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Carotenes produced by alkaliphilic orange- pigmented strain of *Microbacterium arborescens* - AGSB isolated from coastal sand dunes

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Collections of gram positive bacteria from coastal sand dune vegetation, *Ipomoea pes-caprae* showed a predominance of orange pigmented colonies of *Microbacterium arborescens*-AGSB. The pigment was identified using a combination of UV/visible spectral data and HPLC retention time as a lycopene type carotenoid pigment with λmax at 468 nms. These bacteria may be accumulating carotenoids as part of their responses to various environmental stresses and thus aiding their survival in this stressed habitat.

[**Key words**: *Microbacterium arborescens*, sand dunes, carotenoids, bacterium, HPLC, TLC]

Introduction

Carotenoid pigments are present in all of the photosynthetic organisms as well as in some bacteria, fungi, and yeast. A more general role applicable to both photosynthetic and non-photosynthetic cell is protection from photodynamic action. At least 700 carotenoids have been characterized from the two known carotenoid biosynthetic pathways. Most widely distributed is the C_{40} pathway, which is shared by thousands of plant and microbial species[1]. Carotenoids are relatively hydrophobic molecules typically associated with membranes and/or noncovalently bound to specific proteins. In addition, the occurrence of carotenoids in archaebacteria, i.e. in *Halococcus* and *Halobacteria* has been reported[1]. Among non-photosynthetic bacteria carotenoids and their glycosides can be found in cytoplasmic and cell wall membranes which might influence membrane fluidity[2]. From a commercial point of view, there is an increasing demand of special carotenoids as food colorants, as precursors of vitamin A and as animal feed[3]. The demand and market for carotenoids is anticipated to change drastically with the discovery that carotenoids exhibit significant anti-carcinogenic activities[4]. In bacterial systematics, carotenoids serve as important chemotaxonomic markers in genera such as *Micrococcus* and *Flavobacterium*[5].

Gram positive bacteria collected from coastal sand dune vegetation showed a predominance of pigmented isolates. However the nature of these compounds is largely unknown, there have been reports on the analysis of carotenoids from psychrotrophic bacterium *Micrococcus roseus*[6] and from *Staphylococcus aureus*[7] and the analysis of carotenoids from the family *Microbacteriaceae*[8,9]. These pigments form an integral part of the complex membrane structure of a range of mesophilic and thermophilic micro-organisms and influence membrane fluidity, by increasing its rigidity and mechanical strength[2]. It has been suggested that the presence of carotenoids may change the effectiveness of the membrane as a barrier to water, oxygen, and other molecules[10]. The pigment may be aiding the bacteria to survive in this stressed habitat. In this study we report the pigment profile of *Microbacterium arborescens*- AGSB as a carotenoid.

Materials and Methods

The alkaliphilic strain of *Microbacterium arborescens*-AGSB was isolated from the rhizosphere of sand dune creeper, *Ipomoea pes-caprae*, and chosen because of its ability to produce strongly pigmented orange colonies. The organism was grown in polypeptone yeast extract glucose broth (PPYG) (peptone, 5g/l; yeast extract 1.5g/l; disodium hydrogen phosphate, 1.5g/l; sodium chloride, 1.5g/l; magnesium chloride, 0.1 g/l; glucose, 10%; sodium carbonate, 10%, *p*H 10.5). Solid medium was prepared by incorporating 1.5% agar in the medium. Cells for pigment studies were normally grown in,

polypeptone yeast extract glucose broth (*p*H 10.5). The medium occupying one-fifth of the flask volume and flasks were inoculated from an 18 hr broth culture and incubated on a rotary shaker at 160 rpm at 28°C for 48 hrs (conditions which ensure good aeration).

Cells were harvested by centrifugation (8,000 × g, 10 min) and the pellet extracted with acetone by sonication using 0.5 pulses for 3 sec interval for 10 mins. The extraction was repeated if necessary until all pigment had been extracted. Extraction was carried out in the dark to avoid light isomerization. Separation of carotenoids was achieved by HPLC on a C-18 reverse phase column (Waters Spherisorb ODS2 5 ìm, 4.6 mm × 250 mm) column, using acetonitrile: water (solvent A) and ethyl acetate (solvent B) at a flow rate of 1 ml/min. Peaks were monitored with a HPLC-WATERS equiped with waters 2996 Phase diode array detector. The gradient for separation was 0-100% ethyl acetate in acetonitrile/water (9:1) over 25 min with flow rate of 1.2 ml/min.

UV/visible scanning spectra of the acetone extracts containing carotenoids were recorded between 200 and 800 nms on a UV-visible spectrophotometer. Pigments were also purified by thin layer chromatography (TLC) using silica gel plates developed in petroleum ether and ethyl acetate (90:10 v/v). Different solvent systems were used for separation of carotenoids. Petroleum: benzene (98:2 v/v) for separation of carotenoid hydrocarbons, Benzene for separation of carotenoid ketones and benzene: methanol (49:1) for separation of carotenoid hydroxylated[11].

The *Microbacterium arborescens*-AGSB culture was grown in broth and analyzed for its photosynthetic activity using a chlorophyll fluorometer (PAM 101, Walz, Effelrich, Germany). Light intensity was supplied and peak production was observed.

Results

Biochemical characterization of the bacterial isolate

The isolated strain contained gram-positive, non-sporing, rod-shaped cells. The colonies on PPYG showed orange pigmentation (Fig. 1). The biochemical results suggest that the isolated strain belonged to the genus *Microbacterium* (Table 1). As further studies on the strain were needed to identify the isolate, the 16S rRNA gene was sequenced after being amplified by PCR . Sequence analysis using

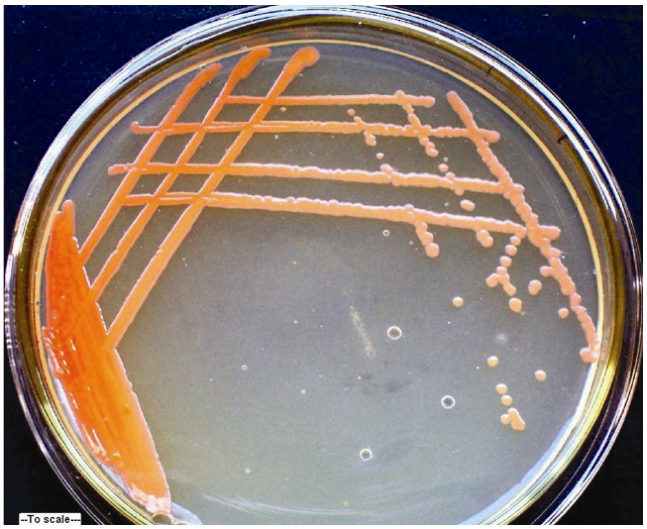

Fig. 1—*Microbacterium arborescens* growing on PPYG at pH 10.5

Table 1—Differential characteristics of *Microbacterium arborescens*

Colour of the colony	orange
Motility	+
Hydrolysis	
Gelatin	+
Starch	-
H_2S production	+
VP test	-
Arginine dihydrolase	-
Assimilation	
Arabinose	+
N-acetyglucosamine	+
Malate	+
Citrate	+
Phenyl acetate	-
Fumarate	+
Propionate	-
Acid from	
Glucose	+
Cell wall diamino acid	Lysine
Major menaquinone acid	MK-11,12

data from the GenBank revealed that the isolate unambiguously belongs to the genus *Microbacterium* being the closest fit (100% similarity). The isolate was proposed as *Microbacterium arborescens*, and the accession number for its 16S rRNA gene in the GenBank Nucleotide Sequence Database is DQ287961. The isolate showed 100% 16S rDNA similarity to *Microbacterium arborescens* (DSM 20754) followed by *Microbacterium* sp. IMCC1739 (DQ664254), *Microbacterium* sp. MAS133 (AJ251194), *Microbacterium schleiferi*

(Y17237), *Microbacterium hominis* (AM181504), *Microbacterium* sp. Atl-19 (EF028128), *Microbacterium* sp. KV488 (AB234027), *Microbacterium lacticum* (AB007415), *Microbacterium hominis* (AB004727), *Microbacterium* sp. KV-483 (AB234026) and *Microbacterium lacticum* (DSM20427) (X77441) (Table 2).

Extraction and analysis of carotenoids

The HPLC profile of *Microbacterium arborescens*-AGSB pigment can be ascertained from Figs 2 and 3. All major peaks in Fig. 2 could be tentatively identified on the basis of their absorption spectra. The principal pigment of *Microbacterium arborescens*-AGSB was found to be lycopene (Peak 2).

UV-visible absorption spectra of the pigment

UV-visible absorption spectra of carotenoid pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids[12]. Table 3 gives the absorption maxima of the pigment in various solvents. The pigment was completely extractable in acetone and partially extractable in other solvents. The UV-visible absorption spectra of the pigment showed absorption

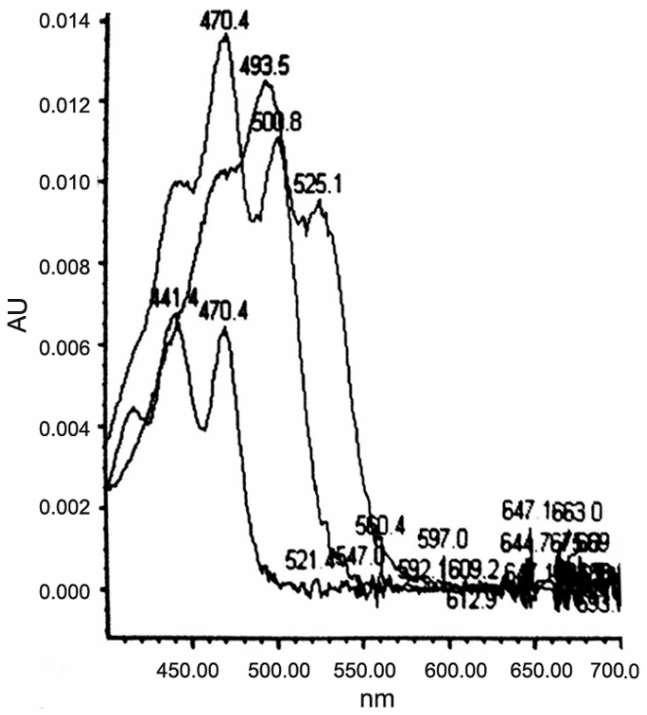

Fig. 2—HPLC chromatogram showing carotenoid fractions separated from the crude pigment extract of *Microbacterium arborescens*-AGSB. Separation achieved using a C-18 Reverse Phase column

Table 2—Percentage 16S rRNA sequence similarities between *Microbacterium arborescens* and some closely related *Microbacterium* species

Strain	Similarity %
Microbacterium arborescens (DSM 20754) (X77443)	100
Microbacterium imperiale (DSM 20530) (X77442)	99
Microbacterium arborescens (AB007421)	99
Microbacterium arborescens SE14 (AY649756)	99
Microbacterium arborescens kr10 (AY238940)	99
Microbacterium imperiale (AB007414)	98
Microbacterium ginsengisoli (AB271048)	98
Microbacterium sp. oral clone AV005b (AF385527)	97
Microbacterium sp. MSCB-7 (EF103204)	97
Microbacterium sp. CNJ743 PL04 (DQ448707)	96
Microbacterium sp. IMCC1739 (DQ664254)	100
Microbacterium sp. MAS133 (AJ251194)	100
Microbacterium schleiferi (Y17237)	100
Microbacterium arborescens (D21339)	99
Microbacterium hominis (AM181504)	100
Microbacterium sp. Atl-19 (EF028128)	100
Microbacterium sp. Ellin174 (AF409016)	96
Microbacterium sp. KV-488 (AB234027)	100
Microbacterium lacticum isolate H193 (EF204396)	98
Microbacterium lacticum isolate D84 (EF204392)	98
Microbacterium lacticum (AB007415)	100
Microbacterium hominis (AB004727)	100
Microbacterium sp. KV-483 (AB234026)	100
Microbacterium sp. 62NP10 (AB242734)	98
Microbacterium lacticum (DSM20427) (X77441)	100

maxima at 533 nm, 468 nm and 341 nm in acetone (Fig. 4). The clear three-band shape of the absorption spectrum of the pigment is characteristic of carotenoids and further reflects its purity. Comparison of the absorption maxima of Peak 2 with the absorption data available for various other carotenoids suggested the presence of 11 conjugated double bonds in Peak 2.

Thin layer chromatography of the pigment

The plates developed in petroleum ether and ethyl acetate showed the separation of the pigment into four spots with different migration rates: yellow spot (Rf 0.15 cms),pink spot (0.20 cms), light yellow spot (0.47 cms) and dark yellow spot (0.77 cms). Carotenoid hydrocarbons showed two spots Rf 0.6cms and Rf 0.86 cms while carotenoid ketones showed 3 spots, intense red (Rf 0.05 cms), pink (Rf 0.31 cms) and peach (Rf 0.75 cms). Carotenoid hydroxylated showed 3 spots, pink (Rf 0.52 cms), yellow (Rf 0.67) and yellow (Rf 0.77 cms).

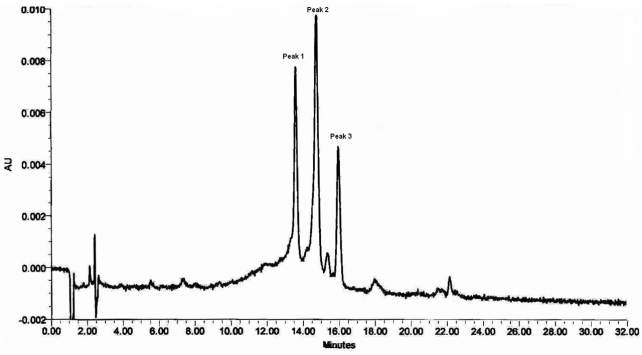

Fig. 3—HPLC profile of the pigment of *Microbacterium arborescens*- AGSB

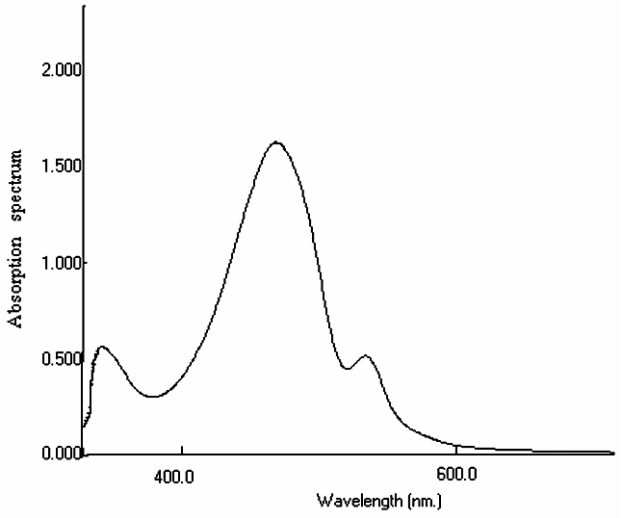

Fig. 4—Absorption spectrum of pigment from alkaliphilic *Microbacterium arborescens* - AGSB in acetone

Photosynthetic activity of the culture

When a light intensity of 4 units was supplied to the *Microbacterium arborescens*-AGSB culture no peak of excitation was observed but on increasing the light intensity to 8 units a peak was observed. The F_0 (initial fluorescence) was calculated as 7 units from the graph suggesting photosynthetic activity of the pigment.

Discussion

The orange colour of *Microbacterium arborescens*-AGSB is due to the presence of C_{40} carotenoids. *Microbacterium* species produce yellow, orange, light yellow and whitish yellow pigments. *Microbacterium aurantiacum, M.chocolatum, M.imperiale* and *M.testaceum* produce orange pigments[13]. Trutko *et al.*[8] reported that pigmented *Microbacterium* strains viz. yellow, orange, and red pigments were found to have absorption spectra typical of C_{40} carotenoids.These compounds were identified using a combination of UV/Visible spectral data and HPLC retention times. Polar organic solvents such as acetone and methanol have been extensively used for the extraction of carotenoids from bacteria[12]. Carotenoids absorb maximally at three wavelengths (533 nm, 468 nm, 341 nm) resulting in three peak spectra which is characteristic of carotenoid pigments. The polyene chromophores of carotenoids, which absorb light in the 400 to 550 nm range, provide the basis for their characteristic yellow-to-red colors and their ability to quench singlet oxygen[14]. Carotenes are readily soluble in petroleum ether, hexane, and toluene; xanthophylls dissolve better in methanol and ethanol[15]. In the present investigation, two extractions with acetone liberated all the pigment from the alkaliphilic *M. arborescens* strain. HPLC was used in the past for the purification of carotenoids from bacteria by employing normal-phase chromatography. However, since normal-phase chromatography is more variable than reverse-phase chromatography, the letter method has now become more popular and has been used in conjunction with a photodiode array detector. Reverse-phase chromatography was used in the present investigation to purify the major carotenoid pigment from a alkaliphilic *Microbacterium arborescens*-AGSB strain from

coastal sand dunes. The carotenoids resolved into distinct pigments, Peak 1 to Peak 3 and the absorption spectra of the pigments were simultaneously recorded with the help of the on-line photodiode array detector. The greater the number of conjugated double bonds, the higher the λmax values. Thus, the most unsaturated acyclic carotenoid lycopene with 11 conjugated double bonds is red and absorbs at the longest wavelengths (λmax at 444 nm, 470 nm and 502 nm). At least 7 conjugated double bonds are needed for a carotenoid to have perceptible color. Thus, β - carotene is light yellow. Being also acyclic, its spectrum has three well-defined peaks, but these are at wavelengths much lower than those of lycopene (λmax at 378 nm, 400 nm, and 425 nm), commensurate with its conjugated system of 7 double bonds. Bicyclic β - carotene, although possessing the same number of conjugated double bonds as lycopene, is yellow orange and has λmax at 450 nm and 477 nm and a mere inflection (shoulder) at 425 nm. Monocyclic β-carotene is red orange and exhibits a spectrum intermediate between those of lycopene and β-carotene in λmax and shape, reflecting a structure that is intermediate between the other two carotenoids. The double bond in the ring of β-carotene is out of conjugation, leaving 10 conjugated double bonds (9 in the polyene chain and 1 in the β ring); thus, this carotenoid is light yellow and its absorption spectrum is more defined with λmax at slightly shorter wavelengths (422 nm, 445 nm, and 473 nm) than those of β-carotene. The λmax values relative to hexane and petroleum ether are practically the same in diethyl ether, methanol, ethanol, and acetonitrile and higher by 2-6 nm in acetone, 10-20 nm in chloroform, 10-20 nm in dichloromethane and 18-24 nm in toluene[10,16].

Umeno *et al*.[1] determined carotenoid pigmentation levels in the culture extracts from the height of absorption maxima (λmax): 470 nm for C^{30} carotenoids and 475 nm for C^{40} carotenoids. Among bacteria, *Micrococci, Mycobacteria,* and *Flexibacters* contain carotenoids with conjugated keto functions on a beta-ionone ring. Canthaxanthin with two such groups was isolated from *Corynebacterium michagenense* and is the principal pigment of *M. roseus*[17]. C_{30} carotenoids are present in the nonphotosynthetic bacteria, such as *Streptococcus faecium*, *Staphylococcus aureus*, and *Methylobacterium rhodinum* and in the photosynthetic *Heliobacterium* species[18]. The thin layer chromatographic analysis of the pigments revealed the presence hydroxylated, hydrocarbon and ketones compounds in the carotenoid pigment.

Interestingly, *Microbacterium arborescens*-AGSB was found to show photosynthetic activity. Scientists at the Oregon State University have studied a bacteria known as SAR11 that can switch to using light much the way plants do to stay alive[19]. The biosynthesis of carotenoids in some representatives of the genera *Agromyces, Leifsonia*, and *Microbacterium* is induced by light[8].

From the spectral scan and the HPLC profile, we can conclude that *Microbacterium arborescens* produces a lycopene type of carotenoid pigment with λmax at 468 nm.

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