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**The PAR promoter expression system: modified *lac*
promoters for controlled recombinant protein production
in *Escherichia coli***

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31 **Highlights.**

- 32 • **The PAR promoter system can express recombinant proteins to many different**
- 33 **levels.**
- 34 • **The system is versatile, functioning in many *E. coli* strains and growth regimes.**
- 35 • **Promoters are tightly regulated, allowing low-level expression of toxic**
- 36 **proteins.**

37

38

39 **ABSTRACT**

40 Many commonly used bacterial promoters employed for recombinant protein production (RPP)
41 in *Escherichia coli* are capable of high-level protein expression. However, such promoter
42 systems are often too strong, being ill suited for expressing proteins that are difficult to fold or
43 proteins that are targeted to the membrane or secreted out of the cytoplasm. To circumvent
44 this we have constructed a suite of bacterial promoters with a range of different promoter
45 strengths, assigning them specific promoter activity ratings (PARs). Selecting three of these
46 PAR promoters, with low, intermediate and high strengths, we demonstrate that the
47 expression of target proteins, such as green fluorescent protein (GFP), human growth
48 hormone (hGH) and single chain variable region antibody fragments (scFvs) can be set to
49 three levels when expressed in *E. coli*. We show the PAR promoter system is extremely
50 flexible, operating in a variety of *E. coli* strains and under various different culture regimes.
51 Furthermore, due to its tight regulation, we show that this system can also express a toxic
52 outer membrane protein, at levels, which do not affect bacterial growth. Thus, the PAR
53 promoter system can be used to tailor the expression levels of target proteins in *E. coli* and
54 maximize RPP.

55

56

57 **Abbreviations:** GFP, green fluorescent protein; hGH, human growth hormone; HRP,
58 horseradish peroxidase; IPTG, isopropyl β -D-1-thiogalactopyranoside; LacI, *lac* operon
59 repressor; MCS, multiple cloning site; PAR, promoter activity rating; RPP, recombinant protein
60 production; scFv, single chain variable region antibody fragment.

61

62

63 **Keywords:** Recombinant protein production, *Escherichia coli*, transcription regulation, *lac*
64 promoter, membrane proteins

65

66

67 Introduction

68 The bacterium *Escherichia coli* has been the cornerstone of recombinant protein
69 production (RPP) for many years. *E. coli* is particularly well suited to its role as a protein
70 production factory as it grows quickly to high density in cheap medium and can be **easily**
71 **manipulated genetically** [1]. As a consequence, many recombinant protein expression
72 systems have been constructed, such as those based on the *E. coli* lactose operon promoter
73 (e.g. the *lac* and *tac* promoters) and the T7 RNA polymerase expression system [2, 3, 4]. In
74 such systems, the gene of interest is usually cloned into a plasmid vector downstream of a
75 strong regulated promoter and RPP is initiated by the addition of an inducer molecule, e.g. the
76 lactose analogue, IPTG (isopropyl β -D-1-thiogalactopyranoside) [5]. In many instances, these
77 systems allow the production of large amounts of high quality protein. However, as RPP
78 expression systems typically have strong inducible promoters, problems can arise when
79 expressing difficult-to-fold targets, membrane proteins and proteins secreted out of the cell or
80 into periplasm. Thus, high-level expression of such proteins often leads to product misfolding,
81 resulting in target degradation or its aggregation into inclusion bodies [6, 7, 8, 9]. In an attempt
82 to increase the amount of soluble target, RPP is often carried out at lower temperatures, or
83 expression levels are decreased by using a weaker promoter or lower inducer concentrations
84 [6, 10, 11]. This often involves trial and error, and may even require switching the expression
85 system, since low-level RPP with some systems, e.g. the T7 RNA polymerase expression
86 system, can be hard to control.

87

88 Ideally, RPP expression systems should be as versatile as possible, being tightly
89 regulated, allowing high- and low-level target expression, and be compatible with many
90 different *E. coli* strains and “helper” plasmids, which can carry various tRNAs or chaperones
91 [1, 10]. Above all, expression systems should be easy to use, with vectors carrying multiple
92 cloning sites (MCS), purification and secretion tags, and, potentially, different antibiotic
93 resistance cassettes. Previously we generated a suite of promoters, which were based on the
94 *lac* and *tac* promoters, and assigned them promoter activity ratings (PAR values) based on
95 their strengths [5]. These PAR promoters (PAR1 to PAR8) show a wide range of promoter
96 activities, stretching from low-level (*i.e.* PAR1) to high-level (*i.e.* PAR8) expression capabilities
97 [5]. Each PAR promoter also carried *lac* operator sequences and were, therefore, repressed
98 by the *lac* operon repressor (LacI) and IPTG-inducible (Fig. 1a). As some of these promoters
99 appeared to be promising for RPP, we have transferred them to easy-to-use vector backbones
100 to generate the PAR promoter expression system. By picking three promoters, which have
101 low, intermediate and high expression capabilities, when fully induced by IPTG, this system is
102 able to express recombinant proteins at three different levels in *E. coli*, allowing the expression
103 level of a target protein to be easily tailored to maximize the production of soluble recombinant

104 protein. In addition, we demonstrate that the PAR promoter system can be tuned by different
105 IPTG concentrations, can be used to express toxic proteins, and is flexible, functioning in
106 different *E. coli* strains, media and at different growth temperatures.

107

108

109 **Materials and methods**

110 *Bacterial strains, plasmids, and materials*

111 *E. coli* strains, XL1 Blue and JCB387, were used for plasmid construction and DNA
112 manipulation, whilst *E. coli* BL21, BL21(DE3), W3110 and SHuffle Express and were used for
113 recombinant protein over-expression (Supplementary Table S1). Strains were grown in LB
114 broth (Sigma), Lennox broth (2% (w/v) peptone (Oxoid), 1% (w/v) yeast extract (Oxoid) and
115 170 mM NaCl) [12] and auto-induction medium [13], with appropriate antibiotic selection
116 (ampicillin 100 µg/ml, kanamycin 50 µg/ml). For RPP, *E. coli* BL21, BL21(DE3) and W3110
117 were routinely grown at 37°C, whilst SHuffle Express was grown at 30°C.

118

119 *Expression vector construction*

120 The PAR promoters, PAR1 to PAR8 (including PAR4L, formerly *lacO3O1*) have been
121 described previously (Browning *et al.*, 2019). Each PAR promoter was amplified from plasmids
122 pRW50/ PAR1 to PAR8 (Supplementary Table S1), using PCR with primers detailed in
123 Supplementary Table S2. Purified PCR products were restricted with BglII and XbaI and
124 cloned into the pET22b and pET26b expression vectors (Novagen), replacing the canonical
125 T7 RNA polymerase promoter (Supplementary Fig. S1). The DNA encoding 6His-GFP, from
126 pET15b/ *6his-gfp*, was cloned into each pET22b and pET26b PAR construct using XbaI and
127 BamHI restriction sites (Supplementary Table S1; Supplementary Figs. S2 and S3). The DNA
128 encoding hGH-6His and anti-IL-1β-6His scFv, from pHAK1 and pYU49, respectively, was
129 cloned into each pET22b PAR construct using NdeI and SacI (Supplementary Figs. S2 and
130 S3) [14, 15]. The DNA encoding the BamA_{ENm} chimeric outer membrane protein was cloned
131 into pET22b PAR1, using NdeI and XhoI (Supplementary Figs. S2 and S3) [16]. The *lacI^q*
132 mutation, which changes a single base in the *lacI* promoter to increase its strength [17, 18],
133 was introduced into pET22b PAR7 and pET26b PAR7 derivatives using the Agilent
134 QuikChange site-directed mutagenesis kit and primers lacIqF/R (Supplementary Table 2). All
135 constructs were verified by Sanger DNA sequencing.

136

137 *Recombinant protein over-expression and detection*

138 Bacterial cultures of *E. coli*, carrying pET expression plasmids containing the PAR
139 promoters and various target genes, were grown with shaking in 10 mL of LB medium, until
140 an optical density (OD₆₀₀) of 0.3 to 0.5. Protein over-expression was induced by the addition

141 of IPTG and samples were taken after three or four hours induction. For the expression of the
142 BamA_{ENm} chimera, BL21 cells were grown in 50 ml Lennox broth, supplemented with glucose,
143 where indicated. Total protein samples were routinely prepared by resuspending normalized
144 amounts of cells in 2X Laemmli loading buffer (Sigma), heating at 95°C for three minutes, and
145 centrifuging prior to loading. Normalized protein samples were resolved by reducing SDS-
146 PAGE and analyzed using Coomassie blue staining and Western blotting, as in our previous
147 work [19]. For Western blotting, 6His-GFP was detected using anti-GFP antiserum raised in
148 mouse (Sigma) and an anti-mouse-HRP secondary antibody (Sigma), hGH-6His was detected
149 using anti-hGH antiserum raised in rabbit [5] and an anti-rabbit-HRP (horseradish peroxidase)
150 secondary antibody (Amersham), and anti-IL-1 β -6His scFv was detected using anti-6His (C-
151 terminal)-HRP (Invitrogen). BamA_{ENm} was detected using anti-BamA antiserum [16] and an
152 anti-rabbit-HRP secondary antibody (Amersham). Blots were developed using Pierce ECL
153 Western blotting substrate and all gels and blots shown are representative. To access the
154 aggregation of product in inclusion bodies, total, soluble and insoluble protein samples were
155 also prepared using Agilent BugBuster, according to the manufacturer's instructions.

156

157 *Cellular fractionation and membrane isolation*

158 50 ml cultures of BL21 cells, carrying pET22b PAR1/ BamA_{ENm}, were grown in Lennox
159 broth, supplemented with 0.2% glucose, in the presence or absence of 1 mM IPTG for three
160 hours. Cells were isolated by centrifugation and pellets were washed with 10 mM Tris-HCl (pH
161 7.4) and resuspended in 20ml of 10 mM Tris-HCl (pH 7.4) containing 2 mM
162 phenylmethylsulfonyl fluoride (PMSF, protease inhibitor). Cell envelopes were disrupted by
163 continuous passage through an Emulsiflex C3 for 5 minutes, and unbroken cells and
164 particulate material was removed by centrifugation for 15 min at 6,000 x g and 4°C. The total
165 membrane fraction (inner and outer membranes) was then isolated by centrifuging the
166 supernatant for 1 hour at 48,000 x g at 4 °C and the soluble fraction, which contains
167 cytoplasmic and periplasmic proteins was retained [16, 19]. Membranes were washed once
168 and resuspended in 0.4 ml of 10 mM Tris-HCl (pH 7.4).

169

170 *Rescue of BamA depletion in E. coli*

171 To determine the ability of the BamA_{ENm} chimera to rescue BamA depletion on solid
172 media, the *E. coli* BamA depletion strain JWD3 (Supplementary Table S1) [20] was grown on
173 LB agar plates, supplemented by 0.2% (w/v) arabinose or 1 mM IPTG, where indicated. For
174 experiments in liquid media, JWD3 cells were grown in 50 ml of Lennox broth with 0.2%
175 glucose, with shaking at 37°C, in the presence or absence of 0.05% (w/v) arabinose. Optical
176 density was monitored and after 2 hours of growth (OD₆₀₀ = 0.3 - 0.4) BamA_{ENm} production was
177 induced by the addition of IPTG. The preparation of normalised total cellular protein samples,

178 after 6 hours of growth, was as in Browning *et al.* [19]. Note that in JWD3 cells chromosomally
179 encoded *E. coli* BamA is only produced in the presence of arabinose, whilst in its absence,
180 BamA expression is prevented, resulting in the cessation of growth and cell death [20].
181 Depletion can be rescued by providing a functional copy of *bamA*, such as that carried by
182 pET22b PAR1/ BamA_{ENm} [16, 19].

183

184 *Flow cytometry.*

185 For flow cytometry analysis, 50 mL cultures of LB medium were incubated with shaking
186 at 37°C until the culture reached OD₆₀₀ ~0.6, and then RPP was induced by addition of IPTG
187 for three to four hours, as stated. Cultures were analysed using a BD Accuri C6 flow cytometer
188 (Becton Dickinson, UK). Samples were mixed with 0.2 µm-filtered PBS and data was collected
189 at a rate of 1000 - 4000 events per second using slow flow and a forward scatter height (FSC-
190 H) threshold of 10000 to eliminate non-cellular material until 20000 events had been recorded
191 per sample. Data were analysed using CFlow software (BD).

192

193

194 **Results and Discussion**

195 *Construction and RPP with the PAR promoters*

196 Previously we generated a suite of PAR promoters, which covered a broad range of
197 promoter activities from low to high expression levels (*i.e.* PAR1 to PAR8) (Fig. 1a) [5]. For
198 ease of use, each PAR promoter was sub-cloned into the medium copy number expression
199 vector pET22b, which carries an extensive MCS to facilitate gene cloning and a functional *lacI*
200 gene to ensure regulation in any *E. coli* host strain (Supplementary Fig. S1 and Table S1). To
201 investigate expression from these new vectors, DNA encoding N-terminally 6His-tagged GFP
202 (*6his-gfp*) (Supplementary Figs. S2 and S3) was cloned downstream of each PAR promoter
203 and recombinant plasmids were transferred into *E. coli* BL21 cells. Cells were grown in LB
204 medium until mid-logarithmic growth (OD₆₀₀ between 0.3 and 0.5) and recombinant PAR
205 promoter expression was induced with 1 mM IPTG for three hours. Levels of total GFP
206 production were then analysed by SDS-PAGE and Western blotting. Results in Fig. 1b show
207 that IPTG-induced GFP production increased with the strength of the PAR promoter (*i.e.* from
208 PAR1 to PAR8). Most PAR promoters were tightly regulated, with little or no expression in the
209 absence of IPTG, but some, *e.g.* PAR6 and PAR8, were found to be leaky (Fig. 1).

210 As we wished to develop vectors with a weak, intermediate and strong promoter, we
211 chose the PAR1, PAR4L and PAR7 constructs, respectively. As expression from the PAR7
212 construct was slightly leaky in the absence of inducer (Figs. 1b and 1c), the *lacI^q* mutation,
213 which increases the expression of LacI, was introduced [17, 18]. This new construct, referred
214 to as pET22b PAR7Q, showed minimal 6His-GFP expression in the absence of IPTG, as

215 judged by Western blotting (results not shown). Expression of 6His-GFP in BL21, driven by
216 the PAR1, PAR4L and PAR7Q promoters, was produced at low, intermediate and high levels
217 respectively, after induction with 1 mM IPTG (Fig. 2a). This was confirmed by flow cytometry
218 (Fig. 2b), which revealed differences in expression after 1 hour of induction.

219

220 Since RPP expression systems should be as flexible as possible, the PAR1, PAR4L and
221 PAR7Q promoters were also introduced into the pET26b expression vector, which carries an
222 alternative plasmid backbone and a kanamycin resistant cassette (Supplementary Table S1).
223 Expression studies, again using *6his-gfp*, demonstrated that graded levels of 6His-GFP
224 expression, as expected, were achieved (Supplementary Fig. S4). Furthermore, our three
225 plasmid PAR promoter system functioned well in the *E. coli* K-12 strain W3110
226 (Supplementary Fig. S5), and with auto-induction medium when cells were grown at different
227 temperatures (*i.e.* 30 and 37°C) for longer periods of time (*i.e.* 23 hrs) (Supplementary Fig.
228 S6) [13]. Thus, we conclude that the PAR promoter system is versatile and can be used to
229 express recombinant proteins to set levels, in different strains and under different growth
230 conditions.

231

232 *Maximizing the solubility of recombinant 6His-GFP using the PAR promoters*

233 In many instances, high level RPP can result in misfolded proteins and aggregation of
234 product into inclusion bodies [6]. We, therefore, hypothesised that expressing target proteins,
235 using the weaker PAR1 and PAR4L promoters, may reduce product aggregation and improve
236 overall protein solubility. To examine this, we analysed soluble and insoluble fractions from
237 BL21 cells expressing 6His-GFP from the pET22b PAR1, PAR4L and PAR7Q constructs.
238 Results illustrated in Fig. 2c, show that, for the strong PAR7Q construct, a large proportion of
239 6His-GFP is found in the insoluble fraction. For the intermediate strength PAR4L promoter,
240 less insoluble 6His-GFP is observed, whilst for the weak PAR1 promoter, all the 6His-GFP
241 was found in the soluble fraction. This shows that the different expression levels achieved with
242 the PAR promoter system can be used to tailor expression levels and minimize insoluble
243 product formation. Note, analysis of the quantity of 6His-GFP in the soluble and insoluble
244 fractions correlates with flow cytometry data (Fig. 2b), whereby the fluorescence of the PAR7Q
245 cultures is only slightly higher than the PAR4L cultures despite containing more total GFP.
246 Flow cytometry has been shown to measure both quantity and folding quality of GFP, with
247 insoluble GFP having low fluorescence [11].

248

249 *Fine-tuning of the PAR promoter response by altering the inducer concentration*

250 In our previous experiments, we used a saturating concentration of IPTG (*i.e.* 1 mM)
251 to ensure that all promoters were fully derepressed. However, for many induction regimes, low

252 inducer concentrations are used to try to decrease RPP and fine-tune expression levels [6].
253 Whilst this works for some expression systems, in other systems this leads to only a proportion
254 of the cells in a culture expressing recombinant protein, which has been termed as an all-or-
255 none phenomenon [21, 22, 23]. Therefore, we examined whether 6His-GFP expression from
256 our pET22b PAR1, PAR4L and PAR7Q constructs was tuneable. Once more, BL21 cells,
257 carrying each plasmid, were grown in LB and induced with different IPTG concentrations (*i.e.*
258 2, 10, 50 and 1000 μ M). The expression of 6His-GFP was then monitored using flow
259 cytometry. Results in Fig. 3 show that for all three promoters different levels of expression
260 could be set in a culture by using different IPTG concentrations. Furthermore, the analysis of
261 individual cells indicated that for each promoter and IPTG concentration tested, GFP induction
262 was homogenous within the bacterial cell population (Supplementary Fig. S7). This is
263 particularly evident for the PAR7Q construct, which produces discrete GFP-expressing
264 populations at many different IPTG concentrations, indicating that expression from this highly
265 active promoter can be effectively tuned by different IPTG concentrations.

266

267 *Expression of different protein targets using the PAR promoter system*

268 To test the versatility of the PAR promoter expression system, we examined the
269 expression of two additional targets, human growth hormone (hGH) and a single chain variable
270 region antibody fragment against interleukin 1 β (anti-IL-1 β scFv). Thus, the DNA encoding
271 each protein, carrying a C-terminal 6His tag, was cloned into pET22b, carrying either the
272 PAR1, PAR4L or PAR7Q constructs (Supplementary Figs. S2 and S3) [14, 15]. The resulting
273 plasmids were then transferred to BL21 cells and RPP was induced by the addition of 1 mM
274 IPTG to mid-logarithmic growing cells. Results in Fig. 4 show that, as anticipated, graded
275 levels of expression were achieved for both hGH-6His and anti-IL-1 β -6His scFv, with the most
276 product produced by cells carrying the PAR7Q construct and the least for PAR1.

277

278 As correct folding of hGH requires the formation of a disulphide bond, we examined
279 whether expressing hGH-6His with the PAR promoters aided its solubility. However, as the *E.*
280 *coli* cytoplasm is a reducing environment that does not favour disulphide bond formation, it
281 was unsurprising to find that the majority of hGH-6His was insoluble (Fig. 5a). To circumvent
282 this problem, hGH-6His expression was carried out in *E. coli* SHuffle Express, a genetically
283 modified *E. coli* strain, which enables cytoplasmic disulphide bond formation. Cells were grown
284 in LB medium at 30°C and RPP induced with 1 mM IPTG for three hours. Results illustrated
285 in Fig. 5b, demonstrate that hGH-6His was successfully induced under this altered induction
286 regime and that for all PAR promoter constructs the majority of recombinant hGH-6His was
287 now found in the soluble fraction. Note that the PAR7Q construct produced the most insoluble

288 product and that the intermediate strength promoter PAR4L gave the best yield of soluble
289 protein with minimal insoluble protein, as detected by Western blotting (Fig. 5b).

290

291 *The PAR1 promoter can be used to express toxic proteins*

292 For some target proteins, very low levels of expression are required, especially when
293 the recombinant product is toxic. As our PAR1 promoter is based on the *lac* promoter, it is
294 subject to catabolite repression and can be inhibited by the inclusion of glucose in the growth
295 medium (Supplementary Fig. S8) [24]. Therefore, to test if the PAR1 promoter could be used
296 to express a toxic protein, we cloned the DNA encoding BamA_{ENm}, a large chimeric outer
297 membrane protein (OMP) from *Neisseria meningitidis*, into our pET22b PAR1 vector
298 (Supplementary Figs. S2 and S3) [16]. This 88 kDa membrane protein has potential as a
299 vaccine candidate against *N. meningitidis* [16, 25]. Results in Fig. 6a, show that
300 overexpression of this construct at 37°C, using the PAR1 promoter with 1 mM IPTG is toxic
301 and leads to the cessation of cell growth. When a lower IPTG concentration was used (*i.e.* 20
302 µM) cells reached a higher optical density but, growth was arrested before the end of the
303 experiment (Fig. 6a). In contrast, induction of BamA_{ENm} expression with 1 mM IPTG in the
304 presence of glucose did not influence bacterial growth (Fig. 6a) and resulted in lower
305 expression levels of BamA_{ENm} without toxicity (Fig. 6b; lane 6). Fractionation of cells into their
306 soluble (cytoplasmic and periplasmic proteins) and membrane components (inner and outer
307 membranes) confirmed that that BamA_{ENm} was located in the membrane fraction (Fig. 6c), as
308 expected for an integral outer membrane protein.

309

310 In *E. coli*, BamA is an essential protein that is responsible for inserting bacterial β-
311 barrel containing OMPs into the bacterial outer membrane [26]. Previously, we demonstrated
312 that very low-level expression of the *N. meningitidis* BamA_{ENm} chimera could function in *E.*
313 *coli*, rescuing the depletion of BamA in the *E. coli* K-12 strain JWD3, where BamA production
314 is absolutely dependent upon arabinose [16]. Results in Supplementary Fig. S9 demonstrate
315 that IPTG induced BamA_{ENm} expression from pET22b PAR1, in the presence of glucose, could
316 also rescue depletion of BamA in JWD3, indicating that under these expression conditions,
317 BamA_{ENm} was folded and functional. It is also of note that, in the absence of IPTG, BamA
318 depletion in JWD3 was not rescued and BamA_{ENm} expression was not detected
319 (Supplementary Fig. S9), indicating that the PAR1 promoter is tightly regulated and suitable
320 for the expression of toxic proteins.

321

322

323

324

325 **Conclusions**

326 Many expression plasmids carry strong promoters. In most instances, this is beneficial,
327 but, in some cases, high-level RPP can result in the accumulation of insoluble protein into
328 inclusion bodies, or cell death if the expressed product is toxic. As obtaining the correct
329 expression levels for problematic proteins can be difficult to achieve, we have developed the
330 PAR promoter system, which consists of three plasmids with low, intermediate and high
331 expression capabilities (PAR1, PAR4L and PAR7Q). Thus, by cloning target DNA into each
332 vector, the most suitable level of expression required for optimal RPP and solubility can be
333 determined quickly. The pET22b and pET26b vectors that we used carry extensive MCS, tags
334 for purification and secretion, and different antibiotic resistance cassettes (Supplementary Fig.
335 S1 and Table S1). Each plasmid also carries the gene encoding the Lac repressor (i.e. *lacI*)
336 and, thus, coordinated regulation can be achieved in any *E. coli* strain regardless of its genotype.
337 Consistent with this, we show that the PAR system functions with different *E. coli* strains
338 commonly used in industry and academia (e.g. BL21, W3110 and SHuffle Express).

339
340 Using the PAR system we have expressed different proteins (GFP, hGH, an scFv and
341 BamA_{ENm}) ranging in size from 23 to 88 kDa (Supplementary Fig. S3). Interestingly, even with
342 GFP, which is often used as a model protein for expression analysis, high-level expression
343 with the PAR7Q construct resulted in substantial product insolubility, with the weaker PAR
344 promoters producing less insoluble product (Fig. 2c). Note that expression levels from our
345 PAR7Q promoter construct rivals that of the highly active T7 expression system and so this
346 effect is to be expected (Supplementary Fig. S10). Cytoplasmic expression of more complex
347 proteins, such as hGH, can be more problematic as it requires disulphide bond formation for
348 correct folding. In this instance, product solubility was greatly improved by employing *E. coli*
349 SHuffle Express as an expression host (Fig. 5), as it allows cytoplasmic disulphide bond
350 formation to occur. Once more, the largest amount of insoluble product was found when using
351 our strongest promoter construct, PAR7Q, with little or no product insolubility observed for the
352 PAR4L and PAR1 constructs. Thus, we show, as others before us, that reducing RPP
353 expression, by using weaker promoters can improve target solubility [6, 10].

354
355 For many experiments, we used high concentrations of IPTG (i.e. 1 mM) to ensure that
356 our expression systems are fully switched on. However, our results show that the level of RPP
357 driven by the PAR promoters can be modulated. Our systems are tuneable, with specific IPTG
358 concentrations producing different expression levels homogeneously within a culture, rather
359 than an all-or-none phenotype that has been observed before (Fig. 3 and Supplementary Fig.
360 S7) [21, 22, 23]. Also, the use of glucose-mediated repression with the PAR1 promoter
361 enabled the expression of the toxic BamA_{ENm} chimera from *N. meningitidis* (Fig. 6). It is of note

362 that BamA_{ENm} is a large outer membrane protein that must traverse the *E. coli* inner membrane
363 and periplasmic space to be inserted into the outer membrane [26]. Overloading of the cellular
364 machinery responsible for these events (*i.e.* the Sec translocase, the periplasmic chaperones,
365 and the nascent Bam complex) will likely result in toxicity and cell death [16, 26]. Thus, due to
366 the tight repression of PAR1 in the absence of inducer and the ability to modulate expression
367 by glucose (Fig. 6; Supplementary Figs. S8 and S9), the PAR1 promoter is ideal for low-level
368 expression of toxic proteins. Finally, all three promoters worked well with auto-induction
369 medium (Supplementary Fig. S6), indicating that inducer exclusion (*i.e.* the ability to prevent
370 lactose uptake when glucose is present in the growth medium) is a feasible way to control and
371 delay RPP induction with the PAR system [13].

372

373 Since its discovery, the *lac* operon promoter and its derivatives have been extensively
374 used in biotechnology [3, 4, 8, 27]. In this work, we have further adapted the *lac* promoter to
375 generate an easy-to-use RPP expression system that allows the expression of target proteins
376 to be quickly set to obtain optimal expression and/ or solubility. Furthermore, we show that the
377 PAR system functions well with many of the common induction regimes used to control both
378 the level and timing of target protein expression. Thus, fine-tuning expression levels from the
379 PAR promoters gives added flexibility. Future research will focus on optimizing the PAR
380 promoter system for use in larger-scale expression and fermenter applications.

381

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389

390 **Author contribution**

391 J.H., T.W.O., S.J.W.B. and D.F.B. devised the research programme, experiments were
392 performed by J.H., R.E.G., C.F. and D.F.B., and the manuscript was written by J.H., S.J.W.B.
393 and D.F.B., with input from all authors.

394

395 **Conflict of interest statement**

396 The Authors declare no conflict of interest.

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472

473 **Figure Legends**

474 **Fig. 1.** Expression of recombinant 6His-GFP protein can be set to different levels using the
475 PAR promoters. (a) The panel shows a schematic representation of the PAR promoters used
476 in this study. Each PAR construct (*i.e.* PAR1 to PAR8) carries -10 and -35 promoter elements,
477 based on either the *lac* or *tac* promoters, **with** flanking *lac* operator DNA sequences, **which**
478 **are either the O1 or O3 operator sequences** [5]. **Base substitutions are defined by the**
479 **position of the base altered, with respect to the *lac* transcription start site (+1), and the**
480 **substituted base introduced.** The LacI repressor protein, binding to each operator target,
481 represses promoter activity (-ve), until the addition of IPTG causes it to release the promoter
482 DNA [28]. Panels (b) and (c) show Coomassie blue stained SDS-PAGE gels and Western
483 blots, respectively, examining 6His-GFP expression in *E. coli* BL21 cells, carrying various
484 pET22b PAR constructs (PAR1 to PAR8). Cells were grown in LB medium and sampled after
485 three hours induction with (or without) 1 mM IPTG. An empty pET22b vector control (EV) was
486 included. In the Western blot in panel (c), recombinant 6His-GFP was detected using anti-
487 GFP antiserum and anti-mouse HRP secondary antibody.

488

489 **Fig. 2.** Analysis of 6His-GFP expression using the three promoter PAR system. (a) The panel
490 shows a Coomassie blue stained SDS-PAGE gel of *E. coli* BL21 cells expressing 6His-GFP,
491 using the pET22b three promoter PAR system (PAR1, PAR4L and PAR7Q). Cells were grown
492 in LB medium and RPP was initiated for three hours by the addition of 1 mM IPTG, where
493 indicated. (b) The panel shows the mean cellular fluorescence of *E. coli* BL21 cells as
494 measured by flow cytometry, expressing 6His-GFP from the pET22b three promoter PAR
495 system. Cells were grown in LB medium and RPP was induced for three hours using 1 mM
496 IPTG. Data are shown as mean green fluorescence values from replica flasks and error bars
497 are \pm the standard deviation. (c) The panel shows a Coomassie blue stained SDS-PAGE gel
498 investigating the solubility of 6His-GFP expressed in *E. coli* BL21 cells using the pET22b three
499 promoter PAR system. Cultures were grown in LB medium and protein production was
500 induced by 1 mM IPTG for three hours. Harvested cells were lysed to prepare total (T), soluble
501 (S) and insoluble (I) protein samples. In panels (a) and (b) empty vector controls (EV) were
502 included.

503

504 **Fig. 3.** Expression from the PAR promoters can be fine-tuned using different IPTG
505 concentrations. The figure shows flow cytometry analysis of mean green fluorescence from
506 BL21 cells carrying pET22b PAR/ 6His-GFP constructs (a) PAR1, (b) PAR4L and (c) PAR7Q.
507 Cells were grown in LB medium and 6His-GFP expression was induced by the inclusion of
508 IPTG at 2, 10, 50 and 1000 μ M. Data are shown as mean green fluorescence values from
509 replica flasks, error bars are \pm the standard deviation.

510

511 **Fig. 4.** Expression of hGH and an anti-IL-1 β scFv using the PAR promoter system. The figure
512 shows Coomassie blue stained SDS-PAGE gels of *E. coli* BL21 cells expressing (a) hGH-6His
513 and (b) anti-IL-1 β -6His scFv, using the pET22b three promoter PAR system (PAR1, PAR4L
514 and PAR7Q). Cells were grown in LB medium and RPP was initiated for three hours by the
515 addition of 1 mM IPTG, where indicated. In each case, an empty vector control (EV) was
516 included.

517

518 **Fig. 5.** Solubility of recombinant hGH-6His expressed in *E. coli* BL21 and *E. coli* SHuffle
519 Express cells. The figure shows Coomassie blue stained SDS-PAGE gels investigating the
520 solubility of hGH-6His expressed in (a) *E. coli* BL21 and (b) *E. coli* SHuffle Express cells using
521 the pET22b three promoter PAR system (PAR1, PAR4L and PAR7Q). Cultures were grown
522 in LB medium and protein production was induced by 1 mM IPTG for three hours. Harvested
523 cells were lysed to prepare total (T), soluble (S) and insoluble (I) protein samples. Empty
524 vector controls (EV) were included. In panel (b) a Western blot is included detailing the
525 detection of hGH-6His in samples, using anti-hGH antiserum and anti-rabbit HRP secondary
526 antibody.

527

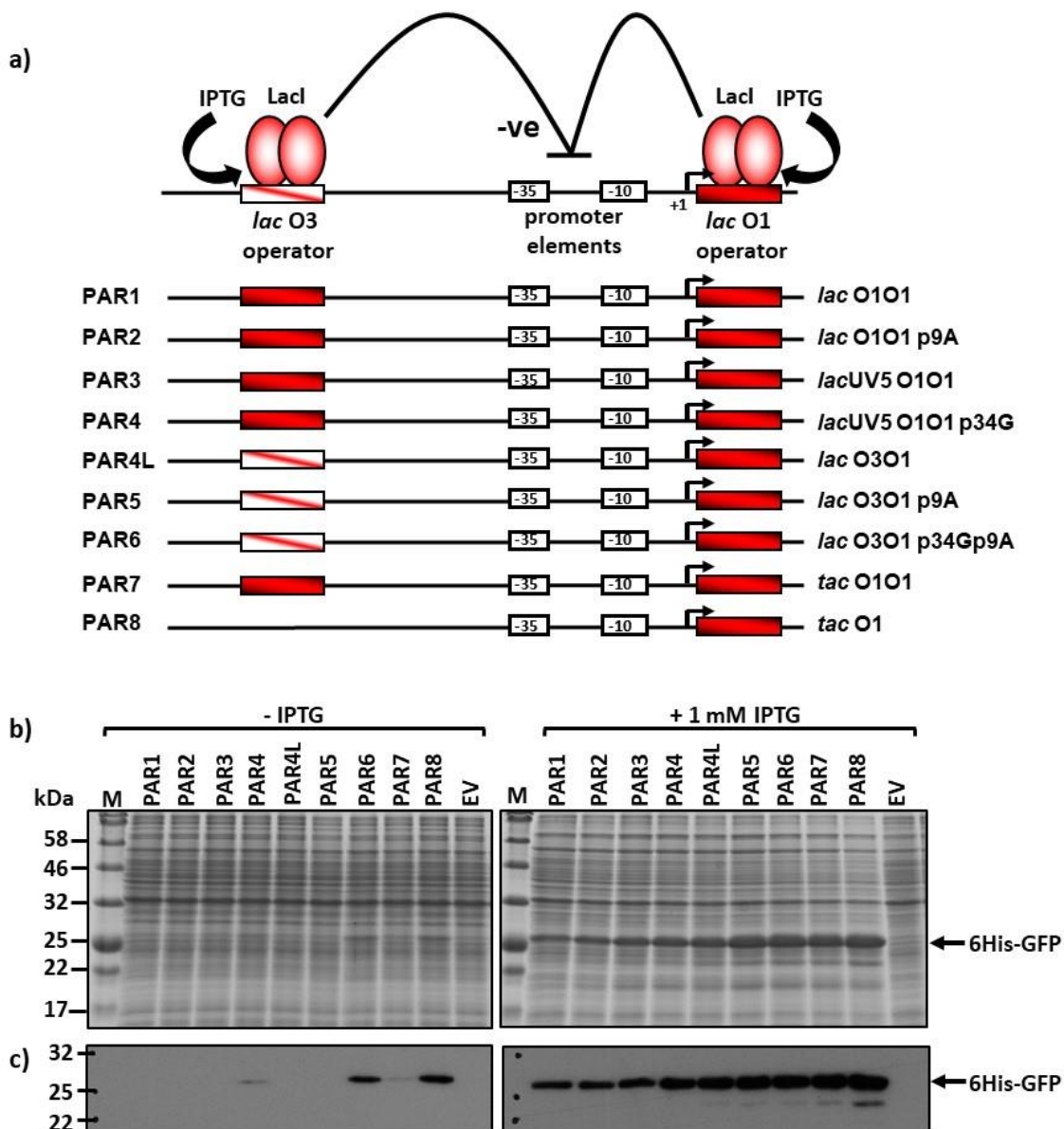
528 **Fig. 6.** Expression of the *N. meningitidis* BamA_{ENm} chimera protein in *E. coli* BL21 cells. (a)
529 The panel shows growth of the *E. coli* BL21 cells carrying either pET22b PAR1 empty vector
530 or pET22b PAR1/ BamA_{ENm} in Lennox broth, supplemented with 0.2% glucose (G 0.2%),
531 where indicated. Expression of BamA_{ENm} was induced after three hours growth by the addition
532 of IPTG at either 20 μ M or 1 mM, where indicated. (b) Detection of BamA_{ENm} chimera
533 expression. The panel shows a Coomassie blue stained gel of normalised total cell protein
534 from the BL21 cells in panel (a), carrying either pET22b PAR1 empty vector or pET22b PAR1/
535 BamA_{ENm} after three hours induction. Culture numbering in panel (a) is the same for the
536 loading of samples in panel (b) (denoted as *). The gel was loaded as follows: lane 1, BL21
537 pET22b PAR1 empty vector (EV) grown in Lennox broth; lane 2, BL21 pET22b PAR1/
538 BamA_{ENm} grown in Lennox broth (uninduced); lane 3, pET22b PAR1/ BamA_{ENm} grown in
539 Lennox broth with 0.2% glucose (uninduced); lane 4, BL21 pET22b PAR1/ BamA_{ENm} grown in
540 Lennox broth and induced with 1 mM IPTG; lane 5, BL21 pET22b PAR1/ BamA_{ENm} grown in
541 Lennox broth and induced with 20 μ M IPTG; lane 6, BL21 pET22b PAR1/ BamA_{ENm} grown in
542 Lennox broth with 0.2% glucose and induced with 1 mM IPTG. (c) Detection of BamA_{ENm} in
543 membrane fractions from BL21 pET22b PAR1/ BamA_{ENm} cells. The panel shows a Coomassie
544 blue stained gel and Western blot of soluble (Sol) and membrane (Mem) fractions from the
545 BL21 cells in panel (a), carrying pET22b PAR1/ BamA_{ENm} grown in Lennox broth with 0.2%
546 glucose in the presence or absence of 1 mM IPTG after 3 hours. For the Coomassie blue

547 stained gel, 5 µg of soluble and 3 µg of membrane protein was loaded, and for the Western
548 blot 0.5 µg and 0.3 µg of protein were loaded, respectively. BamA_{ENm} was detected by probing
549 with anti-*E. coli* BamA POTRA antiserum and anti-rabbit HRP secondary antibody.

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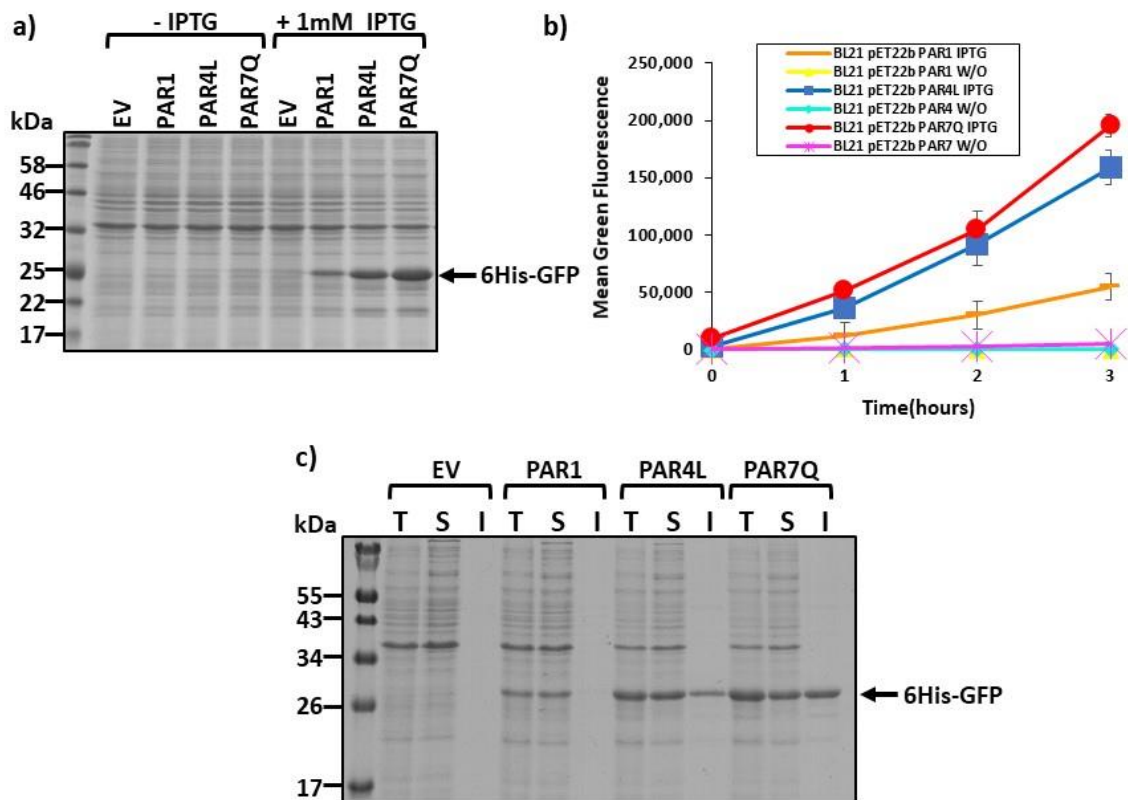
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Fig. 1. Hothersall *et al.* (2021)



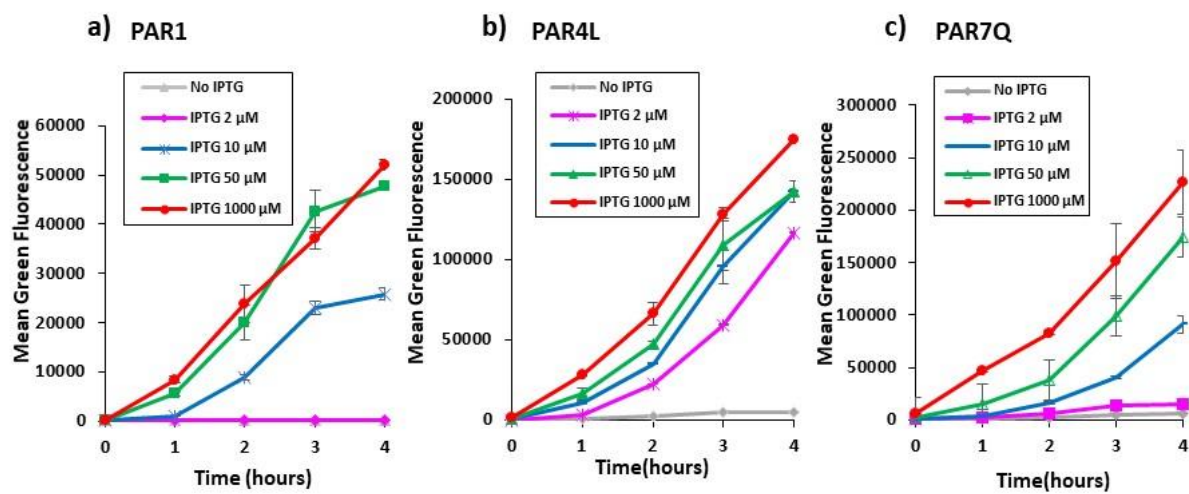
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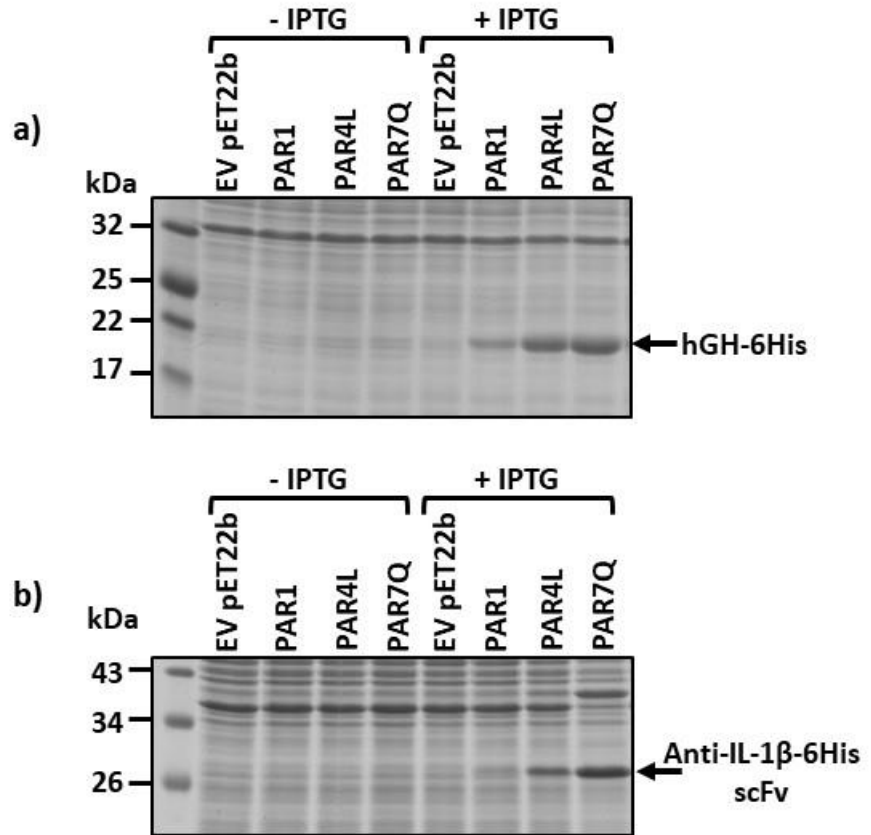
Fig. 3. Hothersall *et al.* (2021)



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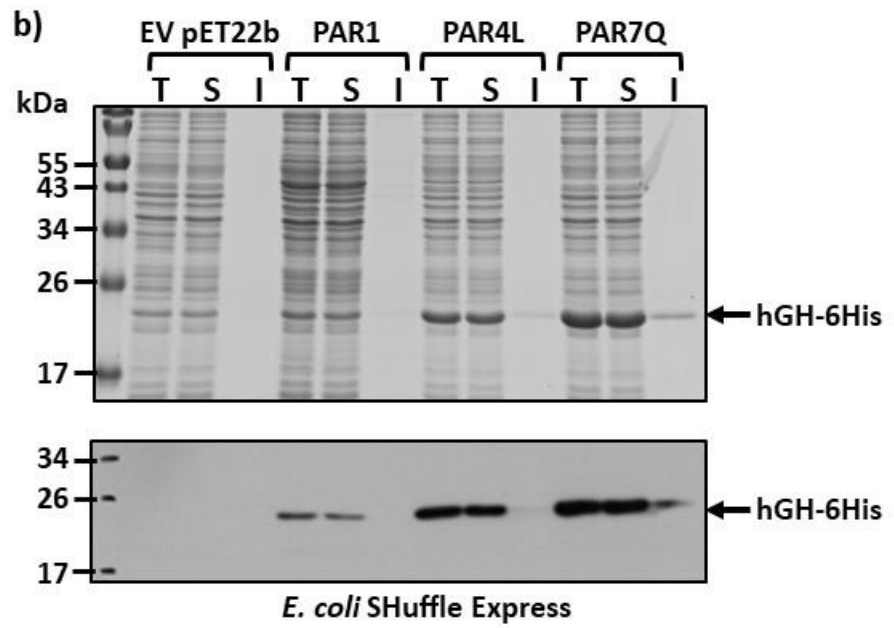
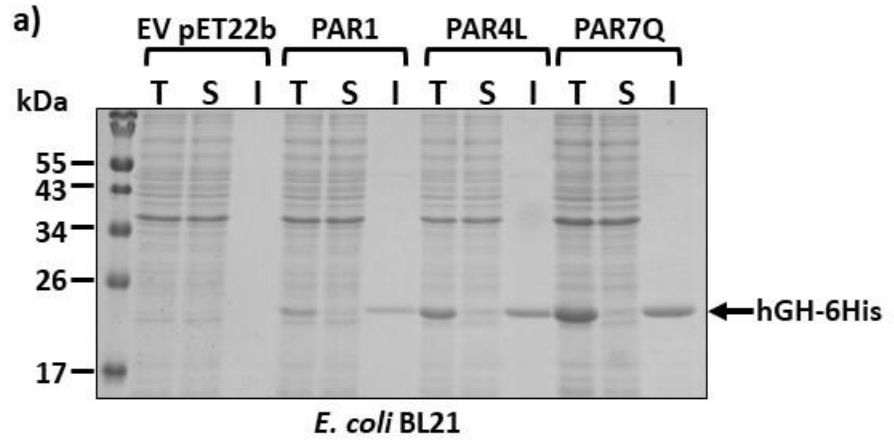
Fig. 4. Hothersall *et al.* (2021)



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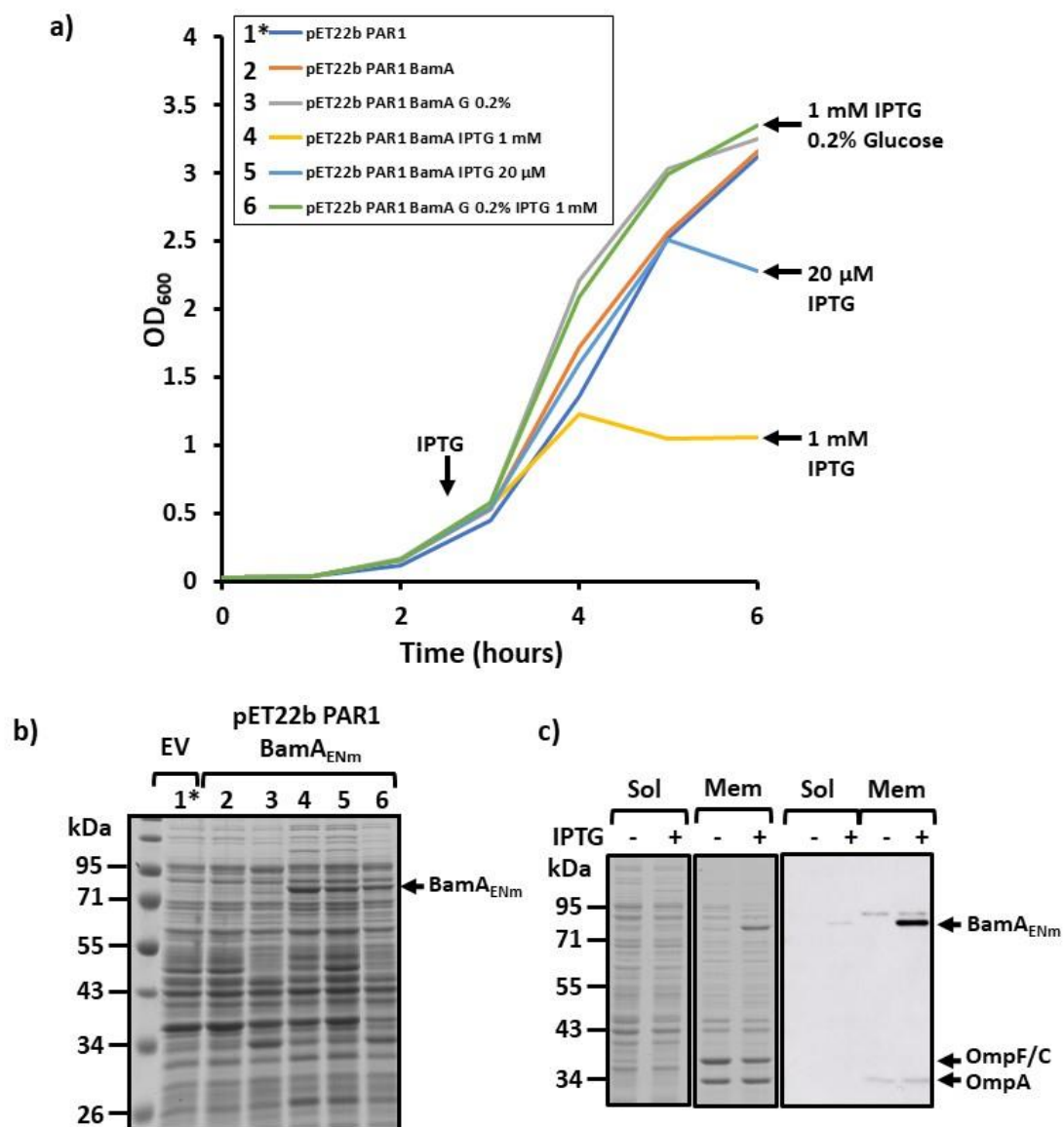
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Fig. 5. Hothersall *et al.* (2021)



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Fig. 6. Hothersall *et al.* (2021)



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The PAR promoter expression system: modified *lac* promoters for controlled recombinant protein production in *Escherichia coli*

Supplementary Material.

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592

593 **Supplementary Table S1.** Bacterial strains and plasmid used in this study.

Name	Details	Source
Strains		
BL21	<i>fhuA2 [lon] ompT gal [dcm] ΔhsdS</i>	Novagen
BL21(DE3)	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i> λ DE3 = λ <i>sBamHI</i> Δ <i>EcoRI-B</i> <i>int::(lacI::PlacUV5::T7 gene1) i21 Δnin5</i>	Novagen
JCB387	Δ <i>nir</i> , Δ <i>lac</i>	[1]
JWD3	<i>E. coli</i> K-12 BamA depletion strain	[2]
SHuffle Express	<i>F' lac, pro, lacI^q / Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC (Spec^R, lacI^q) ΔtrxB rpsL150(Str^R) Δgor Δ(malF) λ⁻, IN(rrnD-rrnE)1, rph-1</i>	NEB
W3110	<i>recA1, endA1, gyrA96, thi-1, hsdR17, supE44,</i>	[3]
XL1 Blue	<i>relA1, lac, [F proAB lacI^qΔM15 Tn10 (Tet^R)]</i>	Agilent
Plasmids		
pET15b	T7 RNA polymerase expression vector (Amp ^R , N-terminal His tag). Carries the <i>lacI</i> gene.	Novagen
pET22b	T7 RNA polymerase expression vector (Amp ^R , <i>pelB</i> signal sequence, C-terminal His tag). Carries the <i>lacI</i> gene.	Novagen
pET26b	T7 RNA polymerase expression vector (Kan ^R , <i>pelB</i> signal sequence, C-terminal His tag). Carries the <i>lacI</i> gene.	Novagen
pET15b/ 6his-gfp	pET15b expressing 6His N-terminal GFP fusion	Dr David Lee.
pYU49	pET23 based vector with <i>ptac</i> promoter expressing TorAsp anti-IL-1β-6His scFv.	[4]
pHAK1	pET23 based vector with <i>ptac</i> promoter expressing TorAsp hGH-6His	[5]
pRW50	<i>lacZ</i> transcription fusion plasmid (Tet ^R)	[6]
pRW50/ PAR1	pRW50 carrying the PAR1 promoter	[7]
pRW50/ PAR2	pRW50 carrying the PAR2 promoter	[7]
pRW50/ PAR3	pRW50 carrying the PAR3 promoter	[7]
pRW50/ PAR4	pRW50 carrying the PAR4 promoter	[7]
pRW50/ PAR4L	pRW50 carrying the PAR4L promoter	[7]
pRW50/ PAR5	pRW50 carrying the PAR5 promoter	[7]
pRW50/ PAR6	pRW50 carrying the PAR6 promoter	[7]
pRW50/ PAR7	pRW50 carrying the PAR7 promoter	[7]
pRW50/ PAR8	pRW50 carrying the PAR8 promoter	[7]

594

595

596 **Supplementary Table S2.** Primers used in this study

Primer	Sequence (5` to 3`)
ptac(BglII)	GGGGG <u>AGATCT</u> GATAATGTTTTTTGCGCCGACATCATAACGG
ptacO3(BglII)	GGGGG <u>AGATCT</u> GGCAGTGAGCGCAACGCAATTATCATAACGGTTCTGGC
placO1(BglII)	GGGGG <u>AGATCT</u> AATTGTGAGCGGATAACAATTAATGTGAGTTAGCTCACTC
placO3(BglII)	GGGGG <u>AGATCT</u> GGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTC
placRV(XbaI)	GGGGG <u>TCTAGA</u> CTGTTTCCTGTGTGAAATTGTTATCCG
lacIqF	GACACCATCGAATGG <u>T</u> GCAAAACCTTTGCGG
lacIqR	CGCGAAAGGTTTTGC <u>A</u> CCATTTCGATGGTGTC

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598

599 **Supplementary Figure Legends.**

600 **Supplementary Fig. S1.** The pET22b and pET26b multiple cloning site (MCS). The figure
601 shows the DNA sequence of the T7 promoter region and MCS of pET22b and pET26b
602 (Novagen). Note that the same region in each plasmid is identical. The location of the 6His
603 purification tag and the *peIB* leader sequence, which allows secretion of protein into the *E. coli*
604 periplasm, is highlighted. Restriction enzyme recognition sites are bold. The location of the T7
605 promoter, the *lac* operator, the ribosome binding site (RBS) and the T7 terminator primer
606 (Novagen) are also indicated. Amino acid sequence is also shown below the relevant DNA
607 sequence.

608

609 **Supplementary Fig. S2.** The DNA sequences of target proteins expressed in this study. The
610 figure shows the DNA sequences of (a) *6his-gfp* (b) *hgh-6his* (c) *IL-1 β -6his scFv* and (d)
611 *bamA_{ENm}* used in this study. Restriction enzyme recognition sites (XbaI, NdeI, BamHI, SacI
612 and XhoI) used to clone each fragment into vectors are shown bold and underlined. For each
613 construct the translation initiation codon (AUG) is green, the DNA encoding the 6His tag is
614 purple and the translation stop codon (TAA) is red. Note the *bamA_{ENm}* construct used in this
615 work encodes the BamA_{ENm} chimera protein, which is a fusion of the *E. coli* BamA N-terminal
616 domain and BamA C-terminal β -barrel domain from *N. meningitidis* [8].

617

618 **Supplementary Fig. S3.** The amino acid sequences of target proteins expressed in this study.
619 The figure shows the amino acid sequences of the (a) 6His-GFP (b) hGH-6His (c) anti-IL-1 β -
620 6His scFv and (d) BamA_{ENm} proteins used in this study. For each protein the 6His tag is purple
621 and predicted molecular weight (Mw) of each protein is given. Note the BamA_{ENm} protein
622 chimera is a fusion of the *E. coli* BamA N-terminal domain and BamA C-terminal β -barrel
623 domain from *N. meningitidis* [8].

624

625 **Supplementary Fig. S4.** Expression of 6His-GFP using the PAR promoter system in the
626 pET26b vector backbone. The figure shows a Coomassie blue stained SDS-PAGE gel
627 analysing the expression of 6His-GFP from the three promoter PAR system (PAR1, PAR4L
628 and PAR7Q) cloned into the pET26b vector system in *E. coli* BL21 cells. Cells were grown in
629 LB medium at 37°C and RPP was induced for three hours by the addition of 1 mM IPTG,
630 where appropriate. An empty vector control (EV) was included.

631

632 **Supplementary Fig. S5.** Expression of 6His-GFP using the PAR promoter system in *E. coli*
633 K-12 strain W3110. The figure shows a Coomassie blue stained SDS-PAGE gel analysing the
634 expression of 6His-GFP from the three promoter PAR system (PAR1, PAR4L and PAR7Q)
635 cloned into the pET22b vector in *E. coli* K-12 strain W3110. Cells were grown in LB medium

636 at 37°C and RPP was induced for 3 hours by the addition of 1 mM IPTG, where appropriate.
637 An empty vector control (EV) was included.

638

639 **Supplementary Fig. S6.** Expression of 6His-GFP using the PAR promoter system in auto-
640 induction medium at different growth temperatures. The figure shows Coomassie blue stained
641 SDS-PAGE gels analysing the expression of 6His-GFP in *E. coli* BL21 cells, using the three
642 promoter PAR system (PAR1, PAR4L and PAR7Q) when cloned into pET22b. Cells were
643 grown in auto-induction medium [9] at either (a) 37°C or (b) 30°C and samples were taken 3,
644 6 and 23 hours after sub-culturing (O/N overnight).

645

646 **Supplementary Fig. S7.** Homogeneous green fluorescence intensity of cells expressing
647 6His-GFP from the PAR promoters. Flow cytometry analysis of green fluorescence from BL21
648 pET22b PAR/ 6His-GFP constructs (a) PAR1, (b) PAR4L and (c) PAR7Q grown in LB medium
649 with 2 µM to 1000 µM IPTG induction for four hours. Data are plotted as histograms showing
650 number of cells with different green fluorescence (FL1-A) values.

651

652 **Supplementary Fig. S8.** Glucose represses expression from the PAR1 promoter. The figure
653 shows the mean green fluorescence intensities of *E. coli* BL21 cells, carrying pET22b
654 PAR1/ *6his-gfp*, for the first three hours after IPTG induction, in the presence and absence of
655 0.5% glucose. Dotted lines correspond to expression levels in cells grown in the absence of
656 glucose, whilst the solid ones represent cells grown in its presence. 6His-GFP expression was
657 induced by the inclusion of IPTG at 2, 10, 50, 100 and 1000 µM. Data are shown as
658 mean green fluorescence values from replica flasks, error bars are ± the standard deviation.

659

660 **Supplementary Fig. S9.** Rescue of BamA depletion by the expression of the *N. meningitidis*
661 BamA_{ENm} chimera protein. (a) The panel shows growth of the *E. coli* BamA depletion strain,
662 JWD3, on LB agar plates in the presence of arabinose (+Ara), the absence of arabinose (-Ara)
663 or the presence of only 1 mM IPTG (+IPTG). Cells carried either pET22b PAR1 empty vector
664 or pET22b PAR1/ BamA_{ENm}. Note that in JWD3 cells the chromosomally-encoded *E. coli*
665 BamA is only produced in the presence of arabinose, whilst in its absence, BamA expression
666 is prevented, resulting in cell death [2]. Depletion can be rescued by providing a functional
667 copy of *bamA* [8, 10], such as that carried by pET22b PAR1/ BamA_{ENm}. (b) The panel shows
668 the growth of JWD3 cells, carrying either pET22b PAR1 empty vector or pET22b PAR1/
669 BamA_{ENm}, in Lennox broth medium, supplemented with 0.2% glucose, in the absence of
670 arabinose, the presence of arabinose (Ara) or the presence of 1 mM IPTG (IPTG). (c)
671 Detection of BamA_{ENm} chimera expression. The panel shows a Coomassie blue stained gel

672 and Western blot of normalised total cell protein from the JWD3 cells carrying either pET22b
673 PAR1 empty vector or pET22b PAR1 BamA_{ENm}, after 480 minutes of growth in Lennox broth
674 with 0.2% glucose supplemented with 0.05% arabinose or 1 mM IPTG, where indicated. Blots
675 were probed with anti-*E. coli* BamA POTRA antiserum and anti-rabbit HRP secondary
676 antibody to detect BamA_{ENm}.

677

678 **Supplementary Fig. S10.** Comparison of 6His-GFP production using standard T7 RNA
679 polymerase-driven expression and the PAR7Q promoter construct. The figure shows a
680 Coomassie blue stained SDS-PAGE gel analysing the expression of 6His-GFP in *E. coli*
681 BL21(DE3) cells, using pET15b/ *6his-gfp*, and in *E. coli* BL21, using pET22b PAR7Q/ *6his-*
682 *gfp*. Cells were grown in LB medium at 37°C with shaking and recombinant protein production
683 was induced for three hours by the addition of 1 mM IPTG, where appropriate.

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722

723

724 **Supplementary Fig. S1.**

725

726

727 **BglIII** **T7 promoter** **lac operator**
 728 AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAA

729

730 **XbaI** **RBS** **NdeI**
 731 TTCCCCTCTAGAAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAATACCTG
 732 M K Y L

733

734 **pelB leader** **NcoI**
 735 CTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCCATGGAT
 736 L P T A A A G L L L L A A Q P A M A M D

737

738
 739 **BamHI** **EcoRI** **SacI** **SalI** **HindIII** **NotI** **XhoI**
 740 ATCGGAATTAATTCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG
 741 I G I N S D P N S S S V D K L A A A L E

742

743 **6His tag**
 744 CACCACCACCACCACCACTGAATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG
 745 H H H H H H **End**

746

747 GCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAACGGGTCTTG
 748 ← **T7 Terminator Primer**

749

750 **Supplementary Fig. S2.**

751 **(a) *6his-gfp***

752 TCTAGAAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATG GGCAGCAGCCATCATCATCATCACAGCA
753 GCGGCCTGGTGCCGCGCGGCAGCCATATGGTGAGCAAGGGCGAGGAGCTGTTACCCGGGGTGGTGCCCATCCTGG
754 TCGAGCTGGACGGCGACGTAAACGGCCACAAGTTTACGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA
755 AGCTGACCCCTGAAGTTTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTGACCACCCCTGACCT
756 ACGGCGTGCAGTGCTTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAG
757 GCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGG
758 GCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC
759 TGGAGTACAACACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAGTTCA
760 AGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCCTACCAGCAGAACACCCCATCGGCGACG
761 GCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCG
762 ATCACATGGTCTGCTGGAGTTCTGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGGAT
763 cc

764

765 **(b) *hgh-6his***

766 CATATGTTCCCAACCATTCCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTG
767 GCCTTTGACACCTACCAGGAGTTTGAAGAAGCCTATATCCCAAAGGAACAGAAGTATTCATTCCAGCAACCC
768 CAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCCTCCAACAGGGAGGAAACACAACAGAAATCCAACCTA
769 GAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCCTCAGGAGTGTCTTCGCC
770 AACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAGGCATCCAACG
771 CTGATGGGGAGGCTGGAAGATGGCAGCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACA
772 AACTCACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGTC
773 GAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCATCATCATCATCATCACTAA
774 TAAAGGATCCGAATTCGAGCTC

775

776 **(c) *IL-1β-6his scFv***

777 CATATGGATATCCAGATGACGCAGAGCCCGAGCAGCCTGAGCGCCAGCGTGGGTGACCGTGTGACCATTACCTGT
778 CGTACCAGCGGCAACATTATAACTATCTGACCTGGTACCAGCAGAAACCGGGCAAAGCGCCGAGCTGCTGATT
779 TATAATGCAAAAACCCCTGGCAGATGGTGTGCCGAGCCGCTTTAGCGGCAGCGGTAGCGGTACCCAGTTACCCCTG
780 ACGATCAGCAGCCTGCAGCCGGAAGACTTTGCCAACTATTACTGCCAGCACTTCTGGAGCCTGCCGTTTACCTTC
781 GGTACAGGGCACGAAAGTGGAAATTAACGTAACCGCGGTGGCGGTAGCGGCGGTGGCGGTAGCGGCGGTGGCGGT
782 AGCGGCGGTGGCGGTAGCGAGGTGCAGCTGGTTGAAAGCGGCGGTGGCCTGGTTACCGGGTGGCAGCCTGCGT
783 CTGAGCTGTGCGGCCAGCGGCTTTGATTTACGCCGTTATGACATGAGCTGGGTGCGTCAGGCACCGGGTAAACGT
784 CTGGAATGGGTTGCCTATATTAGCAGCGGTGGCGGTAGCACCTACTTCCGGATACGGTGAAAGGCCGCTTACC
785 ATCAGCCGTGACAACGCAAAAAATACGCTGTACCTGCAGATGAACAGCCTGCGCGCCGAAGATAACCGCAGTTTAT
786 TACTGCGCCCGTCAGAATAAAAACTGACGTGGTTTCGACTACTGGGGTTCAGGGCACGCTGGTTACGGTTAGCAGC
787 CATCATCATCATCACTAATAAAGGATCCGAATTCGAGCTC

788

789 **(d) *bamA_{ENm}***

790 CATATGGCGATGAAAAAAGTCTGATCGCGTCTCTGCTGTTCTCTTCTGCGACCGTTTACGGTGCTAGCGAAGGT
791 TTCGTTGTTAAAGACATCCACTTTGAAGGTCTGCAACGTGTTGCGGTTGGTGCGGGCGTGTGTCTATGCCGGTT
792 CGTACCGGCGACACCGTGAACGACGAAGACATCTCTAACACCATCCGTGCGCTGTTTCGCGACCGGCAACTTTGAA
793 GACGTTTCGTGTTCTGCGTGACGGTGCACCCCTGCTGGTTTCAAGTTAAAGAAGCTCCGACCATCGCGTCTATCACC
794 TTCTCTGGTAACAAATCTGTTAAAGACGACATGCTGAAACAGAACCTGGAAGCGTCTGGTGTTCGTGTTGGTGAA
795 TCTCTGGACCGTACCACCATCGCGGACATCGAAAAAGGTCTGGAAGACTTCTACTACTCTGTTGGTAAATACTCT
796 GCGTCTGTTAAAGCGGTTGTTACCCCGCTGCCGCGTAACCGTGTGACCTGAAACTGGTTTTCCAGGAAGGTGTT
797 TCTGCGGAAATCCAGCAGATCAACATCGTTGGTAACCACGCTTTCACCACCGACGAACGATCTCTCACTTCCAA
798 CTGCGTGACGAAGTTCCGTGGTGAACGTGGTTGGTGACCGTAAATACCAGAAACAGAAACTGGCGGGTGACCTG

799 GAAACCCTGCGTTCTTACTACCTGGACCGTGGTTACGCGGTTTCAACATCGACTCTACCCAGGTTTCTCTGACC
800 CCGGACAAAAAAGGTATCTACGTTACCGTGAACATCACCGAAGGTGACCAGTACAAACTGTCTGGTGTGAAGTT
801 TCTGGTAACCTGGCGGGTCACTCTGCGGAAATCGAACAACTGACCAAAATCGAACCGGTGAACGTATAACGGC
802 ACCAAAGTTACCAAAATGGAAGACGACATCAAAAACTGCTGGGTGCTTACGGTTACGCTTACCCGCGTGTTCAG
803 TCTATGCCGGAAATCAACGACGCGGACAAAACCGTTAAACTGCGTGTGAACGTGGACGCGGGTAACCGTTTCTAC
804 GTTCGTAAAAATCCGTTTTGAAGGTAACGACACCTCTAAAAGACGCGGTTCTGCGTCGTGAAATGCGTCAGATGGAA
805 GGTGCGTGGCTGGGTTCTGACCTGGTTGACCAGGGTAAAGAACGTCTGAACCGTCTGGGTTTCTTTGAAACCGTT
806 GACACCGACACCCAGCGTGTTCGGGTTCCCCGGACCAGGTTGACGTTGTTTACAAAGTTAAAGAACGTAACACC
807 GGATCCCTGGACCTGTCTGCGGGTTGGGTTACAGGACACCGGCCTGGTTATGTCTGCGGGTGTTCCTCAGGACAAC
808 CTGTTCCGGCACCGGCAAATCTGCGGCGCTGCGTGCCTCTGTTCTAAAACCACCTGAACGGTTCTCTGTCTTTC
809 ACCGACCCGTACTTCACCGCTGACGGTGTTCCTCTGGGTTACGACGTTTACGGTAAAGCGTTTCGACCCGCGTAAA
810 GCGTCTACCTCTATCAAACAGTACAAAACCACCACCGCTGGTGCGGGTATCCGTATGTCTGTTCCGGTTACCGAA
811 TACGACCGTGTGAACTTCGGTCTGGTTGCGGAACACCTGACCGTGAACACCTACAACAAAGCGCCGAAACACTAC
812 GCGGACTTCATCAAAAAATACGGTAAAACCAGCGCACCGACGGTTCTTTCAAAGGTTGGCTGTATAAAGGCACC
813 GTTGGTTGGGGTCGTAACAAAACCGACTCTGCGCTGTGGCCGACCCGTGGTTACCTGACCGGCGTGAACGCGGAA
814 ATCGCGCTGCCGGGTTCTAAACTGCAATACTACTCTGCGACCCACAACCAGACCTGGTTCTTCCCGCTGTCTAAA
815 ACCTTCACCCTGATGCTGGGTGGTGAAGTTGGTATCGCGGGTGGTTACGGTCGTACCAAAGAAATCCCGTTCTTT
816 GAAAACCTTCTACGGTGGTGGTCTGGGTTCTGTTGTTGTTACGAATCTGGCACCTGGGTCCGAAAGTTTACGAC
817 GAATACGGTGAAAAAATCTCTTACGGTGGTAACAAAAAAGCGAACGTGTCTGCGGAACTGCTGTTCCCGATGCCG
818 GGTGCGAAAGACGCGCGTACCGTTTCGTCTGTCTCTGTTTCGCGGACGCGGGTTCTGTTTGGGACGGTAAAACCTAC
819 GACGACAACCTCTTCTTCTGCGACCGGCGGTGCTGTTCAGAACATCTACGGTGCGGGTAACACCCACAAATCTACC
820 TTCACCAACGAACTGCGTTACTCTGCGGGTGGTGCGGTTACCTGGCTGTCTCCGCTGGGGCCCATGAAATTTCTCT
821 TACGCTTACCCGCTGAAAAAAAACCGGAAGACGAAATCCAGCGTTTCCAGTTCCAACCTGGGCACCACCTTCTAA
822 TGAGGGCCCATGAAGTTTAGCTATGCCTATCCATTAAGAAGAAGCCAGAGGATGAGATTCAAAGATTTCAATTT
823 CAATTAGGTACTACTTTTGGCGGCAGATCTCTCGAG
824
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827

828 **Supplementary Fig. S3.**

829 **(a) 6His-GFP Mw 29105 Da**

830 MGSSHHHHHSSGLVPRGSHMVSKEELFTGVVPILEVELDGDVNGHKFSVSSEGEEDATYGKLTCLKFI
831 CTTGKLPVWPPTLVTTLLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
832 GDTLVNRIELKGIIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQ
833 QNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDMVLLLEFVTAAGITLGMDELYK
834

835 **(b) hGH-6His Mw 23083 Da**

836 MFPTIPLSRLFDNAMLRHRLHQLAFDITYQEFEEAYIPKEQKYSFLQNPQTSCLCFSESIPTPSNREET
837 QQKSNLELLRISLLLIQSWLEPVQFLRSVFNLSVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSPRT
838 QQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMKVETFLRIVQCRSVEGSCGFHHHHHH
839

840 **(c) Ant-IL-1 β -6His scFv Mw 27495 Da**

841 MDIQMTQSPSSLSASVGDRTTITCRTSGNIHNYLTWYQQKPGKAPQLLIYNAKTLADGVPSRFSGSGS
842 GTQFTLTISLQPEDFANYCQHFWSLPFTFGQGTKVEIKRTGGGGSGGGGSGGGGSGGGGSEVQLVE
843 SGGGLVQPGGSLRLS CAASGFDFSRYDMSWVRQAPGKRLEWVAYISSGGGTYFPDTPVKGRFTISRDN
844 AKNTLYLQMNLSRAEDTAVYYCARQNKKLTWFDYWGQTLVTVSSHHHHHH
845

846 **(d) BamA_{ENm} Mw 87992 Da**

847 MAMKLLIASLLFSSATVYGASEGFVVKDIHFEGQLQRVAVGAALLSMPVRTGDTVNDEDISNTIRALF
848 ATGNFEDVRVLRDGD TLLVQVKERPTIASITFSGNKS VKDDMLKQNL EASGVRVGESLDRTTIADIEK
849 GLEDFYYSVGKYSASVKAVVTP LPRNRVDLKL VFOEGVSAEIQQINIVGNHAF TTDELISHFQLRDEV
850 PWWNVVGD RKYQKQKLAGDLET LRSYYLDRGYARFNIDSTQVSLTPDKKGIYVTVNITEGDQYKLSGV
851 EVSGNLAGHSAEIEQLTKIEPGELYNGTKVTKMEDDIKLLGRYGYAYPRVQSMPEINDADKTVKLRV
852 NVDAGNRFYVRKIRFE GNDTSKDAVLRREMRQMEGAWLGS DLVDQGERLNRLGFFETVDTDTQRVPG
853 SPDQVDVYKVKERNTGSLDLSAGWVQDTGLVMSAGVSQDNLFGTGKSAALRASRSKTTLNGLSFTD
854 PYFTADGVSLGYDVYKAFDPRKASTSIKQYKTTTAGAGIRMSVPVTEYDRVNFGLVAEHLTVNTYNK
855 APKHYADFIKKYKGT DGTGDSFKGWLYKGTVGWGRNKTD SALWPTRGYLTGVNAEIALPGSKLQYYSA
856 THNQTWFFPLSKTFTLMLGGEVGIAGGYGRTKEIPFFENFYGGGLG SVRGYESGTLGPKVYDEYGEKI
857 SYGGNKKANVSAELLFPMPGAKDARTVRLSLFADAGSVWDGKTYDDNSSSATGGRVQNIYGAGNTHKS
858 TFTNELRYSAGGAVTWLSPLGPMKFSYAYPLKKKPEDEIQRFQFLGTTF
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861 **Supplementary Fig. S4.**

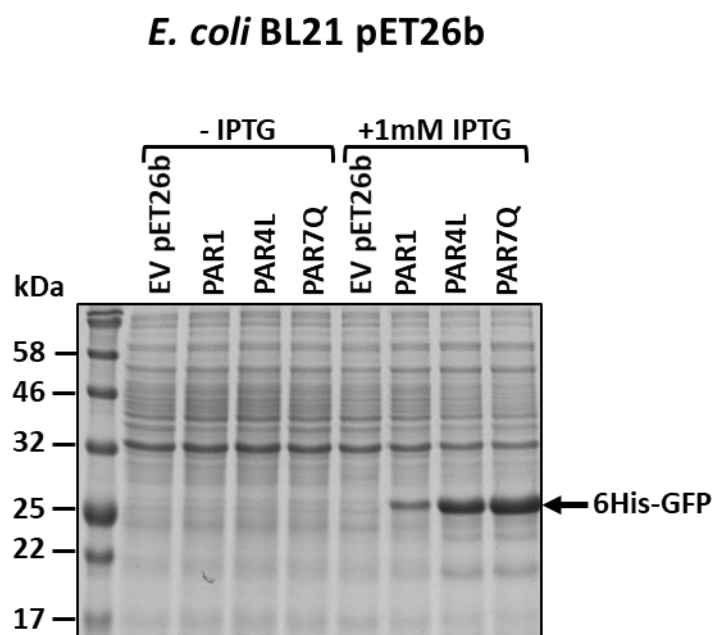
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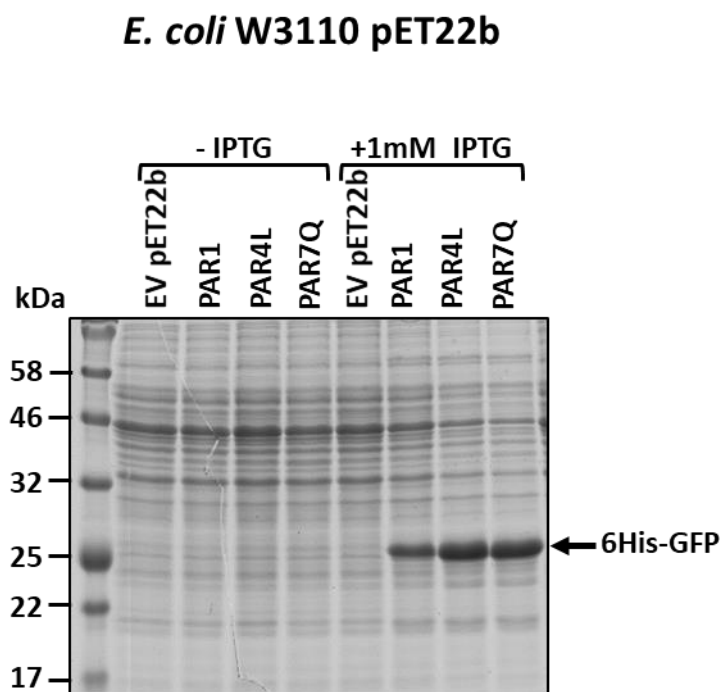
868 **Supplementary Fig. S5.**

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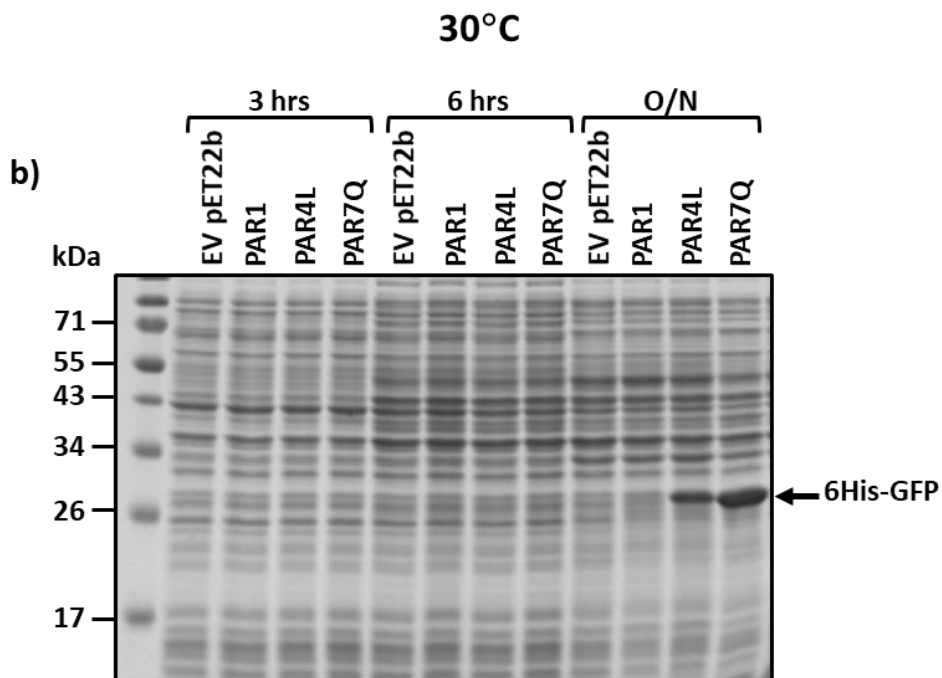
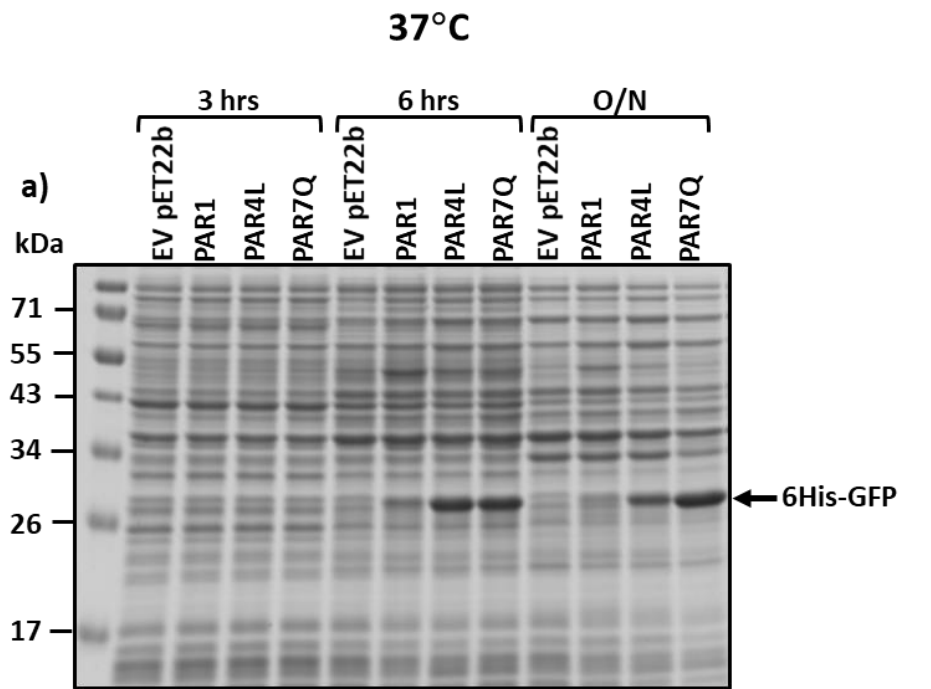
873 **Supplementary Fig. S6.**

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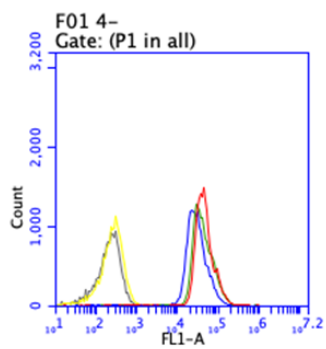


878 **Supplementary Fig. S7.**

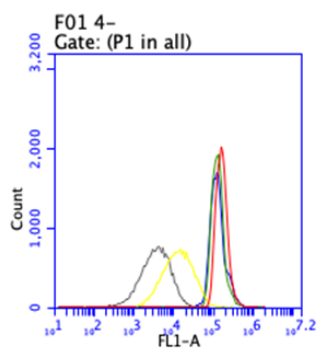
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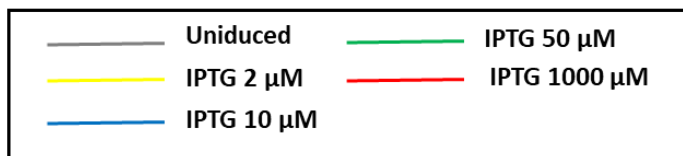
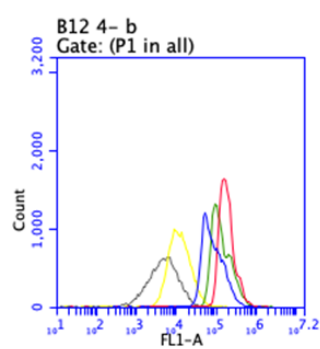
a) PAR1



b) PAR4L



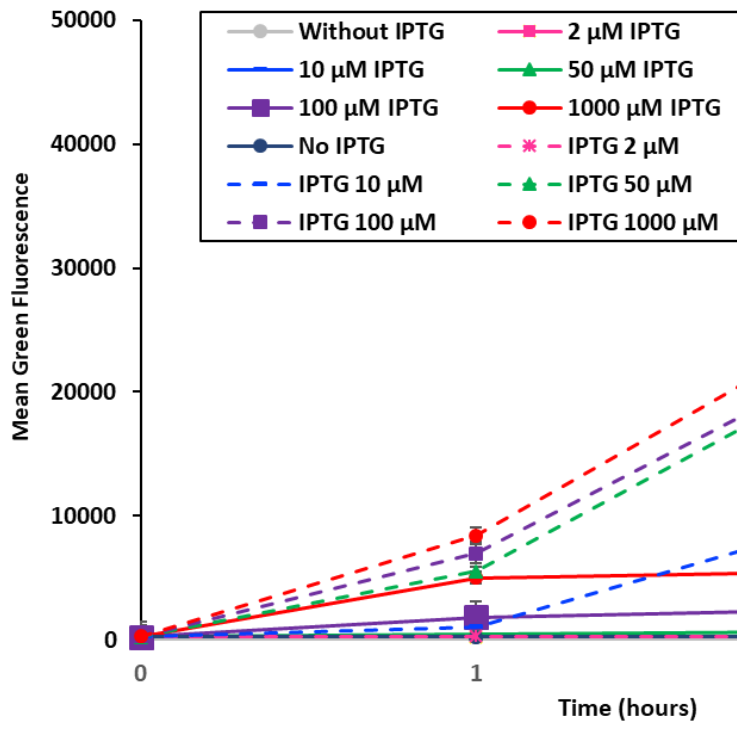
c) PAR7Q



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882 **Supplementary Fig. S8.**

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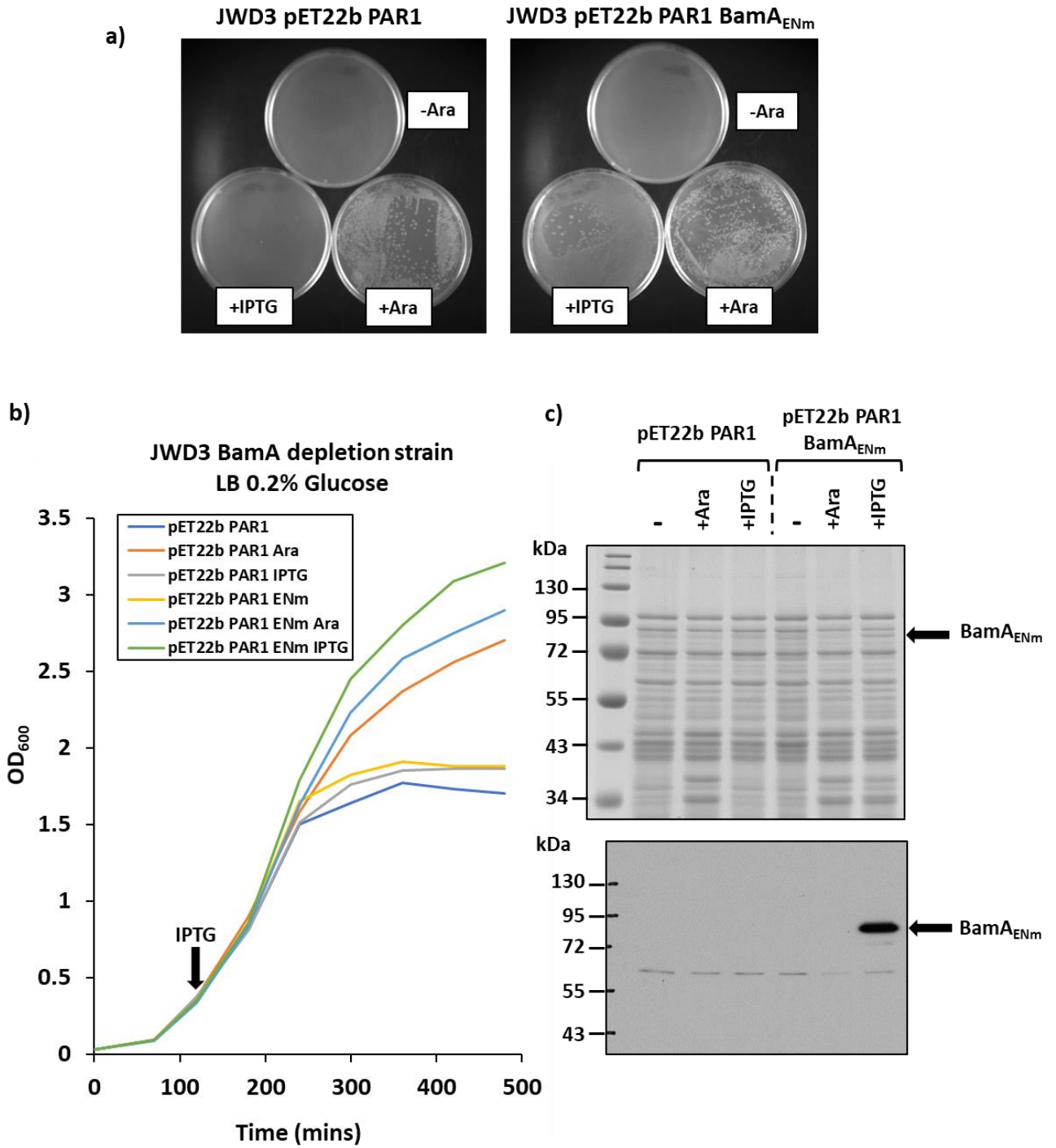
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886 **Supplementary Fig. S9.**

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890 **Supplementary Fig. S10.**

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