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Possible origin of zeaxanthin in the marine sponge, *Reniera japonica*

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Abstract. As part of a study to clarify the origins of biologically active substances in marine sponges, the carotenoids produced by two species of marine bacteria, *Flexibacter* sp. strain number DK30213 and DK30223, associated with the marine sponge, *Reniera japonica*, were investigated. Both bacteria were found to produce zeaxanthin [(3*R*,3′*R*)-dihydroxy-β,β-carotene] which is widely distributed in marine organisms. This carotenoid was also detected in the host sponge, suggesting the transport of zeaxanthin from the microorganisms to the host. As zeaxanthin plays the role of a quencher and scavenger for active species of oxygen, it is presumed that the sponge accumulates the bacterial product as a defense substance against the active oxygen species produced under irradiation by strong sunlight. It is thought that the bacteria are symbionts of the host sponge and act by obtaining the solid substrate and medium needed for settlement and growth from the host, and by producing and transmitting the biologically active substance to the host. Zeaxanthin-producing bacteria are also considered to have potential for practical uses by the aquacultural, pharmaceutical and food industries.
Key words. Carotenoid; marine sponge; *Reniera japonica*; sponge-associated bacteria; *Flexibacter* sp.; zeaxanthin; active oxygen species.

Carotenoids are known as quenchers and/or scavengers of active oxygen species[1]. A number of carotenoids have been isolated from marine animals which lack the ability to biosynthesize the pigments de novo. It is considered that these carotenoids are biosynthesized by microorganisms or algae and transported to the animals by oral and other routes. Animals can metabolize the supplemented carotenoids into their most suitable form and store them.

In relation to marine sponges, a number of active carotenoids such as renieratene, isorenieratene, reniera-purpurin and other aromatic types have been isolated and chemically characterized[2–5], although their origins still remain unknown. It has not yet been elucidated whether sponges can metabolize the dietary carotenoids into a specific structure suitable by enzymatic bioconversion, or whether the precursors and/or carotenoids themselves are biosynthesized by symbiotic or associated microorganisms, before being transmitted to the host sponge and stored. We[6] have recently reported the isolation of the novel C_{50}-carotenoid, okadaxanthin, as a possible precursor of aromatic carotenoids, although the results were preliminary . In additon to the aromatic carotenoids, astaxanthin, zeaxanthin, and other β-carotene-type carotenoids have also frequently been detected in the marine sponge[7], indicating quenching and/or scavenging activities against the active oxygen species[1]. However, a study of the origins of these carotenoids remains to be done. In the present investigation, we isolated the sponge-associated bacterium which produces carotenoids in order to provide clearer evidence on the relationship between a host sponge and its associated bacteria with respect to the transfer of related substances.

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Material and methods

Host sponge. A marine sponge, *Reniera japonica*, which is commonly found off the southern part of the Japanese coast, was collected from the Aburatsubo area of Miura Peninsula in Sagami Bay of Kanagawa Prefecture in July 1990. The specimen was quickly transported to our laboratory in a cooler at 0 °C for use as the host organism.

Isolation and identification of bacteria. The fresh sponge (5 g) was rinsed with distilled water, homogenized with 10 ml of sterilized seawater and diluted in order to prepare a series with 10-fold dilution. Each diluted sample was seeded onto an agar medium in a mixture composed of 750 ml of seawater (pH 7.7), 250 ml of distilled water, 5.0 g of Bacto-pepton (Difco), 1.0 g of Bacto-yeast extract (Difco), 15.0 g of agar (Nacalai Tesque) and 0.04 g of $FeSO_4$. After incubating at 20 °C for four days, all the colored colonies with different pigmentation were chosen for isolation. The isolated bacteria were cultured in the same medium without agar, supplemented with 2 g of glucose, at 20 °C for

three days. Among the isolates, two colonies of bacteria with tentative strain numbers DK30213 and DK30223 were found to be pigmented with the typical β-carotene-type of golden–yellowish color. The strains were identified according to the discription given in Bergey's Manual of Systematic Bacteriology[8].

Isolation of the bacterial carotenoid. A 1L culture broth after three days' incubation of bacterium DK30213 was centrifuged at 8000 rpm for 15 min to give a pellet, which was extracted with acetone under ultrasonic excitation. The extract was concentrated under reduced pressure and separated into two layers by adding ethyl acetate and distilled water. The yellowish pigment was recovered from the ethyl acetate layer, washed several times with distilled water, dried over anhydrous sodium sulfate and concentrated in vacuo to an oily drop. Visible absorption spectrum-guided isolation of the concentration by silica gel column chromatography (Silica gel 60, 230–400 mesh, Merck) eluted with a mixture of 20% of acetone in hexane resulted in the isolation of a yellowish oily pigment. Further purification by HPLC on silica gel (Cosmosil 5SL, $8\phi \times 250$, Nacalai Tesque) with the same solvent, led to the isolation of 0.37 mg of the pigment as a golden–yellowish oil. The same procedure was also performed on bacterium DK30223, resulting in the isolation of 0.48 mg of an oily pigment with similar color.

Identification of the pigment. The pigment was identified mainly by comparing the Rf value from TLC and the retention time from HPLC with the value for an authentic specimen, and by spectroscopic analysis. The visible absorption spectrum (VIS) in benzene was recorded with a Shimadzu UV-2100S recording spectrophotometer. Electron impact mass spectrometry (EIMS) was measured by a JEOL JMS-SX102 mass spectrometer. Authentic carotenoids with the β-ionon end group were then prepared. β-Carotene was purchased from Wako Pure Chemical Ind. and purified. Zeaxanthin was extracted and purified from the ovaries of the mackerel, *Pneumatophorus japonicus*[9], the absolute configuration of this carotenoid being identified as $3R,3'R$ by comparing the retention time in a optical-resolution column (Sumichiral OA-2000, Sumitomo Chemical, $4.0\phi \times 250$ mm) with that of the current data in the literature[10]. $(3S,3'S)$-Astaxanthin was obtained from the fermentation broth of marine bacterium, *Agrobacterium aurantiacum* sp. nov.[11] by a process of extraction and isolation. The purity of each of these carotenoids was monitored by HPLC and found to be greater than 99%.

Carotenoids of the host sponge. The fresh sponge (500 g) was cut into small pieces and extracted with acetone after mixing with sea sand. The extract was concentrated under reduced pressure and separated into two layers by adding ethyl acetate and distilled water. Carotenoids were recovered in the ethyl acetate layer, which was then washed several times with distilled water, dried over anhydrous sodium sulfate and concentrated in vacuo to an oily drop. The oily pigment was dissolved in benzene and made up to a 50 ml volume, the total carotenoid content being determined by the method of McBeth.[12] The whole pigment was concentrated again in vacuo and subjected to column chromatography on silica gel (Silica gel 60, 230–400 mesh, Merck) eluted with a mixture of 20% of acetone in hexane. Subsequent HPLC on silica gel (Cosmosil 5SL, Nacalai Tesque), using a suitable mixture of hexane, benzene and acetone, led to the isolation of each carotenoid. Each carotenoid in the sponge was identified according to methods described above and by comparison with descriptions in the current literature[2–4,6,7].

Result and discussion

Identification of the bacteria. The colony of the bacteria with the tentative strain number DK30213 became spreading swarms with long branched extensions at the edge. The strain was an aerobic Gram-negative rod, slender and flexible ($3–5 \times 0.2$ μm), and motile by gliding without flagella. Oxidase and gelatin tests were positive, whereas the glucose test was negative, and the strin required seawater salts for growth. The colony of the bacterium with tentative strain number DK30223 became also spreading swarms with long branched extensions at the edge. The strain was an aerobic Gram-negative rod, slender and flexible ($6–10 \times 0.5$ μm), and also motile by gliding without flagella. The oxidase test was positive, whereas the glucose and gelatin tests were negative. This strain also required seawater salts for growth. These observations, especially regarding shape and manner of motility, and the description in Bergey's Manual of Systematic Bacteriology[8], enabled both strains to be identified as of *Flexibacter* sp.

Identification of the bacterial pigments. The isolated carotenoids from each bacterial strain indicated the VIS maxima at 435 (shoulder), 462 and 492 nm in benzene, typical of the β-carotene type. Co-TLC on silica gel 60 of the pigments with authentic specimens afforded the same Rf values as that of zeaxanthin by developing with 30% acetone in hexane. This result was also supported by the co-HPLC retention time with a 1.5 ml/min flow of 20% acetone in hexane. These results suggested that both the bacterial carotenoids were zeaxanthin. The M^+ peak by EIMS of each carotenoid was also identical at 568, supporting this identification. The absolute configuration of each carotenoid was found to be $3R,3'R$ by agreement of the retention time with that of authentic $(3R,3'R)$-zeaxanthin in an optical-resolution column by HPLC[10]. From these data, we identified each carotenoid to be $(3R,3'R)$-zeaxanthin (fig.).

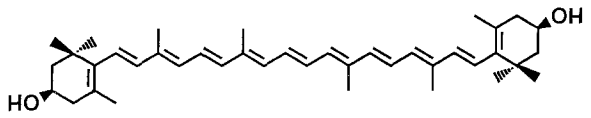

Figure. Structure of (3*R*,3′*R*)-zeaxanthin.

Carotenoids in the host sponge. The total carotenoid content was determined to be 7.8 mg/100 g of wet sponge, and the least six carotenoids could be detected and isolated.

α-Carotene (**1**): Detected as a minor component. **1** was isolated by silica gel column chromatography as a hydrocarbon fraction, and then by silica gel HPLC, using 20% benzene in hexane as the solvent. **1** showed VIS maxima at 420, 444 and 475 nm in hexane as typical of α-carotene. The M^+ peak by EIMS of **1** was detected at 536, indicating the molecular formula to be $C_{40}H_{56}$. These data coincided well with those for authentic α-carotene which was purchased from Nacalai Tesque and purified. We thus identified **1** as α-carotene, and this identity was also supported by the agreement in retention times by HPLC between **1** and the authentic specimen.

β-Carotene (**2**): Detected as one of the major carotenoids. **2** was isolated by silica gel column chromatography and silica gel HPLC according to the same procedure as that used for the isolation of **1**. **2** showed VIS maxima at 425 (shoulder), 450 and 480 nm in hexane, typical of β-carotene. The M^+ peak by EIMS of **2** was detected at 536, indicating the molecular formula to be $C_{40}H_{56}$. The coincidence of these data with those of authentic β-carotene enabled **2** to be identified as β-carotene. This identification was also supported by the agreement of retention times by HPLC between **2** and an authentic specimen.

Isorenieratene (**3**): Detected as one of the major components. **3** was isolated by silica gel column chromatography as a hydrocarbon fraction, and then by silica gel HPLC, using 40% benzene in hexane as the solvent. **3** showed VIS maxima at 450 (shoulder), 484 and 520 nm in carbon disulfide as is typical of φ-carotene. The M^+ peak by EIMS of **3** was detected at 528, indicating the molecular formula to be $C_{40}H_{48}$. These data were considered to match those of isorenieratene in the literature[4], and we thus tentatively identified **3** as isorenieratene.

Renieratene (**4**): Detected as a dominant component. **4** was isolated by the same chromatographic procedure as that used for **3**. **4** showed VIS maxima at 461 (shoulder), 495 and 531 nm in carbon disulfide as is typical of φ-carotene. The M^+ peak by EIMS of **4** was detected at 528, indicating the molecular formula to be $C_{40}H_{48}$. **4** was tentatively identified as renieratene by comparing these data with those of the carotenoid in the literature[3].

Renierapurpurin (**5**): Detected as a minor component, and isolated by the same procedure as that used for **3** and **4**. **5** showed VIS maxima at 474 (shoulder), 503 and 544 nm in carbon disulfide. Although further examination could not be made due to the small amount of **5**, it is possible to identify **5** as renierapurpurin by the typical long-wavelength shift in absorption maxima and by comparing the behavior of **5** during chromatography with that of this carotenoid in the literature[2].

Zeaxanthin (**6**): Detected as a substantial component. **6** was isolated by column chromatography, using 20% acetone in hexane, and then by HPLC with the same solvent. **6** indicated VIS maxima at 435 (shoulder), 462 and 492 nm in benzene, typical of β-carotene. Co-TLC on silica gel 60 of **6** with authentic zeaxanthin afforded the same Rf value by developing with 30% acetone in hexane. The identification was also supported by co-HPLC with authentic zeaxanthin, resulting in similar retention times with a 1.5 ml/min of 20% acetone in hexane as the solvent. These data confirmed that **6** was zeaxanthin, the M^+ peak by EIMS of **6** of 568 supporting this identification. The absolute configuration of **6** was found to be 3*R*,3′*R* by the agreement in retention time with that of authentic (3*R*,3′*R*)-zeaxanthin with optically resolved HPLC[10].

Two carotenoids other than those just described, isocryptoxanthin and astaxanthin, were also detected as trace constituents, but the small amount of each carotenoid forced us to abandon further investigation. The relative percentage of each carotenoid in *R. japonica* is shown in the table. The carotenoids detected could clearly be divided into two groups, approximately 70% being aromatic carotenoids (**3**, **4** and **5**) and approximately 20% being of the β-carotene group (**2** and **6**). Tanaka[7], Matsuno[5] and other researchers have suggested that one of the precursors of aromatic carotenoids should be tethyanine (3,4-didehydro-β,φ-carotene). Tanaka[7] has also proposed the 3,4-dihydroxy-β end group in tethyanine as an intermediate from the 3-hydroxy-β end group to the φ end group. We[6] have presumed that reductive deacylation of such C_{50}-carotenoids as okadaxanthin should result in an aromatic carotenoid. We also tentatively considered sponge-associated bacteria to be producers of C_{50}-carotenoids, which are transferred to and bioconverted in the host sponge. These assumptions still remain tenuous and will be clarified by further enzymatic and tissue culture studies.

Table. Carotenoid composition in the host sponge, *R. Japonica*.

Carotenoid	%
α-Carotene (**1**)	1–2
β-Carotene (**2**)	10–15
Isorenieratene (**3**)	22–25
Renieratene (**4**)	40–45
Renierapurpurin (**5**)	+
Zeaxanthin (**6**)	8–10
Unidentified	10–20

+ = trace.

In the case of the β-carotene-type carotenoids, zeaxanthin (**6**) was isolated from both the host sponge and its associated bacteria. Although we cannot positively assert that zeaxanthin (**6**) is synthesized exclusively in the bacteria, the detection of the carotenoid suggests that the bacteria are the origin of the carotenoid in the host sponge. As zeaxanthin (**6**) has exhibited quenching activity against singlet molecular oxygen approximately 500 times greater than that of α-tocopherol in deuterated chloroform[13], and scavenging activity against organic free radicals approximately 8 times greater than that of α-tocopherol in 50% aqueous dimethylsulfoxide[14], it is presumed that the host sponge uses the bacterial product as a defense substance against active oxygen species produced under irradiation by strong sunlight. It is also considered that the bacteria are symbionts of the host sponge, obtaining a solid substrate and medium for settlement and growth from the host and producing and transferring the biologically active substance to the host.

Zeaxanthin (**6**) is also important for the pigmentation of cultured fish such as the sweet smelt[15]. The blue-green alga, *Spirulina maxima*, has been used as a dietary supplement for fish, the alga containing a large amount of chlorophyll. As the oxidized derivatives may cause disease in fish, it is important to maintain the alga correctly until used. The marine bacteria isolated from sponge are free from chlorophyll and easier to cultivate and maintain than alga. Consequently, these bacteria are considered to have potential practical uses in the aquacultural, pharmaceutical and food industries.

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