Acyl carrier protein of *Azospirillum brasilense*: properties of the purified protein and sequencing of the corresponding gene, *acpP*

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Acyl carrier protein (ACP) plays a crucial role in bacterial fatty acid synthesis. Cloning genes encoding ACPs from Gram-negative bacteria in *Escherichia coli* is difficult due to adverse effects of the cloned gene on host cell viability, and we were unsuccessful in cloning the full length ACP gene (*acpP*) from *Azospirillum brasilense* using conventional methods. Therefore, ACP from *A. brasilense* was purified to homogeneity and a part of the *acpP* gene was cloned using the polymerase chain reaction (PCR) technique with two primers, one designed from the N-terminal amino acid sequence of the purified ACP and the other from the highly conserved amino acid sequence of bacterial ACPs. The nucleotide sequence of the gene was obtained by cloning and sequencing inverse PCR products containing the *acpP* region generated by two oppositely oriented internal primers designed from the partial *acpP* gene sequence using restriction-enzyme-digested, self-circularized chromosomal DNA fragments as templates. Characterization of the purified ACP and analysis of the derived amino acid sequence of the *acpP* gene of *A. brasilense* revealed that: (a) the mature ACP, composed of 78 amino acids, is a highly expressed protein (about $2.0\text{--}3.0 \times 10^4$ molecules per cell), (b) compared to *E. coli* ACP, it has a more compact structure and contains significantly more hydrophobic amino acid residues and (c) the potential mRNA sequence of the ACP gene has some structural features typical of a stable mRNA.

Keywords: *Azospirillum brasilense*, acyl carrier protein, reverse genetics, inverse PCR cloning, *acpP*

INTRODUCTION

Acyl carrier protein (ACP) plays a pivotal role as a cofactor in *de novo* fatty acid synthesis in bacteria and plant plastids. The *Escherichia coli* ACP (Magnuson *et al.*, 1993) is one of the most abundant proteins in the cells. In spite of the fact that ACP is a small protein (about 9 kDa) and the amino acid sequence of the ACP from *E. coli* was determined 28 years ago (Vanaman *et al.*, 1968), cloning of the gene encoding ACP from Gram-negative bacteria has proved to be very difficult. Only recently was the *acpP* gene from *E. coli* cloned (Rawlings & Cronan, 1992).

ACPs have been purified from a number of bacteria including two Gram-negative aerobic diazotrophs, *Rhodobacter sphaeroides* (Cooper *et al.*, 1987) and *Rhizobium meliloti* (Platt *et al.*, 1990), and two cyanobacteria (Froehlich *et al.*, 1990); amino acid sequences of these proteins were also determined. However, the only constitutively expressed ACP gene cloned from Gram-negative bacteria is that from *E. coli* (Rawlings & Cronan, 1992). We wanted to clone and sequence the gene encoding the ACP of *Azospirillum brasilense* to analyse the gene structure, which was expected to throw new light on ACP gene expression in a Gram-negative aerobic bacterium.

In this report we describe an approach based on reverse genetics that yielded the full-length sequence of *acpP*, the gene encoding ACP of *A. brasilense*, involving purification of ACP, determination of the N-terminal amino acid sequence of the protein to construct authentic, ACP-specific degenerate primers, partial cloning of the gene employing the polymerase chain reaction (PCR), and

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Abbreviation: ACP, acyl carrier protein.

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complete sequencing of the *acpP* gene with its flanking region using inverse PCR techniques.

METHODS

Bacterial strains and growth conditions. Growth and maintenance of *A. brasilense* RG, the strain used in this study, were described previously (Mukherjee & Ghosh, 1987). For purification of ACP from *A. brasilense* RG, the bacteria were grown at 32 °C in an 8 l fermentor with vigorous aeration in fructose-salt minimal medium nearly to stationary phase (OD_{590} 4.1). The cells were collected by centrifugation after washing with 0.02 M Tris/HCl (pH 8.0) and the packed cells (77 g) stored frozen at -20 °C until used. *E. coli* cells were grown at 37 °C in LB liquid medium (Sambrook *et al.*, 1989) in a rotary shaker, or on LB agar plates containing appropriate antibiotics when necessary at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 50; tetracycline, 20; streptomycin, 50; kanamycin, 25.

Assay of *A. brasilense* ACP. The assay procedure for *A. brasilense* ACP was adapted from the method of Rock & Cronan (1980). Acyl-ACP synthetase was purified from *E. coli* C-600 following the procedure of Rock & Cronan (1979), up to the final hydroxyapatite step. 1 μl of this preparation could synthesize 0.45 nmol palmitoyl-ACP min^{-1} under the standard conditions of assay. The reliability of the assay was checked with *E. coli* ACP (Sigma).

The ACP assay mixture (final vol. 20 μl) contained Tris/HCl (0.1 M, pH 8.0), LiCl (0.4 M), ATP (5 mM), MgCl_2 (10 mM), DTT (2 mM), Triton X-100 (2%, v/v), [^{14}C]palmitate [60 mCi mmol^{-1} (2.22 GBq mmol^{-1}), Amersham] (60 μM), 2 μl acyl-ACP synthetase preparation and cell extracts containing ACP. The reaction was initiated by adding acyl-ACP synthetase. After incubation at 37 °C for 30 min, the reaction mixtures were immediately cooled to 0 °C and 15 μl aliquots were spotted onto Whatman 3MM filter paper discs. The discs were air-dried, washed with three changes of CHCl_3 :methanol (1:2) to remove radiolabelled free fatty acid, air-dried and the radioactivity retained in the discs measured in a liquid scintillation counter. One activity unit was defined as that capable of forming 0.1 nmol [^{14}C]palmitoyl-ACP (equivalent to 13000 c.p.m.) under the assay conditions. Protein was determined by the method of Bradford (1976).

Purification of ACP of *A. brasilense*. Complete purification of ACP was achieved by a five-step procedure as shown in Table 1.

Briefly, 38.5 g frozen *A. brasilense* cells were thawed, mixed with 75 ml suspension buffer [100 mM Tris/HCl (pH 8.0), 100 mM glycine, 25 mM EDTA (pH 8.0), 14 mM 2-mercaptoethanol, 0.33% Triton X-100], lysed in the presence of 20 mg lysozyme at 37 °C for 30 min and the mixture homogenized by sonication (Braunsonic Sonicator, model 1510) to prepare the crude extracts. An equal volume of 2-propanol was added to the sonicated crude extracts and the 2-propanol supernatant fraction (150 ml) was purified by DEAE-Sephacel (Pharmacia) ion-exchange chromatography using increasing concentrations of NaCl in 20 mM Tris/HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol, first collecting the active fraction by batchwise elution and then subjecting it to gradient elution. The combined peak fractions eluting at about 0.26 M NaCl by gradient chromatography were dialysed against 1 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7.0), 14 mM 2-mercaptoethanol, freeze-dried, redissolved in dialysis buffer and then purified by a preparative native-PAGE treatment. Only the fastest running protein band in the native gel was associated with ACP activity. The unstained gel band containing ACP activity (after identification by parallel staining) was extracted with 0.6 M NaCl in 20 mM Tris/HCl (pH 7.5), the extract dialysed (preparative native-PAGE), freeze-dried and stored in a refrigerator. This freeze-dried preparation served as the source of pure ACP of *A. brasilense* (Fig. 1).

Determination of N-terminal amino acid sequence. The pure ACP preparation after SDS-PAGE was blot-transferred to a PVDF membrane following the method of Matsudaira (1987). N-terminal amino acid sequencing was performed with an automated microsequencer (Model 477A, Applied Biosystems).

Recombinant DNA techniques. Procedures for DNA manipulations, such as plasmid DNA purification, *in situ* colony hybridization, Southern hybridization, DNA band elution from low-melting-point agarose, cloning, subcloning, construction of a genomic library and recombinant plasmid DNA transformation into *E. coli*, were as described by Sambrook *et al.* (1989) with minor modifications. For the construction of a cosmid genomic library, pLAFR3 was used as vector and *E. coli* S17.1 was used as host (Chattopadhyay *et al.*, 1994). Total chromosomal DNA from *A. brasilense* was isolated following the technique of Marmur (1961); the isolated DNA was purified by RNase treatment and a phenol/chloroform extraction procedure. The method yielded high-molecular-mass DNA which was easily digested by all restriction enzymes tested.

Table 1. Purification of ACP from *A. brasilense*

Purification step	Total volume (ml)	Protein concentration (mg ml^{-1})	Total ACP (μg)	Purification (-fold)	Yield (%)
1. Crude extracts	100	37.70	2070*	1	100
2. 2-Propanol supernatant	200	2.89	—	—	—
3. DEAE-Sephacel-I (batch elution)	12	0.56	642	173	31
4. DEAE-Sephacel-II (gradient elution)	9	0.39	625	319	30
5. Preparative native PAGE	4.35	0.10†	435	1823	21

—, Not determined.

* Determination of ACP in the crude extracts was performed with the heat-treated soluble supernatant fraction.

† In the step 5 fraction, ovalbumin was used as a standard in protein determination instead of BSA.

Design of degenerate primers, amplification of a part of the ACP gene by PCR and cloning of the PCR product. Based on the available codon usage pattern of *A. brasilense* (Zimmer *et al.*, 1991), two degenerate oligonucleotides were chemically synthesized (Fig. 2a, b). One (named dgN) was deduced from the N-terminal amino acid sequence of the purified ACP of *A. brasilense* and the other (named dgC) was designed from the conserved amino acid sequence of ACPs of a few Gram-negative bacteria. The primer dgN is a 30-mer having 16-fold degeneracy with a *Bam*HI site at the 5' end, and the primer dgC is a 31-mer having 64-fold degeneracy with an *Eco*RI site at its 5'-end. PCR was carried out in a DNA thermal cycler (Perkin-Elmer-Cetus) using reaction conditions optimized according to Innis *et al.* (1990). The PCR products obtained were purified and digested with *Bam*HI and *Eco*RI. The digested products were ligated to the compatible restriction sites of a pUC18 vector and transformed into *E. coli* strain DH5 α (BRL). The desired transformants (pMG plasmids) with a 163 bp insert derived from the ACP gene (see Fig. 4) were identified by restriction enzyme analysis followed by sequencing.

Construction of a subgenomic library and screening for ACP gene clones. Total genomic DNA of *A. brasilense* was completely digested with different restriction enzymes: *Eco*RI, *Pst*I, *Sal*I, *Nar*I, *Apa*I, *Bss*HII and *Xma*I. The digested DNA fragments were subjected to gel electrophoresis, capillary-transferred onto a nylon membrane (Sigma) and UV-fixed. The partial *acpP* DNA (163 bp *Eco*RI–*Bam*HI DNA fragment from pMG) was radiolabelled with [α -³²P]dATP by nick translation. Southern blot experiments were carried out following the procedures of Singh & Jones (1984) hybridizing with the denatured radiolabelled probe at high stringency (in 4 $\times$ SET buffer with heparin at 72 °C for 18–20 h) followed by washing (1st wash in 2 $\times$ SSC+0.2% SDS at 55 °C for 10 min; 2nd wash in 0.1 $\times$ SSC+0.2% SDS at 50 °C for 10 min; 3rd wash in 0.1 $\times$ SSC at 45 °C for 10 min).

Based on Southern analysis data (Fig. 3a), a *Sal*I-digested subgenomic library was constructed with a pUC18 vector (Yanisch-Perron *et al.*, 1985) in *E. coli* DH5 α . The library was screened by *in situ* colony hybridization with the labelled 163 bp DNA fragment and the positive clones were further confirmed by purifying the recombinant plasmid DNA followed by hybridizing with the labelled probe.

Amplification and cloning of inverse PCR products to identify the full-length coding sequence of ACP. The inverse PCR method followed was as described by Triglia *et al.* (1988). A pair of divergent oligonucleotide primers, IP-21 and IP-22, were designed from the internal sequenced region (102 bp) of the *acpP* gene (shown in Fig. 4) in such a way that they annealed to the target DNA template 26 bp apart, and their 3'-termini were in opposite directions. *A. brasilense* genomic DNA was completely digested with individual restriction enzymes (*Apa*I, *Bss*HII, *Eco*RI, *Sal*I, *Sma*I and *Xma*I). After enzyme inactivation (65 °C, 5 min), the DNA fragments were precipitated from the digests and washed twice with 70% (v/v) ethanol, dried under vacuum, resuspended in ligation buffer at a low concentration of DNA [about 500 ng DNA fragments (ml buffer)⁻¹] and self-circularized by ligase treatment. After a pre-heating step (95 °C, 5 min), inverse PCR reactions were performed with the self-circularized DNA samples using the standard reaction mixture containing dNTPs, primers (IP-21 and IP-22), buffer and Vent DNA polymerase (New England Biolabs) in the thermocycler. Three inverse PCR products from *Apa*I, *Bss*HII and *Xma*I digests showing unique DNA bands in agarose gel (Fig. 3b) after purification were ligated to the *Sma*I site of the pUC18 vector and transformed into the *E. coli* host SURE (Stratagene) yielding plasmids pIGM-A, pIGM-B and pIGM-X, respect-

ively. The desired recombinant clones were checked by sequencing of the target DNA region.

Sequencing of DNA. Both strands of the target DNAs of PCR-, subgenomic- and inverse PCR clones were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using 7-deaza-dGTP to reduce the G/C band compressions arising from the high G+C content of the *A. brasilense* DNA. Sequencing reactions were performed with Sequenase version 2.0 (US Biochemicals) following the manufacturer's instructions except that the labelling and termination reactions were performed at 20 °C for 5 min and 45 °C for 15 min, respectively.

Computer analysis. DNA and protein sequence analyses were performed using selected programs of the University of Wisconsin Genetic Computer Group and PC/Gene (IntelliGenetics) software packages.

RESULTS

Purification of ACP from *A. brasilense*, and determination of its N-terminal amino acid sequence and its expression level in cells

As shown in Table 1, the five-step procedure starting from 38.5 g frozen *A. brasilense* cells yielded a pure preparation of ACP after 1800-fold purification with about 20% yield. Unlike ACP from *E. coli*, *A. brasilense* ACP did not show anomalous migration in SDS-PAGE (Fig. 1b, lanes 1 and 2) indicating its more compact structure. Although the most purified ACP preparation (preparative native-PAGE fraction) showed a single band in native PAGE (Fig. 1a, lane 1), in SDS-PAGE it clearly

Fig. 1. PAGE analysis of purified ACP from *E. coli* and *A. brasilense*. (a) Native-PAGE (15%, w/v, acrylamide), performed according to Jackowski & Rock (1983). Lanes: 1, *A. brasilense* ACP (preparative native-PAGE fraction); 2, *E. coli* ACP and BSA. (b) SDS-PAGE (15%, w/v, acrylamide), performed according to Laemmli (1970). Lanes: 1, *A. brasilense* ACP (preparative native-PAGE fraction); 2, *E. coli* ACP; 3, molecular mass markers.

(a) N-terminal amino acid sequence of *A. brasiliense* ACP

Minor upper gel band: S D V A E R V K K I V V D
 Major lower gel band: A D V A E R V K K I V V D

5'-CGGGATCC GT(C/G) GC(C/G) GAG CG(C/G) GT(C/G) AAG AAG A-3'
*Bam*HI

Degenerate primer dgN

(b) Conserved amino acid sequences of Gram-negative bacterial ACPs

E. coli : S L D T V E L V M A L E E E F D T E I P D E
R. meliloti : S L D T V E L V M A F E E E F G V E I P D D
R. sphaeroides : S L D T V E L V M A F E E E F G I E I P D D

3'-AC CG(G/C) AA(G/C) CT(C/T) CT(C/T) CT(C/T) AAG C(C/T)G CTTAAGGC 5'
*Eco*RI

Degenerate primer dgC

Fig. 2. Design of two degenerate oligonucleotide primers (dgN and dgC) for cloning and sequencing of a part of the ACP gene of *A. brasiliense*. (a) Nucleotide sequence of the N-terminal degenerate primer dgN with a *Bam*HI restriction site. (b) Nucleotide sequence of the C-terminal degenerate primer dgC with an *Eco*RI restriction site.

resolved into two closely spaced bands – a faster migrating major band and a slower migrating minor band (Fig. 1b, lane 1). The N-terminal amino acid sequences of the two polypeptides (Fig. 2a) were identical except for the N-terminal residues, which were Ser and Ala for the minor and the major polypeptides, respectively.

In the quantitative assay of ACP using *E. coli* acyl-ACP synthetase and radiolabelled [1-¹⁴C]palmitate, a linear response of [1-¹⁴C]palmitoyl-ACP synthesis was obtained in the range 50–200 pmol pure *A. brasiliense* ACP (results not shown). Under our assay conditions, approximately 0.20 pmol [1-¹⁴C]palmitoyl-ACP was formed per pmol pure *A. brasiliense* ACP compared to 0.25 pmol [1-¹⁴C]palmitoyl-ACP in the case of *E. coli* ACP within the same range. Since ACP is heat-stable, the concentration of ACP in the crude extracts was determined using heat-treated supernatant of the extracts. The number of ACP molecules per cell of *A. brasiliense* was calculated to be approximately $2.0\text{--}3.0 \times 10^4$ compared to $5\text{--}6 \times 10^4$ for *E. coli* ACP (Magnuson *et al.*, 1993). Any co-precipitation of the soluble ACP with heat-coagulated cellular protein was not tested for but would cause an underestimation of the calculated value. These results show that ACP is a highly expressed protein in *A. brasiliense*.

Cloning and sequencing of an internal part of the *acpP* gene using PCR

Analysis of three separate PCR-derived pMG plasmid clones (see Methods) showed that they all contained a 163 bp insert having an identical sequence in the 102 bp internal region flanked by 30-mer and 31-mer PCR primer sequences (Figs 2, 4). Since the two primers were degenerate, the nucleotide sequences of the three insert

Fig. 3. Southern hybridization of *A. brasiliense* genomic DNA fragments with the labelled 163 bp partial *acpP* gene probe, and amplification of the *acpP* locus by inverse PCR. E, *Eco*RI; P, *Pst*I; S, *Sal*I; N, *Nar*I; A, *Ap*aI; B, *Bss*HII; X, *Xma*I; M, molecular mass markers (*Dra*I-digested λ DNA). (a) Total chromosomal DNA digested with different restriction enzymes and Southern hybridization performed as described in Methods. (b) Inverse PCR products, obtained with different restriction-enzyme-digested, self-circularized chromosomal DNA fragments, and *acpP*-specific IP-21 and IP-22 primers, after gel electrophoresis and ethidium bromide staining.

DNAs of the three clones were slightly different (G replaced by C, C by G, and C by T, without altering the coded amino acids) in the region where the primers

GCTGGTCAACGCTGTAAAAATGCTTTATCGCAGGGGGCTTTTTGTGGCAGGAAAGCACGC 60

AGCCGCGTGTGAGCCGCCGAATTCGCCCCGAGCGCTCTCAAGGATTGGAAGGCTTTAAA 120

AAATGAGCGATGTCGCCGAGCGCGTTAAGAAGATCGTTGTGGACCACCTCGGGGTCGAGG 180

M S D V A E R V K K I V V D H L G V E E

IP-22 IP-21

AGTCGAAGGTGACGGAGAACGCCCTCCTTCATCGACGATCTCGGCGCCGACAGCCTCGACA 240

S K V T E N A S F I D D L G A D S L D T

CGGTCGAGCTGGTTATGGCTTTTCGAGGAAGAGTTCCGGCTGCGAGATTCCGGACGACGCCG 300

V E L V M A F E E E F G C E I P D D A A

CCGAGAAGATTTTGACGGTGAAGGACGCCATCGACTTCATCAAGGCGAACGCCGCCGCCCT 360

E K I L T V K D A I D F I K A N A A A *

GATCAGGCGCTGGCGTGAGAGGCCAGGCAGCTTTCCAAAGTGGCCTGGCCGTCGTTGAGT 420

TTTCCGAGGAAAGGTCAAGGAAT 443

Fig. 4. Nucleotide and deduced amino acid sequence of *A. brasilense acpP*. The nucleotide residues are numbered on the right. The potential ribosome-binding site is double-underlined. An asterisk denotes the stop codon. Two potential hair-pin structures, one around the stop codon and the other in the 3' flanking sequence, are indicated by inverted-arrow-headed dashed overlines. The *NarI* restriction site within the coding sequence is underlined. The T-rich sequence downstream of the putative transcription terminator is indicated by a dashed underline. An A-T rich region between the putative ribosome-binding site and the ATG start codon is shown in bold. Overhead vertical arrows at positions 153 and 256 delimit the core 102 bp region within the 163 bp fragment of *acpP* cloned in pMG. Overhead arrowed lines indicate the sequences used for constructing IP-21 and IP-22 primers.

annealed. The deduced 33 amino acid sequence corresponding to the 99 bp core region matched well with the known amino acid sequences in the same region of three bacterial ACPs, *R. meliloti*, *R. sphaeroides* and *E. coli* (28 out of 33, 29 out of 33 and 23 out of 33 identical amino acid residues, respectively) (alignment not shown).

Attempts to isolate a full-length ACP gene clone from a subgenomic library of *A. brasilense*

As shown in Fig. 3(a), each of the restriction enzyme digests of *A. brasiliense* DNA hybridized with a unique *acpP*-specific hybridizing band (except the *NarI* digest which has a restriction site within *acpP*). This suggests that the *acpP* gene may be present as a single copy in the *A. brasiliense* genome. In order to obtain a full-length ACP gene clone, a number of putative *acpP*-positive clones were selected from the *SalI*-digested subgenomic library (see Methods) for identification of the ACP gene by sequencing. It was observed, however, that none of these clones contained the full-length sequence of the ACP gene (all had a terminally deleted *acpP*). This was not unexpected as the full-length ACP gene, cloned in a high-copy plasmid, could be unstable in the *E. coli* host (Magnuson *et al.*, 1993).

Sequencing the *acpP* gene locus using inverse PCR

Inverse PCR with the two gene-specific primers, IP-21 and IP-22, yielded no amplified product in some cases (*Sma*I-, *Sma*I- and *Eco*RI-digested fragments), but in other cases (*Apa*I-, *Bss*HII- and *Xma*I-digested fragments) unique inverse PCR products with discrete sizes were obtained (Fig. 3b), that matched with the results of Southern experiments shown in Fig. 3(a) (about 1.6 kb for *Apa*I, 1.3 kb for *Bss*HII and 3 kb for *Xma*I). Partial sequencing of both strands in the target region of the cloned recombinant plasmids (pIGM-A, pIGM-B and pIGM-X), derived independently from inverse PCR products, showed identical nucleotide sequences flanking the 102 bp core *acpP* region (Fig. 4). This proved the reliability of the inverse PCR strategy for cloning and sequencing of the full-length *acpP* gene.

Analysis of the nucleotide sequence of *acpP* and the deduced primary structure of *A. brasilense* ACP

The complete nucleotide sequence (Fig. 4) of the *A. brasiliense acpP* gene was compiled from the overlapping sequences of the PCR and inverse PCR products, revealing an ORF encoding a polypeptide of 79 amino acid

residues. The G+C content in the third base of the codons of this ORF is 88.6 mol% which is in agreement with the previous report (Zimmer *et al.*, 1991) of high G+C content of other genes of this bacterium. It has the putative ribosome-binding site GAAGG at 10–14 nt upstream (Fig. 4, positions 109–113) from the ATG start codon. A probable transcription terminator region, 58 nt downstream from the stop codon, has a T-rich sequence preceded by a G+C-rich sequence with dyad symmetry (position 381–410 in Fig. 4). This could form a stable stem-loop structure once transcribed, and could be a rho-independent transcription terminator. No consensus sequences (–10, –35) for σ^{70} recognition sites could be identified upstream of the ATG start codon.

The deduced amino acid sequence (Fig. 4) reveals that the ACP has 79 amino acid residues with a high sequence homology with other bacterial ACPs (e.g. 81% and 68% identity with ACPs of *R. meliloti* and *E. coli*, respectively). The Ser residue predicted at position 2 differs from the Ala residue detected in the protein sequence of the major polypeptide. This suggests post-translational modification by methionyl aminopeptidase and covalent modification of the resulting N-terminal residue as has been observed previously (Hirel *et al.*, 1989; Kawakami & Ohmori, 1994). *A. brasilense* ACP contains a high number of hydrophobic, and more acidic than basic amino acid residues (35, 20 and 8 out of 79, respectively). The calculated molecular mass of the mature ACP of *A. brasilense* comprising 78 amino acid residues is 8.752 kDa (compared with 8.847 kDa for *E. coli* ACP), taking into account the addition of a 4' phosphopantetheine prosthetic group at Ser-36.

DISCUSSION

We found it almost impossible to clone the full-length ACP gene of *A. brasilense* in *E. coli*. A search for a clone containing the ACP gene in the library failed even when a homologous *acpP* gene fragment was used as a probe. Moreover, our attempts to isolate a full-length *acpP* clone from a pUC18-based subgenomic library of *A. brasilense* were unsuccessful. This is in contrast to the finding of Revill & Leaday (1991) who reported high-level expression of the *Saccharopolyspora erythraea* gene encoding ACP cloned in *E. coli*. However, cloning and sequencing of part of the *A. brasilense* ACP gene was achieved by determining the N-terminal amino acid sequence of purified ACP and using this sequence to construct *A. brasilense*-specific degenerate primers. The gene was then cloned by inverse PCR using primers based on the sequence determined from the initial PCR products.

The presence of two ACP protein bands having different N-terminal amino acid residues suggested the possibility of two ACPs of similar structure. However, when several independently derived clones were sequenced no evidence was found for more than one ACP gene. The nucleotide sequence of the 5' and 3' flanking region of the ACP gene revealed features suggestive of a stable ACP transcript. For example, in addition to the stem-loop structure of a typical rho-independent terminator at the 3' downstream

sequence, the second potential hair-pin structure around the stop codon (position 349–376, Fig. 4) could be a barrier to 3' endonucleolytic degradation (Higgins, 1991). The nine nucleotide A-T sequence between the start site and the proposed ribosome-binding site, and the A following the ATG, might also impart mRNA stability as suggested from a study of the highly stable *lpp* mRNA of *E. coli* by Nakamura *et al.* (1980). A stable transcript might be a contributory factor to the high level of expression of ACP.

The role of ACP in bacteria is an enigma. It is not only essential as a cofactor for *de novo* fatty acid biosynthesis in bacteria, but it (or its acyl derivative) also plays important roles in some essential and accessory cellular functions (Beyreuther *et al.*, 1978; Niki *et al.*, 1992; Shearman *et al.*, 1986; Therisod & Kennedy, 1987; Geiger *et al.*, 1991; Platt *et al.*, 1990). However, diverse functional roles of constitutive ACPs have not been investigated to any significant extent in bacteria other than enterobacteria.

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