNA that positively and negatively regulates a variety of genes involved in virulence.[33] Several reports describe a function of the *agr* regulatory system in biofilm formation,[34] although this is markedly strain dependent.[35]

Here we report that the CshA DEAD-box protein from *S. aureus* controls the stability of the *agr* mRNA, and thereby, virulence factor gene expression.

Results

CshA displays RNA-dependent ATPase activity. The *S. aureus* genome encodes two DEAD-box RNA helicases, SA1885 and SA1387, which are homologous to the *B. subtilis* CshA (YdbR) and CshB (YqfR), respectively (**Fig. S1**). The core sequence of the *S. aureus* CshA contains all the sequence motifs characteristic of DEAD-box proteins.[1] In addition, C-terminal of motif VI it possesses a highly charged (39+, 26-) region of 170 amino acids that could be required for the interaction with RNA or other protein partners (**Fig. S1**).

The *B. subtilis* CshA protein was shown to possess RNA dependent ATPase and dsRNA unwinding activity.[36] To assess the enzymatic activity of the staphylococcal CshA, we purified recombinant His_6-tagged protein from *E. coli* and tested for ATPase activity.[37] CshA showed a clear RNA-dependant ATPase activity, typical of members of the DEAD-box family (**Fig. 1**). Michaelis-Menten kinetic analysis was performed using variable ATP concentrations under conditions of saturating RNA. The measured K_M (ATP) value for CshA was 3.7 ± 0.47 mM with a K_{cat} value of 140 min^{-1}. The ATPase activity of CshA carrying a mutation in motif I (Walker A motif; CshA-K52A; **Fig. S1**), that prevents ATP hydrolysis,[38,39] was indistinguishable from that seen with buffer alone (**Fig. 1**).

The *cshA* mutant exhibits a cold-sensitive growth phenotype. Mutations in many bacterial RNA helicase genes cause a cold sensitive growth phenotype. We therefore tested growth

of the *cshA*::mini-Mu mutant strains at different temperatures. Results demonstrated a cold-sensitive phenotype, with partial growth inhibition at 30°C and complete inhibition at room temperature (**Fig. 2**). To confirm that this phenotype was due to the mutation, we complemented the strain with the *cshA* gene under the control of a xylose-inducible promoter in the presence of 1% Xylose.[23] This restored growth to almost wild type at all temperatures tested.

To ascertain that it is the enzymatic activity of the DEAD-box RNA helicase, and not a structural function within a complex, that was responsible for the observed phenotypes, we complemented the *cshA*::mini-Mu mutation with the K52A construct. Complementation by the mutant allele was not observed at room temperature (**Fig. 2**), although western blot analysis confirmed expression of the mutant protein (**Fig. S2**).

The *cshA* mutant is reduced in biofilm formation and shows increased hemolysis. The mini-Mu insertion in the *cshA* gene was originally isolated in a screen for biofilm-deficient mutants in the hyper-biofilm producing strain S30.[23] Biofilm formation in the mutant strain was reduced to approximately 30% of the wild type in microtiter plates or in polystyrene tubes, despite similar amounts of cells in the culture assay (**Fig. 3A**). Complementation with a wild type *cshA* gene restored biofilm formation, whereas complementation with the *cshA*-K52A mutant did not, showing that enzymatic activity is needed for biofilm formation (**Fig. 3A**).

To further characterize the *cshA*::mini-Mu mutant, hemolysis was examined by spotting overnight cultures onto blood agar plates, which were incubated overnight at 37°C and then transferred to 4°C for a further two days to visualize hemolysis. The S30 *cshA* mutant showed drastically increased hemolysis on rabbit and horse blood plates, indicating an increase in alpha and delta toxin production, respectively (**Fig. 3B**). Western blot analysis from exponential cultures confirmed the increased hemolysin synthesis (**Fig. S4**).

The *agr* mRNA is stabilized in the *cshA* mutant. The reduction in biofilm formation and increase in hemolysis would be consistent with an upregulation of the *agr* mRNA and its RNAIII target.[40,41] To test if this was indeed the case, we measured the steady-state levels of *agrA* mRNA by qRT-PCR in exponential phase cultures. The *agrA* mRNA was increased 2.9-fold in the *cshA*::mini-Mu mutant strain relative to the wild type (**Fig. 4A**).

In the light of the role of the *E. coli* RhlB DEAD-box protein in the degradosome and the bacterial two-hybrid interactions of the *B. subtilis* and *S. aureus* CshA proteins with components of the putative Gram-positive degradosome, the increase of the *agrA* mRNA might be caused by an mRNA degradation defect in the *cshA* mutant strain. To measure the stability of the *agr* mRNA in the parental and mutant strains, RNA was extracted at different time points after blocking transcription with rifampicin and *agrA*, *agrB* and *agrC* mRNA levels were quantified by qRT-PCR using *HU* mRNA as an internal reference. The data show a significant stabilization of the *agr* mRNA in the *cshA*::mini-Mu mutant strain compared with its parental counterpart (**Fig. 4B**; **Fig. S5**). Furthermore, the fact that all three analyzed regions of the *agrBDCA* operon mRNA behaved similarly makes a fortuitous stabilization of a particular region of the mRNA very unlikely. Thus, the stabilization of *agr* mRNA correlates with the steady-state increase of *agr* mRNA and, as shown below, also increases the level of RNAIII (**Fig. S6E**).

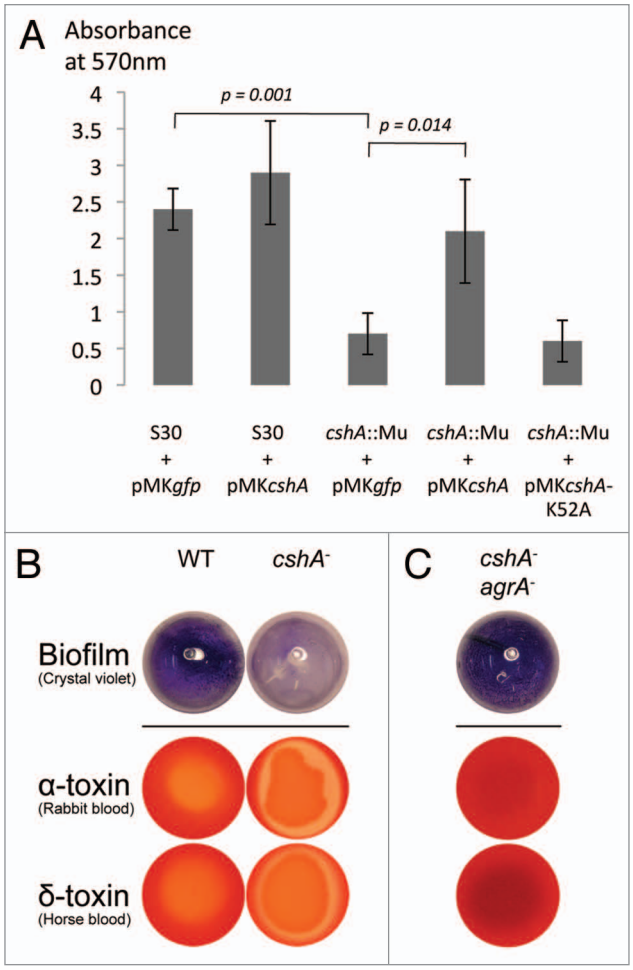

Figure 3. CshA is required for biofilm formation and reduces hemolysis. (**A**) Biofilm formation was analyzed by the crystal violet (CV) assay in polystyrene tubes after growth for 6 h in TSB medium with equal amounts of inoculated cells. The amount of biofilm was measured after solubilization of the CV in ethanol and measuring the absorbance at 570 nm. The error bars (standard deviation) are from three independent experiments. The differences between S30 + pMK*gfp vs cshA*::Mu + pMK*gfp* and *cshA*::Mu pMK*gfp vs cshA*::Mu pMK*cshA* were significant ($p = 0.001$, $p = 0.014$, respectively). (**B**) S30 wild type strain and its *cshA* mutant derivative were spotted onto rabbit and horse blood agar plates. Shown is one representative result from five independent experiments. (**C**) Inactivation of *agrA* in the *cshA* mutant restores biofilm formation and reduces hemolysis.

Inactivation of AgrA suppresses the *cshA* biofilm and hemolysis phenotypes. To check whether the biofilm defect is mediated through *agr* or via another CshA-dependent pathway, we disrupted the *agrA* open reading frame in the parental wild type and the *cshA* mutant strains using the targetron method.[42] Both biofilm formation and hemolysis on blood agar plates in the *cshA agrA* double mutant reverted to wild type (**Fig. 3C**; **Fig. S4**), with a slight increase in biofilm formation and a complete abrogation of hemolysis.

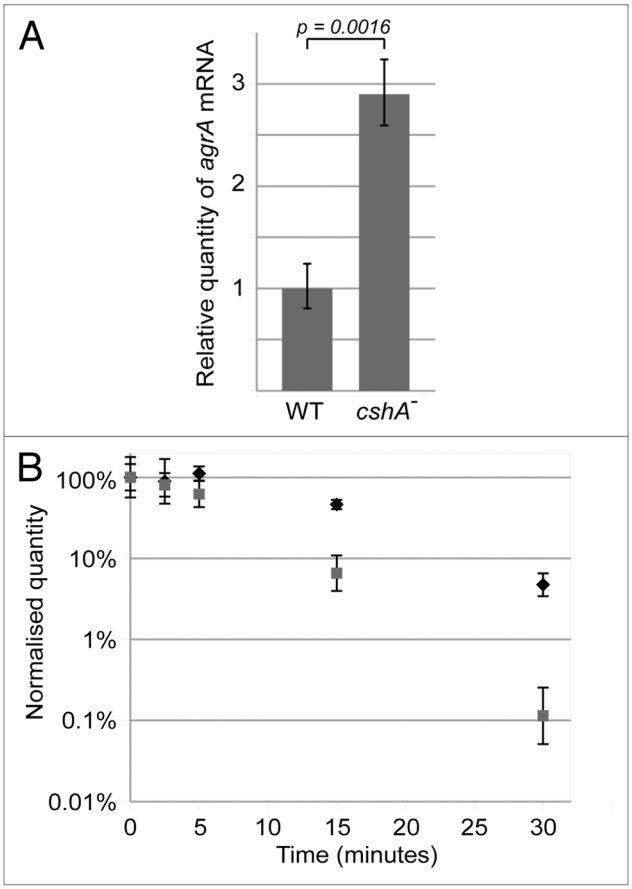

Figure 4. The *agrA* mRNA level is increased and stabilized in the *cshA* mutant strain. (**A**) RNA was isolated from exponentially growing cultures (approx. OD_{600} = 0.4) of the S30 wild-type and the *cshA* mutant. qRT-PCR was performed to determine the level of *agr* mRNA, using the *HU* mRNA as an internal reference. An unpaired T-test was performed to show that the difference in *agr* levels was significant (p = 0.0016). Error bars show the standard deviation. (**B**) Cultures of S30 (gray squares) and the *cshA* mutant (black diamonds) were rifampicin treated to block de novo RNA synthesis. Samples were taken for RNA isolation at 0, 2.5, 5, 15 and 30 min after treatment, and qRT-PCR was performed using primers and probe specific for *agrA*, and using *HU* mRNA as an internal reference. The quantity of *agr*, relative to *HU*, was normalized to 100% at time zero, and plotted in the graph. Error bars represent the 99% confidence level. The figure shows a single experiment out of three biological replicates.

The observed phenotypes can be reproduced in a different clinical isolate. The clinical strain S30 was observed to be a hyperbiofilm producer.[23] Therefore, to confirm that the observations made were not specific to this strain, we repeated the experiments with a different clinical isolate, SA564,[43] in which we inactivated the *cshA* gene by a targetron insertion[42] and by gene replacement using the pyrFE/5-FOA counter selection method.[44]

The analysis showed that growth, biofilm formation, and *agr* RNA data were very similar to the S30 strain (**Fig. S6**). Deletion of *cshA* caused a cold sensitive phenotype for growth and, although SA564 does not produce a lot of biofilm, the *cshA* mutation reproducibly reduced biofilm formation as measured by the crystal violet assay. Most importantly, *agr* mRNA steady-state levels were increased and mRNA decay was reduced in the *cshA* mutant compared with the parent. These results show that the CshA-mediated regulation of *agr* occurs in diverse genetic backgrounds. Since an increase in *agr* levels should result in increased RNAIII expression, we also tested steady-state levels and RNAIII degradation after rifampicin treatment in the wild type and the *cshA* mutant strains. Whereas, as expected, the steady-state levels were increased, the rifampicin experiment revealed a rather stable RNAIII that was not further stabilized in absence of CshA (**Fig. S6**).

Discussion

We have shown that *S. aureus cshA* mutants are cold sensitive, growing poorly already at 30°C, display decreased biofilm production, and show increased hemolysin production. Cold sensitivity is observed for many DEAD-box helicase mutants across many species, and could be explained by the general inability to resolve one or more secondary RNA structures thereby blocking the expression of essential genes. Correspondingly, *cshA* was identified by transposon mutagenesis in a screen for genes that are important for growth at low temperatures in the food poisoning bacterium *Bacillus cereus*.[45,46] Interestingly, a *cshA* mutation in *Bacillus subtilis* was reported to grow normally at low temperatures in one report,[47] but to be cold sensitive in another,[36] a discrepancy that may be strain dependent and needs further analysis.

We have demonstrated an increased stability of *agr* mRNA in the *cshA* mutant, which would account for the higher steady-state levels of the mRNA. This, in turn, induces synthesis of the regulatory RNAIII, an event that explains the increase of hemolysis and reduction of biofilm formation.[40,41,48]

Biofilm formation and hemolysin production are directly, but in an opposing manner, influenced by a specific set of genes and conditions.[40,49,50] Since growth of the *cshA* mutant was almost like wild type at 37°C under conditions in which the amount of bacterial cells was carefully controlled, decreased biofilm was not the consequence of a general growth defect. Moreover, the increased hemolysin production in the mutant is in perfect agreement with the increased *agr*/RNAIII-levels.

To confirm that the observed hemolysis and biofilm phenotypes were indeed a result of changes in *agr* levels, and not due to another unknown effect of the *cshA* mutation, we constructed a *cshA agrA* double mutant that eliminated *agr* quorum sensing. In the double mutant we observed a higher level of biofilm formation compared with wild type and a complete abolishment of hemolysis. We propose that the complete block in *agr* function caused by the *agrA* mutation is the reason why the phenotypes of the *agrA* mutants appear to be more drastic than a simple reversion to wild type. Thus, the absence of the CshA DEAD-box protein affects the regulation of the central *agr* regulatory system and places *cshA* genetically upstream of *agr*. The observed phenotypes are in line with epidemiological observations that bacteria from persistent infections are often deficient in the *agr* regulon.[34,51-55] Nevertheless, an additional effect of CshA on gene expression downstream of the *agr* system cannot be excluded.

A role of CshA in RNA decay is consistent with recent bacterial two-hybrid data demonstrating that the CshA proteins

from *B. subtilis* and *S. aureus* interact with components of a proposed Gram-positive degradosome.[24,25] Interestingly, RNase J1 and RNase J2 were also identified with CshA in the screen for biofilm-deficient mutants.[23] The biochemical analysis of CshA from *S. aureus* showed low ATPase activity compared with other DEAD-box proteins.[56] This was approximately 1.8 fold higher in the presence of RNase J2 (data not shown), although a direct interaction between these proteins was not found in the bacterial two-hybrid system.[25] This stimulation is much weaker than the one observed for RhlB by RNase E.[14] At present the precise role of CshA in RNA degradation is not known but several hypotheses can be formulated. The DEAD-box protein could present a sequence specific interaction with target RNAs and thereby target RNases. However, so far, the only DEAD-box protein with clear sequence specificity is DbpA, involved in ribosome biogenesis. A more likely function could be in assisting the RNA turnover machinery to overcome inhibitory secondary structures. It was shown for the *E. coli* RhlB that this RNA helicase is required for RNAs with secondary structures to allow the activity of the PNPase to proceed. Future experiments will be necessary to assess the function of the DEAD-box protein in presence of one or more components of the putative degradosome.

Finally, regulation of the quorum sensing system by RNA turnover may be a more general phenomenon. Indeed, in order to change quickly from high-level *agr* expression to low level of quorum sensing readout, it will be necessary to rapidly turn down the system (**Fig. 5**). Otherwise, a bacterial cell that is released from a dense population of Staphylococcal cells would not rapidly enough be able to turn on surface protein expression, necessary for adhesion and immune escape. Diluting out the mRNA by cell division or degradation with the bulk mRNA pool are probably not sufficient to obtain a rapid adaption to a new situation. Further analyses in *Staphylococcus* and other bacteria may reveal other similar situations, where RNA turnover is primordial to rapid adaptation.

In conclusion, our data show that the CshA DEAD-box protein is an important player in the regulation of *S. aureus* virulence factors through its involvement in mRNA turnover, in particular the mRNA of the central regulator *agr*. Whereas a DEAH-box RNA helicase was found involved in global gene regulation in *Borrelia burgdorferi*[57] and a DEAD-box protein of the Dhh1 subfamily in regulation of virulence associated genes in the pathogenic fungus *Cryptococcus neoformans*,[57] it is to the best of our knowledge the first time that a DEAD-box RNA helicase is implicated in the regulation of virulence factor expression in a pathogenic bacterium and that a functional CshA protein is important for the regulatory role of the *agr* quorum sensing system in *S. aureus*.

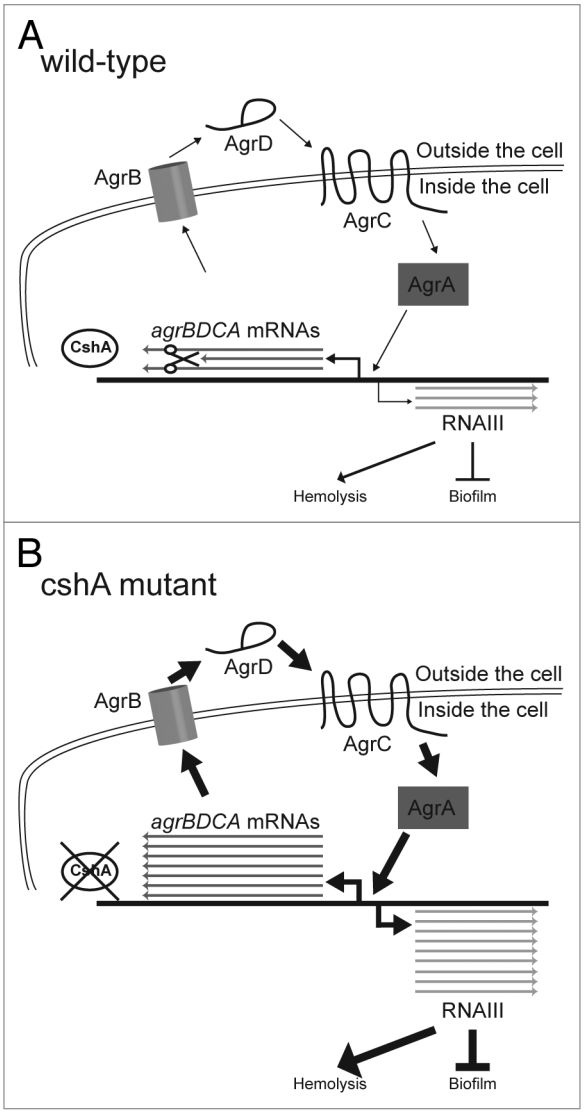

Figure 5. Model of CshA in the degradation of the *agr* mRNA. (**A**) In the wild type, *agrBDCA* mRNAs are produced. However, in the presence of CshA we hypothesize that the degradosome is able to degrade a significant portion of them. Thus the quorum sensing system is working correctly and only small amounts of RNAIII is produced leading to low stimulation of hemolysis and normal biofilm formation. (**B**) In absence of CshA, we propose that the degradosome is unable to degrade the *agrBDCA* mRNAs correctly, leading to a much higher level of Agr proteins, resulting in elevated RNAIII levels. The RNAIII, in turn, strongly stimulates production of hemolysins and extracellular proteases, and inhibits the production of biofilm components.

Material and Methods

Strains and plasmids. Bacteria were grown under standard laboratory conditions.[23,58] For the inactivation of the *agrA* gene, a targetron construct for the integration of a type II intron in anti-sense orientation was generated in a pNL9162 plasmid,[42] and a SphI-NarI fragment containing the intron and the intron encoded protein (IEP) was recloned into the SmaI site of pMK4[59] to avoid incompatibility problems between pNL9162 and the naturally resident pT181 plasmid in S30. After transformation of the parental and the S30 *cshA*::mini-Mu strain with this pMK4-*agrA*-targetron plasmid, colonies were screened by PCR for disruption of *agrA* by integration of the intron after coordinate 442 of the *agrA* open reading frame. The strain PR01Δ*cshA* was constructed using the *pyrFE*/5-FOA counter-selection system.[44] For complementation, the c*shA* gene was cloned under control of a xylose inducible promoter in plasmid pMK4[23] into the KpnI and PstI sites using PCR amplified DNA from strain S30, resulting in

Table 1. Strains

	Strains	Description	Reference
E. coli strains	DH10B	Standard laboratory cloning strain	Invitrogen
	Rosetta(DE3)pLysS	*E. coli* expression strain	Novagen
S. aureus strains	SA564	*S. aureus* clinical isolate	43
	SA564 *cshA*::*tt*	SA564 *cshA*::LI.LtrB group II intron after nucleotide 97	This study
	S30	*S. aureus* clinical isolate	23
	S30 *agrA*::tt	S30 having *agrA* disrupted by group II intron after nucleotide 442	This study
	S30 *cshA*::Mu	S30 cshA::mini-Mu, clone Ω4536	23
	Ω4536-C	S30 *cshA*::mini-Mu, clone Ω4536 complemented with CshA	23
	S30 *cshA*::Mu *agrA*::tt	S30 *cshA*::mini-Mu *agrA*::tt group II intron after nucleotide 442	This study
	PR01	SA564 disrupted for two restriction systems and deleted *pyrFE*	61
	PR01Δ*cshA*	PR01 with *cshA* deleted	This study

Table 2. Plasmids

Name	Description	Reference
pMK4Xyl	*E. coli-S. aureus* shuttle vector pMK4 with a xylose-inducible promoter	23
pMK4*gfp*	pMK4Xyl with *gfp*	23
pMK4*cshA*	pMK4Xyl with *cshA* from S30	23
pMK4*cshA*-K52A	pMK4Xyl with *cshA*-K52A	This study
pNL9162	pT181 based plasmid LI.LtrB group II intron targetron	42
pNL9162-*cshA*	pNL9162 modified to insert the intron into *cshA* (anti-sense)	This study
pMK4-targetron-*agrA*	LI.LtrB group II targetron in pMK4 for disruption of *agrA*	This study
pCN47	pT181 based shuttle vector with an Erm cassette	62
pCN38	pT181 based shuttle vector with a cat194 cassette	62
pEB01	pCN47 with the cat194 cassette from pCN38 replacing the Erm cassette	This study
pEB07	pEB01 with *cshA* gene from SA564 and 542 bp upstream, cloned between the BamHI and the SmaI-site	This study
pET15b	*E. coli* expression plasmid for purification of proteins with N-terminal His_6-tag	Novagen
pET22b	*E. coli* expression plasmid for purification of proteins with C-terminal His_6-tag	Novagen
pET22b-*cshA*	cshA cloned in NdeI and BamHI sites	This study
pET22b-cshA-K52A	cshA cloned in NdeI and BamHI sites	This study

pMK4*cshA*. For the construction of the K52A mutant, a fusion-PCR product was made using the mutagenic cshA-GAT oligonucleotides and the same external oligonuclotides resulting in pMK4*cshA*-K52A. Strains and plasmids are listed in **Tables 1 and 2**. Oligonucleotides are listed in **Table S1**. For overexpression in *E. coli*, the CshA open reading frame was PCR amplified and cloned into the NdeI and BamHI sites of pET22b (Novagen). For the recombinant K52A mutant gene, the same K52A-mutant oligonucleotides were used.

Standard molecular biology methods for plasmid and strain constructions were employed according to either the manufacturer's instructions or Sambrook and Russell.[58]

Purification of proteins and ATPase activity. Overexpression and purification of CshA and CshA-K52A were performed as follows: overnight culture of *E. coli* Rosetta (DE3) carrying pET22b-CshA was diluted in 200 mL LB with 100 μg/mL ampicillin and grown at 30°C. Expression of the protein was induced with 0.5 mM IPTG at OD_{600} 0.5 and incubated for 2 h. Purification of proteins was adapted from Tanner et al.[37] To check the purity of the protein, an aliquot of each eluate was loaded on a 10% SDS-PAGE. ATPase activity was tested at different pH (6.5, 7.5, 8), in different buffers (potassium acetate HEPES, KCl TRIS-HCl, potassium actate TRIS-HCl), different potassium acetate concentrations (40, 50, 60 and 80 mM), and different magnesium acetate concentrations (1, 2, 3 and 4 mM). Optimal reaction conditions for CshA activity were determined to be 50 mM potassium acetate, 20 mM HEPES (pH 7.5), 2 mM magnesium acetate at 37°C. ATPase activity in the presence of 1 mM ATP was measured using the Malachite green method for detection of released Pi and monitored at 630 nm.[37] Michaelis-Menten kinetics of the ATPase reaction were performed using 70 nM of CshA protein, in presence of saturating *E. coli* rRNA (1 μg/μL) and varying ATP concentration from 0 to 4 mM. K_M and K_{cat} were calculated using Kaleidagraph software (Synergy). The standard deviations were derived from the curve fits based on three independent sets of experiments.

Biofilm formation. Optical densities (OD) of bacteria from over night culture grown at 37°C with agitation were measured

at 600 nm. Bacterial suspensions were diluted to an OD of 0.1 into 1 ml fresh TSB medium and inoculated in polystyrene tubes (BD Biosciences 352057). After incubation for 6 h at 37°C without agitation, biofilms attached to the surface were stained for 10 min with a solution of 1% (wt/vol) Crystal Violet (CV) 2% (vol/vol) ethanol, rinsed carefully with water, and air-dried. Residual biofilm-associated CV dye was dissolved with 400 µl ethanol, diluted with 600 µl H_2O and transferred to a new tube for absorbance measurement at 570 nm.

Hemolysin assay. Mueller-Hinton medium with 10 g/l agar was autoclaved and cooled to 43°C, whereupon 50 ml/l 40% xylose and 70 ml/l blood (preheated to 43°C) was added, and the plates were immediately poured. De-fibrinated rabbit and horse blood was obtained from TSC Biosciences (Buckingham, UK). Spots of 10 µl over night culture were deposited on the plates and allowed to dry, incubated for about 20 h at 37°C, and two days at 5°C before scoring the extent of the hemolysis zone. At least five independent assays were performed, with mutants and wild type always on the same plate.

For Western Blot analysis, overnight cultures were diluted in MH medium to 1/100. Ten milliliters of each culture were collected after 4h (exponential phase; 0D = 0.6) of growth at 37°C under shaking conditions. Samples were centrifuged at 10,000 rpm and supernatants were incubated in the presence of Trichloroacetic acid (12% final) at 4°C overnight. Precipitates were centrifuged at 14,000 rpm, washed three times in acetone, resuspended in 50 µl Laemmli buffer and migrated on a 10% PAGE. Western blot analysis was performed with anti-*Staphylococcus* α hemolysin antibodies (abcam, ab50536) diluted to 1/20,000, according to the manufacturer's instructions.

Choice of reference for RNA quantification. Since it cannot be excluded that CshA also participates in ribosome biogenesis and since the 16S rRNA has extensive secondary structure, which might possibly be interacting with an RNA helicase such as CshA, we chose a highly stable mRNA, *HU*, which has been used as a reference in other studies.[60] To evaluate whether *HU* mRNA remains mostly unaffected by the *cshA* mutation, the decay-rate of *HU* mRNA was compared with ten other mRNAs (*asp23, deoD, dapA, dapB, asd, lytM, ilvC, sspA, purA)* by qRT-PCR in a manner similar to the *agrBDCA* operon. Out of the ten, which were chosen by using primers and TagMan-probes that were already available from other projects, six (asp23, dapB, *deoD, dapA, asd, lytM)* exhibited similar decay characteristics as *HU* mRNA, i.e., no change between wild type and *cshA* mutant when compared with the *HU* mRNA (data not shown). To further validate the use of HU as reference mRNA, we measured the amount of HU mRNA in the total RNA pool and found it to be relatively stable and unaffected by the *cshA* mutation (**Fig S3**).

RNA stability assay. *S. aureus* strains from 2 ml overnight cultures grown in MHB were diluted 1:200 into 20 ml of fresh medium and were incubated at 37°C under agitation. At an OD_{600} = 0.4 (in some experiments after 4 h), Rifampicin (200 µg/ml) was added to the culture and 1 ml was taken at different time points: 0, 2.5, 5, 15 and 30 min. Each sample was immediately centrifuged for 20 sec at 8,000 g and then the pellet was rapidly fixed in 500 µl of acetone:ethanol (1:1) and stored at -80°C. Total RNA was extracted as described below. For SA564, we performed the assays using 400 µg/ml antibiotic. In SA564 wild type the *agr* mRNA levels were, under these conditions, down to basal level after only 15 min, and therefore the time points taken were at 0, 2.5, 5, 10 and 15 min.

Total RNA extraction. Bacterial samples were harvested and washed in TE buffer (10 mM TRIS-HCl, 1 mM EDTA [pH 8.0]). Samples were lysed in TE containing lysostaphin (250 µg/ml) for 10 min at 37°C. RNA was extracted using the RNeasy mini Kit (Qiagen, 74104) and QIAshredder columns (Qiagen, 79654) following the manufacturer's instructions. DNA was removed from the RNA preparations by treatment with DNase I (Qiagen, 79254). Purified RNA samples were analyzed by using the RNA NanoLab chip kit (Agilent) on the 2100 Bioanalyser instrument (Agilent). At least two independent cultures and RNA extractions were performed for each strain.

Real-time RT-PCR. mRNA levels were determined by quantitative RT-PCR using the Brilliant II QRT-PCR Master Mix Kit, 1-Step (Stratagene). Primers and probes (**Table S1**) were designed using the PrimerExpress software 3.0 (Applied Biosystems) and obtained from Sigma, Applied Biosystems or Eurogentec. Reactions were performed using 1.25 ng of total RNA in a final volume of 10 µl, with the primers and probes (**Table S1**) at concentrations of 0.2 µM and 0.1 µM, respectively. RT-PCR mixtures were incubated for 30 min at 50°C, followed by incubation for 10 min at 95°C and then 40 cycles of 15 sec at 95°C and 1 min at 60°C in a StepOne Plus instrument (Applied Biosystems). The mRNA levels of target genes extracted from the different strains were normalized relative to their *HU* mRNA levels (see above). To minimize experimental variations, samples taken at different times from the same culture were treated simultaneously (RNA extraction and qRT-PCR analysis).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

Conceived and designed the experiments: P.R., P.F., J.S., P.L. Designed reference gene: P.F., J.S. Performed the experiments: S.O., P.R., A.C., J.P.D., E.B., C.G., M.G. Analyzed the data: P.R., J.P.D., P.F., J.S., P.L. Wrote the paper: P.R., P.L.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/22899

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