Phosphoenolpyruvate carboxylase from *Streptomyces coelicolor* A3(2): purification of the enzyme, cloning of the *ppc* gene and over-expression of the protein in a streptomycete

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Phosphoenolpyruvate carboxylase [PEPC; orthophosphate:oxaloacetate carboxy-lyase (phosphorylating); EC 4.1.1.31] is a major anaplerotic enzyme in the polyketide producer *Streptomyces coelicolor* A3(2). PEPC was purified from *S. coelicolor* and the amino acid sequences of four tryptic peptides were determined. Synthetic oligonucleotides based on the sequences of two of the peptides hybridized to the same bands in various restriction-enzyme digests of *S. coelicolor* genomic DNA. This hybridization allowed molecular cloning of an 8 kb *Bam*HI fragment of genomic DNA. Partial DNA sequencing of this fragment showed that it could encode amino acid sequences similar to those of PEPC from other microorganisms. A *Bam*HI/*Pst*I fragment was subcloned into the streptomycete high-copy-number plasmid vector pIJ486 and transferred into *Streptomyces lividans*. The resulting strain over-expressed PEPC activity 21-fold and also over-expressed a protein with a subunit of 100000 M_r, the same as that of purified *S. coelicolor* PEPC.

INTRODUCTION

Antibiotics and other secondary metabolites produced by streptomycetes have great therapeutic importance (Omura, 1992) and consequently the pathways by which these molecules are synthesized have been studied extensively (reviewed in O'Hagan, 1991).

Secondary metabolites have a wide variety of chemical structures and are all derived from precursors synthesized by primary metabolic pathways. The precursors of several secondary metabolites have been identified by following the fates of radiolabelled substrates after their addition to cells which are synthesizing the metabolite (Gorst-Allman et al., 1981; Yue et al., 1987). The definition and characterization of the pathways which supply these precursors are important not only to provide an understanding of the primary metabolism of streptomycetes but also to facilitate the manipulation of precursor supply, which may enable the overproduction of secondary metabolites. The work described in this paper is part of an investigation into the pathways by which precursors of secondary metabolites are synthesized in the model species *Streptomyces coelicolor* A3(2).

The genetics of *S. coelicolor* has been studied in detail by Hopwood and co-workers who, with others, have developed systems which allow manipulation of streptomycete genes *in vivo* (Hopwood et al., 1985). A combined genetic and physical map (Kieser et al., 1992) of the *S. coelicolor* chromosome is now available and growth conditions which allow control of the physiology of the organism have been established (Hobbs et al., 1989; Strauch et al., 1991). This species provides therefore a good model system in which to study biosynthesis and in which to manipulate enzymes *in vivo*.

S. coelicolor produces at least four secondary metabolites (Lakey et al., 1983) of which two are derived partly (in the case of undecylprodigiosin, Wasserman et al., 1976) or fully (in the case of actinorhodin, Gorst-Allman et al., 1981) from polyketide chains. Polyketides are synthesized by the condensation of acyl-CoA precursors in a series of reactions analogous to those occurring during fatty acid biosynthesis (Birch, 1967; O'Hagan, 1991). Actinorhodin is a dimer of polyketide chains each of which is synthesized from an acetyl-CoA starter unit and seven malonyl-CoA extender units (Gorst-Allman et al., 1981).

When *S. coelicolor* is grown using glucose as sole carbon source phosphoenolpyruvate carboxylase (PEPC: EC 4.1.1.31) is the only anaplerotic enzyme whose activity can be detected using standard protocols (Cannata and Stoppani, 1963; McClure et al., 1971). Since anaplerotic enzymes give rise to branchpoints between energy-producing and biosynthetic pathways their activity is likely to be regulated at the switch from growth to production of secondary metabolites.

Behal et al. (1977) and Deklava and Strohl (1988) have detected PEPC activity in cell-free extracts of other polyketide-producing streptomycetes (*S. aureofaciens* and *Streptomyces* C5 respectively). These groups also reported an increase in the activity of the enzyme during polyketide biosynthesis and Behal et al. (1977) postulated that the oxaloacetate produced by PEPC is converted directly into the polyketide precursor malonyl-CoA. However, labelling studies and ^{13}C-n.m.r. spectroscopy carried out by Wang et al. (1986) indicate that the malonyl-CoA incorporated into polyketides in *S. aureofaciens* is in fact synthesized from acetyl-CoA.

We have found an increase in PEPC activity during actinorhodin biosynthesis in *S. coelicolor* (approx. 2-fold, results not shown) but obtained no evidence for the conversion of oxaloacetate into malonyl-CoA by cell-free extracts of the organism. The role of PEPC activity during secondary metabolite biosynthesis in *S. coelicolor* thus remains unclear and one of our objectives is to define this role. If the activity of PEPC is directly involved in the supply of polyketide precursors, manipulation of this activity by over-expression or gene disruption may affect the yield of product. In this paper we describe work carried out to provide the tools with which to analyse the role of PEPC, that is

Abbreviations used: PEPC, phosphoenolpyruvate carboxylase (EC 4.1.1.31); PEP, phosphoenolpyruvate; PMSF, phenylmethanesulphonyl fluoride; TFA, trifluoroacetic acid.

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the purification of PEPC protein and the cloning of the *ppc* gene from *S. coelicolor*.

EXPERIMENTAL

Materials

Phosphoenolpyruvate (PEP), acetyl-CoA, NADH and malate dehydrogenase were obtained from Boehringer. DEAE-Sepharose and phenyl-Sepharose were from Pharmacia and trypsin was from Worthington Biochemical Corporation, Freehold, NJ, U.S.A. H.p.l.c. solvents were prepared using purified water from a Milli Q reagent water system. DNA restriction enzymes were obtained from Gibco BRL and sequencing enzymes and reagents, as well as DNA ligase, were obtained from Promega. All other reagents used were of analytical grade.

PEPC assay

PEPC activity was determined spectrophotometrically at 30 °C by coupling oxaloacetate production to NADH oxidation using malate dehydrogenase (Yoshinaga et al., 1970). The assay contained; 47 μmol Tris/HCl, pH 7.8, 4.7 μmol $MgCl_2$, 10 μmol $NaHCO_3$, 2 μmol PEP, 0.2 μmol NADH, 0.1 μmol acetyl-CoA, 10 units of malate dehydrogenase and 10–30 μl of extract in a final volume of 1 ml. The background rate of NADH oxidation was measured, by following the decrease in optical absorbance at 340 nm, after addition of extract to the assay mixture without PEP and the assay was then initiated by the addition of PEP. One unit of enzyme activity catalysed the oxidation of 1 μmol of NADH per minute in this assay.

Protein assay

Protein concentrations were determined by the method of Bradford (1976).

Cell growth and preparation of extracts

S. coelicolor A3(2) 1147 (*SCP1*$^+$, *SCP2*$^+$) was grown on a defined minimal medium containing glucose as the sole carbon source (Hobbs et al., 1989). Cultures were inoculated with fresh spores which had been prepared by growth on soya-mannitol-agar plates (Hobbs et al., 1989) for 8–10 days at 30 °C. Spores were harvested into sterile distilled water (5–10 ml/plate) and liquid medium was inoculated with approx. 1×10^7 spores/ml. Cultures of 400 ml were grown at 30 °C with shaking at 250 rev./min for 40 h. For purification of PEPC eight 400 ml cultures were used.

Cells were harvested by centrifugation at 15000 *g* for 15 min and the pellets were resuspended in buffer A [50 mM phosphate buffer, pH 7, containing 5 mM EDTA, 1.2 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine and 0.4 mM dithiothreitol]. The cells were disrupted by passaging twice through a French pressure cell at 98 MPa internal pressure. The lysate was cleared by centrifugation at 48000 *g* for 60 min and the supernatant was used in protein purification.

Purification of PEPC

All procedures were carried out at 4 °C except for f.p.l.c. which was carried out at room temperature.

$(NH_4)_2SO_4$ fractionation

The cell-free extract was adjusted to 30% satn. of $(NH_4)_2SO_4$ and stirred for 30 min. The supernatant obtained after centrifugation at 27000 *g* for 15 min was taken to 60% satn. by addition of further $(NH_4)_2SO_4$, the mixture was stirred for 30 min and centrifuged at 27000 *g* for 15 min. The pellet was resuspended in a minimum volume of buffer A and dialysed overnight into buffer A without PMSF (buffer B).

DEAE-Sepharose chromatography

A DEAE-Sepharose column (40 ml bed volume) was equilibrated in buffer B at a flow rate of 30 ml/h. The dialysed $(NH_4)_2SO_4$ pellet was loaded on to the column at the same flow rate and the column was washed with buffer B until the absorbance of the eluant at 280 nm was the same as that of buffer B. Proteins bound to the column were eluted with a linear gradient of 0–1 M KCl in buffer B. The total volume of the gradient was 600 ml and 4 ml fractions were collected and assayed for PEPC activity.

Phenyl-Sepharose chromatography

Fractions eluted from the DEAE-Sepharose column between 0.28 M and 0.37 M KCl contained PEPC activity. These fractions were pooled and $(NH_4)_2SO_4$ was added to a concentration of 0.7 M. The pooled sample was loaded on to a phenyl-Sepharose column (20 ml bed volume) which had been equilibrated in buffer B containing 0.7 M $(NH_4)_2SO_4$ at a flow rate of 15 ml/h. Unbound material was washed off the column with equilibration buffer and bound material eluted with (i) a linear gradient (300 ml) of 0.7–0 M $(NH_4)_2SO_4$ in buffer B, followed by (ii) 40 ml of buffer B, (iii) a linear gradient (300 ml) of 0–50% (v/v) ethanediol in buffer B and (iv) 40 ml of buffer B containing 50% (v/v) ethanediol. Fractions (4 ml) were collected and assayed for PEPC activity. PEPC eluted isocratically at 50% (v/v) ethanediol; active fractions were pooled and dialysed against buffer B overnight.

Mono Q f.p.l.c.

A 1 ml Mono Q column (Pharmacia) was equilibrated in buffer B at a flow rate of 1 ml/min. The dialysed fraction which eluted from phenyl-Sepharose was loaded on to the column and unbound material removed by washing with buffer B. Proteins bound to the column were eluted with a linear gradient of 0–1 M KCl in buffer B; 0.5 ml fractions were collected and assayed for PEPC activity. The activity was eluted between 0.48 M and 0.54 M KCl.

PAGE

The purity of samples having PEPC activity was assessed by PAGE as described by Laemmli (1970). Resolving gels contained 8% (w/v) acrylamide and proteins were visualized by staining with silver (Poro et al., 1982).

Peptide preparation

Purified samples of PEPC were dialysed overnight against 0.05% $(NH_4)HCO_3$. Trypsin (tosylphenylalanylchloromethane-treated) was added at 2% (w/w) and the mixture was incubated at 37 °C for 10 h with shaking at 100 rev./min.

Peptide purification

Peptides were purified by reverse-phase h.p.l.c. on a Waters μBondapak C-18 column which had been equilibrated at 1 ml/min in acetonitrile/water (30:70, v/v) containing 0.1% trifluoroacetic acid (TFA). After washing the column with equilibration buffer, peptides were eluted with a linear gradient of 30–80% acetonitrile in water containing 0.1% TFA. The absorbance of the eluant at 220 nm was determined and fractions showing peaks of absorbance were collected.

Amino acid sequencing

Fractions from the C-18 column were loaded directly on to an Applied Biosystems model 4774 pulsed-liquid-phase sequencer. Amino acid thiohydantoins were detected using an on-line analyser (Applied Biosystems model 120A). Sequencing was carried out by Drs. M. Cusack and G. B. Curry (Department of Geology, Glasgow University, Glasgow, U.K.)

Molecular biological techniques

The procedures involved in the cloning of DNA in *Escherichia coli* were carried out as described by Sambrook et al. (1989) and those involved in the cloning of DNA in streptomycetes, as well as the preparation of *S. coelicolor* genomic DNA, were carried out as described by Hopwood et al. (1985). When cloning DNA into *S. lividans* the high-copy-number plasmid pIJ486, which carries the thiostrepton-resistance marker (Ward et al., 1986), was used as vector. Thiostrepton-resistant transformants were screened for the presence of insert DNA by determining the size of plasmid which they contained.

Digests of genomic DNA were transferred to Hybond N filters (supplied by Amersham) in alkali transfer buffer as described in 'Blotting and Hybridisation to Membranes' (Amersham Protocol, Amersham International, Amersham, Bucks., U.K.) Filters were prehybridized at 65 °C for at least 3 h before probing was carried out.

Purification of DNA

DNA fragments, except for those used in the primary cloning described below, were purified from agarose gels as described in Sambrook et al. (1989).

S. coelicolor genomic DNA which had been digested with *Bam*HI was purified by h.p.l.c. on a Waters Gen Pak Fax column. Material was loaded on to the column in 25 mM Tris/HCl pH 7.5 at a flow rate of 0.5 ml/min. Fragments of DNA were eluted with a linear gradient of 0–0.75 M NaCl followed by isocratic elution at 0.75 M NaCl in starting buffer. The absorbance of the eluant at 260 nm was determined and DNA recovered from fractions showing peaks of absorbance by ethanol precipitation without any added salt and this was then resuspended in 5 μl of TE buffer (10 mM Tris/HCl, pH 8/1 mM EDTA, pH 8) (Sambrook et al., 1989). These samples were analysed by agarose gel electrophoresis (Sambrook et al., 1989) to determine the size and concentration of the DNA.

Nucleotide sequencing

Double-stranded template was denatured using alkali (Murphy and Ward, 1989) before sequencing. Double-stranded template was sequenced using Taq DNA polymerase at 70 °C in the presence of 7-deazanucleotide triphosphates in order to sequence through regions of dG + dC-rich DNA. Single-stranded template was sequenced using T7 DNA polymerase at 37 °C. Sequences were compiled and analysed using programs of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

RESULTS AND DISCUSSION

Protein purification and amino acid sequencing

PEPC was purified by a combination of $(NH_4)_2SO_4$ precipitation and three chromatographic steps. The results of the purification are given in Table 1 and Figure 1 shows analysis of the purification by SDS/PAGE, which indicates that the final material was at least 95% pure. Like PEPCs from other bacteria (Yoshinaga et al., 1970; Mori and Shiio, 1985), the *S. coelicolor* enzyme was dependent on acetyl-CoA for activity and had a subunit of 100000-M_r.

We were unable to obtain any amino acid sequence from a purified sample of PEPC which contained approx. 10 nmol of protein (100 pmol of protein is normally sufficient to obtain clear sequence information), indicating that the N-terminal amino acid of the protein is modified and therefore resistant to Edman degradation. A second sample of purified PEPC was digested with trypsin. The products were separated by reverse-phase h.p.l.c. and an amino acid sequence was obtained from three of the most abundant peptides (tp3, tp4 and tp5). Peptide tp5, although apparently well resolved by h.p.l.c., in fact contained two peptides which were sequenced simultaneously. The sequences of the four tryptic peptides of *S. coelicolor* PEPC are given in Figure 2.

Alignment of peptide tp5/1 with sequences from other PEPCs is shown in Figure 3. The degree of similarity strongly suggests that this peptide is indeed derived from a PEPC. The other tryptic peptides could not be aligned with other PEPC sequences (not shown), suggesting that they may be derived from less conserved regions of the protein.

Oligonucleotide design and gene cloning

Oligonucleotides 1, 2 and 3 were designed using the sequences of tp4, tp5/1 and tp5/2 respectively, taking into account the known codon usage of 26 streptomycete genes (Seno and Baltz, 1989). Where ambiguity existed in the amino acid sequence the more abundant amino acid was used. Each of the oligonucleotides (shown in Figure 4) contains a limited number of degeneracies and oligo-1 contains three inosine residues in place of a codon where no amino acid could be positively identified in tp4 (see Figure 2).

Oligo-1 and oligo-2 were shown to hybridize to the same unique band in various restriction-endonuclease digests of *S. coelicolor* genomic DNA under stringent conditions (5 × SSC, 60 °C; SSC contains 0.15 M NaCl and 0.015 M sodium citrate). Hybridization to oligo-1 gave the strongest signals and no signal was obtained on hybridization with oligo-3 under stringent conditions.

Fragments of genomic DNA digested with *Bam*HI of approx. 8 kb in length gave hybridization signals with both oligo-1 and oligo-2 and were purified as described in the Experimental section. These fragments were ligated to pUC18, which had been digested with *Bam*HI, and cloned into *E. coli* TG1. Cells which had been transformed with recombinant plasmid were screened by hybridization to oligo-1 and oligo-2. Out of 137 clones examined two, designated p119 and p25, gave hybridization signals with both probes (in 5 × SSC at 60 °C) and p119 was used in subsequent experiments.

Mapping and partial sequencing of p119

In order to confirm that the insert in p119 contains the *ppc* gene it was necessary to demonstrate that the gene product is PEPC. The binding site for oligo-1 in p119 was determined by double-strand sequencing. The amino acid sequence around the binding site was deduced by translation of the nucleotide sequence (the correct frame being that which contained the sequence of tp4). The sequence obtained was similar to the C-terminal region of the PEPCs from *Corynebacterium glutamicum* (O'Regan et al., 1989) and *E. coli* (Fujita et al., 1984) with 11 amino acid residues

Table 1 Purification of PEPC from *S. coelicolor*

Details of each purification step are given in the Experimental section. The increase in yield of activity seen after phenyl-Sepharose chromatography is thought to be due to the presence of ethanediol in the eluant, since it is known that PEPC activity is stimulated by organic solvents (Sanwal et al., 1966).

Sample	Protein concentration (mg/ml)	Total protein (mg)	PEPC specific activity (unit/mg)	PEPC total activity (units)	Yield of activity (%)	Purification factor (fold)
Cell-free extract	4.04	1212	0.082	99	100	1
30–60% $(NH_4)_2SO_4$ pellet	6.9	440.2	0.261	114.8	116	3.2
DEAE-Sepharose eluate	1.15	65.6	1.75	114.6	116	21.3
Phenyl-Sepharose eluate	0.043	7.74	19.77	153	155	241
Mono Q eluate	0.83	0.83	42.9	35.6	36	523

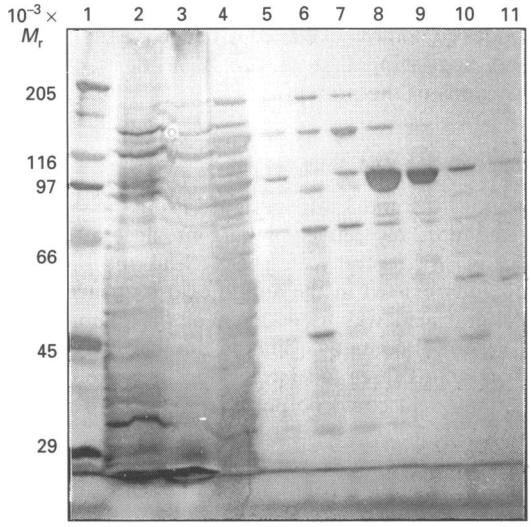

Figure 1 SDS/PAGE analysis of samples taken at each stage of the purification of PEPC from *S. coelicolor*

Tracks contain the following samples; lane 1, M_r markers; lane 2, cell-free extract of *S. coelicolor*; lane 3, 30–60% $(NH_4)_2SO_4$ pellet; lane 4, DEAE-Sepharose eluate; lane 5, phenyl-Sepharose eluate; lanes 6–11, adjacent Mono Q elutate fractions. The fractions shown in lanes 8 and 9 contained 56% and 37% respectively of the total PEPC activity recovered from the Mono Q column and these fractions were combined for amino acid sequencing.

	Residue number														
Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
TP3	A/S	Y	L	P	T	A	Q								
TP4	–	G	L	D	T	V	L	D	E	M	–	Q	Q		
TP5/1	A	A	V	T/E	I	L	E	D/Q	L	L	A	D/F	P	S/V	Y
TP5/2	D	L	D	E/T	F	P	W	Q	P	A	Y	D/M	L		

Figure 2 The amino acid sequences of peptides of *S. coelicolor* PEPC

At some positions we could not distinguish between two residues with complete certainty; both residues are given, with the first being the more abundant; – indicates that the amino acid has not been identified.

identical to *E. coli* PEPC and 10 residues identical to *C. glutamicum* PEPC out of 31 residues of deduced amino acid sequence. As noted above the region covered by peptide tp4 did not itself show significant sequence similarity to other PEPCs (two residues identical to *E. coli* PEPC over 10 residues of tp4 sequence). A *Bam*HI/*Pst*I fragment of p119 which hybridized to oligo-1 and oligo-2 was subcloned into *E. coli* TG1 in pBluescript. The recombinant plasmid obtained, designated pB30, was then mapped by restriction-endonuclease digestion and the map of the insert is shown in Figure 5. Three fragments of pB30, shown in Figure 5, were subcloned into M13mp18 and M13mp19 and the recombinant phages were used as templates for single-strand sequencing.

The position of potential protein coding regions within the nucleotide sequences obtained was determined using the FRAME analysis program (Bibb et al., 1984) and the deduced amino acid sequence was compared with those of *C. glutamicum* and *E. coli* PEPCs. Two regions, of 153 amino acids in total including the sequence around tp4 mentioned above, could be aligned with the C-termini of the known PEPC sequences with significant degrees of similarity, as shown in Table 2.

The nucleotide sequence obtained is from the region around the *Sst*I site in pB30 (see Figure 5) and the open reading frame of the putative *ppc* gene from *S. coelicolor* would be transcribed in the direction shown in Figure 5. The subunit M_r of *S. coelicolor* PEPC is 100000 (see Figure 1) and the protein would be encoded by a gene of approx. 3 kb in length. The amount of DNA in the insert of pB30 is sufficient therefore to contain the entire *ppc* gene as well as promoter sequences (assuming that the gene is not part of an operon).

Over-expression of PEPC in *Streptomyces lividans* TK64

DNA which has been propagated in most strains of *E. coli* cannot be maintained stably by *S. coelicolor* (Hopwood et al., 1985). The restriction system responsible for degradation of *E. coli* DNA in *S. coelicolor* is thought to be methylation specific and a similar system in *Streptomyces avermitilis* has been described (MacNeil, 1988). However, *S. lividans*, a closely related organism, is able to maintain and modify *E. coli* DNA and was used therefore as host for cloning of the putative *ppc* gene into a streptomycete.

Since the insert in pB30 was expected to contain the whole of the *ppc* gene it was transferred into *S. lividans* TK64 using the vector pIJ486. Of seven thiostrepton-resistant colonies five con-

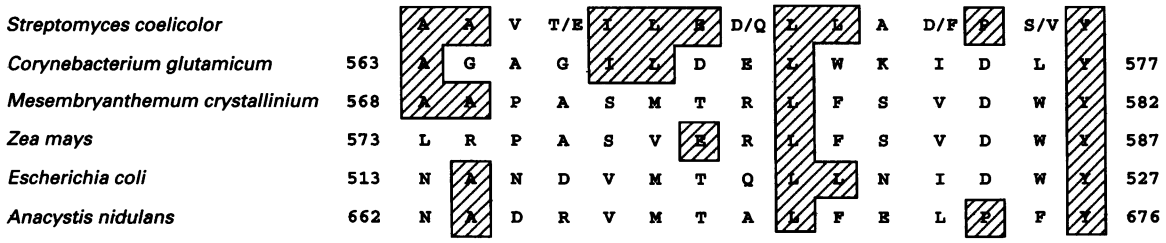

Figure 3 Alignment of the amino acid sequence of tp5/1 with those of PEPCs from a variety of species

Two amino acids are shown at positions where the amino acid assignment in tp5/1 was ambiguous; the first residue was the more abundant (see Figure 2).

Peptide	Oligonucleotide	Amino acid and nucleotide sequences									
TP4	Oligo-1	D GAC	T ACC/G	V GTC/G	L CTC/G	D GAC	E GAG	M ATG	– III	Q CAG	Q CAG
TP5/1	Oligo-2	T ACC	I ATC	L CTG/C	E GAG	D GAC	L CTG/C	L CTG/C	A GCC/G	D GAC	P CC
TP5/2	Oligo-3	D GAC	L CTG/C	D GAC	E GAG	F TTC	P CCC/G	W TGG	Q CAG	P CC	

Figure 4 The sequences of oligonucleotide probes and the peptide sequences from which they were designed

In each case only part of the peptide sequence was used to design the oligonucleotide in order to minimize the degeneracy of the probes.

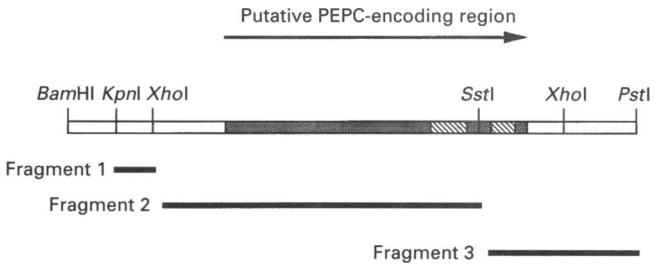

Figure 5 Map of the insert contained in plasmid pB30

Fragments 1, 2 and 3 (0.33 kb, 3.3 kb and 1.66 kb respectively) were each cloned into *E. coli* TG1 in M13mp18 and M13mp19 phage to produce the templates used in single-stranded sequencing reactions. Those regions for which the sequence is known are shown in light hatching (▨) within the putative PEPC encoding region (▩). The length of this region is based on the subunit M_r of *S. coelicolor* PEPC and its position is based on the similarity of deduced amino acid sequence from *S. coelicolor* DNA to the C-terminal regions of known PEPC sequences. The exact sites at which transcription is initiated and terminated have not been determined.

Table 2 Percentage identities of deduced amino acid sequence from the insert in pB30 with the amino acid sequences of known PEPCs

Sequence aligned with deduced amino acid sequence of *S. coelicolor* DNA insert of pB30	Residues over which sequences align	Percentage of identical residues
Escherichia coli PEPC	702–771	41
	805–883	32.1
Corynebacterium glutamicum PEPC	741–822	35.4
	848–919	31.5

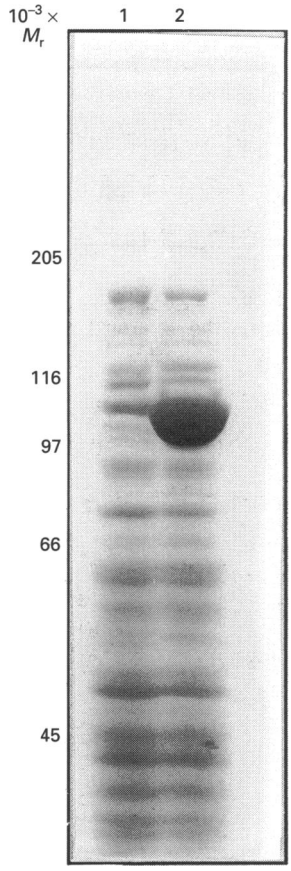

Figure 6 SDS/PAGE analysis of *S. lividans* TK64 cell-free extracts

Extract in lane 1 is from cells transformed with pIJ486 and that in lane 2 is from cells transformed with pIJ486 containing insert.

tained plasmid with insert. One of these, colony 5A3, was grown in liquid culture, as was a control colony containing pIJ486 without insert. The recombinant strain grew more slowly than the control, suggesting that the presence of insert has some physiological effect. The PEPC activity in cell-free extracts of 5A3 was 2.5 units/mg, 21-fold higher than the control (0.12 unit/mg) and a 100000-M_r protein was over-expressed in these extracts (Figure 6). The plasmid pIJ486 has no promoter able to direct transcription from DNA cloned into its polylinker,

and so the increased expression of PEPC must be directed by the *ppc* gene promoter. The insert in pB30 therefore seems to contain both the entire coding sequence and the promoter of the *ppc* gene.

The reduced growth rate of cells over-expressing PEPC protein could be due to sequestration of PEP from other pathways of central metabolism, and may delay the onset of secondary metabolism. In order to investigate directly the role of PEPC in secondary metabolism it will be necessary to over-express PEPC in a controlled manner after the cessation of growth.

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