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Fourier transform infrared spectroscopic study of intact cells of the nitrogen-fixing bacterium *Azospirillum brasilense*

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Abstract

The data of Fourier transform infrared (FTIR) spectroscopic measurements performed on intact cells of the soil nitrogen-fixing bacterium *Azospirillum brasilense* grown in a standard medium and under the conditions of an increased metal uptake are compared and discussed. The structural FTIR information obtained is considered together with atomic absorption spectrometry (AAS) data on the content of metal cations in the bacterial cells. Some methodological aspects concerning preparation of bacterial cell samples for FTIR measurements are also discussed. © 1997 Elsevier Science B.V.

Keywords: Fourier transform infrared spectroscopy; *Azospirillum brasilense*; Bacterial cultures; Atomic absorption spectrometry; Intact cells; Metal uptake

1. Introduction

Over the last decade, it has been shown that Fourier transform infrared (FTIR) spectroscopy applied to the study of biological objects and, in particular, microbial cells is a highly convenient and powerful tool which may be used for differentiation and classification of diverse microbial species and strains, as well as for non-destructive identification and physicochemical characterization of certain cell components and related compounds (see, for example, [1–4] and references cited therein).

In this communication, the results of FTIR spectroscopic investigations are discussed concerning intact cells of the soil diazotroph *Azospirillum brasilense* which is known 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 bacterium–plant association [5–7]. The structural FTIR information obtained is compared with atomic absorption spectrometry (AAS) data on the content of metal cations in the bacterial cells grown in a standard medium and under the conditions of an increased metal uptake [8]. The latter finding is paid special attention since metal cations have been acknowledged to play a vitally important role in the processes of microbial metabolism [9,10], being, for example, specifically responsible for regulating microbial enzymatic activity [10,11]. Some methodological aspects concerning preparation of bacterial cell samples for FTIR measurements are also discussed.

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2. Materials and methods

2.1. Preparation of bacterial cultures

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 at 32°C for 18 h in a standard synthetic medium containing (in g l^{-1}, with respect to anhydrous salts): NaCl 0.1, KH_2PO_4 2.0, K_2HPO_4 3.0, $MgSO_4$ 0.2, $CaCl_2$ 0.02, sodium malate 5.0 (prepared by titrating a malic acid solution with sodium hydroxide 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). A separate sample was prepared by growing the bacterium under the same conditions and in the same medium to which 2×10^{-4} M $CuSO_4 \cdot 5H_2O$ had been added (see below). 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 cultures was controlled by spectroturbidimetric measurements [12] performed after 18 h of cultivation. Bacterial cells were separated from the supernatant by centrifugation (3000 rev min^{-1}, rotor radius 50 cm, 50 min) at 4°C, washed three times with 0.85% NaCl solution and then with doubly distilled water and dried in air at 50°C for 8 h, or under vacuum (0.05 torr, 16 h, 40–45°C) prior to measurements.

2.2. Sample preparation and FTIR spectra acquisition

Air-dried or vacuum-dried bacterial cell samples (see above) 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} 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.

2.3. AAS analyses

Some of the essential (Mg, Ca, Mn, Fe) and added (Cu) metals present in the cultural medium were determined in air-dried (50°C, 8 h) precisely weighed (0.4 to 3.2 mg of dry biomass each) bacterial cell samples after their digestion with spectroscopically pure HNO_3 and deionized water in an acid digestion bomb (Parr Instruments Company; 1 h, 110°C) by flame atomic absorption spectrometry (FAAS; acetylene–air flame) using a Perkin-Elmer spectrometer (Model 3110). Unless otherwise stated, all measurements were conducted at 295 ± 3 K.

3. Results and discussion

In the literature dealing with FTIR spectroscopy of bacterial cell samples (see, for example, [1–4]), considerable attention is paid to the sample preparation procedures. For instance, the data presented in [4] showing a good reproducibility of FTIR room-temperature spectra of bacterial samples provide evidence that the latter dried over P_2O_5 or under high vacuum (10^{-5} torr) exhibit essentially the same qualitative spectral features with the only noticeable differences in the regions of stretching and bending vibrations of adsorbed water, the absorption intensity being somewhat decreased after vacuum drying. In view of that, we measured FTIR spectra of intact cell samples dried both in air (at 50°C) and under vacuum (0.05 torr) for the bacteria grown in a standard medium (Fig. 1, spectra 1a and 1b, respectively). Recently, it was found [8] that the addition of 0.2 mM Cu^{2+} to the medium led to a synergistic increase in the uptake of four essential cations (Mg, Ca, Mn, Fe) by the bacterium as compared to the blank experiments (Table 1), so we also obtained FTIR spectra of both air-dried and vacuum-dried samples of azospirilla grown under the same conditions with the above Cu^{2+} addition to the medium leading to an increased metal content in its cells (Fig. 1, spectra 2a and 2b, respectively).

The FTIR spectra of intact cells are obviously complex additive images of their overall chemical composition which comprise the relative contributions from different functional groups and chemical substructures, also reflecting all possible interactions (H-bonding, complexing, etc.) as well as possible changes occurring during sample preparation [1–3]. Comparison of the spectra in Fig. 1 shows that in both cases (1 and 2) vacuum-drying resulted in a slight but noticeable decrease in the contributions from adsorbed and/or bound water, namely, the ν(O–H) and δ(H–O–H) absorption at about 3440–3460 and

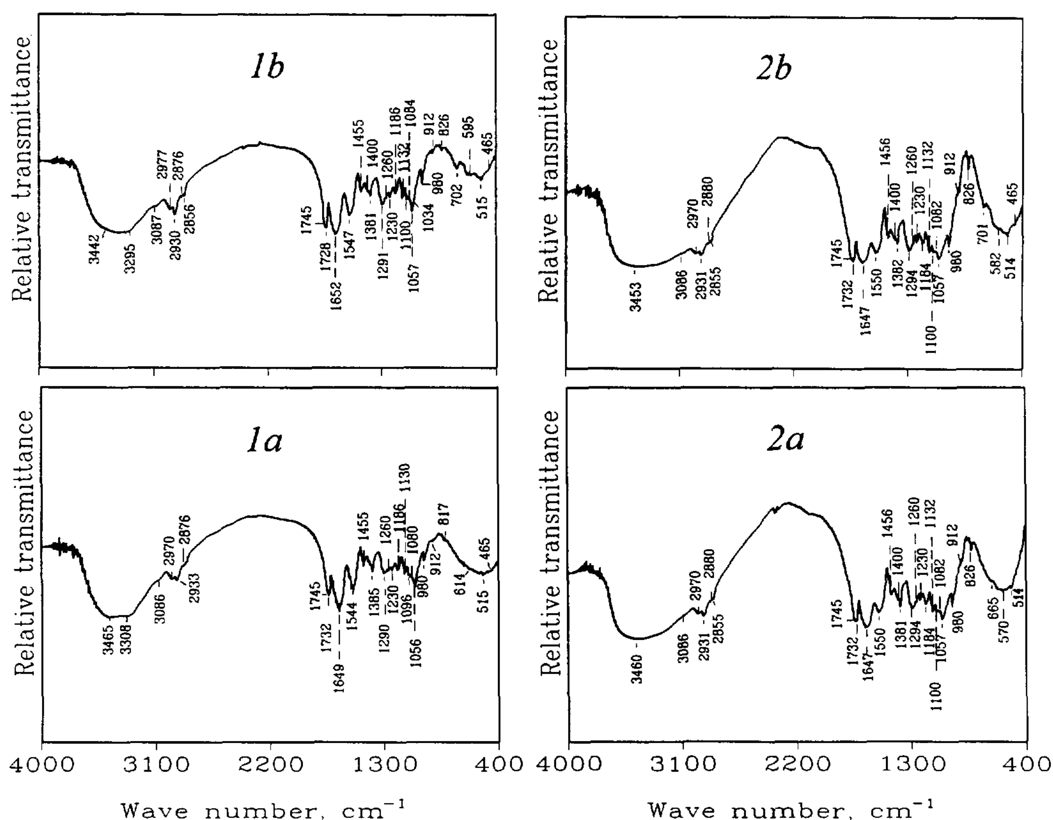

Fig. 1. Fourier transform infrared spectra of (a) air-dried and (b) vacuum-dried intact bacterial cells of *Azospirillum brasilense* Sp 245 grown (1) in a standard medium and (2) in the presence of 0.2 mM Cu^{2+} added to the cultivation medium (see also Table 1). Samples pressed in pellets with spectroscopically pure KBr.

1640 cm^{-1}, respectively, as well as δ(O–H) at about 600 cm^{-1}, which reveals distinct bands at 702 and 515 cm^{-1} and a shoulder at 465 cm^{-1} (see spectra 1b and 2b). As other spectral regions for both couples (spectra "a" and "b") remained essentially similar, it may be concluded that for rapid FTIR spectral analysis air-drying at moderately elevated temperatures (50°C) may well be used, while for a more detailed study the bacterial cell samples should be more extensively dried (e.g. under vacuum).

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

Cultivation medium[a]	Content of metals/mg per g of dried biomass					Total[b]	
	Mg	Ca	Mn	Fe	Cu	mmol g^{-1}	mg g^{-1}
Without Cu^{2+}	1.0	9.5	1.3	4.3	–	0.38	16
With 0.2 mM Cu^{2+}	5.0	19.0	3.5	14.9	2.0	1.04	44

[a] See Section 2.1.
[b] Excluding alkali metals.

We also emphasize the presence of a strong band at 1730–1745 cm^{-1} featuring the $\nu(C{=}O)$ vibrations of carbonyl in (poly)ester and/or non-ionized carboxylic groups which, owing to their obvious inequality, may partially overlap giving rise to a noticeable splitting and/or broadening of the band. It is noteworthy that this band is either absent or much less intensive in FTIR spectra of many other bacteria [1–4] and obviously reflects certain differences in the content of, for example, polyester compounds in cells of azospirilla as compared to other microorganisms [3].

Comparing spectra 1b and 2b for vacuum-dried samples of azospirilla grown in a standard medium and with the Cu^{2+} addition, respectively, it may be noted that for spectrum 2b absorption in the region of broad O–H stretching vibrations at about 3450 cm^{-1} is more pronounced. Considering the approximately threefold increase in the metal content (see Table 1) for this sample, the latter effect may be related to H-bound water obviously entrapped together with the excess of metal cations in their hydration shells [13]. As a result of this (and also of possible coordination of metals with, for example, α-aminoacids [9,13,14]), the broad N–H stretching band at about 3300 cm^{-1} is also somewhat masked. Nevertheless, other amide bands (at 3086 cm^{-1} [13], amide I and II at about 1650 and 1550 cm^{-1} featuring cellular proteins), as well as the regions of different C–H stretching modes (2800–3000 cm^{-1}) and phosphate-carrying components, oligo- and polysaccharides of the cell wall (under 1300 cm^{-1}) are very similar. Being rather specific, these regions may obviously be used for further studies aimed at differentiation and classification of microbial species and strains [1,2,4], as well as for non-destructive identification and physicochemical characterization of certain cell components [3] (our FTIR experiments on various strains of azospirilla grown under different conditions are currently in progress).

Finally, it should also be noted that in spectrum 2b the region of lower wavenumbers (under 700 cm^{-1}) is featured by a relatively higher absorption (see spectrum 2a). This may be attributed to an increased contribution from both the intense $\nu(M{-}O)$ and $\delta(O{-}M{-}O)$ bands (M = metal cation) usually observed below 600 cm^{-1} (the corresponding M–N vibration bands reflecting possible coordination of metals with N-containing bioligands are usually essentially less intense) [14] and the $\delta(O{-}H)$ vibration mode of bound water entrapped with additionally uptaken cations (see above and Table 1) at about 600 cm^{-1}.

4. Conclusions

Analysis of the FTIR data obtained based on comparing the spectra in characteristic regions, and considering the data of atomic absorption analyses for the content of metal cations in cells of *A. brasilense* Sp 245, has shown that the increased metal uptake by the bacterium observed under special conditions leads to some notable alterations in the FTIR spectra of the bacterial cells, which basically involve the absorption regions of bound water entrapped with additionally uptaken metal cations, as well as metal–oxygen vibrations. Vacuum-drying has been shown to somewhat decrease the contribution of stretching and bending vibrations of adsorbed water, improving the resolution of other bands observed in the relevant regions. The fingerprint spectral regions nevertheless remained quite similar for the above bacterial cell samples, making it principally possible to apply FTIR spectroscopy for non-destructive identification and physicochemical characterization of certain cell components of azospirilla, as well as for differentiation and classification of its strains.

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