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1 **Seawater salt-trapped *Pseudomonas aeruginosa* survives for years and gets**
2 **primed for salinity tolerance**

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33 **Abstract**

34 **Background:** In nature, microorganisms have to adapt to long-term stressful conditions
35 often with growth limitations. However, little is known about the evolution of the
36 adaptability of new bacteria to such environments. *Pseudomonas aeruginosa*, an
37 opportunistic pathogen, after natural evaporation of seawater, was shown to be trapped
38 in laboratory-grown halite crystals and to remain viable after entrapment for years.
39 However, how this bacterium persists and survives in such hypersaline conditions is not
40 understood.
41 **Results:**In this study, we aimed to understand the basis of survival, and to characterise
42 the physiological changes required to develop salt tolerance using *P. aeruginosa* as a
43 model. Several clones of
44 *P. aeruginosa* were rescued after fourteen years in naturally evaporated marine salt
45 crystals. Incubation of samples in nutrient-rich broth allowed re-growth and subsequent
46 plating yielded observable colonies. Whole genome sequencing of the *P. aeruginosa*
47 isolates confirmed the recovery of the original strain. The re-grown strains, however,
48 showed a new phenotype consisting of an enhanced growth in growing salt
49 concentration compared to the ancestor strain. The intracellular accumulation of K^+ was
50 elicited by high concentration of Na^+ in the external medium to maintain the
51 homeostasis.Whole transcriptomic analysis by microarray indicated that seventy-eight
52 genes had differential expression between the parental strain and derivative clones.
53 Sixty-one transcripts were up-regulated, while seventeen were down-regulated. Based
54 on a collection of single-gene knockout mutants and gene ontology analysis, we
55 suggest that the adaptive response in *P. aeruginosa* to hyper-salinity relies on multiple

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56 gene product interactions.

57 **Conclusions:** The individual gene contributions build up the observed phenotype, but
58 do not ease the identification of salinity-related metabolic pathways. The long-term
59 inclusion of *P. aeruginosa* in salt crystals primes the bacteria, mediating a readjustment
60 of the bacterial physiology to growth in higher salt concentrations. Our findings provide
61 a starting point to understand how *P. aeruginosa*, a relevant environmental and
62 pathogenic bacterium, survives to long-term salt stress.

63 **Keywords:** *Pseudomonas aeruginosa;* gene expression; salt priming; long-term stress.

64

65

66 Background

67 In their natural environments, microbes often have to cope with stressful conditions. The
68 limitation of nutrients, intense competition for resources and a variety of abiotic stresses
69 such as radiation, temperature, pH, oxygen-derived radicals, antibiotics and high
70 osmolarity are commonly experienced by bacteria [1].

71 Reports on the extreme longevity of microbes in salt are controversial [2]. Hypersaline
72 environments have been a significant reservoir for the long-term evolution of specially
73 adapted microorganisms [3]. Additionally, saline environments may aid the survival of
74 microorganisms, protecting them from desiccation by trapping the cells in fluid
75 inclusions, a phenomenon that occurs in salt crystals upon evaporation [4]. Several
76 studies on ancient microbes are consistent with laboratory experiments and studies on
77 other modern surface halite deposits, which suggest that microorganisms persist inside
78 fluid inclusions in halite for many year [5–7].

79 In a previous study, *Pseudomonas aeruginosa* cells were shown to get trapped in fluid

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80 inclusions pockets of saturated brine in laboratory-grown halite crystals and to remain
81 viable after entrapment [5]. The ability of *P. aeruginosa* to colonise and thrive in myriad
82 environments correlates with its relatively large genome and genetic complexity [8]. An
83 exceedingly high number of assigned open reading frames are transcriptional regulators
84 or members of two-component regulatory systems in comparison to other bacteria [9].
85 This large proportion of regulatory genes also facilitates *P. aeruginosa* adaptability and
86 sensing diverse environmental stresses [8–11]. Potassium is the major intracellular
87 cation in bacteria and plays an important role to maintain homeostasis. In osmotic
88 conditions, bacterial cells accumulate K^+ by a number of different transport systems that
89 vary in kinetics, energy coupling, and regulation [12].
90 However, despite advances in the understanding of the immediate response to
91 hyperosmotic shock in *P. aeruginosa* [13], the physiological mechanisms that allow the
92 bacterial persistence in highly saline environments are still poorly understood. Molecular
93 basis of this persistence may be of great interest to both clinical and environmental
94 microbiology. In the present study, we assess the phenotypic and genotypic changes of
95 *P. aeruginosa* ATCC 27853 after fourteen years of entrapment in seawater salt crystals
96 to characterise the required physiological changes that allow salt tolerance.
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103

104 **Results**

105 **Evaluation of fitness in different salt conditions**

106 In this work, we study the effects of long-term incubation in extremely salty conditions
107 on *P. aeruginosa* using microarrays and salt-tolerance assays. After fourteen years of
108 inclusion in evaporated seawater
109 (37 g/l of salts), different clones of *P. aeruginosa* were recovered and cultivated. The
110 revitalisation of the culture in nutrient broth at 37°C rendered bacterial suspensions that
111 reached an average of
112 OD_{600nm}: 0.9±0.06, after 48 hours of incubation. The ancestor strain (T0 or control) and
113 its derivative 48-hour clones (T48), did not show significant differences in growth rate
114 when cultured with NaCl 8.5 mM (normal concentration of NaCl in DM medium (Table
115 1). However, the final OD was significantly higher in the recovered T48 strain. This
116 implies a growth advantage in the stationary phase demonstrating the adaptability of the
117 recovered cells to extremes conditions such as starvation. After the long period in salt
118 crystals, supposing the selection of more adapted mutants, we also cultured the
119 bacteria on increasing concentrations of NaCl. The variants T48 showed an improved
120 growth rate at concentrations of 250 or 500 mM (Table 1). At 1M, the ancestor strain
121 was not able to grow, whereas T48 clones reached the highest optical density of all
122 conditions (Figure 1). These first results suggested that derivative T48 clones acquired
123 the ability to thrive in high-salt environments, even at NaCl concentrations that were
124 restrictive for the original strain. However, no significant differences were found when
125 comparing growth rates (*r*) between T0 and T48 variants at other evaluated NaCl

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126 concentration (Table 1). Moreover, the addition of 100 mM KCl to bacterial cultures of
127 the T0 strain, inhibited by 1M of NaCl, restored the growth of this strain and allowed T48
128 strain to growth even at 2M NaCl (Figure 2), indicating that growth inhibition not only
129 depends on salt concentration but also on the composition of growth media. The
130 tolerance to NaCl is then influenced by the level of KCl or maybe the ability of the cell to
131 control K^+ transport. Hence, our expectations were to find the role of K^+ and Na^+
132 transporters or regulators in *P. aeruginosa* to long-term hypertonic conditions. All these
133 observations demonstrate that after incubation of *P. aeruginosa* in seawater crystals for
134 a long period (14 years), the cells adapted and became more tolerant to higher salt
135 concentrations.
136
137 **Whole genome sequencing after recovery from salt**
138 To characterise possible genomic adaptations to salt in *P. aeruginosa*, we sequenced
139 five independent clones and the reference strain using a whole genome sequencing
140 approach based on the Illumina MiSeq platform. Seven non-synonymous substitutions
141 were found in the sequenced clones. These mutations were present in one aromatic
142 amino acid transporter, prepilin-type N-terminal cleavage/methylation domain-containing
143 protein, FHA domain-containing protein, hybrid sensor histidine kinase/response
144 regulator and two hypothetical proteins. In addition, three SNPs resulting in a
145 synonymous substitution (c.795G>C p.Arg265Arg; c.34T>C p.Leu12Leu and c.54C>T
146 p.Ser18Ser) respectively in DNA polymerase III subunit beta and in two hypothetical
147 proteins were also detected by WGS (Table S1). Three other mutations were identified
148 in intergenic sites. The products of the genes with non-synonymous mutations do not

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149 seem to contribute in salt stress response. Overall, the lack of convergence in the
150 mutations makes difficult to assign any effect to these SNPs. Suggesting that the
151 changes on salt resistance in the studied strain were probably associated with adaptive
152 response based on changes in global gene expression.
153

154 **Transcriptome profiling of salt-trapped *P. aeruginosa***

155 As the phenotype of T48 clones cannot be easily explained by mutations, differences
156 between T0 and T48 may be due to physiological changes that remain after the
157 recovery from the seawater salt crystals. To investigate the differential gene expression
158 between the wild-type T0 and its salt-tolerant derivate T48, transcriptome analysis by
159 microarrays rendered 78 genes with significant changes in their expression level. From
160 these 78 genes, 25 are genes related to cellular metabolism, 18 are associated to
161 virulence factors, 14 are hypothetical proteins, 11 are associated to transporters and
162 regulatory peptides, 4 are membrane proteins, 4 are implicated in post translational
163 modification and 2 are chaperones and heat shock proteins (Table 2). A global view of
164 all differentially expressed genes is presented in the MA plot in the Figure S1.
165 From the 25 genes encoding enzymes or proteins implicated in the cellular metabolism
166 which are differentially expressed between T0 and T48, 20 genes are up-regulated and
167 five are down-regulated. When the 18 genes associated with expression of virulence
168 factors are analysed, all Type III Secretion System (T3SS) proteins and two cytotoxin
169 secretion factor exoenzymes, ExoS (PA3841) and ExoT (PA0044) are up-regulated
170 (Table 2). A remarkable increase of the expression of H^+ transport T3SS ATPase (*pscN*
171 – PA1697) was also observed in T48 strains. Interestingly, its product can be

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172 responsible for Na^+ extrusion in *P. aeruginosa*. A substantial homeostatic capacity is
173 necessary for adaptation and tolerance to a change in the external environment.
174 There are eight genes encoding transporters that were up-regulated in T48. Except for
175 the genes related to the symport of Na^+/Alanine/Glycine (PA2252), transport of sulphate
176 (*cysW*– PA0281 and *cysT*– PA0282), and the C5-dicarboxylate transport (PA5530), all
177 transporters are putative components of ABC transporters (PA2204, PA3019), and
178 putative amino acid permeases (PA3641, PA0789). Additionally, three genes (a
179 probable AGCS Na^+/alanine/glycine symporter – PA2252, a probable amino acid
180 permease – PA3641, and *nqrB* – PA2998) involved in Na^+ ions transport (GO:0006814)
181 were induced in the T48 variants (fold change 3.10, 2.42 and 2.11 respectively; Table
182 S1 and S2).
183 There is also a set of genes (14 in total) coding for unknown functions that are
184 differentially regulated, half of them are up-regulated and the rest down-regulated
185 (Table 2). Interestingly, when these results are linked to the genome sequence analysis,
186 none of the differentially regulated hypothetical proteins had mutations in the ORF or
187 the promoter. One of these up-regulated hypothetical proteins is *yjjT* (PA4627), which
188 product could be a putative rRNA (Guanin-N2)-methyltransferase (GO:0008990)
189 according to the Gene Ontology analysis.
190

191 **Gene Ontology Analysis**

192 A global analysis of the differentially regulated genes by Gene Ontology (GO) was
193 performed. Such analysis revealed that the majority of the proteins are grouped
194 according to the "catalytic activity" and "binding" biological functions (Figure 4A, Table

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195 S3). When analysed the cellular component category of the GOs, “cell part” which
196 comprises the “plasma membrane” and “intracellular” categories and “macromolecular
197 complex” represent the two groups (Figure 4B). Additionally, the regulated genes could
198 be involved in two biological processes: “metabolic process” and “cellular process”
199 (Figure 4C). When analysed, the product genes’ functions, “transferases”,
200 “oxidoreductases”, “hydrolases”, and “lyases” are the most abundant protein functions
201 (Figure 4D). Stressed bacteria followed a complex adaptive response that involves
202 different biological processes such as the regulation of oxidation-reduction process,
203 regulation of cell shape, transmembrane transport systems and cell redox homeostasis.
204

205 **Salt tolerance assay of *P. aeruginosa* mutants**

206 The detection of a large number of genes differentially regulated in the T48 variant
207 (Table 2) confirmed the hypothesis that high salt resistance in T48 is linked to many
208 genes that participate together in the adaptive response of *P. aeruginosa*. However, it is
209 difficult to determine the individual contribution of each gene in the adaptation to
210 hypersalinity. For this reason, to investigate the individual contribution of each one of
211 the differentially regulated genes in the T48 variant, we decided to explore their
212 available mutants in the
213 *P. aeruginosa* PA14 transposon insertion library [21]. From 78 differentially regulated
214 genes, mutants for 39 genes could be recovered from the library (Table S2). These
215 individual knock-out mutants were tested for growth at different NaCl concentrations.
216 Our results indicated that only mutants in *ccoO2* (cytochrome *c* oxidase subunit),
217 PA4517 (conserved hypothetical protein), *dnaK* (chaperone), and *hslU* (ATP-dependent

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218 protease) showed a significant difference with the wild type when grown in 8.5 and 500
219 mM NaCl (Figure3). Some mutants (in PA1556, PA4517 and PA4761) grew worse than
220 the parental strain in low salt concentration (8.5 mM NaCl), while all they did grow better
221 in high salinity medium (500 mM).
222

223 **Discussion**

224 The results of this study show that *P. aeruginosa* can survive and adapt to prolonged
225 extreme stress conditions. The obtained data suggest that the differential response to
226 salt stress between T0 and T48 variants is not linked to specific mutational events. This
227 in line with previous finding with these clones recovered from salt, where has been
228 shown that after several passages, bacteria recover their normal phenotype [14].
229 However, we cannot discard that some of the detected mutations could play a role in
230 the observed phenotypes. Genetic manipulation of *P. aeruginosa* at single nucleotide
231 level is nowadays still a challenge.
232 The gene expression analysis revealed that many genes are differentially regulated in
233 the stressed cells. The differential induction of membrane transporters may reflect
234 altered ion fluxes between the bacterial cell and the surrounding medium to maintain
235 homoeostasis. In fact, the primary response of bacteria to a highly osmotic environment
236 is the accumulation of certain solutes, like K^+, glutamate, trehalose, proline, and glycine
237 betaine, at concentrations that are proportional to the osmolarity of the medium [15].
238 When bacteria face a growing concentration of Na^+, they actively transport K^+ ions [15].
239 A recent study demonstrated that a steady K^+ supply, even under unfavourable
240 energetic conditions, plays a key role in long-term survival and desiccation tolerance for

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241 *Halobacteriumsalinarum* within salt crystal [16]. This is consistent with the fact that Na^+/
242 K^+ transporters were slightly induced in the T48 variant, including, the glutathione-
243 regulated K^+-efflux system protein KefB (PA1207; 1.5-fold regulation) and a putative K^+
244 channel (PA1496; 1.52-fold regulation). K^+ transporters are regulated by an increase in
245 environment osmolarity regardless of the solute used and turgor. This response is
246 modulated by the external concentrations of Na^+. The K^+ ions act as a cytoplasmic-
247 signaling molecule, activating and/or inducing enzymes and transport systems that
248 allow the cell to adapt to elevated salinity [12, 15].
249 Additionally, three genes involved in Na^+ ion transport (GO:0006814) were induced in
250 the T48 variants (PA2252, PA3641 *nqrB* – PA2998; fold change 3.10, 2.42 and 2.11
251 respectively; Tables S1 and S2). The product of *nqrB* gene is a unique energy-
252 transducing complex, widely distributed among marine and pathogenic bacteria. It
253 converts the energy from the oxidation of NADH and the reduction of quinone into an
254 electrochemical Na^+-gradient that can provide energy to the cell [17]. In addition, it
255 allows the Na^+ ion to pass through the hydrophobic core of the membrane and provides
256 cation specificity to the translocation system [18]. These results are complementary with
257 the 6 up-regulated and 13 down-regulated transporters (Table 2). From these 6 up-
258 regulated transporters, *rnfC* (PA3491) is related with the electron transport complex,
259 which is overexpressed when IscR is up-regulated [19]. In contrast, *rnfE* (PA3494), the
260 putative periplasmic component of the RNF system [20], is underexpressed in T48
261 variants, a contradiction as the RNF system is very close to the Na^+-pumping
262 NADH:ubiquinone oxidoreductase [20, 21]. Another important up-regulated transporter
263 is *czcB* (PA2521), which is associated with resistance to heavy metals [22–24], but

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264 recently it was discovered that it is also responsible for Ca^{2+} ions homeostasis [25].
265 Other up-regulated transporters are *narK1* (PA3877; related with the nitrate respiration
266 under anaerobic conditions [25, 26]), *ompI* (PA3894; related to the aminoglycoside
267 resistance [27]), and two more probable transporters with unknown associated
268 metabolites (PA1876, *yhiH* – PA5231). Despite the fact that the gene coding for OmpI is
269 up-regulated in T48 (Table 2), MexCD (PA4598 and PA4599) transporters, which are
270 related to fluoroquinolones resistance [81, 82], are down-regulated. Similarly, LptG
271 (PA3827), a lipopolysaccharide export system permease, is also down-regulated.
272 Regarding transporters related to carbohydrate transport, RbsA (PA1947) and YhhS
273 (PA1993) are also down-regulated. Finally, the rest of the down-regulated transporters
274 are putative MFS and ABC type transporters of unknown metabolites, except for a
275 putative K^+ channel (PA1496) and YdfC (PA2777), a putative formic/nitrite transporter
276 which was also found to be expressed under antibiotic stress [28, 29].
277 Four members of the 8-gene operon *iscR*-PA3808 the ferredoxin Fdx2 (PA3809), the
278 co-chaperone HscB (PA3811), the L-cysteine desulfuraseIscS (PA3814), and the iron-
279 sulphur cluster assembly transcription factor IscR (PA3815) are up-regulated,
280 supporting previous observations about their expression in high concentration of salts
281 [19]. Surprisingly, we did not find any significant up-regulation of the IscR-regulated
282 ferredoxin FprB (PA4615), which are also associated with salt stress [30]. Other up-
283 regulated genes are implicated in post-translational modifications such as *trmD*
284 (PA3743; tRNA guanosine methyltransferase) and *endA* (PA2749; DNA-specific
285 endonuclease I). Methylation of coding or non-coding RNA might play an important role
286 in gene expression regulation [31]. Moreover, the S-adenosylmethionine decarboxylase

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287 proenzyme (*speD*; PA0654), involved in spermidine biosynthesis, was up-regulated in
288 the T48 variants. Interestingly, previous works reported that spermidine is effective in
289 alleviating the adverse effect of salt stress on plants [32, 33]. A recent finding indicated
290 that spermidine priming treatments enhanced the antioxidant systems in plants exposed
291 to salt stress and contributed to improved ion homeostasis [32–34]. Similarly, in *P.*
292 *aeruginosa,* Johnson *et al* [34] reported that spermidine plays an important function as
293 an organic polycation to bind lipopolysaccharide and to stabilize the cell surface. It
294 protects the outer membrane from aminoglycoside antibiotics, antimicrobial peptides,
295 and oxidative stress.

296 Another up-regulated gene coding for a two-component system response regulator
297 PmrA (PA4776), was identified in this study, which was recently reported to be
298 associated with polymyxin resistance and hence osmotic stress [35].

299 The *rsmA* gene, known to be a regulator of the secondary metabolism and a carbon
300 storage regulator, is down-regulated in salt-tolerant clones (Table 2). RsmA was found
301 to play a very important role in early pathogenesis, especially in early colonisation and
302 dissemination [36], due to its relevance in the expression of Type VI Secretory System
303 (T6SS) [37, 38]. Moreover, *rsmA*-knockouts strains of *P. aeruginosa* have altered the
304 expression of genes involved in a wide variety of pathways, including iron acquisition,
305 formation of multidrug efflux pumps and motility [39]. The genes coding for TssK1
306 (PA0079) and IcmF2 (PA1669) are also down-regulated. Both proteins are fundamental
307 for the pathogenesis of *P. aeruginosa*, as the former is implied in the assembly of T6SS
308 complex [40] while the latter is involved in the virulence [41].

309 Most of these T3SS proteins are members of the 12-gene operon *popN*-*popD*, which

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310 are expressed not only in pathogenesis but also under different environmental stresses
311 such as low concentration of Ca^{2+} or direct contact with host cells [42]. Additionally,
312 ExoS and ExoT have an ADP-ribosyltransferase activity, playing an important role in the
313 bacterial survival and dissemination in clinical strains [43, 44, 45].

314 Salt stress also showed impact on three metabolic genes that were up-regulated: the
315 cytochrome *c* oxidase (*coxA*; PA0106), a putative acyl-CoA dehydrogenase (PA0508),
316 and the anthranilate-coenzyme A ligase (*pqsA*; PA0996). Previously, the putative acyl-
317 CoA dehydrogenase, a gene associated with changes in membrane fluidity [46], was
318 found overexpressed in *Burkholderia pseudomallei* when treated with NaCl. The fact
319 that *pqsA* is up-regulated may indicate that the *Pseudomonas* quinolone signal (PQS)
320 could be also overexpressed during salt encapsulation. This protein shapes bacterial
321 population structure to survive under stressful environments and kills sensitive bacteria
322 at a time that promotes anti-oxidative stress response [47].

323 When transcriptional regulators are analysed, only a putative transcriptional regulator
324 (PA0547) is up-regulated in T48, having a potential role in the differential regulation of
325 gene expression. However, *hslU* (PA5054) and *dnaK* (PA4761) genes, which encode
326 for chaperone activity are found significantly down-regulated. This observation is in
327 contrast with previous studies, where *dnaK* was overexpressed, being relevant in salt
328 resistance in *Lactococcuslactis* [48]. Moreover, *dnaK* was also found up-regulated in
329 marine bacteria allowing the adaptation to cold environments [49]. Possibly, these
330 chaperones were up-regulated in salt-trapped bacteria but, once the T48 variant was
331 recovered, these genes are quickly down-regulated due to other salt stress adaptations,
332 the same maybe true for other genes.

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333 CgrA (PA2127), which is found to be related to the expression of RsmN and, thus, the
334 repression of RsmA [38], is up-regulated in T48 variant. The *cgrA* gene plays a key role
335 in the expression of fimbrial genes and is related to MvaT mutants or anaerobic growth
336 [50]. Despite the fact that only *narK1* (PA3877) was found to be up-regulated, results
337 indicate that most of the up-regulated genes in T48 are associated to aerobic growth.
338 Additionally, no mutations in the MvaT transcriptional regulator were revealed by whole
339 genome analysis.
340 According to our results, salt resistance could be considered as a priming response, i.e.
341 as a physiological process by which organisms prepare themselves for more quick or
342 aggressive situations to future biotic or abiotic stress [51]. Although this phenomenon
343 has been studied mostly in plants, there are also some examples of priming in the
344 bacterial world. One critical issue is to explore how the signals that induce priming are
345 received and transduced by the cells and prepare the bacteria for long-term persistence
346 if growth is not possible. In plants, priming to salinity plays an important role as adaptive
347 phenotypic strategy [52]. This process could develop different defence mechanisms in
348 the cells against salinity stress such as antioxidant defence systems, the repair of
349 membranes and the osmotic adjustment [53]. This kind of response is characterised,
350 essentially, by the slow induction of many genes that together contribute to the
351 acquisition of quick and effective adaptive strategy against stressor conditions. In such
352 cases, molecular mechanisms responsible for priming effects are involved in the
353 accumulation of signalling proteins or transcription factors [54], as well as epigenetic
354 mechanisms [55, 56]. These epigenetic mechanisms are thought to bring a faster and
355 more potent response to subsequent exposure to stress. This idea is supported for the

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356 Gene Ontology analysis, which suggested that some genes were involved in regulation
357 of transcription, methylation process, response to stimulus, RNA metabolic processes
358 and quorum sensing.
359 Interestingly, neither the mutants nor the wild-type were able to grow in DM with 1
360 M NaCl. All these genes showed a decreased transcription in the T48 variant and,
361 consequently, if they are involved in adaptation to hypersalinity, a better growth
362 under high NaCl concentration is expected when inactivated. Moreover, no
363 sequence changes between the five genes from T0 and T48 were found, suggesting
364 that the differences in growth under high salt conditions may be due to differential
365 regulation, which requires further research to be clarified. The preservation of a
366 long-lasting phenotype is not new in bacteria. For instance, the lactose
367 metabolization response in *Escherichia coli* is maintained during more than ten
368 generations after the removal of lactose due to the inheritance of very stable
369 proteins [57].
370

371 **Conclusions**

372 *Pseudomonas aeruginosa* can survive in inclusions of seawater crystals for many years.
373 Upon recovery, this bacterium shows a better ability to grow in highly saline conditions,
374 and the adaptation seems to be only phenotypic but not genetic, indicating a 'priming'
375 phenomenon in this plastic bacterium. Although we have identified several genes
376 potentially involved in adaptation to saline environments, the exact mechanisms which
377 are responsible for priming in *P. aeruginosa* remain unclear. Our study provides a good
378 start toward a deep understanding of the long-term salt stress behaviour of *P.*
379 *aeruginosa*.

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380

381

382

383

384 **Methods**

385 **Bacterial model and growth conditions.** *P. aeruginosa* ATCC 27853 (wild-type: T0 in
386 this study) was grown overnight at 37°C in nutrient broth, centrifuged at 13,000 rpm for
387 10 min, washed three times and then suspended in filtered sterilise seawater to a final
388 concentration of approximately 10^9 CFU/ml in three independent replicas. Cells were
389 incubated during fourteen years in closed Erlenmeyer flasks at room temperature. The
390 concentration of salts in the used seawater was 37 g/l. The water was allowed to
391 evaporate, the saline crystals were apparent after eight months, and the culture became
392 completely desiccated after ten months. The initial number of CFU/ml was confirmed by
393 serial decimal dilutions in nutrient agar.

394

395 **Revitalisation of the bacterial cells.** *P. aeruginosa* cells, maintained during fourteen
396 years in sterilised seawater, were revitalised by the addition of 100 ml of sterilised
397 nutrient broth to the salt crystal in the Erlenmeyer flask and incubated at 37°C with 100
398 rpm of shaking. Subsequent plating of an aliquot from this culture on nutrient agar
399 yielded observable colonies. A few isolated colonies from the different replicas were
400 recovered and saved for further analysis. Biochemical profiles of *P. aeruginosa* ATCC

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401 27853 and the resuscitated cells (T48 variant in this study) were characterised using
402 API 20NE system (bio-Merieux, France).
403
404 **Evaluation of fitness in different salinity conditions.** Bacterial growth curves were
405 carried out in flat-bottomed 96-well microplates (Nunc, Denmark). Each well was filled
406 with 100 µl of Davis Minimal medium (DM: Na2HPO4 6.78g/l; KH2PO4 3g/l; NaCl 0.5g/l;
407 NH4Cl 1g/l; 1 mM MgSO4; 0.1 mM CaCl2, 0.28% Glucose and 0.25% casamino acids),
408 supplemented with NaCl to final concentrations of 2M, 1M, 500mM, 250mM and 8.5mM.
409 Overnight cultures of T0 and T48
410 *P. aeruginosa* cells were added to a final OD595 of 0.04. The growth of T0 and T48
411 variants was followed with four replicas of each one in the same concentrations of NaCl.
412 Microplates were incubated in an Infinite F200 TECAN microplate reader for 24 hours at
413 37°C with 15s of shaking duration, 3mm of shaking amplitude. The interval time of
414 absorbance measurements at 595nm was 15min. The same manipulation was repeated
415 adding 100mM KCl to selected concentrations of NaCl.
416
417 **Whole genome sequencing after recovery from salt.** Libraries were prepared using a
418 TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) and were sequenced on
419 anIllumina-MiSeq system using a 600-cycle v3 reagent kit, resulting in 300-bp paired-
420 end reads. Sequence data are available from the NCBI database under Bioproject
421 accession PRJNA420955. A reference genome for strain
422 *P. aeruginosa* ATCC 27853 was assembled using A5-miseq version 20140604 and
423 annotated using prokka version 1.12-beta [59]. Snippy version 3.2 [60] was used to

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424 identify variants in strains c1-5 (clone1 to clone5) relative to the reference complete
425 genome of *P. aeruginosa* ATCC 27853 (Genbank accession CP015117). Assembly of
426 1,818,724 error-corrected reads (estimated 47.68-fold coverage) resulted in 46 contigs
427 with an N50 of 353 kb and a total size of 6.79 Mb.
428
429 **Transcriptome profiling of salt-trapped *P. aeruginosa*.** To find out what genes
430 are involved in the differential salinity resistance, global transcription profile of
431 cultures of *P. aeruginosa* T0 and its derivative T48 variant were carried out using
432 microarray technology. Bacterial cells were grown overnight in Davis minimal
433 medium (DM) at 37ºC under 200 rpm of shaking. Three independent 1/50 dilutions
434 of each of them were grown until they reached an optical density of 0.5 at 600 nm.
435 The cells were washed and resuspended in DM supplemented with RNA protect
436 reagent (Qiagen, Germany). Cell lysis and total RNA extractions were performed
437 with the RNeasy mini kit according to the manufacturer's recommendations
438 (Qiagen, Chatsworth, CA), except that 1 mg/ml of lysozyme was used to lyse
439 *Pseudomonas* cells. DNase digestions were carried out on the column by adding 82
440 units of Kunitz enzyme (Qiagen) with incubation at room temperature for 15 min. An
441 additional DNase digestion was performed on the purified RNA to ensure the
442 absence of DNA. RNA quality was checked through agarose electrophoresis before
443 cDNA synthesis. Fluorescently labelled cDNA for microarray hybridisation was
444 obtained by using the SuperScript Indirect cDNA Labelling System (Invitrogen) as
445 recommended by the supplier. Briefly, 20 µg of total RNA was transformed to cDNA
446 with Superscript III reverse transcriptase using random hexamers as primers and
447 including aminoalyl-modified nucleotides in the reaction mixture. After cDNA
448 purification, Cy3 or Cy5 fluorescent dye (Amersham Biosciences) was coupled to the

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449 amino-modified first-strand cDNA. The labelling efficiency was assessed by using a
450 NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). Equal amounts of
451 Cy3- or Cy5-labelled cDNAs, one of them corresponding to the control and the other
452 to the problem under analysis, were mixed and dried in a Speed-Vac. Labelled cDNA
453 was hybridised to *P. aeruginosa* microarray slides version 2 from the Pathogen
454 Functional Genomics Resource Center from J. Craig Venter Institute Microbial
455 Hybridization of Labelled Probes protocol. Following hybridization, the slides were
456 washed, dried, and scanned using a ScanArray Express scanner and software
457 (Packard BioScienceBioChip Technologies). For the analysis of DNA microarray
458 slides, background correction and normalization of expression data were performed
459 using LIMMA [61]. To avoid the exaggerated variability of log ratios for low-intensity
460 spots during local background correction, we used the *normexp* method in LIMMA to
461 adjust the local median background estimates. The resulting log ratios were print-
462 tip LOESS normalised for each array [62]. Only genes that exhibited changes
463 compared to the wild-type control of two-fold and more, as well as P values of
464 ≤0.05, were considered in the study. Finally, to explore the functional roles of the
465 regulated genes, the Gene Ontology (GO) analysis was performed through the
466 PANTHER online software [63] and QuickGO tool [64].

467 **Salt tolerance assay of *P. aeruginosa* mutants.** The desired mutants were isolated
468 from PA14 transposon insertion mutants [65]. The selected *P. aeruginosa* PA14
469 mutants, with deletions in genes showing transcriptional variation in the microarray
470 experiments, were used. Salt tolerance of these mutants was measured and compared
471 to the wild-type strain PA14. The salt tolerance assay was performed on 96-well
472 polystyrene plates. Each well was filled with 100 μl of DM minimal medium
473 supplemented with NaCl to final concentrations of 8.5 mM (DM with no NaCl added),

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474 250 mM and 500 mM, with four replicas for each NaCl concentration. The microplates
475 were incubated at 37°C, and the optical density at 600 nm was measured after 24
476 hours. For mutants showing statistically significant differences in growth respect the
477 wild-type strain on NaCl (>25%), salt tolerance assay was repeated for each sodium
478 chloride concentration.
479
480 **Statistical analysis.** All parameters for the growth curves were estimated using
481 Growthcurver [66]. Using this data, all model parameters —carrying capacity, initial
482 population size, growth rate, doubling time and the empirical area under the curve—for
483 all growth curves of both variants, T0 and T48, were compared using Student's *t* test
484 according to the different NaCl and KCl concentrations. Additionally, two-sided
485 Kolmogorov-Smirnov tests were applied to compare the growth curves per treatments.
486 *P* values less than or equal to 0.05 were considered statistically significant. All statistical
487 tests were performed in R v. 3.4.4 [67].
488
489 **Acknowledgements**

490 We are grateful to Prof. Jens Rolff for support and helpful comments from Yeliz Karatas,
491 Arpita Nath from Freie Universität Berlin and Dr Paul Smith.
492
493 **Funding**
494

495 This work was supported by Grants PI10/00105 and REIPI RD06/0008, both from
496 Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, the last co-financed by
497 European Development Regional Fund "A way to achieve Europe" ERDF, Spanish
498 Network for Research in Infectious Diseases (REIPI RD06/0008) and by the PAR
499 project (Ref 241476) from the EU 7th Framework Programme. ARR was also supported
500 SFB 973 (Deutsche Forschungsgemeinschaft, project C5).

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Availability of data and materials

All data are available in the manuscript and supplementary material. The sequences and genomic data were deposited as indicated in the manuscript.

Authors' contributions

EH, EGT, CIQ, AB, KG and ARR carried out the experimental work. ARR and JB designed the experimental work. PJ contributed with genome sequencing and analysis. All authors conducted analytical work. EH and ARR drafted the manuscript with input from all authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

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540 **References**

541 1. Hengge-Aronis R. The general stress response in Escherichia coli. In: Hengge-Aronis
542 GS and R, editor. Bacterial Stress Responses. Washington, D.C.: ASM Press,; 2000. p.
543 161–78.
544 2. Lowenstein TK, Schubert BA, Timofeeff MN. Microbial communities in fluid inclusions
545 and long-term survival in halite. GSA Today. 2011;21:4–9. doi:10.1130/GSATG81A.1.
546 3. McGenity TJ, Gemmell RT, Grant WD, Stan-Lotter H. Origins of halophilic
547 microorganisms in ancient salt deposits. Minireview. Environ Microbiol. 2000;2:243–50.
548 doi:10.1046/j.1462-2920.2000.00105.x.
549 4. Norton CF, Grant WD. Survival of Halobacteria Within Fluid Inclusions in Salt
550 Crystals. J Gen Microbiol. 1988;134:1365–1.
551 5. Adamski JC, Roberts JA, Goldstein RH. Entrapment of Bacteria in Fluid Inclusions in
552 Laboratory-Grown Halite. Astrobiology. 2006;6:552–62. doi:10.1089/ast.2006.6.552.
553 6. Fendrihan S, Legat A, Pfaffenhuemer M, Gruber C, Weidler G, Gerbl F, et al.
554 Extremely halophilic archaea and the issue of long-term microbial survival. Re/views

bioRxiv preprint doi: https://doi.org/10.1101/649152; this version posted May 24, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

555 Environ Sci bio/technology. 2006;5:203–18. doi:10.1007/s11157-006-0007-y.
556 7. Schubert BA, Lowenstein TK, Timofeeff MN, Parker MA. Halophilic *Archaea* cultured
557 from ancient halite, Death Valley, California. Environ Microbiol. 2010;12:440–54.
558 doi:10.1111/j.1462-2920.2009.02086.x.
559 8. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al.
560 Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic
561 pathogen. Nature. 2000;406:959–64. doi:10.1038/35023079.
562 9. Rodrigue A, Quentin Y, Lazdunski A, Méjean V, Foglino M. Cell signalling by
563 oligosaccharides. Two-component systems in Pseudomonas aeruginosa: why so
564 many? Trends Microbiol. 2000;8:498–504.
565 10. Langton Hewer SC, Smyth AR. Antibiotic strategies for eradicating Pseudomonas
566 aeruginosa in people with cystic fibrosis. Cochrane Database Syst Rev.
567 2017;4:CD004197.
568 11. Snitkin ES, Segre JA. Pseudomonas aeruginosa adaptation to human hosts. Nat
569 Genet. 2015;47:2–3. doi:10.1038/ng.3172.
570 12. Epstein W. The roles and regulation of potassium in bacteria. Prog Nucleic Acid Res
571 Mol Biol. 2003;75:293–320. http://www.ncbi.nlm.nih.gov/pubmed/14604015. Accessed 4
572 May 2018.
573 13. Aspedon A, Palmer K, Whiteley M. Microarray analysis of the osmotic stress
574 response in Pseudomonas aeruginosa. J Bacteriol. 2006;188:2721–5. doi:188/7/2721
575 [pii] 10.1128/JB.188.7.2721-2725.2006.
576 14. Romsang A, Duang-Nkern J, Leesukon P, Saninjuk K, Vattanaviboon P,
577 Mongkolsuk S. The Iron-Sulphur Cluster Biosynthesis Regulator IscR Contributes to

bioRxiv preprint doi: https://doi.org/10.1101/649152; this version posted May 24, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

578 Iron Homeostasis and Resistance to Oxidants in Pseudomonas aeruginosa. PLoS One.
579 2014;9:e86763.
580 15. Csonka LN. Physiological and genetic responses of bacteria to osmotic stress.
581 Microbiol Rev. 1989;53:121–47. http://www.ncbi.nlm.nih.gov/pubmed/2651863.
582 Accessed 4 May 2018.
583 16. Kixmüller D, Greie J-C. An ATP-driven potassium pump promotes long-term survival
584 of Halobacterium salinarum within salt crystals. Environ Microbiol Rep. 2012;4:234–41.
585 doi:10.1111/j.1758-2229.2012.00326.x.
586 17. Juárez O, Barquera B. Insights into the mechanism of electron transfer and sodium
587 translocation of the Na(+)-pumping NADH:quinone oxidoreductase. Biochim Biophys
588 Acta. 2012;1817:1823–32. doi:10.1016/j.bbabio.2012.03.017.
589 18. Juárez O, Athearn K, Gillespie P, Barquera B. Acid residues in the transmembrane
590 helices of the Na+-pumping NADH:quinone oxidoreductase from Vibrio cholerae
591 involved in sodium translocation. Biochemistry. 2009;48:9516–24.
592 doi:10.1021/bi900845y.
593 19. Romsang A, Duang-Nkern J, Wirathorn W, Vattanaviboon P, Mongkolsuk S.
594 Pseudomonas aeruginosa IscR-Regulated Ferredoxin NADP(+) Reductase Gene (fprB)
595 Functions in Iron-Sulfur Cluster Biogenesis and Multiple Stress Response. PLoS One.
596 2015;10:e0134374.
597 20. Hreha TN, Mezic KG, Herce HD, Duffy EB, Bourges A, Pryshchep S, et al.
598 Complete topology of the RNF complex from Vibrio cholerae. Biochemistry.
599 2015;54:2443–55.
600 21. Reyes-Prieto A, Barquera B, Juárez O. Origin and Evolution of the Sodium -

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601 Pumping NADH: Ubiquinone Oxidoreductase. PLoS One. 2014;9:e96696.

602 22. Li K, Pidatala RR, Ramakrishna W. Mutational, proteomic and metabolomic analysis

603 of a plant growth promoting copper-resistant *Pseudomonas* spp. FEMS Microbiol Lett.

604 2012;335:140–8.

605 23. Jain S, Bhatt A. Molecular and in situ characterization of cadmium-resistant

606 diversified extremophilic strains of Pseudomonas for their bioremediation potential. 3

607 Biotech. 2014;4:297–304.

608 24. Chong TM, Yin W-F, Chen J-W, Mondy S, Grandclément C, Faure D, et al.

609 Comprehensive genomic and phenotypic metal resistance profile of Pseudomonas

610 putida strain S13.1.2 isolated from a vineyard soil. AMB Express. 2016;6:95.

611 25. Khanam S, Guragain M, Lenaburg DL, Kubat R, Patrauchan MA. Calcium induces

612 tobramycin resistance in Pseudomonas aeruginosa by regulating RND efflux pumps.

613 Cell Calcium. 2017;61:32–43.

614 26. Sharma V, Noriega CE, Rowe JJ. Involvement of NarK1 and NarK2 Proteins in

615 Transport of Nitrate and Nitrite in the Denitrifying Bacterium Pseudomonas aeruginosa

616 PAO1. Appl Environ Microbiol. 2006;72:695–701.

617 27. Jo JTH, Brinkman FSL, Hancock REW. Aminoglycoside efflux in Pseudomonas

618 aeruginosa: involvement of novel outer membrane proteins. Antimicrob Agents

619 Chemother. 2003;47:1101–11.

620 28. Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, et al.

621 Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa. Antimicrob

622 Agents Chemother. 2008;52:3648–63.

623 29. Zhu Y, Czauderna T, Zhao J, Klapperstueck M, Maifiah MHM, Han M-L, et al.

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624 Genome-scale metabolic modeling of responses to polymyxins in Pseudomonas
625 aeruginosa. Gigascience. 2018;7.
626 30. Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA Modifications in Gene
627 Expression Regulation. Cell. 2017;169:1187–200. doi:10.1016/j.cell.2017.05.045.
628 31. Li S, Jin H, Zhang Q. The Effect of Exogenous Spermidine Concentration on
629 Polyamine Metabolism and Salt Tolerance in Zoysiagrass (Zoysia japonica Steud)
630 Subjected to Short-Term Salinity Stress. Front Plant Sci. 2016;7:1221.
631 doi:10.3389/fpls.2016.01221.
632 32. Liu HP, Dong BH, Zhang YY, Liu ZP, Liu YL. Relationship between osmotic stress
633 and the levels of free, conjugated and bound polyamines in leaves of wheat seedlings.
634 Plant Sci. 2004;166:1261–7. doi:10.1016/J.PLANTSCI.2003.12.039.
635 33. Li J, Hu L, Zhang L, Pan X, Hu X. Exogenous spermidine is enhancing tomato
636 tolerance to salinity–alkalinity stress by regulating chloroplast antioxidant system and
637 chlorophyll metabolism. BMC Plant Biol. 2015;15:303. doi:10.1186/s12870-015-0699-7.
638 34. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-Localized
639 Spermidine Protects the Pseudomonas aeruginosa Outer Membrane from Antibiotic
640 Treatment and Oxidative Stress. J Bacteriol. 2012;194:813–26. doi:10.1128/JB.05230-
641 11.
642 35. Lou Y-C, Weng T-H, Li Y-C, Kao Y-F, Lin W-F, Peng H-L, et al. Structure and
643 dynamics of polymyxin-resistance-associated response regulator PmrA in complex with
644 promoter DNA. Nat Commun. 2015;6:8838.
645 36. Mulcahy H, O'Callaghan J, O'Grady EP, Maciá MD, Borrell N, Gómez C, et al.
646 Pseudomonas aeruginosa RsmA plays an important role during murine infection by

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647 influencing colonization, virulence, persistence, and pulmonary inflammation. Infect
648 Immun. 2008;76:632–8.
649 37. Allsopp LP, Wood TE, Howard SA, Maggiorelli F, Nolan LM, Wettstadt S, et al.
650 RsmA and AmrZ orchestrate the assembly of all three type VI secretion systems in
651 Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2017;114:7707–12.
652 38. Romero M, Silistre H, Lovelock L, Wright VJ, Chan K-G, Hong K-W, et al. Genome-
653 wide mapping of the RNA targets of the Pseudomonas aeruginosa riboregulatory
654 protein RsmN. Nucleic Acids Res. 2018.
655 39. Burrowes E, Baysse C, Adams C, O'Gara F. Influence of the regulatory protein
656 RsmA on cellular functions in Pseudomonas aeruginosa PAO1, as revealed by
657 transcriptome analysis. Microbiology. 2006;152:405–18.
658 40. Zoued A, Durand E, Bebeacua C, Brunet YR, Douzi B, Cambillau C, et al. TssK is a
659 trimeric cytoplasmic protein interacting with components of both phage-like and
660 membrane anchoring complexes of the type VI secretion system. J Biol Chem.
661 2013;288:27031–41.
662 41. Lin J, Cheng J, Chen K, Guo C, Zhang W, Yang X, et al. The icmF3 locus is
663 involved in multiple adaptation- and virulence-related characteristics in Pseudomonas
664 aeruginosa PAO1. Front Cell Infect Microbiol. 2015;5:70.
665 42. Yang H, Shan Z, Kim J, Wu W, Lian W, Zeng L, et al. Regulatory role of PopN and
666 its interacting partners in type III secretion of Pseudomonas aeruginosa. J Bacteriol.
667 2007;189:2599–609.
668 43. Sun Y, Karmakar M, Taylor PR, Rietsch A, Pearlman E. ExoS and ExoT ADP
669 ribosyltransferase activities mediate Pseudomonas aeruginosa keratitis by promoting

bioRxiv preprint doi: https://doi.org/10.1101/649152; this version posted May 24, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

670 neutrophil apoptosis and bacterial survival. J Immunol. 2012;188:1884–95.
671 44. Rangel SM, Diaz MH, Knoten CA, Zhang A, Hauser AR. The Role of ExoS in
672 Dissemination of Pseudomonas aeruginosa during Pneumonia. PLOS Pathog.
673 2015;11:e1004945.
674 45. Li K, Xu C, Jin Y, Sun Z, Liu C, Shi J, et al. SuhB is a regulator of multiple virulence
675 genes and essential for pathogenesis of Pseudomonas aeruginosa. MBio.
676 2013;4:e00419-13.
677 46. Pumirat P, Cuccui J, Stabler RA, Stevens JM, Muangsombut V, Singsuksawat E, et
678 al. Global transcriptional profiling of Burkholderia pseudomallei under salt stress reveals
679 differential effects on the Bsa type III secretion system. BMC Microbiol. 2010;10:171.
680 47. Häussler S, Becker T. The Pseudomonas Quinolone Signal (PQS) Balances Life
681 and Death in Pseudomonas aeruginosa Populations. PLoS Pathog. 2008;4:e1000166.
682 doi:10.1371/journal.ppat.1000166.
683 48. Sévin DC, Stählin JN, Pollak GR, Kuehne A, Sauer U. Global Metabolic Responses
684 to Salt Stress in Fifteen Species. PLoS One. 2016;11:e0148888.
685 49. Kilstrup M, Jacobsen S, Hammer K, Vogensen FK. Induction of heat shock proteins
686 DnaK, GroEL, and GroES by salt stress in Lactococcus lactis. Appl Environ Microbiol.
687 1997;63:1826–37.
688 50. García-Descalzo L, Alcazar A, Baquero F, Cid C. Identification of in vivo HSP90-
689 interacting proteins reveals modularity of HSP90 complexes is dependent on the
690 environment in psychrophilic bacteria. Cell Stress Chaperones. 2011;16:203–18.
691 51. Vallet-Gely I, Sharp JS, Dove SL. Local and global regulators linking anaerobiosis to
692 cupA fimbrial gene expression in Pseudomonas aeruginosa. J Bacteriol.

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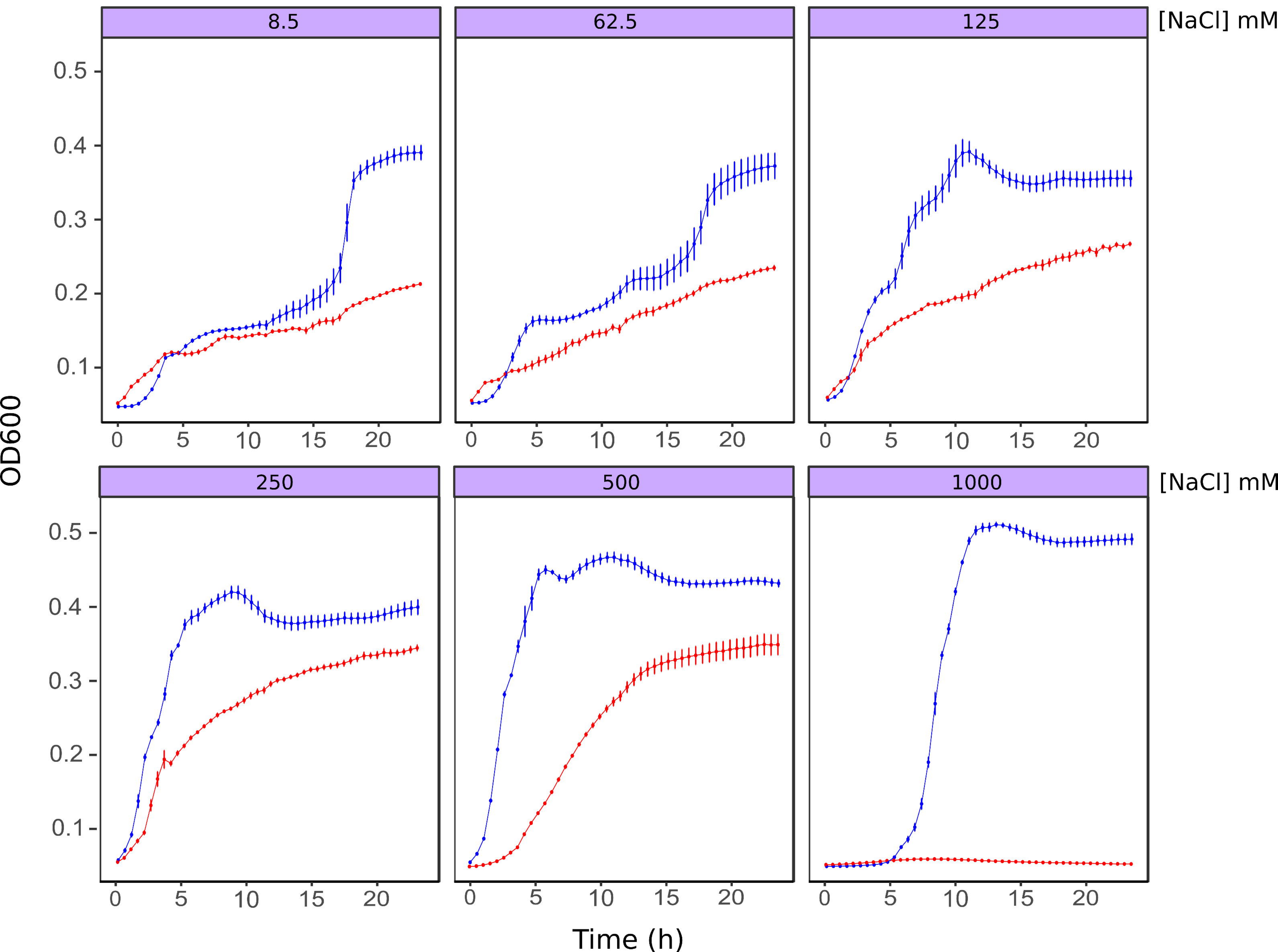
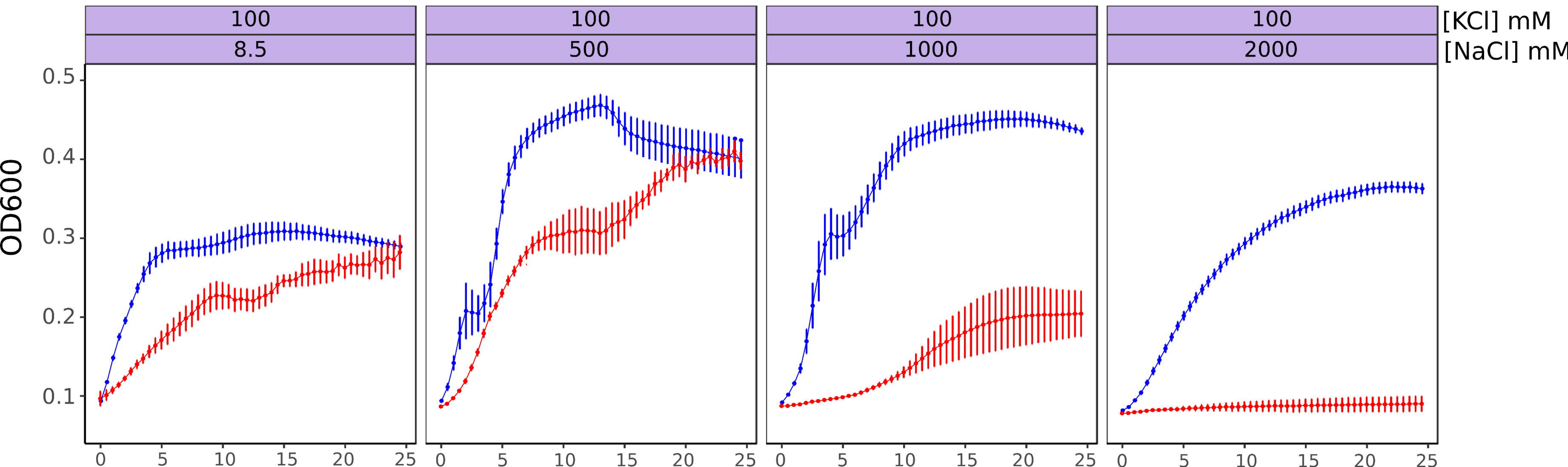
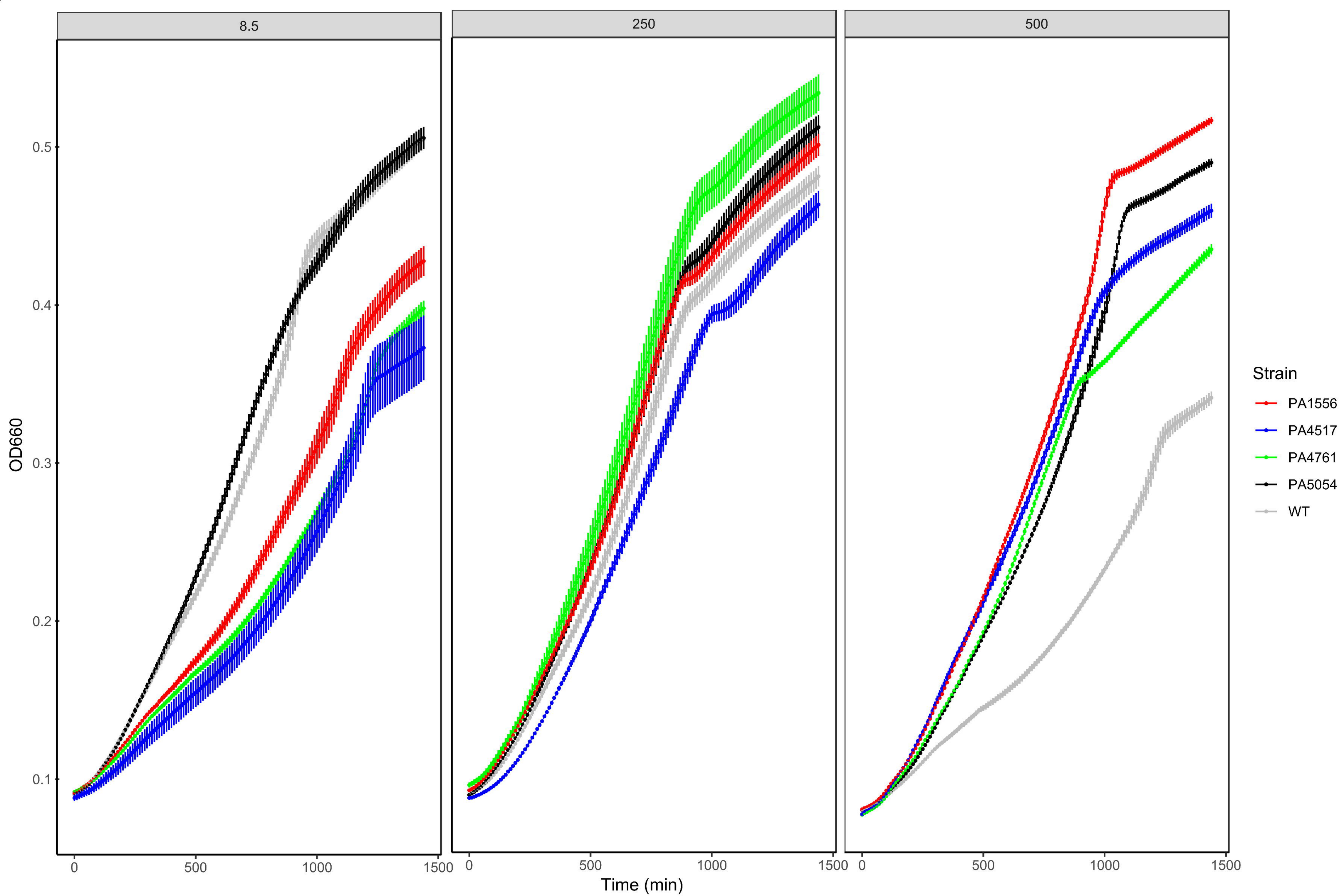
693 2007;189:8667–76.
694 52. Hilker M, Schwachtje J, Baier M, Balazadeh S, Bäurle I, Geiselhardt S, et al.
695 Priming and memory of stress responses in organisms lacking a nervous system. Biol
696 Rev Camb Philos Soc. 2015. doi:10.1111/brv.12215.
697 53. Molassiotis A, Tanou G, Diamantidis G. NO says more than "YES" to salt tolerance:
698 Salt priming and systemic nitric oxide signaling in plants. Plant Signal Behav.
699 2010;5:209–12. http://www.ncbi.nlm.nih.gov/pubmed/20061805. Accessed 26 Jun 2018.
700 54. Ibrahim EA. Seed priming to alleviate salinity stress in germinating seeds. J Plant
701 Physiol. 2016;192:38–46. doi:10.1016/j.jplph.2015.12.011.
702

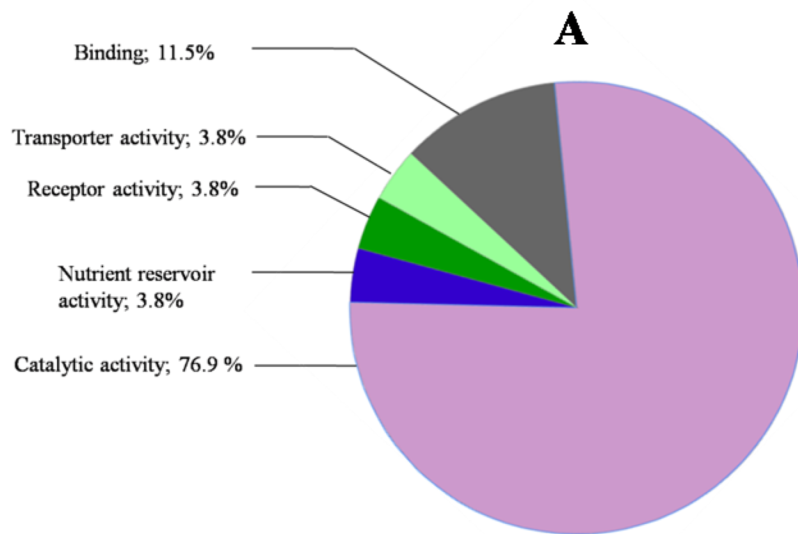

Strains
T0 (original WT)
T48 (14 years in seawater salt crystals)
8.5
62.5
125
250
500
1000
[NaCl] mM
[NaCl] mM
OD600
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
Time (h)

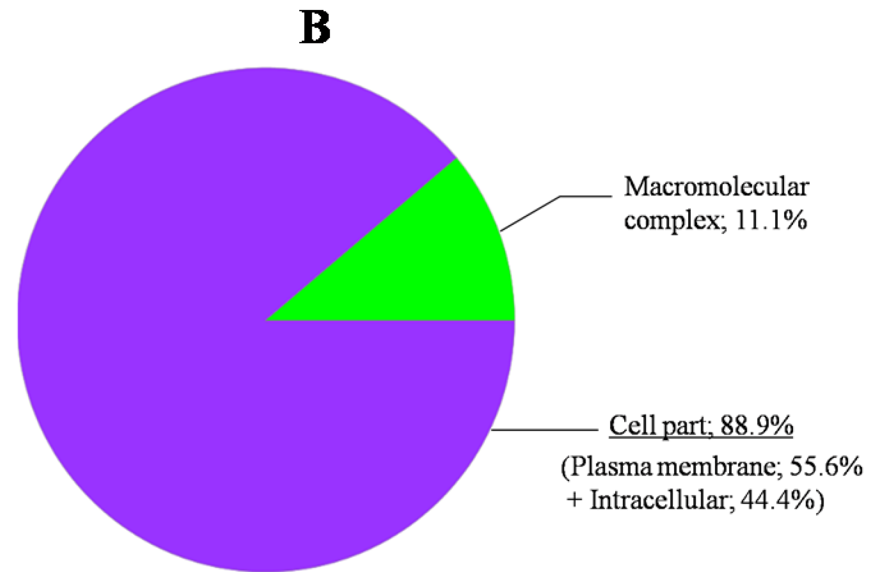


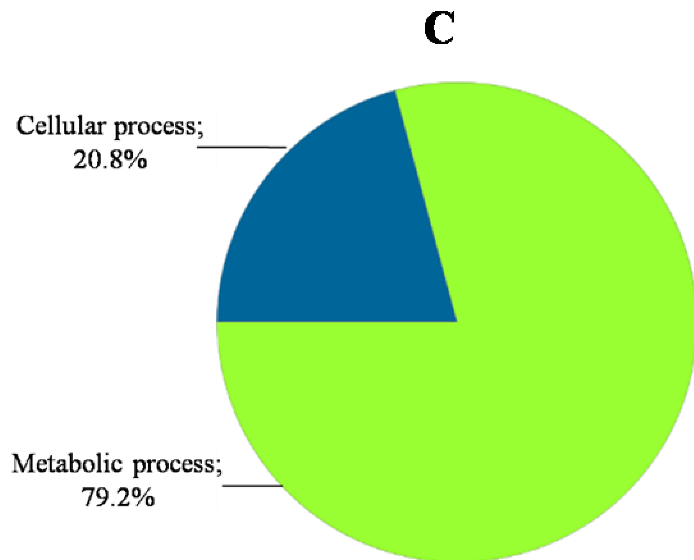
Strains
T0 (original WT)
T48 (14 years in seawater salt crystals)
100
8.5
100
500
100
1000
100
2000
[KCl] mM
[NaCl] mM
OD600
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25

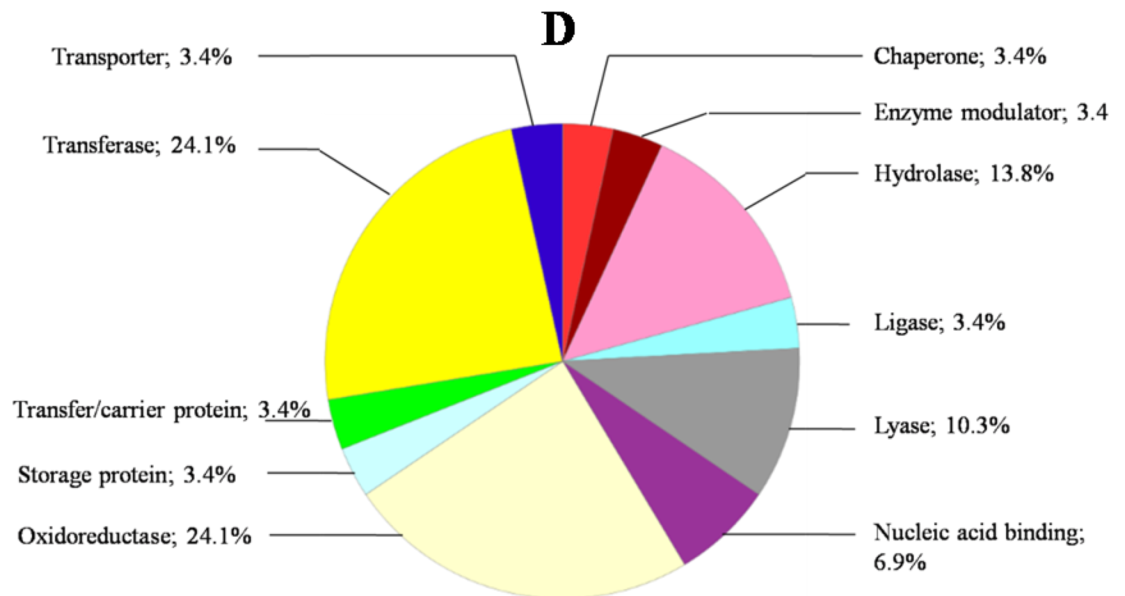


8.5
250
500
OD660
0.1
0.2
0.3
0.4
0.5
0
500
1000
1500
Time (min)
Strain
PA1556
PA4517
PA4761
PA5054
WT


A
Binding; 11.5%
Transporter activity; 3.8%
Receptor activity; 3.8%
Nutrient reservoir activity; 3.8%
Catalytic activity; 76.9 %


B
Macromolecular complex; 11.1%
Cell part; 88.9%
(Plasma membrane; 55.6% + Intracellular; 44.4%)


C
Cellular process; 20.8%
Metabolic process; 79.2%


D
Transporter; 3.4%
Chaperone; 3.4%
Enzyme modulator; 3.4
Transferase; 24.1%
Hydrolase; 13.8%
Ligase; 3.4%
Transfer/carrier protein; 3.4%
Lyase; 10.3%
Storage protein; 3.4%
Oxidoreductase; 24.1%
Nucleic acid binding; 6.9%