Stringent response-mediated control of rRNA processing via the ribosomal assembly GTPase Era

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ABSTRACT
P-loop GTPases are widely conserved across all domains of life. These enzymes act as molecular switches, cycling between inactive GDP-bound, and active GTP-bound states. Our previous work identified the *Staphylococcus aureus* GTPase Era as a binding target for the stringent response alarmone (p)ppGpp. Here we show that, unlike in *Escherichia coli*, Era is not essential in *S. aureus* but is important for 30S ribosomal subunit assembly. We employ bacterial two-hybrid and split luciferase approaches to show that Era interacts with the endonuclease YbeY, a protein of unknown function YbeZ, and the DEAD-box RNA helicase CshA in *E. coli* and natively in *S. aureus*. We determine that both Era and CshA are cold shock proteins required for virulence and rRNA processing. Era and CshA also form direct interactions with the (p)ppGpp synthetase RSH, an interaction required for controlling (p)ppGpp levels in response to cold shock. Taken together, we conclude that Era acts as an intermediary protein, directing enzymes involved in rRNA maturation to their site of action, an activity which, under stress, is controlled by the stringent response.

INTRODUCTION

Ribosomes are macromolecular machines responsible for the synthesis of proteins in all living cells. In bacteria these complexes consist of two subunits, with the large 50S subunit containing 33 large proteins (L1-36) and two rRNAs, and the 30S small subunit containing 21 small proteins (S1-21) and the 16S rRNA. As such, assembly of the ribosome is a tightly regulated process, with correct maturation requiring the help of assembly cofactors, one class of which are the P-loop GTPases. These enzymes are widely conserved across all domains of life and act as molecular switches, cycling between inactive GDP-bound, and active, effector-binding GTP-bound states. Within the P-loop GTPase class lies the Era family (*Escherichia coli* Ras-like protein), members of which are characterised by the presence of a distinct derivative of a KH domain (1). Era, the protein after which this family is named, is highly conserved throughout the bacterial kingdom, although is missing in *Chlamydia* and mycobacterial species (1). This GTPase is essential in *E. coli* (2-5), *Salmonella* Typhimurium (6) and in some strains of *Bacillus subtilis* (7,8). Reported functions for this GTPase include cell cycle control and chromosome segregation, as well as carbon and nitrogen metabolism (9-11). In addition, the ability of the 16S rRNA methyltransferase KsgA to suppress a cold-sensitive phenotype of an Era E200K mutant led to the first indication that Era was involved in ribosomal biogenesis (12).

Era is composed of two domains, an N-terminal GTPase domain and a C-terminal RNA-binding K-homology domain (13). A cryo-electron micrograph structure of Era in complex with the 30S subunit of the ribosome reveals that Era binds into the same pocket as small subunit protein S1 (14). In the absence of S1, Era interacts with proteins S2, S7, S11 and S18, as well as with a number of helices of the 16S rRNA. In addition, Era interacts with h45 and nearby residues 1530-1534 (GAUCA) in the 3' minor domain of the 16S rRNA via its CTD region (14). These residues are close to the anti-Shine Dalgarno sequence, critical for the formation of the 30S preinitiation complex. Including Era in *in vitro* reconstitutions of the 30S ribosome increases the affinity of several late-stage ribosomal proteins for the RNA (15,16). Consequently, it has been proposed that Era functions as a checkpoint protein, and that by binding to the 16S rRNA the formation of the initiation complex is prevented until the appropriate time (14). In addition to interacting with ribosomal proteins, Era has also been reported to interact with a number of proteins involved in 16S rRNA maturation. One of these, YbeY, is an endonuclease required in *E. coli* for the maturation of the 3' end of the 16S rRNA (17,18). It is proposed that the binding of YbeY to Era and S11 guide the endonuclease to its site of action (17).

The stringent response is a bacterial signalling system used by bacteria to cope with a variety of environmental stresses, the best characterised of which is nutrient deprivation. The opportunistic pathogen *Staphylococcus aureus* contains three enzymes, RSH, RelP and RelQ, which upon sensing a stress synthesise the nucleotides guanosine tetra- and pentaphosphate ((p)ppGpp) (19,20). Once produced, this alarmone controls cellular responses to aid survival. Our previous work identified Era, as well as three other GTPase enzymes from *S. aureus*, as target proteins for (p)ppGpp and demonstrated that the production of (p)ppGpp has a negative impact on mature 70S assembly (21). Here, we examine the role of Era as an enzyme required for ribosome biogenesis. We determine that this GTPase is not essential for the growth of *S. aureus*, but that mutant cells are defective in 30S subunit maturation. We identify the endonuclease YbeY and the DEAD-box RNA helicase CshA as interaction partners for Era in *S. aureus*, and show that both Era and CshA are crucial for rRNA homeostasis, especially at cold temperatures. We additionally demonstrate that both Era and CshA interact with the (p)ppGpp synthetase RSH, and that RNA processing is controlled in a (p)ppGpp-dependent manner. With this, we identify Era as a protein that facilitates the interactions between a number of rRNA processing and degrading enzymes and the 30S subunit/16S rRNA, and show that under stress conditions the stringent response is a key regulator of this process.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains were grown in LB and *S. aureus* strains in TSB at 37°C with aeration. Strains and primers used are listed in Tables S1 and S2. Information on strain construction is provided in SI Methods.

β-galactosidase activity assays in *E. coli.* *E. coli* cultures were diluted to an OD_{600} of 0.05 and grown in the presence of 1 mM IPTG until mid-exponential phase. 1 ml aliquots were suspended in ABT buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4 pH 7, 100 mM NaCl, 1% Triton-X-100). 100 µl was mixed with 20 µl 0.4 mg/ml 4-methyl umbelliferyl β-D-galactopyranoside (MUG) using a black 96-well polystyrene plate. Following incubation for 60 min in the dark, the sample was diluted 1:10 in ABT buffer and fluorescence detected at an excitation wavelength of 336 nm and emission wavelength of 445 nm. A standard curve was generated using dilutions of a known concentration of 4-methylumbilliferone (MU) in ABT buffer.

Luciferase assay. Overnight cultures of *S. aureus* strains were diluted to an OD_{600} of 0.05 and grown for 90 min in the presence of 100 ng/ml anhydrotetracycline (Atet). Strains were normalised to an OD_{600} of 0.1 and luciferase activity measured according to the Nano-Glo Luciferase Assay System Protocol (Promega).

Pull-down experiments and western blotting. GST beads were washed 5 times in 1 x wash solution (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). 1 µM of GST or GST-CshA were coupled to the beads by incubating in 1 x wash buffer for 4 h at 4^oC. Unbound protein was removed by washing in 1 x wash solution. Protein-bound beads were incubated with 1 µM His-tagged Era in the presence of 1 x binding buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100) at 4^oC overnight. After washing, attached proteins were eluted from the beads with 50 µl elution buffer (25 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 10 mM reduced glutathione). Samples were mixed 1:1 with 2 x SDS protein sample buffer. Aliquots were separated on 12% SDS-polyacrylamide gels, and proteins subsequently transferred to PVDF membranes. His-tagged Era was detected using HRP-conjugated anti-His antibodies (Sigma) at a 1:4,000 dilution. Blots were developed by enhanced chemiluminescence and imaged using a ChemiDoc MP imager (Bio-Rad).

Construction of bacterial two-hybrid library. Genomic DNA of *S. aureus* was extracted and partially digested by incubation with Sau3AI at 37^oC for 20 min. Digested DNA was run on a 0.8% agarose gel and fragments of 500 to 1000 bp and 1000 to 3000 bp gel extracted and purified. This was repeated five times from separate genomic preps and DNA fragments pooled. The vector pUT18C was digested overnight at 37^oC with BamHI and dephosphorylated with antarctic phosphatase for 90 min. Genomic DNA fragments were ligated into the pUT18C linearized vector using T4 ligase, transformed into *E. coli* DH5α competent cells (New England Biolabs) and plated onto LB agar with carbenicillin. Plates were scraped and transformant plasmids isolated using the GeneJET plasmid purification kit (Thermo scientific).

Bacterial two-hybrid. Bacterial two hybrid plasmids containing genes of interest were co-transformed into *E. coli* BTH101 cells, plated on LB agar containing 150 µg/ml carbenicillin and 30 µg/ml kanamycin and incubated at 30°C overnight. Colonies were isolated, grown overnight at 30°C in LB broth containing 0.5 mM IPTG and 5 µl spotted onto LB agar containing 0.5 mM IPTG and 40 µg/ml X-gal. Plates were incubated at 30°C for up to 48 h. In some cases, BTH101 transformants were incubated overnight with 0.5 mM IPTG and spotted directly onto LB agar containing IPTG, X-gal and appropriate antibiotics.

***Galleria mellonella* survival assays.** *S. aureus* strains were grown overnight in TSB, pelleted at 17,000 x g, washed twice in sterile PBS and diluted to an OD_{600} of 0.1 (2-4 x 10^7 cfu/ml). Colony numbers were confirmed by dilution and plating on TSA. Final larval stage *G. mellonella* weighing 250-350 mg (Live foods direct, Sheffield, UK) were stored in the dark at 4^oC and used within 7 days. 5 µl of *S. aureus* inoculum was injected into the hemocoel through the last pro-leg using a Hamilton syringe 701 RN. Larvae were incubated in sealed plastic petri dishes at 37^oC and the number of surviving individuals recorded daily.

RNA extraction. Strains of *S. aureus* were grown overnight at 37°C and diluted to an OD_{600} of 0.05. Cultures were grown to an OD_{600} of 0.4 at either 37 or 25°C and harvested. RNA was extracted using the RiboPure RNA Purification Kit (Invitrogen) as per guidelines. RNA was visualised using a modified agarose gel containing 0.7% agarose and 0.9% Synergel (Diversified Biotech) in 0.5 X TBE (890 mM Tris, 20 mM EDTA pH 8, 890 mM boric acid) as per Wachi *et al* 1999 (22). RNA was visualised using EvaGreen fluorescent nucleic acid dye (Biotium) on a ChemiDoc MP imager (Bio-Rad).

Methods for adapted assays as listed below are available in SI Methods: Growth curves, MIC determination, protein purifications, synthesis of (p)ppGpp, DRaCALA binding assays, measurement of ppGpp levels in *S. aureus*, ribosomal profiles from *S. aureus* cell extracts, helicase and GTPase assays.

RESULTS

Era is not essential in *S. aureus* but is required for normal growth and ribosome assembly

Previous reports have suggested that *era* is an essential gene in multiple bacterial species (3,6). In agreement with this, transposon insertions in this gene have not been identified in a number of published *S. aureus* transposon libraries (23-25). Closer inspection of the transposon insertion hits within the *era* operon obtained in the Nebraska *S. aureus* mutant library reveals insertions in *ybeZ*, a gene of unknown function, and at the 3' end of the diacylglycerol kinase *dgkA* but not within *ybeY*, *cdd*, *era* or *recO*, suggestive of an additional promoter upstream of *era* and *cdd* (Fig. 1a). In order to rule out polar effects and examine whether *era* in isolation is essential, we replaced the entire coding sequence with the Tet-encoding *tetAM* open reading frame in the presence of the Atet-inducible covering plasmid pCN55iTET-*era*. Following deletion of the chromosomal copy of *era*, we attempted to phage transduce this mutation into *S. aureus* strains containing the empty vector pCN55iTET or the complementing plasmid pCN55iTET-*era*. Transduction efficiencies were similar with both recipients, albeit smaller colony sizes in the absence of *era*, indicating that this gene is not essential in *S. aureus*. We next transduced the *era* deletion into the community-associated methicillin resistant *S. aureus* strain LAC* to rule out secondary mutations and used this strain for further studies. Analysis of growth rates revealed that an *era* mutant strain, while viable, does have a significant growth defect, which can be complemented by the expression of Era from pCN55iTET-*era* (Fig. 1b).

A role for Era as a ribosomal subunit assembly cofactor has been reported in both *E. coli* and *B. subtilis*, where depletion leads to a decrease in 70S ribosomes and an accumulation of individual 50S and 30S subunits (26,27). We reasoned that the growth defect observed in *S. aureus* may be due to defects in ribosome assembly. To investigate this, we analysed the cellular ribosomal content of wild-type, mutant and complemented strains by sucrose density centrifugation. This revealed that the *era* mutant strain contained fewer polysomes with a concurrent build-up of 50S subunits, a defect that was reversed in the presence of the complementing plasmid (Fig. 1c). As Era interacts with the 30S subunit (14), an excess of free 50S is, in and of itself, an indication that there is a defect in small subunit biogenesis. The levels of 70S ribosomes remained similar between the wild-type and mutant, potentially explaining why Era is not as important for growth in *S. aureus* as it is in other species. We reasoned that this defect in ribosome assembly might make the *era* mutant strain more susceptible to ribosome-targeting antibiotics. In agreement with this, we observed that the minimum inhibitory concentration (MIC) for the Δ*era* strain decreased 2-4-fold when exposed to the 30S-targeting antibiotic spectinomycin (Fig. S1a). However, the MIC was unaffected by the 50S targeting antibiotic chloramphenicol (Fig. S1b). Together this indicates that while Era is not an essential protein in *S. aureus*, it is important for optimal growth and maturation of the small ribosomal subunit.

Era protein interaction dynamics in a native background

YbeY is an endoribonuclease implicated in the maturation of the 3' terminus of 16S rRNA, as well as being a quality control checkpoint protein that together with RNase R is involved in eliminating defective 70S ribosomes (18,28). Era interacts with YbeY in *E. coli* (17), although these two genes are not encoded in the same operon in that organism (Fig. 1a). To determine whether Era from *S. aureus* interacts with YbeY, and/or any other proteins encoded in the *S. aureus era* operon, we first used a bacterial two-hybrid approach, heterologously expressing Era in combination with YbeZ, YbeY, DgkA, Cdd or RecO in *E. coli*. Using this approach, we observed that Era does interact with YbeY, as well as with YbeZ (Fig. 2a). In order to look at this interaction in a native background, we adapted the split-luciferase system developed to analyse protein-protein interactions in *Clostridium difficile* for use in *S. aureus* (Fig. 2b) (BioRxiv: https://doi.org/10.1101/426809). The *era* gene, in combination with each of the operon genes, were cloned into the split-luciferase plasmid pAP118 and introduced into *S. aureus* strain LAC*. Induction of protein expression with Atet revealed a positive interaction for Era and YbeY (2 x above the control), although no significant interaction for YbeZ was observed (1.40 x above the control) (Fig. 2c). Adaption of this system now allows for confirmation of protein-protein

interactions in a native *S. aureus* background and has confirmed an interaction between Era and the endonuclease YbeY.

Era interacts with the DEAD-box RNA helicase CshA

In addition to interacting with YbeY and YbeZ, we wished to shed further light on the role played by Era in the cell and sought to identify unknown interaction partners using a genome-wide bacterial two-hybrid screen. A library of *S. aureus* genomic DNA fragments cloned into pUT18C was screened against pKT25N-*era.* Of the 17 hits obtained, 14 contained fragments mapping to *cshA*, a gene encoding a DEAD-box RNA helicase reported to have functions in mRNA protection and RNA decay in *S. aureus* (29,30), while the homologue CsdA from *E. coli* is known to functions in the cold shock degradosome (31). To confirm the interaction between Era and CshA, full-length *cshA* was cloned into both pUT18 and pUT18C. Upon co-transformation, interactions occurred between CshA-T18 and both T25-Era and Era-T25 (Fig. 3a). When quantifying this interaction using β–galactosidase activity we noted it was more apparent at 25°C than 37°C (Fig. S2).

To confirm the validity of this interaction, we performed pulldown assays using glutathione beads coupled to GST-tagged full-length CshA incubated with His-tagged Era, revealing that while His-Era did not interact with the control GST protein, it was pulled down in the presence of CshA (Fig. 3b). Finally, to analyse and confirm this interaction in the context of the native host, we used a split luciferase assay (Fig. 2b). Assaying for luminescence revealed a 4-fold increase in luciferase activity upon expression of Era and CshA (Fig. 3c). This interaction appeared to be temperature independent when assayed using this system (Fig. 3c).

The N-terminal region of CshA is crucial for interacting with Era but binding does not affect the enzymatic activity of either protein

CshA is a DEAD-box RNA helicase with an N-terminal helicase core containing two RecA-like domains and a disordered C-terminal (CT) region involved in RNA binding (Fig. 3d) (30). This protein is capable of unwinding both double stranded RNA and RNA-DNA hybrids and is required for both the stabilisation and the degradation of mRNA, the latter of which occurs via interactions of the CT region with components of the RNA degradosome (29,30).

To determine which regions of both Era and CshA are important for binding, shorter domain constructs comprising only RecA1 (aa 1-221), RecA2 (aa 222-382), the entire core helicase domain (aa 1-382) or just containing the disordered CT-region (aa 383-506) were cloned, together with Era, into pAP118. Interactions with full-length Era were only apparent in the presence of the full core helicase domain (Fig. 3e), indicating that the RNA-binding CT region of CshA is dispensable for this interaction. To determine whether the GTPase domain of Era is required for binding, a shorter construct comprising the N-terminal G domain (aa 1-180) was cloned into pAP118 alongside full-length CshA. Luciferase assays reveal that the G domain is sufficient for this interaction (Fig. 3f).

CshA is an RNA helicase, while Era has GTPase activity. To investigate whether the interaction between these two proteins affects the enzymatic activity of either one, we first performed helicase assays. A double stranded RNA oligo was incubated with each protein singly or in combination. While CshA was able to unwind the dsRNA, the addition of Era had no effect on its activity (Fig. S3a). In addition, the GTPase activity of Era was unaltered in the presence of CshA (Fig. S3b), indicating that while these two proteins do interact, this binding has no effect on their enzymatic functions.

CshA and Era are both cold shock proteins required for virulence and rRNA processing

In *E. coli* CshA has been linked to survival at low temperatures (32), while strains with depleted levels of Era are sensitive to both cold and heat shock (9,12). While both the *S. aureus era* and *cshA* null mutants have significant growth defects at 37°C (Fig. 1b, S4a), the growth of both is severely compromised at 25°C (Fig. 4a, S4b). This defect is enhanced in a double Δ*era cshA* mutant, which

fails to grow at 25°C even after 48 h (Fig. 4a, S4b), highlighting that these two proteins play significant roles in helping bacteria cope with cold shock.

Era is a ribosome-associated GTPase (RA-GTPase), which, along with a second RA-GTPase RsgA, binds to the 30S ribosomal subunit (14,33). While mutations in both genes result in growth defects (Fig. 1b, (21)), we noted that expression of Era could complement a deletion of *rsgA*, suggesting that they may have over-lapping functions in ribosome assembly (Fig. S5a). This prompted us to use cross-complementation of the Δ*era* and Δ*cshA* mutations to investigate whether Era and CshA act in the same pathway and could functionally complement each other. However, cross-complementation did not improve growth at 25°C (Fig. S5b, S5c), indicating that while these proteins interact, they have separate cellular functions. We next assessed the contribution of each gene to the virulence of *S. aureus* using *Galleria mellonella* as an invertebrate model of systemic infection. While the wild-type bacteria were fully virulent, both the *era* and *cshA* mutant strains displayed reduced killing, with the combined double mutant being completely avirulent (Fig. 4b).

To ascertain what effect Era and CshA have on cellular rRNA *in vivo*, and understand why they are so important for normal growth and survival, we sought to access the impact of deleting these genes on the cellular rRNA. We observed that RNA extracted from both the single Δ*era* and double Δ*era cshA* strains grown at 37°C had a decrease in the amount of 16S rRNA, as well as a degradation intermediate product migrating slightly below the 16S band (Fig. 4c). When accessing the rRNA content from cells grown at 25°C we also observed a defect for the Δ*cshA* mutant strain, which migrates at a similar size to unprocessed 17S rRNA (Fig. 4c). In addition, multiple additional bands were evident in the Δ*era cshA* double mutant, which could represent either processing intermediates from the 16S, or degradation intermediates from both the 23S and the 16S. Taken together we propose that Era acts as an intermediary protein on the 30S ribosomal subunit, enabling proteins such as the endonuclease YbeY, and the cold shock responsive helicase CshA to access the 16S rRNA, allowing for processing (maturation or degradation as appropriate) of rRNA under normal and stress conditions, such as growth at cold temperatures.

RSH interacts with both Era and CshA and is important for rRNA processing

Our previous work demonstrated that the GTPase activity of Era is inhibited by the stringent response alarmone ppGpp (21). To examine whether ppGpp also interacts with CshA we performed DRaCALA assays, revealing that this nucleotide does not interact with CshA (Fig. S6a), nor does the affinity of ppGpp for Era alter in the presence of CshA (K_d of 3.123 ± 0.4267 μM in the absence versus 3.152 ± 0.3324 μM in the presence of CshA) (Fig. S6b). To investigate whether the stringent response has any additional points of interaction with either Era and CshA, we examined whether one of the (p)ppGpp synthetases might directly interact with either protein via bacterial two-hybrid. This revealed that RSH, but not RelP or RelQ, interacts with both CshA and Era (Fig. 5a,b). By expressing truncated variants of RSH, we were able to determine that the synthetase domain is important for the interaction with CshA, while the synthetase, TGS and ACT domains all seemed to be involved in interacting with Era (Fig. 5c).

Next we wished to ascertain whether the interactions between RSH and either Era or CshA are important for controlling induction of the stringent response under cold shock conditions. To address this, ppGpp levels were measured in the wild-type LAC* and in both the Δ*era* and Δ*cshA* mutant strains. While no induction of the stringent response was observed from strains grown at 37°C, we observed significant increases in ppGpp production from both mutants after a 1 h shock at 25°C when compared to the wild-type (Fig. 5d). This indicates that Era and CshA may help to regulate RSH-mediated (p)ppGpp synthesis in response to cold shock.

To analyse the importance of RSH for rRNA processing, we made use of the (p)ppGpp-defective mutant rsh_{syn}, which has an amino acid substitution in the synthetase domain of the bifunctional (p)ppGpp synthetase enzyme RSH, affecting (p)ppGpp production but not hydrolysis. We grew both the wild-type *S. aureus* LAC* and the rsh_{syn} mutant at 25°C and shocked with mupirocin, an antibiotic

that inhibits the isoleucyl-tRNA synthetase and increases ppGpp synthesis (34). Upon examination of the rRNA, we observed multiple additional bands in the rRNA isolated from the *rsh*$_{syn}$ mutant when the stringent response was activated, that were lacking in the wild-type (Fig. 5e). Bands below the 16S are likely degradation intermediates of the 16S and/or 23S, while the additional bands that appear between the 23 and 16S can be either degradation intermediates of the 23S or processing intermediates of the 16S (Fig. 5e). From this we propose that RSH directly interacts with components of the ribosomal assembly and turnover machinery, and under conditions of stress, such as cold shock, Era and CshA, and potentially other proteins involved in rRNA processing, fail to function optimally in the absence of a functional stringent response.

DISCUSSION

The synthesis of ribosomes and proteins consumes approximately 40% of the energy within a growing bacterial cell (35). Ribosomal assembly cofactors are, therefore, an essential group of enzymes involved in coordinating biogenesis in as efficient a way as possible. Members of this group include RNA helicases, rRNA and protein modification enzymes, chaperones and RA-GTPases. *S. aureus* contains over 11 RA-GTPases, each with potentially varying roles in the biogenesis of the 50S and 30S subunits, although the precise functions of each is unclear.

Era is one such RA-GTPase, and while this protein has been extensively studied, its precise function in the cell is unknown. Whilst cryo-electron microscopy shows Era interacting with the 30S ribosomal subunit, this protein has also been implicated in numerous other cellular processes (14). Era-depleted *B. subtilis* or *E. coli* cells are elongated, with defects in septum formation. These cells also contain diffuse nucleoid material, implicating Era in cell division and chromosome segregation (5,11). In *B. subtilis,* Era-depleted cells have defects in spore formation (7), while growth defects in strains containing Era variants with reduced nucleotide binding abilities can be rescued by truncating either *rpoN* or *ptsN* (10). RpoN is required for nitrogen assimilation and fixation, while PtsN is involved in sugar transport, suggesting that Era is also involved in regulating carbon and nitrogen metabolism. Localisation studies have indicated that this protein is present at both the membrane and in the cytoplasm (36), and so it has been suggested that this enzyme cycles between the membrane and the ribosome in response to cellular triggers. In addition to ribosomal biogenesis factors, Era has been reported to interact with MazG, Ndk, Pk and YggG (37-39). Ndk, a nucleoside diphosphate kinase and Pk, a pyruvate kinase, from *Pseudomonas aeruginosa* were both shown to form a complex with Era, further implicating Era in energy metabolism (38). MazG is a nucleoside triphosphate pyrophosphohydrolase, while YggG is a membrane-associated heat shock protein. The significance of these interactions with Era is unknown.

Era has also been implicated in helping cells cope with cold stress. Conditional cold-sensitive mutants of Era have been constructed in *E. coli* (9) and these mutations could be suppressed by the overexpression of the 16S rRNA methyltransferase KsgA, providing one of the first links between Era and ribosome biogenesis (12). In addition, Era also interacts with YbeY in *E. coli* (17). YbeY is a universally conserved endonuclease that, while dispensable in *E. coli*, is essential in *B. subtilis* and potentially in *S. aureus*, given the lack of transposon mutants available (18,25,28). This endonuclease is required for the maturation of the 3' end of the 16S rRNA, and in strains depleted of YbeY the 70S ribosomes are targeted for degradation with the help of RNase R (18,28). Era and YbeY are fused in a polypeptide in clostridial species, further highlighting a link between these two proteins. Although not encoded in the same operon as *era* in *E. coli*, this prompted us to examine the interactions of all other proteins encoded in the *era* operon in *S. aureus* with Era. As in *E. coli*, YbeY from *S. aureus* interacts with Era, as does YbeZ, although to a lesser degree (Fig. 2). YbeZ has been shown to interact with YbeY in *E. coli* (17). This protein has an ATPase domain and an RNA binding motif, and so is likely to also bind to the 16S rRNA and may form a complex with Era and YbeY to aid in processing the 16S rRNA.

Here we use interaction studies to show that Era interacts with a protein involved in cold shock survival, the DEAD-box RNA helicase CshA. In *B. subtilis,* CshA is one of the most abundant RNA helicases produced at low temperatures (40) and in *B. subtilis* and *E. coli* it has been implicated in multiple processes, including ribosome biogenesis and interacting with components of the RNA degradosome (30-32,40). In *S. aureus,* CshA has also been linked to controlling the turnover of mRNA in the cell (29). Deletion of *cshA* results in the stabilisation of some mRNA transcripts, such as the *spa* mRNA, but CshA also protects a number of other mRNA and sRNA transcripts under stress conditions (29). In addition the CT region of CshA has been shown to be required for interactions with the degradosome (30). Our analysis shows that Era and CshA interact *in vitro* and when expressed heterologously in *E. coli* (Fig. 3a,b). We additionally adapted a split luciferase assay to show this interaction natively in *S. aureus* (Fig. 3c). Using this assay has also allowed us to determine that the

CT disordered region of CshA, which has previously been shown as important for binding to mRNA and is required for interactions of CshA with the degradosome (30), is dispensable for interacting the Era (Fig. 3).

Mutations in Era have been shown to lead to 17S unprocessed rRNA intermediates (26). Here we see degradation intermediates in the Δ*era* mutant at both 37 and 25°C (Fig. 4c), as well as defects in rRNA processing in a strain lacking CshA, which become apparent at 25°C. A double Δ*era cshA* mutant has multiple processing and degradation defects (Fig. 4c). From this we propose that Era acts as an intermediary protein, allowing proteins such as the endonuclease YbeY and the RNA helicase CshA, and potentially others, access to their substrate rRNA. It has been suggested that CshA helps RNase components of the degradosome access cleavage sites that may be otherwise inaccessible (41), and so it is tempting to speculate that Era is responsible for recruiting CshA to target misfolded ribosomal subunits for degradation under stress conditions.

Cellular levels of rRNA are controlled by the stringent response (42). Upon activation of the stringent response, transcription from rRNA promoters is drastically reduced, either by direct binding of ppGpp to the RNA polymerase in Gram negatives, or by tight control of cellular GTP levels in Gram positives (43,44). Here we see an additional level of regulation by the stringent response on rRNA. We show a direct interaction between the (p)ppGpp synthetase RSH and both CshA and Era (Fig. 5). We also observe that the RSH-mediated control on rRNA processing is essential to rRNA stability, as starved cells defective in RSH-mediated (p)ppGpp synthesis have increased processing and degradation defects (Fig. 5e), possibly due to uncontrolled association of processing enzymes with the ribosome. The precise dynamics of processing-enzyme association under stress conditions requires further in-depth study.

Taken together, we demonstrate a cellular function for Era as a protein important for coordinating 30S ribosomal biogenesis. It is possible that defects in ribosomal assembly lead to altered protein translation, which may be the reason for the plethora of other phenotypes associated with depletion of Era. As Era can complement a defect in RsgA, as well as RbfA (26), we propose a broader role for RA-GTPases as scaffolding proteins. It is now of interest to determine what processing events are coordinated by the other RA-GTPases in *S. aureus*, as well as more broadly in other prokaryotes.

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Conflicts of interest

The authors declare no conflict of interest

REFERENCES

1. Leipe, D.D., Wolf, Y.I., Koonin, E.V. and Aravind, L. (2002) Classification and evolution of P-loop GTPases and related ATPases. *J Mol Biol*, **317**, 41-72.
2. March, P.E., Lerner, C.G., Ahnn, J., Cui, X. and Inouye, M. (1988) The *Escherichia coli* Ras-like protein (Era) has GTPase activity and is essential for cell growth. *Oncogene*, **2**, 539-544.
3. Inada, T., Kawakami, K., Chen, S.M., Takiff, H.E., Court, D.L. and Nakamura, Y. (1989) Temperature-sensitive lethal mutant of *era*, a G protein in *Escherichia coli*. *J Bacteriol*, **171**, 5017-5024.
4. Takiff, H.E., Chen, S.M. and Court, D.L. (1989) Genetic analysis of the rnc operon of *Escherichia coli*. *J Bacteriol*, **171**, 2581-2590.
5. Gollop, N. and March, P.E. (1991) A GTP-binding protein (Era) has an essential role in growth rate and cell cycle control in *Escherichia coli*. *J Bacteriol*, **173**, 2265-2270.
6. Anderson, P.E., Matsunaga, J., Simons, E.L. and Simons, R.W. (1996) Structure and regulation of the *Salmonella* Typhimurium rnc-*era-recO* operon. *Biochimie*, **78**, 1025-1034.
7. Minkovsky, N., Zarimani, A., Chary, V.K., Johnstone, B.H., Powell, B.S., Torrance, P.D., Court, D.L., Simons, R.W. and Piggot, P.J. (2002) Bex, the *Bacillus subtilis* homolog of the essential *Escherichia coli* GTPase Era, is required for normal cell division and spore formation. *J Bacteriol*, **184**, 6389-6394.
8. Morimoto, T., Loh, P.C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S. and Ogasawara, N. (2002) Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*. *Microbiology*, **148**, 3539-3552.
9. Lerner, C.G. and Inouye, M. (1991) Pleiotropic changes resulting from depletion of Era, an essential GTP-binding protein in *Escherichia coli*. *Mol Microbiol*, **5**, 951-957.
10. Powell, B.S., Court, D.L., Inada, T., Nakamura, Y., Michotey, V., Cui, X., Reizer, A., Saier, M.H., Jr. and Reizer, J. (1995) Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. Enzyme IIANtr affects growth on organic nitrogen and the conditional lethality of an erats mutant. *J Biol Chem*, **270**, 4822-4839.
11. Britton, R.A., Powell, B.S., Dasgupta, S., Sun, Q., Margolin, W., Lupski, J.R. and Court, D.L. (1998) Cell cycle arrest in Era GTPase mutants: a potential growth rate-regulated checkpoint in *Escherichia coli*. *Mol Microbiol*, **27**, 739-750.
12. Lu, Q. and Inouye, M. (1998) The gene for 16S rRNA methyltransferase (*ksgA*) functions as a multicopy suppressor for a cold-sensitive mutant of *era*, an essential RAS-like GTP-binding protein in *Escherichia coli*. *J Bacteriol*, **180**, 5243-5246.
13. Johnstone, B.H., Handler, A.A., Chao, D.K., Nguyen, V., Smith, M., Ryu, S.Y., Simons, E.L., Anderson, P.E. and Simons, R.W. (1999) The widely conserved Era G-protein contains an RNA-binding domain required for Era function *in vivo*. *Mol Microbiol*, **33**, 1118-1131.
14. Sharma, M.R., Barat, C., Wilson, D.N., Booth, T.M., Kawazoe, M., Hori-Takemoto, C., Shirouzu, M., Yokoyama, S., Fucini, P. and Agrawal, R.K. (2005) Interaction of Era with the 30S ribosomal subunit implications for 30S subunit assembly. *Mol Cell*, **18**, 319-329.
15. Tamaru, D., Amikura, K., Shimizu, Y., Nierhaus, K.H. and Ueda, T. (2018) Reconstitution of 30S ribosomal subunits *in vitro* using ribosome biogenesis factors. *RNA*, **24**, 1512-1519.
16. Bunner, A.E., Nord, S., Wikstrom, P.M. and Williamson, J.R. (2010) The effect of ribosome assembly cofactors on in vitro 30S subunit reconstitution. *J Mol Biol*, **398**, 1-7.
17. Vercruysse, M., Kohrer, C., Shen, Y., Proulx, S., Ghosal, A., Davies, B.W., RajBhandary, U.L. and Walker, G.C. (2016) Identification of YbeY-Protein Interactions Involved in 16S rRNA Maturation and Stress Regulation in *Escherichia coli*. *MBio*, **7**.
18. Baumgardt, K., Gilet, L., Figaro, S. and Condon, C. (2018) The essential nature of YqfG, a YbeY homologue required for 3' maturation of *Bacillus subtilis* 16S ribosomal RNA is suppressed by deletion of RNase R. *Nucleic Acids Res*, **46**, 8605-8615.

19. Lemos, J.A., Lin, V.K., Nascimento, M.M., Abranches, J. and Burne, R.A. (2007) Three gene products govern (p)ppGpp production by *Streptococcus mutans*. *Mol Microbiol*, **65**, 1568-1581.
20. Cassels, R., Oliva, B. and Knowles, D. (1995) Occurrence of the regulatory nucleotides ppGpp and pppGpp following induction of the stringent response in staphylococci. *J Bacteriol*, **177**, 5161-5165.
21. Corrigan, R.M., Bellows, L.E., Wood, A. and Grundling, A. (2016) ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc Natl Acad Sci U S A*, **113**, E1710-1719.
22. Wachi, M., Umitsuki, G., Shimizu, M., Takada, A. and Nagai, K. (1999) *Escherichia coli cafA* gene encodes a novel RNase, designated as RNase G, involved in processing of the 5' end of 16S rRNA. *Biochem Biophys Res Commun*, **259**, 483-488.
23. Fey, P.D., Endres, J.L., Yajjala, V.K., Widhelm, T.J., Boissy, R.J., Bose, J.L. and Bayles, K.W. (2013) A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio*, **4**, e00537-00512.
24. Chaudhuri, R.R., Allen, A.G., Owen, P.J., Shalom, G., Stone, K., Harrison, M., Burgis, T.A., Lockyer, M., Garcia-Lara, J., Foster, S.J. *et al.* (2009) Comprehensive identification of essential *Staphylococcus aureus* genes using Transposon-Mediated Differential Hybridisation (TMDH). *BMC Genomics*, **10**, 291.
25. Santiago, M., Matano, L.M., Moussa, S.H., Gilmore, M.S., Walker, S. and Meredith, T.C. (2015) A new platform for ultra-high density *Staphylococcus aureus* transposon libraries. *BMC Genomics*, **16**, 252.
26. Inoue, K., Alsina, J., Chen, J. and Inouye, M. (2003) Suppression of defective ribosome assembly in a *rbfA* deletion mutant by overexpression of Era, an essential GTPase in *Escherichia coli*. *Mol Microbiol*, **48**, 1005-1016.
27. Loh, P.C., Morimoto, T., Matsuo, Y., Oshima, T. and Ogasawara, N. (2007) The GTP-binding protein YqeH participates in biogenesis of the 30S ribosome subunit in *Bacillus subtilis*. *Genes Genet Syst*, **82**, 281-289.
28. Jacob, A.I., Kohrer, C., Davies, B.W., RajBhandary, U.L. and Walker, G.C. (2013) Conserved bacterial RNase YbeY plays key roles in 70S ribosome quality control and 16S rRNA maturation. *Mol Cell*, **49**, 427-438.
29. Kim, S., Corvaglia, A.R., Leo, S., Cheung, A. and Francois, P. (2016) Characterization of RNA Helicase CshA and Its Role in Protecting mRNAs and Small RNAs of *Staphylococcus aureus* Strain Newman. *Infect Immun*, **84**, 833-844.
30. Giraud, C., Hausmann, S., Lemeille, S., Prados, J., Redder, P. and Linder, P. (2015) 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*. *RNA Biol*, **12**, 658-674.
31. Prud'homme-Genereux, A., Beran, R.K., Iost, I., Ramey, C.S., Mackie, G.A. and Simons, R.W. (2004) Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol Microbiol*, **54**, 1409-1421.
32. Charollais, J., Dreyfus, M. and Iost, I. (2004) CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic Acids Res*, **32**, 2751-2759.
33. Daigle, D.M. and Brown, E.D. (2004) Studies of the interaction of *Escherichia coli* YjeQ with the ribosome *in vitro*. *J Bacteriol*, **186**, 1381-1387.
34. Reiss, S., Pane-Farre, J., Fuchs, S., Francois, P., Liebeke, M., Schrenzel, J., Lindequist, U., Lalk, M., Wolz, C., Hecker, M. *et al.* (2012) Global analysis of the *Staphylococcus aureus* response to mupirocin. *Antimicrob Agents Chemother*, **56**, 787-804.

35. Wilson, D.N. and Nierhaus, K.H. (2007) The weird and wonderful world of bacterial ribosome regulation. *Crit Rev Biochem Mol Biol*, **42**, 187-219.
36. Gollop, N. and March, P.E. (1991) Localization of the membrane binding sites of Era in *Escherichia coli*. *Res Microbiol*, **142**, 301-307.
37. Zhang, J. and Inouye, M. (2002) MazG, a nucleoside triphosphate pyrophosphohydrolase, interacts with Era, an essential GTPase in *Escherichia coli*. *J Bacteriol*, **184**, 5323-5329.
38. Chopade, B.A., Shankar, S., Sundin, G.W., Mukhopadhyay, S. and Chakrabarty, A.M. (1997) Characterization of membrane-associated *Pseudomonas aeruginosa* Ras-like protein Pra, a GTP-binding protein that forms complexes with truncated nucleoside diphosphate kinase and pyruvate kinase to modulate GTP synthesis. *J Bacteriol*, **179**, 2181-2188.
39. Huang, Y., Zhang, B., Dong, K., Zhang, X., Hou, L., Wang, T., Chen, N. and Chen, S. (2007) Up-regulation of *yggG* promotes the survival of *Escherichia coli* cells containing Era-1 mutant protein. *FEMS Microbiol Lett*, **275**, 8-15.
40. Lehnik-Habrink, M., Rempeters, L., Kovacs, A.T., Wrede, C., Baierlein, C., Krebber, H., Kuipers, O.P. and Stulke, J. (2013) DEAD-Box RNA helicases in *Bacillus subtilis* have multiple functions and act independently from each other. *J Bacteriol*, **195**, 534-544.
41. Lehnik-Habrink, M., Pfortner, H., Rempeters, L., Pietack, N., Herzberg, C. and Stulke, J. (2010) The RNA degradosome in *Bacillus subtilis*: identification of CshA as the major RNA helicase in the multiprotein complex. *Mol Microbiol*, **77**, 958-971.
42. Cashel, M., Gentry, D.R., Hernandez, V.J., and Vinella, D. (1996) *The stringent response. In E. coli and Salmonella*. Washington DC: ASM press.
43. Kriel, A., Bittner, A.N., Kim, S.H., Liu, K., Tehranchi, A.K., Zou, W.Y., Rendon, S., Chen, R., Tu, B.P. and Wang, J.D. (2012) Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Mol Cell*, **48**, 231-241.
44. Ross, W., Sanchez-Vazquez, P., Chen, A.Y., Lee, J.H., Burgos, H.L. and Gourse, R.L. (2016) ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response. *Mol Cell*, **62**, 811-823.

FIGURE LEGENDS

Fig. 1. Role of Era in bacterial growth and ribosome assembly. A) Schematic representation of the *era*-containing operons from *S. aureus*, *B. subtilis* and *E. coli*. The *era* operon in *S. aureus* consists of: *ybeZ* - a gene of unknown function; *ybeY* – a 16S rRNA endoribonuclease; *dgkA* - a diacylglycerol kinase involved in lipid metabolism; *cdd* - a cytidine deaminase involved pyrimidine metabolism; *era*, and *recO* encoding for a DNA repair protein. Arrows indicate the site of transposon insertions in the *S. aureus* Nebraska Transposon library. B) Growth of *S. aureus* strains LAC* iTET, LAC* Δ*era* iTET and LAC* Δ*era* iTET-*era*. Overnight cultures were diluted to an OD_{600} of 0.05 and grown in the presence of 100 ng/ml Atet for 8 h. Growth curves were performed in triplicate, with averages and standard deviations shown. C) Ribosome profiles from LAC* iTET, LAC* Δ*era* iTET and LAC* Δ*era* iTET-*era.* Normalised extracts from each strain were layered onto 10-50% sucrose gradients. Gradients were fractionated and analysed for RNA content at an absorbance of 260 nm. 30S, 50S, 70S and polysomes-containing fractions are indicated. Experiments were performed in triplicate with one representative graph shown.

Fig. 2. Era interacts with other proteins encoded in the same operon. A) Bacterial two-hybrid showing a positive interaction between Era-T25 and both T18-YbeZ and YbeY. T25 and T18-encoding empty vectors are used as negative controls. B) Schematic representation of the luciferase vectors used for native protein-protein interaction studies. pAP118 contains an Atet-inducible promoter upstream of two genes – SB encodes for the small bit of the luciferase protein, while LB encodes for the large bit. When genes encoding for interacting proteins (A & B) are translationally fused to SB and LB they associate to produce luciferase. pAP256 and pAP257 are control vectors. C) Split luciferase assay demonstrating a significant interaction between Era and YbeY in *S. aureus*. The negative is pAP256-$era_{SB\text{-}LB}$, which has Era fused to the SB but nothing fused to LB. The negative is normalised to a relative luminescence unit (RLU) of 1 (dashed line). The average values and standard deviations of triplicate experiments are plotted.

Fig. 3. Era interacts with the DEAD-box helicase CshA. A) Bacterial two-hybrid showing an interaction between CshA-T18 and both Era-T25 and T25-Era. Era is known to form a dimer and an Era-Era interaction is used as a positive control. B) Affinity pulldown assay using GST or GST-tagged CshA coupled to glutathione beads. Beads were incubated with His-tagged Era and after washing bound protein was detected using HRP-conjugated anti-His antibodies. C) Split luciferase assay demonstrating an interaction between Era and CshA in *S. aureus*. Dimers resulting from Era fused to both the small (SB) and large bits (LB) of the luciferase gene function as a positive control. The negative, which is normalised to a relative luminescence unit (RLU) of 1 (dashed line), has Era fused to the SB but nothing fused to LB. Cells were grown to an OD_{600} of 0.2 in the presence of 100 ng/ml Atet at either 37 or 25°C before being normalised. The average values and standard deviations of triplicate experiments are plotted. D) Schematic representation of CshA and Era. The sizes of each domain construct are indicated by the black lines and numbering. E) Split luciferase assay with Era fused to SB and various truncated domain constructs of CshA fused to LB. F) Split luciferase assay with the G domain of Era (1-180) fused to SB and full-length CshA fused to LB. The average values and standard deviations of triplicate experiments are plotted.

Fig. 4. Era and CshA are required for cold shock survival, virulence and rRNA processing. A) Serial dilutions of the wild-type LAC*, Δ*era*, Δ*cshA* and Δ*era cshA* were spotted onto TSA agar plates and incubated at either 37 or 25°C for the times indicated. B) Survival of *G. mellonella* larvae inoculated into the hind leg with 1 x 10^5 cfu of wild-type LAC* and mutant Δ*era*, Δ*cshA* and Δ*era cshA* strains. Larvae were incubated at 37°C for 4 days. Experiments were repeats in triplicate, with one graph shown. C) rRNA profiles from *era* and *cshA* mutant strains. 500 ng of RNA extracted from LAC*,

Δ*era*, Δ*cshA* and Δ*era cshA* grown to an OD_{600} of 0.4 at either 37 or 25°C, were run on 0.7% agarose/0.9% synergel gels and stained with Evagreen dye. * highlights the presence of either processing or degradation intermediates.

Fig. 5. The stringent response synthetase RSH interacts with both Era and CshA. A) Bacterial two-hybrid showing an interaction between CshA-T18/T18-CshA and both RSH-T25 and T25-RSH. CshA did not interact with the small (p)ppGpp synthetase enzymes RelP or RelQ. B) Bacterial two-hybrid showing an interaction between RSH-T25 and both Era-T18 and T18-Era. C) Schematic representation and bacterial two-hybrid of the domains of RSH. Constructs containing each of the 4 domains within RSH were used in bacterial two-hybrid assays to determine which were required to binding to both CshA and Era. D) Measurement of intracellular (p)ppGpp levels in LAC*, LAC*Δ*era* and LAC*Δ*cshA* grown in the presence of ^{32}P-labeled H_3PO_4. The production of ppGpp after shock at 25°C was monitored by TLC. E) rRNA extracted from LAC* and LAC* rsh_{syn}. Strains were grown at 25°C until an OD_{600} of 0.4. Cells were split and exposed to 60 µg/ml mupirocin for 1 h. 500 ng of extracted RNA were run on 0.7% agarose/0.9% synergel gels and stained with Evagreen dye. The black bar highlights the presence of degradation products.

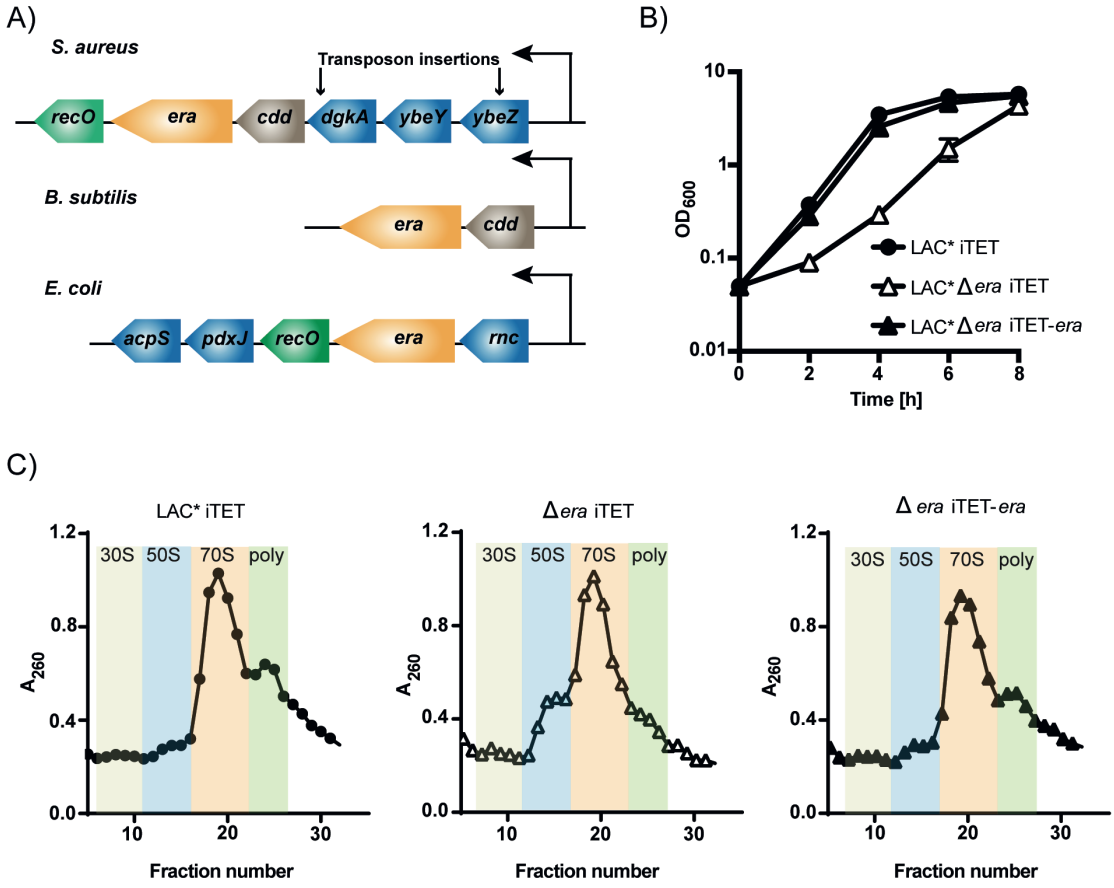
A)
S. aureus
Transposon insertions
recO
era
cdd
dgkA
ybeY
ybeZ
B. subtilis
era
cdd
E. coli
acpS
pdxJ
recO
era
rnc
B)
OD600
Time [h]
LAC* iTET
LAC* Δera iTET
LAC* Δera iTET-era
C)
LAC* iTET
Δera iTET
Δ era iTET-era
30S
50S
70S
poly
A260
Fraction number

Figure 1

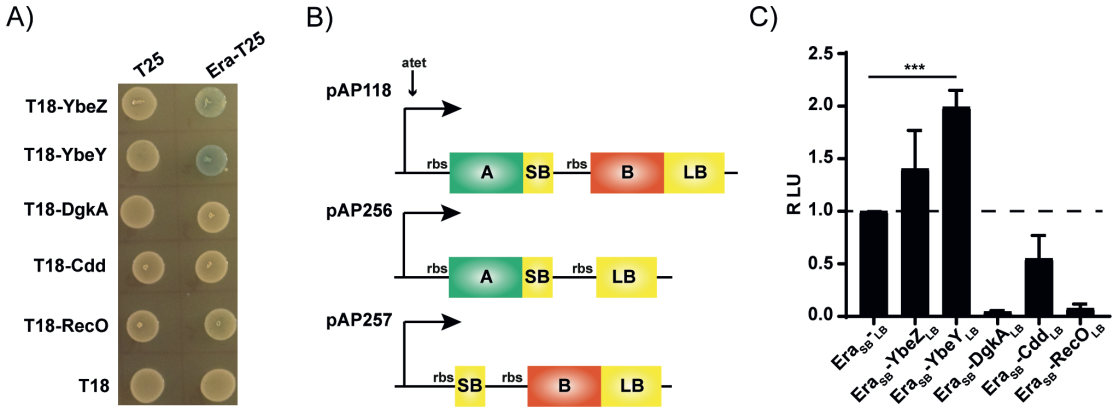
A)
T25
Era-T25
T18-YbeZ
T18-YbeY
T18-DgkA
T18-Cdd
T18-RecO
T18
B)
atet
pAP118
rbs
A
SB
rbs
B
LB
pAP256
rbs
A
SB
rbs
LB
pAP257
rbs
SB
rbs
B
LB
C)
***
R LU
2.5
2.0
1.5
1.0
0.5
0.0
Era$_{SB}$-$_{LB}$
Era$_{SB}$-YbeZ$_{LB}$
Era$_{SB}$-YbeY$_{LB}$
Era$_{SB}$-DgkA$_{LB}$
Era$_{SB}$-Cdd$_{LB}$
Era$_{SB}$-RecO$_{LB}$

Figure 2

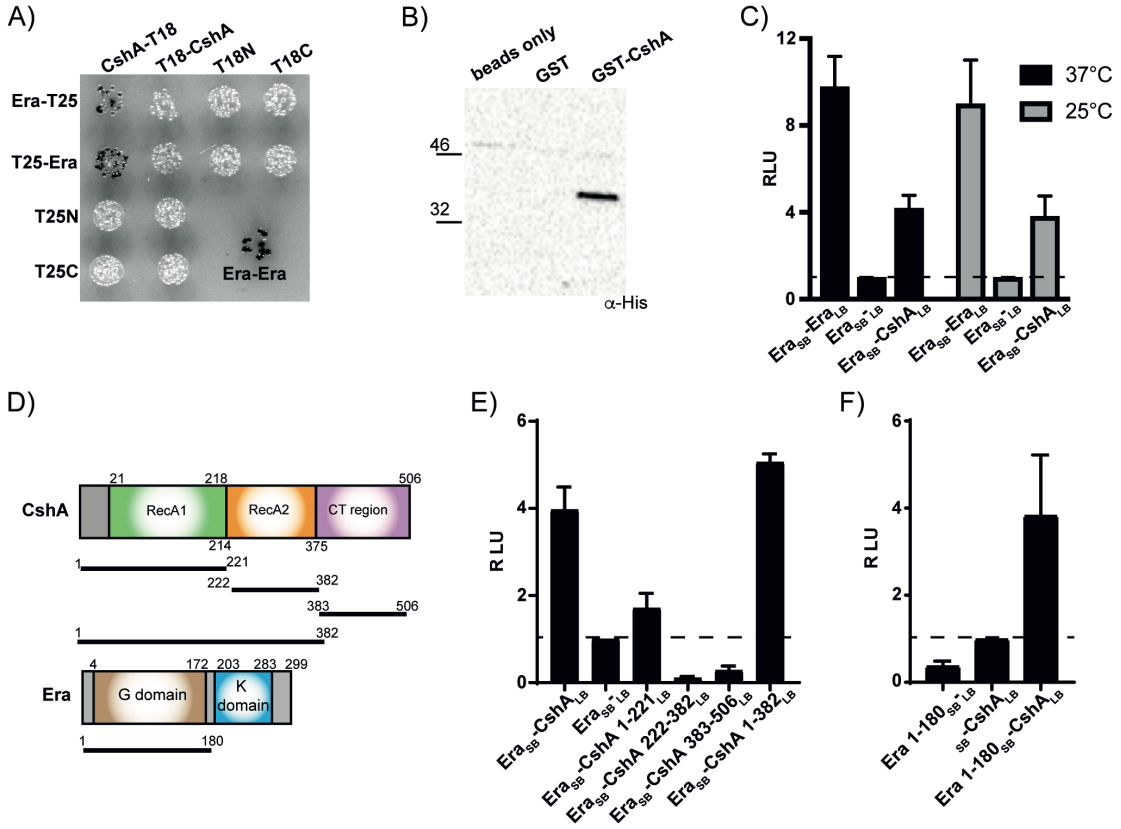
A)
CshA-T18
T18-CshA
T18N
T18C
Era-T25
T25-Era
T25N
T25C
Era-Era
B)
beads only
GST
GST-CshA
46
32
α-His
C)
37°C
25°C
RLU
D)
CshA
RecA1
RecA2
CT region
Era
G domain
K domain
E)
R LU
F)
R LU

Figure 3

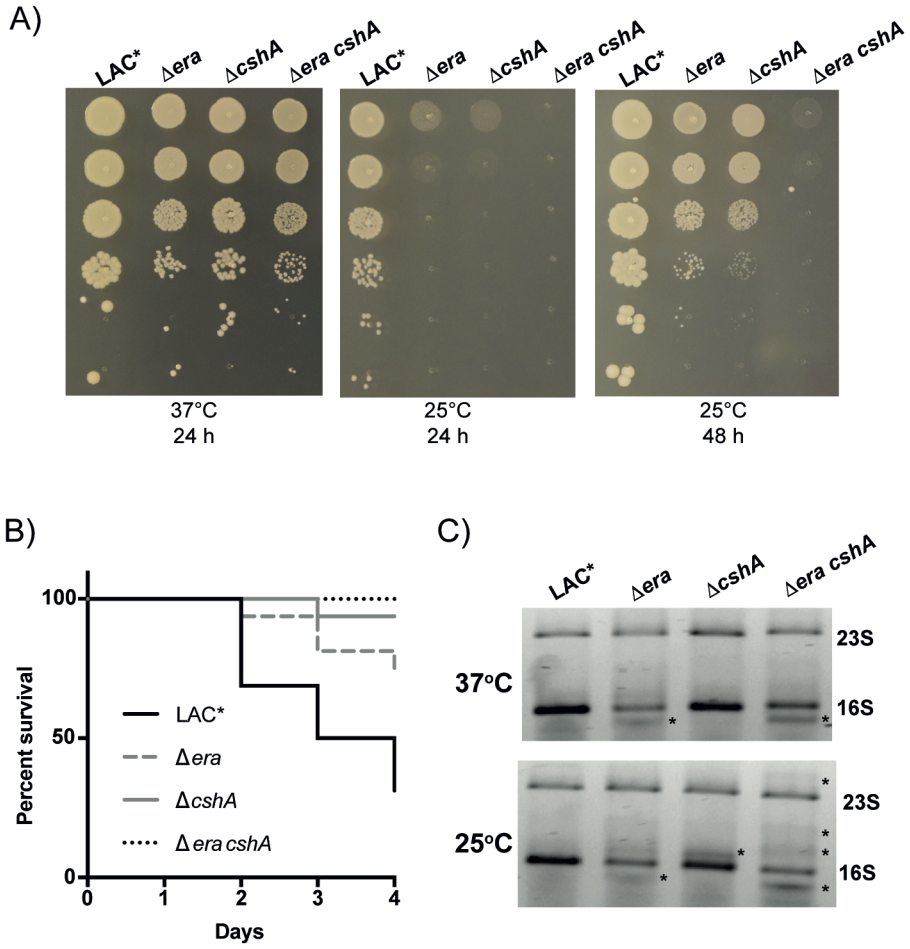
A)
LAC*
Δera
ΔcshA
Δera cshA
37°C
24 h
25°C
24 h
25°C
48 h
B)
Percent survival
100
50
0
0
1
2
3
4
Days
LAC*
Δera
ΔcshA
Δera cshA
C)
LAC*
Δera
ΔcshA
Δera cshA
37°C
25°C
23S
16S

Figure 4

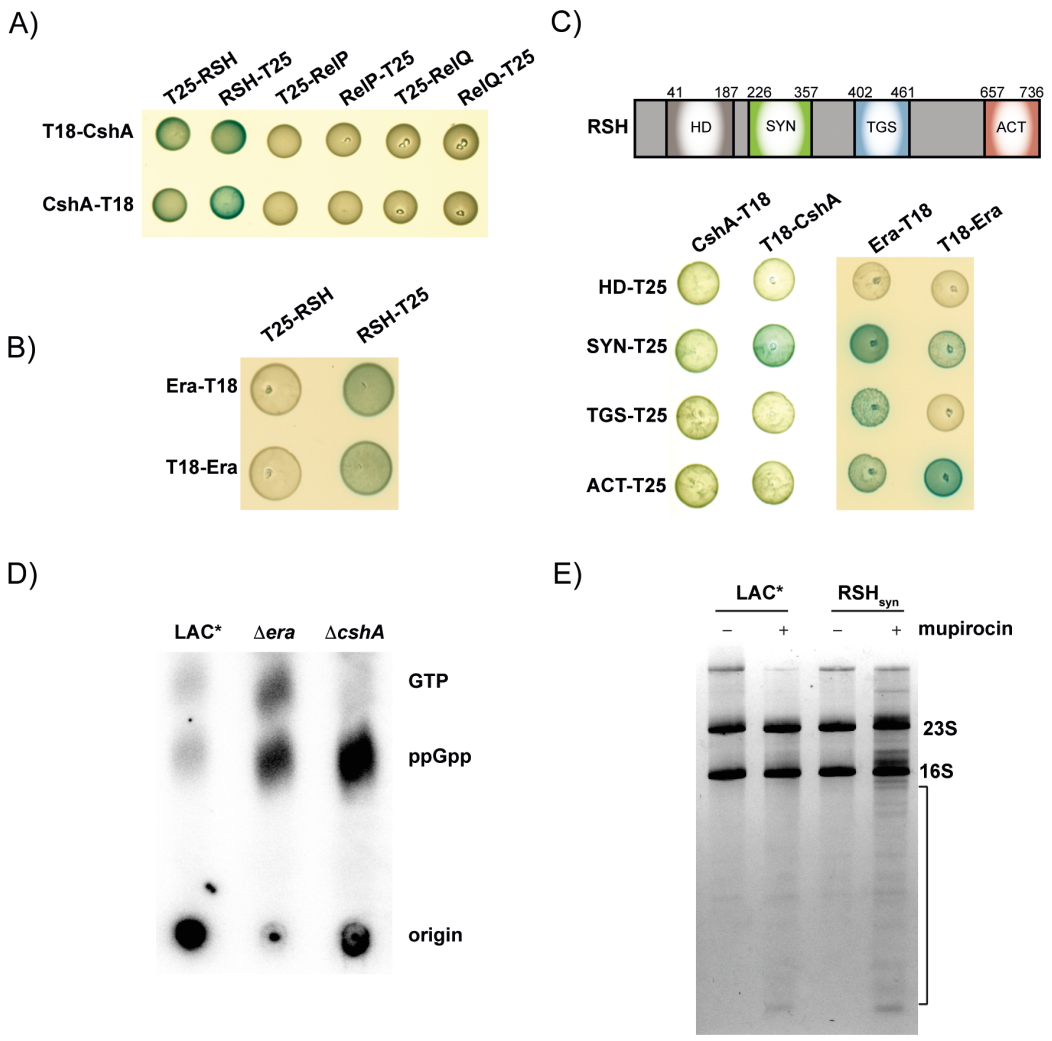
A)
T25-RSH
RSH-T25
T25-RelP
RelP-T25
T25-RelQ
RelQ-T25
T18-CshA
CshA-T18
B)
T25-RSH
RSH-T25
Era-T18
T18-Era
C)
RSH
41
187
226
357
402
461
657
736
HD
SYN
TGS
ACT
CshA-T18
T18-CshA
Era-T18
T18-Era
HD-T25
SYN-T25
TGS-T25
ACT-T25
D)
LAC*
Δera
ΔcshA
GTP
ppGpp
origin
E)
LAC*
RSHsyn
–
+
–
+
mupirocin
23S
16S

Figure 5