

Raman Spectroscopy of Microbial Pigments

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Raman spectroscopy is a rapid nondestructive technique providing spectroscopic and structural information on both organic and inorganic molecular compounds. Extensive applications for the method in the characterization of pigments have been found. Due to the high sensitivity of Raman spectroscopy for the detection of chlorophylls, carotenoids, scytonemin, and a range of other pigments found in the microbial world, it is an excellent technique to monitor the presence of such pigments, both in pure cultures and in environmental samples. Miniaturized portable handheld instruments are available; these instruments can be used to detect pigments in microbiological samples of different types and origins under field conditions.

A great diversity of pigments is found in the microbial world. There are many different classes of pigments present in prokaryotic and eukaryotic microorganisms, and these compounds have very different functional roles. The best known are light-harvesting pigments for photoautotrophic and photoheterotrophic growth and compounds that serve to protect cells against harmful radiation in the visible and UV wavelength range. In other cases, the function of the pigmentation is still unknown. Among the best-known pigments are the chlorophylls (Chls) of oxygenic phototrophs, bacteriochlorophylls (Bchls) in anoxygenic phototrophic prokaryotes, pheophytins (chlorophyll molecules depleted of the magnesium atom in their tetrapyrrole ring), and the phycobiliproteins in cyanobacteria. Some halophilic *Archaea* possess the retinal-containing pigments bacteriorhodopsin and halorhodopsin that serve as light-driven proton and chloride pumps; similar light-driven proton pumps (proteorhodopsin) are known in the *Bacteria* domain. In many groups of prokaryotes, carotenoids and flexirubins absorb radiation in the blue range of the spectrum, while scytonemin and mycosporin-like amino acids protect the cells against harmful UV radiation (1–6).

In recent years, Raman spectroscopy has become a popular analytical tool for the qualitative and quantitative assessment of inorganic and organic compounds, including biomolecules and microbial pigments. The Raman effect, discovered by Sir Chandrasekhar Venkata Raman in 1928 for which he was awarded the Nobel Prize in 1930, arises from the inelastic scattering of photons giving rise to energy changes. When a sample is illuminated with electromagnetic radiation, the light can be scattered by a molecule or by a crystal. In Raman scattering, the scattered photons have an energy which is either smaller (Stokes) or greater (anti-Stokes) than the energy of the incident light. The Raman effect occurring in a sample upon irradiation with a monochromatic laser is related to the population of the molecular rotational and vibrational energy levels. The Raman spectrum reflects the molecular transitions and contains Raman bands whose wavenumber shifts from the laser excitation wavenumber are indicative of particular chemical bonds and their molecular environments, molecular interactions, and overall molecular structures. This forms the basis of diagnostic qualitative and quantitative analytical Raman spectroscopy. A particular advantage of Raman spectroscopy for quantitative

studies is the linear relationship between species concentration and band intensity. Raman scattering is not perceived to be a highly sensitive molecular scattering analytical technique compared with some other techniques; nevertheless, it has several advantages to make it the first choice for combined analytical procedures, and these will be described below.

Raman microspectrometers, i.e., systems in which an optical microscope is coupled to a spectrometer, were developed as the MOLE (molecular optical laser examiner) by Delhaye and Dhamelincourt in 1975 (7). These provided for the first time the ability to obtain a molecular chemical signature from an optical image. The combination of Raman spectroscopy with optical microscopy for the study of single cells was pioneered by Puppels et al. (8); in microbiology, Edwards et al. in 1991 used the combination to analyze the colonization of rock substrates by extremophilic lichens (9). In recent years, Raman spectroscopy has been widely applied in microbiology research. For example, the method has enabled the differentiation of enterococci from various sources (10) and the rapid identification of *Candida* species (11). Many additional microbiological applications have been reported (12–17).

Recent reviews (18, 19) show the power of the applications of Raman spectroscopy in the study of microorganisms. These reviews, however, do not focus on the Raman detection of pigments. Microbial pigments generally give very strong and distinct Raman signals (Table 1). Therefore, the technique has been used extensively in recent years to monitor the presence of different types of pigments in microorganisms in pure cultures and in environmental samples. Such studies have used high-resolution laboratory Raman spectrometers (Fig. 1a) and more recently, portable handheld instruments that can be used in the field (Fig. 1b).

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TABLE 1 Main Raman bands of microbial pigments

Pigment	Origin	Prominent Raman bands observed (band positions, cm ⁻¹) ^a	Reference	
Carotenoids				
Bacterioruberin	<i>Halobacterium</i> sp. strain NRC-1 culture	1,505 s, 1,152 s, 1,000 s	51	
	<i>Halococcus dombrowskii</i> DSM14522 ^T culture	1,507 s, 1,152 s, 1,002 s	101	
	<i>Halorubrum sodomense</i> ATCC 33755 ^T culture	1,506 s, 1,152 s, 1,001 s	54	
	<i>Haloarcula vallismortis</i> ATCC 29715 ^T culture	1,506 s, 1,151 s, 1,000 s	54	
	<i>Rubrobacter radiotolerans</i> DSM 5868 ^T culture	1,503 s, 1,150 s, 1,000 s	52	
Bacteriorhodopsin	Pure protein	1,526 s, 1,199 m, 1,168 m, 1,150 m, 1,007 s	122	
β-Carotene	<i>Dunaliella tertiolecta</i> CS175 culture	1,524 s, 1,155 s, 1,001 m	123	
	<i>Synechocystis</i> sp. strain PCC 6803 culture	1,518 s, 1,155 s, 1,005 m	28	
Decapreno β-carotene	<i>Natromonas pharaonis</i> , Wadi Natrun, Egypt	1,503 m, 1,152 m, 1,000 m	124	
Dodecapreno β-carotene	<i>Natromonas pharaonis</i> , Wadi Natrun, Egypt	1,503 m, 1,447 w, 1,389 w, 1,316 w, 1,285 w, 1,210 w, 1,147 m	124	
Astaxanthin	<i>Hematococcus pluvialis</i> CCALA 883 culture	1,520 s, 1,275 w, 1,192 w, 1,157 s, 1,007 m	50	
Salinixanthin	<i>Salinibacter ruber</i> DSM 13855 ^T culture	1,512 s, 1,155 s, 1,003 s	54	
Sarcinaxanthin	<i>Micrococcus luteus</i> DSM 348 ^T culture	1,532 s, 1,157 s, 1,005 s	114	
Deinoxanthin	<i>Deinococcus radiodurans</i> culture	1,510 s, 1,152 s, 1,003 s	33	
Xanthomonadin	<i>Xanthomonas axonopodis</i> pv. <i>dieffenbachiae</i> culture	1,529-1,531 s, 1,135-1,136 s, 1,004 s	41	
Neurosporene	<i>Rhodobacter sphaeroides</i> G1C culture	1,527 s, 1,288 w, 1,214 w, 1,158 s, 1,006 s	62	
Spheroidene	<i>Rhodobacter sphaeroides</i> RCO2 culture	1,524 s, 1,238 w, 1,159 s, 1,170 s, 1,003 w, 951 w	125	
Flexirubin	<i>Flavobacterium johnsoniae</i> DSM 2064 ^T culture	1,529 s, 1,154, 1133s 1,004 m	57	
	<i>Flexibacter elegans</i> DSM 3317 ^T culture	1,515 s, 1,155 s, 996 m	57	
Chlorophylls				
Chlorophyll <i>a</i>	<i>Dunaliella tertiolecta</i> CS175 culture	1,554 m, 1,437 m, 1,325 m, 1,289 m, 1,233 m, 1,186 m, 986 m, 915 m, 757 m	123	
Chlorophyll	Microbial crust, Atacama Desert, Chile	1,445 m, 1,327 m, 915 m, 745 m, 508 w	69	
Bacteriochlorophyll <i>a</i>	<i>Rhodobacter sphaeroides</i> G1C culture	1,640 w, 1,441 w, 1,371 w, 1,173 s, 1,020 m, 900 s, 773 w, 733 s	62	
Phycobiliprotein	Microbial crust, Atacama Desert, Chile	1,631 w, 1,583 w, 1,370 w, 1,283 w, 1,235 w, 874 w, 815 w, 665 w	69	
Scytonemin	<i>Chlorogleopsis</i> culture O-89-Cgs	1,590 w, 1,556 m, 1,549 w, 1,172 m	80	
	<i>Lyngbya aestuarii</i> from native mat, Laguna Ojo de Liebre, Guerrero Negro, Baja California Sur, Mexico	1,595 m, 1,554 m, 1,173 m	75	
	Microbial crust, Atacama Desert, Chile	1,596 s, 1,558 s, 1,321 s, 1,172 m	69	
	<i>Nostoc</i> sp.	1,630 s	126	
Mycosporine-like amino acid	Jarosite superficial crust, Rio Tinto, Spain	1,493, 1,414, 1,340, 1,293, 1,215, 1,181, 1,150, 920, 845, 485	68	
Pulvinic acid derivatives	Microbial crust, Atacama Desert, Chile	1,484, 1,414, 1,350	69	
	Rhizocarpic acid	<i>Acarospora chlorophana</i> (lichen)	1,031, 1,002, 945, 619, 597	19
	Calycin	<i>Candelariella</i> sp. (epilithic lichen)	1,605, 1,580, 1,390, 1,365, 1,295, 1,152, 990, 965, 482	127
	Atranorin	<i>Cladonia coniocraea</i> (lichen)	1,674 s, 1,629, 1,605 s, 1,453, 1,406 s, 1,379, 1,311, 1,281, 1,115, 1,033, 1,000, 980, 956, 869, 826, 743 m, 704, 615, 503, 303	78
	<i>Lepraria</i> spp. (epilithic lichen)	1,674 s, 1,629, 1,605 s, 1,406 s, 1,379, 1,311, 1,281, 1,033, 1,000, 980, 956, 869, 826, 743 m, 704, 615, 503, 300	127	
Gyrophoric acid	<i>Dirinaria aegialita</i> (lichen)	1,663, 1,627, 1,615, 1,453, 1,437, 1,385, 1,302, 1,287, 1,067, 1,046, 855, 782, 587, 567	78	
Anthraquinones				
Parietin	<i>Xanthoria elegans</i> (epilithic lichen)	1,671, 1,605, 1,380, 1,180, 970, 926	128	
	<i>Caloplaca crosbyae</i> (epilithic lichen)	1,670, 1,608, 1,551, 1,370, 1,275, 1,256, 1,215, 1,198, 1,181, 978, 925, 725, 610, 570, 518, 465, 458, 398	78	

^a s, strong bands; m, medium bands; w, weak bands.

We review here the many recent studies that have shown the power of Raman spectroscopy as a tool for the characterization of microbial pigments in a variety of settings.

Not included in this minireview are studies using enhancement

of the Raman signal due to the generation of surface plasmon effects when the sample is deposited on Ag, Au, or other nanoparticles (surface enhanced Raman spectroscopy [SERS]). This technique has been applied to biomolecules (20–22), but it requires

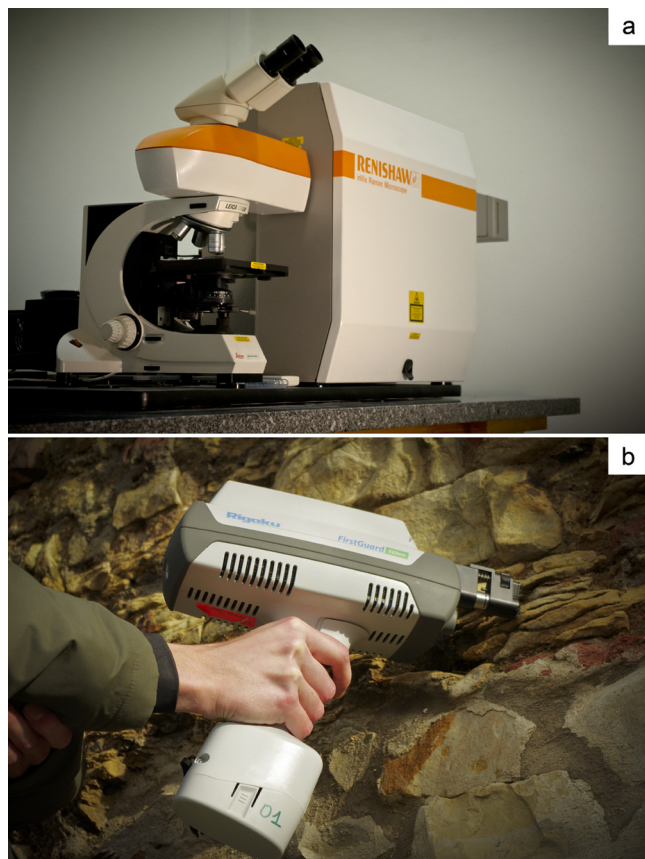


FIG 1 (a) Conventional confocal Raman microspectrometer. The laser irradiates the object selected in the optical microscope (laser spot $\approx 2 \mu\text{m}$), the scattered light from the sample is collected by the optics of the microscope passing through holographic filters, pinhole, and monochromator to be detected by a charge-coupled device (CCD). (b) Handheld Raman spectrometer allows direct measurements on outcrops (cyanobacteria on siliciclastic rocks at Albertov, Prague, Czech Republic). The rugged probe head permits laser illumination of the object and collection of the scattered light.

complex sample treatment, and it has mainly been used in clinical biomedical studies.

RAMAN SPECTROMETRY FOR PIGMENT ANALYSIS—HISTORICAL OVERVIEW

The application of Raman resonance spectroscopy to research on natural pigments, mainly carotenoids and chlorophylls, which became popular in the 1980s (23), dates back to the pioneer work of Euler and Hellstrom in 1932 (24). Their work came just a few years after the discovery of the Raman effect. It was followed in 1970 by the publication of Raman spectra of the carotenoid pigments lycopene and β -carotene in intact plant tissues (25). The technique was later used to assess the presence of light-harvesting bacteriochlorophyll and bacteriochlorophyll-protein complexes in purple photosynthetic bacteria (26). In 1986, it was documented that carotenoids can be easily detected in trace amounts in complex material and even within a single living cell on a very short time scale (27). Resonance Raman spectra (RRS) of astaxanthin-protein complexes published soon after showed some details of carotenoid-protein interactions (28).

RAMAN SPECTROMETRY FOR MICROBIOLOGICAL PIGMENT ANALYSIS—ADVANTAGES AND CRITICAL ISSUES

Raman spectroscopic techniques enable the direct interrogation of living colonies without detaching them from their organic or mineral substrates, affording a unique opportunity to derive molecular chemical structural information about individual components. Raman spectroscopy can thus complement pigment analysis in biological and geobiological matrices using classical techniques, such as absorption spectroscopy and high-performance liquid chromatography (HPLC), mass spectrometry (MS) including liquid chromatography (LC)-MS and gas chromatography (GC)-MS, and nuclear magnetic resonance (NMR) techniques largely used for precise conformational and structural studies. However, in these cases, extraction and sometimes extensive preparative isolation and purification work is needed, which can be quite time-consuming. Raman spectroscopy often does not require extraction and other preliminary sample preparation techniques and can additionally be employed under field conditions (with handheld mobile devices) when the use of other analytical techniques is not feasible.

The linear correlation between Raman signal intensity and chemical species concentration is very useful for the determination of relative concentrations of molecular species in mixtures, but itself has a disadvantage in that the signal response in the Raman spectrum is low compared with other techniques such as UV-visible (Vis) and Fourier transform infrared spectroscopy (FTIR), wherein the low-concentration species in a sample can be accessed using larger specimen path lengths according to the Beer-Lambert law. In Raman microspectroscopy, the use of a microscope to study micrometer-sized specimen regions obviates this disadvantage somewhat, but this usually means that many more spectral sampling points are needed to study large samples, especially when the specimens are heterogeneous. A particular advantage of Raman spectroscopy is that the bands obtained from host inorganic mineral matrices can also be accessed simultaneously with the organic moieties, which means that the molecular interactions between the biological material and their host lattices can be determined. Another advantage of Raman spectroscopy is the insensitivity of the technique to the presence of water. Other Raman spectroscopic techniques are now being explored for development and application in the microbiological field, such as spatially offset Raman spectroscopy (SORS), SERS, and RRS (29–31). Whereas these techniques may require some surface preparation, the application of RRS to microbiological pigments is well established especially for the detection of carotenoids through their signal enhancement provided by laser excitation wavelength near 500 nm, which results in a band intensity increase of several orders of magnitude over the normal nonresonance situation. Wavelength selection for excitation is extremely important for accessing quality Raman spectral data from microbiological samples, and often a multiwavelength approach is necessary to fully determine the composition of a complex specimen. It is critically important to monitor the irradiance of the specimen using different laser illumination configurations, as the focusing of only relatively small laser powers of several milliwatts into micrometer-diameter footprints for spectral interrogation can produce irradiances of milliwatts per square centimeter, enough to seriously deteriorate a living specimen or even to carbonize it. Hence, it is appropriate for the experienced Raman spectroscopist to first study the specimens

using minimally small laser irradiances and then to increase the power levels as necessary to obtain the spectral data.

In some cases, the use of large incident laser irradiance levels is utilized to undertake the so-called “photobleaching” of fluorescent emission which is the bane of Raman spectroscopic studies in biology—here, bleaching or reduction of fluorescent emission intensity by imposing a high irradiance at one spot on the sample actually gives an improved Raman spectral response (32). This phenomenon is not well understood and is commonly referred to as “laser burnout.”

RAMAN SPECTROMETRY OF MICROBIAL PIGMENTS—PURE CULTURE STUDIES

Carotenoids. Carotenoids play many vital roles in a large number of prokaryotic and eukaryotic microorganisms: they participate in energy-harvesting complexes, protect cells against damage by short-wavelength visible and UV radiation, and help repair cellular damage (33). They represent a diverse group of chemicals with a conjugated polyene skeletal backbone. The presence of this polyene backbone enhances the Raman signals, permitting the detection of extremely small amounts of carotenoids (33, 34). The first RRS of carotenoids were reported from 1970 on (23, 26). Tuning the excitation wavelength to the electronic absorption spectrum can produce selective enhancement of certain Raman bands (34, 35). 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. Carotenoids have two strong Raman bands due to in-phase $\nu_1(\text{C}=\text{C})$ and $\nu_2(\text{C}-\text{C})$ stretching vibrations of the polyene chain (23, 26, 36). For example, the Raman spectrum of β -carotene (11 conjugated double bonds) is characterized by the ν_1 band located at $1,515\text{ cm}^{-1}$ and the ν_2 band at $1,157\text{ cm}^{-1}$. A feature of medium intensity occurs at $1,008\text{ cm}^{-1}$ (ν_3), corresponding to the in-plane rocking modes of the CH_3 groups attached to the polyene chain. The wavenumber positions of both ν_1 and ν_2 bands depend on the length of the polyene chain (the number of conjugated double bonds) (37–40). The shift in band position is much more pronounced in the case of the ν_1 band; a longer conjugated polyene chain causes a shift in the ν_1 band to lower wavenumber positions and vice versa (37). When carotenoids are present in combination with other organic molecules, the excitation wavelength of 785 nm becomes an ideal compromise retaining a relatively high sensitivity toward carotenoids (41, 42).

A nice example of the use of Raman spectroscopy for the detection and identification of carotenoids in a heterotrophic prokaryote is the study of the pigments of *Mycoplasma pneumoniae* and the successful discrimination between different *M. pneumoniae* strains (38).

Carotenoid binding within the biomass, which affects the main polyene chain, can cause a significant shift of the ν_1 band position due to change in electronic delocalization (42). Other factors affecting the band shifts in carotenoid Raman spectra are substitutions at the terminal end groups of the molecule (which result in very small wavenumber changes) and isomerism and molecular conformation in the solid and liquid state, respectively (37, 42–44). Moreover, the ν_1 band position of carotenoids depends on the laser wavelength used for excitation (45, 46). This means that the unambiguous identification of particular carotenoids in complex organic material on the basis of Raman spectra alone may some-

times be difficult unless special interpretative appreciation has been given allowing for the various effects delineated above.

Binding of carotenoids to proteins in antenna complexes within photosystems leads to specific changes when carotenoid molecules are distorted from their planar configuration (46–49). In the cyanobacterium *Anacystis nidulans* (*Synechococcus elongatus*), an interaction between phycocyanin and a carotenoid was documented on the basis of a RRS investigation (48). In the case of *Rhodobium marinum*, preferential binding of spirilloxanthin to the photosynthetic reaction center was shown, while anhydrovibrin binds to light-harvesting system I of the pigment-protein complex (49).

Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione), found in plants, bacteria, fungi, and microalgae, has been the object of a number of Raman studies. A Fourier transform Raman spectrometer equipped with a 1,064-nm laser was used to monitor the changes in structures of pure astaxanthin standard and *in situ* in *Haematococcus* cells subjected to thermal stress. Discernible changes were monitored in the Raman spectra upon heating from -100°C systematically up to 150°C (50).

The pigmented extremely halophilic or halotolerant microorganisms that inhabit hypersaline environments such as saltern crystallizer ponds provide interesting opportunities for RRS studies of carotenoids and related pigments. Bacterioruberin is an example of a carotenoid with a long polyene chain (13 conjugated double bonds). RRS of *Halobacterium salinarum* pigmented pinked by bacterioruberin (51) are characterized by the presence of bands at $1,505$, $1,152$, and $1,000\text{ cm}^{-1}$. There is also a published report on the possible presence of bacterioruberin, confirmed by Raman spectroscopy, as the major carotenoid present in actinobacteria related to *Rubrobacter radiotolerans* isolated from medieval frescoes in the Crypt of the Original Sin (Matera, Italy) (52).

A minor component of the hypersaline brine ecosystem is often *Salinibacter ruber* (*Bacteroidetes*). This red-colored bacterium produces a major carotenoid pigment named salinixanthin. It is a 40-carbon carotenoid with one terminal ring and one open end group bound to a glucose moiety which is esterified with a branched 15-carbon fatty acid (53). The Raman spectrum of this unusual pigment was recently published. The $\text{C}=\text{C}$ stretching band is present at $1,512\text{ cm}^{-1}$, the $\text{C}-\text{CH}_3$ mode band is observed at $1,003\text{ cm}^{-1}$, and the $\text{C}-\text{C}$ stretching vibration lies at $1,155\text{ cm}^{-1}$ (54).

Flexirubins. *Flexibacter* and *Flavobacterium* (*Bacteroidetes*) contain yellow to orange pigments called flexirubins. Flexirubin pigments are aryl polyenes containing a polyenoic acid chromophore terminated by a *p*-hydroxyphenyl group and esterified with a dialkylated resorcinol (55, 56). Raman spectroscopy is a suitable tool for the sensitive detection of flexirubin pigments in bacterial colonies and cell extracts of *Flavobacterium johnsoniae* and *Flexibacter elegans*, organisms that contain different flexirubins differentiated by the position of the ν_1 band ($1,527$ to $1,529\text{ cm}^{-1}$ and $1,512$ to $1,516\text{ cm}^{-1}$, respectively) and by the features in the second-order region of the spectrum (57).

Chls and Bchls. Chlorophylls (Chls), including bacteriochlorophylls (Bchls), are the main functional and structural components in all photosystems of oxygenic and anoxygenic phototrophs. Raman spectra obtained from BChls from green photosynthetic bacteria are similar to those of the water-soluble Bchl-protein complexes but differ from spectra of monomeric and aggregated Bchls *in vitro* (58, 59). Fourier transform (FT)

Raman spectroscopy using 1,064-nm laser excitation permits one to obtain Raman spectra suppressing the accompanying Bchl fluorescence emission; thus, key Raman band signatures of the chlorophyll porphyrin skeletal vibrations could be assigned, which can be used to identify chlorophylls in complex cultures (60). The conjugated aromatic pyrrole rings coordinated with the central magnesium(II) ion give characteristic Raman bands in the region of 400 to 1,600 cm^{-1} , and these features have been adopted to identify the presence of chlorophylls in microbial colonies inhabiting rocks in cold-, UV-, and drought-stressed environments (61–63). The application of FT Raman spectroscopy at 1,064 nm to *Rhodobacter sphaeroides* G1C cultures demonstrated the presence of both bacteriochlorophyll *a* and neurosporene (64).

MAAs. The mycosporine-like amino acids (MAAs) are condensation derivatives of a cyclohexenone ring and amino acid or imino alcohol residues (65). Based on the characteristics of UV absorption by MAAs, several studies have suggested that these compounds can act as natural screens of UV radiation and should be considered photoprotectants (66, 67). The Raman spectrum assigned to a mycosporine-like amino acid compound was reported for a jarosite matrix originating from Rio Tinto, Spain (68) and from hyperarid (Atacama, Chile) shallow subsurface layers (69).

Scytonemin. Scytonemin is an effective UV radiation screening pigment exclusively synthesized by some cyanobacteria (70, 71). It is a yellow-brown lipid-soluble pigment, produced as part of the extracellular sheath (72). It may have originated in a common ancestor of the current groups of cyanobacteria or in an early member of this lineage (73, 74). Raman spectra of scytonemin were first investigated by Edwards et al. (75) using FT Raman spectroscopy. The spectra show characteristic corroborative bands of the molecule at 1,590 [$\nu(\text{CCH})$ aromatic ring quadrant stretch], 1,549 [$\nu(\text{CCH})$ *p*-disubstituted aromatic ring], 1,323 [$\nu(\text{C}=\text{N})$ indole ring], and 1,172 cm^{-1} [$\nu(\text{C}=\text{C}-\text{C}=\text{C})$ system (*trans*)].

RAMAN FEATURES OF PIGMENTS FROM NATURAL BIOGEOLOGICAL SAMPLES

Raman spectroscopy of lichen pigments. Lichens, a symbiosis of unicellular green algae or cyanobacteria and fungi, may contain different unique pigments: rhizocarpic acid, parietin, and usnic acid. A comprehensive listing of the lichen pigments identified by Raman spectroscopy is given in Table 1. The first Raman spectrum of a lichen was reported in 1991 in a study of the biodegradation of 16th century Italian Renaissance frescoes in the Palazzo Farnese, Caprarola, Italy, by *Dirina massiliensis* forma *sorediata* (9). The major advantage of the application of Raman spectroscopy here is the ability to interrogate the lichen *in situ* without detachment from its substratum. Use of a Raman microprobe across a range of available laser excitation wavelengths enables the acquisition of molecular information from organic (acids and pigments) and inorganic components of the lichen encrustation with a spectral footprint of several micrometers in diameter (76–79). The distribution of pigments (zeaxanthin, gyrophoric acid, atranorin, and others) was determined in different species of lichens and reported from many areas of differing climatic and physicochemical and substrate conditions (80–86) (Table 1).

Raman spectroscopy of pigments of endoliths. When environmental conditions do not permit microbes to survive on the rock surface, they colonize internal niches between or inside min-

eral grains in rocks. Under such harsh physical or chemical conditions, endoliths can accumulate special chemicals (e.g., pigments and compatible solutes) that help them to live under such extremes. Raman microspectrometry permits the direct study of pigment type and distribution in endolithic zones in the frame of natural stony samples without sample treatment or extractions. Microbial communities inhabiting rocks from various extreme environments were studied: Antarctic habitats (82–84), volcanic rocks as well as travertines on Svalbard (Norway) (80, 85) as well as halophiles from a hot desert (68, 86). Raman spectroscopy revealed differences in pigment distribution in chasmolithic and endolithic colonizations of differing lithologies, including those from dolomite, gypsum, or altered orthoquartzite (83, 86). Raman microspectrometry was able to detect scytonemin as well as chlorophyll, carotenoids and mycosporine-like amino acids in a series of samples of halite endoliths from Yungay and Salar Grande sites in the Atacama Desert in Chile (69). Detailed information on gypsum colonizations in the Atacama Desert was also gathered: *in situ* Raman microspectrometry revealed differences between cyanobacteria and eukaryotic algae, without any sample preparation, and facilitated the estimation of the physiological state of the cyanobacteria, showing a depletion of phycobiliprotein signals relative to carotenoids and chlorophyll within decayed cells (87).

Identification of microbial pigments in halite inclusions by Raman spectroscopy. Crystallizing halite can easily entrap microorganisms and organic debris in liquid inclusions, and there they can retain their viability for prolonged periods (88–92). The presence of cells resembling *Dunaliella* within brine inclusions in ancient halite, 9 thousand years to 1.44 millions of years ago, from borehole cores from Death Valley, Saline Valley, and Searles Lake, California, was corroborated by Raman microspectrometry (with excitation at 514.5 nm). Well-preserved carotenoids occurred within fluid inclusions as yellowish to red-brown amorphous and crystalline masses associated with spheroidal algal cells resembling *Dunaliella* (93). Raman microspectrometry also showed β -carotene accumulations originating from *Dunaliella* in halite inclusions from shallow deposits in extremely acidic and saline Lake Magic (western Australia) (94). Laboratory simulation studies have been performed to follow the entrapment of nine different strains of halophilic *Archaea* within growing halite crystals. Two laser excitation wavelengths were used: nonresonant 1,064 nm and resonant 514.5 nm. The main peaks collected (1,507, 1,152, and 1,002 cm^{-1}) could be mainly assigned to bacterioruberin (95).

ADVANCED RAMAN SPECTROSCOPIC TECHNIQUES IN MICROBIOLOGY

Recently, Raman micromapping has been employed in microbiological research, as it can rapidly provide useful detailed information at the molecular level. Maps of the occurrence of a given compound can be obtained collecting huge series of individual point RS and representing the presence/intensity of a characteristic Raman band as two-dimensional maps. However, in this approach, very small sample areas are mapped, and long times of accumulation may be needed to obtain distribution maps. Planar samples are preferred, and very precise microscopic stages are needed for controlled programmed stepwise scanning of the sample (*x* and *y* directions). Generally, a *z*-direction automatic focusing to the surface at each point permits correct spectrum

collection. Fluorescence effects and spectroscopic artifacts have to be avoided by proper calibration and advanced confocality of the instruments.

Advanced Raman instrumentation provides the possibility to learn more about the spatial distribution of pigments within the cell. Thus, the presence of β -carotene and chlorophyll *a* was demonstrated *in vivo* in individual cells within colonies of *Dunaliella tertiolecta* using confocal Raman microscopy with excitation at 532 nm (96). Confocal Raman spectroscopy was used to differentiate and map the relation between a carotene-containing yeast and *Pseudomonas aeruginosa* cells in living, hydrated, composite biofilms on the basis of their spectral signatures (97). The presence of carotenoids, chlorophyll, and triglycerides was monitored in healthy and starved colonies of the algae *Chlorella sorokiniana* and *Neochloris oleoabundans* (98). The distribution of carotenoids in different microalgae (*Dunaliella*, *Phaeodactylum*, *Haematococcus pluvialis*, *Thermosynechococcus elongatus*) was mapped at the cellular level (99–103) using slightly different instrumental settings (Fig. 2). Raman spectroscopy was used to monitor the production of astaxanthin in the yeast *Phaffia rhodozyma* in fed-batch experiments (104). The use of laser tweezers Raman spectroscopy (LTRS) provides further possibilities (105). It enabled the quantification of the carotenoid content of the yeast *Rhodotorula glutinis* based on the relative intensity of the ν_1 band (106). Coherent anti-Stokes Raman scattering microscopy (CARS) has been identified as an additional technique for advanced imaging of carotenoids in microalgae and cyanobacteria. In this technique, interfering fluorescence effects are efficiently suppressed (107–109).

Finally, Raman spectroscopy of pigments can also be a powerful tool in chemotaxonomy studies. Rösch et al. (110) proposed the use of micro-Raman analysis together with a data classification approach they called the support vector machine (SVM) technique. They tested it on a series of microorganisms originating from airborne particles of clean-room samples. Raman microspectrometry allowed the characterization of bacteria as well as their main cellular components, including pigments in a few cases.

Miniaturization/field applications of Raman spectroscopy in geobiology and astrobiology. Raman spectroscopy is an extremely promising technique for life detection systems in space exploration. Therefore, the development of miniaturized instruments and their exploitation for the analysis of geological samples on Earth will prepare the possible future deployment of Raman instruments to test for pigments such as carotenoids deposited by extinct or extant organisms in the Mars regolith (9, 34, 35, 62, 63, 111–118). Very recent publications review the state of the art of the use of portable and handheld Raman instruments and experience using these instruments (119–121).

Raman spectrometry with 785-nm excitation is useful for the study of biological samples as that wavelength is optimal for the identification of chlorophyll *a*. Small and lightweight (<2 kg) handheld battery-powered Raman instruments have been developed only recently (first 785-nm diodes, more recently 532 nm), and they enable the researcher to obtain spectra outdoors under field conditions. The spectral resolution of commonly marketed portable instruments is around 8 cm^{-1} , and the operational wavenumber shift range is generally $2,000$ to 200 cm^{-1} . Miniaturized Raman instruments have already been applied to the study of lichens *in situ*. Examples are the detection of different biomolecules associated with the yellow Antarctic lichen *Acarospora chloro-*

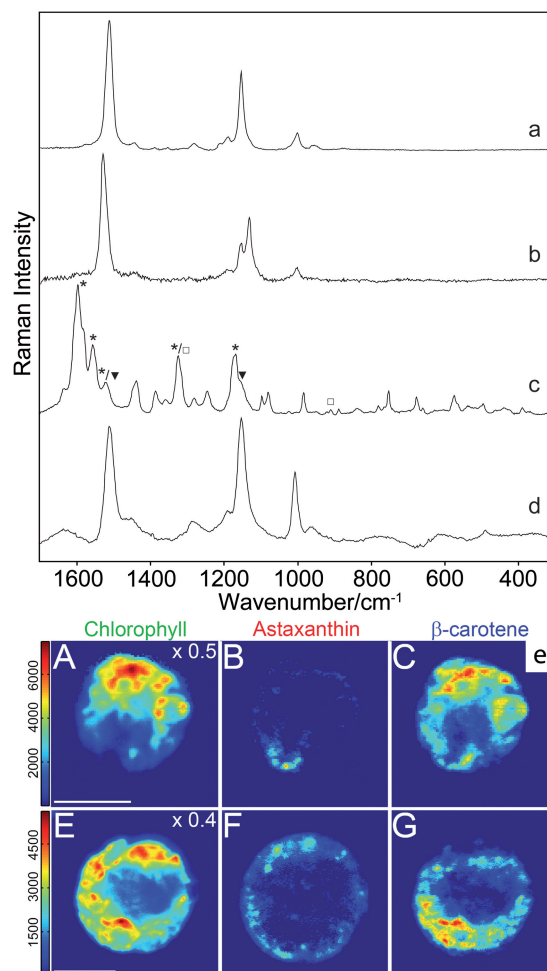


FIG 2 Examples of microbiological application of Raman spectroscopy using different instrumentation. (a to c) Raman spectra obtained using a laboratory Raman microspectrometer. Raman spectra of salinixanthin from a culture of *Salinibacter ruber* (a) (532-nm wavelength; image modified from reference 54), of flexirubin from a culture of *Flavobacterium johnsonae* (b) (532-nm wavelength; image modified from reference 57), and of chlorophyll (□), carotenoid (▼), and scytonemin (*) from a native gypsum crust (Yungay, Atacama, Chile) (c) (785-nm wavelength; image modified from reference 69). (d) Raman spectrum collected using a handheld Raman spectrometer of a spirilloxanthin-like carotenoid of the red layer of the bottom gypsum crust from a saltern at Eilat (Israel) colonized by *Halochromatium*-like microorganisms (532-nm wavelength; image modified from reference 117). (e) Example of Raman mapping—distribution of carotenoids (astaxanthin, β -carotene, chlorophyll) in the frame of flagellated motile cell obtained using a confocal Raman spectrometer (532-nm wavelength; image modified from reference 100, which was published under a Creative Commons agreement). The intensities within the concentration map represent relative component concentrations. Bars, 10 μm .

phana with 852-nm excitation, using one of the first prototype instruments (113). Portable systems with 785-nm excitation performed well in the investigation of the degradation of rhizocarpic acid, parietin, and β -carotene in *Xanthoria parietina* during irradiation experiments (114). Another type of handheld instrument (excitation wavelength of 785 nm) was used to probe lichen samples from a hyperarid area of the Tabernas Desert (Spain). Native lichens were investigated under wet conditions, and several photosynthetic pigments were documented in *Squamarina lentigera*,

Diploschistes diacapsis, as well as in *Lepraria crassissima* (115). Portable and miniaturized Raman spectrometers were further applied to the detection of microbial biomarkers in halite from the hyper-arid region at Yungay (Atacama Desert, Chile). Measurements were performed directly on the rock and on homogenized, powdered rock samples. For carotenoid analysis, excitation at 532 nm was found to be superior for the analysis of powdered specimens, but 785-nm excitation enabled the improved detection of scytonemin, another pigment present in the material examined (116). Using 1,064-nm laser excitation from a semiportable instrument facilitated the detection of scytonemin, carotenoids, and chlorophyll in evaporitic cyanobacterial colonization in the Atacama Desert and carotenes in the epilithic lichen *Acarospora* sp. (112).

With a handheld spectrometer (514-nm excitation) (Rigaku), Raman spectra of pigments in autotrophic (cyanobacteria and purple sulfur bacteria) and heterotrophic halophilic microorganisms (*Archaea* of the family *Halobacteriaceae* and *Salinibacter*) were recorded. Common and less common carotenoids, including α -bacterioruberin (*Haloferax*, *Haloarcula*, and *Halobacterium*) (*Archaea*), salinixanthin (*Salinibacter*) (*Bacteroidetes*), and spirilloxanthin-like (*Ectothiorhodospira*) (*Gammaproteobacteria*) were detected in cell pellets of laboratory cultures. Bacterioruberin was identified as the dominant carotenoid in pellets of cells collected from the saltern crystallizer ponds in Eilat, Israel. Raman band positions correspond well (within 4 cm^{-1}) with data recorded on standards or using high-performance dispersive laboratory microspectrometers. Raman analysis of the colored microbial community layers in a benthic gypsum crust in the saltern evaporation ponds also identified myxoxanthophyll and echinenone carotenoids as well as spirilloxanthin in different layers (117).

CONCLUDING REMARKS

Raman spectroscopy has an excellent sensitivity for detecting polyenes, including carotenoids, because of the strong spectral signatures of the conjugated C—C and C=C functional modes. In particular, this is enhanced by several orders of magnitude through resonance Raman excitation effects occurring between 500- and 532-nm laser wavelengths. In spite of the limitations as outlined in this minireview, Raman spectroscopy has several advantages for pigment and carotenoid work. The technique is non-destructive and very fast, and in the microspectrometry mode, spectral information can be obtained about specific molecules of interest even in complex mixtures. Also, it can be combined with optical imaging of samples at the micrometric level, which is advantageous for the direct *in situ* interrogation of biological inclusions in mineralogical matrices. The astrobiological potential is now well realized, and it is planned to take miniaturized Raman spectrometers to Mars in future space missions.

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