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

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Heat shock of *Bacillus subtilis* CU1147, a strain lysogenic for SP β c2, 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 SP β 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 SP β -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 SP β , *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 SP β -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1980), yielding derivatives affected in growth which frequently undergo massive spontaneous cell lysis (C. Mauël, unpublished). The SP β -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regions of the latter exhibit a high sequence homology to segments of the SP β genome (Zahler, 1993; A. Regamey, unpublished), a situation already described for the λ -*Escherichia coli* system (Campbell, 1996).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 SP β (Zahler, 1982). However, isolation of the SP β c2 mutant, with a thermosensitive repressor, has allowed massive SP β

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 SP β (Foster, 1993).

In the present contribution, we report the sequencing of the prophage SP β 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-

The GenBank accession number for the sequence reported in this paper is AF021803.

Table 1. Bacterial strains, phage and plasmids

Strain, phage or plasmid	Genotype or description	Origin
<i>Escherichia coli</i>		
DH5	F ⁻ <i>endA1 hsdR17</i> (r ⁻ m ⁺) <i>supE44</i> <i>thi-1 λ⁻ recA1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>)U169 ϕ 80 <i>lacZ</i> ΔM15	Gibco-BRL
BL21(DE3)	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Novagen
<i>Bacillus subtilis</i>		
CU1147	<i>trpC2</i> (SPβC2)	Rosenthal <i>et al.</i> (1979)
CU1147 <i>blyA</i>	<i>trpC2</i> (SPβC2::pDIA-C/D'-H3)	This work; insertion of plasmid pDIA-C/D'-H3 into CU1147
QB928	<i>arol906 purB33 dal-1 trpC2</i>	Dedonder <i>et al.</i> (1977)
168	<i>trpC2</i>	Burkholder & Giles (1947)
Phage		
SPβC2	Heat-inducible mutant of SPβ	Rosenthal <i>et al.</i> (1979)
Plasmids		
pDIA 5304	Ap ^r Cm ^r	Glaser <i>et al.</i> (1993)
pET-16b	Ap ^r	Novagen
pDIA-C/D	2644 bp <i>HindIII</i> - <i>XbaI</i> insert of SPβ prophage in pDIA 5304	This work*
pDIA-C/D'	1748 bp <i>XbaI</i> - <i>Clal</i> insert of SPβ prophage in pDIA 5304	This work*
pDIA-C/D'-H3	428 bp <i>HindIII</i> fragment of pDIA-C/D' in pDIA 5304	This work

* The inserts of pDIA-C/D and pDIA-C/D' have an overlap of 767 bp (*HindIII*-*Clal* fragment). The combined sequence corresponds to a 3625 bp *XbaI*-*XbaI* SPβ 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 OD₆₀₀ (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^r 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 (Biofinex 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 (Biofinex) according to the supplier's instructions. 5'-[α-³⁵S]Deoxythioadenosine triphosphate (>1000 Ci mmol⁻¹, >37 TBq mmol⁻¹) was from Amersham. Oligo-

nucleotides 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 SPβ 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' AGATATACATATGTCAGTTTTCACATAATAGCTAC 3') and downstream (5' ATTCGGATCCTTAGATTGTTGATCCAAACTGTCC 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-Bind 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 μ g chloramphenicol ml⁻¹. The prophage 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 50 °C for 6 min, was further incubated at 37 °C so as to allow phage 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 Na₂N₃, centrifuged, resuspended in 1/20 sample vol. 0.02 M Na₂N₃ 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 ($\sim 8 \times 10^7$ cells ml⁻¹) were collected by centrifugation, washed with ice-cold double-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 Mauël & 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-Acetylmuramoyl-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 μ g 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 SP β

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 SP β 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 accommodate transcription signals suggested that *blyA*, *bhlA* and *bhlB* belong to the same operon, a conclusion confirmed by sequencing and analysis of the whole SP β 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 SP β 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 SP β -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 highly similar to those of *Bacillus licheniformis* peptidoglycan hydrolases CwlL, an amidase, and OrfL3, probably also an amidase. The molecular mass of BlyA (40 kDa) hardly differs from those of CwlL (39 kDa) and OrfL3 (38.3 kDa). The other lytic enzymes exhibiting significant homologies to BlyA (see Discussion) have molecular masses around 30 kDa. Finally, BlyA is devoid of an export signal peptide (von Heijne, 1986).

The 70 amino acid polypeptide encoded by *bhlA* has almost the same size and exhibits 42% identity with the product of *wiB*, a *Clostridium perfringens* gene associated with the export process of the BCN5 toxin (Garnier & Cole, 1988a, b). The deduced amino acid sequences of *bhlA* and *wiB* 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

Fig. 1. Genetic map of the 3625 bp *XbaI* SP β prophage fragment cloned into pDIA-C/D and pDIA-C/D'. Arrows correspond to putative ORFs oriented in the direction of transcription. All ORFs, except *orf5*, are complete. The putative terminator ($\blacktriangleleft$) 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, *Clal*.

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

Feature	ORF:			
	<i>blyA</i>	<i>bhlA</i>	<i>bhlB</i>	<i>orf4</i>
Size (bp)	1101	210	264	444
Start codon	AUG	AUG	AUG	AUG
Stop codon	UAA	UAG	UAG	UGA
ΔG of RBS (kcal mol ⁻¹)*	-10.9	-15.5	-13.9	-2.8
G + C content (mol %)	38	33	33	28
Length (amino acids)	367	70	88	148
Molecular mass (kDa)	39.6	8.4	10.1	17.1
Isoelectric point	10.4	4.8	10.1	9.9

* 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 & Bläsi, 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 hydrophathy 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 SP β -induced lysis**

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

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

Protein	Strain	Enzyme activity	Molecular mass (kDa)	Overall identity/similarity with BlyA (%)	Reference
CwlL	<i>B. licheniformis</i>	Amidase	39	38/52	Oda <i>et al.</i> (1993)
OrfL3	<i>B. licheniformis</i>	Probable amidase	38.3	36/53	Lee <i>et al.</i> (1991)
Ply21	<i>B. cereus</i>	Amidase	29.5	29/44	Loessner <i>et al.</i> (1997)
CwlA	<i>B. subtilis</i>	Amidase	30	28/45	Kuroda & Sekiguchi (1990); Foster (1991)
YqeE	<i>B. subtilis</i>	Probable amidase	27.6	26/46	Mizuno <i>et al.</i> (1996)
XlyA	<i>B. subtilis</i>	Amidase	31.9	26/45	Longchamp <i>et al.</i> (1994)
CwlSP	<i>Bacillus</i> sp.	Amidase	28.5	26/43	Potvin <i>et al.</i> (1988)

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 SP β *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.

(Rosenthal *et al.*, 1979). At the beginning of lysis, i.e. 20 min after the heat shock (Fig. 2), cells were harvested and crude lysates prepared. Samples corresponding to 1 ml culture were processed and analysed by electrophoresis on SDS-PAGE gels containing *B. subtilis* 168 cell walls (Fig. 3). At time 0, zymograms revealed four lytic bands. Two of them, A1 and A2, corresponding to the 90 kDa glucosaminidase (Margot *et al.*, 1994) and the 49 kDa main vegetative amidase (Margot & Karamata, 1992; Lazarevic *et al.*, 1992), respectively, were hardly detectable, while bands A3 and A4, with molecular masses of approximately 35 and 32 kDa, respectively, were clearly visible confirming a previous observation (Foster, 1993). At 20 min, an additional 40 kDa lytic enzyme, previously identified by Foster (1993), was present while band A4 appeared considerably more intense than at time 0.

To confirm that the SP β -specific 40 kDa lytic enzyme is encoded by *blyA*, the latter gene was disrupted with the integrational plasmid pDIA-C/D'-H3, a subclone of pDIA-C/D' containing the 428 bp *Hind*III fragment of *blyA* (Table 1; Fig. 1). Heat shock treatment of CU1147 *blyA* led to growth arrest but not to cell lysis which normally accompanies SP β induction of the parent strain (Fig. 2). Samples of heat-shocked CU1147 and CU1147 *blyA* were processed and examined on a renaturing cell-wall-containing gel (Fig. 3). At time 0, zymograms of both strains were similar, mainly exhibiting lytic bands A3 and A4. However, at 20 min, the SP β -specific 40 kDa lytic band was present in the strain CU1147 lysate, but absent from that of strain CU1147 *blyA*, confirming that *blyA* is the structural gene of the 40 kDa endolysin. Occasionally, a slight activity corresponding to a protein

Fig. 3. Identification of lytic activities associated with heat induction of prophage SP β c2 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 SP β 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-

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 6 contains 1 µl of a tenfold dilution of purified BlyA protein. The affinity of BlyA for the glycan chains was assessed by incubating the purified enzyme with SDS-deproteinized *B. subtilis* 168 cell walls. Following centrifugation, proteins present in the supernatant and in the cell wall pellet were analysed. Lane 1, 20% of proteins precipitated from the supernatant; lane 2, 20% of proteins extracted from cell walls; lane 3, protein standards (Pharmacia), with molecular masses indicated on the right. The gel was stained after electrophoresis with Coomassie brilliant blue.

L-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 OD₄₅₀. 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).

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-acetylmuramoyl-L-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 highly similar 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 fulfil 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 & Bläsi, 1995). However, while in so-far identified phage lysis systems, holin-like genes precede the endolysin gene (Young & Bläsi, 1995; Young, 1992; Longchamp *et al.*, 1994; Loessner *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 (López *et al.*, 1992; Croux *et al.*, 1993; Ghuysen *et al.*, 1994). The N-terminal deduced primary structure of BlyA exhibits extensive homologies with the catalytic domains of a family of *Bacillus* N-acetylmuramoyl-L-alanine amidases consisting of: Ply21 of the *Bacillus cereus* bacteriophage TP21 (Loessner *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

Fig. 5. Comparison of analogous regions of SP β , PBSX and genomes of *B. licheniformis*, *B. subtilis* and *Bacillus* sp. Different ORFs, their sense of transcription ($\leftarrow$), putative promoters ($\leftarrow$), and the putative terminators ($\circ$) are indicated. Shading ($\square$, $\boxtimes$, $\boxdot$ and $\boxminus$) 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 CwIL 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 CwIL 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 SP β -mediated cell lysis. Indeed, thermoinduction of CU1147 *blyA*, bearing the SP β c2 *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 SP β development remains unaccounted for. Apparently, there is no SP β 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 SP β 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 SP β c2 *blyA* mutant.

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