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RESEARCH ARTICLES

GENETIC MANIPULATION OF PENICILLIN ACYLASE IN *BACILLUS* SP.

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ABSTRACT

Localization of the Bacillus megaterium *penicillin acylase (PAC) gene on plasmid pMLV101 was done by using the transposition inactivation technique. The PAC gene was found to be about 3 Kb in length. Partial digestion of pMLV101 with restriction enzyme HpaII produced a DNA fragment (F1) of 2.8 Kb. The F1 DNA and the plasmid vector pACYC184 (pBA3 and pBA32) allowed* Escherichia coli *transformants to produce PAC enzyme. A recombinant plasmid of F1 and the shuttle vector pHV33-2, pHBA33, allowed both* E. coli *and* B. subtilis *host cells to produce PAC. All* E. coli *subclones (pBA3, pBA32 and pHBA33) produced about the same level of PAC enzyme activity as did* E. coli *which carried the plasmid pMLV101. Recombinant* B. subtilis *which carried plasmid pHBA33 produced PAC extracellularly, but its activity was slightly lower than that produced by* B. megaterium *UN-1.*

INTRODUCTION

Penicillin G acylase (PAC) cleaves the side chain of penicillin G to yield 6-amino penicillin acid (6-APA)[1, 2]. 6-APA is a key intermediate for producing many penicillin derivatives effectively used in treating infectious diseases. PAC is produced by many bacteria including *E. coli, B. sphaericus* and *B. megaterium*[3]. The gene encoding the enzyme from *E. coli* has been cloned and sequenced[4, 5]. However, up to now only our group and a group in China (personal communication) have been successful in cloning the gene from *B. megaterium*[6]. Previous work showed that recombinant *E. coli* harbouring the PAC gene of *B. megaterium* produced a low level of enzyme and only in the cell bound form[3]. Since isolation and purification of enzymes is easier with extracellular enzymes from culture broth than with intracellular enzymes, the aim of our study was to produce PAC extracellularly from recombinant bacteria in high

amounts. Our approach began with characterization and manipulation of the cloned *B. megaterium* penicillin acylase gene and its subcloning into *B. subtilis* in order to increase extracellular enzyme production.

MATERIALS AND METHODS

Microorganisms and maintenance

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Stock cultures were grown on LB agar (1% tryptone 0.5% NaCl, 0.5% yeast extract and 1.5% agar) at 37°C overnight for *E. coli* or 2 days for *Bacillus* sp. Cells were kept at 4°c and periodically subcultured. For extended storage, cells were kept in 15% glycerol at −70°c.

Plasmid preparation

Plasmids from *E.coli* or *Bacillus* sp. were prepared by the alkaline SDS method[15]. For small scale preparation, cells from 1.5 ml of overnight culture were pelleted and 100 μl of solution I (50 mM glucose, 25 mM Tris HCl buffer pH 8.0, 10 mM EDTA with 2 mg/ml lysozyme) was added. The combination was incubated at room temperature for 5 min before addition of 200 μl of solution II (1% Sodium dodecyl sulfate in 0.2 N Sodium hydroxide) and further incubation at 0°C for 5 min followed by addition of 150 μl of 3M Sodium acetate pH 4.8. After mixing and further incubation at 0°C for 60 min, the supernatant was collected following centrifugation. The DNA in the supernatant was precipitated with ethanol at a final concentration 70%. The DNA pellet was redissolved in TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA) and 7.5 M ammonium acetate was added to obtain a final concentration of 2.5 M before reprecipitation with ethanol. The DNA was reprecipitated 1-2 times more to obtain partially purified DNA for further use. For large scale DNA preparation, greater quantities of cells were used and the extracted DNA was isolated and purified by using cesium chloride gradient centrifugation (Hitachi 70P-72, Japan) at 48,000 rpm 18°C for 40 hours.

DNA restriction and gel electrophoresis

Restriction enzyme digestion of plasmid DNA was performed using the buffers and temperatures recommended by the manufacturers. The digestion period was 1-2 hours. Electrophoresis was carried out by using 1% agarose gel (Sigma, USA) in TBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA, pH 8.0). The agarose gel was stained with 5 μg/ml ethidium bromide solution and visualized by UV transillumination (TL33 Ultraviolet Production Inc., USA) and photographed using Kodak Technical Pan film.

Gene localization with transposon Tn1000

To locate the penicillin acylase gene on plasmid pMLV101, the transposition inactivation technique described by Guyer[16] was used. Plasmid pMLV101 was transformed into *E.coli* 169 (F^+), and the transformants were conjugated with *E.coli* 1L910 (F^-,

TABLE 1 Characteristics of bacteria used in this study.

Strain	Genotype	Reference
E. coli DH1	r_k^-, m_k^-, thi−1, recA	7
E. coli 1L9	F^+, prototroph	6
E. coli 1L910	gyrA, metB, $hsdr_k^-m_k^+$, F^-	6
B. subtilis MI111	r_m^-, m_m^-, leuA8m arg15	8
B. megaterium UN-1	pac^+	9
S. marcescens ATCC27117	$PenG^R$, $6\text{-}APA^S$	10

Pen G^R	=	Penicillin G resistance.	arg	=	Arginine auxotroph.
$6\text{-}APA^S$	=	Sensitive to 6-Aminopenicillanic acid.	met	=	Methionine auxotroph.
pac^+	=	Penicillin acylase producing ability.	F^+	=	Fertility (F-plasmid) present.
r^-	=	Restriction system deficient.	F^-	=	Fertility (F-plasmid) absent.
m^-	=	Modification system deficient.	recA	=	Rec A protein producing ability.
thi	=	Thiamine auxotroph.	gyrA	=	Gyrase enzyme producing ability.
leu	=	Leucine auxotroph.			

TABLE 2 Characteristics of plasmids used in this study.

Plasmid	Characteristic (host)	Reference
pACYC184	Cm^R, Tc^R *(E.coli)*	11
pMLV101	Tc^R, pac^+ *(E.coli)*	6
pHV33	Ap^R, Tc^R, Cm^R *(E.coli)* Cm^R, *(B.subtilis)*	12
pUB110	Km^R, *(B.subtilis)*	13
pTF6	Cm^R, Km^R, *(B.subtilis)*	14
pHV33-2	Cm^R, *(E.coli, B.subtilis)*	This study
pBA3	Cm^R, pac^+, *(E.coli)*	This study
pBA32	Cm^R, pac^+, *(E.coli)*	This study
pHBA33	Cm^R, pac^+, *(E.coli, B.subtilis)*	This study
pBA310	Km^R, pac^+, *(E.coli, B.subtilis)*	This study
pBA401	Km^R, pac^+, *(B.subtilis)*	This study

Ap^R	=	Ampicillin resistance.	Km^R	=	Kanamycin resistance.
Tc^R	=	Tetracyclins resistance.	pac^+	=	Penicillin acylase producing ability.
Cm^R	=	Chloramphenicol resistance.			

rif^R). Then, transconjugants were selected as those with tetracycline and rifampicin resistant characteristics. The selected transconjugants were tested for ability to produce penicillin acylase. Both penicillin acylase producing and non-producing clones were selected and their plasmid DNA's were tested for Tn1000 insertion sites by restriction mapping.

Recovery of DNA fragments

For recovery and separation of DNA fragments of various sizes, samples were electrophoresed in 1% agarose gel. After staining, the desired DNA bands were cut, separately placed in an electrophoretic chamber and overlayered with 1% low temperature agarose gel. Following electrophoresis, the DNA bands moved to the low temperature gel. Part of this gel containing DNA was separated and mixed with one volume of TE buffer. The mixture was melted at 65°C for about 10 min and 1 volume of saturated phenol was added. Then, the mixture was mixed vigorously and centrifuged. The aqueous phase containing DNA was removed and re-extracted with phenol till no white precipitate was observed between the aqueous and the phenol phases. The aqueous phase was carefully transferred to a clean tube. Contaminating phenol in the aqueous phase was eliminated by extraction with diethyl ether. The DNA was reprecipitated with ethanol, dried and resuspended in TE buffer.

Ligation of DNA fragments

Ligation of DNA fragments and plasmid vectors was done in the presence of ligation buffer (65 mM Tris-HCl pH 7.5, 10 mM $MgCl_2-6H_2O$, 10 mM dithiothreitol and 1 mM ATP) and T4 DNA ligase (0.5-1.0 unit). For blunt end ligation, 1mM hexamine cobalt (III) chloride was added to the ligation mixture to increase ligation efficiency[8].

Transformation of *E.coli*

Ligated DNA was transformed into competent *E.coli* prepared by $CaCl_2$ treatment[17]. Ligated plasmids (1-2 μg) were mixed with 200 μl competent *E.coli*. The mixtures were kept in an ice bath for 30 min before being heated at 42°C for 90 seconds. Then, they were quickly cooled in an ice bath for a few min and 1.0 ml of LB broth was added before further incubation at 37°C for 90 min. The transformed cells were then spread on LB agar supplemented with appropriate antibiotics.

Transformation of *B.subtilis*

Transformation of *B.subtilis* was done using the competent transformation method[18]. *B.subtilis* MI111 was grown in minimal medium [0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $MgSO_4.7H_2O$, 0.5% glucose, 0.2% casamino acids and 50 μg/ml leucine and arginine] at 37°C for 16 hours. The culture was used as seed inoculum for fresh medium. After incubation at 37°C for 4-6 hours, the cells were then harvested and resuspended in fresh minimal medium containing additional 0.072%, $MgSO_4.7H_2O$. The cell suspension (1.0 ml) was then mixed with 0.5-3.0 μg of plasmid DNA and the mixture was incubated in an incubator

shaker (180 rpm) at 37°C for 90 min. At the and of the incubation period, the culture was spread on LB-agar containing appropriate antibiotics.

Screening for penicillin acylase producing bacteria.

Penicillin acylase producing bacteria could be distinguished from others which were unable to produce the enzyme by using a biological assay method described by Meevootisom *et al*[19]. To detect the ability to produce penicillin acylase, bacteria were grown on nutrient agar plus 0.15% phenylacetic acid (PAA) (for *E.coli*) or LB-agar (for *Bacillus* sp.) for 2 days at 28°C. Then, the cultures were overlayered with 5 ml of nutrient agar containing 4 mg/ml penicillin-G and 1% (v/v) overnight culture of *S.marcescens*. The overlayered plates were incubated at 28°C overnight and at the end of the incubation period, they were observed for the presence of clear zones in the red carpet of *S.marcesserus* cells. These developed when penicillin acylase underlying bacterial colonies produced penicillin acylase since *S.marcescens* is not sensitive to penicillin G but is sensitive to 6-APA produced by the action of penicillin acylase on penicillin G.

Penicillin acylase activity among subclones.

Production of penicillin acylase enzyme among subclones was compared. Bacterial cells were grown in LB-broth containing appropriate antibiotics and 0.15% phenylacetic acid. the medium was adjusted to pH 7.0 prior to autoclaving. After 48 hours of cultivation on a rotary shaker at 28°C, the cells were centrifuged and resuspended in 0.05 M Tris-HCl buffer pH 8.4. Cell concentration was adjusted to an OD of 1.0 at 600 nm. One ml of the cell suspension was centrifuged and the cell pellet was used as an enzyme source in an assay reaction mixture. The 6-APA product was assayed by the p-dimethylaminobenzaldehyde method (pDAB)[10] which forms a colored Schiff's base. Briefly, 0.5 ml of the reaction mixture was added to 3.5 ml of assay solution containing 0.5 ml of 0.5% (w/v) pDAB in absolute methanol and 3.0 ml of a solution made from 2.0 ml of 20% glacial acetic acid and 1.0 ml of 0.05 M sodium hydroxide. The assay mixture was read immediately at 415 nm against a blank reaction mixture which employed distilled water instead of the enzyme. For extra-cellular enzyme assays, 500 μl of culture broth was used as an enzyme source instead of a cell suspension. One unit of penicillin acylase activity was defined as the amount of enzyme required to form 1 μmole of 6-APA per minute at 40°C.

Stability of the plasmid under nonselective conditions

The capability of host cells to carry various plasmids containing the pencillin acylase gene under nonselective conditions was studied. One loopful of a single bacterial colony was used to inoculate LB broth containing no antibiotic. After overnight incubation, suitable dilutions were prepared before spreading on LB agar plates to obtain single colonies. One hundred colonies were picked and transferred to other LB agar plates with or without appropriate antibiotics. Penicillin acylase enzyme production

of the colonies tested was observed by overlayering with NA agar containing penicillin G and *S.marcescens* as previously described. The process was repeated daily using culture of the previous day as inoculum to the new broth for a period of 7 days. Stability of the test plasmids was reported as percentage of PAC producing colonies as compared to total colonies.

RESULTS

Localization of the penicillin acylase gene on plasmid pMLV101

In 1987, Meevootisom and Saunders[6] cloned a 7.7 kb DNA fragment of *B.megaterium* UNI into pACYC184 and the plasmid was designated pMLV101. Localization of penicillin acylase on the chimeric plasmid by the transposition inactivation technique showed that the penicillin acylase gene was located between the 2 PvuII sites flanking the 2 HindIII sites on the inserted fragment (Fig. 1).

Subcloning the PAC gene in *E.coli*

When pMLV101 was subjected to HpaII restriction cutting, several DNA fragments were obtained (Fig. 2). The largest fragment (2.8 kb) was designated the F1 fragment. It was later shown to contain the complete penicillin acylase gene by subcloning into the ClaI site on plasmid pACYC184. The resulting plasmid, designated pBA3 of 6.8 kb (Fig. 3), allowed its transformants to produce penicillin acylase as detected by the overlayer method. However, the plasmid was found to be unsuitable for further manipulation because of limited restriction sites. Therefore, an attempt was made to flank F1 with a few restriction sites on pACYC184. This was accomplished by treating F1 fragments with S1 nuclease and DNA polymerase to form blunt ends. Then the fragments were joined with pACYC184 at the EcoRV site and transformed into *E.coli*. The transformants were found to exhibit both Cm resistance and the ability to produce penicillin acylase enzyme. The plasmid was designated pBA32 (Figure 4). This plasmid (6.8 kb) allowed recovery of a 3.0 kb BamHI-XbaI DNA fragment containing the peinicillin acylase gene.

Subcloning the PAC gene in *B.subtilis*

Because of low expression of the *Bacillus* PAC gene in *E.coli* and because of the inability of *E.coli* to secrete the product, we attempted to transfer the gene into *B.subtilis* both directly and by using an *E.coli-Bacillus* shuttle vector. The shuttle vector pHV33[13] was used with some modification. Modification of this plasmid was necessary since it carried a β-lactamase gene which destroys Penicillin G. The gene was removed by Bal31 treatment after the plasmid was linearized with PstI. Moreover, to obtain a smaller plasmid, the tetracycline resistance gene was cleaved off by deleting a 2.5 kb PvuII fragment. The resulting plasmid, pHV33-2 was 4.2 kb in size (Fig. 5) and could express Cm resistance and replicate both in *E.coli* and *B.subtilis*.

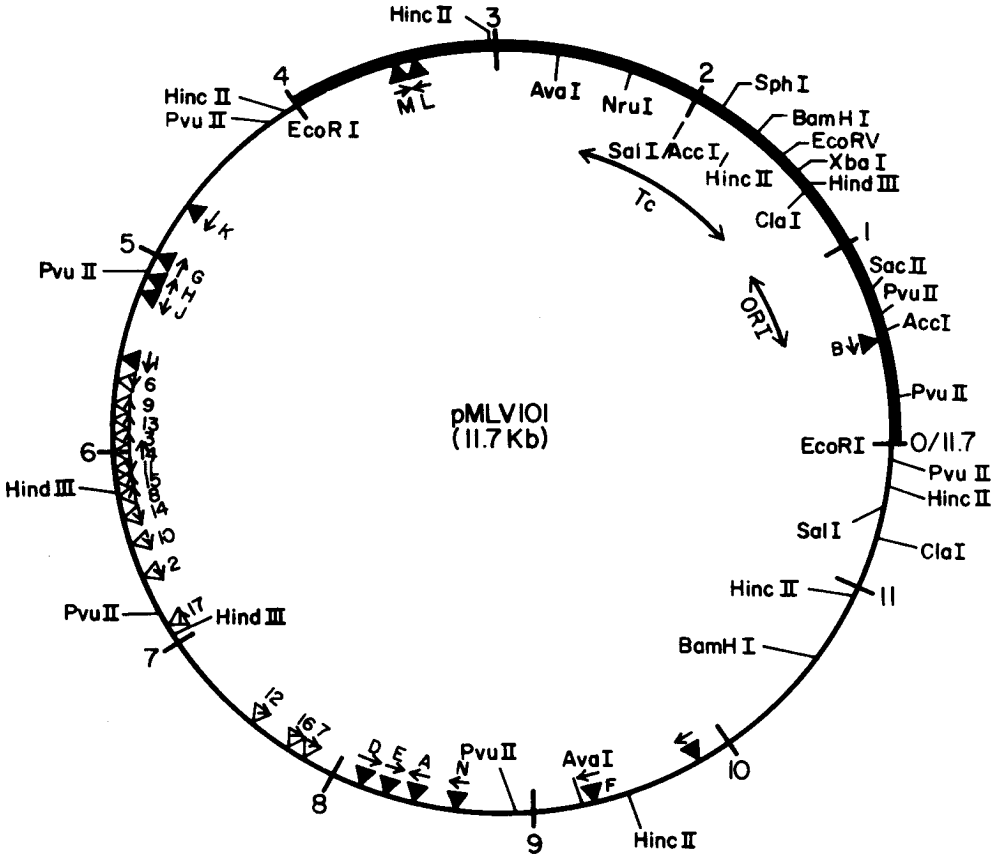

Fig. 1 Physical map of pMLV101. The thick line indicates pACYC184 vector and the thin line indicates the inserted fragment from *B.megaterium*. The triangle shows inserted points of Tn1000, Δ indicates the insertions that resulted in no penicillin acylase production, ▲ represents the insertion points which showed no effect on penicillin acylase production.

: pACYC184

: penicillin acylase (PAC) gene

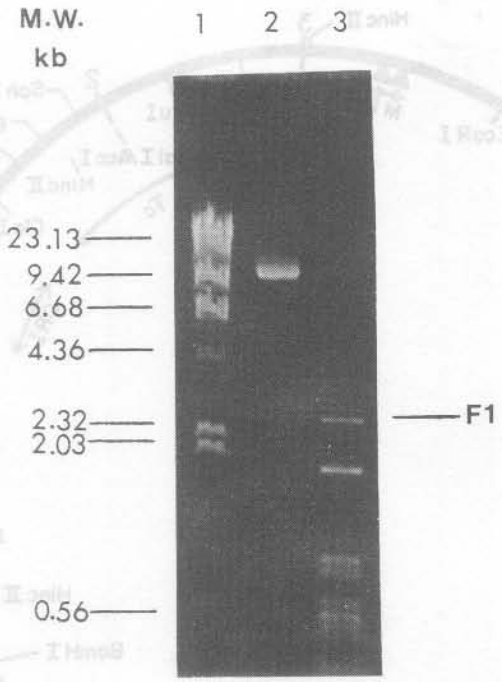

Fig. 2 Agarose gel electrophoresis of pMLV101. F1 indicats fragment with penicillin acylase gene (2.8 kb).
lane 1 : λ DNA digested with HindIII
lane 2 : pMLV101, undigested
lane 3 : pMLV101 digested with HpaII

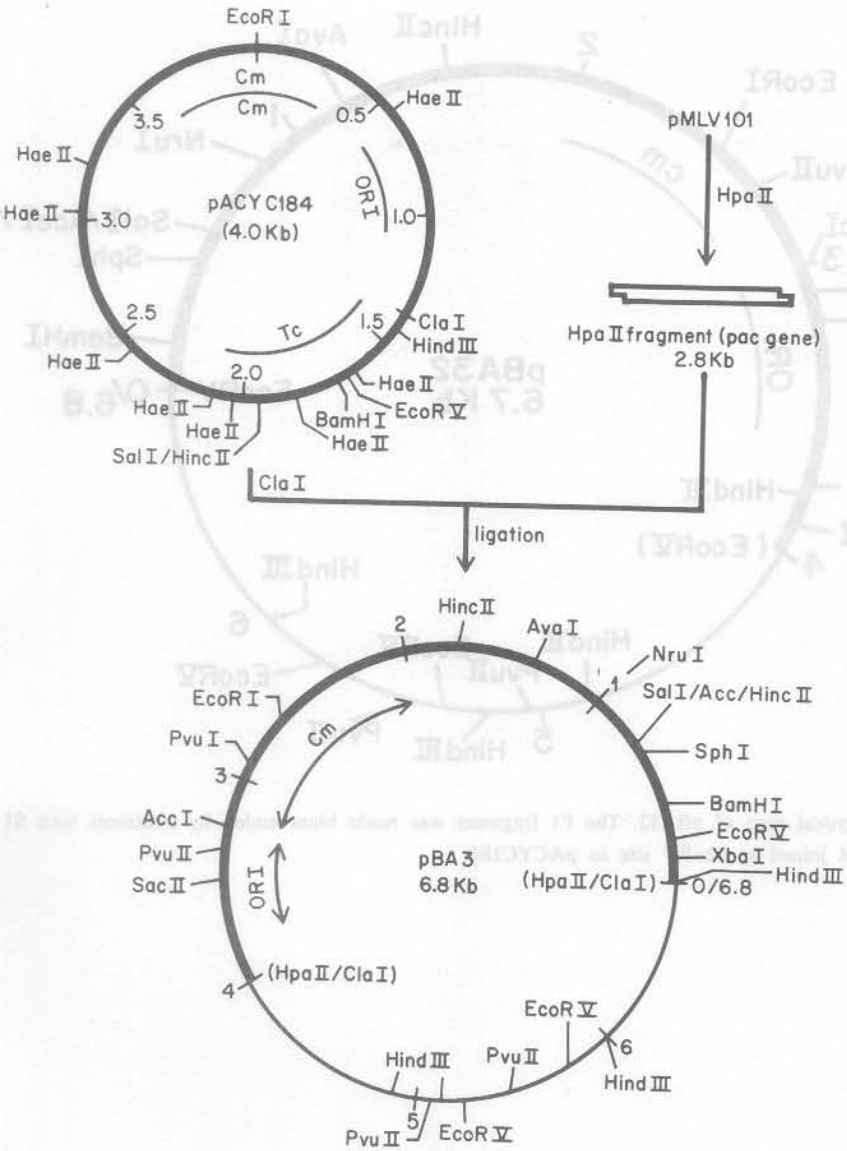

Fig. 3 Construction of pBA3, F1 fragment (2.8 kb) from pMLV101 was cloned into ClaI site of pACYC184.

□ : pACYC184

——— : penicillin acylase (PAC) gene

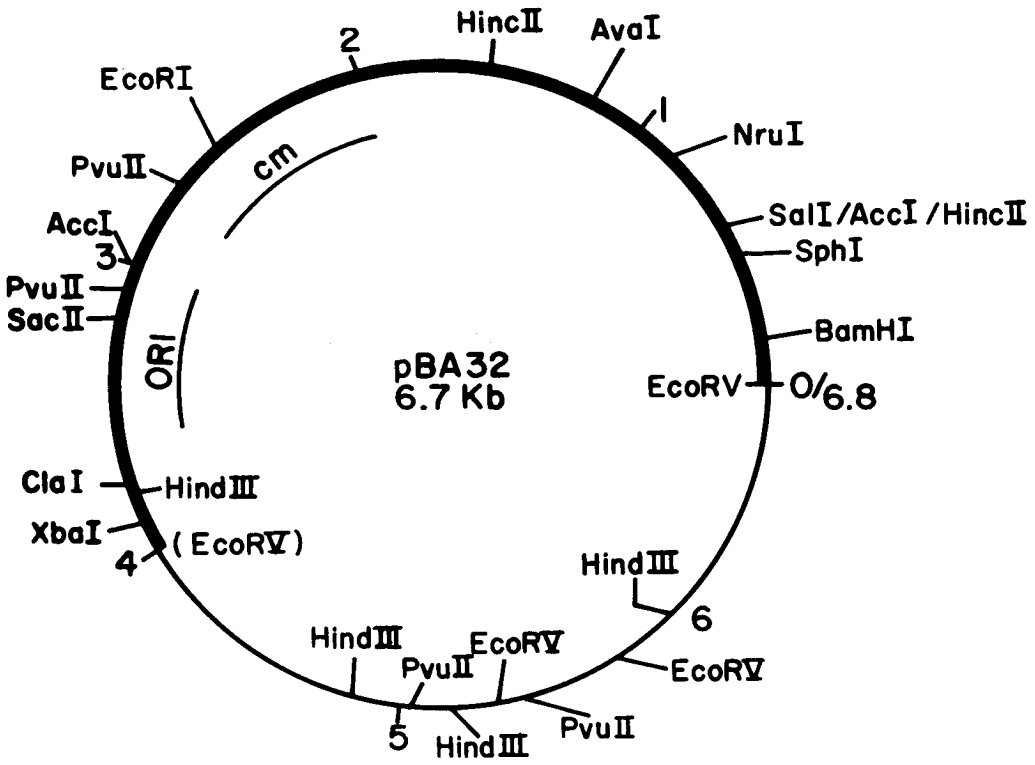

Fig. 4 Physical map of pBA32. The F1 fragment was made blunt ended by treatment with S1 nuclease and joined at EcoRV site to pACYC184

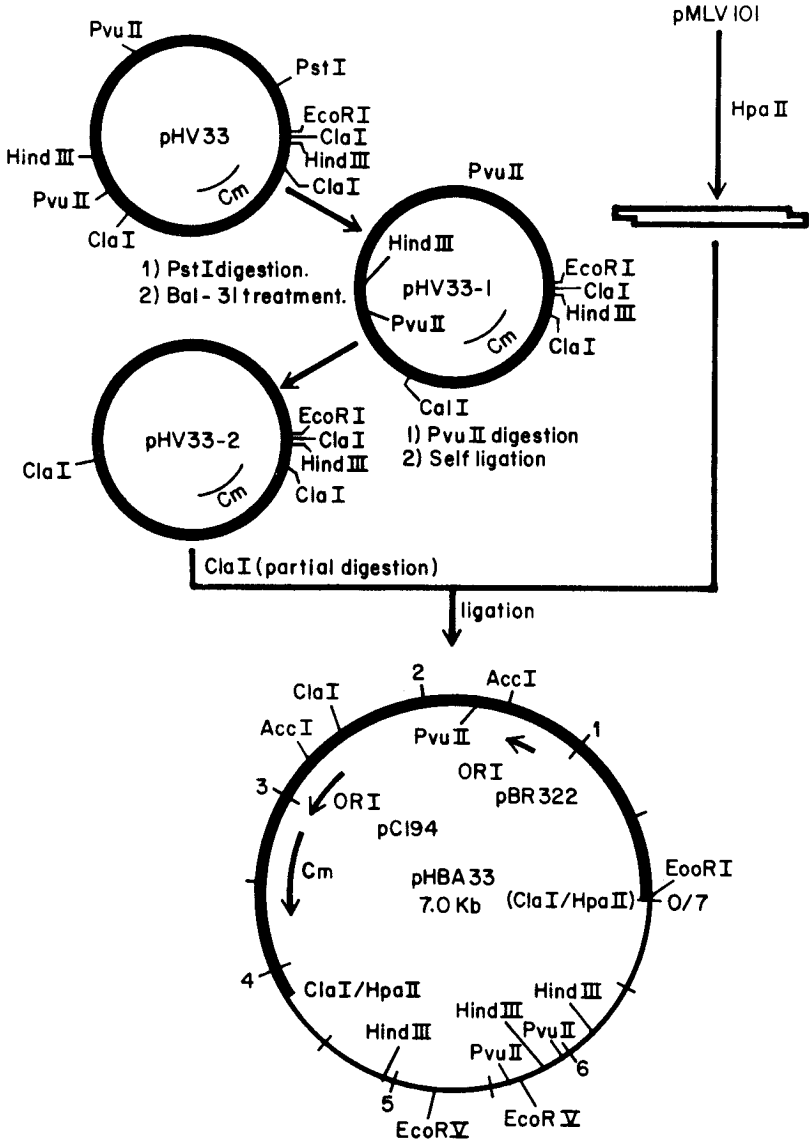

Fig. 5 Construction of pHBA33. The shuttle vector pHV33 was deleted to make pHV33-2 which still could replicate and express chloramphenicol resistance in both *E.coli* and *B.subtilis* pHV33-2 was partially digested with ClaI and joined with F1. The resulting plasmid, pHBA33 enabled both *E.coli* and *B.subtilis* to produce penicillin acylase.

pHV33-2 was partially digested with ClaI and ligated to the F1 fragment. The ligated DNA was then used to transform *B.subtilis*. One transformant which was pac$^+$ and Cm^r was selected and its plasmid DNA was analysed (Fig. 6). It was found that the 0.17 kb ClaI fragment of pHV33-2 was replaced by the F1 fragment as demonstrated by only one ClaI site was remained (Fig. 6, lane 4) since ClaI-HpaII ligating site will not be able to be cleaved by ClaI. And the remaining ClaI site, as expected, was about 2.5 kb away from the EcoRI site. (Fig. 6, lane 5 the lower band). This plasmid was named pHBA33. Its construction and a physical map of pHBA33 are shown in Fig. 5.

The F1 fragment was also cloned directly into the *Bacillus* vectors, pUB110[14] and pTF6[15]. This was done by digesting both pUB110 and pBA3 with BamHI and EcoRI and by ligating the mixed digested products. The products were used to transform *B.subtilis*, and Km^r transformants were selected and screened for pac$^+$ producing clones. One pac$^+$ Km^r clone was isolated and its plasmid was designated pBA310 (Fig. 7). Another attempt involved cloning the F1 fragment into the PstI site of pTF6. pTF6 contains a strong promoter from the β-galactosidase gene of *B.thermoliquefaciens*[15]. The cloning was achieved by making F1 to blunt ended with DNA polymerase and joining it to a PstI linker (BRL, USA). The F1-PstI linker was then ligated to PstI linearized pTF6. The ligated mixture was then used to transform *B.subtilis*. A transformant with Km^r and pac$^+$ was obtained and its plasmid was designated pBA401. Construction of pBA401 is shown in Fig. 8. Comparison of the penicillin acylase activity of genetically engineered strains was shown in Table 3. The activity of penicillin acylase in genetically engineered *E.coli* strains was relatevely low. They gave a range of 1.2-2.3 units per gram cell wet weight (U/g). Those *E.coli* harboring pHBA33 gave the highest enzyme activity (2.3 U/g cells). The penicillin acylase produced by *B.subtilis* (pHBA33) was found to be of the extracellular type, similar to those produced by other *Bacillus* transformants (pBA310 and pBA401) and by *B. megaterum* UN-1. The enzyme activity produced by *B.subtilis* (pHBA33) was found to be 0.3 (U/ml) or around one-third of that produced by *B.megaterium* UN-1. Higher penicillin acylase activities were found with *B.subtilis* harboring pBA310 and pBA401 (1 and 1.3 U/ml respectively). By biological assay method as shown in Fig. 9, clones harboring the penicillin acylase gene showed growth inhibition of *S.marcescens* around their colonies. Even though the method did not quantitate, enzyme activity, around clones with more enzyme activity such as *B.subtilis* (pBA401), larger clear zone will be observed (Fig. 9 and Table 3).

Plasmid stability

Studies on plasmid stability in host cells employed conditions where recombinant clones were grown on growth media plus or minus a suitable antibiotic. Under selective conditions where an antibiotic was used, stability of the plasmids in the host cells was seen throughout the period of this study. Under nonselective conditions,

TABLE 3 Penicillin acylase activity of genetically engineered strains, hosts, and *B.megaterium* UN-1.

Strain	Penicillin acylase activity	
	cell bound (U/g cell wet weight)	extracellular (U/ml)
E.coli (pMLV101)	1.2	0
E.coli (pBA3)	1.2	0
E.coli (pBA32)	1.2	0
E.coli (pHBA33)	2.3	0
B.subtilis (pHBA33)	0	0.3
B.subtilis (pBA310)	0	1.0
B.subtilis (pBA401)	0	1.3
B.megaterium UN1	0	0.7
B.subtilis MI111	0	0

1 Unit of enzyme activity is defined as the amount of the enzyme which can convert penicillin G to 1 μmole of 6-APA per minute at 40°C.

1g *E.coli* cell wet weight is equal to 625 ml of culture giving an O.D.600 of 1

TABLE 4 Stability of plasmids containing the penicillin acylase gene under non-selective conditions as shown by percentage of clones producing the enzyme after daily subculture.

Clones	Percentage of clones producing penicillin acylase (Stability of plasmid)						
	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7
pMLV101	100	98	92	80	33	30	22
pBA3	100	100	99	99	99	90	70
pBA32	98	99	95	98	99	87	66
pHBA33 *(E.coli)*	100	100	100	100	94	ND	97
pHBA33 *(B.subtilis)*	91	89	76	ND	18	12	0

ND = NOT DETERMINED

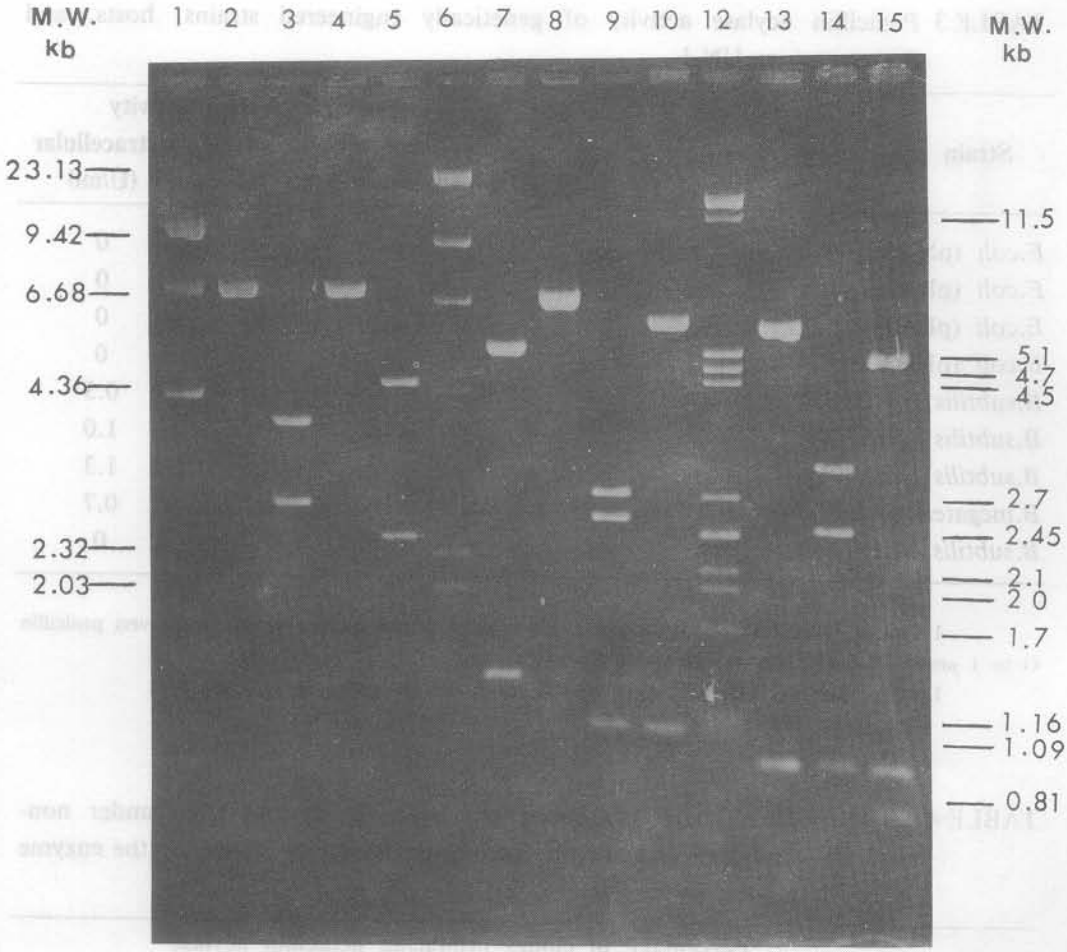

Fig. 6 Agarose gel electrophoresis of plasmid pHBA33 digested products.

lane 1 : intact pHBA33
lane 2 : pHBA33-EcoRI
lane 3 : pHBA33-ClaI + EcoRV
lane 4 : pHBA33 – ClaI
lane 5 : pHBA33-ClaI + EcoRI
lane 6 : Standard λ-HindIII
lane 7 : pHBA33-EcoRV + EcoRI
lane 8 : pHBA33-EcoRV
lane 9 : pHBA33-EcoRV + AccI
lane 10 : pHBA33-AccI
lane 11 : Standard λ-PstI
lane 12 : pHBA33-HindIII
lane 13 : pHBA33-HindIII + ClaI
lane 14 : pHBA33-HindIII + EcoRI

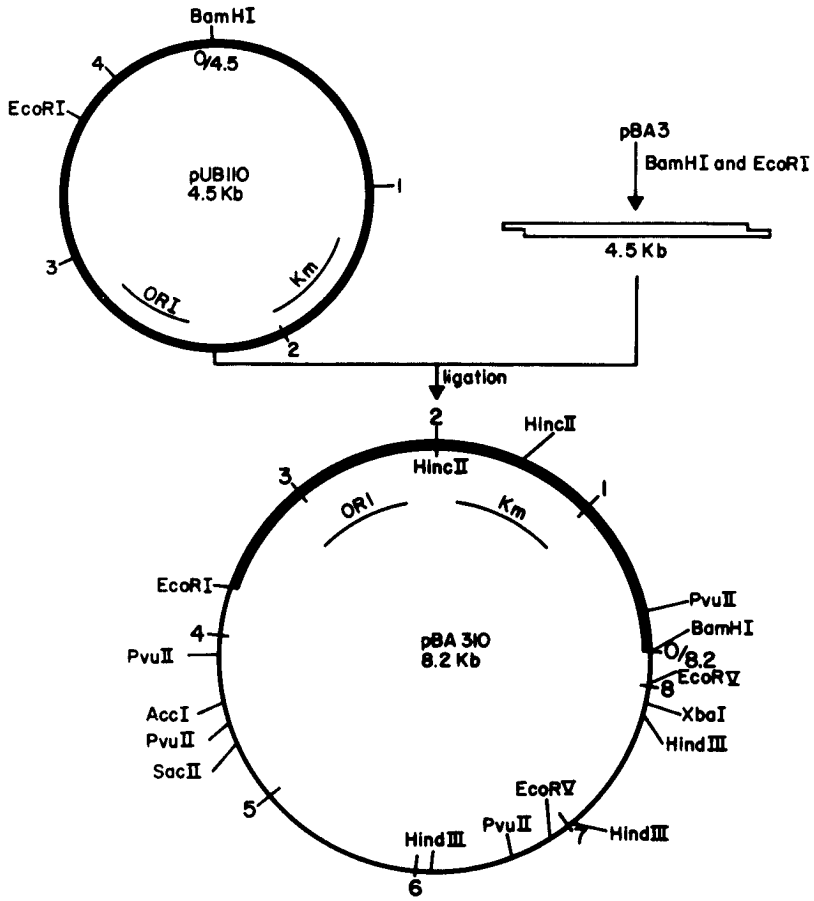

Fig. 7 Construction of pBA310. Both pUB110 (Km^r) and pBA3 (Cm^R, pac^+) were digested with BamHI and EcoRI, ligated and transformed into *B.subtilis*. The Km^r transformants were selected and screened for pac^+ clone. The plasmid from PAC^+ was prepared and analysed for the physical map. The BamHI-EcoRI fragment of pUB110 was replaced by another BamHI-EcoRI fragment harboring PAC gene of pBA 3.

□ : pTF6

_____ : PAC gene

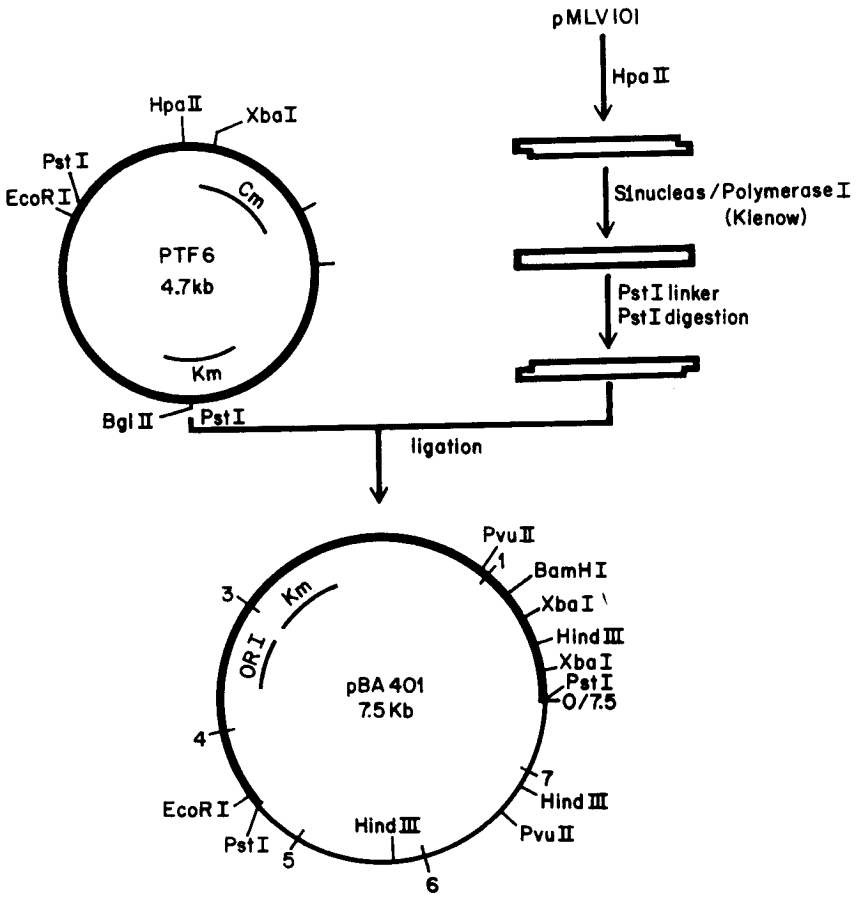

Fig. 8 Construction of pBA401. The F1 fragment was made blunt ended with DNA polymerase (Klenow fragment) and joined with PstI linker and then inserted to PstI site of pTF6. The plasmid was used to transform *B.subtilis* and pac$^+$ producing clone was selected and plasmid was analysed.

□ : pTF6
____ : PAC gene

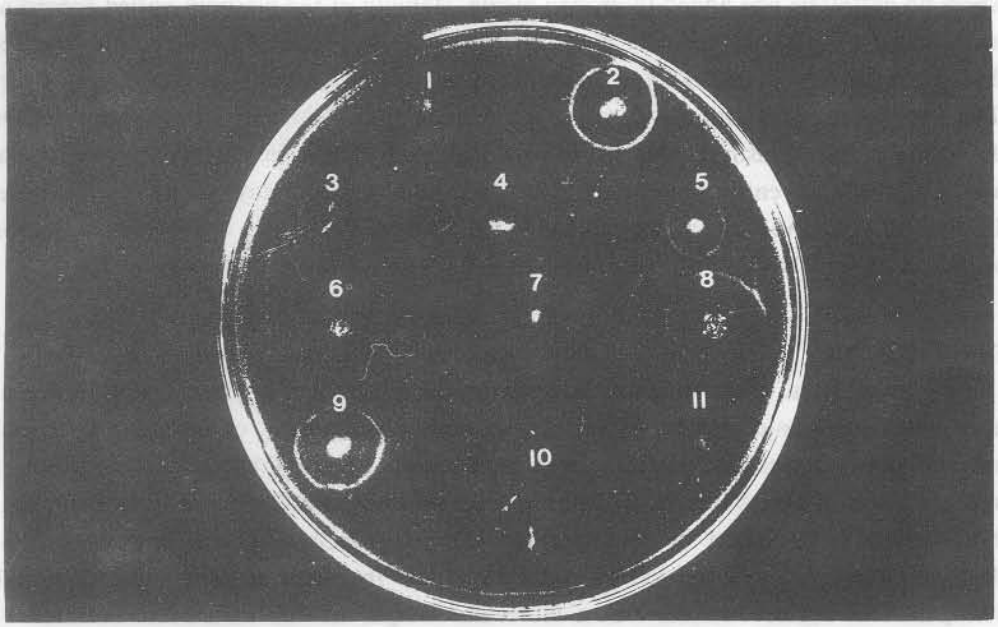

Fig. 9 Clear inhibition zone of transformants harboring recombinant plasmid with PAC gene. The cells were grown on LB agar at 37°C for 24 hours and overlayed with 5 ml of nutrient agar containing 4 mg/ml penicillin-G and 1% (v/v) *S.marcescens* ATCC 27117. The colonies with clear inhibition zone indicated penicillin acylase producing activity.

1. *E.coli* (pACYC184)
2. *B.megaterium* UN-1
3. *E.coli* (pMLV101)
4. *E.coli* (pBA3)
5. *E.coli* (pBA32)
6. *B.subtilis* MI111
7. *B.subtilis* (pBA310)
8. *B.subtilis* (pBA401)
9. *B.subtilis* (pHBA33)
10. *B.subtilis* (pUB110)
11. *B.subtilis* (pTF6)

where no antibiotic was added to growth media, stability of the plasmids varied according to the type of plasmid and the host cell under study (Table 4). The results showed that plasmids pBA3, pBA32 and pHBA33 were relatively stable in *E.coli,* i.e., more than 66% of the population still retained plasmids after 7 days of daily subculture. On the other hand, plasmid pMLV101 was rather unstable in the *E.coli* host; that nearly all (78%) of entire population lost its plasmids within 7 days on daily subculture. The plasmid pHBA33 was more stable in *E.coli* than in *B.subtilis.* The whole population of *B.subtilis* (pHBA33) lost this plasmid within 7 days on daily subculture.

DISCUSSION

Since it has been shown that plasmids carrying large inserted DNA fragments have reduced plasmid stability[20], removal of unnecessary parts of inserted DNA may increase plasmid stability. Results from localization of the PAC gene on 7.7 kb of inserted DNA in pMLV101 showed that the gene was around 2.8 kb in size. Therefore, the inserted DNA could be trimmed by 4.9 kb. Subcloning experiments revealed that the entire gene was located on a 2.8 kb HpaII fragment called F1 (pBA3 Fig. 3) The penicillin acylase enzyme has a molecular weight of 120 kDa as determined by polyacrylamide gel electrophoresis without SDS and by Sephadex G-100 column chromatography. However, it showed a molecular weight of 58 kDa by SDS-PAGE[9]. Therefore it was assumed that the native enzyme existed in a dimeric form and a 2.8 kb fragment is large enough to encode a 58 kDa protein. As stated above, the result of plasmid stability study (Table 4) showed that plasmids with smaller PAC gene insert (pBA3, pBA32) were more stably maintained in *E.coli* cells than that plasmids with large insert (pMLV101).

Expression of *Bacillus* genes in *E.coli* is reported to be relatively inefficient but handling of genetic work with *E.coli* is easier than with *B.subtilis.* Therefore, a shuttle vector pHV33-2 which could propagate in both *E.coli* and *B.subtilis* was constructed. The plasmid was used to clone F1 and a recombinant plasmid, pHBA33 could express pac^+ in both *E.coli* and *B.subtilis.* Harboring this plasmid, they produced enzyme at 2.3 U/gm cells and 0.25 U/ml, respectively. The plasmid was more stably maintained in *E.coli* than in *B.subtilis* (Table 4). The instability of pHBA33 in *B.subtilis* (Table 4) might occur by losing the entire plasmid or rearrangement in the plasmid genome which often observed when introducing a recombinant plasmid in *B.subtilis*[21, 22]. However, the exact mechanism of pHBAB3 instability in *B.subtilis* was not investigated. Comparison of penicillin acylase produced by *E.coli* and *B. subtilis* transformants demonstrated that *E.coli* clones produced lower enzyme activity than *B.subtilis* clones (Table 3). The low activity may have resulted from low efficiency at either the transcriptional or translational levels or both as has been shown with other studies[23-25]. It was found that some of the *B.subtilis* transformants produced higher penicillin acylase levels than *B.megaterium* UN-1, but recombinants harboring pHBA33 produced lower enzyme activities. The differences in penicillin acylase

activity amongst these recombinants likely reflected differences in plamid copy number and/or expression of the gene. Because the number of penicillin acylase genes present in recombinants was higher (around 10-50 copies) than in *B.megaterium* UN-1, it would be expected that the recombinants would produce much higher enzyme activity if the expression were similar to that in *B.megaterium* UN-1. Since the results showed that the highest enzyme producers among the recombinants yielded only 4 times higher activity than *B.megaterium* UN-1, it indicated that the efficiency of PAC gene expression in *B.subtilis* was much lower than that in *B.megaterium* UN-1. Other explanations include differences in efficiency of enzyme transport out of the cells and differences in rates of protease degradation of the enzyme[26, 27]. Expression of the PAC gene in recombinant *B.subtilis* was shown to be under the control of its own promoter (ie. the *B.megaterium* PAC gene promoter) in pHBA33 and pBA310. The higher enzyme activity observed in cells harboring pBA401 could have resulted from activity of the β-galactosidase promoter in the plasmid. Therefore, proper manipulation and a suitable vector system could lead to increased enzyme production, and the use of *B.megaterium* instead of *B.subtilis* as the host might further increase the yield.

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บทคัดย่อ

จากการศึกษาโดยเทคนิค transposition inactivation พบว่า ยีนส์ที่สร้างเอนไซม์เพนนิซิลลินเอซิเลส (Penicillin acylase, PAC) ซึ่งแยกได้จากจุลินทรีย์ *Bacillus megaterium* และได้ถูกโคลนอยู่บน plasmid pMLV101 มีขนาด ประมาณ 2.8 Kb เมื่อทำการตัด plasmid pMLV101 ด้วยเอนไซม์ตัดจำเพาะชนิด HpaII แบบไม่สมบูรณ์ จะได้ชิ้นส่วน DNA ขนาดประมาณ 2.8 Kb ซึ่งให้ชื่อว่า F1 พบว่า F1 นี้ มียีนส์เพนนิซิลลินเอซิเลสอยู่ครบ เมื่อทำการ subclone ชิ้นส่วน F1 นี้กับ plasmid พาหะ pACYC184 จะได้ plasmid ลูกผสม pBA3 และ pBA32. เมื่อนำ plasmid ลูกผสมนี้เข้าสู่จุลินทรีย์ *Escherichia coli* พบว่า *E. coli* ที่ได้รับ plasmid ลูกผสม เหล่านี้ สามารถสร้างเอนไซม์เพนนิซิลลินเอซิเลส ในการศึกษา ได้สร้าง Plasmid pHBA33 ซึ่งเกิดจากการเชื่อมต่อชิ้นส่วน DNA F1 เข้ากับ shuttle vector pHV33-2 ที่สามารถเพิ่มจำนวนในจุลินทรีย์ *E. coli* และ *B. subtilis* ได้. พบว่าทั้งจุลินทรีย์ *E. coli* และ *B. subtilis* ที่ได้รับ plasmid pHBA33 จะสร้างเอนไซม์เพนนิซิลลินเอซิเลสได้. โดยที่ *E. coli* จะสร้างเอนไซม์แบบ intracellular แต่ *B. subtilis* จะสร้างเอนไซม์แบบ extracellular เช่นเดียวกับ *B. megaterium*. Subclone *E. coli* ทั้งหมด (pBA3, pBA32 และ pHBA33) ให้ปริมาณเอนไซม์ activity ใกล้เคียงกัน activity ของ *E. coli* ที่มี plasmid pMLV101 แต่ *B. subtilis* มี plasmid pHBA33 จะสร้างเพนนิซิลลินเอซิเลส และปล่อยออกนอกเซลล์ โดยที่มี activity ต่ำกว่า activity ของเชื้อ *B. megaterium* UN-1 เล็กน้อย