Cloning and Characterization of the *Bacillus licheniformis* Gene Coding for Alkaline Phosphatase

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The structural gene for alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) of *Bacillus licheniformis* MC14 was cloned into the PsiI site of pMK2004 from chromosomal DNA. The gene was cloned on an 8.5-kilobase DNA fragment. A restriction map was developed, and the gene was subcloned on a 4.2-kilobase DNA fragment. The minimum coding region of the gene was localized to a 1.3-kilobase region. Western blot analysis was used to show that the gene coded for a 60,000-molecular-weight protein which cross-reacts with anti-alkaline phosphatase prepared against the salt-extractable membrane alkaline phosphatase of *B. licheniformis* MC14.

A number of biochemical, physiological, and immunological studies concerning the synthesis and localization of alkaline phosphatase (APase; orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) in *Bacillus subtilis* and *Bacillus licheniformis* have been reported. APase of *B. licheniformis* has been identified in a secreted form (F. M. Hulett, K. Stuckmann, D. Spencer, T. Sanopoulou, and F. Abedinpour, submitted for publication), a cell-bound form released by lysozyme (8, 9), a salt-extractable membrane form (10, 17), a detergent-extractable membrane form (19), and an enzymatically inactive form in the cytosol (unpublished data). These various APase species are immunologically related. It has been shown that culturing conditions significantly affect both the distribution and the amount of synthesis of APase (8, 15, 19). Although there are less extensive localization studies of APase in *B. subtilis*, seemingly contradictory reports of cell-bound (22, 24) or membrane-associated (inner leaflet) (7) and secreted APase (4, 27) in the organism have also been made.

It is difficult to interpret the localization data (active dimer associated with inner leaflet of cytoplasmic membrane [6, 21] versus active dimer secreted [Hulett et al., submitted for publication]) based on any of the current models for protein secretion (3, 7, 23–25) or insertion of proteins into membranes (13, 18).

There are at least two possibilities which would explain these data. Either there is a single structural gene which is under uniquely complex transcriptional and translational regulatory control or there are multiple structural genes for APase which account for the synthesis of different species directed to different locations depending on the growth conditions. (The latter possibility is supported by the fact that there have been no APase-negative mutants isolated in the *Bacillus* strains which are due to mutation in the structural gene [phoA].)

We have cloned the *phoA* gene of *B. licheniformis* (MC14) on multicity plasmid pMK2004 to facilitate regulatory and structural studies on APase. This paper reports the steps involved in cloning and subcloning the *phoA* gene, a restriction map defining a minimum coding region, and evidence of relatedness of the cloned gene product to the APase isolated and characterized previously.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used are given in Table 1. *Escherichia coli* Xph90a, which contains deletion E15 within *phoA* (phoA8), was obtained from J. Beckwith via P. Berg (1, 11).

**DNA isolation.** Small-scale plasmid DNA isolation was done by a modification of the method of Birnboim and Doly (2). Cesium chloride equilibrium centrifugation DNA isolation was used for large-scale purification of plasmid DNA.

**Media.** Antibiotic screening was performed on *Luria* agar plates, using penicillin G at 150 µg/ml, kanamycin (Kan) at 50 µg/ml, or tetracycline at 30 µg/ml.

Screening and selection were carried out on 1% Neopeptone plates (Difco Laboratories) containing 1.5% Noble agar, 0.1 M Tris Base (pH 7.5), 1% NaCl, 50 µg of kanamycin per ml, and 50 µg of 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine (XP) (Neo-XP-Kan plates). The indicator dye (XP) was obtained from Sigma Chemical Co. When selection on penicillin G was required, screening was carried out as a second step on the above Neopeptone plates with an antibiotic. It has been reported (1) and we confirmed that XP significantly decreases the effect of penicillin concentration.

**Enzymes.** Restriction enzymes were obtained from Bethesda Research Laboratories or Amersham Corp. Digestions were carried out in high, medium, or low buffer as described previously (14). Digestions were at 37°C for 1 h.

Ligation was performed with T4 ligase at 4°C overnight in 66 mM Tris-hydrochloride (pH 7.9)–33 mM NaCl–10 mM MgCl2–1 mM β-mercaptoethanol–25 mM ATP.

**Transformation.** Transformation was carried out by the method of Cohen et al. (5).

**Gel electrophoresis.** DNA fragments generated by restriction enzyme digests were analyzed in 0.8% agarose gels in 40 mM Tris–20 mM sodium acetate. Smaller fragments were analyzed on acrylamide gels in 0.089 M Tris-borate buffer containing 2 mM EDTA.

**Insertional Tn5 mutagenesis.** *E. coli* Xph90a carrying plasmid pMH81 was infected with bacteriophage lambda carrying transposon Tn5. The multiplicity of infection was one. Adsorption was carried out on ice for 30 min before the cells were grown under plasmid selection (penicillin) at 30°C for 3 h. Cells were then plated on *Luria* plates containing 400 µg of kanamycin per ml. (Tn5 carries a kanamycin resistance gene.) We had previously determined that *E. coli* with Tn5 inserted into the chromosome were killed by concentrations of kanamycin of 200 µg/ml but that *E. coli* cells carrying Tn5 on a multicopy plasmid could grow in the presence of 600 to 800 µg of kanamycin per ml. Therefore, the *Luria* plates
containing 400 μg of kanamycin select for cells containing Tn5 inserts on pMH81. The colonies on Luria plates containing 400 μg of kanamycin were washed off, and the plasmids were isolated from the combined colonies. The resulting plasmid collection was used to transform strain Xph90a. Screening and selection were carried out on Neo-XP-Kan plates as described above. White colonies represent colonies carrying plasmid pMH81 with Tn5 inserted into the APase gene, and blue colonies represent colonies carrying plasmid pMH81 with inserts external to the gene.

**ExoIII-SI deletion mapping.** Deletion mapping was carried out by using a slight modification of the procedure described by Roberts and Lauer (16). pMH81 (10 μg) was digested with XhoI, ethanol precipitated, and suspended in ExoIII buffer (16) containing 50 mM NaCl. Thirty units of ExoIII nuclease were added and incubated at 22°C for 90 min. Samples were taken between 30 and 90 min, and the reaction was stopped by adding an equal volume of 2X SI buffer (16). Five units of SI was added and incubated for 30 min at 20°C. The DNA was then phenol-chloroform extracted, ethanol precipitated, ligated, and used to transform strain Xph90a. Screening for APase activity was carried out on Neo-XP plates.

**Preparation of DTP paper and Western blotting of protein from gel to DTP paper.** The procedures have been outlined previously (26). The only modification was that iodinated goat anti-rabbit immunoglobulin G was substituted for iodinated protein A for labeling the primary antibody. The primary antibody was made to the salt-extractable, membrane-associated APase (10, 17).

**RESULTS**

**Cloning and restriction mapping of the phoA gene.** To clone the APase gene of *B. licheniformis* MC14, we constructed a *PstI* chromosomal DNA fragment library containing 6,000 independently isolated clones. *B. licheniformis* MC14 chromosomal DNA was cut with *PstI* and mixed with the vector pMK2004 (kindly provided by P. Matsumura) which had been cut with *PstI* and pretreated with calf intestinal APase. These DNA fragments were ligated and used to transform *E. coli* Xph90a (Table 1). Selection and screening were carried out by using 1% Neopeptone plates containing kanamycin and XP. In addition, colonies were screened for ampicillin and tetracycline resistance. Since only one *PstI* recognition site exists in pMK2004 and this site is in the gene coding for ampicillin resistance, loss of ampicillin resistance was an indication of insertional inactivation. In these experiments it was found that between 80 and 85% of colonies which were kanamycin and tetracycline resistant were ampicillin sensitive. Putative APase colonies turned blue. Approximately 1 in 3,000 colonies was blue. Plasmid DNA from the blue colonies was isolated by a modified method of Birnboim and Doly (2) and cut with *PstI*. The sizes of the two DNA fragments observed corresponded to linear pMK2004 (5.2 kilobases [kb]) and to an insertion fragment of 8.45 kb.

When the plasmid DNA was used to transform *E. coli* Xph90a, all transformed cells (kanamycin and tetracycline resistant, ampicillin sensitive) were blue on XP indicator plates. This plasmid, pMH8, is shown in Fig. 1 with restriction sites indicated. The following deletion plasmids and subclones were generated to determine the locus of the phosphatase gene (Table 2). An *EcoRI*-*EcoRI* deletion (pMH8Δ1) of pMH8 resulted in a plasmid 2.75 kb smaller

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Xph90a</td>
<td>F'- lacZ624 phoAE15</td>
<td>J. Beckwith (11)</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>phoA*</td>
<td>F. M. Hulet (10)</td>
</tr>
<tr>
<td>MC14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMK2004</td>
<td>Amp'- Tet' Kan'- Whitea</td>
<td>M. Khan (12)</td>
</tr>
<tr>
<td>pMH8</td>
<td>Amp'- Tet' Kan'- Blue</td>
<td>This study</td>
</tr>
<tr>
<td>pMH8Δ1</td>
<td>(EcoRI2-EcoRI1)</td>
<td></td>
</tr>
<tr>
<td>pMH8Δ2</td>
<td>Amp'- Tet' Kan'- White</td>
<td>This study</td>
</tr>
<tr>
<td>pMH8Δ1</td>
<td>(Xho1-EcoRI1)</td>
<td></td>
</tr>
<tr>
<td>pMH8Δ2</td>
<td>Amp'- Tet' Kan'- White</td>
<td>This study</td>
</tr>
<tr>
<td>pMH8Δ2</td>
<td>(PvuII2-PvuII3)</td>
<td></td>
</tr>
<tr>
<td>pMH8Δ1</td>
<td>Amp'- Tet' Kan'- White</td>
<td>This study</td>
</tr>
<tr>
<td>pMH8Δ1</td>
<td>(HindIII1-HindIII1)</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates the ability to cleave XP on indicator plates.

![FIG. 1. Restriction map of pMH8 (13.65 kb). Vector DNA is represented by a heavy solid line. Insert DNA is represented by double lines. Fragments are referred to by the flanking restriction site numbers, e.g., Xho1-PvuII1 identifies the region which includes the phoA gene.](https://jb.asm.org)

**Table 2. Deletion and subclones of pMH8 and pMH81**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Construction</th>
<th>Fragment</th>
<th>Cloned into: Xph90a phenotype (colony)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMH8</td>
<td><em>EcoRI</em></td>
<td>10.95-0</td>
<td>Blue</td>
</tr>
<tr>
<td>pMH8</td>
<td><em>PvuII</em></td>
<td>5.9-8.3</td>
<td>White</td>
</tr>
<tr>
<td>pMH8</td>
<td><em>PvuII</em></td>
<td>5.9-8.3</td>
<td>Smal site of pMK2004</td>
</tr>
<tr>
<td>pMH8</td>
<td>*Xho1-<em>RI</em></td>
<td>6.4-10.95</td>
<td>RI-Xho1 site of pMK2004</td>
</tr>
<tr>
<td>pMH81</td>
<td><em>HindIII</em></td>
<td>4.1-7.2</td>
<td>White</td>
</tr>
<tr>
<td>pMH81</td>
<td><em>BglII</em></td>
<td>6.0-7.0</td>
<td>Blue</td>
</tr>
</tbody>
</table>

* indicates the ability to cleave XP on indicator plates.
above. Deletion plasmids were used to transform strain Xph90a. Plasmids from blue and white colonies were isolated, and the extent of the deletion in each was mapped. Colonies containing plasmids with deletions from Xhol2 to 3.6 (on pMH81) remained blue. Plasmids with larger deletions (from Xhol3), past the point (3.64 kb) at which Tn5 insertion caused inactivation of the APase gene on pMH81, showed no APase production when used to transform Xph90a. This locates one terminus of the gene between 3.60 and 3.64 kb. The minimum size of the coding region is calculated to be 1.3 kb with the right terminus of the gene in pMH81 close to 3.64 kb and the left terminus of the gene containing the PvuII3 site. Preliminary transcription mapping studies indicate that transcription starts at least 150 bases before the PvuII3 at 5 kb.

Expression of B. licheniformis gene in E. coli. Although the blue colony color of cells (Xph90a) containing pMH8 or pMH81 on X plate is easily detected after 2 days, no APase production in growing cultures could be measured. Western blot analysis was used to determine (i) the relatedness to the APase species previously studied and (ii) the size of the cloned gene product. Figure 4 shows an autoradiogram of a Western blot which had been treated with rabbit anti-APase, followed by 125I-labeled goat anti-rabbit immunoglobulin G. Lanes containing purified APase (lane 1) or cell lysates of Xph90a carrying pMH81 (lane 3) show a 60,000-molecular-weight band (Fig. 4). (The subunit size of B. licheniformis MC14 APase is 60,000 [10, 17].) Lane 2 (Fig. 4) which contains a lysate of Xph90a carrying pMK2004 does not show this band.

DISCUSSION

We have cloned the structural gene for APase into an E. coli plasmid, pMK2004. The original DNA fragment (8.45
These and other purposes, in vitro transcription, S1 nuclease mapping, and sequencing studies are being initiated.

ACKNOWLEDGMENTS

I thank Philip Matsumura for his generous contributions of bacterial stains, plasmids, enzymes, and equipment, as well as technical guidance and innumerable helpful discussions. I am grateful to Jung-Wan K. Lee for restriction mapping of pMH81. I thank Diana Rivera for her excellent technical assistance.

LITERATURE CITED


FIG. 4. Cross-reactivity of the APase of B. licheniformis and of pMH81-encoded protein with anti-APase. Purified APase and cell lysates of strain Xph90a carrying pMK2004 or pMH81 were resolved on a sodium dodecyl sulfate-polyacrylamide gel, electrophoretically transferred to DPT paper, and reacted with anti-APase antiserum and 125I-labeled goat anti-rabbit antibody. The blot was subjected to autoradiography for 48 h. Lane 1, purified APase; lane 2, pMK2004; lane 3, pMH81.