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Overexpression of Bacterial Hemoglobin Causes Incorporation of Pre-β-Lactamase into Cytoplasmic Inclusion Bodies

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The expression of *Vitreoscilla* hemoglobin (VHb) in *Escherichia coli* JM101(pRED2) causes the incorporation of the TEM β-lactamase precursor into cytoplasmic inclusion bodies (IBs). Less pre-β-lactamase is translocated and processed to its mature, periplasmic form in the strain coexpressing VHb than in the control strain *E. coli* JM101(pUC19) not expressing VHb. When cells are grown in a special fed-batch procedure, the formation of cytoplasmic IBs consisting of pre-β-lactamase is also inducible in the control strain. Comparative microscopic and compositional analyses of IBs generated in *E. coli* JM101(pUC19) and JM101(pRED2) under identical growth conditions strongly suggest that pre-β-lactamase and VHb coaggregate into common IBs in *E. coli* JM101(pRED2).

Inclusion body (IB) formation is frequently observed when heterologous proteins are expressed in *Escherichia coli* (25, 28, 35). However, native proteins of *E. coli* such as the cytoplasmic protein β-galactosidase (8, 31) or the periplasmic protein TEM β-lactamase (3, 4, 31) can also aggregate into IBs. Culture conditions such as temperature (5, 7, 29, 30, 36), pH (38, 39), substrate supply (16, 31), and induction conditions (5, 16, 18) are known to affect in vivo aggregation of recombinant proteins. In addition, the folding properties of recombinant proteins influence the extent of IB formation within the bacterial cell (11, 22). Single amino acid substitutions can alter in vivo folding pathways and, consequently, change the partition of recombinant protein into soluble and IB fractions (17, 27, 32, 43). Finally, there is experimental evidence that members of the chaperone protein family can facilitate correct folding and assembly of recombinant proteins by preventing in vivo aggregation (1, 6, 10, 23, 44).

The presence of plasmid-encoded antibiotic resistance proteins in IB preparations has been reported previously (12, 14, 37). However, the nature of the interrelationship between these proteins and other cloned proteins in IB preparations remains unclear. In this study, we show that overexpression of *Vitreoscilla* hemoglobin (VHb) causes incorporation of pre-β-lactamase into cytoplasmic IBs. The implication of expression vector design on downstream processes is discussed.

MATERIALS AND METHODS

Bacterial strain and plasmids. *E. coli* JM101 and the plasmid pUC19 are described by Yanisch-Perron et al. (45). The plasmid pRED2 was constructed by inserting the VHb gene under the control of its native promoter into pUC19 (15).

Cell growth. Cells were grown at 37°C in complex medium containing 10 g of Bacto Tryptone, 5 g of yeast extract, 5 g of NaCl, 3 g of K_2HPO_4, and 1 g of KH_2PO_4, each per liter. This medium was supplemented with 100 mg of ampicillin per liter (except for JM101 cultures). Capped 2-liter flasks (Kimax) containing 1 liter of medium each were inoculated with 5 ml of an overnight culture. Cells were grown in a Lab-Line Orbit Environ-Shaker at 225 rpm. After 15 h of growth, feed medium consisting of 110 g of Bacto Tryptone, 110 g of yeast extract, and 110 g of glucose, each per liter, was added to a final concentration of 1% (vol/vol). Growth was continued for an additional 6 h with a reduced shaker speed of 175 rpm. These fed-batch conditions were used as a means of comparing pre-β-lactamase IBs generated under identical culture conditions in *E. coli* JM101(pUC19) and JM101(pRED2) [aggregation of pre-β-lactamase in *E. coli* JM101(pUC19) occurs only in response to feeding in the stationary growth phase]. Cell growth was monitored by determining the A_{590} of the culture broth with a Bausch & Lomb Spectronic 21 spectrophotometer. Samples were diluted appropriately to maintain the absorbance below 0.3.

Preparation of soluble and insoluble cell fractions. All procedures were carried out at 4°C unless otherwise indicated. Cells were harvested by centrifugation. Cell pellets were resuspended in buffer A (100 mM Tris HCl [pH 8], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) and lysed by sonication on ice. Soluble and insoluble cell lysates were collected by centrifugation. Details are specified in the captions to the figures. To distinguish between membrane or membrane-bound proteins and IBs, differential centrifugations of lysed cells collected at the end of the cultivation were carried out. Crude cell lysates were centrifuged at 6,000 × *g* for 10 min, and the pellet fraction was collected (6,000 × *g* insoluble lysate). The supernatant (6,000 × *g* supernatant) was centrifuged at 12,500 × *g* for 15 min, and the pellet fraction was collected (12,500 × *g* insoluble lysate). The remaining supernatant (12,500 × *g* supernatant) was centrifuged at 26,000 × *g* for 1 h, and the pellet fraction was collected (26,000 × *g* insoluble lysate). The supernatant (26,000 × *g* supernatant) was centrifuged at 40,000 × *g* for 2 h, and the pellet fraction was collected (40,000 × *g* insoluble lysate). The remaining

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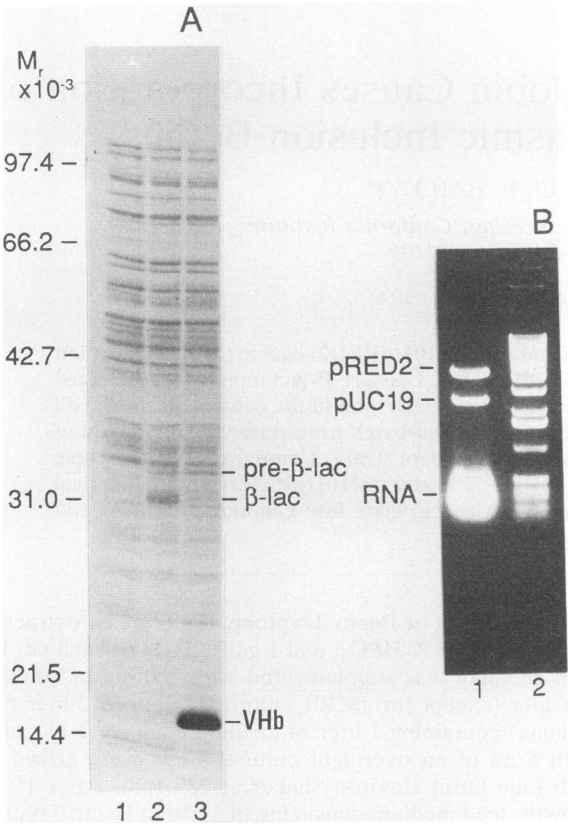

FIG. 1. (A) SDS-PAGE analysis of total cell protein. Samples were collected at the end of the cultivation. Cells were resuspended in buffer A to the same absorbance. Aliquots were taken and prepared for SDS-PAGE as described in Materials and Methods. Lanes 1 to 3 show the total cell protein of JM101, JM101(pUC19), and JM101(pRED2), respectively. Molecular weight markers are shown on the left of panel A (values have already been multiplied by 10^{-3}). (B) Plasmid preparation of JM101(pUC19) and JM101(pRED2). Again, cells were resuspended to the same absorbance. Aliquots were taken and plasmids were isolated from the cell mixture. Plasmid DNA was digested with *Eco*RI and run on a 0.75% agarose gel (lane 1). Marker DNA (1-kb DNA ladder) was from Bethesda Research Laboratories (lane 2).

supernatant was collected as soluble cell lysate. All samples were stored at −70°C until further analysis.

β-Lactamase assay. β-Lactamase activity was measured by using ampicillin as the substrate (42). One unit of β-lactamase activity was defined as 1 μmol of ampicillin hydrolyzed per min at 30°C and pH 7.0.

Polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20). Samples were boiled for 10 min in 4% (wt/vol) SDS–20% (wt/vol) glycerol–150 mM dithiothreitol–125 mM Tris HCl (pH 6.8) and immediately electrophoresed on a 9 to 16% polyacrylamide gradient gel. Gels were silver stained as described by Hochstrasser et al. (13). Electroblotting of proteins to nitrocellulose membranes was done by the method described by Towbin et al. (40). Rabbit anti-TEM β-lactamase antiserum (J. Richards, California Institute of Technology, Pasadena) and a commercially available kit (Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif.) were used for immunodetection of (pre)-β-lactamase.

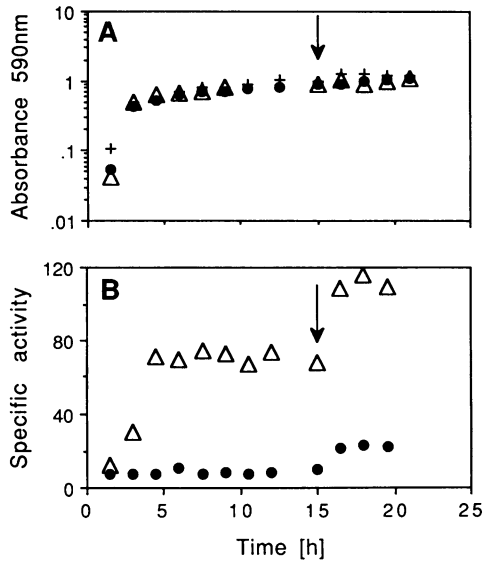

FIG. 2. (A) Growth data of JM101 (+), JM101(pUC19) (△), and JM101(pRED2) (●); (B) profiles of active β-lactamase formation in JM101(pUC19) (△) and JM101(pRED2) (●). Specific β-lactamase activities are reported as total units of enzyme corresponding to 1 ml of culture broth that was concentrated or diluted to an A_{590} of 1. The arrows indicate the time of feeding.

Immunostaining was done by the protocol of Vector Laboratories.

Plasmid preparation. Plasmid preparation, restriction, and agarose gel electrophoresis were carried out as described by Sambrook et al. (34).

Transmission electron microscopy. Cell samples were prepared as described previously (12) and examined with a Phillips 420 transmission electron microscope.

RESULTS

E. coli JM101 carrying the plasmid pUC19 and that carrying its derivative pRED2 were grown under identical conditions. Both plasmids carry the *bla* gene encoding TEM β-lactamase under the control of its natural promoter. The plasmid pRED2 additionally encodes VHb. Both proteins can aggregate into IBs or can be processed to their respective mature forms, periplasmic β-lactamase and dimeric hemoglobin containing the prosthetic group protoheme IX.

When cells were grown in a fed-batch procedure as described in Materials and Methods, mature β-lactamase accumulated up to 1 to 2% of total cell protein in *E. coli* JM101(pUC19) (Fig. 1A). However, β-lactamase was hardly detectable in the total cell extract of *E. coli* JM101(pRED2), although the overall plasmid content did not seem to be affected by the additional presence of the *VHb* gene and its product (Fig. 1B). Both strains exhibited a similar growth pattern, but the amount of β-lactamase activity produced by JM101(pRED2) was only 15% that produced in JM101 (pUC19) (Fig. 2A and B, respectively). Gel electrophoretic analyses of insoluble cell lysates and electron microscopy revealed the appearance of periplasmic IBs composed of β-lactamase in *E. coli* JM101(pUC19) but not in JM101 (pRED2) (Fig. 3 and 4, respectively). (It was estimated that approximately 3% of the cells carried one or two IBs localized in the periplasm; however, the majority appeared to be free of IBs.) Instead, aggregation of pre-β-lactamase

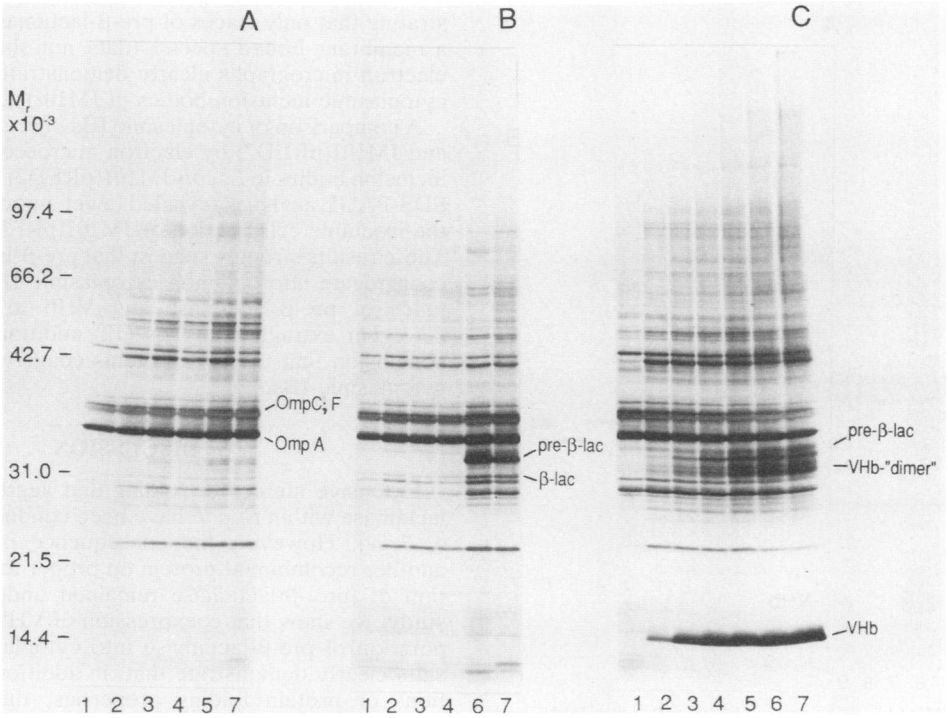

FIG. 3. Cell samples of JM101 (A), JM101(pUC19) (B), and JM101(pRED2) (C) were collected over the course of the cultivation. Cells were resuspended to the same absorbance and lysed by sonication on ice. The insoluble lysates were prepared by centrifugation at 16,000 × *g* for 30 min. Aliquots of the debris fractions were analyzed by SDS-PAGE. Lanes 1 to 7 show the insoluble lysates from 3, 6, 9, 12.5, 15, 18, and 21 h postinoculation, respectively. The insoluble lysates in lanes 6 and 7 were collected after the addition of the feed medium. The outer membrane proteins OmpA, OmpC, and OmpF are indicated in panel A. Molecular weight markers are shown on the left of the figure (values have already been multiplied by 10^{-3}).

into cytoplasmic IBs was observed in *E. coli* JM101(pRED2) (Fig. 3C and 4C). In *E. coli* JM101(pUC19), aggregation of pre-β-lactamase into cytoplasmic IBs was observed only in response to the addition of feed medium in the stationary growth phase (Fig. 3B, lanes 6 and 7; Fig. $4B_2$). To distinguish between membrane-bound pre-β-lactamase and cytoplasmic pre-β-lactamase IBs, differential centrifugations of lysed cells collected at the end of the fed-batch procedure

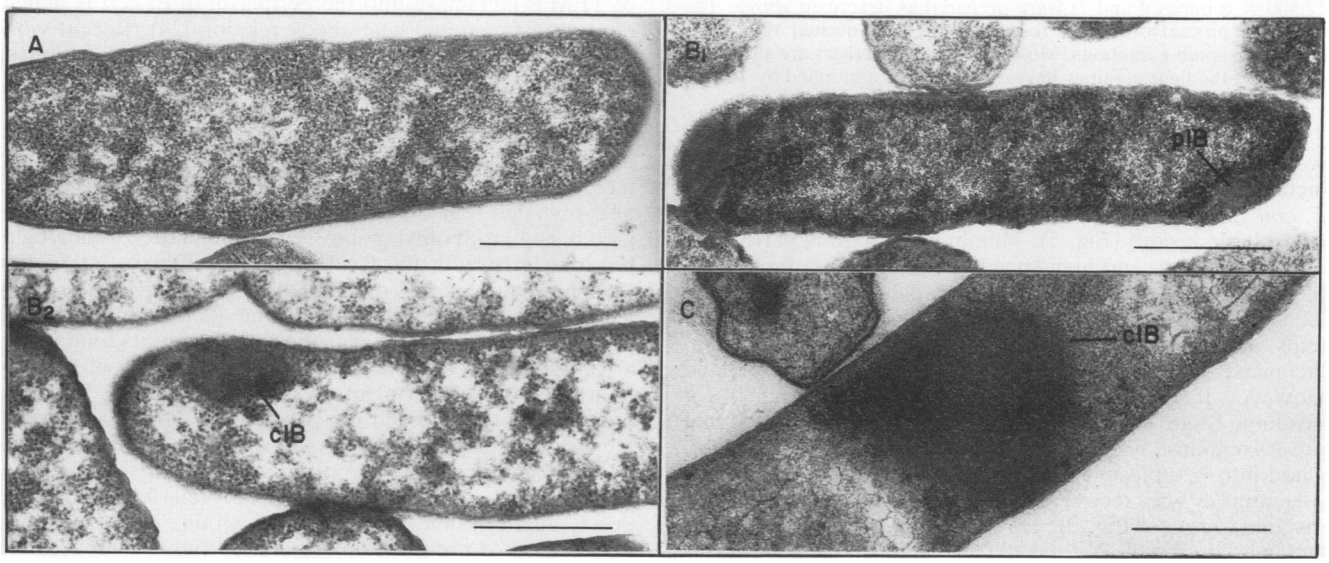

FIG. 4. Transmission electron micrographs of JM101 (A), JM101(pUC19) (B_1 and B_2), and JM101(pRED2) (C). Bars represent 500 nm. The cells shown in panel B_1 were collected prior to feeding; the other micrographs represent cells collected at the end of the cultivation. Periplasmic IBs containing β-lactamase were produced in JM101(pUC19) prior to feeding (B_1); cytoplasmic IBs containing pre-β-lactamase appeared after the addition of feed medium (B_2). Only cytoplasmic IBs were formed in JM101(pRED2) (C). pIB, periplasmic IBs; cIB, cytoplasmic IBs.

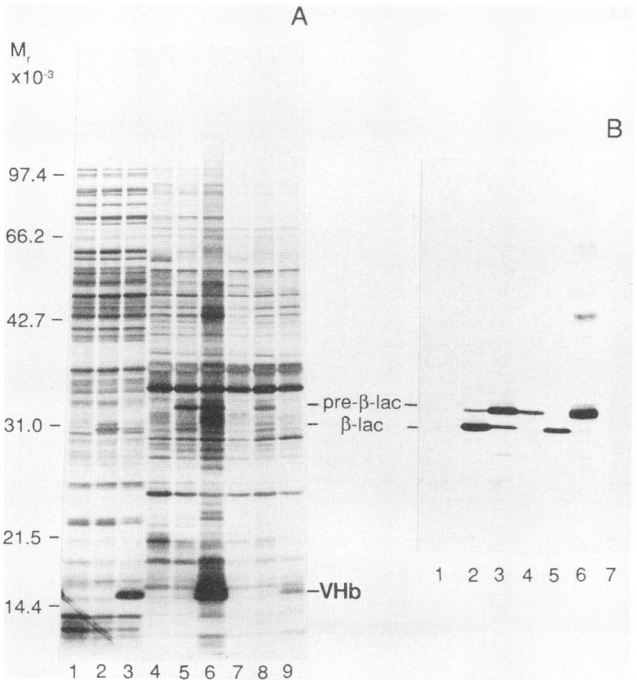

FIG. 5. SDS-PAGE (A) and immunoblots (B) of soluble and insoluble cell lysates. (A) Samples of JM101 (lanes 1, 4, and 7), JM101(pUC19) (lanes 2, 5, and 8), and JM101(pRED2) (lanes 3, 6, and 9) were collected at the end of the cultivation, resuspended to the same absorbance, and lysed by sonication on ice. Insoluble lysates were prepared by centrifugation of crude cell lysates at 6,000 × *g* for 10 min (lanes 4 to 6) and centrifugation of the remaining supernatant at 12,500 × *g* for 15 min (lanes 7 to 9). Soluble lysates were obtained as supernatants after additional centrifugations at 26,000 × *g* for 1 h and 40,000 × *g* for 2 h (lanes 1 to 3). Aliquots were analyzed by SDS-PAGE. Insoluble fractions were concentrated four times relative to respective soluble fractions. (B) Samples of JM101 (lane 1), JM101(pUC19) (lanes 2 to 4), and JM101(pRED2) (lanes 5 to 7) were also analyzed by immunoblotting. Soluble lysates (lanes 2 and 5) and insoluble lysates collected at 6,000 × *g* (lanes 3 and 6) and 12,500 × *g* (lanes 4 and 7) were prepared as described above. The total cell protein of JM101 (lane 1) was concentrated two times relative to soluble fractions. Molecular weight markers are shown on the left of the figure (values have already been multiplied by 10^{-3}).

were carried out. These experiments revealed that pre-β-lactamase was completely recovered in the pellet fraction of *E. coli* JM101(pRED2) by low-speed centrifugation (6,000 × *g* insoluble lysate) (Fig. 5). Membrane fractions partly precipitated at low- and high-speed centrifugations. For example, outer membrane proteins were present in 6,000 × *g* and 12,500 × *g* insoluble lysates (Fig. 5; outer membrane proteins are labelled in Fig. 3A). Membrane-bound pre-β-lactamase should be present in low- and high-speed pellets. However, the absence of pre-β-lactamase in the 12,500 × *g* insoluble lysate of JM101(pRED2) clearly demonstrated that pre-β-lactamase was not bound to the membrane but aggregated into cytoplasmic IBs. In *E. coli* JM101(pUC19), pre-β-lactamase was recovered in 6,000 × *g* and 12,500 × *g* insoluble lysates (Fig. 5). However, the presence of pre-β-lactamase in 26,000 × *g* and 40,000 × *g* insoluble lysates was not apparent on silver-stained gels (data not shown). A minute and barely detectable amount of pre-β-lactamase was visualized in 26,000 × *g* and 40,000 × *g* insoluble lysates of JM101(pUC19) by overstaining of the immunoblots, demonstrating that only traces of pre-β-lactamase were present as a membrane-bound species (data not shown). In addition, electron micrographs clearly demonstrated the presence of cytoplasmic inclusion bodies in JM101(pUC19) (Fig. $4B_2$).

A comparison of cytoplasmic IBs in *E. coli* JM101(pUC19) and JM101(pRED2) by electron microscopy revealed larger inclusion bodies in *E. coli* JM101(pRED2) (Fig. 4). However, SDS-PAGE analyses revealed lower amounts of precursor in the insoluble cell fraction of JM101(pRED2) (Fig. 3 and 5). These results strongly suggest that pre-β-lactamase and VHb coaggregate into common cytoplasmic IBs. Identical resistance of pre-β-lactamase and VHb to solubilization by detergent extraction of IBs (12) additionally supports our conclusion that the two proteins coaggregate into common cytoplasmic IBs.

DISCUSSION

Extensive studies of folding and aggregation of (pre)-β-lactamase within *E. coli* have been conducted previously (3, 4, 7, 9). However, the consequence of coexpression of another recombinant protein on proper folding or IB formation of (pre)-β-lactamase remained undetermined. In this study, we show that coexpression of VHb causes the incorporation of pre-β-lactamase into cytoplasmic IBs. Our results clearly demonstrate that, in addition to culture conditions or protein folding properties, the coexpression of another recombinant protein is a factor influencing the extent of in vivo aggregation of a recombinant protein within the bacterial cell. Culture conditions causing aggregation of pre-β-lactamase in the cytoplasm of the strain coexpressing VHb do not induce the formation of cytoplasmic pre-β-lactamase IBs in the control strain. When cells are grown under identical conditions, more β-lactamase is translocated and processed to its mature form in the strain not expressing VHb.

In vivo aggregation of pre-β-lactamase in the cytoplasm of *E. coli* must occur in competition to translocation or to folding (or complexing) into a translocation-competent form. Translocation occurs posttranslationally (41), and the export of TEM β-lactamase into the periplasm of *E. coli* is dependent on a functional heat shock regulon (24). Recent work suggested that the in vivo translocation of TEM β-lactamase in *E. coli* is chaperoned by the heat shock proteins GroEL and GroES (19). In vitro studies revealed that newly synthesized pre-β-lactamase associates with the GroEL protein (2) and that the folding pathway of pre-β-lactamase is modulated by the GroE heat shock proteins (21). In vitro studies also revealed that the addition of misfolded forms of myoglobin such as apomyoglobin or heat-denatured apomyoglobin interfered with the GroEL–pre-β-lactamase interaction (2). Thus, overexpression of VHb in JM101(pRED2), clearly leading to misfolded forms of the globin, may deprive pre-β-lactamase of its chaperone and prevent its folding into a translocation-competent form. Overburdening of the protein-folding machinery caused by overexpressing VHb and competition of VHb and pre-β-lactamase folding intermediates for mutual folding helpers may cause the incorporation of pre-β-lactamase into cytoplasmic IBs mainly composed of VHb. However, an overload on the protein-folding machinery is also possible in the control strain, resulting in the aggregation of pre-β-lactamase caused by its increased expression in response to the addition of feed medium.

Recovery of active proteins from IBs requiring solubilization and refolding procedures is common practice (14, 25, 26, 33). To simplify recombinant protein purification, proteins

arising from coprecipitation of membrane fractions such as the outer membrane proteins are removed from crude IB preparations by detergent extraction prior to the solubilization and refolding procedures. However, identical resistance of pre-β-lactamase and VHb to detergent extraction of IBs (12) interferes with the purification of VHb prior to solubilization and refolding. Although it is general opinion that the native β-lactamase promoter is constitutive and weak, our studies reveal that more (pre)-β-lactamase is produced than required to provide resistance against β-lactam antibiotics to cells carrying the plasmid pUC19 or its derivatives. The observed phenomenon may also extend to other binary expression systems, and vectors with lower expressions of antibiotic resistance proteins should be employed to simplify purification of the target protein produced in the form of IBs.

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