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1 **The complex development of psoralen-interstrand crosslink resistance in**
2 ***Escherichia coli* requires AcrR inactivation, retention of a *marbox* sequence, and one**
3 **of three MarA, SoxS, or Rob global regulators**
4
5
6 **Abstract**
7 Crosslinking agents, such as psoralen and UVA radiation, can be effectively used
8 as antimicrobials and for treating several dysplastic conditions in humans, including some
9 cancers. Yet, both cancer cells and bacteria can become resistant to these compounds,
10 making it important to understand how resistance develops. Recently, several mutants
11 were isolated that developed high-levels of resistance to these compounds through
12 upregulation of components of the AcrAB-TolC-efflux pump. Here, we characterized
13 these mutants and found that resistance specifically requires inactivating mutations of the
14 *acrR* transcriptional repressor which also retain the *marbox* sequence found within this
15 coding region. In addition, the presence of any one of three global regulators, MarA,
16 SoxS, or Rob, is necessary and sufficient to bind to the *marbox* sequence and activate
17 resistance. Notably, although psoralen is a substrate for the efflux pump, these regulators
18 are not naturally responsive to this stress as neither psoralen, UVA, nor crosslink
19 induction upregulates *acrAB* expression in the absence of mutation.
20
21 **Highlights**
22 - Psoralen crosslink resistance requires AcrR inactivation and MarA/SoxS/Rob activation
23 - Psoralen crosslink resistance is mediated by upregulating the AcrAB-TolC efflux pump
24 - AcrAB-TolC can utilize psoralen as a substrate but not upregulated by this stress
25 - Acquiring resistance to DNA interstrand crosslinks requires mutation
26
27 **Keywords:** DNA interstrand crosslinks, psoralen-UVA, antimicrobial, cancer therapeutic
28
29

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30 **1. Introduction**

31 Psoralen in the presence of UVA irradiation forms DNA interstrand crosslinks
32 and is used in the treatment of psoriasis and vitiligo, as well as in the treatment of
33 cutaneous T-cell lymphoma [1, 2]. The potency of this treatment and similar therapeutics
34 is attributed to its ability to form lethal lesions known as DNA interstrand crosslinks [3–
35 6]. In *E. coli*, a single DNA interstrand crosslink in the genome is sufficient to inactivate
36 the cell [7, 8]. However, the use of psoralen-UVA and other crosslinking agents as
37 antimicrobials and chemotherapeutics can be compromised by the emergence of cells
38 resistant to these drugs [9, 10]. In *Escherichia coli*,several highly resistant mutants to
39 psoralen-UVA interstrand crosslinks have been isolated whose resistance is driven by
40 increased expression of the AcrAB-TolC efflux pump, which protects the DNA and
41 effectively prevents these lethal lesions from forming when psoralen is present in the
42 media [11].

43 AcrAB-TolC belongs to a highly conserved RND (Resistance-Nodulation-
44 Division) efflux pump family found in Gram-negative bacteria [12–16]. The efflux pump
45 consists of a proton-driven transporter AcrB, a periplasmic adapter protein AcrA, and the
46 TolC transmembrane channel [17–20]. AcrAB-TolC is capable of effluxing a wide
47 variety of structurally dissimilar substrates, including many dyes, detergents, and
48 antibiotics [21–25], making it a primary driver of multiple-antibiotic resistance [23].

49 The highly resistant mutants were each found to have mutations in in the
50 transcriptional regulator AcrR [11]. *acrR* encodes a TetR family transcriptional regulator
51 that is located immediately upstream of *acrAB* and is divergently transcribed [26]. Based
52 on *lacZ*-fusion and gel mobility shift assays, Ma et. al. demonstrated that AcrR functions
53 as a repressor of *acrAB* that releases upon binding a recognized substrate [27]. Consistent
54 with this, some substrates of the efflux pump, such as rhodamine, ethidium bromide, and
55 proflavine, bind to AcrR [28, 29] and this correlates with a loss of DNA binding activity
56 in vitro [30].

57 Surprisingly however, deletion of *acrR*'s coding region does not increase
58 resistance to psoralen interstrand crosslinks, suggesting a more complex mechanism of
59 regulation than a simple repressor function is involved [11]. We noted that the first 20
60 nucleotides of *acrR*'s coding region contains a *marbox*-binding sequence for three closely
61 related global stress regulators, MarA, SoxS and Rob [31]. These three regulators share
62 approximately 50% sequence identity [32, 33] and regulate expression of approximately
63 50 genes, including *acrA* and *acrB*, in response to various environmental stressors and
64 toxins (Fig. 1 and [31–40]. Using a *lacZ*-reporter construct and gel mobility shift assays,
65 several groups demonstrated that protein binding to the *marbox* upstream of *acrAB*
66 correlated with its expression [27, 40]. This led to a general model that these global
67 stress activators drive *acrAB* expression, with AcrR serving as a secondary repressive
68 modulator.

69 Given the importance of DNA interstrand crosslinks in antimicrobial and
70 chemotherapeutic therapies, here we sought to characterize the mechanism by which
71 resistance was achieved in these mutants acquired their resistance. We found that
72 although the pump confers resistance to crosslinks, it is not naturally responsive or
73 upregulated in their presence. Resistance relies on mutations that inactivate the AcrR
74 repressor but retain the *marbox* sequence within the gene's coding region. The resistance

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75 can then be activated by the presence of any one of the three global activators, MarA
76 SoxS or Rob.
77
78 **2. Materials and Methods**
79 *2.1 Bacterial Strains.*
80 All strains utilized in this study were derived from BW25113, which is the parent
81 strain of the Keio collection [41], from which the *marA*, *soxS*, and *rob* deletion mutants
82 were obtained. The *acrR* deletion mutant was originally obtained from the Keio
83 collection but was reconstructed by P1 phage transduction into wild-type BW25113. The
84 *acrR*(L34Q) mutant was constructed in our previous study [11]. The *marA*, *soxS*, and *rob*
85 deletions were transduced into *acrR*(L34Q) using a standard PI phage transduction. The
86 *marAsoxSrob* triple mutant was constructed by using FLP recombinase expression from
87 the pCP20 plasmid to remove the kan^R cassette from the *marA* deletion mutant,
88 transducing the *soxS* deletion into the *marA* deletion mutant, and then repeating the above
89 process to also delete *rob*. This process was repeated in the *acrR*(L34Q) mutant to
90 generate the *acrR*(L34Q)*marAsoxSrob* quadruple mutant. The presence of all three
91 deletions was confirmed using PCR. Strains CL5415 - CL5422 were constructed by
92 transforming pBAD33, pBAD33-acrAB, pNN387, or pNN608 plasmids into
93 electrocompetent JW5249, JW4023, JW4359. For the deletion of *acrR* past the *marbox*
94 sequence, the Kan^R cassette was recombineered into BW25113 using primers
95 5'AGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTG
96 GCTCTACGTCTTTATGATTCCGGGGATCCGTCGACC3'and 5'CAGG
97 AAAAATCCTGGAGTCAGATTCAGGGTTATTCGTTAGTGGCAGGATT
98 TGTAGGCTGGAGCTGCTTCG3'All strains used in this study are listed in Table 1.
99
100 *2.2 Psoralen-UVA (PUVA) survival.*
101 10-µL aliquots of 10-fold serial dilutions from overnight cultures were spotted
102 onto LBthy plates containing 20 µg/mL 8-methoxypsoralen. Plates were then exposed to
103 UVA irradiation at an incident dose of 6.5 J/m2/s for the indicated dose and incubated
104 overnight at 37°C. Surviving colonies at each dose were then counted and compared to
105 the non-exposed plates to calculate percent survival.
106 For overexpression of *acrAB* from expression vectors, 5 mL LB subcultures were
107 inoculated with 50 µL of overnight cultures containing the expression plasmid, pBAD33-
108 acrAB, or its parent vector, pBAD33, and grown in a 37°C shaking water bath to OD600
109 of 0.4. 1 mM L-arabinose was added to subcultures for the last 30 minutes of incubation
110 before proceeding with survival assays as described above.
111
112 *2.3 acrAB-lacZ expression.*
113 10-µL aliquots of 10-fold serial dilutions from overnight cultures containing pNN608
114 (*acrABp*-lacZ) or pNN387 (empty vector) were spotted onto LBthy plates supplemented
115 with 120 µg/mL 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-Gal) either with
116 or without 20 µg/mL 8-methoxypsoralen. Two plates each of LB X-GaL and LB X-Gal +
117 20 µg/mL 8-methoxypsoralen were then exposed to 3.8 kJ/m^2 UVA radiation as
118 described above for survival assays. Plates were then compared to unexposed plates and
119 photographed.

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120
121 **3. Results**
122 *3.1 Global Regulators MarA, SoxS, and Rob are required for full resistance to psoralen-*
123 *UVA.*
124 In previous work, three mutations in the transcriptional repressor *acrR* were
125 isolated and found to confer high-level resistance to psoralen-UVA through the
126 upregulation of *acrA* and –*B*, encoding components of the AcrAB-TolC efflux pump
127 (Fig. 2A and [11]). However, when we deleted the entire *acrR* coding region, we found
128 that unlike the other *acrR* mutations, no resistance was conferred (Fig. 2A). The
129 observation argues that the loss of the AcrR repressor is insufficient to confer resistance
130 to psoralen interstrand crosslinks and a more complex mechanism is involved in the
131 acquisition of resistance.
132 Common to all three of the resistance-conferring *acrR* mutants that were islaoted
133 is that they retain the initial third of *acrR*'s coding sequence but alter or remove the latter
134 two-thirds of the protein. The first third of the gene encodes the DNA-binding domain
135 for the AcrR regulator. However, we also noted this region also encodes a MarA, SoxS,
136 and Rob binding sequence, termed *marbox*, which has been reported to positively
137 regulate the *acrA -B* operon [27, 31]. Thus, it is possible that the mutations confer
138 psoralen resistance either through altering AcrR's DNA binding properties or through
139 activation of *acrAB* by MarA, SoxS, or Rob.
140 If the psoralen resistance is mediated through the *marbox*, then deletion of the
141 *marA*, *soxS*, and *rob* genes would be expected to impair resistance in these strains. To test
142 this possibility, we examined the ability of mutants deleted for these genes to survive
143 psoralen-UVA treatment. Ten-fold serial dilutions of an overnight culture were spotted
144 on plates containing 20 μg/mL 8-methoxypsoralen and exposed to increasing doses of
145 UVA. Following overnight incubation at 37°C, surviving colonies were counted and
146 compared to the unexposed plate to determine percent survival. Figure 2B shows that
147 deletion of either *marA*, *soxS*, or *rob* renders cells more sensitive than WT to psoralen-
148 UVA irradiation, indicating that all three of these genes are important for psoralen-UVA
149 resistance. Notably, the contribution of each was not additive, as the absence of any
150 single regulator resulted in hypersensitivity that was similar to the *marA soxS rob* triple
151 mutant (Fig. 2C). Given that MarA, SoxS, and Rob all share a single *marbox* binding
152 sequence within *acrR*, it is unexpected and remains unclear why deleting of any one of
153 these three proteins renders cells hypersensitive. However, the observation indicates that
154 all three proteins are required to maintain resistance to psoralen-UVA interstrand
155 crosslinks, despite sharing a single DNA binding sequence.
156
157 *3.2 MarA, SoxS, and Rob contribute to psoralen-UVA resistance primarily through*
158 *upregulation of acrAB.*
159 MarA, SoxS, and Rob upregulate expression of approximately 50 genes in
160 response to various cellular stresses [31]. Thus, although the results of Fig. 2 indicate that
161 MarA, SoxS, and Rob are required for full resistance to psoralen, they do not establish if
162 this contribution can be attributed directly to the upregulation of *acrAB* or if resistance is
163 conferred by other *marbox*-regulated genes. To test this, we used an arabinose-inducible
164 *acrAB* plasmid to overexpress *acrAB* in the *marA*, *soxS*, *rob* deletion mutants, which

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165 would result in upregulation of *acrAB*, but not any other *marbox*-regulated genes.
166 Actively growing cultures containing the plasmid were incubated with arabinose for 30
167 minutes to induce *acrAB* expression prior to psoralen-UVA treatment. Figure 3 shows
168 that plasmids containing the *acrAB* sequence increase resistance in *marA*, *soxS*, and *rob*
169 mutants to near wild-type levels. By contrast, mutants containing an identical plasmid
170 lacking the *acrAB* sequence remain hypersensitive to psoralen-UVA treatment. The
171 results indicate that MarA, SoxS, and Rob contribute to psoralen-UVA resistance
172 primarily through upregulation of *acrAB* expression and that the idea that loss of this
173 upregulation in the *acrR* deletion mutant could be responsible for its inability to confer
174 resistance.
175
176 *3.3 MarA, SoxS, and Rob activation and AcrR de-repression contribute additively to*
177 *psoralen interstrand crosslink resistance*
178 MarA, SoxS, and Rob-mediated upregulation of *acrAB* is required for full
179 resistance to psoralen-UVA (Fig. 2 and 3). Since the highly resistant *acrR*(L34Q) mutant
180 retains the *marbox* sequence, it is possible that the high level of resistance requires
181 activation by MarA, SoxS, or Rob. If true, we would expect that deletion of *marA*, *soxS*,
182 or *rob* would significantly reduce psoralen-UVA resistance in the *acrR*(L34Q) strain. As
183 shown in Fig. 4, *acrR*(L34Q) mutants remained resistant to psoralen-UVA, when either
184 *marA*, *soxS*, or *rob* was deleted. However, the loss of all three genes reduced the
185 resistance of *acrR*(L34Q) mutants to levels similar to wild-type cells and the *acrR*
186 deletion mutant. Taken together with the previous observations, the results support the
187 idea that both de-repression by AcrR and activation by MarA, SoxS, or Rob are required
188 to achieve resistance to psoralen interstrand crosslinks.
189 To confirm these requirements directly, we used recombineering to generate a
190 complete deletion of the *acrR* coding sequence with the exception of the first 21
191 nucleotides encoding the *marbox* sequence. Figure 5 shows that the *marbox* sequence
192 alone is sufficient to restore full resistance to *acrR* deletion mutants, mimicking the
193 resistance seen in the *acrR*(L34Q) mutant.
194
195 *3.4 acrAB expression is not induced by psoralen, UVA, or psoralen-UVA irradiation.*
196 The results above demonstrate that the AcrR transcriptional regulator and
197 activation by either MarA SoxS or Rob are required toupregulate *acrAB* and confer to
198 crosslink resistance. However, how *acrAB* is regulated in wild-type cells during
199 challenge with psoralen-UVA is unknown. Previous studies have shown that exposure to
200 others stressors and agents- including ethidium bromide, cadaverine, ethanol, or high
201 osmolarity, can induce expression of *acrAB* to increase resistance [27, 42]. To examine
202 if *acrAB* expression is responsive to psoralen-UVA treatments, we used a plasmid that
203 contained the *acrAB* promotor region fused to *lacZ*. The cloned promoter region contains
204 both the AcrR binding site as well as the first 102 nucleotides of *acrR* coding sequence
205 which contains the *marbox* binding site. To test if psoralen, UVA irradiation, or the
206 presence of interstrand crosslinks can serve to induce *acrAB* expression, cultures
207 containing the plasmid were spotted in 10-µL serial dilutions on X-Gal plates that were
208 left untreated or exposed to either psoralen, UVA, or psoralen-UVA. As shown in Figure
209 6A, in the presence of the *acrABp-lacZ* reporter, the parental strain detectably expressed

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210 the *acrAB* genes as indicated by the partially blue colonies, relative to the control
211 plasmid. As controls, we also examined the *acrR*(L34Q) resistant mutant and the
212 sensitive *marA soxS rob* deletion mutant. As expected, *acrAB* expression was elevated in
213 *acrR*(L34Q) mutant as indicated by the intensely blue colonies, correlating with the
214 increased expression of *acrAB* and resistance in this strain. Similarly, colonies were
215 noticeably less blue in the *marA soxS rob* deletion background which correlates with
216 reduced *acrAB* expression and hypersensitivity.
217 Notably however, expression did not increase in the presence of either psoralen,
218 UVA, or psoralen plus UVA treatments. The results imply that psoralen, UVA, or the
219 combination do not generate substrates that activate *acrAB* and suggest these regulators
220 are not normally responsive to this challenge, in the absence of mutation.
221

222 **4. Discussion**

223 The results demonstrate that all three of the related global regulators MarA, SoxS,
224 and Rob have a significant role in psoralen-UVA resistance. However, regulation by
225 these activators was found to be complex. Deletion of any single global effector gene in
226 wild-type cells had a similar impact on psoralen-UVA resistance as deleting all three
227 genes. This result is unexpected for several reasons. First, although *rob* is expressed
228 constitutively, *marA* and *soxS* are expressed at relatively low levels until a specific
229 stressor induces their expression [31, 32, 34, 35, 39, 43]. Additionally, the activity of Rob
230 has been shown to be responsive to its recognition of various substrates and its release
231 from sites of sequestration in the cell [40, 44]. Yet despite these regulators responding to
232 different stressors, Fig. 2 demonstrates that no single effector is responsible for initiating
233 the stress response to psoralen-UVA irradiation. Second, given the high level of
234 homology between MarA, SoxS, and Rob, and their ability to bind the same *marbox* sites
235 across the genome, one might expect that loss of one regulator could be offset by the
236 presence of the other two [31, 32, 34, 35, 39, 45]. Yet this is not observed in wild-type
237 cells. On the other hand, if no redundancy existed, one might expect that deleting *marA*,
238 *soxS*, and *rob* would have an additive effect on psoralen-UVA sensitivity. This is also not
239 observed (Fig. 2). Thus, the apparent any-and-all requirement for MarA, SoxS, and Rob
240 could suggest that crosstalk between these activators is particularly important in psoralen-
241 UVA resistance. Alternatively, it is possible that the sensitivity of this assay to
242 distinguish phenotypic differences decreases as the limits of detectability are approached.
243 Irrespective of the crosstalk, the AcrAB-TolC transporter appears to be the causal
244 target for MarA, SoxS, and Rob generating psoralen crosslink resistance, since the
245 hypersensitive phenotype of these mutants can be suppressed by overexpression of
246 AcrAB alone and does not require any of the other approximately 50 genes under their
247 regulation [31–40]and Fig 3).
248 The results may also suggest a more complex mechanism of regulation by AcrR
249 than that of a simple repressor. The *acrR*(L34Q) point mutation is resistant to psoralen-
250 UVA treatment and retains the *marbox* activation sequence, yet a deletion of the *acrR*
251 open reading frame that deletes the *marbox* activation sequence renders cells sensitive. If
252 AcrR acts as a basic repressor, then the simplest model would be that *acrR*(L34Q) is a
253 null mutation, and that upregulating *acrAB* expression enough to provide full resistance
254 requires both removal of the AcrR repressor and activation by MarA, SoxS, and Rob. The

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255 finding that removal of all three proteins renders the *acrR*(L34Q) mutant similar in
256 resistance to the *acrR* deletion supports this model (Figure 4D). In contrast to what was
257 seen in wild-type cells (Fig 2), deletion of *marA*, *soxS*, or *rob* individually was
258 insufficient to noticeably reduce resistance in *acrR*(L34Q). As mentioned above, one
259 possible explanation would be that the sensitivity of the survival assays used in this study
260 are insufficient to distinguish small differences between hypersensitive strains, as their
261 numbers approach the lower end of detectability. If true, increasing the background level
262 of resistance through de-repression of *acrAB* expression as in Figure 4 could make the
263 differences between individual *marA*, *soxS*, and *rob* deletion mutants and the
264 *marAsoxSrob* triple mutant more detectable.
265 The most prominent model for AcrR repressor function is that it releases upon
266 binding a recognized substrate [27–29, 46, 47]. These initial studies used both LacZ
267 reporter constructs and gel-shift binding assays to provide strong evidence that AcrR can
268 repress expression of *acrAB* when bound to its promoter [27]. However, other aspects of
269 this study also suggested more complexity in its function. Transcription of the *acrR*
270 repressor was also induced by the same stressors that upregulated *acrAB* transporter
271 expression, and *acrAB* upregulation during ethanol stress or growth phase occurred
272 independently of MarA and SoxS, similar to what we observe in the presence of psoralen
273 [27]. The repressor model of AcrR activity is based on its similarity to other TetR family
274 transcriptional regulators. It proposes that upon ligand binding, AcrR releases from DNA
275 to allow transcription. However the AcrAB-TolC transporter is active on a wide range of
276 structurally divergent substrates [42, 47], making it unclear how the protein could
277 effectively recognize this diverse range of toxic substrates. The few substrates which
278 have been examined and shown to promote AcrR release from oligos *in vitro* have been
279 DNA intercalators [28, 29, 46] which makes it difficult to determine if release is due to
280 ligand binding or changes to the DNA structure of the oligos used. Further, studies
281 looking for upregulation of AcrAB following treatments with known substrates of the
282 pump have seen modest to no effect [42, 47]. Thus, the mechanism of regulation and
283 natural substrate for induction of the efflux pump remains unclear.
284 This ambiguity of the regulatory mechanism also holds true for resistance to
285 psoralen interstrand crosslinks. Although psoralen is clearly a substrate for the efflux
286 pump [11], AcrR does not appear to be naturally responsive to this stress, as neither
287 psoralen, UVA, nor crosslink induction upregulates *acrAB* expression. This perhaps
288 makes sense given that the cell must acquire mutations that disrupt AcrR repression of
289 the efflux pump to achieve resistance. Importantly, we show here that full resistance is
290 only achieved when *acrR* null mutations preserve the *marbox* sequence (Figure 5), as in
291 *acrR*(L34Q). This is particularly relevant to the emergence of multi-drug resistance in
292 Gram-negative bacteria, as mutations in *acrR* are known to drive multi-drug resistance
293 and are commonly found in clinical isolates [48–52]. Additionally, the original studies
294 that characterized AcrR used insertion mutants that disrupted the protein after the
295 *marbox*, while later studies used the complete deletion mutant of *acrR* from the Keio
296 collection [26, 27, 41, 42, 47]. Our results demonstrate that it will be important in the
297 future to consider the impact of *acrR* mutations on the *marbox* when assessing their
298 phenotypic effects. Finally, it is also notable that the resistance to psoralen interstrand
299 crosslinks is achieved by preventing this drug from forming this lesion. No mutations

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300 upregulating repair pathways or proteins were observed in the initial screen, consistent
301 with previous studies that found cells lack effective repair mechanisms for this form of
302 damage [6–8, 53].
303
304 **Competing interests:** None declared.
305
306 **Ethical approval:** Not required.
307
308 **Data Availability**.
309 All data is contained within the manuscript. Data, strains, and plasmids used in
310 this work are available upon request to the corresponding author.
311
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316

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317 **Table 1.** List of strains used in this study.

Strain	Relevant Genotype	Source or Construction
BW25113	*lacIq rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78*	[54]
JW0453	*acrR*::FRT-minikan	[41]
JW5249	*marA*::FRT-minikan	[41]
JW4023	*soxS*::FRT-minikan	[41]
JW4359	*rob*::FRT-minikan	[41]
CL5312	*marA*::FRT	pCP20-mediated [55] removal of minikan from JW5249
CL5317	*marA*::FRT soxS::FRT-minikan	P1 transduction of *soxS*::FRT-minikan from JW4023 into CL5312
CL5322	*marA*::FRT *soxS*::FRT	pCP20-mediated [55] removal of minikan from CL5317
CL5414	*marA*::FRT *soxS*::FRT *rob*::FRT-minikan	P1 transduction of *rob*::FRT-minikan from JW4359 into CL5322
CL5230	*acrR*(L34Q)	[11]
CL5323	*acrR*(L34Q) *soxS*::FRT-minikan	P1 transduction of *soxS*::FRT-minikan from JW4023 into CL5230
CL5324	*acrR*(L34Q) *marA*::FRT-minikan	P1 transduction of *marA*::FRT-minikan from JW5249 into CL5230
CL5325	*acrR*(L34Q) *rob*::FRT-minikan	P1 transduction of *rob*::FRT-minikan from JW4359 into CL5230
CL5433	*acrR*(L34Q) *marA*::FRT	pCP20-mediated [55] removal of minikan from CL5324
CL5436	*acrR*(L34Q) *marA*::FRT *soxS*::FRT-minikan	P1 transduction of *soxS*::FRT-minikan from JW4023 into CL5433
CL5438	*acrR*(L34Q) *marA*::FRT *soxS*::FRT	pCP20-mediated [55] removal of minikan from CL5436
CL5440	*acrR*(L34Q) *marA*::FRT *soxS*::FRT *rob*::FRT-minikan	P1 transduction of *rob*::FRT-minikan from JW4359 into CL5438
CL5442	*acrR* (aa 7 – 215)::FRT-minikan	Recombineering to replace amino acids 7 – 215 of *acrR* in BW25113 with FRT-minikan
CL5333	pBAD33	[11]
CL5334	pBAD33-acrAB	[11]
CL5415	*marA*::FRT-minikan pBAD33	Transformation of pBAD33 [56] into JW5249
CL5416	*marA*::FRT-minikan pBAD33-*acrAB*	Transformation of pBAD33-*acrAB* [56] into JW5249
CL5417	*soxS*::FRT-minikan pBAD33	Transformation of pBAD33 [56] into JW4023
CL5418	*soxS*::FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* [56] into JW4023
CL5419	*rob*::FRT-minikan pBAD33	Transformation of pBAD33 [56] into JW4359
CL5420	*rob*::FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* [56] into JW4359
DH7169	pNN387	[57]
CR6000	pNN608	[27]
CL5402	BW25113 + pNN387	Transformation of pNN387 [57] into BW25113
CL5403	BW25113 + pNN608	Transformation of pNN608 [27] into

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		BW25113
CL5421	*acrR*(L34Q) + pNN387	Transformation of pNN387 [57] into CL5230
CL5422	*acrR*(L34Q) + pNN608	Transformation of pNN608 [27] into CL5230
CL5530	*marA*::FRT *soxS*::FRT *rob*::FRT-minikan + pNN387	Transformation of pNN387 [57] into CL5414
CL5531	*marA*::FRT *soxS*::FRT *rob*::FRT-minikan + pNN608	Transformation of pNN608 [27] into CL5414
CL5532	*acrR*(L34Q) *marA*::FRT *soxS*::FRT *rob*::FRT-minikan +pNN387	Transformation of pNN387 [57] into CL5440
CL5533	*acrR*(L34Q) *marA*::FRT *soxS*::FRT *rob*::FRT-minikan +pNN608	Transformation of pNN608 [27] into CL5440

318
319 **References**
320
321
322
323 FIGURE LEGENDS
324 **Figure 1.** Current model of MarA, SoxS, and Rob global gene regulation. Green, MarA;
325 blue, SoxS; purple, Rob; red, AcrR; yellow, DNA binding sites; orange, Mar/Sox/Rob
326 binding site (*marbox*). Arrows indicate activation, while interruption of the end of a line
327 indicates repression (derived from models and data presented in [26, 31, 33, 36, 37, 45]).
328
329 **Figure 2.** *acrR*(L34Q), but not deletion of *acrR*, confers resistance to psoralen–UVA,
330 while deletion of *marA*, *soxS*, or *rob* renders cells hypersensitive. A) The survival of
331 wild-type cells (filled squares); Δ*acrR* (filled circles), and *acrR*(L34Q) mutants (open
332 circles), B) Δ*marA* (filled triangles), Δ*soxS* mutant (filled inverted triangles), Δ*rob*
333 mutants (filled diamonds) and C) Δ*marA* Δ*soxS* Δ*rob* mutants (open diamonds) is plotted
334 following UVA irradiation at the indicated doses in the presence of 20 µg/mL 8-
335 methoxypsoralen. Plots represent the average of at least two independent experiments.
336 Error bars represent the standard error of the mean. Wild type is replotted in each graph
337 for comparison.
338
339 **Figure 3**. Overexpression of AcrAB alone is sufficient to restore psoralen-UVA
340 resistance in Δ*marA*, Δ*soxS*, and Δ*rob* mutants. The survival of wild type (squares),
341 Δ*marA* (triangles), Δ*soxS* (inverted triangles), and Δ*rob* (diamonds) containing either an
342 empty pBAD33 expression vector (filled symbols) or an AcrAB expression vector (open
343 symbols) is plotted following UVA irradiation at the indicated doses in the presence of 20
344 µg/mL 8-methoxypsoralen. Plots represent the average of at least two independent
345 experiments. Error bars represent the standard error of the mean. Wild type is replotted in
346 each graph for comparison.
347
348 **Figure 4**. *acrR*(L34Q) psoralen-UVA resistance requires MarA, SoxS, and Rob
349 activation. The survival of wild-type cells (filled squares), *acrR*(L34Q) (open circles),
350 Δ*marA* (closed triangles), *acrR*(L34Q) Δ*marA* (open triangles); (B) Δ*soxS* (filled inverted
351 triangles), *acrR*(L34Q) Δ*soxS* (open inverted triangles); (C) Δ*rob* (filled diamonds), and

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352 *acrR*(L34Q) Δ*rob* (open diamonds); (D) Δ*marA* Δ*soxS* Δ*rob* mutants (open squares),
353 *acrR*(L34Q) Δ*marA*Δ*soxS*Δ*rob* (filled circles) in the presence of 20 μg/mL 8-
354 methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at
355 least two independent experiments. Error bars represent the standard error of the mean.
356 Wild type is replotted in each graph for comparison.
357
358 **Figure 5.** The *marbox* sequence is necessary and sufficient to induce resistance to
359 psoralen interstrand crosslinks in the absence of the AcrR repressor. The survival of wild-
360 type cells (filled squares), Δ*acrR* (filled circles), *acrR*(L34Q) (open circles), and Δ*acrR*
361 (aa7-215) (filled triangles) in the presence of 20 μg/mL 8-methoxypsoralen at the
362 indicated UVA doses is plotted. Plots represent the average of at least two independent
363 experiments. Error bars represent the standard error of the mean.
364
365 **Figure 6**. *acrAB* expression is not upregulated by psoralen, UVA, or psoralen-UVA. 10
366 μL spots of 10^4 cells of wild type, *acrR*(L34Q), or Δ*marA* Δ*soxS* Δ*rob*, mutants
367 containing a LacZ reporter plasmid fused with the *acrAB* promoter region (p-*acrAB-lacZ*)
368 or no promoter region (empty vector) were plated on LB plates containing X-Gal. Plates
369 contained 20 μg/mL 8-methoxypsoralen and were UVA irradiated with 3.8 kJ/m2 as
370 indicated. LacZ expression from the plasmids is indicated by blue color in colonies.

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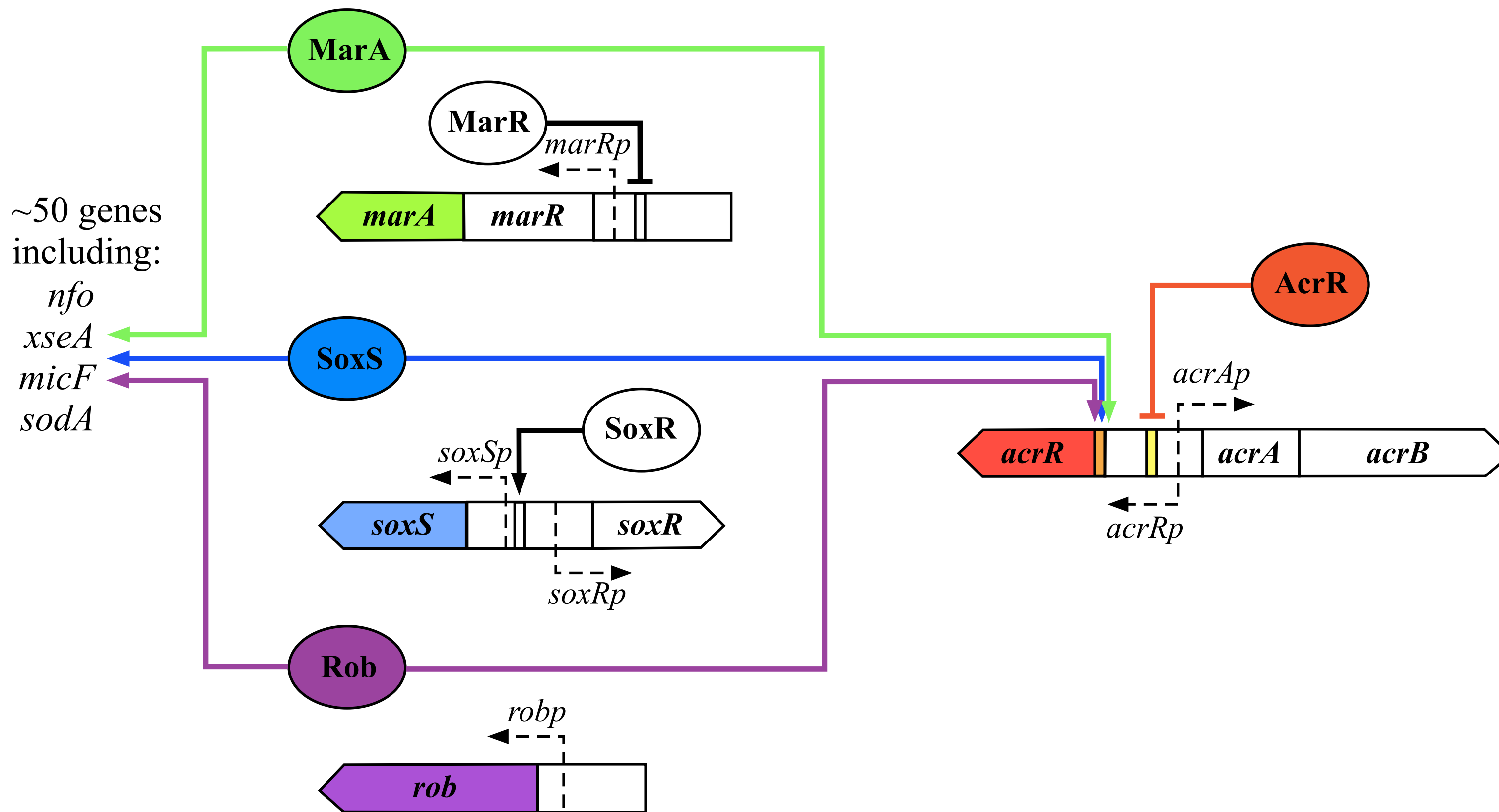

Figure 1. Current model of MarA, SoxS, and Rob global gene regulation. Green, MarA; blue, SoxS; purple, Rob; red, AcrR; yellow, DNA binding sites; orange, Mar/Sox/Rob binding site (*marbox*). Arrows indicate activation, while interruption of the end of a line indicates repression (derived from models and data presented in {Ma et al., 1995; Martin et al., 2000; Martin and Rosner, 2002; Skarstad et al., 1993; Greenberg et al., 1990; Nunoshiba, 1996).

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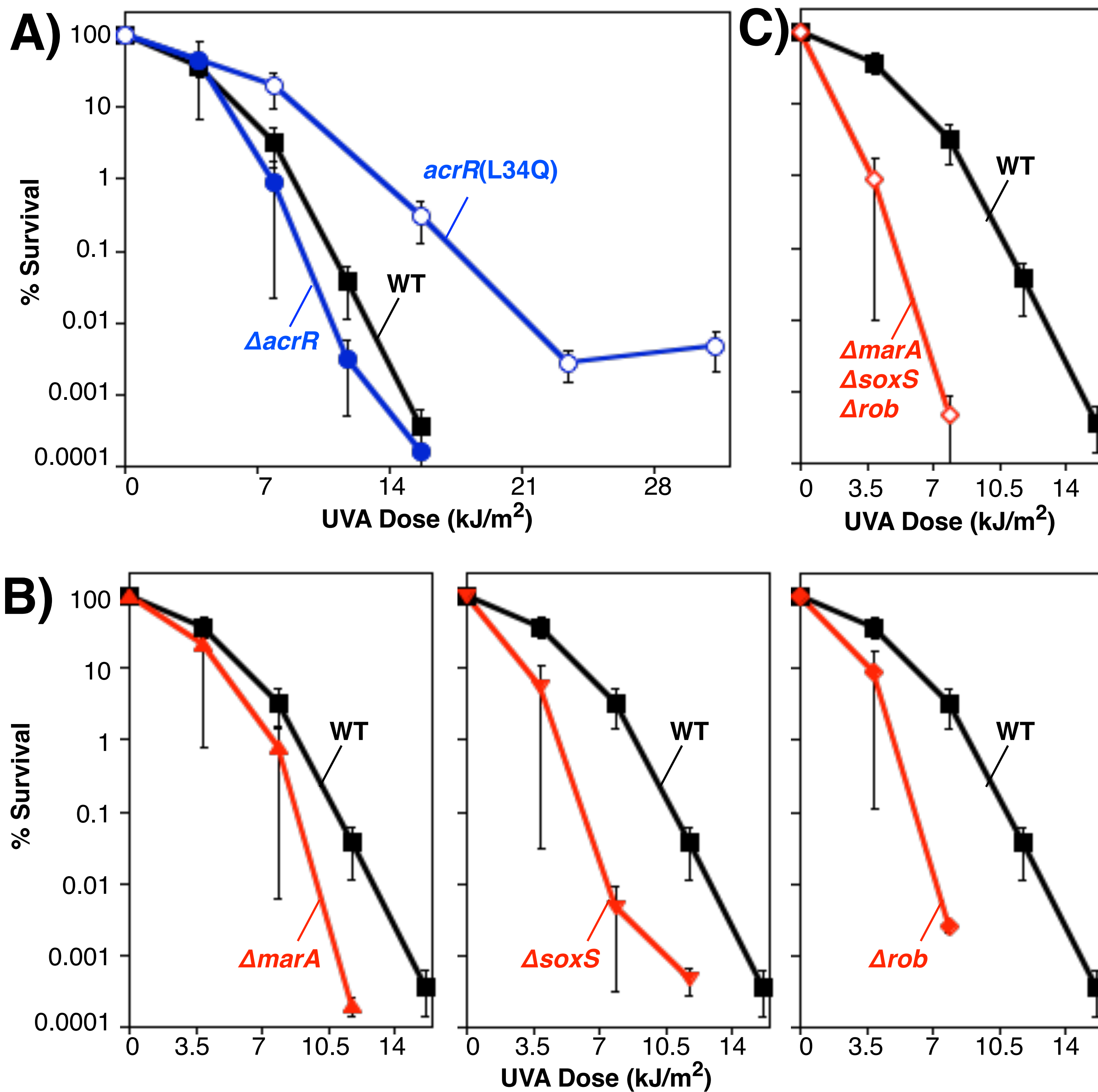

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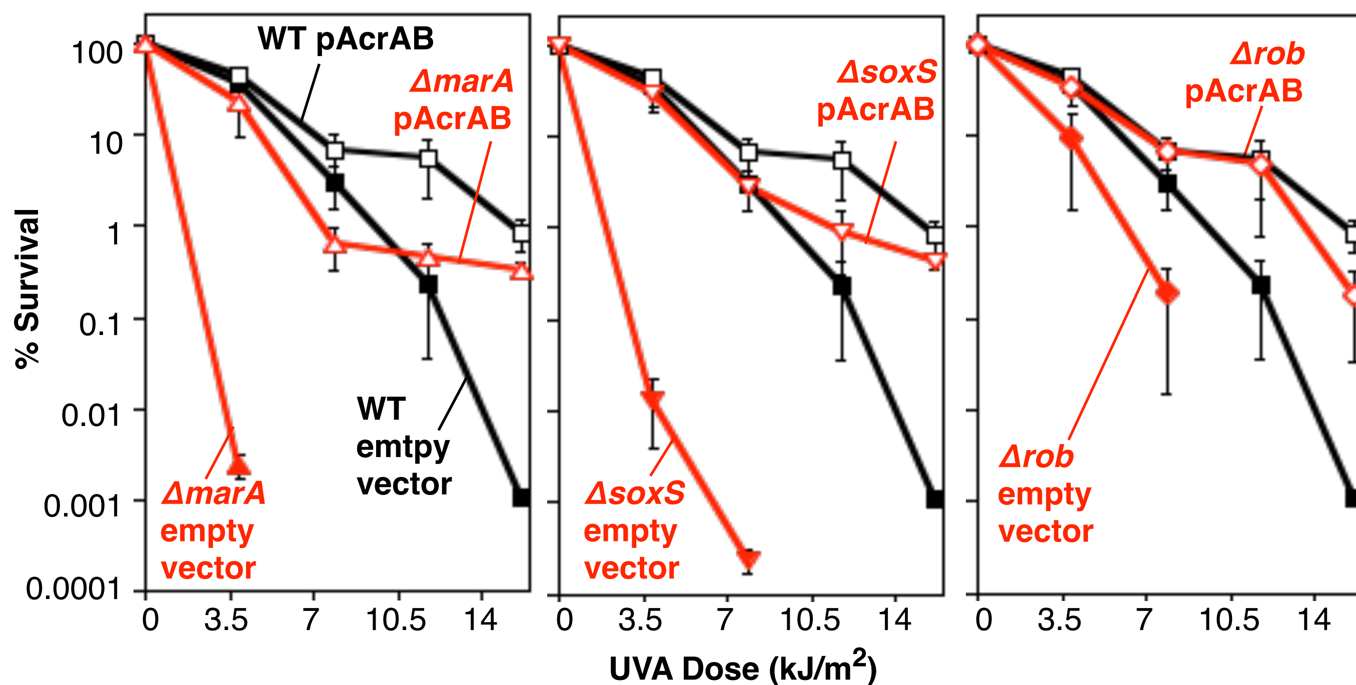

Figure 3. Overexpression of AcrAB alone is sufficient to restore psoralen-UVA resistance in Δ*marA*, Δ*soxS*, and Δ*rob* mutants. The survival of wild type (squares), Δ*marA* (triangles), Δ*soxS* (inverted triangles), and Δ*rob* (diamonds) containing either an empty pBAD33 expression vector (filled symbols) or an AcrAB expression vector (open symbols) is plotted following UVA irradiation at the indicated doses in the presence of 20 μg/mL 8-methoxypsoralen. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean. Wild type is replotted in each graph for comparison.

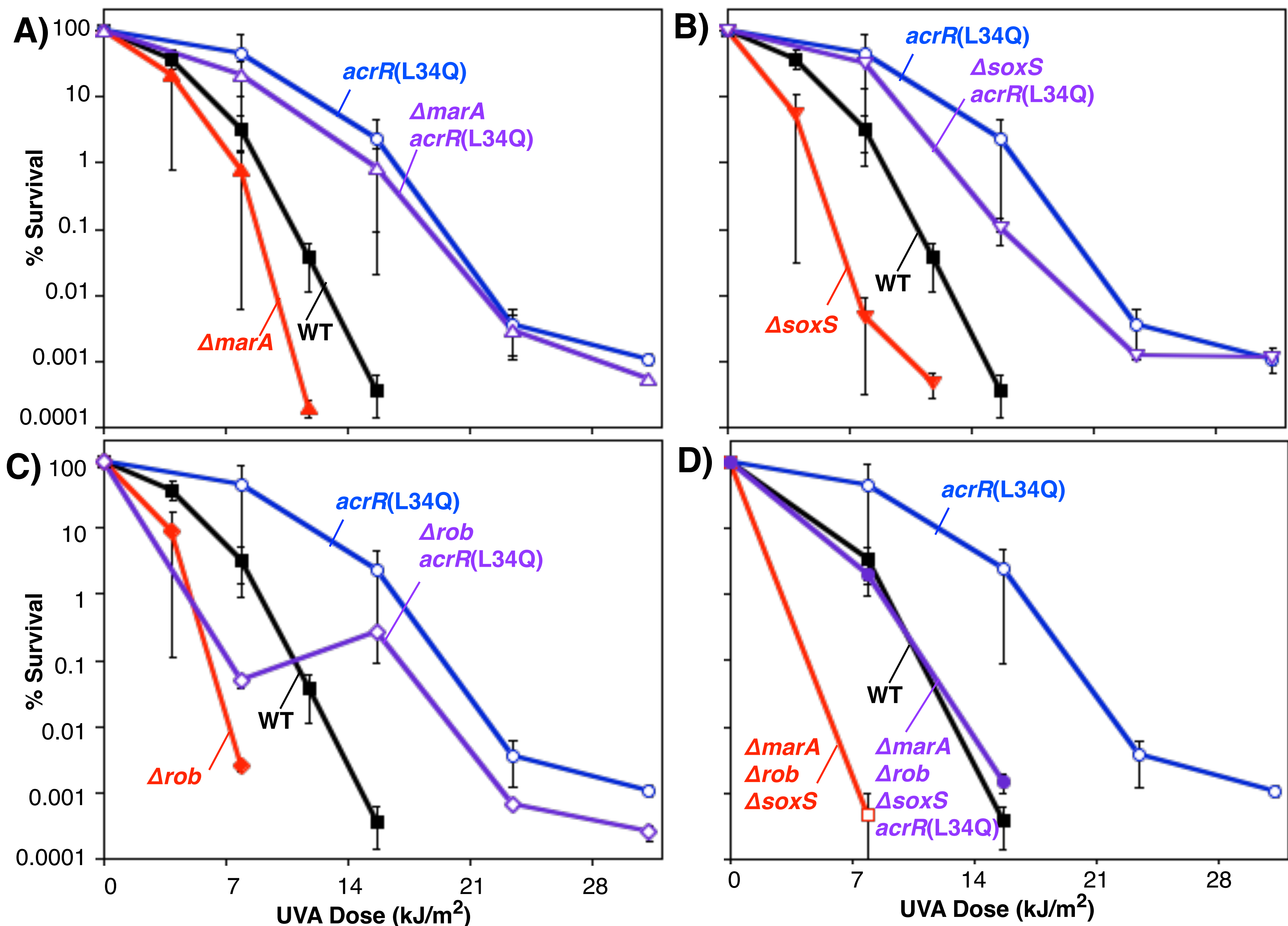

Figure 4. *acrR*(L34Q) is dependent on MarA, SoxS, and Rob for full confers psoralen-UVA resistance. independent of MarA, SoxS, and Rob activation. The survival of wild-type cells (filled squares), *acrR*(L34Q) (open circles), ΔmarA (closed triangles), *acrR*(L34Q) ΔmarA (open triangles); (B) Δ*soxS* (filled inverted triangles), *acrR*(L34Q) Δ*soxS* (open inverted triangles); (C) Δ*rob* (filled diamonds), and *acrR*(L34Q) Δ*rob* (open diamonds); (D) Δ*marA* ΔsoxS Δ*rob* mutants (open squares), *acrR*(L34Q)Δ*marA*Δ*soxS*Δ*rob* (filled circles) in the presence of 20 µg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean. Wild type is replotted in each graph for comparison.

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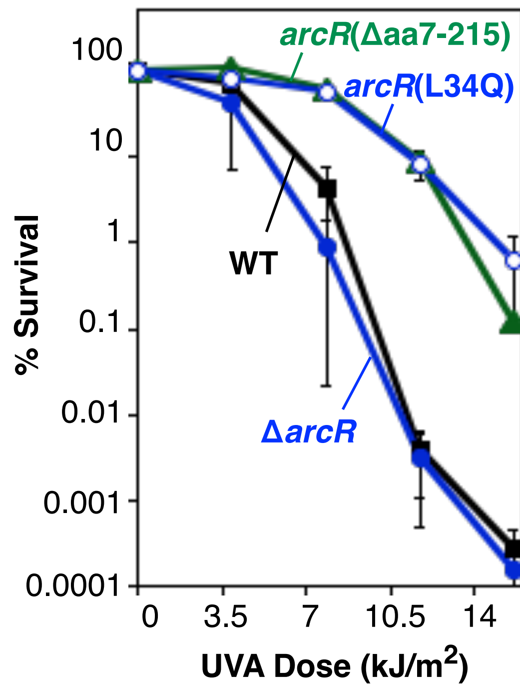

Figure 5. Only *acrR* null mutations that preserve the *marbox*, like *acrR*(L34Q), confer resistance to psoralen-UVA. The survival of wild-type cells (filled squares), Δ*acrR* (filled circles), *acrR*(L34Q) (open circles), and Δ*acrR* (aa7-215) (open triangles) in the presence of 20 µg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean.

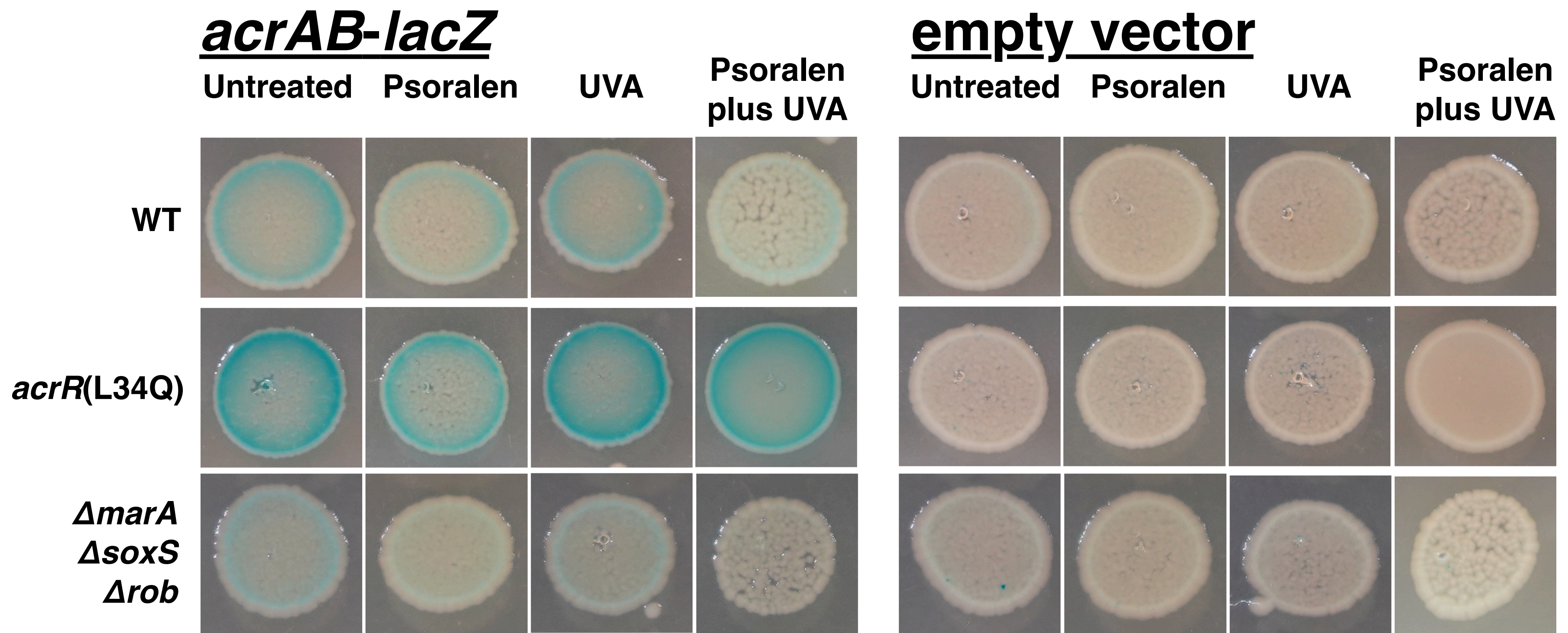

Figure 6. *acrAB* expression is not upregulated by psoralen, UVA, or psoralen-UVA. 10 μL spots of 10^4 cells of wild type, *acrR*(L34Q), and Δ*marA* Δ*soxS* Δ*rob*, mutants containing a LacZ reporter plasmid fused with the *acrAB* promoter region (p-*acrAB*-lacZ) or no promoter region (empty vector) were plated on LB plates containing X-Gal. Plates contained 20 μg/mL 8-methoxypsoralen and were UVA irradiated with 3.8 kJ/m2 as indicated. LacZ expression from the plasmids is indicated by blue color in colonies.