Evolutionary relationship of phototrophic bacteria in the \(\alpha\)-Proteobacteria based on farnesyl diphosphate synthase

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Partial sequences of farnesyl diphosphate (FPP) synthase genes derived from the Rhodobacter–Rhodovulum group and from the Rhodopseudomonas palustris–Bradyrhizobium japonicum group of the \(\alpha\)-Proteobacteria were subjected to phylogenetic analysis to investigate the relationships of phototrophic and non-phototrophic bacteria in the \(\alpha\)-Proteobacteria. The four Rhodovulum species formed a monophyletic group within the Rhodobacter cluster, and Agrobacterium ferrugineum IAM 12616\(^T\) intermingled with the Rhodobacter species. This topology is in good agreement with the 16S rRNA phylogeny, although the FPP synthase gene was more divergent than the 16S rRNA. On the other hand, strains of the phototrophic Rps. palustris formed a cluster far from that of the non-phototrophic Bradyrhizobium japonicum strains. Moreover, Rps. palustris strains were differentiated from the nodule-forming B. japonicum, Mesorhizobium loti MAFF 303099 and Sinorhizobium sp. NGR 234 in the FPP synthase phylogeny. This relationship does not agree with the 16S rRNA phylogeny, wherein Rps. palustris was more closely related to B. japonicum than to strains of the Rhodobacter–Rhodovulum group. These results suggest that the FPP synthase gene of Rps. palustris diverged from that of B. japonicum.

Keywords: Rhodobacter, Rhodopseudomonas, nodule-forming bacteria, phylogeny, isoprenyl diphosphate synthase

INTRODUCTION

Phototrophic bacteria are distinguished from other Proteobacteria by their photosynthetic ability. They do not, however, constitute a phylogenetically homogeneous taxon based on 16S rRNA sequences, and are found in three of the five Proteobacteria classes, namely \(\alpha\)-, \(\beta\)- and \(\gamma\)-Proteobacteria (Stackebrandt et al., 1996), intermingling with non-phototrophic bacteria. For example, Rhodopseudomonas palustris, a phototrophic purple non-sulfur bacterium belonging to the \(\alpha\)-2-Proteobacteria, is phylogenetically close to the nodule-forming, non-phototrophic Bradyrhizobium japonicum (Inui et al., 2001; Fleischman et al., 1995; Wong et al., 1994; Young et al., 1991). Moreover, Rhodobacter species in the \(\alpha\)-3 Proteobacteria are closely related to the non-phototrophic marine Agrobacterium ferrugineum (Uchino et al., 1998). This type of relationship requires further investigation, since the evolution of phototrophic ability among the phototrophic members of Proteobacteria has not been accurately described. Studies on the evolution of photosynthesis have dwelt only on the evolution of the photosynthetic apparatus among the phototrophic species (Xiong et al., 1998, 2000; Nagashima et al., 1997); the diversification of phototrophic bacteria in the Proteobacteria has not been addressed in such studies, and remains unclear.

Farnesyl diphosphate (FPP) synthase, a key enzyme involved in the synthesis of isoprenoid compounds (Ogura et al., 1997), is well conserved among microorganisms (Fujisaki et al., 1990; Koyama et al., 1993; Cunillera et al., 1996; Song & Poulter, 1994; Anderson et al., 1989). This enzyme plays an important role in photosynthesis as well as in the synthesis of other physiologically important compounds. Construction of the FPP synthase gene tree showed similar groupings as...
in the 16S rRNA tree at the suprageneric level within the eubacterial branch; the FPP synthase gene thus appeared to behave in the manner of the 16S rRNA gene (Cantera et al., 2002). Here, we extended our study on the phylogenetic relationship of the FPP synthase genes among phototrophic and non-phototrophic bacteria belonging to the α-Proteobacteria. We also compared the phylogeny of the FPP synthase genes with that of the 16S rRNA, and showed that the FPP synthase genes of some species from the α-2 Proteobacteria may have been acquired by gene transfer.

METHODS

Organisms and growth conditions. Rps. palustris ATCC 17001T, Rhodobacter blasticus NCIMB 11576T, Rps. palustris VA2-2, Rhodobacter sp. AP-10 and Rhodovulum sp. CP-10 were grown as described previously (Kawasaki et al., 1993). Rhodobacter azotoformans JCM 9340T was cultured in medium 200 (Nakase, 1999) while Rhodovulum strictum JCM 9220T and JCM 9221 were grown in medium 194 (Nakase, 1999). All phototrophic cultures were grown under light illumination (2000 lx) at 30 °C for 3–7 days (Kawasaki et al., 1993). A. ferrugineum IAM 12616T was grown aerobically in marine broth (Difco) at 28 °C for 2 days, and B. japonicum IAM 12608T in yeast extract-mannitol (YM) medium for 5–7 days with shaking at 28 °C. Escherichia coli strains used for recombinant DNA manipulations were cultured overnight in Luria–Bertani (LB) medium at 37 °C.

DNA preparation and amplification of FPP synthase genes. Chromosomal DNA was prepared as described by Ausubel et al. (1995). Degenerate PCR primer combinations, which are bound between nucleotide positions 730 and 1170 (E. coli FPP synthase gene numbering; Fujisaki et al., 1990), were FPPaF3 (‘5–CAG GAC GAY MTG CCC KSV ATG GA-3’) and either FPPaR1 (‘5–CYK YSR CRT CSA GAA TGT CGT C-3’) or FPPaR3 (‘5–CYT CGA CRT CSA GAA TRT CRT C-3’). They were designed from the highly conserved regions (regions II and VI) in FPP synthase genes of Rhodobacter sphaeroides ATCC 11167T (accession no. AB028044), Rhodobacter capsulatus ATCC 11660T (AB028046), Rhodovulum sulfidophilum W4T (AB028047) and B. japonicum USDA 110 (U12678) to amplify an approximately 420 bp product. Additional primers with lesser degeneracy and more specific for Rhodobacter–Rhodovulum species, FPPRbaF1 (‘5–CAT GAC GAY ATG CCC TGY ATG GA-3’) and FPPR4 (‘5–TCG TCS GCC AYC TGG AAS GCM AGS C-3’), were later designed from almost exactly the same conserved regions as more sequences became available. The 25 µl reaction mixture consisted of bacterial DNA as the template (10–100 ng), 2.5 mM dNTP mix, 25 pmol of each primer and 1.25 U Taq polymerase (TakaRa Shuzo). The reaction conditions for the primer pair FPPaF3/FPPaR1 were 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min. For the primer pairs FPPaF3/FPPaR3 and FPPRbaF1/FPPR4, initial denaturation was followed by either 6 or 10 cycles of 96 °C for 1 min, 37 °C for 2 min and 72 °C for 1 min, followed by 25 cycles of 96 °C for 1 min, 2 min at either 55 or 57 °C, and 72 °C for 1 min before the final elongation step. This step-up PCR modification was done to increase the number of putative FPP synthase gene products at low annealing temperature, followed by a high annealing temperature to specifically amplify the gene. PCR reaction was carried out using the GeneAmp PCR system 9700 (PE Applied Biosystems).

Sequencing of FPP synthase genes. Purified PCR fragments approximately 400–450 bases long were cloned using a TA cloning kit (Invitrogen) and transformed into competent cells according to the manufacturer’s protocol. DNA sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977) with a BigDye Terminator cycle sequencing kit (PE Applied Biosystems) using an ABI PRISM 310 Genetic analyser (PE Applied Biosystems). Sequence data were collected by means of the program ABI PRISM (Perkin-Elmer) and assembled with an ABI Auto Assembler (Perkin-Elmer).

In silico and phylogenetic analyses. Nucleotide sequences obtained in this work were converted to amino acid sequences after removing the gap-containing and the primer-annealing regions using Genetix-Win software (version 3.1.0). Seven FPP synthase gene sequences obtained from the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/) were combined with the partial sequences for sequence and phylogenetic analyses. A total of 93 amino acids (excluding gaps) were used to generate phylogenetic trees by the neighbour-joining method with CLUSTAL X (Thompson et al., 1997; Jeanmougin et al., 1998) (with 1000 bootstrap replicates using default parameters) and by the maximum-likelihood method (100 replicates using the Jones, Taylor and Thornton method) with MOLPHY version 2.3 (Adachi & Hasegawa, 1996). NJPlot (Perriere & Gouy, 1996) and TreeView (Page, 1996) were used to analyse the phylogenetic relationships of FPP synthase genes from various species. E. coli ispA (the FPP synthase gene in E. coli) was included in the analyses and used as an outgroup.

Nucleotide sequence accession numbers. The sequences determined in this study were submitted to the DDBJ/EMBL/GenBank databases and assigned accession numbers AB053173–AB053178, AB053180 and AB062882–AB062884 (Table 1).

RESULTS AND DISCUSSION

Comparison of the FPP synthase gene sequences of members of the α-Proteobacteria

BLAST searching revealed homologies of as high as 92% between our partial sequences and the FPP synthases from Rba. capsulatus ATCC 11165T, Rba. sphaeroides ATCC 11167T and Rdv. sulfidophilum W4T. The sequences also shared high homology with the FPP synthases (geranyltransferase) of Zymomonas mobilis and B. japonicum USDA 110, thus confirming that our sequences corresponded to those of FPP synthase. Alignment of the partial FPP synthase amino acid sequences with other FPP synthase sequences from several members of the α-2 and α-3 Proteobacteria available in the database showed three of the seven conserved regions (excluding the primer regions) common to all prenyltransferases (Koyama et al., 1993). The resulting alignment produced a high overall amino acid sequence similarity between species of Rhodobacter (75–93.9%) and Rhodovulum (86.8% to as high as 100%) (Table 2). These results suggest that the FPP synthase genes of members of the Rhodobacter–Rhodovulum species are related, and arose from the same ancestral lineage. The non-phototrophic marine A. ferrugineum IAM 12616T on the other hand, is related to the Rhodobacter and Rhodovulum strains, having a mean sequence homology of 69.3±3.5% and 63.0±
0.4%, respectively. Although the gene exhibited high amino acid sequence similarities among species of Rhodobacter and Rhodovulum, the amino acid sequence of FPP synthase is likely to be appropriate for comparisons at the genus and species levels due to the high sequence variation among species of both the Rhodobacter genus and the Rhodovulum genus (ranging from an approximately 5-4% difference between Rba. sphaeroides ATCC 11167 T and Rba. azotoformans JCM 9340 T to as high as 19.4% between Rba. blasticus NCIMB 11576 T and Rhodobacter sp. AP-10). Moreover, the FPP synthase sequence analysed here is not the entire sequence of the gene, and thus it is possible that additional nucleotide bases may be different in the FPP synthase sequence.

The partial FPP synthase sequence of Rps. palustris ATCC 17001 T determined in this study was identical with the FPP synthase (ispA gene, encoding FPP synthase/geranyltranferase) sequence available in the Rps. palustris genome sequence (http://spider.jgi-psf.org/), confirming that the sequence we amplified encodes FPP synthase. The same sequence differed from that of Rps. palustris VA2-2 by at least 3 amino acids, suggesting that FPP synthase is highly conserved among these strains of Rps. palustris. Nevertheless, the three FPP synthases from Rhodopsseudomonas species showed a very low sequence similarity (only 36.8%) with the closest relatives of this species, B. japonicum IAM 12608 T and USDA 110, whereas the FPP synthases of the nodule-forming rhizobia (B. japonicum strains IAM 12608 T and USDA 110, M. loti MAFF 303099 and Sinorhizobium sp. NGR 234) shared a high degree of homology with each other, ranging from 93.9% to 100%. In fact, the FPP synthase of the rhizobial strains had the highest homology values among the strains examined. The very high FPP synthase sequence similarity among the rhizobial strains suggests that the FPP synthase genes of these rhizobia are highly conserved; the FPP synthase gene is not necessarily appropriate for differentiating the nodule-forming species.

**Phylogenetic analysis of the FPP synthase sequence**

The results of the phylogenetic tree inference analysis by the neighbour joining method are shown in Fig. 1. The relationship among species in the α-3 Proteobacteria...

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**Table 1. List of strains and GenBank accession numbers of FPP synthase and 16S rRNA/DNA sequences used in this study**

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<th>Species</th>
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<th>Strain designation*†</th>
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<td>Escherichia coli</td>
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<td>K-12 T</td>
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* Type strain designation (T) may have differed between the FPP synthase DNA and 16S rRNA/dDNA depending on the culture collection from which the strains were acquired.
† Culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; USDA, United States Department of Agriculture, Washington DC, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Japan; IAM, Institute of Applied Microbiology, Univ of Tokyo, Tokyo, Japan; NCDO, National Collection of Dairy Organisms, Aberdeen, UK; JCM, Japan Collection of Microorganisms, Saitama, Japan.
‡ Determined in this study.
§ Isolated and characterized by Kawasaki (1997).
|| Isolated and characterized by H. Kawasaki (unpublished results); this strain shares a high DNA–DNA homology (> 70%) with Rps. palustris ATCC 17001 T.
Table 2. Amino acid sequence similarity (%) of the FPP synthase gene of selected members of α-Proteobacteria used in this study

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Fig. 1. Phylogeny of the FPP synthase gene (a) and 16S rDNA (b) showing the relationships among the α-2 and α-3 Proteobacteria constructed using the neighbour-joining method. Branches supported by 90% or more using the neighbour-joining method are shown in bold; bootstrap values greater than 90% are shown above the branch. Bootstrap values greater than 90% from the maximum-likelihood method are shown below the branch. Arrows point to the positions of each group in the 16S rRNA tree. Bar, 10 nucleotide or amino acid substitutions per 100 nucleotides.
based on the FPP synthase amino acid sequences is consistent with the results obtained from 16S rRNA sequences.

**FPP synthase tree supports the grouping of the α-3 Proteobacteria**

Representative members (Rhodobacter and Rhodovulum spp. and A. ferrugineum IAM 12616) of the α-3 subgroup of the Proteobacteria formed a highly supported phylogenetic cluster that is clearly distinct from other taxa. This suggests that the FPP synthase genes of members of the α-3 Proteobacteria have the same evolutionary origin. This was further supported by the high sequence similarity among the representative members, and is thus consistent with our initial analysis (to be published elsewhere) that the FPP synthase gene reflects the species phylogeny. Our phylogenetic analysis also showed a monophyletic grouping of the marine Rhodovulum spp. separated from the freshwater Rhodobacter spp. that confirms the result of the 16S rRNA phylogeny (Hiraishi & Ueda, 1994). The same tree also confirms the close association of Rba. sphaeroides and Rba. azotoformans, as well as Rba. blasticus and Rba. capsulatus.

One notable deviation of the FPP synthase phylogeny from the 16S rRNA phylogeny is the positioning of the non-phototrophic A. ferrugineum IAM 12616 outside the Rhodobacter clade. This organism is closely related to the members of the phototrophic Rhodobacter genus, and its closest neighbors are Rba. azotoformans and Rba. sphaeroides (Uchino et al., 1997) based on 16S rRNA phylogeny, although it differs from the species of Rhodobacter in its G + C content, and by the absence of photosynthetic abilities, bacteriochlorophyll ε and intracytoplasmic membrane systems, which are defining characteristics of the genus Rhodobacter (Uchino et al., 1998). The result of the analysis using the maximum-likelihood method, however, does not support the position of this species outside the Rhodobacter clade, instead placing A. ferrugineum closer to Rba. azotoformans and Rba. sphaeroides. This implies that the position of A. ferrugineum IAM 12616 in the FPP synthase gene tree is uncertain. Nevertheless, our data demonstrate the phenotypic diversity of species in the Rhodobacter–Rhodovulum group, as exemplified by the close association of the non-phototrophic A. ferrugineum IAM 12616 with phototrophic species of the Rhodobacter and Rhodovulum genera in the FPP synthase tree. It is imperative that more detailed taxonomic and phylogenetic studies using other molecular genes as markers, or comparison of the genome contents between this species and species of Rhodobacter, be performed in order to determine an adequate taxonomic position for A. ferrugineum IAM 12616. Our results clearly show that FPP synthase is a useful molecule not only for differentiating Rhodobacter species from Rhodovulum species but also for revealing variations between A. ferrugineum IAM 12616 and the species of Rhodobacter.

**Unusual clustering of representative members of the α-2 Proteobacteria**

The FPP synthase gene of the phototrophic members of the α-2 subgroup, namely Rps. palustris ATCC 17001 and Rps. palustris VA2-2, formed a separate cluster from the two strains of the non-phototrophic B. japonicum, i.e. strains IAM 12608T and USDA 110. As supported by the high homology among the FPP synthase protein sequences, the distance analysis placed the two Rps. palustris strains far from these two strains of B. japonicum (Fig. 1a). This suggests that the FPP synthase genes of these two species are not closely related and probably evolved from different ancestors. Sequence analysis of 16S rRNA placed Rps. palustris and B. japonicum into a single group despite their differing phenotypes: Rps. palustris is phototrophic, whereas B. japonicum is a non-phototrophic, nodule-forming bacterium.

The two B. japonicum strains, on the other hand, tended to cluster and formed a monophyletic group together with the other non-phototrophic bacteria M. loti MAFF 303099 and Sinorhizobium sp. NGR 234, and this cluster was supported by a high bootstrap value of 100%. These organisms, collectively referred to as rhizobia (Van Rijn & Vanderleyden, 1995), are capable of eliciting nodules on their leguminous hosts. Although these rhizobia shared several morphological and biochemical properties, particularly the ability to form nodules in their hosts, they differed from each other in terms of host specificity and did not form a monophyletic group in the 16S rRNA phylogeny, but rather were placed in several well-separated genera. Reconstruction of the evolutionary history from 16S rRNA gene sequence divergence showed that B. japonicum IAM 12608T and B. japonicum USDA 110 are more related to Rps. palustris strains than to either Sinorhizobium sp. NGR234 or M. loti MAFF 303099 (Fig. 1b). In the FPP synthase tree, however, the positioning of M. loti MAFF 303099 closer to Sinorhizobium sp. NGR 234 than to the B. japonicum strains was similar to the positioning in the 16S rDNA phylogeny. Hence, it is interesting to note that in the FPP synthase gene tree, the non-phototrophic rhizobia (B. japonicum IAM 12608\(^T\), B. japonicum USDA 110, Sinorhizobium sp. NGR234 and M. loti MAFF 303099) were all included in a single cluster supported by a significant bootstrap value of 100%, suggesting that their FPP synthase genes share a common ancestor. This was further supported by the high mean sequence similarity value of their FPP synthases. The longer branch length in the phylogenetic tree that supports the cluster of rhizobia, and the higher homology values of the FPP synthase genes as compared to the 16S rDNAs of rhizobia, are suggestive of a recent lateral gene transfer event before their diversification into their present genera. The lateral transfer of symbiotic genes mediated by plasmids and phages in rhizobia has been reported to be common (Sullivan et al., 1995; Sullivan & Ronson, 1998), and it is not impossible that FPP synthase gene transfer occurred among these rhizobia. Analysis of the M. loti MAFF
303099 chromosome and the plasmid of Sinorhizobium sp. NGR 234 showed that the FPP synthase genes of these rhizobia are located in the symbiotic island region, flanked by highly conserved genes/ORFs (> 90% similarity), and included in a cluster containing the nitrogen fixation and nodulation genes (data not shown). Based on the nucleotide sequence analyses, the FPP synthase gene of B. japonicum strains would have been transferred before the divergence of M. loti MAFF 303099 and Sinorhizobium sp. NGR 234. However, the mechanism of FPP synthase gene transfer in this case remains to be clarified. Alternatively, since the FPP synthase gene is within the symbiotic island region proximal to the nitrogen fixation and nodulation genes, the gene might have played a major role in nodulation; the high similarity of the FPP synthase gene could be due to high selection pressure restricting its divergence.

In summary, the overall FPP synthase tree topology is in good agreement with the 16S rRNA phylogeny, although the FPP synthase gene was more divergent than the 16S rRNA. The greater sequence variations of the FPP synthase gene than the 16S rRNA make this gene useful for indicating the phylogenetic relationships among species belonging to a genus, and relationships among several genera. The use of the FPP synthase gene to clarify phylogenetic relationships at the inter- and intragenic levels may not be appropriate for rhizobial strains due to the high homology among FPP synthases of rhizobia. Nevertheless, the topology of the FPP synthase tree is useful for identifying unusual evolutionary processes that probably occurred during the diversification of the Proteobacteria, and particularly in the case of the α-2 Proteobacteria, as observed from the FPP synthase genes of Rps. palustris and B. japonicum. The discrepancies detected between the FPP synthase phylogeny and the 16S rRNA phylogeny suggest that lateral gene transfer had occurred. FPP synthase sequences therefore offer one of the best opportunities to establish the relationships among the phototrophic Rps. palustris and the nodule-forming bacteria.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Encouragement of Young Scientist (12760056) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, to H. Kawasaki-Nakagawa.

This paper represents a portion of the dissertation submitted by J. J. L. Cantera to Osaka University in partial fulfillment of the requirements for a PhD degree.

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Received 16 January 2002; revised 5 March 2002; accepted 11 March
2002.