The N-acetylmuramoyl-L-alanine amidase encoded by the *Bacillus subtilis* 168 prophage SPβ

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Heat shock of *Bacillus subtilis* CU1147, a strain lysogenic for SPβc2, a prophage with a thermosensitive repressor, results in phage induction and subsequent cell lysis. Cloning in *Escherichia coli* and sequencing of a DNA fragment of prophage SPβ led to the identification of blyA, the gene encoding a 367 amino acid polypeptide with a molecular mass of 39.6 kDa. Purified BlyA obtained from the *E. coli* clone exhibited an N-acetylmuramoyl-L-alanine amidase activity. Insertional mutagenesis confirmed that the latter enzyme was associated with SPβ-phage-mediated cell lysis. Analysis of the neighbouring sequence suggested that the two ORFs immediately downstream of blyA and belonging to the same operon encode polypeptides which may be involved in the release of the endolysin. The presence on the chromosomes of *B. subtilis* or related *Bacillus* spp. of other, similar genes, and their possible relationship, is discussed.

**Keywords:** *Bacillus subtilis* phage, prophage SPβ, N-acetylmuramoyl-L-alanine amidase, endolysin, holin

**INTRODUCTION**

*Bacillus* strains have been found to be lysogenic for one or several bacteriophages (Zahler, 1993). Defective PBSX-like phages, as well as the large SPβ-type phages, appear to be ubiquitous among *Bacillus subtilis* isolates. Attempts to cure *B. subtilis* 168 of PBSX-type phages have been unsuccessful or inconclusive (Buxton, 1980), yielding derivatives affected in growth which frequently undergo massive spontaneous cell lysis (C. Mauel, unpublished). The SPβ-type phages also seem to be closely related to their host. Indeed, certain chromosomal regions of the latter exhibit a high sequence homology to segments of the SPβ genome (Zahler, 1993; A. Regamey, unpublished), a situation already described for the λ-*Escherichia coli* system (Campbell, 1996).

Treatment with inducing agents such as mitomycin C leads to induction of defective PBSX-type phages (Okamoto *et al*., 1968), leaving apparently little room for the induction or the development of SPβ (Zahler, 1982). However, isolation of the SPβc2 mutant, with a thermosensitive repressor, has allowed massive SPβ induction by a heat shock (Rosenthal *et al*., 1979). Development of PBSX (Longchamp *et al*., 1994) was shown to be accompanied by the synthesis of autolytic enzymes with molecular masses of 32 and 34 kDa, whereas a 43 kDa lytic enzyme was associated with the induction of prophage SPβ (Foster, 1993).

In the present contribution, we report the sequencing of the prophage SPβ fragment forming the junction between the chromosomal segments C and D of *B. subtilis* 168 (Anagnostopoulos, 1990; A. Regamey, unpublished). The sequencing of this fragment revealed, among others, three ORFs which encode an N-acetylmuramoyl-L-alanine amidase and two holin-like proteins. The purification and characterization of the endolysin are described. Specific aspects of the phage amidase, the genomic environment of the corresponding gene and its comparison to other similar genes are discussed.

**METHODS**

**Bacterial strains and plasmids.** These are listed in Table 1.

**Media and growth conditions.** *E. coli* and *B. subtilis* strains were grown in LB, and LB or SA media (Karamata & Gross, 1970), respectively. When required, the media were supple-
Table 1. Bacterial strains, phage and plasmids

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Genotype or description</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5</td>
<td>F- endA1 hsdR17(r-m-) supE44</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td></td>
<td>thi-1 Δ recA1 gyrA96 relA1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔargF-lacZYA)U169 φ80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lacZAM15</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F- ompT hsdS rII3(m~) gal dcm</td>
<td>Novagen</td>
</tr>
<tr>
<td></td>
<td>(DE3)</td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CU1147</td>
<td>trpC2 (SPPc2)</td>
<td>Rosenthal et al. (1979)</td>
</tr>
<tr>
<td>CU1147 6lyA</td>
<td>trpC2 (SPPc2::pDIA-C/D'-H3)</td>
<td>This work; insertion of plasmid pDIA-C/D'-H3 into CU1147</td>
</tr>
<tr>
<td>QB928</td>
<td>aro906 purB33 dal-1 trpC2</td>
<td>Dedonder et al. (1977)</td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Burkholder &amp; Giles (1947)</td>
</tr>
<tr>
<td><strong>Phage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPPc2</td>
<td>Heat-inducible mutant of SPPβ</td>
<td>Rosenthal et al. (1979)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDIA 5304</td>
<td>Ap' Cm'</td>
<td>Glaser et al. (1993)</td>
</tr>
<tr>
<td>pET-16b</td>
<td>Ap'</td>
<td>Novagen</td>
</tr>
<tr>
<td>pDIA-C/D</td>
<td>2644 bp HindIII-XbaI insert of SPPβ prophage in pDIA 5304</td>
<td>This work*</td>
</tr>
<tr>
<td>pDIA-C/D'</td>
<td>1748 bp XbaI-ClaI insert of SPPβ prophage in pDIA 5304</td>
<td>This work*</td>
</tr>
<tr>
<td>pDIA-C/D'-H3</td>
<td>428 bp HindIII fragment of pDIA-C/D' in pDIA 5304</td>
<td>This work</td>
</tr>
</tbody>
</table>

* The inserts of pDIA-C/D and pDIA-C/D' have an overlap of 767 bp (HindIII-ClaI fragment). The combined sequence corresponds to a 3625 bp XbaI-XbaI SPP prophage fragment.

mented with either 50 μg ampicillin ml⁻¹ or 5–20 μg chloramphenicol ml⁻¹. Cultures, aerated by bubbling or shaking, were grown at 37 °C. Cell concentration was followed by nephelometric density (EEL nephelometer, Diffusion Systems) or OD₉₀₀ (Pharmacia Novaspec II spectrophotometer).

**Transformation.** Transformation of B. subtilis was performed according to Karamata & Gross (1970), with about 1 μg transforming DNA ml⁻¹. Plasmids were introduced into E. coli DH5 by the procedure of Chung et al. (1989). Transformants containing an integrated plasmid with a Cm' marker were selected on LB agar supplemented with 5 μg chloramphenicol ml⁻¹. They were purified by successive transfer onto plates containing 10, 15 and 20 μg chloramphenicol ml⁻¹.

**DNA manipulations.** Restriction endonucleases, T4 DNA ligase and T7 DNA polymerase (Biofina or Pharmacia) were used according to the supplier’s instructions. For subcloning, DNA fragments were recovered from agarose gels by centrifugation through glass wool (Heery et al., 1990). Plasmid DNA isolation was according to Del Sal et al. (1988). Chromosomal mini-preparations of B. subtilis DNA were according to Noirot et al. (1987).

**DNA sequencing.** Both strands were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) with the T7 Sequencing kit (Biofina) according to the supplier’s instructions. 5'-[α-³²P]Deoxythioadenosine triphosphate (> 1000 Ci mmol⁻¹, > 37 TBq mmol⁻¹) was from Amersham. Oligonucleotides were synthesized with an Oligo 1000 DNA synthesizer (Beckman). Sequagel-6 (National Diagnostics) was used for the sequencing gels.

**Sequence analysis.** Analysis of the DNA sequence was performed with the GCG software package (Genetics Computer Group, University of Wisconsin, USA). Alignment of sequences was made using the GAP program (Needleman & Wunsch, 1970). Comparisons of nucleotide and amino acid sequences to databases were performed with the Basic Local Alignment Search Tool (BLAST) software (Altschul et al., 1990). Determination of the free energy of any postulated ribosome-binding sites (RBS) was achieved with the Oligo 4.01 program (Primer Analysis software, National Biosciences).

**Cloning and expression of the SPPβ endolysin.** The 1101 bp endolysin gene was PCR-amplified on strain 168 DNA with the GeneAmp XL PCR kit (Perkin Elmer Cetus). The upstream (5'-AGATAATACATATGTCAGTTTTCACTAATAGCTAC 3') and downstream (5'-ATTCGGATCCTTAGATTGTTGATCCAACTGTC 3') primers were defined according to the endolysin gene sequence. Upstream and downstream primers included a 5' NdeI and BamHI site (underlined), respectively. After digestion, the PCR product was cloned in E. coli DH5 using the pET-16b vector (Novagen). The target gene, under the control of a bacteriophage T7 transcription signal, was induced by providing a source of T7 RNA polymerase in the host cell, i.e. target genes, initially cloned in
a non-expression host, were transferred into the expression host containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control. The culture was induced at an OD600 of 0.6 by addition of 1 mM IPTG, while a stretch of ten consecutive histidine residues was added to the N terminus of the target protein, allowing rapid affinity purification on a His-Blue metal chelation resin (Novagen). The protein solution was concentrated by ultrafiltration through an anisotropic membrane (Centricon-10, Amicon), and used directly for gel electrophoresis or enzyme assays. The expression of target genes was assessed by analysis of total cell protein by SDS-PAGE.

Preparation of crude lysates for SDS-PAGE. Crude lysates were prepared essentially according to Longchamp et al. (1994). An overnight culture of strains CU1147 or CU1147 blyA was resuspended in LB medium supplemented, when necessary, with 20 μg chloramphenicol ml⁻¹. The prophase was induced at an OD₆0₀ of 0.3–0.4 by addition of 1/3 vol. boiling LB medium (Rosenthal et al., 1979). The culture, held at 37 °C for 6 min, was further incubated at 37 °C so as to allow phase multiplication. The extent of lysis, generally complete at about 40 min, depended on the turbidity of the culture at the time of shift. After 20 min at 37 °C, a sample withdrawn from the culture was added to 2.5 sample vols ice-cold 0.02 M NaN₃, centrifuged, resuspended in 1/20 sample vol. 0.02 M NaN₃ containing 0.5 mg lysozyme ml⁻¹, and incubated for 30 min at 37 °C. Upon lysis, double-strength sample buffer was added and the sample submitted to SDS-PAGE. Non-induced control cultures, grown in parallel, were prepared.

Preparation of cell walls. Cell walls were obtained according to Studer & Karamata (1988). Cells, grown in SA medium at 37 °C to a nephelometric density of 100 (~ 8 x 10⁷ cells ml⁻¹) were collected by centrifugation, washed with ice-cold distilled water and disrupted by a French press (138 MPa; SLM Instruments). Walls were deproteinized by boiling for 15 min in 2% SDS, and further purified by differential centrifugation (Studer & Karamata, 1988). Finally, they were resuspended in water, frozen and lyophilized. ¹⁴C-l-alanine-labelled cell walls of QB928 were used as substrate for the amidase assay (Margot et al., 1991).

Lytic enzyme activity in SDS-PAGE under renaturing conditions. SDS-PAGE was performed essentially as described by Maue & Karamata (1984). Cell walls were incorporated at a concentration of 0.05% into 11% polyacrylamide gels. Proteins separated by electrophoresis were renatured with 0.1% Triton X-100, 10 mM MgCl₂ and 25 mM Tris/HCl (pH 7.5) for 16 h at 37 °C (Foster, 1992). Following clearing of the cell wall substrate revealing bands endowed with peptidoglycan hydrolase activities, gels were stained with 0.1% methylene blue, and photographed. Subsequently, they were fixed and stained with Coomassie blue.

N-Acetylthiramoyl-l-alanine amidase assay. The lysis and the amidase assay were according to Margot et al. (1991). ¹⁴C-l-alanine-labelled cell walls were added at a concentration of 1.1 mg ml⁻¹ to 2.5 ml of the enzyme solution and incubated at 37 °C. The affinity of the amidase for the cell wall was assessed according to Studer (1988). Walls in enzyme solution were incubated for 10 min at 0 °C and collected by centrifugation (48000 g, 10 min, 4 °C). Proteins of the supernatant were precipitated with 5% TCA and 50 μg yeast RNA ml⁻¹. Pelleted walls were washed with 0.9% NaCl in ice-cold double-distilled water, and twice with water. Finally, they were resuspended in water, frozen and lyophilized. Walls were resuspended in the sample buffer, boiled and centrifuged. The supernatant was collected, the extraction repeated once and the pooled samples were analysed by SDS-PAGE.

RESULTS

Nucleotide sequence of a segment of prophage SPβ

Analysis of B. subtilis trpE26 and 168 strains (for a review, see Anagnostopoulos, 1990) led to the isolation of a B. subtilis 168 chromosomal fragment which was shown by Southern hybridization to be part of the SPβ prophage genome (Table 1; A. Regamey, unpublished). Both DNA strands of this 3625 bp fragment were sequenced using synthesized primers and five ORFs were defined (Fig. 1). Two of them – orf5, an incomplete gene and orf4 – were transcribed in the direction of DNA replication, while genes blyA, bhlA and bhlB were transcribed in the opposite direction. Absence of intergenic space sufficient to accomodate transcription signals suggested that blyA, bhlA and bhlB belong to the same operon, a conclusion confirmed by sequencing and analysis of the whole SPβ genome (V. Lazarevic, unpublished). General properties of completely sequenced genes are summarized in Table 2. Their G + C content is close to 34 mol %, the mean G + C content of the SPβ genome (V. Lazarevic, unpublished). A potential terminator between bhlB and the convergently transcribed orf4 (Fig. 1), with an estimated free energy of -11.0 kcal (-46 kJ) mol⁻¹ (Freier et al., 1986), could form a stem–loop structure and simultaneously stop the transcription of operons containing orfs 4 and 5, and genes blyA, bhlA and bhlB, respectively.

Proteins encoded by genes blyA, bhlA and bhlB are likely to be involved in SPβ-mediated cell lysis

The deduced amino acid sequence of blyA exhibits more or less extensive homologies to a family of amidases encoded by several Bacillus strains, as well as to most, if not all, of those encoded by Bacillus phages (Table 3). Homologies are essentially confined to the N-terminal moiety of BlyA where four domains (positions 7–47, 53–79, 93–138 and 144–213) are well-conserved and associated with the export process of the BCNS toxin (von Heijne, 1986). The deduced amino acid sequences of bhlA and uviB have near identical hydropathy profiles (Kyte & Doolittle, 1982), with a hydrophobic N-terminal and a C-terminal moiety rich in charged amino acids (not presented). BhlA has a "dual start" motif with two potential Met starts separated by
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![Genetic map of the 3625 bp XbaI SPβ prophage fragment cloned into pDIA-CD and pDIA-CD'. Arrows correspond to putative ORFs oriented in the direction of transcription. All ORFs, except orf5, are complete. The putative terminator (+) common to the convergently transcribed units, consisting of blyA, bhlA and bhlB, and orfs 4 and 5, respectively, is indicated. Subcloning of the 428 bp HindIII fragment of blyA into integrational vector pDIA-5304 yielded pDIA-C/D'-H3. Restriction sites: X, XbaI; H, HindIII; C, ClaI.]

**Table 2.** Features of the sequenced ORFs and their deduced products

<table>
<thead>
<tr>
<th>Feature</th>
<th>ORF:</th>
<th>blyA</th>
<th>bhlA</th>
<th>bhlB</th>
<th>orf5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (bp)</td>
<td>1101</td>
<td>210</td>
<td>264</td>
<td>444</td>
<td></td>
</tr>
<tr>
<td>Start codon</td>
<td>AUG</td>
<td>AUG</td>
<td>AUG</td>
<td>AUG</td>
<td></td>
</tr>
<tr>
<td>Stop codon</td>
<td>UAA</td>
<td>UAG</td>
<td>UAG</td>
<td>UGA</td>
<td></td>
</tr>
<tr>
<td>ΔG of RBS (kcal mol⁻¹)*</td>
<td>-10.9</td>
<td>-15.5</td>
<td>-13.9</td>
<td>-2.8</td>
<td></td>
</tr>
<tr>
<td>G + C content (mol%)</td>
<td>38</td>
<td>33</td>
<td>33</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Length (amino acids)</td>
<td>367</td>
<td>70</td>
<td>88</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
<td>39.6</td>
<td>8.4</td>
<td>10.1</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>10.4</td>
<td>4.8</td>
<td>10.1</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated with the Oligo 4.01 program (Primer Analysis software, National Biosciences). 1 kcal = 4.184 kJ.

A glutamic acid, a feature characteristic of several holin genes (Young & Blasi, 1995). The 88 amino acid polypeptide encoded by bhlB is nearly of the same size as holin-like proteins such as XhlB (Lee et al., 1991; Longchamp et al., 1994) with which it shows 56% identity. In addition, the bhlB product is highly similar to those of *B. licheniformis* genes orf2 (Oda et al., 1993) and orfL2 (Lee et al., 1991), exhibiting identities of 51% and 49%, respectively. All of the above-listed proteins have near identical hydropathy profiles: a hydrophobic N-terminal region is separated from a second hydrophobic domain by a short hydrophilic one, which in turn is followed by a charge-rich hydrophilic C-terminus. They all have a basic isoelectric point (not presented).

Comparison of the nucleotide and deduced amino acid sequences of orf4 and orf5 with data in the public databases did not reveal any significant homology (not presented).

**blyA encodes a lytic enzyme associated with SPβ-induced lysis**

Lytic enzyme activities accompanying SPβ induction were identified on strain CU1147 which, due to its heat-sensitive SPβ repressor, can be induced by a heat shock

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**Table 3.** Features of BlyA homologous proteins and percentage of identity and similarity between them

<table>
<thead>
<tr>
<th>Protein</th>
<th>Strain</th>
<th>Enzyme activity</th>
<th>Molecular mass (kDa)</th>
<th>Overall identity/similarity with BlyA (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CwIL</td>
<td><em>B. licheniformis</em></td>
<td>Amidase</td>
<td>39</td>
<td>38/52</td>
<td>Oda et al. (1993)</td>
</tr>
<tr>
<td>OrfL3</td>
<td><em>B. licheniformis</em></td>
<td>Probable amidase</td>
<td>38.3</td>
<td>36/53</td>
<td>Lee et al. (1991)</td>
</tr>
<tr>
<td>Ply21</td>
<td><em>B. cereus</em></td>
<td>Amidase</td>
<td>29.5</td>
<td>29/44</td>
<td>Loessner et al. (1997)</td>
</tr>
<tr>
<td>CwIA</td>
<td><em>B. subtilis</em></td>
<td>Amidase</td>
<td>30</td>
<td>28/45</td>
<td>Kuroda &amp; Sekiguchi (1990); Foster (1991)</td>
</tr>
<tr>
<td>YqgE</td>
<td><em>B. subtilis</em></td>
<td>Probable amidase</td>
<td>27.6</td>
<td>26/46</td>
<td>Mizuno et al. (1996)</td>
</tr>
<tr>
<td>XlyA</td>
<td><em>B. subtilis</em></td>
<td>Amidase</td>
<td>31.9</td>
<td>26/45</td>
<td>Longchamp et al. (1994)</td>
</tr>
<tr>
<td>CwISP</td>
<td>Bacillus sp.</td>
<td>Amidase</td>
<td>28.5</td>
<td>26/43</td>
<td>Potvin et al. (1988)</td>
</tr>
</tbody>
</table>
Sequencing of the SPβ amidase gene

Fig. 2. Growth (OD₆₀₀) of B. subtilis CU1147 with (■) or without (□) heat-shock treatment, as well as that of heat-shocked CU1147 blyA (●), in which the SPβ N-acetylmuramoyl-L-alanine amidase gene blyA was disrupted with integrational plasmid pDIA-C/D'-H3. Samples taken at the beginning of lysis, i.e., 20 min after the end of the temperature shock, were analysed by zymograms.

Fig. 3. Identification of lytic activities associated with heat induction of prophage SPβc2 in strains CU1147 and CU1147 blyA. Twenty minutes after the temperature shock, 1 ml samples were harvested for the preparation of crude lysates. They were analysed by SDS-PAGE on 11% polyacrylamide gels containing purified, SDS-deproteinized, B. subtilis 168 cell walls as substrate for lytic enzymes. Upon protein renaturation and staining with 0.1% methylene blue, clearing zones, corresponding to lytic enzyme bands, were identified. Lanes: 1 and 2, uninduced and induced strain CU1147, respectively; 3 and 4, uninduced and induced strain CU1147 blyA, respectively. Major lytic bands are indicated on the right.

with a molecular mass of about 38 kDa was detected in CU1147 blyA lysates.

Cloning and expression of the SPβ endolysin: blyA encodes an N-acetylmuramoyl-L-alanine amidase

blyA was amplified by PCR on strain 168 DNA and cloned in E. coli DH5 using the pET-16b vector. The recombinant plasmid was introduced into E. coli BL21(DE3) and expression induced by addition of IPTG. The target protein was purified on a His-Bind metal chelation resin and concentrated by ultrafiltration through a membrane (Centricon-10, Amicon).

Total cell proteins of the induced and uninduced expression host, as well as the purified target protein, were examined by SDS-PAGE (Fig. 4). A lytic enzyme with an apparent molecular mass of 42 kDa was present in the lysate of the induced host and in the purified sample but absent from the uninduced host. The presence of ten histidine residues at the N-terminal extremity of the cloned endolysin accounted for its apparently increased molecular mass.

Sequence homologies, suggesting that the polypeptide encoded by blyA was an N-acetylmuramoyl-L-alanine amidase, were confirmed by the radioassay of Margot et al. (1991). The reaction mixture consisted of 1-1 mg ¹⁴C-
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**DISCUSSION**

The fragment of the SPβ genome forming the junction between C and D segments of the 168 chromosome (Anagnostopoulos, 1990) contains four complete ORFs. One of the latter, blyA, encodes an N-acetylumuramoylt-alanine amidase involved in SPβ-associated host lysis, and previously described as the SPβ-specific lytic enzyme (Foster, 1993). The downstream-located bhlA and bhlB genes, belonging to the same operon as blyA, encode small peptides similarly to the C. perfringens gene uviB (Garnier & Cole, 1988a, b) and the B. subtilis gene xhlB (Lee et al., 1991; Longchamp et al., 1994). The C. perfringens operon uviAB is followed by gene bcn, which encodes bacteriocin BCN5 (Garnier & Cole, 1988a, b). Because of its predicted primary structure and apparent toxicity, uviB was assumed to play a role in bacteriocin secretion (Garnier & Cole, 1988a). Protein XhlB, encoded by the B. subtilis prophage PBSX (Lee et al., 1991; Longchamp et al., 1994), is believed to correspond to a holin (Longchamp et al., 1994). Therefore, both bhlA and bhlB are likely to be involved in a protein export process, probably required for the targeting of BlyA. Indeed, the combined product BhLA and BhlB could fulfill a holin function, i.e. allow the release of endolysin by generating transmembrane lesions. This observation is in agreement with the fact that BlyA is devoid of a putative signal peptide. Incidentally, the fact that endolysins synthesized without a secretory signal sequence accumulate in the cytoplasm in an active form was very useful for the expression of blyA in E. coli, and the purification of this enzyme from the crude soluble fraction. The holin-endolysin system has been adopted by a majority of bacteriophages of Gram-positive as well as Gram-negative bacteria (Young & Blasi, 1995). However, while in so-far identified phage lysis systems, holin-like genes precede the endolysin gene (Young & Blasi, 1995; Young, 1992; Longchamp et al., 1994; Loesner et al., 1995; Zink et al., 1995; Steiner et al., 1993; Platteeuw & de Vos, 1992), SPβ represents an exception since the putative holin-encoding genes are localized downstream of the endolysin gene (Fig. 5).

The BlyA amidase has a molecular mass of 40 kDa and seems to consist of two domains, a catalytic and a substrate recognition domain, in agreement with the modular organization of many so-far characterized peptidoglycan-hydrolysing enzymes (Lopez et al., 1992; Croux et al., 1993; Ghysen et al., 1994). The N-terminal deduced primary structure of BlyA exhibits extensive homologies with the catalytic domains of a family of Bacillus N-acetylumuramoylt-alanine amidases consisting of: Ply21 of the Bacillus cereus bacteriophage TP21 (Loesner et al., 1997), XlyA of the B. subtilis prophage PBSX (Longchamp et al., 1994), CwlL (Oda et al., 1993), OrfL3 (Lee et al., 1991), CwlA (Kuroda & Sekiguchi, 1990; Foster, 1991) and CwlSP (Potvin et al., 1988). The genes encoding the latter four enzymes were obtained by cloning gene banks in E. coli and selecting colonies exhibiting lytic activity. Therefore, all these BlyA-homologous lytic enzymes may

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**Fig. 4.** SDS-PAGE of a crude protein extract from E. coli BL21(DE3) cells harbouring the blyA expression vector pET-16b and of purified BlyA protein. Lanes 4 and 5 contain total cell proteins corresponding to 50 µl of uninduced and induced E. coli BL21(DE3) cell cultures, respectively. Lane 3, protein standards (Pharmacia), with molecular masses indicated on the right. The gel was stained after electrophoresis with Coomassie brilliant blue.

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L-a-alanine-labelled cell wall of strain QB928 ml⁻¹, to which 2.5 ml of a purified enzyme solution were added so as to achieve complete cell wall hydrolysis in about 25 min. Cell wall degradation was followed at OD₅₅₀. Duplicate samples were withdrawn at regular time intervals and amidase activity was measured as the release of free L-alanine NH₂ groups reacting with fluorodinitrobenzene to yield dinitrophenyl (DNP) alanine (not presented).

The binding of the SPβ-specific amidase to peptidoglycan was assessed by incubating a solution of purified enzyme and cell wall for 10 min at 0 °C. After centrifugation, proteins present in the supernatant and in the pellet were analysed by SDS-PAGE (Fig. 4). The 42 kDa band, corresponding to the SPβ-specific amidase, was present in the pellet, but not detectable in the supernatant. The amidase affinity for the wall was tested by washing the pellet with increasing salt concentrations. Even at 5 M NaCl, the amidase was not released from the cell wall (not presented). To eliminate the possibility of amidase polymerization and co-sedimentation with the cell wall, the enzyme preparation was incubated without the wall. As expected, the amidase did not sediment during centrifugation (not presented).
Sequencing of the SPβ amidase gene

\[
\begin{align*}
\Delta G &= -11 \text{ kcal mol}^{-1} \\
\text{bhlB} & \quad \text{bhlA} & \quad \text{blyA} & \quad \text{SPβ} \\
\text{1 kb} \\
\Delta G &= -22 \text{ kcal mol}^{-1} \\
\text{cwlL} & \quad \text{orf2} & \quad \text{orf1b} & \quad \text{B. licheniformis} \\
\Delta G &= -13 \text{ kcal mol}^{-1} \\
\text{orfA} & \quad \text{orfL3} & \quad \text{orfL2} & \quad \text{orfL1} & \quad \text{orfR7} & \quad \text{B. licheniformis} \\
\Delta G &= -17 \text{ kcal mol}^{-1} \\
\text{orf5} & \quad \text{orf4} & \quad \text{cwlA} & \quad \text{orf2} & \quad \text{orf1} & \quad \text{B. subtilis} \\
\Delta G &= -21 \text{ kcal mol}^{-1} \\
\text{xlyA} & \quad \text{xhIb} & \quad \text{xhlA} & \quad \text{xepA} & \quad \text{PBSX} \\
\Delta G &= -17 \text{ kcal mol}^{-1} \\
\text{Bacillus sp hydrolase} & \quad \text{Bacillus sp} \\
\end{align*}
\]

**Fig. 5.** Comparison of analogous regions of SPβ, PBSX and genomes of B. licheniformis, B. subtilis and Bacillus sp. Different ORFs, their sense of transcription (→), putative promoters (←), and the putative terminators (§) are indicated. Shading (HI, df and m) represents regions of similarity.

Belong to an endolysin family, possibly of phage origin. They all possess a very well-conserved consensus motif SIGVEIC (SIGVEMC at position 104–110 of BlyA), which may correspond to the active site of Bacillus-phage-encoded amidases. However, the consensus motif GSNRY (Lazarevic et al., 1992), common to host amidases present during exponential growth as well as to certain phage amidases, was not found in BlyA.

Although BlyA has a strong affinity for peptidoglycan, its C-terminal moiety, presumably responsible for substrate recognition, neither shares a pronounced homology with any of the so-far sequenced peptidoglycan hydrolases, nor does it contain an obvious repetitive pattern associated with peptidoglycan enzyme binding. For instance, the general consensus motif of a potential peptidoglycan-binding site (Ghuysen et al., 1994) present in the C-terminal non-catalytic domain of CwlL and OrfL3 amidases, otherwise similar to BlyA, is absent from the latter enzyme. Moreover, the consensus motif DGXYGP found in the non-catalytic regions of CwlL and in several cell wall hydrolases (Oda et al., 1993), is not present in BlyA. Nevertheless, some kind of a repetitive motif, which may be required for binding to peptidoglycan, can be recognized in the C-terminal moiety of BlyA: residues 286–295 and 330–339. Interestingly, these segments are located in a threonine-serine rich region (8 out of 10 and 5 out of 10, respectively), which seems to be frequently present in the peptidoglycan-binding domain of cell-wall-bound proteins (Margot et al., 1998).

BlyA is likely to be specifically involved in SPβ-mediated cell lysis. Indeed, thermoinduction of CU1147 blyA, bearing the SPβc2 blyA mutation, does not lead to massive cell lysis despite unimpeded phage development (A. Regamey, unpublished). The presence of an uncharacterized lytic activity of a molecular mass of about 38 kDa and its role, if any, in SPβ development remains unaccounted for. Apparently, there is no SPβ gene encoding such an enzyme (V. Lazarevic, unpublished). This lytic activity, possibly due to the induction of a distant gene under the control of the SPβ repressor or under the control of another thermolabile repressor, does not seem to be able to provoke, on its own, significant cell lysis of the SPβc2 blyA mutant.
ACKNOWLEDGEMENTS

We thank V. Lazarevic for information on the Spβ genome sequence and P. Margot for instruction on relevant methods.

This work was submitted by A. Regamey in partial fulfillment for a PhD degree of the University of Lausanne, Switzerland.

REFERENCES


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Received 1 September 1997; revised 24 November 1997; accepted 28 November 1997.