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SPECTROSCOPIC CHARACTERIZATION OF THE UPTAKE OF ESSENTIAL AND XENOBIOTIC METAL CATIONS IN CELLS OF THE SOIL BACTERIUM *AZOSPIRILLUM BRASILENSE*

A.A. Kamnev*[1a], M.-F. Renou-Gonnord[2], L.P. Antonyuk[1b], M. Colina[3], A.V. Chernyshev[1b], I. Frolov[2] and V.V. Ignatov[1b]

[1a] *Laboratory of Structural Research Methods* and [1b] *Laboratory of Biochemistry, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, 410015 Saratov, RUSSIA*

[2] *Laboratoire des Mecanismes Reactionnels, Departement de Chimie, Ecole Polytechnique, CNRS URA 1307, F-91128 Palaiseau Cedex, FRANCE*

[3] *Laboratorio de Quimica Ambiental, Facultad Experimental de Ciencias, Universidad del Zulia, 4011 Maracaibo, Zulia, VENEZUELA*

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Summary. – The results of flame (FAAS) or graphite furnace atomic absorption spectrometric (GFAAS) analyses are presented and discussed on the accumulation of essential metals (Mg, Ca, Mn and Fe contained in the cultivation medium) and traces of each one of the conventionally xenobiotic elements from the group V, Co, Ni, Cu, Zn or Pb, added to the medium in concentrations (0.2 mM) which do not essentially suppress growth of the bacterial culture, in cells of the plant root-associated nitrogen-fixing bacterium *Azospirillum brasilense*. Along with the essential cations assimilated by the bacterium, Zn and Cu were found to effectively accumulate in the biomass from the environment. The uptake of Co and Ni was significantly less pronounced, whereas Pb and V appeared to be present in cells in much lower concentrations than in the cultivation medium evidently showing no tendency to be assimilated by azospirilla. The effect of the above xenobiotics on the uptake level of the four essential elements provided evidence that they may compete for the formation of biologically active complexes with substances of both intracellular and extracellular localization. The analytical data obtained are compared with Fourier transform infrared (FTIR) spectra of intact vacuum-dried bacterial cells grown in a standard medium and under the conditions of an increased metal uptake.

Key Words: metal cations, *Azospirillum brasilense*, atomic absorption spectrometry, Fourier transform infrared spectroscopy

INTRODUCTION

In biological systems, the role of metal ions has been acknowledged to be of undoubted vital importance, including those elements which are conventionally considered as toxic (*e.g.*, heavy metals) and are present in the biological matter in trace and ultratrace quantities [1–4]. In particular,

* Author to whom correspondence should be addressed.

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the processes of microbial metabolism involve a number of metal cations which play a significant and specific role in regulating the enzymatic activity [3,5,6]. This fact shows the importance of their correct analytical determination and comparison of their content in bacterial cells grown under different conditions.

Recently [7] the effect of a series of metal ions on the activity of glutamine synthetase, a key bacterial enzyme of nitrogen metabolism, in the soil diazotroph *Azospirillum brasilense* was reported. This bacterium is known [8–10] to colonize roots of higher plants stimulating their growth and development owing to its nitrogen-fixing activity and phytohormone production, as well as a number of other features beneficial for the plant-microbial association.

Previously [11] we reported on the redistribution of inorganic components, including metal cations, between the cultivation medium of *A. brasilense* Sp 245 and an insoluble phosphate-containing biomineral formed therein under certain conditions. It was concluded [11] that "immobilization" of metal ions entrapped in such an insoluble phase produced during cultivation of the bacterium may obviously result in their exclusion from the processes of microbial metabolism and, on the other hand, considering the bacterium-plant association, it might present an alternative way contributing to the known schemes of resistance of plants to heavy metals [12].

In the present communication, we report the results of analyses aimed at investigating the uptake of essential metals (Mg, Ca, Mn and Fe contained in a standard cultivation medium) in the absence (blank) and in the presence of a conventionally xenobiotic element (V, Co, Ni, Cu, Zn or Pb added to the medium in concentrations which do not at all, or at least essentially, suppress growth of the bacterial culture) in cells of *A. brasilense* Sp 245 using flame (FAAS) or graphite furnace atomic absorption spectrometry (GFAAS). Special emphasis is put on the effect of the above metals of the second group on the uptake level of the four essential elements necessary for the growth of azospirilla, observed in the process of their assimilation. The analytical data obtained are compared with Fourier transform infrared (FTIR) spectra of intact vacuum-dried bacterial cells grown in a standard medium and under the conditions of an increased metal uptake.

EXPERIMENTAL

Materials. *Azospirillum brasilense* Sp 245 (the Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia) was cultivated with shaking at 32°C for 18 h in a synthetic medium containing (g l^{-1}, with respect to unhydrous salts): KH_2PO_4 2.0, K_2HPO_4 3.0, NaCl 0.1, $MgSO_4$ 0.2, $CaCl_2$ 0.02, sodium malate 5.0 (prepared by titrating a malic acid solution with NaOH up to pH 6.86), Na_2MoO_4 0.002, $FeSO_4$ 0.02, $MnSO_4$ 0.1, yeast extract 0.1 (initial pH adjusted to 6.86 prior to autoclaving), to which none (blank) or one of the following salts was added up to the final concentration equal to $2 \cdot 10^{-4}$ M: $VOSO_4$ $2H_2O$, $CoCl_2$ $6H_2O$, $NiSO_4$ $7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4$ $7H_2O$, $Pb(NO_3)_2$. All chemicals used were of analytical or reagent grade. The pH values in solutions were monitored with an OP 264/1 digital pH-meter (Hungary). Growth of the culture was controlled by spectroturbidimetric measurements [13] performed after 18 h of cultivation. After that, bacterial cells were separated

from the supernatant by centrifugation (3000 rpm, rotor radius 50 cm, 50 min) at 4°C, washed 3 times with 0.85% NaCl solution and then with doubly distilled water and dried at 50°C for 8 h.

Methods. The above metal cations present in or added to the cultural medium were determined in dried bacterial cells after digestion of precisely weighed samples (0.4 to 3.2 mg of dry biomass each) in a Parr Acid Digestion Bomb No. 4745 (total volume 23 ml, Parr Instruments Company) by flame (FAAS; acetylene-air flame) or, for lowest metal concentrations, graphite furnace atomic absorption spectrometry (GFAAS) using a Perkin-Elmer spectrometer (Model 3110) with a graphite furnace (Perkin-Elmer, model HGA 600) and an autosampler (Perkin-Elmer, Model AS-60).

All experiments on the culture growth under certain conditions (the addition of no (blank) or of a certain xenobiotic), including further separation of cells and their spectral (AAS) analyses, were carried out in three parallel replications in order to assess the biological reproducibility, and the results of the analyses for each cation in parallel sets of experiments were averaged.

FTIR spectra were obtained in the transmission mode (mid-infrared region, 4000–400 cm^{-1}) using a Perkin-Elmer FTIR spectrometer (Model 2000) coupled with a personal computer loaded with an IR Data Manager Program supplied by the manufacturer (Perkin-Elmer). Vacuum-dried (0.05 Torr, 16 h, 40–45°C) bacterial cell samples were carefully pressed into pellets with spectroscopically pure KBr (Merck). Spectra were collected with a total of 60 scans at a resolution of 4 cm^{-1}. Unless otherwise stated, all measurements were conducted at 295±3 K.

RESULTS AND DISCUSSION

The experimental work on accumulation of metal ions in intact cells of *A.brasilense* was undertaken in order to assess, from the viewpoint of their interchangeability, the possibility for essential and xenobiotic microelements, including heavy metals which are commonly regarded as toxic (being, nevertheless, in many cases vitally necessary for the living matter in trace quantities), to be involved in biochemical processes of the bacterium. Besides the essential ecological aspect, this might appear important for a deeper insight into general principles that regulate the functioning of biosystems on the level of a probabilistic approach to the metal ion interchangeability in the latter.

In view of that, we analysed the uptake of several essential metals (Mg, Ca, Mn, Fe) in cells in the absence (blank) and in the presence of a certain xenobiotic from the group V, Co, Ni, Cu, Zn or Pb, traces of which were also determined in the biomass, and compared the results (Table 1). It should be noted that in the presence of the above essential elements (and other constituents of the cultivation medium, as listed in the Experimental section) 0.2 mM of each of the xenobiotics under study did not lead to a noticeable or at least essential suppression of culture growth. However, special experiments showed that in the absence of the four "physiological" metals the latter was always negligible, which is quite natural.

Considering the weight concentrations of the added xenobiotics in the medium (*ca.* 10 to 13 μg per ml, except 41 μg per ml for Pb), as compared with their weight content in cells, it follows from Table 1 that Zn and Cu most effectively accumulate in the biomass from the environment. This is in principle not surprising, since both of them are known to be essential components of a wide diversity of enzymes [3,4].

Table 1. Content of metals, present in or added to the cultivation medium, in dried cells of *Azospirillum brasilense* Sp 245 determined using atomic absorption spectrometric analysis.

Added cation	Content (SD) of metals, mg per gram of dried biomass				
	Mg	Ca	Mn	Fe	Added cation
– (blank)	1.01(0.42)	9.5(3.1)	1.34(0.71)	4.3(2.9)	–
Pb	2.8(1.9)	8.0(5.8)	1.43(0.24)	7.0(2.4)	<0.01[a]
Zn	2.1(1.0)	9.5(8.6)	1.60(0.58)	7.0(3.6)	4.5(2.8)
Cu	5.0(2.5)	19.0(5.6)	3.5(3.1)	14.9(10.6)	2.0(1.3)
Ni	5.3(3.2)	n.t.[b]	2.5(1.9)	0.49(0.16)	0.50[c]
Co	3.4(1.3)	n.t.	1.45(0.98)	0.33(0.14)	0.59(0.30)
V	1.22(0.54)	n.t.	0.60(0.47)	0.50(0.18)	0.010(0.005)[a]

[a] In μg per gram of dry biomass.

[b] Not tested.

[c] Single measurement.

The uptake of Co and Ni is significantly less pronounced, although still noticeable (see Table 1), which correlates with their role reported for different microorganisms. In particular, nickel is required (in relatively small total quantities [4]) for the functioning of several specific bacterial enzymes [14,15]. Cobalt(II) was recently shown [7] to essentially support the activity of glutamine synthetase (GS) of *A.brasilense* in the absence of Mg or Mn, the traditional activating cations for GS (20 other cations showed a significantly lower efficiency), while it gave a 3-fold sinergistic increase in the Mg-supported GS activity (other cations were experimentally divided into groups which do not inhibit, partially inhibit or totally suppress the latter) [7]. A similar specific activating effect of Co^{2+} in concentrations 0.05 to 0.5 mM, in contrast to a series of other bivalent cations, was observed towards Mg-supported GS of other origin [5].

As also follows from the data of Table 1, the concentrations of Pb and V in cells have appeared to be 3 orders of magnitude lower than in the corresponding cultivation media, evidently showing no tendency for these cations to be assimilated by azospirilla. These results are still more noteworthy since they provide an indirect evidence refuting the principally possible alternative explanation for "accumulation" of the cations in intact cells due to adsorption of their hydrolysed or otherwise coordinated forms at the cell surface or due to co-sedimentation of their possible colloidal forms in the centrifugate together with bacterial cells. It is obvious that, if the latter cases had taken place as the main reason for the cation "uptake", both Pb^{2+} and vanadyl (VO^{2+}) species would have also been found at least in quantities comparable with those for other metals studied. Nevertheless,

further experiments aimed at assessing the content of metal cations in desintegrated bacterial cells and cell components (*e.g.* at the bacterial membranes) are in progress.

The uptake of the "physiological" elements (Mg, Ca, Mn and Fe) was essential in all cases (see Table 1) reflecting their vital importance for the bacterial growth. We note that in general in these experiments the biological reproducibility was not high, as is often the case for biological samples. Nevertheless, in some cases statistically significant differences are observed.

Comparing the contents of the alkali-earth cations, it may be noted that, although in bacteria Mg is generally regarded as the most abundant bivalent metal while Ca is contained in considerably smaller quantities than in other living organisms [4], in all our measurements calcium was found to be noticeably predominant.

One of the most interesting results is the effect of the added xenobiotic cations on the uptake of the essential metals (see Table 1). Most pronounced is a drastic decrease (of an order of magnitude) in the uptake of Fe observed in the presence of Co, Ni or V, as compared to the blank experiment (without xenobiotics). This could possibly be related to the fact that these metals may form even stronger complexes with substances responsible for iron transport from the environment into the cell, *e.g.* catecholates (phenolates) and hydroxamates [16–18] comprising the diversity of bacterial siderophores (including that reported for *A. brasilense* [19]). Note also that cobalt was determined [20] to possess some stimulating effect on the growth of another diazotroph, *Rhizobium leguminosarum*, under iron deficiency.

The uptake of manganese was relatively less affected, slightly decreasing in the presence of VO^{2+} and somewhat increasing in the presence of copper (see below). Accumulation of Mg was enhanced 2- to 5-fold in the presence of all xenobiotics, except vanadium.

The addition of Cu to the medium gave rise to a marked sinergistic increase in the uptake of all the above four "physiological" cations (Mg, Ca, Mn, Fe) as compared to the blank experiment. This phenomenon may obviously be connected in general with the response of azospirilla to the antagonism of cations [4] which are significantly accumulated by the bacterium. As follows from Table 1, in the presence of Cu the total metal content is maximal (*ca.* 1 mmol per gram) exceeding that of the blank by *ca.* 3 times. This correlates with an increased infrared absorption in the region of stretching OH vibrations (about 3400 cm^{-1}, Fig. 1) which may be related to H-bound water obviously entrapped together with the excess of metal cations in their hydration shells (the latter may be partly or, for more strongly coordinated ligands, even completely substituted). As far as it can be seen from the FTIR spectra (see Fig. 1), this is the most essential difference noticeable for the two samples. Note that both in the region of different C–H stretching modes (2700–3100 cm^{-1}) and in the fingerprint spectral region (under 2000 cm^{-1}) comprising the characteristic absorption of carbonyl (*ca.* 1740 cm^{-1}), cellular proteins between 1500 and 1700 cm^{-1} (the amide I and amide II bands), carboxylates (*ca.* 1600 and 1400 cm^{-1}), phosphate-carrying components, oligo- and polysaccharides of the cell wall (under 1300 cm^{-1}), the spectra are very similar and, being rather specific, may obviously be

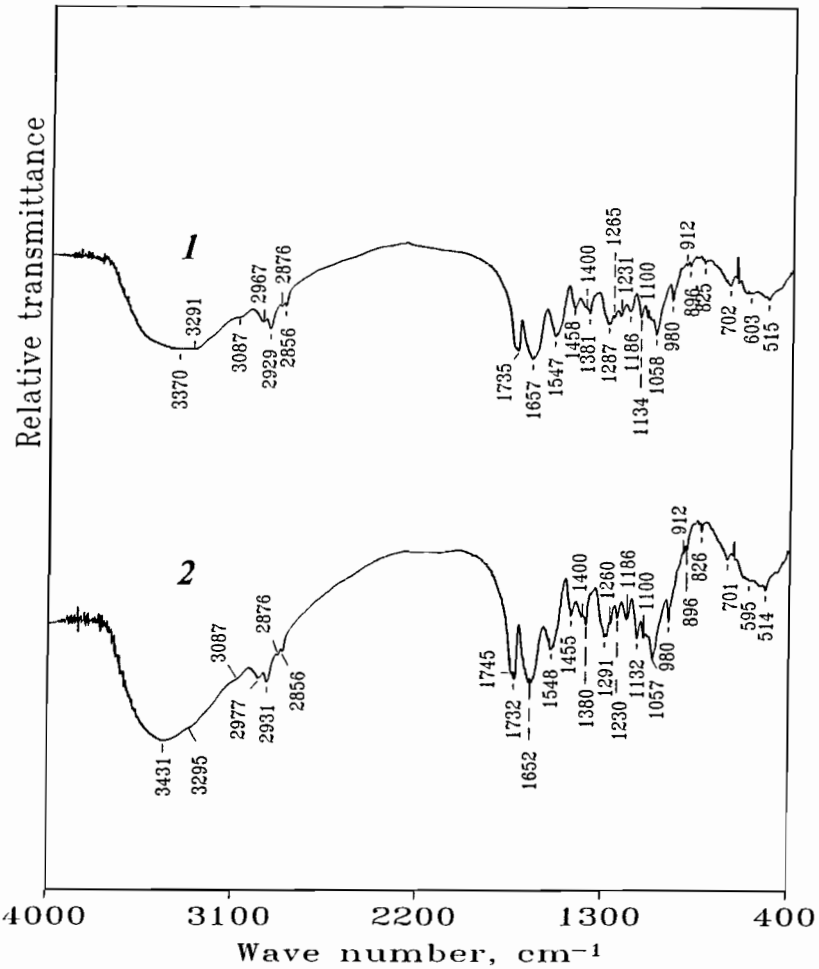

Figure 1. Fourier transform infrared spectra of vacuum-dried intact cells of *Azospirillum brasilense* Sp 245 grown *(1)* in the absence of xenobiotics and *(2)* in the presence of 0.2 mM Cu^{2+} in the cultivation medium (see also Table 1). Samples pressed in pellets with KBr.

used for differentiation and classification of diverse microbial species and strains [21–24], as well as for non-destructive identification and physicochemical characterization of certain cell components [25]. These experiments involving various strains of azospirilla are currently in progress.

It is also noteworthy that the content of certain essential elements (mostly, Fe, Mg and/or Mn) in the biomass was noticeably affected even in the presence of those metals which themselves accumulate up to much lower levels (Co, Ni) or even do not at all (V, Pb; see above). This result suggests that the latter four cations may compete with the essential metals for the formation of biologically active complexes with substances excreted by azospirilla into the environment.

CONCLUSIONS

The data of atomic absorption analyses for four essential metals (Mg, Ca, Mn and Fe) and each of the heavy metals from the group V, Co, Ni, Cu, Zn or Pb in cells of *Azospirillum brasilense* Sp 245, as well as the effect of the latter xenobiotics on the uptake of the four essential cations manifested in the assimilation process, lead to the following conclusions.

Along with the essential cations assimilated by the bacterium in more or less noticeable quantities, Zn and Cu were found to effectively accumulate in the biomass from the environment, the uptake of Co and Ni was significantly lower, whereas Pb and V showed no tendency to be assimilated by azospirilla. In the presence of Co, Ni or V the uptake of Fe was considerably decreased, whereas the addition of Cu to the medium gave rise to a marked sinergistic increase in the content of all the above four "physiological" cations in cells resulting in certain alterations in their Fourier transform infrared spectra. Nevertheless, the fingerprint spectral region remained essentially unchanged and may therefore be used for identification of bacterial cultures grown under different conditions. The uptake of certain essential metals was found to be noticeably affected even in the presence of those cations which themselves accumulate up to much lower levels (Co, Ni) or even do not at all (V, Pb).

The results obtained show that, along with the essential elements vitally necessary for the growth of azospirilla, certain conventionally xenobiotic microelements, including toxic heavy metals, may be effectively involved in both intracellular and extracellular biochemical and physicochemical processes accompanying its metabolism.

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