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Alteration of product specificity of Aeropyrum pernix farnesylgeranyl diphosphate synthase (Fgs) by directed evolution

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Directed evolution of the C25 farnesylgeranyl diphosphate synthase of Aeropyrum pernix (Fgs) was carried out by error-prone PCR with an in vivo color complementation screen utilizing carotenoid biosynthetic pathway enzymes. Screening yielded 12 evolved clones with C20 geranylgeranyl diphosphate synthase activity which were isolated and characterized in order to understand better the chain elongation mechanism of this enzyme. Analysis of these mutants revealed three different mechanisms of product chain length specificity. Two mutants (A64T and A64V) have a single mutation at the 8th amino acid upstream of a conserved first aspartate-rich motif (FARM), which is involved in the mechanism for chain elongation reaction of all prenyl diphosphate synthases. One mutant (A135T) carries a single mutation at the 7th amino acid upstream of another conserved region (G141,Q142), which was recently found to be another important region controlling chain elongation of a type III C20 geranylgeranyl diphosphate synthase and Escherichia coli C15 farnesyl diphosphate synthase. Finally, one mutant carrying four mutations (V84I, H88R, I177 M and M191V) is of interest. Molecular modeling, site-directed mutagenesis and in vitro assays of this mutant suggest that product chain-length distribution can be also controlled by a structural change provoked by a cooperative interaction of amino acids.

Keywords: carotenoid/in vitro evolution/FGPP synthase/prenyl diphosphate synthase

Introduction

Prenyl diphosphate synthases catalyze the sequential head-to-tail condensation reactions of isopentenyl diphosphate (IPP, C5) with different lengths of allylic diphosphates to produce linear isoprenoid derivatives (Figure 1). The isoprenoids of different chain lengths are then transformed or modified into bioactive compounds such as steroids, quinones, prenylated proteins, lipid components and carotenoids. Depending on the stereochemistry of the double bond present in the final product, cis- and trans-type synthases can be distinguished (Liang et al., 2002). trans-Prenyl diphosphate synthases can further be classified into short (C15–C25), medium (C30–C50) and long chain (C40–C50) based on the chain lengths of the isoprenoid product. A number of different trans-prenyl diphosphate synthases, especially farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) synthases, from different sources have been studied structurally and enzymatically (Kellogg and Poulter, 1997).

Short-chain prenyl diphosphate synthases precisely control a definite chain length for their final product, which can be geranyl diphosphate (GGP, C10), farnesyl diphosphate (FPP, C15), geranylgeranyl diphosphate (GGPP, C20) or farnesylgeranyl diphosphate (FGPP, C25) (see Figure 1). Even though the chain lengths of the final products of these enzymes vary, they share similar tertiary structures and conserved regions in their amino acid sequences. Two highly conserved aspartate-rich motifs, namely a first aspartate-rich motif (FARM) and a second aspartate-rich motif (SARM), and also five other conserved regions can be identified (Chen et al., 1994; Wang and Ohnuma, 1999). The crystal structure of avian FPP synthase (Tarshis et al., 1994) showed that the aspartate-rich motifs (FARM and SARM) are involved in substrate binding and catalysis with a cofactor metal ion such as Mg2+. Structure-based mutational analysis of a number of short-chain prenyl diphosphate synthases indicated that amino acids upstream of the FARM, especially the 11th, 8th and 5th positions, are of importance in determining

Fig. 1. Isoprenoid precursor pathway for carotenoid biosynthesis in recombinant E.coli. Steps within the native E.coli isoprenoid biosynthesis pathway are replaced with the heterologous FGPP synthase and combined with carotenoid biosynthetic pathway enzymes (cit) to generate a visible color screen. The enzymes involved in the above pathway are isopentenyl diphosphate (IPP) isomerase (IDI); farnesyl diphosphate synthase (IspA); farnesylgeranyl diphosphate (FGPP) synthase; geranylgeranyl diphosphate (GGPP) synthase (CrtE); phytoene synthase (CrtB); in vitro evolved phytoene desaturase (CrtI4).
the chain lengths of isoprenoids (Ogura and Koyama, 1998). Studies of short-chain prenyl diphosphate synthases have so far mostly been focused on C15 FPP synthases and C20 GGPP synthases of *Saccharomyces cerevisiae* (Hemmi et al., 2003), *Salinobacter ruber* (Ohnuma et al., 1996a) and *Bacillus stearothermophilus* (Ohnuma et al., 1996b).

Recently, a short-chain prenyl diphosphate synthase with novel chain length specificity for farnesylgeranyl diphosphate (crtB, C25) has been characterized from the hyperthermophilic archaeon *Aeropyrum pernix* (Tachibana et al., 2000). To investigate the mechanisms of product chain length specificity of *A.pernix* C25 GGPP synthase (Fgs), we chose to use an in *vitro* evolution approach to alter the chain length specificity of Fgs from C25 GGPP to C20 GGPP. A directed evolution approach does not rely on assumptions about enzyme activity drawn from known structures and can therefore provide novel insights into a catalytic mechanism of interest and identify novel structural regions for prenyl diphosphate activity.

To screen the generated Fgs mutant libraries for the desired phenotype, we utilized a visual screen in which GGPP production is linked to carotenoid biosynthesis in recombinant *Escherichia coli* cells (see Figure 1), similar to a screen developed previously for a B.stearothermophilus FPP synthase (FGPP synthase) library generated by chemical random mutagenesis (Ohnuma et al., 1996b). Molecular modeling studies and site-directed mutagenesis were utilized to provide insight into specific structural mechanisms of chain-length determination.

**Materials and methods**

**Genes, plasmids and culture conditions**

The genes and plasmids described in this section are summarized in Table I. For assembly of *crtB* and *crtI14*, genes encoding phytoene synthase (*crtB*) or mutant phytoene desaturase (*crtI14*) were subcloned from pUCmod into the SalI site (crtB) and HindIII (crtI14) sites of pACmod (Schmidt-Dannert et al., 2000) by amplification of the genes together with the modified constitutive lac promoter, using primers that introduce the corresponding restriction enzyme sites at both ends, to give pAC_crtB_crtI14 where *crtB* and *crtI14* have the same orientation as the disrupted tetracycline resistance gene (Table I).

For carotenoid production, recombinant *E.coli* JM109 were cultivated for 48 h in the dark at 28°C in Luria–Bertani (LB) medium (100 or 500 ml of medium) supplemented with the appropriate selective antibiotics chloramphenicol (50 μg/ml) and/or carbenicillin (100 μg/ml).

**Error-prone PCR mutagenesis**

The *fgs* gene was amplified under optimized mutagenic PCR conditions with PCR primers (5′-CCGACTTGAGAAGCCG-3′ and 5′-AGAAGCCGTCACGGG-3′) flanking the gene and promoter region. The PCR reaction mix consisted of 1× Promega Mg^2+^ free thermophilic buffer (Promega, Madison, WI), 10 ng/ml template plasmid, 1 μM of each primer, 2.5 U Taq DNA polymerase, 2.5 mM MgCl2, 25 μM MnCl2, 0.3 mM

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<td>pUCmod</td>
<td>Constitutive expression vector modified from pUC19, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Schmidt-Dannert et al. (2000)</td>
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<td>pACmod</td>
<td>Cloning vector modified from pACYC184 for constitutive expression of <em>crt</em> genes subcloned (including their individual promoters) from pUCmod, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Schmidt-Dannert et al. (2000)</td>
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<tr>
<td>pET20b</td>
<td>Inducible expression vector, T7 promoter, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<td>pACmod expressing <em>crtB</em> and <em>crtI14</em></td>
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dGTP/dATP and 0.25 mM dTTP/dCTP. PCR was carried out with a program of 95°C for 4 min followed by 32 cycles of 94°C for 1 min, 50°C for 40 s and 72°C for 1 min and finally 72°C for 7 min. The 1.3 kb PCR product was purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA), digested with EcoRI and NotI, ligated into the corresponding sites of vector pUCmod and transformed into competent E.coli JM109 [pAC_crtB_crtI14]. Transformants were plated on LB plates supplemented with 100 µg/ml carbenicillin and 50 µg/ml chloramphenicol. After 18 h of incubation at 30°C in the dark, colonies were replicated using a white nitrocellulose membrane and transferred on to fresh LB plates containing the same antibiotics. Colonies were screened visually for color variants after an additional 24 h of incubation (or until color developed) at room temperature. Overnight cultures (3 ml of LB) were inoculated with selected colonies for analysis of carotenoid synthesis. Plasmids from positive clones (showing a strong red/pink color) were isolated and retransformed into fresh competent E.coli cells [pAC_crtB_crtI14]. Mutations of fgs sequence were confirmed by DNA sequencing of the entire plasmid inserts.  

Expression and purification of the wild-type and mutated FGPP synthases

The wild-type and mutated fgs genes (M1 and M4) were amplified with a forward primer containing at its 5' end a NdeI site (underlined) (5'-GGAATTCCATATGCTCATAGACCAC-3') and a reverse primer containing at its 5' end a XhoI site (underlined) (5'-CCGCTGACGCTCACCTCCTCAC-3') flanking the gene. The PCR products were purified and digested with the restriction enzymes NdeI and XhoI. The DNA fragments were ligated into the NdeI/XhoI-treated pET20b (Invitrogen) to construct pET_fgsW_His, pET_fgsM1_His and pET_fgsM4_His, which express His<sub>6</sub>-tagged wild-type and mutant FGPP synthase. E.coli BL21(DE3) having each of the plasmids were grown at 37°C in 500 ml of LB supplemented with 100 µg/ml carbenicillin. The recombinant cells were induced with isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM when A<sub>600</sub> reached 0.5. Cells were incubated at 28°C for 12 h after induction and were harvested by centrifugation. The harvested cells were washed with 50 mM sodium phosphate buffer (pH 8.0) containing 50 mM NaCl and 5 mM imidazole. Ten units of DNase were added to 10 ml of the cell suspension and incubated on a rocking plate at 150 r.p.m. at room temperature for 15 min. Cells were then sonicated (Branson Digital Sonifier 250) on ice (30% power with a period of 4×20 s pulse-on and 4×10 s pulse-off) and cell debris was removed by centrifugation at 13 000 g for 30 min at 4°C. The supernatant was loaded onto a pre-equilibrated Ni-agarose column (7.5 ml; Qiagen) and washed with buffer A (pH 8.0, 50 mM sodium phosphate, 300 mM NaCl) and buffer B (pH 8.0, 50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole). The proteins were eluted with buffer C (pH 8.0, 50 mM sodium phosphate, 300 mM NaCl, 300 mM imidazole). Fractions containing the recombinant protein (15 ml) were immediately pooled, concentrated and then diluted repeatedly with 50 mM Tris–HCl buffer (pH 8.0) in a Centricon YM-10 centrifugal filter (Millipore) and finally concentrated to 0.2 mg/ml. Proteins were quantified by the Bradford method and homogeneity was confirmed by SDS–PAGE with Coomassie Brilliant Blue staining (data not shown).  

Prenyltransferase activity and product analysis by HPLC and LC–mass spectrometry (MS)  

The activity of wild-type and mutated FGPP synthases was assayed in a 200 µl reaction volume of 70 mM Tris–HCl buffer (pH 7.6), 5 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 100 µM allylic substrates GPP and IPP and 20 µg of the purified recombinant enzyme. The reaction mixture was incubated at 37°C for 2 h. After filtration, the reaction mixture was directly applied to a Zorbax RX-C18 column (4.6×150 mm, 5 µm; Agilent Technologies) without further purification and eluted under gradient conditions with acetonitrile and 25 mM NH<sub>4</sub>HCO<sub>3</sub> at a flow rate of 1 ml/min (Zhang and Poulter, 1993) using an Agilent 1100 HPLC system equipped with a photodiode-array detector. A linear gradient was used for the separation of phosphorylated isoprenoids (0–5 min, 25 mM NH<sub>4</sub>HCO<sub>3</sub>; 5–30 min, 10–100% acetonitrile) and the eluate was monitored at 214 nm. The reaction samples were further analyzed on an LCQ mass spectrometer (Thermo Finnigan) equipped with a Zorbax RX-C18 column (4.6×250 mm, 5 µm; Agilent Technologies). Molecular ions (M + H<sup>+</sup>) of phosphorylated isoprenoids (GPP, FPP, GGPP, FGPP) were detected in the negative ion electrospray ionization (ESI) mode. Retention times and mass spectra of GPP, FPP and GGPP were compared with those of authentic samples purchased from Sigma-Aldrich and retention times and mass spectra of FGPP were compared with published data (Zhang and Poulter, 1993) as it is not commercially available.  

Site-directed mutagenesis

Site-directed mutagenesis was carried out with mutagenic primers following a QuikChange protocol (Stratagene). Introduction of the mutation was confirmed by DNA sequencing.  

Isolation and analysis of carotenoids

Wet cells from a 100 ml culture (~250 mg) were repeatedly extracted at 4°C with a total volume of 35 ml of acetone until all visible pigments had been extracted. After centrifugation (4°C, 4000 r.p.m.), the colored supernatants were further purified as described (Lee et al., 2003). Aliquots of 1–3 µl of the fractions and the crude extracts were subjected to thin-layer chromatography (Whatman silica gel plate, 4.5 mm particle size, 200 mm thickness) for initial analysis of the crude extract using hexane–chloroform (100:5) as the mobile phase. For the HPLC analysis of carotenoids, 20 µl of the crude extracts were applied to a Zorbax SB-C18 column (4.6×150 mm, 5 µm; Agilent Technologies) and eluted under isocratic conditions with a solvent system containing 80% acetonitrile, 15% methanol and 5% 2-propanol at a flow rate of 1 ml/min using an Agilent 1100 HPLC system equipped with a photodiode-array detector. Carotenoids were identified by a combination of HPLC retention times, absorption spectra and mass spectra. The relative amounts of carotenoids were calculated by comparing integrated peak areas from the chromatograms and results from three separate cultures averaged for each analysis. Mass spectra were monitored in a mass range of m/z 200–800 on an LCQ mass spectrometer (Thermo Finnegan) equipped with an atmospheric pressure chemical ionization (APCI) interface.  

Alignment and modeling

The model structure of Fgs was obtained with the recently published E.coli IspA (PDB database entry 1RQJ) (Hosfield...
et al., 2004) as a known X-ray structure using SWISS-PDB software (Schwede et al., 2003).

Results

In vivo activity of wild-type A. pernix FGPP synthase in E. coli

The fgs gene encoding A. pernix farnesylgeranyl diphosphate (FGPP) synthase was cloned into pUCmod (Schmidt-Dannert et al., 2000) for constitutive expression in E. coli. Co-formation with a compatible plasmid pAC_crtB_crtI14 constitutively expressing crtB (encoding a phytoene synthase) and crtI14 [encoding a mutant phytoene desaturase (Schmidt-Dannert et al., 2000)] was used to detect positively evolved FGPP synthases producing C20 GGPP instead of C25 FGPP. As shown in Figure 1, if C20 GGPP is supplied (by CrtE in the native biosynthetic pathway), CrtB and CrtI14 will sequentially catalyze the head-to-head condensation of two molecules of C20 GGPP to produce colorless C20 phytoene and the desaturation of phytoene to produce colored carotenoids. In our visual screening system, the previously phytoene and the desaturation of phytoene to produce colored carotenoids. In our visual screening system, the previously reported mutant CrtI14 was utilized as it produces a mixture of the pink/red carotenoid tetradehydrolycopene (hmax 493, 516, 540 nm) and lycopene (hmax 449, 475, 507 nm), which generates a pink/red color phenotype which is more easily detected visually on a white nitrocellulose membrane when compared with wild-type CrtI, which produces the yellow/orange carotenoid lycopene alone.

E. coli [pAC_crtB_crtI14] colonies co-expressing Fgs appeared very weakly colored compared to the white E. coli [pAC_crtB_crtI14] colonies, indicating that a very small amount of C20 GGPP is released from Fgs during the stepwise prenyl chain elongation to synthesize C25 FGPP (see Supplementary data available at PEDS Online).

Random mutagenesis of A. pernix FGPP synthase

The fgs gene encoding FGPP synthase was amplified under mutagenic PCR conditions and the mutated fgs gene library was transformed as pUC_fgs into E. coli cells [pAC_crtB_crtI14]. Among ~7000 clones visually screened on white membrane filters, 12 distinguishable clones with a strong pink/red phenotype were isolated and the plasmid insert DNA sequenced. Based on the deduced amino acid sequence of the isolated 12 mutant clones (M1 to M12), four Fgs mutants were identified to have unique mutations in their amino acid sequence (Table II): three single-point mutants (A64T, A64V or A135T) and one multi-point mutant (V84I, H88R, I177M and M191V).

Because the dynamic range (upper visual detection limit) of the carotenoid color screen is limited, carotenoid production of the two clones at levels similar to those observed in the presence of the heterologous CrtE gene encoding GGPP synthase (Lee et al., 2003) strongly indicates that the Fgs mutant clones produce significant amounts of C20 GGPP and therefore have new GGPP synthase activity.

Analysis of in vitro prenyl diphosphate synthase activity of Fgs variants

To analyze the actual prenyl product spectrum of evolved Fgs variants in comparison with the wild-type enzyme, mutants M1 and M4 producing different carotenoid levels (Figure 2) were chosen for an in vitro assay. The His6-tagged enzymes were expressed on pET_fgsW_His, pET_fgsM1_His and pET_fgsM4_His in E. coli BL(DE3) cells and purified by Ni-metal affinity chromatography.

Purified wild-type Fgs produced only C25 FGPP ([M – H]+ = 517.1) without C20 GGPP ([M – H]+ = 449.1), C30 HexPP ([M – H]+ = 585.1) or longer products as detected by HPLC and LC–negative ESI-MS (Figure 3). Similarly, the purified mutant M1 produced in vitro only C20 GGPP ([M – H]+ = 449.1) as a final product without C15 FPP ([M – H]+ = 381.1) or C25 FGPP (Figure 3). Interestingly, mutant M4 generated a mixture of C15 FPP ([M – H]+ = 381.1) and C20 GGPP ([M – H]+ = 449.1) without the formation of FGPP and the total amount of the products was less than that of other mutants (Figure 3).

Sequence analysis and molecular modeling of wild-type and mutant Fgs

In order to evaluate the mechanisms by which the observed amino acid substitutions influence product chain length,
structural models of the mutant and wild-type Fgs were constructed based on the recently solved crystallographic structure of IspA from E.coli (Hosfield et al., 2004). Although the overall homology between the two enzymes is relatively low (~30% amino acid sequence identity), comparison to the known structure of IspA revealed a structural core model for Fgs with a typical 13-helix configuration and a central substrate-binding cavity formed by 10 helices. The outer sphere of Fgs, especially the N- and C-termini, had little structural homology to IspA, whereas the core region around the substrate-binding cavity (Figure 4). For example, the calculated distance between Ala64 and Ala64 in wild-type Fgs is 4.90 Å whereas the distance between Thr64/Val64 and Ala135 in M12 is reduced to 3.82 and 3.97 Å, respectively (Figure 4). Hence the mutants M1 and M12 seem to block further chain elongation of GGPP to FGPP and produce C20 GGPP exclusively as detected in vitro (Figure 3).

Unlike other mutants and the wild-type enzyme, M4 (V84I, H88R, I177M and M191V) produced a mixture of C15 FPP and C20 GGPP (Figure 3) and consequently synthesized in vivo less C40 carotenoids derived from the condensation of 2 × C20 GGPP. Sequence alignment shows that even though V84I and H88R are the 8th and 12th amino acids, respectively, downstream of the extended FARM region (Ala64) upstream of FARM and the 7th amino acid (Ala135) in M12 is reduced to 3.82 and 3.97 Å, respectively (Figure 4). Hence the mutants M1 and M12 seem to block further chain elongation of GGPP to FGPP and produce C20 GGPP exclusively as detected in vitro (Figure 3).

Site-directed mutagenesis of in vitro evolved Fgs mutants

Site-directed mutagenesis was carried out on the wild-type and mutant Fgs clones in order to understand better the function of the altered amino acids. The importance of the 8th amino acid (Ala64) upstream of FARM and the 7th amino acid (Ala135) upstream of the conserved region (A141GQ142) were further examined by changing Ala64 and Ala135 into the smaller amino acid Gly64 or Gly135 by site-directed mutagenesis to examine the determining factors in the altered product chain-length of this mutant (see below).
prenyl diphosphate synthase activity. This may be the result of either a loss of enzymatic activity or a reduction in the expression or stability of the enzyme.

Based on the mutational studies, isoprenoid product spectrum and the model structure, the mutant M4 shows unusual characteristics. Therefore, eight additional daughter mutants (Table II) were created by reverting mutations present in M4 and co-expressed in E.coli [pAC_crtB_crtI14] to identify the critical amino acid(s) responsible for the altered activity of M4. First, the four mutants S6, S7, S8 and S12 were created by reverting individually each of the four mutated amino acids in M4 to the wild-type sequence. Among the four mutants, only S12 (V84I, H88R and I177M) showed a similar level of carotenoid production to M4 whereas the remaining mutants S6, S7 and S8 showed complete loss of color phenotype, suggesting the complete loss of prenyl diphosphate synthase activity (Table II). However, the additional four mutants S9, S10, S11 and S13, each carrying only one of the mutated amino acids present in M4, showed background carotenoid production levels similar to wild-type Fgs, suggesting that these single amino acid mutants retain FPP synthase activity but have no GGPP synthase activity (Table II).

Discussion

In this study, we demonstrated for the first time the directed evolution of the novel short-chain prenyl diphosphate synthase, C25 FGPP synthase (Fgs), from the hyperthermophile A.pernix, with a visual screening system where the in vivo product profile of the FGPP synthase is quantitatively and qualitatively linked with engineered tetradehydrolycopene biosynthetic enzymes (CrtB and CrtI14). New information on a mechanism for controlling the chain elongation of FGPP synthase was obtained by a series of in vitro assays, mutational studies and structural models of mutants generated by error-prone PCR.

Twelve positively evolved Fgs mutants (M1 to M12) having GGPP synthase activity were isolated from the library. Four Fgs mutants (M1, M2, M4 and M12) have a unique mutation in their amino acid sequence. The mutants M1 (A64T) and M12 (A64V) demonstrate clearly the significance of the 8th amino acid upstream of the FARM (72 DDIMD76) conserved in all prenyl diphosphate synthases. So far, most studies have been intensively aimed at the conserved FARM, especially the 5th, 6th, 8th and/or 11th amino acid upstream of the FARM, present in C15 FPP and C20 GGPP synthases (Liang et al., 2002).

In vitro and in vivo assays of mutant M1 clearly showed that the 8th amino acid upstream of the FARM is an important determinant in controlling the chain elongation of C25 FGPP synthase as in the case of the well-studied C15 FPP synthases and C20 GGPP synthases (Ohnuma et al., 1998; Kharel et al., 2001; Hemmi et al., 2002). The presence of two alanine residues in these particular positions is a unique feature of wild-type Fgs. Among all short-chain prenyl phosphate synthase sequences, only one other example (Streptomyces argenteolus) (Kawasaki et al., 2003) has an alanine in the position corresponding to A64 and a very few examples
have an alanine in the position corresponding to A135; almost all other sequences hold leucine, isoleucine, cysteine, threonine or serine residues in both positions. Our results suggest that if one of the two positions hosts a larger residue than alanine, the product length is limited to C20 or shorter isoprenoids. If both residues are small amino acids, larger products can be expected as in the case of the C25-producing wild-type Fgs.

Additional new information was obtained from mutant M2 (A135T), carrying a single mutation at the position of Ala135, the 7th amino acid upstream of a conserved region (141GQ142) present in short-chain prenyl diphosphate synthases. A model structure of M2 showed that Ala135 is positioned in an adjacent helix exactly opposite to Ala64 substituted in mutants M1 and M12. Structural modeling and in vivo assays of M1, M2 and M12 suggest that the mutation of either of these particular alanine residues sufficiently blocks the further elongation of GGPP to FGPP by narrowing the substrate-binding channel, thus functioning as its gatekeeper. Recently, Hemmi et al. (2003) showed that *S.cerevisiae* GGPP synthase product specificity can be influenced by mutations in an equivalent region. Therefore, mutant M2 confirms that chain elongation reactions of short chain prenyl diphosphate synthases (C15–C25) can be controlled by changing amino acids near the conserved region (141GQ142), providing an additional control mechanism to the 5th, 6th, 8th and/or 11th amino acids upstream of the FARM.

Mutant M4 carries multiple mutations of amino acids (V84I, H88R, I177M and M191V) and produces in vitro a mixture of C15 FPP and C20 GGPP not observed with the other evolved mutants. Because the mutated amino acids do not have any apparent relationship with known conserved regions and no structural explanation for their influence on enzyme function was found, site-directed mutagenesis was performed to investigate mutant M4. The single mutants S9, S10, S11 and S13, each carrying one of the four mutated amino acids in M4, showed only background C20 GGPP synthase activity, as shown by the cell color phenotype and HPLC traces. Furthermore, the three triple mutants S6, S7 and S8 showed a complete loss of color phenotype, suggesting the complete loss of prenyl diphosphate synthase activity. Therefore, we suspect that a cooperative structural rearrangement provoked by those amino acids may alter the substrate-binding channel and thus control the chain elongation reaction without alteration of amino acids within the substrate-binding region.

In summary, directed evolution was used to create mutant FGPP synthases that confirm the importance of amino acids upstream of the FARM of prenyl diphosphate synthases and demonstrate the significance of mutations upstream of an additional conserved region (141GQ142), which has recently been recognized in only a type III C20 GGPP synthase and *E.coli* C15 FPP synthase.

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**References**


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**Directed evolution of the Fgs of A.pernix**

![Fig. 4. Three-dimensional model structure of Fgs. Molecular model shows FGPP synthase’s substrate-binding cavity with residues mutated in this study and the FARM region. Also shown are the bound substrate isopentenyl diphosphate (IPP) and the unceavable substrate analog dimethylallyl S-thiolodiphosphate (DMSPP) present in the E.coli IspA structure (PDB 1IRQ).](image-url)