SYNTHESIS AND EXPORT OF *tem1* β-LACTAMASE IN *ESCHERICHIA COLI*

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1. Introduction

The mechanism of synthesis and secretion of the plasmid encoded β-lactamase has recently received considerable interest. The nucleotide sequence of the gene [1] as well as the protein sequence of the enzyme [2] have been determined. This protein is produced in a precursor form that bears a signal peptide of 23 amino acid residues [1].

According to the signal sequence hypothesis [3], one might expect that this periplasmic protein of *E. coli* is produced in membrane-bound polysomes of *E. coli* like the maltose and arabinose binding proteins [4] and co-translationally transferred across the cytoplasmic membrane. However, two puzzling results that do not fit this hypothesis were recently reported. On the one hand, in addition to its periplasmic location, β-lactamase could be found associated to the inner and outer membranes [5]. On the other hand, results suggesting a post-translational transfer and a synthesis in free-polysomes have been reported [6].

The present work was initiated in order to clarify these problems. Evidence is presented that β-lactamase is really located in the periplasmic space of *E. coli* cells and produced in membrane-bound polysomes.

2. Materials and methods

2.1. *Strains and bacteriological techniques*

The strain 0600 pBR322 r_K^- m_K^- *recBC*$^-$ *lacy*$^-$ was used throughout this study. Media, osmotic shock and spheroplast formation were done as in [7]. In vivo labelling of proteins with [^{35}S]methionine (Amersham, 20 μCi/ml) was done in minimal medium supplemented with methionine assay medium (Difco).

2.2. *Preparation of free and membrane-bound polysomes*

The technique in [4] was used without major modifications. Sonication of cell suspensions at 0°C in a rosette for two 6 s periods on scale 4 of a Branson Sonifier (model S110) with 45 s interval ensured an efficient cell disruption and well-conserved polysomes. All buffers and materials used were sterile.

2.3. *Cell-free protein synthesis*

The system programmed by free and membrane-bound polysomes as in [4] was used. [^{35}S]Methionine was present at 0.04–0.24 mCi/ml in the final mixture. Syntheses were done for 25 min at 37°C and stopped by adding puromycin (10 μg/ml) and non-radioactive methionine (1 mM). The proteins were analyzed in a SDS–10–15% polyacrylamide gradient gel.

2.4. *Enzyme assays*

β-Galactosidase [8] and cyclic phosphodiesterase [9] were assayed as described.

2.5. *Antisera and immunoprecipitation procedures*

The antisera against the matrix protein, the elongation factor Tu (EF-Tu) and against the β-lactamase were generous gifts from Dr J. Rosenbuch and G. Caesarini, respectively. Indirect immunoprecipitations using protein A–Sepharose (Pharmacia) were carried out. Immunoprecipitates were analyzed on 12.5% SDS–polyacrylamide gels.

2.6. *Electron microscopy*

Cells harvested in log phase, were washed once with fresh medium, fixed in 2% paraformaldehyde and 0.2% glutaraldehyde, for 60 min at 4°C, washed in 10 mM phosphate buffer (NaCl 0.15 M) (pH 7.4). After centrifugation, the pellet was embedded in

Table 1
Cellular location of β-lactamase[a]

Preparation procedure	Cell compartment	Total proteins cpm × 10^{-6}	EF-Tu cpm × 10^{-6}	β-Galactosidase cpm × 10^{-6}	β-Lactamase cpm × 10^{-6}	c-Phosphodiesterase units/ml
Lysozyme EDTA	Cytoplasm/membrane	3.91 (79)	7.32 (74)	3.10 (81)	0.257 (4)	15 (15)
	Periplasm	1.039 (21)	2.57 (26)	0.729 (19)	6.177 (96)	85 (85)
Osmotic shock	Cytoplasm/membrane	2.52 (74)	1.63 (24)	2.36 (90)	0.488 (11)	20.6 (20)
	Periplasm	0.868 (26)	5.18 (76)	0.26 (10)	3.946 (89)	82.4 (80)

[a] Figures in parenthesis correspond to percentages

100 μl bovine serum albumin (15%) and polymerized in 2.5% glutaraldehyde. Frozen thin sections were carried out and incubated with rabbit specific antisera (overnight at 4°C) and then with ferritin-labelled anti-rabbit IgG antibodies (2 h at 20°C) raised in goats, as in [10].

3. Results

3.1. *Location of β-lactamase within* E. coli *cells*

Proteins that are truly periplasmic are released both after osmotic shock and spheroplast formation in contrast to cytoplasmic proteins like EF-Tu located near the inner face of the cytoplasmic membrane,

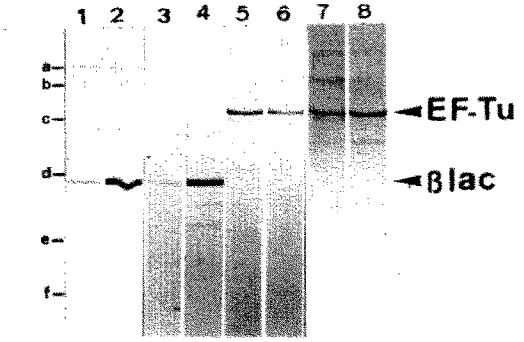

Fig.1. Cellular location of β-lactamase. After in vivo labelling with [^{35}S]methionine, ~10^{10} cells were either converted to spheroplasts by addition of lysozyme and EDTA (lanes 1,2,5,6) on submission to osmotic shock (lanes 3,4,7,8). 10^7 cpm were incubated in the presence of specific antiserum. The following samples were loaded on a 12.5% SDS–polyacrylamide gel: immunoprecipitates carried out on cytoplasmic (lanes 1,3,5,7) and periplasmic (lanes 2,4,6,8) compartments with serum anti-β-lactamase (lanes 1–4) and serum anti-EF-Tu (lanes 5–8). M_r standards: (a) phosphorylase *b* (94 000); (b) bovine serum albumin (67 000); (c) ovalbumin (43 000); (d) carbonic anhydrase (30 000); (e) soybean trypsin inhibitor (20 000); (f) α-lactalbumin (14 400). Arrows indicate the positions of β-lactamase and EF-Tu.

that are only released by osmotic shock [11].

The extent of lysis in the two procedures was determined by assaying β-galactosidase (table 1). About 19% and 10% lysis occurred during spheroplasting and osmotic shock that were achieved with yields of 85% and 80%, respectively, as assayed by release of cyclic phosphodiesterase. Both techniques allowed an efficient release of β-lactamase. Even the eventual membrane-bound enzyme was taken into account in the radioimmunoassay since detergent was added prior to immunoprecipitation. In contrast to the determination by assaying β-lactamase activity [5] (66% membrane-bound and 33% periplasmic), very little enzyme was found associated to the fraction containing the cytoplasm and membranes.

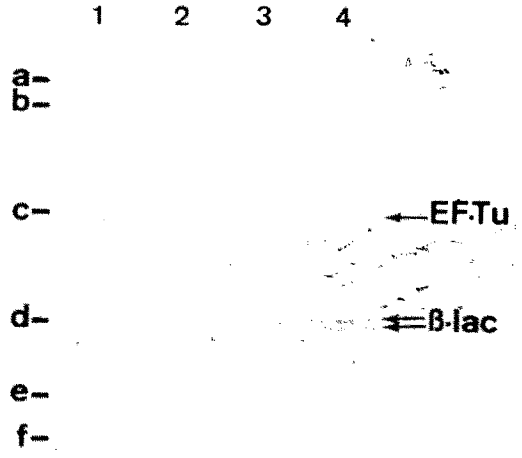

Fig.2. Cell-free synthesis in systems programmed by free and membrane-bound polysomes. The products of in vitro synthesis were immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis (10–15% gradient): lanes 1,3, products from free polysomes; lanes 2,4, products from membrane-bound polysomes. The following immunoprecipitates were applied: lanes 1,2, EF-Tu; lanes 3,4, β-lactamase.

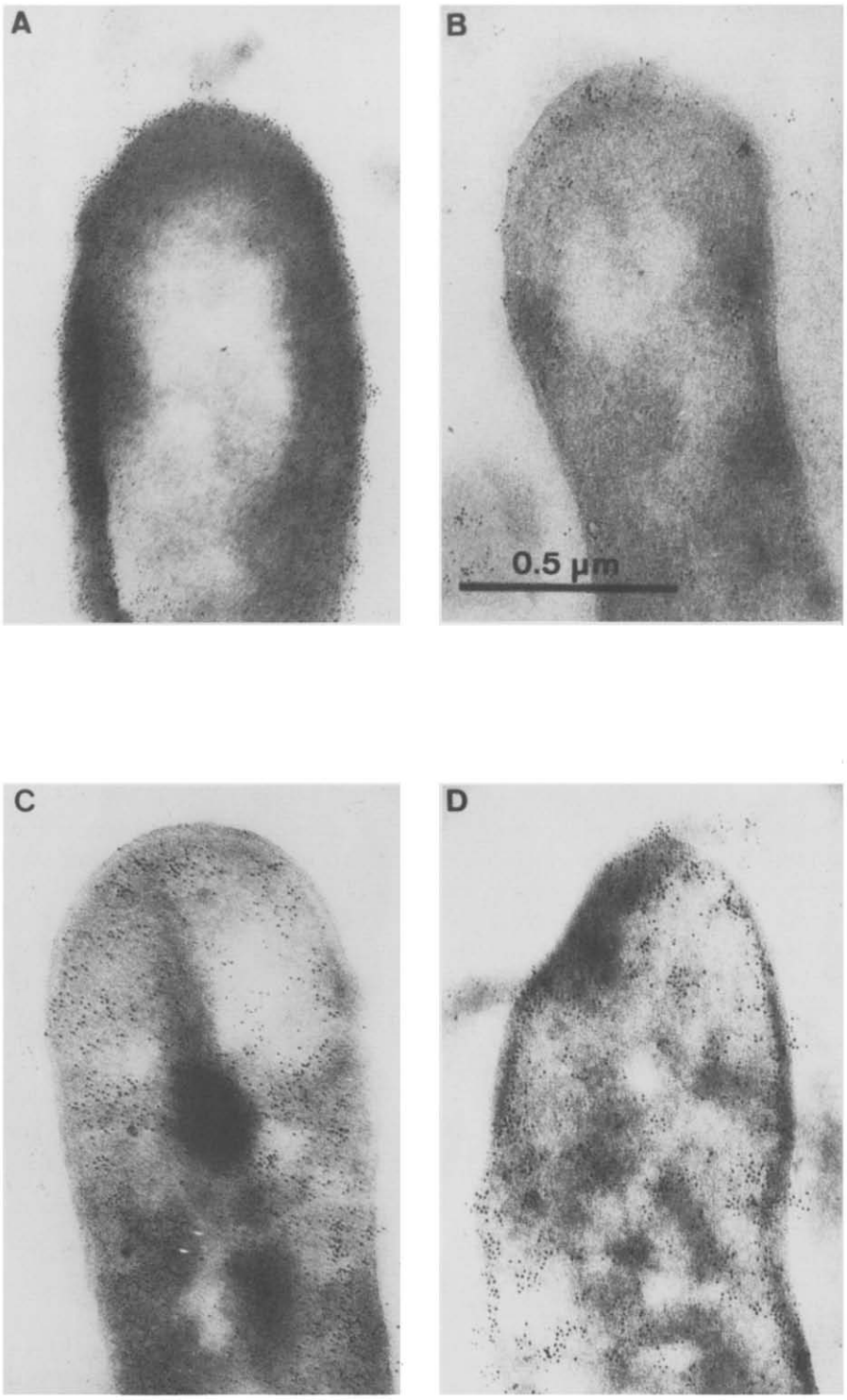

Fig.3. β-Lactamase is periplasmic: electron microscopic localization. (A) Matrix protein; (B) β-lactamase; (C) EF-Tu; (D) aminopeptidase N. The dilutions of specific antisera used were 1/1000 in A and C 1/500 in B and D, respectively.

The cellular distribution was also checked by SDS–polyacrylamide gel electrophoresis (fig.1). Again β-lactamase was immunoprecipitated from the periplasmic compartment. The distribution of EF-Tu between the various compartments reflects that presented in table 1.

The final evidence for a periplasmic localization was obtained through electron microscope studies (fig.2). A representative protein from each compartment was chosen and located on frozen thin sections of cells through immunoferritin studies. As expected, the matrix protein and EF-Tu were located in the cell envelope and cytoplasm, respectively. In contrast, β-lactamase was located in the periplasmic space and chiefly at the cell poles. However, in contrast to aminopeptidase N [7] it did not appear to be bound to the outer face of the cytoplasmic membrane.

3.2. *Synthesis in membrane-bound polysomes*

Free and membrane-bound polysomes were prepared according to [4]. These were used to program cell-free synthesis systems in which polypeptide synthesis that had been initiated in vivo, was terminated in vitro in the presence of radiolabelled methionine. In this system no reinitiation occurs [4].

The quality of the separation of free and membrane-bound polysomes was evidenced by the fact that products obtained from both fractions were clearly different (not shown). The elongation factor Tu, chosen as a representative protein from the cytoplasm, was exclusively produced in the free-polysomes (compare lanes 1 and 2). In contrast, β-lactamase was produced in membrane-bound polysomes (compare lanes 3 and 4). Two bands were resolved for β-lactamase produced in the cell-free system. This is not surprising, since it has been demonstrated that both precursor and mature forms can be detected for the arabinose and maltose binding proteins when they are produced in the same system [12].

4. Discussion

Analysis of the synthesis and secretion of the β-lactamase encoded by the plasmid pBR 322 in *E. coli,* can be summarized as follows:

(i) The mature enzyme is located in the periplasmic space and we have not found evidence that any significant amount is bound to inner and outer membranes, in contrast to [5];

(ii) This protein is produced in membrane-bound polysomes and not in free polysomes as suggested in [6,13].

(iii) Two forms, presumably the precursor and mature forms that differs by ~2500 M_r are produced in the cell-free system.

These results must be discussed with regards to [6]. According to [6], β-lactamase is processed post-translationally and secretion of this protein requires the carboxy-terminal amino acid sequence. It is therefore suggested that the presence of the signal sequence is not sufficient to ensure secretion. Cellular location of mature and precursor forms showed that the latter one that had a half-life of ~60 s, was partly cytoplasmic and that a substantial fraction was membrane-bound. The mature form, in agreement with our results, was found to be periplasmic. From these results it was suggested that β-lactamase might be produced in free-polysomes in the cytoplasm and exported post-translationally [6,13].

To accommodate these results with those presented above the following hypothesis can be presented:

The protein would be produced in membrane-bound polysomes in accordance with the presence of a signal peptide [1] used to initiate ribosome binding to the membrane. However, the polypeptide would not be transferred co-translationally in the periplasmic space and a transfer intermediate located in the cytoplasmic membrane would form.

The processing of the signal peptide would occur post–translationally in this location. This feature is not unusual and was in fact previously observed for another periplasmic protein in *E. coli* [14].

The carboxy-terminal amino acid sequence would be required for the release from the cytoplasmic membrane and transfer in the periplasmic space. The transitory binding to the cytoplasmic membrane might explain why some β-lactamase activity was detected in this fraction [5].

Acknowledgements

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