Identification of four unique clones encoding 10 kDa proteins from *Bacillus* that cause phenotypic complementation of a *phoA* mutant strain of *Escherichia coli*

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A number of clones have been isolated from two *Bacillus* species which complement the PhoA⁻ phenotype of *Escherichia coli* mutants under conditions that induce the expression of alkaline phosphatase (APase). These clones were initially thought to carry XPases because the transformed host could hydrolyse a common APase substrate, XP (5-bromo-4-chloro-3-indolyl-phosphate). The sequences of the open reading frames responsible for the phenotypic complementation showed no sequence similarity to APases of *E. coli*, human (bone-liver-kidney, intestinal or placental) or *Bacillus*. Therefore, these clones were designated as XPA (for X Phosphatase Activity) clones. Four of the clones encoded small (10 kDa), basic, hydrophobic proteins. Two of these, *xpaB* from *B. subtilis* 168 and *xpaL2* from *B. licheniformis* MC14, shared 62% identity at both the DNA and the predicted amino acid sequence level. The fact that homologues from two *Bacillus* strains were cloned indicated that the screen was specific, but not for APase genes. It is clear that phenotypic complementation with cloned DNA from another genus does not ensure the identification of an APase gene. Possible mechanisms for the abnormal phenotypic complementation are discussed.

Introduction

Two *Bacillus* species, *B. licheniformis* MC14 and *B. subtilis* have been used in our laboratory to study alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1; APase] of *Bacillus*. Biochemical and localization studies have indicated that there are multiple species of vegetative APase in each *Bacillus* species. The pattern of distribution of APases in these two *Bacillus* species is very similar. A salt-extractable APase is found on both the inner and outer leaflets of the cytoplasmic membrane phospholipid bilayer (Ghosh *et al.*, 1971, 1977; McNicholas & Hulett, 1977; Yamane & Maruo, 1978; Spencer & Hulett, 1981), and a detergent-extractable APase is imbedded in the outer leaflets of the cytoplasmic membrane (Spencer *et al.*, 1982). One of the secreted

forms of APase is cell bound, but can be released as a soluble APase upon removal of the cell wall with lysozyme (Hansa *et al.*, 1981). The other secreted form is found outside the cells (Yamane & Maruo, 1978; Spencer *et al.*, 1981; Hulett *et al.*, 1990). These APases are active dimers with two identical subunits (Hulett & Campbell, 1971*b*; Hulett *et al.*, 1976, 1986) except for one secreted APase from *B. subtilis* which is an active monomer (Yamane & Maruo, 1978; Hulett *et al.*, 1990). APases from *B. licheniformis* MC14 have a subunit molecular mass of 60 kDa (Hulett & Campbell, 1971*b*; Hansa *et al.*, 1981; Spencer *et al.*, 1982). The size of *B. subtilis* APase subunits varies from 45 to 60 kDa (Le Hegarat & Anagnostopoulos, 1973; Ghosh *et al.*, 1977; Yamane & Maruo, 1978). The APases from the two *Bacillus* species are related to each other immunologically and biochemically.

The expression of vegetative APases in both *Bacillus* species is derepressed as the concentration of phosphate decreases below 0.1 mM in the culture medium during vegetative growth (Spencer *et al.*, 1981; Hulett & Jensen, 1988). Genetic studies in *B. subtilis* suggested that *pho* regulon genes, *phoP* and *phoR*, are involved in the regulation of vegetative APases (Miki *et al.*, 1965) as are two genes which contribute to the control of initiation of

Abbreviations: APase, alkaline phosphatase; ORF, open reading frame; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside; XP, 5-bromo-4-chloro-3-indolyl phosphate; XPA, X phosphatase activity (see text).

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers M37167 (*Xho*I₂–*Pvu*II₄ fragment of *B. licheniformis* MC14) and M37168 (*xpaB* gene of *B. subtilis* 168).

sporulation, *spoOA* and *spoOH* (Hulett & Jensen, 1988). These findings suggest that there is overlapping regulation in the expression of vegetative APase by two different sets of regulatory genes. In addition to multiple vegetative APases, there is another APase species produced during sporulation (Glenn & Mandelstam, 1971; Glenn, 1975). The expression of sporulation APase is independent of the phosphate concentration in the culture and is affected by a different set of mutations (Piggot & Taylor, 1977; Bookstein *et al.*, 1990).

All of these data have led us to two alternative hypotheses: (i) there is a single structural gene for APase in *Bacillus* which is under complex regulation by multiple genes, or (ii) there are multiple structural APase genes, each of which is under different regulatory control(s). However, our recent data from partial protein sequencing of *B. subtilis* APases has indicated that there are at least two structural genes for APase in *B. subtilis* (Hulett *et al.*, 1990). Therefore, the isolation of structural genes for APase from two *Bacillus* species was necessary for further studies on gene regulation, protein translocation, and molecular evolution of APase in *Bacillus*.

In attempts to isolate APase structural genes from *Bacillus* species, we have used an *Escherichia coli* APase-negative strain as a cloning and screening host since there has been no *Bacillus* APase negative mutant isolated which has a mutation in the structural gene (Glenn & Mandelstam, 1971; Le Hegarat & Anagnostopoulos, 1973; Grant, 1974; Glenn, 1975). *E. coli* *phoA* mutants have been used in various laboratories as cloning hosts to clone genes involved in the *pho* regulon, since it has been the only bacterium for which such mutants were available (McCarter & Silverman, 1987; Filloux *et al.*, 1988; Xie *et al.*, 1989). We isolated putative APase clones from *B. licheniformis* MC14 (Hulett, 1984) and *B. subtilis* (Hulett *et al.*, 1988) by using the same method that had been employed in cloning the APase structural gene (*phoA*) of *E. coli* (Berg, 1981; Inouye *et al.*, 1981). However, further analysis of the clones has indicated that none of those *Bacillus* clones contained a structural gene for APase. Even though they did not carry APase genes, the *Bacillus* clones could hydrolyse XP and gave rise to a blue colony phenotype on low-phosphate-XP indicator plates. The phenotype was lost when the clones were interrupted.

Methods

Bacterial strains. *E. coli* strains Xph90a (F^- *lacZ*624 *phoAE15 proC*⁺ *phoR*⁺ *trp rpsL*; from P. Berg) and BW12720 [*DE3(lac)*x74 Δ (*phoA532 PvuII*) *phnD*(*EcoB*) *phoM*(wt), *arcA1655 fmr-1655*; from B. Wanner] were used as host strains for XPA clones. *E. coli* POI 1734 [MAL103 with Mu *dI* 1734 (Km^r)] and POII 1734 [MC1040 (Mu *cts*)] with Mu *dII*

1734 *lac*⁺] were used to create transcriptional and translational fusions, respectively, between the *lacZ* gene and p1881. *B. licheniformis* MC14 was the wild-type strain used to isolate APase (XPA) genes (Hulett & Campbell, 1971a). *B. subtilis* 168 (*trpC2*) and *B. subtilis* *phoP* (*trpC2 phoP12*) strains were used as hosts for the expression study.

Plasmids and recombinant DNA techniques. The integration expression vector, pDH32 (Shimotsu & Henner, 1986), contained an *amyE* gene interrupted by a promoter-less *lacZ* gene fused to a ribosome-binding site of the *spoVG* gene and the Gram-positive chloramphenicol resistance gene. It also carried a penicillin resistance gene and an origin for replication in *E. coli*. The *E. coli* vector, pTTQ19, was purchased from Amersham (Stark, 1987). It contained the composite *tac* promoter preceded by a polylinker, the *lacI*^a gene, and a penicillin resistance gene. Transformation of *E. coli* with plasmid DNA was done according to Cohen *et al.* (1972). Transformation of *B. subtilis* was done by the method of Dubnau *et al.* (1969). The restriction enzymes and T4 ligase were purchased from Bethesda Research Laboratories and used according to the manufacturer's recommendations. Plasmid DNA was isolated from *E. coli* according to Maniatis *et al.* (1982).

Growth media. To screen putative APase clones, neopeptone-XP medium or low-phosphate MOPS medium was used. Neopeptone-XP medium was a low-phosphate complex medium and prepared as described by Hulett *et al.* (1985). Low-phosphate MOPS minimal medium was prepared as described by Neidhardt *et al.* (1974). 5-Bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (XP, Sigma) was added at 50 μ g ml⁻¹. Antibiotic selection of *E. coli* transformants was done on Luria-agar plates containing penicillin G at 150 μ g ml⁻¹, kanamycin at 50 μ g ml⁻¹, or tetracycline at 30 μ g ml⁻¹. *Bacillus* transformants were selected on TBABG plates (Tryptose Blood Agar Base, Difco, containing 0.1% glucose) supplemented with chloramphenicol at 5 μ g ml⁻¹. Low-phosphate defined medium (DM) was prepared as described previously (Hulett & Jensen, 1988); high-phosphate DM was the same as low-phosphate DM except that it contained 45 mM-K₂HPO₄. Schaeffer's sporulation medium was prepared according to Schaeffer *et al.* (1965). 5-Bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) was added at 50 μ g ml⁻¹.

DNA sequencing. DNA sequence analysis of p1881 was done by the dideoxy chain-termination method (Sanger *et al.*, 1977) using M13 clones. The DNA sequence of *xpaB* was determined by plasmid sequencing using Sequenase kits purchased from United States Biochemical and following the manufacturer's instructions. The sequences of both DNA strands were determined and all sequence junctions overlapped.

Mu d fusion study. To obtain a transcriptional fusion between the *lacZ* gene and the insert of p1881, *E. coli* POI 1734 carrying Mu *dI* 1734 (Castilho *et al.*, 1984) was transformed with p1881. The phage lysate was prepared as described by Groisman & Casadaban (1986) after the transformants had been induced to allow transposition of the phage. The phage lysate was transduced into *E. coli* Xph90a, and the transductants were selected and screened on neopeptone-XP plates supplemented with kanamycin and also on McConkey agar plates with kanamycin. For translational fusion, POII 1734 carrying Mu *dII* 1734 (Castilho *et al.*, 1984) was used in the same way.

Bal31 exonuclease deletion mapping. Bal31 exonuclease was purchased from Bethesda Research Laboratories and the deletion mapping was carried out as described by Silhavy *et al.* (1984). p1881 was linearized at the unique *PstI* site in the polylinker of the vector and treated with Bal31 exonuclease for various lengths of time and used to transform *E. coli* Xph90a after religation. Transformants with blue or white colony phenotypes on low-phosphate-XP indicator plates were analysed for the extent of deletion by restriction and DNA sequence analysis.

Construction of subclones. The subclone with ORFL1 was constructed by inserting a 460 bp *Hae*III fragment, which contained upstream sequence of ORFL1, ORFL1, and about half of ORFL2, at the *Sma*I site of pTTQ19, 3' of the *tac* promoter. In order to subclone various lengths of the downstream sequence of ORFL1 behind the *tac* promoter, p1881 was linearized at the *Pst*I site and treated with Bal31 exonuclease. The Bal31 exonuclease treated p1881 was digested with *Eco*RI and the resulting DNA was ligated to pTTQ19 at the *Eco*RI and *Sma*I sites. In order to construct the subclone of ORFL2 (pKLQL2), the *Hind*III_{3a}-*Hind*III₄ fragment of pKLQ13 was deleted by digesting the clone with *Hind*III and religating the plasmid. The subclone of ORFL3 (pKLQL3) was constructed using the *Xho*II site located in the middle of ORFL2 and the *Bgl*II site located 500 bp downstream of ORFL3. The *Xho*II-*Bgl*II₂ fragment was ligated to pTTQ19 at the *Bam*HI site. XPA of these pTTQ19 subclones was tested on neopeptone phosphate plates containing XP with incubation at 37 °C for 16–24 h after transformation into *E. coli* Xph90a. IPTG (isopropyl β-D-thiogalactopyranoside) was added to the plates at the final concentration of 0.5 mM to induce the *tac* promoter. If the presence of IPTG in the plates killed the cells, the cells were allowed to grow overnight at 37 °C and a drop of IPTG (0.1 M) was applied to the colony. The subclone of ORFR7 was constructed by cloning *Bcl*I₂-*Xho*I₂ fragment on pMK2004 digested with *Bam*HI and *Xho*I. The phenotype of the transformants was determined on neopeptone plates supplemented with XP and kanamycin.

Results and Discussion

Cloning of p1881 and *xpaB*

In order to isolate APase genes, genomic DNA of either *B. licheniformis* MC14 or *B. subtilis* 168 was digested with *Pst*I and shotgun cloned in an *E. coli* vector, pMK2004. Putative APase clones were selected and screened on low-phosphate complex medium plates (1% neopeptone) supplemented with XP and penicillin after transformation into an *E. coli* *phoA* mutant, Xph90a. Five thousand transformants from the *B. licheniformis* ligation and 20000 from the *B. subtilis* ligation were screened. Blue colonies which were positive for XPA on the indicator plate were picked and analysed further as putative APase clones. From the three putative APase clones isolated from *B. licheniformis* MC14 and 15 putative APase clones from *B. subtilis* 168, two clones, p1881 and *xpaB*, were selected for further analysis and are discussed in this paper. p1881 is a subclone of pMH8. pMH8 contains one of the original *Pst*I fragments cloned into pMK2004 (Hulett, 1984), which was isolated from *B. licheniformis* MC14. A part of the insert on pMH8 was subcloned first in pMK2004 (pMH81) and subsequently moved into pUC18 (p1881) for better phenotypic expression in *E. coli*. p1881 had a 4 kb *Xho*I-*Eco*RI insert (Fig. 1a). The region which was responsible for the phenotype was mapped between 300 bp before *Xho*I₂ and 150 bp before *Pvu*II₄ as previously described (Hulett *et al.*, 1985). The *xpaB* clone was isolated from *B. subtilis* 168 and had a *Pst*I insert of 0.45 kb (Fig. 1a). The insert of the *xpaB* clone was also transferred into pUC18 from pMK2004. When the 0.45 kb *Pst*I fragment was inserted

Fig. 1. (a) The restriction maps of p1881 and *xpaB*. The inserts are presented as thin lines and the portions of the vectors (pUC18) as thick lines. The relative locations and orientations of the *lac* promoter and operator (*lac*_{op}) and the *lacZ* gene of pUC18 to each insert are indicated above the lines. For the construction of p1881, the 4 kb *Xho*I₂-*Eco*RI₂ fragment was inserted into pUC18 at the *Sa*I and *Eco*RI sites. For *xpaB*, the 0.45 kb *Pst*I fragment was ligated with pUC18 digested with *Pst*I. The region responsible for the XPA-positive phenotype is indicated under the line for each clone using a bidirectional arrow (p1881) or an arrow (*xpaB*). Abbreviations for restriction sites are: B, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pvu*II; Ps, *Pst*I; S, *Sa*I; X, *Xho*I. (b) Strategy for DNA sequencing of the *Xho*I₂-*Pvu*II₄ fragment of p1881. *Sau*3AI or *Hpa*II was used to generate subclones for DNA sequencing. Some other restriction enzymes were also used. The arrowheads above the line indicate the *Sau*3AI restriction sites, and those below the line indicate the *Hpa*II restriction sites. Bidirectional arrows indicate that both strands of the fragment were sequenced. Restriction sites abbreviated as in (a), plus R, *Rsa*I.

in one orientation in pUC18, the resulting clone was positive for XPA, but in the reverse orientation, the phenotype of the clone was negative for XPA. This indicated either that the fragment did not contain a promoter for the *xpaB* gene or that the promoter for the gene was not functioning in *E. coli*, and that the *lac* promoter of the vector was necessary for the expression of the *xpaB* gene. The DNA fragment inserted on p1881 was long enough to encode a *B. licheniformis* APase. However, the DNA fragment inserted on the *xpaB* clone was too short to encode an APase, even though it had *phoA*-complementing activity. In order to understand how each of these clones complemented the *PhoA*⁻ phenotype in *E. coli*, nucleotide sequence analysis of both clones was carried out.

DNA sequencing analysis of p1881 and *xpaB* clones

The 2.3 kb *Xho*I₂-*Pvu*II₄ fragment of p1881 and the 0.45 kb *Pst*I fragment of *xpaB* were sequenced. Restriction maps for p1881 and *xpaB* are shown in Fig. 1(a).

(a) <u>XhoI</u> : CTCAGCGCAAGTTGGCGAACTTAACCTTGGCACTTTCCAAAGCGTTTAAACGAATGGT	60 V F P	<u>BclI</u> : AAT CAG ACG TTG ATC ATG TTT GGA AAG CCG GTT TTG CCG GTC GCT	773
TAATATTCCGACACACACTAAAGTTCTGTTATACAAAAGATGCCACAGGTACGGTATTGT	120 <u>RsaI</u> : N I I R V A S F N T I C F I G C T R Y K	GAA GAT CAG ATT CAT ACA TTG GCG GAT GCC CTG TAT TCG GCC GGA	799
TGAAGTGCAATAGCAAAAGGAAAATTGGTTTAAATATACCTGCATTCGATTGCCGAA	180 N F T C Y C F P F N T K I Y R C E Y Q R	TCT GCG GCT TTT ACG ATC GCT GCA TCG CTC GTT GCC TGG TAT AAG	863
AGATATCGCGCGTCCCGCTTTTCAGTTTGTAGGTAGCATCTCAATTGGGCATGTC	240 F I D A T G R K E T Q L H L M E I P C T	AAC AAC TAT GTG ACG AGC AAA GGG AAA ATG CAA AAA GAA GTT TTA	908
AATACTCGCAGTATCATAGCTCCAGTGCAGTGTGATGCTAATGCTGATCGAATAAC	300 W Y E A T D Y A A L S H Q H Y H R S R F	Asn Asn Tyr Val Thr Ser Lys Gly Lys Met Gln Lys Glu Val Leu	
TGCTCAATACAGTAATCCAAATGAATATATCATGTTTAGGATTAGTTTTAAGCCGCA	360 S S L Y L L D M	CAG AAA AAA GGT TTA ACG AAA TAAAGCTTCCTTCAGACTTCCAGAAATGAAT	962
TAAATATGAAGAGGAATTAATAATG CCG GAA GCA CCA GAA CTG GAT ATA	411 SD1 Met Pro Glu Ala Pro Glu Leu Asp Ile	TTGAAAGGAATGATGAAGTTTG GTC AAA GTA ATC AAT AAC TTT GTA AAA	1012
TTC CAA AAA GAA GTT CAG GAG ATG AAA GCT GAT CAA AAA TCA CTC	456 <u>BclI</u> : Phe Gln Lys Glu Val Gln Glu Met Lys Ala Asp Gln Lys Ser Leu	GTC AAT CAA TAC GAC CGT CCC GGG CTG AAG CTT GCG GCA GTC AAA	1057
GAA CAG CGC GTC AGT ACA CTC GAA ACA ACA TCA GAC CGC CAC GAT	501 <u>RsaI</u> : Glu Gln Arg Val Ser Thr Leu Glu Arg Thr Ser Asp Arg His Asp	GGC ATC GTC ATG CAT TGG ACG GCG ACG CCC GGG GCT TCG GCA TTG	1102
CAG CAA ATC ATC TCG ATT AAC GAA AAG CTG AAC AAA ATC GAA GAG	546 Gln Gln Ile Ile Ser Ile Asn Glu Lys Leu Asn Lys Ile Glu Glu	AAT GAG CGA AAT TAT TTC AAT GGC ACA TGC ATT GCC GAC AAG CGT	1147
AAT ACA ACC TGG ATT AAG CGC AGC ATC ACA GGC GCG ATA ATT ACA	591 Asn Thr Thr Trp Ile Lys Arg Ser Ile Thr Gly Ala Ile Ile Thr	TAT GCG TCG GCT CAT TAT TTT GTC GAC CGC CAT GAG GCG CAG CAT	1192
GCG GTC AGC ACC GGC ATC ATC GGC GGC GCG ATC GCT GTT TTT TAT	636 Ala Val Ser Thr Gly Ile Ile Gly Gly Ala Ile Ala Val Phe Tyr	ATT ATT CCC CAT CAT GAA GTC GCA TAT CAT GCG CAT GAT CAA AAC	1237
AAT TTA TTG CAG AAA TAGGGAGGACATCATC	683 Asn Leu Leu Gln Lys *** SD2 Met Lys His Ile Asp	CGC TGC TAT GTC AGC TTT CTG AAG CCG AAT GCC AAT ACC ACG GCT	1282
AAA GGC ACG GTC AGG ACG GTG CTT TTT ATT GCG CTG GTC	728 Lys Gly Thr Val Val Arg Thr Val Leu Leu Phe Ile Ala Leu Val	CTC GGG GTC GAA ATG TGC GTG GAA AAA GAC GGT ACC ATC CAT GAA	1327
		GAG ACG ATT CGC AAT GCG GCA GAG CTT GTA GCG GAT TTG TGC AAG	1372

Fig. 2. Nucleotide sequences of the *XhoI*₁-*PvuII*₄ fragment of p1881 and the *xpaB* gene. The DNA sequence of one strand of each DNA fragment is shown. Relevant restriction sites are also indicated. Numbering begins at the 5' end of the sequence. (a) The nucleotide sequence of the *XhoI*₁-*PvuII*₄ fragment is shown with the predicted amino acid sequences of four open reading frames in three-letter code. ORFR7 is oriented away (as indicated by an arrow) from the rest of the ORFs. Therefore, the non-coding strand of ORFR7 is shown and its amino acid sequence is presented in single-letter code. Putative ribosome-binding sites are underlined (SD1, SD2, SD3, SD4). The two regions of dyad symmetry at the end of ORFL3 are underlined. The putative promoter sequences of ORFL4 are shown in comparison with the consensus sequence. (b) Nucleotide sequence of the 426 bp *PstI* fragment which contains the *xpaB* gene. The predicted amino acid sequence of the *xpaB* gene (87 amino acids) is shown in three-letter code. The putative ribosome binding site is underlined (SD).

Fig. 1(b) shows sequencing strategy for p1881. *xpaB* was sequenced by a plasmid sequencing technique using universal forward or reverse primer under various restriction conditions. The DNA sequence of one strand of each clone is presented in Fig. 2. The sequenced DNA fragment of p1881 was 2318 bp long and contained several possible open reading frames on each strand of DNA. Mini Mu-*lacZ* transcriptional and translational fusions were used to determine the direction of transcription. Mu *dI-lacZ* fusions indicated that divergent transcription occurred, as summarized in Fig. 3. Five different transcriptional fusions and one translational fusion (using Mu *dII*) indicated transcriptional activity in the direction from *XhoI*₂ toward *PvuII*₄. Each of four ORFs found in this direction (translated from left to right, Fig. 3) was preceded by consensus translational signals. ORFL1, which could encode 89 amino acid residues, had an ATG as a putative start codon at position 385 and was preceded by a potential ribosome-binding site, GAAAGAGGGAA, which was complementary to the sequence near the 3' terminus of 16S rRNA (Shine & Dalgarno, 1974). ORFL2 could encode a protein with 87 amino acid residues, had an ATG at position 669, and was preceded by an AAGGGAGGA sequence. ORFL3 was the longest open reading frame found in the sequenced region, with 354 amino acid residues, and had a TTG at position 983 which was preceded by a putative ribosome-binding site,

Fig. 3. Bal31 deletion mapping of the XPA coding region and transcriptional or translational fusion between Mu *d-lacZ* and the insert cloned on p1881. The sequenced region is shown as a thick line, the rest of the insert as a thin line, and the portion of vector as a dashed line. All of the predicted open reading frames from the nucleotide sequence are shown as boxes. The dark box preceding the box represents the position of the putative translational signal (ribosome-binding site and appropriately spaced start codon). ORFRs are translated from right to left on one DNA strand and ORFLs are translated from left to right on the other strand. The extent of Bal31 deletion is shown as boxes with slashed lines along with their associated XPA phenotypes. Dotted lines lead to the deletion junction of the XPA-positive clone with the longest deletion or the XPA-negative clone with the shortest deletion. The phage Mu is represented by a triangle with a tail. A filled triangle represents translational fusion and an open triangle represents transcriptional fusion. Arrows above the triangles indicate the direction of transcription or translation of the *lacZ* gene. Abbreviations for restriction sites are: B, *BclI*; E, *EcoRI*; H, *HindIII*; P, *PvuII*; Ps, *PstI* on the vector; R, *RsaI*; S, *SalI*; X, *XhoI*.

GAAAAGGAA. There was a DNA sequence at the end of ORFL3 which could form a stem and loop structure, followed by a stretch of six thymine residues. This might indicate that ORFL1, L2 and L3 form a transcriptional unit. In support of this interpretation, the *PvuII*₃-*PvuII*₄ fragment contained promoter activity for ORFL4 (Fig. 2; C. Bookstein & F. M. Hulett, unpublished data). Two different transcriptional fusions mapping very close to *XhoI*₂ indicated transcriptional activity in the opposite direction. Among several ORFRs (translated from right to left), ORFR7 was located downstream of the transcriptional fusions in that orientation (Fig. 3). ORFR7 was not preceded by a sequence resembling a ribosome-binding site, but it could encode a protein with 91 amino acid residues starting with a TTG codon at position 321. *In vitro* transcription-translation studies or *E. coli* minicell expression studies using subclones of the ORFs showed that these ORFs encoded proteins (data not shown).

xpaB contained an open reading frame encoding 87 amino acids which started with an ATG at position 26 and was preceded by a potential ribosome-binding site, AAAGGGGG ($\Delta G = -14.6$ kcal mol⁻¹; -61.1 kJ mol⁻¹). Assuming that the first methionine is the amino terminus of the *xpaB* gene, the sequence data indicated that an intact promoter for this gene could not be present on this clone and that the gene required the *lac* promoter for expression as suggested above.

Homology of predicted amino acid sequences of XPA clones with other proteins

None of the predicted translation products of the ORFs mentioned above showed significant homology to any of the APases or other types of phosphatases whose sequence is known. The product of the longest open reading frame, ORFL3, showed 49% identity at the carboxyl terminus with the amino acid residues between 16 and 75 of muramoyl-pentapeptide carboxypeptidase from *Streptomyces albus* (Dideberg *et al.*, 1982; Joris *et al.*, 1983) (Fig. 4a). This enzyme catalyses carboxypeptidation and transpeptidation reactions involved in bacterial cell wall metabolism. The catalytic activity is located in the carboxyl-terminal domain, one of its two globular domains. The function of the amino-terminal domain (amino acids 1–76), which shares homology with the carboxyl terminus of ORFL3, is not known. The products of the other ORFs showed no significant homology to any other proteins with known sequence. However, ORFL2 from *B. licheniformis* MC14 and *xpaB* from *B. subtilis* 168 were very similar to each other. They shared 62% identity both at the DNA sequence level and at the predicted amino acid sequence level (Fig. 4b). They encoded basic proteins with hydrophobic amino terminal regions. Computer analysis of the predicted amino acid sequences using the method of Klein *et al.* (1985) indicated that both of them as well as ORFL1 could be integral membrane proteins.

Identification of three open reading frames with XPA on p1881

To determine the ORF(s) responsible for the XPA in the direction from *Xho*I₂ toward *Pvu*II₄, Bal31 exonuclease deletion mapping was carried out as described in Methods. Transformants with blue or white colony phenotypes on XP indicator plates were analysed for the extent of deletion, and the results are shown in Fig. 3. The deletion junctions of a clone with the shortest deletion exhibiting a white colony phenotype and a clone with the longest deletion exhibiting a blue colony phenotype were determined by DNA sequencing. Clones containing deletions which ended less than 31 bp before ORFL1 maintained XPA. However, when the deletion extended 83 bp into ORFL1 or further, the resulting clones lost the XPA. Three possible explanations of these results are: (i) ORFL1 might be the only ORF responsible for XPA, (ii) there might be more than one ORF responsible for XPA, with all ORFs being expressed from a promoter in front of ORFL1, or (iii) this is a polycistronic operon in which a distal gene has XPA. It could not be determined if ORFR7 had XPA from the Bal31 exonuclease deletion mapping study.

(a)	
ORFL3	G T A V T Q I Q T Q L A A L Y Y Y P D K 301
	* * * * *
MPC	G E A V R Q L Q I R V A G - Y - - P G T 32
ORFL3	G A K N N G I D G Y Y G M K T A N A V K 327
	* * * * *
MPC	G A Q L A - I D G Q F G P A T K A A V Q 51
ORFL3	R F Q L M Y G L G A D G I Y G P K T K A 347
	* * * * *
MPC	R F Q S A Y G L A A D G I A G P - T F N 70
ORFL3	K M L S L 352
	* *
MPC	K I Y Q L 75
(b)	
ORFL2	M K H I D K G T V V R T V L L F I A L V N Q T L I
	* * * * *
XPAB	M N T F D K G T V I R T V L L I A L I N Q T M L
ORFL2	M F G K P V L P V A E D Q I H T L A D A L Y S A G
	* * * * *
XPAB	M L G K S P L D I Q E E Q V N Q L A D A L Y S A G
ORFL2	S A A F T I A A S L V A W Y K N N Y V T S K G K M
	* * * * *
XPAB	S I A F T I G T T L A A W F K N N Y V T E K G K K
ORFL2	Q K E V L Q K K G L T K
	* * * *
XPAB	Q R D L L R D N N L T K

Fig. 4. Amino acid sequence homology. (a) Predicted amino acid sequence homology between the product of ORFL3 and muramoyl-pentapeptide carboxypeptidase (MPC) of *Streptomyces albus*. The amino acid sequence between 288 and 352 of the ORFL3 product and that between 16 and 75 of muramoyl-pentapeptide carboxypeptidase are shown in comparison with each other. The identical residues between the two sequences are marked with asterisks. They share 49% identity in a 65 amino acid residue overlap. (b) Predicted amino acid sequence homology between the products of ORFL2 and *xpaB*. The identical residues between the two sequences are marked with asterisks. They show 62% identity over the entire amino acid sequence (87 amino acid residues).

To determine which ORFL was responsible for XPA, subclones of each ORFL were constructed using pTTQ19 as described in Methods. The subclone containing ORFL1 (pKLQL1) showed XPA without induction of the *tac* promoter with IPTG, indicating that the fragment contained its own promoter for the gene (Fig. 5). The subclones containing various extent of deletions into ORFL1, ORFL2 and ORFL3 (pKLQ13, pKLQ14 and pKLQ15, Fig. 5) lost XPA on low-phosphate neopeptone-XP indicator plates when they were transformed into Xph90a. None of the white colonies carrying those subclones turned blue upon induction of the *tac* promoter. One of them (pKLQ13), however, did not

grow on the plate with IPTG. The colony carrying the clone was grown on a neopeptone-XP plate overnight and a drop of 0.1 M-IPTG was applied to the colony. The clone turned blue within an hour of incubation at 37 °C. pKLQ13 contained ORFL2, ORFL3, and further downstream sequence. ORFL2 was determined as another complementing gene by applying a drop of IPTG on the colony carrying the ORFL2 subclone (pKLQ2, Fig. 5). The colony harbouring the ORFL3 subclone (pKLQ13, Fig. 5) remained white on neopeptone-XP plates in the presence or absence of IPTG. This result indicated that ORFL3 was not an XPA gene. The ORFR7 subclone (pCER7, Fig. 5) turned bright blue on neopeptone-XP plates, indicating that ORFR7 is another XPA gene. The genes cloned on pKLQ1, pKLQ2, and pCER7 were designated as *xpaL1*, *xpaL2* and *xpaR7*, respectively. All three XPA genes isolated from *B. licheniformis* MC14 and one XPA gene, *xpaB*, isolated from *B. subtilis* 168 could encode small proteins rich in basic amino acids. XP-agarose overlay assay showed that the *xpaL2* and *xpaB* genes complemented APase activity with an optimum pH of 8 in two *E. coli phoA* strains tested (data not shown). The optimum pH of *E. coli* APase was determined as 8 by Garen & Levinthal (1960). Having shown that the *phoA* complementation was not due to an APase gene, we explored possible mechanisms responsible for the phenotypic complementation.

Characterization of the promoter for two XPA genes, *xpaL1* and *xpaL2*: a phosphate starvation inducible promoter in *B. subtilis*

From the data described above, it was likely that *xpaL1*, *xpaL2*, and ORFL3 could form a transcriptional unit

Fig. 5. Subclones of the 4 kb insert of p1881 on pTTQ19 or pMK2004. The DNA fragments inserted behind the *tac* promoter (P_{tac}) on pTTQ19 or behind the promoter for the tetracycline resistance gene (P_{tet}) on pMK2004 are shown as lines. The phenotype of each clone on low-phosphate medium plates after transformation into Xph90a is also shown. IPTG was added to the medium to induce the *tac* promoter on pTTQ19 clones. B (blue) indicates the XPA-positive phenotype and W (white) indicates the XPA-negative phenotype. (B) indicates that the XPA phenotype was observed when IPTG was dropped on the colony. IPTG in the plate prevents the growth of the cells in such cases. The position and the direction of each open reading frame is shown above the restriction map with an arrow. The relative position and the direction of the *tac* or *tet* promoter to each insert is also indicated with an arrow. Abbreviations for restriction sites are: B, *BclI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; Ha, *HaeIII*; P, *PvuII*; X, *XhoI*; XII, *XhoII*. (See Methods for the construction of the above clones.)

expressed from a promoter which was located upstream of *xpaL1*. The promoter activity was tested in *B. subtilis* 168 by creating a fusion between the *RsaI*₁–*RsaI*₂ fragment (Fig. 3) and the *lacZ* gene on the integration expression vector, pDH32 (Shimotsu & Henner, 1986). The resulting expression clones (pKL11 and pKL12, Fig. 6) were transformed into *B. subtilis* 168 after linearization at the unique *PstI* site. The transformants of pKL11

Fig. 6. Map of the expression clones, pKL11 or pKL12. The 350 bp *RsaI* fragment containing the promoter for the *xpaL1*, *xpaL2*, and ORFL3 genes (P_{xpaL}) was ligated first at the *HincII* site of pUC7 and then inserted at the *BamHI* site of pDH32 using the two flanking *BamHI* sites in the polylinker of pUC7. pKL11 has the insert in such orientation that the *lacZ* gene could be expressed from the promoter for the *xpaL1*, *xpaL2* and ORFL3 genes. pKL12 has the insert in the other orientation. The orientation of each gene on the vector is indicated by an arrow. The interrupted *B. subtilis* *amyE* gene on the plasmids facilitates homologous recombination at the *amyE* locus on the chromosome, creating gene replacement. The transformants, which were chloramphenicol resistant and negative for α -amylase production, were screened on low-phosphate defined medium plates supplemented with X-Gal to detect β -galactosidase activity.

(MH1451), in which the *lacZ* gene was being expressed by the promoter in front of *xpaL1*, had a blue colony phenotype on low-phosphate defined medium plates. Neither the colony carrying the vector (MH1001) nor the colony carrying the clone with the *RsaI* fragment in the reverse orientation (MH1452) showed any detectable β -galactosidase production when integrated into the *amyE* locus. However, none of the transformants with each expression clone showed β -galactosidase production on either high-phosphate defined medium plates or Schaeffer's sporulation medium plates. PhoP is the homologue of PhoB in *E. coli*, the positive activator of the two-component phosphate regulatory system (Makino *et al.*, 1989; Seki *et al.*, 1988). In a *B. subtilis* *phoP* strain, *phoP12* (Le Hegarat & Anagnostopoulos, 1973), the promoter for *xpaL1* and *xpaL2* was active on both low- and high-phosphate defined medium plates. These results agreed well with those from liquid culture assays (data not shown). These data indicate that the promoter for *xpaL1*, *xpaL2* and ORFL3 is a phosphate starvation inducible (*psi*) promoter whose expression is regulated by PhoP.

Three possible mechanisms for the phenotypic complementation

Three possible mechanisms for the phenotypic complementation by XPA clones were considered: (i) the *Bacillus* XPA clones might encode small phosphatases, (ii) the clones might encode regulatory proteins which increased production of phosphatases in *E. coli* other than APase encoded by the *phoA* gene, or (iii) the proteins encoded by the XPA genes might affect the membrane permeability, so that XP entered the cell more readily and was hydrolysed by cellular phosphatases such as serine phosphatase. The attempt to test the first possibility by assaying phosphatase activity of the XPA gene products which were synthesized using an *in vitro* translation kit (purchased from Amersham) was not successful because the S-30 cell extract of the translation kit contained phosphatase activity itself. However, the XPA gene products may not be phosphatases, since extensive computer search did not reveal any significant homology between the XPA genes and the known phosphatase genes. The isolation of such small phosphatases from prokaryotes has not been reported either. The second possibility was tested by Western blotting of the *E. coli* cell lysates harbouring various clones with antibodies raised against APase either from *E. coli* or from *B. licheniformis* MC14. The cell lysates with various XPA clones including *xpaL2* and *xpaB* did not show consistent differences in protein band patterns from that of *E. coli* cell lysate without an XPA clone. In some cases, we could detect a 60 kDa or a 45 kDa protein cross-

reacting with the antibodies in the presence of an XPA clone, but the detection of the proteins was subject to day to day variation (data not shown). Previously, Hulett *et al.* (1985) had detected a 60 kDa protein cross-reacting with *B. licheniformis* MC14 anti-APase antibody in the *E. coli* cell lysates that were transformed with pMH81 (p1881). However, the detection of the 60 kDa protein has shown the same variation with the current polyclonal antibody preparation. The third possibility was supported by the computer analysis predicting *xpaL1*, *xpaL2*, and *xpaB* as genes encoding integral membrane proteins and the observation that overexpression of these genes was detrimental to host *E. coli* cells. Recently, W. Metcalf & B. Wanner identified an *E. coli* gene which apparently allowed transport of organophosphates, including XP, which were subsequently hydrolysed by cytoplasmic phosphatases in *E. coli* (unpublished data and personal communication). Failure to isolate chromosomal mutations which caused the loss of the XPA phenotype in the presence of these genes in both laboratories suggested that there might be more than one phosphatase hydrolysing XP in the cytoplasm of *E. coli*.

E. coli phoA mutants have been used in various laboratories, including ours, as hosts to clone genes of the *pho* regulon. An indole phosphate hydrolase gene was isolated from *Nostoc commune* UTEX 584 using an *E. coli phoA* mutant and low-phosphate-XP medium (Xie *et al.*, 1989). The expression of the gene was altered in *E. coli*. McCarter & Silverman (1987 and personal communication) attempted to clone genes regulated by phosphate concentration in *Vibrio parahaemolyticus* using *E. coli* as the screening system and XP as the substrate. They isolated a clone of a phosphate-regulated major outer-membrane protein and some clones with phosphatase activity. However, the phosphatase clones were not APase clones even though they were transcribed from mRNAs which were phosphate starvation inducible. Cloning of *phoA*, *phoB* or *phoR* equivalents of *Pseudomonas aeruginosa* using similar screening systems was reported by Filloux *et al.* (1988). However, from our data, phenotypic or genetic complementation of the *phoA*, *phoB* or *phoR* mutations by the clones from a foreign organism does not always represent the isolation of *phoA*, *phoB* or *phoR* clones. Complementation of various *pho* mutants of *E. coli* by clones from other species must be analysed at the protein level or the DNA sequence level. Since amino acid sequences of APases seem to be conserved among various organisms (Kam *et al.*, 1985; Kim & Wyckoff, 1989; Hulett *et al.*, 1990), DNA sequencing analysis of putative APase clones would be an excellent tool to confirm the isolation of APase gene from an organism. Recently, we have cloned portions of APase structural genes from *B. licheniformis* MC14 and *B. subtilis* using NH₂-terminal protein sequencing data

of purified *Bacillus* APases (Bookstein *et al.*, 1990; Kapp *et al.*, 1990).

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