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Fusion-type lycopene β-cyclase from a thermoacidophilic archaeon *Sulfolobus solfataricus*

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Abstract

Examination of the sequence of a hypothetical gene with an unknown function included in the carotenogenic gene cluster in the genome of a thermoacidophilic archaeon *Sulfolobus solfataricus* led to the prediction that the gene encodes a novel-type lycopene β-cyclase, whose N- and C-terminal halves are homologous to the subunits of the bacterial heterodimeric enzymes. The recombinant expression of the gene in lycopene-producing *Escherichia coli* resulted in the accumulation of β-carotene in the cells, which verifies the function of the gene. Homologues of the archaeal lycopene β-cyclase from various organisms such as bacteria, archaea, and fungi have been reported. Although their primary structures are clearly specific to the biological taxa, a phylogenetic analysis revealed the unexpected complicity of the evolutional route of these enzymes.

Keywords: Lycopene β-cyclase; Cyclase; Lycopene; β-Carotene; Zeaxanthin; Carotenoid; Archaea; *Sulfolobus*; Evolution; Phylogeny

Carotenoids are a group of hydrophobic polyene pigments produced by green plants and some types of fungi, bacteria, and archaea. Some are often utilized as co-pigments associated with photosynthesis. Many carotenoids also act as anti-oxidant agents, leading to their use as food additives and drugs. The majority of carotenoids are synthesized from lycopene (ψ, ψ-carotene), the C_{40} acyclic intermediate, and over 600 variants are generated by various biochemical reactions such as terminal cyclization, hydroxylation, and glycosylation. Among these, β-carotene (β, β-carotene) is well-known as the precursor of vitamin A [1]. This carotenoid compound is biosynthesized directly from lycopene by β-cyclization at both termini, and the reaction is catalyzed by lycopene β-cyclase (LC). LCs are widely distributed in many organisms and divided into two groups based on their amino acid sequences [2]. There is no similarity between the sequences of enzymes belonging to the different groups. One group is mainly comprised of enzymes from gram-negative bacteria, cyanobacteria, and plants, and are generally designated as CrtY. In this paper, we refer to them as type-1 LC because they were discovered first. The enzymes are known to possess a dinucleotide binding motif in their sequences and require NADPH as a co-factor [3]. The other group, type-2 LC, mainly consists of LCs from gram-positive bacteria and fungi, although large structural differences exist between them. Bacterial type-2 LC was initially identified in a study using a gram-positive coryneform bacterium *Brevibacterium linens* [4]. This enzyme is composed of two small homologous subunits referred to as CrtYc and CrtYd, and their homologues were found to be encoded in the genomes of *Mycobacterium* sp. and, exceptionally, a gram-negative bacterium *Myxococcus xanthus* [2,5]. On the other hand, fungal LCs, generally designated as CrtYB, are expressed as a fusion protein with phytoene synthase, which catalyzes an earlier step in catotenoid biosynthesis [6–9]. Its C-terminal ca. 400 amino acids have phytoene synthase activity, and the residual N-terminal region, ca. 200 amino acid residues, comprise the LC-catalytic domain, which is a tandem fusion of the homologues of CrtYc and CrtYd. The catalytic mechanism and co-factor requirements of this group of enzymes are currently unclear.

The production of carotenoids has also been reported in archaea. It is known that many halophilic archaea utilize retinal, which is normally synthesized from

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β-carotene, as the chromophore of light-driven ion pump proteins such as bacteriorhodopsin [10]. *Halobacterium salinarium* also produces C_{50} carotenoids such as bacterioruberin [11]. On the other hand, several types of zeaxantin glycosides were isolated from a thermoacidophilic archaeon *Sulfolobus shibatae*, which has no photosynthetic system, and, as a result, the functions of these carotenoids are unclear [12]. Zeaxantin is thought to be produced from β-carotene via the action of β-carotene hydroxylase (Fig. 1). In this study, we report on an examination of a cluster of putative carotenoid biosynthetic genes in the genome of *Sulfolobus solfataricus*. Although most genes of the cluster have high similarities with known carotenoid biosynthetic genes such as phytoene synthase, phytoene desaturase, and β-carotene hydroxylase, the function of one gene in the cluster, SSO2904, had not been assigned because the entire amino acid sequence of its predicted gene product does not show a high similarity with known proteins. However, through further analyses, we found that the N-terminus and the C-terminus of the protein encoded by SSO2904 are homologous with CrtYc and CrtYd from bacteria, respectively. This fact strongly suggests that the gene encodes a type-2 LC although its structure is different from those of bacterial and fungal type-2 LCs. The function of the gene was confirmed by recombinant expression with other carotenogenic genes in *Escherichia coli*. Moreover, a phylogenic analysis of type-2 LCs revealed an unexpected complicity of their evolutional route.

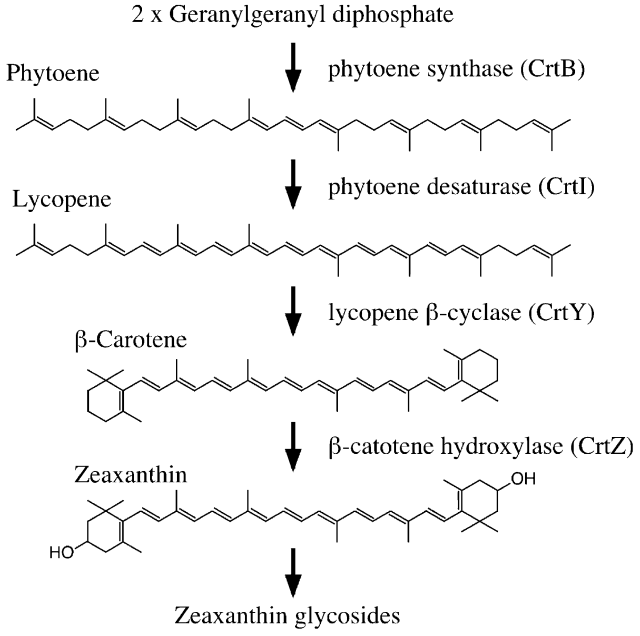

Fig. 1. The hypothetical pathway of carotenoid biosynthesis in *S. solfataricus*.

Materials and methods

Materials. Authentic lycopene and β-carotene were purchased from Funakoshi. For high performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) analyses, HPLC grade solvents were used. All other chemicals were of analytical grade.

General procedures. Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook et al. [13].

Cultivation of archaea. S. solfataricus was cultured in an ATCC 1304 broth at 70 °C and harvested at the late log phase.

Isolation of the LC gene from S. solfataricus. A detailed homology search using the BLAST program revealed that the open reading frame (ORF) SSO2904 would encode a type-2 LC. This ORF was amplified by the polymerase chain reaction using primers specific to the 5′ and 3′ ends; 5′-TTTCGCATGCTGCAGTCTATGGAGTTTAAACCCG-3′ and 5′-TATATAGGATCCTTATTTCTGTTTCATCCATAAACTG-3′, respectively. The restriction sites newly introduced in the primers, the *Pst*I and *Bam*HI sites, are indicated by underlines. The genome of *S. solfataricus* as the template and KOD DNA polymerase (TOYOBO) were used for the reaction. The amplified fragment was extracted from an 0.8% agarose gel after electrophoresis and was digested with *Pst*I and *Bam*HI and then ligated into the *Pst*I–*Bam*HI sites of the pUC119 vector. The resulting plasmid was designated as pUC-ssY.

Extraction of carotenoids from transformed E. coli. E. coli DH5α transformed with pUC-ssY and pACYC-IBE, a plasmid for lycopene-production [14], was cultivated into 100 mL M9YG broth supplemented with ampicillin (100 μg/ml), tetracycline (20 μg/ml), and 1 mM isopropyl 1-thio-β-D-galactoside (IPTG). After cultivation over 24 h, the cells were harvested and stored at −20 °C. The following experimental procedures, when possible, were carried out in darkness. The frozen cells were suspended in 5 ml acetone, and the solution was shaken for 20 min at 50 °C and then allowed to settle. After the cell debris had settled to the bottom, the supernatant was recovered. The acetone extract was condensed by evaporation and then partitioned between 3 ml hexane and 2 ml of H_2O saturated with NaCl. The hexane layer was recovered and washed with 2 ml H_2O. The hexane layer was evaporated and the residue dissolved in 800 μl of HPLC grade ethanol.

HPLC analysis of carotenoids. The carotenoids extracted from recombinant *E. coli* were loaded on the Lichrosorb RP-18 column (5 μm, Kanto Chemical) and eluted isocratically with acetonitrile/methanol/1-propanol (85:10:5, v/v) at a flow rate of 1 ml/min. The elution of carotenoids was monitored by measuring the absorption at 460 nm. The absorption spectra of carotenoids were simultaneously observed using a photodiode assay UV–VIS detector SPD-M6A (Shimadzu).

LC–MS analysis. The carotenoids extracted from *E. coli* were fractionated with the HPLC system under the conditions described above, and carotenoid compounds having an elution time the same as β-carotene were recovered. An LC–MS analysis of the carotenoid was performed with an MStation JMS-700 mass spectrometry system (JEOL) using atmospheric pressure chemical ionization in the positive ion mode. Samples were loaded on the COSMOSIL $5C_4$-AR-300 column (4.6 × 150 mm, Nacalai Tesque) and eluted with methanol/H_2O (96:4, v/v) at a flow rate of 0.25 or 0.5 ml/min.

Phylogenetic analysis. The phylogenetic analysis of type-2 LCs obtained from public databases was constructed using the CLUSTAL W 1.8 program on the DDBJ website (http://spiral.genes.nig.ac.jp/homology/welcome-e.shtml). All parameters used in these programs were set at default.

Results and discussion

The entire genome sequence of *S. solfataricus* was determined and the putative roles of the proteins encoded in many ORFs have been assigned based on

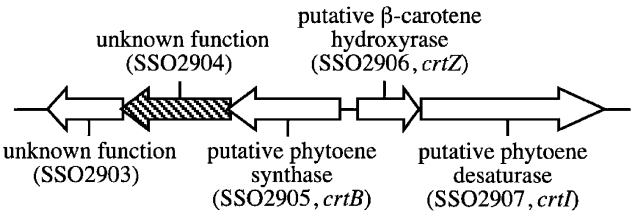

Fig. 2. The cluster of putative carotenoid biosynthetic genes in the genome of *S. solfataricus*.

automated homology search results against protein databases [15]. The genes encoding putative carotenoid biosynthetic enzymes form a cluster in the genome (Fig. 2). Two ORFs aligned tandem, i.e., SSO2906 and SSO2907, encode a putative β-carotene hydroxylase and a putative phytoene desaturase, respectively. SSO2905, the ORF located next to SSO2906 but in the opposite direction, encodes a putative phytoene synthase. On the other hand, the gene cluster still seems to contain two hypothetical ORFs, the putative roles of which have not been assigned. SSO2904 is located below SSO2905 with a slight overlap and SSO2903 follows them. As the result of a homology search using the BLAST program, however, we found that there is a slight similarity between the N-terminal region of the hypothetical protein encoded in SSO2904 and the putative CrtYc subunit of *Mycobacterium marinum*. Thus, we attempted a similarity search using its N-terminal and C-terminal halves and, in consequence, found that they show a high similarity with bacterial CrtYc and CrtYd, respectively. This strongly suggests that the ORF encodes for a type-2 LC and that its primary structure is analogous to a fusion of the two subunits of a bacterial LC. (On the other hand, no information regarding the sequence of SSO2903 could be found because its homologue is hardly found in the genomes of other organisms.)

Based on this hypothesis, we attempted to determine whether the ORF encodes for a LC. We isolated the ORF SSO2904 using the polymerase chain reaction and inserted it into a plasmid vector for *E. coli*. The resultant plasmid pUC-ssY was introduced into the *E. coli* DH5α strain that had held a carotenogenic plasmid pACYC-IBE, which contains the genes of three carotenoid biosynthetic enzymes, i.e., geranylgeranyl diphosphate synthase, phytoene synthase, and phytoene desaturase, from *Erwinia uredovora* and is sufficient for producing lycopene in *E. coli* [14]. The cells of *E. coli* transformed with pACYC-IBE and pUC-ssY formed colonies that were red-orange in color, while the colonies of those transformed with pACYC-IBE and pUC-119 were red. HPLC analyses of non-polar lipids extracted from the transformants clearly showed that the composition of the carotenoids was altered by the introduction of the ORF 2904 (Fig. 3A). The absorption spectrum of the

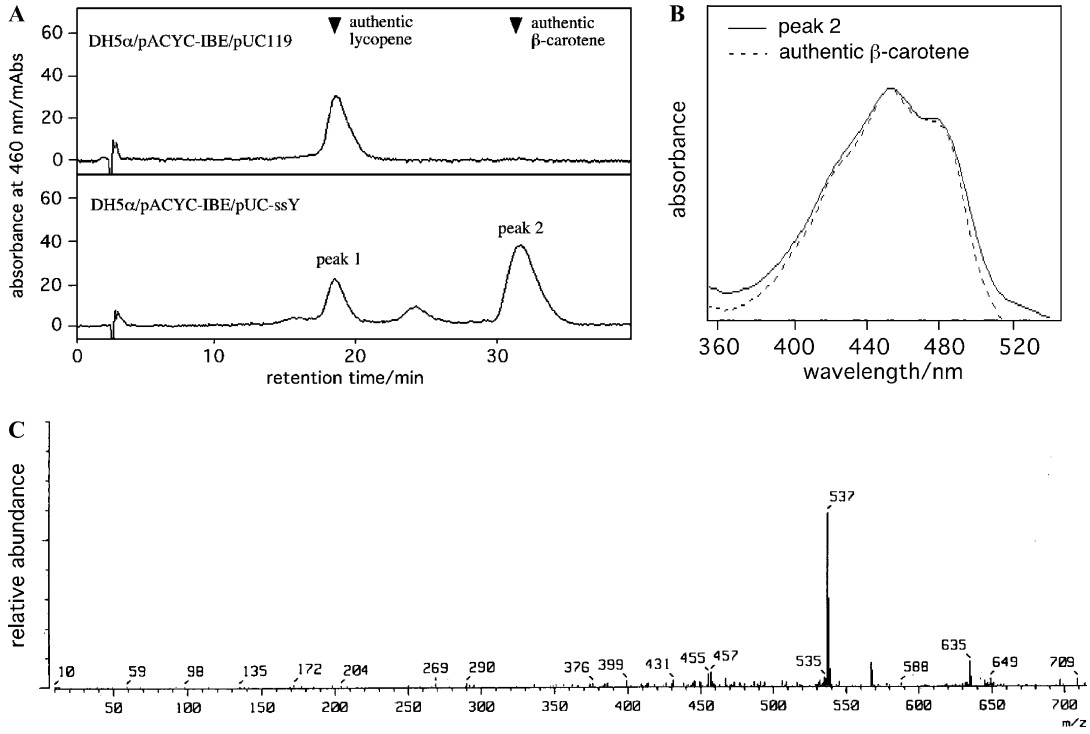

Fig. 3. Analyses of carotenoids produced in *E. coli* cells transformed with carotenogenic genes. The conditions used in each analysis were described under "Materials and methods." (A) HPLC elution profiles of carotenoids extracted from the recombinant *E. coli*. The positions of the elution of authentic carotenoids, i.e., lycopene and β-carotene, were indicated with arrowheads. (B) Visible spectrum of the pigment (peak 2) eluted from *E. coli* DH5α/pACYC-IBE/pUC-ssY. The spectrum was compared with that of the authentic β-carotene. (C) LC–MS analysis of the carotenoid in the peak 2.

newly generated peak (peak 2) is almost superimposable with that of authentic β-carotene (Fig. 3B), while that of peak 1, which is also present in the chromatogram of the extracts from DH5α/pACYC-IBE/pUC119, is similar to that of lycopene (data not shown). The origin of the small peak observed between peak 1 and peak 2 is likely γ-carotene (β, ψ-carotene) produced by β-ionone ring formation at only one terminus of the lycopene. As shown in Fig. 3C, a molecular ion for peak 2 was observed at m/z 537 in an LC–MS analysis, which corresponds to the molecular weight of β-carotene [16].

These results clearly indicate that the ORF SSO2904 encodes for a fusion-type LC, the N-terminal and C-terminal halves of which correspond to the two subunits of bacterial type-2 LC (Fig. 4). The archaeal LC resembles, but differs from fungal LCs, because the latter are the result of a further fusion between the former and phytoene synthase. Thus, the archaeal enzyme was shown to be involved in the group of type-2 LCs. Recently, Peck et al. [17] reported that a halophilic archaeon *Halobacterium salinarum* possesses a LC with a similar fusion-type structure with that from *S. solfataricus*, which led us to hypothesize that the structure

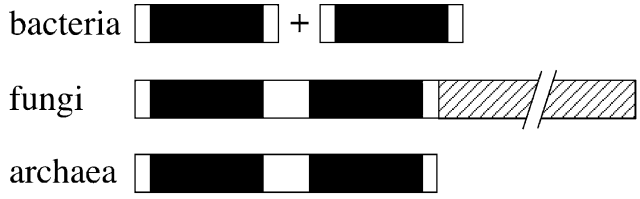

Fig. 4. Models of the primary structures of type-2 LCs from bacteria, archaea, and fungi. Homologous subunits and regions are indicated by boxes in black. The phytoene synthase-catalytic region in fungal LC is indicated by a hatched box.

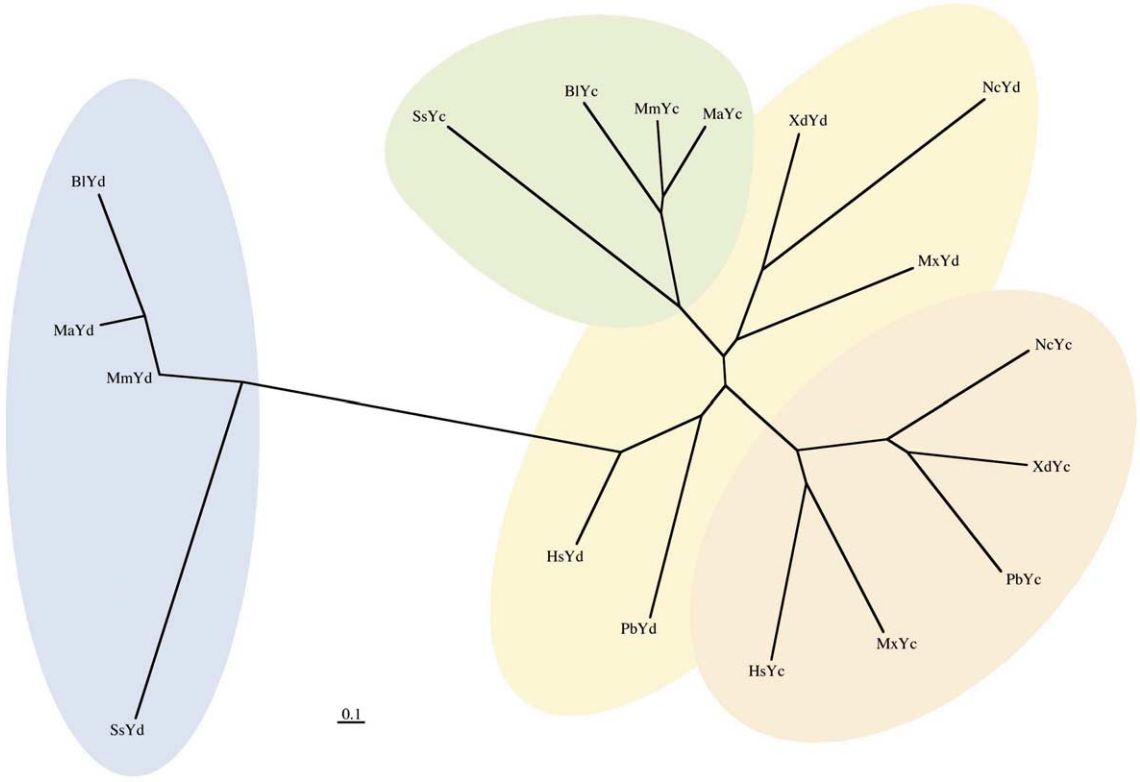

Fig. 5. The phylogenetic tree of the amino acid sequences of the subunits or domains of type-2 LCs. The conditions for the phylogenetic analysis were described under "Materials and methods." The abbreviation and accession number of the subunits and domains are as follows: SsYc and SsYd, the regions at 8–101 and 129–225 of *S. solfataricus* LC (AAK43013), respectively; HsYc and HsYd, the regions at 38–138 and 171–269 of *H. salinarum* LC (CrtY, [17]), respectively; NcYc and NcYd, the regions at 38–138 and 171–269 of *N. crassa* LC (Al-2, P37295), respectively; PbYc and PbYd, the regions at 38–138 and 171–269 of *Phycomyces blakesleeanus* LC (CarRA, CAB86388), respectively; XdYc and XdYd, the regions at 38–138 and 171–269 of *Xanthophyllomyces dendrorhous* LC (CrtYB, CAB51949), respectively; BlYc, the region at 6–103 of the CrtYc subunit of *B. linens* LC (AAF65588); BlYd, the region at 5–105 of the CrtYd subunit of *B. linens* LC (AAF65587); MaYc, the region at 1–93 of the CrtYc subunit of *Mycobacterium aurum* LC (CAB94796); MaYd, the region at 6–106 of the CrtYd subunit of *M. aurum* LC (CAB94797); MmYc, the region at 7–104 of the putative CrtYc subunit of *M. marinum* LC (hypothetical protein-33, AAB71429); MmYc, the region at 100–200 of the putative CrtYd subunit of *M. marinum* LC (hypothetical protein-34, AAB71430); MxYc, the region at 3–102 of the putative CrtYc subunit (encoded in orf7 of the carotenoid biosynthetic gene cluster) of *M. xanthus* LC (S74258); and MxYd, the region at 8–110 of the putative CrtYd subunit (encoded in orf8) of *M. xanthus* LC (S68195). The bar indicates 0.1 amino acid substitution per site. Differently colored areas indicate CrtYc or CrtYd subunits (or regions) of two subgroups of type-2 LCs.

would be common to all archaeal LCs. We, therefore, conducted a phylogenetic study of type-2 LCs derived from various organisms. Because the CrtYc and CrtYd subunits of a bacterial LC are known to be homologous, we divided the archaeal LCs into their N-terminal and C-terminal halves (for convenience, these domains are denoted as CrtYc and CrtYd, respectively). Similarly, the LC-catalytic domain of the fungal CrtYB proteins was divided into halves. All of the amino acid sequences of the homologous subunits and domains were provided for the calculations with the CLUSTAL W program. To exclude the effects of extended N- or C-terminals (or linker domains of fusion proteins), only the sequences of relatively homologous regions were selected, based on the result of the initially performed multiple alignment. The phylogenetic tree for these selected sequences with the nearly same lengths clearly showed the existence of two subgroups (Fig. 5). One (subgroup A) contains LCs of *S. solfataricus*, *B. linens*, and *Mycobacterium* sp., and the other (subgroup B) contains those of *H. salinarum*, *M. xanthus*, and fungi. The CrtYd subunits (or domain) of subgroup A, highlighted with light blue, are clearly separated from the others (the bootstrap value was 993 for 1000 trials). The CrtYc subunits of subgroup A and those of subgroup B also seem to construct respective clusters (indicated with the pale green and pink areas, respectively) branched out from that of the CrtYd subunits of subgroup B (the yellow area). It should be noted that the phylogenetic tree constructed with the sequences containing extended N- or C-terminals showed a similar pattern to that in Fig. 5 (data not shown).

Based on a comparative analysis of the sequences of LCs, Peck et al. proposed that the genes of the subunits of bacterial type-2 LC arose from the duplication of a single gene encoding a homodimeric enzyme and that the resultant heterodimeric LCs evolved into fusion-type archaeal LCs and then into fungal LCs by fusing with phytoene synthase. However, our result strongly suggests an evolutional independence between the two subgroups, into which the fusion-type archaeal LCs are divided. Bacterial LCs are also separated into two subgroups. This leads to the possibility that the evolutional ancestor of the fungal LCs would be related to *H. salinarum* LC, not to *S. solfataricus* LC. On the other hand, *S. solfataricus* LC should be closely related to LCs from *B. linens* and *Mycobacterium* sp. This fact easily leads to the possibility that the archaeal enzyme might have arisen from the fusion of the bacterial subunits. It should be noted that carotenogenic genes form clusters in the genomes of these organisms, suggesting the occurrence of horizontal gene transmission. However, this hypothesis is based on the supposition that the common fusion-type structure of two archaeal LCs is simply an accidental coincidence. At this time, we cannot exclude the possibility that the archaeal fusion-type LC is also ancestral to some bacterial enzymes.

The physiological function of carotenoids in *Sulfolobus* sp. is currently unclear. Kull et al. [12] isolated zeaxanthin glycosides from *S. shibatae* and speculated that they might play roles as membrane reinforcers. However, this view is not persuasive because, in the membrane of *Sulfolobus* sp., which is largely constructed of dipolar tetraether lipids, the effect of the carotenoids as trans-membrane 'rivets' would not be expected [18]. Moreover, Kull et al. used a mutant of *S. shibatae*, whose carotenoid production is light-regulated, in their report. Thus, we conclude that the carotenoids might act as radical scavengers in *Sulfolobus* sp. Interestingly, the entire genome sequencing of *Sulfolobus tokodaii* proved that the organism does not possess a carotenogenic gene cluster [19]. This suggests that carotenoids, which should be secondary metabolites in *Sulfolobus* sp., are not essential for the growth of all *Sulfolobus* sp. Here, we should mention that our attempt to extract and detect carotenoid compounds from *S. solfataricus* failed probably because they are present in extremely low levels in the cells (data not shown). Although the issue of whether carotenoid biosynthetic enzymes are actively expressed in *S. solfataricus* is unclear, they would attract more interest because they would be expected to provide important information regarding the complicated evolutional routes of carotenoid biosynthesis.

Acknowledgments

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