

# Export and secretion of overproduced OmpA- $\beta$ -lactamase in *Escherichia coli*

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The export of  $\beta$ -lactamase to the periplasm of *Escherichia coli* can be directed by the OmpA signal peptide in the secretion cloning vector pIN-III. The overproduction of the hybrid precursor specifically induces a delay in the onset of processing of newly synthesized polypeptide chains. However, when the processing starts, no alteration in the rate of cleavage itself is observed. Our results suggest that the temporal mode of processing (which reflects translocation) does not depend on the nature of the signal peptide but rather depends on the nature of the polypeptide chain exported.

Hybrid protein; Protein export; Signal peptide; Protein processing; Periplasmic protein

## 1. INTRODUCTION

Periplasmic proteins of Gram-negative bacteria constitute a simple model to study in vivo the mechanism of polypeptide translocation across membrane. It has become increasingly clear that the co-translational model of protein export is purely operational and does not invoke an obligate coupling between polypeptide elongation and translocation [1]. A post-translational transfer in vitro [2,3] for proteins that are co-translationally processed in vivo [4] has been demonstrated in *Escherichia coli*.

The synthesis and the export of  $\beta$ -lactamase, a periplasmic protein [5,6] conferring on cells resistance to ampicillin, have been extensively studied. TEM- $\beta$ -lactamase, the most common R factor-mediated  $\beta$ -lactamase, has a higher than normal rare-codon usage and the average rate of

elongation of its polypeptide chain is particularly low [7]. This elongation occurs on membrane-bound polysomes [8]. In contrast to most exported proteins of *E. coli* that have been studied, TEM- $\beta$ -lactamase is processed entirely post-translationally and newly synthesized polypeptide chains are translocated across the inner membrane after completion of their translation [4,9]. Energy is required for the translocation and maturation of the precursor form [10–12]. The half-life of the precursor of  $\beta$ -lactamase is about 42 s [13]. The formation of the single disulfide bond occurs concomitant with processing [14]. More recently, evidence for a distinct release step from the outer side of the cytoplasmic membrane, associated with a conformational change has been obtained [15].

Secretion cloning vectors using the OmpA signal peptide have been constructed. The cloned gene product can be synthesized in large amounts and very efficiently secreted across the cytoplasmic membrane [16]. When the  $\beta$ -lactamase gene was cloned into one of these secretion vectors (pIN-III), the protein was produced at a level of 20% of total cellular proteins and was accumulated in the

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periplasmic space [17]. Although the OmpA signal peptide was used instead of the authentic  $\beta$ -lactamase signal sequence, processing occurs at the normal cleavage site [17]. In this study, we have used the secretion cloning vector pIN-III containing the OmpA- $\beta$ -lactamase construct (pJG108) to characterize further the molecular and kinetic details of the export pathway of the hybrid precursor.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strain, plasmid and medium

*E. coli* strain JA221 (*hdsM*<sup>+</sup>, *trpE5*, *leuB6*, *lacY*, *recA1/F*<sup>-</sup>, *lacI*<sup>q</sup>, *lac*<sup>+</sup>, *pro*<sup>+</sup>) was used in all experiments [18]. The plasmid pJG108 has been described in [17]. A minimal medium M9 supplemented with required nutrients and chloramphenicol (10  $\mu$ g/ml) was used as previously

reported [15]. Isopropyl- $\beta$ -D-thiogalactoside (IPTG) was added at 2 mM (final concentration) to induce hybrid gene expression.

### 2.2. Expression of the ompA- $\beta$ -lactamase gene

At various times after addition of IPTG, cells were harvested by centrifugation. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting/immunoprinting as reported in [19,20].

### 2.3. Pulse labelling and pulse-chase experiments

At various times after induction (0, 30, 60, 90 and 180 min), samples (10<sup>9</sup> cells) were labelled for 2 min with [<sup>35</sup>S]methionine (3  $\mu$ Ci/ml). Cells were centrifuged and pellets were analyzed by SDS-PAGE. Fluorography and densitometer scanning of the fluorograms were carried out as described by Pagès and Lazdunski [11].

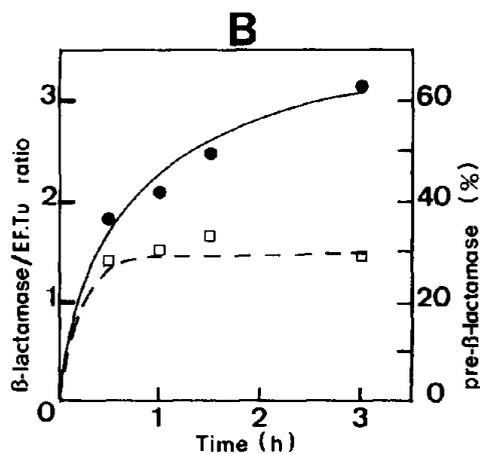
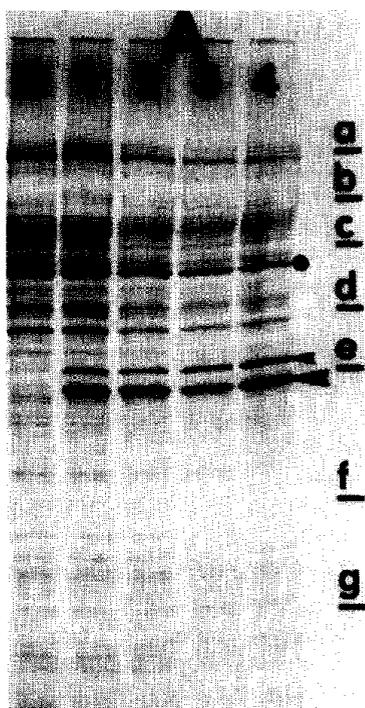


Fig. 1. Synthesis of  $\beta$ -lactamase during overproduction. At various times after induction, samples (10<sup>9</sup> cells) of *E. coli* JA221 pJG108 were labelled for 2 min with [<sup>35</sup>S]methionine. Total cellular proteins were analyzed by SDS-PAGE (A). Gels were prepared for fluorography. Lanes: 0, non-induced cells; 1, 30 min; 2, 60 min; 3, 90 min and 4, 180 min after induction. Small and large arrowheads indicate respectively the migration of precursor and mature forms of  $\beta$ -lactamase; the dot indicates the migration of EF-Tu. Relative molecular mass standards (kDa): (a) 94; (b) 67; (c) 46; (d) 36; (e) 30; (f) 14. The total  $\beta$ -lactamase/EF-Tu (●—●) and precursor/total  $\beta$ -lactamase (□--□) ratios were respectively determined by densitometer scanning analyses (B).

For pulse-chase experiments [ $^{35}\text{S}$ ]methionine (100  $\mu\text{Ci}/\text{ml}$ ) was added, after 15 s an excess of non-radioactive methionine (10 mg/ml) was added and samples were removed after 15, 30, 60, 90, 120, 180, 300 and 600 s. Samples were collected into trichloroacetic acid (15%, w/v, final concentration) plus chloramphenicol (150  $\mu\text{g}/\text{ml}$ ) and immediately frozen in liquid nitrogen [13]. The trichloroacetic acid precipitates were solubilized and immunoprecipitations with various antisera were carried out as reported in [13,20]. The immunoprecipitates were analyzed by SDS-PAGE and fluorography.

### 3. RESULTS

#### 3.1. Level of synthesis of $\beta$ -lactamase during overproduction

To evaluate the level of production, the cells were labelled for 2 min with [ $^{35}\text{S}$ ]methionine at various times after induction. The pattern of total radiolabelled proteins is shown in fig.1A. From three independent experiments, it was determined that the maximum rate of synthesis was reached after about 20–30 min of induction (fig.1B). The level of  $\beta$ -lactamase was calculated using the elongation factor Tu (EF-Tu) as an internal standard [19]. We observed that fully induced cells produce about 3-times as much  $\beta$ -lactamase as EF-Tu which corresponds about to  $2\text{--}2.5 \times 10^5$  copies per cell. After 30 min of induction, the precursor of  $\beta$ -lactamase was already detected in the cell. The accumulation of the precursor form was evaluated as about 30% of the total labelled  $\beta$ -lactamase and this ratio was stable even during further times of induction (fig.1B).

#### 3.2. The high level of $\beta$ -lactamase does not cause accumulation of precursors of other exported proteins

A general effect of hyperproduction of a periplasmic protein, PhoS, on the maturation of exported proteins has been shown [13,20]. Thus, it was of interest to determine if the overproduction of  $\beta$ -lactamase resulted in any significant saturation of exported sites [21]. The amounts of mature and precursor forms of  $\beta$ -lactamase, histidine binding protein, OmpA and OmpF were evaluated during induction by immunoprinting, performed with specific antisera after Western blotting

(fig.2). Two results emerged from this analysis: (i) a significant accumulation of precursor starting about 30 min after induction (fig.2A); (ii) precursor forms were not detected for other exported proteins, OmpA, OmpF and HisJ in contrast to the previous results reported with PhoS hyperproduction [13,20]. The detection of pre- $\beta$ -lactamase is consistent with the results from pulse labelling (fig.1).

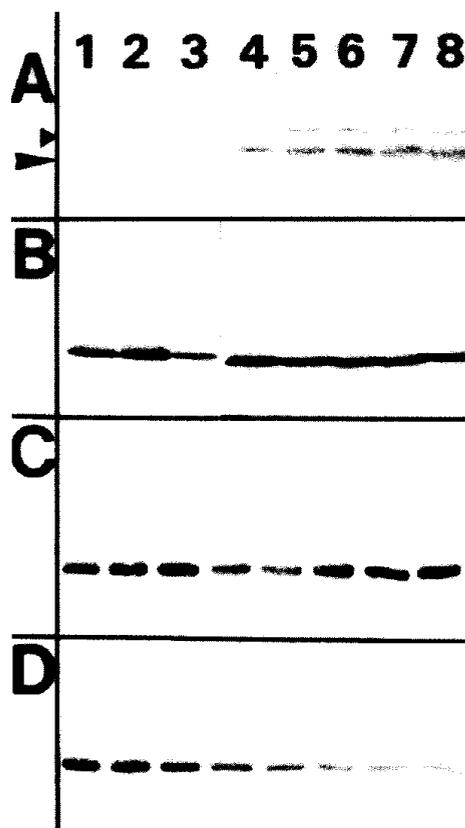


Fig.2. Immuno-blot analyses of various exported proteins. Aliquots of the samples were subjected to SDS-PAGE. After migration, the proteins were electrotransferred to nitrocellulose and the immunodetections were carried out as described in [19]. In A, B, C and D antisera directed against  $\beta$ -lactamase, HisJ, OmpA and OmpF, respectively, were applied. Lanes 1–3, JA221; lanes 4–8, JA221 pJG108. Times of induction: 0 min (lanes 1 and 4); 30 min (lanes 2 and 5); 60 min (lane 6); 90 min (lane 7); 180 min (lanes 3 and 8). The small and large arrowheads indicate respectively the migration of precursor and mature form of  $\beta$ -lactamase.

### 3.3. Temporal mode of protein translocation/maturation

The effect of both, use of the OmpA signal sequence and overproduction of the protein, on the

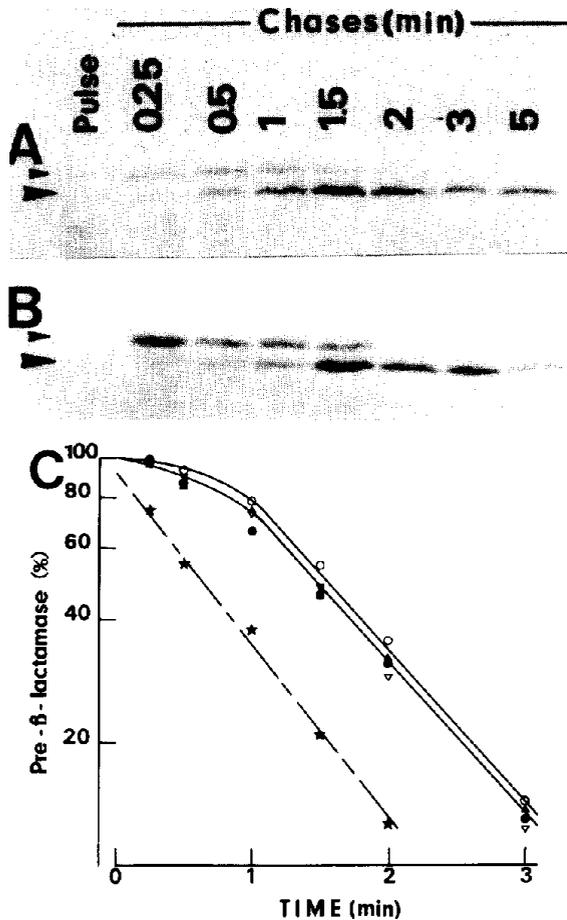


Fig.3. Quantitative analysis of the pulse-chase experiments. At various times after induction, pulse labelling and chases were performed as described in section 2. After solubilization of total cellular proteins, immunoprecipitations with an antiserum directed against  $\beta$ -lactamase were carried out. The immunoprecipitates were analyzed by SDS-PAGE and fluorography: (A) pulse-chase labelling with non-induced cells; (B) 1 h after induction. The relative percentages of pre- $\beta$ -lactamase to total  $\beta$ -lactamase were evaluated by densitometer scanning analyses of fluorograms (C). (★) Non-induced cells; (○) 30 min; (●) 60 min; (▽) 90 min; (▲) 180 min after induction. Small and large arrowheads indicate respectively the migration of precursor and mature forms of  $\beta$ -lactamase. (A) An over-exposed film (obtained with the non-induced cells).

processing step was investigated. The half-life of hybrid precursor at 37°C was determined at various times during induction by short pulse labelling (15 s) and chase experiments (fig.3). The precursor form of  $\beta$ -lactamase did not appear immediately after the pulse but only during the chase (fig.3A and B). This is caused by the presence of smaller discrete intermediate nascent chains (not shown) which are converted into precursor forms during the chase [7,15]. It has been previously reported that  $\beta$ -lactamase is expressed at a basal level before IPTG addition. The kinetics of processing before induction indicated a precursor half-life of 40 s (fig.3A,C). This rate of cleavage corresponds to the processing of the normal pre- $\beta$ -lactamase, 42 s, reported in [13]. Thus the exchange of signal peptide did not induce any modification of the kinetics of processing before overproduction.

Under conditions where the hybrid precursor was accumulated after induction, there was a 1 min delay before the onset of maturation. The overproduction caused stalling in the conversion of precursor to the mature form, the half-life of the hybrid precursor being increased to 100 s. However, the rate of cleavage itself which was

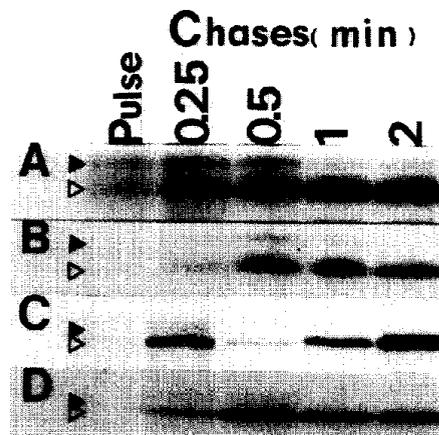


Fig.4. Processing of precursor forms of OmpA and OmpF. The same samples as those described in fig.3 were subjected to immunoprecipitations with antisera directed either against OmpF (A,B) or against OmpA (C,D) and the products were analyzed by SDS-PAGE. (A,C) Non-induced cells; (B,D) 1 h after induction. The closed and open triangles indicate respectively the migration of precursor and mature forms.

evaluated from the slope of the curve (fig.3C) was unaltered after this delay. Thus, the overproduction induced a translation of the curve with a 1 min shift.

The kinetics of processing of other exported proteins have been investigated during the induction of  $\beta$ -lactamase. Although in the case of OmpA the same signal peptide was used, we failed to detect any significant alteration in the processing step of precursor forms of OmpA and OmpF (fig.4) in agreement with the absence of precursor forms demonstrated in fig.2. Again, because of the short pulse times and the time required to finish nascent chains, precursor forms were hardly detectable in the samples taken immediately after the pulse.

#### 4. DISCUSSION

It has previously been shown that  $\beta$ -lactamase using the OmpA signal peptide is very efficiently expressed and exported, under the control of both the *lpp* promoter and the *lac* promoter-operator, in the secretion vector constructed by Ghrayeb et al. [17].

Using immunoblots and radiolabels, we have demonstrated here that although the pre- $\beta$ -lactamase is not detectable by Coomassie blue staining, the precursor begins accumulating between 10 and 30 min after induction. This effect is not due to the nature of the signal sequence since we have found no accumulation of pre-OmpA. Moreover, the precursor is not detected when  $\beta$ -lactamase is not overproduced (before induction). Furthermore, it has been demonstrated that this signal peptide is correctly removed [17]. In addition, we have observed that the enzymatic activity of  $\beta$ -lactamase in cell lysates increased by more than 20-times after 3 h induction.

This system appeared well suited to study the effect of the  $\beta$ -lactamase overproduction on the cleavage of the signal sequence. Previous results have suggested that the temporal mode of processing depends upon the nature of the polypeptide chain exported [4]. The synthesis of pre- $\beta$ -lactamase is completed before processing and it has been proposed that the precursor first becomes associated with the inner side of the cytoplasmic membrane, the polypeptide chain being transferred independently of its synthesis [4,8,22]. We have shown here that despite the substitution of

the authentic signal sequence of pre- $\beta$ -lactamase for that of pre-OmpA, the half-life of the precursor form remains the same (40 s) [9,13] before the overproduction. This result clearly demonstrated that the temporal mode of processing (and probably translocation) can be entirely ascribed to the nature of the mature part of the polypeptide chain.

The overproduction of  $\beta$ -lactamase does result however, in a significant delay in the onset of maturation. This delay of about 60 s can be interpreted as meaning that the leader peptidase has no access to the signal peptide for some time after completion of synthesis of the polypeptide chain. This elongation time by itself is rather long since the average rate of amino acid assembly is only about 7 amino acids per s [7]. Thus, the second step, the translocation, requiring energy [10–12], is presumably the delayed one. Although a lag is observed before the onset of cleavage, the rate of processing itself corresponding to the slope of the curve (fig.3C) is the same regardless of the extent of  $\beta$ -lactamase production. This result suggests that the leader peptidase is not saturated during overproduction. Moreover, we have previously shown that under conditions of PhoS hyperproduction, the leader peptidase activity is not rate limiting [13]. Thus, the most likely effect appears to be at the energy-dependent translocation of newly synthesized polypeptide chains. Why the overproduction might alter this possibly rate-limiting step is not clear at present. It does not appear that any common component of the export machinery becomes titrated although this situation can occur [23,24], since the processing of other proteins, OmpA and OmpF, remains unaltered.

It is of interest to note that hyperproduction of the PhoS protein caused a saturation of export sites and induced the accumulation of precursor forms of  $\beta$ -lactamase, PhoA and PhoE [13,20]. This difference may be ascribed to the difference in levels of production ( $2 \times 10^5$  copies per cell of  $\beta$ -lactamase as compared to  $2 \times 10^6$  copies per cell of PhoS). However, it is also possible that the temporal mode of translocation between the two polypeptide chains caused the variation in the effect of overproduction,  $\beta$ -lactamase being post-translationally transferred in contrast to PhoS.

To conclude, the results presented provide evidence that the temporal mode of processing

does not depend upon the nature of the signal peptide but rather depends on the nature of the polypeptide chain exported. The overproduction of  $\beta$ -lactamase specifically induces a delay in the onset of processing of newly synthesized polypeptide chains of this protein. Further experiments are in progress with the aim of determining the mechanism by which this delay occurs.

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