

Luan Tao · Henry Yao · Hiroaki Kasai
Norihiko Misawa · Qiong Cheng

A carotenoid synthesis gene cluster from *Algoriphagus* sp. KK10202C with a novel fusion-type lycopene β -cyclase gene

Received: 11 January 2006 / Accepted: 23 March 2006 / Published online: 20 April 2006
© Springer-Verlag 2006

Abstract A carotenoid synthesis gene cluster was isolated from a marine bacterium *Algoriphagus* sp. strain KK10202C that synthesized flexixanthin. Seven genes were transcribed in the same direction, among which five of them were involved in carotenoid synthesis. This cluster had a unique gene organization, with an isoprenoid gene, *ispH* (previously named *lytB*), being present among the carotenoid synthesis genes. The lycopene β -cyclase encoded by the *crtY_{cd}* gene appeared to be a fusion of bacterial heterodimeric lycopene cyclase CrtY_c and CrtY_d. This was the first time that a fusion-type of lycopene β -cyclase was reported in eubacteria. Heterologous expression of the *Algoriphagus crtY_{cd}* gene in lycopene-accumulating *Escherichia coli* produced bicyclic β -carotene. A biosynthesis pathway for monocyclic flexixanthin was proposed in *Algoriphagus* sp. strain KK10202C, though several of the carotenoid synthesis genes not linked with the cluster have not yet been cloned.

Keywords Gene fusion · Lycopene β -cyclase · β -Carotene ketolase · *Algoriphagus* · Flexixanthin

Introduction

Over 700 carotenoids are widely distributed in nature including bacteria, fungi, algae, and plants (Britton et al.

2004). They carry out important functions in photosynthesis, nutrition, and protection against oxidative damage. Most naturally occurring carotenoids are hydrophobic tetraterpenoids containing a C₄₀ methyl-branched hydrocarbon backbone derived from successive condensation of eight C₅ isoprene units. The C₅ isoprene unit, isopentenyl pyrophosphate (IPP), can be generated from acetyl-CoA precursor by the mevalonate pathway (Wilding et al. 2000) or from the pyruvate and glyceraldehydes 3-phosphate precursors by the non-mevalonate pathway (Rohmer et al. 1996). Several new genes including *ispH* (Rohdich et al. 2002) have recently been identified in the non-mevalonate isoprenoid pathway. IPP is then isomerized to dimethylallyl pyrophosphate (DMAPP) by IPP isomerase encoded by the *idi* gene. IPP is condensed with DMAPP to form the C₁₀, geranyl pyrophosphate (GPP), and elongated to the C₁₅, farnesyl pyrophosphate (FPP). In the carotenogenic organisms, FPP is extended to common C₄₀ carotenoids (Fig. 1) such as lycopene by geranylgeranyl pyrophosphate synthase (CrtE), phytoene synthase (CrtB), and phytoene dehydrogenase (CrtI). The aliphatic ends of lycopene (ψ,ψ -carotene) can be modified by enzymes such as hydratase (CrtC) and desaturase (CrtD). They can also be cyclized by lycopene cyclase and further modified by enzymes such as β -ionone ring ketolase (CrtW) and β -ionone ring hydroxylase (CrtZ) to generate a variety of C₄₀ carotenoids.

Both structural and functional diversity has been reported for lycopene β -cyclases. Four different types of lycopene β -cyclases have been described in a review (Krubasik and Sandmann 2000b): the common monomeric bacterial CrtY-type (Misawa et al. 1995; Schnurr et al. 1996; Pasamontes et al. 1997; Krugel et al. 1999; Hannibal et al. 2000); the monomeric plant CrtL-type or Lcy-b type (Cunningham et al. 1994, 1996; Huguency et al. 1995; Pecker et al. 1996); the heterodimeric CrtY_c and CrtY_d type (Krubasik and Sandmann 2000a; Viveiros et al. 2000); and the bifunctional fungal CrtYB type (Verdoes et al. 1999; Velayos et al. 2000; Arrach et al. 2001, 2002). The lycopene cyclase domain of the fungal

Communicated by G. Klug

L. Tao · H. Yao · Q. Cheng (✉)
Biological and Chemical Sciences and Engineering,
Central Research and Development,
E. I. DuPont, de Nemours Inc., Experimental Station,
E328/B48, Wilmington, DE 19880-0328, USA
E-mail: qiong.cheng@usa.dupont.com
Tel.: +1-302-6959952
Fax: +1-302-6951829

H. Kasai · N. Misawa
Marine Biotechnology Institute,
3-75-1, Kamaishi-shi 026-0001, Japan

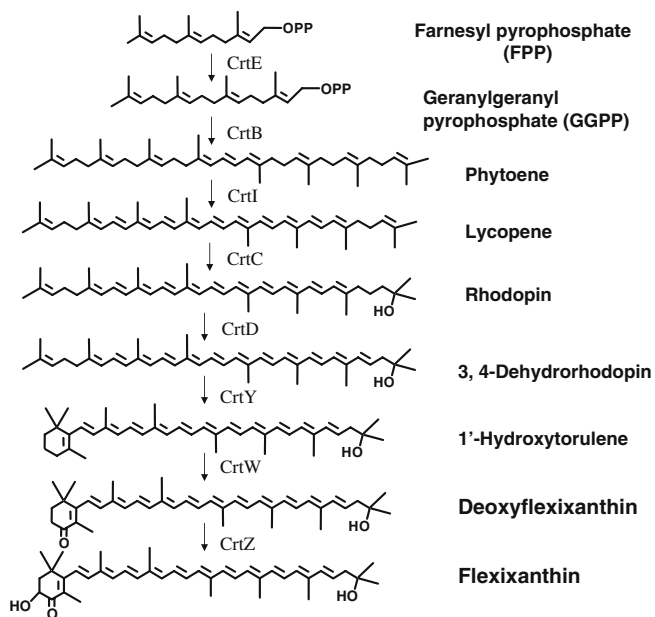


Fig. 1 Proposed pathway for synthesis of flexixanthin in *Algoriphagus* sp. KK10202C. CrtE: geranylgeranyl pyrophosphate synthase, CrtB: phytoene synthase, CrtI: phytoene dehydrogenase, CrtC: hydratase, CrtD: desaturase, CrtY: lycopene β -cyclase, CrtW: 4,4'- β -ionone ring ketolase, CrtZ: 3,3'- β -ionone ring hydroxylase

CrtY_B share homology with the heterodimeric CrtY_c and CrtY_d from gram-positive bacteria, however, they don't show homology with the monomeric lycopene cyclases. CrtY_c and CrtY_d encode two proteins, which have to interact as heterodimers for lycopene cyclization. Recently, a novel fusion-type of lycopene β -cyclases has been described in archaeon (Peck et al. 2002; Hemmi et al. 2003), whose N- and C-terminal halves are homologous to the CrtY_c and CrtY_d subunits of the bacterial heterodimeric enzymes. Most of the lycopene β -cyclases are bicyclases, which cyclize both ends of lycopene to produce bicyclic β -carotene. Two novel lycopene β -monocyclases, the CrtY_m (Teramoto et al. 2003) and the CrtL_m (Tao et al. 2004), were recently isolated from bacteria that synthesized exclusively monocyclic carotenoids. The lycopene β -monocyclases asymmetrically cyclize only one end of lycopene to produce monocyclic γ -carotene.

Algoriphagus sp. belongs to the *Flexibacteraceae* family within the bacterial phylum *Bacteroidetes*. The structures of carotenoids (flexixanthin and deoxyflexixanthin) produced in certain flexibacteria were elucidated several decades ago (Aasen and Jensen 1966). Flexixanthin is a monocyclic carotenoid that possesses one oxygenated β -ionone ring analogous to that of astaxanthin and one hydroxylated aliphatic end group as found in demethylspheroidene. However, no pathway has been proposed for flexixanthin synthesis and no carotenoid synthesis gene has been cloned from flexibacteria. Here we report the isolation of a carotenoid synthesis gene cluster from a marine isolate *Algoriphagus* sp.

strain KK10202C. Five of the genes in the cluster were involved in flexixanthin synthesis. One of them encoded a novel fusion-type lycopene β -cyclase that was first reported in eubacteria.

Materials and methods

Strain isolation

A marine bacterium *Algoriphagus* sp. strain KK10202C was isolated from marine sponge, *Halichondria okadai*, which was collected at the coastal area in Numazu, Japan. For strain isolation, 0.1 ml homogenates of the specimen were transferred onto plates of marine agar 2216 (Difco, Detroit, MI, USA). From a number of strains, an orange-pigmented bacterium, KK10202C, was isolated and purified on the same medium.

Carotenoid identification

Cultivation of the bacterium KK10202C and the extraction of carotenoids produced were carried out by the same methods as previously described (Yokoyama and Miki 1995). The main carotenoid extracted was purified by column chromatography on silica gel 60 (Nakarai Tesque, Japan) with chloroform and methanol (9:1, V/V), and HPLC on Cosmosil 5-SL (Nakarai Tesque) with hexane and acetone (8:2, V/V). Three mg of the pigment was isolated from 12 l of the culture. The purified carotenoid was identified by visible spectrum, ¹H-NMR spectrum, and mass spectrum (Aasen and Jensen 1966). Authentic standard of lycopene, β -carotene was purchased from Sigma (St. Louis, MO, USA).

Library construction and screening

A small insert library of strain KK10202C was prepared by partial restriction digest method. Genomic DNA of KK10202C was partially digested with *HincII* and separated on a 0.8% agarose gel. The 4–6-kb fraction was excised from the gel and extracted using Qiagen MinElute Gel-Extraction kit. The extracted DNA was ligated to pEZseq vector using pEZSeq Blunt Cloning kit (Lucigen, Middletown, WI, USA). The ligation mixture was electroporated into freshly prepared competent cells of *Escherichia coli* 10G containing a β -carotene producing plasmid pBHR-crt1 (Tao et al. 2004). Transformants were plated on LB plates with 100 μ g/ml ampicillin and 50 μ g/ml kanamycin. Positive clones were screened by orange color of the colonies.

A cosmid library of KK10202C was constructed using the pWEB cosmid cloning kit from Epicentre Technologies (Madison, WI, USA). Genomic DNA was sheared by passing it through a 261/2 G syringe needle three times. The sheared DNA was end-repaired and size-selected on a low-melting-point agarose gel. DNA fragments approximately 40-kb in size were purified and

ligated into the blunt-ended pWEB cosmid vector. The library was packaged using ultra-high efficiency MaxP-lax Lambda Packaging Extracts, and titered with EPI100 *E. coli* cells. Approximately 600 cosmid clones were grown in LB with 100 µg/ml ampicillin. They were pooled and screened by PCR using primers for the *crtW* gene identified from the small insert library.

Sequencing of the positive clones

From an orange positive clone, the library plasmid pEZ-HY1 was separated from the β-carotene reporter plasmid by selecting for ampicillin resistant and kanamycin sensitive clones. The insert on the pEZ-HY1 was sequenced by random transposon insertion using the EZ-TN <TET-1> kit (Epicentre, Madison, WI, USA) and/or primer walking. The insert on the positive cosmid was also sequenced using the EZ-TN <TET-1> kit. The sequences were assembled with the Sequencher program (Gene Codes Corp., Ann Arbor, MI, USA).

Construction of the expression clone

The lycopene cyclase gene *crtY_{cd}* from KK10202C was expressed in *E. coli* using the pTrcHis2-TOPO expression vector. It was amplified using primers 5'-ATGGGA AACTATCTCTACCTAGC-3' and 5'-CTA ATA ACT TTTAGTTTGTGAATTGTTTTCCG-3'. The resulting plasmid pDCQ250 containing the *crtY_{cd}* gene in the forward orientation was confirmed by restriction analysis and sequencing. Its function was tested in lycopene-accumulating *E. coli* DH10B containing pDCQ51 (Tao et al. 2004).

Phylogenetic analysis

The almost-complete 16S rDNA sequence (1,443 nucleotides) of *Algoriphagus* sp. KK10202C was deposited in GenBank under accession number AB086623. Aligned 16S rRNA gene sequences of representative strains of family *Flexibacteriaceae* were obtained from the databases of RDP-II release 9 (Cole et al. 2005). The sequences were aligned with those from the GenBank database based on the sequences from the RDP database by using the profile alignment function of ClustalX v. 1.83 (Thompson et al. 1997). Phylogenetic analysis was conducted using MEGA 3.1 (Kumar et al. 2004). Evolutionary distance was calculated based on Kimura 2-parameter model (Kimura 1980). Neighbor-joining method (Saitou and Nei 1987) was used for tree construction. The reliability of an inferred tree was tested by 1,000 replicates of bootstrap method (Felsenstein 1985).

Nucleotide accession number

The carotenoid synthesis gene cluster isolated from *Algoriphagus* sp. KK10202C has been deposited in GenBank under accession number DQ286432.

Results

Isolation of *Algoriphagus* sp. that synthesized flexixanthin

Strain KK10202C was an orange-pigmented bacterium isolated from marine sponge. It was gram-negative and rod-shaped. Its approximate size was $1.3 \times 0.6 \mu\text{m}^2$. This strain utilized glucose oxidatively, but could not degrade gelatin. Phylogenetic analysis (Fig. 2) revealed that strain KK10202C belongs to the family *Flexibacteriaceae* (Garrity and Holt 2001). Its 16S rDNA sequence has 98% identity to the 16S rDNA sequences of several other bacteria in the family of *Flexibacteriaceae* such as *Algoriphagus* and *Cyclobacterium*. Those close relatives of KK10202C were also isolated from the marine environment (Matsuo et al. 2003; Nedashkovskaya et al. 2004). The major carotenoid in KK10202C was identified as flexixanthin by the data of visible spectrum (λ_{max} : 481, 509), 1H-NMR spectrum (data not shown), and mass spectrum (*m/e*: 582). Since flexixanthin was shown to be synthesized by *Algoriphagus* sp. (Aasen and Jensen 1966), strain KK10202C was named as *Algoriphagus* sp., which belongs to the *Algoriphagus* genus of the *Flexibacteriaceae* family. This strain *Algoriphagus* sp. KK10202C has been deposited in Marine Biotechnology Institute Culture collection (MBIC) as MBIC01539 (previously named as *Cytophaga* sp.). We propose the pathway for synthesis of flexixanthin by *Algoriphagus* sp. KK10202C as shown in Fig. 1. Presence of carotenoids such as lycopene (λ_{max} : 448, 473, 505; *m/e*: 536), 3, 4-dehydro-rhodopin (λ_{max} : 454, 477, 509; *m/e*: 552) and deoxyflexixanthin (λ_{max} : 481, 509; *m/e*: 566) as minor intermediates in *Algoriphagus* sp. KK10202C supported the proposed pathway.

Cloning of a carotenoid synthesis gene cluster from *Algoriphagus* sp

The major carotenoid synthesized by *Algoriphagus* sp. KK10202C is a keto-carotenoid flexixanthin. KK10202C presumably contains a 4,(4')-β-ionone ring ketolase, which could be identified by turning β-carotene producing *E. coli* cells from yellow to orange. Approximately 30,000 *E. coli* transformants were obtained from the small insert library of KK10202C and several orange colonies were identified. HPLC analysis showed that ketocarotenoids (canthaxanthin and echinenone) were produced in the positive *E. coli* clones. One of the positive clone, pEZ-HY1, was shown to contain an insert of ~3 kb. Sequencing of the insert confirmed that it contained an intact 4,(4')-β-ionone ring ketolase gene (*crtW*). To obtain sequences of other carotenoid synthesis genes that were adjacent to the *crtW*, a cosmid library was constructed and screened for the presence of the *crtW* by PCR. A 22-kb DNA fragment spanning the *crtW* gene was assembled from cosmid sequencing. Table 1 shows the organization and homology analysis

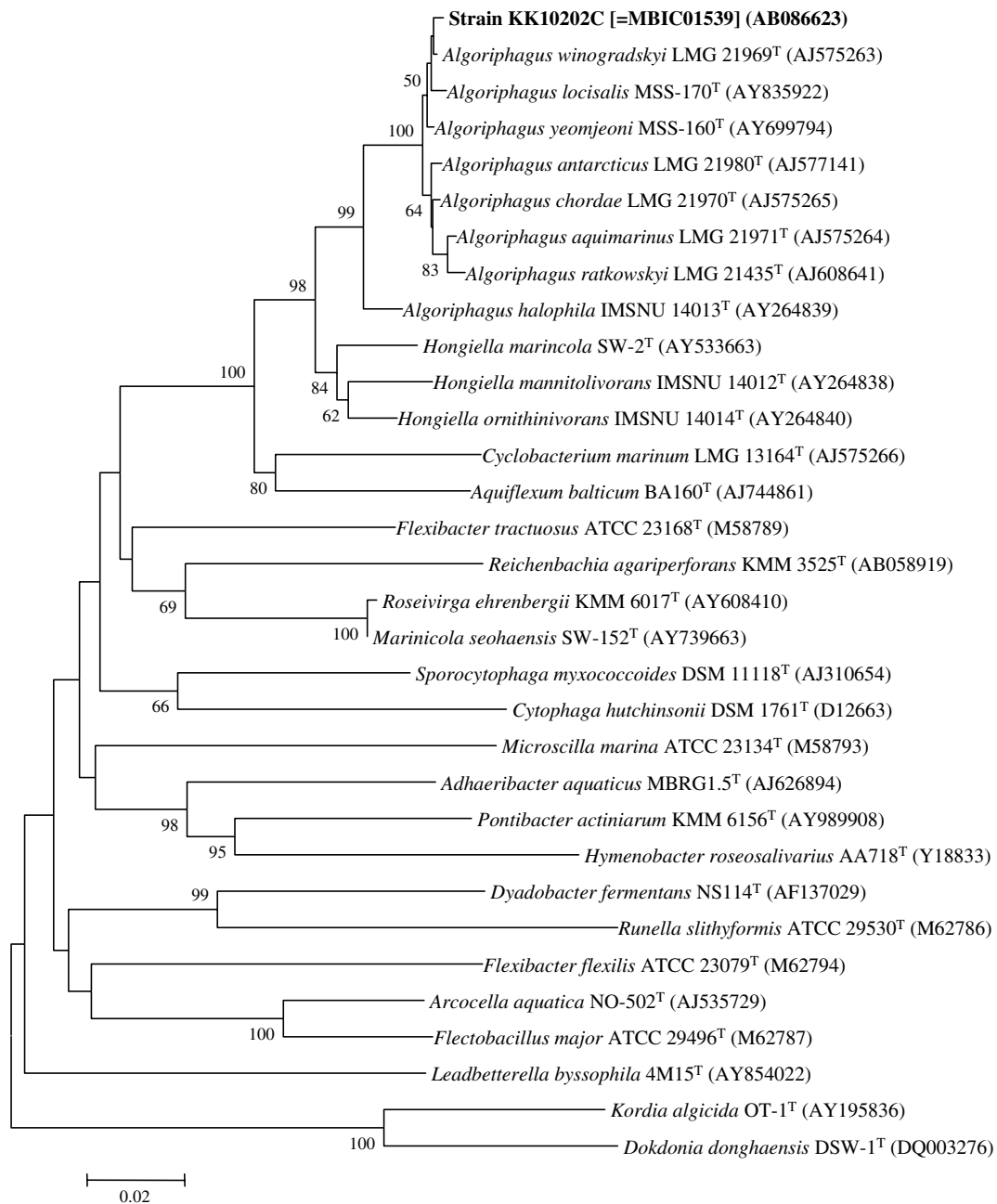



Fig. 2 Phylogenetic tree based on the 16S rRNA gene sequences of strain KK10202C and representative strains of the family *Flexibacteriaceae*. The tree was constructed by using the neighbor-joining method. Bootstrap values (more than 50%) expressed as percentages

of 1,000 replications are shown at the *branch points*. Numbers in *parentheses* are DDBJ accession numbers. The *scale bar* represents 2 nucleotide substitutions per 100 nucleotides

of the carotenoid synthesis genes and several flanking genes in this region.

Seven genes were transcribed in the same direction, among which five of them were involved in carotenoid synthesis. ORF2 (designated as CrtI) contained 492 amino acids, which showed high sequence similarities to phytoene desaturases/ dehydrogenases from many different organisms. ORF3 (designated as CrtB) contained 280 amino acids, which showed high sequence similarities to many phytoene synthases. ORF4 (designated as CrtY_{cd})

contained 236 amino acids, which showed sequence similarities to hypothetical proteins and lycopene cyclases from different organisms (more details in next section). ORF5 (designated as IspH) contained 281 amino acids, which showed high sequence similarities to many penicillin tolerance proteins (LytB). ORF6 (designated as CrtW) contained 256 amino acids, which showed sequence similarities to 4,(4')-β-ionone ring ketolases from many different organisms. No ORFs on the sequenced 22-kb insert showed homology to geranylgeranyl

Table 1 Organization and homology analysis of the genes in the carotenoid synthesis gene cluster in *Algoriphagus* sp. KK10202C


Name	Top hit ^a	Description	E value ^b	Identity (%) ^c	Similarity (%) ^d	Function confirmed
ORF1	AAO77490	RNA polymerase ECF-type sigma factor [<i>Bacteroides thetaiotaomicron</i> VPI-5482]	4e-35	43	65	
CrtI (ORF2)	BAC77668	Phytoene desaturase [marine bacterium P99-3]	e-147	52	70	
CrtB (ORF3)	ZP_00310775	Phytoene/squalene synthetase [<i>Cytophaga hutchinsonii</i>]	8e-86	57	74	
CrtY _{cd} (ORF4)	ZP_00310778	Hypothetical protein [<i>C. hutchinsonii</i>]	e-46	42	58	Yes
IspH (ORF5)	ZP_00307983	Penicillin tolerance protein LytB [<i>C. hutchinsonii</i>]	7e-87	59	72	
CrtW (ORF6)	BAB74888	beta-carotene ketolase [<i>Nostoc</i> sp. PCC 7120]	3e-48	47	62	Yes
ORF7	CAH07052	Putative TonB dependent outer membrane protein [<i>Bacteroides fragilis</i> NCTC 9343]	e-171	40	61	

^a The accession number of the top hit identified by BLAST search (Altschul et al. 1990)

^b The expect value estimates the statistical significance of the match, specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance

^c Identity is defined as percentage of amino acids that are identical between the two proteins

^d Similarity is defined as percentage of amino acids that are identical or conserved between the two proteins

pyrophosphate synthases (CrtE) or 3, (3')-β-ionone ring hydroxylases (CrtZ). They presumably are located elsewhere in the chromosome. The genes responsible for the aliphatic end modification of flexixanthin (*crtCD*) (Giraud et al. 2004) were also not found in this region.

Identification and characterization of the fusion-type lycopene β-cyclase gene

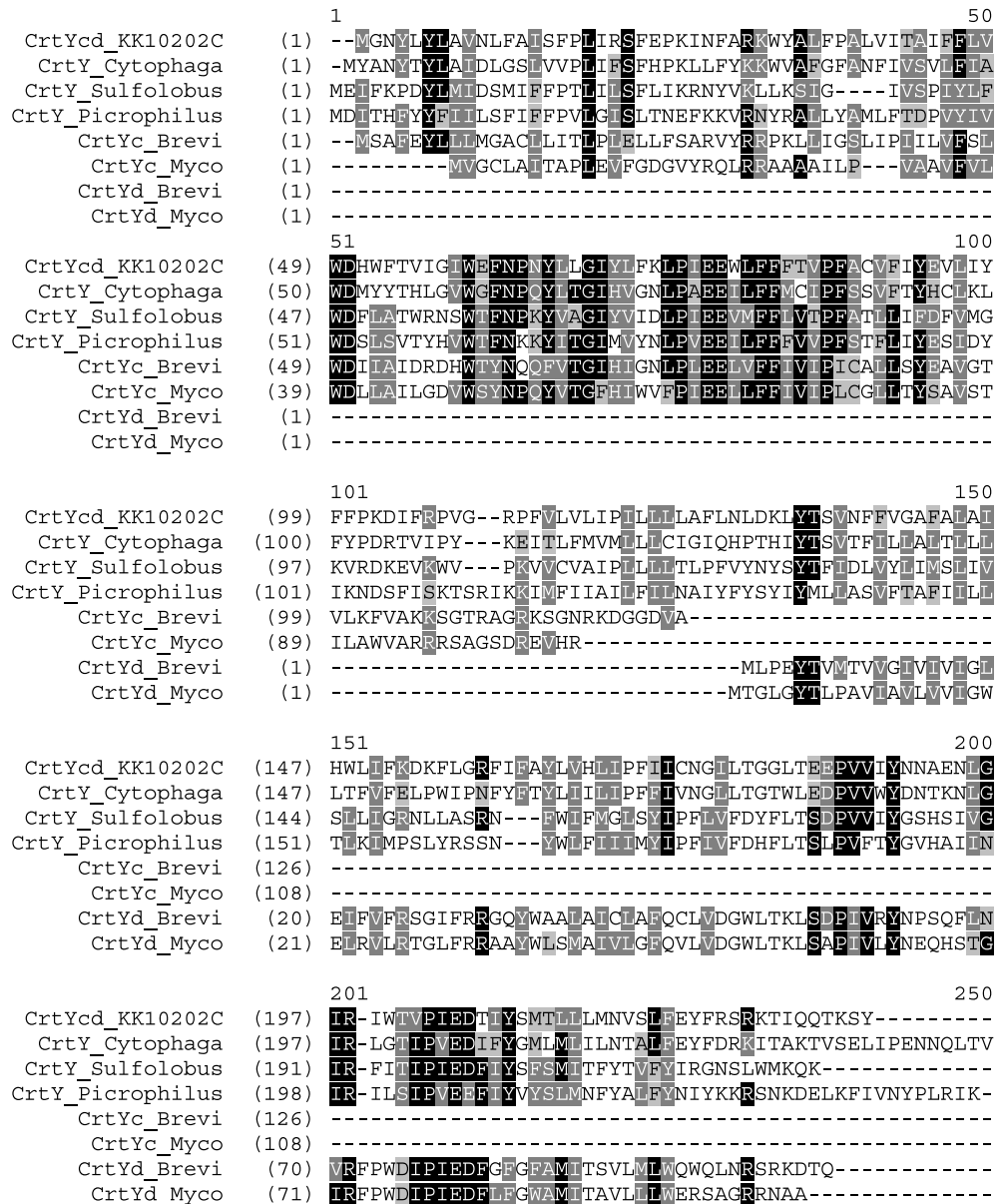
BLAST analysis (Altschul et al. 1990) showed that ORF4 (236 amino acids) from *Algoriphagus* sp. KK10202C had the highest similarity (42% amino acid identity) to a hypothetical protein Chut02000358 from *Cytophaga hutchinsonii*. This 245-amino acid hypothetical protein from *C. hutchinsonii* was derived from automated computational analysis of its genomic sequence. ORF4 also had moderate similarities (25–28% amino acid identities) to a functionally confirmed lycopene β-cyclase from *Sulfolobus solfataricus* (Hemmi et al. 2003) and a putative lycopene β-cyclase from *Picrophilus torridus* (Futterer et al. 2004). The 226-amino acid lycopene β-cyclase from thermoacidophilic archaeon *S. solfataricus* was shown to be a novel fusion-type enzyme, whose N- and C-terminal halves are homologous to the subunits of bacterial heterodimeric lycopene β-cyclases (CrtY_c and CrtY_d). The N- and C-terminal halves of ORF4 also showed homology to the CrtY_c and CrtY_d from *Mycobacterium aurum* (Viveiros et al. 2000) and *Brevibacterium linens* (Krubasik and Sandmann 2000a). Multiple sequence alignment of ORF4 with the archaeal fusion-type lycopene β-cyclases and the bacterial heterodimeric enzymes is shown in Fig. 3. ORF4 was designated as CrtY_{cd} to reflect the fusion of CrtY_c and CrtY_d. This is the first time that the fusion-type lycopene β-cyclase was identified in eubacteria. The lycopene cyclization function of the *crtY_{cd}* from

Algoriphagus sp. strain KK10202C was confirmed by heterologous expression in a lycopene-accumulating *E. coli*. β-carotene was produced as the predominant carotenoid and γ-carotene as a minor carotenoid upon expression of *crtY_{cd}*.

Discussion

This paper reports the cloning of a carotenoid synthesis gene cluster from a marine *Algoriphagus* isolate and first identification of a fusion-type of lycopene β-cyclase in eubacteria. Heterodimeric type of lycopene β-cyclases were previously reported in gram-positive bacteria (Krubasik and Sandmann 2000a; Viveiros et al. 2000). The novel fusion-type of lycopene β-cyclases, whose N- and C-terminal halves are homologous to the CrtY_c and CrtY_d subunits of the bacterial heterodimeric enzymes, were recently described in archaeon (Peck et al. 2002; Hemmi et al. 2003). In the thermoacidophilic archaeon *S. solfataricus*, the fusion-type *crtY* was located in a cluster with putative *crtB*, *crtZ*, and *crtI* genes. In the halophilic archaeon *Halobacterium salinarum*, the fusion-type *crtY* was upstream of *blh* and was required for bacteriorhodopsin biogenesis. The CrtY_{cd} from *Algoriphagus* showed significant homology to the fusion-type CrtY from *Sulfolobus*, but no significant homology to the fusion-type CrtY from *Halobacterium*, whose function was also confirmed experimentally. Phylogenetic analysis was previously performed with the respective subunits or domains of bacterial heterodimeric lycopene cyclases, the archaeal fusion-type lycopene cyclases and the fungi lycopene cyclase-phytoene synthase fusion proteins (Hemmi et al. 2003). Two subgroups emerged which separated the two functionally confirmed archaeal lycopene cyclases. Subgroup A contained the enzymes from

Fig. 3 Multiple sequence alignment of the fusion-type lycopene β -cyclases with the heterodimeric lycopene β -cyclases. The sequences used for the alignment are as follows: the fusion-type lycopene β -cyclase from *Algoriphagus* sp. KK10202C (accession number DQ286432); the putative fusion-type lycopene β -cyclase from *Cytophaga hutchinsonii* (accession number ZP_00310778); the fusion-type lycopene β -cyclase from *Sulfolobus solfataricus* (accession number NP_344223); the fusion-type lycopene β -cyclase from *Picrophilus torridus* (accession number YP_024312); the heterodimeric lycopene β -cyclase from *Brevibacterium linens* (accession number AF139916); the heterodimeric lycopene β -cyclase from *Mycobacterium aurum* (accession number AJ133724). The alignment was generated by AlignX, a component of Vector NTI Suite 7.0 (Informax Inc., Bethesda, MD, USA). The identical or conservative residues were highlighted in *black boxes*; the similar residues in *gray boxes*



Sulfolobus, *Brevibacterium*, and *Mycobacterium*, and subgroup B contained those from *Halobacterium*, *Myxococcus* (putative), and fungi. It appears that the *Algoriphagus* CrtY_{cd} belongs to the subgroup A based on sequence homology. It was proposed that the fusion type of lycopene cyclases might be evolutionary intermediates for the fungal CrtYB, which the fusion CrtY was further fused with phytoene synthase CrtB.

Aside from homology with the known lycopene β -cyclases, the CrtY_{cd} from *Algoriphagus* sp. had the highest similarity to a hypothetical protein Chut02000358 from *C. hutchinsonii*. Phylogenetic analysis of the strains in Fig. 2 showed that *Cytophaga* is closely related to *Algoriphagus* and belongs to the same *Flexibacteraceae* family as *Algoriphagus*. Chut02000358 from *C. hutchinsonii* would be another fusion-type of

CrtY_{cd} from bacteria if its function were demonstrated to be a lycopene β -cyclase.

The bicyclase activity of the CrtY_{cd} from KK10202C was somewhat unexpected since only monocyclic carotenoids were detected in strain KK10202C. It appears that presence of monocyclic carotenoids in strain KK10202C was not due to the monocyclase activity of the lycopene cyclase as reported in several other bacteria (Teramoto et al. 2003; Tao et al. 2004). It is likely that in the native KK10202C host, CrtCD competes with CrtY_{cd} for the lycopene substrate (Fig. 1). After one end of acyclic lycopene was modified by CrtCD, only the other linear end was available for cyclization by CrtY_{cd} to synthesize monocyclic carotenoids such as flexixanthin.

It is interesting that the isoprenoid gene *ispH* was found for the first time to be organized with the carotenoid

genes in this cluster. IspH was renamed from LytB since it was recently shown to encode 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, which is part of the non-mevalonate isoprenoid pathway (Rohdich et al. 2002) to synthesize the IPP precursor. IspH (LytB) of *E. coli* is essential for cell growth (McAteer et al. 2001). Heterologous expression of *ispH* (*lytB*) in *E. coli* was shown previously to be able to increase carotenoid production (Cunningham et al. 2000). Linking of isoprenoid genes with carotenoid synthesis genes was observed previously in *Pantoea agglomerans* with a different isoprenoid gene *idi* encoding isopentenyl pyrophosphate isomerase (Hahn et al. 1999), which was located with the *crtEXY-IBZ* genes as a cluster (GenBank accession number M87280).

Several genes (*crtE*, *crtZ*, *crtC*, and *crtD*) of the carotenoid synthesis pathway in *Algoriphagus* sp. KK10202C were not located in the cluster shown in Table 1. Neither of them was identified in the 15-kb region downstream of *crtW* from cosmid sequencing. Degenerate PCR approach was attempted to clone *crtZ* and was not successful. Another approach could be used is to screen the activity of the individual gene in an appropriate reporter strain. The *crtE* gene could be screened to complement the function of a *crt* cluster containing a *crtE* knockout mutation. The *crtZ*, *crtC* or *crtD* would probably have to be screened by HPLC or TLC. Cloning and characterization of the remaining carotenoid synthesis genes would give a complete picture of carotenoid synthesis in *Algoriphagus* sp. KK10202C. The functionalization genes (*crtY*, *crtW*, *crtZ*, *crtC*, and *crtD*) could also be exploited for combinatorial synthesis of novel carotenoids and heterologous production of desirable carotenoids.

Acknowledgments We thank DuPont Macromolecule Analysis Lab for DNA sequencing.

References

- Aasen AJ, Jensen SL (1966) Bacterial carotenoids: the structures of flexixanthin and deoxy-flexixanthin. *Acta Chem Scand* 20:1970–1988
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Arrach N, Fernandez-Martin R, Cerda-Olmedo E, Avalos J (2001) A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in *Phycomyces*. *Proc Natl Acad Sci USA* 98:1687–1692
- Arrach N, Schmidhauser TJ, Avalos J (2002) Mutants of the carotene cyclase domain of al-2 from *Neurospora crassa*. *Mol Genet Genomics* 266:914–921
- Britton G, Liaaen-Jensen S, Pfander H (eds) (2004) Carotenoids handbook. Birkhauser Verlag, Basel
- Cole JR et al (2005) The ribosomal database project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* 1:D294–D296
- Cunningham FX Jr, Lafond TP, Gantt E (2000) Evidence of a role for LytB in the nonmevalonate pathway of isoprenoid biosynthesis. *J Bacteriol* 182:5841–5848
- Cunningham FX Jr, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E (1996) Functional analysis of the beta and epsilon lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell* 8:1613–1626
- Cunningham FX Jr, Sun Z, Chamovitz D, Hirschberg J, Gantt E (1994) Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942. *Plant Cell* 6:1107–1121
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Futterer O et al (2004) Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proc Natl Acad Sci USA* 101:9091–9096
- Garrity GM, Holt JG (2001) Taxonomic outline of the archaea and bacteria. In: Boone DR, Castenholz RW (eds) *Bergey's manual of systematic bacteriology*. Springer, Berlin Heidelberg New York, pp 155–166
- Giraud E et al (2004) Two distinct *crt* gene clusters for two different functional classes of carotenoid in *Bradyrhizobium*. *J Biol Chem* 279:15076–15083
- Hahn FM, Hurlburt AP, Poulter CD (1999) *Escherichia coli* open reading frame 696 is *idi*, a nonessential gene encoding isopentenyl diphosphate isomerase. *J Bacteriol* 181:4499–4504
- Hannibal L et al (2000) Isolation and characterization of canthaxanthin biosynthesis genes from the photosynthetic bacterium *Bradyrhizobium* sp. strain ORS278. *J Bacteriol* 182:3850–3853
- Hemmi H, Ikejiri S, Nakayama T, Nishino T (2003) Fusion-type lycopene beta-cyclase from a thermoacidophilic archaeon *Sulfolobus solfataricus*. *Biochem Biophys Res Commun* 305:586–591
- Hugueney P et al (1995) Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum* chromoplasts. *Plant J* 8:417–424
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Krubasik P, Sandmann G (2000a) A carotenogenic gene cluster from *Brevibacterium linens* with novel lycopene cyclase genes involved in the synthesis of aromatic carotenoids. *Mol Gen Genet* 263:423–432
- Krubasik P, Sandmann G (2000b) Molecular evolution of lycopene cyclases involved in the formation of carotenoids with ionone end groups. *Biochem Soc Trans* 28:806–810
- Krugel H, Krubasik P, Weber K, Saluz HP, Sandmann G (1999) Functional analysis of genes from *Streptomyces griseus* involved in the synthesis of isorenieratene, a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. *Biochim Biophys Acta* 1439:57–64
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5:150–163
- Matsuo Y, Suzuki M, Kasai H, Shizuri Y, Harayama S (2003) Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. *Environ Microbiol* 5:25–35
- McAteer S, Coulson A, McLennan N, Masters M (2001) The *lytB* gene of *Escherichia coli* is essential and specifies a product needed for isoprenoid biosynthesis. *J Bacteriol* 183:7403–7407
- Misawa N et al (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J Bacteriol* 177:6575–6584
- Nedashkovskaya OI et al (2004) Description of *Algoriphagus aquimarinus* sp. nov., *Algoriphagus chordae* sp. nov. and *Algoriphagus winogradskyi* sp. nov., from sea water and algae, transfer of *Hongiella halophila* Yi and Chun 2004 to the genus *Algoriphagus* as *Algoriphagus halophilus* comb. nov. and emended descriptions of the genera *Algoriphagus* Bowman et al. 2003 and *Hongiella* Yi and Chun 2004. *Int J Syst Evol Microbiol* 54:1757–1764
- Pasamontes L, Hug D, Tessier M, Hohmann HP, Schierle J, van Loon AP (1997) Isolation and characterization of the carotenoid biosynthesis genes of *Flavobacterium* sp. strain R1534. *Gene* 185:35–41
- Peck RF, Johnson EA, Krebs MP (2002) Identification of a lycopene beta-cyclase required for bacteriorhodopsin biogenesis in the archaeon *Halobacterium salinarum*. *J Bacteriol* 184:2889–2897

- Pecker I, Gabbay R, Cunningham FX Jr, Hirschberg J (1996) Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Mol Biol* 30:807–819
- Rohdich F et al (2002) Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein. *Proc Natl Acad Sci USA* 99:1158–1163
- Rohmer M, Seemann M, Horbach S, Bringer-Meyer S, Sarm H (1996) Glyceraldehyde-3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *J Am Chem Soc* 118:2564–2566
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schnurr G, Misawa N, Sandmann G (1996) Expression, purification and properties of lycopene cyclase from *Erwinia uredovora*. *Biochem J* 315(Pt 3):869–874
- Tao L, Picataggio S, Rouviere PE, Cheng Q (2004) Asymmetrically acting lycopene β -cyclases (CrtLm) from non-photosynthetic bacteria. *Mol Gen Genomics* 271:180–188
- Teramoto M, Takaichi S, Inomata Y, Ikenaga H, Misawa N (2003) Structural and functional analysis of a lycopene beta-monocyclase gene isolated from a unique marine bacterium that produces myxol. *FEBS Lett* 545:120–126
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Velayos A, Eslava AP, Iturriaga EA (2000) A bifunctional enzyme with lycopene cyclase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides*. *Eur J Biochem* 267:5509–5519
- Verdoes JC, Krubasik KP, Sandmann G, van Ooyen AJ (1999) Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. *Mol Gen Genet* 262:453–461
- Viveiros M, Krubasik P, Sandmann G, Houssaini-Iraqi M (2000) Structural and functional analysis of the gene cluster encoding carotenoid biosynthesis in *Mycobacterium aurum* A+. *FEMS Microbiol Lett* 187:95–101
- Wilding EI et al (2000) Identification, evolution, and essentiality of the mevalonate pathway for isopentenyl diphosphate biosynthesis in gram-positive cocci. *J Bacteriol* 182:4319–4327
- Yokoyama A, Miki W (1995) Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. *FEMS Microbiol Lett* 128:139–144