JB Accepted Manuscript Posted Online 7 November 2016
J. Bacteriol. doi:10.1128/JB.00708-16
Copyright © 2016, American Society for Microbiology. All Rights Reserved.

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

1 **Novel RpoS-dependent mechanisms strengthen the envelope permeability**
2 **barrier during stationary phase**

3

4 Angela M. Mitchell,[a] Wei Wang,[b] Thomas J. Silhavy[a]#

5

6

7 Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA[a]; Lewis-
8 Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey, USA[b]

9

10

11 Running Title: RpoS-dependent mechanisms to strengthen the envelope

12

13

14 #Address correspondence to Thomas J. Silhavy, tsilhavy@princeton.edu

15

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

Abstract

Gram-negative bacteria have effective methods of excluding toxic compounds including a largely impermeable outer membrane (OM) and a range of efflux pumps. Furthermore, when cells become nutrient-limited, RpoS enacts a global expression change providing cross-protection against many stresses. Here, we utilized sensitivity to an anionic detergent (sodium dodecyl sulfate, SDS) to model probe changes occurring to the cell's permeability barrier during nutrient limitation. *Escherichia coli* is resistant to SDS whether cells are actively growing, carbon-limited, or nitrogen-limited. In actively growing cells, this resistance depends on the AcrAB-TolC efflux pump; however, this pump is not necessary for protection in either carbon-limiting or nitrogen-limiting conditions, suggesting alternative mechanism(s) of SDS resistance. In carbon-limited cells, RpoS-dependent pathways lessen the permeability of the OM preventing the necessity for efflux. In nitrogen-limited, but not in carbon-limited cells, the loss of *rpoS* can be completely compensated for by the AcrAB-TolC efflux pump. We suggest that this difference simply reflects the fact that nitrogen-limited cells have access to a metabolizable energy (carbon) source that can efficiently power the efflux pump. Using a Tn-Seq approach, we identified three genes, *sanA*, *dacA*, and *yhdP,* necessary for RpoS-dependent SDS resistance in carbon-limited stationary phase. Using genetic analysis, we determined these genes are involved in two different envelope-strengthening pathways. These genes have not previously been implicated in stationary phase stress responses. A third novel RpoS-dependent pathway appears to strengthen the cell's permeability barrier in nitrogen-limited cells. Thus, though cells remain phenotypically SDS-resistant, SDS resistance mechanisms differ significantly between growth states.

Accepted Manuscript Posted Online
Journal of Bacteriology
JB

38

39 **Importance**

40 Gram-negative bacteria are intrinsically resistant to detergents and many antibiotics due to
41 synergistic activities of a strong outer membrane (OM) permeability barrier and efflux pumps
42 that capture and expel toxic molecules eluding the barrier. When depleted of an essential
43 nutrient, a program of gene expression is induced providing cross protection against many
44 stresses. Whether this program alters the OM to further strengthen the barrier is unknown.
45 Here, we identify novel pathways dependent on the master regulator of stationary phase
46 further strengthening the OM permeability barrier during nutrient limitation, circumventing the
47 need for efflux pumps. Decreased permeability of nutrient-limited cells to toxic compounds has
48 important implications for designing new antibiotics capable of targeting Gram-negatives that
49 may be in a growth-limited state.

50

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

51 **Introduction**

52 Antibiotic resistance in Gram-negative bacteria is a matter of increasing importance. In fact, five
53 of seven bacterial groups listed by WHO as "bacteria of international concern" are Gram-
54 negative and resistance rates for these bacteria to fluoroquinolones and third generation
55 cephalosporins have exceeded 25% to 50% in countries all over the world (100). Furthermore,
56 the outer membrane (OM) poses a significant challenge to the development of new antibiotics
57 for treatment of Gram-negative bacteria. This barrier consists of an asymmetric bilayer with an
58 inner leaflet consisting of phospholipids and an outer leaflet consisting mainly of
59 lipopolysaccharide (LPS). The tightly packed hydrophilic regions of LPS make the OM a very
60 effective permeability barrier to both large (>700 Daltons) and hydrophobic molecules
61 (reviewed in(87)). As most antibiotics must penetrate this barrier as well as the periplasm and
62 inner membrane (IM) to function, designing new antibiotics for Gram-negative bacteria has
63 been problematic (67).

64 Beyond the strong permeability barrier posed by the OM, antibiotics must also overcome the
65 actions of a wide range of efflux pumps with broad specificity (Reviewed in (49)). *Escherichia*
66 *coli* has 29 efflux pumps and putative efflux pumps of which the AcrAB-TolC pump has the
67 greatest effect on the MIC of toxic compounds (49, 91). Many of the efflux pumps (e.g. AcrAB-
68 TolC) consist of tripartite complexes with an IM pump (AcrB), a periplasmic adaptor (AcrA), and
69 an OM channel (TolC), while others are single-component pumps (e.g. EmrD) that pump
70 compounds to the periplasm instead of the extracellular environment (49). Most of the efflux
71 pumps are driven by the proton motive force although some are ATP-driven (49). While many
72 efflux pumps are constitutively expressed (e.g. *acrAB*, *emrAB*, *emrD*, *mdfA*), the expression of

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

73 others is controlled by stress responses (e.g. *mdtABC*, *mdtD*, *acrD*) or is dependent on growth
74 phase (*mdtEF*) (11, 30, 42). Thus, the combination of a largely impermeable OM and a wide
75 variety of efflux pumps make the envelope of Gram-negative bacteria a significant hurdle for
76 antibacterial activity.

77 One mechanism by which cells survive in the presence of antibiotics that penetrate the
78 envelope is persistence. Persisters are cells without resistance mutations that comprise a small
79 portion of an isogenic bacterial population, remain viable during antibiotic treatment, and can
80 generally resume growth following antibiotic treatment (reviewed in (13)). Clinically, persisters
81 have been found to be important to pathogenesis of infections including persistent
82 tuberculosis, reoccurring uropathogenic *E. coli* infections, and *Pseudomonas aeruginosa*
83 infections in cystic fibrosis patients (47, 60, 74, 85, 90). The rate of persister formation is much
84 higher in stationary phase cells than in actively growing cells (41, 44, 47, 50, 89, 94). In addition
85 to increasing rates of persister formation, stationary phase can also increase rates of resistance
86 and tolerance to antibiotics (18, 53, 61, 77, 89). Although there is an effect of decreased
87 metabolic activity in stationary phase cells, the resistance to and tolerance for antibiotics
88 observed in stationary phase cells is largely due to RpoS, the stationary phase alternative sigma
89 factor, which induces a global gene expression change in nutrient-limited cells preparing cells to
90 survive in stressful conditions for long time periods (reviewed in (62)). As it has been estimated
91 that approximately 60% of the world's biomass is made up of quiescent microbes (46),
92 understanding the changes that occur due to regulatory factors such as RpoS in these non-
93 growing microbes leading to antibiotic resistance and persistence is imperative.

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

94 Stationary phase *E. coli* cells incur changes to their morphology, metabolism, transcriptional
95 programs, and translational programs, which induce cross protection from many stresses
96 including osmotic shock, oxidative stress, heat shock, and acid and base shock (37, 38, 62, 88).
97 These changes include alternations to the cell's envelope to make it more stress resistant. For
98 example, the IM becomes more highly ordered with greater proportion of cyclopropyl fatty
99 acid derivatives and cardiolipin, the thickness and crosslinking of peptidoglycan (PG) increases,
100 and trehalose, membrane derived oligosaccharides, and other stress response factors are
101 secreted into the periplasm (Reviewed in (34, 62)). However, very little is known about changes
102 that may occur to the OM during stationary phase. It has been suggested that, during stationary
103 phase, the overall amount of protein in the OM is decreased and that the OM lipoprotein-PG
104 cross-linking increases (4, 99). However, these studies were limited by the techniques available
105 at the time of their publication. Therefore, we set out to elucidate changes that occur to the
106 cells permeability barrier during stationary phase.

107 We utilized sodium dodecyl sulfate (SDS) resistance to probe the strength of the cell's
108 permeability barrier since it does not rely on the cell's metabolism for its toxicity as many
109 antibiotics do. Previous studies have determined that SDS resistance in *E. coli* correlates well
110 with antibiotic resistance in actively growing cells with mutations in envelope biosynthesis
111 pathways (22, 51, 78, 81). As SDS correlates with antibiotic resistance in these mutants, we
112 focused on SDS resistance to assess the cell's permeability barrier thus avoiding any effects of
113 altered metabolism between different growth states. Here, we demonstrate that the
114 mechanisms of SDS resistance differ between actively growing cells, carbon-limited stationary
115 phase cells, and nitrogen-limited stationary phase cells. Furthermore, we have elucidated genes

Accepted Manuscript Posted Online

116 involved in novel *rpoS*-dependent pathways strengthening the OM in carbon-limited cells. Our
117 results highlight the decreased envelope permeability of non-growing cells, a result with
118 important implications for antibiotic design strategies targeting Gram-negative bacteria.

119

120 **Materials and Methods**

121 **Strains and growth conditions.** All strains used in this study are listed in Table S1. Strains were
122 grown at 37 °C in M63 media (86) supplemented with 1 mM $MgSO_4$ and 100 µg/mL thiamine
123 and with glucose and $(NH_4)_2SO_4$ concentrations appropriate for the growth state. Carbon-
124 limited cells were grown with 0.05% glucose and 0.2% $(NH_4)_2SO_4$, while nitrogen-limited cells
125 were grown with 0.4% glucose and 0.05% $(NH_4)_2SO_4$. Cultures for actively growing cells were
126 grown for carbon-limitation overnight then back diluted 1:200 to M63 with 0.2% glucose and
127 0.2% $(NH_4)_2SO_4$. Strains were constructed by P1*vir* transduction (86). Unless otherwise noted,
128 deletion alleles were derived from the Keio collection (9). Where indicated, the kanamycin
129 resistance cassette was removed as has been previously described (17).

130 **Detergent treatment and viability assay.** For evaluation of SDS resistance, strains were grown
131 overnight to either carbon or nitrogen limitation or were grown for 30 minutes for actively
132 growing cells. Cultures were treated with 10% SDS for a final concentration of 2% SDS or with
133 an equal volume of water for control cultures and incubated at 37 °C. Viability was assessed at
134 the indicated time points by plating on LB and counting colony-forming units. For calculation of
135 fold decrease in viability, the viability of treated samples was compared to untreated samples.
136 Unless otherwise noted, values are the average of at least three independent biological

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

137 replicates. Error bars represent the standard error of the mean (SEM). Significance was
138 calculated using the non-parametric Mann-Whitney test. We observed no indication that cell
139 number directly affects SDS sensitivity.

140 **Tn-Seq sample preparation.** A transposon mutant library was constructed from MG1655 by
141 electroporation of the EZ-Tn5<KAN-2>Tnp transposome (Epicentre) as per manufacture
142 instructions and selected on LB with 25 mg/L kanamycin. Approximately 190 000 individual
143 colonies were pooled for the initial transposon library. LB overnight cultures were grown from
144 the pooled library and used to inoculate cultures for carbon or nitrogen limitation with
145 approximately 100 copies of the library. The cultures were treated with SDS for 24 hours as
146 they were for viability analysis. Samples of $2x10^9$ cells were taken before and after SDS
147 treatment and genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) as per
148 manufacturer instructions. Libraries of transposon junctions were prepared using a method
149 based on the Transposon Directed Insertion Site sequencing (TraDIS) (45), with genomic DNA
150 randomly sheared using a Covaris sonicator. The libraries were pooled and sequenced on the
151 Illumina HiSeq 2500 sequencer in Rapid mode with 67nt single-end reads following the
152 standard manufacturer protocol. The sequencing data is available on the Princeton University
153 HTSEQ database (htseq.princeton.edu).

154 **Tn-Seq data analysis.** The sequencing reads were trimmed to 25nt and mapped to the E coli
155 K12 genome NC_000913.3 using BWA 1.2.3 (48). The number of reads mapped to each gene
156 was quantified using htseq-count 0.6.0 (http://www-
157 huber.embl.de/users/anders/HTSeq/doc/count.html). Tn-Seq reads across the genome were
158 visualized using Integrative Genomics Viewer (80, 93). The average and median reads per gene

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

159 were very similar between the different libraries (Table S2). Using the reads per gene, the log_2
160 fold change between post- and pre-treatment samples was calculated. To ensure the accuracy
161 of fold change data, only genes with at least 700 reads in one or more conditions were analyzed
162 further. Given the standard deviation of the fold values (Table S3), the frequency of transposon
163 insertions in a given gene was considered changed if the change was at least 3-fold.

164

165 **Results**

166 ***E. coli* is SDS resistant regardless of growth stage.** For many stresses, such as heat shock,
167 osmotic shock, and oxidative stress, the resistance of stationary phase *E. coli*, whether carbon
168 or nitrogen starved, is greater than that of exponentially growing cells (37, 38); therefore, we
169 hypothesized that stationary phase cells would be more resistant to SDS than actively growing
170 cells. In order to test this hypothesis, we grew MG1655 cells overnight to stationary phase in
171 minimal media with limiting concentrations of either glucose (carbon-limited) or ammonium
172 sulfate (nitrogen-limited) and treated them with 2% SDS or a vehicle control and assayed
173 viability over 24 hours. For treatment of actively growing cells, we diluted carbon-limited cells
174 into fresh media with excess carbon and nitrogen and added SDS after 30 minutes of
175 adaptation to the new conditions. In contrast to other stresses, actively growing cells showed
176 no decrease in viability or growth rate after 6 hours of SDS treatment relative to untreated cells
177 (Figure 1A). In 24 hours of 2% SDS treatment, carbon-limited cells demonstrated only a minimal
178 decrease in viability (4.9-fold) (Figure 1B) while nitrogen limited cells demonstrated no

Accepted Manuscript Posted Online

JB Journal of Bacteriology

179 decrease in viability (Figure 1C). Given the impressive resistance of the cells in all growth stages
180 to SDS, we then set out to determine the mechanism of SDS resistance in these cells.

181 **The AcrAB-TolC efflux pump is the primary mediator of SDS-resistance in actively growing**
182 **cells but not in stationary phase cells.** When the minimum inhibitory concentration (MIC) of
183 SDS is examined by growth on plates, the presence of the AcrAB-TolC efflux pump is a major
184 determinant of SDS resistance (91). This pump needs AcrA, AcrB, and TolC present in order to
185 function, although AcrA and TolC can also interact with several other IM pump components (3,
186 23, 25). Thus, we deleted *acrA* and *acrB* and examined the effect on SDS resistance of cells in
187 the three growth states. Strains lacking TolC were not used due to the pleiotropic effects of *tolC*
188 mutation on the OM (12, 19, 57, 97, 98) that would confound interpretation of results. As
189 compared to wild type cells where SDS had no effect (Figure 1A), actively growing cells with
190 deletions in *acrA* or *acrB* demonstrated a 120 to 170-fold decrease in viability in 8 h leading to a
191 4000 to 5000-fold difference in viability, respectively, between treated and non-treated cells
192 (Figure 2A). In contrast, in carbon-limited cells, the viability of *acrA* and *acrB* cells treated with
193 SDS was within 2.5-fold of that of wild type cells (Figure 2B). There was, however, a kinetic
194 difference in viability between the wild type and *acrA* or *acrB* strains. Whereas wild type cells
195 did not demonstrate a significant difference in viability between treated and untreated cells
196 until 24 hours post treatment (Figure 1B), *acrA* and *acrB* cells had a significant decrease in
197 viability starting at 1 hour post treatment (Figure 2B). Similarly, nitrogen-limited cells
198 demonstrated a small (1.8 to 2.6-fold) but significant decrease in viability starting at 1 hour post
199 treatment (Figure 2C). Nevertheless, these data illustrate that, unlike actively growing cells, the
200 AcrAB-TolC efflux pump is not the main determinant of SDS resistance in stationary phase

Accepted Manuscript Posted Online

JB Journal of Bacteriology

201 suggesting that stationary phase cells must employ a different mechanism of resistance,
202 perhaps involving strengthening their envelope permeability barrier.

203 **SDS resistance in carbon-limited cells requires an RpoS-dependent mechanism.** There are two
204 possible explanations for SDS resistance in stationary phase. First, the non-growing state
205 induced by lack of nutrients might be directly responsible for the increase in envelope
206 resistance through limitation of cell division or metabolic activity. Second, a specific change in
207 gene expression in stationary phase may produce the increase in resistance through activation
208 of protective pathways. RpoS drives a change in gene expression in stationary phase that
209 affects, directly or indirectly, 10% of the genome (62). Therefore, we investigated the SDS
210 resistance of *rpoS* deletion cells in the three growth phases to determine whether a specific
211 change in gene expression was necessary for stationary phase SDS resistance. In actively
212 growing cells, deletion of *rpoS* had no effect on the SDS resistance (Figure 3A); however, in
213 carbon-limited cells, *rpoS* deletion led to a 940-fold decrease in viability in 24 hours in SDS
214 treated cells demonstrating that *rpoS* is required for SDS resistance in carbon-limited cells
215 (Figure 3B). These data suggest RpoS-dependent pathways activated in carbon-limited cells are
216 responsible for the SDS resistance of carbon-limited cells. Surprisingly, in nitrogen-limited cells,
217 deletion of *rpoS* had no effect on the viability of SDS treated cells (Figure 3C). These data
218 suggest that RpoS is the main determinant of SDS resistance in carbon-limited cells but not in
219 actively growing cells, which are protected by efflux, or in nitrogen-limited cells. This is
220 interesting considering that the levels of RpoS are much lower in nitrogen-limited cells than in
221 carbon-limited cells and are lower still in exponentially growing cells (52).

Journal of Bacteriology

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

222 We next investigated whether the lack of protective effect of RpoS in exponentially growing
223 cells, demonstrated by a decrease in viability with only *acrA* or *acrB* deletion, was due to the
224 low levels of RpoS present in these cells or to protective mechanisms activated by RpoS which
225 can only operate in stationary phase cells. To investigate these possibilities, we utilized *sprE*
226 deletion strains. SprE is responsible for the ClpXP-dependent degradation of RpoS during
227 exponential phase and deleting *sprE* increases RpoS levels in both exponential cells and
228 stationary phase cells (73). In actively growing cells, deletion of *sprE* alone did not cause any
229 SDS sensitivity; however, *acrA sprE* deletion cells were significantly more resistant to SDS than
230 were *acrA* deletion cells (Figure 4A). Deletion of *sprE* was also protective in carbon-limited cells
231 alone or in combination with *acrA* deletion, but was not protective in *rpoS* deletion cells (Figure
232 4B). These data demonstrate that RpoS-dependent mechanisms can be protective in actively
233 growing cells and suggest that wild type levels of RpoS in these cells are too low to be
234 protective. These data also illustrate that increasing RpoS levels in carbon-limited cells can
235 further increase SDS-resistance, suggesting that the effect of RpoS on SDS resistance is
236 dependent on RpoS levels.

237 **In nitrogen-limited cells, the AcrAB-TolC efflux pump can compensate for the loss of *rpoS*-**
238 **dependent resistance mechanisms.** As we had found AcrAB-TolC to be the main determinant of
239 SDS-resistance in actively growing cells and RpoS to be the main determinant of SDS-resistance
240 in carbon-limited cells, we then investigated the effect of simultaneously removing these two
241 factors. Therefore, we examined the SDS resistance of *acrA rpoS* and *acrB rpoS* double mutants
242 in the three growth states. In actively growing cells, *acrA rpoS* and *acrB rpoS* double mutants
243 were even more sensitive to SDS than *acrA* or *acrB* single mutants and viability decreased to

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

244 near our limit of detection by 3 hours of treatment and below our level of detection by 5 hours
245 of treatment (Figure 5A). These results emphasize that, although RpoS-dependent mechanisms
246 can be protective in actively growing cells allowing some remaining viability without the AcrAB-
247 TolC efflux pump, levels of RpoS are too low to be fully protective. Carbon-limited double
248 mutant cells were also more sensitive to SDS than single mutant cells, with viability decreasing
249 to near or below our limit of detection in 24 hours (Figure 5B). These data demonstrate that
250 AcrAB-TolC can play a protective role in carbon-limited cells in the absence of RpoS but that
251 AcrAB-TolC cannot compensate for the loss of RpoS in these cells. The lack of full protection by
252 the AcrAB-TolC efflux pump in carbon-limiting conditions suggests that the RpoS-dependent
253 mechanisms of SDS resistance must be preventing the SDS from penetrating the OM. In
254 nitrogen-limiting conditions, *acrA rpoS* and *acrB rpoS* double mutant cells were SDS-sensitive
255 with 880 000-fold and 4000-fold decreases in viability, respectively, at 7 hours post treatment
256 followed by suppressor mutant outgrowth (Figure 5C). These data combined with the lack of
257 SDS sensitivity in *rpoS*, *acrA*, or *acrB* mutants (Figures 2C & 3C) demonstrate that, in nitrogen-
258 limiting condition, AcrAB-TolC and RpoS-dependent protective mechanisms are fully
259 functioning and that each can fully compensate for the loss of the other. Thus, cells in all three
260 growth phases can be protected by both RpoS-dependent mechanisms and by efflux but the
261 dominance of each mechanism depends on the growth conditions.

262 **Tn-Seq identifies genes causing SDS sensitivity in carbon-limited but not nitrogen-limited**
263 **conditions.** Given the global changes enacted by RpoS in stationary phase (62), we wished to
264 know which RpoS-regulated genes were protecting cells from SDS in stationary phase. In order
265 to address this question, we utilized a Tn-Seq experiment (96). We created a pooled library of

Journal of Bacteriology

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

266 approximately 190,000 mutants each containing an Ez-Tn5 insertion, grew this library overnight
267 to stationary phase in either nitrogen-limiting or carbon-limiting conditions, and collected
268 genomic DNA samples from these cultures before and after 24 hours of 2% SDS treatment
269 (Figure 6A). We isolated the transposon junctions from these mutants and subjected them to
270 deep sequencing to determining the frequency of transposon junctions throughout the genome
271 in the various samples. An example region of the genome is shown in Figure 6B. The average
272 and median number of reads per gene across the library were very similar between pre- and
273 post-treatment samples for both carbon- and nitrogen-limiting conditions (Table S2).

274 In analyzing the resulting data, we took advantage of the differences in SDS resistance
275 mechanisms between carbon- and nitrogen-limiting conditions to distinguish between
276 mutations that cause non-specific envelope defects and those that cause defects in the *rpoS*-
277 dependent mechanism of SDS resistance in stationary phase. Based on our data (Figures 2C, 3C,
278 & 5C), causing SDS sensitivity through the *rpoS*-dependent pathway in nitrogen-limited cells
279 requires at least two mutations: inactivation of the AcrAB-TolC efflux pump and inactivation of
280 a component of the *rpoS*-dependent pathway. In contrast, one mutation that affects envelope
281 biogenesis in a non-specific manner in an *rpoS*-independent pathway could cause SDS
282 sensitivity in N-limited cells. Therefore, to enrich for genes important for the *rpoS*-dependent
283 pathway of SDS sensitivity, we identified genes for which the frequency sequencing reads
284 decreased in the post-treatment carbon-limited sample as compared to the pre-treatment
285 carbon-limited sample but did not change between the nitrogen-limited samples, utilizing these
286 samples as a control for genes where disruption causes envelope defects unrelated to RpoS.
287 We chose a minimum of a 3-fold difference between the pre- and post-treatment samples to

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

288 define a change as this value defined the edges of the main population of the reads (Table S3
289 and Figure S1). An example gene that fit these parameters, *sanA*, is shown in Figure 6B.

290 Overall, we identified 12 envelope related genes (Table 1) and 8 non-envelope related genes
291 (Table S4) that had at least a 3-fold decrease in reads in carbon-limiting conditions and less than
292 a 3-fold change in reads in nitrogen-limiting conditions. We then proceeded to examine the
293 effect of the envelope related genes on SDS resistance in carbon-limiting conditions in a non-
294 competitive environment. *ftsN*, which is an essential gene, for which we identified transposon
295 junctions in the non-essential 3′ region, was not examined further. In single deletion settings,
296 we were able to confirm the SDS sensitivity of five of the examined genes: *rfaH*, *sanA*, *dacA*,
297 *yhdP*, and *ydgH* (Figure 6C). Of these genes, *rfaH*, *sanA*, and *dacA* demonstrated the largest
298 effects on SDS resistance causing 100- to 200-fold decreases in viability in 24 hours with SDS
299 treatment. The two other genes, *yhdP* and *ydgH*, had smaller but significant effects on viability
300 with SDS treatment, demonstrating 23-fold and 18-fold decreases in viability in 24 hours of
301 treatment, respectively. We considered these five genes to be candidates for participants in the
302 *rpoS*-dependent pathway protecting carbon-limited cells from SDS. Interestingly, *wecE* deletion
303 had no effect on SDS sensitivity in carbon-limited cells in a non-competitive environment,
304 despite causing SDS sensitivity in actively growing cells due to accumulation of lipid II bound to
305 enterobacterial common antigen (ECA) (Figure S2, (16)). These data emphasize the increase in
306 envelope strength that occurs in nutrient limited cells, as they are able to overcome the effect
307 of mutations that cause envelope permeability in actively growing cells.

308 **RpoS acts through *sanA*, *dacA*, and *yhdP* to mediate SDS resistance in stationary phase.** To
309 determine whether the genes from the Tn-Seq that cause SDS sensitivity in carbon-limiting

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

310 conditions were on-pathway with *rpoS*, we constructed double mutants of these genes with
311 *rpoS* and with *acrA* to differentiate between additive and non-additive effects of these
312 mutations. For three of the genes, *yhdP*, *dacA*, and *sanA*, the effects of their deletion was not
313 additive with *rpoS* (Figure 7A left panel). These data suggest that RpoS acts through *sanA*, *dacA*,
314 and *yhdP* in order to make carbon-limited cells SDS resistant. Combining *rpoS* and *acrA*
315 deletions leads to a very strong effect on SDS resistance; in fact, the viability of SDS treated
316 cultures is below the limit of detection after 24 hours of treatment (Figure 5B). When we
317 combined deletions in the three genes we identified with *acrA*, only the *acrA sanA* strain
318 recapitulated the strong synthetic phenotype effect of an *acrA rpoS* deletion with viability
319 decreasing to near the limit of deletion in 24 hours (Figure 7A left panel). These data suggest
320 that *sanA* plays a dominant role in RpoS-dependent SDS resistance.

321 In contrast to the three genes mentioned above, *rpoS* mutant cells demonstrated additive SDS
322 sensitivity when combined with *rfaH* and *ydgH* mutations (Figure 7A right panel). As *rfaH* is
323 required for efficient LPS biosynthesis (10, 15, 58, 72), it is likely that *rfaH* mutants have OM
324 permeability unrelated to the pathway affected by RpoS. YdgH is predicted to be a periplasmic
325 protein and its levels have been suggested to be downregulated by MicA, a sRNA that increases
326 upon entry to stationary phase but is transcriptionally regulated by sigma E, the envelope stress
327 sigma factor (6, 28, 40, 70). Thus, YdgH may play a role in stationary phase envelope
328 permeability while not being directly regulated by RpoS.

329 As *sanA*, *dacA*, and *yhdP* appear to work downstream of *rpoS* to mediate SDS resistance in
330 carbon-limited cells, we then wondered whether they work together on the same pathway or
331 through several different mechanisms to mediate SDS resistance. To address this question, we

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

332 constructed all possible double and triple deletion mutants of these genes and determined the
333 effect of these mutations on SDS sensitivity in carbon-limiting conditions. In combination, a
334 *dacA yhdP* double mutant did not show an additive effect over that of either mutant alone,
335 while *sanA* demonstrated an additive effect with both *dacA* and *yhdP* (Figure 7B). These data
336 suggest that *dacA* and *yhdP* work together to mediate SDS resistance in carbon-limiting
337 conditions, while *sanA* works through a separate *rpoS*-dependent pathway. These data
338 correlate well with our *acrA* data which suggest that *sanA* is playing a more important role in
339 strengthening the OM than are *dacA* and *yhdP*. Interestingly, the *yhdP dacA sanA* triple mutant
340 had a similar level of SDS sensitivity to that of *rpoS* deletion cells with a decrease in viability in
341 24 hours of more than 900-fold. In contrast, in nitrogen limiting conditions, *sanA*, *dacA*, and
342 *yhdP* had no effect on SDS sensitivity even when combined with an *acrA* mutation (Figure 7C),
343 suggesting that there may be a third *rpoS*-dependent mechanism of SDS resistance operating
344 specifically in nitrogen-limiting conditions. Taken together, all of these data demonstrate that
345 the mechanisms of SDS resistance vary greatly depending on growth conditions and involve
346 several novel *rpoS*-dependent mechanisms for strengthening the envelope permeability barrier.

347

348 **Discussion**

349 In stationary phase, RpoS enacts a global program of transcriptional regulation that prepares
350 cells for stressful conditions (62). In this work, we have investigated changes occurring to the
351 cells permeability barrier during times of nutrient limitation, using SDS resistance as a model of
352 the strength of the envelope permeability barrier. Gene deletions causing SDS and SDS EDTA

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

353 sensitivity in *E. coli* have previously been investigated in a high-throughput study (65); however,
354 the study examined colony size on plates and so did not differentiate between different growth
355 states. Several studies examining the SDS resistance of *Enterobacteria* have touched on the SDS
356 resistance of *E. coli* (2, 7, 8, 75, 76). However, the studies that examined growth phase-
357 dependent effects were conducted in a W3110 background (7, 8), which has mutations in
358 several stress response pathways including the Rcs pathway and, depending on the source, may
359 lack functional RpoS and/or RpoF (31, 39). In addition, no genes involved in stationary phase
360 SDS resistance were identified.

361 Therefore, we investigated the SDS resistance of wild type MG1655. We have determined that,
362 although actively growing cells, carbon-limited cells, and nitrogen-limited cells are all resistant
363 to SDS (Figure 1), the mechanisms of SDS resistance deployed depend on the growth phase of
364 the cells. In actively growing cells, cells are mainly protected from SDS by the AcrAB-TolC efflux
365 pump (Figure 2). These data suggest that, in these cells, SDS penetrates the OM and then
366 AcrAB-TolC pumps it back out through an energy dependent process (Figure 8A). Interestingly,
367 it has been previously suggested that SDS could be found in the *E. coli* periplasm during SDS
368 treatment (1, 66). Although *rpoS* can play a role in SDS resistance in these cells, it is not the
369 dominant mechanism of resistance employed and is not fully protective (Figures 4 & 5). With
370 ample carbon and nitrogen available, the cells instead rely on the energy intensive process of
371 efflux, which in this case, is driven by the proton motive force (49) (Figure 8A).

372 In carbon-limited cells, the resistance to SDS is primarily dependent on *rpoS* (Figure 3). Our
373 model for these data suggests that RpoS acts to activate pathways of SDS resistance in carbon-
374 limited cells, which prevent SDS from entering the cell by strengthening the permeability

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

375 barrier of the OM (Figure 8B). In the absence of *rpoS*, the cells can be minimally protected by
376 the AcrAB-TolC efflux pump; however, this pump cannot fully protect the cells in the absence of
377 *rpoS* likely due to a lack of energy (Figure 8B). This model explains the kinetic difference in SDS
378 resistance we observed between AcrAB-TolC deficient cells and wild type cells in carbon-
379 limiting conditions wherein decreases in viability were observed at earlier time points for *acrA*
380 and *acrB* cells than for wild type cells (Figure 1B vs Figure 2B), despite equal levels of RpoS
381 present in these cells (Figure S3). It is likely carbon-limited cells can efflux SDS for a brief time,
382 increasing their SDS resistance, but are quickly depleted of protons with which to run efflux
383 resulting in low levels of cell death. This cell death would occur more quickly in cells in which
384 the efflux pump has been removed. Furthermore, *rpoS* deletion cells exhibit significant cell
385 death following SDS treatment even when the AcrAB-TolC efflux pump is present. This inability
386 of efflux to protect carbon-limited cells from SDS emphasizes that RpoS-dependent SDS
387 resistance must involve strengthening the OM permeability barrier preventing the necessity for
388 efflux.

389 We have identified three genes, *sanA*, *dacA*, and *yhdP*, for which deletion decreases SDS
390 resistance in carbon-limited cells and for which the effect of deletion is not additive with *rpoS*
391 (Figure 7). Deletion of these genes has no effect on the levels of RpoS in carbon-limited cells
392 (Figure S3). These data suggest that RpoS acts through SanA, PBP5 (DacA), and YhdP to provide
393 SDS resistance in carbon-limited cells (Figure 8B). Given the strong synthetic phenotype
394 observed with *sanA* and *acrA* deletions (Figure 7), our model suggest that RpoS is working
395 primarily through SanA to strengthen the OM in carbon-limiting conditions. It remains possible
396 that PBP5 and YhdP act to decrease SDS sensitivity by altering some other aspect of the cell's

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

397 envelope (e.g. PG or IM). It is of interest, since RpoS can be protective in exponentially growing
398 cells in the absence of AcrAB-TolC, that deletion of *sanA*, *dacA*, or *yhdP* can increase SDS
399 sensitivity in exponentially growing cultures, although this sensitivity cannot be observed for
400 *sanA* or *dacA* in an efficiency of plating assay on plates with SDS suggesting the exponential
401 phase effect is small (Figure S4). As these genes have no effect in nitrogen-limiting conditions
402 (Figure 7), these data suggest that other regulatory factors, in addition to RpoS, may allow
403 these genes to provide a basal level of protection in actively growing cells. Overall, our data
404 suggest that, in carbon-limited cells, RpoS acts directly or indirectly to activate pathways that
405 strengthen the OM permeability barrier and involve SanA, PBP5, and YhdP (Figure 8B).

406 SanA was first identified as a multicopy suppressor of a unknown mutant with an OM
407 permeability defect, which had a deletion of *sanA* as well as other mutations leading to OM
408 defects (79). SanA has 239 amino acids and is predicted to have an inner membrane localization
409 with a very small N-terminal cytoplasmic domain (6 amino acids), one transmembrane helix,
410 and the remainder of the protein localized in the periplasm (43, 70). The periplasmic domain of
411 SanA contains a DUF218 domain (24). DUF218 domains contain several charged amino acids
412 suggesting enzymatic activity (24) and are found in many species throughout the bacterial
413 domain as well as in some archaea, plants, and fungi, mainly in proteins of unknown function
414 (24).

415 *E.coli* has three homologs of SanA containing DUF218 domains: YgjQ, YdcF, and ElyC (14, 24).
416 YgjQ has no known function though it is predicted to have the same topology as SanA (43, 70).
417 YdcF is predicted to be a cytoplasmic protein, which binds to S-adenosyl-L-methionine, and has
418 been suggested to be directly or indirectly regulated by both the Rcs response and FNR (14, 27,

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

419 43, 82). A deletion mutant of *elyC* (*ycbC*), but not *sanA*, was identified through a high-
420 throughput screen as causing cell lysis at room temperature in LB media with 1% salt (68).
421 Suppressor and complementation assays suggested that ElyC may be involved in balancing
422 undecaprenyl-phosphate (Und-P) use between PG biosynthesis and the biosynthesis of
423 polysaccharides such as ECA (68). Supporting this role, deletions of genes involved in
424 biosynthesis of ECA, a glycolipid with trimeric repeats of N- acetyl-D-glucosamine (GlcNAc), N-
425 acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose
426 (Fuc4NAc), have a suppressive or synthetic phenotype on *elyC* deletion depending on Und-P
427 utilization of the mutants. Thus, deletion of *wecA* acts as a suppressor of lysis with *elyC* deletion
428 as it prevents Und-P use for ECA synthesis by preventing the formation of Und-P-P-GlcNAc
429 (Lipid I^{ECA}), while deletion of *wecE* has a synthetic phenotype with *elyC* deletion as it causes the
430 buildup of Und-P-P-GlcNAc-ManNAcA (Lipid II^{ECA}), preventing the use of Und-P for PG
431 biosynthesis (16, 55, 68). Although these proteins contain DUF218 domains similar to SanA,
432 deletion of *ygjQ*, *ydcF*, or *elyC* had no effect on the SDS sensitivity of carbon-limited cells (Figure
433 S5). In addition, mutation of *wecA* or *wecE* in a *sanA* deletion strain had no effect on SDS
434 sensitivity (Figure S5), demonstrating that alteration of Und-P levels available for PG
435 biosynthesis has no effect on the SDS sensitivity of a *sanA* mutant. These data suggest that
436 SanA plays a different role in strengthening the OM permeability barrier than those played by
437 its paralogs.

438 Deletion of both *sanA* and the *Salmonella* homolog of *sanA*, *sfiX*, has been shown to confer
439 vancomycin sensitivity at 43 °C but not 42 °C, suggesting a temperature-dependent effect of
440 SanA (59, 79). In addition, *sanA* is annotated to have a heat shock-dependent RpoH promoter

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

441 (33). This suggests that, as the *rpoH* promoter can be utilized by RpoS and the levels of RpoH
442 increase upon carbon starvation (35, 36), the effect of RpoS on SanA may be indirect through
443 RpoH. The function of SanA in strengthening the OM remains an open question but our data
444 suggest that SanA is acting independently of PBP5 and YhdP through a mechanism unique from
445 those of its homologs (Figure 8B). As an *acrA sanA* strain shows a strong synergistic effect on
446 SDS sensitivity unlike *acrA dacA* and *acrA yhdP* (Figure 7), the RpoS-dependent pathway
447 involving SanA appears to be the dominant pathway strengthening the OM in carbon-limiting
448 conditions. We are currently investigating this pathway.

449 The second envelope related gene we identified to be involved with the RpoS-dependent
450 mechanisms for decreasing envelope permeability in stationary phase is PBP5. PBP5 is a D-
451 alanyl-D-alanine carboxypeptidase (DD-CPase) that trims the fifth amino acid from the PG chain
452 after the precursor has been polymerized into the cell wall and is thought to be involved with
453 PG remodeling (5, 26, 95). Thus, the PG of exponential phase cells with a deletion of *dacA* has a
454 4-fold increase in pentapeptides as compared to PG of wild type cells (71). PBP5 is under the
455 transcriptional control of BolA, the stationary phase morphogene, suggesting that its link to
456 RpoS is likely mediated by BolA (64, 83). *E. coli* has eight proteins (PBP4, PBP4b, PBP5, PBP6,
457 PBP6b, PBP7, AmpC, and AmpH) capable of removing the terminal D-alanine from PG chains
458 and removal of more than one of them is necessary to cause visible morphological defects (29,
459 54, 63, 95). In fact, cells remain viable when grown in LB after deletion of the genes for at least
460 seven out of the eight proteins responsible for this activity (21). The different proteins with this
461 activity are thought to act in different conditions. For instance, during active growth in neutral
462 conditions PBP5 is the main protein responsible for DD-CPase activity, while at a pH of 5 PBP6b

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

463 is mainly responsible for DD-CPase activity (69). In addition, while PBP5 is most active in early
464 log phase, PBP6 and PBP6b are thought to be most active in mid-log and stationary phase (83,
465 84). It is interesting that, although PBP5 is not the DD-CPase most active in stationary phase, it
466 is involved in mediating SDS resistance in carbon-limiting conditions. We are currently
467 investigating the possibility that PBP5 activity in carbon-limiting conditions acts as a signal for
468 an envelope-strengthening pathway. As the effects of *yhdP* and *dacA* are not additive, we
469 hypothesize that this pathway involves YhdP, the third gene through which RpoS acts to
470 decrease envelope permeability during carbon-limiting conditions.

471 YhdP, like SanA, is predicted to be an IM protein with a small N-terminal cytoplasmic domain (6
472 amino acids), one transmembrane helix, and a large periplasmic domain (amino acids 30
473 through 1266) (43, 70). Homologs of YhdP are common *Gammaproteobacteria* and are also
474 found in *Betaproteobacteria* (92). YhdP's periplasmic domain contains a DUF3971 domain and
475 an AsmA_2 domain, which is similar to the C-terminal domain of AsmA (24). AsmA has been
476 implicated in the assembly of outer membrane porins (20, 56, 101). We are currently
477 investigating whether YhdP may play a similar role. As *yhdP* does not have an annotated
478 promoter, it is unclear whether RpoS acts directly or indirectly on YhdP. Interestingly, YhdP was
479 suggested through a high-throughput screen to bind to YdgH (32), which also affects SDS
480 resistance in carbon-limiting conditions although not in an RpoS-dependent manner (Figures 7).
481 A double mutant of *yhdP* and *ydgH* was quite sensitive to SDS in carbon limiting conditions
482 (Figure S6) and we are further investigating the possibility of functional interactions between
483 these proteins.

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

484 In contrast to carbon-limiting conditions, nitrogen-limited cells lacking either *rpoS* or *acrA/B*
485 alone are resistant to SDS (Figures 2 & 3); instead, both *rpoS* and *acrA* or *acrB* deletions are
486 needed in order to cause SDS sensitivity. These data suggest that, in wild type cells, RpoS-
487 dependent mechanisms prevent SDS entry in to nitrogen-limited cells but that when *rpoS* is
488 removed the AcrAB-TolC efflux pump can compensate for the loss by pumping SDS out of the
489 cell (Figure 8C). Moreover, the ability of AcrAB-TolC to compensate for loss of RpoS suggests
490 that the nitrogen-limited cells are not energy limited likely because of the inhibitory effect of
491 nitrogen-limitation on protein synthesis, a highly energy-dependent process (Figure 8C).
492 Intriguingly, deletion of *acrA* and *sanA*, *dacA*, or *yhdP* does not cause SDS sensitivity in nitrogen-
493 limited cells (Figure 7), suggesting that the mechanism of RpoS-dependent SDS resistance
494 present in nitrogen-limited cells is different than that found in carbon-limited cells (Figure 8C).
495 We are currently investigating this mechanism, which may involve efflux and/or strengthening
496 the OM's or IM's resistance to SDS. Our data for SDS resistance are in contrast to other stress
497 such as heat shock, osmotic stress, and oxidative stress where RpoS plays the same role in
498 carbon- and nitrogen-limited cells (37, 38). Thus, SDS resistance is an interesting model for
499 investigating the effects of varying RpoS levels found with different nutrient limitations (52).

500 In summary, we have demonstrated that mechanisms of SDS resistance differ between cells in
501 different growth states. Whereas actively growing cells rely on efflux, stationary phase cells
502 utilize RpoS-dependent mechanisms to strengthen their envelope permeability barrier and may
503 play a role in the persistence, tolerance, and resistance to antibiotics observed during
504 stationary phase. As the vast majority of microbes in the environment are in a quiescent state
505 (46), insights into changes to the cell's permeability barrier that occur in non-growing cells have

Accepted Manuscript Posted Online

JB Journal of Bacteriology

506 important implications for the design of new antibiotics targeting Gram-negative bacteria for
507 which envelope permeability is a major hurtle. Furthermore, we identified several novel RpoS-
508 dependent pathways through which the cell's envelope permeability barrier can be
509 strengthened. Further investigation of these pathways should lead to insights into the biology
510 of the cell's envelope permeability barrier.

511

512 **Acknowledgments**

513 We thank members of the Silhavy laboratory for productive discussions. We also thank Jessica
514 Wiggins at the Princeton University Genomics Core Facility for preparing libraries and
515 performing sequencing for the Tn-Seq experiment. This work was funded by the National
516 Institute of General Medical Science Grants GM065216 and GM118024 and by a National
517 Institute of General Medical Science Fellowship F32GM116188.

518

519

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

520 **References**

521 1. **Adamowicz, M., T. Conway, and K. W. Nickerson.** 1991. Nutritional complementation
522 of oxidative glucose metabolism in *Escherichia coli* via pyrroloquinoline quinone-
523 dependent glucose dehydrogenase and the Entner-Doudoroff pathway. Applied and
524 Environmental Microbiology **57:**2012-2015.
525 2. **Adamowicz, M., P. M. Kelley, and K. W. Nickerson.** 1991. Detergent (sodium dodecyl
526 sulfate) shock proteins in *Escherichia coli*. Journal of Bacteriology **173:**229-233.
527 3. **Aires, J. R., and H. Nikaido.** 2005. Aminoglycosides are captured from both periplasm
528 and cytoplasm by the AcrD multidrug efflux transporter of *Escherichia coli*. Journal of
529 Bacteriology **187:**1923-1929.
530 4. **Allen, R. J., and G. K. Scott.** 1979. Biosynthesis and turnover of outer-membrane
531 proteins in *Escherichia coli* ML308-225. Biochem J **182:**407-12.
532 5. **Amanuma, H., and J. L. Strominger.** 1980. Purification and properties of penicillin-
533 binding proteins 5 and 6 from *Escherichia coli* membranes. Journal of Biological
534 Chemistry **255:**11173-11180.
535 6. **Argaman, L., R. Hershberg, J. r. Vogel, G. Bejerano, E. G. H. Wagner, H. Margalit, and S.
536 Altuvia.** 2001. Novel small RNA-encoding genes in the intergenic regions of *Escherichia
537 coli*. Current Biology **11:**941-950.
538 7. **Aspedon, A., and K. W. Nickerson.** 1994. The energy dependence of detergent
539 resistance in *Enterobacter cloacae*: a likely requirement for ATP rather than a proton
540 gradient or a membrane potential. Canadian Journal of Microbiology **40:**184-191.

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

541 8. **Aspedon, A., and K. W. Nickerson.** 1993. A two-part energy burden imposed by growth
542 of *Enterobacter cloacae* and *Escherichia coli* in sodium dodecyl sulfate. Can J Microbiol
543 **39:**555-61.
544 9. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M.**
545 **Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame,
546 single-gene knockout mutants: the Keio collection. Molecular Systems Biology
547 **2:**2006.0008-2006.0008.
548 10. **Bailey, M. J. A., V. Koronakis, T. Schmoll, and C. Hughes.** 1992. *Escherichia coli* HlyT
549 protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by
550 the rfaH (sfrB) locus required for expression of sex factor and lipopolysaccharide genes.
551 Molecular Microbiology **6:**1003-1012.
552 11. **Baranova, N., and H. Nikaido.** 2002. The BaeSR two-component regulatory system
553 activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in
554 *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. Journal of
555 Bacteriology **184:**4168-4176.
556 12. **Bleuel, C., C. Große, N. Taudte, J. Scherer, D. Wesenberg, G. J. Krauß, D. H. Nies, and G.**
557 **Grass.** 2005. TolC is involved in enterobactin efflux across the outer membrane of
558 *Escherichia coli*. Journal of Bacteriology **187:**6701-6707.
559 13. **Brauner, A., O. Fridman, O. Gefen, and N. Q. Balaban.** 2016. Distinguishing between
560 resistance, tolerance and persistence to antibiotic treatment. Nat Rev Micro **14:**320-
561 330.

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

562 14. **Chao, K. L., K. Lim, C. Lehmann, V. Doseeva, A. J. Howard, F. P. Schwarz, and O.
563 Herzberg.** 2008. The *Escherichia coli* YdcF binds S-adenosyl-L-methionine and adopts an
564 α/β-fold characteristic of nucleotide-utilizing enzymes. Proteins: Structure, Function,
565 and Bioinformatics **72:**506-509.

566 15. **Creeger, E. S., T. Schulte, and L. I. Rothfield.** 1984. Regulation of membrane
567 glycosyltransferases by the sfrB and rfaH genes of *Escherichia coli* and *Salmonella*
568 *typhimurium*. Journal of Biological Chemistry **259:**3064-3069.

569 16. **Danese, P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998.
570 Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate
571 stimulates *degP* transcription in *Escherichia coli*. Journal of Bacteriology **180:**5875-5884.

572 17. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes
573 in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of
574 Sciences **97:**6640-6645.

575 18. **Davey, P., M. Barza, and M. Stuart.** 1988. Tolerance of *Pseudomonas aeruginosa* to
576 killing by ciprofloxatin, gentamicin and imipenem in vitro and in vivo. Journal of
577 Antimicrobial Chemotherapy **21:**395-404.

578 19. **Deininger, K. N. W., A. Horikawa, R. D. Kitko, R. Tatsumi, J. L. Rosner, M. Wachi, and J.
579 L. Slonczewski.** 2011. A Requirement of TolC and MDR Efflux Pumps for Acid Adaptation
580 and GadAB Induction in *Escherichia coli*. PLoS One **6:**e18960.

581 20. **Deng, M., and R. Misra.** 1996. Examination of AsmA and its effect on the assembly of
582 *Escherichia coli* outer membrane proteins. Molecular Microbiology **21:**605-612.

Accepted Manuscript Posted Online

JB Journal of Bacteriology

583 21. **Denome, S. A., P. K. Elf, T. A. Henderson, D. E. Nelson, and K. D. Young.** 1999.
584 *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding
585 proteins: Viability, characteristics, and implications for peptidoglycan synthesis. Journal
586 of Bacteriology **181:**3981-3993.
587 22. **Dwyer, R. S., D. P. Ricci, L. J. Colwell, T. J. Silhavy, and N. S. Wingreen.** 2013. Predicting
588 functionally informative mutations in *Escherichia coli* BamA using evolutionary
589 covariance analysis. Genetics **195:**443-455.
590 23. **Elkins, C. A., and H. Nikaido.** 2003. Chimeric analysis of AcrA function reveals the
591 importance of its C-terminal domain in its interaction with the AcrB multidrug efflux
592 pump. Journal of Bacteriology **185:**5349-5356.
593 24. **Finn, R. D., A. Bateman, J. Clements, P. Coggill, R. Y. Eberhardt, S. R. Eddy, A. Heger, K.**
594 **Hetherington, L. Holm, J. Mistry, E. L. L. Sonnhammer, J. Tate, and M. Punta.** 2014.
595 Pfam: the protein families database. Nucleic Acids Research **42:**D222-D230.
596 25. **Ge, Q., Y. Yamada, and H. Zgurskaya.** 2009. The C-terminal domain of AcrA is essential
597 for the assembly and function of the multidrug efflux pump AcrAB-TolC. Journal of
598 Bacteriology **191:**4365-4371.
599 26. **Ghosh, A. S., C. Chowdhury, and D. E. Nelson.** 2008. Physiological functions of D-alanine
600 carboxypeptidases in *Escherichia coli*. Trends in Microbiology **16:**309-317.
601 27. **Hagiwara, D., M. Sugiura, T. Oshima, H. Mori, H. Aiba, T. Yamashino, and T. Mizuno.**
602 2003. Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB
603 phosphorelay system in *Escherichia coli*. Journal of Bacteriology **185:**5735-5746.

Journal of Bacteriology

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

604 28. **Hammann, P., D. Parmentier, M. Cerciat, J. ReimegÃ¥rd, A.-C. Helfer, S. Boisset, M.
605 Guillier, F. o. Vandenesch, E. G. H. Wagner, P. Romby, and P. Fechter.** 2014. A method
606 to map changes in bacterial surface composition induced by regulatory RNAs in
607 *Escherichia coli* and *Staphylococcus aureus*. Biochimie **106:**175-179.

608 29. **Henderson, T. A., K. D. Young, S. A. Denome, and P. K. Elf.** 1997. AmpC and AmpH,
609 proteins related to the class C beta-lactamases, bind penicillin and contribute to the
610 normal morphology of *Escherichia coli*. Journal of Bacteriology **179:**6112-21.

611 30. **Hirakawa, H., Y. Inazumi, T. Masaki, T. Hirata, and A. Yamaguchi.** 2005. Indole induces
612 the expression of multidrug exporter genes in *Escherichia coli*. Molecular Microbiology
613 **55:**1113-1126.

614 31. **Höltje, J. V., W. Fiedler, H. Rotering, B. Walderich, and J. van Duin.** 1988. Lysis
615 induction of *Escherichia coli* by the cloned lysis protein of the phage MS2 depends on
616 the presence of osmoregulatory membrane-derived oligosaccharides. Journal of
617 Biological Chemistry **263:**3539-3541.

618 32. **Hu, P., S. C. Janga, M. Babu, J. J. Díaz-Mejía, G. Butland, W. Yang, O. Pogoutse, X. Guo,
619 S. Phanse, P. Wong, S. Chandran, C. Christopoulos, A. Nazarians-Armavil, N. K. Nasseri,
620 G. Musso, M. Ali, N. Nazemof, V. Eroukova, A. Golshani, A. Paccanaro, J. F. Greenblatt,
621 G. Moreno-Hagelsieb, and A. Emili.** 2009. Global functional atlas of *Escherichia coli*
622 encompassing previously uncharacterized proteins. PLoS Biol **7:**e1000096.

623 33. **Huerta, A. M., and J. Collado-Vides.** 2003. Sigma70 promoters in *Escherichia coli*:
624 specific transcription in dense regions of overlapping promoter-like signals. Journal of
625 Molecular Biology **333:**261-278.

Accepted Manuscript Posted Online
Journal of Bacteriology
JB

626 34. **Huisman, G. W., D. A. Siegele, M. M. Zambrano, and R. Kolter.** 1996. Morphological
627 and physiological changes during stationary phase. ASM Press, Washington, D.C.

628 35. **Janaszak, A., B. Nadratowska-Wesołowska, G. Konopa, and A. Taylor.** 2009. The P1
629 promoter of the *Escherichia coli* rpoH gene is utilized by σ70-RNAP or σS-RNAP
630 depending on growth phase. FEMS Microbiology Letters **291:**65-72.

631 36. **Jenkins, D. E., E. A. Auger, and A. Matin.** 1991. Role of RpoH, a heat shock regulator
632 protein, in *Escherichia coli* carbon starvation protein synthesis and survival. Journal of
633 Bacteriology **173:**1992-1996.

634 37. **Jenkins, D. E., S. A. Chaisson, and A. Matin.** 1990. Starvation-induced cross protection
635 against osmotic challenge in *Escherichia coli*. Journal of Bacteriology **172:**2779-2781.

636 38. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross protection
637 against heat or H2O2 challenge in *Escherichia coli*. J Bacteriol **170:**3910-4.

638 39. **Jishage, M., and A. Ishihama.** 1997. Variation in RNA polymerase sigma subunit
639 composition within different stocks of *Escherichia coli* W3110. Journal of Bacteriology
640 **179:**959-63.

641 40. **Johansen, J., A. A. Rasmussen, M. Overgaard, and P. Valentin-Hansen.** 2006. Conserved
642 small non-coding RNAs that belong to the σE regulon: Role in down-regulation of outer
643 membrane proteins. Journal of Molecular Biology **364:**1-8.

644 41. **Knudsen, G. M., Y. Ng, and L. Gram.** 2013. Survival of bactericidal antibiotic treatment
645 by a persister subpopulation of *Listeria monocytogenes*. Applied and Environmental
646 Microbiology **79:**7390-7397.

Journal of Bacteriology

Accepted Manuscript Posted Online
JB
Journal of Bacteriology

647 42. **Kobayashi, A., H. Hirakawa, T. Hirata, K. Nishino, and A. Yamaguchi.** 2006. Growth
648 phase-dependent expression of drug exporters in *Escherichia coli* and its contribution to
649 drug tolerance. Journal of Bacteriology **188:**5693-5703.
650 43. **Krogh, A., B. r. Larsson, G. von Heijne, and E. L. L. Sonnhammer.** 2001. Predicting
651 transmembrane protein topology with a hidden markov model: application to complete
652 genomes1. Journal of Molecular Biology **305:**567-580.
653 44. **Kuczyńska-Wiśnik, D., K. Stojowska, E. Matuszewska, D. Leszczyńska, M. M. Algara, M.**
654 **Augustynowicz, and E. Laskowska.** 2015. Lack of intracellular trehalose affects
655 formation of *Escherichia coli* persister cells. Microbiology **161:**786-796.
656 45. **Langridge, G. C., M.-D. Phan, D. J. Turner, T. T. Perkins, L. Parts, J. Haase, I. Charles, D.**
657 **J. Maskell, S. E. Peters, G. Dougan, J. Wain, J. Parkhill, and A. K. Turner.** 2009.
658 Simultaneous assay of every *Salmonella Typhi* gene using one million transposon
659 mutants. Genome Research **19:**2308-2316.
660 46. **Lewis, D. L., and D. K. Gattie.** 1991. The ecology of quiescent microbes. ASM American
661 Society for Microbiology News **57:**27-32.
662 47. **Lewis, K.** 2007. Persister cells, dormancy and infectious disease. Nat Rev Micro **5:**48-56.
663 48. **Li, H., and R. Durbin.** 2009. Fast and accurate short read alignment with Burrows-
664 Wheeler transform. Bioinformatics **25:**1754-1760.
665 49. **Li, X.-Z., P. Plésiat, and H. Nikaido.** 2015. The challenge of efflux-mediated antibiotic
666 resistance in Gram-negative bacteria. Clinical Microbiology Reviews **28:**337-418.

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

667 50. **Luidalepp, H., A. JÃµers, N. Kaldalu, and T. Tenson.** 2011. Age of inoculum strongly
668 influences persister frequency and can mask effects of mutations implicated in altered
669 persistence. Journal of Bacteriology **193:**3598-3605.
670 51. **Malinverni, J. C., and T. J. Silhavy.** 2009. An ABC transport system that maintains lipid
671 asymmetry in the Gram-negative outer membrane. Proceedings of the National
672 Academy of Sciences **106:**8009-8014.
673 52. **Mandel, M. J., and T. J. Silhavy.** 2005. Starvation for different nutrients in *Escherichia*
674 *coli* results in differential modulation of RpoS levels and stability. Journal of Bacteriology
675 **187:**434-442.
676 53. **McKay, S. L., and D. A. Portnoy.** 2015. Ribosome hibernation facilitates tolerance of
677 stationary-phase bacteria to aminoglycosides. Antimicrobial Agents and Chemotherapy
678 **59:**6992-6999.
679 54. **Meberg, B. M., A. L. Paulson, R. Priyadarshini, and K. D. Young.** 2004. Endopeptidase
680 penicillin-binding proteins 4 and 7 play auxiliary roles in determining uniform
681 morphology of *Escherichia coli*. Journal of Bacteriology **186:**8326-8336.
682 55. **Meier-Dieter, U., K. Barr, R. Starman, L. Hatch, and P. D. Rick.** 1992. Nucleotide
683 sequence of the *Escherichia coli* rfe gene involved in the synthesis of enterobacterial
684 common antigen. Molecular cloning of the rfe-rff gene cluster. Journal of Biological
685 Chemistry **267:**746-753.
686 56. **Misra, R., and Y. Miao.** 1995. Molecular analysis of asmA, a locus identified as the
687 suppressor of OmpF assembly mutants of *Escherichia coli* K-12. Molecular Microbiology
688 **16:**779-788.

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

689 57. **Misra, R., and P. R. Reeves.** 1987. Role of micF in the tolC-mediated regulation of OmpF,
690 a major outer membrane protein of *Escherichia coli* K-12. Journal of Bacteriology
691 **169:**4722-4730.
692 58. **Moores, A., S. Chipper-Keating, L. Sun, G. McVicker, L. Wales, K. Gashi, and I. C.**
693 **Blomfield.** 2014. RfaH suppresses small RNA MicA inhibition of fimB expression in
694 *Escherichia coli* K-12. Journal of Bacteriology **196:**148-156.
695 59. **Mouslim, C., A. D. Cano, and J. Casadesús.** 1998. The sfiX, rfe and metN genes of
696 *Salmonella typhimurium* and their involvement in the Hisc pleiotropic response.
697 Molecular and General Genetics MGG **259:**46-53.
698 60. **Mulcahy, L. R., J. L. Burns, S. Lory, and K. Lewis.** 2010. Emergence of *Pseudomonas*
699 *aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis.
700 Journal of Bacteriology **192:**6191-6199.
701 61. **Murakami, K., T. Ono, D. Viducic, S. Kayama, M. Mori, K. Hirota, K. Nemoto, and Y.**
702 **Miyake.** 2005. Role for rpoS gene of *Pseudomonas aeruginosa* in antibiotic tolerance.
703 FEMS Microbiology Letters **242:**161-167.
704 62. **Navarro Llorens, J. M., A. Tormo, and E. Martinez-Garcia.** 2010. Stationary phase in
705 gram-negative bacteria. FEMS Microbiol Rev **34:**476-95.
706 63. **Nelson, D. E., and K. D. Young.** 2000. Penicillin binding protein 5 affects cell diameter,
707 contour, and morphology of *Escherichia coli*. Journal of Bacteriology **182:**1714-1721.
708 64. **Nguyen, L. H., and R. R. Burgess.** 1997. Comparative analysis of the interactions of
709 *Escherichia Coli* σS and σ70 RNA polymerase holoenzyme with the stationary-phase-
710 specific bolAp1 promoter. Biochemistry **36:**1748-1754.

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

711 65. **Nichols, R. J., S. Sen, Y. J. Choo, P. Beltrao, M. Zietek, R. Chaba, S. Lee, K. M.
712 Kazmierczak, K. J. Lee, A. Wong, M. Shales, S. Lovett, M. E. Winkler, N. J. Krogan, A.
713 Typas, and C. A. Gross.** 2011. Phenotypic landscape of a bacterial cell. Cell **144:**143-156.

714 66. **Nickerson, K. W., and A. Aspedon.** 1992. Detergent-shock response in enteric bacteria.
715 Molecular Microbiology **6:**957-961.

716 67. **Nikaido, H.** 2003. Molecular basis of bacterial outer membrane permeability revisited.
717 Microbiology and Molecular Biology Reviews **67:**593-656.

718 68. **Paradis-Bleau, C., G. Kritikos, K. Orlova, A. Typas, and T. G. Bernhardt.** 2014. A
719 genome-wide screen for bacterial envelope biogenesis mutants identifies a novel factor
720 involved in cell wall precursor metabolism. PLoS Genet **10:**e1004056.

721 69. **Peters, K., S. Kannan, V. A. Rao, J. Biboy, D. Vollmer, S. W. Erickson, R. J. Lewis, K. D.
722 Young, and W. Vollmer.** 2016. The redundancy of peptidoglycan carboxypeptidases
723 ensures robust cell shape maintenance in *Escherichia coli*. mBio **7**.

724 70. **Petersen, T. N., S. Brunak, G. von Heijne, and H. Nielsen.** 2011. SignalP 4.0:
725 discriminating signal peptides from transmembrane regions. Nat Meth **8:**785-786.

726 71. **Potluri, L.-P., M. A. de Pedro, and K. D. Young.** 2012. *Escherichia coli* low-molecular-
727 weight penicillin-binding proteins help orient septal FtsZ, and their absence leads to
728 asymmetric cell division and branching. Molecular Microbiology **84:**203-224.

729 72. **Pradel, E., and C. A. Schnaitman.** 1991. Effect of rfaH (sfrB) and temperature on
730 expression of rfa genes of *Escherichia coli* K-12. Journal of Bacteriology **173:**6428-6431.

731 73. **Pratt, L. A., and T. J. Silhavy.** 1996. The response regulator SprE controls the stability of
732 RpoS. Proceedings of the National Academy of Sciences **93:**2488-2492.

Journal of Bacteriology

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

733 74. **Putrinš, M., K. Kogermann, E. Lukk, M. Lippus, V. Varik, and T. Tenson.** 2015.
734 Phenotypic heterogeneity enables uropathogenic *Escherichia coli* to evade killing by
735 antibiotics and serum complement. Infection and Immunity **83:**1056-1067.

736 75. **Rajagopal, S., N. Eis, M. Bhattacharya, and K. W. Nickerson.** 2003. Membrane-derived
737 oligosaccharides (MDOs) are essential for sodium dodecyl sulfate resistance in
738 *Escherichia coli*. FEMS Microbiology Letters **223:**25-31.

739 76. **Rajagopal, S., N. Sudarsan, and K. W. Nickerson.** 2002. Sodium dodecyl sulfate
740 hypersensitivity of clpP and clpB mutants of *Escherichia coli*. Applied and Environmental
741 Microbiology **68:**4117-4121.

742 77. **Rami, A., C. M. Toutain, and A. Jacq.** 2005. An increased level of alternative sigma
743 factor RpoS partially suppresses drug hypersensitivity associated with inactivation of the
744 multidrug resistance pump AcrAB in *Escherichia coli*. Research in Microbiology **156:**356-
745 360.

746 78. **Ricci, D. P., C. L. Hagan, D. Kahne, and T. J. Silhavy.** 2012. Activation of the *Escherichia*
747 *coli* beta-barrel assembly machine (Bam) is required for essential components to
748 interact properly with substrate. Proceedings of the National Academy of Sciences
749 **109:**3487-3491.

750 79. **Rida, S., J. Caillet, and J. H. Alix.** 1996. Amplification of a novel gene, sanA, abolishes a
751 vancomycin-sensitive defect in *Escherichia coli*. Journal of Bacteriology **178:**94-102.

752 80. **Robinson, J. T., H. Thorvaldsdottir, W. Winckler, M. Guttman, E. S. Lander, G. Getz, and**
753 **J. P. Mesirov.** 2011. Integrative genomics viewer. Nat Biotech **29:**24-26.

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

754 81. **Ruiz, N., T. Wu, D. Kahne, and T. J. Silhavy.** 2006. Probing the barrier function of the
755 outer membrane with chemical conditionality. ACS Chemical Biology **1:**385-395.
756 82. **Salmon, K., S.-p. Hung, K. Mekjian, P. Baldi, G. W. Hatfield, and R. P. Gunsalus.** 2003.
757 Global gene expression profiling in *Escherichia coli* K12: The effects of oxygen availability
758 and Fnr. Journal of Biological Chemistry **278:**29837-29855.
759 83. **Santos, J. M., M. Lobo, A. P. A. Matos, M. A. De Pedro, and C. M. Arraiano.** 2002. The
760 gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal
761 morphology in *Escherichia coli*. Molecular Microbiology **45:**1729-1740.
762 84. **Sarkar, S. K., M. Dutta, C. Chowdhury, A. Kumar, and A. S. Ghosh.** 2011. PBP5, PBP6
763 and DacD play different roles in intrinsic β-lactam resistance of *Escherichia coli*.
764 Microbiology **157:**2702-2707.
765 85. **Schilling, J. D., and S. J. Hultgren.** 2002. Recent advances into the pathogenesis of
766 recurrent urinary tract infections: the bladder as a reservoir for uropathogenic
767 *Escherichia coli*. International Journal of Antimicrobial Agents **19:**457-460.
768 86. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions.
769 Cold Spring Harbor Laboratory.
770 87. **Silhavy, T. J., D. Kahne, and S. Walker.** 2010. The bacterial cell envelope. Cold Spring
771 Harbor Perspectives in Biology **2**.
772 88. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and
773 base resistance in *Escherichia coli* and *Shigella flexneri*: role of rpoS and growth pH.
774 Journal of Bacteriology **176:**1729-1737.

Accepted Manuscript Posted Online
JB Journal of Bacteriology
Journal of Bacteriology

775 89. **Spoering, A. L., and K. Lewis.** 2001. Biofilms and planktonic cells of *Pseudomonas*
776 *aeruginosa* have similar resistance to killing by antimicrobials. Journal of Bacteriology
777 **183:**6746-6751.

778 90. **Stewart, G. R., B. D. Robertson, and D. B. Young.** 2003. Tuberculosis: a problem with
779 persistence. Nat Rev Micro **1:**97-105.

780 91. **Sulavik, M. C., C. Houseweart, C. Cramer, N. Jiwani, N. Murgolo, J. Greene, B.**
781 **DiDomenico, K. J. Shaw, G. H. Miller, R. Hare, and G. Shimer.** 2001. Antibiotic
782 susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes.
783 Antimicrobial Agents and Chemotherapy **45:**1126-1136.

784 92. **Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M.**
785 **Simonovic, A. Roth, A. Santos, K. P. Tsafou, M. Kuhn, P. Bork, L. J. Jensen, and C.**
786 **vonÂ Mering.** 2015. STRING v10: protein–protein interaction networks, integrated over
787 the tree of life. Nucleic Acids Research **43:**D447-D452.

788 93. **Thorvaldsdóttir, H., J. T. Robinson, and J. P. Mesirov.** 2013. Integrative Genomics
789 Viewer (IGV): high-performance genomics data visualization and exploration. Briefings in
790 Bioinformatics **14:**178-192.

791 94. **Tkachenko, A. G., N. M. Kashevarova, E. A. Karavaeva, and M. S. Shumkov.** 2014.
792 Putrescine controls the formation of *Escherichia coli* persister cells tolerant to
793 aminoglycoside netilmicin. FEMS Microbiology Letters **361:**25-33.

794 95. **Typas, A., M. Banzhaf, C. A. Gross, and W. Vollmer.** 2012. From the regulation of
795 peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Micro **10:**123-
796 136.

Accepted Manuscript Posted Online

JB Journal of Bacteriology

797 96. **van Opijnen, T., D. W. Lazinski, and A. Camilli.** 2001. Genome-wide fitness and genetic
798 interactions determined by Tn-seq, a high-throughput massively parallel sequencing
799 method for microorganisms, Current Protocols in Molecular Biology. John Wiley & Sons,
800 Inc.

801 97. **Vega, D. E., and K. D. Young.** 2013. Accumulation of periplasmic enterobactin impairs
802 the growth and morphology of *Escherichia coli* tolC mutants. Molecular Microbiology
803 **91:**508-521.

804 98. **Wandersman, C., and P. Delepelaire.** 1990. TolC, an *Escherichia coli* outer membrane
805 protein required for hemolysin secretion. Proceedings of the National Academy of
806 Sciences **87:**4776-4780.

807 99. **Wensink, J., N. Gilden, and B. Witholt.** 1982. Attachment of lipoprotein to the murein
808 of *Escherichia coli*. Eur J Biochem **122:**587-90.

809 100. **World Health Organization.** 2014. Antimicrobial resistance : global report on
810 surveillance.

811 101. **Xiong, X., J. N. Deeter, and R. Misra.** 1996. Assembly-defective OmpC mutants of
812 *Escherichia coli* K-12. Journal of Bacteriology **178:**1213-5.

813

814

815

Journal of Bacteriology

Accepted Manuscript Posted Online

JB Journal of Bacteriology

816 **Figure Legends**

817 **Figure 1. *E. coli* is resistant to SDS regardless of growth stage.** Cells prepared to be actively
818 growing (**A**), carbon-limited (**B**), or nitrogen-limited (**C**) were treated with 2% SDS and viability
819 was assayed at the indicated time points. Closed data points indicate untreated samples while
820 open data points indicate SDS treated samples. * $p<0.05$ comparing treated and untreated
821 samples

822 **Figure 2. The AcrAB-TolC efflux pump is responsible for SDS resistance in exponential phase.**
823 Actively growing (**A**), carbon-limited (**B**), or nitrogen-limited (**C**) cells of the indicated *acrA* or
824 *acrB* deletion strains were treated with 2% SDS and viability was assayed at the indicated time
825 points. Closed data points indicate untreated samples while open data points indicate SDS
826 treated samples. * $p<0.05$ compared to initial time point for *acrA* strain; ɨ $p<0.05$ compared to
827 initial time point for *acrB* strain

828 **Figure 3. RpoS mediates SDS resistance in carbon-limited cells.** Actively growing (**A**), carbon-
829 limited (**B**), or nitrogen-limited (**C**) *rpoS* deletion cells were treated with 2% SDS and viability
830 was assayed at the indicated time points. Closed data points indicate untreated samples while
831 open data points indicate SDS treated samples. * $p<0.05$ comparing treated and untreated
832 samples

833 **Figure 4. RpoS can be protective in exponential phase. A.** Actively growing cells of the
834 indicated strains were treated with 2% SDS and viability was assayed at 0 and 7 hours. Fold
835 change between the 0 and 7 hour time points (7 hour sample/0 hour sample) are shown. Solid
836 bars indicate untreated samples while open bars indicate treated samples. * $p<0.05$ for both

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

837 between the treated and untreated samples and the indicated strains. **B.** Carbon-limited cells of
838 the indicated strains were treated with 2% SDS and viability was assayed after 24 hours. The
839 fold decrease in the treated samples versus the untreated samples is shown (untreated
840 sample/treated sample). * $p<0.05$ comparing the indicated strains

841 **Figure 5. AcrAB-TolC can compensate for the loss of RpoS in nitrogen-limited cells.** Actively
842 growing (**A**), carbon-limited (**B**), or nitrogen-limited (**C**) cells of the indicated strain (*acrA rpoS* or
843 *acrB rpoS*) were treated with 2% SDS and viability was assayed at the indicated time points.
844 Closed data points indicate untreated samples while open data points indicate SDS treated
845 samples. Dotted line indicates the limit of detection. * $p<0.05$ comparing treated and untreated
846 samples for *acrA rpoS* strain; ǂ $p<0.05$ comparing treated and untreated samples for *acrB rpoS*
847 strain

848 **Figure 6. Tn-Seq to identify genes responsible for SDS resistance in C-limited cells. A.**
849 Experimental strategy for Tn-Seq experiment: A large library of Tn5 mutants was grown under
850 either carbon- or nitrogen-limitation then treated with 2% SDS for 24 hours. DNA samples were
851 collected and transposon junctions were sequenced both pre- and post-treatment. **B.**
852 Sequenced transposon junctions were mapped to the MG1655 genome and a histogram for an
853 example region of the genome is shown. The boxed region indicates a gene, *sanA*, for which
854 sequencing reads decreased more than 3-fold in carbon-limiting conditions but did not change
855 in nitrogen-limiting conditions. Scale 0-500 reads. **C.** Strains with deletions in the indicated
856 genes were grown under carbon-limitation and treated with 2% SDS. Viability was assayed at 24
857 h of treatment. The fold decrease in the treated samples versus the untreated samples is
858 shown (untreated sample/treated sample). Open bars indicate control strains while closed bars

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

859 represent strains with deletions in genes identified in the Tn-Seq experiment as causing SDS
860 sensitivity only in carbon-limiting conditions. Dotted line indicates the fold decrease in wild type
861 MG1655. * p<0.05 compared to MG1655

862 **Figure 7. *rpoS* is epistatic to *sanA*, *dacA*, and *ydhP*. A.** Carbon-limited cells of the indicated
863 strains were treated with 2% SDS and viability was assayed at 24 h post-treatment. The fold
864 decrease in the treated samples versus the untreated samples is shown (untreated
865 sample/treated sample). Open bars indicates strains in a wild type background while black bars
866 indicate strains on an *rpoS* background and blue bars indicate strains on an *acrA* background.
867 Control samples (MG1655, *rpoS*, *acrA*) are identical between the two panels and include 6
868 biological replicates. Strains are separated by those that are not additive with *rpoS* (left) and
869 those that are additive with *rpoS* (right). * p<0.05 one tailed for increase compared to the
870 parent strain with the greater effect **B.** Carbon-limited cells of the indicated strains were
871 treated with 2% SDS and viability was assayed at 24 h post-treatment. The fold decrease in the
872 treated samples versus the untreated samples is shown (untreated sample/treated sample).
873 The control strain is indicated by a white bar, the single mutants by black bars, double mutants
874 by blue bars, and the triple mutant by an orange bar. * p<0.05 one tailed for increase compared
875 to parent; n/s no significant difference between the indicated strains. **C.** Nitrogen-limited cells
876 of the indicated strains were treated with 2% SDS and viability was assayed at 3 h post-
877 treatment. The fold decrease in the treated samples versus the untreated samples is shown
878 (untreated sample/treated sample) on a linear scale. * p<0.05 one tailed for increase in strain
879 without *acrA* compared to strain with *acrA*

Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology

880 **Figure 8. Model of interactions between efflux and envelope permeability in SDS resistance. A.** In
881 actively growing cells, SDS is able to penetrate the OM and is then removed by the AcrAB-TolC efflux
882 pump. This energy intensive process, which relies on the proton motive force, is made efficient by the
883 abundance of nutrients available to the cell. **B.** In carbon-limited cells, an RpoS-dependent decrease in
884 OM permeability through pathways that involve SanA, PBP5, and YhdP prevents SDS from entering the
885 cell. Early in treatment any SDS that does penetrate the OM is removed by the AcrAB-TolC efflux pump;
886 however, lack of energy in carbon-limited cells eventually leads the efflux pump to fail and causes a low
887 level of cell death. When *rpoS* is removed, SDS penetrates the OM and inefficiency in the efflux pumps
888 due to lack of energy causes the cells to die quickly. **C.** In nitrogen-limited cells, an RpoS-dependent
889 mechanism that is different than that employed in carbon-limited cells prevents SDS entry into the cell;
890 however, the loss of *rpoS* can be compensated for by the activity of the AcrAB-TolC efflux pump,
891 suggesting that, although there is a lack of available nitrogen for making new proteins, nitrogen-limited
892 cells have sufficient energy available in the form of the proton motive force for efficient efflux over the
893 time scale of the experiment.

894

895 **Table 1. Envelope related Tn-Seq hits causing SDS sensitivity only in carbon-limited cells**

	Carbon-limited Reads per Kbp[b]		Log2 Fold Post/Pre treatment[c]	
Gene[a]	**Pre-treatment**	**Post-treatment**	**Carbon-limited**	**Nitrogen-limited**
ydgH	758	60	-3.7	0.7
bssR	2000	279	-2.8	0.9
tatB	3841	558	-2.8	0.2
wecA (rfe)	2043	323	-2.7	-0.3
ftsN	10134	1618	-2.6	-0.5
ompA	2108	354	-2.6	-0.1
dacA	867	162	-2.4	-1.2
sanA	3261	625	-2.4	-0.2
ytfK	7816	2135	-1.9	0.9
wecE	1834	531	-1.8	0.9
rfaH	3155	1004	-1.7	0.4

yhdP	2386	760	-1.7	0.3

896 [a] Genes with at least 700 sequence reads, decreasing at least 3-fold during treatment in carbon-

897 limiting conditions, and changing less than 3-fold in nitrogen limiting conditions

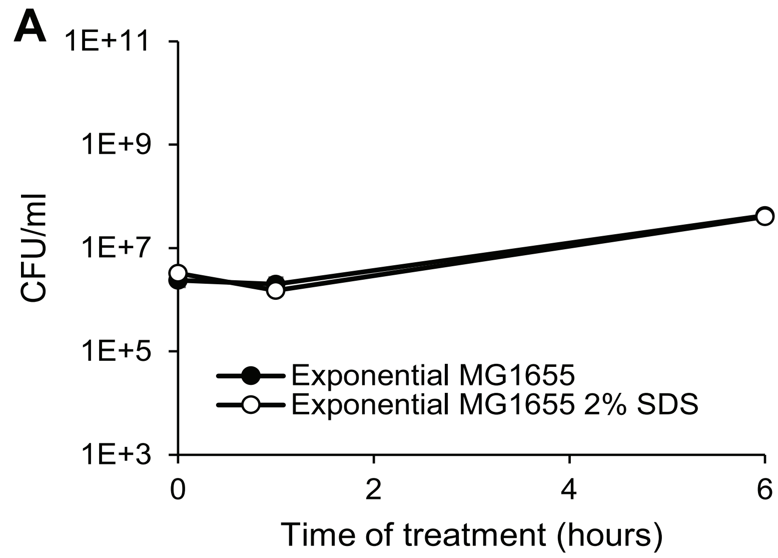
898 [b] The number of sequence reads for each gene was normalized to the length of the gene

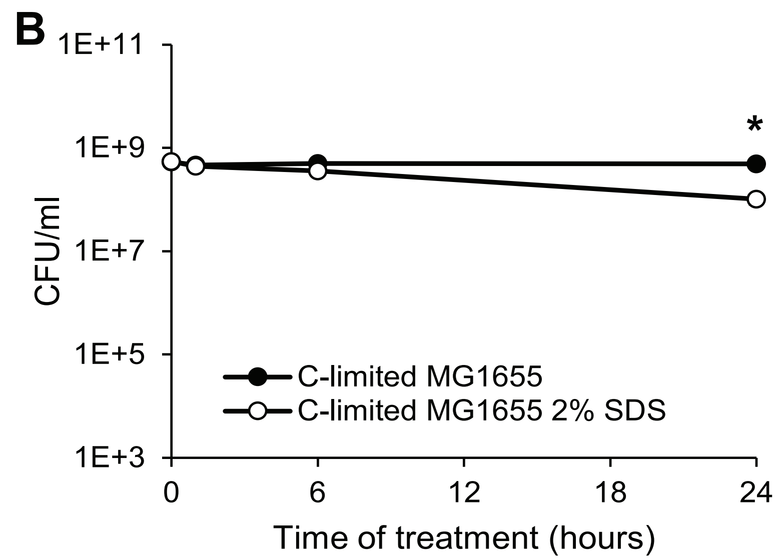
899 [c] The fold change in read number before and after SDS treatment in either carbon- or nitrogen-

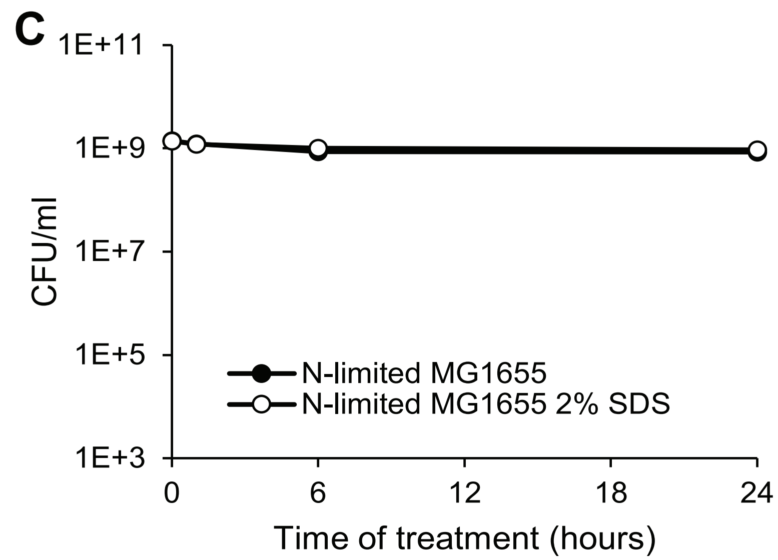
900 limiting conditions

Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology

Accepted Manuscript Posted Online



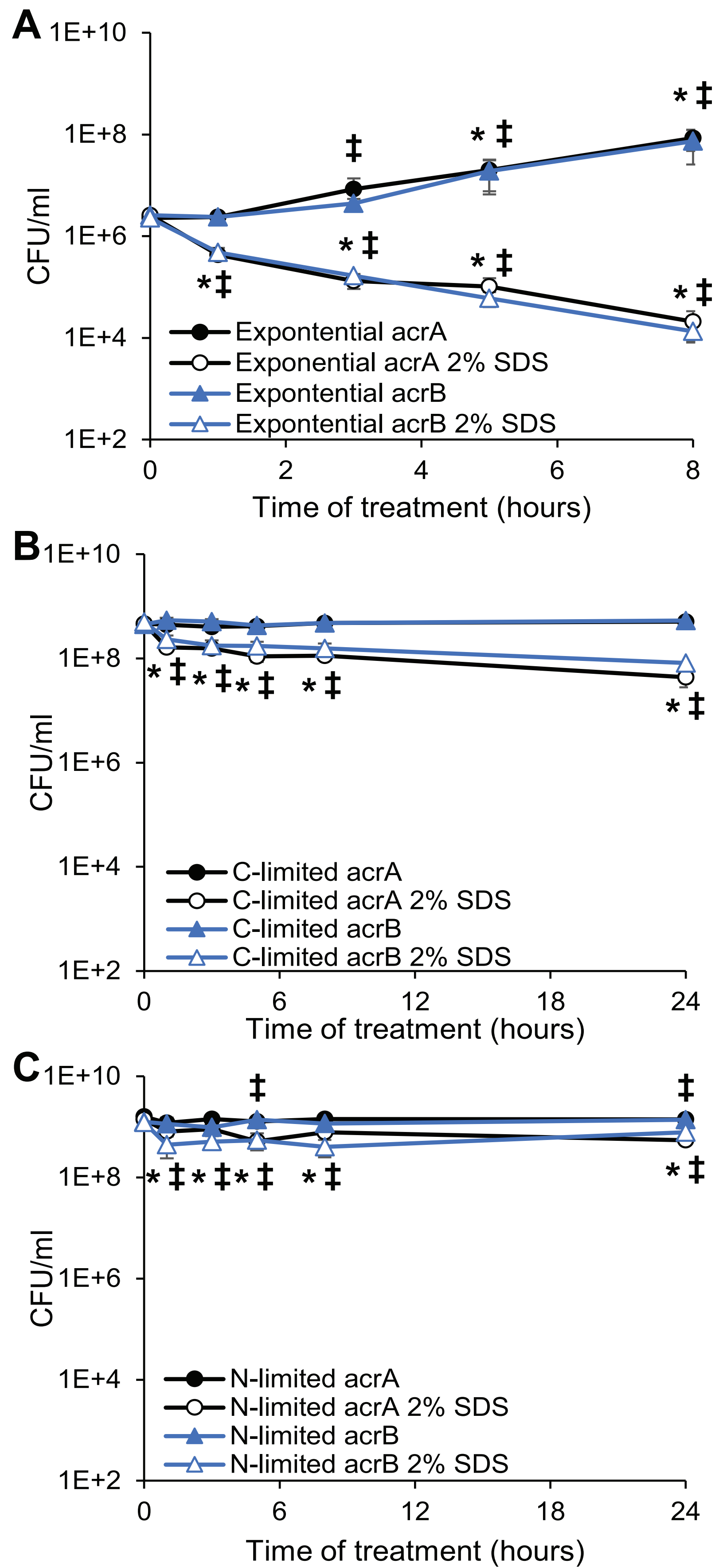




Accepted Manuscript Posted Online

Journal of Bacteriology

JB



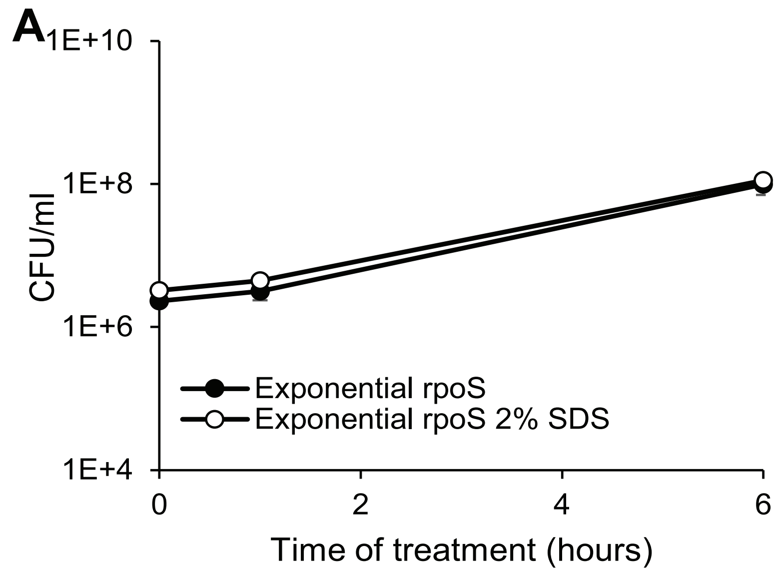
Journal of Bacteriology

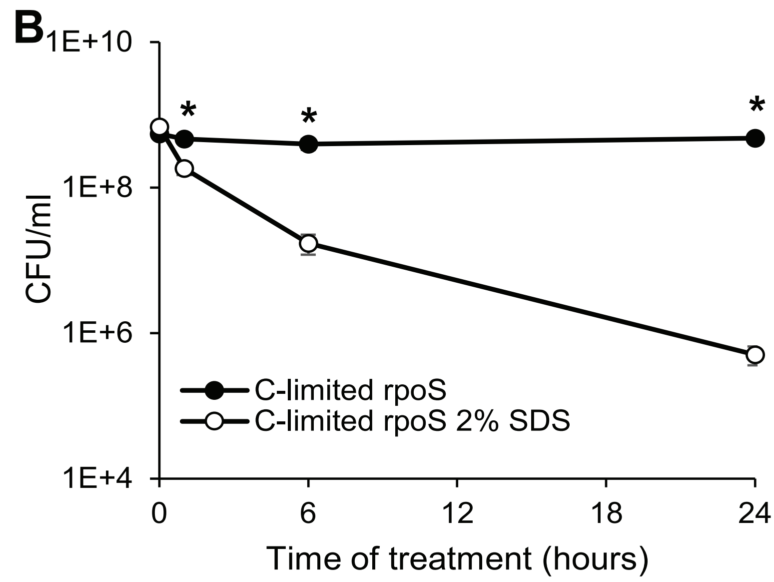
Accepted Manuscript Posted Online

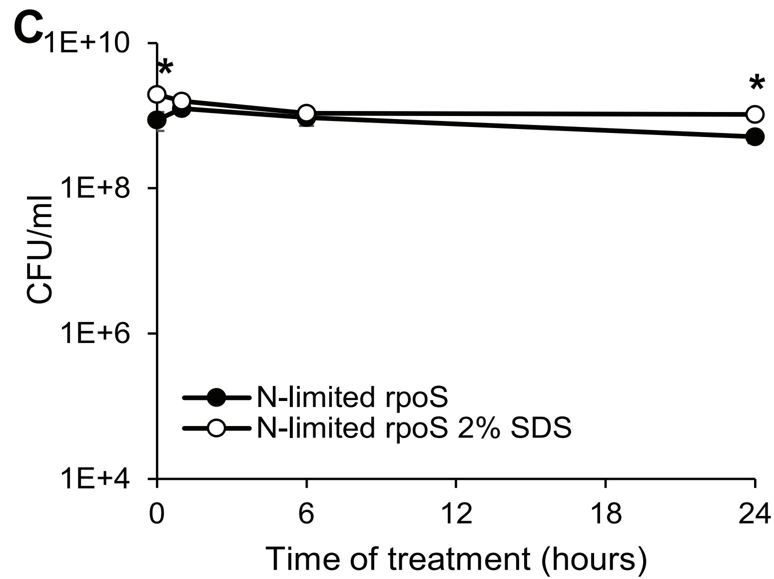
Journal of Bacteriology

JB

Journal of Bacteriology



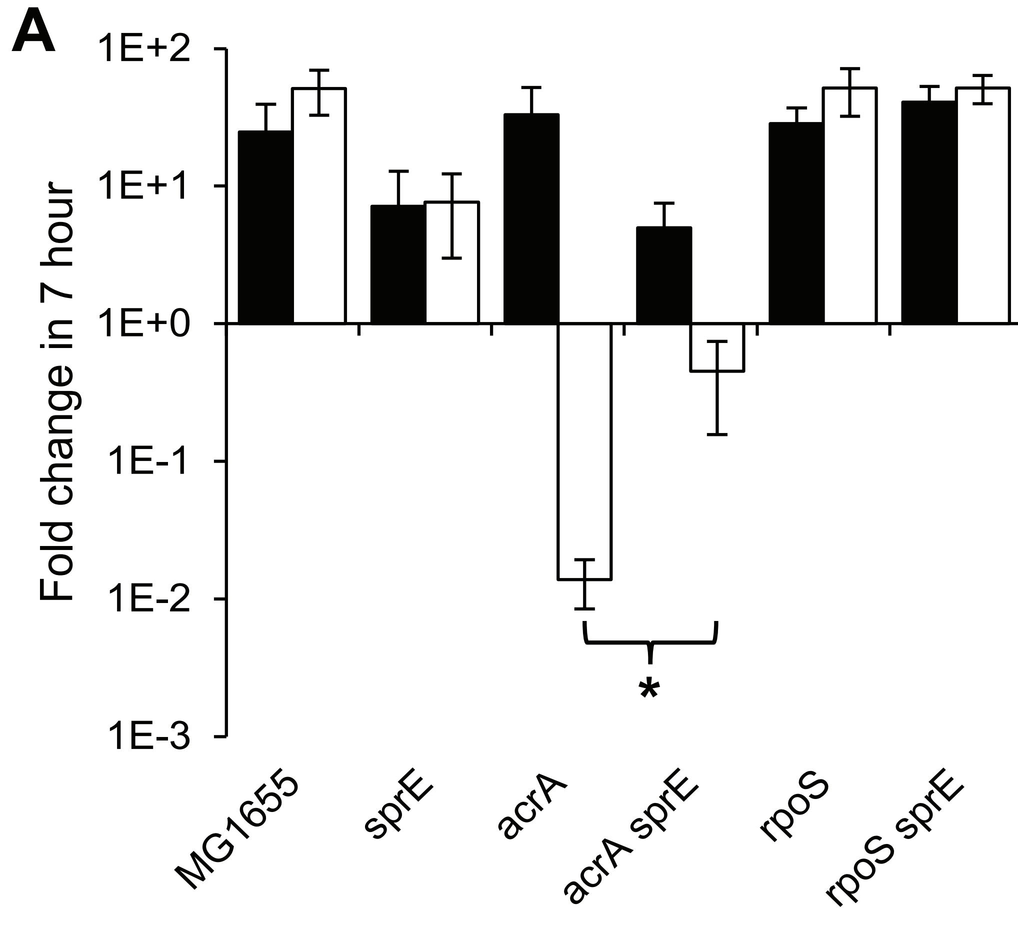


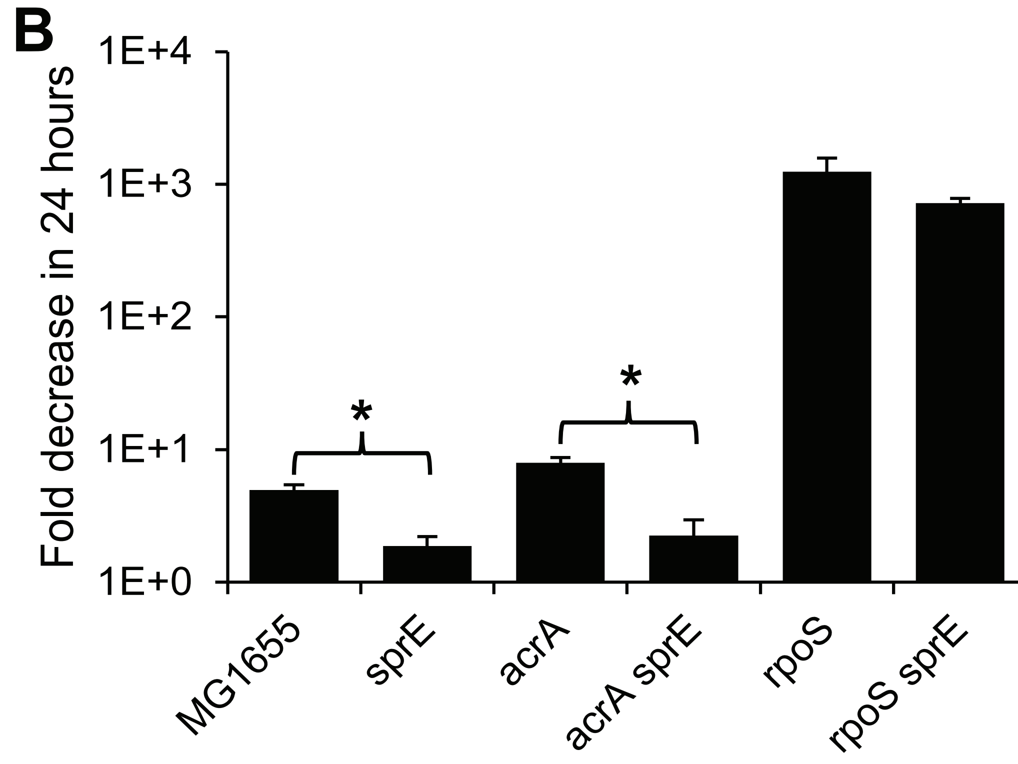


Accepted Manuscript Posted Online

Journal of Bacteriology

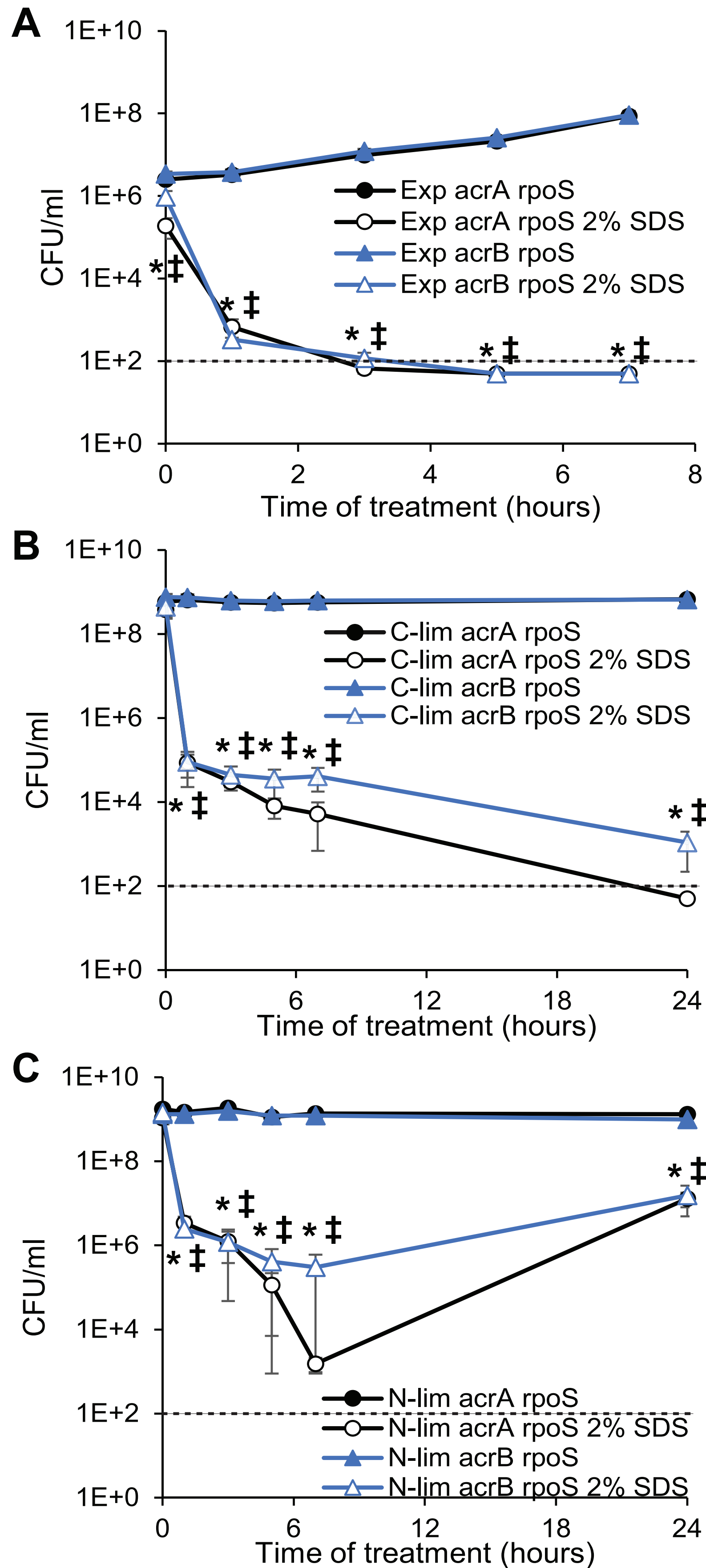
JB





Journal of Bacteriology

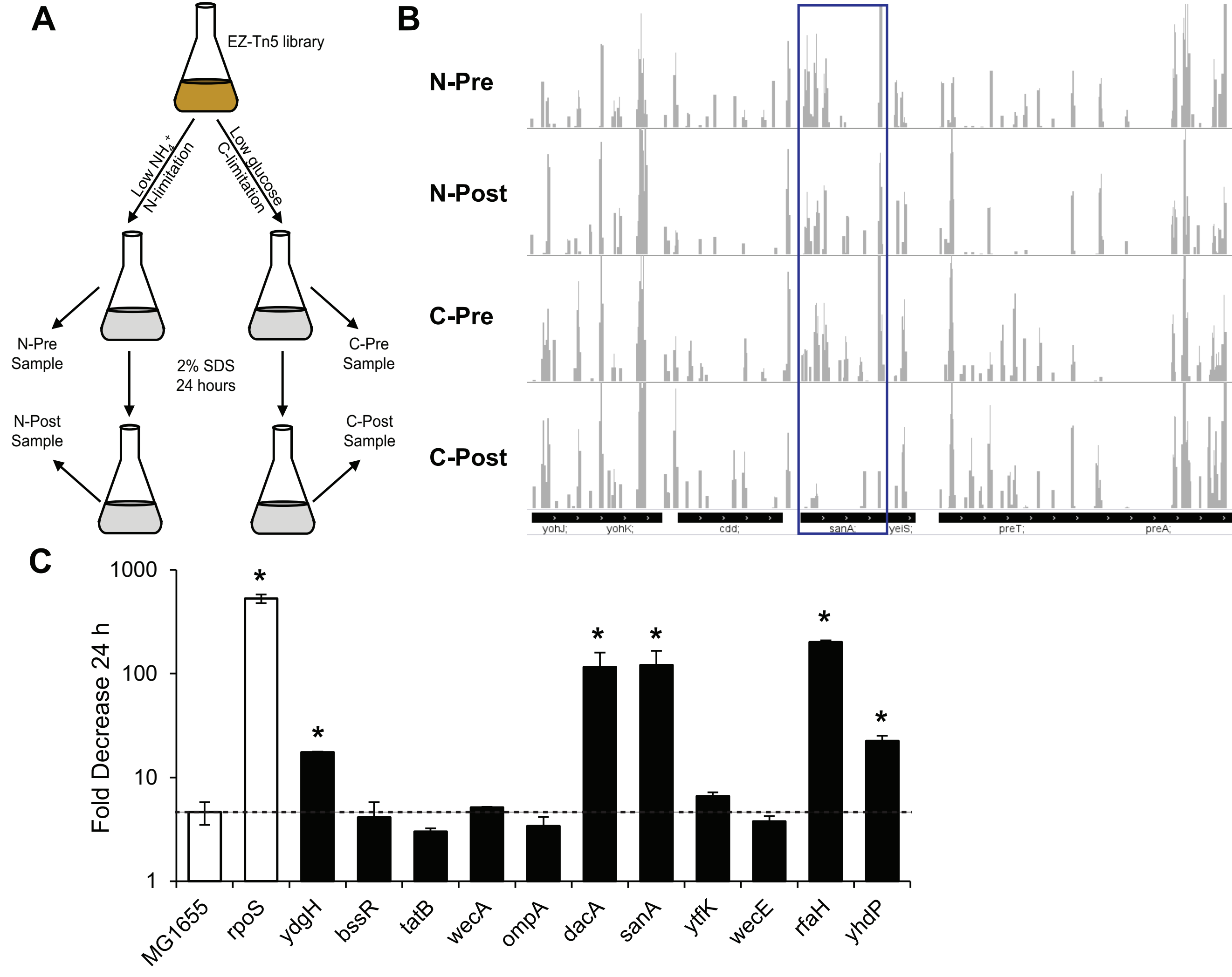
Accepted Manuscript Posted Online
Journal of Bacteriology
JB
Journal of Bacteriology



Accepted Manuscript Posted Online

Journal of Bacteriology

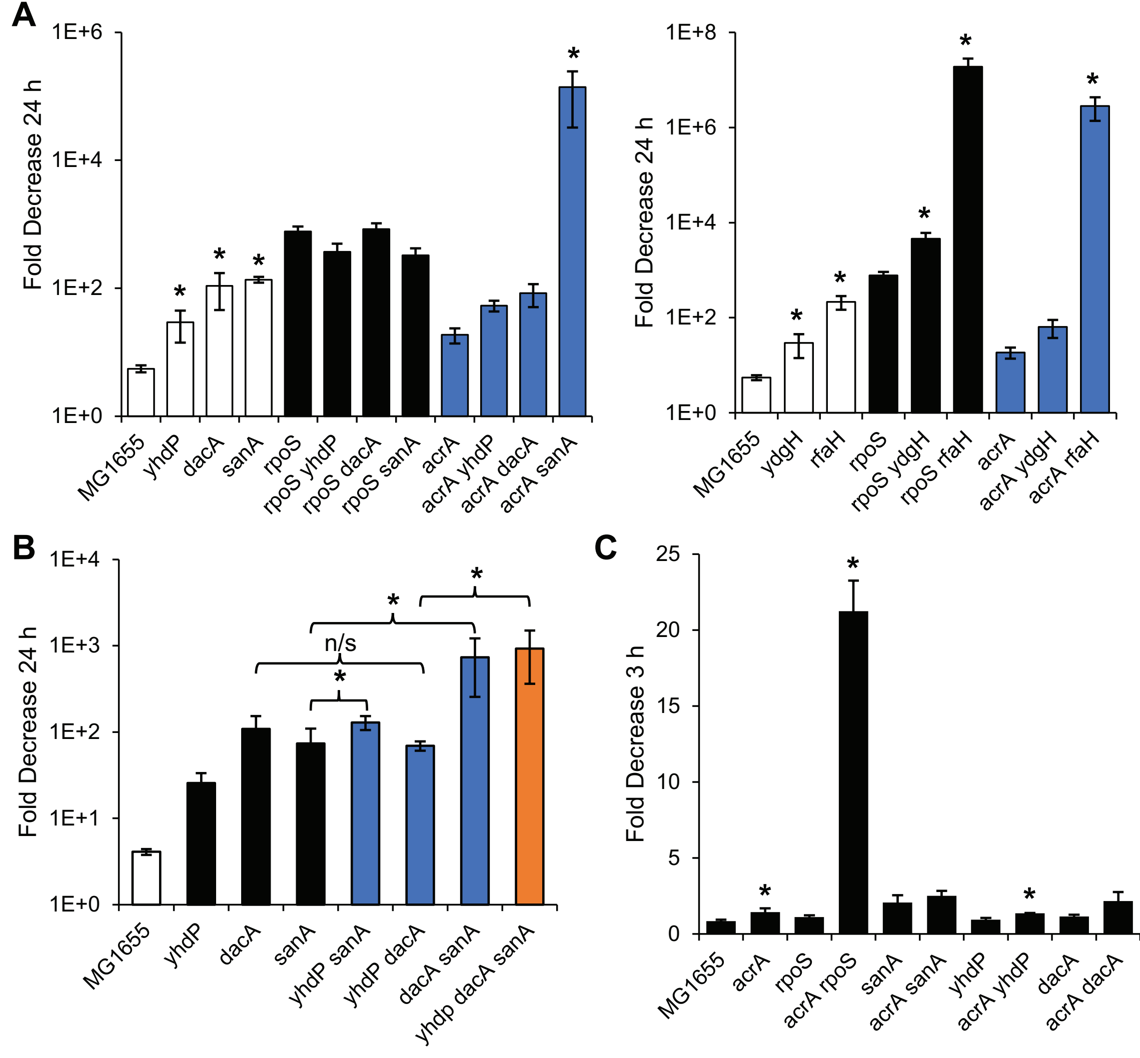
JB



Journal of Bacteriology

Accepted Manuscript Posted Online

JB Journal of Bacteriology



Journal of Bacteriology

Accepted Manuscript Posted Online

Journal of Bacteriology

JB

Journal of Bacteriology