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Use of the *Bacillus subtilis* Subtilisin Signal Peptide for Efficient Secretion of TEM β-Lactamase during Growth

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We report the development of an efficient *Bacillus subtilis* secretory system, with the secreted product stably maintained in the medium for 100 h. The system is based on characterization of the subtilisin signal peptidase cleavage site and promoters, catabolite repression of sporulation, presence of a vegetative secreting mechanism, and availability of a protease-deficient strain.

Bacillus subtilis serves as an excellent system for studying the expression and secretion of foreign proteins, since *B. subtilis* is able to secrete a wide variety of enzymes, such as proteases, α-amylase, and β-glucanase, directly into the medium at high levels (8, 13, 17, 18). A large number of both procaryotic and eucaryotic genes have been successfully fused to the regulatory regions and signal peptide sequences derived from the *Bacillus* system for expression and secretion studies (3, 4, 6, 9, 10, 12). The reported levels of secreted proteins ranged from less than 1 to 50 mg/liter for rat proinsulin and staphylococcal nuclease, respectively. In most of the reported cases, the low yield and poor quality of secreted heterologous proteins were mainly due to the simultaneous production of at least three extracellular proteases (neutral protease, subtilisin, and esterase) during the sporulation or stationary phase (1, 3, 9, 15). These proteases significantly reduced both the physical integrity and the biological activities of the secreted foreign proteins. In this report we describe the use of a combination of a secretion vector system, an improved double protease mutant strain (5), and an enriched medium to achieve efficient synthesis of biologically active TEM β-lactamase, which can be stably maintained in the growth medium for at least 100 h without any significant degradation.

With the in vitro and in vivo characterization of both the transcription regulatory region (16) and the signal peptidase cleavage site for the subtilisin gene (15a), we were able to dissect the transcription regulatory element and the signal peptide sequence from the subtilisin gene and fuse them to a DNA sequence encoding the mature β-lactamase enzyme in a pUB110 derivative plasmid to form the pSHL-7 vector. The detailed construction of pSHL-7 plasmid has been reported elsewhere (15a). This vector was used to study the effect of host proteases on the stability of secreted β-lactamase. The pSHL-7 plasmid was transformed into isogenic *B. subtilis* DB101 (*his*), DB102 (*his nprR2 nprE18*), and DB104 (*his nprR2 nprE18 aprA3*). The cells were grown in the 2XSG sporulation medium (7), and the secreted β-lactamase activity was determined following growth by using PADAC (CalBiochem) as the β-lactamase substrate. As shown in Fig. 1 and Table 1, the peak level of secreted β-lactamase activity from strain DB102 was slightly higher than that from strain DB101. However, both showed much lower activity than DB104. At 5 h after the cell enters the stationary phase, the β-lactamase activity from DB101 and DB102 dropped significantly, almost to the background level, while that from DB104 remained reasonably high (Table 1). The rapid decrease in β-lactamase activity for DB101 and DB102 was clearly caused by the excretion of extracellular proteases into the medium. The slight decrease in β-lactamase activity for DB104 can be due either to the production and secretion of the extracellular esterase during sporulation phase or to lysis of the spore mother cell, resulting in the release of intracellular proteases into the medium.

To overcome these protease problems, we decided to exchange the subtilisin promoter cluster in the pSHL-7 vector with a strong, glucose-insensitive, vegetative-phase promoter so that we could grow cells in superrich medium (2), catabolite-repress the induction of esterase and sporulation (11), and synthesize the β-lactamase during growth. Thus, the problems with the production of extracellular esterase and the formation of leaky sporulation mother cells could be avoided. Whether the β-lactamase could be synthesized and secreted during growth was unpredictable.

For the isolation of strong, glucose-insensitive, vegetative-phase promoters, an expression probe plasmid was developed. The removal of the subtilisin transcription control element and the resulting construction of promoter probe plasmid pSL-4 is shown in Fig. 2. This vector has a unique *Bam*HI site preceding the ribosomal binding site and signal peptide sequence derived from the subtilisin gene fused to the mature β-lactamase sequence. *Sau*3A-digested

TABLE 1. Comparison of β-lactamase activity in the medium for strains DB101, DB102, and DB104[a]

Strain	β-Lactamase activity[b]			
	Maximum level		Stage T_5[c]	
	mU/ml	% of DB104 level	mU/ml	% of DB104 level
DB104	1,080	100	900	100
DB102	380	35	30	3.3
DB101	280	26	40	4.4

[a] Cells were grown in 2XSG sporulation medium.
[b] As a control for basal enzyme activity, strain DB104 containing plasmid pEL1 (this plasmid contains the mature β-lactamase gene without its promoter or signal peptide [15a]) produced no β-lactamase activity.
[c] I.e., 5 h after cells entered stationary phase.

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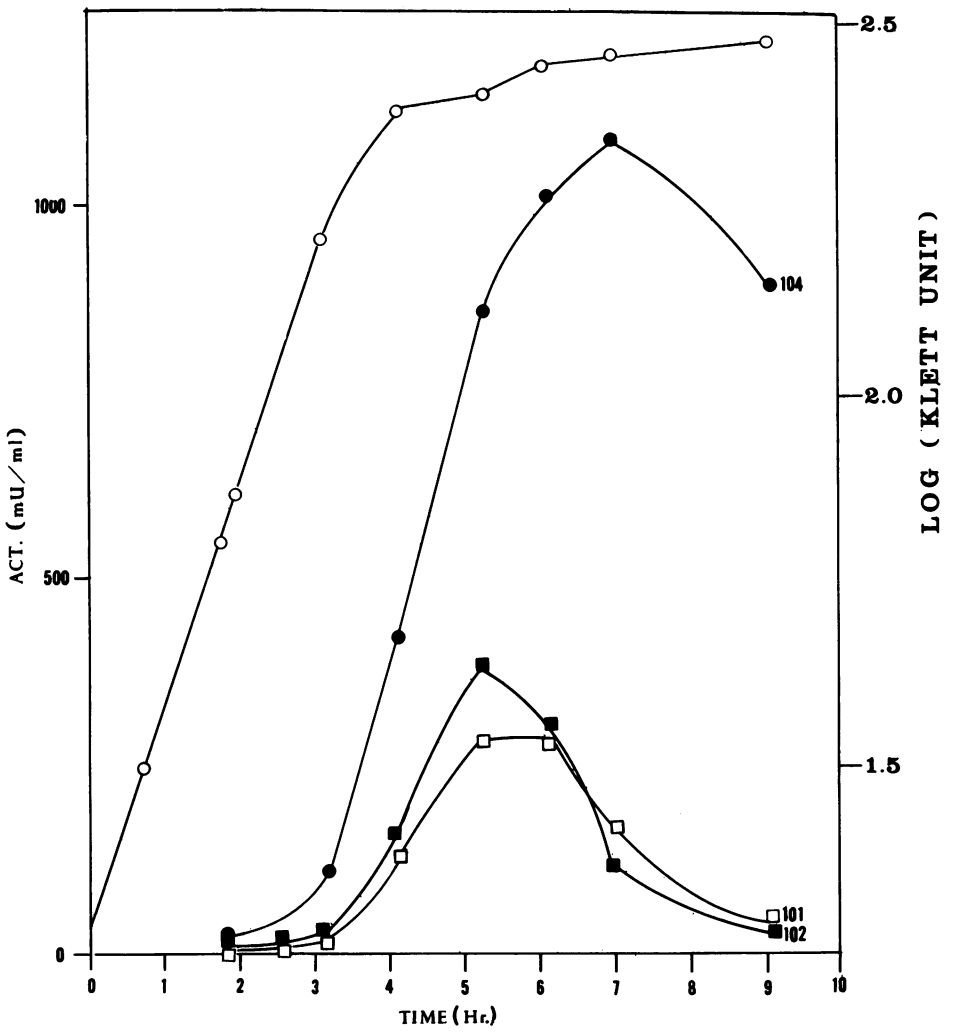

FIG. 1. Expression and secretion of β-lactamase into the medium during growth by different *B. subtilis* strains carrying the pSHL-7 plasmid. All cells were grown in 2XSG sporulation medium with kanamycin (5 μg/ml). At different developmental stages, 300 μl of cells were harvested and pelleted by centrifugation; 20 μl of supernatant was used for the β-lactamase assay (15a). One unit of β-lactamase activity is defined as the hydrolysis of 1 μmol of substrate per min. Symbols: ○, growth of *B. subtilis* in 2XSG medium; β-lactamase activity in the medium from strains DB104 (●), DB102 (■), and DB101 (□).

B. subtilis chromosomal DNA fragments were ligated to the *Bam*HI-cut pSL-4 plasmid and transformed into *B. subtilis* DB104. Colonies secreting the β-lactamase were identified by use of the ampicillin screen filter paper on TBAB agar plates containing 5 μg of kanamycin per ml as described in the brochure from Bethesda Research Laboratories. Among the 70 colonies active in secreting β-lactamase, clones carrying a strong, vegetative, glucose-resistant promoter in the promoter probe plasmid were selected by comparing the level of β-lactamase production on TBAB agar plates with and without 3% glucose. One clone carrying plasmid pVSL-33 was selected for further characterization because of its high-level expression and secretion of β-lactamase in the high-glucose medium. The growth curve of this clone in superrich medium and the β-lactamase activity curve are shown in Fig. 3. Even after the cell had been grown for 100 h, the secreted β-lactamase activity still remained at high levels. Western blot analysis (14) and Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gel-resolved β-lactamase are shown in Fig. 4. The high yield and high quality of undegraded β-lactamase could be observed in the medium even after 100 h of growth. At early times during growth the secreted protein was quite pure, since most extracellular protein genes are repressed during growth. The success of this system depended on the following three key elements: (i) a secretion vector containing a strong, glucose-resistant, vegetative promoter inserted upstream of the ribosomal binding site and signal peptide sequence derived from the *B. subtilis* subtilisin gene; (ii) a double protease mutant strain, *B. subtilis* DB104, which produced only low levels of glucose-repressible extracellular esterase; and (iii) a superrich medium containing 3% glucose that supported cell growth to high density, maintained the cells in a nonsporulating stage, which reduced the probability of extracellular esterase production and cell lysis, and allowed high production of recombinant gene product starting from the logarithmic phase of growth.

These basic concepts should be helpful in the study of the synthesis, secretion, and stabilization of many heterologous gene products from *B. subtilis*.

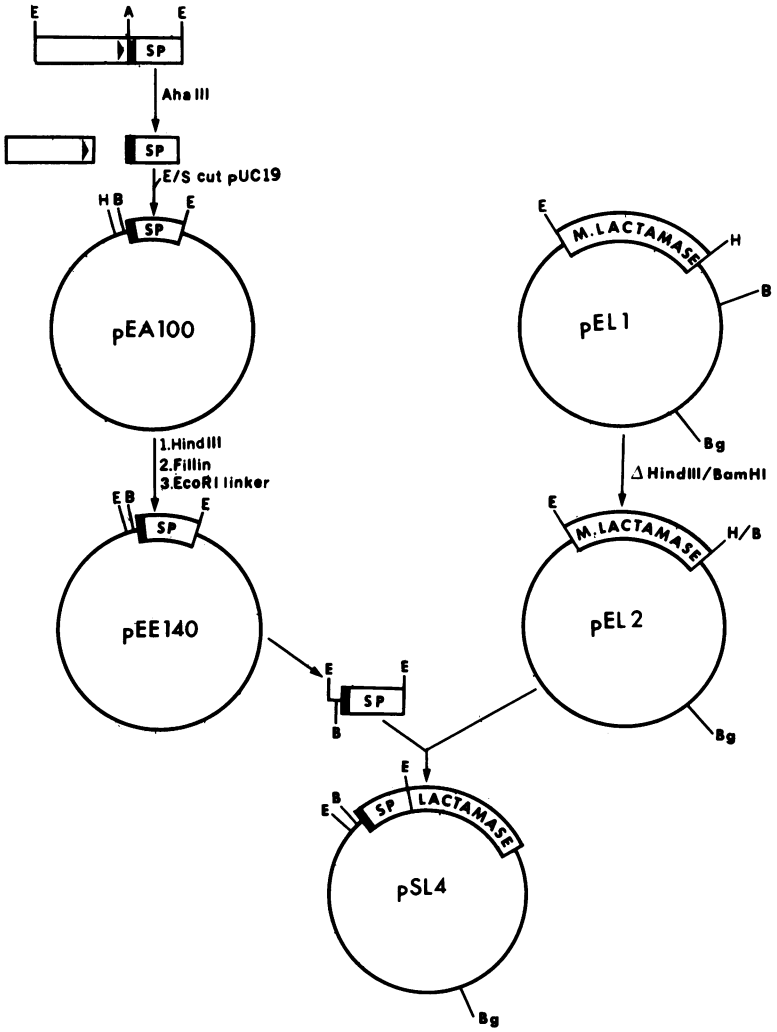

FIG. 2. Construction of the pSL-4 expression vector. The 370-base-pair (bp) *Eco*RI fragment carrying the subtilisin promoter, ribosomal binding site, and signal peptide was isolated from pSHL-7. The promoter portion was removed by *Aha*III digestion. The 100-bp *Aha*III-*Eco*RI fragment was ligated to *Eco*RI-*Sma*I-cut pUC19 to yield pEA100. The *Hin*dIII site in this plasmid was converted to an *Eco*RI site to form plasmid pEE140. The 140-bp *Eco*RI fragment from pEE140 was then ligated to pEL-2 to yield pSL-4. pEL-2 is a derivative of pEL-1 (15a). It was constructed by *Bam*HI-*Hin*dIII digestion of pEL-1 followed by a fill-in reaction and ligation. Abbreviations and symbols: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Bg, *Bgl*III; M. lactamase, mature lactamase sequence; SP, signal peptide; ▶, σ^{37} promoter of the subtilisin gene; ▮, ribosomal binding site.

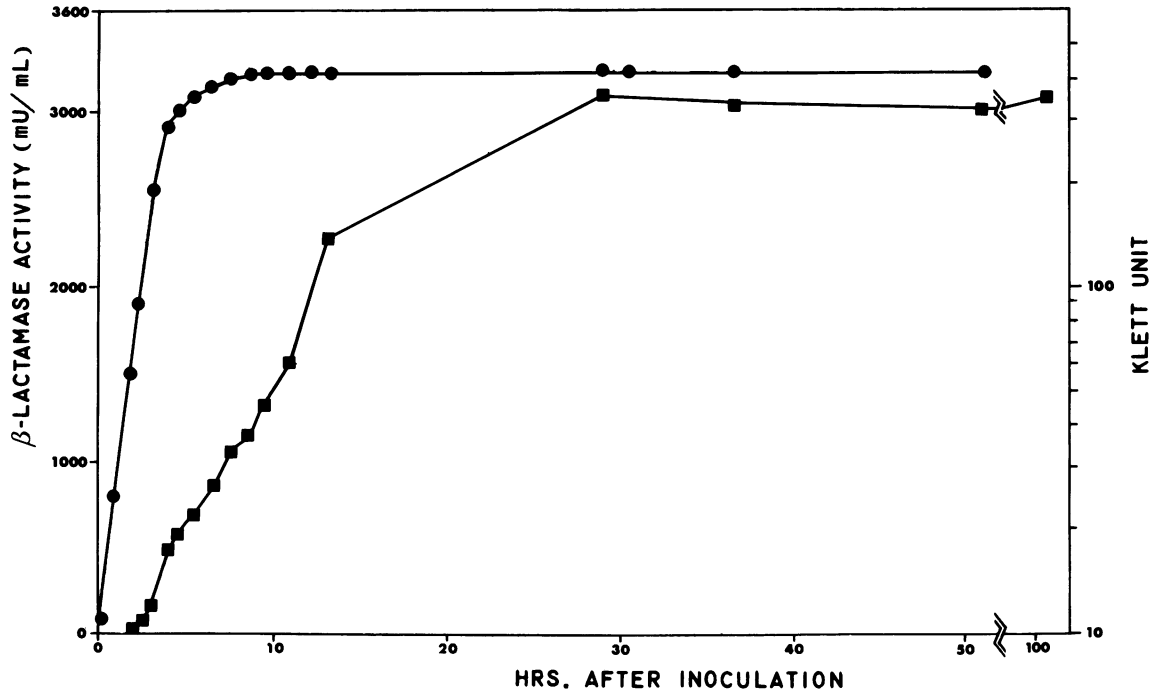

FIG. 3. Growth curve (●) and β-lactamase activity curve (■) of *B. subtilis* DB104(pVSL-33) in superrich medium.

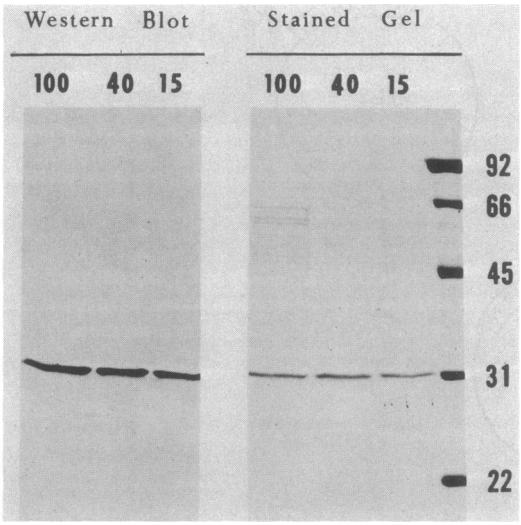

FIG. 4. SDS-polyacrylamide gel electrophoresis and Western blot analysis of secreted β-lactamase in the medium. Cells were harvested at 15, 40, and 100 h after inoculation as indicated above the lanes. Supernatant (300 μl) from each stage was applied to the 12% SDS–polyacrylamide gel for electrophoresis. Half of the gel was stained with Coomassie blue, and the other half was used for Western blot analysis with anti-β-lactamase antibody. Sizes are indicated to the right (in kilodaltons).

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