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Involvement of β-Lactamase Inhibitory Protein, BLIP-II, in Morphological Differentiation of *Streptomyces exfoliatus* SMF19

Kim, Eun Sook[1], Ju Yeon Song[1], Dae Wi Kim[1], Eun Ji Ko[1], Susan E. Jensen[2], and Kye Joon Lee[1]*

[1]*School of Biological Sciences, Seoul National University, Seoul 151-747, Korea*
[2]*Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada*

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The β-lactamase inhibitory protein, BLIP-II, found in the culture supernatant of *Streptomyces exfoliatus* SMF19, shows no discernible sequence identity with other β-lactamase inhibitory proteins identified in *Streptomyces* spp. A null mutant of the gene encoding BLIP-II (*bliB::hyg*^{r}) showed a bald appearance on solid media. Although BLIP-II was initially isolated from the supernatant of submerged cultures, sites of BLIP-II accumulation were seen in the cell envelope. Mutation of *bliB* was also associated with changes in the formation of septa and condensation of the chromosomal DNA associated with sporulation. The *bliB* mutant exhibited infrequent septa, showing dispersed chromosomal DNA throughout the mycelium, whereas the condensed chromosomes of the wild-type were separated by regularly spaced septa giving the appearance of a string of beads. Therefore, on the basis of these results, it is suggested that BLIP-II is a regulator of morphological differentiation in *S. exfoliatus* SMF19.

Keywords: *Streptomyces exfoliatus* SMF19, BLIP-II, morphological differentiation

The widespread use of β-lactam antibiotics has led to the evolution of β-lactamase-mediated resistance in bacteria, which is now a serious threat to antibiotic therapy [16, 19]. Thus, new β-lactam antibiotics that are not susceptible to β-lactamases, and small-molecule β-lactamase inhibitors, such as clavulanic acid [2], thienamycin [7], and olivanic acid [3], have been developed as an effective way to overcome this problem. Large-molecule β-lactamase inhibitors have also been investigated following the initial discovery of a β-lactamase inhibitory protein (BLIP) in *S. clavuligerus* [5, 20, 26]. Two different β-lactamase inhibitory proteins, abbreviated as BLIP-I and BLIP-II, were also identified in the culture supernatant of *S. exfoliatus* SMF19 [8, 11, 12, 18]. Although BLIP-I shows a high similarity to the BLIP of *S. clavuligerus* [8], the structure of BLIP-II is unrelated. Instead, BLIP-II is a rare example of a prokaryotic protein with a very similar structure to that of the regulator chromosome condensation factor family (RCC1) of proteins [13, 18].

Small-molecule β-lactamase inhibitors have relatively poor inhibitory activity, and their usefulness is now threatened by the emergence of pathogens producing inhibitor-resistant β-lactamases [1, 27]. However, BLIPs show exceptionally potent inhibitory activity against β-lactamases, with Ki values in the nano- to picomolar range. Understanding the mechanism of interaction between BLIPs and β-lactamases at the molecular level could provide a basis for the design of novel β-lactamase inhibitors to fight pathogens resistant to β-lactam antibiotics [13, 21, 22, 28].

Accordingly, this study investigated the physiological roles of BLIP-II, and found that a BLIP-II-deficient mutant of *S. exfoliatus* SMF19 exhibited defects in sporulation and septum formation, including a failure to undergo nucleoid condensation and BLIP-II accumulation in the cell envelope. Based on these data, it is proposed that BLIP-II is involved in morphological differentiation.

Materials and Methods

Microorganisms

S. exfoliatus SMF19 [11] was used as the wild-type strain producing BLIP-II. A null mutant deficient in BLIP-II (SMF1902) was also constructed as reported previously [13]. The strains were cultured on sporulation media (Bennett), and the resulting spores were collected and stored in 20% (v/v) glycerol at −70°C [10].

Microscopy

The colonies growing on an MM containing 2% glucose (MMG) agar medium (20 g glucose, 2 g NH_4Cl, 10 g MOPS, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $CaCl_2$, 0.02 g $ZnSO_4$, 0.007 g $FeSO_4$, 0.002 g $MnCl_2$, 0.004 g $CuSO_4$, and 0.004 g $CoCl_2$ per liter, pH 7.2) [8] were observed using a Zeiss Stemi 2000-C microscope. Phase contrast and fluorescence microscopy of solid-medium-grown cultures were conducted using sterile

*Corresponding author
Phone: 82-2-880-1382; Fax: 82-2-882-9956;
E-mail: lkj12345@snu.ac.kr

glass coverslips inserted at a 45° angle into MMG agar plates that had been inoculated with spores and mycelia of the *S. exfoliatus* strains [10]. For the fluorescence microscopy to visualize peptidoglycan, the mycelia grown on glass coverslips at 30°C for 48 h were fixed by overlaying 4 ml of 2.8% paraformaldehyde and 0.0045% glutaraldehyde in phosphate-buffered saline (PBS) on the coverslips and incubating for 15 min at 21°C. The fixative was then aspirated off and each slide was gently washed twice with PBS and allowed to dry thoroughly. The cells were rehydrated with PBS for 5 min and then treated with lysozyme at 2 mg/ml in GTE (50 mM glucose, 20 mM Tris-Cl, pH 8.0, 10 mM EDTA) for 1 min. After aspirating off the lysozyme solution, the samples were washed with PBS and then blocked in 2% bovine serum albumin (BSA) in PBS for 5 min. The treated samples were stained with fluorescein-conjugated wheat germ agglutinin (WGA; Texas Red-X, Molecular Probes) at 5 μg/ml for 1 h for visualization of the cell wall [25]. After staining and washing five times with PBS, the ProLong Gold anti-fade medium (Molecular Probes) was added to the specimens. Coverslips were then mounted and the samples werecured for 24 h and examined under a Carl Zeiss Axiovert 200 M fluorescent microscope.

The sites in the mycelia where BLIP-II accumulated were visualized by immunofluorescence microscopy, as reported by Schwedock *et al.* [25] with slight modifications. The mycelia formed on the glass coverslips were treated as described above for staining with Fluo-WGA, except that the cells were fixed for 30 min with 4% (w/v) ice-cold glutaraldehyde in PBS and then exposed to lysozyme treatment for 15 min before washing and blocking with BSA. The cells were then incubated with mouse anti-BLIP-II polyclonal antibodies (diluted 1/500 [v/v] in a blocking buffer) for 1 h at 4°C. The polyclonal antibodies for BLIP-II were prepared by injecting purified BLIP-II into mice, as reported previously [18]. The glass coverslips were washed three times for 5 min each with ice-cold PBS and then incubated for 3 h at 4°C with the secondary antibody, goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC), which was diluted 1/100 in a blocking buffer. The coverslips were then washed three times with ice-cold PBS and mounted on glass slides using the ProLong Gold anti-fade medium. Confocal images were acquired using a Bio-Rad confocal microscope optimized for fluorescence. The samples were also stained with 1 mg/ml of propidium iodide (PI) to visualize the DNA.

For the scanning electron microscopy, the colonies formed on the plates of MMG were processed and examined as previously described [13]. For the transmission electron microscopy, the colonies grown on

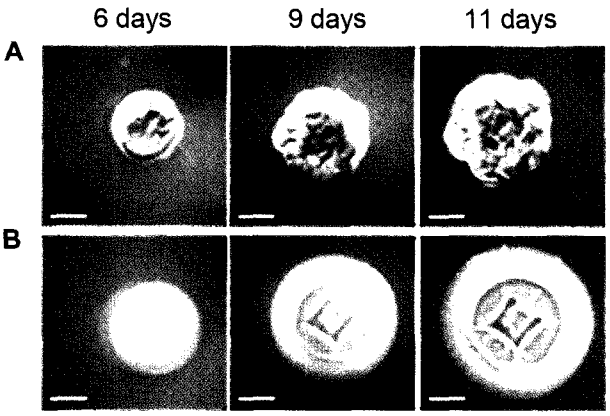

Fig. 1. Morphological comparison of *S. exfoliatus* SMF19 (wild type) and SMF1902 (*bliB* mutant).
S. exfoliatus SMF19 (**A**) and SMF1902 (**B**) were cultured on MMG. Size bars correspond to 2 mm.

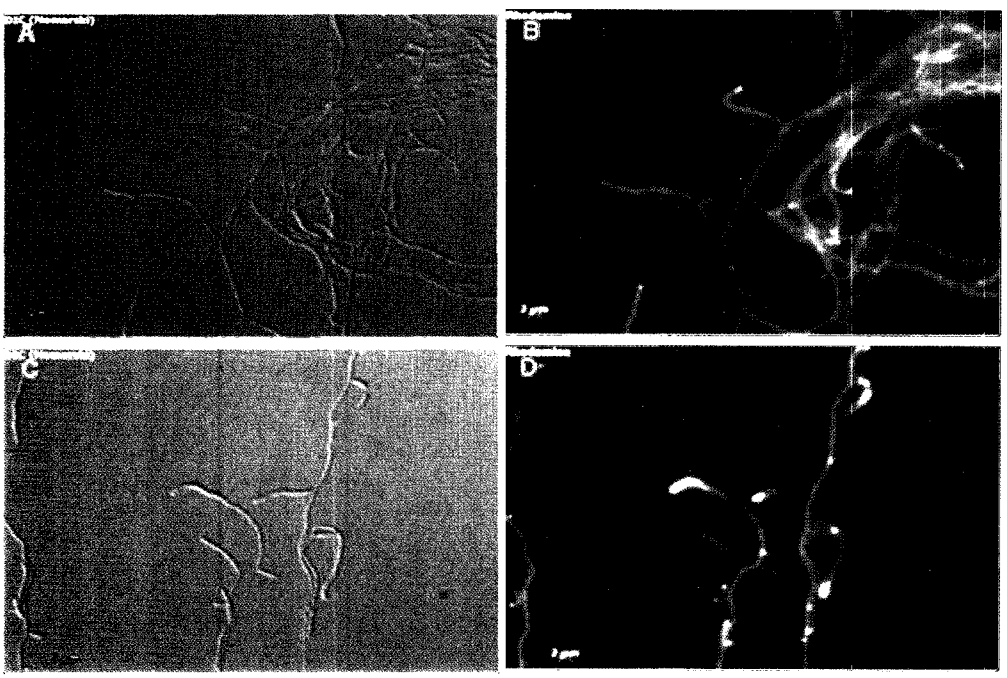

Fig. 2. Microscopic appearance of *S. exfoliatus* SMF19 (wild type) and SMF1902 (*bliB* mutant).
SMF19 and SMF1902 were cultured on MMG for 2 days. The mycelia formed on glass coverslips were fixed with 2.8% paraformaldehyde and 0.0045% glutaraldehyde in PBS, and then washed with PBS and stained with WGA. **A** and **C**. Phase-contrast images; **B** and **D**. WGA fluorescence images.

the MMG were fixed as previously described [6], stained with 0.5% uranyl acetate citrate, and embedded in resin. The blocks were sectioned with a microtome, and then mounted on copper grids and restained with 2% uranyl acetate and lead citrate for 5 min each. Finally, the sections were examined under a Carl Zeiss LIBRA 120 transmission electron microscope.

Results

Mutational Inactivation of BLIP-II has Effects on Mycelium Differentiation

The morphological differentiation of a wild-type strain and *bliB* null mutant (SMF1902) of *S. exfoliatus* SMF19 was compared in cultures growing on MMG agar plates (Fig. 1). The wild-type strain developed aerial mycelia and spores showing the typical growth pattern of *Streptomyces* spp. (Fig. 1A), whereas the *bliB* null mutant demonstrated a "bald" appearance due to the production of substrate mycelia rather than aerial mycelia or spores (Fig. 1B). The growth characteristics of the *bliB* mutant and wild-type strain grown on the surface of glass coverslips were also compared under a phase contrast microscope. The wild-type strain formed large amounts of well-branched mycelia (Fig. 2A), whereas the null mutant mycelia were more sparse with only a few branches (Fig. 2C). Staining with WGA, a stain specific for incompletely cross-linked peptidoglycan, showed that the wild-type strain

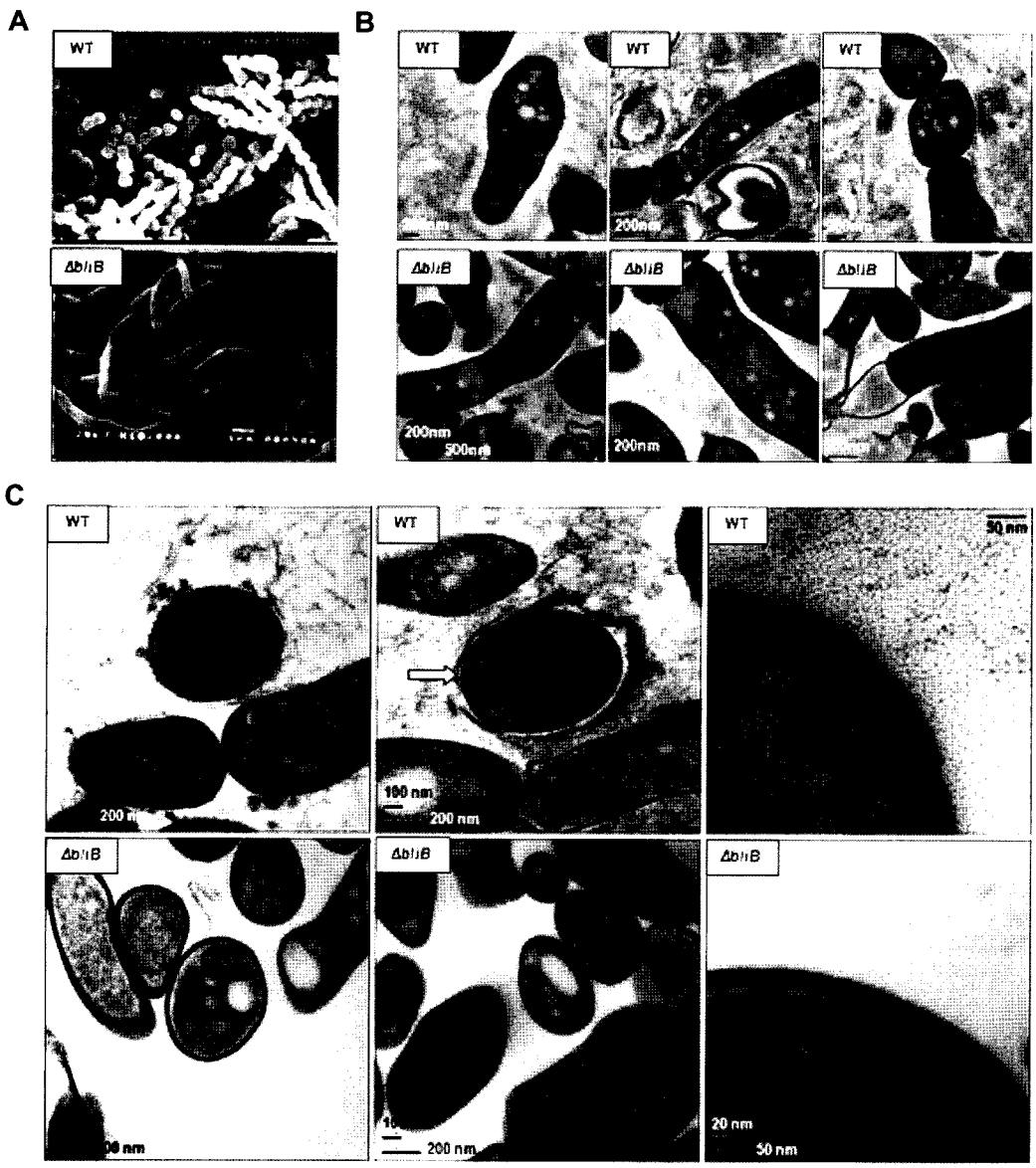

Fig. 3. Electron micrographs of *S. exfoliatus* SMF19 (wild-type) and SMF1902 (*bliB* mutant).
A. Scanning electron micrographs of SMF19 (top) and SMF1902 strains (bottom). **B** and **C**. Transmission electron micrographs of SMF19 (top) and SMF1902 strains (bottom).

formed hyphae with a relatively uniform thickness (Fig. 2B), whereas the hyphae of the *bliB* mutant had somewhat swollen ends that stained strongly (Fig. 2D).

BLIP-II Affects the Formation of Septum and Spores

The morphological differentiation in the wild-type and *bliB* mutant strains was also compared by examining cultures grown on an MMG solid medium for 6 days using both scanning and transmission electron microscopies. Whereas the scanning electron microscopy, abundant spores were observed in the wild-type strain (Fig. 3A top, taken from [13]), while only mycelia and no spores were seen in the *bliB* null mutant (Fig. 3A bottom), confirming the bald phenotype, as shown in Fig. 1. Longitudinal sections of hyphae showed septa in the wild-type strain moving inward from the cell surface to create individual units that eventually separated into chains of spores (Fig. 3B, top). In contrast, septum formation was much less frequent in the *bliB* null mutant and never progressed to separated cells or spores (Fig. 3B, bottom). From cross-sectional views (Fig. 3C), the wall thickness of the hyphae was estimated to be about 20 nm in both the wild-type strain and the *bliB* null mutant (Fig. 3C, left). However, circular forms with thick walls (indicated by arrow in Fig. 3C, top-middle) were also observed in the wild type, but not in the *bliB* null mutant, which represented spores rather than hyphae. The wild-type spores measured about 700–1,000 nm in diameter, and the thickness of the spore cell wall was 80–100 nm (Fig. 3C, right).

BLIP-II Accumulates in the Cell Envelope

Although BLIP-II was originally isolated as an extracellular product, the BLIP-II antigen was seen by fluorescence microscopy as a weak signal evenly distributed over the hyphae, with stronger signals across the septal regions of developing spore chains in the wild-type strain (Fig. 4A). No staining was detected in the mycelia of the *bliB* null mutant (Fig. 4B). The chromosomal DNA was also visualized in the same samples based on a reaction with propidium iodide, and in the developing spore chains of the wild-type strain was observed to be tightly condensed to give the appearance of strings of red-stained beads regularly separated by green-stained septa (Fig. 4A). Meanwhile, in the *bliB* null mutant, no spore septa or chains were evident, and the chromosomal DNA was dispersed throughout the mycelium in an uncondensed state, typical of vegetative mycelia (Fig. 4B).

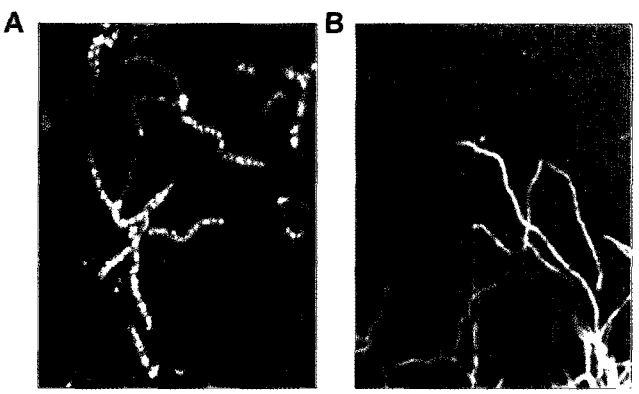

Fig. 4. Cellular location of BLIP-II.
S. exfoliatus SMF19 (**A**) and SMF1902 (**B**) were cultured on glass coverslips. After fixation, the cells were treated with a polyclonal antibody against BLIP-II, and FITC secondary antibody (green). To visualize the DNA, the cells were stained with 1 mg/ml of propidium iodide (PI) (Red). The images were acquired using a BIO-Rad confocal microscope with fluorescence.

DISCUSSION

The BLIP-II protein has a seven-bladed β-propeller structure with a unique blade motif consisting of three antiparallel β-strands. The overall fold is highly similar to the core structure of the human regulator of chromosome condensation (RCC1), although BLIP-II only shares a low sequence identity (21%) with RCC1 [13, 18]. The RCC1 family of proteins is found exclusively in the nuclei of eukaryotes, where they act as the guanine nucleotide exchange factor (GEF) for the GTPase, Ran [4, 14, 15, 17, 24]. BLIP-II is the first protein characterized from prokaryotic cells with this unique protein structure, suggesting that the BLIP-II protein is evolutionarily related to the RCC1 family [13]. In view of the differences observed in the arrangement of the chromosomal DNA in the wild-type and *bliB* mutant strain, this similarity of BLIP-II to RCC1 family proteins was of particular interest. Although the failure of chromosomal DNA condensation in the *bliB* mutant may just have been a secondary effect of the bald phenotype, it is also possible that BLIP-II could be involved in the regulation of intracellular processes.

This study demonstrated that the production of BLIP-II was closely associated with morphological differentiation in *S. exfoliatus* SMF19 (Figs. 1–3). Furthermore, BLIP-II was also clearly associated with the cell envelope (Fig. 4). The localization of BLIP-II in the septum, as well as the lateral cell wall, may have reflected its binding to enzymes related to septum biosynthesis, thereby interfering with morphological differentiation, particularly at the stage of the emergence of aerial hyphae and consequently spore formation (Fig. 4). The enzymes involved in peptidoglycan biosynthesis, such as transpeptidases and carboxypeptidases, are commonly referred to as penicillin-binding proteins (PBPs) and are evolutionarily related to β-lactamases [9, 23]. Since BLIP-II is a protein that inhibits β-lactamase, it may normally interact with these enzymes to regulate mycelium development in the producer organism. To investigate this further, the screening of two yeast hybrid was conducted to identify the molecules that interacted with BLIP-II. A cDNA library from *S. coelicolor*, rather than *S. exfoliatus*, was used for these experiments, as the unsequenced genome of *S. exfoliatus* would make the

subsequent characterization of any interacting partners difficult, and since it was anticipated that enzymes, such as transpeptidases and carboxypeptidases, would interact with BLIP-II. These types of enzyme are highly conserved across *Streptomyces* spp. However, the screening revealed that BLIP-II interacted strongly with SCO4677, a 144 amino acid putative regulatory protein from *S. coelicolor* (data not shown), thereby raising the possibility that BLIP-II may be involved in the regulation of intracellular processes.

Therefore, when taken together, the present results suggest that BLIP-II in *S. exfoliatus* SMF19 may exist in the cytoplasm for some period before export and thereby regulate certain intracellular processes, such as septum and aerial hyphae formation, through interaction with proteins similar to SCO4677.

Acknowledgments

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