

Investigation of the in vivo interaction between β -lactamase and its inhibitor protein

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Abstract: The affinity of β -lactamase inhibitory protein (BLIP) for TEM-1 β -lactamase has raised hopes in the challenge of protein-based inhibitor discovery for β -lactamase-mediated antibiotic resistance. Currently, the effect of the formation of the β -lactamase:BLIP complex in vivo in β -lactam resistant bacteria is an open question. The scarcity of information to the extent to which BLIP can impair β -lactamase activity inside cells has urged us to assess the in vivo efficacy of BLIP as a potent β -lactamase inhibitor. To this end, β -lactamase and BLIP were coexpressed in *Escherichia coli*. Simultaneous expression of β -lactamase and BLIP and the formation of the TEM-1 β -lactamase:BLIP complex in the periplasmic space of *E. coli* were verified by electrophoretic and Western blot techniques. Growth profiles of the cells expressing both β -lactamase and its protein inhibitor, complemented with β -lactamase activity measurements, suggested that BLIP synthesis retarded cell growth and reduced β -lactamase activity. Although co-expression of β -lactamase and its protein inhibitor did not completely impair cell growth, the specificity of BLIP enabled it to bind β -lactamase in the bacterial periplasm, regardless of the crowding components.

Key words: β -lactamase, antibiotic resistance, BLIP

1. Introduction

β -lactam antibiotics hold great importance in healing diverse bacterial infections. The function of these wonder drugs is to block the synthesis of the bacterial cell wall by binding the periplasmic bacterial transpeptidases (also known as penicillin-binding proteins, PBPs) that are responsible for the formation of oligopeptide cross-links in the peptidoglycan layer of the bacterial cell wall. The interruption of the peptidoglycan formation results in loss of cell wall integrity and ultimately the cells die. Unfortunately, the irresponsible use of these antibiotics has led to a rise in the number of antibiotic resistant bacteria (Essack, 2001; Majiduddin et al., 2002; Adelowo and Fagade, 2012; Chanda et al., 2013). One mechanism by which these bacteria acquire resistance is by β -lactamases. The production of this enzyme renders the antibiotic ineffective by hydrolyzing the amide bond of the β -lactam ring before the antibiotic can bind PBPs (Frère, 1995; Matagne et al., 1998). The rapid transfer of the β -lactamase gene between organisms via plasmids is a major clinical problem since it contributes to an increase in the number of resistant bacteria (Blazquez et al., 1993).

TEM-1 β -lactamase is one of the most prevalent plasmid-encoded β -lactamases and its presence constitutes the main reason for antibiotic resistance in gram-negative bacteria. This periplasmic enzyme retains hydrolytic activity against a broad range of antibiotics (Huang et al., 1996). One strategy to counteract β -lactamase-mediated antibiotic resistance is combination therapies of β -lactam antibiotics with β -lactamase inhibitors such as clavulanic acid (Martinez et al., 1987). These combination therapies have proven to be effective in combating antibiotic resistance; however, bacteria continue to evolve their defense mechanisms against available therapies by producing inhibitor resistant β -lactamases (Blazquez et al., 1993). In this context, protein-based inhibitors hold great promise since they interact with the target proteins with high affinity.

One protein inhibitor of β -lactamases that has been extensively studied is the β -lactamase inhibitory protein (BLIP), isolated from *Streptomyces clavuligerus* (Doran et al., 1990). BLIP is a 17.5 kDa protein that binds TEM-1 β -lactamase and hinders its hydrolytic activity (Strynadka et al., 1994). The well-established affinity between TEM-1

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β -lactamase and BLIP has been the focus of a number of recent studies to design new tools. Using this interaction, Yuan et al. (2009) proposed a genetic screen for BLIP function, Hanes et al. (2010) developed a simple and robust pulse proteolysis method focusing on binding affinity, and Fryszczyn et al. (2011) offered an improvement in phage display technology. FRET-based techniques that utilize the TEM-1 β -lactamase:BLIP interaction were used to measure binding in the cytoplasm of HeLa cells to test protein association in a crowded environment, and for high-throughput analysis of protein-protein interactions (Khait and Schreiber, 2012; Phillip et al., 2012a, 2012b). The TEM-1:BLIP interface served as a model in the development of methods for predicting protein-protein interactions and for protein docking (Strynadka et al., 1994; Robert and Janin, 1998).

Furthermore, the interaction of BLIP with TEM-1 guided BLIP based peptide design efforts (Huang et al., 2003; Phichith et al., 2010; Alaybeyoglu et al., 2015). Even though these peptides promise great potential as drugs and easier cell penetration than a protein, their inhibition constant values remain at around 100 micromolars, which is too low for clinical studies. The delivery of proteins inside the cell remains a huge obstacle in the design of protein drugs. However, the inhibition constant of BLIP is 0.3 nM (Rudgers et al., 2001), which makes it is an attractive protein for future drug design and delivery efforts.

In an effort to describe and characterize the in vivo behavior of the TEM-1:BLIP interaction, the two proteins were expressed simultaneously in *E. coli* and the extent to which this interaction affected cell viability was investigated. The key objectives of this study were to: 1) demonstrate the binding between β -lactamase and

BLIP in the periplasmic extract; 2) achieve simultaneous β -lactamase and BLIP synthesis; and 3) evaluate the effect of the in vivo interaction between β -lactamase and BLIP on cell viability. The findings obtained in this study contribute to a better understanding of the in vivo inhibition of β -lactamase by BLIP and open up a new perspective for investigating the potential BLIP derived inhibitors binding to β -lactamase.

2. Materials and methods

2.1. Chemicals

The chemicals and solutions used in this study were purchased from Applichem (Germany), Merck (Germany), Molekula (Germany), Sigma (USA), and Roche (Germany). Ampicillin and kanamycin were obtained from Sigma (USA). Protein size markers were supplied by Fermentas (USA).

2.2. Bacterial strains and plasmids

E. coli BL21(DE3) strain, used for simultaneous expression of β -lactamase and BLIP, was obtained from TÜBİTAK-GEPI. This strain is compatible with the pET protein expression vectors that are under the control of the T7 promoter. The strain used for purification of β -lactamase was *E. coli* TB1 (from our laboratory stock). Plasmids pUC18 and pET-26SJ were used as expression vectors for the synthesis of β -lactamase and BLIP, respectively. The pUC18 plasmid carried the *bla* gene of TEM-1 β -lactamase. The pET-26SJ plasmid was constructed by inserting the BLIP *bli* gene with its native leader sequence into pET-26b(+). The pET-26b(+) was used in the control cells.

E. coli strains and plasmids used in this study are summarized in Table 1.

Table 1. *E. coli* strains and plasmids used in this study.

<i>E. coli</i> strain	Feature	Recombinant protein/s synthesized	Reference
<i>E. coli</i> -Pu	<i>E. coli</i> BL21(DE3) with pUC18	β -lactamase	This study
<i>E. coli</i> -Pet	<i>E. coli</i> BL21(DE3) with pET-26b(+)	No β -lactamase and BLIP production	This study
<i>E. coli</i> -Sj	<i>E. coli</i> BL21(DE3) with pET-26SJ	BLIP with native leader signal sequence	This study
<i>E. coli</i> -PuPet	<i>E. coli</i> BL21(DE3) with pUC18 & pET-26b(+)	β -lactamase	This study
<i>E. coli</i> -PuSj	<i>E. coli</i> BL21(DE3) with pUC18 & pET-26SJ	β -lactamase and BLIP with native signal sequences	This study
<i>E. coli</i> TB1-Pu	<i>E. coli</i> TB1 with pUC18	β -lactamase	This study
Plasmid	Features (Resistance marker)	Expressed gene/s	Reference
pUC18	Cloning vector (ampicillin)	<i>bla</i> _{TEM-1} gene encoding β -lactamase	Lab stock
pET-26b(+)	Cloning vector (kanamycin)	No expression of <i>bla</i> _{TEM-1} and <i>bli</i> genes	Lab stock
pET-26SJ	<i>bli</i> gene from <i>Streptomyces clavuligerus</i> cloned into pET-26b(+) (kanamycin)	<i>bli</i> gene encoding BLIP with its native eader signal sequence	SJ*

* Plasmid pET-26SJ was a kind gift from Susan Jensen (University of Alberta)

2.3. Cell growth

A single colony of *E. coli* was cultured in 5 mL of LB medium (per liter: 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) at 37 °C and 180 rpm. The medium was supplemented with 100 µg/mL of ampicillin and 50 µg/mL of kanamycin to retain plasmids pUC18 and pET-26b(+)/pET-26SJ, respectively. The preculture, grown to an OD₆₀₀ of 0.7, was used to inoculate fresh LB medium. Cells were incubated at 37 °C and 180 rpm. Cell growth was monitored by measuring OD₆₀₀. The values obtained from triplicate readings were averaged to analyze cell growth profiles. Culture volume was always kept at one fifth of the flask volume for proper aeration.

2.4. Production of β-lactamase and BLIP

Synthesis of β-lactamase and BLIP was achieved at 37 °C. β-lactamase was constitutively expressed in LB medium with 100 µg/mL ampicillin by *E. coli* cells harboring the pUC18 plasmid. For purification of β-lactamase, *E. coli*-Pu cells were grown to a final OD₆₀₀ of 1.50 at 37 °C. BLIP was expressed in LB medium supplemented with 50 µg/mL kanamycin by *E. coli* cells harboring the pET-26SJ plasmid. Expression was induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when the absorbance of cells reached 0.5 at 600 nm. Cells were allowed to grow for 3 h after induction.

2.5. Extraction of periplasmic proteins

Periplasmic β-lactamase and BLIP were extracted from *E. coli* cells by the osmotic shock procedure (Nossal and Heppel, 1966). Cells were centrifuged at 4 °C and 7000 rpm and then resuspended in 20% sucrose solution prepared in 30 mM Tris-HCl (pH of 8.0) with 1 mM ethylenediaminetetraacetic acid (EDTA). Following incubation at room temperature for 20 min, cells were centrifuged at 9000 rpm. The pellet was resuspended in 5 mM ice-cold MgCl₂ and gently incubated for 20 min. Cell debris was removed from the periplasmic protein extract by centrifugation at 9000 rpm.

Total protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard.

2.6. Purification of β-lactamase

Periplasmic protein extract containing β-lactamase was dialyzed against 10 mM Tris-HCl buffer (pH 7.0) and then concentrated by ultrafiltration through a polyethersulfone membrane (Amicon) with a 10,000 molecular weight cut-off. The concentrated protein extract was applied to a DEAE-cellulose column equilibrated with 10 mM Tris-HCl buffer (pH 7.0). Elution was achieved with 150 mM Tris-HCl buffer (pH 7.0). Fractions with β-lactamase activity were pooled and the purity of the enzyme was verified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Mass spectrometric analysis of β-lactamase

The β-lactamase band was excised from the Coomassie Brilliant Blue stained gel and mass spectrometric analysis of the protein was performed as previously described (Shevchenko et al., 2007; Ozbalci et al., 2010).

2.8. Measurement of β-lactamase activity

The β-lactamase activity was measured by monitoring the hydrolysis of its substrate CENTA (Calbiochem, Cat. No. 219475). Upon CENTA hydrolysis, the color of the reaction mixture turned from yellow to chrome yellow (Jones et al., 1982; Bebrone et al., 2001). This change was followed by measuring the absorbance at 405 nm.

The reaction was carried out at 25 °C in a total volume of 1 mL in 50 mM potassium phosphate buffer (pH 7.0) with 47 µM CENTA. For the inhibition experiments, periplasmic protein extract containing BLIP was added to the assay mixture and the mixture was incubated for 1 h at 37 °C prior to activity measurement. One Unit (U) of β-lactamase activity was defined as the amount of enzyme that hydrolyzed 1 µmol of CENTA per minute at 25 °C.

Protein bands from native electrophoretic gels were assayed for β-lactamase activity after the gel pieces were cut into small pieces. The gels were mixed with 47 mM CENTA in potassium phosphate buffer (pH 7.0) in a total volume of 1 mL. This mixture was incubated for 2 days at 25 °C. Changes in the color of the mixture were monitored spectrophotometrically.

2.9. Measurement of cell viability

Viabilities of induced and uninduced cultures of *E. coli*-PuPet and *E. coli*-PuSj were measured after induction with IPTG. Bacterial samples from actively growing cultures were plated on LB plates with serial dilution. Colonies formed on the plates were counted after an overnight incubation at 37 °C. The number of colonies obtained from triplicate experiments was averaged to get the number of colony forming units (CFU).

2.10. Electrophoretic analysis of proteins

2.10.1. Denaturing gel electrophoresis

SDS-PAGE was used to analyze periplasmic proteins in denatured form according to the method of Laemmli (1970). Gels were stained with Coomassie G-250 dye protocol to visualize the proteins (Blakesley and Boezi, 1977).

2.10.2. Native gel electrophoresis

Non-denaturing gel electrophoresis was used to confirm the presence of the β-lactamase:BLIP complex. Extracted periplasmic proteins were mixed with a sample buffer without SDS and then loaded to the non-denaturing gels (12.5% gels without SDS).

2.11. Western blot analysis of proteins

Electrophoretic transfer of native polyacrylamide gel resolved proteins onto the PVDF membrane was carried

out by semidry electroblotting. The unoccupied sites on the PVDF membrane were blocked with 5% (w/v) nonfat milk powder for 2 h at room temperature. The membrane was washed three times for 10 min, each with PBS-Tween, and then incubated with β -lactamase mouse anti-*E. coli* monoclonal antibody (LSBio, Cat. No. LS-C74803) overnight at 4 °C. This membrane was again washed three times with PBS-Tween and then incubated for 2 h with donkey anti-mouse-IgG-HRP conjugate (Santa Cruz Biotechnology, Cat. No. sc-2318). Antibody detection of β -lactamase was accomplished by the ECL method (Western Blotting Detection Reagents, Amersham).

3. Results and discussion

3.1. β -Lactamase and BLIP synthesis

The first part of this work involved the analysis of the expression of TEM-1 β -lactamase and BLIP in separate hosts.

The β -lactamase synthesized in *E. coli*-Pu and *E. coli* TB1-Pu cells was extracted from the periplasm using the osmotic shock procedure. Periplasmic extracts from *E. coli* BL21(DE3) or *E. coli* TB1 cells were used as controls. Figure 1 shows the electrophoretic analysis of the extracted periplasmic proteins from *E. coli* TB1 and *E. coli* TB1-Pu cells on SDS-PAGE. The 28.9 kDa protein band corresponding to the mature β -lactamase was visualized only in the periplasmic extracts of β -lactamase expressing cells.

The periplasmic extract was assayed for β -lactamase activity and its yield in the crude extract was measured as 148.5 U/L culture. No measurable activity was detected

in the cytoplasmic fractions or in the culture filtrates. In order to confirm that the 28.9 kDa band visualized on the electrophoretic gels was the protein possessing β -lactamase activity, β -lactamase was further purified from the periplasmic extract. To this end, the crude periplasmic extract was applied to a DEAE-cellulose column. Electrophoretic analysis of the pooled fractions from the column possessing β -lactamase activity revealed a single protein band of 28.9 kDa (Figure 2). The estimated specific activity, yield, and purification fold of β -lactamase before and after purification are summarized in Table 2. For β -lactamase, 5.1-fold purification was achieved with a yield of 21%. Total protein yield was 0.12 mg of β -lactamase from 1 L of culture. Following tryptic digestion, mass spectrometric analysis of the purified protein band returned 10 peptides matching the TEM-1 β -lactamase. The results of the mass spectrometric analysis are summarized in Table 3.

Periplasmic BLIP was expressed by *E. coli*-Sj cells. Electrophoretic analysis of the periplasmic extract obtained from these cells is presented in Figure 3, Lane 4. Upon induction with IPTG, cells expressed a protein with a molecular weight of 17.5 kDa. This protein band is missing in the absence of induction, suggesting that the band corresponds to BLIP. The native signal peptide allowed its transport to the periplasm.

3.2. In vitro inhibition of β -lactamase with BLIP

In order to test whether the synthesized β -lactamase would be inhibited by the synthesized BLIP, β -lactamase activity was measured in the absence and in the presence of the inhibitor. Keeping the β -lactamase amount

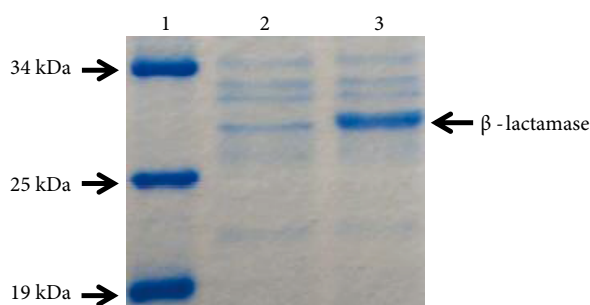


Figure 1. SDS-PAGE analysis of periplasmic protein extracts of the cells. Lane 1: Protein size marker (34 kDa/25 kDa/19 kDa); Lane 2: Periplasmic proteins obtained from *E. coli* TB1 cells; Lane 3: Periplasmic proteins obtained from *E. coli* TB1-Pu cells.

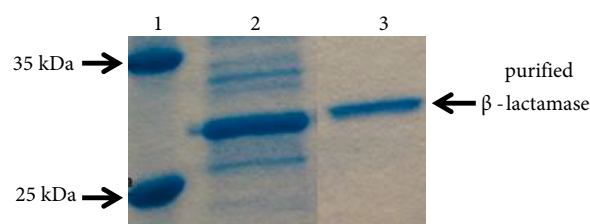


Figure 2. Analysis of the purified β -lactamase on SDS-PAGE. Lane 1: Protein size marker (34 kDa/25 kDa); Lane 2: Periplasmic proteins after ultrafiltration; Lane 3: β -lactamase eluted from a DEAE-cellulose column with 150 mM Tris-HCl buffer (pH 7.0).

Table 2. Summary for β -lactamase purification from *E. coli* TB1-Pu cells.

	Specific activity (U/mg protein)	Yield (%)	Fold
Crude extract	50.8	100	1.0
Purified enzyme	256.9	21	5.1

Table 3. Mass spectrometric analysis for β -lactamase.

SwissProt accession number	Protein name	Molecular weight	Matched peptide sequences
BLAT_ECOLX	Beta-lactamase TEM	31495	VKDAEDQLGAR/ VGYIELDLNSGK/ ILESFRPEER/ VDAGQEQLGR/ IHYSQNDLVEYSPVTEK/ LLTGELLTLASR/ QQLIDWMEADKVGAPLLR/ GIIAALGPDGKPSR/ IVVIYTTGSQATMDER/ QIAEIGASLIK

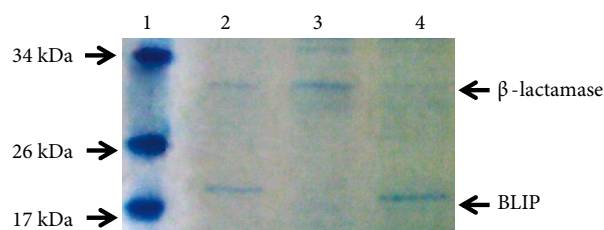


Figure 3. Electrophoretic analysis of periplasmic protein extracts. Lane 1: Molecular weight marker; Lane 2: Protein extract from *E. coli*-PuSj cells induced with 0.2 mM IPTG; Lane 3: Protein extract from uninduced *E. coli*-PuSj cells; Lane 4: Protein extract from *E. coli*-Sj induced with 0.2 mM IPTG.

constant, the amount of BLIP in the reaction mixture was increased. The results presented in Table 4 show that even in the presence of the crowding components such as other proteins and ions in the periplasmic extract, incubation of β -lactamase with BLIP resulted in β -lactamase inhibition and an increase in the BLIP amount increased the extent of β -lactamase inhibition.

Inhibition of β -lactamase by BLIP was further investigated with the purified enzyme. The results demonstrated that pure β -lactamase was more effectively inhibited by BLIP. This could be explained by the absence of the crowding components of the crude periplasmic extract.

3.3. In vivo β -lactamase inhibition by BLIP

3.3.1. Co-expression of BLIP and β -lactamase in *E. coli*-PuSj

Following confirmation of β -lactamase and BLIP expression in separate *E. coli* hosts and demonstration of β -lactamase inhibition by BLIP using crude cellular extracts, simultaneous expression of β -lactamase and its inhibitor protein in *E. coli*-PuSj cells was achieved. Under uninduced conditions, only β -lactamase was expressed in *E. coli*-PuSj cells (Figure 3, Lane 3), while both BLIP and β -lactamase were expressed by the same cells under induced conditions (Figure 3, Lane 2). Electrophoretic analysis also showed that there was no leaky BLIP expression under uninduced conditions and that BLIP synthesis was induced only with the IPTG addition.

3.3.2. β -lactamase and BLIP complex formation in the periplasm

The presence of β -lactamase and its complex with BLIP in the periplasm of *E. coli*-Pu cells and *E. coli*-PuSj cells was analyzed by Western blot analysis (Figure 4). Upon induction, only β -lactamase was present in the periplasmic extracts of *E. coli*-PuPet cells (Figure 4, Lane 1). Two bands were present in the extract of *E. coli*-PuSj cells: one corresponding to β -lactamase and the other corresponding to the size of the complex (Figure 4, Lane 2). This result demonstrates the formation of the BLIP: β -lactamase complex in the *E. coli* periplasm when these two proteins were co-expressed in the same host.

Table 4. Activity of β -lactamase with and without BLIP.

Extract from <i>E. coli</i> -Pu	Extract from <i>E. coli</i> -Sj	% inhibition
7.8 μ g	-	
7.8 μ g	7.8 μ g	69
7.8 μ g	31.2 μ g	90
Purified β -lactamase		
7.8 μ g	7.8 μ g	90

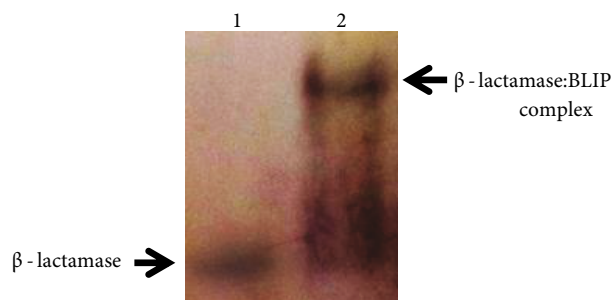


Figure 4. Western Blot analysis of periplasmic extracts from *E. coli*-Pu (Lane 1) and *E. coli*-PuSj cells (Lane 2).

Bands of β-lactamase observed by Western blot in native gels were also assayed, as described in the methods section. Gel pieces containing the enzyme turned the color of the reaction mixture to yellow, whereas the color of the reaction mixture with the control gel pieces remained unchanged.

3.3.3. Effect of periplasmic β-lactamase:BLIP complex formation on cell growth

The formation of the complex was determined by electrophoretic analysis of the periplasmic extract of bacteria co-expressing the two proteins. However, this did not give any clue about the extent of intracellular β-lactamase inhibition by BLIP. Hence, β-lactamase inhibition also required verification. The inhibition of β-lactamase by its inhibitor protein is expected to retard cell growth since β-lactamase that is engaged with an inhibitor will not be able to hydrolyze the β-lactam antibiotic, and this antibiotic will impose its lethal effect on the peptidoglycan layer.

The growth rate of the control *E. coli*-PuPet cells remained unchanged with IPTG induction (Figure 5). The growth rate of *E. coli*-PuSj cells decreased when BLIP synthesis was initiated with IPTG (Figure 6). Under all conditions, there was a constitutive β-lactamase expression in both cells.

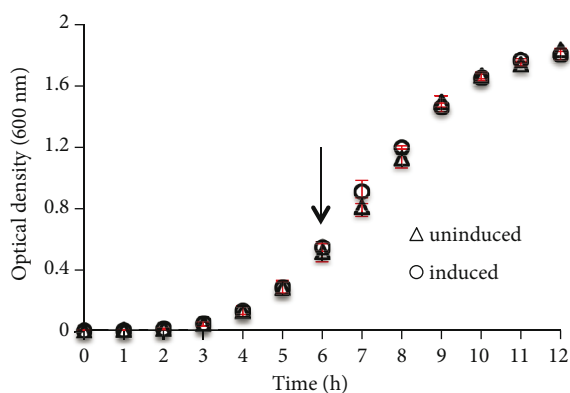


Figure 5. Growth of *E. coli*-PuPet (b) cells. Arrow indicates induction with 0.2 mM IPTG.

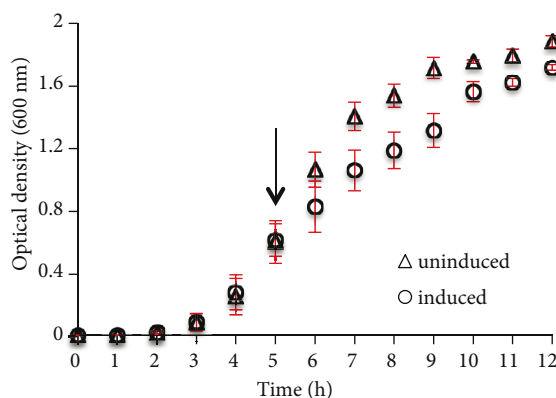


Figure 6. Growth of *E. coli*-PuSj cells. Arrow indicates induction with 0.2 mM IPTG.

The findings from the analysis of growth rates indicated the formation of the β-lactamase:BLIP complex and the inhibition of β-lactamase by BLIP. This should be the reason for the observed decrease in the growth rate.

3.3.4. Effect of periplasmic β-lactamase:BLIP complex on cell viability

Following the growth rate by simply measuring the absorbance can sometimes be misleading since this does not provide any information about cell viability. All medium components, including dead cells and waste products, contribute (along with culturable cells) to the absorbance. To evaluate retardation of cell growth by the β-lactamase:BLIP complex formation, the number of viable cells was quantified on solid media after initiating BLIP synthesis (Figure 7). The viable cell count dropped with the induction of BLIP synthesis and decreased as a function of induction time. This also points to the binding of the inhibitor BLIP to β-lactamase.

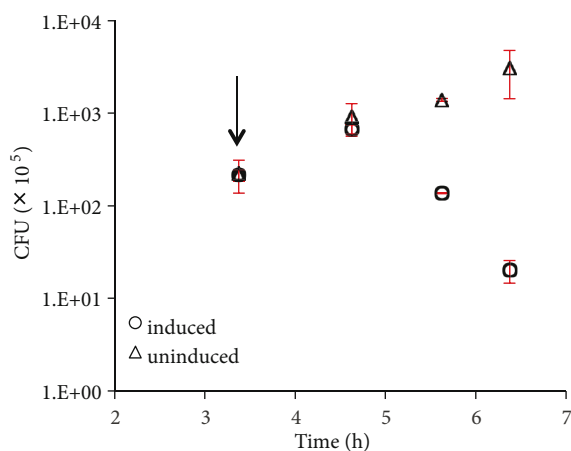


Figure 7. Colony forming units of *E. coli*-PuSj cells under induced and uninduced conditions. Arrow indicates induction with 0.2 mM IPTG.

3.3.5. Periplasmic β -lactamase activity with BLIP synthesis

Analysis of growth rates demonstrated the effect of the β -lactamase:BLIP complex formation in the periplasm. It showed that cell growth has not ceased completely. For this reason we tried to evaluate the ratio of bound to unbound β -lactamase by measuring enzyme activity in the periplasmic extracts of *E. coli* PuPet and *E. coli* PuSj cells, both induced with IPTG. In other words, since it is harder to measure the amount of β -lactamase bound to the inhibitor, we quantified the free enzyme by measuring residual activity with BLIP expression. The obtained value was compared with the total enzyme activity measured in the extracts of *E. coli*-PuPet cells harboring the identical parent plasmids (pUC18 and pET-26b(+)) without the *bli* gene.

In *E. coli*-PuPet cells, total β -lactamase activity was 91.8 U/L. However, when the extract from *E. coli*-PuSj cells was assayed, total β -lactamase activity was only 4.3 U/L. This suggests that, with BLIP expression, about 95% of the β -lactamase is found as complexed to BLIP.

The main goal of the current study was to investigate β -lactamase inhibition by BLIP in live cells. The *E. coli*-PuSj construct, which harbored both pUC18 and pET-26Sj plasmids, was used as the model organism for simultaneous expression of β -lactamase and BLIP. After co-expression of these proteins in *E. coli* cells, analyses were conducted to detect complex formation within cells. Detection of complex formation can be facilitated by tagging one or both proteins or by using fusion proteins,

but these methods may alter the structure and binding properties of the proteins. Hence, we used native TEM-1 β -lactamase and BLIP to investigate their interaction. The results of this study demonstrated that BLIP formed a complex with β -lactamase in the *E. coli* periplasm, but this interaction was not sufficient to completely stop cell growth. This could be related to the difference in the copy number of the plasmids. The pUC18 is a high copy number (500–700) plasmid, whereas the pET-26b(+) is a low copy number (~40) plasmid. This may result in excess β -lactamase. Furthermore, the in vitro inhibition constant of BLIP is 0.3 nM; in the presence of crowding agents, the inhibitor potential of BLIP may decrease.

Our results show that the protein based β -lactamase inhibitor BLIP acts as an inhibitor even in the presence of crowding agents. This is promising in terms of potential utilization of other protein based inhibitors. Stoichiometry is the key in providing the extent of inhibition and a quantitative analysis of in vivo inhibition will be a valuable future study.

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