Identification of a Novel Gene for Biosynthesis of a Bacteroid-Specific Electron Carrier Menaquinone

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Abstract

Ubiquinone (UQ) has been considered as an electron mediator in electron transfer that generates ATP in Rhizobium under both free-living and symbiosis conditions. When mutated, the dmtH gene has a symbiotic phenotype of forming ineffective nodules on Astragalus sinicus. The gene was isolated from a Mesorhizobium huakuii 7653R transposon-inserted mutant library. The DNA sequence and conserved protein domain analyses revealed that dmtH encodes demethylmenaquinone (DMK) methyltransferase, which catalyzes the terminal step of menaquinone (MK) biosynthesis. Comparative analysis indicated that dmtH homologs were present in only a few Rhizobia. Real-time quantitative PCR showed dmtH is a bacteroid-specific gene. The highest expression was seen at 25 days after inoculation of strain 7653R. Gene disruption and complementation tests demonstrated that the dmtH gene was essential for bacteroid development and symbiotic nitrogen fixation ability. MK and UQ were extracted from the wild type strain 7653R and mutant strain HK116. MK-7 was accumulated under microaerobic condition and UQ-10 was accumulated under aerobic condition in M. huakuii 7653R. The predicted function of DmtH protein was confirmed by the measurement of methyltransferase activity in vitro. These results revealed that MK-7 was used as an electron carrier instead of UQ in M. huakuii 7653R bacteroids.

Introduction

In bioenergetic chains, diverse chemical types of quinones, such as ubi-, plasto-, mena-, rhodo-, calciallega- or sulfolobus-quinones have been identified as membrane-bound, mobile hydrogen carriers in different species. Ubiquinone (UQ) is the dominant quinone species in α/β/γ-proteobacteria. The majority of strictly aerobic Gram-negative bacteria contain exclusively UQ [1,2,3]. Menaquinone (MK) is most widely distributed in most Gram-positive bacteria and anaerobic Gram-negative bacteria. Because MK is the only quinone in the early branching of phylogenetic tree archaecaal and bacterial phyla, MK was speculated to be the ancestral type of quinone in bioenergetic systems [4]. It was deduced that the evolutionary transition of bioenergetic chains from high-potential MK to low-potential UQ in the proteobacterial phylum had drove by rising levels of dioxygen 2.5 billion years ago, and bioenergetic ambivalence of the respective organisms which work both on MK-and on UQ-pools, was necessary in transition proceed [5]. For example, E. coli and closely related species can switch between MK-and UQ-based bioenergetic chains in response to varying growth conditions. In E. coli, UQ and MK perform distinct functions. UQ-8 is used as the main quinone in aerobic respiration [6]. However, under anaerobic conditions, MK-8 and demethylmenaquinone-8 (DMK-8) are synthesized as electron transferring quinones [7,8].

In most bacteria, MK is derived from chorismate which is comest from the shikimate pathway. Chorismate is initially converted into isochorismate and then into 2-succinyl-6-hydroxyl-2, 4-cyclohexadiene-1-carboxylate (SHCHC). SHCHC is dehydrated into o-uccinylenzoate, and linked with CoA, then converted into 1,4-dihydroxy-2-naphthoate(DHNA). DHNA is added prenyl side chain into DMK, and DMK is converted into MK by S-adenosylmethionine-dependent methylation[9,10]. In this pathway, DMK methyltransferase are encoded by menG genes (Figure1). Recently, an alternative microbial MK biosynthetic pathway was discovered whose genes have no homology to men genes, which were identified in Streptomyces [10]. In E. coli, the ubiE gene catalyzes the common C methylation step in MK and UQ biosynthesis [11]. The E. coli mutant strain AN70 containing the ubiE401 mutation lacks both UQ and MK and instead produces only DMK [12].

Rhizobium, classified as the Gram-negative α-proteobacteria, is strictly aerobic under free-living but is microaerobic as bacteroids in nodules. Rhizobium contains diverse and complicated respiratory electron transport systems to cope with free-living and symbiosis conditions. Under aerobic conditions, BradyRhizobium japonicum transfers electrons from the ubiquinone (UQ) pool to the O2-reducing aa3-type terminal oxidase via the FeS protein-cytochrome bc1 complex and a membrane-bound cytochrome c [13,14]. Under microaerobic and bacteroid conditions diverse electron transport pathways were reported for B. japonicum, and one of the mainstream chains was that a cbhl-type-terminal oxidase transferred electrons from FeS/bc1 to O2 [15]. In a different study, when nitrous oxide was supplied as the terminal electron acceptor, electrons flowed from the UQ pool to nitrous oxide via cytochrome bc1 [16]. In the electron transport pathway of
Rhizobium trifolii, flavoprotein lies between NADH and UQ, and b-cytochromes transfer electrons from UQ to O₂ [17]. These reports suggested that UQ was an electron mediator to oxidize NAD(P)H in Rhizobium cultured under different conditions.

Mesorhizobium huakuii can establish a nitrogen-fixing symbiosis with Astragalus sinicus and form indeterminate nodules. M. huakuii has a strict specificity for A. sinicus which makes this species important for studies on the molecular mechanism of symbiotic nitrogen fixation. We previously constructed a transposon mutagenesis library by random insertion of the Tn5-B12S into the M. huakuii 7653R genome [18]. A new gene, dmtH, was isolated from the mutagenesis library which mutant formed ineffective nodules on A. sinicus. The DNA sequence and conserved protein domain analyses revealed that dmtH encodes DMK methyltransferase, which catalyzes the terminal step of MK biosynthesis. The result is interesting, because no Rhizobium has been reported to encode electron transfer containing both MK and UQ thus far. We hypothesized that a few of Rhizobium may use MK as electron carrier under symbiosis conditions. In this study, we identified dmtH function by symbiotic phenotype analysis and protein enzymatic activity assays in vitro. The results suggested that dmtH encoded a DMK methyltransferase and was biosynthesized in M. huakuii bacteroids. The implications of these findings for the functions of dmtH and MK in bacteroid metabolism and symbiosis are also discussed.

Results

Cloning and sequence analysis of the dmtH gene

The flanking sequence of the transposon insertion site was amplified by TAIL-PCR, from the mutant strain HK115 (nod”fix”). After sequencing, a homology search revealed that a consensus sequence was present only in some isolates of Rhizobium. Since the genome of M. huakuii has not still been sequenced, a pair of specific primers, dmtUP and dmtLOW were designed, which were based on the homologous sequences of M. loti MAFF303099, to amplify a 750-bp fragment including the complete ORF sequences from the genome of wild-type strain 7653R. This gene was designated as dmtH (GenBank accession no. JN400272). The dmtH gene is predicted to encode a 249-amino acid polypeptide with an expected molecular mass of 27.3 kDa and a pI value of 5.4. The homology analysis of the DmtH protein showed that had 99% identity to the function-unknown gene mll2332 from M. loti MAFF303099, and another similar proteins were annotated as demethylmenaquinone methyltransferase proteins in M. opportuni-stum WSM2075 (94% identity), R. leguminosarum bv. trifolii WSM1325 (90% identity) and R. etli CFPN42 (88% identity). DmtH homology protein also presented in other bacteria as demethylmenaquinone methyltransferase, such as Amycolatopsis mediterranei, U32 (59% identity), Roseiflexus sp. RS-1 (49% identity) (Figure S1, S2). The dmtH gene was also discovered that had no homology to men genes, whereas had 47% identity and 64% Positives with annotated demethylmenaquinone methyltransferase gene of Streptomyces lividans TK24. In Streptomyces, MK biosynthetic pathway was identified and whose genes have no homology to men genes(10). We thus assumed that the DmtH protein encoded DMK methyltransferase, which catalyzes methylation at the terminal step in MK biosynthesis.

Construction of dmtH null mutant HK116 and symbiotic phenotype identification

To confirm that the function of the dmtH gene in nodulation and nitrogen fixation ability was disrupted by the transposon insertion, a dmtH gene null mutant was constructed by the homologous recombination method, and one mutant strain, HK116, which formed ineffective nodules on A. sinicus, was obtained. The disruption of dmtH in mutant strain HK116 was verified by PCR. The symbiotic phenotype of the disruptant HK116 was examined by plant growth test (Figure 2 and Table 1). The results showed that HK116 formed small abnormal white nodules with a host plant, but lacked nitrogen-fixing ability. Nitrogenase activity of the nodules induced by HK116 was undetectable, and the number of nodules remarkably decreased compared to wild type 7653R.

Microscopic analysis in nodules

Paraffin section of the nodules induced by HK116 showed a clearly decreased number of bacteroids in nodules (Figure 3). Since a few symbiosomes were contained in the nodule infection zone (II zone) and the II–III interzone, further ultrastructural comparisons between nodules induced by HK116 and by 7653R were performed by transmission electron microscopy. Consistent with the light microscope observation, bacteroids were mainly located in the II zone and were degraded in the nitrogen fixation zone (III). Many symbiosomes in the nodules induced by HK116 were aberrant and the bacteroid membrane showed incrassation. Bacteroid sizes were smaller than in the wild type nodules. In the bacteroids, much poly-β-hydroxybutyrate (PHB) was distinctly observed (Figure 3). These changes showed that the dmtH gene is necessary to achieve efficient symbiotic interaction between M. huakuii and A. sinicus.

Complementation of the disruptant HK116

Complementation studies were carried out by cloning the dmtH gene into the broad host range vector pBBRgus. The disruptant strain HK116 transformed with pBBRgus-dmtH was inoculated on A. sinicus. HK116 harboring pTRgus-dmtH, which was designated HK116C, formed normal nodules and restored nitrogen-fixing ability to the host plant (Table 1). Light and transmission electron microscopy showed that bacteroids in root nodules induced by HK116C had similar numbers and configuration to wild type nodules, except for a slightly smaller size (Figure 3).

Figure 1. Methylation reactions in the biosynthetic pathway of MK. The length of the isoprenoid side chain (n) varies depending on the species. SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; DMK, demethylmenaquinone; MK, menaquinone.
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Quantification of dmtH gene expression by real-time RT-PCR

The relative expression level of the dmtH gene in root nodules collected at several time points after inoculation, and in free-living cultures was estimated by quantitative real-time PCR (Figure 4). The results show that the dmtH gene had much lower expression levels under free-living conditions, and was specifically expressed in bacteroids during symbiosis. The expression of the dmtH gene achieved the highest level in nodules at 25 days after inoculation (dai). These results indicated that dmtH gene expression was induced by symbiosis, and was indispensable for bacteroid development and nitrogen fixation, but was not needed for the free-living growth and metabolism of Rhizobia.

Analysis of menaquinone and ubiquinone in 7653R and mutant HK116

Recent studies have shown no evidence of the presence of MK in Rhizobium. The function of DmtH protein was hypothesized to participate in the biosynthesis of MK in 7653R, and the dmtH gene was specifically expressed in bacteroids. Therefore, we compared and identified the electron carrier used under free-living and symbiotic conditions. Quinone was extracted from 7653R and mutant HK116 growing under aerobic and microaerobic conditions and extracts were analyzed by reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry (MS). The results demonstrated that UQ-10 accumulated in 7653R and HK116 under aerobic conditions, but when growing conditions were switched from aerobic to microaerobic, MK-7 was confirmed to be produced in the wild type strain 7653R but was not in the extract from mutant HK116, in which the content of UQ-10 was also significantly decreased (Figure 5), and the result of MS further confirmed MK-7 and UQ-10 were accumulated in 7653R in microaerobic respiration. These results indicated that MK was synthesized only in bacteroids of wild type 7653R and not produced in free-living cells, implying that the bacteroids use MK as the specific electron carrier under symbiosis conditions to accommodate metabolic changes during development and nitrogen fixation.

Methyltransferase activity measurement of DmtH protein in vitro

To identify the enzymatic activity of the DmtH protein as a DMK methyltransferase that catalyzes methylation in MK biosynthesis, an overexpression plasmid pETdmt for the dmtH gene was constructed and transformed into recipient strain E. coli Rosetta 2(DE3). The target protein was overexpressed after inducing with isopropyl β-D-thiogalactopyranoside and purified (Figure 6).

Because the substrate specificity of the methyltransferase is not stringent for the prenyl side chain of demethyl-MK [9,19], DMK-8 of E.coli JC7623A4-1, which accumulated only DMK-8 but not MK-8 (Figure 7A), was used as the substrate of DmtH protein activity test. A crude homogenate of E.coli JC7623A4-1 was prepared. S-adenosyl methionine (SAM) was used as the methyl donor. The reaction system, with the crude E.coli JC7623A4-1 homogenate, purified DmtH protein and SAM, was incubated at

Table 1. Symbiotic phenotypes of M. huakuii wild type strain 7653R, mutant strain HK116 (Nod+ fix+), and complemented strain HK116C.

| Strain M. huakuii | Plant dry wt. (mg plant⁻¹) | No. of nodules per plant | Nodule fresh wt. (mg plant⁻¹) | Acetylene reduction activity (nmol of ethylene plant⁻¹ h⁻¹) |
|-------------------|-----------------------------|--------------------------|-------------------------------|-------------------------------------------------|
| 7653R             | 44.28⁺                      | 21.50⁺                   | 27.38⁺                        | 2.55⁺                                           |
| HK116             | 12.45⁺                      | 11.08⁺                   | 6.9⁺                          | 0                                               |
| HK116C            | 43.71⁺                      | 22.44⁺                   | 26.81⁺                        | 2.47⁺                                           |
| Control           | 11.08⁺                      | 0                        | 0                             | 0                                               |

*Data are the average of five replicates
⁺⁺⁺Values in each column followed by the same letter are not significantly different (P<0.05).
Control: plants not inoculated with rhizobia strain.
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37°C for 1 h, and the products analyzed by HPLC and MS. MK-8 was found in the reaction products in addition to DMK-8 (Figure 7B, 8 and Figure S5). This showed that DmtH had methyltransferase activity to transfer a methyl group from SAM to DMK-8, indicating that the \textit{dmtH} gene had a function similar to the \textit{ubiE} gene.

**Discussion**

In 1960 s–1980 s, studies were carried out on the electron transfer mechanisms of \textit{Rhizobium}. UQ was considered to be the electron carrier operating electron transfer in free-living cells and bacteroids. In vitro, menadione could substitute for UQ to reduce cytochrome b [20], but no evidence was obtained for the presence of MK in \textit{Rhizobium} [21]. In previous reports, work focused on the electron transfer pathway of \textit{BradyRhizobium} sp., but with little on \textit{Mesorhizobium}.

In this work, the function of the \textit{dmtH} gene was identified and characterized by gene disruption, symbiotic phenotype, functional complementation, gene expression pattern and DMK methyltransferase activity measurement in vitro. The results showed that...
UQ-10 accumulated under aerobic conditions, while synthesis of MK-7 was induced under microaerobic conditions in strain 7653R when NO3\(^+\) was supplied as terminal electron acceptor. Since the \textit{dmtH} gene was mainly expressed during symbiosis, we assume that MK-7 was produced only in the bacteroids with little in free-living cells.

Also supporting MK as a quinone pool in bacteroids was the ultrastructural of nodules induced by HK116, in which PHB was accumulated and the membrane in bacteroids was incrassated. \textit{M. huakuii} form indeterminate nodules on host plants and do not accumulate visible PHB granules during symbiosis. PHB granules are synthesized during the initial stage of invasion and used in the process of bacteroid differentiation [22]. Intracellular PHB may fuel cell division and growth during root infection and invasion [23]. PHB granules accumulating in bacteroids implied that the metabolism of the carbon source or energy was shifted. Normally, intracellular PHB as a carbon and energy source can be metabolized in the TCA cycle for NADH production, which then flows into the electron transfer chain for ATP formation during root infection and invasion. However, in the symbiosis of the disruptant HK116, MK could not be synthesized, which led to obstruction of the electron transfer chain. To allow continued operation of the TCA cycle, NAD(P)H was channeled into other biosynthesis reactions, such as PHB synthesis, for use as reducing equivalents.

During nitrogen fixation, the bacteroid is supplied with C\(_4\)-dicarboxylates as a carbon resource by the plant [24], and bacteroids carry the carbon resources into the TCA cycle to produce reducing equivalents and ATP for driving nitrogen fixation [25,26]. Therefore, NADH synthesized from metabolites supplied by host plant, did not flow into the electron delivery chain to produce ATP for nitrogen fixation in the disruptant HK116 bacteroids lacking DMK methyltransferase. Instead, the NADH was used for synthesis of PHB and long-chain fatty acids, which further facilitated their accumulation. Thus, abnormal morphologies and structures of bacteroids, such as smaller size, membrane incrassation and PHB granules, were observed (Figure 4). Therefore, the obstruction of MK biosynthesis and the electron transfer chain in bacteroids, caused impairment of the generation of ATP, and \textit{Rhizobia} could not carry out the normal differentiation program in nodules, losing nitrogen fixation ability.

In \textit{E. coli}, UQ is the electron-transferring quinone in aerobic respiration, and MK in anoxic conditions. Correspondingly, two dehydrogenases that transfer electrons from NADH to the respiratory chain are present in cells. NDH-1 type dehydrogenase, which is a multisubunit complex, is expressed under anaerobic conditions, reducing fumarate and using MK as an electron acceptor. NDH-2 dehydrogenase, a monomeric protein smaller than NDH-1, oxidizes succinate and reduces UQ [27,28,29]. If MK was used as an electron carrier in bacteroids of \textit{Rhizobia}, two dehydrogenases would also be expected to participate in oxidation-reduction reactions in free-living and symbiosis modes, as reported for \textit{R. leguminosarum} [30]. This study isolated two different NADH-dependent dehydrogenases, DH1 and DH2, from \textit{R. leguminosarum}. The DH1 complex, like the NDH-1 type dehydrogenases with a molecular mass of 550 kDa, is a specific-bacteroid membrane component. The DH2 complex, with a molecular mass of

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Figure 5. HPLC analyses of MK and UQ from wild type strain 7653R and disruptant HK116 under aerobic and microaerobic conditions. (A) UQ-10 was accumulated in 7653R under aerobic condition. (B) UQ-10 was accumulated in HK116 under aerobic condition. (C) MK-7 was accumulated in wild type strain 7653R when culturing conditions were shifted to microaerobic for 24 hours. (D) MK-7 was not produced in disruptant HK116 under microaerobic condition.

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110 kDa, is present in both bacteroids and free-living bacteria. This implies that MK could act as an electron carrier in bacteroids. In agreement with our findings, the dntH gene was distributed only in a few of *Rhizobium* genomes including *Mesorhizobium* sp., *R. leguminosarum* and *R. etli*, by homology analysis and Southern blotting (Figure S3), which showed that MK was not a commonly trait in most *Rhizobia*.

In previous report, MK was considered to represent the ancestral type of quinone in proteobacteria, and MK-to-UQ transition which had occurred 2 billion years ago probably proceeded via ambivalent organisms which can use both types of bioenergetic chains[5]. The phenomena was observed in *E. coli* and closely related species. Recently the γ-proteobacterium *Shewanella* ANA-3 was reported to perform bioenergetic electron transport from lactate to arsenate based on a MK-pool[31]. The *Halorhodospira* species *H. halophila*, one of photosynthetic members, was also proved to contain both UQ and MK, and operate the photosynthetic cycle based on MK-pool which was not a common trait of *Halorhodospira/Ectothiorhodospira*, and other subclasses of *Halorhodospira* worked on UQ bioenergetic chains when grown phototrophically [5]. According to the findings, we guessed *M. huakuii* maybe one of ambivalent organisms. In proceeding of the *Rhizobium* evolvement, the difference of host plants appears to influence bacteroid physiological transition. *Rhizobium* could form indeterminate nodules in galegoid legumes and determinate nodules in nongaleoid legumes. Using recombinant *Rhizobium* strains nodulating both two legume types, bacteroid cell cycle and differentiation had shown to be controlled by the host plant in the *Rhizobium*-legume symbiosis [32]. *M. huakuii* has a strict host specificity and only form nodules in *A. sinicus* which imply *M. huakuii*...
and two branched pathways in *Rhizobium trifolii* [17]. In our studies, MK-7 was confirmed to play an important role as a quinone pool in *M. huakuii* bacteroids, which implies that more complicated branched electron transfer networks exist in *Rhizobia*. Detailed information on the full respiratory chain of *Mesorhizobium* particularly the pathway used under symbiosis, requires further research.

**Materials and Methods**

**Media and growth conditions**

*E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C. *Rhizobia* were grown in tryptone yeast medium (TY) at 28°C. When required, the following antibiotics were used: ampicillin (Ap) 100 μg/ml; spectinomycin (Spe) 50 μg/ml; tetracycline (Tet) 15 μg/ml; gentamicin (Gm) 20 μg/ml; and kanamycin (Kan) 50 μg/ml.

**Cloning and sequence analysis of *dmtH* gene**

The genome of *M. huakuii* has not still been sequenced at present, hence thermal asymmetric interlaced PCR (TAIL-PCR) was used to amplify the flanking regions of Tn5-B12S from the genome of the original symbiosis defective mutant HK115 (mol“ix”). Techniques were performed as previously described [18]. TAIL-PCR products were cloned into plasmid vector pMD-18T (Takara, DaLian, China) and sequenced. This gene was designated as *dmtH* (GenBank accession no. JN400272). Two oligonucleotides, *dmtUP* (5'-ATGACGCGATCCGCGACGA-3') and *dmtLOW* (5'-CTAATTTGGGTTCCCCAAGC-3') were designed to amplify the 750-bp DNA sequences containing the full-length ORF of *dmtH* gene from total DNA of *M. huakuii* 7563R. Nucleotides and protein homology searches were carried out by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). The protein-conserved domains were predicted using the InterProScan server of EBI (http://www.ebi.ac.uk/InterProScan).

**Construction of *dmtH* null mutant**

Primers used to PCR-amplify a segment of 7653R genomic DNA containing the *dmtH* ORF with 200 bp of flanking sequence on either side of the coding sequence were: *dmtUP1*: 5'-TCAATTTCAACCGGAGTCC-3'; *dmtLOW1*: 5'-GACCTGG-TTTCGCGCGCG-3'. The resulting PCR product (1.1 kb) was ligated into pMD-18T to generate pMD*dmtH*. A 950-bp fragment containing a Km-resistance cassette was amplified from plasmid pMH1701 by PCR with primers kanNcoIU (5'-TTTCCATGGG-GAAAGCCACGTTGTTGTCT-3'; the NcoI restriction site is underlined) and kanNcoIL (5'-TTTCCATGGGAAAAACTGATCGAGCA-3'; the NsiI restriction site is underlined). The Km-resistance cassette was inserted into the unique *NcoI* site of the *dmtH* gene in pMD*dmtH* to create plasmid pMD*dmtH-Km*. A 2.1-kb fragment containing *dmtH* and the Km cassette was cut from pMD*dmtH-Km* and subcloned into pJQ200 mp18 using PstI and EcoRI restriction sites. The resulting suicide plasmid, pJQ*dmtH-Km*, was conjugated into *M. huakuii* 7563R by tri-parental mating with a helper plasmid pRK2013 [33]. A *dmtH* double-crossover recombinant was selected using the sac mutagenesis strategy as previously described [34]. The disruption of *dmtH* in mutant strain HK116 was verified by PCR and plant nodulation experiment.

**Complementation**

For the symbiotic function complementation test, the *dmtH* gene was amplified with 7653R total DNA as the template using the following two primers: *dmtUP2*: 5'-ACTCTCGAGTAAATGTA-GACTGAACGAGGAGGTCGACGATGACGCATAC-3' (the
XhoI restriction site is underlined), dmtLOW2: 5’- ACTCTCGAGCTATTTGTTCCCAAA-3’ (the XhoI restriction site is underlined). The amplified fragment digested with XhoI was cloned into the previously modified vector pBBRgas, in which gusA was inserted into the broad-host-range plasmid pBBR1MCS5 under the Lac promoter [35]. The recombinant plasmid pBBRgus-dmt was obtained, and transferred into mutant strain HK116 by triparental mating. Transconjugants were used to inoculate seedlings of A. sinicus to test for ability to restore the symbiotic phenotype of HK116.

Plant growth and nodulation experiment

Seeds of A. sinicus cultivar XY202 were surface sterilized for 5 min in 75% ethanol, soaked for 20 min in 2% sodium hypochlorite, and rinsed extensively with sterile water. Plants were grown in pots filled with sterile sand containing nitrogen-free Faheyraeus solution [36]. Cultivation was carried out in a controlled-environment chamber (10 h light and 6 h darkness at 22 and 20°C, respectively) for 35 days. Nodules were collected for paraffin-embedded section slides, electron microscopy and nitrogenase activity examination. Nitrogenase activity was measured by gas chromatography (GC) as acetylene reduction activity as previously described [37].

Microscopic analysis

Light and transmission electron microscopic studies of nodules were performed as follows. For paraffin sections, root nodules were dehydrated in ethanol and xylene, embedded in paraffin wax, serial-cut longitudinally, and slides were stained with toluidine blue, then observed with a light microscope. For electron microscopy, after nodules were fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, rinsed, dehydrated and embedded in London resin white, ultra-thin sections were taken and examined in a Hitachi H-7650 transmission electron microscope.

Quantification of dmtH gene expression by real-Time RT-PCR

To determine the relative dmtH gene expression level, total RNA was isolated with Trizol reagent from free-living M. huakuii 7653R cultured in TY liquid medium and plant nodules, which were harvested from A. sinicus inoculated with wild type strain 7653R after 10, 15, 20, 25, 30, 35 and 40 days. Quantitative real-time PCR was performed using the SYBR Premix ExTaq (Takara, 7635R after 10, 15, 20, 25, 30, 35 and 40 days. Quantitative real-time PCR was performed using the SYBR Premix ExTaq (Takara, Dalian, China) on the iCycler iQ5 Multicolor Real-Time PCR Detection System (primers: 5’-CATCTATCCCAATGCCGTCAAC-3’ and 5’-GCCAGACCTCTTCCCAATCTGGT-3’) (Bio-Rad, Hercules, CA, USA). Expression levels were normalized using rnpB gene as internal control (primers: 5’- ATAACTGGTTTACCGTGCCGC-3’). The amplified fragment digested with XhoI restriction site is underlined) were synthesized, and used to amplify dmtH. PCR products were cloned into the pET28a vector digested with XhoI and XhoI. The resulting plasmid, named pETdmt, was transformed into E. coli Rosetta 2(DE3) for overexpression of the target protein DmtH. Expression, extraction and purification of DmtH protein was carried out by standard methods on Bio-Rad Biologic LP apparatus [39].

E. coli JC7623A4-1, which contains only DMK but no MK, was kindly supplied by Professor Catherine F. Clarke of University of California. Crude homogenates of E. coli JC7623A4-1 cells were prepared as a reaction substrate for DmtH protein activity assays. As described previously, JC7623A4-1 cells grown into late exponential phase in LB medium were centrifuged, suspended in a solution of 25 mM Tris-HCl buffer pH 7.7 containing 1 mM EDTA and 10 mM 2-mercaptoethanol (2 ml/g of wet cells), and disrupted with a Branson sonifier [9].

The DmtH protein reaction mixture contained, in a total volume of 10 ml, 4 ml of crude homogenate of JC7623A4-1 cells, 0.1 M Tris-HCl buffer (pH 8.0), 10 mM MgCl2, 10 mM dithiothreitol, 10% aqueous Triton X-100 (to give a final concentration of 0.5% Triton X-100), 10 nmol S-adenosyl-L-methionine (SAM) and 1 μM dmtH product. After incubation at 37°C for 1 h, the reaction was stopped by addition of 10 ml of 0.1 M acetic acid in methanol. The reaction products were extracted with 40 ml methanol/chloroform (1:1) and evaporated in vacuo. The lipid extracts were resuspended in 0.5 ml of chloroform/liter of culture, and analyzed by HPLC and MS.

Supporting Information

Figure S1 Multiple sequence alignment of the DmtH protein and the homologous sequence. Columns that are dark represent identical residues, lighter black represent similar residues in function. The numbers on the upper indicate the positions of amino acids. Protein names are indicated to the left of the alignment. These proteins come from Mesorhizobium loti MAFF303099 (mlr2332), Rhizobium loti ORG11089 (AO102630), Agrobacterium tumefaciens (AraR_10170), K. pneumoniae (Kp3048), Streptomyces coelicolor A3(2) (smeR_3954), Thermobifida fusca DSM 20689 (40625), Xanthomonas campestris pv. glycines DSM 427 (Xcu00805), Staphylococcus aureus (nc1023), and several other bacteria. (TIF)
Figure S2 Homology tree of DmtH homologs. The homology tree was constructed using MEGA5.04 software (Tamura K, 2011). The scale bars indicate 10% substitutions per site. (TIF)

Figure S3 Southern blot analysis of dmtH gene in different Rhizobium genomes. The total genomic DNA of tested strains were digested for at least 3 h with 5-10 U of restriction enzyme PstI and BamHI per μg of DNA. Digested genomes were subjected to electrophoresis and transfer to nylon membranes, then hybridized to a 32P-labeled probe. The tested strains were Agrobacterium tumefaciense, Sinorhizobium saheli, Sinorhizobium medicae, Azorhizobium caulinodans HAMBI 1552, Rhizobium leguminosarum LRP 5045 (R. leg), Sinorhizobium fredii HN01 (S. fre), Mesorhizobium loti 541 (M. lot), Sinorhizobium arboris HAMBI 1532, Acidorhizobium caulinodans USDA 4892 (A. cau). (A) the electrophoresis map of DmtH-digested genomic DNA; (B) the electrophoresis map of PstI-digested genomic DNA. (C) BamHI-digested DNA hybridised with probe dmtH. (D) PstI-digested DNA hybridised with probe dmtH. The Southern blot shows that dmtH gene is present in R. leguminosarum LRP 5045 and M. lot 541. (TIF)

References
1. Collins MD, Jones D (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol Rev 45: 316–354.
2. Sobale R, Poole RK (1999) Microbial ubiquinones: multiple roles in respiration, gene regulation and oxidative stress management. Microbiology 145 (Pt 8): 1817–1830.
3. Nosivcova B, Kruk J (2010) Occurrence, biosynthesis and function of isoprenoid quinones. Biochim Biophys Acta 1797: 1587–1605.
4. Schutz M, Brugna M, Lebrun E, Baymann F, Huber R, et al. (2000) Early evolution of cytochrome bc complexes. J Mol Biol 300: 665–675.
5. Schoepp-Cothenet B, Lieutaud C, Baymann F, Vermeglio A, Friedrich T, et al. (2006) Menaquinone as pool quinone in a purple bacterium. PNAS 103: 1849–1854.
6. Brondijk TH, Figgen D, Richardson DJ, Cole JA (2002) Roles of NapF, NapG and NapH, subunits of the Escherichia coli periplasmic nitrate reductase, in ubiquinol oxidation. Mol Microbiol 44: 245–255.
7. Wraith WH, King A, Under G (1990) The specific functions of menaquinone and dimethylnaphthoquinone in anaerobic respiration with fumarate, dimethylsulfoxide, trimethylamine N-oxide and nitrite by Escherichia coli. Arch Microbiol 154: 60–66.
8. Bekker M, Alexeeva S, Laan W, Soeters G, de Mattos JT, et al. (2010) The ArchA two-component system of Escherichia coli is regulated by the redox state of both the ubiquinone and the menaquinone pool. J Bacteriol 192: 746–754.
9. Kooke-Takeshita A, Koyama T, Ogura K (1997) Identification of a novel gene cluster participating in menaquinone (vitamin K2) biosynthesis. Cloning and sequence determination of the 2-heptaprenyl-1-naphthoquinone menaquinone transmethylase gene of Bacillus stearothermophilus. J Biol Chem 272: 12380–12383.
10. Hitrusaka T, Furuhita K, Ishikawa J, Yamashita H, Inoh N, et al. (2008) An alternative menaquinone biosynthetic pathway operating in microorganisms. Science 321: 1670–1673.
11. Wissenbach U, Ternes D, Under G (1992) An Escherichia coli mutant containing only dimethylnaphthoquinone, but no menaquinone: effects on fumarate, dimethylsulfide, trimethylamine N-oxide and nitrite respiration. Arch Microbiol 158: 68–73.
12. Lee PT, Hsu AY, Ha HT, Clarke CF (1997) A C-methyltransferase involved in fumarate, dimethylsulfoxide, trimethylamine N-oxide and nitrate respiration. Arch Microbiol 154: 60–66.
13. Gabel C, Maier RJ (1990) Nucleotide sequence of the coxA gene encoding the subunit I of cytochrome aa3 of Bradyrhizobium japonicum. Nucleic Acids Res 18: 4143–4148.
14. Bott M, Preiss O, Hennecke H (1992) Genes for a second terminal oxidase in Bradyrhizobium japonicum. Arch Microbiol 158: 335–343.
15. Preiss O, Zaffreery R, Thony-Meyer L, Appleby CA, Hennecke H (1996) A high-affinity cbb3-type cytochrome oxidase terminates the sbb-158428symbiotic respiratory chain of Bradyrhizobium japonicum. J Bacteriol 178: 1552–1538.
16. Richardson DJ (2009) Bacterial respiration: a flexible process for a changing environment. Microbiology 156 (Pt 3): 551–571.
17. de Hollander JA, Stouthamer AH (1980) The electron transport chain of Rhizobium trifolii. Eur J Biochem 111: 473–478.
18. Cheng GJ, Li YG, Zhou JC (2006) Cloning and identification of opa22, a new gene involved in nodulation formation by Mesorhizobium huakuii. FEBS Microbiol Lett 257: 152–157.
19. Swarzening JW Jr., Fuentes DE, Araya MA, Plisker MF, Saavedra CP, et al. (2006) Expression of the ubiB gene of Geobacillus stearothermophilus V in Escherichia coli K-12 mediates the evolution of salinophile compounds into the headspace of salinophile and salinophile-remodeled cultures. Appl Environ Microbiol 72: 963–967.
20. O’Brien MR, Maier RJ (1982) Electron transport components involved in hydrogen oxidation in free-living Rhizobium japonicum. J Bacteriol 152: 422–430.
21. Daniel RM (1979) The occurrence and role of ubiquinone in electron transport to oxygen and nitrate in aerobically, anaerobically and symbiotically grown Rhizobium japonicum. J Gen Microbiol 119: 333–337.
22. Lodwig EM, Leonard M, Marroqui S, Wheeler TR, Findlay K, et al. (2005) Role of polynhydroxybutyrate and glycerol as carbon storage compounds in pea and bean bacteroids. Mol Plant Microbe Interact 18: 67–74.
23. Trainer MA, Charles TC (2006) The role of PHB metabolism in the symbiosis of rhizobia with legumes. Appl Microbiol Biotechnol 71: 372–386.
24. Bolon E, Higgison B, Harrington A, O’Gara F (1966) Dicarboxylate acid transport in Rhizobium meliloti: isolation of mutants and cloning of dicarboxylic acid transport genes. Arch Microbiol 144.
25. Prell J, Poole P (2006) Metabolic changes of rhizobia in legume nodules. Trends Microbiol 14: 161–168.
26. White J, Prell J, James EK, Poole P (2007) Nutrient sharing between symbionts. Plant Physiol 144: 604–614.
27. Young RG, Rogers BL, Campbell HD, Jaworowski A, Shaw DC (1981) Identification and Characterization of a Bacteroid-Specific Dehydrogenase Gene Involved in Nodule Formation by Mesorhizobium huakuii. FEMS Microbiol Lett 25: 152–157.
28. Tram QH, Bongaerts J, Vlad D, Under G (1997) Requirement for the presequence of NADH dehydrogenase I of Escherichia coli in respiration of NADH to fumarate and its bioenergetic implications. Eur J Biochem 244: 155–160.
29. Nantapong N, Otofuji A, Migita CT, Adachi O, Toyama H, et al. (2005) Role of polyhydroxybutyrate and glycerol as carbon storage compounds in pea and bean bacteroids. Mol Plant Microbe Interact 18: 67–74.
30. Trainer MA, Charles TC (2006) The role of PHB metabolism in the symbiosis of rhizobia with legumes. Appl Microbiol Biotechnol 71: 372–386.
31. Kooke-Takeshita A, Koyama T, Ogura K (1997) Identification of a novel gene cluster participating in menaquinone (vitamin K2) biosynthesis. Cloning and sequence determination of the 2-heptaprenyl-1-naphthoquinone menaquinone transmethylase gene of Bacillus stearothermophilus. J Biol Chem 272: 12380–12383.
32. Mergaert P, Uchiumi T, Alunni B, Evanno G, Cheron A, et al. (2006) Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-leguminosae symbiosis. Microbiol Rev 45: 316–354.

Figure S4 HPLC of the pure standard MK-4, MK-7, MK-9 and UQ-10. (TIF)

Figure S5 Mass spectrum of quinine compounds from E.coli JC7623ΔA1-1 and the reacting system of testing DmtH protein activity. (A) Mass spectrum of DMK-8 which was accumulated in E.coli JC7623ΔA1-1 but no MK-8’ MS found. (B) Mass spectrum of MK-8 which was tested from in reaction system. The result showed MK-8 was synthesized in the reaction system and DmtH had the the function of methyltransferase. (TIF)

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Author Contributions
Conceived and designed the experiments: FX YL. Performed the experiments: FX GX HX 2W. Analyzed the data: LL. Wrote the paper: FX YL.
34. Cheng G, Li Y, Xie B, Yang C, Zhou J (2007) Cloning and identification of lpsH, a novel gene playing a fundamental role in symbiotic nitrogen fixation of Mesorhizobium huakuii. Curr Microbiol 54: 371–375.

35. Xie B, Chen DS, Zhou K, Xie YQ, Li YG, et al. (2006) Symbiotic abilities of Sinorhizobium fredii with modified expression of purL. Appl Microbiol Biotechnol 71: 505–514.

36. Nutman PS (1970) The modified Fahraeus slide technique, appendix III. In: Vincent JM, ed. A manual for the practical study of root-nodule bacteria. Oxford, UK: Blackwell Scientific. pp 144–145.

37. Hardy RWF, Bum RC, Holstein RD (1973) Application of C2H2-C2H4 assay for measurement of nitrogen fixation. Soil Biology and Biochemistry 5: 47–81.

38. Unden G (1988) Differential roles for menaquinone and demethylmenaquinone in anaerobic electron transport of E. coli and their fur-independent expression. Arch Microbiol 150: 499–503.

39. Wu FF, Provost R (2006) Automated Three-Dimensional Purification Method for Histidine-Tagged Proteins Using the BioLogic DuoFlow Maximizer™ Chromatography System. BioRadiations 119: 30–32.