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The Chaperone ClpX Stimulates Expression of Staphylococcus aureus Protein A by Rot Dependent and Independent Pathways

Lotte Jelsbak1, Hanne Ingmer1, Lukás Valihrach1*, Marianne Thorup Cohn1, Mie H. G. Christiansen2, Birgitte H. Kallipolitis2, Dorte Frees1*

1 Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark
2 Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Abstract
The Clp ATPases (Hsp100) constitute a family of closely related proteins that have protein reactivating and remodelling activities typical of molecular chaperones. In Staphylococcus aureus the ClpX chaperone is essential for virulence and for transcription of spa encoding Protein A. The present study was undertaken to elucidate the mechanism by which ClpX stimulates expression of Protein A. For this purpose, we prepared antibodies directed against Rot, an activator of spa transcription, and demonstrated that cells devoid of ClpX contain three-fold less Rot than wild-type cells. By varying Rot expression from an inducible promoter we showed that expression of Protein A requires a threshold level of Rot. In the absence of ClpX the Rot content is reduced below this threshold level, hence, explaining the substantially reduced Protein A expression in the clpX mutant. Experiments addressed at pinpointing the role of ClpX in Rot synthesis revealed that ClpX is required for translation of Rot. Interestingly, translation of the spa mRNA was, like the rot mRNA, enhanced by ClpX. These data demonstrate that ClpX performs dual roles in regulating Protein A expression, as ClpX stimulates transcription of spa by enhancing translation of Rot, and that Rot additionally is required for full translation of the spa mRNA. The current findings emphasize that ClpX has a central role in fine-tuning virulence regulation in S. aureus.

Introduction
Staphylococcus aureus is an opportunistic pathogen capable of causing a variety of diseases in humans, ranging from localized infections of skin and soft tissue to life-threatening systemic infections [1]. The pathogenicity of S. aureus relies on a wide array of surface-bound and secreted virulence factors that provide the bacterium with the ability of tissue binding, tissue destruction, and immune evasion [2]. These virulence factors are coordinately produced in a growth phase dependent manner. The cell-surface associated factors are primarily expressed during exponential growth phase, whereas expression of the secreted factors is induced upon transition to stationary phase. Central for this regulation is the quorum sensing agr locus [3].

Protein A is a major surface-bound virulence factor found in all examined strains of S. aureus [4]. It is well-known for its ability to bind the Fc-region of IgG from several mammalian species [5]. Additionally, Protein A can bind von Willebrand factor, and is capable of inducing inflammatory responses in the host [5,6]. Accordingly, the importance of Protein A in infections has been demonstrated in several animal models [7–9].

Expression of Protein A is regulated by growth phase and is controlled by complex regulatory networks acting at both the transcriptional, translational and post-translational levels [10–13]. The complex regulation of Protein A expression has been schematically depicted in Fig. 1. At the pinnacle of this regulatory network is the Agr quorum sensing system reviewed in [14]. The effector molecule of the agr quorum sensing system is RNAIII, a small regulatory RNA that is strongly induced in the post-exponential growth phase [14]. RNAIII is 514 nucleotides long and folds into a complex secondary structure comprising 14 distinct stem-loops [15]. Recent evidence supports that RNAIII fulfills its role as a global virulence regulator primarily by controlling translation of target genes [12,16–18]. In regard to Protein A expression, RNAIII acts both directly and indirectly to reduce synthesis of the protein. Directly, RNAIII down regulates expression of Protein A by binding to the spa mRNA [12]. This binding reduces Protein A synthesis at two levels, as it both inhibits translation and promotes degradation of the spa mRNA [12]. Indirectly, RNAIII reduces transcription of spa by inhibiting translation of Rot [17]. As illustrated in Fig. 1, Rot activates spa transcription directly by binding to the spa promoter, and additionally, by enhancing transcription of sarS, encoded directly upstream of spa [11,13]. Similar to Rot, SarS activates spa transcription through direct interactions with the spa promoter region [10,11,13,19]. The activator activities of Rot and SarS are counteracted by the
Bacterial strains and growth conditions

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table 1. *S. aureus* 8325-4 and SA564 were used as wild-type strains. As primary recipient for plasmids the restriction deficient strain *S. aureus* RN4220 [29,30] was used. *S. aureus* strains were grown in Tryptic Soya Broth media (TSB; Oxoid) under vigorous agitation (200 rpm) at 37°C. Usually 20 ml of medium was inoculated in 200 ml flasks to allow efficient aeration of the medium. For solid medium, 1.5% agar was added to give TSA plates. Erythromycin (5 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹), or tetracycline (5 µg ml⁻¹) was added as required. Upon receipt of the low-passage isolate SA564 (from G. Somerville) the strain was cultured once and stored frozen at −80°C. In all experiments we used SA564 streaked freshly from this stock. *Escherichia coli* strains Top10, Top10F' or StrataClone Solopack Competent Cells were used for DNA cloning and were grown in Luria–Bertani (LB; Oxoid) broth or on LB agar plates at 37°C. Tetracycline (10 µg ml⁻¹), kanamycin (50 µg ml⁻¹), chloramphenicol (35 µg ml⁻¹) or ampicillin (50 µg ml⁻¹) was added as required.

Plasmid and strain construction

**SA564ΔclpX.** For construction of SA564ΔclpX, pSaΔclpX from RN4220 [21] was electroporated into competent SA564 cells at 30°C as described in [31]. A strain containing a 655 bp in frame deletion in the *clpX* gene was obtained by allelic replacement as described in [21], except that the final plasmid-loss was performed at 37°C. Verification of the chromosomal *clpX* deletion was done as described in [21].

**Restoration of the chromosomal clpX locus in 8325-4ΔclpX and SA564 ΔclpX.**

As we were unable to clone an error-less clpX gene in *E. coli* an alternative strategy was employed to complement the *clpX* deletion in 8325-4 and SA564:

In the course of attempting to create a *clpX* deletion in the strain Newman following the same procedure as described in the present manuscript and in [21], we obtained the desired chromosomal deletion of *clpX* (D. Frees, unpublished data). However, following replacement recombination these colonies

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**Figure 1. Model depicting the regulatory network controlling Protein A expression.** Transcription of *spa* is positively regulated by the transcriptional activators Rot and SarS and negatively regulated by SarA [10,11,13,41]. Translation of *rot* and *spa* mRNAs is inhibited by RNAIII [12]. We hypothesized that ClpX stimulates Protein A expression by stimulating either synthesis or activity of Rot (indicated by the dotted arrow). See text for further details. Solid arrows indicate activation, while solid T-bars indicate repression. Proteins are indicated by spheres, mRNAs are indicated by wavy lines, Promoters are indicated by bent arrows.

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still maintained a plasmid-borne copy of the intact \textit{clpX} gene (and its promoter). This plasmid was transformed into SA564 and 8325-4, and used to restore the chromosomal \textit{clpX} locus by doing the same double cross-over procedure as initially used to create the \textit{clpX} deletion \cite{21}. Colonies containing an intact chromosomal copy of the \textit{clpX} gene were identified by colony PCR using the primers SaclpX385f (5'-GACGATGCAGAACAACGTG) and SaclpX2447r (5'-CCATACCCTGGAACATCCAC), and subsequently plasmid loss was achieved by growing at the non-permissive temperature.

\begin{table}
\centering
\caption{Bacterial strains and plasmids used.}
\begin{tabular}{|l|l|l|}
\hline
\textbf{S. aureus strains} & \textbf{Relevant genotype} & \textbf{Reference} \\
\hline
8325-4 & Reference strain & \cite{29} \\
RN4220 & Restriction-deficient mutant of 8325-4 & \cite{30} \\
RN6911 & 8325-4, \textit{agr::tetM} (Tet\textsuperscript{R}) & \cite{46} \\
HI2209 & 8325-4, \textit{△clpX} & \cite{21} \\
WA525 & 8325-4, \textit{rot::erm} & \cite{11} \\
HI2433 & 8325-4, \textit{rot::TN917} & \cite{47} \\
DF2269 & 8325-4, \textit{△clpX, agr::tetM} (Tet\textsuperscript{R}) & \cite{20} \\
LJ93 & 8325-4/pLOJ132, (Erm\textsuperscript{R}) & This work. \\
LJ97 & \textit{agr/pLOJ132, (Erm\textsuperscript{R}) (Tet\textsuperscript{R})} & This work. \\
LJ94 & \textit{△clpX/pLOJ132, (Erm\textsuperscript{R})} & This work. \\
LJ135 & \textit{△clpX, agr/pLOJ132, (Erm\textsuperscript{R}) (Tet\textsuperscript{R})} & This work. \\
LJ98 & 8325-4/pLOJ133, (Erm\textsuperscript{R}) & This work. \\
LJ102 & \textit{agr/pLOJ133, (Erm\textsuperscript{R}) (Tet\textsuperscript{R})} & This work. \\
LJ99 & \textit{△clpX/pLOJ133, (Erm\textsuperscript{R})} & This work. \\
LJ133 & \textit{△clpX, agr/pLOJ133, (Erm\textsuperscript{R}) (Tet\textsuperscript{R})} & This work. \\
LJ113 & 8325-4/pLUG520, (Erm\textsuperscript{R}) & \cite{12} \\
LJ117 & \textit{agr/pLUG520, (Erm\textsuperscript{R}) (Tet\textsuperscript{R})} & \cite{12} \\
LJ115 & \textit{△clpX/pLUG520, (Erm\textsuperscript{R})} & This work. \\
LJ121 & \textit{△clpX, agr/pLUG520, (Erm\textsuperscript{R}) (Tet\textsuperscript{R})} & This work. \\
HI2438 & 8325-4, \textit{hfq mutant} (Cam\textsuperscript{R}) & This work. \\
LJ118 & WAS25/pWA163. & This work. \\
SA564 & Clinical isolate & \cite{37} \\
HI2781 & SA564::\textit{△clpX} & This work. \\
HI2670 & 8325-4::\textit{△clpX+clpX} & This work. \\
HI2871 & SA564::\textit{△clpX+clpX} & This work. \\
\hline
\textbf{E. coli strains} & & \\
\hline
Top10 & Cloning strain & Invitrogen \\
Top10F\textsuperscript{R} & Cloning strain & Invitrogen \\
ER2566 & Protein expression strain & NEB \\
Strataclone cells & Cloning strain & Stratagene \\
\hline
\textbf{Plasmids} & & \\
\hline
\textit{pET28a\textsuperscript{+}} & His-tag vector (Kan\textsuperscript{R}) & Novagen \\
\textit{pET100/D} & His-tag vector (Amp\textsuperscript{R}) & Invitrogen \\
pRMC2 & shuttle-vector with tet-inducible promoter (Cam\textsuperscript{R}) & \cite{32} \\
pLOJ104 & \textit{pET28a\textsuperscript{+}} derivative with \textit{rot -his\textsubscript{L}}, (Kan\textsuperscript{R}) & This work. \\
pET100clpX & \textit{pET100/D-derivative with clpX-his\textsubscript{L}}, (Amp\textsuperscript{R}) & This work \\
pLUG520 & \textit{Prop0::spa lacZ translational fusion}, (Erm\textsuperscript{R}) & \cite{12} \\
pLOJ132 & \textit{Prop0::rot lacZ translational fusion}, (Erm\textsuperscript{R}) & This work. \\
pLOJ133 & \textit{Prop0::lacz transcriptional fusion}, (Erm\textsuperscript{R}) & This work. \\
pUC19 & \textit{E. coli cloning vector} (Amp\textsuperscript{R}) & This work. \\
pLOJ131 & \textit{Prop0::rot lacz fusion in pUC19} (Amp\textsuperscript{R}) & This work. \\
pWA163 & \textit{Prop0::rot (Tet\textsuperscript{R})} & \cite{11} \\
pASrot & \textit{Ptet0::rot (anti-sense)} (Cam\textsuperscript{R}) & This work. \\
\hline
\end{tabular}
\end{table}
Construction of pASrot expressing anti-sense rot from an inducible promoter

To vary the Rot content in S. aureus we used the expression vector pRMC2 [32]. This plasmid harbors an improved copy of the tetO promoter in pALC2073 that ensures complete repression when non-induced and high expression when induced by anhydrotetracycline. The rot gene was PCR-amplified from 3235-4 chromosomal DNA using the primers KpmI-rotF (5'-TTACCATGGTACACCATG) and SacI-rotR (5'-AATGAGAAGCCTGCAGC-3') which, except for the added restriction sites (underlined), are the same primers that J. Oscarsson used to clone rot in pWA163 [11]. The PCR-fragment was TOPO-cloned (Invitrogen), and after confirming that the resulting plasmid contained an error-less rot-gene, it was cloned into pRMC2 in the anti-sense orientation using SacI and EcoRI. The plasmid was first introduced into RN4220, purified from this strain using the Qiagen Plasmid Midi prep kit and then introduced into SA564 by electroporation as described by [31].

**pLOJ132.** A fragment containing the *rhoB*-promoter region, the first part of the *rhoB*-leader, but leaving out the Shine-Dalargino and startcodon was PCR amplified using the primers Ppsofsd (5'-AATTGAATTCGTTAAAGGAAAGTGATGC-3') and PrpoRevNy (5'-TCAGGTACAAAAATATATGGTACGCT-3'), digested with EcoRI and *KpnI* and inserted into pUC19 generating pLOJ130. A fragment containing part of the *rho*-leader [33] and the first 3 aa codons was PCR amplified using the primers o-loj-rotmfw (5'-GGGTTACCATGGATGTTTTACTATTAGTTAATTAAATTGCC-3'), digested with EcoRI and *KpnI* and inserted into pUC19 generating pLOJ130. A fragment containing the first part of the *rpoB* leader was gel purified (GFX) and ligated into pLUG520 where the *rpoB*-leader was translationally fused to lacZ. This resulted in pLOJ132; *rhoB*-rhoB-leader translationally fused to lacZ in a pLUG520 derivative.

**pLOJ133.** A fragment containing the *rhoB*-promoter region, the *rhoB*-leader, and the Shine-Dalargino and startcodon was PCR amplified using the primers Ppsofsd and PrpoRev2 (5'-GGGTTATGCACTCCTGCAAACAGATT-CAC-3'), digested with EcoRI and *BamHI* and ligated into pLUG520 where the *rhoB*-rhoB-leader fragment had been removed using the same enzymes. This resulted in pLOJ133; *rhoB* translationally fused to lacZ in a pLUG520 derivative.

The plasmids were introduced into *S. aureus* strain RN4220 by electroporation and purified using the miniprep kit from Omega. The plasmid isolation procedure was modified by incubating the cell suspension in solution I containing 50 mM TrisHCl, pH = 7.4 to a calculated OD 600 of 10.0. PMSF, 1 lysostaphin and collected. The cell pellets were thawed on ice, resuspended in 50 mM TrisHCl, pH = 7.4 to a calculated OD 600 of 10.0 and then induced by adding IPTG to a final concentration of 1.0 mM for 2 h. The overexpressed proteins were purified on a Nickel NTA resin as described by the manufacturer (Qiagen) and 200 μg of pure protein was used to immunize a rabbit (antibodies were produced by CovalAB Company) using standard procedures (Sambrook et al., 1989).

Overexpression and purification of Hfq protein and generation of anti-Hfq-antibodies

For purification of Hfq, the intein system (Impact-CN; New England Biolabs) was used. Protein purification was performed as described previously [33]. Anti-Hfq-antibody was produced by Charles River Laboratories by immunizing rabbits with purified Hfq protein. The specificity of the antibody was tested by Western blot analysis on purified Hfq protein and protein extracts.

Western blotting

*S. aureus* strains were grown in TSB as specified above. Exponential cells were harvested at OD600 = 1.0 (+/-0.1), and used for isolation of intracellular proteins. One ml aliquots were harvested and kept at -80°C until all samples were collected. The cell pellets were thawed on ice, resuspended in 50 mM TrisHCl, pH = 7.4 to a calculated OD600 of 10.0. PMSF, DNase, RNase and lysostaphin were added to the samples, and they were incubated at room-temperature for 15 min. Cellular debris was removed by centrifugation and the protein concentration of each sample was measured using the Bradford dye-binding method from Bio-Rad. For immunoblotting a total of 5 μg (or 10 μg for Hfq-immunoblotting) of each sample was loaded on NuPAGE® 4–12% Bis-Tris gels (Invitrogen) using MOPS-Buffer (Invitrogen). The proteins were blotted onto a polyvinylidene difluoride membrane using the XCell II blotting module (Invitrogen). The membranes were pre-blocked with IgG. The Rot and ClpX proteins were probed using Rabbit-anti-Rot-antibody or Rabbit-anti-ClpX-antibody at a 1:2000 dilution. Protein A was probed using monoclonal Mouse-anti-Protein-A antibodies (Sigma) at a 1:10000 dilution. The Hfq protein was probed using anti-Hfq antibodies at a 1:25000 dilution. Bound antibody was detected with the WesternBreeze Chemiluminescent Anti-Rabbit kit or Anti-mouse kit (Invitrogen). All Western blots were repeated at least three times with similar results.

RNA extraction and Northern blot analysis

Cells were grown in TSB at 37°C with shaking to the indicated OD600. 1 ml aliquots were harvested and immediately frozen and
stored at -80°C. Cells were lysed mechanically using the FastPrep system (Bio101; Q-biogene), and RNA was isolated using the RNeasy mini kit (QIAGEN, Valencia, Calif) according to the manufacturer’s instructions. Total RNA was quantified by spectrophotometric analysis (λ = 260 nm), and 5 μg of RNA of each preparation was loaded onto a 1% agarose gel and separated in 10 mM sodium phosphate buffer as described previously [20]. RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by capillary blotting as described by Sambrook et al. Hybridization was performed according to [20] using gene-specific probes that had been labeled with [32P]dCTP using the Ready-to-Go DNA-labeling beads from Amersham Biosciences. Internal fragments of the rot gene (amplified with the primers o-loj-rot-7 and o-loj-rot-10), spa (amplified with the primers described in [20]) or the madd gene (amplified with the primers 5'- GATCACAGAGATGTTATGG + 5'-CATAGCAGTGATCCAAGG) were used as template in the labeling reactions. All steps were repeated in three independent experiments giving similar results.

Measurements of β-galactosidase activity

Cells were grown in TSB as described above to the indicated OD_{600}. 1 ml aliquots were harvested and immediately frozen and stored at -20°C. Cell pellets were resuspended in 300 μl 0.9% NaCl containing 5 μg/ml lysothain and incubated at 37°C for 15 min until the samples became clear indicating lysis was complete. Cellular debris was removed by centrifugation and the samples were transferred to fresh tubes. The protein concentration of each sample was measured using the Bradford dye-binding procedure from Bio-Rad. Expression of β-galactosidase was quantified by adding 400 μl Z-buffer containing 1 mg/ml ONPG to 100 μl of each sample as described previously [36]. Samples were incubated at 37°C until the solution had become yellow or not longer than 180 min. The reaction was stopped by adding 500 μl 1 M NaCO₃ and β-galactosidase activity was measured at OD_{420} and the specific activity (1 unit = 1 nmole o-nitrophenol produced per min per mg protein) was calculated. All steps were repeated in three independent experiments giving similar results.

Statistical analysis. All significant values were determined using the 2 sample t-test with unequal variances. Error bars represent standard deviations.

Results

Cells lacking ClpX contain reduced levels of the Rot transcriptional regulator

Similar to the transcriptional regulator Rot, ClpX is required for transcription of the spa gene, encoding Protein A [20,21]. Based on this observation we proposed that the chaperone activity of ClpX is required either to activate Rot, or, to control expression of Rot [20]. To estimate the level of Rot protein in the clpX mutant cells, we prepared polyclonal antibodies against Rot. The Rot antibodies were used to determine the relative amount of Rot protein in wild-type cells and in cells carrying deletions in clpX, clpP, or agr. As translation of the rot mRNA is inhibited by RNAIII [17], the Rot level was assayed both in exponential growth phase (prior to induction of RNAIII) and in post-exponential growth phase (RNAIII induced) (Fig. 2). The anti-Rot antibodies recognized a protein of the expected 16 kDa that was absent in protein extracts derived from the rot mutant (Fig. 2A). In wild-type cells the Rot protein was most abundant during exponential growth phase (Fig. 2A), which is consistent with the inhibitory role of RNAIII on rot translation [17,18]. In post-exponential cells the Rot level was gradually reduced, reaching 75% and 50% of the exponential level at OD_{600} = 2.0 and 3.0, respectively. In accordance with the proposed model, the down regulation of Rot was clearly dependent on agr. Interestingly, cells lacking ClpX contained 2–3 fold less Rot protein than wild type-cells at all assayed time-points (Fig. 2A). As restoration of the chromosomal clpX locus, resulted in wild-type expression of both Rot and Protein A we conclude that ClpX indeed has a stimulatory effect on Rot synthesis (Fig. 2B and data not shown).

Figure 2. Expression of Rot is decreased in cells lacking ClpX.

Proteins from wild-type, and clpX, rot, or agr mutant cells, (A), wt, clpX mutant cells and clpX complemented clpX mutant cells (ΔclpX+clpX) (B), and wild-type and clpP mutant cells (C) were extracted from exponential (OD_{600} = 1.0 (+/- 0.1)) or post-exponential cells (OD_{600} = 2.0 (+/- 0.1) and OD_{600} = 3.0 (+/- 0.1) as indicated. In each blot extracted proteins were separated by SDS-PAGE, blotted onto a PVDF membrane and probed with Rot antibody (A, B, central panel and, C) and ClpX antibody (B, upper panel). To visualize equal loading, signals detected from non-specific binding of the Rot antibody to an unknown cellular protein are shown. The Rot protein levels in (A) and (C) were quantified relative to the wild-type Rot in exponential cells (100%) using NIH imageJ 1.40. The ClpX protein, Rot protein and loading control protein levels in (B) were quantified relative to the wt level (100%) using Gene-snap from Perkin Elmer. The Western blots were repeated in at least 4 independent experiments giving similar results.

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The post-exponential down regulation of Rot was still observed in the clpX mutant, indicating that the effect of ClpX and RNAIII on Rot expression is mediated by different pathways. Moreover, cells lacking clpP contained wild-type levels of Rot protein at all assayed time-points (Fig. 2C), signifying that ClpX enhances Rot expression independently of ClpP.

**Expression of spa is activated above a threshold level of Rot**

After confirming that ClpX is required for full expression of Rot, we examined whether the 2–3 fold reduction of the Rot level can explain the dramatically reduced Protein A level of the clpX mutant [20]. To control the Rot-level, we employed a rot mutant that harbours a plasmid carrying the rot gene transcribed from a xylose inducible promoter [11]. Protein samples were derived from exponential cultures grown with varying amounts of xylose, and were used for Western blot detection of Rot and Protein A. As seen in Fig. 3, upper panel, Rot expression increased with increasing concentrations of xylose, and with 4% added xylose the Rot level approached the wild-type level. The experiment clearly demonstrated that Protein A synthesis was dependent on the Rot levels (Fig. 3, central panel). Specifically, Protein A expression remained low until the level of Rot protein constituted approximately 75% of the wild-type level. As Rot controls Protein A synthesis at the level of transcription, this finding indicates that a threshold concentration of Rot must be exceeded to obtain induction of **spa** transcription. Importantly, in the clpX mutant, the Rot level was below this threshold level (the level was comparable to the Rot level in cells grown in the presence of 0.5–1% xylose). Hence, this experiment confirms that the reduced level of Rot in the clpX mutant can explain the severely reduced **spa** transcription in this strain [20]. Additionally, our results suggest that Protein A expression is regulated by a threshold mechanism.

**ClpX Stimulates Translation**

The essential roles of ClpX and Rot in Protein A expression are conserved in a clinical strain

The increasing focus on virulence gene regulation in clinical strains has emphasized that regulatory models established in laboratory strains may not be representative of the regulatory mechanisms observed in clinical isolates [37–39]. To assess if the role of ClpX in Rot and Protein A synthesis is conserved in clinical isolates, we made the 651 bp in-frame deletion of the clpX gene in the low-passage isolate SA564 derived from a patient with toxic shock syndrome [37]. Expression of Protein A and Rot in the SA564ΔclpX strain and SA564 wild type strain was compared using Western blot analysis (Fig. 4A). Strikingly, the stimulatory role of ClpX on Rot/ProteinA synthesis appears even more pronounced in the SA564 background, as deletion of clpX in SA564 substantially reduced (approximately 6 fold) the cellular level of Rot, and concomitantly decreased **spa** transcription and Protein A synthesis to less than 5% of the wild-type level (Fig. 4A). Upon complementation of the clpX locus, Protein A and the Rot level were returned to wild-type levels (Fig. 4B). We conclude, that the positive roles of ClpX on Rot and Protein A synthesis are conserved in a low-passage clinical strain, suggesting that the underlying mechanism is conserved among **S. aureus** strains.

The SA564 strain expresses considerably more Protein A than 8325–4, and we therefore examined if Protein A expression is dependent on a threshold-level of Rot, as was observed for 8325–4 (Fig. 3). To reduce the cellular Rot level in SA564, the rot gene was cloned in the anti-sense orientation under control of the **tisO** promoter in the expression vector pRMC2 (see Materials and Methods for details; 32). As can be seen in Fig. 5, we could achieve a minor reduction (2 fold at the highest concentration of anhydrotetracycline) in the cellular Rot concentration by inducing transcription of the **rot** anti-sense mRNA. Interestingly, this modest reduction of Rot reduced expression of Protein A as much as 10 fold. This finding supports that below a threshold level of Rot, Protein A synthesis is greatly reduced.

**rot** transcription is slightly reduced in **clpX** and **clpP** mutant cells

We next attempted to pin-point the level at which ClpX mediates its effect on Rot synthesis. We first examined if ClpX controls Rot synthesis at the level of transcription. To this end, Northern blot analysis was performed to determine the level of **rot** transcript in exponential and post-exponential wild-type, **clpX** mutant and **clpP** mutant cells (Fig. 6). The Northern blot revealed that both **clpX** and **clpP** mutant cells contained **rot**-transcript levels that were slightly reduced compared to wild-type levels. As described above, the Rot protein level in the **clpP** mutant strain was comparable to the wild-type level, suggesting that the reduced **rot** transcription in **clpX** and **clpP** mutant strains is not reflected at the protein level. The Northern blot also revealed that in all strains transcription of **rot** increased in the post-exponential growth phase, as also reported by others [18]. The post-exponential induction of **rot** transcription occurs concomitantly with the down-regulation of Rot at the protein level emphasizing the significance of post-transcriptional regulation in Rot expression. From this experiment, we conclude that ClpX controls Rot synthesis mainly at the post-transcriptional level.

**ClpX does not control Rot expression by stimulating transcription of RNAIII**

Next, we addressed if ClpX controls Rot expression by enhancing expression of RNAIII that is an inhibitor of Rot translation [17]. To this notion, the level of RNAIII in wild-type,

![Figure 3. Rot performs threshold regulation of Protein A expression. The effect of the Rot concentration on Protein expression A was assayed by growing a rot-mutant expressing rot from a xylose inducible promoter in five parallel cultures supplemented with increasing amounts of xylose. Samples were collected at OD_{600} \approx 1.0 and total protein was extracted, equal amounts of protein in each lane was separated by SDS-PAGE, blotted onto a PVDF membrane, and probed to Rot polyclonal antibody (upper panel) and Protein A monoclonal antibody (lower panel). For comparisons, the Rot- and Protein A levels were determined in wild-type cells and in cells lacking either clpX or spa. To visualize equal loading, signals detected from non-specific binding of the Rot antibody to an unknown cellular protein are shown in the bottom panel. The Rot protein, Protein A, and loading control protein levels were quantified relative to the respective wild-type level in exponential cells (100%) using NIH imageJ 1.40. Each experiment was performed three times with similar results.](https://www.plosone.org/article?id=10.1371/journal.pone.0012752.g003)
agr, and clpX mutant cells in exponential and post-exponential growth phase was determined by Northern blot analysis (Fig. 7). The experiment demonstrated that the RNAIII level in the clpX mutant does not deviate from the wild-type levels. Hence, we ruled out that the reduced Rot level in the clpX mutant is accomplished through an increase in RNAIII synthesis.

ClpX stimulates translation of rot mRNA

To investigate directly whether ClpX regulates Rot translation, a translational fusion between the 5′-rot-leader and lacZ was constructed as depicted in Fig. 8A. This construct encompasses the RNAIII interacting region, the ribosome binding site, and the first three amino acid codons of the rot leader translationally fused to lacZ [17,33]. Transcription of the fusion is driven by the constitutive promoter PrpoB. The plasmid harbouring the rot-translational fusion was introduced into wild-type, and agr, clpX, clpP, and agr;clpX mutant strains, and β-galactosidase activity was assayed in samples derived from post-exponential cultures (Fig. 8B). The assay revealed that β-galactosidase activity was almost five fold higher in the agr mutant than in the wild-type (Fig. 8B). This suppressive effect of RNAIII on rot-translation is in the same range as observed by others and, thus, validates the constructs [17]. While samples derived from clpP mutant cells expressed wild-type levels of β-galactosidase (data not shown), β-galactosidase activity was reduced approximately two fold compared to wild-type in clpX mutant cells. This two-fold difference was statistically significant (wild-type different from clpX; P-value = 0.008), indicating that ClpX indeed has a positive effect on translation of rot. Interestingly, β-galactosidase activity in the agr, clpX double mutant was substantially reduced compared to the activity measured in the agr single mutant strain. This suggests that even though the inhibitory effect of RNAIII is relieved by a deletion of the agr locus, translation of Rot still depends on ClpX. Taken
together; these results specify that ClpX stimulates \( \text{rot} \) translation, and that the role of ClpX in \( \text{rot} \) translation is not linked to the RNAIII mediated inhibition of \( \text{rot} \) translation.

ClpX stimulates translation of \( \text{spa} \) mRNA but not of \( \text{rpoB} \) mRNA

Huntzinger et al. showed that translation of \( \text{spa} \) (Protein A), similar to translation of Rot, is inhibited by RNAIII [12]. To examine if ClpX has a role in translation of Protein A, the plasmid, pLUG520, carrying the \( \text{PrpoB-\text{spa}} \)-\( \text{lacZ} \) translational fusion used by Huntzinger et al. [12] was introduced into wild-type and mutant strains. This construct encompasses the constitutive \( \text{rpoB} \) promoter and the first 63 nucleotides of the \( \text{spa} \) mRNA (including the sites for RNAIII interaction) fused in frame with \( \text{lacZ} \) deleted of its ribosomal binding site, and thus specifically measures the translational activity of the \( \text{spa} \)-leader. In samples derived from post-exponential cultures, \( \beta \)-galactosidase activity was approximately 1.5 fold higher in the \( \text{agr} \) background than in the wild-type background, in accordance with previous results obtained by Huntzinger et al. [12] (Fig. 8C). Importantly, the absence of ClpX reduced \( \beta \)-galactosidase activity expressed from the \( \text{spa-lacZ} \) translational fusion approximately 5 fold, both in wild-type and in cells devoid of \( \text{agr} \)/RNAIII. We conclude that translation of \( \text{spa} \) mRNA, similar to translation of \( \text{rot} \) mRNA, is positively affected by ClpX and that the positive effect of ClpX on \( \text{spa} \) and \( \text{rot} \) translation occurs independently of RNAIII.

To investigate if ClpX also contributes to translation of mRNAs not interacting with RNAIII, we examined expression of the house-keeping gene \( \text{rpoB} \) in the \( \text{clpX} \) mutant by using a translational fusion between the \( \text{rpoB} \) promoter and \( \text{lacZ} \) (pLOJ133). With this plasmid, \( \beta \)-galactosidase activity was similar in samples derived from all strains demonstrating that neither RNAIII nor ClpX regulates expression of RpoB (Fig. 8D).

**Discussion**

Protein A is an abundant surface protein that by its ability to bind the Fc fragment of immunoglobulins from mammalian species is believed to be important for immune evasion of \( S. \text{aureus} \) [5]. Previously, we have shown that ClpX (but not ClpP), similar to the transcriptional regulator Rot, is essential for transcription of the \( \text{spa} \) gene encoding Protein A, and therefore we hypothesized that the chaperone activity of ClpX is required for either synthesis or activity of Rot [20]. In the present study we confirm that ClpX is indeed required for full expression of Rot, as cells devoid of ClpX contain approximately 2–3 fold less Rot protein than wild-type cells. This finding raised the question whether the relatively modest 2–3 fold reduction in the cellular Rot level is sufficient to abolish \( \text{spa} \) transcription. We confirmed this by varying Rot expression from an inducible promoter and demonstrated that a 2 fold reduction in Rot expression causes a dramatic 90% reduction in Protein A expression in both 8325-4 and the low-passage clinical isolate SA564 [40]. This finding emphasizes that major changes in Protein A expression may be achieved by small reductions in the Rot level and suggests that a threshold level of Rot is needed for synthesis of Protein A.

Rot stimulates Protein A transcription both directly, by binding to the \( \text{spa} \) promoter, and in-directly by enhancing transcription of...
Figure 8. Translation of Rot and Protein A is decreased in the absence of ClpX. (A) Schematic drawing of the lacZ translational fusion points in pLOJ132 and pLOJ133. pLOJ132 is a PrpoB-rot-lacZ translational fusion. Transcription is driven from the constitutive PrpoB promoter. The rot-leader encompassing the RNAIII interacting region from +162 to +250 (numbered with respect to the transcriptional start site) is translationally fused to the lacZ gene. pLOJ133 is a PrpoB-lacZ translational fusion. (B) β-Galactosidase activity measured in post-exponential (OD$_{600}$ = 2.0) wild-type- and, clpX, agr, agr;clpX mutant strains harbouring pLOJ133. The specific activities are presented as a percentage of the wild-type activity (100%). P-values were calculated using the two-sample t-test with unequal variances: wild-type different than clpX: P-value = 0.008; agr different than agr;clpX: P-value = 0.0004. The statistical calculations were based on five independent experiments. (C) Protein A translation was measured as β-Galactosidase activity expressed from pLUG520 in wild-type- and clpX, agr, and agr;clpX mutant cells in the post-exponential growth phase (OD$_{600}$ = 2.0). Specific
activities are presented relative to the wild-type level (100%). [D] β-Galactosidase activity measured in post-exponential (OD_{600} = 2.0) wild-type and mutant cells that harbour pOJ133 carrying a translational fusion between spa and lacZ. The specific activities have been calculated relative to the wild-type level (100%). Each experiment was performed at least four independent times with similar results.

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**ClpX Stimulates Translation**

**Supporting Information**

**Figure S1** Hfq could not be detected in Western blot analysis. Cells were harvested in early stationary phase or late stationary phase. Cells were lysed as described in materials and methods and total protein extract were separated on an SDS-gel. 10 μg protein was loaded (0.5 μg purified S. aureus Hfq protein), and samples were heated to 95 °C for 20 min prior to loading. For comparison of protein sizes, the PageRuler TM Plus Prestained Protein Ladder (Fermentas) was included (not shown). The Hfq antibody specifically recognizes the monomeric and multimeric species of the purified Hfq protein, whereas no bands at corresponding sizes were observed in any of the cellular protein samples tested.

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**Author Contributions**

Conceived and designed the experiments: LJ HI MTC BK DF. Performed the experiments: LJ LV MC DF. Analyzed the data: LJ LV MTC MC BK DF. Contributed reagents/materials/analysis tools: DF. Wrote the paper: LJ HI LV MC BK DF.
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