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/phosphinothricin/acetyltransferase/herbicide resistance/bar

Introduction

Streptomyces which produce antibiotics 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 (brpa, 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 dimethylphosphinothricin (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 PstI (1.7 kb, pBG3) or BamHI (2.0 kb, pBG4) fragments (Figure 1).

The bar gene was subcloned in E. coli W31108 using the vector pUC19 (Norlander et al., 1983). DNA inserted in the polylinker region of pUC19 can be transcribed from the lac promoter in the vector. In W31108, transcription from the lac promoter is strongly repressed but can be induced with isopropyl-thiogalactoside (IPTG). The orientation of the gene was indicated by subcloning the BamHI fragment from the original streptomycete isolate, pBG4, in both orientations into the BamHI site of pUC19 to generate pBG195 and pBG196 (see Figure 1). W31108 was slightly resistant to bialaphos when it contained pBG196; however, increased levels of resistance could not be induced by IPTG. In contrast, with W31108/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. Sau3aI fragments of the pBG195 insert were cloned into the BamHI site of the expression vector, pJ680 (Hopwood et al., 1985). A plasmid, pBG106, which conferred resistance to bialaphos contained an ~620-bp Sau3aI insert.

The Sau3aI fragment was also subcloned from pBG195 into the BamHI site of pUC19. Two plasmids, pBG93 and pBG94,
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 Sau3A1 subclone), generated proteins of 28 and slightly more than 22 kd, respectively. Plasmids pBG195 and pBG196 containing the 2.0 kb BamHI 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 Sau3A1 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 Sau3A1 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 2).
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.

To test the hypothesis that the acetylation was catalysed by the product of the bar gene, an extract of *S. lividans* containing pBG106 was 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 Sau3A1 fragment coded for the acetyltransferase activity.

In order to study the overexpression of bar in *S. hygroscopicus*, the PstI fragment from pBG3 was subcloned into the broad host-range, multicopy vector, pJ385 (Hopwood et al., 1985) to generate pBG20. HP632 containing this plasmid was used to define the kinetics of DPAT/PAT synthesis. DPAT/PAT activities were 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 over-expressed 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 $K_M$ 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 hydroxysine 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 W3110/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 W3110/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 W3110/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 (bar), including bar, are transcriptionally activated by a regulatory gene called brp (Anzai et al., 1987). Experiments designed to test the hypothesis that the repeated nucleotide 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* bar 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 Sau3A1 frag-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (mM)</th>
<th>Formula</th>
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<tr>
<td>PPT</td>
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<td>R$\equiv$CH$_2$CH$_2$CHOH</td>
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<tr>
<td>Demethyl-PPT</td>
<td>2.</td>
<td>R$\equiv$CH$_2$CH$_2$CHOH</td>
</tr>
<tr>
<td>Bialaphos</td>
<td>22$^a$</td>
<td>R$\equiv$CH$_2$CH$_2$CHO$_2$Ala-Ala</td>
</tr>
<tr>
<td>Demethyl-bialaphos</td>
<td>no reaction</td>
<td>R$\equiv$CH$_2$CH$_2$CHO$_2$Ala-Ala</td>
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<tr>
<td>Glutamate</td>
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<td>COOH$\equiv$CH$_2$CH$_2$CHOH</td>
</tr>
<tr>
<td>Methionine sulfoximine</td>
<td>36.</td>
<td>NH$\equiv$CH$_2$CH$_2$CHOH</td>
</tr>
<tr>
<td>$\delta$-hydroxysine</td>
<td>56.</td>
<td>NH$\equiv$CH$_2$CH$_2$CHOH</td>
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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 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. hypogriseus HPI632 is a bialaphos-overproducing derivative of S. hypogriseus ATCC21705 which was provided by Meiji Seika Kaisha, Ltd. Streptomyces vectors pJ385 and pJ680 (Hopwood et al., 1985) were obtained from the John Innes Culture Collection.

Streptomyces 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 streptomycin minimal medium (containing per litre: 5 g soluble starch, 2.6 g NaNO₃, 1.5 g KH₂PO₄, 1 mg CoCl₂·6H₂O, 50 mg MgSO₄·7H₂O, 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 band in gradients of CsCl/ethidium bromide. Streptomyces 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). Comparisons 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 absorption at 412 nm was divided by 3.6 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, DMPPT, bialaphos and demethylbialaphos were provided by Meiji Seika Kaisha, Ltd.

Preparation of acetylated product

S. lividans strain 1326/pBG20 mycelia were grown in YEML 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/pBG20 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. H₂SO₄ 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 elute was then applied to a Sephadex G-10 column (equilibrated in H₂O). 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% KH₂PO₄, 0.0001% CoCl₂, pH 6.4). After 3 days of growth at 28°C, the mycelia were pelleted, washed twice and resuspended in TEM buffer (10 mM Tris, pH 7.5, 0.005 mM β-mercaptoethanol, 10 mM MgCl₂) 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 band was blotted onto polybren coated glass fibre sheets and the 22-kd band was applied to an amino acid sequencer for N-terminal amino acid analysis as described by Vandenkerkhove 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 streptomyces extracts (Thompson et al., 1984).

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References

Herbicide-resistance gene


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