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Research Paper

Carotenoid Analysis of Halophilic Archaea by Resonance Raman Spectroscopy

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ABSTRACT

Recently, halite and sulfate evaporate rocks have been discovered on Mars by the NASA rovers, *Spirit* and *Opportunity*. It is reasonable to propose that halophilic microorganisms could have potentially flourished in these settings. If so, biomolecules found in microorganisms adapted to high salinity and basic pH environments on Earth may be reliable biomarkers for detecting life on Mars. Therefore, we investigated the potential of Resonance Raman (RR) spectroscopy to detect biomarkers derived from microorganisms adapted to hypersaline environments. RR spectra were acquired using 488.0 and 514.5 nm excitation from a variety of halophilic archaea, including *Halobacterium salinarum* NRC-1, *Halococcus morrhuae*, and *Natrinema pallidum*. It was clearly demonstrated that RR spectra enhance the chromophore carotenoid molecules in the cell membrane with respect to the various protein and lipid cellular components. RR spectra acquired from all halophilic archaea investigated contained major features at approximately 1000, 1152, and 1505 cm^{-1}. The bands at 1505 cm^{-1} and 1152 cm^{-1} are due to in-phase C═C (ν_1) and C–C stretching (ν_2) vibrations of the polyene chain in carotenoids. Additionally, in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds occur in the 1000 cm^{-1} region. We also investigated the RR spectral differences between bacterioruberin and bacteriorhodopsin as another potential biomarker for hypersaline environments. By comparison, the RR spectrum acquired from bacteriorhodopsin is much more complex and contains modes that can be divided into four groups: the C═C stretches (1600–1500 cm^{-1}), the CCH in-plane rocks (1400–1250 cm^{-1}), the C–C stretches (1250–1100 cm^{-1}), and the hydrogen out-of-plane wags (1000–700 cm^{-1}). RR spectroscopy was shown to be a useful tool for the analysis and remote *in situ* detection of carotenoids from halophilic archaea without the need for large sample sizes and complicated extractions, which are required by analytical techniques such as high performance liquid chromatography and mass spectrometry. Key words: Biomarkers—Halophilic Archaea—Mars—Raman spectroscopy—Spectroscopic biosignatures. Astrobiology 7, 631–643.

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INTRODUCTION

Recently, halite and sulfate evaporate rocks have been discovered on Mars by the NASA rovers, *Spirit* and *Opportunity*. This suggests that brine pools may have been relatively common on the surface of that planet and, thus, may have provided regions of high salt concentration. Consequently, it is reasonable to propose that halophilic microorganisms could have potentially flourished under such hypersaline conditions (for example, Landis, 2001; Wierzchos *et al.*, 2006). Thus, modern terrestrial salt basin and cultured salt-tolerant microbes are good analogues for conditions under which life might have evolved on Mars (for example, Landis, 2001; Wierzchos *et al.*, 2006). If so, biomolecules found in microorganisms adapted to high salinity and basic pH environments on Earth may also be reliable biomarkers for detecting life on Mars.

Halophilic archaea are chemo-organotrophs that belong to the class Euryarchaeota. These microbes are often the predominant microorganism present in salt lakes, pools of evaporating seawater, solar salterns, and other hypersaline environments with salt concentrations as high as halite saturation (Oren, 2002). Halophiles also live in a variety of warm and cold environments. Extremely halophilic archaea have been noted for their bright red or purple color. The pigments responsible for these colors consist of isoprenoid-derived or retinal-protein compounds (Kushwaha *et al.*, 1974). The pigment responsible for the purple color is a retinal-protein complex, bacteriorhodopsin, while the isoprenoid-derived carotenoid pigment, bacterioruberin, gives rise to a bright pinkish-red color.

Bacteriorhodopsin converts the energy of green light (500–650 nm) into an electrochemical proton gradient, which in turn is used for ATP production by ATP-synthases. For example, the plasmic membrane of *Halobacterium salinarum* NRC-1 contains membrane patches known as the purple membrane with a protein:lipid ratio of 75:25. The only protein in the purple membrane is bacteriorhodopsin, which forms a hexagonal 2-dimensional crystal that consists of bacteriorhodopsin trimers (Haupts *et al.*, 1999). Bacterioruberin is a ubiquitous and abundant pinkish-red pigment in moderately (Rønnekleiv and Liaaen-Jensen, 1995) to extremely halophilic archaea (Liaaen-Jensen, 1979). This red pigment, located in the membrane of halophilic archaea, not only plays a role in the photoprotection system (Cockell and Knowland, 1999), but is also important for the adaptation of membrane fluidity to changing osmotic conditions (D'Souza *et al.*, 1997).

Raman spectroscopy is mostly viewed as a specialist laboratory or research technique. However, in recent years several systems have been specifically developed for field-based applications. Coupling recent advances in laser sources, optical elements, spectrometers, and detectors has led to the development of robust, compact, and miniaturised Raman systems. Consequently, the potential use of Raman spectroscopy in planetary exploration as part of a rover or lander instrumentation package, particularly for the exploration of Mars, is now being realized. NASA and ESA currently consider Raman spectroscopy, either separately or in combination with laser-induced breakdown spectroscopy, or fluorescence, as a fundamental next-generation instrument for the characterization of mineralogical and organic material during the exploration of Mars. Instrumentation for robotic missions is probably the most important consideration for Mars exploration. It is important to note that Raman applications, which add to the knowledge of Mars, cover other aspects, such as the study of potential terrestrial martian analogues. Hence, it is crucial to construct a Raman database of biosignatures of potential martian analogue microbial life found in extreme environments on Earth to facilitate the detection of biosignatures on Mars. This work has already commenced; for example, there are a number of recent pioneering studies on cyanobacterial biomolecules using Raman spectroscopy, predominantly NIR FT-Raman spectroscopy by Wynn-Williams and Edwards, (2000), Edwards *et al.* (2005, 2004), Villar *et al.* (2005), and, for a more extensive review, Villar and Edwards (2006).

Motivated by the growing importance of being able to detect biomolecules during planetary exploration, this study aimed to investigate the potential of resonance Raman (RR) spectroscopy of carotenoids biosynthesized by halophilic archaea as a potential new biomarker for hypersaline environments on Mars.

RESONANCE RAMAN SPECTROSCOPY OF CAROTENOIDS

Raman spectroscopy is a form of vibrational spectroscopy that has long been routinely used

to identify and quantify chemical compounds. A Raman spectrum is a spectrum of the light scattered from a sample, which is irradiated with monochromatic radiation in the visible or near-infrared region. The light may be scattered either elastically (Rayleigh scattering) or inelastically (Raman scattering) as shown schematically by the possible consequences of a photon-molecule interaction (Carey, 1982) in Fig. 1. In the case of Raman scattering, the emergent light is shifted from its original frequency by a quantum of energy that corresponds to a molecular transition of the sample. The transition may be translational, rotational, vibrational, or electronic in nature. For chemical and biological purposes, the vibrational Raman effect is the most important. For a vibrational mode to be Raman active, a change in polarizability is needed as the molecule vibrates. Molecules consist of a nuclear structure surrounded by a complex field or cloud of electrons. Application of a potential field causes the electrons to ebb and flow so that they are slightly concentrated toward the + and away from the − of the applied field. The ease at which electrons respond to a given field is described as polarizability. If the polarizability changes as the molecule vibrates, we observe Raman active modes shown as Raman bands in the spectrum.

If a biological molecule absorbs light in the visible portion of the electromagnetic spectrum, then new possibilities are opened up for Raman spectroscopy for these compounds of interest, via the resonance Raman effect (Spiro, 1987). Judicious selection of tuning the excitation wavelength to the electronic absorption spectrum can produce selective enhancement of certain Raman bands (Carey, 1982; Spiro, 1987) (Fig. 1). These Raman bands correspond to vibrational modes that involve motions of the atoms in the chromophore, which is that portion of the molecule where the electronic transition is localized. There are two types of resonance-enhanced vibrational modes, which are termed type A (A-term) and B (B-term) (Carey, 1982; Spiro, 1987). Type A modes connect the ground state to the resonant excited state through the Franck-Condon overlap, while type B modes couple the resonant excited state to another excited state at higher frequency through vibronic mixing. Type A modes are totally symmetric, since the ground state wave function has the full symmetry of the molecule. Type B modes, however, may possess any symmetry that is a result of the direct product of the two electronic

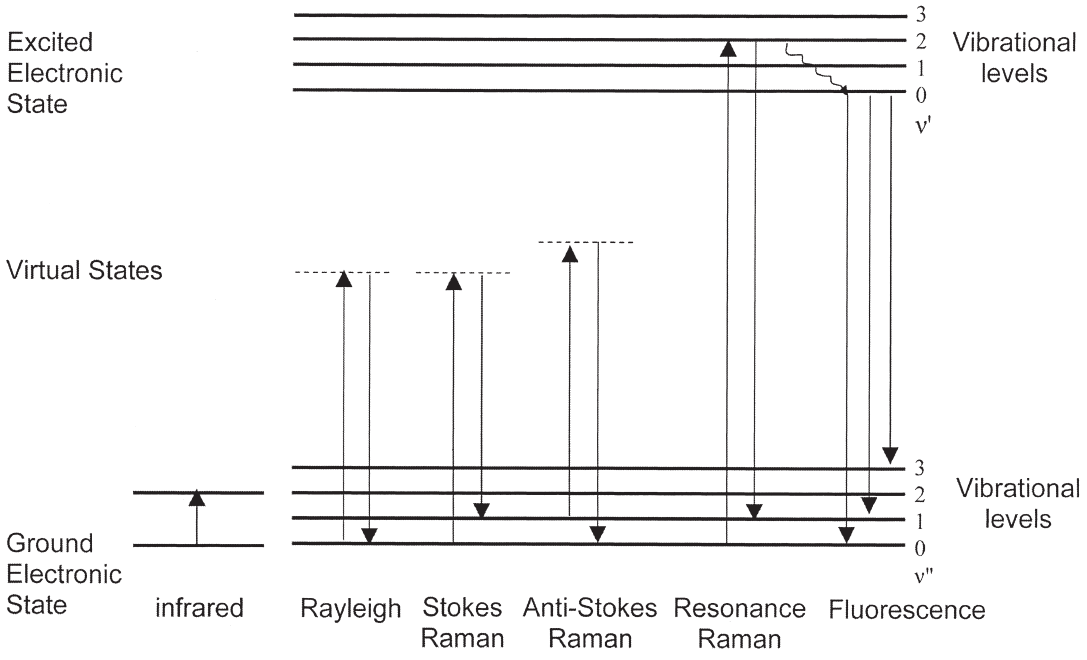


FIG. 1. Some of the possible consequences of a photon-molecule interaction. The lengths of the upward arrows are proportional to the frequencies of the incoming light while the lengths of the downward arrows are proportional to the frequencies of the scattered light. The vibrational quantum numbers in the upper and lower electronic states are ν' and ν'' respectively (modified from Carey, 1982).

transition representations. Therefore, RR spectroscopy provides a means whereby vibrations of biological chromophores can be distinguished from many of the vibrational modes associated with the complex biological matrix. Significantly, the resonance enhancement factor can be quite large, in the order of 10^3 to 10^6 orders of magnitude and, thereby, allow the analysis of chromophore concentrations as low as 10^{-4} to 10^{-6} *M*. The chromophore vibrations completely dominate the spectrum. Consequently, RR spectroscopy could be used to identify and delineate potential biomarker compounds, particularly at the trace quantity level.

Carotenoids are π-electron-conjugated carbon-chain molecules and are similar to polyenes with regard to their structure and optical properties. Structurally, these molecules are a linear, chain-like conjugated carbon backbone that consists of alternating carbon single (C–C) and double bonds (C═C) with varying numbers of conjugated double bonds and a varying number of attached methyl side groups. For example, the molecular structure of β-carotene and bacterioruberin, which are the most important carotenoids in cyanobacteria and halophilic archaea, respectively, are shown in Fig. 2. Carotenoids are strongly colored as they have an allowed π-π^* (or S_0-S_2) transition that occurs in the visible region of the electromagnetic spectrum. This color is dependent on the number of conjugated double bonds in the main linear chain. Red shifting of this π-π^* absorption band indicates an increase in the conjugation length, which is reflected in its color, progressing from yellow to orange to red. For example, β-carotene has 11 conjugated double bonds and is orange in color, while bacterioruberin has 13 conjugated double bonds and is red in color (Fig. 2). Significantly, for Raman spectroscopic applications, when the wavelength of laser excitation coincides with an allowable π-π^* electronic transition of carotenoids, RR spectra are obtained.

MATERIALS AND METHODS

Organisms and growth conditions

Halophilic archaea investigated in this study include *Halobacterium salinarum* NRC-1, *Halococcus morrhuae*, and *Natrinema pallidum*. The strain *Hbt. salinarum* NRC-1 was a gift from Professor Helga Stan-Lotter. *Hcc. morrhuae* and *Nnm. pallidum* were gifts from Professor Masahiro Kamekura. All strains, except *Hbt. salinarum* NRC-1, were cultured in DSM 97 medium (DasSarma *et al.*, 1995) with 150 g of NaCl and supplemented with 7.23 g $MgCl_2 \times 6\ H_2O$ and 2.70 g $CaCl_2 \times 2\ H_2O$ per liter, pH 7.4 (DSM 97 modified). *Hbt. salinarum* NRC-1 was cultured in ATCC 2185 medium as described at http://www.atcc.org/mediapdfs/2185.pdf. Cultures were incubated at 37 °C on a rocking platform for up to two weeks.

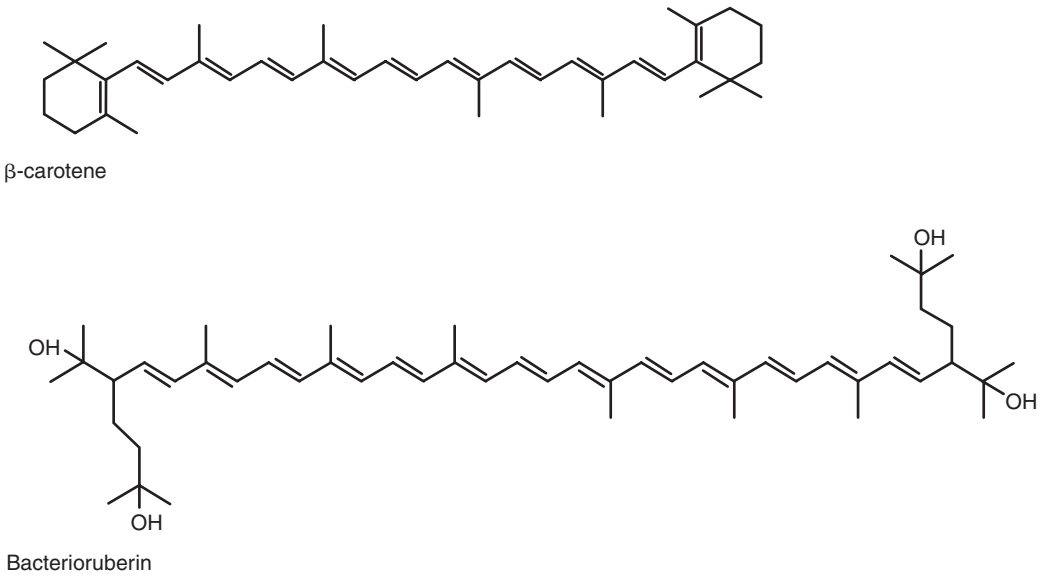


FIG. 2. A comparison of the molecular structures of β-carotene and bacterioruberin carotenoids.

Carotenoid extraction

The bacterioruberin carotenoid pigment was extracted from ~0.2 mg of *Hbt. salinarum* NRC-1 cells using 5 ml of cold acetone. The acetone-soluble extract obtained from *Hbt. salinarum* NRC-1 was identified by its electronic absorption spectrum and compared with previously published electronic spectra obtained from bacterioruberin (Britton, 1985; D'Souza *et al.*, 1997). Bacteriorhodopsin (99% purity) isolated from the purple membranes of *Hbt. salinarum* was purchased from Sigma.

Electronic absorption spectroscopy

Electronic absorption spectra were obtained from the acetone-soluble extract of *Hbt. salinarum* NRC-1 biomass using a Cary 5 UV-Vis spectrometer in the 350–600 nm range.

Resonance Raman spectroscopy

A Renishaw *InVia* Reflex Raman microprobe with a multi-wavelength facility operating at 488.0 and 514.5 nm was used to assess the effect of excitation wavelength on the recording of spectra from the halophilic archaea. The collection optics are based on a Leica DMLM microscope. A refractive glass 50× objective lens was used to focus the laser onto a 2 μm spot to collect the backscattered radiation. The 488.0 and 514.5 nm line of a 5W Ar^+ laser (Spectra-Physics Stabilite 2017 laser) orientated normal to the sample was used to excite the sample. For both excitation lines, the following spectra acquisition parameters were used for performing direct comparisons: 10 seconds exposure time, 5 accumulations, and ~1.2 mW of laser power at the sample. The scan ranges were 800–1800 cm^{-1} in the fingerprint region of resonance-enhanced Raman spectra obtained from carotenoids. The halophilic archaea were smeared as a one-cell layer onto a clean aluminum microscope slide and irradiated with the laser to obtain spectra.

RESULTS AND DISCUSSION

1. Electronic absorption spectra

The representative electronic absorption spectrum shown in Fig. 3 was obtained from the acetone-soluble extract of *Hbt. salinarum* NRC-1 biomass. The red pigment was identified by its characteristic absorption spectrum as bacterioruberin, with absorption maxima (λ_{max}) at 388, 468, 495, and 530 nm in acetone. These results are in accordance with the observations of Britton (1985) and D'Souza *et al.* (1997). Electronic absorption spectroscopy not only allows for pig-

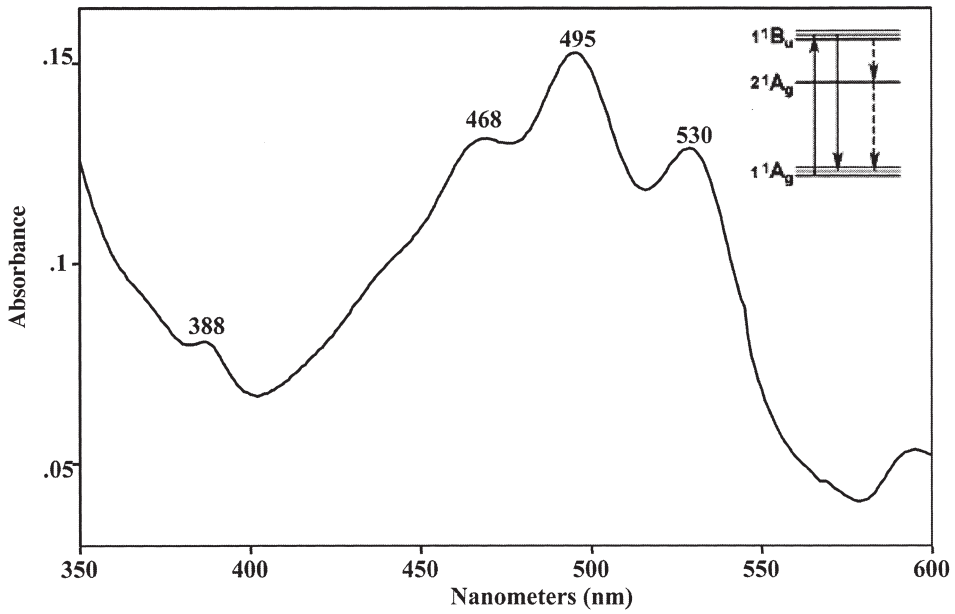


FIG. 3. The electronic absorption spectrum of acetone-soluble extract of *Hbt. salinarum* NRC-1 cells. Absorptions occur as broad bands in the blue/green spectral range with λ_{max} at 468, 495, and 530 nm characteristic for an absorption spectrum for bacterioruberin. The insert shows an energy level diagram for carotenoids.

ment identification, but it also delineates whether the compound of interest exhibits absorption bands due to electronic dipole transitions of the molecules within a certain portion of the visible wavelength range. When illuminated with monochromatic light overlapping one of these absorption bands, the Raman scattered light will exhibit a substantial resonance enhancement in the order of 10^5 and, therefore, allow judicious selection of the excitation wavelength to achieve this phenomenon.

The electronic absorptions occur as broad bands (~150 nm wide) in the blue/green visible spectral range. Carotenoid molecules feature an unusual even parity excited state (see insert, Fig. 3). Consequently, absorption transitions are electronic-dipole allowed in these molecules, but spontaneous emission is forbidden. The resulting absence of any strong fluorescence in carotenoids is the main reason for the possibility of applying RR spectroscopy for these analyses (shown as a downward pointing arrow—optical transition in the insert in Fig. 3). This absorption shows a clearly resolved vibronic substructure due to a strong electron-phonon coupling. Strong electric-dipole allowed absorption transitions occur between the molecules' delocalized π orbitals from the $1\ ^1A_g$ singlet ground state to the $1\ ^1B_u$ singlet excited state (insert, Fig. 3). Optical excitation within the $1\ ^1A_g \rightarrow 1\ ^1B_u$ absorption band led to only weak luminescence transitions (Thrash *et al.*, 1977; Shreve *et al.*, 1991). It can be noted from the insert in Fig. 3 that the extremely low quantum efficiency of the luminescence was caused by the existence of a second excited singlet state, a $2\ ^1A_g$ state, which lies below the $1\ ^1B_u$ state. Following excitation of $1\ ^1B_u$ state, the carotenoid molecule relaxes very rapidly within ~200 to 250 fs (Shreve *et al.*, 1991), via non-radiative transitions, to the lower $2\ ^1A_g$ state from which electronic emission to the ground state is parity-forbidden. This is shown by the dashed downward pointing upper arrow in the insert of Fig. 3. The resulting low $1\ ^1B_u \rightarrow 1\ ^1A_g$ luminescence efficiency (10^{-5} to 10^{-4}), and absence of $2\ ^1A_g \rightarrow 1\ ^1A_g$ fluorescence of the molecules, enabled the detection of the RR spectral response of the molecular vibrations clearly shown as a solid downward pointing arrow in the insert of Fig. 3. Specifically, RR spectroscopy detects the stretching vibrations of the conjugated backbone as well as the methyl side groups (Thrash *et al.*, 1977; Shreve *et al.*, 1991).

2. Resonance Raman spectroscopy

2.1 Choice of excitation wavelength

Figure 4 shows the representative RR spectra in the region of 800–1800 cm^{-1} acquired from a one-cell-layer smear of *Hbt. salinarum* NRC-1 cells recorded at 488.0 and 514.5 nm excitation wavelengths. It should be noted that a properly configured Raman spectrometer for Mars exploration would be configured to encompass 50–4000 cm^{-1} Raman shift. This would enable, for example, the observation in samples of bound and unbound water (νOH) stretching modes between 3000 and 3800 cm^{-1} if present. This is also significant in the astrobiological prospecting of Mars. However, the RR spectra shown here are displayed in the region of 800–1800 cm^{-1} since bands occurring above 2000 cm^{-1} are due overtone and combination bands, which in this case provide little to no information and obscure any stretching vibrational modes associated with water. Therefore, the fingerprint region for the resonance enhancement of carotenoids was chosen for all spectra shown herein. The stacked RR spectra in Fig. 4 contain major features at approximately 1000, 1152, and 1505 cm^{-1}. The bands at 1505 cm^{-1} and 1152 cm^{-1} are due to in-phase C═C (ν_1) and C–C stretching (ν_2) vibrations of the polyene chain in carotenoids. Additionally, in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds occurred in the 1000 cm^{-1} region.

Due to the absorption bands that occur as broad bands in the blue/green spectral range, we selected to illuminate with monochromatic light overlapping these absorption bands with both 488.0 and 514.5 nm excitation lines. The mechanism for resonance enhancement of carotenoids is attributable to the A-term with involvement of a single excited electronic state ($\pi \rightarrow \pi^*$). This process leads to a large intensity gain for those modes at 1505 and 1152 cm^{-1} from stretching of the (ν_1) C═C and (ν_2) C–C bonds, respectively, since these bond distances change appreciably in the π^* state. However, the C–H bonds show little change when passing from the $\pi \rightarrow \pi^*$. Consequently, C–H stretching modes show minimal intensity variation as the resonance condition is approached. Most notably, when the excitation line is changed from 488.0 to 514.5 nm, the ν(C–C) and δ(C═CH) decrease in intensity with a concomitant increase in the ν(C═C) intensity. This

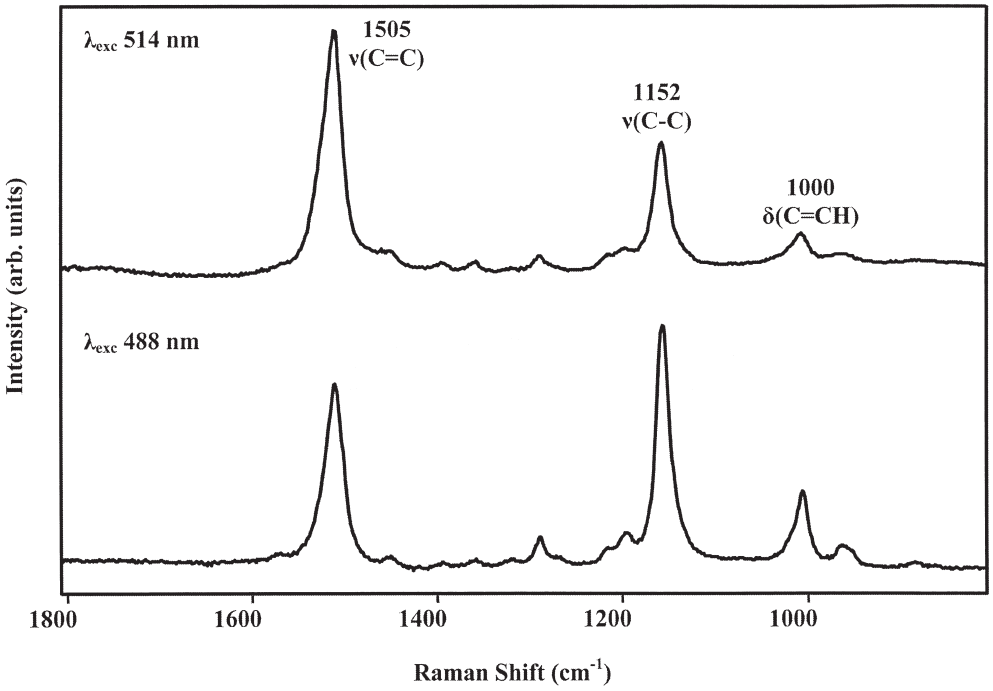


FIG. 4. Stacked resonance Raman spectra of a smear of bacterioruberin cells showing resonance enhancement recorded at 488.0 and 514.5 nm excitations. Collection parameters for both 488.0 and 514.5 nm excitations are 10 s exposure, 5 accumulations, and 1.2 mW laser power at the sample on an *InVia* Reflex Renishaw Raman spectrometer. RR-enhanced modes at 1505 cm^{-1} and 1152 cm^{-1} are due to in-phase C═C (ν_1) and C–C stretching (ν_2) vibrations of the polyene chain in carotenoids, and in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds occur in the 1000 cm^{-1} region.

feature is not observed for botryoxanthin and β-carotene for RR spectra obtained from cyanobacteria and micro-algae using 488.0 and 514.5 nm excitation (Marshall *et al.*, 2006a). This phenomenon does not, however, have any significant impact on excitation choice for obtaining biomarker data pertaining to bacterioruberin biosynthesised by halophilic archaea. The 514.5 nm excitation line was chosen for the RR spectroscopic investigation of the other halophilic archaea.

2.2 Comparison between different halophilic archaea

Spectra acquired from the three Halobacteria cultures, namely *Hbt. salinarum* NRC-1, *Hcc. morrhuae*, and *Nnm. pallidum,* contained major features at approximately 1000, 1152, and 1505 cm^{-1} (Fig. 5). It can be seen that there were no spectral differences between RR analyses of the three different halophilic archaea. The bands at 1505 cm^{-1} and 1152 cm^{-1} are due to in-phase C═C (ν_1) and C–C stretching (ν_2) vibrations from the polyene chain of carotenoids. Additionally, in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds occurred in the 1000 cm^{-1} region. Weaker features between 920–980 and 1170–1450 cm^{-1} were also noted.

Carotenoids range in color from pale yellow to bright orange to deep red; color is directly related to their structure. As the number of conjugated (C═C) bonds increases, the wavelength of the absorbed light also increases, which gives the pigment an increasingly red appearance. The Raman shift (cm^{-1}) of the ν_1 band is strongly dependent on the length of the carotenoid chain. It has been shown (Thomas *et al.*, 1990; Veronelli *et al.*, 1995; Withnall *et al.*, 2003; Schulz *et al.*, 2005) that a correlation between ν_1 Raman shift (cm^{-1}) and effective conjugated chain length occurs. These investigators used this correlation to determine the number of double bonds in the polyconjugated main chains of carotenoids, including dodecapreno-β-carotene, decapreno-β-carotene, lycopene, β-carotene, crocetin, and retinal. Consequently, the ν_1 (C═C) stretching mode is an important marker band by which to elucidate the structure of carotenoids. Application of this to our data acquired from the various halophilic archaea that show a Raman shift (cm^{-1}) of 1505

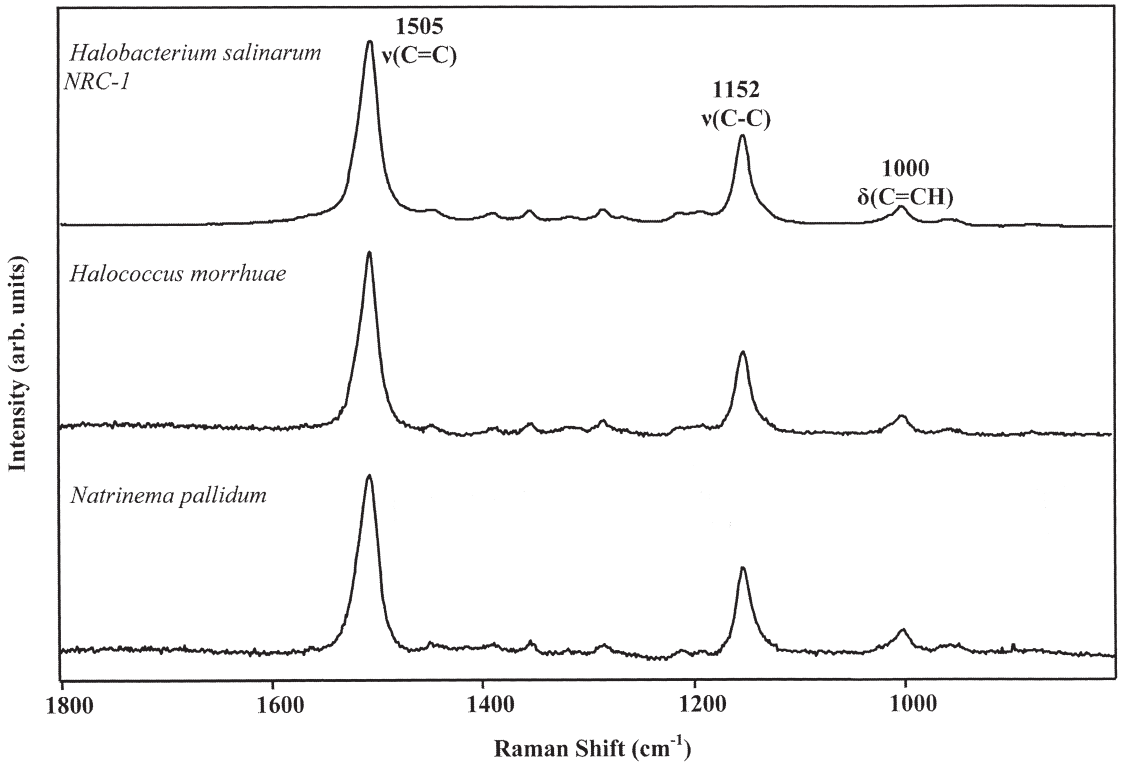


FIG. 5. Stacked resonance Raman spectra of a smear of *Hbt. salinarum* NRC-1, *Hcc. morrhuae*, and *Nnm. pallidum* cells. Collection parameters for the three different archaea are 514.5 nm excitation, 10 s exposure, 5 accumulations, and 1.2 mW laser power at the sample on an *InVia* Reflex Renishaw Raman spectrometer. RR-enhanced modes at 1505 cm^{-1} and 1152 cm^{-1} are due to in-phase C═C (ν_1) and C–C stretching (ν_2) vibrations of the polyene chain in carotenoids, and in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds occur in the 1000 cm^{-1} region.

cm^{-1} for the ν_1 (C═C) band indicated a number of 13 double bonds in the polyconjugated main chain for bacterioruberin. This result is in agreement with the structure for bacterioruberin (Fig. 2). Resonance Raman spectroscopy reveals that the different halophilic archaea analyzed in this investigation all biosynthesize bacterioruberin.

2.3 Comparison between bacterioruberin and bacteriorhodopsin

The differences between bacteriorhodopsin and bacterioruberin are somewhat unclear. For example, Ellery and Wynn-Williams (2003) noted that halobacteria biosynthesize a red pigment bacteriorhodopsin, which is based on a C_{50} carotenoid that could be detected by Raman spectroscopy. Our electronic absorption and RR spectral results show that bacterioruberin is the pink-ish-red carotenoid and this C_{50} carotenoid can be used as a halophilic archaeal biomarker using RR spectroscopy. Since bacteriorhodopsin is another possible biomarker for hypersaline environments, we undertook a comparison of the RR spectra produced by both bacterioruberin and bacteriorhodopsin (Fig. 6).

Figure 6 shows representative stacked RR spectra acquired from a culture of *Hbt. salinarum* NRC-1 and the isolated bacteriorhodopsin. The spectrum acquired for bacterioruberin is less complex than that acquired for bacteriorhodopsin. RR spectra of carotenoproteins such as bacteriorhodopsin generally exhibit more vibrational bands than the free carotenoids. No bands assignable to the protein component are observed, though. The RR spectrum acquired from the bacteriorhodopsin standard contains several modes that can be divided into four groups: the C═C

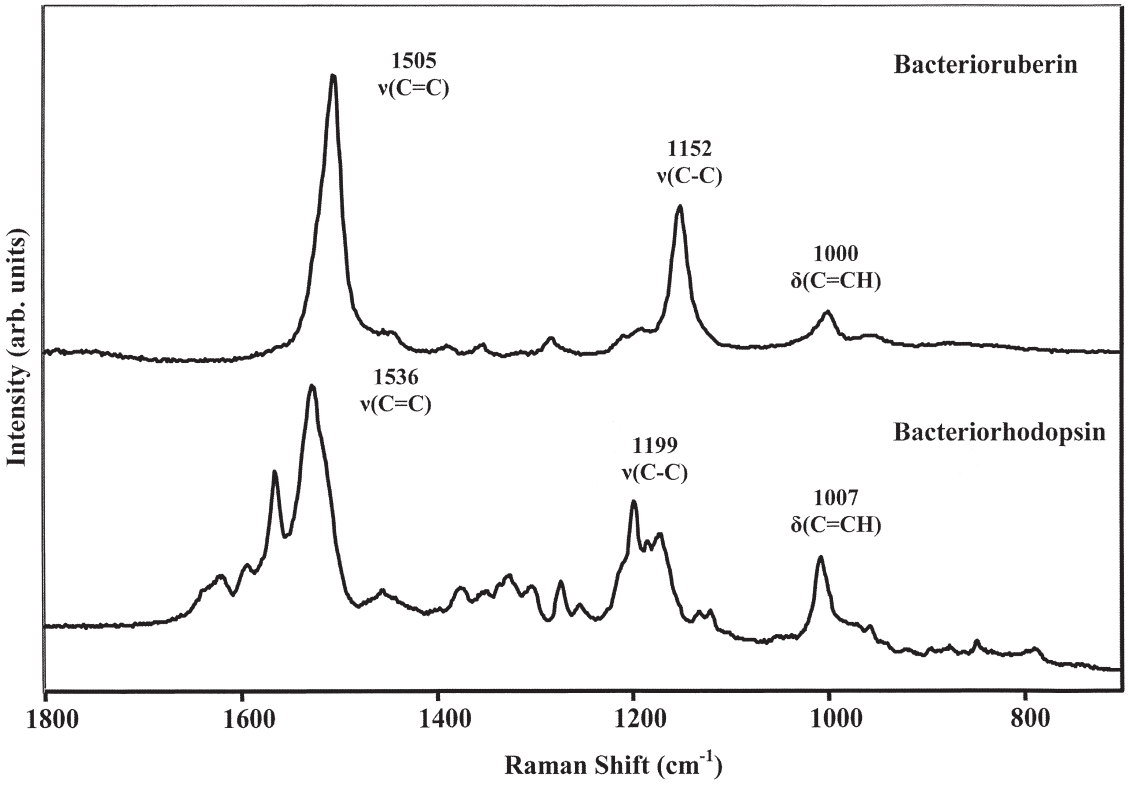


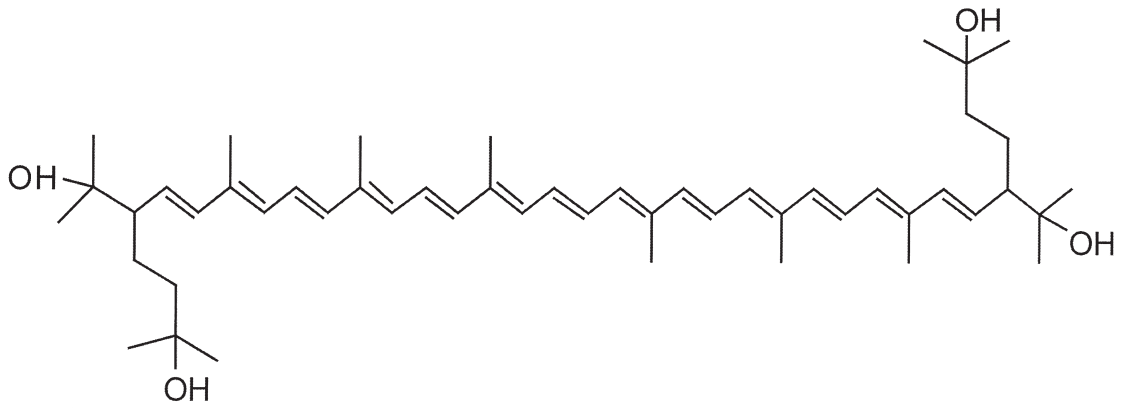
FIG. 6. Stacked resonance Raman spectra of bacterioruberin and bacteriorhodopsin acquired from *Hbt. salinarum* NRC-1. Collection parameters for both spectra are 514.5 nm excitation, 10 s exposure, 5 accumulations, and 1.2 mW laser power at the sample on an *InVia* Reflex Renishaw Raman spectrometer. RR-enhanced modes at 1505 cm^{-1} and 1152 cm^{-1} are due to in-phase C═C (ν_1) and C–C stretching (ν_2) vibrations of the polyene chain in carotenoids, and in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds occur in the 1000 cm^{-1} region and can be observed for bacterioruberin. While four vibrational modes can be observed for bacteriorhodopsin: the C═C stretches (1600–1500 cm^{-1}), the CCH in-plane rocks (1400–1250 cm^{-1}), the C–C stretches (1250–1100 cm^{-1}), and the hydrogen out-of-plane wags (1000–700 cm^{-1}).

stretches (1600–1500 cm^{-1}), the CCH in-plane rocks (1400–1250 cm^{-1}), the C–C stretches (1250–1100 cm^{-1}), and the hydrogen out-of-plane wags (1000–700 cm^{-1}). For more detailed assignments in this region refer to Marshall *et al.* (2006b).

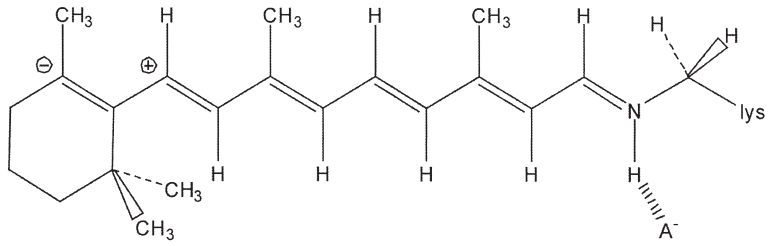
These results highlight the difference between the two biomarker compounds from hypersaline environments. This confusion is surprising given the macromolecular complexity and protein-retinal nature of bacteriorhodopsin (MW 26,000 Dalton) in comparison with bacterioruberin, a C_{50} carotenoid. The differences between Raman spectra acquired from bacterioruberin and bacteriorhodopsin were due to molecular structure and composition (Fig. 7). Application of the well-established relationship of the Raman shift (cm^{-1}) position of the marker ν_1 (C═C) band, the Raman shift of 1536 cm^{-1}, indicated a polyconjugated chain length for the chromophore portion probed by 514.5 nm consists of 5 conjugated double bonds.

3. Astrobiological implications of resonance Raman spectroscopy of carotenoids

Currently, there are a number of analytical techniques employed for carotenoid identification and structural elucidation. Unfortunately, most of these techniques must rely on tedious separation methods (Patzlaff and Barry, 1996; Wilhelm *et al.*, 1995; Berkaloff *et al.*, 1990). Typically, carotenoids need to be isolated by centrifugation and solubilisation. Separation techniques such as high performance liquid chromatography, reverse-phase liquid chromatography, or thin layer chromatography are also required in



Bacterioruberin



Bacteriorhodopsin

FIG. 7. A comparison of the molecular structure of bacterioruberin and the structure of the retinal chromophore of the retinal-protein complex of bacteriorhodopsin.

order to purify the carotenoids (Wilhelm *et al.*, 1995). Additionally, a large biomass is required since the pigments are in trace quantities within the cell. The purified carotenoid extracts are subsequently analyzed by techniques that range from mass spectrometry to optical spectroscopy and fluorescence excitation and emission (Fawley, 1989). Apart from the large amount of biomass required, another problem is that most separation and detection methods are slow. Moreover, the extraction process can be destructive to the carotenoid and retinal-protein complexes, which may be the only unique biomarkers present. From an astrobiological perspective, none of the methods that require sample preparation allow for remote detection.

Vibrational spectroscopy as a means for the detection of potential martian biomolecules is especially attractive because of its speed, nondestructive nature, and very small (single cell) sample size. However, certain limitations arise due to the complex chemical composition of microorganisms. For example, microbial cells consist of macromolecules that are distributed in various cellular components, including the cytoplasm (dominated by proteins and nucleic acids), the cytoplasmic cell membrane (composed predominantly of phospholipids), and the cell wall (composed predominantly of peptidoglycan). On average, the composition of microbial cells are fairly homogenous: ~40–60% proteins, ~15–20% nucleic acids, ~10–15% polysaccharides, ~10–15% lipids, and ~3% or less other organic and inorganic components. Therefore, within a typical mixture, it is nearly impossible to extract conventional infrared or Raman vibrational spectra of biological markers. From an astrobiological perspective, it is desirable to target organic molecules that are clearly distinguished from abiogenic compounds that are widely distributed throughout the cosmos. For example, an infrared spectrum of protein does not necessarily indicate biogencity. A more useful spectroscopy for the identification of molecules that are only synthesized by biological processes would be one that can selectively and sensitively excite biogenic markers, such as carotenoids. Since potential martian microbes could conceivably contain pho-

tosynthetic and photo-protective pigments, there is great potential to use visible light lasers to excite RR spectra of chromophore molecules selectively within the whole cell. We have demonstrated that the RR spectra of haloarchaea are solely due to bacterioruberin and this carotenoid can be used as a biomarker for hypersaline environments.

The selection of excitation wavelengths for miniaturized Raman spectrometers for the exploration of Mars is important. Ideally, the choice of excitation wavelength should be suitable for the analysis/detection of both minerals and biomolecules. The blue/green visible portion of the electromagnetic spectrum probes the S_2, which rapidly converts to S_1, and the weakness of the S_1 to S_0 radiative transition explains the lack of strong fluorescence from carotenoids (Shreve *et al.*, 1991). We have demonstrated that 488.0 and 514.5 nm excitations are ideal for carotenoid detection, as the spectra show only vibrational modes associated with carotenoids and, more importantly, with no fluorescence swamping the Raman signal. Moreover, shorter wavelength lasers excite Raman scattering more efficiently (ν^4 dependence). For example, Raman scattering that uses 514.5 nm is five times more efficient compared to 780 nm and, thereby, provides quicker scan times with stronger signals. In addition, these shorter wavelengths are also ideal for mineralogical measurements by Raman spectroscopy. Most minerals will not fluoresce using this excitation. Withnall *et al.* (2003), for example, collected high quality RR spectra of carotenoids in a carbonate matrix.

The occurrence and spatial distribution of preserved pigments or their derivatives in hypersaline environments on Mars should be detectable *in situ* by non-destructive RR spectroscopy, as it is on Earth. It has been demonstrated that both bacteriorhodopsin and bacterioruberin generate distinctive RR spectra. This work adds to the expanding biomarker data for extremophile microorganisms that have been collected by previous Raman spectroscopic analyses of cyanobacteria as potential martian biomarkers. The fossil equivalent or the diagenetic alteration of bacterioruberin (perhydrobacterioruberin) has yet to be discovered in geological samples (Brocks and Summons, 2004). However, the abundance and ubiquity of bacterioruberin in haloarchaea makes this molecule or the diagenetically modified version a potential highly diagnostic biomarker for haloarchaea in sedimentary rocks of ancient hypersaline deposits.

Resonance Raman spectroscopy can also be used to obtain information about physiological responses *in situ*. In addition, to provide information about key biomolecules, RR spectroscopy can afford data about changes in the environment. Such indirect information is available when a physiological alteration occurs in response to an environmental trigger. For example, RR spectroscopy could offer a potential metabolic test for extant life on the martian surface. Solar radiation is the primary energy source for surface planetary life; thus pigments (that is, carotenoids and chlorophyll) are fundamental components of any surface-dwelling phototrophic microorganism. The production of bacterial and archaeal photosynthetic pigments responds to changes in luminance. Potentially, RR spectroscopy could be used to estimate the change in pigment concentration across day-night boundaries and, hence, provide a novel technique as a metabolic test for extant life.

CONCLUSION

The potential of RR spectroscopy for the detection of molecular biosignatures from halophilic archaea has been demonstrated. In particular, RR spectroscopy has been shown to be a useful tool for the analysis and remote detection of carotenoid pigments *in situ* of halophilic archaea without the need for large sample sizes and extraction, which is required by analytical techniques such as high performance liquid chromatography and mass spectrometry.

The favorable wavelength for excitation should be centered at 514.5 nm for the observation of the RR spectra of carotenoids and retinal-protein complexes from halophilic archaea. The 514.5 nm line induced no fluorescence emission that swamps Raman bands. Therefore, the resulting absence of any strong fluorescence in carotenoids compared to fluorescence in other organic molecules raises the possibility that resonance Raman spectroscopy could be used to detect molecules of biogenic origin on Mars or in other astrobiologically significant settings.

It is possible to obtain molecular information that pertains to the effective polyconjugation chain length of the carbon backbone structure of the carotenoid. This was clearly shown by the cor-

relation of the Raman shift (cm^{-1}) of the ν_1 mode and effective conjugation length of the C═C in the main chain, which, in the case of bacterioruberin, the 1505 cm^{-1} C═C mode equates to 13 C═C structures in the main chain. This correlated extremely well with the known molecular structure of bacterioruberin. We have highlighted RR spectroscopic differences between bacterioruberin and bacteriorhodopsin. Both of these carotenoid and retinal-protein complexes can be used as potential biomakers for hypersaline microorganisms/environments and for the future astrobiological exploration of Mars.

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ABBREVIATIONS

RR, resonance Raman spectroscopy.

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