

Gene and gene product for the production of beta-lactam-compounds

The invention relates to the gene and gene product of a key enzyme, which is new in its structure and multivalent functionality, and catalyses the last stage of biosynthesis of penicillin G and penicillin V, in purified and isolated form.

Already in 1953, Kato observed (J. Antibiot. Ser. Tokyo 6, 130-136, 1953) in precursorless penicillin fermentations an accumulation of the penicillin nucleus. This β -lactam was later isolated by Batchelor et al. (Nature 183, 257-258, 1959) and identified as 6-aminopenicillanic acid (6-APA). 6-APA is accumulated especially in precursorless penicillin fermentations. Erickson and Bennett (Appl. Microbiol. 13, 738-742, 1965) regarded 6-APA as a secondary metabolite which is obtained by enzymatic 7-desacylation of isopenicillin N in the absence of phenyl- or phenoxyacetic acid as a precursor, or resp. is formed by enzymatic cleavage of natural hydrophobic penicillins. It was later discovered that the last stage of penicillin biosynthesis consisted of an exchange of the L- α -aminoadipic acid side chain of isopenicillin N for phenyl- or phenoxyacetic acid, whereby the side chain acids enter the reaction as acyl-coenzyme A-compounds. An enzyme which catalyses this acyl transfer was identified by Loder (Postepy Hig. Med. Dosw. 26, 493-500, 1972) in raw extracts of *P. chrysogenum* and was called penicillin acyl transferase (PAT). This enzyme is found in all penicillin-forming fungi and is intracellularly localised. The works of Fawcett et al. (Biochem. J. 151, 741-746, 1975) confirm the transacylation of isopenicillin N to penicillin G with enzyme raw extracts of *P. chrysogenum* in the presence of phenylacetyl-coenzyme A.

The importance of PAT for the biosynthesis of penicillin G or V is verified by observations made by Brunner et al. (Hoppe-Seyler's Z. Physiol. Chem. 349, 95-103, 1968), Spencer (Biochem. Biophys. Res. Commun. 31, 170-175, 1968), Gatenback and Brunsberg

(Acta Chem. Scand. 22, 1059-1061, 1968), Spencer and Maung (Biochem. J. 118, 29-30, 1970), Meesschaert et al. (J. Antibiotics 33, 722-730, 1980), Kogekar et al. (Indian J. of Biochem. and Biophys. 20, 208-212, 1983), Frederiksen et al. (Biotechn. Letters 6, 549-554, 1984), Abraham (in Regulation of Secondary Metabolite Formation, Kleinkauf et al., eds., Proceedings of the 16th Workshop Conferences Hoechst, Gracht Castle, 12.-16.5.85, Vol. 16, Weinheim, p. 115-132, 1986), Luengo et al. (J. Antibiotics 39, 1565-1573 and 1754-1759, 1986), as well as Alvarez et al. (Antimicrob. Agents and Chemotherapy 31, 1675-1682, 1987). The thiol-dependent enzyme can also catalyse acyl exchange within various penicillins, and is encountered to a greater extent in penicillin strains having high synthesis capacity rather than in low-producing strains (Pruess and Johnson, J. Bact. 94, 1502-1508, 1967).

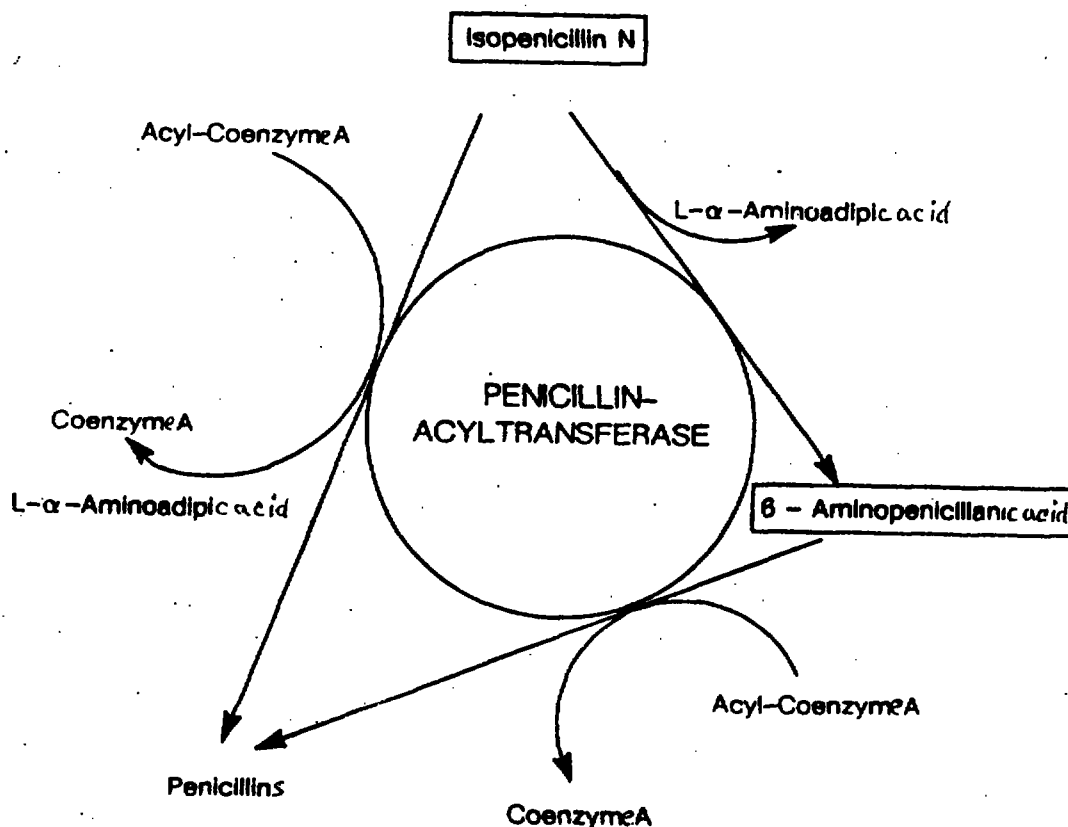
The present invention relates to the isolation, characterisation and explanation of the sequence of gene and gene product of this important biosynthesis enzyme, which will give essential prerequisites for genetic engineering work both regarding titre increase and regarding hybrid- resp. mutasynthesis resp. disclosure of new enzymatic β -lactam-transformation ways.

The protein sequencing is known according to Laursen, Methods Enzymology 25, 344-359 (1972). It is similarly a present state of the art to localise the genes coding for proteins (Rothstein et al., Cold Spring Harbor Symposia on Quantitative Biology, Vol. XLV, 99-105, 1981) and to sequence them (Maxam and Gilbert, Methods Enzymology 65, 499-560, 1980). The attained coupling of a gene to a strong, optionally inducible promoter is known according to Pribnow, Biolog. Regulation and Development, Vol. 1, 231-277, Plenum Press No.4 (Goldberger et al., 1979).

Several enzymes of β -lactam biosynthesis have already been isolated in pure form and characterised according to structure. Similarly, the coding gene regions of several of these enzymes

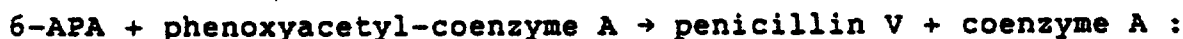
have in the meantime been isolated, sequenced, incorporated into corresponding expression vectors which are suitable for transformation and cloned (e.g. the isopenicillin N synthetase from *cephalosporium acremonium*: Samson et al., Nature 318, 191-194, 1985; Baldwin et al., J. Antibiotics 40(5), 652-659, 1987; EP 200 425, Eli Lilly, 1985; the isopenicillin N synthetase from *penicillium chrysogenum*: Carr et al., Gene 48, 257-266, 1986; EP 225 128, Eli Lilly, 1985; the expandase from *cephalosporium acremonium*: Samson et al., Biotechn. 5, 1207-1214, 1987; as well as several enzymes from *streptomyces clavuligerus* which catalyse β -lactam biosynthesis: EP 233 715, Beecham, 1986). The first transformation of a β -lactam producing microorganism, carried out with such expression vectors, has similarly already been described (Skatrud et al., Curr. Genet. 12(5), 337-348, 1987).

The PAT which catalyses the last step of penicillin biosynthesis, namely the transacylation of isopenicillin N, resp. its cleavage to 6-APA and its acylation to penicillin, in accordance with the following reaction scheme:



has already been identified earlier (see above) in penicillin-producing fungus strains, and its multivalent catalytic functionality described, but because of its extreme instability, until now it could not be isolated noticeably purely and characterised according to structure. The thiol-dependent PAT which is highly unstable and slightly acidic, and is increasingly formed in penicillin-forming strains of *penicillium chrysogenum* during 40 to 100 hours fermentation, catalyses on the one hand the acyl transfer of coenzyme A activated fermentative side chains (e.g. acyl-coenzyme A-compounds of the hydrophobic precursor substances phenoxyacetic acid, phenylacetic acid, hexanoic acid, octanoic acid etc.) to natural penicillins (e.g. isopenicillin N, penicillin V, penicillin G, 6-APA etc.), and on the other hand catalyses acyl transfer of natural penicillins among one another, i.e. during the acyl transfer which operates by way of an acyl enzyme complex, the natural penicillins function both as an acyl donor and as an acyl acceptor, while the coenzyme A activated hydrophobic precursor compounds may only enter the enzymatic reaction as an acyl donor. Since the acyl transfer takes place in an aqueous medium, water as a nucleophile also competes with the acyl enzyme complex, whereby the acyl donors which are rich in energy can be simply cleaved hydrolytically. Thus, 6-APA, the β -lactam nucleus of natural penicillins is always found in the culture broth of penicillin fermentations.

Detection of PAT may take place using conventional methods of analysis, such as radio-active, microbiological or chromatographic test methods. The following three different testing methods were prepared e.g. to determine the 6-APA-phenoxyacetyl-coenzyme A-acyl transferase activity of PAT, which catalyses the enzymatic reaction



- a) Detection of penicillin V by microbiological agar diffusion test with specifically product-sensitive test strains, e.g.

micrococcus luteus ATCC 9341 has antibacterial sensitivity towards penicillin which is more than three decimal powers greater than towards 6-APA.

b) When using S 35-labelled 6-APA (enzymatically from S 35-penicillin G) detection of extractable S 35-penicillin V in the β -counter.

c) Detection of extracted penicillin V using HPLC.

The enzymatic transacylation reaction is effected in microtitre plates with the following mixture:

6-APA (2.5 mg/ml in the reaction buffer)	20 μ l
PaCoA (ca. 15-20 mg/ml in bidist. water)	30 μ l
enzyme resp. reaction buffer (0.1 M phosphate buffer pH 7.8 incl. 1 mM DTT)	150 μ l

Immediately, resp. at various times within a one hour stationary incubation period, 20 μ l of aliquot was respectively taken, then diluted in the same microtitre plate 1:5 with 50% aqueous ethanol (= stopping of the enzyme reaction) resp. 1:5 with 5 U/ml penicillinase (= β -lactam control), and tested for penicillin V in the agar diffusion test on test plates which have been seeded with *micrococcus luteus* ATCC 9341 resp. with the lactam-supersensitive strain *pseudomonas aeruginosa* BC 248 (seeding rate 0.5%, antibiotic medium 1 of DIFCO, 50 μ l per well, incubation 15 hours at 37°C).

At various times, aliquots are taken from the enzymatic transacylation preparation (same mixture as above with 2 ml total volume), then extracted 1:1 with diisopropylether at pH 2.0 (HCl), the organic phase evaporated off with nitrogen, the residue re-suspended in the same volume of reaction buffer and tested for penicillin V by HPLC (conditions as for PaCoA content determination, but with an eluant of 40% methanol and 60% 0.01 M TBAS in 0.025 M phosphate buffer pH 7.0).

Retention times (min)	phenoxyacetic acid	1.10
	penicilloic acid G	1.62
	penicilloic acid V	1.92
	penicillin G	3.00
	penicillin V	4.92
	3-desacetoxycephalosporin V	2.46
	cephalosporin V	3.24

The other activities of the multispecific PAT enzyme may similarly be determined by HPLC, whereby 0.001 M TBAS in 0.025 M phosphate buffer pH 7.0 is used solely as the eluant for the polar substrates resp. products.

Retention times (min)	isopenicillin N	1.67
	6-APA	4.00
	DTT red.	2.41
	DTT oxid.	4.60

Isopenicillin N can be recovered from precursorless penicillin fermentations using adsorber resin (DIAION HP 20) and reverse phase chromatography (Nucleosil C 18, 10 μ m).

Depending on the enzyme preparation, attention must be paid especially to the choice of indicator reaction, with which the actual activity of the multispecific biosynthesis enzyme is to be detected. For example, it is not especially purposeful to use 6-APA-phenoxyacetyl-coenzyme A acyltransferase activity as the indicator reaction for PAT in the raw extract, since disturbing activities present in this raw enzyme preparation both rapidly hydrolyse phenoxyacetyl-coenzyme A (= PaCoA) and penicillin V, and also acylate 6-APA with the rich-in-energy side chain derivative PaCoA to penicillin V, which e.g. known proteolytic enzymes such as chymotrypsin catalyse. These interfering activities however are clearly differentiated from the actual thiol-dependent PAT. They have e.g. different chromatographic elution behaviour, stability profile and inhibition pattern.

These interfering, disturbing activities explain on the one hand the very low specific PAT activity previously found in raw preparations (about 1-10 μ U/mg protein), as well as similarly the discovery which appears as a paradox, that the 6-APA-PaCoA-acyltransferase activity in the raw extract increases greatly as enzyme dilution increases, up to a maximum activity. An essential factor in the detection of PAT is the presence of reducing compounds, such as dithiothreitol or β -mercaptoethanol, which activate resp. stabilise the highly oxidation-sensitive SH-enzyme.

The high instability to temperature of PAT similarly makes enzyme purification considerably more difficult. For example, a freshly prepared enzyme preparation loses the entire thiol-dependent 6-APA-PaCoA acyltransferase activity after one hour's stationary incubation at 37°C, despite the presence of various stabilisers (e.g. 5 mM dithiothreitol + 5% sorbitol + 0.1 mM phenylmethane-sulphonyl fluoride). It is therefore fitting to carry out all purification work in a cooling laboratory at 4°C with protection against oxidation. Coarse, pre-purified enzyme solutions can be frozen (-196°C, -20°C) for several weeks in the presence of stabilisers without any noticeable loss of activity, whereby however several freeze-thaw steps immediately disactivate the PAT activity. Ammonium sulphate precipitates are similarly more or less stable for a few days resp. weeks at 4°C in the presence of the said stabiliser mixture.

From young, penicillin-positive penicillin mycelium, the purification process for PAT used in the invention yields, after mycelium decomposition, nucleic acid precipitation, protein precipitation, hydrophobic interaction- and affinity-chromatography, relatively stable, at least 50% active, enzyme preparations, as are necessary for enzyme-kinetic resp. protein-chemical characterisation. Working up to a relatively storable protein precipitation may be carried out using conventional purification processes, e.g. by cell decomposition with a high-

pressure homogenizer or glass ball-mill resp. nucleic acid precipitation with cetyltrimethylammonium bromide, streptomycin sulphate or protamine sulphate. For protein precipitation, ammonium sulphate is suitable, among others, whereby a 50% saturation is sufficient to precipitate all the detectable PAT activity. Starting with the ammonium sulphate precipitate, the thiol-dependent PAT is bonded to phenylsepharose CI 4 B by hydrophobic interaction, and after removing over 90% of the ballast protein, the product is eluted with an aqueous stabiliser solution of low ion strength, concentrated by a 10kD cut off ultrafiltration membrane and further purified preferably by affinity chromatography resp. alternately by a gel filtration step (Ultrogel Aca 54) by anion exchanger chromatography (DEAE-sepharose FF) resp. adsorption chromatography (hydroxylapatite) to form an at least 50% active enzyme preparation, which corresponds to a concentration factor of ca. 1000, based on the ammonium sulphate precipitate.

The usual macroporous carrier materials such as sepharose, cellulose, polymer materials, stabilised silica gel, aluminium oxide etc., may be used as the affinity matrix. Suitable affinity ligands, which are bonded to the carrier by a C₂ to C₁₀ spacer or resp. without a spacer covalently or by hydrophobic resp. ionogenic reciprocal action, are preferably acyl- resp. amino- β -lactam compounds. Binding of these ligands to the spacer resp. the matrix must be effected such that the binding site at the ligand remains accessible to the enzyme to be purified. In accordance with the invention, an especially strong purifying effect is shown in particular by affinity matrices with terminal amino-spacers such as AH-sepharose 4 B resp. HMD-Ultrogel Aca 34, to which are bonded, by means of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), ligands such as natural penicillins, preferably 6-APA, penicillin V or penicillin G resp. analogous stable 3-desacetoxycephalosporin compounds such as 7-amino-3-desacetoxycephalosporanic acid (7-ADCA) resp. 7-phenoxyacetamido- or 7-phenylacetamido-3-desacetoxycephalosporanic acid (3-des-

acetoxyccephalosporin V and G). Thus, the amino- β -lactam affinity matrices may be produced from the corresponding phenylacetamido- β -lactam matrices without expensive protecting-group technology with soluble penicillin G-acylase of *E. coli*.

The required microorganisms for enzyme production are cultivated in the usual nutrient media for penicillin formation. Suitable constituents of nutrient media are all substrates which are generally employed for cultivating fungi. In addition to these nutrients, additives which promote growth of the microorganisms and increase PAT activity can be used in an appropriate combination. Additives are e.g. magnesium sulphate, sodium chloride, calcium carbonate, phosphates and similar inorganic salts, as well as growth substances, vitamins and trace elements. By adding various inductors, preferably phenoxy- resp. phenyl-acetic acid and their derivatives or analogues, the achieved enzyme titre can be considerably increased.

Culture takes place predominantly submers under aerobic conditions at a temperature in the range of 15 to 35°C and at a pH value of between 4 and 8. The time taken for the culture broth to reach maximum enzyme activity depends on the type of micro-organism used, and the optimum time for cultivation is therefore preferably evaluated separately for each individual strain. In general, the duration of cultivation ranges from preferably 2 to 5 days. For transacylation, the culture broth resp. an active secondary preparation prepared therefrom can be used. Examples of such active secondary preparations are the unmodified resp. permeabilised cells which have been recovered from the culture broth and washed; cell-free extracts which are obtained by means of physical, chemical and enzymatic treatment of the mycelium (for example the cell homogenates which are obtained by decomposition or by ultrasound treatment of the mycelium, and cell lysates which are formed by treatment with surface-active substances or enzymes); partially or wholly purified preparations of the desired enzyme, which are obtained by purifying the cell-

free extracts with the aid of conventional enzyme purification methods, for example by salting out, by fractional precipitation, by dialysis, by gel filtration, by ion exchange-, adsorption- and affinity-chromatography; stabilised PAT preparations which are obtained in such a way that the enzyme resp. the unmodified resp. permeabilised cells are immobilised either physically or chemically with high-molecular-weight carrier substances that are insoluble in water, by means of adsorption, covalent formation, cross-linking, enclosure or encapsulation.

Enzymatic resp. physicochemical characterisation of the above-resulting enzyme concentrates may take place using classic methods such as chromatography, electrophoresis, research regarding substrate specificity, N-terminal sequencing, pH and temperature profile for activity and stability, studies of activation, inhibition and stabilisation etc.

There exist numerous methods of isolating genes. A general review is given in the books of Watson et al. (Rekombinierte DNA - Eine Einführung, Heidelberg, 1983) and Winnacker (Gene und Klone - Eine Einführung in die Gentechnologie, Weinheim, 1984). In the case of the pat-gene (gene for the enzyme PAT), it appears most significant to use synthetic DNA-probes, since the necessary amino acid sequences exist. A DNA sequence based on the genetic code can be derived from the amino acid sequence of polypeptide 1 of PAT, and the corresponding oligonucleotides can be chemically synthesized. Figure 5 shows the arrangement of the amino acid radicals 2 to 22 of polypeptide 1 of PAT. Since 2, 3, 4 or 6 codons are assigned to a few amino acids, there results a nucleotide sequence as given under the amino acid sequence. The section which corresponds to amino acid residues 16 to 21 would be e.g. especially suitable, since here only 16 different gene sequences are possible. These 16 oligonucleotides, which are complementary to the RNA, can be synthesized as four mixtures each with four oligonucleotides (oligonucleotide mixtures 1, 2, 3 and 4 in figure 5). More certain identification of the gene is probably

not possible with these oligonucleotides, since further DNA sequences occur in the genom of penicillium chrysogenum, which are very similar to these DNA sequences. Differentiation between these varying DNA sequences can be very complicated. It is therefore advisable to have a further oligonucleotide available as a tracer sequence.

The strategy of isolating genes using DNA probes is discussed by Lathe (J. Mol. Biol. 183, 1-12, 1985). For example, if information is known about the frequency of certain codons or certain dinucleotide series, a longer DNA sequence can be synthesized. For penicillium chrysogenum, such information is not available. In spite of this, in order to obtain a longer oligonucleotide as a probe, the available oligonucleotides can be used as a sequencing primer: the enzyme reverse transcriptase can synthesize a DNA strand which is complementary to a given RNA, but only when a suitable starter molecule (= primer) is present. In the case described, oligonucleotide mixtures 1 to 4 can be used as primer. It is thus possible to sequence only the pat mRNA, although numerous other mRNAs are present in the preparation. The sequencing reaction is carried out in the presence of base-specific chain-breaking reagents (dideoxynucleoside triphosphates). Thus, numerous extended oligonucleotides are produced, the gel-electrophoretic analysis of which allows the determination of the base series of a DNA which is complementary to the pat mRNA. The advantage of this method is that the sequence information obtained allows the direct synthesis of new oligonucleotides. In addition, it is very significant that this sequence must conform with the sequence which is derived from the amino acid sequence, and thus ensures that a pat-specific sequence does in fact exist.

The actual isolation of the gene may then be effected from a gene bank. A gene bank is a collection of recombinant DNA vectors which respectively contain a small part of the DNA of penicillium chrysogenum (in the case of a λ -EMBL3 gene bank, this is ca.

0.05% per recombinant molecule). By means of plaque hybridisation, λ -clones can be isolated, and these hybridise with the radioactively labelled DNA probe. A physical map can be prepared from the DNA of these clones. Such a restriction map indicates the arrangement of restriction endonuclease intersection sites which may serve as an aid to orientation. Using DNA hybridisation techniques, it is possible to determine with which part region of the DNA the DNA probes hybridise. The corresponding part fragments are incorporated into plasmid- or bacteriophage- vector molecules and further characterised by molecular-genetic methods. Cloning of a DNA segment into the vector M13mp19 then allows the determination of the DNA sequence, whereby the pat-specific DNA probe can be used as primer. Further sequencing of the DNA enables the coding part of the gene and the amino acid sequence of PAT derived therefrom to be ascertained. The already-determined amino acid part sequences may supply the required information.

Based on the DNA sequence information, the pat-Gen of penicillium chrysogenum can be incorporated into an expression vector, which allows good expression of the PAT into well known expression organisms (Reznikoff and Gold, Maximizing Gene Expression, Boston, 1986). So as to make precise construction possible, in which the ATG comes exactly into the position of the E.coli starting codon, a NcoI intersection site can be introduced by site directed mutagenesis. Prior to this however, a complete cDNA clone must be isolated from a corresponding gene bank. Example 19 (see also Fig. 7) describes the details of construction of the plasmid pBC2001. In order to do this, there is first of all cloning of the DNA fragment from a pat-cDNA-clone into M13mp19. Thus, there is available a single-strand DNA which is used for the site directed mutagenesis to introduce the NcoI intersection site; in addition, a HindIII intersection site which is necessary for cloning. The plasmid pBC2001 is produced when the NcoI-Hind-III-fragment is incorporated into the vector pKK233-2. It contains effective signals for expression in E.coli (ptrc,

rrnBT1T2) and an ampicillin-resistance gene (bla), as E.coli selection index.

The DNA of the pat-gene can be incorporated into vectors which permit transformation of penicillium chrysogenum or other β -lactam-producing microorganisms. Since transformants frequently contain the introduced DNA in several copies (e.g. Kolar et al.), stronger expression of the gene is possible, which can lead to increased penicillin formation. Example 20 describes the construction of the plasmid pBC2002, which can be used for the transformation of penicillium chrysogenum. pBC2002 (see also Fig. 8) contains the complete penicillium chrysogenum pat gene on a 4.8kb Sall restriction fragment, which has been incorporated into the modified vector pHS103. The phleomycin resistance gene (ble) allows selection in penicillium chrysogenum; the ampicillin resistance gene (bla) allows selection in E.coli.

Furthermore, manipulation of the DNA is possible. The exchange of promoter segments can similarly improve the transcription in penicillium chrysogenum. Site directed mutagenesis of the ATGs can increase the translation in penicillium chrysogenum. The exchange of special amino acid radicals by site directed or fragment specific - undirected but gene-specific -mutagenesis make it possible to isolate special mutants which change the stability of the enzyme or its activity or specificity.

Screening of randomly or non-randomly selected microorganisms - in which the DNA of such organisms is examined for specific hybridisation with radio-active pat DNA - is also an obvious use of the isolated DNA segment. The result of such screening may be strains which previously produced unknown β -lactams, or strains which contain the PAT-similar enzymes but have other properties.

The DNA of the present invention was isolated from penicillium chrysogenum. The procedure is described in examples 10 to 13 and is illustrated by figs. 4, 5 and 6. Starting from the amino acid

sequence of polypeptide 1 of PAT (fig. 5), four oligonucleotides were synthesized (fig. 5: oligonucleotide mixtures 1 to 4, fig. 6C), which are used as a sequencing primer (example 11). The first 29 bases of the sequence thus determined (fig. 6D) conform with the postulated pat mRNA sequence (fig. 6B). As a result of this information, a 30mer was synthesized (fig. 6E), which was used as a radioactive probe for isolating a λ -clone from the penicillium chrysogenum gene bank (examples 12 and 13). After subcloning into M13 vectors, the DNA was sequenced (fig. 6F). The first 77 bases of this sequence conform with bases 35 to 111 of the sequence which is complementary to mRNA (fig. 6D). The connections between these individual sequences demonstrate that the pat-gene has in fact been isolated.

The PAT has a molecular weight of ca. 38,000 D. From an average amino acid molecular weight of 110 D, a size of 1100 bp can be estimated for the coding part of the gene. Since genes of filamentous fungi usually contain only few, and then small, introns (Ballance, Yeast 2,229 1986), it may be assumed that the complete gene lies on the described DNA molecule, and that control regions are similarly present in complete form.

The following examples illustrate the invention without limiting it. In the examples, the abbreviations ml, l, mg, g, rpm, DS, Wv% and U stand for millilitres, litres, milligrams, grams, revolutions per minute, dry substance, weight/volume percent and international enzyme unit (1 international enzyme unit = 1 μ mol substrate/min). The parts by weight are related to parts by volume as g to ml, the temperature is given in degrees celsius. Characterisation of the products was carried out using the following techniques: high-power thin-layer chromatography (HPTLC), high-pressure liquid chromatography (HPLC), ultraviolet spectrophotometry (UV), infrared spectrometry (IR) and nuclear magnetic resonance (NMR). The numbers added to the strain names listed in the examples correspond to the registered strain symbols of those culture collections in which the strains are

filed as such, e.g. for ATCC (American Type Culture Collection, Rockville, Maryland, USA).

Production and analysis of phenoxyacetyl-coenzyme A (PaCoA):

Principle: coenzyme A + phenoxyacetic acid chloride → PaCoA

901 mg of CoA are dissolved whilst cooling with ice in 15 ml of bidistilled water and set at pH 7.0 with sodium hydroxide (2.56 ml 1 N NaOH and 1.60 ml 0.5 N NaOH). Subsequently, a total of 235 mg of phenoxyacetic acid chloride are added in portions with vigorous stirring, and the pH value is kept at 7.0 (1.8 ml 1 N NaOH). After stirring for one hour whilst cooling with ice, the solution is extracted three times, each time with 40 ml of di-ethylether, at pH 2.0 (6 resp. 1 N HCl), then the aqueous phase is neutralised with NaOH, the residual ether is removed at room temperature in a rotary evaporator, the mixture is gassed for a short time with nitrogen, then diluted 1:50 with bidistilled water, made into ampoules in portions of 500 µl, and frozen in liquid nitrogen. Analysis is effected using HPLC, whereby phenyl-acetyl-coenzyme A (PeCoA) is used as the standard.

HPLC system: HP 1090 Liquid Chromatogram (Hewlett Packard)
column: Hypersil ODS 5 µm, C 18, 150x200 mm
integrator: Model 3392 (Hewlett Packard) / ATT 3
flow rate: 0.5 ml/min
detection: 230 nm
eluant: 35% methanol
65% 0.01 M tetrabutylammonium sulphate (TBAS)
in 0.025 M phosphate buffer pH 7.0

evaluation:

sample	retention time (min)	area	height
400 µg/ml PeCOA	9.41	8466900	210692
200 --	9.68	4105400	107689

100	-"	9.88	2092100	54990
PaCoA stock soln. 1:200		10.53	1966600	47737
PaCoA stock soln. 1:50		10.68	7850700	172983
PaCoA stock soln. 1:40		10.47	10389000	222475

Area integration → 19.2 mg PaCoA/ml
 height evaluation → 16.8 mg PaCoA/ml
 average value → 18.0 mg PaCoA/ml

Example 1: Preparation of the enzymatically active biomass:

The contents of an ampoule with lyophilised spores of penicillium chrysogenum P2 / ATCC 48271 are suspended in 8 ml of medium A (composition per litre: 15 g lactose, 0.11 g nitrogen from Cornsteep Liquor, 5 g Witte peptone, 4 g NaCl, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 g KH_2PO_4 , 5 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, the total ad 1000 ml with distilled H_2O , pH 4.85, sterilisation: 20 mins. at 120°). A 2 l Erlenmeyer flask with 225 ml barley is seeded with 2 ml of this spore suspension. Prior to filling, the barley is washed with water until clear, left to swell in medium A for 20 to 30 minutes, filtered over a sieve and dried for ca. one hour on filter paper, subsequently filled to 225 ml in a 2 l Erlenmeyer flask, then sterilised three times at 100° for ca. one hour at one-day intervals, and prior to seeding dried for 2 days at 40 to 45° . After seeding with spores, the Erlenmeyer flask is shaken for a short time, then incubated for approximately 8 days at $24 \pm 1^\circ$ and $60 \pm 10\%$ relative humidity.

After this stationary incubation period, the fungi spores are re-suspended for 3 to 5 minutes at 140 rpm with 250 ml 0.9% NaCl + 0.1% Tween 80 on a vertical agitator. After the further addition of 250 ml 0.9% NaCl solution (without Tween 80), the spore suspension is sterilely decanted into a 1 l seeding canister. 50 2 l Erlenmeyer flasks each with 200 ml medium B (composition per liter: 9 g potassium phenoxy acetate, 150 g lactose monohydrate, 2.25 g nitrogen as pharmamedia, 10.5 g $(\text{NH}_4)_2\text{SO}_4$, 4 g Na_2SO_4 ,

0.5 g KH_2PO_4 , 10 ml animal oil (lard oil), 25 g CaCO_3 , the total ad 1000 ml with distilled H_2O ; pH 6.5, sterilisation: 20 mins. at 120°) are each respectively seeded with 8 ml of this seeding material which can be stored at 4° for ca. one month, and then agitated for three days at $25 \pm 1^\circ$ at 260 rpm. After this agitating incubation, 1820 g of enzymatically active biomass is harvested. The penicillin titre is 1.9 g penicillin V per litre pulp.

Example 2: Production of an enzymatically active raw enzyme preparation:

1786 g of moist mycelium (= 220 g DS), obtained in example 1, are resuspended in 3.5 l 0.1 M phosphate buffer pH 7.5 including 5 mM dithiothreitol (DTT), 0.1% Triton X 100 and 0.1 mM phenylmethane-sulphonyl fluoride (PMSF), and homogenised continuously whilst cooling to $2/3^\circ$ in a glass ball mill (600 ml steel milling container with 510 ml 500-750 μm glass pearls, rotary speed 2000 rpm, 0.1 mm friction gap width, flow rate 30 l/hour, forward motion 2 to 3° , return motion 5° , brine cooling with forward motion $-20/8^\circ$ and return motion $-5/-1^\circ$). The homogenate is subsequently centrifuged for 30 minutes at 15,000 rpm and at 4° , the supernatant mixed at 0° with 0.5 WV% cetyl trimethylammonium bromide (CTAB) and stirred for 30 minutes. The precipitated nucleic acid is separated by centrifugation (10 minutes, 15,000 rpm, 4°), the supernatant brought to 50 WV% saturation by adding solid ammonium sulphate, and held at 4° over night in order to complete protein precipitation. The precipitate is then centrifuged, washed with cold saturated ammonium sulphate solution, and after adding 5 WV% sorbitol, 5 mM DTT, 2 mM EDTA and 0.1 mM PMSF, is stored at 4° until the next chromatography step. Decomposition resp. the two precipitation steps take place using protein determination (Bradford) resp. activity determination (microbiological detection of 6-APA-PaCoA-acyltransferase activity with the lactam-supersensitive strain *Pseudomonas aeruginosa* BC 248). The values found are listed in Table 1.

Example 3: Microbiological detection of transacylation activity resp. penicillin V cleavage activity in the enzyme preparation AS-precipitate with resp. without the addition of inhibitors:

The raw enzyme preparation produced as described in example 2 is tested as follows in the microbiological agar diffusion test for transacylation of 6-APA resp. isopenicillin N with PaCoA and cleavage of potassium penicillin V with resp. without the addition of an inhibitor:

test organism: *micrococcus luteus* ATCC 9341/BC 85, 0.5% seeding
test medium: antibiotic medium 1 (DIFCO), 110 ml
test plate: Nunc 243x243x18 mm
sample: 50 μ l per well (6 mm diameter)
incubation: 37°, 15 hours
enzyme: As-precipitate (produced as described in example 2): 1 ml centrifuged (20,000 rpm/10 mins.), supernatant discarded, pellet resuspended in 20 ml RP + Z.
RP + Z: 0.1 M phosphate buffer pH 7.5 + 1 mM DTT + 10 mM $MgCl_2$

A. Transacylation/acylation

Preparation in microtitre plates:

substrate 6-APA resp. isopenicillin N (2.5 mg/ml)	10 μ l
PaCoA (ca. 15 mg/ml)	15 μ l
enzyme resp. RP + Z with resp. without inhibitor	75 μ l

Inhibitor: a) without inhibitor (= control)
b) 2 mM iodoacetamide
c) 2 mM PMSF

Immediately and resp. after 7, 30 and 60 minutes incubation time, 20 μ l aliquots are taken from the preparations, diluted 1:5 with 50% ethanol (enzyme reaction stops) and tested for penicillin content in the agar diffusion test.

Transacylation of isopenicillin N (# 28 & 36) with PaCoA (HH Ø mm)

Isopenicillin N #	a)				b)				c)			
	s	7'	30'	60'	s	7'	30'	60'	s	7'	30'	60'
No. 28	22.5	25.5	26.0	23.0	21.1	20.8	18.3	14.7	22.7	24.7	25.7	20.7
No. 36	27.0	26.3	30.6	28.7	25.2	25.0	23.6	22.1	26.3	27.2	27.7	26.7
	Isopenicillin N h µg/ml				Penicillin V h µg/ml							
	50	25	12.5		2.0	1.5	1.0	0.5	0.25			
No. 28	20	16.5	12.4		25.3	24.0	21.7	17.8	9.7			
No. 36	24.5	22.1	18.4									

Acylation of 6-APA with PaCoA in dependence on enzyme dilution

1:5				1:10				1:50				1:100			
s	7'	30'	60'	s	7'	30'	60'	s	7'	30'	60'	s	7'	30'	60'
18.3	24.4	24.3	15.7	18.6	25.4	28.5	25.4	16.0	24.2	30.7	30.7	14.5	21.3	26.7	27.0
1:500				1:1000				Penicillin V h µg/ml							
s	7'	30'	60'	s	7'	30'	60'	2.0	1.5	1.0	0.5	0.25			
14.8	17.8	20.8	22.8	0	9.6	10.5	11.1	25.3	24.0	21.7	17.8	9.7			

B. Penicillin V cleavage

Preparation in microtitre plates:

potassium penicillin V 100 µg/ml 10 µl

0.1 M phosphate buffer pH 7.5 15 µl

enzyme resp. RP + Z (+ inhibitor) 75 µl

a) without inhibitor (= control)

b) + 2 mM iodoacetamide

c) + 2 mM ethylmaleimide

d) + 2 mM PMSF

e) RP + Z

Cleavage of potassium penicillin V (HH Ø mm) in the presence of various inhibitors

a)				b)				c)				d)			
s	7'	30'	60'	s	7'	30'	60'	s	7'	30'	60'	s	7'	30'	60'
25.7	21.8	0	0	25.7	23.1	0	0	25.0	23.0	0	0	25.3	0	0	0
e)				potass. Penicillin V h µg/ml				transacylation for comparison							
s	7'	30'	60'	2.0	1.5	1.0	0.5	0.25	6-APA + PaCoA →		Penicillin V				
26.8	25.8	26.2	26.4	27.4	24.7	23.4	18.4	11.9			17.0	23.2	26.9	25.9	

Example 4: Activation, inhibition, stabilisation and pH profile of PAT:

The PAT-ammonium sulphate precipitate produced as described in example 2 is diluted 1:10 with 0.1 M reaction buffer, in the presence of various activators resp. inhibitors or stabilisers, and tested for 6-APA-PaCoA acyltransferase activity immediately resp. after one-day incubation at 4° resp. -20° in the microbiological test model. The results are summarised in Table 2.

Example 5: Temperature stability of PAT:

The PAT-ammonium sulphate precipitate produced as described in example 2 is diluted 1:20 with 0.1 M phosphate buffer pH 7.5, including 1 resp. 10 mM DTT, then rigorously centrifuged, the supernatant undergoes stationary incubation at -196°, -20°, +4°, +20° and +37°, and is tested for 6-APA-PaCoA acyltransferase activity immediately resp. after 1, 4, 26 and 120 hours in the microbiological test model. The results are listed in Table 3.

Example 6: Purification of PAT by means of hydrophobic interaction chromatography on phenyl sepharose Cl-4B (HIC flow) and affinity chromatography on 6-APA-AH-sepharose 4 B (affinity flow):

240 g of the ammonium sulphate precipitation obtained as described in example 2 are dissolved in 2000 ml of 50 mM phosphate buffer pH 7.5 including 1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM DTT, and added in a laboratory cooling chamber at +4° to a BP 113-column with 2000ml equilibrated phenyl sepharose Cl-4B. The column is subsequently washed at a flow rate of 50 ml/min with 3 base volumes of 50 mM phosphate buffer pH 7.5 including 1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM DTT, then the ballast protein is removed with a further 2 base volumes of 50 mM phosphate buffer pH 7.5 including 1 mM DTT, then the PAT-enzyme is eluted with 3 base volumes of de-ionised water ($\text{E-H}_2\text{O}$) including 1 mM DTT, the active fractions

are concentrated to a tenth of the volume in a Pellicon cassette system using a polysulphone ultrafiltration membrane with a cut off of 10 kD, stabilised with 1 WV% sorbitol, 5 mM DTT, 2 mM EDTA and 0.1 mM PMSF and frozen at -20° until the next chromatography step. The microbiologically established 6-APA-PaCoA-acyl-transferase activity and the total protein content of the individual fractions comprising one base volume are summarised in the following table:

Sample	Microbiological PAT-Activity (mm HM $\odot$ on <i>Pseudomonas aeruginosa</i> BC 248)			total protein/Bradford (mg)
	immed	30 min	30 min + Penase	
charge	19	24	0	5483
PL 150/1	17	18	0	230
PL 150/2	16	15	0	102
PL 150/3	14	15	0	98
PL 150/4	0	0	0	2750
PL 150/5	0	0	0	390
PL 150/6	trace	15	0	52
PL 150/7	18	31	0	389
PL 150/8	0	0	0	14

The active retentate of the HIC fraction is thawed under careful conditions on the next day and added to a K 26/40 Pharmacia column with 50 ml affinity matrix 6-APA-AH-sepharose 4 B, which was produced by coupling penicillin G to AH-sepharose 4B (Pharmacia) with EEDQ according to the LKB-record "Practical guide for use in affinity chromatography and related techniques", Reactifs IBF-Societe Chimique Pointet-Girard, France 1933, page 133, and by subsequently cleaving the phenylacetic acid with soluble penicillin G-amidase of E.coli. The column is then eluted at 4° at a flow rate of 3 ml/min. with the following media:

AL 278/1: 12 base volumes 50 mM phosphate buffer pH 7.5 + 1 mM DTT + 0.1 M NaCl

AL 278/2: 3 base volumes of the same buffer + 1 mM DTT + 0.1 M NaCl + 1 mM 6-APA

AL 278/3: 6 base volumes of the same buffer + 1 mM DTT + 1 M NaCl

+ 1 mM 6-APA

PAT activity eluted in fraction 278/2 with the specific eluant 6-APA is concentrated to one tenth of the volume in the Minitan cassette system using a polysulphone-UF-membrane with a cut off of 10 kD, mixed again with the stability mixture 1 WV% sorbitol, 5 mM DTT, 2 mM EDTA and 0.1 mM PMSF, and deep-frozen in portions. The substrate specificity of this enzyme preparation which is at least 50% pure, determined by HPLC, is recorded in Table 4.

Example 7: Chromatofocussing of the PAT enzyme preparation produced by affinity chromatography on Mono P/Pharmacia (Chromatofocussing AL 310);

A 1 ml portion of the PAT enzyme preparation, which is purified as described in example 6 by means of affinity chromatography and concentrated, is thawed carefully, then undergoes buffer exchange over an equilibrated PD 10 sephadex G 25 finished column for 25 mM bis tris/HCl buffer pH 6.3, is then added to a 4 ml FPLC Mono P finished column which is equilibrated with the same buffer, subsequently immediately eluted at a flow rate of 60 ml/hour with 100 ml of a 1:10 diluted polybuffer 74 set at pH 4.0, then fractionated peak-wise and the fractions undergo analysis, either in original form or resp. concentrated ten times by Centricon 10, by means of RPC, SDS-gradient polyacrylamide gel electrophoresis, isoelectric focussing based on immobiline, and gel filtration.

Fig. 1 shows the elution profile of the PAT-enzyme on the Mono P. The multifunctional enzyme is split into at least three isoelectric forms (PAT 310/3, PAT 310/4 and PAT 310/5). These enzyme variants with different charges may illustrate either true isoenzymes or different redox forms of the same enzyme.

As can be seen from Fig. 2, the thiol-dependent PAT of penicillium chrysogenum breaks down under the denaturizing conditions of RPC (column = Biorad Hi Pore RP 304, 250x4.6 mm;

HPLC-parameters: 0.5 ml/min, 45 bar, 280 nm, 40°, 25 μ l injection volume; linear gradient between eluant A: 35% aqueous acetonitrile with 0.01% trifluoroacetic acid and eluant B: 80% aqueous acetonitrile with 0.01% trifluoroacetic acid, 30 minutes) into one polypeptide 1, and depending on the iso- resp. redox-variant, into one resp. two variably hydrophobic polypeptide variants 2a and 2b.

In SDS-gradient polyacrylamide gel electrophoresis (see Fig. 3a, conditions according to Laemmli, Nature 227, 680-685, 1970; however gradient gel with 8-20% T), the PAT-enzyme breaks down into two polypeptides of varying size, with MW of about 30 and 8 kD. Using RPC and subsequent SDS-gradient-PAGE, it may be demonstrated that polypeptide 1 firstly eluted in the RPC is identical with the ca. 30 kD component, and the two stronger retained, hydrophobic polypeptides 2a and 2b are identical with the 8 kD component. Such multidimensional analysis may further show that the small polypeptides 2a and 2b on the SDS-gradient-PAGE have the same migration, while the larger, ca. 30 kD polypeptide 1 of the basic iso- resp. redox-variant migrates more quickly than the corresponding polypeptide 1 of the more acidic form.

During the isoelectric focussing (IEF) based on ampholine (see fig. 3b, methodic execution according to LKB Application Note 1804), the active affinity pool shows three bands lying close together with an isoelectric point at 5.1.

In the narrow pH range of the highly solvent IEF based on immobililine (see fig. 3c, methodic execution according to LKB Application Note 1819), the PAT-variants separated by chromatofocussing have p_i -values of 5.15, 5.06 and 5.32.

When determining the MW by gel filtration, on Superose 12 of Pharmacia (eluant: 0.1 M phosphate buffer, pH 7.5 including 1 mM DTT, 0.4 ml/min) a MW of 36.5 kD is determined, resp. on the TKS-

G2000 SW of LKB (the same eluant with 0.5 ml/min) a MW of 38 kD is determined, which correlates well with the sum of the two denaturizing polypeptides 1 and 2.

Example 8: N-terminal sequencing of polypeptides 1 and 2a, as well as 2b:

A further portion (2 mg protein) of the PAT-enzyme preparation, which is purified by affinity chromatography as described in example 6, is separated twice by RPC after careful thawing (the same column and conditions as given in example 7, but with a flatter increasing gradient: min/% B = 0/0, 8/0, 20/12, 25/12, 30/100, 33/100, 36/0, 40/0), the fractions lyophilised with polypeptides 1, as well as 2a and 2b, and N-terminal sequenced in the protein-sequenator.

N-terminal amino acid sequence:

30 kD unit / polypeptide 1: part sequence 1

1	5	10	15	20
__-Thr-Thr-Ala-Tyr-Cys-Gln-Leu-Pro-Asn-Gly-Ala-Leu-Gln-Gly-Gln-Asn-Trp-Asp-Phe-				
	25	30	35	37
Phe-Ser-Ala-Thr-Lys-Glu-Asn-Leu-Ile-Arg-__-__-__-__-Gln-__-Gly-				

8 kD unit I / polypeptide 2a: part sequence 6

1	5	10	15	20
Met-Leu-His-Ile-Leu-Cys-Gln-Gly-Thr-Pro-Phe-Glu-Ile-Gly-Tyr-Glu-His-Gly-Ser-Ala-				
	25	30	34	
Ala-Lys-Ala-Val-Ile-Ala-Arg-Ser-Ile-Asp-Phe-Ala-Val-Asp-				

8 kD unit II / polypeptide 2b: part sequence 6

1	5	10	15	20
Met-Leu-His-Ile-Leu-Cys-Gln-Gly-Thr-Pro-Phe-Glu-Ile-Gly-Tyr-Glu-His-Gly-Ser-Ala-				
	25	30	35	40
Ala-Lys-__-Val-Ile-Ala-Arg-__-Ile-Asp-Phe-Ala-Val-Asp-Leu-__-__-Gly-__-Thr-				

As in the SDS-gradient-PAGE, the small polypeptides 2a and 2b which have differing retention in the RPC are not differentiated in the sequenced molecule section.

Example 9: Sequencing of proteolytic peptide fragments of polypeptide 1 and 2:

A lyophilised fraction of polypeptide 1 resp. 2 which is produced as described in example 8 is enzymatically cleaved according to J.M.Wilkinson (Practical Protein Chemistry - A Handbook, edited by A.Darbre, 1986) with trypsin, resp. lysyl-endopeptidase, individual peptide fragments are isolated by RPC and sequenced in the protein-sequenator. The following amino acid part sequences are obtained, among others:

AA-part sequence 2 (tryptic peptide fragment of polypeptide 1):

1 5 10 13

Gly-Ala-Thr-Leu-Phe-Asn-Ile-Ile-Tyr-Asp-His-Ala-Arg-

AA-part sequence 3 (tryptic peptide fragment of polypeptide 1):

1 5 10

Pro-Thr-Asn-Pro-Asp-Glu-Met-Phe-Val-Met-Arg

AA-part sequence 4 (tryptic peptide fragment of polypeptide 1):

1 5 10

Glu-Leu-Asp-Pro-Leu-Pro-Asp-Ser-Trp-Asn-Arg

AA-part sequence 5 (tryptic peptide fragment of polypeptide 1):

1 5 10 12

Met-Glu-Phe-Leu-_-Asp-Gly-Phe-Asp-Gly-Thr-Lys

AA-part sequence 7 (tryptic peptide fragment of polypeptide 1):

1 5 10 14

Ile-Ala-Leu-Glu-Ser-Thr-Ser-Pro-Ser-Gln-Ala-Tyr-Asp-Arg

AA-part sequence 8 (tryptic peptide fragment of polypeptide 1):

1 5 10 15

Val-Gly-Phe-Asn-Ser-Ala-Gly-Val-Ala-Val-Asn-Tyr-Asn-Ala-Leu-His

20 25 30 32

Leu-Gln-Gly-Leu-Arg-Pro-Thr-Gly-Val-Pro-Ser-His-Ile-Ala-Leu-Arg

AA-part sequence 9 (tryptic peptide fragment of polypeptide 2):

1 5 8

Thr-Glu-Phe-Ala-Tyr-Gly-Leu-Lys

AA-part sequence 10 (tryptic peptide fragment of polypeptide 2):

1 5

Tyr-Tyr--Glu-Ile-Arg

AA-part sequence 11 (tryptic peptide fragment of polypeptide 2):

1

Trp-Pro-Lys

AA-part sequence 12 (tryptic peptide fragment of polypeptide 2):

1 5 10

Ser-Ile-Asp-Phe-Ala-Val-Asp-Leu-Ile-Arg

AA-part sequence 13 (tryptic peptide fragment of polypeptide 2):

1 5 10

Asp-Val-Ser-Glu-Ile-Val-Met-Leu-Asn-Thr-Arg

AA-part sequence 14 (tryptic peptide fragment of polypeptide 2):

1 5 8

Gln-Val-Leu-Ser-Gln-Leu-Gly-Arg

AA-part sequence 15 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

1 5

Cys-Gln-Gly-Thr-Pro-Phe-Glu

AA-part sequence 16 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

1 5 10

Tyr-Tyr-Glu-Glu-Ile-Arg-Gly-Ile-Ala-Lys

AA-part sequence 17 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

1 5 10 15

Gln-Val-Leu-Ser-Gln-Leu-Gly-Arg-Val-Ile-Glu-Glu-Arg--Pro-Lys

AA-part sequence 18 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

1 5 10 15

Gly-Ala-Glu-Arg-Asp-Val-Ser-Glu-Ile-Val-Met-Leu-Asn-Thr-Arg

AA-part sequence 19 (tryptic peptide fragment of polypeptide 2):

1 5

Ala-Val-Ile-Ala-Arg

AA-part sequence 20 (tryptic peptide fragment of polypeptide 2):

1 5

Lys-Thr-Asp-Glu-Glu-Leu-Lys

AA-part sequence 21 (tryptic peptide fragment of polypeptide 2):

1 4

Gly-Ile-Ala-Lys

AA-part sequence 22 (tryptic peptide fragment of polypeptide 2):

1 5

Val-Ile-Glu-Glu-Arg

AA-part sequence 23 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

1 5 10

Asn-Thr-_-Thr-Glu-Phe-Ala-Tyr-Gly-Leu-Lys

AA-part sequence 24 (tryptic peptide fragment of polypeptide 2):

1 3

Ala-Ala-Arg

**Example 10: Isolation of poly(A)⁺RNA from mycelium of penicillium
chrysogenum:**

A spore suspension of penicillium chrysogenum P2/ATCC 48271 is incubated in an agitator at 250 rpm for about 70 hours at 25° in 5 Erlenmeyer flasks (each of 1000 ml) each containing 100 ml of CDS-medium (for 1 l CDS-medium, in accordance with autoclave treatment, there are mixed: 900 ml solution A: 3 g NaNO₃, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, 0.1 g FeSO₄·2H₂O, 5 g yeast extract, 10 g casein peptone, 20 g saccharose, pH 5.8 and 100 ml solution B: 250 mM K₂HPO₄/KH₂PO₄, pH 5.8). 12 Erlenmeyer flasks (each of 1000 ml) each containing 300 ml CDLP-medium (composition as CDS-medium, but instead of 20 g saccharose, 150 g lactose/l and 50 ml 10% phenoxyacetic acid [dissolved in water, pH 7.5 set with KOH]) are each seeded with 30 ml of the preculture and incubated in the agitator at 250 rpm for 40 hours at 25°. The mycelium is filtered using a Büchner funnel, washed briefly with TE (10 mM tris/HCl, pH 8.1, 1 mM EDTA) and pulverised in liquid nitrogen to a fine powder. This powder is suspended in lysis buffer (5 M guanidine

monothiocyanate, 10 mM EDTA, 50 mM tris/HCl, pH 7.5, 8% β -mercaptoethanol) (3 ml lysis buffer per g moist mycelium mass) and stirred for 1 hour at room temperature. Isolation of the total RNA takes place according to a described method (Cathala et al., DNA 2, 329-335, 1983). 60 g of moist mycelium mass enables about 18 g of raw-RNA to be isolated. This is precipitated with ethanol and stored at -70° . Concentration of the poly(A)⁺RNA takes place by means of oligo (dT) cellulose-affinity chromatography, as is described in Maniatis et al., Molecular Cloning, Cold Spring Harbor, 1982. For this purpose, the raw-RNA is centrifuged, dried and dissolved in water. After chromatography, fractions containing the poly(A)⁺RNA are again precipitated with ethanol and stored at -70° . The soundness of the poly(A)⁺RNA is tested by gel electrophoresis in the presence of glyoxal (Maniatis et al.), and the concentration by UV-absorption.

Example 11: Partial sequencing of RNA coding for the enzyme PAT:

On the basis of the genetic code, a DNA-sequence is derived from the amino acid sequence of polypeptide 1 of PAT (example 8) (fig. 5). A range of 17 bp, which allows a minimum number of different sequences, is selected for the synthesis of oligonucleotide mixtures. 4 mixtures of 4 different oligonucleotides, which are complementary to the intended strand and thus also to the RNA, are synthesized (oligonucleotide mixtures 1, 2, 3 and 4 in fig. 5). These oligonucleotides are enzymatically phosphorylated and thus radioactively labelled (Maniatis et al). 150 ng oligonucleotides are incubated with 16 units of polynucleotide kinase for 30 minutes at 37° , in 12 μ l reaction preparation (50 mM tris/HCl, pH 9.5, 10 mM $MgCl_2$, 5 mM DTE, 5% glycerol) with 250 μ Ci ATP (Amersham PB 10218). 10 μ g of the poly(A)⁺RNA (example 10) in 250 mM KCl, 10 mM tris/HCl, pH 8.3 are mixed with 2 μ l 32 P-labelled oligonucleotide mixture in a 0.5 ml reaction container. The mixture is heated for 2 minutes to 75° and then warmed for 45 minutes at 50° . To each of 4 Eppendorf containers are added 3.3 μ l reaction buffer (24 mM tris/HCl, pH

8.3, 16 mM $MgCl_2$), 8 mM DTE, 0.4 mM dATP, 0.8 mM dGTP, 0.4 mM dCTP, 0.4 mM dTTP, 6 units reverse transcriptase and in addition each has 1 μ l 1 mM dATP or 1 μ l 1 mM dGTP or 1 μ l 1 mM dCTP or 1 μ l 1 mM dTTP. After the addition of 2 μ l poly(A)⁺RNA-oligonucleotide mixture, the contents of the Eppendorf container are centrifuged for a short time and heated for 45 minutes at 50°. The reaction is terminated by adding 2 μ l stop-buffer (99% formamide, 0.3% bromophenol blue, 0.3% xylene-cyanol) and boiling for three minutes. 4 μ l of each sample is applied to a 8% polyacrylamide/urea gel and opened up for 2.5 hours at 40 W (Sequi Gen, Biorad). Thereafter, the gel is placed for 20 minutes in 5% methanol, 5% acetic acid, and dried for 1 hour at 80°. The cooled gel is wrapped in domestic foil. An X-ray film (Kodak XOMat AR) is applied for 20 hours. The sequence that can be read from this X-ray film is reproduced:

	10	20	30	40	50	80
5'	-CCATTTCGAAGTTGACAATAGGCAGTGGTGCAGCCATCACGGGCTGCCTTGAGCCCGTAT					
	70	80	90	100	110	
	GCAAATTCGGTGCCGGTATTAAGCATGACAATCTCGGAGACATCGCGTTCA-3'					

Positions 1 to 29 are complementary to the DNA-sequence, which is derived from the amino acid sequence of polypeptide 1 of the PAT (fig. 5, 6D). An oligonucleotide which embraces the first 30 nucleotides of this sequence is synthesized.

Example 12: Construction of a genomic gene bank of penicillium chrysogenum:

DNA is isolated from the mycelium of a penicillium chrysogenum culture (example 10) grown in CDS-medium. The mycelium is pulverised in liquid nitrogen, then added to 1% sarcosyl, 0.1 M EDTA, pH 8, 100 μ g proteinase K/ml (1 g mycelium/25 ml) and agitated for 48 hours at 37°. The mixture is extracted three times with phenol, and after adding 0.1 parts by volume of 3 M sodium acetate pH 5.2, is precipitated with ethanol. CsCl-EtBr centrifugation is subsequently carried out (Maniatis et al.), and after extraction with isoamylalcohol and dialysis on TE, the

concentration is determined by UV absorption. In addition, the DNA is tested by agarose-gel electrophoresis. 300 μ g penicillium chrysogenum DNA is cleaved in 5 U Sau3A (BRL) for 60 minutes at 37° in 10 mM tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTE, 50 mM NaCl. The extent of cleavage is tested on agarose-gel. It is desirable for a large part of the DNA to be of the size between 10 and 20 kb. The DNA is then applied to two NaCl gradients (produced by freezing and then thawing 2 Beckmann-SW28.1 ultracentrifuging tubes with 20% NaCl in TE [10 mM tris/HCl, pH 8.0, 1 mM EDTA]) and is centrifuged for 16 hours at 14,000 rpm in the ultracentrifuge in the rotor SW 28.1. The contents of the tube are fractionated. Fractions with DNA greater than 10 kb are combined and dialysed on TE. After concentrating the DNA (500 μ g/ml), it is combined with λ -EMBL3-DNA (Frischhauf et al., J.Mol.Biol. 170, 827-832, 1983) which has been cleaved with BamH1 and EcoR1. After adding 0.5 U T4-DNA-ligase, it is then ligated over night at 16° in 30 mM tris/HCl, pH 7.5, 10 mM MgCl₂, 10 mM DTE, 2.5 mM ATP (DNA concentration 200 μ g/ml, vector to penicillium chrysogenum-DNA in molar ratio 1:1). The ligation mixture is packed in vitro with the assistance of protein extracts ("packaging mixes", Maniatis et al.). The resultant λ -lysates are titrated on the indicator strain NM539 (Frischhauf et al.). 10⁶ pfu are obtained.

Example 13: Isolation of λ -clones which hybridise with a PAT-specific oligonucleotide:

40,000 recombinant phages of the penicillium chrysogenum gene bank in λ -EMBL3 (example 12), with the strain NM538 (Frischhauf et al.) are discharged onto 90 mm TB-plates in 0.7% agarose (TB medium contains 10 g bacto-trypton and 5 g NaCl per litre, the pH of 7.5 is set with NaOH). Two impressions of these plates are made on nylon filter (Amersham Hybond N-filter). After UV-fixing of the DNA, these filters are used for hybridisation. The 30mer described in example 11, whose sequence is complementary to the DNA-sequence which is from the amino acid sequence of polypeptide 1 of the PAT, is radioactively labelled (T4-polynucleotide-kinase

reaction as in example 11). Hybridisation is carried out for about 20 hours at 37° in 6xSSPE (1xSSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 30% formamide, 5x Denhard, 0.1% SDS, 100 µg/ml herring sperm DNA, 0.1 mM ATP, 3 ng/ml 32-P-labelled oligonucleotide. The filters are then washed three times for 10 minutes at room temperature in 2xSSC (1xSSC is 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, and then three times for 20 minutes at 56° in 1xSSC, 0.1% SDS, and after drying are autoradiographed. Regions in the agarose layer of the original plate, which correspond to positive hybridisation signals on the X-ray film, are taken out with a sterile Pasteur pipette and re-suspended in SM-buffer (5.8 g NaCl, 2 g MgSO₄.7H₂O and 50 ml 1 M tris/HCl, pH 7.5). An appropriate dilution with NM 538 as indicator strain is again discharged onto TB-plates. The phages on these plates are transferred onto nylon filter and hybridised as described above. The corresponding recombinant λ-phages are isolated from plaques which give positive signals for the second time. The purified DNA of these phages is used for restriction analysis and southern hybridisation. In addition, subcloning is carried out in plasmids (pUC 12, Messing, Methods Enzymol. 101, 20, 1983) and M13-vectors (M13mp18, M13mp19, Norrander et al., Gene 26, 101, 1983). Sequencing of a M13mp19-subclone with the oligonucleotide gives the following sequence:

	10	20	30	40	50
5'-	CATC	ACGG	GCTGC	CTTG	AGCC
	60	70	80	90	100
	ATGA	CAAT	CTCG	GAGAC	ATCC
	110	120	130	140	150
	TTCG	CGGTC	ATCG	CTCACC	ATTG
	160	170	180	190	200
	GTGC	CACTC	ACCG	CGAAT	CTCCT
	CGTAGTATTTGCGCCATCTTCTCGATC-3'				

Nucleotides 35 to 111 of the DNA sequence, which were ascertained using RNA (example 11), conform with nucleotides 1 to 77 of this DNA sequence (fig. 6F).

Example 14: DNA sequence of the PAT gene:

In fig. 4, the position of this DNA sequence is marked by two horizontal lines (A), 1 corresponding to the first nucleotide and 1972 to the last.

```

      10      20      30      40      50      60      70
GTTGATGTCCCATCAGTGTCACTGCTATCGTCCCAGATTGGTGGCTACGCCAATATAAAATCTCAGCATCCA

      80      90     100     110     120     130     140
GTTCCGCGCTGCATGATCATCCCCAGGACGCCGTTTGTTCATCTCCGTCAAGCCAGGTCTCAGTTGTTTACCC

     150     160     170     180     190     200     210
ATCTTCCGACCCGCCAGCAGAAATGCTTCACATCCTCTGTCAAGGCACTCCCTTTGAAGTAAGTGGTCCAC

     220     230     240     250     260     270     280
TGAATACCAGATTTTTTCTTCTGAATCTTCCGAGTTCTGACCTGATCCAGATCGGCTACGAACATCGCT

     290     300     310     320     330     340     350
CTGCTGCCAAAGCCGTGATAGCCAGAAGCATTGACTTCGCCGTGATCTCATCCGAGGGAAGCAAGAA

     360     370     380     390     400     410     420
GACGGACGAAGAGCTTAACAGGTAATCTCGCAACTGGGGCGGTGATCGAGGAAGATGGCCCAATAC

     430     440     450     460     470     480     490
TACGAGGAGATTCCCGGTGAGTCCCACTTCGGTCTTTCTTACATTTTCTGCACCAATGCTGACCGATGAC

     500     510     520     530     540     550     560
CCCCGAAAAACCAGGTATTGCAAAGGGCGCTGAACCGGATGTCTCCGAGATTGTTCATGCTTAATACCCCG

     570     580     590     600     610     620     630
ACCGAATTTGCATACGGGCTCAAGCCAGCCCGTGATGGCTGCACCACTGCCTATTGTCAACTTCCAAATG

     640     650     660     670     680     690     700
GAGCCCTCCAGGCCCAAACTGGGATGTACGTTAAGAGATTTTACCTCCTCATTTTATTCCATCGAATTT

     710     720     730     740     750     760     770
CGCGCCGACTAATTTGGTTGTTCAAGTTCTTTTCTGCCACCAAGAGAACCCTGATCCGGTTAACGATCCGT

     780     790     800     810     820     830     840
CAGGCCCGACTTCCCACCATCAAATTCATAACCGAGGCTCGAATCATCCGGAAGGTTGGATTTAACAGTG

     850     860     870     880     890     900     910
CGGGGGTCCCGTCAATTACAACGCCCTTCACCTTCAAGGTCTTCGACCCACCGGAGTTCCTTCGCATAT

     920     930     940     950     960     970     980
TGCCCTCCCATAGCCCTCGAAAGCACTTCTCCTTCCCAGCCCTATGACCGGATCGTGGAGCAAGGCCGA

     990    1000    1010    1020    1030    1040    1050
ATGCGCGCCAGCCCTTTTATCATGGTGGGCAATCGCCACGAGGCATTGTTTGAATTCTCCCCACCA
```

```
1060      1070      1080      1090      1100      1110      1120
GCATCCGAAAGCAGGTGCTCGACCGGAATGGTAGGATGGTGACACCAACCACTGCTTCGCTTCAGCACGG

1130      1140      1150      1160      1170      1180      1190
CAAAAATGAGAAAGAGCTCGATCCCTTACCGGACTCATGGAATCGCCACCAGCGTATGGAGTTCTCTCTC

1200      1210      1220      1230      1240      1250      1260
GACGGGTTGACCGGCACCAAACAGGCATTTGCCAGCTCTGGCCCGACGAAGACAATTATCCCTTTAGCA

1270      1280      1290      1300      1310      1320      1330
TCTGCCCGCCTTACGAGGAGGGCAAGAGCAGAGGCGGACTCTGTTCAATATCATCTACGACCATGCCCC

1340      1350      1360      1370      1380      1390      1400
TAGAGAGGCAACGGTGCGGCTTGGCCGGCCGACCAACCCTGATGAGATGTTTGTTCATGCCGTTTGACGAG

1410      1420      1430      1440      1450      1460      1470
GAGGACGAGAGGTCTCGGCTCAACGCCAGGCTTTGAAGCTCTTCATGACGAGCCAATGCATCTTTTCTA

1480      1490      1500      1510      1520      1530      1540
TGTAGCTTCAACCGACTCCGTCTTCACTTCTTCCGCCCGACTGCCTACCGTTTGTACCATCTGACTCATA

1550      1560      1570      1580      1590      1600      1610
TAAATGTCTAGCCCCCTACCTACACTATACCTAAGGGAGAGAAGCGTAGAGTGATTAACTACGGGCCTAT

1620      1630      1640      1650      1660      1670      1680
AGTACCCCGATCTCTAGATAGAACATTTAGTAGAGATTAGGATGCCTAACTAATTTAACTTGAGCATTGT

1690      1700      1710      1720      1730      1740      1750
CCCGTTTCATATTGATTTTCATCCATTATACACTCTTAATCGTTTCCCGGTAGAAGCCGNATATATACGAC

1760      1770      1780      1790      1800      1810      1820
CATAGCGTGTGGAGAACACGGCTTCCCGTCTGCTTGGCCGTAAGCTATATATTCTACACGCCCAAT

1830      1840      1850      1860      1870      1880      1890
ACTCAATGTGCCCTTAGCACCTAAGCGGCACTCTAGGGTAAGTCCGGGTGATATACGTGAGAAGTCTTAA

1900      1910      1920      1930      1940      1950      1960
GACTGAAGACAGGATATCACGCGTTACCCTGCACCGTACCTACTACCTTCAATATCACTCTTTCAGGATG

1970
GACAGCGTCCGAC
```

Example 15: DNA sequence of the PAT gene:

In this DNA sequence, the coding range was established by a comparison of the experimentally determined amino acid part sequences with the coding capacity of this DNA sequence. It is thus shown that three introns are present in this gene. They were additionally identified by intron-extron consensus sequences (Rambosek and Leach, Critical Reviews in Biotechnology 6, 357-393, 1987).

10 20 30 40 50 60 70
GTTGATGTCATCATGCTATGGTCCCAGATTGGTGGCTACGGCAATATAAATCTCAGCATGCA

80 90 100 110 120 130 140
GTTCCCGCTGCATGATCATCCCCAGGACCGCGTTTGTCTATCTCCGTCAAGCCAGGTCTCAGTTGTTTACCC

150 160 170 180 190 200 210
ATCTTCCGACCCGACGAGAAATGCTTCACATCCTCTGTCAAGGCACTCCCTTTGAAGTAAGTGCTCCAC
MetLeuHisIleLeuCisGlnGlyThrProPheGlu

220 230 240 250 260 270 280
TGAATACCAGATTTTTTCTTCTGAATCTTCCGAGTTCTGACCTGATCCAGATCCGCTACGAACATGGCT
IleGlyTyrGluHisGlyS

290 300 310 320 330 340 350
CTGCTGCCAAAGCCGTGATAGCCAGAAGCATTGACTTCGCCGTGATCTCATCCGAGGAAAAACGAAGAA
erAlaAlaLysAlaValIleAlaArgSerIleAspPheAlaValAspLeuIleArgGlyLysThrLysLy

360 370 380 390 400 410 420
GACCGACGAAGAGCTTAAACAGGTACTCTCCAACTGGCGCGGTGATCGAGGAAAGATGGCCCAAATAC
sThrAspGluGluLeuLysGlnValLeuSerGlnLeuGlyArgValIleGluGluArgTrpProLysTyr

430 440 450 460 470 480 490
TACGAGGAGATTCCGGTGAGTCCCACTTCGGTCTTTCTACATTTTCTGCACCAATGCTGACCGATGAC
TyrGluGluIleArgG

500 510 520 530 540 550 560
CCCCGAAAAACCAGGTATTGCAAAGCGCGCTGAACGGATGTCTCCGAGATTGTCTATGCTTAATACCCGC
lyIleAlaLysGlyAlaGluArgAspValSerGluIleValMetLeuAsnThrArg

570 580 590 600 610 620 630
ACCGAATTTGCATACGGGCTCAAGGCAGCCCGTGATGGCTGCACCACTGCCTATTGTCAACTTCCAAATG
ThrGluPheAlaTyrGlyLeuLysAlaAlaArgAspGlyCysThrThrAlaTyrCysGlnLeuProAsnG

640 650 660 670 680 690 700
GAGCCCTCCAGGGCCAAACTGGGATGTACGTTAAGAGATTTTACCTCCTCATTATTATCCATCGAATT
lyAlaLeuGlnGlyGlnAsnTrpAsp

710 720 730 740 750 760 770
CGCCCGACTAATTTGGTTGTTCAAGTTCTTTTCTGCCACCAAAAGAAACCTGATCCGGTTAACGATCCGT
PhePheSerAlaThrLysGluAsnLeuIleArgLeuThrIleArg

780 790 800 810 820 830 840
CAGCCCGACTTCCCAACATCAAATTCATAACCGAGGCTCGAATCATCCGGAAGGTTGGATTTAACAGTG
GlnAlaGlyLeuProThrIleLysPheIleThrGluAlaGlyIleIleGlyLysValGlyPheAsnSera

850 860 870 880 890 900 910
CGGGCGTCCCGTCAATTACAACGCCCTTCACCTTCAAGGTCTTCGACCCACCGAGTTCTTCCGATAT
laGlyValAlaValAsnTyrAsnAlaLeuHisLeuGlnGlyLeuArgProThrGlyValProSerHisIl

920 930 940 950 960 970 980
TGGCCTCCCATACCGCTCGAAAGCACTTCTCCTTCCAGGCCTATGACCGGATCGTGAGCAAGGCCGA
eAlaLeuArgIleAlaLeuGluSerThrSerProSerGlnAlaTyrAspArgIleValGluGlnGlyGly

990 1000 1010 1020 1030 1040 1050
 ATGCCCCCAGCGCTTTTATCATGCTGGCCAATGGCCACGAGGCATTTGCTTTGGAATTCTCCCCCACC
 MetAlaAlaSerAlaPheIleMetValGlyAsnGlyHisGluAlaPheGlyLeuGluPheSerProThrS

1060 1070 1080 1090 1100 1110 1120
 GCATCCGAAAGCAGGTGCTCGACGCGAATCGTAGGATGGTCCACCAACCACTGCTTGCTTCAGCACCG
 erIleArgLysGlnValLeuAspAlaAsnGlyArgMetValHisThrAsnHisCysLeuLeuGlnHisGl

1130 1140 1150 1160 1170 1180 1190
 CAAAAATGAGAAAGAGCTCGATCCCTTACCGGACTCATGGAATCCCCACCAGCGTATGGAGTTCTCTCTC
 yLysAsnGluLysGluLeuAspProLeuProAspSerTrpAsnArgHisGlnArgMetGluPheLeuLeu

1200 1210 1220 1230 1240 1250 1260
 GACGGGTTCGACGGGCACCAACAGGCATTTGCCAGCTCTCGCCGACGAAGACAATTATCCCTTTAGCA
 AspGlyPheAspGlyThrLysGlnAlaPheAlaGlnLeuTrpAlaAspGluAspAsnTyrProPheSerI

1270 1280 1290 1300 1310 1320 1330
 TCTGCCCGCGCTTACGAGGAGGCAAGACAGAGCGCCGACTCTGTTCAATATCATCTACGACCATGCCCC
 leCysArgAlaTyrGluGluGlyLysSerArgGlyAlaThrLeuPheAsnIleIleTyrAspHisAlaAr

1340 1350 1360 1370 1380 1390 1400
 TAGAGAGGCAACGGTGCCGGCTTGGCCCGCCGACCAACCCTGATGAGATGTTTGTCTATGCCGTTTGACGAG
 gArgGluAlaThrValArgLeuGlyArgProThrAsnProAspGluMetPheValMetArgPheAspGlu

1410 1420 1430 1440 1450 1460 1470
 GAGGACGAGAGGTCTGCCCTCAACCCACGCTTTGAAGGCTCTTCATGACGAGCCAAATGCCATCTTTTGTA
 GluAspGluArgSerAlaLeuAsnAlaArgLeu***

1480 1490 1500 1510 1520 1530 1540
 TGTAGCTTCAACCGACTCCGTCTTCACTTCTTCCCGCCGACTGCCTACCGTTTGTACCATCTGACTCATA

1550 1560 1570 1580 1590 1600 1610
 TAAATGTCTAGCCCCTACCTACACTATACCTAAGGGAGAGAAGCGTAGAGTGATTAACGTACCGCCCTAT

1620 1630 1640 1650 1660 1670 1680
 AGTACCCCGATCTCTAGATAGAACATTTAGTAGAGATTACGATGCCTAACTAATTTAACTTGACCATTGT

1690 1700 1710 1720 1730 1740 1750
 CCCGTTTCATATTGATTTTCATCCATTATACACTCTTAATCGTTTCCCGGTAGAAGCCGNATATATACGAC

1760 1770 1780 1790 1800 1810 1820
 CATAGGGTGTGAGAACAGCGCTTCCCGTCTGCTTGGCCGTACTTAACTATATATTCTACACCGCCAAT

1830 1840 1850 1860 1870 1880 1890
 ACTCAATGTGCCCTTAGCACCTAAGCCCGACTCTAGGGTAAGTCCCGGTGATATAGGTGAGAAGTCTTAA

1900 1910 1920 1930 1940 1950 1960
 CACTGAAGACAGGATATCACCGGTTACCCTGCACCGTACCTACTACCTTCAATATCACTCTTTACGATG

1970
 CACAGGCTCGAC

Example 16: Amino acid sequence of PAT, derived from the DNA sequence:

This polypeptide has a molecular weight of 40,000 D. If it is cleaved between amino acid radical 102 and 103, two polypeptides are obtained: One polypeptide with 11,500 D and the N-terminal amino acid sequence of polypeptide 2; all peptide fragments may be found again in this amino acid sequence (AA part sequences 9 to 24). This is thus the "8kD component of PAT".

One polypeptide with 28,500 D and the N-terminal amino acid sequence of polypeptide 1; all peptide fragments may be found again in this amino acid sequence (AA part sequences 2, 3, 4, 5, 7, 8). This is thus the "30kD component of PAT".

10	20
MetLeuHisIleLeuCysGlnGlyThrProPheGluIleGlyTyrGluHisGlySerAla	
30	40
AlaLysAlaValIleAlaArgSerIleAspPheAlaValAspLeuIleArgGlyLysThr	
50	60
LysLysThrAspGluGluLeuLysGlnValLeuSerGlnLeuGlyArgValIleGluGlu	
70	80
ArgTrpProLysTyrTyrGluGluIleArgGlyIleAlaLysGlyAlaGluArgAspVal	
90	100
SerGluIleValMetLeuAsnThrArgThrGluPheAlaTyrGlyLeuLysAlaAlaArg	
110	120
AspGlyCysThrThrAlaTyrCysGlnLeuProAsnGlyAlaLeuGlnGlyGlnAsnTrp	
130	140
AspPhePheSerAlaThrLysGluAsnLeuIleArgLeuThrIleArgGlnAlaGlyLeu	
150	160
ProThrIleLysPheIleThrGluAlaGlyIleIleGlyLysValGlyPheAsnSerAla	
170	180
GlyValAlaValAsnTyrAsnAlaLeuHisLeuGlnGlyLeuArgProThrGlyValPro	
190	200
SerHisIleAlaLeuArgIleAlaLeuGluSerThrSerProSerGlnAlaTyrAspArg	
210	220
IleValGluGlnGlyGlyMetAlaAlaSerAlaPheIleMetValGlyAsnGlyHisGlu	
230	240
AlaPheGlyLeuGluPheSerProThrSerIleArgLysGlnValLeuAspAlaAsnGly	

	250	260
ArgMetValHisThrAsnHisCysLeuLeuGlnHisGlyLysAsnGluLysGluLeuAsp		
	270	280
ProLeuProAspSerTrpAsnArgHisGlnArgMetGluPheLeuLeuAspGlyPheAsp		
	290	300
GlyThrLysGlnAlaPheAlaGlnLeuTrpAlaAspGluAspAsnTyrProPheSerIle		
	310	320
CysArgAlaTyrGluGluGlyLysSerArgGlyAlaThrLeuPheAsnIleIleTyrAsp		
	330	340
HisAlaArgArgGluAlaThrValArgLeuGlyArgProThrAsnProAspGluMetPhe		
	350	
ValMetArgPheAspGluGluAspGluArgSerAlaLeuAsnAlaArgLeu		

Example 17: Comparison of the amino acid sequence of PAT derived from the DNA with the experimentally determined amino acid sequences:

30kD unit/polypeptide 1 (N-terminal amino acid sequence)

2	ThrThrAlaTyrCysGlnLeuProAsnGlyAlaLeuGlnGlyGlnAsnTrpAspPhePhe	
104	ThrThrAlaTyrCysGlnLeuProAsnGlyAlaLeuGlnGlyGlnAsnTrpAspPhePhe	
22	SerAlaThrLysGluAsnLeuIleArg	30
124	SerAlaThrLysGluAsnLeuIleArg	132

AA part sequence 2 (tryptic peptide fragment of polypeptide 1):

1	GlyAlaThrLeuPheAsnIleIleTyrAspHisAlaArg	13
311	GlyAlaThrLeuPheAsnIleIleTyrAspHisAlaArg	323

AA part sequence 3 (tryptic peptide fragment of polypeptide 1):

1	ProThrAsnProAspGluMetPheValMetArg	11
333	ProThrAsnProAspGluMetPheValMetArg	343

AA part sequence 4 (tryptic peptide fragment of polypeptide 1):

1	GluLeuAspProLeuProAspSerTrpAsnArg	11
258	GluLeuAspProLeuProAspSerTrpAsnArg	268

AA part sequence 5 (tryptic peptide fragment of polypeptide 1):

1	MetGluPheLeu—AspGlyPheAspGlyThrLys	12
272	MetGluPheLeuLeuAspGlyPheAspGlyThrLys	283

AA part sequence 7 (tryptic peptide fragment of polypeptide 1):

1	IleAlaLeuGluSerThrSerProSerGlnAlaTyrAspArg	14
187	IleAlaLeuGluSerThrSerProSerGlnAlaTyrAspArg	200

AA part sequence 8 (tryptic peptide fragment of polypeptide 1):

1	ValGlyPheAsnSerAlaGlyValAlaValAsnTyrAsnAlaLeuHisLeuGlnGlyLeu	
155	ValGlyPheAsnSerAlaGlyValAlaValAsnTyrAsnAlaLeuHisLeuGlnGlyLeu	
21	ArgProThrGlyValProSerHisIleAlaLeuArg	32
175	ArgProThrGlyValProSerHisIleAlaLeuArg	186

8kD unit I/polypeptide 2a

1	MetLeuHisIleLeuCysGlnGlyThrProPheGluIleGlyTyrGluHisGlySerAla	
1	MetLeuHisIleLeuCysGlnGlyThrProPheGluIleGlyTyrGluHisGlySerAla	
	AlaLysAlaValIleAlaArgSerIleAspPheAlaValAsp	34
	AlaLysAlaValIleAlaArgSerIleAspPheAlaValAsp	34

8kD unit II/polypeptide 2b

1	MetLeuHisIleLeuLeyCysGlnGlyThrProPheGluIleGlyTyrGluHisGlySerAla	
1	MetLeuHisIleLeuCysGlnGlyThrProPheGluIleGlyTyrGluHisGlySerAla	
	AlaLysAlaValIleAlaArgSerIleAspPheAlaValAspLeuIleArgGlyLysThr	40
	AlaLys__ValIleAlaArg__IleAspPheAlaValAspLeu____Gly__Thr	40

AA part sequence 9 (tryptic peptide fragment of polypeptide 2):

1	ThrGluPheAlaTyrGlyLeuLys	8
90	ThrGluPheAlaTyrGlyLeuLys	97

AA part sequence 10 (tryptic peptide fragment of polypeptide 2):

1	TyrTyrAnyGluIleArg	6
65	TyrTyrGluGluIleArg	70

AA part sequence 11 (tryptic peptide fragment of polypeptide 2):

1	TrpProLys	3
62	TrpProLys	64

AA part sequence 12 (tryptic peptide fragment of polypeptide 2):

1	SerIleAspPheAlaValAspLeuIleArg	10
28	SerIleAspPheAlaValAspLeuIleArg	37

AA part sequence 13 (tryptic peptide fragment of polypeptide 2):

1	AspValSerGluIleValMetLeuAsnThrArg	11
79	AspValSerGluIleValMetLeuAsnThrArg	89

AA part sequence 14 (tryptic peptide fragment of polypeptide 2):

1	GlnValLeuSerGlnLeuGlyArg	8
49	GlnValLeuSerGlnLeuGlyArg	56

AA part sequence 15 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

6	CysGlnGlyThrProPheGlu	12
1	CysGlnGlyThrProPheGlu	7

AA part sequence 16 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

65	TyrTyrGluGluIleArgGlyIleAlaLys	74
1	TyrTyrGluGluIleArgGlyIleAlaLys	10

AA part sequence 17 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

49	GlnValLeuSerGlnLeuGlyArgValIleGluGluArgTrpProLys	64
1	GlnValLeuSerGlnLeuGlyArgValIleGluGluArg__ProLys	16

AA part sequence 18 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

75	GlyAlaGluArgAspValSerGluIleValMetLeuAsnThrArg	89
1	GlyAlaGluArgAspValSerGluIleValMetLeuAsnThrArg	15

AA part sequence 19 (tryptic peptide fragment of polypeptide 2):

23	AlaValIleAlaArg	27
1	AlaValIleAlaArg	5

AA part sequence 20 (tryptic peptide fragment of polypeptide 2):

42	LysThrAspGluGluLeuLys	48
1	Lys__AspGluGluLeuLys	7

AA part sequence 21 (tryptic peptide fragment of polypeptide 2):

71	GlyIleAlaLys	74
1	GlyIleAlaLys	4

AA part sequence 22 (tryptic peptide fragment of polypeptide 2):

57	ValIleGluGluArg	61
1	ValIleGluGluArg	5

AA part sequence 23 (peptide fragment of polypeptide 2 with
lysyl-endopeptidase):

87	AsnThrArgThrGluPheAlaTyrGlyLeuLys	97
1	AsnThr____ThrGluPheAlaTyrGlyLeuLys	11

Summary:

AA part sequence	position of AA in the AA- sequence derived from the DNA
N-terminal AA sequence 30 kD unit/polypeptide 1	104-113
N-terminal AA sequence 8 kD unit/polypeptide 2a	1- 34
N-terminal AA sequence 8 kD unit/polypeptide 2b	1- 40
AA part sequence 2 (tryptic peptide fragment of polypeptide 1):	311-323
AA part sequence 3 (tryptic peptide fragment of polypeptide 1):	333-343
AA part sequence 4 (tryptic peptide fragment of polypeptide 1):	258-268
AA part sequence 5 (tryptic peptide fragment of polypeptide 1):	272-283
AA part sequence 7 (tryptic peptide fragment of polypeptide 1):	187-200
AA part sequence 8 (tryptic peptide fragment of polypeptide 1):	155-186
AA part sequence 9 (tryptic fragment of polypeptide 2):	90- 97
AA part sequence 10 (tryptic fragment of polypeptide 2):	65- 70
AA part sequence 11 (tryptic fragment of polypeptide 2):	62- 64
AA part sequence 12 (tryptic fragment of polypeptide 2):	28- 37
AA part sequence 13 (tryptic fragment of polypeptide 2):	79- 89
AA part sequence 14 (tryptic fragment of polypeptide 2):	49- 56
AA part sequence 15 (lysyl-e. of polypeptide 2):	6- 12
AA part sequence 16 (lysyl-e. of polypeptide 2):	65- 74
AA part sequence 17 (lysyl-e. of polypeptide 2):	49- 64
AA part sequence 18 (lysyl-e. of polypeptide 2):	75- 89
AA part sequence 19 (tryptic fragment of polypeptide 2):	23- 27
AA part sequence 20 (tryptic fragment of polypeptide 2):	42- 48
AA part sequence 21 (tryptic fragment of polypeptide 2):	71- 74
AA part sequence 22 (tryptic fragment of polypeptide 2):	57- 61
AA part sequence 23 (lysyl-e. of polypeptide 2):	87- 97

Example 18: part sequence of a DNA fragment which lies between the gene for isopenicillin N synthetase (ips) and the gene for PAT:

This sequence begins with "GGATCC", the recognition sequence of the restriction enzyme BamH1, which lies in the ips gene. The last 34 (C-terminal) codons of this gene are shown; they have been identified by comparison with the published DNA sequence (Carr et al., Gene 48, 257-266, 1986). After an intermediate region, position 1 of the pat-DNA sequence follows at 1442. This DNA part sequence is marked in fig. 4 (B).

This DNA part sequence is important, since it proves the narrow coupling between ips- and pat-gene, and because all essential control elements for transcription and translation of the pat-gene lie on this fragment.

```

      10      20      30      40      50
GGATCCTAGCAAGGAAGACGGCAAGACCGATCAGCGGCCAATCTCGTACG
AspProSerLysGluAspGlyLysThrAspGlnArgProIleSerTyrG

      60      70      80      90     100
GGACTATCTGCAGAACGGATTAGTTAGTCTAATCAACAAGAACGCCAG
lyAspTyrLeuGlnAsnGlyLeuValSerLeuIleAsnLysAsnGlyGln

     110     120     130     140     150
ACATGAAAGGGCCCATGGATGGGACCGGGATGGAAATCCCGGACTCTGAG
ThrEnd

                                           850
NNNNNNNNNNNNNNNNNNNN 1250 bp NNNNNNNNNNNNNNNNNNNNN

     1410     1420     1430     1440     1450
CAAGACTAGCGCGATGCAGCAGGGATACTCGAGGTCCCGGAGTTGATGTC

     1460     1470     1480     1490     1500
CCATCAGTGTTCATGCTATGGTCCCAGATTGGTGGCTACGCCCAATATAAA

     1510     1520     1530     1540     1550
TCTCAGCATCCAGTTCCGCCTGCATGATCATCCCAGGACCCCCGAGCA

     1560     1570     1580     1590
GAAATGCTTCACATCCTCTGTCAAGGCACTCCCTTTGAAGTA
MetLeuHisIleLeuCysGlnGlyThrProPheGlu

```

Example 19: Construction of the plasmid pBC2001 and expression of the pat-gene in E.coli:

a) Construction of a cDNA gene bank of penicillium chrysogenum:

5 μ g of the poly(A)⁺-RNA, the isolation and purification of which are described in example 10, are reacted using reverser transcriptase with an oligo dT-primer in the presence of the four desoxynucleoside triphosphates to form a complementary single-stranded DNA. A double-stranded molecule is formed therefrom by means of the enzyme RNaseH and DNA polymerase (Gubler and Hoffman, Gene 25, 263, 1983). After adding appropriate EcoR1 adaptors, for which purpose the enzymes polynucleotide kinase and T4-DNA ligase are used, a linear, double-stranded cDNA is obtained, which can be built into cloning vectors. For these reactions, a commercial cDNA synthesis kit (Fa. Pharmacia) is favourably used. It contains the most important enzymes and the adaptor oligonucleotide. The reaction is carried out in accordance with the manufacturer's instructions. The double-stranded cDNA thus synthesized, with EcoR1 ends, is cloned into the vector gt10 (Huynh et al., DNA cloning, Glover, D.M. ed., Oxford, 1, 49, 1985). 80 μ l of the cDNA preparation is mixed with 16 μ l of gt10-DNA (8 μ g), which has previously been cleaved with EcoR1 and treated with alkaline phosphatase (Maniatis et al.). After adding 3 μ l 3 M sodium acetate (pH 5.2) and 250 μ l ethanol, the mixture is precipitated for 20 hours at -20° and subsequently dissolved in 60 μ l of 10 mM tris-HCl (pH 7.5), 1 mM EDTA. Ligation is effected at 12° for 20 hours in 66 mM tris-HCl (pH 7.5), 1 mM spermidine, 10 mM MgCl₂, 15 mM dithiothreitol, 0.2 mg/ml BSA, 0.5 mM ATP, by adding 6 U T4-DNA ligase. The ligation mixture is packed in vitro with protein extracts ("packaging mix") and plated with the E.coli strain C600hfl (Huynh et al.). The necessary methods are described (Maniatis et al.). In such a test, more than $5 \cdot 10^5$ plaques may be obtained.

b) Isolation of the pat-specific cDNA clones and subcloning in M13mp19.

Plaque hybridisation is carried out as described in example 13, using about 40,000 plaques of the penicillium chrysogenum cDNA gene bank. The E.coli strain C600hfl (Huynh et al.) is used as the indicator strain. After partial cleavage with EcoR1, the DNA of the recombinant gt10-phages is ligated with EcoR1-cleaved M13mp19-RF-DNA and transfixed. Individual clones of recombinant M13mp19-RF-DNA are identified and confirmed by restriction mapping.

c) Site-directed mutagenesis of the DNA of the recombinant M13mp19 clone to insert a NcoI intersection site.

A 41mer oligonucleotide with the following sequence is synthesized:

5'-TCCGACCCGCAGCAGCCATGGTTCACATCCTCTGTCAAGGC-3'

The DNA sequence has altered compared with the DNA sequence of the pat-cDNA, in that a NcoI intersection site is obtained, which encloses the ATG corresponding to the N-terminal methionine radical. To the right are 20, to the left 15 nucleotides identical to the pat-cDNA sequence. 5 pmol of the phosphorylated oligonucleotide and 0.5 pmol of the single-stranded M13mp19-DNA are mixed and heated in 10 μ l 20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, for 5 minutes at 65° and for 20 minutes at 42°. Treatment with Klenow polymerase, T4-DNA-ligase and S1-nuclease is effected according to Eghtedarzadeh and Henikoff (Nucl. Acids Res. 14, 5115, 1986): 10 μ l solution with 2 units Klenow polymerase and 3 units T4-DNA-ligase in 20 mM tris-Cl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.8 mM of each of the four dNTPs, 1 mM ATP, for 1 hour at 42°. The reaction is stopped by adding EDTA (final concentration 25 mM) and is heated for 10 minutes to 70°. After precipitation with ethanol, the pellet is dissolved in 10 μ l tris-HCl (pH 8), 1 mM EDTA. After adding

15 μ l 30 mM potassium acetate (pH 4.6), 0.25 M NaCl, 1 mM ZnCl₂, 5% glycerol, 7 units S1 nuclease, incubation is effected for 20 minutes at room temperature. After transfection of an appropriate E.coli strain (e.g. JM101; Yanisch-Perron et al., Gene 33, 103, 1985) with the reaction preparation, and isolation of RF-DNA, the changed DNAs can be identified by cleavage with NcoI.

d) Cloning of the NcoI-HindIII fragment with the pat cDNA into the plasmid pKK233-2.

The RF-DNA of the recombinant M13 clone, which contains the pat cDNA with the inserted NcoI intersection site, is cleaved with NcoI and HindIII. The plasmid pKK233-2 (Amann and Brosius, Gene 40, 183, 1985) is similarly cleaved with NcoI and HindIII. Ligation of the two DNAs takes place for 20 hours at 14° in 66 mM tris-HCl (pH 7.5), 1 mM spermidine, 10 mM MgCl₂, 15 mM dithiothreitol, 0.2 mg/ml BSA, 0.5 mM ATP, with the addition of 2 U T4-DNA-ligase. After transformation of an appropriate E.coli strain (e.g. JM83; Yanisch-Perron et al., Gene 33, 103, 1985), individual plasmid-DNAs are isolated and characterised by restriction mapping. The plasmid which contains the NcoI-HindIII restriction fragment with the pat cDNA in the plasmid pKK233-2 is called pBC2001.

c) Expression of the pat-gene in E.coli

E.coli RB791 (Amann and Brosius, Gene 40, 183, 1985) is transformed with pBC2001. 50 ml LB medium (per litre: 10 g bacto tryptone, 8 g NaCl, 5 g yeast extract; pH 7.5 is set with NaOH) is seeded with an individual colony of the strain RB791(pBC2001) and agitated at 200 rpm at 37° until an optical density of 0.5 has been attained (measured at 600 nm). 5 ml of a 0.1 M IPTG (isopropyl- β -D-thiogalactoside) are added. Agitation is subsequently effected at 200 rpm for 3 hours at 37°. The cells are then centrifuged (10 minutes, 5000 rpm, 20° Beckmann JA20)

and worked up for identification of the protein expressed in *E.coli*. This identification may be effected by SDS polyacrylamide gel-electrophoresis of the *E.coli* total protein, by Western-Blot using PAT-specific antibodies or by enzymatic detection.

Example 20: Transformation of the pat-gene for penicillium chrysogenum

a) Construction of the plasmid pBC2002:

The plasmid pHS103 (Kolar et al., Gene 62, 127, 1988) is totally cleaved with *EcoRI* and ligated again in the presence of *EcoRI*-*Sall*-cleaved M13mp19-RF-DNA (66-mM tris-HCl (pH 7.5), 1 mM spermidine, 10 mM $MgCl_2$, 15 mM dithiothreitol, 0.2 mg/ml BSA, 0.5 mM ATP, adding 2 U T4-DNA-ligase at 14° for 20 hours). After transformation, a plasmid is available which is suitable for the cloning of *Sall* fragments. This plasmid is cleaved with *Sall*, just as a recombinant plasmid with a 4.8 kb *Sall* fragment with the complete pat-gene (see example 13). After ligation and transformation, a plasmid can be identified, which consists of the modified pHS103 and the 4.8 kb *Sall* fragment with the pat-gene; this plasmid is called pBC2002.

b) Isolation of penicillium chrysogenum protoplasts and transformation:

2 ml of a dense spore suspension of penicillium chrysogenum P2/ATCC are added to 200 ml of sterile minimum medium for penicillium chrysogenum in a 1 l Erlenmeyer flask (per litre: 3 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 10 mg $FeSO_4 \cdot 7H_2O$, 20 g saccharose, 13 g KH_2PO_4 and 1 ml trace element mixture (for 100 ml: 0.1 g $FeSO_4 \cdot 7H_2O$, 0.9 g $ZnSO_4 \cdot 7H_2O$, 0.04 g $CuSO_4 \cdot 5H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g H_3BO_3 , 0.01 g $Na_2HPO_4 \cdot 2H_2O$)) and agitated at 250 rpm for 20 hours at 25°. Isolation and purification of the protoplasts takes place according to Yelton et al. (Proc. Natl. Acad. Sci. U.S.A., 81, 1470, 1984).

The mycelium is centrifuged off and washed twice in 0.9 M NaCl and resuspended in 20 ml 0.9 M NaCl with 5 mg/ml novozyme 234. Incubation at 30° for 1.5 hours. The reaction preparation is centrifuged (Beckman cooling centrifuge JS 7.5, 1500 rpm, 20°, 5 minutes). The protoplast pellet is washed twice with 0.9 M NaCl and then once in 1.0 M sorbitol, 50 mM CaCl₂. The protoplasts are resuspended in 0.2 ml 1.0 M sorbitol, 50 mM CaCl₂ (about 0.5 to 5.10⁸ protoplasts/ml).

5 µg pBC2002 in 10 mM tris-HCl (pH 7.5), 1 mM EDTA are used for transformation and added to the protoplast suspension. 12.5 µl 25% polyethylene glycol (BDH), 10 mM tris-HCl pH 7.5, 50 mM CaCl₂ (sterile-filtered) are added. Incubation for 20 minutes in ice. 0.5 ml of 12.5 µl 25% polyethylene glycol (BDH), 10 mM tris-HCl (pH 7.5), 50 mM CaCl₂ (sterile-filtered) are added; the mixture is incubated for 5 minutes at 20°. After adding 1 ml 0.9 M NaCl, 50 mM CaCl₂, thorough mixing takes place, and the mixture is added to 3 ml of minimum medium with 0.5% agar and 20 µg phleomycin/ml (48°). The transformation preparations are plated onto minimum medium plates (minimum medium as described above, with 1.6% agar and 20 µg phleomycin/ml).

DNA which is isolated from various colonies can be characterised by southern hybridisation with radioactively-labelled pat-DNA. Of the transformants, those which contain several copies of the pat-gene due to multiple integration occurrences are discovered. Such strains are subsequently tested by test fermentations for increased penicillin formation.

Table 1: Decomposition and precipitation of thiol-dependent PAT from penicillium chrysogenum P2/ATCC 48271

Purification	duration of decomposition	total volume (ml)	Protein		Microbiological 6-APA-PaCoA-Acyltransferase-Activity (mm HH Ø on BC 248)								
					¹⁾ supernatant undiluted			supernatant 1:10			supernatant 1:50		
			(mg total)	(mg/ml)	immediately	30'	30' + penase	immediately	30'	30' + penase	immediately	30'	30' + penase
Cell decomp. with Dyno mill Type KDL (1786 g Bfm =220 g DS ln3.5 l) 0.1 M Phosphate buffer pH 7.5+5 mM DTT +0.1% Triton X 100+0.1 mM PMSF; 600 ml steel milling container; 510 ml 500-750 µm glass pearls	0	4900	676	0.138	0	0	0	0	0	0	0	0	0
	30		20776	4.24	trace	18.0	0	0	24.4	0	0	trace	0
	60		25431	5.19	trace	17.9	0	trace	27.0	0	0	15.7	0
in continuous operation reversed speed: 2000 rpm(= 6.7 m/sec); friction gap width 0.1mm flow rate for grinding matter=30l/h Temp. PM 2/3°C RM 5°C; Temp. cooling PM -20/-8°C RM -5/-1°C	90		29204	5.96	15.2	22.0	0	13.8	29.9	0	0	16.5	0
	120		29302	5.98	trace	17.1	0	0	24.8	0	0	0	0

Table 1 continuation: Decomposition and precipitation of thiol-dependent PAT from penicillium chrysogenum P2/ATCC 48271

Purification	duration of decomposition	total volume (ml)	Protein		Microbiological 6-APA-PaCoA-Acyltransferase-Activity (mm HH Ø on BC 248)								
					¹⁾ supernatant undiluted			supernatant 1:10			supernatant 1:50		
			(mg total)	(mg/ml)	immediately	30'	30' + penase	immediately	30'	30' + penase	immediately	30'	30' + penase
Supernatant after nucleic acid precipitation with 0.5% CTAB		4650	12927	2.78	14.4	26.0	0	0	25.8	0	0	0	0
Protein precipitate with 50 WV % Ammonium sulphate		480 (= 544 g)	10965	22.8	27.4 ²⁾	28.8	0	18.4	28.2	0	16.1	27.1	0

¹⁾ Prior to activity testing, the undiluted decomposed samples undergo buffer exchange over PD 10 columns (separation of residual content of penicillin V. 2.5 ml supernatant sample with 3.5 ml 0.1M phosphate buffer pH 7.5 + 1mM DTT)

²⁾ (NH₄)₂SO₄ precipitate prior to activity testing with 0.1 M phosphate buffer pH 7.5 + 1 mM DTT in 1:5 dilution

Penase = penicillinase

Table 2: Stability respectively activity of thiol dependent PAT from penicillium chrysogenum P2/ATCC 48271 in dependence on temperature, time, pH value and stabilizer additives

Test	immediately			24 h at + 4°C			24 h at -20°C		
Stabilisation condition	Microbiological 6-APA-PaCoA-Acylation activity (mm HH O on Micrococcus luteus ATCC 9341/BC 85)								
	immediately	30'	30' + penase	immediately	30'	30' + penase	immediately	30'	30' + penase
Activation									
without additive	11	15.5	0	-	-	0	-	-	0
+ 1 mM DTT	23.5	37.1	0	0	trace	0	15	38	0
+ 10 mM DTT	22.7	39.0	0	16.6	36	0	16	33	0
+ 10 mM β-ME	15.4	27.8	0	0	trace	0	trace	28.3	0
+ 10 mM MgCl ₂	9.8	17.1	0	0	trace	0	18.5	15	0
+ 2 mM EDTA	0	16.0	0	-	-	0	-	-	0
+ 1 mM DTT + 10 mM MgCl ₂	26.9	36.7	0	0	trace	0	12.2	37	0
+ 2 mM EDTA + 10 mM MgCl ₂	16.3	25.4	0	0	14.8	0	trace	23	0
+ 5% Sorbitol + 1 mM DTT + 2 mM EDTA + 1 mM PMSF	26.0	38.6	0	0	trace	0	11.3	33	0
+ 5% Sorbitol + 1 mM DTT + 1 mM PMSF	25.5	36.6	0	0	trace	0	16.8	36	0
pH profil									
0.1 M HEPES pH 7.7 + 1 mM DTT	26.6	37.3	0	0	trace	0	12.5	33.5	0
0.1 M NaH ₂ PO ₄ /Na ₂ HPO ₄ pH 6.0 + 1 mM DTT	0	17.3	0	-	-	0	-	-	0
0.1 M NaH ₂ PO ₄ /Na ₂ HPO ₄ pH 7.0 + 1 mM DTT	22.1	32.7	0	0	11.2	0	0	29.3	0
0.1 M NaH ₂ PO ₄ /Na ₂ HPO ₄ pH 8.0 + 1 mM DTT	33.8	35.0	0	0	13	0	17	36.5	0
0.1 M TRIS/HCl pH 8.0 + 1 mM DTT	14.3	19.6	0	0	0	0	0	16	0
0.1 M TRIS/HCl pH 9.0 + 1 mM DTT	0	trace	0	-	-	0	-	-	0

Table 2 continuation: Stability respectively activity of thiol dependent PAT from penicillium chrysogenum P2/ATCC 48271 in dependence on temperature, time, pH value and stabilizer additives

Test	immediately			24 h at + 4°C			24 h at -20°C		
Stabilization condition	Microbiological 6-APA-PaCoA-Acylation activity (mm HH O on Micrococcus luteus ATCC 9341/BC 85)								
	immediately	30'	30' + penase	immediately	30'	30' + penase	immediately	30'	30' + penase
Inhibitors									
without additives	22.6	33.6	0	trace	trace	0	11.6	34	0
+ 2 mM PMSF	22.1	32.2	0	0	trace	0	13.8	27	0
+ 2 mM DIPFP	22.6	34.5	0	0	trace	0	10	35	0
+ 2 mM 2,2'-Bipyridyl	0	0	0	-	-	0	-	-	0
+ 2 mM Hydroxyquinoline	n.	34.5	0	0	12	0	13.5	38	0
+ 2 mM o-Phenanthroline	18.9	29.9	0	trace	trace	0	11	33	0
+ 2 mM N-Ethylmaleinimide	0	0	0	-	-	0	-	-	0
+ 2 mM Iodacetamide	0	0	0	-	-	0	-	-	0
+ 2 mM 4-Hydroxymercuri-Na-benzoate	19.4	32.3	0	0	11.6	0	12.2	39	0
Stabilization									
without additive	21.5	33.4	0	0	trace	0	15.2	38	0
+5% Sorbitol	23.3	34.1	0	0	12.2	0	16.3	38	0
+ 20% Sorbitol	21.9	34.2	0	0	12	0	15.2	37	0
+ 1 mM PMSF	22.0	33.7	0	0	trace	0	15	40	0
+ 1 % BSA	trace	20.0	0	0	0	0	0	18	0
+ 10% Glycerol	22.0	33.1	0	0	11.5	0	15.2	38	0
+ 5 % Mannitol	24.0	36.2	0	0	trace	0	16.8	38	0
+ 100 µg 6-APA/ml	26.8	35.4	0	15	24	0	17.2	38	0

Table 2 continuation

Test	immediately			24 h at + 4°C			24 h at -20°C		
Stabilization condition	Microbiological 6-APA-PaCoA-Acylation activity (mm HH O on Micrococcus luteus ATCC 9341/BC 85)								
	immediately	30'	30' + penase	immediately	30'	30' + penase	immediately	30'	30' + penase
	µg/ml Penicillin V Standard			mm HH O on BC 85					
	2			27.4					
	1.5			24.7					
	1.0			23.4					
	0.5			18.4					
	0.25			11.9					

Enzyme: PAT-ammonium sulphate precipitate

Storage and testing: 1 + 10 with 0.1 M HEPES reaction buffer pH 7.5 under various stabilization conditions

Table 3: Temperature stability of PaCoA-6-APA-acyltransferase activity of penicillium chrysogenum

DTT (mM)	duration of incu- bation	-196°C			-20°C			+4°C			RT			37°C		
		Microbiological 6-APA-PaCoA Transacylation activity (mm HH O on Micrococcus luteus ATCC 9341 BC 85)														
		imme diate ly	30'	30' + pe- nase	imme diate ly	30'	30' + pe- nase	imme diate ly	30'	30' + pe- nase	imme diat.	30'	30' + pe- nase	imme diate ly	30'	30' + pe- nase
1	immediat.	14	40	0	14	40	0	14	40	0	14	40	0	14	40	0
	1 h	14	36	0	trace	38	0	trace	38	0	10	37	0	0	trace	0
	4 h	12	37	0	17	36	0	12	36	0	0	16	0	0	0	0
	26h	17	33	0	17	35	0	10	32	0	trace	14	0	0	0	0
	120 h	26	32	0	23	34	0	0	trace	0	0	0	0	0	0	0
10	immediat.	24	40	0	24	40	0	24	40	0	24	40	0	24	40	0
	1 h	15	35	0	11	35	0	14	34	0	13	34	0	0	17	0
	4 h	-	-	0	12	34	0	11	32	0	11	28	0	0	0	0
	26 h	18	32	0	17	34	0	11	27	0	0	trace	0	0	0	0
	120h	-	-	0	28	32	0	0	trace	0	0	0	0	0	0	0

Enzyme: PAT-ammonium sulphate precipitate

Storage and testing: 1 + 10 with 0.1 M HEPES reaction buffer pH 7.5 under various stabilization conditions

Table 4: Substrate specificity of purified thiol-dependent PAT from *Penicillium chrysogenum* P2/ATCC 48271 (active fraction AL 278/2 following affinity chromatography)

Substrate		Product	Specific enzyme activity determined by HPLC (mU/mg Protein) A1 278/2
Acyl acceptor	Acyl donor		
Isopenicillin N	PaCoA	Penicillin V	146
Isopenicillin N	PaCoA	Penicillin G	80
6-APA	PaCoA	Penicillin V	393
6-APA	PaCoA	Penicillin G	329
7-ADCA	PaCoA	3-Desacetoxy-cephalosporin V	49
7-ACA	PaCoA	Cephalosporin V	0
Cephalosporin C	PaCoA	Cephalosporin V	0
Penicillin G	PaCoA	Penicillin V	25
Penicillin V	PaCoA	Penicillin G	99
H ₂ O	Isopenicillin N	6-APA	150
H ₂ O	Penicillin V	6-APA	75
H ₂ O	Penicillin G	6-APA	17
H ₂ O	7-Glutaryl-APA	6-APA	106

Figure 1: Purification of thiol-dependent PAT from *Penicillium chrysogenum* P2/ATCC 48271
 Chromatofocussing of affinity-chromatographically produced PAT-enzyme preparation AL 278/2
 on Mono P = AL 310

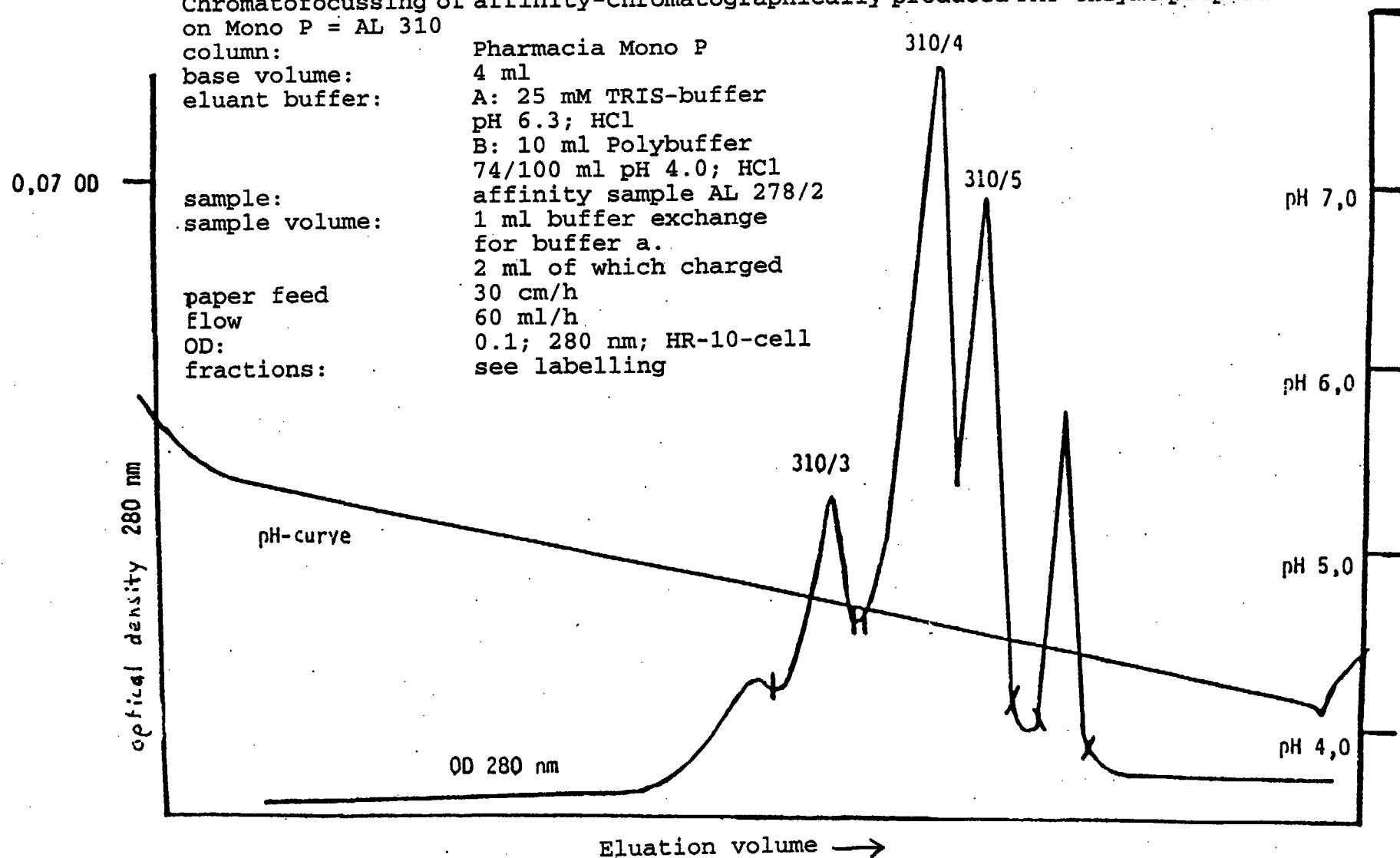


Figure 2: Purification of thiol-dependent PAT of *P. chrysogenum* P2/ATCC 48271

RPC (MN Nucleosil 300-5/C₄) of fractions 3-5 from AL 310/-
chromatofocussing of the active pool AL 278/2 (following affinity
chromatography) on Mono P

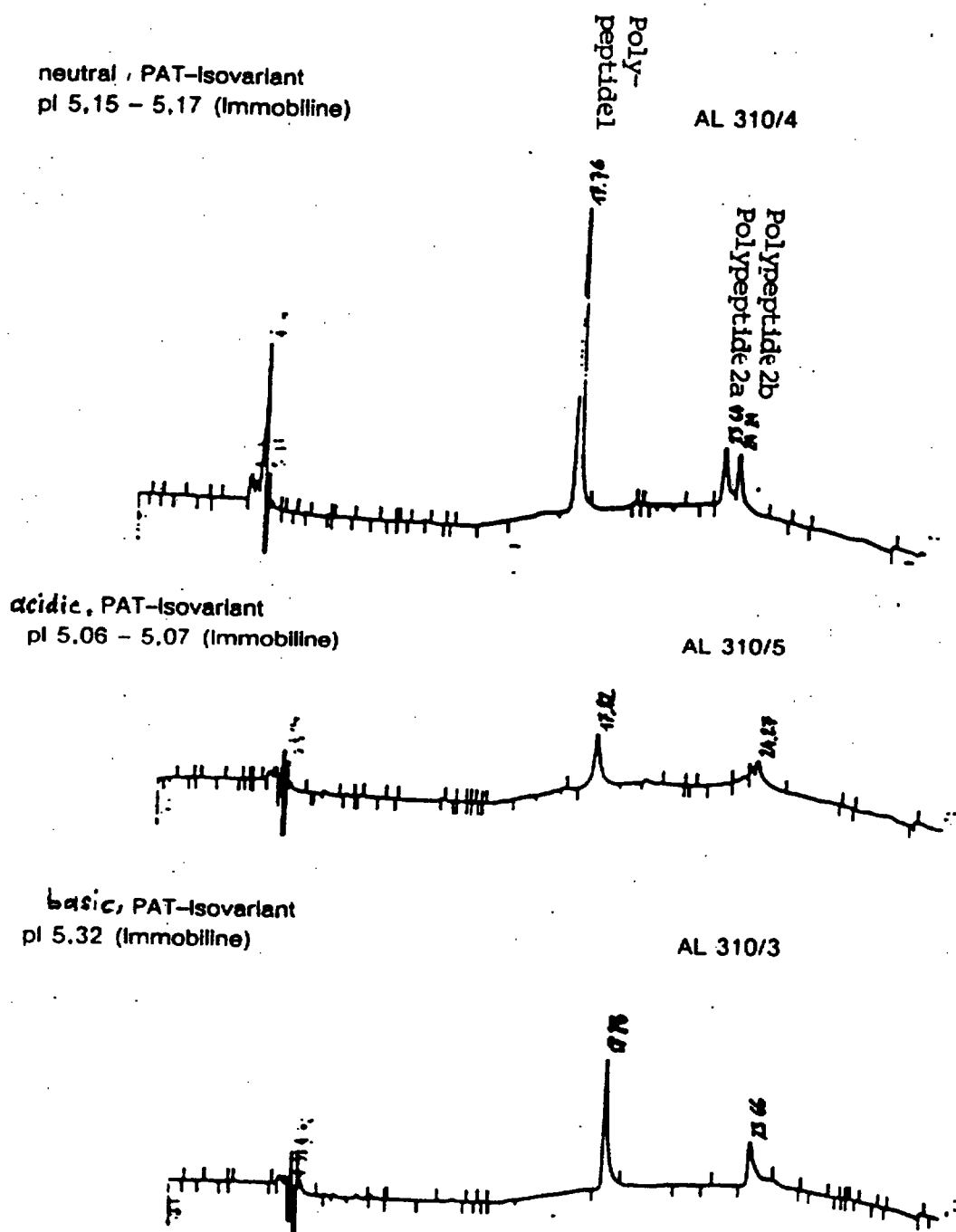
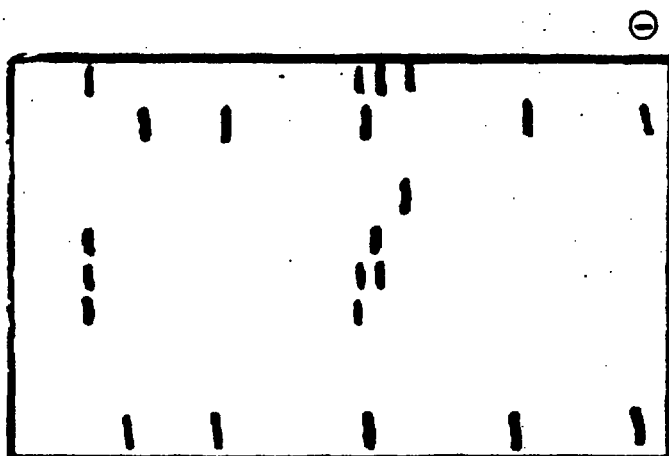


Figure 3: Electrophoretic characterisation of thiol-dependent PAT of penicillium chrysogenum P2/ATCC 48271

Gradient SDS-polyacrylamide gel electrophoresis and isoelectric focussing on ampholine (pH 3.5 - 9.5) resp. immobililine (pH 4.5 - 6.5) of AL 310/chromatofocussing of the affinity-chromatographically produced PAT-enzyme preparation AL 278/2 on Mono P.

3a: Gradient SDS-PAGE (8-20% T)

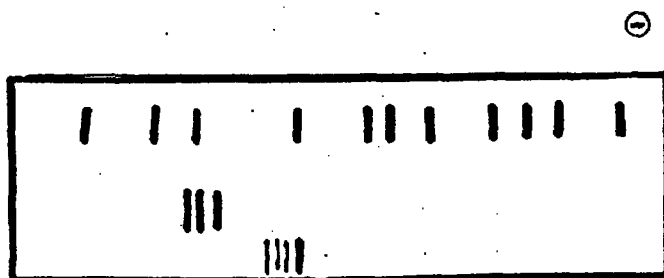


trace	symbol
1	charge 278/2
2	Low-MW-std. 66. 45. 31. 22. 14 kD
3	AL 310/7
4	AL 310/6
5	AL 310/5
6	AL 310/4
7	AL 310/3
8	AL 310/2
9	AL 310/1
10	Low-MW-std. 66. 45. 31. 22. 14 kD

pH-range of
6.0 - 4.0 (→)

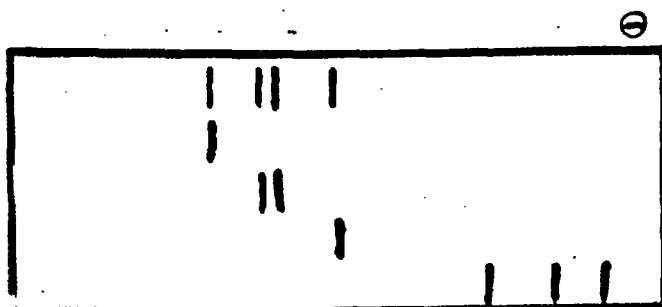
A 25 mM bis-TRIS/HCl pH 6.3
B Polybuffer 74 10%lg pH 4.0

3b: Ampholine (pH 3.5 - 9.5)



trace	symbol
1	pI study Pharmacia from - to + (9.30: 8.65: 8.45: 8.15: 7.35: 6.85: 6.55: 5.85: 5.20: 4.55: 3.50)
2	AL 278/2
3	Carboanhydrase newly prepared

3c: Immobililine (pH 4.5 - 6.5)



trace	symbol	origin
1	AL 278/2	Affinity chromatogr.. 6-APA-AH-Seph.
2	AL 310/5	Chromatofocussing
3	AL 310/4	
4	AL 310/3	
5	Carboanhydrase	

Figure 4: Restriction map of penicillium chrysogenum, which embraces the pat-gene

Restriction maps are approximate reproductions of restriction intersection sites in DNA molecules. The distances shown of the restriction intersection sites are proportional to the actual distances, but the distances actually observed may differ from these. Not all restriction intersection sites are given, further intersection sites may be present throughout.

- A = sequence of examples 14 & 15
B = sequence of example 18

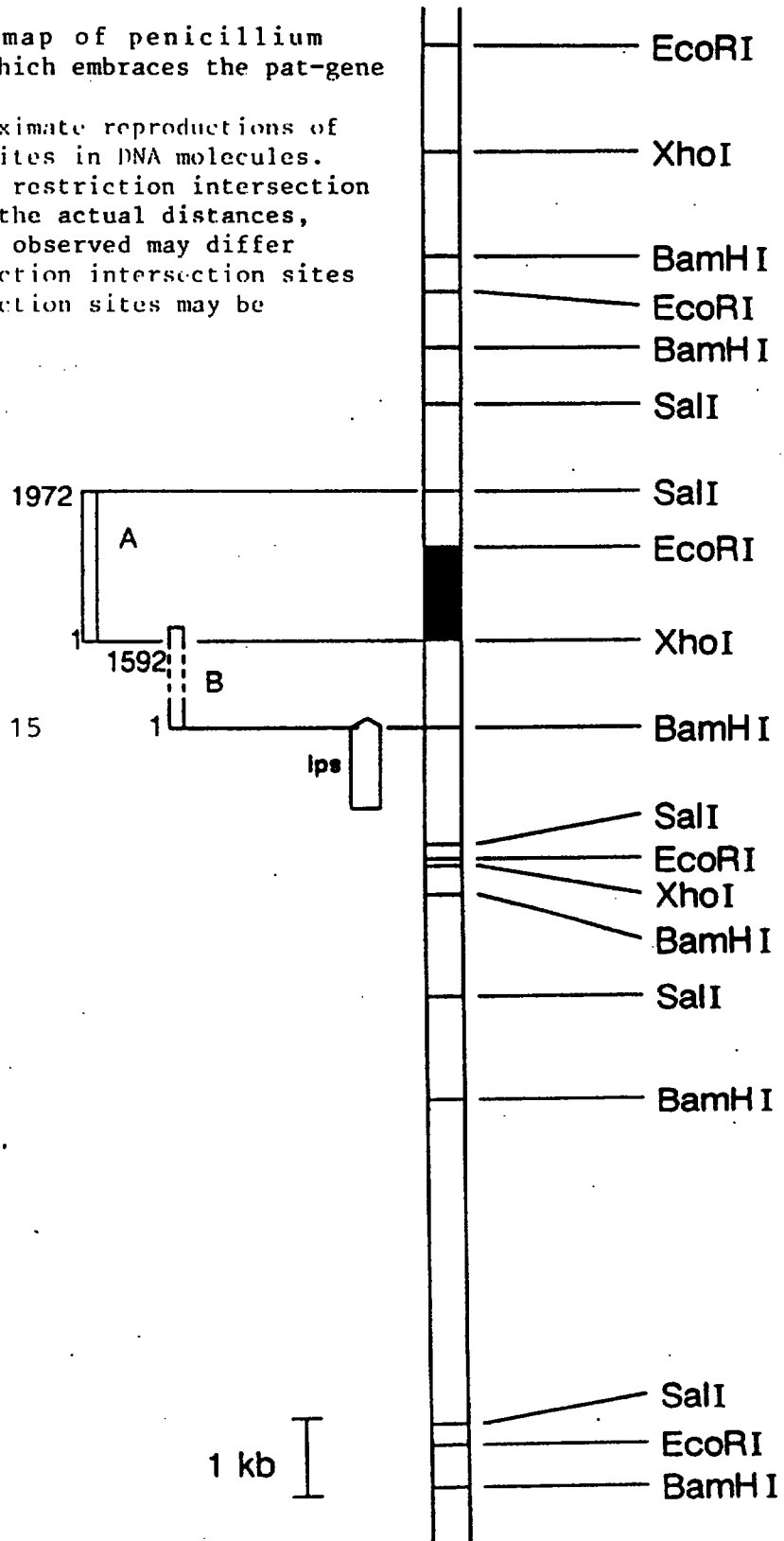


Figure 5: Arrangement and sequence of the chosen oligonucleotide mixtures

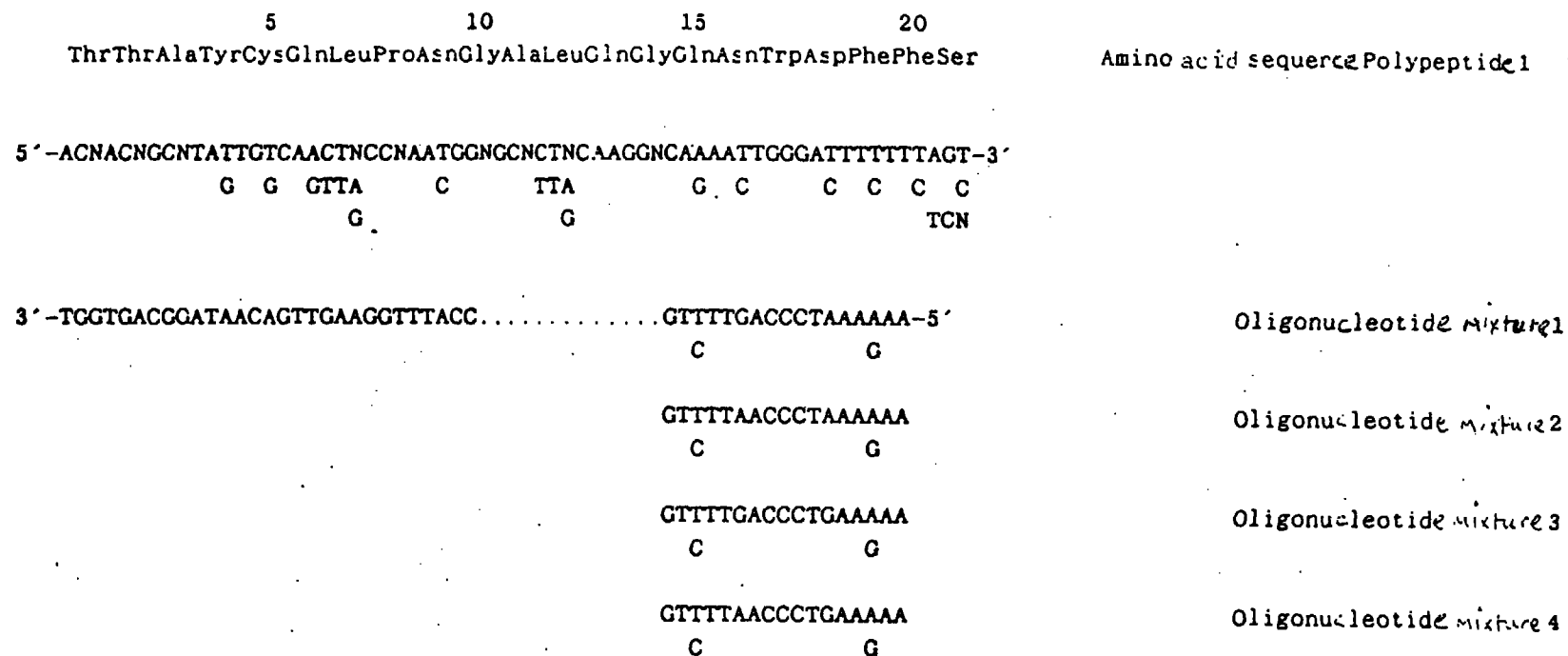


Figure 6: Schematic illustration of the relationships between the sequenced regions (D,F,G), the oligonucleotides (C,E), the polypeptide 1 of PAT and the sequence of the coding DNA-strand (B) derived therefrom.

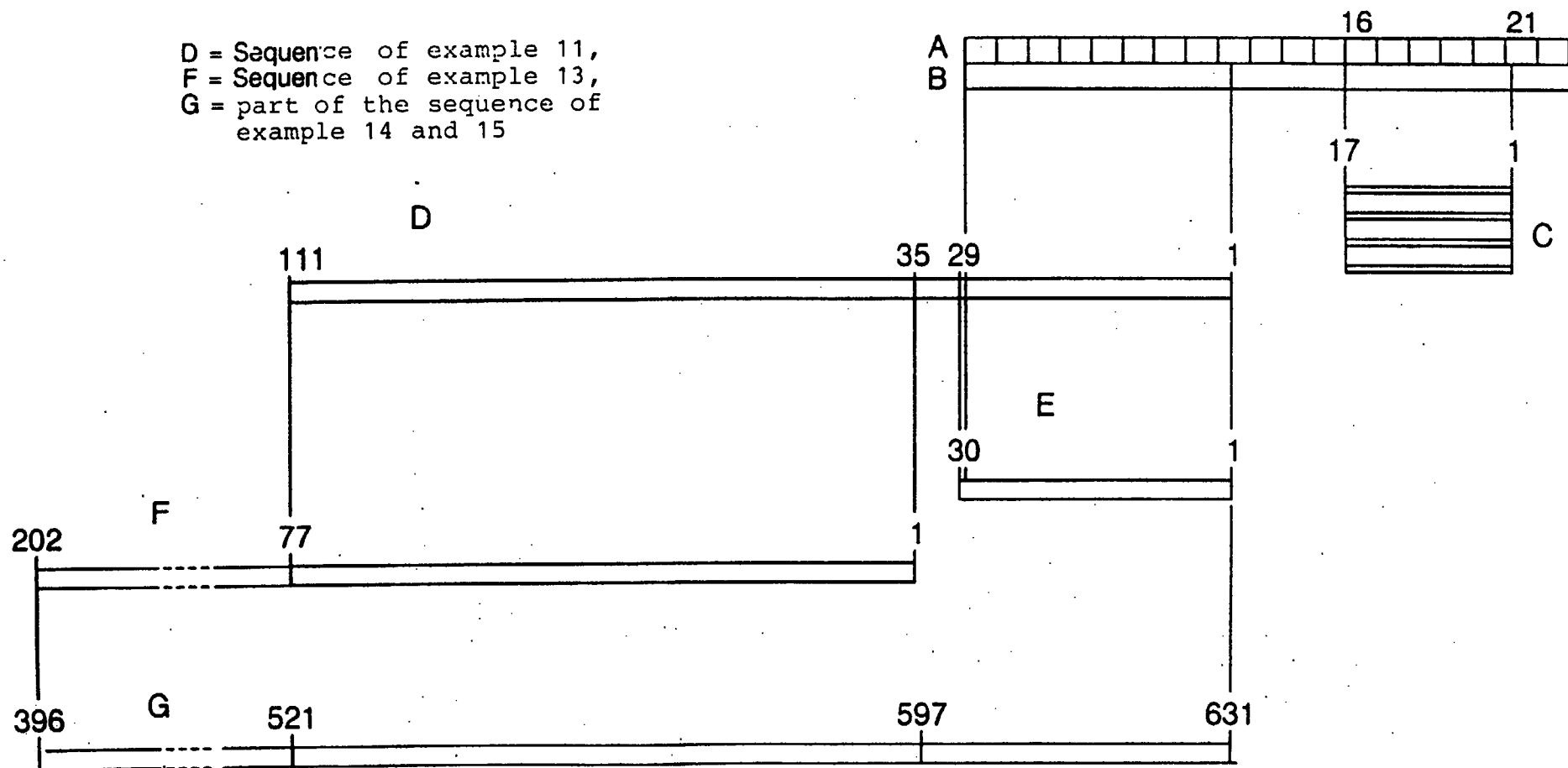
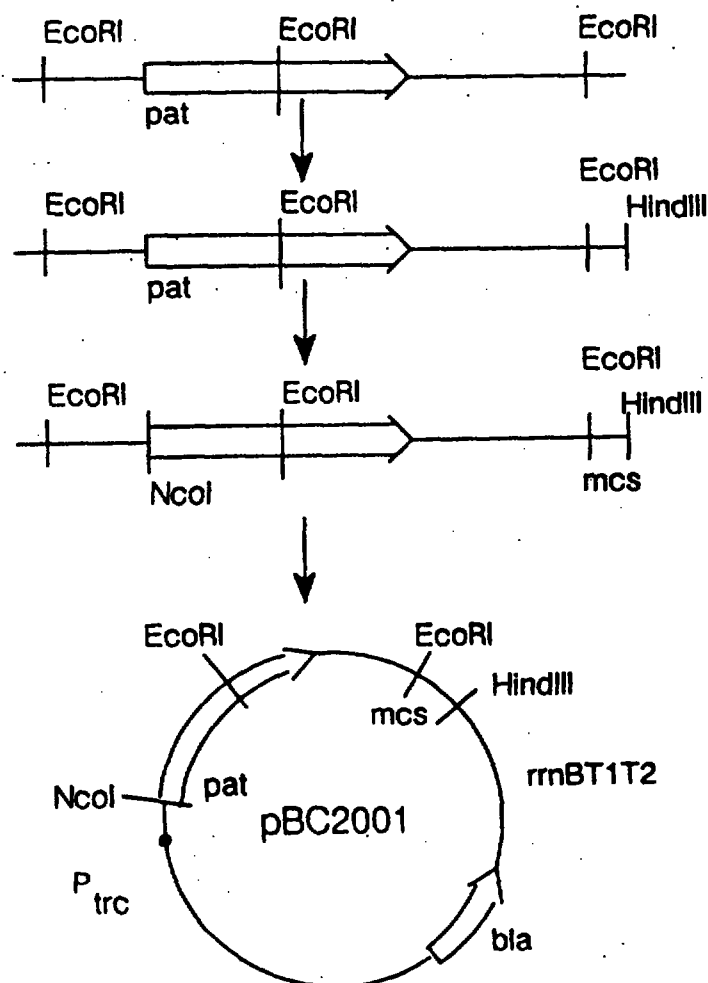


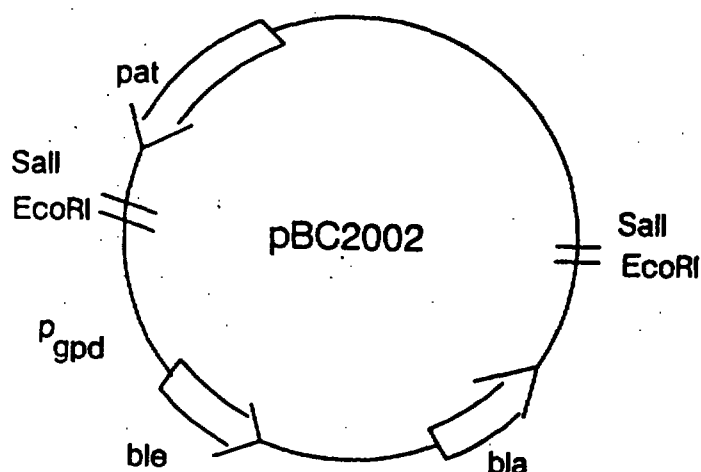
Figure 7: Construction of the plasmid pBC2001.



Starting with the DNA of a complete *pat*-cDNA clone (top lines), the two *EcoRI* fragments are cloned into vector M13mp19. A *NcoI* intersection site is inserted using an oligonucleotide. The *NcoI*-*HindIII*-fragment is then built into the expression vector pKK233-2.

EcoRI, *HindIII*, *NcoI* indicate the intersection sites of the corresponding enzymes; *mcs* multiple cloning site of M13mp19; *p_{trc}* trp-lac fusion promoter of pKK233-2; *rrnBT1T2* transcription terminator of pKK233-2; *bla* ampicillin resistance gene of pKK233-2.

Figure 8: The plasmid pBC2002



Construction of this plasmid starts with pHS103 (Kolar et al.), which contains a fusion of the aspergillus nidulans promoter p_{gdp} (promoter of the glycerol aldehyde phosphate dehydrogenase gene) with the phleomycin resistance gene (ble). The ampicillin resistance gene (bla) serves as a selection mark in E.coli. In pHS103, the 4.8 kb Sall fragment is built in with the penicillium chrysogenum pat-gene. Sall, EcoRI are the intersection sites of the corresponding restriction enzymes.



Case 970-9757

Claims:

1. A DNA sequence in purified and isolated form that codes for the enzyme penicillin acyl transferase which has the following sequence:

```

10                               20
MetLeuHisIleLeuCysGlnGlyThrPropheGluIleGlyTyrGluHisGlySerAla
30                               40
AlaLysAlaValIleAlaArgSerIleAspPheAlaValAspLeuIleArgGlyLysThr
50                               60
LysLysThrAspGluGluLeuLysGlnValLeuSerGlnLeuGlyArgValIleGluGlu
70                               80
ArgTrpProLysTyrTyrGluGluIleArgGlyIleAlaLysGlyAlaGluArgAspVal
90                               100
SerGluIleValMetLeuAsnThrArgThrGluPheAlaTyrGlyLeuLysAlaAlaArg
110                              120
AspGlyCysThrThrAlaTyrCysGlnLeuProAsnGlyAlaLeuGlnGlyGlnAsnTrp
130                              140
AspPhePheSerAlaThrLysGluAsnLeuIleArgLeuThrIleArgGlnAlaGlyLeu
150                              160
ProThrIleLysPheIleThrGluAlaGlyIleIleGlyLysValGlyPheAsnSerAla
170                              180
GlyValAlaValAsnTyrAsnAlaLeuHisLeuGlnGlyLeuArgProThrGlyValPro
190                              200
SerHisIleAlaLeuArgIleAlaLeuGluSerThrSerProSerGlnAlaTyrAspArg
210                              220
IleValGluGlnGlyGlyMetAlaAlaSerAlaPheIleMetValGlyAsnGlyHisGlu
230                              240
AlaPheGlyLeuGluPheSerProThrSerIleArgLysGlnValLeuAspAlaAsnGly
250                              260
ArgMetValHisThrAsnHisCysLeuLeuGlnHisGlyLysAsnGluLysGluLeuAsp
270                              280
ProLeuProAspSerTrpAsnArgHisGlnArgMetGluPheLeuLeuAspGlyPheAsp
290                              300
GlyThrLysGlnAlaPheAlaGlnLeuTrpAlaAspGluAspAsnTyrProPheSerIle
310                              320
CysArgAlaTyrGluGluGlyLysSerArgGlyAlaThrLeuPheAsnIleIleTyrAsp
330                              340
HisAlaArgArgGluAlaThrValArgLeuGlyArgProThrAsnProAspGluMetPhe
350
ValMetArgPheAspGluGluAspGluArgSerAlaLeuAsnAlaArgLeu.
```



2. A DNA sequence according to claim 1 which includes the following sequence:

```

      10      20      30      40      50      60      70
GTTGATGTCCCATCAGTGTCTATCGTCCCAGATTGGTGGCTACCCCAATATAAATCTCACCATGCA
      80      90     100     110     120     130     140
GTTCCCCCTGCATGATCATCCCCAGGACCCCGTTTGTCTCTCCGTCAACCAGGTCTCAGTTGTTTACCC
     150     160     170     180     190     200     210
ATCTTCCGACCCGCCACCAGAAATGCTTCACATCCTCTGTCAAGCCACTCCCTTTGAAGTAAGTCTCCAC
     220     230     240     250     260     270     280
TGAATACCAGATTTTTTCTTCTGAATCTTCCGAGTTCTGACCTGATCCAGATCCGCTACCAACATCGCT
     290     300     310     320     330     340     350
CTGCTGCCAAAGCCGTGATAGCCAGAAGCATTGACTTCCCCCTCCATCTCATCCGAGGAAAAACGAAGAA
     360     370     380     390     400     410     420
GACCGACGAAGAGCTTAAACAGTACTCTCCCAACTGGCCCCCGTGATCCGAGAAAGATGCCCCAAATAC
     430     440     450     460     470     480     490
TACGAGCAGATTCCCGGTGAGTCCCACCTTCGGTCTTTCTTACATTTTCTCCACCAATGCTGACCGATGAC
     500     510     520     530     540     550     560
CCCCGAAAAACCAGCTATTCCAAAGCCCGCTCAACCCGATGTCTCCGAGATTGTCTCTTAATACCCGC
     570     580     590     600     610     620     630
ACCGAATTTCCATACCCGCTCAAGCCAGCCCGTGATCCCTCCACCACTCCCTATTGTCAACTTCCAAATG
     640     650     660     670     680     690     700
GAGCCCTCCAGGGCCAAACTCGGATGTACGTTAAGAGATTTTACCTCCTCATTTTATTCATCGAATTT
     710     720     730     740     750     760     770
CCCCCCTAATTTGCTTGTTCAGTTCTTTTCTGCCACCAAAAGACAACCTCATCCGTTAACCATCCGT
     780     790     800     810     820     830     840
CAGCCCCGACTTCCACCATCAAATTCATAACCGAGCGTCCGAATCATCCGAAGGTTGGATTTAACAGTG
     850     860     870     880     890     900     910
CGGGCGTCCCGTCAATTACAACCCCTTCACCTTCAAGGTCTTCCGACCCACCGAGTTCCCTCCCATAT
     920     930     940     950     960     970     980
TCCCTCCGCATACCGCTCGAAAGCACTTCTCCTTCCCACGCCCTATGACCCGATCGTGGACCAAGCCCGA
     990    1000    1010    1020    1030    1040    1050
ATCCCCCCCAGCGCTTTTATCATGGTCCGCAATGGGCACGAGGCATTTGCTTTGGAATTTCCCCCACCA
    1060    1070    1080    1090    1100    1110    1120
CCATCCGAAAGCAGGTCTCCACCGCAATGTAGCATCGTCCACACCAACCACTGCTTCTTACGACCGG
    1130    1140    1150    1160    1170    1180    1190
CAAAAATGAGAAAGAGCTCGATCCCTTACCCGACTCATCGAATCCCCACCACCGTATCGACTTCTCTCTC
    1200    1210    1220    1230    1240    1250    1260
GACCGGTTCCAGGGCACCAAAACAGGCATTTGCCACGCTCTCCCCGACCAAGACAATTATCCCTTTAGCA
    1270    1280    1290    1300    1310    1320    1330
TCTCCCGCCCTTACGACGACCCCAAGAGCAGAGCGCGGACTCTGTTCAATATCATCTAGGACCATGCCCC
    1340    1350    1360    1370    1380    1390    1400
TAGAGAGCCAACCGTCCCGCTTCCCGCGCCGACCAACCCTGATGAGATCTTTGTCTATCCGCTTTCACCAG
    1410    1420    1430    1440    1450    1460    1470
GAGGACGAGAGGTCTCCGCTCAACCCACGCTTTGAAGCCTCTTCATGACGAGCCAATGCATCTTTTGTA
    1480    1490    1500    1510    1520    1530    1540
TGTAAGTTCAACCGACTCCGTCTTCACTTCTTCCCGCCCACTCCCTACCGTTTGTACCATCTGACTCATA
    1550    1560    1570    1580    1590    1600    1610
TAAATGTCTAGCCCTACCTACACTATACCTAAGCGAGAGAACCGTAGAGTGATTACGTACGCCCTAT

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1820 1830 1840 1850 1860 1870 1880
AGTACCCCGATCTCTAGATAGAACATTTAGTAGAGATTACGATGCCCTAACTAATTTAAGTTGACCATTTGT
1690 1700 1710 1720 1730 1740 1750
CCCGTTTCATATTGATTTTTCATCCATTATACACTCTTAATCGTTTCCCGGTAGAAGCCGNATATATACGAC
1760 1770 1780 1790 1800 1810 1820
CATACCGTCTCGAGAACAGCGCTTCCCGTCTGCTTCCCGTACTTAACTATATATTCTACACGCCCAAT
1830 1840 1850 1860 1870 1880 1890
ACTCAATCTGCCCTTAGCACCTAAGCGCCACTCTACCGTAAGTCCGGCTCATATACGTCAGAACTCTTAA
1900 1910 1920 1930 1940 1950 1960
CACTGAAGACAGGATATCAGCGCTTACCCTGCACCGTACCTACTACCTTCAATATCACTCTTTCACCATG
1970
CACACCGTCCAC.

3. A DNA sequence according to claim 1 or 2 which includes the sequences necessary for the transcription and translation of the *Penicillium chrysogenum* PAT gene.
4. A DNA sequence according to claim 3 in which the sequences which are necessary for the transcription and translation of the *Penicillium chrysogenum* PAT gene are the sequences between the Isopenicillin N-synthetase gene and the *Penicillium chrysogenum* PAT gene.
5. A protein which is coded for by a DNA sequence according to any one of claims 1 to 4, in purified an isolated form.
6. A vector which contains a DNA sequence that codes for the enzyme penicillin acyltransferase (PAT); the enzyme penicillin acyltransferase having the following sequence:

	10	20
MetLeuHisIleLeuCysGlnGlyThrProPheGluIleGlyTyrGluHisGlySerAla		
	30	40
AlaLysAlaValIleAlaArgSerIleAspPheAlaValAspLeuIleArgGlyLysThr		
	50	60
LysLysThrAspGluGluLeuLysGlnValLeuSerGlnLeuGlyArgValIleGluGlu		
	70	80
ArgTrpProLysTyrTyrGluGluIleArgGlyIleAlaLysGlyAlaGluArgAspVal		
	90	100
SerGluIleValMetLeuAsnThrArgThrGluPheAlaTyrGlyLeuLysAlaAlaArg		
	110	120
AspGlyCysThrThrAlaTyrCysGlnLeuProAsnGlyAlaLeuGlnGlyGlnAsnTrp		
	130	140
AspPhePheSerAlaThrLysGluAsnLeuIleArgLeuThrIleArgGlnAlaGlyLeu		



150 160
ProThrIleLysPheIleThrGluAlaGlyIleIleGlyLysValGlyPheAsnSerAla
170 180
GlyValAlaValAsnTyrAsnAlaLeuHisLeuGlnGlyLeuArgProThrGlyValPro
190 200
SerHisIleAlaLeuArgIleAlaLeuGluSerThrSerProSerGlnAlaTyrAspArg
210 220
IleValGluGlnGlyGlyMetAlaAlaSerAlaPheIleMetValGlyAsnGlyHisGlu
230 240
AlaPheGlyLeuGluPheSerProThrSerIleArgLysGlnValLeuAspAlaAsnGly
250 260
ArgMetValHisThrAsnHisCysLeuLeuGlnHisGlyLysAsnGluLysGluLeuAsp
270 280
ProLeuProAspSerTrpAsnArgHisGlnArgMetGluPheLeuLeuAspGlyPheAsp
290 300
GlyThrLysGlnAlaPheAlaGlnLeuTrpAlaAspGluAspAsnTyrProPheSerIle
310 320
CysArgAlaTyrGluGluGlyLysSerArgGlyAlaThrLeuPheAsnIleIleTyrAsp
330 340
HisAlaArgArgGluAlaThrValArgLeuGlyArgProThrAsnProAspGluMetPhe
350
ValMetArgPheAspGluGluAspGluArgSerAlaLeuAsnAlaArgLeu.

7. A vector according to claim 6 in which the DNA sequence that codes for the enzyme penicillin acyltransferase (PAT), includes the following sequence:

10 20 30 40 50 60 70
CTTGATGTCCCATCACTCTCATGCTATCGTCCCAGATTGGTCCCTACCCCAATATAAATCTCACCATCCA
80 90 100 110 120 130 140
GTCCCCCTGCATGATCATCCCCAGGACCCCGTTTGTTCATCTCCGTACCCACGGTCTCAGTTGTTTACCC
150 160 170 180 190 200 210
ATCTTCCGACCCCAACCAGAAATCCTTCACATCCTCTGTCAAGCCACTCCCTTTGAAGTAAGTCTCCAC
220 230 240 250 260 270 280
TGAATACCAGATTTTTTCTTCTCAATCTTCCGAGTTCTGACCTGATCCAGATCCCGCTACGAACATGCCT
290 300 310 320 330 340 350
CTGCTGCCAAAGCCGTGATAGCCAGAACATTGACTTCCCCGTCCATCTCATCCGACCGAAACCAAGAA
360 370 380 390 400 410 420
CACCGACCAAGAGCTTAAACAGGTACTCTCCAACTGCGCCCGGTGATCCGACCAAGATCGCCCAATAC
430 440 450 460 470 480 490
TACGACGAGATTCGCCGTGAGTCCCACTTCCGTCTTTCCTACATTTTCTCCACCAATCTGACCGATCAC



500 510 520 530 540 550 560
CCCCAAAAACCAGCTATTGCAAAGCGCGCTGAACCGCATGCTCCGAGATTGTCATCCTTAATACCCGC
570 580 590 600 610 620 630
ACCGAATTTGCATACCGGCTCAAGGCAGCCCGTCATCCCTCCACCACTGCCTATTGTCAACTTCCAAATG
640 650 660 670 680 690 700
GACCCCTCCAGCCCCAAAACTCCGATGTACGTTAAGAGATTTTACCTCCTCATTTTATTCCATCCAATTT
710 720 730 740 750 760 770
CCCCCGACTAATTTGCTTGTTCAGTTCTTTTCTGCCACCAAAGAGAACCTGATCCCGTTAACGATCCGT
780 790 800 810 820 830 840
CAGCCCGGACTTCCCACCATCAAATTCATAACCGAGCGCTCGAATCATCCGGAAGGTTGGATTTAACAAGTG
850 860 870 880 890 900 910
CCCCCGTCCCGTCAATTACAACCGCCCTTCACCTTCAACGCTCTTCGACCCACCGGAGTTTCTTCCCATAT
920 930 940 950 960 970 980
TCCCTCCCGATAGCCCTCCAAAGCACTTCTCCTTCCCAGCCCTATGACCCGATCGTGGACCAAGCCGA
990 1000 1010 1020 1030 1040 1050
ATGCCCCCGAGCGCTTTTATCATGGTGGGCAATGGCCACCAAGGCATTTGCTTTGCAATTTCTCCCCACCA
1060 1070 1080 1090 1100 1110 1120
GCATCCGAAAGCAGGTGCTCCAGCCCAATGTACGATCGTGCACACCAACCACTGCTTGTTCACCAAGG
1130 1140 1150 1160 1170 1180 1190
CAAAAATGAGAAAGACCTCGATCCCTTACCGACTCATGCAATCCGACCAAGCGTATCGAGTTCTCTCTC
1200 1210 1220 1230 1240 1250 1260
GACCGGTTCCAGCGCACCAAAACAGGCATTTGCCCAGCTCTGCCCCGACCAACACAATTATCCCTTTAGCA
1270 1280 1290 1300 1310 1320 1330
TCTGCCCGGCTTACGAGGAGCGCAAGAGCAGACGCGGCACTCTGTTCAATATCATCTACGACCATGCCCCG
1340 1350 1360 1370 1380 1390 1400
TAGAGAGCCAAACCGTCCCGCTTCCCGCCCGACCAACCCTCATGAGATGTTTGTCTATCCCGTTTCACCAG
1410 1420 1430 1440 1450 1460 1470
GAGGACGAGAGGTCTCCGCTCAACCCGAGCGTTTGAAGCGCTCTTCATGACGAGCCAATGCATCTTTTGT
1480 1490 1500 1510 1520 1530 1540
TGTAGCTTCAACCGACTCCGTCTTCACTTCTTCCCCCGCACTCCCTACCGTTTGTACCATCTGACTCATA
1550 1560 1570 1580 1590 1600 1610
TAAATGCTTACCCCTACCTACACTATACCTAAGCAGAGAAACCGTAGAGTGATTAACGTACCGCCCTAT
1620 1630 1640 1650 1660 1670 1680
AGTACCCCGATCTCTAGATAGAACATTTAGTAGAGATTACGATGCCCTAACTAATTTAACTTGACCATTCT
1690 1700 1710 1720 1730 1740 1750
CCCGTTTATATTGATTTTCATCCATTATACACTCTTAATCGTTTCCCGGTAGAAGCCGNATATATACGAC
1760 1770 1780 1790 1800 1810 1820
CATACCGTGTGAGAACAGCGCTTCCCGTCTCCTTCCCGTACTTAACTATATATTCTACACCGCCCAAT
1830 1840 1850 1860 1870 1880 1890
ACTCAATGTCCCGTTACCACTAAGCCGCACTCTACCGTAAGTCCCGGTATATACGTGAGAAGTCTTAA
1900 1910 1920 1930 1940 1950 1960
GACTGAAGACAGGATATACCGGTTACCCTCCACCGTACCTACTACCTTCAATATCACTCTTTCACCATG
1970
GACACCGTCCAC

8. A vector according to claim 6 or claim 7 which contains further genes that are necessary for the transcription and translation of the *Penicillium chrysogenum* PAT gene.



9. A vector according to any one of claims 6 to 8 which contains further genes that permit the selection of the plasmid in β -lactam producing microorganisms.
10. A vector according to any one of claims 6 to 9 which contains a further gene that codes for the enzyme Isopenicillin N synthetase.
11. A process for the production of the enzyme penicillin acyltransferase (PAT), comprising transforming a host with a vector according to any one of claims 6 to 10 and isolating the expressed PAT.
12. Cells which contain a vector according to any one of claims 6 to 10.
13. A process for improving the production of penicillin characterized in that a penicillin producing cell is transformed with a vector according to any one of claims 6 to 10, and the penicillin is isolated.