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ORIGINAL PAPER

Emission (^{57}Co) Mössbauer spectroscopy as a tool for probing speciation and metabolic transformations of cobalt(II) in bacterial cells

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Abstract The emission (^{57}Co) variant of Mössbauer spectroscopy, rarely used in biology-related studies, was applied to study binding and possible transformations of $^{57}Co^{II}$ traces in live and dead (hydrothermally treated) cells of the rhizobacterium *Azospirillum brasilense* (strain Sp7) at T= 80 K in frozen aqueous suspensions and as their dried residues. The Mössbauer parameters calculated from the spectra were compared with the similarly obtained data reported earlier for another *A. brasilense* strain, Sp245 (which differs from strain Sp7 by the ecological niche occupied in the rhizosphere and was found earlier to exhibit different metabolic responses under similar environmental conditions). Similarly to strain Sp245, live cells of strain Sp7, rapidly frozen 2 min and 1 h after their contact with $^{57}Co^{2+}$ (measured in frozen suspensions), showed marked differences in their Mössbauer parameters, reflecting metabolic transformations of $^{57}Co^{2+}$ occurring within an hour. However, the parameters for strains Sp7 (this work) and Sp245 (reported earlier), obtained under similar conditions, were found to significantly differ, implying dissimilarity in their metabolic response to Co^{2+}. This is in line with their different metabolic responses to several heavy metals, including Co^{2+}, detected earlier using Fourier transform infrared spectroscopy.

Keywords Cobalt(II) metabolism · *Azospirillum brasilense* · ^{57}Co emission Mössbauer spectroscopy · Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy

Abbreviations

DRIFT	Diffuse reflectance infrared Fourier transform (spectroscopy)
EMS	Emission Mössbauer spectroscopy
FTIR	Fourier transform infrared (spectroscopy)
MS	Mössbauer spectroscopy
PHB	Poly-3-hydroxybutyrate

Introduction

Cobalt is a trace element which has a wide variety of important physiological and biochemical functions (see,

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e.g. [1, 2] and references therein). Nevertheless, like many transition metals, at higher concentrations, cobalt can become toxic. Interaction of bacterial cells with cobalt ions, along with its diverse scientific aspects [2–4], is also of ecological significance. In particular, this is due to biogeochemical problems related to bioleaching of the ^{60}Co radioisotope from nuclear waste disposal sites facilitated by microorganisms [5, 6].

The emission (^{57}Co) variant of Mössbauer spectroscopy (^{57}Co EMS) is a highly sensitive and informative tool, which has yet scarcely been used in biology-related studies [2, 3, 7, 8], primarily owing to methodological difficulties of using a radionuclide in samples under study. Nevertheless, about a decade ago, ^{57}Co EMS has been shown for the first time to be applicable to probing and distinguishing different cation-binding sites within the active centres of a sophisticated enzyme, bacterial glutamine synthetase [9, 10]. As for live cells, ^{57}Co EMS was applied for monitoring the state of cobalt(II) in roots of the water hyacinth *Eichhornia crassipes* [11], as well as in cells of a cyanobacterium (the blue-green alga *Synechococcus vulcanus*) [12] and Gram-negative bacteria (*Escherichia coli* [13] and *Azospirillum brasilense* (strain Sp245; see below) in the freeze-dried state [9] or in frozen aqueous suspensions [14]).

The aforementioned nitrogen-fixing rhizobacterium *A. brasilense*, the most studied species among the bacteria of the genus *Azospirillum*, has long been under intensive investigation worldwide owing to its plant-growth stimulating potential [15, 16]. This bacterial species also represents a remarkable model for studying its responses to various ecological factors. Among its wild-type strains, strain Sp245 is a facultative endophyte (capable of penetrating into and colonising the plant root tissues), whereas strain Sp7 is an epiphyte (colonising the root surface only) [16]. Thus, the two strains of the same species occupy essentially different ecological niches. As a consequence of their different adaptive strategies, they have often been compared and documented to show notable differences in behaviour under similar environmental conditions [3, 16–19]. In particular, they exhibited different metabolic responses to the effects of heavy-metal ions (including Co^{2+} [3]), which was revealed using Fourier transform infrared (FTIR) spectroscopy (or its diffuse reflectance variant, diffuse reflectance infrared Fourier transform (spectroscopy), DRIFT) of cell biomass [3, 19].

In this work, ^{57}Co emission Mössbauer spectroscopy was utilised as a sensitive tool to study binding and possible transformations of $^{57}Co^{II}$ traces in live and dead cells of *A. brasilense* (epiphytic strain Sp7) at T=80 K in frozen $^{57}Co^{2+}$-containing aqueous suspensions and as their dried residues (a preliminary brief account of part of this work has recently been reported in [20]). DRIFT spectroscopy was used to monitor the overall cell composition of the bacterium (prior to the experiments on binding of $^{57}Co^{2+}$ traces) with regard to cellular proteins and other biopolymers [3, 14, 21]. The results are compared with similarly obtained FTIR (DRIFT) and ^{57}Co EMS data reported earlier for the 'ecologically different' *A. brasilense* strain Sp245 [3, 14].

Materials and methods

The bacterium *A. brasilense* Sp7 from the Collection of IBPPM RAS, Saratov, was cultivated at 31 °C for 18 h under aeration on a rotary shaker (180 rpm) in a standard phosphate–malate medium [3, 21] with 0.5 gl^{-1} NH_4Cl as a source of bound nitrogen. For DRIFT spectroscopic measurements (see below), live cells were collected by centrifugation (2,100×*g*, 25 min), washed three times with physiological solution (aqueous 0.85 % NaCl) to remove culture medium components and dried in air at room temperature until constant mass. For comparison, two additional samples of *A. brasilense* Sp7 cells, similarly grown (for 2 days with 0.5 gl^{-1} NH_4Cl and without NH_4Cl, i.e. under nutritional stress in the latter case) and prepared, were used in DRIFT spectroscopic measurements.

For ^{57}Co emission Mössbauer measurements, live cells (grown as above) or dead cells (hydrothermally treated for 1 h at 90 °C using a water bath) were prepared as reported in [3]. The culture was collected by centrifugation in Eppendorf tubes (10,000×*g*, 10 min), washed three times with physiological solution (aqueous 0.85 % NaCl) to remove culture medium components and kept at a density of 1.4×10^9 cellsml^{-1} overnight at +4 °C. Prior to emission Mössbauer measurements, the culture was incubated for 2 h at room temperature, kept in contact with $^{57}Co^{2+}$ in dense aqueous suspensions (0.2 ml; 1.4×10^9 cellsml^{-1}; 1.2 mCi $^{57}CoCl_2$, corresponding to 1.2×10^{-5}M $^{57}Co^{2+}$, which is of very weak toxicity to *A. brasilense* Sp7 [3, 22]) for 2 min or 1 h and then rapidly frozen in liquid nitrogen.

Emission spectra were obtained using a conventional Mössbauer spectrometer WISSEL (FRG) for the resulting frozen aqueous suspensions and their dried residues. Samples, which in EMS are sources of γ-radiation, were kept in a specially designed cryostat (with a window for γ-rays) filled with liquid nitrogen (at T=80 K). The absorber ($K_4[^{57}Fe(CN)_6]\cdot 3H_2O$) was attached to a vibrator in the γ-ray beam path (sample–absorber–detector). The experimental data obtained using a conventional detector and computer-operated multichannel analyser as described elsewhere [3] were processed using the MOSSWINN program [23]. Standard computer-based statistical analysis consisted of fitting the experimental data obtained (converted into a form compatible with that of absorption ^{57}Fe Mössbauer measurements) as a sum of Lorentzians using a least squares minimisation procedure for χ^2 [3]. The values of isomer shift (δ; relative to α-Fe at room temperature), quadrupole

splitting (ΔE), linewidth (Γ; full width at half maximum) and relative areas of spectral components (S_r) were thus calculated for each spectrum.

In order to check and compare the overall composition of cellular biomass, DRIFT spectra were obtained and processed for the dried biomass sample (prepared prior to ^{57}Co EMS measurements) and for the two samples of dried biomass used for comparison (grown as above for 2 days without and with the nutritional stress of bound nitrogen deficiency) using a Nicolet 6700 FTIR spectrometer (Thermo Electron Corporation, USA) with a DRIFT accessory and a Micro sampling cup (Spectra-Tech, USA) as reported elsewhere [19, 21].

Results and discussion

DRIFT spectroscopic measurements

In order to control the overall composition of cellular biomass, which may show some specific structural and compositional differences in cellular biomacromolecules in case of stresses or unfavourable conditions [3, 19, 21], FTIR spectroscopy in the diffuse reflectance mode (DRIFT) was used. Note that azospirilla (including *A. brasilense*), being commonly microaerophilic [16], nevertheless grow relatively well also under aeration [21].

The DRIFT spectrum of the dried biomass (prepared prior to ^{57}Co EMS measurements; Fig. 1, spectrum 1) is well comparable with that obtained for *A. brasilense* Sp7 cells grown similarly for 2 days without nutritional stress (Fig. 1, spectrum 2) and resembles those of many other Gram-negative bacteria [24, 25]. In particular, the vibration regions featuring cellular proteins (amide I and amide II bands within ca. 1,700–1,500 cm^{-1}) are very similar in spectra 1 and 2 (some slight differences in the region under ca. 1,200 cm^{-1} are attributable to cellular polysaccharides of highly diverse and variable composition [21, 24]). However, for the sample grown without NH_4Cl (i.e. under nutritional stress), the DRIFT spectrum is significantly different (Fig. 1, spectrum 3). In particular, a number of additional bands can be observed, featuring an enhanced accumulation of carbon-and-energy storage material, poly-3-hydroxybutyrate (PHB), which is commonly induced by nitrogen deficiency and some other stresses [3, 19, 21, 24]. Note for comparison that for *A. brasilense* strain Sp245 grown under stressed conditions, along with PHB accumulation under nitrogen deficiency, in DRIFT spectra, some splitting was detected in the amide I band [21] (see the amide I and amide II bands of cellular proteins in Fig. 1), which is known to be sensitive to the secondary structure of proteins [21, 24], as compared to the control cells.

In view of the aforementioned, a relatively weak shoulder at ca. 1,740 cm^{-1} (see Fig. 1, spectrum 1), featuring the

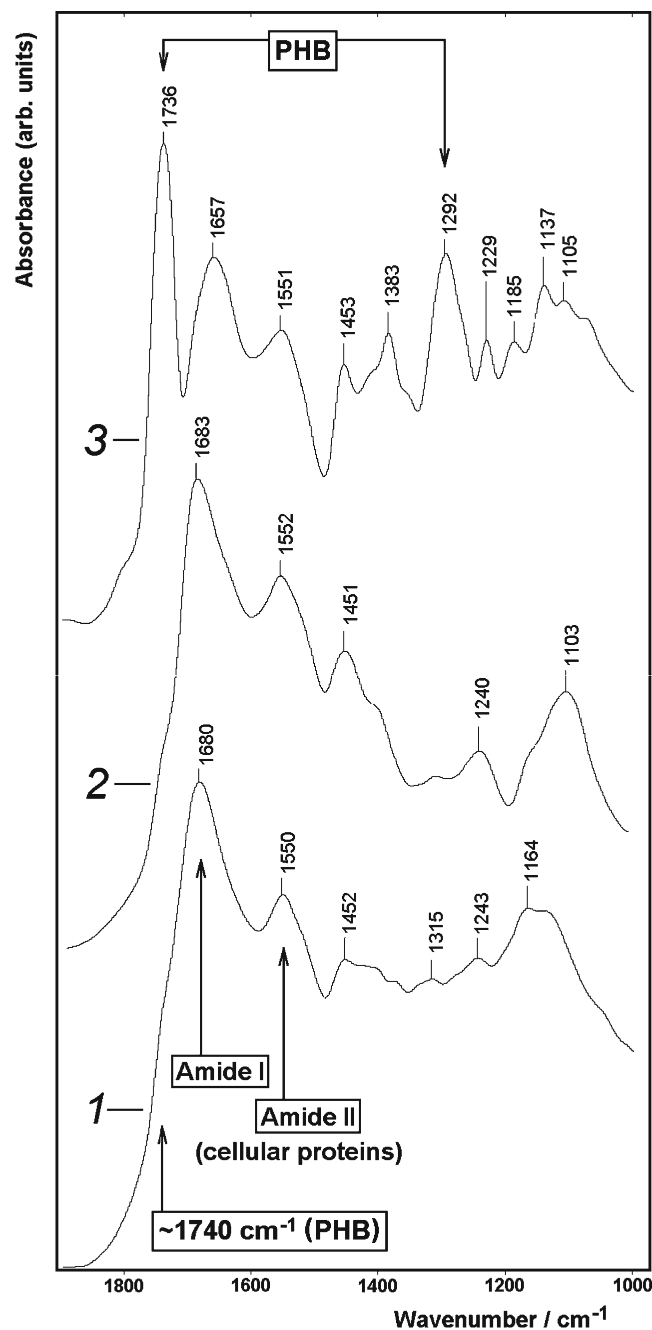

Fig. 1 DRIFT spectra of *A. brasilense* strain Sp7 cells (*1*) grown for 18 h with 0.5 gl^{-1} NH_4Cl and dried prior to the experiments on $^{57}Co^{2+}$ binding, as well as of dried cell biomass used for comparison: similarly grown for 2 days (*2*) with 0.5 gl^{-1} NH_4Cl (no nutritional stress) and (*3*) without NH_4Cl (under nutritional stress). The positions of main bands of cellular proteins, amide I and amide II, and of the most pronounced bands related to poly-3-hydroxybutyrate (PHB, intracellular storage polyester) are indicated with *arrows*

stretching C=O mode of cellular polyester compounds, shows that under the cultivation conditions applied for this sample, *A. brasilense* Sp7 cells were not subjected to any appreciable nutritional stress which could induce PHB

accumulation [19]. Thus, the biomass prepared for ^{57}Co EMS measurements was evidently in its state close to 'normal'.

^{57}Co emission Mössbauer spectroscopic measurements

The ^{57}Co→^{57}Fe nuclear transformation, proceeding via electron capture from inner electronic shells by the ^{57}Co nucleus (which thereby turns into ^{57}Fe), is accompanied by subsequent concatenated processes of consecutively filling in the vacancies in inner electronic shells of the resulting ^{57}Fe atom (the so-called Auger cascade developing within 10^{-14} to 10^{-15} s). This is followed by emission of a 14.4-keV γ-quantum (ca. 10^{-7} s after the electron capture). Within this extremely short time interval, no chemical transformations can occur. Registering fine differences in the energy of the emitted γ-quanta (by nuclear γ-resonance using a standard ^{57}Fe-containing absorber which is moved (vibrated) to achieve energy modulation using the Doppler effect) provides information on all possible hyperfine interactions in the sample and, accordingly, on the properties of the ^{57}Co sites (for more methodological details, see, e.g. [2] and references therein). Thus, the resulting information on the substance under investigation using the ^{57}Co EMS technique may be described as a 'snapshot' of a ^{57}Fe complex substituted for the 'parent' ^{57}Co binding site (at the moment of γ-quantum emission). This daughter ^{57}Fe complex retains (and, therefore, reflects in the emission spectrum) the 'parent' coordination, geometry and charge, while some proportion of a differently charged ^{57}Fe species may form owing to aftereffects [2]. For example, for $^{57}Co^{II}$ sites, along with stabilised daughter $^{57}Fe^{II}$, some part of aliovalent stabilised nucleogenic $^{57}Fe^{III}$ may appear owing to the interaction (capture) of some Auger electrons within the immediate chemical environments of the atom, reflecting their electron-acceptor properties.

Some typical emission Mössbauer spectra for live and dead (hydrothermally treated) cells are shown in Figs. 2 and 3, respectively. The main Mössbauer parameters calculated from all the emission spectra are listed in Table 1. Similar to strain Sp245 [9, 14], for strain Sp7 studied in this work in all cases, two quadrupole doublets of nucleogenic (daughter) high-spin $^{57}Fe^{II}$ components were found corresponding to two main chemical forms of parent $^{57}Co^{II}$. This correlates with the existence of various functional groups on the bacterial cell wall [26] which could form different kinds of complexes (with different microenvironments), in particular, upon initial binding [14].

Dead Sp7 cells, both in suspension (*S*) (see Fig. 3) and dried (*D*), gave very close Mössbauer parameters (see Table 1). Slight differences in ΔE and S_r for their doublet 2 may be attributed to changes in the $^{57}Co^{2+}$ microenvironment caused by drying. Similarity of the parameters for dead cells reflects the purely chemical nature of cobalt(II) binding, in the absence of ongoing metabolic transformations (see below)

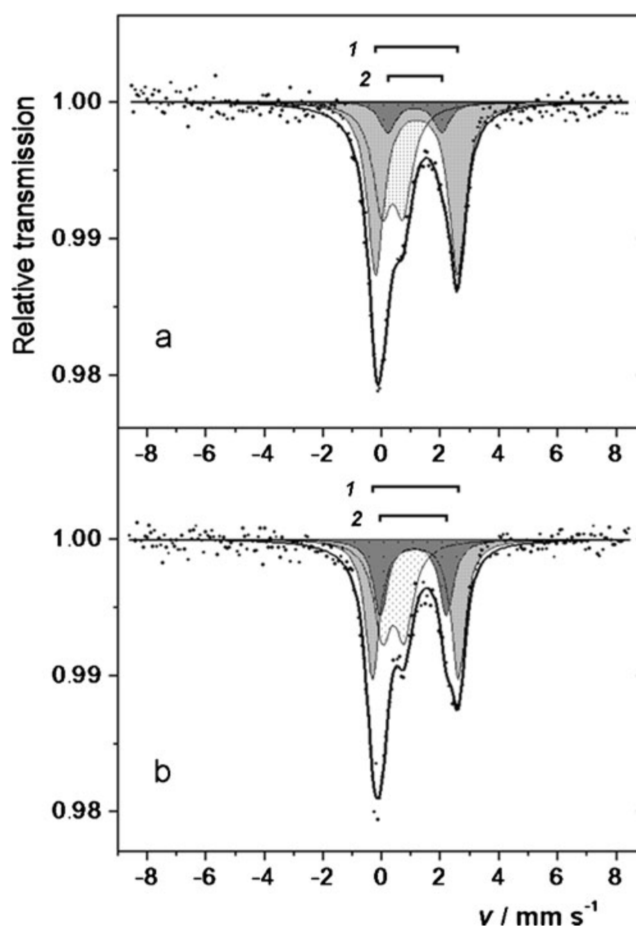

Fig. 2 Emission Mössbauer spectra of *A. brasilense* Sp7 live cells (0.2 ml, dense culture) incubated with 1.2 mCi $^{57}CoCl_2$ **a** for 2 min or **b** for 1 h, then rapidly frozen in liquid nitrogen and dried in air (measured as dried suspensions at T=80 K). The positions of the two main quadrupole doublets (*dark shaded*, *1* and *2*) corresponding to daughter $^{57}Fe^{II}$ forms (see Table 1, samples "Live (2 min), *D*" and "Live (1 h), *D*") are shown above the spectra (the third light-shaded narrow doublets correspond to daughter $^{57}Fe^{III}$ resulting from aftereffects of the ^{57}Co→^{57}Fe transition)

which could continue in the process of drying [14]. Thus, in suspensions (*S*), dead cells gave the parameters statistically indistinguishable from those for live cells frozen after 1 h, while for dried samples (*D*), the parameters for dead cells are much closer to those for live cells frozen after 2 min. This can logically be ascribed to possible gradual changes still occurring in the latter while drying.

It should be noted that the contributions from aftereffects of the ^{57}Co→^{57}Fe nuclear transformation (see, e.g. the third narrow doublets in both spectra in Figs. 2 and 3 related to the nucleogenic $^{57}Fe^{III}$ [2, 3]) markedly increased upon drying for live cells frozen 2 min after their contact with $^{57}Co^{2+}$ (from 25 % (*S*) to 35 % (*D*); see the data in Table 1, footnote a, for the residual doublets related to daughter $^{57}Fe^{III}$) as well as for dead cells (from 27 % (*S*) to 36 % (*D*)). These contributions, which are comprised by different

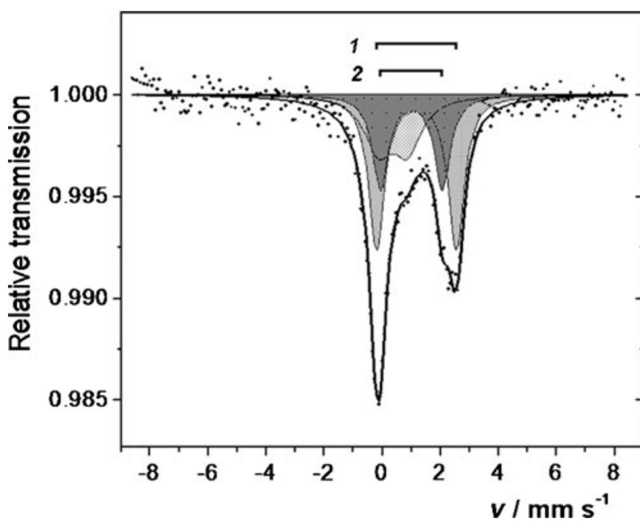

Fig. 3 Emission Mössbauer spectrum of *A. brasilense* Sp7 dead cells (0.2 ml, dense culture; hydrothermally treated for 1 h at 90 °C) incubated with 1.2 mCi $^{57}CoCl_2$ for 1 h, then rapidly frozen in liquid nitrogen (measured in frozen suspension at T=80 K). The positions of the two main quadrupole doublets (*dark shaded*, *1* and *2*) corresponding to daughter $^{57}Fe^{II}$ forms (see Table 1, sample "Dead (1 h), *S*") are shown above the spectra (the third light-shaded narrow doublet corresponds to daughter $^{57}Fe^{III}$ resulting from aftereffects of the $^{57}Co \rightarrow ^{57}Fe$ transition)

yields of aftereffects from each ^{57}Co microenvironment in a sample, depend on the electron-acceptor properties of the ligands, i.e. of the nearest coordination microenvironment [2]. The latter may evidently change upon drying, which inevitably alters the hydration of the coordinating functional groups (ligands) and probably also of the cation in the complexes. Note that a similar effect (i.e. increasing yields of stabilised nucleogenic $^{57}Fe^{III}$ in ^{57}Co emission Mössbauer spectra of $^{57}Co^{2+}$-doped bacterial cells registered in dried biomass, as compared to those in frozen aqueous suspensions) was observed also for strain Sp245 of this bacterium (see [3] and the corresponding data for strain Sp245 cited in Table 1). These findings imply that the electron-acceptor properties of 'less hydrated' $^{57}Co^{II}$ microenvironments in dried bacterial cell samples are generally more pronounced as compared to those in the aqueous medium [2], inducing higher yields of aliovalent stabilised nucleogenic $^{57}Fe^{III}$.

Note also that the aforementioned changes in the two different types of the nearest coordination microenvironments of $^{57}Co^{II}$ (represented by doublets 1 and 2, see Table 1) induced by drying can result in a redistribution of $^{57}Co^{II}$ between the latter owing to possible changes in their relative stability. This may account, at least in part, for changes in the areas of doublets 1 and 2 upon drying. In particular, for doublets 1 or 2 (cf. samples *S* and *D*, 1 h), their corresponding δ and ΔE values are close in going from sample *S* to sample *D*, but their respective areas (S_r) change oppositely upon drying, assuming different hydration of the Co sites.

One of the most important findings is that for live cells frozen 2 min and 1 h after their contact with $^{57}Co^{2+}$ (measured

Table 1 Mössbauer parameters calculated from ^{57}Co emission spectra for live or dead cells of *A. brasilense* Sp7 (in brackets, the corresponding data are presented for *A. brasilense* strain Sp245 taken from [3]), incubated with $^{57}CoCl_2$ for specified periods of time and then rapidly frozen in liquid nitrogen (measured at T=80 K), in aqueous suspension (*S*) or dried (*D*)

Samples of bacterial cells	Multiplet[a]	δ,[b] mms^{-1}	ΔE,[c] mms^{-1}	S_r,[d] %
Live (2 min), *S*	Doublet 1	1.10 (1.26)	2.59 (3.00)	56 (44)
	Doublet 2	0.89 (1.20)	2.00 (2.23)	19 (20)
Live (1 h), *S*	Doublet 1	1.16 (1.26)	2.84 (2.89)	35 (51)
	Doublet 2	1.02 (1.16)	2.18 (2.03)	31 (20)
Dead (1 h), *S*	Doublet 1	1.17 (1.24)	2.75 (3.00)	45 (44)
	Doublet 2	1.00 (1.17)	2.13 (2.18)	28 (27)
Live (2 min), *D*	Doublet 1	1.18 (1.24)	2.79 (3.08)	55 (19)
	Doublet 2	1.12 (1.14)	1.84 (2.35)	10 (23)
Live (1 h), *D*	Doublet 1	1.14 (1.22)	2.93 (2.84)	43 (38)
	Doublet 2	1.07 (1.00)	2.25 (2.03)	23 (8)
Dead (1 h), *D*	Doublet 1	1.17	2.78	57
	Doublet 2	1.04	1.95	7

[a] Main doublets corresponding to daughter $^{57}Fe^{II}$ forms stabilised after the $^{57}Co \rightarrow ^{57}Fe$ nuclear transition, with linewidth (Γ) values ranging within 0.6–0.8 mms^{-1} (the residual $^{57}Fe^{III}$ forms resulting from aftereffects had δ ~0.36–0.39 mms^{-1}, ΔE ~0.7–1.0 mms^{-1}; their S_r values are 100 % minus the corresponding S_r values for doublets 1 and 2)

[b] Isomer shift (relative to α-Fe at room temperature)

[c] Quadrupole splitting

[d] Relative resonant absorption area. Calculated errors: for δ, ±0.02 mms^{-1}; for ΔE and Γ, ±0.05 mms^{-1}; for S_r, ±7 rel.%

both as *S* and *D*), notable differences in corresponding Mössbauer parameters were found, reflecting metabolic transformations of $^{57}Co^{2+}$ occurring within an hour. (Similar metabolic changes were detected earlier in emission Mössbauer spectra for live cells of strain Sp245 [14].) Also, the increased contributions of doublet 2 in going from 2-min to 1-h samples suggest their relation to ongoing metabolic processes.

However, the Mössbauer parameters for strains Sp245 [14] and Sp7 (see Table 1), obtained under similar conditions, show significant differences both in δ and ΔE for both quadrupole doublets 1 and 2. This suggests essential dissimilarities in their metabolic response to Co^{2+}, which is in line with the data obtained earlier using FTIR spectroscopy [3].

Finally, for strain Sp7 studied in this work, it has to be emphasized that, while the Mössbauer parameters of doublet 1 in all samples (δ>1.1 mm s^{-1}, ΔE≈2.6–2.9 mm s^{-1} at 80 K) may be ascribed to octahedrally coordinated $^{57}Co^{II}$ (resulting in similar daughter high-spin $^{57}Fe^{II}$ microenvironments upon the $^{57}Co \rightarrow ^{57}Fe$ transformation), the parameters for doublet 2 have significantly smaller values of both δ (within ca. 0.9–1.1 mm s^{-1}) and ΔE (within ca. 1.8–2.2 mm s^{-1}). These values may suggest tetrahedral coordination of $^{57}Co^{II}$ which is quite typical for many complexes of cobalt(II) with various biological molecules (see, e.g. [2, 27–30]) and, in particular, facilitates isostructural substitution of Co^{II} in tetrahedral sites, e.g. for Zn [30, 31] (cf. also Mössbauer parameters for similar Fe^{II} carboxylate-rich complexes with the T_d coordination symmetry [32]).

An alternative explanation for the lower δ and ΔE values for doublet 2 might be partial involvement of sulphur as donor atoms in $^{57}Co^{II}$-binding ligands (along with possible O or N donor atoms), which can result in similarly decreased δ and ΔE values [33]. Note that 1 h of Co^{II} interaction with live cells may be insufficient for the molecular mechanism involved in Co^{2+} uptake to develop any toxicity-related effects (e.g. in [13], $^{57}Co^{II}$ was supposed to remain within the *E. coli* cell wall for 24 h). However, considering the changes in Mössbauer parameters observed for $^{57}Co^{II}$ within 1 h, this may have implications for the recently discovered molecular basis for cobalt(II) toxicity in bacteria [4], with the proven involvement of Co^{2+} in reactions with labile [Fe–S] clusters during their de novo biosynthesis or repair. This has yet to be tested by more long-term ^{57}Co EMS measurements on bacterial cells and comparisons with the EMS data on model $^{57}Co^{II}$ complexes with different donor atoms and having different coordination structures. Nevertheless, it is noteworthy that the Mössbauer parameters for one of the four Fe atoms in the $[4Fe–4S]^{2+}$ cluster in the LytB protein (also called IspH; the last enzyme in the methylerythritol phosphate (MEP) pathway) in LytB-overexpressing *E. coli* were reported to be δ=0.89 mm s^{-1} and ΔE=1.97 mm s^{-1} at T=77 K [33]. These and similar values reported in [33] and in references cited therein, which correspond to high-spin $^{57}Fe^{II}$ with three S and three O or N donor atoms in its coordination sphere (while 5-fold coordination cannot be excluded), are remarkably close to those found for doublet 2 in *A. brasilense* Sp7 cells (see Table 1).

Conclusions

At the present stage of methodological development, the ^{57}Co EMS technique applied in biology-related fields has still to be regarded as being in its infancy. This is largely due to insufficiency of data for various model or more sophisticated complexes of $^{57}Co^{2+}$ with different biological molecules. Although, because of that, it is not possible at present to reliably ascribe the emission Mössbauer parameters, obtained from the spectra of bacterial cells, to particular chemical species, still some sound conclusions from such ^{57}Co EMS measurements can be drawn. First of all, it is undoubted that ^{57}Co EMS 'feels' the metabolic transformations of $^{57}Co^{II}$ within the live cell which occur within an hour. This has been confirmed both for *A. brasilense* strain Sp245 studied earlier [3, 9, 14] and for its strain Sp7 studied in this work. Nevertheless, the Mössbauer parameters for these two strains, which are known to respond differently to moderate heavy-metal stress [3, 19] including cobalt(II) [3], have been found to be significantly different. While it is yet to be elucidated what molecular mechanisms underlie these differences, this finding definitely correlates with the different ecological behaviour of these strains. Thus, ^{57}Co EMS is a promising tool for cobalt(II) speciation analysis in diverse biological systems, from simple [29, 34] or more sophisticated biocomplex models [2, 9, 10] to live cells.

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