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A vector for promoter trapping in *Bacillus cereus*

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Abstract

We constructed a promoter-trap plasmid, pAD123, for *Bacillus cereus*. This plasmid contains a promoterless gene that encodes a mutant version of the green fluorescent protein, GFPmut3a, that is optimized for fluorescence-activated cell sorting [Cormack, B.P., Valdivia, R.H., Falkow, S., 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173, 33–38.]. The plasmid replicates and confers drug resistance in both *Escherichia coli* and *B. cereus*. We constructed a library in pAD123, which consists of 29 000 clones containing chromosomal DNA from *B. cereus* strain UW85. A portion of the library (988 clones) was screened for GFP expression in *B. cereus* UW85 using a 96-well microtiter dish assay. GFP expression was detected by visual inspection with a fluorimager. We identified 21 clones as fluorescing in the initial screen, and further characterized these clones by restriction analysis, sequencing, and quantification of fluorescence intensity. Flow cytometry and cell sorting efficiently separated *B. cereus* cells expressing GFP from a 10 000-fold excess of non-expressing cells. Selected clones provided useful markers to follow *B. cereus* populations on plant surfaces. Our results indicate that GFP and pAD123 are useful tools for identifying regulatory sequences in *Bacillus cereus*, and that flow cytometry and cell sorting is a useful method for screening large libraries constructed in this vector. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: GFP; Gene regulation

1. Introduction

The study of gene expression in *Bacillus cereus* is a largely unexplored area of biology although *B. cereus* has diverse biological activities in the environment. *B. cereus* is ubiquitous in soil and on plant roots (Stabb et al., 1994), and certain strains of *B. cereus* produce antibiotics (Wakayama et al., 1984; Kamogashira et al., 1988; Tschén and Tseng, 1989; Silo-Suh et al., 1994) and enterotoxins (Turnbull, 1986), and some antibiotic-producing strains suppress plant disease (Tschén and Lee, 1988, Silo-Suh et al., 1994). The diversity of roles of *B. cereus* in the environment stimulates significant interest in the study of how this organism regulates gene

expression in response to particular environmental conditions, and how this gene expression affects its biological activities.

A tool that is lacking for study of gene expression in *B. cereus* is a promoter-trap vector. Promoter-trapping involves the use of a specifically designed vector with the key feature being a multiple cloning site adjacent to a promoterless reporter gene. Libraries containing fragments of DNA cloned in front of a reporter gene can be used to study promoter activity under various environmental conditions. A particularly useful reporter gene is *gfp*. Unlike the use of *gus* or *lacZ* reporters, *gfp* allows the study of gene expression in environmental samples without the addition of exogenous substrates, and allows the use of techniques such as fluorescence-activated cell sorting (FACS) for rapid and efficient screening of libraries. *gfpmut3a* is a promoterless version of the *Aequorea victoria* green fluorescent protein gene that has been optimized for FACS (Cormack et al., 1996).

Our interests focus on regulation of gene expression in *B. cereus* strain UW85 (ATCC 52522), which colonizes plant roots (Halverson et al., 1993), suppresses

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Abbreviations: Ap, ampicillin; Cm, chloramphenicol; GFP, green fluorescent protein; LB, Luria–Bertani medium; MCS, multiple cloning site; NCBI, National Center for Biotechnology Information; nt, nucleotide; ORF, open reading frame; PMT, photo-multiplier tube; RBS, ribosome binding site; RGB, red green blue; TSA, tryptic soy agar; TSB, tryptic soy broth.

diseases caused by plant pathogens on tobacco, alfalfa, cucumber, soybean, and peanut (Handelsman et al., 1990, 1991; Smith et al., 1993; Osburn et al., 1995; Phipps, 1992), and produces several antibiotics (Silo-Suh et al., 1994) including zwittermicin A, an aminopolyol that represents a new class of antibiotic (He et al., 1994). Here, we present the construction of a promoter-trap plasmid for *B. cereus* containing an improved *gfp* gene, the use of the plasmid for constructing a promoter library of *B. cereus* UW85, and the results from initial screening of the library. We demonstrate the use of flow cytometry and cell sorting for efficient screening of pAD123 libraries and the use of an isolated clone as a marker organism for laboratory experiments involving plant–microbe interactions.

2. Materials and methods

2.1. General

All enzymes were purchased from Promega (Madison, WI) unless indicated otherwise. Methods for agarose gel electrophoresis and basic cloning were adapted from Current Protocols in Molecular Biology (Ausubel, 1997). DNA fragments were gel purified using the QIAGEN QIAquick PCR Purification Kit. Plasmid preparation was performed using the QIAGEN QIAprep Spin Miniprep Kit. *E. coli* strains were electroporated as directed in the operating manual for the Gene Pulser transfection apparatus (Bio-Rad Laboratories). pAD123 was introduced into *Bacillus subtilis* 168 by electroporation using method 3.13 in Molecular Biological Methods for *Bacillus* (Harwood and Cutting, 1990).

2.2. Construction of pAD123

pAD123 (Fig. 1) was constructed from portions of the plasmids pKK232-8 (Brosius, 1984), pHP13 (Haima et al., 1987), and pFPV25 (Valdivia and Falkow, 1996). A gel-purified *Pvu*II restriction fragment of pKK232-8 containing the gene for ampicillin resistance and the *Escherichia coli* pBR322 origin of replication was ligated (T4 DNA ligase) to a gel-purified *Hpa*I and T4 DNA polymerase-blunted *Bgl*I restriction fragment of pHP13, containing chloramphenicol resistance and the *Bacillus* replication origin from pTA1060. The resulting plasmid was then digested with *Eco*RI and *Hind*III, treated with shrimp alkaline phosphatase (Boehringer Mannheim), and ligated to a gel-purified *Eco*RI and *Hind*III restriction fragment of pFPV25 containing the promoterless *gfp*mut3a, resulting in pAD123.

2.3. Construction of a promoter library of *B. cereus* UW85

Chromosomal DNA from *B. cereus* UW85 was isolated using a protocol described previously (Silo-Suh et al., 1994). A partial *Sau*3A digest of purified *B. cereus* UW85 chromosomal DNA was ligated (T4 DNA ligase) to *Bam*HI-digested, shrimp alkaline phosphatase-treated pAD123. This ligation mixture was introduced into *E. coli* strain GM2929 by electroporation, and cells were plated on LB agar containing 10 µg/ml of Cm and 100 µg/ml of Ap. Approximately 200–300 colonies grew per plate. Colonies on each plate were scraped off the plate, pooled in 2 ml of LB broth containing 10 µg/ml of Cm and 100 µg/ml of Ap, and incubated at 37°C with shaking for 2 h. The culture was then divided, and 0.8 ml was added to 0.2 ml of 100% glycerol for storage at –80°C. Plasmid DNA was then purified from 1 ml and stored at –20°C. The library contains approximately 29 000 clones stored in 129 pools. Seven plasmid pools were introduced into *E. coli* DH5α by electroporation. From each pool, five clones were isolated, and plasmids were purified from these clones. The purified plasmids were digested with *Pst*I, and agarose gel electrophoresis was performed to determine the proportion of clones containing inserts.

2.4. Initial screen of the promoter library

Forty-five randomly selected plasmid pools were introduced into *B. cereus* UW85 by electroporation using a method described previously (Silo-Suh et al., 1994). Approximately 24 clones were picked at random from each pool, resulting in a total of 988 clones. The clones were screened in a 96-well microtiter dish assay for GFP expression in response to growth in 100 µl each of MESAA1 minimal medium (Milner et al., 1995), 1/10-strength TSB, and 1/2-strength TSB (all containing 10 µg/ml of Cm). Microtiter dishes were incubated at 25°C with gentle shaking. Induction of gene expression, measured by visual inspection of GFP fluorescence, was monitored throughout growth with a Molecular Dynamics FluorImager595 (scanning parameters: 200-µm pixel size, normal detection sensitivity, 650 PMT voltage, 16-bit digital resolution, emission filter 530DF30). Visually detectable fluorescence is defined as detection on the FluorImager595 without the aid of quantification software.

2.5. Characterization of GFP-expressing clones

Plasmids were isolated from *B. cereus* using a QIAGEN QIAprep Spin Miniprep protocol according to the manufacturer's instructions, except that cells were resuspended in buffer P1 containing 2 mg/ml of lysozyme, with incubation at 37°C for 20 min prior to

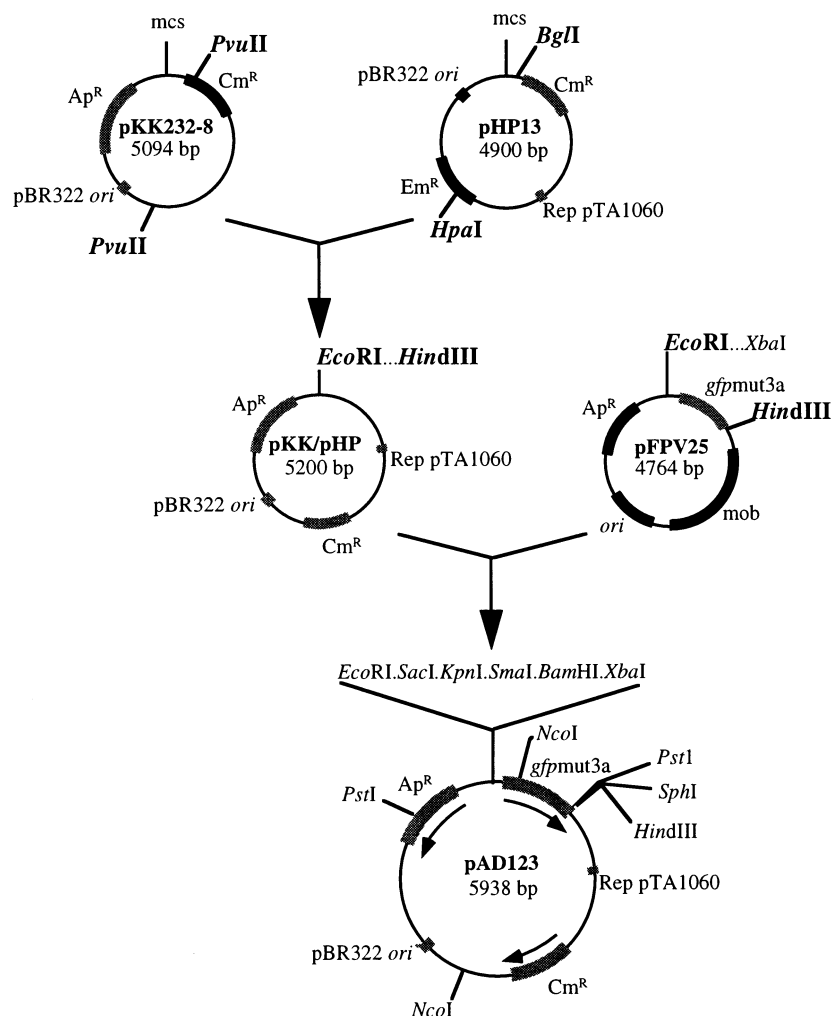


Fig. 1. Construction of pAD123. A restriction fragment of pKK232-8 containing Ap^R and the pBR322 *ori* was ligated to a restriction fragment of pHP13 containing Cm^R and Rep pTA1060, resulting in pKK/pHP. The *EcoRI*/*HindIII* restriction fragment of pFPV25 containing *gfpmut3a* was inserted into pKK/pHP, resulting in pAD123. *EcoRI*...*HindIII*, first and last restriction site in mcs; Ap^R , ampicillin resistance gene (*bla*); Cm^R , chloramphenicol resistance gene (*cam*); Em^R , erythromycin resistance gene (*erm*).

adding buffer P2. The purified plasmids were re-introduced into UW85 by electroporation to confirm GFP expression. Additionally, the plasmids were introduced into *E. coli* DH5 α by electroporation, and then purified from *E. coli*. A sample of the purified plasmid was then digested using *PstI*, and agarose gel electrophoresis was performed to determine the approximate insert size. Plasmids isolated from *E. coli* were the template for sequencing, performed with the ABI Prism[®] Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), using primers flanking the multiple cloning site; left: 5'ACC TGA CGT CTA AGA ACC CAT TAT T3', right: 5'GGG ACA ACT CCA GTG AAA AGT TCT T3'. Sequencing gels were run at the University of Wisconsin-Madison Biotechnology Center, on an ABI 373 Automated DNA Sequencer. Each sequencing reaction produced approximately 300–500 bases of readable sequence. The sequences were

analyzed with BLASTN and BLASTX for nucleotide or protein sequence similarity and searched for open reading frames using the ORF Finder program found on the NCBI home page (<http://www.ncbi.nlm.nih.gov/>). Putative ribosome binding sites were identified by defined optimal *B. subtilis* ribosome-binding sites (Mountain, 1989). EditSeq (DNASTAR, Inc.) was used to search clone sequences for -35 (TTGaca) and -10 (TATaAT) recognition sequences for the *B. subtilis* sigma factor, σ^A (Helmann, 1995).

2.6. Quantification of fluorescence in selected clones

Ten milliliters of 1/10-strength or 1/2-strength TSB containing 10 $\mu\text{g/ml}$ of Cm in 250-ml side-arm flasks were inoculated with 100 μl of a culture (a 12-h LB broth culture containing 10 $\mu\text{g/ml}$ of Cm grown at 28°C with shaking) of either UW85 containing pAD123, clone

31-26, or clone 43-25. The flasks were shaken at room temperature (25°C with gentle shaking). The absorbance of the cultures at 600 nm was measured using a Spectronic 20⁺ (Milton Roy), and three 100- μ l samples of each culture were transferred to a 96-well microtiter dish and scanned into the FluorImager595 using the parameters described above. The fluorescence intensity was determined using the volume report option in the ImageQuaNT quantification software (Molecular Dynamics). The values obtained for pixel intensity in an ellipse of constant width and height positioned directly inside the well wall were averaged for the three samples (this value is referred to as the average fluorescence intensity). Three replicate cultures were tested.

2.7. Fluorescence microscopy and photomicrographs of clones

Cultures of clones 31-26 and 43-25 were grown as described in Section 2.6. At 5 and 10 h, the A_{600} of the cultures was recorded, and three 100- μ l samples were placed into a 96-well microtiter dish and scanned into the FluorImager595. A photomicrograph was taken of the cultures on microscope slides using an Olympus BX-60 microscope equipped for epifluorescence microscopy with an HBO 100-W mercury arc lamp. Images were captured using an Optronics DEI-750 digital camera and IPLabSpectrum (Signal Analytics). A U83000 FISH Filter Set (Olympus) was used with an exposure time of 1/8 s. Images were stored as 24-bit RGB tagged image format files, and converted from analog to digital format using Adobe Photoshop Version 3.0. Images were printed on a Sony UP-5600MD color video/digital printer.

2.8. Flow cytometry and cell sorting

One-milliliter overnight cultures of *B. cereus* UW85 containing pAD123, clone 43-25, and clone 44-12 were grown at 25°C with gentle shaking (in 1/2-strength TSB containing 10 μ g/ml of Cm) in three replicates. One hundred microliters of each culture were used to inoculate 10 ml of the same medium type, and allowed to grow for 4 h or until early the log phase of growth. Samples of the cultures were then combined in a volume ratio of 0.5:0.5:10 000 (clone 43-25:clone 44-12:UW85 containing pAD123). The mixtures were sorted into GFP-expressing and non-GFP-expressing fractions using an EPICS Elite[™] flow cytometer/cell sorter (Coulter Corporation). The 488-nm air-cooled argon ion laser was set to 15 mW, and green fluorescence was detected using a 525-nm bandpass filter. The GFP-expressing fraction from each replicate was collected in 500 μ l of 1/2-strength TSB containing 10 μ g/ml of Cm and plated onto 1/10-strength TSA containing 10 μ g/ml of Cm. To verify the presence or absence of GFP

expression in the sorted fraction, plates were scanned into the FluorImager 595 using the parameters described in Section 2.4.

Additionally, a sort was performed on the mixture to separate the fraction of the culture containing the most fluorescent cells. These cells were collected and grown overnight in the same medium described above, and the plasmids were isolated as described in Section 2.5. A restriction-enzyme digestion was performed, as described in Section 2.5, to determine the approximate insert size as an indicator of plasmid identity (clone 43-25 contains an insert of approximately 1.0 kb, and clone 44-12 contains an insert of 3.6 kb).

2.9. Confocal scanning laser microscopy of clone 44-12

A 10-ml culture of clone 44-12 was grown in 1/2-strength TSB for 5 days at 25°C with gentle shaking until sporulated. One milliliter of the culture was washed three times with sterile distilled water and diluted 1/10 in sterile distilled water. Fifteen milliliters of this solution were used to moisten RI37 tomato seeds (Smith et al., in press) placed in sterile vermiculite in a sterile 100 \times 15 mm polystyrene petri dish (Fisher Scientific). The dishes were sealed with Parafilm 'M' (American National Can), and the seeds were allowed to germinate and grow at room temperature under normal light conditions. After 16 days, a seedling was removed from the vermiculite, placed on a microscope slide and covered with a 22 \times 50 mm coverslip. The image was captured with a Leica TSC-NT confocal microscope optimally configured for FITC/GFP analysis using the 488-nm excitation laser and a 40 \times 1.0NA oil PL Fluotar objective. The image was printed as described in Section 2.7.

2.10. Plasmid stability assay

Six replicate cultures of clone 44-12 were grown overnight in 1/2-strength TSB containing 10 μ g/ml of Cm. One hundred microliters of each were subcultured into 10 ml of 1/2-strength TSB and incubated for 10 h until the cells were in stationary phase (approximately 12 generations). This was repeated for a total of three sequential subcultures or 35 generations. The absorbance at 600 nm was measured as described in Section 2.6 after subculturing and at stationary phase. At each stationary-phase point, a 12.5- μ l sample was taken, placed into a 9-ml water dilution blank and spiral-plated (Autoplate model 3000, Spiral Biotech) onto 1/10-strength TSA and 1/10-strength TSA containing 10 μ g/ml of Cm. Plates were counted, as described in the Autoplate 3000 User Guide, and from these data, the percentage of Cm-resistant bacteria was determined.

3. Results and discussion

3.1. Construction of pAD123 and the UW85 library

Genetic tools are needed to study *B. cereus*, whose gene expression patterns are of interest due to its diverse biological activities. To facilitate the study of gene expression in *B. cereus*, we constructed a promoter-trap vector, pAD123 (Fig. 1). pAD123 is a shuttle vector with selectable markers and origins of replication for both *E. coli* and *B. cereus*. The replication origin was previously shown to be active in *B. subtilis* (Uozumi et al., 1980), *B. methanolicus* (Cue et al., 1997) and *B. cereus* (results presented here), which represent genetically diverse *Bacillus* species, and pAD123 is functional in both *B. subtilis* and *B. cereus*. The plasmid copy number is greater than 25 in *E. coli* (Bolivar et al., 1977) and five to six in *B. subtilis* (Bron, 1990).

pAD123 contains *gfpmut3a* as the promoterless reporter gene. The mutant protein encoded by *gfpmut3a* was optimized by Cormack et al. (1996) for use in fluorescence-activated cell sorting, and has an optimal excitation wavelength of 498 nm. GFPmut3a fluoresces more brightly and folds more efficiently than the protein encoded by the wild-type gene isolated from *Aequorea victoria* (Cormack et al., 1996). These characteristics make *gfpmut3a* a useful reporter gene in studies in which flow cytometry and cell sorting or a FluorImager595 are used to detect gene expression. Although the *gfp* gene and various red-shifted mutants have been used successfully in *B. subtilis* (Webb et al., 1995; Lewis and Errington, 1996), to our knowledge, there are no reports of the use of a FACS-optimized *gfp* in existing *B. subtilis* promoter-trap vectors or the use of *gfp* in *B. cereus*.

To test the utility of pAD123 in *B. cereus*, we constructed a library of chromosomal DNA from *B. cereus* UW85 and stored the library of 29 000 clones in pools of 200–300. Of the 35 clones tested, 31 contained DNA inserts (89%), ranging in size from 0.1 to 0.6 kb.

3.2. Initial screening of the UW85 library

To assess our ability to identify GFP expression in the library, we screened 988 randomly selected clones in a 96-well microtiter dish assay. Twenty-one clones expressed GFP and showed expression in minimal medium, 1/10-strength TSB, and 1/2-strength TSB.

To compare the efficiency of detection of fluorescence by visual inspection on the FluorImager595 with fluorescence microscopy, we screened 35 randomly selected clones in a 96-well microtiter dish assay for GFP expression. After overnight growth in 1/2-strength TSB, the dish was scanned into the FluorImager595 and four clones expressed GFP when inspected visually using the fluorimager. A total of eight clones fluoresced sufficiently to be detected by fluorescence microscopy. These results

suggest that visual assessment with a fluorimager is less sensitive than fluorescence microscopy.

3.3. Sequence analysis of the GFP-expressing clones

The insert sizes of the clones ranged from 0.063 to 3.6 kb. The clones with inserts greater than 0.4 kb were sequenced using primers originating from both regions flanking the multiple cloning site, and clones with inserts less than 0.4 kb were sequenced using a primer in the right-flanking region or the left-flanking region. Of the 18 clones sequenced, 11 had a similarity to protein or nucleotide sequences, open reading frames, and/or putative promoter sequences (Fig. 2). In three of these clones, the direction of expression of the similar gene or protein was in the opposite orientation to the expression of *gfpmut3a* (30-20, 31-25, and 44-12). Four clones (7-11, 10-1, 20-21, 44-12) contained inserts with a similarity to the beginning portion of hypothetical or known proteins. Five clones (12-10, 20-21, 27-20, 30-20, and 31-25) contained ORFs leading into the right flanking region of the vector, in the same orientation as expression of *gfpmut3a*. Clones 6-24 and 47-26 contained recognizable promoter-like sequences.

The data indicate that GFP expression is often associated with sequences that resemble authentic promoters. In some cases, GFP expression is associated with sequences that have promoter-like activity, but contain no recognizable promoter sequence. More conclusive tests for promoters will be performed on clones of interest isolated during future screens of the library.

3.4. Quantification of fluorescence intensity

To determine the effect of culture age on GFP expression, we measured the fluorescence intensity of clones 31-26 and 43-25 during growth in liquid culture. Whereas clone 43-25 expressed GFP throughout growth, clone 31-26 expressed GFP only when the cells reached the stationary phase (Fig. 3). The pattern of GFP expression for each clone was similar in 1/10-strength and 1/2-strength TSB. Though the sequenced portions of clone 31-26 did not contain a putative ORF or promoter sequence, the insert contains DNA that directs stationary-phase GFP expression.

To further relate the average fluorescence intensity to the amount of fluorescence visible to the eye, photomicrographs of individual bacterial cells of clones 31-26 and 43-25 were taken after 5 and 10 h of growth (Fig. 4). The fluorescence observed in the cells was directly related to the average fluorescence intensity for each clone, as shown in Fig. 3, verifying the measurements obtained for fluorescence intensity using the ImageQuANT software.

Clone	Insert Size (kb)	Putative RBS	Sequence Homology	Diagram
6-24 ¹	0.084		nonent21..TTGGCA..nt17..TACAAT..nt34....
7-11 ^{1,2}	1.4		A=last 1/3 of hypothetical <i>Lactobacillus helveticus</i> protein (36%) B=first 1/6 of hypothetical proteins, <i>Bacillus subtilis</i> (49%)	A → B →
10-1 ^{1,2}	1.0		<i>Bacillus isreali</i> malate dehydrogenase (79%)	→
12-10 ²	0.3	—	none→
20-21 ^{1,2}	2.4	+ *	probable <i>Bacillus subtilis</i> acetyltransferase (79%)→
27-20 ²	0.2	—	middle portion of <i>Bacillus subtilis</i> BEX protein (63%)→
30-20 ²	0.2	+ **	last 1/5 of <i>Bacillus</i> sp. BSID723 16srRNA gene (99%)	←→
31-25 ²	0.3	+ **	last 1/5 of <i>Bacillus</i> sp. BSID723 16srRNA gene (92%)	←→
31-26 ^{1,2}	1.0		middle portion of <i>Salmonella typhimurium</i> cysteine synthase B (63%)	←
44-12 ^{1,2}	3.6	—	A=first 2/3 of <i>Bacillus subtilis</i> transcription regulator, similar to transcriptional regulator (GntR family) (44%) B= <i>Clostridium difficile</i> transposon group II intron with potential coding region (88%)	A → B →
47-26 ¹	0.25		nonent52..TTGGTG..nt18..TATATA..nt117....

Fig. 2. Sequence analysis of 11 of the 21 isolated clones. The diagrams show the UW85 chromosomal DNA inserts (single line) containing the gene or protein sequence similarity, as determined by BLASTN or BLASTX searches, with the direction of transcription/translation (arrow), putative ORFs (dotted arrow), and/or putative promoter sequence (nt, number of nucleotides). DNA inserts are not drawn to scale relative to each other. The thick line denotes the regions of pAD123 flanking the multiple cloning site (*gfpmut3a* is adjacent to the right flanking region); ¹Sequencing primer 1 in the left flanking region; ²sequencing primer 2 in the right flanking region; *putative RBS of AAGGA; **putative RBS of GGAGG.

3.5. Flow cytometry and cell sorting

As a model for future screens of the pAD123 library, we performed a series of flow cytometry and cell sorting to determine the efficiency of flow cytometry and cell sorting in separating *B. cereus* UW85 cells expressing GFP from non-expressing cells. We performed three sorts of a cell mixture containing a 10 000-fold excess of cells not expressing GFP over the GFP-expressing cells. The fluorescent fraction was collected and plated, resulting in greater than 95% fluorescent colonies. We consistently sorted GFP-expressing *B. cereus* cells from those not expressing GFP.

As a test for the ability to sort *B. cereus* cells based on fluorescence intensity, a mixture of *B. cereus* UW85 cells containing pAD123 (a negative control), clone

43-25, and clone 44-12 was sorted to isolate the fraction of the mix with the greatest fluorescence intensity. Microscopic examination prior to the sort showed that clone 44-12 fluoresced with a greater intensity than clone 43-25. The restriction digestion pattern of the flow cytometry and cell sorting isolated bacteria was identical to that of clone 44-12.

We sorted GFP-expressing *B. cereus* UW85 cells from non-expressing cells rapidly using flow cytometry and cell sorting. Although induced gene expression may not result in 'on/off' phenotypes, flow cytometry and cell sorting has the potential to sort based on fluorescence intensity. Multiple sorts can separate clones with DNA inserts containing sequences whose transcription is regulated in response to conditions of interest from those not regulated under identical conditions. Such possibilit-

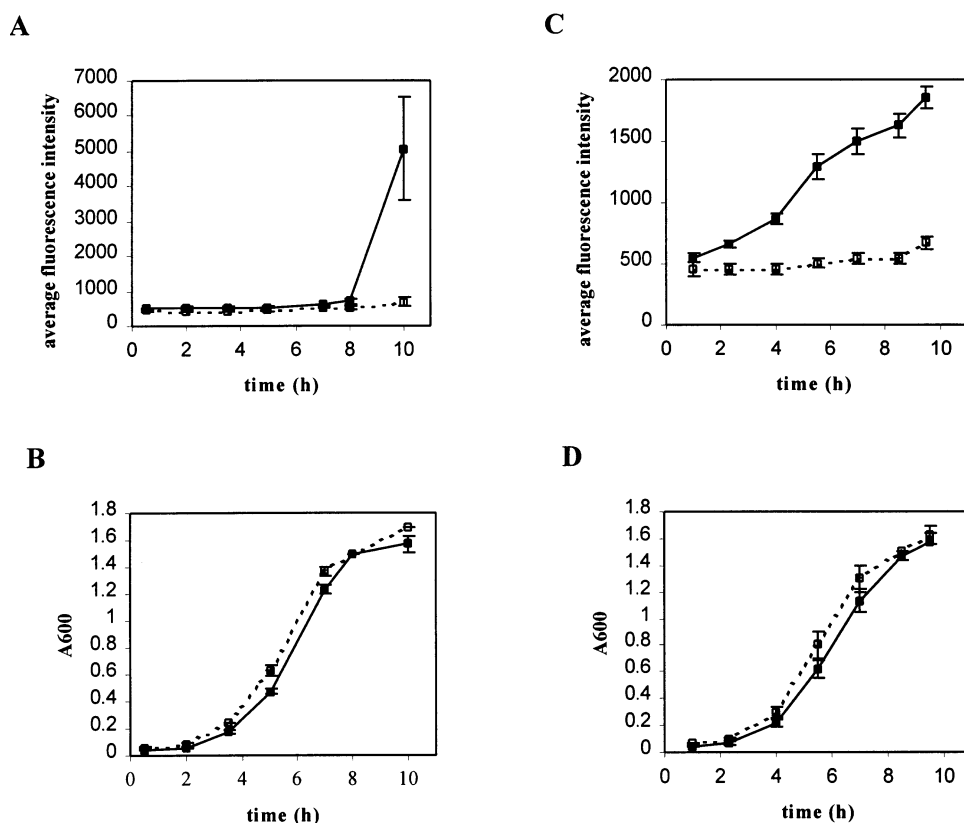


Fig. 3. Fluorescence intensity and growth curves of representative clones, grown in 1/2-strength TSB. In all graphs, *B. cereus* UW85 containing pAD123 is represented by a broken line, and the clone (either 31-26 or 43-25) is represented by a solid line. (A) Average fluorescence intensity over time for clone 31-26 and *B. cereus* UW85 containing pAD123. (B) Growth curves of clone 31-26 and *B. cereus* UW85 containing pAD123. (C) Average fluorescence intensity over time for clone 43-25 and *B. cereus* UW85 containing pAD123. (D) Growth curves of clone 43-25 and *B. cereus* UW85 containing pAD123. The standard error of the mean is reported using error bars. Where not visible, the error bars are found within the symbol for the data point. Average fluorescence intensity was measured using the ImageQuANT analysis software standard with the FluorImager595 (Molecular Dynamics). Fluorescence patterns were similar in 1/10-strength TSB.

ies have been shown with *Salmonella typhimurium* and GFP expression in response to growth in murine macrophages (Valdivia and Falkow, 1997).

3.6. Use of clone 44-12 as a marker organism

Our initial screen identified clones that expressed GFP constitutively under the conditions tested. One constitutively expressing clone was used to monitor the interactions between tomato plants and *B. cereus*. We coinoculated spores of clone 44-12 with germinating tomato seeds. Seedling roots were viewed using confocal scanning laser microscopy to identify bacteria expressing GFP attached to roots. A typical root is shown in Fig. 5. In this case, a single plane scan was combined with a pseudo-colored transmitted light image. The apparent variation in fluorescence intensity can be explained by bacteria partially contained in the scanned plane.

Chloramphenicol was not used as a selection for plasmid retention in the presence of the plant due to the potential for adverse developmental effects in the

tomato seedlings. A plasmid stability assay was performed to monitor the percentage of bacteria containing the plasmid during approximately 35 generations of growth without selection. At 10 h (approximately 12 generations), an average of 74.86% (range of 42.39–105.45%) of the bacteria were Cm-resistant, at 20 h (approximately 24 generations), an average of 43.3% (range of 30.33–60.38%) were Cm-resistant, and at 30 h (approximately 35 generations), an average of 29.33% (range of 19.28–50.75%) were Cm-resistant.

It would be desirable to construct a mutant of *B. cereus* UW85 that contains a stable copy of GFP on the chromosome to be used as a reliable marker organism for laboratory studies involving the interactions of *B. cereus* UW85 with plants. Recent work in *Azoarcus* sp. BH72 (Egener et al., 1998) showed the utility of GFP fusions for visualizing single cells in soil and on plant roots. Some of the promoter-like sequences identified in the initial screen have the potential to be used as chromosomally maintained drivers of GFP expression if introduced into the chromosome.

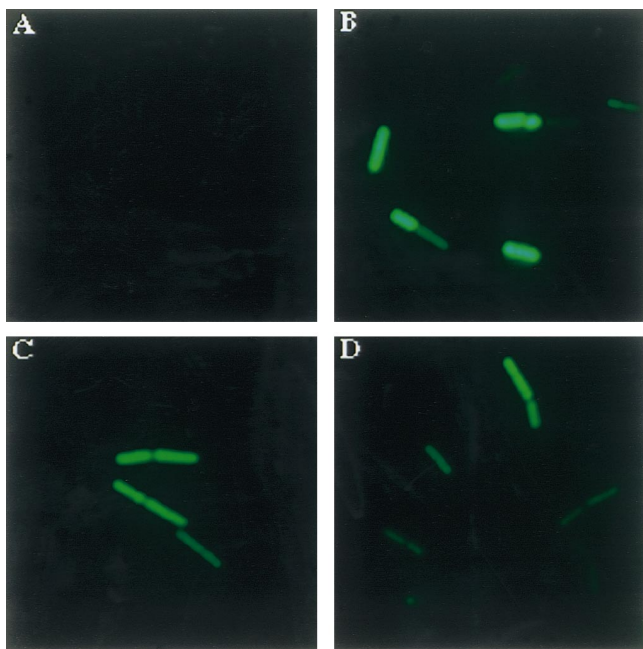


Fig. 4. Photomicrographs of clones 31-26 and 43-25 grown in 1/2 strength TSB. (A) Clone 31-26 after 5 h of growth. (B) Clone 31-26 after 10 h of growth. (C) Clone 43-25 after 5 h of growth. (D) Clone 43-25 after 10 h of growth. The fluorescence intensity shown here is consistent with the average fluorescence intensity at the indicated time points for the same clones reported in Fig. 3.

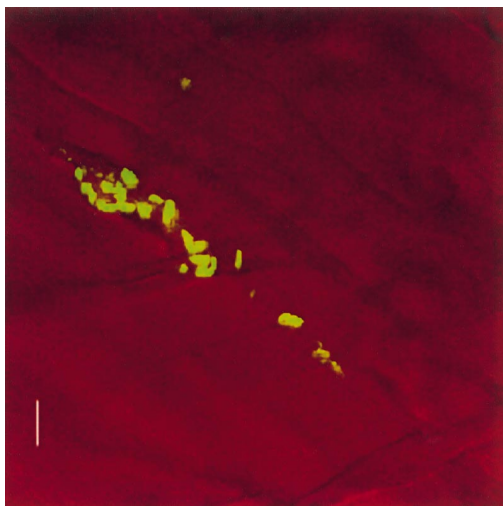


Fig. 5. Confocal scanning laser microscopy of clone 44-12 associated with a 16-day-old tomato seedling root. The image is a single-plane scan combined with a transmitted light-microscopic view pseudo-colored red. Bacteria can be seen in green. The white bar represents 10 μm .

3.7. Conclusions

Through this initial screen of the *B. cereus* UW85 chromosomal DNA library, we have shown the utility of pAD123 and *gfpmut3a* in *B. cereus*. To our knowledge, this is the first report of the use of GFP in *B. cereus*. A powerful application of a promoter-trap vector such as pAD123 is identification of promoters and/or

genes regulated in response to environmental conditions. The *B. cereus* library will be screened using flow cytometry and cell sorting for inserts containing DNA that promotes expression of GFP under specific growth conditions, with a particular focus on expression regulated in the presence of plants. pAD123 will be a powerful tool to unravel the story of gene expression in this versatile organism.

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