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Fourier transform infrared spectroscopic characterisation of heavy metal-induced metabolic changes in the plant-associated soil bacterium *Azospirillum brasilense* Sp7

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Abstract

Structural and compositional features of whole cells of the plant-growth-promoting rhizobacterium *Azospirillum brasilense* Sp7 under standard and heavy metal-stressed conditions are analysed using Fourier transform infrared (FTIR) spectroscopy and compared with the FT-Raman spectroscopic data obtained previously [J. Mol. Struct. 563–564 (2001) 199]. The structural spectroscopic information is considered together with inductively coupled plasma-mass spectrometric (ICP-MS) analytical data on the content of the heavy metal cations (Co^{2+}, Cu^{2+} and Zn^{2+}) in the bacterial cells. As a bacterial response to heavy metal stress, all the three metals, being taken up by bacterial cells from the culture medium (0.2 mM) in significant amounts (ca. 0.12, 0.48 and 4.2 mg per gram of dry biomass for Co, Cu and Zn, respectively), are shown to induce essential metabolic changes in the bacterium revealed in the spectra, including the accumulation of polyester compounds in bacterial cells and their enhanced hydration affecting certain IR vibrational modes of functional groups involved.

Keywords: Fourier transform infrared spectroscopy; inductively coupled plasma-mass spectrometry; *Azospirillum brasilense*; Heavy metal stress

1. Introduction

Over the past decade, vibrational spectroscopy based on the rapidly expanding use of modern Fourier-transform (FT) IR and Raman spectrometers has been shown to be a valuable and informative tool for the structural characterisation of diverse biological objects, starting from functional groups of biopolymers and supramolecular assemblies up to intact cells [1–9]. Some of our previous research was focused on structural spectroscopic and analytical studies on whole cells and cell constituents of the plant-associated soil bacterium *Azospirillum brasilense* [10–14] which, among other *Azospirillum* species, attracts the world-wide attention of researchers owing to its plant growth-promoting effects (for reviews see [15–20]). In line with our data previously obtained using FT-Raman

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spectroscopy [13], in the present work we analyse heavy metal-induced metabolic changes in *A. brasilense* (wild-type strain Sp7) reflected by certain FTIR spectroscopic features of its whole cells. The structural spectroscopic data are complemented by inductively coupled plasma-mass spectrometric (ICP-MS) analyses of the cell samples of the bacterium, grown both in a standard medium and in the presence of a certain metal cation (Co^{2+}, Cu^{2+} or Zn^{2+}).

2. Experimental

2.1. Preparation of bacterial cultures

Azospirillum brasilense (wild-type strain Sp7; the collection of IBPPM RAS, Saratov, Russia) was cultivated in a standard synthetic phosphate- and malate-containing medium as reported elsewhere [10–13], with 0.5 g/l NH_4Cl as a bound nitrogen source (pH 6.9), under aeration by stirring on a rotary shaker. Along with the standard medium (control), the bacterium was similarly cultured also in the same media to which $CoCl_2$, $CuSO_4$ or $ZnSO_4$ had been added up to 2.0×10^{-4} M. Bacterial cells were harvested by centrifugation, washed and dried in air as reported earlier [10,11].

2.2. Sample preparation and FTIR spectra acquisition

Bacterial cell samples (see Section 2.1), vacuum-dried prior to FTIR measurements at 0.1–0.5 Torr overnight at ambient temperature, were carefully pressed into pellets with spectroscopically pure KBr (Merck). FTIR spectra were collected with a total of 60 scans at a resolution of 4 cm^{-1} in the transmission mode (4000–400 cm^{-1}) using a Perkin–Elmer FTIR spectrometer (Model 2000) coupled to a PC loaded with an IR Data Manager Program supplied by the manufacturer.

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 spectroscopic measurements. Precisely weighed portions of the dried bacterial biomass (10–37 mg) were digested as described earlier [10–12] and analysed using a Hewlett-Packard ICP-MS spectrometer (model 4500). If not indicated otherwise, all measurements were performed at ambient temperature (295 ± 3 K).

3. Results and discussion

Binding of heavy metals by the cell surface in Gram-negative bacteria, to which azospirilla belong, is mediated primarily by capsular polysaccharide (PS) and lipopolysaccharide (LPS) materials [21–23]. The latter are characteristic for *A. brasilense* and, along with its outer-membrane proteins, are believed to be involved in contact interactions with host plant roots [17,24,25] and other surfaces [24,26], as well as in bacterial cell aggregation [17,27,28].

Our previous studies have shown [10–12] that enhanced metal uptake by *A. brasilense* Sp245 cells from the medium induces some structural and/or compositional changes noticeable in FTIR spectra of both intact cells and cell membranes. In this work, we deal with another strain of this bacterium, Sp7, which is known to colonise the plant root surface (in contrast to endophytic strains, e.g. *A. brasilense* Sp245 studied earlier [10–12,14], capable of colonising the root interior) [15]. Therefore, strain Sp7 always occurs in close contact with soil components in the rhizosphere, including occasional heavy metal species. This feature accounts for the importance of studying environmentally induced metabolic responses of the bacterium.

The three metals (Co^{2+}, Cu^{2+} and Zn^{2+}) which are considered as conventionally toxic were chosen for the following reasons. Being involved in diverse enzymatic activities in bacteria in trace amounts (see, e.g. [11] and references therein), each of them was also found to noticeably accumulate in cells of strain Sp245, especially Zn^{2+} (up to ca. 0.5% dry weight), from the medium with 0.2 mM of a metal added [10–12]. Cu^{II} was found to induce drastic changes in the electrophysical properties of the *A. brasilense* Sp245 cell surface [29]. Co^{II} plays a role in regulating the activity of glutamine synthetase (GS, a key enzyme of nitrogen metabolism) in azospirilla [30]. We have shown recently using emission ^{57}Co Mössbauer spectroscopy that Co^{2+} is rapidly bound by *A. brasilense* Sp245 cells and then further metabolised; also, insertion of $^{57}Co^{2+}$ cations in GS showed their binding to two active sites of the enzyme with different coordination and affinity [14].

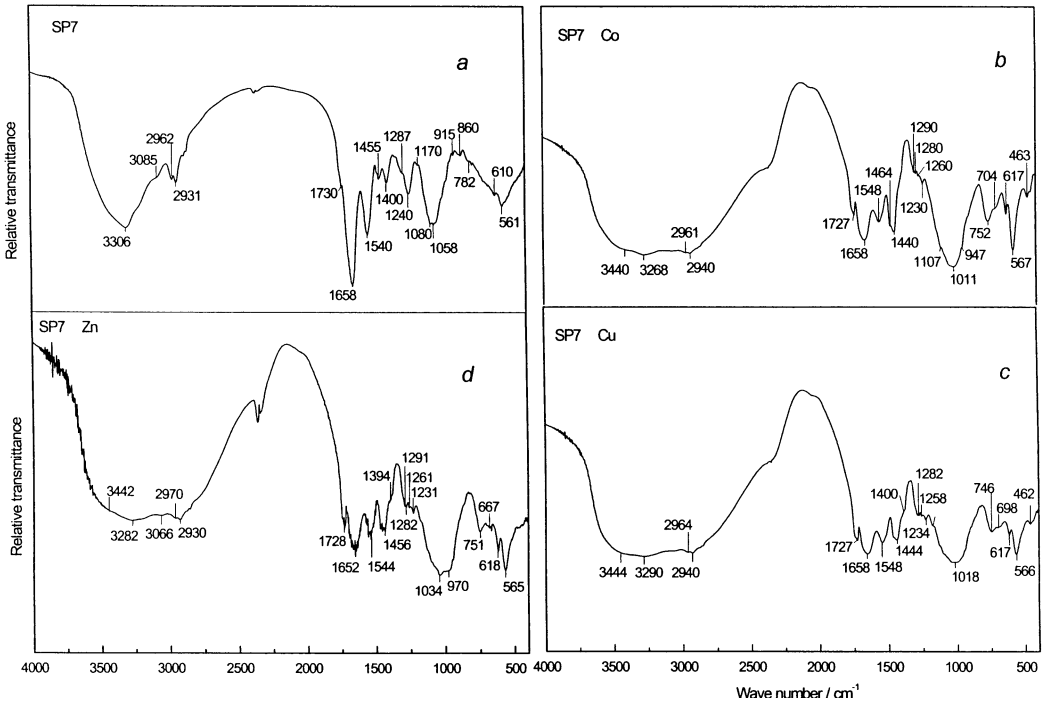

Fig. 1. Fourier transform infrared spectra of vacuum-dried whole cells of *Azospirillum brasilense* Sp7 grown (a) in a standard phosphate-malate medium (control) as well as in the same medium in the presence of 2.0×10^{-4} M (b) Co^{2+}, (c) Cu^{2+} and (d) Zn^{2+}.

Previously [13], we found that FT-Raman images of intact cells of *A. brasilense* Sp7 grown in the presence of Co^{2+}, Cu^{2+} or Zn^{2+} showed some specific features suggesting some metabolic changes induced by the metals. In particular, the profiles of the ν(C–H) vibrations at 3000–2800 cm^{-1} were affected, and an additional band in the C–C–O vibration region (at about 943 cm^{-1}) appeared reflecting some changes in the cell composition as a result of heavy metal stress.

Table 1
Content of metals, either present in the standard cultivation medium as impurities or added to the medium (see Section 2.1), in cells of *Azospirillum brasilense* Sp7 determined using ICP-MS

Cultivation medium	Content of metals (μg/g of dried cells)			Figure
	Co	Cu	Zn	
Standard (control)	0.5	47	25	1a
With 0.2 mM Co^{2+}	118	2	27	1b
With 0.2 mM Cu^{2+}	0.7	477	35	1c
With 0.2 mM Zn^{2+}	0.6	7	4240	1d

Fig. 1a–d shows striking differences in the overall FTIR profiles of the cells of strain Sp7 grown in a standard medium and in the presence of each one of the three heavy metals studied. The uptake level of the latter (Table 1) shows that each added cation (0.2 mM in the medium) is taken up by cells in significantly higher amounts than from the medium where it is present as a natural impurity. Nevertheless, the absolute content of each metal is low enough, so that the major FTIR spectral differences are to be ascribed to metabolic changes that occurred in the bacterium during growth under heavy metal stress.

All the three types of metal-stressed cell samples exhibit a very broad enhanced absorption in the region of strong ν(O–H) vibrations of bound water at about 3400–3000 cm^{-1} (see Fig. 1b–d) reflecting a higher hydration of the cells, which was also assumed considering the profiles of their FT-Raman spectra [13]. This agrees with the shift of the $\nu_{as}(PO_2^-)$ band from 1240 cm^{-1} (Fig. 1a) to 1230–1234 cm^{-1} (Fig. 1b–d) featuring the transition from the dehydrated or

medium-hydrated state to a higher hydration of phosphate moieties [31]. Also, the weak ν(N–H) band of amide NH moieties centred at 3306 cm^{-1} in Fig. 1a is found at lower wavenumbers (3268, 3290 and 3282 cm^{-1}) in Fig. 1b–d, which may result from both H-bonding (owing to enhanced hydration) and metal binding.

Note that divalent cations may induce disparate effects on the hydration level of LPS, PL and other amphiphilic biopolymers [31,32]; note, however, that Zn^{2+} was reported to enhance LPS phosphate hydration (in contrast to, e.g. Mg^{2+}) [31]. These processes may be regulated by the formation of relatively stable H-bond networks and/or salt bridges [31,33] between the phosphate and protonated amide moieties, e.g. in phosphatidylethanolamine (PE) [33] (the major phospholipid in Gram-negative bacteria including azospirilla [12,34]), which could be affected by certain metal cations. Interestingly, the formation of exocellular PE was reported for another Gram-negative soil bacterium, *Pseudomonas fluorescens* [35], thus mediating its tolerance to millimolar quantities of metals (Al^{3+}, Fe^{3+}, Ca^{2+}, Ga^{3+} and Zn^{2+}) simultaneously present in the culture medium.

Another striking feature of the metal-stressed cells is the appearance of a relatively strong and well-resolved ester ν(C=O) band at about 1727 cm^{-1} (see Fig. 1b–d), which in the control cells is seen only as a weak shoulder (see Fig. 1a). In line with our assumptions based on FT-Raman spectra [13], this reflects an enhanced production of poly-3-hydroxybutyrate (PHB) in metal-stressed cells, whereas under normal conditions, with aeration and in N-supplemented media its biosynthesis is decreased [36]. This biopolymer is known to accumulate in cells of azospirilla under unfavourable conditions playing a role in bacterial tolerance to environmental stresses [34,36]. The position of the ν(C=O) band (under 1730 cm^{-1}) corresponds to that of pure PHB found in other bacterial cells [37,38], whereas other polyhydroxyalkanoates (PHAs) including medium-chain-length products exhibit bands at 1732–1740 cm^{-1} [37]. Considering the 'left-hand' asymmetry of the ester ν(C=O) band in Fig. 1b–d with maxima at about 1727 cm^{-1}, PHB is likely to dominate; nevertheless, the presence of other PHAs is possible.

Also increased is absorption in the region of C–O–C and C–C–O vibrations (1150–1000 cm^{-1}) in Fig. 1b–d as compared to that in Fig. 1a, relative to the marker amide I and amide II bands of cellular proteins at ca. 1650 and 1550 cm^{-1}. Note that the amide I band (mainly peptidic ν(C=O) vibrations of cellular proteins), which is rather symmetric in Fig. 1a, is noticeably broadened in all metal-stressed cells. This is likely to reflect some partial changes in the secondary structure of the cellular proteins from dominating α-helix (1657 cm^{-1}) to other possible conformations which give overlapping bands in the range of wavelengths from 1620 to 1690 cm^{-1} [2,8]. An increase in absorption in the region of various δ(C–H) modes (at 1440–1460 cm^{-1}) and about 750–700 cm^{-1} (rocking CH_2 mode) in Fig. 1b–d may result from both the PHB and phospholipid accumulation (e.g. PE; vide supra) as a response to metal stress [12]. Note that the weak ν(C–H) region of methyl and methylene chain groups (3000–2800 cm^{-1}), relatively well resolved in Fig. 1a, is somewhat masked by the enhanced broad ν(O–H) absorption in Fig. 1b–d.

4. Conclusions

The results obtained, based on FTIR spectra of whole bacterial cells, in line with the assumptions made on the basis of previously reported FT-Raman data [13], have revealed essential metabolic changes induced by heavy metal cations (Co^{2+}, Cu^{2+} or Zn^{2+}) taken up by the bacterium from the medium. It is proposed that, along with a possible increase in PL synthesis under metal stress which was assumed earlier for another *A. brasilense* strain (Sp245) [12] and reported for another soil bacterium [35], metal-induced accumulation of PHB may be another feature contributing to the bacterial response to heavy metal stress.

It should be noted that this way of enhancing PHB accumulation in bacterial cells may also be promising for further applied research and basic studies of control mechanisms involved in the biosynthesis of this commercially attractive easily degradable biopolymer [36,39].

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