Expression of *Bacillus amyloliquefaciens* amylase and *Vibrio alginolyticus* protease A fusion genes

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Previously we reported [Deane, S. M., Maharaj, R., Robb, F. T. & Woods, D. R. (1987) *Journal of General Microbiology* 133, 2295–2302] that the production of a *Vibrio alginolyticus* SDS-resistant alkaline serine protease (Pro A) cloned in *Escherichia coli* was characterized by a 12 h delay between the synthesis of an inactive precursor and secretion of active Pro A. Replacement of the *V. alginolyticus* promoter region by the α -amylase promoter region from *Bacillus amyloliquefaciens* resulted in the simultaneous synthesis and secretion of Pro A in *E. coli*. The *V. alginolyticus* *proA* gene cloned on a shuttle vector did not produce active Pro A in *Bacillus subtilis*. Although Pro A has a typical Gram-positive signal sequence, it was not functional in *B. subtilis*. Replacement of the Pro A signal sequence with the α -amylase signal sequence resulted in the production of active Pro A in *B. subtilis*.

Introduction

Vibrio alginolyticus produces a collagenase and seven extracellular alkaline serine proteases (Reid *et al.*, 1978, 1980; Long *et al.*, 1981; Hare *et al.*, 1981, 1983; Deane *et al.*, 1986, 1987a). One of these proteases, Pro A, is SDS-resistant, Ca^{2+} -dependent and is produced by *V. alginolyticus* cells grown in complex media (Deane *et al.*, 1987a). The gene (*proA*) encoding Pro A was cloned on a 7.1 kb fragment of *V. alginolyticus* chromosomal DNA, and its expression was studied in *Escherichia coli* (Deane *et al.*, 1987b). The nucleotide sequence of the *proA* gene was determined, and the deduced amino acid sequence of Pro A had a high degree of similarity to the subtilisin group of proteases (Deane *et al.*, 1989). The *V. alginolyticus* *proA* gene was expressed from its own regulatory region in *E. coli*. Pro A contained a putative signal peptide and was secreted by *E. coli* containing the *proA* gene, without cell lysis. Although Pro A was produced during exponential growth in *E. coli* expressing *proA*, the intracellular enzyme was inactive, and was secreted in an active form during the late stationary growth phase. There was a delay of approximately 12 h between the synthesis of the inactive precursor and secretion of active Pro A by *E. coli* cells.

Pro A has characteristics that make it potentially suitable for industrial and laboratory use. It is resistant to

detergents such as SDS (> 2.5%, w/v) and shows activity over a broad temperature range (20–55 °C) (Deane *et al.*, 1987a), making it suitable for use in household laundry detergent formulations. It could also be used as an alternative for proteinase K, a protease commonly used to remove contaminating proteins from DNA preparations.

Industrial production of Pro A would require a suitable micro-organism. *Bacillus subtilis* is non-pathogenic and has been used for the production of foodstuffs (Debaov, 1982). Fermentation technology for *B. subtilis* already exists and genetic manipulation of this organism is well established. More significantly, it secretes proteins into the medium, and has also been shown to secrete specific foreign proteins relatively free of other cellular proteins (Doi *et al.*, 1986). Doi *et al.* (1986) pointed out a number of disadvantages in the use of *B. subtilis*, such as the production of endogenous proteases and the specificity of its transcription and excretion mechanisms. However, these disadvantages have now been overcome. Efficient expression of foreign genes can be obtained in *B. subtilis* by fusing the gene of interest in-frame to regulation signals of an endogenous *B. subtilis* gene. Secretion vectors which use the regulatory signals of *B. subtilis* α -amylase (Sarvas, 1986; Schein *et al.*, 1986; Saunders *et al.*, 1987) and subtilisin (Vasanth & Thompson, 1986; Saunders *et al.*, 1987; Wang *et al.*, 1988) have been constructed, as well as an inducible system using the *B. subtilis* levansucrase gene promoter and the region encoding its signal sequence (Joliff *et al.*,

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Abbreviations: Pro A, protease A; SD, Shine-Dalgarno sequence.

1989; Petit *et al.*, 1990). Overbeeke *et al.* (1990) have developed a medium that suppresses the residual protease activity of protease-deficient *B. subtilis* DB104 (Kawamura & Doi, 1984), thus improving the expression of fusion gene constructs.

We tested the ability of *B. subtilis* DB104 to produce and secrete active Pro A by subcloning the gene, and its regulatory signals, into a multicopy *B. subtilis* shuttle vector. Active Pro A could not be detected in *B. subtilis* DB104 cells or culture containing the recombinant plasmid. We describe the construction and expression in *E. coli* and *B. subtilis* of *Bacillus amyloliquefaciens* α -amylase and *proA* gene fusions.

Methods

Bacterial strains, plasmids and growth conditions. *E. coli* strain LK111 (a *lacH*, *lacZDM15*, *lacY*⁺ derivative of K514) (Zabeau & Stanley, 1982) was used as a host for plasmid manipulations. *B. subtilis* DB104 (*his*, *nprR2*, *nprE18*, Δ *aprA3*) (Kawamura & Doi, 1984) was used as a host for all experiments involving *B. subtilis*. The α -amylase gene and promoter were cloned as a single fragment from *B. amyloliquefaciens* NCP1 (Corfield *et al.*, 1984) (plasmid pVC102), which was a gift from J. A. Thomson. The DNA sequence of the gene and its promoter have been determined (J. A. Thomson, personal communication) and shown to be >99% identical to a previously sequenced *B. amyloliquefaciens* α -amylase gene (Palva *et al.*, 1981). DNA fragments were subcloned into pUC19 (Yanisch-Perron *et al.*, 1985) or Bluescript (Stratagene) vectors for subcloning and the generation of deletions and fusions. Selected constructs were then ligated into the *B. subtilis* shuttle vector pEB1, which was constructed by one of us (H.Z.) and has been described elsewhere (Lin *et al.*, 1990). pEB1 is an *E. coli*-*B. subtilis* shuttle vector with positive selection for inserted DNA fragments in *E. coli*. *E. coli* and *B. subtilis* were grown in 2 $\times$ YT medium (Sambrook *et al.*, 1989) containing 10 mM-CaCl₂. Peptone water agar, containing Difco Peptone (2.5%, w/v), agar (1.5%, w/v) and skim milk (1%, w/v), was used as solid medium for the selection of Pro-A-producing colonies. Zones of hydrolysis (clearing) appeared around colonies producing active Pro A after 24 h incubation at 37 °C. Antibiotics added to media for plasmid selection and maintenance were ampicillin (100 μ g ml⁻¹) for *E. coli* and chloramphenicol (20 μ g ml⁻¹) for *B. subtilis*.

Transformation of *E. coli* and *B. subtilis*. *E. coli* was transformed with plasmid DNA by the CaCl₂-shock method of Dagert & Ehrlich (1979). *B. subtilis* was transformed using the protoplast method of Rubenstein *et al.* (1988).

Preparation of DNA, recombinant DNA techniques and DNA sequencing. Plasmid DNA was prepared from *E. coli* by the alkaline lysis method of Ish-Horowitz & Burke (1981) followed by CsCl equilibrium centrifugation in the presence of ethidium bromide. The method used for *B. subtilis* plasmid isolation was identical, except that Solution I contained 5 mg lysozyme ml⁻¹, and cells were incubated for 10 min at 37 °C in Solution I before continuing with the standard method. Deletions were generated using the nuclease BAL-31 (New England Biolabs) (Misra, 1985). Subcloning was performed using standard techniques (Sambrook *et al.*, 1989). Enzymes were used according to the manufacturer's specifications. DNA sequencing was accomplished using a Sequenase kit (Sequenase Version 2.0, United States Biochemical Corp., Cleveland, Ohio). One unique primer with the sequence 5'-GCACGCTGTTATTTGTC-3' was synthesized in an

Applied Biosystems 381A DNA synthesizer. The primer was used to determine the nucleotide sequence over α -amylase-*proA* gene fusion regions.

Production of proteases. Overnight cultures (grown with shaking at room temperature, approx. 22 °C) of *E. coli* or *B. subtilis* containing recombinant plasmids were diluted in fresh prewarmed (37 °C) 2 $\times$ YT medium to an OD₆₀₀ of 0.01–0.02 (approximately 1 in 200 dilution). The diluted cultures were incubated at 37 °C with shaking, and OD₆₀₀ and Pro A production were monitored throughout the growth curve.

Azocasein Pro A Assay. Pro A activity was assayed using the synthetic substrate azocasein (Sigma) and a modified method of Long *et al.* (1981). The assay volume was reduced, but proportions of solutions were identical. Samples (1.5 ml) were taken from cultures at intervals and bacterial cells removed by centrifugation (1 min) in a microfuge. A sample (0.1 ml) was combined with 0.1 ml of azocasein solution [2%, w/v, azocasein in 0.1 M-Tris/HCl (pH 9.0), 0.4 M-NaCl, 2 mM-CaCl₂] and incubated for 30 min at 37 °C. Ice-cold TCA (10%, v/v) was added, and the solution kept on ice for a further 30 min. The precipitated azocasein was pelleted by centrifugation (1 min) in a microfuge and 0.3 ml supernatant was transferred to another microfuge tube. Care was taken to avoid contamination of the supernatant with particles of precipitated azocasein. An equal volume of NaOH (0.5 M) was added and the A₄₄₀ was determined. One unit (U) ml⁻¹ of Pro A activity was defined as the amount of enzyme that gave an increase in absorbance of 0.1 at 440 nm (1 cm path length) in 30 min at 37 °C.

Cell fractionation and enzyme assay. *E. coli* periplasmic and cytoplasmic cell fractions were prepared by the osmotic-shock method of Willis *et al.* (1974). *B. subtilis* cytoplasmic fractions were prepared by sonicating washed cells for three 30 s bursts at 4 °C in 0.05 M-potassium phosphate buffer (pH 7.0). The sonicated cells were centrifuged at 4 °C for 15 min in an Eppendorf microfuge, and the supernatant fluid was retained for enzyme assays. Pro A, glutamine synthetase (Bender *et al.*, 1977) and β -lactamase (Sykes & Nordstrom, 1972) were assayed in *E. coli* cell-free culture supernatants, cytoplasmic and periplasmic cell fractions. Pro A and sucrase (Scholle *et al.*, 1987) were assayed in *B. subtilis* cell-free culture supernatants and cytoplasmic fractions.

Gelatin-PAGE protease assay. Gelatin-PAGE protease assays were done as described by Deane *et al.* (1987b). SDS (2.5%, w/v) and glycerol (2.0%, v/v) were added to the samples which were then held at 37 °C for 30 min. The proteases were assayed by PAGE in slab gels containing SDS and gelatin as a copolymerized substrate. After PAGE, the gels were washed in Triton X-100 (2.5%, v/v) for 1 h at room temperature to remove the SDS, and then incubated in 0.1 M-glycine buffer (pH 9.0) for 3 h at 37 °C. Bands or zones of proteolytic activity were detected after staining with 0.2% (w/v) amido black. Alternatively, to detect SDS-resistant Pro A only, the gels were stained immediately with amido black after PAGE.

Results

Construction and analysis of pBAP plasmids containing α -amylase-*proA* fusion genes

A 1.85 kb *Pst*I-*Hind*III fragment of *V. alginolyticus* chromosomal DNA from pVP100, encoding the entire *proA* structural gene and promoter region, was subcloned into pUC19 and the *B. subtilis* shuttle vector pEB1 to generate the plasmids pVP19 (Fig. 1) and pBPV100 respectively.

Fig. 1. Partial restriction map of pVP19 (After Deane *et al.*, 1989). The thin line represents vector DNA; the box, insert DNA. Only the region coding for the N-terminal region of the protein is shown in detail. The start point and direction of BAL-31 nuclease deletions are indicated by a horizontal arrow. Relevant features are shown as follows: protease A promoter; leader peptide; structural gene.

Fig. 2. Partial restriction map of pAmyPL and pAmyP. The thin line represents vector DNA; the box, insert DNA. The *Bg*II-*Eco*RI fragment was subcloned from pVC102 (Corfield *et al.*, 1984). The start and direction of BAL-31 deletions are indicated with a horizontal arrow. Relevant features are shown as follows: α -amylase promoter; α -amylase leader peptide; α -amylase structural gene.

α -Amylase-*proA* fusion genes in the *B. subtilis* shuttle vector pEB1 were constructed using two intermediate steps and *E. coli* as the host for recombinant plasmids. Plasmids carrying deletions of *proA* were constructed by subcloning the *Pst*I-*Eco*RI fragment from pVP19 (Fig. 1) into pUC19 and generating progressive deletions from the *Pst*I site using BAL-31 nuclease. Eight recombinant plasmids carrying deletion fragments were sequenced to determine the extent of the deletion. These shortened fragments were then used to replace the *Pst*I-*Eco*RI fragment in pVP19 to generate deletion plasmids pP-3, pP1, pP12, pP24, pP26, pP28, pP30 and pP31, each with a specific deletion in the region coding for the N-terminal end of Pro A (the first set of intermediate plasmids). pP-3 contained 9 bp upstream of the *proA* start codon which encoded an additional 3 amino acids. pP1 through pP31 contained deletions in the *proA* structural gene, such that pP1 and pP31 encoded proteins lacking 1 and 31 N-terminal amino acids respectively.

A 718 bp *Eco*RI-*Bg*II fragment from pVC102, encoding the *B. amyloliquefaciens* α -amylase promoter, Shine-Dalgarno (SD) sequence and signal peptide, including 103 N-terminal amino acids of the α -amylase, was subcloned into Bluescript^R to generate the plasmid

pAmyPL (Fig. 2). The plasmid pAmyP carried a DNA fragment which encoded the α -amylase promoter, SD and the first amino acid codon of the α -amylase gene, and was constructed by treating *Eco*RI digested pAmyPL with BAL-31 nuclease and subcloning the shortened fragments into pUC19. One recombinant plasmid (pAmyP) was selected and sequenced to confirm that it only contained the α -amylase promoter, SD, and first amino acid codon of the α -amylase gene.

α -Amylase-*proA* fusion gene plasmids pAP-3, pAP12, pAP24, pAP26, pAP28, pAP30 and pAP31 (the second set of intermediate plasmids) were constructed by subcloning the *Xba*I-*Acc*I fragment of pAmyPL (Fig. 2) and the corresponding deleted *proA* fragment in plasmids pP-3, pP12, pP24, pP26, pP28, pP30 and pP31 (intermediate step 1 plasmids described above) simultaneously into pUC19. The sequence of each fusion region was confirmed by DNA sequencing using the unique primer described in Methods. The entire fusion DNA fragment for each clone was then subcloned into the *E. coli*-*B. subtilis* shuttle vector pEB1 to generate the final constructs pBAP-3, pBAP12, pBAP24, pBAP26, pBAP28, pBAP30 and pBAP31 (Fig. 3). This series of shuttle plasmids contained the α -amylase DNA sequence encoding the promoter, SD and signal peptide, including

40	*	†	1	13		Proteolytic activity in <i>E. coli</i> <i>B. subtilis</i>		
F E W Y	P	E H F	M L K K L L S C C I T S A L C F H S S . .		pBAP-3	+	+	
				↓				
. . F E W Y	P	A L C F H S S L A F S Q P N E I A D S A E L Q . .			pBAP12	+	+	
				↓				
. . F E W Y	P	P N E I A D S A E L Q Q A P D T L P A T L M L . .			pBAP24	+	+	
				↓				
. . F E W Y	P	E I A D S A E L Q Q A P D T L P A T L M L A P . .			pBAP26	+	+	
				↓				
. . F E W Y	P	A D S A E L Q Q A P D T L P A T L M L A P D D . .			pBAP28	+	+	
				↓				
. . F E W Y	P	S A E L Q Q A P D T L P A T L M L A P D D I A . .			pBAP30	+	+	
				↓				
. . F E W Y	P	A E L Q Q A P D T L P A T L M L A P D D I A I . .			pBAP31	+	+	
				↓				
. . . G A G A G G A G A G G A A A C A T G A A T				G G C C G	T T A A A G A A A C T A C T A . .	pBAP1	+	-
--SD--				M I	G P	L K K L L		
				1	*	2		

Fig. 3. Structure of the α -amylase-Pro A fusion genes. The amino acid sequence at the junction between the genes is shown (in single letter code) for all plasmids. For pBAP1, the nucleotide sequence is also shown. Amino acid 40 of the α -amylase sequence (including the leader peptide) is indicated (numbering after Palva *et al.*, 1981). Pro A amino acid numbering for pBAP-3 (starting from the first methionine residue) is according to Deane *et al.* (1989). Vertical lines within each amino acid sequence demarcate the fusion region. Lines linking one plasmid and the next show the extent of the deletion in the following construct. The vertical arrow above pBAP12 shows the predicted Pro A leader peptide cleavage site. The asterisk indicates an amino acid residue encoded by additional bases originating from the pUC19 multiple cloning site. The dagger indicates amino acids encoded by translation of 9 bp immediately upstream of the Pro A start codon. Proteolytic activity of *E. coli* or *B. subtilis* carrying the constructs is indicated by + (activity) or - (no activity).

approximately 40 N-terminal amino acids fused to specific N-terminal deletions of Pro A. The plasmid pBAP1 was constructed in a similar manner using the deletion fragment from pAmyP (Fig. 3). pBAP1 contained the α -amylase DNA sequence encoding the promoter, SD and the first amino acid codon fused to the start of the *proA* structural gene. All the gene fusion plasmids contained DNA encoding extra amino acids between the end of the α -amylase and start of the *proA* coding regions. This DNA originated from remnants of the pUC19 multiple cloning site or *V. alginolyticus* DNA from regions upstream of the *proA* structural gene (Fig. 3) and was translated as part of the in-frame gene fusions.

Production of Pro A and α -amylase-Pro A fusion proteins in *E. coli* and *B. subtilis*

The production of Pro A and α -amylase-Pro A fusion proteins in *E. coli* and *B. subtilis* was determined by plating the strains carrying the various plasmids on peptone water medium containing skim milk and scoring for zones of proteolytic activity. pBVP100 (carrying the entire *proA* gene under the control of its own promoter) produced Pro A activity in *E. coli* cells but did not

produce Pro A activity in *B. subtilis* cells. Restriction mapping of re-isolated recombinant plasmids confirmed that the lack of proteolytic activity in *B. subtilis* cells was not due to DNA fragment rearrangements.

Production of Pro A by *E. coli* and *B. subtilis* cells transformed with pBAP plasmid constructs which contained the amylase DNA encoding the promoter region and the signal peptide fused to the *proA* structural gene (pBAP-3 to pBAP31, Fig. 3), resulted in zones of proteolytic activity on peptone milk agar. Pro A production in *E. coli* cells was initiated by the Gram-positive α -amylase promoter and Pro A activity in *E. coli* cells was not affected by the presence or absence of the α -amylase signal peptide (Fig. 3). Background zones of proteolytic activity produced by the *B. subtilis* protease-deficient host were markedly reduced in size and began to appear 24 h later than the host strain containing the recombinant plasmids. *B. subtilis* cells transformed with pBAP1 (the α -amylase promoter and SD sequence fused to the *proA* structural gene with its own DNA encoding the *V. alginolyticus* signal peptide) did not produce zones of proteolytic activity on peptone milk agar (Fig. 3). Restriction mapping of re-isolated recombinant plasmids confirmed that DNA fragment rearrangements had not occurred.

Fig. 4. Pro A production by *E. coli* cells carrying various α -amylase-*proA* fusion genes. Filled symbols represent no addition of chloramphenicol, open symbols addition of $100 \mu\text{g}$ chloramphenicol ml^{-1} . The vertical arrow shows the time at which antibiotic was added. ●, ○, Growth of *E. coli* LK111; ■, □, Pro A production by *E. coli* LK111(pBAP-3); ▲, △, Pro A production by *E. coli* LK111(pBAP24); ◆, ◇, Pro A production by *E. coli* LK111(pBAP1). The growth curves of each of the *E. coli* strains were determined, but the presence of the different recombinant plasmids did not affect the growth of the *E. coli* cells, and all the growth curves were similar to that of the *E. coli* LK111 strain. The experiment was repeated three times (a representative example is shown).

Production of Pro A by *E. coli* carrying the pBAP plasmids

E. coli strains carrying different pBAP plasmids were grown in $2 \times \text{YT}$ medium. The OD_{600} and production of Pro A were monitored over 30 h. Preliminary experiments showed that expression of pBAP plasmid fusion genes in *E. coli* could be grouped according to Pro A activity. *E. coli* cells transformed with pBAP-3 or pBAP12 showed similar, relatively low levels of activity (group 1). *E. coli* cells transformed with pBAP24, 26, 28, 30 or 31 showed similar, relatively high levels of Pro A activity (group 2). Within this group, *E. coli* cells carrying pBAP24 or pBAP26 showed the highest Pro A activity levels. *E. coli* cells transformed with pBAP1 showed intermediate levels of Pro A activity (Group 3). Therefore Pro A production by *E. coli*(pBAP-3), *E. coli*(pBAP24) and *E. coli*(pBAP1) was examined in liquid media as representative of each group (Fig. 4). These three strains produced 6.6, 35.7 and 16.7 U ml^{-1} of Pro A activity, respectively, after 24 h. In each case, active Pro A was detected in the medium approximately 10–12 h after inoculation. The addition of chloramphenicol ($100 \mu\text{g ml}^{-1}$) to *E. coli* cells containing these plasmids inhibited growth and caused a decrease in the levels of Pro A relative to the uninhibited control. Previously we showed that in *E. coli* (pVP100) cells the level of Pro A in chloramphenicol-treated cultures did not decrease relative to the uninhibited control (Deane *et al.*, 1987b).

Fig. 5. Pro A production by *B. subtilis* carrying α -amylase-*proA* fusion genes. Filled symbols represent no addition of tetracycline, open symbols addition of $100 \mu\text{g}$ tetracycline ml^{-1} . The vertical arrow shows the time at which antibiotic was added. ●, ○, Growth by *B. subtilis* DB104; ▼, residual protease production by *B. subtilis* DB104; ■, □, protease production by *B. subtilis* DB104(pBAP12); ▲, △, protease production by *B. subtilis* DB104(pBAP24). The growth curves of each of the *B. subtilis* strains were determined, but the presence of the different recombinant plasmids did not affect the growth of the *B. subtilis* cells, and all the growth curves were similar to that of the *B. subtilis* DB104 strain. The experiment was repeated three times (a representative example is shown).

Production of Pro A by *B. subtilis* DB104 carrying the pBAP plasmids

B. subtilis strains carrying different pBAP plasmids were grown in $2 \times \text{YT}$ medium. The OD_{600} and production of Pro A were monitored over a 30 h period. As for *E. coli*, preliminary experiments showed that expression of pBAP plasmids in *B. subtilis* could be grouped according to Pro A activity. *B. subtilis*(pBAP-3) and *B. subtilis*(pBAP12) produced similar, relatively low levels of Pro A activity (group 1). *B. subtilis* carrying pBAP24 or pBAP26 produced relatively high levels of Pro A activity (group 2), while *B. subtilis* carrying pBAP28, pBAP30 or pBAP31 produced intermediate levels of activity (group 3). Active Pro A was not detected in cultures of *B. subtilis* DB104(pBAP1). The production of Pro A in *B. subtilis*(pBAP12) and *B. subtilis*(pBAP24) was examined in liquid media as representative of groups 1 and 2, respectively (Fig. 5). *B. subtilis*(pBAP12) produced 3.6 U ml^{-1} , and *B. subtilis*(pBAP24), 10.6 U ml^{-1} of Pro A activity after 24 h. There was a lag of approximately 7 h before active Pro A was detected in the culture medium. Addition of tetracycline (protein synthesis inhibitor) to cultures during the exponential growth phase resulted in the inhibition of growth and Pro A production, relative to the uninhibited control.

Table 1. Localization of Pro A activity in stationary phase *E. coli* LK111(pBAP24) and *B. subtilis* DB104(pBAP24) cells.

E. coli and *B. subtilis* were grown in the complete medium containing 10 mM-CaCl₂ for 21.5 h. Sucrose (0.2%, w/v) was added to the *B. subtilis* cultures to induce sucrase activity.

Bacterium	Enzyme	Enzyme activity*		
		Cytoplasm	Periplasm	Extracellular supernatant
<i>E. coli</i>	Glutamine synthetase	100.0	0.0	0.0
<i>E. coli</i>	β -Lactamase	3.9	86.9	9.2
<i>E. coli</i>	Pro A	7.6	38.4	54.0
<i>B. subtilis</i>	Sucrase	68.0	—	32.0
<i>B. subtilis</i>	Pro A	1.6	—	98.4

* Enzyme activities are expressed as a percentage of the total enzyme activity.

Localization of protease activity

Previously, we reported that *E. coli* LE392 cells containing pVP100, which contains the *V. alginolyticus* signal sequence, secreted ProA without cell lysis (Deane *et al.*, 1987b). Experiments with stationary phase *E. coli* LK111 cells containing the fusion plasmid pBAP24 indicated that Pro A fused to a *Bacillus* signal sequence was also secreted without cell lysis (Table 1). The cytoplasmic (glutamine synthetase) and periplasmic (β -lactamase) marker enzymes were predominantly located in the cytoplasm and periplasm, respectively. Experiments with stationary-phase *B. subtilis* DB104(pBAP24) cells indicated that the majority of the cytoplasmic enzyme marker (sucrase) was in the cytoplasm (68%) but some cell lysis occurred, as 32% of the sucrase activity was located in the supernatant fraction (Table 1). Secretion of the majority of the Pro A by *B. subtilis* DB104(pBAP24) cells was demonstrated by the presence of 98.4% of the Pro A activity in the supernatant fraction.

Gelatin-PAGE protease assay

Extracellular supernatant and cellular fractions from stationary-phase cultures of *B. subtilis* DB104(pBAP24), *B. subtilis* DB104(pEB1) and a wild-type *B. subtilis* strain, 1A2, were analysed using gelatin-SDS-PAGE gels (Fig. 6). The gels were stained either after removal of the SDS by soaking in Triton X-100 and incubation in 0.1 M-glycine buffer (pH 9.0) (Fig. 6a) or immediately after gelatin-PAGE (Fig. 6b). Previously, we had investigated the production of Pro A by *E. coli*(pVP100), using the gelatin-PAGE protease assay, and reported a characteristic broad zone of protease activity at the top of the gel (Deane *et al.*, 1987b). Since Pro A is SDS-resistant, it is active during electrophoresis in SDS-gelatin gels, and the zone of protease activity is apparent on staining immediately after gelatin-PAGE. Analysis of *B. subtilis*

DB104(pBAP24) supernatant and cellular fractions revealed the presence of the majority of the SDS-resistant Pro A in the supernatant fraction, and a relatively small amount in the cellular fraction (Fig. 6, lanes 1 and 2). A minor band of protease activity was present in the supernatant fraction after soaking, but was not apparent after staining immediately. This minor band was not present in the other *B. subtilis* preparations. Control experiments with supernatant and cellular fractions of *B. subtilis* DB104, containing the shuttle plasmid pEB1 without the *proA* gene, indicated that this host bacterium did not produce a protease which was active in the presence of SDS (Fig. 6b, lanes 3 and 4). Faint bands of protease activity were observed in these fractions after soaking, but the relative activity in comparison with the equivalent fractions from *B. subtilis* DB104(pBAP24) cells was minimal. These results indicate that the recombinant plasmid pBAP24 did not induce an increase in endogenous protease. Furthermore, a wild-type *B. subtilis* strain with respect to protease production did not produce a SDS-resistant protease (Fig. 6b, lanes 5 and 6).

Discussion

Deane *et al.* (1987b) reported that when the *V. alginolyticus* Pro A gene was expressed in *E. coli*, under the control of its own regulatory regions and signal peptide, synthesis of Pro A took place during the exponential growth phase, whereas active extracellular protease was detected only in stationary phase cultures after 18 h. Addition of chloramphenicol to these cultures during the latter half of the exponential growth phase (6 h) did not affect the kinetics or levels of active Pro A production (Deane *et al.*, 1987b). It was concluded that in *E. coli*, the *V. alginolyticus* Pro A is synthesized during

Fig. 6. Analysis of SDS-resistant protease production by *B. subtilis* DB104(pBAP24), *B. subtilis* DB104(pEB1) and *B. subtilis* 1A2 cells. Gelatin-SDS-PAGE gels were stained after removal of the SDS by soaking in Triton X-100 and incubation in 0.1-M glycine buffer (pH 9.0) (a) or immediately after gelatin-PAGE (b). Lanes 1 and 2, *B. subtilis* DB104(pBAP24) extracellular supernatant and cytoplasmic fraction, respectively; lanes 3 and 4, *B. subtilis* DB104(pEB1), extracellular supernatant and cytoplasmic fraction, respectively; lanes 5 and 6, *B. subtilis* 1A2, extracellular supernatant and cytoplasmic fraction, respectively.

the exponential growth phase as an inactive precursor and secreted as active Pro A during the stationary growth phase. Active intracellular Pro A was not detected in *E. coli* cells containing the *proA* gene. When the *V. alginolyticus* DNA encoding the *proA* promoter and signal peptide were replaced by the DNA encoding an α -amylase promoter and signal peptide (pBAP-3, pBAP12 to 31) or the *proA* promoter was replaced by the

α -amylase promoter, active Pro A was detected in the culture media of *E. coli* cultures containing these constructs, approximately 8 h earlier than in *E. coli* cultures containing the entire *V. alginolyticus proA* gene. Furthermore, production of Pro A by *E. coli* cells containing the α -amylase-*proA* fusion genes was markedly affected by the inhibition of protein synthesis during the exponential growth phase. The addition of chloramphenicol to *E. coli* cells containing the entire *proA* gene did not affect the production of Pro A (Deane *et al.*, 1987b). It appears that Pro A production by the α -amylase-*proA* fusion genes in *E. coli* did not involve a 12 h lag between synthesis and secretion, and that Pro A synthesis and secretion occurred simultaneously. These results suggest that the *V. alginolyticus* promoter region is responsible for the kinetics of Pro A production in *E. coli*, since the replacement of this promoter region by the α -amylase promoter altered the kinetics of Pro A production. The *V. alginolyticus* Pro A signal peptide does not appear to be involved in the delayed secretion process, since the construct containing the DNA encoding the α -amylase promoter, the Pro A signal peptide and the *proA* structural gene (pBAP1) showed simultaneous Pro A synthesis and secretion in *E. coli* cells. Since simultaneous synthesis and secretion appears to be controlled by the α -amylase promoter, it is suggested that transcription and translation may take place in close association with membrane components, but in the case of delayed secretion with the protease promoter, transcription and translation may not involve membrane components and there is a consequent delay while the secretory process is activated.

B. subtilis cells containing the entire *V. alginolyticus proA* gene carried on pBVP100 did not produce active Pro A. This was presumably due to differences in promoter consensus requirements between Gram-positive and Gram-negative bacteria (Graves & Rabinowitz, 1986).

The Pro A protein has a signal peptide of 21 amino acids and an alkaline phosphatase fused to the Pro A protein was shown to be transported to the periplasm (Deane *et al.*, 1989). Two positively charged amino acids are followed by a core of hydrophobic and small neutral amino acid residues. A putative processing site is situated between Ala-21 and Phe-22, in agreement with the rules of von Heijne (1983). It was, therefore, surprising that this signal sequence was non-functional in *B. subtilis*. Active extracellular Pro A was not detected in supernatant samples of cultures of *B. subtilis*(pBAP1). pBAP1 contains the α -amylase promoter fused to the *proA* structural gene, including the sequence encoding the Pro A signal peptide. Replacement of the Pro A signal peptide code with that for the α -amylase signal peptide (pBAP24 to 31) resulted in the production of

active extracellular Pro A. Active Pro A was not detected by gelatin-PAGE analysis in cell extracts from *B. subtilis*(pBAP1) cells (results not shown). Although restriction mapping of the recombinant plasmid indicated that gross DNA rearrangements did not occur, small deletions or rearrangements could have occurred, which would not have been detected in restriction analyses, but may have rendered the gene or gene product inactive. Alternatively, Pro A was either not synthesized or was inactive in the cytoplasm of *B. subtilis*(pBAP1) cells.

Deletion of N-terminal amino acids of Pro A affected the levels of Pro A production in both *E. coli* and *B. subtilis*. In both species, deletion of the first 12 amino acids resulted in relatively low levels of Pro A activity, whereas deletion of 24 or more amino acids resulted in higher levels of Pro A activity. Processing may be important in both the secretion and activity of Pro A. Pro A shares homology with the subtilisin group of serine proteases (Deane *et al.*, 1989), and subtilisins are synthesized in a pre-pro-form (Powers *et al.*, 1986; Ikemura *et al.*, 1987). Furthermore, the α -amylase gene used for the fusions in this work does not encode a propeptide (Palva *et al.*, 1981). It is, therefore, possible that Pro A is synthesized in a pre-pro-form.

We have shown that the original *proA* gene and its regulatory region was not expressed in *B. subtilis*. Fusion of this gene without its signal peptide sequence to a *B. amyloliquefaciens* DNA encoding an α -amylase promoter and signal peptide resulted in the production of active Pro A in *B. subtilis* culture supernatants. The expression of this gene in *B. subtilis* for purposes of industrial production could probably be enhanced by the use of other DNA sequences encoding promoter/leader peptide combinations such as the *B. subtilis* *spo2* promoter DNA and *B. amyloliquefaciens* α -amylase DNA encoding a signal peptide (Overbeeke *et al.*, 1990). Alternatively, the use of integration vectors and multiple insertion techniques could result in higher production and stability (Calogero *et al.*, 1989; Petit *et al.*, 1990).

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