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

HAROLD ZAPPE, GREGORY L. BLATCH and DAVID R. WOODS*

Department of Microbiology, University of Cape Town, P B Rondebosch 7700, South Africa

(Received 18 March 1991; revised 14 October 1991; accepted 17 October 1991)

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 was 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, Ca²⁺-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* chromosom 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 ([Vasantha & 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.,...])

* Author for correspondence. Tel. (021) 650 2173/4; fax (021) 650 2138.

Abbreviations: Pro A, protease A; SD, Shine-Dalgarno sequence.
Developed a medium that suppresses the residual protease activity of protease-deficient \textit{B. subtilis} DB104 (Kawamura & Doi, 1984), thus improving the expression of fusion gene constructs.

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

\textbf{Methods}

\textit{Bacterial strains, plasmids and growth conditions.} \textit{E. coli} strain LK111 (a lacI, lacZDM15, lacY) derivative of K514 (Zabeau & Stanley, 1982) was used as a host for plasmid manipulations. \textit{B. subtilis} DB104 (his, napR2, napE18, napA3) (Kawamura & Doi, 1984) was used as a host for all experiments involving \textit{B. subtilis}. The \(\alpha\)-amylase gene and promoter were cloned as a single fragment from \textit{B. amyloliquefaciens} NCP1 (Corfield et al., 1984) (plasmid pVCI02), 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 \textit{B. amyloliquefaciens} \(\alpha\)-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 \textit{B. subtilis} shuttle vector pEBI, which was constructed by one of us (H.Z.) and has been described elsewhere (Lin et al., 1990). pEBI is an \textit{E. coli}–\textit{B. subtilis} shuttle vector with positive selection for inserted DNA fragments in \textit{E. coli}. \textit{E. coli} and \textit{B. subtilis} were grown in 2 \times YT medium (Sambrook et al., 1989) containing 10 \text{mM-CaCl}_2, Peptone water agar, containing Difco Peptone (2.5\% w/v), agar (1.5\% w/v) and skim milk (1.25\% 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\(^\circ\)C. Antibiotics added to media for plasmid selection and maintenance were ampicillin (100 \text{\mu g ml}^{-1}) for \textit{E. coli} and chloramphenicol (20 \text{\mu g ml}^{-1}) for \textit{B. subtilis}.

\textbf{Transformation of \textit{E. coli} and \textit{B. subtilis}.} \textit{E. coli} was transformed with plasmid DNA by the CaCl\(_2\)-shock method of Dagenet & Ehrlich (1979). \textit{B. subtilis} was transformed using the protoplast method of Rubenstein et al. (1988).

\textbf{Preparation of DNA, recombinant DNA techniques and DNA sequencing.} Plasmid DNA was prepared from \textit{E. coli} by the alkaline lysis method of Ish-Horowicz & Burke (1981) followed by CaCl\(_2\) equilibrium centrifugation in the presence of ethidium bromide. The method used for \textit{B. subtilis} plasmid isolation was identical, except that Solution 1 contained 5 mg lysozyme ml\(^{-1}\), and cells were incubated for 10 min at 37\(^\circ\)C in Solution 1 before continuing with the standard method. Deletions were generated using the nuclelease 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'–GGACGCGTGTATTTGTC-3' was synthesized in an Applied Biosystems 381A DNA synthesizer. The primer was used to determine the nucleotide sequence over \(\alpha\)-amylase–\textit{proA} gene fusion regions.

\textbf{Production of proteases.} Overnight cultures (grown with shaking at room temperature, approx. 22\(^\circ\)C) of \textit{E. coli} or \textit{B. subtilis} containing recombinant plasmids were diluted in fresh prewarmed (37\(^\circ\)C) 2 \times YT medium to an OD\(_{600}\) of 0.01–0.02 (approximately 1 in 200 dilution). The diluted cultures were incubated at 37\(^\circ\)C with shaking, and OD\(_{600}\) and Pro A production were monitored throughout the growth curve.

\textbf{Azocasein \textit{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-CaCl\(_2\)] and incubated for 30 min at 37\(^\circ\)C. Ice-cold TCA (10\% w/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 A\(_{440}\) 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\(^\circ\)C.

\textbf{Cell fractionation and enzyme assay.} \textit{E. coli} periplasmic and cytoplasmic cell fractions were prepared by the osmotic-shock method of Willis et al. (1974). \textit{B. subtilis} cytoplasmic fractions were prepared by sonicating washed cells for three 30 s bursts at 4\(^\circ\)C in 0.05 M-potassium phosphate buffer (pH 7.0). The sonicated cells were centrifuged at 4\,000 \text{rpm} 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 \(\beta\)-lactamase (Sykes & Nordstrom, 1972) were assayed in \textit{E. coli} cell-free culture supernatants, cytoplasmic and periplasmic cell fractions. Pro A and Sucrase (Scholle et al., 1987) were assayed in \textit{B. subtilis} cell-free culture supernatants and cytoplasmic fractions.

\textbf{Gelatin-PAGE protease assay.} Gelatin-PAGE protease assays were done as described by Deane et al. (1987b). SDS (2.5\% w/v) and glycercol (2.0\% w/v) were added to the samples which were then held at 37\(^\circ\)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\% w/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\(^\circ\)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.

\textbf{Results}

\textbf{Construction and analysis of \textit{pBAP} plasmids containing \(\alpha\)-amylase–\textit{proA} fusion genes.} A 1.85 kb \textit{PstI–HindIII} fragment of \textit{V. alginolyticus} chromosomal DNA from pVP100, encoding the entire \textit{proA} structural gene and promoter region, was subcloned into pUC19 and the \textit{B. subtilis} shuttle vector pEBI to generate the plasmids pVP19 (Fig. 1) and pBVP100 respectively.
Expression of amylase-protease fusion genes

\[ \text{expression of amylase-protease fusion genes} \]

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**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: \( \square \), protease A promoter; \( \mathbb{R} \), leader peptide; \( \mathbb{G} \), structural gene.

**Fig. 2.** Partial restriction map of pAmyPL and pAmyP. The thin line represents vector DNA; the box, insert DNA. The BglII–EcoRI 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: \( \square \), \( \alpha \)-amylase promoter; \( \mathbb{R} \), \( \alpha \)-amylase leader peptide; \( \mathbb{G} \), \( \alpha \)-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 PstI–EcoRI fragment from pVP19 into pUC19 and generating progressive deletions from the PstI 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 PstI–EcoRI 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 EcoRI–BglII fragment from pVC102, encoding the B. amyloliquefaciens \( \alpha \)-amylase promoter, Shine-Dalgarino (SD) sequence and signal peptide, including 103 N-terminal amino acids of the \( \alpha \)-amylase, was subcloned into Bluescript\(^{\text{K}} \) to generate the plasmid pAmyPL (Fig. 2). The plasmid pAmyP carried a DNA fragment which encoded the \( \alpha \)-amylase promoter, SD and the first amino acid codon of the \( \alpha \)-amylase gene, and was constructed by treating EcoRI 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 \( \alpha \)-amylase promoter, SD, and first amino acid codon of the \( \alpha \)-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 XbaI–AccI 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 \( \alpha \)-amylase DNA sequence encoding the promoter, SD and signal peptide, including...
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 (pBAP1 to pBAP3, 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.
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Production of Pro A by E. coli carrying the pBAP plasmids

E. coli strains carrying different pBAP plasmids were grown in 2 x 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 µg 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).

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

B. subtilis strains carrying different pBAP plasmids were grown in 2 x 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.
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* LA111 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 on staining is apparent 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)

**Table 1. Localization of Pro A activity in stationary phase *E. coli* LA111(pBAP24) and *B. subtilis* DB104(pBAP24) cells.**

*E. coli* and *B. subtilis* were grown in the complete medium containing 10 mM-CaCl₂ for 21-25 h. Sucrose (0.2%, w/v) was added to the *B. subtilis* cultures to induce sucrase activity.

<table>
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<th>Bacterium</th>
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<th>Periplasm</th>
<th>Extracellular supernatant</th>
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<tr>
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<td>Pro A</td>
<td>1.6</td>
<td>–</td>
<td>98.4</td>
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* Enzyme activities are expressed as a percentage of the total enzyme activity.

**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...
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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 pro-peptide (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).

References


Expression of amylase-protease fusion genes


