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, 1971b; 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, 1971b; 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 (XhoI-PvuII, 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; Fillolux 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+ lacZΔ24 phoEA15 proC+ phoR+ trp rpsL; from P. Berg) and BW12720 (DE3(lac)74 Δ(phaA532 PspI1) phoD(EcoB) phoM(wt), araC1655 frr-1655; from B. Wanner) were used as host strains for XPA clones. E. coli POI 1734 [MAL103 with Mu dI 1734 (KmR)] and POI 1734 [MCI1040 (Mu cts)] with Mu dI 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 poly linker, the lacP 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-indoly l phosphate (B-poluidine salt (XP, Sigma) was added at 50 µg ml−1. Antibiotic selection of E. coli transformants was done on Luria-agar plates containing penicillin G at 150 µg ml−1, kanamycin at 50 µg ml−1, or tetracycline at 30 µg ml−1. Bacillus transformants were selected on TBAG plates (Tryptose Blood Agar Base, Difco, containing 0.1% glucose) supplemented with chloramphenicol at 5 µg 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-K$_2$HPO$_4$. Schaeffer’s sporulation medium was prepared according to Schaeffer et al. (1965). 5-Bromo-4-chloro-3-indoly l β-d-galactoside (X-Gal) was added at 50 µg 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 spaB 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, POI 1734 carrying Mu dI 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 poly linker 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.
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 PstI and shotgun cloned in an E. coli vector, pMK2004. Putative APase clones were selected and screened on low-phosphate complex medium 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 ml) was applied to the colony. The subclone of ORFR7 was constructed by cloning BclII-XhoI fragment on pMK2004 digested with HindIII, -HindIII fragment of pKLQ13 was deleted by digesting the clone with HindIII and religating the plasmid. The subclone of ORFL3 containing XhoI-PvuII, PstI fragment was inserted into pTTQ19 at the BamHI site. XPA clones were selected and screened on neopeptone-kanamycin. In order to isolate APase genes, genomic DNA of either B. subtilis 168 was linearized at the PstI site and treated with Bal31 exonuclease. The Bal31 exonuclease treated p1881 was digested with EcoRI and the resulting DNA was ligated to pTTQ19 at the EcoRI and SmaI sites. In order to construct the subclone of ORFL2 (pKLQ12), the HindIII fragment of pKLQ13 was deleted by digesting the clone with HindIII and religating the plasmid. The subclone of ORFL3 (pKLQ13) was constructed using the XhoI site located in the middle of ORFL2 and the BglII site located 500 bp downstream of ORFL3. The XhoI-BglII fragment was ligated to pTTQ19 at the BamHI site. XPA clones were selected for further analysis and are discussed above the lines. For the construction of p1881, the 4 kb XhoI-EcoRI fragment was inserted into pUC18 at the SauI and EcoRI sites. For xpaB, the 0.45 kb PstI fragment was ligated with pUC18 digested with PstI. 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, BglII; E, EcoRI; H, HindIII; P, PstI; S, SalI; X, XhoI. (b) Strategy for DNA sequencing of the XhoI-PvuII fragment of p1881. Sau3AI or HpaII was used to generate subclones for DNA sequencing. Some other restriction enzymes were also used. The arrowheads above the line indicate the Sau3AI restriction sites, and those below the line indicate the HpaII restriction sites. Bidirectional arrows indicate that both strands of the fragment were sequenced. Restriction sites abbreviated as in (a), plus R, Rsal.

DNA sequencing analysis of p1881 and xpaB clones

The 2.3 kb XhoI-PvuII fragment of p1881 and the 0.45 kb PstI fragment of xpaB were sequenced. Restriction maps for p1881 and xpaB are shown in Fig. 1(a).
(a) Xhol.

CTCGAAGCCAAATGGCGCAACTTACCTTGCGCAGCTTTCACACGCTTTAAACGGATGCTG 60

V F P

Real

TAAATTATGCGGACACGACCCTAAAAGCTTGTTTACACAAAAGATGCCCCAGGCTGATTGATTGT 120

N I R V A S F N T I C F G T R Y K

TGAAGGCTGCAATAGCGAAAAATTTGATATATATACCTCTGCCATCTGTATGCGCAAA 180

N F T C Y C F F P F H T K I Y R C Y Q R

AGATATGCGGCTGGCCGCGTTTTTTAGTTAGGTTAGCTACCTAACTGGCGATCTTTG 240

F I D A T G R K E T Q L H L M E I P C T

AAATCTCAGAGTATGATCGTCAAGTGGGTATGTGTAAGTGGTACGCGGACGCA 300

W Y E A T D Y A A L S H Q H Y H R S R F

S S L Y L L D M

← ORF7

TAAAATATGGAAGAAGGAGGATTAAATATCCG GAA GCA CCA GAA CTG GAT ATA 411

Met Pro Glu Ala Pro Glu Leu Asp Ile

Real.

TTC CAA AAA GAA GTT CAG GAG ATG 456

Phe Gln Lys Val Glu Gln Glu Met

Real.

GAA CAG GGC GTC AGT ACA CTC GAA AAA ACA CCA GAC CAC GAT 501

Glu Gln Arg Val Ser Thr Leu Glu Arg Thr Ser Asp Arg His Asp

CAG CAA ATC ATC TCG ATT AAC GAA AAG CTG AA AAT ACA GAA GAG 546

Gln Glu Ile Ile Pro Ser Leu Gln Lys Gln Asp Ile Gln

AAT ACC ACC TGG ATT AAG GCC AGC GTC ACA GCG GCG GTA ATT ACA 591

Asn Thr Thr Trp Ile His Tyr Val Glu Asp Arg Ser Ile Gly Thr Ala Val

GGC GTC ACC ACC ATC GTC GGC GCC GCC ACC ATC GCT GGT TTT TAT 636

Ala Val Ser Thr Gly Ile Ile Gly Gly Ala Ile Ala Val Phe Tyr

ORF2

AAC GAG GAT GTC GCG AGC ATG GCG GTT TTA AGG GCC GCA GTC 683

Lys Gly Thr Val Val Arg Leu Leu Leu Leu Phe Ile Ala Leu Val

Real.

AAA GGC ACG GTC GTC AGG ACG GTG CCT TTT TTT ATT GCG GTG 728

Lys Gly Thr Val Val Arg Thr Val Val Phe Ile Ala Leu Val

Real.

AAAT CAC ACG TTA ACC AAG AGA ATG CAC GAT 773

Met Phe Gln Gly Pro Val Val Leu Pro Val Ala

GAA GAT CAG ATT CAT ACA TGG GCC GAT GCC CTG TAT TGG GCC GGA 799

Glu Asp Glu Ile His Thr Leu Ala Asp Leu Tyr Ser Ala Gly

TCT GCC GCT TTT AAC ATC GTC GAA TCG TCC TGT GCG TCG TGA AAG 863

Ser Ala Ala Phe Ile Ala Ala Ser Leu Val Ala Trp Tyr Lys

AAG AAC TAT GTG AGC AAG AAA GGG AAA ATG CAA AAA GAT TTA 908

Asn Asn Tyr Val Thr Gln Gly Asp Ala AAA AAG Glu Ala Met

Real.

CAG AAA AAA GGT TTA AAC AAA TAAAGCTCCTTCCAGACTTTCCAAGAATTTG 962

Gln Lys Gln Trp Leu Asp Ala

ORFL3

TTAAAAAGGAAATGATGAGTTTG GTC AAA GTA TTA ATC GAT CAA 1012

Met Val Leu Val Ile Asn Phe Val Leu

Real.

GTC AAT CAA TAC GAC GCT GGG GCC GTC TTT ACG ATC GCT GCA TTG 1057

Val Asn Gln Tyr Arg Ala Gly Val Phe Thr Ile Ala Ala Leu

GCG ATC GTC ATG CAT TGG ACC GGG GCG GCT GTA CGT 1102

Gly Ile Val Met His Trp Thr Ala Thr Gln Asp Ala Leu

AAT GAG CCA AAT TAT TAC AAC GCA TAC ATT GCC GAG ACC GCT 1147

Asn Glu Arg Thr Tyr Phe Asn Ala Asn Ala Ala Gln

Real.

ATT TGG GCT TGT CAT TAT TGG GCC GAC CAT GAG GAG CAT 1192

Tyr Ala Ser Ala His Tyr Phe Val Asp Arg Glu Ala Glu

Real.

ATT CCC GAT CAT GAA GTC GCA TAT CAT GCG GAT CAT GAG CTT TGT GAG 1237

Ile Ile Pro His Glu Val Ala Tyr His Ala His Asp Glu

Real.

CAG GCC TAT GTC GAT CCG CGC CAT GAG GCG CAG CAT 1282

Arg Cys Tyr Val Ser Phe Leu Lys Pro Asn Ala Asn Thr Thr Ala

Real.

CAG AGC ATT GCC AAT GCG GCA GAG CTT GTA GCC GAT TGG TCA AAG 1327

Glu Thr Ile Arg Asn Ala Ala Ala Asp Leu Cys Lys

Glu Thr Ile Arg Asn Ala Ala Ala Asp Leu Cys Lys
Fig. 2. Nucleotide sequences of the *XhoI*–*PstI* fragment of p1881 and the *xpaB* gene. The DNA sequence 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*–*PstI* 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 sequences. (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 *dl1–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* toward *PvuII* 

**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*, *RsuI*; *S*, *SauI*; X, *XhoI*.

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, GAAAAGGA. 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–PvuII* fragment contained promoter activity for ORFL4 (Fig. 2; C. Bookstein & F. M. Hulett, unpublished data). Two different transcriptional fusions mapping very close to *XhoI* indicated transcriptional activity in the opposite direction. Among several ORFLs (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).
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 *XhoI* toward *PvuII*, 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.

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

**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 amino acid sequence between 288 and 352 of the MPC are shown in comparison with each other. The identical residues between the two sequences are marked with asterisks. They share 49% identity over the entire amino acid sequence (87 amino acid residues). (b) Predicted amino acid sequence homology between the product 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).
The colony carrying the clone was grown on a neopeptide-XP plate overnight and a drop of 0.1 m-IPTG was applied to the colony. The colony harbouring the ORFL3 subclone (pKLQL3, Fig. 5) remained white on neopeptide-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 neopeptide-XP plates, indicating that ORFR7 is another XPA gene. The genes cloned on pKLQL1, pKLQL2, 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 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-RsaI 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

![Diagram of expression clones](Image)

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 BamH1 site of pDH32 using the two flanking BamH1 sites in the polylinker of pUC7. pKL11 has the insert in such an 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 Rsal 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 ORF13 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 attempt to test the second possibility by assaying phosphatase activity 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.
References


