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A general strategy for the production of difficult-to-express inducer-dependent bacterial repressor proteins in *Escherichia coli*

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ABSTRACT

Inducer-dependent prokaryotic transcriptional repressor proteins that originally evolved to orchestrate the transcriptome with intracellular and extracellular metabolite pools, have become universal tools in synthetic biology, drug discovery, diagnostics and functional genomics. Production of the repressor proteins is often limited due to inhibiting effects on the production host and requires iterative process optimization for each individual repressor. At the example of the *Streptomyces pristinaespiralis*-derived streptogramin-dependent repressor PIP, the expression of which was shown to inhibit growth of *Escherichia coli* BL21*, we demonstrate that the addition of the PIP-specific streptogramin antibiotic pristinamycin I neutralizes the growth-inhibiting effect and results in >100-fold increased PIP titers. The yield of PIP was further increased 2.5-fold by the engineering of a new *E. coli* host suitable for the production of growth-inhibiting proteins encoded by an unfavorable codon usage. PIP produced in the presence of pristinamycin I was purified and was shown to retain the antibiotic-dependent binding to its operator *pir* as demonstrated by a fluorescence resonance energy transfer (FRET)-based approach. At the example of the macrolide-, tetracycline- and arsenic-dependent repressors MphR(A), TetR and ArsR, we further demonstrate that the production yields can be increased 2- to 3-fold by the addition of the cognate inducer molecules erythromycin, tetracycline and As^{3+}, respectively. Therefore, the addition of inducer molecules specific to the target repressor protein seems to be a general strategy to increase the yield of this interesting protein class.

Prokaryotic repressor proteins responsive to allosteric regulation by small molecules are coordinating the bacterial transcriptome with changing environmental conditions to optimize nutrient utilization or to defend toxic insults [1–4]. For example, the transcriptional repressors TetR [3], PIP [2] and MphR(A) [4] dissociate from their cognate operators (*tetO*, *pir* and *etr*, respectively) in the presence of tetracycline, streptogramin and macrolide antibiotics thereby de-repressing the expression of resistance genes to enable the export or neutralization of the antibiotic compound and survival of the host cell.

The antibiotic-dependent interaction between a repressor protein and its cognate operator was applied in the design of inducible gene switches in mammalian cells and mammals [5–8], in the production of biopharmaceuticals [9,10], in drug discovery [11,12], in diagnostics [13,14], in synthetic biology [15–19] as well as in many basic and applied research studies [20–22]. For example, the TetR, PIP and MphR(A) repressor proteins, evolutionary optimized to specifically detect their target antibiotic within complex samples, were recently incorporated into an ELISA-like configuration for the class-specific quantification of tetracycline-, macrolide- and streptogramin antibiotics in food samples [13,14]. For this aim, the operator was immobilized in a microtiter plate, loaded with its cognate repressor and exposed to antibiotic-containing samples resulting in an antibiotic-dependent dissociation of the repressor from its operator. Residual operator-bound repressors were quantified by enzyme-linked antibodies and the antibiotic concentration in the sample was determined using a calibration curve. Integrating TetR, PIP and MphR(A) into this setup enabled the rapid detection and quantification of different tetracycline, streptogramin and macrolide antibiotics below the maximum residual limit allowed for food stuff [14]. Large scale manufacturing of such diagnostic devices requires the production of the respective repressor proteins at mg–g scale, a challenging task given the low titers often obtained for transcriptional repressor proteins in *Escherichia coli* host cells. Several studies describe circuitous production methods for repressor proteins like the expression in the uninduced state (without IPTG, [23]), the fusion to bulky protein partners like

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GST [4] or expression at low temperature for several days [23]. Despite these approaches have been shown to be promising, they commonly prolong or complicate production and purification (e.g. cleaving off GST partner) and overall yields are rather low [24]. In an attempt to overcome these limitations, the Wurm group expressed the tetracycline-dependent repressor TetR, difficult-to-produce in *E. coli* [25], in suspension-adapted HEK-EBNA cells using transient gene expression and yielded in 18 mg purified protein per liter culture volume [25]. Despite this success, protein production in mammalian cells might still be too expensive for the manufacturing of raw materials for diagnostic devices and therefore economic strategies for the high-yield production of this protein class in *E. coli* would be highly desirable.

In this study we demonstrate that the yield of the streptogramin-dependent repressor PIP produced in *E. coli* was increased >100-fold by the addition of the PIP-specific inducer molecule, the streptogramin antibiotic pristinamycin I. Similarly, the inclusion of a repressor-specific inducer molecule into the culture medium, was shown to increase the production of the tetracycline-, macrolide- and arsenic-dependent repressors TetR, MphR(A) and ArsR, demonstrating the general applicability of this strategy.

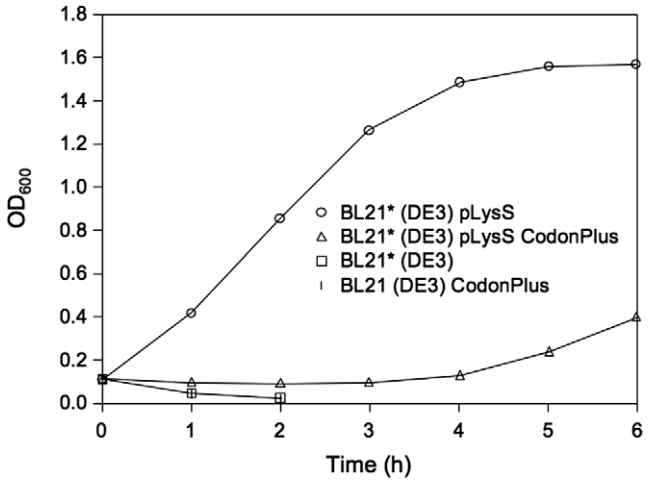

Fig. 1. Comparison of different *E. coli* hosts for the production of PIP. The indicated *E. coli* hosts were transformed with plasmid pWW312, grown over night and used as inoculum for the production culture. Growth was monitored for 6 h. At an optical density (600 nm) of 1, gene expression was induced by the addition of 1 mM IPTG.

Materials and methods

Plasmid construction

The *arsR* expression plasmid pMK1 was constructed by amplifying the *E. coli arsR* gene (Accession No. NC_010473) from *E. coli* K-12 substr. DH10B chromosomal DNA using oligos OMK1 (5′-CCCATGAATTC *AGGAGAT*CCACC**ATG**TCATTTCTGTTACCCATCCAATT-3′, EcoRI site underlined, Shine Dalgarno sequence in italics, start codon in bold) and OMK2 (5′-GGGTATCTGCGGCCGCTTA*ATG ATGGTGGTGATGGTG*CGCGCGGCTGTACGCGGAACTGCAAATGTTCTTA CTGTCCC-3′, NotI site underlined, hexahistidine tag in italics) and subsequent cloning (EcoRI/NotI) into pWW29 [7] thereby placing the *arsR* gene under the control of the phage T7 promoter (P_{T7}). The expression vectors for hexahistidine-tagged *pip*, *mphR*(*A*) *and tetR* have been described previously (pWW312, P_{T7}-*pip*-*his*$_6$; pWW313, P_{T7}-*mphR*(*A*)-*his*$_6$, pWW307, P_{T7}-*tetR*-*his*$_6$) [14]. All expression vectors further harbor an ampicillin resistance determinant.

Expression hosts and culture conditions

The following hosts were used for protein production: *E. coli* BL21* (DE3) (Invitrogen, Carlsbad, CA, Cat. No. C6010-03), *E. coli* BL21* (DE3) pLysS (Invitrogen, Cat. No. C6020-03) and *E. coli* BL21 (DE3) CodonPlus®-RIPL (Stratagene, La Jolla, CA, Cat. No. 230280). The *E. coli* BL21* (DE3) pLysS CodonPlus host was constructed by isolating the plasmids encoding extra copies of *argU*, *ileY*, *proL* and *leuW* tRNA genes from *E. coli* BL21 (DE3) CodonPlus®-RIPL and subsequent transformation into *E. coli* BL21* (DE3) pLysS (plasmids harbor compatible origins of replication). The presence of the *proL* gene in *E. coli* BL21* (DE3) pLysS CodonPlus was confirmed by PCR on a plasmid miniprep using oligonucleotides OEC15 (5′-GTAATTCATTAAGCATTCTGCC-3′) and OEC16 (5′-TGGTCGGCACGAGAGGATT-3′).

All cultures were performed at 37 °C and shaking at 250 rpm in LB medium. The medium was optionally supplemented with chloramphenicol (34 μg/ml for pLys-containing cells) or streptomycin (1 μg/ml for CodonPlus-transformed cells). Production cultures were optionally supplemented with inducers for PIP (pristinamycin I, PI contained in pyostacine®, Sanofi-Aventis, Paris, France), MphR(A) (erythromycin, Sigma, St. Louis, MO, Cat. No. E5389), ArsR (phenylarsine oxide, PAO, Sigma, St. Louis, MO, Cat. No. P3075) or TetR (Tetracycline, Sigma, Cat. No. T7660).

For transformation, chemically competent *E. coli* were used. Following heat-shock-based transformation, the cells were shaken for 1 h prior to the addition of 10 ml medium and further shaking over night (medium supplemented with 100 μg/ml ampicillin and optionally streptomycin and chloramphenicol). The next day, the culture was used to inoculate the production cultures.

Protein purification and analysis

Cells were harvested by centrifugation (6000*g*, 5 min, 4 °C), resuspended in Lysis buffer (1/5 of initial culture volume, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by passing twice through a French press (Thermo Scientific, Wohlen, Switzerland, Cat. No. FA-032). The supernatant was cleared by centrifugation (20,000*g*, 20 min, 4 °C) and purified using gravity flow Ni^{2+} affinity chromatography (Qiagen, Hilden, Germany, Cat. No. 30230). Unspecific proteins were eliminated by washing (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) while hexahistidine-tagged proteins were eluted with increasing imidazole concentrations (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, supplemented with 100–250 mM imidazole). For analysis, protein samples were supplemented with reducing SDS loading buffer (10% (v/v) glycerol, 50 mM Tris/HCl, 20 g/L sodium dodecylsulfate, 2 g/L bromphenolblue, 10% (v/v) β-mercaptoethanol, pH 6.8), boiled for 5 min and subsequently separated by SDS–PAGE (15% acrylamide). Equal amounts of total protein were loaded per lane. The gel was either Coomassie-stained or blotted onto a PVDF membrane followed by detection using a primary anti-his$_6$ antibody (Novagen, Madison, WI, Cat. No. 70796) and a secondary anti-mouse IgG conjugated to horseradish peroxidase (Amersham Life Science, NJ, USA, Cat. No. NA931V). Equal amounts of protein per lane were confirmed by staining the blotting membrane with Ponceau S. Chemiluminescence-based detection was performed using ECL-plus (GE Healthcare Europe GmbH, Dübendorf, Switzerland, Cat. No. RPN2106) on an Intas chemilux system (Göttingen, Germany). Bands were quantified using the Gel-Pro Analyzer Versions 4.5 software package (Media Cybernetics, Bethesda, MD). Protein concentrations were determined using the Bradford method (Bio-Rad, München, Germany, Cat. No. 500-0006) with BSA as standard.

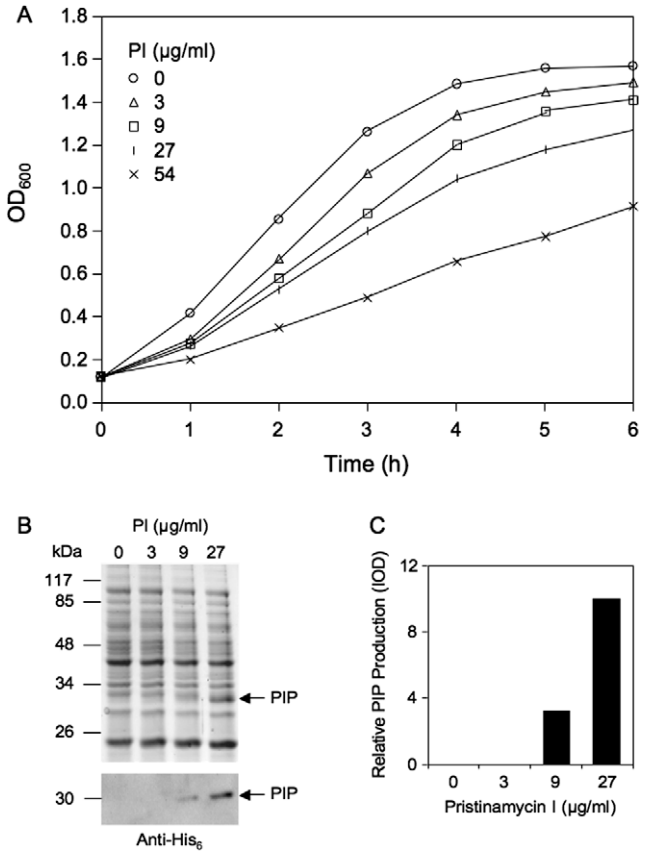

Fig. 2. Effect of pristinamycin I (PI) on cell growth and PIP production in *E. coli* BL21* (DE3) pLysS. (A) *E. coli* BL21* (DE3) pLysS cells transformed with pWW312 were grown over night and subsequently used to inoculate five cultures containing PI at the indicated concentrations. At an optical density (600 nm) of 1 PIP production was induced by the addition of 1 mM IPTG. (B) SDS–PAGE and Western blot analysis of PIP production in the crude cell lysate. (C) Quantification of PIP production from the bands in the Western blot. The value at 27 µg/ml PI corresponds to a yield of 7.5 mg purified PIP protein per liter initial culture volume. IOD, integrated optical density.

Functional validation of PIP

For evaluating the pristinamycin I (PI)[2]-responsive binding of PIP to its operator *pir*, the following oligos were used and pairwise annealed (OWW1700/OWW1702 and OWW1701/OWW1703, annealing conditions: 10 µM oligonucleotides in 100 µl 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0, were heated for 3 min at 95 °C and cooled down to 25 °C over 2–3 h). The sequences for the oligonucleotides are OWW1700, 5′-GACCAGAAAGTGTTGTA CAGT^{F}G-3′; OWW1701, 5′-TATAGGAAAGCCGGTG-3′; OWW1702, 5′-TACAACACTTTCTGGTC-3′ and OWW1703, 5′-CACCGGCTTTCCTAT ACACT^{D}G-3′; T^{F}, fluorescein coupled to thymidine; T^{D}, dabcyl coupled to thymidine. The reaction setup contained 200 nM of each annealed oligo pair, 40 µM purified PIP, 50 mM Tris/HCl, 500 mM NaCl, 1 mM EDTA, 600 µg/ml competitive DNA (sheared salmon sperm DNA, Roche Applied Science, Rotkreuz, Switzerland, Cat. No. 11467140001), pH 8.0 in a total volume of 30 µl. PI was added as stock solution in DMSO (0–30 µg/ml). In all samples DMSO was added to reach the same final concentration (10%, v/v). As mock control, PIP was replaced with BSA (Calbiochem, San Diego, CA, Cat. No. 402406). The mix was incubated for 1 h at room temperature prior to fluorescence quantification in a 384-well plate (Greiner bio-one, Frickenhausen, Germany, Cat. No. 781076) on an Infinite 200 (Tecan, Männedorf, Switzerland) reader (excitation, 490 nm; emission, 520 nm).

[2] *Abbreviations used:* PI, pristinamycin I; FRET, fluorescence resonance energy transfer.

Results and discussion

The PIP repressor is growth-inhibiting for the E. coli production host

The *Streptomyces pristinaespiralis*-dervied pristinamycin I-dependent repressor PIP [2] was shown to be a suitable biosensor for the detection of forbidden antibiotics in food samples [13,14]. In order to enable mass production of such PIP-based analytical devices, we evaluated the production of C-terminal hexahistidine-tagged PIP in different *E. coli* hosts: first attempts to produce PIP in *E. coli* BL21* (DE3) failed since the production culture did not grow (Fig. 1) even without IPTG induction. This PIP-dependent growth inhibition was overcome by switching to *E. coli* BL21* (DE3) pLysS host cells (Fig. 1) engineered for lower leaky expression in the uninduced state, resulting in production of PIP following IPTG induction as verified by Western blotting [14]. However, PIP was not detected on a Coomassie-stained gel (neither in the soluble nor insoluble fraction) and Ni^{2+} affinity chromatography

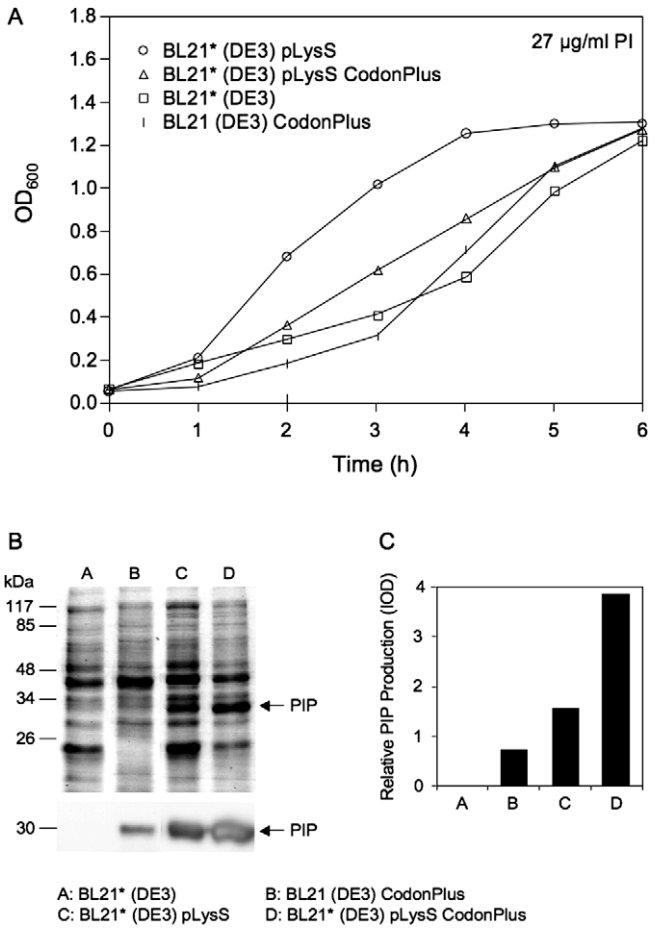

Fig. 3. Comparison of different *E. coli* hosts for the production of PIP in the presence of pristinamycin I (PI). (A) Plasmid pWW312 was transformed into the indicated hosts with addition of 27 µg/ml PI directly after the heat shock. The cultures were shaken over night and used the next morning to inoculate production cultures also containing 27 µg/ml PI. At an optical density (600 nm) of 1, 1 mM IPTG was added to induce PIP production. (B) SDS–PAGE and Western blot analysis of PIP production in the crude cell lysate. (C) Quantification of PIP production from the Coomassie-stained gel. The value for *E. coli* BL21* (DE3) pLysS CodonPlus corresponds to 18 mg purified PIP per liter initial culture volume. IOD, integrated optical density.

did not result in the enrichment of a specific band (data not shown). One reason for low PIP production could be unfavorable codon usage of the *Streptomyces*-derived *pip* gene containing two rare CCC codons for proline. In order to overcome codon bias, we evaluated *E. coli* BL21 (DE3) CodonPlus-RIPL cells harboring extra tRNA genes (*argU*, *ileY*, *leuW* and *proL*) however, as for *E. coli* BL21* (DE3) the production culture did not grow. In order to reduce growth-inhibiting leaky expression of the *pip* gene in the absence of IPTG and to simultaneously address the unfavorable codon usage, we isolated tRNA-encoding plasmids from *E. coli* BL21-CodonPlus (DE3)-RIPL cells and transformed it into *E. coli* BL21* (DE3) pLysS resulting in the new production host *E. coli* BL21* (DE3) pLysS CodonPlus. This host showed slow growth upon transformation with the *pip* expression plasmid (Fig. 1) suggesting that the optimized codon usage increases growth-inhibiting leaky PIP production levels. Also, no PIP-specific bands were detected on Coomassie-stained gels. Alternative strategies to produce the PIP protein as used for other transcriptional repressors [4] like the N-terminal fusion to a GST-tag also did not improve production levels (data not shown).

Pristinamycin I reverts PIP-dependent growth inhibition and increases PIP production

One reason for PIP-dependent growth inhibition and overall low production titers might be non-specific binding of the transcriptional repressor PIP to *pir*-like sites in the *E. coli* genome leading to interference with the bacterial transcriptome. In order to prevent DNA-binding of PIP in *E. coli* BL21* (DE3) pLysS host cells, we added increasing concentrations of the PIP-specific inducer pristinamycin I (PI) to the production culture and followed growth and PIP titers (Fig. 2). Gradually increasing PI concentrations dose-dependently compromised cell growth [26] (Fig. 2A), but correlated with a dose-dependent (0–27 μg/ml PI) increase in PIP production as determined by Coomassie-stained SDS–PAGE gel and Western blotting (Fig. 2B and C). At 27 μg/ml PI, 7.5 mg purified PIP were obtained per liter of initial culture volume.

Synergy of PI addition and optimized codon usage

In order to address suboptimal codon usage in the *pip* gene, we performed production runs in the four host cells described above (Fig. 1) with the addition of 27 μg/ml PI directly after the heat shock applied for transformation (PI addition one hour later already compromised growth of pLysS-devoid hosts as in Fig. 1, data not shown). All four production hosts were growing (Fig. 3A) and IPTG was added at an optical density (600 nm) of 1 resulting in PIP-specific bands on a Coomassie-stained SDS–PAGE for all strains except for *E. coli* BL21* (DE3). The highest yield (18 mg/L initial culture volume) was obtained for the newly engineered host *E. coli* BL21* (DE3) pLysS CodonPlus (Fig. 3B and C) reflecting the better

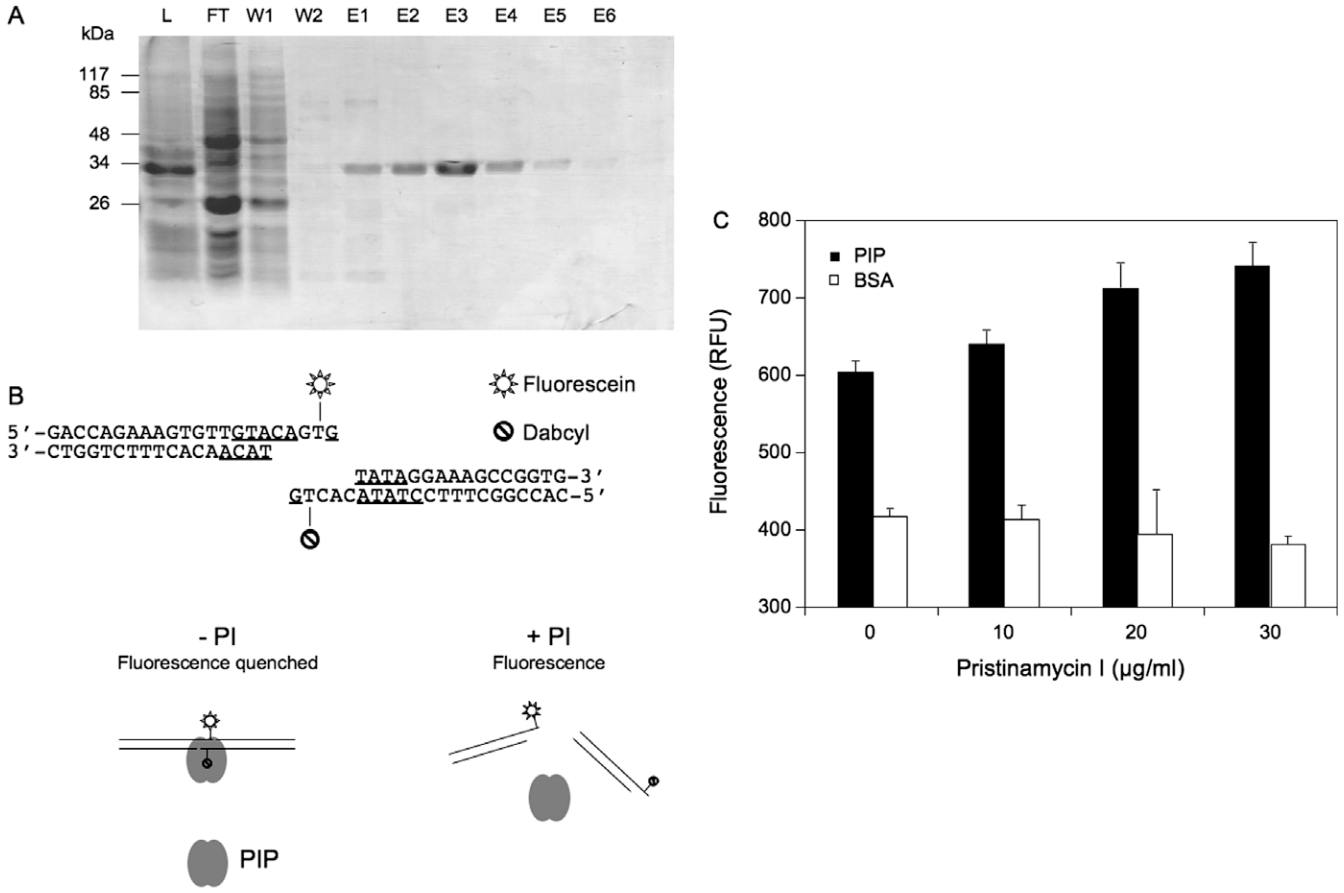

Fig. 4. Pristinamycin I (PI)-dependent binding of PIP to its operator *pir*. (A) Analysis of PIP purification on Coomassie-stained SDS–PAGE. PIP produced in *E. coli* BL21* (DE3) pLysS CodonPlus was purified by Ni^{2+} affinity chromatography. L, crude cell lysate; FT, flow-through of affinity column; W1, W2, wash steps; E1, elution 100 mM imidazole; E2: elution 175 mM imidazole; E3-E6, elution 250 mM imidazole. Fraction volume: 1 ml. (B) Schematic representation of the molecular beacon approach for validating the pristinamycin I-dependent interaction between PIP and its operator *pir*. *pir* was split into two parts, one conjugated to fluorescein, the other to fluorescence-quenching dabcyl. Binding of dimeric PIP triggers association of both parts resulting in quenched fluorescence. Addition of PI dose-dependently triggers dissociation of PIP from its operator segments which are no longer associated with each other resulting in increased fluorescence readout. (C) Validation of the PI-dependent association of PIP with its operator *pir*. The fluorescein- and dabcyl-labeled *pir* fragments were incubated with PIP in the presence of increasing PI concentrations for 1 h prior to fluorescence measurement (excitation, 490 nm; emission, 520 nm).

agreement of codon usage in the *pip* gene and tRNA availability in the production host.

Functional validation of PIP produced in the presence of PI

In order to evaluate whether PIP produced in the presence of PI retained the antibiotic-dependent binding activity to its cognate operator *pir*, PIP was purified by Ni^{2+} affinity chromatography (Fig. 4A) and subjected to a fluorescence resonance energy transfer (FRET)-based assay in analogy to the one described previously [27]. Therefore, two *pir* operator fragments were synthesized, one labeled with fluorescein and one with the fluorescence quencher dabcyl (Fig. 4B). Binding of PIP to both *pir* fragments approaches the molecular beacons resulting in quenched fluorescence whereas PI addition triggers dissociation of PIP from the *pir* fragments which subsequently dissociate and result in a dose-dependent increase in fluorescence levels (Fig. 4C). This pristinamycin I-dependent binding of PIP to *pir* shows that the purified PIP protein has retained its functionality.

General strategy for the increased production of inducer-dependent repressor proteins

In order to evaluate whether the production of transcriptional repressor proteins can in general be increased by the addition of repressor-specific inducer molecules, we performed production runs for the repressors TetR [3], ArsR [28] and MphR(A) [4] in the presence of increasing concentrations of the specific inducers tetracycline, As^{3+} and erythromycin, respectively. For the production of TetR, *E. coli* BL21* (DE3) pLysS were transformed with a tetR expression plasmid (pWW307) and grown in the presence of increasing tetracycline concentrations with induction of gene expression at $OD_{600} = 1$ (Fig. 5A). *E. coli* cells grew in the presence of 0.3 μg/ml tetracycline, whereas higher antibiotic concentrations significantly inhibited growth. Despite these antibiotic-mediated effects, TetR production increased 2-fold in the presence of 0.3 μg/ml tetracycline (Fig. 5A).

Prior to the production of ArsR, *E. coli* BL21* (DE3) pLysS cells transformed with the ArsR expression plasmid pMK1 were first

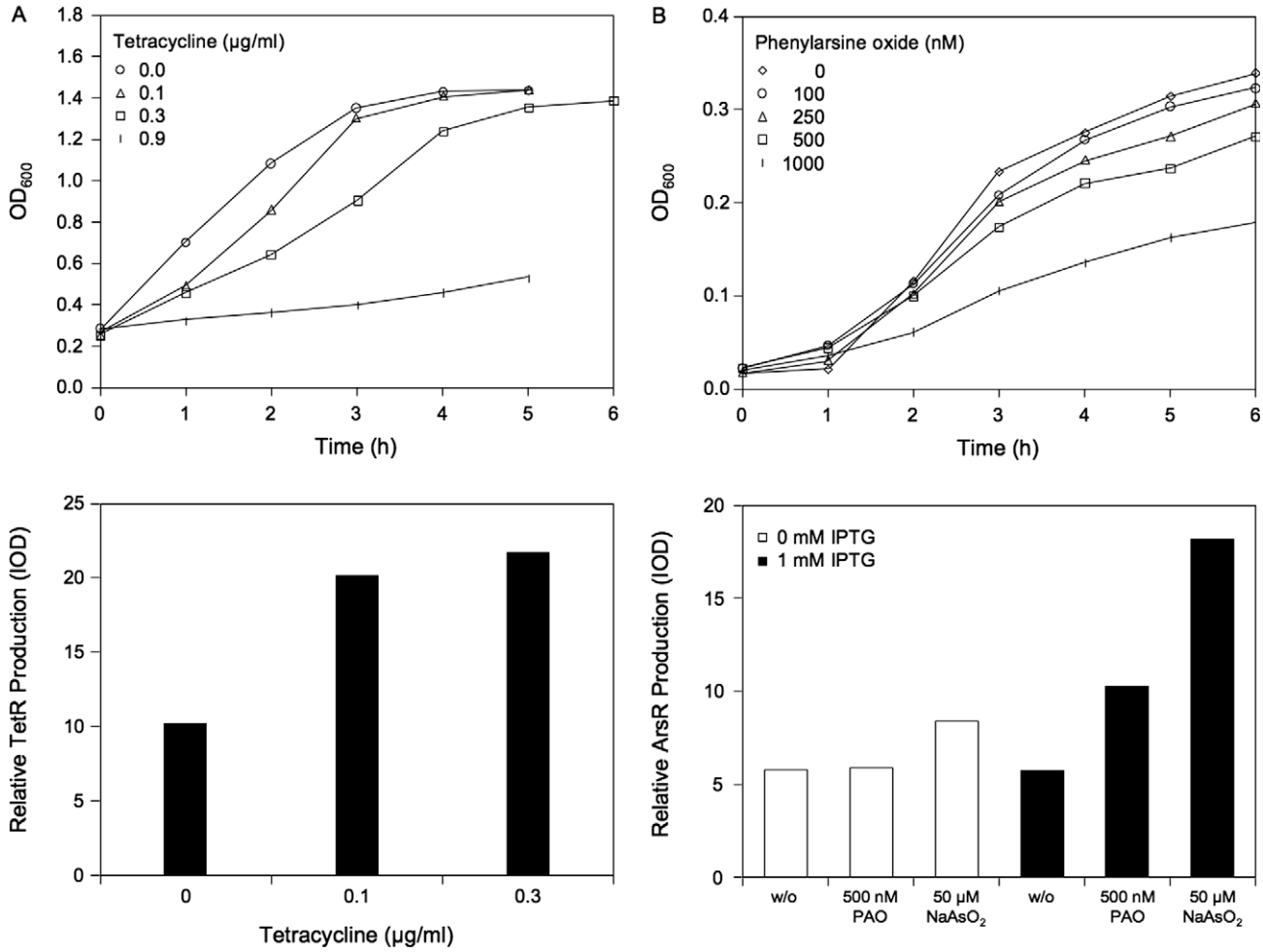

Fig. 5. A general strategy to increase the production of allosterically regulated bacterial repressor proteins in *E. coli*. (A) Production of the tetracycline-dependent repressor TetR. An overnight culture of *E. coli* BL21* (DE3) pLysS transformed with pWW307 was used to inoculate three cultures containing increasing tetracycline concentrations. TetR production was induced at $OD_{600} = 1$ by 1 mM IPTG and quantified on a Coomassie-stained SDS–PAGE (IOD, integrated optical density). The value at 0.3 μg/ml tetracycline corresponds to 96 mg purified TetR per liter of culture volume. (B) Production of the arsenite-dependent repressor ArsR. Growth of *E. coli* BL21* (DE3) pLysS transformed with pMK1 was followed in the presence of increasing phenylarsine oxide (PAO) concentrations. For production of ArsR, pMK1-transformed *E. coli* BL21* (DE3) pLysS were grown in the absence or presence of 500 nM PAO or 50 μM sodium arsenite. At $OD_{600} = 0.5$, 1 mM IPTG was added to half of the cultures and ArsR production was quantified by Western blotting. (IOD, integrated optical density). (C) Production of the erythromycin-dependent repressor MphR(A). *E. coli* BL21* (DE3) pLysS transformed with pWW313 were grown until $OD_{600} = 0.95$ prior to the addition of 1 mM IPTG and increasing erythromycin concentrations. MphR(A) production was quantified by Western blot analysis, the value at 300 μg/ml eryhtromycin corresponds to a yield of 15.2 mg purified MphR(A) protein per liter initial culture volume. IOD, integrated optical density.

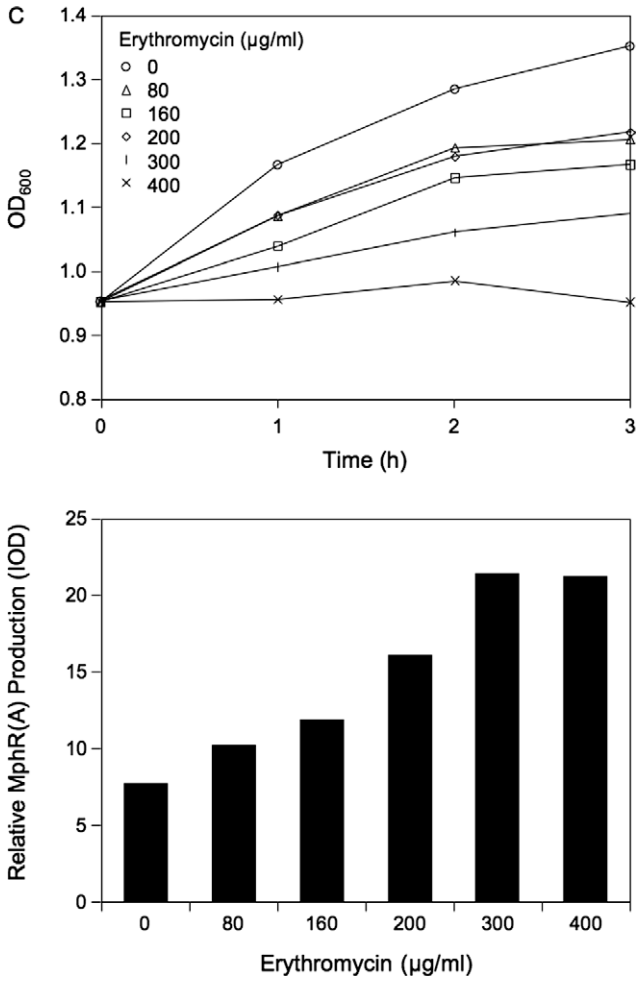

Fig 5. (*continued*)

grown in the presence of increasing concentrations of the ArsR-specific inducer phenylarsine oxide (PAO) revealing that 500 nM PAO were the maximum tolerated dose (Fig. 5B). As second inducer we evaluated sodium arsenite, which has been described to be tolerated by *E. coli* cells at concentrations of 50 µM [29]. ArsR was produced in pMK1-transformed *E. coli* BL21* (DE3) pLysS cells in the presence or absence of PAO or sodium arsenite until an optical density of 0.5 prior to inducing protein production with 1 mM IPTG. In parallel we also performed production runs in the absence of IPTG according to a previous study [23]. ArsR, as quantified by Western blotting, was produced at similar levels in the absence or presence of IPTG, however, addition of PAO or sodium arsenite resulted in up to 3-fold increased ArsR titers (Fig. 5B).

The erythromycin-responsive repressor MphR(A) was produced in pWW313-transformed *E. coli* BL21* (DE3) pLysS cells in the presence of increasing erythromycin concentrations (0–162 µg/ml), however, no effect on MphR(A) yields at growth-permitting erythromycin concentrations (0–135 µg/ml) was observed (data not shown). Since growth of *E. coli* BL21* (DE3) pLysS cells containing the *mphR(A)* expression vector pWW313 was not compromised, we added increasing erythromycin concentrations only together with IPTG (Fig. 5C) resulting in a 2.7-fold increase in MphR(A) production in the presence of 300 µg/ml erythromycin (Fig. 5C).

Conclusion

In this study we have devised a novel approach for the production of difficult-to-express prokaryotic transcriptional repressor proteins in *E. coli* provided a specific inducer is available preventing DNA-binding of the target protein. At the example of the pristinamycin-induced protein PIP showing an unfavorable codon usage and strong growth inhibition on the production host, we demonstrated that the combination of both the engineering of a new production host overcoming codon bias and the application of the inducer molecule PI resulted in increased PIP production levels by more than two orders of magnitude. The observation that the production titers of TetR, ArsR and MphR(A) were 2- to 3-fold increased in the presence of tetracycline, arsenite and erythromycin, suggest that the addition of inducer molecules specific to the target repressor protein represents a general strategy to increase the yield of this difficult-to-express protein class. In order to evaluate whether this approach has the potential to lower protein production costs, the price for the inducer molecule must be charged up against the gain in yield, for example, the addition of tetracycline increases the production costs of TetR by EUR 0.02 per gram TetR.

To conclude, the approach presented in this work has the potential to enable the economic manufacturing of allosterically regulated repressor proteins for the cost-effective production of diagnostic devices.

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