#### 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  $\alpha$  subunit, (iii) positions 706 - 867 coding for a spacer peptide, and (iv) positions 868 - 2538 coding for the  $\beta$  subunit. Plasmids were constructed which direct the synthesis of a pac gene product lacking the signal peptide, and the synthesis of the  $\alpha$  subunit or the  $\beta$  subunit. The following results were obtained: (i) The two dissimilar subunits are processing products of a single precursor polypeptide; the spacer peptide 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  $\alpha\beta$  heterodimer and is localized in the periplasmic space of producing <u>E. coli</u> 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).

# Nucleic Acids Research

In this communication we report on the detailed geneprotein relationship of the enzyme. It is shown that the penicillin acylase structural gene (<u>pac</u>) consists of the following functional segments: (i) a 78 bp leader peptide sequence; (ii) 627 bp coding for the  $\alpha$  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  $\beta$  subunit. This structure has been proven by the construction of plasmids which overexpress either the  $\alpha$  or  $\beta$  subunits alone. Moreover, the pathway of processing was delineated by in vitro experiments employing a genetically engineered <u>pac</u> gene lacking the signal sequence information.

# MATERIALS AND METHODS

#### Bacterial strains and plasmids

E. coli strain 54-2 which is  $\Delta(pro-lac)$ , recA, rpsL/F' lacI<sup>q</sup> Z  $\Delta$  M15 proA<sup>+</sup>B<sup>+</sup> 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 pHMl2 (4) carries the <u>pac</u> gene together with the homologous upstream control region. The HindIII fragment of pHMl2 which contains the <u>pac</u> gene was cloned into the HindIII site within the <u>lac</u> UV5 promoter of plasmid pEl (gift of U. Ruether). The resulting plasmid pEl-ll contains the <u>pac</u> gene under the control of the <u>lac</u> UV5 promoter. Plasmid pKK177-3 is an expression vector bearing a hybrid <u>trp-lac</u> 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  $\alpha$  and  $\beta$  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 holoenzyme, 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, Sl 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 pHMl2 (4). The

1 -52	يم	GCTT	CGT	rgcī	AGT	TCA	ATTCO	CT A	TTA	RACA	CTG	CAG	SD \GGA		Met ATG	Ly S AAA	Asn AAT	Arg AGA	Asn AAT	Arg CGT	Met ATG	Ile ATC	Val GTG	Asn AAC	Cys TGT	Val GTT	Thr ACT
14 40	Ala S GCT T	nd II er L CC C	eu ł TG /	Met NTG	Tyr TAT	Tyr TAT	Trp TGG	Ser AGC	Leu TTA	Pro CCT	Ala GCA	Leu CTG	Ala GCT	Glu GAG,	Gln CAG	Ser TCG	Ser TCA	Ser AGT	Glu GAG	Ile ATA	Lys AAG	Ile ATT	Val GTT	Arg CGC	Asp GAT	Glu GAA	Tyr TAC
41	Gly M	et P	ro I	lis	Ile	Tyr	Ala	Asn	Asp	Thr	Trp	H15	Leu	Phe	Tyr	G1y	Tyr	Gly	Tyr	Val	Val	Ala	Gln	Asp	Arg	Leu	Phe
121	GGC A	TG C	CG C	Cat	ATT	TAT	GCC	AAT	GAT	ACA	TGG	CAC	CTA	TTT	TAT	GGC	TAT	GGC	TAT	GTA	GTA	GCA	CAA	GAT	CGC	CTT	TTT
68 202	Gln M CAG A	et G TG G	lu P	let ATG	Ala GCA	Arg CGT	Arg CGC	Ser AGT	Thr ACT	Gln CAA	Gly GGG	Thr ACT	Val GTC	Ala GCG	Glu GAA	Val GTG	Leu CTT	Gly GGC	Lys AAA	Авр Бат	Phe TTT	Val GTG	Lys AAA	Phe TTT	Asp GAT	LYS	Asp GAT
95	Ile A	TG A	rg /	Asn	Tyr	Trp	Pro	Asp	Ala	Ile	Arg	Ala	Gln	Ile	Ala	Ala	Leu	Ser	Pro	Glu	Asp	Met	Ser	Ile	Leu	Gln	Gly
283	ATC, C		GT /	AAC	TAC	TGG	CCG	GAT	GCT	ATC	CGG	GCG	CAA	ATT	GCT	GCC	CTT	TCC	CCA	GAG	GAT	ATG	TCC	ATT	CTG	CAA	GGC
122	TYT A	la A	Sp (	G1 y	Met	Asn	Åla	Trp	Ile	Asp	Lys	Val	Λsn	Thr	Asn	Pro	Glu	Thr	Leu	Leu	Pro	Lys	Gln	Phe	Asn	Thr	Phc
364	TAC G	CT G		GGA	ATG	AAT	GCC	TGG	ATT	GAT	AAG	GTA	AΛT	ACC	AAT	CCA	GAG	ACG	CTC	TTA	CCA	AAA	CAG	TTT	AAT	ACA	TTT
149	Gly P	he T	hr I	Pro	Lys	Arg	Trp	Glu	Pro	Phe	Asp	Val	Ala	Met	Ile	Phe	Val	Gly	Thr	Met	Ala	Asn	Arg	Phe	Ser	Asp	Ser
445	GGC T	TT A	CT (	CT	AAG,	CGC	TGG	GAA	CCG	TTT	GAT	GTC	GCG	ATG	ATA	TTT	GTG	GGC	ACC	ATG	GCA	AAC	CGC	TTC	TCT	GAT	AGC
176	Thr S	er G	lu I	lle	Asp	Asn	Leu	Ala	Leu	Leu	Thr	Ala	Leu	Lys	Asp	Lys	Tyr	Gly	Val	Ser	Gln	G1y	Met	Ala	Val	Phe	Asn
526	ACT A	GC G		ATT	GAT	AAT	CTG	GCA	CTG	CTA	ACG	GCT	TTA	AAA	GAT	Ana	TAT	GGT	GTA	TCA	CAA	GGC	ATG	GCG	GTA	TTT	AAT
203	Gln L	eu L	Y 8 7	Frp	Leu	Val	Àsn	Pro	Ser	Ala	Pro	Thr	Thr	Ile	Ala	Val	Gln	Glu	Ser	Asn	Tyr	Pro	Leu	Lys	Phe	Asn	Gln
607	CAG T	TG A		FGG	CTG	GTA	AAC	CCA	TCA	GCG	CCA	ACC	ACT	ATT	GCC	GTA	CAA	GAG	AGT	AAC	TAC	CCA	CTT	AAA	TTT	AAT	CAG
230	Gln A	sn S	er (	Gln	Thr	Ala	Ala	Leu	Leu	Pro	Arg	Tyr	Asp	Leu	Pro	Ala	Pro	Met	Leu	∧вр	Arg	Pro	Ala	Lys	Gly	Ala	Asp
688	CAA A	AC T	CG (	CAA	ACA	GCA	GCT	CTG	TTG	CCA	CGC	TAC	Gat	TTA	CCT	GCA	CCA	ATG	CTT	GAС	CGA	CCA	GCA	AAA	GGG	GCG	GAT
257	Gly A	la L	eu I	Leu	Ala	Leu	Thr	Ala	G 1 y	Lys	Asn	∧rg	Glu	Thr	Ile	Val	∧la	Gln	Phe	Λla	Gln	Gly	Gly	Ala	Asn	Gly	Leu
769	GGC G	CA C	TG (	CTG	GCG	TTA	ACA	GCA	GGG	AAG	AAC	CGG	GAA	ACT	ATT	GTT	GCA	CAA	TTT	GCA	CAG	GGT	GGT	GCC	AAT	GGT	CTG
284	Ala G	ly T	yr I	Pro	Thr	Thr	Ser	Asn	Met	Trp	Val	Ile	Gly	Lys	Ser	Lys	Ala	Gln	Asp	Ala	Lys	Ala	ile	Met	Val	Asn	G1y
850	GCG G	GG T	At C	CC <b>A</b>	ACG	ACC	AGC	AAT	ATG	TGG	GTT	ATC	GGC	AAA	AGC	AAA	GCC	CAG	GAT	GCG	AAA	GCA	ATC	ATG	GTA	AAT	GGT
311	Pro G	ln P	he (	Gly	Trp	Tyr	Ala	Pro	Ala	Tyr	Thr	Tyr	Gly	Ile	Gly	Leu	H18	Gly	Ala	Gly	Tyr	Asp	Val	Thr	G1y	Asn	Thr
931	CCG C	AG T	TT (	GGC	TGG	Tat	GCG	CCT	GCG	TAT	ACT	Tat	GGT	ATT	GGT	CTG	CAC	GGT	GCT	GGT	TAT	GAT	GTC	ACT	GGC	AAT	
338	Pro P	he A	la 1	Fyr	Pro	Gly	Leu	Val	Phe	Gly	His	Λsn	Gly	Val	Ile	Ser	Trp	Gly	Ser	Thr	Ala	Gly	Phe	Gly	Asp	Asp	Val
1012	CCA T	TT G	CC 1	Fat	CCT	GGG	CTG	GTT	TTT	GGT	CAT	AAT	GGT	GTG	ATT	TCC	TGG	GGA	TCA	ACG	GCA	GGT	TTC	GGC	GAT	GAT	GTC
365	Asp I	le P	he /	Ala	Glu	Arg	Leu	Ser	Ala	Glu	LYS	Pro	G1y	Tyr	Tyr	Leu	His	Asn	Gly	Ly s	Trp	Val	Lys	Met	Leu	Ser	Arg
1093	GAT A	TT T	TT (	GCT	GAA	CGG	CTG	TCG	GCA	GAG	AAA	CCA	GGC	TAC	TAC	TTG	CAT	AAT	GGT	AAG	TGG	GTG	AAA	ATG	TTA	AGC	CGT
392	Glu G	LU T	hr 1	lle	Thr	Val	Lys	Asn	Gly	Gln	Ala	Glu	Thr	Phe	Thr	Val	Trp	Arg	Thr	Val	H18	Gly	Asn	Ile	Leu	Gln	Thr
1174	GAG G		CC /	ATT	ACG	GTG	AAA	AAT	GGT	CAG	GCA	GAG	ACC	TTT	ACT	GTC	TGG	CGT	ACG	GTG	CAT	GGC	AAC	ATT	CTC	CAA	ACT
419	Asp G	In T	hr 1	Thr	Gln	Thr	Ala	Tyr	Ala	Lys	Ser	Λrg	Ala	Trp	Asp	Gly	Lys	Glu	Val	Ala	Ser	Leu	Leu	Ala	Trp	Thr	His
1255	GAC C	AG A	CG /	ACA	CAA	ACG	GCT	TAC	GCT	AAA	TCC	CGC	GCA	TGG	GAT	GGT	AAA	GAG	GTG	GCG	TCT	TTG	CTG	GCC	TGG	ACT	CAT
446	Gin M	et L	YS AG	Ala	Ly S	Asn	Trp	Gln	Glu	Trp	Thr	Gln	Gln	Ala	Ala	Lys	Gln	Ala	Leu	Thr	Ile	Asn	Trp	Tyr	Tyr	Ala	Asp
1336	CAG A	rg A		GCC	AAA	AAT	TGG	CAG	GAG	TGG	ACA	CAG	CAG	GCA	GCG	AAA	CAA	GCA	CTG	ACC	ATC	AAC	TGG	TAC	TAT	GCT	GAT
473	Val A	BN G	ly /	\sn	Ile	Gly	TY T	Val	H15	Thr	Gly	Ala	Tyr	Pro	Asp	Arg	Gln	Ser	Gly	HIS	Asp	Pro	Arg	Leu	Pro	Val	Pro
1417	GTA A	AC G	GC /	\AT	ATT	GGT	TAT	GTT	CAT	ACT	GCT	GCT	TAT	CCA	GAT	CGT	CAA	TCA	GGC	CAT	GAT	CCG	CGA	TTA	CCC	GTT	CCT
500	GIY T	hr G	ly I	Lys	Trp	Asp	Trp	Lys	G1y	Leu	Leu	Pro	Phe	Glu	Met	Asn	Pro	Lys	Val	Tyr	Asn	Pro	Gln	Ser	Gly	Tyr	Ile
1 <b>498</b>	GGT A	CG G	GA J	AAA	TGG	GAC	TGG	AAA	GGG	CTA	TTG	CCT	TTT	GAA	ATG	AAC	CCT	AAG	GTG	TAT	AAC	CCC	CAG	TCG	GGA	Tat	ATT
527	Ala A	an T	rp /	Asn	Asn	Ser	Pro	Gln	LYS	Asp	Tyr	Pro	Ala	Ser	Asp	Leu	Phe	Ala	Phe	Leu	Trp	Gly	Gly	Ala	Asp	Arg	Val
1579	GCT A		GG /	AAC	AAT	TCT	CCC	CAA	AAA	GAT	Tat	CCC	GCT	TCA	GAT	CTG	TTT	GCC	TTT	TTG	TGG	GGT	GGT	GCA	GAT	CGC	GTT
554	Thr G	lu I	le /	Asp	Arg	Leu	Leu	Glu	Gln	Lys	Pro	Arg	Leu	Thr	Ala	Asp	Gln	Ala	Trp	Asp	Val	Ile	Arg	Gln	Thr	Ser	Arg
1660	ACG G	AG A	TC (	GAC	CGA	CTG	CTT	GAG	CAA	AAG	CCA	CGC	TTA	ACT	GCT	GAT	CAG	GCA	TGG	GAT	GTT	ATT	CGC	CAA	ACC	AGT	CGT
581	Gln A	SP L	eu /	Asn	Leu	Arg	Leu	Phe	Leu	Pro	Thr	Leu	Gln	Ala	Ala	Thr	Ser	Gly	Leu	Thr	Gln	Ser	Asp	Pro	Arg	Arg	Gln
1741	CAG G	AT C	TT /	AAC	CTG	AGG	CTT	TTT	TTA	CCT	ACT	CTG	CAA	GCA	GCG	ACA	TCT	GGT	TTG	ACA	CAG	AGC	GAT	CCG	CGT	CGT	CAG
608	Leu V.	al G	lu 1	Thr	Leu	Thr	Arg	Trp	Asp	Gly	Ile	Asn	Leu	Leu	Asn	Asp	Asp	Gly	Lys	Thr	Trp	Gln	Gln	Pro	Gly	Ser	Ala
1822	TTG G	TA G	AA J	ACA	TTA	ACA	CGT	TGG	GAT	GGC	ATC	AAT	TTG	CTT	AAT	GAT	GAT	GGT	AAA	ACC	TGG	CAG	CAG	CCA	GGC	TCT	GCC
635	Ile L	eu A	an N	Val	Trp	Leu	Thr	Ser	Met	Leu	Lys	λrg	Thr	Val	Vāl	Ala	Ala	Val	Pro	Met	Pro	Phe	Авр	Lys	Trp	Tyr	Ser
1903	ATC C	TG A		STT	TGG	CTG	ACC	AGT	ATG	TTG	AAG	CGT	ACC	GTA	GTG	GCT	GCC	GTA	CCT	ATG	CCA	TTT	Бат	AAG	TGG	TAC	AGC
662	Ala S	er G	ly 1	Fyr	Glu	Thr	Thr	Gln	Asp	G1y	Pro	Thr	Gly	Ser	Leu	Asn	Ile	Ser	Val	G1y	Ala	Lys	Ile	Leu	Tyr	Glu	Ala
1984	GCC A	GT G	GC 1	FAC	GAA	ACA	ACC	CAG	GAC	GGC	CCA	ACT	GGT	TCG	CTG	AAT	ATA	AGT	GTT	GGA	GCA	AAA	ATT	TTG	TAT	GAG	GCG
689	Val G	ln G	ly J	Asp	Ly S	Ser	Pro	Ile	Pro	Gln	Ala	Val	Asp	Leu	Phe	Ala	Gly	LYS	Pro	Gln	Gln	Glu	Val	Val	Leu	Ala	Ala
2064	GTG C	AG G	GA (	GAC	AAA	TCA	CCA	ATC	CCA	CAG	GCG	GTT	GAT	CTG	TTT	GCT	GGG	AAA	CCA	CAG	CAG	GAG	GTT	GTG	TTG	GCT	GCG
716	Leu G	lu A	SP 1	Thr	Trp	Glu	Thr	Leu	Ser	Ly S	Arg	Tyr	G1y	Asn	Asn	Val	Ser	۸sn	Trp	Lys	Thr	Pro	Ala	Met	Ala	Leu	Thr
2146	CTG G	AA G		NCC	TGG	GAG	ACT	CTT	TCC	AAA	CGC	Tat	GGC	AAT	AAT	GTG	AGT	۸AC	TGG	AAA	ACA	CCT	GCA	ATG	GCC	TTA	ACG
743 2227	Phe A TTC C	rg A GG G	la /	Asn	Asn AAT	Phe TTC	Phe TTT	G1y GGT	Val GTA	Pro CCG	Gln CAG	Ala GCC	Ala GCA	Ala GCG	Glu GAA	Glu GAA	Thr ACG	Arg CGT	His CAT	Gln CAG	Ala GCG	Glu GAG	TYT TAT	Gln CAA	ABD AAC	Arg CGT	G1y GGA
770 2308	Thr G ACA G		an J	Asp GAT	Met ATG	Ile ATT	Val GTT	Phe TTC	Ser TCA	Pro CCA	Thr ACG	Thr ACA	Ser AGC	Asp Gat	Arg CGT	Pro CCT	Val GTG	Leu CTT	Ala GCC	Trp TGG	Asp GAT	Val GTG	Val GTC	Ala GCA	Pro CCC	Gly GGT	Gln CAG
797	Ser G	ly P	he 1	Ile	Ala	Pro	Asp	Gly	Thr	Val	Asp	Lys	His	Tyr	Glu	Asp	Gln	Leu	Lys	Met	Tyr	Glu	Asn	Phe	G1y	Arg	Lys
2389	AGT G	GG T	TT /	ATT	GCT	CCC	GAT	GGA	ACA	GTT	GAT	AAG	CAC	Tat	GAA	GAT	CAG	CTG	AAA	ATG	TAC	GAA	AAT	TTT	GGC	CGT	AAG
824 2470	Ser L TCG C	eu T TC T	rp I GG_1	Leu ITA Hoa I	Thr ACG	Lys AAG	Gln CAG	Λsp GAT	Val GTG	Glu GAG	Ala GCG	His CAT	Lys AAG	Glu GAG	Ser TCG	Gln CAG	Glu GAA	Val GTG	Leu TTG	His CAC	Val GTT	Gln CAG	Arg AGA	таа			

Fig. 1. Nucleotide sequence of the penicillin acylase structural gene.  $\alpha$  and  $\beta$  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  $\alpha$  and  $\beta$  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.

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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  $\alpha$  and  $\beta$  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  $\alpha$  and  $\beta$  subunits, respectively. The initial three amino acid residues of the  $\alpha$  and  $\beta$  subunits are given, along with the final three residues of the  $\alpha$  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 <u>pac</u> gene at the 5' side, there is a sequence motif recognized in <u>E. coli</u> 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 <u>pac</u> gene (3). Apart from a few deviations there is general agreement with the respective <u>pac</u> sequence portions reported in this communication.

To correlate the nucleotide sequence of the <u>pac</u> gene with the sequence of the protein products, namely with those of the  $\alpha$  and  $\beta$  subunits of penicillin acylase, the N-terminal and Cterminal amino acid positions of the two subunits were determined. The following results were obtained:

		<u>N-terminus</u>	<u>C-terminus</u>
α	subunit:	H <sub>2</sub> N-Glu-Gln-Ser-Ser-Ser-	-Gln-Thr-Ala-COOH
ß	subunit:	H <sub>2</sub> N-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 2. Each sequence occurred only once and, therefore, un-Fiq. equivocally indicates the start and termination sites of the coding information for the respective subunits. Accordingly,  $\alpha$  is encoded by the aminoterminal and ß by the carboxyterminal part of the pac open reading-frame. The C-terminus of the  $\alpha$  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  $\alpha$  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 <u>pac</u> 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  $\alpha$  or  $\beta$  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 <u>pac</u> 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 pEl-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 <u>pac</u> gene portion, beyond the EcoRV restriction site, and the <u>lac</u>Z 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  $\alpha$  subunit and reaches into the lacZ coding area



Fig. 4. Constructions of plasmid pBT702 coding for the  $\alpha$  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 Sl 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 this construction in order to check whether the for manipulations at the DNA level (Klenow or Sl treatment, religation) 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 % SDSpolyacrylamide 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 125I-labeled S. aureus protein A. The resulting autoradiographs are shown above. Lanes 2 give the position of the a and B 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<sub>2</sub> N-Met-Tyr-Tyr-Phe) preceding the sequence coding for mature  $\alpha$  subunit (to be published elsewhere).

Plasmids were also constructed which code for the presumptive  $\alpha$  or  $\beta$  part of the <u>pac</u> gene product. With the aid of the plasmids, it is possible to definitely prove that the  $\alpha$  and  $\beta$  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  $\alpha$  subunit, the EcoRI/HpaI 720 bp fragment of the pac gene carried by



Immunoblots of cell lysates from E. coli strain 54-2 Fig. 6. 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 subunit coding region) and two tandem stop α codons and а HindIII restriction site were added bv 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 Fiq. 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  $\beta$  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  $\alpha$  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  $\alpha$  and ß subunit within the same cell , or by genetically extending the N-terminus of the  $\alpha$  subunit by an additional four amino acids (H2N-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  $\alpha$  or  $\beta$  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 <u>pac</u> cross-reacting material was present in cell extracts in a particular and sedimentable state. As the <u>pac</u> 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,  $\alpha$  and  $\beta$  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 <u>pac</u> precursor (Fig. 5A). When the cells were broken in the French press prior to the addition of the detergent,  $\beta$  and  $\alpha$  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  $\alpha$  subunit. Consistently, cross-



Fig. 8. Processing pathway of the penicillin acylase precursor as delineated by in vitro maturation experiments.  $\alpha$  ( $\alpha$ \*) and  $\beta$  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  $\alpha$  subunit and indicated a precursor-product relationship (Fig. 7A). To analyze whether these polypeptide species might constitute processing intermediates of the  $\alpha$  subunit (which may contain the <u>pac</u> endopeptide or part of it), the immunoblots were developed with antibodies specific for  $\beta$  or  $\alpha$  subunit polypeptides. Fig. 7B and C, indeed, show that the putative intermediates only react with anti- $\alpha$  subunit but not with anti- $\beta$  subunit antibodies.

The interpretation of the in vitro processing experiments of Fig. 7 requires the following information: (i) The anti- $\alpha$ sera - in contrast to the anti- $\beta$  directed ones - are not pure. They contain anti- $\beta$  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  $\beta$ subunit always co-migrates in the  $\alpha$ -peak; even after several runs,  $\alpha$  subunit preparations contain traces of  $\beta$  subunit which are immunogenic. Our present interpretation is that  $\beta$  subunit cannot be completely denatured by SDS and a certain percentage (possibly in a more compact state) migrates into the  $\alpha$ -peak. The reaction of the anti- $\alpha$  sera with  $\beta$  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,  $\alpha$  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  $\alpha$  and  $\beta$  subunits, penicillin acylase activity emerged (not shown).

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

#### 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 unique for prokaryotes. Processing hitherto requires translocation of the pac gene product through the cytoplasmic at present it is unknown whether the proteolytic membrane; attack takes place during the export process or within the The fact that precursor polypeptide periplasmic space. 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  $\alpha$  and  $\beta$  chains via the spacer endopeptide exerts a profound effect on the physical and biochemical properties of the <u>pac</u> 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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