

1 **Hierarchical Model for the Role of J-Domain Proteins in Distinct**
2 **Cellular Functions**

3 **Running head:** Role of J-domain proteins

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21 **ABSTRACT**

22 In *Escherichia coli*, the major bacterial Hsp70 system consists of DnaK, three
23 J-domain proteins (JDs: DnaJ, CbpA, and DjlA), and one nucleotide exchange
24 factor (NEF: GrpE). JDs determine substrate specificity for the Hsp70 system;
25 however, knowledge on their specific role in bacterial cellular functions is limited.
26 In this study, we demonstrated the role of JDs in bacterial survival during heat
27 stress and the DnaK-regulated formation of curli—extracellular amyloid fibers
28 involved in *E. coli* biofilm formation. Genetic analysis with a complete set of
29 JD-null mutant strains demonstrated that only DnaJ is essential for survival at
30 high temperature, while DnaJ and CbpA are indispensable in DnaK regulation of
31 curli production. Additionally, we found that DnaJ and CbpA are involved in the
32 expression of the master regulator CsgD through the folding of MlrA; this keeps
33 CsgA in a translocation-competent state by preventing its aggregation in the
34 cytoplasm. Our findings support a hierarchical model wherein the role of JDs in
35 the Hsp70 system differs according to individual cellular functions.

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37 **Keywords:** amyloid/ curli/ Hsp70/ JDs/ hierarchy

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43 **INTRODUCTION**

44 Proteostasis is the maintenance of protein homeostasis in cells and is essential

45 for all life. Key players in proteostasis include molecular chaperones, which
46 assist in protein folding, refolding of denatured and aggregated proteins, protein
47 transport, and quality control of regulatory proteins. Therefore, molecular
48 chaperones are involved in diverse cellular activities including cell division, DNA
49 replication, stress response, organelle functions, and autophagy (Hipp et al.,
50 2019).

51 The 70-kDa heat shock proteins (Hsp70s) are ubiquitous molecular
52 chaperones involved in a wide variety of cellular functions (Mayer and Kityk,
53 2015). Hsp70s are ATP-dependent molecular chaperones that consist of an
54 N-terminal nucleotide-binding domain (NBD) and a 25 kDa C-terminal
55 substrate-binding domain (SBD) (Zhu et al., 1996). Hsp70s function via
56 nucleotide-regulated substrate binding and release cycles (Szabo et al., 1994;
57 McCarty et al., 1995). In the ATP-bound state, Hsp70s exhibit low affinity toward
58 substrates; therefore, their rates of substrate binding and release are rapid. In
59 contrast, the ADP-bound state exhibits high substrate affinity, with consequent
60 low rates of substrate binding and release. In the nucleotide-dependent
61 chaperone cycle, Hsp70s require cofactors, known as co-chaperones. Among
62 co-chaperones, J-domain proteins (JDPs), also referred to as Hsp40s, stimulate
63 the ATPase activity of Hsp70s and the binding of substrate proteins (Gässler et
64 al., 1998; Suh et al., 1998, 1999). Conversely, nucleotide exchange factors
65 (NEFs), another type of co-chaperone, induce ADP dissociation from the NBD of
66 Hsp70s and substrate-release from the SBD (Brehmer et al., 2004). Although
67 the amplification and diversification of Hsp70s may be involved in their functional
68 versatility, JDPs far outnumber Hsp70s in the vast majority of life forms

69 (Kampinga and Craig, 2010), and the multiplicity of JDPs drives the functional
70 diversity of Hsp70s (Craig and Marszalek, 2017). Six JDPs (DnaJ, CbpA, DjlA,
71 HscB, DjlB, and DjlC) have been identified in *Escherichia coli*, 22 in
72 *Saccharomyces cerevisiae*, and 41 in humans, whereas there are three Hsp70s
73 in *E. coli* (DnaK, HscA, and HscC), 16 in *S. cerevisiae*, and 17 in humans (Table
74 EV1) (Powers and Balch, 2013). In *E. coli*, three JDPs (DnaJ, CbpA, and DjlA)
75 productively interact with DnaK and play redundant roles in the regulation of
76 DnaK chaperone activity (Sell et al., 1990; Ueguchi et al., 1994; Genevoux et al.,
77 2001; Gur et al., 2004). These observations suggest that DnaJ, CbpA, and DjlA
78 share overlapping functions.

79 Previously, we demonstrated that DnaK (a bacterial Hsp70) serves an
80 important role in the formation of *E. coli* biofilms—well-organized microbial
81 communities that form on surfaces—and that the production of
82 curli—extracellular functional amyloid fibers—relies on DnaK functions
83 (Arita-Morioka et al., 2015). Curli fibers play crucial roles in biofilm organization
84 and host colonization by adhering to surfaces and holding bacterial cells in a
85 self-produced extracellular matrix (Olsén et al., 1989; Chapman et al., 2002).
86 Secretion and assembly of curli is mediated by a characteristic secretion
87 pathway, known as the nucleation-precipitation mechanism or the type VIII
88 secretion system (Desvaux et al., 2009). In *E. coli*, seven proteins encoded by
89 two dedicated operons, the curli-specific genes *BAC* (*csgBAC*) and *DEFG*
90 (*csgDEFG*) operons, are involved in the expression, export, and assembly of the
91 amyloid fibers (Hammar et al., 1995). An alternative sigma factor, RpoS, also
92 known as σ^S or σ^{38} , activates expression of the *csgDEFG* operon (Hammar et al.,

93 1995; Dudin et al., 2014). In addition, CsgD, the master transcriptional regulator
94 of curli synthesis, directly promotes transcription of the *csgBAC* operon
95 (Hammar et al., 1995; Zakikhany et al., 2010). CsgA and CsgB are the major and
96 the minor curli subunits, respectively. Following transport across the cytoplasmic
97 membrane via the Sec translocon, the CsgA and CsgB subunits are exported
98 across the outer membrane in a manner dependent on CsgG, a curli-specific
99 translocation channel (Goyal et al., 2014; Cao et al., 2014). After secretion,
100 CsgB nucleates CsgA subunits into amyloid fibrils (Shu et al., 2012). In addition,
101 CsgE, a periplasmic accessory protein, directs CsgA to CsgG for secretion
102 (Nenninger et al., 2011). CsgF, an extracellular accessory protein, is required for
103 the specific localization and/or nucleation activity of CsgB (Nenninger et al.,
104 2009).

105 Recently, we found that DnaK multitasks to maintain homeostasis of the
106 key players in curli biogenesis, including regulation of the quantity and *de novo*
107 folding of RpoS and CsgD, and export of CsgA (Sugimoto et al., 2018); however,
108 curli production was not affected by single knockout of JDPs (DnaJ, CbpA, and
109 DjIA), all of which are known to functionally interact and cooperate with DnaK
110 (Genevaux et al., 2001, 2007). These findings motivated us to investigate
111 whether DnaK works alone or together with specific JDPs in distinct cellular
112 functions, such as curli biogenesis and survival at high temperature. Our results
113 will further our understanding of the role of JDPs and the activity of the DnaK
114 chaperone system.

115

116 **RESULTS**

117 **Either DnaJ or CbpA is indispensable for curli biogenesis**

118 To address which JDPs are essential for curli biogenesis, we used the Keio
119 collection, a widely used *E. coli* single-gene knockout library (Baba et al., 2006).
120 We also constructed a complete set of JDP double- and triple-null mutants of the
121 K-12 strain BW25113 (Table EV2) by the one-step method for inactivation of
122 chromosomal genes (Datsenko and Wanner, 2000). Curli production was
123 detected on Congo Red-containing YESCA agar (CR-YESCA: 1% casamino
124 acids, 0.1% yeast extract, and 2% agar) plates at 25°C for 2 days. In the strains
125 $\Delta cbpA \Delta dnaJ$ and $\Delta cbpA \Delta djIA \Delta dnaJ$, curli production was reduced, as well as
126 in the strains $\Delta dnaK$, $\Delta csgA$, $\Delta csgD$, $\Delta csgG$, and $\Delta rpoS$, while it was not affected
127 in the others (Fig 1A). Curli was also evaluated by immunoblotting for CsgA
128 monomers as described below.

129 To confirm the responsibility of JDPs in the observed phenotypic
130 changes, we conducted a trans-complementation assay using the
131 JDP-expression plasmids (Table EV2). The plasmids carrying DnaJ and CbpA
132 restored curli production in BW25113 derivatives $\Delta cbpA \Delta dnaJ$ (Fig 1B). In
133 contrast, neither DjIA nor DjIA^{ΔTM}, which lack the transmembrane domain,
134 restored curli production. These results indicate that either DnaJ or CbpA, but
135 not DjIA, is essential for curli production and that DnaJ and CbpA work
136 redundantly in this process.

137

138 **DnaJ is essential for survival at high temperature**

139 Previously, the requirement for JDPs in the survival of *E. coli* at high temperature
140 was reported using MC4100 and its isogenic mutants (Sell et al., 1990; Ueguchi

141 et al., 1994; Genevoux et al., 2001). These reports showed that deletion of JDPs
142 was not lethal at 30°C and that DnaJ, but neither CbpA nor DjIA, was essential
143 for the growth of MC4100 at 43°C. We revisited the requirement of JDPs in the
144 survival of BW25113 at high temperature using newly constructed null mutant
145 strains (Table EV2). Our study also revealed that all JDPs were dispensable for
146 growth of BW25113 at 30°C, and only DnaJ was indispensable for survival at
147 43°C (Fig EV1A). In addition, complementation analysis demonstrated that
148 expression of DnaJ rescued the growth of BW25113 $\Delta dnaJ \Delta cbpA$ as well as
149 BW25113 $\Delta dnaJ \Delta cbpA \Delta djIA$ at 43°C (Fig 1C and EV1B). Expression of CbpA
150 or DjIA^{ΔTM} partially recovered the survival of BW25113 $\Delta dnaJ \Delta cbpA$ and $\Delta dnaJ$
151 $\Delta cbpA \Delta djIA$ at 43°C (Fig 1C and EV1B). These results indicate that DnaJ plays
152 the most pivotal role in survival of *E. coli* at high temperature.

153

154 **Diversity of DnaK chaperone activities required for distinct phenotypes**

155 Our results indicate that under physiological conditions, only DnaJ is essential
156 for the growth of *E. coli* at high temperature, while DnaJ and CbpA work
157 redundantly in curli production. These findings imply the presence of a diversity
158 of DnaK chaperone activities required for different cellular processes. If this is
159 the case, some DnaK mutants with reduced chaperone activity would support
160 curli production but not in survival at high temperature. Here, we focused on a
161 mutant of DnaK with reduced interaction with JDPs. Substitutions of Tyr-145,
162 Asn-147, and Asp-148 to Ala in DnaK (DnaK^{YND}) (Fig 2A) caused reduced
163 interaction with DnaJ via its J-domain (Gässler et al., 1998), suggesting that this
164 mutant DnaK interacts very weakly with JDPs. We also focused on defective

165 allosteric communication between the NBD and SBD. Previously, two charged
166 residues, a surface-exposed, positively charged residue in the NBD (Lys-155)
167 and a negatively charged residue in the linker connecting NBD and SBD
168 domains (Asp-393), were shown to be important for interdomain communication
169 (Vogel et al., 2006). One of the mutants, DnaK^{K155D}, in which Lys-155 in NBD is
170 replaced with Asp, greatly reduces the stimulation of the substrate dissociation
171 rate by ATP while retaining substrate- and DnaJ-mediated stimulation of ATPase
172 activity (Vogel et al., 2006). In contrast, another mutant, DnaK^{D393A}, in which
173 Asp-393 is substituted to Ala, drastically reduces the stimulation of substrate
174 dissociation rate by ATP, as well as the substrate- and DnaJ-mediated
175 stimulation of ATPase activity (Vogel et al., 2006).

176 In our study, expression of these mutant DnaK proteins did not induce
177 recovery from the growth defect of the $\Delta dnaK$ strain at high temperature (Fig 2A
178 and B). This indicates that interaction of DnaK with JDPs and allosteric
179 communication between the NBD and SBD of DnaK is indispensable for survival
180 under heat stress conditions, as previously reported (Gässler et al., 1998; Vogel
181 et al., 2006). However, notably, expression of DnaK^{YND}, DnaK^{K155D}, and
182 DnaK^{D393A} fully restored curli production in the $\Delta dnaK$ strain (Fig 2C).

183 These results support our hypothesis that there is a functional diversity
184 of the DnaK system required for distinct cellular functions.

185

186 **Loss of DnaJ and CbpA reduces expression of CsgD**

187 Based on our data, we hypothesized that either DnaJ or CbpA plays an
188 important role for expression and folding of certain proteins associated with curli

189 biogenesis. To address this, we investigated the expression levels of several
190 proteins in the BW25113 strains by immunoblotting.

191 Firstly, we confirmed the expression of chaperone proteins (Fig 3A). As
192 expected, DnaK, DnaJ, and CbpA were not detected in the respective mutant
193 strains, revealing that deletion of these proteins was successfully conducted.
194 Only DjlA was not detected because of the absence of an available specific
195 antibody. The cellular level of DnaK was slightly increased in the strains lacking
196 DnaJ, while that of DnaJ was much higher in the strain $\Delta dnaK$ compared to the
197 other strains. These phenomena can be accounted by the accumulation of RpoH,
198 also known as σ^{32} or σ^H , in these mutant strains, which induces expression of
199 DnaK and DnaJ (Tatsuta et al., 2000). These results are also consistent with the
200 previous observation (Gur et al., 2004). In addition, the cellular level of CbpA in
201 the strain $\Delta rpoS$ was lower than that in the wild-type strain, confirming that the
202 expression of CbpA is positively regulated by RpoS during the stationary growth
203 phase as previously reported (Yamashino et al., 1994).

204 Secondly, to elucidate the mechanisms of how either DnaJ or CbpA
205 affects the biogenesis of curli, we investigated cellular levels of CsgA, CsgD, and
206 CsgG. In agreement with the results of CR-plate assays (Fig 1A), no CsgA was
207 detected in the strains $\Delta cbpA \Delta dnaJ$ and $\Delta cbpA \Delta djIA \Delta dnaJ$ as in the
208 curli-negative strains $\Delta csgA$, $\Delta csgD$, $\Delta csgG$, and $\Delta rpoS$ (Fig 3A and B). Likewise,
209 a low level of CsgD was detected in the strains $\Delta cbpA \Delta dnaJ$ and $\Delta cbpA \Delta djIA$
210 $\Delta dnaJ$. In addition, the cellular levels of CsgG in the strains $\Delta cbpA \Delta dnaJ$ and
211 $\Delta cbpA \Delta djIA \Delta dnaJ$ were lower than those in the wild-type strain. These data for
212 the strains $\Delta cbpA \Delta dnaJ$ and $\Delta cbpA \Delta djIA \Delta dnaJ$ are similar to those of the strain

213 $\Delta dnaK$. Our previous study indicated that expression of the *csgDEFG* operon
214 was reduced in the strain $\Delta dnaK$ at the transcription level, which resulted in a
215 reduction in the expression of the *csgBAC* operon (Sugimoto et al., 2018).
216 Collectively, these results suggest that reduced expression of *csgDEFG* operon
217 leads to the decreased expression of *csgBAC* operon in the strains $\Delta cbpA$
218 $\Delta dnaJ$ and $\Delta cbpA \Delta djIA \Delta dnaJ$.

219 Next, we analyzed the cellular levels of RpoS, the stationary
220 phase-specific sigma factor, in the BW25113 derivatives as it positively regulates
221 the expression of the *csgDEFG* operon. A slight reduction in the RpoS level was
222 observed in the strains $\Delta dnaK$, $\Delta cbpA \Delta dnaJ$, and $\Delta cbpA \Delta djIA \Delta dnaJ$ (Fig 3).
223 The decreased level of RpoS in the strain $\Delta dnaK$ may be due to accelerated
224 proteolytic degradation by ClpXP (Rockabrand et al., 1998). Consistent with
225 these results, the cellular activity of RpoS through the measurement of catalase
226 activity was reduced in these mutant strains (Fig EV2), suggesting that a partially
227 reduced RpoS level resulted in a decrease in the expression of the *csgDEFG*
228 operon. However, the activity of RpoS in the strains $\Delta dnaK$, $\Delta cbpA \Delta dnaJ$, and
229 $\Delta cbpA \Delta djIA \Delta dnaJ$ was not low enough to shut the expression of the *csgDEFG*
230 operon down. Therefore, other mechanisms may account for the drastic
231 reduction of the cellular CsgD level in the strain $\Delta cbpA \Delta dnaJ$ and $\Delta cbpA \Delta djIA$
232 $\Delta dnaJ$.

233

234 **Either DnaJ or CbpA is involved in folding of transcriptional regulator MlrA**

235 To investigate whether either DnaJ or CbpA is important for the expression of
236 CsgD (Fig 3), we tested the contribution of these JDPs to the folding of MlrA *in*

237 *vitro*. MlrA was synthesized using an *in vitro* translation system (PURE System;
238 Shimizu et al., 2001), in the presence or absence of complete and incomplete
239 sets of DnaK/DnaJ/GrpE (KJE) or DnaK/CbpA/GrpE (KAE). *De novo*
240 synthesized MlrA readily formed aggregates and a complete set of KJE or KAE
241 assisted the folding of MlrA (Fig 4A and EV3A). Incomplete sets of DnaK/DnaJ
242 (KJ), DnaJ/GrpE (JE) and DnaJ alone (J) also promoted the solubility of MlrA,
243 indicating that the folding of MlrA strongly relied on DnaJ. It should be noted that
244 CbpA can compensate for DnaJ in KJE-assisted folding of MlrA (Fig 4A and
245 EV3A). In contrast, DnaK alone (K), DnaK/CbpA (KA), DnaK/GrpE (KE),
246 CbpA/GrpE (AE), CbpA alone (A), and GrpE (E) showed no or only slight
247 stimulation of the solubility of MlrA (Fig 4A and EV3A). These results indicate
248 that either DnaJ or CbpA is required for the DnaK chaperone system to efficiently
249 fold MlrA. In addition, these findings are in good agreement with those already
250 described, indicating that either DnaJ or CbpA is indispensable for efficient curli
251 production (Fig 1–3).

252 Next, we examined whether *de novo* folding of CsgD required either
253 DnaJ or CbpA, because its folding is assisted by the complete DnaK chaperone
254 system (KJE) (Sugimoto et al., 2018). Our study revealed that CsgD formed
255 aggregates in the absence of the chaperones and that KJE and KAE promoted
256 CsgD folding (Fig 4B and EV3B). In contrast, DnaK alone did not support CsgD
257 folding under the tested conditions (Fig 4B and EV3B). Interestingly, incomplete
258 sets of the DnaK systems (KJ and KE) moderately enhanced the solubility of
259 CsgD *in vitro* (Fig 4B and EV3B). DnaJ, CbpA, GrpE, and combinations of DnaJ
260 and GrpE or CbpA and GrpE did not assist the folding of CsgD (Fig 4B and

261 EV3B). These results indicate that these incomplete DnaK systems (KJ and KE)
262 can act as molecular chaperones and that JDPs are dispensable at least for the
263 folding of CsgD.

264

265 **Either DnaJ or CbpA is required for maintenance of CsgA in a**
266 **translocation-competent state**

267 Transport of CsgA across the cytoplasmic membrane is pivotal for curli
268 biogenesis. In this process, DnaK acts on the transport precursor of CsgA to
269 maintain its transport competent state via direct interaction with its N-terminal
270 aggregation-prone signal peptide (Sugimoto et al., 2018). Firstly, we examined
271 whether co-expression of CsgBAEFG was able to complement the defect of curli
272 production in the strain BW2513 $\Delta dnaJ \Delta cbpA$, in which CsgA was not detected,
273 as shown in Fig 3. Previously, we showed that introduction of the plasmid
274 pCsgBAEFG was able to recover the production of curli in the BW25113 strains
275 $\Delta csgA$, $\Delta csgB$, $\Delta csgE$, $\Delta csgF$, and $\Delta csgG$, confirming the plasmid was functional
276 (Sugimoto et al., 2018). However, expression of CsgBAEFG did not restore the
277 production of curli in $\Delta dnaJ \Delta cbpA$ (Fig 5A), suggesting that secretion of CsgA
278 was defective at the step of translocation from the cytoplasm to the periplasm or
279 from the periplasm to the extracellular milieu.

280 Secondly, we investigated the requirements of JDPs for the translocation
281 of CsgA from the cytoplasm to the periplasm using an *in vivo* visualization
282 system (Sugimoto et al., 2018). Previously, we expressed a CsgA-sfGFP fusion
283 protein in the BW25113 parental strain and its isogenic $\Delta dnaK$ strain.

284 Subsequently, we observed that CsgA-sfGFP localized at the periplasm of the

285 parental strain, whereas it formed aggregates in the cytoplasm of the $\Delta dnaK$
286 mutant (Sugimoto et al., 2018). In this study, we expressed the fusion protein in
287 BW25113 derivative strains $\Delta dnaJ$, $\Delta cbpA$, and $\Delta dnaJ \Delta cbpA$. Fluorescence of
288 sfGFP was observed at the periphery of the single null strains $\Delta dnaJ$ and $\Delta cbpA$,
289 indicating that CsgA-sfGFP was translocated to the periplasm. In contrast, it
290 formed aggregates in the cytoplasm of the double knockout strain $\Delta dnaJ \Delta cbpA$
291 (Fig 5B). These results suggest the requirement of either DnaJ or CbpA for
292 translocation of CsgA from the cytoplasm to the periplasm.

293 Taken together, our results indicate that either DnaJ or CbpA is required
294 for DnaK to assist the translocation of CsgA to the periplasm during curli
295 biogenesis.

296

297 **DISCUSSION**

298 Previously, we showed that DnaK is essential for curli biogenesis and biofilm
299 formation via the maintenance of certain important proteins including RpoS,
300 CsgD, and CsgA (Sugimoto et al., 2018). Here, we demonstrated that DnaJ and
301 CbpA were essential for this process, while DjIA was not essential and that DnaJ
302 and CbpA work redundantly in this process (Fig 1–3 and EV1). DnaK cooperates
303 with DnaJ to assist in protein folding and to repair damaged proteins under
304 harmful conditions, such as high temperature at 43°C, which causes
305 denaturation and aggregation of numerous proteins (Fig 1C and EV1). The
306 DnaK system efficiently prevents the aggregation of diverse thermolabile
307 proteins, including 150–200 species, corresponding to 15–25% of detected
308 proteins, under physiological heat stress conditions (Mogk et al., 1999). In

309 addition, the disaggregation activities of the DnaK/CbpA/GrpE and
310 DnaK/DjlA/GrpE systems were lower than that of the DnaK/DnaJ/GrpE system
311 (Gur et al. 2004), suggesting that only DnaJ is essential for survival under
312 severe stress conditions (e.g., heat stress at $\geq 43^{\circ}\text{C}$). Our model is consistent
313 with the current body of research in several respects: (1) In curli biogenesis, only
314 a subset of proteins (minimally RpoS, CsgD, and CsgA) require DnaK for their
315 correct folding or export (Sugimoto et al., 2018). Our study also suggests that
316 efficient folding of MlrA needs the DnaK system including DnaK, GrpE, and
317 either DnaJ or CbpA (Fig 3–5). (2) DnaK mutants (DnaK^{YND}, DnaK^{K155D}, and
318 DnaK^{D393A}) with reduced basal activity were functional in the curli production but
319 not in survival at high temperature of 43°C (Fig 2). (3) *E. coli* produces curli
320 during the stationary phase of growth, in which expression of CbpA is induced
321 via RpoS (Fig 3A). Therefore, the contribution of CbpA to the promotion of the
322 DnaK function may be emphasized during curli biogenesis. This growth
323 phase-specific selection of JDPs is a reasonable strategy for improving bacterial
324 survival and fitness under nutrient-depleted conditions (e.g., during stationary
325 phase) and adaptation during host colonization. (4) DjlA is an inner membrane
326 anchoring JDP in *E. coli* (Clarke et al., 1996). Therefore, DjlA may be involved in
327 the quality control of membrane proteins and trafficking of exported proteins
328 (Kelley and Georgopoulos, 1997). However, DjlA is dispensable for maintenance
329 of proteins associated with curli production.

330 Based on these observations, we propose a hierarchical model whereby
331 Hsp70 chaperone activities regulate proteostasis in distinct cellular functions
332 (Fig 6). When a large amount/variety of proteins in the cell are injured by severe

333 stress conditions (e.g., heat stress at $\geq 43^{\circ}\text{C}$), full specification of the DnaK
334 system (DnaK/DnaJ/GrpE) prevents their aggregation and the repair of toxic
335 protein aggregates. In contrast, under certain conditions (e.g., curli biogenesis),
336 a moderately active DnaK system (DnaK/CbpA/GrpE) fulfills its chaperone
337 function by handling only a subset of proteins. Although DnaK/DjlA/GrpE and
338 DnaK alone did not support the production of curli, they may function weakly by
339 holding substrate proteins (Evans et al., 2011), which might be associated with a
340 specific phenotype such as colanic acid production (Kelley and Georgopoulos,
341 1997) and other unknown cellular functions. This model is also supported by the
342 results of DnaK mutants in which DnaK^{YND}, DnaK^{K155D}, and DnaK^{D393A} restored
343 the curli production in the $\Delta dnaK$ strain despite the failure to support the growth
344 at high temperature (Fig 2). In addition, neither DnaK^{K70A} which possesses a
345 defective ATPase activity nor DnaK^{V436F} which retains decreased substrate
346 affinity was able to rescue the thermosensitivity as well as the deficiency in curli
347 production of the $\Delta dnaK$ strain (Sugimoto et al., 2018).

348 This study focused on the requirement of JDPs in the production of
349 bacterial amyloid fibers. In addition to JDPs, DnaK cooperates with GrpE, a
350 well-conserved bacterial NEF. We were also interested in the requirement of
351 GrpE in curli biogenesis. However, deletion of the *grpE* gene from the genome of
352 BW25113 was difficult because of its lethality in *E. coli* (Ang and Georgopoulos,
353 1989). Genetic analysis using an available *grpE* null strain derived from *E. coli*
354 strain C600 (Ang and Georgopoulos, 1989; Sugimoto et al., 2008) suggested
355 that GrpE may be dispensable for curli production (Sugimoto et al., unpublished).
356 Further careful study is needed to clarify this observation.

357 Our data provide insight into the evolution of molecular chaperones and
358 proteostasis. It has been previously suggested that Hsp70 chaperone systems
359 co-evolved with the proteome to regulate the physiological state of the cell
360 (Powers and Balch, 2013). The number of Hsp70s increases roughly linearly as
361 the size of the genome increases (Powers and Balch, 2013). In addition, the
362 number of JDPs is often much higher than that of Hsp70s in almost all life forms
363 (Kampinga and Craig, 2010). Moreover, in contrast to Hsp70s, JDPs show a
364 greater degree of sequence and structural divergence. These insights imply that
365 they may play a major role in driving the multi-functionality of Hsp70 chaperone
366 systems (Craig and Marszalek, 2017). In contrast, the single set of
367 DnaK-DnaJ-GrpE is well conserved in Gram-positive bacteria, such as
368 *Staphylococcus aureus* (Table EV1) (Warnecke, 2012). Generally, the genome
369 sizes of Gram-positive bacteria are smaller than those of Gram-negative
370 bacteria. Therefore, smaller numbers of Hsp70s and JDPs in Gram-positive
371 bacteria may be anticipated, as the proteome size is also small, minimizing the
372 work of the Hsp70 system. In the cases of microorganisms with extremely small
373 genomes, such as *Candidatus Hodgkinia cicadicola* and *Candidatus Carsonella*
374 *ruddii*, only either DnaJ or GrpE is present, and there is a single Hsp70/DnaK
375 (Table EV1). How do such incomplete DnaK systems (so called proto-DnaK
376 systems) work in maintaining proteostasis? These organisms possess quite
377 small numbers of proteins; therefore, the roles of the DnaK systems must be
378 minimal. In this situation, activity of proto-DnaK systems (DnaK/DnaJ and
379 DnaK/GrpE) may be sufficient for regulating a limited proteome to aid survival of
380 these microorganisms (Fig 6). This notion is consistent with our results that

381 incomplete sets of the *E. coli* DnaK chaperone system (DnaK/DnaJ and
382 DnaK/GrpE) can contribute to folding of certain proteins (e.g., MlrA and CsgD)
383 (Fig 4B and EV3B). Whether other cellular processes require either
384 full-specification DnaK systems or only proto-DnaK systems remains unclear;
385 some proteins may require DnaK and either DnaJ or GrpE for their folding. It is
386 expected that proteins with lower affinity with DnaK need only DnaJ, since DnaK
387 alone is not able to capture them and these proteins can be released
388 spontaneously from DnaK despite the absence of GrpE. In contrast, folding of
389 proteins with higher affinity with DnaK may depend on GrpE because, although
390 DnaK alone is able to bind them, the release of proteins tightly bound to DnaK
391 requires GrpE. The lower affinity proteins may be expressed predominantly in
392 Candidatus bacteria that possess the DnaK/DnaJ system, whereas the higher
393 affinity proteins may be expressed in Candidatus bacteria that possess the
394 DnaK-/GrpE system. These insights into diversification and evolution of the
395 Hsp70 chaperone system, in combination with our data, imply that primitive
396 organisms may use proto-DnaK systems to manage their small proteomes.

397

398 **METHODS AND MATERIALS**

399 **Bacterial strains**

400 The *E. coli* strains used in this study are listed in Table EV2. All strains were
401 cultivated in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or
402 YESCA medium (1% casamino acid, 0.1% yeast extract). When appropriate, the
403 medium was supplemented with 30 µg/ml chloramphenicol, 50 µg/ml kanamycin,
404 or 100 µg/ml ampicillin.

405

406 **Construction of *E. coli* null mutant strains**

407 The JDP-null mutant strains of BW25113 (Table EV2) were constructed by the
408 one-step method for inactivation of chromosomal genes (Datsenko and Wanner,
409 2000; Baba et al., 2006). The plasmids and primers used for gene knockout are
410 listed in Tables EV2 and EV3, respectively.

411

412 **Construction of plasmids**

413 The ASKA clone plasmids (pASKA-DnaJ, pASKA-CbpA, pASKA-DjIA,
414 pASKA-CsgD, and pASKA-MlrA) were provided by the National Institute of
415 Genetics (Shizuoka, Japan). For construction of pDjIA^{ΔTM} (Table EV2), the DNA
416 encoding the transmembrane domain was deleted by inverse PCR using KOD
417 Plus Neo DNA polymerase (Toyobo, Osaka, Japan), pASKA-DjIA as a template,
418 and a primer set DjIA-deltaTM-F and DjIA-deltaTM-R (Table EV3).

419 For construction of plasmids expressing DnaK mutants
420 (DnaK^{YND}, DnaK^{K155D}, and DnaK^{D393A}), site-directed mutagenesis was
421 performed by inverse PCR using KOD Plus Neo DNA polymerase
422 (Toyobo, Osaka, Japan), pDnaK^{WT} as a template, and the following
423 primer sets: dnaK-YND-F/dnaK-YND-R, dnaK-K155D-F/dnaK-K155D-R,
424 and dnaK-D393A-F/dnaK-D393A-R. The resultant plasmids were
425 termed pDnaK^{YND}, pDnaK^{K155D}, and pDnaK^{D393A}, respectively (Table
426 EV2).

427 The plasmids were analyzed by DNA sequencing (Eurofins Genomics,
428 Tokyo, Japan). Primers used in this study were synthesized by Thermo Fisher

429 and are summarized in Table EV3.

430

431 **Protein purification**

432 Recombinant DnaK, DnaJ, and GrpE were purified as described previously
433 (Niwa et al., 2012). CbpA was expressed in *E. coli* BL21(DE3). Cells harboring
434 pCU60 were grown at 30°C in 2x YT medium containing 100 µg/ml ampicillin,
435 and expression of CbpA was induced by adding IPTG (1 mM) and incubating at
436 30°C for 3 h. Cells from 2-L culture were harvested by centrifugation and
437 resuspended in 50 ml buffer A [10 mM Tris-HCl (pH 8.0), 1 mM DTT, 10%
438 glycerol] supplemented with a protease inhibitor cocktail. After sonication on ice,
439 cell lysates were centrifuged at 12,000 xg for 60 min at 4°C, and the supernatant
440 was loaded onto a 5-ml bed volume of HiTrap Heparin column (GE Healthcare,
441 Pittsburgh, PA, USA) pre-equilibrated with buffer A. CbpA was eluted using a 0–
442 1,000 mM NaCl gradient in buffer A. Each fraction containing CbpA was pooled
443 and further purified by chromatography using a HiTrap Q column (GE
444 Healthcare) and a 0–1,000 mM NaCl gradient in buffer A. Purified CbpA was
445 confirmed by LC-MS/MS and quantified using a Bradford Assay Kit.

446

447 **Antibodies**

448 Rabbit anti-DnaJ and rabbit anti-RpoH antisera were gifted by Dr. B. Bukau
449 (Gamer et al., 1992). The other antibodies were prepared as previously reported
450 (Arita-Morioka et al., 2018; Sugimoto et al., 2018).

451

452 **Congo Red (CR)-binding assay**

453 Curli formation was assayed at 25°C on CR-containing YESCA (1% casamino
454 acid, 0.1% yeast extract, 2% agar) plates as previously reported (Arita-Morioka
455 et al., 2018; Sugimoto et al., 2018). When needed, 30 µg/ml chloramphenicol
456 was added to supplement select transformants.

457

458 **Thermosensitivity assay**

459 *E. coli* BW25113 derivative cells were grown at 30°C in LB medium overnight.
460 Overnight cultures were serially diluted 10-fold, and 5 µl of these dilutions were
461 spotted onto LB agar plates. If required, 30 µg/ml chloramphenicol was added to
462 supplement select transformants. These plates were incubated at 30°C or 43°C
463 for 24 h.

464

465 **Immunoblotting**

466 For detection of CsgA, CsgD, CsgG, RpoS, RpoD, RpoH, DnaK, DnaJ, CbpA,
467 and His-tagged MlrA, immunoblotting was performed as previously reported
468 (Arita-Morioka et al., 2018; Sugimoto et al., 2018). After SDS-PAGE, proteins
469 were transferred to polyvinylidene difluoride membranes using the iBlot 2 dry
470 blotting system (Thermo Fisher) following the manufacturer's instructions.
471 Membranes were blocked with blocking solution [1–5% skimmed milk,
472 Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T)] for at least 1 h at
473 25°C or overnight at 4°C. After gentle washing with TBS-T, the membrane was
474 incubated with appropriate primary antibodies for at least 1 h at 25°C or
475 overnight at 4°C. Membranes were subsequently probed with appropriate
476 secondary HRP-conjugated antibodies for 1 h at 25°C or overnight at 4°C. After

477 washing the membrane three times with TBS-T, signals were detected using the
478 ECL Prime Western Blotting Detection Reagent (GE Healthcare) and an
479 ImageQuant LAS-4000 system (GE Healthcare). When required, signal
480 intensities were quantified with ImageQuant TL software version 7.0 (GE
481 Healthcare).

482 Primary antibodies were diluted into CanGet Signal 1 (Toyobo) as
483 follows: anti-CsgA (1/1,000), anti-CsgD (1/200), anti-CsgG (1/5,000–1/1,000),
484 anti-RpoS (1/10,000–1/1,000), anti-RpoD (1/10,000–1/1,000), anti-RpoH
485 (1/2,000), anti-DnaK (1/10,000), anti-DnaJ (1/10,000), anti-CbpA (1/10,000), and
486 anti-His (1/10,000). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated
487 goat anti-mouse IgG secondary antibodies were diluted 1/50,000 and 1/10,000,
488 respectively, in CanGet Signal 2 (Toyobo).

489 For detection of CsgA monomers, curli fibers were depolymerized into
490 subunits by treatment with hexafluoroisopropanol (HFIP) before SDS-PAGE
491 (Sugimoto et al. 2018). Bacterial cells (1 mg) were suspended in 10 µl STE
492 buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA] and mixed well with
493 50 µl HFIP. After brief sonication, samples were vacuum dried using a SpeedVac
494 vacuum concentrator (Thermo Fisher) at 45°C for more than 30 min. The
495 HFIP-treatment was repeated. The dried materials were dissolved in 40 µl 8 M
496 urea solution. After brief sonication in a water bath for 5 min at room temperature,
497 the solutions were mixed with an equal volume of 2x SDS sample buffer [150
498 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol].
499 Proteins were separated by 15% SDS-PAGE.

500

501 ***In vitro* protein folding assay**

502 *De novo* folding of CsgD and MlrA was analyzed using the PURE System
503 (Shimizu et al., 2001) as previously reported (Niwa et al., 2012; Sugimoto et al.,
504 2018). The *csgD* gene was amplified from the CsgD-expression plasmid
505 pASKA-CsgD (Table EV2) by PCR using KOD Plus DNA polymerase v. 2
506 (Toyobo) and the primer set Pure-Niwa-F and Pure-CsgD-R (Table EV3). The
507 *mlrA* gene was amplified from the MlrA-expression plasmid pASKA-MlrA (Table
508 EV2) by PCR using Phusion High-Fidelity DNA polymerase (New England
509 Biolabs, Tokyo, Japan) and the primer set Pure-Niwa-F and Pure-Niwa-R (Table
510 EV3). The amplified DNA fragments were purified and incubated with PURE*frex*
511 solution (GeneFrontier Corp., Chiba, Japan) at 37°C for 3–4 h according to the
512 manufacturer's instructions. When required, reaction mixtures (20–40 µl) were
513 supplemented with DnaK (5 µM), DnaJ (1 µM), CbpA (1 µM), and/or GrpE (1 µM).
514 After incubation, aliquots (10–20 µl) of the solution were obtained as the total
515 fractions and were centrifuged at 20,000 ×g for 30 min at 4°C to separate the
516 soluble and insoluble fractions. The equivalent volumes of the total, soluble, and
517 insoluble fractions were mixed with 2× SDS sample buffer. After boiling at 95°C
518 for 5 min, proteins were resolved by 15% SDS-PAGE and stained with CBB. For
519 detection of CsgD and MlrA, immunoblotting was performed as described above.

520

521 **Fluorescence microscopy**

522 Transport and aggregation of CsgA-sfGFP was observed in *E. coli* as previously
523 reported (Sugimoto et al., 2018) with a slight modification. *E. coli* expressing
524 CsgA-sfGFP were grown in LB medium supplemented with 100 µg/ml ampicillin

525 at 30°C overnight. A small aliquot of the overnight cultures was placed on a slide
526 and covered with a coverslip. Fluorescence of sfGFP was observed under a
527 fluorescence microscope (Nikon, Tokyo, Japan) equipped with B2 (excitation
528 filter, 450–490 nm; barrier filter, 520 nm) and G2A (excitation filter, 510–560 nm;
529 barrier filter, 590 nm) filters. In this study, no arabinose was supplemented into
530 the media, because leaky expression from the plasmid was sufficient to visualize
531 fluorescence.

532

533 **Statistical analysis**

534 One-way ANOVA with Dunnett's post hoc test was used to determine whether
535 any of the groups exhibited a statistically significant difference in the solubility of
536 MlrA and CsgD analyzed by the PURE System. All experiments were performed
537 at least three times. For all analyses, $P < 0.05$ was considered statistically
538 significant.

539

540

541

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558

559 **AUTHOR CONTRIBUTIONS**

560 S.S., K.Y., and T.O. planned the project. S.S. designed the experiments and
561 developed the assay. S.S. and T.N. purified proteins. S.S. performed the
562 experiments and analyzed the data. Y.K. and Y.M. supported the project. S.S.
563 and K.Y. wrote the paper with input from all co-authors.

564

565 **CONFLICT OF INTEREST**

566 There is no conflict of interest.

567

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769

770 **FIGURE LEGENDS**

771 **Figure 1. Effects of JDP deletion on curli biogenesis**

772 (A) Curli production in *E. coli* BW25113 and its isogenic mutants were examined
773 on Congo Red (CR)-containing YESCA plates (CR-YESCA). Strains were grown
774 at 25°C for 2 days. The strains $\Delta csgA$, $\Delta csgD$, $\Delta csgD$, and $\Delta rpoS$ were used as
775 negative controls. (B) Curli production in BW25113 and its isogenic $\Delta cbpA$
776 $\Delta dnaJ$ strains transformed with pCA24N (empty vector) and the indicated
777 JDP-expression plasmids were examined on CR-YESCA plates supplemented
778 with chloramphenicol. Strains were grown at 25°C for 2 days. (C)
779 Thermosensitivity of the indicated strains was assayed on LB agar plates
780 supplemented with chloramphenicol. Ten-fold serial dilutions of the overnight
781 cultures were spotted on the plates. Plates were incubated at 30°C or 43°C for
782 24 h.

783

784 **Figure 2. Complementation of the *dnaK*-null strain with defective DnaK 785 mutants in cooperation with JDPs and interdomain communication**

786 (A) The domain structure of DnaK and mutants used in this study. Arrowheads
787 represent mutation sites. (B) The thermosensitivity of the indicated strains was
788 assayed on LB agar plates supplemented with chloramphenicol. Ten-fold serial

789 dilutions of the overnight cultures were spotted on the plates. Plates were
790 incubated at 30°C or 43°C for 24 h. (C) Curli production in the strains was
791 examined on CR-YESCA plates supplemented with chloramphenicol. The plates
792 were incubated at 25°C for 2 days.

793

794 **Figure 3. Effects of JDPs on expression of curli-related proteins**

795 (A) BW25113 and its isogenic null mutants were grown on YESCA plates at 25°C
796 for 2 days. Expression of curli-related proteins, chaperones, and sigma factors
797 was analyzed by immunoblotting. CsgA monomers were depolymerized with
798 hexafluoroisopropanol (HFIP). All experiments were conducted using total
799 protein samples. Relative protein levels of CsgA, CsgD, CsgG, and RpoS were
800 quantified based on the band intensity of immunoblots. All experiments were
801 repeated at least three times to ensure accuracy and averaged values with
802 standard deviations were calculated. The band intensities in the BW25113
803 parental strain were defined as 100%.

804

805 **Figure 4. Effects of complete and incomplete DnaK chaperone systems on** 806 ***de novo* folding of transcriptional regulators**

807 (A, B) *De novo* folding of MlrA and CsgD was analyzed in the absence (Control)
808 or presence of the indicated chaperone proteins using a cell-free translation
809 system (PURE System). The solubilities (%) of MlrA (A) and CsgD (B) were
810 calculated based on the band intensity of immunoblots. All experiments were
811 repeated at least three times to ensure accuracy and averaged values with
812 standard deviations were calculated. K, DnaK; KJ, DnaK/DnaJ; KA, DnaK/CbpA;

813 KE, DnaK/GrpE; KJE, DnaK/DnaJ/GrpE; KAE, DnaK/CbpA/GrpE; JE,
814 DnaJ/GrpE; AE, CbpA/GrpE; J, DnaJ; A, CbpA; E, GrpE. ***, $P < 0.001$. *, $P <$
815 0.05. NS, not significant.

816

817 **Figure 5. Effects of DnaJ and CbpA on translocation of CsgA-sfGFP**

818 (A) Curli production in the indicated strains was examined on CR-YESCA plates
819 supplemented with chloramphenicol as described in Fig 1B. The plates were
820 incubated at 25°C for 2 days. (B) Translocation of CsgA across the cytoplasmic
821 membrane was analyzed in BW25113 derivatives using a CsgA-sfGFP fusion
822 protein (Sugimoto et al., 2018). *E. coli* cells were grown in LB medium
823 supplemented with 100 µg/ml ampicillin. Scales, 10 µm.

824

825 **Figure 6. A hierarchical model of DnaK chaperone activities**

826 The proposed model for DnaK chaperone activities showing that the most
827 powerful DnaK/DnaJ/GrpE system is essential for survival under severe stress
828 conditions (e.g., high temperature) for dealing with a wide variety of substrate
829 proteins. The middle active DnaK/CbpA/GrpE system (Gur et al. 2004) can
830 perform specific cellular functions (e.g., curli biogenesis). The modest active
831 DnaK/DjlA/GrpE system is not able to support curli production but may work for
832 unknown cellular activities. DnaK alone engages in holding substrate proteins,
833 but its cellular functions remain elusive. Respective DnaK mutants with
834 hierarchical activities are indicated based on the results presented in this study
835 and our previous one (Sugimoto et al., 2018).

836

837 **EXPANDED VIEW LEGENDS**

838 **Figure EV1. Effects of JDP deletion on thermotolerance of *E. coli* strain**

839 **BW25113**

840 (A) Thermosensitivity of the indicated strains was assayed on LB agar plates.

841 Ten-fold serial dilutions of the overnight cultures were spotted on the plates and

842 incubated at 30°C or 43°C for 24 h. (B) BW25113 and its isogenic $\Delta cbpA \Delta dnaJ$

843 $\Delta djlA$ strains were transformed with pCA24N (empty vector) or the indicated

844 JDP-expression plasmids. The strains were grown at 30°C or 43°C for 24 h on

845 LB agar plates supplemented with chloramphenicol.

846

847 **Figure EV2. Activity of RpoS in BW25113 derivative strains**

848 Relative RpoS activity was assayed by measuring catalase activity as previously

849 reported (Sugimoto et al., 2018). All experiments were repeated at least three

850 times to ensure accuracy and averaged values with standard deviations were

851 calculated. The activity of the BW25113 parental strain was defined as 100%.

852

853 **Figure EV3. Effects of complete and incomplete DnaK chaperone systems**

854 **on de novo folding of transcriptional regulators MlrA and CsgD**

855 (A, B) De novo folding of MlrA and CsgD was analyzed in the absence (Control)

856 or presence of the indicated chaperone proteins using a cell-free translation

857 system (PURE System). His-tagged MlrA (A) and CsgD (B) were detected using

858 anti-His antibody and anti-CsgD antibody, respectively. All experiments were

859 repeated at least three times to ensure accuracy and representative images are

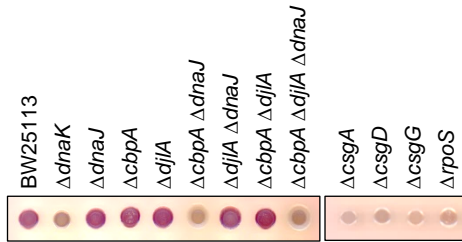
860 shown. K, DnaK; KJ, DnaK/DnaJ; KA, DnaK/CbpA; KE, DnaK/GrpE; KJE,

861 DnaK/DnaJ/GrpE; KAE, DnaK/CbpA/GrpE; JE, DnaJ/GrpE; AE, CbpA/GrpE; J,

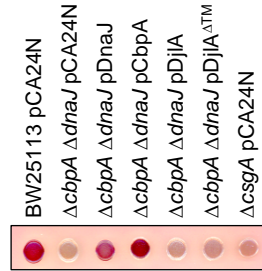
862 DnaJ; A, CbpA; E, GrpE.

Sugimoto *et al.* Figure 1

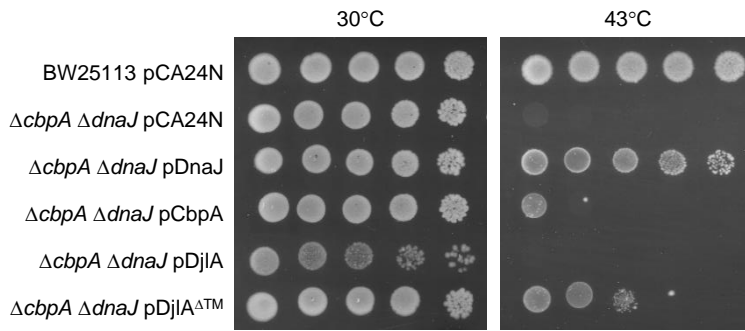
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B

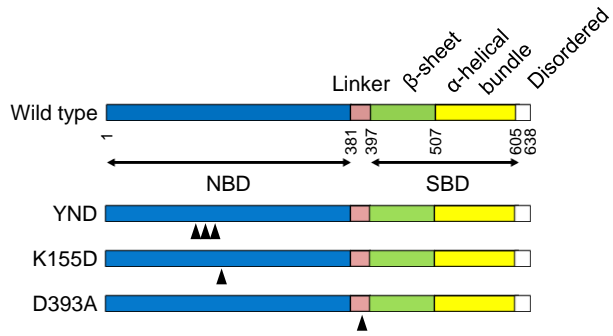


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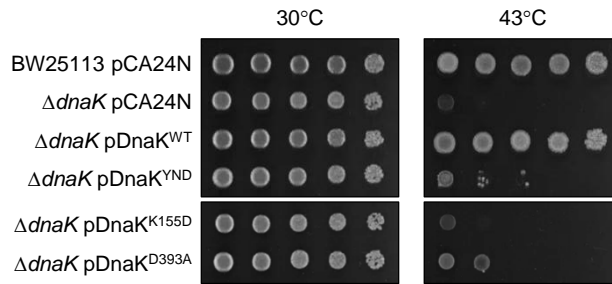


Sugimoto *et al.* Figure 2

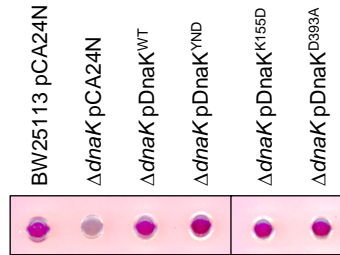
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B

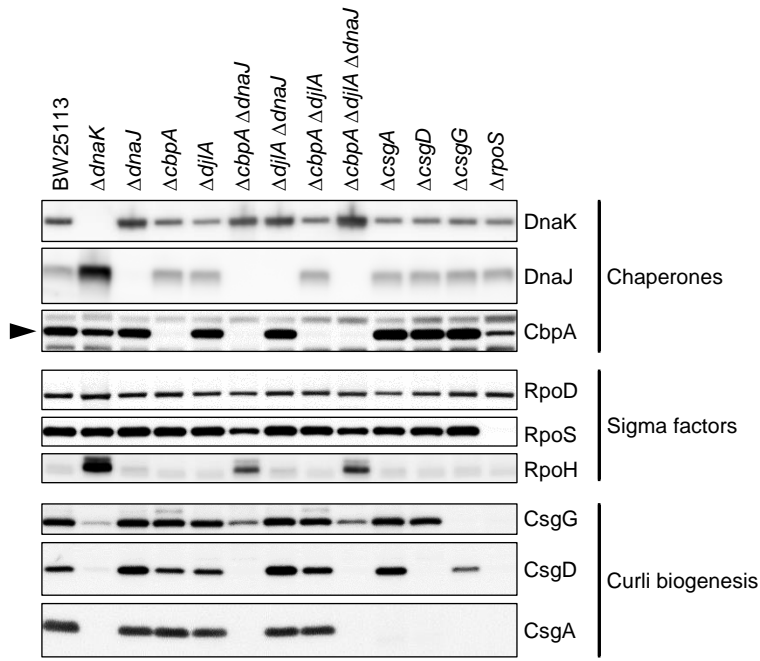


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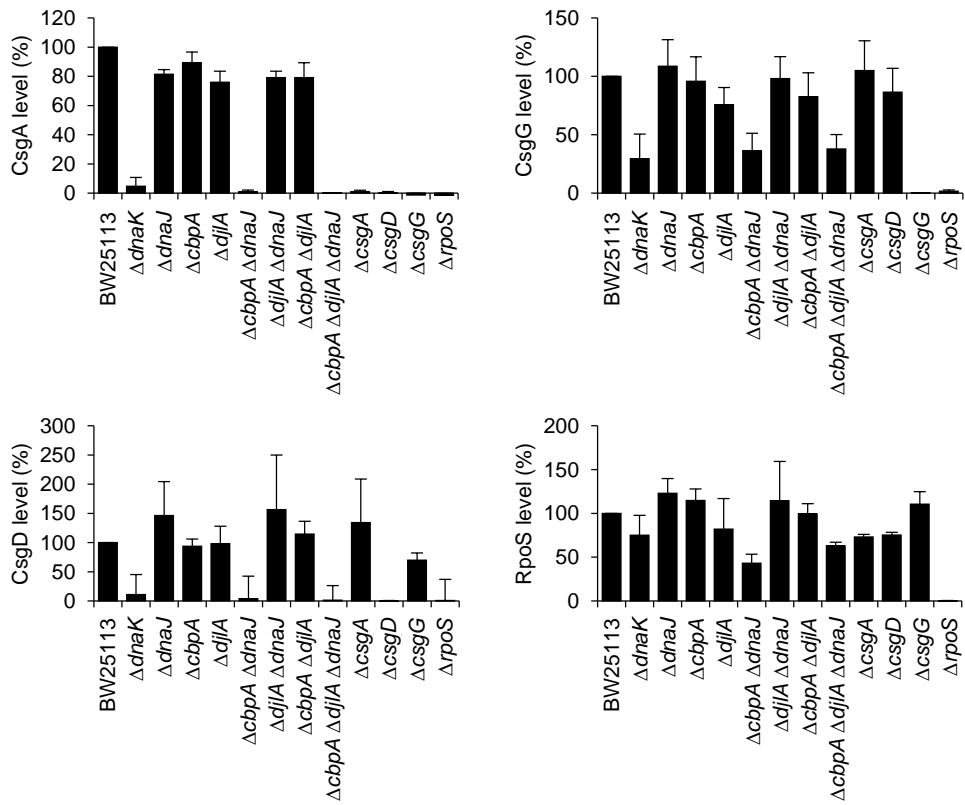


Sugimoto et al. Figure 3

A

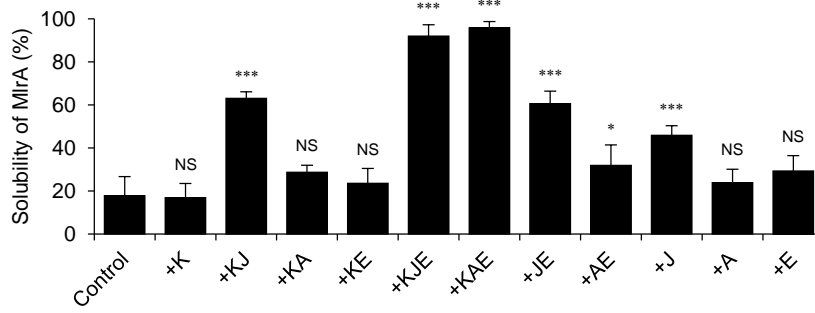


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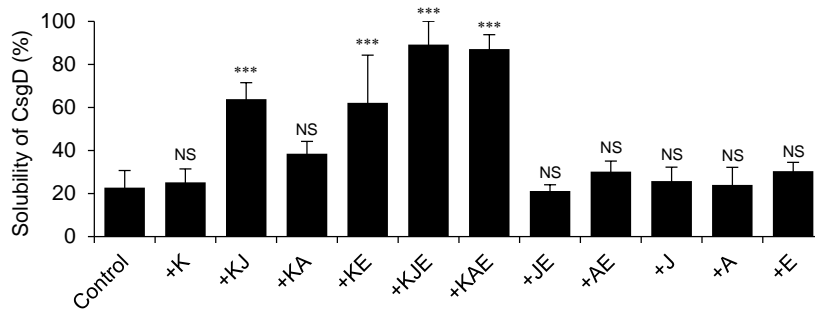


Sugimoto *et al.* Figure 4

A



B

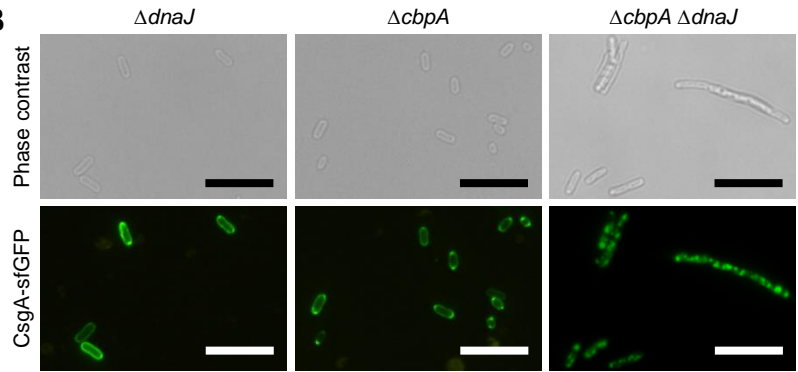


Sugimoto *et al.* Figure 5

A



B



Sugimoto *et al.* Figure 6

