

## Instrumental analysis of bacterial cells using vibrational and emission Mössbauer spectroscopic techniques

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### Abstract

In biosciences and biotechnology, the expanding application of physicochemical approaches using modern instrumental techniques is an efficient strategy to obtain valuable and often unique information at the molecular level. In this work, we applied a combination of vibrational (Fourier transform infrared (FTIR), FT-Raman) spectroscopic techniques, useful in overall structural and compositional analysis of bacterial cells of the rhizobacterium *Azospirillum brasilense*, with <sup>57</sup>Co emission Mössbauer spectroscopy (EMS) used for sensitive monitoring of metal binding and further transformations in live bacterial cells. The information obtained, together with ICP-MS analyses for metals taken up by the bacteria, is useful in analysing the impact of the environmental conditions (heavy metal stress) on the bacterial metabolism and some differences in the heavy metal stress-induced behaviour of non-endophytic (Sp7) and facultatively endophytic (Sp245) strains. The results show that, while both strains Sp7 and Sp245 take up noticeable and comparable amounts of heavy metals from the medium (0.12 and 0.13 mg Co, 0.48 and 0.44 mg Cu or 4.2 and 2.1 mg Zn per gram of dry biomass, respectively, at a metal concentration of 0.2 mM in the medium), their metabolic responses differ essentially. Whereas for strain Sp7 the FTIR measurements showed significant accumulation of polyhydroxyalkanoates as storage materials involved in stress endurance, strain Sp245 did not show any major changes in cellular composition. Nevertheless, EMS measurements showed rapid binding of cobalt(II) by live bacterial cells (chemically similar to metal binding by dead bacteria) and its further transformation in the live cells within an hour. © 2006 Elsevier B.V. All rights reserved.

**Keywords:** Bacterial cells; Heavy metals; Metabolic processes; Spectroscopic analysis; Fourier transform infrared (FTIR) spectroscopy; Emission Mössbauer spectroscopy

### 1. Introduction

In diverse fields of biological sciences and biotechnology, the expanding application of physicochemical approaches using modern instrumental techniques is an efficient strategy to obtain valuable and often unique bioanalytical information at the molecular level. Various modifications of vibrational (Fourier transform infrared (FTIR), FT-Raman) spectroscopy have been extensively used for structural and compositional analysis of

diverse biological materials [1–6]; in particular, as convenient and sensitive tools for monitoring both macroscopic changes in the cellular composition and fine structural rearrangements of cellular constituents [7–15].

The bioanalytical information jointly obtained by a combination of independent instrumental techniques may often be of advantage, especially when comparing the data on overall cellular metabolic changes (e.g., using vibrational spectroscopy) with analyses for microelements (e.g., trace metal uptake) and/or their chemical forms (speciation analysis). One of the extremely sensitive techniques is the emission variant of Mössbauer (nuclear gamma-resonance) spectroscopy (EMS) that has so far been relatively rarely used in bioscience [16]. Though nuclear analytical

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methods are generally not capable of speciation analysis [17], the EMS technique (not commonly regarded as analytical) can provide quantitative information on the content of chemical species of the Mössbauer-active element. The main limitation of EMS is that its use is restricted to a few such elements, the most convenient nuclide for EMS being the radioactive  $^{57}\text{Co}$  isotope. Nevertheless, cobalt as a trace element with a broad range of biochemical functions is of paramount importance for many organisms [18,19]. It also attracts attention owing to biogeochemical problems related to bioleaching of the radioactive  $^{60}\text{Co}$  isotope from disposal sites [20,21] facilitated by possible microbial dissimilatory reduction of  $\text{Co}^{\text{III}}$  oxide-containing minerals [22,23]. The EMS technique can readily be adapted for in situ studies, giving valuable quantitative information on the structure and rearrangements of cation-binding sites in biomolecules and metalloproteins [16,24,25].

In this work, we compared the results of FTIR spectroscopic analyses of whole cells of different bacterial strains under moderate heavy metal stress (induced by cobalt(II) as well as some other divalent cations (Cu, Zn) at submillimolar concentrations), with the data of emission Mössbauer spectroscopic monitoring of primary binding of  $^{57}\text{Co}$ -cobalt(II) by bacterial cells and its further transformations in live cells. The subject of this study was the plant-associated rhizobacterium *Azospirillum brasilense* that attracts attention owing to its phytostimulating potential [26], including its strain Sp245 which is known to be a facultative endophyte (capable of penetrating to and colonising the plant root interior), and non-endophytic strain Sp7 (colonising the root surface only), that occupy different ecological niches and show some differences in behaviour [27,28].

## 2. Experimental

### 2.1. Preparation of bacterial cultures

The bacteria *A. brasilense* (wild-type strains Sp7 and Sp245; the Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia) were cultivated in a standard synthetic phosphate- and malate-containing medium as reported elsewhere [28,29], with  $3\text{ g l}^{-1}$   $\text{NH}_4\text{Cl}$  as a bound nitrogen source and 0.6% sodium malate as a carbon source (pH 6.9), under aeration by stirring on a rotary shaker. For FTIR spectroscopic measurements, along with using the standard medium (control), the bacteria were similarly cultured also in the same medium to which  $\text{CoCl}_2$ ,  $\text{CuSO}_4$  or  $\text{ZnSO}_4$  had been added up to 0.2 mM.

### 2.2. Sample preparation and FTIR spectra acquisition

For FTIR in the transmission mode, cell samples were mixed with KBr (Merck) or, for diffuse reflectance infrared Fourier transform (DRIFT) measurements, used as dry finely ground powder in a Micro sampling cup (Spectra-Tech Inc., USA). FTIR studies were performed using a Perkin-Elmer (Model 2000) or (for DRIFT) a Nicolet spectrometer (model Magna-IR 560 E.S.P.) with a total of up to 100 scans (resolution  $4\text{ cm}^{-1}$ ). Other details of spectra acquisition were reported earlier [28,30].

### 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 [29,31] and analysed using a Hewlett-Packard ICP-MS spectrometer (model 4500). Unless indicated otherwise, all measurements were performed at ambient temperature ( $295 \pm 3\text{ K}$ ).

### 2.4. Sample preparation and EMS measurements

For EMS measurements, the culture of *A. brasilense* Sp245 was grown as described above in the standard phosphate-malate mineral medium supplemented with 5 mM  $\text{NH}_4\text{Cl}$  as a nitrogen source (pH 6.9). The cell density in the growing culture was controlled by spectroturbidimetric measurements [31] up to ca.  $2.4 \times 10^8$  cells  $\text{ml}^{-1}$  (approximately mid-exponential growth phase). Optical microscopic observations confirmed the motility of all cells in the culture. The culture obtained was stored for 1 day in Eppendorf tubes at  $4^\circ\text{C}$  and, just prior to adding  $^{57}\text{Co}^{2+}$ , incubated at room temperature ( $20\text{--}23^\circ\text{C}$ ) for 1 h. To prepare dead cells, aliquots of the culture were kept in small plastic Eppendorf tubes (ca. 1.5 ml) in a water bath at  $90^\circ\text{C}$  for 1 h and then, just prior to adding  $^{57}\text{Co}^{2+}$ , cooled down to room temperature. Aliquots of the cell suspensions (1.0 ml), grown and treated as above, were then placed into PTFE sample holders each containing 1 mCi of radioactive  $^{57}\text{CoCl}_2$  free from natural  $\text{Co}^{2+}$  (obtained from the Centre for Radionuclide Diagnostics, Moscow State University, Moscow, Russia), that had been dried from aqueous solution (final  $^{57}\text{Co}^{2+}$  concentration ca.  $2 \times 10^{-6}\text{ M}$ ), thoroughly mixed, closed to prevent evaporation, and after 2 or 60 min of incubation at room temperature the corresponding samples were rapidly frozen in liquid nitrogen at ca. 80 K (further used for EMS measurements either as a frozen suspension or as a freeze-dried powder). A sample with dead cells prepared as above was processed identically (60 min of incubation with  $^{57}\text{Co}^{2+}$ ; rapidly frozen suspension). A similar sample (60 min of incubation with  $^{57}\text{Co}^{2+}$ ) was prepared using transparent cell-free supernatant liquid separated from the bacterial cells by centrifugation (3000 rpm, rotor radius 50 cm, 50 min) immediately after growth and rapidly frozen in liquid nitrogen.

EMS measurements were performed by placing the  $^{57}\text{Co}$ -containing sample (source) in a cryostat filled with liquid nitrogen (at ca. 80 K) using a conventional constant-acceleration Mössbauer spectrometer (absorber  $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ ) combined with a PC-operated multichannel analyser. Standard PC-based statistical analysis consisted of fitting the experimental data obtained (converted into a form compatible with that of absorption  $^{57}\text{Fe}$  Mössbauer measurements) as a sum of Lorentzians using a least squares minimisation procedure, which resulted in  $\chi^2$  minimisation (in all cases,  $1 < \chi^2 \leq 1.2$ ). This enabled determination of the isomer shift (IS; relative to  $\alpha\text{-Fe}$  at room temperature), quadrupole splitting (QS), linewidth (i.e. full width at half maximum, FWHM) and relative areas of spectral components ( $S_i$ ). Other details of methodology and data treatment were reported elsewhere [16,24,25].

### 3. Results and discussion

#### 3.1. Vibrational spectroscopic measurements

Previous studies have shown that azospirilla, widely studied representatives of plant root-associated phytostimulating rhizobacteria [26,27], are relatively tolerant to submillimolar concentrations of heavy metals which, in that case, did not substantially suppress growth of the bacterial culture [31]. As moderate concentrations of heavy metals in soil can often be found as a result of contamination [32,33], assessing their impact on the bacterial metabolism is of importance for a deeper insight into their biology and presents an obvious biotechnological and agricultural interest, considering the plant-growth-promoting abilities of azospirilla [26,34].

Our FT-Raman spectroscopic studies on the wild-type strain of *A. brasilense* Sp7 showed that in metal-stressed cells (for  $\text{Co}^{2+}$  and  $\text{Cu}^{2+}$  in the culture medium), besides an enhanced overall hydration, some decrease in the unsaturation degree of fatty acid residues was revealed in the region of stretching C–H vibrations (around  $3000\text{ cm}^{-1}$ ) [29], and a new band was observed at ca.  $945\text{ cm}^{-1}$  in the region of C–C–O vibrations. It was supposed that under moderate heavy metal stress, the bacterial metabolism may be altered, leading to macroscopic changes in the cellular composition.

As FTIR spectroscopy may be more sensitive to certain functional groups (e.g., including polar bonds) as compared to FT-Raman spectroscopy, we attempted FTIR analyses of whole bacterial cells grown in a standard medium (control) and in the presence of each one of the heavy metals ( $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ ). In Fig. 1, FTIR spectra are shown in the so-called fingerprint region (under  $2000\text{ cm}^{-1}$  down to ca.  $400\text{ cm}^{-1}$ ) for cells of strain Sp7 grown in the standard medium and with  $0.2\text{ mM}$  of each of the above cations. There are striking differences in the overall FTIR profiles between the control cells (Fig. 1a) and cells grown under metal stress (Fig. 1b–d). A most prominent feature of the metal-stressed cells is the appearance of a relatively strong and well-resolved ester  $\nu(\text{C}=\text{O})$  band at about  $1727\text{ cm}^{-1}$  (see Fig. 1b–d). In the control cells (see Fig. 1a), where the amide I and amide II bands of cellular proteins (at about  $1650$  and  $1540\text{ cm}^{-1}$ , respectively) dominate, there is only a weak shoulder at ca.  $1730\text{ cm}^{-1}$ . Together with an increased FTIR absorption in the regions of  $\text{CH}_2$  bending vibrations (at  $1460$ – $1440\text{ cm}^{-1}$ ), as well as C–O–C and C–C–O vibrations (at  $1150$ – $1000\text{ cm}^{-1}$ ) and  $\text{CH}_2$  rocking vibrations (at ca.  $750\text{ cm}^{-1}$ ) in the metal-stressed cells (cf. Fig. 1a and b–d), these spectroscopic changes provide evidence for the accumulation of polyester compounds in cells of strain Sp7 as a response to metal stress.

Note that the position of the  $\nu(\text{C}=\text{O})$  band under  $1730\text{ cm}^{-1}$  corresponds to that of pure poly-3-hydroxybutyrate (PHB) found in many bacterial cells [35–37] including azospirilla (known to accumulate it under subnormal nutritional conditions) [38,39], whereas other polyhydroxyalkanoates (PHAs) including medium-chain-length products exhibit bands at  $1732$ – $1740\text{ cm}^{-1}$  [35]. Considering the noticeable “left-hand” asymmetry of the ester  $\nu(\text{C}=\text{O})$  band in Fig. 1b–d with maxima

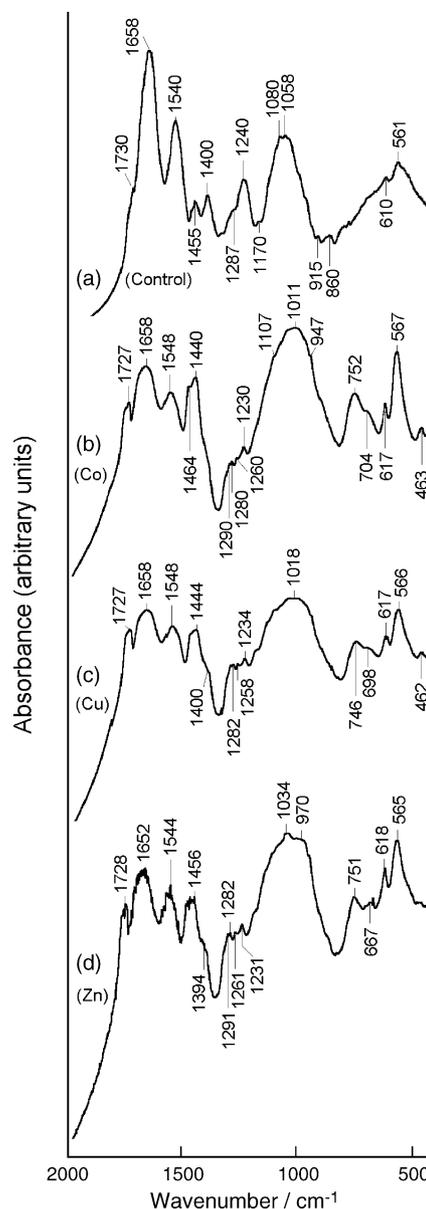


Fig. 1. FTIR spectra (in the transmission mode) of dried biomass of *Azospirillum brasilense* (non-endophytic strain Sp7) grown (a) in a standard phosphate-malate medium (control) as well as in the same medium in the presence of  $0.2\text{ mM}$   $\text{Co}^{2+}$  (b),  $\text{Cu}^{2+}$  (c) and  $\text{Zn}^{2+}$  (d).

at about  $1727\text{ cm}^{-1}$ , the presence of PHAs other than the dominating PHB is possible. PHB is known to accumulate in cells of azospirilla under unfavourable conditions playing a role in bacterial tolerance to environmental stresses, whereas under normal conditions, with aeration and in N-supplemented media, its biosynthesis is decreased [38,39]. Thus, the induction of biosynthesis and accumulation of PHB and other PHAs under normal nutritional conditions by heavy metals is a novel feature for bacteria, which is in line with the overall strategy of their responses to stresses.

In addition, the position of the  $\nu_{\text{as}}(\text{PO}_2^-)$  band changed from  $1240\text{ cm}^{-1}$  (Fig. 1a) to  $1230$ – $1234\text{ cm}^{-1}$  (Fig. 1b–d), thus featuring the transition from the dehydrated or medium-hydrated state to a higher hydration of phosphate moieties [40]. This find-

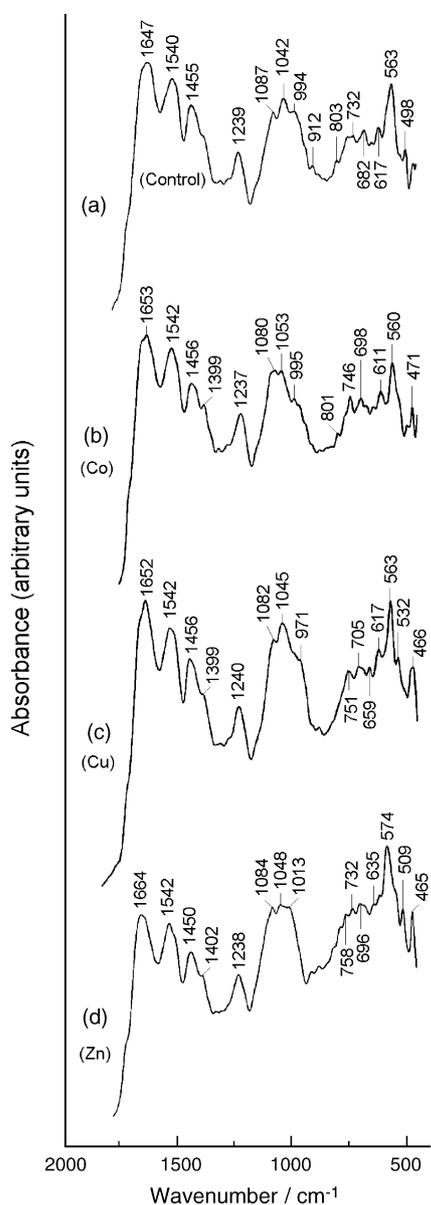


Fig. 2. FTIR spectra (in the DRIFT mode) of dried biomass of *Azospirillum brasilense* (facultatively endophytic strain Sp245) grown (a) in a standard phosphate-malate medium (control) as well as in the same medium in the presence of 0.2 mM  $\text{Co}^{2+}$  (b),  $\text{Cu}^{2+}$  (c) and  $\text{Zn}^{2+}$  (d).

ing is in line with the overall hydration of metal-stressed Sp7 cells revealed in FT-Raman spectra [29] and in FTIR spectra within the correspondingly broadened  $\nu(\text{OH})$  region of bound water at about  $3400\text{--}3000\text{ cm}^{-1}$  (not shown).

Comparing FTIR spectroscopic images of the other strain, Sp245, grown under similar conditions in the standard medium and in the presence of the three cations (0.2 mM), shows no major differences between them (Fig. 2a–d). In all the four samples, there is a weak shoulder at about  $1730\text{ cm}^{-1}$  (ester  $\nu(\text{C}=\text{O})$  band), but in metal-stressed cells there occurs no accumulation of PHA that was found under similar conditions in strain Sp7 (see Fig. 1). Moreover, the position of the representative  $\nu_{\text{as}}(\text{PO}_2^-)$  band of cellular phosphate moieties is constant within the relatively narrow region  $1237\text{--}1240\text{ cm}^{-1}$ , thus confirming relative stability of the state of these functional groups both in the control and under metal stress. This finding is remarkable, especially considering the comparable uptake level of each of the cations studied in the bacterial cells (see Table 1).

### 3.2. ICP-MS analyses of cell samples for metal cations

The results of ICP-MS analyses of cell samples used for FTIR measurements (Table 1) show that both strains Sp7 and Sp245 take up noticeable amounts of heavy metals from the medium (at metal concentrations 0.2 mM in the cultural liquid): ca. 0.12 and 0.13 mg Co, 0.48 and 0.44 mg Cu, 4.2 and 2.1 mg Zn per gram of dry biomass, respectively. This is 1–3 orders of magnitude higher than their content in cells grown in the medium with background impurities of the metals (see Table 1).

It is noteworthy that the amount of cobalt accumulated by strain Sp7 up to ca. 0.12 mg per gram of dry biomass induces approximately the same metabolic response of the bacterium as a ca. fourfold higher amount of copper or a ca. 36-fold higher amount of zinc (cf. Table 1, Fig. 1a–d). It is clear that such amounts of metal complexes per se cannot give any noticeable FTIR absorption related to their intrinsic functional groups. This means that in strain Sp7, moderate heavy metal stress induces noticeable metabolic transformations revealed in their FTIR spectra as macroscopic compositional changes. In its turn, this suggests direct participation of cobalt(II) as well as the other cations, taken up by the bacterial cells, in cellular processes as a result of their assimilation. However, for strain Sp245, despite the levels of metal uptake comparable with those for strain Sp7,

Table 1  
Content of metals in cell samples of *Azospirillum brasilense* (strains Sp7 and Sp245) used for FTIR measurements (see Figs. 1 and 2) determined using ICP-MS<sup>a</sup>

Cultivation medium used for growing the bacteria	Content of metals (mg per gram of dried cells) in cells of strains Sp7 and Sp245					
	Co		Cu		Zn	
	Sp7	Sp245	Sp7	Sp245	Sp7	Sp245
Standard (control)	0.0005	0.0031	0.047	0.021	0.025	0.041
With 0.2 mM $\text{Co}^{2+}$	<b>0.118</b>	<b>0.134</b>	0.002	0.033	0.027	0.058
With 0.2 mM $\text{Cu}^{2+}$	0.0007	0.0008	<b>0.477</b>	<b>0.438</b>	0.035	0.053
With 0.2 mM $\text{Zn}^{2+}$	0.0006	0.0016	0.007	0.033	<b>4.24</b>	<b>2.14</b>

<sup>a</sup> The metals analysed in cell samples were either present in the standard cultivation medium as impurities (data for cell samples given in normal font) or added to the medium up to 0.2 mM (data for cell samples given in bold font).

this is not obvious, considering the lack of noticeable compositional changes revealed by FTIR spectroscopy (see Fig. 2).

### 3.3. Emission Mössbauer spectroscopic measurements

In order to check whether in strain Sp245 cobalt(II) traces are only bound by the cell surface in a purely chemical process or cobalt(II) is assimilated, EMS measurements were performed using traces of  $^{57}\text{Co}^{2+}$  salt. It should be mentioned that the Mössbauer effect is observed for solids only, so that aqueous solutions, suspensions or liquids are commonly studied rapidly frozen [41]. Rapid freezing often allows crystallisation of the liquid (solvent) to be avoided, so that the structure of the resulting glassy solid matrix represents that of the solution. It is also of importance that upon freezing, biochemical (metabolic) processes in live cells, tissues or other biological samples cease at a certain point. This allows different states of the processes to be analysed separately by studying samples frozen at different moments of time, starting from a few seconds for ordinary laboratory equipment, or even much shorter periods (reported for modern rapid freeze-quench methodology which has so far been used in conjunction with transmission  $^{57}\text{Fe}$  Mössbauer spectroscopy) [42].

In Fig. 3a–b, emission Mössbauer spectra are presented for aqueous suspensions of live Sp245 cells rapidly frozen after 2 or 60 min of contact with  $^{57}\text{Co}^{2+}$ ; similar spectra are presented in Fig. 4a–b for freeze-dried cells of strain Sp245 that were similarly kept in contact with  $^{57}\text{Co}^{2+}$  for 2 and 60 min. For each spectrum, the relevant spectral components (subspectra) are shown which contributed to the resulting spectrum (solid-line envelope) obtained by computer fitting to the experimental data (points with vertical error bars). The positions of the spectral components (quadrupole doublets) are indicated by horizontal square brackets above the zero line.

Mössbauer parameters calculated from the experimental data are listed in Table 2. For each of the aforementioned samples, there are two EMS components corresponding to two chemical forms of high-spin  $^{57}\text{Co}^{\text{II}}$  featured by their isomer shift (IS) and quadrupole splitting (QS) values. (The presence of the third component with the parameters typical for high-spin nucleogenic iron(III), stabilised after nuclear decay of the parent  $^{57}\text{Co} \rightarrow ^{57}\text{Fe}$  nuclear transformation. This phenomenon typical for emission Mössbauer spectra is discussed in more detail elsewhere [41,43,44] and therefore is not considered here.)

The presence of at least two major cobaltous forms (with different IS and QS values, see Table 2) revealed in the spectra of bacterial cells may be related to the availability of different functional groups (also with possibly different donor atoms) as ligands at the azospirillum cell surface (see, e.g., [26–31] and references therein). It should be noted that the main parameters (IS, QS and even FWHM) of the corresponding (+2)-forms for live bacterial cells measured in the dry state and in frozen aqueous suspension are statistically indistinguishable or very close both for 2 min (see Table 2, sample 1; cf. FAS and DB) and for 1 h of contact with  $^{57}\text{Co}^{2+}$  (see Table 2, sample 2; cf. FAS and DB). This finding shows that freeze-drying does not

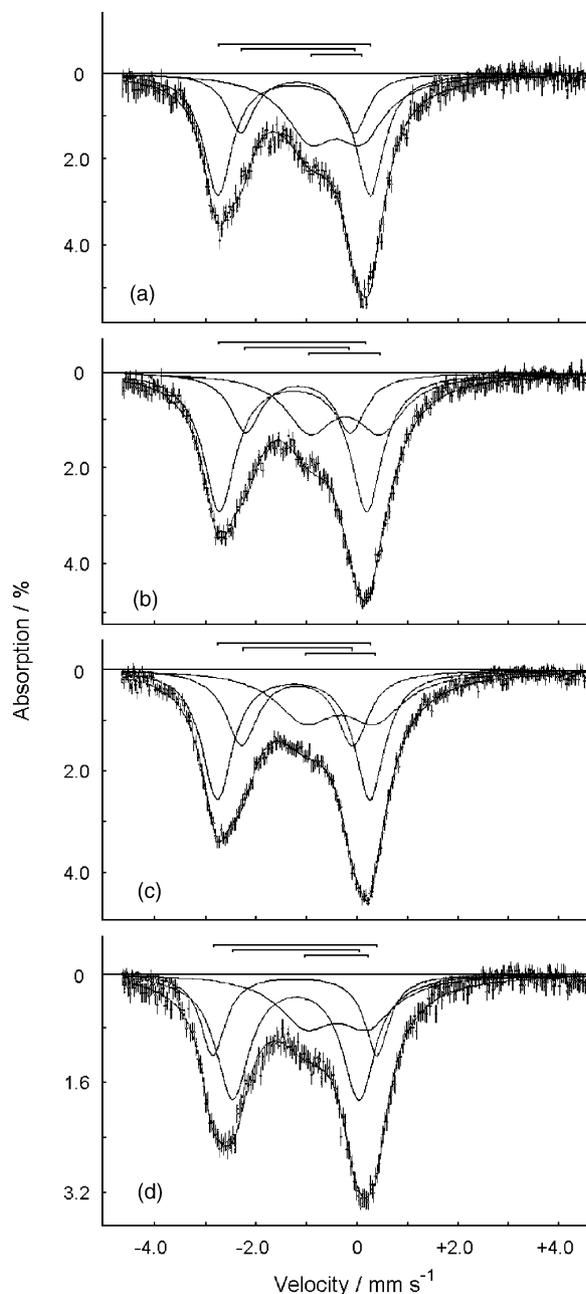


Fig. 3. Emission Mössbauer spectra of frozen aqueous suspensions of live (a and b) and dead (c) cells of *Azospirillum brasilense* Sp245 in the culture medium as well as of the cell-free supernatant liquid (d), that were incubated with  $^{57}\text{CoCl}_2$  for 2 min (a) and 60 min (b–d) at ambient temperature and then rapidly frozen in liquid nitrogen (spectra collected at  $T = 80$  K; see also Table 2).

significantly affect the chemical state of the cobalt(II) species bound at the surface or within the membrane of the bacterium, once their parameters remain virtually unchanged. Some redistribution of the relative contents of the forms (featured by the  $S_r$  values; see Table 2) in going from the hydrated (FAS) state to the dry (DB) state (samples 1 and 2; in particular, the increased  $S_r$  values for  $^{57}\text{Fe}^{3+}$  for the DB state) may be related to changes in the yield of the (+3)-form (resulting from after-effects) in going from the aqueous medium to the dry biomass (e.g., due to possible changes in the outer coordination sphere of  $^{57}\text{Co}^{2+}$

Table 2  
Mössbauer parameters<sup>a</sup> calculated from emission Mössbauer spectroscopic data for aqueous suspensions of live and dead cells of *Azospirillum brasilense* Sp245 in the <sup>57</sup>Co<sup>II</sup>-containing culture medium, dried biomass as well as for the cell-free supernatant liquid, which were incubated with <sup>57</sup>CoCl<sub>2</sub> for specified periods of time at ambient temperature and then rapidly frozen in liquid nitrogen (spectra collected at *T* = 80 K)

Sample	State <sup>b</sup>	Oxidation state <sup>c</sup>	IS <sup>d</sup> (mm s <sup>-1</sup> )	QS <sup>e</sup> (mm s <sup>-1</sup> )	FWHM <sup>f</sup> (mm s <sup>-1</sup> )	S <sub>r</sub> <sup>g</sup> (%)	Fig.
(1) Live bacterial cells (frozen 2 min after adding <sup>57</sup> CoCl <sub>2</sub> to the culture medium)	FAS	+2	1.26 (1)	3.00 (3)	0.69 (3)	44	Fig. 3a
		+2	1.20 (6)	2.23 (6)	0.65 (8)	20	
		+3	0.45 (5)	1.0 (1)	1.2 (1)	36	
	DB	+2	1.24 (3)	3.08 (6)	0.70 (10)	19	Fig. 4a
		+2	1.14 (3)	2.35 (9)	0.83 (13)	23	
		+3	0.35 (5)	1.26 (8)	1.43 (12)	58	
(2) Live bacterial cells (frozen 60 min after adding <sup>57</sup> CoCl <sub>2</sub> to the culture medium)	FAS	+2	1.26 (1)	2.89 (2)	0.78 (2)	51	Fig. 3b
		+2	1.16 (1)	2.03 (4)	0.73 (6)	20	
		+3	0.24 (2)	1.40 (3)	1.13 (6)	29	
	DB	+2	1.22 (4)	2.84 (7)	0.88 (10)	38	Fig. 4b
		+2	1.00 (5)	2.03 (9)	0.5 (2)	8	
		+3	0.26 (5)	1.55 (7)	1.36 (16)	54	
(3) Dead bacterial cells (frozen 60 min after adding <sup>57</sup> CoCl <sub>2</sub> )	FAS	+2	1.24 (1)	3.00 (2)	0.73 (2)	44	Fig. 3c
		+2	1.17 (1)	2.18 (4)	0.76 (4)	27	
		+3	0.33 (3)	1.39 (6)	1.4 (1)	29	
(4) Supernatant liquid (frozen 60 min after adding <sup>57</sup> CoCl <sub>2</sub> )	FAS	+2	1.22 (1)	3.23 (5)	0.60 (2)	24	Fig. 3d
		+2	1.21 (1)	2.48 (3)	0.80 (3)	48	
		+3	0.40 (6)	1.22 (3)	1.3 (2)	28	

<sup>a</sup> Errors (in the last digits) are given in parentheses.

<sup>b</sup> FAS, frozen aqueous suspension (or frozen solution in case of sample 4) and DB, dried biomass.

<sup>c</sup> Oxidation states of the nucleogenic <sup>57</sup>Fe components stabilised after nuclear decay of the parent <sup>57</sup>Co<sup>II</sup>.

<sup>d</sup> Isomer shift (relative to α-Fe) converted to the normal absorption convention (positive with regard to α-Fe).

<sup>e</sup> Quadrupole splitting.

<sup>f</sup> Full line width at half maximum (assumed to be equal for both the lines of a doublet).

<sup>g</sup> Relative resonant absorption areas (relative error ±4%) of the relevant spectral components, which represent relative contents of the corresponding nucleogenic Fe forms assuming a common recoilless fraction (Mössbauer effect probability) for all forms in a sample contributing to the spectrum.

complexes occurring upon removal of excess water), as well as partly by nonequal changes in the recoilless emission probability (Mössbauer–Lamb factor) for different forms upon drying.

Comparing the EMS data for different periods (2 min and 1 h) of contact of live bacteria with <sup>57</sup>Co<sup>2+</sup> traces, one can see essential differences in the corresponding QS values (see Table 2) for the two (+2)-forms. This finding shows that within an hour, after primary rapid adsorption onto the cell surface, cobalt(II) undergoes further transformation, most probably occurring within the cell membrane. For comparison, earlier (and virtually first) EMS measurements on the accumulation of <sup>57</sup>Co<sup>2+</sup> complex with a bacterial hexadentate iron(III)-chelating agent, enterochelin (a cyclic trimer of *N*-2,3-dihydroxybenzoylserine), in *Escherichia coli* [45] showed that part of the <sup>57</sup>Co<sup>2+</sup> complex absorbed by the bacteria was located within the cell membrane (a quasi-solid cellular structure) for at least 24 h, thus giving some weak but noticeable Mössbauer effect even above the freezing point (at +3 °C).

The parameters obtained from the emission spectrum of dead (thermally killed) cells (see Fig. 3c and Table 2, sample 3) are very close to those for frozen aqueous suspension of live cells after 2 min of contact with <sup>57</sup>Co<sup>2+</sup> for both the (+2)-forms and their relative contents (see Table 2, sample 1, FAS). The statistically insignificant differences in the parameters of both the (+2)-forms and their close relative contents indicate that the processes of primary adsorption of cobalt(II) by live cells is

chemically similar to its interaction with thermally killed bacteria, which in the latter case obviously represents a purely chemical interaction. Note that some differences in the parameters for the stabilised (+3)-form, resulting from aftereffects, for samples 1 (FAS) and 3 in Table 2, and its lower content in sample 3 might be related to some possible changes in the properties of cell-surface biopolymers induced by the hydrothermal treatment.

Neither of the (+2) components found in all of the samples, including cell-free supernatant liquid (see Table 2, sample 4), evidently corresponds to the aquo complex [<sup>57</sup>Co(H<sub>2</sub>O)<sub>6</sub>]<sup>2+</sup>, as the latter is featured by IS = 1.3–1.4 mm s<sup>-1</sup> and QS = 3.3–3.4 mm s<sup>-1</sup> in frozen aqueous solutions (see, e.g., [41], chapters 4, 5). Note that the parameters for the cell-free medium (see Table 2, sample 4) are different from those found for all other samples (bacterial cells). Thus, we conclude that in the cell-free supernatant liquid the chemical state of Co<sup>2+</sup> trace species (i.e., Co<sup>2+</sup> complexes) is different from those in cell samples. In its turn, this shows that in the presence of bacterial cells, Co<sup>2+</sup> traces are completely (and, considering sample 1, also rapidly, within 2 min) bound by the cells.

In the cell-free culture solution, the most likely ligands which could bind Co<sup>2+</sup> are phosphate and possibly malate anions (present in the initial standard growth medium at concentrations of the order of 10<sup>-2</sup> M [28–31]), ammonia (from NH<sub>4</sub><sup>+</sup> added to the initial medium at ~5 mM), as well as various (probably

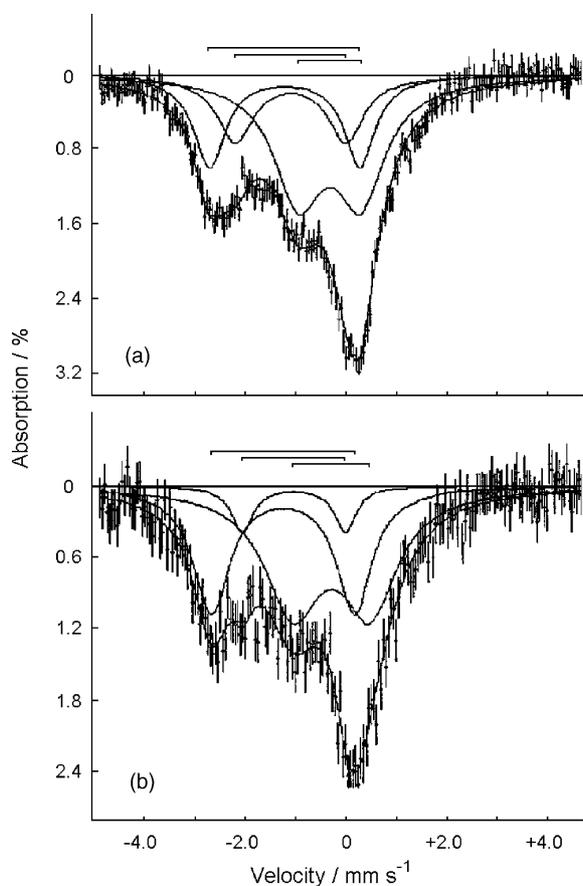


Fig. 4. Emission Mössbauer spectra of dried cells of *Azospirillum brasilense* Sp245 that were incubated as live cells with  $^{57}\text{CoCl}_2$  for 2 min (a) and 60 min (b) at ambient temperature, then rapidly frozen in liquid nitrogen and freeze-dried prior to EMS measurements (spectra collected at  $T = 80\text{ K}$ ; see also Table 2).

acidic) exopolysaccharides dissolving from the cell surface [46]. Note that both malate and  $\text{NH}_4^+$  are gradually consumed by the growing bacteria as sources of carbon and bound nitrogen. Thus, the majority of donor atoms in the first coordination spheres of  $^{57}\text{Co}^{2+}$  complexes in the cell-free solution are likely to be represented by oxygen (including that of hydration water molecules) and probably nitrogen.

Note that direct identification of metal complexes in such sophisticated systems as bacterial cells is so far difficult for several reasons. First, there is still lack of experimental EMS data on model cobalt biocomplexes [16] which could facilitate interpretation of EMS parameters. Second, various chemical species with structurally (with regard to the coordination symmetry and arrangement of donor atoms around the cation) and compositionally (with regard to the nature of donor atoms of the ligands) similar coordination microenvironments can give similar Mössbauer parameters. Also, in the emission variant of Mössbauer spectroscopy, the Auger electrons formed during the Auger cascade [16,41,44] inevitably influence to some extent the coordination environment of the nuclide, even if its chemical state remains unchanged, which, in particular, results in some noticeable line broadening in  $^{57}\text{Co}$  emission spectra (as compared to absorption spectra of the corresponding  $^{57}\text{Fe}$  compounds) [44].

#### 4. Conclusions

FTIR spectroscopic analyses of whole bacterial cells performed for different strains of *A. brasilense*, compared with FT-Raman spectroscopic data obtained earlier, together with ICP-MS trace analyses of metal uptake by the bacteria, have shown that moderate heavy metal stress induces a noticeable rearrangement in the metabolism of the non-endophytic strain Sp7. As a specific response to heavy metal stress, this strain accumulates polyhydroxyalkanoates (with dominating poly-3-hydroxybutyrate). The response of the endophytic strain Sp245 to a moderate heavy metal stress was found to be much less pronounced than that of the non-endophyte Sp7. These dissimilarities in their behaviour may be related to different adaptation abilities of the strains under stress conditions owing to their different ecological status. In particular, an enhanced accumulation of polyester storage compounds is known to play a role in bacterial tolerance to environmental stresses. However, polyhydroxyalkanoates usually accumulate in cells under nutritional stress (e.g., a high C/N ratio) [38,39]. PHB and/or PHA biosynthesis and accumulation in bacterial cells induced by heavy metal stress, as found for strain Sp7, is a novel feature. In the non-endophytic strain, it may be a specific flexible adaptation strategy related to the localisation of the bacteria on the rhizoplane, i.e. in direct contact with rhizospheric soil components. This corresponds to the documented capability of strain Sp7 to outcompete other co-inoculated strains [27].

EMS measurements in frozen aqueous solutions have shown that live *A. brasilense* Sp245 cells rapidly (within 2 min) and completely adsorb traces of  $^{57}\text{Co}^{2+}$  from the medium, with its further transformations occurring within an hour. Comparison of the Mössbauer parameters for live and dead bacterial cells indicates that the processes of primary rapid adsorption of cobalt(II) by live cells are chemically similar to its interaction with dead (hydrothermally killed) bacteria.

The results obtained demonstrate that EMS is a valuable tool for the monitoring of trace cobalt uptake and its transformations in such complicated biological systems as living bacterial cells (in vivo) with ongoing metabolic processes. The combination of the highly sensitive trace metal speciation using EMS technique with ICP-MS analysis of metal uptake and vibrational spectroscopic analysis of overall cellular composition provides complementary information on bacterial behaviour under heavy metal stress.

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