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Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*

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A gene which confers resistance to the herbicide bialaphos (*bar*) has been characterized. The *bar* gene was originally cloned from *Streptomyces hygroscopicus*, an organism which produces the tripeptide bialaphos as a secondary metabolite. Bialaphos contains phosphinothricin, an analogue of glutamate which is an inhibitor of glutamine synthetase. The *bar* gene product was purified and shown to be a modifying enzyme which acetylates phosphinothricin or demethylphosphinothricin but not bialaphos or glutamate. The *bar* gene was subcloned and its nucleotide sequence was determined. Interspecific transfer of this *Streptomyces* gene into *Escherichia coli* showed that it could be used as a selectable marker in other bacteria. In the accompanying paper, *bar* has been used to engineer herbicide-resistant plants.

Key words: bialaphos/phospinothricin/acetyltransferase/herbicide resistance/*bar*

Introduction

Streptomyces which produce antibotics have evolved mechanisms to avoid the toxicity of their own products. Such strains often contain modifying enzymes which can inactivate the antibiotics they produce. Genes which code for these enzymes have been isolated from various antibiotic-producing organisms by cloning them selectively in the antibiotic sensitive host, *S. lividans* (reviewed by Hopwood *et al.*, 1986). The resistance genes have served as interesting and useful model systems to study the regulation of the biochemical and morphological differentiation processes which occur when streptomycetes enter stationary phase. Antibiotic resistance genes have also been used as probes to isolate clusters of antibiotic biosynthetic genes (Chater and Bruton, 1985; Stanzak *et al.*, 1986; Murakami *et al.*, 1986). Since *Streptomyces* spp. produce hundreds of different antibiotics (Berdy *et al.*, 1980, 1981) their resistance genes have been a rich source of selectable markers for the construction of both bacterial (reviewed in Hopwood *et al.*, 1986) and animal cell vectors (Vara *et al.*, 1986). With these applications in mind we have cloned the *bar* gene which confers resistance to an antibiotic called bialaphos (Murakami *et al.*, 1986).

Bialaphos is now being used in agriculture as a non-selective herbicide. It is a tripeptide which is composed of two L-alanine residues and an analogue of glutamic acid known as phosphinothricin (PPT) (Ogawa *et al.*, 1973; Kondo *et al.*, 1973). While PPT is an inhibitor of glutamine synthetase in both plants and bacteria, the intact tripeptide has little or no inhibitory activity *in vitro* (Bayer *et al.*, 1972; Tachibana *et al.*, 1986a). In both bacteria and plants, intracellular peptidases remove the alanine residues and release active PPT.

S. hygroscopicus is used for the commercial production of bialaphos. Imai *et al.* (1984, 1985) and Seto *et al.*, (1982, 1983a, 1983b, 1984) have shown that the bialaphos is synthesized from three carbon precursors (probably pyruvate or phosphoenolpyruvate) in a series of at least thirteen conversions. Many of the genes which code for these enzymes as well as a function which positively regulates their transcription (*brp*A, Anzai *et al.*, 1987) have been defined by blocked mutants. Cloned DNA fragments which either restore productivity to these mutants or confer resistance to bialaphos (i.e. *bar*) mapped to an 18-kb gene cluster (Murakami *et al.*, 1986). Although enzyme assays for most of these steps have not been developed, Imai *et al.* (1985) have shown that *S. hygroscopicus* contains an acetyl-coenzyme A-dependent activity which can modify either demethylphosphinothricin (DMPT), an intermediate in the pathway, or PPT itself.

If *bar* could be introduced and used as a resistance gene in crop plants, bialaphos could be used more selectively in the field. Here we describe the subcloning, sequencing and biochemical characterization of *bar* and its gene product; the accompanying paper describes the use of this gene as a selectable marker in plants.

Results

Subcloning the bar gene

A gene which confers bialaphos resistance (*bar*) has been cloned from *S. hygroscopicus* genomic DNA (Murakami *et al.*, 1986). The *bar* gene was isolated as overlapping *Pst*I (1.7 kb, pBG3) or *Bam*HI (2.0 kb, pBG4) fragments (Figure 1).

The *bar* gene was subcloned in *E. coli* W3110i^q using the vector pUC19 (Norrander *et al.*, 1983). DNA inserted in the polylinker region of pUC19 can be transcribed from the *lac* promoter in the vector. In W3110i^q, transcription from the *lac* promoter is strongly repressed but can be induced with isopropylthiogalactoside (IPTG). The orientation of the gene was indicated by subcloning the *Bam*HI fragment from the original streptomycete isolate, pBG4, in both orientations into the *Bam*HI site of pUC19 to generate pBG195 and pBG196 (see Figure 1). W3110i^q was slightly resistant to bialaphos when it contained pBG196; however, increased levels of resistance could not be induced by IPTG. In contrast, with W3110i^q/pBG195, the levels of resistance could be induced from 0.005 to >0.05 mg/ml.

The resistance gene was further localized by subcloning in *S. lividans*. *Sau*3aI fragments of the pBG195 insert were cloned into the *Bam*HI site of the expression vector, pIJ680 (Hopwood *et al.*, 1985). A plasmid, pBG106, which conferred resistance to bialaphos contained an ~620-bp *Sau*3aI insert.

The *Sau*3aI fragment was also subcloned from pBG195 into the *Bam*HI site of pUC19. Two plasmids, pBG93 and pBG94,

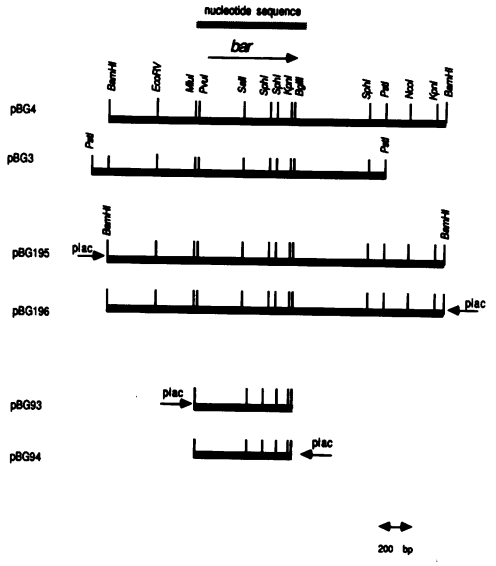

Fig. 1. Subclones used to determine the orientation and location of *bar*. The *bar* gene was originally cloned in the streptomycete plasmids pBG3 and pBG4 (Murakami *et al.*, 1986). The gene was subcloned into pUC19 in both orientations with respect to the lactose promoter (*plac*). The nucleotide sequence shown in Figure 2 is represented by the stippled region and the location of the *bar* structural gene is indicated by the arrow.

were selected which contained the fragment in alternative orientations (see Figure 1); both conferred resistance. The observation that resistance to bialaphos could be induced from 0.001 mg/ml to >0.010 mg/ml with IPTG in pBG93 but not pBG94 confirmed the orientation of *bar* and indicated that the C terminus was located near the *Bgl*II site (also *Sau*3aI) termini.

The size of bar gene product

The size of the gene product was first studied using a streptomycete coupled transcription/translation system (Thompson *et al.*, 1984). Plasmids pBG93 and pBG94 (containing the ~620-bp *Sau*3AI subclone), generated proteins of 28 and slightly more than 22 kd, respectively. Plasmids pBG195 and pBG196 containing the 2.0 kb *Bam*HI fragment in alternative orientations both generated gene products of 22 kd. The suggestion that pBG93 and pBG94 coded for fusion proteins and that an intact *bar* gene contained in pBG195 and pBG196 coded for the gene product of 22 kd was confirmed by the nucleotide sequence.

Nucleotide sequence of bar

The nucleotide sequence of the region including and adjacent to the *Sau*3aI fragment which conferred resistance was determined using the Maxam–Gilbert procedure (Figure 2). Computer analysis of the six possible reading frames indicated that the most likely open reading frame containing the codons used in streptomycete structural genes coded for a 21-kd protein. Its orientation was in agreement with the induction experiments described above (Figure 1).

The gene product was purified from a strain of HP632 which overexpressed *bar* (see below) in order to determine the position of the coding sequence on the *Sau*3aI fragment. When this 22-kd band was applied to a protein sequenator, amino acid residues could not be detected in the first two cycles of the Edman degradation. The N-terminal sequence which was derived for the third to twelfth cycle corresponded to the beginning of the open reading frame which codon distribution had indicated to be the *bar* structural gene (Figure 2). This open reading frame was preceded by an overlapping direct repeated sequence (Figure

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GCTCGCTGTCATTTTCGAGACGCCATCTTTGGAAGCGGTGGCCGAATCCGTACTGCGCGG    60

ACTCGACGACGCGTAAAACGATCGACCACGTACACGAGTCCGGACACGGGGCGAGGAGGC   120
                  PvuI
                                          ProGluArgArgPro
CCGGTTCCGGCACCGAGGAAGACCGAAGGAAGACCACACGTGAGCCCAGAACGACGCCCG   180

AlaAspIleArgArgAlaThrGluAlaAspMetProAlaValCysThrIleValAsnHis
GCCGACATCCGCCGTGCCACCGAGGCGGACATGCCGGCGGTCTGCACCATCGTCAACCAC   240

TyrIleGluThrSerThrValAsnPheArgThrGluProGlnGluProGlnGluTrpThr
TACATCGAGACAAGCACGGTCAACTTCCGTACCGAGCCGCAGGAACCGCAGGAGTGGACG   300

AspAspLeuValArgLeuArgGluArgTyrProTrpLeuValAlaGluValAspGlyGlu
GACGACCTCGTCCGTCTGCGGGAGCGCTATCCCTGGCTCGTCGCCGAGGTGGACGGCGAG   360

ValAlaGlyIleAlaTyrAlaGlyProTrpLysAlaArgAsnAlaTyrAspTrpThrAla
GTCGCCGGCATCGCCTACGCGGGCCCCTGGAAGGCACGCAACGCCTACGACTGGACGGCC   420

GluSerThrValTyrValSerProArgHisGlnArgThrGlyLeuGlySerThrLeuTyr
GAGTCGACCGTGTACGTCTCCCCCCGCCACCAGCGGACGGGACTGGGCTCCACGCTCTAC   480

ThrHisLeuLeuLysSerLeuGluAlaGlnGlyPheLysSerValValAlaValIleGly
ACCCACCTGCTGAAGTCCCTGGAGGCACAGGGCTTCAAGAGCGTGGTCGCTGTCATCGGG   540

LeuProAsnAspProSerValArgMetHisGluAlaLeuGlyTyrAlaProArgGlyMet
CTGCCCAACGACCCGAGCGTGCGCATGCACGAGGCGCTCGGATATGCCCCCCGCGGCATG   600

LeuArgAlaAlaGlyPheLysHisGlyAsnTrpHisAspValGlyPheTrpGlnLeuAsp
CTGCGGGCGGCCGGCTTCAAGCACGGGAACTGGCATGACGTGGGTTTCTGGCAGCTGGAC   660

PheSerLeuProValProProArgProValLeuProValThrGluIle***
TTCAGCCTGCCGGTACCGCCCCGTCCGGTCCTGCCCGTCACCGAGATCTGAACGGAGTGC   720
                                        BglII
GCGTGGGCATCGCCCGAGTTGGAGCTGGTACGGGAACTCATCGAACTCAACTGGCATACC   780

CGCAATGGTGAGGTGGAACCGCGGCGGATCGCGTACGACCGTGCCCAGGAGGCCT
                                                    StuI
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Fig. 2. The nucleotide sequence of *bar*. Edman degradation products of the N-terminal region of the *bar* gene product are boxed. A repeated sequence which preceded the *bar* gene is indicated by arrows. Note that the *Sau*3aI sites can be cleaved with *Pvu*I (5′) and *Bgl*II (3′).

2). The *bar* structural gene began with a GTG and ended with TGA stop signal which was located one nucleotide after the *Sau*3aI (*Bgl*II) cleavage site.

The demonstration that the 625-bp *Sau*3aI fragment does not contain a translational stop signal predicts that pBG93, pBG94 and pBG106 must code for fusion gene products. The pUC19 nucleotide sequence adjacent to the *Bam*HI cloning site contains translational stop signals in the reading frame coding for the *bar* gene product which account for the 21- and 28-kd gene products observed in pBG93 and pBG94. In pBG106, the nucleotide sequence shows that the *bar* gene product is fused in frame to a 24-kd C-terminal fragment of the aminoglycoside phosphotransferase protein (Thompson and Gray, 1983). These experiments suggest that C-terminal fusions to the *bar* gene might retain enzymatic activity. This has been demonstrated by more detailed analyses of *bar* fusion proteins (J.Botterman *et al.*, in preparation).

The enzymatic activity of the bar gene product

It is known from the studies of Imai *et al.* (1985) that acetylation of DMPT is a step in the bialaphos biosynthetic pathway. Since acetyltransferases often inactivate antibiotics, we thought that this biosynthetic enzyme might also be able to acetylate PPT and confer resistance to bialaphos. We adapted a spectrophotometric acetyltransferase assay (Shaw, 1975) as a convenient and quantitative assay of the enzyme which can acetylate PPT or DMPT. Since in subsequent communications this activity will be discussed either as a PPT resistance determinant or as a DMPT converting activity in the bialaphos biosynthetic pathway, we will

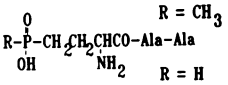
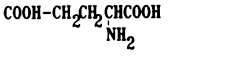
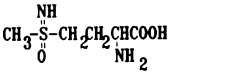
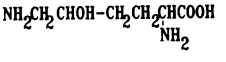
Substrate	Km (mM)	Formula
PPT	0.06	 R = CH_3
Demethyl-PPT	2.	R = H
Bialaphos	22.[a]	 R = CH_3
Demethyl-bialaphos	no reaction	R = H
Glutamate	240.	
Methionine sulfoximine	36.	
δ-hydroxylysine	56.	

Fig. 3. Substrate specificity of purified PAT. The K_M of PAT for different substrates was determined using the spectrophotometric assay described in Materials and methods. [a]The apparent PAT-catalysed acetylation of bialaphos probably resulted from contamination of the bialaphos preparation with PPT.

refer to it here as DPAT/PAT (DMPT or PPT acetyltransferase).

To test the hypothesis that the acetylation was catalysed by the product of the *bar* gene, an extract of *S. lividans* containing pBG106 were assayed for DPAT/PAT activity. The transformants contained DPAT/PAT activity which was not found in the parent. The observation described below that pBG93 contained an IPTG inducible DPAT/PAT confirmed that the *bar* gene subcloned on the *Sau*3aI fragment coded for the acetyltransferase activity.

In order to study the overexpression of *bar* in *S. hygroscopicus*, the *Pst*I fragment from pBG3 was subcloned into the broad host-range, multicopy vector, pIJ385 (Hopwood *et al.*, 1985) to generate pBG20. HP632 containing this plasmid was used to define the kinetics of DPAT/PAT synthesis. DPAT/PAT activities was present at low levels (0.12 units/mg protein) during the growth phase (1 day). After the culture entered stationary phase and antibiotic production had begun, activity increased to 0.88 units/mg protein (2 days) and accumulated with time to 1.04 units/mg protein (4 days) and 2.26 units/mg protein (8 days). Without pBG20, the strain produced 15 times less DPAT/PAT at day 8; SDS−PAGE gels indicated that a 22-kd protein which accumulated after day 1 was also being overexpressed in the strain containing pBG20.

Substrate specificity of the acetyltransferase

Purified DPAT/PAT which was produced by an *E. coli* strain harbouring a plasmid directing enhanced levels of expression of *bar* (J.Botterman, in preparation) was analysed for its ability to acetylate PPT and chemically related compounds. Although it appeared that k_{cat} for the various compounds were the same, their K_M values were significantly different (Figure 3). The lowest K_M was obtained for PPT. The affinity of DPAT/PAT for DMPT was almost 30 times lower. Although the table indicates that the enzyme catalyses the acetylation of bialaphos, we think that the measured enzymatic activity is an artefact due to low levels of PPT in the bialaphos preparation. No enzymatic reaction was detected with demethylbialaphos as substrate. Glutamate and analogues such as methionine sulfoximine and hydroxylysine were enzymatically modified by DPAT/PAT, but were poor substrates. These results indicate that DPAT/PAT has a very specific substrate requirement.

The acetylated reaction product

Enzyme extracts were prepared from *S. lividans*, *S. lividans*/pBG20 and W3110i^q/pBG93 (induced and uninduced with IPTG) and incubated with a reaction mix containing acetyl-coenzyme A and PPT. The reaction product was purified and then analysed by mass spectroscopy. Extracts of *S. lividans*/pBG20 and W3110i^q/pBG93 (induced) contained reaction products which corresponded in molecular weight to an acetyl-substituted PPT derivative (223 daltons). The observation that this product no longer reacted with ninhydrin indicated that the amino group had been acetylated. *S. lividans* or W3110i^q/pBG93 extracts (uninduced) did not modify PPT.

Discussion

Although *bar* was initially isolated as a gene which conferred resistance to bialaphos, it may serve as both an antibiotic-resistance and an antibiotic-biosynthetic gene which has interesting applications in either context. The nucleotide sequence of *bar* will facilitate the use of the gene as a model system to understand the control of antibiotic-biosynthetic genes and also as a selectable marker.

Expression of the *bar* gene is activated as *S. hygroscopicus* cultures enter stationary phase and bialaphos production begins. Many bialaphos biosynthetic genes (*bap*), including *bar*, are transcriptionally activated by a regulatory gene called *brp* (Anzai *et al.*, 1987). Experiments designed to test the hypothesis that the repeated nucleoside sequence which precedes the *bar* structural gene is a recognition site for the *brp* gene product are now underway.

The substrate specificity of DPAT/PAT suggests that it may serve a dual purpose in *S. hygroscopicus* cultures producing bialaphos. Studies of intermediates accumulated by *S. hygroscopicus bap* mutants have shown that DMPT is an intermediate (Imai *et al.*, 1985) in the pathway. In addition, this strain contains peptidase activity which degrades the tripeptide and releases PPT (Imai *et al.*, 1985). PPT and DMPT are inhibitors of *S. hygroscopicus* glutamine synthetase (Imai, personal communication) and both can be acetylated by the *bar* gene product. We have not rigorously demonstrated that DPAT/PAT actually serves either or both purposes during antibiotic production. Using a recently developed gene replacement technique, Kumada has specifically inserted a mutant *bar* allele into the *S. hygroscopicus* chromosome (personal communication). This *bar*$^-$ strain is bialaphos sensitive and accumulates DMPT. Thus, in addition to being a conventional biosynthetic enzyme, DPAT/PAT serves to prevent accumulation of PPT by recycling it into the biosynthetic pathway.

Since bialaphos has broad spectrum activity against bacteria, fungi and plants, it will be useful for the construction of vectors in many organisms. We have shown that the *S. hygroscopicus bar* gene can be used as a dominant selectable marker in bacteria. The availability of the nucleotide sequence will make it possible to express *bar* under the control of other prokaryotic or eucaryotic transcriptional and translational signals. The observation that fusion proteins such as those synthesized from the *Sau*3aI frag-

ment also confer bialaphos resistance suggests that it could also be used to select for transfer and expression of genes fused to *bar*.

In contrast to its bacteriostatic activity on minimal medium, the herbicide activity of bialaphos does not result from glutamine starvation and cannot be reversed by externally applied glutamine (Tachibana *et al.*, 1986a). Instead, inhibition of chloroplast glutamine synthetase results in toxic levels of ammonium accumulation which occur within hours of application (Tachibana *et al.*, 1986b; De Block *et al.*, 1987). This characteristic makes bialaphos especially effective against plants.

In the accompanying paper (De Block *et al.*, 1987), the use of *bar* as a selectable marker in transgenic plants has been evaluated. Although aminoglycoside-resistance genes have already been used as selectable marker genes in plants (Herrera-Estrella *et al.*, 1983; Van den Elzen *et al.*, 1985), the *bar* gene offers special advantages. Most importantly, there is a major biotechnological interest in engineering herbicide-resistant plants. In contrast to several genes which can generate tolerance to herbicide (Comai *et al.*, 1985; Shah *et al.*, 1986), the expression of *bar* in plants generates plants which are fully resistant to bialaphos and PPT.

Materials and methods

Bacterial strains and vectors

E. coli strains ED8767 (Murray *et al.*, 1977) and a laci^q derivative of W3110 (Campbell *et al.*, 1978) as well as the vectors pBR322 (Bolivar *et al.*, 1977) and pUC19 (Norrander *et al.*, 1983) were obtained from the Biogen culture collection. *S. lividans* 66 came from the John Innes Culture Collection (assigned number 1326). *S. hygroscopicus* HP632 is a bialaphos-overproducing derivative of *S. hygroscopicus* ATCC21705 which was provided by Meiji Seika Kaisha, Ltd. Streptomycete vectors pIJ385 and pIJ680 (Hopwood *et al.*, 1985) were obtained from the John Innes Culture Collection.

Streptomycetes were maintained on either nutrient agar (Difco) or NE medium (Murakami *et al.*, 1986); *E. coli* was grown in Luria broth or on Luria agar (Maniatis *et al.*, 1982). In order to use bialaphos as an antibiotic, strains were grown on streptomycete minimal medium (containing per litre: 15 g soluble starch, 2.6 g $NaNO_3$, 1.5 g KH_2PO_4, 1 mg $CoCl_2$-$6H_2O$, 50 mg $MgSO_4$-$7H_2O$, 20 g agar, pH 7.0) or M9 minimal medium for *E. coli* (Maniatis *et al.*, 1982). Plates containing gradients of bialaphos were used to estimate relative levels of bialaphos resistance. The *lac* promoter was induced with 0.1 mM IPTG.

Nucleic acid procedures

Plasmid DNA was isolated from *E. coli* using the protocol of Birnboim and Doly (1979) and then banded in gradients of CsCl/ethidium bromide. Streptomycete plasmids were isolated according to Kieser (1984). Recombinant plasmids were constructed using enzymes supplied by BRL and standard protocols for *E. coli* or streptomycete transformation (Hopwood *et al.*, 1985). The DNA sequence was obtained from pBG195 using the Maxam and Gilbert procedure (1980). Compressions in the sequencing ladder are sometimes encountered when sequencing GC-rich streptomycete DNA. In order to resolve these artefacts, fragments which had undergone the Maxam–Gilbert sequencing reactions were subjected to a reaction which opens the cytosine ring and prevents GC pairing (Ambartsumyan and Mazo, 1980). The sequence was analysed using a program provided by the University of Wisconsin. A program called 'Codon Preference' used a library of codons representing those found in streptomycete structural genes to locate the *bar* structural gene.

Acetyltransferase assay

The chloramphenicol acetyltransferase assay (Shaw, 1975) was adapted for the quantification of acetyltransferase using other substrates. The reaction was carried out at 22°C in a mix which contained Tris–HCl (50 mM, pH 7.5), 0.4 mg/ml 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 0.1 mM acetyl-coenzyme A. After establishing a baseline rate of DTNB reduction, the reaction was started by the addition of substrate (0.2 mM PPT). The rate of substrate-specific increase in adsorption at 412 nm was divided by 13.6 to give the activity in micromoles of substrate acetylated per minute (units). In order to determine specific activity, the protein concentration was determined by Bio-Rad Protein Assay using bovine serum albumin as standard.

Substrates PPT, DMPT, bialaphos and demethylbialaphos were provided by Meiji Seika Kaisha, Ltd.

Preparation of acetylated product

S. lividans strain 1326/pBG20 mycelia were grown in YEME liquid medium (200 ml) containing 34% sucrose for 48 h at 30°C (Hopwood *et al.*, 1985). Mycelium was collected by centrifugation and washed with 50 mM phosphate buffer (pH 6.5) containing 50 μM β-mercaptoethanol. It was then resuspended in the same buffer, disrupted by sonication, and clarified by centrifugation (17 000 *g*, 15 min, 4°C). *E. coli* strain ED8767/pBG93 was grown in LB medium (200 ml) at 37°C and induced during exponential growth with 0.5 mM IPTG for 3 h. Extracts were prepared as described above. PPT (0.5 mg/ml) and acetyl-coenzyme A (1.0 mg/ml) were added to 10 ml of *E. coli* or *S. lividans* extract and the reaction mix was incubated at 30°C for 3 h. H_2SO_4 was added to adjust the pH to 2.0 and precipitated proteins were removed by centrifugation (10 min, 17 000 *g*, 4°C). The supernatant was then passed through a Dowex 50 column (H^+ form) to adsorb residual PPT. The effluent was neutralized (pH 7) with NaOH, adsorbed to a Dowex-1 (Cl^- form) column, washed with 0.5% NaCl, and eluted with 3.0% NaCl. The eluate was then applied to a Sephadex G-10 column (equilibrated in H_2O). Fractions were lyophilized to obtain pure crystalline material. The molecular weight of the reaction product was defined by fast atom bombardment mass spectroscopy in positive and negative ion modes.

DPAT/PAT purification

DPAT/PAT was isolated from mycelia of HP632/pBG20 containing the *bar* gene cloned on a high copy number plasmid. A lyophilized culture of the strain was inoculated into seed medium and grown at 28°C for 40 h. Five millilitres of this culture were used to inoculate 1 litre of bialaphos production medium (7% glucose, 3.9% wheat germ, 2.5% soluble vegetable protein, 0.3% KH_2PO_4, 0.0001% $CoCl_2$, pH 6.4). After 3 days of growth at 28°C, the mycelia were pelleted, washed twice and resuspended in TM buffer (10 mM Tris, pH 7.5, 0.005 mM β-mercaptoethanol, 10 mM $MgCl_2$) and then disrupted by sonication. The extract was clarified by centrifugation (17 000 *g*, 20 min, 4°C). Protein was precipitated in 90% saturated ammonium sulphate and separated by SDS–PAGE. The gel was blotted onto polybrene coated glass fibre sheets and the 22-kd band was applied to an amino acid sequenator for N-terminal amino acid analysis as described by Vandekerckhove *et al.* (1985).

Enzymatically active DPAT/PAT was purified from a strain of *E. coli* containing a plasmid which directed high level expression of the *bar* gene product (J.Botterman *et al.*, in preparation).

In vitro transcription/translation

The size of gene products were determined in an *in vitro* system for transcription and translation using streptomycete extracts (Thompson *et al.*, 1984).

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