ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 6, pp. 809–811. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © A.A. Kamnev, A.V. Tugarova, L.P. Antonyuk, 2007, published in Mikrobiologiya, 2007, Vol. 76, No. 6, pp. 908–911.

SHORT COMMUNICATIONS

Endophytic and Epiphytic Strains of *Azospirillum brasilense* Respond Differently to Heavy Metal Stress

A. A. Kamnev[1], A. V. Tugarova, and L. P. Antonyuk

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia

Received May 07, 2007

DOI: 10.1134/S0026261707060239

The fundamental aspects of the production of intracellular polyhydroxyalkanoates (PHA) by bacteria attract much attention. These commercially important biological materials have specific properties, including thermoplasticity and capacity for complete biodegradation [1, 2]. Biosynthesis of PHA, including poly-3-hydroxybutyrate (PHB), by a number of soil bacteria is known to be a specific cellular response to certain types of stress [3]. For example, PHB biosynthesis and accumulation by *Azospirillum brasilense* has been reported at high C : N ratios (nitrogen deficiency); PHB accumulation is believed to be one of the factors promoting rhizobacterial survival under unfavorable conditions [3, 4].

We have no knowledge of any publications dealing with the induction of PHA biosynthesis under stress caused by the presence of heavy metals (HM) alone (see, for example, reviews [1, 3]). In *A. brasilense* cells in the presence of some HM ions we have observed, however, among the other metabolic changes detectable by vibrational spectroscopy, an indication of the accumulation of polyester compounds [5–7]. Infrared (IR) spectroscopy is most sensitive to polar molecules, including polyesters, which have specific intense absorption bands. Thus, whole cell preparations can be analyzed; isolation and purification of their components is not required [7–10].

The goal of the present work is a comparative investigation of the effect of a number of heavy metals on the metabolism of *A. brasilense* epiphytic and endophytic strains.

IR spectroscopy was used to analyze the cells of strain Sp7 (capable of root surface colonization only) and strain Sp245 (a facultative endophyte) grown either on a malate–salt medium (control) or under moderate stress caused by 0.2 mM of Co^{2+}, Cu^{2+}, or Zn^{2+} in the medium.

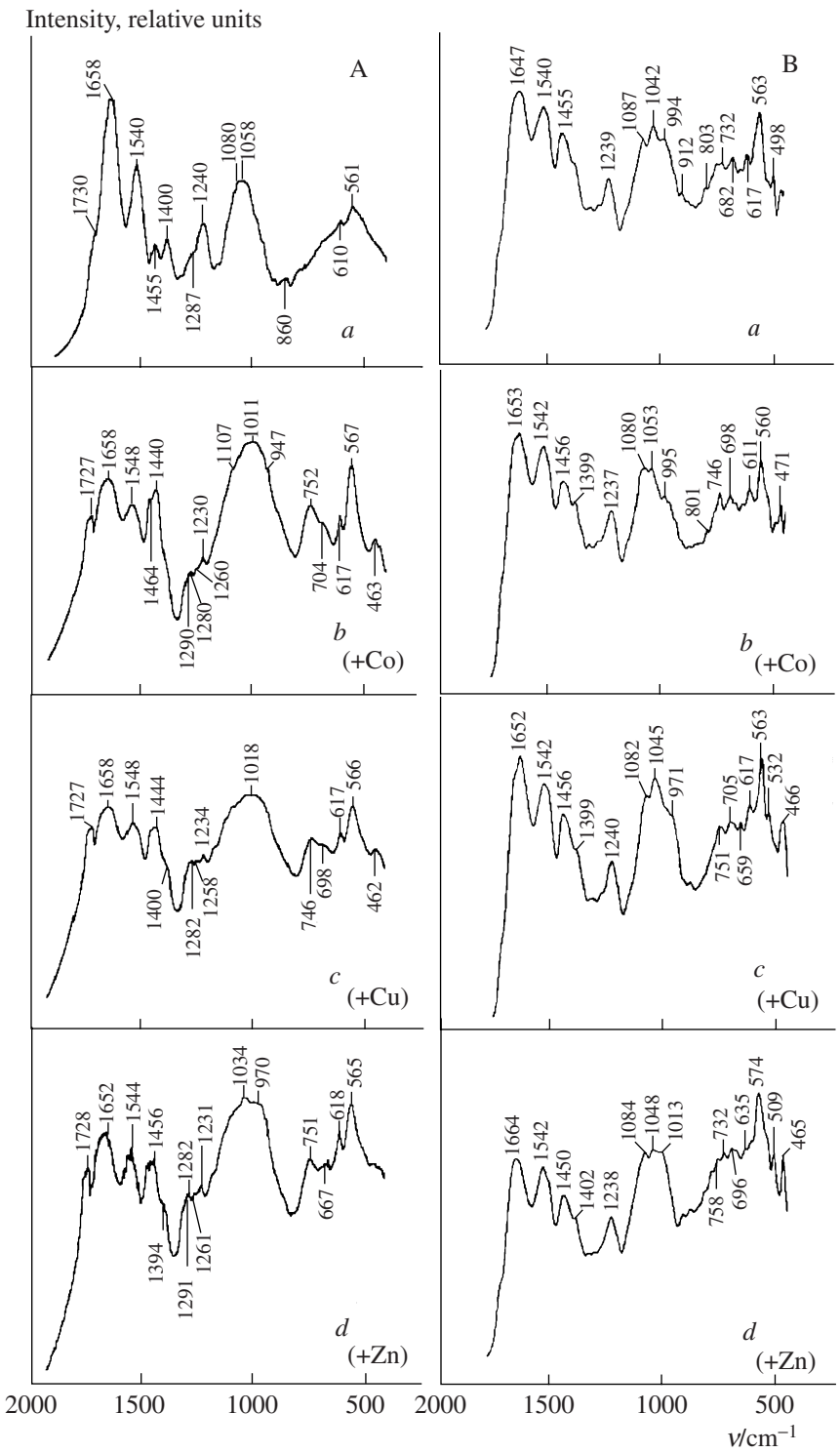
A. brasilense strains Sp7 and Sp245 were obtained from the collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences. The cultures were grown in the liquid synthetic malate medium of the following composition (g/l): K_2HPO_4, 3.0; KH_2PO_4, 2.0; NaCl, 0.1; malic acid, 3.76; NaOH, 2.24; NH_4Cl, 0.5; yeast extract, 0.1; $MgSO_4 \times 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeSO_4 \times 7H_2O$, 0.02; $MnSO_4 \times 5H_2O$, 0.1; $Na_2MoO_4 \times 2H_2O$, 0.002 (pH 6.8–7.0). The inoculum and the cells for analysis were grown in 250-ml conical flasks with 100 ml of the medium under shaking (180 rpm) at 32°C. In all the experiments the initial cell density was 5×10^7 cells/ml. The cultures were grown on this medium (control) and on the same medium supplemented with heave metal salts (experiment). The salts of heavy metals ($CoCl_2 \times 6H_2O$, $CuSO_4 \times 5H_2O$, or $ZnSO_4 \times 7H_2O$) as sterile solutions were added up to the final metal concentration of 0.2 mM.

For IR spectroscopic analysis, the cells were collected by centrifugation (15 min, 7000 *g*), washed three times with 15-ml portions of phosphate buffer saline (pH 7.0–7.2), and dried at room temperature to constant weight. IR Fourier spectrometers Perkin–Elmer model 2000 (United Kingdom) or Nicolet model Magna-IR 560 E.S.P (United States) were used to obtain the spectra. The spectra were obtained in a KBr matrix (Merck) in transmittance and diffuse reflectance modes in the range of 4000 to 400 cm^{-1} with accumulation of up to 100 spectral scans at 4 cm^{-1} resolution at 23 ± 2°C. The spectral data were treated using the standard enclosed software packages.

The IR spectra of *A. brasilense* Sp7 cells grown on the control medium differed significantly from those of the cells grown in the presence of HM (Fig. A, spectrum *a*; cf. with spectra *b–d*). In the spectra of cells grown in control media, the typical amide I and amide II bands can be seen (ca. 1660 and 1540 cm^{-1}, respectively) of the proteinaceous compounds, as well as the vibrational band of the phosphate group (1240 cm^{-1}), the vibrational range of C–O–C, C–C–O, and C–O–P bands of polysaccharides and other macromolecules (ca. 1100–950 cm^{-1}), and the region of superimposed vibrations of various functional groups (below 900 cm^{-1}) [9].

The IR spectra of *A. brasilense* Sp7 cells grown in the presence of heavy metals (Fig. A, spectra *b–d*) contained a pronounced characteristic band at 1727 cm^{-1} of

[1] Corresponding author; e-mail: aakamnev@ibppm.sgu.ru



IR spectra of whole cells of *Azospirillum brasilense* strains Sp7 (A) and Sp245 (B) grown in the standard NH_4^+-containing medium (*a*) and in the presence of 0.2 mM Co^{2+} (*b*), Cu^{2+}(*c*), or Zn^{2+} (*d*).

the ν(C=O) vibrations of polyesters, which was present in control samples (spectrum *a*) as a weakly pronounced shoulder. In the region of ca. 1440–1450 cm^{-1} (deformational vibrations of CH_2 and CH_3 groups) and 1100–950 cm^{-1} (stretching vibrations of C–O–C and C–C–O), an absorption increase can also be seen compared to the amide I and amide II bands. These changes indicate PHB accumulation in the cells of strain Sp7

grown in the presence of HM. In particular, according to [10], the PHB band has a maximum at no higher than 1730 cm^{-1}, while other PHA have bands at ca. 1732–1744 cm^{-1}. The position of the ν(C=O) band with a maximum at ca. 1727 cm^{-1} and its evident asymmetry (Fig. A, spectra *b–d*) suggest that PHB predominates in this case, while other PHA may also be present.

A low-frequency shift of the phosphate group band from 1240 cm^{-1} (Fig. A, spectrum *a*) by 6–10 cm^{-1} (Fig. A, spectra *b–d*) should also be mentioned; it indicates an increased hydration of these groups which accompanies the previously reported overall increase in hydration of bacterial cells accumulating HM from the medium [5, 6].

The cells of *A. brasilense* Sp245 grown in the presence of HM under similar conditions did not differ significantly from the control (Fig. B, spectra *a* and *b–d*). In particular, apart from a weak shoulder present in all the samples, including the control ones, no characteristic ν(C=O) band was observed at ca. 1730 cm^{-1}. It should be noted that no substantial differences in HM accumulation were revealed between *A. brasilense* strains Sp7 and Sp245 [7]. Due to the relatively low intracellular HM concentration, the changes in IR spectra of Sp7 whole cells cannot be caused by the metal compounds as such; they instead reflect a reorganization of bacterial metabolism induced by the heavy metals.

Thus, PHB accumulation by *A. brasilense* Sp7 was demonstrated for the first time for bacteria in the presence of the heavy metals mentioned above in the strain grown on complete NH_4^+-containing malate medium. Under the same conditions, *A. brasilense* strain Sp245 did not respond with increased PHB synthesis. The heavy metal-induced PHB accumulation by the nonendophytic *A. brasilense* Sp7 indicates its more flexible adaptive capabilities. These are probably the result of its localization on the root surface, in contact with the soil components of the rhizosphere, unlike the endophytic strain *A. brasilense* Sp245, which can penetrate into plant roots [11].

The authors are grateful to P.A. Tarantilis and M.G. Polissiou (Athens, Greece) for collaboration.

The work was partially supported by the grants from NATO (LST.CLG.977664, LST.NR.CLG.981092, ESP.NR.NRCLG 982857) and from the Russian Academy of Sciences Youth Committee (grant no. 205, VI Expert contest).

REFERENCES

1. Lenz, R.W. and Marchessault, R.H., Bacterial Polyesters: Biosynthesis, Biodegradable Plastics and Biotechnology, *Biomacromol.*, 2005, vol. 6, no. 1, pp. 1–8.
2. Volova, T.G., Gladyshev, M.I., Trusova, M.Yu., and Zhila, N.O., Degradation of Polyhydroxyalkanoates and the Composition of Microbial Destructors under Natural Conditions, *Mikrobiologiya*, 2006, vol. 75, no. 5, pp. 682–688 [*Microbiology* (Engl. Transl.), vol. 75, no. 5, pp. 593–598].
3. Kadouri, D., Jurkevitch, E., Okon, Y., and Castro-Sowinski, S. Ecological and agricultural significance of bacterial polyhydroxyalkanoates, *Crit. Rev. Microbiol.*, 2005, vol. 31, no. 2, pp. 55–67.
4. Kadouri, D., Jurkevitch, E., and Okon, Y., Involvement of the Reserve Material Poly-β-hydroxybutyrate in *Azospirillum brasilense* Stress Endurance and Root Colonization, *Appl. Environ. Microbiol.*, 2003, vol. 69, no. 6, pp. 3244–3250.
5. Kamnev, A.A., Tarantilis, P.A., Antonyuk, L.P., Bespalova, L.A., Polissiou, M.G., Colina, M., Gardiner, P.H.E., and Ignatov, V.V., Fourier Transform Raman Spectroscopic Characterisation of Cells of the Plant-Associated Soil Bacterium *Azospirillum brasilense* Sp7, *J. Mol. Struct.*, 2001, vol. 563-564, pp. 199–207.
6. Kamnev, A.A., Antonyuk, L.P., Tugarova, A.V., Tarantilis, P.A., Polissiou, M.G., and Gardiner, P.H.E., Fourier Transform Infrared Spectroscopic Characterisation of Heavy Metal-Induced Metabolic Changes in the Plant-Associated Soil Bacterium *Azospirillum brasilense* Sp7, *J. Mol. Struct.*, 2002, vol. 610, nos. 1-3, pp. 127–131.
7. 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., Instrumental Analysis of Bacterial Cells Using Vibrational and Emission Mössbauer Spectroscopic Techniques, *Anal. Chim. Acta*, 2006, vol. 573–574, pp. 445–452.
8. Schmitt, J. and Flemming, H.-C., FTIR-Spectroscopy in Microbial and Material Analysis, *Int. Biodeterior. Biodegrad.*, 1998, vol. 41, no. 1, pp. 1–11.
9. Naumann, D, Infrared Spectroscopy in Microbiology, in *Encyclopedia of Analytical Chemistry*, Meyers, R.A., Ed., Chichester: Wiley, 2000, pp. 102–131.
10. Hong, K., Sun, S., Tian, W., Chen, G.Q., and Huang, W., A Rapid Method for Detecting Bacterial Polyhydroxyalkanoates in Intact Cells by Fourier Transform Infrared Spectroscopy, *Appl. Microbiol. Biotechnol.*, 1999, vol. 51, no. 4, pp. 523–526.
11. Rothballer, M., Schmid, M., and Hartmann, A., In situ Localization and PGPR-Effect of *Azospirillum brasilense* Strains Colonizing Roots of Different Wheat Varieties, *Symbiosis*, 2003, vol. 34, pp. 261–279.