CsrA-controlled proteins reveal new dimensions of *Acinetobacter baumannii* desiccation tolerance

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ABSTRACT

Hospital environments are excellent reservoirs for the opportunistic pathogen *Acinetobacter baumannii* in part because it is exceptionally tolerant to desiccation. We found that relative to other *A. baumannii* strains, the virulent strain AB5075 was strikingly desiccation resistant at 2% relative humidity (RH), suggesting that it's a good model for studies of the functional basis of this trait. Consistent with results from other *A. baumannii* strains at 30% RH, we found the global post-transcriptional regulator CsrA to be critically important for desiccation tolerance of AB5075 at 2% RH. To identify CsrA-controlled proteins that may contribute to desiccation tolerance we used proteomics to identify proteins that were differentially present in wild type and *csrA* mutant cells. Subsequent mutant analysis revealed nine genes that were required for wild type levels of desiccation tolerance, five of which had modest phenotypes. Catalase and a universal stress protein gene were moderately important for desiccation tolerance and two genes of unknown function had very strong desiccation phenotypes. The predicted amino acid sequence of one of these genes predicts an intrinsically disordered protein. This category of proteins is widespread in eukaryotes but less so in prokaryotes. Our results suggest there may be mechanisms responsible for desiccation tolerance that have not previously been explored in bacteria.

IMPORTANCE

Acinetobacter baumannii is commonly found in terrestrial environments but can cause nosocomial infections in very sick patients. A factor that contributes to the prevalence of *A. baumannii* in hospital settings is that it is intrinsically resistant to dry conditions. Here, we established the virulent strain *A. baumannii* AB5075 as a model for studies of desiccation tolerance at very low relative humidity. Our results show that this trait depends on two proteins of unknown function, one of which is predicted to be an intrinsically disordered protein. This category of protein is critical for the small animals named tardigrades to survive desiccation. Our

27 results suggest that *A. baumannii* may have novel strategies to survive desiccation that have
28 not previously been seen in bacteria.
29

30 **INTRODUCTION**

31 Hospital-acquired infections are an important healthcare concern and economic burden (1, 2)
32 and environmental persistence plays a critical role in the transmission of bacteria that cause
33 these infections (3–6). One such bacterium is *Acinetobacter baumannii,* an opportunistic
34 pathogen that infects very sick patients. It is responsible for about 2% of nosocomial infections
35 in the United States and Europe and the frequencies are higher in the rest of the world. *A.*
36 *baumannii* is especially problematic because on a global basis, about 45% of isolates are multi-
37 drug resistant (7). A factor that contributes to the prevalence of *A. baumannii* in hospital settings
38 is desiccation tolerance. *A. baumannii* can survive in a desiccated state on inanimate dry
39 surfaces for days to several months (8–10). These surfaces include materials that are often
40 encountered in the hospital, such as polyvinyl chloride, rubber, and stainless steel (11) .
41

42 When desiccated, bacteria must respond to diverse stresses that include accumulation of
43 reactive oxygen species, loss of cytoplasmic volume, and loss of cell membrane integrity (12,
44 13). Proteomics analysis of *A. baumannii* showed that desiccated cells had higher levels of
45 proteins involved in protein stabilization, antimicrobial resistance, and reactive oxygen species
46 detoxification (14). Attributes of *A. baumannii*, that have been shown to be associated with
47 desiccation tolerance include biofilm formation (15, 16) and protein aggregation (17). $LpxM_{AB}$-
48 dependent acetylation of lipid A is essential for survival of *A. baumannii* ATCC17978 at 40% RH
49 (18), and a *recA* mutant of ATCC17978, defective in DNA repair, had a pleiotropic phenotype,
50 including a defect in desiccation tolerance (19). *katE*, encoding catalase also contributes to
51 desiccation tolerance (20). Despite these observations, the number of genes identified in *A.*
52 *baumannii* that are specifically involved in desiccation tolerance is small. This could be because

53 *A. baumannii* cells have evolved modified cell structures that are both essential for viability and
54 important for desiccation tolerance. The genetic basis for this would be difficult to uncover in
55 mutant screens. It is also possible that the conditions of desiccation used, typically 30% RH in
56 studies to date, were not sufficiently severe to allow identification of some desiccation tolerance
57 genes.
58
59 With the goal of identifying new desiccation tolerance genes, we established *A. baumannii* strain
60 AB5075 as a model for studying desiccation tolerance under severe conditions of 2% RH. We
61 then followed up on a recent report showing that *csrA,* which encodes a global post-
62 transcriptional regulator, is important for desiccation tolerance (21) and identified nine CsrA-
63 controlled proteins that confer desiccation tolerance on AB5075 at 2% RH. One of these has
64 predicted properties that suggest new dimensions of desiccation tolerance.
65
66 **RESULTS**
67 **Desiccation assay.** Previous studies have shown that *A. baumannii* can survive in a desiccated
68 state for days to several months (8–11, 20). For these and other desiccation studies,
69 investigators worked with a variety of strains and usually incubated cells at either 30% RH or in
70 room air, which varied between 25 and 61% RH in one study (20). These differences can make
71 it difficult to compare desiccation phenotypes between studies. Thus we thought it important to
72 establish a robust desiccation assay that reduces experimental variables like choice of strains,
73 drying times, and RH during desiccation, using a highly desiccation resistant strain.
74
75 Following from previous reports, saturated calcium chloride hexahydrate solution placed in a
76 sealed plastic Snapware container caused the RH inside the container to rapidly equilibrate to
77 30% (9). We found that use of DRIERITE instead of calcium chloride, resulted in an RH of 2%.
78 To test desiccation tolerance under different conditions, we grew bacteria to a desired density in

79 Trypticase Soy Broth (TSB), harvested them, washed them twice, and resuspended them in
80 phosphate buffer to a final OD_{600} of 1. Drops of cell suspension were placed on polycarbonate
81 membranes and filtered to allow for rapid drying. The membranes were then placed in
82 uncapped 15 ml conical centrifuge tubes and incubated in desiccation containers. After various
83 periods of incubation, buffer was added to each centrifuge tube followed by 5 min of shaking on
84 a rotary shaker. Viable cell numbers were then determined by plating on TSB agar. To control
85 for the stress of filtration we did viable counts immediately following filtration and took this as our
86 "day 0" time point.
87
88 **Relative desiccation tolerance of *A. baumannii* strains**. As shown in Fig 1A, *A. baumannii*
89 strain AB5075 and *Escherichia coli* strain W3100 each survived desiccation at 30% RH far
90 better than *Pseudomonas aeruginosa* strain PAO1. However at 2% RH, *A. baumannii* survived
91 far better than either *E. coli* or *P. aeruginosa*. As has been reported (20, 22), we found that *A.*
92 *baumannii* stationary phase cells were much more tolerant to desiccation than actively growing
93 cells (Fig. S1) and so we routinely used stationary phase cells in our desiccation assays. We
94 compared the desiccaton tolerance of AB5075 to two additional frequently used laboratory
95 strains of *A. baumannii*, ATCC17978 and ATCC19606. We found that AB5075 was strikingly
96 more resistant than the others at 2% RH and somewhat mores resistant at 30% (Fig. 1B).
97 Given the strength of its phenotype, these findings indicate that AB5075 is a good model for
98 studing desiccation tolerance. This strain was isolated from a surgical wound, is multidrug
99 resistant and is highly virulent in an animal model (23). A comprehensive ordered mutant library
100 of AB5075 is available that has two to three seqenced Tn insertions in each gene and is called
101 the three-allelle library (24).
102
103 **CsrA is critical for desiccation tolerance.** We examined the contribution of the post-
104 transcriptional regulator CsrA to desiccation tolerance of AB5075 by constructing a *csrA*

105 deletion mutant (Δ*csrA*). We found that the mutant grew poorly on TY agar and had an
106 elongated cell morphology when grown in TY broth (Fig. 2A). On agar plates, large colonies
107 frequently appeared on a backgound of poor growth, likely due to occurance of second site
108 suppressor mutations in the Δ*csrA* strain. The Δ*csrA* strain was also defective in growth on other
109 nutrient-rich media, including LB broth, nutrient broth, and TSB broth. A similar sensitivity to
110 growth in complex media was reported by Farrow et al. for several *A. baumannii* strains
111 including strain AB5075 (21). In agreement with Farrow et al., a Δ*csrA* mutant grew as the wild
112 type in defined medium, in our case, M9 minimal medium with 10 mM succinate as a sole
113 carbon source (M9/succinate), and it had close to a wild type cell morphology (Fig 2A). A
114 *Yersinia enterocolitica csrA* mutant, has a growth defect in LB due to the presence of 90 mM of
115 NaCl (25). However, the *A. baumannii* Δ*csrA* mutant was not sensitive to this level of NaCl. In
116 fact, the mutant grew in M9/succinate supplemented with up to 100 mM of NaCl without a
117 significant reduction of growth compared to the wild type.
118
119 When desiccated after growth in M9/succinate to stationary phase, the AB5075 Δ*csrA* mutant
120 lost almost all viability over 6 days (Fig. 2B and Table 1). The desiccation phenotype was
121 complemented by expressing *csrA in trans*. Δ*csrA* mutant cells incubated for 6 days after being
122 filtered and resuspended in PBS remained fully viable (Fig 2B).
123
124 **CsrA affects mutiple cellular processes in *A. baumannii*.** To identify genes whose
125 translation might be regulated by CsrA, we compared the proteomes of wild-type and Δ*csrA*
126 cells (Table S2A). There were 97 proteins present at higher levels in the Δ*csrA* mutant
127 compared to the wild type (ratio of Δ*csrA*/WT ≥2.5, Table S2B). Among these were proteins for
128 type IV pilus assembly, synthesis of the siderophore ferric acinetobactin, and a
129 glutamate/aspartate transporter. The Δ*csrA* mutant also had elevated levels of enzymes for the

130 catabolism of hydroxcinnamates, phenylacetate and quinate. Levels of an alcohol
131 dehydrogenase (ABUW_1621) and an aldehyde dehydrogenase (ABUW_1624) were also
132 elevated. The Δ*csrA* mutant was defective in pilus-mediated twiching motility as assessed by
133 movement aross a soft-agar plate (Fig. 3A). The mutant also had a severe growth defect when
134 grown on succinate in the presence of ethanol (Fig. 3B). One possible explanation for this is that
135 the *csrA* mutant metabolized ethanol to form toxic acetaldehyde to levels that slowed growth.
136
137 There were 106 proteins present in lower amounts in the Δ*csrA* mutant compared to the wild
138 type (ratio of WT/Δ*csrA* ≥ 2.5, Table S2C). A large proportion of these (39%) are annotated as
139 hypothetical proteins. Several membrane proteins, and proteins annotated as involved in *β*-
140 lactam antibiotic resistance (ABUW_1194, 2619, and 3497), trehalose synthesis (ABUW_3123)
141 and possibly biofilm formation (ABUW_0916) were in lower abundance in the Δ*csrA* mutant
142 compared to wild type. As reported previously, a Δ*csrA* mutant did not form biofilms (21). This
143 phenotype was complemented by expressing the *csrA* gene *in trans* (Fig. 3C). The Δ*csrA*
144 proteome profile also suggested that CsrA is invovled in promoting the expression of proteins
145 invovled in oxidative stress, including peroxidase (ABUW_0628) and catalase (*katE*,
146 ABUW_2436). Indeed the Δ*csrA* mutant lacked detectable catalase activity (Fig. 3D).
147
148 **Genes important for desiccation tolerance in *A. baumannii* AB5075.** We took advantabge
149 of the three-allele transposon library to test how important some of the gene transcripts that
150 were likely to be controlled by CsrA were for desiccation tolerance (Tables 1 and S1). When
151 possible, we tested two different transposon mutants (transposon insertions in different
152 positions of the gene) for each gene. *A. baumannii* AB5075 produces opaque and translucent
153 colony variants that interconvert at high frequency and reflect changes in the thickness of
154 capsular exopolysaccharide (26). AB5075 cells with decreased capsule production are about

155 2.5-fold more sensitive to desiccation (27). Here, we used only opaque colonies of AB5075 and
156 its mutant derivatives in our desiccation assays.
157
158 We found that *katE* and *ABUW_2639* mutants were about 5-fold more sensitive to desiccation
159 than the wild type, whereas *ABUW_2433* and *ABUW_2437* mutants were greater than 100-fold
160 more sensitive to desiccation than the wild type (Table 1, Fig 4). The phentoytpes of
161 *ABUW_2433* and *ABUW_2437* mutants could be complemented (Fig S2). The ABUW_2433
162 protein has 411 amino acids and is annotated as a KGG domain-containing protein. The KGG
163 domain comprises a small region in the N-terminus of the protein and the remainder of the
164 protein is annotated by InterPro as a disordered region that includes a series of AT_hook DNA
165 binding motifs (SMART SM00384). The full length ABUW_2433 sequence was predicted to be
166 intrinsically unstructured when queried with the IUPred3 tool (https://iupred.elte.hu) (28).
167 ABUW_2437 is annotated as an iron-containing redox enzyme or a heme-oxygenase -like
168 protein (Fig 4). The predicted *ABUW_2437* transcript has traits characterisitic of a target of CsrA
169 post-transcriptional regulation. The DNA sequence predicts a relatively long 5' untranslated
170 region (316 bp) and there is a predicted CsrA binding motif (GGA) in the ribosome binding site
171 of the transcript. *ABUW_2639* is annoated as belonging to a universal stress protein A family. It
172 has been shown to protect *A. baumannii* ATCC17987 from oxidative stress of hydrogen
173 peroxide (29).
174
175 We wondered if *ABUW_2433* and *ABUW_2437* might play a role in promoting desiccation
176 tolerance of the two *A. baumannii* strains, ATCC17978 and ATCC19606, that do not survive
177 well at 2% RH (Fig 1B). ATCC19606 has the gene region shown in Fig 4 intact, but the gene
178 that is homologous to *ABUW_2433*, encoding the KGG domain-containing protein, is annotated
179 as a pseudogene. ATCC17978 appears to be missing a gene in homologus to *ABUW_2433*.
180 However it has conitguous *katE* and iron-containing redox protein genes (*A1S_1386 and*

181 *A1S_1385*). Expression of the two AB5075 genes *in trans* improved the survival of the two
182 ATCC strains at 2% RH (Fig 5), providiing evidence that ABUW_2433 and ABUW_2437 are
183 generally important for desiccaton tolerance.
184
185 **Other possible desiccation tolerance genes**. As shown in Table 1, we identified an
186 additional six genes that are likely regulated by CsrA, that may have a small role in desiccation
187 tolerance. *ABUW_0916*, encoding a biofilm-associated protein and *otsA*, encoding tehalose-6-
188 phosphate synthase, are the only two of the six for which we can hypothesize some connection
189 to desiccation. Biofilms have been shown to be important for desiccation tolerance of bacteria
190 (15, 16) and trehalose has been shown to play a significant role in desiccation tolerance of
191 eukaryotes and bacteria (30, 31). When added extrinsically to cultures, trehalose increased the
192 desiccation tolerance of *A. baumannii* ATCC 19606 (22). However, a *ΔmtlD-otsB* mutant of
193 ATCC19606, defective in endogenous production of the compatible solutes, mannitol, and
194 trehalose, was not more sensitive to desiccation than the wild type (22).
195
196 **DISCUSSION**
197 Depletion of water during desiccation leads to loss of membrane integrity and accompanying
198 disruption of aerobic respiration results in the generation of reactive oxygen species, including
199 hydrogen peroxide (32). So, it makes sense that *katE*, encoding catalase, contributes to
200 desiccation tolerance. Proteomics analyses of *A. baumannii* showed that proteins involved in
201 redox defense including catalase, alkyl peroxidase reductases and superoxide dismutase were
202 elevated in stationary-phase cells (33), which is consistent with the observation made by many
203 that cells stationary-phase cells survive desiccation much better than exponentially growing
204 cells.
205

206 Since the desiccation-tolerance genes *ABUW_2433* and *ABUW_2437* are near or adjacent to
207 *katE*, on the genome and all are likely to be controlled by CsrA, it seemed important to consider
208 that they might somehow mediate oxidative stress tolerance even though the amino acid
209 sequences of the encoded proteins don't have motifs typically associated with reactive oxygen
210 species detoxification. However, we were unable to demonstrate that *ABUW_2433*::Tn and
211 *ABUW_2437*::Tn mutants were sensitive to hydrogen peroxide and paraquat - both powerful
212 oxidizing agents. In addition, a study that looked at effects of hydrogen peroxide exposure on
213 gene expression in *A. baumannii,* found that *katE* but not *ABUW_2433* or *ABUW_2437*, was
214 expressed at elevated levels and neither of these genes is part of the OxyR regulon that
215 controls the response to oxidative stress in *A. baumannii* (34). Thus, we do not think that either
216 of these proteins, which have the greatest defects in desiccation found to date, are likely to
217 function by protecting cells against oxidative stress
218
219 The predicted physical properties of ABUW_2433 provide suggestions as to how it may
220 function. It is an intrinsically disordered protein (IDP) that is highly hydrophilic, with 27%
221 positively charged amino acids residues and 31% negatively charged residues. It is also
222 predicted to assume a collapsed or extended conformation, likely depending on its context
223 (ROBETTA PFRMAT TS prediction; https://robetta.bakerlab.org). ABUW_2433 has 13 repeated
224 AT-hook DNA binding motifs that extend across about 70% of the protein. This motif
225 preferentially binds to AT-rich sequences in the minor groove of DNA. AT-hook DNA binding
226 motifs are found primarily in eukaryotic proteins, many of which have roles in transcriptional
227 regulation (35, 36). Only 8.5% of annotated AT hook DNA binding motifs are found in bacteria,
228 but about half of these are found in gamma proteobacteria, the group to which *A. baumannii*
229 belongs. We hypothesize that ABUW_2433 binds to *A. baumannii* DNA and somehow protects
230 it from desiccation-induced damage. IDPs are critical for the microscopic animals called
231 tardigrades to survive desiccation. When desiccated, some of these proteins vitrify and probably

232 trap desiccation sensitive molecules in a noncrystalline amorphous matrix, thereby protecting
233 them from denaturation or other forms of destruction (37, 38). IDPs or proteins with IDP
234 domains are less common in prokaryotes than in eukaryotes, but drawing from work on
235 eukaryotes, they are proposed to play a central role in cellular process in bacteria that may
236 depend on the formation of molecular condensates (39). It is possible that this is important for
237 the viability of desiccated *A. baumannii*.
238
239 Although not much work has been done on *Acinetobacte*r CsrA, based on what is known for
240 other gamma proteobacteria, we hypothesize that a set of ncRNAs that is induced by a GacSA
241 (ABUW_3306 and ABUW_3639) two-component regulatory system, controls the repressor
242 activity of CsrA by sequestering it (40). We can draw a link between the GasSA system and
243 CsrA because they both appear to control catabolism of the aromatic compound phenylacetate.
244 An *A. baumannii* Δ*gacA* mutant is unable to catabolize phenylacetate (41), and our proteomics
245 results suggest that CsrA acts to repress the synthesis of at least one enzyme required for
246 phenylacetate degradation. In fact, we have determined that the Δ*csrA* mutant grows better on
247 phenylacetate; with a doubling time of 39 min, than the wild type, which has doubling time of 50
248 min. We hypothesize that a Δ*gacA* mutant does not synthesize ncRNAs that would normally
249 "sponge-up" CsrA, thus allowing CsrA to bind to the 13 -gene (ABUW_2524 to ABUW_2536)
250 phenylacetate mRNA transcript to repress its translation. At this point we do not have a clear
251 understanding of the inventory of *A. baumannii* ncRNAs that may bind to CsrA, but ncRNAs are
252 abundant in AB5075, and several of them are expressed at extremely high levels (42). The
253 desiccation phenotype of CsrA appears to depend on its ability to activate translation and
254 although it's difficult to reconcile this activity with a model where CsrA is sequestered by
255 ncRNAs, it is known that ncRNA turnover can occur resulting in the release of free CsrA (43).
256 Most of what is known about mechanisms of CsrA action centers on its role as a repressor of

257 translation (44–47) and it may be of interest to probe its capability as an activator in *A.*
258 *baumannii*.
259
260 We found that *A. baumannii* AB5075 survived desiccation for six days at 2% RH much better
261 than two other *A. baumannii* strains that we tested, but it is important to note that most studies
262 of desiccation tolerance have been carried out at about 30% RH or in room air and the
263 emphasis has been on the number of days or months that a particular strain remains viable
264 when desiccated. When Farrow et al (20) tested the survival of several strains that were dried
265 and incubated at an RH of 25–61% (mean 46%) they found AB5075 to have an average
266 survival time of 90 days, whereas strains ATCC19606 and ATCC17978 had average survival
267 times of 3 and 34 days respectively. Even though AB5075 is tolerant to desiccation over
268 months at a mean RH of 46% and over days at 2% RH, we cannot necessarily conclude that the
269 same sets of genes are needed for desiccation tolerance under these two different conditions.
270 For example, Farrow et al. (20) found that the response-regulator protein BmfR was important
271 for desiccation tolerance of ATCC17978 in long term desiccation assays, whereas we did not
272 observe a role of *bmfR* in protecting AB5075 from desiccation in shorter term incubations at
273 2%RH (Table S1).
274
275 Here we established a robust assay for desiccation tolerance of a highly virulent strain of *A.*
276 *baumannii* and identified two genes, *ABUW_2433* and *ABUW_2437*, that are extremely
277 important for desiccation tolerance. Outside of the *Acinetobacter* genus, *ABUW_2437* has
278 homologs in *Pseudomonas stutzeri* (40% amino acid identity). However, except for a partial
279 homolog found in *Enterobacteriaceae* bacterium TzEc051 (99% identity over 38% of the
280 AB5075 protein), *ABUW_2433* does not appear outside *Acinetobacter*. This and the unusual
281 predicted physical properties of ABUW_2433 as anIDP, suggest that there is novel physiological
282 basis for desiccation tolerance in *A. baumannii* that remains to be explored.

283

284 **MATERIALS AND METHODS**

285 **Bacterial strains and growth conditions.** Strains used in this study are listed in Table S3A.
286 Strain AB5075 (AB5075-UW) was used as a wild type (24) and individual transposon mutants
287 were obtained from the Manoil lab comprehensive ordered transposon mutant library at the
288 University of Washington (24). All strains except for the Δ*csrA* mutant were routinely grown and
289 maintained in TY (10 g Tryptone, 5 g Yeast extract, and 8 g NaCl in 1000 ml) medium or BBL
290 Trypticase Soy Broth (TSB) media at 37°C, unless otherwise stated. The Δ*csrA* mutant was
291 grown in M9/succinate.

292

293 **Desiccation assay.** Strains from a frozen stock (-80°C) were streaked onto TY plates and
294 incubated at 37°C. Colonies (three to five) were picked and inoculated into 2 ml of TSB, and
295 cultures were grown overnight at 37°C with a shaking speed of 200rpm. Overnight cultures were
296 diluted to yield an initial OD_{600} of 0.025 in 10 ml TSB in a 50 ml Erlenmeyer flask. Cultures were
297 grown at 37°C with a shaking speed of 200 rpm to mid-exponential-phase (OD_{600}=0.4 to 0.6) or
298 stationary-phase (24 hours after inoculation). Cells were harvested by centrifugation and
299 washed twice with Dulbecco's phosphate-buffered saline (DPBS, Gibco), and cell density was
300 adjusted to OD_{600}=1 (about 5 x 10^8 cells/ml) with DPBS. Cell suspension (2 spots of 50 µl each
301 per membrane) was filtered onto a 0.4 µm Whatman nucleopore polycarbonate track-etched
302 membranes (25 mm diameter) that had been placed in Nalgene analytical filter units, and the
303 membranes were then placed into 15 ml uncapped centrifuge tubes. To obtain the T0 (baseline)
304 viable cell number, 1 ml of DPBS was immediately added to one tube and incubated for 5 min at
305 room temperature (24 ± 2°C) on a rotary shaker. Viable cell numbers were determined by
306 plating on TSB agar. For desiccation, tubes with membranes were placed in a Snapware
307 containers (2.3 x 6.3 x 8.4 inches) that contained DRIERITE in the lids of 50 ml centrifuge tubes
308 (x4, 7.5 g of DRIERITE desiccant in each lid) or saturated calcium chloride hexahydrate solution

309 in 5 ml beaker (x8) to yield the RHs of 2% or 30% (± 2), respectively. The Snapware containers
310 were incubated at room temperature. Digital hygrometers (VWR International Ltd) were placed
311 in each container to monitor the RH. At desired time points, tubes containing membranes were
312 removed from the containers, 1 ml of DPBS was added to each tube, and incubated for 5 min at
313 room temperature on a rotary shaker. Viable cell counts were determined on TSB agar. For
314 each strain, a minimum of three biological replicates of desiccation assays were performed
315 except for individual transposon mutants, which were assayed twice for each allele.
316
317 **Construction of the Δ*csrA* mutant.** In-frame deletion of the *csrA* (*ABUW_2750*) gene was
318 generated by overlap extension PCR as described (48). PCR primers are listed in Table S3B.
319 PCR product was cloned into mobilizable suicide vector pEX2-TetRA and transformed into *E.*
320 *coli* NEB 10-beta (New England Bio Labs). The sequence-verified deletion construct was
321 transformed into *E. coli* S17-1, and further mobilized into *A. baumannii* strain AB5075 by
322 conjugation on TY agar. Single recombinant conjugants were first selected on M9/succinate
323 plate containing 20 µg/ml Tc, and Tc resistant colonies were further plated onto M9/succinate
324 plate containing 5% sucrose. Sucrose resistant and Tc sensitive colonies were screened by
325 colony PCR and sequencing to validate the expected chromosomal in-frame deletion of the *csrA*
326 gene.
327
328 To complement the Δ*csrA* mutant, the full length *csrA* gene plus the 15 bp upstream that
329 contains the putative ribosome binding site was PCR amplified and cloned into pMMB67EH-
330 TetRA. The construct was transformed into *E. coli* NEB 10-beta (New England Bio Labs). The
331 sequence-verified construct was transformed into *E. coli* S17-1, and further mobilized into the
332 Δ*csrA* mutant by conjugation on M9/succinate plates containing 20 µg/ml Tc. As a negative
333 control, empty vector pMMB67EH-TetRA was used. The same procedure was used to clone
334 *ABUW_2433* and *ABUW_2437* for complementation experiments.

335

336 **Phenotypic characterization of the Δ*csrA* mutant.** M9/succinate was used in all experiments.
337 For motility assays, an overnight culture (16 to 18 hours) was diluted to yield OD_{600}=0.5, and 2
338 µl of sample was spotted onto the freshly prepared M9/succinate plate and incubated at 37°C
339 for 24 h. For biofilm assays, an overnight culture was inoculated into 100 µl of M9/succinate in
340 Costar vinyl 96 well "U" bottom plates (initial OD_{600}=0.05), and the plates were sealed with
341 Breath-Easy sealing membranes. After incubation at room temperature for 48 h, culture was
342 removed, the plate was rinsed with tap water twice, and 150 µl of 0.1% crystal violet solution
343 was added to each well. After incubating at room temperature for 15 min, crystal violet solution
344 was removed, the plate was rinsed with tap water 5 times, and the plate was dried at room
345 temperature. For catalase assays, cells were harvested at OD_{600}=0.5, supernatant was
346 removed, and cells were resuspended in DPBS to yield 10 mg wet cell/100 µl DPBS. 100 µl of
347 cell suspensions were placed in 13 x 100 mm borosilicate glass tubes. Then 100 µl of 1% Triton
348 X-100 and 100 µ1 of 30% hydrogen peroxide were added, mixed thoroughly, and incubated for
349 15 min at room temperature (49) .

350

351 **Label-free protein quantification.** Since the Δ*csrA* mutant had a severe growth defect on TY
352 medium, both the wild type and Δ*csrA* mutant were grown in M9/succinate. Two biological sample
353 replicates were prepared for each strain. Cells from each culture were harvested at OD_{600}=0.5 by
354 centrifugation, washed twice with DPBS, and cells were stored in -80°C before further analysis.
355 Cells were lysed in buffer containing 4% SDS, 100 mM Tris pH8.0, 10 mM DTT by heating at
356 95°C for 5 min. After cooling to room temperature, the lysates were sonicated with ultrasonication
357 probe on ice to shear DNA. Total protein concentration was determined by the BCA assay
358 (Thermo Pierce, Rockford, IL). 500 µg of each protein lysate was reduced and alkylated, diluted
359 in 8 M urea solution, and the SDS was removed with a 3kD molecular weight cutoff filter. After
360 buffer exchange, the protein lysates were digested with trypsin (Promega, Madison, WI) at 37°C

361 overnight and the digested samples were desalted with 1cc C18 Sep-Pak solid phase extraction
362 cartridges (Milford, MA, Waters). The eluted samples were vacuum dried and resuspended in
363 0.1% formic acid. Reverse phase nanoLC-MS analysis of the protein digests was carried out with
364 a Thermo Easy-nanoLC coupled to a Thermo Q-Exactive Plus Orbitrap mass spectrometer.
365 Triplicate top 20 data-dependent acquisition runs were acquired for each sample, and 1 µg of
366 protein digest was loaded for each run. The peptides were separated by a 50 cm x 75 µm I.D C8
367 column (5 µm diameter, 100 Å pore size C8 MichromMagic beads) with a 90 min 10 to 30% B
368 gradient (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile, flow rate
369 300 nl/min). The MS data acquisition parameters were set as follows: full MS scan resolution 70k,
370 maximum ion injection time 100 mS, AGC target 10^6, scan range of 400 to 2000 m/z; MS/MS scan
371 resolution 17.5 k, maximum ion injection time 100 mS, AGC target 5^4, isolation window 1.6 m/z,
372 HCD NCE 35 scan range of 200 to 2000 m/z; loop count 20, intensity threshold 5^3, underfill ratio
373 1%, dynamic exclusion 10 sec. High resolution MS^2 spectra were searched against a target-decoy
374 proteome database of strain AB5075 (a total of 7678 sequences) downloaded from Uniprot
375 (Oct17, 2017) using Comet (version 2015.02 rev. 1) (50) with following parameters: precursor
376 peptide mass tolerance 20 ppm, allowing for -1, 0, +1, +2, or +3 ^{13}C offsets; fragment ion mass
377 tolerance 0.02 Da; static modification, carbamidomethylation of cysteine (57.0215 Da); variable
378 modification, methionine oxidation (15.9949 Da). The search results were further processed by
379 PeptideProphet (51) for probability assignment to each peptide-spectrum match, and
380 ProteinProphet (52) for protein inference and protein probability modeling. The output pepXML
381 files from three technical replicates were grouped for subsequent spectral counting analysis using
382 Abacus (53). The pepXML and protXML files for each sample, combined ProteinProphet file from
383 all samples were parsed into Abacus for spectral counting of each protein. The following filters
384 were applied for extracting spectral counts from MS/MS datasets: (1) the minimum
385 PeptideProphet score the best peptide match of a protein must have maxIniProbTH=0.99; (2) The
386 minimum PeptideProphet score a peptide must have to be even considered by Abacus,

387 iniProbTH=0.50; (3) The minimum ProteinProphet score a protein group must have in the
388 COMBINED file, minCombinedFilePw=0.90. Spectral counts for 1616 proteins were reported
389 across four sample groups (two strains and two biological replicates) with estimated protein false
390 discovery rate of 1.94%. The protein expression fold changes between wild type AB5075 and
391 Δ*csrA* mutant were computed from adjusted spectral counts output from Abacus. The mass
392 spectometry data have been deposited to the ProteomeXchange Consortium via the PRIDE
393 partner repository with the dataset identifier xxxxxxxxx..
394
395

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398 sharing the pEX2-TetRA and pMMB67EH-TetRA vectors. We thank Indranil Biswas for alerting
399 us to the possibility that intrinsically disordered proteins could be involved in desiccation
400 tolerance in *A. baumannii*.
401

402 **FUNDING**
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404 Infectious Diseases under Grant 1U19AI107775-01.
405
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408

409 **Table 1. Genes that contribute to desiccation tolerance of *A. baumannii* AB5075.**

Gene	Gene name	Gene annotation	Relative viability loss ratio (day 6/day 0 desiccation)[a]	Protein abundance (ratio of WT/*ΔcsrA*)
AB5075 (WT)			1.0	
ABUW_0916		Biofilm-associated protein	1.5	2.6
ABUW_2433		KGG domain-containing protein	125	4.3
ABUW_2436	*katE*	Catalase	7.5	3.2
ABUW_2437		Heme oxygenase-like protein	150	10.5
ABUW_2639		Universal stress protein family	6.0	4.4
ABUW_2724		Hypothetical protein	4.0	2.4
ABUW_2750	*csrA*	Carbon storage regulator	480	8.5
ABUW_3123	*otsA*	Trehalose-6-phosphate synthase	2.0	10.5
ABUW_3346	*acnA*	Aconitate hydratase 1	1.5	3.2

410 [a] All mutants were except the ΔcsrA strain were grown in TSB broth to the stationary phase of growth
411 prior to being desiccated. The viability loss of wt day6/day 0 was 17 for the WT grown in TSB broth. The
412 viability loss of WT grown in M9/succinate was 276
413
414

415 **FIGURE LEGENDS**

416 **Figure 1.** Desiccation tolerance of *A. baumannii*. (A) Comparison of AB5075, *E. coli* W3110,
417 and *P. aeruginosa* PAO1 at 30% RH or 2% RH. (B) Desiccation tolerance of *A. baumannii*
418 strains AB5075, ATCC17978, and ATCC19606 after 0 days (control) and 6 days of desiccation
419 at 30% RH or 2% RH. The cell numbers represent the total number of viable cells recovered
420 from each membrane. The data are the average of three or more biological replicates, and
421 standard deviations are shown as error bars.

422

423 **Figure 2.** Growth and desiccation tolerance of a Δ*csrA* mutant (A) Comparison of wild type (WT)
424 and a Δ*csrA* mutant on TY and M9/succinate agar and in TY and M9/succinate broth. (B)
425 Desiccation tolerance of WT, Δ*csrA* mutant, Δ*csrA* mutant with pMMB (empty vector), and Δ*csrA*
426 mutant with *csrA* expressed in trans (pAB2750) at day 0 and at day 6 of desiccation at 2% RH
427 or at 6 days in PBS. The cell number represents the total number of viable cells recovered from
428 each membrane. The data are the average of three or more biological replicates, and standard
429 deviations are shown as error bars.

430

431 **Figure 3.** Phenotypes of the Δ*csrA* mutant: (A) twitching motility; (B) growth of wild type (black
432 symbol) or the Δ*csrA* mutant (red symbol) in M9/succinate in the absence (closed symbol) or
433 presence (open symbol) of 0.5% ethanol. The data shown are representative of each strain and
434 condition; (C) crystal violet staining of biofilms from wild type, Δ*csrA* mutant, Δ*csrA* mutant with
435 pMMB (empty vector), or Δ*csrA* mutant with pAB2750; and (D) catalase activity of wild type,
436 Δ*csrA* mutant, Δ*csrA* mutant with pMMB (empty vector), or Δ*csrA* mutant with pAB2750.

437

438 **Figure 4.** *A. baumannii* genes important for desiccation tolerance. (A) Genes and (B)
439 Desiccation tolerance of mutants at 2% RH. The cell number represents the total number of

440 viable cells recovered from each membrane. The data are the average of three or more
441 biological replicates, and standard deviations are shown as error bars.
442
443 **Figure 5.** Effect of expression of *ABUW_2433* (red bars) or *ABUW_2337* (blue bars) gene *in*
444 *trans* on desiccation tolerance of *A. baumannii* (A) ATCC17978 and (B) ATCC19606 after 0
445 days (control) and 6 days of desiccation at 2% RH. Empty vector (pMMB, black bars) was used
446 as a control. The data are the average of three or more biological replicates, and standard
447 deviations are shown as error bars.
448
449
450

SUPPLEMENTAL MATERIAL

Figure S1. Desiccation tolerance of *A. baumannii* AB5075 cells from the log- and stationary-phases of growth. The cell number represents the total number of viable cells recovered from each membrane. The data are the averages of three or more biological replicates, and standard deviations are shown as error bars.

Figure S2. Complementation of desiccation phenotypes of (A) *ABUW_2433*::Tn and (B) *ABUW_2437*::Tn after 0 days (control) and 6 days of desiccation at 2% RH. Empty vector (pMMB) was used as a control. The data are the average of three or more biological replicates, and standard deviations are shown as error bars.

Table S1. Mutants from the three-allele library tested for desiccation tolerance

Table S2. (A) Label-free protein quantification of wild type and the Δ*csrA* mutant. (B) List of proteins up-regulated (ratio of Δ*csrA*/WT ≥2.5 and Δ*csrA* read count ≥7.5) in the Δ*csrA* mutant compared to wild type. (C) List of proteins down-regulated (ratio of Δ*csrA*/WT ≤0.4 and WT read count ≥7.5) in the Δ*csrA* mutant compared to wild type.

Table S3. (A) Bacterial strains used in this study. (B) Plasmids and primers used in this study

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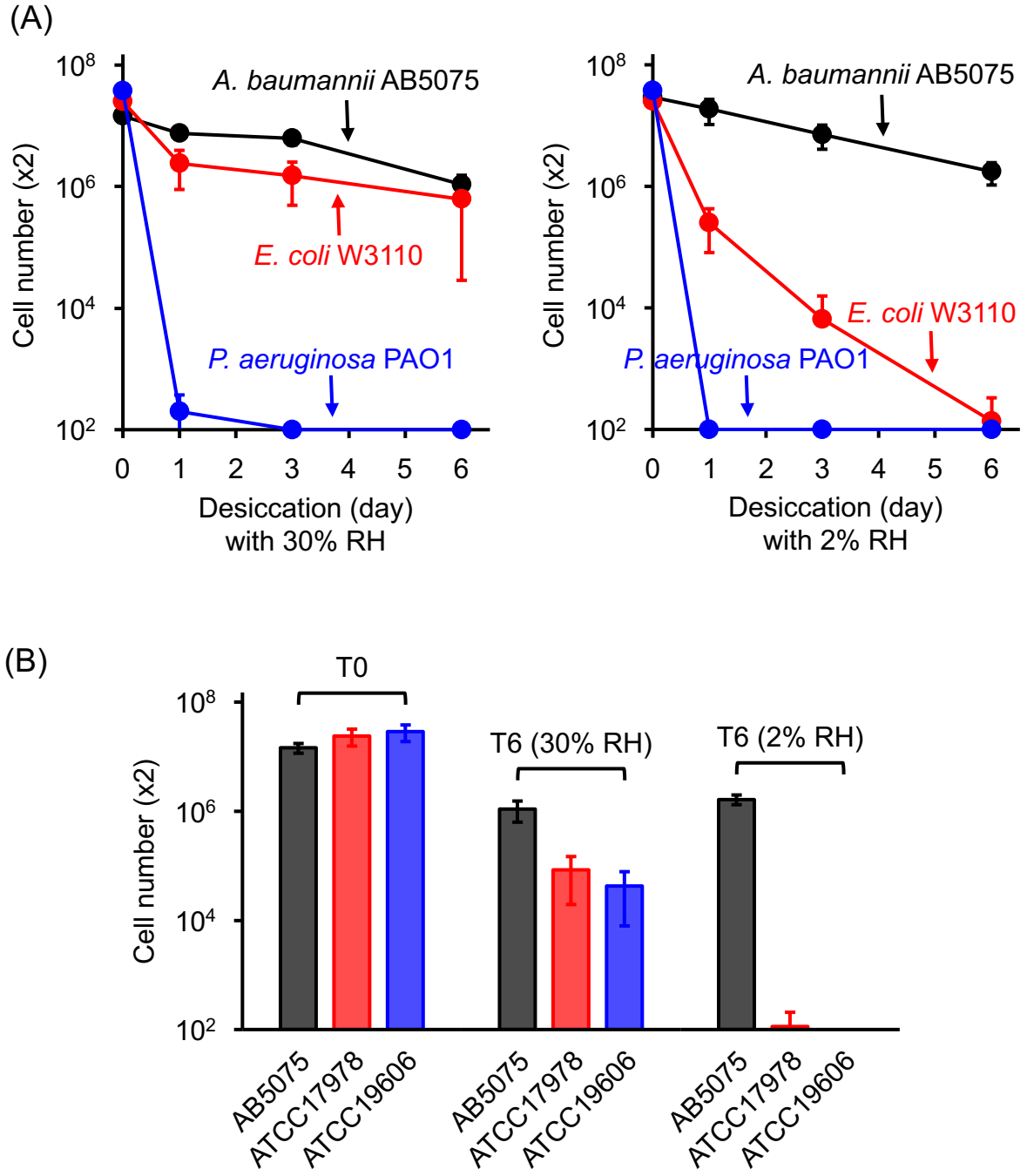


Figure 1. Desiccation tolerance of *A. baumannii*. (A) Comparison of AB5075, *E. coli* W3110, and *P. aeruginosa* PAO1 at 30% RH or 2% RH. (B) Desiccation tolerance of *A. baumannii* strains AB5075, ATCC17978, and ATCC19606 after 0 days (control) and 6 days of desiccation at 30% RH or 2% RH. The cell numbers represent the total number of viable cells recovered from each membrane. The data are the average of three or more biological replicates, and standard deviations are shown as the error bars.

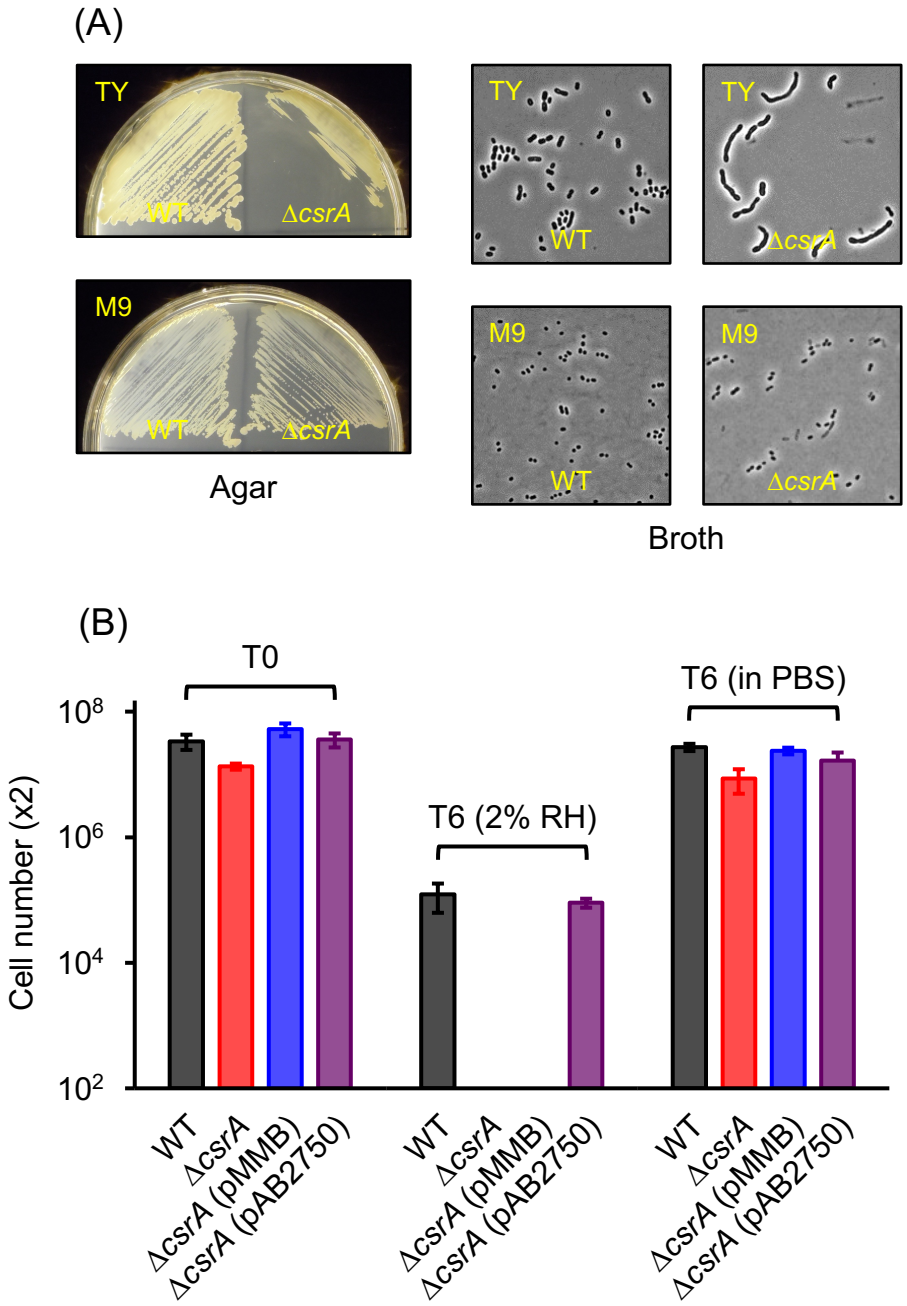


Figure 2. Growth and desiccation tolerance of the Δ*csrA* mutant (A) Comparison of wild type (WT) and the Δ*csrA* mutant on TY and M9/succinate agar and in TY and M9/succinate broth. (B) Desiccation tolerance of WT, Δ*csrA* mutant, Δ*csrA* mutant with pMMB (empty vector), and Δ*csrA* mutant with *csrA* expressed in trans (pAB2750) at day 0 and at day 6 of desiccation at 2% RH or at 6 days in PBS. The cell number represents the total number of viable cells recovered from each membrane. The data are the average of three or more biological replicates, and standard deviations are shown as the error bar.

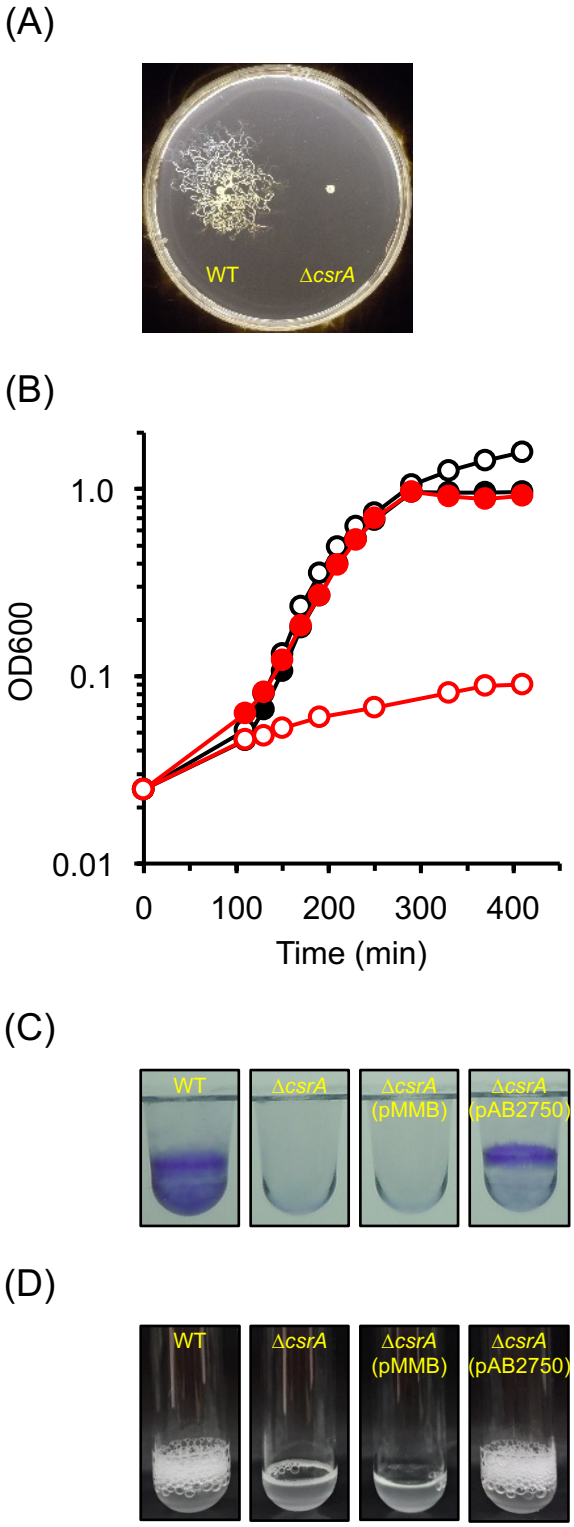


Figure 3. Phenotypes of the Δ*csrA* mutant: (A) twitching motility; (B) growth of wild type (black symbol) or the Δ*csrA* mutant (red symbol) in M9/succinate in the absence (closed symbol) or presence (open symbol) of 0.5% ethanol. The data shown are the representative of each strain and condition; (C) crystal violet staining of biofilms from wild type, Δ*csrA* mutant, Δ*csrA* mutant with pMMB (empty vector), or Δ*csrA* mutant with pAB2750; and (D) catalase activity of wild type, Δ*csrA* mutant, Δ*csrA* mutant with pMMB (empty vector), or Δ*csrA* mutant with pAB2750.

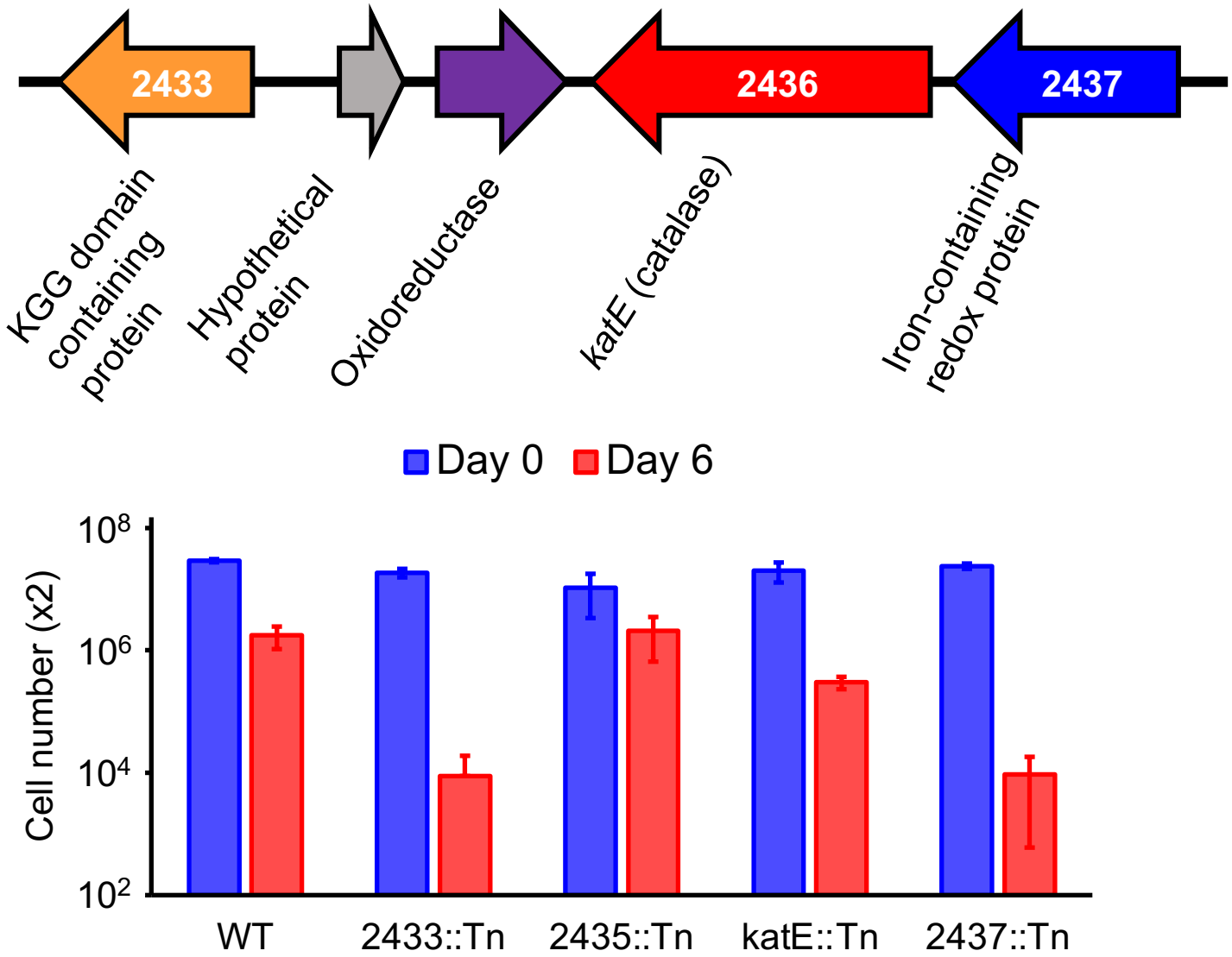


Figure. 4. *A. baumannii* genes important for desiccation tolerance. (A) Genes (B) Desiccation tolerance of mutants at 2% RH. The cell number represents the total number of viable cells recovered from each membrane. The data are the average of three or more biological replicates, and standard deviations are shown as the error bar.

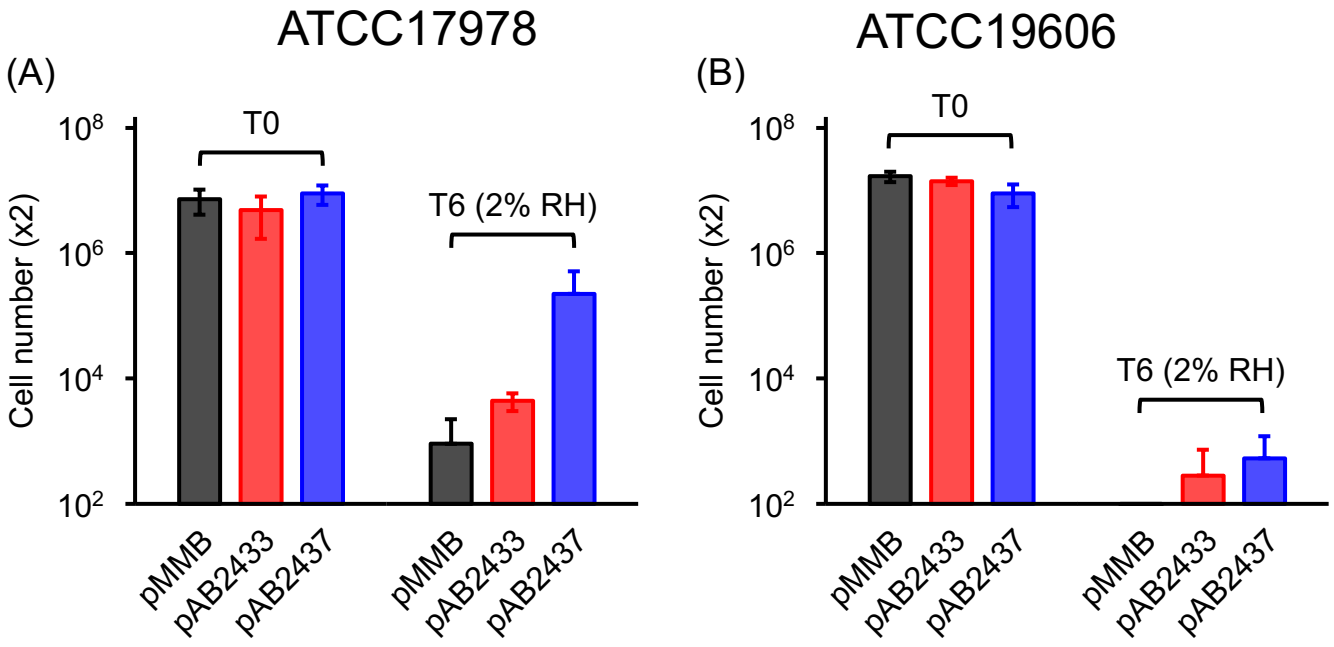
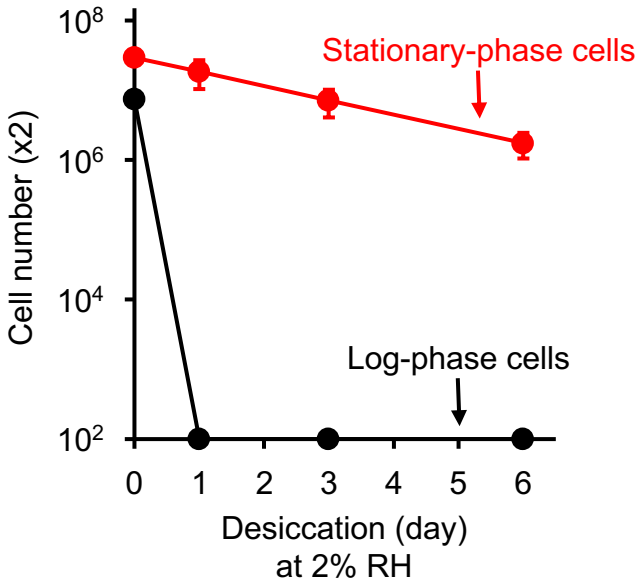
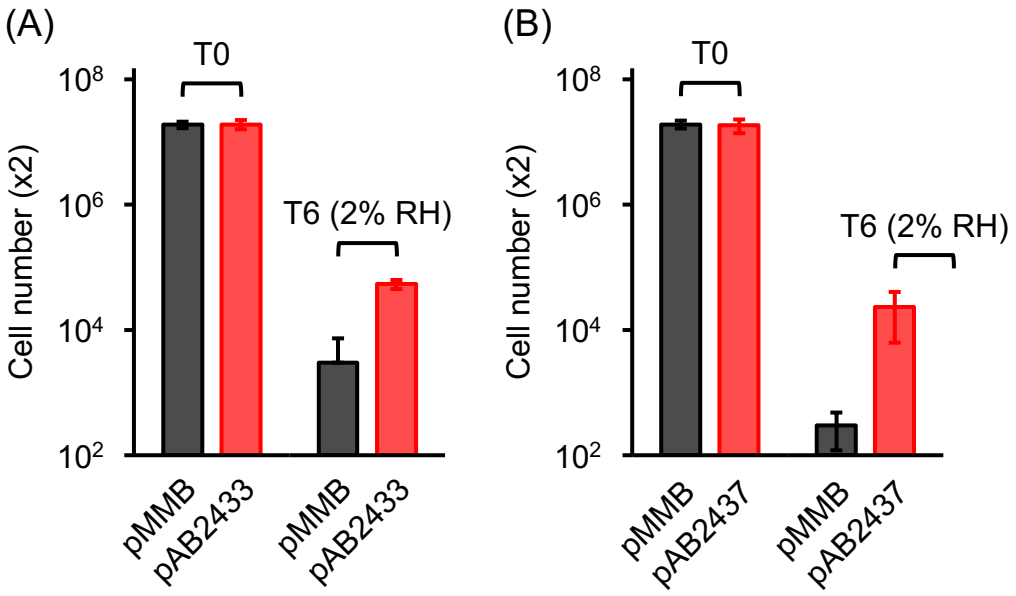


Figure 5. Effect of expression of ABUW_2433 (red bar) or ABUW_2337 (blue bar) gene *in trans* desiccation tolerance *A. baumannii* (A) ATCC17978 and (B) ATCC19606 after 0 days (control) and 6 days of desiccation at 2% RH. Empty vector (pMMB, black bar) was used as a control. The data are the average of three or more biological replicates, and standard deviations are shown as the error bar.



Supplementary Figure 1. Desiccation tolerance of *A. baumannii* AB5075 cells from the log-phase and stationery-phase of growth. The cell number represents a total number of viable cells recovered from each membrane. The data are the averages of three or more biological replicates, and standard deviations are shown as error bars.



Supplementary Figure 2. Complementation of desiccation phenotypes of (A) ABUW_2433::Tn and (B) ABUW_2437::Tn after 0 days (control) and 6 days of desiccation at 2% RH. Empty vector (pMMB) was used as a control. The data are the average of three or more biological replicates, and standard deviations are shown as the error bars.