Bacillus licheniformis* APase I gene promoter: a strong well-regulated promoter in *B. subtilis

JUNGWAN K. LEE, CHARLES W. EDWARDS and F. MARION HULETT*

Laboratory for Molecular Biology, University of Illinois at Chicago, PO Box 4348, Chicago, IL 60680, USA

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The 5' regulatory region and the portion of the structural gene coding for the amino-terminal sequence of alkaline phosphatase I (APase I) were isolated from *Bacillus licheniformis* MC14 using a synthetic oligodeoxynucleotide deduced from the amino acid sequence of the enzyme. The DNA sequence analysis of this region revealed an open reading frame of 129 amino acids containing the amino-terminal sequence of the mature APase protein. The protein sequence was preceded by a putative signal sequence of 32 amino acid residues. The predicted amino acid sequence of the partial APase clone as well as the experimentally determined amino acid sequence of the enzyme indicated that *B. licheniformis* APase retains the important features conserved among other APases of *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*, and various human tissues. Heterologous expression studies of the promoter using a fusion with the *lacZ* gene indicated that it functions as a very strong inducible promoter in *B. subtilis* that is tightly regulated by phosphate concentration.

Introduction

Bacillus licheniformis MC14, a facultatively thermophilic Gram-positive bacterium, was chosen for the study of alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1; APase] based on its capability of hyper-production of APase (Hulett & Campbell, 1971). In the presence of Co²⁺, *B. licheniformis* MC14 produces 35 times more APase activity than reported for other *Bacillus* species (Hydrear *et al.*, 1977) and 99% of the APase synthesized is efficiently secreted into the culture medium (Spencer *et al.*, 1981).

Biochemical studies of *B. licheniformis* MC14 APase have indicated that there exist multiple APase species in this bacterium whose expression can be derepressed upon phosphate starvation during vegetative growth (McNicholas & Hulett, 1977; Hansa *et al.*, 1981; Spencer & Hulett, 1981; Spencer *et al.*, 1982; Hulett *et al.*, 1986). The expression and localization of APase enzymes in the cell are dependent on the age of the culture as well as the

culture conditions. The major APase species in *B. licheniformis* MC14 has a molecular mass of 60 kDa (Hulett & Campbell, 1971). The amino acid compositions are very similar among the APase species, but not identical. It has been our working hypothesis that there are multiple structural genes responsible for each APase species in *B. licheniformis* MC14. However, it is not likely that the hyper-production of APase in *B. licheniformis* MC14 compared to other *Bacillus* species is due solely to the existence of multiple APase genes, since it has been shown recently that there are multiple APase genes in *B. subtilis* (Hulett *et al.*, 1990). *B. subtilis* produces (or secretes) only one-sixteenth of the total APase activity produced by *B. licheniformis* MC14 under similar culture conditions (Hulett & Jensen, 1988). Therefore, the nature of the hyper-production of APase in *B. licheniformis* MC14 is more likely to be the result of a difference in the promoter strength of one or more of the APase structural genes.

Efforts to isolate APase structural genes from *Bacillus* by complementation of an *Escherichia coli* *phoA* strain have not been successful (Hulett *et al.*, 1988; Lee *et al.*, 1991). Previous problems in sequencing the APase protein were overcome by employing a new technique using the PVDF (polyvinylidene difluoride) immobilon (Matsudaira, 1987). In this paper, we report the cloning of the amino terminus of the APase structural gene from *B. licheniformis* MC14 using the protein sequence data.

Abbreviations: APase, alkaline phosphatase; PVDF, polyvinylidene difluoride.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M37169.

The possible usage of the promoter of the APase gene is also discussed based on the results of the expression study of the promoter.

Methods

Bacterial strains and plasmids. *B. licheniformis* MC14 was used to isolate APase protein (Hulett & Campbell, 1971). *B. subtilis* IS401 (*trpC2 pheA1*) (from I. Smith) was the wild-type strain used for the expression study. *E. coli* DH5 α [F⁻ ϕ 80 Δ lacZ Δ M15 Δ (*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r⁻m⁺) *supE44* λ ⁻ *thi-1 gyrA relA1*] was used as a recipient for cloning and culturing recombinant plasmids. The *E. coli* vector, pJM103, was generated by ligating pUC19 and the Gram-positive chloramphenicol resistance gene, and was used in cloning the APase gene (Ferrari & Hoch, 1989). The integration expression vector, pDH32, contained an *amyE* gene interrupted by a promoterless *lacZ* gene fused to the ribosome-binding site of the *spoVG* gene and the Gram-positive chloramphenicol resistance gene (Shimotsu & Henner, 1986). It also carried a penicillin resistance gene and an origin for replication in *E. coli*.

Purification of membrane-associated APase. APase enzyme was purified from *B. licheniformis* MC14 as described by Hulett *et al.* (1976).

Amino acid sequencing. APase for sequencing was run on a 7.5% (w/v) SDS-PAGE gel and blotted onto PVDF immobililon as described by Hulett *et al.* (1990). The 60 kDa protein band containing APase protein was cut out after staining and destaining of the membrane and analysed by the Harvard Microchemistry facility on a 470A protein sequencer equipped with a 120A on-line PTH-AA analyser (Applied Biosystems).

Oligodeoxynucleotide synthesis. Oligodeoxynucleotides were synthesized on a Biosearch 8700 DNA synthesizer and cartridge purified. The oligodeoxynucleotides synthesized for plasmid sequencing were 15 nucleotides long and based on previous sequencing data. The oligodeoxynucleotide synthesized for DNA hybridization, SOKL56, was 56 nucleotides long and deduced from the amino acid sequencing data. Where codons were redundant, the one most likely to be used by *Bacillus* (Piggett & Hoch, 1985) was chosen to be included in the sequence.

Recombinant DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories (BRL) and used according to the manufacturer's recommendations. Plasmid DNA was isolated from *E. coli* according to Maniatis *et al.* (1982). *Bacillus* chromosomal DNA was isolated as described by Bookstein *et al.* (1990).

Southern hybridization. Southern hybridizations were carried out on Hybond-N nylon membranes according to Amersham membrane transfer detection methods. The probes were prepared either by labelling plasmids with [α -³²P]CTP using the nick-translation kit from BRL or by end-labelling the oligodeoxynucleotide, SOKL56, with T4 polynucleotide kinase from BRL. To end-label the synthetic oligodeoxynucleotide, 100 pmol SOKL56 was incubated with 100–150 pmol [γ -³²P]ATP [specific activity >4500 Ci mmol⁻¹ (>166.5 TBq mmol⁻¹), Amersham, or >7000 Ci mmol⁻¹ (>259 TBq mmol⁻¹), ICN] and T4 polynucleotide kinase at 37 °C for 30 min (Maniatis *et al.*, 1982). Conditions of stringency for hybridization and wash were determined as described by Bookstein *et al.* (1990). According to that method, 59 °C represented 100% stringency and 47 °C represented 90% stringency.

Construction and screening of the sub-genomic DNA libraries. In order to construct sub-genomic DNA libraries, *B. licheniformis* MC14 chromosomal DNA was digested by the restriction enzyme of interest and separated on an agarose gel by electrophoresis. To increase the

possibility of cloning the right fragment, DNA fragments of an appropriate size range as determined by Southern analysis were eluted electrophoretically onto a strip of DEAE cellulose membrane (Schleicher & Schuell, NA-45) according to the manufacturer's instructions. The DNA fragments were then ligated to an *E. coli* vector, pJM103, after elution from the DEAE membrane. Each sub-genomic DNA library was transformed into a *recA E. coli* strain, DH5 α , and spread directly onto Amersham nylon membranes. Preparation of filters with fixed colonies and colony blot hybridization of the filters were done essentially as described by Lampe *et al.* (1988) with the modifications made by Bookstein *et al.* (1990). Usually, 2000–3000 colonies were screened for each sub-genomic library, using 10⁷ c.p.m. of the ³²P end-labelled SOKL56 as a probe.

Transformation. Transformations of *E. coli* DH5 α with sub-genomic DNA libraries were carried out by the method of Hanahan (1985). Transformation of *B. subtilis* was carried out by a modification of the method of Dubnau *et al.* (1969).

DNA sequencing. The nucleotide sequence of the amino-terminal region of the APase I gene and its 5'-flanking region was determined by plasmid sequencing using Sequenase kits purchased from United States Biochemical and following the manufacturer's instructions. Both strands of DNA sequence were determined using universal primers and oligonucleotides synthesized based on previous sequencing data.

APase assay. APase assays of *B. subtilis* strains were performed on culture supernatants from cells grown in defined medium as described previously (Spencer *et al.*, 1981; Hulett & Jensen, 1988). Units of activity were calculated as μ mol *p*-nitrophenol phosphate hydrolysed min⁻¹ at pH 9.5 in 1 M-CHES buffer (Sigma) with 0.1 mM-CoCl₂, 70 mM-MgSO₄ and 10⁻⁵ mM-ZnCl₂. Specific activity was calculated as units of activity per ml culture divided by OD₅₂₅.

β -Galactosidase assay. The β -galactosidase plate assay was done using defined medium agar plates supplemented with 50 μ g 5-bromo-4-chloro-3-indolyl β -D-galactosidase (X-Gal) ml⁻¹, containing either low phosphate (0.4 mM) or high phosphate (45 mM). β -Galactosidase assay was also done on cultures grown in defined medium containing either low phosphate (0.4 mM) or high phosphate (10 mM) as described by Ferrari *et al.* (1986). β -Galactosidase specific activity was expressed in units (mg protein)⁻¹. The unit used was equivalent to 0.33 nmol orthonitrophenol produced min⁻¹.

Results and Discussion

Amino acid sequencing of *B. licheniformis* MC14 APase I

Previous attempts to sequence the amino terminus of *B. licheniformis* MC14 APase protein yielded results suggesting that the amino terminus may be blocked for protein sequencing. However, the new protein blotting technique using an immobilon (Matsudaira, 1987) made it possible to determine the amino acid sequence of the enzyme in the amino-terminal region. The 60 kDa membrane-associated APase was purified from *B. licheniformis* MC14 by Mg²⁺ extraction (Hulett *et al.*, 1976; Spencer & Hulett, 1981; Spencer *et al.*, 1982) and its first 21 amino acid residues at the amino terminus were determined. The sequence (Fig. 2, underlined) showed homology to the sequence of the first β -sheet strand, strand A, and the aspartic acid residue providing

Fig. 1. Restriction map of the 2.2 kb *Hind*III fragment and DNA sequencing strategy. The arrow above the map indicates the direction of the APase I gene. The 593 bp *Hae*III fragment which was isolated separately is shown by a thick line. The 625 bp *Rsa*I fragment used for the expression study of the promoter is indicated by a line binding the two sites. The nucleotide sequence of the 1020 bp *Rsa*I–*Hae*III fragment was determined by plasmid sequencing using universal primers or the synthetic oligonucleotides.

a ligand to the metal-binding site B of *E. coli* APase (Wyckoff *et al.*, 1983). These domains are known to be conserved among APases from various organisms including two *B. subtilis* APases (Kim & Wyckoff, 1989; Hulett *et al.*, 1990). A 56-nucleotide oligodeoxynucleotide was synthesized based on the amino acid sequence data, using the codon with the highest usage in *Bacillus* for each amino acid (Piggot & Hoch, 1985). The following is the sequence of the synthetic oligodeoxynucleotide, SOKL56: 5'-AAA-AAA-AAA-GAA-GAA-CCG-AAA-GTT-AAA-AAT-GTT-ATT-ATG-ATG-GTT-ATG-GAG-GAA-AC-3'.

Southern analysis of *B. licheniformis* MC14 genomic DNA

To determine the sizes of *B. licheniformis* MC14 chromosomal DNA fragments containing the amino terminus of the APase structural gene, the chromosomal DNA was digested with various restriction enzymes and probed with the synthetic oligodeoxynucleotide SOKL56. *B. licheniformis* MC14 chromosomal DNA digested with *Pst*I, *Hind*III, *Pvu*II, *Hae*III or *Rsa*I showed a 4.5 kb, a 2.2 kb, a 3.5 kb, a 593 bp or a 625 bp fragment, respectively, when probed with end-labelled SOKL56 at a stringency of 90% (data not shown). *B. subtilis* chromosomal DNA digests showed three *Pst*I DNA fragments (2.8 kb, 4.4 kb and 6.0 kb) hybridizing to SOKL56 at a stringency lower than 80%, but none was detected at higher stringency (data not shown). The 2.8 kb and the 4.4 kb fragments were the sizes of the *Pst*I DNA fragments containing the APase III and APase IV genes of *B. subtilis* (Hulett *et al.*, 1990, and unpublished data). This suggested that APase genes in these two *Bacillus* species were similar to each other at the DNA

sequence level. *Bacillus* APases are immunologically related to each other, but not to *E. coli* APase (Hulett *et al.*, 1990).

Cloning the 5' regulatory and structural terminus of the APase I gene of *B. licheniformis*

To avoid problems associated with DNA rearrangements which we experienced in cloning the complete *B. subtilis* APase structural genes in *E. coli*, a sub-genomic DNA library was constructed in an *E. coli* vector, pJM103, using the *Hae*III fragments of *B. licheniformis* MC14 genomic DNA in the range 0.5–1 kb. Two thousand colonies were screened by colony hybridization using the end-labelled SOKL56, and three of them with strong signals were found to contain the same 593 bp *Hae*III DNA fragment. The Southern analysis of *B. licheniformis* MC14 genomic DNA digests using the *Hae*III clone as the probe ensured the cloning of the correct DNA fragment, later confirmed by DNA sequencing analysis (see below). The 2.2 kb *Hind*III fragment was also cloned in a similar way by screening the *Hind*III sub-genomic library with SOKL56. The restriction mapping of these clones indicated that the 593 bp *Hae*III fragment was internal to the 2.2 kb *Hind*III fragment as shown in Fig. 1. The *Hind*III clone provided 5' APase I flanking DNA required for assessing the promoter function of this gene.

Sequencing analysis of the 593 bp *Hae*III fragment and flanking 5' DNA

The nucleotide sequence of the 593 bp *Hae*III fragment region was determined to ensure that we had cloned the portion of the gene encoding the amino-terminal region of the APase I protein of *B. licheniformis* MC14. The sequence of its 5' flanking DNA which was used for promoter expression study was also determined (see below). The sequencing strategy is shown in Fig. 1 and the sequence is shown in Fig. 2. The nucleotide sequence of the 593 bp *Hae*III fragment showed an open reading frame of 129 amino acids containing a predicted amino acid sequence identical to the amino acid sequence determined experimentally (Fig. 2, underlined). The amino-terminal sequence of the mature APase was preceded by a putative signal sequence of 32 amino acid residues. The putative signal sequence contained two positively charged arginine residues, at positions –26 and –28, and a stretch of 25 hydrophobic amino acid residues. The putative cleavage site had an alanine, which is a typical residue found at the cleavage site in Gram-positive bacterial signal sequences (von Heijne & Abrahmsen, 1989). The probable translation initiation

<u>RsaI</u>	
GTACCTTCTGAGGATTTGGCTCTAGAGGACTGGAACCGGTCATATCAACCAATTTGACGGG	63
ATGTTCTAGGATGCCGGAAGCGATCGGCTATATGCTCGACCAATAATTAAAGGCTCGGTC	126
	<u>HaeIII</u>
ATCAATATGTCACGCTCCATCAGCAAAATCCCGTGGCGCATTTTGTCCATTATCGGCAACG	189
AAGGCGGGGCAAAATGCTGACGGAACCTTGCCTTGAATATGCCCGAAAGGAATCGG	252
	<u>HaeIII</u>
GCTAAACGCGATCGGCGCGGCGCAATCGATACGCGGATTAACGCGGAAATTCGCTGATCC	315
CGAATGAAAAAAGCGGTATTGAATTGATTCTATAGGCTATATCGGAAGCGGAGGAAT	378
	<u>HaeIII</u>
CGGACGCTCGGCGGCTCGCTAGCATCCGGAAGCAAGCATGCTGACGGCGCTCAGGCTCTA	441
	<u>SmaI</u>
TCTACAGCGGGGATGACAAAATACCGCGGATTCGAAGCGGAAAGGTTGAAAAGAGCGCG	504
ATGCTTCTTTTCATCATAAGCCCATGAAATTTACATAAGATTAGAAAGCAAAAGTCT	567
	<u>RsaI</u>
GTTTACATAGCAATGATAGTCTAGGCTGTCTCAACAAAATGCAGTTATCGAGCGACTACATAAG	630
	rbs
A ATG CGT TTC TTA CGC AAC AGA ATC GTC GGC ATT ACA CTG GCA GGC	676
Met Gly Phe Leu Arg Asn Arg Ile Val Gly Ile Thr Leu Ala Gly	15
GCA GTC GGT TTA GGA TCG CGC GGA ACA GGC AGT CGC GCA ATG GAA ACA	724
Ala Val Ala Leu Gly Ser Ala Gly Thr Gly Ser Ala Ala Met Glu Thr	31
CGC AAA AAA AAA GAA GAG CGC AAG GTC AAA AAC CTG ATC ATG ATG GTC	772
Ala Lys Lys Lys Glu Glu Pro Lys Val Lys Asn Val Ile Met Met Val	47
ATG CAT GGC ACA AGC TCT TCT GCA ACA ACT TTG GCA CGC TGG TAT AAA	820
Met Asp Gly Thr Ser Ser Ser Ala Thr Thr Leu Ala Arg Trp Tyr Lys	63
CGC GAA CGC CTC GCA ATG GAC CAG ATC GTC AGC GGC GGT GTC CGG ACG	868
Gly Glu Pro Leu Ala Met Asp Gln Ile Val Ser Gly Gly Val Arg Thr	79
	<u>PstI</u>
TAT TCG GCA GAA TCG GGC ATC ACT GAT TCT GCA CCT GCA GCA ACT GGC	916
Tyr Ser Ala Glu Ser Ala Ile Thr Asp Ser Ala Pro Ala Ala Thr Ala	95
ATG GCA ACG GGA CGA AAA TCA AAT TCT TCC TAT ATC GGT GTG CTG CCT	964
Met Ala Thr Gly Arg Lys Ser Asn Ser Ser Tyr Ile Gly Val Leu Pro	111
TCA AAA GTG AAT ACA CGA GGA GTC GAG AAG GTC CGT GAA AAA GAT CAT	1012
Ser Lys Val Asn Thr Pro Gly Val Glu Lys Val Arg Glu Lys Asp His	127
	<u>HaeIII</u>
TAC CGG CC	1020
Tyr Arg	129

Fig. 2. Nucleotide sequence of the 593 bp *HaeIII* fragment and its 5' flanking DNA. The sequence contained the amino-terminal portion of the APase I gene and the promoter region that was used for the expression study. Numbering begins at the 5' end of the sequence. The predicted amino acid sequence of the APase I gene is shown below the DNA sequence in three-letter code, beginning at 632. The putative ribosome-binding site is underlined and marked as rbs. The predicted amino acid sequence that is identical to the amino acid sequence determined experimentally is underlined and preceded by an alanine, which is the typical residue found at the cleavage site of a signal sequence.

site was a methionine, ATG, at position 632, which was preceded by a potential ribosome-binding site, GGAGGGAGT, 8 bp upstream. The predicted amino acid sequence of the mature APase showed homology to the amino-terminal sequences of APases from other organisms including *E. coli* (Chang *et al.*, 1986), *B. subtilis* (Hulett *et al.*, 1991), *Saccharomyces cerevisiae* (Kaneco *et al.*, 1987), and human (Kam *et al.*, 1985; Weiss *et al.*, 1988) (Fig. 3). The amino acid sequence of *B. licheniformis* APase, in this region, showed approximately 24–29% identity with the amino acid sequence of

	-----/\///----- ---A---*---	
ECO :	RTPEMPVLENRAAQGDITAPGGARRLTGDQTAALRDSLSDK-PAKNIILLIGDGMG	55
BLI :	-----KKKEEP--KV-KNVIMVMDGTS	20
BS3 :	-----Q--EKKKGND--EIKNVIVLIGDGMG	23
BS4 :	-----KK--QDKAEIRNVIVMIGDGMG	20
SCE :	KKRRISKRSKIIIVTVVCI-GLLLVLVQLAFPSFALRSASHKKKNVIFVTDGMG	55
HPL :	-----IIPYEEENPDFWNREAAEALGAACKLPAQTC-AKNIILFLDGMG	45
HBLK :	-----LVPEKEKDPKYWRDQAQETLKYALELQKLNTNVAKNVIMFLDGMG	46
	/\///\///-----/\///---B----- --/@@/\	
ECO :	DSEITAARNYAEGAGGFFKGIDALPLTGQYTHY-ALNKKTKG--PDY-VTDSAAASA	107
BLI :	SSATTTLARWY-----K-GE--PLAMDQI--VSGGVRTYS--AESAITDSAPAA	61
BS3 :	VSYTSAYR-YLKNKKTKV-VE--PTAFDQYL-V-GQQTTPDDPEQNVITDSAAA	73
BS4 :	TPYIRAYRSM-KNNG--DTPNNPKLTFDRNL--TGMMTHPDDPDYNTDSAAA	71
SCE :	PASLSMARSFN--QHVNDLPIDD-ILTLDEH--FIGSSRTRSSDSL--VTDSAAAGA	104
HPL :	VSTVTAAR-ILKQKKKDLGPE-IPLMDRFFYVALS-KTYNVDKH--VPDSCATA	96
HBLK :	VSTVTAAR-ILKQQLHHPGEE-TRLEMDKFFFYVALS-KTYNTNAQ--VPDSAGTA	97
	/\///\///----- ----- -----	
ECO :	TAWSTGVKTYNGALGVDIH-----EKDH--	130
BLI :	TAMATGRKSNSSYIGVLPKVNTP-GVEKVRKDHRY	97
BS3 :	TAMSAGIKTYNNAIVD-----ND-GSE-----	95
BS4 :	TALATGVKTYNNAIGVD--K-N--G-K--K----	93
SCE :	TAFACALKSYNGAIGVDPHHR--PCG-----	128
HPL :	TAYLCGVKGNFQITGLSAAARFNQCNTRKNE-----	128
HBLK :	TAYLCGVKANEGTVGVSAAATERSRCNTTQNE-----	129

Fig. 3. Comparison of the amino-terminal amino acid sequence of APase I with other APases. The sequence of the first 97 amino acid residues of the mature *B. licheniformis* APase I protein (BLI) is highly homologous to conserved regions of *E. coli* (ECO), *B. subtilis* (BS3 and BS4), *S. cerevisiae* (SCE), human placenta (HPA), and human bone-liver-kidney (HBLK) APases. The , = , * , and @ marks represent the α -helix, β -sheet, a ligand to metal-binding site B, and phosphorylation site, respectively, as determined by the analysis of crystallized *E. coli* APase (Wyckoff *et al.*, 1983; Hulett *et al.*, 1991).

each of the above APases, being most closely similar to *B. subtilis* APases. Some structurally and functionally important features as determined from X-ray diffraction study of the crystallized *E. coli* APase are conserved in this region (Kim & Wyckoff, 1989). The first two β -sheet strands, A and B, are conserved among APases. The aspartic acid providing one of the ligands to the metal-binding site B and the serine residue at the phosphorylation site are conserved in all APases. It is interesting that, like other *Bacillus* APases, *B. licheniformis* APase is also truncated such that the first β -sheet strand is very close to the amino-terminus (Hulett *et al.*, 1990). The results of the DNA sequencing and comparison analysis of the predicted amino acid sequence indicated that we had cloned the DNA fragment for the amino-terminal portion of the APase structural gene (APase I gene) from *B. licheniformis* MC14 and its 5' flanking region.

Expression study using the promoter for the APase I gene in *B. subtilis*

The expression and the strength of the promoter for the APase I gene of *B. licheniformis* MC14 were studied in *B. subtilis* IS401. For this purpose the 625 bp *RsaI* fragment (Fig. 1) was cloned in an integration vector pDH32 (Shimotsu & Henner, 1986; Fig. 4) in an orientation which resulted in the construction of a transcriptional fusion between the APase I gene promoter and the promoterless *lacZ* structural gene. The 625 bp *RsaI*

Fig. 4. Map of the expression clone, pKL14. The 625 bp *RsaI* fragment was ligated to the *SmaI* site of pJM103 first, and then cloned between the *EcoRI* and *BamHI* sites of pDH32 (9.8 kb) using the *EcoRI* and *BamHI* sites in the polylinker of pJM103. The arrow indicates the direction of transcription of each gene or the promoter. The *lacZ* gene lacks its own promoter and is fused to the ribosome-binding site of the *spoVG* gene. The penicillin resistance gene (*Pen^R*) was derived from pBR322 and the chloramphenicol resistance gene (*Cm^R*) from pC194.

fragment which contains the putative ribosome-binding site and further upstream sequence (Fig. 1) was first ligated to the *SmaI* site of pJM103 and then the insert was cloned into pDH32 at the *EcoRI* and *BamHI* sites using the *EcoRI* and *BamHI* sites in the polylinker of pJM103. The resulting expression clone, pKL14, was linearized at the unique *PstI* site and used to transform competent cells of *B. subtilis* IS401. The linearized DNA was integrated into the chromosomal DNA by gene replacement at the *amyE* locus. The construct was selected by chloramphenicol resistance and screened for the loss of α -amylase synthesis on starch agar plates. The proper rearrangement in the chromosomal DNA was confirmed by Southern analysis (data not shown). MH1000 and MH1400 contained the *amyE* portion of pDH32 and pKL14, respectively, at the *amyE* locus on the chromosome. Assay of MH1400 for β -galactosidase production on low-phosphate defined agar medium supplemented with X-Gal showed a blue colony phenotype. This promoter activity was not detected on high (45 mM) phosphate defined agar medium. Both MH1000 and the parental strain, IS401, remained white on both types of agar. These data indicated that the *RsaI* fragment in front of the APase I open reading frame retained phosphate-starvation-inducible (*psi*) promoter activity in *B. subtilis*.

In order to study the expression pattern of the APase I gene promoter during the course of vegetative growth and to measure the strength of the promoter, β -galactosidase activity of MH1400 was monitored by liquid culture assay in low-phosphate defined medium

(Fig. 5). In order to avoid the excessive APase and β -galactosidase activity in the initial inoculum carried over from overnight culture, cells were grown in low-phosphate defined medium to early exponential phase. Cells were collected by centrifugation, suspended in low-phosphate defined medium and used to inoculate fresh defined medium containing either low phosphate (0.4 mM) or high phosphate (10 mM). The *B. licheniformis* APase I promoter initiated expression of β -galactosidase activity 1 h later than the derepression of *B. subtilis* vegetative APase after 3 h growth, as the culture OD_{525} exceeded 1.0 (Fig. 5a, b). β -Galactosidase specific activity increased from <10 to 7800 between 3 h and 5 h (Fig. 5a). The specific activity of β -galactosidase directed by the APase promoter reached a maximum of 7900 after 8 h growth, indicating that it may be at least more than five times stronger than the APase III promoter of *B. subtilis* (C. Bookstein and others, unpublished data). The *B. licheniformis* APase I gene promoter was completely repressed by phosphate in the growth medium until 3 h (Fig. 5c). The repression factor was greater than 10^3 when fully induced β -galactosidase activity (Fig. 5a, 6 h) was compared to that of phosphate-repressed APase I promoter at the same hour of growth (Fig. 5c, 6 h). This result agreed with the result of a separate phosphate repression experiment in which the culture grown in low-phosphate defined medium was split into two flasks when the cells reached the end of exponential growth; excess phosphate (10 mM) was added to one of the flasks. The culture which received additional phosphate resumed exponential growth and ceased to produce APase and β -galactosidase immediately (data not shown). The culture which received no additional phosphate entered stationary phase and continued to express APase and β -galactosidase activities similar to those of the culture in Fig. 5(a). The β -galactosidase activity in MH1000, the strain containing the vector with a promoterless *lacZ* gene, remained near zero throughout the vegetative growth of the cells in defined medium containing either high or low phosphate (Fig. 5b, d). The medium (DM) used in these studies is a complete medium in which growth of *B. subtilis* ceases because of limiting phosphate (Hulett & Jensen, 1988). Induction of the APase I promoter was dependent on the phosphate concentration and was initiated during exponential growth between 3 and 4 h, when the phosphate concentration was 0.1–0.08 mM. Altering growth conditions to slow the growth rate increased the length of time between induction of the APase I promoter and the beginning of stationary phase. The timing of APase induction and the beginning of stationary phase remained dependent on the phosphate concentration, but the concentrations required for initiation of each of the two events were different. Forms

Fig. 5. Expression of the APase I gene promoter in *B. subtilis*. Cells carrying the pKL14 fusion (MH1400; a) or pDH32 (MH1000; b) were grown in the low-phosphate defined medium that maximized derepression of vegetative APases. To check the effect of a high concentration of phosphate on the promoter activity, cells were grown in the high-phosphate defined medium containing 10 mM- K_2HPO_4 (MH1400; c; MH1000; d). ●, Growth; ■, specific APase activity; ▲, specific β -galactosidase activity. Specific activities are expressed as described in Methods.

of regulation other than phosphate depletion that may be involved in the expression of vegetative APase have not yet been determined for either *B. subtilis* or *B. licheniformis*.

The data presented in this communication indicate that the promoter of the *B. licheniformis* APase I gene functions in *B. subtilis* as a very strong promoter regulated by the phosphate concentration of the medium. Therefore, this promoter has potential for use as a powerful tool in constructing expression and secretion vectors for *B. subtilis*, especially when the overexpression of a gene product is detrimental to the host cells.

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