The cloning and characterization of the *arom* gene of *Pneumocystis carinii*

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The *arom* gene, encoding a single polypeptide that catalyses five consecutive steps of the pre-chorismate aromatic amino acid biosynthetic pathway, has been cloned from the opportunistic pathogen *Pneumocystis carinii*. There is a single open reading frame of 4788 bp which includes an intron of 45 bp that does not introduce a stop codon into the sequence. Thus, the derived amino acid sequence consists of 1581 residues, which is highly homologous to all fungal AROM proteins studied to date. These data support the view that *P. carinii* is a fungus and imply that its aromatic amino acid biosynthesis is conventionally organized.

Introduction

Pneumocystis carinii is the pathogen responsible for a pneumonia which affects the immunosuppressed and is the principal cause of death amongst AIDS sufferers in Europe and the USA. Despite increased clinical interest little is known about the biology of the organism.

Recent evidence strongly suggests that *P. carinii* is a fungus (Edman *et al.*, 1988; Wakefield *et al.*, 1992; Ympa-Wong *et al.*, 1992), rather than a protozoon. Aromatic amino acid biosynthesis in the fungi is dependent on the pre-chorismate pathway. Steps 2 to 6 of this pathway are catalysed by a single pentafunctional protein called AROM which is encoded by a single gene (Fig. 1). The aromatic amino acid biosynthetic pathway is found in bacteria, plants and lower eukaryotes, but is absent from mammals.

The existence of a pentafunctional AROM protein has been demonstrated in *Neurospora crassa* (Giles *et al.*, 1967) and *Schizosaccharomyces pombe* (Strauss, 1979). The AROM encoding genes, *arom* and *ARO1*, have been cloned from *Aspergillus nidulans* and *Saccharomyces cerevisiae* respectively (Charles *et al.*, 1986; Duncan *et al.*, 1987). In plants, the five enzymes are found as three separate polypeptides and one bifunctional protein that contains the 3-dehydroquinate (DHQ) dehydratase and 3-dehydroshikimate (DHS) dehydrogenase activities. The corresponding enzymes from *E. coli* and other bacteria are monofunctional, encoded by separate genes dispersed around the genome (Bachmann, 1983). The fungal *arom* locus is thought to have arisen through gene fusions (Hawkins, 1987) as the protein is homologous with the five monofunctional bacterial enzymes, and is made up of functional domains which fold independently or semi-independently.

This paper describes the cloning and characterization of the *arom* gene from *P. carinii*. The strategy employed in cloning the gene used mixed primer polymerase chain reaction (PCR). The sequences of the mixed primers were derived from the back-translation of conserved blocks of amino acids from the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzymes from other organisms, with bias toward the fungal sequences.

Methods

Isolation of P. carinii DNA. *P. carinii* infection was induced in Sprague-Dawley rats by steroid immunosuppression (Wakefield *et al.*, 1988) and the organisms were purified from infected rat lung using the

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Abbreviations: DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase.

The GenBank accession number for the sequence reported in this paper is L18918.

Fig. 1. (a) Reactions of the early common (pre-chorismate) pathway of aromatic amino acid biosynthesis. The enzymes catalysing these steps are: (1) 3-deoxy-D-arabino-heptulosonic acid-7-phosphate synthase (EC 4.1.2.15); (2) 3-dehydroquinate synthase (EC 4.6.1.3); (3) 3-dehydroquinase (EC 4.2.1.10); (4) shikimate dehydrogenase (EC 1.1.1.25); (5) shikimate kinase (EC 2.7.1.71); (6) 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19); (7) chorismate synthase (EC 4.6.1.4). DAHP, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate. The AROM pentafunctional enzyme catalyses steps 2 to 6 of the pathway as shown. The gene symbols shown are from *E. coli*. (b) Use of chorismate in the biosynthesis of aromatic compounds.

method described by Peters *et al.* (1992). Rat-derived *P. carinii* material was digested with proteinase K (0.5 mg ml⁻¹) in the presence of 0.2% SDS at 50 °C for 16 h. Genomic DNA was isolated by phenol extraction followed by ethanol precipitation.

Oligonucleotides and PCR amplification. Two synthetic mixed oligonucleotides were made, AroPCR1 and AroPCR6, the sequences of which were derived from the back-translation of conserved amino acid motifs from the bacterial and plant EPSPS sequences and the EPSPS domains from the fungal AROM proteins. The sequence of AroPCR1 was derived from the peptide LGNAGTA (residues 496–502 from the *S. cerevisiae* AROM protein) and the nucleotide sequence was 5'-T¹TNGGNAAT¹GCNGGNACNGC (5' to 3' on the coding strand). The sequence of AroPCR6 was based on the peptide LMCAPYA (which is conserved in the fungi, residues 588–594 from *S. cerevisiae* AROM protein) and the nucleotide sequence was 5'-GC¹ATANGGNGC¹CA CATNA (5' to 3' on the non-coding strand). The final concentrations for the reagents in the amplification reactions were: 10 mM-Tris/HCl, 50 mM-KCl, 0.4 mM-dNTPs, 0.1 mg gelatin ml⁻¹, 0.025 units Taq DNA polymerase (Boehringer Mannheim) µl⁻¹ and the concentration of MgCl₂ was varied between 1.5 and 4 mM. The PCR reaction cycle consisted of: 94 °C for 1.5 min, 48 °C for 1.5 min and 72 °C for 2 min, and was repeated 40 times.

Two further oligonucleotides were made from specific *P. carinii* sequences. These were Aroseq1 (5'-GCACTTATAGAGGATTGGC) and Aro15 (5'-CATCAATAGAAACAATGTGAGC). The positions of these primers are illustrated in Fig. 2. The conditions for the PCR amplification cycles were as described previously and the concentration of MgCl₂ was 3 mM. These primers were used in PCR experiments in which the template was *P. carinii* genomic DNA and in other experiments in which a *P. carinii* cDNA library served as template. The cDNA library was obtained from Dr C. J. Delves (Department of Molecular Sciences, The Wellcome Laboratories, Beckenham, UK). The construction of this library was described by Dyer *et al.* (1992).

Resolution of PCR products by PAGE. A 6% (w/v) non-denaturing polyacrylamide gel (29:1, acrylamide/bisacrylamide) was used for separation of PCR products. DNA was visualized by staining the gel with ethidium bromide (1 µg ml⁻¹) and viewed under UV illumination. Candidate bands were excised from the gel and the DNA was extracted using the crush and soak method described by Sambrook *et al.* (1989). The DNA was then treated with Klenow DNA polymerase followed by T4 polynucleotide kinase to allow ligation into *EcoRV* digested and phosphatase treated pBluescriptII (Stratagene).

Sequencing. Recombinant plasmids were sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977) using T7 DNA

Fig. 2 (following three pages). Complete nucleotide sequence of the *P. carinii* *arom* gene and translation of the ORF into the amino acid sequence. The underlined motifs in the 5' untranslated regions show a potential TATA box (TATAA) and a ribosome binding site (GAAG) immediately before the ATG translation initiation codon. The underlined sequence in the 3' untranslated sequence may constitute a polyadenylation signal (AATAA). The region marked IVS indicates the nucleotides that represent an intron. The sequence from which the oligonucleotide Aroseq1 was derived is indicated (—→) and the sequence from which Aro15 was derived is also shown (←———). Aro15 was from the anti-sense strand. The sequence between -233 and -225 is potentially a GCN4 binding site.

GCN4

ttattataaatataaattattttaaacacacatctgactaaagtatttttaaacagaagtattaatcaaaatatcattttaaaaaaattact -174
 aaaaataaaaaatagtaaaataataataatataataatttttccaaagatttattataagttatatttaaactcataagagtgcatattat -84
 aaatttaagcataaaagactttctcatctaaaaatttgagaaaatacataaattattcatattaaactgctttcttaaatgaaggaATGAAG 6
M K 2

GAAATATCAAACTTAGTATTTTAGGGAAAGATTCTATTCATATTGGGCTTCACTTATGGCCACATATAACTAATGAACATTATTACATGT 96
 E I I K L S I L G K D S I H I G L H L W P H I T N E L F T C 32

ATATTTTCTCCAACATATGTAATCATTACGGATTCAAATATTGAAACCTTATATATTCCTAGTTTCAAAACATATTTTCAATTTCTATGGCA 186
 I F S P T Y V I I T D S N I E T L Y I P S F K T Y F I S M A 62

Aroseq1

AAACAAAGATCTATTAATTCTCGTCTTTTATTTTACAATTCCTCTGGAGAAAAATCAAAATCAAGAAAAACAAAGCACTTATAGAG 276
 K Q R S I N S R L L F F T I P P G E K S K S R K T K A L I E 92

GATTGGCTTTTATCAGAAAAATGCACAAGAGATACTGTTATAATAGCTATAGGGGGCGGTGTTATAGGCGATCTCGTAGGTTATGTCTCT 366
 D W L L S E K C T R D T V I I A I G G G V I G D L V G Y V S 122

GCAACATTTATGAGAGGAGTTCTGTTTATTCAAATTCCTACAACCTCTTTTAGCTATGGTAGATTTCATCTATTGGAGGGAAAAATTCCTATA 456
 A T F M R G V R F I Q I P T T L L A M V D S S I G G K N S I 152

AATACATCTTATGGAAAAATGCAATTGGTACTATTGGCAACCGGAAAGAATTTTATTGATTTTACATTTCTTGAAACACTTACAGAA 546
 N T S Y G K N A I G T I W Q P E R I F I D F T F L E T L T E 182

AAAGAATTTATAAATGGTATAGCAGAATAATCAAGgttataaaaaataaattaatttttatataatttttaatttccagACTATTATT 636
 K E F I N G I A E L I K ◀IVS..... ▶ T I I 197

ATATGGGATGAATCAGAATTTGCATCTCTAGAAAACATTTTCAAGAAAAATTCGGAACAGTGAGATCAATGTCATTAACATCTAATAAA 726
 I W D E S E F A S L E N I S E K I A K T V R S M S L T S N K 227

Aro15

CATTCAAAATTTAATGAAATATAAAAGATCTTAAAGATATATTATATCTTCGATAAAAAATTAAGCTCACATTGTTTCTATTGATGAA 816
 H S K F N E I I K D L K R Y I I S S I K I K A E I V S I D E 257

AAAGAAAAAGATCTTAGAACTCTTTTAAATTTTGGCCATTCTATAGACATGCAATAGAAACAGTTCTAGCTCCTTATATTCTTCATGGA 906
 K E K D L R T L L N F G H S I G E A I E T V L A P Y I L E G 387

GAATCTATTTCTATTGGAATGGTCAAAGAAGCAGAGCTTTCTAGGCATTTAGGTATACTAAATCCTAATGTAGTATCACGACTCATCAA 996
 E S I S I G M V K E A E L S R H L G I L N P N V V S R L I K 317

TGTTTAAACACATGGGGTTTACCTACATCTTTTAAAGATAGGCGATTAAAGAAGTAATTTTGGGAAAAAACATTTAATAGAAGATATC 1086
 C L N T W G L P T S F K D R R F K E V I L G K K H L I E D I 347

CTTGAAATATGCTCTATAGACAAAAAAATGATAGTAATAATAAAAAATAGTCCTTCTTTTCAAGCAATAGGAAAAACATATGAAAAAAA 1176
 L E I M S I D K K N D S N N K K I V L L S A I G K T Y E K K 377

GCATCCTCAGTATCTGACGATGATATTAGAACTATTTTATCACAATAATTCTTTTATATGGTATTCCATTAAATGCATTTCAAAAACAC 1266
 A S S V S D D D I R T I L S Q N I L L Y G I P L N A F Q K H 407

ACTACAATTACTCTTCCAGGATCTAAGTCTATATCCAATAGAGCATTAATTTTAGCTTCACTTTCTAACGGAATTTGTTATTTAAAAAAT 1356
 T T I T L P G S K S I S N R A L I L A S L S N G I C Y L K N 437

TTTTTGCAATTCAGATGATACATATTACATGCTTTCTGCTTTGGAAAACTAAATGCAGCAGAATTCAAATGGGAACAAGATGGAGATGTA 1446
 F L H S D D T Y Y M L S A L E K L N A A E F K W E Q D G D V 467

TTAGTCGTTAAAGGTAAATCAGGATATCTTGAAAACCCCTCAAATGGAATTATATCTGGGAAATTCAGGAACAACAGCTAGATTTTAAACC 1536
 L V V K G K S G Y L E N P Q M E L Y L G N S G T T A R F L T 497

AGTATCTGTACACTAGTACAACCTAATTCACGTGAAAAATCATTTAATACTGACAGGAGTAATCGTATGAAGCAGAGACCTATCGGCCCT 1626
 S I C T L V Q P N S R E N H L I L T G S N R M K Q R P I G P 527

TTAGTAGATGCTCTTAAAAATAATGGATGTTGTATAGAATATCTTGAGTTAGAAAAATGCTTACCATTATTAAATTAACCAAAAAGAAATA 1716
 L V D A L K N N G C C I E Y L E L E N C L P L L I K P K E I 557

GGGCTTTATGGGGGAATATCAATCTTTCCGCTACTGTCTCATCCCAATATGTATCATCAATTTTAAATGTGTTCCCATATGCAAAAAACA 1806
 G L Y G G N I N L S A T V S S Q Y V S S I L M C S P Y A K T 587

CAAGTAACTCTCTCATTAATTGGAGGAAAACCAATATCTCAACCCTATATAGATATGACTATTTCTATGATGTCATCATTTGGTATTAAA Q V T L S L I G G K P I S Q P Y I D M T I S M M S S F G I K	1896 617
GTAACCAGATCTCATTTCAAAGGAAAATACATATTATATTTCCAAAAGGATGTTACACCTGTCCCTCTGAATATATAAATTGAAGGCGATGCA V T R S H S K E N T Y Y I P K G C Y T C P S E Y I I E G D A	1986 647
ACTTCAGCTACTTATCCTTTAGCAATTGCTGCAATAACGGGTGGAAGCTGCACTATATCTAATGTTGGTAGTGCTTCATTACAAGGAGAC T S A T Y P L A I A A I T G G S C T I S N V G S A S L Q G D	2076 677
TCTAAATTTTTCAGAATATATATTAAACCTATGGGATGTGAAGTGGTCCAATCGCCTACAACCTACATATATAAAGGGCCCTCCAAAAGGG S K F S E Y I L K P M G C E V V Q S P T T T Y I K G P P K G	2166 707
AAACTCAAATCATTAGGGTCTATAAATATGGAATCAATGACTGATACCTTTTTAACAGCAGCAGTTTGTAGCCTCTGTGCTTATGAAGAG K L K S L G S I N M E S M T D T F L T A A V L A S V A Y E E	2256 737
TCAAAACCATATGTTACAAAATACACGGGATTTCTAATCAAAGAATTAAAGAATGCAATAGAATCAATGCTATGGTTTGTGAACCTAAA S K P Y V T K I T G I S N Q R I K E C N R I N A M V C E L K	2346 767
AAATTTGGAATAGAAGCAGGTGAACCTCCAGATGGAATATATGTAAGCATTGAATACTTCCAATCTCCATATTAGTAGAAGGAATA K F G I E A G E L P D G I Y V K A L N T S N L P Y S V E G I	2436 797
AATTGCTATAATGATCACAGAATTGCAATGAGTTTGTAGTGTGTTAGCATGCATCTCTTCCAAACCACTACTATTCTAGATAAAGCTTGT N C Y N D H R I A M S F S V L A C I S S K P T T I L D K A C	2526 827
GTCAATAAAACCTGGCCTTATTGGTGGGATATTTTAAATTCACCTTTTAAAGTCAAATGAAAGGAATTGAATTGCATCTAAACCTACA V N K T W P Y W W D I L N S T F K V Q M K G I E F D L N P T	2616 857
ATCAATTCATCTATATTGCATCATCCATCAGAATGTACTATTTTTCTTATTGGAATGAGAGGAGCTGGGAAAACAACCTTTAGGACAGCTT I N S S I L H E P S E C T I F L I G M R G A G K T T L G Q L	2706 887
GCAGCTAACTTTCTTGGCGGGAATTTATTGACTTAGACTCAATTATTGAAGAAGATATTAAAAACAACCTAGAAATTCATTCAAAAA A A N F L G R E F I D L D S I I E E D I K T T I L E F I Q K	2796 917
TATAGTTGGGATGTTTTTGAAGAAAAGAACTACATTTTCTTAAACCTCTTTTAAACAGAAAAAAGAAAACCTATATTATATCTTGTGGA Y S W D V F R R K E L H F L K T L L T E K K E N Y I I S C G	2886 947
GGTGGCATTATTGAACTGAAGAAGCTAGAGATCTCTTAATATCTTATATAGAAGCTAATGGAATTGTTATACATATTCATCGTAATATT G G I I E T E E A R D L L I S Y I E A N G I V I E I H R N I	2976 977
CAGGATATTATTAAATATTTAAATACTGACAAAACACGTGCTCCTTATCAAGATAATATAATGCATGTATGGGAAAGACGAAAAGATGG Q D I I K Y L N T D K T R A P Y Q D N I M H V W E R R K R W	3066 1007
TATAATTTATGCAGTTCTCATCAATTTACATGACTTCAACAGATCCTACAGAATTTAAAAAATAATACCGTTAAATCTTAAATCCTCC Y N L C S S E Q F H M T S T D P T E F K K N I P L N L K S S	3156 1037
TTTAATAATTTTAAAGAACAAATTACAGGAAAAATAATATATTTTCTCTAATTTTGAAGAAAAAAGAAAGTTATTTTATCTTTAGCA F N N F L R T I T G K N N I F S L I L K K K R S Y F L S L A	3246 1067
TTTTCTGACCTTGAACATTTTCTTTTACTTGATATTATAACAGCTGGATGTGATGCAATTGAGATAAGAATCGATTTATTTCAAAAA F S D L E N I F S L L D I I T A G C D A I E I R I D L F Q K	3336 1097
CCTGAGGAAATTGACAAATATCCAAGCTTAGAATATATAGCAGAGAAAATATTTCTACTTCGTCAAAAACTAGTCTTCTTTGATTAT P E E I D K Y P S L E Y I A E K I F L L R Q K T S L P L I Y	3426 1127
ACGTTGCGTACAACGAATCATGGAGGATCATTTCTTTCTTGAAAAAAATAGCGAAGAAATATATATTACATGGTGCAAAATGGGGA T L R T T N H G G S F L S S E K K L A K E Y I L H G A K W G	3516 1157
TTCCGAATTTCTTGATATAGAATTAGATATAGCATCTGAATTATTTAAGACCATAAATACTCTTGGCCATATACAAAGATAATTGCATCT F E F L D I E L D I A S E L F K T I N N S W P Y T K I I A S	3606 1187
TATCATAATATAGAAAAACCAATATCTTGCGATGATTTTGAATGGATACAAAAGTACAAAGAAGCTCAACACTACGGTCACATTATAAAA Y H N I E K P I S C D D F E W I Q K Y K E A Q H Y G H I I K	3696 1217
TTAGTGGGAACTTCATCATCAATTGAAGATAATTTTTCTTAGAAGAATTTAAAAGCAAAATTTATTAATAAAAAAGTACCTTCAATAATA L V G T S S S I E D N F F L E E F K S K F I N K K V P S I I	3786 1247
ATAAACACAGGCATAAAAGGCCAATTATCTAGAATAATGAATACATTTTATGACGCCTGTACTCATCCATCGCTACCATCCAAAATAGCA I N T G I K G Q L S R I M N T F M T P V T H P S L P S K I A	3876 1277

CCTGGACAACATCAATTAAGAAATTAACACTGCTCTTCATATAATGGGTCTTCTCCAGAAAAAATATTTCTTTTGGAAAACCT	3966
P G Q L S I K E I N T A L E I M G L L P E K K Y F L F G K P	1307
ATTAAGCATTCTCAATCACCTAATATTCATAATTTAGGTTTGAATTTAGGTCTTCCATATAAATCAATTGTTTGAACAGATTCT	4056
I K H S Q S P N I H N L G F E I L G L P Y K Y Q L F E T D S	1337
ATTAGTGAACCTTAAAGAAATCTTACATTTAGAAGAATTCGGGGGAGCTTCTGTTACTATTCCACTAAAAACAAATATCAATTTTATTA	4146
I S E L K E I L H L E E F G G A S V T I P L K T N I S I L L	1367
GATGAAATATCAGATCATGCAGCATTAATTTGGTTCTGTTAATACTATAACAAGACGTATAATAATGGACAATATATTCTTAAAGGTGAA	4236
D E I S D H A A L I G S V N T I T R T Y N N G Q Y I L K G E	1397
AATACGGATTGGCAAGGAATAATAAGCAATAAAGAATTTTAACAAATTTGAAAAATCCTTTGAAAAATTTCTCAGGGTTTATAATTGGA	4326
N T D W Q G I I K A I K N F N K F E K S F E N F S G F I I G	1427
GCAGGAGGAGCAAGTAGGGCAGCTATATATGCATTACTTTCTCTTGGAAATATCTCCAATTTATCTTATAAATCGATCTAAAGATAAACTA	4416
A G G A S R A A I Y A L L S L G I S P I Y L I N R S K D K L	1457
AATAAATATATCATTTTAAAAACAAATCACATAATTTCCCATACAGAATATCATGAATTAATAATATAAATTTTGATATTGGAATA	4506
N K L Y E F F N T N H I I P I T E Y H E L N N I N F D I R I	1487
GGAATTTCTACAATACCAACAGATAATCCAATAGATCCTTCTGTTCTAGAAATAGCAAAAAATTTCTTTAATTTAAAAAGAAAATCATCA	4596
G I S T I P T D N P I D P S V L E I A K I F F N L K R K S S	1517
GAAGGTATATTTTATAGATATGGCATATGGATCAAAATACACAGACCTTACTATTATTGCAAAAGCGTCAATTGGAAAAATTATACATGGT	4686
E G I F L D M A Y G S N T T D L T I I A K A C N W K I I H G	1547
TTAGAAATTTTGCTAGAACAGGCTCAGAACAATTTTATTATGGACCGAAACATATATCCCTTATAATCAAGTGAATATGCTATATTA	4776
L E I L L E Q G S E Q P L L W T E T Y I P Y N Q V K Y A I L	1577
GGACCAAAACAAATAaccgttatTTtagacaagtataaattcaaaacaaatccacgattttcttcaa	4842
G P N K *	1581

polymerase (Pharmacia) and the protocol described by Tabor & Richardson (1987). Single stranded template was generated using alkaline denaturation (Chen & Seeburg, 1985). The University of Wisconsin Genetics Computer Group (UWCGC) programs (Devereux *et al.*, 1984) were used in the analysis of sequence data.

Construction of a rat-derived *P. carinii* genomic library. *P. carinii* genomic DNA was partially digested with the restriction enzyme *Mbo*I and fragments between 23 and 9 kbp were size selected following electrophoresis on a 0.7% agarose gel (SeaPlaque-GTG from FMC). The gel slice was treated with agarase (Boehringer) to digest the agarose, releasing the *Mbo*I fragments which were then ligated into phosphatase treated λ EMBL3 arms which had been prepared by digestion with *Bam*HI. The ligation was packaged using the Packagene (Promega) system followed by transfection into the *E. coli* host strain LE392. A total of 1.5×10^4 plaques were screened by hybridization using the 300 bp cloned section of the putative *P. carinii* arom locus.

Pulsed field gel electrophoresis. Agarose gel blocks were prepared by adding *P. carinii* organisms (a mixture of cysts and trophozoites) in 1 ml of phosphate-buffered saline (PBS; 0.15 M-NaCl, 0.016 M- Na_2HPO_4 , 0.004 M- NaH_2PO_4 , pH 7.3) to 1 ml of molten agarose (2%, w/v, SeaPlaque-GTG in 125 mM-EDTA). The blocks were cast in a Bio-Rad block former. Solidified blocks were incubated in 500 mM-EDTA (pH 8), 1% *N*-laurylsarcosine and 1 mg proteinase K ml^{-1} at 50 °C for 48 h. Pulsed field gel electrophoresis was performed using a CHEF (contour clamped homogeneous electric field) DRII (Bio-Rad) system. *P. carinii* chromosomes were separated by electrophoresis on a 0.9% SeaKem-GTG (FMC) in $0.5 \times \text{TBE}$ ($1 \times \text{TBE}$ is 0.089 M-Tris base, 0.089 M-boric acid, 0.002 M-EDTA) for 48 h at 180 V in $0.5 \times \text{TBE}$ with an initial switching time of 10 s increasing to a final switching time of 40 s. Gels were stained with ethidium bromide and photographed under UV illumination. Transfer to nylon membrane was preceded by depurination in 0.25 M-HCl for 30 min, followed by denaturation and

neutralization as described by Sambrook *et al.* (1989). DNA hybridization was carried out in 25% (v/v) formamide at 42 °C and the filter was then washed at 55 °C in $1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7) and 0.1% SDS.

In situ hybridization. A probe which consisted of the cloned EPSPS coding region from *A. nidulans* (a gift from Dr A. R. Hawkins, University of Newcastle upon Tyne, UK) was labelled with biotin 16-dUTP (Boehringer) using the random prime method (Amersham Multiprime kit). A cloned section of the putative *P. carinii* arom locus was labelled with biotin 16-dUTP by use of PCR, according to the method of Emanuel (1991). Sections were taken from a formalin fixed, paraffin embedded *P. carinii* infected rat lung onto silane coated slides. The sections were treated with proteinase K (0.5 mg ml^{-1}) for 30 min at 37 °C to expose the DNA in the sample. The hybridization and subsequent detection were carried as described by Burns *et al.* (1987). The stringent wash was $1 \times \text{SSC}$ at 55 °C.

Results and Discussion

PCR amplification using mixed oligonucleotides

The mixed oligonucleotide primers AroPCR1 and AroPCR6, derived from blocks of highly conserved amino acids from EPSPS sequences, were used to amplify a section of the *P. carinii* gene coding for EPSPS from genomic DNA. The high level of degeneracy of the primers and the contamination of *P. carinii* DNA template with rat DNA resulted in the generation of numerous products around the expected size (about 300 bp) as determined by agarose gel electrophoresis.

Thus, a 6% polyacrylamide gel was run to enable discrete bands in the 300 bp size range to be resolved. DNA from these bands was cloned and sequenced. The deduced amino acid sequence of one of the cloned products was very similar to published EPSPS sequences in the region between the sites of the mixed primers.

*Isolation of a bacteriophage λ clone carrying the *P. carinii* *arom* locus*

About 1.5×10^4 plaques from an unamplified *P. carinii* genomic library constructed in λ EMBL3 were screened with the cloned portion of the *P. carinii* EPSPS coding gene generated by the mixed oligonucleotide PCR. One plaque gave a positive hybridization signal. This plaque was purified and replated, and the progeny phage were again positive after hybridization. One of the resultant plaques was picked, named λ Pcaro, and the DNA was extracted for further analysis. Digestion of λ Pcaro with *Sal*I released an insert of about 12 kb which was gel purified then digested with *Xba*I generating fragments which were cloned into pBluescriptII. One of these subclones contained a 6.5 kb insert and was named pSB30 and another, pSB31, contained a 5.5 kb insert, the result of incomplete digestion with *Xba*I.

*Sequencing of the *P. carinii* *arom* locus*

The insert of pSB31, which hybridized to the 300 bp cloned section of the EPSPS gene, was sequenced entirely on both strands, revealing an open reading frame (ORF) of about 4 kb with the N-terminal region of the putative protein absent. The deduced amino acid sequence from this ORF was homologous to the AROM proteins of *S. cerevisiae* and *A. nidulans*, and by analogy with these sequences it was determined that about 700 bp of coding sequence was missing from the 5' end of the locus. A section of pSB30 was sequenced and a putative ATG initiation codon was identified. Amplification from *P. carinii* genomic template with the primers Aroseq1 and Aro15 generated a ~550 bp product which was cloned and sequenced, showing that the inserts of pSB30 and pSB31 were contiguous. The complete nucleotide sequence of the *P. carinii* *arom* locus and the deduced amino acid sequence are shown in Fig. 2. The ORF

appears to consist of 4788 bp of nucleotide sequence coding for a peptide of 1596 residues. However, examination of the alignment of the amino acid sequence of the *P. carinii* protein with those of *S. cerevisiae* and *A. nidulans* and a composite of the sequences of the five monofunctional enzymes from *E. coli* (Fig. 3) indicated that an intron of 45 bp could have been present in the DHQ synthase domain. This was confirmed experimentally through amplification from cDNA template using the primers Aroseq1 and Aro15, resulting in a product of ~500 bp which was cloned and sequenced. The dinucleotide GT (nucleotide positions 583–584) is at the 5' splice site within the intron and AG (nucleotide positions 626–627) is at the 3' border. There is a polypyrimidine tract near the 3' end of the intron and a sequence (YTRAT) that has been identified as a putative lariat forming motif in other *P. carinii* intervening sequences (Zhang & Stringer, 1993) is present. The occurrence of small introns has been reported in other *P. carinii* genes (Edman *et al.*, 1989b; Volpe *et al.*, 1992).

The coding sequence of the *arom* locus is highly AT rich (70.5%) and 85% of the codons contain an A or a T in the third position. These data agree well with the base composition from other *P. carinii* genes that have been sequenced (Edman *et al.*, 1989a; Kovacs *et al.*, 1993). The 5' untranslated region contains the sequence TATAA (located at –86 to –82 relative to the 5' methionine codon), which is a good match for the consensus TATA box. There is also a possible ribosome binding site GAAG at nucleotide positions –5 to –2. At nucleotides 4810–4814 the sequence AATAA is a close match for the consensus polyadenylation signal. A further feature of the nucleotide sequence is the presence of a putative GCN4 binding site about 230 bp upstream of the 5' methionine. The optimum sequence for this motif, usually located at 100–600 bp upstream of the transcriptional start site, is the palindrome ATGA(C/G)TCAT, but the sequence found upstream of the *P. carinii* *arom* locus (CTGACTAAA) shows conservation of the essential bases. The binding of GCN4 to its target sites mediates the 'general amino acid control' mechanism of *S. cerevisiae* in which starvation for a particular amino acid can lead to the up-regulation of expression of more than 30 genes involved in amino acid biosynthesis (Fink, 1986). The *ARO1* (AROM encoding) locus of *S.*

Fig. 3 (following three pages). Comparison of the amino acid sequences from the *A. nidulans* (Anarom), *S. cerevisiae* (Scarom), and *P. carinii* (Pcarom) AROM enzymes and also a composite of the *E. coli* monofunctional enzymes which have been arranged in the following order: DHQ synthase (residues 1–363) (Millar & Coggins, 1986); EPSP synthase (364–789) (Duncan *et al.*, 1984); shikimate kinase (790–963) (Millar *et al.*, 1986); DHQ dehydratase (964–1215) and shikimate dehydrogenase (1216–1487) (Anton & Coggins, 1988). The sequence of DHQ dehydratase has been amended from that originally published (Duncan *et al.*, 1986) following the corrections specified by Chaudhuri *et al.* (1991). The sequence of the *A. nidulans* *qutR* protein (Hawkins *et al.*, 1992) is also shown in the alignment (labelled Anqutr).

Pcarom	MKEIKIKLSILGKDSIHIGLHLWPH-ITNELFTCIFFS- - - - -PTTYVIIT	42
Anarom	MSNPPTKIKSILGRESIIADFGGLWRNYVAKDLISDCSS- - - - -TTYVLVT	43
Scarom	MVQLAKIVPILGNDIIVHVGYNHHDHLV-ETIIKHCPSS- - - - -STTYVICN	42
Ecara	- - - - -MERIVVTLGERSYPITIASGLFNEPASAFLPLKKSGEQVMLVT	41
Anqutr	- - - - -	0
Pcarom	DSNIETLYIIPSFKTYFISMAKQRSINSRRLFFFTIPPGEKSKSRKTKALIE	92
Anarom	DTNIGSIYTPSPFEEAFRRKRAAEITPSPRLLIYNRPPGEVSKSRQTKADIE	93
Scarom	DTNLSKV- -PYQQQLVLEFKASLPEGSRLLTYVVKPGETSKSRRETKAQLE	90
Ecara	NETLAPLYLDKVRGVLEQ- -AGVNVDSVIL- - - - -PDGEQYKSLAVLDTVF	85
Anqutr	- - - - -	0
Pcarom	DWLLSEK- -CTRDTVIIIAIGGGVIGDLVGVVVSATFMRGVRFIOIPTTLLA	140
Anarom	DWMLSQNPPCGRDTVVIALGGGVIGDLTGFAVASTYMRGVRYVQVPTTLLA	143
Scarom	DYLLVE- -GCTRDTVMVVIAIGGGVIGDMLIGFAVASTFMRGVRYVQVPTSLA	138
Ecara	TALL- -QKPHGRDRTLVAIGGGVIGDLTGFAAASVQRGVRFIOVPTTLLS	133
Anqutr	- - - - -	0
Pcarom	MVDSSSIGGKNSINTSYGKNATIGTIWQPERIFIDFTFLETLTTEKEFINGIA	190
Anarom	MVDSSSIGGKTAIDITPLGKNLIGAIWQPTKIYIDLEFLETLPVREFINGMA	193
Scarom	MVDSSSIGGKTAIDITPLGKNFAGAFVQPKFVLVDIKWLETLAKREFINGMA	188
Ecara	QVDSSSVGGKTAVNHPPLGKNMIGAFYQPASVVDLDCLEKTLPPRELASGLA	183
Anqutr	- - - - -	0
Pcarom	ELIKTTIIIIWDESEFASLENISEKIAKTVRS- - - - -MSLTSNKHHS	229
Anarom	EVIKTTAAISSSEEEFTALEENNAETILKAVR- - - - -REVTTPGEH-	230
Scarom	EVIKTACIWNADEFTRLLESNASLFLNVVNGAKNVKVNTNQLTNEIDEISNT	238
Ecara	EVIKYGIILDGAFFFNWLEENLDALLRLDGP- - - - -	214
Anqutr	- - - - -	0
Pcarom	KFNEI IKDLKRYIISSIKIKAHIVS IDEKEKDLRTLLNF GHSSIGHAIEITV	279
Anarom	RFEGETEELKARILASARHKAYVVVSADDEREGGLRNLLNFWGHSSIGHAIEAI	280
Scarom	DEAMLDHTYKLVLESIKVKAEVVSADDERESSLRNLLNFWGHSSIGHAYEAI	288
Ecara	- - - - -MAYCIRRCCELKAEVVAADDERETGLRALNLLGHSTFGHAIEAE	256
Anqutr	- - - - -	0
Pcarom	LA- -PYILHGESISIGMVKEAEELSRHLGLIDNPNVVSRLIKCLNTWGLPFTSF	328
Anarom	LT- -PQILHGECEVAIGMVKEAEELARHLGLIKGVAVSRIVKCLAAAYGLPFTSL	329
Scarom	LT- -PQALHGECEVSIIGMVKEAEELSRYPFGILSPTQVAVRLSKILVAYGLPFTSP	337
Ecara	HGYGNWLHGEAAVAAAGMVMAARTSERLGQFSSAETQRIITLLKRAAGLP- - -	303
Anqutr	- - - - -	0
Pcarom	KDRRFKEVILGKKHLLIEDILEIMSIDKKNDSSNNKKIVLLSAIGKTYEKKKA	378
Anarom	KDARIRKLTAGKKHCSPLDQLMFNMALDKKNDGPKKKIVLLSAIGTPYETRA	379
Scarom	DEKWFKELTTHKKTPLDILKKKMSIDKKNEGSKKKVVLLESIIGKCYGDSAA	387
Ecara	- - - - -VNGPREMSAQAYLPHMLRDKKVLAGEMRLILPLAIGKS-EVRS	345
Anqutr	- - - - -	0
Pcarom	SSVVSDDDIRTTLSQNILLYGIPLNAFQK-HTTITLPGSKSISNRALILAS	427
Anarom	SVVANEDIRVVLLAPSIEVH- -PGVAHSS-NVICAPPGSKSISNRALVLAAS	426
Scarom	QFVVSDEDLRFITLTDETLVYPFKDIPADQ-QKVVIIPGSKSISNRALILAA	436
Ecara	GVSHELVLNAIAADCQSAMESLTLOPIARVDGTINLPGSKTVSNRALLLAAS	395
Anqutr	- - - - -	0
Pcarom	LSNGICCYLKNFLHSDDDTYYMLSALEKLNAAAEFKWEQDGDVLVVKGKSGG-Y	476
Anarom	LGSIGTCRIKNLLHSDDDTEVMNLNALERLGAATFSWEEEGEVLVVNGKGG-N	475
Scarom	LEGEGQCKIKNLLHSDDDTKHMLTAVHELKGAATISWEDNGETLVVVEGHGGST	486
Ecara	LAHGKTVLTNLLDSDDVVRHMLNALTAL-GVSYTSLSADRTRCEIIGNGGPL	444
Anqutr	- - - - -	0
Pcarom	LENPQMBLYLGNSGTTARFLTSTICTLVQPNSSRENHLILTGSNRMKQRPIG	526
Anarom	LQASSSPLYLGNAAGTASRFLTSTVATLANSSSTVDS- -VLTGNNRMKQRPIG	524
Scarom	LSACADPLLYLGNAAGTASRFLTSLAALVNSTSSQKYIVLTGNARMQORPIA	536
Ecara	HAEGALELFLGNAAGTAMRPLAALCLGS- - - - -NDIIVLTGEPRMKKEPPIG	489
Anqutr	- - - - -	0
Pcarom	PLVDALKNNGCCTIEYLELENCLPLLIKPKKEIGLYGGNINLSATVSSQYVVS	576
Anarom	PLVDALTANVLPNTSKGRASLPLKIAASG-GFAGGNINLAAKVVSSQYVVS	573
Scarom	PLVDSILRANGTKTEYLLNNGSLPLIKVYTDSDV-FKGGRIELAAKVVSSQYVVS	585
Ecara	HLVDALRLGGGAKITYLEQENYFPLRLQG- - -GFTGGNVDDVDSVSSSQFLT	536
Anqutr	- - - - -	0

Pcarom	S I L M C A S P Y A K T Q V T L S L I G G K P I S Q P Y I D M T I S M M S S F G I K V T R S H S K E N	676
Anarom	S I L M C A P Y A K E P V T L L A L V G G K P I S Q P Y I D M T I T A M M E K S F G I D V Q K S T T E E H	623
Scarom	S I L M C A P Y A K E E P V T L L A L V G G K P I S Q P Y I D M T I K M M E K S F G I N V E Q K S T T E P Y	635
Ecaro	A L L M T A P L A P E D - T V I R I K G D L V S K P Y I D T L N L M K T F G V E I E N Q H Y Q Q -	584
Anqutr	- -	0
Pcarom	T Y Y I P K G - - C Y T C P S E Y I I E G D A T S A T Y P L A I A A I T G G S C T I S N V G S S A S L	674
Anarom	T Y H I P Q G - - R Y V N P A E Y V I E S D A S C A T Y P L A V A A V T G T T C T V P N I G S A S L	671
Scarom	T Y Y I P K G - - H Y I N P S E Y V I E S D A S S A T Y P L A F A A M T G T T V T V P N I G F E S L	683
Ecaro	- - F V V K G G Q S Y Q S P G T Y L V E G D A S S A S Y F L A A A A I K G G T V K V T G I G R N S M	632
Anqutr	- M F H A F S S Q Y I	10
Pcarom	Q G D S K F S E Y I L K P M G C E V V Q S P T T T T Y I K G P P K G K L K S L G - - - S I N M E S M	720
Anarom	Q G D A R F A V E V L R P M G C K T V E Q T A T S T T V T G P S D G I L R A T S K R G Y G T N D R C V	721
Scarom	Q G D A R F A R D V L K P M G C K T I T O T A T S T T V S G P V G T L K P L K H - - - V D M E M P	729
Ecaro	Q G D I R F A - D V L E K M G A T I C W G D D - - Y I S C T R G E L N A I D - - - M D M N H I	673
Anqutr	T C Q P - - - N M I S T F G C G I I R R L A I S I L V R P P K R R L A D T E N - - - - - - - - -	46
Pcarom	T D T F R L T A A V L A S V A Y E E S K - - P Y V T K I T G I S N Q R I K E C N R I N A M V C E L K K	768
Anarom	P R C F R T G - - S H R P M E K S Q - - T T P P V S S G I A N Q R V K E C N R I K A M K D E L A K	766
Scarom	T D A F L T A C V V A I S H D S D P N S A N T T T I E G I A N Q R V K E C N R I L A M A T E L A K	779
Ecaro	P D A A M T I A T A A L F A K G - - - - - T T R L R N I Y N W R V K E T D R L F A M A T E L R K	716
Anqutr	- - - - - - - - - - - - - - - - - L D Q N H R L F - - - - - - - - -	54
Pcarom	F G I E A G E L P D G T Y V K A L N T - S N L P Y S V E - - - G I N C Y N D H R I A M S F S V L	812
Anarom	F G V I C R E H D D G L E I D G I D R - S N L R Q P V G - - - G V F C Y D D H R V A F S F S V L	810
Scarom	F G V A E T E L P D G I Q V H I G L N S I K D L K V P S D S G P V G V C T Y N D H R V A M S F S L	829
Ecaro	V G K E V E E G H D Y I R I T - - - - - P P P E K L N F A E I A T Y N D H R V A M C F S L V	756
Anqutr	Y G I S A R E T A S - - - T P I N T S A D Y G R F D E R P G S G D G S R Y A S P F Q E L S S S Q G	100
Pcarom	A C I - - - - - S S K P T T I L D K A C V N K T W P Y G W D I L L N S T F K V Q M K G I E F D	853
Anarom	S L V - - - - - T P Q P T L I L E K E C V G K T W P G W W D T L L R Q L F K V K L E G K E L E	851
Scarom	A G M V N S Q N E R D E V A N P V R I L E R H C T G K T W P G W W D V L L H S E L G A K L D G A E -	877
Ecaro	A L - - - - - S D T P V T I L D P K C T A K T F P D Y F E Q L A R I S Q A A M T Q - - -	792
Anqutr	S L - - - - - - - T R V E D S L Q T R R K F P - - - - - - - - -	116
Pcarom	L N P T I N S S I L H H P S E C T I F L I G M R G A G K T T L G Q L A A N F L G R E F I D L D S I I	903
Anarom	E E P V A A S G - - P D R G N A S I V I I G M R G A G K S T A G N W V S K A L N R P P F V D L D T E L	899
Scarom	- - - P L E C T S K K N S K K S I V I I G M R A G K T T I S K W C A S A L G Y K L V D L D E L F	923
Ecaro	- - - - - - - - - - - P L F L I G P R G C G K T T V G M A L A D S L N R R F V D L D T D Q W L	826
Anqutr	- - - - - P N A S I V L I G I R G T G K S S L A V M L A A S Y G R R V I E A D L Y F	153
Pcarom	E E D - I K T T I L E F I Q K Y S W D V F R R K E L H F L K R T L T E - K K E N Y I I T S C G G G T I	951
Anarom	E T V - E G M T I P D I I K T R G W Q G F R N A E L E I L K R T L L K E - R S R D G Y V F A C G G G V V	947
Scarom	E Q Q H N N Q S V K Q F V V E N G W E K F R E E E T R I F K E V I Q N Y G S D G Y V F S T G G G I V	973
Ecaro	Q S Q - L N M T V A E I V E R E E W A G F R A R E T A A L E A V - - - T A P S T V I A T G G G I I	871
Anqutr	H R V - T G R C R G V Y K R E H T L S E Y R R Q E A I V M E S L L M E - H Q E N C V I V C G F G D V	201
Pcarom	E T E E A R D L L I S Y I E A N G I V I H I H R N I Q D I I K Y L N T D K - - - - - T R A P Y	993
Anarom	E M P E A R K L L T D Y H K K G N V L L H M R D I K K I I M D F L S I D K - - - - - S R P A Y	989
Scarom	E S A E S R K A L L K D F A S S G G Y V L L H R D I E E T I V F L Q S D P - - - - - S R P A Y	1015
Ecaro	L T E F N R H F M - - - Q N N G I V I V Y L C A P V S V L V N R L Q A A P E E D L R P T L T G K P L	917
Anqutr	E R N G Q M R - L R E Y A K T H P - V I H I V R D L E S I Q S Y L K A R D - - - - -	236
Pcarom	Q D N I M H V W E R R K R W Y N L C S S H Q P H M T S T D P T E F K K N I P L N L K S S F N N F - -	1041
Anarom	V E D M M G V W L R R K P W F Q E C S N I Q Y Y S R D A S P S G L A R A - - - - S E D F N R F - -	1032
Scarom	V E E I R E V W N R R E G W Y K E C S N F S P F A P H C S - - - - A E A E F Q A L R R S F S K Y - -	1059
Ecaro	S E E V Q E V L E E R D A L Y R E V A H I I I D A T N E P S Q V I S E - - - - I R S A L A Q T - -	960
Anqutr	T E K V R R F L E L S G P I Y R S C S N L E F F - - N V S E K G I S D Q P S A K D S Q H Y T Q W D A	284
Pcarom	- L R T I T G K N N I F S L I L K K K R S Y - - F L S L A F S D L E N I - F S L - - - - -	1077
Anarom	- L Q V A T G Q I D S L S I I K E K E H S F - - F A S L L T P D D L R E A - G D I - - - - -	1068
Scarom	- I A T I T G V R E - - I E I P S G R S A - - F V Q L T F D D L T E Q - T E N - - - - -	1092
Ecaro	- I N C M K T V T V K D L V I G T G A P K I - - I V S L M A K D I A S V K S E A - - - - -	997
Anqutr	E V D Q R T Q T T T P F L M L K R L Q R D F L R F V A L A T G N I P E L R N Q L S P F P L H M Q P I	334
Pcarom	- - - - - - - - - - - - - - - - - L D I I T A G C D A I E I R I D L F Q K P E E I D K Y P	1105
Anarom	- - - - - - - - - - - - - - - - - L E E V C V G S D A V E L R V D L L K D P A S N N D I P	1096
Scarom	- - - - - - - - - - - - - - - - - L T P I C Y G C B A V E W R V D H L A N - - - - - Y	1113
Ecaro	- - - - - - - - - - - - - - - - - L T A Y R E A D T A D I L E W R V D H Y A D - - - - - L S	1019
Anqutr	E S R K F T Y A A T V P I S H L L E N D V D I E E L E S T A D A F E L K I D V S A A P S A R L G T E	389

Pcarom	S	-	L	E	Y	I	A	E	K	I	F	L	R	Q	K	T	-	S	L	P	L	I	Y	T	L	R	T	N	H	G	G	S	F	L	S	S	E	K	K	L	-	A	K	E	Y	I	L	1151							
Anarom	S	-	V	D	Y	V	S	K	Q	L	S	F	L	R	S	R	V	-	T	L	P	I	I	F	T	I	R	T	Q	S	Q	G	G	R	F	P	D	N	A	H	D	A	-	A	L	E	L	Y	R	1142					
Scarom	S	-	A	D	F	V	S	K	Q	L	S	I	L	R	K	A	T	-	D	S	I	P	I	I	F	T	V	R	T	M	K	Q	G	G	N	F	P	D	E	E	F	K	T	-	L	R	E	L	Y	D	1160				
Ecaro	N	-	V	E	S	V	M	A	A	A	K	I	L	R	E	T	M	P	E	K	P	L	L	F	T	F	R	S	A	K	E	G	G	E	-	Q	A	I	S	T	E	A	-	Y	I	A	L	N	R	1065					
Anqutr	S	N	L	A	D	S	I	S	H	T	V	A	T	V	R	N	I	-	I	V	P	M	I	Y	H	V	E	S	S	V	F	P	D	S	A	P	L	R	R	S	D	A	S	Y	L	E	L	V	L	433					
Pcarom	H	G	A	K	W	G	-	F	E	F	L	D	I	E	L	D	I	A	S	E	L	F	K	T	I	N	-	N	S	W	P	Y	T	K	I	I	A	S	Y	H	N	I	E	K	P	I	-	S	C	1197					
Anarom	L	A	F	R	S	G	-	C	E	F	V	D	L	D	I	A	F	P	E	D	M	L	R	A	V	T	-	E	M	K	G	F	S	K	I	I	A	S	H	H	D	P	K	G	E	L	-	S	W	1188					
Scarom	I	A	L	K	N	G	-	V	E	F	L	D	L	E	L	T	L	P	T	D	I	Q	Y	E	V	I	-	N	K	R	G	N	T	K	I	I	G	S	H	H	D	F	Q	G	L	Y	-	S	W	1206					
Ecaro	A	A	I	D	S	G	L	V	D	M	T	D	L	E	L	F	T	G	D	D	Q	V	K	E	T	V	A	Y	A	H	A	H	D	V	K	V	M	S	N	H	D	F	H	K	T	P	-	E	A	1114					
Anqutr	H	G	L	R	L	G	P	-	E	F	V	T	V	D	L	S	F	E	D	S	I	L	S	Q	I	I	-	G	T	K	G	S	K	V	I	G	H	Y	S	Q	T	Q	P	P	P	Q	G	W	480						
Pcarom	D	D	F	E	W	I	Q	K	Y	K	E	A	Q	H	Y	G	-	H	T	I	K	L	V	G	T	S	S	S	I	E	D	N	F	-	F	L	E	E	F	K	S	-	K	F	I	N	-	K	K	1242					
Anarom	A	N	M	S	W	I	K	P	Y	N	K	A	L	E	Y	G	-	D	I	I	K	L	V	G	V	A	R	N	I	D	D	N	T	-	A	L	R	K	F	K	N	-	W	A	A	E	A	H	D	1234					
Scarom	D	D	A	E	W	E	N	R	F	N	Q	A	L	T	D	V	D	V	K	F	V	G	T	A	V	N	F	E	D	N	L	-	R	L	E	H	F	R	D	-	-	-	-	T	H	K	N	1250							
Ecaro	E	E	I	-	I	A	R	L	R	K	M	Q	S	F	D	A	D	I	P	K	I	A	L	M	P	Q	S	T	S	D	V	L	T	L	L	A	A	T	L	E	-	-	M	Q	E	Q	Y	A	D	1160					
Anqutr	S	D	P	E	Y	E	A	I	Y	E	R	A	K	K	L	G	C	D	M	V	R	L	T	Q	P	A	T	T	I	D	N	F	-	A	V	E	R	F	R	H	Q	I	K	T	L	P	G	P	Q	529					
Pcarom	V	P	S	I	I	N	T	G	I	K	Q	L	S	R	I	M	N	T	F	M	T	-	P	V	T	H	P	S	L	P	S	K	I	A	P	G	-	-	Q	L	S	I	K	E	I	N	T	A	1289						
Anarom	V	P	L	I	A	I	N	M	G	D	Q	G	Q	L	S	R	I	L	N	G	F	M	T	-	P	V	S	H	P	S	L	P	F	K	A	A	P	G	-	-	Q	L	S	A	T	E	I	R	K	G	1281				
Scarom	K	P	L	I	A	V	N	M	T	S	K	G	S	I	S	R	V	L	N	N	V	L	T	-	P	V	T	S	D	L	L	P	N	S	A	A	P	G	-	-	Q	L	T	V	A	Q	I	N	K	M	1297				
Ecaro	R	P	I	I	T	M	S	M	A	K	T	G	V	I	S	R	L	A	G	E	V	F	G	S	A	A	T	F	G	A	V	K	K	A	S	A	P	G	-	-	Q	I	S	V	N	D	L	R	T	V	1208				
Anqutr	L	P	V	I	A	Y	N	S	G	P	L	G	R	Q	S	C	F	N	P	V	L	T	P	V	I	P	R	S	L	I	S	Q	S	G	T	K	G	L	P	S	I	T	I	Q	E	A	Q	E	A	579					
Pcarom	L	H	I	M	G	L	L	P	E	K	Y	F	L	F	G	K	P	I	K	H	S	Q	S	P	N	I	H	N	L	G	F	E	I	L	G	L	P	Y	K	Y	Q	L	F	E	T	D	S	I	S	1339					
Anarom	L	S	L	M	G	E	I	K	P	K	K	F	A	I	F	G	S	P	I	S	Q	S	A	P	Q	L	S	T	T	P	Y	L	P	R	S	A	S	P	I	T	T	P	A	W	R	L	R	T	P	K	1331				
Scarom	Y	T	S	M	G	C	I	E	P	K	E	L	F	V	V	G	K	P	I	G	H	S	R	S	P	I	L	H	N	T	G	Y	E	I	L	G	L	P	H	K	F	D	K	F	E	T	E	S	A	Q	1347				
Ecaro	L	T	I	L	H	Q	A	M	E	-	T	Y	A	V	F	G	N	P	I	A	H	S	K	S	P	F	I	H	Q	P	A	Q	Q	L	N	I	E	H	P	Y	G	R	V	L	A	P	I	N	D	1257					
Anqutr	L	Y	S	S	F	V	L	D	P	M	Q	F	F	V	F	G	R	K	H	N	I	Q	F	V	P	S	N	A	Q	C	R	L	Q	G	A	G	N	A	D	I	Y	R	I	H	Q	S	P	T	L	R	629				
Pcarom	E	L	K	E	I	L	-	-	H	L	E	B	F	G	G	A	S	V	T	I	P	L	K	T	N	I	S	I	L	L	D	E	I	S	D	H	A	-	-	-	A	L	I	G	S	V	N	T	-	1382					
Anarom	M	C	R	S	S	-	-	A	L	L	T	S	A	A	P	S	V	T	I	R	S	S	T	S	C	P	F	S	T	K	L	P	R	K	P	R	S	S	E	L	L	T	Q	S	F	P	C	R	-	1379					
Scarom	L	V	K	E	K	L	D	G	N	K	N	F	G	G	A	A	V	T	I	P	L	K	L	D	I	M	Q	Y	M	D	E	L	T	D	A	A	K	-	-	-	V	I	G	A	V	N	T	V	1393						
Ecaro	F	I	N	T	L	N	A	F	S	A	G	G	K	G	A	N	V	T	V	P	F	K	E	E	A	F	A	R	A	D	E	L	T	E	R	A	-	-	-	A	L	A	G	A	V	N	T	L	1303						
Anqutr	G	I	N	-	-	Y	L	V	E	N	P	N	F	G	G	T	S	V	S	L	P	Y	K	T	E	V	I	P	L	L	H	S	M	S	P	H	A	R	A	-	-	-	I	G	A	V	N	T	L	673					
Pcarom	I	T	R	T	Y	N	N	G	Q	Y	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	K	G	E	N	T	D	W	Q	G	I	K	A	I	K	N	F	N	K	F	E	-	1415			
Anarom	L	A	R	T	L	H	H	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	V	G	R	N	T	D	W	Q	G	M	I	L	S	L	R	K	A	G	V	Y	G	P	K	1411	
Scarom	I	P	-	-	-	-	-	L	G	N	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	K	G	D	N	T	D	W	L	G	I	R	N	A	L	I	N	N	G	V	P	E	Y	-	1423	
Ecaro	M	-	-	-	-	-	-	R	L	E	D	G	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	L	G	D	N	T	D	G	V	G	L	L	S	D	L	E	R	L	S	F	I	R	P	G	1334
Anqutr	I	P	I	R	N	L	E	G	S	T	D	N	A	L	D	L	E	K	N	R	A	G	P	I	K	G	L	H	G	D	N	T	D	W	I	G	I	G	I	C	T	R	-	-	G	L	S	P	A	N	-	722			
Pcarom	K	S	F	E	N	F	S	G	F	I	I	G	A	G	G	A	S	R	A	A	I	Y	A	L	L	S	L	G	I	S	P	I	Y	L	I	N	R	S	K	D	K	L	N	K	L	Y	H	F	F	-	1464				
Anarom	R	K	D	Q	E	Q	S	A	L	V	I	G	G	G	G	T	A	R	A	A	I	Y	A	L	H	N	M	G	Y	S	P	I	Y	I	V	G	R	T	P	S	K	L	E	N	M	V	S	T	F	-	1460				
Scarom	-	-	V	G	H	T	A	G	L	V	I	G	A	G	G	T	S	R	A	A	L	Y	A	L	H	S	L	G	C	K	K	I	F	I	I	N	R	T	T	S	K	L	K	P	L	I	E	S	L	-	1470				
Ecaro	L	R	I	-	-	-	-	L	L	I	G	A	G	G	A	S	R	G	V	L	L	P	L	L	S	L	D	C	-	A	V	T	I	T	N	R	T	V	S	R	A	E	E	L	A	K	L	F	-	1377					
Anqutr	A	I	R	P	S	T	T	G	L	I	I	G	A	G	G	N	A	R	A	G	I	Y	A	M	I	H	L	G	V	Q	N	I	F	I	W	N	R	T	V	A	N	A	E	K	L	A	Q	H	Y	M	772				
Pcarom	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1501							
Anarom	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1495							
Scarom	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1508							
Ecaro	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1416							
Anqutr	R	L	N	L	C	T	L	G	G	S	G	S	A	S	Y	T	I	H	V	L	K	S	L	Q	E	S	W	P	A	N	Y	K	Q	P	T	I	V	V	S	G	I	P	A	H	R	I	G	D	Q	P	822				
Pcarom	V	L	E	I	A	K	I	F	F	N	L	K	R	K</																																									

cerevisiae has been shown to be under general amino acid control (Duncan *et al.*, 1988).

The regulation of expression of the *arom* gene in *A. nidulans* is complicated by the presence of the inducible quinic acid utilization pathway which shares a common reaction with the aromatic amino acid biosynthetic pathway. Two of the intermediates from the pre-chorismate pathway, DHQ and DHS, are also substrates in the quinic acid utilization pathway (known as *qut* in *A. nidulans*), which is responsible for the catabolism of quinic acid to protocatechuate and is found in fungi such as *A. nidulans* and *N. crassa* but is absent from *S. cerevisiae*. The pentafunctional character of the AROM protein has been attributed to the need to channel DHQ and DHS in order to keep the intracellular pools of these metabolites separate (Giles, 1978). However, it has recently been demonstrated that the AROM protein is leaky *in vivo* and there is normally no significant channelling function (Lamb *et al.*, 1991). However, under non-optimal conditions of nutrient and oxygen availability the channelling function may become significant so that a minimum flux through the aromatic biosynthetic pathway is maintained (Lamb *et al.*, 1992). It has been proposed that expression of *arom* is linked to a starvation response for aromatic amino acids. The *P. carinii* *arom* locus seems to possess a GCN4 binding site indicating that it may be under general control and that the expression would be up-regulated in response to starvation for any amino acid, and thus would act to maintain a minimal level of aromatic amino acid biosynthesis even under conditions of nutrient depletion. However, it is not yet known whether *P. carinii* possesses a *qut* pathway.

Confirmation that the cloned arom locus originates from the P. carinii genome

The cloning strategy employed for the isolation of this AROM encoding gene combined with the unavoidable impurity of the *P. carinii* genomic DNA samples allowed for the possibility that the locus was not of *P. carinii* origin. Experiments involving the use of pSB31 as a probe for *in situ* hybridization and hybridization to *P. carinii* chromosomes were carried out to address this question.

DNA from a *P. carinii* enriched fraction extracted from infected rat lung was electrophoresed so that chromosomes in the size range 200 kb to 1 Mb would be resolved. The karyotype obtained was considered to be that of *P. carinii* (Cushion *et al.*, 1993). Under the CHEF conditions used, rat chromosomes were too large to be resolved and remained above the band of limiting mobility. Prior to extraction of *P. carinii*, impression smears of the infected lung were examined by light

microscopy and no evidence of infection by any microbe other than *P. carinii* was observed. *S. cerevisiae* chromosomes were also separated by the CHEF procedure and transferred to a nylon membrane. The filters were probed with a ³²P-labelled portion of the putative *P. carinii* *arom* locus. Under stringent conditions this fragment hybridized to one of the chromosomes of *P. carinii* (about 420 kb) whereas there was no hybridization to the *S. cerevisiae* chromosomes (Fig. 4).

The same probe was biotinylated by means of a PCR reaction and was found to hybridize specifically to *P. carinii* DNA when used in an *in situ* hybridization experiment on sections of *P. carinii* infected rat lung. The thin walled, single nucleated trophozoite form of the organism develops through a series of stages involving thickening of the outer wall and division of the nucleus to form the mature cyst which contains up to eight intracystic bodies, each with a single nucleus. The sections from the *in situ* hybridization experiment were examined by light microscopy and the hybridization signal was characteristic of the distinctive arrangement of the nuclei of the intracystic bodies within the cysts (Wakefield *et al.*, 1988). Signal was largely restricted to spherical bodies identified as cysts (Fig. 5). The EPSPS coding region from the *A. nidulans* *arom* gene was also biotin-labelled and used as a control in the *in situ* hybridization experiment on serial sections of *P. carinii* infected rat lung. No evidence of hybridization to *P. carinii* was observed. The background signal produced through the detection system for the biotinylated probes was assessed through the use of a control in which there was no probe added. The background signal was negligible.

Analysis of the P. carinii AROM polypeptide

The deduced amino acid sequence of the *P. carinii* AROM protein was compared with those from *A. nidulans* and *S. cerevisiae* and over the entire length there was significant similarity between the three sequences. The locations of the domains in the *P. carinii* protein, corresponding to the five enzyme activities, was determined by alignment of the amino acid sequence of the AROM protein with the sequences of the five monofunctional *E. coli* enzymes (Fig. 3). The order of the functional domains from the N- to the C-terminus was DHQ synthase, EPSPS, shikimate kinase, DHQ dehydratase and shikimate dehydrogenase. The comparison also highlighted some regions with very little homology between the proteins, particularly in some of the interdomain regions, such as that between EPSPS and shikimate kinase. The alignment also includes the amino acid sequence of the *qut* repressor from *A. nidulans*, which is homologous to the three domains at the C-

Fig. 4. (a) Electrophoretic karyotype of *P. carinii* from the rat host (lanes 2 and 3), *S. cerevisiae* (lanes 4 and 5) and λ genome molecular size markers (lanes 1 and 6). The size of the smallest visible band (291 kb) in these markers is indicated and represents a concatamer of six λ genomes. Each successive band represents an increase of 48.5 kb. (b) Autoradiography after transfer of samples shown in (a), probed with pSB31.

Fig. 5. *In situ* hybridization of a *P. carinii* *arom* biotin labelled probe (pSB31) to sections of *P. carinii* infected rat lung, showing hybridization signal in the distinct arrangement of the nuclei of the intracystic bodies within the *P. carinii* cysts (labelled 'C'), and absent from the surrounding rat tissue.

termini of the AROM proteins. This relationship is interesting in both functional and evolutionary terms. The repressor is thought to be capable of binding

quininate, DHQ and DHS such that the binding of any of these would lead to the inactivation of the *qutR* protein. However, in the absence of these metabolites the

activator of the quinate utilization pathway (the *qutA* protein) cannot bring about the induction of the enzymes required for quinate catabolism. The kinase activity of the *qut* repressor is thought to be involved in the inactivation of the activator (Hawkins *et al.*, 1992). The homology between the *qut* repressor and AROM suggests an evolutionary link between the two genes.

Throughout the *P. carinii* AROM protein there are important amino acid motifs that are conserved in equivalent enzymes across fungal, bacterial and plant species. The motif LGNAGTA contains a universally conserved glycine (Gly⁹⁶ in *E. coli*) implicated as being involved in the interaction of phosphoenolpyruvate with EPSPS (Padgett *et al.*, 1991), and the universally contained group DHR contains the histidine residue (His³⁸⁵ in *E. coli*) which was shown to be at or near the active site of EPSPS by Huyhn (1987). A highly conserved sequence motif, GXGXXG, associated with polypeptides that bind dinucleotide (Rossman *et al.*, 1974) is found in all DHQ synthase enzyme sequences (Fig. 6a). The type A motif, ^GXXXXGK^T, identified in purine nucleotide binding proteins (Walker *et al.*, 1982) is found in all shikimate kinase sequences (Fig. 6b).

The homology between the deduced amino acid sequence from the putative *arom* gene from *P. carinii* and the other AROM protein sequences together with the conservation of motifs identified as functionally important suggests very strongly that the gene encodes a functional AROM protein *in vivo*. The existence of an mRNA molecule that has been derived from the *P. carinii* *arom* gene further strengthens this view. However, formal proof that the gene encodes a functional protein awaits the expression of the cloned *P. carinii* gene in a heterologous system such as *S. cerevisiae*, using *ARO1* mutants and demonstrating the ability of the *P. carinii* gene to complement such mutations.

Multifunctional enzymes

Multifunctional enzymes catalysing consecutive steps in metabolic pathways have been identified in a wide range of organisms but are especially common in lower eukaryotes. An example of this phenomenon has recently been discovered in *P. carinii* by Volpe *et al.* (1992), who cloned the folic acid synthesis gene (*fas*) which has two identifiable domains, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase and dihydropteroate synthase. However, it was shown by Edman *et al.* (1989b) that the dihydrofolate reductase and thymidylate synthase genes of *P. carinii* were unfused, which differs from the protozoan arrangement but is in accord with the fungi.

The multifunctional AROM enzyme has been identified in all fungi investigated to date and also the unicellular green alga *Euglena gracilis* (Berlyn *et al.*,

(a)							
<i>S. cerevisiae</i>	G	G	G	V	I	G ¹¹³	
<i>A. nidulans</i>	G	G	G	V	I	G ¹¹⁸	
<i>P. carinii</i>	G	G	G	V	I	G ¹¹⁵	
<i>E. coli</i>	G	G	G	V	V	G ¹⁰⁸	
<i>M. tuberculosis</i>	G	G	G	A	A	T ¹¹⁰	
Motif associated with dinucleotide binding proteins	G	X	G	X	X	G	
(b)							
<i>S. cerevisiae</i>	G	M	R	A	A	G	K T ⁹⁰²
<i>A. nidulans</i>	G	M	R	G	A	G	K S ⁸⁷⁸
<i>P. carinii</i>	G	M	R	G	A	G	K T ⁸⁸²
<i>E. coli</i> (<i>aroL</i>)	G	P	R	G	C	G	K T ¹⁶
<i>E. coli</i> (<i>aroK</i>)	G	P	M	G	C	G	K S ⁴⁶
<i>A. nidulans</i> <i>qutR</i>	G	I	R	G	T	G	K S ¹³²
Type A motif associated with proteins that bind purine nucleotides	G	X	X	X	X	G	K T
	A						S

Fig. 6. Motifs in the *P. carinii* *arom* complex. (a) Consensus sequence for a dinucleotide binding protein and corresponding motifs from DHQ synthase enzymes. The *Mycobacterium tuberculosis* sequence originates from Garbe *et al.* (1991). (b) The type A purine nucleotide binding motif and corresponding sequences from shikimate kinase enzymes. The *aroL* gene product is shikimate kinase II and *aroK* codes for shikimate kinase I (Løbner-Olesen & Marinus, 1992). The *A. nidulans* *qut* repressor sequence shown is derived from the N-terminal portion of this protein that is homologous to shikimate kinase.

1970). Thus the identification of the multifunctional protein in *P. carinii* is in accordance with recent evidence placing the organism in the fungal kingdom. The discovery of the pentafunctional AROM protein in *P. carinii* represents an extension in the knowledge of the metabolism of the organism. A high proportion of *P. carinii* genes cloned to date have had connections with folate metabolism, since many of the drugs currently in use act at this pathway. In addition to shedding light on the nature of *P. carinii* aromatic amino acid biosynthesis, the cloning of the *P. carinii* *arom* locus may suggest a novel approach to therapy. Since the mammalian hosts of *P. carinii* do not possess any of the enzymes of the pre-chorismate pathway, specific inhibitors of these enzymes might be antimicrobial without any adverse effects on the host. In addition, since the AROM protein is multifunctional it may present several targets for the design of inhibitors.

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