UNIVERSITY^{OF} BIRMINGHAM University of Birmingham Research at Birmingham

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 DOI:

10.1016/j.nbt.2021.05.001

Document Version Early version, also known as pre-print

Citation for published version (Harvard):

Hothersall, J, Godfrey, R, Fanitsios, C, Overton, T, Busby, S & Browning, D 2021, 'The PAR promoter expression system: modified *lac* promoters for controlled recombinant protein production in *Escherichia coli*, *New Biotechnology*, vol. 64, pp. 1-8. https://doi.org/10.1016/j.nbt.2021.05.001

Link to publication on Research at Birmingham portal

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.

•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.

•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?) •Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

1	PREPRINT
2	
3	
4	
5	The PAR promoter expression system: modified lac
6	promoters for controlled recombinant protein production
7	in Escherichia coli
8	
9	
10	
11	
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 *
14	
15	
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: <u>j.hothersall@bham.ac.uk</u> Tel: +44 (0)121 414 5434
25	Email: <u>s.j.w.busby@bham.ac.uk</u> Tel: +44 (0)121 414 5439
26	Email: <u>d.f.browning@bham.ac.uk</u> Tel: +44 (0)121 414 5434
27	

Highlights.

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

36 ABSTRACT

Many commonly used bacterial promoters employed for recombinant protein production (RPP) 37 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 strengths, assigning them specific promoter activity ratings (PARs). Selecting three of these 42 PAR promoters, with low, intermediate and high strengths, we demonstrate that the 43 expression of target proteins, such as green fluorescent protein (GFP), human growth 44 45 hormone (hGH) and single chain variable region antibody fragments (scFvs) can be set to three levels when expressed in *E. coli*. We show the PAR promoter system is extremely 46 47 flexible, operating in a variety of *E. coli* strains and under various different culture regimes. Furthermore, due to its tight regulation, we show that this system can also express a toxic 48 outer membrane protein, at levels, which do not affect bacterial growth. Thus, the PAR 49 50 promoter system can be used to tailor the expression levels of target proteins in E. coli and 51 maximize RPP.

52

53

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

58

59

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

- 62
- 63

64 Introduction

65 The bacterium Escherichia coli has been the cornerstone of recombinant protein production (RPP) for many years. E. coli is particularly well suited to its role as a protein 66 production factory as it grows quickly to high density in cheap medium and can be manipulated 67 easily [1]. As a consequence, many recombinant protein expression systems have been 68 constructed, such as those based on the E. coli lactose operon promoter (e.g. the lac and tac 69 70 promoters) and the T7 RNA polymerase expression system [2, 3, 4]. In such systems, the gene of interest is usually cloned into a plasmid vector downstream of a strong regulated 71 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 expression systems typically have strong inducible promoters, problems can arise when 75 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 to increase the amount of soluble target, RPP is often carried out at lower temperatures, or 79 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

Ideally, RPP expression systems should be as versatile as possible, being tightly 85 regulated, allowing high- and low-level target expression, and be compatible with many 86 different E. coli strains and "helper" plasmids, which can carry various tRNAs or chaperones 87 [1, 10]. Above all, expression systems should be easy to use, with vectors carrying multiple 88 cloning sites (MCS), purification and secretion tags, and, potentially, different antibiotic 89 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 their strengths [3, 4, 5, 12]. These PAR promoters (PAR1 to PAR8) show a wide range of 92 promoter activities, stretching from low-level (*i.e.* PAR1) to high-level (*i.e.* PAR8) expression 93 94 capabilities [5]. Each PAR promoter also carried two lac operator sequences and were, therefore, repressed by the lac operon repressor (Lacl) and IPTG-inducible (Fig. 1a). As some 95 of these promoters appeared to be promising for RPP, we have transferred them to easy-to-96 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

E. coli strains, XL1 Blue and JCB387, were used for plasmid construction and DNA manipulation, whilst *E. coli* BL21, BL21(DE3), W3110 and SHuffle Express and were used for recombinant protein over-expression (Supplementary Table S1). Strains were grown in LB broth (Sigma), Lennox broth (2% (w/v) peptone (Oxoid), 1% (w/v) yeast extract (Oxoid) and 170 mM NaCl) [13] and auto-induction medium [14], with appropriate antibiotic selection (ampicillin 100 μ g/ml, kanamycin 50 μ g/ml). For RPP, *E. coli* BL21, BL21(DE3) and W3110 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 *lac*O3O1) 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 BgIII and Xbal and cloned into the pET22b and pET26b expression vectors (Novagen), replacing the canonical 121 T7 RNA polymerase promoter (Supplementary Fig. S1). The DNA encoding 6His-GFP, from 122 pET15b/ 6his-gfp, was cloned into each pET22b and pET26b PAR construct using Xbal and 123 BamHI restriction sites (Supplementary Table S1; Supplementary Figs. S2 and S3). The DNA 124 encoding hGH-6His and anti-IL-1β-6His scFv, from pHAK1 and pYU49, respectively, was 125 cloned into each pET22b PAR construct using Ndel and Sacl (Supplementary Figs. S2 and 126 S3) [15, 16]. The DNA encoding the BamA_{ENm} chimeric outer membrane protein was cloned 127 into pET22b PAR1, using Ndel and Xhol (Supplementary Figs. S2 and S3) [17]. The lacl^q 128 mutation, which changes a single base in the *lacl* promoter to increase its strength [18, 19], 129 was introduced into pET22b PAR7 and pET26b PAR7 derivatives using the Agilent 130 131 QuikChange site-directed mutagenesis kit and primers laclqF/R (Supplementary Table 2). All constructs were verified by Sanger DNA sequencing. 132

133

134 Recombinant protein over-expression and detection

Bacterial cultures of *E. coli*, carrying pET expression plasmids containing the PAR promoters and various target genes, were grown with shaking in 10 mL of LB medium, until 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 amounts of cells in 2X Laemmli loading buffer (Sigma), heating at 95°C for three minutes, and 141 centrifuging prior to loading. Normalized protein samples were resolved by reducing SDS-142 PAGE and analyzed using Coomassie blue staining and Western blotting, as in our previous 143 work [20]. For Western blotting, 6His-GFP was detected using anti-GFP antiserum raised in 144 mouse (Sigma) and an anti-mouse-HRP secondary antibody (Sigma), hGH-6His was detected 145 using anti-hGH antiserum raised in rabbit [5] and an anti-rabbit-HRP (horseradish peroxidase) 146 secondary antibody (Amersham), and anti-IL-1β-6His scFv was detected using anti-6His (C-147 terminal)-HRP (Invitrogen). BamA_{ENm} was detected using anti-BamA antiserum [17] and an 148 anti-rabbit-HRP secondary antibody (Amersham). Blots were developed using Pierce ECL 149 150 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 also prepared using Agilent BugBuster, according to the manufacturer's instructions. 152

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-HCI (pH 7.4) and resuspended in 20ml of 10 mM Tris-HCl (pH 7.4) containing 2 mM 158 phenylmethylsulfonyl fluoride (PMSF, protease inhibitor). Cell envelopes were disrupted by 159 continuous passage through an Emulsiflex C3 for 5 minutes, and unbroken cells and 160 particulate material was removed by centrifugation for 15 min at 6,000 x g and 4°C. The total 161 membrane fraction (inner and outer membranes) was then isolated by centrifuging the 162 supernatant for 1 hour at 48,000 x g at 4 °C and the soluble fraction, which contains 163 cytoplasmic and periplasmic proteins was retained [17, 20]. Membranes were washed once 164 and resuspended in 0.4 ml of 10 mM Tris-HCl (pH 7.4). 165

166

167 Rescue of BamA depletion in E. coli

To determine the ability of the BamA_{ENm} chimera to rescue BamA depletion on solid media, the *E. coli* BamA depletion strain JWD3 (Supplementary Table S1) [21] was grown on LB agar plates, supplemented by 0.2% (w/v) arabinose or 1 mM IPTG, where indicated. For experiments in liquid media, JWD3 cells were grown in 50 ml of Lennox broth with 0.2% glucose, with shaking at 37°C, in the presence or absence of 0.05% (w/v) arabinose. Optical density was monitored and after 2 hours of growth ($OD_{600} = 0.3 - 0.4$) BamA_{ENm} production was induced by the addition of IPTG. The preparation of normalised total cellular protein samples, after 6 hours of growth, was as in Browning *et al.* (2013). Note that in JWD3 cells chromosomally encoded *E. coli* BamA is only produced in the presence of arabinose, whilst in its absence, BamA expression is prevented, resulting in the cessation of growth and cell death [21]. Depletion can be rescued by providing a functional copy of *bamA*, such as that carried by pET22b PAR1/ BamA_{ENm} [17, 20].

180

181 Flow cytometry.

For flow cytometry analysis, 50 mL cultures of LB medium were incubated with shaking at 37°C until the culture reached $OD_{600} \sim 0.6$, and then RPP was induced by addition of IPTG for three to four hours, as stated. Cultures were analysed using a BD Accuri C6 flow cytometer (Becton Dickinson, UK). Samples were mixed with 0.2 µm-filtered PBS and data was collected at a rate of 1000 - 4000 events per second using slow flow and a forward scatter height (FSC-H) threshold of 10000 to eliminate non-cellular material until 20000 events had been recorded 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 ease of use, each PAR promoter was sub-cloned into the medium copy number expression 195 vector pET22b, which carries an extensive MCS to facilitate gene cloning and a functional lacl 196 gene to ensure regulation in any *E. coli* host strain (Supplementary Fig. S1 and Table S1). To 197 investigate expression from these new vectors, DNA encoding N-terminally 6His-tagged GFP 198 199 (6his-gfp) (Supplementary Figs. S2 and S3) was cloned downstream of each PAR promoter and recombinant plasmids were transferred into E. coli BL21 cells. Cells were grown in LB 200 medium until mid-logarithmic growth (OD₆₀₀ between 0.3 and 0.5) and recombinant PAR 201 promoter expression was induced with 1 mM IPTG for three hours. Levels of total GFP 202 production were then analysed by SDS-PAGE and Western blotting. Results in Fig. 1b show 203 204 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 absence of IPTG, but some, e.g. PAR6 and PAR8, were found to be leaky (Fig. 1). 206

As we wished to develop vectors with a weak, intermediate and strong promoter, we chose the PAR1, PAR4L and PAR7 constructs, respectively. As expression from the PAR7 construct was slightly leaky in the absence of inducer (Figs. 1b and 1c), the *lacl^q* mutation, which increases the expression of Lacl, was introduced [18, 19]. This new construct, referred 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 respectively, after induction with 1 mM IPTG (Fig. 2a). This was confirmed by flow cytometry 214 (Fig. 2b), which revealed differences in expression after 1 hour of induction. 215

216

Since RPP expression systems should be as flexible as possible, the PAR1, PAR4L and 217 PAR7Q promoters were also introduced into the pET26b expression vector, which carries an 218 alternative plasmid backbone and a kanamycin resistant cassette (Supplementary Table S1). 219 Expression studies, again using 6his-gfp, demonstrated that graded levels of 6His-GFP 220 expression, as expected, were achieved (Supplementary Fig. S4). Furthermore, our three 221 plasmid PAR promoter system functioned well in the E. coli K-12 strain W3110 222 (Supplementary Fig. S5), and with auto-induction medium when cells were grown at different 223 temperatures (*i.e.* 30 and 37°C) for longer periods of time (*i.e.* 23 hrs) (Supplementary Fig. 224 225 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 overall protein solubility. To examine this, we analysed soluble and insoluble fractions from 233 234 BL21 cells expressing 6His-GFP from the pET22b PAR1, PAR4L and PAR7Q constructs. Results illustrated in Fig. 2c, show that, for the strong PAR7Q construct, a large proportion of 235 6His-GFP is found in the insoluble fraction. For the intermediate strength PAR4L promoter, 236 237 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 cultures is only slightly higher than the PAR4L cultures despite containing more total GFP. 242 Flow cytometry has been shown to measure both quantity and folding quality of GFP, with 243 insoluble GFP having low fluorescence [11]. 244

245

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

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

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-ornone phenomenon [22, 23, 24]. Therefore, we examined whether 6His-GFP expression from 252 our pET22b PAR1, PAR4L and PAR7Q constructs was tuneable. Once more, BL21 cells, 253 carrying each plasmid, were grown in LB and induced with different IPTG concentrations (*i.e.* 254 2, 10, 50 and 1000 µM). The expression of 6His-GFP was then monitored using flow 255 cytometry. Results in Fig. 3 show that for all three promoters different levels of expression 256 could be set in a culture by using different IPTG concentrations. Furthermore, the analysis of 257 258 individual cells indicated that for each promoter and IPTG concentration tested, GFP induction was homogenous within the bacterial cell population (Supplementary Fig. S7). This is 259 particularly evident for the PAR7Q construct, which produces discrete GFP-expressing 260 261 populations at many different IPTG concentrations, indicating that expression from this highly active promoter can be effectively tuned by different IPTG concentrations. 262

263

264 Expression of different protein targets using the PAR promoter system

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

274

As correct folding of hGH requires the formation of a disulphide bond, we examined 275 whether expressing hGH-6His with the PAR promoters aided its solubility. However, as the E. 276 coli cytoplasm is a reducing environment that does not favour disulphide bond formation, it 277 278 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 modified *E. coli* strain, which enables cytoplasmic disulphide bond formation. Cells were grown 280 in LB medium at 30°C and RPP induced with 1 mM IPTG for three hours. Results illustrated 281 in Fig. 5b, demonstrate that hGH-6His was successfully induced under this altered induction 282 regime and that for all PAR promoter constructs the majority of recombinant hGH-6His was 283 284 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

The PAR1 promoter can be used to express toxic proteins 288

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

306

307 In *E. coli*, BamA is an essential protein that is responsible for inserting bacterial βbarrel containing OMPs into the bacterial outer membrane [27]. Previously, we demonstrated 308 that very low-level expression of the N. meningitidis BamA_{ENm} chimera could function in E. 309 310 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 depletion in JWD3 was not rescued and BamA_{ENm} expression was not detected 315 (Supplementary Fig. S9), indicating that the PAR1 promoter is tightly regulated and suitable 316 317 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, but, in some cases, high-level RPP can result in the accumulation of insoluble protein into 324 inclusion bodies, or cell death if the expressed product is toxic. As obtaining the correct 325 expression levels for problematic proteins can be difficult to achieve, we have developed the 326 PAR promoter system, which consists of three plasmids with low, intermediate and high 327 expression capabilities (PAR1, PAR4L and PAR7Q). Thus, by cloning target DNA into each 328 vector, the most suitable level of expression required for optimal RPP and solubility can be 329 determined quickly. The pET22b and pET26b vectors that we used carry extensive MCS, tags 330 331 for purification and secretion, and different antibiotic resistance cassettes (Supplementary Fig. S1 and Table S1). Each plasmid also carries the gene encoding the Lac repressor (i.e. *lacl*) 332 and, thus, coordinated regulation can achieved in any E. coli strain regardless of its genotype. 333 334 Consistent with this, we show that the PAR system functions with different E. coli strains commonly used in industry and academia (e.g. BL21, W3110 and SHuffle Express). 335

336

Using the PAR system we have expressed different proteins (GFP, hGH, an scFv and 337 338 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 effect is to be expected (Supplementary Fig. S10). Cytoplasmic expression of more complex 343 proteins, such as hGH, can be more problematic as it requires disulphide bond formation for 344 correct folding. In this instance, product solubility was greatly improved by employing E. coli 345 346 SHuffle Express as an expression host (Fig. 5), as it allows cytoplasmic disulphide bond formation to occur. Once more, the largest amount of insoluble product was found when using 347 our strongest promoter construct, PAR7Q, with little or no product insolubility observed for the 348 PAR4L and PAR1 constructs. Thus, we show, as others before us, that reducing RPP 349 expression, by using weaker promoters can improve target solubility [6, 10]. 350

351

For many experiments, we used high concentrations of IPTG (*i.e.* 1 mM) to ensure that our expression systems are fully switched on. However, our results show that the level of RPP driven by the PAR promoters can be modulated. Our systems are tuneable, with specific IPTG concentrations producing different expression levels homogenously within a culture, rather than an all-or-none phenotype that has been observed before (Fig. 3 and Supplementary Fig. S7) [22, 23, 24]. Also, the use of glucose-mediated repression with the PAR1 promoter 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, and the nascent Bam complex) will likely result in toxicity and cell death [17, 27]. Thus, due to 362 the tight repression of PAR1 in the absence of inducer and the ability to modulate expression 363 by glucose (Fig. 6; Supplementary Figs. S8 and S9), the PAR1 promoter is ideal for low-level 364 expression of toxic proteins. Finally, all three promoters worked well with auto-induction 365 medium (Supplementary Fig. S6), indicating that inducer exclusion (*i.e.* the ability to prevent 366 lactose uptake when glucose is present in the growth medium) is a feasible way to control and 367 delay RPP induction with the PAR system [14]. 368

369

Since its discovery, the *lac* operon promoter and its derivatives have been extensively 370 371 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 PAR system functions well with many of the common induction regimes used to control both 374 the level and timing of target protein expression. Thus, fine-tuning expression levels from the 375 376 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

379 Acknowledgements

J.H. was generously supported by an Industrial Biotechnology Catalyst (Innovate UK, BBSRC, EPSRC) (BB/M018261/1) to support the translation, development and commercialisation of innovative Industrial Biotechnology processes and by a BBSRC IAA Follow-on-Fund award (BBSRC IAA BB/S506709/1). D.F.B was supported by BBSRC grants BB/M018261/1 and BB/R017689/1. C.F. was supported by a studentship from the BBSRC Midlands Integrative Biosciences Training Programme.

386

387 Author contribution

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

391

392 **Conflict of interest statement**

- 393 The Authors declare no conflict of interest.
- 394
- 395

396 **References**

- 397[1]Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and
challenges. Front Microbiol 2014;5:172. doi: 10.3389/fmicb.2014.00172.
- 399[2]Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-400level expression of cloned genes. J Mol Biol 1986;189(1):113-30.
- 401[3]Amann E, Brosius J, Ptashne M. Vectors bearing a hybrid *trp-lac* promoter useful for402regulated expression of cloned genes in *Escherichia coli*. Gene 1983;25(2-3):167-78.
- 403[4]de Boer HA, Comstock LJ, Vasser M. The *tac* promoter: a functional hybrid derived from the404*trp* and *lac* promoters. Proc Natl Acad Sci U S A 1983;80(1):21-5.
- 405 [5] Browning DF, Godfrey RE, Richards KL, et al. Exploitation of the *Escherichia coli lac* operon
 406 promoter for controlled recombinant protein production. Biochem Soc Trans
 407 2019;47(2):755-763. doi: 10.1042/bst20190059.
- 408[6]Overton TW. Recombinant protein production in bacterial hosts. Drug Discov Today4092014;19(5):590-601. doi: 10.1016/j.drudis.2013.11.008.
- 410 [7] Wagner S, Klepsch MM, Schlegel S, et al. Tuning *Escherichia coli* for membrane protein
 411 overexpression. Proc Natl Acad Sci U S A 2008;105(38):14371-6. doi:
 412 10.1073/pnas.0804090105.
- 413 [8] Browning DF, Richards KL, Peswani AR, et al. *Escherichia coli* 'TatExpress' strains super414 secrete human growth hormone into the bacterial periplasm by the Tat pathway. Biotechnol
 415 Bioeng 2017;114(12):2828-2836. doi: 10.1002/bit.26434.
- 416 [9] Quick M, Wright EM. Employing *Escherichia coli* to functionally express, purify, and
 417 characterize a human transporter. Proc Natl Acad Sci U S A 2002;99(13):8597-601. doi:
 418 10.1073/pnas.132266599.
- 419 [10] Gopal GJ, Kumar A. Strategies for the production of recombinant protein in *Escherichia coli*.
 420 Protein J 2013;32(6):419-25. doi: 10.1007/s10930-013-9502-5.
- 421 [11] Vera A, González-Montalbán N, Arís A, et al. The conformational quality of insoluble
 422 recombinant proteins is enhanced at low growth temperatures. Biotechnol Bioeng
 423 2007;96(6):1101-6. doi: 10.1002/bit.21218.
- 424 [12] Müller-Hill B. The *lac* Operon. A Short History of a Genetic Paradigm. Berlin New York:
 425 Walter de Gruyter; 1996.
- 426 [13] Squire DJ, Xu M, Cole JA, et al. Competition between NarL-dependent activation and Fis427 dependent repression controls expression from the *Escherichia coli yeaR* and *ogt* promoters.
 428 Biochem J 2009;420(2):249-57. doi: BJ20090183 [pii] 10.1042/BJ20090183.
- 429 [14] Studier FW. Protein production by auto-induction in high density shaking cultures. Protein
 430 Expr Purif 2005;41(1):207-34. doi: 10.1016/j.pep.2005.01.016.
- 431 [15] Alanen HI, Walker KL, Lourdes Velez Suberbie M, et al. Efficient export of human growth
 432 hormone, interferon alpha2b and antibody fragments to the periplasm by the *Escherichia*433 *coli* Tat pathway in the absence of prior disulfide bond formation. Biochim Biophys Acta
 434 2015;1853(3):756-63. doi: 10.1016/j.bbamcr.2014.12.027.
- 435 [16] Matos CF, Robinson C, Alanen HI, et al. Efficient export of prefolded, disulfide-bonded
 436 recombinant proteins to the periplasm by the Tat pathway in *Escherichia coli* CyDisCo
 437 strains. Biotechnol Prog 2014;30(2):281-90. doi: 10.1002/btpr.1858.
- 438[17]Browning DF, Bavro VN, Mason JL, et al. Cross-species chimeras reveal BamA POTRA and439beta-barrel domains must be fine-tuned for efficient OMP insertion. Mol Microbiol4402015;97(4):646-59. doi: 10.1111/mmi.13052.
- 441 [18] Calos MP. DNA sequence for a low-level promoter of the *lac* repressor gene and an 'up'
 442 promoter mutation. Nature 1978;274(5673):762-5.
- 443 [19] Muller-Hill B, Crapo L, Gilbert W. Mutants that make more *lac* repressor. Proc Natl Acad Sci
 444 U S A 1968;59(4):1259-64.

- 445 [20] Browning DF, Matthews SA, Rossiter AE, et al. Mutational and topological analysis of the
 446 *Escherichia coli* BamA protein. PLoS One 2013;8(12):e84512. doi:
 447 10.1371/journal.pone.0084512.
- Lehr U, Schütz M, Oberhettinger P, et al. C-terminal amino acid residues of the trimeric
 autotransporter adhesin YadA of *Yersinia enterocolitica* are decisive for its recognition and
 assembly by BamA. Mol Microbiol 2010;78(4):932-46. doi: 10.1111/j.13652958.2010.07377.x.
- 452 [22] Novick A, Weiner M. Enzyme Induction as an all-or none phenomenon. Proc Natl Acad Sci U
 453 S A 1957;43(7):553-66.
- 454 [23] Maloney PC, Rotman B. Distribution of suboptimally induces -D-galactosidase in *Escherichia* 455 *coli*. The enzyme content of individual cells. J Mol Biol 1973;73(1):77-91.
- 456 [24] Khlebnikov A, Risa O, Skaug T, et al. Regulatable arabinose-inducible gene expression system
 457 with consistent control in all cells of a culture. J Bacteriol 2000;182(24):7029-34.
- Kaur J, Kumar A, Kaur J. Strategies for optimization of heterologous protein expression in *E. coli*: Roadblocks and reinforcements. Int J Biol Macromol 2018;106:803-822. doi:
 10.1016/j.ijbiomac.2017.08.080.
- 461[26]Guan Q, Wang X, Wang X, et al. In silico analysis and recombinant expression of BamA462protein as a universal vaccine against Escherichia coli in mice. Appl Microbiol Biotechnol4632016;100(11):5089-98. doi: 10.1007/s00253-016-7467-y.
- 464[27]Konovalova A, Kahne DE, Silhavy TJ. Outer Membrane Biogenesis. Annu Rev Microbiol4652017;71:539-556. doi: 10.1146/annurev-micro-090816-093754.
- 466[28]Makoff AJ, Oxer MD. High level heterologous expression in *E. coli* using mutant forms of the467*lac* promoter. Nucleic Acids Res 1991;19(9):2417-21.
- 468 [29] Wilson CJ, Zhan H, Swint-Kruse L, et al. The lactose repressor system: paradigms for
 469 regulation, allosteric behavior and protein folding. Cell Mol Life Sci 2007;64(1):3-16. doi:
 470 10.1007/s00018-006-6296-z.

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

485

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

500

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

507

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

514

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

524

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

- blot 0.5 μ g and 0.3 μ g 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.









Fig. 4. Hothersall et al. (2021)



Fig. 5. Hothersall et al. (2021)



Fig. 6. Hothersall et al. (2021)



ion system: modified <i>lac</i> mbinant protein production
ion system: modified <i>lac</i> mbinant protein production
ion system: modified <i>lac</i> mbinant protein production
mbinant protein production
• •
chia coli
ry Material.
hristos Fanitsios ^{2,3} , Tim W. Overton ² ,
Douglas F. Browning ^{1*}
ol of Biosciences, University of Birmingham,
of Microbiology and Infection, University of
versity of Warwick, Coventry, CV4 7AL, UK.
:
414 5434
414 5439
1 414 5434

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

Name	Details	Source
Strains		
BL21	fhuA2 [lon] ompT gal [dcm] ΔhsdS	Novagen
BL21(DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ∆hsdS λ DE3 = λ sBamHlo ∆EcoRI-B int::(lacl::PlacUV5::T7 gene1) i21 ∆nin5	Novagen
JCB387	Anir, Alac	[1]
JWD3	<i>E. coli</i> K-12 BamA depletion strain	[2]
SHuffle Express	F ['] lac, pro, lacl ^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 , lacl ^q) ΔtrxB rpsL150(Str ^R) Δgor Δ(malF) λ^{-} , IN(rrnD-rrnE)1, rph-1	NEB
W3110	recA1 endA1 avrA96 thi-1 hsdR17 sunE44	[3]
XL1 Blue	relA1, lac, [F proAB lacl $^{9}Z\Delta M15$ Tn10 (Tet ^R)]	Agilent
Plasmids		
pET15b	T7 RNA polymerase expression vector (Amp ^R , N-terminal His tag). Carries the <i>lacl</i> gene	Novagen
pET22b	T7 RNA polymerase expression vector (Amp ^R , <i>pelB</i> signal sequence, C-terminal His tag).	Novagen
pET26b	T7 RNA polymerase expression vector (Kan ^R , <i>pelB</i> signal sequence, C-terminal His tag).	Novagen
pET15b/ 6his-gfp	pET15b expressing 6His N-terminal GEP fusion	Dr David Lee.
pYU49	pET23 based vector with <i>ptac</i> promoter	[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]
	1	1

Supplementary Table S2. Primers used in this study

Primer	Sequence (5` to 3`)
ptac(BgIII) ptacO3(BgIII) placO1(BgIII) placO3(BgIII) placRV(XbaI) lacIqF lacIqR	GGGGGAGATCTGATAATGTTTTTTGCGCCGACATCATAACGG GGGGGAGATCTGGCAGTGAGCGCAACGCAA

592 Supplementary Figure Legends.

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

601

Supplementary Fig. S2. The DNA sequences of target proteins expressed in this study. The 602 figure shows the DNA sequences of (a) 6his-gfp (b) hgh-6his (c) IL-1 β -6his scFv and (d) 603 604 bamA_{ENm} used in this study. Restriction enzyme recognition sites (Xbal, Ndel, BamHI, Sacl 605 and Xhol) 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 purple and the translation stop codon (TAA) is red. Note the *bamA_{ENm}* construct used in this 607 608 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

511 **Supplementary Fig. S3.** The amino acid sequences of target proteins expressed in this study. 512 The figure shows the amino acid sequences of the (a) 6His-GFP (b) hGH-6His (c) anti-IL-1β-513 6His scFv and (d) BamA_{ENm} proteins used in this study. For each protein the 6His tag is purple 514 and predicted molecular weight (Mw) of each protein is given. Note the BamA_{ENm} protein 515 chimera is a fusion of the *E. coli* BamA N-terminal domain and BamA C-terminal β-barrel 516 domain from *N. meningitidis* [8].

617

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

624

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

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

631

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

638

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
pET22b PAR/ 6His-GFP constructs (a) PAR1, (b) PAR4L and (c) PAR7Q grown in LB medium
with 2 μM to 1000 μM IPTG induction for four hours. Data are plotted as histograms showing
number of cells with different green fluorescence (FL1-A) values.

644

Supplementary Fig. S8. Glucose represses expression from the PAR1 promoter. The figure shows the mean green fluorescence intensities of *E. coli* BL21 cells, carrying pET22b PAR1/ *6his-gfp*, for the first three hours after IPTG induction, in the presence and absence of 0.5% glucose. Dotted lines correspond to expression levels in cells grown in the absence of glucose, whilst the solid ones represent cells grown in its presence. 6His-GFP expression was induced by the inclusion of IPTG at 2, 10, 50, 100 and 1000 μ M. Data are shown as 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, JWD3, on LB agar pates in the presence of arabinose (+Ara), the absence of arabinose (-Ara) 655 or the presence of only 1 mM IPTG (+IPTG). Cells carried either pET22b PAR1 empty vector 656 657 or pET22b PAR1/ BamA_{ENm}. Note that in JWD3 cells the chromosomally-encoded E. coli BamA is only produced in the presence of arabinose, whilst in its absence, BamA expression 658 is prevented, resulting in cell death [2]. Depletion can be rescued by providing a functional 659 660 copy of *bamA*[8, 10], such as that carried by pET22b PAR1/ BamA_{ENm}. (b) The panel shows the growth of JWD3 cells, carrying either pET22b PAR1 empty vector or pET22b PAR1/ 661 BamA_{ENm}, in Lennox broth medium, supplemented with 0.2% glucose, in the absence of 662 663 arabinose, the presence of arabinose (Ara) or the presence of 1 mM IPTG (IPTG). (c) Detection of BamA_{ENm} chimera expression. The panel shows a Coomassie blue stained gel 664

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

670

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

677 Supplementary References

- 678 [1] Page L, Griffiths L, Cole JA. Different physiological roles of two independent pathways for
 679 nitrite reduction to ammonia by enteric bacteria. Archives of microbiology. 1990;154(4):349680 54. PubMed PMID: 2173895; eng.
- Lehr U, Schütz M, Oberhettinger P, et al. C-terminal amino acid residues of the trimeric
 autotransporter adhesin YadA of *Yersinia enterocolitica* are decisive for its recognition and
 assembly by BamA. Molecular microbiology. 2010 Nov;78(4):932-46. doi: 10.1111/j.13652958.2010.07377.x. PubMed PMID: 20815824; eng.
- [3] Hayashi K, Morooka N, Yamamoto Y, et al. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. Molecular systems biology. 2006;2:2006.0007. doi:
 10.1038/msb4100049. PubMed PMID: 16738553; PubMed Central PMCID:
 PMCPmc1681481. Eng.
- 689 [4] Matos CF, Robinson C, Alanen HI, et al. Efficient export of prefolded, disulfide-bonded
 690 recombinant proteins to the periplasm by the Tat pathway in *Escherichia coli* CyDisCo
 691 strains. Biotechnology progress. 2014 Mar-Apr;30(2):281-90. doi: 10.1002/btpr.1858.
 692 PubMed PMID: 24376243; eng.
- Alanen HI, Walker KL, Lourdes Velez Suberbie M, et al. Efficient export of human growth
 hormone, interferon alpha2b and antibody fragments to the periplasm by the *Escherichia coli* Tat pathway in the absence of prior disulfide bond formation. Biochimica et biophysica
 acta. 2015 Mar;1853(3):756-63. doi: 10.1016/j.bbamcr.2014.12.027. PubMed PMID:
 25554517; eng.
- 698 [6] Lodge J, Fear J, Busby S, et al. Broad host range plasmids carrying the *Escherichia coli* lactose
 699 and galactose operons. FEMS microbiology letters. 1992 Aug 15;74(2-3):271-6. PubMed
 700 PMID: 1526459; eng.
- 701 [7] Browning DF, Godfrey RE, Richards KL, et al. Exploitation of the *Escherichia coli lac* operon
 702 promoter for controlled recombinant protein production. Biochemical Society transactions.
 703 2019 Apr 30;47(2):755-763. doi: 10.1042/bst20190059. PubMed PMID: 30971435; eng.
- 704 [8] Browning DF, Bavro VN, Mason JL, et al. Cross-species chimeras reveal BamA POTRA and
 705 beta-barrel domains must be fine-tuned for efficient OMP insertion. Molecular microbiology.
 706 2015 Aug;97(4):646-59. doi: 10.1111/mmi.13052. PubMed PMID: 25943387; PubMed
 707 Central PMCID: PMCPmc4950039. Eng.
- 708 [9] Studier FW. Protein production by auto-induction in high density shaking cultures. Protein
 709 expression and purification. 2005 May;41(1):207-34. doi: 10.1016/j.pep.2005.01.016.
 710 PubMed PMID: 15915565; eng.
- 711[10]Browning DF, Matthews SA, Rossiter AE, et al. Mutational and topological analysis of the712*Escherichia coli* BamA protein. PloS one. 2013;8(12):e84512. doi:
- 713 10.1371/journal.pone.0084512. PubMed PMID: 24376817; PubMed Central PMCID:
 714 PMCPmc3871556. Eng.
- 715
- 716

717 Supplementary Fig. S1.

ACA			י א יייר	ccc		חתת	T 7 קידייי	pr	CDC	oter	<u>ر</u> سم	Ͳ᠕᠊ᢚ		[דע עי		ope	erat	or
AGA	.10.		AIC		JUGA			ATAC	GAC	, I C A	IC I A	IAG	GGG	IAAI	IGI	GAG		JA1
		х	baI									RBS			ľ	IdeI	:	
TTC	CC	CTC	TAG	AAA	TAA	TTT	TGT	TTA	ACI	TTA	AGA	AGG	AGA	TAT	'A C	TAT	'GAA	AT
																М	K	Y
						pel	LB 1	lead	ler									Nc
CTG	CCC	GAC	CGC	TGC	TGC	TGG	GTCI	GCI	GCI	CCI	CGC	TGC	CCA	GCC	GGG	CGAT	'GG C	CA
L	Ρ	Т	A	А	A	G	L	L	L	L	А	А	Q	Ρ	А	М	A	M
					Ban	HI	Ecc	RI	Sa	сI	s	alI	Hi	ndI	II	Not	I	
ATC	GGZ	AAT	'TAA	TTC	GGA	TCC	GAA	ATTC	GAG	GCTC	CGT	'CGA	CAA	GCI	TGC	CGGC	CGC	- ZAC
I	G	I	Ν	S	D	Ρ	N	S	S	S	v	D	K	L	A	А	А	I
	6H:	is	taq															
CAC	CA	CCA	CCA	CCA	ACCA	CTG	GAGA	ATCC	CGGC	CTGC	TAA	CAA	AGC	CCG	GAAA	AGGA	AGC	TG
Н	Н	Н	Н	Н	Н	Er	nd											
	GC	ГGС	CAC	CGC	TGA	GCA	ATA	ACI	AGC	ATA	ACC	CCT	TGG	GGC	СТС	CTAA	ACO	GGG
GCT	00.																	

743 Supplementary Fig. S2.

744 (a) 6his-gfp

745 **TCTAGA**AATAATTTTGTTTAACTTTAAGAAGGAGATATACC**ATG**GGCAGCAGCC**ATCATCATCATCATCAC**AGCA 746 GCGGCCTGGTGCCGCGCGGCAGC**CATATG**GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGG 747 TCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA 748 AGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCT 749 ACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAG 750 GCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGG 751 GCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC 752 TGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCA 753 AGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCCATCGGCGACG 754 GCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCCAACGAGAAGCGCG 755 ATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG**TAAGGAT** 756 CC

757

758 (b) hgh-6his

759 **CATATG**TTCCCAACCATTCCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTG 760 761 CAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCTCCAACAGGAGGAAACACAACAGAAATCCAACCTA 762 GAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCTCAGGAGTGTCTTCGCC 763 AACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAGGCATCCAAACG 764 CTGATGGGGAGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACA 765 766 767 **TAA**GGATCCGAATTC**GAGCTC**

768

769 (c) *IL-1β-6his scFv*

770 **CATATG**GATATCCAGATGACGCAGAGCCCGAGCAGCCTGAGCGCCAGCGTGGGTGACCGTGTGACCATTACCTGT 771 CGTACCAGCGGCAACATTCATAACTATCTGACCTGGTACCAGCAGAAACCGGGCAAAGCGCCGCAGCTGCTGATT 772 TATAATGCAAAAACCCTGGCAGATGGTGTGCCGAGCCGCTTTAGCGGCAGCGGTAGCGGTACCCAGTTCACCCTG 773 ACGATCAGCAGCCTGCAGCCGGAAGACTTTGCCAACTATTACTGCCAGCACTTCTGGAGCCTGCCGTTTACCTTC 774 775 AGCGGCGGTGGCGGTAGCGAGGTGCAGCTGGTTGAAAGCGGCGGTGGCCTGGTTCAGCCGGGTGGCAGCCTGCGT 776 ${\tt CTGAGCTGTGCGGCCAGCGGCTTTGATTTCAGCCGTTATGACATGAGCTGGGTGCGTCAGGCACCGGGTAAACGT}$ 777 ${\tt CTGGAATGGGTTGCCTATATTAGCAGCGGTGGCGGTAGCACCTACTTTCCGGATACGGTGAAAGGCCGCTTCACC}$ 778 ATCAGCCGTGACAACGCAAAAAATACGCTGTACCTGCAGATGAACAGCCTGCGCGCCGAAGATACCGCAGTTTAT 779 TACTGCGCCCGTCAGAATAAAAAACTGACGTGGTTCGACTACTGGGGTCAGGGCACGCTGGTTACGGTTAGCAGC 780 **CATCATCATCATCACTAATAA**GGATCCGAATTC**GAGCTC**

781

782 (d) bamA_{ENm}

783 **CATATG**GCGATGAAAAAACTGCTGATCGCGTCTCTGCTGTTCTCTGCGACCGTTTACGGTGCTAGCGAAGGT 784 TTCGTTGTTAAAGACATCCACTTTGAAGGTCTGCAACGTGTTGCGGTTGGTGCGGCGCTGCTGTCTATGCCGGTT 785 CGTACCGGCGACACCGTGAACGACGACGACACATCTCTAACACCATCCGTGCGCTGTTCGCGACCGGCAACTTTGAA 786 GACGTTCGTGTTCTGCGTGACGGTGACACCCTGCTGGTTCAGGTTAAAGAACGTCCGACCATCGCGTCTATCACC 787 TTCTCTGGTAACAAATCTGTTAAAGACGACATGCTGAAACAGAACCTGGAAGCGTCTGGTGTTCGTGTTGGTGAA 788 TCTCTGGACCGTACCACCATCGCGGACATCGAAAAAGGTCTGGAAGACTTCTACTACTCTGTTGGTAAATACTCT 789 GCGTCTGTTAAAGCGGTTGTTACCCCGCTGCCGCGTAACCGTGTTGACCTGAAACTGGTTTTCCAGGAAGGTGTT 790 TCTGCGGAAATCCAGCAGATCAACATCGTTGGTAACCACGCTTTCACCACCGACGAACTGATCTCTCACTTCCAA 791 CTGCGTGACGAAGTTCCGTGGTGGAACGTGGTTGGTGACCGTAAATACCAGAAACAGAAACTGGCGGGTGACCTG

792	GAAACCCTGCGTTCTTACTACCTGGACCGTGGTTACGCGCGTTTCAACATCGACTCTACCCAGGTTTCTCTGACC
793	CCGGACAAAAAAGGTATCTACGTTACCGTGAACATCACCGAAGGTGACCAGTACAAACTGTCTGGTGTTGAAGTT
794	TCTGGTAACCTGGCGGGTCACTCTGCGGAAATCGAACAACTGACCAAAATCGAACCGGGTGAACTGTATAACGGC
795	ACCAAAGTTACCAAAATGGAAGACGACATCAAAAAACTGCTGGGTCGTTACGGTTACGCTTACCCGCGTGTTCAG
796	TCTATGCCGGAAATCAACGACGCGGACAAAACCGTTAAACTGCGTGTGAACGTGGACGCGGGTAACCGTTTCTAC
797	GTTCGTAAAATCCGTTTTGAAGGTAACGACACCTCTAAAGACGCGGTTCTGCGTCGTGAAATGCGTCAGATGGAA
798	GGTGCGTGGCTGGGTTCTGACCTGGTTGACCAGGGTAAAGAACGTCTGAACCGTCTGGGTTTCTTTGAAACCGTT
799	GACACCGACACCCAGCGTGTTCCCGGGTTCCCCGGACCAGGTTGACGTTGTTTACAAAGTTAAAGAACGTAACACC
800	GGATCCCTGGACCTGTCTGCGGGTTGGGTTCAGGACACCGGCCTGGTTATGTCTGCGGGTGTTTCTCAGGACAAC
801	CTGTTCGGCACCGGCAAATCTGCGGCGCGCGCGCGCGCGC
802	ACCGACCCGTACTTCACCGCTGACGGTGTTTCTCTGGGTTACGACGTTTACGGTAAAGCGTTCGACCCGCGTAAA
803	GCGTCTACCTCTATCAAACAGTACAAAACCACCACCGCTGGTGCGGGTATCCGTATGTCTGTTCCGGTTACCGAA
804	TACGACCGTGTGAACTTCGGTCTGGTTGCGGAACACCTGACCGTGAACACCTACAACAAAGCGCCGAAACACTAC
805	GCGGACTTCATCAAAAAATACGGTAAAACCGACGGCACCGACGGTTCTTTCAAAGGTTGGCTGTATAAAGGCACC
806	GTTGGTTGGGGTCGTAACAAAACCGACTCTGCGCTGTGGCCGACCCGTGGTTACCTGACCGGCGTGAACGCGGAA
807	ATCGCGCTGCCGGGTTCTAAACTGCAATACTACTCTGCGACCCACAACCAGACCTGGTTCTTCCCGCTGTCTAAA
808	ACCTTCACCCTGATGCTGGGTGGTGAAGTTGGTATCGCGGGTGGTTACGGTCGTACCAAAGAAATCCCGTTCTTT
809	GAAAACTTCTACGGTGGTGGTCTGGGTTCTGTTCGTGGTTACGAATCTGGCACCCTGGGTCCGAAAGTTTACGAC
810	GAATACGGTGAAAAAATCTCTTACGGTGGTAACAAAAAGCGAACGTGTCTGCGGAACTGCTGTTCCCGATGCCG
811	GGTGCGAAAGACGCGCGTACCGTTCGTCTGTCTCTGTTCGCGGACGCGGGTTCTGTTTGGGACGGTAAAACCTAC
812	GACGACAACTCTTCTTCTGCGACCGGCGGTCGTGTTCAGAACATCTACGGTGCGGGTAACACCCACAAATCTACC
813	TTCACCAACGAACTGCGTTACTCTGCGGGTGGTGCGGTTACCTGGCTGTCTCCGCTGGGGCCCATGAAATTCTCT
814	TACGCTTACCCGCTGAAAAAAAACCGGAAGACGAAATCCAGCGTTTCCAGTTCCAACTGGGCACCACCTTC TAA
815	TGAGGGCCCATGAAGTTTAGCTATGCCTATCCATTAAAGAAGAAGCCAGAGGATGAGATTCAAAGATTTCAATTT
816	CAATTAGGTACTACTTTTGGCGGCAGATCT CTCGAG
817	

821 Supplementary Fig. S3.

822 (a) 6His-GFP Mw 29105 Da

823 MGSSHHHHHHHSSGLVPRGSHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFI
 824 CTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
 825 GDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQ
 826 QNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

827

828 (b) hGH-6His Mw 23083 Da

829 MFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREET
 830 QQKSNLELLRISLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSPRT
 831 GQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEGSCGFHHHHHHH

832

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

834 MDIQMTQSPSSLSASVGDRVTITCRTSGNIHNYLTWYQQKPGKAPQLLIYNAKTLADGVPSRFSGSGS
 835 GTQFTLTISSLQPEDFANYYCQHFWSLPFTFGQGTKVEIKRTGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVE
 836 SGGGLVQPGGSLRLSCAASGFDFSRYDMSWVRQAPGKRLEWVAYISSGGGSTYFPDTVKGRFTISRDN
 837 AKNTLYLQMNSLRAEDTAVYYCARQNKKLTWFDYWGQGTLVTVSSHHHHHH

838

839

(d) BamA_{ENm} Mw 87992 Da

840 MAMKKLLIASLLFSSATVYGASEGFVVKDIHFEGLQRVAVGAALLSMPVRTGDTVNDEDISNTIRALF 841 ATGNFEDVRVLRDGDTLLVOVKERPTIASITFSGNKSVKDDMLKONLEASGVRVGESLDRTTIADIEK 842 GLEDFYYSVGKYSASVKAVVTPLPRNRVDLKLVFQEGVSAEIQQINIVGNHAFTTDELISHFQLRDEV 843 PWWNVVGDRKYQKQKLAGDLETLRSYYLDRGYARFNIDSTQVSLTPDKKGIYVTVNITEGDQYKLSGV 844 EVSGNLAGHSAEIEQLTKIEPGELYNGTKVTKMEDDIKKLLGRYGYAYPRVQSMPEINDADKTVKLRV NVDAGNRFYVRKIRFEGNDTSKDAVLRREMRQMEGAWLGSDLVDQGKERLNRLGFFETVDTDTQRVPG 845 846 SPDQVDVVYKVKERNTGSLDLSAGWVQDTGLVMSAGVSQDNLFGTGKSAALRASRSKTTLNGSLSFTD 847 PYFTADGVSLGYDVYGKAFDPRKASTSIKQYKTTTAGAGIRMSVPVTEYDRVNFGLVAEHLTVNTYNK 848 APKHYADFIKKYGKTDGTDGSFKGWLYKGTVGWGRNKTDSALWPTRGYLTGVNAEIALPGSKLQYYSA 849 THNOTWFFPLSKTFTLMLGGEVGIAGGYGRTKEIPFFENFYGGGLGSVRGYESGTLGPKVYDEYGEKI 850 SYGGNKKANVSAELLFPMPGAKDARTVRLSLFADAGSVWDGKTYDDNSSSATGGRVQNIYGAGNTHKS 851 TFTNELRYSAGGAVTWLSPLGPMKFSYAYPLKKKPEDEIQRFQFQLGTTF 852

E. coli BL21 pET26b



861 Supplementary Fig. S5.

E. coli W3110 pET22b



a)

kDa

71 · 55 ·

867

868

869

















