1   **RqcH supports survival in the absence of non-stop ribosome rescue factors**

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Abstract

Ribosomes frequently translate truncated or damaged mRNAs due to the extremely short half-life of mRNAs in bacteria. When ribosomes translate mRNA that lacks a stop codon (non-stop mRNA), specialized pathways are required to rescue the ribosome from the 3' end of the mRNA. The most highly conserved non-stop rescue pathway is *trans*-translation, which is found in greater than 95% of bacterial genomes. In all Proteobacteria that have been studied, the alternative non-stop ribosome rescue factors, ArfA and ArfB, are essential in the absence of *trans*-translation. Here, we investigate the interaction between non-stop rescue pathways and RqcH, a ribosome quality control factor that is broadly conserved outside of Proteobacteria. RqcH does not act directly on non-stop ribosomes but adds a degron tag to stalled peptides that obstruct the large ribosomal subunit, which allows the stalled peptide to be cleared from the ribosome by peptidyl-tRNA hydrolase (PTH). We show that *Bacillus subtilis* can survive without *trans*-translation and BrfA (Bacillus ArfA homolog), due to the presence of RqcH. We also show that expression of RqcH and its helper protein RqcP rescues the synthetic lethality of Δ*ssrA*Δ*arfA* in *Escherichia coli*. These results suggest that non-stop ribosome complexes can be disassembled and then cleared because of the tagging activity of RqcH, and that this process is essential in the absence of non-stop ribosome rescue pathways. Moreover, we surveyed the conservation of ribosome rescue pathways in >14,000 bacterial genomes. Our analysis reveals a broad distribution of non-stop rescue pathways, especially *trans*-translation and RqcH, and a strong co-occurrence between the ribosome splitting factor MutS2 and RqcH. Altogether, our results support a role for RqcH in non-stop ribosome rescue and provide a broad survey of ribosome rescue pathways in diverse bacterial species.

40 **Importance**

41 Ribosome stalling on damaged mRNA is a major problem in bacteria. It is estimated that 2-4% of

42 all translation reactions terminate with the ribosome stalled on a damaged mRNA lacking a stop

43 codon. Mechanisms that rescue these ribosomes, such as *trans*-translation, are often essential

44 for viability. We investigated the functional overlap between RqcH and the non-stop ribosome

45 rescue systems (ArfA and *trans*-translation) that are present in both *E. coli* and *B. subtilis*. Since

46 these two species are extremely distant relatives, our work is likely to have wider implications for

47 understanding ribosome rescue in bacteria. Furthermore, we used a bioinformatics approach to

48 examine the conservation and overlap of various ribosome rescue systems in >14,000 species

49 throughout the bacterial domain. These results provide key insights into ribosome rescue in

50 diverse phyla.

Introduction

Truncated mRNAs that lack a stop codon (non-stop mRNAs) are a major problem in bacteria (1, 2). The release factors (RF1 and RF2) require stop codon recognition to terminate translation (3, 4). Therefore, if an mRNA lacks a stop codon, the ribosome becomes stalled at the 3' end of the message (5). Non-stop mRNAs arise from premature transcription termination, mRNA degradation by nucleases, and stop codon readthrough. Bacteria have evolved ways to rescue ribosomes from non-stop mRNAs. The primary rescue system found in nearly all bacteria, *trans*-translation, is mediated by transfer-messenger RNA (tmRNA) encoded by the *ssrA* gene, and its partner protein, small protein B (SmpB) (5, 6). SmpB bound to tmRNA senses the empty mRNA channel of the non-stop ribosome and translation then resumes on the mRNA like-domain of tmRNA (7, 8) . A degradation tag, encoded by the short reading frame of tmRNA, is appended to the nascent peptide that will target the truncated protein for degradation by proteases (5, 9, 10). A stop codon at the end of the tmRNA reading frame recruits release factors for translation termination. *trans*-Translation is essential in many bacterial species, including *Mycobacterium tuberculosis*, *Shigella flexneri*, and *Neisseria gonorrhoeae* (11–13)*.

An alternative non-stop rescue factor, ArfA, was uncovered from a synthetic lethal screen in *E.coli* lacking *ssrA* (14). ArfA recognizes the empty mRNA channel and recruits RF1 or RF2 to terminate translation independent of a stop codon (15–17). A second alternative rescue factor, ArfB, is essential in *Caulobacter crescentus* in the absence of *trans*-translation (18). *C. crescentus* does not encode ArfA. And, although *E. coli* does encode ArfB, it is poorly expressed and not sufficient to rescue Δ*ssrA*Δ*arfA* (19). The essentiality of *trans*-translation in many bacteria and the conditional essentiality of ArfA and ArfB in the absence of *trans*-translation indicate that rescuing ribosomes from truncated mRNAs is essential (20).

Bacillus subtilis can survive without *trans*-translation, and encodes an alternative rescue factor called *Bacillus* ribosome rescue factor A (BrfA) (21). BrfA is homologous to ArfA, sharing 21.54% protein sequence identity when aligned with Clustal Omega (22). Like ArfA, BrfA

77 recognizes non-stop ribosomes and recruits RF2 to terminate translation (21). BrfA is also

78 negatively regulated by *trans*-translation, since the *brfA* transcript contains an RNase III

79 cleavage site upstream of the stop codon and is therefore translated from a non-stop mRNA

80 (21).

81 In addition to *trans*-translation and BrfA, *B. subtilis* also encodes the Ribosome quality

82 control Homolog RqcH . RqcH is broadly distributed in bacteria but is absent in most Alpha-,

83 Beta-, and Gamma- proteobacteria including *E. coli* (23). RqcH acts on 50S ribosomal subunits

84 obstructed with peptidyl-RNA. If the 70S ribosome dissociates from an mRNA and separates

85 into 30S and 50S subunits before translation termination, the 50S subunit remains obstructed

86 with the P-site tRNA still covalently bound to the nascent polypeptide in the exit tunnel. When

87 this occurs, RqcH recruits alanine-charged tRNAs to the obstructed large subunit, allowing the

88 large subunit to catalyze addition of an alanine tag to the stalled peptide in a template-

89 independent manner. The alanine tail serves as a degron tag to target the stalled peptide for

90 degradation (24, 25). The alanine tail also exposes the amino-acyl bond between the peptide

91 and the tRNA to the cytoplasm, where the enzyme PTH can then hydrolyze this bond and thus

92 free the nascent chain from the tRNA (26). The nascent peptide then diffuses, making the large

93 subunit available for another round of translation.

94 Here we show that *B. subtilis* can survive without *trans*-translation and the alternative rescue

95 factor BrfA because it encodes RqcH. We further show that RqcH and the helper protein RqcP

96 (27) from *B. subtilis* are sufficient to rescue deletion of Δ*arfA*Δ*ssrA* in *E. coli*. Finally, we report a

97 bioinformatic analysis of >14,000 species across the bacterial domain to determine the

98 prevalence and distribution of *trans*-translation, ArfA/BrfA, ArfB, and RqcH. We report a strong

99 co-occurrence between RqcH and the ribosome splitting factor MutS2. Our findings provide

100 insight into the conservation and functional interaction between ribosome rescue systems.

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Results

ΔsmpBΔrqcH exhibits a severe growth defect similar to ΔsmpBΔbrfA in *Bacillus subtilis*.

Ribosomes stall on truncated mRNAs that lack a stop codon (non-stop mRNA) since translation termination by release factors requires the presence of a stop codon in the ribosomal A-site. *B. subtilis* can rescue non-stop ribosomes using either *trans*-translation (encoded by *ssrA* and *smpB*) or BrfA (encoded by *brfA*). Deleting *brfA* and *smpB* causes a severe synthetic growth defect (Fig. 1), consistent with previously published results (21). The maximum growth rate of wild-type cells was 1.30±0.14 hour^{-1} whereas the maximal growth rate of ΔsmpBΔbrfA was 0.88±0.4 hour^{-1} at 37˚C. ΔsmpBΔbrfA cells also exhibited a longer lag phase than wild-type cells (2.50±0.54 hours and 0.94±0.13 hours, respectively) when transferred from an overnight culture into fresh media.

  B. subtilis encodes the ribosome quality control factor RqcH. In contrast to *trans*-translation and BrfA, RqcH does not act directly on ribosomes stalled on non-stop mRNAs but adds alanine residues to the stalled peptide which is subsequently hydrolyzed from the tRNA by PTH (23, 26). Single deletion of *rqcH* had no measurable effect on growth in LB at 37˚C (Fig. 1). However, the ΔsmpBΔrqcH strain exhibited a severe growth defect with a maximum growth rate of 0.64±0.16 hour^{-1} at 37˚C. ΔsmpBΔrqcH also exhibited an increased lag time compared to wild-type cells. Wild-type cells took 0.94±0.13 hours to exit lag phase, whereas ΔsmpBΔrqcH took 3.94±0.75 hours to exit lag phase. When comparing ΔsmpBΔrqcH to ΔsmpBΔbrfA, the ΔsmpBΔrqcH strain exhibited a significantly slower maximal growth (p = 0.0241) and significantly longer lag phase (p =0.0233). These data suggest that RqcH plays a significant role in ribosome rescue in the absence of *trans*-translation in *B. subtilis*.

SmpB is required for cell growth in the absence of *brfA* and *rqcH* in *B. subtilis*.

Deleting *smpB* and *brfA* from *B. subtilis* causes a severe, but non-lethal growth defect (Fig. 1). In contrast, deleting *smpB* and *arfA* from *E. coli* is lethal (14). Therefore, we hypothesized that *B. subtilis* can survive deletion of *smpB* and *brfA* because it encodes RqcH, which is absent in *E.*

129 *coli*, and that deletion of all three factors (*smpB*, *brfA* and *rqcH*) would be lethal to *B. subtilis*. To

130 test this, we used CRISPR interference (CRISPRi) (28, 29) to deplete SmpB from cells lacking

131 *rqcH* and/or *brfA*. We expressed nuclease deficient Cas9 (dCas9) under the control of a xylose

132 inducible promoter while constitutively expressing a guide RNA targeting *smpB* (sgRNA^{smpB}).

133 Knocking down *smpB* from Δ*brfA* or Δ*rqcH* cells decreased colony size, consistent with the growth

134 defect observed in liquid culture, but was not lethal (Fig. 2). In contrast, when *smpB* expression

135 was blocked in the Δ*rqcH*Δ*brfA* strain, colonies failed to form on plates at both 37°C and 30°C

136 (Fig. 2B), indicating that *trans*-translation becomes essential in the absence of BrfA and RqcH.

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138 **B. subtilis RqcH and RqcP rescues the synthetic lethal phenotype of Δ*ssrA*Δ*arfA* in E. coli.**

139 *E. coli* lacks the RQC system but can survive deletion of *trans*-translation because of the

140 alternative non-stop ribosome rescue system, ArfA (14). To determine whether *E. coli* lacking

141 *ssrA* and *arfA* could survive when expressing components of the *B. subtilis* RQC pathway, we

142 transformed MG1655Δ*ssrA::cat^R* with a plasmid encoding *rqcH* and the helper protein *rqcP*

143 (from *B. subtilis)* under the control of an arabinose inducible promoter. We then used phage

144 lysate generated from MG1655Δ*arfA::kan^R* to transduce the Δ*arfA::kan^R* deletion into

145 MG1655Δ*ssrA::cat^R* *E. coli* strains, harboring either empty vector or vector encoding RqcH and

146 RqcP (pRqcH-RqcP). No colonies were recovered when Δ*arfA::kan^R* was transduced into the

147 Δ*ssrA* strain containing empty vector. In contrast, colonies were recovered on LB with 1%

148 arabinose when Δ*arfA::kan^R* was transduced into the Δ*ssrA* strain containing plasmid encoding

149 arabinose-inducible RqcH and RqcP. These cells did not grow on plates in the absence of

150 arabinose, indicating that expression of RqcH/RqcP is required for survival in this condition (Fig

151 3A). In liquid culture, Δ*arfA*Δ*ssrA* containing pRqcH-RqcP grew better in the presence of

152 arabinose (Fig 3B). These results demonstrate that RqcH and RqcP are sufficient to support *E.*

153 *coli* survival when *trans*-translation and ArfA are absent.

154

155 **Ribosome rescue pathways are broadly conserved in bacteria.**

156 *B. subtilis* RqcH/RqcP rescued the synthetic lethality of Δ*ssrA*Δ*arfA* in *E. coli*, a species which

157 lacks RqcH. To investigate the conservation of various ribosome rescue pathways in bacteria, we

158 surveyed 14,479 representative reference genomes for the presence of genes encoding tmRNA,

159 SmpB, ArfA, ArfB, and RqcH. Consistent with previous reports (30), we detected the genes

160 encoding *trans*-translation in most bacterial genomes (Fig. 4) (Table 1). *smpB* was detected in

161 95.3% of the representative genomes surveyed and *ssrA* was detected in 94.2%. 95.8% of

162 genomes contained either *smpB* or *ssrA* (Fig. 4B) (Table 1). Genomes that lacked *ssrA* were

163 found mainly in Mycoplasmatota and Bacteria incertae sedis. However, most of these genomes

164 still encoded *smpB*, indicating *ssrA* may still be present, but was not identified in our search. *arfB*

165 is widely distributed across the bacterial domain and was conserved in 54.7% of surveyed

166 genomes. Most Gram-positive phyla, including Firmicutes, lack *arfB*. *arfA* was the least conserved

167 rescue pathway, and is restricted specifically to Gammaproteobacteria, some Betaproteobacteria,

168 and 10 species in the *Bacillus subtilis* group. *rqcH* was identified in 23.6% of the surveyed

169 genomes and was found in phyla across the bacterial domain, except Proteobacteria. The broad

170 conservation of RqcH and the presence of an RqcH homolog in eukaryotes are consistent with

171 RqcH being present in the last universal common ancestor (31).

172

| 173 | Table 1: Conservation of ribosome rescue pathways across 14,479 genomes. |

Phylum	total genomes	*smpB*	*ssrA*	*arfB*	*arfA*	*smrB*	*rqcH*	*mutS2*
174 Phylum	total genomes	*smpB*	*ssrA*	*arfB*	*arfA*	*smrB*	*rqcH*	*mutS2*
175 Acidithiobacillia	9	9	9	0	0	0	9	3
176 Acidobacteriota	49	48	48	4	0	0	9	35
177 Actinobacteriota	3066	2975	2969	2055	0	0	1	1
178 Alphaproteobacteria	2327	2218	2215	1886	0	0	0	0
179 Aquificota + Campylobacterota +								
180 Deferribacterota	197	192	193	6	0	0	163	192
181 Armatimonadota	4	4	4	1	0	0	3	4
182 Atribacterota	1	1	1	0	0	0	1	1
183 Bacteria incertae sedis	12	11	8	0	0	0	2	1
184 Bdellovibrionota	14	14	14	6	0	0	5	12
185 Betaproteobacteria	907	837	804	586	30	1	0	4
186 Caldisericota/Cryosericota group	2	2	1	0	0	0	0	2
187 Chloroflexota	50	48	48	15	0	0	30	44
188 Chrysiogenota	3	3	3	0	0	0	2	3
189 Coprothermobacterota	2	2	2	0	0	0	2	2
190 Cyanobacteriota/Melainabacteria group	144	132	128	69	0	0	132	129

191	Deinococcota	97	86	85	0	0	0	86	52
192	Deltaproteobacteria	8	8	8	5	0	0	6	6
193	Desulfuromonadota + Desulfobacterota	201	197	197	130	0	0	74	124
194	Dictyoglomota	2	2	2	0	0	0	2	2
195	Elusimicrobiota	3	3	3	0	0	0	0	0
196	FCB group	1791	1712	1705	1262	0	0	346	1559
197	Firmicutes	2437	2310	2304	5	10	0	2307	2312
198	Fusobacteriota	36	30	32	0	0	0	30	32
199	Gammaproteobacteria	2429	2293	2317	1700	569	851	0	0
200	Hydrogenophilia	6	6	6	0	0	0	0	0
201	Mycoplasmatota	188	169	51	0	0	0	16	16
202	Myxococcota	57	57	55	55	0	0	50	38
203	Nitrospinae/Tectomicrobia group	1	1	1	0	0	0	0	1
204	Nitrospirota	16	15	15	7	0	0	8	13
205	PVC group	211	208	206	100	0	0	11	8
206	Spirochaetota	142	139	136	18	0	0	57	54
207	Synergistota	19	19	19	0	0	0	19	19
208	Thermotogota	39	39	39	0	0	0	39	39

209	Zetaproteobacteria	6	6	6	6	0	0	0	0
210	unclassified Bacteria	3	3	3	2	0	0	0	0
211	Total	14479	13799	13637	7918	609	852	3410	4708

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RqcH and MutS2 frequently co-occur in bacterial genomes.

215   Two newly discovered ribosome quality control proteins are SmrB and MutS2. Both

216   proteins contain SMR domains and bind collided ribosomes (31–33). MutS2 splits stalled

217   ribosomes from the mRNA, leaving an obstructed large ribosomal subunit that is a substrate for

218   RqcH (31). MutS2 and RqcH are both found throughout the bacterial domain and have homologs

219   in eukarya (31). Since MutS2 generates substrates for RqcH, we investigated how frequently

220   MutS2 and RqcH co-occur in bacterial genomes. 96% of genomes that encoded RqcH also

221   encoded MutS2 (Fig. 4C). Of the genomes that have lost RqcH, 87% have also lost MutS2. These

222   data indicate that there is a high degree of co-occurrence between RqcH and MutS2.

223

RqcH ribosome rescue activity is decreased, but not abolished, in the absence of MutS2.

225   Our bioinformatic analysis revealed a strong co-occurrence between RqcH and MutS2 in

226   bacterial genomes, suggesting that RqcH is dependent on the ribosome splitting activity of

227   MutS2. To test this, we compared the impact of SmpB depletion from Δ*brfA*Δ*rqcH* cells to SmpB

228   depletion from Δ*brfA*Δ*mutS2* cells. In the Δ*brfA* background, when SmpB is depleted, cells are

229   totally reliant on RqcH. In three independent experiments, SmpB depletion from Δ*brfA*Δ*mutS2*

230   exhibited a more severe fitness defect than SmpB depletion from the Δ*brfA* single deletion,

231   indicating that MutS2 is a major splitting factor acting upstream of RqcH (Fig. 5). However, this

232   fitness defect was not as severe as SmpB depletion from Δ*brfA*Δ*rqcH*. These data indicate that

233   RqcH retains some function in the absence of MutS2, and that there may be additional factors

234   capable of splitting ribosomes from nonstop messages prior to rescue by RqcH.

235

236

Discussion

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238 Previous work demonstrates that bacteria can survive without *trans*-translation only if they

239 encode one or more alternative non-stop rescue factors, either ArfA or ArfB (12, 14, 18).

240 However, these studies were restricted mainly to Proteobacteria, which lack the recently

241 discovered ribosome rescue factor RqcH. RqcH is highly conserved in bacterial phyla outside of

242 Proteobacteria (Fig. 4). Therefore, we investigated the essentiality of the non-stop rescue

243 factors in *B. subtilis*, a model Firmicute that encodes RqcH. We found that *B. subtilis* lacking all

244 the canonical non-stop rescue factors (*trans*-translation, ArfA and ArfB) can survive because it

245 encodes RqcH (Fig. 1 and 2). Moreover, expression of RqcH and its helper protein RqcP in *E.*

246 *coli* rescued the well-documented synthetic lethality of Δ*ssrA*Δ*arfA* in this species (Fig. 3). These

247 results suggest that RqcH/RqcP can support viability in the absence of *trans*-translation and the

248 alternative non-stop ribosome rescue pathways.

249 RqcH does not act directly on non-stop ribosomes but can still support cell survival in the

250 absence of *trans*-translation and *arfA* in both *B. subtilis* and *E. coli*. These results suggest that

251 ribosomes can dissociate from non-stop messages and subsequently be rescued by the activity

252 of RqcH and PTH. How do ribosomes dissociate from non-stop mRNAs? Recently, MutS2 was

253 identified as a ribosome splitting factor that can remove collided ribosomes from mRNAs (31).

254 Since MutS2 generates substrates for RqcH, we determined how frequently MutS2 and RqcH

255 co-occur in bacterial genomes. We found an extremely high co-occurrence of MutS2 and RqcH

256 in genomes across the bacterial domain (Fig. 4). However, experimentally, we found that RqcH

257 does not have a strict requirement for MutS2 (Fig. 5). SmpB depletion from Δ*brfA*Δ*mutS2* cells

258 exhibited decreased fitness, but not as severe as depletion from Δ*brfA*Δ*rqcH* cells (Fig. 5).

259 Moreover, RqcH/RqcP were sufficient to rescue viability of Δ*arfA*Δ*ssrA* in *E. coli*, a species that

260 does not encode MutS2 (Fig. 3). Therefore, MutS2 is unlikely to be the only ribosome splitter.

261 One potential ribosome splitter is YbnA/HflX, a universally conserved GTP-binding protein that

262 functions as a ribosome splitting factor and is important during stressful conditions such as heat

263 shock where ribosome stalling may increase (34). However, HflX is only known to act on

264 hibernating ribosomes or on ribosomes carrying a deacylated tRNA in the P-site (35, 36).

265 Neither RqcH or MutS2 were detected in the Proteobacteria we surveyed (Table 1).

266 Gamma-proteobacteria encode SmrB, an SMR domain-containing protein which binds to

267 collided ribosomes but does not split them (33). Instead, SmrB possesses endonuclease activity

268 that cleaves mRNA between collided ribosomes. *trans*-Translation, ArfA, and ArfB all require

269 truncated mRNA and their rescue activity is undetectable on ribosomes that are stalled mid-

270 message (37–40). Therefore, in the absence of RqcH, cells are more reliant on *trans*-translation

271 and ArfA or ArfB to rescue ribosomes, and it becomes more important for mRNA cleavage to

272 occur. Thus, loss of RqcH could be the pressure that selected for the nuclease activity of SmrB.

273 Although RqcH can support viability in the absence of *trans*-translation and is conserved

274 in 24% of genomes, the genes encoding *trans*-translation are present in nearly all bacterial

275 genomes (>95%) (Table 1). Even obligate intracellular bacteria with reduced genomes such as

276 *Rickettsia rickettsii*, *Chlamydia trachomatis*, *Coxiella burnetii,* and *Buchnera aphidicola* retain

277 *trans*-translation. *trans*-Translation provides a means to both rescue the stalled ribosome and

278 target the stalled peptide for degradation by proteases in one pathway. In contrast, the RQC

279 pathway requires a ribosome splitter, addition of a degron tag to the stalled peptide by RqcH,

280 and the activity of PTH to hydrolyze the stalled peptidyl-tRNA and clear the obstructed

281 ribosome. Therefore, *trans*-translation provides the most efficient mechanism to rescue non-

282 stop ribosomes, which may explain its near-total conservation.

283 While the mRNA cleavage activity of SmrB allows *trans*-translation to be the dominant

284 ribosome rescue pathway in *E. coli*, *B. subtilis* has evolved to also rely heavily on RqcH. It is

285 notable that the growth defect of ΔsmpBΔbrfA in *B. subtilis* exhibits similar severity to

286 ΔsmpBΔrqcH. This finding indicates that obstructed 50S ribosomal subunits are a major

287 problem in *B. subtilis*. Future work is needed to identify the major sources of these obstructed

288 subunits and to identify factors that split stalled ribosomes in *B. subtilis*.

Materials and Methods

Strains and media. Strains were derived from *Bacillus subtilis* 168 *trpC2* and grown in LB media (10g tryptone, 5g yeast extract, 5g NaCl per liter) at 30˚C or 37˚C with aeration where indicated (Table 1). Gene deletions were made by transforming genomic DNA from the BKK collection (41) into the lab's naturally competent wild type. The pDR244 marker loop-out plasmid was used to create a marker less *smpB* deletion from the BKE collection and a marker less *brfA* from the BKK collection. Final concentrations of antibiotics used for selection included 100ug/mL ampicillin, 20ug/mL kanamycin, 100ug/mL spectinomycin, 5ug/mL chloramphenicol, and 1X MLS (1ug/mL erythromycin and 25ug/mL lincomycin).

Growth curves for *B. subtilis*. Cells were grown overnight in LB with aeration and back diluted to a starting OD600 of 0.5 and serially diluted to OD600 of 0.05, 0.005, and 0.0005. The cells were grown for 24h at 37˚C, shaking at 2mm amplitude using Thermo Scientific 96-well flat bottom plates (Cat.No. 167008). OD600 values were obtained in 15min intervals from the BioTek Synergy H1 microplate reader, Gen5 3.11. Growth rates at OD600=0.05 were measured using non-linear regression of logistic growth and statistical analysis was performed using an unpaired t-test followed by a Welch's correction using GraphPad Prism version 10.1.1 for macOS.

Plasmid construction. RqcH from *Bacillus subtilis* was amplified using primers KC53 & KC54 and gel extracted. pBAD322 digested with EcoRI/NcoI was used as the plasmid backbone for Gibson assembly (42) of the PCR product resulting in pKC353. IDT gblock of RqcP with homology to pKC353 was then Gibson assembled after pKC353 digestion with NcoI/XbaI to create pKC426 (Genbank ID). Plasmids were transformed into Dh5alpha cells and selected on ampicillin plates containing 1% arabinose. Whole Plasmid Sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation.

CRISPR interference. To create the sgRNA targeting SmpB, we amplified pJMP2 using primer sp60 (29) and reverse primer HRH175 (43). The Phusion PCR product was DpN-1 treated for 2.5hrs at 37˚C followed by PNK treatment for 1h. The resulting band was gel extracted and ligated

315 overnight at room temperature using T4 DNA ligase (NEB). pKC349 was transformed into

316 Dh5alpha and selected on ampicillin and sequencing was performed by Plasmidsaurus. Genomic

317 DNA of *B. subtilis* harboring dCas9 under an inducible xylose promoter at *lacA* (43) was

318 transformed into Δ*rqcH::kan,* Δ*brfA,* and Δ*brfA*Δ*rqcH::kan* cells. pKC349 was ScaI digested and

319 transformed into the above backgrounds for integration into *amyE.* Strains were grown in LB for

320 4h at 37˚ and back diluted to a final OD600 of 0.05 in 1XTbase + 1mM MgS04. The cultures were

321 serially diluted and 10ul were spotted onto LB agar containing 1% xylose and incubated at 30˚C

322 or 37˚C overnight.

323 **Transduction.** Phage lysate was obtained from *E. coli* Δ*arfA::kan*. MG1655Δ*ssrA::cat* was

324 electroporated with pKC353 or pKC426 and grown on LB agar containing ampicillin. Recipient

325 cells were transduced with phage lysate (44–49) and selected on media with and without 1%

326 arabinose to induce expression of RqcH (pKC353) and RqcH with RqcP (pKC426).

327 **Growth curves for *E. coli*.** Cells were grown overnight in LB and strains harboring the

328 pRqcH/RqcP plasmids were grown with 100ug/ml ampicillin and 1% arabinose to maintain the

329 plasmid. The overnight cultures were back diluted to a starting OD600 of 0.05. The cells were

330 grown in the presence of 1% arabinose or 1% glucose where indicated for 24h at 30˚C or 37˚C,

331 shaking at 2mm amplitude using Thermo Scientific 96-well flat bottom plates (Cat.No. 167008).

332 OD600 values were obtained in 15min intervals from the BioTek Synergy H1 microplate reader,

333 Gen5 3.11.

334 **Gene detection.** Genes were detected in a database of 14,479 representative prokaryotic

335 genomes from NCBI RefSeq (50) using HMMER v3.3 (nhmmer) (hmmer.org) with an E-value

336 cutoff of 0.05. HMMER profiles were built using ≥ 5 genes as annotated by the NCBI Prokaryotic

337 Genome Annotation Pipeline (50). At least one gene sequence from every taxa expected to have

338 the gene based on a preliminary search was included. In Alphaproteobacteria like *Caulobacter*

339 *crescentus*, the *ssrA* gene is interrupted by an internal loop that is excised from the final RNA

340 product (51). Thus, two separate HMMER profiles were built and searches were performed for

341   *ssrA* to separately probe Alphaproteobacteria and other phyla. Proteins closely resembling *B.*

342   *subtilis* RqcH are described in NCBI as "NFACT RNA binding domain-containing proteins." Only

343   genes for proteins with this description were used when building the *rqcH* HMMER profile. The

344   domain architecture of MutS2 is similar to that of MutS1, but the homology is only shared over

345   approximately 1000 bp. Therefore, hits were filtered for a coverage of >1250 bp to remove

346   erroneous *mutS1* hits. Similarly, *arfB* shares approximately 100 bp of homology to *prfB*, so hits

347   were filtered for a coverage of >150 bp. SMR domains in other proteins resemble the C-terminus

348   of *smrB*, so hits were filtered for a coverage of >400 bp.

349   **Phylogenetics.** 16S rRNA sequences of all genomes were identified and acquired using BLAST

350   v2.13.0 (52), aligned using MAFFT v7.453 (53), and applied to FastTree v2.1.11 (54) to infer a

351   maximum likelihood tree. FastTree produces unrooted phylogenies, so trees were midpoint rooted

352   using the phangorn v2.11.1 package (55). Representatives from each phyla were randomly

353   selected and used to subset the built 16S tree. The identities of the randomly selected genomes

354   can be found in Table 1. Taxonomic classification was assigned to genomes using the NCBI

355   Taxonomy database (56). Phyla were named using the conventions in Coleman et al. 2021 (57).

356   The tree was visualized using ggtree v3.6.2 (58).

357

358

364

365 Table 1

Strain (strain number)	Description	Source
367 HAF1	168 *trpC2 B. subtilis* wild type	(59)
368 KC118	168 *trpC2* Δ*rqcH*::kan	This study
369 KC145	168 *trpC2* Δ*smpB*	This study
370 KC153	168 *trpC2* Δ*smpB*Δ*rqcH*::kan	This study
371 KC158	168 *trpC2* Δ*brfA*	This study
372 KC167	168 *trpC2* Δ*brfA*Δ*rqcH*::kan	This study
373 HRH774	168 *trpC2 lacA*:: P_{xyl}-*dCas9*-mls	(43)
374 KC357	168 *trpC2 lacA*:: P_{xyl}-*dCas9*-mls *amyE*::P_{veg}-sgRNA[smpB]	This study
375 KC379	168 *trpC2* Δ*brfA*Δ*mutS2::*kan *lacA*:: P_{xyl}-*dCas9*-mls *amyE*::P_{veg}-sgRNA[smpB]	This study
376 KC381	168 *trpC2* Δ*rqcH::*kan *lacA*:: P_{xyl}-*dCas9*-mls *amyE*::P_{veg}-sgRNA[smpB]	This study
377 KC383	168 *trpC2* Δ*brfA*Δ*rqcH::*kan *lacA*:: P_{xyl}-*dCas9*-mls *amyE*::P_{veg}-sgRNA[smpB]	This study
378 KC385	168 *trpC2* Δ*brfA lacA*:: P_{xyl}-*dCas9*-mls *amyE*::P_{veg}-sgRNA[smpB]	This study
379 KC462	168 *trpC2* Δ*mutS2::*kan *lacA*:: P_{xyl}-*dCas9*-mls *amyE*::P_{veg}-sgRNA[smpB]	This study
380 KCK19	MG1655 WT	Ken Keiler
381 KCK18	MG1655Δ*ssrA*::cat	Ken Keiler
382 KCK 345	BW25113 Δ*arfA*::kan (Keio collection)	(60)
383 KC444	MG1655Δ*ssrA*::catΔ*arfA*::kan pBAD322-RqcH-RqcP	This study

384

	Plasmid	Description	Source
385	Plasmid	Description	Source
386	pDR244	Cre-lox plasmid	(41)
387	pJMP1	pJMP1 *lacA*::P_{xyl}-*dCas9*	(29)
388	pKC349	pJMP2 *amyE*::P_{veg}-sgRNA^{smpB}	(21)
389	pOPO646	pBAD322-arapBAD	DQ119282.1
390	pKC353	pBAD322-RqcH	This study
391	pKC426	pBAD322-RqcH-RqcP	This study
392			

	Primer	Sequence	Source
393	Primer	Sequence	Source
394	sp60	5'-GCTCGTGTTGTACAATAAATGTAGGAATCCTTAAGGTTTAC	
395		GGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC -3'	(21)
396	HRH175	5'-ACATTTATTGTACAACACGAGCC-3'	(43)
397	HAF1	5'-CTACCGCTTTGACGAACAGCATTTCG-3'	This study
398	HAF2	5'-GATATGCCTGAAACAGCTCATTGCAG-3'	This study
399	brfAchkF	5'-GCACGGAGATTAATCATGATATGC -3'	This study
400	brfAchkR	5'-CTGGCTGATGGTCATACTGTTGAC-3'	This study
401	KC31	5'-TCGTTTGGTCCATGCTTGTC-3'	This study
402	KC32	5'-GAAAGAGCTTGCTGAAAACGTGG-3'	This study
403	KC53	5'-TGGGCTAGCAGGAGGAATTCATTGCATATGTCGTTTGATGGC	
404		ATGTTTACATAC-3'	This study

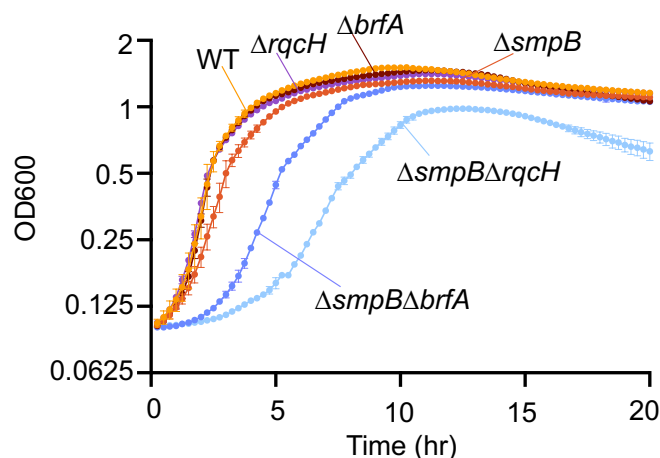| 405	KC54	5'-GGATCCCCGGGTACCATGGTCAGCTTTTTTTGAGCTTGATGAC	
406		AGTATCAGC-3'	This study
407	MG1655_ssrA_F	5'-CTGGTCATGGCGCTCATAAATCTGGTATAC-3'	This study
408	MG1655_ssrA_R	5'-TCGGATGACTCTGGTAATCACCGATGGAG-3'	This study
409	MG1655_arfA_F	5'-GTTGTTGATTTTTTGCACTGGCAGG-3'	This study
410	MG1655_arfA_R	5'-ATTCGTGATTTGCTGAAAGAGCAGAATAAC-3'	This study
411	KC61	5'-TCGCAACTCTCTACTGTTTCTCCATACCCG-3'	This study
412	KC62	5'-GAAAATCTTCTCTCATCCGCCAAAACAGCC-3'	This study
413			
414			

415 <u>Accession numbers of representative genomes for 16S tree</u>

416	Acidobacteriota	NZ_LAIJ01000000	Deinococcota	NZ_CP038512
417	Actinobacteriota	NZ_AP022562	Desulfuromonadota + Desulfobacterota	NZ_JACADJ010000000
418	Alphaproteobacteria	NZ_JACIIU010000000	FCB group	NZ_CP083387
419	Aquificota + Campylobacterota + Deferribacterota	NZ_AP013035	Firmicutes	NZ_CP017326
420	Bacteria incertae sedis	NC_021219	Fusobacteriota	NZ_CP028102
421	Bdellovibrionota	NC_016026	Gammaproteobacteria	NZ_KB899143
422	Betaproteobacteria	NZ_CAJQUM010000000	Mycoplasmatota	NZ_JAOOPZ010000000
423	Chloroflexota	NZ_LGCL01000000	Myxococcota	NZ_JAKCFA010000000
424	Cyanobacteriota/Melainabacteria group	NC_009925	Nitrospirota	NZ_CZPZ01000000

425	PVC group	NZ_CP042912	Spirochaetota	NZ_RQER01000000
426	Synergistota	NC_014011	Thermotogota	NZ_JAHC01000000
427				
428				

429   **Figures**

430
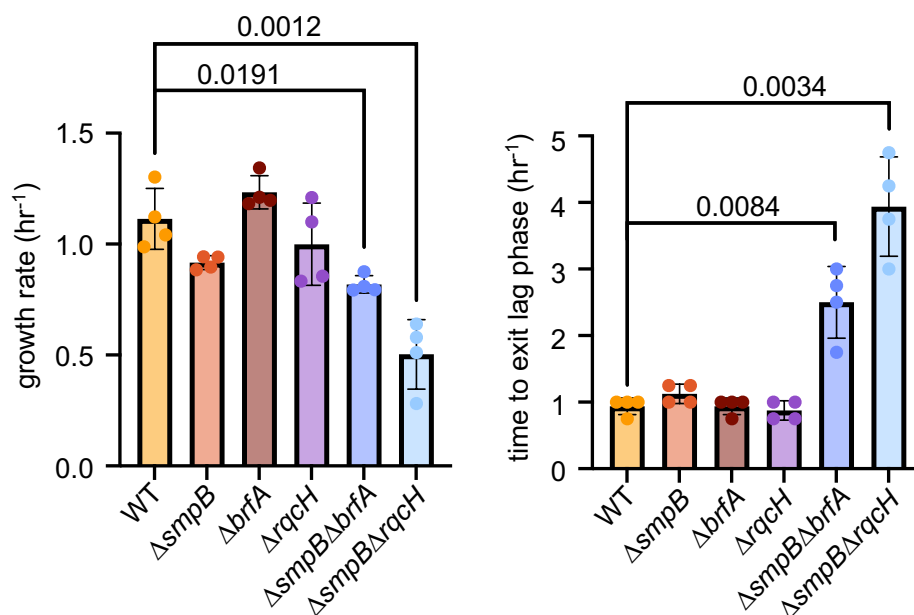
431   **Figure 1. Growth of wild-type *B. subtilis,* Δ*smpB*, Δ*brfA*, Δ*rqcH::kan^R*, Δ*smpB*Δ*brfA::kan^R*,**
432   **and Δ*smpB*Δ*rqcH::kan^R* cells.** (A) Δ*smpB*Δ*brfA::kan* and Δ*smpB*Δ*rqcH* cells exhibit a more
433   severe growth defect and longer lag-phase under aerobic growth in LB at 37˚C. (B) Bar graphs
434   show maximal growth rates and time for cells to exit lag phase (time at which OD exceeds
435   0.125). Δ*smpB*Δ*rqcH* cells have an increased lag phase and slower maximal growth rate than
436   wild type. Error bars represent the standard deviation of four independent replicates performed
437   on different days. p-values represent results of an unpaired t-test with Welch's correction.
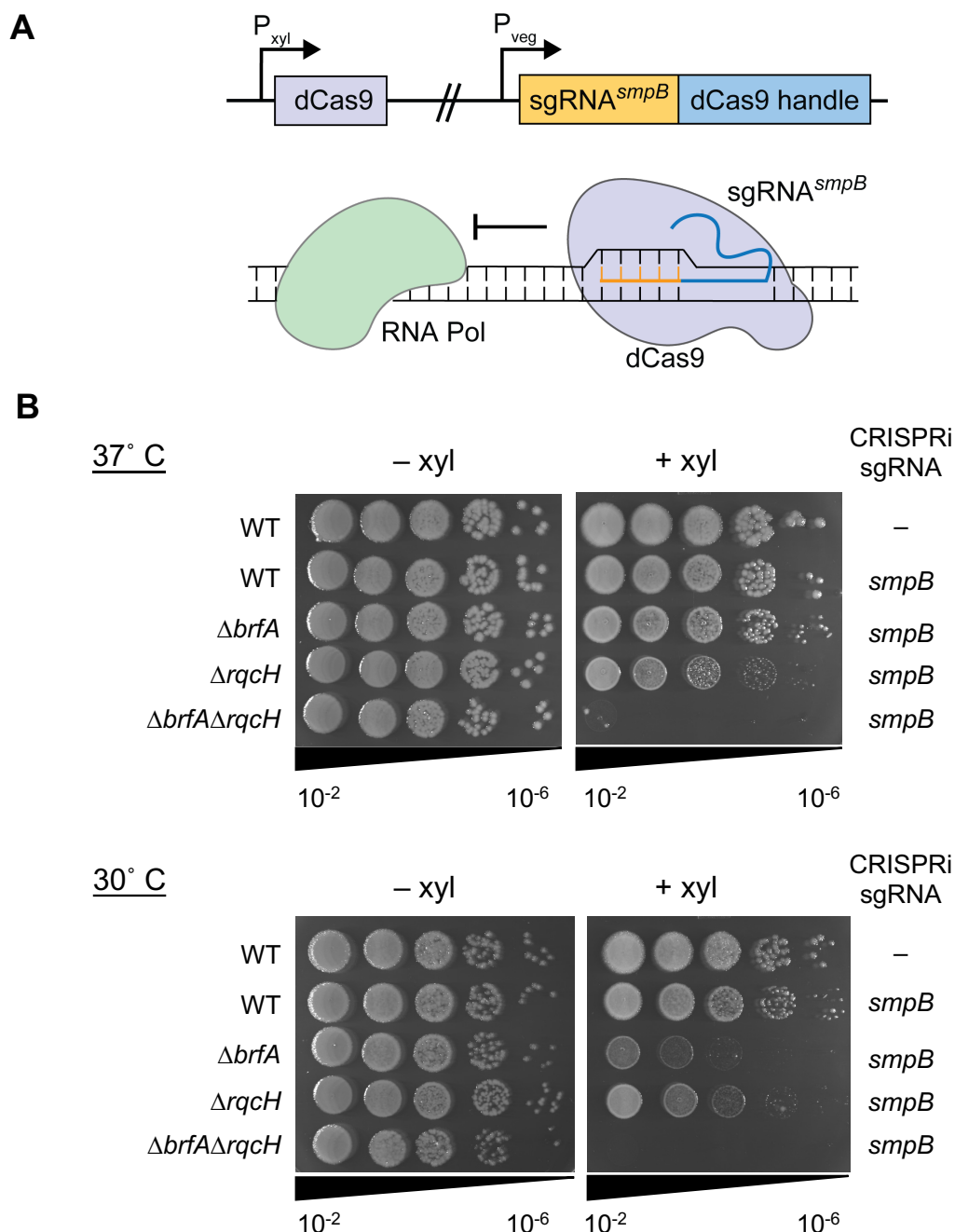438

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Figure 2. Impact of *smpB* depletion from cells lacking *brfA* & *rqcH*. (A) Schematic showing depletion of *smpB* using CRISPRi. Guide RNA targeting *smpB* (sgRNA^{smpB}) under the vegetative promoter in *B. subtilis* is co-expressed with deactivated Cas9 (*dCas9*) under the control of a xylose inducible promoter resulting in transcriptional repression. (B) Strains harboring sgRNA^{smpB} and *dCas9* were serially diluted and spot plated on LB agar with or without 1% xylose at 37°C and 30°C. SmpB depletion from Δ*brfA*Δ*rqcH* is more severe than depletion of SmpB from the single deletions. SmpB depletion from the Δ*brfA* or Δ*rqcH* single deletions is more severe at 30°C than at 37°C. Images are representative of three independent experiments.
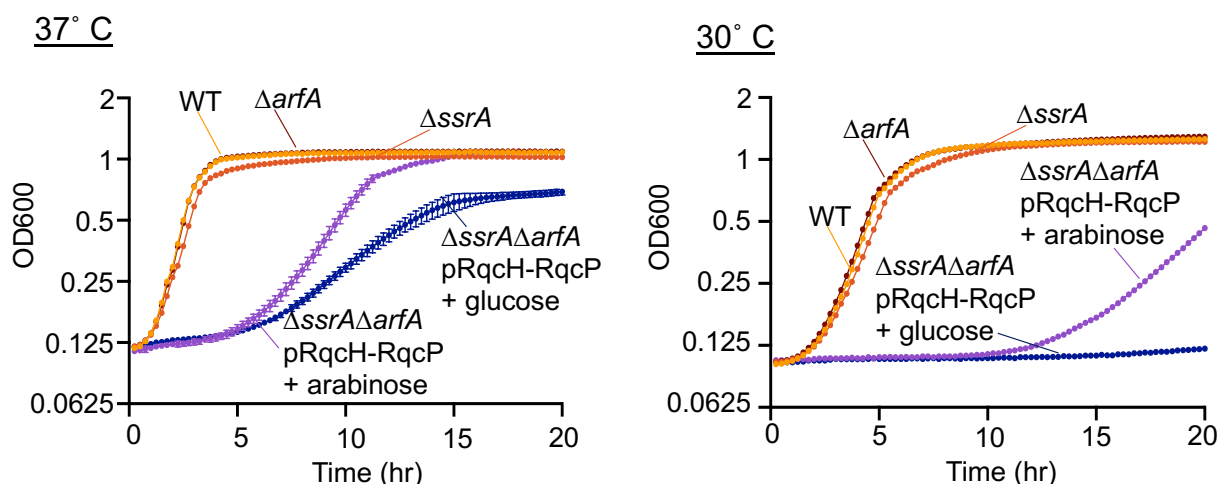
448
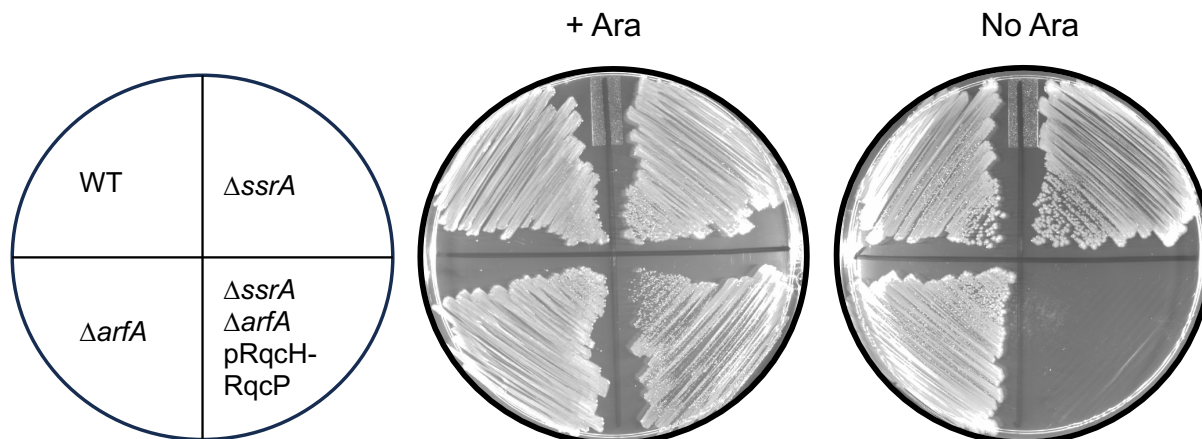
Figure 3. *E.coli* can survive the synthetic lethality of $\Delta ssrA::cat^R \Delta arfA::kan^R$ when provided with *B. subtilis rqcH and rqcP*. (A) Strains depicted in the schematic were grown on LB plates with or without 1% arabinose to induce expression of RqcH/RqcP. (B) Growth curves in LB of *E. coli* $\Delta arfA::kan^R$, $\Delta ssrA::cat^R$ and $\Delta ssrA::cat^R \Delta arfA::kan^R$ strain harboring the plasmid expressing RqcH and RqcP. $\Delta ssrA \Delta arfA$ with pRqcH-RqcP grows better with arabinose induction of RqcH and RqcP. However, cells are not recovered to the wild type growth rate. Error bars represent the standard deviation of cells grown from independent colonies in triplicate.
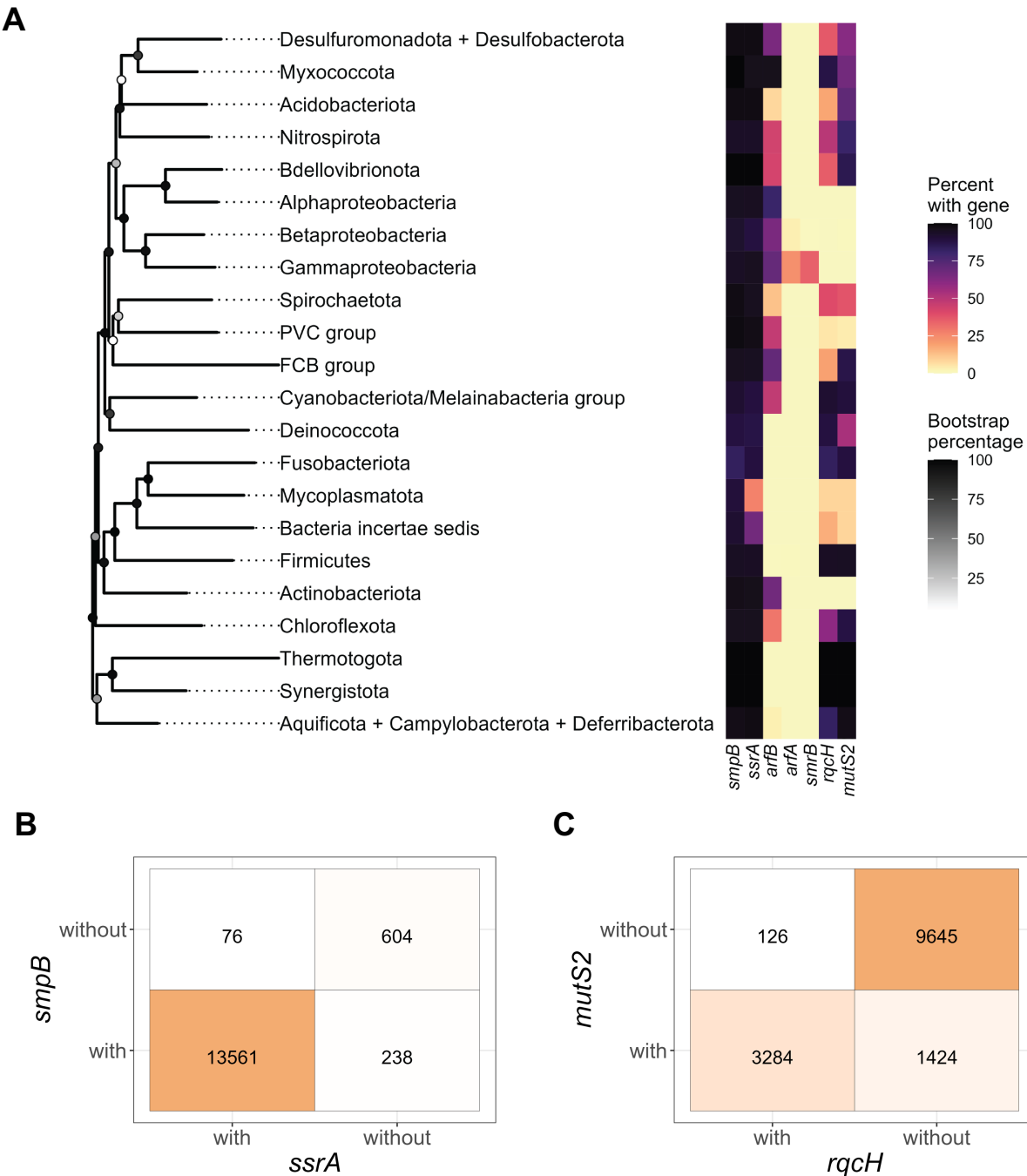
Figure 4. Conservation of ribosome rescue pathways across >14,000 bacterial genomes. *rqcH* and *mutS2* co-occur across bacterial phyla. (A) Heatmap showing the percent of genomes per phyla containing *smpB*, *ssrA*, *arfA*, *arfB*, *smrB*, *rqcH*, and *mutS2*. The tree was built using 16S rRNA genes from a single representative species for each phylum. NCBI accession numbers for the representatives are listed in Table 1. Bootstrap percentages are shown as darkened circles at each node. (B) Co-occurrence matrix of *smpB* and *ssrA*. The values represent the number of genomes with or without these genes. (C) Co-occurrence matrix of *rqcH* and *mutS2*. The values represent the number of genomes with or without these genes.
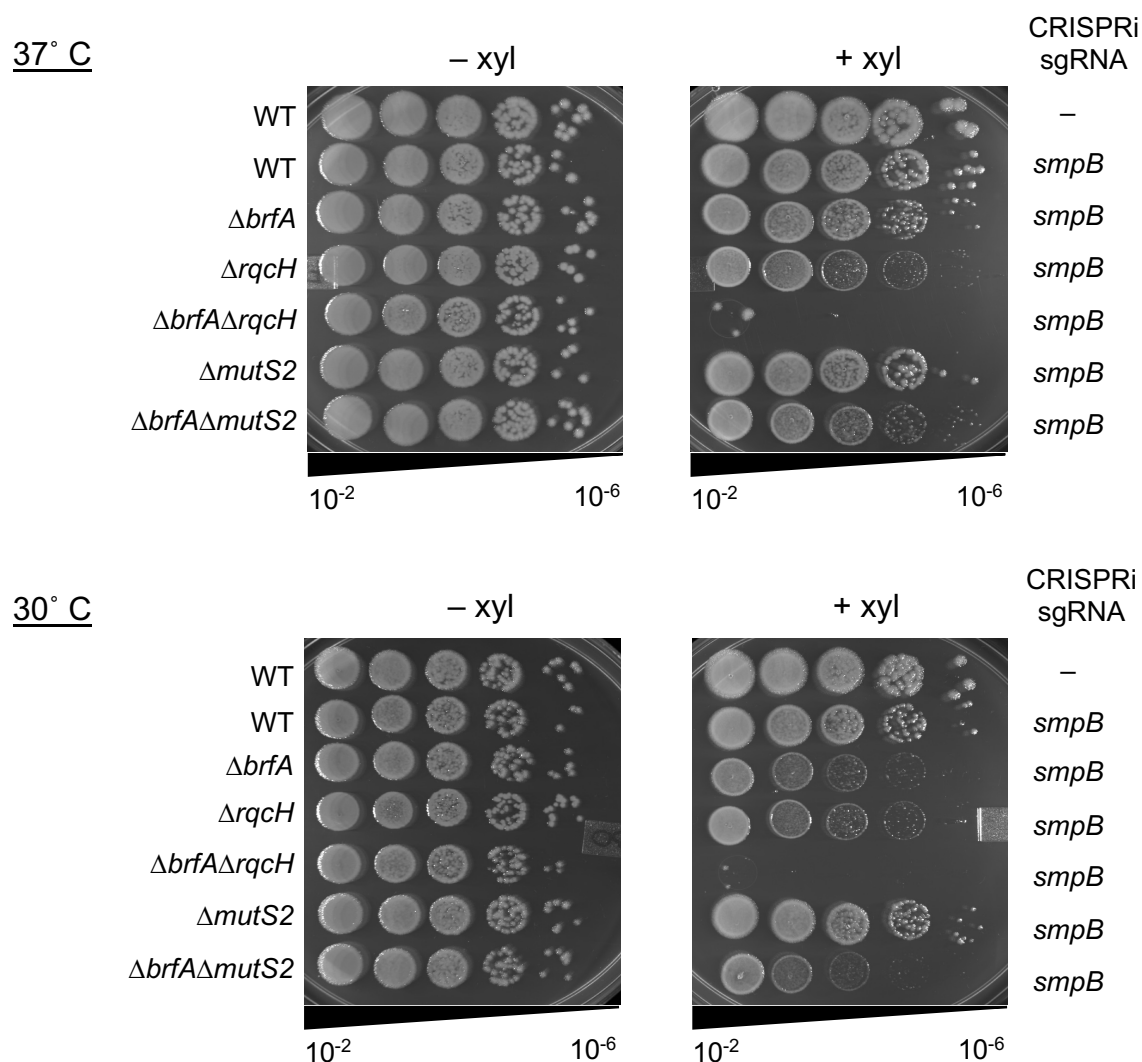
Figure 5. The RQC pathway can still function in the absence of MutS2. Strains harboring sgRNA^{smpB} and *dCas9* were serially diluted and spot plated on LB agar with or without 1% xylose at 37˚C and 30˚C. Depleting SmpB from Δ*rqcH* cells resulted in a severe growth and survival defect at both 30˚C and 37˚C. In contrast, depleting SmpB from Δ*mutS2* cells results in a modest growth defect in wild-type cells at both 37˚C and 30˚C. Similarly, depleting SmpB from Δ*brfA*Δ*mutS2*, resulted in growth and survival defects that were more severe than depleting SmpB from Δ*brfA*, but not as severe as depleting SmpB from Δ*brfA*Δ*rqcH*, indicating that RqcH is not absolutely dependent on the activity of MutS2. Image shows a representative from three independent experiments.

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