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New vectors for urea-inducible recombinant protein production

Joanne Hothersall [a,*], Alexander Osgerby [b], Rita E. Godfrey [a], Tim W. Overton [b], Stephen J.W. Busby [a,*], Douglas F. Browning [a,c,**]

[a] Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK
[b] School of Chemical Engineering, University of Birmingham, Birmingham B15 2TT, UK
[c] College of Health & Life Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK

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ABSTRACT

We have developed a novel urea-inducible recombinant protein production system by exploiting the *Proteus mirabilis* urease *ureR-ureD* promoter region and the *ureR* AraC-family transcriptional regulator. Experiments using the expression of β-galactosidase and green fluorescent protein (GFP) showed that promoter activity is tightly regulated and that varying the concentration of urea can give up to 100-fold induction. Production of proteins of biopharmaceutical interest has been demonstrated, including human growth hormone (hGH), a single chain antibody fragment (scFv) against interleukin-1β and a potential *Neisserial* vaccine candidate ($BamA_{ENm}$). Expression levels can be fine-tuned by temperature and different urea concentrations, and can be induced with readily available garden fertilisers and even urine. As urea is an inexpensive, stable inducer, a urea-induced expression system has the potential to considerably reduce the costs of large-scale recombinant protein production.

1. Introduction

Recombinant protein production (RPP), where a gene of interest is cloned into an expression vector and transferred to a particular host, enables the expression of large amounts of quality protein with experimental, industrial and therapeutic applications (for a review see [1]). In particular, the model organism *Escherichia coli*, which can be easily genetically manipulated, is a popular choice of host, allowing rapid growth to high cell densities on inexpensive carbon sources [2,3]. Bacterial cultures are typically grown in rich medium to mid-logarithmic phase, and over-expression of heterologous proteins initiated with the addition of an inducer molecule. The target gene is often cloned downstream of a strong promoter, such as a *lac*-based promoter, which is inducible with isopropyl β-*D*-1-thiogalactopyranoside (IPTG) [4–6]. Similarly, IPTG induction is used with the T7 RNA polymerase expression system to direct high level protein expression from genes cloned under the control of a strong T7 promoter [7,8]. However, high levels of protein production are not always desirable as proteins may mis-fold, aggregating into inclusion bodies or becoming degraded. Thus, conditions often need to be optimised through careful expression system and host selection, altering culture conditions (*e.g.*, growth temperature), or even engineering the target protein itself [9,10].

The main limitations of using IPTG induction for RPP are its cost, in particular for large-scale production, and its stability, with the requirement for a refrigerated supply chain [11]. Other over-expression systems have been developed that utilise alternative inducer molecules, such as the pBAD expression system, which is induced by arabinose, through the activity of the AraC regulator [12]; and the *ogt101* and *narG* promoters, which are regulated by the NarL activator protein, through induction by cheap and stable inorganic nitrate ions [11]. Alternatively, autoinduction medium, containing lactose, as defined in [8], can be used to switch on expression from *lac*-based promoters without the need to monitor cell density and some systems can utilise temperature shifts [13].

Urea is a cheap, stable and readily available molecule and therefore would be an ideal inducer to promote inexpensive RPP. In *Proteus*

Abbreviations: AU, artificial urine; BAM, β-barrel assembly machinery; GFP, green fluorescent protein; hGH, human growth hormone; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, lysogeny broth; OMP, outer membrane protein; ONPG, O-nitrophenyl-β-D-galactopyranose; PI, propidium Iodide; POTRA, polypeptide transport-associated; RPP, recombinant protein production; scFv, single chain variable region antibody fragment.

* Corresponding authors.
** Corresponding author at: Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK.
E-mail addresses: j.hothersall@bham.ac.uk (J. Hothersall), s.j.w.busby@bham.ac.uk (S.J.W. Busby), d.browning@aston.ac.uk (D.F. Browning).

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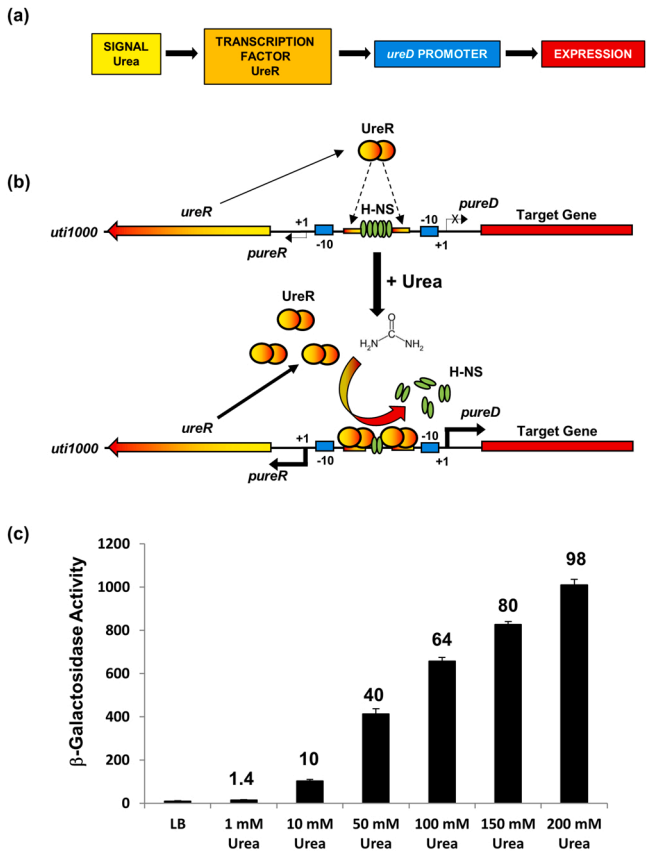

Fig. 1. Experiments with the *uti1000* promoter fragment. (a) Control of gene expression by urea and the UreR transcription activator protein. In the presence of urea, UreR activates transcription from the *ureD* promoter to drive transcription of downstream genes. (b) A schematic representation of the *uti1000* promoter and its regulation by UreR. In the absence of urea, a low basal level of UreR is present allowing the system to be poised and triggered rapidly in response to urea. UreR binds weakly to the two UreR-binding sites within the *ureR-ureD* regulatory region and competes directly with the nucleoid associated protein H-NS. In the presence of urea, UreR undergoes a conformational change and occupies both of its binding sites within the *ureR-ureD* intergenic region, outcompeting H-NS to activate transcription from the *ureD* and *ureR* promoters [16,19]. The *ureR* and *ureD* −10 promoter elements are shown as rectangles, and transcript start sites (+1) are indicated by bent arrows. (c) The panel shows measured β-galactosidase activities in wild-type JCB387 cells, carrying the *uti1000* promoter fragment cloned into pRW50. Cells were grown in LB medium and supplemented with various concentrations of urea, as indicated. β-Galactosidase activities are expressed as nmol ONPG hydrolysed min^{-1} mg^{-1} dry cell mass and represent the average of three independent experiments. Error bars show standard deviation. Values above bars indicate the fold induction at each urea concentration.

mirabilis, urea induces the expression of the seven gene *ureDABCEFG* operon required for the synthesis and maturation of urease [14]. Induction is due to the activity of UreR, an AraC-family transcription factor, encoded by the *ureR* gene, located divergently upstream of *ureD* (Fig. 1). In the absence of urea, there is a basal low-level of UreR within cells. Under these conditions, UreR is able to bind weakly to two UreR-binding sites within the *ureR-ureD* regulatory region but is unable to activate transcription. In the presence of urea, UreR undergoes a conformational change, enabling it to bind tightly to its sites and activate transcription of both *ureR* and *ureDABCEFG* [15–17]. Additional temperature-dependent regulation is mediated by H-NS, an abundant nucleoid associate protein, that represses by binding to a poly(A) tract in the *ureR-ureD* intergenic region (Fig. 1). H-NS binding causes DNA bending that interferes with UreR binding in the absence of urea [18, 19]. In this study, a novel urea-induced expression cassette, comprised of the UreR transcriptional regulator and the *ureR-ureD* regulatory region, has been developed. Its use for over-expressing recombinant proteins in *E. coli* is described.

2. Materials and methods

2.1. Bacterial strains, plasmids, and materials

Details of bacterial strains used in this study are listed in Supplementary Table S1. *E. coli* strains XL1 Blue and JCB387 were used for DNA manipulation and plasmid construction, whilst JCB387, BL21, W3110 and SHuffle® Express were used for RPP. Cultures were grown in lysogeny broth (LB) (Sigma, Gillingham, UK) supplemented with appropriate antibiotics (ampicillin 100 μg/ml, tetracycline 15 μg/ml) as required. Details of plasmids and promoter fragments used and generated in this study are listed in Supplementary Table S1.

2.2. Vector construction

The *uti1000* expression cassette was amplified from the genome of *P. mirabilis* NCTC 10975 by PCR, using primers ureDFw and ureRRev (Supplementary Tables S1 and S2). The purified PCR fragment was restricted with *Eco*RI and *Bam*HI and cloned into expression vector pRW50 [20]. The DNA sequence of the *uti1000* expression cassette is shown in Supplementary Fig. S1. The *uti101* DNA fragment was amplified by PCR with primers uti101(*Bgl*II) and uti101(*Xba*I) using pRW50/ *uti1000* as template. Purified DNA was cut with *Bgl*II and *Xba*I and cloned into the Novagen pET20b expression vector (Merck Life Science, Watford, UK), and pET15b/ *6his-gfp* [5], replacing the T7 RNA polymerase promoter. The DNA encoding hGH-6His and anti-IL-1β-6His single-chain variable fragment (scFv), from pHAK1 and pYU49, respectively, was cloned into pET20b/ *uti101* using *Nde*I and *Sac*I [21, 22]. The DNA encoding the chimeric $BamA_{ENm}$ protein was cloned into pET20b/ *uti101*, using *Nde*I and *Xho*I [23]. All constructs were verified by Sanger DNA sequencing. The amino acid sequences of target proteins are shown in Supplementary Fig. S2.

2.3. β-galactosidase assays

The low copy number *lacZ* transcription fusion plasmid, pRW50, containing the *uti1000* expression cassette was transferred into *E. coli* JCB387 and β-galactosidase expression was measured using the Miller protocol that assays enzyme activity [24], as in previous work [25]. Overnight cultures were inoculated into LB medium, supplemented with a range of urea concentrations (0–200 mM), and grown to OD_{650} 0.4–0.6. β-Galactosidase activities are expressed as nmol O-nitrophenyl-β-D-galactopyranose (ONPG) hydrolysed/min/g dry cell mass.

2.4. Recombinant protein over-expression and detection

For RPP, bacterial cultures carrying pET vectors, containing various target genes under the control of the *uti101* promoter (and *lac*-based promoters), were grown in 10 ml LB with shaking. *E. coli* strains JCB387, BL21 and W3110 were routinely grown at 37 °C, except where stated, whilst SHuffle® Express cells was grown at 30 °C. Recombinant protein over-expression from cultures in mid-logarithmic phase (OD_{600} 0.3–0.5) was routinely induced from the *uti101* promoter with 100 mM urea and from *lac*-based promoters with 1 mM IPTG. Samples were taken after 3 h induction. Where garden fertilisers were used as alternative inducers, solutions of BabyBio (SBM Life Science, Ecully, France), Miracle-Gro All Purpose and Miracle-Gro LiquaFeed (Evergreen Garden Care, Surrey, UK) were added to a final concentration of 1% (v/v or w/v). Where artificial urine (AU) was used as an alternative inducer (prepared as in [26], with the addition of peptone 1 g/L (Oxoid), yeast extract 5 mg/L (Oxoid), pH 7.0), cultures were grown to mid-logarithmic phase in 2X LB, then induced with an equal volume of AU, or with distilled water for

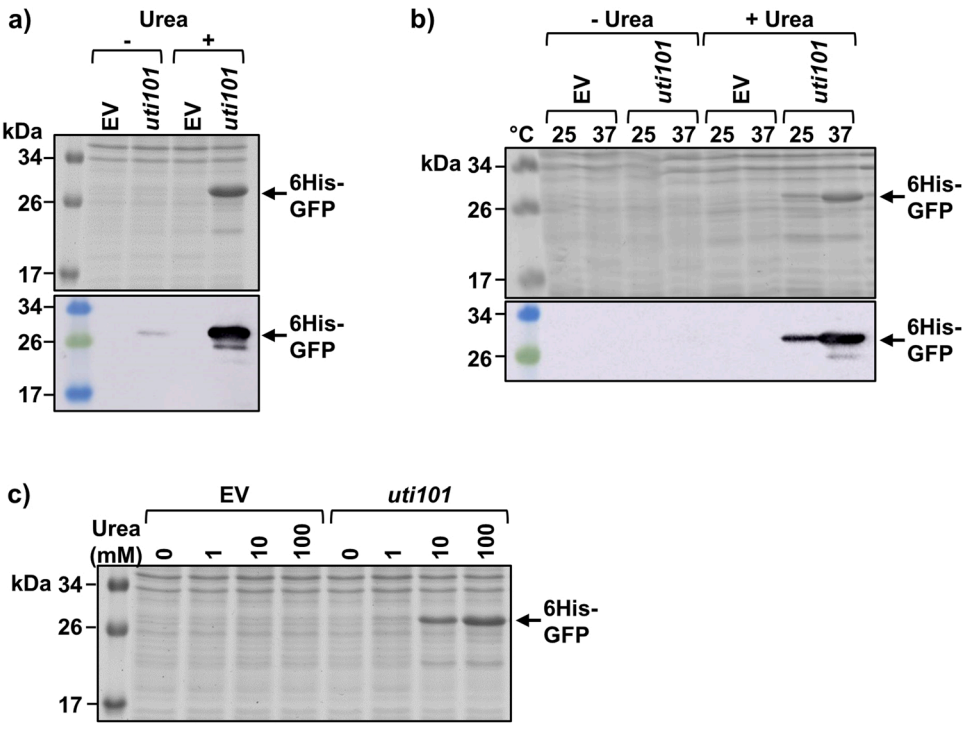

Fig. 2. Recombinant protein production with the *uti101* promoter fragment. The figure shows 6His-GFP expression from the *uti101* promoter in JCB387 cells grown in LB at different temperatures and induced with various urea concentrations for 3 h. SDS-PAGE gels were stained with Coomassie blue (above) and Western blots detected with anti-GFP antibody (below). (a) 6His-GFP expression was induced (+) with 100 mM urea and cells grown at 37 °C. (b) Shows the effect of temperature (25 °C vs 37 °C) on expression from the *uti101* promoter induced (+) with 100 mM urea. (c) Shows the effect of induction with a range of different urea concentrations, as indicated, at 37 °C. Empty vector controls (EV) were included. 6His-GFP protein was detected using mouse anti-GFP antibody (Sigma, catalogue number G6795) at a 1:5000 dilution and sheep anti-mouse HRP secondary antibody (Sigma, catalogue number A6782) at a 1:10,000 dilution.

a no induction control.

Total protein samples were prepared by resuspending normalised amounts of cells in 2X Laemmli loading buffer (Sigma), heating at 95 °C for 3 min then centrifuging prior to loading as in [27]. Soluble and insoluble protein samples were prepared by lysis of bacterial cells by BugBuster® Master Mix (Novagen, Merck Life Science), according to the manufacturer's recommended procedure. Samples were resolved by reducing sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by Coomassie blue staining and Western blotting. For Western blotting analysis, 6His-GFP was detected with a mouse anti-GFP antibody (Sigma) (catalogue number G6795, Lot # 127M4785V) (1:5000 dilution) and sheep anti-mouse-HRP (horseradish peroxidase) secondary antibody (Sigma) (catalogue number A6782, Lot # SLBX6429) (1:10000 dilution). Recombinant hGH-6His was detected using rabbit anti-hGH antiserum [6] (1:10000 dilution) with donkey anti-rabbit-HRP secondary antibody (Amersham, GE Healthcare, Little Chalfont, UK) (catalogue number NA934, Lot # 16976257) at a 1:10000 dilution. Anti-hGH antiserum specificity was previously demonstrated in [6]. Recombinant anti-IL-1β-6His scFv was detected using anti-6His (C-terminal)-HRP monoclonal antibody (3D5) (Invitrogen, Carlsbad, CA, USA, catalogue number R931–25) (1:10000 dilution). The $BamA_{ENm}$ chimera was detected using rabbit anti-BamA POTRA (polypeptide transport-associated) antiserum (1:5000 dilution) [27] with donkey anti-rabbit-HRP secondary antibody (Amersham, GE Healthcare) (1:10000 dilution). Anti-*E. coli* BamA POTRA antiserum specificity was demonstrated previously in [27]. Blots were developed using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA) and all gels and blots shown are representative of replica experiments.

2.5. Flow cytometry analysis

For flow cytometry analysis, 50 ml LB cultures of JCB387 carrying pET15b/ *uti101*/ *6his-gfp* were incubated with shaking at 37 °C until the culture reached OD_{600} 0.4–0.6, and then RPP was induced by the addition of 0–500 mM urea, as stated. Samples were analysed using a BD Accuri C6 flow cytometer (Becton Dickinson, UK). Samples were diluted with phosphate-buffered saline (PBS) (0.2 μm-filtered) prior to analysis to permit a data rate of 1000–4000 events sec^{-1} at a slow flow rate using a forward scatter height (FSC-H) threshold of 10,000 to distinguish between bacteria and particulate noise. The sample was illuminated with a 488 nm laser and green fluorescence from GFP detected using a 533/30 BP filter (channel FL1). Dead cells were detected using the membrane-impermeant dye propidium iodide (PI) at a concentration of 4 μg/ml as previously described [28]; PI fluorescence was detected using a 670 LP filter (channel FL3). Data were analysed using CFlow (BD).

3. Results

3.1. Construction and RPP with the uti1000 and uti101 promoters

A 1477 kb fragment (*uti1000*), encoding the *ureR* transcription activator, and containing the intergenic bidirectional promoter between *ureR* and *ureD*, was amplified from the DNA of *P. mirabilis* strain NCTC 10975. This was cloned into the *lacZ* reporter plasmid pRW50 and transferred to *E. coli* K-12 JCB387 cells to be assessed for urea inducible promoter activity (Fig. 1). β-Galactosidase assays showed that the activity of the *ureD* promoter in this fragment is tightly regulated, and that varying the concentration of urea from 1 to 200 mM resulted in up to a 100-fold induction.

A shorter version of the *uti1000* promoter fragment (*uti101*), lacking most of the untranslated region of the *ureD* transcript (Supplementary Fig. S1), was amplified from *uti1000* and cloned into pET15b, containing the N-terminally 6-His-tagged green fluorescent protein (6His-GFP). This organisation enables the use of the strong ribosome binding site found in pET vectors, as well as their extensive multiple cloning sites. Expression of 6His-GFP from the *ureD* promoter in the *uti101* fragment was then investigated by transferring the plasmid to *E. coli* JCB387, growing the cells in LB medium to mid-logarithmic growth at 37 °C, and inducing with 100 mM urea for 3 h. 6His-GFP production was analysed by Coomassie blue-stained SDS-PAGE and Western blotting (Fig. 2a) and showed high expression of 6His-GFP with urea induction, with only very low-level expression in the absence of urea.

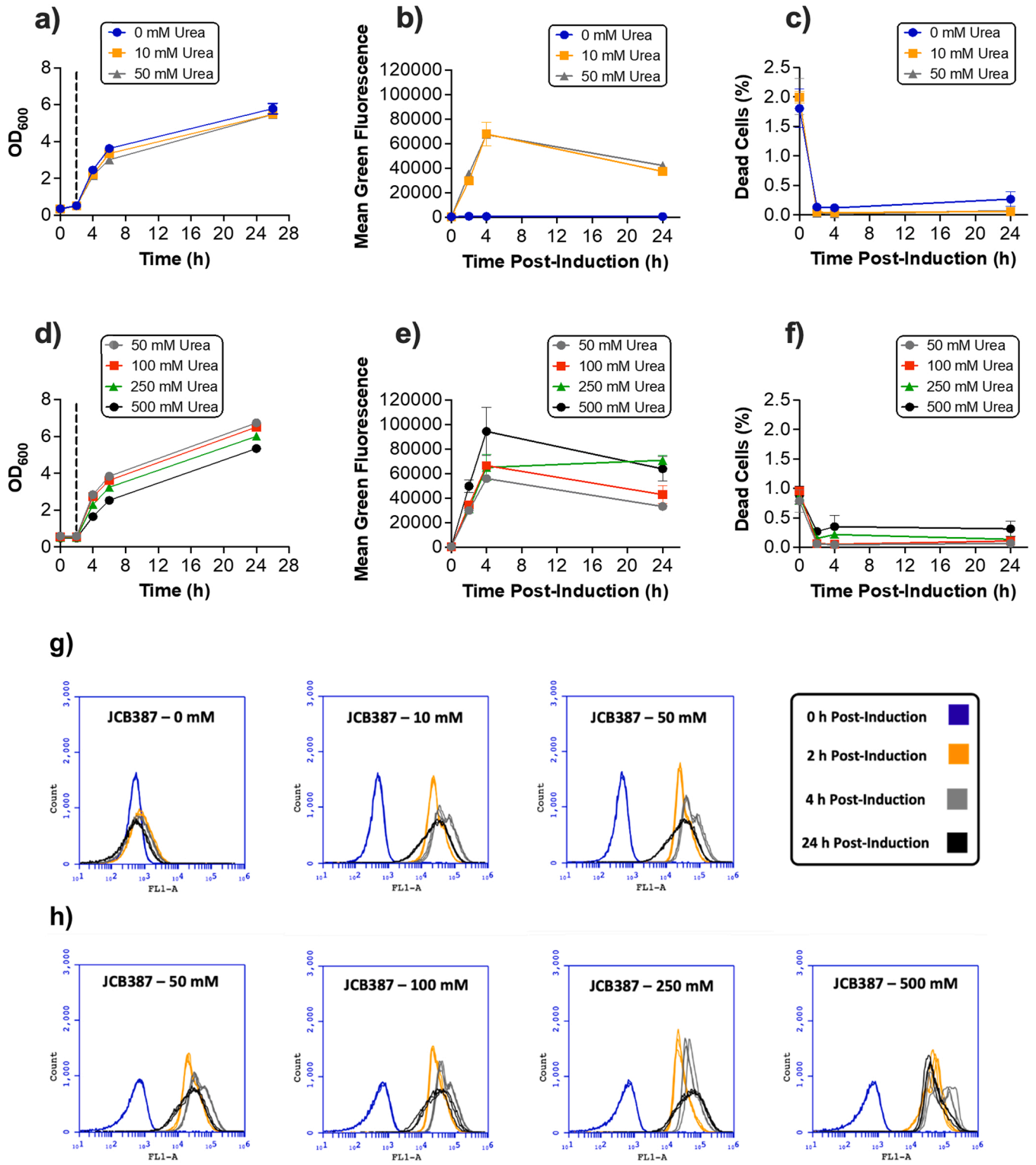

Fig. 3. Flow cytometry analysis of 6His-GFP expression from the *uti101 promoter* induced with a range of urea concentrations. JCB387 cells were grown in LB at 37 °C for 28 h and induced with different urea concentrations (as indicated in each key) after 2 h growth. Panels (a, d) show the effect on cell growth, with the time point of urea induction shown as a dotted line; (b, e) show mean green fluorescence indicating 6His-GFP expression; and (c, f) show the % of cells that are judged to be dead after PI staining. Error bars represent standard deviation; n = 3. (g, h) Show flow cytometry analyses of green fluorescence. Data is plotted as histograms showing the number of cells with different green fluorescence (FL1-A) values.

3.2. Manipulating RPP from the uti101 promoter fragment: temperature, inducer concentration and host effects

RPP systems need to be flexible and sometimes require fine-tuning to achieve lower levels of expression, for example if the recombinant protein is toxic to the host or difficult to fold. Previously Poore and Mobley [19] noted that both temperature and urea concentration are factors in regulation of the urease gene cluster, through the activity of

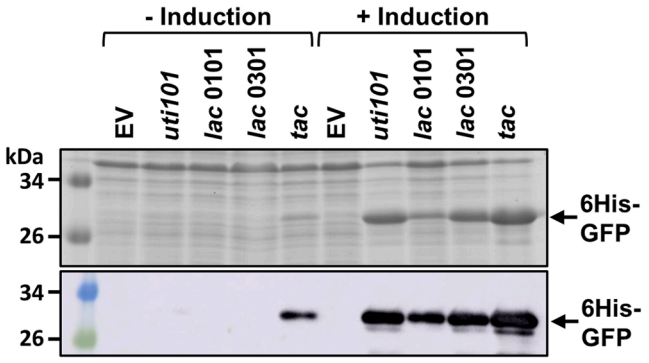

Fig. 4. Comparison of RPP from the *uti101* system with other *lac*-based expression systems. This figure shows a Coomassie blue-stained SDS-PAGE gel of BL21 cells carrying 6His-GFP cloned under the control of either the *uti101* promoter, weak or medium strength *lac*-based promoters *lac* 0101 and *lac* 0301, respectively, or the strong *tac* promoter. The cells were grown in LB medium and induced (+) with either 100 mM urea (*uti101*) or 1 mM IPTG (*lac* and *tac*) at mid-logarithmic growth. Empty vector controls (EV) were included.

H-NS repression and UreR activation (Fig. 1). In particular, urease expression and activity increased when cultures were grown at 37 °C compared to 25 °C and this effect was observed in *E. coli* cells carrying the complete *P. mirabilis* urease gene cluster [19]. Therefore, 6His-GFP expression from the *uti101* promoter was compared between JCB387 cells grown at 25 °C and 37 °C, and analysed by Coomassie blue-stained SDS-PAGE and Western blotting. As predicted, levels of 6His-GFP were lower at 25 °C than 37 °C (Fig. 2b). Furthermore, a range of 6His-GFP expression levels could be achieved by varying the concentration of urea inducer from 1 to 500 mM (Fig. 2c and Fig. 3). Low levels of 6His-GFP expression were induced by as little as 1 mM urea. Conversely, a high level of expression could be achieved with 500 mM urea, without adverse effects on bacterial cell growth (Fig. 3 and Supplementary Fig. S3). Live/dead cell staining with propidium iodide revealed that even at 500 mM, urea did not lead to significant bacterial death (Fig. 3c, f). When expression was left for longer periods of time, 6His-GFP RPP was maintained, with green fluorescence detected 24 h after induction (Fig. 3b, e). It was also evident, through monitoring 6His-GFP levels in individual cells by flow cytometry, that 6His-GFP induction is broadly homogeneous within the bacterial cell population, at each urea concentration and timepoint (Fig. 3 g, h).

To examine the flexibility of the u*ti101* promoter system, 6His-GFP expression was determined in a range of host strains, including the *E. coli* K-12 strain W3110 and the *E. coli* B strain BL21 (Supplementary Fig. S4). In each case, 6His-GFP expression was seen to be comparable. Additionally, the strength of the *uti101* promoter was compared with other *lac*-based promoters (*i.e.,* the weak *lac* 0101 promoter, the intermediate strength *lac* 0301 promoter and the strong but leaky *tac* promoter) [4–6]. Levels of 6His-GFP from the *uti101* promoter in BL21 with 100 mM urea induction were equivalent to expression levels from the intermediate strength *lac* 0301 promoter, when fully induced with 1 mM IPTG (Fig. 4). Note that cell growth was similar with the different promoter expression systems (Supplementary Fig. S5), indicating that the urea-mediated induction did not impose a critical burden on cells.

3.3. RPP of biopharmaceuticals

To demonstrate the versatility of the urea-inducible RPP system for expressing products of biopharmaceutical interest, the *uti101* promoter fragment was cloned into a series of pET20b vectors encoding C-terminally 6-His tagged human growth hormone (hGH-6His), or an scFv antibody fragment against interleukin-1β (anti-IL-1β-6His scFv). The resulting constructs were transferred into JCB387 cells, grown at 37 °C and induced with 100 mM urea at mid-logarithmic phase. Coomassie

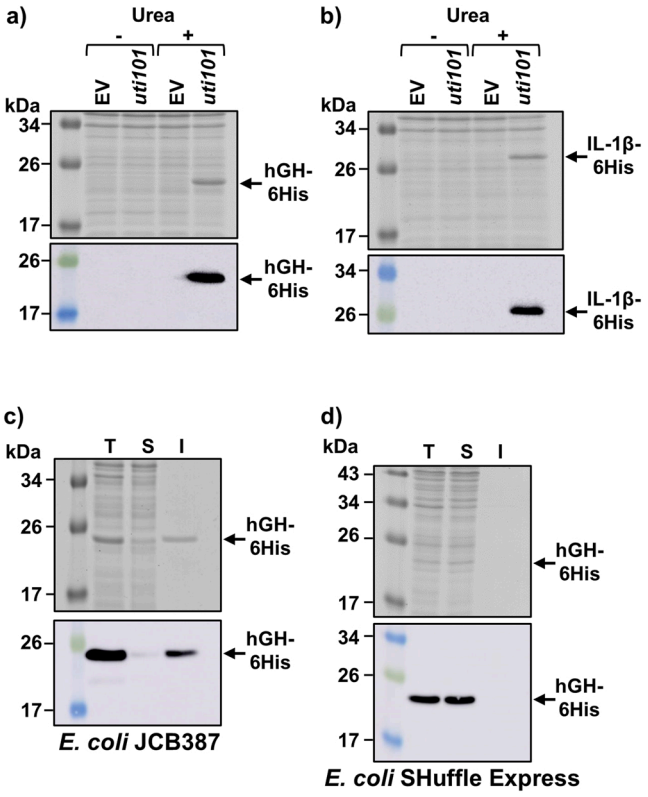

Fig. 5. RPP of biopharmaceuticals using the *uti101* system. (a, b) show Coomassie blue-stained SDS-PAGE gels (above) and Western blots (below) detailing expression of recombinant proteins from JCB387 cells carrying (a) pET20b/ *uti101/ hgh-6his* and (b) pET20b/ *uti101/ anti-IL-1β-6his scFv*. Cells were grown in LB at 37 °C and induced (+) with 100 mM urea at mid-logarithmic growth. Empty vector controls (EV) were included. To assess protein solubility cells carrying pET15b/ *uti101/ hgh-6his* harvested from (c) JCB387, grown and induced as above, and from (d) *E. coli* SHuffle® Express, grown and induced as above but at 30 °C. Cells were lysed to prepare total (T), soluble (S) and insoluble (I) protein samples. Coomassie blue-stained SDS-PAGE gels (above) and Western blots (below) detail expression of recombinant hGH-6His and anti-IL-1β-6His scFv. Recombinant hGH-6His was detected, using rabbit anti-hGH antiserum (1:10000 dilution) and donkey anti-rabbit HRP secondary antibody (Amersham, GE Healthcare, catalogue number NA934) (1:10000 dilution). The specificity of anti-hGH antiserum was demonstrated in [6]. Anti-IL-1β-6His scFv was detected using anti-6His (C-terminal)-HRP monoclonal antibody (3D5) (Invitrogen, catalogue number R931–25) (1:10000 dilution).

blue stained SDS-PAGE and Western blotting clearly indicated that substantial levels of each of target were produced (Fig. 5). hGH requires disulphide bond formation to fold correctly and avoid inclusion body aggregation [5,11]. However, the normal reducing environment of the *E. coli* cytoplasm does not promote disulphide bond formation. Thus, unsurprisingly, fractionation of JCB387 cells, expressing hGH-6his, into their soluble and insoluble protein components, indicated that the majority of hGH-6His protein was insoluble (Fig. 5c). Therefore, pET20b/ *uti101/ hGH-6his* was transferred to *E. coli* SHuffle® Express, which is genetically modified to enable cytoplasmic disulphide bond formation [29]. Remarkably, in the SHuffle® Express host strain, all detectable hGH-6His was in the soluble fraction (Fig. 5d), highlighting the flexibility of the *uti101* expression system in operating in different strains.

In addition, the *uti101* promoter fragment was also tested for its ability to express a potential *Neisserial* vaccine candidate, $BamA_{ENm}$, a large chimeric outer membrane protein (OMP) from *Neissieria meningitidis* [5,23]. Note that, in Gram-negative bacteria, the BAM complex (β-barrel assembly machinery) is required for insertion of β-barrel-containing OMPs into the outer membrane [30]. The BamA protein is an important part of this complex being an outer membrane protein and

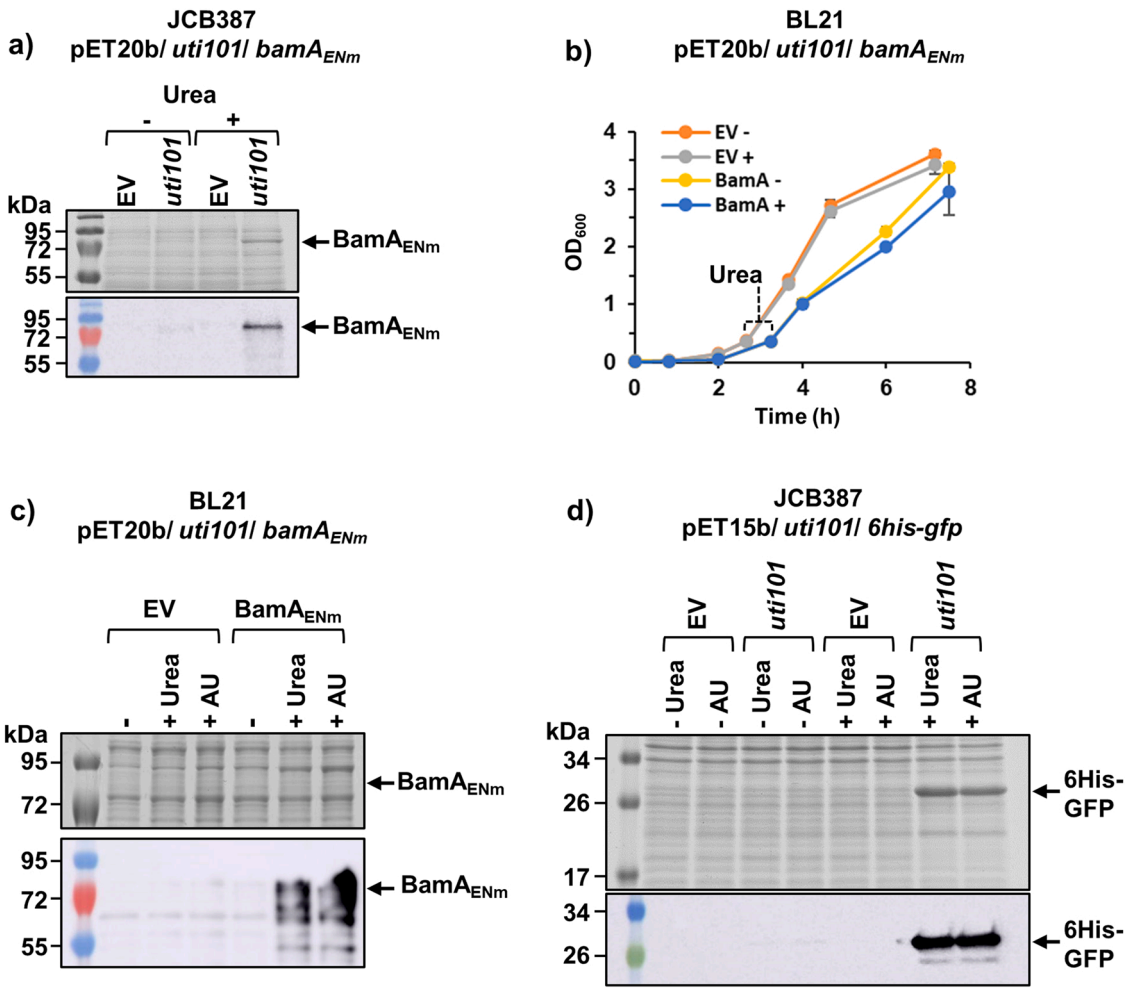

Fig. 6. Expression of a potential *Neisserial* vaccine candidate $BamA_{ENm}$ and induction with artificial urine. (a) Coomassie blue-stained SDS-PAGE gel (above) and Western blot (below) detail the detection of $BamA_{ENm}$, which is a chimeric protein of the *N. meningitidis* C-terminal β-barrel domain and the *E. coli* N-terminal polypeptide transport-associated (POTRA) domains. JCB387 cells carrying pET20b/ *uti101/ bamA*$_{ENm}$ were grown in LB at 37 °C and induced (+) with 100 mM urea at mid-logarithmic growth. Panel (b) shows the effect of expressing $BamA_{ENm}$ on BL21 cell growth. Cells carrying pET20b/ *uti101/ bamA*$_{ENm}$ were grown in LB and induced with 100 mM urea at mid-logarithmic growth (OD_{600} = 0.3–0.5), as indicated by a black dotted line. (c, d) Comparisons of recombinant protein expression levels when the *uti101* promoter was induced with 100 mM urea or artificial urine (AU) are shown for (c) $BamA_{ENm}$ in BL21 and (d) 6His-GFP in JCB387. Empty vector controls (EV) were included. $BamA_{ENm}$ was detected using rabbit anti-BamA POTRA antiserum (1:5000 dilution) and a donkey anti-rabbit-HRP secondary antibody (Amersham, GE Healthcare) (1:10000 dilution). The specificity of the anti-*E. coli* BamA POTRA antiserum was demonstrated in [27]. 6His-GFP was detected with mouse anti-GFP antibody (Sigma, catalogue number G6795) (1:5000 dilution) and sheep anti-mouse HRP secondary (Sigma, catalogue number A6782) (1:10000 dilution).

viewed as a potential vaccine target [5,31]. Thus, the DNA encoding $BamA_{ENm}$ was cloned into pET20b/ *uti101*, transferred into JCB387 cells and expression was induced with 100 mM urea. SDS-PAGE and Western blotting confirmed that the 88 kDa $BamA_{ENm}$ was expressed (Fig. 6a), however, during the experiment, cell cultures stopped growing (results not shown), confirming previous results that the over-expression of this target can be toxic [5,23]. To circumvent this, pET20b/ *uti101/ bamA*$_{ENm}$ was transferred to BL21 cells and the effect on bacterial growth, during $BamA_{ENm}$ urea-induced expression, was monitored. In this situation, production of $BamA_{ENm}$ was detected, but had very little effect on growth (Fig. 6b, c).

3.4. Induction with artificial urine and inexpensive garden fertilisers

A potential application of any urea-inducible RPP system would be to generate a delivery system that specifically targets the human urinary tract. This would require the system to be induced with urine. Thus, to investigate whether the *uti101* promoter could be induced in this manner, both BL21 containing pET20b/ *uti101/ bamA*$_{ENm}$, and JCB387 containing pET15b/ *uti101/ 6his-gfp*, were grown to mid-logarithmic phase and induced with the addition of artificial urine (AU). Expression of both $BamA_{ENm}$ and 6His-GFP from the *uti101* fragment was found to be induced well with AU (Fig. 6c, d).

Although purified urea is relatively inexpensive, a desirable outcome would be to generate an industrial-scale expression system that would benefit locations where infrastructure is limiting and where maintaining a cold supply chain is difficult. Since the urea content of garden fertilisers is high, it was investigated whether such products could be used to switch on the *uti101* promoter. Results in Fig. 7 show that 1 % solutions of Miracle-Gro, BabyBio and LiquaFeed fertilisers induce 6His-GFP production to levels similar to those found with 100 mM urea.

4. Discussion

In this study, a novel urea-inducible bacterial expression system has been developed that exploits the bi-directional promoters of the *P. mirabilis* intergenic *ureR* and *ureD* regulatory region and the *ureR* gene, encoding the UreR transcription activator (Fig. 1). Previously, UreR-

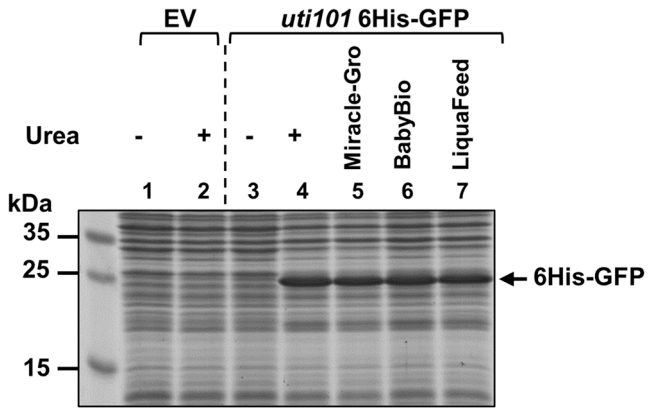

Fig. 7. Induction of the *uti101* promoter using various garden fertilisers. The figure shows a Coomassie blue strained SDS-PAGE gel, detailing 6His-GFP expression in JCB387 cells carrying the pET15b/ *uti101/ 6his-gfp* expression plasmid. Cells were grown in LB medium and RPP was induced for 3 h by the addition of 100 mM urea or various household fertilisers (Miracle-Gro All Purpose, BabyBio and Miracle-Gro LiquaFeed) to a final concentration of 1% (w/v or v/v). Empty vector controls (EV) have been included.

dependent activation of the *ureD* promoter in *E. coli* was demonstrated [32], and it is known that while urea can alter gene expression profiles, *E. coli* growth is largely unaffected by even high levels of urea [33]. The present experiments showed that, in our constructs, the *ureD* promoter is tightly regulated and up to 100-fold activation can be achieved with induction by urea. With full induction, 6His-GFP expression levels were comparable to medium strength expression systems such as the IPTG inducible *lac* O3O1 promoter [5]. Expression levels could also be fine-tuned by varying urea inducer concentration. Very low levels of induced protein production were detected with just 1 mM urea and high levels were achieved with 500 mM, without adverse effects on cell growth. Flow cytometry showed that 6His-GFP expression levels were uniform in cell populations at each urea concentration, i.e. the whole population was expressing at a particular level rather than the mixed populations of fully induced and uninduced cells that have been seen in other systems [34,35]. Expression is also thermoregulated, with lower expression observed at 25 °C compared to 37 °C, as found for the native *P. mirabilis* urease cluster [19]. Thus, the *uti101* promoter expression system reported here is a flexible system, allowing different levels of expression to be set as required for the target protein. To demonstrate its potential as an industrially relevant expression system, various biopharmaceutical targets were used, including hGH, an scFv antibody fragment, and a potential *Neisserial* vaccine target, $BamA_{ENm}$. Production of these was achieved and, when toxicity impaired bacterial growth with $BamA_{ENm}$ over-expression, the problem was overcome by selecting a different host. Similarly, hGH solubility was greatly improved by switching strains to the SHuffle® Express cells, to promote cytoplasmic disulphide bond formation [29]. Note that SHuffle hosts have previously been used to express soluble antibodies, including scFv fragments [5,11, 36].

The cost of popular IPTG induced expression systems can be prohibitive, whereas urea is cheap, plentiful and stable, making the *uti101* promoter expression system an inexpensive system that could potentially reduce the cost of large-scale industrial RPP. Due to their high urea content, easily available garden fertilisers can be used for induction, making a system that can benefit locations where infrastructure is limiting. Furthermore, as urine can be used as an inducer, the *uti101* promoter expression system has potential to target the urinary tract, thereby facilitating bacterial delivery of therapeutic proteins to the bladder. Future experimentation will therefore involve examining the expression of additional biotherapeutics and urea-induced expression in large scale fermenters.

CRediT authorship contribution statement

J.H., T.W.O., S.J.W.B. and D.F.B. devised the research programme, experiments were performed by J.H., A.O., R.E.G. 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.

Declaration of Competing Interest

The Authors declare no conflict of interest.

Data availability

All data relating to this article are present in the article and the accompanying Supplementary Material.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.10.003.

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