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Biochemical and genetic characterization of an early step in a novel pathway for the biosynthesis of aromatic amino acids and *p*-aminobenzoic acid in the archaeon *Methanococcus maripaludis*

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Summary

***Methanococcus maripaludis* is a strictly anaerobic, methane-producing archaeon and facultative autotroph capable of biosynthesizing all the amino acids and vitamins required for growth. In this work, the novel 6-deoxy-5-ketofructose-1-phosphate (DKFP) pathway for the biosynthesis of aromatic amino acids (AroAAs) and *p*-aminobenzoic acid (PABA) was demonstrated in *M. maripaludis*. Moreover, PABA was shown to be derived from an early intermediate in AroAA biosynthesis and not from chorismate. Following metabolic labelling with [U-^{13}C]-acetate, the expected enrichments for phenylalanine and arylamine derived from PABA were observed. DKFP pathway activity was reduced following growth with aryl acids, an alternative source of the AroAAs. Lastly, a deletion mutant of *aroA′*, which encodes the first step in the DKFP pathway, required AroAAs and PABA for growth. Complementation of the mutants by an *aroA′* expression vector restored the wild-type phenotype. In contrast, a deletion of *aroB′*, which encodes the second step in the DKFP pathway, did not require AroAAs or PABA for growth. Presumably, methanococci contain an alternative activity for this step. These results identify the initial reactions of a new pathway for the biosynthesis of PABA in methanococci.**

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Introduction

Methanococcus maripaludis is a strictly anaerobic, methane-producing archaeon. It is a mesophile that utilizes H_2 or formate for the reduction of CO_2 to methane as a source of energy. Although *M. maripaludis* is an autotroph that can grow with CO_2 as its sole source of carbon, it also can assimilate acetate and amino acids (Jones *et al*., 1983a; Shieh and Whitman, 1987; 1988; Whitman *et al*., 1987). Many factors make *M. maripaludis* a useful model in which to study the function of genes *in vivo*. First, genetics tools have been developed, including selectable resistance markers, efficient transformation systems, gene deletion methods involving substitutions with resistance markers or markerless in frame deletions, and expression vectors (Tumbula *et al*., 1994; Gardner and Whitman, 1999; Porat *et al*., 2004; Moore and Leigh, 2005). Second, *M. maripaludis* grows quickly compared with many other methanogens and is easily cultured on plates (Jones *et al*., 1983b). Third, the sequence of the *M. maripaludis* genome is relatively small, containing only 1722 open reading frames (ORFs) (Hendrickson *et al*., 2004).

The canonical pathway for the biosynthesis of aromatic amino acids (AroAAs) starts with erythrose-4-phosphate and phosphoenol pyruvate. In this pathway, chorismate is the branch point for the biosynthesis of phenylalanine and tyrosine, tryptophan, *p*-aminobenzoate (PABA), vitamins E and K, ubiquinone and certain siderophores (Bentley, 1990). The first two steps in the common pathway are catalysed by 3-deoxy-D-arabino-2-heptulosonate-7-phosphate (DAHP) synthase and 3-dehydroquinate (DHQ) synthase. In many organisms, the DAHP synthase is precisely regulated by the AroAAs (Gosset *et al*., 2001; Helmstaedt *et al*., 2005).

Four lines of evidence indicate that *M. maripaludis* and other euryarchaeotes possess a novel pathway for the initial steps of AroAAs biosynthesis. First, DAHP synthase activity is not detectable in cell-free extracts of *Methanohalophilus mahii* (Fisher *et al*., 1993). Second, the metabolic labelling of pentoses in *M. maripaludis* is not consistent with erythrose-4-phosphate as a precursor for AroAAs (Tumbula *et al*., 1997). Third,

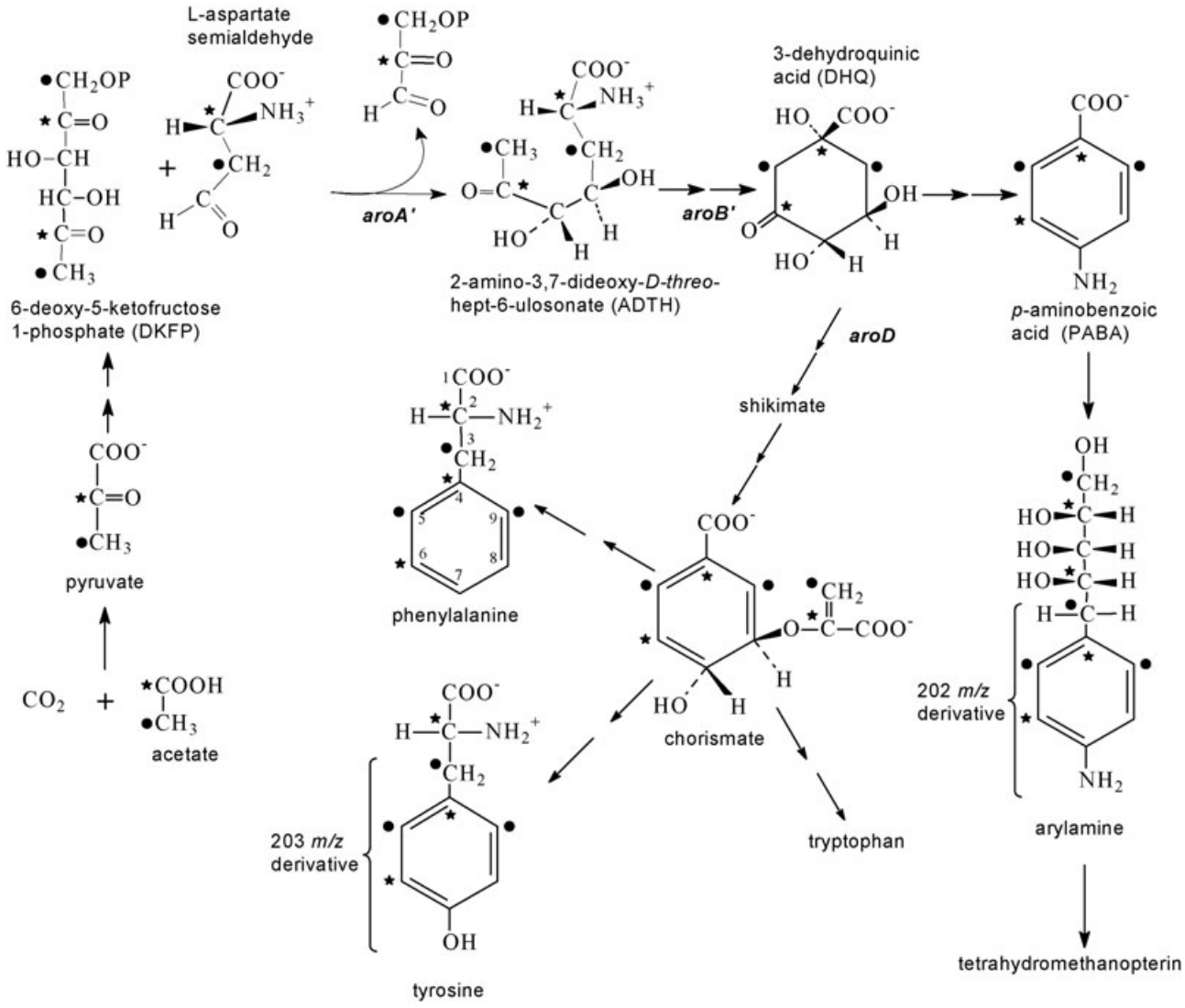

Fig. 1. Proposed *de novo* pathway for the biosynthesis of AroAAs and PABA for tetrahydromethanopterin biosynthesis in *M. maripaludis*. The AroAAs are biosynthesized through shikimate and chorismate; tetrahydromethanopterin is biosynthesized through PABA and arylamine. The expected labelling patterns from [U-^{13}C]-acetate are shown (C_1, ●; C_2, ★). The sources of the 203 m/z fragment of tyrosine and the 202 m/z fragment of arylamine are marked.

homologues to the first two steps of the canonical pathway are absent from most of the genome sequences of the euryarchaeotes (Gosset *et al*., 2001; Xie *et al*., 2003; Hendrickson *et al*., 2004). Fourth, previously unidentified ORFs of *Methanocaldococcus jannaschii*, MJ0400 and MJ1249, possess the biochemical activities to convert 6-deoxy-5-ketofructose-1-phosphate (DKFP) and L-aspartate semialdehyde to DHQ (White, 2004). These two enzymatic reactions were recently named 2-amino-3,7-dideoxy-D-*threo*-hept-6-ulosonate (ADTH) synthase and DHQ synthase II and are encoded by the genes *aroA′* and *aroB′* respectively (R.A. Jensen, C.A. Bonner and J. Song, submitted for publication). These enzymes represent an alternative for the early steps in chorismate and AroAAs biosynthesis in many euryarchaeotes (Fig. 1, White, 2004).

In contrast to the initial steps, *M. maripaludis* possesses the subsequent steps of the canonical pathway starting with DHQ dehydratase (AroD). Homologues for all the genes necessary to convert DHQ to chorismate and to the AroAAs are present in the genome sequence (Hendrickson *et al*., 2004). Deletion of the *aroD* gene yielded an AroAA auxotroph (Porat *et al*., 2004). In this mutant, the aryl acids (phenylacetate, *p*-hydroxyphenylacetate and indoleacetate) fulfil the requirement of AroAAs for growth. Labelling and additional mutagenesis studies further elucidate a second pathway for the biosynthesis of AroAAs in which the aryl acids are activated to the coen-

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zyme A thioesters prior to reductive carboxylation and amination to AroAAs (Xing and Whitman, 1992).

Surprisingly, the Δ*aroD* mutant of *M. maripaludis* does not require PABA for growth, indicating that PABA may not be formed from chorismate (Porat *et al*., 2004). In addition, *M. maripaludis* does not possess homologues to the *Escherichia coli* genes *pabA*, *pabB* and *pabC*, required for the biosynthesis of PABA from chorismate in bacteria (Green and Nichols, 1991; Viswanathan *et al*., 1995; Hendrickson *et al*., 2004). Thus, *M. maripaludis* may possess a novel pathway for the biosynthesis of PABA.

In the present work, a common pathway for the biosynthesis of PABA and AroAAs is demonstrated by metabolic labelling. Moreover, PABA was shown to be derived from an early intermediate in AroAA biosynthesis and not from chorismate. Lastly, the role of the DKFP pathway in *M. maripaludis* was tested *in vivo* by mutagenesis.

Results

Metabolic labelling of phenylalanine

To determine if the DKFP pathway could be present in *M. maripaludis*, the labelling of phenylalanine by [U-^{13}C]-acetate was examined by proton nuclear magnetic resonance (NMR). In this experiment, the ratio of ^{13}C/^{12}C was determined from the ratio of the protons adjacent to either ^{13}C or ^{12}C atoms (Fig. 2). The enrichments of the C_2 and C_3 of phenylalanine, which corresponded to the C-1 and C-2 of acetate, were 63% and 72% respectively. These values were close to two-thirds of the enrichment of the exogenous acetate, consistent with previous observations that autotrophic acetate biosynthesis continued in the presence of readily assimilated organic carbon (Yang *et al*., 2002). Similarly, the lower enrichment of carbon derived from the C_1 of acetate was consistent with a partial exchange of carbon at this position with unlabelled carbon from CO_2 catalysed by acetyl CoA synthase and was diagnostic of carbon derived by this route (Yang *et al*., 2002). Because the H atoms at C-5 and C-9 of the aromatic ring were equivalent by NMR, the observed enrichment was the sum of the enrichment of each carbon. The enrichment of 144%, or twice that of the C_2 of acetate, was consistent with their biosynthesis from the C-2 of acetate. Similarly, the carbons 6 and 8 of the aromatic ring were enriched by 62%, consistent with the formation of one-half of the carbon from the C_1 of acetate and one-half of the carbon from unlabelled CO_2. The carbon at position 7 was not labelled. Although expected to be labelled, the enrichment of carbon 4 was not measured because of the absence of a H atom on this carbon. These results were consistent with formation of phenylalanine by the DKFP pathway as proposed in Fig. 1 as well as the canonical pathway common in bacteria.

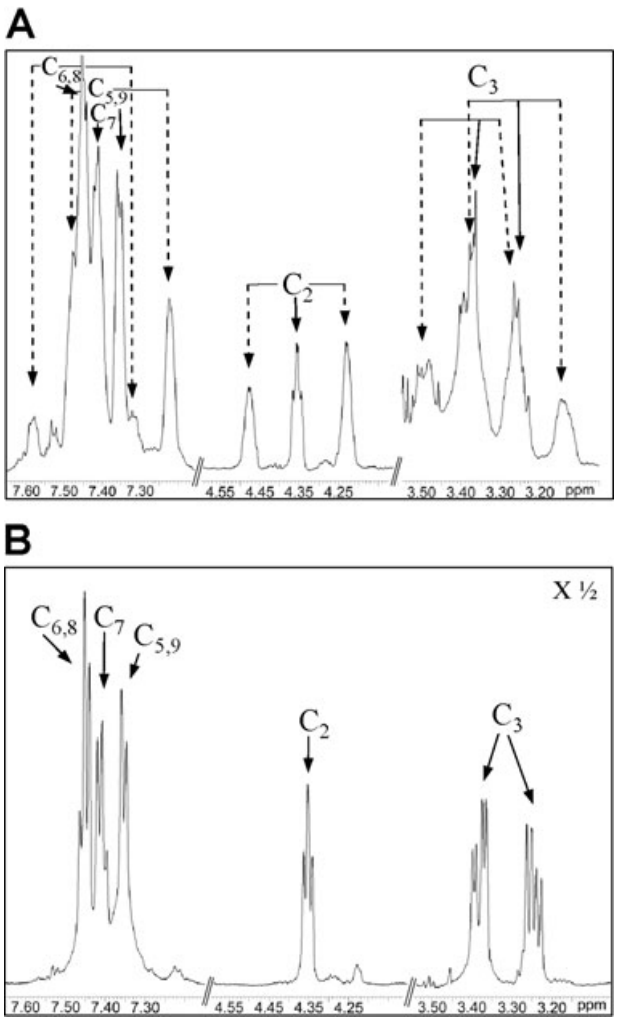

Fig. 2. Proton NMR spectra of phenylalanine from *M. maripaludis* S2 following growth on [U-^{13}C]-acetate. Coupled (A), decoupled (B). The resonances from protons on ^{12}C atoms are marked with solid arrows (A, B). The protons coupled to ^{13}C atoms are marked with dashed arrows (A). The scale of B is twice that of A.

Activity and regulation of the DKFP pathway

Aryl acids are readily incorporated into AroAAs and regulate the *de novo* pathway in *M. maripaludis* (Porat *et al*., 2004). However, the target of this regulation has not been identified. In many prokaryotes and yeast, the initial reaction DAHP synthase is a key regulatory check point for AroAA biosynthesis (Hall *et al*., 1983; Koll *et al*., 1988; Panina *et al*., 2001; 2003). Assuming that the DKFP pathway is the early step in the *de novo* pathway in methanococci (White, 2004), its expression might be regulated by the aryl acids. To test this hypothesis, *M. maripaludis* was cultured in minimal medium with acetate; with acetate and aryl acids; and with acetate, aryl acids and Casamino acids. The relative activity of the DKFP pathway was determined from the accumulations of shikimate and dihydroshikimate following incubation of

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cell-free extracts with the substrates [U-^{13}C]glucose-6-phosphate, L-homoserine and NAD (White, 2004). In the extract from the acetate-grown cells, the shikimate and dihydroshikimate pools were 90% labelled with a C_3 unit, indicating a high activity for the DKFP pathway in this extract (data not shown). For the extracts from cells grown with aryl acids or in the combination of aryl acids and Casamino acids, the production of labelled shikimate and dihydroshikimate was reduced to 1% or $< 0.2\%$ respectively. Therefore, the activity of the DKFP pathway was greatly reduced following growth with aryl acids. This apparent regulation was consistent with a role in an early step in AroAA biosynthesis.

Common precursors for the biosynthesis of AroAAs and PABA

A possible explanation for the absence of a PABA requirement for growth of an *aroD* mutant was that PABA was formed from tyrosine or from the tyrosine precursor, *p*-hydroxyphenylacetate. *M. maripaludis* S87 strain, an *aroD::pac* mutant (Porat *et al*., 2004), was grown in minimal medium with acetate, [$^2H_6$]-*p*-hydroxyphenylacetate, phenylacetate and indoleacetate. The distribution of deuterium incorporated into the tyrosine extracted from cells was the same as the distribution of deuterium in the [$^2H_6$]-*p*-hydroxyphenylacetate added to the medium as well as the [$^2H_6$]-*p*-hydroxyphenylacetate isolated from the spent culture medium (data not shown). These results confirmed that this aryl acid was a precursor for tyrosine. However, no label was found in the arylamine derived from PABA. Therefore, PABA was not formed from this aryl acid.

The second hypothesis tested was that PABA was formed from an early intermediate in the AroAA pathway prior to DHQ dehydratase, which was encoded by *aroD*. *M. maripaludis* wild-type cells (S2) grown in the presence of [U-^{13}C]-acetate readily incorporated label into the arylamine derived from PABA and the AroAAs tyrosine and phenylalanine. The distribution of the label in the 203 m/z fragment from tyrosine derivative was identical to the distribution found in the 202 m/z fragment of the arylamine derivative (Table 1). Similarly, the distribution of the label in phenylalanine was identical to that in tyrosine (data not shown). To insure that PABA was formed *de novo* during the labelling experiment, a second metabolic labelling was performed in which cells were grown in minimal medium with [U-^{13}C]-acetate and [^{15}N]-ammonium sulphate as a nitrogen source. Again, a similar labelling distribution was obtained for the 203 m/z fragment of tyrosine and for the 202 *m/z* fragment of arylamine, only now the masses were increased in the arylamine by one due to the incorporation of ^{15}N (data not shown). The similarities in the labelling patterns of the AroAAs and the arylamine derived from PABA provided strong evidence that these compounds shared a common precursor. Moreover, it eliminated a trivial explanation for the absence of PABA requirement by the Δ*aroD* mutant, that *M. maripaludis* did not make PABA.

Table 1. Distribution of ^{13}C in selected fragments of the arylamine and tyrosine derivatives following growth with [U-^{13}C]-acetate.[a]

	Distribution of ^{13}C following growth with [U-^{13}C]-acetate (% of total) in:	
Number of ^{13}C	202 m/z fragment ion of arylamine derivative[b]	203 m/z fragment ion of the tyrosine derivative
0	17.3	17.9
1	12.0	13.1
2	20.7	22.2
3	15.3	16.6
4	18.7	13.4
5	16.0	16.9

a. *Methanococcus maripaludis* S2 was cultured in McN medium with 8.7 mM [U-^{13}C]-acetate. The data have been corrected for the natural abundance of ^{13}C.
b. The arylamine was prepared from tetrahydromethanopterin as described in *Experimental procedures*.

Distribution of the homologues of ADTH synthase and DHQ synthase II from M. maripaludis

In *M. jannaschii*, the ORFs MJ0400 and MJ1249 were proposed to play a key role in an alternative pathway of DHQ biosynthesis (White, 2004). In *M. maripaludis*, the homologues to these genes were Mmp0686 and Mmp0006 respectively. Homologues were also widely distributed in the genomic sequences of the euryarchaeotes as well as some bacteria (Fig. 3). Several lines of circumstantial evidence support a role for these genes in a novel pathway of AroAA biosynthesis. All of these genomes lack homologues for DAHP synthase; and except for *Frankia* sp. and *Polaromonas* sp. they lack homologues for DHQ synthase. These genes encode the first and second steps of the canonical pathway. Except for the partial sequence of the uncultured soil crenarchaeote, these genomes possess homologues to either the type I or type II DHQ dehydratase, confirming that these organisms can biosynthesize AroAAs. In addition, except for the genomes of *Methanothermobacter thermautotrophicus*, *Frankia* sp., *Methanosphaera stadtmanae*, *Methanococcoides burtonii*, *Archaeoglobus fulgidus* and the partial sequence of the uncultured soil crenarchaeote, these genomes possess homologues to either the type I or type II of fructose-1,6-bisphosphate (FBP) aldolase. The type I FBP aldolases are homologous to the ADTH synthases (Siebers *et al*., 2001) and difficult to distinguish on the basis of sequence similarity alone (see later). Thus, the presence of a paralogous aldolase is expected if the

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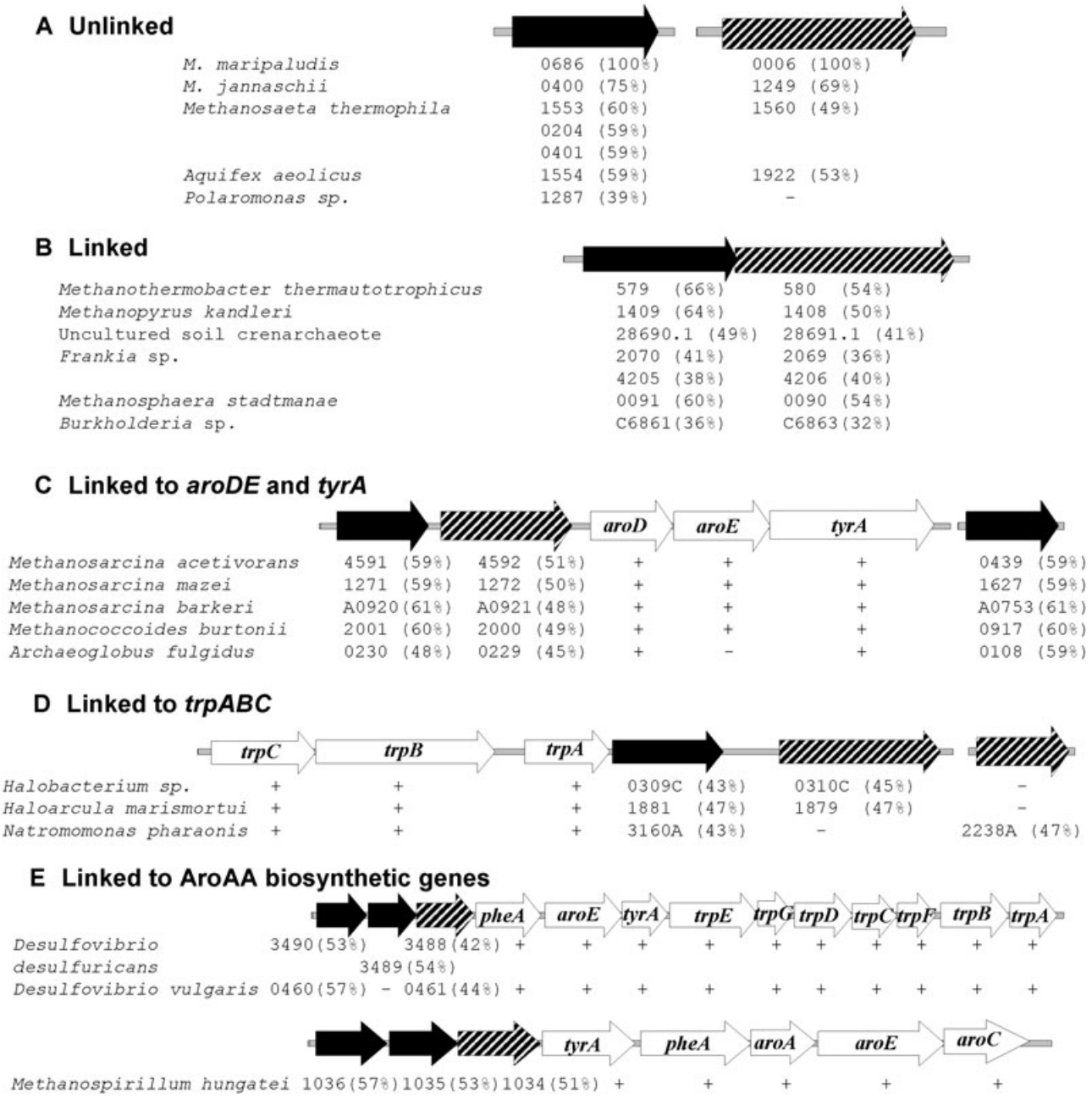

Fig. 3. AroAA biosynthetic genes from organisms that contain homologues to ADTH synthase and DHQ synthase II. Numbers are the ORF designations from the complete genomic sequences or from the draft genomic sequences at the NCBI database. The presence or absence of the corresponding homologue is showed in + or – respectively. Black, homologues to ADTH synthase (encoded by *aroA′*); striped, homologues to DHQ synthase II (encoded by *aroB′*); and white, other genes for AroAA biosynthesis. The percentages of amino acid identity to the *M. maripaludis* ORFs are in parenthesis.
A. Unlinked: organisms where the homologues of *aroA′* and *aroB′* are unlinked.
B. Linked: organisms where the homologues of *aroA′* and *aroB′* were adjacent to each other.
C. Linked to *aroD:* organisms where the homologues of *aroA′* and *aroB′* were adjacent to *aroDE* and *tyrA*.
D. Linked to *trpA*: organisms that contain the homologues of *aroA′* and *aroB′* adjacent to *trpCBA* genes.
E. Linked to AroAA biosynthetic genes: organisms that contain the homologues of *aroA′* and *aroB′* adjacent to many AroAA biosynthetic genes.

ADTH synthase is present. In addition, in many genomes, the *aroA′* and *aroB′* homologues are linked to other genes in the AroAA biosynthetic pathway, strongly suggesting that these genes are involved in the same function. The fact that these genes are unlinked in the methanococci does not lessen the weight of this argument because few

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biosynthetic genes are linked in these organisms (Bult *et al*., 1996; Hendrickson *et al*., 2004). Lastly, more than one *aroA′* homologue were found in some organisms (Fig. 3). In some cases, one *aroA′* homologue is linked to other AroAA biosynthetic genes, supporting its assignment to this pathway. However, many of these genes are unlinked, and it is not possible to decide if they are isozymes or encode another activity.

Mutagenesis of aroA′ *and* aroB′ *genes in* M. maripaludis

To further test the role of the DKFP pathway in *M. maripaludis*, mutations were constructed in key genes. First, the internal portion of *aroA′* was replaced with the puromycin resistance or *pac* cassette to construct S162 (Fig. 4A). The genotype of S162 was confirmed by Southern blotting (data not shown). The increase in size of the ScaI fragment from 2.0 kb for the wild type (S2) to 2.9 kb for the mutant (S162) was consistent with replacement of a 0.5 kb internal portion of the *aroA′* gene with the 1.3 kb *pac* cassette. The S162 mutant required both AroAAs and PABA for growth (Fig. 5A). No growth was observed in medium with either PABA or the AroAAs omitted. However, in the presence of PABA and AroAAs, growth of the S162 mutant lagged about 30 h behind that of wild-type strain S2 (Fig. 5A). This lag was similar in magnitude to the lag observed with the *aroD* mutant on AroAAs (Porat *et al*., 2004). In this case, the lag was shown to be due to the poor uptake of tyrosine.

The phenotype of the S162 mutant could have resulted in part from a polar effect on transcription of the downstream ORF Mmp0687, which encoded a triosephosphate isomerase homologue. Therefore, an in frame deletion of *aroA′* was constructed in which an internal 576 bp was deleted and the downstream reading frame was restored for the remainder of the gene (Fig. 4B). The resulted polymerase chain reaction (PCR) amplifications of 243 bp for the mutant S170 compared with the 819 bp for the wild-type strain S2 confirmed the genotype (data not shown). In addition, the *aroA′* locus was sequenced from strains S2 and S170, and it was exactly as expected. Like the gene replacement mutant S162, the in frame deletion mutant S170 also required both PABA and AroAAs for growth and possessed a growth lag when compared with the wild-type (Fig. 5A). In addition, the possibility that the delayed growth of the mutant S170 was the result of selection for a spontaneous revertant was rejected by transferring two times to the same medium and obtaining a similar lag (data not shown).

Finally the role of the ADTH synthase in the DKFP pathway in *M. maripaludis* was confirmed by complementing the S162 and S170 mutants with *aroA′* expressed from a methanococcal shuttle vector. Strains S162 (Δ*aroA′::pac*) and S170 (Δ*aroA′*) were transformed with

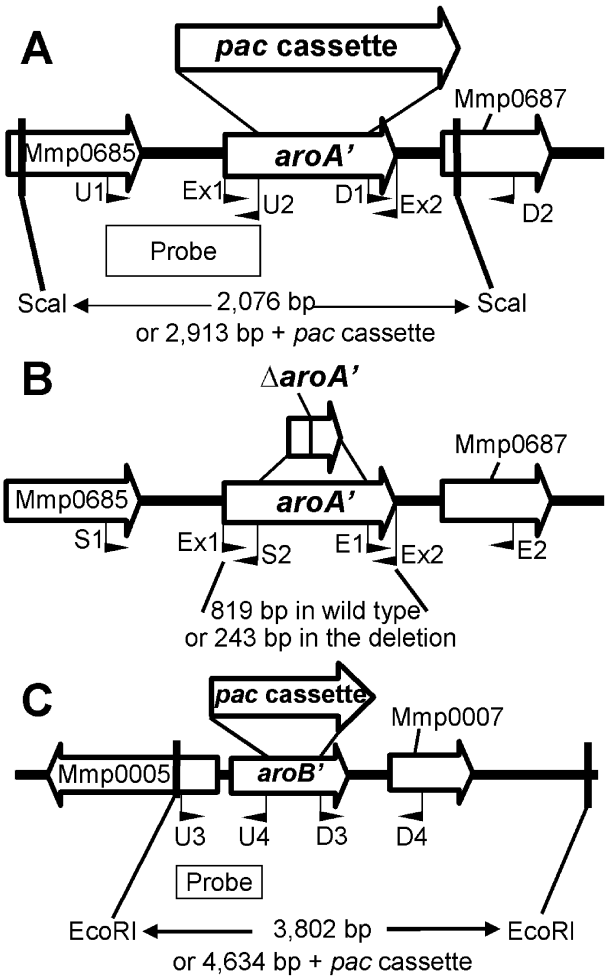

Fig. 4. Construction of mutations in *aroA′* and *aroB′*.
A and B. The region of the *M. maripaludis* genome encoding *aroA′*. The ORFs Mmp0685 and Mmp0687 were annotated as ribosomal protein L11 and triosephosphate isomerase respectively (Hendrickson *et al*., 2004). A. The primers U1, U2, D1 and D2 were used for cloning the upstream and downstream regions flanking the *aroA′* gene for construction of the gene replacement. B. The primers S1, S2, E1 and E2 were used for cloning the flanking regions of the *aroA′* for construction of the in frame deletion. The primers Ex1 and Ex2 were used for amplification and cloning of the *aroA′* in the expression vector.
C. The region of the *M. maripaludis* genome encoding *aroB′*. The ORFs Mmp0005 and Mmp0007 were annotated as hypothetical proteins (Hendrickson *et al*., 2004). The primers U3, U4, D3 and D4 were used for cloning the upstream and downstream regions flanking the *aroB′* gene for construction of the gene replacement.

pMEV2-aroA′ to yield S165 and S176 respectively. Both strains S165 and S176 grew in minimal medium with acetate only (Fig. 5B and data not shown). In conclusion, the ADTH synthase, encoded by *aroA′*, was clearly involved in an early step of the biosynthesis of AroAAs and PABA in *M. maripaludis.*

The ORF Mmp0006 was the *M. maripaludis* homologue to MJ1249, which was proposed to encode DHQ synthase II, the next gene in the DKFP pathway (Fig. 1, White,

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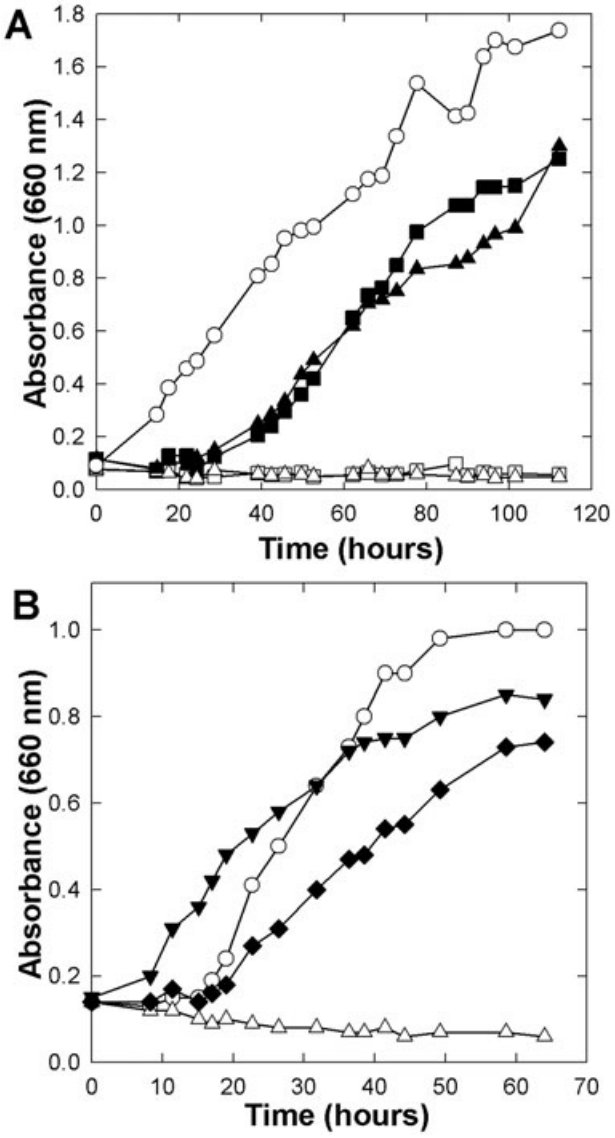

Fig. 5. Phenotype of the *aroA′* and *aroB′* deletion mutants.
A. The *aroA′* deletion mutants, S162 and S170, required both AroAAs and PABA for growth. Growth in McNA medium in the presence of AroAAs (1 mM) and/or PABA (0.5 mM) as indicated. When the growth responses were very similar for more than one condition, only the average growth is shown. Shown are growth of the wild-type S2 with AroAAs and PABA, or with AroAA only, or with PABA only, or with no addition (○); Δ*aroA′::pac* mutant, S162, with AroAAs and PABA (■); S162 with AroAAs only, or with PABA only, or with no addition (□); Δ*aroA′* mutant, S170, with AroAAs and PABA (▲); S170 with AroAAs only, or with PABA only, or with no addition (△).
B. The wild-type phenotype was restored in the S176 strain (complementation of S170 with *aroA′* expressed in a vector). The Δ*aroB′::pac* mutant (S140) did not require AroAAs or PABA for growth. Shown are the growth of the strains S2 (○), S140 (▼), S170 (△) and S176 (◆) in McNA medium.

2004). This gene was deleted by replacement with the *pac* cassette (Fig. 4C). The genotype of the Δ*aroB′::pac* mutant S140 was confirmed by Southern blotting (data not shown). The increase in the size of the EcoRI fragment from 3.8 kb in the wild-type strain S2 to 4.6 kb in the S140 mutant confirmed the replacement of a 0.5 kb fragment of *aroB′* with the 1.3 kb *pac* cassette. Unexpectedly, S140 did not require either AroAAs or PABA for growth, and growth was similar to that of the wild-type strain in minimal medium with acetate only (Fig. 5B). In addition, the relative activity of the DKFP pathway was determined for the S140 strain as described earlier. Using acetate-grown cells, the shikimate and dihydroshikimate pools in extracts of S140 were 96% labelled or nearly the same as the wild-type strain. Therefore, this gene was not required for AroAA biosynthesis in *M. maripaludis.*

Aryl acids inhibit the growth of the aroA′ *mutants*

Aryl acids provide an alternative source of AroAAs and replace the requirement for AroAAs in an *aroD* mutant S87 (Porat *et al*., 2004). Surprisingly, the aryl acids did not replace the requirement of AroAAs in the *aroA′* mutants (Fig. 6A and data not shown). The growth of S170 was completely inhibited in minimal medium with acetate, PABA and aryl acids. However, this inhibition was partially rescued when AroAAs were added, indicating that the inhibition is related to the biosynthesis of AroAAs (Fig. 6A). In contrast, the wild-type strain S2 was not inhibited by the aryl acids under this condition. Moreover, inhibition of S170 was mostly due to the aryl acid *p*-hydroxyphenylacetate. Growth of the S170 mutant was largely unaffected by either phenylacetate or indoleacetate in the presence of the remaining AroAAs (Fig. 6B). However, when tyrosine was replaced by *p*-hydroxyphenylacetate, the growth was severely inhibited. These results also indicated that the aryl acid pathway was not inhibited in the S170 mutant.

Identification of the methanococcal FBP aldolase

Based upon its similarity to the genes from *Pyrococcus furiosus* and from *Thermoproteus tenax*, bioinformatic analysis classified MJ0400 from *M. jannaschii* as FBP aldolase (Siebers *et al*., 2001; Verhees *et al*., 2003). With the discovery of ADTH synthase activity for this gene, the identity of FBP aldolase was uncertain. *M. jannaschii* and *M. maripaludis* contain a second homologue for FBP aldolase, MJ1585 and Mmp0293 respectively (Siebers *et al*., 2001). To determine if MJ1585 possessed FBP aldolase activity, the recombinant protein was expressed in *E. coli* from the plasmid pMJ1585 vector. Even though most of the recombinant protein formed inclusion bodies, heat-stable FBP aldolase activity of 540 mU mg^{-1} was present in cell extracts. In contrast, after heat treatment no detectable FBP aldolase activity was found in an extract of *E. coli* without the expression vector. Similarly, FBP aldolase activity in the purified MJ0400 protein was undetect-

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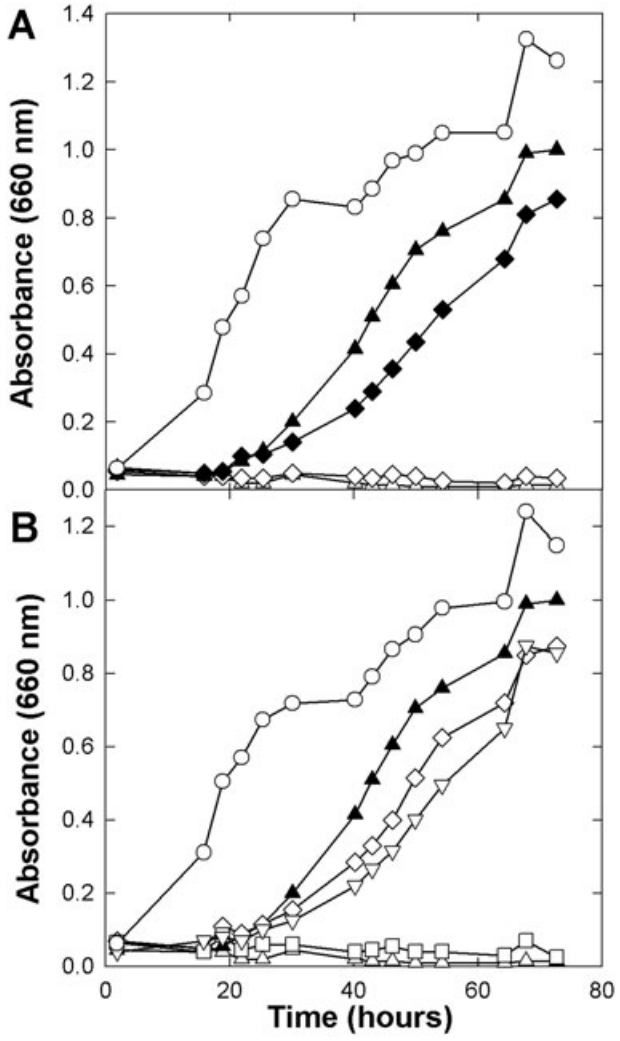

Fig. 6. Effect of the aryl acids on growth of the Δ*aroA′* mutant S170. The McNA medium contained 1 mM aryl acids (phenylacetate, *p*-hydroxyphenylacetate and indoleacetate), 1 mM AroAAs (phenylalanine, tyrosine and tryptophan) and 0.5 mM PABA as indicated. When the growth responses were very similar for more than one condition, only the average growth is shown.
A. Aryl acids inhibited the growth of S170. Shown are growth of the wild-type S2 with aryl acids, AroAAs and PABA, or with aryl acids and PABA, or with AroAAs and PABA, or with no addition (○); and growth of Δ*aroA′* mutant S170, with aryl acids, AroAAs and PABA (◆), with aryl acids and PABA (◇), with AroAAs and PABA (▲), and with no addition (△).
B. *p*-Hydroxyphenylacetate inhibited the growth of S170 strain. Shown are growth of the wild-type S2 with AroAAs and PABA, or with phenylalanine, tyrosine, indoleacetate and PABA, or with phenylalanine, *p*-hydroxyphenylacetate, tryptophan and PABA, or with phenylacetate, tyrosine, tryptophan and PABA, or with no addition (○); and growth of Δ*aroA′* mutant S170, with AroAAs and PABA (▲), or with phenylalanine, tyrosine, indoleacetate and PABA (◇), or with phenylalanine, *p*-hydroxyphenylacetate, tryptophan and PABA (□), or with phenylacetate, tyrosine, tryptophan and PABA (▽), or with no addition (△).

able, < 0.1 mU mg^{-1}. These results clearly identified MJ1585 as FBP aldolase in *M. jannaschii*. To confirm these results in *M. maripaludis*, the FBP aldolase activity was measured in cell-free extracts of the wild- type S2, S170 (*ΔaroA′*) and S176 (Δ*aroA′*/pMEV2-aroA′). All three extracts possessed similar FBP aldolase activities of 6.6–7.2 mU mg^{-1}, confirming that *aroA′* did not encode FBP aldolase activity.

FBP aldolase from *M. jannaschii* has been proposed to also biosynthesize DKFP (R.H. White, submitted for publication). If true, the expression of this enzyme might be regulated by aryl acids. In cell-free extracts from wild-type cells grown in McNA medium in the absence or presence of the aryl acids, the specific activities for FBP aldolase were 3.9 and 0.34 mU mg^{-1} respectively. These results supported a role for FBP aldolase in the DKFP pathway.

Discussion

The *in vivo* role of the DKFP pathway for AroAA biosynthesis in *M. maripaludis* is strongly supported by the data presented here. First, the labelling of phenylalanine following metabolic incorporation of [U-^{13}C]-acetate is in agreement with this novel pathway. Second, labelled shikimate and dehydroshikimate were detected following their formation from [U-^{13}C]glucose-6-phosphate, L-homoserine and NAD in cell-free extracts of *M. maripaludis*. This result was identical to that used to initially demonstrate the DKFP pathway in extracts of *M. jannaschii* (White, 2004). Third, the aryl acids are substrates of a second pathway for the biosynthesis of AroAA in *M. maripaludis* and regulate the *de novo* biosynthetic pathway (Porat *et al*., 2004). However, DHQ dehydratase (encoded by *aroD*) activity is only reduced to one-third following growth with aryl acids (Porat *et al*., 2004). Therefore, the nearly complete absence of labelled shikimate and dehydroshikimate production following growth with aryl acids indicates that a step between glucose-6-phosphate and DHQ is strongly regulated. Regulation by aryl acids supports a role for these reactions in the *de novo* pathway. Fourth, the requirement for the ADTH synthase, encoded by *aroA′*, was demonstrated genetically. Deletion mutants of *aroA′* required AroAAs for growth, and genetic complementation restored the wild-type phenotype.

Previously, a bioinformatic analysis annotated the ORFs MJ0400 and MJ1585 from *M. jannaschii* as type I FBP aldolases (Siebers *et al*., 2001). However, as shown here, only MJ1585 and its *M. maripaludis* homologue Mmp0293 are likely to be actual FBP aldolases. Recently, White proposed that DKFP is formed by an additional activity catalysed by FBP aldolase in *M. jannaschii* (Fig. 7A; R.H. White, submitted for publication). Consistent with this hypothesis, the expression of FBP aldolase was greatly reduced following growth of *M. maripaludis* with aryl acids. These results not only linked FBP aldolase

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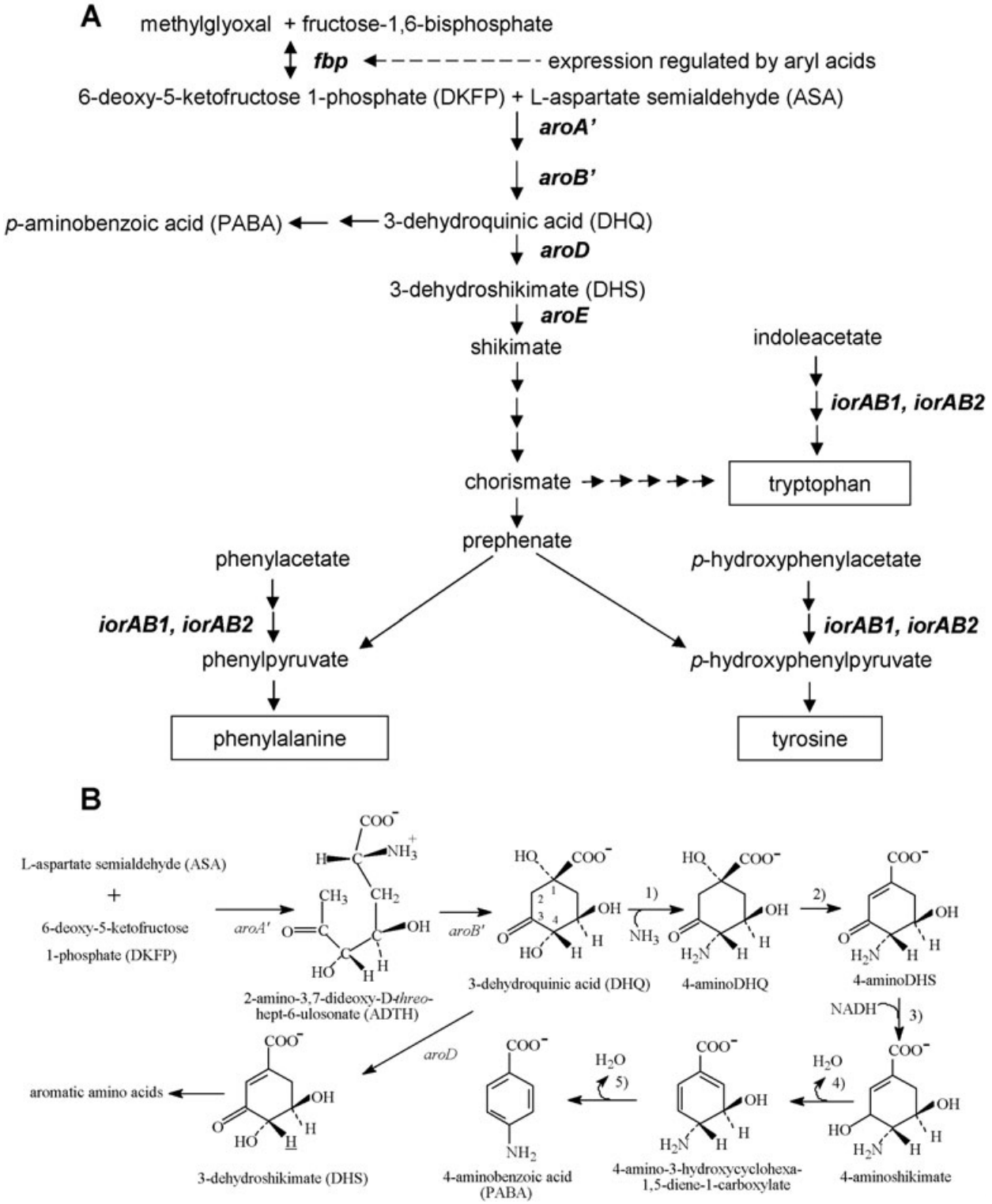

Fig. 7. A. Pathways for the biosynthesis of aromatic amino acids and for the biosynthesis of *p*-aminobenzoic acid. The genes included in the scheme are: *fbp*, *aroA′*, *aroB′*, *aroD*, *aroE*, *iorAB1* and *iorAB2*, encoding for fructose-1,6-bisphosphate aldolase, 2-amino-3,7-dideoxy-D-*threo*-hept-6-ulosonate synthase, DHQ synthase II, DHQ dehydratase, shikimate dehydrogenase and indolepyruvate oxidoreductase I and II respectively.
B. Proposed pathway for the biosynthesis of *p*-aminobenzoic acid in archaea. Five proposed new reactions are numbered: 1, 3-dehydroquinate aminotransferase; 2, 4-aminodehydroshikimate synthase; 3, 4-aminoshikimate dehydrogenase; 4, 4-aminoshikimate dehydratase; and 5, 4-aminobenzoic acid synthase.

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to this novel pathway, but also suggested a possible site for regulation by the aryl acids.

Previously reported metabolic labelling experiments in other methanogens do not distinguish between the canonical and the proposed DKFP pathway (Ekiel *et al*., 1983; 1985a,b; Eisenreich *et al*., 1991; Patel *et al*., 1993; Choquet *et al*., 1994). Following the incorporation of [^{13}C]acetate, $^{13}CO_2$ or [^{13}C]pyruvate, the expected labelling of the AroAAs by both pathways is the same. However, the bioinformatic analysis of the distribution of homologues for the key genes *aroA′* and *aroB′* suggests that the DKFP pathway is widely distributed in the methanogens. In many genomes, these homologues are linked to other AroAAs biosynthetic genes. In addition, none of these genomes possess homologues for DAHP synthase, the first step in the canonical pathway. Interestingly, this analysis also suggests that the DKFP pathway may also be present in some bacteria, such as *Desulfovibrio*. While additional evidence is necessary to confirm this conclusion, it implies that the DKFP pathway may be more widely distributed than originally believed.

This work also demonstrated that PABA is formed from the DKFP pathway *in vivo* and suggested that DHQ is the branch point for the biosynthesis of PABA (Fig. 7A). This conclusion is based on the following evidence. First, the labelling pattern from [U-^{13}C]-acetate was the same for the AroAA tyrosine and the arylamine derived from PABA (Fig. 1), suggesting a common origin. Second, the *aroA′* deletion mutants were PABA auxotrophs, confirming that both AroAAs and PABA share a common source. Third, a Δ*aroD* mutant does not require PABA for growth, and PABA cannot be formed from subsequent steps in the pathway, such as chorismate (Porat *et al*., 2004). Fourth, homologues for the genes required for PABA biosynthesis from chorismate, *pabA*, *pabB* or *pabC* (Green and Nichols, 1991; Viswanathan *et al*., 1995), are absent in the genome of *M. maripaludis* as well as that of other euryarcheotes likely to possess the DKFP pathway (data not shown). This is the first report of an alternative to the chorismate pathway for PABA biosynthesis in a living organism.

A possible new pathway for the biosynthesis of PABA is proposed in Fig. 7B. The critical first step would be the conversion of DHQ into 4-aminodehydroquinic acid (4-aminoDHQ). The subsequent reaction would convert 4-aminoDHQ into 4-aminodehydroshikimate (4-aminoDHS), and this product could be converted into 4-aminoshikimate (step 3 in Fig. 7B) by shikimate dehydrogenase (encoded by *aroE*, Fig. 7A). This suggestion is based on the similarity of the substrates, DHS and 4-aminoDHS; and the possible requirement of NADH for catalysing the formation of 4-aminoshikimate. The last two steps in this proposed pathway would involve two dehydration reactions leading to PABA. Then, except of the possible additional function for AroE, the rest of genes for this new pathway have not been identified in the genome of *M. maripaludis.*

Incorporation of phenylacetate into phenylalanine in the Δ*aroD::pac* mutant previously demonstrated the presence of the second pathway for the biosynthesis of AroAAs in *M. maripaludis* (Fig. 7A and Porat *et al*., 2004). In this pathway, the aryl acids are converted into AroAAs via indolepyruvate oxidoreductases (encoded by *iorAB1* and *iorAB2*). Here, *p*-hydroxyphenylacetate was shown for the first time to be incorporated as an intact unit into tyrosine in the Δ*aroD::pac* mutant. Surprisingly, the aryl acids could not replace the AroAAs requirement for growth of the Δ*aroA′* mutants. In contrast, the aryl acids supported growth of the Δ*aroD::pac* mutant of *M. maripaludis* better than the AroAAs, suggesting that they were assimilated more readily than the AroAAs themselves (Porat *et al*., 2004). The reason for this unexpected phenotype of the Δ*aroA′* mutant is still unclear. However, one possible explanation may be related to the regulation of FBP aldolase by the aryl acids. FBP aldolase is proposed to catalyse the formation of DKFP from methylglyoxal and FBP (Grochowski *et al*., 2006; R.H. White, submitted for publication). In the Δ*aroA′* mutants, lower expression of FBP aldolase as well as reduced demand for DKFP may lead to an accumulation of methylglyoxal. Methylglyoxal is very toxic, and many organisms detoxify it by excretion to the medium or metabolism to other compounds (Ferguson *et al*., 1998; Kalapos, 1999; Booth *et al*., 2003). Thus, the AroAA pathway could be a major sink for methylglyoxal in methanococci. In contrast, the growth of the Δ*aroD::pac* mutant may not be inhibited by the aryl acids because PABA biosynthesis continues to act as a sink for DKFP and, hence, methylglyoxal.

In contrast to the *aroA′* mutants, the Δ*aroB′::pac* did not require AroAAs or PABA for growth. In previous work, White (2004) demonstrated that the recombinant *M. jannaschii* proteins ADTH synthase (AroA′) and DHQ synthase II (AroB′) were required to convert DKFP and aspartate semialdehyde to DHQ *in vitro* (Fig. 7A). While it is possible that the *aroB′* is not involved in the DKFP pathway *in vivo*, the bioinformatic analysis supports a role for this gene. One possible explanation is that a second, presently unidentified ORF complements the function of *aroB′* in the *M. maripaludis*. Because the genome does not possess obvious homologues to *aroB′*, complementation must be by a non-orthologous gene.

Experimental procedures

Synthesis of chemicals

[$^{2}H_2$]*p*-Aminobenzoic acid was prepared as previously described (White, 1985). For the synthesis of [$^{2}H_6$]-*p*-hydroxyphenylacetic acid, *p*-Hydroxyphenylacetic acid

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Table 2. Bacterial strains and plasmids.

Bacterial strain or plasmid	Genotype or description	Source or reference
Methanococcus maripaludis		
S2	Wild-type	Whitman *et al.* (1986)
S87	Δ*aroD::pac*	Porat *et al.* (2004)
Mm900	Δ*hpt*	Moore and Leigh (2005)
S162	Δ*aroA′::pac*	This work
S170	Δ*aroA′*	This work
S140	Δ*aroB′::pac*	This work
S165	Δ*aroA′::pac*/pMEV2-aroA′	This work
S176	Δ*aroA′*/pMEV2-aroA′	This work
Escherichia coli		
DH5α	F^- (*ϕ80dlacZΔM15*), *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* ($r_k^-m_k^+$) *supE44*, Δ(*lacZYA-argF*) U169	Hanahan (1983)
BL21-CodonPlus (DE3)-RIL	*E. coli* B F^-*ompT hsdS*(r_B-m_B-) dmc + Tet^r *E. coli gal* λ(DE3) *endA* Hte[*argU ileY leuW* Cam^r]	Stratagene
BL21-CodonPlus (DE3)-RIL/pMJ1585	*E. coli* BL21-CodonPlus (DE3)-RIL with pMJ1585	R.H. White, submitted for publication
Plasmids		
pIJA03	Pur^r methanogen integration vector	Stathopoulos *et al.* (2001)
pIJA03-aroA′	pIJA03 with the upstream and downstream regions of the *aroA′* gene	This work
pIJA03-aroB′	pIJA03 with the upstream and downstream regions of the *aroB′* gene	This work
pCRPrtNeo	Neo^r marker for positive selection and *hpt* gene for negative selection	Moore and Leigh (2005)
pCRPrtNeo-aroA′	pCRPrtNeo with the upstream and downstream regions of aroA′ gene	This work
pMEV2	Neomycin shuttle vector	Lin and Whitman (2004)
pMEV2-aroA′	pMEV2 with aroA′ gene	This work
pMJ1585	*M. jannaschii* MJ1585 cloned into pT 7-7, under T7 promoter	R.H. White, submitted for publication

(50 mg) was dissolved in 0.3 ml of [U-$2H_4$]-acetic acid (Sigma/Aldrich, St Louis, MO) and 0.8 ml of 20% DCl in ^2H_2O (Sigma/Aldrich). The sample was placed in a sealed glass tube and heated at 190°C for 5 h. The volatile solvents were removed by evaporation with a stream of nitrogen gas, and the sample was washed with water. Yield was essentially quantitative, and the product contained, as measured by mass spectrometry, the following distribution of deuterium 0.1% $^2H_0$, 0.52% $^2H_1$, 2.5% $^2H_2$, 11.1% $^2H_3$, 30.0% $^2H_4$, 36.8% $^2H_5$, 18.9% $^2H_6$. These results indicated that all of the carbon-bonded hydrogens in the molecule had exchanged.

Bacterial strains, plasmids, media, culture conditions and extract preparation

The bacterial strains and plasmids used in this work are listed in Table 2. *E. coli* was grown in Luria–Bertani medium with ampicillin (100 μg ml^{-1}) or chloramphenicol (30 μg ml^{-1}) when needed. *M. maripaludis* was grown with 276 kPa H_2/CO_2 gas [80:20 (v/v)] at 37°C in mineral medium (McN, Whitman *et al.*, 1986) with 10 mM sodium acetate (McNA). When indicated aryl acids (phenylacetate, *p*-hydroxyphenylacetate and indoleacetate, 1 mM each), PABA (0.5 mM), Casamino acids [0.4% (w/v)] or yeast extract [0.2% (w/v)] were added to McNA medium. McCV medium [McNA with Casamino acids, 0.2% (w/v), yeast extract, 0.2% (w/v) and vitamins solution] (Balch *et al.*, 1979) was used when starting growth from colonies or for revival of frozen cultures. Puromycin (2.5 μg ml^{-1}) or neomycin (500 μg ml^{-1}) was added when needed. Only linear growth curves are presented because the rate of transfer of H_2 gas to the liquid medium limits the exponential growth phase even at low cell densities.

For preparation of cell extracts from *M. maripaludis*, cultures were grown in bottles with 100 ml of McNA medium and 138 kPa of H_2-CO_2 gas [80:20 (v/v)] at 37°C. Cell-free extracts were prepared as previously described (Shieh and Whitman, 1987; Lin *et al.*, 2003), except as noted below. For testing FBP aldolase activity, the cells were resuspended in 100 mM Tris/HCl, pH 7.5, 1 mM DTT and 1 mM EDTA. For the detection of shikimate and dehydroshikimate production, cells were resuspended in an extraction buffer of 50 mM TES/KOH pH 7.2, 10 mM $MgCl_2$ and 2 mM DTT. For the analysis of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane, ~0.8 g (wet weight) of *M. maripaludis* wild-type cells was suspended in 2.3 ml of extraction buffer, sonicated at 3°C for 3 min, and centrifuged (14 000 *g*, 10 min) to generate the cell extract.

For preparation of cell extracts from *E. coli* strains, cells were grown in 25 ml of LB medium. For strains with plasmids, the medium also contained chloramphenicol (30 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). To reduce the formation of inclusion bodies, cultures were incubated at 30°C overnight without induction, reaching a final absorbance (660 nm) of 2.0. Cultures were harvested by centrifugation at 8000 *g* for 30 min at 4°C. Then the cells were frozen at –20°C. Upon thawing, the cells were resuspended in 100 mM Tris/HCl pH 7.5, 1 mM EDTA and 1 mM DTT buffer (1.5 vol × wet weight) and passed through a chilled French pressure cell at 110 MPa.

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DNase, 10 U, was added, and the suspension was incubated for 15 min at 37°C. The cell-free extract was obtained by separation of the cell debris and the unbroken cells by centrifugation at 12 000 *g* for 30 min at 4°C. Partial purification of thermal stable protein was performed by incubation of the cell-free extract at 75°C for 20 min and centrifugation in a microfuge at 14 000 r.p.m. for 10 min at room temperature.

Construction of mutants

The mutants S162 (Δ*aroA′::pac*) and S140 (Δ*aroB′::pac*) were constructed as previously described (Porat *et al*., 2004) using the suicide vector pIJA03 (Stathopoulos *et al*., 2001). The primers U1 and U2 and D1 and D2 (*Supplementary material* Table S1) were used to amplify the upstream and downstream regions of *aroA′*; and the primers U3 and U4 and D3 and D4 (*Supplementary material* Table S1) were used to obtain the corresponding fragments for *aroB′*. The upstream fragments were cloned into the XhoI-BglII and XhoI-XbaI sites of pIJA03 for *aroA′* and *aroB′* respectively. The downstream fragments of both ORFs were cloned into the KpnI-NheI sites of pIJA03. The resulting plasmids, pIJA03-aroA′ and pIJA03-aroB′, were transformed into *M. maripaludis* S2 as described previously (Tumbula *et al*., 1994).

The in frame deletion mutant (Δ*aroA′*) was constructed as described by Moore and Leigh (2005). The pCRPrtNeo-aroA′ plasmid was constructed by cloning the upstream and downstream regions of *aroA′*, amplified using the primers S1 and S2 and E1 and E2, into the KpnI-BamHI and BamHI-AflII sites of pCRprtNeo (Moore and Leigh, 2005). In the resulting plasmid, pCRPrtNeo-aroA′, the first 111 bp were fused to the last 132 bp of *aroA′* in frame through an engineered BamHI site, deleting about 70% of the internal portion of the gene. To obtain the initial integration of the entire plasmid in the chromosomal DNA, transformants were plated in McCV medium with aryl acids and neomycin. Colonies obtained were verified by PCR using the primers Ex1 and Ex2. Isolated colonies were inoculated into 5 ml of McCV broth with aryl acids and PABA, incubated overnight and plated on McCV plates with aryl acids, PABA and 8-azahypoxanthine (0.25 mg ml^{-1}) in order to obtain the in frame deletion mutant. The genotype was confirmed by PCR using the primers Ex1 and Ex2. A single colony with the correct genotype, named strain S170, was isolated in this fashion. The DNA sequence at the *aroA′* locus of the strains S2 and S170 was confirmed following amplification of the region with the primers U1 and D2 and the Herculase polymerase (Stratagene) and sequencing with the primers U1, Ex1, D1, D2, Ex2 and U2.

The plasmid pMEV2-aroA′ for complementation of the mutants S162 and S170 was constructed as previous described (Porat *et al*., 2004) using the primers Ex1, Ex2 and Herculase polymerase. The PCR product was cloned into the NsiI-XbaI sites of the methanococcal expression vector pMEV2. Because of a NsiI site in the *aroA′* gene, it was cloned following partial digestion by NsiI. The transformation, plating and storage of the resulting mutants were performed as previously described (Porat *et al*., 2004).

Assay for initial steps of the DKFP pathway

The assay was similar to that used to demonstrate the activity in extracts of *M. jannaschii* (White, 2004). To cell extracts (100 μl) were added 10 μl of [U-^{13}C]-glucose-6-phosphate, 10 μl of 0.1 M L-homoserine, 10 μl of 0.05 M NAD and 10 μl of 0.05 M NADP. The samples were incubated for 30 min at 37°C under argon. After cooling to room temperature, 10 μl of a 5.3 M solution of $NaBH_4$ in water was added. After incubation for 15 min at room temperature, 100 μl of 1 M HCl was slowly added, and the precipitated proteins were removed by centrifugation (14 000 *g*, 10 min). The resulting sample was evaporated to dryness with a stream of nitrogen gas. Methanol, 0.5 ml, was added, and the sample was again evaporated to dryness. This step was repeated an additional three times to remove the borate. Samples were then dissolved in 200 μl of water, passed through a Dowex 50W-X8 H^+ column (2 × 5 mm), evaporated to dryness, and treated for 12 h with 0.5 ml of 1 M HCl in methanol at room temperature. After evaporation of the methanol/HCl with a stream of nitrogen, the entire sample was purified by preparative thin layer chromatography using a solvent consisting of acetonitrile-water-formic acid (88%), 19:2:1 v/v/v. In this solvent system, the methyl ester of (3*R*, 4*S*, 5*R*) shikimate and its (3*S*, 4*S*, 5*R*) isomer (3-*epi*-shikimate), formed by reduction of 3-dehydroshikimate, both had an R_f of 0.59. The area of the plate containing these compounds was removed, and these compounds were eluted with 70 μl of 70% methanol. After evaporation to dryness, the sample was treated with 100 μl of a 50% v/v solution of trifluoroacetic anhydride in methylene chloride for 2 h. After evaporation to dryness, the sample was suspended in 20 μl of methyl acetate for analysis by gas chromatography-mass spectrometry (GC-MS), as previously described (White, 2004).

Analysis of 5-(p-aminophenyl)-1,2,3,4-tetrahydroxypentane portion of methanopterin

To 1 ml of cell extract from *M. maripaludis* S2 was added 0.2 ml of 6 M HCl, and the sample was centrifuged (14 000 *g*, 10 min) to remove the precipitated proteins. After centrifugation, a solution of I_2 in methanol was added to the supernatant until the sample was visibly coloured by the I_2. A few milligrams of Zn dust were added, and the sample was shaken for 10 min. After separation of the Zn by centrifugation (14 000 *g*, 5 min), the sample was heated for 1 min at 100°C and evaporated to dryness with a stream of nitrogen gas. It was then dissolved in 100 μl of water, applied to a Dowex 50W-X8-H^+ column (2 × 10 mm), washed with 500 μl of water, and eluted with 300 μl of 6 M NH_4OH. After evaporation of the ammonia, the sample was reacted with 100 μl of 50% trifluoroacetic anhydride in methylene chloride for 2 h. After evaporation of the solvent, the sample was suspended in 20 μl of methylene chloride in preparation for GC-MS analysis. Some aspects of this procedure have been previously reported (White, 1997; 1998). Mass spectral data for the trifluoroacetyl derivative 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane have been previously reported (White, 1986). This procedure also converted a portion of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane into a cyclic sugar derivative that also contained the 202 m/z fragment, which was used to further confirm the position of label incorporated into the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane.

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Metabolic labelling and analyses of whole cells incubated with [$^2H_6$]-p-hydroxyphenylacetate

For labelling, 300 ml of culture were grown in McNA medium with 0.1 mM phenylacetate, 0.1 mM [$^2H_6$]-*p*-hydroxyphenylacetate and 0.02 mM indoleacetate. Cells were harvested by centrifugation (10 000 *g*, 30 min at 4°C). For the analysis of the deuterium distribution in [$^2H_6$]-*p*-hydroxyphenylacetate remaining in the medium after growth, 20 ml of the growth medium was concentrated to 1 ml by evaporation with a stream of nitrogen gas, acidified to a pH < 1 by the addition of 6 M HCl and extracted two times with 1 ml of methyl acetate. The extracts were combined, dried with Na_2SO_4, evaporated to dryness, converted into the methyl ester trifluoroacetyl derivatives, and analysed as described above. The cells (0.65 g wet weight) were suspended in 2 ml of extraction buffer, sonicated and centrifuged as described above. To 400 µl of this cell extract was added 40 µl of 6 M HCl, and the proteins were removed by centrifugation (14 000 *g*, 10 min). The clear layer was removed and evaporated to dryness with a stream of nitrogen gas. The sample was dissolved in 100 µl of water and passed through a Dowex 50W-X8-H^+ column (1×4 cm). The column was then washed with 10 ml of water and eluted with 10 ml of 6 M NH_4OH. This fraction contained all of the amino-containing compounds present in the cells including tyrosine. After evaporation of the solvent, the residue was dissolved in 0.5 ml of water and applied to a second Dowex 50W-X8-H^+ column (3×10 mm). This column was washed with 900 µl of 1 M HCl and eluted with 600 µl of 6 M HCl. This elution procedure was specifically designed to isolate the PABA and anthranilic acid (*o*-aminobenzoic acid), but it also resulted in the isolation of several of the other amino acids, which were separated during the GC-MS analyses. The 6 M HCl eluant or a portion of the NH_4OH eluant from the first column was evaporated to dryness, converted into the methyl ester trifluoroacetyl derivatives, and assayed by GC-MS as previously described (Zheng *et al*., 1994).

Metabolic labelling of whole cells with [U-^{13}C]-acetate or with [U-^{13}C]-acetate and [^{15}N]-ammonium sulphate

For labelling with [U-^{13}C]-acetate, 800 ml of culture was grown in McN medium with 8.7 mM sodium [U-^{13}C]-acetate (99% of ^{13}C, Sigma/Aldrich), as previously described (Tumbula *et al*., 1997). For labelling with [U-^{13}C]-acetate and [^{15}N]-ammonium sulphate, 120 ml of culture was grown in side-arm bottles of McN medium with 9 mM sodium [U-^{13}C]-acetate and 4.6 mM [^{15}N]-ammonium sulphate as previously described (Tumbula *et al*., 1997; Xia *et al*., 2006). Cells were harvested by centrifugation at 10 000 *g* for 30 min at 4°C. A small part of the [U-^{13}C]-acetate labelled cell pellet (50 mg) was mixed with 1 ml of 6 M HCl, heated at 100°C for 12 h, evaporated to dryness and dissolved in 200 µl of water. The amino acid tyrosine and arylamine were then purified on a Dowex 50W-X8 H^+ column and assayed as described earlier. The same procedure was performed to detect arylamine and tyrosine from 0.79 g of the [U-^{13}C]-acetate and [^{15}N]-ammonium sulphate-labelled cells.

The remainder of the [U-^{13}C]-acetate-labelled cells (2.4 g wet weight) were resuspended in 7 ml of extraction buffer and passed through a chilled French pressure cell at 110 MPa. DNase, 10 U, was added, and the suspension was incubated for 15 min at 37°C. The cell-free extract (6 ml) was obtained by removal of the cell debris and the unbroken cells by centrifugation at 12 000 *g* for 30 min at 4°C. Then 600 µl of 6 M HCl was added to the supernatant. After 30 min at 0°C, the insoluble material was collected by centrifugation at 8700 *g* for 20 min at 4°C.

The insoluble material, which was composed mostly of protein, was washed and acid-hydrolysed as previously described (Porat *et al*., 2004). The aromatic amino acids were separated and purified from the pool of acid-hydrolysed proteins using a Dowex 50W-X8 H^+ cation exchange resin (Baker Analyzed Reagent, Phillipsburg, NJ) packed in 1×15 cm column (Bio-Rad, Hercules, CA). Amino acids were eluted with a step-wise gradient of 1.5 N (10 ml), 2.5 N (35 ml) and 4 N (35 ml) HCl at a flow rate of 1 ml min^{-1} (Stein and Moore, 1950). The amino acids were identified in the 1 ml fractions by thin layer chromatography using silica gel plates and butanol : acetic acid : water (1:2:1) as the solvent (Branner *et al*., 1969). Fractions containing phenylalanine, which eluted after about 20 ml of 4 N HCl, were pooled and evaporated to dryness. After suspending in water, multivalent cations were removed upon passage through 1 ml of chelating resin (iminodiacetic acid, Sigma/Aldrich) and an adsorption resin (4 g, aluminium oxide, Type WN-3: Neutral; Sigma/Aldrich). A sample, containing about 15 µg of phenylalanine, was dried and resuspended in 250 µl of D_2O/DCl.

Proton NMR analysis of ^{13}C-enriched phenylalanine

All ^{1}H NMR data were acquired at 25°C on a Varian Inova 600 MHz spectrometer (599.8 MHz, ^{1}H) using a cryogenic triple-resonance probe. ^{1}H chemical shifts at 25°C were referenced to DSS (2,2-dimethyl-2-silapentane-5-sulphonic acid) via the HDO resonance frequency at 4.81 p.p.m. The ^{13}C decoupling during the acquisition for the decoupled ^{1}H experiments was performed by the GARP method or globally optimized alternating-phase rectangular pulses (Shaka *et al*., 1985). The typical parameters for the experiments were 6000 Hz spectral window, 60 s predelay time, 360 scans and an acquisition time of 2 s for the coupled and 0.5 s for decoupled experiments.

Both the coupled and decoupled spectra were used to calculate the ^{13}C enrichment at each position of phenylalanine with a C-H bond. Following integration, the intensities were normalized between the spectra. To calculate the fraction of ^{13}C atoms, the coupled spectrum was corrected by subtraction of the intensities of contaminating unlabelled compounds from the decoupled spectrum. Because the resonance overlap of the absorbance of the protons from the aromatic and C-3 carbons made it difficult to resolve all the peaks, the ^{1}H-$^{13}C_3$ intensities were compared with the C-2 atom to determine the fractional enrichment.

FBP aldolase assay

The FBP aldolase activity was determined in the catabolic direction in a coupled assay with glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) and triose-phosphate isomerase (EC

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5.3.1.1) as previously described (Siebers *et al*., 2001). The continuous assays were linear with time for at least 10 min. All values reported were the average of three independent determinations. The specific activity is given in mU mg^{-1} [or nanomoles min^{-1} (milligram of protein)$^{-1}$]. The FBP aldolase assays for the recombinant *M. jannaschii* enzyme were performed at 50°C. The *M. maripaludis* activity was assayed at 37°C.

The protein concentration was determined using the bicinchoninic acid assay kit (Pierce, Rockford, IL) after incubation at 90°C in 0.1 M NaOH for 30 min.

Bioinformatic comparison of organisms containing homologues with ADTH synthase and DHQ synthase II

First, BLAST searches at the NCBI website using the ADTH synthase (*M. maripaludis* Mmp0686) sequence were performed. Genomic sequences that contained homologues to ADTH synthase were then tested for homologues of DHQ synthase II (searched with *M. maripaludis* Mmp0006), type I DHQ dehydratases (searched with *M. maripaludis* Mmp1394), or type II DHQ dehydratases (searched with *Desulfovibrio vulgaris Hildenborough* DVU1665), DAHP synthase (searched with *P. furiosus* PF1690), DHQ synthase (searched with *P. furiosus* PF1691), type I FBP aldolase (searched with Mmp0293) and type II FBP aldolase (searched with *D. vulgaris Hildenborough* DVU2143). The percentage of identical amino acids was then obtained by pairwise alignments to the ADTH synthase and DHQ synthase II sequences of *M. maripaludis* by the Bestfit program of the GCG package.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. List of primers.

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