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ORIGINAL PAPER

Use of Raman spectroscopy for identification of compatible solutes in halophilic bacteria

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Abstract We explored the use of Raman spectroscopy to detect organic osmotic solutes as biomarkers in the moderately halophilic heterotrophic bacterium *Halomonas elongata* grown in complex medium (accumulation of glycine betaine) and in defined medium with glucose as carbon source (biosynthesis of ectoine), and in the anoxygenic phototrophic *Ectothiorhodospira marismortui* known to synthesize glycine betaine in combination with minor amounts of trehalose and *N*-α-carbamoyl glutamineamide. We tested different methods of preparation of the material: lyophilization, two-phase extraction of water-soluble molecules, and perchlorate extraction. Raman signals of glycine betaine and ectoine were detected; perchlorate extraction followed by desalting the extract on an ion retardation column gave the best results. Lyophilized cells of *E. marismortui* showed strong signals of carotenoid pigments, and glycine betaine could be detected only after perchlorate extraction and desalting. The data presented show that Raman spectroscopy is a suitable tool to assess the mode of osmotic adaptation used by halophilic microorganisms.

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Introduction

Most halophilic and halotolerant microorganisms that grow at elevated salt concentrations synthesize and/or accumulate organic osmotic solutes ('compatible solutes') to adjust intracellular water activity and to provide osmotic balance between the cytoplasm and the surrounding medium (Brown 1990; Galinski 1993, 1995; Welsh 2000). An alternative strategy of accumulating inorganic ions to osmotically balance the outside salt concentration is used by a few specialized groups of prokaryotes only: the aerobic archaea of the family *Halobacteriaceae*, the aerobic *Salinibacter* (*Bacteroidetes*) which physiologically resembles the halophilic archaea, and the *Halanaerobiales*, an order of anaerobic fermentative bacteria (Oren 2002; Oren et al. 1999, 2002). Accumulation of organic compatible solutes does not require special adaptations of the intracellular machinery and provides the cells with a large extent of flexibility as the intracellular solute concentrations can be rapidly adjusted following changes in salinity of the surrounding medium.

Many different organic molecules have been shown to act as osmotic solutes in microorganisms. These include amino acids (proline, glutamic acid) and amino acid derivatives (glycine betaine, ectoine, hydroxyectoine), simple sugars (sucrose, trehalose), polyols (glycerol), and others (Galinski 1995; Imhoff 1986; Roberts 2005; Ventosa et al. 1998). Since glycine betaine was first detected as an osmotic solute in the phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski and Trüper 1982),

there were initial reports that the compound is the predominant osmotically active molecule in halophilic and halotolerant members of the bacteria (Imhoff and Rodriguez-Valera 1984). However, only very few non-phototrophic prokaryotes can synthesize glycine betaine de novo, but they will accumulate it from the medium when present. Much more widespread compatible solutes are ectoine [(*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid], first detected in the *Halorhodospira halochloris* (Galinski et al. 1985), and its 5-hydroxy derivative. Most heterotrophic bacteria produce ectoine and hydroxyectoine when grown in nutrient-poor media that lack ready-made suitable compounds that can be accumulated from the medium to be used for osmotic stabilization (Galinski 1995; Severin et al. 1992; Ventosa et al. 1998; Wohlfarth et al. 1990).

To qualitatively and quantitatively assess the presence of organic osmotic solutes, different techniques are used. They are all based on the extraction of the cells using different protocols, followed by analysis using techniques such as high performance liquid chromatography and ^{13}C and ^{1}H nuclear magnetic resonance (Motta et al. 2004; Roberts 2006; Severin et al. 1992; Wohlfarth et al. 1990). These techniques are relatively complicated and often require large samples and long preparation times. We are therefore exploring the use of Raman spectroscopy for the qualitative detection of organic osmotic solutes.

Raman spectroscopy is a non-destructive technique, it requires small samples only, and the existence of portable miniaturized Raman spectrometers enables application of the method outside the laboratory as well. Confocal dispersive Raman spectroscopy has been successfully applied to the study of microorganisms, and the FT-Raman mode with 1064 nm excitation was found to be particularly useful. Raman spectra of carotenoids were obtained from halophilic archaea (family *Halobacteriaceae*) (Marshall et al. 2006; Fendrihan et al. 2009). Different modes of Raman microspectroscopy were applied to identify biomarkers in different microorganisms. Heraud et al. (2006) demonstrating the presence of β-carotene and chlorophyll *a* in vivo in individual cells within colonies of *Dunaliella tertiolecta*. Huang et al. (2009) showed presence of carotenoids, chlorophyll and triglyceride and followed their distribution in healthy and starved colonies of the algae *Chlorella sorokiniana* and *Neochloris oleoabundans* using confocal Raman microscopy at 532 nm. Buijtels et al. (2008) used Raman spectra (785 nm excitation) for the identification of mycobacteria. The feasibility of Raman spectroscopy was also proven for the detection of various organic compounds from recent and fossilized microorganisms in rock samples (Wynn-Williams and Edwards 2000; Edwards et al. 2005; Marshall et al. 2007; Vítek et al. 2009).

As organic osmotic solutes are often present in molar concentrations intracellularly, they should be relatively easy to detect using Raman spectroscopy. We have earlier established a database of Raman spectra of some of the most commonly encountered compatible solutes as well as some less common ones: ectoine, hydroxyectoine, glycine betaine, glycerol, sucrose, trehalose, glucosylglycerol, mannosylglycerate, and di-myo-inositol phosphate (Jehlička et al. 2012). We here report the result of experiments to assess the presence of different organic osmotic solutes in two model halophilic bacteria. We selected the heterotrophic bacterium *Halomonas elongata* known to produce ectoine in high-salt minimal media and to accumulate glycine betaine when grown in complex media (Imhoff and Rodriguez-Valera 1984; Severin et al. 1992; Wohlfarth et al. 1990) and the halophilic anoxygenic phototrophic sulfur bacterium *Ectothiorhodospira marismortui* earlier shown to produce glycine betaine together with minor amounts of other solutes (trehalose and *N*-α-carbamoyl glutamineamide (Galinski and Oren 1991; Oren et al. 1991).

Materials and methods

Cultures

Halomonas elongata ATCC 33173^{T} (Vreeland et al. 1980) was grown with shaking (100 rpm) at 30 °C in 250-ml Erlenmeyer flasks with 100-ml portions of complex medium containing yeast extract or minimal medium with glucose as carbon and energy source. The complex medium contained (g l^{-1}): NaCl, 100; KCl, 1.0; $MgSO_4 \cdot 7H_2O$, 10; yeast extract (Difco), 20; Tris, 15; pH 7.5 adjusted with HCl. The composition of the minimal medium was (g l^{-1}): NaCl, 100; $MgSO_4 \cdot 7H_2O$, 2.8; NH_4Cl, 4.5; K_2HPO_4, 1.1; $FeSO_4 \cdot 7H_2O$, 0.01; Tris, 15; glucose (added from a separately autoclaved concentrated solution), 2.0; pH 7.5 adjusted with HCl. *Ectothiorhodospira marismortui* EG-1^{T} (DSM 4180) was grown in the light (25 µmol quanta m^{-2} s^{-1}) at 35 °C in 300-ml serum bottles completely filled with medium containing (g l^{-1}): NaCl, 100; Na_2SO_4, 2.5; $CaCl_2 \cdot 2H_2O$, 0.33; Na-acetate, 0.5; yeast extract, 1.0; Na_2CO_3, 1.5; $Na_2S \cdot 9H_2O$, 0.125, and further nutrients and trace elements as described, pH 7.0 (Oren et al. 1989, 1991). Cells in the late exponential growth phase were harvested by centrifugation (10 min, 12000×*g*, 4 °C). *Halomonas* cells were washed once with salt solutions (pH 7.5) containing NaCl, KCl, and $MgSO_4 \cdot 7H_2O$ in concentrations equal to those used for growth.

Preparation of cells and cell extract for Raman spectroscopy

To identify the best approach for the detection of compatible solutes by Raman spectroscopy, different modes of

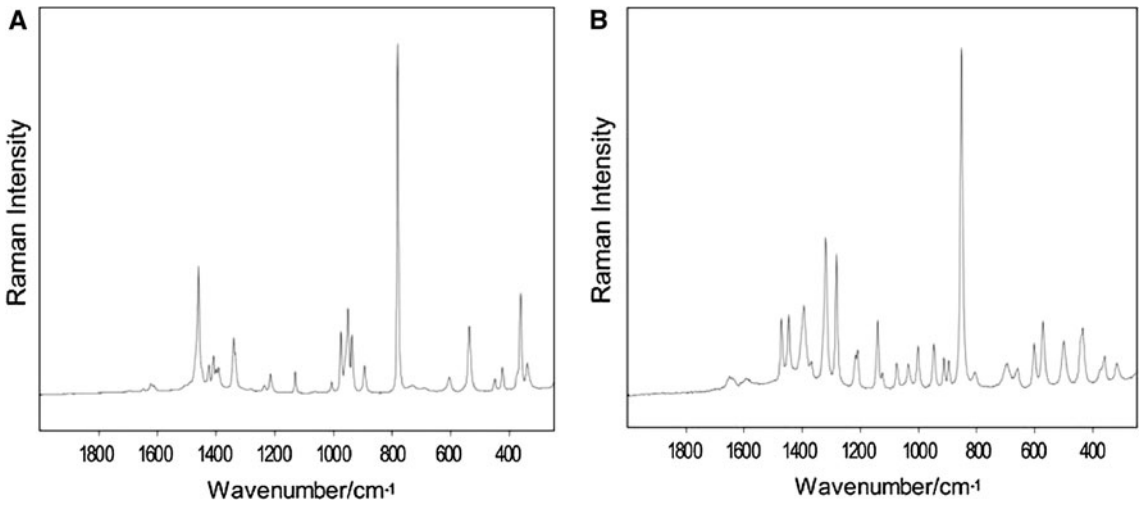

Fig. 1 Raman spectra (20–2000 cm^{-1}) of glycine betaine (**a**) and ectoine (**b**). For excitation a diode laser (785 nm) was used

preparation were tested: (1) drying of cells by lyophilization in a Speedvac system; (2) fixation of the wet pellet by addition of 10 mM $HgCl_2$; (3) a two-phase extraction with methanol and chloroform, based on the Bligh and Dyer (1959) lipid extractions, and (4) perchlorate extraction following the protocol given by Roberts (2006), and the same, followed by (5) desalting over an ion retardation column. For method (3), 3.75 ml of methanol–chloroform (2:1 v/v) was added to a cell pellet (originating from 100 to 200 ml of culture for *H. elongata*, 1 l for *E. marismortui*) resuspended in 1 ml distilled water. After 4 h extraction at room temperature, cell debris were removed by centrifugation, 1.25 ml of chloroform and 1.25 ml water was added to the supernatant, and after thorough mixing the phases were separated by centrifugation. The upper (aqueous) phase was collected and dried by lyophilization. For method (4), similar cell pellets were extracted in the cold for 30 min with 2 ml of 7 % perchloric acid. After centrifugation to remove cell debris, the supernatant was brought to pH 7 with 1 M KOH, the precipitate of potassium perchlorate was removed by centrifugation, and the supernatant was frozen and dried by lyophilization. In part of the experiments (method 5), the perchlorate extract was desalted on an ion retardation column (Bio-Rad AG 11A8/DOWEX® Retardion® 11A8; 2.2 × 50 cm, eluted with distilled water at a rate of 7.5 ml/min), and the desalted fraction was dried by lyophilization.

Recording of Raman spectra

Micro-Raman analyses of powders were performed on a multichannel Renishaw In Via Reflex spectrometer coupled with a Peltier-cooled CCD detector. Excitation was provided by the 785 nm line of a diode laser or by the 514.5 nm Ar laser. Typically, during compatible solutes work, the near-infrared 785 nm excitation was used; excitation by a 514 nm laser permitted to obtain better results for pigments detection. To achieve enhanced signal-to-noise ratios, 10–30 scans were accumulated, each of 20 s exposure time with laser power ranging between 30 and 100 mW. Spectra were recorded at a spectral resolution of 2 cm^{-1} between 100 and 1800 cm^{-1} (first order spectra) and 2000 and 4000 cm^{-1} (second order spectra). Second order spectra were observable only for samples treated with perchloric acid and desalted on an ion retardation column. Between 7 and 10 measurements were taken at different parts of the sample. As standards, we used individual grains of ectoine and glycine betaine (Sigma Aldrich), their first order Raman spectra are given in Fig. 1. Polystyrene, sulfur, ε-caprolactone, acetonitrile/toluene (50/50 v/v), cyclohexane, stearic acid, and glycerol were used to check the band position calibration.

Raman spectra were exported into the Galactic *.SPC format. Spectra were then compared using GRAMS AI (Version 8.0, Thermo Electron Corp., Waltham, MA, USA). Raman spectra were not subjected to any data manipulation or processing techniques and are reported as collected, except for the baseline correction performed in a few cases.

Results

Comparison of modes of extraction

Raman spectra recorded on samples of the same organism obtained using different ways of pretreatment differ in general aspects, as shown in Fig. 2 for *H. elongata* cells grown in complex medium; data for *H. elongata* cells grown in defined medium and for *E. marismortui* are given in Figs. 3 and 4, respectively. In all cases, a significant background was observed related to fluorescence effects. However, in all cases, the majority of strong and medium

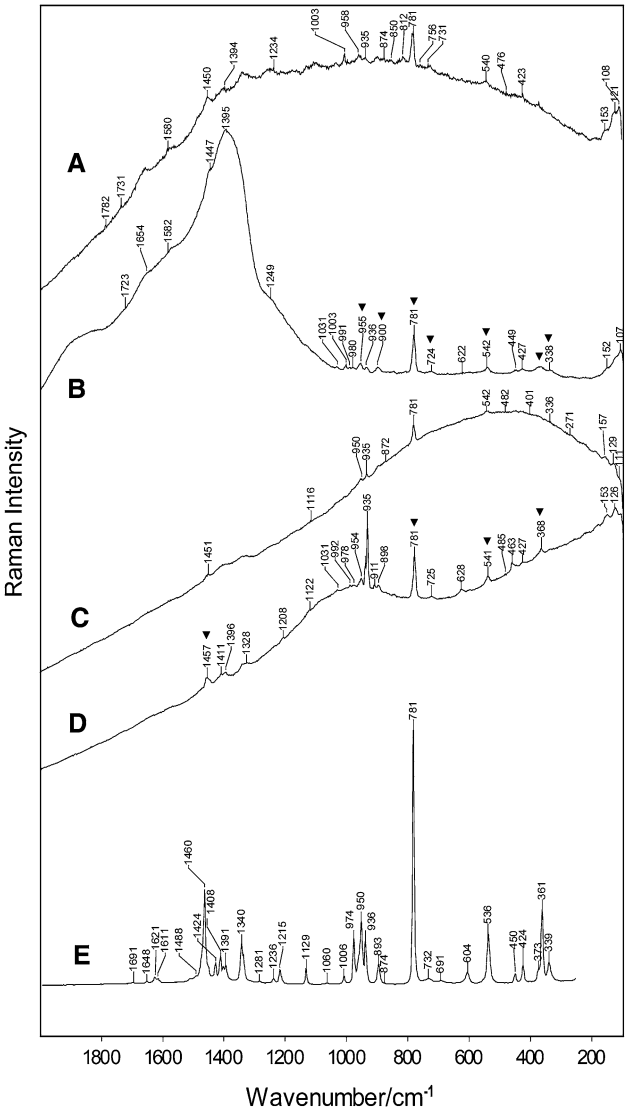

Fig. 2 Raman spectra (excitation at 785 nm) of material originating from a culture of *Halomonas elongata* grown in complex medium containing 10 % NaCl: lyophilized cells (*A*), a wet preparation of cells fixed with $HgCl_2$ (*B*), the lyophilized water phase from a two-phase extraction with methanol/chloroform/water (*C*), and a lyophilized perchloric acid extract (*D*). A spectrum of the perchloric acid extract after desalting on an ion retardation column is shown in Fig. 4a. For details about the preparation protocols see the "Materials and methods" section. Signals attributable to glycine betaine are marked with *arrows*. For comparison the Raman spectrum of glycine betaine is shown (*E*). Further data are given in Supplementary Table 1

intensity Raman bands corresponding to the bands of reference pure compatible solutes (Jehlička et al. 2012) were recorded at correct band positions ±3–4 cm^{-1}.

Figure 2A and B shows a comparison of Raman spectra obtained using a lyophilized pellet of untreated *H. elongata* cells grown in complex medium, and using a wet cell pellet fixed with $HgCl_2$, respectively. The lyophilization mode may be advantageous from the point of view of simplicity: numerous Raman features are observed in this type of samples, especially in the 400–1200 cm^{-1} region and after fluorescence subtraction. Lyophilization of the water phase from a two-phase chloroform/methanol/water extraction resulted in an important concentration of compatible solutes; however, residual fluorescence background was still present (Figs. 2C, 3A). Following perchloric acid extraction, residual traces of perchlorate remained present (Figs. 2D, 3B). Raman bands were found in the spectrum corresponding to K-perchlorate (bands at 1124, 1086, 941, 641, 634, 628, and 464 cm^{-1}, bands marked *x* in Supplementary Table 2), and these can compromise the reading of the Raman spectra. Intense and well-separated Raman bands were only obtained after passing the perchloric acid extract through the ion retardation column followed by concentration by lyophilization (Fig. 5; column E in Supplementary Tables 1 and 2). Raman features in the second order were recorded as well in samples subjected to this treatment.

Halomonas elongata grown in rich medium

Figure 2 shows the Raman spectra corresponding to *Halomonas elongata* grown in a rich medium containing yeast extract. Raman bands observed are listed in Supplementary Table 1. Glycine betaine is the compound identified by interpretation of the spectra, agreeing well with the earlier observations using other analytical techniques (Imhoff and Rodriguez-Valera 1984; Ventosa et al. 1998). Carboxyl ion vibrations reported for crystalline betaine at 1624, 1614, 691, 605 and 539 cm^{-1} (Ilczyszyn and Ratajczak 1996; Jehlička et al. 2012) were found at 1621, 1611, 689, 600 and 540 cm^{-1} in preparations of *H. elongata*. Antisymmetric and symmetric C_3N stretching modes occur in the obtained spectra at 1007, 974 and 781 cm^{-1} bands corresponding to deformation modes at 450 and 424 cm^{-1} (antisym) and 361 and 339 cm^{-1} (sym). All band positions correspond well to those previously published for glycine betaine. Some bands, such as the carboxyl ion vibrations at and above 1600 cm^{-1} were very faint or absent in most extracts. Raman bands of medium and strong intensity were consistently found. The most characteristic feature for betaine is the presence of the 781 symmetric C_3N stretching mode band. This band does not occur in other compatible solutes and can be used as a marker for the presence of betaine in a sample. The best result was obtained using a lyophilized perchloric acid extract after desalting on the ion retardation column. In this case, not only a complete series of first order Raman bands of glycine betaine was recorded but also the bands from the second order spectra area (see Supplementary Table 1).

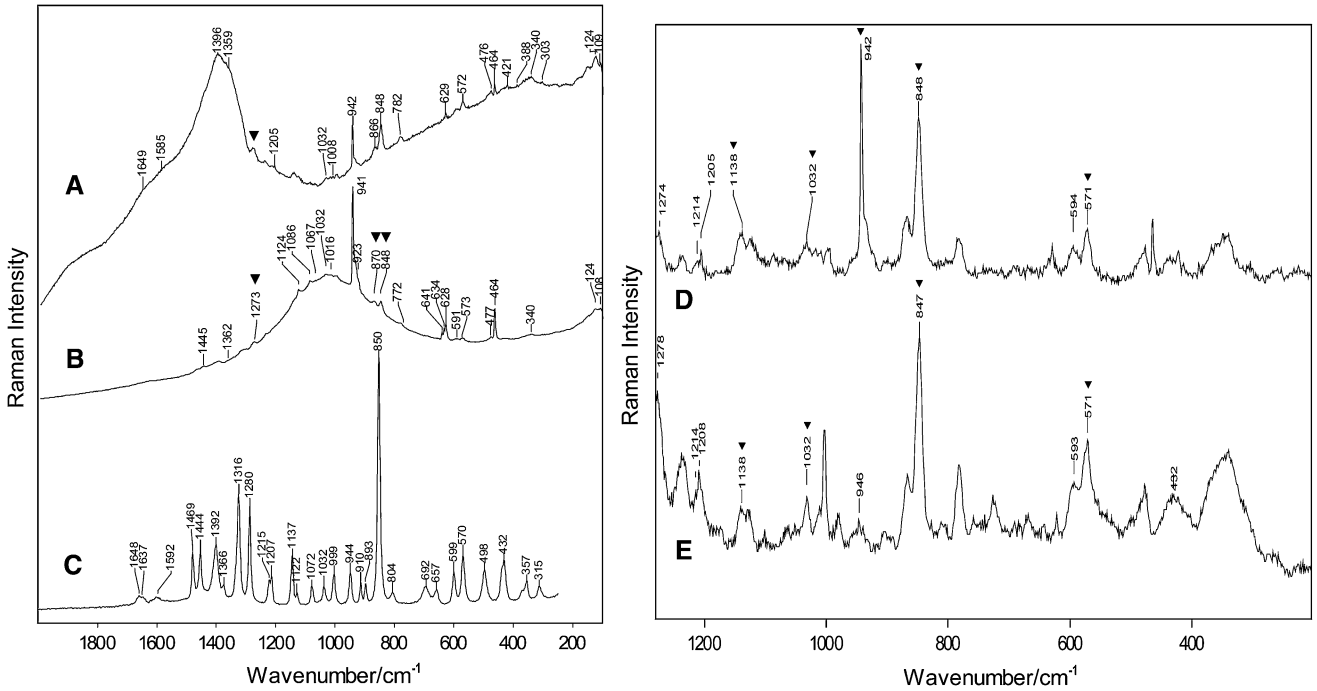

Fig. 3 Raman spectra (excitation at 785 nm) of material originating from a culture of *Halomonas elongata* grown in minimal medium containing 10 % NaCl: the lyophilized water phase from a two-phase extraction with methanol/chloroform/water (*A*, *D*), a lyophilized perchloric acid extract (*B*) and a wet preparation of cells fixed with $HgCl_2$ (*E*). For comparison the Raman spectrum of ectoine is shown (*C*). Spectra shown in *D* and *E* are background corrected spectra. Signals attributable to ectoine are marked with *arrows*. A spectrum of the perchloric acid extract after desalting on an ion retardation column is shown in Fig. 4B. Further data are given in Supplementary Table 2

Halomonas elongata grown in poor medium

Raman bands observed in preparations of *H. elongata* grown in defined medium with glucose as carbon source are given in Supplementary Table 2. Figure 3 shows Raman spectra of culture preparations [two-phase chloroform/methanol/water extraction (A) and perchloric acid extraction (B)], as well as a reference Raman spectrum of pure ectoine. Strong and medium Raman bands of ectoine (Fig. 3C) occur at the correct positions (± 5 cm^{-1}) in the spectra of the cell preparations. In the case of perchloric acid extraction, weak bands at 1125, 1087, 941, 641, 634, 628, and 464 cm^{-1} corresponding to K-perchlorate are well seen in Fig. 3B. Lower numbers of medium- and low-intensity bands were recorded in comparison with the glycine betaine spectra obtained from *Halomonas* grown in a rich medium (Fig. 2). The observation of characteristic medium- and strong-intensity bands permits the unambiguous identification of the compatible solute present in the cell as ectoine. A complete series of Raman bands corresponding to ectoine was obtained from the lyophilized perchloric acid extract after passing through the ion retardation column. In addition, three second-order bands were collected (Supplementary Table 2).

Ectothiorhodospira marismortui

Raman analysis of lyophilized and $HgCl_2$-fixed wet cells of *E. marismortui*, using the 785 nm laser, only showed an intense fluorescence background, and no Raman features were observed. Sharp and well-separated Raman bands were only obtained after a perchlorate extraction and desalting over the ion retardation column (Fig. 4; Supplementary Table 3). In this case, a complete series of sharp bands corresponding to glycine betaine was recorded, including bands in the second order area.

In addition, we recorded Raman spectra using a 514.5 nm laser. Figure 5 shows the spectrum obtained for an untreated lyophilized sample; corresponding band positions are given in Supplementary Table 4. Strong carotenoid signals were observed at 1514, 1152 and 1003 cm^{-1}, together with weaker bands at 1569, 1448, 1282, 1212, 1198, 1184, and 863 cm^{-1}. The major bands can be assigned to in-phase vibrations of the polyene chain in carotenoids: ν_1 (C=C) at 1514 cm^{-1}, ν (C–C) at 1152 cm^{-1}, and to in-plane rocking modes of CH_3 groups attached to the polyene chain coupled with C–C bonds ω (CH_3) at 1003 cm^{-1} (Withnall et al. 2003). However, the use of the Ar ion, 514.5 nm excitation, prevented the observation of Raman bands of compatible solutes.

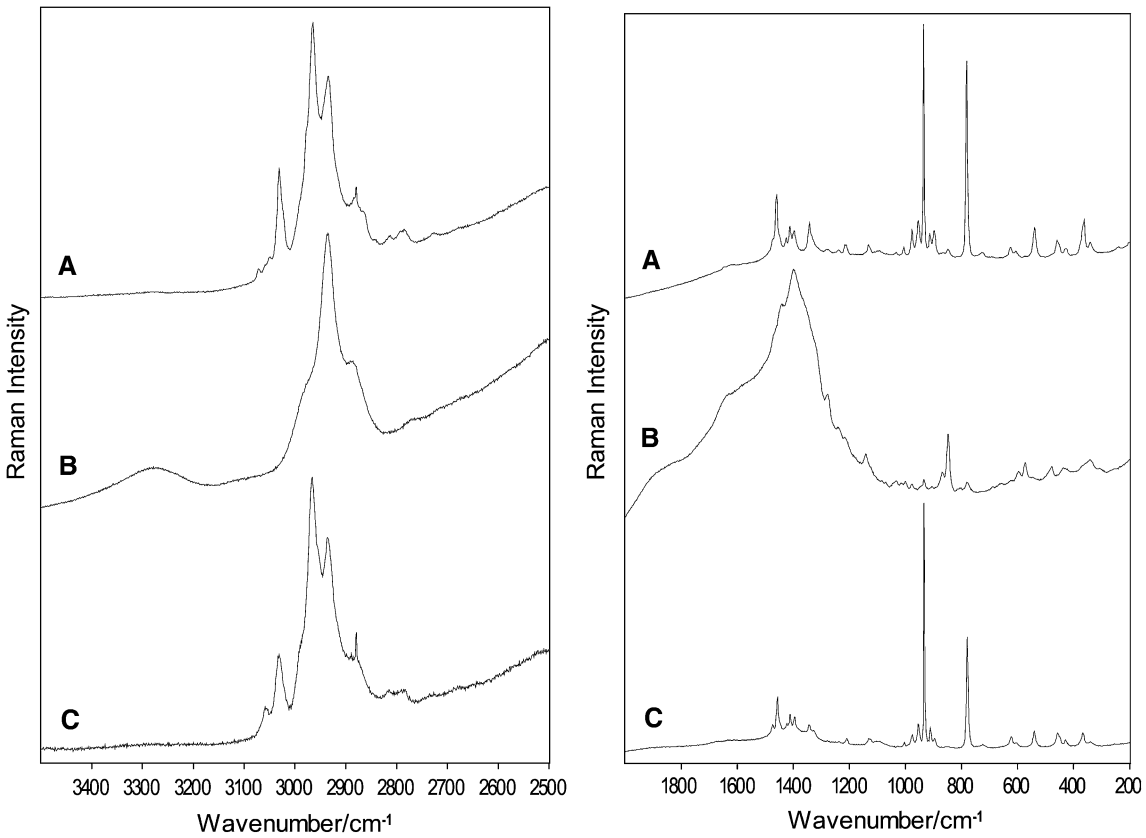

Fig. 4 Raman spectra (excitation at 785 nm, second order *left*, first order *right*) of lyophilized material from a culture of *Halomonas elongata* grown in minimal medium (*A*), *Halomonas elongata* grown in complex medium (*B*), and *Ectothiorhodospira marismortui* (*C*), after perchloric acid extraction and desalting on an ion retardation column. Further data are given in Supplementary Table 3

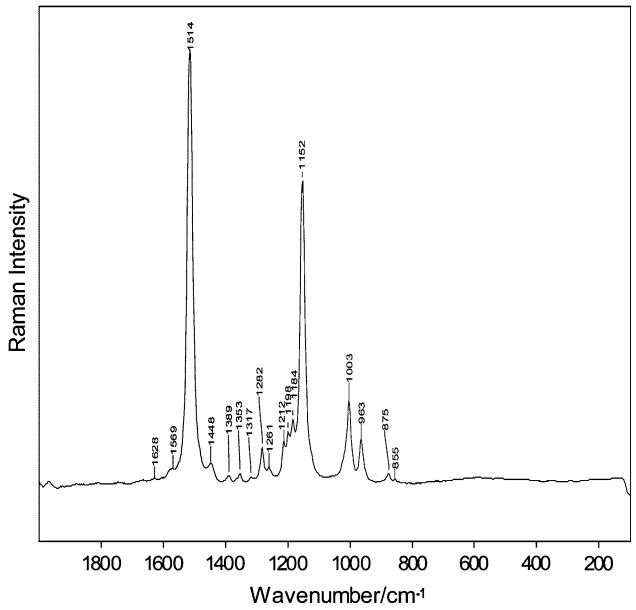

Fig. 5 Raman spectrum (excitation at 514 nm) of lyophilized material from a culture of *Ectothiorhodospira marismortui* grown in medium containing 10 % NaCl. Signals at 1152 and 1514 cm^{-1} can be attributed to the spirilloxanthin carotenoids. Further data are given in Supplementary Table 4

Discussion

The results presented above show that the Raman spectroscopy is a suitable technique for the detection of organic compatible solutes in halophilic bacteria. To the best of our knowledge, the approach has not been used before. In a previous report (Jehlička et al. 2012), we established a database of Raman spectra of pure compatible solutes, using both 514 and 785 nm excitation lasers. In our current study, we have demonstrated that the near-infrared 785 nm lasers are especially suitable for the identification of compatible solutes in cultures of halophilic microorganisms. The 514 nm excitation is recommended for carotenoid studies, as strong Raman bands as well as pre-resonance and resonance bands are obtained for many pigments.

Earlier studies have shown that *H. elongata* accumulates glycine betaine when present in the medium and synthesizes ectoine when no suitable solutes are found in the growth medium (Imhoff and Rodriguez-Valera 1984; Severin et al. 1992; Wohlfarth et al. 1990); *E. marismortui* is known to produce glycine betaine as the major osmotic solute, together with minor amounts of trehalose and

N-α-carbamoyl glutamineamide (Galinski and Oren 1991; Oren et al. 1991). Using Raman spectroscopy, we easily detected glycine betaine and ectoine in *H. elongata*, and the position of the bands obtained corresponding relatively well to pure reference compounds (Jehlička et al. 2012). Slight differences are commonly observed between the general shape (width) as well as band positions obtained from Raman spectra of pure (crystalline) chemicals and the same compounds in biological materials, due to solvent effects and binding of compounds to other cellular structures (Marshall and Marshall 2010; de Oliveira et al. 2010).

The presence of carotenoid and other pigments can easily interfere with the recording and interpretation of Raman spectra for compatible solute detection, as our example of *E. marismortui* shows. *E. marismortui* is known to contain bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series (Oren et al. 1989). The bands observed in intact cells and lyophilized cell preparations using 514 nm excitation (Fig. 5) are dominated by carotenoid signals. Only after perchlorate extraction and desalting, we obtain clear signals of the major osmotic solute glycine betaine, using the 785 nm laser. The method used was not sufficiently sensitive to also detect trehalose (a compound included in the reference spectra library (Jehlička et al. 2012) and/or signals that can be attributed to the unique minor compound *N*-α-carbamoyl glutamineamide, a compound not available in pure form as a reference.

Our data show that Raman spectroscopy can be used as an additional, relatively simple and robust analytical tool to complement methods of HPLC, ^{1}H-NMR, natural abundance ^{13}C-NMR and other techniques in use for the qualitative and quantitative detection of organic osmotic solutes in microorganism (Galinski 1993, 1995; Roberts 2006). A clear advantage of Raman spectroscopy is the rapid analysis: recording spectra as shown in Figs. 2, 3, 4 and 5 typically takes a few minutes only. To achieve similar results using HPLC and NMR techniques, much more time is needed, as well as expensive chemicals (HPLC-grade solvents, deuterated water, etc.). Raman signals to be attributed to the major osmotic compounds can already be detected with no or minimal sample preparation. However, further purification steps, that require more preparation time, allow a more sensitive and reliable assessment of the types of osmotic compounds present.

We are currently exploring the use of the techniques developed in the current study for the assessment of the presence of different osmotic solutes within stratified microbial communities as those that occur within sediments in hypersaline systems, e.g., saltern evaporation ponds (Oren et al. 1995). The results will be reported elsewhere.

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