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Biochemical and Biophysical Research Communications 303 (2003) 387–392
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Functional characterization of β-ketoacyl-ACP reductase (FabG) from *Plasmodium falciparum*☆

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Received 12 February 2003

Abstract

The malaria parasite, *Plasmodium falciparum*, unlike its human host, utilizes type II fatty acid synthesis, in which steps of fatty acid biosynthesis are catalyzed by independent enzymes. Due to this difference, the enzymes of this pathway are a potential target of newer antimalarials. Here we report the functional characterization of *Plasmodium* FabG expressed in *Escherichia coli*. The purified recombinant FabG from *P. falciparum* is soluble and active. The K_m of the enzyme for acetoacetyl-CoA was estimated to be 75 μM with a V_{max} of 0.0054 μmol/min/ml and a k_{cat} value of 0.014 s^{-1}. NADPH exhibited negative cooperativity for its interaction with FabG. We have also modeled *P. falciparum* FabG using *Brassica napus* FabG as the template. This model provides a structural rationale for the specificity of FabG towards its cofactor, NADPH.

Keywords: *Plasmodium falciparum*; Malaria; Acyl carrier protein; Fatty acid biosynthesis; β-ketoacyl-ACP-reductase; FabG; Coenzyme A; Acetoacetyl coenzyme A

Malaria remains a major cause of mortality and morbidity in a large part of the world. Two million deaths occur due to infection with *Plasmodium falciparum*, which causes the most severe form of the disease [1]. Many drugs used for treating malaria such as chloroquine, mefloquine, primaquine, artemisinin, methotrexate, pyrimethamine, etc. either act by poorly understood mechanisms or work also on the biochemical machinery of the host [2–4]. Hence, they exhibit harmful side effects. Also the emergence of resistant parasites that are no longer susceptible to the current antimalarials demands the development of new antimalarials that target the parasite specific metabolic pathways alone [4–6]. In this context the fatty acid biosynthesis of the parasite appears to be an attractive target.

Fatty acids are constituents of phospholipids and sphingolipids that make up the plasma membrane and the membranes of intracellular organelles [7,8]. They are also critical for several essential cellular functions including cell signaling and energy storage through the formation of triglycerides [9]. Hence, all the organisms with the exception of Mycoplasmas synthesize them from simple precursors. *P. falciparum* has recently been shown to synthesize fatty acids de novo [10]. The fatty acid synthesis is organized into two distinct ways based on the architecture of the enzymes involved. Type I synthases found in fungi and mammals are large multifunctional enzymes with multiple domains catalyzing various reactions of fatty acid synthesis [11]. Plants, bacteria, and apicomplexans on the other hand use type II systems in which each reaction of fatty acid synthesis is catalyzed by a discrete enzyme [10,11]. During the elongation cycle of FAS II, the acyl chain covalently attached to acyl carrier protein (ACP) is elongated successively by two carbon units by the action of four enzymes acting consecutively. First, β-ketoacyl ACP synthase (either FabB or FabF) elongates the acyl-ACP of C_n acyl chain to a C_{n+2}

☆ *Abbreviations:* ACP, acyl carrier protein; CoA, coenzyme A; PfFabG, *Plasmodium falciparum* β-ketoacyl-ACP-reductase; PfFabI, *P. falciparum* enoyl ACP reductase; FAS, fatty acid synthase.

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0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00321-8

β-ketoacyl form. The β-ketoacyl-ACP thus formed is reduced to β-hydroxyacyl-ACP by an NADPH dependent β-hydroxyacyl-ACP reductase (FabG). The β-hydroxyacyl group is then dehydrated to an enoyl-ACP by a β-hydroxyacyl-ACP dehydratase (FabZ or A). The reduction of enoyl group by an enoyl-ACP reductase (FabI, FabK or FabL) finally produces C_{n+2} acyl-ACP that is ready to reenter the cycle typically until a C14 or a C16 fatty acid is synthesized. The fatty acid thus synthesized is hydrolyzed from ACP for the synthesis of phospholipids or sphingolipids or get diverted for other modifications. Since the parasite employs the type II fatty acid synthesis pathway, taking place in the plastid, which is strikingly different from that of its human host, its inhibition is unlikely to be harmful to the host [10].

Fatty acid synthesis broadly consists of two steps: initiation and elongation. Synthesis of malonyl-CoA by acetyl-CoA carboxylase, transfer of malonyl group to ACP by malonyl-coenzyme A:ACP transacylase (FabD), and the condensation reaction by β-ketoacyl-ACP synthase (FabH) constitute the initiation step, whereas the reactions catalyzed by FabG, FabZ/FabA, FabI, and FabB/FabF comprise the elongation step of FAS. While FabD, FabH, and ACP, which are involved in the initiation of FAS of *P. falciparum*, have received attention of other researchers [12–14], enzymes of elongation cycle of the parasite are being studied in detail in our laboratory. In this context we have reported earlier the expression and characterization of FabI of *P. falciparum* [10,15]. Here we report the cloning, expression, characterization, and modeling of FabG from *P. falciparum*.

FabG has been identified in few organisms [16–18] and a detailed characterization of the enzyme is available only from *B. napus* and *E. coli*. The crystal structures of FabG have recently become available from these two organisms [19,20]. FabG is the only enzyme known to catalyze the reduction of a β-keto group. As compared to the other enzymes of the pathway (FabB, FabI, and FabZ), which are reported to have more than one isoform, until now only a single isoform has been identified for FabG. It was shown earlier that the genes encoding several key fatty acid biosynthetic enzymes are clustered on the *E. coli* chromosome known as the *fab* cluster. Insertion of a transcription terminator cassette between the *fabD* and *fabG* genes of the *E. coli* chromosome abolished *fabG* transcription and blocked cell growth, thus demonstrating that *fabG* is an essential gene [21]. Thus, there is a need to study this enzyme from malaria parasite in greater detail.

Materials and methods

Materials. Media components were obtained from Difco (BD, NJ, USA). Acetoacetyl-CoA, β-NADPH, imidazole, and SDS–PAGE reagents were obtained from Sigma Chemical, St. Louis, MO. Protein molecular weight markers and the prestained markers were from MBI Fermentas. His-bind resin and anti-His tag-HRP conjugate were obtained from Novagen. Phenyl-Sepharose column was from Amersham Biosciences, Uppsala, Sweden. All other chemicals used were of analytical grade.

Strains and plasmids. pGEM-T vector (Promega) and *E. coli* DH5α cells (Novagen) were used for the cloning of the gene. pET-28a(+) vector (Novagen) and BL21 (DE3) cells (Novagen) were used for the expression of FabG.

Cloning of FabG. Total RNA was isolated from asynchronous cultures of *P. falciparum*, treated for 45 min at 37 °C with RNase free DNase (Promega; 1 U/μg RNA), and was repurified by phenol:chloroform extraction and ethanol precipitation. It was then subjected to RT-PCR using one step RT-PCR kit (Qiagen). PCR was performed with primers PfFabGfor (5′-CCGGAATTCTATTGTGGGGAAAA TAAAGTTGCT-3′) and PfFabGrev (5′-CCTGCTCGAGTAGGT GATAGTCCACCGTCTATTACGAAAACTCG-3′). PCR product of the expected size (723 base pairs) was cloned in pGEM-T vector and the clones were confirmed by dideoxy sequencing using T7 promoter on an ABI Prism Model 377 sequencer. The deduced amino acid sequence revealed a protein of 241 amino acid residues with a predicted molecular mass of 28.0 kDa. The DNA sequence encoding the mature protein was amplified using gene specific primers and cloned in *Bam*HI and *Xho*I sites of pET-28a(+) vector.

Expression and purification of FabG in E. coli. The constructs were transformed into *E. coli* BL21 DE(3), primary cultures were grown overnight at 37 °C and the secondary cultures at 20 °C for 12 h followed by induction with 1 mM IPTG at 12 °C for 12 h. The cells were harvested and resuspended in 50 mM Tris, pH 7.4, containing 150 mM NaCl. The supernatant obtained after lysing the cells was loaded onto His-bind resin (Novagen). The column was initially washed with lysis buffer and the protein was eluted using 50 mM imidazole in the same buffer. The eluate was loaded onto Phenyl-Sepharose column (Amersham Biosciences) in buffer containing 0.5 M ammonium sulphate. The protein eluted in the wash-through and the purity of the fractions was determined by SDS–PAGE.

Enzyme assay. The functional activity of the expressed protein was monitored by the decrease in absorbance at 340 nm due to oxidation of NADPH to $NADP^+$ in the presence of the substrate, acetoacetyl-CoA. The standard reaction mixture in a final volume of 100 μl contained 50 mM Tris–Cl buffer, pH 7.5, 18 μg purified protein, and 250 μM acetoacetyl-CoA. The reaction was started by the addition of 125 μM

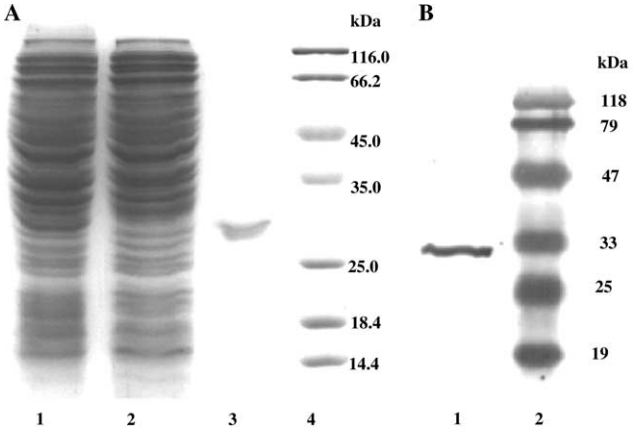

Fig. 1. (A) SDS–PAGE showing the expression of PfFabG in *E. coli* cultures transformed with pET-28a(+)-FabG. Lane 1: crude cell lysate; lane 2: supernatant; lane 3: pure FabG; and lane 4: protein molecular weight markers. (B) Western blot of PfFabG using anti-His antibodies. Lane 1: PfFabG band; lane 2: Prestained protein molecular weight markers.

NADPH. The K_m for each substrate was determined by varying the concentrations of that substrate while keeping the concentration of the other substrate constant. The kinetic parameters were determined by nonlinear regression analysis. The data were also evaluated by double reciprocal plots. The reverse reaction, i.e., the oxidation of β-hydroxybutyryl-CoA to acetoacetyl-CoA was also characterized by reduction of $NADP^+$ to NADPH, as observed by an increase in absorbance at 340 nm.

Modeling of P. falciparum FabG. We modeled *P. falciparum* FabG (PfFabG) using MOE (Molecular Operating Environment) [22]. The PfFabG sequence was compared with the pre-aligned families of protein sequences in MOE-PDB using Blosum62 substitution matrix. The PfFabG model was built using FabG from *B. napus* (PDB code: 1EDO) as template. There is 48% sequence identity between the two enzymes. Ten intermediate models were generated and subjected to molecular mechanics energy minimization to a RMS gradient of 1. Finally the one which scored best according to MOE's packing evaluation function was chosen.

Results and discussion

Expression and purification of FabG

It has been demonstrated earlier that the nuclear encoded plastid proteins identified in *Plasmodium* exhibit long amino-terminal extensions similar to those observed in plants, required for targeting of the protein to apicoplast [12]. Hence we PCR amplified, cloned, and expressed the sequence corresponding to the mature FabG protein.

The cultures grown at 37 °C to an OD_{600} of 0.6 and induced with 0.2 mM IPTG for 3 h at the same temperature led to the expression of the protein in inclusion bodies. However, when the primary culture of the

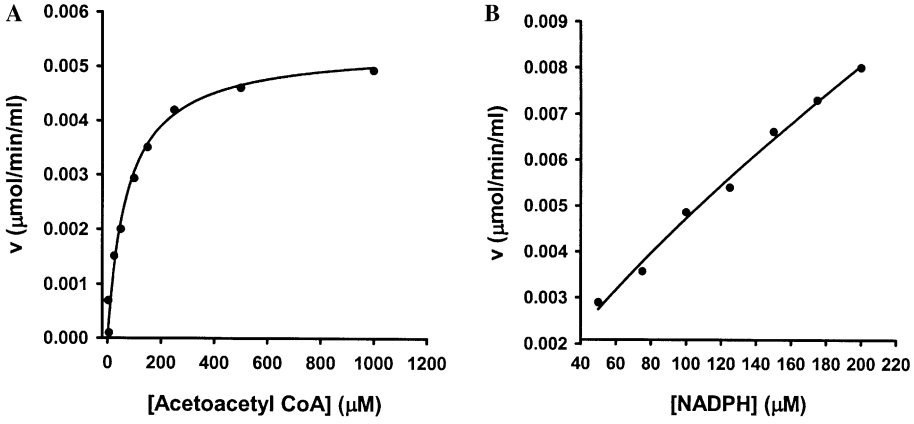

Fig. 2. (A) Determination of K_m by nonlinear regression analysis. Plots using varying concentrations of acetoacetyl-CoA and 125 µM NADPH and (B) varying concentrations of NADPH at 250 µM acetoacetyl-CoA.

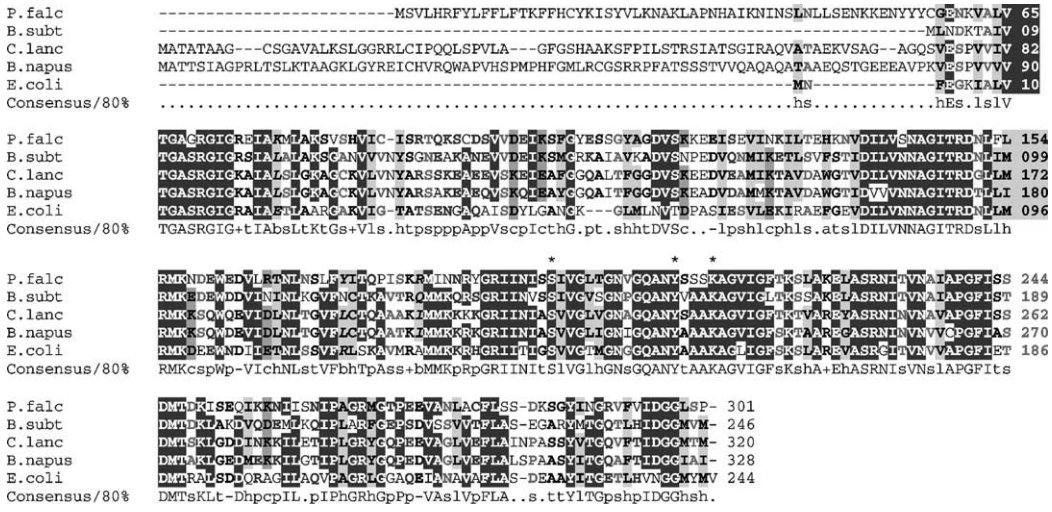

Fig. 3. Multiple sequence alignment of FabG sequences: P.falc (*P. falciparum*, Accession No. AAK83686), B.subt (*Bacillus subtilis*, Accession No. AAC44307), C.lanc (*Cuphea lanceolata,* Accession No. P28643), B.napus (*Brassica napus*, Accession No. CAC41363), and E.coli (*Escherichia coli*, NP_415611). * indicates the residues of active site triad. Color scheme: Conserved residues shown in bold with black background, negative residues indicated by −, positive by +, aliphatic by l, aromatic by a, tiny by t, small by s, big by b, charged by c, polar by p, and hydrophobic by h (all below the alignment).

PfFabG clone was grown overnight at 37 °C and the secondary cultures at 20 °C for 12 h induced with 1 mM IPTG at 12 °C for 12 h, most of the PfFabG was found in the supernatant as observed by SDS–PAGE analysis (Fig. 1A). The expressed protein was confirmed by western blot analysis using anti-His antibodies (Fig. 1B). The protein was purified by affinity and hydrophobic interaction chromatography and gave a band of Mr. 28,000 corresponding to the PfFabG (Fig. 1A, lane 3).

Enzyme assay

A unit of the enzyme is defined as the amount of protein required to catalyze the reduction of one μmol of acetoacetyl-CoA per minute. Under these conditions, acetoacetyl-CoA was converted quantitatively to β-hydroxybutyryl-CoA. The K_m for acetoacetyl-CoA as calculated from nonlinear least squares regression analysis (Fig. 2A) and double reciprocal plots (data not shown) was 75 and 111 μM, respectively. Thus demonstrating that PfFabG has a higher affinity for acetoacetyl-CoA as compared to its counterpart from *B. napus* that has a K_m of 251 μM. [23]. The V_{max} of PfFabG with respect to acetoacetyl-CoA was 0.0054 μmol/min/ml with a k_{cat} value of 0.014 s^{-1}.

The v versus [S] plot for the reaction utilizing increasing concentrations of NADPH, at constant acetoacetyl-CoA (250 μM) concentrations, demonstrated a cooperative behavior (Fig. 2B). This is consistent with the observation made for the *E. coli* enzyme [19]. The relationship between binding and catalytic rate is most simply realized in the concept that the rate is proportional to the formation of enzyme–substrate complex, which reflects the binding affinity. Thus catalytic rate will be proportional to the binding reaction. The data fit well to a sigmoidal plot leading to a Hill coefficient of 0.8, demonstrating negative cooperativity. Also, higher concentrations of acetoacetyl-CoA (750 μM) did not have any effect on the activity of FabG.

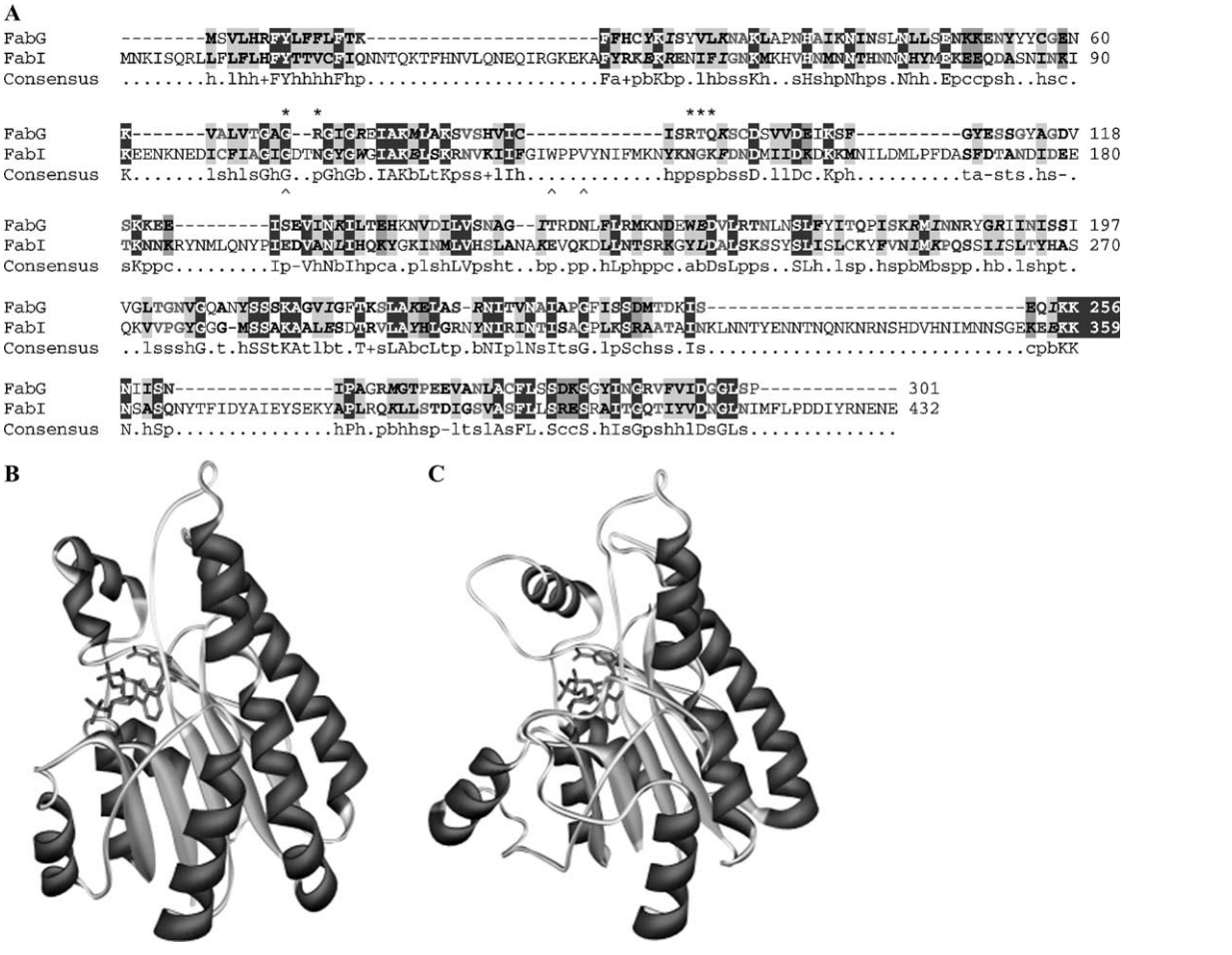

Fig. 4. (A) Pairwise sequence alignment of FabG (Accession No. AAK83686) and FabI (Accession No. AAK83687) from *P. falciparum*. * indicates residues in FabG sequence involved in cofactor binding, ^ indicates the corresponding residues in FabI. Color scheme as in Fig. 3. (B) The overall fold of *Plasmodium falciparum* β ketoacyl-ACP reductase and enoyl-ACP reductase (C) with their respective cofactors $NADP^+$ and NAD^+ in the active site (shown in sticks).

For, β-hydroxybutyryl-CoA a K_m value of 3.73 mM with a V_{max} of 0.011 μmol/min/ml (data not shown) was obtained. For the reverse reaction 26 μM protein was used leading to a k_{cat} value of 0.007 s^{-1}. Thus, the enzyme catalyzes the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA more efficiently. Other intermediates of the elongation cycle such as an enoyl-CoA (crotonyl-CoA) or any of the acyl CoAs—i.e., malonyl-CoA or acetyl-CoA were ineffective as substrates. Likewise, neither NADH nor NAD^+ could replace NADPH or $NADP^+$ as cofactors. In this respect PfFabG is similar to its counterparts in *B. napus* and *E. coli* but is remarkably distinct from the acetoacetyl-CoA reductase from *Alcaligenes eutrophus*, which can utilize both NADH and NADPH [24].

Sequence comparison and modeling

The sequence of *P. falciparum* FabG was blast searched and compared for its similarity with FabG from other organisms using ClustalW [25]. As shown in Fig. 3 the sequence of PfFabG is most similar to those of plants and bacteria consistent with its evolutionary linkage to a photosynthetic bacterium and its location in the apicoplast of the parasite. PfFabG also has the characteristic sequence motif formed by a triad of Ser196, Tyr209, and Lys213 residues that are involved in the catalytic mechanisms of the enzymes of the short-chain alcohol dehydrogenase (SDR) family. So far FabG enzyme from only a few sources has been characterized and more so the structure of the enzyme from only *B. napus* and *E. coli* is available [19,20]. PfFabG forms the typical NAD(P) binding Rossmann fold. The model of PfFabG as shown in Fig. 4 exhibits considerable similarity with that of PfFabI. Such similarity has been noted between the *B. napus* counterparts as well [26]. The modeling studies were used to seek an explanation for its specificity for NADPH.

The phosphate moiety bound to the 2′ adenine ribose hydroxyl nestles in a pocket formed by the sidechains of Gly69, Arg70, and the loop residues Arg92, Thr93, and Gln94. The positively charged guanidino group of Arg70 helps to stabilize the negative charge of the phosphate group. Lack of this phosphate group in NADH is responsible for its poor binding to this cofactor, explaining the specificity of PfFabG for NADPH. Conversely, in PfFabI, the 2′-hydroxyl group of the adenine ribose occupied a small depression flanked by Gly106, Trp131, and Val134, which resulted in a tight fit for the NAD^+/NADH cofactor. This spatial arrangement leaves no room for the extra phosphate group of NADPH, hence, PfFabI has specificity for NADH alone [27].

In conclusion, this paper reports the cloning and expression of FabG from *P. falciparum*. This study provides a platform for further work towards understanding the mechanism of its catalysis, structure activity relationship, and aid in the development of an antimalarial targeted to FabG.

Acknowledgment

The authors acknowledge Department of Biotechnology, Government of India for financial support to N.S.

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