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Effects of heavy metals on plant-associated rhizobacteria: Comparison of endophytic and non-endophytic strains of *Azospirillum brasilense*

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Abstract

The plant-associated nitrogen-fixing rhizobacterium *Azospirillum brasilense* attracts world-wide attention owing to its plant growth-promoting activities. Among hundreds of its strains known up to date, wild-type strain Sp245 has been proved to be capable of colonising both the plant-root interior and exterior (i.e. a facultative endophyte), whereas others are non-endophytes colonising the root surface only. Thus, the different ecological niches occupied by these strains in the rhizosphere suggest that their responses to environmental conditions might differ as well. In this study, responses of *A. brasilense* strains Sp245 and Sp7 to several heavy metals (Co^{2+}, Cu^{2+}, Zn^{2+}), present in the medium at tolerable concentrations (up to 0.2 mmol/l) and taken up by the bacteria, were compared. Fourier transform infrared (FTIR) spectroscopy was used for controlling the compositional features of whole cells. The results obtained show that in strain Sp7 (non-endophyte) the heavy metals induced an enhanced accumulation of polyester compounds (poly-3-hydroxybutyrate; PHB). In contrast, the response of the endophytic strain Sp245 to heavy metal uptake was found to be much less pronounced. These dissimilarities in their behaviour may be caused by different adaptation abilities of these strains to stress conditions owing to their different ecological status. It was also found that adding 0.2 mmol/l Cu^{2+} or Cd^{2+} in the culture medium resulted in noticeably reducing the levels of indole-3-acetic acid (IAA, auxin) produced by both the strains of the bacterium. This can directly affect the efficiency of associative plant–bacterial symbioses involving *A. brasilense* in heavy-metal-contaminated soil.

Keywords: Heavy metals; *Azospirillum brasilense*; Fourier transform infrared (FTIR) spectroscopy; Indole-3-acetic acid (auxin) production

Introduction

The functioning of associative plant–bacterial symbioses in heavy-metal-polluted soil can be affected from the side of both the micropartner (plant-associated bacteria) and the host plant (see, e.g. [1–4] and references therein). In this case, the adaptation capabilities of both the partners of the associative symbiosis as well as the bioremediation potential of the microsymbiont are of importance to minimise the detrimental effect of heavy-metal pollution.

The plant-associated soil diazotroph *Azospirillum brasilense* has been attracting world-wide attention over the last decades owing to its plant growth-promoting activities (for the latest comprehensive reviews see, e.g.

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[5,6]). Even within this species, there is an endophytic strain (e.g. wild-type strain Sp245 capable of colonising the root interior) and non-endophytes colonising the root surface only [7]. Thus, the different ecological niches occupied by these strains [8,9] suggest that their responses to environmental conditions might differ as well.

Our previous studies [10–13] have shown that *A. brasilense* is tolerable to several conventionally toxic heavy metals (at concentrations under 1 mmol/l), some of which (e.g. Co, Cu, Zn, etc.), however, in trace amounts are involved in diverse enzymatic activities and therefore are necessary for bacteria. The aim of this study was to compare the responses of endophytic and non-endophytic strains to the above heavy metals using Fourier transform infrared (FTIR) spectroscopy as a probe for structural and compositional changes in whole bacterial cells. In addition, the effects of the common heavy-metal pollutants, Cu^{2+} and Cd^{2+}, on the production of indole-3-acetic acid (IAA, a phytohormone of the auxin series) by the strains were comparatively studied. It should be mentioned that IAA production by rhizobacteria is believed to play an important role in plant–bacterial interactions [14]. Therefore, any direct influence on IAA production by bacteria may in turn affect their phytostimulating efficiency.

Materials and methods

A. brasilense (wild-type strains Sp7 and Sp245; the Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia) were cultivated in a malate salt medium (MSM) [10] in all cases. For all spectroscopic experiments, the MSM was supplemented with 3 g/l NH_4Cl (control cultures). To study the effects of heavy metals, the MSM was supplemented with $CoCl_2$, $CuSO_4$ or $ZnSO_4$ added up to 2.0×10^{-4} mol/l, the cells were harvested by centrifugation, washed and dried as described elsewhere [10,13]. For FTIR in the transmission mode, cell samples were mixed with KBr (Merck) or, for diffuse reflectance infrared Fourier transform (DRIFT) measurements, used as dry finely ground powder in a Micro sampling cup (Spectra-Tech Inc., USA). FTIR studies were performed using a Perkin–Elmer (Model 2000) or (for DRIFT) a Nicolet spectrometer (model Magna-IR 560 E.S.P.) with a total of up to 100 scans (resolution 4 cm^{-1}). Other details of spectra acquisition were reported earlier [10–13].

For IAA analysis, the bacteria were grown for 72 h in the MSM with L-tryptophan (200 mg/l) as an IAA precursor and the only source of combined nitrogen, in the presence and in the absence (control) of 2×10^{-4} mol/l $CdCl_2$ or $CuSO_4$. The IAA content in the culture medium was measured by HPLC using a liquid chromatograph equipped with a UV spectrophotometric detector and a 150×4.6 mm i.d., 5-μm-particle Luna 5u C18(2) analytical column (Phenomenex, USA). The mobile phase used was methanol/distilled water/acetic acid (36/64/1 volume ratio). The mobile phase flow rate was 0.7 ml min^{-1}. The loop volume was 20 μl, and IAA was monitored at 290 nm by UV spectrophotometric detection.

The number of viable cells in the cultures (as colony-forming units, CFUs) was determined by the standard plate method.

Results and discussion

The effects of heavy metals on bacterial metabolism

Vibrational spectroscopy is a convenient and sensitive tool for monitoring macroscopic changes in the cellular composition and can be useful for various applications [15–17]. In our previous work [12], comparison of FT-Raman spectra of whole cells of the non-endophytic strain *A. brasilense* Sp7 grown in the MSM (control) and in the presence of 0.2 mmol/l Co^{2+}, Cu^{2+} or Zn^{2+} showed that, besides an enhanced overall hydration and (for Co and Cu only) some decrease in the unsaturation degree of fatty acid residues revealed in the region of stretching C–H vibrations [12], for metal-stressed cells, a new band was observed at ca. 945 cm^{-1} in the region of C–C–O vibrations.

As shown in Fig. 1, in FTIR spectra of metal-stressed cells there appears a well-resolved $\nu(C=O)$ band at ca. 1727 cm^{-1} featuring polyester carbonylic groups, which in control cells is present as a relatively weak shoulder only. These metal-induced changes, together with an increased FTIR absorption in the regions of CH_2 bending vibrations (1440–1460 cm^{-1}; cf. Fig. 1a–d), as well as C–O–C and C–C–O vibrations (1000–1150 cm^{-1}) and CH_2 rocking vibrations (ca. 750 cm^{-1}) [12,13] provide evidence for the accumulation of polyester compounds in cells of strain Sp7 as a response to metal stress. Note that the $\nu(C=O)$ band with a maximum under 1730 cm^{-1} corresponds to poly-3-hydroxybutyrate (PHB), whereas other polyhydroxyalkanoates (PHAs) give bands at 1732–1740 cm^{-1} [17].

It should be mentioned that, in principle, very similar FTIR bands correspond to lipids containing essentially similar chemical functional groups. However, accumulation of such amounts of lipids in bacterial cells is abnormal and may be ruled out. Also, the uptake level of heavy metals in the cells, though noticeable as compared to the metal concentration in the medium reaching several mg/g of dry cells [13], is still insufficient

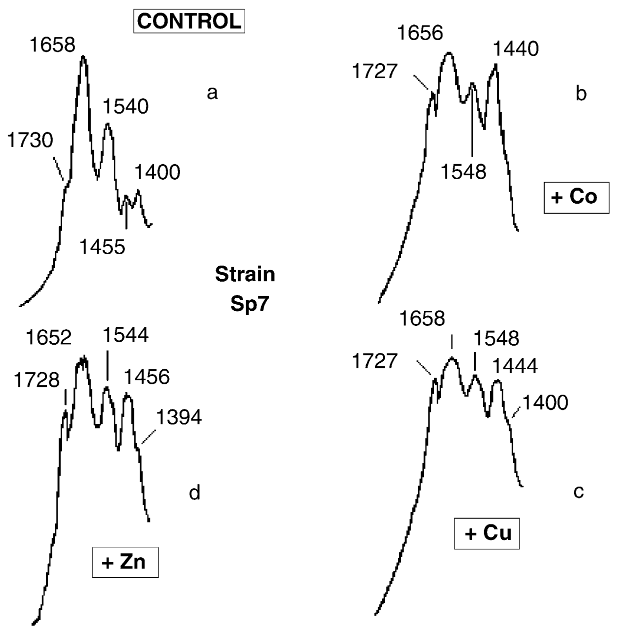

Fig. 1. Fourier transform infrared spectra of whole cells of *A. brasilense* Sp7 (non-endophyte) grown (a) in the MSM (control) and in the medium with 0.2 mmol/l Co^{2+} (b), Cu^{2+} (c) or Zn^{2+} (d).

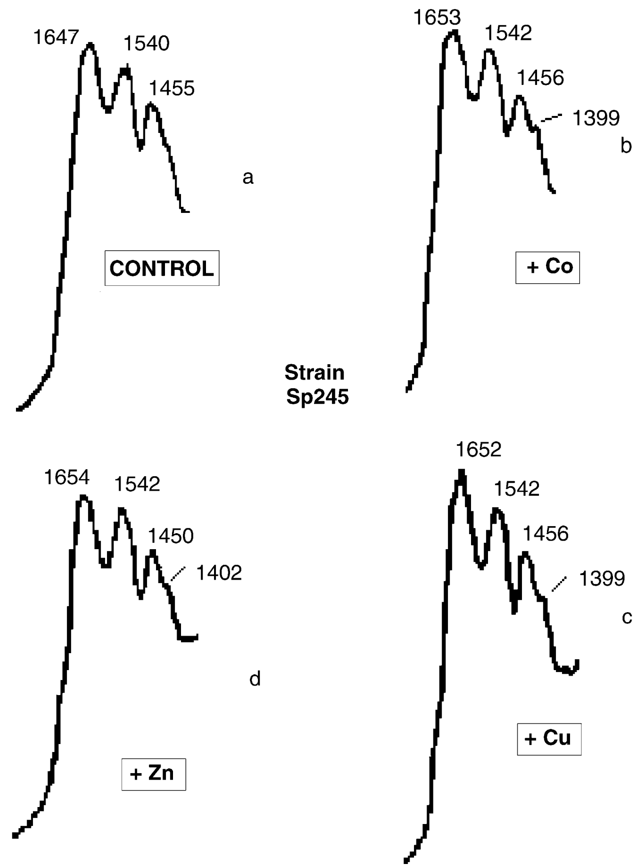

Fig. 2. Fourier transform infrared spectra of whole cells of *A. brasilense* Sp245 (facultative endophyte) grown (a) in the MSM (control) and in the medium with 0.2 mmol/l Co^{2+} (b), Cu^{2+} (c) or Zn^{2+} (d).

to give any noticeable spectroscopic features. Thus the new bands reflect the accumulation of some compounds within the cells as a result of metabolic changes induced by heavy metals.

Considering the asymmetry of the ν(C = O) band at ca. 1727 cm^{-1} (see Fig. 1b–d), PHB seems to dominate, which has been documented for azospirilla under unfavourable conditions (see [18] and references therein), but the presence of other PHAs is also possible.

In order to compare the response to heavy-metal stress of the endophytic strain Sp245, the latter was grown under identical conditions, and the harvested cells were studied using FTIR spectroscopy in the DRIFT mode. It can be seen from the spectra (Fig. 2) that the presence of 0.2 mmol/l Co^{2+}, Cu^{2+} or Zn^{2+} in the NH_4^+-complemented MSM medium did not result in any essential alterations in cell composition. In particular, the ν(C = O) region around 1730 cm^{-1} is quite similar both for the control (Fig. 2a) and metal-stressed cells (Fig. 2b–d) represented by a relatively weak shoulder as compared to the typical amide I and amide II bands of cellular proteins at ca. 1650 and 1540 cm^{-1}, respectively.

Thus, the results obtained demonstrate that the response of the endophytic strain Sp245 to a moderate heavy-metal stress is much less pronounced than that of the non-endophytic strain Sp7. These dissimilarities in their behaviour may be caused by different adaptation abilities of the strains to stress conditions owing to their different ecological status. In particular, an enhanced accumulation of polyester storage compounds is known to play a role in bacterial tolerance to environmental stresses. However, PHAs usually accumulate in cells under nutritional stress (e.g. a high C/N ratio) [18]. PHB biosynthesis and accumulation in bacterial cells induced by heavy-metal stress is a novel feature [13]. In the non-endophytic *A. brasilense* strain Sp7, it may be a specific flexible adaptation strategy related to the localisation of this strain on the rhizoplane, i.e. in direct contact with rhizospheric soil components. In particular, this corresponds to the documented capability of strain Sp7 to outcompete other co-inoculated strains [7].

The effects of heavy metals on indole-3-acetic acid production by *Azospirillum*

It has been well documented that the biosynthesis of auxins with their excretion into soil makes a major contribution to bacterial plant-growth-promoting effect [1,4,5]. Some plant-associated bacteria were shown to improve host plant growth and development in heavy-metal-contaminated soil by mitigating toxic effects of

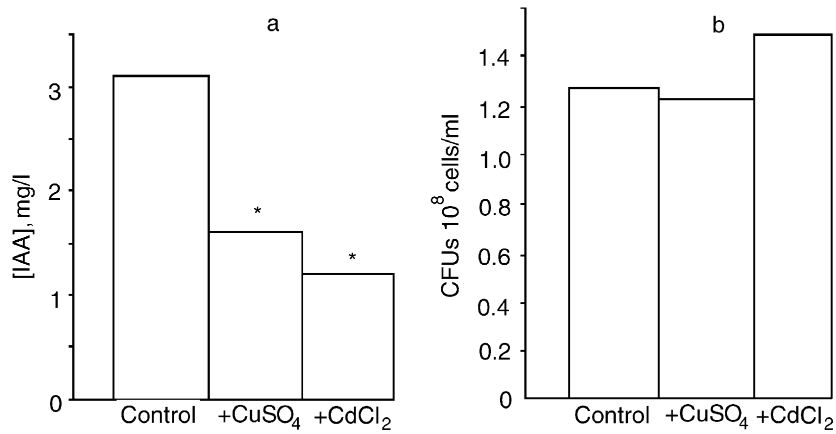

Fig. 3. Indole-3-acetic acid production levels (a) and bacterial growth rate (b) for *A. brasilense* Sp7 (non-endophyte) grown in the MSM (control) and in the medium with 0.2 mmol/l Cu^{2+} or Cd^{2+}. Asterisks show statistically significant differences from the control.

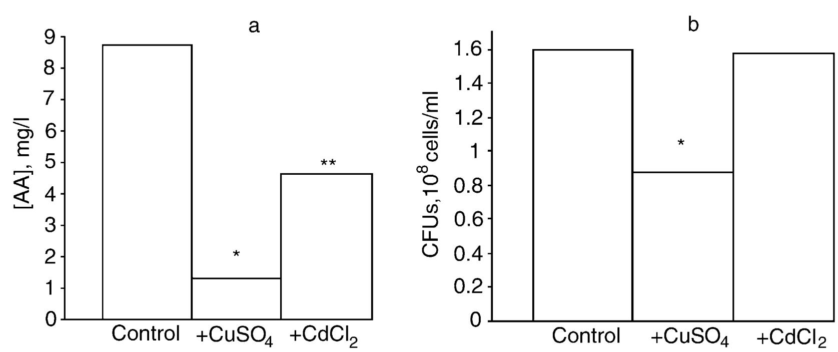

Fig. 4. Indole-3-acetic acid production levels (a) and bacterial growth rate (b) for *A. brasilense* Sp245 (facultative endophyte) grown in the MSM (control) and in the medium with 0.2 mmol/l Cu^{2+} or Cd^{2+}. Asterisks show statistically significant differences from the control.

heavy metals on the plants [19]. In this work, we compared the levels of IAA production by control cells and in the presence of $CuSO_4$ or $CdCl_2$ (0.2 mmol/l) for each of the two strains studied. In parallel, the bacterial growth rate (CFUs) was also assessed.

For strain Sp7, both Cu^{2+} and Cd^{2+} ions were found to significantly decrease the level of IAA production (Fig. 3a), whereas the bacterial growth rate was virtually not affected (Fig. 3b). For strain Sp245, the overall level of IAA production in the control was approximately 3 times higher than for Sp7 (Fig. 4a; cf. Fig. 3a). Nevertheless, strain Sp245 in this respect appeared to be more sensitive to heavy metals: a noticeable decrease in IAA production was observed under the effect of both metals, especially with Cu^{2+} (see Fig. 4a). Note, however, that for strain Sp245 copper was somewhat more toxic decreasing also the bacterial counts (Fig. 4b).

Thus, the observed decrease in IAA production by the bacteria under the influence of copper and cadmium may directly affect the efficiency of associative plant–bacterial symbioses in heavy-metal-polluted soils. Whereas for the non-endophytic strain Sp7, this detrimental influence of the soil pollutant is inevitable, it has still to be confirmed experimentally whether the possibility for the endophytic localisation of strain Sp245 would be advantageous to resist heavy-metal toxicity.

Note that the strains under study have shown some heavy-metal tolerance, as in three cases out of four (cf. Figs. 3b and 4b) the bacterial growth rate was not inhibited. Nevertheless, azospirilla are relatively less studied, so that the mechanism of heavy-metal resistance reported for better-studied microorganisms [20] has still to be clarified.

Conclusions

It has been found that non-endophytic and facultatively endophytic strains of *A. brasilense* (Sp7 and Sp245, respectively) show different responses to moderate heavy-metal stress. In particular, the presence of 0.2 mmol/l Co^{2+}, Cu^{2+} or Zn^{2+} in the growth medium induced the accumulation of PHB in strain Sp7 reflected in FTIR spectra of whole cells, whereas for strain Sp245 the response was much less pronounced.

Cu^{2+} and Cd^{2+} were found to significantly suppress the production of IAA (auxin) by both strains, which can directly affect the plant-growth-stimulating efficiency of azospirilla.

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