Lytic enzymes associated with defective prophages of *Bacillus subtilis*: sequencing and characterization of the region comprising the *N*-acetylmuramoyl-L-alanine amidase gene of prophage PBSX

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Prophage induction in *Bacillus subtilis* strains 168, S31 and W23 is accompanied by synthesis of two endolysins. The synthesis of those of strain 168, with molecular masses of 32 and 34 kDa, was shown to be controlled by the repressor of the defective phage PBSX. The 32 kDa protein corresponds to an *N*-acetylmuramoyl-L-alanine amidase, and plays the major role in PBSX-mediated lysis. Its structural gene, *xlyA*, is the last in the PBSX late operon, whose four most distal open reading frames have been cloned and sequenced. Analysis of the nucleotide sequence suggests that the two open reading frames preceding *xlyA*, designated *xhIA* and *xhIB*, encode polypeptides whose combined action could play the role of a holin. The open reading frame upstream of *xhIA*, designated *xepA*, encodes an exoprotein. The phage amidase, although endowed with a signal peptide, is apparently, like Xep, exported by a holin-like mechanism which does not involve the cleavage of the signal peptide. The presence on the *B. subtilis* chromosome of other, similar, genes, and their possible widespread occurrence, is discussed.

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

INTRODUCTION

All known isolates of *Bacillus subtilis* and of related spore-forming bacilli, such as *B. natto*, *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens*, are lysogenic for morphologically similar defective bacteriophages, characterized by contractile tails with fibres (Ionesco *et al.*, 1964; Tsutsumi *et al.*, 1990; Huang & Marmur, 1970; Steensma *et al.*, 1978). Their treatment with inducing agents leads to phage development and cell lysis (Okamoto *et al.*, 1968). The liberated phage particles kill susceptible hosts, while being apparently unable to infect and either lysogenize or give rise to a lytic cycle (Okamoto *et al.*, 1968). Efforts to cure cells of these prophages have remained unsuccessful and inconclusive (Buxton, 1980; D. Karamata, unpublished), leaving open the possibility that a function essential to cell economy is under phage control. The

ubiquity of these prophages and their extensive morphological and physiological similarities are compatible with their having a common ancestor, possibly as ancient as that of the spore-forming Firmicuta.

The most studied of these phages are PBSX, harboured by *B. subtilis* 168 (Ionesco *et al.*, 1964; Seaman *et al.*, 1964), and, to a lesser extent, PBSZ and PBSY, found in strains W23 and S31 (Okamoto *et al.*, 1968). The isolation and characterization of PBSX mutants (Siegel & Marmur, 1969; Thurm & Garro, 1975b), as well as studies of phage development (Thurm & Garro, 1975a; Mauël & Karamata, 1984), were followed, more recently, by the sequencing of parts of the prophage genome and the identification of an early and a late prophage operon (Wood *et al.*, 1990a, b).

We report here the identification of PBSX-associated endolysins, and the sequencing of the prophage region consisting of four open reading frames (ORFs), which encode the *N*-acetylmuramoyl-L-alanine amidase, two components of a possible holin-like protein, and an exoprotein. Specific aspects of the phage amidase and the

Abbreviations: Cm, chloramphenicol; MMC, mitomycin C; ND, nephelometric density; rbs, ribosome-binding site.

The GenBank accession number for the nucleotide sequence reported in this paper is L25924.

Table 1. Bacterial strains, phages and plasmids

Genotype or description		Origin
Escherichia coli		
P ₂ 392	F ⁻ <i>hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55P2</i> lysogen	Genofit (Geneva, Switzerland)
DH5	F ⁻ <i>endA1 hsdR17(r⁻m⁺) supE44 thi-1 λ-recA1 Δ(argF-lacZYA)U169 φ80 lacZΔM15</i>	Gibco BRL
Bacillus subtilis		
W23	Prototroph, Sm ^R	Farmer & Rothman (1965)
S31	Prototroph	Ionesco <i>et al.</i> (1964)
168 strains		
168	<i>trpC2</i>	Burkholder & Giles (1947)
L 4590	<i>trpC2, PBSX::p517.5</i>	This work; insertion of plasmid p517.5 in 168 <i>trpC2</i>
QB 928	<i>aroI 906 purB33 dal-1 trpC2</i>	Dedonder <i>et al.</i> (1977)
Phages		
λ C	Clone from PBSX prophage	Wood <i>et al.</i> (1990a)
λ 517	Clone from PBSX prophage	P. Longchamp, University of Lausanne
Plasmids		
pMTL20EC	Ap ^R Cm ^R Em ^R	Chambers <i>et al.</i> (1988)
pMTL21	Ap ^R	Oultram <i>et al.</i> (1988)
pWD1	Cm ^R	H. Wood, Trinity College Dublin
pLP1C	Ap ^R Cm ^R	1.1 kbp <i>EcoRI</i> fragment containing the Cm ^R gene from pWD1 into the <i>EcoRV</i> site of pMTL21
pC2	Ap ^R Cm ^R Em ^R	<i>SacI</i> - <i>SalI</i> fragment C2 (Fig. 2) in pMTL20EC
p517.9	Ap ^R Cm ^R	<i>SacI</i> - <i>BamHI</i> fragment 517.9 (Fig. 2) in pLP1C
p517.5	Ap ^R Cm ^R	<i>SacI</i> - <i>PstI</i> fragment 517.5 (Fig. 2) in pLP1C
p517.1	Ap ^R Cm ^R Em ^R	<i>SacI</i> - <i>SphI</i> fragment 517.1 (Fig. 2) in pMTL20EC
p517.13	Ap ^R Cm ^R Em ^R	<i>SacI</i> - <i>SalI</i> fragment 517.13 (Fig. 2) in pMTL20EC
p517.2	Ap ^R Cm ^R	<i>SacI</i> - <i>SacI</i> fragment 517.2 (Fig. 2) in pLP1C
p517.6	Ap ^R Cm ^R	<i>PstI</i> - <i>PstI</i> fragment 517.6 (Fig. 2) in pLP1C
p517.8	Ap ^R Cm ^R	<i>SacI</i> - <i>PstI</i> fragment 517.8 (Fig. 2) in pLP1C

widespread occurrence of regions similar to that encompassing the amidase are discussed.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. *B. subtilis* clones in λ EMBL3 (Frischauf *et al.*, 1983), [λ C (Wood *et al.*, 1990a) and λ 517 (P. F. Longchamp, unpublished; see Fig. 2)], were subcloned into vectors pMTL20EC (Chambers *et al.*, 1988), pMTL21 (Oultram *et al.*, 1988) or pLP1C (Table 1).

Media and growth conditions. *B. subtilis* strains were grown in SA, SAT or TS medium (Karamata & Gross, 1970). When required, up to 20 µg chloramphenicol ml⁻¹ was added. Cultures, aerated by bubbling, were grown at 37 °C. The cell concentration was followed using an EEL nephelometer (Diffusion system, London, UK). A nephelometric density (ND) of 100 corresponds to 8 × 10⁷ cells ml⁻¹. *E. coli* DH5 was grown in L medium supplemented, when necessary, by 50 µg ampicillin ml⁻¹.

Transformation. Transformation of *B. subtilis* was performed according to Karamata & Gross (1970), with linear bacterial or

circular plasmid DNA (each 1 µg ml⁻¹). Competent cells were kept frozen (Cutting & Vander Horn, 1990). 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 LA plates 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, T4 DNA polymerase, calf intestinal alkaline phosphatase and Klenow polymerase were obtained from Biofinex (Switzerland), and used according to the supplier's instructions. DNA fragments for subcloning or preparation of probes were recovered from agarose gels by centrifugation through siliconized glass wool (Heery *et al.*, 1990). Plasmid DNA was isolated from *E. coli* by the boiling method followed by cetyltrimethylammonium bromide precipitation (Del Sal *et al.*, 1988). Chromosomal minipreparations of *B. subtilis* DNA were obtained by phenol-extraction from lysozyme- and SDS-treated cells (Noirot *et al.*, 1987). Minipreps of phage λ DNA were obtained by the method of Grossberger (1987).

DNA sequencing. Sets of deleted derivatives of relevant subclones introduced into the pUC-related vectors pMTL20EC,

pMTL21 and pLP1C (Chambers *et al.*, 1988; Oultram *et al.*, 1988; Table 1) were obtained using the double-strand Nested Deletion Kit (Pharmacia). DNA sequencing was performed on both strands by the dideoxy-chain termination method (Sanger *et al.*, 1977) with the Sequenase version 2.0 kit (USB), according to the supplier's instructions. 5'-[α - 35 S]Deoxythioadenosine-triphosphate (> 1000 Ci mmol $^{-1}$, > 37 TBq mmol $^{-1}$) was obtained from Amersham. M13 primer 17mer (−20) and M13 reverse primer 16mer (−24) were supplied by Biofinex. Oligonucleotides were synthesized with an SM oligonucleotide synthesizer (Beckman). Sequencing gels were prepared using Sequagel-6 from National Diagnostics. The sequence was compiled and analysed by the University of Wisconsin Computer Group Software (Devereux *et al.*, 1984).

Hybridization procedures. For Southern hybridization (Southern, 1975), size-fractionated DNA samples were transferred from agarose to Hybond-N nylon membranes (Amersham) by alkali blotting (Reed & Mann, 1985). Probes were prepared either by the random priming labelling method (Feinberg & Vogelstein, 1983) using [α - 32 P]dNTP (3000 Ci mmol $^{-1}$) from Amersham or with the Dig-DNA labelling and detection kit from Boehringer Mannheim.

Preparation of crude lysates for SDS-PAGE. An overnight culture was resuspended in SAT medium supplemented, when necessary, with 20 μ g chloramphenicol ml $^{-1}$. At an ND of about 30, the culture was induced by addition of mitomycin C [MMC; 2 μ g ml $^{-1}$ (final concn)]. After 12 min, cells were harvested by filtration (0.45 μ m membrane filter, Millipore), washed and resuspended in fresh SAT medium. Induction of the strain carrying an *xbi* mutation (thermosensitive PBSX repressor) was achieved by 15 min incubation at 48 °C. At 50 min after either MMC addition or temperature shift (ND = 70), a 0.4 ml sample was withdrawn from the culture and added to 1 ml of ice-cold 0.02 M NaN $_3$, centrifuged, resuspended in 30 μ l 0.02 M NaN $_3$, and incubated for 30 min at 37 °C. As stated in the Results, lysis occurred through the action of induced phage lytic enzymes or added lysozyme (1 mg ml $^{-1}$). Upon lysis, 30 μ l of double-strength sample buffer was added and the samples were analysed by SDS-PAGE. Non-induced control cultures, grown in parallel, were treated with lysozyme.

Pulse labelling of proteins in MMC-induced cultures. Labelling of proteins with 14 C-amino acids was performed in TS medium, according to the protocol of Mauël & Karamata (1984).

Preparation of cell walls. Cell walls were obtained according to Studer & Karamata (1988). Cells, grown in SA medium at 37 °C to an ND of 100, were collected by centrifugation, washed with cold double-distilled water, disrupted by the French press (SLM Instruments), and centrifuged (15000 r.p.m. for 15 min, Sorvall SS34-rotor). The pellet, deproteinized by boiling three times in 3% (w/v) SDS, was washed three times in boiling water to remove the SDS. Walls thus obtained were resuspended in water, frozen and lyophilized. Cell walls used as substrate for the amidase assay (see below) were labelled by 14 C-L-alanine incorporation into *B. subtilis* strain QB 928, essentially according to 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 0.1% into 12% gels. Proteins separated by electrophoresis were renatured with 0.1% Triton X-100, 10 mM MgCl $_2$ and 25 mM Tris/HCl (pH 7.5) for 16 h at 37 °C (Foster, 1992). Following clearing of the cell wall substrate, achieved by lytic activities, gels were fixed, stained with 0.1% methylene

blue, and photographed. Subsequently, they were stained with Coomassie blue and processed for autoradiography. Hyperfilm-MP (Amersham) was used; exposure time was 1–2 d.

N-Acetylmuramoyl-L-alanine amidase assay. Crude extracts were prepared as described above. Fifty minutes after MMC induction, 4 ml of the culture was withdrawn and passed through a French press. Disrupted cells were centrifuged (15000 r.p.m. for 15 min, Sorvall SS34-rotor at 4 °C). The supernatant was filtered (0.45 μ m membrane filter, Millipore) and used as enzyme stock. For the reaction, 2.6 mg of 14 C-L-alanine-labelled cell walls, at a concentration of 1.1 mg ml $^{-1}$, were added to 2.5 ml crude extract and incubated at 37 °C. The lysis and the amidase assay were according to Margot *et al.* (1991).

RESULTS

Lytic enzymes associated with the induction of defective prophages of *Bacillus subtilis*

To determine the lytic activities which accompany prophage induction, cultures of *B. subtilis* strains 168 (PBSX) $^{+}$, W23 (PBSZ) $^{+}$ and S31 (PBSY) $^{+}$, in early

Fig. 1. Identification of lytic activities induced by MMC treatment of *B. subtilis* strains S31, W23 and 168. Crude extracts from control and MMC-induced cultures were analysed by SDS electrophoresis on 12% polyacrylamide gels containing purified SDS-deproteinized *B. subtilis* 168 cell walls, as substrate for the lytic enzymes. Upon protein renaturation and staining with 0.1% methylene blue, clearing zones, corresponding to lytic enzyme bands, were identified. Samples were taken 50 min after removal of MMC. Control samples were from non-induced cultures at a comparable ND. Lysis conditions are specified below. The positions and molecular masses of protein standards, separated on the same gel, are indicated. Lanes 1, 4 and 7: non-induced cultures of strains S31, W23 and 168, respectively. Lanes 3, 6 and 9: MMC-induced cultures of strains S31, W23 and 168. Lanes 2, 5, and 8: MMC-induced and lysozyme-treated cultures of strains S31, W23 and 168, respectively.

Fig. 2. Physical map of the region encompassing *xlyA*, the structural gene of the PBSX *N*-acetylmuramoyl-L-alanine amidase. Relevant fragments of λ clones 517 and C were subcloned into integrational vectors pLP1C and pMTL20EC, and introduced by transformation into *B. subtilis* 168. Selected Cm^r recombinants, carrying insertions of plasmids pC2, p517.9, p517.1 and p517.13 (□) were treated with MMC. Induced cells were radioactively labelled with a ¹⁴C-labelled amino acid cocktail, and separated on a 12% SDS gel containing deproteinized cell walls of *B. subtilis* 168. The phage protein pattern (Fig. 3) and the lytic activities (Fig. 4) were analysed to determine the presence (+) or absence (—) of PX34, PX32 and PX31. The map positions of PX32 (*xlyA*) and PX31 (*xepA*) are deduced from (i) the positions of the inserts, (ii) the presence or the absence of endolysin bands and those of other phage proteins and (iii) the known direction of transcription of the 18 kb PBSX late operon (Wood *et al.*, 1990a). The position of *xre*, the PBSX repressor gene, as well as that of the putative terminator (◊) of the 18 kb transcription unit (←) are indicated. The nucleotide sequencing strategy of the chromosomal DNA region containing *xlyA* is also shown; the arrows indicate the origin, the extent, and the direction of sequence determination, as well as the nature of the primer used, i.e. M13 (→) or synthetic (←). Restriction sites are denoted: B, *Bam*HI; E, *Eco*RI; Sa, *Sal*I; S, *Sac*I; P, *Pst*I; Sp, *Sph*I.

exponential growth phase (ND = 30), were treated for 12 min with 2 μ g MMC ml⁻¹, a treatment leading to the development of the defective phages (Mauël & Karamata, 1984). At 50 min, i.e. 10 min before the onset of lysis, 400 μ l samples were processed and analysed by electrophoresis on SDS-polyacrylamide gels containing native *B. subtilis* 168 cell walls (Leclerc & Asselin, 1989; Foster, 1992). After renaturation of the proteins, each of the examined strains showed two bands associated with lytic activity (Fig. 1). The molecular masses of apparently analogous proteins were very similar: 32.5 and 33.5 kDa (PX32 and 34) for strain 168 (Fig. 1, lane 9); 33 and 34 kDa (PZ33 and 34) for W23 (Fig. 1, lane 6); and 32 and 33 kDa (PY32 and 33) for S31 (Fig. 1, lane 3). The very weak bands corresponding to endolysins present in non-induced cultures were most likely due to spontaneous prophage induction (Fig. 1, lanes 1, 4 and 7). Treatment of MMC-induced cells with lysozyme did not significantly alter the profile of the autolytic activity on renatured gels (Fig. 1, lanes 8, 5 and 2 for 168, W23 and S31, respectively).

To assess if MMC-induced lytic enzymes are under the control of prophage regulatory elements, we used mutants deficient in *xre*, the structural gene of the PBSX repressor (Wood *et al.*, 1990b). Upon heat-induction, mutants bearing *xhi*, a mutation associated with a heat-labile repressor (Buxton, 1976), exhibited an endolysin SDS-PAGE profile identical to that of MMC-induced strains, while mutation *xin*, associated with a repressor insensitive to MMC or UV induction (Thurm & Garro, 1975b), prevented the appearance of the PX32 and PX34 bands in MMC-treated as well as in non-induced cultures (not shown). The latter observation confirmed that the low endolytic activity in non-induced cultures was due to spontaneous PBSX induction. Thus, the MMC-induced lytic enzymes of strain 168 are related to PBSX, suggesting, by analogy, that those of strains S31 and W23 are also likely to be under prophage control.

Occasionally, the main clearing zones were accompanied by additional, weak, lytic bands. However, the most frequently encountered ones were *xre*-controlled. They

Fig. 3. Proteins synthesized during PBSX development. Samples were withdrawn 50 min after MMC induction, pulsed for 10 min with ^{14}C -labelled amino acids and centrifuged. Upon lysis, enhanced by addition of lysozyme (1 mg ml^{-1}), the pellets were subjected to electrophoresis on a 12% acrylamide gel and autoradiographed for 2 d. The positions of previously identified proteins (Mauël & Karamata, 1984) are indicated. Lane 1: *B. subtilis* 168, non-induced, control. Lanes 2, 3, 4 and 5: MMC-induced cultures of *B. subtilis* 168, and of strains carrying integrated plasmids pC2, p517.1 and p517.9 (see Fig. 2), respectively.

Fig. 4. Lytic activities associated with the induction of *B. subtilis* 168. Strains carrying an integrated plasmid were grown in the presence of chloramphenicol ($20\text{ }\mu\text{g ml}^{-1}$). Samples (0.4 ml), withdrawn 50 min after MMC induction, were processed for SDS-PAGE and loaded on a gel (12% polyacrylamide) containing vegetative *B. subtilis* 168 SDS-treated cell walls (0.1%). Following electrophoresis, proteins were renatured and the gel was stained with 0.1% methylene blue. Positions of relevant proteins are indicated. Lanes 1, 3, 5 and 7: untreated strains; 168 and strains carrying inserted plasmids pC2, p517.1 and p517.13, respectively. Lanes 2, 4, 6 and 8: MMC-induced strains; 168 and strains carrying inserted plasmids pC2, p517.1 and p517.13, respectively.

appeared as satellites to PX34 (not shown), corresponding most likely to degradation products of this rather labile protein (P. F. Longchamp, unpublished).

Physical mapping of the genes encoding PBSX bacteriolysins

To localize the structural genes of PX32 and PX34, the PBSX late transcription unit, shown to be responsible for phage-induced cell lysis (Wood *et al.*, 1990a), was dis-

rupted with integrational plasmids. Relevant inserts from λC (Wood *et al.*, 1990a) and λ517 were subcloned into the pUC derivatives pMTL20EC or pLP1C, yielding plasmids pC2, p517.9, p517.1 and p517.13 (Fig. 2). The latter were used to transform strain 168 with selection for Cm^r recombinants. Integration occurred by recombination in the homologous PBSX prophage region, as confirmed by Southern hybridization (not shown). The Cm^r recombinants carrying insertions were induced by MMC and examined for (i) cell lysis, (ii) the presence of PX32- and PX34-associated lytic activity (Figs 2 and 4), and (iii) the presence of proteins associated with PBSX induction (Mauël & Karamata, 1984) (Fig. 3). In the latter experiments, MMC-induced cells were labelled with a ^{14}C -labelled amino acid cocktail, and analysed by SDS-PAGE and autoradiography.

Insertion of pC2 (Fig. 3, lane 3) led to the disappearance of some of the virion's structural proteins, such as X35, and of those not incorporated into the mature phage particle, such as PX31, 32 and 36, previously denoted P31, 32 and 36, respectively (Mauël & Karamata, 1984). Insertion of p517.1 led to the absence of PX32 only (Fig. 3, lane 4). In both cases, the phage endolysin protein profile was characterized by the absence of PX32 and the presence of PX34 (Fig. 4, lanes 4 and 6). Inactivation of PX32 was associated with very poor lysis of the culture, compared to that obtained with 168 (PBSX) $^+$ strains (Mauël & Karamata, 1984). Insertion of the most distal clone, p517.13, had no effect on cell lysis and phage endolysin protein profile (Fig. 4, lane 8), suggesting that the relevant insert was either overlapping the extremity of the PX32 structural gene or was located downstream of it. Therefore, the latter gene was assigned to a 2 kb segment (Fig. 2), encompassing the left *SacI* site of the fragment inserted into p517.2. This conclusion is supported by the observation that *E. coli* DH5, carrying plasmid p517.2, undergoes spontaneous lysis during the stationary growth phase. None of the insertions affected the presence and the lytic activity of PX34, revealing that its structural gene is not located in the investigated PBSX late operon.

Insertion of p517.9 led to the absence of PX32 and PX31 (Fig. 3, lane 5), revealing that the structural gene of PX31, an exported protein (Mauël & Karamata, 1984), is located, at least in part, between inserts 517.9 and 517.1, i.e. upstream of the structural gene of PX32.

Nucleotide sequence of the PBSX genome region comprising *xlyA*, the gene encoding PX32

The endolysin-encoding region, defined above, was sequenced on both strands according to the strategy outlined in Fig. 2. The sequenced segment (Fig. 5) contains four open reading frames. *orf4*, denoted *xlyA* for PBSX lytic enzyme A (see below), encodes a polypeptide of 297 amino acids with a molecular mass of 32 kDa. It is preceded by a ribosome-binding site (rbs) with an estimated free energy of $-13.2\text{ kcal } (-55.23\text{ kJ})\text{ mol}^{-1}$ (Shine & Dalgarno, 1974; Tinoco *et al.*, 1973), and followed by a potential terminator with an estimated free

Fig. 5. Nucleotide sequence of the region encompassing *xlyA*, the structural gene of the PBSX *N*-acetylmuramoyl-L-alanine amidase. Putative ribosome binding sites (rbs) are underlined. The inverted repeat corresponding to a probable terminator is shown (>>><<<). The deduced amino acid sequence is given above the nucleotide sequence. Asterisks denote stop codons. The main restriction sites and the position of the potential cleavage site (↓) of a putative leader sequence (von Heijne, 1986) are given. The dual translational signal MKM is indicated in italic.

Table 2. Features of the *xepA*, *xhIA*, *xhIB* and *xlyA* genes and their deduced products

Property	PBSX			
	<i>xepA</i>	<i>xhIA</i>	<i>xhIB</i>	<i>xlyA</i>
Size of ORF (bp)	843	267	261	891
Start codon	AUG	AUG	AUG	AUG
Stop codon	UAA	UAA	UAA	UGA
ΔG of RBS* (kJ mol ⁻¹)	-84.94	-75.31	-61.1	-55.23
TA/GC (%)	52/48	52/48	55/45	53/47
Length (amino acids)	281	89	87	297
Molecular mass (kDa)	31	10	9.7	31.9
Isoelectric point	6.9	5.1	9.9	5.6

* Calculated according to Tinoco *et al.* (1973).

energy of -21 kcal (-87.86 kJ) mol⁻¹. The latter, which could form a stem-loop structure followed by a stretch of six thymine residues, probably ends the 18 kb transcription unit identified as the late phage operon (Wood *et al.*, 1990a).

orf 1, *2* and *3* encode polypeptides of 281, 89 and 87 amino acids with molecular masses of 31, 10 and 9.7 kDa, respectively. They are preceded by putative rbs with estimated free energies of -20.3, -18 and -14.6 kcal (-84.94, -75.31 and -61.1 kJ) mol⁻¹, respectively. *orf1* encodes the previously identified exported protein PX31 and was accordingly designated *xepA* for PBSX exported protein. *orf2* and *orf3* were denoted respectively *xhIA* and *xhIB* for PBSX holin-like protein (see below). The general properties of the four sequenced genes are summarized in Table 2.

The sequence of the *PstI*-*PstI* fragment (bp 1204-1627), encompassing *xhIB* and the beginning of *xlyA*, was previously reported by Lee *et al.* (1991). They showed that this fragment complemented the Pho⁻ phenotype of *E. coli* *phoA* strains under conditions inducing the expression of alkaline phosphatase. Therefore, they designated *xhIB* as *xpaB* for X Phosphatase Activity. Our sequence (Fig. 5) and that of Lee *et al.* (1991) differ in the following points: GGCT instead of CGTG at bases 1555-1558; C instead of G at base 1568; and, more significantly, CCGG instead of CCG at bases 1531-1534. The latter discrepancy, possibly due to a sequencing error, introduced an apparent frame-shift mutation which prevented Lee *et al.* from identifying the beginning of the *xlyA* gene.

Deduced amino acid sequences of XlyA, XepA, XhIA and XhIB; search for homologies

Computer analysis of the deduced amino acids sequence of XlyA revealed three domains with homologies to several polypeptides (Figs 6 and 10). The N-terminal

region, encompassing amino acids 53-99, is highly similar to the N-terminal region of CwlA (Kuroda & Sekiguchi, 1990; Foster, 1991), a *B. subtilis* amidase, and to that of the *Bacillus* sp. hydrolase (Potvin *et al.*, 1988) (Fig. 6a). It contains a consensus motif present in N-acetylmuramoyl-L-alanine amidases (Lazarevic *et al.*, 1992), as well as in several muramidases (Garcia *et al.*, 1988, 1990). This motif is, however, absent from the *B. licheniformis* amidases, i.e. CwlL (Oda *et al.*, 1993), and its highly similar gene *orfL3* (Lee *et al.*, 1991) (Fig. 6a: bold symbols). That the site of the amidase activity is confined to the N-terminal region is in agreement with the observation that plasmid p517.2, containing *xlyA* truncated at its C-terminal (amino acids 216 to end), provokes lysis of *E. coli* DH5 in stationary phase. This conclusion is in line with the results establishing that the C-terminal domain is not necessary for the activity of homologous proteins, CwlA (Kuroda & Sekiguchi, 1990) and the *Bacillus* sp. hydrolase (Potvin *et al.*, 1988). The C-terminal of proteins XlyA, CwlA and the product of the *B. licheniformis* *orfL3* (Lee *et al.*, 1991) is another region of high similarity (Figs 6c and 10, amino acids 198 to end). No similarity was found with the C-terminal end of the *Bacillus* sp. hydrolase. The middle region (amino acids 148-220, Fig. 6b) is similar to several proteins involved in cell lysis: product of gene 15 of the *B. subtilis* bacteriophage ϕ 29 (Garvey *et al.*, 1986), Eh muram, the muramidase-2 from *Enterococcus hirae* (Chu *et al.*, 1992), and Sf cwhyd, the *Streptococcus faecalis* bacterial cell wall hydrolase (Beliveau *et al.*, 1991). In addition, this region exhibits similarity to Ec dniR, a regulatory protein encoded by the *dniR* gene of *E. coli* (Kajie *et al.*, 1991), and to Sa ProtA, the protein A of *Staphylococcus aureus* Cowan1 (Shuttleworth *et al.*, 1987). Finally, the comparison of the whole protein sequence revealed an identity of 44% between XlyA and CwlA, 38% between XlyA and OrfL3 of *B. licheniformis*, and 33% between XlyA and the *Bacillus* sp. hydrolase.

Protein XlyA has a typical export signal peptide with a potential cleavage site (von Heijne, 1986) located after the Ala₄₀ residue (Fig. 5) and the dual translational start signal MKM (Fig. 5). Such a signal, also present in XepA, plays a role in the expression of gene *S* of λ (Young, 1992). Inspection of the N-terminal amino acid sequence of XlyA (Foster, 1993) revealed that transcription begins at the second Met residue. Although no data are available for *xepA*, the position of the putative rbs, overlapping the first M codon, as in *xlyA*, would suggest that its translation also starts at the second M codon.

The two small polypeptides, encoded by genes *xhIA* and *xhIB*, located between *xepA* and *xlyA*, are highly similar to and have almost identical sizes to the *B. licheniformis* proteins encoded by genes *orfL1* and *orfL2* located upstream of *orfL3* (Lee *et al.*, 1991). The identity between *xhIA*, *xhIB* and *orfL1*, *orfL2* is almost 63% (Figs 7 and 10). No such similarity was found within the region upstream of the *B. subtilis* *cwlA* gene. However, the 100 amino acids encoded by the partially sequenced C-terminal end of *orf1*, upstream of *cwlA*, are highly similar (58% identity) to the corresponding region of XepA (Figs 8 and 10).

(a)		
CwlA:	50 SSSTSFHFVDDKQVIQGIPTNRNAWHTGDGT NGTGNRK SIGVEICYSKS 99	
XlyA:	53 DTTTSWHFTVDDTEIYQHLPLNENGWHAGDG NGSGNR ASIGIEIC..EN 99	
Bs Hy:	48 STGTSFHIAVDDKEAIQLIPFNRNAWHAGDGT NGRGNR HSIGVEICYSQS 97	
(b)		
XlyA:	148 SFKAGIGGGGSQTYVVKQGDTLTSIAFAFGVTVAQLQEWN NIEDPNLIRVGQVLIVSAPSAAEKPELYPLPD 220	
φ29 gene 15:	149 VSRGTSSTKTTTPKYVKVSGDNLTKIAKKHNTTVATLLKLNPSIKDPNMIRVGQTVNVTGSGGK..... 212	
Eh muram:	284 GNSGGSATTGTTYTVKSGDSVWGISHSFGITMAQLIEWNNIKN-NFIYPGQKLTIKGGQSAGSSTTNTGNNA 356	
Sf cwhyd:	596 GGSNNNQSGTNTYYTVKSGDTLNKIAAQYGVTVANLRSWNGISG-DLIFVGQKLIVKKGTSNGTGGSSNGGSN 667	
Ec dniR:	295 TLVADNTPLNSRVYTVRSGDTLSSIASRLGVSTKDLQQWNKLRGSKL-KPGQSLTIGAGSSAQLANNSDSIT 366	
Sa ProtA:	634 DGNKPGKEDGNGVHVVKPGD TVNDIAKANGTTADKIAADNKLADKNMIKPGQELVVDKKQPANHADANKAQAAL 706	
(c)		
CwlA:	171 SKASVAKKKTNTTSSKKTSYALPSGIFKVKSPMMRGEKVTQIQKALAAL. 219	
XlyA:	198 RVGQVLIVSAPSAAEKPELYPLPDGIIQLTTPYTSGEHVQVQRALAAL. 246	
OrfL3:	580 STEKALSAAEASAAGKSKTWTLPDGIYKVNPLMKGTAVTQIQTALAAL. 628	
CwlA:	220 YFYPDKGAKNNGIDGVYGPKTADAIRRFQSMYGLTQDGIYGPKTAKLEA 269	
XlyA:	247 YFYPDKGAVNNGIDGVYGPKTADAVARFQSVNGLTADGIYGPATKEKIAA 296	
OrfL3:	629 YYYPDKGAKNNGIDGYGMKTANAVKRFQLMYGLGADGIYGPKTAKMLS 678	
CwlA:	270 LLKX 272	
XlyA:	297 QLSX 299	
OrfL3:	679 LLKX 682	

Fig. 6. Alignment of the N-terminal (a), middle (b) and C-terminal (c) deduced amino acid sequence of XlyA with CwlA, an *N*-acetylmuramoyl-L-alanine amidase of *B. subtilis* (Kuroda & Sekiguchi, 1990; Foster, 1991); *Bs Hy*, a *Bacillus* sp. cell wall hydrolase (Potvin *et al.*, 1988); OrfL3, a protein of *B. licheniformis* (Lee *et al.*, 1991); gene 15, the endolysin of bacteriophage φ29 (Garvey *et al.*, 1986); Eh muram, the muramidase-2 from *Enterococcus hirae* (Chu *et al.*, 1992); Sf cwhyd, the *Streptococcus faecalis* bacterial cell wall hydrolase (Beliveau *et al.*, 1991); Ec dniR, encoded by the *dni R* gene of *E. coli* (Kajie *et al.*, 1991), and Sa Prot A, the gene encoding protein A from *Staphylococcus aureus* Cowan1 (Shuttleworth *et al.*, 1987). The consensus motif, endowed with an *N*-acetylmuramoyl-L-alanine amidase, defined by Lazarevic *et al.* (1992), is in bold characters. Identical and similar amino acids are indicated by vertical lines and colons, respectively.

PX32 encodes an *N*-acetylmuramoyl-L-alanine amidase

Preliminary results (Jaunin, 1987), suggesting that PX32 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 ml⁻¹ of cell wall labelled with ¹⁴C-L-alanine, to which was added 2.5 ml samples of French-press-broken MMC-induced cells, collected 50 min after induction. Samples were withdrawn

at regular intervals and the increase of the free L-[¹⁴C]alanine amino groups, generated by the amidase activity, was determined. Compared to the amidase activity of strain 168, that of strain L4590::p517.5 was substantially reduced (Fig. 9), revealing that inactivation of *xlyA* was associated with diminished amidase activity in the extract of the phage lysate. The residual amidase activity was likely to be due to the host enzyme LytC (CwlB) (Kuroda & Sekiguchi, 1991; Lazarevic *et al.*, 1992), poorly solubilized during the preparation of the

(a)	
<i>xhIA</i> :	1 MQQEADVNVFQQDLADMKGHEHKALEQRVSALE RVSDRQDQQIMTLNEKLN 50 : : : : . . . : :
<i>orfL1</i> :	1 MPEAPELDIFQKEVQEMKADQKSLEQRVSTLERTSDRHDQQIISINEKLN 50
<i>xhIA</i> :	51 KIEENTTWIKRTITGAIITAVSTGIIAEP SPL 82 . : : : : :
<i>orfL1</i> :	51 KIEENTTWIKRSITGAIITAVSTGIIGGAI AV 82
(b)	
<i>xhIB</i> :	1 MNTFDKGTVIRTVLLLI ALINQ TMLMLGKSP LD IQEEQVNQLADALYSAG 50 . : : : : : : . . . : : :
<i>orfL2</i> :	1 MKHIDKGTVVRTVLLF IALVNQ TLIMFGKPVLPVAEDQIHTLADALYSAG 50
<i>xhIB</i> :	51 SIAFTIGTTLA AWFKN NYVTEKGKKQ RDLLRDNNLTK 87 : . . : . . : : : . . .
<i>orfL2</i> :	51 SAAFTIAASLVAWYKNNYVTSKGKMQKEVLQKKGLTK 87

Fig. 7. Alignment of the deduced amino acid sequences of the *xhIA* and *xhIB* products with those of *orfL1* (a) and *orfL2* (b) from *B. licheniformis* (Lee *et al.*, 1991). Identical and similar amino acids are shown by vertical lines and colons, respectively.

<i>xepA</i> :	175	RKTFDFVFHENAESA SEGAALPVDGAAHLLVEVYGTAE MSEVKFWGKSVSGQKLP	229
<i>orf1</i> :	xxx	SKTMDIVFHDKTETIGEGNPFTVGSFKTL LIEVYGTAE TSELKFWGKSLSGTKRA	xxx
<i>xepA</i> :	230	IRGVKTDDATTASSTLGKAEAWAFDIKGFKEIIMEI ISITGGT LSVKGTAVS	end
<i>orf1</i> :	xxx	LRGQKVDDGTFATSTKGKSEAWSFSITGFKEI VMELTALTNGNFSVRGTAVS	end

Fig. 8. Alignment of the deduced amino acid sequence of the *xepA* product with that of the partly sequenced *orf1* from the *B. subtilis* *cwlA* region. Identical and similar amino acids are shown by vertical lines and colons, respectively.

crude extract, rather than to CwlA (Kuroda & Sekiguchi, 1990; Foster, 1992), an amidase not induced by MMC treatment (Foster, 1993). Finally, since lysis of MMC-induced cells of strain L4590::p517.5 is very poor (not presented), it follows that PX32 is the main phage endolysin responsible for the degradation of the host cell wall.

DISCUSSION

The late operon of the *B. subtilis* bacteriophage PBSX (Wood *et al.*, 1990a), like that of other similar bacteriophages, contains genes involved in phage morphogenesis, as well as those responsible for host lysis. The latter PBSX genes were localized at the distal end of this transcription unit (Fig. 10). In this respect, PBSX resembles $\phi 29$, a virulent *B. subtilis* phage (Garvey *et al.*, 1986), but differs from coliphages λ , μ , T4 and P1, where analogous genes occupy the proximal end of the late operon (Calendard, 1988). In both λ and $\phi 29$, the unit responsible for cell lysis encodes two proteins, a so-called holin and an endolysin.

Their structural genes are *S* and *R* for λ , and *g14* and *15* for $\phi 29$ (Steiner *et al.*, 1993), respectively. The holin is transcribed before the endolysin, a lytic transglycosylase for λ and a murein hydrolase for $\phi 29$.

The four PBSX ORFs possibly involved in host lysis were localized between the gene encoding PX76 (Longchamp, 1990), the tail fibre protein, and the terminator of the late operon (Fig. 10). The most distal ORF, *xlyA*, was shown to encode an *N*-acetylmuramoyl-L-alanine amidase, which corresponds to the previously described protein P32 (Mauël & Karamata, 1984). The enzyme, with an isoelectric point of 5.6, has a strong affinity for teichoic-acid-containing walls (Mauël, 1984), and was shown to play the main role in the degradation of host cell wall.

The *xlyA* gene is preceded by two small ORFs designated *xhIA* and *xhIB*, each of which resembles a different part of the holins so far described (Young, 1992). Indeed, *xhIB* encodes a protein of 9.7 kDa, which, like the λ holin (gene *S*), possesses (i) two hydrophobic domains, separated by a short hydrophilic domain containing a reverse-turn Pro

Fig. 9. *N*-acetylmuramoyl-L-alanine amidase assay with crude lysates of induced *B. subtilis* strains 168 and L4590::p517.5. Samples were treated as described in Methods. The amidase activity was measured as the release of free L-alanine NH₂ groups which reacted with fluorodinitrobenzene to yield dinitrophenyl (DNP) alanine (Margot *et al.*, 1991). The percentage of amidase-cleaved *N*-acetylmuramoyl-L-alanine bonds was calculated as follows: $100 \times [\text{DNP-L-alanine}/(\text{total DNP-L-alanine} + \text{L-alanine})]$. Non-induced (open symbols) and MMC induced (filled symbols) strains 168 (□, ■) and L4590::p517.5 (○, ●).

residue, (ii) a charge-rich, hydrophilic C-terminus, and (iii) a basic isoelectric point. Protein XhlA of 10 kDa presents a marked α helix-turn- α helix secondary structure.

xhlA and *xhlB* differ from the structural gene of the λ holin, since, unlike the latter, they do not have a dual MKM start motif. This motif, however, was shown to play a role in the modulation of the holin activity but not in its function as such. Therefore, it would seem that the combined action of these polypeptides could fulfil the function of a holin, i.e. release the endolysin by generating transmembrane lesions. This interpretation is strongly supported by observations of Lee *et al.* (1991), who showed that *xhlB*, named *xpaB* in their study, can complement certain alkaline-phosphatase-deficient mutants of *E. coli*. Incidentally, *orfL1* and L2 of *B. licheniformis* (Fig. 10), similar to *xhlA* and *xhlB*, also complemented certain alkaline phosphatase-deficient mutants of *E. coli*. Lee *et al.* (1991) suggested that complementation was due to altered membrane permeability. In view of our observations, we believe that Xpa-like proteins correspond to holins, and that Lee *et al.* (1991) have actually devised a relatively general test for the detection of such proteins.

The first of the four sequenced ORFs (*xep*, for PBSX exported protein) encodes the previously described PBSX protein P31. This protein, shown to be exported during phage development (Mauël & Karamata, 1984), did not exhibit lytic activity (see above) and had no affinity for the cell wall (C. Mauël, unpublished). Its function remains unknown. However, the measured and the deduced molecular mass of P31 are identical, suggesting that this protein, devoid of the export signal, may also be extruded by a holin type mechanism, possibly that responsible for XlyA translocation.

Sequence similarity analysis revealed that the genetic

Fig. 10. Comparison of the relevant similar regions between PBSX and the genomes of *B. subtilis*, *B. licheniformis* and *Bacillus* sp. The ORFs described in different organisms, their putative terminator structure (Ω), sense of transcription (←), and putative promoters (→) are specified. Symbols (▨, ▩, ▪, ▫, ▬) represent regions of similarity, while asterisks denote genes complementing the Pho⁻ phenotype of *E. coli* *phoA* mutants. 1 kcal = 4.184 kJ.

entity comprising *xylA* is present in more than one copy in the *B. subtilis* 168 genome. Indeed, the deduced amino acid sequence of *xylA* is 44% similar to that of *cwlA*, an amidase-encoding gene located near *aroD* at about 226° on the *B. subtilis* genetic map (Foster, 1991). Both genes are flanked by a strong terminator, while the region upstream of *cwlA*, so far sequenced, reveals a similar organization to that upstream of *xylA* (Fig. 10). *cwlA* is preceded by *orf2*, which, like *xhlB*, encodes a basic protein with two hydrophobic domains and a charged C-terminus, and which, in turn, is preceded by a partly sequenced gene transcribed in the same direction and similar to the C-terminal end of *xepA*. Preliminary Southern hybridization experiments (not presented) revealed that a *xylA*-derived probe hybridized with three, possibly four, different loci, suggesting the presence of one or two copies of this gene, in addition to *xylA* and *cwlA*. Presence of multiple copies of endolysin genes has been reported in *B. licheniformis* (Oda *et al.*, 1993) as well as in *Streptococcus pneumoniae*, where the close relationship between host and phage enzymes (Romero *et al.*, 1990; Diaz *et al.*, 1992) has provided strong support for the concept of the modular organization of proteins. In conclusion, these situations may be related to that of bacteriophage λ , several degenerate copies of which – *rac*, *qsr'* and *qin* prophages – were identified on the *E. coli* chromosome (Espion *et al.*, 1983; Strathern & Herskowitz, 1975).

Inspection of the data banks revealed that entities encompassing holins and *xylA*-like genes are widespread among bacilli. A *Bacillus* sp. hydrolase and a *B. licheniformis* gene exhibit homologies to *xylA* and *cwlA* (see above). It is, however, impossible to unambiguously attribute such entities to PBSX-like phages or to their degenerate copies. Indeed, if the unit described in *B. licheniformis* consists of a gene with some similarity to *xylA*, flanked on one side by a terminator and on the other by two small ORFs, it is preceded by a divergently transcribed gene. Therefore, from the structural point of view, this entity forms a divergon and is unlikely to be part of a late phage transcription unit. The question remains as to whether such holin and endolysin-encoding units are of phage origin or whether the holin, and possibly endolysin genes, are chromosomal in origin and somehow brought under phage control.

The PBSX amidase presents an interesting feature regarding its translocation. On the one side, like the ϕ 29 and λ lytic enzymes, *xylA* is preceded by two genes encoding proteins which probably fulfil a holin function, while, on the other, it is endowed with a signal peptide, an unusual property for a bacteriophage endolysin (Steiner *et al.*, 1993). Apparently, both export mechanisms are likely to be used. The N-terminal amino acid sequence of XlyA (Foster, 1993) corresponds to that encoded by the signal peptide, implying that the uncleaved protein was exported, probably through a holin-like system. However, a protein similar to XlyA, the CwlA amidase which can be cloned and expressed in *E. coli*, is cleaved and possibly exported independently of the holin. Should this observation be confirmed for *xylA*, which so far has been difficult to clone in *E. coli*, it would suggest a versatile

system capable of using different strategies for lysing different hosts, i.e. Gram-positive and Gram-negative bacteria. This might reveal some ancestral phage with a wide host spectrum.

Finally, PX34, the other endolysin identified on cell-wall-containing renaturing gels, possibly endowed with a muramidase activity (Ward *et al.*, 1982), and shown to have an affinity for cell wall (C. Mauël, unpublished), does not seem to be encoded by the PBSX late operon. Although the possibility remains that the structural gene of PX34 is part of contiguous prophage regions which have not been cloned yet, it cannot be ruled out that this gene, controlled by the PBSX repressor is located elsewhere on the chromosome implying that the PBSX genome is scattered, i.e. that it forms a regulon.

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