A novel engineering tool in the *Bacillus subtilis* toolbox: inducer-free activation of gene expression by selection-driven promoter decryptification

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*Bacillus subtilis* is a Gram-positive bacterium that is easy to manipulate genetically. Several methods for genome engineering have been developed that helped to extend our understanding of how the *B. subtilis* cell operates. Consequently, the bacterium has become one of the best-studied organisms. *B. subtilis* has also been engineered for industrial applications. Moreover, great progress has been achieved in promoter engineering to improve the performance of production strains. To expand the toolbox for engineering *B. subtilis*, we have constructed a system for the inducer-free activation of gene expression. The system relies on spontaneous mutational activation of a cryptic promoter and selection-driven enrichment of bacteria harbouring the mutated promoter. The synthetic promoter is cryptic due to a perfect direct repeat, separating the binding motifs of the RNA polymerase housekeeping sigma factor. The promoter can be fused to genes for industrial applications and to a growth-promoting gene that, upon mutational activation of the promoter, allows enrichment of the engineered bacteria due to a selective growth advantage.

INTRODUCTION

The Gram-positive soil-dwelling bacterium *Bacillus subtilis* has the remarkable ability to adapt to and survive in a variety of ecological niches (Schilling et al., 2007; Buescher et al., 2012; Nicolas et al., 2012). Moreover, *B. subtilis* is capable of forming robust endospores allowing survival in unfavourable environments (McKenney et al., 2013). In the course of its domestication the genetic competence of *B. subtilis* has been enhanced considerably, making manipulations of the genome much easier (Burkholder & Giles, 1947). Consequently, *B. subtilis* has become one of the best-studied bacteria (Sonenshein et al., 2002). Because the organism is ‘generally regarded as safe’ it is an attractive host for industrial applications (Schallmey et al., 2004). Indeed, the bacterium has been engineered and used for the production of vitamins and enzymes (Perkins et al., 1999; Manabe et al., 2013; Commichau et al., 2014). *B. subtilis* has also been subjected to systematic genome reduction to identify the essential gene set that is needed to sustain life and to obtain minimal cell factories for industrial use (Juhas et al., 2014; http://minibacillus.org). Several methods have been developed for biochemical studies and for genome engineering (Herzberg et al., 2007; Kumpfmüller et al., 2013). Moreover, great progress has been achieved in promoter engineering to expand the transcriptional capacities of engineered organisms and to tightly control gene expression (Brautaset et al., 2009; Blazek & Alper, 2013; Radeck et al., 2013; Vogl et al., 2014). Current knowledge about the control of gene expression has undoubtedly helped to enhance the production levels of many engineered bacteria.

Populations of rapidly growing bacteria such as *B. subtilis* can reach high cell densities, and small fractions of the populations always accumulate spontaneous mutations. Some mutations may become important for bacteria if they ensure survival of unpredictable environmental changes. DNA sequences such as direct repeats (DRs) are mutational hotspots that impact the potential of an organism to adapt to the environment by spontaneous mutagenesis (Zhou et al., 2014). Several micro-organisms can exploit the instability of DRs to reversibly shut down genes, and to activate or to modulate gene expression (Belitsky & Sonenshein, 1998; Martin et al., 2005; Flores et al., 2013). To further expand the

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*Abbreviations*: ACN, acetonitrile; DR, direct repeat; GDH, glutamate dehydrogenase.

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toolbox for engineering *B. subtilis*, we have designed a novel system for inducer-free activation of gene expression. The system is based on spontaneous mutational activation of a synthetic core promoter that is cryptic due to a perfect DR, separating the binding motifs of the RNA polymerase.

A cryptic promoter is fused to an artificial expression unit that, when expressed, provides the bacteria with a selective growth advantage. Those cells that have spontaneously ‘decrypted’ the synthetic promoter and express the growth-promoting gene together with additional genes encoding proteins that are of interest for industrial applications can be enriched during growth under selection.

**METHODS**

**Chemicals, media and DNA manipulation.** Primers were purchased from Sigma-Aldrich (see Table S1, available in the Online Supplementary Material, for the sequences). Chromosomal DNA was isolated from *B. subtilis* using the DNeasy Blood & Tissue kit (Qiagen). Plasmids were isolated using the Nucleospin Extract kit (Macherey & Nagel). DNA fragments generated by PCR were purified using the PCR Purification kit (Qiagen). Phusion DNA polymerase, restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific and used according to the manufacturer’s instructions. Miscellaneous chemicals and media were purchased from Sigma-Aldrich, Carl Roth and Becton Dickinson. Plasmids were sequenced using Sanger sequencing services by SeqLab Sequence Laboratories.

**Plasmid construction.** To allow expression of genes in single copy from the *lacA* locus of the *B. subtilis* chromosome, we constructed the plasmid pBP106. Plasmid pBP106 (Fig. S1) contains the *aphA3* kanamycin resistance gene and has a small multiple-cloning site containing six recognition sites for single-cutting restriction enzymes. The *aphA3* kanamycin resistance gene was amplified from plasmid pDG780 (Guéroult-Fleury *et al.*, 1995) using the oligonucleotides KG47 and FC200 (Table 1; see also Table S1). The PCR product was digested with the enzymes *Bgl*II and *Sma*I, and ligated to plasmid pGP882 (Dietmaier *et al.*, 2011) cut with the enzymes *Bam*HI and *Sma*I. The active *P*⁺ and the cryptic *P*CR promoters were generated by hybridization of the oligonucleotide pairs ST5/ST6 and ST7/ST8, respectively. To obtain *P*⁺-*lacZ* and *P*CR-*lacZ* fusions, the promoters were ligated to plasmid pAC6 (Stülke *et al.*, 1997) cut with the enzymes *Eco*RI and *Bam*HI. The plasmids harbouring the *P*⁺-*lacZ* and *P*CR-*lacZ* fusions were designated pBP300 and pBP311, respectively. For integrating the *P*CR-*gudB* fusion into the *amyE* locus of the *B. subtilis* chromosome, we constructed the plasmid pBP302. For this, the *gudB*⁺ allele was amplified using the primer pair KG92/KG134 and chromosomal DNA of strain GP801. The PCR product was cut with *Bam*HI and ligated to plasmid pBP302 digested with the same enzyme. The correct orientation of the *gudB* gene was confirmed by PCR. Plasmid pBP168 for the integration of the *P*CR-*gudB* fusion into the *lacA* locus of the *B. subtilis* chromosome was constructed as follows. The *P*CR-*gudB* fusion was amplified from plasmid pBP302 by PCR using oligonucleotides LS32 and LS33. The PCR product was digested with the enzymes *Pst*I and *Mfe*I and ligated to plasmid pBP106 cut with the enzymes *Pst*I and *Eco*RI (Figs S1 and S2). For integrating the *P*CR-*gudB–gfp* fusion into the *lacA* locus we constructed the plasmid pBP169. The *gfp* gene was amplified by PCR using oligonucleotides LS34 and LS35 and the plasmid pSG1154 (Lewis & Marston, 1999) as a template. The PCR product was digested with *Xho*I and *Eco*RI and ligated to plasmid pBP168 cut with the same enzymes. Plasmid pBP172 was constructed to integrate the entire artificial *P*CR-*gudB–pdxST–Strep–gfp* fusion into the *lacA* locus. The *pdxST–Strep* fusions were amplified by PCR using oligonucleotides LS36 and LS37 and chromosomal DNA of the *B. subtilis* wild-type strain 168. The PCR product was digested with *Sal*I and *Bgl*II and ligated to plasmid pBP169 cut with the same enzymes giving plasmid pBP172 (see Figs S2 and S3).

**Strains and cultivation conditions.** *Escherichia coli* or *B. subtilis* (Tables 2 and 3) were grown in Luria–Bertani (LB) and SP medium or in C minimal medium supplemented with carbon sources, nitrogen sources and auxotrophic requirements (at 50 mg l⁻¹) as indicated (Sambrook *et al.*, 1989). C-Glc medium is C minimal medium supplemented with 0.5 % (w/v) glucose (Wacker *et al.*, 2007a). LB and SP plates were prepared by the addition of 17 g Bacto agar l⁻¹ (Difco) to LB and SP (8 g nutrient broth l⁻¹, 1 mM MgSO₄, 13 mM KCl, supplemented after sterilization with 2.5 μM ammonium ferric citrate, 500 μM CaCl₂ and 10 μM MnCl₂), respectively. When required, media were supplemented with antibiotics at the following concentrations: ampicillin (100 μg ml⁻¹), kanamycin (10 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹) and spectinomycin (150 μg ml⁻¹). The chromogenic substrate X-Gal was dissolved in N,N-dimethylformamide to a final concentration of 80 mg ml⁻¹ and agar plates were prepared with 80 μg X-Gal ml⁻¹. Deletion of the *gudB* gene including its native promoter was achieved by transformation with a deletion cassette constructed by long-flanking homology PCR using oligonucleotides (Table S1) to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes (Guéroult-Fleury *et al.*, 1995), as described previously (Wach, 1996). *B. subtilis* was transformed with plasmid and chromosomal DNA according to a previously described two-step protocol (Kunst & Rapoport, 1995).

**β-Galactosidase assay.** Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: cells were grown in SP medium. Cells were harvested at an OΔOD₆₀₀ of 0.6–0.8. β-Galactosidase-specific activities were determined with cell extracts obtained by lysozyme treatment, as described previously (Kunst & Rapoport, 1995). One
unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of o-nitrophenol min⁻¹ at 28 °C.

**Isolation of suppressor mutants.** To isolate suppressor mutants with the ‘decryptified’ P⁺CR promoters that express the gudB gene from the artificial operons, strains BP205, BP206 and BP216 lacking native rocG and gudB genes (see Table 3) were propagated on SP complex medium. The plates were incubated for 48 h at room temperature. Emerging papilla were isolated and characterized by fluorescence microscopy. Moreover, excision of one part of the DR of the cryptic P⁺CR promoters was confirmed by DNA sequencing (Fig. S4).

**Fluorescence microscopy.** For fluorescence microscopy, cells were grown in LB medium to optical densities as indicated, harvested and resuspended in PBS (pH 7.5; 50 mM). Fluorescence images were obtained with an Axioskop 40 FL fluorescence microscope, equipped with an AxioCam MRm digital camera and AxiosVision Rel software. The applied filter set was Lumar 38 for EGFP detection microscope (Zeiss) equipped with the ZEN lite 2011 (blue edition) and served as primary antibodies to detect PdxT-Strep, GFP and GudB proteins (Commichau et al., 2007b) (PromoKine; MBL). The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega) and the CDP-Star detection system (Roche Diagnostics), as described previously (Commichau et al., 2007b).

**Isolation of the PdxST enzyme complex from B. subtilis.** To isolate the Strep-tagged PdxST enzyme complex from B. subtilis, a first preculture was grown in LB containing 0.5% glucose for 10 h at 37 °C. This preculture was used to inoculate 1 litre of LB medium containing 0.5% glucose to an OD600 of 0.1. This culture was grown at 37 °C until the optical density had reached 2.0. The cells were harvested and disrupted using a French press (20 000 p.s.i., 138 MPa; Spectronic Instruments) and the enzyme complex was purified using the CDP-Star detection system (Roche Diagnostics), as described previously (Commichau et al., 2007b).

**Western blotting.** For Western blot analysis, proteins present in 20 μg cell-free crude extract were separated by 12.5% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) by electroblocting. Anti-Strep-tag II, anti-GFP and anti-GudB polyclonal antibodies were diluted 1:1000, 1:10 000 and 1:15 000, respectively, and served as primary antibodies to detect PdxT-Strep, GFP and GudB proteins (Commichau et al., 2007b) (PromoKine; MBL). The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega) and the CDP-Star detection system (Roche Diagnostics), as described previously (Commichau et al., 2007b).

**Protein identification by MS.** Silver nitrate-stained gel slices were destained by incubation in 30 mM K₃[Fe(CN)₆] and 100 mM Na₂S₂O₃ until colourless and washed three times in water before processing gel slices as previously described (Thiele et al., 2007). Briefly, gel pieces were washed twice with 200 μl of 20 mM NH₄HCO₃ and 50% (v/v) acetonitrile (ACN) for 30 min at 37 °C.

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**Table 1.** Plasmids

<table>
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<tr>
<th>Plasmid</th>
<th>Purpose</th>
<th>Parent plasmid</th>
<th>Reference</th>
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<td>pAC6</td>
<td>Integration of DNA into the B. subtilis amyE locus</td>
<td>–</td>
<td>Stülke et al. (1997)</td>
</tr>
<tr>
<td>pBP106</td>
<td>Integration of DNA into the B. subtilis lacA locus</td>
<td>pGP882</td>
<td>This study</td>
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<td>pBP168</td>
<td>Expression of the P⁺CR–gudB fusion from the B. subtilis lacA locus</td>
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<td>This study</td>
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<td>pBP169</td>
<td>Expression of the P⁺CR–gudB–gfp fusion from the B. subtilis lacA locus</td>
<td>pBP168</td>
<td>This study</td>
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<td>pBP172</td>
<td>Expression of the P⁺CR–gudB–pdxST–Strep–gfp fusion from the B. subtilis lacA locus</td>
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<td>This study</td>
</tr>
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<td>pAC6</td>
<td>This study</td>
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<td>pAC6</td>
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<td>This study</td>
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<tr>
<td>pGP882</td>
<td>Integration of DNA into the B. subtilis lacA locus</td>
<td>–</td>
<td>Dietmaier et al. (2011)</td>
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**Table 2. E. coli strains used in this study**

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5x</td>
<td>F⁺ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR mupG φ80dilacZΔM15 Δ(lacY1-argF)U169, hsdR17(λlacZΔM15 $\Delta\text{lacZ}{\text{argF}}\text{U169, hsdR17}(\lambda\text{lacZ})\text{M15}$), λ− endA1 gyrA96(nda2) thi-1 recA1 relA1 lac glnV44 F[α- Tn10 proAB+ lacI4 Δ(lacZ)159] hsdR17(λ− $\Delta\text{lacZ}{\text{argF}}\text{U169, hsdR17}(\lambda$−))</td>
<td>Laboratory collection, used for cloning</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>ΔlacY1-argF)U169, hsdR17(λlacZΔM15 $\Delta\text{lacZ}{\text{argF}}\text{U169, hsdR17}(\lambda\text{lacZ})\text{M15}$), λ− endA1 gyrA96(nda2) α− Tn10 proAB+ lacI4 Δ(lacZ)159] hsdR17(λ− $\Delta\text{lacZ}{\text{argF}}\text{U169, hsdR17}(\lambda$−))</td>
<td>Laboratory collection, used for cloning</td>
</tr>
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</table>
The conserved elements of prokaryotic promoters, i.e. the −35 and −10 regions TTGACA and TATAAT, respectively, bound by sigma factor σ70 are well studied and can be rationally engineered (Blazeck & Alper, 2013). To obtain an inactive promoter that could be activated by spontaneous mutagenesis, we increased the spacing between the −10 regions TTGACA and TATAAT, respectively. Glutamine at 10 mM served as the nitrogen source. The formation of pyridoxal phosphate was monitored at a wavelength of 414 nm using the Synergy MX multi-well plate reader (BioTek). Samples lacking the purified enzyme complex served as control.

**RESULTS AND DISCUSSION**

**Construction and evaluation of a cryptic core promoter**

The conserved elements of prokaryotic promoters, i.e. the −35 and −10 regions TTGACA and TATAAT, respectively, bound by sigma factor σ70 are well studied and can be rationally engineered (Blazeck & Alper, 2013). To obtain an inactive promoter that could be activated by spontaneous mutagenesis, we increased the spacing between the −35 and −10 regions of a synthetic promoter by duplication of 10 regions TTGACA and TATAAT, respectively. Glutamine at 10 mM served as the nitrogen source. The formation of pyridoxal phosphate was monitored at a wavelength of 414 nm using the Synergy MX multi-well plate reader (BioTek). Samples lacking the purified enzyme complex served as control.
its performance, we expected the PCR promoter to be transcriptionally inactive (cryptic). To determine its activity, we fused the PCR promoter to a lacZ reporter gene encoding \( \beta \)-galactosidase. The isogenic \( P^+ \) promoter lacking one unit of the 18 bp DR served as the control (Fig. 2a). Both plasmids carrying the promoter lacZ fusions were introduced into the amyE locus of the B. subtilis wild-type strain 168, giving strains BP472 (PCR–lacZ) and BP429 (\( P^+–lacZ \)) were propagated on SP-rich medium plates containing with 80 \( \mu \)g X-Gal ml\(^{-1} \) and the plates were incubated for about 48 h at 37 °C. (c) \( \beta \)-Galactosidase assay to quantify activities of the translational promoter–lacZ fusions. Representative results from three independent experiments are shown. The maximum deviation of the series of representative data did not exceed 10%.

Activation of gene expression by selection-driven promoter decryptification

A B. subtilis strain lacking the glutamate dehydrogenase (GDH) rocG gene encoding the active GDH RocG shows a severe growth defect on rich medium (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The growth defect of the rocG mutant strain is relieved by the rapid emergence of suppressor mutants that have activated the cryptic gudBCR gene by the precise excision of 9 bp of an 18 bp DR. The DR is present in the ORF of the gudBCR gene and renders the encoded GudBCR protein unstable and inactive (Belitsky & Sonenshein, 1998; Gunka et al., 2012). The ‘decryptified’ gudB gene encodes the functional GDH GudB that restores glutamate homeostasis of the cell (Gunka et al., 2013). It has also been shown that the cryptic gudBCR gene is stably inherited because a lower GDH activity provides the bacteria with a selective growth advantage when exogenous glutamate is scarce (Gunka et al., 2013; Stannek et al., 2014). Thus, depending on the supply with glutamate (e.g. growth on rich medium or not) either the cryptic gudBCR gene is inherited among the bacteria or the gene spontaneously ‘decryptifies’ and the active gudB gene is propagated in a cell population.

The observation that the DR in the gudBCR gene harbours a mutational hotspot provoked us to design and construct a system for inducer-free activation of gene expression in bacteria. This system is based on the instability of the DR in the PCR promoter that, if mutated, drives the expression of the growth-promoting gudB gene in the background of a GDH-deficient strain. For this, we fused the cryptic PCR promoter to the decryptified gudB gene and integrated the construct into the chromosome of the B. subtilis strain BP200 (ΔgudB), giving strain BP201 (see Tables 1 and 3 for the construction of plasmids and strains, respectively). Next we inactivated the second GDH-encoding rocG gene by transformation using chromosomal DNA of the rocG gene strain GP747. The resulting strain BP205 contains only the functional GDH-encoding gudB gene fused to the mutable PCR promoter (Fig. 3a). To illustrate the application potential of the expression system, we constructed two additional artificial operons and integrated them into the chromosome of a GDH-deficient strain, giving strains BP207 and BP216 (Fig. 3a; Table 3). In addition to the gudB gene, strain BP207 harbours the gfp gene encoding GFP to facilitate the identification of mutant bacteria that have mutated the cryptic PCR promoter. In strain BP216 the cryptic PCR promoter controls the expression of the gudB and gfp genes, and the native pdxST operon encoding the tagged PdxST vitamin B6 synthase complex for purification via the Strep-tag protein purification system (Fig. 3a). The full sequence of the artificial PCR gudB pdxST-tag gfp operon is given in Fig. S3. As described above, expression of the gudB gene in the background of a GDH-deficient strain provides the bacteria with a strong selective growth advantage on rich medium. Therefore, it can be expected that once the PCR promoter has been spontaneously ‘decryptified’ the selective pressure acting on the bacteria results in the rapid clonal expansion of mutants harboring the active P+ promoter. Indeed, visual inspection of ageing colonies of strains BP205.
isolated suppressor mutants including that of strain BP206 (derived from strain BP205 lacking the gfp gene) was verified by DNA sequencing analysis (Fig. S4). In all analysed suppressor mutants one 9 bp unit of the DR in the P\textsuperscript{CR} promoter was precisely excised. To verify that the suppressor mutant strain BP219 (P\textsuperscript{+} gudB pduST-tag gfp) produces the GDH GudB, the PdxT-Strep subunit of the vitamin B6 PdxST enzyme complex and GFP, we performed Western blot analyses (Fig. 3d). The parent strain BP216 (P\textsuperscript{CR} pduST-tag gfp) served as the negative control. While the parent strain BP216 did not produce the three proteins, the mutant strain BP219 harbouring the decrypted P\textsuperscript{+} promoter synthesized the GudB and PdxT-Strep proteins as well as GFP (Fig. 3d). Next, we tested whether the functional vitamin B6 synthase PdxST-Strep can be isolated from cell-free crude extract of strain BP219 (P\textsuperscript{+} gudB pduST-tag gfp). For this, we performed a Strep-tag protein purification experiment and

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**Fig. 3.** Characterization of the engineered *B. subtilis* strains harbouring the artificial operons. (a) Operon structures of the engineered *B. subtilis* strains having inactivated the *rocG* and the *gudB* genes encoding the native glutamate dehydrogenases *RocG* and *GudB*, respectively (Table S4). (b) The emergence of suppressor mutants (white papillae) in ageing colonies of the *B. subtilis* strains that are shown in (a) was visualized by bright-field (BF) microscopy. The suppressor mutants have decrypted the P\textsuperscript{CR} promoters. Suppressor mutants derived from strains BP207 and BP216 harbouring the gfp gene were identified by monitoring the GFP fluorescence signal. Exposure time, 1.5 s; bar, 1 mm. (c) Single-cell characterization of strains harbouring the cryptic and the spontaneously activated promoters P\textsuperscript{CR} and P\textsuperscript{+}, respectively, by phase-contrast (PC) and fluorescence microscopy. Exposure time, 3 s; bar, 5 \( \mu \text{m} \). (d) Western blot analysis to confirm the synthesis of GudB, PdxT-Strep and GFP in strain BP219 (P\textsuperscript{+} gudB pduST-tag gfp). Cell-free crude extracts (20 \( \mu \text{l} \)) were separated by SDS-PAGE and the proteins were identified using GudB, GFP and Strep-tag antisera. The parent strain BP216 (P\textsuperscript{CR} gudB pduST-tag gfp) harboured the cryptic P\textsuperscript{CR} promoter served as the negative control. (e) Purification of the Strep-tagged PdxST vitamin B6 synthase complex from the *B. subtilis* strain BP219 (P\textsuperscript{+} gudB pduST-tag gfp) by Strep-tag affinity chromatography. The proteins in 20 \( \mu \text{l} \) of the elution fractions E1 and E2 were separated by SDS-PAGE and visualized by silver staining. (f) Enzyme assay to demonstrate the activity of the purified Strep-tagged PdxST vitamin B6 synthase complex shown in (e). A representative measurement from three independent experiments using a concentration of 40 \( \mu \text{M} \) of the PdxST enzyme complex is shown.
evaluated the elution fractions by SDS-PAGE (Fig. 3e). In the elution fraction 2 (E2) two proteins were identified with molecular masses of 21 and 32 kDa that correspond to the PdxT-Strep (Sample 2) and PdxS (Sample 1) subunits of the vitamin B6 synthase complex, respectively. MS analysis revealed that the two isolated proteins were indeed the PdxT-Strep and PdxS proteins from *B. subtilis* (Fig. 3e and Table S2). Finally, we performed an enzyme assay to test whether the isolated enzyme complex was capable of forming vitamin B6. As shown in Fig. 3(f), the isolated enzyme complex was active and formed significant amounts of vitamin B6. In conclusion, the system we present here is suitable for inducer-free activation of gene expression and for the production of proteins, as illustrated by the isolation of a functional enzyme complex from *B. subtilis*.

There are several applications for the inducer-free expression system in synthetic biology because the enrichment of mutant bacteria producing a protein of interest can be achieved with any gene that provides the cells with a selective growth advantage. Moreover, for wastewater treatment one could envisage constructing strains harbouring multiple growth advantage. Moreover, for wastewater treatment one mutant bacteria producing a protein of interest can be


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