**yveB**, encoding endolevanase LevB, is part of the *sacB–yveB–yveA* levansucrase tricistronic operon in *Bacillus subtilis*

Yannick Pereira, Marie-Françoise Petit-Glatron and Régis Chambert

Institut Jacques Monod, Laboratoire Génétique et Membranes, CNRS – Universités Paris 6 et Paris 7, Tour 43, 2 place Jussieu, 75251 Paris Cedex 05, France

**Transcription of *sacB*, *yveB* and *yveA*, three clustered genes on the *Bacillus subtilis* chromosome, is simultaneously induced by sucrose. Northern blotting analyses with specific probes showed three distinct mRNAs: a monocistronic 1·7 kb *sacB* mRNA, a bicistronic 3·3 kb *sacB–yveB* mRNA and a tricistronic 4·9 kb *sacB–yveB–yveA* mRNA. These results indicate that *sacB*, encoding levansucrase, is the proximal gene of a sucrose-inducible operon that includes the two other genes. The yield of the full-length transcript is lower than that of the bicistronic transcript, whose yield is itself lower than that of the monocistronic transcript. This suggested that the 3' terminal parts of *sacB* and *yveB* genes worked as internal terminator structures. The protein encoded by *yveB*, which remains anchored to the membrane, displays an endolevanase activity, which, coupled with exolevanase activity of SacB, leads to a complete degradation of levan, a branched fructosyl polymer. It is proposed to rename *yveB* as *levB*.

**Keywords**: levanase, levansucrase operon, sucrose metabolism, *degU32(Hy)* mutant

**INTRODUCTION**

Identification of the various genes involved in the metabolism of sucrose by *Bacillus subtilis* is mainly based on the seminal work of Lepesant *et al.* (1972). Of the seven loci characterized, the *sacB* locus was predicted to include only the gene encoding levansucrase and was not expected to be organized into a transcriptional unit involving the adjacent genes. Moreover, the sequence of a 2 kb fragment of *B. subtilis* genome containing the structural gene encoding levansucrase (Steinmetz *et al.*, 1985) showed that the coding frame of this protein was followed by a region that included a dyad symmetry 13 nt long, which was thought to be a strong rho-independent transcription terminator. Such a result supported the hypothesis proposed by Lepesant *et al.* (1972) that the *sacB* locus is monogenic. However, examination of the complete sequenced genome of *B. subtilis* (Kunst *et al.*, 1997) showed that no potential promoter was discernible in the short intergenic region between the stop codon of *sacB* and the start codon of the predicted downstream gene *yveB*. Since *yveB* encodes a protein displaying a similarity to levanase (encoded by *sacC*), it can be anticipated that the product of *yveB* plays a role in the metabolism of sucrose. Furthermore, *yveA*, the adjacent coding frame of *yveB*, could be part of the same transcription unit, given its proximity to the stop codon of *yveB*. The protein coded by *yveA* displays all the features of a membrane protein. Therefore, we anticipated that the *sacB*, *yveB* and *yveA* genes constitute an operon. Using Northern blot analysis, we tested the hypothesis that the transcription of the three genes was simultaneously induced by sucrose. The positive result obtained prompted us to characterize the cellular location of the protein YveB and its catalytic specificity.

**METHODS**

**Bacterial strains and culture.** *Bacillus subtilis* QB112 [sacA321, degU32(Hy)] was used in this work (Chambert & Petit-Glatron, 1984). Aerobic cultures were run in a 1·5 l fermenter (Bioflo 3000, New Brunswick Scientific). Cells were grown in minimal medium, supplemented with 1·5 % (w/v) glucose as carbon source at the beginning of growth and with 60 mM sucrose added at an OD<sub>600</sub> of 0·2 when necessary. When the bacterial suspension reached an OD<sub>600</sub> of 6, fresh minimal medium containing 1 % (w/v) glucose was fed at a constant rate equal to that of the cell suspension outflow (660 ml h<sup>−1</sup>). pH and temperature were controlled and maintained at values of 7·0 and 37 °C, respectively. Solubilized O<sub>2</sub> was measured with a Clark electrode and 85 % O<sub>2</sub> saturation was maintained by a bubbler through the culture. The speed of agitation was 800 r.p.m. Under these conditions,
the specific growth rate, $\mu$, remained maximum and constant, at 0.69 h$^{-1}$. Growth yield was approximately 78 g biomass (mol glucose)$^{-1}$.

*Escherichia coli* XL-1 Blue strain was grown in TerB rich medium (Sambrook *et al.*, 1989) containing 150 µg ampicillin ml$^{-1}$.

**Isolation and purification of total RNA.** RNAs were prepared as described previously (Pereira *et al.*, 2001) from bacteria (5 ml) growing exponentially in the presence or absence of sucrose. All traces of genomic DNA were eliminated during an additional step using a high pure RNA isolation kit (Roche).

**Northern blotting.** Northern blotting experiments were done essentially as described by Pereira *et al.* (2001). The *sacB* probe (859 nt) was obtained by an *Eco*I and *Kpn*I digestion of plasmid pL5S0 (Steinmetz *et al.*, 1985). The probes *yveB* and *yveA* of, respectively, 817 nt and 818 nt, were prepared by PCR using the oligonucleotides shown in Table 1. Co-migration with a molecular mass marker mixture (RNA molecular weight markers, Fermentas) made it possible to estimate the length of the transcripts. DNA bands were revealed by Phosphorimaging and quantified with Image-Quant software (Molecular Dynamics).

**Construction of a sac–yveB fusion and expression of YveB in *E. coli*.** The *B. subtilis* yveB gene corresponds to an ORF encoding a putative protein of 516 amino acids located 73 nt after the C-terminal stop codon of *sacB* gene. This gene was amplified by PCR with primers yveB3 and yveB4 including restriction sites *Aat*II and *Eco*RV, respectively (Table 1), from the chromosomal DNA of strain QB112 isolated as previously described (Leloup *et al.*, 1995). The amplified blunt-ended fragment was inserted into the pCR(+) vector at the *Srf*I site after appropriate treatment according to the supplier’s recommendations (Stratagene). The *yveB* fragment was isolated after digestion with *Aat*II and *Eco*RV and ligated into the pCR(+) vector pGMC9 (Leloup *et al.*, 1999a) digested with the same enzymes. The resulting plasmid pGMC13 was used to transform strain *E. coli* XL-1 Blue. One transformant was selected and the complete sequence of the gene fusion *sacR–yveB* was checked by sequencing the double-stranded recombinant DNA with the appropriate synthetic oligonucleotides. This strain was grown in parallel with the same strain transformed with pCR(+) vector at 37 °C. A sample of the cell suspensions (6 ml) was withdrawn at an OD$_{500}$ of 2.5 and centrifuged. The cell pellets were resuspended in 1 ml 0.1 M sodium phosphate pH 6.5 containing 100 µg chloramphenicol ml$^{-1}$. After a freeze–thaw cycle, the cells were disrupted by sonication and centrifuged. Levanase activity was assayed in the supernatants as described below.

**Substrate and levanase assay.** Uniformly labelled $[^{14}C]$levan was obtained by the action of immobilized levanucrase on $[^{14}C]$sucrose (Chambert & Petit-Glatron, 1993). Levanase was assayed as described by Leloup *et al.* (1999a).

**Fractionation of *B. subtilis* cells.** The cell suspension (250 ml) was centrifuged at an OD$_{500}$ of 2.5 and the bacteria were washed with 10 ml cold buffer containing 2 M KCl. After centrifugation, the cell pellet was resuspended in 10 ml 0.1 M sodium phosphate pH 7 and lysed by incubation for 10 min at room temperature in the presence of a lysozyme/ DNase mixture (1 mg lysozyme ml$^{-1}$, 0.1 mg DNase ml$^{-1}$, 0.02 mg MgCl$_2$ ml$^{-1}$). Disruption was completed by sonication.

The membrane fraction was obtained by centrifuging the suspension twice, first at 5000 g for 10 min and then, after discarding the cell debris, at 40000 g for 30 min. The membrane pellet was resuspended in 2 ml 0.1 M sodium phosphate, pH 7.

**RESULTS**

Three different transcripts: *sacB* mRNA, *sacB–yveB* mRNA and *sacB–yveB–yveA* mRNA, are present in the *degU32(Hy)* strain growing in the presence of sucrose

DNA probes specific to *sacB*, *yveB* and *yveA* were used to probe Northern blots performed with total RNAs prepared from *degU32(Hy)* cells grown in minimal medium supplemented with glucose in the presence or absence of sucrose. Three bands were observed in the lanes hybridized with the *sacB* probe (Fig. 1, lanes 2 and 6) corresponding to transcripts of 1.7, 3.3 and 4.9 kb. Two bands were observed in the lane hybridized with the *yveB* probe (Fig. 1, lane 4), corresponding to transcripts of 3.3 and 4.9 kb; only one band of 4.9 kb was observed in the lane hybridized with the *yveA* probe (Fig. 1, lane 8). No band corresponding to either the *yveB* or the *yveA* transcript was observed, suggesting that there were no specific promoters of these two genes nor any subsequent processing of a primary tricistronic transcript into three monocistronic units. These results are consistent with a tricistronic organization of *sacB*,

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’−3’)*</th>
<th>Restriction site at 5’ end</th>
</tr>
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<tr>
<td>yveA1</td>
<td>GGGATGGGAGCCATCTTTGGATCGCGTGGCT</td>
<td>Smal</td>
</tr>
<tr>
<td>yveA2</td>
<td>GCAAGCCAGCCTAAACCGAGCATGCC</td>
<td>Smal</td>
</tr>
<tr>
<td>yveB1</td>
<td>ACACCCGGGCGCATGCCCCGCTCAGAGG</td>
<td>AatII</td>
</tr>
<tr>
<td>yveB2</td>
<td>TCGCCCGGGAGCGGAGCTGTATGTTTCCATCC</td>
<td>EcoRV</td>
</tr>
<tr>
<td>yveB3</td>
<td>GGAGACGTCAATGGAACATATATAAAAGACAGGCAAATGTAACCG</td>
<td></td>
</tr>
<tr>
<td>yveB4</td>
<td>CCGATATCGCCCAATATGTAACAGAATACCCG</td>
<td></td>
</tr>
<tr>
<td>SS RNA probe</td>
<td>ACTACCATGGCGGCTGAAGA</td>
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</tr>
</tbody>
</table>

* Restriction sites are in bold.

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**Table 1. Oligonucleotides used in this study**
yveB and yveA, transcribed exclusively from a common promoter upstream of sacB. In the frame of such a hypothesis, the lower intensity of the bands corresponding to sacB–yveB and sacB–yveB–yveA would be the result of partial arrests of the RNA polymerase at the transcription termination loops after sacB and yveB. Using the MFOLD program (Zuker et al., 1999) we evaluated the free energies of these terminators to be $-18.7$ and $-20.4$ kcal mol$^{-1}$ ($-78.2$ and $-85.4$ kJ mol$^{-1}$), respectively.

**Organization of the levansucrase operon**

The region upstream of sacB has been shown to include a sigma vegetative promoter (Steinmetz et al., 1985), a short region of dyad symmetry (RAT) (Aymerich & Steinmetz, 1992; Tortosa & Le Coq, 1995), a target of the antiterminator SacY (Crutz et al., 1990), whose active dephosphorylated form is induced in the presence of sucrose, and a structure similar to a transcription terminator (Shimotsu & Henner, 1986; Steinmetz & Aymerich, 1986). The structural gene sacB, encoding a precursor of 473 amino acid residues which includes a signal sequence of 29 residues, is immediately followed by a stretch of 73 nt (Fig. 2). This DNA fragment includes a terminator structure and a Shine–Dalgarno consensus sequence (AAAGGTG) 8 nt distant from the ATG start codon of yveB. The structural gene of yveB, encoding a protein of 516 amino acid residues, revealed a putative signal sequence according to SignalP, as reported by Tjalsma et al. (2000). The following open reading frame of 520 amino acid residues encoded by...
yveA is 107 nt distant from the stop codon of yveB. This intergenic region includes a terminator structure and a Shine–Dalgarno sequence (AAAGGAG), 7 nt before the start codon of yveA (Fig. 2).

Characterization of YveB as an endolevanase

In view of the amino acid sequence similarity of YveB with levanase SacC, we could predict a levanase activity for YveB (SubtiList database: http://genolist.pasteur.fr/SubtiList/). Therefore, we tested the presence of levanase activity in the crude extracts obtained from an E. coli strain expressing the cloned yveB gene (see Methods). The crude extracts were incubated in the presence of high-molecular-mass (>$10^7$ Da) levan uniformly labeled (Chambert & Petit-Glatron, 1993). Products released by the enzyme action were analysed either by thin-layer chromatography (Fig. 3a), which indicated that the action of YveB on levan released small amounts of fructose and oligofructan with a low degree of polymerization (DP), or by chromatography on a Biogel P60 column (Fig. 3b), illustrating the size distribution of the products. The results show that oligofructosides spanning a molecular mass range of 1 kDa to 10 kDa (corresponding to a DP of fructosyl residues lower than 50) are the main products of the enzyme action. It can be concluded that YveB mainly displays an endolevanase activity. The catalytic specificity of YveB is therefore different from that of SacC, which has been proved to be an exolevanase releasing only free fructose from its action on levan (Wanker et al., 1991). From this enzymic characterization, we hereafter re-name YveB as LevB.

Since LevB is co-synthesized with SacB, we anticipated that these two enzymes could act synergistically on levan. Indeed, SacB has the dual catalytic activity of a levan polymerase and a levan hydrodase (Rapoport & Dedonder, 1963). However, this latter exolevanase activity is arrested at the 2 → 1 branch points of the polymer levan. Thus, only 30% of available fructose is released by prolonged action of the enzyme on levan. When LevB and SacB acted together on levan, we observed (not shown) that the release of fructose was greater than that obtained by each enzyme separately with the same substrate since more than 60% of the polymer is hydrolysed to free fructose. This means that the endolevanase activity of LevB releases new sites on levan for the exolevanase activity of SacB. This property was used to characterize the localization of LevB in B. subtilis. In fact, the synergy of LevB and SacB activities mimics the activity of SacC, whose synthesis is induced by fructose during the stationary phase of growth. The possible effect of this property under in vivo conditions is discussed below.

Localization of LevB in B. subtilis

Culture supernatants, cytoplasmic and membrane fractions of induced and non-induced bacteria were obtained as described in Methods. In each fraction, levan hydrolytic activity was tested (Fig. 4a). Levanase activity of SacB was present in the culture supernatant and in the 2 M KCl washing supernatant of induced bacteria. In contrast, the catalytic activity of LevB, characterized by oligofructose release from levan, remained mainly associated with the membrane fraction...
Levansucrase operon of *Bacillus subtilis*

(a) (b)

**Fig. 4.** Analysis of the cellular distribution of SacB and LevB in *B. subtilis* degU32(Hy). A cell suspension at an OD<sub>600</sub> of 0.2 was divided into equal portions in flasks with (+) or without (−) sucrose, final concentration 50 mM. After 2 h growth (OD<sub>600</sub> 1.0), cell suspensions (250 ml) were treated as described in Methods. (a) Levanase activity was tested by incubating 10 µl [U<sup>14</sup>C]levan solution with 10 µl of (1) culture supernatant, (2) 2 M KCl washing supernatant of the cells, (3) cytoplasmic fraction, or (4) membrane fraction. After 4 h incubation at 30 °C, products were analysed by paper chromatography with n-butanol/acetic acid/water (4:1:1, by vol.) as developing solvent. (b) Levanase activity present in culture supernatant (1) and in membrane fraction (4) was assayed in the presence of 50 µg pure levansucrase (SacB) ml<sup>−1</sup>.

of induced cells. This result was confirmed by the synergy of SacB and the membrane fraction, which led to an increase in levan hydrolysis (Fig. 4b). 30% fructose was released by SacB alone and 60% by a mixture of SacB and the membrane fraction. This result provided evidence that the hydrophobic N-terminal sequence of this protein, which was proposed to be a cleavable signal peptide according to SignalP (Nielsen *et al*., 1997), could not be processed and consequently was more likely to be a membrane anchor as predicted by SignalP (Nakai & Kanehisa, 1991). This hypothesis is substantiated by the observation that, for the potential precursor form of this protein, the cleavage site of the leader peptidase is clearly unconventional when compared to that of SacB, a typical exocellular enzyme, secreted by the Sec-dependent pathway (Leloup *et al*., 1999b).

**DISCUSSION**

Northern blotting performed with the *B. subtilis* degU32(Hy) mutant indicated that transcription of sacB, levB (yveB) and yveA, three clustered genes, was triggered by sucrose. Hybridization of total RNA with specific probes showed that the transcript of sacB, the proximal gene, is synthesized at a higher level than that of the other two genes. These latter were detected at lower levels as only bicistronic sacB–levB or tricistronic sacB–levB–yveA transcripts, suggesting that these three genes constitute a sucrose-inducible tricistronic operon.

The difference in transcript levels in a same operon can be the result of two different mechanisms: either processing of the primary transcript and different stabilities of the processing products, or partial termination of transcription at internal termination structures. The absence of any levB and yveA transcripts led us to favour the latter hypothesis. Furthermore, the intergenic regions between sacB–levB and levB–yveA were screened for the potential presence of a sigma A promoter consensus (Haldenwang, 1995). Allowing four mismatches and a spacer of 17 nt between the −35 and −10 sites, one promoter can be predicted in the intergenic region between levB and yveA, but in this case the −10 site would be only 7 nt distant from the Shine–Dalgarno sequence, making a transcriptional start site in this region highly improbable, in agreement with the Northern blot analysis.

Modulation of the transcriptional level of the three genes of a same operon by partial termination of transcription at internal termination structures is perhaps related to a difference in the required quantities of the proteins encoded by the various genes. The protein products of sacB and levB are both involved in sucrose metabolism, sacB encoding levansucrase, a well-characterized exocellular enzyme (Chambert *et al*., 1974), and levB encoding a protein which displays an endolevanase activity as shown in this study. The fact that LevB remains membrane associated could explain why its production is lower than that of levansucrase, since this latter exocellular enzyme is rapidly diluted in the cell environment and could therefore contribute to cell metabolism shortly after its synthesis.

The function of YveA, the third protein encoded by the
operon, is unknown. Its numerous predicted transmembrane segments suggest a strong membrane association but the proposed amino acid permease similarity does not fit with the function of the two other genes, unless this protein mediates chemotactic responses to a variety of compounds like the Tar chemoreceptor of E. coli, which senses two quite different chemoeffectors, aspartate and maltose (Slocum & Parkinson, 1983). Further studies are required to understand a possible link between the two metabolic pathways.

A similar operon organization of clustered genes encoding proteins involved in sucrose metabolism has been postulated from the DNA sequence of several micro-organisms, Bacillus stearothermophilus (Li et al., 1997; Naumoff, 1999), Gluconacetobacter diazotrophicus (L. Hernandez and others, unpublished; see GenBank L141732) and Zymomonas mobilis (Song et al., 1999). Unfortunately, a lack of information about both the mechanism of the operon transcription and the enzyme specificity of the encoded proteins strongly limits any fruitful comparison with the B. subtilis operon characterized in this study.

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