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Comparing poly-3-hydroxybutyrate accumulation in *Azospirillum brasilense* strains Sp7 and Sp245: The effects of copper(II)

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ABSTRACT

Intracellular accumulation of poly-3-hydroxybutyrate (PHB) was comparatively studied in the plant-growth-promoting bacterium (PGPB) *Azospirillum brasilense* (epiphytic strain Sp7 and endophytic strain Sp245) grown microaerobically for 2 days under nitrogen deficiency in the standard malate salt medium in the absence (control) or presence of copper (0.1 mM Cu^{2+}). For quantitative determination of PHB content of cells, diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy of whole-cell biomass samples was used. After 2 days in control cells, PHB accumulation in strain Sp7 reached ca. 24% of dry cell mass (d.c.m.). In strain Sp245, the PHB content in the control was over 1.3-fold higher (32% d.c.m.) than in strain Sp7. In the presence of copper(II), PHB accumulation was notably enhanced in strain Sp7 (over 1.6-fold, up to ca. 39% d.c.m.), whereas in strain Sp245 it changed insignificantly (from 32% up to ca. 35% d.c.m.). The levels of copper(II) uptake were comparable in both strains. These findings are in line with our earlier observations that even in rich NH_4^+-supplemented medium, some heavy metals, including copper(II), induce PHB biosynthesis in *A. brasilense* strain Sp7, but not in Sp245. The dissimilarities in the levels of PHB accumulation and in the effects induced by copper(II) in the two strains are attributed to their different adaptive potentials owing to different ecological niches they occupy in the rhizosphere.

1. Introduction

Among soil bacteria, many are known to accumulate intracellular granules of polyhydroxyalkanoates (PHA, carbon and energy storage materials) under various stress conditions (Castro-Sowinski et al., 2010; Mayet et al., 2010; Rehm, 2007). These biopolymer materials are represented by poly-3-hydroxybutyrate (PHB), its copolymers with 3-hydroxyvalerate, as well as some other short- and medium-chain-length PHA (Chen, 2010; Khanna and Srivastava, 2005). Such biopolymers, besides their applicability as biodegradable plastics (Chen, 2010), have been documented to play an important role in bacterial sustainability, tolerance to stresses and survival under unfavourable conditions (Castro-Sowinski et al., 2010; Kadouri et al., 2005; Ratcliff et al., 2008). In the plant-growth-promoting bacterium (PGPB) *Azospirillum brasilense*, stress-induced biosynthesis of PHA storage materials has been proved to be represented by homopolymer PHB only, the relative quantities of which can reach over 80% of dry cell biomass (Itzigsohn et al., 1995; Kadouri et al., 2003, 2005).

The bacteria of the genus *Azospirillum* have long been under investigation worldwide owing to a number of their beneficial traits applicable in agriculture and environmental biotechnology to promote plant growth (for recent reviews see, e.g. Bashan and de-Bashan, 2010; Veresoglou and Menexes, 2010; Biró et al., 2006; Bashan et al., 2004); the species *A. brasilense* has been most intensively studied. This species is a good model for studying environmental effects and ecological behaviour, particularly as it comprises, among many others, two strains, Sp7 (epiphyte, colonizing plant root surface only) and Sp245 (a facultative endophyte capable of penetrating into the root tissue). The two strains have often been compared and documented to show various behavioural differences under similar conditions (Bashan et al., 2004; Kamnev et al., 2006; Mulyukin et al., 2009; Pogorelova et al., 2009; Khalsa-Moyers, 2010).

In our earlier study (Tugarova et al., 2006) it was found that 0.1 mM Cu^{2+} is a minimum growth-inhibiting concentration (MIC) for both *A. brasilense* strains Sp7 and Sp245 (while 0.5 mM was

Abbreviations: DRIFT, diffuse reflectance infrared Fourier transform (spectroscopy); d.c.m., dry cell mass; FTIR, Fourier transform infrared (spectroscopy); ICP-MS, inductively coupled plasma mass spectrometry; IR, infrared; MIC, minimum growth-inhibiting concentration; MLC, minimum lethal concentration; PGPB, plant-growth-promoting bacterium; PHA, polyhydroxyalkanoate(s); PHB, poly-3-hydroxybutyrate.

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Table 1
Content of poly-3-hydroxybutyrate (PHB) and copper (Cu) in dry cell samples of *Azospirillum brasilense* strains Sp7 and Sp245 grown microaerobically in the synthetic NH_4^+-free malate salt medium for 2 days in the absence and in the presence of 1.0×10^{-4} M Cu^{2+} (see also Fig. 1).

Strain of *A. brasilense*	Cu^{2+} added to the growth medium	PHB content,[a] % d.c.m. (S.E.)	Cu content,[b] mg g^{-1} d.c.m. (S.E.)	Spectrum in Fig. 1
Sp7	–	24.2 (1.0)	0.038 (0.006)	*a1*
Sp7	0.1 mM	39.1 (2.8)	0.24 (0.03)	*a2*
Sp245	–	31.9 (0.6)	0.021 (0.006)	*b1*
Sp245	0.1 mM	35.2 (1.8)	0.26 (0.05)	*b2*

[a] Determined from the DRIFT spectra (see text and Fig. 1); S.E., standard error.
[b] The presence of copper in cells in the absence of Cu^{2+} added to the growth medium is due to its background impurities in the culture medium components.

found to be a minimum lethal concentration, MLC, for them). For comparison, some strains of other associative rizosphere bacteria, *Azospirillum lipoferum*, *Arthrobacter mysorens*, *Agrobacterium radiobacter* and *Flavobacterium* sp., were reported (Belimov et al., 2004) to show the MIC and MLC values for Cu^{2+} to be in the range of 0.05–0.2 mM and 0.15–0.7 mM, respectively. Thus, 0.1 mM Cu^{2+} for the two *A. brasilense* strains under study (as well as evidently for many other associative rhizosphere bacteria) may be regarded as a moderately toxic heavy-metal concentration.

In this communication, we compare the levels of PHB accumulation and the effects of submillimolar copper(II) (featuring a moderate heavy-metal stress) on the latter in *A. brasilense* strains Sp7 and Sp245 grown microaerobically under bound-nitrogen deficiency (nitrogen-fixing conditions). The PHB content was analysed using Fourier transform infrared (FTIR) spectroscopy, which has been documented to be a sensitive and convenient tool for analysing bacterial biomass samples (Kamnev et al., 1999, 2006, 2008; Kansiz et al., 2000; Naumann, 2000; Mayet et al., 2010; Pistorius et al., 2009). The hypothesis of this study is that the two strains with different ecological statuses, which have been documented to show different behaviour under similar conditions (see above), might show different responses to nitrogen deficiency and to a mild heavy-metal stress (presence of Cu^{II}) expressed via PHB accumulation.

2. Materials and methods

A. brasilense strains Sp7 (Tarrand et al., 1978; ATCC 29145, Gherna et al., 1992) and Sp245 (Baldani et al., 1983), taken from the Collection of the IBPPM RAS, Saratov, were grown microaerobically (without stirring) at 32 °C in the synthetic NH_4^+-free malate salt medium (Day and Döbereiner, 1976), slightly modified as reported earlier (Kamnev et al., 2008), for 2 days either in the absence (control) or in the presence of 1.0×10^{-4} M $CuSO_4$ in the medium.

For FTIR spectroscopic analyses, cells were separated by centrifugation ($7000 \times g$, 15 min), washed three times with phosphate buffered saline (pH 7.1) and dried at ambient temperature (25 ± 3 °C) up to a constant mass. The resulting dry biomass was powdered; DRIFT spectra (with a ± 4 cm^{-1} resolution) were recorded on a Nicolet 6700 FTIR spectrometer (Thermo Electron Corporation, USA) by placing ca. 0.5 mg of the dry powdered biomass in a Micro sampling cup (Spectra-Tech Inc., USA) as reported earlier (Kamnev et al., 2008), without using potassium bromide (KBr) in sample preparation, and mounting the sampling cup onto the DRIFT accessory sample holder of the spectrometer. All the data, obtained in triplicate, were well reproducible. The percentage of PHB in dry biomass samples was determined from the spectra by calculating the intensity ratio of the ν(C=O) band of PHB at around 1730 cm^{-1} to that of the amide II band at ca. 1550 cm^{-1}, as described by Naumann (2000). For calibration, similar ratios calculated using the IR spectroscopic data (Kansiz et al., 2000) for PHB-accumulating *Escherichia coli* and the data of PHB determinations by gas chromatography also reported by Kansiz et al. (2000) were used.

Copper(II) was determined in dry bacterial samples as described earlier (Kamnev et al., 2006) using a Hewlett–Packard ICP-MS spectrometer (model 4500). Unless indicated otherwise, all measurements were performed at ambient temperature (25 ± 3 °C).

3. Results and discussion

Our previous studies have shown that azospirilla are relatively tolerant to submillimolar concentrations of heavy metals which, in that case, did not substantially suppress growth of the bacterial culture (see, e.g. Kamnev et al., 1999, 2006; Tugarova et al., 2006, and references therein; see also Bashan et al., 2004). Moderate concentrations of heavy metals can often be found in soil as a result of contamination or intensive use of heavy-metal containing bactericides (Biró et al., 2006; Khan et al., 2009; Rutgers, 2008). Assessing their impact on the bacterial metabolism is of importance for a deeper insight into their ecology. In the case of azospirilla, considering their plant-growth-promoting abilities (Bashan and de-Bashan, 2010; Veresoglou and Menexes, 2010), this also presents an obvious agricultural and biotechnological interest.

PHB, accumulating in bacterial cells under some unfavourable conditions, is known to be featured by its main IR absorption bands at about 1730 cm^{-1} (stretching C=O vibrations of the ester moieties), 1450 and 1380 cm^{-1} (various C–H bending vibrations of methyl and methylene groups), $1285 \div 1300$ cm^{-1} (C–C–O/C–O–C polyester fragment vibrations), as well as some other vibration modes (Kamnev et al., 2006, 2008; Kansiz et al., 2000; Naumann, 2000; Mayet et al., 2010; Wróbel-Kwiatkowska et al., 2009). The most prominent PHB band corresponds to the polyester carbonyl stretching mode, ν(C=O), at about 1730 cm^{-1}. Thus, the ratio of its intensity to those of the typical protein bands, either "amide I" at about 1660 cm^{-1} (Kansiz et al., 2000) or "amide II" at about 1550 cm^{-1} (Naumann, 2000), can be used for PHB quantification in bacterial cells. It has to be noted that a similar stretching ν(C=O) mode of the ester moieties (at about 1730 cm^{-1}) is typical also for other cellular polyesters (lipids) largely represented by lipopolysaccharides and phospholipids. Nevertheless, their content is limited by a few percent of d.c.m. and relatively constant, and the proportion of the carbonyl ester moieties in them is low.

The representative DRIFT spectra (an averaged spectrum for each sample's three parallels) in the most informative "fingerprint" region, under 1800 cm^{-1} (Naumann, 2000), are shown in Fig. 1. Comparing spectra *a1* and *a2* for strain Sp7 (grown in the absence and presence of copper(II), respectively), a clearly noticeable enhancement in the corresponding PHB regions, as shown above the spectra, can be seen in spectrum *a2*, as compared to the typical amide I and amide II bands of cellular proteins (at ca. 1660 and 1550 cm^{-1}, respectively), reflecting an enhanced PHB accumulation in the latter case.

To assess the percentage of PHB in the dry biomass samples from the DRIFT spectra, the methodology was applied of calculating the intensity ratio of the ν(C=O) band of PHB at around 1730 cm^{-1} to that of the amide II band at ca. 1550 cm^{-1}, as described by Naumann (2000). Together with that, similar ratios calculated using the IR spectroscopic data (Kansiz et al., 2000) for PHB-accumulating *E. coli*

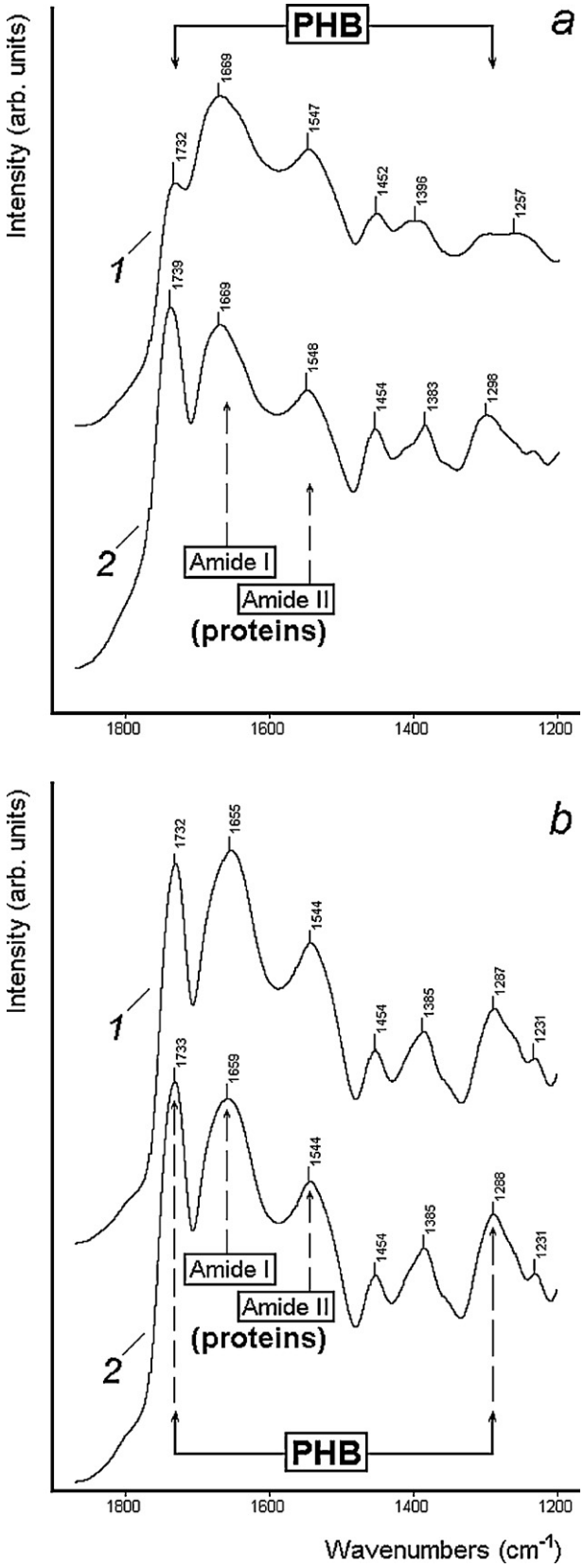

Fig. 1. Diffuse reflectance infrared Fourier transform (DRIFT) spectra of dry cells of *Azospirillum brasilense* strains Sp7 (*a*) and Sp245 (*b*) microaerobically grown at 32 °C in the synthetic NH_4^+-free malate salt medium for 2 days (*1*) in the absence and (*2*) in the presence of 1.0×10^{-4} M Cu^{2+}. The positions of the two main poly-3-hydroxybutyrate (PHB) bands (polyester carbonyl stretching mode, ν(C=O), at about 1730 cm^{-1}, and C–C–O/C–O–C polyester fragment vibrations at ca. 1290 cm^{-1}) are indicated, together with the positions of amide I and amide II bands of cellular proteins at ca. 1660 and 1550 cm^{-1}, respectively (see text).

and the data of PHB determinations by gas chromatography, also reported by Kansiz et al. (2000), were used in the present work for calibration. The results of PHB determination, together with the data on copper(II) contents in dry biomass samples, are presented in Table 1.

According to our calculations, *A. brasilense* strain Sp7 microaerobically grown in the NH_4^+-free medium (corresponding to a high C:N ratio, i.e. under nutritional stress) accumulated ca. 24% d.c.m. PHB after 2 days (see Table 1, Fig. 1, spectrum *a1*). Similar amounts of PHB were reported for *A. brasilense* strains Sp7 and Cd under N_2-fixing conditions (see, e.g. Tal and Okon, 1985 and references therein). However, significantly (over 1.6-fold) more PHB was found in cells grown the presence of 0.1 mM Cu^{2+} (i.e. under additional moderate heavy-metal stress; see Table 1 and spectrum *a2*).

In strain Sp245, the PHB content in the control was over 1.3-fold higher than in strain Sp7, albeit it changed insignificantly in the presence of copper(II) (see Table 1 and Fig. 1b). Note that the levels of copper uptake were comparable in both strains (see Table 1), similarly to the data obtained under different growth conditions (Kamnev et al., 2006).

These findings are in line with our earlier observations that even in rich NH_4^+-supplemented medium (at low C/N ratios unfavourable for PHB accumulation), some heavy metals, including copper(II), still induce PHB biosynthesis and accumulation in *A. brasilense* strain Sp7, but not in Sp245 (Kamnev et al., 2006). From the results obtained in this work, it follows that in nitrogen-fixing conditions (under a nutritional stress related to bound-nitrogen deficiency), an additional mild heavy-metal stress (0.1 mM Cu^{2+}) markedly enhances the rate of PHB accumulation in the epiphytic strain Sp7 within the early stationary growth phase, while this factor has an insignificant effect on the PHB accumulation rate in the endophyte Sp245. This could be interpreted as a reflection of their different ecological statuses. Strain Sp7 (epiphyte) is located in the rhizosphere and, therefore, is always in contact with the rhizospheric soil and, in particular, with its contaminants (e.g. heavy metals). Thus, strain Sp7 may be generally more tolerant to nitrogen deficiency (accumulating less PHB) than strain Sp245, but better "feels" the additional mild stress (0.1 mM Cu^{2+}) and, under the effect of Cu^{2+}, increases the rate of PHB accumulation significantly more than strain Sp245 does. In contrast, strain Sp245, being a facultative endophyte, may be defended by plant root tissue from the 'outer' soil contaminants and therefore does not appreciably enhance PHB accumulation under the effect of Cu^{2+} (i.e. does not use this strategy as a defense mechanism). Note that the role of PHB accumulation as a general strategy of microbial defense from various stresses has been mentioned above (Castro-Sowinski et al., 2010; Kadouri et al., 2005; Ratcliff et al., 2008).

The observed dissimilarities in the levels of PHB accumulation, as well as in the effects on PHB accumulation rate induced by copper(II), in the two strains can thus be attributed to their different adaptive potentials owing to different ecological niches they occupy in the rhizosphere.

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