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Fourier transform Raman spectroscopic characterisation of cells of the plant-associated soil bacterium *Azospirillum brasilense* Sp7

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Abstract

Structural and compositional features of bacterial cell samples and of lipopolysaccharide–protein complex isolated from the cell surface of the plant-growth-promoting rhizobacterium *Azospirillum brasilense* (wild-type strain Sp7) were characterised using Fourier transform (FT) Raman spectroscopy. The structural spectroscopic information obtained is analysed and considered together with analytical data on the content of metal cations (Co^{2+}, Cu^{2+} and Zn^{2+}) in the bacterial cells grown in a standard medium as well as in the presence of each of the cations (0.2 mM). The latter, being taken up by bacterial cells from the culture medium in significant amounts, were shown to induce certain metabolic changes in the bacterium revealed in FT-Raman spectra, which is discussed from the viewpoint of bacterial response to environmental stresses.

Keywords: Fourier transform Raman spectroscopy; *Azospirillum brasilense*; Lipopolysaccharide–protein complexes; Metal cations

1. Introduction

Over the 1990s, a representative series of reports have appeared on various aspects of vibrational spectroscopic studies of diverse biological objects [1–15], based on the rapidly expanding use of modern Fourier-transform spectrometers. Owing to the greatly enhanced sensitivity and resolution of the latter and improved overall characteristics, Fourier transform infrared (FTIR) and FT-Raman spectroscopies allow a number of fine structural effects to be revealed, starting from the levels of functional groups of macromolecules and complicated supramolecular structures [4,7,9–16] up to whole cells [1–3,5,6,8,16]. In particular, in the field of microbiology, the FTIR and FT-Raman techniques applied to whole-cell samples have been shown to be capable of discriminating between certain cell components and, on the other hand, different bacterial species and strains as well as their modifications [1–3,5,6,8,16–18].

Some of our earlier reports [19–22] were focussed on FTIR spectroscopic studies of intact cells, cell membranes and their major constituents of the soil bacterium *Azospirillum brasilense* cultured under

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different conditions, which were complemented by the use of other analytical techniques. The results revealed certain effects of a range of conventionally toxic heavy metals (some of which in trace amounts are essential components of various enzymes) added to the medium in concentrations (0.2 mM) which did not cause any significant suppression of culture growth. Those investigations, along with the basic scientific interest in studying bacterial responses to certain stress factors, are also in line with the worldwide attention of researchers to the bacterial genus *Azospirillum* (see, e.g. [23–31], including recent reviews [24–26,29,30], and references therein) and, in particular, to one of its species known to date, viz. *A. brasilense*. Azospirilla are free-living plant-associated soil bacteria known to proliferate in the rhizosphere of various plant species (but tending to gradually die out in bulk non-planted soil [23]) and stimulate host plant growth and development. This feature accounts for the importance of studying environmentally, nutritionally or otherwise induced metabolic responses of azospirilla [19–23,31–34]. Different strains of *A. brasilense*, along with certain similarities between some of them [30], are known to occupy different ecological niches, e.g. essentially differing in the mode of plant root colonisation [30] and in responses of the host plant induced by the latter process [31].

In the present work, we applied FT-Raman spectroscopy to the study of cells of the bacterium *A. brasilense* (wild-type strain Sp7) grown both in a standard medium and in the presence of certain metal cations (Co^{2+}, Cu^{2+} and Zn^{2+}), complemented by inductively coupled plasma-mass spectrometric (ICP-MS) analyses of the biomass samples for the metals taken up by the bacterial cells. The FT-Raman spectroscopic information obtained is considered from the viewpoint of structural organisation of the bacterial cell envelope and its major constituents, including the effects of metal cations.

2. Experimental

2.1. Preparation of bacterial cultures

A. brasilense (wild-type strain Sp7) from the collection of IBPPM RAS (Saratov, Russia) was cultivated in a standard synthetic phosphate- and malate-containing medium as reported elsewhere [19–21], with addition of 0.5 g/l NH_4Cl as a bound nitrogen source (pH 6.9), in 200-ml flasks containing 100 ml of the cultural media under excessive aeration (stirring on a rotatory shaker). Along with the experiments in the standard medium (control), bacteria were similarly cultured also in the same media, to which 0.2 mM of $CoCl_2$, $CuSO_4$ or $ZnSO_4$ had been added. Bacterial cells were harvested by centrifugation, washed and dried in air as reported earlier [19,20].

2.2. Sample preparation, FT-Raman spectra acquisition and data treatment

FT-Raman spectra were measured using a Nicolet spectrometer (model Magna IR 750) with a FT-Raman accessory (Nd^{3+}-YAG laser, 1064 nm excitation wavelength with ca. 0.48–0.50 W laser power at the sample and a total of 250 scans applied; InGaAs detector; beamsplitter: CaF_2). Samples of dried bacterial biomass (25–35 mg) were mixed with 70 mg KBr (Merck, spectroscopic purity; dried in air over P_2O_5 at 50°C) and ground in a small agate mortar for 5 min. The resulting mixture was pressed with a glass rod in a standard NMR tube (4 mm internal diameter), closed with a plastic cap, attached to the sample holder, and the laser beam of the spectrometer was focussed at the centre of the sample. (It appeared impossible to obtain FT-Raman spectra of pure cell samples without KBr even at lower laser power values owing to immediate local decomposition of the sample under laser irradiation). Treatment of the spectra was performed using the OMNIC 3.1 software (Nicolet) accompanying the equipment.

If not indicated otherwise, all measurements were performed at ambient temperature (295 ± 3 K).

2.3. Analyses of bacterial samples for metal cations

Metal cations (Co, Cu and Zn) were determined in the same bacterial samples that were used for FT-Raman measurements. Precisely weighed portions of the dried bacterial biomass (10–37 mg) were digested as described earlier [19–21] and analysed by ICP-MS using an ICP-MS spectrometer (Hewlett–Packard 4500).

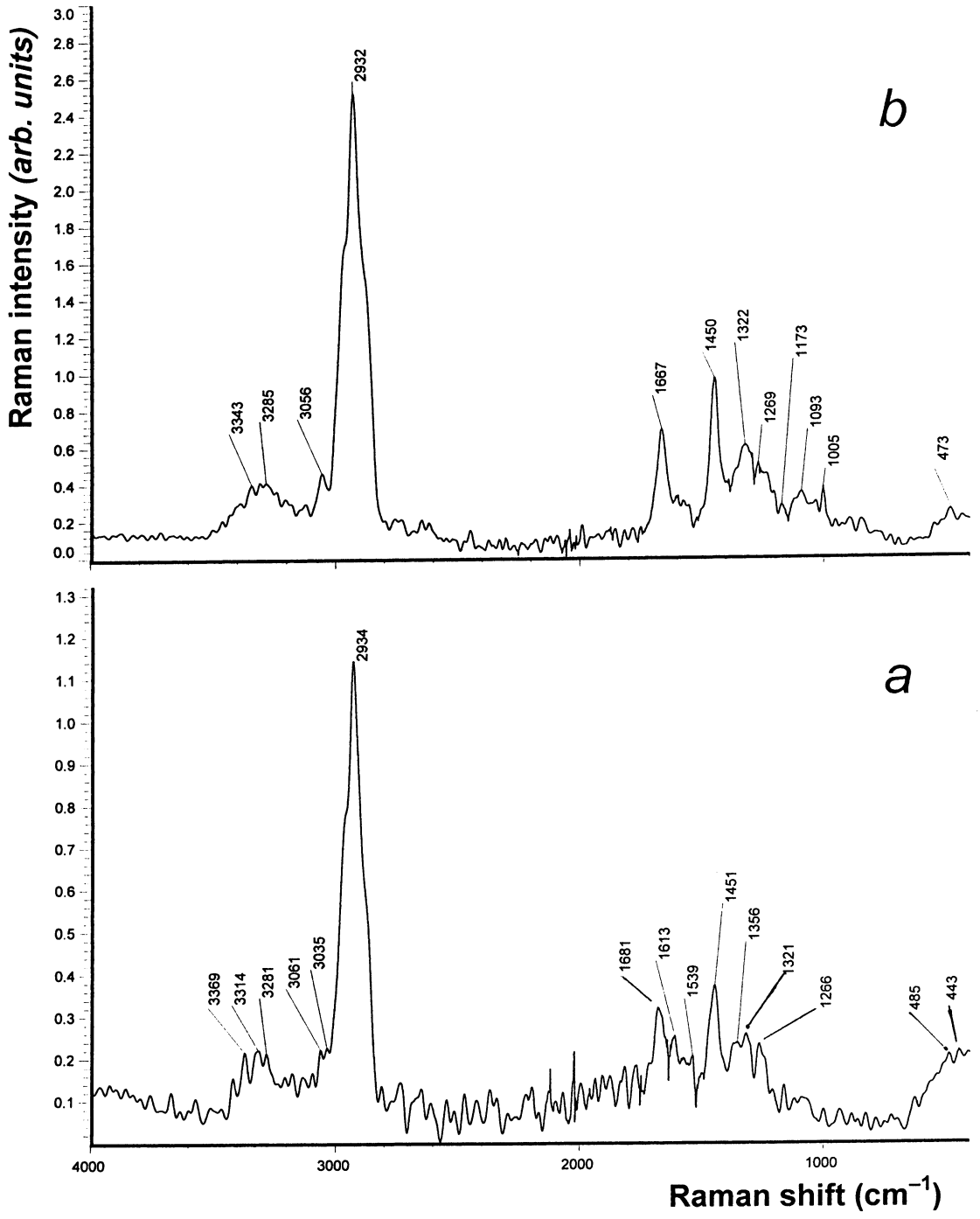


Fig. 1. Overall view of Fourier transform Raman spectra of (a) cells of *Azospirillum brasilense* Sp7 and (b) lipopolysaccharide–protein complex isolated from the bacterial cell surface.

Table 1
Content of metals, added to the cultivation medium (see Section 2.1), in dried cells of *Azospirillum brasilense* Sp7, determined using ICP-MS

Cultivation medium	Content of metals/mg per g of dried biomass		
	Co	Cu	Zn
Standard (control)	0.0005	0.047	0.025
With 0.2 mM Co^{2+}	0.118	0.002	0.027
With 0.2 mM Cu^{2+}	0.0007	0.477	0.035
With 0.2 mM Zn^{2+}	0.0006	0.007	4.24

3. Results and discussion

Raman spectroscopic images of whole cells represent the overall cellular composition and are controlled mainly by two factors [5], viz. (*i*) the relative abundance of all the cellular components contributing to the spectrum and (*ii*) the physical factors governing peak intensities. The former point is obviously the same for IR spectroscopy, whereas the latter is essentially different [35,36]. Thus, if polar groups (and water in particular) give stronger absorption bands in IR and weaker in Raman, less polar or non-polar fragments (e.g. C–H, C–C, C=C) exhibit much stronger Raman scattering bands and a weaker IR absorption, owing to which these two 'counterparts' of vibrational spectroscopy provide complementary information [5,36]. For FT-Raman spectroscopy of bacterial samples, the near-IR excitation wavelength used (1064 nm) provides for fluorescence-free spectra even in the presence of highly coloured cell constituents (e.g. carotenoids which are present in azospirilla [37]).

An overall FT-Raman spectrum of *A. brasilense* Sp7 cells (4000–400 cm^{-1}) is presented in Fig. 1a. As could be expected for bacterial cells, the most intensive scattering region is at ca. 3100–2800 cm^{-1} featuring various C–H stretching modes of =CH–, $–CH_2–$ (methylene) and terminal methyl ($–CH_3$) groups mainly of fatty acid chains of lipopolysaccharide (LPS) and phospholipid (PL) constituents of *Azospirillum* cell envelope [21,22,26,33,34,38–40]. The other representative (fingerprint) region, under 2000 cm^{-1}, features the amide I band (ν(C=O) in peptides) overlapping with ν(C=C) of unsaturated fatty acid (UFA) chains at about 1650–1680 cm^{-1}; C–H deformation bands (largely the typical $\delta(CH_2)$ scissoring band at about 1450 cm^{-1}); ν(C–C), δ(C–C–H) and methylene twisting bands (1360–1300 cm^{-1}); amide III (ν(C–N) in peptides) overlapping with δ(=C–H) in UFA at 1270–1250 cm^{-1}; skeletal C–C, C–O and stretching C–C–O bands (1200–800 cm^{-1}) [5,35,36].

For comparison, in Fig. 1b is shown a FT-Raman spectrum of LPS–protein complex (LPS–PC) obtained from the cell surface of *A. brasilense* Sp7, which was characterised by FTIR previously [22]. The obvious similarity of both spectra (see Fig. 1a and b), including the fingerprint region, corresponds to the essential role in the overall cell composition played by major LPS- and protein-containing components [34,38]. Concerning the bacterial cell surface mainly composed of polysaccharides (PS), LPS–PC and PS–lipid complexes typical for azospirilla [21,26,39,40], both LPS and protein components are believed to be involved in associative contact interactions of azospirilla with plant roots [26,32,39,40] and other supports [32,41], as well as in its cell aggregation [33,34,42]. Moreover, certain extracellular PS components of azospirilla specifically interacting with cereal root lectins [26,39] may have a signalling function [43,44].

Metal uptake and related effects in *A. brasilense* are of essential interest and are paid special attention in this study since metal cations have been commonly acknowledged to play a vitally important role in the processes of microbial metabolism, in particular, for this bacterium [19–21,45]. In our previous reports [19–21], we have shown that enhanced metal uptake by *A. brasilense* cells induces certain structural and/or compositional changes revealed in FTIR spectra of intact cells and cell membranes. Here we analyse the effects of cobalt(II), copper(II) and zinc(II) cations added to the culture media (0.2 mM) on the FT-Raman spectroscopic images of bacterial cells in various spectral regions, as well as their accumulation in cells during cultivation (Table 1). It can be seen that each of the three metals, when added to the culture medium, is taken up in essentially higher quantities than when present as a trace impurity in the standard medium. This corresponds to our data on metal uptake in strain Sp245 of this bacterium [19,20]. Note that in the presence of 0.2 mM Co^{2+} or Zn^{2+} the uptake of trace amounts of copper from the medium tends to

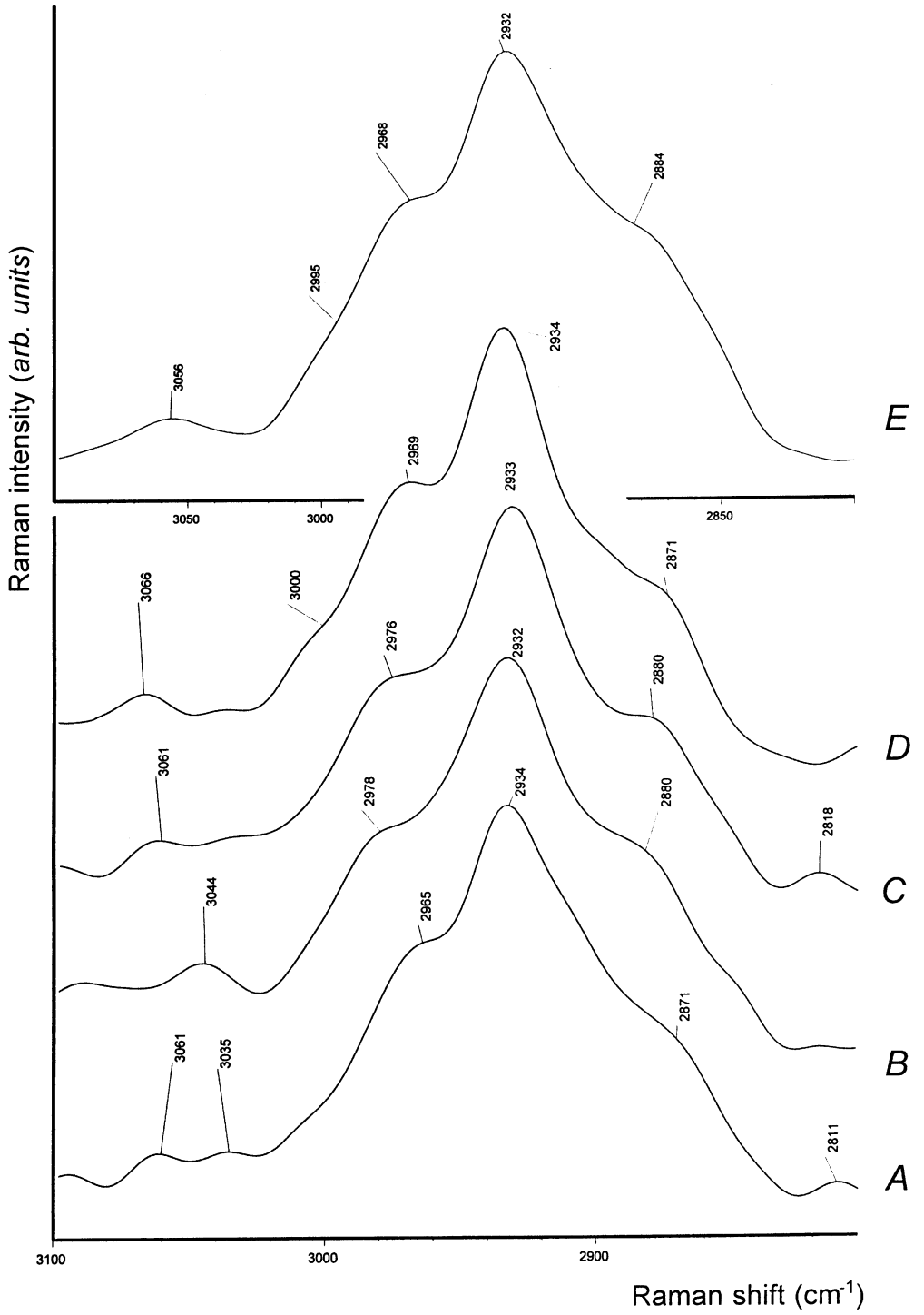
diminish as compared to the control (see Table 1) which can be related to a redistribution of binding sites in the excess of a certain cation.

The choice of these conventionally toxic heavy metals is based on the fact that firstly, all of them are involved in diverse enzymatic activity in bacteria (see [20] and references therein); secondly, they were found to noticeably accumulate in cells of *A. brasilense* (wild-type strain Sp245) from the medium with 0.2 mM of each of them, especially Zn (up to ca. 5 mg per g of dry biomass) [19,20]. Moreover, Cu^{2+} has recently been shown to induce drastic changes in the electrophysical properties of the *A. brasilense* Sp245 cell surface monitored by electro-optical spectroscopy of cell suspensions at different frequencies of the orienting electric field [46]. As for cobalt(II), besides its role in regulating the activity of glutamine synthetase (GS, a key enzyme of nitrogen metabolism) in azospirilla [45], our recent studies using emission ^{57}Co Mössbauer spectroscopy in frozen solutions have shown that the metal is rapidly complexed by *Azospirillum* cells and, within an hour, is further metabolised; also Co^{2+} interaction with GS showed its binding to two sites with different coordination symmetry (A.A. Kamnev, L.P. Antonyuk et al., in preparation) which is in line with biochemical data on GS activated by divalent cations [45].

Fig. 2 shows the expanded region of C–H stretching bands (3100–2800 cm^{-1}) for cells of *A. brasilense* grown in a standard medium and in the presence of Co^{2+}, Cu^{2+} and Zn^{2+} (A–D, respectively) and for LPS–PC (E) added for comparison. This spectral region comprises several contributions from different CH-containing groups mainly of fatty acid acyl chains in LPS and PL, including the most abundant: methylene symmetric and antisymmetric modes $\nu_s(CH_2)$ at 2850–2860 cm^{-1} and $\nu_{as}(CH_2)$ at 2930–2935 cm^{-1}, methyl $\nu_s(CH_3)$ at 2870–2890 cm^{-1} and $\nu_{as}(CH_3)$ at 2965–2980 cm^{-1}, as well as ν(=CH) at 2995–3015 cm^{-1} featuring UFA [35,36]. It should be noted that, despite the much lower intensities of the bands related to C–H stretching vibrations in IR, a number of reports focussed on the use of the $\nu_s(CH_2)$ band (around 2850 cm^{-1} in IR) to monitor acyl chain packing and order–disorder transformations [3,13,14]. While such FTIR studies both in model membranes [13,14] and in intact cells [3] can undoubtfully provide valuable information, however, Brandenburg et al. [15] have recently shown that the IR position of the $\nu_s(CH_2)$ band for some LPS forms may be influenced by externally added cations or at reduced water content indicating an increase in acyl chain packing density, whereas the X-ray data provide evidence that the latter does not change. It has also been concluded [15] that different head groups in LPSs affect the $\nu_s(CH_2)$ frequencies even if their lipid moieties are identical, which impedes their quantitative comparison. Anyway, the higher Raman intensities of the C–H stretching bands mentioned above, in principle allow the FA composition to be compared.

The profiles of the overall ν(CH) band envelope for *A. brasilense* cells (see Fig. 2A–D) are slightly but distinctly different. In particular, it can be seen that in the case of Co^{2+} (B) and Cu^{2+} (C) the contribution of ν(=CH) vibrations featuring UFA (around 3000–3010 cm^{-1}) is decreased. This may reflect a decrease in UFA content in metal-stressed bacteria, thus decreasing the relative susceptibility of the component FA moieties within the cell envelope to metal-induced lipid peroxidation [47] and membrane permeability. Nonetheless, for Zn^{2+} (see Fig. 2D) this effect is not observed, which is in line with its stable oxidation state.

Fig. 3a–d shows the fingerprint regions of FT-Raman spectra of control (a) and metal-stressed cells (b–d). Besides an obvious higher hydration of the latter as compared to the control, reflected by the higher broad 'humps' in the regions of water vibrations (the ν(OH) region over 3200 cm^{-1} not shown) in Fig. 3b–d, one can see some other spectral differences. Thus, a new band at 943–945 cm^{-1} appears, which is most prominent in the case of Co^{2+} (see Fig. 3b), noticeable in the case of Cu^{2+} (c) and weaker for Zn-stressed cells (d). This band lying in the region of C–C–O vibrations may reflect accumulation of some ester compound, e.g. poly-β-hydroxybutyrate (PHB) which is known to accumulate in cells of azospirilla under unfavourable conditions in high amounts, playing a role in increasing bacterial tolerance to environmental stresses [33,34,48]. The presence of PHB in *Azospirillum* cells evidently accounts for the strong ν(C=O) ester carbonyl band in their FTIR spectra at 1730–1750 cm^{-1} [16,19–21], which is


Raman intensity (arb. units)
Raman shift (cm-1)
3100
3000
2900
3050
3000
2850
E
3056
2995
2968
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D
3066
3000
2969
2934
2871
C
3061
2976
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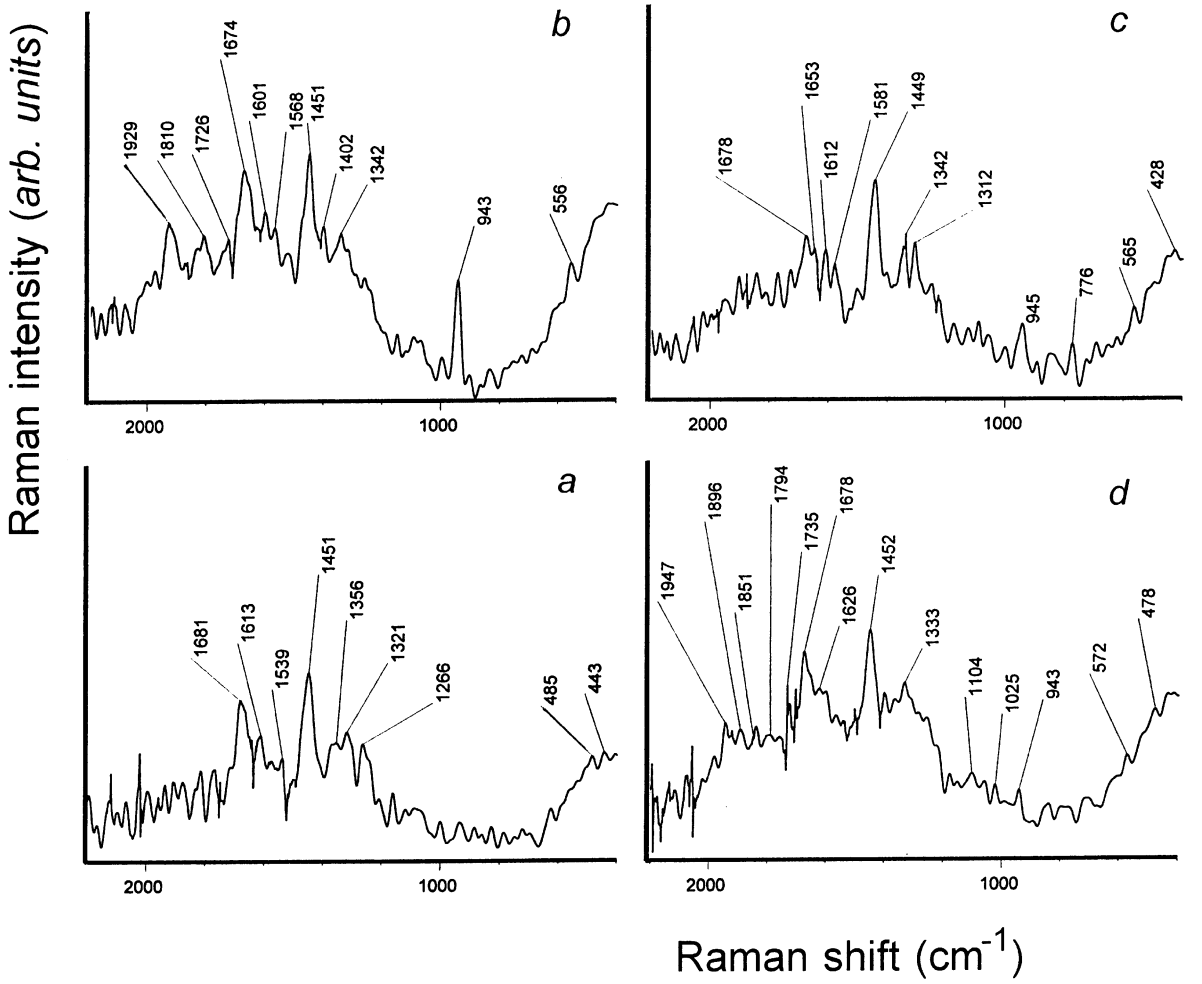

Fig. 3. Fourier transform Raman spectra (in the fingerprint region) of cells of *Azospirillum brasilense* Sp7 grown (a) in a standard medium, as well as in the presence of 0.2 mM of (b) Co^{2+}, (c) Cu^{2+} and (d) Zn^{2+}.

either absent or much weaker in FTIR spectra of many other bacteria [1–6]. It is essential that metal stress induces metabolic changes leading to the accumulation of certain organics within the cell revealed in FT-Raman spectra, as the band at 943 cm^{-1} cannot be caused by any metal compound owing to insufficient overall metal concentrations (see Table 1).

Considering the hydration effects, it should be noted that divalent metal cations may induce different effects on LPS and PL. For example, Mg^{2+} was shown to induce dehydration of the LPS phosphate groups with a concomitant increase in the hydration of the ester carbonyl groups, whereas Zn^{2+}, on the contrary, induced phosphate hydration [11]. These processes may be regulated by the formation of relatively stable

Fig. 2. Fourier transform Raman spectra (in the region 3100–2800 cm^{-1}) of cells of *Azospirillum brasilense* Sp7, grown (A) in a standard medium and in the presence of 0.2 mM of (B) Co^{2+}, (C) Cu^{2+} and (D) Zn^{2+}, as well as of lipopolysaccharide–protein complex isolated from the bacterial cell surface (E).

networks of H-bonds and/or salt bridges between the phosphate and protonated amide moieties, e.g. in phosphatidylethanolamine [49] (the major phospholipid in Gram-negative bacteria including azospirilla [21,34]), which could be affected by certain metal cations.

4. Conclusions

Cells of the plant-associated rhizobacterium *A. brasilense* (wild-type strain Sp7) and LPS–PC isolated from its cell surface were comparatively characterised using FT-Raman spectroscopy. Analysis of FT-Raman spectra of cells of *A. brasilense* Sp7 grown in a standard medium and in the presence of 0.2 mM cobalt(II), copper(II) or zinc(II), complemented by ICP-MS analytical data for the cations, have revealed certain metabolic changes induced by the latter. It is proposed that the metal cations induce, albeit to a different extent, a decrease in unsaturated fatty acids (Co, Cu) and accumulation of polyester compounds (e.g. PHB) in bacterial cells as a response to metal stress.

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