CsrA-controlled proteins important for *Acinetobacter baumannii* desiccation tolerance

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ABSTRACT

Hospital environments serve as excellent reservoirs for the opportunistic pathogen *Acinetobacter baumannii* in part because it is exceptionally tolerant to desiccation. To understand the functional basis this trait, we used transposon sequencing (Tn-seq) to identify genes contributing to desiccation tolerance in *A. baumannii* strain AB5075. We identified 142 candidate desiccation tolerance genes, one of which encoded the global post-transcriptional regulator CsrA. We characterized CsrA in more detail by using proteomics to identify proteins that were differentially present in wild type and *csrA* mutant cells. Among these were a predicted universal stress protein A, an iron-containing redox protein, a KGG-domain containing protein, and catalase. Subsequent mutant analysis showed that each of these proteins was required for *A. baumannii* desiccation tolerance. The amino acid sequence of the KGG-domain containing protein predicts that it is an intrinsically disordered protein. Such proteins are critical for desiccation tolerance of the small animals called tardigrades. This protein also has a repeat nucleic acid binding amino acid motif, suggesting that it may protect *A. baumannii* DNA from desiccation-induced damage.

19 **INTRODUCTION**

20 Hospital-acquired infections are an important healthcare concern and economic burden (1, 2)
21 and environmental persistence plays a critical role in the transmission of bacteria that cause
22 these infections (3–6). One such bacterium is *Acinetobacter baumannii,* an opportunistic
23 pathogen that infects very sick patients. It is responsible for about 2% of nosocomial infections
24 in the United States and Europe and the frequencies are higher in the rest of the world. *A.*
25 *baumannii* is especially problematic because on a global basis, about 45% of isolates are multi-
26 drug resistant (7). A factor that contributes to the prevalence of *A. baumannii* in hospital settings
27 is desiccation tolerance. *A. baumannii* can survive in a desiccated state on inanimate dry
28 surfaces for days to several months (8–10). These surfaces include materials that are often
29 encountered in the hospital, such as polyvinyl chloride, rubber, and stainless steel (11) .
30
31 When desiccated, bacteria must respond to diverse stresses that include accumulation of
32 reactive oxygen species, loss of cytoplasmic volume, and loss of cell membrane integrity (12,
33 13). Proteomics analysis of *A. baumannii* showed that desiccated cells had higher levels of
34 proteins involved in protein stabilization, antimicrobial resistance, and reactive oxygen species
35 detoxification (14). Attributes of *A. baumannii*, that have been shown to be associated with
36 desiccation tolerance include biofilm formation (15, 16) and protein aggregation (17). $LpxM_{AB}$-
37 dependent acetylation of lipid A is essential for survival of *A. baumannii* ATCC17978 at 40%
38 humidity (18), and a *recA* mutant of ATCC17978, defective in DNA repair, had pleiotropic
39 effects, including a defect in desiccation tolerance (19). *katE*, encoding catalase also
40 contributes to desiccation tolerance (20). To further probe the functional basis for desiccation
41 tolerance in *A. baumannii* we applied transposon sequencing (Tn-seq), an unbiased, high-
42 throughput genetic screening approach, to generate a comprehensive list of genes that may be
43 important for desiccation survival. From 142 candidate genes identified in the Tn-seq screen,
44 single mutant analysis of a small subset revealed six genes that likely contribute to desiccation

45 tolerance, one of which was *csrA* (ABUW_2750). *csrA* encodes a global post-transcriptional
46 regulator found in gamma-proteobacteria and was recently reported by another group as
47 important for desiccation tolerance of *A. baumannii* (21) . Here we expanded on this recent
48 report and identified CsrA-controlled proteins, several of which turned up in the Tn-seq screen,
49 that play a role desiccation survival.
50
51
52 **RESULTS**
53 **Desiccation assay.** Previous studies have shown that *A. baumannii* can survive in a desiccated
54 state for days to several months (8–11, 20). For these and other desiccation studies,
55 investigators worked with a variety of strains and usually incubated cells at either 30% relative
56 humidity (RH) or in room air, which varied between 25 and 61% RH in one study (20). These
57 differences can make it difficult to compare desiccation phenotypes between studies. Thus we
58 thought it important to establish a robust desiccation assay that reduces experimental variables
59 like choice of strains, drying times, and RH during desiccation.
60
61 Following from previous reports, saturated calcium chloride hexahydrate solution placed in a
62 sealed plastic Snapware container caused the RH inside the container to rapidly equilibrate to
63 30% (9). We found that use of DRIERITE instead of calcium chloride, resulted in an RH of 2%.
64 To test desiccation tolerance, we grew bacteria to a desired density in tryptone-yeast extract
65 (TY) broth, harvested them, washed them twice with phosphate buffer, and resuspended them
66 in buffer to a final OD_{600} of 1. Drops of cell suspension were placed on polycarbonate
67 membranes and filtered to allow for rapid drying. The membranes were placed in uncapped 15
68 ml conical centrifuge tubes and incubated in desiccation containers. After various periods of
69 incubation, buffer was added to each centrifuge tube followed by 5 min of shaking on a rotary
70 shaker. Viable cell numbers were then determined by plating on TY agar. To control for the

71 stress of filtration we did viable counts immediately following filtration and took this as our "day
72 0" time point.
73
74 **Relative desiccation tolerance of *A. baumannii* strains**. As shown in Fig 1A, *A. baumannii*
75 strain AB5075 and *Escherichia coli* strain W3100 each survived desiccation at 30% RH far
76 better than *Pseudomonas aeruginosa* strain PAO1. However at 2% RH, *A. baumannii* survived
77 far better than either *E. coli* or *P. aeruginosa*. As has been reported (20, 22), we found that *A.*
78 *baumannii* stationary phase cells were much more tolerant to desiccation than actively growing
79 cells (Fig. S1) and so we routinely used stationary phase cells in our desiccation assays. We
80 also tested the desiccaton tolerance of two additional frequently used laboratory strains of *A.*
81 *baumannii* , ATCC17978 and ATCC19606, and found that they did not survive well when
82 desiccated at 2% RH for six days (Fig. 1B). However, these strains survived when the RH was
83 30%, which is similar to the RH of many hospital environments. Strain-to-strain variation in
84 desiccation tolerance has previously been reported (10, 20).
85
86 In our desiccation experiments we wanted to use a condition under which *A. baumannii* survived
87 better than *E. coli* and we also wanted to provide strong selection pressure in our screen for
88 genetic determinants of desiccation tolerance. To satisfy these requirements we used *A.*
89 *baumannii* strain AB5075 and 2% RH in subsequent experiments. This strain was isolated from
90 a surgical wound, is multidrug resistant and is highly virulent in an animal model (23). A
91 comprehensive ordered mutant library of AB5075 is available that has two to three seqenced Tn
92 insertions in each gene and is called the three-allelle library (24).
93
94 **Unbiased screening to identify desiccation sensitive mutants by Tn-seq.** To begin to
95 define genetic mechanisms underlying desiccation tolerance in *A. baumannii*, we created a
96 saturating high-density transposon mutant pool containing approximately 400,000 individual *A.*

97 *baumannii* mutants. We grew the transposon mutant pool to stationary-phase and plated cells
98 on TY medium at day 0 and day 6 of desiccation at 2%RH. After a brief period of growth, cells
99 were harvested, pooled and processed for mapping of Tn insertions. The transposon mutant
100 pool survived desiccation as well as wild-type strain AB5075, indicating that the transposon
101 itself did not affect desiccation tolerance (Fig. S2).
102
103 Excluding genes that were not well-represented (less than100 Tn reads/kb) in the day 0 sample,
104 we found that 142 genes showed a 2.5-fold or more decrease in abundance of Tn insertions
105 after 6 days of desiccation (Table S1). We picked 12 genes that had a 10-fold or more decrease
106 in transposon insertions after a 6-day period of desiccation to validate their desiccation
107 phenotypes. When possible, we tested two different transposon mutants (transposon insertions
108 in different positions of the gene) for each of these 12 genes from the three-allele library. *A.*
109 *baumannii* AB5075 produces opaque and translucent colony variants that interconvert at high
110 frequency and reflect changes in the thickness of capsular exopolysaccharide (25). AB5075
111 cells with decreased capsule production were as much as 100-fold more sensitive to desiccation
112 (26). Here, we used only opaque colonies of AB5075 and its mutant derivatives in our
113 desiccation assays. We found that when mutated, six of the 12 genes we tested had 2-fold or
114 greater defects in desiccation tolerance (Table 1). We were interested to see that one of the
115 genes was *csrA*. CsrA, also called RsmA in some bacteria, is an important global regulator of
116 mRNA translation in gamma proteobacteria. It has diverse effects on many processes including
117 motility, biofilm formation, quorum sensing and secretion of virulence factors, depending on the
118 species (27–30).
119
120 **CsrA is critical for desiccation tolerance** *csrA* mutants from the three-allele library were
121 only two-fold more sensitive to desiccation than the wild type. To try to confirm its importance for
122 desiccation tolerance we constructed a *csrA* deletion mutant (Δ*csrA*). We found that the Δ*csrA*

123 strain grew poorly on TY agar and had an elongated cell morphology when grown in TY broth
124 (Fig. 2A). On agar plates, large colonies frequently appeared on a backgound of poor growth,
125 likely due to occurance of second site suppressor mutations in the Δ*csrA* strain. The Δ*csrA*
126 strain was also defective in growth on other nutrient-rich media, including Luria broth, nutrient
127 broth, and tryptone soy broth. A similar sensitivity to growth in complex media was reported by
128 Farrow et al for a several *A. baumannii* strains including strain AB5075 (21). In agreement with
129 Farrow et al., a Δ*csrA* mutant grew as the wild type in defined medium, in our case, M9 minimal
130 medium with 10 mM succinate as a sole carbon source (M9/succinate), and it had close to a
131 wild type cell morphology (Fig 2A). A *Yersinia enterocolitica csrA* mutant, has a growth defect in
132 LB due to the presence of 90 mM of NaCl (31). However, the *A. baumannii* Δ*csrA* mutant was
133 not sensitive to NaCl. In fact, the mutant grew in M9/succinate supplemented with up to 100 mM
134 of NaCl without a significant reduction of growth compared to the wild type.
135
136 When desiccated after growth in M9/succinate to stationary phase, the ΔcsrA mutant lost almost
137 all viability over 6 days (Fig. 2B and Table 1). The desiccation phenotype was complemented
138 by expressing *csrA in trans*. Δ*csrA* mutant cells incubated for 6 days after being filtered and
139 resuspended in PBS remained fully viable (Fig 2B). As we were preparing this paper for
140 publication, Farrow et al reported that *csrA* mutants of *A. baumannii* strains AB09-003 and
141 ATCC 17961 were sensitive to desiccation in room air over a period of 14d. CsrA in these
142 strains was also required for biofilm formation and virulence in a *Galleria melonella* infection
143 model (21).
144
145 **CsrA affects mutiple cellular processes in *A. baumannii*.** To identify genes whose
146 translation might be regulated by CsrA, we compared the proteomes of wild-type and Δ*csrA*
147 cells (Table S3A). There were 97 proteins present at higher levels in the Δ*csrA* mutant

148 compared to the wild type (ratio of Δ*csrA*/WT ≥2.5, Table S3B). Among these were proteins for
149 type IV pilus assembly, synthesis of the siderophore ferric acinetobactin, and a
150 glutamate/aspartate transporter. The Δ*csrA* mutant also had elevated levels of enzymes for for
151 the catabolism of hydroxcinnamates, phenylacetate and quinate. Levels of an alcohol
152 dehydrogenase (ABUW_1621) and an aldehyde dehydrogenase (ABUW_.1624) were also
153 elevated. The Δ*csrA* mutant was defective in pilus-mediated twtiching motility as assessed by
154 movement aross a soft-agar plate (Fig. 3A). The mutant also had a severe growth defect when
155 grown on succinate in the presence of ethanol (Fig. 3B). One possible explanation for this is that
156 the *csrA* mutant metabolized ethanol to form toxic acetaldehyde to levels that slowed growth,
157 and insufficient aldehyde dehydrogenase activity was present in cells to relieve this toxicity.
158
159 There were 106 proteins present in lower amounts in the Δ*csrA* mutant compared to the wild
160 type (ratio of WT/Δ*csrA* ≥ 2.5, Table S3C). A large proportion of these (39%) are annotated as
161 hypothetical proteins. Several membrane proteins, and proteins annotated as involved in *β*-
162 lactam antibiotic resistance (ABUW_1194, 2619, and 3497), trehalose synthesis (ABUW_3123)
163 and possibly biofilm formation (ABUW_0916) were in lower abundance in the Δ*csrA* mutant
164 compared to wild type. As reported previously, a Δ*csrA* mutant did not form biofilms, (Farrow
165 2020) and this phenotype was complemented by expressing *csrA* gene *in trans* (Fig. 3C). The
166 Δ*csrA* proteome profile also suggested that CsrA was invovled in promoting the expression of
167 proteins invovled in oxidative stress, including peroxidase (ABUW_0628) and catalase (*katE*,
168 ABUW_2436). When tested for catalase activity, we found that the Δ*csrA* mutant lacked this trait
169 (Fig. 3D).
170
171 **Genes important for desiccation tolerance in *A. baumannii* AB5075.** We took advantabge
172 of the three-allele transposon library to test how important some of the gene transcripts that
173 were likely to be controlled CsrA were for desiccation tolerance. All the genes that we found to

174 possibly be important for desiccation tolerance are listed in Table 1. The results of desiccation
175 assays for all the mutant strains that we tested in this study are listed in Table S2. *katE*, and
176 *ABUW_2639* mutants were about 5-fold more sensitive to desiccation than the wild type,
177 whereas *ABUW_2433* and *ABUW_2437* mutants were greater than 100 fold more sensitive to
178 desiccation than the wild type (Table 1, Fig 4). The phentoytpes of *ABUW_2433* and
179 *ABUW_2437* mutants could be complemented (Fig S3). The ABUW_2433 protein has 411
180 amino acids and is annotated as a KGG domain-containing protein. The KGG domain
181 comprises a small region in the N-terminus of the protein and the remainder of the protein is
182 annotated by InterPro as a disordered region that includes a series of AT_hook DNA binding
183 mofits (SMART SM00384). The full length ABUW_2433 sequence was predicted to be
184 intrinsically unstructured when queried with the IUPred3 tool (https://iupred.elte.hu) (32).
185 ABUW_2437 is annotated as an iron-containing redox enzyme or a heme-oxygenase -like
186 protein (Fig 4). The predicted *ABUW_2437* transcript has traits characterisitic of a target of
187 CsrA post-transcriptional regulation. The DNA sequence predicts a relatively long (316 bp) 5'
188 untranslated region (316 bp) and there is a predicted CsrA binding motif (GGA) in the ribosome
189 binding site of the transcript. ABUW_2639 is annoated as belonging to a universal stress
190 protein A family. It has been shown to protect *A. baumannii* ATCC17987 from oxidative stress of
191 hydrogen peroxide (33).
192
193 We wondered if ABUW_2433 and ABUW_2437 might play a role in promoting desiccation
194 tolerance of the two *A. baumannii* strains, ATCC17978 and ATCC19606, that do not survive
195 well at 2% RH (Fig 1B). ATCC19606 has the gene region shown in Fig 4 intact, but the gene
196 that is homologous to *ABUW_2433*, encoding the KGG domain-containing protein, is annotated
197 as a pseudogene. ATCC 17978 appears to be missing a gene in homologus to *ABUW_2433*.
198 However it has conitguous *katE* and iron-containing redox protien genes (*A1S_1386 and*
199 *A1S_1385*). Expression of the two AB5075 genes *in trans* improved the survival of the two

200 ATCC strains at 2% RH (Fig 5), providiing evidence that ABUW_2433 and ABUW_2437 are
201 generally important for desiccaton tolerance.
202
203 **Other possible desiccation tolerance genes**. As shown in Table 1, we identified an
204 additional five genes, some but not all of which are likely regulated by CsrA, that may have a
205 small role in desiccation tolerance. *otsA*, encoding tehalose-6-phosphate synthase, is the only
206 one of the five for which we can hypothesize some connection to desiccation. Trehalose has
207 been shown to play a significant role in desiccation tolerance of eukaryotes and bacteria (34,
208 35) and trehalose added extrinsically to cultures increased the desiccation tolerance *A.*
209 *baumannii* ATCC 19606 (22). However, a *ΔmtlD-otsB* mutant of ATCC19606, defective in
210 endogenous production of the compatible solutes, mannitol and trehalose, was not more
211 sensitive to desiccation than the wild type (22).
212
213 **DISCUSSION**
214 Depletion of water during desiccation leads to loss of membrane integrity and accompanying
215 disruption of aerobic respiration results in the generation of reactive oxygen species, including
216 hydrogen peroxide (36). That *katE*, encoding catalase, contributes to desiccation tolerance
217 makes sense in this context. Proteomics analyses of *A. baumannii* showed that proteins
218 involved in redox defense including catalase, alkyl peroxidase reductases and superoxide
219 dismutase were elevated in stationary-phase cells (37), which is consistent with the observation
220 made by many that cells stationary-phase cells survive desiccation much better than
221 exponentially growing cells.
222
223 Since the desiccation -tolerance genes *ABUW_2433* and *ABUW_2437* are near or adjacent to
224 *katE*, it seemed important to consider that they might somehow mediate oxidative stress

225 tolerance even though the amino acid sequences of the encoded proteins don't have motifs
226 typically associated with reactive oxygen species detoxification. However, we were unable to
227 demonstrate that *ABUW_2433*::Tn and *ABUW_2437*::Tn mutants were sensitive to hydrogen
228 peroxide, nitrous oxide or paraquat - all powerful oxidizing agents. In addition, a study that
229 looked at effects of hydrogen peroxide exposure on gene expression in *A. baumannii,* found that
230 *katE* but not *ABUW_2433* or *ABUW_2437*, was expressed at elevated levels and neither of
231 these genes is part of the OxyR regulon that controls the response to oxidative stress in *A.*
232 *baumannii* (38).
233
234 The physical properties and cellular function of ABUW_2433 will be fascinating to explore. It is
235 an intrinsically disordered protein that is highly hydrophilic, with 27% positively charged amino
236 acids residues and 31% negatively charged residues. It is also predicted to assume a collapsed
237 or extended conformation, likely depending on its context (ROBETTA PFRMAT TS prediction;
238 https://robetta.bakerlab.org). ABUW_2433 has 13 repeated AT-hook DNA binding motifs that
239 occupying about 70% of the protein. This motif preferentially binds to AT-rich sequences in the
240 minor groove of DNA. AT-hook DNA binding motifs are found primarily in eukaryotic proteins,
241 many of which have roles in transcriptional regulation (39, 40). Only 8.5% of annotated AT hook
242 DNA binding motifs are found in bacteria, but about half of these are found in gamma
243 proteobacteria, the group to which *A. baumannii* belongs. We hypothesize that ABUW_2433
244 binds to *A. baumannii* DNA and somehow protects it from desiccation-induced damage. IDPs
245 are critical for the microscopic animals called tardigrades to survive desiccation. When
246 desiccated, some of these proteins vitrify and probably trap desiccation sensitive molecules in a
247 noncrystalline amorphous matrix, thereby protecting them from denaturation or other forms of
248 destruction (41, 42). IDPs or proteins with intrinsically disordered regions are less common in
249 prokaryotes than in eukaryotes, but drawing from work on eukaryotes, they have been proposed
250 to play a central role in cellular process in bacteria that may depend on the formation of

251 molecular condensates (43). It is possible that this is important for the viability of desiccated *A.*
252 *baumannii*.
253
254 Although not much work has been done on *Acinetobacte*r CsrA, based on what is known for
255 other gamma proteobacteria, we hypothesize that a set of ncRNAs that is induced by a GacSA
256 (ABUW_3306 and ABUW_3639) two -component regulatory system, controls the repressor
257 activity of CsrA by sequestering it (44). We can draw a link between the GasSA system and
258 CsrA because they both appear to control catabolism of the aromatic compound phenylacetate.
259 An *A. baumannii* Δ*gacA* mutant is unable to catabolize phenylacetate (45), and our proteomics
260 results suggest that CsrA acts to repress the synthesis of at least one enzyme required for
261 phenylacetate degradation. We hypothesize that a Δ*gacA* mutant does not synthesize ncRNAs
262 that would normally "sponge-up" CsrA, thus allowing CsrA to bind to the 13 -gene phenylacetate
263 mRNA transcript to repress its translation. At this point we do not have a clear understanding of
264 the inventory of *A. baumannii* ncRNAs that may bind to CsrA, but ncRNAs are abundant in
265 AB5075, with several of them expressed at extremely high levels (46). The desiccation
266 phenotype of CsrA appears to depend on its ability to activate translation and although it's
267 difficult to reconcile this activity with a model where CsrA is sequestered by ncRNAs, it is known
268 that ncRNA turnover can occur resulting in the release of free CsrA (29). Most of what is known
269 about mechanisms of CsrA action centers on its role as a repressor of translation (30, 47–49)
270 and it may be of interest to probe its capability as an activator in *A. baumannii*.
271
272 We found that *A. baumannii* AB5075 survived desiccation for six days at 2% RH much better
273 than two other *A. baumannii* strains that we tested, but it is important to note that most studies
274 of desiccation tolerance have been carried out at about 30% RH or in room air and the
275 emphasis has been on the number of days or months that a particular strain remains viable

276 when desiccated. When Farrow et al (20)tested the survival of several strains that were dried
277 and incubated at a relative humidity of 25–61% (mean 46%) they found AB5075 to have an
278 average survival time of 90 days, whereas strains ATCC19606 and ATCC17978 had average
279 survival times of 3 and 34 days respectively. Even though AB5075 is tolerant to desiccation
280 over months at a mean RH of 46%T and over days at 2% RH, we cannot necessarily conclude
281 that the same sets of genes are needed for desiccation tolerance under these two different
282 conditions. For example, Farrow et al. (20) found that the response-regulator protein BmfR was
283 important for desiccation tolerance of ATCC17978 in long term desiccation assays, whereas we
284 did not observe a role of *bmfR* in protecting AB5075 from desiccation in shorter term
285 incubations at 2%RH (Table S2).
286
287 The emergence of *A. baumannii* is great threat in healthcare facilities worldwide, and there is an
288 urgent need for development of new antibiotics and new strategies for infection control and
289 prevention. Since CsrA is involved in several clinically important traits, including desiccation
290 tolerance, biofilm formation, and pathogenesis, the development of modulators of CsrA-RNA
291 interactions may have potential for improving the efficacy of biocides or for treatment of *A.*
292 *baumannii* infections (50).
293
294 **MATERIALS AND MESTHODS**
295 **Bacterial strains and growth conditions.** Strains used in this study are listed in Table S4A.
296 Strain AB5075 (AB5075-UW) was used as a wild type (24) and individual transposon mutants
297 were obtained from the Manoil lab comprehensive ordered transposon mutant library at the
298 University of Washington (24). All strains except for the Δ*csrA* mutant were routinely grown and
299 maintained in TY (10 g Tryptone, 5 g Yeast extract, and 8 g NaCl in 1000 ml) medium or BBL
300 Trypticase Soy Broth (TSB) media at 37°C, unless otherwise stated. The Δ*csrA* mutant was
301 grown in M9/succinate.

302

303 **Desiccation assay.** Strains from a frozen stock (-80°C) were streaked onto TY plates and
304 incubated at 37°C. Colonies (three to five) were picked and inoculated into 2 ml of TSB, and
305 cultures were grown overnight at 37°C with a shaking speed of 200rpm. Overnight cultures were
306 diluted to yield an initial OD_{600} of 0.025 in 10 ml TSB in a 50 ml Erlenmeyer flask. Cultures were
307 grown at 37°C with a shaking speed of 200 rpm to mid-exponential-phase (OD_{600}=0.4 to 0.6) or
308 stationary-phase (24 hours after inoculation). Cells were harvested by centrifugation and
309 washed twice with Dulbecco's phosphate-buffered saline (DPBS, Gibco), and cell density was
310 adjusted to OD_{600}=1 (about 5 x 10^8 cells/ml) with DPBS. Cell suspension (2 spots of 50 µl each
311 per membrane) was filtered onto a 0.4 µm Whatman nucleopore polycarbonate track-etched
312 membranes (25 mm diameter) that had been placed in Nalgene analytical filter unit, and the
313 membranes were then placed into 15 ml uncapped centrifuge tubes. To obtain the T0 (baseline)
314 viable cell number, 1 ml of DPBS was immediately added to one tube and incubated for 5 min at
315 room temperature (24 ± 2°C) on a rotary shaker. Viable cell numbers were determined by
316 plating on TSB agar. For desiccation, tubes with membranes were placed in a Snapware
317 containers (2.3 x 6.3 x 8.4 inches) that contained DRIERITE in the lids of 50 ml centrifuge tubes
318 (x4, 7.5 g of DRIERITE desiccant in each lid) or saturated calcium chloride hexahydrate solution
319 in 5 ml beaker (x8) to yield the RHs of 2% or 30% (± 2), respectively. The Snapware containers
320 were incubated at room temperature. Digital hygrometers (VWR International Ltd) were placed
321 in each container to monitor the RH. At desired time points, tubes containing membranes were
322 removed from the containers, 1 ml of DPBS was added to each tube, and incubated for 5 min at
323 room temperature on a rotary shaker. Viable cell counts were determined on TSB agar. For
324 each strain, a minimum of three biological replicates of desiccation assays were performed
325 except for individual transposon mutants, which were assayed twice for each allele.

326

327 **Screening of desiccation sensitive mutant by Tn-seq.** We created a high-density transposon
328 mutant pool containing approximately 400,000 *A. baumannii* mutants by combining two
329 separately constructed transposon mutant pools. The first transposon mutant pool was
330 previously constructed and selected on LB agar (24) The second transposon mutant pool was
331 constructed on Neidhardt MOPS minimal medium (51) excluding NaCl, and sodium succinate
332 was used as a sole carbon source as follows: T26 mutagenesis was carried out as previously
333 described (24), except following electroporation, cells were incubated in 1 ml of minimal medium
334 with rolling at 37°C for 2 hours. Cells were flash frozen in 5% dimethyl sulfoxide (DMSO) and
335 stored at -80°C for later selection. For selection, frozen cell aliquots were thawed on ice,
336 pelleted, re-suspended in 1 ml fresh minimal medium, and plated onto a Q-tray (Genetix)
337 containing 250 ml minimal medium, 1.2% agarose, and 12.5 µg/ml tetracycline (Tc). After
338 incubating for 16 h at 37°C, cells were scraped, resuspended in 3.5 ml of minimal medium
339 supplemented with 10% glycerol, and aliquots were flash frozen and stored at -80°C. This
340 yielded a density of approximately 1,000 to 2,000 transposon mutants per Q-tray. We repeated
341 these procedures a total of 69 times, and all 3.5 ml aliquots were thawed and mixed to create
342 the final transposon mutant pool, which resulted in a total of approximately 120,000 individual
343 transposon mutants from minimal medium. Finally, 1 ml aliquots of the final transposon mutant
344 pool were flash frozen and stored at -80°C.

345 Aliquots of the transposon mutant pools derived from LB and minimal media were thawed
346 on ice. To account for differences in cell density, 100 µl of the LB transposon mutant pool and 1
347 ml of the minimal medium transposon mutant pool were pelleted and resuspended in 1 ml TSB,
348 and the cells densities for each transposon mutant pool were adjusted to OD_{600}=1.0. These
349 samples were diluted 1:200 in 12.5 ml of TSB in a 250 ml Erlenmeyer flask and incubated at
350 37°C for 24 hours. Cells from each culture were harvested by centrifugation, washed twice with
351 DPBS and cell density was adjusted to OD_{600}=1 with DPBS. The transposon mutant pools were
352 combined in a 2:1 ratio of LB to minimal medium transposon mutant pool. Cell suspension (2

353 spots of 50 µl) was filtered and membranes were treated as described above, except in addition
354 to viable counts, the remaining cells that were resuspended from the membrane (about 900 µl)
355 were plated onto TSB in a Q-tray and incubated for 4-5 hours, which resulted in a thin film of
356 growth. Cells were scraped, resuspended in 1 ml DPBS, centrifuged, and frozen at -80°C for
357 Tn-seq library preparation. Two biological replicates of Tn-seq were performed.
358
359 **Tn-seq library preparation, Illumina sequencing, and data analysis.** Genomic DNA from
360 samples desiccated for 0 or 6 days were extracted using the DNeasy Blood and Tissue Kit
361 (Qiagen). Tn-seq libraries were prepared using the terminal deoxynucleotidyl transferase (TdT)
362 method as previously described (24, 52), and sequencing was carried out on an Illumina Miseq
363 platform. Total reads were ranged from approximately 3.8 to 7.7 million. Oligonucleotides used
364 in this study are listed in Table S5B. Reads were normalized, mapped, and counted using
365 custom Python scripts available at Github (https://github.com/elijweiss/Tn-seq). Reads were
366 further normalized to gene length. To identify candidate genes important for desiccation
367 resistance, reads from both biological replicates were averaged for samples desiccated for 0 or
368 6 days.
369
370 **Construction of the Δ*csrA* mutant.** In-frame deletion of the *csrA* (ABUW_2750) gene was
371 generated by overlap extension PCR as described (53). PCR primers are listed in Table S5B.
372 PCR product was cloned into mobilizable suicide vector pEX2-TetRA and transformed into *E.*
373 *coli* NEB 10-beta (New England Bio Labs). The sequence-verified deletion construct was
374 transformed into *E. coli* S17-1, and further mobilized into *A. baumannii* strain AB5075 by
375 conjugation on TY agar. Single recombinant conjugants were first selected on M9/succinate
376 plate containing 20 µg/ml Tc, and Tc resistant colonies were further plated onto M9/succinate
377 plate containing 5% sucrose. Sucrose resistant and Tc sensitive colonies were screened by

378 colony PCR and sequencing to validate the expected chromosomal in-frame deletion of the *csrA*
379 gene.
380
381 To complement the Δ*csrA* mutant, the full length *csrA* gene including the 15 bp upstream that
382 contains the putative ribosome binding site was PCR amplified and cloned into pMMB67EH-
383 TetRA. The construct was transformed into *E. coli* NEB 10-beta (New England Bio Labs). The
384 sequence-verified construct was transformed into *E. coli* S17-1, and further mobilized into the
385 Δ*csrA* mutant by conjugation on M9/succinate plate containing 20 µg/ml Tc. As a negative
386 control, empty vector pMMB67EH-TetRA was used. The same procedure was used to clone
387 ABUW_2433 and ABUW_2437 for complementation experiments.
388
389 **Label-free protein quantification.** Since the Δ*csrA* mutant had severe growth defect on TY
390 media, both wild type and Δ*csrA* mutant were grown in M9/succinate. Two biological sample
391 replicates were prepared for each strain. Cells from each culture were harvested at OD600=0.5
392 by centrifugation, washed twice with DPBS, and cells were stored in -80°C before further
393 analysis. Cells were lysed in buffer containing 4% SDS, 100 mM Tris pH8.0, 10 mM DTT by
394 heating at 95°C for 5 min. After cooling to room temperature, the lysates were sonicated with
395 ultrasonication probe on ice to shear DNA. Total protein concentration was determined by the
396 BCA assay (Thermo Pierce, Rockford, IL). 500 µg of each protein lysate was reduced and
397 alkylated, diluted in 8 M urea solution, and the SDS was removed with a 3kD molecular weight
398 cutoff filter. After buffer exchange, the protein lysates were digested with trypsin (Promega,
399 Madison, WI) at 37°C overnight and the digested samples were desalted with 1cc C18 Sep-Pak
400 solid phase extraction cartridges (Milford, MA, Waters). The eluted samples were vacuum dried
401 and resuspended in 0.1% formic acid. Reverse phase nanoLC-MS analysis of the protein
402 digests was carried out with a Thermo Easy-nanoLC coupled to a Thermo Q-Exactive Plus
403 Orbitrap mass spectrometer. Triplicate top 20 data-dependent acquisition runs were acquired

404 for each sample, and 1 µg of protein digest was loaded for each run. The peptides were
405 separated by a 50 cm x 75 µm I.D C8 column (5 µm diameter, 100 Å pore size C8
406 MichromMagic beads) with a 90 min 10 to 30% B gradient (solvent A: 0.1% formic acid in water,
407 solvent B: 0.1% formic acid in acetonitrile, flow rate 300 nl/min). The MS data acquisition
408 parameters were set as follows: full MS scan resolution 70k, maximum ion injection time 100
409 mS, AGC target 10^6, scan range of 400 to 2000 m/z; MS/MS scan resolution 17.5 k, maximum
410 ion injection time 100 mS, AGC target 5^4, isolation window 1.6 m/z, HCD NCE 35 scan range of
411 200 to 2000 m/z; loop count 20, intensity threshold 5^3, underfill ratio 1%, dynamic exclusion 10
412 sec. High resolution MS^2 spectra were searched against a target-decoy proteome database of
413 strain AB5075 (a total of 7678 sequences) downloaded from Uniprot (Oct17, 2017) using Comet
414 (version 2015.02 rev. 1) (54) with following parameters: precursor peptide mass tolerance 20
415 ppm, allowing for -1, 0, +1, +2, or +3 ^{13}C offsets; fragment ion mass tolerance 0.02 Da; static
416 modification, carbamidomethylation of cysteine (57.0215 Da); variable modification, methionine
417 oxidation (15.9949 Da). The search results were further processed by PeptideProphet (55) for
418 probability assignment to each peptide-spectrum match, and ProteinProphet (56) for protein
419 inference and protein probability modeling. The output pepXML files from three technical
420 replicates were grouped for subsequent spectral counting analysis using Abacus (57). The
421 pepXML and protXML files for each sample, combined ProteinProphet file from all samples
422 were parsed into Abacus for spectral counting of each protein. The following filters were applied
423 for extracting spectral counts from MS/MS datasets: (1) the minimum PeptideProphet score the
424 best peptide match of a protein must have maxIniProbTH=0.99; (2) The minimum
425 PeptideProphet score a peptide must have to be even considered by Abacus, iniProbTH=0.50;
426 (3) The minimum ProteinProphet score a protein group must have in the COMBINED file,
427 minCombinedFilePw=0.90. Spectral counts for 1616 proteins were reported across four sample
428 groups (two strains and two biological replicates) with estimated protein false discovery rate of

429 1.94%. The protein expression fold changes between wild type AB5075 and Δ*csrA* mutant were
430 computed from adjusted spectral counts output from Abacus.
431
432 **Phenotypic characterization of the Δ*csrA* mutant.** M9/succinate was used in all experiments.
433 For motility assays, an overnight culture (16 to 18 hours) was diluted to yield OD_{600}=0.5, and 2
434 µl of sample was spotted onto the freshly prepared M9/succinate plate and incubated at 37°C
435 for 24 h. For biofilm assays, an overnight culture was inoculated into 100 µl of M9/succinate in
436 Costar vinyl 96 well "U" bottom plates (initial OD_{600}=0.05), and the plates were sealed with
437 Breath-Easy sealing membranes. After incubation at room temperature for 48 hours, culture
438 was removed, the plate was rinsed with tap water twice, and 150 µl of 0.1% crystal violet
439 solution was added to each well. After incubating at room temperature for 15 min, crystal violet
440 solution was removed, the plate was rinsed with tap water 5 times, and the plate was dried at
441 room temperature. For catalase assays, cells were harvested at OD_{600}=0.5, supernatant was
442 removed, and cells were resuspended in DPBS to yield 10 mg wet cell/100 µl DPBS. 100 µl of
443 cell suspensions were placed in 13 x 100 mm borosilicate glass tubes. Then 100 µl of 1% Triton
444 X-100 and 100 µ1 of 30% hydrogen peroxide were added, mixed thoroughly, and incubated for
445 15 min at room temperature (58).
446 .
447

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450 sharing the pEX2-TetRA and pMMB67EH-TetRA vectors. We thank Indranil Biswas for alerting
451 us to the possibility that intrinsically disordered proteins could be involved in desiccation
452 tolerance in *A. baumannii*.
453

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456 Infectious Diseases under Grant 1U19AI107775-01.

457 **FIGURE LEGENDS**

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

464

465

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

473

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

480

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

483 recovered from each membrane. The data are the average of three or more biological
484 replicates, and standard deviations are shown as the error bar.
485
486 **Figure 5.** Effect of expression of *ABUW_2433* (red bar) or *ABUW_2337* (blue bar) gene *in trans*
487 on desiccation tolerance *A. baumannii* (A) ATCC17978 and (B) ATCC19606 after 0 days
488 (control) and 6 days of desiccation at 2% RH. Empty vector (pMMB, black bar) was used as a
489 control. The data are the average of three or more biological replicates, and standard deviations
490 are shown as the error bar.
491
492
493

SUPPLEMENTAL MATERIAL

Figure S1. 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.

Figure S2. Desiccation tolerance of wild type (black symbol) and the transposon mutant pool (red symbol). The cell number represents a total number of viable cells recovered from each membrane. The data are the average of three (for wild type) or two (for transposon mutant pool) biological replicates, and standard deviations from wild-type data are shown as error bars.

Figure S3. 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.

Table S1. Screening of desiccation sensitive mutant by Tn-seq.

Table S2. Mutants from the three-allele library tested for desiccation tolerance

Table S3. (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 S4. Validation of desiccation phenotype of transposon mutant derived from the Δ*csrA* mutant proteomics analysis.

Table S5. Bacterial strains used in this study (A). Plasmids and primers used in this study (B).

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Table 1. *Acinetobacter baumannii* strain AB5075 genes identified as important for desiccation tolerance

Gene	Survival ratio of mutant (day 0/day 6 desiccation)[a]	Gene name	Gene annotation	Ratio of Tn insertions from Tn-seq screen (day 0/day 6 desiccation)	Protein abundance (ratio WT/ΔcsrA)
AB5075 (WT)	17				
ABUW_2389	32	*cioA*	cytochrome bd ubiquinol oxidase, subunit I	12.1	ND[b]
ABUW_2433	2,102		KGG domain-containing protein	6.9	4.3
ABUW_2436	128	*katE*	catalase	1.3	3.2
ABUW_2437	2,540		Iron-containing redox protein	11.0	10.5
ABUW_2639	103		universal stress protein family	19.4	4.4
ABUW_2724	43		hypothetical protein	0.6	2.4
ABUW_2750	43	*csrA*[c]	carbon storage regulator	19.9	8.5
ABUW_2954	68		putative transcriptional regulator	13.6	ND
ABUW_3123	32	*otsA*	trehalose-6-phosphate synthase	1.3	10.5
ABUW_3837	44	*ampD*	N-acetylmuramoyl-L-alanine amidase, family 2	12.7	0.5

[a] All strains were grown in TY broth to the stationary phase of growth prior to being desiccated

[b] ND, protein not detected

[c] The survival ratio (day 0/ day 6 desiccation) of a Δc*srA* mutant grown in M9/succinate was 133,750. The survival ratio of WT grown in M9/succinate was 276.

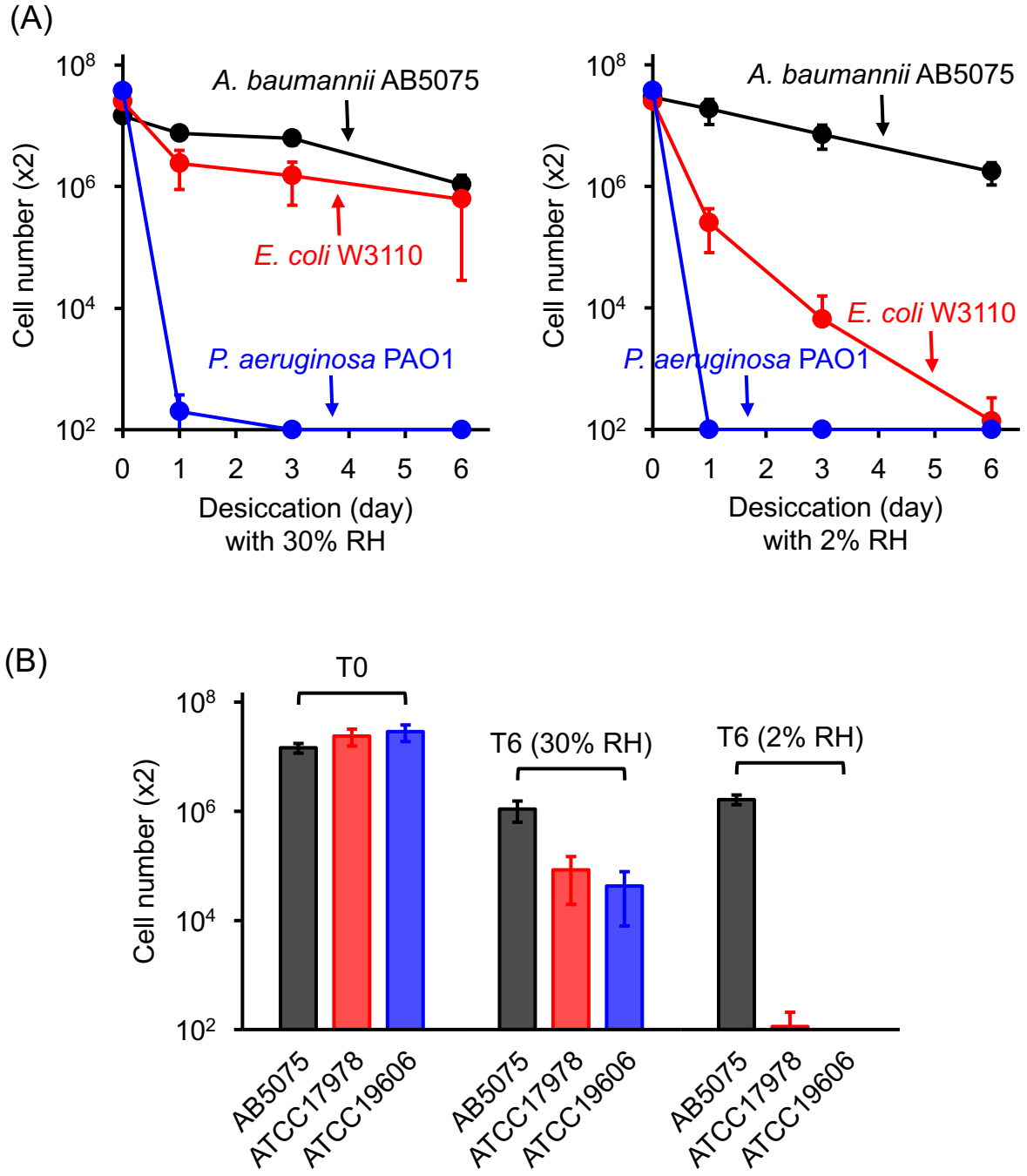


Figure 1. Desiccation tolerance of *A. baumannii*. (A) Comparison of AB5075, *E. coli* W3110, and *P. aeruginosa* PAO1 at 30% RH or 2% RH. (B) Desiccation tolerance of *A. baumannii* strains AB5075, ATCC17978, and ATCC19606 after 0 days (control) and 6 days of desiccation at 30% RH or 2% RH. The cell 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.

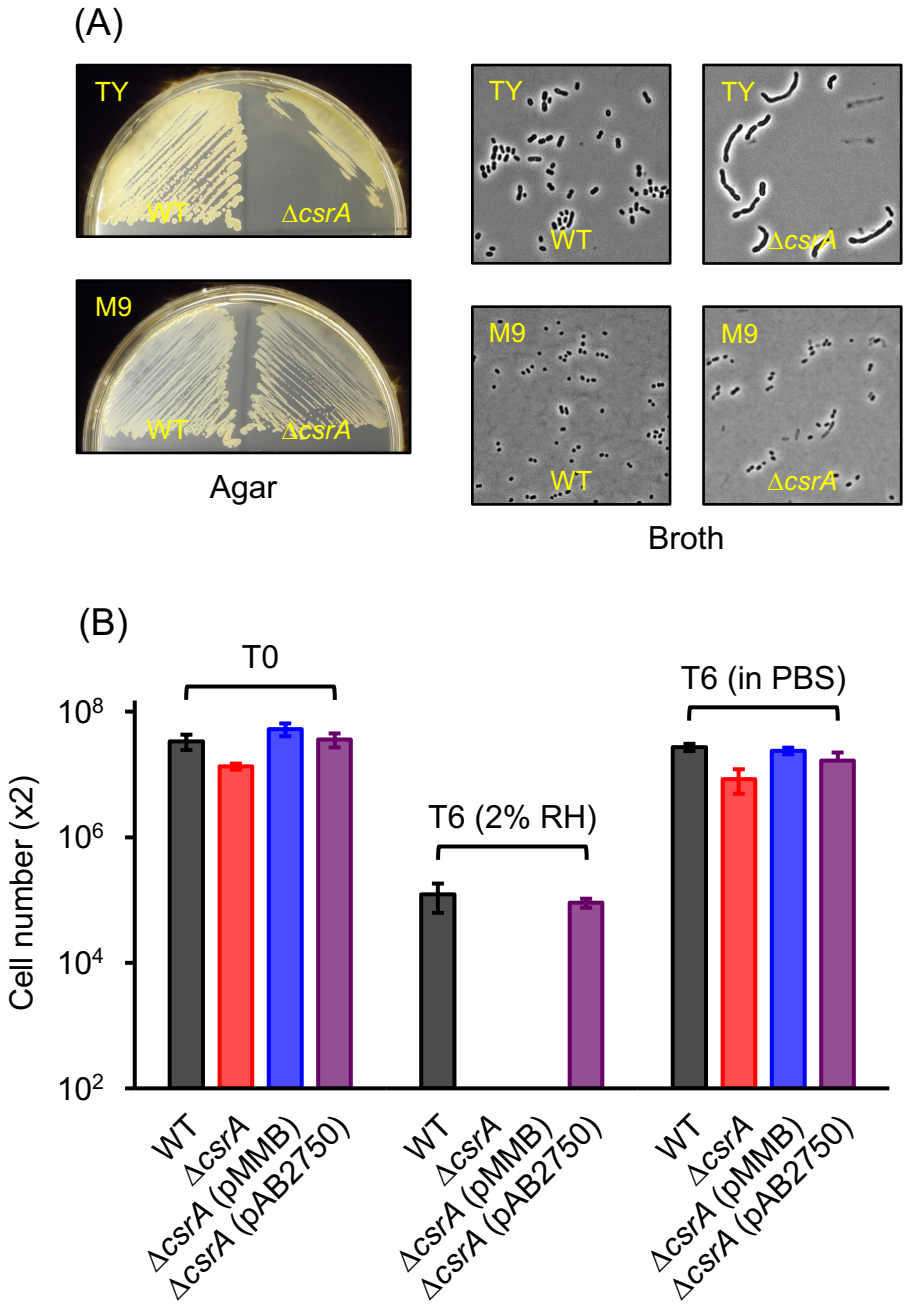


Figure 2. Growth and desiccation tolerance of the Δ*csrA* mutant (A) Comparison of wild type (WT) and the Δ*csrA* mutant on TY and M9/succinate agar and in TY and M9/succinate broth. (B) Desiccation tolerance of WT, Δ*csrA* mutant, Δ*csrA* mutant with pMMB (empty vector), and Δ*csrA* mutant with *csrA* 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.

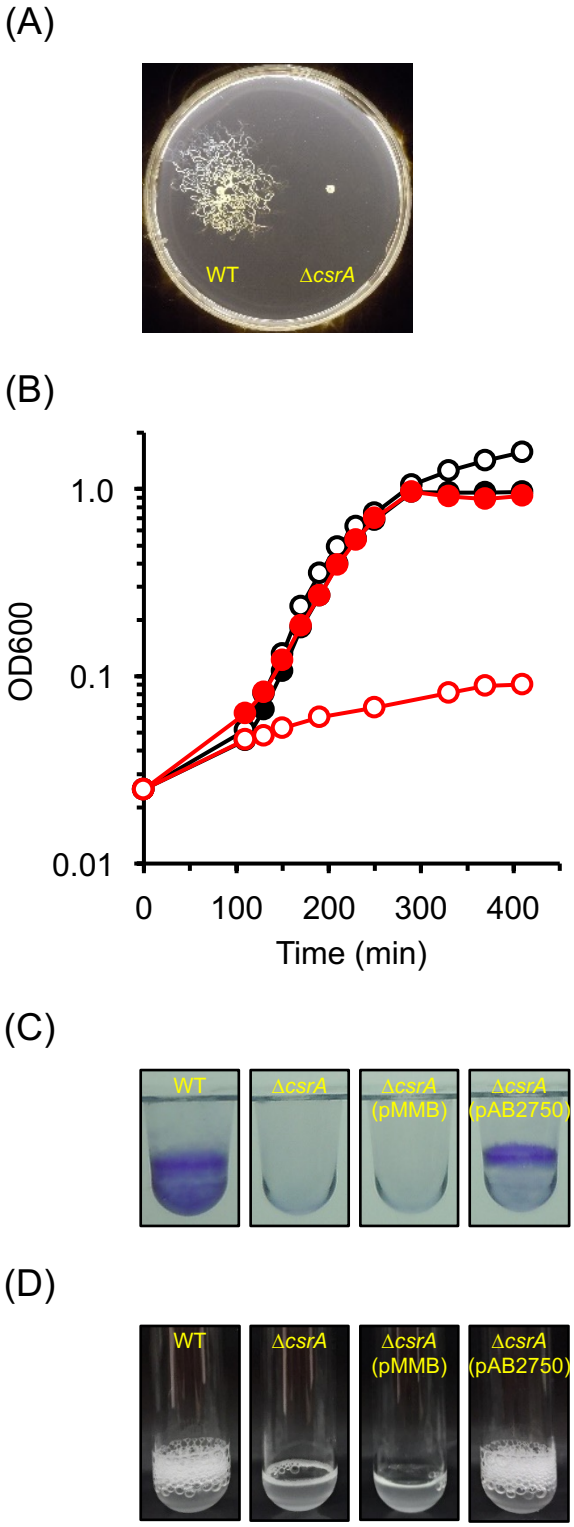


Figure 3. Phenotypes of the Δ*csrA* mutant: (A) twitching motility; (B) growth of wild type (black symbol) or the Δ*csrA* mutant (red symbol) in M9/succinate in the absence (closed symbol) or presence (open symbol) of 0.5% ethanol. The data shown are the representative of each strain and condition; (C) 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.

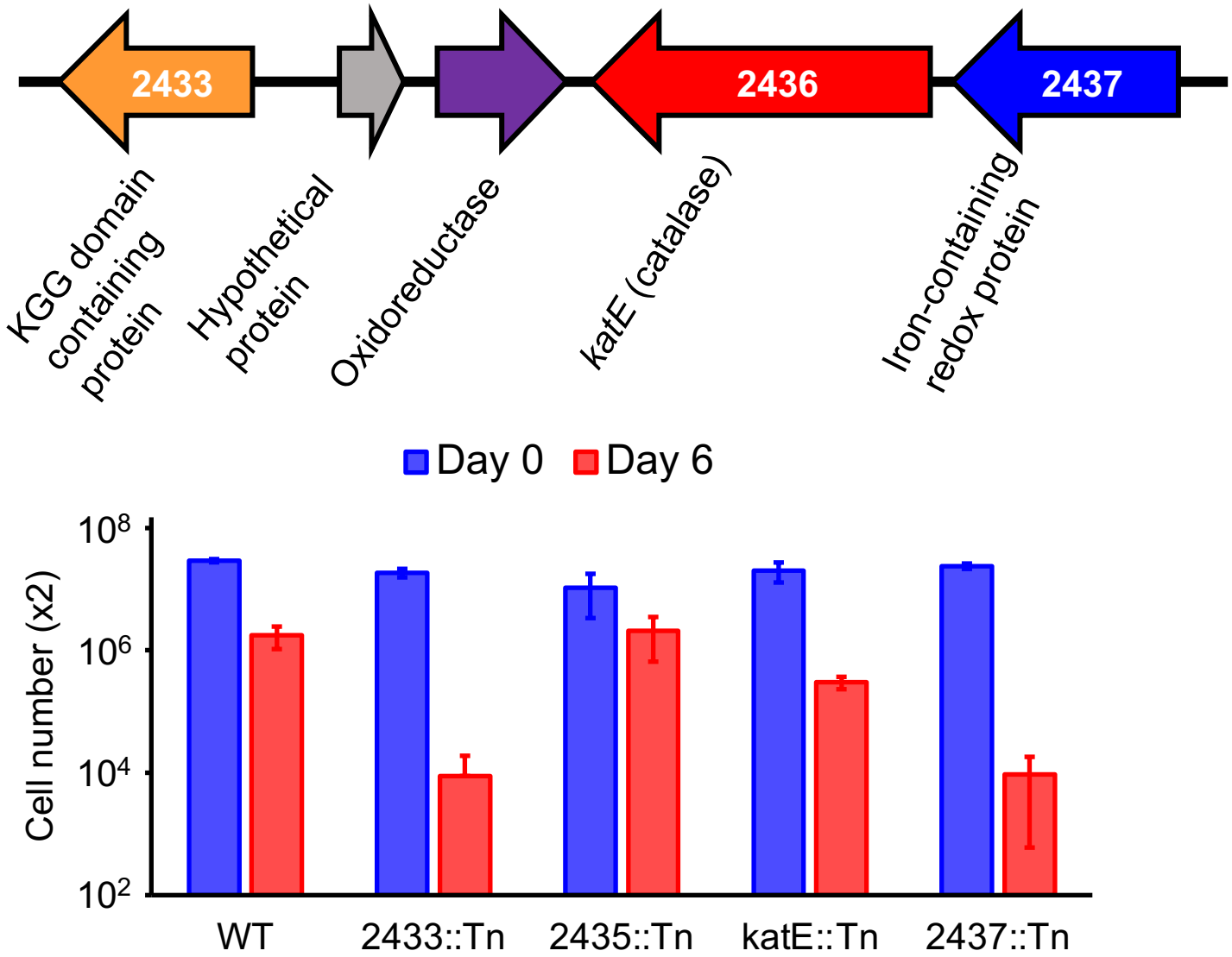


Figure. 4. *A. baumannii* genes important for desiccation tolerance. (A) Genes (B) Desiccation tolerance of mutants at 2% RH. The cell number represents the total number of viable cells recovered from each membrane. The data are the average of three or more biological replicates, and standard deviations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

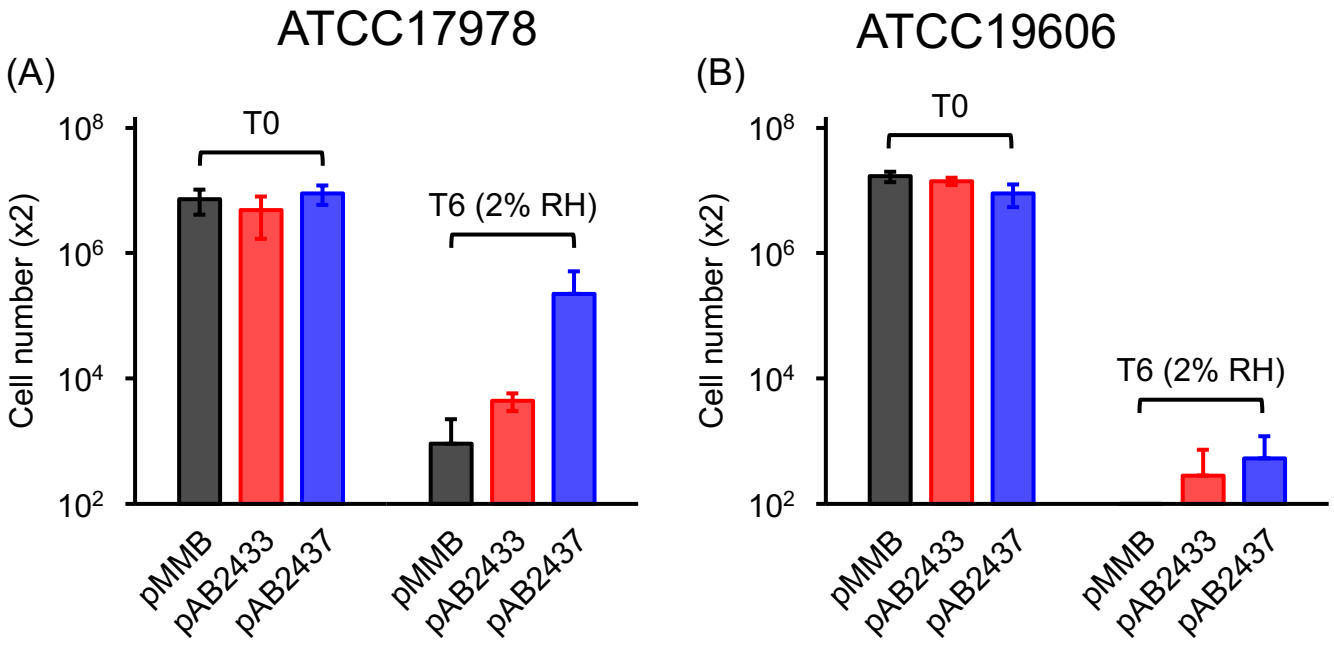
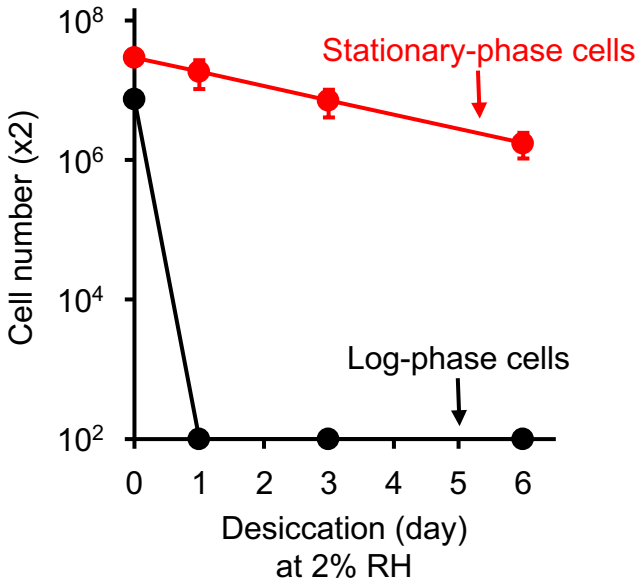
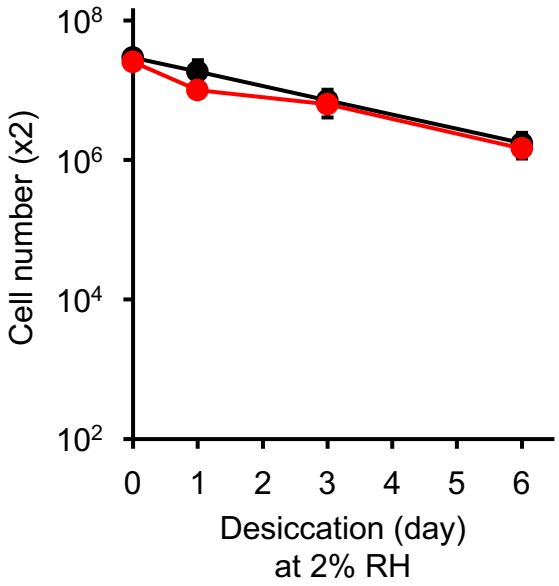


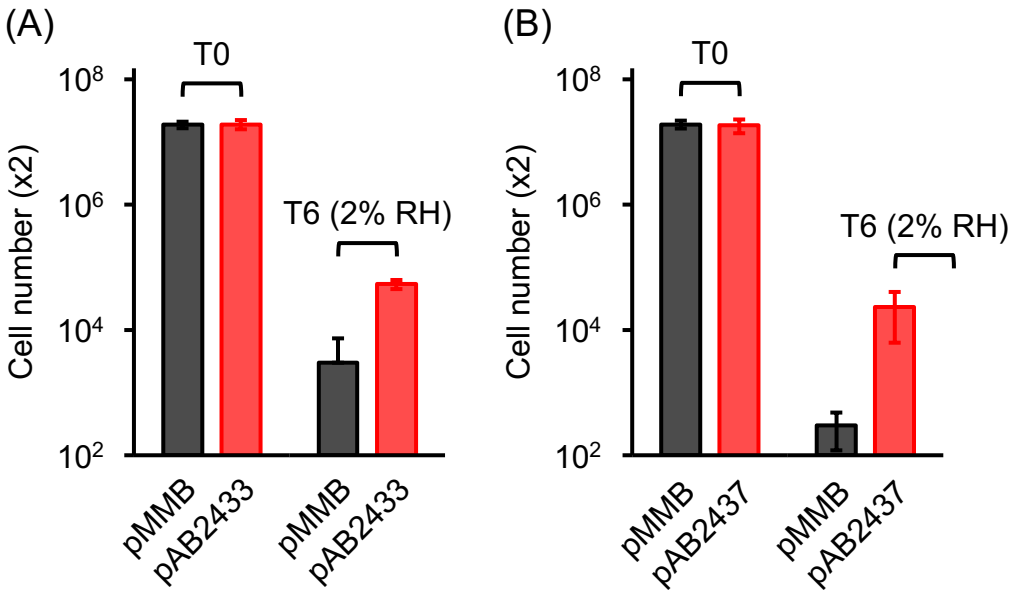
Figure 5. Effect of expression of ABUW_2433 (red bar) or ABUW_2337 (blue bar) gene *in trans* desiccation tolerance *A. baumannii* (A) ATCC17978 and (B) ATCC19606 after 0 days (control) and 6 days of desiccation at 2% RH. Empty vector (pMMB, black bar) was used as a control. The data are the average 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. Desiccation tolerance of wild type (black symbol) and the transposon mutant pool (red symbol). The cell number represents a total number of viable cells recovered from each membrane. The data are the average of three (for wild type) or two (for transposon mutant pool) biological replicates, and standard deviations from wild-type data are shown as error bars.



Supplementary Figure 3. 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.