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Carotenoids of an Antarctic psychrotolerant bacterium, *Sphingobacterium antarcticus*, and a mesophilic bacterium, *Sphingobacterium multivorum*

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Abstract The major carotenoid pigments of an Antarctic psychrotolerant bacterium, *Sphingobacterium antarcticus*, and a mesophilic bacterium, *Sphingobacterium multivorum*, were identified as zeaxanthin, β-cryptoxanthin, and β-carotene. Analysis was based on ultraviolet-visible spectroscopy, mass spectroscopy, and reversed-phase HPLC. Photoacoustic spectroscopy of intact bacterial cells revealed that the bulk of the pigments in *S. antarcticus* and *S. multivorum* was associated with the cell membrane. In vitro studies with synthetic membranes of phosphatidylcholine demonstrated that the major pigment was bound to the membranes and decreased their fluidity. The relative amounts of polar pigments were higher in cells grown at 5 °C than in cells grown at 25 °C. In the mesophilic strain, the synthesis of polar carotenoids was quantitatively less than that of the psychrotolerant strain.

Key words Carotenoids · Antarctica · Psychrotolerant bacterium · Membrane interaction

Introduction

Carotenoid pigments are present in a wide variety of bacteria, algae, fungi and plants (Bramely and Mackenzie 1988; Goodwin 1980). In photosynthetic bacteria, carotenoids help in harvesting and transferring light energy to chlorophyll and protect against photodynamic damage (Krinsky 1968; Siefirmann-Harms 1987). In non-photosynthetic bacteria, carotenoids may provide protection against ultraviolet (UV) radiation (Becker-Hapak et al. 1997; Goodwin 1980; Siefirmann-Harms 1987). In bacterial systematics, carotenoids serve as important chemotaxonomic markers in genera such as *Micrococcus* and *Flavobacterium* (Holmes et al. 1984; Kocur, 1986).

Recent in vitro studies have demonstrated that zeaxanthin, a polar carotenoid, confers more rigidity to a membrane than β-carotene, a non-polar carotenoid (Subczynski et al. 1992). Hence it appears that carotenoids play a role in modulation of membrane fluidity in bacteria. Earlier studies indicated that microorganisms grown at suboptimal temperatures synthesise a greater proportion of unsaturated fatty acids than when they are grown at higher temperatures in order to maintain the fluidity of their membranes (Nichols and Russell 1996; Russell 1984). They also exhibit certain temperature-dependent changes, such as phosphorylation of membrane proteins and lipopolysaccharides (Ray et al. 1994a, 1994b, 1994c). Studies have also indicated that several psychrotrophic bacteria from Antarctica contain cartotenoid pigments (Shivaji et al. 1988, 1989a, 1989b, 1992), and synthesis of these pigments increases in bacteria grown at 5 °C compared to at 25 °C (Chattopadhyay et al. 1997). Carotenoid pigments can also bind and alter the fluidity of synthetic membranes (Jagannadham et al. 1991, 1996a, 1996b). Thus it is possible that microorganisms modulate membrane fluidity by regulating the biosynthesis of carotenoids, depending on the growth temperature.

In the present investigation, carotenoid pigments of a psychrotolerant bacterium, *Sphingobacterium antarcticus*, isolated from a soil sample from Antarctica and characterised in a previous study (Shivaji et al.1992), were purified and chemically characterised. In addition, their in vivo localisation, temperature-dependent biosynthesis and interaction with synthetic membranes were also studied. *Sphingobacterium multivorum*, a mesophilic and yellow-pigmented strain (Shivaji et al. 1992), was used for comparison. Based on various phenotypic characteristics, such as the G+C content of DNA, DNA-DNA relatedness and nature of the sphingolipids, *S. antarcticus* closely resembles *S. multivorum*. This report shows that simulta-

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neous enhancement of the synthesis of unsaturated and branched-chain fatty acids and polar carotenoids helps to maintain an optimum membrane fluidity in psychrotolerant bacteria.

Materials and methods

Bacterial strains and growth conditions

Sphingobacterium antarcticus (MTCC 675, ATCC 51970; Shivaji et al. 1992) was cultured in Antarctic bacterial medium (ABM), containing peptone (0.5% w/v) and yeast extract (0.2% w/v), with continuous shaking in an incubator at 5 °C and 25 °C. The mesophilic type culture of *S. multivorum* (NCTC 11343) was obtained from the National Collection of Type Cultures (National Chemical Laboratory, Pune, India) and was grown in ABM at 30 °C (Shivaji et al. 1988, 1989a, 1989b, 1992). Cultures of both strains were incubated for 6 days and then used for extraction of the pigments.

Extraction and analysis of the pigments

The yellow pigments from *S. antarcticus* and *S. multivorum* were extracted with methanol from freeze-dried cell pellets until the latter became colourless. All manipulations during preparation of the carotenoids were carried out rapidly and the purified carotenoids were used immediately; therefore, there was no need to use antioxidants (Schiedt and Liaaen-Jensen 1995). The effects of temperature and light were avoided by carrying out the extractions at room temperature in glassware covered with aluminium foil. The detrimental effects of oxygen were avoided by flushing all solutions with nitrogen. The pigments thus obtained were pooled, concentrated by rotary evaporation and purified by HPLC on a μ-Bondapak C-18 reversed-phase column (3.9×300 mm), using a gradient from 80% (v/v) aqueous methanol (solvent A) to 100% methanol (solvent B) at a flow rate of 1 ml/min (HPLC-1). The solvent combination from 0 to 5 min was varied from 0 to 70% B, and from 6 min until the end of the run (40 min) it was 100% B. Peaks were monitored at 450 nm with a HP1090 HPLC (Hewlett Packard, Waldbronn, Germany) equipped with a diode array detector. The crude extracts were also analysed on a Zorbox ODS column by isocratic, non-aqueous, reversed-phase liquid chromatography as described earlier (Nelis and DeLeenheer 1989). The mobile phase consisted of acetonitrile, methanol and ethyl acetate in a ratio of 40:50:10 (by vol), and the flow rate was 1 ml/min (HPLC-2). The detector was set at 450 nm. Pigments were also purified by preparative TLC using precoated Silica gel plates (Merck, Bombay, India) and heptane:acetone (1:1, vol/vol) as solvent. An average extinction coefficient of $\varepsilon\,^{1\%}_{1\,cm} = 2500$ was used for the quantitation of pigments (Davies 1976).

UV-visible, infrared, mass, ^{1}H-NMR and photoacoustic spectra

UV-visible spectra of the pigments were recorded using a Hitachi 150–20 spectrophotometer (Tokyo, Japan). Mass spectra of the pigments were recorded on a VG Autospec M mass spectrometer (Micromass, Manchester, UK) at 70 eV. ^{1}H-NMR spectra were recorded on a Bruker AM 300 MHz spectrometer (Spectrospin, Fallenden, Switzerland) by using $CDCl_3$, with tetramethyl silane as an internal standard. Infrared (IR) spectra were recorded using KBr pellets in a Perkin Elmer IR instrument (Warrington, UK).

Photoacoustic spectroscopy is a simple and convenient technique that has been used to obtain absorption spectra of optically opaque and light-scattering biological samples, such as skin, malarial parasite, algal cells, lobster shell and bacteria (Anjo and Moore 1984; Balasubramanian et al. 1984; Jagannadham et al. 1996b; Lechaine et al. 1993). The advantage of this technique is that it facilitates in vivo studies on pigments such as carotenoids, thereby eliminating the need to isolate the carotenoids (Jagannadham et al. 1996b; Narayanan et al. 1997). The carotenoids were localised in the cells by recording the photoacoustic spectra of signals from the surface (in-phase spectra) and inside (quadrature spectra) of the cells (Jagannadham et al. 1996b; Narayanan et al. 1997) using an OAS-400 photoacoustic spectrometer (EDT Research, London, UK).

Acetylation of the pigments

The hydroxy groups of the pigments from *S. antarcticus* were acetylated essentially as described earlier (Davies 1976) using about 200 μg of the carotenoid pigment. Aliquots of the acetylated products were taken at different time intervals and analysed by HPLC on a reversed-phase column using a gradient of 80–100% methanol at a flow rate of 1 ml/min as described earlier (HPLC-1).

Isolation of bacterial membranes and interaction of pigments with synthetic membrane vesicles

The bacterial cell membranes were prepared as described earlier (Ray et al. 1994c). Interaction of pigments from *S. antarcticus* with small unilamellar vesicles of phosphatidylcholine (PC) was monitored using 8-anilino-1-naphthalene sulfonate (ANS) and pyrene as the fluorescent probes, as described earlier (Jagannadham et al. 1991; Chattopadhyay et al. 1997). A stock solution of ANS in methanol was added to either 1 ml of buffer (5 mM HEPES, pH 7.4) or 1 ml of buffer containing 150 μM PC vesicles such that the final concentration of ANS was 25 μM. ANS was excited at 370 nm and the spectra were recorded from 420 to 550 nm. Pyrene was incorporated into the vesicles by rapidly mixing a stock solution of pyrene (2 mM in methanol) with the vesicles (150 μM in 5 mM HEPES, pH 7.4); the final concentration of pyrene was 4 μM. Earlier studies have demonstrated that 4 μM pyrene is soluble in an aqueous buffer such as 5 mM HEPES, pH 7.4 (Vanderkooi and Callis 1974; Vijayasarathy et al. 1982). The concentration of methanol was not allowed to exceed 1%. Pyrene was excited at 333 nm and the emission spectra were recorded from 360 to 600 nm. The excitation and emission slit widths were 5 nm each (Jagannadham et al. 1991) in all the experiments.

Membrane fluidity of intact cells of *S. antarcticus* grown at 5 °C and 25 °C

Pyrene was used to monitor the fluidity of intact cells as described earlier (Jagannadham et al. 1991; Shivaji 1986). Bacterial cells were grown both at 5 °C and 25 °C to the late exponential phase, harvested by centrifugation, washed, and resuspended in 5 mM HEPES buffer (pH 7.4) and incubated at room temperature (~25 °C) for 4 h in the presence of 4 μM pyrene. Cells without pyrene served as a control. In all the experiments the same cell concentration was used (OD_{600}=0.5).

Preparation and analysis of fatty acid methyl esters

The fatty acid methyl esters of *S. antarcticus* and *S. multivorum* were prepared as described earlier (Morrison et al. 1964) and analysed on a GLC Hewlett Packard 5890 gas chromatograph equipped with a flame ionisation detector and a HP-5 column (30 m×0.32 mm×0.25 μm column). The carrier gas was nitrogen. The nitrogen flow rate was approximately 2 ml/min, the flow rate of hydrogen to the detector was 40 ml/min and the air flow rate was 450 ml/min. The detector temperature was maintained at 280 °C, and the oven temperature was increased from 120 °C to 250 °C at a rate of 5 °C/min. Commercially obtained methyl esters (Sigma, St. Louis, Mo.) served as standards.

The fatty acid methyl esters of *S. antarcticus* were also analysed by GC-MS on a VG autospec-M mass spectrometer coupled with a HP 5890 series II gas chromatograph (Hewlett Packard); the fatty acids were identified according to Suutari and Laakso (1993) and Tornabene et al. (1967). A HP-5 capillary column was used for the analysis. Helium was used as the carrier gas at a flow rate of approximately 2 ml/min. The sample (1 µl), containing the fatty acid methyl ester in chloroform, was injected under a split ratio of 1:100. The GC conditions were as follows: 100 °C from 0 to 5 min; increased to 220 °C by 17 min at 10 °C/min and maintained at 220 °C until 20 min. Mass spectra were scanned between 20 and 600 Da. The instrument was controlled by OPUS V3.1x software (Micromiass, Manchester, UK). The electron impact spectra were recorded at a source temperature of 250 °C, a trap current of 200 µA, and at an electron energy of 70 eV.

Chemicals and reagents

All chemicals and reagents were of analytical grade. Methanol (HPLC grade) was obtained from Spectrochem (Bombay, India). Phosphatidylcholine, pyrene, and β-carotene were from Sigma. (3*R*,3′*R*)-Zeaxanthin was a gift from Dr. Reinhard Zell (Hoffman-La Roche, Basel). 8-Anilino-1-napthalene sulfonate (ANS) was from Aldrich. The chemicals used for bacterial culture were obtained from Loba (Bombay, India).

Results

UV-visible absorption spectra and HPLC of the pigments of *S. antarcticus* and *S. multivorum*

UV-visible absorption spectra of the methanol extracts containing the total pigments of *S. antarcticus* and *S. multivorum* were identical and showed multiple absorption peaks at 427, 448, and 475 nm, which are characteristic of carotenoids. Separation by HPLC on a µ-Bondapak C-18 reversed-phase column (HPLC-1) or on a Zorbax ODS column (HPLC-2) resolved the total pigments into five distinct peaks (PS1–PS5; Table 1).

Table 1 Quantitative changes in the carotenoid content of *Sphingobacterium antarcticus* grown at 5 °C and 25 °C. Pigments (PS1–PS5) were separated by HPLC on a Zorbax ODS column and the peak areas corresponding to each pigment were obtained directly from the HPLC integrator. The retention times of PS1–PS5 resolved by HPLC on a C-18 reversed-phase column were 12.40, 14.40, 23.33, 25.10 and 35.04 min, respectively. The pigments PS1–PS5 of *Sphingobacterium multivorum* separated with retention times identical to the five pigments of *S. antarcticus*. Data were obtained from three independent experiments and are reported as mean±standard deviation (*SD*). The areas of the *cis/trans* isomers of a particular pigment were added together for quantitation

Pigment	Retention time (min)	Relative quantity (%)	
		5 °C	25 °C
PS1 Unidentified	3.76	6.12±2.92	3.69±1.75
PS2 Zeaxanthin	4.38	79.23±3.63	49.28±2.45
PS3 β-Cryptoxanthin	8.86	12.84±1.88	30.19±0.82
PS4 Unidentified	10.08	0.61±0.22	1.78±0.53
PS5 β-Carotene	24.00	1.71±0.20	15.04±2.80

Temperature-dependent biosynthesis of the pigments of *S. antarcticus*

The total carotenoid content of *S. antarcticus* grown at 5 °C and 25 °C was 0.02% and 0.03% (w/w), respectively, of the wet mass of the cell pellet. Furthermore, in *S. antarcticus*, the amount of pigments PS1 and PS2 was higher in cells grown at 5 °C than in cells grown at 25 °C, whereas the amount of PS3, PS4 and PS5 was lower in cells grown at 5 °C than in cells grown at 25 °C (Table 1). However, at both temperatures, PS2 was the most predominant pigment. Concentration-normalised HPLC chromatograms (60 pg of the crude pigment) revealed that pigment PS2 was more abundant in *S. antarcticus* than in the mesophile *S. multivorum* (data not shown).

Identification of the pigments of *S. antarcticus* and *S . multivorum*

Both PS2 from *S.antarcticus* and authentic zeaxanthin eluted at 14.4 min and at 4.18 min after HPLC-1 or HPLC-2, respectively. TLC also confirmed that PS2 was a single pigment and that PS2 comigrated with synthetic zeaxanthin with an R_f of 0.44 when the solvent system consisted of chloroform and acetone (9:1, v/v), and with an R_f of 0.52 when dichloromethane and ethyl acetate (4:1, v/v) were used. Further, based on the UV-visible, IR, NMR, and mass spectra, PS2 was shown to be identical to pure authentic zeaxanthin (see Discussion).

PS3 eluted at 23.3 min using HPLC-1 and at 8.52 min using HPLC-2 and exhibited a fine-structure in its UV-visible absorption spectrum characteristic of carotenoids, with absorption maxima at 425, 446, and 477 nm, similar to zeaxanthin. However, PS3 differed from zeaxanthin since, when PS3 was acetylated, it produced a monoacetyl derivative and the mass spectrum exhibited a molecular ion [M^+] at 552 *m/z* and other peaks at 534 [M^+–18], 460 [M^+–92], and 446 *m/z* [M^+–106] characteristic of β-cryptoxanthin. These results are in good agreement with those published earlier on β-cryptoxanthin (Takaichi et al. 1990).

PS5 eluted at 35.01 min using HPLC-1 and exhibited in its UV-visible absorption spectrum fine-structures characteristic of carotenoids; the absorption maxima at 426, 446 and 476 nm were very similar to those of authentic β-carotene. Further, HPLC using two different solvent systems (HPLC-1 and HPLC-2) demonstrated that PS5 and synthetic β-carotene eluted with identical retention times.

The five pigments, PS1–PS5, of *S. multivorum* were identical to the corresponding pigments of *S. antarcticus* with respect to their retention times on the µ-Bondapak C-18 reversed-phase column and Zorbax ODS column; they also had identical absorption spectra. Furthermore, PS2 and PS5 co-eluted with zeaxanthin and β-carotene, respectively, as in *S. antarcticus*, and PS3 behaved like β-cryptoxanthin. PS1 (with absorption maxima at 426, 446, and 447 nm) and PS4 were present in very minute amounts and could not be identified.

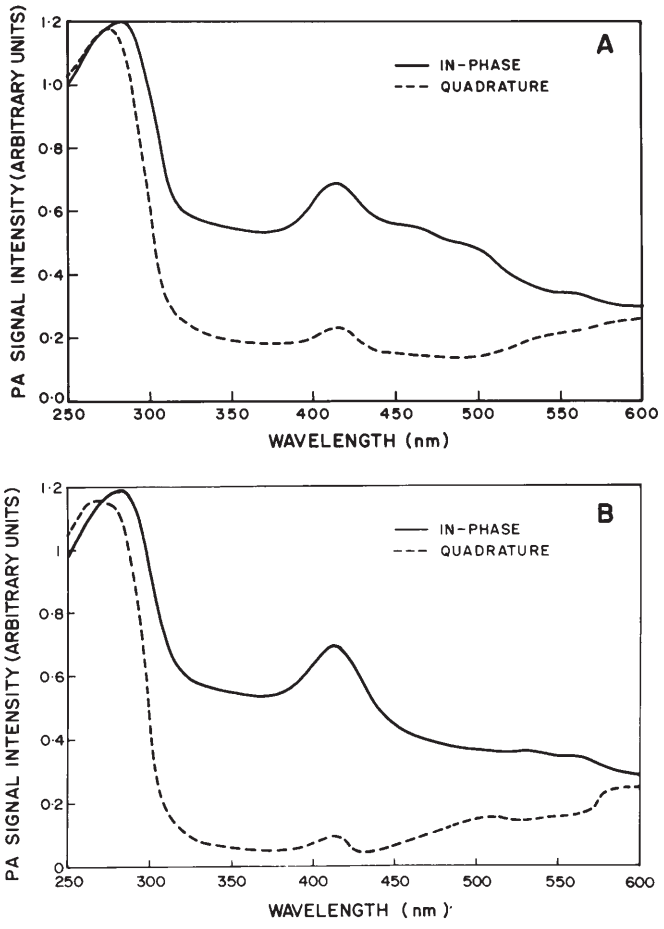

Fig. 1 Photoacoustic spectra of the surface (*straight line*) and inside (*broken line*) of psychrotolerant *Sphingobacterium antarcticus* (**A**) and mesophilic *Sphingobacterium multivorum* (**B**)

Phase-dependent photoacoustic spectroscopy of *S. antarcticus* and *S. multivorum*

The photoacoustic spectra of *S. antarcticus* and *S. multivorum* showed bands at 480 nm and 524 nm, characteristic of carotenoids, and a strong band around 415 nm, indicative of the presence of another chromophore. In both microorganisms, the signal from the pigment was clearly seen in the surface spectrum but was not prominent in the interior spectrum. A prominent absorption peak at 280 mm was also seen in the surface spectrum, but it was slightly blue-shifted compared to the interior spectrum (Fig. 1 A,B). The ratio of the pigment peak to the protein peak at 280 nm is taken to be indicative of the pigment content in each phase. The ratio in both the psychrotolerant and the mesophilic bacterium was high in the in-phase, but very low in the quadrature. These findings indicate that the pigment is associated with the membranes. This is further confirmed by the observation that isolated cell membranes were yellow in colour whereas the cytosol was colourless.

Interaction of zeaxanthin (PS2) and β-carotene (PS5) with PC vesicles

Using ANS as the fluorescent probe, the interaction of zeaxanthin and β-carotene with PC vesicles was monitored. Free ANS in buffer exhibited a fluorescence emission peak at 515 nm. Upon binding to PC vesicles, the fluorescence emission intensity increased substantially and the emission peak was blue-shifted from 515 nm to 485 nm. Addition of zeaxanthin or β-carotene to PC vesicles in the presence of ANS quenched the fluorescence inten-

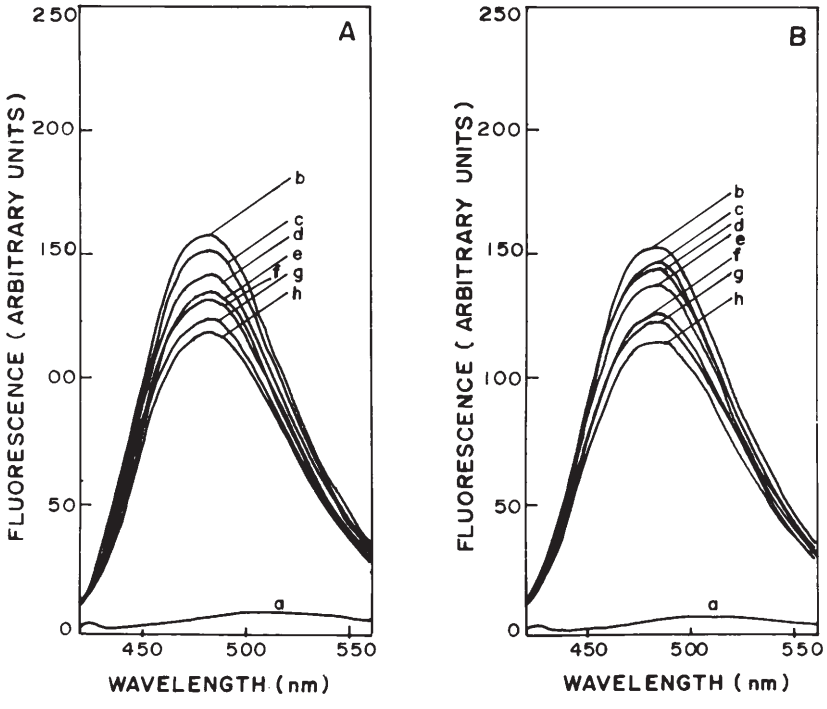

Fig. 2 Influence of zeaxanthin (**A**) and β-carotene (**B**) on the fluorescence emission spectra of 8-anilino-1-napthalene sulfonate (*ANS*) incorporated into phosphatidylcholine (*PC*) vesicles. In both **A** and **B**, 25 μM of ANS and 150 μM of PC vesicles were used. The excitation wavelength was 370 nm. The spectra in **A** and **B** are as follows: *a* 25 μM free ANS in buffer (5 mM HEPES, pH 7.4), *b* 25 μM ANS in buffer plus 150 μM of PC vesicles, *c–h* successive addition of 1 μg of zeaxanthin (**A**) or β-carotene (**B**) to 25 μM ANS plus 150 μM of PC vesicles

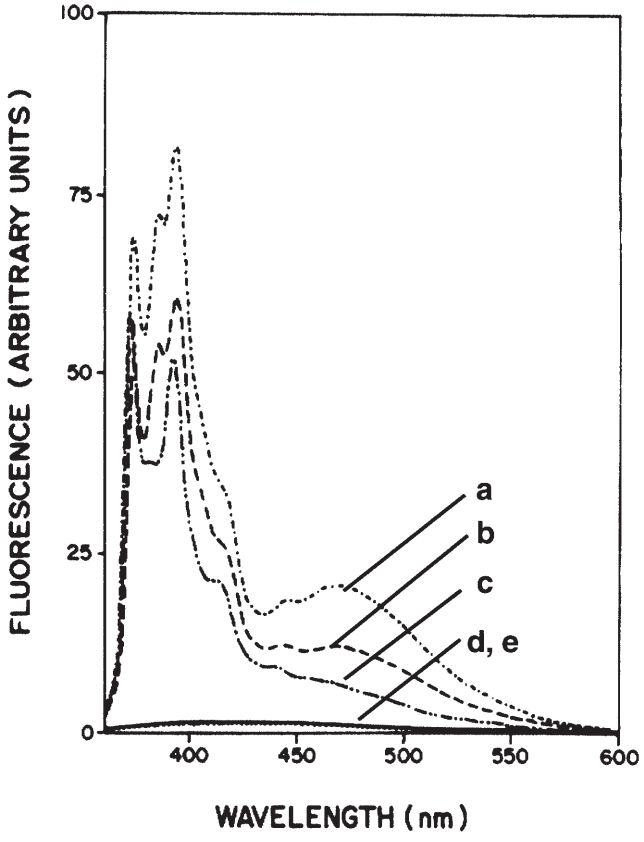

Fig. 3 The fluorescence emission spectra of 4 μM pyrene incorporated into the cells of *S. antarcticus* grown at 5 °C (*a*) or 25 °C (*b*), and spectra of cells without pyrene grown at 5 °C (*d*) and 25 °C (*e*). The spectrum of pyrene in 5 mM HEPES, pH 7.4, the buffer which was used for recording the above spectra, is shown in *c*

sity of ANS in a concentration-dependent manner, indicating that the pigments were bound to the vesicles (Fig. 2). The pigments, when added directly to 25 μM ANS in buffer (5 mM HEPES, pH 7.4), did not alter the fluorescence intensity of ANS. These results indicate that both zeaxanthin and β-carotene are bound to membranes (Fig. 2).

The interaction of zeaxanthin and β-carotene with synthetic membrane vesicles was also studied using the fluorescent probe pyrene, whose excimer peak could be conveniently monitored as a function of membrane fluidity (Vanderkooi and Callis 1974; Vijayasaradhy et al. 1982). This method is based on the principle that pyrene, dissolved in an aqueous solution and excited at 333 nm, gives two monomer peaks, one at 372 and the other at 392 nm. When the fluorophore pyrene is in a hydrophobic environment, its free movement is restricted, thus leading to the formation of a dimer peak at 470 nm. The ratio of the fluorescence intensity of the dimer peak (470 nm) to the monomer peak (392 nm) is a measure of the degree of fluidity of the membranes; an increase of the value indicates an increase in fluidity. In the present study, both zeaxanthin and β-carotene, at a concentration of 4 μg/ml, decreased the emission intensity ratio between the dimer and the monomer of pyrene in PC vesicles by about 30%, indicating a decrease in fluidity of the vesicles in the presence of carotenoids. The pigments on their own did not decrease the dimer-to-monomer ratio of pyrene (data not shown). The study also demonstrates that the membrane fluidity of *S. antarcticus* cells, as determined by the dimer-to-monomer intensity ratio of pyrene, was similar at 5 °C and 25 °C. The dimer-to-monomer intensity ratio of pyrene was 0.24 in the cells grown at 5 °C as compared to 0.21 in the cells grown at 25 °C (Fig. 3).

Table 2 Fatty acid composition (% of total) of psychrotrophic *S. antarcticus* grown at 5 °C and 25 °C. The *number before the colon* represents the number of carbon atoms in the chain, and the *number after the colon* represents the number of double bonds in the chain. The letters *i* and *a* indicate iso and anteiso branching, respectively. Data were obtained from three independent experiments and are expressed as mean±standard deviation

Fatty acid	Fatty acid content (%)	
	5 °C	25 °C
12:0	2.12±0.53	5.54±0.39
14:0	0.96±0.22	0.65±0.02
15:1	1.08±0.07	0.67±0.25
i15:0	10.11±0.48	7.43±0.59
a15:0	5.51±0.27	0.69±0.10
15:0	0.6 ±0.10	0.52±0.06
i16:1	1.27±0.65	0.47±0.15
16:1 Δ5[a]	44.57±0.61	32.06±0.22
16:1 Δ9[a]	5.48±0.31	0.83±0.03
16:0	8.05±0.52	17.65±0.3
i17:1	3.24±0.35	3.22±0.17
a17:1	7.96±0.52	2.86±0.17
i17:0	0.35±0.03	1.28±0.17
a17:0	1.39±0.19	–
17:0	–	1.93±0.12
i18:1	0.70±0.39	0.63±0.24
18:1	5.66±0.56	19.76±0.66
18:0	0.74±0.04	1.33±0.05
Unidentified	0.21	2.48

[a]Double bond positions were tentatively identified based on the chromatographic behaviour

Fatty acid analysis

The total cellular fatty acids of S. antarcticus grown at 5 °C and 25 °C were identified as $C_{16:0}$, $C_{16:1\Delta5}$, $C_{16:1\Delta9}$, $C_{17:1}$, $C_{18:1}$ and $iC_{15:0}$ (Table 2). However, in cells grown at 5 °C, the amount of unsaturated and branched chain fatty acids increased compared to the amount in cells grown at 25 °C.

Discussion

In vitro studies based on the interaction of carotenoids with model membranes have clearly demonstrated that polar carotenoids decrease and non-polar carotenoids increase the fluidity of membranes (Gabrielska and Gruszecki 1996). Therefore it is logical to assume that

carotenoids also modulate membrane fluidity in vivo. With this in view, the carotenoid pigments of psychrotolerant *S. antarcticus* and mesophilic *S. multivorum* were characterised in the present study and various aspects such as their in vivo location, biosynthesis and in vitro interaction with membranes were studied. The pigments were identified by two independent chromatographic methods, thus ensuring the purity and identity of the samples. Structural characterisation of PS2 as zeaxanthin was based on the following criteria: (1) it exhibited a fine-structure in its absorption spectrum and was prone to a solvent-induced bathochromic shift; (2) it had a polyene chain of 11 conjugated double bonds with cyclic end groups; (3) both end groups were hydroxylated; (4) it was a C_{40} carotenoid with a molecular mass of 568 Da; (5) its ^{1}H-NMR spectrum indicated that it had olefinic, allylic, and aliphatic hydrogen; and (6) it co-eluted with authentic zeaxanthin in different chromatographic systems. All the spectral features mentioned above are consistent with those from studies carried out earlier with authentic zeaxanthin (Englert et al. 1991). Pigments PS3 and PS5 were identified as β-crypoxanthin and β-carotene based on their chromatographic behaviour, absorption spectrum, and mass spectrum. Formation of a monoacetyl derivative after acetylation also confirmed the structure of PS3 as β-cryptoxanthin. Pigments PS1 and PS4 could not be identified since they were present in minute quantities.

Many in vitro studies have been directed towards localisation and orientation of carotenoids in the lipid bilayer (Jezowska et al. 1994; Johansson et al. 1981; N'Soukpoe-Kossi et al. 1988). However, in the present investigation, photoacoustic spectroscopy was used to demonstrate the location of carotenoids in vivo in the bacterial cell membrane. Furthermore, the strong absorption band at 415 nm in *S. antarcticus* was not due to carotenoids, since in our previous study (Chauhan and Shivaji 1994) it was observed that mutants of *S. antarcticus* incapable of producing carotenoid pigments still exhibited a band around 415 nm. Our earlier study on the localisation of carotenoid pigments in psychrotrophic and mesophilic *Micrococcus roseus*, using photoacoustic spectroscopy, also indicated that carotenoid pigments were associated with membranes (Jagannadham et al. 1996b).

In *S. antarcticus*, the amount of pigment synthesised increased with an increase in the growth temperature (Chauhan and Shivaji 1994). However, the previous study did not reveal whether the observed increase was due to increased synthesis of all the individual carotenoid pigments or only some of them. In the present study, it was observed that *S. antarcticus* grown at 5 °C synthesised a greater proportion (together ~85%) of the polar carotenoids PS1 and PS2 (zeaxanthin) than cells grown at 25 °C. This confirms an earlier study on psychrotrophic *M. roseus*, in which the relative amount of polar carotenoids was higher in cells of *S. antarcticus* grown at 5 °C than in cells grown at 25 °C (Chattopadhyay et al. 1997). Simultaneously, we observed an increase in the biosynthesis of unsaturated and branched chain fatty acids in cells of *S. antarcticus* grown at 5 °C compared to cells grown at 25 °C (Table 2). Taken together, these results suggest that in cells grown at 5 °C the unsaturated and branched chain fatty acids increase the fluidity of the membrane, whereas the polar carotenoids counterbalance this effect by stabilising the membrane (Jagannadham et al. 1991, 1996a). This indeed could be a mechanism by which cells maintain homeoviscous adaptation, viz. an optimum membrane fluidity. In fact, in the present study, the membrane fluidity of intact cells of *S. antarcticus* was similar in cells grown at 5 °C and 25 °C.

As yet, it is not known whether polar carotenoids are required for the survival of pigmented chemotrophic bacteria at low temperatures. However, since psychrotolerant bacteria synthesise higher amounts of polar carotenoids when grown at 5 °C compared to 25 °C, it is possible that these pigments modulate membrane fluidity depending on the growth temperature. Thus, by switching over the synthesis of carotenoids from one type (polar) to another type (non polar), a bacterium could alter the fluidity of its membrane and thus influence homeoviscous adaptation.

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