Gain-of-function mutation of sapB that affects formation of alkaline phosphatase by *Bacillus subtilis* in sporulation conditions

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**INTRODUCTION**

Sporulation of *Bacillus subtilis* is a primitive system of cell differentiation. The system involves both spatial and temporal controls of gene expression (Errington, 1993; Losick & Stragier, 1992). An event associated with the asymmetric division that is defined as stage II of sporulation (Ryter, 1965) is the induction of alkaline phosphatases (APases) (Piggot & Coote, 1976). This induction is not a response to phosphate starvation, in contrast to APase production during vegetative growth. The same structural genes appear to be involved in producing the activity during both growth and sporulation, but the controls of expression differ (Glenn & Mandelstam, 1971; Grant, 1974; Bookstein et al., 1990).

Sporulation APase production is dependent on the activation of the sporulation-associated RNA polymerase sigma factor σ^E^, and it is not detected in strains with mutations in *spolIG* (Piggot & Coote, 1976), which encodes σ^K^ (Stragier et al., 1984). Not is it seen in strains with mutations in *spolIIA* (Piggot & Coote, 1976), the locus encoding the sporulation-associated factor σ^F^, which is required, among other things, for the activation of σ^E^ (reviewed by Errington, 1993). Chesnut et al. (1991) have shown that *phoB*, which encodes the APase responsible for the majority of the sporulation APase, is transcribed from two different promoters: P_σ^F_ during sporulation, and P_σ^E_ during vegetative growth. P_σ^E_ is thought to be recognized by RNA polymerase that contains σ^E^, and P_σ^F_ by RNA polymerase that contains σ^F^.

Piggot & Taylor (1977) investigated the link between the spo genes and sporulation APase expression. They identified a locus, sapB, defined by suppressor mutations that restored formation of sporulation-associated APase to *spolI* and *spolII* mutants. Additionally, the sapB mutants showed similar timing of APase induction to *spoI* and *spoIIA* strains. Thus sporulation APase expression was rendered independent of σ^F^ and σ^K^.

The result was surprising, as these sigma factors appear to be largely responsible for transcription at stage II of sporulation (Errington, 1993). Birkey et al. (1994) have recently shown that in *spolI* and *spolIIA* double mutants, *phoB* is transcribed from P_σ^F_ instead of P_σ^E_ in sporulation conditions, and that this expression requires PhoP and PhoR, the sensor–effector system responsible for monitoring extracellular phosphate levels (Seki et al., 1987, 1988). Their result explains why sporulation APase is induced in the absence of functional σ^F^. However, it does not explain how the timing of induction of...
sporulation APase in \textit{spoIIA sapB} mutants is similar to the timing in \textit{spo+ sapB+} strains. Here we report the cloning, sequencing and further study of \textit{sapB} and explore the induction of APase during sporulation in \textit{sapB} mutants.

**METHODS**

**Bacterial strains.** The \textit{B. subtilis} strains used in this work were derivatives of 168 \textit{trpC2} (Table 1). The \textit{Escherichia coli} strains used were DH5a and HB101. Bacteria were routinely grown in Luria–Bertani (LB) broth. Sporulation was promoted by either the exhaustion method using modified Schaeffer’s sporulation medium (MSSM) (Piggot & Curtis, 1987) or the resuspension system of Sterlini & Mandelstam (1969). Selections for antibiotic resistance were with 5 \mu g chloramphenicol ml⁻¹, 5 \mu g neomycin ml⁻¹, 10 \mu g kanamycin ml⁻¹ or 100 \mu g ampicillin ml⁻¹ as appropriate.

**DNA techniques and methods of genetic exchange.** \textit{B. subtilis} was transformed by the method of Young & Wilson (1974) as modified by Piggot et al. (1984). In most transformations, 50 ng DNA ml⁻¹ was used. When congression was desired, 5 \mu g DNA ml⁻¹ was used. DNA-mediated transformations of \textit{E. coli} strain DH5α were performed by a calcium chloride/rubidium chloride method modified from Hanahan (1985). PBS1-mediated transductions were performed as described by Hoch (1991). Transposon mutagenesis was conducted using the modified Tn917 (Tomich et al., 1980) contained in pLT7 (Camilli et al., 1990). Insertion-liberty construction and cloning of transposon-proximal DNA were performed as described by Youngman (1985). The plasmids pPP376 and pPP388 were cloned directly in \textit{E. coli} from the Tn917 inserts in \textit{B. subtilis} as described by Camilli et al. (1990). The plasmid pPP427 was produced by digesting pPP388 with BamHI and SalI, followed by treatment with Klenow fragment and self-ligation. SalI cuts in the vector 351 bp from the Tn917 junction with the insert. To facilitate sequencing of the \textit{sapB} region, the 1.2 kbp \textit{PstI} fragment and 250 bp \textit{BamHI-PstI} fragments derived from pPP376 were cloned into pBluescriptKS (Stratagene) at those sites in the multiple-cloning region to produce pPP379 and pPP380, respectively.

Routine manipulations of DNA were as described by Sambrook \textit{et al.} (1989). DNA was sequenced with a Sequenase kit (USB) according to the manufacturer’s instructions. The method is based on the dideoxy chain-termination method of Sanger \textit{et al.} (1977). DNA to be sequenced was purified with a Qiagen column. Usually, 50 ng synthetic primer and 3–5 \mu g purified template DNA were included in the sequencing reaction. Vector-based primers were obtained with the PHD program from EMBL (Rost \textit{et al.}, 1994).

**Inactivation of \textit{sapB}.** The \textit{sapB} gene was inactivated by the insertion of the \textit{neo} gene from pBest501 (Itaya \textit{et al.}, 1989) into the \textit{BamHI} site within the coding region of \textit{sapB}. To obtain the \textit{neo} marker with flanking \textit{BamHI} sites, pBest501 was digested with \textit{SmaI}, and the 1.3 kbp fragment containing the \textit{neo} cassette was isolated and purified by Gene-Clean; this fragment was ligated into pBluescript SK which had also been cut with \textit{SmaI}. As the \textit{neo} gene confers kanamycin resistance in \textit{E. coli}, recombinant plasmids were isolated from the Kan’Amp’ transformants of \textit{E. coli}. The \textit{neo} cassette was isolated from one such plasmid after digestion with \textit{BamHI}, and cloned into pPP388, which had also been digested with \textit{BamHI}, to construct pPP492. This plasmid was linearized with \textit{SalI} and used to transform \textit{B. subtilis} strain MB24, selecting for Neo’. Chromosomal DNA from a Neo’/Cmr’ transformant, SL6216, was isolated, and the disruption of \textit{sapB} by \textit{neo} insertion was verified by Southern blotting. The structure of \textit{sapB:neo} strains produced by transformation with chromosomal DNA from SL6216 was verified by PCR using primers mw201 (5’-CTTTGATATCTCTTCTTTTC3’) and 202r (5’-GAGCTGGTATATTGATCC-3’) that flank the site of insertion.

**Detection of enzyme production in solid and liquid medium.** APase production was monitored by growing strains on Schaeffer’s sporulation agar in the presence of 80 \mu g bromochloro-indoyl phosphate ml⁻¹. \beta-Galactosidase activity on plates was visualized by using 40 \mu g X-Gal ml⁻¹. Protease activity was determined by incubating bacteria on skimmed milk agar (1.5% skimmed milk by weight) at 37 °C for 18 h (Tanaka & Kawata, 1988). Spores were counted in the manner

**Table 1. \textit{B. subtilis} strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>MB24</td>
<td>\textit{trpC2 metC3 rif2}</td>
<td>Piggot &amp; Curtis (1987)</td>
</tr>
<tr>
<td>MH 5124</td>
<td>\textit{trpC2 phoR::tet}</td>
<td>Hulet \textit{et al.} (1994)</td>
</tr>
<tr>
<td>MLK 351</td>
<td>\textit{trpC2 lys3 amyE::erm}</td>
<td>M. L. Karow</td>
</tr>
<tr>
<td>QB 693</td>
<td>\textit{purB6 tre12}</td>
<td>Dedonder \textit{et al.} (1977)</td>
</tr>
<tr>
<td>SL 311</td>
<td>\textit{sapB2 spoIIA69 rif2}</td>
<td>Piggot &amp; Taylor (1977)</td>
</tr>
<tr>
<td>SL 373</td>
<td>\textit{spoIIA69 sapB10 rif2}</td>
<td>Piggot &amp; Taylor (1977)</td>
</tr>
<tr>
<td>SL 650</td>
<td>\textit{trpC2 rif2 sapB10}</td>
<td>Piggot &amp; Taylor (1977)</td>
</tr>
<tr>
<td>SL 6216</td>
<td>\textit{trpC2 metC3 rif2 sapB::neo}</td>
<td>This study</td>
</tr>
<tr>
<td>SL 6564</td>
<td>\textit{spoIIA69 trpC2 sapB::neo}</td>
<td>This study</td>
</tr>
<tr>
<td>SL 6926</td>
<td>\textit{trpC2 metC3, amyE::sapB-lacZ}</td>
<td>This study</td>
</tr>
<tr>
<td>SL 6927</td>
<td>\textit{trpC2 metC3, amyE::sapB-lacZ}</td>
<td>This study</td>
</tr>
<tr>
<td>SL 7015</td>
<td>\textit{trpC2 sapB10 amyE::sapB-lacZ}</td>
<td>This study</td>
</tr>
<tr>
<td>SL 7016</td>
<td>\textit{trpC2 sapB::neo amyE::sapB-lacZ}</td>
<td>This study</td>
</tr>
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</table>
of Nicholson & Setlow (1990) from dilutions of overnight cultures grown in MSSM or the Sterlini–Mandelstam resuspension medium. β-Galactosidase activity in B. subtilis was determined according to the method of Miller (1972) as modified by Nicholson & Setlow (1990). Aβase determinations were done according the protocol of Grant (1974).

RESULTS AND DISCUSSION

The cloning and sequencing of sapB

The sapB locus was cloned by exploiting the fact that it had previously been mapped by PBS1 transduction (Piggot & Taylor, 1977). As the gene maps near purB, a selectable auxotrophic marker for purine requirement, a Tn917 insertion library was prepared with a pur+ strain and then phage PBS1 propagated on the library. A pur- mutant was transduced to pur+ Cmr to select those strains that contained a transposon near (i.e. within 200 kb) purB. The linkage of the transposon to sapB was then tested by transformation into a sapB mutant. Of the 33 transductants tested, in three cases the transposon showed strong (> 70% co-transformation) linkage to sapB. By using features of the modified Tn917 (Camilli et al., 1990), both the wild-type and the sapB10 alleles were cloned in plasmids which were named pPP376 and pPP379, respectively. Restriction enzyme sites are shown by the arrowheads. Restriction enzyme sites used to construct sapB-lacZ fusions in strains SL6926 and SL6927 are indicated at the bottom of the figure. The fusions were integrated into the B. subtilis chromosome at amyE as described by Shimotsu & Henner (1986). The site of insertion of the neo cassette in sapB is also shown. Restriction enzyme abbreviations: E, EcoRI; H, HindIII; B, BamHI; P, PstI; Hc, HincII.

Fig. 1. Partial restriction map of the 5.9 kbp EcoRl fragment from MB24 showing the position of the Tn917 insertion used to clone sapB and the inserts in derived plasmids. The presence (+) or absence (−) of sapB10 is also indicated in the right panel. Coding regions are indicated by arrows. Regions that were used to construct sapB-lacZ fusions in strains SL6926 and SL6927 are indicated at the bottom of the figure. The fusions were integrated into the B. subtilis chromosome at amyE as described by Shimotsu & Henner (1986). The site of insertion of the neo cassette in sapB is also shown. Restriction enzyme abbreviations: E, EcoRI; H, HindIII; B, BamHI; P, PstI; Hc, HincII.

Fig. 2. Nucleotide sequence and deduced amino acid sequence of sapB. The final 133 amino acid residues of OpuE are also shown. A possible RBS is indicated by underlining, and bases modified by Nicholson & Setlow (1990). APase determinations were done according the protocol of Grant (1974).

pPP380 of B. subtilis

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pPP388 and pPP376, respectively. A number of plasmids which contained deletions into the cloned region were then constructed to determine the site of the sapB10 mutation. The sapB10 mutation was mapped to a 250 bp BamHI PstI fragment contained in pPP380 (Fig. 1), and the region was sequenced. These plasmids were used to sequence sapB and the sapB10 mutant allele. The sapB2 allele was sequenced by a PCR-based method.
Table 2. Sequences similar to SapB detected by BLAST (Altschul et al., 1990)

The length of similarity shows the total number of residues contained in the regions that are similar between the sequences. Percentage identity and similarity refer to the number of identical amino acid residues or conserved substitutions between SapB and each deduced protein sequence over the length of similarity. Minimum sum probability is the probability of occurrence by chance of the two sequences having equal or greater similarity than the observed similarity as calculated by the Karlin-Altschul statistic (Karlin & Altschul, 1990).

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Source organism</th>
<th>Total length of protein (aa)</th>
<th>Total length of similarity (aa)</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
<th>Minimum sum probability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIIU32748-1 1 (HinF)</td>
<td>Haemophilus influenzae</td>
<td>240</td>
<td>148</td>
<td>64</td>
<td>77</td>
<td>1.3e−57</td>
<td>Fleischmann et al. (1995)</td>
</tr>
<tr>
<td>P26606 (OrfC)</td>
<td>Escherichia coli</td>
<td>216</td>
<td>139</td>
<td>34</td>
<td>59</td>
<td>2.1e−23</td>
<td>Mizuno &amp; Tanaka (1993)</td>
</tr>
<tr>
<td>MgtC</td>
<td>Salmonella typhimurium</td>
<td>228</td>
<td>115</td>
<td>40</td>
<td>66</td>
<td>1.9e−18</td>
<td>Snavely et al. (1991); Tao et al. (1995)</td>
</tr>
<tr>
<td>SrpB</td>
<td>Synecococcus sp.</td>
<td>182</td>
<td>107</td>
<td>39</td>
<td>64</td>
<td>1.2e−16</td>
<td>Nicholson &amp; Laudenbach (1995)</td>
</tr>
</tbody>
</table>

Fig. 3. Comparison of deduced amino acid sequences that are similar to SapB. Sequences were aligned using the PILEUP program (GCG) and drawn by the Box-Shade program. Residues conserved in three or more proteins are shown as boxed positions. The asterisk shows the site of the sapB10 mutation.

Sequence analysis of sapB

The sequenced region contained an ORF which spanned the pPP380 insert. The ORF had a UUG initiation codon that was preceded by a putative RBS. The deduced sequence of the sapB gene product is a 232 amino acid residue, 26 kDa protein (Fig. 2). No well-characterized functional domains were located by the motifs program (GCG). BLAST similarity searching detected four similar hypothetical proteins in the combined GenBank/EMBL non-redundant database (Table 2). While the source organisms of these sequences are widely distributed within the euobacterial kingdom, no function has been determined for any of the putative proteins. The proteins are of similar size. The majority of conserved residues map in the N-terminal region as shown by multiple sequence alignment (Fig. 3).

The sapB locus was defined by two independently derived mutations, sapB2 and sapB10 (Piggot & Taylor, 1977). Both mutations were found to be a G→A transition, resulting in a predicted Ala111-Thr change, indicated with an asterisk in Fig. 3. Interestingly, this position is occupied by a hydroxyl-containing residue in the other sequences.

The sequence of the region 5′ to sapB suggested the presence of another gene which similarity searches predicted to encode a proline permease. C. von Blohn & E. Bremer (personal communication), in the course of their study of the proline permeases of B. subtilis, have cloned and sequenced this gene and demonstrated that it is a proline transporter. They have kindly provided us with the sequence for the purpose of comparison and have named the permease gene opuE. No similar ORF to OpuE was detected adjacent to the SapB-like ORFs in the species listed in Fig. 3. Sequence analysis using the terminator program (Brendel et al., 1984) suggested the presence of a weak ρ-independent terminator 5′ to sapB, and a stronger terminator 3′ to sapB.

Transcription of sapB

For the purposes of examining the transcription of sapB and of delimiting the promoter, two sapB–lacZ fusions were constructed using the back-to-front amyl system (Shimotsu & Henner, 1986) (Fig. 1). The expression of sapB in rich medium which allows (MSSM) or prevents (LB) sporulation is shown in Fig. 4. In both media, sapB is induced toward the end of vegetative growth and is expressed maximally during the first hour of stationary phase. Determination of the transcription of sapB–lacZ in strains SL6926, SL7015 and SL7016, which contain either the wild-type sapB (SL6926), the sapB10 (SL7015) or null (SL7016) alleles, were indistinguishable, suggesting that sapB is not auto-regulated (data not shown).
P-galactosidase was expressed in strain SL6926 but not sapB-lacZ in SL6927, suggesting that the reporter gene fusion. Squares and circles indicate Blohn containing either a full (SL6926) or truncated (SL6927) part promoter within or upstream of sapB, as shown by P-galactosidase activity in LB medium as shown by β-galactosidase activity [nM ONPG hydrolysed min⁻¹ (mg cell dry wt)⁻¹] in strains containing either a full (SL6926) or truncated (SL6927) part of the cloned region 5' to sapB (see Fig. 4). Open squares show MB24, filled triangles show SL6926 and open circles show SL6927. Time 0 is defined as the end of exponential growth. Data shown are means from three independent determinations.

Despite the presence of a possible terminator 5' to sapB, β-galactosidase was expressed in strain SL6926 but not in SL6927, suggesting that sapB is transcribed from a promoter within or upstream of opuE (Fig. 5). C. von Blohn & E. Bremer (personal communication) have located another ORF upstream of opuE, and pointing in the opposite direction; it seems plausible that opuE and sapB form an operon.

Eubacteria take up proline principally for metabolism or for osmotic protection (reviewed by Maloy, 1987; Csonka, 1989). The fact that sapB is 3' to opuE prompted testing whether either proline levels or osmotic shock had an impact on the transcription of sapB-lacZ. The NaCl concentration previously used in the study of the stress response in B. subtilis (Boylan et al., 1993) and the proline concentrations used in the study of putP of E. coli (Maloy, 1987) and twofold excess of each (0.6 M NaCl and 0.5% proline) were used, but neither NaCl nor proline affected the transcription of sapB (data not shown).

**Mutation of sapB**

The sapB gene was disrupted by inserting a neo cassette as described in Methods. Surprisingly, the sapB::neo strain was indistinguishable from the spoIIA69 sapB” strains with respect to APase production. As noted earlier, Birkey et al. (1994) reported an analysis of the phoB promoter in which they demonstrated that sapB2 mutants transcribe phoB from P₆, the vegetative or phosphate starvation-associated promoter, during sporulation. They also showed that this expression requires PhoP/PhoR and speculated that SapB is a negative regulator of PhoR activation. Apparently only the sapB10/sapB2 allele allows the activation of PhoR. That is, the mutant allele sapB2/sapB10 confers a gain-of-function to SapB, allowing the activation of PhoR, and hence transcription of phoB from P₆.

It was noted that APase is expressed at the highest level in a spoIIA sapB10 mutant when the bacteria were grown in Sterlini–Mandelstam resuspension medium. The fact that this medium contains 0.5 mM phosphate prompted testing whether SapB10-related activation of PhoR/PhoP is sensitive to the amount of phosphate in the medium by using the same resuspension medium, but with 5 mM NaPO₄. The results suggest that this activation is indeed phosphate-sensitive (Fig. 6), yet this concentration is not sufficient to impede sporulation, as the MB24 cultures sporulated well (> 60% heat-resistant spores from samples taken at t₉). The change in phosphate concentration did not affect sapB transcription (data not shown). Taken together, these data suggest that the SapB10 protein acts through PhoR in a manner that is inhibited by the presence of excess phosphate.

This conclusion also raises the question of what is the normal role of SapB in the cell. If the sapB2/10 mutation represents a gain-of-function that allows the activation of PhoR in a phosphate-repressible manner, the possibility emerges that SapB may normally communicate with another sensor-class protein. The most plausible candidate would be ResD, the sensor in the ResD ResE system that governs the respiration of B. subtilis (Sun et al., 1996; Hullet, 1996). The primary structures of ResD...
Fig. 6. Expression of APase in strains harbouring various alleles of sapB in the sporulation/resuspension system of Sterlini & Mandelstam (1969). Also shown is the effect of extra (5 mM) phosphate on APase production. Units are nM PNPP hydrolysed min⁻¹ mg cell dry wt⁻¹. Time 0 is the time of resuspension. Strains shown are as follows: open circles show SL373 (sapB10 spoIIA69). Filled circles show SL373 in resuspension medium containing 5 mM NaPO₄. Squares show MB24 (spoI1A sapB⁺) in either resuspension medium alone (open) or resuspension medium plus 5 mM phosphate (filled). Open triangles show SL650 (spoI1A69 sapBAneo), and filled triangles show SL6564 (spoI1A69 sapBAneo), both in resuspension medium. Data are means from three independent determinations.

REFERENCES


Received 13 September 1996; accepted 25 September 1996.