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# Raman spectroscopic study of the photoprotection of extremophilic microbes against ultraviolet radiation

H.G.M. Edwards[1], R. Moeller[2,3], S.E. Jorge Villar[1,4]*, G. Horneck[2] and E. Stackebrandt[3]

[1]*Chemical and Forensic Sciences, School of Life Sciences, University of Bradford, Bradford BD7 1DP, UK*
*e-mail: h.g.m.edwards@bradford.ac.uk*
[2]*German Aerospace Center, Institute of Aerospace Medicine, Radiation Biology Division, Cologne, Germany*
[3]*German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany*
[4]*Area Geodinamica Interna, Facultad de Humanides y Educacion, Universidad de Burgos, Calle Villadiego s/n, 09001 Burgos, Spain*
*e-mail: susanajorgevillar@hotmail.com, seju@ubu.es*

**Abstract**: Extremophiles use a range of pigments for protection against low-wavelength radiation in exposed terrestrial habitats and photoaccessory materials are synthesized for the effective harnessing of photosynthetically active radiation. Raman spectroscopy has been demonstrated to be a useful probe for information on the survival strategies employed by extremophilic bacteria through the identification of key biomolecular signatures of the suite of protective chemicals synthesized by the organisms in stressed environments. Raman spectroscopic analyses of *Bacillus* spp. spores, *Bacillus atrophaeus* (DSM 675: deep red) and *Bacillus subtilis* (DSM 5611: light grey and DSM 7264: dark grey), *Deinococcus radiodurans* (pink) and *Natronomonas pharaonis* (red), of visually different pigmentation showed the presence of different carotenoids and other protectant biomolecules, which assist microorganisms against UVA radiation. The implications for the survival of extremophilic microbes in extraterrestrial habitats and for the detection of the protectant biomolecules by remote, robotic Raman spectroscopic instrumentation in an astrobiological search for life context are discussed.

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**Key words**: astrobiology, *Bacillus atrophaeus*, *Bacillus subtiis*, *Deinococcus radiodurans*, *Natronomonas pharaonis*, Raman spectroscopy, carotenoids, endospores.

## Introduction

The study of the survival strategies of extremophilic organisms that have colonized terrestrial niche environments which can be otherwise deemed to be unsupportable of life is receiving much interest in the search for habitable sites in our Solar System and beyond (Horneck 1995; Brack *et al.* 1999; Horneck & Baumstarck-Khan 2000; Wynn-Williams and Edwards 2000a,b; Carcichidi 2002). The announcement by NASA and the European Space Agency (ESA) that novel analytical instrumentation is now being considered for deployment in a life-detection role on Martian planetary landers and rovers prior to the human exploration of Mars (Aurora: ExoMars Project 2001) gives further impetus for the consideration of the survivability of biological material that might be expected on the planet, in specimens from sample return missions and also with regards to planetary protection for spacecraft. The key issues in the successful occupation of terrestrial geological niches by extremophilic organisms and bacteria are recognized to be the synthesis of protective biochemicals and their adaptation to the environment for local survival (Cockell and Knowland 1999; Cockell 2000; Wynn-Williams and Edwards 2000b). In this regard, the identification of a range of terrestrial sites and habitats has been essential for the understanding of colonization strategies used by extremophilic organisms in 'limits of life' scenarios.

Of the major stresses that are known to affect the ability of extremophilic organisms to survive harsh environments, those of radiation insolation, desiccation, temperature and barometric and hydrological pressure are recognized contributors and, of these, the exposure of organisms and microbes to high-energy radiation has been identified as a highly significant parameter affecting their survival strategies (Cockell and Knowland 1999; Horneck 2000; Wynn-Williams and Edwards 2000b). The most successful terrestrial extremophiles produce a suite of radiation-protective biochemicals and accessory pigments to harness photosynthetically active radiation whilst at the same time protecting the colony against harmful electromagnetic radiation of higher energy in the ultraviolet (UV) region (Cockell 1999;

* Corresponding author.

Wynn-Williams and Edwards 2000a). The dualistic role (Cockell and Knowland 1999) of carotenoids as pigments for protection against low-wavelength radiation and as antioxidants in the cell DNA-repair process is an example of such a survival strategy adopted by extremophiles subjected to radiation stress.

Raman spectroscopy has been demonstrated to be a viable analytical technique for the examination of the key protective biochemicals and biogeologically modified host matrices produced by extremophiles in a variety of terrestrial locations (Newton and Edwards 1999; Edwards 2004; Jorge Villar and Edwards 2006). It is significant also that Raman spectroscopy is being adopted as a novel analytical technique by the ESA in its ExoMars/AURORA programme for inclusion in missions to Mars as part of its life-detection instrumentation suites. Hence, it is critically important that the Raman spectroscopic information obtained from a diverse range of extremophilic scenarios can be evaluated in terms of the recognition of the protective biomolecular spectral signatures that occur in the geological record as evidence of extinct or extant life.

Raman spectroscopy, alone and in combination with chemometric techniques, has been shown to be specific for the taxonomic classification of microorganisms (Naumann *et al.* 1991, 1995; Naumann 2001). Maquelin *et al.* (2000) used a confocal Raman microscope for the identification of microbes in the micrometre range. Often, however, the fluorescence associated with bacterial colonies generated using visible radiation can be problematic. Recently, Rosch *et al.* (2003) applied confocal Raman microscopy to the identification of microorganisms using an excitation wavelength of 785 nm. They found that spectral differences were noted between colourless colonies of *Bacillus subtilis* and *Bacillus sphaericus*, that no carotenoid bands could be observed and the spectrum from the *Bacillus subtilis* colonies gave bands at 1660 (amide 1), 1445 ($CH_2$ scissors) and 1002 $cm^{-1}$ (ring breathing) modes from protein. In our present study, Raman spectroscopy at excitation wavelengths of 1064, 785, 514 and 488 nm were used to detect the carotene pigment *in situ* in the viable endospores.

In this paper we consider for the first time the analytical detection capabilities of Raman spectroscopic techniques for the identification of the photoprotective pigments that are produced by extremophilic microbes under radiation stress. We have selected *Bacillus* spp. endospores (Moeller *et al.* 2005) vegetative cells of *Deinococcus radiodurans* (Pogoda de la Vega *et al.* 2005) and the halophilic archaea *N. pharaonis* (Quint *et al.* 2002) as an example of terrestrially radiation-resistant bacteria for this study. The response behaviour of these extremophile bacteria assessed by Raman spectroscopy will inform the database of terrestrial extremophile activity.

Endospores of *Bacillus* spp., *D. radiodurans and Natronomonas pharaonis* are highly resistant to a variety of environmental stresses, such as toxic chemical agents, desiccation, high and low pressure, temperature extremes and high doses of ionizing or UV radiation (Nicholson *et al.* 2000a). They are ubiquitous, inhabit soils and rocks (Nicholson and Law 1999) and are easily disseminated by wind and water. Their high resistance to environmental extremes also makes these spores ideal model systems for testing their responses to extraterrestrial conditions, such as outer space (Horneck 1993) or simulated planetary conditions (Nicholson *et al.* 2002b). Among fungal and bacterial spores collected at high altitudes up to 77 km, pigmented forms dominated (Imshenetsky *et al.* 1978); it seems that endogenous pigments, such as carotenoids and melanins, might provide a selective advantage to these microorganisms by screening environmental UV radiation, which, at these high altitudes, comprises the full extraterrestrial spectrum, including the UVC and UVB ranges. In various microorganisms, endogenous production of pigments has been shown to protect against oxidative damage caused by UV or ionizing radiation by scavenging free radicals (Sadasivan and Neyra 1987; Shivaprasad and Page 1989).

Our major focus in this study is the characterization by Raman spectroscopy of the pigments in the intact spores and cells of *Bacillus* spp., *D. radiodurans* and *N. pharaonis*, and understanding the role of endogenous pigments in their relevance to UV radiation.

## Experimental

### *Microorganism growth conditions*

The specimens were obtained from the German Collection of Microorganisms and Cells (DSMZ), Braunschweig, Germany: *N. pharaonis* (DSM 3395) and *Bacillus atrophaeus* (DSM 675) producing red pigmented spores, *B. atrophaeus*$^T$ (DSM 7264), formerly known as *Bacillus subtilis* var. *niger*, producing dark-grey spores and *B. subtillis* (DSM 5611) producing light-grey spores. Spores were harvested from cultures in a sporulation medium after 4–5 days of incubation at 37 °C, when a sporulation rate of over 90% was reached. Free spores were purified by centrifugation (10 000 × ***g***, 20 min at 4 °C) and treatment with $MgSO_4$ (2.5 μg $ml^{-1}$), lysozyme (200 μg $ml^{-1}$) and DNAse (2 μg $ml^{-1}$) for 30 min at 37 °C in order to destroy the residual vegetative cells. The enzymes were inactivated by heating for 10 min at 80 °C. After repeated centrifugation and washing in distilled water, the purified spores (approx. $10^{10}$ spores $ml^{-1}$) were stored in aqueous suspension at 4 °C. *D. radiodurans* were obtained from the stock culture of the Department of Pathology at the Uniformed Services University of Health Sciences (Bethesda, MD). *D. radiodurans* cells were grown aerobically in TGY broth (0.5% Bacto Tryptone, 0.3% Bacto Yeast extract; both Bacto products were purchased from BD Becton, Dickinson and Co, Difco Laboratories, Sparks, MD) as well as 0.1% D(+)–Glucose–Monohydrate (Merck KGaA, Darmstadt, Germany) and shaken at their temperature optimum of 30 °C for up to 48 h. For colony counts (*Bacillus* spp. and *D. radiodurans*), the medium was solidified by adding 1.5% agar. *N. pharaonis* were grown in a halophil-alkaline medium (HAM – 1% Tryptone, 1.5% $Na_2CO_3$, 1.5% Casamino acid, 0.5% yeast, 0.25% KCl, 0.6% EDTA-Tris, 20.0% NaCl; pH 9.5). Vegetative cells for the irradiation

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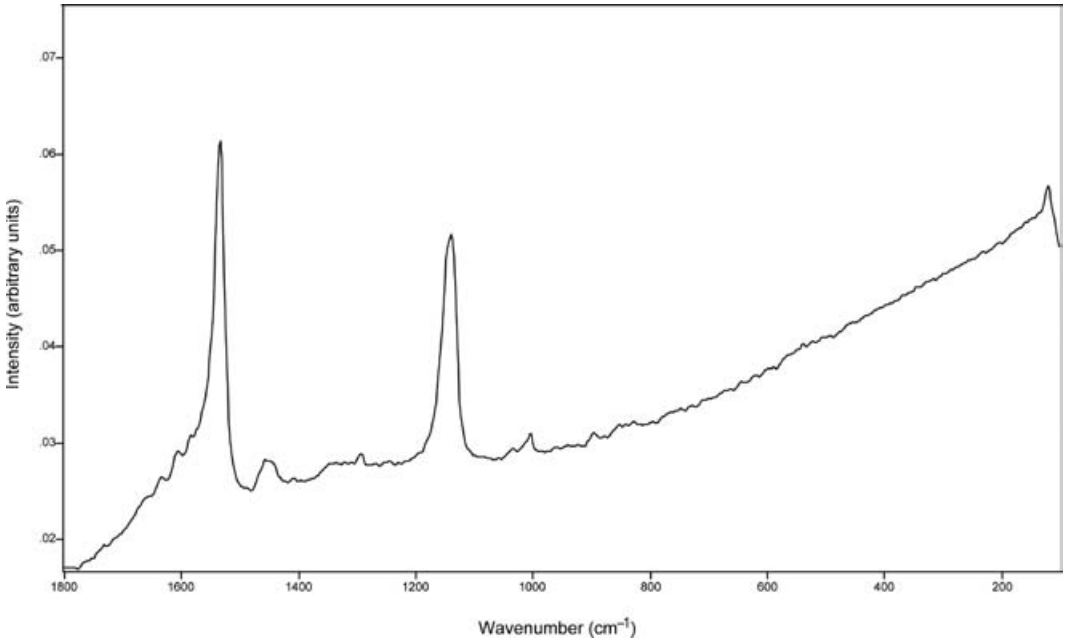


**Fig. 1.** Raman spectrum collected on *B. atrophaeus* showing bands of neoxanthin.

were taken from the late exponential and alternatively early stationary growth phase and washed twice with strain-specific buffered saline (*D. radiodurans* – 0.7% $Na_2HPO_4 \times 2H_2O$, 0.3% $KH_2PO_4$, 0.4% NaCl, pH 7.5) and halophil-alkaline buffered saline (*N. pharaonis* – 0.25% KCl, 0.6% EDTA-Tris, 20.0% NaCl, pH 9.5) and stored at 4 °C. For the *in situ* Raman spectroscopy, 30 µl aqueous bacteria suspension ($5 \times 10^7$ spores or cells) were plated as a monolayer on 7 mm diameter quartz discs (Horneck *et al.* 1993) and placed in a desiccator for 4 days. The experiments were carried out in triplicate, i.e. three independent trials were performed.

### *Raman spectroscopy*

The Raman spectra of several microrganism strains have been analysed. For the pigmentation studies, spectra were recorded using a Bruker IFS 66 Fourier-transform Raman spectrometer (Bruker IR Analytische GmbH, Karlsruhe, Germany) with an FRA 106 Raman module attachment and $Nd^{3+}$/YAG laser excitation at 1064 nm in the macroscopic mode of analysis wi th a 100 µm footprint. Using a laser power of about 10–20 mW, 4000 spectral scans were accumulated with a 4 $cm^{-1}$ spectral resolution over a period of about 2 h. A Renishaw *In Via* confocal Raman microscope (Renishaw plc, Wootton-under-Edge, UK) was used with 785 (near infrared), 514 (green) and 488 nm (blue) laser excitations to obtain spectra using ×20 and ×50 microscope lenses; the specimen footprint was approximately 5 and 2 µm, respectively. An exposure time of 10 s was used and the laser power range va ried from 0.5 to 25 mW depending on the excitation wavelength used. The scan accumulations ranged from between 30 and 50 over approximately 20 min accumulation time.

## Results

The Raman spectrum of *B. atrophaeus* (DSM 675) spores with a dark red colouration showed strong bands at 1534 and 1139 $cm^{-1}$ and weaker bands at 1636, 1605, 1456, 1294, 1004 and 895 $cm^{-1}$ characteristic of neoxanthin (Fig. 1), a carotene with 10 C═C bonds derived from violaxanthin (violaxanthin has 10 C═C bonds). The bands at 1534 and 1139 $cm^{-1}$ are characteristic of a carotenoid with seven or eight C═C bonds in conjugation (Veronelli *et al.* 1995) and, clearly, the carotenoid is not beta-carotene, which has a different Raman biosignature with bands at 1516, 1156 and 1007 $cm^{-1}$, and the carotenoid (C—C) spectral band here is very similar to the spectral pattern given for demethylated beta-carotene as reported by Okamoto *et al.* (1984). The C═C bond position at 1534 $cm^{-1}$ is best described as exhibiting a crocetin-type structure and matches closely that of neoxanthin. Other weaker spectral features are assignable to aromatic ring modes and proteins. An older strain of *B. atrophaeus* (DSM 675: light red) gave similar results, and the carotene neoxanthin was also identified by its Raman spectrum. There were no results for a positive carotene detection from *B. atrophaeus*$^T$ (DSM 7264) with dark-grey coloured spores. This may be ascribed to the sample being a thin deposit. No carotenoid bands appear in any spectra of *B. subtilis* (DSM 5611), but in the Fourier-transform Raman spectrum bands at 1575, 1399, 1010 and 823 $cm^{-1}$ appear along with weaker bands at 1656, 1610, 1449, 1339, 1034 and 1004 $cm^{-1}$ (Fig. 2) and they are all attributed to calcium dipicolinate. Calcium dipicolinate is recognized as a spore specific biomineral complex with a DNA-protective function. A broad unresolved band centred at 2935 $cm^{-1}$ is characteristic of aliphatic CH groups. In the Raman spectrum obtained using 785 nm excitation, bands from calcium

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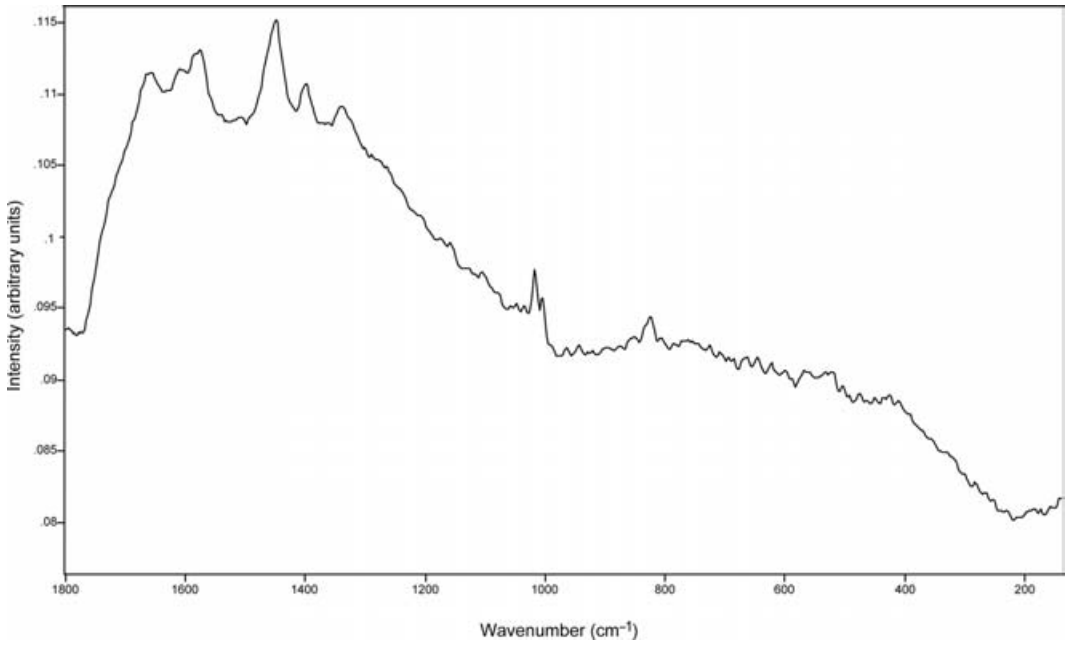


**Fig. 2.** Calcium dipicolinate Raman spectrum achieved on *B. subtilis*.

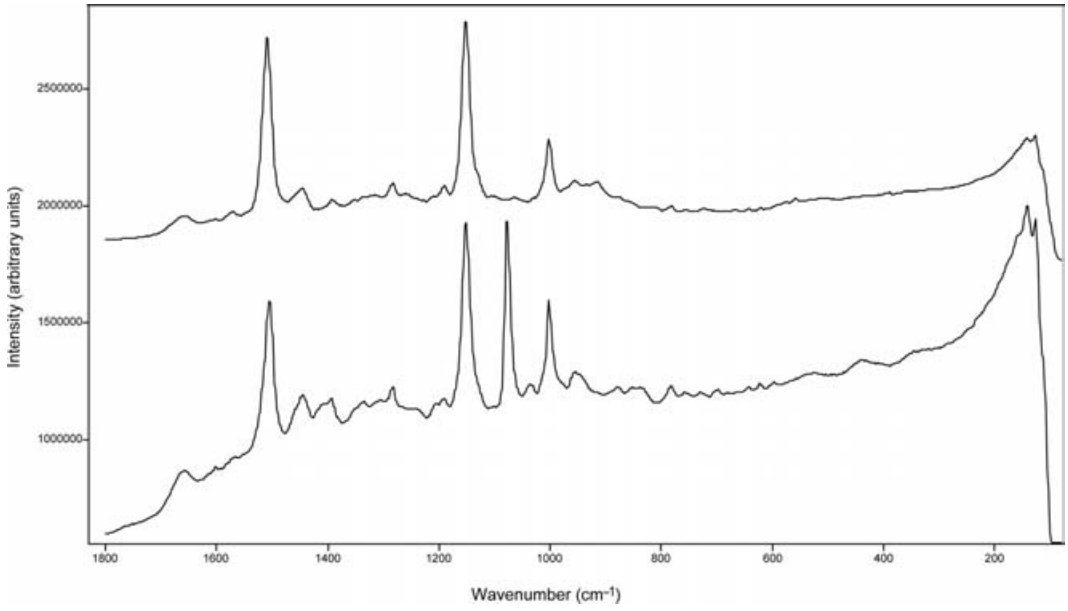


**Fig. 3.** A comparison of spectra collected on *D. radiodurans* (top) and *N. pharaonis* (bottom).

dipicolinate at 1445, 1396, 1335, 1016, 1002 and 823 $cm^{-1}$ are also visible.

*D. radiodurans*, the most resistant organisms against radiation, give a Raman spectrum with bands at 1656, 1572, 1509, 1447, 1392, 1284, 1190, 1151, 1001, 956, 933 and 915 $cm^{-1}$ (Fig. 3). The significative carotene band at 1509 $cm^{-1}$ shows shoulders at 1515, 1520 and 1505 $cm^{-1}$ (Fig. 4); we could assign this spectrum to a mixture of beta-carotene or canthaxanthin, zeaxanthin and decapreno-beta-carotene, respectively. Although the carotene astaxanthin has the strongest bands at the same position (1510 and 1151 $cm^{-1}$), the medium intensity band, here at 1001 $cm^{-1}$ (1005 $cm^{-1}$ in astaxanthin), together with the weakest bands lead us to dismiss the presence of astaxanthin in *D. radiodurans*.

The *N. pharaonis* spectrum shows bands at 1658, 1505, 1446, 1394, 1284, 1192, 1151, 1001, 956 and 935 $cm^{-1}$ (Fig. 3), all of which are assigned to decapreno-beta-carotene, but the spectrum also shows weaker shoulders at 1520, 1515 and 1495 $cm^{-1}$ (Fig. 5) in the band at 1505 $cm^{-1}$ which could be related to zeaxanthin, beta-carotene or canthaxanthin and dodecaprene-beta-carotene; the signature at 1077 $cm^{-1}$ has been assigned to sodium carbonate.

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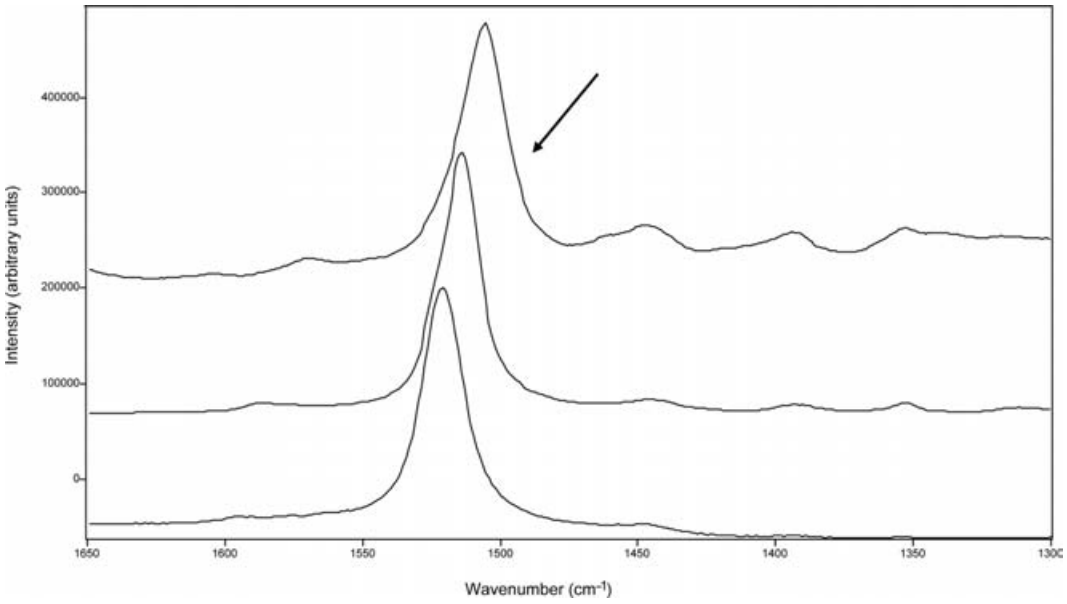


**Fig. 4.** Detail of the 1650–1300 $cm^{-1}$ wavenumber region of the strong Raman band at 1509 $cm^{-1}$ (shoulders at 1520, 1515 and 1505 $cm^{-1}$) on *D. radiodurans* (top) and comparison with neoxanthin (middle) and beta-carotene (bottom). The arrow shows the shoulder at 1005 $cm^{-1}$ assigned to decapreno-beta-carotene.

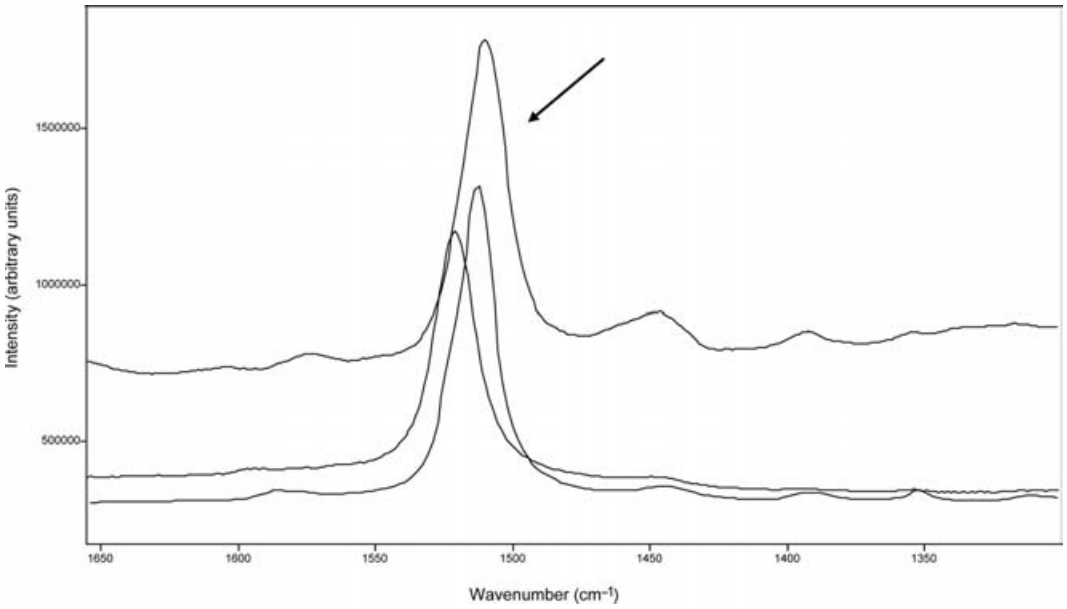


**Fig. 5.** Detail of the 1650–1300 $cm^{-1}$ wavenumber region of the strong Raman band at 1505 $cm^{-1}$ (shoulders at 1520, 1515 and 1495 $cm^{-1}$) on *D. radiodurans* (top) and comparison with neoxanthin (middle) and beta-carotene (bottom). The arrow shows the shoulder at 1005 $cm^{-1}$ assigned to decapreno-beta-carotene.

## Conclusions

Previous radiation biological studies with the selected microorganisms have indicated that these bacteria are ideal model systems for studying their photobiological response to terrestrial and extraterrestrial UV radiation (Moeller *et al.* 2005; Pogoda de la Vega *et al.* 2005; Quint *et al.* 2002). In the case of the pigmented spores of *Bacillus* spp., red pigmented endospores of *B. atrophaeus* (DSM 675) were 10 times more resistant to UVA radiation than those of the other two investigated strains, whereas the responses to the more energetic UV(A + B) and UVC radiation were identical in all different pigmented spores of *Bacillus* spp. (Moeller *et al.* 2005). In this study we have used Raman spectroscopy for the identification, without pigment extraction, of carotenoids and other organic compounds used as protective pigments by *B. atrophaeus* (DMS 675, 676 and 7264), *B. subtilis* (DMS 5611), *N. pharaonis* and *D. radiodurans*. Carotene is the most common pigment used by most of the microorganisms studied here; it is significant that some of the strains use more

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than one carotene. The antioxidant, UV screen and DNA repair functions of carotene appear to play an important role in the resistance of these microorganisms to environmental stress.

The presence of neoxanthin in the red and the dark red strains (DSM 675 and DMS 676, respectively) of *B. atrophaeus* appears to play an important role in the high resistance to UVA radiation demonstrated by these microorganisms (Moeller *et al*. 2005). No carotenes were detected in the two varieties with dark grey colouration (*B. atrophaeus* DSM 7264 and *B. subtilis* DSM 5611), only *B. subtilis* looks to use calcium dipicolinate as a UV radiation protective pigment; the red coloured *B. atrophaeus* (DSM 675) is more resistant to UV radiation than those with grey colouration.

The high resistance to UV radiation of *D. radiodurans* could be related to the presence of three different carotenes, zeaxanthin, beta-carotene/canthaxanthin and decapreno-beta-carotene, whereas in *N. pharaonis* decapreno-beta-carotene appears together with zeaxanthin, beta-carotene/canthaxanthin and dodecapreno-beta-carotene.

The data from this study demonstrate that Raman spectroscopy is a suitable analytical technique for the detection of the traces of life, even from non-treated and vital microorganisms, i.e. biosignatures, of essential biochemicals of life in a variety of terrestrial and extraterrestrial locations such as Mars.

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