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BIOCHEMISTRY

A dedicated thioesterase of the Hotdog-fold family is required for the biosynthesis of the naphthoquinone ring of vitamin K_1

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Abstract

Phylloquinone (vitamin K_1) is a bipartite molecule that consists of a naphthoquinone ring attached to a phytyl side chain. The coupling of these 2 moieties depends on the hydrolysis of the CoA thioester of 1,4-dihydroxy-2-naphthoate (DHNA), which forms the naphthalenoid backbone. It is not known whether such a hydrolysis is enzymatic or chemical. In this study, comparative genomic analyses identified orthologous genes of unknown function that in most species of cyanobacteria cluster with predicted phylloquinone biosynthetic genes. The encoded approximately 16-kDa proteins display homology with some Hotdog domain-containing CoA thioesterases that are involved in the catabolism of 4-hydroxybenzoyl-CoA and gentisyl-CoA (2,5-dihydroxybenzoyl-CoA) in certain soil-dwelling bacteria. The *Synechocystis* ortholog, encoded by gene slr0204, was expressed as a recombinant protein and was found to form DHNA as reaction product. Unlike its homologs in the Hotdog domain family, Slr0204 showed strict substrate specificity. The *Synechocystis slr0204* knockout was devoid of DHNA-CoA thioesterase activity and accumulated DHNA-CoA. As a result, knockout cells contained 13-fold less phylloquinone than their wild-type counterparts and displayed the typical photosensitivity to high light associated to phylloquinone deficiency in cyanobacteria.

Keywords: cyanobacteria, isoprenoids, photosynthesis, phylloquinone

Vitamin K encompasses a class of fat-soluble compounds formed from a naphthoquinone ring attached to a polyisoprenyl chain of variable length and saturation. Its main natural forms are vitamin K_1 (phylloquinone), which contains a partially saturated C-20 phytyl side chain, and vitamin K_2 (menaquinone), whose usually longer side chain is fully conjugated (Figure 1). Phylloquinone is synthesized by plants and most cyanobacteria (1), whereas menaquinone is synthesized by facultative anaerobic bacteria, archaea, and some cyanobacteria (1, 2). Vitamin K plays very different roles in the organisms that synthesize it compared to those that do not. Facultative anaerobic bacteria use menaquinone as an electron transporter in their respiratory chain (3). Similarly, in cyanobacteria and plants, phylloquinone serves as the one-electron carrier at the A1 site of photosystem I (4). In contrast, vertebrates, which do not synthesize vitamin K, use it as a cofactor for certain carboxylases involved in blood coagulation, bone and vascular metabolism, and cell cycle regulation (5). In mammals, vitamin K is also known to act as a signaling molecule that regulates the transcription of genes involved in its own metabolism (6).

Depending on the organisms, there are 2 different routes for the biosynthesis of the naphthoquinone moiety of vitamin K. Both start from chorismate. In the first pathway, that is specific to certain menaquinone-synthesizing microorganisms, the naphthalenoid backbone originates from a chorismate-inosine conjugate called futalosine (7). In the second pathway, which includes all of the phylloquinone producers, the core of the naphthoquinone ring comes from 1,4-dihydroxy-2-naphthoate (DHNA). It is subsequently coupled to the polyisoprenyl side chain and then methylated (Figure 1). The DHNA-pathway was initially described in *Escherichia coli* (8), and later using genetic approaches in cyanobacteria (9–11) and plants (12–16). In this pathway, chorismate is first converted into isochorismate, to which a succinyl side chain is added at the C2 position. After elimination of pyruvate and aromatization of the cyclohexadiene

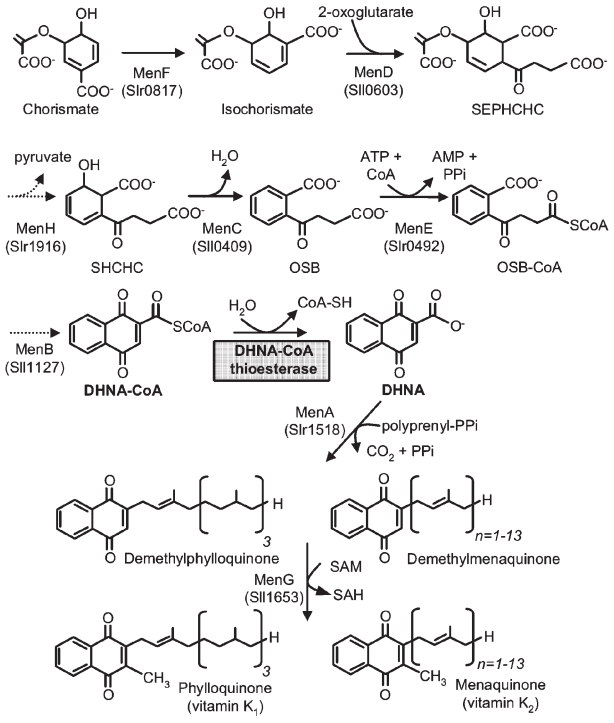

Figure 1. The biosynthetic pathway of vitamin K in *E. coli* and *Synechocystis* sp. PCC 6803. The *E. coli* Men enzymes and the protein number of their orthologs in *Synechocystis* (brackets) corresponding to each known step are indicated. MenF, isochorismate synthase; MenD, SEPHCHC synthase; MenH, SHCHC synthase (note that this enzyme is currently mis-annotated in most databases as DHNA-CoA thioesterase); MenC, OSB synthase; MenE, OSB-CoA ligase; MenB, DHNA synthase; MenA, DHNA prenyltransferase; MenG, demethylmenaquinone/demethylphylloquinone methyltransferase. SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid; SHCHC, (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid.

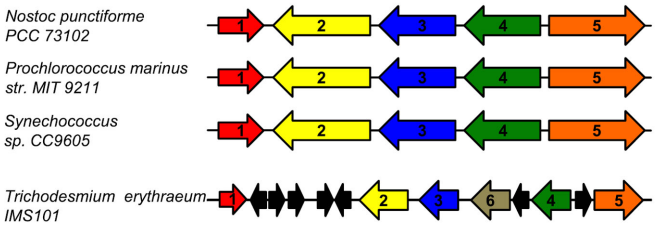

Figure 2. Clustering of a putative CoA thioesterase with predicted phylloquinone biosynthetic genes in cyanobacteria. Matching colors and numbers indicate orthology. 1, putative CoA-thioesterase; 2, OSB-CoA ligase; 3, OSB synthase; 4, DHNA prenyltransferase; 5, isochorismate synthase; 6, SHCHC synthase. Black arrows indicate genes of unknown function or whose function is a priori not directly connected to phylloquinone biosynthesis. Note that the gene cluster in *T. erythraeum* is not to scale with that of the other species.

ring, the succinyl chain is activated by ligation with CoA, and then cyclized, yielding DHNA-CoA (Figure 1). The subsequent removal of CoA is of special interest, for it frees the carboxyl group of DHNA and allows its conjugation to the polyisoprenyl chain for anchoring into biological membranes. The hydrolysis of DHNA-CoA has long been a puzzling mystery of vitamin K biosynthesis, and it is actually not clear whether this step is enzyme-driven or a purely chemical process (1). It was first thought to occur concomitantly to the cyclization step catalyzed by DHNA synthase, hence the name of this enzyme (17). Such a mechanism was later invalidated when it was shown that the product of the DHNA synthase-catalyzed reaction was not DHNA but DHNA-CoA (18). The identification in the menaquinone biosynthetic operon of *E. coli* of a gene encoding for a protein termed MenH (8), whose α/β-fold type 1 motif is commonly found among thioesterases compounded the confusion. Despite the lack of direct evidence that MenH or its orthologs displayed some activity against DHNA-CoA, the protein was assumed to correspond to the missing CoA thioesterase (8). It is not until recently that MenH was shown to lack thioesterase activity and to catalyze in fact an earlier step in the pathway (Figure 1) (19), reopening de facto the search for a putative DHNA-CoA thioesterase.

In this study, we used comparative genomics to infer the existence of DHNA-CoA thioesterase in the phylloquinone biosynthetic pathway of cyanobacteria. We provide biochemical and genetic evidence that the encoding gene is required for phylloquinone biosynthesis.

Results

Cyanobacteria Contain Putative CoA Thioesterases That Cluster with Predicted Phylloquinone Biosynthetic Genes. Because prokaryotic genes that are involved in the same cellular process tend to cluster together (20), we used the on-line database for comparative genomics SEED and its tools (http://theseed.uchicago.edu/FIG/index.cgi) to search for conserved physical associations between known vitamin K biosynthetic genes and genes whose function is unknown or a priori unrelated. These in silico searches detected a group of cyanobacterial orthologs that cluster with the near complete set of phylloquinone biosynthetic genes (Figure 2). The encoded proteins (approximately 16-kDa) have unknown function, but feature a conserved domain (cd03440), named Hotdog, that is found in certain thioesterases (21). Consistent with this, the cyanobacterial proteins share 17–25% identity with *Pseudomonas* sp. strain CBS-3 4-hydroxybenzoyl-CoA thioesterase (4-HBT; EC 3.1.2.23) and 16%–23% identity with *Bacillus halodurans* C-125 gentisyl-CoA (2,5-dihydroxybenzoyl-CoA) thioesterase, 2 members of the superfamily of Hotdog domain-containing proteins (22, 23). Because these 2 enzymes belong to catabolic pathways that are specific to a few clades of soil-dwelling bacteria, we hypothesized

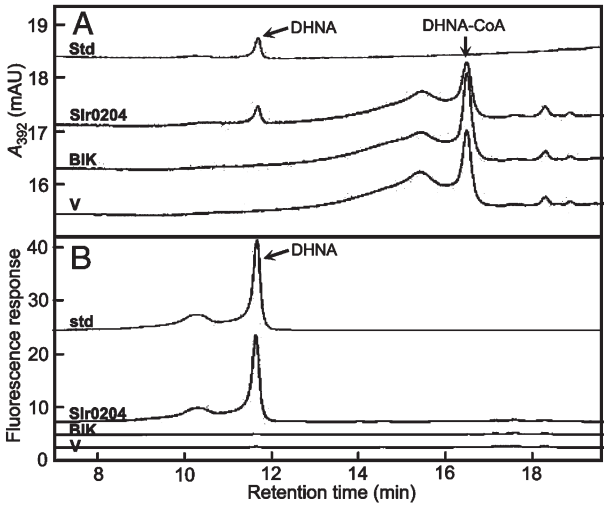

Figure 3. HPLC assays of DHNA-CoA thioesterase activity in desalted extracts of *E. coli*. (*A*) Detection at A_{392} nm. (*B*) Selective detection of DHNA by fluorometry (Ex = 260 nm, Em = 450 nm). Extract of cells overexpressing *Synechocystis* Slr0204 protein (Slr0204), overexpressor extract boiled for 15 min before the assay (Blk), vector alone control extract (V). Assays contained 126 μM DHNACoA and 0.14 μg protein and were carried out for 10 min at 30 °C. The uppermost trace in each frame corresponds to 100 pmol authentic DHNA(Std).

that their cyanobacterial homologs were likely to have a different function and were good candidates for the missing DHNA-CoA thioesterase of phylloquinone biosynthesis.

***Synechocystis* Protein Slr0204 Displays Highly Specific DHNA-CoA Thioesterase Activity.** BLASTp searches using the protein sequences of the candidates previously identified in SEED detected a single ortholog encoded by gene slr0204 in *Synechocystis* sp. PCC 6803. To determine whether the corresponding protein bore DHNA-CoA thioesterase activity, gene slr0204 was cloned into bacterial expression vector pET43.1a, and the protein was overexpressed in *E. coli* BL21 (DE3). DHNA-CoA thioesterase activity was assayed in desalted extracts of the overexpressor and the vector-alone control by monitoring the consumption of DHNA-CoA and the simultaneous formation of DHNA (Figure 3). The hydrolysis of DHNA-CoA was readily detected in the extract of the overexpressor, but not in the vector-alone control (Figure 3), providing initial biochemical evidence for DHNA-CoA thioesterase activity associated with Slr0204. No activity was detected when the extracts were boiled before the addition of the substrate (Figure 3), confirming that the formation of DHNA associated with the overexpressor extract was not simply due to the chemical hydrolysis of DHNA-CoA. To corroborate this result and to test for the substrate specificity of Slr0204, the 6×his-tagged protein was isolated by affinity chromatography. The purified enzyme hydrolyzed DHNA-CoA, but unlike its 4-HBT and gentisyl-CoA thioesterase homologs (23, 24), it did not display any activity against benzoyl-CoA and phenylacetyl-CoA (Table 1). Nor was any activity detected against aliphatic acyl-CoA thioesters (Table 1). The cyanobacterial enzyme thus appears to have stringent substrate specificity.

***slr0204* Knockout Lacks DHNA-CoA Thioesterase Activity and Accumulates DHNA-CoA.** To investigate directly the function of slr0204 in *Synechocystis* cells, we replaced its entire sequence by a marker cassette using homologous recombination. After selection on solid media, a homogenously knockout strain was isolated and transferred to a liquid culture. Although DHNA-CoA thioesterase activity was readily detected in the desalted extracts of wild-type cells, no activity was observed in the extracts of their knock-

Table 1. Substrate specificity of *Synechocystis* DHNA-CoA thioesterase

	Activity, μmol·h^{-1}·mg^{-1}
DHNA-CoA (65 μM)	102 ± 14
Benzoyl-CoA (30 μM)	<0.001
Benzoyl-CoA (120 μM)	<0.001
Phenylacetyl-CoA (50 μM)	<0.001
Phenylacetyl-CoA (100 μM)	<0.001
Succinyl-CoA (14 μM)	<0.001
Palmitoyl-CoA (11 μM)	<0.001

Purified recombinant Slr0204 (1.3–8 μg) was assayed with various CoA thioester substrates. The formation of DHNA was quantified by HPLC; for other substrates, the release of CoA was measured spectrophotometrically using a DTNB-based dosage, as described in Materials and Methods. Data are means ± SE of three replicates.

out counterparts (Figure 4*A*). Moreover, the lack of DHNA-CoA thioesterase activity in knockout cells was paralleled by an approximately 30-fold increase of their pool size of DHNA-CoA compared to wild-type ones (Figure 4*B*), confirming that the Slr0204 protein also functions as a DHNA-CoA thioesterase in vivo.

***slr0204* Knockout Lacks Phylloquinone and Is Photosensitive.** As in plants, vitamin K is not the sole quinone derived from DHNA (25); we verified that Slr0204 was indeed involved in phylloquinone biosynthesis by measuring the level and redox status of this metabolite in the knockout and wild-type cells. The knockout cells were found to contain 13-fold less phylloquinone than the wild-type ones (Figure 5 *A*) and displayed lower phylloquinone (oxidized)/phylloquinol (reduced) ratio (Figure 5*A*). Addition of DHNA restored the phylloquinone content of the mutant (Figure 5*A*), whereas no significant changes were seen when *o*-succinylbenzoate (OSB)—a biosynthetic intermediate located upstream the DHNA-CoA-catalyzed reaction—was added (Figure 5). Transformation of the knockout with plasmid pSynExp2 containing the sequence of slr0204 under the control of an endogenous cyanobacterial promoter restored phylloquinone content and redox status to levels similar to that of wild-type cells (Figure 5*A*), verifying that the lack of phylloquinone in the knockout was not caused by a secondary mutation. Compared to wild-type and complemented cells, the knockout strain displayed severe growth retardation at high light intensity, but grew normally in low light (Figure 5*B*). This phenotype is characteristic of phylloquinone deficiency in *Synechocystis* (9).

Discussion

We report here the identification of a cyanobacterial protein specifying the missing DHNA-CoA thioesterase of phylloquinone biosynthesis. This brings the total number of known phylloquinone biosynthetic genes to 9 and completes the identification of the enzymes involved in the formation of the naphthoquinone ring in cyanobacteria. Unlike their orthologs in *Nostoc punctiforme*, *Prochlorococcus marinus*, *Trichodesmium erythraeum*, and S*ynechococcus*, none of the phylloquinone biosynthetic genes of *Synechocystis* sp. PCC 6803 clusters with each other. This feature actually guided our choice to work with this species, as it permitted us to knock out the DHNA-CoA thioesterase candidate without creating a negative polar effect on other phylloquinone biosynthetic genes.

Phylloquinone-synthesizing eukaryotes display homologs of cyanobacterial DHNA-CoA thioesterase (Figure S1). The corresponding proteins are either plastid- or chromatophore-encoded in rhodophytes and amoeba, respectively, or nuclear-encoded in green algae and flowering plants, but then display predicted chloroplast-targeting peptides. Such a distribution is notable, for it strictly follows that of the other phylloquinone biosynthetic genes

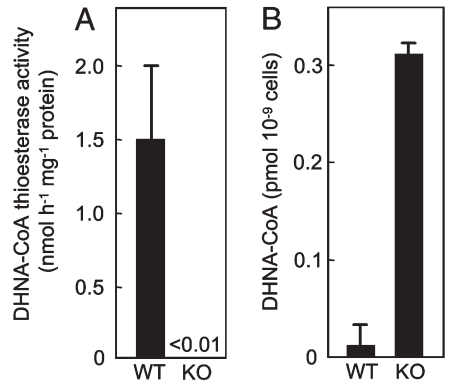

Figure 4. The slr0204 mutant lacks DHNA-CoA thioesterase activity and accumulates DHNA-CoA. (*A*) Desalted extracts of wild-type (WT) and *slr0204* knockout (KO) *Synechocystis* cells were assayed for DHNA-CoA thioesterase activity for 10 min at 30 °C. Assays with wild-type and knockout extracts contained 15 and 14 μg protein, respectively. (*B*) DHNA-CoA present in theextracts of wild-type and knockout cells was quantified by HPLC-fluorescence after enzymatic conversion to DHNA with an excess of recombinant Slr0204 protein. DHNA was then extracted by phase partitioning and chemically reduced to confer fluorescent properties. The data are means of 3 replicates (DHNA-CoA thioesterase activity) and 2 replicates (DHNA-CoA level) ± SE.

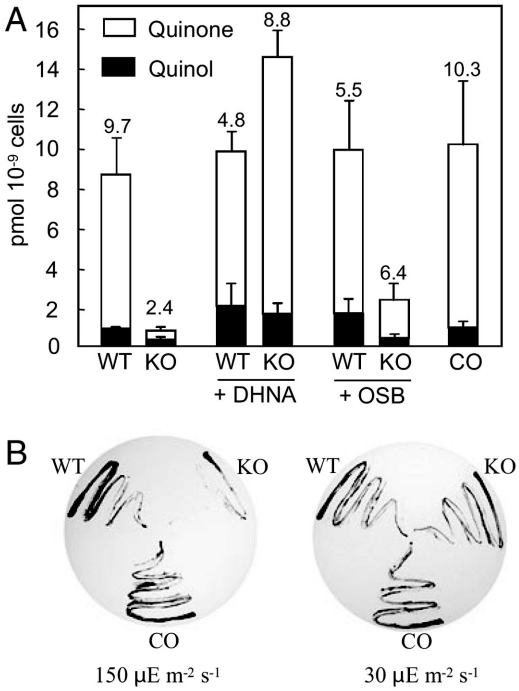

Figure 5. The slr0204 mutant lacks phylloquinone and displays photosensitivity to high light intensities. (*A*) Level and redox status of phylloquinone in *Synechocystis* cells. The final concentration of DHNA and OSB in the culture media was 100 μM. Values above the bars indicate the quinone/quinol ratio. Data are means ± SE of 3 replicates. (*B*) Growth of *Synechocystis* cells in high and low light intensities. Similar numbers of cells have been plated on BG-11 medium containing glucose and no antibiotics. Plates have been incubated for 10 days (150 μE m^{-2}·s^{-1}) or 14 days (30 μE m^{-2}·s^{-1}) at 22 °C. WT, wild type; KO, slr0204 knockout; CO, complemented mutant.

in these species, and plastids are the site of phylloquinone biosynthesis (12–15). Importantly, the rhodophytes and amoeba homologs are arranged in clusters of phylloquinone biosynthetic genes

as occurs in their cyanobacterial ancestors (Figure S2*A*). In the case of green algae and flowering plants, the genomic context of the slr0204 homologs does not permit to infer a similar functional linkage with known phylloquinone biosynthetic genes, and therefore the orthology assignment of these predicted CoA thioesterases merits further investigation.

Although slr0204 homologs are also detected in prokaryotes outside the cyanobacterial lineage, the genomic neighborhood of these genes does not indicate that they indeed encode for such an enzyme. For instance, in several facultative anaerobic bacteria, these homologs are localized in an operon-like structure comprising of components of the Tol membrane transport system and subunits of cytochrome ubiquinol oxidase (Figure S2*B*). In our view, the identity of DHNA-CoA thioesterase remains therefore to be established in these microorganisms. There are nonetheless 2 notable exceptions in proteobacteria and verrucomicrobia species, in which the slr0204 homologs cluster with predicted menaquinone biosynthetic genes (Figure S2*B*).

The identification of DHNA-CoA thioesterase adds a functional member to the thioesterase family of Hotdog domain-containing proteins, which includes various acyl-CoA thioesterases, gentisyl-CoA thioesterase, and 4-HBT (PROSITE PF03061). DHNA-CoA thioesterase is most closely related to these last 2 members. Gentisyl-CoA thioesterase is thought to be involved in the oxidation of gentisate (2,5-dihydroxybenzoate) (23), whereas 4-HBT is part of the degradation pathway of the halogenated pollutant 4-chlorobenzoate (22). Interestingly, 4-chlorobenzoate-CoA ligase and 4-chlorobenzoyl-CoA dehalogenase, the 2 enzymes preceding 4-HBT in the degradation pathway of 4-chlorobenzoate (26), display striking homologies with OSB-CoA ligase and DHNA-synthase, respectively (Figure S3). An attractive hypothesis would be that the catabolism of such a chlorinated aromatic compound has diverged from the naphthoquinone branch of vitamin K biosynthesis.

The absence of detectable activity of DHNA-CoA thioesterase against benzoyl-CoA and phenylacetyl-CoA, whereas its homologs, 4-HBT and gentisyl-CoA thioesterase, show some activity with these substrates (23, 24), is of much interest from the standpoint of the structural determinants of substrate recognition in this enzyme family. Indeed, alignment of cyanobacterial and plant DHNA-CoA thioesterases with 4-HBT and gentisyl-CoA thioesterase, reveals that the Asp residue deemed essential for substrate binding in these last 2 enzymes has been replaced by a His in DHNA-CoA thioesterase (Figure S1). On the other hand, the nucleophilic catalyst Asp of 4-HBT and gentisyl-CoA thioesterase is strictly conserved in DHNA-CoA thioesterase (Figure S1). It is possible that the stringent substrate specificity of DHNA-CoA thioesterase reflects the existence, upstream in the pathway, of another aromatic CoA derivative (OSB-CoA), whose enzymatic hydrolysis would create a futile cycle and annihilate the biosynthetic flux of phylloquinone.

The slr0204 knockout is devoid of DHNA-CoA thioesterase activity and accumulates DHNA-CoA. The trace levels of phylloquinone detected in this mutant are therefore most likely attributable to the chemical decomposition of DHNA-CoA rather than to the action of moonlighting enzymes. Arguing in favor of this hypothesis, we have routinely observed that at physiological pH, pure preparations of DHNA-CoA spontaneously decomposed into DHNA and CoA. Whether enzymatic or chemical, the background hydrolysis of DHNA-CoA in vivo is not by itself sufficient to sustain adequate phylloquinone production. It results in a profound change of phylloquinone redox homeostasis and growth impairment at high light intensity. Similar phototoxicity because of the absence of phylloquinone has been reported for the *Synechocystis* mutants corresponding to DHNA phytyl transferase and DHNA synthase (the *menA* and *menB* homologs, respectively), and has been attributed to an excess reduction of photosystem II (9). The demonstration that a dedicated enzyme hydrolyses DHNA-CoA in cyanobacteria, together with the availability of a corresponding knockout mutant, opens the door to the functional characterization of the homologs of such an enzyme in green algae and flowering plants and to its identification in facultative anaerobic bacteria and archaea.

Methods

Chemicals and Reagents. DHNA-CoA synthesis was modified from Jiang et al. (19); a detailed procedure is given in "Supplemental Materials and Methods". OSB was prepared by dissolving 4 mg of OSB-dilactone in 550 μL 0.68 M NaOH. The solution was incubated for 3 min at 100 °C, and then neutralized with 100 μL 3.2 M HCl. OSB-dilactone was synthesized from phtalique anhydride and succinic acid in the presence of potassium acetate as described (27). Benzoyl-CoA, phenylacetyl-CoA, phylloquinone, and DHNA were from Sigma. All other chemicals were from Fisher Scientific.

Production of Recombinant Slr0204 Protein. The slr0204 gene minus its stop codon was amplified from *Synechocystis* sp. PCC 6803 genomic DNA by using the primers 5′-*CATATG*GGGACATTTACCTATGA-3′(forward) and 5′-*CTCGAG*CGGGTGATCCAAATCACAA-3′ (reverse), which contained the *Nde*I and *Xho*I restriction sites (italicized), respectively. The amplified products were ligated into pGEM-T Easy (Promega), and the construct was verified by sequencing. The 416-bp *Nde*I/*Xho*I-digested fragment was then subcloned into the corresponding sites of pET43.1a (Novagen) to yield an in-frame fusion upstream a 6×His-tag. This construct then was introduced in BL21 (DE3) cells. The expression and the purification of recombinant Slr0204 are detailed in "Supplemental Materials and Methods".

Enzyme Assays. DHNA-CoA thioesterase assays (50 μL) contained 20 mM NaH_2PO_4 (pH 7.0), 100 mM NaCl, 60–150 μM DHNA-CoA, and 0–15 μg proteins and were run for 5–30 min at 30 °C protected from light. External standards of DHNA were incubated in the same conditions minus DHNA-CoA and enzyme. Reactions were stopped on ice with 150 μL 95% EtOH (vol/vol). The samples were centrifuged (14,000× *g* for 10 min at 8 °C) and immediately analyzed by HPLC using the same conditions as those described for the purification of DHNA-CoA ("Supplemental Materials and Methods"). DHNA was detected by fluorescence (Ex = 260 nm, Em = 450 nm) at a retention time of 11.6 min and was quantified relative to the external standards of DHNA. Disappearance of DHNA-CoA was monitored in parallel at A_{392}. The hydrolysis of benzoyl-CoA, phenylacetyl-CoA, palmitoyl-CoA, and succinyl-CoA was measured spectrophotometrically with DTNB. Assays (250 μL) contained 20 mM NaH_2PO_4 (pH 7.0), 100 mM NaCl, 11–120 μM substrates, and 7–8 μg recombinant DHNA-CoA thioesterase. Assays with palmitoyl-CoA contained in addition 3 μM BSA. Blank samples contained no enzyme. Reactions were incubated for 30–120 min at 30 °C and were then mixed with 250 μL of an aqueous solution of 400 μM DTNB. Change in A_{412} compared to blank samples was measured immediately.

Generation of *slr0204* Knockout and Functional Complementation. Gene slr0204 was deleted by replacing its entire sequence with the *aadA* spectinomycin marker (14). For that, the flanking regions of slr0204 were amplified from *Synechocystis* sp. PCC 6803 genomic DNA by using the following pairs of primers: Pair 1, 5′-ATTGGGCGTAAATTC-GCAAA-3′ (forward)/5′-GTAAACTTGCCTTTCATAGG-3′ (reverse) and Pair 2, 5′-CCTATGAAAGGCAAGTTTAC*CGTACG*GCGATTTGT-GATTTGG-3′ (forward) containing a *Bsi*WI site (italicized)/5′-AAGGCGT-GGTGATGAAATC-3′ (reverse) for the upstream and downstream regions, respectively. The 2 PCR products were combined and annealed to serve as a template in a third round of amplification with forward primer of Pair 1 and reverse primer of Pair 2, and the amplified product was cloned into pGEM-T Easy (Promega). The *aadA* gene was isolated from the pMenG-Spec-KO vector (14) using *Bsi*WI and was ligated in the pGEM-T Easy construct, which had been linearized with the same enzyme. Transformation of wild-type *Synechocystis* sp. PCC 6803 with this final construct and isolation of homogenous knockout clones was performed as described (28) using spectinomycin-containing medium. Replacement of slr0204 by the *aadA* marker was verified by PCR amplification of genomic DNA. For the functional complementation of the *slr0204* knockout, gene slr0204 was amplified from *Synechocystis sp.* PCC 6803 genomic DNA by using the primers 5′-GGAATTCTG*CATATG*GGGACATTTACCTATG-3′ (forward) and 5′-CG*GGATCC*TCACGGGTGATCCAAATC-3′ (reverse), which contained

the *Nde*I and *Bam*HI restriction sites (italicized), respectively. The amplified product was cloned into *Nde*I/*Bgl*II-digested pSynExp-2 vector, for expression under the control of the *psbA2* cyanobacterial promoter (29). This construct was introduced into the *slr0204* knockout, and transformed cells were selected on BG-11 medium containing spectinomycin and chloramphenicol. Incorporation of slr0204 was verified by PCR on genomic DNA. For growth comparison experiments, *Synechocystis* cells were grown on solid BG-11 medium containing 5 mM glucose and no antibiotics.

Metabolite Analyses. Cells were grown at a light intensity of 80 $\mu E.m^{-2}.s^{-1}$ to A_{730} approximately 0.8–1.2, in liquid BG-11 medium containing 5 mM glucose with or without addition of DHNA or OSB, and with the appropriate antibiotics. Subsequent operations were at room temperature and protected from light to avoid photodegradation of naphthoquinones. The analysis of phylloquinol and phylloquinone in *Synechocystis* cells has been adapted from that described for plant tissues (30) and is detailed in "Supplemental Materials and Methods". DHNA-CoA in *Synechocystis* extracts was quantified after its enzymatic conversion into DHNA; a detailed procedure is given in "Supplemental Materials and Methods".

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References

1. Sakuragi Y, Bryant DA (2006) in *Photosystem I: The Light-Driven Plastocyanin:Ferredoxin Oxidoreductase. Advances in Photosynthesis and Respiration*, ed Golbeck JH (Springer, Dordrecht), pp. 205–222.
2. Collins MD, Jones D (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol Rev* **45**:316–354.
3. Wallace BJ, Young IG (1977) Role of quinones in electron transport to oxygen and nitrate in *Escherichia coli.* Studies with a *ubiA*$^-$ *menA*$^-$ double quinone mutant. *Biochim Biophys Acta* **461**:84–100.
4. Fromme P, Grotjohann I (2006) in *Photosystem I: The Light-Driven Plastocyanin:Ferredoxin Oxidoreductase. Advances in Photosynthesis and Respiration*, ed Golbeck JH (Springer, Dordrecht), pp. 47–69.
5. Vermeer C, et al. (2004) Beyond deficiency: Potential benefits of increased intakes of vitamin K for bone and vascular health. *Eur J Nutr* **43**:325–335.
6. Ichikawa T, Horie-Inoue K, Ikeda K, Blumberg B, Inoue S (2006) Steroid and xenobiotic receptor SXR mediates vitamin K2-activated transcription of extracellular matrix-related genes and collagen accumulation in osteoblastic cells. *J Biol Chem* **281**:16927–16934.
7. Hiratsuka T, et al. (2008) An alternative menaquinone biosynthetic pathway operating in microorganisms. *Science* **321**:1670–1673.
8. Meganathan R (2001) Biosynthesis of menaquinone (vitamin K_2) and ubiquinone (coenzyme Q): A perspective on enzymatic mechanisms. *Vitam Horm* **61**:173–218.
9. Johnson TW, et al. (2000) Recruitment of a foreign quinone into the A(1) site of photosystem I. I. Genetic and physiological characterization of phylloquinone biosynthetic pathway mutants in *Synechocystis* sp. pcc 6803. *J Biol Chem* **275**:8523–8530.
10. Wade Johnson T, et al. (2003) The *menD* and *menE* homologues code for 2-succinyl-6-hydroxyl-2,4-cyclohexadiene-1-carboxylate synthase and O-succinylbenzoic acid-CoA ligase in the phylloquinone biosynthetic pathway of *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta* **1557**:67–76.
11. Sakuragi Y, et al. (2002) Insertional inactivation of the menG gene, encoding 2-phytyl-1,4-naphthoquinone methyltransferase of *Synechocystis* sp. PCC 6803, results in the incorporation of 2-phytyl-1,4-naphthoquinone into the A(1) site and alteration of the equilibrium constant between A(1) and F(X) in photosystem I. *Biochemistry* **41**:394–405.
12. Shimada H, et al. (2005) Inactivation and deficiency of core proteins of photosystems I and II caused by genetical phylloquinone and plastoquinone deficiency but retained lamellar structure in a T-DNA mutant of Arabidopsis. *Plant J* **41**:627–637.
13. Gross J, et al. (2006) A plant locus essential for phylloquinone (vitamin K1) biosynthesis originated from a fusion of four eubacterial genes. *J Biol Chem* **281**:17189–17196.
14. Lohmann A, et al. (2006) Deficiency in phylloquinone (vitamin K_1) methylation affects prenyl quinone distribution, photosystem I abundance, and anthocyanin accumulation in the *Arabidopsis* AtmenG mutant. *J Biol Chem* **281**:40461–40472.
15. Kim HU, van Oostende C, Basset GJ, Browse J (2008) The AAE14 gene encodes the *Arabidopsis* o-succinylbenzoyl-CoA ligase that is essential for phylloquinone synthesis and photosystem I function. *Plant J* **54**:272–283.
16. Lefebvre-Legendre L, et al. (2007) Loss of phylloquinone in *Chlamydomonas* affects plastoquinone pool size and photosystem II synthesis. *J Biol Chem* **282**:13250–13263.
17. Sharma V, Suvarna K, Meganathan R, Hudspeth ME (1992) Menaquinone (vitamin K_2) biosynthesis: Nucleotide sequence and expression of the *menB* gene from *Escherichia coli. J Bacteriol* **174**:5057–5062.
18. Truglio JJ, et al. (2003) Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in vitamin K_2 biosynthesis. *J Biol Chem* **278**:42352–42360.
19. Jiang M, et al. (2008) Identification and characterization of (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase in the menaquinone biosynthesis of *Escherichia coli. Biochemistry* **47**:3426–3434.
20. Overbeek R, Fonstein M, D'Souza M, Pusch GD, Maltsev N (1999) The use of gene clusters to infer functional coupling. *Proc Natl Acad Sci USA* **96**:2896–2901
21. Dillon SC, Bateman A (2004) The Hotdog fold: Wrapping up a superfamily of thioesterases and dehydratases. *BMC Bioinf* **5**:109.
22. Thoden JB, Holden HM, Zhuang Z, Dunaway-Mariano D (2002) X-ray crystallographic analyses of inhibitor and substrate complexes of wild-type and mutant 4-hydroxybenzoyl-CoA thioesterase. *J Biol Chem* **277**:27468–27476.
23. Zhuang Z, Song F, Takami H, Dunaway-Mariano D (2004) The BH1999 protein of *Bacillus halodurans* C-125 is gentisyl-coenzymeAthioesterase. *J Bacteriol* **186**:393–399.
24. Song F, Zhuang Z, Dunaway-Mariano D (2007) Structure-activity analysis of base and enzyme-catalyzed 4-hydroxybenzoyl coenzyme A hydrolysis. *Bioorg Chem* **35**:1–10.
25. Dansette P, Azerad R (1970) A new intermediate in naphthoquinone and menaquinone biosynthesis. *Biochem Biophys Res Commun* **40**:1090–1095.
26. Chang K-H, Liang P-H, Beck W, Scholten JD, Dunaway-Mariano D (1992) Isolation and characterization of the three polypeptide components of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS-3. *Biochemistry* **31**:5605–5610.
27. RoserW(1884) About phtalylderivatives (*transl. from German*). II. *Ber Deut Chem Ges* **17**:2770–2775.
28. Williams JGK (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods Enzymol* **167**:766–778.
29. Sattler SE, Cahoon EB, Coughlan SJ, DellaPenna D (2003) Characterization of tocopherol cyclases from higher plants and cyanobacteria. Evolutionary implications for tocopherol synthesis and function. *Plant Physiol* **132**:2184–2195.
30. Oostende C, Widhalm JR, Basset GJ (2008) Detection and quantification of vitamin K(1) quinol in leaf tissues. *Phytochemistry* **69**:2457–2462.

[Supplemental Materials and Methods follows.]

Supplemental Materials and Methods

DHNA-CoA Synthesis. All steps were protected from light. DHNA (204 mg), 230 mg *N*-hydroxysuccinimide (NHS), and 384 mg 1–1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were mixed in 5 mL tetrahydrofuran under N_2 atmosphere, and stirred for 8 h at room temperature. The mixture was then evaporated to dryness, resuspended in 2 mL dichloromethane, and applied to a silica gel 60 column (50 cm × 5 cm). DHNANHS was eluted from the column with approximately 70 mL ethyl acetate/hexane (5:1) and evaporated to dryness. DHNANHS (16 mg) was then resuspended in 1.5 mL tetrahydrofuran and mixed with 1.5 mL of an aqueous solution of 0.013 M Na_2CoA-SH (pH 8.0) under N_2 atmosphere. The mixture was stirred for 15 min at room temperature, while keeping pH approximately 8.0 with 0.2 M NaOH. Tetrahydrofuran was evaporated with a gentle stream of N_2 at 4 °C, and insoluble materials were removed by centrifugation before storage at –80 °C. DHNA-CoA was purified by HPLC from thawed reaction mixtures using a Zorbax Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm). The column was eluted at 30 °C with a linear gradient, starting from 20% methanol and 80% 50 mM sodium acetate, pH 5.9, to 100% methanol over 30 min at a flow rate of 0.75 mL·min^{-1}. Absorbance was monitored at 392 nm; DHNACoA eluted at 16.5 min. DHNA-CoA containing fractions were collected, evaporated with gaseous N2, and resuspended in 0.2 mL degassed 20 mM NaH_2PO_4 (pH 7.0), 100 mM NaCl to be used immediately.

Expression and Purification of Recombinant Slr0204. Starter cultures (5 mL) grown in LB medium containing 150 μg·mL^{-1} ampicillin were used to inoculate 500 mL prewarmed LB medium without antibiotic. When A_{600} reached approximately 0.9, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 100 μM, and the incubation was continued for 6 h at 28 °C. Subsequent operations were at 4 °C. Cells were harvested by centrifugation, resuspended in 8 mL extraction buffer [50 mM NaH_2PO_4, (pH 8.0), 300 mM NaCl, 10% glycerol (vol/vol), 10 mM imidazole], and disrupted with 0.1 mm zirconia/silica beads in a MiniBeadbeater (BioSpec Products) at 5,000 rpm for 5 × 20 s. The extracts were centrifuged (14,000 × *g* for 10 min), and the histidine-tagged protein was isolated under native conditions with Ni-NTA His-Bind resin (Novagen) following the manufacturer's recommendations. The purified protein was immediately desalted on a PD-10 column (GE Healthcare) equilibrated in 20 mM NaH_2PO_4 (pH 7.0), 100 mM NaCl, 10% glycerol (vol/vol). The desalted fraction was frozen in liquid N2 and stored at –80 °C, which preserved the enzymatic activity.

Quantification of Phylloquinol and Phylloquinone in Synechocystis Extracts. For phylloquinone analysis, cells from 1.8-mL culture aliquots were harvested by centrifugation, washed once with 1 mL BG-11 medium, and finally resuspended in 250 μL BG-11 medium. A 100-μL aliquot was then added to 550 μL 95% (vol/vol) ethanol containing 142 mM β-mercaptoethanol, spiked with 0.25–0.5 nmol menaquinol/menaquinone mixture as an internal standard and vortexed briefly. The lysate was cleared by centrifugation and immediately analyzed by HPLC with postcolumn chemical reduction coupled to fluorescence detection (Excitation: 238 nm, Emission: 426 nm). Cells were quantified by absorbance at 730 nm on a dilution of the remaining resuspended pellet and using the formula 0.25 unit $A_{730} \Leftrightarrow 10^8$ cells.

DHNA-CoA Analysis in Synechocystis Extracts. Cells from 500-mL cultures were harvested by centrifugation, washed with 50 mL BG-11 medium, resuspended in 8mL 50mMNaH_2PO_4 (pH 7.0), 100 mM NaCl, 5 mM DTT, and then disrupted with 0.1 mm zirconia/silica beads in a MiniBeadbeater (5,000 rpm for 5 × 20 s). The lysates were cleared by centrifugation and then split in half. The first half was incubated for 1 h at 30 °C with 21 μg recombinant Slr0204, whereas the second half was kept on ice. At the end of the incubation, the lysates were acidified to pH 4.0 with HCl, and were extracted twice with 12 mL ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness with gaseous N_2. The residue was resuspended in 250 μL 100% methanol and analyzed by HPLC-fluorescence using the same solvent system as that described for the purification of DHNACoA ("Supplemental Materials and Methods"). The level of DHNA-CoA was calculated by subtracting the amount of DHNA detected in the enzyme-treated sample (hydrolyzed DHNA-CoA + endogenous DHNA) with that of the sample kept on ice (endogenous DHNA).

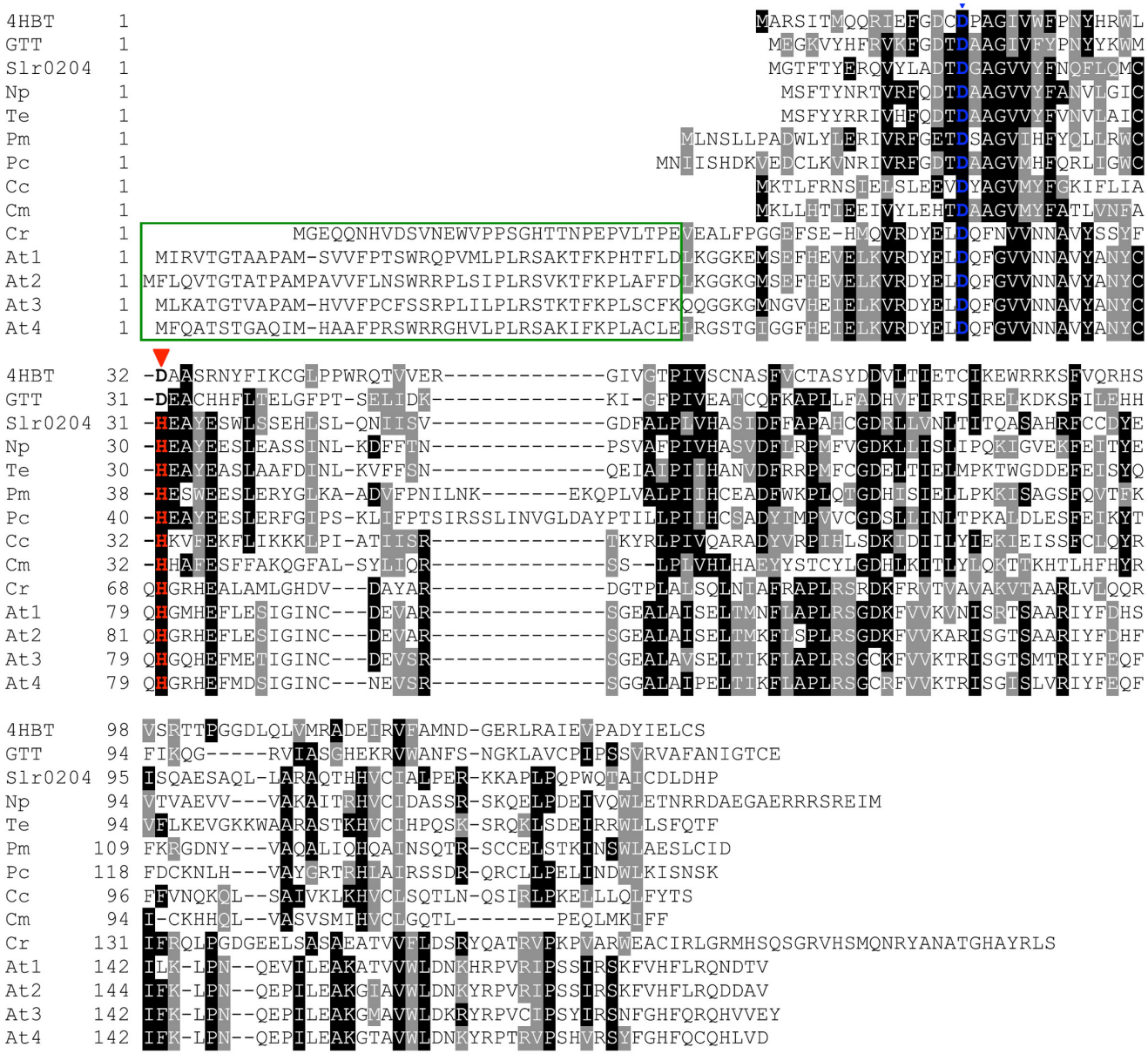


Figure S1. Alignment of the protein sequences from *Pseudomonas* sp. CBS3 4-HBT (4HBT, ABQ44580), *B. halodurans* C-125 gentisyl-CoA thioesterase (GTT, NP_242865), cyanobacterial DHNA-CoA thioesterases from Chroococcales (*Synechocystis* sp. PCC 6803, Slr0204, NP_442358), Nostocales (*N. punctiforme* PCC 73102, Np, YP_001869668), Oscillatoriales (*T. erythraeum* IMS101, Te, YP_723549), and Prochlorales (*P. marinus* str. MIT9211, Pm, YP_001550076), and their eukaryotic homologs in amoeba (*P. chromatophora*, Pc, YP_002048985), rhodophytes (*C. caldarium* RK1, Cc, NP_045103; *C. merolae* 10D, Cm, NP_849030), green algae (*C. reinhardtii*, Cr, XP_001703093), and *Arabidopsis* (At1, NP_176995; At2, NP_564926; At3, NP_564457; At4, NP_174759). The *Arabidopsis* proteins are paralogs that share over 70% identity with each other. Dashes symbolize gaps introduced to maximize alignment. Identical residues are shaded in black, similar ones in gray. The blue arrowhead indicates the conserved Asp residue known to trigger the nucleophilic attack in 4-HBT. The red arrowhead points to the Asp residue that has been proposed to be important for substrate binding in 4-HBT and gentisyl-CoA thioesterase; note that the cyanobacterial and eukaryotic proteins have a His residue at this position. The N-terminal extensions that are predicted to encode plastid targeting peptides in *C. reinhardtii* and *Arabidopsis* are boxed.

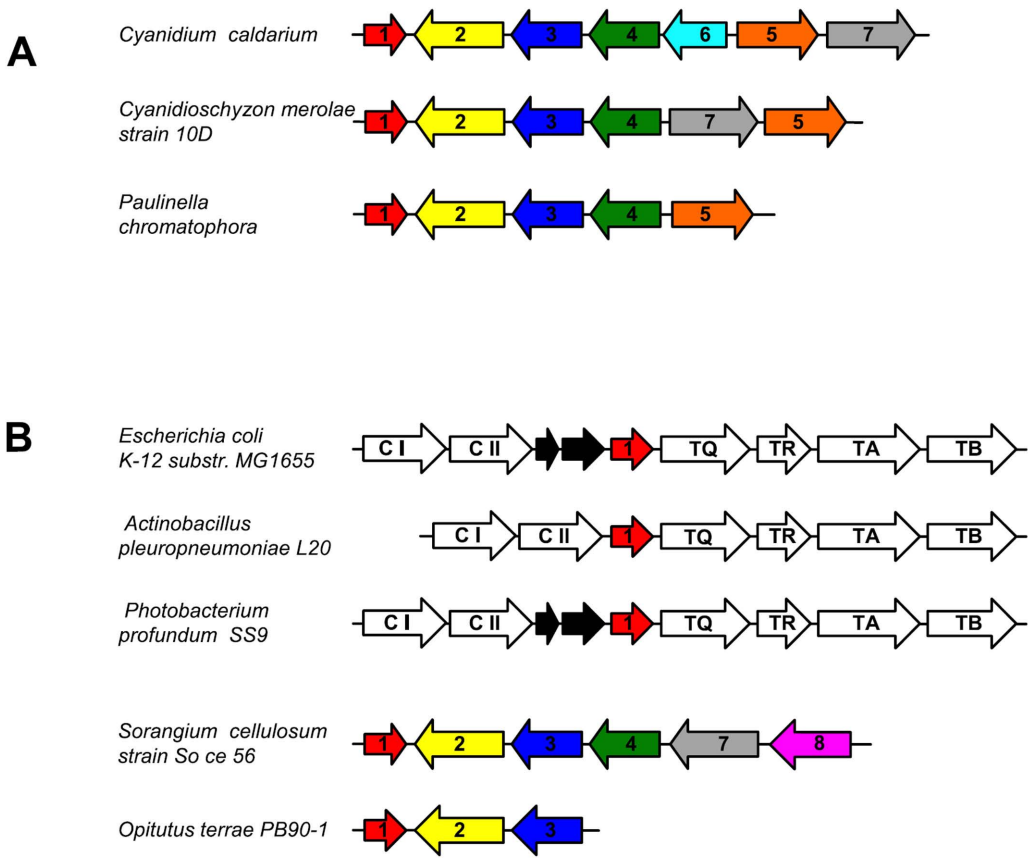
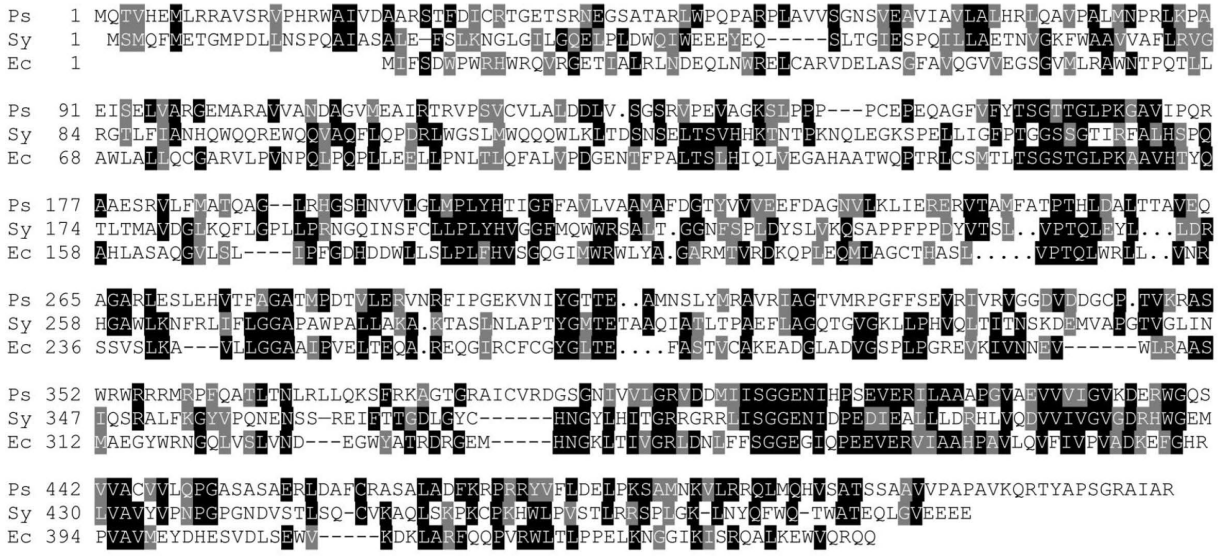


Figure S2. Genomic context of the homologs of cyanobacterial DHNA-CoA thioesterase in eukaryotes and prokaryotes. (*A*) Clustering with phylloquinone biosynthetic genes in the rhodophytes *C. caldarium* and *C. merolae*, and the amoeba *P. chromatophora*. (*B*) Presence of the homolog of cyanobacterial DHNA-CoA thioesterase in the Tol operon-like structure of facultative anaerobes or in a cluster with menaquinone biosynthetic genes in the Proteobacteria *S. cellulosum* and the Verrucomicrobia *O. terrae*. 1, putative CoA-thioesterase; 2, OSB-CoA ligase; 3, OSB synthase; 4, DHNA prenyltransferase; 5, isochorismate synthase; 6, DHNA synthase; 7, SEPHCHC synthase; 8, demethylmenaquinone methyl transferase. C I and CII, cytochrome ubiquinol oxidase subunit I and II, respectively; TQ, TR, TA, and TB, Tolmembranetransport system component Q, R, A, and B, respectively. Black arrows indicate genes encoding proteins annotated as hypothetical.

A



B

C

4-chlorobenzoate ligase	OSB-CoA ligase	
Pseudomonas	*Synechocystis*	*E. coli*
Identity/Similarity	22%/34%	20%/34%

4-chlorobenzoate dehalogenase	DHNA synthase	
Pseudomonas	*Synechocystis*	*E. coli*
Identity/Similarity	28%/44%	24%/35%

Figure S3. Sequence comparisons of *Pseudomonas* sp. CBS3 4-chlorobenzoate-CoA ligase and 4-chlorobenzoate dehalogenase with OSB-CoA ligase and DHNA synthase, respectively. (*A*) *Pseudomonas* sp. CBS3 4-chlorobenzoate-CoA ligase (Ps, B42560), *Synechocystis* sp. PCC 6803 OSB-CoA ligase (Sy, NP_442252), *E. coli* OSB-CoA ligase (Ec, NP_416763). (*B*) *Pseudomonas* sp. CBS3 4-chlorobenzoate dehalogenase (Ps, A42560), *Synechocystis* sp. PCC 6803 DHNA synthase (Sy, NP_440855), *E. coli*DH-NAsynthase (Ec, NP_416765). Dashes symbolize gaps introduced to maximize alignment. Identical residues are shaded in black, similar ones in gray. (*C*) Levels of sequence homology between 4-chlorobenzoate-CoA ligase and OSB-CoA ligase, and between 4-chlorobenzoate dehalogenase and DHNA synthase.