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Carotenoid Pigments of an Antarctic Psychrotrophic Bacterium *Micrococcus Roseus:* Temperature Dependent Biosynthesis, Structure, and Interaction with Synthetic Membranes

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Pigmentation in a psychrotrophic *M.roseus* was found to be increased when the bacteria were grown at 5°C as compared to its pigmentation at 25°C. In addition more polar pigments were synthesised at low temperature. The pigments were identified as bacteriorubberins and were demonstrated to bind to synthetic membranes of phosphatidylcholine with almost equal affinity, irrespective of the polarity of the pigments.

Adaptation of microorganisms to low temperatures is a hitherto unexplained phenomenon. Investigations performed in this laboratory on some psychrotrophic bacterial strains, isolated from Antarctic soil, have offered some clues [1,2]. Earlier studies on the taxonomy of the Antarctic bacteria revealed a predominance of the pigmented isolates belonging to the genera *Pseudomonas* [3], *Sphingobacterium* [4], *Micrococcus* an, *Planococcus* [5], *Arthrobacter* [6], *Janthinobacterium* [7], *Flectobacillus* [8], *Halomonas* [9], *Deinococcus* [10] and *Cornybacterium* [11]. Hence a role of the pigments in cold adaptation of the bacteria was implied. In a previous study the major pigment in an Antarctic psychrotroph, *Micrococcus roseus* (45R) was isolated, localised and identified to be a carotenoid by us and the interaction of this carotenoid pigment with synthetic and natural membranes was studied [12,13,14]. Investigations performed in other laboratories revealed that carotenoids influence membrane fluidity and that polar and non-polar carotenoids alter membrane fluidity in a differential manner [15]. Hence, the present investigation was undertaken to make an in depth study on the carotenoid pigments in a psychrotrophic *M.roseus* with respect to the temperature dependent biosynthetis of the pigments, their structure and their interaction with membranes. This report highlights for the first time that biosynthesis of carotenoid pigments with varying polarities is temperature dependent. It also demonstrates that the polarities of the pigment do not alter their binding affinities to synthetic membranes.

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MATERIALS AND METHODS

Chemicals and reagents. All chemicals used for bacterial cultures such as yeast extract, peptone etc. were obtained from Loba Chemie (Bombay, India). Methanol (HPLC grade) and Phosphorous oxychloride (analytical grade) were obtained from Spectrochem (Bombay, India). Acetic anhydride and pyridine were obtained from Emerck (Bombay, India) and $CDCl_3$ was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Bacterial strains and growth conditions. The bacterial strain under investigation (45R) was a psychrotrophic, red coloured, Gram-positive cocci isolated from Antarctic soil, identified earlier as *Micrococcus roseus* [5]. Both the mesophilic (NCTC 07523) and psychrotrophic strains of *Micrococcus roseus* (MTCC 678, IMTECH, Chandigarh, India) were grown in Antarctic bacterial medium (peptone, 0.5% w/v, yeast extract, 0.2% w/v). The mesophilic strain was grown at 30°C. Effect of temperature on carotenogenesis in *M.roseus* (45R) was observed by growing the cells at 5°C and 25°C on a rotary shaker. Methanolic extracts of pigments from cells grown at different temperatures, were used for spectroscopic studies and High Performance Liquid Chromatography (HPLC).

Purification and binding of pigments to membranes. The pigments of *M.roseus* were isolated and purified as described earlier [12]. Pigments P3, P4 to P5 were also prepared by preparative TLC using precoated Silica gel plates obtained from Emerck (Bombay, India) using acetone : heptane (1:1) as the solvent system. Binding of the pigments to membranes was studied using small unilamellar vesicles of phosphatidylcholine as a model system [12]. Binding constant (K_d) and number of binding sites (n) were calculated by Scatch-

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ard analysis of the data obtained from competitive binding studies involving 8-anilino-1-napthalene sulfonic acid [16].

Chemical methods. Acetylation of the pigments [17] and dehydration reactions were done according to described procedures [18].

Physical methods. UV-Vis absorption spectra of the pure pigments in various solvents were recorded using a Hitachi 150-20 spectrophotometer. The IR spectra were recorded by using KBr pellets in a Nicolet 740 FTIR spectrometer. The electron impact (EI) mass spectra of the pigments P3, P4 and P5 were recorded on a VG Autospec-M at 70 eV, with an ion source temperature of 250°C and with the probe set at 220-230°C. Liquid Secondary Ion (LSI) mass spectra of the pigments P2 and P3 were recorded by maintaining the ionisation source at room temperature on the same instrument. Cesium ions of 22 KV energy were used for bombardment. The carotenoid samples were mixed with 1 - 2 μl of matrix, 2-nitrobenzyl alcohol, on the target for each analysis. ^{1}H-Nuclear Magnetic Resonance spectra (^{1}H-NMR) were recorded on a Bruker AM300 Mz spectrometer using $CDCl_3$ with tetramethyl silane as an internal standard.

RESULTS

The yield of total carotenoid pigment was higher in the cells grown at 5°C than in the cells grown at 25°C (Figure 1). But, this data did not reveal whether the increase observed was due to increased synthesis of all the individual carotenoid pigments or only some of them. Further, it did not provide any clue to the nature of the pigments. Hence, attempts were made to purify the pigments, establish their structure and to quantify their levels in cells grown at 5 and 25°C.

Methanolic extracts of psychrotrophic *M.roseus* (45R), containing the total carotenoid pigments, following HPLC on a C-18 reverse phase column, separated into five distinct pigments (designated P1 to P5) with retention times of 9.66, 10.75, 12.19, 15.66 and 15.93 min respectively (Fig. 2A). Mesophilic *M.roseus* also contained at least five carotenoid pigments with retention times of 12.19, 13.14, 16.42, 20.61 and 32.67 min respectively (Fig. 2B). Thus it is obvious that pigments P1 and P2 which were more polar in nature were present only in psychrotrophic *M.roseus* and absent in mesophilic *M.roseus*. Further, it was also observed that the biosynthesis of these polar carotenoids (P1 and P2) was significantly higher in psychrotrophic *M.roseus* grown at 5°C compared to cells grown at 25°C (Table 1). In contrast, pigment P3 was quantitatively higher in cells grown at 25°C compared to cells grown at 5°C.

All the pigments (P1 to P5) of psychrotrophic *M.roseus* (45R) exhibited similar absorption spectra with a fine structure of three absorption peaks characteristic of carotenoids (Table 2) and also exhibited solvent induced bathochromic shift in the absorption maxima [12] another feature common to all carotenoids. Based on the UV-visible spectra, mass spectra, IR spectra, ^{1}H-NMR spectra, relevant chemical reactions and chromatographic behaviour, pigments P1 to P5 of psychrotrophic *M.roseus* were identified (Table 2 and Fig. 3). P3, the major pigment in psychrotrophic *M.roseus*, exhibited maxima at 466, 493 and 523 nm in methanol suggesting the presence of 13 conjugated double bonds. In addition, the appearance of a double *cis* peak (365

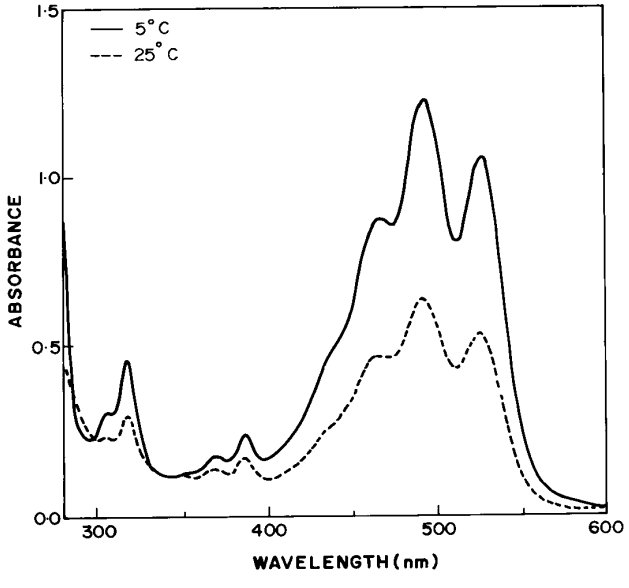

FIG. 1. UV-visible absorption spectra of the methanolic extract of psychrotrophic *Micrococcus roseus* (45R) grown at 5°C (-) and 25°C (---). Equal amounts of cells were extracted with equal volumes of methanol.

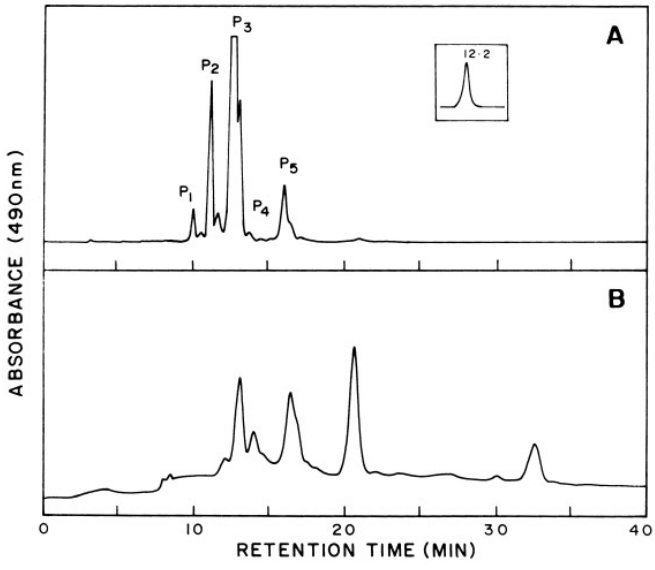

FIG. 2. HPLC profile of the methanolic extracts of psychrotrophic (A), and mesophilic (B) strains of *M.roseus* grown at 25°C and 30°C respectively, chromatographed on a C-18 reverse phase column. Pigments from the psychrotroph were labelled P1 to P5, based on increasing retention times (i.e., decreasing polarity). The *cis*-isomers (not labelled in the diagram) which eluted subsequent to the all-*trans* isomers are clearly visible in A. The inset indicates peak P3 which was rechromatographed.

TABLE 1

Temperature Dependent Variation in the Synthesis of Different Carotenoid Pigments in Psychrotrophic *M.roseus* (45R)

Growth temperature (°C)	% of total pigment[a]				
	P1	P2	P3	P4	P5[b]
5	5.74 ± 1.52	22.99 ± 3.27	62.34 ± 2.52	6.35 ± 0.16	~2.00
25	3.93 ± 0.63	14.5 ± 1.06	72.95 ± 0.71	7.76 ± 0.83	~2.00

[a] The relative amount of different pigments was calculated as the ratio of the area of individual pigment peak to the area of all the pigment peaks resolved by HPLC. The areas of the *trans*/*cis* isomers were added together. Each value represents the arithmetic mean ± standard deviation of three independent analyses.

[b] P5 was obtained in small amounts thus making data acquisition difficult.

and 385 nm) was characteristic of non-cyclic aliphatic chromophores. The infrared spectra of P3 showed bands at ~ 3436 cm^{-1} (broad) suggesting the presence of hydroxy groups, and at 2957 cm^{-1}, 2929 cm^{-1} and 2840 cm^{-1} respectively due to C-H stretching of methyl and methylene groups and a weak band at 1640 cm^{-1} due to C═C stretching and many other bands.

Pigment P3, however did not undergo acetylation. After dehydration with phosphorous oxychloride and subsequent HPLC analysis on a reverse phase HPLC column, four major peaks were obtained in addition to the peak of the unreacted P3. The absorption spectra of all the peaks were identical. Hence it was inferred that the hydroxy groups were tertiary in nature and no allylic hydroxy group was present. Moreover the prsence of terminal methylene groups in the deyhydration products was also confirmed.

The mass spectrum of P3 displayed a molecular ion [M^+] at m/z 740 corresponding to a molecular formula $C_{50}H_{76}O_4$ [$C_{50}H_{72}(OH)_4$] and exhibited peaks at [M-92] and [M-106] characteristic of carotenoids [19].

The ^{1}H-NMR spectrum of P3 exhibited the presence of a conjugated polyene system (δ 6.0 - 6.8, 18 H, δ 5.43, 2H) and methyl groups (δ 1.9, 6H, δ 1.96, 6H). The signal for methyl groups attached to the hydroxy groups (δ 1.23) corresponded to 8 methyl groups. Thus, based on the chemical reactions and spectroscopic data the major pigment P3 was identified as bacterioruberin (Fig. 3). In fact, bacterioruberin isolated from *H.salinarium* [20] and P3 co-eluted on a reverse phase column and exhibited identical absorption spectra (data not shown).

P4 exhibited similar absorption spectra as P3 but the mass spectra exhibited molecular ion at m/z 722. The other peaks at m/z 704, 686 and 668 indicated the presence of three hydroxy groups in the molecule. Based on the absorption spectra, mass spectral data and the relative polarity P4 was identified as monoanhydrobacterioruberin (Fig. 3).

P5 exhibited absorption maxima at 455, 480 and 513 nm suggesting the presence of 12 conjugated double bonds. Further, the mass spectra indicated a molecular ion [M^+] at m/z 738 with characteristic peaks corresponding to haloxanthin at m/z 720 (M-18), 702 (M-18-18), 646 (M-92), 638 (M-100, 632 (M-106) and 602 (M-18-18-100) [21]. Based on these characteristics P5 was

TABLE 2

Absorption[a] and Mass Spectral Characteristics of the Carotenoid Pigments of *M.roseus* (45R)

Pigment	Identification	Absorption spectra[b] (nm)	Mass spectra[c]
P1	Bacterioruberin diglycoside	465, 493, 525	—
P2	Bacterioruberin monoglycoside	466, 492, 525	902[d] (M^+)
P3	Bacterioruberin	466, 493, 523	740[d] (M^+), 682 (M-58), 664 (M-18-58) 646 (M-92), 634 (M-106), 616 (M-18-106), 582 (M-158), 562 (M-18-18-18-18-106)
P4	Monoanhydrobacterioruberin	465, 492, 524	722 (M^+), 704 (M-18), 686 (M-18-18), 668 (M-18-18-18), 664 (M-58), 646 (M-18-58), 630 (M-92), 616 (M-106)
P5	Haloxanthin	455, 480, 513	738 (M^+), 720 (M-18), 702 (M-18-18-18), 646 (M-92), 632 (M-106), 628 (M-100), 602 (M-18-18-100)

[a] Spectra were recorded in methanol on a Hitachi 150-20 UV-vis spectrophotometer. The absorption maxima given are accurate to within ± 2 nm.

[b] After exposure to light, samples develop a double *cis* peak at ~140 nm below the maximum of highest wavelength.

[c] Diagnostically important peaks in the upper mass region obtained in EI mass spectra of he pigments.

[d] Molecular ions were also confirmed by LSI-MS.

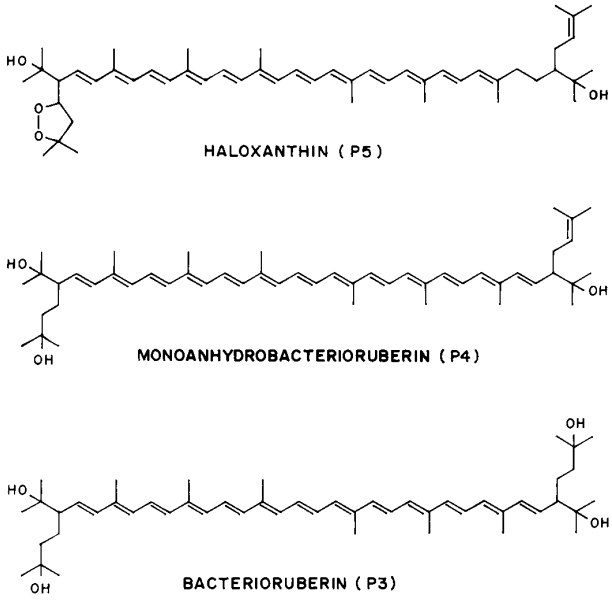

FIG. 3. Structures of the pigments P3, P4 and P5 of psychrotrophic *M.roseus*.

identified as haloxanthin (Table 2, Fig. 3). In the present study, P1 and P2 were the most polar pigments, had absorption spectra similar to bacterioruberins (Table 2) and were glycosidic (as indicated by IR spectral bands at 3345 cm^{-1}, 1069 cm^{-1} and 964 cm^{-1}). Further, acetylation studies indicated the presence of four accessible hydroxy groups in P2 (data not shown). Mass spectral studies of P2 displayed a molecular ion $[M^+]$ at m/z 902 corresponding to bacterioruberin monoglycoside. Based on similar reasoning bacterioruberin glycosides were identified earlier [22,23]. P1 was tentatively identified as bacterioruberin diglycoside based on its relative polarity and absorption spectra.

Since the biosynthesis of P1 and P2 increased in cells grown at 5°C compared to cells grown at 25°C, it was tempting to speculate that the binding of these pigments to membranes might also be temperature dependent. But, binding studies involving HPLC purified P1, P2 and P3 and small unilamellar vesicles made of phosphatidylcholine at 10°C and 25°C revealed that all of them bound to membranes and there was no significant variation in the values of dissociation constants (K_d) and number of binding sites (n). P4 and P5 were also capable of binding to membranes at the above temperatures (data not shown).

DISCUSSION

Regulation of membrane fluidity is a crucial strategy adopted by living cells to survive changes in environmental temperature. In bacteria, the fluidity of membranes is increased at low temperatures by an increase in the synthesis of unsaturated fatty acids. However, as yet a strategy which could decrease membrane fluidity or maintain an optimum rigidity of the membrane, is still unknown. It is widely believed that in prokaryotes carotenoids perform the role of cholesterol as a membrane spanning agent and thus confer some membrane rigidity [15,24]. Our earlier study established a membrane rigidifying role for the major carotenoid pigment of psychrotrophic *M.roseus* (45R) [12] but it did not provide any information with respect to the interaction of the other pigments with membranes. Thus, the present investigation is a continuation of the previous study in search of further clues for the effect of the other pigments of *M.roseus* (45R) on the bacterial membranes.

The synthesis of total carotenoid was significantly influenced by the temperature at which the organism was grown. The quantity of total pigment was substantially higher in cells grown at 5°C than cells grown at 25°C (Fig. 1). This corroborates with the earlier observation of low temperature induced carotenogenesis in *Staphylococcus* [25]. We had earlier demonstrated by

TABLE 3

Binding of ANS with Phosphatidylcholine Liposomes at Different Temperatures in the Presence or Absence of Carotenoid Pigments of Psychrotrophic *M. roseus* (45R)[a]

	10°C		25°C	
Pigment	n	K_d	n	K_d
No pigment	16.59 ± 2.23	1.28 ± 0.35	17.3 ± 2.49	1.27 ± 0.57
P1	13.0 ± 1.29	2.88 ± 1.34	12.52 ± 3.18	1.75 ± 0.42
P2	12.75 ± 1.19	1.72 ± 1.17	11.92 ± 2.32	1.70 ± 1.26
P3	14.19 ± 3.06	1.76 ± 0.86	14.99 ± 2.81	1.75 ± 0.26

[a] Fixed amount of liposome suspension (small unilamellar vesicles) in 5 mM HEPES-buffer (pH 7.4) was titrated in the presence or absence of pigments (P1, P2 and P3) with ANS at different temperatures. Number of binding sites (n) and dissociation constants (K_d) were calculated by modified Scatchard analysis. Each value is the arithmetic mean ± standard deviation of values obtained from three independent experiments.

HPLC (on a μ-bondapak C_{18} reverse phase column) that the total carotenoid pigment of psychrotrophic *M.roseus* (45R) consisted of five fractions (P1 to P5) eluting in a sequential manner starting with P1 [12,14]. Thus, in the light of the observation that more pigment was synthesised at 5°C compared to the yield at 25°C, it appeared worthwhile to know whether all or only some of the five pigments increased in quantity. Interestingly, the relative amount of P1 and P2 increased whereas that of P3 and P4 decreased (Table 1). Thus it was tempting to speculate that P1 and P2 were required more at low temperatures than at 25°C. Further, since P1 and P2 were the more polar fractions, a preferential increase in the synthesis of these polar pigments at low temperature was indicated. With this assumption, a preponderance of the less polar carotenoid pigments was expected in the mesophilic strain. The methanolic extracts of the psychrotrophic and mesophilic *M.roseus*, when analysed on a reverse phase HPLC column under identical conditions, corroborated our postulation. Comparison of the chromatograms clearly indicated that the content of polar carotenoids was higher in the psychrotroph when compared to the mesophilic *M.roseus*, in which all the pigments eluted with retention times greater than that of P1 and P2 (Fig. 2B). To our knowledge this is the first report of a temperature dependent variation in the production of carotenoid pigments with different polarities in any bacterium.

On the basis of chemical reactions (acetylation or dehydration) and spectral data (UV-visible, IR, ^{1}H-NMR and mass) the pigments of psychrotrophic *M.roseus* (45R) were identified as bacterioruberins (Table 2, Fig. 3) and were similar to that identified in halophilic bacteria [21]. Bacterioruberins were previously detected in *Halobacterium salinarium* and *Halobacterium cutirubrum* [18,27] and bacterioruberin monoglycoside and bacterioruberin diglycoside were isolated from a halophilic bacterium, which was an inhabitant of glacial mud [23].

Earlier it was reported that a polar carotenoid conferred more rigidity to lipid membranes compared to a non-polar carotenoid [24,26] but the study did not reveal whether this observed differential effect of the carotenoids on the membranes was due to differences on binding affinity of the pigments to membranes. The present study demonstrates that both the more polar (P1 and P2) and the less polar pigments (P3) bound to the membranes with almost equal affinity (Table 3) thus implying that variation in the binding affinity of the pigments to membranes was probably not the reason for the differential effect of the polar and nonpolar carotenoids on the rigidity of membranes. The pigments may be useful to the bacteria in reinforcing the membrane as shown earlier in reconstituted lipid membrane of *Halobacterium* [28]. Thus the present study demonstrated temperature dependent variation in the synthesis of pigments of different polarities in a psychrotrophic bacterium and also showed that binding of these pigments with membranes is not dependent on the polarity of the pigment. Furhter studies on the ability of the individual pigments to modulate the fluidity of membranes may provide new clues to our understanding of the mechanism of cold adaptation.

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