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Penicillin acylase from *E. coli*: unique gene–protein relation

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ABSTRACT

The nucleotide sequence of the gene (pac) coding for penicillin G acylase from E. coli ATCC 11105 was determined and correlated with the primary structure of the two constituent subunits of this enzyme. The pac gene open reading frame consists of four structural domains: (i) Nucleotide positions 1 - 78 coding for a signal peptide, (ii) positions 79 - 705 coding for the α subunit, (iii) positions 706 - 867 coding for a spacer peptide, and (iv) positions 868 - 2538 coding for the ß subunit. Plasmids were constructed which direct the synthesis of a pac gene product lacking the signal peptide, and the synthesis of the α subunit or the ß subunit. The following results were obtained: (i) The two dissimilar subunits are processing products of a single precursor polypeptide; the spacer peptide is removed during processing; (ii) the precursor polypeptide lacking the signal sequence is accumulated in the cytoplasm; it is not processed proteolytically in the cytoplasm and it does not display enzyme activity. Processing, therefore, requires translocation through the cytoplasmic membrane; (iii) processing follows a distinct sequential pathway in vitro.

INTRODUCTION

Penicillin acylase (penicillin G amidohydrolase; E.C. 3.5.1.11) displays - apart from its use as a tool for the production of semisynthetic penicillins - a number of interesting basic properties. Catalytically active enzyme is an αß heterodimer and is localized in the periplasmic space of producing E. coli cells (1, 2, 3). The two subunits are derived from a membrane-bound, single polypeptide precursor via a processing pathway hitherto unique in its features for a prokaryotic enzyme (1, 2). It has been postulated that proteolytic processing of the membrane-bound precursor might be mechanistically coupled to the release of the subunits into the periplasm (1, 2).

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In this communication we report on the detailed gene-protein relationship of the enzyme. It is shown that the penicillin acylase structural gene (pac) consists of the following functional segments: (i) a 78 bp leader peptide sequence; (ii) 627 bp coding for the α subunit; (iii) 162 bp coding for a spacer peptide of 54 amino acids which is removed during proteolytic processing, and (iv) 1671 bp coding for the ß subunit. This structure has been proven by the construction of plasmids which overexpress either the α or ß subunits alone. Moreover, the pathway of processing was delineated by in vitro experiments employing a genetically engineered pac gene lacking the signal sequence information.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strain 54-2 which is Δ(pro-lac), recA, rpsL/F' $lacI^q$ Z Δ M15 $proA^+B^+$ was used as host strain for the expression of pac genes carried by plasmids. The medium used contained 1 % yeast extract (w/v), 0.5 % meat extract (w/v) and 0.2 % NaCl (w/v). Cultivation temperature was 37°C throughout.

Plasmid pHM12 (4) carries the pac gene together with the homologous upstream control region. The HindIII fragment of pHM12 which contains the pac gene was cloned into the HindIII site within the lac UV5 promoter of plasmid pE1 (gift of U. Ruether). The resulting plasmid pE1-11 contains the pac gene under the control of the lac UV5 promoter. Plasmid pKK177-3 is an expression vector bearing a hybrid trp-lac promoter, a multi-linker and two consecutive transcription termination sites (5).

Genetic techniques

Standard techniques were employed for preparation of plasmid DNA, for restriction enzyme cleavage of DNA, for S1 nuclease treatment and DNA polymerase I (Klenow fragment) incubation and for ligation and transformation (6, 7).

DNA sequence analysis was carried out by the chemical cleavage method of Maxam and Gilbert (8); both strands were sequenced throughout. Suitable restriction sites for labeling of DNA fragments were created by cleavage of DNA with a non-

-specific double-strand-directed endonuclease and by insertion of an EcoRI linker (9). Deletions were created by subsequent digestion with EcoRI (plasmid pBTE1-11 was used for sequencing which contains a unique EcoRI site) and the sizes of the fragments were analyzed. Clones were chosen the plasmid size of which differed by 200 to 300 bp. The deletion clones were opened at the EcoRI site and labelled at the 5' end with polynucleotide kinase and at the 3' recessed end with Klenow fragment. In most cases, the 5' and 3' labelled strands were analyzed on the same gel (6). Oligodesoxyribonucleotides were synthesized according to Crea et al. (10).

Other techniques

The N-terminal amino acid sequences of penicillin acylase α and ß subunits were determined by the DABITC/PITC double coupling method (11). The C-terminal amino acids were analyzed with the aid of carboxypeptidases B (12) and Y (13).

Antisera were prepared by immunizing rabbits with the small or large subunit of penicillin acylase or with holo-enzyme, respectively. The immunization protocol described by Schmid and Böck (14) was followed. Total cell lysates were obtained by suspending cells in sample buffer (15) and incubating them at 100°C for 4 min. The cell lysates were separated on 12.5 % polyacrylamide slab gels (15) and investigated for cross-reacting material by the immunoblotting technique of Howe and Hershey (16).

Penicillin acylase activity was assayed by following the formation of 6-amino-penicillanic acid with the aid of the colorimetric method of Balasingham et al. (17).

Materials

Restriction endonucleases, S1 nuclease, DNA polymerase I (Klenow fragment) and T4 DNA ligase were supplied by Boehringer Mannheim GmbH. Plasmid pKK177-3 was obtained from J. Brosius.

RESULTS AND DISCUSSION

Nucleotide sequence of the pac gene and correlation with gene products

Previous experiments have shown that the complete coding information for penicillin acylase is located on a 3.0 kbp HindIII restriction fragment of plasmid pHM12 (4). The

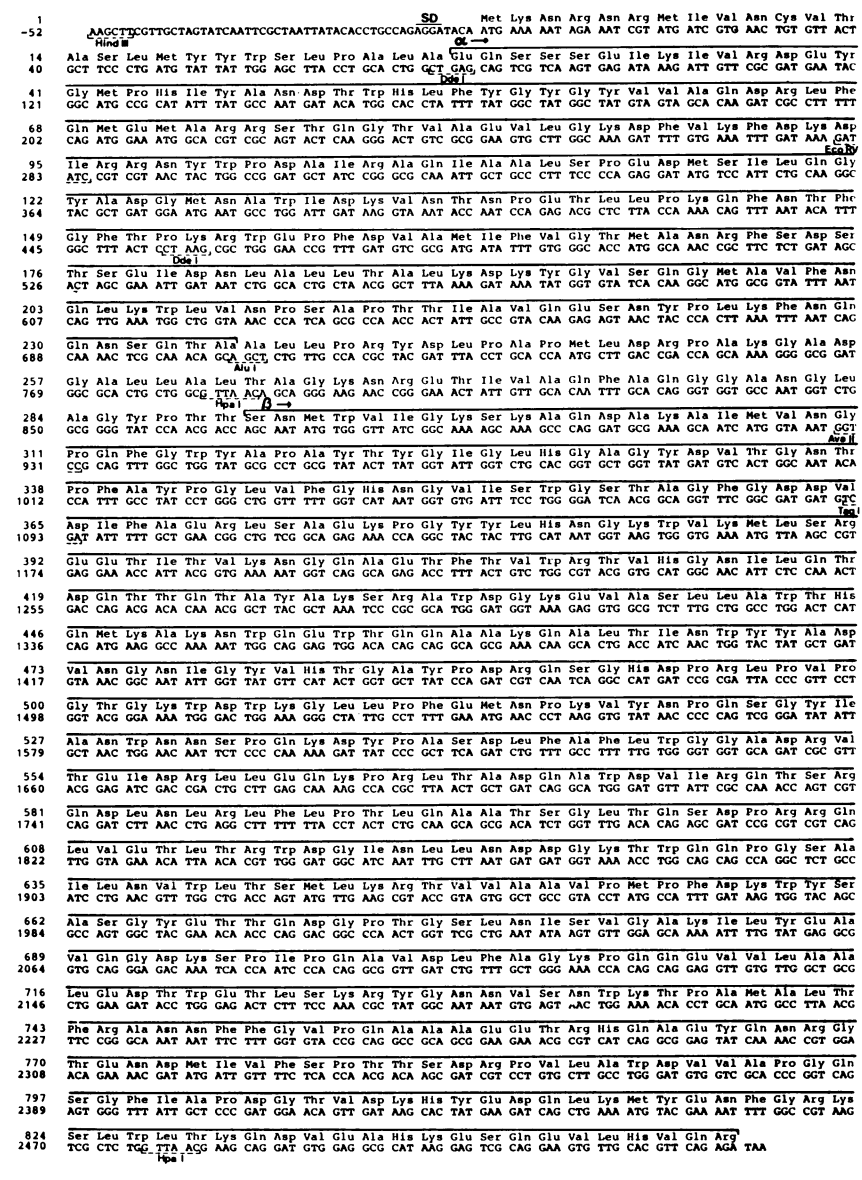

Fig. 1. Nucleotide sequence of the penicillin acylase structural gene. α and ß subunit sequences are indicated by a line above the amino acid sequence. A signal peptide of 26 amino acids is located at the N-terminus of the precursor molecule. The α and ß subunits are separated in the precursor polypeptide by a spacer peptide of 54 amino acids. Restriction sites relevant for the plasmid constructions are given. The ribosomal binding site is indicated by a solid line above the sequence.

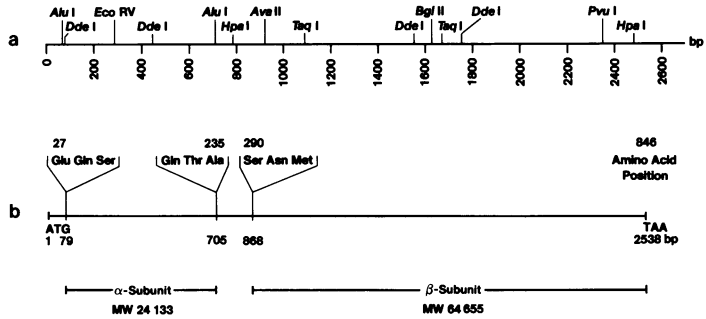

Fig. 2. Gene-protein relationship of penicillin acylase.
a) Restriction map of the nucleotide sequence encoding the penicillin acylase structural genes.
b) Details of the regions coding for the α and ß subunits of penicillin acylase. The lower numbers represent the base-pair sequence as in a), with the translation start-site being labeled +1. The upper numbers refer to the amino acid positions of the initial and final residues of the α and ß subunits, respectively. The initial three amino acid residues of the α and ß subunits are given, along with the final three residues of the α subunit.

nucleotide sequence of this 3.0 kbp fragment was determined; Fig. 1 shows the result. Translation of the sequence into the amino acid sequence indicates that the HindIII fragment contains an open reading frame of 2538 nucleotides with the coding capacity for a protein of 846 amino acids. In the region flanking the pac gene at the 5' side, there is a sequence motif recognized in E. coli as a ribosome binding site (see Fig. 1). At the 3' side the open reading frame ceases with a UAA termination codon. During the preparation of this manuscript a report appeared on the nucleotide sequence of about 1/3 of the pac gene (3). Apart from a few deviations there is general agreement with the respective pac sequence portions reported in this communication.

To correlate the nucleotide sequence of the pac gene with the sequence of the protein products, namely with those of the α and ß subunits of penicillin acylase, the N-terminal and C-terminal amino acid positions of the two subunits were determined. The following results were obtained:

	N-terminus	C-terminus
α subunit:	H_2N-Glu-Gln-Ser-Ser-Ser-	-Gln-Thr-Ala-COOH
ß subunit:	H_2N-Ser-Asn-Met-	-Val-Gln-Arg-COOH

A computer search for the occurrence of these sequences within the pac open reading frame gave the positions summarized in Fig. 2. Each sequence occurred only once and, therefore, unequivocally indicates the start and termination sites of the coding information for the respective subunits. Accordingly, α is encoded by the aminoterminal and ß by the carboxyterminal part of the pac open reading-frame. The C-terminus of the α subunit is separated from the N-terminus of the ß subunit by a "spacer peptide" consisting of 54 amino acids. The relevance of this "endopeptide" - as we shall address it - for processing and for protein folding is discussed below. The nucleotide sequence coding for the α subunit is preceded by a sequence possessing the consensus features for a signal peptide (18) which is in accordance with the fact that penicillin acylase is exported into the periplasmic space.

It should be mentioned that the sizes of the pac precursor polypeptide and of the subunits deduced from the DNA sequence are in excellent agreement with those determined previously at the protein level (1, 2). Apart from the cysteine residue within the leader peptide, this amino acid is neither represented in the subunits nor in the endopeptide sequence.

Construction of a plasmid encoding the sequence of pac lacking the signal sequence and of plasmids directing the synthesis of either the α or ß subunits

In order to investigate whether translocation of the pac gene product to the periplasmic space is indeed linked to proteolytic processing, we have constructed a plasmid with the coding information for pac lacking the signal sequence, i.e. the 78 bp region at the 5' end. As a consequence, leader-less precursor should accumulate in the cytoplasm and should provide an answer to the question of whether there is processing without export. Fig. 3 gives details of the crucial steps of the construction.

In short, plasmid pE1-11 was cut with HpaI and the 6 kbp fragment obtained was shortened from each end by approximately 500 bp with Bal31 exonuclease. An in-frame fusion was constructed between the N-terminal pac gene portion, beyond the EcoRV restriction site, and the lacZ gene. The fusion plasmid

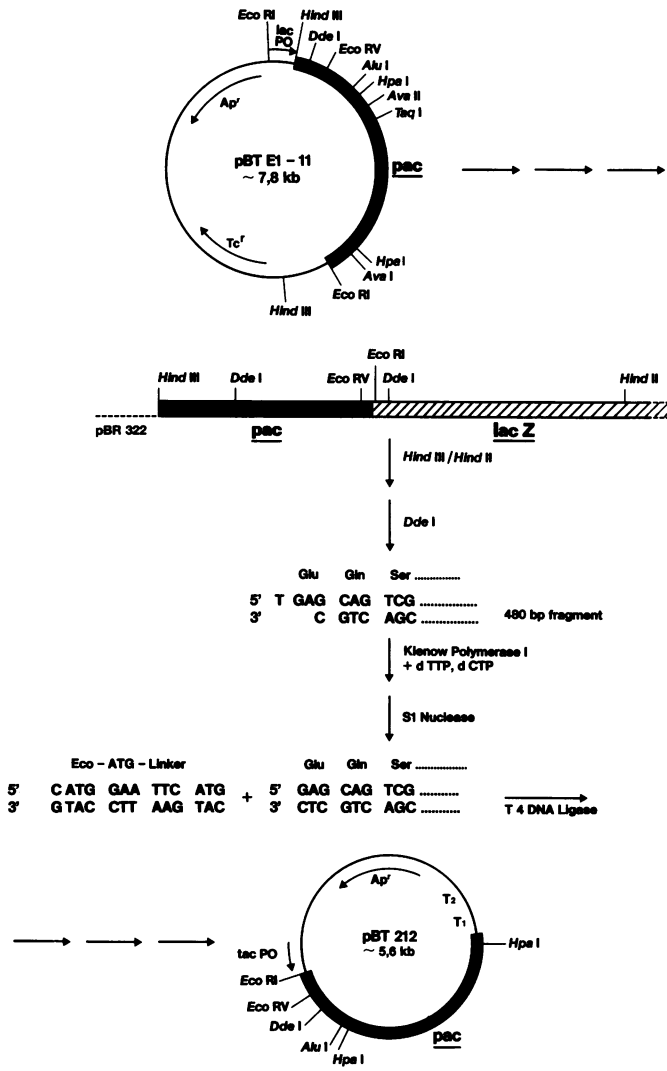

Fig. 3. Construction of plasmid pBT212 coding for a penicillin acylase precursor which is lacking the signal peptide. For details see text. For sake of clarity only those restriction sites are given which are important for delineating the construction strategy.

was cut with HindIII and HindII and the relevant 800 bp fragment was digested with DdeI to obtain a 480 bp DdeI fragment which contains the coding information for the N-terminus of the α subunit and reaches into the lacZ coding area

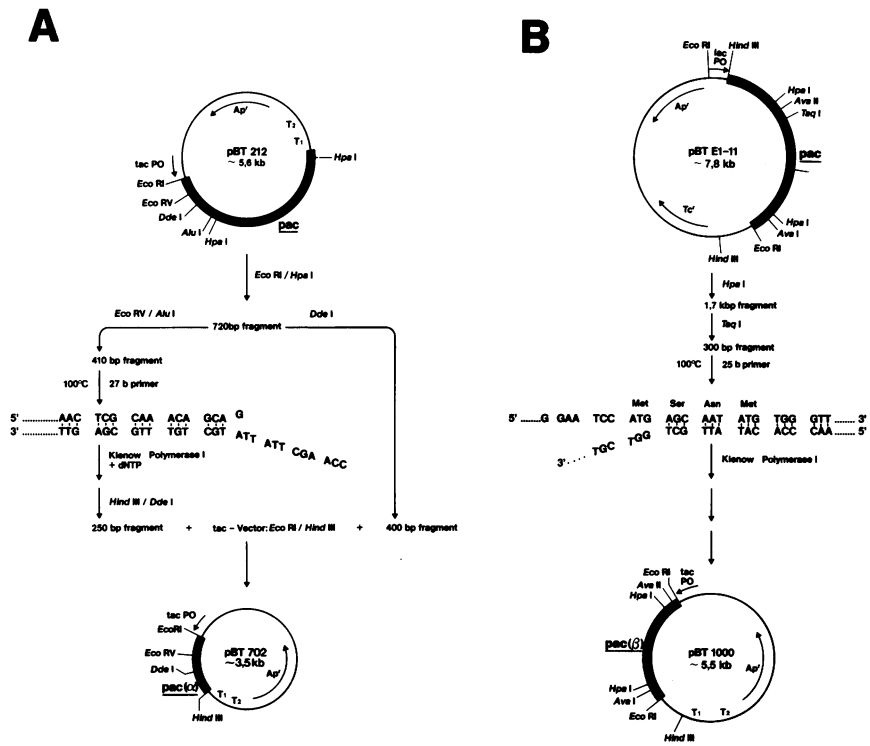

Fig. 4. Constructions of plasmid pBT702 coding for the α subunit of penicillin acylase (A) and plasmid pBT1000 encoding the ß subunit (B). See text for details.

(see Fig. 3). Two bases were filled in at the "sticky" end with polymerase I and the residual dT was removed with S1 nuclease. An Eco-ATG-linker was added by blunt end ligation. The resulting construct was cut with EcoRI and EcoRV and ligated to the "downstream" EcoRV-AvaI fragment of the pac gene and inserted into plasmid pKK177-3. The pac-lacZ fusion was chosen for this construction in order to check whether the manipulations at the DNA level (Klenow or S1 treatment, re-ligation) kept the gene in frame. This was confirmed by screening the colonies on X-gal plates after re-ligating the leader-less pac gene to the lacZ part. The 5' EcoRI site of the lacZ gene was used for this construction. The resulting plasmid, pBT212, contains the leader-less pac gene under expression control of the tac hybrid promoter (5).

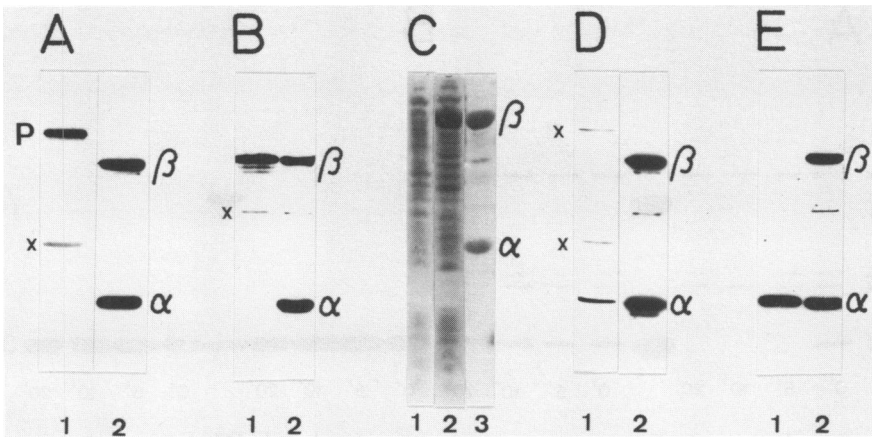

Fig. 5. Immunoblot analysis of total cell lysates of E. coli strain 54-2 harbouring plasmid pBT212 (A; lane 1), plasmid pBT1000 (B; lane 1), plasmid pBT702 (D; lane 1) and plasmid pBT702II (E; lane 1). Cells were grown in the presence of 1 mM IPTG and the cell lysates were separated on 12.5 % SDS-polyacrylamide gels. The proteins were blotted to nitrocellulose by electrotransfer and the blotted filters were incubated with antiserum directed against penicillin acylase holoenzyme. Detection of cross-reacting material was carried out using ^{125}I-labeled S. aureus protein A. The resulting autoradiographs are shown above. Lanes 2 give the position of the α and ß subunits of purified enzyme. P denotes the migration of the precursor molecule, x marks bands which react unspecifically with the antiserum used.
C shows total cell lysates of 54-2/pBT1000, grown without addition of IPTG (lane 1), three hours after induction with IPTG (lane 2) and purified penicillin acylase (lane 3). Cell lysates were separated on 12.5 % SDS polyacrylamide gels.
Plasmid pBT702II is like plasmid pBT702 with the exception that it contains the coding information for the additional four amino acids (H_2 N-Met-Tyr-Tyr-Phe) preceding the sequence coding for mature α subunit (to be published elsewhere).

Plasmids were also constructed which code for the presumptive α or ß part of the pac gene product. With the aid of the plasmids, it is possible to definitely prove that the α and ß subunits are proteolytically derived from a single precursor polypeptide; in addition, they provide a tool for the analysis of the function of the endopeptide in the subunit folding pathway and in subunit association. The essential steps in the construction procedure are summarized in Fig. 4.

For the construction of a plasmid expressing the α subunit, the EcoRI/HpaI 720 bp fragment of the pac gene carried by

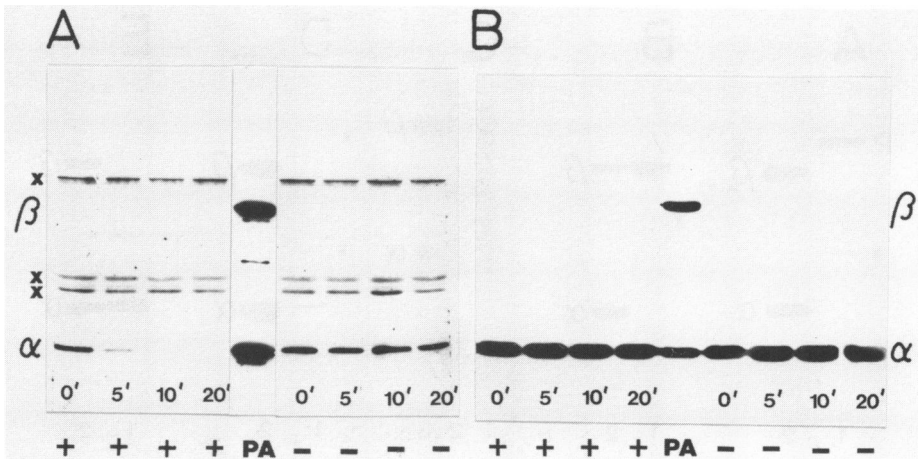

Fig. 6. Immunoblots of cell lysates from E. coli strain 54-2 harbouring plasmid pBT702 (A) and plasmid pBT702II (B). Cells were grown in presence of 1 mM IPTG. 100 µg chloramphenicol was added, per ml of culture, at an OD_{420} of 0.8 and samples were taken at the times indicated (lanes marked +). The immunoblot analysis was carried out as described for legend of Fig. 5. The filters were treated with anti-holoenzyme antibodies. Cultures without chloramphenicol served as control (lanes marked -). Purified penicillin acylase is shown in lanes designated PA. For explanation of x see legend for Fig. 5.

pBT212 was isolated (Fig. 4A). A 410 bp fragment was obtained from it by EcoRV-AluI digestion (the AluI site covers the 3' end of the α subunit coding region) and two tandem stop codons and a HindIII restriction site were added by oligonucleotide repair synthesis (19). The final construct, pBT702, was obtained by ligating a 250 bp HindIII-DdeI fragment and a 400 bp EcoRI-DdeI fragment into EcoRI-HindIII digested tac-vector pKK177-3.

The construction of a plasmid expressing the ß subunit part of the pac gene followed a similar procedure. It is outlined in Fig. 4B. The crucial step consisted of the oligonucleotide-directed addition of an ATG codon and of an EcoRI restriction sequence to the 5' end of the pac ß subunit coding region.

All constructs (pBT212, pBT702, pBT1000) were analzyed for their identity by DNA sequencing over the 5' and 3' fusion

points. From the details of construction it is obvious that an ATG codon precedes the respective open reading frames; the derived gene products, therefore, are supposed to contain an N-terminal methionine residue not present in the wild-type gene products.

Expression studies

Plasmids pBT212, pBT702 and pBT1000 were transformed into E. coli strain 54-2. The transformants were grown to the exponential phase, induced by the addition of IPTG and the SDS lysates of cells were analyzed for relevant pac gene products by the immunoblotting procedure described (1, 2). Fig. 5 shows the results. Each plasmid directed the synthesis of the expected protein product. It was particularily interesting to observe that the precursor protein accumulated by the pBT212 containing transformant was not subject to any processing steps (Fig. 5A). The amount of ß subunit produced by pBT1000/54-2 was considerable and amounted to 40 to 50 % of the total cellular protein (Fig. 5B/5C). The expression of α subunit, as directed by pBT702, resulted in the accumulation of less material (Fig. 5D). A more detailed analysis showed that the subunit undergoes rapid degradation in the cytoplasmic compartment as visualized by the disappearance of cross-reacting material when synthesis is blocked by an antibiotic (Fig. 6A). This degradation of the subunit could be prevented or reduced by expressing both α and ß subunit within the same cell , or by genetically extending the N-terminus of the α subunit by an additional four amino acids (H_2N-Met-Tyr-Tyr-Phe-) from the lacZ-lacY gene border (to be published elsewhere). This tetrapeptide stabilizes the subunit (Fig. 6B); as a consequence this subunit is accumulated to an extent comparable to that of the ß subunit in the pBT1000 transformants (Fig. 5E).

Transformants synthesizing either α or ß subunit alone did not exhibit any penicillin acylase activity, either in vivo or in vitro. Under conditions of gross overproduction they contained between 1 and 4 refractile inclusion bodies. The major part of pac cross-reacting material was present in cell extracts in a particular and sedimentable state. As the pac protein does not contain any cysteine residues the formation of

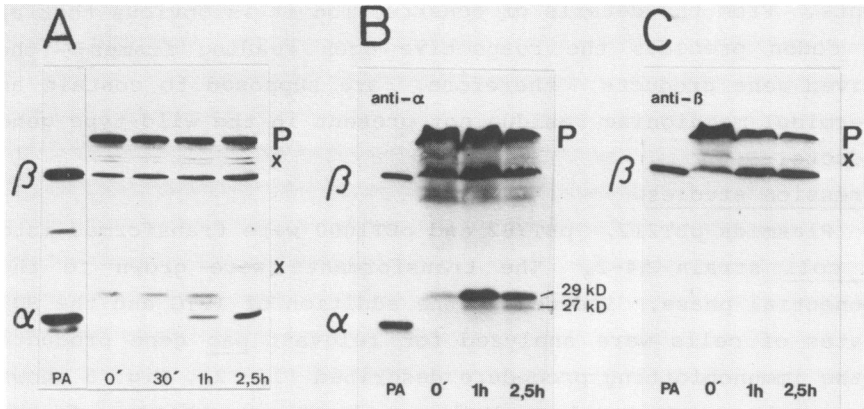

Fig. 7. Immunoblots of cell lysates from E. coli strain 54-2 harbouring plasmid pBT212. Cells were grown in presence of 1 mM IPTG. Crude extracts were prepared as described in the text and incubated at 30°C. Samples were taken at the times indicated. Purified penicillin acylase is shown in lanes designated PA. A, B and C give the reaction with antiserum directed against holoenzyme, α and ß subunit, respectively. For explanation of P and x see legend for Fig. 5.

refractile bodies cannot be due to the formation of Cys-Cys interaction.

Processing of the pBT212 encoded precursor polypeptide

pBT212/54-2 cells, when harvested and immediately lysed with SDS, contained only pac precursor (Fig. 5A). When the cells were broken in the French press prior to the addition of the detergent, ß and α subunits appeared on the immunoblots (Fig. 7A).

A substantial fraction of intact precursor in the cellular homogenate is present in the cytoplasmic supernatant, the remainder sediments with the membrane fraction. There is no cross-reacting material in the periplasmic fraction (not shown) prepared with the chloroform permeabilization procedure (20). Altogether, these findings support the notion that pac precursor is accumulated in the cytoplasm in soluble form and that it is only proteolytically processed when cell compartmentalization is disrupted.

In the in vitro processing kinetics ß subunit was detectable "earlier" than α subunit. Consistently, cross-

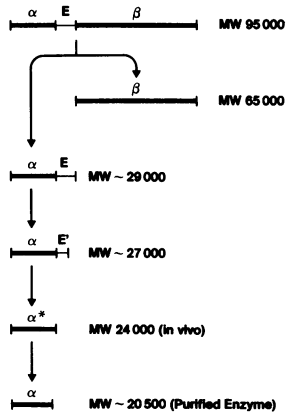

Fig. 8. Processing pathway of the penicillin acylase precursor as delineated by in vitro maturation experiments. α (α*) and ß denote the small and large subunit, respectively, which form the active enzyme. E designates the spacer peptide and E' a spacer peptide shortened at the C-terminus.

reacting material of 29 and 27 kD preceded the appearance of α subunit and indicated a precursor-product relationship (Fig. 7A). To analyze whether these polypeptide species might constitute processing intermediates of the α subunit (which may contain the pac endopeptide or part of it), the immunoblots were developed with antibodies specific for ß or α subunit polypeptides. Fig. 7B and C, indeed, show that the putative intermediates only react with anti-α subunit but not with anti-ß subunit antibodies.

The interpretation of the in vitro processing experiments of Fig. 7 requires the following information: (i) The anti- α sera - in contrast to the anti-ß directed ones - are not pure. They contain anti-ß antibodies (Fig. 7B). The reason is that purification of the subunits can only be achieved by gel chromatography in the presence of SDS. A certain portion of ß subunit always co-migrates in the α-peak; even after several runs, α subunit preparations contain traces of ß subunit which are immunogenic. Our present interpretation is that ß subunit cannot be completely denatured by SDS and a certain percentage (possibly in a more compact state) migrates into the α-peak. The reaction of the anti- α sera with ß subunit can be

"quenched" by the addition of purified ß subunit (not shown). (ii) The kinetics of processing vary, possibly depending on the extent of cell breakage in the French press (see Fig. 7A, versus Fig. 7B). As a consequence, the appearance of mature subunit also varied. In any case, however, α precursor preceded the appearance of the mature subunit form.

In vitro processing of the pBT212 encoded precursor was substantiated by experiments following the appearance of penicillin acylase activity. Permeabilized or gently lysed pBT212/54-2 cells did not exhibit any enzyme activity. However, under conditions of in vitro maturation of α and ß subunits, penicillin acylase activity emerged (not shown).

Fig. 8 summarizes the events of the formation of penicillin acylase α and ß subunits as delineated by the in vitro studies. ß subunit, apparantly by a specific endoproteolytic cleavage, is removed from the common precursor and α subunit is derived by C-terminal proteolysis to α* (2) and α.

CONCLUSIONS

The results presented above demonstrate that the two constituent subunits of penicillin acylase are formed via proteolytic processing of a precursor polypeptide in a manner hitherto unique for prokaryotes. Processing requires translocation of the pac gene product through the cytoplasmic membrane; at present it is unknown whether the proteolytic attack takes place during the export process or within the periplasmic space. The fact that precursor polypeptide expressed from the wild-type pac gene can only be detected in the membrane-bound state (2) argues for the former possibility.

The covalent linking of the α and ß chains via the spacer endopeptide exerts a profound effect on the physical and biochemical properties of the pac gene product. It appears that the endopeptide directs the folding of the polypeptide into a certain pathway predetermining the correct fit of the subunits. Expression of each subunit separately, seems to direct the folding into an aberrant pathway which results in the formation of insoluble products which cannot be readily associated into the active holoenzyme. The cloning and overexpression of the subunits and precursor reported not only open the way for mass

production of the enzyme, but also provide the tools for further dissection of this unique export and processing mechanism.

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