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A rapid reporter system using GFP as a reporter protein for identification and screening of synthetic stationary-phase promoters in *Escherichia coli*

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Abstract To develop a rapid reporter system for the screening of stationary-phase promoters in *Escherichia coli*, the expression pattern of the green fluorescent protein (GFP) during bacterial cultivation was compared with that of the commonly used β -galactosidase. Using GFP with enhanced fluorescence, the expression pattern of both reporter systems GFP and β -galactosidase were similar and showed a typical induction of gene activity of the reporter genes, i.e. increase of expression at the transition from exponential to stationary phase. The expression was affected by the culture medium, i.e. in contrast to the complex medium (LB medium), the stationary-phase specific induction was only observed in synthetic medium (M9) when amino acids were added, whereas there was generally no induction in MOPS medium. To develop a rapid screening method on agar plates for stationary-phase promoters, a photographic approach was used, continued with computational image treatment. A screening method is presented which enables an on-line monitoring of gene activity.

Introduction

For large-scale production of recombinant proteins, new expression vectors that include metabolically regulated promoters provide cost-effective alternatives to establish improved expression systems (Sawers and Jarsch 1996). Besides nutrient-regulated promoters (Wanner et al. 1988; Carter et al. 1992; Su et al. 1990; Kasahara et al. 1991),

carbon-source-regulated promoters (Cagnon et al. 1991; Lobell and Schleif 1991; Müller et al. 1989; Death and Ferenci 1994), oxygen-regulated promoters (Khosla and Bailey 1988; Dikshit et al. 1990; Schroeckh et al. 1992; Sawers 1993; Oxer et al. 1991) or promoters regulated in response to osmolarity (Herbst et al. 1994), stationary-phase promoters represent a further class of regulated promoters which could potentially be exploited for designing expression vectors. These vectors could be extremely useful for the cost-effective production of recombinant proteins. However, natural stationary-phase promoters seem to be relatively weak (Becker and Hengge-Aronis 2001), and their regulation is often complex. Therefore, many promoters cannot be used independently on the host strain or culture medium. To overcome the limitations, we constructed libraries of synthetic stationary-phase promoters for *Escherichia coli* (Miksch et al. 2005). The promoters selected in this way cover a wide range of promoter activities and have different induction times.

For an effective screening of large bacterial populations, a rapid screening system which can also be automated would be advantageous to detect and characterise bacterial clones containing stationary-phase promoters. Commonly, reporter genes are used to monitor transcription indirectly by putting genes of enzymes under the control of promoters of interest.

Until now, an overwhelming number of experiments dealing with the characterisation and monitoring of stationary-phase promoters were carried out with β -galactosidase as a reporter system (Notley and Ferenci 1996; Barth et al. 1995; Hiratsu et al. 1995; Becker and Hengge-Aronis 2001; Mellis et al. 1994; Weichart et al. 1993; Wise et al. 1996; Lange and Hengge-Aronis 1991; Schweder et al. 1996; Pratt and Silhavy 1996; Hengge-Aronis et al. 1993; Loewen et al. 1993; Brondsted and Atlung 1996; Aldea et al. 1989, 1990; Lange et al. 1993; Yim et al. 1994; Yamagishi et al. 1993; Lomovskaya et al. 1994). However, a major limitation of this system is that it is invasive, requiring permeabilization of bacterial cells. Inasmuch as the efficiency of cell permeabilization varies, observed differences in β -galactosidase activity can re-

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flect altered substrate uptake rather than changing levels of gene expression, preventing the exact analysis of individual clones (Nwoguh et al. 1995). In addition, because the β -galactosidase assay comprises several steps for preparing bacterial cells, this reporter system cannot be automated. Generally, experiments addressing real-time measurements of gene expression tend to use fluorescence-emitting reporter systems, which have also the greatest sensitivity (Hautefort and Hinton 2000). Therefore, we decided to use the green fluorescent protein (GFP) from *Aequorea victoria* (jellyfish) as a reporter protein, which naturally emits green fluorescence after excitation by blue light (Cubitt et al. 1995; Prasher et al. 1992; Ward 1998). Until now, there is only one report where GFP was used for monitoring stationary-phase promoters (Makinoshima et al. 2002). GFP does not require the addition of any substrate or co-factor, it promises real-time visualization of gene expression, and measuring can be automated. Initially, we were not successful by using *gfp* as a reporter gene for bacterial promoter activity, because fluorescence intensity was too weak. However, by

using a bright mutant of GFP obtained by DNA shuffling (Cramer et al. 1996), we got satisfactory results.

Here we compare the use of GFP with that of the β -galactosidase assay for monitoring of the expression pattern of stationary-phase promoters and investigate the effect of different culture conditions. Using GFP together with a computational approach, we present a practical reporter system for identifying and characterising stationary-phase promoters with the basis for automation.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K12 strain DH5 α [F^- *gyrA96* (Nal^r) *recA1 relA1 endA1 thi-1 hsdR17* ($r_k^- m_k^+$) *glnV44 deoR* Δ (*lacZYA-argF*) *U169* (ϕ 80f Δ [*lacZ*] *M15*)] was used as a host strain for all expression vectors. For construction of a GFP-*lacZ* expression vector, the promoter probe vector of clone 372-35 (Miksch et al. 2005) containing GFP as a reporter protein

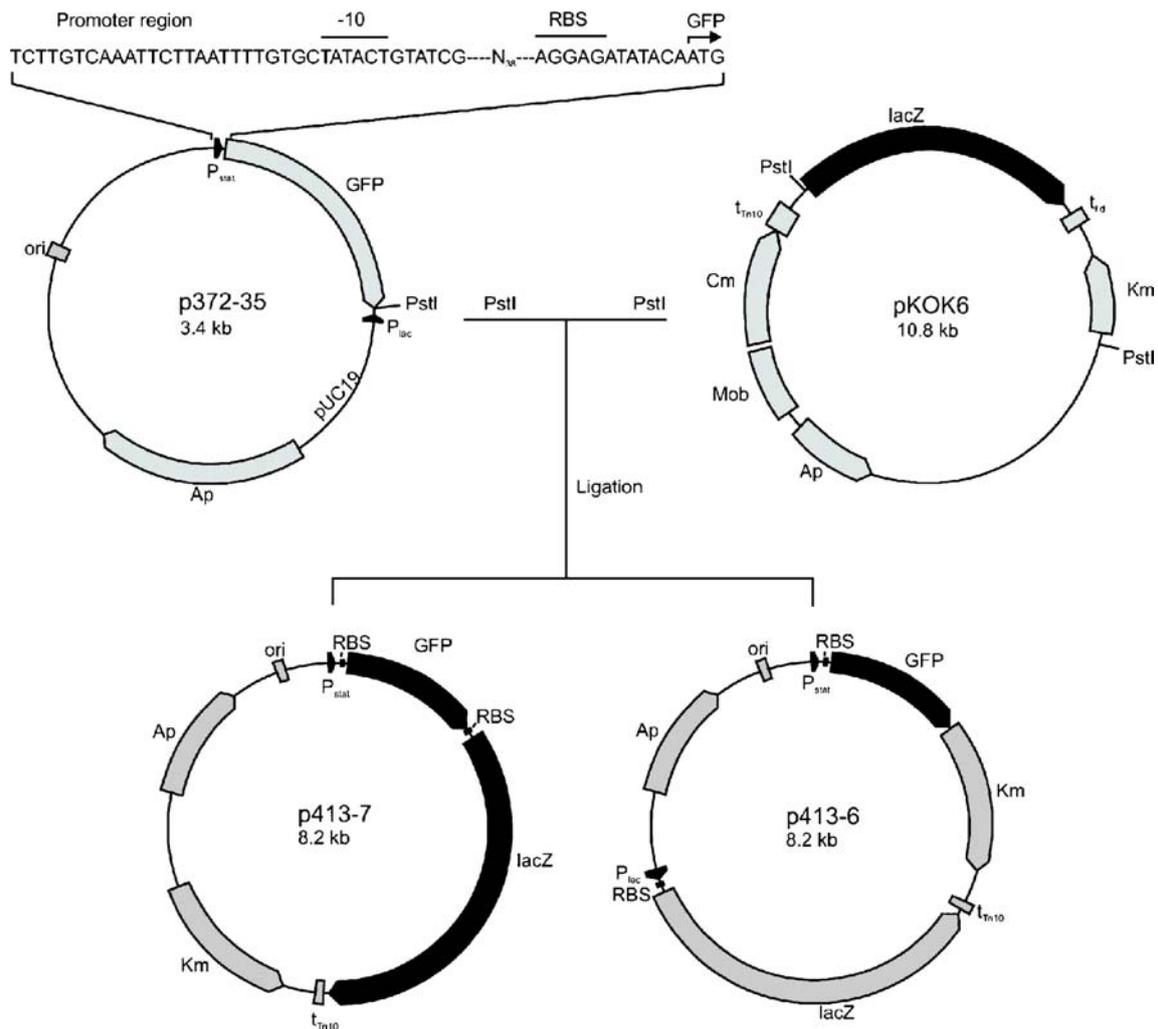


Fig. 1 Construction of a GFP-*lacZ*-expression cassette (p413-7). Both reporter genes *gfp* and *lacZ* are transcribed by the same stationary-phase promoter. In plasmid p413-6, *gfp* is transcribed by the stationary-phase promoter, whereas *lacZ* is transcribed by the *lac* promoter

was used. This vector was selected from a library of synthetic stationary-phase promoters cloned upstream of GFP. The *gfp* gene of the vectors originates from pBAD-GFP (Crameri et al. 1996). A 4.7-kb fragment containing a lacZ-kanamycin cassette with a promoterless *lacZ* gene, including its natural ribosome binding site as well as a transcription termination signal and the gene for kanamycin resistance, was cleaved out from plasmid pKOK6 (Kokotek and Lotz 1989) by *Pst*I digestion and inserted into the single *Pst*I site immediately downstream of the *gfp* gene. Thus, in theoretically 50% of insertions, both *gfp* and *lacZ* were transcribed as a transcriptional unit by the same promoter (Fig. 1). The resulting plasmid was designated p413-7. On the other hand, when the fragment was inserted in opposite direction (plasmid p413-6), the *lacZ* gene was expressed by the LacZ promoter after induction with IPTG.

To demonstrate a constitutive expression pattern of GFP on agar plates, plasmid pBAD-GFP (Crameri et al. 1996) was used. In this case, bacteria were cultivated on agar plates containing 0.2% arabinose. As a control for non-expressed *gfp* gene, the promoter cloning vector p363 containing the *gfp* gene without promoter was used.

β -Galactosidase assay

The assay was carried out as described by Miller (1972). Cultures carrying the plasmids p413-7 and p413-6, respectively, were grown in LB medium (Sambrook et al. 1989), supplemented with ampicillin ($200 \mu\text{g ml}^{-1}$). The results presented are averages of measurements of the activities of three individual cultures.

Fluorescence assay

To compare the induction pattern of fluorescence caused by the expression of GFP with that of β -galactosidase, as well as the effect of cultivation medium on promoter activities, the cultivation of selected clones was carried out in 1-l Erlenmeyer flasks. One hundred and fifty milliliters of LB medium was inoculated 1:100 with an overnight culture grown in LB medium. For experiments to investigate the effect of different media onto bacterial growth and expression pattern of GFP, LB and two synthetic media, M9 (Sambrook et al. 1989) and MOPS (Neidhardt et al. 1974), were used in a modified form, i.e. both media were supplemented with all the 20 proteinogenic amino acids (in mg l^{-1} : alanine 430, L-arginine 5,400, asparagine 320, aspartic acid 320, cysteine 80, glutamic acid 530, L-glutamine 530, glycine 360, L-histidine 190, L-isoleucine 314, L-leucine 630, L-lysine 350, L-methionine 180, L-phenylalanine 400, L-proline 280, DL-serine 6,300, L-threonine 290, tryptophan 120, L-tyrosine 220, L-valine 420; Ajinomoto, Raleigh, USA) and nucleotides (in mg l^{-1} : adenine 160, guanine 180, cytosin 130, uracil 130), as well as thiamine (20 mg l^{-1}) and calcium panthothenate (28 mg l^{-1}). The flasks were shaken at 150 rpm (rotary

shaker model Certomat-R, Braun, Melsungen, Germany) and 37°C . Ampicillin was added to a concentration of $200 \mu\text{g ml}^{-1}$. Samples were taken at the time intervals indicated. For measuring the optical density and fluorescence, $250 \mu\text{l}$ of bacterial culture was filled into the wells of 96-well microtiter plates (black with transparent bottom; Greiner, Frickenhausen, Germany). For each strain, eight repetitions were used with LB medium as a reference. Optical density and fluorescence were measured consecutively with the multi-functional plate reader Spectrafluor Plus (Tecan, Crailsheim, Germany), i.e. optical density was measured as OD_{600} , and fluorescence was measured with an excitation at 395 nm and an emission at 509 nm. When the optical density of bacterial cultures exceeded 0.5, the OD was taken after 1:5 dilution in LB. For all measurements of fluorescence, a gain of 55 was used. The gain is an indication of the sensitivity of the reader. Specific fluorescence was calculated as quotient of relative fluorescence units (rFU) (average of eight repetitions) and OD_{600} and designated as specific relative fluorescence units, sRFU = $\text{rFU}/\text{OD}_{600}$. Controlling the reader and data acquisition can be managed by the software tool XFLUOR4, which is an Excel-macro. On the basis of this tool, the programme 'Analyzer' (Bettenworth, unpublished) was developed to compute the data and to analyse the expression pattern of different clones (see Results).

Cultivation of bacteria for digital imaging

For overnight cultures, 3 ml LB medium supplemented with ampicillin ($200 \mu\text{g ml}^{-1}$) was inoculated in culture tubes ($16 \times 100 \text{ mm}$, Omnilab, Hamburg, Germany) and shaken on the shaker KS125 (IKA Labortechnik, Staufen, Germany). Overnight cultures were diluted 1:100, and $1 \mu\text{l}$ of diluted bacterial suspension was dotted on rectangular agar plates containing LB medium (8×12 positions) so that each clone had eight repetitions. The dotted plates were incubated for 4 h at 37°C and then analysed by digital imaging.

Results

GFP reporter system vs. β -galactosidase assay

To compare the commonly used β -galactosidase with GFP as a reporter system, we constructed a GFP-lacZ cassette where both genes are located on the same transcription unit on the expression vector p413-7., i.e. both genes were expressed by the same stationary-phase promoter (Fig. 1). In contrast, when the lacZ-kanamycin cassette from pKOK6 was inserted in the opposite direction (as expected in 50% of clones), the *gfp* gene was also transcribed by the stationary-phase promoter, whereas the *lacZ* gene was transcribed by the inducible lac promoter located on the vector region coming from pUC19.

Optical density, fluorescence intensity and β -galactosidase activity were determined during cultivation. When

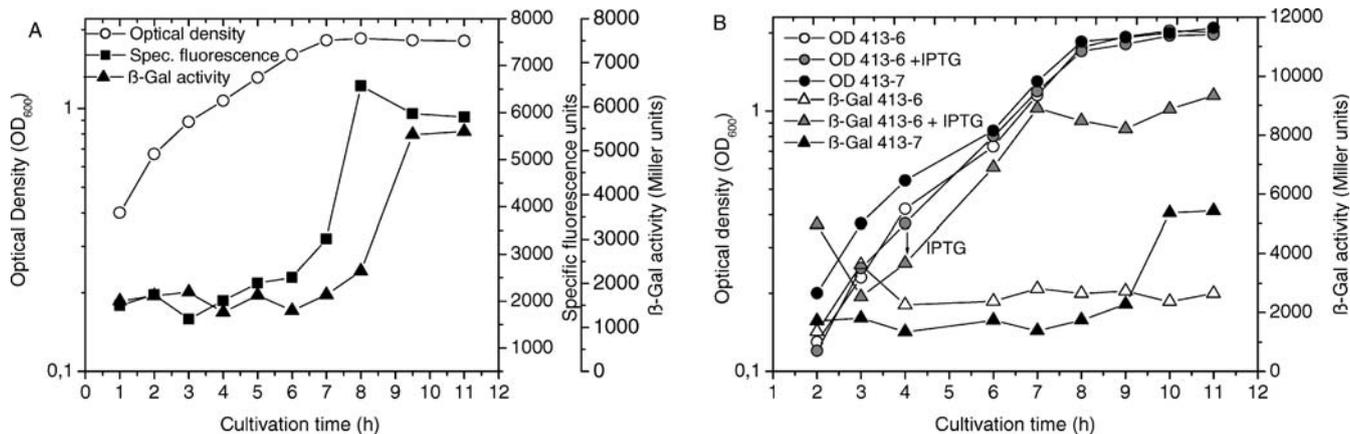


Fig. 2 Expression pattern of GFP and β -galactosidase in *E. coli* during cultivation in LB medium. **a** Cell density and specific expression of both genes *gfp* and *lacZ* located on plasmid p413-7 by

the same stationary-phase promoter. **b** Cell density and specific expression of *lacZ* located on plasmid p413-6 by the *lac* promoter with and without addition of IPTG

both reporter genes were transcribed by the stationary-phase promoter, the expression pattern of GFP and β -galactosidase were very similar, i.e. the induction of both gene activities began at the transition from exponential to stationary phase (Fig. 2). In contrast, when the *lacZ* gene was transcribed by the *lac* promoter (p413-6), no increase in β -galactosidase activity was observed without induction. However, as expected, β -galactosidase activity increased rapidly when IPTG as an inducing agent was added in early exponential phase. Figure 2 also shows that the maximal expression level in stationary phase was approximately 1.6 fold of that of the stationary-phase promoter. The reason can be that the stationary-phase promoter used is one of the weakest of the promoter library. On the other hand, the *lac* promoter is well known as a strong one.

The GFP reporter system is affected by culture conditions

Stationary-phase promoters are induced under starvation conditions or a reduced growth rate. To analyse the kind of starvation and the time of starving during cultivation, it is necessary to use defined synthetic media for bacterial growth. We compared the expression pattern of GFP expressed by a stationary-phase promoter during cultivation in a complex medium (LB) and two modified synthetic media (M9 and MOPS). Initial experiments had shown that in normal M9 and MOPS medium, GFP was expressed from the beginning of cultivation (data not shown), indicating that a low growth rate of bacteria is sufficient to cause the induction of a stationary-phase promoter. Therefore, all proteinogenic amino acids as well as nucleotides were added to the synthetic media to prevent stress caused by amino acid synthesis. In contrast to the normal M9 medium (which is a minimal medium), a rapid increase in fluorescence intensity at the transition from exponential to stationary phase was observed during cultivation of bacteria in supplemented M9 medium. However, the fluores-

cence level was lower than in LB medium (Fig. 3). Using the supplemented MOPS medium, no increase in fluorescence intensity was observed. This shows that the stationary-phase promoter was induced in the modified M9 medium, however, not in the modified MOPS medium. The reason for this is unknown. In contrast, the bacterial growth was not affected in modified MOPS medium compared to that of the modified M9 medium. It might be that 3-morpholinopropanesulfonic acid as the main component of the medium is toxic for the expression of GFP or for the synthesis of the sigma factor σ^S .

Digital imaging and data analysis

The transformation of promoter probe vectors containing promoter regions into *E. coli* results in a large number of different bacterial clones on many agar plates. These clones must be screened for promoters showing expression in the stationary phase using the fluorescence intensities of GFP.

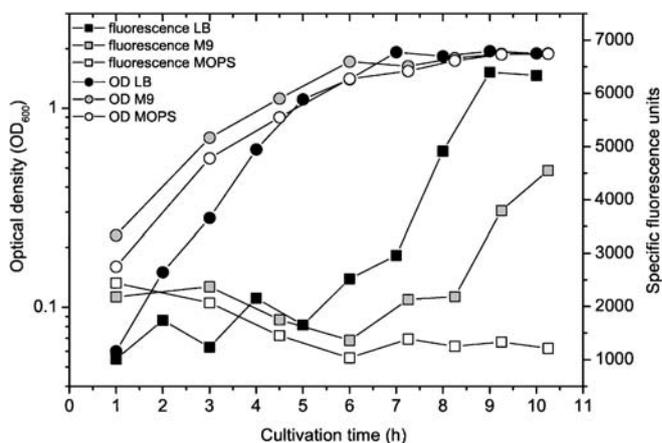


Fig. 3 Effect of the medium composition on the expression of a stationary-phase promoter. Comparison of cell density and specific expression of *gfp* in LB medium and two modified synthetic media (M9 and MOPS, both with amino acids)

Therefore, a rapid screening method allowing characterisation of many bacterial colonies or growth spots at the same time is desirable. In addition, first informations about induction time and promoter strength would be advantageous. Thus, we developed a method for on-line monitoring of fluorescence intensities of bacteria by digital imaging. A special dark-cultivation chamber containing a thermostate, two UV lamps, equipment for digital camera and electronical units was constructed. The cultivation chamber was connected with a PC. The temperature in the growth chamber could be kept constant ($\pm 1^\circ\text{C}$) and controlled by an external digital device. The UV lamp was controlled by a serial relais and connected via an RS232 interface with the PC. To set user-defined parameters for the UV lamps, a GUI was developed. Onset and time interval of UV light could be programmed by the user. A Canon PowerShot G2 with a green filter controlled by commercial remote capturing software (Remote Version 2.7, Canon) was used for image acquisition. The number and time of intervals could be defined.

The image evaluation was based on the determination of grey level intensities of fluorescent areas (Fig. 4). The range of grey values depended on the bit depth of the image. Twenty-four bit color images (8 bit per channel in the RGB model) can differentiate between 256 grey values per pixel and channel. Grey values (0–255) were used for image processing and analysis operations, e.g. to separate fluorescent and non-fluorescent areas. Figure 4 shows the flow diagram for image capturing and image analysis. After set up of UV light and camera functions (see left side of Fig. 4) image processing was characterised by several steps: image registration, Hough transformation, segmentation and morphological operations.

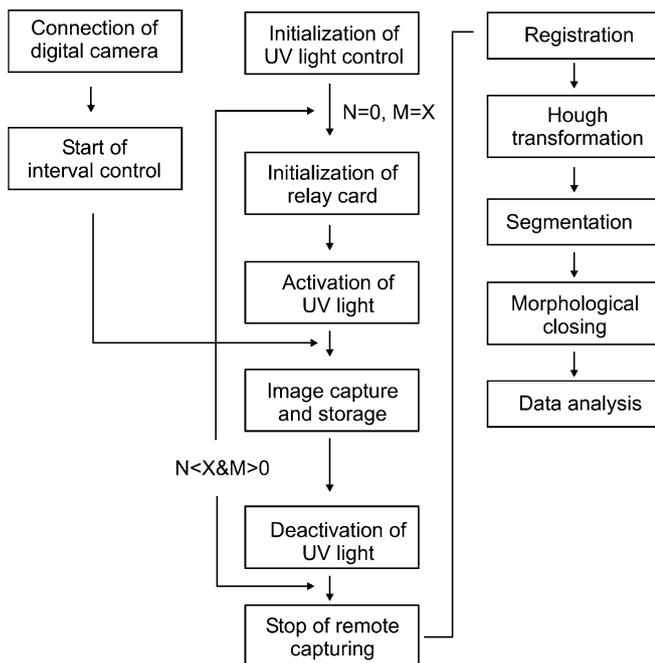


Fig. 4 Flow diagram of digital imaging and image processing

For image processing, a spatial congruence of all points of the image sequence is necessary. To compensate for possible shifts eventually occurring during cultivation, a rigid image registration was used. Using a similarity measure based on a normalised correlation coefficient, the rotation angles and translation components of a transformation were estimated by a gradient descent resulting in the highest correlation between two images.

The Hough transformation (Hough 1962) can detect arbitrary shapes of interest. It is not affected by image noises and systematical errors. In addition, the algorithm is able to detect partial structures. To identify single spots in agar plates, Hough transformation was used on the gradient magnitude image from the last one of the sequence. The position of spots can be estimated on the basis of the peaks in the Hough accumulator.

To separate fluorescent regions from non-fluorescent background, a threshold value was calculated from the last image of the sequence by the Otsu method (Otsu 1979). Using this method, the intensity histogram can be automatically divided into two distinct regions without requiring any pre-information. The Otsu method uses the histogram of image intensities and selects the threshold which maximises the ratio of inter-class and inner-class variances of fluorescent and non-fluorescent regions. The application of the threshold results in a binary mask which can be used for the detection of fluorescent regions. In addition, a morphological closing operator is used to remove small holes and gaps in the binary mask. Using the detected spots in combination with the binary mask, the average signal time curve for each spot of the image sequence could be calculated. After passing the steps of image processing, data analysis results in an average fluorescent signal for the clones tested.

The screening method for stationary-phase promoters described here was tested by using ten different clones with stationary-phase promoters. For comparison of the expression patterns, a constitutively expressed promoter and the promoter probe vector containing GFP without promoter were also involved. To investigate the reliability of fluorescence measurements, eight repetitions per strain were spotted (Fig. 5). The spots were set as a lattice net (corresponding to the pattern of 96-well microtiter plates) to guarantee the same distance between the spots and to simplify image registration.

As expected, fluorescence of bacteria containing GFP with stationary-phase promoters appeared generally later than those with a constitutively expressed GFP (Fig. 5a). A comparison of images of different times during the cultivation shows that even different onsets of fluorescence were visible. So the expression of GFP in clone 8 begins relatively late, whereas in clones with similar promoter strength (clones 108, 59 and 119), the fluorescence was already visible after 13 h (Fig. 5b,c). Thus, it was relatively easy to detect clones with stationary-phase promoters. To get more information about promoter strength and onset of expression, the plates were treated by the procedure of image processing described above (Fig. 6). During cultivation, all stationary-phase promoters showed a similar

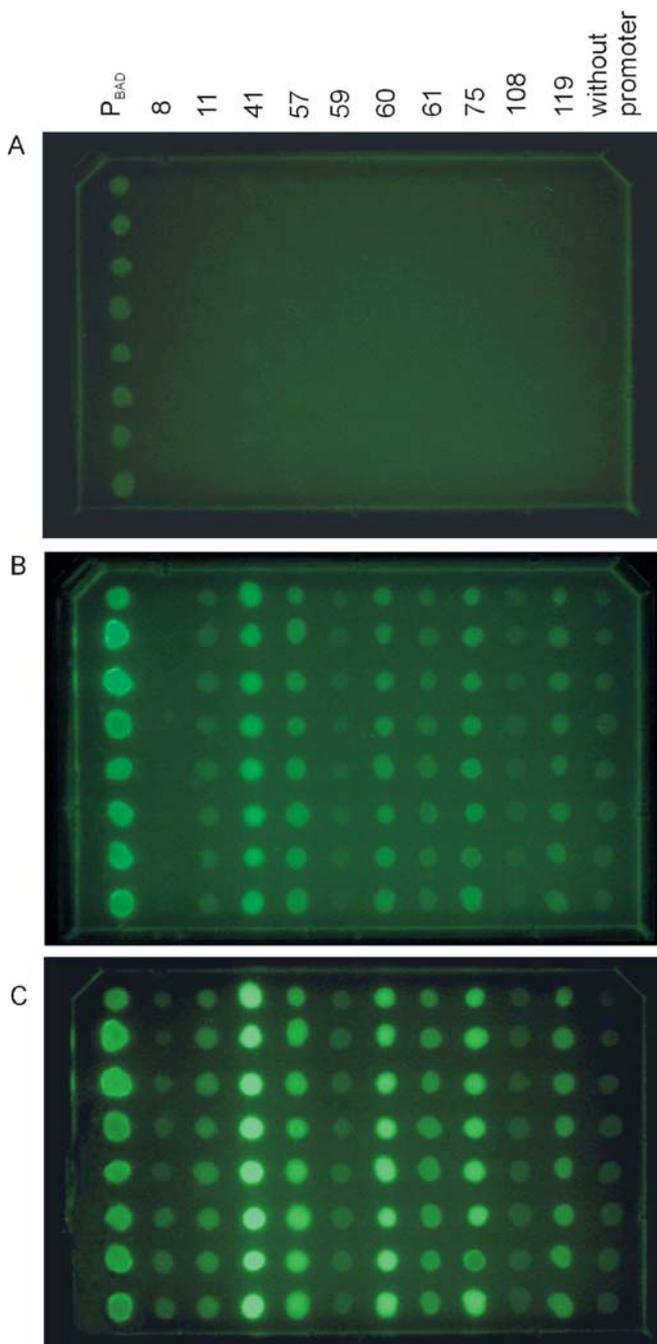


Fig. 5 Fluorescence of bacteria from different clones containing GFP expressed by stationary-phase promoters (8, 11, 41, 57, 59, 60, 61, 75, 108, 119), by a constitutively expressed promoter (P_{BAD}) and GFP without promoter at different times during cultivation on agar plates. **a** 4 h after spotting; **b** 13 h after spotting; **c** 24 h after spotting

delayed development of fluorescence, whereas the fluorescence of the constitutively expressed promoter increased rapidly after spotting of bacteria (Fig. 6a). The calculation of the standard errors indicated that the difference of fluorescence at the end of cultivation (after 24 h) was significant among several clones, i.e. at least four groups could be differentiated (Fig. 6b): very low fluorescence (clones 108, 59, 8 and 119), medium fluorescence (clones

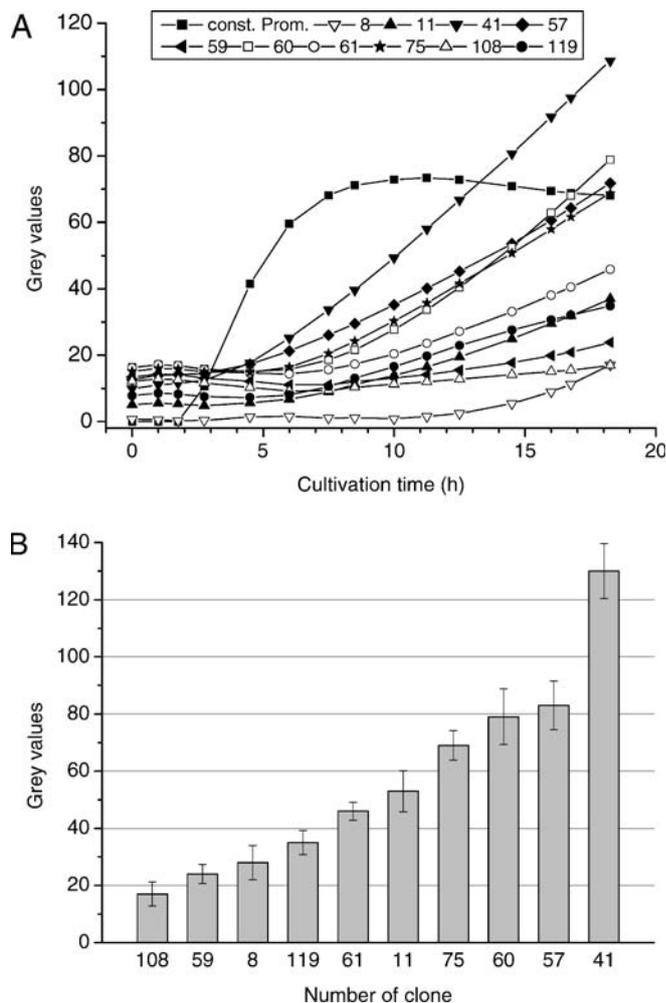


Fig. 6 Promoter activities of stationary-phase promoters on agar plates. Promoter activities were presented as grey values derived from fluorescence intensities by the method of image processing. **a** Promoter activities of 10 stationary-phase promoters and a constitutively expressed promoter (P_{BAD}) during cultivation. Values of the control without promoter were subtracted at each time. **b** Promoter activities of 10 stationary-phase promoters 24 h after spotting of bacteria on agar plates (see also Fig. 5c). The promoters were ordered according to their activities (SEM $P=0.05$)

61 and 11), higher fluorescence (clones 75, 60 and 57) and one clone with very high fluorescence (clone 41). The experiment was repeated several times resulting in similar relations between the clones.

Discussion

In the past, the LacZ reporter system was used primarily to demonstrate the expression profile of genes. To overcome the limitations of this reporter system and to establish a rapid screening system which can be automated, we developed a method based on the use of GFP with enhanced fluorescence and a computational approach. The comparison of expression profiles shows that the GFP reporter system can be used similarly as the β -galactosi-

dase assay for monitoring gene expression. In contrast to the β -galactosidase assay, GFP can be used for real-time measurements. On-line detection of gene expression is particularly desirable in fermentation processes. Furthermore, for the screening of large amounts of different clones such as in libraries, it would be desirable to have a rapid and simple detection method for gene expression. Therefore, the GFP reporter system is a suitable tool for our purposes.

The expression level of GFP was lower in modified M9 medium than in LB medium. A possible reason could be the sub-optimal synthesis of RpoS protein. Transcription of *rpoS* (σ^s) increases gradually throughout exponential phase with a very substantial increase following the transition to stationary phase (Mulvey et al. 1990). Investigations with *lacZ* fusions in different media revealed a fivefold stimulation of *rpoS* transcription in complex medium during entry into stationary phase, but little or no stimulation in minimal medium (Mc Cann et al. 1991; Lange and Hengge-Aronis 1991, 1994). By addition of amino acids, we could adjust the growth rate to that of LB; however, there are apparent regulation mechanisms which do not function in M9 medium. In general, the regulation of cellular levels of σ^s is not yet fully understood.

Although the expression level is lower than in LB medium, a typical stationary-phase expression pattern was recognized in modified M9 medium. Thus, this medium is suitable when cultivation or stress factors leading to stationary-phase induction are to be investigated.

Among the labyrinth of regulatory mechanisms, control factors and environmental factors, carbon, phosphate or nitrogen starvation and the corresponding onset of stationary phase were the first stresses identified (Gentry et al. 1993; Lange and Hengge-Aronis 1994; Jishage and Ishihama 1995; Muffler et al. 1997; Zgurskaya et al. 1997). Furthermore, acid shock (Gorden and Small 1993; Small et al. 1994), heat shock (Jishage and Ishihama 1995; Muffler et al. 1997) as well as high osmolarity (Muffler et al. 1996; Pratt and Silhavy 1996) can cause the increase of σ^s and enable stationary-phase behaviour. Furthermore, we suggest that the modified M9 medium may be a convenient synthetic medium to investigate whether there are specific promoter sequences for different induction modes.

To develop a method for the rapid screening of large amounts of colonies on agar plates, a photographic approach was used, followed by a computational image treatment. Using this approach, we could demonstrate that clones containing promoters with different expression levels and/or different expression profiles can be significantly distinguished. It was also shown that a rough impression of fluorescence intensity by eyes could be transformed into a reliable method by image processing. Using large petri dishes with single colonies, large amounts of clones can be screened. Furthermore, we suggest that the use of GFP and image processing as described here can be a powerful method to characterise any gene activity.

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