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Expression of Bacillar Glutamyl Endopeptidase Genes in *Bacillus subtilis* by a New Mobilizable Single-Replicon Vector pLF

Alexei B. Shevelev,*[,1] Vladimir V. Aleoshin,† Lesya A. Trachuk,* Alexei E. Granovsky,* Yakov N. Kogan,* Leonid M. Rumer,* Anna V. Serkina,* Elena V. Semenova,† Anastassia M. Bushueva,* Vitaly A. Livshits,* Sergey V. Kostrov,‡ Alexander S. Shcheglov,* Svetlana I. Novikova,§ and Galina G. Chestukhina*

**Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia; †Institute of Physiological, Biochemical, and Nutritional Problems of Farm Animals, Borovsk, Kaluzhskaya Region, Russia; ‡Institute of Molecular Genetics, Moscow, Russia; and §Moscow Institute of Physics and Technology—Technical University, Moscow, Russia*

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The pLF1311 natural plasmid from *Lactobacillus fermentum* 1311 was used to construct a single-replicon vector suitable for rapid cloning in a wide range of gram-positive hosts and *Escherichia coli.* The new vector is capable of conjugative mobilization from *E. coli* to various hosts by conjugal transfer. The final vector (3.4 kb) showed a high segregational and structural stability and a high copy number. Glutamyl endopeptidase genes from *Bacillus licheniformis* (*gse*BL) and *B. intermedius* (*gse*BI) were cloned in both pLF9 and pLF14 vectors and introduced to *B. subtilis.* The yield of enzymes in the pLF-derived producers was 6- to 30-fold more than in the natural producers and reached 100–150 mg/L of mature protease.

Key Words: mating; secretion; glutamyl endopeptidase; *Bacillus subtilis, B. licheniformis, B. intermedius.*

1. INTRODUCTION

Bacterial glutamyl endopeptidases represent a recently discovered group of enzymes distantly related to animal chymotrypsin. Their genes were described for several genera like *Staphylococcus* (Yoshikawa *et al.,* 1992), *Bacillus, Streptomyces* (Barbosa *et al.,* 1996), *Thermoactinomyces* (Rebrikov *et al.,* 1999), *Mycoplasma* (Fraser *et al.,* 1995), and *Haemophilus* (Fleischmann *et al.,* 1995). Nevertheless, the biological role of glutamyl endopeptidases is still unclear. In contrast with digestive extracellular proteases in *Bacillus,* glutamylendopeptidases are characterized with a low expression independent of sporulation. Glutamyl endopeptidases are acknowledged as a tool for peptide mapping and other uses (Barbosa *et al.,* 1996). V8 proteinase of *S. aureus* (Yoshikawa *et al.,* 1992) is the most known commercially available representative of glutamyl endopeptidases. Other glutamyl endopeptidases are not broadly applied regardless of their higher substrate specificity perhaps due to the poor yields of these enzymes which never exceed 30 mg/L in the natural producers (Kakudo *et al.,* 1992b). A goal of the present work was to improve the production of glutamyl endopeptidase from *B. licheniformis* and *B. intermedius* by increasing the dosage of cloned genes in *B. subtilis* on the basis of a high copy number plasmid.

A number of plasmid vectors have been engineered for expression in gram-positive hosts on the basis of widespread and well-characterized rolling-cycle replicons (RCR)[2] of bacilli and cocci (Alikhanian *et al.,* 1981; Gryczan, 1982; Radford and Hodgson, 1991; del Solar *et al.,* 1993). In contrast to most known replicons from gram-negative bacteria, RCR are sensitive to insertions and revealed structural instability increasing proportionally to the size of the con-

[1] To whom correspondence should be addressed at Laboratory of Protein Chem., GosNIIgenetika, 1st Dorozhny pr. 1, Moscow 113545, Russia. Fax: 7 (095) 315 05 01. E-mail: shevel_a@hotmail.com.

[2] Abbreviations used: Cm, chloramphenicol; Em, erythromycin; Gse, glutamyl endopeptidase; PAGE, polyacrylamide gel electorphoresis; RCR, rolling cycle replicon; Z-Glu-pNA, carbobenzoxy-glutamylparanitroanilide.

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struct (Ballester *et al.,* 1989). This observation encouraged works directed toward developing bacillar vectors of greater capacity on the basis of naturally large θ-replicons: pAMβ1 from *Streptococcus* (Bruand *et al.,* 1993; Rabinovitch *et al.,* 1985) and *cry*-gene containing plasmids from *B. thuringiensis* (Baum *et al.,* 1990). These vectors demonstrated a good segregation stability and a practically unlimited capacity to admit foreign DNA (Ceglowski *et al.,* 1993). But θ-replicons failed to provide the desirable multiplication of expressed genes (Bruand *et al.,* 1993). There are several reports about vectors combining high copy number with high stability, but this feature was strictly allocated to the natural host of the replicon (Gamel and Piot, 1992; Wang and Lee, 1997).

We intended to develop a simple and universal cloning system designed for fast generation of a large series of recombinants, e.g., for site-directed mutagenesis experiments. We tried to combine the advantages of a high copy number of RCR with a high stability.

A pLF1311 natural plasmid from *Lactobacillus fermentum* 1311 revealed a useful feature to be supported in both gram-positive and gram-negative hosts (Livshits *et al.,* 1989). The sequence of pLF1311 was not published previously and there were no data about its use for expression of any genes. Hereby the minimal-size version of pLF replicon with a universal *E. coli–Bacillus* determinant of Cm resistance was constructed. Expression of the *gse* gene encoding for glutamyl endopeptidase from *B. licheniformis* (GseBL) and *B. intermedius* (GseBI) was performed on pLF-based vectors. The expression of *gse* genes was simultaneously performed at the pCB *E. coli*/bacillar shuttle vector on the basis of pSM19035 equipped with either the Em or the Cm resistance marker. Segregational stability, yield of the recombinant protein, and its dependence on antibiotic selection were assessed.

2. MATERIALS AND METHODS

2.1. Strains and Plasmids Used

The following strains were used: *E. coli:* TG1 (Sambrook *et al.,* 1989), C600 (RP4) *thi-1, thr-1, leuB6, lacY1, ton21, hsdM, hsdR; B. licheniformis;* B-6839 is a natural isolate (Trachuk *et al.,* 1996); *B. subtilis* AJ73: *amy*E4, *npr*512, *apr*73 was kindly supplied by Dr. J. Jomantas.

Plasmids. pUK21 plasmid was generously supplied by Dr. J. Messing (Vieira and Messing, 1991). *E. coli*/bacilli shuttle vector with Em resistance pCB22 (Sorokin *et al.,* 1986) was provided by Dr. S. Kostrov. pLF31311 and its derivative pLFmob76 were described previously (Livshits *et al.,* 1989). Sequences of GseBI (Kakudo *et al.,* 1992a) and GseBI gene and source constructs with it (Rebrikov *et al.,* 1999) were published previously.

2.2. Primers for Site-Directed Mutagenesis of pLF

Primers for site-directed mutagenesis of PLF of the following structure were used.

```
primer CK      TGA TTG GAC AGT ACC TCG T
primer BS      CCC TTA AAG CCT AGC AAT TTG G
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PCR amplification of the *gse*BL gene was performed by primers of the following structure.

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primer gap H   TTC CCG TGC GCC TCC GGG
primer gap T   GGG GAT CCT TCA AAT TTC ACG AT
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2.3. Transformation of B. subtilis and E. coli

The cells of *E. coli* were transformed following a standard Ca-mediated procedure (Sambrook *et al.,* 1989). The transformants were selected and further maintained at LB medium supplemented with 30 mg/L Cm or 100 mg/L ampicillin. The cells of *B. subtilis* were transformed using a modified Spizeisen protocol (Canosi *et al.,* 1978) and selected on Lennox agar (Difco) supplemented with 10 mg/L Cm or Em.

Assembling of pLF- and pCB-based plasmids with *gse*BL gene was performed in *E. coli* TG1 and then they were introduced to *B. subtilis* by either conjugative mobilization or transformation. Assembling of *gse*BI-containing plasmids was carried out directly in *B. subtilis* AJ73 under selection of the clones on antibiotic/skim milk agar plates by formation of a halo as described previously (Rebrikov *et al.,* 1999).

TABLE 1

Measurement of the Copy Number in pLF Derivatives

	pUC19	pLF9	pLF14	pCB20
E. coli	450/200	180/80	240/105	330/145
B. subtilis	—	120/32	154/41	15/4

Note. The yield of plasmid (μg/100-ml culture) and calculated copy number per cell are shown in each well (yield/copy number).

2.4. Measurement of Plasmid Copy Number

The copy number of pLF and other plasmids was estimated on the basis of their yield relative to control plasmid pUC19 in *E. coli* (Sambrook *et al.,* 1989) and pCB20 in *B. subtilis* (Sorokin *et al.,* 1986). These experiments were performed with both hosts on the basis of large-scale plasmid purification in a CsCl gradient as described previously (Sambrook *et al.,* 1989). The yield (μg) from 100 ml of culture is shown in Table 1.

2.5. Cultivation of B. subtilis Strains

Both natural and recombinant bacillar strains were cultivated on LB Lennox agarized or liquid medium (Difco) when used for transformation, storage, and plasmid. Fermentation for generation of Gse was performed in DPS medium of the following composition: 2% maize meal, 3% dry yeast BVK (Factory of Enzymatic Preparations, Vyshny Volochek, Russia), 0.3% $CaCO_3$, pH 7.4.

2.6. Measurement of Protease Activity

Gse activity was measured toward a synthetic chromogenous substrate Z-Glu-pNA dissolved in a concentration of 0.5 mg/ml in 0.05 M Tris–HCl, pH 8.2, supplemented with 1 mM $CaCl_2$. The enzyme in 10–100 μl was placed into 1 ml of the final volume of the reaction mixture and the reaction was performed for 30–250 min at 37°C and stopped by the addition of 500 μl of 1 M Na-citrate buffer containing 20% dimethylformamide, pH 3.0. Measurement was perfomed at the wavelength of 410 nm. One unit of activity was calculated as the amount of enzyme producing 1 mmol of nitroaniline for 1 min. Subtilisin activity was assessed toward synthetic Z-Ala-Ala-Leu-pNA as described by Gololobov *et al.* (1991).

2.7. Purification of the Mature Glutamyl Endopeptidases

The cells were displaced from cultural liquid by centrifugation and the total protein was precipitated with isopropanol at a final concentration of 70%. The pellet was solubilized in a minimal volume of 0.05 M Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ dialyzed against the same buffer and applied to a 20-ml bacitracin–Sepharose column. The column was washed with the application buffer. Elution of Gse was carried out by the same buffer supplemented with 1 M NaCl.

3. RESULTS

3.1. Construction of pLF9 Expression Vector

Plasmid pLFmob76 (Fig. 1) was used as a source of replicon for the present work (Livshits *et al.,* 1989). It contains the replication origins (*ori*+ and *ori*−[3]), genes *rep*A and *rep*B (involved in replication and its control), the pC194-*cat* gene (Ballester *et al.,* 1990), and the *oriT* mobilization origin of plasmid RP4 (Simon, 1984). The *repA* and *repB* genes were transcribed from the same promoter located upstream of *repA.* Between the *ori*− region and *oriT* there was a nonfunctional fragment of the *kan* gene derived from Tn903 containing the promoter, the translation initiation site, and 12 triplets of the coding region (Vieira and Messing, 1991).

A residual fragment of the *kan* gene was used to create a cloning-expression unit in pLFmob76. To this end we inserted a strong sporulation-dependent BT1–BT2-type promoter of *cry*1Aa δ-endotoxin gene from *B. thuringiensis* ssp *kurstaki* to the unique *Pst*I of pLFmob76. The promoter is included within a 220-bp *Nsi*I–*Nsi*I fragment from pOK2 plasmid (Osterman *et al.,* 1989) containing the full-length *cry*1Aa gene (Brown and Whiteley, 1988). The BT pro-

[3] ori+ designation is applied to the double-strand replication origin and ori− means a single-strand origin in RCR.

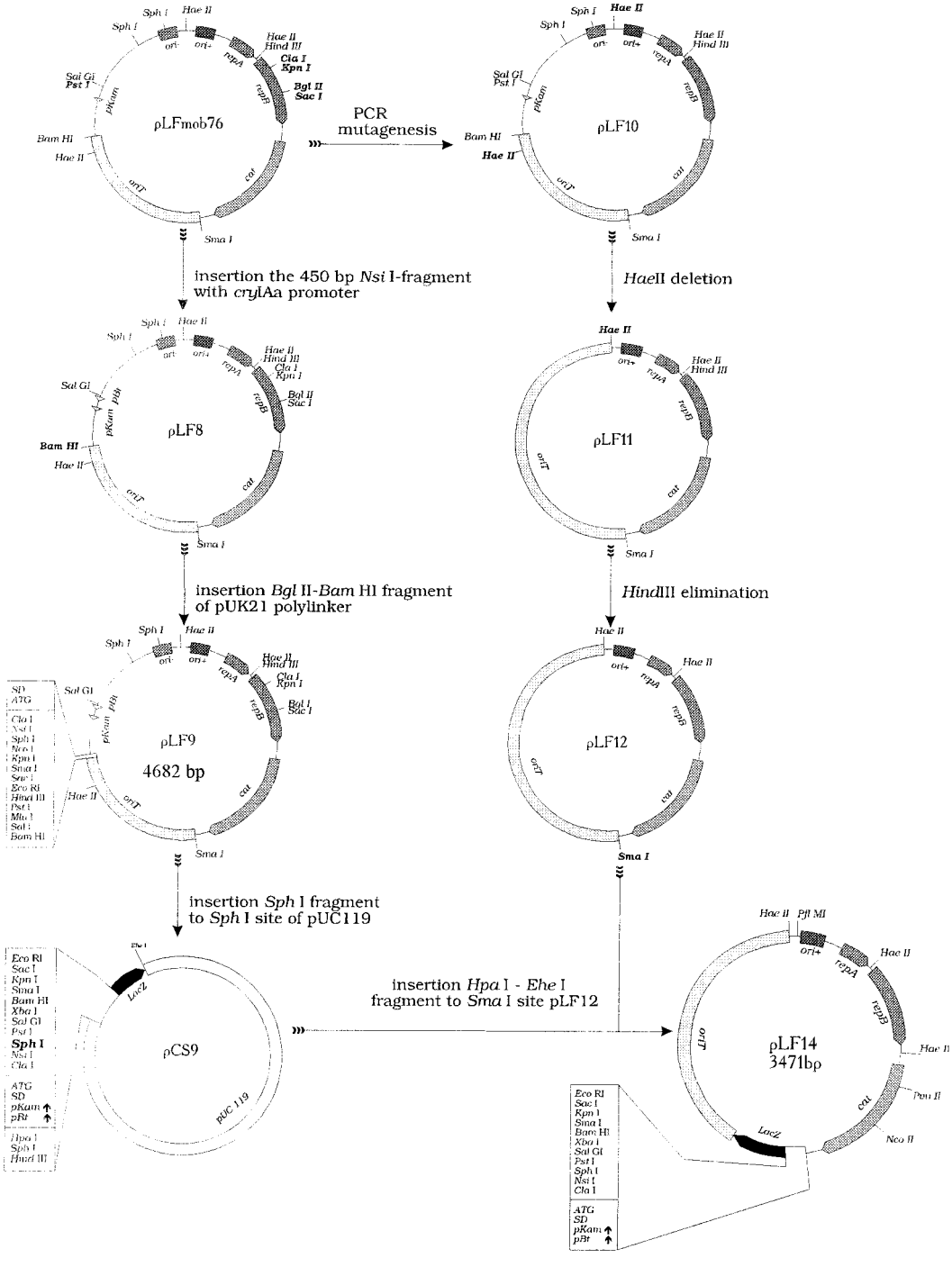

FIG. 1. Scheme of the generation of pLF family vectors. See detailed description in the text.

moter had the same orientation as the promoter of the *kan* gene. The resulting plasmid with the double promoter was named pLF8.

The fragment of pUK21 polylinker (Vieira and Messing, 1991) flanked with *Bgl*II and *Bam*HI sites was inserted to the unique *Bam*HI

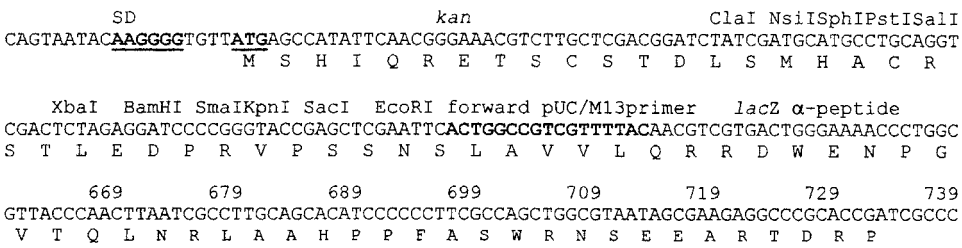

FIG. 2. Cloning/expression unit in pLF14. Translation initiation site derived from the *kan* gene (SD, Shine–Delgarno sequence, and ATG start codon are in bold and underlined), hybrid ORF (*kan*-polylinker-*lacZ*), multiple cloning site, and the pUC/M13 17-bp standard primer annealing site are depicted.

site of pLF8. It contained 13 restriction sites; 5 of them (*Nsi*I, *Pst*I, *Eco*RI, *Mlu*I, and *Bam*HI) were single. This plasmid was named pLF9.

The structure of pLF9 allowed expression (under the control of the *kan* or BT promoter) of the inserted DNA in a translational fusion with the kanamycin phosphotransferase due to the absence of stop codons between SD-ATG of the *kan* gene and the sites of the polylinker. Nevertheless the application of pLF9 for cloning was complicated by the duplication of the polylinker sites *Sma*I, *Sal*I, *Nco*I, *Sac*I, *Kpn*I, *Cla*I, *Hind*III, and *Sph*I in the plasmid. The choice of sites available was limited; besides there were some nonessential regions which could be deleted to increase the replicative capacity. Thus we undertook further manipulations to displace these disadvantages.

3.2. Construction of a Universal Expression-Cloning Unit for E. coli–bacilli Shuttle Vectors

The elements of pLF9 contained the BT-promoter, the *kan*-promoter, the *kan* translation initiation point, and the polylinker pUK21 (Fig. 1). We isolated these elements from pLF9 in the *Sph*I–*Sph*I fragment and cloned them into the *Sph*I site of pUC119. The translational fusion of the *kan* gene with *lacZ* restored activity of β-galactosidase in cells containing the recombinant pCS9 plasmid. We used this construct as a source of the expression-cloning unit for the final variant of pLF. The unit was isolated from pCS9 in the *Hpa*I–*Ehe*I fragment. *Hpa*I is located 40 bp upstream of the BT promoter and the *Ehe*I site (*Nar*I) limited the *lacZ* coding region of pUC119 at the 3′-end. It contained both BT and *kan* promoters, the translation initiation point of *kan*, partially modified polylinker pUC119, and *lacZ.*

3.3. Construction of pLF14 Cloning–Expression Vector

The unique sites *Sac*I, *Bgl*II, *Kpn*I, and *Cla*I are located in pLFmob76 in the coding region of the *repB* gene. PCR mutagenesis was used to modify the sequence of the sites. Two oligonucleotides (CK and BS), overlapping the sites *Sac*I, *Bgl*II, *Kpn*I, and *Cla*I, were used to amplify a fragment of the *repB* gene. The changes in the nucleotide sequence did not lead to any amino acid change in the encoded protein. The source plasmid was cut with the restrictases *Cla*I and *Ecl*136.II and treated with mung bean nuclease. Then the amplified fragment was cloned instead of the removed fragment. After ligation and transformation, clones without *Sac*I, *Cla*I, *Bgl*II, and *Kpn*I were selected. The resulting plasmid was designated pLF10.

After PCR mutagenesis we subsequently carried out *Hae*II deletion of pLF10 and filling in of the *Hind*III site between the *rep*A and *rep*B genes. An 800-bp fragment removed with the *Hae*II deletion contained a nonessential part of *oriT,* the rest of the *kan* gene, and *ori*−. Deletion of the last fragment presumably would not impair stability of the replication in *B. subtilis* because of the high specificity of ori− to the homologous species only (Meijer *et al.,* 1995; Wang and Lee, 1997), whereas the natral host of pLF1311 was *L. fermentum.*

The *Hpa*I–*Ehe*I fragment from pCS9 was inserted into the unique *Sma*I site of pLF12, resulting in construct pLF14 (Fig. 2). As pre-

dicted, *E. coli* TG1 (pLF14) formed blue colonies on X-gal/IPTG-containing medium. Its nucleotide sequence of 3471 bp was deposited in the EMBL Gene Bank under Accession Number X85430.

3.4. Cloning of gseBL-gene in pCB22, pLF9, and pLF14 Shuttle Vectors

A natural isolate of *B. licheniformis* B-6839 was previously shown to produce chitinases (Trachuk *et al.,* 1996). Glutamyl endopeptidase activity was found in its growth medium by hydrolysis of the specific synthetic substrate Z-Glu-pNA. The determined N-terminal amino acid sequence of the discovered enzyme was similar to one described earlier (Svendsen and Breddam, 1992; Nienaber *et al.,* 1993). We used the published DNA sequence to order oligonucleotide primers specific to *gse*BL gene (Kakudo *et al.,* 1992a). The primers were used for PCR amplification of the gene directly from the genome of *B. licheniformis* (see Materials and Methods). The primers were chosen in respect to covering a complete coding and promoter region of *gse*BL. The amplified fragment (1100 bp) was digested with *Mfe*I and *Bam*HI and cloned into *Eco*RI/*Bam*HI sites of vector pUK21. The inserted DNA in the resulting plasmid pGAP1 was completely sequenced and found to be identical with the reported sequence (Kakudo *et al.,* 1992a).

The *Nsi*I–*Bam*HI fragment from pGAP1 was transferred to *Pst*I–*Bam*HI sites of the polylinker pLF9. pLF9-derived plasmids with the cloned *gse*BL gene were designated pLFgap9. The *Sph*I–*Bam*HI fragment from pGAP1 was cloned into the same sites of pLF14. The recombinant clone was named pLFgap14. The *Bgl*II–*Bam*HI fragment of pGAP1 was cloned to the *Bgl*II site of the pCB22 vector and the resulting plasmid, pCB22-gap1, was transformed to the *B. subtilis* AJ73 protease-deficient strain.

3.5. Conjugative Mobilization of pLF Derivatives into B. subtilis Cells and Stability Tests

Triparental matings were used to transfer the recombinant constructs from *E. coli* to *B. subtilis* and *B. licheniformis.* Overnight cultures of *E. coli* TG1 harboring pLFgap9 and pLFgap14 constructs (10^8 cells), *E. coli* C600 harboring RP4 natural plasmid (10^8 cells), and *B. subtilis* AJ73 or *B. licheniformis* B-6839 (10^7 cells) were mixed and inoculated onto M9 minimal salt agar plates containing 0.04% glucose with no antibiotic and were incubated overnight at 30°C. Then bacteria were washed from the plate with fresh LB broth and inoculated to selective plates with 10 mg/L of Cm and polymyxin. Transconjugative colonies appeared after 40 h of growth at 37°C. The effectivity reached 10^{-5} colonies per donor cell and 10^{-6}–10^{-7} per recipient cell when tested on both pLFgap constructs.

The presence of the plasmid constructs in the cells was tested by PCR amplification with the primer pairs annealing to the *gse*BL gene and by purification of plasmid DNA.

The frequency of pLFgap9 and pLFgap14 loss was tested after 100 generations (10 days of subsequent passages in liquid LB medium without antibiotic; the culture was inoculated by 1/1000 of the final volume of 3 ml twice a day). One colony of 960 tested turned out to be Cm sensitive when assessed with pLFgap14. No loss of resistance was observed in the same experiment with pLFgap9. Thus instability of pLF derivatives did not exceeded 10^{-5} per generation. Instability tests on *B. subtilis* (pCBgap1) demonstrated a 5×10^{-3} loss of plasmid per generation.

3.6. Assay of Production of gseBL-Containing Clones

A single colony of *B. subtilis* harboring pLFgap9 or pLFgap14 or plasmidless *B. licheniformis* B-6839 was inoculated to 3 ml LB agar slant containing 10 mg/L Cm and cultivated for 24–48 h at 37°C. *B. subtilis* AJ73 (pCBgap1) was cultivated under the same conditions in the presence of 10 mg/L Em. The cells were washed from the agar slant with liquid LB medium and used to inoculate 1–3 flasks with 75 ml of LB medium (either with no antibiotic or Em) and cultivated for 48 h at 37°C with a vigorous agitation. Aliquots of the cultural liq-

TABLE 2

Production of Secreted GseBL by the Natural Strain of *B. licheniformis* B-6839 and Recombinant Strains of *B. subtilis* AJ73 Transformed by Recombinant pLF- and pCB22-Based Constructs in the Presence and Absence of either Cm or Em in Medium

Strain	24 h	48 h
B. licheniformis B-6839[a]	0.009	0.011
AJ73 (pLFgap9), Cm+	0.000	0.000
AJ73 (pLFgap9), Cm−	0.150	0.170
AJ73 (pLFgap14), Cm+	0.000	0.001
AJ73 (pLFgap14), Cm−	0.200	0.210
AJ73 (pCB-gap1), Em+	0.003	0.003
AJ73 (pCB-gap1), Em−	0.003	0.003

Note. Activity toward Z-Glu-pNA is given in U/ml of cultural medium.

[a] Data presented were obtained in a single experiment. The GseBL productivity of the natural strain may vary in the range of 0.006–0.035 U/ml.

uid were picked up after 24 and 48 h of growth, and specific proteinase activity was measured.

Production of the Gse was quantified toward Z-Glu-pNA synthetic substrate. Results of the tests are shown in Table 2. They show essentially better yield of GseBL in strains on the basis of pLF in comparison with pCB22-based strains and the natural producer. A complete repression of GseBL synthesis was observed in both pLFgap9 and pLFgap14 producers in the presence of Cm in medium, whereas there was no difference in production of the enzyme by the pCB-based strain in the presence and in the absence of Em.

3.7. Preparative Purification of GseBL from pLFgap9 and pLFgap14 Cultures

B. subtilis AJ73 (pLFgap 9) and AJ73 (pLFgap14) inoculum were generated by 24-h growth of the cultures on LB slant agar containing 10 mg/L Cm. The cells were washed and put into flasks containing 75 ml DPS medium without antibiotic. Fermentation was performed at 37°C under vigorous agitation for 32 h. Cells were removed by centrifugation and glutamyl endopeptidase activity was measured. It reached 280 U/L. The clear cultural medium was used for further protein purification as described under Materials and Methods. Purity of the enzyme was estimated by SDS–PAGE (Fig. 3) and the concentration measured by D_{280}. The total amount of pure enzyme yielded from 800 ml was 120 mg. We estimate the protein production to be no less than 150 mg/L of cultural liquid in both strains constructed.

3.8. Cloning and Expression of the Glutamyl Endopeptidase Gene of B. intermedius (GseBI) in pLF and pCB22 Vector

Cloning and sequencing of the *gse*BI gene in the composition of p58.21 plasmid based on the pCB22-type replicon with resistance to Cm was described previously (Rebrikov *et al.*, 1999). A *Pst*I–*Eco*RI fragment of p58.21 was cloned in the pLF14 vector and introduced to the BG2036 strain of *B. subtilis.* Fermentation was performed in DPS medium with or without Cm at a concentration of 10 mg/L at 30°C. Results of fermentation are shown in Table 3. A 1.5-fold

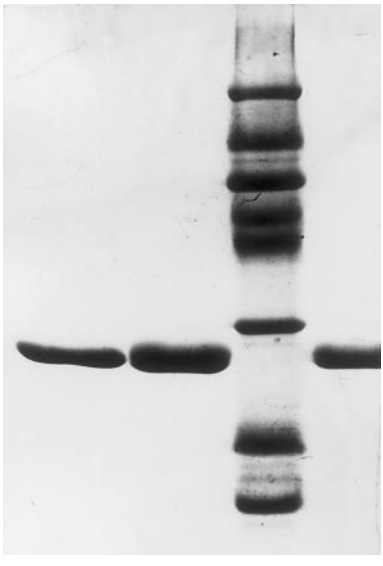

FIG. 3. Electrophoretic assay of the purity of GseBL and GseBI preparations obtained from recombinant strains of *B. subtilis* based on pLF vectors. SDS–PAGE was performed on a denaturing 12.5% gel. The slab is stained with Coomassie R250. The following samples were loaded into the slots: 1. Positive control—GseBL purified from wild-type *B. licheniformis* B-6839, 10 μg; 2. Molecular mass standard—14, 22, 31, 40, 43, 55, 66, and 97 kDa; 3. GseBI purified from cultural liquid of AJ73(pLFbip14), 20 μg; 4. GseBL purified from cultural liquid of AJ73(pLFgap14), 15 μg.

TABLE 3

Kinetics of Accumulation of Secreted GseBI in Recombinant Strains of *B. subtilis* BG2036 Transformed by Recombinant pLF- and pCB22-Based Constructs in the Presence and Absence of Cm in Medium

	18 h	30 h	48 h	52 h
pLFbip2, Cm+	0.000	0.000	0.000	0.002
pLFbip2, Cm−	0.005	0.010	0.040	0.060
p58.21, Cm+	0.000	0.000	0.000	0.000
p58.21, Cm−	0.002	0.004	0.020	0.040

Note. Activity toward Z-Glu-pNA is given in U/ml of cultural medium.

increase of the yield was observed with the pLF-based replicon in comparison with the pCB-type. The recombinant strain of *B. subtilis* on the basis of pLF14 rendered 6-fold more yield than natural producers of GseBI (Leshchinskaya *et al.,* 1997). The performed experiment shows complete repression of *gse*BI expression in the presence of Cm regardless of replicon type.

4. DISCUSSION

The most serious problem in the practical use of shuttle vectors for bacilli and other gram-positive hosts is still a high level of structural and segregative instability. Thus the ability of pLF family vectors to support the cloned gene in *E. coli* as well as in the final host was tested in the course of cloning and expression of two different *gse* genes. The universality of the structural elements composing pLF9 and pLF14 vectors minimized their own size and the replicative capacity was sufficient to clone at least 3 kb of gene while the plasmid would be filled in to natural size. The segregative stability of pLF vectors harboring the well-expressed *gse* gene was as high as 10^{-5} or less per generation in the absence of Cm selection. This feature can be useful for construction of the producer strains.

The conjugative mobilization procedure represents a simple technique for introducing recombinant DNA to a wide range of bacteria including *Bacillus, Lactobacillus, Lactococcus, Corynebacterium* (Trieu-Cuot *et al.,* 1991), and other species which cannot be naturally transformed. The efficiency and recovery of conjugative mobilization are high. The use of three-strain mating allows us to resolve the problem of interference between pLF and RP4 helper plasmids and to avoid the donor strain construction step.

A noteworthy but not quite clear phenomenon found on the model of *gse* gene expression in both the pLF and the pCB vector is its sensitivity to the presence of Cm but not Em. This phenomenon does not depend on the replicon type used and may be restricted by *gse* genes only. This effect could essentially decrease the yield of Gse when pSM19035 derivatives are used in combination with Cm resistance due to rapid loss of the plasmid under no selection. Since the pLF vector is quite stable in the absence of antibiotic selection, it seems to be preferable over other types of vectors for *gse* gene expression in combination with the Cm resistance determinant.

The yield of glutamyl endopeptidases reached in the present work is better than has been reported earlier for this group of enzymes (Kakudo *et al.,* 1992a,b; Nienaber *et al.,* 1993) and shows a certain advantage to the cloning-expression system used.

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