

Spectroscopic characterization of cell membranes and their constituents of the plant-associated soil bacterium *Azospirillum brasilense*

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Abstract

Structural and compositional features of bacterial membranes and some of their isolated constituents (cell surface lipopolysaccharide, phospholipids) of the plant-growth-promoting diazotrophic rhizobacterium *Azospirillum brasilense* (wild-type strain Sp245) were characterized using Fourier transform infrared (FTIR) spectroscopy and some other techniques. FTIR spectra of the cell membranes were shown to comprise the main vibration modes of the relevant lipopolysaccharide and protein components which are believed to be involved in associative plant–bacterium interactions, as well as of phospholipid constituents. The role and functions of metal cations in the structural organization and physicochemical properties of bacterial cell membranes are also discussed considering their accumulation in the membranes from the culture medium. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bacterial cell membranes; *Azospirillum brasilense*; Metal cations; Fourier transform infrared spectroscopy; Atomic absorption spectrometry

1. Introduction

The application of Fourier transform (FT) vibrational spectroscopic techniques to extremely complex

and diverse biological objects, which was progressively expanding over the last decade, greatly facilitates non-destructive physicochemical characterization of the systems under study, in particular, on the level of their certain constituents (see, e.g. [1–4] and references cited therein). Concerning bacterial cultures, the Fourier transform infrared (FTIR) and FT Raman spectroscopic techniques have shown their power for discriminating, on the one hand, between different cell components and, on

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the other hand, between different bacterial species and strains [2–8]. Our recent studies using FTIR and analytical atomic spectroscopy (AAS) [9,10], as applied to the plant-growth-promoting soil bacterium *Azospirillum brasilense* (wild-type strain Sp245), have revealed some essential methodological aspects of sample preparation and treatment of intact bacterial cells, as well as the effects of metals uptake from the cultivation medium on their structural characteristics manifested in the spectra. In this paper, we also comparatively analyse the structural and compositional features of *A. brasilense* cell membranes and some of their isolated constituents using FTIR and AAS data, as well as some other techniques. The role and functions of metal cations in the structural organization and physicochemical properties of bacterial cell membranes are also discussed considering their accumulation in the membranes from the medium.

2. Materials and methods

2.1. Preparation of bacterial cultures

Azospirillum brasilense Sp245 (taken from 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 phosphate- and malate-containing synthetic salt medium (pH 6.86) as described elsewhere [9–11]. A separate sample was prepared by growing the bacterium under the same conditions and in the same medium to which 0.2 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ had been added (described later). All chemicals used were of analytical or reagent grade.

2.2. Preparation of bacterial membranes

Bacterial membranes were obtained by treating cells, grown as described earlier and separated from the supernatant by centrifugation (3000 rpm, rotor radius 50 cm, 50 min) at 4°C and then resuspended in a tris-HCl buffer solution (pH 7.0) cooled with ice, using a UD-20 ultrasonic disintegrator (mode 5; 10 cycles of 2 min each with 2 min pauses). The resulting cell debris were washed, separated by

centrifugation as described earlier and dried in air at ca. 40°C (4 h).

2.3. Isolation and analysis of phospholipids

Lipid constituents were extracted from wet cell precipitate, after its three-fold washing with bidistilled water, with organic solvents [12,13]. The total fraction of phospholipids was about 7% w/w of dry cell biomass. Chromatographic separation, identification and quantitative determination of individual phospholipids were performed as described in detail elsewhere [13–15]; colorimetric measurements were made using a Specol-221 photocolormeter at the wavelength of 610 nm.

2.4. Isolation of lipopolysaccharide

Lipopolysaccharide (LPS) from the bacterial cell surface of the late-log-phase culture was extracted with EDTA [16] as described elsewhere [17]. Monosaccharide composition of capsular polysaccharides of *A. brasilense* strains (including strain Sp245 studied here) and some other of their physicochemical properties were reported in detail earlier [18,19].

2.5. Sample preparation and FTIR spectra acquisition

Bacterial membrane or LPS samples (described earlier) vacuum-dried prior to FTIR measurements (0.05–0.1 Torr, 40°C–45°C, minimum 1 h) [9,11] 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 to a personal computer loaded with an IR Data Manager Program supplied by the manufacturer.

2.6. AAS analyses

Some of the essential (Mg, Ca, Mn, Fe) and added (Cu) metals present in the culture medium were determined in air-dried (50°C; 8 h) precisely weighed (0.4–1.5 mg of dry biomass each) cell membrane samples (see Section 2.2) after their digestion with spectroscopically pure HNO_3 and deionized water in an acid digestion bomb (Parr Instruments Company;

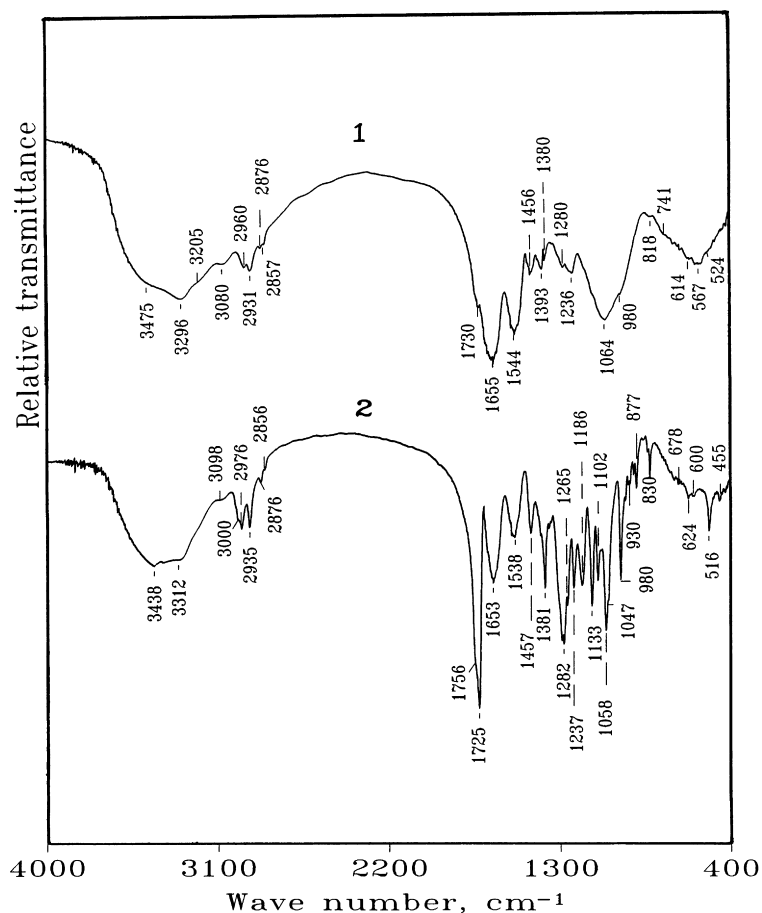


Fig. 1. Fourier transform infrared spectra of vacuum-dried cell membrane samples obtained from *Azospirillum brasilense* Sp245 cells grown (1) in a standard medium and (2) in the presence of 0.0002 M Cu^{2+} added to the cultivation medium (see Section 2.1, Section 2.2 and Section 2.5). Samples pressed in pellets with spectroscopically pure KBr (the same for Fig. 2).

1 h, 110°C) by flame atomic absorption spectrometry (acetylene–air flame) with a Perkin-Elmer spectrometer (Model 3110). Three parallel analyses of samples, prepared from independently grown bacterial cultures in relevant media, were averaged for each metal. Unless otherwise stated, all measurements were conducted at 295 ± 3 K.

3. Results and discussion

FTIR absorption spectra of supramolecular cellular structures (e.g., intact cells or cell components) are obviously highly complex additive images of their overall chemical composition reflecting also all

possible interactions [2–11]. We have recently found [10] that the addition of 0.2 mM Cu^{2+} to the culture medium of *A. brasilense* Sp245 results in its noticeable assimilation (up to 2 mg Cu per gram of dry cell biomass) accompanied by a synergistic increase in the uptake of essential metals (Mg, Ca, Mn, Fe). Comparison of FTIR spectra of intact cells grown in a standard medium and under the conditions of an increased metal uptake has shown [9] that the latter effect leads to some alterations in FTIR spectra that basically involve the absorption regions of bound water entrapped with additionally uptaken metal cations, as well as signs of metal-oxygen vibrations. In order to follow possible effects which the metal-stress conditions

Table 1

Phospholipid composition of *Azospirillum brasilense* Sp245 cell membranes at the exponential (12 h) and stationary (18 h) growth phases

Phospholipids	Phospholipid content, relative percentage	
	Exponential phase	Stationary phase
Phosphatidylethanolamine	64.4	60.7
Phosphatidylglycerol	19.5	18.2
Cardiolipin	9.2	11.9
Phosphatidic acid	5.1	7.8
Phosphatidylinositol	1.8	1.4
Total (percentage of dry cell biomass)	6.8	7.2

described earlier might have exerted on the azospirillum cell membrane, it might be helpful to compare also the spectra of the latter.

In Fig. 1, FTIR spectra are presented for vacuum-dried cell membranes that were obtained from cells of azospirillum grown in the standard medium (spectrum 1) and under the conditions of an increased metal uptake by the bacterium induced by the presence of Cu^{2+} [9,10] (spectrum 2). It is noteworthy that the latter effect leads to essential differences in the FTIR spectra of membranes. Most striking is the presence of a strong C=O stretching band at 1725–1756 cm^{-1} (see spectrum 2) characteristic of polyester carbonyl (and/or possibly of non-ionized carboxy) its asymmetric shape may be attributed to inequality of different carbonyls, which are obviously not essentially involved in hydration and/or H-bonding owing to its relatively high frequency [20]. This band is also present in spectrum 1 as a weak but definitely noticeable shoulder at ca. 1730 cm^{-1} . As

was noted earlier [9], this $\nu(\text{C}=\text{O})$ band which reproduces well in FTIR spectra of intact azospirillum cells, being comparable by intensity to those assigned to the usual amide I (ca. 1650 cm^{-1}) and amide II (ca. 1540 cm^{-1}) modes of cellular proteins, is either virtually absent or much less intensive in IR spectra of many other microorganisms [3–7,21,22]. This might reflect certain differences in the content of polyester compounds in azospirillum cells as compared to other microbial cells [5,9].

Considering the membrane spectra in Fig. 1, along with the polyester $\nu(\text{C}=\text{O})$ band which may also be attributed in part to the lipid constituents [23] of azospirillum membrane phospholipids (Table 1), there are also some more intensive bands in spectrum 2, as compared to spectrum 1, in particular, within the phosphate region, viz., $\nu(\text{P}=\text{O})$ at ca. 1280 cm^{-1} (which may be split and/or broadened owing to its partial involvement in H-bonding [24]), $\nu_{\text{as}}(\text{PO}_2)$ at 1237 cm^{-1} and $\nu_{\text{s}}(\text{PO}_2)$ at 1058 cm^{-1} , $\nu(\text{P}-\text{O}-)$ at 980 cm^{-1} (which appears under 1000 cm^{-1} for phosphate attached to longer methylene chains [24]). The $\nu_{\text{as}}(\text{C}-\text{O}-\text{C})$ and $\nu_{\text{s}}(\text{C}-\text{O}-\text{C})$ bands at 1133 and 1047 cm^{-1} , respectively, are more noticeable as well in spectrum 2. Also, the C–H vibration regions in the latter case are more pronounced, including the $\nu_{\text{as}}(\text{CH}_2)$ and $\nu_{\text{s}}(\text{CH}_2)$ bands at ca. 2935 and 2856 cm^{-1} , respectively; $\nu_{\text{as}}(\text{CH}_3)$ and $\nu_{\text{s}}(\text{CH}_3)$ bands at ca. 2976 and 2876 cm^{-1} , respectively (note that in spectrum 2 the shoulder at 3000 cm^{-1} , which is commonly characteristic for the $\nu(\text{CH})$ mode in $\text{C}=(\text{CH})-\text{R}$, may reflect an increased content of lipid residues containing unsaturated hydrocarbon chains), as well as the bending CH (1381 cm^{-1}) and CH_2 scissoring mode (1457 cm^{-1}).

Table 2

Content of metals, present in or added to the cultivation medium of *Azospirillum brasilense* Sp245, in cell membranes^a determined using atomic absorption spectrometry

Cultivation medium ^b	Content of metals/mg per g of dried membrane biomass				
	Mg	Ca	Mn	Fe	Cu
Without Cu^{2+}	0.13 ± 0.01	6.9 ± 2.5	8.7 ± 2.2	33.8 ± 6.8	–
With 0.2 mM Cu^{2+}	0.82 ± 0.39	5.4 ± 3.2	5.6 ± 0.7	25.1 ± 5.9	1.02 ± 0.48

^a Average (± S.D.) of three parallel analyses of samples, each of which was prepared from independently grown bacterial cultures in appropriate media (see Section 2.1, Section 2.2 and Section 2.6) without Cu^{2+} and with 0.2 mM Cu^{2+} added (see also Fig. 1, spectra 1 and 2, respectively).

^b See Section 2.1.

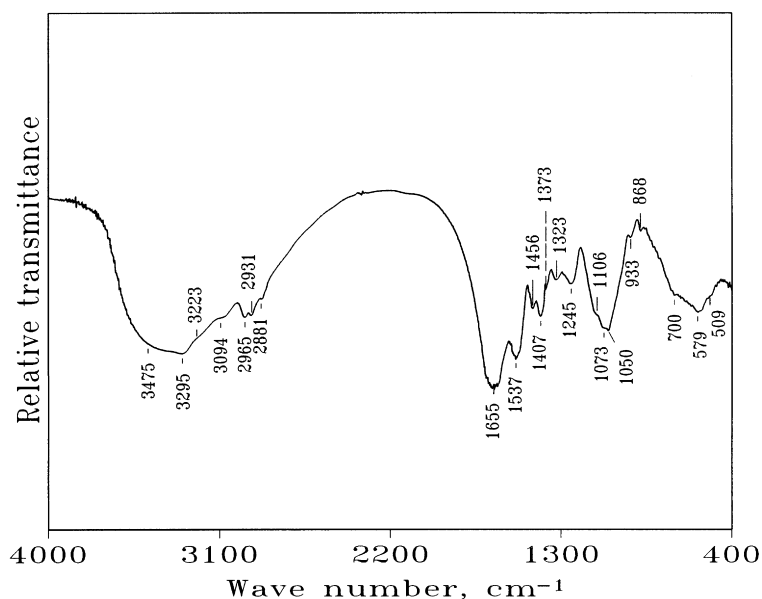


Fig. 2. Fourier transform infrared spectrum of a vacuum-dried sample of lipopolysaccharide complex isolated from the cell surface of *Azospirillum brasilense* Sp245 (see Section 2.4 and Section 2.5).

The effects described earlier might indicate that the addition of Cu^{2+} to the culture medium of *A. brasilense* inducing an enhanced uptake of essential metals by the bacterium cells [10] leads also to some noticeable alteration of the membrane composition and/or properties. It should be noted, for example, that Cu is directly involved in the biochemical processes that control Fe transport through the membranes and its uptake by other microorganisms [25,26], which may be accompanied by expression of certain membrane proteins [26,27]. Moreover, the membrane may contain significant amounts of metals (in particular, bivalent cations), which play an essential role in maintaining its structural organization and in its resistibility to stress conditions [28–30], including mechanical stress.

In view of that, considering also the fact that some additional and/or more intensive bands detected for the membranes of “metal-rich” cells (see Fig. 1) may well represent also the spectral features of phospholipids (as described earlier and in Table 1), this may in part be connected with some reinforcement of the membrane in the latter case, a contribution to which may be made by entrapped bivalent metal cations. However, analyses of the membrane samples,

corresponding to spectra 1 and 2 in Fig. 1, for metal cations (Table 2) show a significant difference only for Mg, though a noticeable accumulation of copper, added to the medium, in the membrane (cf. its similar uptake in intact *A. brasilense* Sp245 cells [9,10]) could be important as well. Note also that the *A. brasilense* Sp245 phospholipids, constituting ca. 7% w/w of the dry cell biomass, constitute an essential part of its membranes and are represented by a set of the three major phospholipids (see Table 1) typical for Gramnegative bacteria [31], with the commonly observed predominance of phosphatidylethanolamine, at both the exponential and stationary growth stages with minor quantitative differences between them.

An FTIR spectrum of LPS isolated from the azospirillum cell surface (see Section 2.4) is shown in Fig. 2. Along with the polysaccharide vibration modes including, in particular, the broad $\nu(\text{O-H})$ envelope (about $3200\text{--}3500\text{ cm}^{-1}$), C–C and C–O vibrations region (ca. $950\text{--}1200\text{ cm}^{-1}$), it also features a significant contribution of specific modes of bound proteins: stretching N–H at 3295 with a shoulder at 3094 cm^{-1} , intensive amide I [$\nu(\text{C=O})$ in peptides] and amide II [$\nu(\text{C-N})$ mixed with $\delta(\text{N-H})$] bands at 1655 and

1537 cm^{-1} , respectively (cf. also Fig. 1). This reflects the formation of LPS-protein complexes typical for azospirilla [18,19]. However, it should also be noted that the FTIR spectrum of LPS (see Fig. 2) does not contain any definitely resolved signs of the characteristic $\nu(\text{C}=\text{O})$ vibrations of polyester (e.g., lipid) or protonated carboxylic groups at ca. 1730–1760 cm^{-1} , both of which were mentioned (but not shown) in [18] for IR spectra of surface polysaccharide complexes isolated from a number of *A. brasilense* strains (including Sp245). Nevertheless, in the sample studied (see Fig. 2) they can be present in lesser amounts, and/or the polyester carbonyl may be involved in H-bonding which decreases its vibration frequency [20,24] thus overlapping with the peptide carbonyl band (note the markedly broadened amide I band at 1655 cm^{-1} in Fig. 2 as compared to those in Fig. 1). Besides that, any spectral differences for azospirillum cell surface LPS complexes might be explained in part by some differences in the isolation procedures applied and/or by the fact that different types of polysaccharides can be produced by azospirilla even under the same conditions [32]. Comparing Figs. 1 and 2, it is also evident that the spectra of the cell membranes comprise the main vibration modes of the relevant lipopolysaccharide and protein components, both of which are believed to be involved in associative azospirillum-plant root interactions [17–19,32–35] starting with primary adhesion which seems to be regulated by physicochemical rather than biological mechanisms [35,36], possibly also involving some metal cations [25–30,37,38].

4. Conclusions

Structural and compositional features of bacterial membranes and some of their isolated constituents (cell surface lipopolysaccharide, phospholipids) of the plant-growth-promoting diazotrophic rhizobacterium *A. brasilense* (wild-type strain Sp245) were characterized using FTIR and some other techniques. FTIR spectra of the cell membranes were shown to comprise the main vibration modes of the relevant lipopolysaccharide and protein components which are believed to be involved in associative plant-bacterium interactions, as well as of phospholipid constituents. The five *A. brasilense* Sp245 membrane

phospholipids found, constituting ca. 7% w/w of the dry cell biomass, are represented by the three major phospholipids (comprising over 90% of the total phospholipids content) typical for Gram-negative bacteria, with the commonly observed predominance of phosphatidylethanolamine, at both the exponential and stationary growth stages with only minor quantitative differences between them.

FTIR spectrum of *A. brasilense* Sp245 membranes obtained from the bacterial culture grown in the presence of 0.2 mM Cu^{2+} in the medium (which has earlier been shown to induce an increased uptake of some other essential metals by the bacterium from the medium), as compared to that obtained from the culture grown in the Cu^{2+} -free medium, represents more intensive spectral features of phospholipids, which may in part be connected with some reinforcement of the membrane in the case of the “metal stress”, a contribution to which may be made by entrapped bivalent metal cations. However, AAS analyses of the two membrane samples for metal cations (Mg, Ca, Mn, Fe) show a significant difference only for Mg, along with a noticeable accumulation of Cu^{2+} , when added to the medium, in the membrane (similar to its uptake in intact *A. brasilense* Sp245 cells observed earlier).

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Note added in proof

Considering the FTIR spectroscopic data on azospirillum membrane phospholipids discussed in this paper, we would like to note a most recent publication which appeared in October 1998 (A. Masia, S.V. Avery, M.A. Zoroddu, G.M. Gadd, FEMS Microbiol. Lett. 167 (1998) 321–326; see also references cited therein), on the correlation of the content of polyunsaturated fatty acids in microbial membrane lipids with their metal sensitivity (and

uptake) connected with the extent of membrane permeabilization during metal stress.

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