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Carotenoid Pigments of Facultatively Anaerobic Spirochetes

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Carotenoid pigments were purified from a previously undescribed, red, halophilic spirochete (spirochete RS1), and from *Spirochaeta aurantia* strain J1. Both spirochetes are facultative anaerobes and produce pigments when growing aerobically. The major pigments of the two spirochetes were identified by means of chromatographic analysis, absorption spectroscopy, hydride reduction, acetylation and silylation experiments, and mass spectrometry. It was concluded that the major pigment from spirochete RS1 was 4-keto-1',2'-dihydro-1'-hydroxytorulene. This conclusion was further supported by infrared spectroscopy and additional analytical data. The evidence showed that the major pigment from *S. aurantia* was 1',2'-dihydro-1'-hydroxytorulene. Chromatographic and spectrophotometric evidence indicated that this pigment was also present, as a minor carotenoid component, in spirochete RS1. These pigments have been previously detected almost exclusively in gliding bacteria, such as species of *Flexibacter*, *Stigmatella*, and *Myxococcus*. The occurrence of 4-keto-1',2'-dihydro-1'-hydroxytorulene and 1',2'-dihydro-1'-hydroxytorulene in both spirochetes and gliding bacteria may have significance with respect to the evolutionary development of these organisms.

Most species of the genus *Spirochaeta* are obligately anaerobic and form colonies which are not pigmented (8). At present, the only known facultatively anaerobic species in this genus is *Spirochaeta aurantia*, a spirochete commonly found in fresh-water bodies in the United States (7; J. A. Breznak, and E. Canale-Parola, Arch. Microbiol., in press). This organism produces a yellow-orange pigment under aerobic conditions, but shows no pigmentation when grown anaerobically. Recently we isolated, from the mud of a solar pond in Israel, a previously undescribed facultatively anaerobic spirochete to which we refer as "spirochete RS1". Aerobically grown colonies of this spirochete are red, whereas colonies grown in the absence of O_2 are white. Spirochete RS1 differs from *S. aurantia* morphologically, and also because it is a halophile which grows optimally when relatively high concentrations of NaCl (0.75 M), $MgSO_4$ (0.2 M), and $CaCl_2$ (0.01 M) are present in the medium. Spirochete RS1 fails to grow when any one of these salts is omitted from the medium (see below). Furthermore, the temperature range for optimum growth of spirochete RS1 is 35 to 40 C, as compared to *S. aurantia* strains which grow abundantly between 25 and 30 C, but poorly or not at all at 37 C. The morphological and physiological characteristics of spirochete RS1 will be described in greater detail elsewhere (E. P. Greenberg and E. Canale-Parola, manuscript in preparation).

Spirochete RS1 and *S. aurantia* are the only known spirochetes which form pigmented colonies. Previous workers (7) found that the major pigment of *S. aurantia* strain J1 was a carotenoid with an absorption spectrum similar to that of lycopene. However, the chemical structure of this carotenoid was not determined.

In the study reported here, we have determined the molecular structures of the major pigments of spirochete RS1 and of *S. aurantia* J1. The main objectives of our work were to achieve a greater understanding of the phylogenetic relatedness between these two spirochetes, and to obtain information on the evolutionary relationship of facultatively anaerobic spirochetes to other procaryotes.

MATERIALS AND METHODS

Organisms and culture conditions. *S. aurantia* J1 was cultured by the technique of Breznak and Canale-Parola (7). Spirochete RS1 was cultured in inorganic salts-maltose (ISM) medium containing 0.2 g of peptone (Difco) and 0.4 g of yeast extract (Difco) per 100 ml of inorganic salts solution. The inorganic salts solution had the following composition: $CaCl_2$, 0.01 M; NaCl, 0.75 M; and $MgSO_4$, 0.2 M. In preparing the salts solution the salts were added to distilled water in the order in which they are listed, to avoid the formation of a precipitate. The dihydrate form of

$CaCl_2$ and the heptahydrate form of $MgSO_4$ were used. After adjusting the pH to 7.5 with KOH the medium was sterilized by autoclaving. Sterile 25% maltose was subsequently added to a final concentration of 0.5%. Spirochete RS1 was grown in 10-liter batches at 37 C on a Microferm laboratory fermentor (New Brunswick Scientific Co.) operated at 100 rpm and aerated at a rate of 3 liters of air per min. The inoculum was 5 ml of aerobically grown culture (cell density, 4×10^8 cells/ml) per 100 ml of ISM medium. Cells were harvested by batch centrifugation at 5 C when the density reached 7×10^8 cells/ml.

Pigment purification. Batches of pigments from spirochete RS1 were purified as follows. The pigments from about 20 g (wet weight) of cells were extracted with 500 ml of acetone-methanol (7:3,vol/vol). Concentration of the extracted pigments by flash evaporation at 35 C resulted in formation of red crystals which were separated from the remaining colorless solution by filtration, and were then redissolved in approximately 10 ml of carbon disulfide. This material was applied directly to an activity grade III neutral aluminum oxide column (1 by 20 cm) and eluted with increasing concentrations of acetone in petroleum ether (Table 1). The major pigment eluted from the column (Table 1, fraction 3) was crystallized from methanol and water, lyophilized, and stored in the dark under N_2 at -25 C. The crystallization step, lyophilization step, and up to 1-month storage had no effect on the chromatographic properties or the absorption spectrum of the pigment. All analyses of the pigment eluted in fraction 3 (Table 1, major red band) were carried out on the purified crystals. Analyses on fraction 2 (Table 1, minor orange band) were carried out immediately after elution from the aluminum oxide column. Both the major pigment from *S. aurantia* J1 and lycopene from tomato puree were purified and stored as described by Breznak and Canale-Parola (7) except that the saponification steps were omitted. In all cases, during and after pigment extraction, preparations were kept in the dark or in very dim light and handled under N_2 as much as possible. All pigment preparations used for the analyses were chromatographically homogeneous (thin-layer chromatography [TLC] and aluminum oxide column chromatography).

TABLE 1. *Chromatographic properties of spirochete RS1 pigments*

Fraction[a]	Pigment	Eluant[b]	R_f value[c]
1	Minor yellow band	0	
2	Minor orange band[d]	7	0.23
3	Major red band	14	0.08
	Tomato lycopene[e]	4	0.68

[a] Fractions 1 to 3 extracted from spirochete RS1. Lycopene extracted from tomato puree.

[b] Percentage of acetone in petroleum ether. Activity grade III neutral aluminum oxide columns were used.

[c] TLC Silica Gel G. Solvent system: 12% (vol/vol) acetone in petroleum ether.

[d] The major carotenoid extracted from *S. aurantia* J1 had the same properties.

[e] Used as a reference.

Spectra. Absorption spectra were determined with a Beckman recording spectrophotometer, model DK-1A. Infrared spectra of pigment samples in KBr pellets were recorded with a Perkin-Elmer model 257 grating infrared spectrophotometer. Mass spectra were obtained by the methods of Enzell et al. (12) with a Hitachi Perkin-Elmer model RMU-6L mass spectrometer. High-resolution mass determinations were obtained with a Dupont (CEC) model 110 mass spectrometer and the data were analyzed with an IBM 1800 computer to determine elemental compositions (9).

Other analytical procedures. TLC was on Silica Gel G with the solvent system acetone-petroleum ether (12:88, vol/vol). Hydride reductions were by the technique of Liaaen-Jensen (19). Partition ratios were determined quantitatively by the method of Petracek and Zechmeister (22). Acetylation tests (3, 20) were performed by dissolving pigments in 1 ml of dry pyridine, adding 0.1 ml of acetic anhydride, and incubating at room temperature for 24 h. Silylation tests (20) were carried out by dissolving pigments in Sigma-Sil-A (Sigma Chemical Co., St. Louis, Mo.) at room temperature. The presence of acetylation or silylation products was determined by TLC with the solvent system previously mentioned. The melting point was determined with a Mel-Temp capillary melting-point apparatus (Laboratory Devices, Cambridge, Mass.). Temperature was increased at a rate of 4 to 6 C per min and observed readings were corrected against melting-point standards.

Chemicals. All reagents were of analytical grade. Acetone and petroleum ether were redistilled (boiling range 40 to 70 C). Lithium aluminum hydride was purchased from Bradford Scientific, Inc., Marblehead, Mass. Silica Gel G plates (250-μm thick) were purchased from Analtech Inc., Newark, Del. Neutral aluminum oxide AG-7 (100 to 200 mesh) was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Pigments from spirochete RS1. Column chromatography of pigments extracted from spirochete RS1 yielded one major and two minor colored bands which were eluted as separate fractions (Table 1). The major band accounted for at least 90% of the total pigment content (absorbance at 474 nm). The pigment which constituted the minor orange band (Table 1, fraction 2) was indistinguishable chromatographically from the major pigment of *S. aurantia* J1 (see below).

The major pigment from spirochete RS1 (Table 1, fraction 3) had an absorption maximum at 477 nm and a shoulder at 504 nm in petroleum ether or hexane (Fig. 1). The spectrum showed little fine structure, the shape being indicative of a keto carotenoid (25). After reduction of the major pigment with lithium

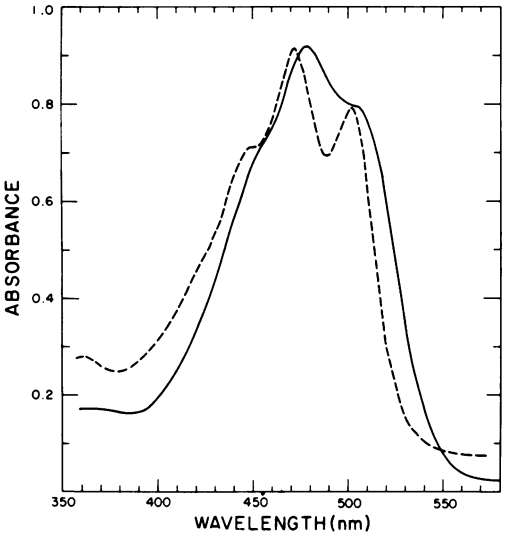

FIG. 1. *Absorption spectra in visible light of the major pigment from spirochete RS1* (—) *and the hydride reduction product of the major pigment from spirochete RS1* (- - -). *Solvent, petroleum ether.*

aluminum hydride the absorption spectrum exhibited maxima at 502 and 471 nm, as well as shoulders at 446 and 361 nm (Fig. 1). Shifts of this nature occur when a carbonyl function is present in position four of a cyclohexene end group (16, 21, 25).

High-resolution mass spectra of the major pigment from spirochete RS1 exhibited the molecular ion at *m/e* 566.41370. Computer analysis indicated that this mass number corresponded to the elemental composition $C_{40}H_{54}O_2$ (calculated value, 566.41238). The upper part of the mass spectrum (Fig. 2A) showed peaks at M-92 and M-106, which are characteristic of carotenoids (12, 13). The presence of a tertiary hydroxyl end group was indicated by the significant peaks at M-18 and M-58 (13). Acetylation and silylation tests supported a tertiary hydroxyl assignment (20). As judged by TLC, no acetates of the major pigment from spirochete RS1 were formed. However, a trimethylsilyl ether, R_f value 0.43 in TLC was formed.

The evidence described above indicated that the major pigment of spirochete RS1 was 4-keto-1',2'-dihydro-1'-hydroxytorulene (Fig. 3A), which has been called deoxy-flexixanthin by Aasen and Liaaen-Jensen (3). This conclusion was supported by comparing additional properties of the major pigment of spirochete RS1 with properties of deoxy-flexixanthin reported by other authors (3), as described below. The infrared spectrum of the major pigment from spirochete RS1 (Fig. 4) was essentially superimposable with the infrared spectrum of deoxy-flexixanthin determined by Aasen and Liaaen-Jensen (3). Both spectra showed an absorption band at 1,650 cm^{-1}, which is characteristic of a conjugated carbonyl group (3, 21). The presence of this absorption band was consistent with results of hydride reduction experiments described above. Furthermore, weak absorption at approximately 1,140 cm^{-1} supported mass spectrometric and chemical evidence described above. Furthermore, weak absorption at approximately 1,140 cm^{-1} supported mass spectrometric and chemical evidence described above indicating the presence of a tertiary hydroxyl group in the molecule (3, 21).

The major pigment from spirochete RS1 had a melting point of 155 to 160 C. Partition ratios were 38:62 in petroleum ether/95% methanol, and 80:20 in petroleum ether/85% methanol. These melting-point and partition ratio data are in agreement with values reported for deoxy-flexixanthin (3).

Major pigment from S. aurantia J1. The behavior of the pigments from *S. aurantia* J1 on neutral aluminum oxide chromatographic columns has been described previously (7). As mentioned above, the major pigment from *S. aurantia* J1 was indistinguishable chromatographically (TLC and column chromatography) from the minor orange pigment (Table 1, fraction 2) of spirochete RS1. Both pigments in petroleum ether or hexane had absorption maxima at 502,473 nm and shoulders at 446 to 450 and 361 nm (Fig. 5). As pointed out by previous workers (7), this absorption spectrum is similar to that of lycopene, but is not as sharply defined (Fig. 5). The less pronounced fine structure of the absorption spectrum suggested the presence of a cyclic end group (21, 25).

Comparison of the R_f value of lycopene with that of the major pigment from *S. aurantia* J1 (Table 1) raised the possibility that the latter pigment contained oxygen (21). The presence of oxygen in the major pigment from *S. aurantia* J1 was confirmed by analysis of high-resolution mass spectra, which exhibited the molecular ion at *m/e* 552.43219. Computer analysis indicated that this mass number corresponded to the elemental composition $C_{40}H_{56}O$ (calculated value, 552.43311).

The fine structure of the absorption spectrum of the major pigment from *S. aurantia* J1 (Fig. 5) was not indicative of a keto carotenoid (25). To determine chemically whether a carbonyl group was present in the molecule, the major pigment from *S. aurantia* J1 was treated with lithium aluminum hydride. As judged by TLC and absorption characteristics in petroleum ether, no product was formed. These observations were consistent with the conclusion that

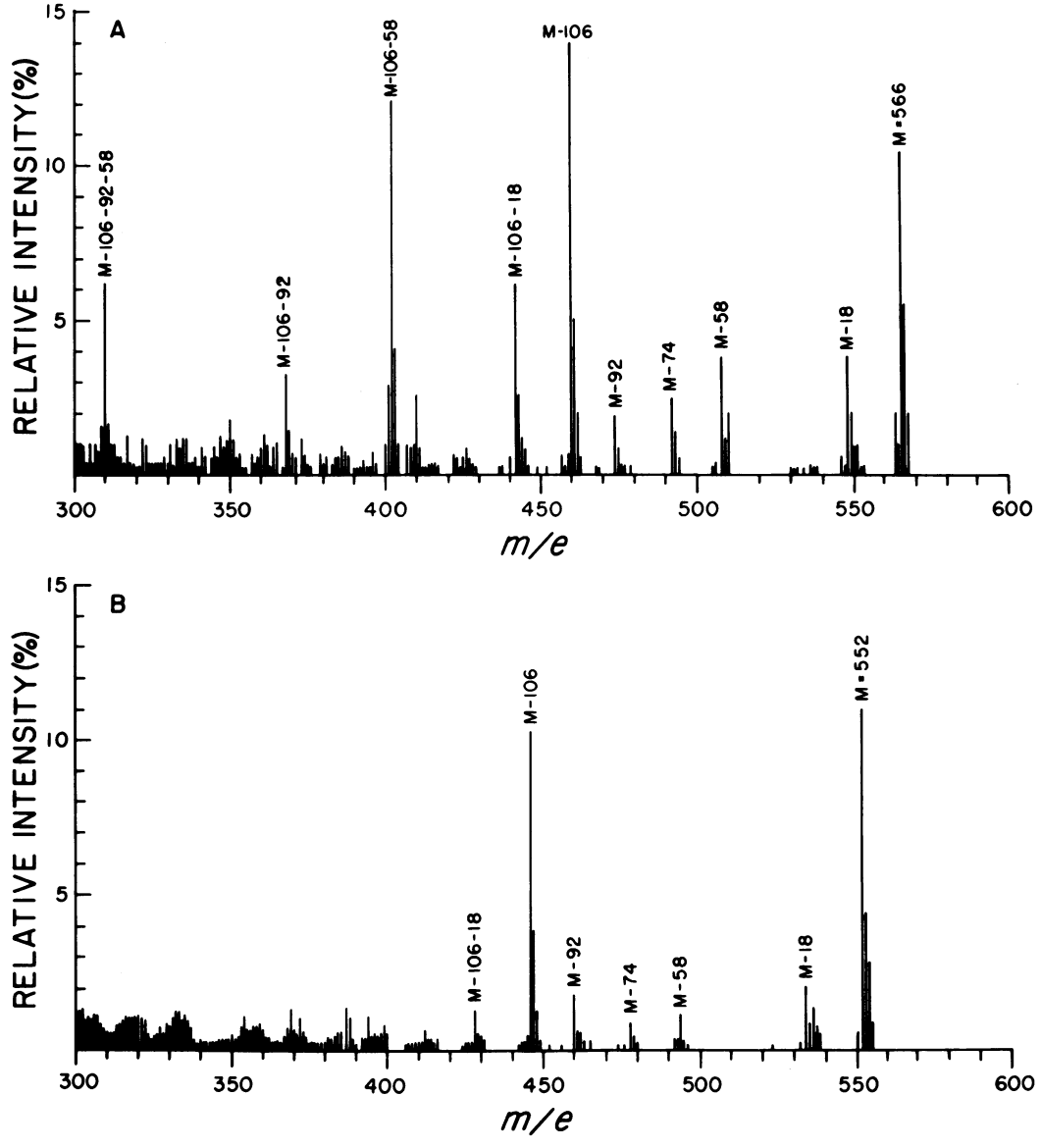

FIG. 2. *High-mass region of mass spectrum of the major pigment from spirochete RS1 (A). Base peak occurred at m/e 59; relative abundance at m/e 91 was 60%. High-mass region of mass spectrum of major pigment from S. aurantia J1 (B). Base peak occurred at m/e 91; relative abundance at m/e 59 was 76.2%.*

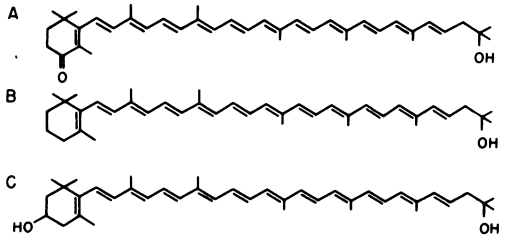

FIG. 3. *Deoxy-flexixanthin (A), 1',2'-dihydro-1'-hydroxytorulene (B) and saproxanthin (C).*

carbonyl groups were not present in the molecule (21, 25). Evidence for a tertiary hydroxyl end group (13) was obtained by mass spectrometry, which showed significant peaks at M-18 and M-58 (Fig. 2B). Acetylation and silylation experiments (20) supported mass spectrometric evidence for a tertiary hydroxyl group. Acetates of the major pigment from *S. aurantia* J1 were not detected but a trimethylsilyl ether with an R_f value of 0.68 in TLC was formed. Thus the data indicated that, in the major pigment from *S. aurantia* J1, the oxygen was present in a

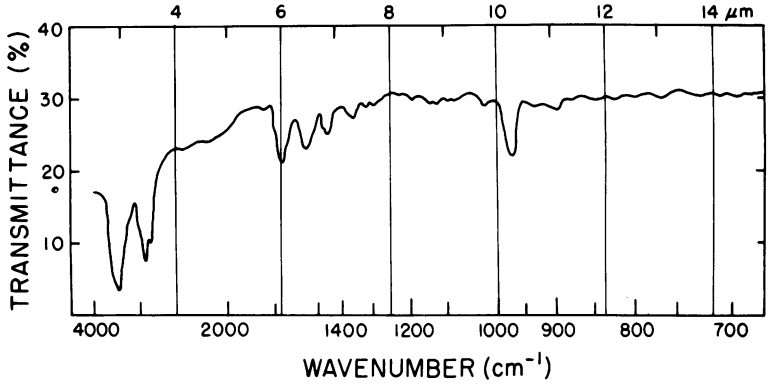

FIG. 4. *Infrared spectrum (solid in KBr) of the major pigment from spirochete RS1.*

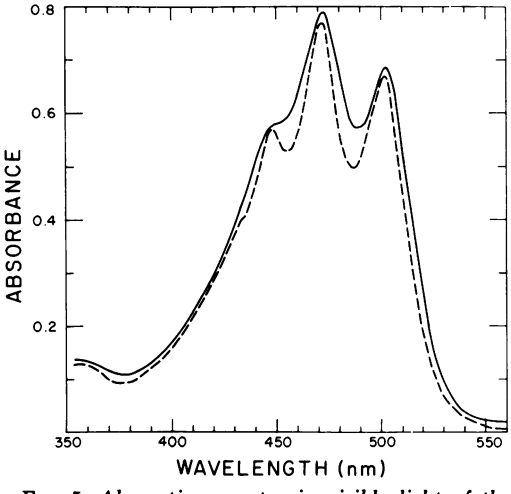

FIG. 5. *Absorption spectra in visible light of the major pigment from S. aurantia J1* (—) *and lycopene from tomatoes* (- - -). *Solvent, petroleum ether.*

tertiary hydroxyl group. The mass spectrum (Fig. 2B) had peaks at M-92 and M-106 which, as previously mentioned, are characteristic of carotenoids.

The absorption spectrum of 1',2'-dihydro-4, 1'-dihydroxytorulene which was the reduction product of deoxy-flexixanthin (Fig. 1) (3), and the absorption spectrum of the major pigment from *S. aurantia* J1 (Fig. 5) were indistinguishable. This indicated that these compounds had identical chromophores, thus suggesting that the major pigment from *S. aurantia* J1 differed from deoxy-flexixanthin only in a substitution at position four of the cyclohexene ring. It should also be noted that the significant peaks in the mass spectrum of the major pigment from *S. aurantia* J1 (Fig. 2B) were at *m/e* values, 14 mass units lower than corresponding peaks in the spectrum of deoxy-flexixanthin (Fig. 2A). Such lower values were consistent with the conclusion that the two molecules differed only because of the presence of the carbonyl group in deoxy-flexixanthin.

On the basis of the evidence described above we consider the major pigment from *S. aurantia* J1 to be 1',2'-dihydro-1'-hydroxytorulene (Fig. 3B).

DISCUSSION

The evidence we report in this paper indicates that the major pigments of spirochete RS1 and *S. aurantia* J1 are deoxy-flexixanthin and 1',2'-dihydro-1'-hydroxytorulene, respectively. These carotenoids are similar in chemical structure, differing only in a substitution in the cyclohexene ring (Fig. 3).

In addition to deoxy-flexixanthin, two other carotenoid pigments were detected in cells of spirochete RS1. One of these pigments (Table 1, fraction 2) appeared to be identical with the major pigment from *S. aurantia* J1 (1',2'-dihydro-1'-hydroxytorulene). It seems likely that, in cells of spirochete RS1, the latter carotenoid serves as a biosynthetic precursor to deoxy-flexixanthin.

Although results of comparative studies on the physiology and morphology of spirochete RS1 and *S. aurantia* indicate that these organisms represent two different bacterial species (E. P. Greenberg and E. Canale-Parola, manuscript in preparation), the similarities in carotenoid composition are in agreement with the possibility that the two spirochetes are closely related phylogenetically.

The carotenoid pigments identified in spirochete RS1 are dissimilar in chemical structure from the carotenoids of other known halophilic bacteria (1, 17, 18). Thus, it may be speculated that the halophilic spirochete RS1 and the fresh-water isolate *S. aurantia* J1 evolved from a common ancestral spirochete, rather than

from nonspirochetal halophilic and fresh-water ancestors.

Deoxy-flexixanthin or 1',2'-dihydro-1'-hydroxytorulene have been detected previously in certain gliding bacteria, such as a species of *Flexibacter* (3), *Stigmatella aurantiaca* (15), and *Myxococcus fulvus* (24). However, in these bacteria the pigments were present as minor carotenoid components. In *St. aurantiaca* and *M. fulvus* deoxy-flexixanthin is bound to glucose forming 4-keto-1',2'-dihydro-1'-hydroxytorulene glucoside (16, 24), which has been named myxobacton by Kleinig et al. (16). Esters of myxobacton constitute a major pigment fraction in *St. aurantiaca* and *M. fulvus* (16, 24). Furthermore, myxobacton and the glucoside of 1',2'-dihydro-1'-hydroxytorulene have been found in the gliding photosynthetic bacterium *Chloroflexus aurantiacus* (14, 23). Saproxanthin, a carotenoid remarkably similar in chemical structure (Fig. 3C) to the identified pigments of spirochete RS1 and *S. aurantia* J1, was reported to be the major carotenoid of *Saprospira grandis* (2). The latter is a gliding bacterium previously considered to be a spirochete (6, 8).

With one exception, we have not found reports in the scientific literature indicating that deoxy-flexixanthin or 1',2'-dihydro-1'-hydroxytorulene have been detected in organisms other than gliding bacteria. The exception is a report stating that flexixanthin and small quantities of deoxy-flexixanthin occur in a nongliding bacterium of uncertain taxonomic position (4). It was suggested that the carotenoid composition might indicate a relationship between this nongliding organism and the gliding flexibacteria (4). The presence of deoxy-flexixanthin and 1',2'-dihydro-1'-hydroxytorulene in both spirochetes and gliding bacteria suggests that the phylogenetic relationship between these two types of bacteria may be closer than it was previously believed. Spirochetes swim in liquid environments exhibiting rotation around their longitudinal axis and a variety of flexing movements. In addition to swimming in liquids, at least some spirochetes perform "creeping" or "crawling" translational motion on solid surfaces (5, 10). Comparative studies between this type of motility and the motility of gliding bacteria may yield information on the evolutionary relationship between the latter organisms and spirochetes.

Dworkin (11) has reported that the growth of gliding bacteria generally is inhibited by relatively low concentrations of actinomycin D, whereas that of other gram-negative bacteria usually is not. We found that the growth of spirochete RS1 and *S. aurantia* J1, both of them gram-negative bacteria, was strongly inhibited by actinomycin D under experimental conditions similar to those used by Dworkin (11) to test gliding bacteria. The response to actinomycin D may reflect similarities in cell surface composition between spirochetes and gliding bacteria.

The guanine plus cytosine contents of the deoxyribonucleic acid of spirochetes and gliding bacteria vary considerably from taxon to taxon (8). However, we feel that comparisons of guanine plus cytosine contents are of limited value in the study of bacterial phylogeny, inasmuch as they may reveal dissimilarities between present-day bacterial genomes, but do not shed light on the evolutionary processes which led to the development of these genomes.

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