Mapping of a transcription promoter located inside the priA gene of the Bacillus subtilis chromosome

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The genome sequence of the Gram-positive soil bacterium Bacillus subtilis was completed in 1997 (Kunst et al., 1998) and the results included the identification of a putative transcription unit encompassing the yloI to yloS genes. Within this region of the B. subtilis chromosome 11 putative open reading frames were found with a wide diversity of probable functions. In this work we have analyzed transcription in the region of the priA-cpgA genes and we have mapped a promoter which is located inside the priA gene and its activity directs transcription of the def- yloM genes. Moreover, this transcript can be extended at low level to the prpC-prik-cpgA genes. Analysis of the sequence in proximity of the transcription start site revealed a sequence suitable for the housekeeping σA subunit of RNA polymerase. Analysis of the β-glactosidase activity of transcription fusions revealed that the identified promoter is active at low level and its activity is increased during late exponential phase of growth.

Keywords: Bacillus subtilis, transcription, weak promoters

INTRODUCTION

Regulation of the transcription is the most common way of controlling gene expression and is necessary for all living organisms to adapt to the continuously changing environmental conditions. Most of this regulation takes place at the level of initiation. Appropriate organization of the genome is therefore very important in order to optimize the transcription process.

The Gram-positive soil bacterium Bacillus subtilis encounters various conditions in its natural environment. Its genome has been sequenced and annotated (Kunst et al., 1997) and also an initial functional analysis was performed (Kobayashi et al., 2003). However, many regulation aspects remain undiscovered. From the genome sequencing project, a 28-kbp DNA segment ranging from 138.9° to 142.1° on the genome map (position 1 635 434 to 1663 803) was found to contain 27 ORFs. In this fragment a transcription unit yloK-yloS containing 11 ORFs was proposed (Foulger & Errington, 1998). Four of these ORFs have been cloned and their products characterized: PriA, a primosomal replication factor Y (Marians et al., 1999), Def, a protein deformylase (Huntington et al., 2000), PrpC, a protein Ser/Thr phosphatase (Obuchowski et al., 2000), PrkC, a protein Ser/Thr kinase (Madec et al., 2002) and CpgA, a GTP binding protein, essential for viability (Cladiere et al., 2006). The prpC gene overlaps by 3 bp with prkC and this couple is followed by an essential gene, cpgA. The organization of the genes prpC, prkC and cpgA is conserved in several Gram-positive bacteria. Previous analysis of this region revealed that at least two promoters are present in front of the prpC and cpgA genes (Iwanicki et al., 2005) (see Fig. 1). Interestingly, genes located within this region appear to encode proteins involved in many different functions (Table 1), including priA required for restarting replication forks following DNA damage (Marians et al., 1999). This region contains a pair of genes, prpC and prkC, encoding a protein phosphatase and a protein kinase, respectively (Madec et al., 2002). Autophos-

Abbreviations: dNTP, mixture of deoxynucleoside triphosphates; DTT, dithiothreitol; ONP, 2-nitrophenol; ONPG, 2-nitrophenyl-β-D-galactopyranoside; ORF, open reading frame.
phorylated form of PrkC is efficiently dephospho-
ylated by the PrpC phosphatase (Obuchowski et al., 2000). Notably, this type of association of the
two genes is found in several bacterial species. The
conservation of these two adjacent genes encoding
enzymes with opposite activities could suggest that
they participate together in the regulation of one or
more cellular processes. Recently, the structure of
CpgA (formerly YloQ) was published and it turned
out to be a GTP-binding protein (Levdikov et al.,
2003).

The recent work done by de Hoon and co-
workers showed a lack of Rho-independent tran-
scription terminators within the priA-yloS region (de
Hoon et al., 2005). This data supports the hypothesis
about the presence of another promoter upstream of
prpC-prkC-cpgA, which might be responsible for
the basal level of transcription. Previously described
promoters pO and pQ are activated only at specific
conditions and their activities do not explain the ob-
served constant level of the PrpC and PrkC proteins
(Madec et al., 2002, Obuchowski et al., 2000, Iwanicki
et al., 2005).

Here we present an analysis of a transcription
start site within the region of priA-def based on a
real-time PCR method, β-galactosidase activity as-
says and primer extension analysis. This is another
yet transcription start site which can drive expres-
sion of the prpC-prkC-cpgA genes (Iwanicki et al.,
2005).

MATERIALS AND METHODS

Bacterial strains, plasmids and cultivation.
All strains and plasmids used are listed in Table 2. Bacteria were grown either in Luria-Bertani rich me-
dium (Miller, 1972) or in minimal medium: 0.015 M
K₂SO₄, 0.08 M K₂HPO₄, 0.044 M KH₂PO₄, 3.4 mM
sodium citrate, 0.8 mM MgSO₄, 0.4% glucose, 0.005%

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Table 1. Similarity of the products of genes from the yloI-yloS region to other known proteins.

Results of previous BLAST search were taken from Foulger and Errington (1998). Positions of ORF were taken from SubtiList (http://genolist.pasteur.fr/SubtiList/).

<table>
<thead>
<tr>
<th>ORF</th>
<th>Position in B. subtilis chromosome</th>
<th>ORF</th>
<th>Position in B. subtilis chromosome</th>
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<td>1646724</td>
<td>yloR</td>
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Figure 1. Position of cloned fragments used in this study. Open boxes, fragments transcriptionally inactive. Black boxes, fragments transcriptionally active. Arrows, promoters identified in previous work (Iwanicki et al., 2005). For details see text and Table 1.
Transcription in Bacillus subtilis in priA-cpgA region

- l-tryptophan, 0.2% l-glutamine, 4 μg FeCl₃ ml⁻¹, 0.2 μg MnSO₄ ml⁻¹. Spectinomycin was added for cultivation of B. subtilis strains harbouring a spectinomycin resistance cassette up to the final concentration of 100 μg/ml. X-gal plates contained 12.5 μg/ml of this compound.

**Escherichia coli DH5α** (Hanahan, 1983) was used as a host for cloning.

**Total RNA isolation.** Total RNA isolation was performed using a modified hot phenol method described previously (Volker et al., 1994). Cultures were grown in appropriate media and cells were harvested at indicated times. Cell pellets were suspended in 4 ml of 65°C hot TE buffer, then glass beads (125–250 μm) and 4 ml of hot phenol (65°C, pH 4.8) were added. The mixture was incubated for 2 min at 65°C, vortexed for 2 min and cooled on ice for 3 min. After cooling the mixture was centrifuged and the aqueous phase was collected. Three extractions were made with 1 vol. of phenol, phenol/chloroform (1:1, v/v) and chloroform, respectively (3 × 1 min, with 1 min incubation on ice). After each extraction the mixture was centrifuged and the aqueous phase was collected. Finally, 1/10 vol. of 9 M LiCl and 3.5 vol. of cold 95% ethanol were added. RNA was precipitated, pelleted by a 30 min centrifugation, washed with cold 80% ethanol and dried in a vacuum dryer. The dry RNA pellet was dissolved before use in 20 μl of RNase-free water.

**Real-time PCR experiments.** Total RNA (50 ng) from late exponential phase cultures grown in rich media was used for real-time PCR. Prior to real-time PCR, RNA was digested with RNase-free DNase I (Roche) and then the enzyme was thermally inactivated. Experiments were performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) as recommended by the manufacturer. Primers used are shown in Table 3. Products of reactions were analysed for homogeneity by performing a melting
Table 3. Primers used for real-time PCR reactions and RACE PCR.

<table>
<thead>
<tr>
<th>Probe symbol</th>
<th>Gene</th>
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<th>Orientation</th>
<th>Position in B. subtilis chromosome</th>
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<tr>
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<tr>
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<td>R</td>
<td>1645932</td>
</tr>
</tbody>
</table>

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Cloning. For the cloning of selected genome fragments the chromosomal DNA from wild type B. subtilis was used as a template in PCR reactions (for primers see Table 4). Reactions were performed with Pwo polymerase (Roche). The amplified fragment was digested with appropriate enzymes and inserted into the pDG1728 integration vector (Guerrout-Fleury et al., 1996). The resulting plasmid was linearized with Xho1 and used to transform B. subtilis 168.

β-Galactosidase measurements. LB cultures were grown at 37°C with shaking. Samples were taken from various growth phases and stored at –20°C until the enzyme assay was carried out. After thawing, bacterial pellets were suspended in buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4) containing 1 mM DTT, and 1/100 vol. of lysis solution (1 mg/ml DNase, 10 mg/ml lysozyme) was added. The mixture was incubated for 20 min at 37°C and then centrifuged at 4°C. The supernatant was used for measurement of protein concentration and β-galactosidase assay (Miller, 1972). Protein concentration was measured using the Bradford reagent (Bio-Rad) as recommended by the manufacturer. Supernatant (200 µl) was mixed with 600 µl of buffer Z containing 1 mM DTT. Samples were placed in a 37°C water bath and 200 µl of ONPG (4 mg/ml) was added. After 60 min incubation, the reaction was stopped by the addition of 500 µl of 1 M Na2CO3. The absorbance of samples was measured spectro-photometrically at 420 nm. The β-galactosidase activity in nmoles of ONP min–1 mg–1 was calculated using the following formula: (420 nm absorbance × 1.5)/(conc. of protein in mg/ml × volume of sample in ml × reaction time in min × 0.00486).

Primer extension analysis. A total amount of 20 µg of RNA was mixed with 2.5 µl of appropriate primer (1 mM) and adjusted to a volume of 15 µl. The mixture was then incubated at 80°C for 10 min, transferred to 37°C for 30 min and finally left at room temperature for another 30 min. The RNA annealed with the primer was placed on ice and the following reagents were added: reverse transcriptase buffer, a mixture of four dNTPs (final concentration 200 pM), 0.5 µCi (1·35×1010 Bq) of [α-32P]dATP and 200 units of M-MLV Reverse Transcriptase (Promega). The volume of the reaction was adjusted with water to 25 µl. The mixture was incubated at 42°C for 1 h and then precipitated with ethanol. Dried pellets were dissolved in 5 µl of water, mixed with loading buffer and loaded onto a pre-warmed sequencing gel. Sequencing ladders were generated using Reader Sequencing kit (Fermentas) with the use of a control plasmid included in the kit. The gel was run for 120 min, dried and subjected to autoradiography.

RACE PCR. Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and an unknown 5' end. For such an analysis we used the RACE system produced by Invitrogen (version 2.0) and experiments were performed according to the manufactur-
er’s manual. The specific primers for cDNA synthesis (RevT) and PCR amplification (cDAMP and AAP; 9d and AUAP) are listed in Table 3.

RESULTS

Similarity comparison of ORFs in the putative transcription unit priA-cpgA

The sequence of the putative transcription unit priA-cpgA was previously analysed for the presence of ORFs that would encode peptides of at least 67 aa (Foulger & Errington, 1998). A BLAST search was repeated in this study in order to compare sequences with current databases (Table 1). Products of three genes — yloM, yloN and yloS share similarity with proteins from other bacteria, however, they have not been analyzed so far in B. subtilis. Four genes – priA (yloJ), prpC (yloO), prkC (yloP) and cpgA (yloQ) have already been cloned and their products identified. The PriA protein is defined as the primosomal factor Y (for a review see Marians, 1999). PrpC is a protein PPM phosphatase (Obuchowski et al., 2000) and PrkC is a protein Ser/Thr kinase (Maced et al., 2002; 2003). CpgA is a GTPase with perturbed G motifs and is necessary for proper shap-
The level of transcription of individual genes differs across the priA-cpgA region as measured by real-time PCR

Providing that the region priA-yloN forms a single operon, a real-time PCR scan of the level of mRNAs corresponding to the ORFs throughout the whole region should ideally give a relatively constant level of signal. In order to investigate the expression of genes identified in the transcription unit priA-yloN pairs of primers corresponding to different ORFs were first tested in PCR reactions with *B. subtilis* chromosomal DNA as a template to confirm that they were correctly designed. In each case the primers gave similar levels of products in control experiments (not shown). RNA samples were prepared from late exponential phase bacteria cultivated in LB medium. The results presented in Fig. 2 reveal differences in the levels of transcript of genes across the priA-yloN region and this variation is difficult to reconcile with the presence of a single transcript. The presence of a weak signal in the case of the internal part of the priA gene (PCR probe 8-7) in Fig. 2, in contrast to the signal from the def probe (PCR probe 10-9) may indicate the presence of a promoter inside the priA gene, in addition to a presumptive promoter upstream of priA. The transcript detected using probes 10-9, 12-11, 15-14 and 17-16 shows a relatively high, constant level, although the probe 18-17 shows a much lower one. On the basis of these results we postulate that the analysed transcript starts at the distal part of the priA gene and terminates in the proximal part of the yloN gene (Fig. 2).

A possible promoter located in the priA-fmt fragment

The real-time PCR analysis of the priA-cpgA region (Fig. 2) enabled press on the possible location of a promoter(s) upstream of def. On this basis we PCR-amplified fragment B from the *B. subtilis* chromosome (1250 bp, starting from 1645585 to 1646833) and cloned it into the pDG1728 vector upstream of the lacZ gene with translational signals of the *B. subtilis* spoVG gene. The resulting plasmid pB-lacZ was then integrated into the *B. subtilis* chromosome in the non-essential locus amyE. This approach provides the possibility for monitoring the expression of a gene, a promoter or a genome fragment of interest in the host chromosome. An integrant strain was then tested for β-galactosidase activity on LB solid medium containing X-gal. The strain containing the chromosomal B-lacZ fusion showed detectable activity of β-galactosidase after an overnight incubation of the plates at 37°C.

The promoter within the distal part of priA becomes activated in the late exponential phase

The real-time PCR analysis of the priA-yloN unit together with the preliminary β-galactosidase plate test suggested the presence of one or more putative promoters functioning in the priA-fmt region. To confirm this, an integrant strain harbouring the B-lacZ fusion (priA-fmt region) in the amyE locus was screened for β-galactosidase activity during growth in rich liquid medium. The results demonstrated a significant increase in the β-galactosidase activity with
Transcription in Bacillus subtilis in priA-cpgA region

Increasing age of culture (Fig. 3A). In order to identify precisely the position of this putative promoter, the priA-fmt region (fragment “B”) was divided into three parts, each of approx. 500 bp. The corresponding fragments obtained by PCR amplification (for primers used see Table 4, for localization of B1, B2 and B3 fragments, see Fig. 1) were cloned back into the pDG1728 vector and the resulting plasmids pB1-lacZ, pB2-lacZ, pB3-lacZ, carrying respective fusions with lacZ were integrated into the chromosomal amyE locus. The resultant strains were sampled from cultures grown in LB medium and subjected to β-galactosidase assays. The β-galactosidase activity was detected in the case of the strain carrying the lacZ fused to the most proximal cloned fragment B3 (Fig. 3B). The B3 fragment was therefore further divided into three parts overlapping each other to some extent (B3a-222 bp, B3b-209 bp and B3c-205 bp, see Fig. 1 and Table 4) and each of the parts was used to construct fusion with lacZ on pDG1728 plasmid and subsequent integration of the respective fusion into B. subtilis chromosome. The β-galactosidase activity was detected in the case of B3a-lacZ fusion in the most distal part of the priA gene (Fig. 3C). This information enabled the design of specific primer (5’-TAGATATCGCTATCAATGCG-3’), which was used in primer-extension experiment to localize transcription start site in the region encompassed by B3a. A single transcription start region was found. Precise identification of transcription start site was done by RACE method and named pK as indicated in Fig. 5, which correspond to the position 1 645 759 in the B. subtilis chromosome.

The pdef promoter drives transcription of def-yloM genes

In the previous research we observed that introduction of the terminator region into the prpC gene decreased the β-galactosidase activity in the strain harbouring the cpgA-lacZ fusion (Iwanicki et al., 2005). This observation suggested that there might be a transcription promoter upstream of the prpC. The pO (see Fig. 1) was previously described to be not strong enough to explain observed β-galactosidase activity. The mapping of the pK transcription start site enabled us to postulate that transcript starting from that point may go through prpC-prkC-cpgA genes. This will allow explaining observed β-galactosidase activity of fusion with prpC-cpgA genes. To verify this hypothesis we constructed an integration plasmid (pKH30) which had two terminator regions placed up- and downstream of the cloned fragment of the yloM gene. Such plasmid, after integration into the B. subtilis chromosome (strain MM1630), should terminate transcription in either directions. After the introduction of the lacZ fusions with yloN, prpC or cpgA genes into such strain we observed a 3- to 6-fold decrease of β-galactosidase activity (Fig. 4). On this basis we propose that most of the transcripts which begin at the pdef promoter terminate at the yloN gene, however, some of them can continue through, at least, the cpgA gene.
The insertion of pH30 plasmid into the yloM gene results only in an approximately 5-fold decrease in level of the transcripts, downstream of the insertion site as measured by real-time PCR (not shown). The ratio of the β-galactosidase activity measured with the use of the lacZ fusions with prpC, prkC or cpgA genes in strains 168 and MM1630 remains consistent with the real-time PCR analysis.

DISCUSSION

The priA-cpgA region of the B. subtilis chromosome was originally postulated to be transcribed as a single, policistronic mRNA extending from yloI located immediately upstream of priA to the yloS gene (see Fig. 1). This assumption was made solely on the basis of DNA sequence analysis (Foulger & Errington, 1998). However, the ORFs in this region correspond to a wide variety of possible functions. Thus, for example, whilst the product of the priA gene was identified as a primosomal factor Y involved in DNA replication restart (Marians, 1999), prpC and prkC encode proteins apparently important in a signalling pathway involved in development (Obuchowski et al., 2000, Madec et al., 2002), and the products of def and fmt are involved in post-translational modifications (Haas et al., 2001). Such a variety of functions might be difficult to reconcile with a single transcription unit.

The transcription in that region was originally postulated to start upstream of the priA and yloI genes (Foulger & Errington, 1998). In this work we provide evidence for the presence of an internal promoter functioning within the priA gene. First, the transcription start site inside the priA gene was postulated on the basis of real-time PCR analysis of transcript levels in bacterial cells in the late-exponential growth phase in rich medium (LB). The observed increase of the real-time PCR signal in the case of the distal part of this gene coincided with the activity of an internal promoter mapped in this region of the genome (designated the pdef promoter). Activity of this promoter was detected both by the lacZ fusion and by primer extension.

Previous work revealed that at least two promoters function within the unit (Iwanicki et al., 2005; see Fig. 1). However, both identified promoters are weak or become activated only in specific conditions, i.e. the pQ promoter in late exponential phase and pQ after ethanol shock. The results presented here clearly show that the promoter located inside the priA gene may be responsible for the basal level of expression of the prpC-prkC-cpgA genes. This hypothesis is supported by the fact that the identified promoter has a sequence which is recognized by the B. subtilis "housekeeping" σ subunit (σ A) (Handelwag, 1995; Sonenshein et al., 2002).

In conclusion, our analysis of the transcription in the priA-cpgA region of the B. subtilis genome indicated that regulation of the genes in this region may be complex. Although most of the genes in the def-cpgA region appear to be transcribed as a single transcript, at least under conditions tested, the presence of three transcription start sites suggests a much more complicated pattern of transcription. However, from this analysis we cannot exclude the possibility that other promoters and terminators might operate in this region of the B. subtilis genome (Yoshida et al., 2000).

Acknowledgements

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