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Monitoring of cobalt(II) uptake and transformation in cells of the plant-associated soil bacterium *Azospirillum brasilense* using emission Mössbauer spectroscopy

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Abstract

Interaction of cobalt(II) at micromolar concentrations with live cells of the plant-growth-promoting rhizobacterium *Azospirillum brasilense* (strain Sp245) and further transformations of the metal cation were monitored using ^{57}Co emission Mössbauer spectroscopy (EMS). Cell suspensions of the bacterial culture (2.4×10^8 cells ml^{-1}) were doped with radioactive $^{57}CoCl_2$ (1 mCi; final concentration 2×10^{-6} M $^{57}Co^{2+}$), kept under physiological conditions for various periods of time (from 2 min up to 1 hour) and then rapidly frozen in liquid nitrogen. Analysis of emission Mössbauer spectra of the frozen aqueous suspensions of the bacterial cell samples shows that the primary absorption of cobalt(II) at micromolar concentrations by the bacterial cells is rapid and virtually complete, giving at least two major forms of cobalt(II) species bound to the cells. Within an hour, the metal is involved in further metabolic transformations reflected by changes occurring in the spectra. The Mössbauer parameters calculated from the EMS data by statistical treatment were different for suspensions of live and dead (thermally killed) bacterial cells that had been in contact with $^{57}Co^{2+}$ for 1 h, as well as for the cell-free culture medium containing the same concentration of $^{57}Co^{2+}$. Chemical after-effects of the nuclear transition ($^{57}Co \rightarrow {}^{57}Fe$), which provide additional information on the chemical environment of metal ions, are also considered. The data presented demonstrate that EMS is a valuable tool for monitoring the chemical state of cobalt species in biological matter providing information at the atomic level in the course of its uptake and/or metabolic transformations.

Introduction

Cobalt is one of biologically essential microelements with a broad range of physiological and biochemical functions (Williams *et al.* 2000; Williams 2001; Balogh *et al.* 2003); nevertheless, it can be toxic for many organisms at higher concentrations (Dies 1999). In particular, when present in the medium, Co^{2+} (as well as some other heavy metals) is rapidly accumulated in most bacterial cells by the fast and unspecific membrane-integral protein CorA belonging to the metal-inorganic-transport (MIT) family (Dies 1999; Ehrlich 1997). In many microorganisms, cobalt(II) is involved in diverse enzymatic activities (Williams *et al.* 2000; Dies 1999; Ehrlich 1997; Bespalova *et al.* 1999; Antonyuk *et al.* 2001; Kamnev *et al.* 2002, 2004; Wojciechowski *et al.* 2002) and can be included in magnetosomes (Vainshtein *et al.* 2002). Nevertheless, its primary binding to the cell surface in the course of purely chemical and virtually non-selective processes can occur both in live and dead microbial cells (Ehrlich 1997).

Cobalt attracts attention also owing to biogeochemical problems resulting from the microbially me-

diated migration of the radionuclide ^{60}Co from disposal sites (Christensen *et al.* 2001). Such processes can be facilitated by release of cobalt traces entrapped within ferric oxide minerals upon the microbial dissimilatory reduction of iron(III) (Zachara *et al.* 2001) together with possible Co^{3+} species (Lovely *et al.* 2000).

In view of the aforementioned, studying biological transformations of cobalt is warranted both from the analytical viewpoint and for its chemical speciation. The latter, however, presents a challenging problem for bioanalytical chemistry which currently faces the generally increasing significance of elemental speciation (Lobinski 2001; Bettmer 2002), as cobalt participation in physiological processes in biological systems is usually limited by its trace concentrations.

For the emission variant of Mössbauer (nuclear gamma-resonance) spectroscopy (EMS), ^{57}Co is the most widely used radionuclide (with a half-life of 270 d) (Vértes *et al.* 2001). Its nuclear decay via electron capture gives the stable ^{57}Fe isotope and is accompanied by specific physical and chemical after-effects (Vértes *et al.* 1998; Afanasov *et al.* 1985). The resulting recoil energy (ca. 4.6 eV) for the daughter ^{57}Fe nucleus is sufficiently low, so that the nucleogenic iron atoms do not shift from their positions, and in many cases their chemical state and environment reflects that in the parent ^{57}Co species. However, the concatenated processes of consecutively filling in the vacancies in inner electronic shells of the ^{57}Fe atom after the electron capture by the ^{57}Co nucleus (the so-called Auger cascade developing within 10^{-15} to 10^{-14} s) result in the ejection of Auger electrons out of the ^{57}Fe atom, giving a series of its different, mainly short-lived charge states. By the moment of emitting a 14.4-keV γ-quantum by the nucleus (ca. 10^{-7} s after the electron capture), most of the Auger electrons return, giving common charge states of the daughter ^{57}Fe ions. Part of these states correspond to those of the parent ^{57}Co; however, the interaction of some Auger electrons with the chemical environment of the atom can result in the formation of (an)other charge state(s) of the latter (Vértes *et al.* 1998; Afanasov *et al.* 1985). These after-effects, albeit making the emission Mössbauer spectra more complicated, can provide additional information, e.g. on the electron-acceptor properties of the coordination environment of the metal under study (Afanasov *et al.* 1985).

It is also noteworthy that EMS is several orders of magnitude more sensitive than its absorption variant which, nevertheless, has a much richer history of applications in biosciences (see, e.g. (Vértes *et al.* 1979; Ambe 1994; Kilcoyne *et al.* 2000; Oshtrakh 1999, 2004), including recent reviews (Ostrakh 1999, 2004), and references therein). The main restriction of EMS, along with the limited availability of the elements having Mössbauer-active nuclides, is the necessity to work in a specially equipped radiochemical laboratory with samples which contain either an intrinsic radioactive nuclide or that introduced artificially (Vértes *et al.* 1998, 1979). This is connected with obvious experimental and/or methodological difficulties, accounting for the scarcity of reports with EMS data on biological systems (Kamnev *et al.* 2002, 2004; Vértes *et al.* 1979; Ambe 1990).

Though EMS is capable of providing quantitative data on the relative content of different chemical species of the radionuclide, the technique is commonly not regarded as purely analytical; for instance, it is not mentioned in the recent review on nuclear analytical methods (Weise *et al.* 2001). Nevertheless, once the element under study is suitable, EMS provides essential information at the atomic level directly on the chemical state of the trace nuclide. The method is non-destructive and can readily be adapted to be performed *in situ*. As the Mössbauer effect can be observed for a nuclide fixed within (or otherwise attached to) a solid matrix and is negligibly small for motile chemical species, Mössbauer spectra of liquid samples are commonly obtained in the form of frozen solutions (Vértes *et al.* 1998). For suspensions of live bacteria, rapid freezing also ceases the development of metabolic processes and thus allows the biosystem to be fixed at a definite metabolic point.

As for the plant-associated nitrogen-fixing soil bacterium *Azospirillum brasilense* which, among other *Azospirillum* species, attracts world-wide attention owing to its phytostimulating activities (Steenhoudt *et al.* 2000), our previous analytical studies have shown that cobalt (II) added to its culture medium is accumulated to some extent by the bacterial cells (up to 0.1–0.6 mg Co per gram of dry biomass for different *A. brasilense* strains at 0.2 mM Co^{2+} in the culture solution, which corresponds to 0.012 mg Co per gram of solution) (Kamnev *et al.* 1997, 2001). Vibrational spectroscopic studies of whole cells of *A. brasilense* Sp7 have shown (Kamnev *et al.* 2001, 2002) that cobalt(II), as well as some other heavy metals, can induce specific metabolic changes in the bacterium as a response to heavy-metal stress. *In-vitro* biochemical and spectroscopic investigations on glutamine synthetase, a key enzyme of nitrogen metabolism, isolated

from this bacterium have also demonstrated that Co^{2+} is among the divalent cations (along with Mg^{2+} and Mn^{2+}) most effective in supporting the activity of this enzyme at different adenylylation states (Bespalova *et al.* 1999; Antonyuk *et al.* 2001), binding at its two-metal-ion-containing active centres (Kamnev *et al.* 2002, 2004) and playing a role in the organisation of its secondary structure (Antonyuk *et al.* 2001; Kamnev *et al.* 2004).

In the present work, EMS was used to monitor primary absorption of Co^{2+} by bacterial cells and its further metabolic transformations in *A. brasilense* (strain Sp245). The bacterial cells were stored in the culture medium with added traces of $^{57}CoCl_2$ for different periods of time (2 min to 1 h) and then rapidly frozen in liquid nitrogen. Similar comparative EMS measurements were performed for suspensions of dead (thermally killed) cells as well as for the corresponding cell-free culture medium.

Experimental

Preparation of bacterial cultures

The culture of *Azospirillum brasilense* (strain Sp245; The Collection of IBPPM RAS, Saratov, Russia) was grown as described elsewhere (Kamnev *et al.* 1997, 2001, 2002) in a standard phosphate-malate mineral medium supplemented with 5 mM NH_4Cl as a nitrogen source and 0.6% sodium malate as a carbon source (pH 6.9). The bacterial cell density in the growing culture was controlled by spectroturbidimetric measurements (Kamnev *et al.* 1997) up to ca. 2.4×10^8 cells/ml (approximately mid-exponential growth phase). Optical microscopic observations confirmed the viability (motility) of all cells in the culture. The culture obtained was stored for 1 day in Eppendorf tubes at 4 °C and, before adding $^{57}Co^{2+}$, was incubated at room temperature (20° to 23 °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 °C for 1 h and then, just prior to adding $^{57}Co^{2+}$, cooled down to room temperature.

Sample preparation for EMS measurements

Aliquots of the cell suspensions (1.0 ml) were placed into PTFE sample holders each containing 1 mCi of radioactive $^{57}CoCl_2$ free from natural Co^{2+}, i.e. 'carrier-free' (obtained from the Centre for Radionuclide Diagnostics, Moscow State University, Moscow, Russia), that had been dried from aqueous solution (final $^{57}Co^{2+}$ concentration ca. 2×10^{-6} M), thoroughly mixed, closed to prevent evaporation, and after 2, 10 or 60 min of incubation at room temperature the corresponding samples were rapidly frozen in liquid nitrogen. A sample with dead cells prepared as above was processed identically (60 min of incubation with $^{57}Co^{2+}$). A similar sample (60 min of incubation with $^{57}Co^{2+}$) was prepared using transparent cell-free supernatant liquid separated by centrifugation (3000 rpm, rotor radius 50 cm, 50 min) from the bacterial culture immediately after growth.

EMS measurements and data treatment

EMS measurements were performed by placing the ^{57}Co-containing frozen samples (as a source of γ-radiation) in a cryostat filled with liquid nitrogen (at ca. 80 K) using a conventional constant-acceleration Mössbauer spectrometer (with $K_4[Fe(CN)_6]\cdot 3H_2O$ as an absorber) combined with a PC-operated multichannel analyser (256 channels per spectrum). The spectrometer was calibrated using α-Fe foil at room temperature. In order to obtain suitable statistics, each spectrum was collected for 1.5 to 2 days. Standard PC-based statistical analysis consisted of fitting the experimental data obtained to a sum of doublets with Lorentzian-shaped lines using a least-squares minimisation procedure. The Mössbauer parameters calculated from the experimental data were the isomer shift (IS; relative to α-Fe; converted into a form compatible with that of conventional absorption ^{57}Fe Mössbauer measurements, with IS values positive with regard to α-Fe), quadrupole splitting (QS), linewidth (i.e. experimentally obtained full width at half maximum, FWHM) and relative areas of spectral components (S_r). The latter 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 overall spectrum.

General considerations

In our earlier studies on metal uptake in different strains of the Gram-negative plant-associated soil bacterium *A. brasilense* and their cellular responses to different heavy metals (Kamnev *et al.* 1997, 1999, 2001, 2002), their concentrations of 0.2 mM were applied as a model of a moderated heavy-metal stress. At such submillimolar concentrations, the heavy metals studied (V^{4+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}) did

not at all or significantly suppress growth of the bacterial culture (even under nitrogen-limited conditions (Kamnev *et al.* 1997)). Nevertheless, the heavy metal-induced metabolic responses in some cases were well detectable using vibrational spectroscopy of whole cells or cell constituents (Kamnev *et al.* 1997, 1999, 2001, 2002; Tugarova *et al.* 2002). Note for comparison that in the case of cobalt(II), its minimal inhibitory concentrations for Co^{2+}-sensitive and Co^{2+}-resistant strains of Gram-negative bacteria of the genus *Pseudomonas* were reported to be 2 mM and 8 mM, respectively (Ivanov *et al.* 1998).

In this work, the $^{57}Co^{2+}$ concentration used (0.002 mM) was 100 times lower than that (of natural Co^{2+}) which induced some detectable metabolic changes (Kamnev *et al.* 2001, 2002) during growth of *A. brasilense* Sp7, a strain which colonises the surface of plant roots (Kirchhof *et al.* 1997). Moreover, *A. brasilense* strain Sp245 studied in this work, which is known to be a facultative endophyte (capable of penetrating to and colonising the root interior, in contrast to strain Sp7 and many other *A. brasilense* strains) (Kirchhof *et al.* 1997), was found to exhibit a much less pronounced metabolic response to heavy metals (0.2 mM Co^{2+}, Cu^{2+} or Zn^{2+} in the culture medium) than strain Sp7 under similar conditions (Kamnev *et al.* 2002). This may in particular be attributed to different adaptation abilities of the endophytic and non-endophytic strains to stress conditions owing to the different ecological niches they occupy in the rhizosphere. Note also that in our earlier studies on heavy metal uptake by *A. brasilense* and their relevant metabolic effects (Kamnev *et al.* 1997, 1999, 2001, 2002), the heavy metals under study had been present in the medium throughout the whole period of bacterial growth, whereas in this work the period of incubation of the bacterium with cobalt(II) ranged from 2 min up to 1 h.

Considering the aforementioned, the cellular processes occurring in the bacteria under the conditions considered in this work may be regarded as being close to 'normal' metabolism under a virtually insignificant heavy-metal stress. This is essential, as a more pronounced heavy-metal stress could induce biosynthesis of additional (stress-induced) cell-surface biopolymers and/or other metal-binding structures, in contrast to cells under 'normal' metabolism (Kamnev *et al.* 2002; Ivanov *et al.* 1998, 1999).

Primary binding of heavy metals by the cell surface in Gram-negative bacteria is mediated by capsular polysaccharide (PS, particularly carboxylated acidic PS), lipopolysaccharide (LPS, including phosphate LPS moieties) and proteinaceous materials (see, e.g. (Dies 1999; Ehrlich 1997; Ivanov *et al.* 1998, 1999; Beveridge *et al.* 1985) and references therein). In *A. brasilense*, these biopolymers and their covalently bound complexes characteristic for the cell surface (Skvortsov *et al.* 1998; Castellanos *et al.* 1998; Burdman *et al.* 1999, 2000, 2001) are believed to be involved in contact interactions with plant roots (Skvortsov *et al.* 1998; Burdman *et al.* 2001; Yegorenkova *et al.* 2001) and in bacterial cell aggregation (Burdman *et al.* 1999, 2000, 2001). Consequently, these processes essential for the formation of efficient plant-bacterial symbioses can well be affected by heavy metal coordination with the cell-surface functional groups responsible for the relevant biospecific interactions, especially in the case of increased levels of heavy-metal pollution.

In equilibrium, metal binding by bacterial cells was in some cases satisfactorily described by the linearised Freundlich adsorption isotherm (Volesky 1990; Sar *et al.* 1999):

$$\log q_e = \log k + (1/n) \log C_e$$

where q_e is the specific amount of an adsorbed metal expressed per gram of biomass (Sar *et al.* 1999) or sometimes per gram of cell protein (Garcia-Gil *et al.* 1997) (though, strictly speaking, the overall cell composition and, therefore, the relative protein content in the biomass may be significantly affected when live bacteria are exposed to a heavy metal (Kamnev *et al.* 2002; Ivanov *et al.* 1998, 1999)); C_e is the equilibrium metal concentration in solution; while k and n are constants (the $1/n$ value being the slope of the linearised isotherm in the coordinates $\{\log q_e; \log C_e\}$) quantitatively representing the adsorption capacity and intensity, respectively. Interestingly, the highly generalised macroscopic approach using the Freundlich isotherm seems to have adequately described the adsorption of very different metals both by lyophilised biomass (Sar *et al.* 1999) and by various live bacteria (Garcia-Gil *et al.* 1997). The latter case seems especially strange, as for live cells their ongoing metabolism and culture growth (with cell multiplication and the concomitant permanent increase of the total surface area of the 'adsorbent') evidently make the term 'equilibrium' hardly appropriate. This unprecise approach of simple adsorption can probably give satisfactory results only for the short time intervals applied (usually within several minutes (Volesky 1990; Garcia-Gil *et al.* 1997)), within which live bacterial

biomass may, from the physicochemical standpoint, be roughly considered to be in a quasi-steady state. After that, the involvement of a range of both specific and unspecific membrane-bound heavy-metal transporters (Dies 1999; Zehrlich 1997) would inevitably change the 'absorption equilibrium' at the surface of live cells. In order to check this statement for *A. brasilense*, in this work cobalt(II) interaction with bacterial cells was monitored for longer periods (up to 1 h).

Results and discussion

Emission Mössbauer spectra of rapidly frozen suspensions of *A. brasilense* Sp245 live cells (after 2, 10 and 60 min of incubation with $^{57}Co^{2+}$), dead cells (60 min of incubation with $^{57}Co^{2+}$) and cell-free supernatant liquid (60 min of incubation with $^{57}Co^{2+}$) are shown in Figure 1,A–E. The corresponding Mössbauer parameters calculated from the experimental spectroscopic data are presented in Table 1.

In view of the relatively high uptake level of cobalt(II) in *A. brasilense* (Kamnev *et al.* 1997, 2002), it could be expected that $^{57}Co^{2+}$ at the micromolar concentration would be largely or completely bound by its cell surface. This is corroborated by comparing the EMS data for the 2-min incubation of the bacterial cells with $^{57}Co^{2+}$ (Figure 1A; Table 1, sample 1) and for the cell-free supernatant liquid (Figure 1E; Table 1, sample 5). After only 2 min of contact of live bacterial cells with $^{57}Co^{2+}$, the spectrum at 80 K exhibits two high-spin components [19] corresponding to oxidation state +2. Neither of these components evidently corresponds to $[^{57}Co(H_2O)_6]^{2+}$, as the latter shows IS = 1.3–1.4 mm/s and QS = 3.3–3.4 mm/s in frozen aqueous solutions (Vértes *et al.* 1979; Ambe 1994) (see also (Vértes *et al.* 1998), chapters 4, 5). The presence of the third component with the parameters typical for high-spin iron(III) is evidently a result of after-effects(*). Note that the parameters both for the two nucleogenic $^{57}Fe^{II}$ forms and for the stabilised $^{57}Fe^{III}$ form in the cell-free supernatant liquid (sample 5 in Table 1) are distinctly different from those of the bacteria after 2 min of contact with $^{57}Co^{2+}$ (sample 1). The presence of at least two major cobaltous forms (with different IS and QS values, see Table 1) 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 (Steenhoudt *et al.* 2000; Kamnev *et al.* 1999, 2001, 2002; Skvortsov *et al.* 1998; Yegorenkova *et al.* 2001).

In the cell-free culture solution, the most likely ligands which could bind Co^{2+} were phosphate and possibly malate anions (they were present in the initial standard growth medium (Kamnev *et al.* 1997, 1999, 2001, 2002; Tugarova *et al.* 2002) at concentrations of the order of 10^{-2} M), ammonia (from NH_4^+ added to the initial medium at 5 mM), as well as various (probably acidic) exopolysaccharides dissolving from the bacterial cell surface (Skvortsov *et al.* 1998). Note that both malate and ammonium 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 $^{57}Co^{2+}$ complexes in the cell-free solution are likely to be represented by oxygen (including that of hydration water molecules) and probably nitrogen. The largest QS value (QS = 3.23 ± 0.05 mm/s in sample 5) is rather close to that for the ferrous hexaaquo complex (Vértes *et al.* 1979; Ambe 1994); however, the value of IS = 1.22 ± 0.01 mm/s for that form is significantly lower. In view of that, the presence of 'simple' $[^{57}Co(H_2O)_6]^{2+}$ cations in the cell-free culture solution in somewhat noticeable amounts is unlikely. On the other hand, for live bacteria already after 2 min of contact with $^{57}Co^{2+}$ (sample 1), the lower QS values for both the (+2)-forms, as compared to those in the supernatant liquid (sample 5), presume the formation of complexes with other organic O- and N-donor ligands (with more covalent interactions) which are characterised by lower QS values (Vértes *et al.* 1979; Ambe 1994). Note also the significant difference in the relative contents of the stabilised $^{57}Fe^{III}$ forms (cf. sample 1 with S_r = 36% and sample 5 with S_r = 28%) which also reflects the difference in the coordination environment of the parental $^{57}Co^{2+}$ ions.

Thus, the process of primary adsorption of cobalt(II) traces by the bacterial cells is evidently rapid and complete. This finding is in line with the results on other Gram-negative bacteria (Volesky 1990; Garcia-Gil *et al.* 1997; Sar *et al.* 1999) showing, in particular, that bacterial biomass (both as a live culture and lyophilised cell biomass) is promising for

(*)In principle, each chemical form of $^{57}Co^{II}$ in the sample under study can give an individual form of stabilised nucleogenic $^{57}Fe^{III}$ as a result of after-effects. Nevertheless, owing to insufficient resolution of the lines of the daughter $^{57}Fe^{III}$ (with the obvious absence of relaxation phenomena in view of the 'flat' right-hand wings in spectra A–E (Figure 1) at velocities over 2 mm/s asymptotically approaching zero of absorption), its contributions in each spectrum were approximated by one doublet (with consequently somewhat increased linewidths), which did not influence the resolution of the $^{57}Fe^{II}$ lines (see Table 1).

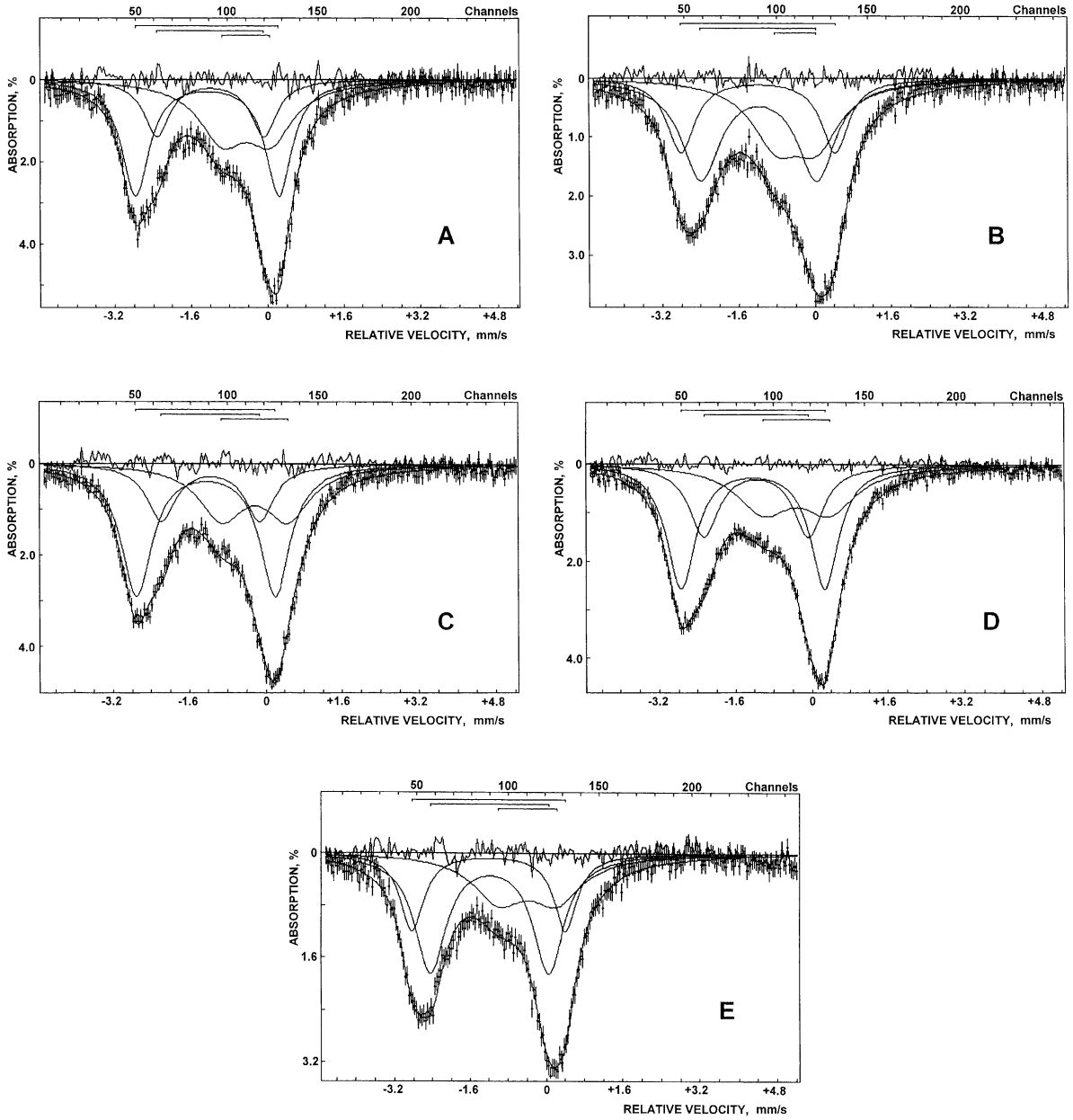

Fig. 1. Emission Mössbauer spectra of aqueous suspensions of live (A, B, C) and dead (D) cells of *Azospirillum brasilense* Sp245 in the culture medium as well as of the cell-free supernatant liquid (E), which were incubated with $^{57}CoCl_2$ for 2 min (A), 10 min (B) and 60 min (C, D, E) at ambient temperature and then rapidly frozen in liquid nitrogen (spectra collected at $T = 80$ K; see also Table 1). 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 brackets above the zero line; the difference curve (the computer-fitted spectrum minus experimental data) is also presented for each spectrum.

Table 1. Mössbauer parameters[a] calculated from emission Mössbauer spectroscopic data for aqueous suspensions of live and dead cells of *Azospirillum brasilense* Sp245 in the culture medium as well as for the cell-free supernatant liquid, which were incubated with $^{57}CoCl_2$ for specified periods of time at ambient temperature and then rapidly frozen in liquid nitrogen (spectra collected at $T = 80$ K)

Sample	Oxidation state[b]	IS,[c] mm/s	QS,[d] mm/s	FWHM,[e] mm/s	S_r,[f] %	Fig.
1. Live bacterial cells (frozen 2	+2	1.26(1)	3.00(3)	0.69(3)	44(1)	
min after adding $^{57}CoCl_2$ to	+2	1.20(6)	2.23(6)	0.65(8)	20(1)	1A
the culture medium)	+3	0.45(5)	1.0(1)	1.2(1)	36(1)	
2. Live bacterial cells (frozen 10	+2	1.20(1)	3.23(2)	0.71(2)	24(1)	
min after adding $^{57}CoCl_2$ to	+2	1.19(4)	2.42(9)	1.00(6)	44(1)	1B
the culture medium)	+3	0.41(9)	0.9(2)	1.2(1)	32(1)	
3. Live bacterial cells (frozen 60	+2	1.26(1)	2.89(2)	0.78(2)	51(1)	
min after adding $^{57}CoCl_2$ to	+2	1.16(1)	2.03(4)	0.73(6)	20(1)	1C
the culture medium)	+3	0.24(2)	1.40(3)	1.13(6)	29(1)	
4. Dead bacterial cells (frozen	+2	1.24(1)	3.00(2)	0.73(2)	44(1)	
60 min after adding $^{57}CoCl_2$	+2	1.17(1)	2.18(4)	0.76(4)	27(1)	1D
to the culture medium)	+3	0.33(3)	1.39(6)	1.4(1)	29(1)	
5. Cell-free supernatant liquid of	+2	1.22(1)	3.23(5)	0.60(2)	24(1)	
the live culture (frozen 60 min	+2	1.21(1)	2.48(3)	0.80(3)	48(1)	1E
after adding $^{57}CoCl_2$)	+3	0.40(6)	1.22(3)	1.3(2)	28(1)	

[a]Errors (in the last digits) are given in parentheses. [b]Oxidation states of the daughter ^{57}Fe components stabilised after nuclear decay of the parent $^{57}Co^{II}$. [c]Isomer shift (relative to α-Fe) converted to the normal absorption convention (positive with regard to α-Fe). [d]Quadrupole splitting. [e]Full line width at half maximum (assumed to be equal for both the lines of a doublet). [f]Relative resonant absorption areas 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.

use in heavy-metal bioremediation. Note also that cobalt(II) (at 0.05 to 0.2 mM) was reported (Ivanov *et al.* 1998) to affect the properties of the outer membrane of *Pseudomonas* strains, which was reflected by changes in electrophysical properties of the cell surface detected already after 15 min of contact with Co^{2+}. (In *A. brasilense* Sp245, similar changes detected in electro-orientational spectra of aqueous cell suspensions at kHz-MHz frequencies of the orienting alternating electric field were observed (Ignatov *et al.* 2001) for cells exposed to 0.2 mM copper(II) for 1 h and more.) In cobalt(II)-resistant *Pseudomonas* strains, as compared to cobalt(II)-sensitive strains, biosynthesis of several new cell-surface proteins was documented which are supposed to be responsible for the protective effect against metal ions (Ivanov *et al.* 1998, 1999).

It is interesting to note that after 10 min and, further, 60 min of incubation of live cells with $^{57}Co^{2+}$ (Figure 1, spectra B and C; samples 2 and 3 in Table 1), the Mössbauer parameters significantly changed as compared to those for the 2-min incubation (sample 1). The observed decrease in QS for the daughter $^{57}Fe^{II}$ component with IS $= 1.26 \pm 0.01$ mm/s and its increased proportion in sample 3 (after 60 min of incubation), as well as the appearance of the $^{57}Fe^{II}$ component with lower IS (1.16 ± 0.01 mm/s) and QS (2.03 ± 0.04 mm/s) indicate further metabolic transformations of $^{57}Co^{II}$, following its primary adsorption. This could be related to the gradual formation of species with a lower coordination number (with O-donor ligands), as well as with more covalent coordination bonds (involving, e.g. ligands with N- or S-donor atoms) for the latter component (Vértes *et al.* 1979, 1998; Burdman *et al.* 1999), e.g. related to cell-wall proteins (Ivanov *et al.* 1998, 1999; Castellanos *et al.* 1998; Burdman *et al.* 1999, 2001). Such processes may also account for a slight gradual decrease in the yield of the stabilised nucleogenic $^{57}Fe^{III}$ component (36%, 32% and 29% samples 1, 2 and 3, respectively; see Table 1).

It should be noted that direct identification of metal complexes in such sophisticated systems as bacterial cells is as yet difficult for several reasons. First, various chemical species with similar coordination microenvironments can give similar Mössbauer parameters (Kuzmann *et al.* 2003). Second, different cell-surface biopolymers may well have rather similar cation-binding functional groups. Also, in the emission variant of Mössbauer spectroscopy, the Auger electrons can influence to some extent the coordination environment of the nuclide, even when its chemical state remains unchanged (Vértes *et al.* 1979, 1998; Baggio-Saitovitch *et al.* 1972), which, in particular, results in some noticeable line broadening in ^{57}Co emission Mössbauer spectra as compared to absorption spectra of the corresponding ^{57}Fe compounds. Thus, at this step, the above conclusions on metabolic transformations of cobalt(II) in live cells remain rather speculative, which is mainly owing to the lack of EMS data on particular bacterial cell-surface structures. Further model EMS experiments with isolated cellular constituents and metabolites could be helpful in analysing the relevant bacterial processes *in situ*.

The data in Table 1 also show that the parameters for dead (thermally killed) cells (sample 4) are very close to those for live cells after 2 min of contact with $^{57}Co^{2+}$ (sample 1; primary absorption), in particular, for both the (+2)-forms and their relative contents. This can well be attributed to the similarity of the primary metal-binding process at the cell surface of live bacteria (with further ongoing metabolic transformations mentioned above detected after larger periods) with the process of metal complexation at the cell surface of dead cells which obviously represents a purely chemical interaction. The overall similarity of the parameters for samples 1 and 4 (see Table 1) is remarkable, considering the preceding hydrothermal treatment of the cells of sample 4. Such treatment could in principle induce some chemical modifications mostly in cellular proteins (e.g., their partial denaturation) and/or some alterations in water content in cell-surface biopolymers. Therefore the statistically insignificant differences in the parameters of both the (+2)-forms in samples 1 and 4 and their close relative contents indicate that the processes of primary adsorption of cobalt(II) by live cells and its interaction with thermally killed bacteria are chemically similar and thus obviously not affected by the hydrothermal treatment applied. The minor differences in the after-effects (somewhat different parameters for the stabilised (+3)-form for samples 1 and 4, and its lower content in sample 4) might be related to some possible decrease in the hydration of cell-surface biopolymers due to the hydrothermal treatment.

For comparison it could be noted that similar earlier EMS studies in *Escherichia coli* showed that ^{57}Co, taken up from enterocholine solution, remained within the cell membrane for 24 h, after which it was found in the interior of the cell (cited from [19], p. 386). In view of that, it may be inferred that the changes observed in the spectra (see Figure 1,A–C and Table 1, samples 1–3), reflecting cobalt(II) metabolic transformations in *A. brasilense* Sp245 within an hour, correspond to processes occurring within the bacterial cell wall involving (lipo)polysaccharide- and protein-containing moieties typical for azospirilla (Steenhoudt *et al.* 2000; Kamnev *et al.* 1999, 2001, 2002; Skvortsov *et al.* 1998; Burdman *et al.* 2001).

Conclusions

EMS measurements in frozen aqueous solutions have shown that live *A. brasilense* Sp245 cells rapidly and completely adsorb traces of $^{57}Co^{2+}$ from the medium, with its further metabolic 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 hydrothermally killed bacteria. The results obtained demonstrate that EMS is a valuable tool for the monitoring of trace cobalt uptake and its metabolic transformations in complicated biological systems including, e.g. living bacterial cells (*in vivo*) with ongoing metabolic processes.

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