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Specific Processing of the Bacterial β-Lactamase Precursor in Saccharomyces cerevisiae

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Synthesis and processing of the bacterial enzyme β-lactamase (E.C. 3.5. 2.6) were studied in Saccharomyces cerevisiae. The 2-μm DNA vector pADH040-2 containing the yeast ADH1 promoter fused to the bacterial gene was used in order to obtain enhanced synthesis of the bacterial protein in yeast transformants. Both precursor and mature β-lactamase were shown to be present in yeast cells, the precursor being the major product. The mature enzyme was purified about 500-fold over crude extracts to apparent homogeneity and thus represents nearly 0.2% of the total yeast protein. No difference in specific activity and molecular weight could be observed when compared with the authentic β-lactamase from Escherichia coli. Specificity of the processing of β-lactamase in yeast cells was verified by partial amino acid sequence analysis demonstrating the removal of the signal peptide at the correct position.

Key words: β-lactamase, Saccharomyces cerevisiae, heterologous gene expression, preprotein, specific processing

The bacterial enzyme β-lactamase (E.C. 3.5. 2.6), encoded by the ampicillin resistance gene (bla) of the plasmid pBR322, is a secretory protein located in the periplasmic space of Escherichia coli. Transport of the enzyme is accomplished via a signal sequence that is cleaved off during membrane passage according to the signal hypothesis and specified models recently proposed [1–3]. The signal sequence comprises 23 amino acids as was shown by the comparison of the nucleotide sequence of the β-lactamase gene and the amino acid sequence of the mature enzyme [4,5].

The use of recombinant DNA techniques allows the study of events such as processing and secretion mechanisms in heterologous systems. Recently we were able to show that the β-lactamase gene is expressed in Saccharomyces cerevisiae and that the active gene product has the same molecular mass as the mature enzyme from E

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coli [6]. These findings suggested that the same recognition and specific cleavage of the signal peptide occurs in yeast as well as in E coli.

In this study we have sequenced the N-terminal amino acids of the mature β-lactamase purified from yeast. The results show that the signal peptide is cleaved off precisely. Furthermore, our analysis of the proteins synthesized from the bla gene in yeast reveals that the precursor is the predominant product. In contrast to mature β-lactamase, however, it exhibits virtually no enzymatic activity, confirming our previously published preliminary data [6].

MATERIALS AND METHODS

Preparative and Analytical Methods

Purification of β-lactamase was performed essentially as described earlier [6] with the addition of a final gel filtration step using Sephacryl-S-200 (Pharmacia, Uppsala, Sweden). As the source of enzyme, the protease-negative strain pep4-3 leu2 [7] containing the plasmid pADH040-2 was routinely used. Protein was determined according to Lowry et al [8] and analyzed by gel electrophoresis using 12.5% or 15% polyacrylamide in the presence of sodium dodecyl sulfate as described [6]. Activity of β-lactamase was visualized directly in gels after renaturation by specific staining with Nitrocefin [6]. Analysis of the β-lactamase protein using specific antibodies [6] was performed by the immune replica technique [9] using nitrocellulose paper and ^{125}I-labeled protein A (Amersham-Buchler, Braunschweig, FRG) for the final labeling.

Amino Acid Sequence Analysis

About 400 μg of purified β-lactamase from yeast transformants was loaded on a polyacrylamide gel in the presence of sodium dodecyl sulfate. After electrophoresis the proteins were visualized by staining with 0.1 M KCl at 4° C. The protein band corresponding to β-lactamase was cut out, eluted in a dialysis bag, and finally dialyzed against 10 mM sodium phosphate buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate. To 1 ml of protein solution (about 200 μg of protein) 1 ml 0.3 M N,N-dimethyl-N-allylamin-trifluoroacetic acid buffer (pH 9.5) in 65% pyridine was added. The protein was then coupled to p-phenylene diisothiocyanate-activated porous glass (230-Å pore diameter) at room temperature overnight. The glass was washed with H_2O, methanol, and ethyl ether, respectively. Amino acid sequence was determined with a solid phase sequencer (model 12, Sequemat, Watertown, USA). The derived thiazolinone amino acids were converted into the phenylthiohydantoin derivatives as described [10] and analyzed by high-pressure liquid chromatography [11].

In Vitro Processing Procedures

Yeast crude extracts in 0.1 M TRIS-HCl, 50 mM NaCl, 2mM dithiothreitol (pH 7.0) were prepared as described [6] and centrifuged at 15,000 g for 30 min. The resulting postmitochondrial supernatant, which contained between 30 and 40 mg protein per ml was centrifuged again at 100,000 g for 1.5 hr. The microsomal pellet was suspended in 1/25 of the original volume using 20 mM HEPES, 50 mM NaCl, and 5 mM dithiothreitol (pH 7.0). Processing of the β-lactamase precursor was performed in a reaction mixture of 12 μl final volume containing 1 μl of in vitro synthesized β-lactamase preprotein labeled with ^{35}S-methionine as described [6], 5 μl

0.1 M TRIS-HCl, 5 mM dithiothreitol (pH 7.0), 4 μl H_2O and 3 μl of yeast microsomes (P100) or 100,000 g supernatant (S100), respectively. Incubation was at 25°C for one hr and labeled polypeptides were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate followed by fluorography [6]. If indicated, 1–10 μg of trypsin (Serva, Heidelberg, FRG) was added and incubation was continued for another 30 min.

RESULTS

Identification of Precursor and Mature β-Lactamase in Yeast Transformants

Yeast transformants harboring plasmid pMP78-1 with the intact bacterial bla gene [12] exhibit low levels of β-lactamase activity [6] due to the weak function of the prokaryotic promoter in yeast [13]. Therefore, analysis of the gene products by polyacrylamide gel electrophoresis was difficult in yeast crude extracts. Consequently, partially purified β-lactamase was used in all studies [6]. However, the use of partially purified enzyme fractions bears the risk of missing original translation products. To avoid such problems, we replaced the prokaryotic promoter of the bla gene by the yeast ADH1 promoter [14] resulting in the plasmid pADH040-2 (Fig. 1). The yeast strain pep4-3 was transformed with this plasmid and used for enzymatic studies. The resulting high β-lactamase activity in such transformants allows identification of β-lactamase activity in sodium dodecyl sulfate polyacrylamide gels using crude extracts. After renaturation of the fractionated proteins in the gel the β-lactamase-specific Nitrocefin color assay revealed two activities (Fig. 2A, lane 3).

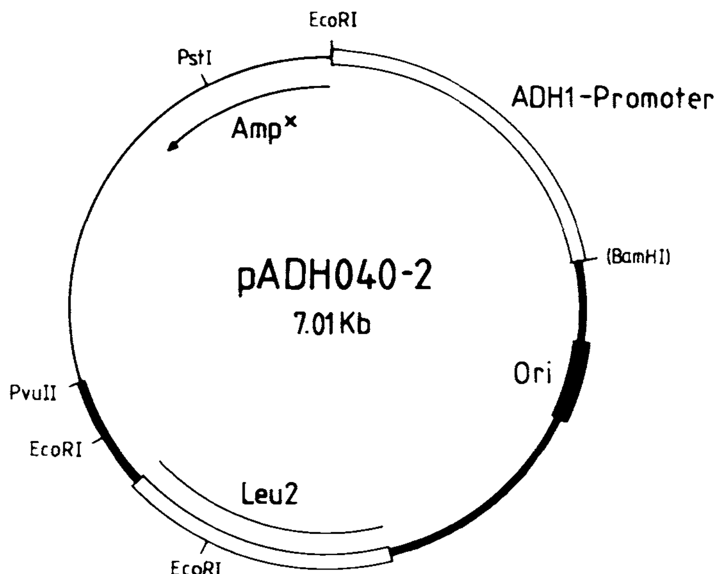
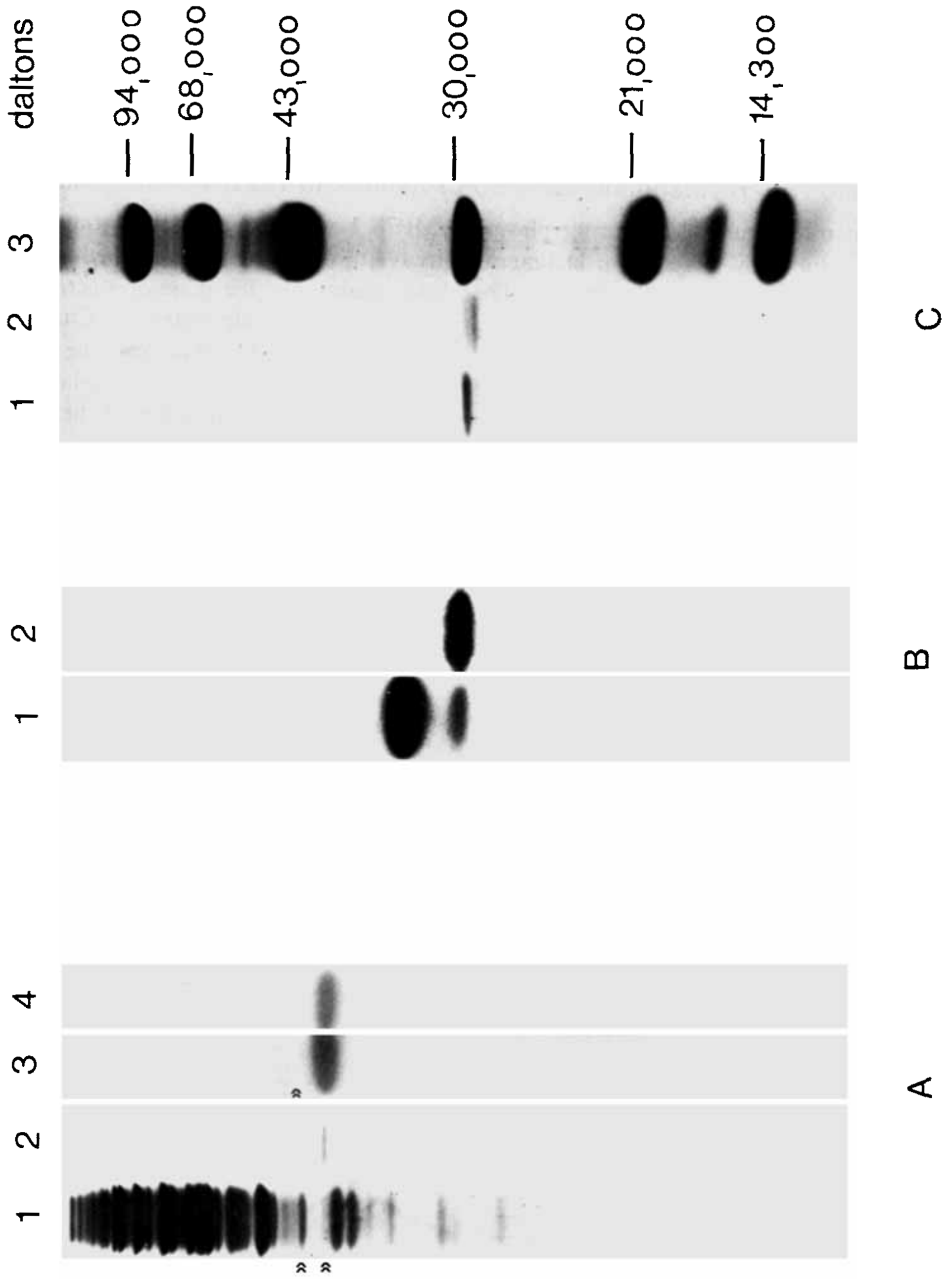


Fig. 1. Plasmid pADH040-2 contains the β-lactamase gene derived from plasmid pBR322 in which the prokaryotic promoter has been replaced by the ADH1 promoter of yeast [16]. For selective expression in yeast, the PvuII-BclI fragment of pJDB219 [15] containing part of 2-μm DNA and the yeast LEU2 gene was used, further details of the plasmid construction given in [16].


daltons
94,000
68,000
43,000
30,000
21,000
14,300
1
2
3
1
2
1
2
3
4
A
B
C

One coincides with the position of mature β-lactamase, the other with that of the preprotein. However, the latter activity was extremely low compared to that of the mature enzyme (Fig. 2A, lane 3) as evident from the opposite relative intensities of the precursor and mature protein bands in immunoblots of the same gels using β-lactamase-specific antibodies. The band at the position of the precursor shows a strong labeling with an intensity of about one order of magnitude higher than that of the mature enzyme (Fig. 2B, lane 1). This difference of intensities is in accordance with the Coomassie blue stain showing a heavily stained band at the position of the preprotein and a weak band at the position of mature β-lactamase. Both protein bands are absent in the nontransformed yeast. It can be concluded that besides mature β-lactamase significant amounts of the preprotein are present in yeast transformants and that β-lactamase activity of the preprotein is hardly detectable, as reported earlier [6]. It should be noted that the precursor of the enzyme cannot be detected in crude extracts derived from E coli cells (Fig. 2B, lane 2).

Properties of Purified β-Lactamase From Yeast Transformants

Crude extracts of yeast transformants carrying the plasmid pADH040-2 exhibit high β-lactamase activities [16] allowing the purification of the enzyme. The final preparation yielded homogeneous protein having the same specific activity and molecular mass as the purified β-lactamase from E coli (Fig. 2C, lane 1, Table I). As discussed previously, β-lactamase appeared on polyacrylamide gels in the presence of sodium dodecyl sulfate as a double band, due to differential denaturation [6]. Our preparations of purified β-lactamase from yeast and E coli (Fig. 2C, lanes 1 and 2) shows that the yeast enzyme is present mainly as the slower migrating form, whereas the main part of the enzyme from E coli is present at the position of the faster-migrating band of the doublet. Possibly, such differences between the enzyme from

TABLE I. Purification of β-Lactamase From Yeast Transformant pep4-3-Carrying Plasmid pADH040-2*

Procedure	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Yield (%)	Purification
Crude extract	9,625	0.36	3,500	100	1
Ultracentrifugation	5,100	0.6	3,060	87	1.7
I. DEAE cellulose	400	2.0	800	23	5.6
II. DEAE cellulose	68	5.2	360	10	14
Sephacryl S-200	1.4	208	298	8.5	580

*Starting material was 60 gm of yeast cells (wet weight). Specific activity of β-lactamase from E coli when purified under the same conditions was 249 units/mg protein. DEAE, diethylaminoethyl.

Fig. 2. Analysis of the β-lactamase gene products of yeast transformant pep4 containing pADH040-2 by sodium dodecyl sulfate gel electrophoresis. A) Lane 1, Coomassie blue staining of proteins from a crude extract (40 μg of protein); small arrows give the position of the activity staining, which is shown in lane 3. Lane 2, purified β-lactamase of E coli (0.2 μg of protein), and lane 4, its activity staining. B) Lane 1, immunoblot of yeast crude extract (40 μg of protein), and lane 2, of crude extract from E coli (28 μg of protein). C) Lane 1, purified β-lactamase from yeast transformant pep4 containing pADH040-2 (1 μg of protein) and lane 2, purified β-lactamase from E coli. Polyacrylamide concentrations were 15% (A) and 12.5% (B and C). Further details are given in Materials and Methods.

yeast and that from E coli might be caused by modifications during membrane passage as suggested by Koshland et al [17].

It can be calculated from the amount of purified enzyme that about 0.2% of total yeast protein is mature β-lactamase (Table I). Based on the rough estimation of a ten times higher amount of precursor (Fig. 1), we judge that about 2% of total yeast protein represents the primary product of the bla gene. The preprotein is separated from the enzyme by column chromatography, and its purification and further analysis will be reported (Roggenkamp and Hollenberg, in preparation). It should be noted in this context that mature β-lactamase could be obtained with similar yields and properties from yeast transformants other than that of the protease-negative yeast strain pep4. However, such preparations were less stable, especially in the presence of sodium dodecyl sulfate. This fact is obviously correlated to vacuolar proteases, which are almost completely absent in the pep4-3 strain [7].

Determination of NH_2-Terminal Amino Acids of Yeast β-Lactamase

The determination of molecular mass of the partially purified β-lactamase from yeast transformants studied so far led to the tentative conclusion that the protein is processed to the same mature form as in E coli [6]. However, the specificity of the cleavage can only be deduced from the NH_2-terminal amino acids. Hence, automated Edman degradation of yeast β-lactamase was performed. The derived sequence in Table II clearly shows that the matured yeast enzyme is identical to that of the prokaryotic one, which has been sequenced completely by Ambler and Scott [5]. The results strongly suggest that yeast contains a signal peptidase that is able to recognize a prokaryotic signal sequence and to remove precisely the signal peptide comprising 23 amino acids.

TABLE II. Automated Edman Degradation* of N-Terminal Amino Acids of Purified Mature β-Lactamase From Yeast Transformant pep4-ADH040-2

Cycle	Amino acid	
1	X	(His)[a]
2	Pro	(Pro)
3	Glu	(Glu)
4	Thr	(Thr)
5	Leu	(Leu)
6	Val	(Val)
7	Lys	(Lys)
8	Val	(Val)
9	Lys	(Lys)
10	Asp	(Asp)
11	Ala	(Ala)

*Analysis of the phenylthiohydantoin derivatives was performed by high-pressure liquid chromatography as described in Materials and Methods.
[a]Amino acids given in parentheses show the N-terminal sequence of the authentic enzyme from E coli. Data are taken from Ambler and Scott [5].

Processing Activities in Yeast Crude Extracts

The β-lactamase preprotein synthesized in an E coli in vitro transcription and translation system [18] can be partially processed to a protein of the same molecular mass as the mature enzyme by the addition of crude extracts derived from yeast cells [6]. Further studies on this cleavage reaction revealed that at least two different activities are involved. One activity was found in the microsomal fraction; the other appeared as a soluble fraction (Fig. 3A,B).

The microsomal activity generates a processed form of β-lactamase, which is at least partially resistant to subsequent tryptic digestion, indicating that processing is coupled with transport of the derived protein into membrane vesicles (Fig. 3A, lanes 5 and 6). This processing activity, however, was always limited to a certain fraction of preprotein and could neither by improved by longer incubations nor by the addition of larger amounts of yeast microsomes (not shown). Instead, the extent of processing was proportional to the amount of added preprotein (Fig. 3A, lanes 2–4), suggesting the additional requirement for a factor that is present in the bacterial in vitro transcription and translation system containing the preprotein. Since in the bacterial system very small amounts of mature form are always present (Fig. 2A, lane 1), we suspected that traces of small bacterial membrane vesicles were possibly involved in the processing reaction. This possibility was confirmed by testing various methods for the preparation of the Zubay system. If the E coli cells were disrupted by glass beads instead of a French press, as described originally by Zubay et al [18], in order

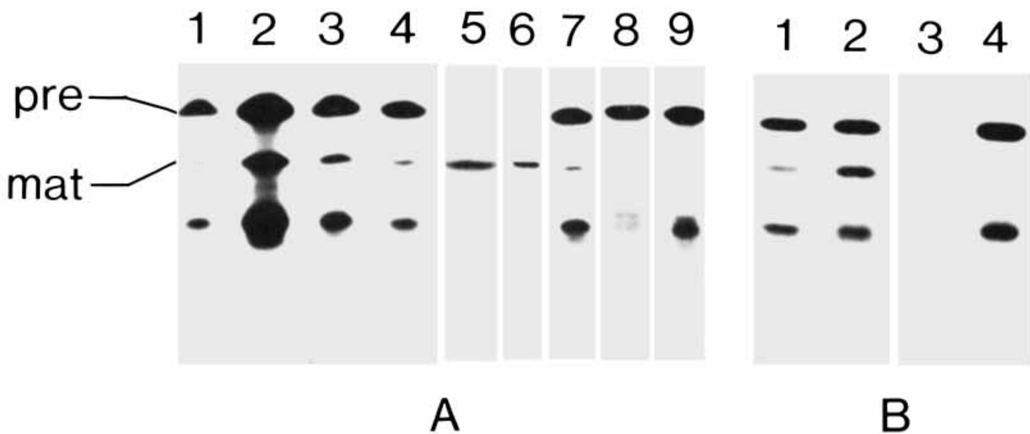


Fig. 3. Processing of the in vitro synthesized precursor of β-lactamase by yeast microsomes and 100,000 g supernatant. See Materials and Methods for experimental details. Pre, precursor; mat, mature form. A) Lane 1, 2 μl of the in vitro synthesized precursor; lanes 2-4, various amounts of in vitro synthesized precursor; ie, 5 μl, 2 μl, and 1 μl with the addition of 3 μl of yeast microsomes, respectively; lane 5, as 2, with the addition of 10 μg of trypsin; lane 6, as 3, with the addition of 10 μg of trypsin; lane 7, as 3, in the presence of 0.3×10^{-3} M N-methyl-maleimide; lane 8, 2 μl of in vitro synthesized precursor, but unlike lanes 1-7, protein mix for synthesis derived by homogenization with glass beads; lane 9, as 8, with addition of 3 μl of yeast microsomes. B) Lane 1, as A, lane 1, but with addition of 3 μl yeast S100 fraction and 0.3×10^{-3} M $ZnCl_2$; lane 2, as lane 1, but $ZnCl_2$ omitted; lane 3, as lane 2, with the addition of 10 μg of trypsin; lane 4, as lane 2, but yeast S100 fraction prepared from the protease-negative strain pep4.

to avoid these contaminants, virtually no processing was observed in the presence of yeast microsomes (Fig. 2A, lanes 8 and 9). We hypothesize that a certain amount of the in vitro synthesized β-lactamase precursor is inserted into the membranes of the bacterial vesicles and that addition of yeast microsomes mediates transport and processing of the protein. Whether the processing that is inhibited by sulfhydryl-blocking reagents (Fig. 3A, lane 7) occurs in bacterial or yeast membranes cannot be answered at this moment. Evidence that the processing is of bacterial origin can be deduced from experiments by Chang et al [19], who were able to show that mere addition of detergents can stimulate the processing of the in vitro synthesized precursor. Under our experimental conditions, however, the activity of the yeast microsomes, which was heat labile, could not be simulated by the use of detergents.

In contrast to the microsome-dependent processing described above, the cleavage activity present in a soluble yeast fraction is independent of factors of bacterial origin and transport through membranes as shown by the trypsin accessibility (Fig. 3B, lanes 2 and 3). The efficiency of processing was both dependent on time and on the amount of the soluble fraction added to the reaction mixture (not shown). The activity was found to be sensitive to Zn^{++} (Fig. 3B, lane 1). Surprisingly this activity was not detectable if crude extracts were prepared from the protease-negative strain pep4-3 (Fig. 3B, lane 4). Since this yeast strain is deficient in the maturation process of a variety of vacuolar enzymes [7], it is evident that either the protease responsible for maturation or one or more of the vacuolar proteases causes the shortening of the β-lactamase preprotein. Although apparently this process is rather specific, it does not play a significant role, if any, in vivo. This is demonstrated by the fact that the activity of β-lactamase in pep4-3 transformants is as high as in other yeast transformants (Table I) [16]. Since, as shown above, the preprotein is almost inactive, a pep4 transformant should exhibit no or a very low enzyme activity if a PEP4-dependent protease would be responsible for in vivo processing.

DISCUSSION

Our previous work had shown that expression of the bacterial β-lactamase gene in yeast is accompanied by the processing of the precursor [6]. The evidence was obtained by determination of the molecular mass of the active enzyme that had been partially purified. Here we have demonstrated the specificity of the processing by determining the amino acid sequence at the NH_2-terminal end. This additional evidence was required because, as we have also shown here, the size of the β-lactamase precursor can be reduced by vacuolar proteases, at least under in vitro conditions. Such nonspecific proteolytic reactions can also be mediated in vitro by trypsin, as has been shown by others [20,21].

The efficiency of maturation of the β-lactamase precursor in yeast transformants is low; our data have revealed that the precursor is the major product. However, it should be emphasized that the absolute amount of the mature form is quite high, being 0.1–0.2% of total yeast protein, as much as that found for invertase, a processed and secreted enzyme in S cerevisiae [22]. Recently performed pulse-chase experiments are consistent with a co-translational type of processing (unpublished results). Therefore, we assume that the β-lactamase precursor is inserted into the endoplasmic reticulum, although this remains to be shown directly. Recent support of this view has been obtained from in vitro experiments which indicated that the preprotein of β-

lactamase, as a eukaryotic protein, is cotranslationally inserted into dog pancreas microsomes [21], and that this process is dependent on the signal recognition particle [23–25].

Given the receptor-mediated processing of β-lactamase in yeast, one could argue that the efficiency of the processing is limited by the number of signal recognition particles due to the high synthetic rate of the protein, about 2% of the total yeast protein. However, that seems not to be the case, since low expression vectors like pMP78-1 [6] show the same ratio of preprotein to mature form (our unpublished observations). Processing of only a fraction of the preprotein could also be the result of a low binding efficiency of the presumptive yeast signal recognition particle to the heterologous signal peptide. In this way the majority of nascent chains would escape mediated control of the signal recognition particle. Thus what factors are involved in β-lactamase processing and whether these are part of the authentic yeast secretory pathway are questions that remain to be answered.

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