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A Circularly Permuted β-Lactamase as a Novel Reporter for Evaluation of Protein Cyclization Efficiency

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Split inteins have been used as a versatile tool in protein engineering to mediate efficient *in vivo* and *in vitro* trans-splicing of a protein. The trans-splicing ability of split inteins was also applied to the *in vivo* cyclization of a protein. However, cyclization efficiency is dependent upon the type of split inteins employed and the conditions under which cyclization occur. In this study, a novel reporter system that easily measures the cyclization efficiency of split inteins was developed. For this purpose TEM-1 β-lactamase was divided into two fragments (24~215 and 216~286 amino acids) and circularly permuted. The circularly permuted β-lactamase expressed in *Escherichia coli* showed little β-lactamase activity, most likely due to the structural modification of the protein. However, when the circularly permuted β-lactamase was cyclized by the *Synechocystis* sp. PCC6803 DnaB split mini-intein, β-lactamase activity both *in vitro* and *in vivo* was recovered. These results suggest that the novel reporter system can be exploited to develop new inteins with high efficiency of *in vivo* protein cyclization.

***Keywords*:** protein cyclization, split intein, circularly permuted β-lactamase

To increase peptide efficacy, biological peptides should possess the necessary properties such as greater conformational integrity, increased enzymatic stability, and prolonged activity. The instability of proteins often limits the number of medical and industrial applications for which these proteins could be used. Cyclization of peptides; however, leads to a reduction of conformational entropy, thereby leading to high thermal stabilities (Williams *et al.*, 2002). A cyclic β-lactamase was found to be stabilized against heat-precipitation and exopeptidase degradation (Iwai and Pluckthun, 1999). Furthermore, studies demonstrated that the cyclization of the N-terminal glutamine residue to pyroglutamic acid in onconase, an anti-cancer chemotherapeutic agent, was reported to increase protein activity as well as stability (Welker *et al.*, 2007). The successful cyclization of recombinant proteins can be facilitated by the use of inteins (Camarero and Muir, 1999; Camarero *et al.*, 2001; Xu and Evans, 2001).

Inteins are protein domains that undergo auto-excision from nascent polypeptide chains and ultimately join the flanking polypeptide sequences, the exteins, via a concomitant peptide bond (Perler *et al.*, 1994). Inteins are found in all three domains of life: Archaea, Bacteria, and Eukarya (Perler, 2002); however, research has shown that their distribution in both species and host is sporadic. There are two major types in inteins: classical inteins with a central endonuclease domain and mini-inteins with a central linker sequence in place of an endonuclease domain (Starokadomskyy, 2007).

Synechocystis sp. PCC6803 DnaB intein can be split and expressed as two inactive halves (split mini-intein: N-intein, IntN and C-intein, IntC). Interestingly, when the inactive IntN and IntC are rejoined, the splicing activity is restored (Mills *et al.*, 1998; Wu *et al.*, 1998; Spotts *et al.*, 2002). The split mini-intein was used for the *in vivo* cyclization of proteins (Williams *et al*., 2002). The study showed that a reporter protein, fused with the split mini-intein in the order of IntC-reporter protein-IntN, was successfully cyclized with its N- and C-termini linked by a normal peptide bond (Scott *et al*., 1999; Evans *et al*., 2000; Siebold and Erni, 2002; Williams *et al*., 2002). In this study we developed a novel reporter system that can be used to evaluate *in vivo* cyclization efficiency of new split mini-inteins. For this purpose TEM-1 β-lactamase was divided into two fragments and circularly permuted. We demonstrate that the circularly permuted TEM-1 β-lactamase developed in this study is a useful reporter that can evaluate new split mini-inteins for their efficiency of both *in vivo* and *in vitro* protein cyclization.

Materials and Methods

Strains and growth medium

Escherichia coli strain DH5α [FΦ80d *lac*Z DM15 Δ(*lac*ZYA-*arg*F) U169 *hsd*R17(rk-mk+) *deo*R recA1 supE44λ-thi-1 *gyr*A96 *rel*A1] was used for routine cloning and *E. coli* strain BL21(DE3) [F- *ompT* $hsdS_B(r_B\text{-}m_B)$*gal dcm* (DE3)] was used for protein expression. *E. coli* was grown in Luria-Bertani-Kanamycin medium containing 5% (w/v) yeast extract, 1% (w/v) Bacto-tryptone, 1% (w/v) NaCl and 35 μg/ml kanamycin.

Cloning of TEM1 β-lactamase gene

The TEM1 β-lactamase (BLA) gene without the signal sequence (24~286 amino acids) was amplified by PCR using

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the primers, for24-1-1 and Re286-2-4 (Table 1), from pRSETa (Invitrogen, USA). The PCR product was sequenced and cloned into the vector pET28a (Novagen) between the *Nco*I and *Hin*dIII sites, resulting in the plasmid pWT-BLA.

Construction of modified DnaB split mini-intein

The split mini-intein used in this study was derived from the *Synechocystis* sp. strain PCC6803 DnaB intein. The N- and C-terminal splicing domains were amplified by PCR using the primer sets, 'BNfusiF and BNhR' and 'BCNcoF and BCEcR' (Table 1), from genomic DNA of *Synechocystis* sp. strain PCC6803. The N-terminal fragment (IntN) included the A and B motifs conserved among inteins and 4 native N-extein amino acid residues, RESG. The C-terminal fragment (IntC) included the conserved F and G motifs and 4 native C-extein amino acid residues, SIEF. The DNA fragments corresponding to the IntC and IntN domains were inserted into the pGEM T-easy vector forming the pT-IntCN vector.

Protein expression, purification, and analysis

E. coli strains harboring plasmids with modified β-lactamase genes were grown to OD_{600}=0.6 at 37°C and protein expression was induced by 0.05 mM IPTG. After 3 h of induction, cells were collected and disrupted by sonication in lysis buffer (50 mM Tris-Cl; pH 8.0, 100 mM NaCl) containing lysozyme (1 mg/ml) and DNase (1 mg/ml). Lysates were cleared by centrifugation at 5,000 rpm for 15 min at 4°C and then loaded on a Hightrap Ni-affinity column (Amersham Biosiences, USA). The column was subsequently washed with 50 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl and 10 mM imidazole. The bound protein was eluted with 200 mM imidazole. The fractions containing proteins were collected and the pool was extensively dialyzed at 4°C for 16 h in order to remove excess imidazole.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli, 1970) using final acrylamide concentrations of 15% and 5% (w/v) for the separating and stacking gels, respectively. After electrophoresis, the proteins were transferred onto a PVDF membrane (Amersham Biosiences, USA) using a semi-dried transfer kit (Hoefer, USA). The proteins were immunostained with an anti-Penta-His tag antibody (QIAGEN, USA) and detected via the DIG method (Boehringer Mannheim, Germany). β-Lactamase activity was measured by the modified acidimetric method (Rubin and Smith, 1973). Zymogram detection of β-lactamase activity was performed as described previously by Massidda (Massidda *et al.*, 1991).

Ampicillin resistance test

β-Lactamases were induced by adding 0.05 mM IPTG (Isopropylthio-β-D-galactoside) into cultures grown up to OD_{600}=0.6. After 5 min, 30 μg/ml ampicillin was added to the cultures and growth was monitored at 37°C.

Results and Discussion

Synthesis of modified TEM-1 β-lactamases

Cyclic peptide or protein libraries are extremely useful as they can be exploited for genetic study or peptide inhibitor screening (Horswill and Benkovic, 2005). Studies have shown that cyclic proteins, with their N- and C-termini linked by a normal peptide bond, are easily generated using split inteins. Indeed, a few split inteins, including the split mini-intein derived from the *Synechocystis* sp. strain PCC6803 DnaB intein, have been used to cyclize peptides or proteins (Scott *et al*., 1999; Evans *et al*., 2000; Siebold and Erni, 2002; Williams *et al*., 2002). Unfortunately, the efficiency of protein cyclization varies with the types of inteins and culture conditions used for this process. Therefore, in order to effectively cyclize a protein via the split intein method without the accumulation of linear forms or splicing intermediates, more work must be completed in this area of research (Williams *et al*., 2002). In an attempt to develop new split

Table 1. Primers used in this study

Name	Sequence (5' → 3')
for24-1-1	CATGCCATGGGACACCCAGAAACGCTGGT
Re286-2-4	CCCAAGCTTCCAATGCTTAATCAGTGAGG
BCNcoF	CATGCCATGGGCTCACCAGAAATAGAAAAGTTG
BCEcR	CGGAATTCGATACTGTTATGGACAATG
BNfusiF	TGGATGGAGGCGGATAAAAGAGAGAGTGGCTGCATC
BNhR	CCCAAGCTTTAATTGTAAAGAGGAGCTTTCTAG
Re215-1-1-9	CGCGGATCCACTCTCACGCGTTTTATCCGCCTCCATCCA
For218-2-1-10	CGCGGATCCATCGAATTCGGACCACTTCTGCGCTC
For(218/cir)-17	CATGCCATGGGCGAATTCGGACCACTTCTG
Re-1(286/cir)-12	TCCCTCGATTCCAGAACCCCAATGCTTAATCAGTGA
Re-1(286/cir)-fusi-13	GATGATGATGAGAGCCCCTTCCCTCGATTCCAGAACC
For-2(24/cir)-14	CATCATCATTCTGGAGGCGGACACCCAGAAACGCTGGTG
For-2(24/cir)-fusi-15	AGGGGCTCTCATCATCATCATTCTGGAGGC
Re(215/cir)-18	CCCAAGCTTACGCGTTTTATCCGCCTC

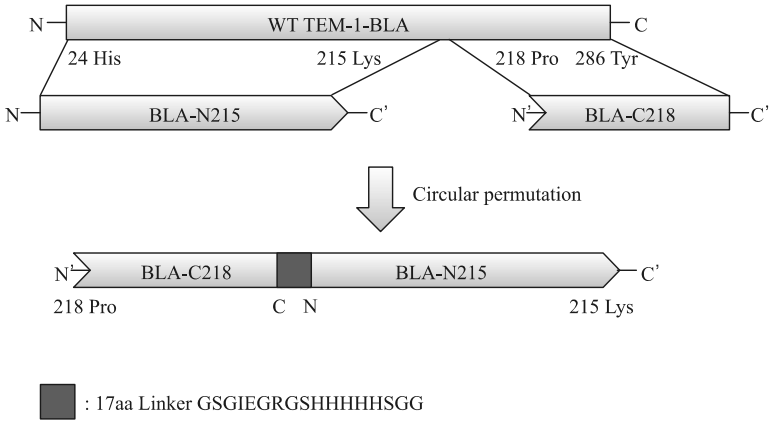

Fig. 1. Construction of the circularly permuted β-lactamase. The circularly permuted β-lactamase was created by joining the N- and C-terminal parts of the β-lactamase with a 17 amino acid linker (GSGIEGRGSHHHHHSGG). The linker contains a penta-His-tag and a Factor Xa cleavage site.

inteins for the successful *in vivo* generation of cyclic peptide libraries, it was determined that a specially designed protein, reporting the *in vivo* cyclization efficiency of new split inteins would facilitate the development process. For this purpose, we chose TEM-1 β-lactamase as a reporter protein, due to the well characterized properties of the protein activity, crystal structure, and stability (Zahn and Pluckthun, 1994; Vanhove *et al.*, 1995). The TEM-1 β-lactamase used for this work was then modified into a circularly permuted form.

The protein cyclization efficiency of a split intein is easily measured *in vivo* through conditional testing. Cyclization efficacy can be monitored via the enhanced cellular survival of cells expressing the cyclic version of the reporter protein, while cells expressing the linear form of the protein show diminished cellular survival under certain growth conditions. Thus, a circularly permuted β-lactamase was designed, which would remain active only in the cyclic form and become inactive when in a linear form. The two DNA fragments corresponding to N-terminal (24∼215 amino acids, BLA-N215) and C-terminal (218∼286 amino acids, BLA-C218) halves of β-lactamase were amplified by PCR using the two primer sets, 'For-2(24/cir)-14, Re(215/cir)-18' and 'For(218/cir)-17, Re-1(286/cir)-12' (Table 1) respectively, from the plasmid pWT-BLA, which contains the β-lactamase gene without the signal sequence (24∼286 amino acids). A linker, RGSHHH HHSGG, that includes a penta-His tag was added at the N-terminus of BLA-N215 through PCR using primers For-2 (24/cir)-fusi-15 and Re(215/cir)-18. Similarly, a second linker, GSGIEGRGSHHH, incorporating a factor Xa cutting site was introduced at the C-terminus of BLA-C218 using primers For(218/cir)-17 and Re-1(286/cir)-fusi-13. A circularly permuted β-lactamase (BLA-C218-BLA-N215) was constructed by reversing the original positions of the N-terminal domain, BLA-215, and the C-terminal domain, BLA-C218 (Fig. 1). Finally, the gene encoding the circularly permuted β-lactamase was cloned into the *Nco*I and *Hin*dIII sites of pET28a to yield pCP-BLA (Fig. 2).

The split mini-intein derived from the *Ssp* DnaB intein provided a means to verify the reporting efficiency of the circularly permuted β-lactamase with regard to *in vivo* protein cyclization (Williams *et al*., 2002). Since the intein mediated splicing process may be affected by residues in the splice junctions, we cloned a few extra sequences along with the *Ssp* DnaB N- and C-terminal domains (IntN and IntC); the amino acids, SIEF, from the *Ssp* DnaB C-extein were included in the IntC domain and the amino acids, RESG, in the IntN domain. The two domains were fused

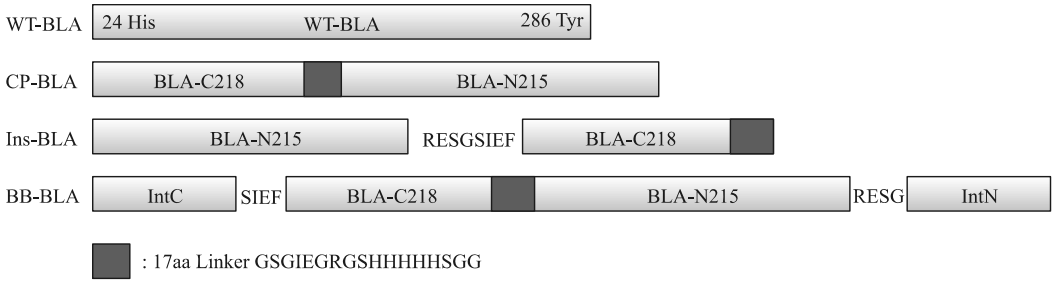

Fig. 2. Schematic representation of modified β-lactamases. The plasmid pWT-BLA has the gene encoding the wild type β-lactamase without signal sequence. The plasmid pCP-BLA contains the gene encoding the circularly permuted β-lactamase. The plasmid pIns-BLA has the gene encoding the RESGSIEF-interrupted β-lactamase. The plasmid pBB-BLA incorporates the gene encoding the circularly permuted β-lactamase flanked with the C- and N-terminal fragments of the split mini-intein (IntC-CP-BLA-IntN).

with the circularly permuted β-lactamase (CP-BLA), generating IntC-CP-BLA-IntN. The gene encoding the IntC-CP-BLA-IntN fragment was cloned into pET28a, which resulted in the plasmid pBB-BLA (Fig. 2).

The cyclic β-lactamase produced from pBB-BLA will contain the extra amino acids, RESGSIEF, between BLA-N215 and BLA-C218. To check whether the insertion of the RESGSIEF sequence between the N- and C-fragments affects the activity of β-lactamase, a second plasmid, Ins-BLA, which expresses the linear BLA-N215-RESGSIEF-BLA-C218 was constructed using the primer sets 'for24-1-1, Re215-1-1-9' and 'For218-2-1-10, Re286-2-4' (Fig. 2) from the plasmid pBB-BLA.

Expression of modified β-lactamases and confirmation of cyclization of β-lactamase

E. coli strains expressing the modified β-lactamases were grown and induced to produce the proteins. It was determined that a small portion of the modified β-lactamases expressed in each *E. coli* strain was in a soluble fraction under the induction condition (data not shown). When the total cell lysates were analyzed through SDS-PAGE and western blotting, the wild type β-lactamase, the circularly permuted β-lactamase and the RESGSIEF-interrupted β-lactamase were detected as prominent bands. However, the putative cyclic β-lactamase was detected as a minor band due to the inefficient cyclization process of the split mini-intein, which generated various intermediate products and the unprocessed whole intein-β-lactamase protein (Fig. 3A).

To confirm that the β-lactamase protein was expressed as a cyclic form in the strain carrying pBB-BLA, the putative cyclic β-lactamase was purified and further treated with the factor Xa. It is generally expected that the linear and cyclic forms of a protein may have different electrophoretic mobility upon SDS-PAGE analysis because of their structural differences (Scott *et al*., 1999). Indeed, the fast moving band, the originally purified protein, was converted into the slow moving band upon treatment with the factor Xa. Further, the amount of the converted protein was found to be proportional to the treatment time (Fig. 3B). This result indicated that the cyclic β-lactamase was formed from the circularly permuted β-lactamase via the circularly permuted *Ssp* DnaB mini-intein in pBB-BLA.

In vitro activity of cyclic β-lactamase

To exploit the cyclic β-lactamase, in order to monitor the cyclization efficiency of new split inteins, a convenient and sensitive detection method for the presence of the cyclic β-lactamase was a prerequisite. The activity of β-lactamase, which hydrolyzes penicillin G to penicilloic acid, is often measured by a color change of the pH indicator phenol red. It is known that the cyclic β-lactamase retains activity; however, the circularly permuted β-lactamase does not possess the same biological activity. Therefore, the spectrometric analysis will verify the efficiency of the cyclization process of a new split intein on the circularly permuted β-lactamase placed between the split intein. While the wild type β-lactamase expressed from pWT-BLA changed the color of the reaction mixture from red to yellow, indicating activity, the circularly permuted β-lactamase from pCP-BLA showed no

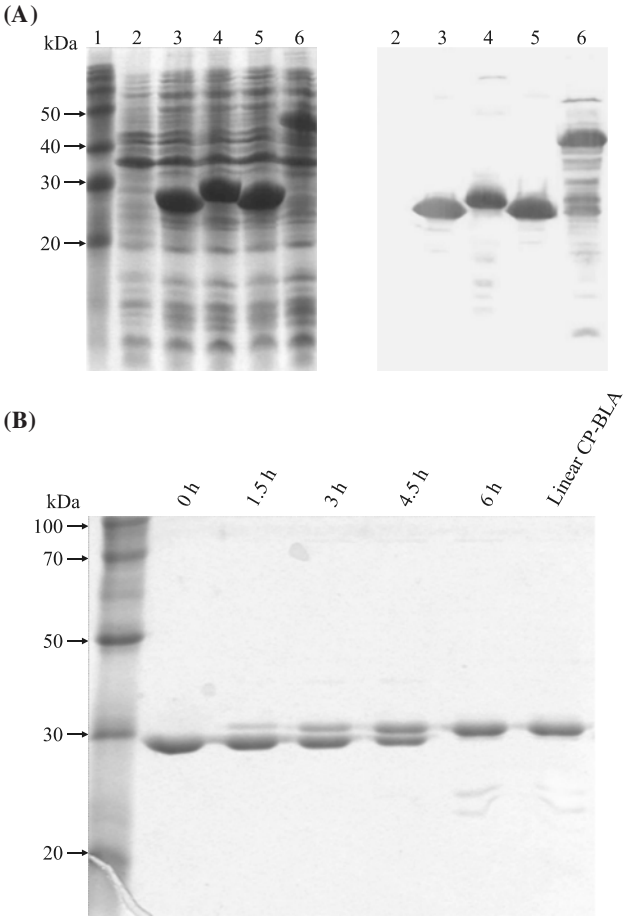

Fig. 3. Analysis of modified β-lactamases. (A) SDS-PAGE and Western blot analysis of the expressed proteins. Lane 2, cell lysate of an *E. coli* strain harboring a control plasmid. Lanes 3~6, cell lysates from *E. coli* strains harboring pWT-BLA (lane 3), pCP-BLA (lane 4), pIns-BLA (lane 5) or pBB-BLA (lane 6). (B) Analysis of *in vivo* cyclization. Purified cyclic β-lactamase (25 μg) was treated with the factor Xa (0.5 μg) at 23°C. Samples were withdrawn after the indicated times and resolved by 15% SDS-PAGE.

color change, which indicates that the circularly permuted β-lactamase had no β-lactamase activity. However, the cyclic β-lactamase expressed from pBB-BLA restored β-lactamase activity, although weaker than that of the wild type β-lactamase (Fig. 4A). Western blot analysis showed that the expression levels of all modified β-lactamases were similar in the soluble fraction of the cell lysates (Fig. 4B).

The β-lactamase activity of the modified proteins by zymogram analysis was further tested. The zymogram analysis of the crude extracts, performed after renaturing SDS-PAGE with the nitrocefin chromogenic substrate, revealed that the wild type β-lactamase, the linear RESGSIEF-interrupted β-lactamase, and the cyclic β-lactamase yielded a band indicating β-lactamase activity. However, a band signifying β-lactamase activity was not detected for the linear circularly permuted β-lactamase (Fig. 4C). This result is consistent with that obtained by the spectrometric assay. Therefore, it is clear that cyclization of the circularly permuted β-lactamase

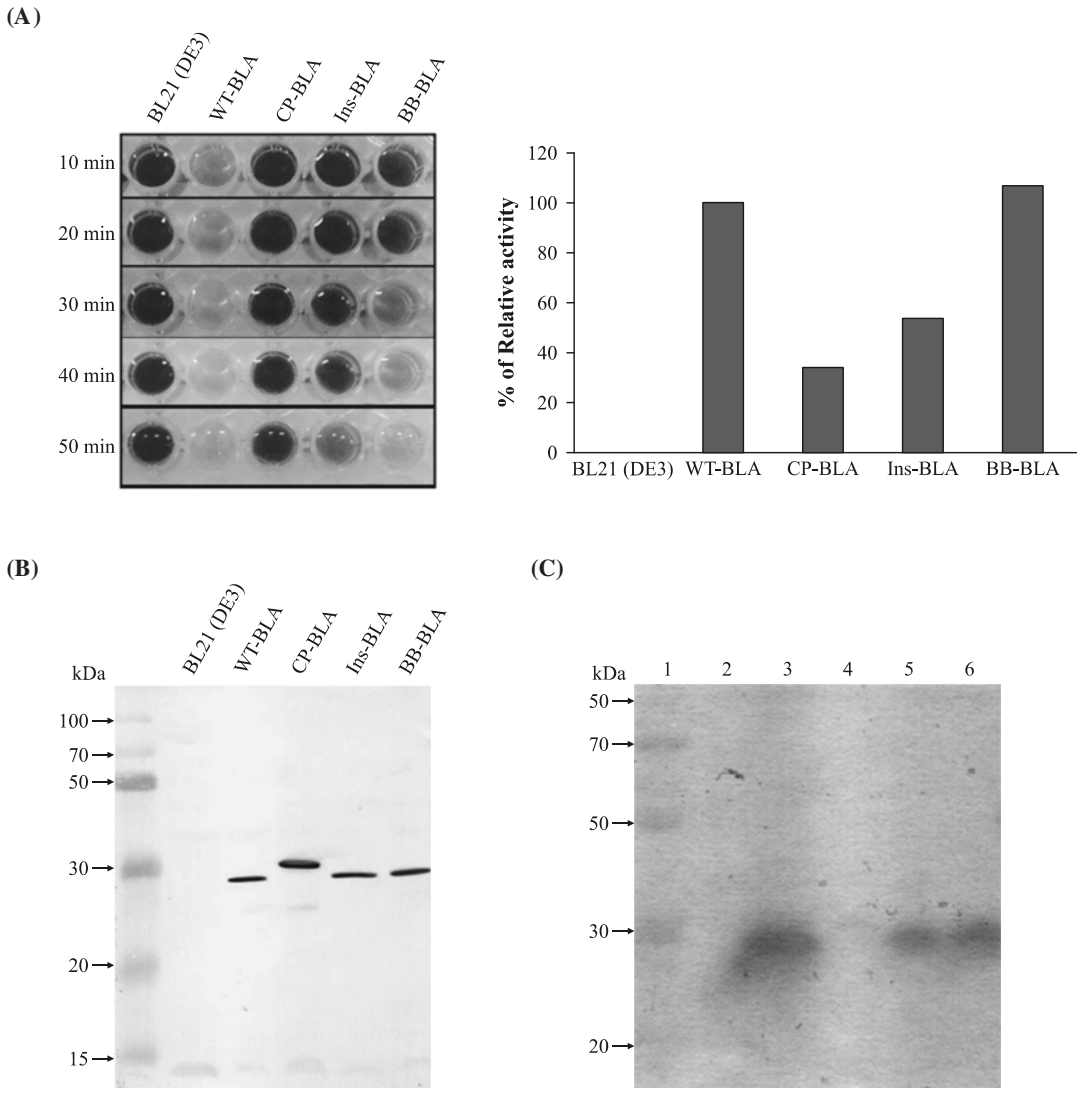

Fig. 4. *In vitro* β-lactamase activity assay. (A) A representative image of the results obtained by the modified acidimetric method. Spectrophotometrical measurements were taken at 240 nm with the 50 min reaction samples. (B) Western blot analysis of the cell lysates used for β-lactamase activity assay in (A). (C) Zymogram analysis. Lane 2, cell lysate of an *E. coli* strain harboring a control plasmid. Lanes 3~6, cell lysates from *E. coli* strains harboring pWT-BLA (lane 3), pCP-BLA (lane 4), pIns-BLA (lane 5), or pBB-BLA (lane 6).

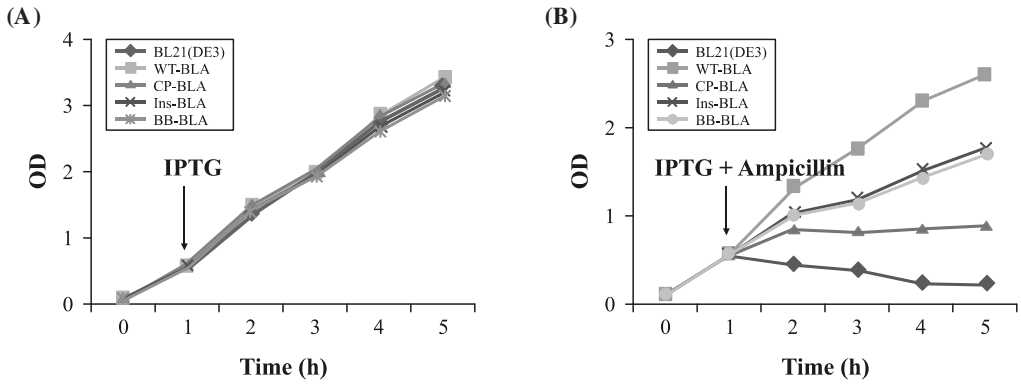

Fig. 5. *In vivo* β-lactamase activity assay. IPTG (0.05 mM) and ampicillin (30 μg/ml) were added to the cultures when cells were grown to OD_{600}=0.6 in LB medium.

through intein splicing can restore β-lactamase activity.

In vivo activity of cyclic β-lactamase

It is thought that cyclic β-lactamase activity *in vivo* protects cell from ampicillin. Therefore, the *E. coli* strain harboring BB-BLA in media containing ampicillin was grown to test this hypothesis. The studies found that the strain expressing the cyclic β-lactamase continued to grow in the presence of ampicillin, which was in contrast with the strains expressing the linear circularly permuted β-lactamase (Fig. 5). The growth patterns of the strains expressing the other modified β-lactamases in ampicillin medium were generally in agreement with the results obtained from the *in vitro* analysis. Collectively, these results demonstrate that the circularly permuted β-lactamase developed in this study can be cyclized if it is flanked by an appropriate split intein and the resulting cyclic β-lactamase will be active against ampicillin. Therefore, the circularly permuted β-lactamase can be used to develop new split inteins for efficient *in vivo* cyclization of proteins or peptides.

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