

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

Hothersall, Joanne; Godfrey, Rita; Fanitsios, Christos; Overton, Tim; Busby, Steve; Browning, Doug

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

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12 Joanne Hothersall ^{1*}, Rita E. Godfrey ¹, Christos Fanitsios ^{2,3}, Tim W. Overton ²,
13 Stephen J. W. Busby ^{1*}, and Douglas F. Browning ^{1*}

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16

17 ¹ Institute of Microbiology and Infection and School of Biosciences, University of Birmingham,
18 Edgbaston, Birmingham, B15 2TT, UK.

19 ² School of Chemical Engineering and Institute of Microbiology and Infection, University of
20 Birmingham, Birmingham, B15 2TT, UK.

21 ³ Present address: Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

22

23 *To whom correspondence should be addressed:

24 Email: j.hothersall@bham.ac.uk Tel: +44 (0)121 414 5434

25 Email: s.j.w.busby@bham.ac.uk Tel: +44 (0)121 414 5439

26 Email: d.f.browning@bham.ac.uk Tel: +44 (0)121 414 5434

27

28 **Highlights.**

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

34

35

36 **ABSTRACT**

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

52

53

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

58

59

60 **Keywords:** Recombinant protein production, *Escherichia coli*, transcription regulation, *lac*
61 promoter, membrane proteins

62

63

64 Introduction

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

84
85 Ideally, RPP expression systems should be as versatile as possible, being tightly
86 regulated, allowing high- and low-level target expression, and be compatible with many
87 different *E. coli* strains and “helper” plasmids, which can carry various tRNAs or chaperones
88 [1, 10]. Above all, expression systems should be easy to use, with vectors carrying multiple
89 cloning sites (MCS), purification and secretion tags, and, potentially, different antibiotic
90 resistance cassettes. Previously we generated a suite of promoters, which were based on the
91 *lac* and *tac* promoters, and assigned them promoter activity ratings (PAR values) based on
92 their strengths [3, 4, 5, 12]. These PAR promoters (PAR1 to PAR8) show a wide range of
93 promoter activities, stretching from low-level (i.e. PAR1) to high-level (i.e. PAR8) expression
94 capabilities [5]. Each PAR promoter also carried two *lac* operator sequences and were,
95 therefore, repressed by the *lac* operon repressor (LacI) and IPTG-inducible (Fig. 1a). As some
96 of these promoters appeared to be promising for RPP, we have transferred them to easy-to-
97 use vector backbones to generate the PAR promoter expression system. By picking three
98 promoters, which have low, intermediate and high expression capabilities, when fully induced
99 by IPTG, this system is able to express recombinant proteins at three different levels in *E. coli*,
100 allowing the expression level of a target protein to be easily tailored to maximize the production

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

104

105

106 **Materials and methods**

107 *Bacterial strains, plasmids, and materials*

108 *E. coli* strains, XL1 Blue and JCB387, were used for plasmid construction and DNA
109 manipulation, whilst *E. coli* BL21, BL21(DE3), W3110 and SHuffle Express and were used for
110 recombinant protein over-expression (Supplementary Table S1). Strains were grown in LB
111 broth (Sigma), Lennox broth (2% (w/v) peptone (Oxoid), 1% (w/v) yeast extract (Oxoid) and
112 170 mM NaCl) [13] and auto-induction medium [14], with appropriate antibiotic selection
113 (ampicillin 100 µg/ml, kanamycin 50 µg/ml). For RPP, *E. coli* BL21, BL21(DE3) and W3110
114 were routinely grown at 37°C, whilst SHuffle Express was grown at 30°C.

115

116 *Expression vector construction*

117 The PAR promoters, PAR1 to PAR8 (including PAR4L, formerly *lacO3O1*) have been
118 described previously (Browning *et al.*, 2019). Each PAR promoter was amplified from plasmids
119 pRW50/ PAR1 to PAR8 (Supplementary Table S1), using PCR with primers detailed in
120 Supplementary Table S2. Purified PCR products were restricted with BglII and XbaI and
121 cloned into the pET22b and pET26b expression vectors (Novagen), replacing the canonical
122 T7 RNA polymerase promoter (Supplementary Fig. S1). The DNA encoding 6His-GFP, from
123 pET15b/ *6his-gfp*, was cloned into each pET22b and pET26b PAR construct using XbaI and
124 BamHI restriction sites (Supplementary Table S1; Supplementary Figs. S2 and S3). The DNA
125 encoding hGH-6His and anti-IL-1β-6His scFv, from pHAK1 and pYU49, respectively, was
126 cloned into each pET22b PAR construct using NdeI and SacI (Supplementary Figs. S2 and
127 S3) [15, 16]. The DNA encoding the BamA_{ENm} chimeric outer membrane protein was cloned
128 into pET22b PAR1, using NdeI and XhoI (Supplementary Figs. S2 and S3) [17]. The *lacI^q*
129 mutation, which changes a single base in the *lacI* promoter to increase its strength [18, 19],
130 was introduced into pET22b PAR7 and pET26b PAR7 derivatives using the Agilent
131 QuikChange site-directed mutagenesis kit and primers lacIqF/R (Supplementary Table 2). All
132 constructs were verified by Sanger DNA sequencing.

133

134 *Recombinant protein over-expression and detection*

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

138 of IPTG and samples were taken after three or four hours induction. For the expression of the
139 BamA_{ENm} chimera, BL21 cells were grown in 50 ml Lennox broth, supplemented with glucose,
140 where indicated. Total protein samples were routinely prepared by resuspending normalized
141 amounts of cells in 2X Laemmli loading buffer (Sigma), heating at 95°C for three minutes, and
142 centrifuging prior to loading. Normalized protein samples were resolved by reducing SDS-
143 PAGE and analyzed using Coomassie blue staining and Western blotting, as in our previous
144 work [20]. For Western blotting, 6His-GFP was detected using anti-GFP antiserum raised in
145 mouse (Sigma) and an anti-mouse-HRP secondary antibody (Sigma), hGH-6His was detected
146 using anti-hGH antiserum raised in rabbit [5] and an anti-rabbit-HRP (horseradish peroxidase)
147 secondary antibody (Amersham), and anti-IL-1 β -6His scFv was detected using anti-6His (C-
148 terminal)-HRP (Invitrogen). BamA_{ENm} was detected using anti-BamA antiserum [17] and an
149 anti-rabbit-HRP secondary antibody (Amersham). Blots were developed using Pierce ECL
150 Western blotting substrate and all gels and blots shown are representative. To access the
151 aggregation of product in inclusion bodies, total, soluble and insoluble protein samples were
152 also prepared using Agilent BugBuster, according to the manufacturer's instructions.

153

154 *Cellular fractionation and membrane isolation*

155 50 ml cultures of BL21 cells, carrying pET22b PAR1/ BamA_{ENm}, were grown in Lennox
156 broth, supplemented with 0.2% glucose, in the presence or absence of 1 mM IPTG for three
157 hours. Cells were isolated by centrifugation and pellets were washed with 10 mM Tris-HCl (pH
158 7.4) and resuspended in 20ml of 10 mM Tris-HCl (pH 7.4) containing 2 mM
159 phenylmethylsulfonyl fluoride (PMSF, protease inhibitor). Cell envelopes were disrupted by
160 continuous passage through an Emulsiflex C3 for 5 minutes, and unbroken cells and
161 particulate material was removed by centrifugation for 15 min at 6,000 x g and 4°C. The total
162 membrane fraction (inner and outer membranes) was then isolated by centrifuging the
163 supernatant for 1 hour at 48,000 x g at 4 °C and the soluble fraction, which contains
164 cytoplasmic and periplasmic proteins was retained [17, 20]. Membranes were washed once
165 and resuspended in 0.4 ml of 10 mM Tris-HCl (pH 7.4).

166

167 *Rescue of BamA depletion in E. coli*

168 To determine the ability of the BamA_{ENm} chimera to rescue BamA depletion on solid
169 media, the *E. coli* BamA depletion strain JWD3 (Supplementary Table S1) [21] was grown on
170 LB agar plates, supplemented by 0.2% (w/v) arabinose or 1 mM IPTG, where indicated. For
171 experiments in liquid media, JWD3 cells were grown in 50 ml of Lennox broth with 0.2%
172 glucose, with shaking at 37°C, in the presence or absence of 0.05% (w/v) arabinose. Optical
173 density was monitored and after 2 hours of growth (OD₆₀₀ = 0.3 - 0.4) BamA_{ENm} production was
174 induced by the addition of IPTG. The preparation of normalised total cellular protein samples,

175 after 6 hours of growth, was as in Browning *et al.* (2013). Note that in JWD3 cells
176 chromosomally encoded *E. coli* BamA is only produced in the presence of arabinose, whilst
177 in its absence, BamA expression is prevented, resulting in the cessation of growth and cell
178 death [21]. Depletion can be rescued by providing a functional copy of *bamA*, such as that
179 carried by pET22b PAR1/ BamA_{ENm} [17, 20].

180

181 *Flow cytometry.*

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

189

190

191 **Results and Discussion**

192 *Construction and RPP with the PAR promoters*

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

207 As we wished to develop vectors with a weak, intermediate and strong promoter, we
208 chose the PAR1, PAR4L and PAR7 constructs, respectively. As expression from the PAR7
209 construct was slightly leaky in the absence of inducer (Figs. 1b and 1c), the *lacI^q* mutation,
210 which increases the expression of LacI, was introduced [18, 19]. This new construct, referred
211 to as pET22b PAR7Q, showed minimal 6His-GFP expression in the absence of IPTG, as

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

216

217 Since RPP expression systems should be as flexible as possible, the PAR1, PAR4L and
218 PAR7Q promoters were also introduced into the pET26b expression vector, which carries an
219 alternative plasmid backbone and a kanamycin resistant cassette (Supplementary Table S1).
220 Expression studies, again using *6his-gfp*, demonstrated that graded levels of 6His-GFP
221 expression, as expected, were achieved (Supplementary Fig. S4). Furthermore, our three
222 plasmid PAR promoter system functioned well in the *E. coli* K-12 strain W3110
223 (Supplementary Fig. S5), and with auto-induction medium when cells were grown at different
224 temperatures (*i.e.* 30 and 37°C) for longer periods of time (*i.e.* 23 hrs) (Supplementary Fig.
225 S6) [14]. Thus, we conclude that the PAR promoter system is versatile and can be used to
226 express recombinant proteins to set levels, in different strains and under different growth
227 conditions.

228

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

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

245

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

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

249 inducer concentrations are used to try to decrease RPP and fine-tune expression levels [6].
250 Whilst this works for some expression systems, in other systems this leads to only a proportion
251 of the cells in a culture expressing recombinant protein, which has been termed as an all-or-
252 none phenomenon [22, 23, 24]. Therefore, we examined whether 6His-GFP expression from
253 our pET22b PAR1, PAR4L and PAR7Q constructs was tuneable. Once more, BL21 cells,
254 carrying each plasmid, were grown in LB and induced with different IPTG concentrations (*i.e.*
255 2, 10, 50 and 1000 μ M). The expression of 6His-GFP was then monitored using flow
256 cytometry. Results in Fig. 3 show that for all three promoters different levels of expression
257 could be set in a culture by using different IPTG concentrations. Furthermore, the analysis of
258 individual cells indicated that for each promoter and IPTG concentration tested, GFP induction
259 was homogenous within the bacterial cell population (Supplementary Fig. S7). This is
260 particularly evident for the PAR7Q construct, which produces discrete GFP-expressing
261 populations at many different IPTG concentrations, indicating that expression from this highly
262 active promoter can be effectively tuned by different IPTG concentrations.

263

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

265 To test the versatility of the PAR promoter expression system, we examined the
266 expression of two additional targets, human growth hormone (hGH) and a single chain variable
267 region antibody fragment against interleukin 1 β (anti-IL-1 β scFv). Thus, the DNA encoding
268 each protein, carrying a C-terminal 6His tag, was cloned into pET22b, carrying either the
269 PAR1, PAR4L or PAR7Q constructs (Supplementary Figs. S2 and S3) [15, 16]. The resulting
270 plasmids were then transferred to BL21 cells and RPP was induced by the addition of 1 mM
271 IPTG to mid-logarithmic growing cells. Results in Fig. 4 show that, as anticipated, graded
272 levels of expression were achieved for both hGH-6His and anti-IL-1 β -6His scFv, with the most
273 product produced by cells carrying the PAR7Q construct and the least for PAR1.

274

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

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

287

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

289 For some target proteins, very low levels of expression are required, especially when
290 the recombinant product is toxic. As our PAR1 promoter is based on the *lac* promoter, it is
291 subject to catabolite repression and can be inhibited by the inclusion of glucose in the growth
292 medium (Supplementary Fig. S8) [25]. Therefore, to test if the PAR1 promoter could be used
293 to express a toxic protein, we cloned the DNA encoding BamA_{ENm}, a large chimeric outer
294 membrane protein (OMP) from *Neisseria meningitidis*, into our pET22b PAR1 vector
295 (Supplementary Figs. S2 and S3) [17]. This 88 kDa membrane protein has potential as a
296 vaccine candidate against *N. meningitidis* [17, 26]. Results in Fig. 6a, show that
297 overexpression of this construct at 37°C, using the PAR1 promoter with 1 mM IPTG is toxic
298 and leads to the cessation of cell growth. When a lower IPTG concentration was used (*i.e.* 20
299 µM) cells reached a higher optical density but, growth was arrested before the end of the
300 experiment (Fig. 6a). In contrast, induction of BamA_{ENm} expression with 1 mM IPTG in the
301 presence of glucose did not influence bacterial growth (Fig. 6a) and resulted in lower
302 expression levels of BamA_{ENm} without toxicity (Fig. 6b; lane 6). Fractionation of cells into their
303 soluble (cytoplasmic and periplasmic proteins) and membrane components (inner and outer
304 membranes) confirmed that that BamA_{ENm} was located in the membrane fraction (Fig. 6c), as
305 expected for an integral outer membrane protein.

306

307 In *E. coli*, BamA is an essential protein that is responsible for inserting bacterial β-
308 barrel containing OMPs into the bacterial outer membrane [27]. Previously, we demonstrated
309 that very low-level expression of the *N. meningitidis* BamA_{ENm} chimera could function in *E.*
310 *coli*, rescuing the depletion of BamA in the *E. coli* K-12 strain JWD3, where BamA production
311 is absolutely dependent upon arabinose [17]. Results in Supplementary Fig. S9 demonstrate
312 that IPTG induced BamA_{ENm} expression from pET22b PAR1, in the presence of glucose, could
313 also rescue depletion of BamA in JWD3, indicating that under these expression conditions,
314 BamA_{ENm} was folded and functional. It is also of note that, in the absence of IPTG, BamA
315 depletion in JWD3 was not rescued and BamA_{ENm} expression was not detected
316 (Supplementary Fig. S9), indicating that the PAR1 promoter is tightly regulated and suitable
317 for the expression of toxic proteins.

318

319

320

321

322 **Conclusions**

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

336

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

351

352 For many experiments, we used high concentrations of IPTG (i.e. 1 mM) to ensure that
353 our expression systems are fully switched on. However, our results show that the level of RPP
354 driven by the PAR promoters can be modulated. Our systems are tuneable, with specific IPTG
355 concentrations producing different expression levels homogeneously within a culture, rather
356 than an all-or-none phenotype that has been observed before (Fig. 3 and Supplementary Fig.
357 S7) [22, 23, 24]. Also, the use of glucose-mediated repression with the PAR1 promoter
358 enabled the expression of the toxic BamA_{ENm} chimera from *N. meningitidis* (Fig. 6). It is of note

359 that BamA_{ENm} is a large outer membrane protein that must traverse the *E. coli* inner membrane
360 and periplasmic space to be inserted into the outer membrane [27]. Overloading of the cellular
361 machinery responsible for these events (*i.e.* the Sec translocase, the periplasmic chaperones,
362 and the nascent Bam complex) will likely result in toxicity and cell death [17, 27]. Thus, due to
363 the tight repression of PAR1 in the absence of inducer and the ability to modulate expression
364 by glucose (Fig. 6; Supplementary Figs. S8 and S9), the PAR1 promoter is ideal for low-level
365 expression of toxic proteins. Finally, all three promoters worked well with auto-induction
366 medium (Supplementary Fig. S6), indicating that inducer exclusion (*i.e.* the ability to prevent
367 lactose uptake when glucose is present in the growth medium) is a feasible way to control and
368 delay RPP induction with the PAR system [14].

369

370 Since its discovery, the *lac* operon promoter and its derivatives have been extensively
371 used in biotechnology [3, 4, 8, 28]. In this work, we have further adapted the *lac* promoter to
372 generate an easy-to-use RPP expression system that allows the expression of target proteins
373 to be quickly set to obtain optimal expression and/ or solubility. Furthermore, we show that the
374 PAR system functions well with many of the common induction regimes used to control both
375 the level and timing of target protein expression. Thus, fine-tuning expression levels from the
376 PAR promoters gives added flexibility. Future research will focus on optimizing the PAR
377 promoter system for use in larger-scale expression and fermenter applications.

378

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386

387 **Author contribution**

388 J.H., T.W.O., S.J.W.B. and D.F.B. devised the research programme, experiments were
389 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.
390 and D.F.B., with input from all authors.

391

392 **Conflict of interest statement**

393 The Authors declare no conflict of interest.

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395

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471

472 **Figure Legends**

473 **Fig. 1.** Expression of recombinant 6His-GFP protein can be set to different levels using the
474 PAR promoters. (a) The panel shows a schematic representation of the PAR promoters used
475 in this study. Each PAR construct (*i.e.* PAR1 to PAR8) carries -10 and -35 promoter elements,
476 based on either the *lac* or *tac* promoters, and two flanking *lac* operator DNA sequences [5].
477 The LacI repressor protein, binding to each operator target, represses promoter activity (-ve),
478 until the addition of IPTG causes it to release the promoter DNA [29]. Panels (b) and (c) show
479 Coomassie blue stained SDS-PAGE gels and Western blots, respectively, examining 6His-
480 GFP expression in *E. coli* BL21 cells, carrying various pET22b PAR constructs (PAR1 to
481 PAR8). Cells were grown in LB medium and sampled after three hours induction with (or
482 without) 1 mM IPTG. An empty pET22b vector control (EV) was included. In the Western blot
483 in panel (c), recombinant 6His-GFP was detected using anti-GFP antiserum and anti-mouse
484 HRP secondary antibody.

485
486 **Fig. 2.** Analysis of 6His-GFP expression using the three promoter PAR system. (a) The panel
487 shows a Coomassie blue stained SDS-PAGE gel of *E. coli* BL21 cells expressing 6His-GFP,
488 using the pET22b three promoter PAR system (PAR1, PAR4L and PAR7Q). Cells were grown
489 in LB medium and RPP was initiated for three hours by the addition of 1 mM IPTG, where
490 indicated. (b) The panel shows the mean cellular fluorescence of *E. coli* BL21 cells as
491 measured by flow cytometry, expressing 6His-GFP from the pET22b three promoter PAR
492 system. Cells were grown in LB medium and RPP was induced for three hours using 1 mM
493 IPTG. Data are shown as mean green fluorescence values from replica flasks and error bars
494 are \pm the standard deviation. (c) The panel shows a Coomassie blue stained SDS-PAGE gel
495 investigating the solubility of 6His-GFP expressed in *E. coli* BL21 cells using the pET22b three
496 promoter PAR system. Cultures were grown in LB medium and protein production was
497 induced by 1 mM IPTG for three hours. Harvested cells were lysed to prepare total (T), soluble
498 (S) and insoluble (I) protein samples. In panels (a) and (b) empty vector controls (EV) were
499 included.

500
501 **Fig. 3.** Expression from the PAR promoters can be fine-tuned using different IPTG
502 concentrations. The figure shows flow cytometry analysis of mean green fluorescence from
503 BL21 cells carrying pET22b PAR/ 6His-GFP constructs (a) PAR1, (b) PAR4L and (c) PAR7Q.
504 Cells were grown in LB medium and 6His-GFP expression was induced by the inclusion of
505 IPTG at 2, 10, 50 and 1000 μ M. Data are shown as mean green fluorescence values from
506 replica flasks, error bars are \pm the standard deviation.

507

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

514

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

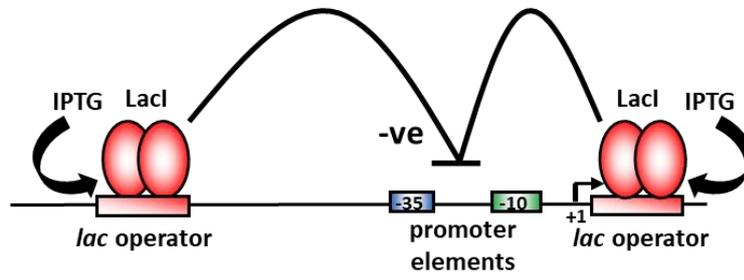
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525 **Fig. 6.** Expression of the *N. meningitidis* BamA_{ENm} chimera protein in *E. coli* BL21 cells. (a)
526 The panel shows growth of the *E. coli* BL21 cells carrying either pET22b PAR1 empty vector
527 or pET22b PAR1/ BamA_{ENm} in Lennox broth,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

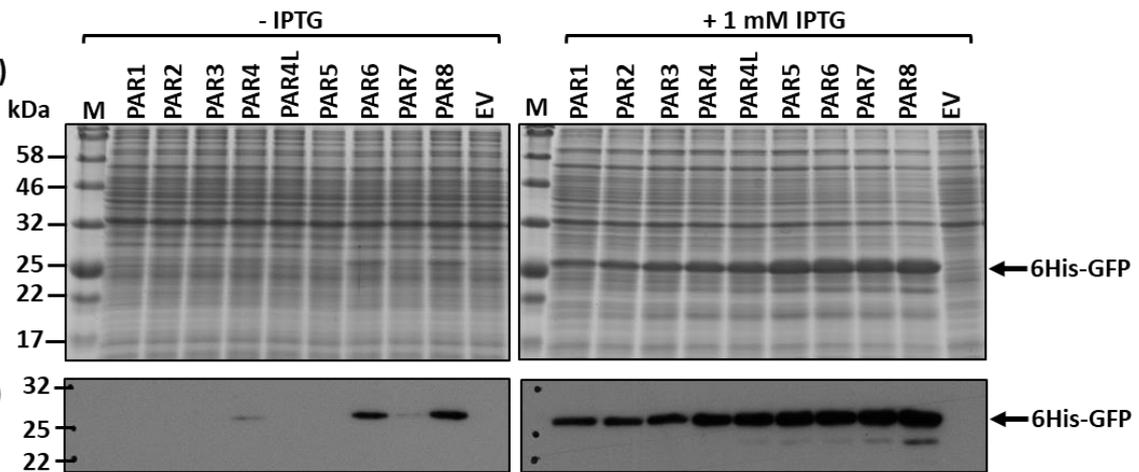
545 blot 0.5 µg and 0.3 µg of protein were loaded, respectively. BamA_{ENm} was detected by probing
546 with anti-*E. coli* BamA POTRA antiserum and anti-rabbit HRP secondary antibody.
547

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

a)



b)



c)

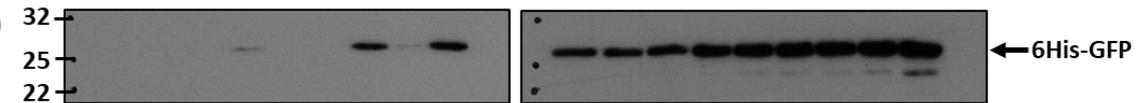
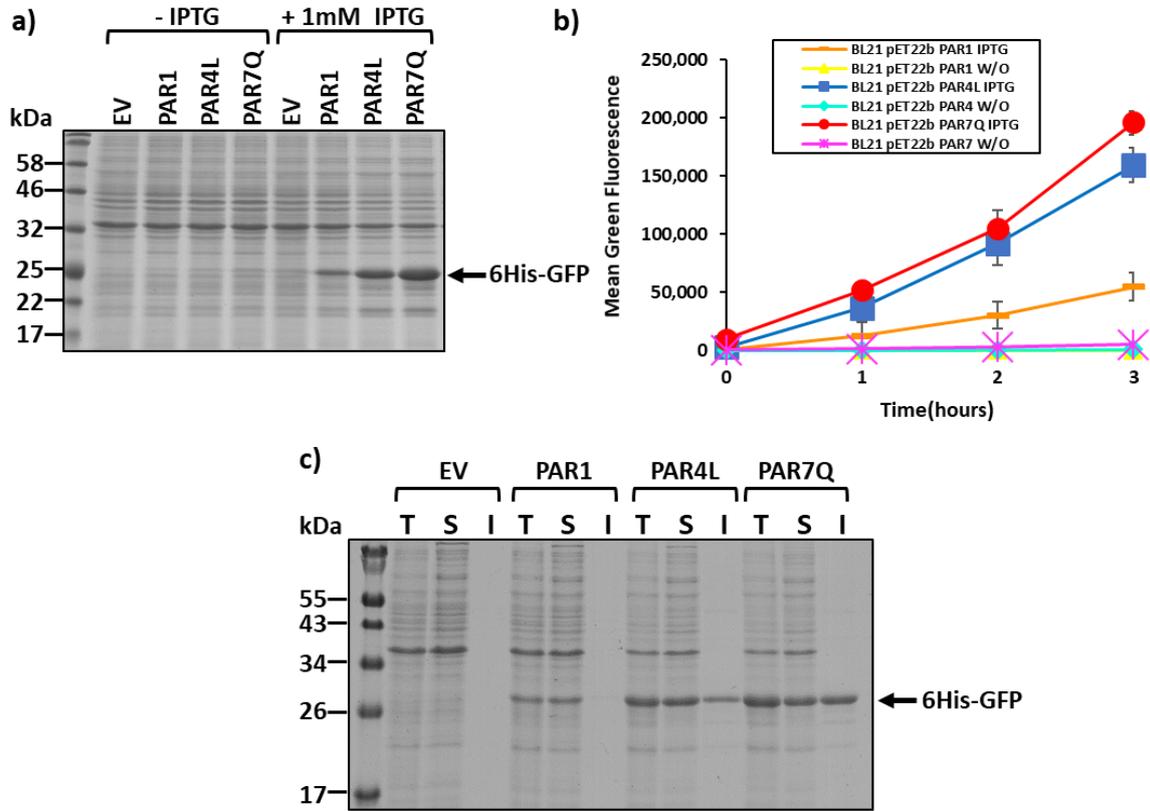
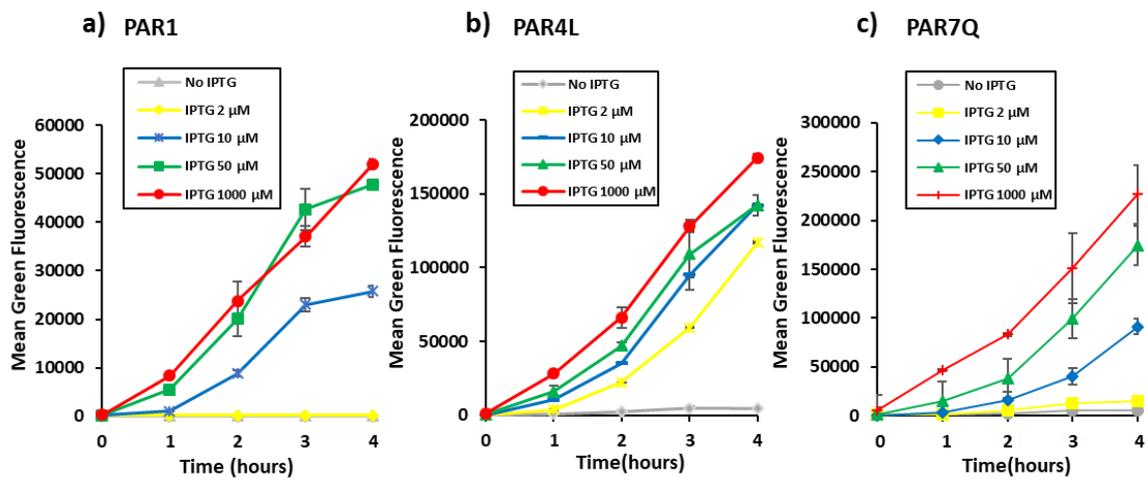


Fig. 2. Hothersall *et al.* (2021)



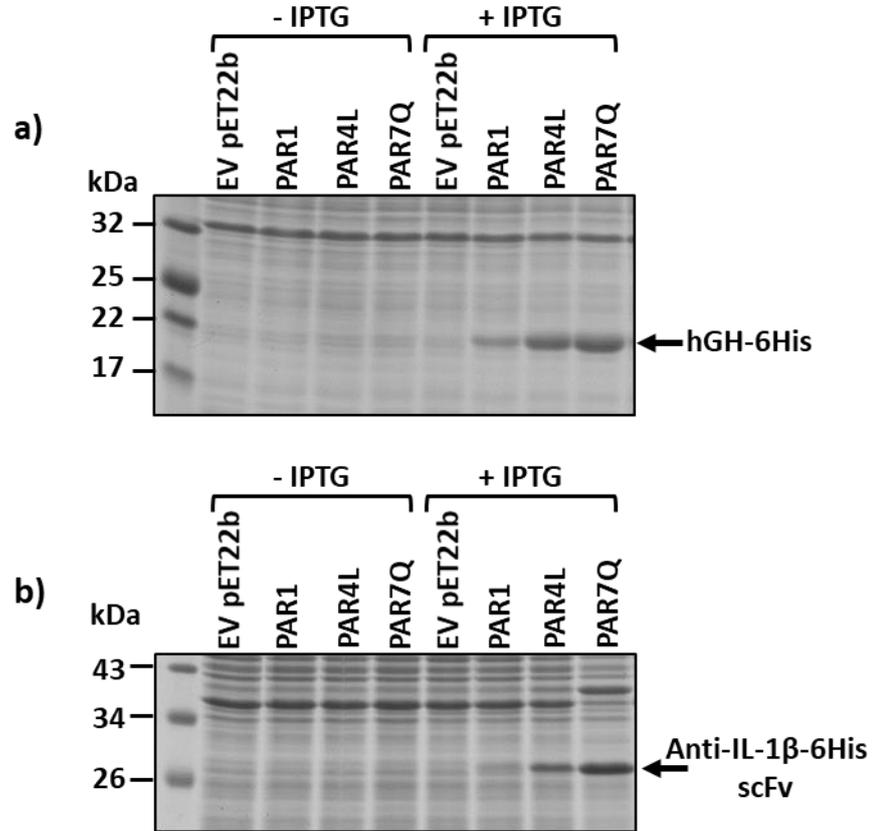
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Fig. 3. Hothersall *et al.* (2021)



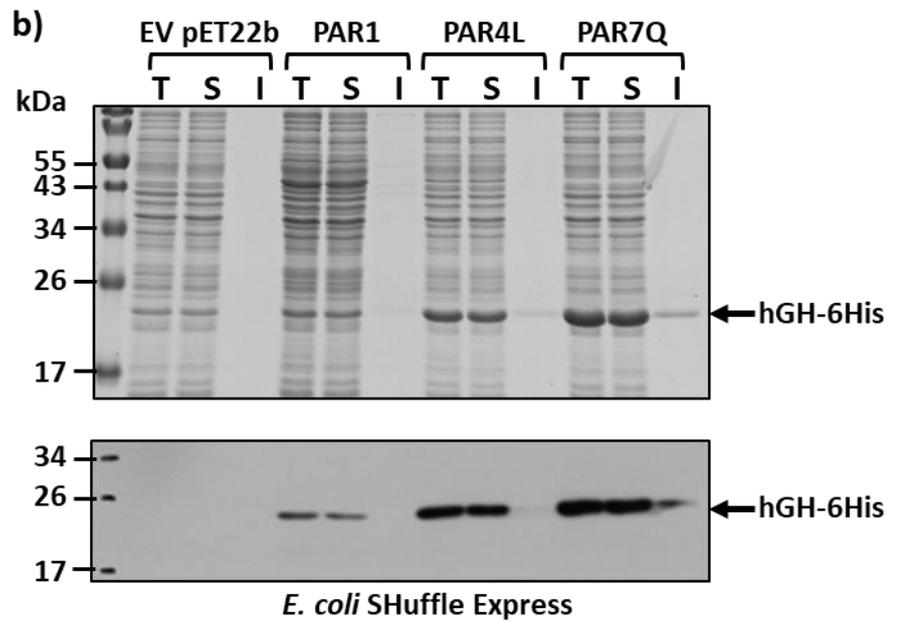
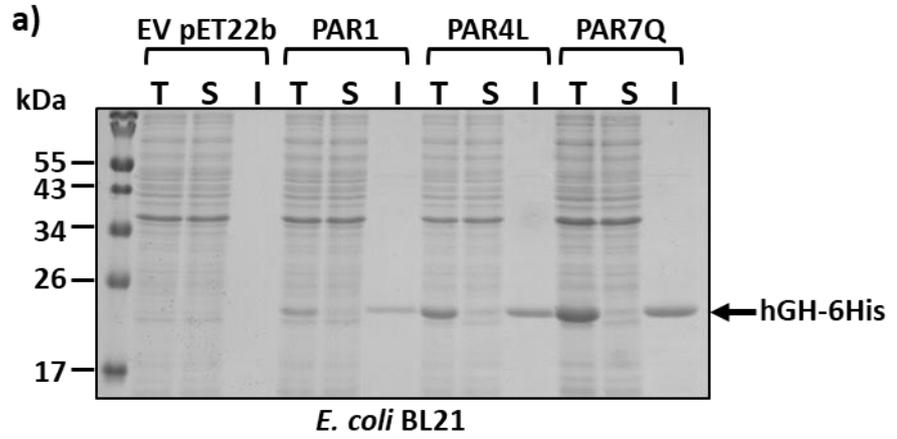
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Fig. 4. Hothersall *et al.* (2021)



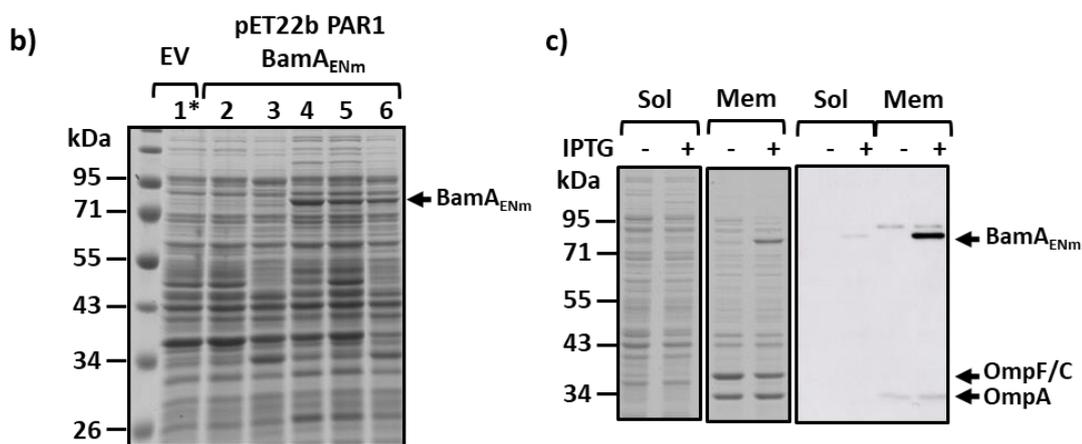
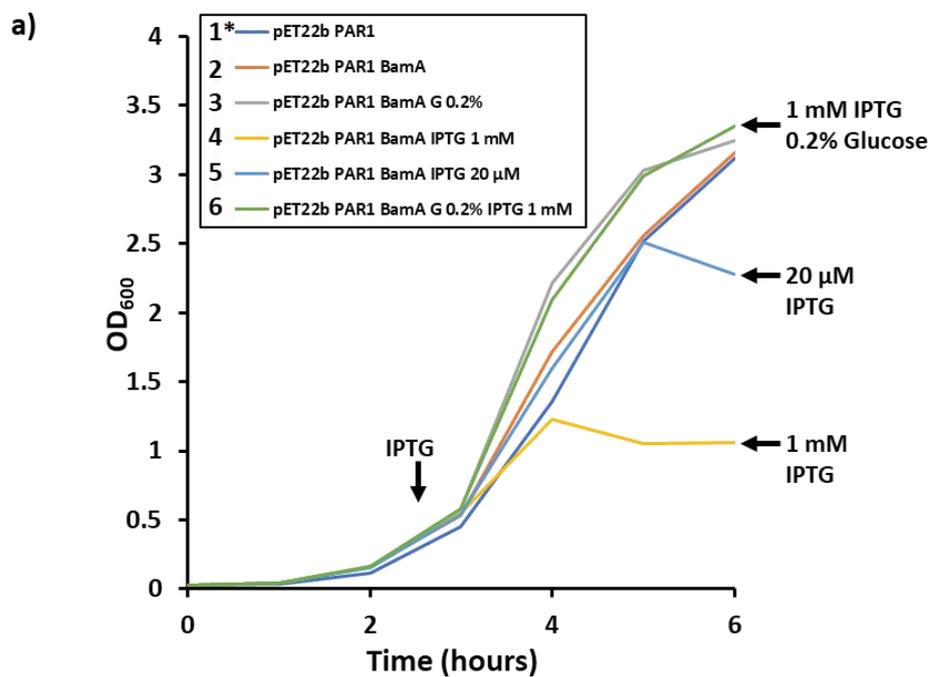
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Fig. 5. Hothersall *et al.* (2021)



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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.

Joanne Hothersall ^{1*}, Rita E. Godfrey ¹, Christos Fanitsios ^{2,3}, Tim W. Overton ²,
Stephen J. W. Busby ^{1*}, and Douglas F. Browning ^{1*}

¹ Institute of Microbiology and Infection and School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

² School of Chemical Engineering and Institute of Microbiology and Infection, University of Birmingham, Birmingham, B15 2TT, UK.

³ Present address: Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

*To whom correspondence should be addressed:

Email: j.hothersall@bham.ac.uk Tel: +44 (0)121 414 5434

Email: s.j.w.busby@bham.ac.uk Tel: +44 (0)121 414 5439

Email: d.f.browning@bham.ac.uk Tel: +44 (0)121 414 5434

585

586 **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]

587

588

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

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

590

591

592 **Supplementary Figure Legends.**

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

601

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

610

611 **Supplementary Fig. S3.** The amino acid sequences of target proteins expressed in this study.
612 The figure shows the amino acid sequences of the (a) 6His-GFP (b) hGH-6His (c) anti-IL-1 β -
613 6His scFv and (d) BamA_{ENm} proteins used in this study. For each protein the 6His tag is purple
614 and predicted molecular weight (Mw) of each protein is given. Note the BamA_{ENm} protein
615 chimera is a fusion of the *E. coli* BamA N-terminal domain and BamA C-terminal β -barrel
616 domain from *N. meningitidis* [8].

617

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

624

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

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

631

632 **Supplementary Fig. S6.** Expression of 6His-GFP using the PAR promoter system in auto-
633 induction medium at different growth temperatures. The figure shows Coomassie blue stained
634 SDS-PAGE gels analysing the expression of 6His-GFP in *E. coli* BL21 cells, using the three
635 promoter PAR system (PAR1, PAR4L and PAR7Q) when cloned into pET22b. Cells were
636 grown in auto-induction medium [9] at either (a) 37°C or (b) 30°C and samples were taken 3,
637 6 and 23 hours after sub-culturing (O/N overnight).

638

639 **Supplementary Fig. S7.** Homogeneous green fluorescence intensity of cells expressing
640 6His-GFP from the PAR promoters. Flow cytometry analysis of green fluorescence from BL21
641 pET22b PAR/ 6His-GFP constructs (a) PAR1, (b) PAR4L and (c) PAR7Q grown in LB medium
642 with 2 µM to 1000 µM IPTG induction for four hours. Data are plotted as histograms showing
643 number of cells with different green fluorescence (FL1-A) values.

644

645 **Supplementary Fig. S8.** Glucose represses expression from the PAR1 promoter. The figure
646 shows the mean green fluorescence intensities of *E. coli* BL21 cells, carrying pET22b
647 PAR1/ *6his-gfp*, for the first three hours after IPTG induction, in the presence and absence of
648 0.5% glucose. Dotted lines correspond to expression levels in cells grown in the absence of
649 glucose, whilst the solid ones represent cells grown in its presence. 6His-GFP expression was
650 induced by the inclusion of IPTG at 2, 10, 50, 100 and 1000 µM. Data are shown as
651 mean green fluorescence values from replica flasks, error bars are ± the standard deviation.

652

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

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

670

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

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715

716

717 **Supplementary Fig. S1.**

718

719

720 **BglIII** **T7 promoter** **lac operator**
 721 AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAA

722

723 **XbaI** **RBS** **NdeI**
 724 TTCCCCTCTAGAAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAATACCTG
 725 M K Y L

726

727 **pelB leader** **NcoI**
 728 CTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCCATGGAT
 729 L P T A A A G L L L L A A Q P A M A M D

730

731 **BamHI** **EcoRI** **SacI** **SalI** **HindIII** **NotI** **XhoI**
 732 ATCGGAATTAATTCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG
 733 I G I N S D P N S S S V D K L A A A L E

734

735 **6His tag**
 736 CACCACCACCACCACCACTGAATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG
 737 H H H H H H **End**

738

739 GCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAACGGGTCTTG
 740 ← **T7 Terminator Primer**

741

742

743 **Supplementary Fig. S2.**

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

745 TCTAGAAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATG GGCAGCAGCCATCATCATCATCACAGCA
746 GCGGCCTGGTGCCGCGCGGCAGCCATATGGTGAGCAAGGGCGAGGAGCTGTTACCCGGGGTGGTGCCCATCCTGG
747 TCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA
748 AGCTGACCCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTGACCACCCCTGACCT
749 ACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAG
750 GCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGG
751 GCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC
752 TGGAGTACAACACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAGTTC
753 AGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCCTACCAGCAGAACACCCCATCGGCGACG
754 GCCCCGTGCTGCTGCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCG
755 ATCACATGGTCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGGAT
756 cc

757

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

759 CATATGTTCCCAACCATTCCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTG
760 GCCTTTGACACCTACCAGGAGTTTGAAGAAGCCTATATCCCAAAGGAACAGAAGTATTCATTCCAGCAACCC
761 CAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCCTCCAACAGGGAGGAAACACAACAGAAATCCAACCTA
762 GAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCTCAGGAGTGTCTTCGCC
763 AACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAGGCATCCAACG
764 CTGATGGGGAGGCTGGAAGATGGCAGCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACA
765 AACTCACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGTC
766 GAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCATCATCATCATCATCACTAA
767 TAAAGGATCCGAATTCGAGCTC

768

769 **(c) *IL-1β-6his scFv***

770 CATATGGATATCCAGATGACGCAGAGCCCGAGCAGCCTGAGCGCCAGCGTGGGTGACCGTGTGACCATTACCTGT
771 CGTACCAGCGGCAACATTCACTAATACTATCTGACCTGGTACCAGCAGAAACCGGGCAAAGCGCCGAGCTGCTGATT
772 TATAATGCAAAAACCCCTGGCAGATGGTGTGCCGAGCCGCTTTAGCGGCAGCGGTAGCGGTACCCAGTTCACCCTG
773 ACGATCAGCAGCCTGCAGCCGGAAGACTTTGCCAACTATTACTGCCAGCACTTCTGGAGCCTGCCGTTTACCTTC
774 GGTACAGGGCACGAAAGTGGAAATTAACGTAACCGCGGTGGCGGTAGCGGCGGTGGCGGTAGCGGCGGTGGCGGT
775 AGCGGCGGTGGCGGTAGCGAGGTGCAGCTGGTTGAAAGCGGCGGTGGCCTGGTTACCGGGTGGCAGCCTGCGT
776 CTGAGCTGTGCGGCCAGCGGCTTTGATTTACGCCGTTATGACATGAGCTGGGTGCGTCAGGCACCGGGTAAACGT
777 CTGGAATGGGTTCCTATATTAGCAGCGGTGGCGGTAGCACCTACTTCCGGATACGGTGAAAGGCCGCTTACC
778 ATCAGCCGTGACAACGCAAAAATACGCTGTACCTGCAGATGAACAGCCTGCGCGCCGAAGATAACCGCAGTTTAT
779 TACTGCGCCCGTCAGAATAAAAACTGACGTGGTTCGACTACTGGGGTCAGGGCACGCTGGTTACGGTTAGCAGC
780 CATCATCATCATCACTAATAAGGATCCGAATTCGAGCTC

781

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

783 CATATGGCGATGAAAAAAGTCTGATCGCGTCTCTGCTGTTCTCTTCTGCGACCGTTTACGGTGTAGCGAAGGT
784 TTCGTTGTTAAAGACATCCACTTTGAAGGTCTGCAACGTGTTGCGGTTGGTGCGGGCGTGTGTCTATGCCGGTT
785 CGTACCAGGCGACACCGTGAACGACGAAGACATCTCTAACACCATCCGTGCGCTGTTTCGCGACCGGCAACTTTGAA
786 GACGTTTCGTGTTCTGCGTGACGGTGCACCCCTGCTGGTTCAGGTTAAAGAAGCTCCGACCATCGCGTCTATCACC
787 TTCTCTGGTAACAAATCTGTTAAAGACGACATGCTGAAACAGAACCTGGAAGCGTCTGGTGTTCGTGTTGGTGAA
788 TCTCTGGACCGTACCACCATCGCGGACATCGAAAAAGGTCTGGAAGACTTCTACTACTCTGTTGGTAAATACTCT
789 GCGTCTGTTAAAGCGGTTGTTACCCCGCTGCCGCGTAACCGTGTGACCTGAAACTGGTTTTCCAGGAAGGTGTT
790 TCTGCGGAAATCCAGCAGATCAACATCGTTGGTAACCACGCTTTCACCACCGACGAACGATCTCTCACTTCCAA
791 CTGCGTGACGAAGTTCCTGGTGGTGAACGTGGTTGGTGACCGTAAATACCAGAAACAGAAACTGGCGGGTGACCTG

792 GAAACCCTGCGTTCTTACTACCTGGACCGTGGTTACGCGGTTTCAACATCGACTCTACCCAGGTTTCTCTGACC
793 CCGGACAAAAAAGGTATCTACGTTACCGTGAACATCACCGAAGGTGACCAGTACAAACTGTCTGGTGTGAAGTT
794 TCTGGTAACCTGGCGGGTCACTCTGCGGAAATCGAACAACTGACCAAAATCGAACCGGTGAACGTATAACGGC
795 ACCAAAGTTACCAAAATGGAAGACGACATCAAAAAACTGCTGGGTGCTTACGGTTACGCTTACCCGCGTGTTCAG
796 TCTATGCCGGAAATCAACGACGCGGACAAAACCGTTAAACTGCGTGTGAACGTGGACGCGGGTAACCGTTTCTAC
797 GTTCGTAAAAATCCGTTTTGAAGGTAACGACACCTCTAAAAGACGCGGTTCTGCGTCGTGAAATGCGTCAGATGGAA
798 GGTGCGTGGCTGGGTTCTGACCTGGTTGACCAGGGTAAAGAACGTCTGAACCGTCTGGGTTTCTTTGAAACCGTT
799 GACACCGACACCCAGCGTGTCCGGGTTCCCCGGACCAGGTTGACGTTGTTTACAAAGTTAAAGAACGTAACACC
800 GGATCCCTGGACCTGTCTGCGGGTTGGGTTTACAGGACACCGGCCTGGTTATGTCTGCGGGTGTTCCTCAGGACAAC
801 CTGTTCCGGCACCGGCAAATCTGCGGCGCTGCGTGCCTCTGTTCTAAAACCACCTGAACGGTTCTCTGTCTTTC
802 ACCGACCCGTACTTACCGCTGACGGTGTTCCTCTGGGTTACGACGTTTACGGTAAAGCGTTTCGACCCGCGTAAA
803 GCGTCTACCTCTATCAAACAGTACAAAACCACCACCGCTGGTGGGGTATCCGTATGTCTGTTCCGGTTACCGAA
804 TACGACCGTGTGAACTTCGGTCTGGTTGCGGAACACCTGACCGTGAACACCTACAACAAAGCGCCGAAACACTAC
805 GCGGACTTCATCAAAAAATACGGTAAAACCGACGGCACCGACGGTTCTTTCAAAGGTTGGCTGTATAAAGGCACC
806 GTTGGTTGGGGTCGTAACAAAACCGACTCTGCGCTGTGGCCGACCCGTGGTTACCTGACCGGCGTGAACGCGGAA
807 ATCGCGCTGCCGGGTTCTAAACTGCAATACTACTCTGCGACCCACAACCAGACCTGGTTCTTCCCGCTGTCTAAA
808 ACCTTCACCCTGATGCTGGGTGGTGAAGTTGGTATCGCGGGTGGTTACGGTCGTACCAAAGAAATCCCGTTCTTT
809 GAAAACCTTCTACGGTGGTGGTCTGGGTTCTGTTGTTGTTACGAATCTGGCACCCCTGGGTCCGAAAGTTTACGAC
810 GAATACGGTGAAAAAATCTCTTACGGTGGTAACAAAAAAGCGAACGTGTCTGCGGAACTGCTGTTCCCGATGCCG
811 GGTGCGAAAGACGCGCGTACCGTTTCGTCTGTCTGTTTCGCGGACGCGGGTTCTGTTTGGGACGGTAAAACCTAC
812 GACGACAACCTCTTCTTCTGCGACCCGGCGGTGCTGTTTACGAAACATCTACGGTGGGGTAAACCCACAAATCTACC
813 TTCACCAACGAACTGCGTTACTCTGCGGGTGGTGCGGTTACCTGGCTGTCTCCGCTGGGGCCCATGAAATTTCTCT
814 TACGCTTACCCGCTGAAAAAACCAGGAAAGACGAAATCCAGCGTTTCCAGTTCCAACCTGGGCACCACCTTCTAA
815 TGAGGGCCCATGAAGTTTAGCTATGCCTATCCATTAAGAAGAAGCCAGAGGATGAGATTCAAAGATTTCAATTT
816 CAATTAGGTACTACTTTTGGCGGCAGATCTCTCGAG
817
818

819
820

821 **Supplementary Fig. S3.**

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

823 MGSSHHHHHSSGLVPRGSHMVSKEELFTGVVPILEVELDGDVNGHKFSVSSEGEEDATYGKLTCLKFI
824 CTTGKLPVPWPTLVTTLLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
825 GDTLVNRIELKGIIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQ
826 QNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDMVLLLEFVTAAGITLGMDELYK
827

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

829 MFPTIPLSRLFDNAMLRHRLHQLAFDITYQEFEEAYIPKEQKYSFLQNPQTSCLCFSES IPTPSNREET
830 QQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSPRT
831 GQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEGSCGFHHHHHH
832

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

834 MDIQMTQSPSSLSASVGDRTTITCRTSGNIHNYLTWYQQKPGKAPQLLIYNAKTLADGVPSRFSGSGS
835 GTQFTLTISLQPEDFANYCQHFWSLPFTFGQGTKVEIKRTGGGGSGGGGSGGGGSGGGGSEVQLVE
836 SGGGLVQPGGSLRLS CAASGFDFSRYDMSWVRQAPGKRLEWVAYISSGGGTYFPDTPVKGRFTISRDN
837 AKNTLYLQMNSLRAEDTAVYYCARQNKKLTWFDYWGQTLVTVSSHHHHHH
838

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

840 MAMKLLIASLLFSSATVYGASEGFVVKDIHFEGLRVAVGAALLSMPVRTGDTVNDEDISNTIRALF
841 ATGNFEDVRVLRDGD TLLVQVKERPTIASITFSGNKS VKDDMLKQNL EASGVRVGESLDRTTIADIEK
842 GLEDFYYSVGKYSASVKAVVTP LPRNRVDLKL VFOEGVSAEIQQINIVGNHAF TTDDELISHFQLRDEV
843 PWWNVVGD RKYQKQKLAGDLET LRSYYLDRGYARFNIDSTQVSLTPDKKGIYVTVNITEGDQYKLSGV
844 EVSGNLAGHSAEIEQLTKIEPGELYNGTKVTKMEDDIKLLGRYGYAYPRVQSMPEINDADKTVKLRV
845 NVDAGNRFYVRKIRFE GNDT SKDAVLRREMRQMEGAWLGS DLVDQGKERLNRLGFFETVDTDTQRVPG
846 SPDQVDVYKVKERNTGSLDLSAGWVQDTGLVMSAGVSQDNLFGTGKSAALRASRSKTTLNGLSFTD
847 PYFTADGVSLGYDVYKAFDPRKASTSIKQYKTTTAGAGIRMSVPVTEYDRVNFGLVAEHLTVNTYNK
848 APKHYADFIKKYKGT DGT DGSFKGWLYKGTVGWGRNKTD SALWPTRGYLTGVNAEIALPGSKLQYYSA
849 THNQTWFFPLSKTFTLMLGGEVGIAGGYGRTKEIPFFENFYGGGLG SVRGYESGTLGPKVYDEYGEKI
850 SYGGNKKANVSAELLFPMPGAKDARTVRLSLFADAGSVWDGKTYDDNSSSATGGRVQNIYGAGNTHKS
851 TFTNELRYSAGGAVTWLSPLGPMKFSYAYPLKKKPEDEIQRFQFLGTTF
852

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854 **Supplementary Fig. S4.**

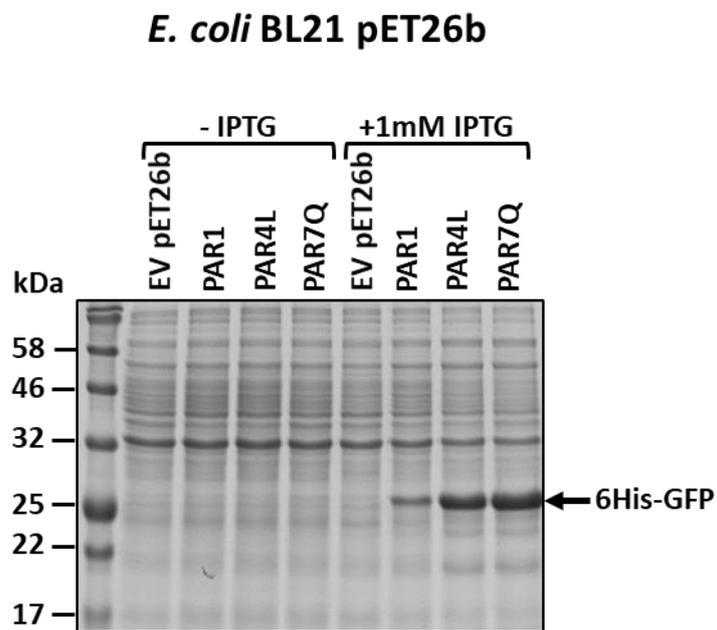
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861 **Supplementary Fig. S5.**

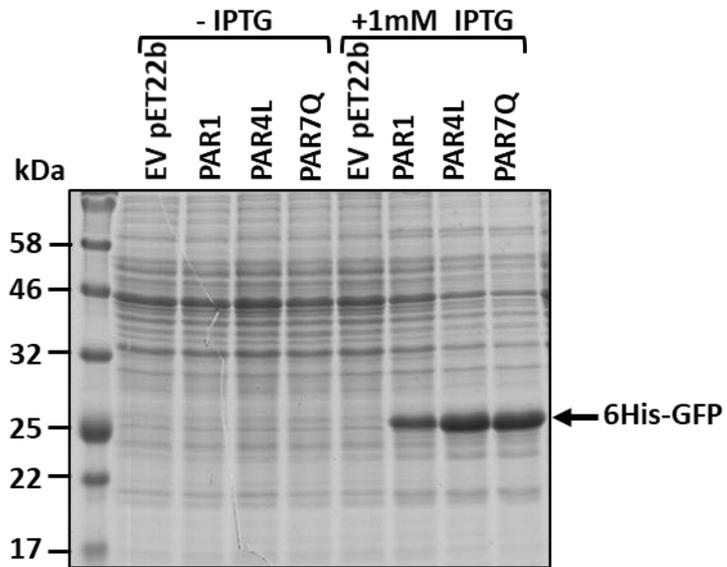
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***E. coli* W3110 pET22b**



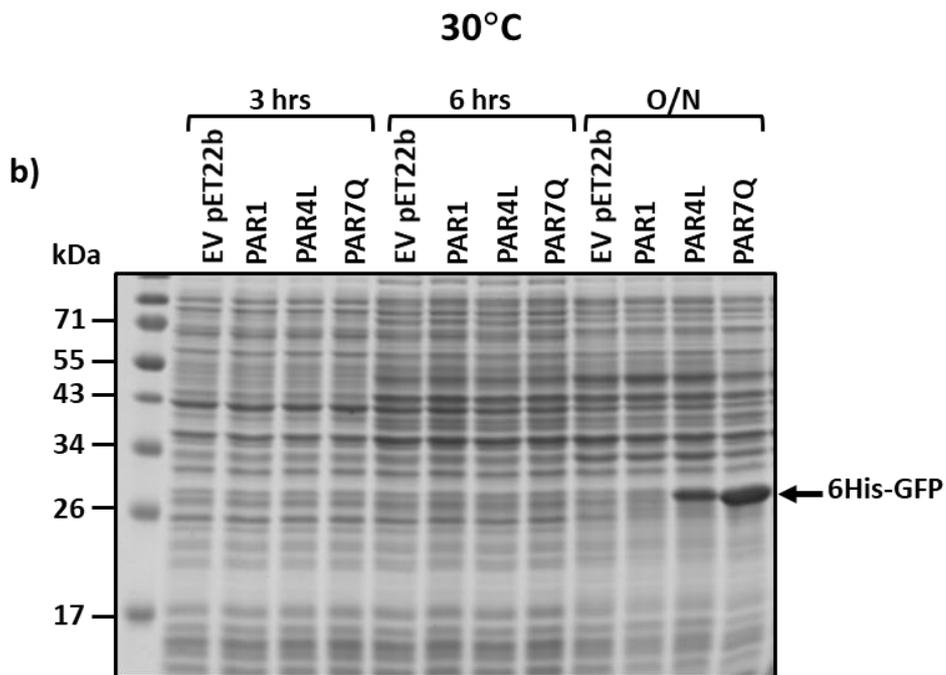
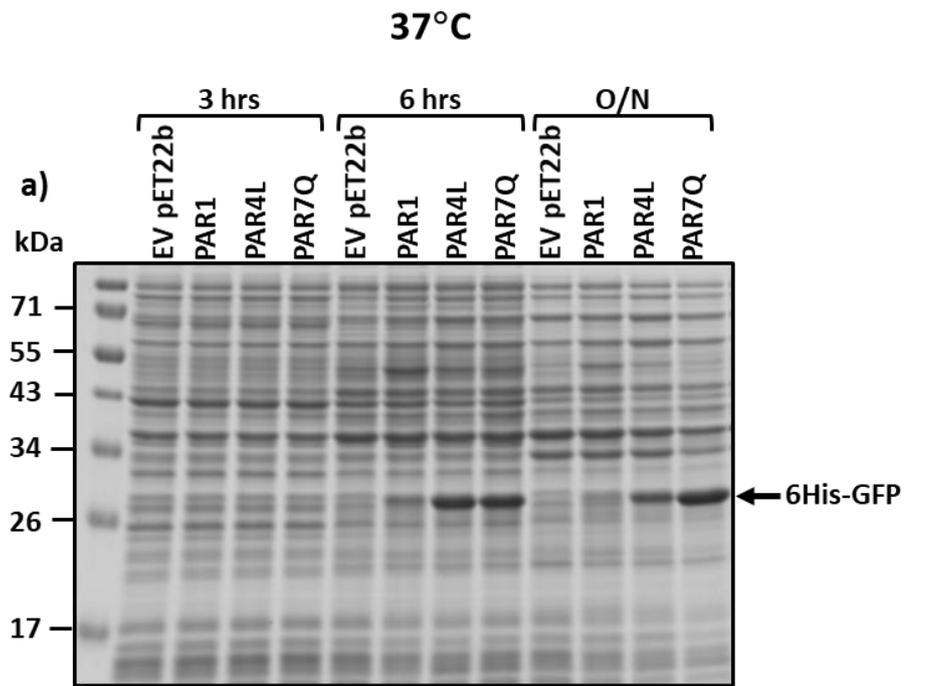
866 **Supplementary Fig. S6.**

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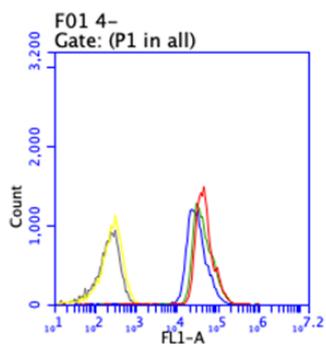


871 **Supplementary Fig. S7.**

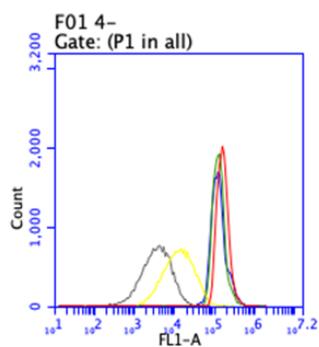
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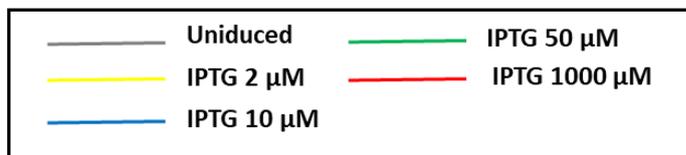
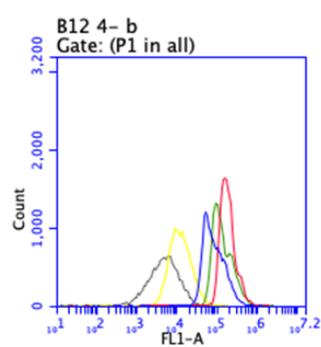
a) PAR1



b) PAR4L

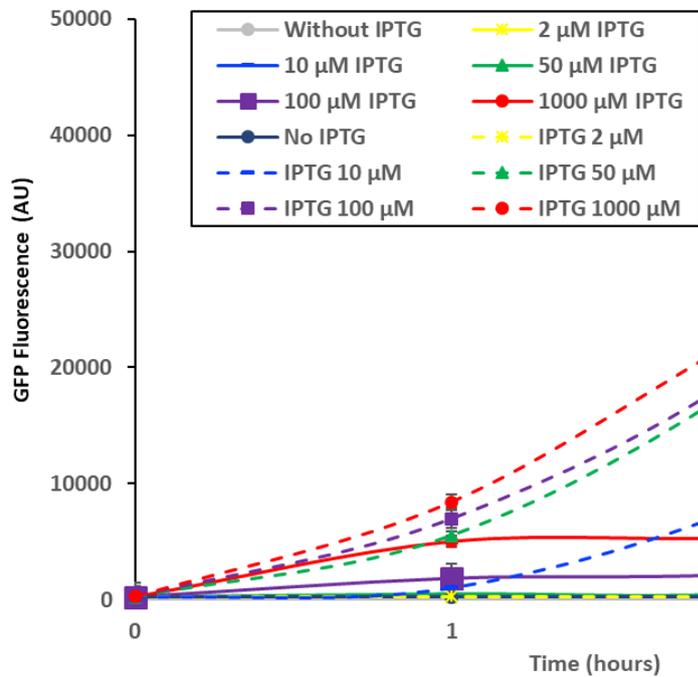


c) PAR7Q



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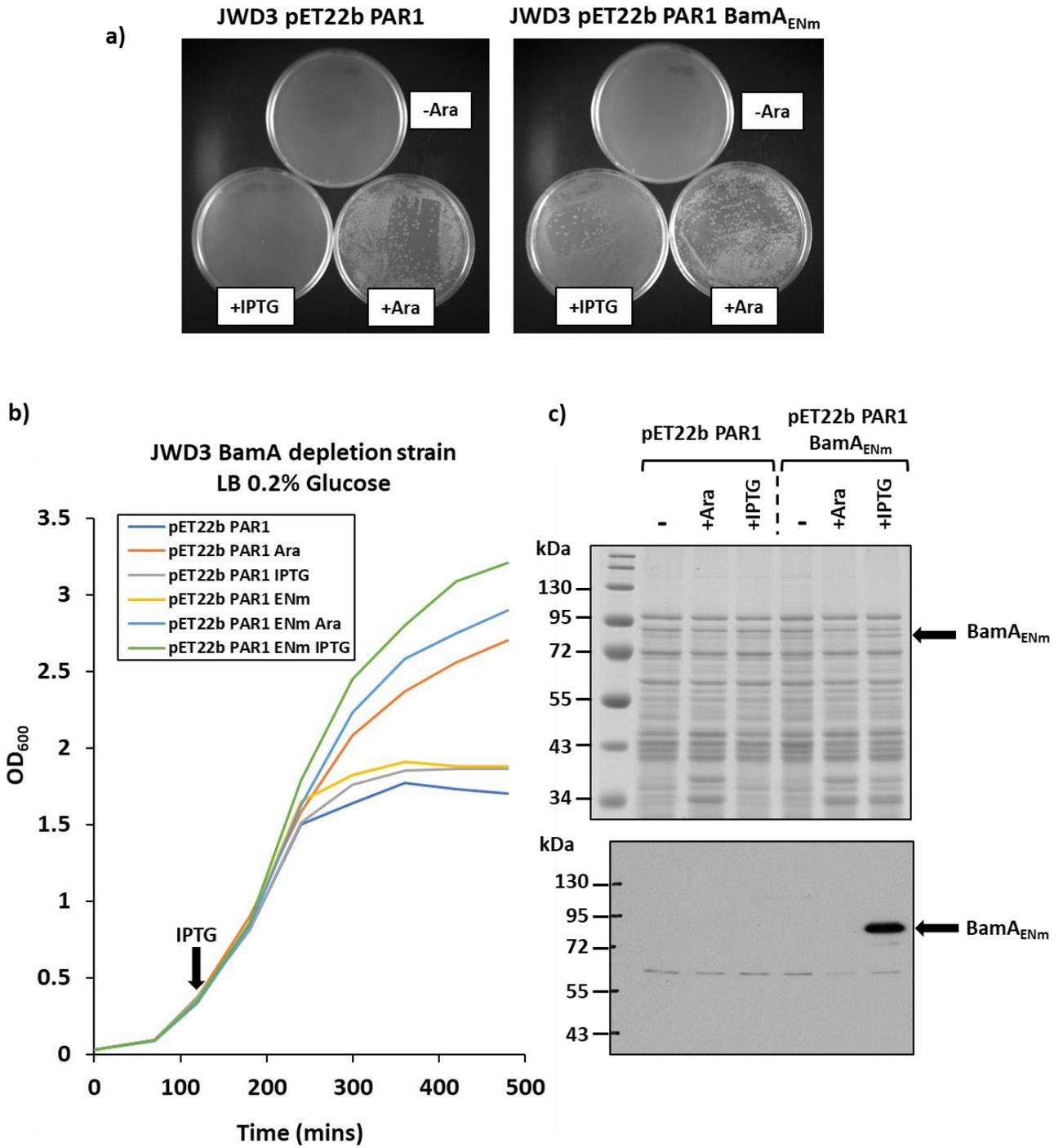
875 **Supplementary Fig. S8.**



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883 **Supplementary Fig. S10.**

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