Molecular Cloning and Expression of Penicillinase Genes from *Bacillus licheniformis* in the Thermophile *Bacillus stearothermophilus*

By MIKIO FUJII, TADAYUKI IMANAKA AND SHUICHI AIBA*

Department of Fermentation Technology, Faculty of Engineering, Osaka University,
Yamada-oka, Suita-shi, Osaka 565, Japan

(Received 18 March 1982)

By using plasmid pTB90, penicillinase genes, *penP* and *penI*, from both the wild-type and constitutive strains of *Bacillus licheniformis* 9945A were cloned and expressed in a thermophile, *Bacillus stearothermophilus*. *Bacillus stearothermophilus* carrying the recombinant plasmid produced a large amount of penicillinase [3300 U (mg cells)⁻¹] at 48 °C, whereas the amount of enzyme produced at 55 °C and 60 °C was reduced to 660 U (mg cells)⁻¹ and 39 U (mg cells)⁻¹, respectively. Active repressor for penicillinase functioned normally. About 10 to 20 % of the total penicillinase was secreted into the culture medium by the transformant of *B. stearothermophilus*.

INTRODUCTION

The genus *Bacillus* represents one of the most important groups of bacteria not only for the production of commercially valuable enzymes but also for the study of the secretion mechanism of extracellular enzymes. Molecular cloning with plasmids has been well documented in some *Bacillus* species. However, only a few examples have been reported for the cloning of structural genes for extracellular enzymes in the genus *Bacillus*, i.e. the cloning of penicillinase genes of *Bacillus licheniformis* in *Bacillus subtilis* and *B. licheniformis* (Gray & Chang, 1981; Imanaka *et al.*, 1981*b*), and the cloning of the α -amylase gene of *Bacillus amyloliquefaciens* in *B. subtilis* (Palva *et al.*, 1981). So far such studies have been restricted to mesophilic bacteria.

The cloning of penicillinase genes of *B. licheniformis* in *Bacillus stearothermophilus* is significant with regard to examining the possibility of producing the enzyme at higher temperatures and of elucidating the difference in secretion of extracellular enzymes between mesophiles and thermophiles. The cloning of such genes would thus be of practical and academic interest.

We have established a transformation system in *B. stearothermophilus* by using a shuttle vector plasmid pTB90 between the thermophile *B. stearothermophilus* and the mesophile *B. subtilis* (Imanaka *et al.*, 1982). The purpose of this paper is to show the expression of penicillinase genes, *penP* and *penI*, from a mesophile in a thermophilic *Bacillus* strain.

METHODS

Media and materials. All the media used in this work have been described by Imanaka *et al.* (1981*a, b*, 1982). The companies and laboratories, from which the antibiotics, restriction enzymes and all other reagents used here were purchased were the same as those used in previous works (Imanaka *et al.*, 1981*a, b*, 1982).

Bacterial strain and plasmids. *Bacillus stearothermophilus* CU21 (streptomycin resistant; Imanaka *et al.*, 1982) was used as the host strain. pTB90 (encoding resistance to both kanamycin and tetracycline, Km^r Tc^r; Imanaka *et al.*, 1982) was used as the vector plasmid. Penicillinase genes from both the constitutive and wild-type strains of *B. licheniformis* 9945A have been cloned in *Escherichia coli* with pMB9 (Tc^r) as vector plasmid. These recombinant plasmids are designated, pTTE11 (Tc^r *penP*⁺ *penI*10) and pTTE21 (Tc^r *penP*⁺ *penI*⁺), respectively (Imanaka *et al.*, 1981*b*). *penP* and *penI* are the structural gene and the repressor gene for penicillinase, respectively, and are coded on the same *EcoRI* fragment (2.8 MDal, Imanaka *et al.*, 1981*b*). The restriction endonuclease cleavage maps of these plasmids are shown in the upper row of Fig. 1.

Preparation of plasmid DNA. Plasmid DNA was prepared from *B. stearothersophilus* by the rapid alkaline extraction method or CsCl-ethidium bromide equilibrium density gradient centrifugation as described previously (Imanaka *et al.*, 1981a, 1982). pTTE11 and pTTE21 were prepared from *Escherichia coli* as described elsewhere (Imanaka *et al.*, 1981b).

Transformation. Transformation of *B. stearothersophilus* protoplasts with plasmid DNA was performed as described previously (Imanaka *et al.*, 1982). Transformants were selected on regeneration agar containing either kanamycin (Km; $25 \mu\text{g ml}^{-1}$) or tetracycline (Tc; $5 \mu\text{g ml}^{-1}$), and stored on L agar containing Km ($5 \mu\text{g ml}^{-1}$) or Tc ($5 \mu\text{g ml}^{-1}$) at 4°C .

Detection of penicillinase-positive colonies on plates. Antibiotic-resistant transformants were transferred by replica plating on L agar containing 0.75% (w/v) polyvinylalcohol and the specific antibiotic (Km or Tc). After colony formation at 48°C , penicillinase-positive (PCase⁺) colonies were detected quickly by adding an iodine solution as described by Sherratt & Collins (1973).

Penicillinase assay. *Bacillus stearothersophilus* strains carrying the cloned penicillinase genes were grown at constant temperatures (48 , 55 and 60°C) in LG broth. Km ($5 \mu\text{g ml}^{-1}$) was added to the culture medium to guarantee the presence of the recombinant plasmid. Penicillinase was assayed in both the culture broth directly and in cell-free culture supernatant by the iodometric method as described previously (Imanaka *et al.*, 1981b). One unit of penicillinase was defined as the quantity required to hydrolyse $1 \mu\text{mol}$ of penicillin G h^{-1} at 30°C . For the inducibility test, $2 \mu\text{g ml}^{-1}$ of cephalosporin C was used as an inducer, since $5 \mu\text{g}$ cephalosporin C ml^{-1} used for *B. licheniformis* (Imanaka *et al.*, 1981b) in LG broth produced bacterial lysis.

Other procedures. Procedures for digestion of plasmid DNA with restriction endonucleases, ligation of DNA and agarose gel electrophoresis are all described elsewhere (Imanaka *et al.*, 1981b, 1982).

RESULTS

Cloning of penicillinase genes, penP and penI, from B. licheniformis in B. stearothersophilus

Cloning of penicillinase genes was attempted in a thermophilic bacterium *B. stearothersophilus* CU21. Partially digested pTB90 DNA with *EcoRI* was mixed with an *EcoRI* digest of

Fig. 1. Restriction endonuclease cleavage maps of plasmids. Cleavage sites of *Bgl*II, *Eco*RI, *Hind*III and *Pst*I are indicated by B, E, H and P, respectively. Outer circles, —, originated from pMB9; —, from pTB90; —, *Eco*RI fragment containing penicillinase genes from *B. licheniformis*. The numbers inside the inner circles indicate molecular size of *Eco*RI fragments in MDal.

pTTE11 (*penP*⁺ *penI*⁻) or pTTE21 (*penP*⁺ *penI*⁺), followed by ligation. The ligation mixtures (pTTE11 + pTB90 and pTTE21 + pTB90) were used to transform *B. stearothermophilus* CU21. Several Km^r Tc^r PCase⁺ transformants were obtained with each ligation mixture. Plasmids were isolated from these transformants and examined by agarose gel electrophoresis. The smallest recombinant plasmids obtained from the ligation mixture of pTTE11 plus pTB90 and pTTE21 plus pTB90 were designated as pLP11 and pLP21, respectively.

These plasmids were digested with several restriction endonucleases and subjected to agarose gel electrophoresis. The results obtained from these analyses (results not shown) were consistent with the restriction endonuclease cleavage maps of pLP11 and pLP21 as shown in Fig. 1.

Expression of penicillinase genes in B. stearothermophilus at elevated temperatures

Penicillinase activity was measured for the whole culture of *B. stearothermophilus* with and without recombinant plasmid carrying penicillinase genes in the absence of inducer (Table 1). No enzyme activities were detected for either *B. stearothermophilus* or the strain carrying only the vector plasmid pTB90. *Bacillus stearothermophilus* cells carrying pLP21 (*penP*⁺ *penI*⁺) produced nearly the same amount of penicillinase [90 to 120 U (mg cells)⁻¹] at 48 and 55 °C, while considerably less activity was detected by cultivation at 60 °C [17 U (mg cells)⁻¹]. On the contrary, a large amount of penicillinase [3300 U (mg cells)⁻¹] was observed for the strain harbouring pLP11 (*penP*⁺ *penI*⁻). However, the enzyme activity sharply decreased with increase in temperature of cultivation. It is evident from these facts that both genes, *penP* and *penI*, were expressed in *B. stearothermophilus*.

As described previously (Imanaka *et al.*, 1982), the copy number of pTB90 in *B. stearothermophilus* CU21 is amplified in the presence of Tc. The copy number of pLP11 should therefore increase, if Tc was added to the medium. *Bacillus stearothermophilus* CU21 carrying pLP11 was cultivated at 48 °C in LG plus Km (5 µg ml⁻¹) containing 1 to 4 µg Tc ml⁻¹ and penicillinase activities were measured for the culture broth (Table 1). With an increase in the amount of Tc added, penicillinase activities were enhanced. The highest penicillinase activity [6000 U (mg cells)⁻¹] was detected when the bacterium was cultivated in the presence of 3 µg Tc ml⁻¹, about a twofold increase of penicillinase compared with the amount found in the absence of Tc [3300 U (mg cells)⁻¹].

Penicillinase activity was also measured for the cell-free supernatant (Table 1). About 10 to 20% of the total penicillinase was secreted into the culture medium at 48 °C.

Table 1. *Penicillinase activity of B. stearothermophilus*

Plasmid	Antibiotic*	Growth temperature (°C)	Penicillinase activity† [U (mg of cells) ⁻¹]	
			Total	Supernatant
None	None	48	ND	—
pTB90	Km (5)	48	ND	—
pLP21	Km (5)	48	90	7.4
pLP21	Km (5)	55	120	—
pLP21	Km (5)	60	17	—
pLP11	Km (5)	48	3300	820
pLP11	Km (5)	55	610	—
pLP11	Km (5)	60	39	—
pLP11	Km (5) + Tc (1)	48	3700	510
pLP11	Km (5) + Tc (2)	48	4700	680
pLP11	Km (5) + Tc (3)	48	6000	700
pLP11	Km (5) + Tc (4)	48	5800	750

—, Not tested.

ND, Not detectable [0.01 U (mg dry cell wt)⁻¹].

* The numbers in parentheses indicate the concentration of the antibiotic used (µg ml⁻¹).

† Similar results were obtained by another series of experiments.

DISCUSSION

We have cloned penicillinase genes, *penP* and *penI*, from a mesophile *B. licheniformis* in a thermophile *B. stearothermophilus* by using the vector plasmid pTB90. The cloned penicillinase genes could be expressed in *B. stearothermophilus* even at 60 °C. The amount of penicillinase produced by *B. stearothermophilus* (pLP11) at 48 °C [3300 U (mg cells)⁻¹] was as much as that produced by the magno-constitutive strain *B. licheniformis* C01 at 37 °C [3000 U (mg cells)⁻¹; Imanaka *et al.*, 1981*b*]. However, the enzyme activity that was expressed decreased with increase in temperature of cultivation. The deterioration of penicillinase expressed at higher temperatures in *B. stearothermophilus* is under further investigation but may be due to the following: (i) heat inactivation of penicillinase, (ii) degradation of penicillinase by a proteolytic enzyme(s), (iii) decline in ability of the secretion mechanism for penicillinase at higher temperatures and (iv) deterioration in gene expression (transcription and translation) of foreign (mesophilic) DNA at elevated temperatures. In addition, the fact that the reduced penicillinase activity at higher temperatures for *B. stearothermophilus* (pLP21) was less marked than that of the pLP11 carrier strain (Table 1), suggests the thermal inactivation of the repressor.

The mode of gene expression for penicillinase in *B. stearothermophilus* is similar to that in *B. subtilis* in the sense that active repressor functioned normally (see Table 1). Penicillinase was not induced (data not shown) presumably because of the absence of the gene for antirepressor, which might titrate the repressor (*penI* gene product) in the presence of inducer, on the chromosome of *B. stearothermophilus* as was suggested for *B. subtilis* (Imanaka *et al.*, 1981*b*).

Both *B. licheniformis* and *B. subtilis* could secrete penicillinase into the culture media (Imanaka *et al.*, 1981*b*). About 10 to 20% of the total penicillinase was secreted by *B. stearothermophilus* carrying penicillinase genes compared with 30% for *B. subtilis* (Imanaka *et al.*, 1981*b*). With respect to the extracellular enzyme, these results might point out that some secretion mechanisms could be shared among these *Bacillus* species.

REFERENCES

- GRAY, O. & CHANG, S. (1981). Molecular cloning and expression of *Bacillus licheniformis* β -lactamase gene in *Escherichia coli* and *Bacillus subtilis*. *Journal of Bacteriology* **145**, 422–428.
- IMANAKA, T., FUJII, M. & AIBA, S. (1981*a*). Isolation and characterization of antibiotic resistance plasmids from thermophilic bacilli and construction of deletion plasmids. *Journal of Bacteriology* **146**, 1091–1097.
- IMANAKA, T., TANAKA, T., TSUNEKAWA, H. & AIBA, S. (1981*b*). Cloning of the genes for penicillinase, *penP* and *penI*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus licheniformis*. *Journal of Bacteriology* **147**, 776–786.
- IMANAKA, T., FUJII, M., ARAMORI, I. & AIBA, S. (1982). Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. *Journal of Bacteriology* **149**, 824–830.
- PALVA, I., PETTERSSON, R. F., KALKKINEN, N., LEHTOVAARA, P., SARVAS, M., SÖDERLUND, H., TAKKINEN, K. & KÄÄRIÄINEN, L. (1981). Nucleotide sequence of the promoter and NH₂-terminal signal peptide region of the α -amylase gene from *Bacillus amyloliquefaciens*. *Gene* **15**, 43–51.
- SHERRATT, D. J. & COLLINS, J. F. (1973). Analysis by transformation of the penicillinase system in *Bacillus licheniformis*. *Journal of General Microbiology* **76**, 217–230.