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pBaSysBioII: an integrative plasmid generating *gfp* transcriptional fusions for high-throughput analysis of gene expression in *Bacillus subtilis*

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Plasmid pBaSysBioII was constructed for high-throughput analysis of gene expression in *Bacillus subtilis*. It is an integrative plasmid with a ligation-independent cloning (LIC) site, allowing the generation of transcriptional *gfpmut3* fusions with desired promoters. Integration is by a Campbell-type event and is non-mutagenic, placing the fusion at the homologous chromosomal locus. Using *phoA*, *murAA*, *gapB, ptsG* and *cggR* promoters that are responsive to phosphate availability, growth rate and carbon source, we show that detailed profiles of promoter activity can be established, with responses to changing conditions being measurable within 1 min of the stimulus. This makes pBaSysBioII a highly versatile tool for real-time gene expression analysis in growing cells of *B. subtilis.*

INTRODUCTION

The live cell array (LCA) technology has proven to be an accurate and versatile method of determining gene expression in bacteria in a high-throughput format (Kalir *et al.*, 2001; Ronen *et al.*, 2002; Zaslaver *et al.*, 2006). This technology involves generating transcriptional fusions with fast-folding fluorescent proteins, such as GFP, and monitoring their accumulation under appropriate conditions. The promoter activity profile of up to 96 individual *gfp* fusions in cells grown in microtitre plates can be obtained at very high resolution by determining the difference in fluorescence levels at successive time points. This approach has many advantages over the complementary transcriptomic methodology: sample preparation is not required prior to measurement; measurements can be made at intervals as short as 1 min over an extended period; data acquisition and analysis are greatly simplified; and the cost is significantly less. Critically, the high resolution with which the kinetics of gene expression can be established in response to particular stimuli allows a detailed understanding of the underlying regulatory mechanisms.

In bacteria, the LCA approach allows expression of all genes involved in particular cellular processes to be monitored simultaneously under specified conditions. In *Escherichia coli*, for example, LCA technology has been used to establish the kinetic profiles of flagellar protein synthesis (Kalir *et al.*, 2001) and the SOS response (Ronen *et al.*, 2002). An impressive example of the use of LCAs is the determination at high resolution of the expression of 2000 *E. coli* promoters in a glucose–lactose diauxic shift experiment (Zaslaver *et al.*, 2006).

The European *Bacillus* community within EU-funded consortia has a record of generating new tools for genetic analysis in bacteria. The *E. coli* strain TP611 was constructed during the *Bacillus* sequencing project as a host to stably clone large fragments of *Bacillus subtilis* chromosomal DNA

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Abbreviations: LCA, live cell array; LIC, ligation-independent cloning.

The GenBank/EMBL/DDBJ accession number for the complete sequence of pBaSysBioII is GU126429.

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(Glaser *et al.*, 1993). The pMUTIN series of plasmids was developed during the subsequent functional analysis programme to generate *lacZ* transcriptional fusions and to generate strains containing genes under IPTG-inducible control (Vagner *et al.*, 1998). Here we report the development of plasmid pBaSysBioII, constructed during the EU-funded BaSysBio systems biology program (http://www.basysbio.eu/) for use in LCA analysis of gene expression in *Bacillus*. It can be used to generate transcriptional fusions of *Bacillus* promoters with *gfp* in a high-throughput manner using ligation-independent cloning (LIC) and integration of each fusion into the chromosome in a non-mutagenic manner. pBaSysBioII was characterized using five strains carrying transcriptional fusions with promoters responsive to growth rate, phosphate availability or carbon source. Our analyses yielded high-resolution definition of the kinetics of promoter activity in response to each stimulus that are consistent with those previously established at lower resolution with conventional approaches.

METHODS

Bacterial strains and growth conditions. *E. coli* TG1 was used for plasmid constructions and transformation using standard techniques (Sambrook *et al.*, 1989). A tryptophan prototrophic *B. subtilis* 168 strain (provided by E. Dervyn and P. Noirot, INRA, Jouy en Josas, France) was transformed using standard procedures (Anagnostopoulos & Spizizen, 1961). Luria–Bertani (LB) broth was used to grow *E. coli* and *B. subtilis*. For LCA experiments, *B. subtilis* was grown in low phosphate defined medium (LPDM), high phosphate defined medium (HPDM) (Muller *et al.*, 1997), or in a modified M9 medium supplemented with 0.4 % malate as the sole carbon source (Harwood & Cutting, 1990). Media were supplemented with antibiotics at the following concentrations, as required: 100 μg ampicillin ml^{-1} or 100 μg spectinomycin ml^{-1}.

Plasmid constructions. Plasmid pBaSysBioII contains an LIC site generated synthetically and the *gfpmut3* gene derived from plasmid pJBA27 (Cormack *et al.*, 1996; Andersen *et al.*, 1998). The *gfpmut3* gene was amplified from plasmid pJBA27 using oligonucleotides that introduced an optimized ribosome-binding site for *B. subtilis* (AAGGAGGAACTACT**ATG**: complementary to the 3′ end of the 16S rRNA and positioned thus, relative to the *gfpmut3* ATG indicated in bold type) and subcloned into a precursor cloning vector. The LIC–*gfpmut3* cassette was amplified using oligonucleotides OBP235A (containing the LIC site upstream of the ribosome-binding site) and OBP238, digested with *Eco*RI and cloned (as a blunt end/*Eco*RI fragment) into pDG1727 (Guérout-Fleury *et al.*, 1995) digested with *Nae*I and *Eco*RI. The resulting plasmid pBaSysBioII was established in *E. coli* with selection for spectinomycin and was completely sequenced. A schematic diagram of the plasmid is shown in Fig. 1(a). Promoter regions (400 bp) of *phoA*, *murAA*, *cggR*, *ptsG* and *gapB* were generated by PCR from genomic DNA using the appropriate primers listed in Table 1, and purified using the GFX purification kit (GE Healthcare). For LIC (Fig. 1b), 0.2 pmol of each fragment was incubated with 2.5 mM dTTP and T4 DNA polymerase. The pBaSysBioII plasmid was linearized using *Sma*I, gel-purified, and treated with T4 DNA polymerase and 2.5 mM dATP. A mix of 5 ng prepared vector and 15 ng inserts was used to transform *E. coli* (Fogg & Wilkinson, 2008; Aslanidis & de Jong, 1990; Bonsor *et al.*, 2006). The resulting plasmids were extracted from *E. coli* and used to transform *B. subtilis*. Three independent clones from each transformation were selected and used for LCA experiments to verify biological reproducibility.

Culture of *B. subtilis* in an LCA. For the phosphate-limitation experiments, *B. subtilis* strains were grown overnight in HPDM at 37 °C in a 96-well plate and then diluted to OD_{600} 0.05 in 100 μl LPDM in a 96-well black plate with optical bottom (Nunc). To convert LPDM to HPDM during the experiment, 5 μl of 73 mM phosphate solution was injected into all wells, giving a final phosphate concentration of 3.65 mM. For carbon source shift experiments, cultures of *B. subtilis* strains growing exponentially in 100 μl LB in 96-well plates were inoculated into M9 medium with 0.4 % malate at a range of dilutions and incubated overnight. The dilution that yielded exponentially growing cultures next morning was used to inoculate 100 μl of the same medium (M9 malate) to OD_{600} 0.05 in a 96-well cell culture plate (CELLSTAR, Greiner Bio-One). The carbon source shift was carried out by injection of a glucose solution to a final concentration of 0.3 % when cells reached OD_{600} 0.4. All plates (with lids to avoid evaporation) were incubated at 37 °C with constant shaking (slow) in a Synergy 2 multimode microplate reader (BioTek) for 10 h. The OD_{600} and fluorescence (excitation 485/20 nm, emission 528/20 nm) were measured at the designated intervals (1 min for carbon shift and 10 min for phosphate limitation). The OD_{900} and OD_{977} were measured at the beginning of the experiment in order to correct the lightpath length of samples to 1 cm using the following equation: $(OD_{977}-OD_{900})/0.18$.

Promoter activity was calculated as follows: background fluorescence of wild-type *B. subtilis* (the mean from six cultures) was determined and subtracted from the raw fluorescence values of reporter strains at the same OD_{600}; promoter activities were calculated taking the derivative of the fluorescence divided by the OD_{600} ($dGFP/dt/OD_{600}$) at each time point (Ronen *et al.*, 2002; Zaslaver *et al.*, 2006). Curves were smoothed by averaging expression values from three time points $(P_1+P_2+P_3)/3$. For the 1 min kinetics, polynomial and exponential functions were used to fit the experimental GFP dataset; promoter activities ($dGFP/dt/OD_{600}$) were consequently calculated using these functions.

Single-cell microscopy. Fluorescence microscopy was performed with a Leica DM5500 B microscope equipped with a motorized stage and a temperature-controlled incubation chamber set at 37 °C. Time-lapse movies were recorded by taking pictures every 5 min. The fluorescence images were acquired using a Leica EL6000 lamp and L5 filter cube. To minimize phototoxicity, the intensity of the lamp was set to 10 %. The exposure time was 1000 ms. M9 agarose medium with glucose or malate was pipetted into a 65 μl Frame-Seal slide chamber (SLF-0601, Bio-Rad). Using a scalpel, two large sections of the solidified M9 agarose were removed, leaving a ~1.5 mm strip in the centre of the frame. This allowed sample aeration, with evaporation being prevented by the seal between frame, slide and coverslip (Veening *et al.*, 2008). A preculture in M9 medium containing 4 g malate l^{-1} as the single carbon source was made from an overnight culture in LB medium (Difco) containing 10 μg spectinomycin ml^{-1}. After reaching OD_{600} 0.2, cells were applied to the M9 agarose slide containing 3 g glucose l^{-1} as the carbon source. Phase-contrast images were false-coloured red and fluorescence pictures were false-coloured green for visualization using the ImageJ software package (http://rsbweb.nih.gov/ij/). Time-lapse images were processed with ImageJ, and statistical analysis of the data was done in Microsoft Excel.

RESULTS AND DISCUSSION

Features of the integrating pBaSysBioII plasmid

The pBaSysBioII plasmid (Fig. 1a) was designed for high-throughput generation of transcriptional fusions with the *gfpmut3* reporter gene to monitor promoter activity using LCAs of *B. subtilis*. Generating transcriptional fusions by a

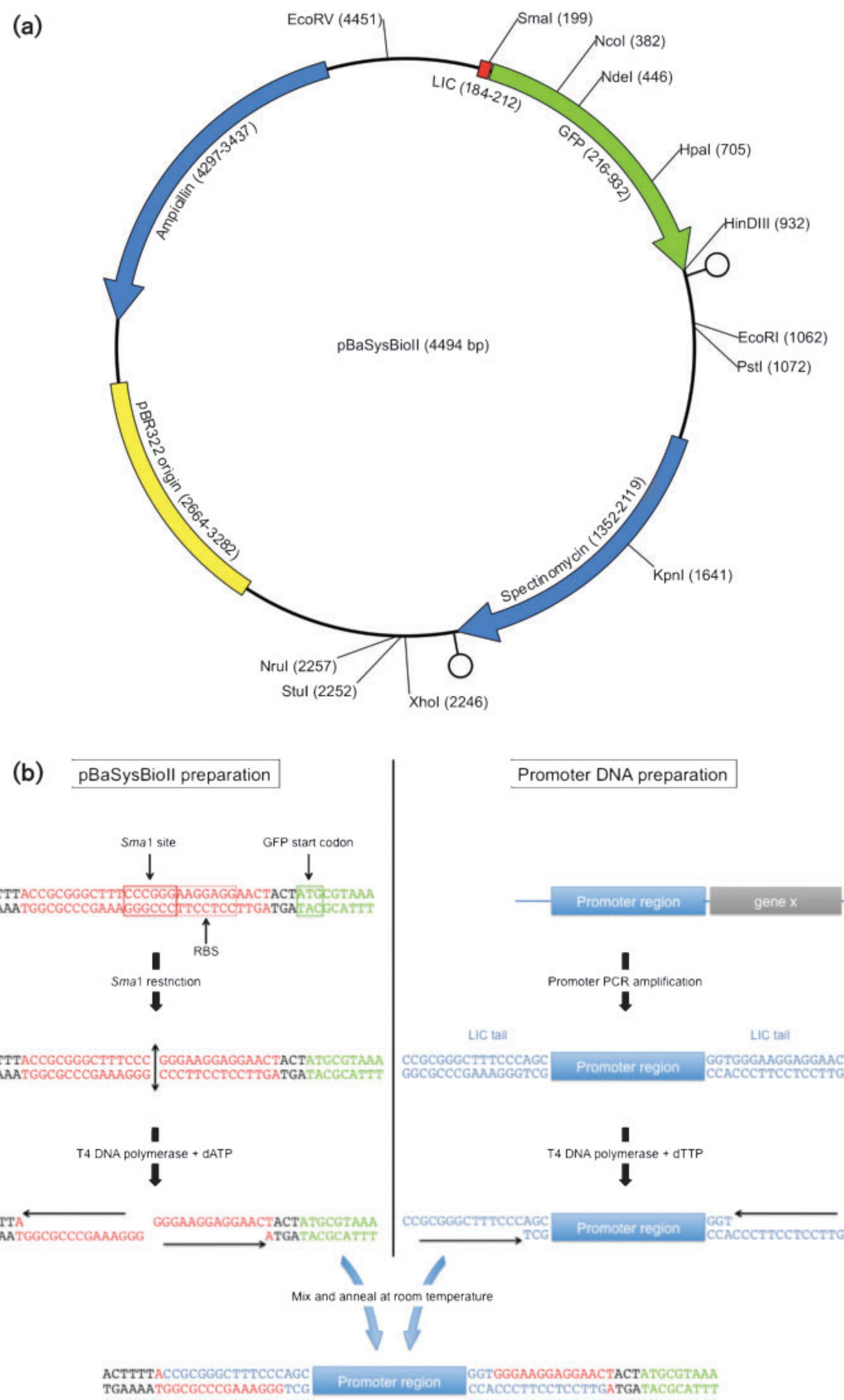

Fig. 1. Schematic diagram of (a) pBaSysBioII and (b) the LIC system. (a) pBaSysBioII plasmid ORFs are indicated by thick coloured arrows, with the direction of transcription indicated. The identity of each ORF is indicated adjacent to each box, followed by its plasmid coordinates. Transcriptional terminators are indicated by lollipops. Relevant restriction sites are also indicated. (b) LIC cleavage of pBaSysBioII with *Sma*I and treatment with T4 DNA polymerase in the presence of dATP generates linearized vector DNA with 14- and 13-base 5′ overhangs, as shown (red). Promoter fragments suitable for high-throughput cloning are generated by amplification of chromosomal DNA using forward and reverse primers with an LIC tail (blue) containing sequences complementary to the LIC sequences of the pBaSysBioII plasmid. Treatment of the PCR-amplified promoter fragments with T4 DNA polymerase in the presence of dTTP generates 5′ single-stranded DNA overhangs that are perfectly complementary to those of the vector. Annealing of the treated vector and PCR fragments at room temperature produces a circular duplex species with staggered nicks on the two strands. The duplex is sufficiently stable for direct introduction into competent *E. coli* strains.

Table 1. Oligonucleotides used in this study

Sequences required for LIC are underlined.

Oligonucleotide	Sequence (5′–3′)
phoAF	CCGCGGGCTTTCCCAGCATTTTCCTCCCCAAATGTTA
phoAR	GTTCCTCCTTCCCACCCCGTTTATCATGTTAGGGAA
murAAF	CCGCGGGCTTTCCCAGCCGACTGATATGCAGTCACAA
murAAR	GTTCCTCCTTCCCACCTTTATGCATTTAAGTCAGAA
gapBF	CCGCGGGCTTTCCCAGCGAGCTGGAAGCGGACGGTG
gapBR	GTTCCTCCTTCCCACCAATTATGCCATCCTAATTTG
cggRF	CCGCGGGCTTTCCCAGCCGCCTATACATTTTGGATC
cggRR	GTTCCTCCTTCCCACCCTTTTTGCTGGACATTATATG
ptsGF	CCGCGGGCTTTCCCAGCAAAAAAGAAGAACCGACTAAAGAACCAG
ptsGR	GTTCCTCCTTCCCACCTTTTACTAGTCTGACCTTACAGCTAAAAATCATG
OBP235A	ACGCGTCGACTTTTACCGCGGGCTTTCCCGGGAAGGAGGAACTACTATGCGTAAAG
OBP238	CCGGAATTCGGTTCTGTAGTCGACTTCTCACC

Campbell-type single crossover event was chosen because plasmid construction is amenable to high-throughput semi-automated DNA manipulation technology, and integration into the chromosome is non-mutagenic, resulting in positioning of the fusion at its homologous site. Promoter-containing fragments were ⩾400 bp in length, i.e. 400 bp fragments were used when the intergenic region was less than or equal to this size, while the full intergenic region was used when it exceeded this size. The 3′ end of all promoter-containing fragments was positioned 18 nucleotides upstream of the start codon of the first cistron of each operon (i.e. it excluded the endogenous ribosome-binding site) to ensure generation of a transcriptional *gfp* fusion. An LIC tail sequence (Fig. 1b) was designed into the oligonucleotides used to amplify each promoter fragment. A similar LIC tail sequence is positioned immediately upstream of the *gfp* ribosome-binding site in pBaSysBioII (Fig. 1b). Thus, after linearization of pBaSysBioII with *Sma*I, treatment of the vector (in the presence of dATP) and promoter-containing fragment (in the presence of dTTP) with T4 DNA polymerase generates DNA molecules with complementary single-stranded ends. Transformation of *E. coli* is performed after mixing and annealing the vector and insert (without DNA ligase addition) at room temperature (Fig. 1b). Advantages of this approach include amenability to high-throughput semi-automated technologies, high transformation frequency with little or no background, and directional cloning of the promoter-containing fragment, thereby generating transcriptional *gfp* fusions. Gfp expression was measured in at least three independent transformants with each transcriptional fusion, since instances were observed where the Gfp level in a particular transformant was exactly twofold higher than that of their co-transformants. This is most likely due to integration of a dimeric plasmid into the chromosome or to amplification of the integrated plasmid.

The GFPmut3 variant is reported to be extraordinarily stable in *E. coli*, with a conservatively estimated half-life of more than one day (Andersen *et al.*, 1998). To estimate the GFPmut3 half-life in exponentially growing *B. subtilis* cells under conditions as close to physiological as possible (i.e. without addition of translation-inhibiting antibiotics), we utilized the fact that transcription of the P_{tuaA}*gfpmut3* and P_{phoA}*gfpmut3* fusions ceases within 30 min of phosphate addition to phosphate-limited cells (Fig. 2a). Assuming a similar decrease of *gfp* transcript level during this time period, then translation of GFP should cease within 30 min of phosphate addition. Therefore, the persistence of GFPmut3 in this exponentially growing culture (Fig. 2a, between t_{360} and t_{480}) reflects the half-life of the protein in cells under these conditions. The estimated half-life of GFPmut3, using data from both these transcriptional fusions that have different expression levels, was approximately 10 h. This level of stability makes it an ideal reporter for promoter activity determination in *B. subtilis*, as detailed below.

Monitoring promoter activity in an LCA during phosphate limitation and in response to changes in carbon source

To test the performance of pBaSysBioII in monitoring promoter activity, and in particular to evaluate its sensitivity in detecting changed gene expression patterns, we chose four promoters of differing strengths whose activity changes significantly in response to altered nutritional conditions. PhoA is an alkaline phosphatase that is induced upon phosphate limitation; MurAA is a UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase, the first enzyme of the peptidoglycan biosynthetic pathway; GapB is the gluconeogenic glyceraldehyde-3-phosphate dehydrogenase; and PtsG is the glucose-specific enzyme II of the carbohydrate : phosphotransferase system (PTS). The activity of the *phoA* and *murAA* promoters increases and decreases, respectively, in response to phosphate limitation, with the reverse profile occurring upon phosphate injection into a culture of phosphate-limited cells. Likewise, the activity of the *gapB* and *ptsG* promoters

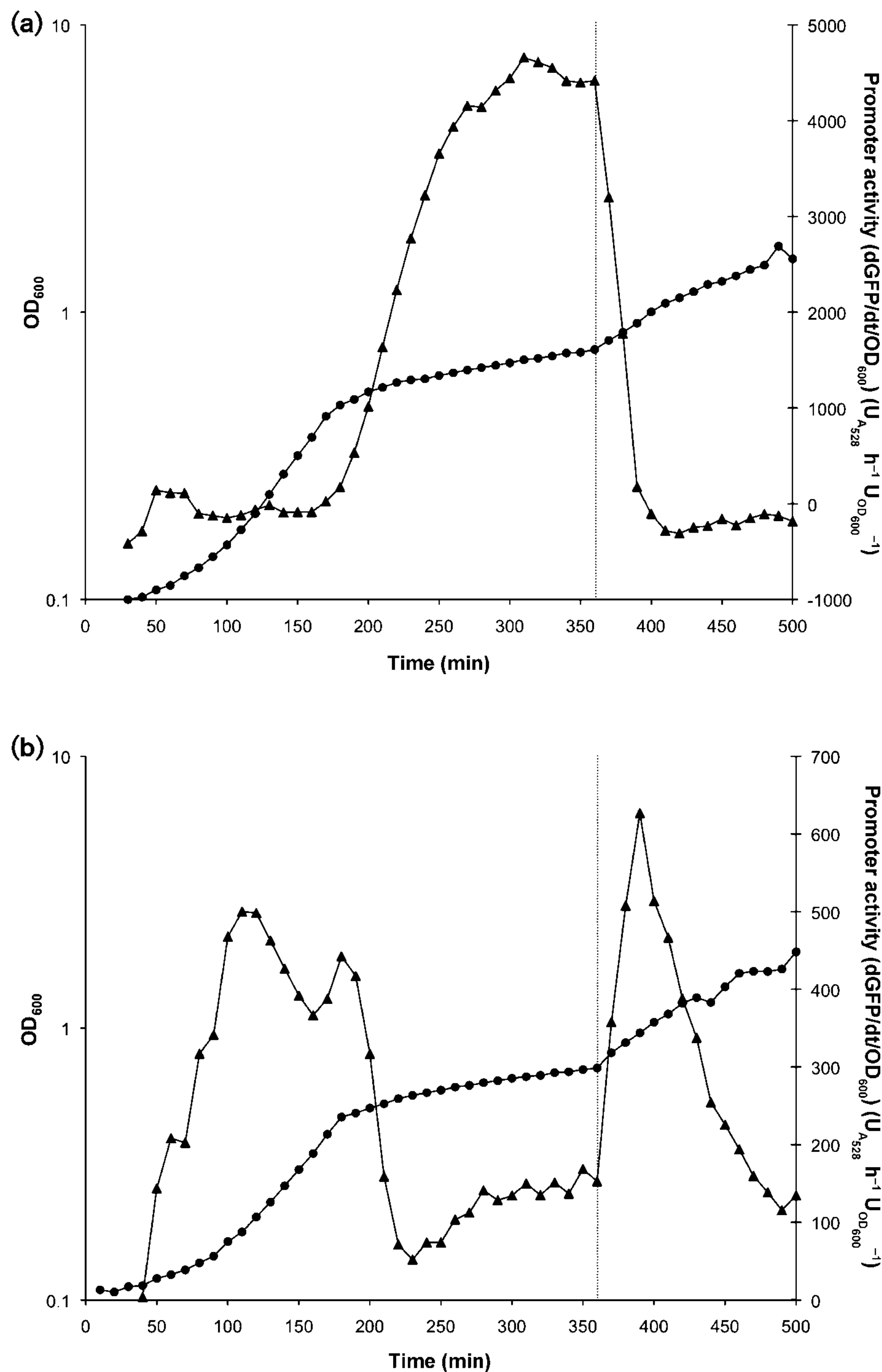

Fig. 2. Growth and promoter activity profiles of wild-type strain 168 carrying (a) the P_{phoA}*gfp* and (b) the P_{murAA}*gfp* transcriptional fusions at their homologous chromosomal locus. Both strains were grown in LPDM and phosphate limitation conditions were continued for 180 min (180–360 min). Phosphate was injected into phosphate-limited cultures at 360 min, indicated by a vertical dotted line. ●, Growth; ▲, promoter activity.

decreases and increases, respectively, when glucose is added to a culture growing on malate as sole carbon source (Fillinger *et al.*, 2000).

The growth profiles and promoter activities of strains carrying the promoter fusions under the relevant conditions are shown in Fig. 2 (phosphate limitation) and Fig. 3

(change of carbon source). Strains carrying $P_{phoA}gfp$ (Fig. 2a) or $P_{murAA}gfp$ (Fig. 2b) transcriptional fusions grew exponentially in LPDM for 180 min until OD_{600} ~0.5, at which point the growth rate decreased significantly due to phosphate limitation (180–360 min). When phosphate was injected into these phosphate-limited cultures at t_{360}, the growth rate increased dramatically within 10 min of phosphate addition. The two promoters of interest (P_{phoA} and P_{murAA}) behave differently during this growth regime. The *phoA* promoter is not active during exponential growth but activity commences upon phosphate limitation, stimulated by the PhoPR two-component system in response to phosphate limitation (at t_{180}), increasing to ~5000 activity units (Fig. 2a). Two aspects of the *phoA* promoter activity profile are of particular note: (i) the point of increased promoter activity coincides with the onset of phosphate limitation, indicated by decreased growth rate; and (ii) promoter activity increases during the initial ~2 h of phosphate limitation, at which point the maximum is achieved. Promoter activity then continues at this maximal level during the remaining phosphate limitation period. This expression profile is consistent with that observed using Northern analysis (Howell *et al.*, 2006; A. Howell and K. M. Devine, unpublished results). When phosphate was added by injection into the phosphate-depleted culture at t_{360} (dotted line), *phoA* promoter activity decreased precipitously within 10 min of phosphate addition and returned to basal levels within 30–40 min of injection (Fig. 2a). MurAA is involved in peptidoglycan biosynthesis; thus, its expression is expected to be maximal during exponential growth. There is a burst of $P_{murAA}gfp$ promoter activity when cells enter the exponential period of growth that reaches a plateau of 500 units and remains at approximately this level (400–500 units) throughout exponential growth (Fig. 2b). Upon entry into phosphate limitation, the point at which growth rate slows, there is a small increase in *murAA* promoter activity followed by a precipitous decrease to approximately one-tenth of its peak level. This rapid decrease in expression upon decreased growth rate is consistent with Northern analysis of *murAA* expression under similar conditions (Kock *et al.*, 2004). However, *murAA* promoter activity is not shut off during phosphate limitation, but increases gradually, consistent with the very low growth rate that occurs during this period (Bisicchia *et al.*, 2010; this study). The promoter activity of *murAA* increases dramatically within 10 min of phosphate addition (t_{360}), coincident with the increased growth rate observed at this time. Thus, altered promoter activity can be detected within 10–20 min of the applied stimuli (phosphate limitation and addition), showing that the transcriptional fusions generated with pBaSysBioII can be used to monitor the kinetics of gene expression during these short time intervals.

The activity of the $P_{ptsG}gfp$ and $P_{gapB}gfp$ promoters upon glucose addition to cultures growing in M9 medium with malate as sole carbon source was also determined (Fig. 3). This situation is distinct from phosphate limitation in that glucose addition to malate-grown cells does not lead to a major change in growth rate. The $P_{ptsG}gfp$ promoter activity is very low, while $P_{gapB}gfp$ promoter activity is high during growth on malate as sole carbon source (t_{-10}–t_0, Fig. 3a, b, respectively). When glucose is added to these cultures (vertical dotted line) at OD_{600} ~0.4, the promoter activity of $P_{ptsG}gfp$ sharply increases while that of $P_{gapB}gfp$ rapidly decreases. These profiles are consistent with results

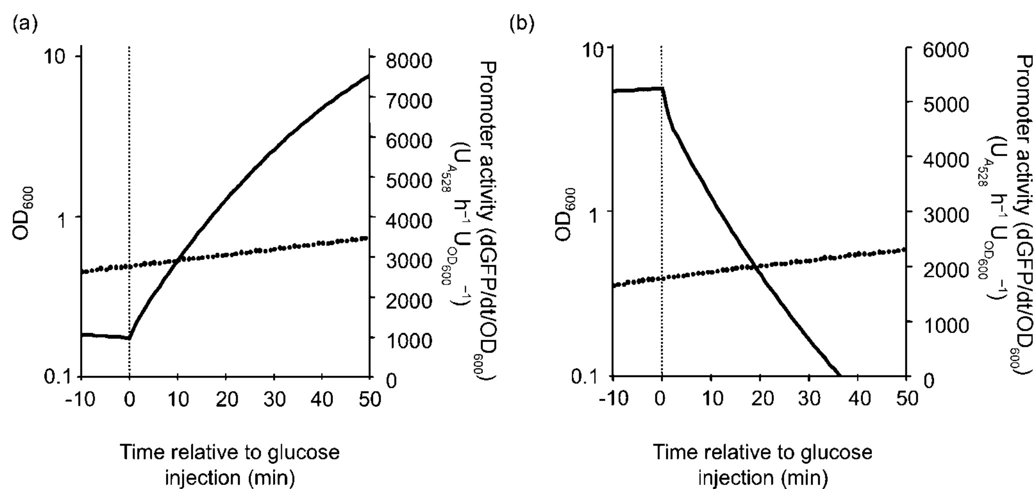

Fig. 3. Growth and promoter activity profiles of wild-type strain 168 carrying (a) the $P_{ptsG}gfp$ and (b) the $P_{gapB}gfp$ transcriptional fusions at their homologous locus. Both strains were grown in M9 medium containing malate as carbon source. Glucose was added to a culture growing exponentially (broken lines) at the time indicated by the vertical dotted lines. To smooth the technical variability related to very short OD_{600} and GFP measurements, exponential fittings were used for the biomass and polynomial fittings were used for the GFP. Growth is indicated by broken lines and promoter activity by solid lines.

previously obtained using *lacZ* transcriptional fusions (Stülke *et al.*, 1997; Fillinger *et al.*, 2000) and transcriptomic arrays (Blencke *et al.*, 2003). However, pBaSysBioII is amenable to establishing a very detailed profile of promoter activity. Here we show the response of these promoters to glucose addition, monitoring growth and expression (OD_{600} and GFP) at 1 min intervals from 10 min prior to injection to 50 min after injection. These detailed measurements show that changes in *ptsG* and *gapB* promoter activity can be detected as soon as 1–3 min after glucose addition (Fig. 3). Furthermore, the kinetic profiles show that the *gapB* promoter is fully turned off within 35±3 min of glucose addition (Fig. 3b). In contrast, *ptsG* promoter activity increases within the first minute after glucose addition, but a plateau has still not been reached almost 1 h after the shift (Fig. 3a). These results show that the GFPmut3 protein can be used in *B. subtilis* to monitor changes in promoter activity over a wide range in an LCA format and that changes in expression can be detected over very short time periods.

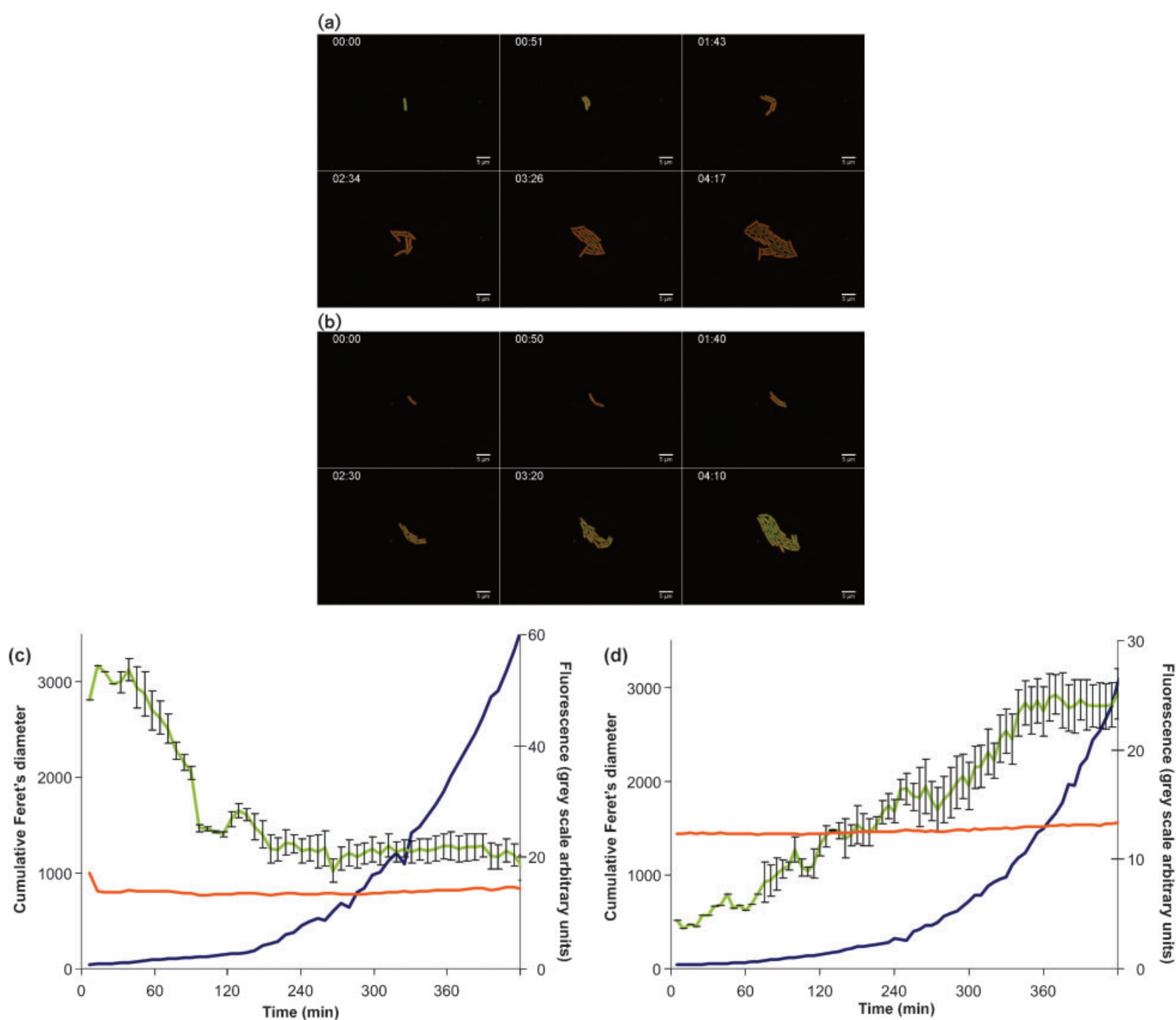

Fig. 4. Time-lapse fluorescence microscopy of cells expressing the P_{gapB}*gfp* (a, c) and P_{cggR}*gfp* (b, d) transcriptional fusions. Cells were precultured in liquid M9 medium containing malate and subsequently transferred to microscope slides with M9 medium containing glucose. (a) *gfp* expression driven by the P_{gapB} promoter; (b) *gfp* expression driven by the P_{cggR} promoter. The time points of image recording after transfer of the cells to the microscope slide are indicated in the upper-left corner of each individual image (h : min). (c) GFP fluorescence and growth of the strain with P_{gapB}*gfp*; (d) GFP fluorescence and growth of the strain with P_{cggR}*gfp*. The background-corrected fluorescence of GFP is plotted in grey scale units (green line with error bars). Background fluorescence is shown as an orange line. Growth was measured by calculating the cumulative Feret's diameter of the cells (blue line). The Feret's diameter of a *B. subtilis* cell is defined as its length from pole to pole.

Monitoring promoter activity by microscopy

We examined expression of the P_{gapB}*gfp* and P_{cggR}*gfp* fusions at the single-cell level upon a carbon source shift from malate to glucose, to ascertain how GFPmut3 accumulates intracellularly and to establish whether there is any heterogeneity of expression within growing micro-colonies. The *cggR* gene encodes the repressor of the hexacistronic *gapA* operon, which includes the *cggR*, *gapA*, *pgk*, *tpi*, *pgm* and *eno* genes. As documented by Doan & Aymerich (2003), binding of the CggR repressor to its single DNA target sequence is modulated by fructose 1,6-bisphosphate. Importantly, the *cggR* and *ptsG* expression profiles are known to be very similar (Doan & Aymerich, 2003). If heterogeneity existed, i.e. if only a proportion of cells expressed Gfp, then the promoter activities determined by LCA would not be a true reflection of the expression levels in all cells of the culture. For fluorescence microscopy, cells precultured in M9 medium with malate were transferred to a micro chamber containing M9 agarose medium with glucose, conditions comparable to the LCA. Individual cells were monitored by time-lapse microscopy (Fig. 4). Crucially, all cells grown on malate expressed *gapB* (Fig. 4a, c), but did not detectably express *cggR* under these conditions (Fig. 4b, d). Upon spotting the malate preculture onto the glucose-containing agarose slide, expression of *gapB* was turned off in all cells (Fig. 4a, c), while *cggR* expression was turned on in all cells (Fig. 4b, d). Thus, there is no heterogeneity of promoter activity in cells growing on malate plus glucose, and gene expression can be monitored in real-time in individual cells. While the trends of Gfp expression observed by time-lapse fluorescence microscopy and plate reader assays are similar, the kinetics appear to differ. This may be due to differences in the physiology of cells growing on an agar surface or in liquid media, to differences in data acquisition by the Biotek plate-reader and microscope, or to other as-yet-unidentified factors.

Concluding remarks

The pBaSysBioII plasmid has been developed for high-throughput analysis of promoter activity in *B. subtilis*. It contains an LIC sequence for high-throughput cloning of *B. subtilis* promoter sequences. Transcriptional fusions are generated using the *gfpmut3* gene as reporter. The GFPmut3 produced is stable in *B. subtilis*, with a half-life of approximately 10 h, allowing promoter activity to be determined in an LCA format in microtitre plates. Changes in promoter activity can be detected within short time intervals (1–10 min) of the stimulus, allowing the kinetics of gene expression to be accurately established. Here we show that the transcriptional response to phosphate limitation and altered carbon source occurs very rapidly, being detected within 10 and 1–3 min of the applied response, respectively. These data are consistent with previously published expression profiles, but significantly refine the kinetics of the responses. Furthermore, expression heterogeneity within each culture can be monitored by single-cell microscopy studies. Thus, the pBaSysBioII plasmid is a new tool to study the expression of genes in *B. subtilis* using high-throughput and single-cell methods. Our ongoing efforts are aimed towards the construction of a pBaSysBioII-based LCA that covers all transcriptionally active regions of the *B. subtilis* chromosome, as recently identified through transcript profiling with high-density arrays (Rasmussen *et al.*, 2009).

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