The (p)ppGpp-binding GTPase Era promotes rRNA processing and cold shock survival in *Staphylococcus aureus*

Short title: Era promotes rRNA processing and bacterial survival

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Abstract

Ribosome assembly cofactors are widely conserved across all domains of life. One such group, the ribosome-associated GTPases (RA-GTPase), act as molecular switches to coordinate ribosome assembly. We previously identified the *Staphylococcus aureus* RA-GTPase Era as a target for the stringent response alarmone (p)ppGpp, although the function of Era in ribosome assembly is unclear. Era is highly conserved throughout the bacterial kingdom and is essential in many species. Here we show that Era is not essential in *S. aureus* but is important for 30S ribosomal subunit assembly. Protein interaction studies reveal that Era interacts with the endonuclease YbeY and the DEAD-box RNA helicase CshA. We determine that both Era and CshA are required for growth at suboptimal temperatures, virulence and 16S rRNA processing. Era and CshA also form direct interactions with the (p)ppGpp synthetase Rel_{SA}, with Rel_{SA} positively impacting the GTPase activity of Era. We propose that Era acts to direct enzymes involved in rRNA maturation and ribosome subunit assembly to their site of action, an activity that is regulated by components of the stringent response.

Author summary

The bacterial ribosome is an essential cellular component and as such is the target for a number of currently used antimicrobials. Correct assembly of this complex macromolecule requires a number of accessory enzymes, the functions of which are poorly characterised. Here we examine the function of Era, a GTPase enzyme involved in 30S ribosomal subunit biogenesis in the important human pathogen *S. aureus*. We uncover that Era is not an essential enzyme in *S. aureus*, as it is in many other species, but is important for correct ribosome assembly. In a bid to uncover the cellular function of this enzyme, we identify a number of protein interaction partners with roles in ribosomal RNA maturation and degradation, supporting the idea that Era acts as an intermediary protein facilitating ribosomal biogenesis. We also uncover a link between Era and the (p)ppGpp synthetase Rel_{SA}, revealing an additional level of control of rRNA levels by the stringent response. With this study we elaborate on the functions of GTPases in ribosomal assembly, processes that are controlled at multiple points by the stringent response.

61 **Introduction**

62 Ribosomes are macromolecular machines responsible for the synthesis of proteins in
63 all living cells. In bacteria these complexes consist of two subunits, with the large 50S
64 subunit containing 33 large proteins (L1-36) and two rRNAs, and the 30S small
65 subunit containing 21 small proteins (S1-21) and the 16S rRNA. As such, assembly of
66 the ribosome is a tightly regulated process, with correct maturation requiring the help
67 of assembly cofactors, one class of which are the P-loop ribosome-associated GTPases
68 (RA-GTPase). These enzymes are widely conserved across all domains of life and act
69 as molecular switches, cycling between inactive GDP-bound, and active, effector-
70 binding GTP-bound states. Within the P-loop GTPase class lies the Era family
71 (*Escherichia coli* Ras-like protein), which is characterised by the presence of a distinct
72 derivative of a KH domain [1]. Era, the protein after which this family is named, is
73 highly conserved throughout the bacterial kingdom, although is missing in *Chlamydia*
74 and mycobacterial species [1]. This GTPase is essential in *E. coli* [2-5], *Salmonella*
75 Typhimurium [6] and in strains of *Bacillus subtilis* [7, 8]. The first indication that Era
76 is involved in ribosomal biogenesis came from the ability of the 16S rRNA
77 methyltransferase KsgA to suppress a cold-sensitive phenotype of an Era E200K
78 mutation [9]. While important for ribosomal maturation, additional phenotypic defects
79 associated with depleted cellular levels of Era include cell cycle control and
80 chromosome segregation, as well as carbon and nitrogen metabolism [10-12].

81 Era is composed of two domains, an N-terminal GTPase domain and a C-
82 terminal RNA-binding K-homology domain [13]. A cryo-electron micrograph
83 structure of Era in complex with the 30S subunit of the ribosome reveals that Era binds
84 into the same pocket as small subunit protein S1 [14]. In the absence of S1, Era
85 interacts with proteins S2, S7, S11 and S18, as well as with a number of helices of the

86 16S rRNA. In addition, Era interacts with h45 and nearby residues 1530-1539
87 (GAUCACCUCC) in the 3' minor domain of the 16S rRNA via its CTD region [14,
88 15]. These residues include the anti-Shine Dalgarno sequence, critical for the
89 formation of the 30S preinitiation complex. Including Era in *in vitro* reconstitutions of
90 the 30S ribosome promotes the incorporation of several late-stage ribosomal proteins
91 for the RNA [16, 17]. Consequently, it has been proposed that Era functions as a
92 checkpoint protein, and that by binding to the 16S rRNA the formation of the initiation
93 complex is prevented until the appropriate time [14]. In addition to interacting with
94 ribosomal proteins, Era has also been reported to interact with a number of proteins
95 involved in 16S rRNA maturation. One of these, YbeY, is an endonuclease required in
96 *E. coli* for the maturation of the 3' end of the 16S rRNA [18, 19]. It is proposed that
97 the binding of YbeY to Era and S11 guide the endonuclease to its site of action [18].

98 The stringent response is a bacterial signalling system used by bacteria to cope
99 with a variety of environmental stresses, the best characterised of which is nutrient
100 deprivation. The opportunistic pathogen *Staphylococcus aureus* contains three
101 enzymes, Rel_{SA}, RelP and RelQ, which upon sensing a stress, synthesise the
102 nucleotides guanosine tetra- and pentaphosphate ((p)ppGpp) [20, 21]. Once produced,
103 this alarmone controls cellular responses to aid survival. Our previous work identified
104 Era, as well as three other GTPase enzymes from *S. aureus*, as target proteins for
105 (p)ppGpp and demonstrated that the production of (p)ppGpp has a negative impact on
106 mature 70S assembly [22]. Here, we examine the role of Era as an enzyme required for
107 ribosome biogenesis. Unlike in *E. coli*, this GTPase is not essential for the growth of *S.*
108 *aureus*, however mutant cells are defective in 30S subunit maturation. We identify the
109 *S. aureus* endonuclease YbeY and the DEAD-box RNA helicase CshA as interaction
110 partners for Era, and show that both Era and CshA are crucial for rRNA homeostasis

111 and growth at low temperatures. We additionally demonstrate that both Era and CshA
112 interact with the (p)ppGpp synthetase Rel_{SA}, and that cellular rRNA processing is
113 controlled in a stringent response-dependent manner. With this, we propose that Era is
114 a protein that facilitates the interactions between a number of rRNA processing and
115 degrading enzymes and the 30S subunit/16S rRNA, and show that under stress
116 conditions the stringent response is important for this process.
117
118

119 Results

120 Era is not essential in *S. aureus* but is required for normal growth and ribosome 121 assembly

122 Previous reports show that *era* is an essential gene in multiple bacterial species [3, 6].
123 In agreement with this, transposon insertions in this gene have not been identified in a
124 number of published *S. aureus* transposon libraries [23-25]. Closer inspection of the
125 transposon insertion hits within the *era* operon obtained in the Nebraska *S. aureus*
126 mutant library [23] reveals insertions in *ybeZ*, a gene of unknown function, and at the
127 3' end of the diacylglycerol kinase *dgkA* but not within *ybeY*, *cdd*, *era* or *recO*,
128 suggestive of an additional promoter upstream of *era* and *cdd* (Fig 1a). To rule out
129 polar effects and examine whether *era* in isolation is essential, we replaced the entire
130 coding sequence with the Tet-encoding *tetAM* open reading frame in the presence of
131 the anhydrotetracycline (Atet)-inducible covering plasmid pCN55iTET-*era*. Following
132 deletion of the chromosomal copy of *era*, we attempted to phage transduce this
133 mutation into *S. aureus* strains containing the empty vector pCN55iTET or the
134 complementing plasmid pCN55iTET-*era*. Transduction efficiencies were similar with
135 both recipients, albeit smaller colony sizes were observed in the absence of *era*,

136 indicating that this gene is not essential in *S. aureus*. We next transduced the *era*
137 deletion into the community-associated methicillin resistant *S. aureus* strain LAC* to
138 rule out secondary mutations and used this strain for further studies. Analysis of
139 growth rates revealed that an *era* mutant strain, while viable, does have a significant
140 growth defect, which can be complemented by the expression of Era from
141 pCN55iTET-*era* (Fig 1b).

142 A role for Era as a ribosomal subunit assembly cofactor has been reported in
143 both *E. coli* and *B. subtilis*, where depletion leads to a decrease in 70S ribosomes and
144 an accumulation of individual 50S and 30S subunits [26, 27]. We reasoned that the
145 growth defect observed in *S. aureus* may be due to defects in ribosome assembly. To
146 investigate this in the context of a complete *era* deletion, we analysed the cellular
147 ribosomal content of wildtype, mutant and complemented strains by sucrose density
148 centrifugation. This revealed that the *era* mutant strain contained fewer polysomes
149 with a concurrent build-up of 50S subunits, a defect that was reversed in the presence
150 of the complementing plasmid (Fig 1c). As cryo-electron microscopy has shown Era
151 interacting with the 30S subunit [14], an excess of free 50S subunits may be, in and of
152 itself, an indication that there is a defect in small subunit biogenesis, leading to a
153 build-up in free 50S. While the peak for polysomes disappeared, the levels of 70S
154 ribosomes remained similar between the wildtype and mutant, however it is not known
155 if these 70S ribosomes are fully functional. We reasoned that this defect in ribosome
156 assembly might make the *era* mutant strain more susceptible to ribosome-targeting
157 antibiotics. In agreement with this, we observed that the minimum inhibitory
158 concentration (MIC) for the Δ*era* strain decreased 2-4-fold when exposed to the 30S-
159 targeting antibiotic spectinomycin (Fig S1a). However, the MIC was unaffected by the
160 50S targeting antibiotic chloramphenicol (Fig S1b). Together this indicates that while

161 Era is not an essential protein in *S. aureus*, it is important for optimal growth and
162 ribosome maturation.
163
164 **Uncovering Era protein interaction dynamics in a native background**
165 In *S. aureus,* Era is encoded in an operon with five other genes, one of which encodes
166 YbeY (Fig 1a). YbeY is an endoribonuclease implicated in the maturation of the 3'
167 terminus of 16S rRNA in *E. coli*, as well as a quality control checkpoint protein that
168 together with RNase R is involved in eliminating defective 70S ribosomes [19, 28].
169 Era interacts with YbeY in *E. coli* [18], although these two genes are not encoded in
170 the same operon in that organism (Fig 1a). To determine whether Era from *S. aureus*
171 interacts with YbeY, and/or any other proteins encoded in the *S. aureus era* operon,
172 we first used a bacterial two-hybrid approach, heterologously expressing Era in
173 combination with YbeZ, YbeY, DgkA, Cdd or RecO in *E. coli*. Using this approach,
174 we observed that Era potentially interacts with YbeY and YbeZ (Fig 2a).
175 In order to look at these interactions natively in an *S. aureus* background, we
176 adapted a split-luciferase system recently developed to analyse protein-protein
177 interactions in *Clostridium difficile* for use in *S. aureus* (Fig 2b) [29]. The *era* gene, in
178 combination with itself and each of the operon genes, were cloned into the split-
179 luciferase plasmid pAP118 and introduced into *S. aureus* strain LAC*. Induction of
180 protein expression with Atet revealed a strong positive interaction for Era with itself
181 (Fig 2c), indicating that Era can form dimers. Dimerisation of Era has previously been
182 observed while solving the crystal structure of the *E. coli* protein [30], and so this
183 interaction confirms the functionality of the split-luciferase system in *S. aureus*. In
184 addition, a significant interaction was also observed for Era and YbeY (2 x above the
185 control), although not for YbeZ (1.40 x above the control) (Fig 2c). In agreement with

186 the bacterial two-hybrid results, no interactions occurred between Era and DgkA, Cdd
187 or RecO. A decrease in luminescence below the Era control pAP256-$era_{SB\text{-}LB}$ occurred
188 when co-expressing Era with DgkA and RecO. The reason for this is unclear but could
189 be due to toxicity upon over-expression of a second copy of these genes from a multi-
190 copy plasmid. To confirm the validity of the Era-YbeY interaction *in vitro*, we
191 performed pulldown assays using glutathione beads coupled to either GST or GST-
192 tagged Era and incubated them with His-tagged YbeY (Fig 2d). Here we observed
193 low-level cross reactivity of YbeY with the gluthatione beads that could not be
194 reduced despite increased washes. However, His-YbeY was pulled down significantly
195 more in the presence of Era (Fig 2d). Altogether the bacterial two-hybrid, split
196 luciferase and affinity pulldown assays confirm an interaction between Era and the
197 endonuclease YbeY from *S. aureus*. With this data we have adapted a luciferase
198 system for confirmation of protein-protein interactions in a native *S. aureus*
199 background.
200
201 **Era interacts with the DEAD-box RNA helicase CshA**
202 We wished to shed further light on the role played by Era in the cell and sought to
203 identify unknown interaction partners using a genome-wide bacterial two-hybrid
204 screen. A library of *S. aureus* genomic DNA fragments cloned into pUT18C was
205 screened against pKNT25-*era.* Of the 17 hits obtained, 14 contained fragments
206 mapping to *cshA*, a gene encoding a DEAD-box RNA helicase. CshA functions in
207 mRNA protection and RNA decay in *S. aureus* [31, 32], while the homologue CsdA
208 from *E. coli* is involved in the cold shock degradosome [33]. To confirm the
209 interaction between Era and CshA, full-length *cshA* was cloned into both pUT18 and

210 pUT18C. Upon co-transformation, interactions occurred between CshA-T18 and both
211 T25-Era and Era-T25 (Fig 3a).
212 To assess this interaction *in vitro*, we performed pulldown assays using
213 glutathione beads coupled to either GST or GST-tagged CshA and incubated them
214 with His-tagged Era, revealing that while His-Era did not interact with the control
215 GST protein, it was pulled down in the presence of CshA (Fig 3b). Finally, to analyse
216 and confirm this interaction in the context of the native host, we used the split
217 luciferase assay (Fig 2b). Assaying for luminescence revealed a 4-fold increase in
218 luciferase activity upon co-expression of Era and CshA (Fig 3c), confirming the
219 identification of a novel Era interaction partner.
220
221 **The N-terminal region of CshA is crucial for interacting with Era**
222 CshA is a DEAD-box RNA helicase with an N-terminal helicase core containing two
223 RecA-like domains and a disordered C-terminal (CT) region involved in RNA binding
224 (Fig 3d) [32]. This protein is capable of unwinding both double stranded RNA and
225 RNA-DNA hybrids and is required for both the stabilisation and the degradation of
226 mRNA, the latter of which occurs via interactions of the CT region with components
227 of the RNA degradosome [31, 32].
228 To determine which regions of both Era and CshA are important for
229 interacting, shorter domain constructs comprising only RecA1 (aa 1-221), RecA2 (aa
230 222-382), the entire core helicase domain (aa 1-382) or just containing the disordered
231 CT-region (aa 383-506) were cloned, together with Era, into the split luciferase vector
232 pAP118. Interactions with full-length Era were only apparent in the presence of the
233 full core helicase domain (aa 1-382: Fig 3e), indicating that the RNA-binding CT
234 region of CshA is dispensable for this interaction. To determine whether the GTPase

235 domain of Era is required for binding, a shorter construct comprising the N-terminal G
236 domain (aa 1-180) was cloned into pAP118 alongside full-length CshA. Luciferase
237 assays reveal that the G domain is sufficient for this interaction (Fig 3f).
238
239 **Interactions between Era and CshA do not affect enzymatic activity**
240 CshA is an RNA helicase, while Era has GTPase activity. To investigate whether the
241 interaction between these two proteins affects the enzymatic activity of either one, we
242 first performed helicase assays. A double stranded RNA oligo was incubated with each
243 protein singly or in combination. While CshA was able to unwind the dsRNA, the
244 addition of Era had no effect on its activity (Fig S2a). In addition, the GTPase activity
245 of Era was unaltered in the presence of either CshA or YbeY (Fig S2b and S2c),
246 indicating that while these proteins do interact, this binding has little effect on their
247 enzymatic functions. As previously proposed, we suggest that these interactions fit
248 with a role for Era as a guide for rRNA/ribosome maturation enzymes to their
249 substrates [14, 18].
250
251 **CshA and Era are both required for growth at suboptimal temperatures,**
252 **virulence and rRNA processing**
253 In *E. coli,* CshA has been linked to survival at low temperatures [34], while strains
254 with depleted levels of Era are sensitive to both cold and heat shock [9, 10]. To
255 examine the importance of these enzymes in *S. aureus*, we utilised deletion strains in
256 the *S. aureus* MRSA strain LAC*. While both the *S. aureus era* and *cshA* null mutants
257 showed significant growth defects at 37°C (Fig 1b and S3a), the growth of both was
258 severely compromised at 25°C (Fig 4a and S3b). This defect was enhanced in a double
259 Δ*era cshA* mutant, which failed to grow at 25°C even after 48 h (Fig 4a and S3b),

260 highlighting that these two proteins are essential for bacteria to cope with growth at
261 suboptimal temperatures.

262 Era is an RA-GTPase that, along with a second RA-GTPase RsgA, binds to the
263 30S ribosomal subunit [14, 35]. In *E. coli,* overexpression of Era can complement a
264 deletion of *rsgA* [36]. After confirming that expression of Era can complement a
265 deletion of *rsgA* in *S. aureus* (Fig S4a), we used cross-complementation of the Δ*era*
266 and Δ*cshA* mutations to investigate whether Era and CshA act in the same pathway
267 and can functionally complement each other. However, cross-complementation did not
268 improve growth at 25°C (Fig S4b and S4c), indicating that while these proteins
269 interact and bacteria lacking both cannot survive cold temperatures, they have separate
270 cellular functions. We next assessed the contribution of each gene to the virulence of
271 *S. aureus* using *Galleria mellonella* as an invertebrate model of systemic infection.
272 While the wildtype bacteria were fully virulent, both the *era* and *cshA* mutant strains
273 displayed reduced killing, with the combined double mutant being completely
274 avirulent (Fig 4b).

275 To understand why Era and CshA are so important for growth at cold
276 temperatures and for virulence, we sought to assess the impact of deleting these genes
277 on the cellular rRNA. We observed that RNA extracted from both the single Δ*era* and
278 double Δ*era cshA* strains grown at 37°C had a processing or degradation intermediate
279 migrating slightly below the 16S band (Fig 4c). At 25°C we also observed a defect for
280 the Δ*cshA* mutant strain and multiple additional bands were evident in the Δ*era cshA*
281 double mutant (Fig 4c), which could represent either processing intermediates from
282 the 16S, or degradation intermediates from both the 23S and the 16S. A build-up of
283 degradation products are expected in the *cshA* mutant due to its role in the RNA
284 degradosome. However, to determine whether processing defects could be occurring

285 in these mutant strains when grown at low temperatures, we performed qRT-PCR with
286 probes that either amplified internally to the 16S rRNA sequence, or either flanked the
287 5' or 3' processing junctions required to convert 17S into mature 16S rRNA (Fig 4d).
288 While the levels of 16S rRNA were not significantly different between wildtype and
289 mutants, large increases in unprocessed 5' and 3' rRNA were evident in the Δ*era* strain
290 (Fig 4d). As Era is known to interact with the 30S ribosomal subunit, this could
291 potentially be due to mis-localisation of endonuclease YbeY in the absence of Era. It is
292 therefore, possible that Era acts as an intermediary protein on the 30S ribosomal
293 subunit, enabling proteins such as the endonuclease YbeY, and the RNA-degradosome
294 helicase CshA to access the 17S rRNA, allowing for processing (maturation or
295 degradation as appropriate) of rRNA under normal and stress conditions, such as
296 growth at cold temperatures.
297
298 **Rel_{SA} interacts with both Era and CshA**
299 Our previous work demonstrated that the GTPase activity of Era is inhibited by the
300 stringent response alarmone ppGpp [22]. To examine whether ppGpp also interacts
301 with CshA we performed DRaCALA assays, revealing that this nucleotide does not
302 interact with CshA (Fig S5a), nor does the affinity of ppGpp for Era alter in the
303 presence of CshA (K_d of 3.1 ± 0.4 μM in the absence versus 3.2 ± 0.3 μM in the
304 presence of CshA) (Fig S5b).
305 To investigate whether the stringent response has any additional points of
306 interaction with either Era and CshA, we examined whether one of the (p)ppGpp
307 synthetases might directly interact with either protein via bacterial two-hybrid. This
308 revealed that Rel_{SA}, but not RelP or RelQ, interacts with CshA and that Rel_{SA} interacts
309 with Era (Fig 5a). To confirm this interaction, we first used affinity pulldown assays,

310 showing that although no significant interaction occurred with Era, CshA and Rel_{SA}
311 interacted quite strongly (Fig 5b). Following this, we again used the split luciferase
312 assay, which confirmed an interaction between Rel_{SA} and CshA (Fig 5c) or Era (Fig
313 5d). It was interesting to note that the CshA-Rel_{SA} interaction only occurred in the
314 $CshA_{SB}$-Rel_{LB} and not the Rel_{SB}-$CshA_{LB}$ orientation, highlighting the importance of
315 creating both types of luciferase fusions.
316

317 **Rel_{SA} positively affects the GTPase activity of Era and is important for rRNA**
318 **processing**

319 We wished to ascertain whether the interactions between Rel_{SA} and Era impact the
320 activity of either enzyme. Recombinantly purified full-length Rel_{SA} is known to be in
321 the synthetase-off/hydrolase-on conformation *in vitro* [37]. Using purified Rel_{SA}, we
322 determined that Era and CshA have no effect on the pppGpp hydrolase activity of this
323 enzyme (Fig S5c). However, upon examination of the GTPase activity of Era, we
324 observed significantly increased activity in the presence of Rel_{SA} (Fig 6a).

325 Through Rel_{SA}, the stringent response appears to interact with components of the
326 ribosome and 16S rRNA processing machinery. To analyse the importance of the
327 stringent response for rRNA processing, we grew the wildtype *S. aureus* LAC* at
328 25°C, a condition under which we have shown that both Era and CshA are important
329 for viability, and induced the stringent response with mupirocin, an antibiotic that
330 inhibits the isoleucyl-tRNA synthetase mimicking amino acid deprivation [38]. We
331 again performed qRT-PCR with the probes as in Fig 4d that either amplified internally
332 to the 16S rRNA sequence, or either flanked the 5' or 3' processing junctions of the
333 17S rRNA. For the probe internal to the 16S rRNA we observed a significant decrease
334 in 16S rRNA in the presence of mupirocin (Fig 6b). This is expected, as it is known

335 that rRNA levels decrease upon activation of the stringent response. However, when
336 we compare the amount of unprocessed 5' or 3' sequences relative to the level of 16S
337 in each strain, we observed a significant increase in the amount of unprocessed leader
338 17S rRNA upon induction of (p)ppGpp (Fig 6c: p = 0.0031). We have previously
339 shown that ppGpp interacts with Era and that mature ribosome formation is inhibited
340 upon induction of the stringent response [22]. Here we propose that the stringent
341 response can also impact RA-GTPase function through direct interactions of Rel_{SA}
342 with both Era and CshA, influencing the GTPase activity of Era and 17S rRNA
343 processing.
344
345
346 **Discussion**
347 The synthesis of ribosomes and proteins consumes approximately 40% of the energy
348 within a growing bacterial cell [39]. Ribosomal assembly cofactors are, therefore, an
349 essential group of enzymes for coordinating biogenesis as efficiently as possible.
350 Members of this group include RNA helicases, rRNA and protein modification
351 enzymes, chaperones and RA-GTPases. *S. aureus* contains over 11 RA-GTPases, each
352 with potentially varying roles in the biogenesis of the 50S and 30S subunits, although
353 the precise functions of each in this process is unclear.
354 While the RA-GTPase Era has been extensively studied, its precise function in
355 the cell is unknown. Cryo-electron microscopy shows Era interacting with the 30S
356 ribosomal subunit, however this protein has also been implicated in numerous other
357 cellular processes [14]. Era-depleted *B. subtilis* or *E. coli* cells are elongated, with
358 defects in septum formation. These cells also contain diffuse nucleoid material,
359 implicating Era in cell division and chromosome segregation [5, 12]. In *B. subtilis,*

360 Era-depleted cells have defects in spore formation [7], while growth defects in strains
361 containing Era variants with reduced nucleotide binding abilities can be rescued by
362 truncating either *rpoN* or *ptsN* [11]. RpoN is required for nitrogen assimilation and
363 fixation, while PtsN is involved in sugar transport, suggesting that Era is also involved
364 in regulating carbon and nitrogen metabolism. Localisation studies have indicated that
365 this protein is present at both the membrane and in the cytoplasm [40], and so it has
366 been suggested that this enzyme cycles between the membrane and the ribosome in
367 response to cellular triggers. In addition to ribosomal biogenesis factors, Era has been
368 reported to interact with MazG, Ndk, Pk and YggG [41-43]. Ndk, a nucleoside
369 diphosphate kinase and Pk, a pyruvate kinase, from *Pseudomonas aeruginosa* were
370 both shown to form a complex with Era, further implicating Era in energy metabolism
371 [42]. MazG is a nucleoside triphosphate pyrophosphohydrolase, while YggG is a
372 membrane-associated heat shock protein. The significance of these interactions with
373 Era is unknown.

374 Era has also been implicated in helping cells cope with cold stress. Conditional
375 cold-sensitive mutants of Era have been constructed in *E. coli* [10] and these mutations
376 could be suppressed by the overexpression of the 16S rRNA methyltransferase KsgA,
377 providing one of the first links between Era and ribosome biogenesis [9]. In addition,
378 Era interacts with YbeY in *E. coli* [18]. YbeY is a universally conserved endonuclease
379 that, while dispensable in *E. coli*, is essential in *B. subtilis* and potentially in *S. aureus*,
380 given the lack of transposon mutants available [19, 25, 28]. This endonuclease is
381 required for the maturation of the 3' end of the 16S rRNA, and in strains depleted of
382 YbeY the 70S ribosomes are targeted for degradation with the help of RNase R [19,
383 28]. Era and YbeY are fused in a polypeptide in clostridial species, further
384 highlighting a link between these two proteins. Although not encoded in the same

385 operon as *era* in *E. coli*, this prompted us to examine the interactions of all other
386 proteins encoded in the *era* operon in *S. aureus* with Era (Fig 2). As in *E. coli*, YbeY
387 from *S. aureus* interacts with Era. YbeZ also interacted by bacterial two-hybrid but not
388 significantly using the quantitative luciferase assay (Fig 2). YbeZ has been shown to
389 interact with YbeY in *E. coli* [18]. This protein has an ATPase domain and an RNA
390 binding motif, and so is likely to also bind to the 17S rRNA and may form a complex
391 with Era and YbeY to aid in processing the 17S rRNA to the mature 16S form.

392 Here we use interaction studies to show that Era interacts with a protein
393 involved in survival at suboptimal temperatures, the DEAD-box RNA helicase CshA
394 (Fig 3). In *B. subtilis,* CshA is one of the most abundant RNA helicases produced at
395 low temperatures [44] and in *B. subtilis* and *E. coli* it has been implicated in multiple
396 processes, including ribosome biogenesis and interacting with components of the RNA
397 degradosome [32-34, 44]. In *S. aureus,* CshA has been linked to controlling the
398 turnover of mRNA in the cell [31]. Deletion of *cshA* results in the stabilisation of some
399 mRNA transcripts, such as the *spa* mRNA, but CshA also protects a number of other
400 mRNA and sRNA transcripts under stress conditions [31]. In addition, the CT region
401 of CshA is required for interactions with the degradosome [32]. Our analysis shows
402 that Era and CshA interact *in vitro* and when expressed heterologously in *E. coli* (Fig
403 3a and 3b). We additionally used an adapted split luciferase assay to show this
404 interaction natively in *S. aureus* (Fig 3c). Using this assay has also allowed us to
405 determine that the CT disordered region of CshA, which is important for binding to
406 mRNA and the degradosome [32], is dispensable for interacting with Era (Fig 3).

407 Using *cshA* and *era* deletion strains we show that both single mutants are
408 important for growth at cold temperatures, with a double mutant unable to survive at
409 25°C (Fig 4). By examining the rRNA of these strains we observe that the *era* mutant

410 strain has increased 17S unprocessed rRNA intermediates (Fig 4d). Additionally, a
411 double Δ*era cshA* mutant has multiple processing and degradation defects (Fig 4c). It
412 has been proposed that Era may act to guide processing enzymes to their site of action
413 [18]. In keeping with this, we have observed that these proteins have little effect on the
414 enzymatic activity of each other. Therefore, we propose a model whereby Era acts as
415 an intermediary protein, allowing proteins such as the endonuclease YbeY and the
416 RNA helicase CshA, and potentially others, access to their substrate rRNA. It has been
417 suggested that CshA helps RNase components of the degradosome access cleavage
418 sites that may be otherwise inaccessible [45], and so it is tempting to speculate that Era
419 is responsible for recruiting CshA to target misfolded ribosomal subunits for
420 degradation under stress conditions.

421 Cellular levels of rRNA are controlled by the stringent response [46]. Upon
422 activation of the stringent response, transcription from rRNA promoters is reduced
423 (Fig 6b), either by direct binding of ppGpp to the RNA polymerase in Gram negatives,
424 or by tight control of cellular GTP levels in Gram positives [47, 48]. Here we see an
425 additional level of regulation by the stringent response on rRNA. We show a direct
426 interaction between the (p)ppGpp synthetase Rel_{SA} and both CshA and Era (Fig 5),
427 and show that Rel_{SA} has a positive effect on the GTPase activity of Era (Fig 6a).
428 Additionally, we observed that the activation of the stringent response results in
429 increased processing defects in a wildtype strain grown at 25°C (Fig 6c). RA-GTPases
430 are reported to have increased association to the ribosome in the GTP-bound form.
431 Hydrolysis of GTP then acts as a signal to promote dissociation, presumably following
432 a maturation event [49, 50]. The increased GTPase activity of Era in the presence of
433 Rel_{SA} could promote premature dissociation of Era from the ribosome, leading to
434 processing defects (Fig 6c) and contributing to the stalled growth phenotype

435 synonymous with the stringent response. The precise dynamics of processing-enzyme
436 association under stress conditions requires further in-depth research but is beyond the
437 scope of this study. For instance, under what growth conditions is Rel_{SA} binding to Era
438 and CshA promoted and how does this affect the localisation of processing proteins to
439 the ribosome?

440 Taken together, we demonstrate a cellular function for Era as a protein
441 important for coordinating 30S ribosomal biogenesis in a stringent response-dependent
442 manner. It is possible that defects in ribosomal assembly lead to altered protein
443 translation, which may be the reason for the plethora of other phenotypes associated
444 with depletion of Era. As Era can complement a defect in RsgA (Fig S4a, [36]), as
445 well as RbfA [26], we propose a broader role for RA-GTPases as intermediary
446 proteins. It is now of interest to determine what processing events are coordinated by
447 the other RA-GTPases in *S. aureus*, as well as more broadly in other prokaryotes.

448

449

450 Materials and methods

451 **Bacterial strains and culture conditions.** *E. coli* strains were grown in Luria Bertani
452 broth (LB) and *S. aureus* strains in tryptic soy broth (TSB) at 37°C with aeration.
453 Strains and primers used are listed in Tables S1 and S2. Information on strain
454 construction is provided in SI Methods.

455

456 **Luciferase assay.** Overnight cultures of *S. aureus* strains were diluted to an OD_{600} of
457 0.05 and grown for 90 min in the presence of 100 ng/ml anhydrotetracycline (Atet).
458 Strains were normalised to an OD_{600} of 0.1 and luciferase activity measured according
459 to the Nano-Glo Luciferase Assay System Protocol (Promega).

460

461 **Pull-down experiments and western blotting.** Glutathione beads were washed 5
462 times in 1 x wash solution (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5%
463 Triton X-100). 1 μM of glutathione-S-transferase (GST), GST-CshA or GST-Era were
464 coupled to the beads by incubating in 1 x wash buffer for 4 h at 4°C. Unbound protein
465 was removed by washing in 1 x wash solution between 5-10 times. Protein-bound
466 beads were incubated with 1 μM His-tagged Era, His-YbeY or His-Rel_{SA} in the
467 presence of 1 x binding buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$,
468 0.5% Triton X-100) at 4°C overnight. After washing, attached proteins were eluted
469 from the beads with 50 μl elution buffer (25 mM Tris pH 8, 150 mM NaCl, 1 mM
470 EDTA, 0.5% Triton X-100, 10 mM reduced glutathione). Samples were mixed 1:1
471 with 2 x SDS protein sample buffer. Aliquots were separated on 12% SDS-
472 polyacrylamide gels and proteins subsequently transferred to PVDF membranes.
473 Bound His-tagged proteins were detected using HRP-conjugated anti-His antibodies
474 (Sigma) at a 1:500 dilution. Blots were developed by enhanced chemiluminescence
475 and imaged using a ChemiDoc MP imager (Bio-Rad).

476

477 **Construction of bacterial two-hybrid library.** Genomic DNA of *S. aureus* was
478 extracted and partially digested by incubation with Sau3AI at 37°C for 20 min.
479 Digested DNA was run on a 0.8% agarose gel and fragments of 500 to 1000 bp and
480 1000 to 3000 bp gel extracted and purified. This was repeated five times from
481 separate genomic preps and DNA fragments pooled. The vector pUT18C was digested
482 overnight at 37°C with BamHI and dephosphorylated with antarctic phosphatase for 90
483 min. Genomic DNA fragments were ligated into the pUT18C linearized vector using
484 T4 ligase, transformed into *E. coli* DH5α competent cells (New England Biolabs) and

485 plated onto LB agar with carbenicillin. Plates were scraped and transformant plasmids
486 isolated using the GeneJET plasmid purification kit (Thermo scientific).
487
488 **Bacterial two-hybrid.** Bacterial two hybrid plasmids containing genes of interest were
489 co-transformed into *E. coli* BTH101 cells, plated on LB agar containing 150 µg/ml
490 carbenicillin and 30 µg/ml kanamycin and incubated at 30°C overnight. Colonies were
491 isolated, grown overnight at 30°C in LB broth containing 0.5 mM IPTG and 5 µl
492 spotted onto LB agar containing 0.5 mM IPTG and 40 µg/ml X-gal. Plates were
493 incubated at 30°C for up to 48 h. In some cases, BTH101 transformants were
494 incubated overnight with 0.5 mM IPTG and spotted directly onto LB agar containing
495 IPTG, X-gal and appropriate antibiotics.
496
497 ***Galleria mellonella* survival assays.** *S. aureus* strains were grown overnight in TSB,
498 pelleted at 17,000 x g, washed twice in sterile PBS and diluted to an OD_{600} of 0.1 (2-4
499 x 10^7 cfu/ml). Colony numbers were confirmed by dilution and plating on TSA. Final
500 larval stage *G. mellonella* weighing 250-350 mg (Live foods direct, Sheffield, UK)
501 were stored in the dark at 4°C and used within 7 days. 5 µl of *S. aureus* inoculum was
502 injected into the hemocoel through the last pro-leg using a Hamilton syringe 701 RN.
503 Larvae were incubated in sealed plastic petri dishes at 37°C and the number of
504 surviving individuals recorded daily.
505
506 **RNA extraction.** Strains of *S. aureus* were grown overnight at 37°C and diluted to an
507 OD_{600} of 0.05. Cultures were grown to an OD_{600} of 0.4 at either 37 or 25°C and
508 harvested. RNA was extracted using the RiboPure RNA Purification Kit (Invitrogen)
509 as per guidelines. RNA was visualised using a modified agarose gel containing 0.7%

510 agarose and 0.9% Synergel (Diversified Biotech) in 0.5 X TBE (44.5 mM Tris, 1 mM
511 EDTA pH 8, 44.5 mM boric acid) as per Wachi *et al.* 1999 [51]. RNA was visualised
512 using EvaGreen fluorescent nucleic acid dye (Biotium) on a ChemiDoc MP imager
513 (Bio-Rad).
514
515 **RT-PCR.** RNA was extracted as described above. Complementary DNA was
516 synthesised from 1.5 µg RNA with transcriptor reverse transcriptase (Sigma) and
517 random primers. RT-PCR was performed on 100 ng of cDNA in triplicate using
518 SYBR Green Jumpstart Taq readymix (Sigma). Primers RMC379/380 (16S internal),
519 RMC570/571 (leader) and RMC572/548 (trailer) were designed to amplify 200-220 bp
520 target regions in the 17S rRNA. The housekeeping gene *rho* was amplified with
521 primers RMC573/574. The *rho* transcript was chosen as it has been shown to be highly
522 stable under nutrient-deficient conditions under which the stringent response would be
523 induced [52]. Cycle threshold values were determined for 3 biological repeats in
524 duplicate. For each reaction, the ratio to *rho* transcript number was calculated as
525 follows: $2^{-(\text{Ct target} - \text{Ct rho})}$.
526
527 **Growth curves.** *S. aureus* strains were grown overnight in TSB medium containing
528 the appropriate antibiotics. Overnight cultures were diluted to a starting OD_{600} of 0.05
529 in the presence of 100 ng/ml Atet where appropriate, incubated at 25°C or 37°C with
530 aeration and OD_{600} values determined at 2 h intervals. Growth curves were performed
531 in triplicate, and averages and standard deviations are plotted.
532
533 **Minimum Inhibitory Concentrations.** Overnight cultures of wildtype LAC* and
534 Δ*era* strains were adjusted to an OD_{600} of 0.05 in Mueller-Hinton broth and 100 µl

535 incubated in 96 well plates with 2-fold dilutions of various antimicrobials at the
536 following starting concentrations: spectinomycin 250 μg/ml and chloramphenicol 64
537 μg/ml. Plates were incubated at 37°C overnight with shaking. Assays were performed
538 in triplicate, and averages and standard deviations are plotted.
539
540 **Protein purifications.** Proteins were purified from 1-2 L *E. coli* cultures. Cultures
541 were grown to an OD_{600} 0.5-0.7, expression induced with 1 mM IPTG and incubated
542 for 3 h at 37°C. Protein purifications were performed by either nickel or glutathione
543 affinity chromatography. For nickel purifications, cell pellets were resuspended in 5
544 ml Buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 10 mM imidazole),
545 lysed with 100 μg lysozyme and sonication and the filtered cell lysate loaded onto a 1
546 ml HisTrap HP Ni^{2+} column (GE Healthcare) before elution using a gradient of Buffer
547 B (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 500 mM imidazole). GST-tagged
548 proteins were resuspended in 5 ml PBS, lysed and the cell lysate was loaded onto a 1
549 ml GSTrap HP column (GE Healthcare) before elution using 50 mM Tris pH 8.0, 10
550 mM reduced glutathione. Protein containing fractions were dialysed in 50 mM Tris pH
551 7.5, 200 mM NaCl, 5% glycerol before storage at -80°C. Protein concentrations were
552 determined by A_{280} readings.
553
554 **Synthesis of (p)ppGpp and differential radial capillary action of ligand assay**
555 **(DRaCALA).** The synthesis of (p)ppGpp and DRaCALA binding assays were
556 performed as described previously [22].
557
558 **Ribosomal profiles from *S. aureus* cell extracts.** Crude isolations of ribosomes from
559 *S. aureus* cell extracts were achieved as described by Chin Loh *et al.* with some

560 modifications [27]. Briefly, 100 ml cultures of the different *S. aureus* strains were
561 grown to an OD_{600} of 0.4 in TSB 100 ng/ml Atet. 100 µg/ml chloramphenicol was
562 added to each culture, before being cooled to 4°C. Pelleted cells were suspended in
563 association buffer (20 mM Tris-HCl pH 7.6, 8 mM $MgCl_2$, 30 mM NH_4Cl and 2 mM
564 β-mercaptoethanol), normalized to an OD_{600} of 15, lysed by the addition of 0.2 µg/ml
565 lysostaphin and 75 ng/ml DNase and incubated for 30 min at 37°C. The extracts were
566 centrifuged at 17,000 x g for 5 min and subsequently 250 µl were layered onto 10-50%
567 sucrose density gradients made in association buffer. Gradients were centrifuged for
568 4.5 h at 192,100 x g. Gradients were fractionated by upwards displacement of 250 µl
569 aliquots, which were analysed for RNA content at an absorbance of 260 nm.
570
571 **Helicase assay.** Reactions were set up as described [53]. Briefly, 250 nM of Cy3-
572 labelled duplex RNA (Cy3-GCUUUACGGUGCUA,
573 AACAAAACAAAAUAGCACCGUAAAGC) purchased from IDT, was incubated
574 with 0.5 µM recombinant protein in reaction buffer (20 mM Tris pH 7.5, 50 mM
575 potassium acetate, 5 mM magnesium acetate, 10 mM DTT, 0.1 mg/ml BSA, 10 U
576 RNasin). The 0.5 µM recombinant protein used is at a concentration similar to that
577 used in the affinity pulldown assays (1 µM) that show an Era-CshA interaction. The
578 reaction was initiated with the addition of 1 mM ATP and incubated at 25°C for 10
579 min. Reactions were terminated by mixing with an equal volume of stop buffer (1%
580 SDS, 0.5 mM EDTA, 20% glycerol) and loaded onto 20% native polyacrylamide gels
581 before being electrophoresed in 0.5 x TBE. Bands were imaged using a ChemiDoc MP
582 imager (Bio-Rad).
583

584 **Nucleotide hydrolysis assays.** The ability of proteins to hydrolyse GTP was
585 determined by incubating 100 nM recombinant Era with equimolar YbeY/CshA or
586 Rel_{SA}, 100 nM *S. aureus* ribosomes, 1 μM GTP and 2.78 nM α-^{32}P-GTP in 40 mM
587 Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ at 37°C for the indicated times. All
588 reactions were set up in the absence of Era as controls. pppGpp hydrolysis assays were
589 performed by incubating 100 nM recombinant Rel_{SA} in the presence and absence of
590 equimolar GST, GST-Era or GST-CshA, 1 μM pppGpp and 2.78 nM α-^{32}P-pppGpp in
591 50 mM Tris pH 8.5, 0.1 mM $MnCl_2$, 20 mM KCl at 37°C for the indicated times. All
592 reactions were set up in the absence of Rel_{SA} as controls. Hydrolysis reactions were
593 inactivated with the addition of formic acid to a final concentration of 1.2 M.
594 Precipitated proteins were pelleted by centrifugation at 17,000 x g for 10 min.
595 Reaction products were then visualized by spotting 1 μl on PEI-cellulose thin layer
596 chromatography (TLC) plates (Merck Millipore) and separated using 1 M KH_2PO_4,
597 pH 3.6 buffer. The radioactive spots were visualised using an LA 7000 Typhoon
598 PhosphorImager and images quantified using ImageJ.
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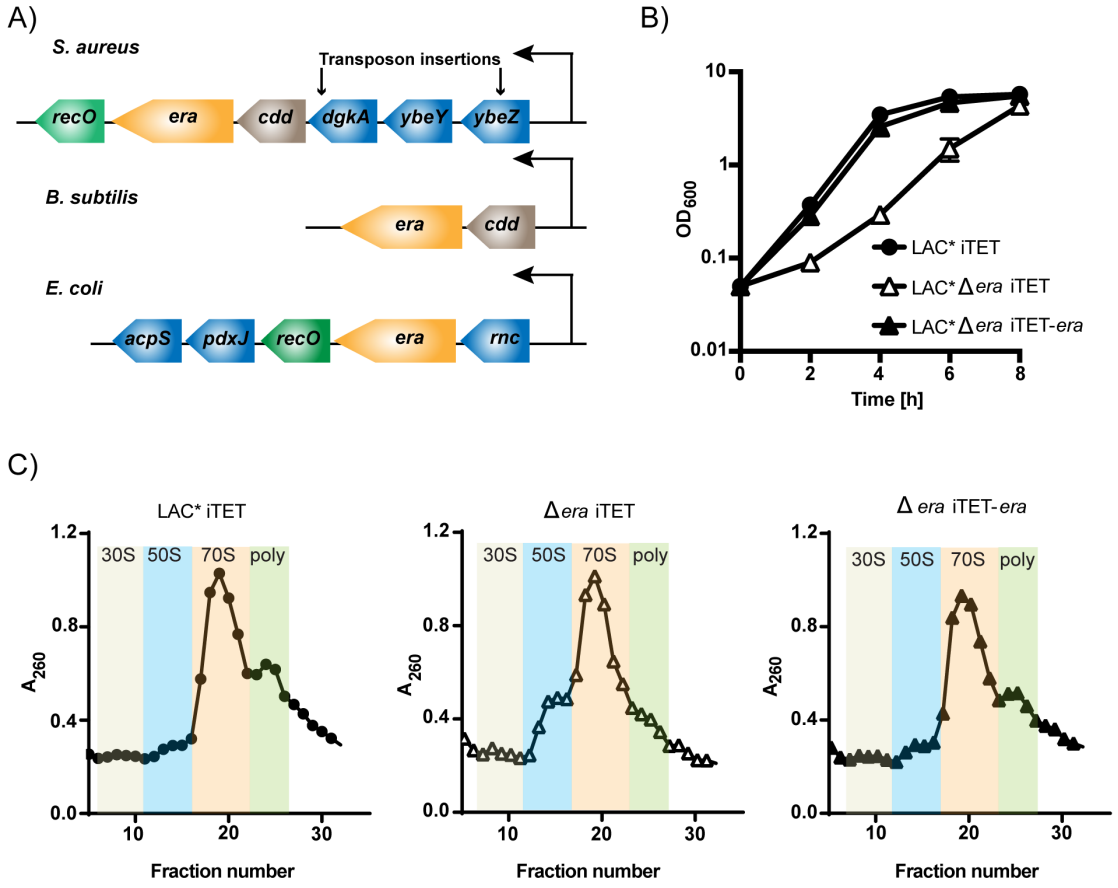

Fig 1. Role of Era in bacterial growth and ribosome assembly. A) Schematic representation of the *era*-containing operons from *S. aureus*, *B. subtilis* and *E. coli*. The *era* operon in *S. aureus* consists of: *ybeZ* – encoding for a protein of unknown function; *ybeY* – a 16S rRNA endoribonuclease; *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 (N=3), with averages and standard deviations shown. C) Ribosome profiles from LAC* iTET, LAC* Δ*era* iTET and

783 LAC* Δ*era* iTET-*era*. Normalised extracts from each strain were layered onto 10-50%
784 sucrose gradients. Gradients were fractionated and analysed for RNA content at an
785 absorbance of 260 nm. 30S, 50S, 70S and polysomes-containing fractions are
786 indicated. Experiments were performed in triplicate (N=3) with one representative
787 graph shown.
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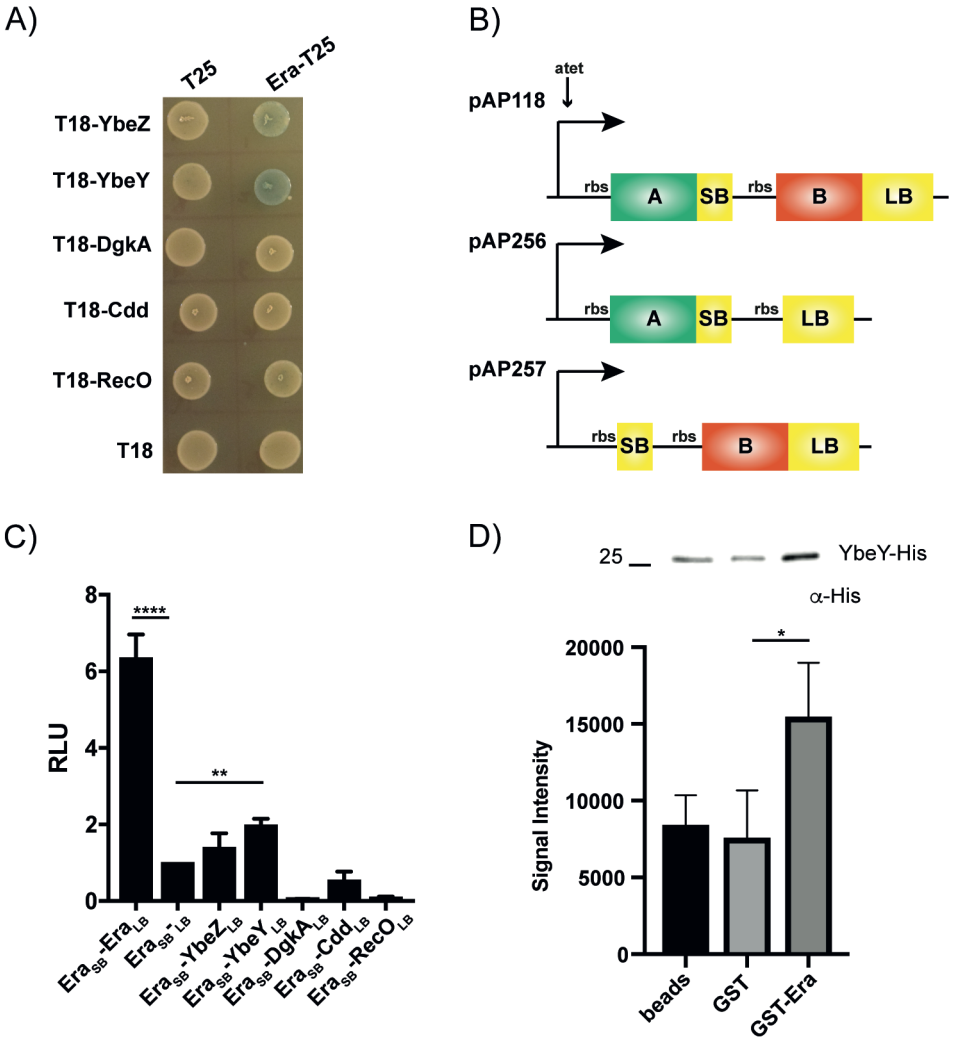

Fig 2. Era interacts with other proteins encoded in the same operon. A) Bacterial two-hybrid showing a positive interaction between Era-T25 and both T18-YbeZ and YbeY. T25 and T18-encoding empty vectors are used as negative controls. N=3, with one representative image shown. B) Schematic representation 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-Era and Era-YbeY in *S. aureus*. The negative is pAP256-*era*$_{SB\text{-}LB}$, which has Era fused to the SB but nothing fused to

808 LB. The negative is normalised to a relative luminescence unit (RLU) of 1. The
809 average values and standard deviations of triplicate experiments are plotted (N=3).
810 Statistical analysis was performed using a one-way ANOVA, followed by Dunnett's
811 multiple comparisons test (** $P < 0.005$, **** $P < 0.0001$). D) Top: affinity pulldown
812 assay using GST or GST-tagged Era coupled to glutathione beads. Beads alone, GST
813 or GST-Era coupled beads were incubated with His-tagged YbeY. After washing,
814 bound YbeY-His was detected using HRP-conjugated anti-His antibodies. N=3 with
815 one representative image shown. Below: the mean signal intensities and standard
816 deviations from the 3 repeats are plotted. Statistical analysis was performed using a
817 one-way ANOVA, followed by Dunnett's multiple comparisons test (* $P < 0.05$).
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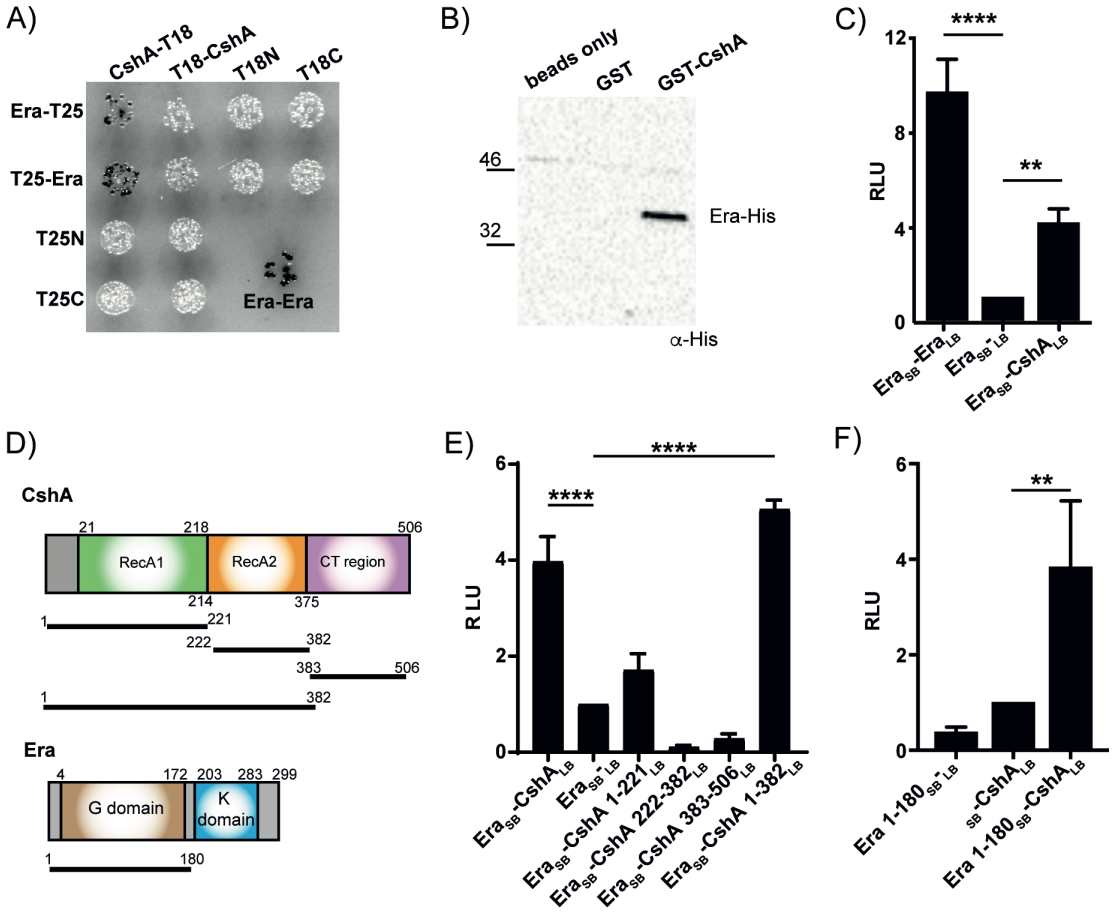

Fig 3. Era interacts with the DEAD-box helicase CshA. A) Bacterial two-hybrid showing an interaction between CshA-T18 and both Era-T25 and T25-Era. Era is known to form a dimer and an Era-Era interaction is used as a positive control. N=3 with one representative image shown. B) Affinity pulldown assay 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. N=3 with one representative image shown. 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, has Era fused to the SB but nothing fused to LB. Cells

were grown in the presence of 100 ng/ml Atet at 37°C before being normalised. The average values and standard deviations of triplicate experiments (N=3) 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 (N=3) for all luciferase assays are plotted. All statistical analysis was performed using a one-way ANOVA, followed by Dunnett's multiple comparisons test (** $P < 0.005$, **** $P < 0.0001$).

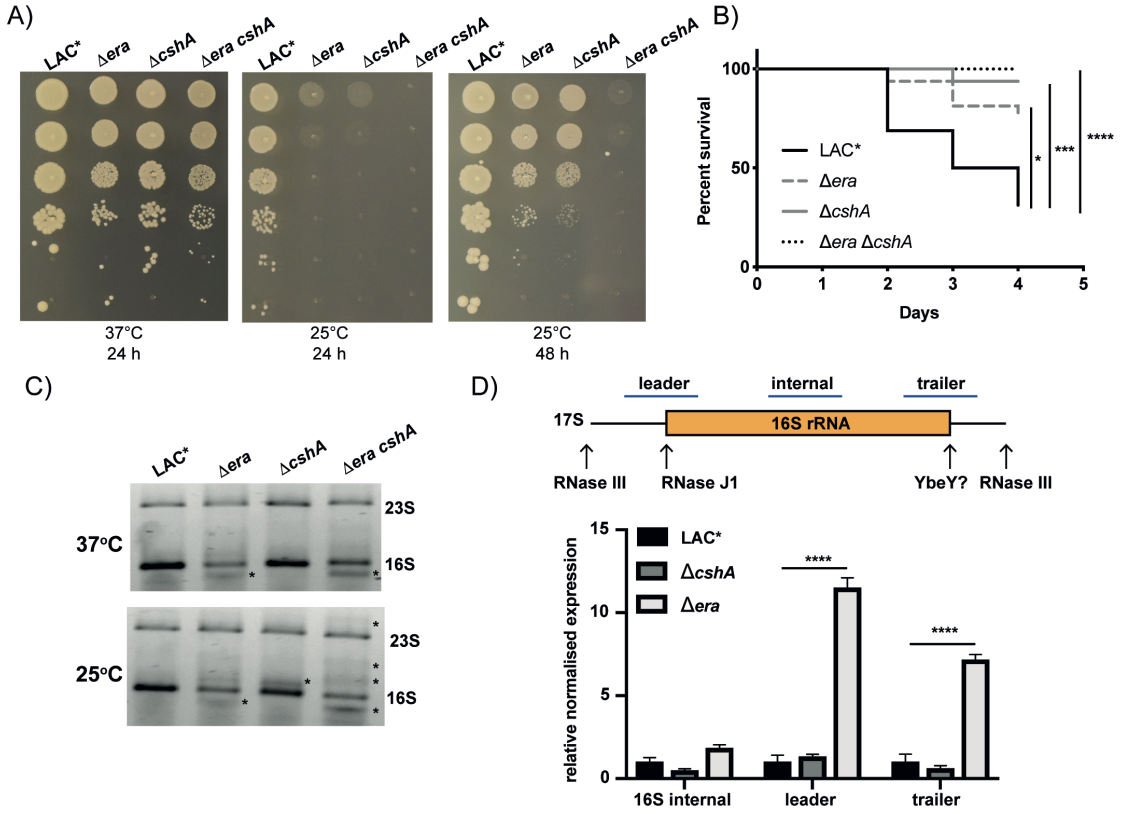

Fig 4. Era and CshA are required for cold shock survival, virulence and rRNA processing. A) Serial dilutions of the wildtype LAC*, Δ*era*, Δ*cshA* and Δ*era cshA* were spotted onto TSA agar plates and incubated at either 37 or 25°C for the times indicated. N=3 with one representative image shown. B) Survival of *G. mellonella* larvae inoculated into the hind leg with 1 x 10^5 cfu of wildtype 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 (N=3), with one graph shown. Survival curves were calculated by Kaplan-Meier analysis, with the log-rank (Mantel Cox) test performed to compare different strains (* $P < 0.05$, *** $P < 0.0005$, **** $P < 0.0001$). 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. N=3 with

876 one representative image shown. D) top: schematic of the 17S rRNA, with arrows
877 indicating processing sites that lead to the formation of the mature 16S rRNA. The
878 blue bars indicate the probe regions amplified by RT-PCR. Bottom: RT-PCR of total
879 RNA from the wildtype LAC*, Δ*era*, and Δ*cshA* strains grown at 25°C using probes
880 as indicated above. rRNA values are expressed as mean relative expression +/- SD
881 normalised to the wildtype and the expression of *rho* RNA as an internal control.
882 Statistical analysis was performed using a two-way ANOVA, followed by Tukey's
883 multiple comparisons test (**** $P < 0.0001$). Cycle threshold values were determined
884 for 3 biological repeats in duplicate (N=3).
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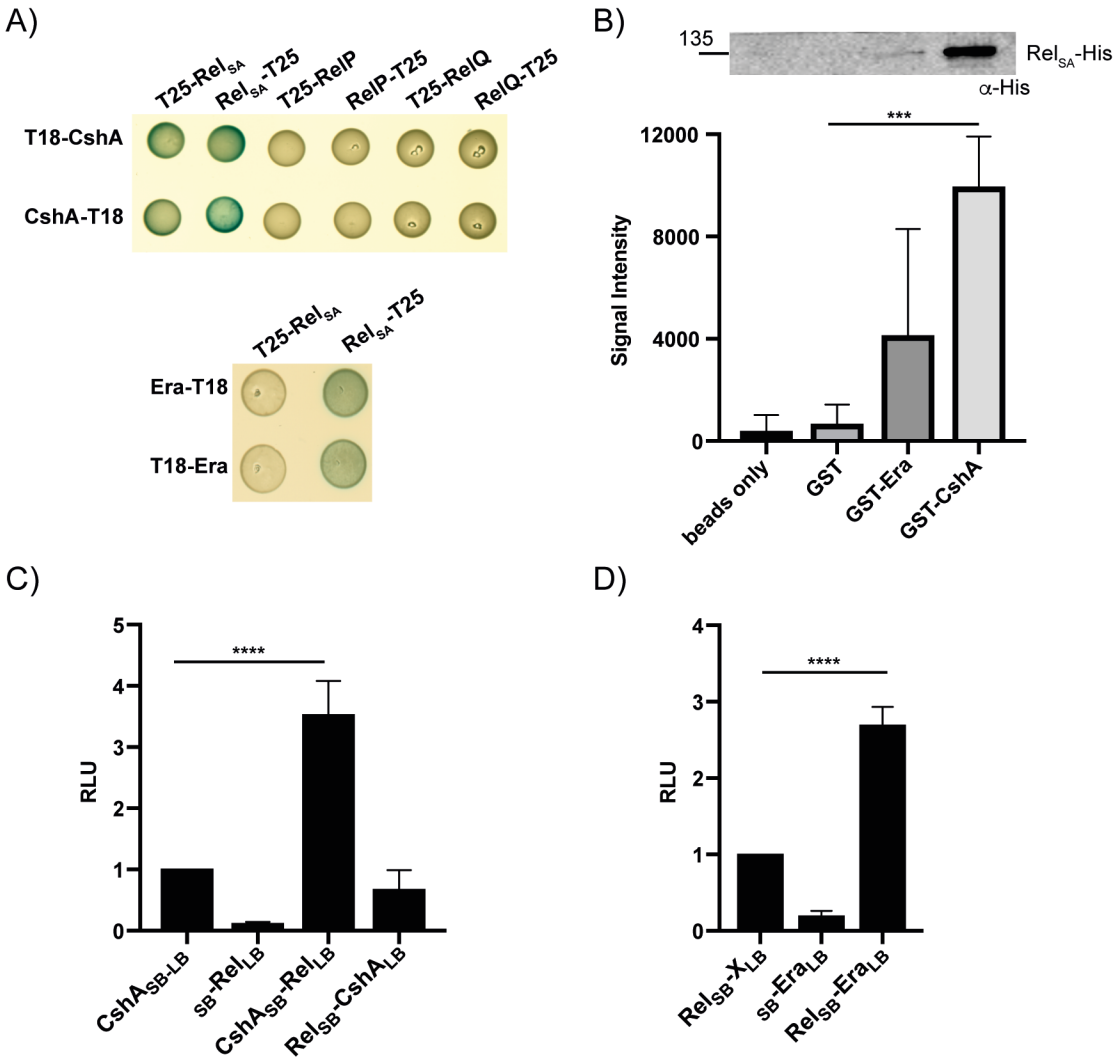

Fig 5. The stringent response synthetase Rel_{SA} interacts with Era and CshA. A) Top: Bacterial two-hybrid showing an interaction between CshA-T18/T18-CshA and both Rel_{SA}-T25 and T25-Rel_{SA}. CshA did not interact with the small (p)ppGpp synthetase enzymes RelP or RelQ. Bottom: Bacterial two-hybrid showing an interaction between Rel_{SA}-T25 and both Era-T18 and T18-Era. N=3 with one representative image shown. B) Affinity pulldown assay using GST, GST-tagged Era and GST-tagged CshA coupled to glutathione beads. Beads alone, GST, GST-Era and GST-CshA coupled beads were incubated with His-tagged Rel_{SA}. After washing, bound Rel_{SA}-His was detected using HRP-conjugated anti-His antibodies. N=3 with one representative image shown above. Below, the mean signal intensities and

907 standard deviations from the 3 repeats are plotted. Statistical analysis was performed
908 using a one-way ANOVA, followed by Dunnett's multiple comparisons test (*** $P <$
909 0.0005). C & D) Split luciferase assay demonstrating an interaction between CshA (C)
910 or Era (D) and Rel_{SA} in *S. aureus*. The negative controls have genes singly fused to
911 either the SB or the LB. The average values and standard deviations of triplicate
912 experiments (N=3) are plotted. All statistical analysis was performed using a one-way
913 ANOVA, followed by Dunnett's multiple comparisons test (**** $P < 0.0001$).
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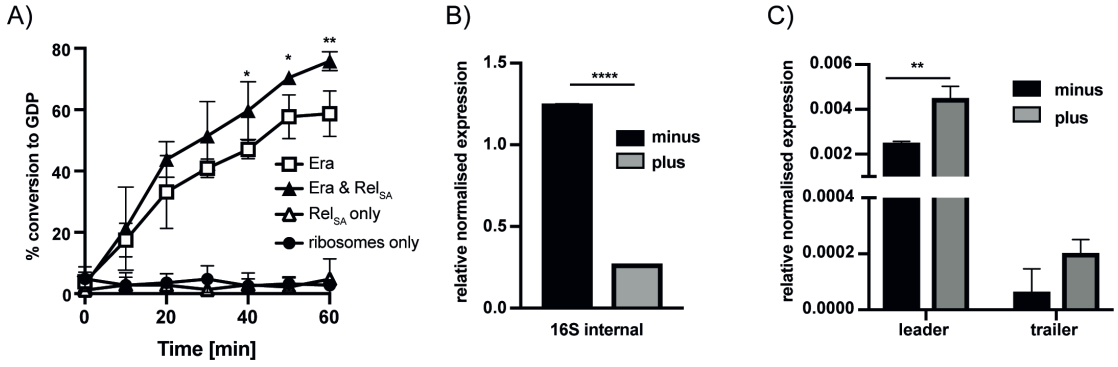

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931 **Fig 6. The stringent response influences Era GTPase activity and rRNA**
932 **processing.** A) The GTPase activity of 100 nM Era was measured in the presence of
933 an equal amount of ribosomes, 100 nM Rel_{SA} and 1 μM GTP. All reactions contained
934 ribosomes. The Rel_{SA} only control reaction contained Rel_{SA} and ribosomes but no Era.
935 Hydrolysis of ^{32}P-GTP was monitored by TLC and the percentage GDP formed
936 quantified using ImageJ. Experiments were repeated three times with means and
937 standard deviations shown. Statistical analysis was performed using a two-way
938 ANOVA, followed by Dunnett's multiple comparisons test (** $p < 0.005$, * $p < 0.05$).
939 B & C) rRNA extracted from LAC*. Strains were grown at 25°C until an OD_{600} of 0.4.
940 Cells were split and half exposed to 60 μg/ml mupirocin for 1 h (plus), which at 25°C
941 corresponds to one doubling. RT-PCR of total RNA using the probes as indicated in
942 Fig. 4d that bind internally to the 16S (B) or to the unprocessed leader and trailer
943 sequences (C) of the 17S rRNA. rRNA values are expressed as mean relative
944 expression +/- SD normalised to the expression of *rho* RNA as an internal control. For
945 comparisons between two groups (B - LAC* minus v plus) a two-tailed, unpaired
946 Student's t-test was performed (**** $p < 0.0001$). For comparisons between more than
947 two groups (C) a two-way ANOVA, followed by Sidak's multiple comparisons test
948 was performed (** $p < 0.005$). Cycle threshold values were determined for 3
949 biological repeats in duplicate (N=3).

950 **Supporting information**

951 **S1 Fig. Antibiotic susceptibility testing**

952 **S2 Fig. Enzyme activity of CshA and Era**

953 **S3 Fig. Growth of Δ*era* and Δ*cshA* strains at 37 and 25°C**

954 **S4 Fig. Cross-complementation of Δ*rsgA*, Δ*era* and Δ*cshA* strains**

955 **S5 Fig. ppGpp does not interact with CshA**

956 **Supplemental methods** – plasmid and strain construction

957 **S1 Table.** Bacterial strains used in this study

958 **S2 Table.** Primers used in this study

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