ISSN 1062-8738, Bulletin of the Russian Academy of Sciences. Physics, 2015, Vol. 79, No. 8, pp. 1036–1040. © Allerton Press, Inc., 2015.
Original Russian Text © A.A. Kamnev, A.V. Tugarova, K. Kovács, E. Kuzmann, Z. Homonnay, L.A. Kulikov, Yu.D. Perfiliev, 2015, published in Izvestiya Rossiiskoi Akademii Nauk. Seriya Fizicheskaya, 2015, Vol. 79, No. 8, pp. 1165–1169.

Mössbauer Spectroscopic Study of Iron and Cobalt Metabolic Transformations in Cells of the Bacterium *Azospirillum brasilense* Sp7

A. A. Kamnev[a], A. V. Tugarova[a], K. Kovács[b], E. Kuzmann[b], Z. Homonnay[b], L. A. Kulikov[c], and Yu. D. Perfiliev[c]

[a] *Institute of the Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, 410049 Russia*

[b] *Institute of Chemistry, Eötvös Loránd University, H-1117 Budapest, Hungary*

[c] *Moscow State University, Moscow, 119991 Russia*

e-mail: aakamnev@ibppm.sgu.ru; a.a.kamnev@mail.ru

Abstract—The transmission (^{57}Fe) and emission (^{57}Co) variants of Mössbauer spectroscopy are used to monitor metabolic transformations of $^{57}Fe^{III}/^{57}Fe^{II}$ and $^{57}Co^{II}$ in cells of the bacterium *Azospirillum brasilense* Sp7. (The spectra are measured at 80 K.) For $^{57}Co^{II}$, metabolic changes in the living cells are observed after 1 h. Two components of high-spin $^{57}Fe^{II}$ are observed for the bacteria grown with $^{57}Fe^{III}$ as a source of iron; the parameters for $^{57}Fe^{III}$ correspond to ferritin-like components.

DOI: 10.3103/S1062873815080110

INTRODUCTION

The transmission (^{57}Fe) variant of Mössbauer spectroscopy (TMS) has long and productively been used to study the states and transformations of iron in cells of different microorganisms [1, 2]. On the other hand, the use of emission (^{57}Co) Mössbauer spectroscopy (EMS) in biology to similarly investigate the state and transformations of cobalt compounds has been limited to only a few studies, despite the considerably higher sensitivity of the emission variant (see review [3]).

Soil bacteria of the *Azospirillum* genus that includes *A. brasilense* have attracted the attention of researchers for more than 35 years because of their occurrence in almost all climatic zones and the significant agrobiotechnological potential associated first of all with their phytostimulating activity [4]. The bacteria of the *A. brasilense* species are also a convenient model object for research in the field of the ecology of microorganisms, since this species includes strains that occupy different ecological niches in the rhizosphere and respond differently to external effects [5–7]. Nevertheless, data on the state of iron in azospirillum cells are few and far between [8, 9]. In one of our recent studies, TMS was applied to azospirilla for the first time to obtain data on the state of iron in living cells of *A. brasilense* (strain Sp245) (after quick freezing of the biomass) [10], and to the lyophilized biomass of that strain at room temperature [11]. We were the first to use EMS to study the structural organization of the cation-binding sites of the metalloenzyme glutamine synthetase from *A. brasilense* Sp245 doped with $^{57}Co^{2+}$ ions [12, 13], and the binding and transformation of cobalt(II) by cells of this bacterium [3, 5].

In this work, we perform a comparative study of the state and metabolic transformations of $^{57}Fe^{III}/^{57}Fe^{II}$ and $^{57}Co^{II}$ in cells of the *A. brasilense* bacterium (strain Sp7 that differs in ecological behavior from strain Sp245 [5–7]) by Mössbauer spectroscopy, using the transmission and emission variants, respectively.

EXPERIMENTAL

Our bacterial culture was grown as described in [10, 11] at 31°C with aeration (in the presence of 0.5 g L^{-1} NH_4Cl as a source of bound nitrogen) for 18 h. To study the state of iron by TMS, the bacterial culture was grown in the presence of 7×10^{-5} M of a complex of $^{57}Fe^{III}$ with nitrilotriacetic acid ($^{57}Fe^{III}$–NTA; 4.0 mg L^{-1} $^{57}Fe^{III}$) in the growth medium that served as the only source of iron. The cells were separated from the culture liquid via centrifugation and washed three times with saline (0.85 wt % NaCl). The wet suspension was then quick-frozen using liquid nitrogen.

To measure the emission Mössbauer spectra, samples grown in the cell culture and washed of the culture liquid as described above were held for 2 min or 1 h in the presence of $^{57}Co^{2+}$ (volume, 0.2 mL; culture density, 1.4×10^9 cells/mL; 1.2 mCi $^{57}CoCl_2$, corresponding to a concentration of 1.2×10^{-5} M $^{57}Co^{2+}$ with low toxicity toward *A. brasilense* Sp7 [5, 14]) and then quick-frozen using liquid nitrogen. Measurements (at a temperature of 80 K) were performed using both

Table 1. Mössbauer parameters[a], calculated using the emission spectra of samples of the cell biomass of *Azospirillum brasilense* (strain Sp7) grown over18 h, quick-frozen in liquid nitrogen after the addition of $^{57}Co^{2+}$ and incubation for 2 min and 1 h (*frozen suspension*), and after drying of the above suspension in air (*dried biomass*). All measurements were performed at a temperature of 80 K (see also Fig. 1).

Samples of cell biomass (time of contact with $^{57}Co^{2+}$)	Spectral components (quadrupole doublets)[b]	δ, mm s^{-1}	Δ, mm s^{-1}	S_r, %
Frozen suspension (2 min)	*1*	1.10	2.59	56
	2	0.89	2.00	19
Frozen suspension (1 h)	*1*	1.16	2.84	35
	2	1.02	2.18	31
Dried biomass (2 min)	*1*	1.18	2.79	55
	2	1.12	1.84	10
Dried biomass (1 h)	*1*	1.14	2.93	43
	2	1.07	2.25	23

[a] δ is the isomer shift (with respect to α-Fe at room temperature), Δ is the quadrupole splitting, and S_r is the area of the spectral component (in % of the total spectrum area).

[b] Quadrupole doublets corresponding to daughter forms of $^{57}Fe^{II}$ stabilized after nuclear transformation $^{57}Co \rightarrow {}^{57}Fe$ with values of the experimentally measured full line width at half of the intensity (W) in the range of 0.6–0.8 mm s^{-1} (the residual forms of $^{57}Fe^{III}$ stabilized as a result of aftereffects had values of δ ~ 0.36–0.39 mm s^{-1} and Δ ~ 0.7–1.0 mm s^{-1}. Their S_r values are 100% minus the corresponding S_r for doublets *1* and *2*). Estimated errors: for δ, ±0.02 mm s^{-1}; for Δ and Γ_{exp}, ±0.05 mm s^{-1}; relative error for S_r, ±7%.

the frozen suspensions and samples obtained from them by drying in air for one day.

Transmission Mössbauer spectra were measured by placing the frozen samples into a cryostat with liquid nitrogen (temperature of measurement, 80 K) [10] on a WISSEL spectrometer (Germany) functioning in the mode of constant acceleration with a $^{57}Co(Rh)$ source. In measuring the emission spectra, the samples frozen and put into the cryostat served as the source of γ radiation, with an absorber ($K_4[^{57}Fe(CN)_6] \cdot 3H_2O$) [7]. The resulting experimental data (in the case of the emission spectra converted into a form compatible with the transmission spectra) were approximated using the least squares method and the MOSSWINN program [15], all the values of the isomer shift being given with respect to α-Fe at room temperature.

RESULTS AND DISCUSSION

A comparison of the Mössbauer parameters calculated using the emission spectra of the cell biomass of *A. brasilense* Sp7 that was in contact with $^{57}Co^{2+}$ for 2 min and 1 h (Table 1; Figure 1 gives as an example the emission Mössbauer spectra of dry biomass measured at 80 K) shows that in the cells of strain Sp7, two components of nucleogenic high-spin $^{57}Fe^{II}$ corresponding to the initial components of $^{57}Co^{II}$ are observed. (The emergence in the emission spectra of an additional quadrupole doublet associated with aliovalent $^{57}Fe^{III}$ corresponds to the aftereffects of the nuclear transformation $^{57}Co \rightarrow {}^{57}Fe$ [3, 5, 12].) In the course of an hour, a metabolic transformation of cobalt(II) occurs with some change in the parameters and a redistribution of relative amounts of both components (see Table 1). It should be noted that although similar changes were observed in the emission Mössbauer spectra of the *A. brasilense* Sp245 strain that had come into contact with $^{57}Co^{2+}$ [5], the parameters of both components of $^{57}Co^{II}$ observed in the spectra of these two strains differ considerably, reflecting the differences between their metabolic responses to cobalt(II) also noted earlier using Fourier transform infrared spectroscopy [5].

For doublets *2* in the emission spectra (see Fig. 1 and Table 1), with their relative contribution growing in proportion to the time of the cells' contact with $^{57}Co^{2+}$ ions, lower values of the isomer shift (δ ~ 0.9–1.1 mm s^{-1}) and quadrupole splitting (Δ ~ 1.8–2.2 mm s^{-1}) compared to doublets *1*, are observed. It should be noted that the above parameters of the emission spectra (which correspond to the daughter cations of $^{57}Fe^{II}$ in the coordination environment of the initial $^{57}Co^{II}$ cations) are very close to the values obtained in Mössbauer transmission measurements for high-spin $^{57}Fe^{II}$ in the composition of iron–sulfur proteins coordinated along with S atoms by O/N donor atoms (see [16] and references reported therein). For one of the four Fe atoms in the cluster $[4Fe–4S]^{2+}$ of the LytB protein isolated from the *E. coli* bacterium superproducing it, parameters δ = 0.89 mm s^{-1} and Δ = 1.97 mm s^{-1} were thus recorded at T = 77 K [16]. This is of particular interest because,

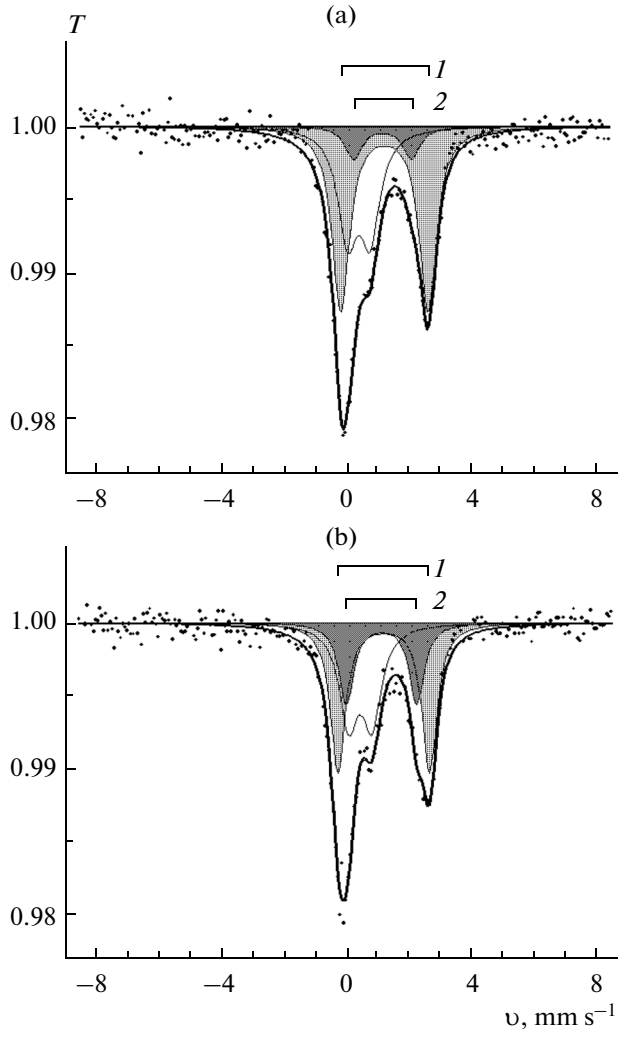

Fig. 1. Emission Mössbauer spectra for samples of the cell biomass of *Azospirillum brasilense* (strain Sp7) grown over 18 h, quick-frozen in liquid nitrogen after the addition of $^{57}Co^{2+}$ and incubation for (a) 2 min and (b) 1 h (measurements were performed at a temperature of 80 K after drying the above suspensions in air (*dried biomass*); see Table 1). The square brackets above the spectra show the positions of the spectral components (quadrupole doublets *1* and *2*) corresponding to the $^{57}Fe^{II}$ components (indicated in the spectra by different shades of hatching). Doublets corresponding to the $^{57}Fe^{III}$ form (as a result of aftereffects) are indicated in the spectra without hatching. The total spectrum (marked by the bold enveloping line) was obtained by approximating the experimental data (points) in the form of the sum of the spectral components (the above quadrupole doublets; see also Table 1); the same for Fig. 2.

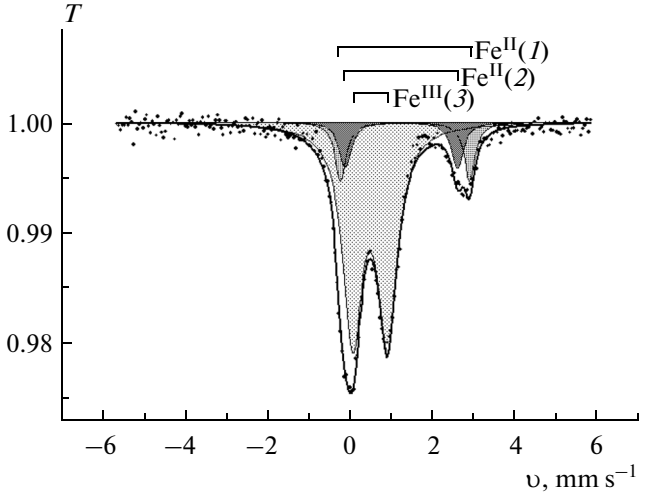

Fig. 2. Mössbauer spectrum of a sample of the cell biomass of *Azospirillum brasilense* (strain Sp7) grown over 18 h (using a nitrilotriacetate complex of $^{57}Fe^{III}$ as the only source of iron), washed of the culture liquid, and quick-frozen in liquid nitrogen (measurements were performed at a temperature of 80 K; see Table 2). The square brackets above the spectrum show the positions of the spectral components, quadrupole doublets *1–3* (indicated by light shading for the $^{57}Fe^{III}$ component and darker shading for the $^{57}Fe^{II}$ components).

as was demonstrated in [17] using *E. coli* as an example, cobalt(II) toxicity to bacteria is due to Co^{2+} cations participating in reactions with labile clusters [Fe–S] during their biosynthesis de novo or repair (also with the participation of special iron-containing proteins, i.e., donors of iron ions in which Fe^{II} is bound much more weakly and with more lability than Fe^{III} [1]). The data obtained in our Mössbauer emission measurements thus indicate the possibility of $^{57}Co^{2+}$ ions participating in similar reactions with the iron-sulfur proteins of azospirilla at the initial stages of interaction. However, additional experiments are required to prove this directly.

Our Mössbauer study of assimilation by cells of *A. brasilense* Sp7 of iron(III) from the complex $^{57}Fe^{III}$–NTA convincingly indicates its partial reduction to iron(II). The lines of the latter are present in our Mössbauer spectrum (Fig. 2) in the form of two quadrupole doublets with different parameters (Table 2; spectral components *1* and *2*) corresponding to high-spin $^{57}Fe^{II}$ in octahedral coordination with a predominantly oxygen (and possibly nitrogen) coordination environment. The relative content of these forms in total (as determined from the S_r values; see Table 2) reaches 22%. We earlier noted a similar reduction of iron(III) from the $^{57}Fe^{III}$–NTA complex for the *A. brasilense* Sp245 strain 18 h after cultivation [10], which also was observed to have two forms of $^{57}Fe^{II}$ (with parameters slightly different from those obtained in this work), their relative content in total reaching 33%. Such behavior is typical of many gram-negative bacteria [18–20] and other microorganisms [11, 21].

The main iron content in the cells of *A. brasilense* Sp7 (78% in the form of $^{57}Fe^{III}$) corresponds to quadrupole doublet *3* (see Fig. 2) with parameters (see Table 2) corresponding to the ferritin-like components observed in other microorganisms [2, 18–23]. The presence of similar ferritin-like components with het-

Table 2. Mössbauer parameters[a], calculated using the transmission spectrum of a sample of the cell biomass of *Azospirillum brasilense* (strain Sp7) grown over 18 h (using a nitrilotriacetate complex of $^{57}Fe^{II}$ as the only source of iron), washed of the culture liquid, and quick-frozen in liquid nitrogen (measurements were performed at a temperature of 80 K; see also Fig. 2).

Spectral components (quadrupole doublets)[b]	δ, mm s^{-1}	Δ, mm s^{-1}	W, mm s^{-1}	S_r, %
(*1*) $^{57}Fe^{II}$	1.32(1)	3.13(3)	0.32(4)	12
(*2*) $^{57}Fe^{II}$	1.23(2)	2.72(4)	0.34(4)	10
(*3*) $^{57}Fe^{III}$	0.47(1)	0.85(1)	0.56(1)	78

[a] The designation of the parameters is the same as in Table 1. Estimated errors (for the last digit) are given in parentheses; the relative error of S_r is ±7%.)

[b] Quadrupole doublets are indicated in Fig. 2 by light shading for form (*3*) of $^{57}Fe^{III}$ and darker shading for forms (*1*, *2*) of $^{57}Fe^{II}$.

erogeneous structure of their cores (which corresponds to the increased width of the lines of quadrupole doublet *3* in Fig. 2; see also Table 2) was demonstrated for *A. brasilense* Sp245 using Mössbauer measurements with a high velocity resolution [11]. Preliminary data from low-temperature Mössbauer measurements (some of which were made in an external magnetic field) of lyophilized biomass grown in the presence of $^{57}Fe^{III}$–NTA as a source of iron prove they are present in the cells of *A. brasilense* Sp7 as well [24].

ACKNOWLEDGMENTS

This work was partially supported by the Russian Foundation for Basic Research, project no. 13-04-01538-a. It was performed as part of the Agreement on Scientific Cooperation between the Russian Academy of Sciences and the Hungarian Academy of Sciences for 2011–2013 (project nos. 28 and 29).

REFERENCES

1. Nobre, L.S., Garcia-Serres, R., Todorovic, S., Hildebrandt, P., Teixeira, M., Latour, J.-M., and Saraiva, L.M., *PLoS ONE,* 2014, vol. 9, no. 4, e95222. doi 10.1371/journal.pone.0095222
2. Matzanke, B.F., *Transition Metals in Microbial Metabolism*, Winkelmann, G. and Carrano, C., Eds., Amsterdam: Harwood Acad. Publ., 1997, p. 117.
3. Kamnev, A.A., *Mössbauer Spectroscopy: Applications in Chemistry, Biology, and Nanotechnology*, Sharma, V.K., Klingelhofer, G., and Nishida, T., Eds., New York: Wiley, 2013, ch. 17, p. 333.
4. Bashan, Y. and de-Bashan, L.E., *Adv. Agron.*, 2010, vol. 108, p. 77.
5. Kamnev, A.A., Tugarova, A.V., Antonyuk, L.P., Tarantilis, P.A., Kulikov, L.A., Perfiliev, Yu.D., Polissiou, M.G., and Gardiner, P.H.E., *Anal. Chim. Acta*, 2006, vols. 573–574, p. 445.
6. Kamnev, A.A., Tugarova, A.V., Tarantilis, P.A., Gardiner, P.H.E., and Polissiou, M.G., *Appl. Soil Ecol.*, 2012, vol. 61, p. 213.
7. Kamnev, A.A., Tugarova, A.V., Kovacs, K., Kuzmann, E., Biro, B., Tarantilis, P.A., and Homonnay, Z., *Anal. Bioanal. Chem.*, 2013, vol. 405, p. 1921.
8. Barton, L.L., Johnson, G.V., and Bishop, Y.M., *Iron Nutrition in Plants and Rhizospheric Microorganisms*, Barton, L.L. and Abadia, J., Eds., Dordrecht: Springer, 2006, ch. 9, p. 199.
9. Bacchawat, A.K. and Ghosh, S., *J. Gen. Microbiol.*, 1987, vol. 133, p. 1759.
10. Kamnev, A.A., Tugarova, A.V., Kovács, K., Biró, B., Homonnay, Z., and Kuzmann, E., *Hyperfine Interact.*, 2014, vol. 226, p. 415.
11. Alenkina, I.V., Oshtrakh, M.I., Tugarova, A.V., Biro, B., Semionkin, V.A., and Kamnev, A.A., *J. Mol. Struct.*, 2014, vol. 1073, p. 181.
12. Kamnev, A.A., Antonyuk, L.P., Smirnova, V.E., Serebrennikova, O.B., Kulikov, L.A., and Perfiliev, Yu.D., *Anal. Bioanal. Chem.*, 2002, vol. 372, p. 431.
13. Kamnev, A.A., Antonyuk, L.P., Smirnova, V.E., Kulikov, L.A., Perfiliev, Yu.D., Kudelina, I.A., Kuzmann, E., and Vertes, A., *Biopolymers*, 2004, vol. 74, p. 64.
14. Tugarova, A.V., Kamnev, A.A., Antonyuk, L.P., and Gardiner, P.H.E., *Metal Ions in Biology and Medicine*, Alpoim, M.C., Morais, P.V., Santos, M.A., Cristovao, A.J., Centeno, J.A., and Collery, Ph., Eds., Paris: John Libbey Eurotext, 2006, vol. 9, p. 242.
15. Klencsár, Z., Kuzmann, E., and Vértes, A., *J. Radioanal. Nucl. Chem.*, 1996, vol. 210, p. 105.

16. Seemann, M., Janthawornpong, K., Schweizer, J., Böttger, L.H., Janoschka, A., Ahrens-Botzong, A., Ngouamegne Tambou, E., Rotthaus, O., Trautwein, A.X., Rohmer, M., and Schünemann, V., *J. Am. Chem. Soc.*, 2009, vol. 131, p. 13184.
17. Ranquet, C., Ollagnier-de-Choudens, S., Loiseau, L., Barras, F., and Fontecave, M., *J. Biol. Chem.*, 2007, vol. 282, p. 30442.
18. Matzanke, B.F., Bill, E., Müller, G.I., Winkelmann, G., and Trautwein, A.X., *Hyperfine Interact.*, 1989, vol. 47, p. 311.
19. Matzanke, B.F., Müller, G.I., Bill, E., and Trautwein, A.X., *Eur. J. Biochem.,* 1989, vol. 183, p. 371.
20. Mielczarek, E.V., Royt, P.W., and Toth-Allen, J., *Biol. Met.*, 1990, vol. 3, p. 34.
21. Andrews, S.C., *Adv. Microb. Physiol.*, 1998, vol. 40, p. 281.
22. Winkler, H., Meyer, W., Trautwein, A.X., and Matzanke, B.F., *Hyperfine Interact.*, 1994, vol. 91, p. 841.
23. Hartnett, A., Bottger, L.H., Matzanke, B.F., and Carrano, C.J., *J. Inorg. Biochem.*, 2012, vol. 116, p. 188.
24. Kovács, K., Pechousek, J., Kamnev, A.A., Tugarova, A.V., Kuzmann, E., Machala, L., Zboril, R., Stichleutner, S., Homonnay, Z., Biró, B., and Lázár, K., *Proc. XIII Int. Conf. "Mössbauer Spectroscopy and Its Applications"* (Suzdal, 2014) *Russia*, Moscow: IMET RAN, 2014, p. 92.

Translated by E. Berezhnaya