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Stable Secretion Vector Derived from the RCR (rolling-circle replication) Plasmid of *Bacillus mesentericus*

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The 5.8 kb pMMH1, rolling-circle replication (RCR) plasmid of the wild type soil *Bacillus mesentericus* was developed into a novel secretion vector system in *Bacillus subtilis*. The pMMH1 turned out to have a replication origin and two open reading frames (ORFs) of the putative γ-GTP and type I signal peptidase (*sipP*). To characterize the regions necessary for plasmid stability and high copy number, five vectors (pPS, pPP, pEN, pMN, pME) were constructed by disruption or deletion of each region in pMMH1. Like pMMH1, all constructed vectors were stable over 100 generations in a non-selective medium. Since pPS was the smallest (2.3 kb)of all, it was selected for the construction of a novel secretion vector. Using the α-amylase promoter/signal sequence of *B. subtilils*, the novel plasmid pJSN was constructed. When β-glucosidase was expressed using pJSN, we found β-glucosidase activity in the medium. This result strongly suggested that plasmid pJSN can be used for the production of bioactive peptides in *B. subtilis*.

Key words: rolling circle replication (RCR) plasmid, *Bacillus mesentericus*, β-glucosidase

Bacillus subtilis, a gram-positive, non-pathogenic aerobic bacterium, has been widely used for the production of enzymes and special chemicals. It provides an attractive alternative to *Escherichia coli* as a host for expression of cloned genes. The greatest advantage of this strain is its ability to secrete of heterologous or homologous proteins into a culture medium. With the accumulation of secreted proteins in a culture medium to a high level in a relatively pure state, the purification of secreted proteins is generally much easier and less costly than that of intracellular proteins. In addition, secreted proteins exist in a soluble and biologically active form (Shiroza *et al.*, 1985; Wong *et al.*, 1994; Jeong and Kim, 2000). However, the disadvantage of this host is a lack of stable cloning vectors (Gibson *et al.*, 1992).

High levels of both segregational and structural instability have impeded the development of efficient host/vector systems for recombinant DNA technology in *B. subtilis* (Bron *et al.*, 1991). The recombinant plasmid based on small, cryptic plasmids from *B. subtilis* is unstable (Meijer *et al.*, 1998). Such instability leads to the reduced overall levels of the desired product in the fermentation, a negative impact on specific activities of proteins, and high production costs (Vyas *et al.*, 1994).

Many attempts have been made to construct stable vectors to take advantage of *Bacillus* as a host for gene expression (Bron *et al.*, 1991). These plasmids are usually based on RCR plasmids derived from staphylococci and streptococci (Gruss and Novick, 1989). Using these plasmids and protease-deficient strains, various foreign proteins have been successfully secreted including β-glucosidase (Tangnu *et al.*, 1981; Kim *et al.*, 1996; Kim *et al.*, 1999), human serum albumin (Saunders *et al.*, 1987), human interleukin-1 (Bellini *et al.*, 1991), antidigoxin single-chain antibody (Wu *et al.*, 1993), and streptokinase (Wong *et al.*, 1986).

In this study, we constructed a new stable expression/secretion vector for *Bacillus* using the pMMH1 plasmid isolated from the wild type *B. mesenetricus* and a *B. subtilis* α-amylase promoter/signal sequence system. The new expression/secretion vector secreted successfully oat β-glucosidase into a culture medium. This result suggests that pMMH1 could be a good base for the construction of a stable expression/secretion vector in *B. subtilis*.

Materials and Methods

Bacterial strains, plasmids, and DNA manipulation

Bacterial strains and plasmids used in this study are sum-

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Table 1. Bacterial strains and plasmids used in this study

Strains or Plasmids	Relevant characteristics	Reference or source
E. coli		
DH5αF′	*SupE44ΔlacU169(ϕ80lacZΔM15)hsdR17recA1gyrA96thi-1relA1*	26
B. subtilis		
DB104	his, nprR2, nprE18, aprA3	24
B. mesntericus	Wild type strain	Unpublished
Plasmids		
pET-Glu1	Amp^r, *Glu1* gene at the *Eco*RI-*Xho*I sites of pET24a(+)	10
p8A1		9
p8A1-α25	*Glu1* gene in p8A1	This work
pKS-α25	α-amylase promoter, signal sequence, and the *Glu1* gene in pBluescript KS	This work
pMMH1	5.8 kb, RCR plasmid isolated from *B. mesentericus*	Unpublished
pGEMC	*Cat* gene (1.4 kb fragment) at the *Sal*I site of pGEM5z(+)	This work
pMMG	Amp^r, hybrid plasmid of pGEM5zf(+) and pMMH1 at the *Nco*I site	This work
pMMGC	*Cat* gene (1.4 kb fragment) at the *Sal*I site of pMMG	This work
pPS	Cm^r, 6.8 kb, 2.3kb *Pst*I-*Spe*I fragment of pMMH1 in pGEMC	This work
pPP	Cm^r, 6.9 kb, 2.4kb *Pst*I-*Sca*I fragment of pMMH1 in pGEMC	This work
pEN	Cm^r, 9.0 kb, pMMGC without γ-GTP	This work
pMN	Cm^r, 8.4 kb, pMMGC without γ-GTP and *sipP* genes	This work
pME	Cm^r, 8.5 kb, pMMGC without γ-GTP and ORF2	This work
pJSN	Cm^r, 9.3kb, α-amylase promoter-signal sequence and β-glucosidase mature gene in pPS	This work

marized in Table 1. Wild type *B. mesentericus* was isolated from a soil sample from Yong-in, south Korea. *E. coli* DH5α *(supE44, lacU169 (80lacZ M15), hsdR17, recA1, gyrA96, thi-1, relA1)* and *B. subtilis* DB104 *(his, nprR2, nprE18, aprA3)* were used as host strains for the DNA manipulation and heterologous gene expression, respectively. Restriction enzymes and *Ex Taq* DNA polymerase were purchased from Takara (Shiga, Japan). Media ingredients were purchased from Difco Laboratories Ltd. (East Molesley, United Kingdom) and other chemicals were obtained from Sigma (Poole, United Kingdom). The enzymes used for DNA manipulation were obtained from several commercial sources and used under the conditions recommended by the manufacturers. All DNA manipulations including restriction digestion, ligation, and agarose gel electrophoresis were carried out as described by Sambrook *et al.* (Sambrook *et al.*, 1989). *B. subtilis* was transformed by the Sadaie and Kada method (Sadaie *et al.*, 1983).

Growth conditions

In general, bacteria strains were grown in an LB medium (tryptophan, 1%, yeast extract 0.5%, NaCl 1%) supplemented with ampicillin (50 μg/ml) or chloramphenicol (10 μg/ml). LB medium containing appropriate antibiotics was used for the selection of *E. coli* and *B. subtilis* transformants and for the segregational stability analysis. For the secretion analysis of β-glucosidase, *B. subtilis* transformants were cultured in a CPGY medium [KH_2PO_4 0.3%, $Na_2HPO_4 \cdot 12H_2O$ 0.7%, $(NH_4)_2SO_4$ 0.3%, casein peptone 3%, glutamate 0.5%, yeast extract 0.5%, glycerol 5%, $MgSO_4 \cdot 7H_20$ 0.1%, sodium citrate 0.1%, pH 7.0, 0.1% (V/V) of a trace mineral solution ($FeSO_47H_20$ 1%, $CaCl_2 \cdot 2H_20$ 0.2%, $CoCl_2 \cdot 6H_2O$ 0.025%, $ZnSO_4 \cdot 7H_2O$ 0.22%, $MnSO_4 \cdot 4H_2O$ 0.05%, $CuSO_4 \cdot 5H_2O$ 0.1%, $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$ 0.001%, $Na_2B_4O_7 \cdot 10H_2O$ 0.002%)] containing chloramphenicol (10 μg/ml). Seed cultures grown in LB broth were transferred to 250-ml flasks and incubated at 25 with shaking at 200 rpm.

Construction of an expression vector system

The parts of the pMMH1 plasmid were deleted or disrupted to see which region is necessary for stable plasmid maintenance with a high copy number. For convenient DNA manipulation, the pMMH1 was inserted into pGEM5zf(+) vector (Promega, U.S.A.) at the *Nco*I site to yield pMMG. The 1.4 kb *Sal*I fragments of the *cat* gene (chloramphenicol acetyltransferase) as a selection marker in *Bacillus* was cloned into pGEM5zf(+) to construct pGEMC. The *Eco*RV-*Nco*I fragments of pGEMC were ligated to the *Pvu*II-*Nco*I fragments of pMMH1 to construct pEN. To construct pMN, pEN was digested with *Nde*I and the resulting 4.0 kb fragments containing DNA binding protein coding region, replication origin, and the secretion protein coding region were ligated into pGEM5zf(+). The coding region of DNA binding protein of pEN was removed by digestion of pEN with *Eco*RI and then self-ligation to yield pME. The 2.4 kb *Pst*I fragments and 2.3 kb *Pst*I-*Spe*I fragments of pMMH1 containing a replication region were isolated from pGEMC and then inserted into the pGEM5zf(+) to construct pPP and pPS, respectively (Fig. 1).

The expression vector pJSN was constructed using the smallest and most stable pPS and α-amylase promoter-signal sequence (Kim *et al.*, 1998) (Fig. 2). To place the β-glucosidase gene (1.5 kb) under the control of α-amylase promoter-signal sequence, the β-glucosidase gene

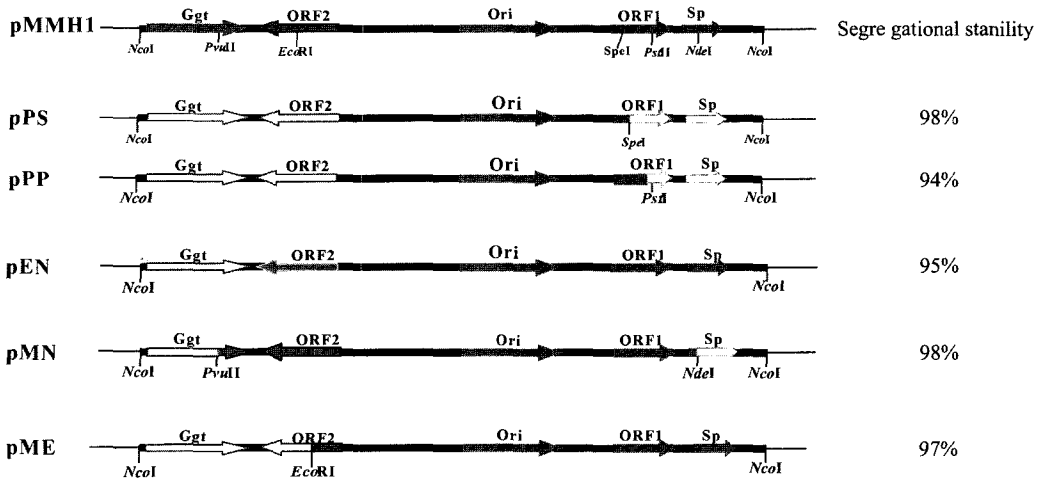

Fig. 1. Constructed vectors and segregational stability. Each region of pMMH1 was disrupted or deleted and then ligated with the *E. coli* pGEM5zf (+) vector. ORF1; secretion protein coding region, ORF2; DNA binding protein coding region, Ggt; gamma-glutamic acid, Ori; replication origin, Sp; type I signal peptidase (*sipP*). Open arrows indicate disrupted or deleted regions. Segregational stability was analyzed two times after 100 generations in a non-antibiotic medium.

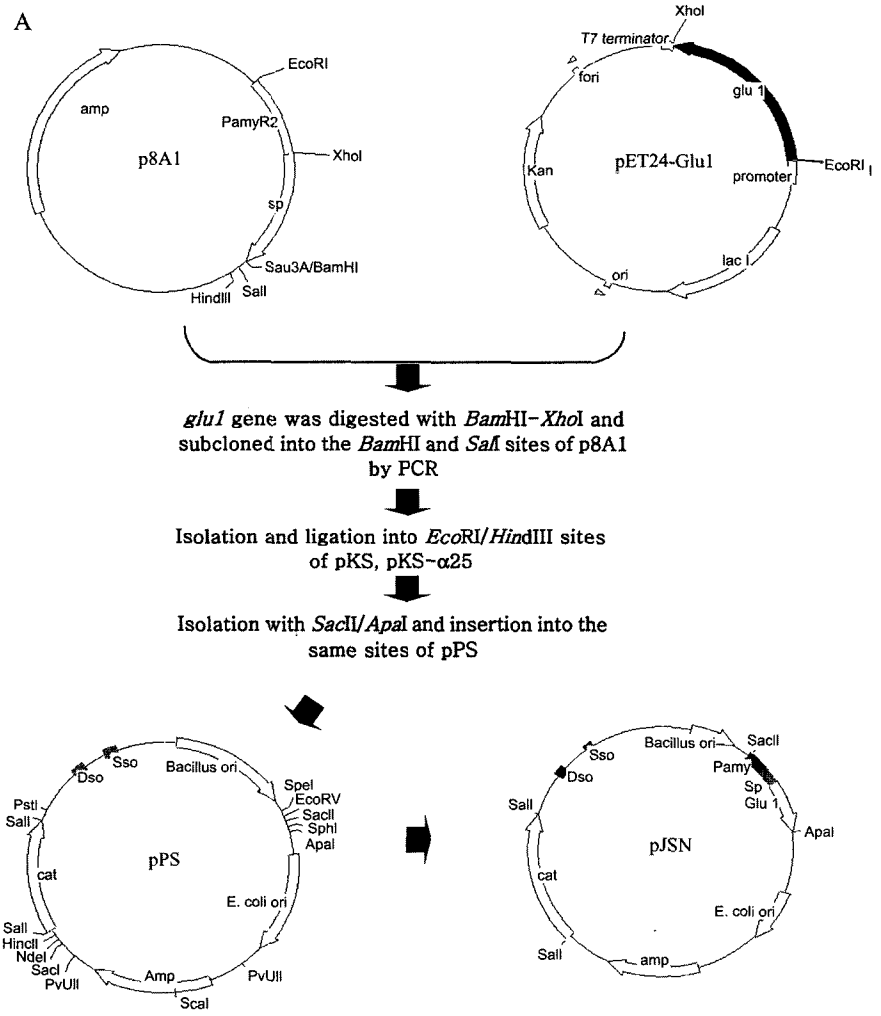

Fig. 2. Scheme for the construction of secretion vector. (A) Schematic diagram of construction of pJSN vector. (B) The nucleotide and amino acid sequence at fusion site. P : α-amylase promoter, RBS : ribosome binding site, tandom repeat of stop codons follows the C-terminal of β-glucosidase mature gene, nucleotide C (boxed) was inserted to keep the frame by PCR.

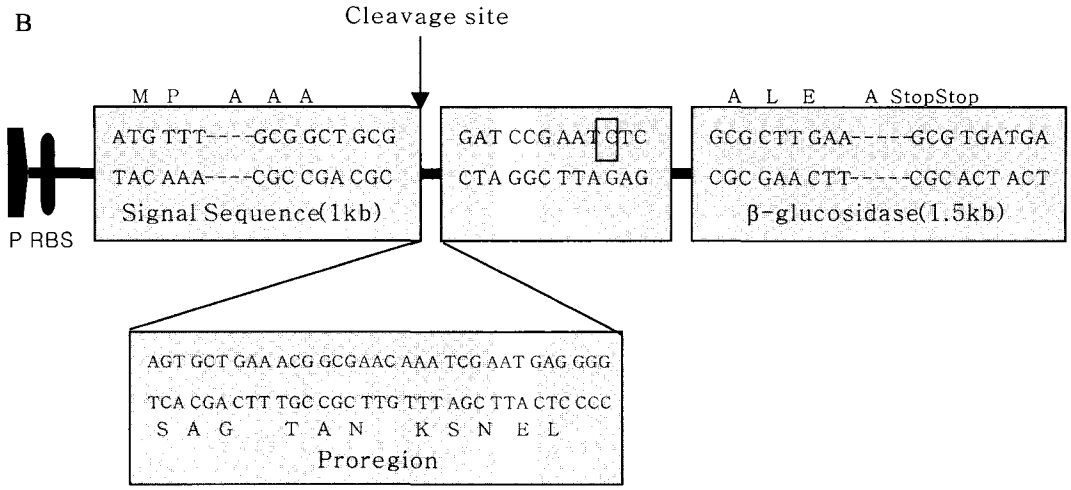

Fig. 2. Continued.

was amplified from pET24-Glu1 (Kim *et al.*, 1999) by PCR and cloned at the *Bam*HI and *Sal*I sites of p8A1 to construct p8A1-α25 (Kim *et al.*, 2000). The forward primer (5'-GGATCCGAATCTCGCGCTTGAAAGT-3') was designed to contain the *Bam*HI site (underlined) for easy cloning and one nucleotide C (underlined) for the in-frame fusion between the α-amylase signal sequence and the N-terminus of the β-glucosidase mature gene. The reverse primer (5'-CTGCAGCTCGAGTCATCACGCGGT-3') was designed to contain the *Xho*I site (underlined) and tandem TGA stop codons (underlined). The p8A1-α25 was digested with *Eco*RI-*Hin*dIII and the 2.5 kb-fragments containing α-amylase promoter-signal sequence-β-glucosidase were subcloned into pBluescript II KS (+) at the same sites to construct pKS-α25. Finally, pKS-α25 was digested with *Sac*II-*Apa*I and then inserted into the same sites of the pPS vector to construct the pJSN expression vector (Fig. 2). The junction sequence of the resulting plasmid was confirmed by DNA sequence analysis.

Assay of plasmid segregational stability

A Single colony of transformants harboring constructed vectors was inoculated into 3 ml of LB medium containing chloramphenicol (10 μg/ml) and incubated at 37°C overnight with shaking. The seed culture (1 ml) was transferred to 100 ml of LB broth without chloramphenicol and then grown for about 100 generations in successive batch cultures. Every 20 generations of growth, the culture was diluted with a prewarmed non-antibiotic medium and plated onto a non-antibiotic LB medium. At least 200 colonies were transferred to plates containing chloramphenicol. The percentage of plasmid-bearing cells was calculated from the ratio of the number of colonies grown on the antibiotic plate to the total colonies transferred.

Assay of β-glucosidase activity

β-glucosidase activity was determined by measuring the liberated *p*-nitrophenol (*p*NP) from paranitrophenyl-β-D-glucopyranoside (*p*NPG). The transformant *B. subtilis* DB104 harboring the pJSN vector was precultured in 3 ml of LB medium containing chloramphenicol (10 μg/ml) overnight. Three ml of preculture was then transferred to 50 ml of CPGY medium in a 250 ml flask and incubated at different growth temperatures of 37°C, 30°C, and 25°C with shaking at 200 rpm. One ml of culture broth was centrifuged at 15000 rpm for 20 min. The supernatant of the culture broth (50 μl) was mixed with 450 μl of 10 mM *p*NPG solution resolved in a 20 mM potassium phosphate buffer (KH_2PO_4, pH 5.5). Reactions were carried out at 37°C for 15 min and stopped by addition of a stop solu-

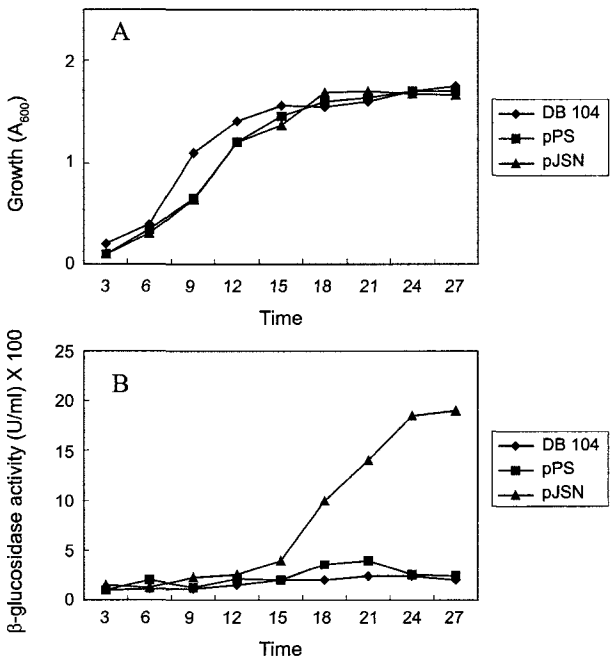

Fig. 3. β-glucosidase activity. Cells was cultured in a CPGY medium and growth (A) and β-glucosidase activity (B) were determined every 3h.

tion (2 M Na_2CO_3 solution). The released *p*NP was determined by reading A_{405} (Fig. 3). One unit was defined as the amount of enzyme which hydrolyzed 1 nmol of *p*NP per min at 37°C (Kim *et al.*, 1996).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The profile of proteins in the culture supernatant was analyzed by SDS-PAGE (11). Proteins in the culture were precipitated using trichloroacetic acid to a final concentration of 10% (w/v). The precipitates were resuspended in 50 μl of 0.1 N NaOH. After adding 10 μl of 5 X sample buffer, samples were boiled for 5 min and loaded on a 10% SDS-polyacrylamide gel.

Results and discussion

Vector construction and plasmid segregational stability

The RCR plasmid, pMMH1 was previously isolated from the wild type *B. mesentericus* (K.-H. Park, I. H. Lee, and J.-W. Suh, unpublished data). Because it is very stable in a non-selective condition, an attempt was made to develop it into a stable vector in *Bacillus*. Sequence analysis revealed that the pMMH1 contained a replication origin and two open reading frames (ORFs) of γ-GTP and type I signal peptidase (*sipP*) (K.-H. Park, I. H. Lee, and J.-W. Suh, unpublished data). To define the regions that are necessary for vector stability of pMMH1, each region of pMMH1 was removed, constructing five vectors (pPS, pPP, pEN, pMN, pME) as *B. subtilis-E. coli* shuttle vectors (Fig. 1). The pEN in which γ-GTP coding sequence was removed showed 98% stability over 100 generations suggesting that γ-GTP coding sequence is dispensable for plasmid stability. The deletion/disruption of ORF2 (pME) or type I signal peptidase in addition (pMN) to γ-GTP coding sequence did not affect plasmid stability significantly. In both pPP and pPS, γ-GTP coding sequence, ORF1, ORF2, and type I signal peptidase were deleted, but a greater portion of ORF1 was deleted in pPS than in pPP. Therefore, the pPS was the smallest with the 2.3 kb of the pMMH1 plasmid.

All constructed plasmids were very stable with over 90% stability after about 100 generations (Fig. 1). Because the deletion/disruption of γ-GTP coding sequence, ORF1, ORF2, and type I signal peptidase did not affect plasmid stability significantly, the SSO (single strand origin) between replication origin and ORF2 seems to be most important for plasmid stability. Meijer *et al.* reported that the SSO is an essential factor in a cloning vector derived from a RCR plasmid for *B. subtilis* (Meijer *et al.*, 1995).

The type I signal peptidase in the pMMH1 plasmid is important for the stability in *Bacillus*. *B. subtilis* has a well-developed extracellular secretion system and type I signal peptidase (type I SPases) plays a main role during secretion and processing of many intracellular molecules through membrane translocases. The type I signal peptidases are located on the chromosomes (SipS, SipT, SipU, SipV, SipW) and plasmid (SipP). Among these, SipS, SipT are very important factors in both preprotein processing and viability. SipP is an also important factor because it can functionally replace both damaged SipS and SipT (Tjalsma *et al.*, 2000). In our study, the deletion of type I signal peptidase does not affect plasmid stability, suggesting that it is not a necessary factor. However, the possibility can not be excluded that the type I signal peptidase in the pMMH1 plasmid is a major factor for its stability in *B. mesentericus*.

Construction of a secretion vector system and expression of β-glucosidase in B. subtilis DB104

Because the pPS was stable and the smallest (6.8 kb), it was chosen for the construction of a secretion vector. The pPS is the hybrid of the pGEM5zf(+) that is a commercial *E. coli* cloning vector (Promega, U.S.A.), a part of pMMH1, and the *cat* (chloramphenicol acetyltransferase) gene. Therefore, the pPS functions in both *E. coli* and *B. subtilis*.

For expression/secretion, an α-amylase promoter-signal sequence system (1 kb) of *B. subtilis* was used. When the oat ß-glucosidase gene was inserted, β-glucosidase was secreted successfully. The activity of β-glucosidase in the supernatant increased steadily after 15 h and was the highest at 27 h (Fig. 3). In SDS-PAGE analysis of proteins in

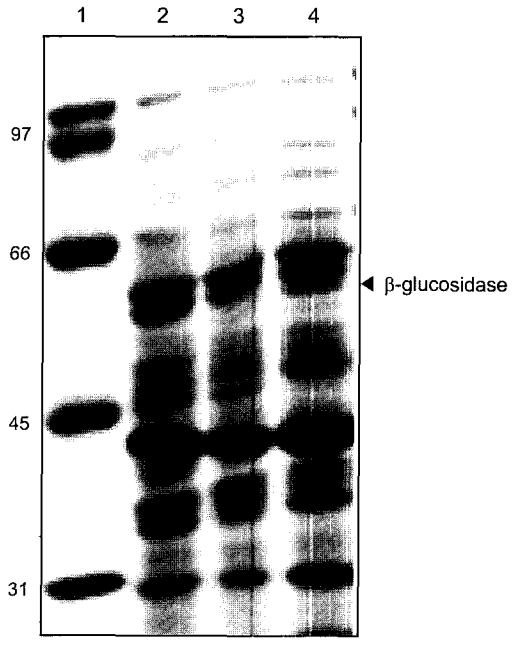

Fig. 4. SDS-PAGE analysis of oat β-glucosidase produced in *B. subtilis* DB104 harboring pJSN vector. Host and recombinant strains were cultured at 25°C for 27 h. Lane 1; molecular mass standard, lane 2; *B. subtilis* DB104 (host), lane 3; *B. subtilis* DB104 (pPS), lane 4; *B. subtilis* DB104 (pJSN). The arrow indicates the secreted mature oat β-glucosidase.

supernatant, the expected β-glucosidase with a size of around 60 kDa was found only in samples from transformants containing an expression/secretion vector, confirming the secretion of ß-glucosidase (Fig. 4).

Most of the developed RCR plasmids have been derived from staphylococci and streptococci, but these plasmids are frequently unstable (Meijer *et al.*, 1998). When the heterologous gene was inserted into an RCR plasmid, it caused segregational instability (Kiewiet *et al.*, 1993). Therefore, RCR plasmids were less attractive as a cloning vector. However, our study suggests that RCR plasmids of *B. mesentericus* can be developed into a useful expression/secretion vector in *Bacillus*.

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