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In Vivo Characteristics and Localisation of Carotenoid Pigments in Psychrotrophic and Mesophilic *Micrococcus roseus* Using Photoacoustic Spectroscopy

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Photoacoustic spectra of cells of *M.roseus* were recorded to study the *in vivo* characteristics and localisation of the carotenoid pigments in these cells. The PA spectra indicated that both the psychrotrophic and mesophilic strains had similar chromophores. The *cis* carotenoids were prominent in the psychrotrophic *M.roseus* whereas shorter polyenes were more prominent in mesophilic *M.roseus.* Further, depth profiling photoacoustic studies revealed that in both the strains of *M.roseus* the bulk of the chromophore was associated with the cell membrane.

Microbial biodiversity studies of soil samples from Antarctica carried out by us had clearly demonstrated that a good proportion of psychrotrophic bacteria were pigmented (1-5). *In vitro* studies demonstrated that the pigments were of the carotenoid type and could bind to membranes and stabilise them (6-8). Earlier studies have indicated that carotenoids are present in a wide variety of bacteria and act as chemotaxonomic markers and also influence functions related to light harvesting, photoprotection against UV and membrane fluidity (9-13). However, data related to the *in vivo* characteristics and localisation of the pigment were lacking. Such *in vivo* studies are important especially because carotenoids are very unstable and are known to change isomerically during the extraction process. Thus, in order to avoid such artefacts in the present investigation, for the first time, photoacoustic spectroscopy (PAS) was used to ascertain the characteristics and localisation of carotenoid pigments *in vivo* in a psychrotrophic and a mesophilic strain of the bacterium *M.roseus.* PAS is a simple and convenient technique which has been effectively used to obtain absorption spectra of optically opaque and light scattering biological samples as varied as skin, malarial parasite, algal cells, lobster-shell etc. (14-17). The PAS results demonstrated that in both the strains the pigment chromophore was similar, the pigments existed both as its *cis* and *trans* isomers and the pigments were associated with the membrane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The psychrotrophic *M.roseus* (MTCC 678; IMTECH, Chandigarh, India) (1) was maintained at 20°C in a medium containing peptone (0.5%), yeast extract (0.2%) and soil extract (0.5%) with continuous shaking for six days. The mesophilic *M.roseus* (NCTC 07523; National Chemical Laboratory, Pune, India) was cultured at 37°C in nutrient broth.

Photoacoustic spectra. The photoacoustic (PA) spectral measurements were made on an edt OAS-400 PA spectrometer to which several modifications were incorporated. Light beam from a 300 Watt Xenon arc lamp was intensity modulated using a mechanical chopper (HMS 222), whose frequency could be varied between 10 Hz to several kHz depending on the chopper blades used. The PA spectra were recorded at 40 Hz. The light beam was then monochromated and passed on to the sample cell to which a Bruel & Kjaer microphone was attached. Microphone signal was sent to a microcomputer (IBM PC) through a preamplifier (EG&G 113, USA) and a lock-in analyzer (EG&G 5206, USA)

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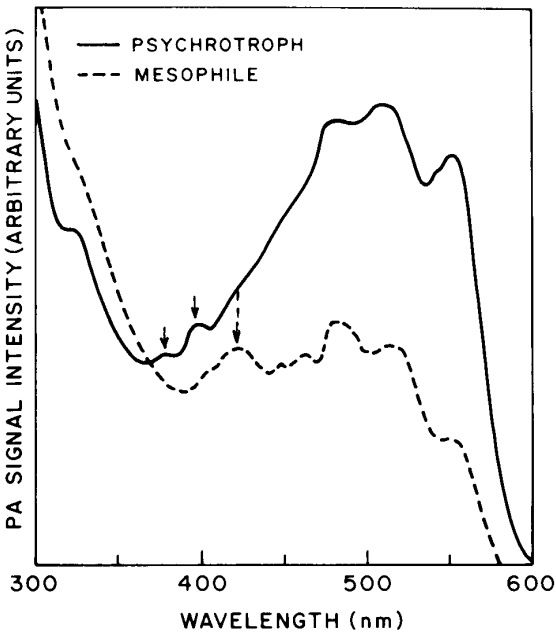

FIG. 1. Photoacoustic spectra of lyophilised cell pellets of psychrotrophic (—) and mesophilic (---) *M.roseus.* The solid arrows indicate absorption at 375 and 398 nm due to the *cis* peak, and the discontinous arrow indicates the absorption peak at 418 nm indicative of shorter polymers.

over a GPIB interface through an A/D converter. The computer controls the spectrometer, processes the data and plots the spectrum. Normalisation of the PA spectra to constant input light intensity was achieved by using the PA spectrum of carbon black. The depth profiling of the bacterial species was carried out by recording the PA spectra of signals with different phase delays in the front surface illumination mode, as described earlier (14,17,18).

Pigment purification and UV-visible spectra. The total carotenoid pigment of *M.roseus* was extracted with methanol and the crude pigment was further purified on a μ-Bondapak C-18 using a Hewlett Packard HPLC (6). The pigments were eluted from the μ-Bondapak C-18 column using solvents A (80% aqueous methanol) and B (methanol). The solvent combination from 0 to 5 minutes was 0% B to 70% B after which it was gradually increased in a linear gradient to 100% B in 40 minutes. The flow rate of the solvent mixture was 1 ml/min. UV-visible spectra of the pigments was recorded using an online diodearray detector.

RESULTS

Photoacoustic Spectra of M.roseus

The normalized PA spectra of the lyophilised powder of psychrotrophic and mesophilic *M.roseus* are shown in Fig. 1. Both the spectra showed similar features with peaks at 478, 510 and 548 nm. In addition, the psychrotrophic strain also showed two more bands at 375 and 398 nm and the mesophilic strain one more band at 418 nm. Freshly pelleted cells of *M.roseus* (psychrotrophic and mesophilic) spread on a filter paper also showed the same PA spectral characteristics.

Separation of cis and trans Carotenoids

The two additional peaks at 375 nm and 398 nm in the PA spectrum of psychrotrophic *M.roseus* suggest that the cells contain *cis* carotenoids *in vivo.* In fact, in psychrotrophic *M.roseus* the *trans* carotenoids (P1 to P5) could be separated from the *cis* isomers with P1 to P5 eluting before the corresponding *cis* isomers (Fig. 2). The *cis* isomers showed absorption peaks at 365 and 385 nm in addition to peaks at 460, 489 and 524 nm (see inset to Fig. 2).

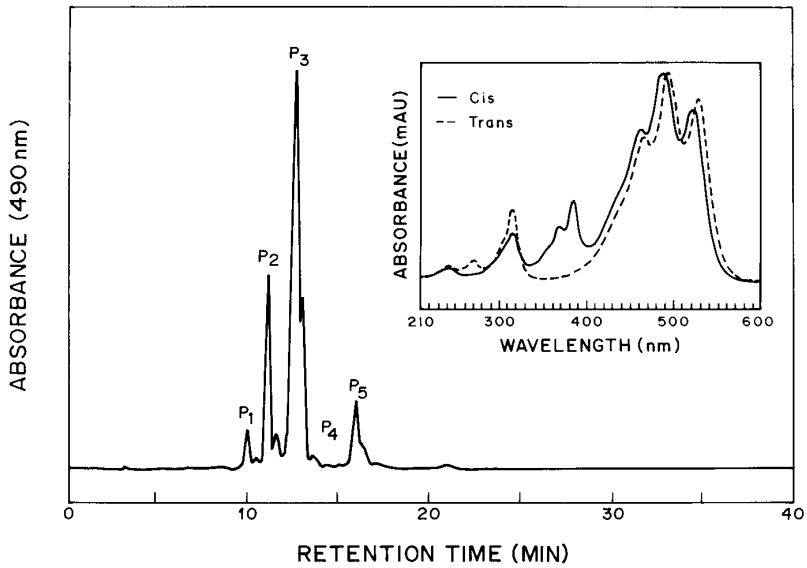

FIG. 2. HPLC profile of the crude pigment of psychrotrophic *M.roseus* chromatographed on a C-18 reverse phase column. P1 to P5 represent the five *trans* isomers of the pigment and the respective *cis* isomers eluted immediately after the *trans* isomers. The inset shows the UV–visible absorption spectra of the *cis* (—) and *trans* (---) isomers of P3 obtained using an online diode array detector.

Phase Dependent PA Spectral Studies on M.roseus

The in-phase (0° phase) and the quadrature (90° phase) PA spectral profiles which are intensity normalized for the 280 nm peak for the psychrotrophic and mesophilic strains of *M.roseus* are shown in Fig. 3. The in-phase (surface) component is first selected by maximising the signal through adjusting the phase there by achieving the maximum discrimination against the signal from the cell interior. The quadrature (interior) spectrum is obtained by giving an additional 90° phase to the above maximised phase (14,17). In both the microorganisms the signal from the pigment was clearly seen in the surface (in-phase) spectrum while it was not very prominent in the interior (quadrature or 90°) spectrum. A 280 nm absorption peak was also prominently seen in the surface spectrum which was, however, blue shifted by about 10 nm in the interior spectrum. The ratio of the 280 nm peak to the pigment peak is taken to be indicative of the presence and extent of pigment in each phase with low values of protein to pigment ratio indicating higher concentration of the pigment. Thus, it is obvious that this ratio in both the psychrotroph and mesophile was extremely low in the in-phase (<2) but the ratio was very high in the quadrature (>6) indicating that the pigment was associated with the membrane region.

DISCUSSION

The PA spectra of the lyophilised cell pellets of psychrotrophic and mesophilic *M.roseus* unambiguously demonstrate that the pigments contained similar chromophores with multiple absorption peaks at 478, 510 and 548 nm characteristic of carotenoids. In fact, it had earlier been demonstrated that the pigments in *M.roseus* were carotenoids (6,19). Further, the two additional peaks at 375 and 398 nm in psychrotrophic *M.roseus* suggests the *in vivo* presence of *cis* carotenoids (9). The all *trans* isomers of carotenoids could be resolved by HPLC from the *cis*-isomers which showed additional bands at 365 and 385 nm characteristic of *cis*-isomers (9). Cis-isomers of carotenoids have earlier been detected in bacteria

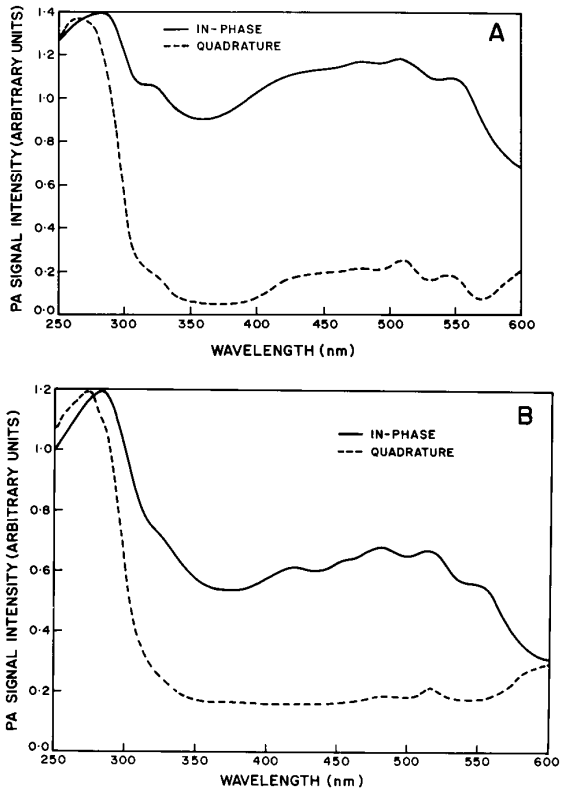

FIG. 3. The photoacoustic depth profile spectra of psychrotrophic (A) and mesophilic (B) *M.roseus* in phase (—) and quadrature (---).

such as *M.roseus,* Brevibacterium sp., *Erwinia herbicola* and *Erythrobacter longus* (6,20-22). Though the *cis* peak appears to be characteristic of psychrotrophic *M.roseus,* an absorption peak at 418 nm indicative of the presence of shorter polyenes (23-24) was distinctly obvious in the mesophilic *M.roseus.* Though, in general, carotenoids have been implicated in various physiological functions nothing is known so far whether these functions are influenced by both or only a particular isomeric form of a carotenoid. Our unpublished data indicate that in psychrotrophic *M.roseus,* the synthesis of both *cis* and *trans* carotenoids increases when the cells are grown at low temperature (5°C) compared to cells grown at 25°C. Since these carotenoids are associated with the membrane it would be logical to assume that *cis* carotenoids would further increase the fluidity of the membrane, an event which normally occurs due to increased synthesis of unsaturated fatty acids in cells grown at low temperature. Carotenoid *cis* isomers have also been detected in human tissues but their biological relevance is still unknown though an inverse relationship was demonstrated between serum carotenoid levels and incidence of cancer (25,26).

In bacterial cells carotenoids are generally associated with the cell membrane. Thus, this system could be well approximated to a two layer model consisting of the surface (which would include the pigments and the proteins) and the rest (which would include proteins and other cell constituents). In the present investigation PAS was used to ascertain the localisation of carotenoid pigments *in vivo* in bacteria. The required information by PAS could be acquired by two different methods. For instance by varying the chopping frequency it is possible to record the depth profiles of specimens provided of course the specimen is large and thermal

diffusivity is accurately known. Thus, this approach is not convenient to bacterial cells which are very small and very high chopping frequencies would be required. Further, the accurate determination of thermal diffusivity of bacterial cells is extremely difficult. Due to these difficulties intrinsic to the specimen being analysed in the present study phase resolved monitoring of PA signals was carried out and the in-phase (surface) and quadrature (interior) spectra were recorded. This, later approach has been effectively used for biological samples (27-29).

From the PA spectra (Figs. 3) it is apparent that the pigments were distributed anisotropically, with most of the pigments being distributed in the cell membrane as evidenced from the fact that the surface component (0° phase) of the PA signal showed all the characteristic absorption peaks of the pigments and a peak at 280 nm due to the proteins, while the quadrature (90° phase) component showed only one major band at around 270 nm which is blue shifted by about 10 nm with respect to the inphase protein absorption peak at 280 nm. This shift may be due to the composite absorption between proteins, nucleic acids and other cellular components. These results clearly show that the bulk of the absorbing chromophores is distributed in the cell membrane and the pigments are present at almost the same depth in both the mesophilic and psychrotrophic species. This *in vivo* localisation of carotenoids in the cell membrane of *M.roseus* confirms earlier *in vitro* studies by Asenzi and Cooney (30) and our own observations (1).

Thus the present study demonstrates that PAS could be used to acquire data related to spectral characteristic of pigments and their localisation in bacterial cells without subcellular fractionation of cells or solvent extraction of cells, From the PA spectra of the cells characteristics such as the presence of the *cis* isomers of carotenoids and shorter polyenes in carotenoids could also be easily discerned. The data also demonstrated that carotenoid pigments are associated with the membrane in the bacterial cell and the localisation of the pigments both in the mesophilic and psychrotrophic strain of the same species was identical.

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