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Two alkaline phosphatase genes positioned in tandem in *Bacillus licheniformis* MC14 require different RNA polymerase holoenzymes for transcription

(*in vitro* transcription/gene duplication/σ factor/protein secretion)

F. MARION HULETT*, PEI-ZHI WANG†, MICHAEL SUSSMAN*, AND JUNG-WAN K. LEE*

*Department of Biological Sciences, Laboratory for Cell, Molecular, and Developmental Biology, University of Illinois at Chicago, P.O. Box 4348, Chicago, IL 60680; and †Department of Biochemistry and Biophysics, University of California at Davis, Davis, CA 95616

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ABSTRACT **Southern transfer analysis of *Bacillus licheniformis* MC14 DNA, using as probe a DNA fragment from within the coding region of a previously cloned alkaline phosphatase (APase) gene, revealed a second area of hybridization adjacent to the cloned APase gene. A second APase gene (*APase II*) was subcloned from the same plasmid clone, pMH8, from which the first APase gene (*APase I*) had been subcloned. The two genes are arranged in tandem with several hundred base pairs separating them. Immunoblot analysis showed that both code for M_r 60,000 proteins that crossreact with anti-APase. Both proteins enzymatically cleave 5-bromo-4-chloro-3-indolyl phosphate. *In vitro* transcription showed that *APase I* and *APase II* are transcribed in the same direction but that the two genes require different forms of *Bacillus* RNA polymerase: σ^{55}- and σ^{37}-containing RNA polymerase holoenzymes, respectively.**

Localization studies have shown that alkaline phosphatase [APase, orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] of *Bacillus licheniformis* MC14 and *Bacillus subtilis* is located intracellularly (1–3) and extracellularly (4). The active intracellular and extracellular APase species of *B. licheniformis* show no significant differences with respect to amino acid composition, M_r, antigenicity, limited proteolysis profiles, or substrate specificity (3, 5–9).

If a single gene is responsible for these APase species, it is difficult to interpret the localization data [active dimer associated with the inner leaflet of the cytoplasmic membrane (2, 3) vs. active dimer secreted (9)] based on any of the current models for protein secretion (10) or insertion of proteins into membranes (11). There have been no APase$^-$ *Bacillus* mutants isolated that are due to mutation in the structural gene (*phoA*) to indicate that there is a single gene for APase.

Hulett (12) has reported the cloning of a structural gene for APase from *B. licheniformis* MC14. We report here the use of an internal probe from the coding region of this gene to identify a second area of hybridization adjacent to the cloned gene. We have cloned a second APase gene, which makes a M_r 60,000 protein that crossreacts with anti-APase and have shown that the gene product has enzymatic activity. These genes are positioned in tandem and are transcribed in the same direction but have promoters that require different forms of *Bacillus* RNA polymerase.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains and plasmids used are given in Table 1. *Escherichia coli* strain Xph90a, which contains deletion E15 within *phoA* (*phoA*8), was obtained from J. Beckwith (Harvard University) through P. Berg (Bethesda Research Laboratories).

DNA Isolation. Small-scale plasmid DNA isolation was done by a modification of the method of Birnboim and Doly (15). Cesium chloride equilibrium centrifugation was used for large scale purification of plasmid DNA. *B. licheniformis* DNA was isolated by the method of Marmur and Doty (16).

Media. Antibiotic screening was done on Luria-agar plates, using penicillin G at 150 μg/ml, kanamycin at 50 μg/ml, or tetracycline at 30 μg/ml.

Screening and selection were carried out on Neo/X*P* plates [1% (wt/vol) Neopeptone (Difco) plates containing 1.5% (wt/vol) noble agar, 0.1 M Tris Cl (pH 7.5), 1% (wt/vol) NaCl, kanamycin (50 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (X*P*) p-toluidine salt (50 μg/ml; Sigma)]. When selection with penicillin G was required, Tris was replaced with Mops [3-(*N*-morpholino)propanesulfonic acid], as described by Guan *et al.* (17).

Enzymes. Restriction enzymes were obtained from Bethesda Research Laboratories or Amersham. Digestions were carried out at 37°C for 1 hr in the buffers recommended by the suppliers.

Ligation was achieved with T4 DNA ligase at 4°C overnight in 66 mM Tris Cl (pH 7.9)/33 mM NaCl/1 mM 2-mercaptoethanol/25 mM ATP.

Transformation. Transformation was carried out by the method of Cohen *et al.* (18).

Gel Electrophoresis. DNA restriction enzyme digests were analyzed in 0.8% agarose gels in 40 mM Tris/20 mM sodium acetate. Smaller fragments were analyzed on polyacrylamide gels in 0.089 M Tris borate buffer containing 2 mM EDTA.

DNA Hybridization. DNA·DNA hybridization was carried out by the method of Southern (19). The hybridization probe was prepared by digesting pMH81 with *Pvu* II and *Hin*dIII. The *Pvu* II_3–*Hin*dIII_3 fragment (see Fig. 1) from within the coding region of the APase gene was isolated on a 3.5% polyacrylamide gel. The fragment was labeled by nick-translation (11).

Exonuclease III/Nuclease S1 Deletion Mapping. Deletion mapping was carried out using a slight modification of the procedure described by Roberts and Lauer (20). Plasmid DNA (10 μg) was linearized with the indicated restriction enzyme, ethanol-precipitated, and suspended in exonuclease

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Abbreviations: APase, alkaline phosphatase; X*P*, 5-bromo-4-chloro-3-indolyl phosphate; Neo/X*P*, peptone/agar medium containing X*P*; kb, kilobase(s); bp, base pairs.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Genotype or phenotype	Source and reference
E. coli Xph90a	F-*lacZ624 phoA*-E15 *proC*$^+$ *phoR*$^+$ *trp rpsL*	J. Beckwith (13)
B. licheniformis MC14	*phoA*$^+$	F.M.H. (7)
pMK2004	Amp^r Tet^r Kan^r	M. Kahn (14)
pMH8	Amp^s Tet^r Kan^r *phoA*$^+$	F.M.H. (12)
pMH81	Amp^r Tet^s Kan^s *phoA*$^+$	F.M.H. (12)
pMH87	Kan^r *phoA*$^+$	This study

III buffer (6.6 mM Tris Cl, pH 7.4/6.6 mM $MgCl_2$/6.6 mM 2-mercaptoethanol) containing 50 mM NaCl. Exonuclease III (30 units/pmol of DNA) was added, and the mixture was incubated at 22°C for 90 min. Samples were taken between 15 and 90 min and the reaction was stopped by adding an equal volume of 2× S1 buffer [exonuclease III buffer plus 0.05 M NaOAc/HOAc (pH 4.0), 0.15 M NaCl, and 6 mM $ZnSO_4$]. Nuclease S1 (5 units/pmol of DNA) was added, and the mixture was incubated at 20°C for 30 min. The DNA was then phenol/chloroform extracted, ethanol-precipitated, ligated, and used to transform Xph90a. Screening for APase activity was carried out on Neo/*XP* plates containing the appropriate antibiotic.

Immunoblotting on Diazophenyl Thioether (DPT) Paper. The procedures have been outlined previously (21). The only modification was that ^{125}I-labeled goat anti-rabbit IgG was substituted for ^{125}I-labeled protein A for detecting the primary antibody. The primary antibody was directed against the salt-extractable, membrane-associated APase.

***In Vitro* Transcription.** The methods described by Goldfarb *et al.* (22) were used.

RESULTS

A Second APase Gene Cloned in pMH8 and pMH87. The APase gene of *B. licheniformis* MC14 was inserted into the *Pst* I site of pMK2004 (pMH8) and cloned as described previously (12). It was further subcloned in a 4.2-kilobase (kb) *Eco*RI/*Xho* I fragment inserted into the *Eco*RI–*Xho* I sites on pMK2004, to yield plasmid pMH81 (Fig. 1). The 0.97-kb *Pvu* II_3–*Hin*dIII_3 fragment within the coding region of this APase gene in pMH81 (Fig. 1) was used as a probe in the Southern transfer analysis. DNA flanking the *Bgl* II_1 site on pMH8 was implicated as a second region of hybridization by the Southern analysis (data not shown). A *Sal* I deletion of pMH8 (pMH87) was constructed (Fig. 1). This deletion plasmid lacks more than half the coding region of the APase gene subcloned in pMH81 (*APase I*) and contains 1.1 kb of the original DNA insert that is also in pMH81. When this deletion plasmid was used to transform *E. coli* Xph90a and

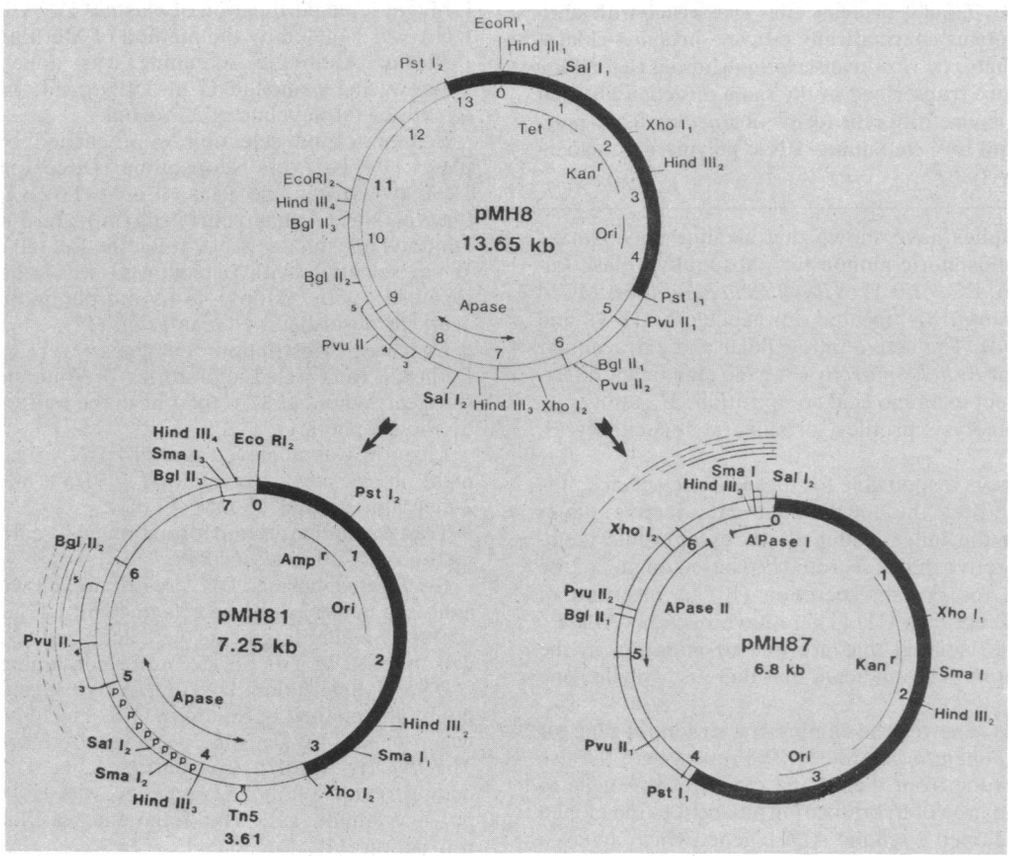

FIG. 1. Subcloning of two fragments from pMH8. The 4.2-kb *Xho* I_2-*Eco*RI_2 fragment from pMH8 was inserted into the *Eco*RI-*Xho* I sites of pMK2004 to yield pMH81 (12). The coding region for *APase I* on pMH81 is indicated. A *Sal* I_2 to *Sal* I_1 deletion of pMH8 yielded pMH87. Xph90a containing pMH87 can also hydrolyze *XP*. The pMH81 fragment used as the probe for Southern hybridization (*Pvu* II_3–*Hin*dIII_3) is indicated by ppp. Exonuclease III/nuclease S1 deletion mapping: for pMH81, solid lines indicate deletion distance from *Bgl* II_2 in pMH81 that resulted in retention of the APase phenotype when the deletion plasmid was used to transform Xph90a and dashed lines indicate lengths of DNA deleted that resulted in APase$^-$ phenotype; for pMH87, solid lines indicate the deletion distance from *Sal* I_2 that allowed retention of the APase phenotype when the constructed plasmid was used to transform Xph90a and dashed lines indicate lengths of DNA deleted that resulted in APase$^-$ phenotype.

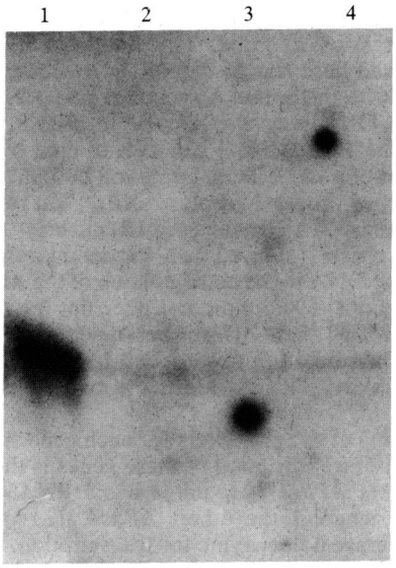

FIG. 2. Crossreactivity of APase of *B. licheniformis* and of pMH81-, pMH8-, or pMH87-encoded protein with anti-APase. Purified APase (lane 1) and cell lysates of Xph90a carrying pMH8 (lane 2), pMH81 (lane 3), or pMH87 (lane 4) were resolved by $NaDodSO_4$/PAGE, electrophoretically transferred to DPT paper, incubated sequentially with rabbit anti-APase antiserum and ^{125}I-labeled goat anti-rabbit IgG antibody, and autoradiographed for 48 hr. The purified APase used was the magnesium-extractable membrane APase (8); the antiserum was raised against this APase species.

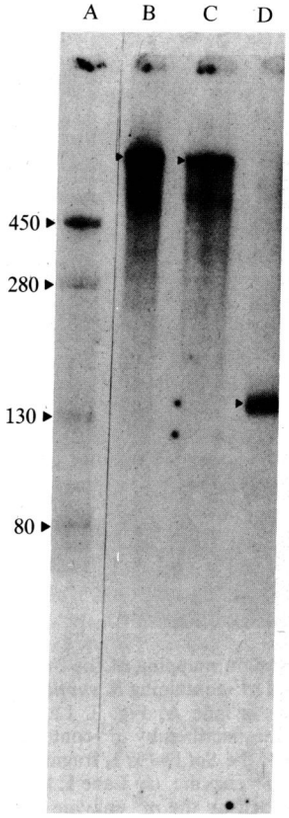

FIG. 3. Transcription mapping of the *Bgl* II_2–*Xho* I_2 fragment of pMH81 with σ^{55}-containing *B. subtilis* RNA polymerase. Lane A: *Hin*dIII fragment of phage ϕ29 DNA transcribed by σ^{55} polymerase to yield transcripts of 450, 280, 130, and 80 nucleotides, used as markers. Lane B: intact *Bgl* II_2–*Xho* I_2 transcribed by σ^{55} enzyme. Lane C: *Bgl* II_2–*Xho* I_2 fragment digested with *Hin*dIII and transcribed by σ^{55} enzyme. Lane D: *Bgl* II_2–*Xho* I_2 fragment digested with *Hin*dIII and *Pvu* II and transcribed by σ^{55} enzyme.

plated on Neo/X*P*/kanamycin plates the colonies turned blue in 1 day. The possibility that the APase phenotype of *E. coli* Xph90a transformed with pMH87 was dependent on DNA previously assigned to the coding region of *APase I* was eliminated by constructing and analyzing deletion plasmids of pMH87 that retained none of the *APase I* gene. Such deletion plasmids of pMH87 complement *phoA*$^-$ *E. coli*.

Deletions of pMH87 were constructed by digestion of pMH87 with *Sal* I, followed by exonuclease III and nuclease S1 digestion as described in *Materials and Methods*. Deletion plasmids were used to transform *E. coli* Xph90a. Plasmids from blue colonies and from white colonies were isolated and the extent of the deletion in each was mapped. Colonies containing plasmids in which all of the DNA from the coding region of the APase gene in pMH81 (*APase I*) was deleted remained blue (Fig. 1). Further deletion (from *Sal* I_1) up to a point approximately 400 bases before *Xho* I_2 also showed APase (APase II) production when used to transform Xph90a. Deletion plasmids in which all DNA from *Sal* I_2 to a point approximately 260 bases before *Xho* I_2 or further was removed showed no APase (APase II) production when used to transform *E. coli* Xph90a (Fig. 1). Therefore, one terminus of the *APase II* gene is mapped at 6.1–6.3 on pMH87.

Both *APase I* and *APase II* Code for M_r 60,000 Proteins that Crossreact with Anti-APase. Previous immunoblot analysis has shown that pMH81 carries a gene that codes for a protein of M_r 60,000 (subunit M_r of *B. licheniformis* APase) (12) that crossreacts with anti-APase. Fig. 2 shows an autoradiogram of an immunoblot that had been treated with rabbit anti-APase followed by ^{125}I-labeled goat anti-rabbit IgG. Lanes containing purified APase (lane 1) or cell lysates of *E. coli* Xph90a carrying pMH81 (lane 2), pMH8 (lane 3), or pMH87 (lane 4) show a M_r 60,000 protein. Therefore, *APase II* codes for a M_r 60,000 protein that crossreacts anti-APase.

Preliminary Characterization of the Promoter for *APase I*. Deletion mapping and *in vitro* transcription studies showed that the promoter for *APase I* lies between *Pvu* II_3 and *Pvu* II_4 on pMH81 and that a σ^{55}-containing RNA polymerase holoenzyme is required for transcription.

Exonuclease III/nuclease S1 deletion mapping was used to locate the terminus of the *APase I* gene in the *Pvu* II_{3-5} region of pMH81. [*Pvu* II_3 is within the coding region of *APase I* (12).] The strategy was to digest pMH81 with *Bgl* II, then digest with exonuclease III followed by nuclease S1 to generate deletion plasmids of pMH81, which then were used to transform Xph90a. Both blue and white colonies were observed on Neo/X*P*/penicillin plates. Deletion-plasmid DNA from blue colonies and white colonies was isolated and the extent of the deletion in each was mapped by digesting the deletion plasmid with *Pvu* II. When the *Pvu* II_5 and *Pvu* II_4 sites were deleted, the APase phenotype was retained (blue colonies). If the *Pvu* II_3 site was deleted, *E. coli* Xph90a transformed with such a plasmid made white colonies. Therefore, the *Pvu* II_4–*Pvu* II_3 fragment contains one end of the coding region of *APase I* (Fig. 1).

The *Bgl* II_2–*Xho* I_2 fragment of pMH81 (*APase I*) was used as template for *in vitro* transcription (Fig. 3). σ^{55} RNA polymerase holoenzyme from *B. subtilis* produced a large transcript (lane B). To better map the promoter and the direction of transcription, the *Bgl* II_2–*Xho* I_2 fragment was digested with *Hin*dIII or *Hin*dIII/*Pvu* II. The transcript was shortened by *Hin*dIII digestion of the template (lane C) and *Hin*dIII/*Pvu*II digestion resulted in a unique 150-nucleotide transcript (lane D). These data, coupled with the results of exonuclease III/nuclease S1 mapping, suggested that the promoter was located toward the right end of the fragment

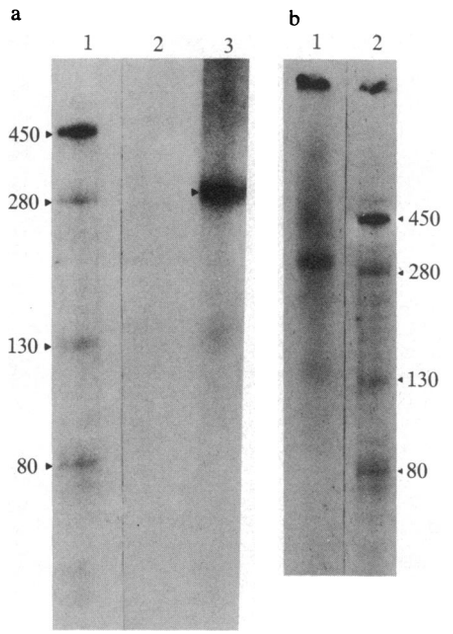

FIG. 4. Transcription mapping of the *Sal* I_2–*Pst* I_1 fragment of pMH87 with σ^{55}- and σ^{37}-containing *B. subtilis* RNA polymerase. (*a*) Lane 1: markers as in lane A, Fig. 3. Lane 2: the *Sal* I_2–*Pst* I_2 fragment intact and transcribed by σ^{55}-containing RNA polymerase holoenzyme. Lane 3, the *Sal* I_2–*Pst* I_1 fragment digested with *Xho* I and transcribed by σ^{37} enzyme. (*b*) Lane 1: the isolated *Sma* I_2–*Xho* I_2 fragment transcribed by the σ^{37} enzyme. Lane 2: markers as in lane A, Fig. 3.

about 150 bases before *Pvu* II_3 and that the *APase I* gene is read rightward by a σ^{55} RNA polymerase holoenzyme.

Preliminary Characterization of the Promoter for *APase II*. Deletion mapping and *in vitro* transcription studies showed that the promoter for *APase II* lies between *Hin*dIII$_3$ and *Xho* I_2 ≈300 base pairs (bp) from *Xho* I_2 and that it requires a σ^{37}-containing RNA polymerase holoenzyme for transcription.

We described above exonuclease III/nuclease S1 deletion mapping that showed that when DNA from *Sal* I_2 (on pMH87) to ≈260 bp before *Xho* I_2 or further was deleted, the *APase II* gene was inactivated. This positioned one terminus of *APase II* between *Sal* I_2 and *Xho* I_2, >260 bp from *Xho* I_2.

In vitro transcription studies were carried out with the *Sal* I_2–*Pst* I_1 fragment of pMH87. σ^{55} holoenzyme of *B. subtilis* did not yield a transcript with this template (Fig. 4*a*, lane 2) but σ^{37} holoenzyme of *B. subtilis* did. *Xho* I digestion of the fragment produced a transcript of ≈300 bp when σ^{37} holoenzyme was used (lane 3). Transcription of the isolated *Xho* I_2–*Sal* I_2 fragment of pMH87 digested with *Sma* I produced the same 300-bp run-off fragment, showing that the run-off transcript (Fig. 4*b*, lane 1) was at the *Xho* I_2 end of the fragment. These data, combined with the deletion mapping data, position the promoter ≈300 bp from the *Xho* I site and indicate that *APase II* is read counterclockwise by a σ^{37}-containing RNA polymerase holoenzyme.

Thus, we have cloned two tandemly positioned APase genes from *B. licheniformis*, one that requires the major polymerase, σ^{55}-containing holoenzyme, and another that requires a minor polymerase, σ^{37}-containing holoenzyme, for transcription.

DISCUSSION

Hulett (12) recently reported the cloning of a structural gene for APase (*APase I*) from *B. licheniformis*. We report here the detection of a second APase gene (*APase II*) by use of a DNA fragment from within the coding region of the cloned gene (*APase I*) as a probe. The Southern hybridization conditions used here should detect DNA sequences >84% homologous with the probe. A second region of homology in the genomic DNA was also cloned in pMH8. A second subclone of pMH8, pMH87, was constructed by making a *Sal* I deletion of pMH8. Transformation of Xph90a resulted in colonies that could hydrolyze X*P*. (We refer to this activity as APase II.) Exonuclease III/nuclease S1 deletion mapping of pMH87 showed two important results. First, deletion of the DNA in the coding region of the *APase I* gene at the 3′ terminus (500 bp) did not interrupt the APase gene cloned in pMH87, nor did further deletion of ≈200 bp. Second, further digestion toward *Xho* I_2 resulted in the loss of APase activity. This positions the two APase genes in tandem.

The mapping of the promoters for each gene suggests that the direction of transcription of these genes is the same and that one gene, *APase I*, requires a σ^{55} RNA polymerase holoenzyme whereas the other, *APase II*, requires a σ^{37} RNA polymerase holoenzyme for transcription. Analysis of these promoters should allow us to determine whether one of these genes is responsible for the APase that is made in small amounts in rich medium during logarithmic growth and localized on the inside of the cytoplasmic membrane (2) whereas the other is responsible for the production of the APase that constitutes 50% of the total protein secreted by this organism when the inorganic phosphate concentration in the medium decreases below 0.075 mM and the culture is entering the stationary phase of growth (23).

Examples of stabilized gene duplication in bacteria are few and for the most part not well characterized. Perhaps the best studied tandem duplicated genes in bacteria are the *hisJ* and *argT* genes of *Salmonella typhimurium* (24, 25). These two genes, which code for two periplasmic amino acid binding proteins of different specificities, are >70% homologous, with regions of >90% homology (25). The leucine transport system in *E. coli* also contains duplicated genes, *livJ* and *livK*, which code for two binding proteins with different specificities. These two binding proteins are ≈80% conserved at the amino acid level but show divergence in both function and regulation (26). Tandem and nontandem duplication of ribosomal genes has been shown in *B. subtilis* (27) and *E. coli* (28), respectively. Other examples of gene duplication include certain genes for enzymes of the aromatic amino acid biosynthetic pathway in *E. coli* (*aroF*, *-G*, and *-H*) (29) and genes encoding elongation factors (*tufA* and *B*) (30, 31) and protein S of *Myxococcus xanthus* (32, 33). In the last two examples, the duplicated gene products are believed to serve identical functions within the cell.

It has been suggested that different genes account for the intracellular serine protease and the extracellular serine protease (subtilisin) of *B. subtilis* and that they arose via gene duplication and divergence (34). As in the case of *Bacillus* APase(s), mutations in the structural gene(s) for subtilisin could not be isolated. [Deletion mutations of subtilisin have recently been constructed *in vitro* (35).] Amino acid sequence homology (50%) was determined by Edman degradation of 50 amino acids of the NH_2 termini of the secreted and nonsecreted proteases (34). Recently, the gene for the secreted protease has been cloned, sequenced, and shown to require a σ^{37} polymerase for transcription (36). To our knowledge, this is the only other *Bacillus* gene of known function thus far isolated that has a σ^{37}-requiring promoter. Wong *et al.* (36) suggest that the role of the σ^{37} promoter may include, in addition to expression of sporulation-specific genes (37–40), expression of genes encoding extracellular enzymes as well as of genes regulated by growth phase. We now can determine whether the APase

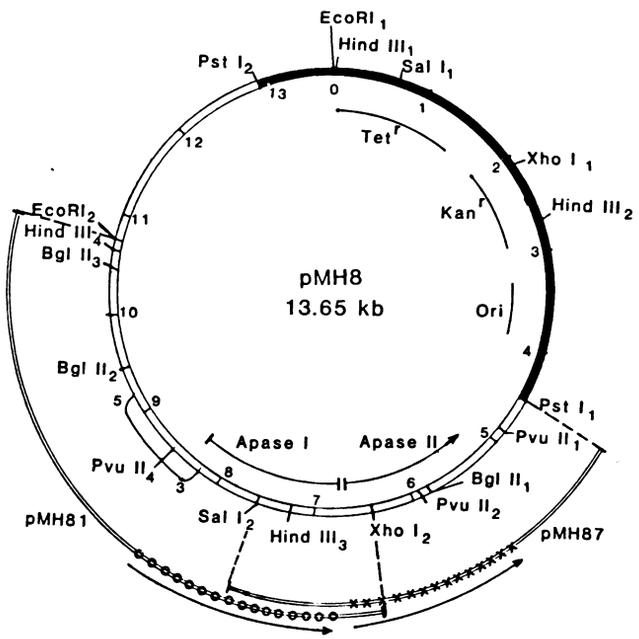

FIG. 5. Tandem APase genes from *B. licheniformis* MC14. *APase I* and *APase II* arranged in tandem on pMH8. *APase I* terminates ≈400 bp after *Hind*III$_3$ (at about 6.9 on pMH8) and transcription initiation for *APase II* starts ≈300 bp before *Xho* I$_2$ at 6.7 on pMH8 and proceeds to the right (counterclockwise). Therefore, the APase genes are separated by ≈200 bp. *APase I* is transcribed by an RNA polymerase from a σ^{55}-requiring promoter (transcription starts 150 bp before *Pvu* II$_3$ and proceeds to the right), whereas *APase II* is transcribed by σ^{37}-containing RNA polymerase. Circles indicate coding region for *APase I* in pMH81; Xs, coding region for *APase II* on pMH87; and arrows, direction of transcription.

gene requiring σ^{37}, *APase II*, is responsible for the secreted APase species of *B. licheniformis* whereas the σ^{55}-requiring *APase I* encodes the internal APase.

Having cloned the APase genes, we now can generate structural-gene mutants in *APase I* or *APase II* in *B. licheniformis* to determine the final destination of each gene product. It will be of interest to determine whether divergence of duplicated genes in *Bacillus* has resulted in altered regulation of the gene and location of the protein product.

In summary, we have cloned two APase genes arranged in tandem on the chromosome of *B. licheniformis* MC14 (Fig. 5). Evidence that the genes code for APase includes enzymatic activity (ability to hydrolyze XP), protein subunit size, and antigenic crossreactivity with anti-APase. *In vitro* transcription mapping of each promoter agrees well with exonuclease III/nuclease S1 deletion mapping of the coding region of each gene. The finding that each gene has its own promoter and that these differ with respect to the σ factor required for transcription suggests that regulation of the two genes differs.

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