Visualization of Differential Gene Expression by Improved Cyan Fluorescent Protein and Yellow Fluorescent Protein Production in Bacillus subtilis

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Received 28 April 2004/Accepted 24 June 2004

The distinguishable cyan and yellow fluorescent proteins (CFP and YFP) enable the simultaneous in vivo visualization of different promoter activities. Here, we report new cloning vectors for the construction of cfp and yfp fusions in Bacillus subtilis. By extending the N-terminal portions of previously described CFP and YFP variants, 20- to 70-fold-improved fluorescent-protein production was achieved. Probably, the addition of sequences encoding the first eight amino acids of the N-terminal part of ComGA of B. subtilis overcomes the slow translation initiation that is provoked by the eukaryotic codon bias present in the original cfp and yfp genes. Using these new vectors, we demonstrate that, within an isogenic population of sporulating B. subtilis cells, expression of the abrB and spoIIA genes is distinct in individual cells.

The use of the green fluorescent protein (GFP) from the jellyfish Aequorea victoria has proven to be a powerful method to study in vivo gene expression in a broad range of hosts (2). Mutagenesis of gfp resulted in variants with different fluorescent properties (4, 14). The use of the distinguishable cyan (cfp) and yellow (yfp) variants of gfp has allowed studies of multiple cellular processes within a single cell (6, 8). One of the best-studied microbial organisms displaying cellular differentiation is the gram-positive bacterium Bacillus subtilis. Because of its ability to develop natural competence, secrete large quantities of proteins, and form highly resistant spores, it has been extensively studied and used as a model for bacterial cellular differentiation (5, 7, 19). Therefore, the availability of easily detectable variants of CFP and YFP in B. subtilis would considerably facilitate studies of multiple expression patterns in the organism.

Previously reported vectors for the production of fluorescent-protein fusions in B. subtilis contain the genes ecfp (Clontech) and eycfp (Clontech) (8) (in this work, we will refer to these genes as cfp and yfp, respectively). However, these fusions frequently display no or weak fluorescent signals when expressed in B. subtilis (reference 20 and this work). Here, we show that the cfp and yfp variants described are not efficiently translated in B. subtilis when used in promoter-cfp or -yfp fusions. In contrast to gfp (18), the codon usage in the cfp and yfp genes has been optimized for use in eukaryotic cell lines (8). Although a strong bias in codon usage has not been observed for B. subtilis (22), it was reported that, particularly at the initial stages of translation, the occurrence of less preferred triplets has an effect on translation efficiency (11, 27, 30, 32). Moreover, highly expressed genes of B. subtilis generally display a codon usage significantly different from that of genes expressed at low levels (22).

In order to obtain stable and efficiently translated variants of CFP and YFP in B. subtilis, vectors encoding CFP and YFP variants having an N-terminal extension were constructed. This N-terminal extension contains the first eight amino acids of ComGA, a strongly expressed B. subtilis protein involved in competence development (12). Our present studies show that the addition of this N-terminal extension overcomes the hammering of the initiation and processivity of translation. As a result, high levels of fluorescent protein can be produced.

Studying the underlying mechanisms of the differentiation of an isogenic population into distinct developmental stages is an important task in developmental biology. The new vectors described in this paper allow the visualization of differential gene expression within a genetically identical population. In this respect, the process of sporulation in B. subtilis has been studied for many years as a model for cellular differentiation. A major role of Spo0A, the key sporulation regulator, is to repress the expression of abrB and activate the transcription of the spoIIA operon (for a review, see reference 31). By visualizing the expression of the abrB and spoIIA promoters using the CFP- and YFP-encoding vectors described in this work, we demonstrate that within an isogenic population of B. subtilis cells, the initiation of sporulation is distinct from expression of the abrB promoter, which is observed in nonsporulating cells. These results demonstrate the practicability of the novel vectors for studying bacterial cellular differentiation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. TY (tryptone-yeast extract) medium contained Bacto-Tryptone (1%), Bacto-Yeast Extract (0.5%), and NaCl (1%). Sporulation medium contained dehydrated nutrient broth (0.8%), NaOH (0.5 mM), MgSO₄ (1 mM), KCl (1 g/liter), Ca(NO₃)₂ (1 mM), NaOH (0.5 mM), MgSO₄ (1 mM), KCl (1 g/liter), Ca(NO₃)₂ (1 mM),
was performed, using the plasmid pSG1186 (cfp) in Table 2. 

Escherichia coli RnlacZ-fw GGTTTTCCCAGTCACGACGTTGTAA 3′/H11032 was supplemented with ampicillin (100 μg/ml); media for B. subtilis were prepared as described by van Dijl et al. (34). Minimal medium for B. subtilis was prepared as described by van Dijl et al. (17). When required, medium for Escherichia coli was supplemented with ampicillin (100 μg/ml); media for B. subtilis were supplemented with chloramphenicol (CHL; 5 μg/ml) or kanamycin (KAN; 10 μg/ml).

**Recombinant DNA techniques and oligonucleotides.** Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of E. coli were carried out as described by Sambrook et al. (28). Enzymes were obtained from Roche (Mannheim, Germany). B. subtilis was transformed as described by Leskelä et al. (17). The oligonucleotides used in this study are listed in Table 2.

**Plasmids.** To construct the plasmid pICFP carrying the “improved” cfp gene (cfp), a PCR with the primers cfp-yfp-comG-F + EcoRI and RnlacZ-fw (Table 2) was performed, using the plasmid pSG1186 (cfp) (8) as a template. To construct the plasmid pYFP carrying the improved yfp gene (yfp), the same primer pair was used in a PCR using pSG1187 (yfp) (8) as a template. Plasmid pSG1186 contains the cfp gene derived from pECPF (Clontech), and pSG1187 contains the yfp gene derived from pEYPF-C1 (Clontech). The amplified fragments were subsequently cleaved with EcoRI and XbaI and ligated into the corresponding sites of pSG1186, replacing the cfp gene with the icfp and yfp genes. This resulted in plasmids pICFP and pYFP, respectively. Sequencing of the multiple cloning sites of pECPF and pYFP showed that the cfp gene corresponded to the original gene, whereas the yfp gene contained a single (A → G) point mutation in nucleotide 557, resulting in an Asp186 → Gly186 (D186G) substitution. However, this point mutation does not appear to affect the fluorescence spectrum or intensity of IYFP compared to those of the previously reported YFP.

To construct plasmids p86-IA and p87-IA, carrying the B. subtilis spoIIA promoter region fused with the cfp or yfp gene, a PCR with the primers IIA-F and IIA-R (Table 2) was performed, using chromosomal DNA of B. subtilis as a template. The amplified fragment was subsequently cleaved with KpnI and Clai and ligated into the corresponding sites of pSG1186 and pSG1187, resulting in plasmids p86-IA and p87-IA, respectively.

To construct plasmids pICFP-IIA and pYFP-IIA, carrying the B. subtilis spoIIA promoter region fused with the icfp or yfp sequence, a PCR with the primers IIA-F-V800 + KpnI and pSpoIIAA-R-HindIII (Table 2) was performed using chromosomal DNA of B. subtilis as a template. The amplified fragment was subsequently cleaved with KpnI and HindIII and ligated into the corresponding sites of pSG1186 and pSG1187, resulting in plasmids pICFP-IIA and pYFP-IIA, respectively. It should be noted that the first 24 bp of the spoIIA promoter region replaced the bgaB gene present on pDK.

To construct plasmids p86-icfpB, p87-icfpB, pICFP-icfpB, and pYFP-icfpB, a PCR with the primers F-abrB and R-abrB (Table 2) was performed, using chromosomal DNA of B. subtilis as a template. The amplified fragment was subsequently cleaved with Clai and EcoRI and ligated into the corresponding sites of pSG1186, pSG1187, pICFP, and pYFP to generate plasmids p86-abrB, p87-abrB, pICFP-abrB, and pYFP-abrB, respectively.

**Strains.** B. subtilis strains 86-IA, 87-IA, icfpB, and yfpB-IA were obtained by a Campbell-type integration (single crossover) of plasmids p86-IA, p87-IA, pICFP-IIA, and pYFP-IIA into the chromosomal spoIIA promoter region of B. subtilis 168. Transformants were selected on TY agar plates containing CHL after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

**Plasmids.** To construct plasmid pAmy-ICFP-IIA, plasmid pICFP-IIA was cleaved with KpnI and XbaI. The resulting 1.3-kb fragment, carrying the P_{spoIIA} gene, was ligated into the corresponding sites of pDK (36); the resulting plasmid was named pAmy-ICFP-IIA. Note that as a result of this cloning strategy, the P_{spoIIA} region replaced the bgaB gene present on pDK.

To construct plasmids p86-icfpB, p87-icfpB, pICFP-icfpB, and pYFP-icfpB, a PCR with the primers F-abrB and R-abrB (Table 2) was performed, using chromosomal DNA of B. subtilis as a template. The amplified fragment was subsequently cleaved with Clai and EcoRI and ligated into the corresponding sites of pSG1186, pSG1187, pICFP, and pYFP to generate plasmids p86-abrB, p87-abrB, pICFP-abrB, and pYFP-abrB, respectively.
B. subtilis strain iyfp-abrB-icfp-IIA-amyE was obtained by transformation of strain iyfp-abrB with chromosomal DNA of strain icfp-IIA-amyE. Transformants were selected on TY agar plates containing CHL and KAN after overnight incubation at 37°C.

**Microscopy.** Cells were prepared for microscopy and applied to agarose slides as described by Glaser et al. (9), and images were acquired using an Axioptot microscope equipped with an AxioVision camera (Zeiss, Oberkochen, Germany). Fluorescence filter sets used to visualize CFP and YFP were obtained from Zeiss. Fluorescent signals of CFP were visualized using set 47 (excitation, 426 to 446 nm; emission, 460 to 500 nm), and fluorescent signals of YFP were visualized using set 46 (excitation, 490 to 510 nm; emission, 520 to 550 nm). AxioVis20 software (Zeiss) was used for image capture, and the figures were prepared for publication using Corel Graphics Suite 11. The ICFP protein displays a fluorescence excitation maximum of 434 nm and an emission maximum of 477 nm. The IYFP protein displays a fluorescence excitation maximum of 514 nm and an emission maximum of 527 nm. CFP fluorescence cannot be visualized using the YFP filter, and likewise, fluorescence of YFP cannot be visualized using the CFP filter (data not shown).

**Western blot analysis and immunodetection.** Cells were separated from the growth medium by centrifugation (20,000 × g; 1 min; room temperature). The pelletted cells were resuspended in protoplast buffer (20 mM potassium phosphate, pH 7.5, 15 mM MgCl2, 2% sucrose, and 1 mM of l-lysine/200 mM) and incubated at 37°C for 30 min. The resulting protoplasts were diluted with 2× sodium dodecyl sulfate (SDS) sample buffer, incubated at 95°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (26). Next, the proteins were transferred to a polyvinylidene difluoride membrane (Roche) as described previously (28). CFP and YFP were detected with commercial anti-GFP antibodies (Molecular Probes, Leiden, The Netherlands) and horseradish peroxidase-anti-rabbit immunoglobulin G conjugate (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturers’ instructions. Anti-GFP antibodies can be used to detect CFP and YFP due to high amino acid sequence conservation among GFP, CFP, and YFP (15).

**Flow cytometric analysis of total cytosolic protein extracts.** Cells were separated from the growth medium by centrifugation (10,600 × g; 2 min; room temperature). The pelletted cells were washed with and resuspended in 50 mM Tris-Cl, pH 7. Next, 0.5 g of glass beads (50- to 105-μm diameter) were added, and the cells were disrupted using a minibeadbeater (twice for 1 min each time; BioSpect Products, Bartville, Wash.). To remove the glass beads, samples were centrifuged (20,000 × g; 5 min; 4°C), and the supernatants were transferred to clean 0.5-ml tubes. Cytosolic proteins were separated from membranes by velocity centrifugation (195,000 × g; rotor TL-A120; 30 min; 4°C). Samples were analyzed on a fluorometer (LS-50 B; Perkin-Elmer, Boston, Mass.) using quartz cuvettes (101 QS; Hellma, Müllheim, Germany). The settings to measure CFP fluorescence were as follows: excitation, 436/10; emission, 480/20. The settings to measure YFP fluorescence were as follows: excitation, 540/10; emission, 570/15. During all measurements, the photomultiplier tube voltage was set at 750 V. The results were normalized using B. subtilis strain 168 as a reference. The data were analyzed using FACS. Volcano Mannheim, Mannheim, Germany). A schematic presentation of the pICFP and pIYFP genes in B. subtilis 168 is shown in Fig. 1. The pICFP and pIYFP plasmids contain a multiple cloning site directly upstream of the TTG start codon. This allows the construction of C-terminal fluorescent protein fusions and/or promoter activity studies. The E. coli strains containing either pICFP or pIYFP (pICFP, ECE180; pIYFP, ECE181) can be ordered from the Bacillus Genetic Stock Center (http://www.bgsc.org/).

**RESULTS AND DISCUSSION**

**Construction of new cfp and yfp variants.** Promoter fusions with the unmodified cfp or yfp gene in B. subtilis resulted in little or no production of fluorescent protein and thus poor in vivo fluorescence (see below). For this reason, we set out to construct improved variants of cfp and yfp. To obtain vectors carrying improved variants of cfp and yfp, the first 24 bp of the coding sequence of comGA were fused to the cfp and yfp genes. The N terminus of ComGA was selected, because this protein is produced at high levels during competence development (1) and was shown to be highly stable when used in fusions with CFP or YFP (data not shown). Using a primer carrying sequences encoding the first eight amino acid residues of ComGA (Table 2), the cfp and yfp genes were amplified and cloned into pSG1186 (8), replacing the original cfp gene with the extended cfp or yfp gene (see Materials and Methods). Using this cloning strategy, the original EcoRI recognition site of pSG1186 was removed and a new EcoRI recognition site was introduced upstream of the comGA sequence. Due to introduction of the comGA sequence, a TTG start codon was employed instead of the ATG start codon present in the original cfp or yfp sequence. The use of the non-ATG start codon TTG is quite common in gram-positive organisms (1) and does not seem to have a substantial influence on translation efficiency (24). A schematic presentation of the pICFP and pIYFP vectors is given in Fig. 1. The pICFP and pIYFP plasmids contain a multiple cloning site directly upstream of the TTG start codon. This allows the construction of C-terminal fluorescent protein fusions and/or promoter activity studies. The E. coli strains containing either pICFP or pIYFP (pICFP, ECE180; pIYFP, ECE181) can be ordered from the Bacillus Genetic Stock Center (http://www.bgsc.org/).

**CFP and YFP are inefficiently produced in B. subtilis.** To compare fluorescent-protein production from fusions made with the previously described vectors (8) and the vectors described above (Fig. 1), the B. subtilis abrB and spoIIA promoter regions were fused with the fluorescent-protein-encoding genes (see Materials and Methods). Expression driven from these promoters was shown to be high under specific growth conditions (29). The promoter fusion plasmids were introduced in B. subtilis 168 and integrated into the chromosome of the organism. Next, strains were examined for fluorescent-protein production by fluorescence microscopy and Western blotting. B. subtilis strains 86-abrB (P_abrB_cfp), icfp-abrB (P_abrB_ycfp), 87-abrB (P_abrB_yfp), and yfp-abrB (P_abrB_ycfp) were grown in TY medium, and samples were withdrawn at the mid-exponential growth phase. In addition, strains 86-spoIIA (P_spoIIA_cfp), icfp-spoIIA (P_spoIIA_ycfp), 87-spoIIA (P_spoIIA_yfp), and yfp-spoIIA (P_spoIIA_yfp) were grown in sporulation medium, and cells were harvested 2 h after entry into the stationary growth phase. The cells were analyzed by fluorescence micros-
copy using the appropriate filters. Strikingly, only strains harboring the comGA-cfp and comGA-yfp fusions showed detectable fluorescence signals (Fig. 2). Therefore, we refer to these variants as improved cfp and yfp (icfp and iyfp). As shown by Western blotting, only minor amounts of CFP and YFP could be visualized in cells containing the original variants, whereas strains expressing the N-terminally extended variants (ICFP and IYFP) showed considerably higher protein production levels (Fig. 3).

To quantify the differences in fluorescent signals shown in Fig. 2, the total fluorescence in cytosolic protein extracts was determined using a fluorimeter. The different B. subtilis strains containing the abrB-promoter fusions were grown in TY medium, and samples were taken at the mid-exponential growth phase. The abrB fusion strains were chosen for this experiment, since abrB is highly expressed during exponential growth. Total cytosolic proteins were isolated, and fluorescence was measured as described in Materials and Methods. As specified in Table 3, ICFP protein extracts showed 20- to 30-times-higher fluorescence than CFP extracts. For IYFP, an improvement of between 50 and 70 times could be measured compared to the fluorescence of YFP.

Taken together, these results demonstrate that production of the modified fluorescent proteins in B. subtilis is considerably higher and is sufficient to be visualized by fluorescence microscopy, in contrast to the unmodified variants.

**Production of cfp and yfp mRNAs in B. subtilis.** To investigate whether the small amounts of CFP and YFP proteins produced in B. subtilis resulted from low mRNA production levels, RNA dot blot experiments were performed. The different B. subtilis strains containing abrB-promoter fusions were grown in TY medium, and samples were withdrawn at the mid-exponential growth phase. RNA was isolated, blotted, hybridized, and analyzed as described in Materials and Methods. As shown in Fig. 4, production levels of cfp and yfp mRNAs did not differ significantly from the levels produced by the icfp and iyfp variants. This result suggests that the insufficient production of CFP and YFP in B. subtilis is not related to an inadequate production of cfp and yfp mRNAs.

**Increased translational efficiencies of ICFP and IYFP in B. subtilis.** To investigate the stability and putative degradation of CFP/YFP and ICFP/IYFP, pulse-chase labeling experiments were performed. The different B. subtilis strains containing the abrB-promoter fusions were grown in S7 medium, and cells were labeled with [35S]methionine-[35S]cysteine for 30 s prior to a chase with an excess of nonradioactive methionine-cysteine. As depicted in Fig. 5, the fluorescent proteins with an N-terminal extension (ICFP and IYFP) are produced more rapidly and in larger quantities than CFP and YFP. While both the ICFP and IYFP proteins were already produced at high levels immediately after the chase of the cells, even small amounts of CFP and YFP were not detectable at that point. As already demonstrated by Western blotting (Fig. 3), significant degradation of the fluorescent proteins could not be observed. This indicates that proteolytic activity by one of the endogenous proteases of B. subtilis is probably not the cause of the low levels of CFP and YFP compared to the ICFP and IYFP protein levels. More likely, the low levels of CFP and YFP production were the result of the low translation efficiencies of the original cfp and yfp genes.

**abrB and spoIIA are distinctly expressed.** To demonstrate the experimental applicability of the vectors described in this work, we examined the expression of the abrB and spoIIA genes within an isogenic population of B. subtilis cells. In B. subtilis, transition state regulator proteins play an essential role in the adaptive capacity and survival of the cell. The transcription regulator AbrB regulates many stationary-phase processes by repressing the expression of genes involved in sporulation...
(e.g., spo0E and spoVG), competence (e.g., comK), degradative enzyme production (e.g., aprE), amino acid utilization (e.g., dpp), and antibiotic production (e.g., tycA) (13, 26, 33). When cells reach the end of exponential growth and various environmental signals promote the activation of the response regulator Spo0A, **abrB** expression is repressed by Spo0A/H11011P (23, 25). Furthermore, Spo0A/H11011P activates 40 genes directly, including the **spoIIA** operon, which contains the early sporulation genes **spoIIAA**, **spoIIAB**, and **sigF** (21). Chung et al. proposed that expression of the **spoIIA** operon (and initiation of sporulation in general) requires a threshold concentration of Spo0A−P (3). Knowing this, it is to be expected that cells that initiate sporulation (i.e., express **spoIIA**) do not express **abrB**. To see whether expression of **abrB** and initiation of sporulation are strictly separated between individual cells, we investigated how the expression of **abrB** and **spoIIA** is distributed between cells within an isogenic population. Since cells that have initiated sporulation (and have not yet formed an asymmetric sep-

![FIG. 2. Visualization of fluorescent-protein production in B. subtilis by fluorescence microscopy. Strains carrying an **abrB** promoter-cfp or -yfp fusion were grown in TY medium, and samples were withdrawn at mid-exponential growth phase (upper panels). Strains containing a **spoIIA** promoter-cfp or -yfp fusion were grown in sporulation medium, and cells were collected 2 h after entry into the stationary growth phase (lower panels). Production of CFP, YFP, ICFP, or IYFP, whose expression was driven by activity of either the **abrB** (P_{abrB}) or the **spoIIA** (P_{spoIIA}) promoter, was visualized by fluorescence microscopy, as described in Materials and Methods.](#)

![FIG. 3. Fluorescent-protein production. Strains carrying an **abrB** and **spoIIA** promoter-cfp or -yfp fusion were grown as described in the legend to Fig. 2. Cells were separated from the growth medium by centrifugation and analyzed by SDS-PAGE and Western blotting using GFP-specific polyclonal antibodies. The arrow indicates GFP-specific signal.](#)

**TABLE 3.** Fluorescence determinations of cytosolic protein extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence at:</th>
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<tbody>
<tr>
<td></td>
<td>1:100</td>
</tr>
<tr>
<td>CFP</td>
<td>ND</td>
</tr>
<tr>
<td>ICFP</td>
<td>ND</td>
</tr>
<tr>
<td>YFP</td>
<td>0.9</td>
</tr>
<tr>
<td>IYFP</td>
<td>69.2</td>
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*Fluorescence is presented in arbitrary units. The results of three dilutions of cytosolic protein extracts are shown. Samples were normalized against a cytosolic protein extract of the parental B. subtilis 168 strain and were prepared and measured as described in Materials and Methods. ND, not determined.*
served in all cells, indicating that initiation of sporulation is a heterogeneous process. This was also demonstrated in a different way by flow cytometry experiments (3). Gonzalez-Pastor and coworkers (10) showed that cells that have initiated sporulation development can delay the commitment to further stages of sporulation by killing their siblings and utilizing the nutrients that are released. The two operons involved in this so-called self-digestion (skf and sdp) are directly regulated by active Spo0A, as is the spoIIA operon (21, 29). The cannibalistic behavior of a sporulating culture could, at least in part, account for the observed heterogeneity in spoIIA expression. We are currently trying to understand the underlying mechanisms involved in this heterogeneous process.

Concluding remarks. We have constructed new cfp and yfp vectors encoding fluorescent proteins with an eight-amino-acid N-terminal extension that can be produced at useful levels in vectors encoding fluorescent proteins with an eight-amino-acid.

FIG. 4. Production of CFP-, ICFP-, YFP-, and IYFP-encoding mRNAs. Strains 168, 86-abrB (P_cfp-abrB), icfp-abrB (P_icfp-abrB), 87-abrB (P_yfp-abrB), and iyfp-abrB (P_iyfp-abrB) were grown in TY medium, and samples for RNA isolation were withdrawn at the mid-exponential growth phase. Twofold serial dilutions of total RNA, starting with 4.5 μg, were applied to the membrane and probed as described in Materials and Methods. As a positive control (Pos.), 30 ng of unlabeled PCR fragment was spotted. Total RNA from the parental B. subtilis 168 strain was spotted as a negative control (Neg.).

acknowledgments. We thank the Bacillus Genetic Stock Centre for providing plasmids pSG1186, pSG1187, and pDK. We thank Reindert Nijland for useful discussions.

J.-W.V. was supported by grant ABC-5587 from NWO-STW. W.K.S. was supported by grant 811.35.002 from NWO-ALW.

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