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ORIGINAL PAPER

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Identification of the 7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase required for coenzyme F_{420} biosynthesis

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Abstract The hydride carrier coenzyme F_{420} contains the unusual chromophore 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO). Microbes that generate F_{420} produce this FO moiety using a pyrimidine intermediate from riboflavin biosynthesis and the 4-hydroxyphenylpyruvate precursor of tyrosine. The *fbiC* gene, cloned from *Mycobacterium smegmatis*, encodes the bifunctional FO synthase. Expression of this protein in *Escherichia coli* caused the host cells to produce FO during growth, and activated cell-free extracts catalyze FO biosynthesis in vitro. FO synthase in the methanogenic euryarchaeon *Methanocaldococcus jannaschii* comprises two proteins encoded by *cofG* (MJ0446) and *cofH* (MJ1431). Both subunits were required for FO biosynthesis in vivo and in vitro. Cyanobacterial genomes encode homologs of both genes, which are used to produce the coenzyme for FO-dependent DNA photolyases. A molecular phylogeny of the paralogous *cofG* and *cofH* genes is consistent with the genes being vertically inherited within the euryarchaeal, cyanobacterial, and actinomycetal lineages. Ancestors of the cyanobacteria and actinomycetes must have acquired the two genes, which subsequently fused in actinomycetes. Both CofG and CofH have putative radical *S*-adenosylmethionine binding motifs, and pre-incubation with *S*-adenosylmethionine, Fe^{2+}, sulfide, and dithionite stimulates FO production. Therefore a radical reaction mechanism is proposed for the biosynthesis of FO.

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Abbreviations *AdoMet (SAM)* *S*-adenosyl-L-methionine · *Compound 6* 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione · *FO* 7,8-Didemethyl-8-hydroxy-5-deazariboflavin · *HPP* 4-Hydroxyphenylpyruvate

Introduction

The hydride transfer coenzyme F_{420} functions analogously to the nicotinamide adenine nucleotide coenzymes, although its deazariboflavin structure is similar to that of riboflavin (Walsh 1986). Named for its intense fluorescence upon excitation with 420 nm light, the oxidized coenzyme F_{420} (*N*-(*N*-L-lactyl-γ-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin) was first discovered in mycobacteria (Cousins 1960) and later purified for structural identification from the methane-producing microorganism *Methanobacterium* sp. strain M.o.H. (Cheeseman et al. 1972; Eirich et al. 1978). Since these original studies, F_{420} has been found in numerous actinomycetes and in all methanogens (Isabelle et al. 2002). Most cyanobacteria and halophilic archaea produce a DNA photolyase enzyme that uses a biosynthetic precursor of F_{420} as a coenzyme: 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (Lin and White 1986; de Wit and Eker 1987; Eker et al. 1990). In spectroscopic studies of electron transfer reactions, 5-deazaflavin analogues have been employed as photoreduction catalysts using the semiquinone radicals as strong $1e^-$ reductants (Hemmerich et al. 1977).

A plausible biosynthetic pathway for F_{420} biosynthesis was inferred through tracer studies of stable isotope incorporation by *Methanothermobacter thermautotrophicus* (Eisenreich et al. 1991). Early on, organisms that produce F_{420} were shown to secrete FO into their growth media, suggesting that FO was the deazariboflavin precursor in F_{420}

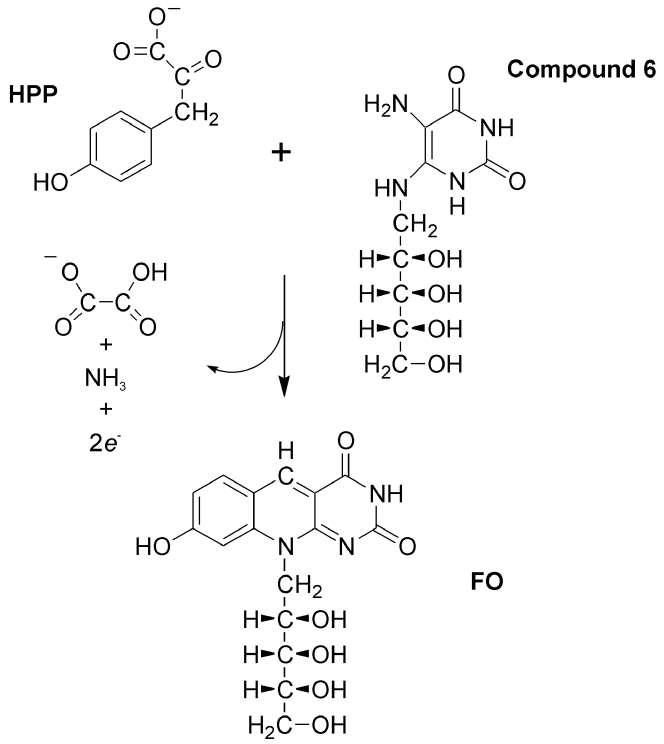

Fig. 1 The FO synthase reaction transfers the hydroxybenzyl group from 4-hydroxyphenylpyruvate (*HPP*) to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (*compound 6*), releasing oxalate and ammonia. After oxidation, the product is 7,8-didemethyl-8-hydroxy-5-deazariboflavin (*FO*)

biosynthesis (Kern et al. 1983). Figure 1 shows that FO is derived from 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (compound 6), an intermediate in riboflavin biosynthesis, and 4-hydroxyphenylpyruvate (HPP), the precursor to tyrosine (Van et al. 1985; Reuke et al. 1992). Most of the enzymes required for riboflavin and F_{420} biosynthesis have been identified (Bacher et al. 2000; Graham and White 2002), but not those responsible for forming the 5-deazariboflavin ring.

Genetic studies with the F_{420}-producing bacterium *Mycobacterium bovis* BCG used transposon mutagenesis to identify genes that were required for F_{420} biosynthesis (Choi et al. 2001, 2002). Mutations in one gene, *fbiC*, abolished both FO and F_{420} production in *M. bovis*. Therefore, it was predicted that this gene would play a role in the early steps of FO biosynthesis (Choi et al. 2002). The complete genome sequence of a hyperthermophilic archaeon that produces F_{420}, *Methanocaldococcus jannaschii* ("*Methanococcus jannaschii*"), includes two genes, at loci MJ0446 (*cofG*) and MJ1431 (*cofH*), that are homologous to the amino-terminal and carboxy-terminal halves of the *M. bovis* gene *fbiC*, respectively (Bult et al. 1996). These two genes are themselves paralogs: both proteins contain a conserved "radical SAM" sequence motif, which is characteristic of enzymes that homolytically cleave *S*-adenosylmethionine (AdoMet) to form a 5′-deoxyadenosyl free radical (Cheek and Broderick 2001; Frey 2001; Sofia et al. 2001).

Here we describe the cloning of the *M. jannaschii cofG* and *cofH* genes for heterologous expression in *Escherichia coli* and functional characterization in vitro. *E. coli* cells that express both *M. jannaschii* CofG and CofH or the bifunctional *Mycobacterium smegmatis* MsFbiC were shown to produce the compound FO, which diffuses into the growth medium. A mixture of *E. coli* extracts containing the MjCofG and MjCofH expressed separately catalyzes the formation of FO from compound 6 and HPP in vitro. Much more FO was produced by the bifunctional MsFbiC and the amount of product was stimulated by the addition of Fe^{2+}, sulfide, and AdoMet to the incubation mixture. Because cells that expressed either CofG or CofH alone did not produce FO and their respective extracts were insufficient to catalyze deazariboflavin ring formation in vitro, we conclude that the FO synthase comprises both the CofG and CofH subunits. This is the first demonstration of activity by the FO synthase enzyme (4-hydroxyphenylpyruvate:5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 4-hydroxybenzyltransferase (deaminating)). The production of FO by a recombinant strain of *E. coli* suggests a facile method for the large-scale production of deazariboflavins that could become economically competitive with a fermentation method using *M. smegmatis* (Isabelle et al. 2002).

Materials and methods

Source of microorganisms and chromosomal DNA isolation

Chromosomal DNA was prepared by standard methods from *Methanocaldococcus jannaschii* JAL-1 (DSM 2661) cells purchased from the University of Illinois Fermentation Facility (Urbana). *Mycobacterium smegmatis* mc^26 cells were a generous gift from Dr. Biswarup Mukhopadhyay (Virginia Bioinformatics Institute). Chromosomal DNA from *M. smegmatis* was prepared by standard methods (Larsen 2000). *Nostoc* sp. PCC 7120 (ATCC 27893) ("*Anabaena* sp.") cells were grown by Dr. Liang Shi (Pacific Northwest National Laboratory).

Gene cloning and protein expression

M. jannaschii cofG (locus MJ0446, Swiss-Prot accession number Q57888), *M. jannaschii cofH* (locus MJ1431, Swiss-Prot accession number Q58826) and *M. smegmatis fbiC* (http://www.tigr.org) were amplified by PCR from genomic DNA using oligonucleotide primers synthesized by Invitrogen. *M. jannaschii cofG* was amplified using the primers: Fwd (5′-GGTGGTCATATGGATAGGATGATAAG-3′) and Rev (5′- GATCGGATCCTTATTCCTTTAAC-3′). *M. jannaschii cofH* was amplified using the primers: Fwd (5′-GGTGGTCATATGGACCCAAATAAATTTAG-3′) and Rev (5′- GATCGGATCCTTACTCTAAAATTTTATAC-3′). Primers for the *M. jannaschii* genes introduced *Nde*I and *Bam*HI restriction sites at the 5′ and 3′ ends of genes. *M. smegmatis fbiC* was amplified with the primers: Fwd (5′-GGTCATATGAACTCCGAGCACGGCGCCGATCTGGGTG-3′) and Rev (5′-GCTAAGCTTCTACGCGGCCAGGGGCGCGTAGGTGGTG-3′). Primers for the *M. smegmatis fbiC* gene introduced *Nde*I and *Hin*dIII restriction sites at the 5′ and 3′ ends of the gene. PCR amplifications were carried out as described previously using an annealing temperature of 50 °C for *M. jannaschii cofG* and *cofH* and 65 °C for *M. smegmatis fbiC* (Graham et al. 2002). The PCR products were purified using a QIAquick spin column (Qiagen) and then digested with *Nde*I and *Bam*HI or *Nde*I and *Hind*III restriction

enzymes (Invitrogen). Bacteriophage T4 DNA ligase (Invitrogen) was used to ligate digested DNA fragments into compatible sites in plasmid pT7–7 (Tabor and Richardson 1985) to make the recombinant plasmids pMjCofG, pMjCofH, and pMsFbiC. To construct plasmid pMjCofH-His, the *cofH* DNA fragment was ligated into compatible sites in plasmid pET19b (Novagen). Each of these plasmids contains a canonical T7 RNA polymerase promoter sequence upstream of the inserted DNA. The DNA sequences were verified by dye-terminator sequencing at the University of Iowa DNA facility. For the co-expression of *M. jannaschii* CofG and CofH, *cofH* was amplified by PCR from the plasmid pMjCofH-His using oligonucleotide primers: Fwd (5′-GAAGATCTTAATACGACTCACTATAGG-3′) and Rev (5′-GAAGATCTGCTAGTTATTGCTCAGCGG-3′). Restriction sites for the *Bgl*II enzyme were introduced in the 5′ and 3′ ends of the PCR product. The purified, digested DNA fragment was ligated into a *Bgl*II site in the plasmid pMjCofG. The recombinant plasmids pMjCofG, pMjCofH, and pMjCofGH were introduced into *E. coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene) and plasmid pMsFbiC was introduced into *E. coli* BL21-CodonPlus (DE3)-RP cells (Stratagene) by transformation. The transformed *E. coli* cells were grown in Luria-Bertani medium (200 ml, Difco) supplemented with 100 µg ampicillin/ml at 37 °C with shaking until they reached optical densities at 600 nm of 0.8–1.0. Heterologous protein expression using host-encoded T7 RNA polymerase was then induced with 28 mM lactose. After an additional 2 h of incubation at 37 °C with shaking, the cells were harvested by centrifugation (4,000×*g*, 5 min) and frozen at –20 °C. M9 minimal medium (with lactose induction) was used in studies of FO production by recombinant cells (Miller 1972).

Isolation and analysis of FO

Different procedures were used for the isolation and analysis of FO depending on the source and amount of FO to be assayed. For the analysis of low concentrations of FO in growth medium (pH 5–6), a 6 ml sample was concentrated 12-fold by evaporation under a stream of nitrogen gas. In the standard method of analysis, the concentrate was applied to a C_{18} silica column (0.5×5 cm) equilibrated in water. The column was washed with 4.3 ml water followed by 2 ml 10% methanol in water. FO was eluted from the column with 2–3 ml 30% methanol until the yellow fluorescent riboflavin started to elute, monitored using a 366 nm hand-held illuminator (UVP, San Gabriel, Calif., USA). The fraction containing FO was concentrated with a stream of nitrogen gas and the FO further purified by preparative TLC on silica gel 60 F_{254} TLC plates (E. Merck, Darmstadt, Germany) using acetonitrile/water/formic acid (88%), 40:10:5 by volume as the developing solvent. FO migrated with a R_f=0.53 and was identified by its blue fluorescence upon illumination with long-wavelength ultraviolet light. The area of the TLC plate that contained FO was physically removed, ground to a powder and the FO eluted with 50% methanol. After concentration by evaporation with a stream of nitrogen gas, FO in the samples was measured by HPLC.

For the analysis of growth medium containing higher amounts of FO, direct quantification was possible by measuring the fluorescence intensity of the samples (excitation wavelength, 420 nm; emission wavelength, 480 nm) compared to standards with known FO concentrations. In each case the presence of FO was confirmed by the standard chromatographic assay.

FO present in cell pellets was isolated by suspending the cells in 50% methanol (3 ml) and warming at 70 °C for 5 min followed by centrifugation. The resulting pellets were then re-extracted in the same manner. The combined clear extracts were mixed and the methanol removed by evaporation with a stream of nitrogen gas. FO in the samples was purified by preparative TLC and assayed by HPLC.

For FO present in cell-free incubations, proteins were precipitated from the incubation mixture with three volumes of methanol and insoluble material was removed by centrifugation (14,000×*g*, 10 min at room temperature). FO contained in the soluble extract was purified by preparative TLC and analyzed by HPLC.

HPLC analyses were done on a Shimadzu SCL-6B HPLC using a C_{18} reversed phase column (AXXI Chrom octyldecyl silane, 5 µm, 4.6 mm×25 cm) preceded by a guard column (RP-18 NEWGUARD, 7 µm, 1.5 cm). A 25 mM sodium acetate buffer (pH 6.0, 0.02% NaN_3) containing 35% methanol was used for isocratic elution at a flow rate of 0.5 ml/min. The eluent was monitored by fluorescence (excitation wavelength, 420 nm, emission wavelength, 480 nm) using a RF-551 fluorescence HPLC monitor (Shimadzu), and by absorbance at 280 nm using an SPD-6AV UV-Vis spectrophotometric detector (Shimadzu). This HPLC method separates FO, which has a retention time of 9.6 min, from all other known F_{420} species (Graupner and White 2003; Li et al. 2003).

Characterization of deazariboflavin compounds in a cyanobacterium

Frozen cells of *Nostoc* sp. PCC 7120 (2 g) were suspended in 5 ml water and heated at 100 °C for 15 min. The resulting material was centrifuged (14,000×*g*, 10 min at room temperature), the soluble green extract was reserved, and the insoluble material was re-extracted by the same procedure. The combined extracts were treated and assayed for FO analogs by three different schemes. The first was a direct analysis: the extract was concentrated to 1 ml and the fluorescent FO was assayed by the standard method. In the second method, the extract was applied to a DEAE column, washed with water, and eluted with NH_4HCO_3 to recover the fluorescent anionic compounds, including FO or any compound containing FO such as F_{420}. Portions of the eluate were either assayed directly by the standard method or subjected to acid hydrolysis (100°C for 1 h in 1 M HCl) before analysis using the standard method. In the third procedure, the extract was hydrolyzed directly (100 °C for 1 h in 1 M HCl) before analysis by the standard method.

Analysis of protein expression

E. coli cells containing the heterologous proteins were sonicated and the insoluble material removed by centrifugation as previously described (Graham et al. 2002). Protein expression was confirmed by SDS-PAGE of total cellular protein and the soluble protein fraction. Sizes of the denatured proteins were measured relative to low-molecular-weight protein standards (Bio-Rad) separated by electrophoresis on a SDS-polyacrylamide gel (12%T, 2.7%C acrylamide) with a Tris/glycine buffer system.

MsFbiC protein was partially purified by salting out under anoxic conditions with ammonium sulfate followed by desalting on a Sephadex G-25 column. The protein precipitating between 25 and 40% ammonium sulfate saturation was used. Extracts containing MjCofG or MjCofH were heated at 70 °C for 10 min before centrifugation to remove denatured, insoluble material. Both of these proteins were both found to be stable to heating under these conditions. Protein concentrations were measured using the BCA total protein assay (Pierce) with bovine serum albumin as a standard.

Sequence alignment and phylogenetic analysis of *cofG* and *cofH* homologs

The MjCofG and MjCofH amino acid sequences were used to query the non-redundant protein database at the National Center for Biotechnology Information using the BLASTP program (Version 2.2.6) (Altschul et al. 1997) with the BLOSUM62 matrix and default gap costs for existence-11 and extension-1. Additional homologs were identified using similar methods to search partial genome sequences. Amino acid sequences from 16 *cofG* homologs and 44 *cofH* homologs were aligned separately using the ClustalW program (Version 1.83) (Thompson et al. 1994). These alignments were combined using the ClustalW profile alignment option. From the full alignment of 60 sequences, 326 amino acid positions that were deemed to be confidently aligned were selected for phylogenetic analysis.

The phylogeny of CofG and CofH homologs was inferred by protein distance and maximum likelihood methods. The protein distance method used the PROTDIST and NEIGHBOR programs (Version 3.6a3) (Felsenstein 2001) with the Jones, Taylor, and Thornton model of amino acid changes and a γ distribution of positional rates of change (coefficient of variation=0.64). Bootstrap proportions were calculated by using SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE programs (Felsenstein 2001) to create and evaluate 500 resampled alignments. An alternative tree was identified by the protein maximum likelihood criteria using the PROML program (Version 3.6a3) (Felsenstein 2001). The PROML program was run with the same parameters used for the PROTDIST program.

Chemical reagents

AdoMet and HPP were obtained from Sigma. Compound 6 was prepared by the catalytic hydrogenation of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine (Bacher 1986). Concentrations of compound 6 were calculated from the absorbance of samples in 0.1 M HCl using the extinction coefficient ε=24,500 M^{-1} cm^{-1} at 268 nm (Plaut and Harvey 1971). Although solutions of compound 6 were very sensitive to air oxidation at neutral pH at room temperature, they can be stored for long periods of time at –70 °C under hydrogen in the presence of Pd catalyst. FO was prepared from 1-deoxy-1-[(3-hydroxyphenyl)amino]-D-ribitol and 6-chlorouracil as previously described (Ashton and Brown 1980).

Results and discussion

Co-expression of CofG and CofH is required for FO production in *E. coli*

To demonstrate that CofG and CofH were sufficient to produce FO, *M. jannaschii cofG* and *cofH* were cloned in *E. coli* for heterologous protein expression. Analyzed by SDS-PAGE, MjCofG and MjCofH migrated with apparent masses of 42 kDa and 40 kDa, close to their expected masses of 41,985 Da and 40,806 Da, respectively. While both proteins were strongly expressed, only MjCofG was substantially soluble in cell-free extract. Therefore, MjCofH was expressed as a fusion protein with an N-terminal polyhistidine tag. MjCofH-His was also strongly expressed in *E. coli* but was slightly more soluble than the untagged protein. Despite these high levels of expression, cells containing either MjCofG or MjCofH produced no more than background levels of FO (Table 1). (A small amount of FO was observed in media from control experiments with *E. coli* lacking any *cof* genes. This low level of synthesis could arise from the chemical condensation of compound 6 and HPP naturally present in *E. coli*; this spontaneous reaction has been observed in vitro (R.H.W., unpublished results) Alternatively, the extracts may contain another fluorescent molecule that co-chromatographs with FO.) However, when *cofG* and polyhistidine-tagged *cofH* were co-expressed, the cells released nearly 50 times more FO into the medium than did control strains (Table 1).

Multienzyme complexes of enzymes that catalyze sequential reactions can facilitate substrate channeling and coordinate regulation (Huang et al. 2001). Often, genes that encode subunits of a complex fuse to encode new bifunctional enzymes, which can have favorable kinetic or regulatory properties (Seo et al. 2000). In the actinomycetes, *cofG* and *cofH* are fused, encoding a single FbiC polypeptide. We cloned the fused *M. smegmatis fbiC* gene and heterologously expressed bifunctional MsFbiC in *E. coli* to test its activity. Although most of the expressed MsFbiC protein was found to be insoluble in cell-free extract, a portion of the enzyme was soluble and migrated during SDS-PAGE with an apparent mass of 88 kDa, close to its expected mass of 93,268 Da. Heterologously expressed FbiC from *Mycobacterium bovis* is also insoluble in cell-free extract (Choi et al. 2002).

Cells expressing MsFbiC released 84-fold more FO into the minimal growth medium than did cells co-expressing MjCofG and MjCofH (Table 1). The same cells grown in minimal medium saturated with tyrosine released fourfold more FO, suggesting that tyrosine was converted into the HPP substrate, stimulating activity. In contrast, cells grown in minimal medium supplemented with 120 mM methionine (a precursor to AdoMet) released 60% less FO into the medium. FO production was enhanced by growing the MsFbiC-expressing cells in complex medium relative to minimal medium: 80% more FO was released into the growth medium and cells contained 39 nmol FO/g dry cell mass. While the amount of FO released into the growth medium by the recombinant *E. coli* strains described here is lower than that reported from cultures of *M. thermautotrophicus* and *M. smegmatis* (Table 1), optimization of gene expression and growth conditions could make heterologous FO production competitive with the large-scale fermentation method using *M. smegmatis* described by Isabelle et al. (2002).

Table 1 7,8-Didemethyl-8-hydroxy-5-deazariboflavin (FO) released into growth medium

Strain	Growth medium	FO concentration (nM)
Escherichia coli (pRIL)	M9+0.5% peptone	0.02
Escherichia coli (pRIL, pMjCofG)	M9+0.5% peptone	0.04
Escherichia coli (pRIL, pMjCofH)	M9+0.5% peptone	0.05
Escherichia coli (pRIL, pMjCofGH)[a]	M9+0.5% peptone	1.9
Escherichia coli (pRP, pMsFbiC)	M9	160
Escherichia coli (pRP, pMsFbiC)	M9+6 mM tyrosine	640
Escherichia coli (pRP, pMsFbiC)	LB	290
Methanothermobacter thermautotrophicus[b]	Minimal medium	7200
Mycobacterium smegmatis[b]	Complex medium	1240

[a]These cells expresses both MjCofG and MjCofH-His
[b]*M. thermautotrophicus* and *M. smegmatis* data are from Isabelle et al. (2002).

Table 2 Production of FO by MsFbiC. Anoxic pH 7.0 solutions of the desired compounds (0.1 or 0.01 M) were added to 40 µl partially purified MsFbiC (184 µg total protein) under an Ar atmosphere. Reactions contained 13 mM dithiothreitol with 6 mM 4-hydroxyphenylpyruvate (HPP) and/or 2.1 mM 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (compound 6) and were incubated for 50 min at the indicated temperature. Enzyme was activated by pre-incubation for 15 min with 6 mM AdoMet, 6 mM dithionite, 0.3 mM $Fe(NH_4)_2SO_4$, and/or 0.3 mM hydrosulfide as indicated. *n.d.* Not detected

Substrates	Enzyme activation	Temperature (°C)	FO produced (pmol)
Compound 6	None	25	n.d.
HPP	None	25	n.d.
Compound 6, HPP	None	25	68
Compound 6, HPP	AdoMet, dithionite	25	86
Compound 6, HPP	Fe^{2+}, HS^-, AdoMet, dithionite	25	660[a]
Compound 6, HPP	Fe^{2+}, HS^-, AdoMet, dithionite	70	580[a]

[a]Identical incubations with cell extracts not containing the MsFbiC gene product produced no FO

FO production in cell extracts

To assay FO production in vitro using the heterologously expressed proteins, cell-free extracts were partially purified to remove contaminating FO that was produced in vivo. When MsFbiC was incubated anoxically with compound 6 and HPP, 68 pmol FO was produced in the 48-µl reaction volume (Table 2). Members of the "radical SAM" group of enzymes contain a labile iron–sulfur cluster that must be reconstituted in vitro for full enzyme activity (Duin et al. 1997; Kulzer et al. 1998). After activation with Fe^{2+}, HS^-, AdoMet and dithionite, MsFbiC activity increased almost ten-fold. Surprisingly, MsFbiC activity was not significantly reduced during incubations at 70 °C, a temperature that caused proteins in the cell-free extract to denature and precipitate. Either the reconstituted protein is more thermostable than the protein in cell-free extract or most of the enzyme activity occurs early in incubation, before the protein denatures. Similar incubations of cell-free extracts containing either MjCofG or MjCofH produced no more FO than was observed in control incubations, whereas incubations with 50 µl of both protein extracts (at 60 °C for 25 min) produced 350 pmol FO.

Identification of FO in *Nostoc* sp. strain PCC 7120

Many cyanobacteria, actinomycetes, and halobacteria contain an unusual type of DNA photolyase that cleaves cyclobutane–pyrimidine dimers formed by DNA damage. In contrast to the proteobacterial and yeast enzymes, this photolyase uses FAD and FO chromophores (Eker et al. 1990). The genome sequence of *Nostoc* sp. strain PCC 7120 encodes FO-dependent DNA photolyase, CofG, and CofH (Kaneko et al. 2001). To test whether this organism produces FO or FO-containing derivatives, cell-free extracts were purified and analyzed by HPLC. Equal amounts of FO were detected in eluates from C_{18} and DEAE columns, corresponding to 0.44 nmol FO/g dry cell mass. Portions of the DEAE column eluate were subjected to acid hydrolysis (100 °C for 1 h in 1 M HCl) before analysis using the standard method; under these conditions, F_{420} species decompose to form FO (Cheeseman et al. 1972). This analysis detected 0.33 nmol FO/g dry cell mass. Direct hydrolysis of cell-free extract before analysis using the standard method identified 0.22 nmol FO/g dry cell mass. Considering losses due to the acid hydrolysis and additional manipulations, these results indicate that the deazariboflavins in *Nostoc* sp. strain PCC 7120 are FO rather than F_{420} species. No other deazariboflavin species were identified in these samples during chromatography (Graupner and White 2003). Therefore this organism likely uses CofG and CofH to produce FO.

Sequence analysis and evolution

CofG and CofH are expressed from diverged paralogous genes. Although the two subunits share only 22–27% amino acid sequence identity, the two families share a number of conserved amino acid positions (Fig. 2). In particular, both proteins contain three conserved cysteine residues in a motif that is characteristic of the unusual iron–sulfur clusters found in enzymes that use AdoMet to initiate radical catalysis (Cheek and Broderick 2001; Sofia et al. 2001): CXXXCXXC. The proteins also share a glycine-rich motif spaced ca. 30 amino acids from the cysteine-rich motif, another characteristic of the "radical SAM" proteins (Sofia et al. 2001). Therefore, these enzymes probably share a partial reaction mechanism involving the iron–sulfur-cluster-mediated homolytic cleavage of AdoMet (Frey 2001). While CofG and CofH share additional conserved residues with the biotin synthase (BioB) and thiazole synthase (ThiH) enzymes (also "radical SAM" proteins), confirmation of the enzymes' relatedness must await future structural characterization.

Orthologs of *cofG* and *cofH* are found in genome sequences of all organisms known to produce coenzyme F_{420} or FO (Fig. 3). Homologs from all F_{420}-producing organisms (the methanogens, *Archaeoglobus fulgidus*, *Halobacterium* sp., and the actinomycetes) group together and appear to have been vertically inherited in each lineage. Therefore, an ancestor of the *Actinomycetales* must have acquired F_{420} biosynthetic genes from the Euryarchaea and *cofG* and *cofH* must have fused during evolution of the actinomycete lineage. Bootstrap support for most branches

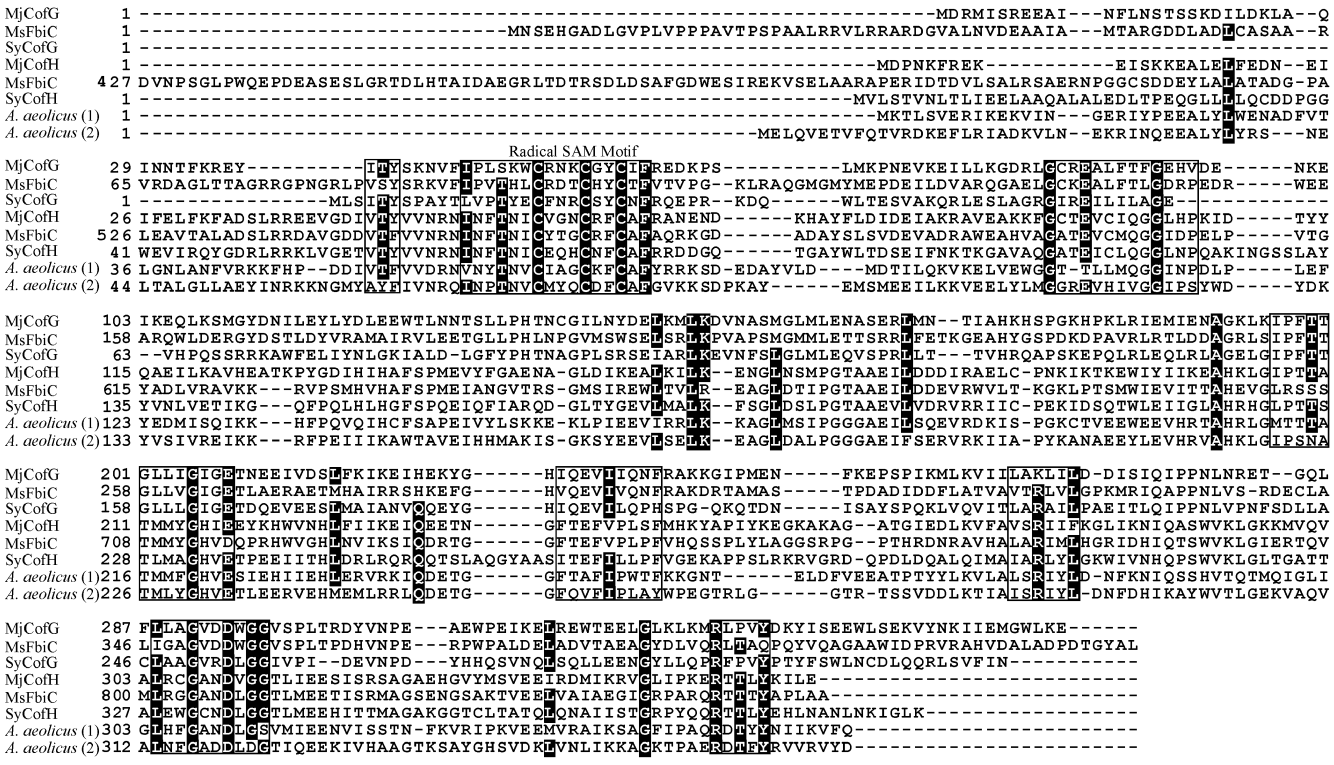

Fig. 2 Alignment of CofG/CofH sequences. MjCofG and MjCofH sequences are from *Methanocaldococcus jannaschii* and SyCofG and SyCofH sequences are from *Synechocystis* sp. strain PCC 6803. The paralogous N-terminal and C-terminal sections of MsFbiC from *Mycobacterium smegmatis* are split and aligned separately. Two paralogs of CofH from *Aquifex aeolicus* are differentiated by *numbers in parentheses*. Positions of identically conserved residues are shown in *white* on *black*, and regions of similarly conserved residues are *boxed*. Sequence accession numbers are as described for Fig. 3

in the CofG and CofH clusters is good, except for those of the *Methanopyrus kandleri* and *Halobacterium* sp. homologs. An alternative phylogenetic tree produced using the protein maximum-likelihood method places the *M. kandleri* homologs closer to those of *M. thermautotrophicus* and *M. jannaschii* (data not shown), consistent with the recently proposed placement of *M. kandleri* in an organismal phylogeny (Slesarev et al. 2002).

Cyanobacteria that produce the FO-dependent DNA photolyase also have homologs of *cofG* and *cofH* in their genomes (Fig. 3). These genomes contain no recognizable homologs of other F_{420} biosynthetic genes and we found no deazariboflavin other than FO in extracts of *Anabaena* sp. Therefore, CofG and CofH in these organisms likely produce FO as an end product. Weak bootstrap support precludes identifying the source of the *cofG* and *cofH* genes that were apparently acquired by a cyanobacterial ancestor, although these sequences are most similar to those of *Halobacterium* sp. While the predominant deazariboflavins in the halobacteria are F_{420} derivatives (Lin and White 1986; de Wit and Eker 1987), the *Halobacterium* sp. genome encodes two proteins that are 33–40% identical to the cyanobacterial FO-dependent DNA photolyase (McCready and Marcello 2003). It remains to be determined whether the halobacterial DNA photolyases use FO or F_{420}.

Surprisingly, genomes from a number of bacteria and archaea contain multiple homologs of *cofH*. Whereas *Methanococcus maripaludis* and members of the *Methanosarcinales* have two *cofH* paralogs that evolved independently from recent gene duplication events, many microorganisms that are not known to produce FO have *cofH* homologs that are anciently diverged (groups A and B in Fig. 3). In the archaea *Aeropyrum pernix*, *A. fulgidus*, *Pyrobaculum aerophilum*, and *Thermoplasma volcanii* and the δ/ε-proteobacteria *Desulfovibrio vulgaris*, *Geobacter metallireducens*, and *Helicobacter pylori* these genes are located close together on the chromosome, suggesting a coordinate function for the proteins. In most other bacteria, however, the homologs are unlinked on the chromosome. The molecular phylogeny of *cofH* homologs does not resolve whether these uncharacterized paralogs arose from a predecessor of the FO-producing *cofH* gene or by recruitment and subsequent duplication of a *cofH* gene involved in FO biosynthesis. The phylogeny inferred using protein maximum-likelihood criteria supports the latter scenario (data not shown).

FO synthase reaction mechanism

By analogy to other enzymes that contain "radical SAM" motifs, the CofG and CofH subunits of the FO synthase are each proposed to catalyze the homolytic cleavage of AdoMet by a single-electron reduction to generate a 5′-deoxymethyl adenosine radical (Frey 2001). These radi-

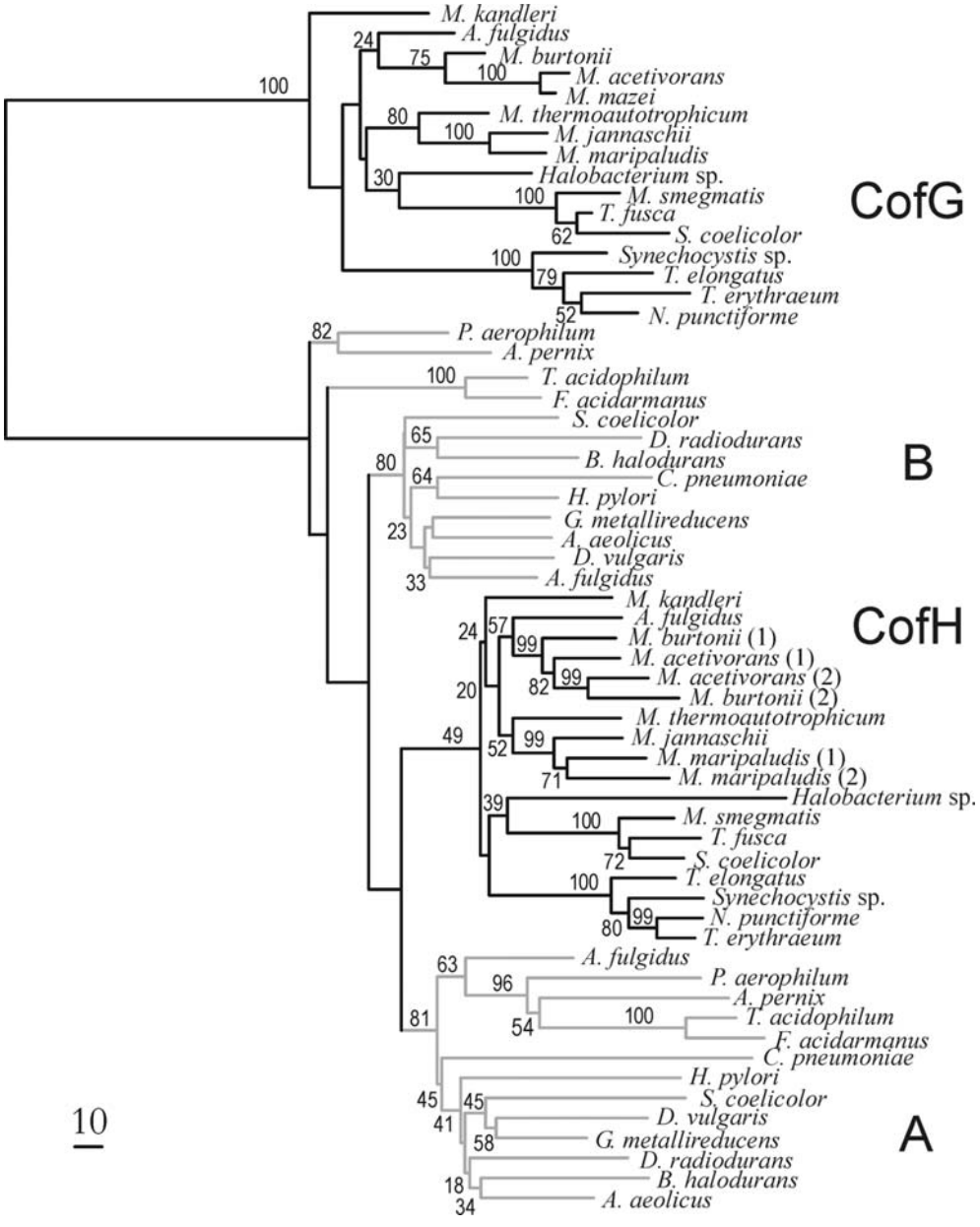

Fig. 3 Phylogeny of CofG/CofH homologs inferred by the neighbor-joining method. FbiC homologs were split as in Fig. 2. Sequences cluster into four groups: CofG and CofH proteins (*black branch lines*) together form the FO synthase; paralogs A and B (*gray branch lines*) are genetically linked in many organisms and the proteins probably form an enzyme complex with undetermined function. Paralogs from several methanogens are distinguished by *numbers in parentheses* following their species name. The tree was rooted using the CofG sequences as an outgroup. Bootstrap percentages are indicated for branches supported by a plurality of bootstrap replicates. Proteins (and their database accession numbers or URLs) are from *Aeropyrum pernix* (dbj|BAA79165.1 and dbj|BAA79162.1), *Aquifex aeolicus* (gb|AAC06943.1 and gb|AAC06853.1), *Archaeoglobus fulgidus* (gb|AAB90441.1, gb|AAB90440.1, gb|AAB90841.1 and gb|AAB90843.1), *Bacillus halodurans* (dbj|BAB07130.1 and dbj|BAB06862.1), *Chlamydophila pneumoniae* (gb|AAD18653.1 and gb|AAD19049.1), *Deinococcus radiodurans* (gb|AAF09948.1 and gb|AAF09653.1), *Desulfovibrio vulgaris* (http://www.tigr.org), *Ferroplasma acidarmanus* (ref|ZP_00001297.1 and ref|ZP_00001299.1), *Geobacter metallireducens* (gb|ZP_00080296.1 and gb|ZP_00080295.1), *Halobacterium* sp. (gb|AAG20121.1 and gb| AAG20122), *Helicobacter pylori* (gb|AAD06182.1 and gb|AAD06180.1), *Methanocaldococcus jannaschii* (sp|Q57888 and sp|Q58826), *Methanococcoides burtonii* (http://www.jgi.doe.gov/), *Methanococcus maripaludis* (http://www.genome.washington.edu/UWGC/methanococus/Methanococcus.html), *Methopyrus kandleri* (gb|AAM02111.1 and gb|AAM02110.1), *Methanosarcina acetivorans* (gb|AAM04905.1, gb|AAM04904.1 and gb|AAM04903.1), *Methanosarcina mazei* (gb|AAM32190.1), *Methanothermobacter thermautotrophicus* (gb|AAB85687.1 and gb|AAB85320.1), *Mycobacterium smegmatis* (http://www.tigr.org), *Nostoc punctiforme* (ref|ZP_00111465.1 and ref|ZP_00107247.1), *Pyrobaculum aerophilum* (gb|AAL63295.1 and gb|AAL63294.1), *Streptomyces coelicolor* (emb|CAB88436.1, emb|CAB88436.1, emb|CAD55486.1 and CAB44543.1), *Synechocystis* sp. PCC 6803 (sp|P73191 and sp|P72811), *Thermobifida fusca* (gb|ZP_00058551.1 and gb|ZP_00058551.1), *Thermoplasma acidophilum* (emb|CAC11361.1 and emb|CAC11363.1), *Thermosynechococcus elongatus* (dbj|BAC09067.1 and dbj|BAC09149.1) and *Trichodesmium erythraeum* (ref|ZP_00072597.1 and ref| ZP_00075129). *Bar* 10 predicted amino acid replacements per 100 positions

cals could then be used to abstract a hydrogen from each substrate, initiating the reaction shown in Fig. 4. Subsequently, the two substrate radicals could couple to generate an intermediate that can cyclize and rearrange to form dihydro-FO (H_2FO). H_2FO is oxidized to form FO. Precedence for radical reactions of compound 6, as well as other diaminouracils, includes its well-known ease of air oxidation (Plaut and Harvey 1971; Hu et al. 2002). These diaminouracils undergo oxidative condensation to produce coupled compounds. For 5,6-diaminouracil, the products are pyrimidopteridines, which are related to Wieland's "bis-alloxazine" product generated by the condensation of aminopyrimidines (Taylor et al. 1955). Electrochemical oxidation of 6-hydroxy-2,4,5-triaminopyrimidine confirms this compound's high reactivity at the C-1 amino group (Goyal et al. 1999). The generation of radicals from HPP has precedent in the biosynthesis of thyroxine, where a hydroperoxide of the enol form of 4-HPP has been identified (Cahnmann and Funakoshi 1970). Finally, the cyclization step is analogous to the chemical addition of amines to quionines (Finley 1974) and is not known to involve radicals. Confirmation of this proposed reaction mechanism will require extensive work.

The data presented here demonstrate that both the CofG and CofH protein subunits are required to heterologously produce FO in *E. coli* and they are sufficient to catalyze the condensation of compound 6 and HPP to produce FO in vitro. The activity of the fused, bifunctional MsFbiC enzyme appears greater than that of the MjCofG and MjCofH subunits, but is still low (ca. 3.6 nmol h^{-1} mg $protein^{-1}$ in cell-free extract). Enzymes that catalyze similar reactions also have relatively low specific activities: the 6,7-dimethyl-8-ribityl-8-ribityllumazine synthase from *M. jannaschii* has a specific activity of 11 nmol h^{-1} mg $protein^{-1}$ (Haase et al. 2003) and riboflavin synthase from *M. thermautotrophicus* has a specific activity of 2700 nmol h^{-1} mg $protein^{-1}$ (Eberhardt et al. 1997). This low activity could result from the constraints imposed by the Haldane relationship on the kinetic rates of enzymes that recognize their substrates with high affinity. Alternatively, the low activity of the FO synthase could be due to sub-optimal assay conditions or the depletion of an unrecognized enzyme or cofactor required for full activity. For example, HPP tautomerase activity may be required to convert the biosynthetically derived keto form of HPP into the enol form (Blasi et al. 1969). This enzyme activity cannot be detected in *E. coli* (R.H.W., unpublished results) but could be present in Archaea. The FO synthase could also require a specific single-electron donor to initiate AdoMet cleavage.

Riboflavin and FO have similar structures; both are derived from compound 6; and *M. jannaschii* produces both using synthases composed of two homologous subunits, with slow reaction rates (Haase et al. 2003). Despite these similarities, cells produce the two coenzymes using unrelated enzymes and reactions. In riboflavin biosynthesis, 6,7-dimethyl-8-ribityl-8-ribityllumazine synthase condenses compound 6 with 3,4-dihydroxy-2-butanone-4-phosphate to produce 6,7-dimethyl-8-ribityl-8-ribityllumazine. Riboflavin synthase then catalyzes the dismutation of two 6,7-dimethyl-8-ribityl-8-ribityllumazine molecules to produce riboflavin and regenerate compound 6 (Bacher et al. 2000). In contrast, the FO synthase uses only one molecule of compound 6 and uses HPP instead of 3,4-dihydroxy-2-butanone-4-phosphate to form the hydroxybenzyl moiety of FO. Thus, the riboflavin and FO biosynthetic pathways diverge after the production of compound 6. Considering the significant differences between the bacterial, archaeal, and eukaryal apparatuses for riboflavin biosynthesis (Bacher et al. 2000; Graupner et al. 2002), these

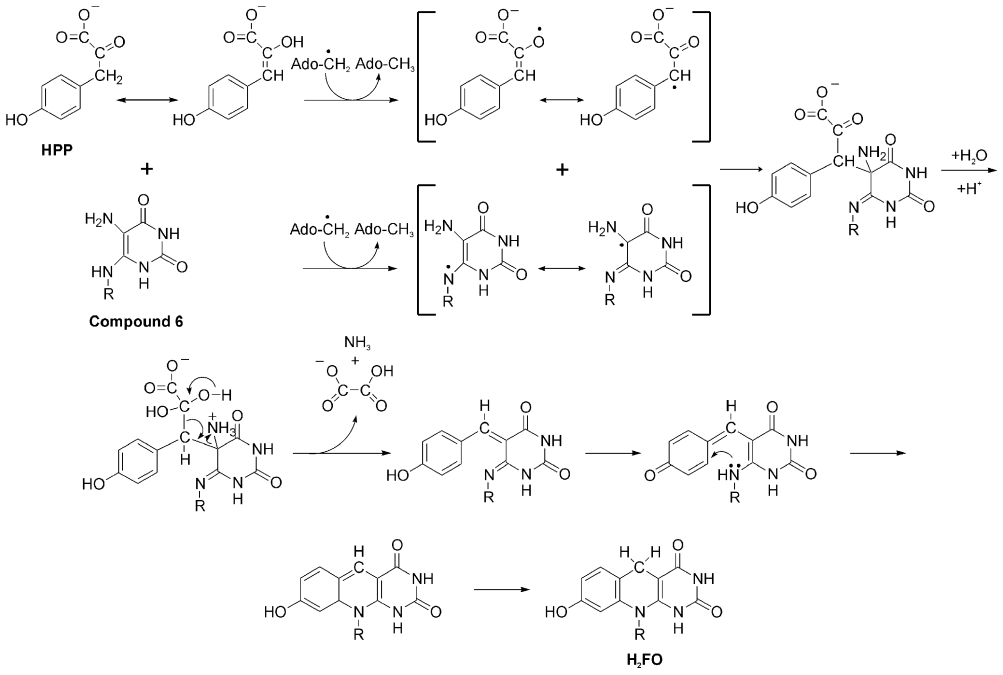

Fig. 4 Proposed radical reaction mechanism for FO synthase. 5′-Deoxyadenosine radicals formed by the homolytic cleavage of *S*-adenosylmethionine could be used to form HPP and compound 6 radicals. These radical intermediates combine and, after the addition of water, undergo the elimination of ammonia and oxalate. Proton rearrangement and ring-closure steps produce dihydro-7,8-didemethyl-8-hydroxy-5-deazariboflavin (H_2FO)

results suggest that flavin and deazaflavin biosyntheses are not immutable. Rather, they have evolved by gene recruitment, duplication, and divergence to use new pathways and, in some cases, even to exploit different chemical reaction mechanisms.

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