Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5582–5586, September 1982
Cell Biology

Secretion of *Escherichia coli* β-lactamase from *Bacillus subtilis* by the aid of α-amylase signal sequence

(cloning in *Bacillus*/secretion vector/α-amylase gene fusions/preprotein processing/gene expression)

ILKKA PALVA*†, MATTI SARVAS†, PÄIVI LEHTOVAARA*, MERVI SIBAKOV†, AND LEEVI KÄÄRIÄINEN*

*Recombinant DNA Laboratory, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, Finland; and †Central Public Health Laboratory, Mannerheimintie 166, SF-00280 Helsinki 28, Finland

Communicated by Daniel Nathans, June 22, 1982

ABSTRACT **We describe a secretion vector system for introducing foreign genes into *Bacillus subtilis*. We constructed secretion vectors from the plasmid pUB110 and the promoter and signal sequence region of the α-amylase gene from *Bacillus amyloliquefaciens*. Foreign structural genes can be inserted into the various vectors after the signal sequence region of the α-amylase gene. Demonstrating secretion of a foreign gene product from *Bacillus*, we here report that the *Escherichia coli* β-lactamase gene, devoid of its own signal sequence coding region, can be expressed in *B. subtilis* by the aid of the secretion vectors so that >95% of the enzyme activity is secreted to the growth medium. Efficient secretion of β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) is observed if the complete signal sequence coding region of the α-amylase gene precedes the β-lactamase structural gene. However, an incomplete α-amylase signal peptide lacking the six carboxy-terminal amino acid residues does not promote secretion of the fused β-lactamase, which remains unprocessed and cell-associated.**

Bacilli are potential hosts for the biosynthesis of foreign gene products in microorganisms by recombinant DNA technology. Compared to *Escherichia coli*, *B. subtilis* has the following advantages: (*i*) it is nonpathogenic, (*ii*) it is well known as an industrial organism, and (*iii*) it can secrete proteins to the culture medium. However, efficient expression of foreign genes and secretion of the gene products in *Bacilli* is still hampered by several problems. For instance, the secretory mechanisms of gram-positive *Bacilli* are not sufficiently well characterized, and particularly the control mechanisms remain unresolved. There also has been a lack of suitable vectors to promote expression and secretion of the product of an inserted gene. Expression of hepatitis B core antigen and of the major antigen of foot-and-mouth disease virus in *B. subtilis* has been obtained (1), but there have been no reports on secretion of foreign gene products by *Bacilli*.

The enzyme α-amylase (1,4-α-D-glucon gluconohydrolase, EC 3.2.1.1) is secreted in large amounts by some *Bacilli* to the culture medium. We have chosen the α-amylase gene as a model system to study the regulatory gene regions involved in efficient expression and secretion. The chromosomal α-amylase gene from *Bacillus amyloliquefaciens*, including the promoter region, has been isolated and cloned in the plasmid pUB110, with *B. subtilis* as host organism (2). The nucleotide sequence of the promoter and signal sequence region of this gene has been determined (3). Here we have constructed a series of secretion vectors for cloning in *B. subtilis* in hopes of obtaining both efficient expression and secretion of foreign gene products. The vectors are derivatives of the plasmid pUB110, and they contain the promoter, ribosome binding site, and signal sequence region of the α-amylase gene in front of the site where a foreign gene can be inserted. It has been shown that expression of foreign genes in *B. subtilis* apparently requires transcriptional and translational initiation signals from *Bacillus* or other gram-positive bacteria (4–6). Here we show that *B. subtilis* can secrete enzymatically active β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) to the culture medium after transformation with hybrid plasmids containing the α-amylase promoter and complete signal sequence region fused to the pBR322 structural gene of β-lactamase.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Bacteria, Plasmids, and Media. *Bacillus subtilis* strain IH6140, a prototrophic derivative of *B. subtilis* Marburg with reduced level of exoprotease activity, was from our collection. Plasmid pHV33, a tetracycline-resistant derivative of pHV14 (7), was obtained from S. D. Ehrlich. Plasmids pKTH38 and pKTH39 were derived from pKTH10 (2, 3) by exonuclease treatment as described below. Plasmid pKN410 carrying the β-lactamase gene (8) was a kind gift from K. Nordström through T. Palva. *B. subtilis* IH6140 transformants were selected on L-broth plates supplemented with 10 μg of kanamycin per ml. To assay the β-lactamase activity in liquid cultures, *B. subtilis* were grown in minimal medium (9) containing 0.5% glycerol, 1% soluble starch, and 10 μg of kanamycin per ml.

Cloning and Plasmid Isolation. *E. coli* K-12 strain HB101 was transformed (10), and plasmids were isolated (11) under standard conditions. Transformation of *B. subtilis* was as described by Gryczan *et al.* (12). To obtain plasmid multimers necessary for the physiological transformation of *B. subtilis*, the ligation was performed at 23°C for 3 hr by using an insert/plasmid ratio of 2:1 and a plasmid concentration of 75 μg/ml. Plasmid isolation in preparative scale was as described (3) and rapid screening was done by the method of Birnboim and Doly (13).

Exonuclease Treatments. Partial digestion of 9 pmol of linearized plasmid DNA with 75 units (18 pmol) of *E. coli* exonuclease III (New England BioLabs) was in 100 μl of 20 mM Tris·HCl, pH 7.6/0.66 mM $MgCl_2$/1 mM 2-mercaptoethanol at 22°C. Aliquots were removed at several time points (10–20 min) to an equal volume of 60 mM sodium acetate, pH 4.5/0.6 M NaCl/6 mM $ZnSO_4$ to stop the reaction. To obtain blunt ends, the pooled DNA was digested with S1 nuclease (0.1 units, P-L Biochemicals) first for 30 min at 37°C and then for 5 min at 4°C, followed by phenol extraction and a fill-in reaction with 10 units of reverse transcriptase (gift of J. Beard, Life Sciences) in 50 mM Tris·HCl, pH 8.3/60 mM KCl/12 mM $MgCl_2$/1 mM dithiothreitol/150 μM dNTPs for 30 min at 37°C.

Partial digestion of 10 pmol of linearized plasmid DNA with 1 unit of BAL-31 (Bethesda Research Laboratories) was in 250

μl of 20 mM Tris·HCl, pH 8.1/0.6 M NaCl/12 mM $CaCl_2$/12 mM $MgCl_2$/1 mM EDTA at 30°C. An excess of EDTA was added to aliquots removed at time points between 5 and 7.5 min, and the pooled DNA was extracted with phenol and filled in with T4 DNA polymerase (P-L Biochemicals) as follows: 3.3 pmol of DNA termini, 10 units of T4 polymerase, 50 μM of each dNTP in 6.3 mM $MgCl_2$/63 mM Tris·HCl, pH 8.1, in 20 μl for 80 min at 11°C. Linkers were ligated to linearized plasmids at 15°C for 16 hr by using a linker-to-plasmid molar ratio of 40:1.

Other Methods. β-lactamase was assayed from the supernatants of cultured cells by the method of Callaghan *et al.* (14). After electrophoresis in 12.5% $NaDodSO_4$/polyacrylamide gels (15), β-lactamase was visualized by an immunoblotting method (16) by using anti-β-lactamase anti-serum and ^{125}I-labeled protein A. DNA sequences were determined by the method of Maxam and Gilbert (17) as described (3).

RESULTS

Construction of Secretion Vectors for Cloning in *Bacillus*. The parental plasmid, pKTH38, is a derivative of the plasmid pKTH10 (2, 3), which contains the cloned chromosomal α-amylase gene from *B. amyloliquefaciens*. In pKTH38, the promoter and signal sequence for the secreted α-amylase is followed by 90 nucleotides from the coding region and an *Eco*RI linker. The plasmid pKTH38 was linearized with *Eco*RI (Fig. 1*A*), partially digested with exonuclease BAL-31, followed by ligation of *Hin*dIII linkers to the digested plasmid population. Linear plasmid monomers with *Hin*dIII linkers at the termini were purified, ligated, and transformed into *B. subtilis*.

The position of *Hin*dIII linkers in the plasmid DNA of 30 individual transformants was determined by sequence determination of the purified DNAs labeled at the *Hin*dIII sites and cleaved with *Cla* I. The sequence ladders A+G and C+T (17), covering nucleotides 1–40 for each sample, unequivocally determined the position of the *Hin*dIII linkers within the previously determined nucleotide sequence region of the α-amylase gene (3). By this screening, we obtained 12 different potential secretion vectors, with the *Hin*dIII linkers attached to the codon at the signal-sequence-processing site (pKTH50) or at various amino-terminal codons of the α-amylase structural gene. The gene coding for mature β-lactamase was fused to the plasmid pKTH50 to construct pKTH78.

We also prepared a pool from 120 isolated transformants containing *Hin*dIII linkers at various sites and characterized the pool by electrophoresis of the *Cla* I/*Hin*dIII double digests (Fig. 2). The size distribution of the *Cla* I/*Hin*dIII fragments showed that the *Hin*dIII linkers are attached within a 70-nucleotide region that should cover also the signal sequence cleavage site of α-amylase. When *B. subtilis* was transformed with plasmids that were recombinants of this pool and the β-lactamase gene, and when the transformants were screened for expression of β-lactamase, the plasmids pKTH83, -84, and -86 were obtained (Fig. 3).

Isolation of the β-Lactamase Gene Devoid of the Signal Sequence. Expression of an inserted foreign gene in *B. subtilis* and secretion of the product was tested by using β-lactamase gene from pBR322. For isolation of the β-lactamase gene devoid of its signal sequence, a fusion plasmid pHV33 (Fig. 1*B*) was linearized with *Eco*RI and partially digested with *E. coli* exonuclease III. The use of this fusion plasmid instead of pBR322 enabled the preservation of the tetracycline-resistance marker of pBR322. Exonuclease III/S1 nuclease-digested plasmids were attached to *Eco*RI linkers, sealed, and transformed into *E. coli*. Plasmids from ampicillin-sensitive transformants were isolated, and the *Eco*RI linkers were localized by nucleotide sequence determination from the *Eco*RI sites. Thus, the plasmid pKTH33 obtained has an *Eco*RI linker attached to the β-lactamase gene that is devoid of its signal sequence.

To obtain a construction with an *Eco*RI linker also near the 3′ end of β-lactamase gene, the *Bst*NI fragment containing this gene was isolated from pKTH33, *Eco*RI linkers were attached to it, and, after *Eco*RI digestion, the fragment was cloned in pBR325. For insertion of this β-lactamase gene into the *Bacillus* secretion vectors, the *Eco*RI linkers were replaced by *Hin*dIII linkers, and the fragment was recloned in pBR322.

Fusion of β-Lactamase Gene with *Bacillus* Secretion Vectors. The β-lactamase gene from pBR322, devoid of its signal sequence and containing *Hin*dIII termini, was ligated with the secretion vector pool. It also was ligated with one of the secretion vectors of known sequence, pKTH50, that had the linker attached to position −1 in the α-amylase signal sequence. The

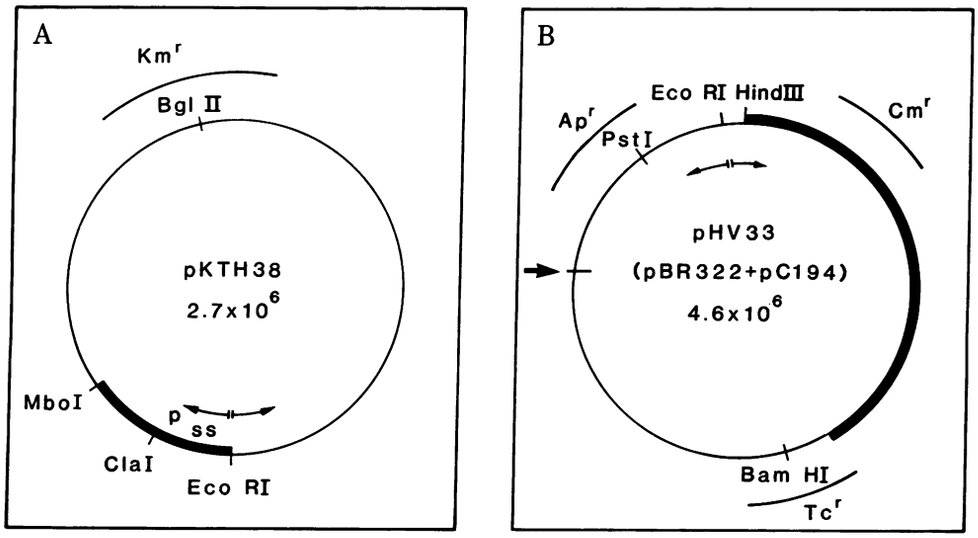

FIG. 1. (*A*) Plasmid pKTH38. The thin line represents pUB110 DNA, and the heavy black line represents a 0.7-kilobase chromosomal DNA insert from *B. amyloliquefaciens*. The *Eco*RI site is formed by a DNA linker at a position coding for amino acid 32 in the mature α-amylase. The arrows ←→ indicate the BAL-31 digestion starting at the *Eco*RI site. P and ss, promoter–signal sequence area of the α-amylase gene. (*B*) Plasmid pHV33. The thin line represents pBR322 DNA, and the heavy black line represents the pC194 DNA. The arrows ←→ indicate the *E. coli* exonuclease III/S1 digestion starting at the *Eco*RI site of pBR322. The position of the relevant *Bst*NI site is shown by the thick arrow (➔).

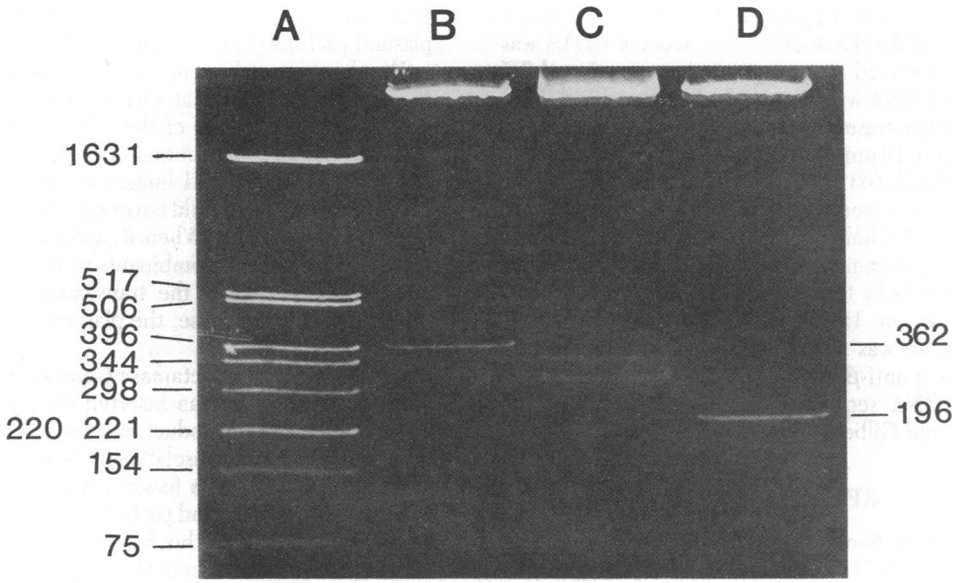

FIG. 2. Polyacrylamide gradient gel electrophoresis (18) of the BAL-31 digest of pKTH38. Lanes: A, *Hin*fI digest of pBR322; B, *Cla* I/*Eco*RI double digest of plasmid pKTH38, which has an *Eco*RI linker 362 base pairs from the *Cla* I site; C, *Cla* I/*Hin*dIII double digest of a plasmid pool isolated from 120 independent BAL-31-digested pKTH38 derivatives; D, *Cla* I/*Eco*RI double digest of plasmid pKTH39, a pKTH10 derivative, which has an *Eco*RI linker 196 base pairs from the *Cla* I site. Sizes are shown in base pairs.

hybrid plasmids were transformed into *B. subtilis*, the kanamycin-resistant transformants were screened for β-lactamase activity, and the sequences of some hybrid plasmids isolated from the β-lactamase-positive transformants were partially determined from the *Hin*dIII sites as described above.

Fig. 3 shows nucleotide sequences at the α-amylase gene–β-lactamase gene junctions of the plasmids pKTH78, pKTH83, pKTH84, and pKTH86. In plasmid pKTH78, only one nucleotide is missing from the full signal sequence of α-amylase, and the linker is attached to the codon for amino acid residue −1 (−1 construction). The plasmids pKTH83 and -84 contain in addition to the complete signal sequence, 11 nucleotides (+4 construction) and 41 nucleotides (+14 construction), respectively, from the α-amylase gene. In plasmid pKTH86, the β-lactamase gene is joined to an incomplete α-amylase signal sequence, which is devoid of 19 nucleotides (−7 construction). The deduced amino acid sequences of these constructions are shown in Fig. 4 together with those of the signal sequence and amino terminus of α-amylase and β-lactamase. In all four hybrid plasmids, an additional tetrapeptide sequence, Gln-Ala-Cys-Pro, coded by the linker construction, precedes the mature β-lactamase that starts from the proline residue at position 2.

Expression of the β-Lactamase Gene Fused to the Vectors. *B. subtilis* carrying the hybrid plasmids were grown in glycerol minimal medium supplemented with starch, and the β-lactamase activity was assayed from the culture supernatants and from sonicated cells (Fig. 5). The activity curves for strains carrying the plasmids pKTH78 and pKTH83 (construction −1 and +4) were almost identical (Fig. 5 *A* and *B*). The maximal activity, about 2,000 units/ml, was obtained 6–8 hr after the logarithmic growth phase.

B. subtilis harboring the plasmids with −1 or +4 vector constructions were able to secrete over 95% of the β-lactamase activity to the growth medium, and those carrying the plasmid pKTH84 (+14 construction) secreted over 90%. The total activity obtained by using the +14 construction was only 20% of

```
                -31 -30                 -25                 -20                 -15                 -10
pKTH10      ATG ATT CAA AAA CGA AAG CGG ACA GTT TCG TTC AGA CTT GTG CTT ATG TGC ACG CTG TTA TTT GTC AGT TTG

                    -5                  -1  +1                  +5                  +10                 +15
            CCG ATT ACA AAA ACA TCA GCC GTA AAT GGC ACG CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG AAC GAC GGC


                                    -4  -3  -2  -1                  +2  +3  +4
pKTH78                  ... AAA ACA TCA GCG CAA GCT TGC CGC  CCA GGA ACG ...

                                    +1  +2  +3  +4
pKTH83                  ... GTA AAT GGC ACG CAA GCT TGC CGC  CCA GGA ACG ...

                                    +11 +12 +13 +14
pKTH84                  ... TGG TAT ACG CCG CAA GCT TGC CGC  CCA GGA ACG ...

                                    -10 -9  -8  -7
pKTH86                  ... GTC AGT TTG CCG CAA GCT TGC CGC  CCA GGA ACG ...

                            α-amylase gene              β-lactamase gene
```

FIG. 3. The nucleotide sequence of the α-amylase gene–β-lactamase gene junction in the hybrid plasmids pKTH78, pKTH83, pKTH84, and pKTH86. The nucleotides from the linkers are encased (G-C-A-A-G-C-T-T-G-C is the *Hin*dIII linker and C-C is from an *Eco*RI linker), and those from the β-lactamase structural gene are given in italics. The complete signal sequence (−31 to −1), and the codons for 17 amino-terminal amino acids in the α-amylase gene contained in pKTH10 (3) are shown.

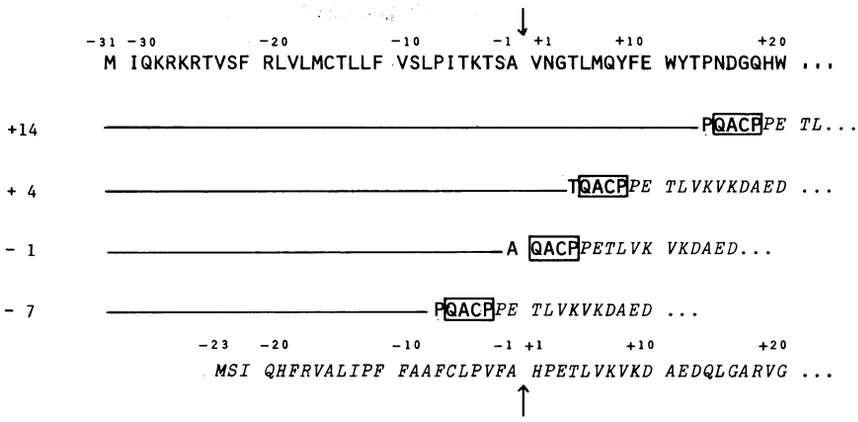

FIG. 4. The deduced amino acid sequences coded by the α-amylase and β-lactamase hybrid constructions +14 (pKTH84), +4 (pKTH83), −1 (pKTH78), and −7 (pKTH86). The sequences coded by the linker are encased, and the β-lactamase sequences are shown in italics. Identity is denoted by a line. The β-lactamase structural gene in the hybrids starts with a codon for proline at position +2. For comparison, the signal peptide and amino terminus of α-amylase (3) is given at the top and those of β-lactamase from pBR322 (19) are given at the bottom of the figure. The arrows indicate the site for processing for α-amylase and β-lactamase. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

that from the −1 or +4 constructions. The −7 construction, in contrast to the three others, did not contain the full signal sequence of α-amylase, and 90% of the β-lactamase activity was found to be cell bound. Also the total activity was low—only 10% of that obtained with the −1 and +4 vector constructions.

The size of the β-lactamase produced was estimated by $NaDodSO_4$/polyacrylamide gel electrophoresis in combination with an immunoblotting technique (Fig. 6). The β-lactamase secreted by all of the constructions −1, +4, and +14 (Fig. 6, lanes 4, 5, and 6, respectively) was somewhat larger than the authentic enzyme purified from *E. coli* carrying plasmid pKN410 (Fig. 6, lanes 3 and 7). This can be understood on the basis of the DNA sequence data, which indicates that the β-lactamase is preceded by 3, 7, or 18 extra amino acids in the constructions −1, +4, and +14, respectively. Apparently the signal sequence of α-amylase is processed at or near the correct position in these constructions. However, the cell-bound β-lactamase produced by construction −7 migrated more slowly than the other products (Fig. 6, lane 1), suggesting that the incomplete signal sequence of α-amylase was not cleaved. Somewhat unexpected was the relatively slow mobility of the "secreted" product from the same construction (Fig. 6, lane 2).

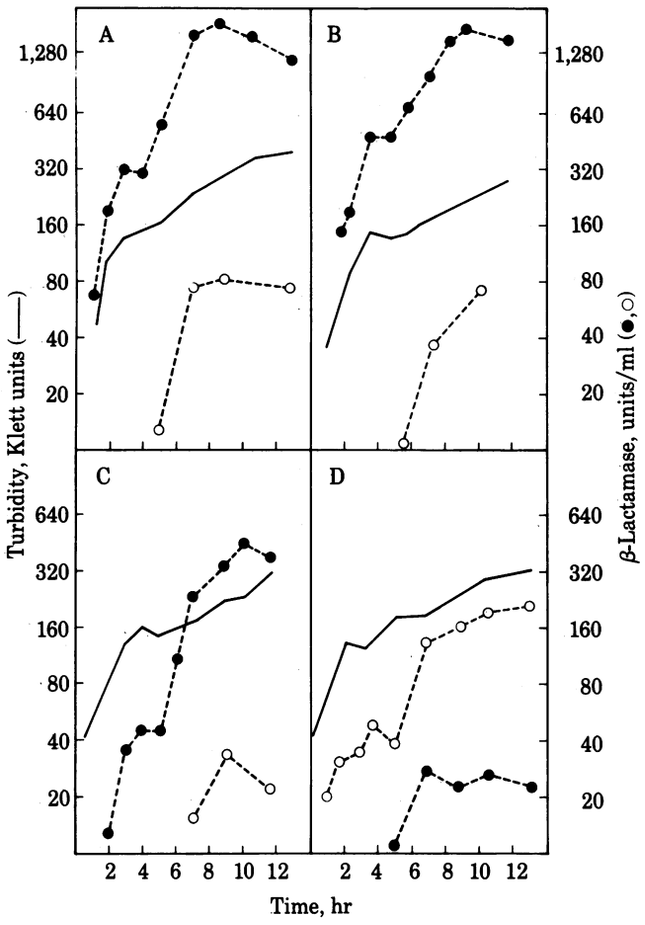

FIG. 5. Activity curves of β-lactamase assayed from the culture supernatants and sonicated cells of the different α-amylase gene–β-lactamase gene hybrid constructions. These constructions were: −1 (*A*); +4 (*B*); +14 (*C*); and −7 (*D*). The cells were grown in Spizizen minimal medium containing 0.5% glycerol, 1% soluble starch, and 10 μg of kanamycin per ml in a rotatory shaker at 37°C. ●----●, β-Lactamase activity in supernatant; ○----○, β-lactamase activity in sonicated cells; ——, turbidity.

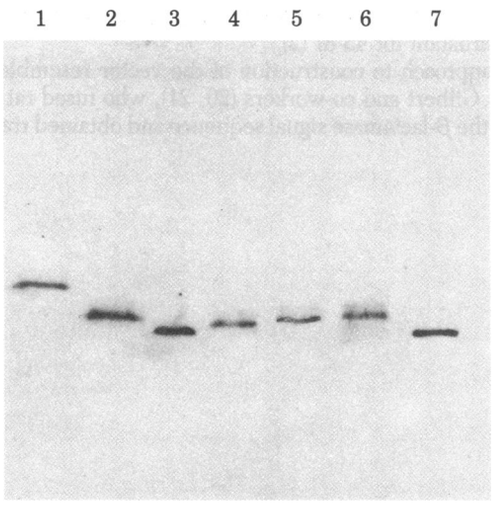

FIG. 6. Immunoblotting analysis of the β-lactamase synthesized by α-amylase gene–β-lactamase gene hybrid constructions. Lanes: 1, cells of −7 construction; 2, supernatant of −7 construction; 3 and 7, β-lactamase isolated from *E. coli* carrying the plasmid pKH410; 4, supernatant of −1 construction; 5, supernatant of +4 construction; and 6, supernatant of +14 construction.

DISCUSSION

We report here the construction of hybrid plasmids to be used as expression and secretion vectors for cloning of foreign genes in *Bacillus subtilis*. The ability of the plasmid constructions to promote secretion was first tested by introducing the β-lactamase gene, devoid of its own signal sequence, from pBR322 into the vectors after the promoter and signal sequence region of α-amylase. The transformed *B. subtilis* strain secreted β-lactamase efficiently to the culture medium, thus demonstrating the potential of this system.

The size estimations of the secreted β-lactamase show that it was processed correctly or at least within a few amino acid residues from the correct position in the constructions −1 and +4. The production curves for these constructions were nearly indistinguishable. This suggests that the amino acid sequence after the signal peptide cleavage site may not be critical for the specificity of the signal peptidase(s) in *B. subtilis* and that the signal sequence of α-amylase is sufficient for the processing and secretion of β-lactamase. Most of the β-lactamase coded by the −7 construction was membrane bound and apparently unprocessed, showing that the carboxyl-terminal six amino acids of the α-amylase signal peptide are necessary for secretion and processing.

The yield of β-lactamase by the +14 construction was surprisingly only 20% of that produced by the −1 and +4 constructions. This could be due to (*i*) a lower specific activity of the enzyme with an amino-terminal extension, (*ii*) a lower rate of synthesis, or (*iii*) greater susceptibility to proteolytic degradation. According to the quantitations from immunoblotting analysis (Fig. 6), all of the different constructions produced β-lactamase with approximately the same specific activity (data not shown), which would exclude the first possibility.

Secretion of β-lactamase begins at the end of the logarithmic growth phase, like the secretion of α-amylase. The maximal amount of extracellular β-lactamase in L broth with starch media was obtained 4–5 hr after the logarithmic phase and was about 6,200 units/ml, corresponding to 20 μg/ml. It seems probable that the proteases emerging at the beginning of the stationary growth phase of *B. subtilis* still prevent us from using the full capacity of this system, even though our host has a reduced level of proteases (unpublished data). *B. subtilis* harboring the plasmid pKTH10 continues to accumulate α-amylase in the supernatant for 45 hr (2).

Our approach to construction of the vector resembled that used by Gilbert and co-workers (20, 21), who fused rat proinsulin to the β-lactamase signal sequence and obtained transport of the proinsulin into the periplasmic space of *E. coli*. The complete signal peptide of α-amylase was in our system sufficient to direct secretion and processing of *E. coli* β-lactamase in *B. subtilis*, but a shortened signal peptide lacking the six carboxyl-terminal amino acid residues was not. The gene for β-lactamase was chosen to test our secretion vector because this enzyme is normally secreted into the periplasmic space of *E. coli*. We also have found that human interferon can be secreted from *B. subtilis* to the growth medium (unpublished data). It remains to be clarified whether this system also can be applied to promote secretion of proteins which normally are not secreted.

We thank Mses. Eila Kujamäki, Hannele Lehtonen, and AnnaLiisa Ruuska for their excellent technical help. This work was funded by the Finnish National Fund for Research and Development (SITRA).

1. Hardy, K., Stahl, S. & Küpper, H. (1981) *Nature (London)* **293**, 481–483.
2. Palva, I. (1981) *Gene*, in press.
3. Palva, I., Pettersson, R. F., Kalkkinen, N., Lehtovaara, P., Sarvas, M. Söderlund, H., Takkinen, K. & Kääriäinen, L. (1981) *Gene* **15**, 43–51.
4. Williams, D. M., Schoner, R. G., Duvall, E. J., Preis, L. H. & Lovett, P. S. (1981) *Gene* **16**, 199–206.
5. Goldfarb, D. S., Doi, R. H. & Rodriguez, R. L. (1981) *Nature (London)* **293**, 309–311.
6. McLaughlin, J. R., Murray, C. L. & Rabinowitz, J. C. (1981) *J. Biol. Chem.* **256**, 11283–11291.
7. Ehrlich, S. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1433–1436.
8. Uhlin, B. E., Molin, S., Gustafsson, P. & Nordström, K. (1979) *Gene* **6**, 91–106.
9. Anagnostopoulos, C. & Spizizen, J. (1961) *J. Bacteriol.* **81**, 741–746.
10. Mandell, M. & Higa, A. (1970) *J. Mol. Biol.* **53**, 159–162.
11. Clewell, D. B. & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA* **62**, 1159–1166.
12. Gryczan, T., Contente, S. & Dubnau, D. (1978) *J. Bacteriol.* **134**, 318–329.
13. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
14. Callaghan, C. H., Morris, A., Kirby, S. & Schnigler, A. H. (1972) *J. Antimicrob. Chemother.* **1**, 283–288.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
17. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
18. Jeppesen, P. G. N. (1980) *Methods Enzymol.* **65**, 305–319.
19. Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3737–3741.
20. Talmadge, K., Stahl, S. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3369–3373.
21. Talmadge, K., Kaufman, J. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3988–3992.