

Responses of *Azospirillum brasilense* to Nitrogen Deficiency and to Wheat Lectin: A Diffuse Reflectance Infrared Fourier Transform (DRIFT) Spectroscopic Study

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Abstract For the rhizobacterium *Azospirillum brasilense*, the optimal nutritional range of C:N ratios corresponds to the presence of malate (ca. 3 to 5 g l⁻¹ of its sodium salt) and ammonium (ca. 0.5 to 3 g l⁻¹ of NH₄Cl) as preferred carbon and nitrogen sources, respectively. This micro-aerophilic aerotactic bacterium is known to have a narrow optimal oxygen concentration range of ca. 3 to 5 μM, which is 1.2% to 2% of oxygen solubility in air-saturated water under normal conditions. In this work, the effects of stress conditions (bound-nitrogen deficiency related to a high C:N ratio in the medium; excess of oxygen) on aerobically grown *A. brasilense* Sp245, a native wheat-associated endophyte, were investigated in the absence and presence of wheat germ agglutinin (WGA, plant stress protein and a molecular host-plant signal for the bacterium) using FTIR spectroscopy of whole cells in the diffuse reflectance mode (DRIFT). The nutritional stress resulted in the appearance of prominent spectroscopic signs of poly-3-hydroxybutyrate (PHB) accumulation in the bacterial cells; in addition, splitting of the amide I band related to bacterial cellular proteins indicated some stress-induced alterations in their secondary structure components. Similar structural changes were observed in the presence of nanomolar WGA

both in stressed *A. brasilense* cells and under normal nutritional conditions. Comparative analysis of the data obtained and the relevant literature data indicated that the stress conditions applied (which resulted in the accumulation of PHB involved in stress tolerance) and/or the presence of nanomolar concentrations of WGA induced synthesis of bacterial cell-surface (glyco)proteins rich in β-structures, that could be represented by hemagglutinin and/or porin.

Abbreviations

ATR	attenuated total reflectance
DRIFT	diffuse reflectance infrared Fourier transform
EPS	extracellular polysaccharides
FT	Fourier transform
FTIR	Fourier transform infrared
IR	infrared
PGPR	plant-growth-promoting rhizobacteria
PHA	polyhydroxyalkanoate
PHB	poly-3-hydroxybutyrate
WGA	wheat germ agglutinin

Introduction

Bacteria of the genus *Azospirillum*, belonging to the α-subclass of proteobacteria, have been under investigation since the late 70s of the twentieth century [11, 60]. Under natural conditions, they commonly colonise roots of higher plants, although their large genome (from 4.8 Mbp for *Azospirillum irakense* up to 9.7 Mbp for *Azospirillum lipoferum*) and considerable adaptive potential [11] allow these bacteria to occupy other ecological niches. The same features evidently account for their occurrence over virtually

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all climatic zones, being of interest to researchers in many fields related to microbiology.

In their natural habitat, azospirilla commonly incur not a single stress factor but are rather under several types of stress. Although stress responses of azospirilla and other bacteria have been attracting the attention of researchers [2, 12, 24, 26, 29, 30, 33, 50, 52, 61, 69], for *Azospirillum brasilense*, the related reports are few [29, 30, 33, 50, 52, 61]. Since *A. brasilense* and *A. lipoferum* are used in bacterial preparations applied as environmentally friendly fertilisers and biocontrol agents [11], knowledge of their adaptation to stresses is not only of basic interest for a better understanding of their survival strategy under natural conditions, but is also of importance for agricultural biotechnology.

As is known, a variety of stress-induced adaptational reactions in bacteria can lead to changes in the quantitative ratio of certain classes of organic constituents of the cell [12, 50]. In earlier studies, such changes could be detected mainly by means of biochemical analysis. However, the development and progress of Fourier transform vibrational spectroscopic techniques over the last two decades [13, 27, 44–47] have made it possible to draw information on the quantitative and qualitative stress-induced changes in bacterial cellular macromolecules from spectroscopic images of whole cells. Such images comprise the absorption bands of chemical functional groups related to all the components that are present in the sample under study in dominating or at least noticeable quantities (e.g., a few percent), including contributions from intermolecular non-covalent interactions.

Microbial cells, being highly compositionally sophisticated objects, have nevertheless been successfully studied using Fourier transform infrared (FTIR) spectroscopy [25, 27, 28, 33, 34, 36, 46, 47, 54, 57, 68]. For instance, FTIR spectra of several species of the genera *Bacillus*, *Staphylococcus*, *Streptococcus*, *Citrobacter*, as well as *Pseudomonas* spp. and *Escherichia coli*, prepared as thin dry films on a zinc selenide (ZnSe) optical plate, were compared with their FT-Raman spectra (as a technique complementary to FTIR absorption, both representing vibrational spectroscopy) and classified using cluster analysis [46]. Basics of FTIR spectroscopy in its various modes, viz transmission, attenuated total reflectance (ATR) and diffuse reflectance (DRIFT; also see below), were briefly reviewed [54], with a few examples of classification of two *Pseudomonas* spp. and biofilm formation. A much more detailed and profound account of FTIR methodology in microbiological analyses, with a wide variety of spectroscopic data and application examples, appeared as a specialised encyclopedic review chapter [47]. Transmission FTIR spectra of purified preparations of different polyhydroxyalkanoates (PHA, sampled from their chloroform solutions as dried films on

IR-transparent windows) were compared with FTIR spectra of several PHA-producing *Pseudomonas* spp. and *Azotobacter vinelandii* (as films of dried cells), offering a simple method of PHA analysis in bacterial cells [27]. Quantitative changes in *Clostridium acetobutylicum* cell components during fermentation, monitored using FTIR spectroscopy (as dry cell biomass films on ZnSe or in pellets with potassium bromide, KBr), were shown [25] to correlate with the cell physiological state and the activity of cellular biochemical pathways. An excellent comprehensive ATR-FTIR spectroscopic study of intact cells of two *Bacillus* spp. and two *Pseudomonas* spp. (grown under various conditions and measured against the background of the culture liquids), as well as of their specially prepared cell wall fragments and soluble fractions of lysed cells, was reported [28], providing a wealth of information on functional group chemistry of bacterial cell surfaces. Whole cells of *Salmonella enteritidis*, *Salmonella typhimurium*, *E. coli*, *Yersinia enterocolitidis* and *Shigella boydii* were studied by FTIR microspectroscopy, as compared to some of their cell envelope samples (each smeared onto a 0.2- μm -thick-gold-coated glass slide) and cytoplasmic extracts (using ATR-FTIR measurements on ZnSe), and differentiated down to the strain level [68]. Recently, transmission FTIR spectroscopy was used to distinguish between the contents of major macromolecular components in cells of two photosynthetic H_2 -producing strains, *Rhodoblastus acidophilus* and *Rhodobacter capsulatus*, as well as their extracellular polymeric substances [57].

In our earlier work, transmission FTIR spectroscopy was used, for the first time for azospirilla, to compare the effects of copper ions (0.2 mM Cu^{2+}) on *A. brasilense* Sp245 (dried biomass ground with KBr and pressed in pellets), grown in an NH_4^+ -free malate salt medium, with the uptake of essential cations (Mg, Ca, Mn and Fe) and copper in cells [34] as well as in cell membranes [32]. Later, comparing our FT-Raman spectroscopic data [35] with those obtained using the same FTIR spectroscopic approach, we found (for the first time for bacteria) that even in the rich NH_4^+ -supplemented medium, several heavy metals (0.2 mM Co^{2+} , Cu^{2+} or Zn^{2+}) induced accumulation of polyester storage compounds (poly-3-hydroxybutyrate, PHB, typical for azospirilla) in cells of another *A. brasilense* strain, Sp7 [33]. Note that, in contrast to the epiphytic *A. brasilense* strain Sp7, under the same conditions, such changes were not detected in the endophytic strain Sp245 [37], which could be ascribed to different ecological niches that these strains occupy in the rhizosphere [11] and, consequently, to their different adaptation capabilities. Nevertheless, in addition to FTIR spectroscopy (that did not show any signs of PHB accumulation in strain Sp245 in the rich NH_4^+ -supplemented medium in the presence of 0.2 mM Co^{2+} , Cu^{2+} or Zn^{2+}), the application of emission

Mössbauer (nuclear γ -resonance) spectroscopy using the ^{57}Co radionuclide provided evidence that cobalt(II) was involved in metabolic transformations in live cells of this strain [36].

The aim of the present study was to monitor changes in composition and/or structure of cellular macromolecular constituents in *A. brasilense* Sp245, a native wheat endophyte, in response to stress conditions (strong nitrogen starvation related to a very high C:N ratio in the medium; excess of oxygen) using FTIR spectroscopy of whole cells in the diffuse reflectance mode (DRIFT). Another aim was to follow possible changes in composition and/or structure of cellular constituents in aerobically grown *A. brasilense* Sp245 under the influence of wheat germ agglutinin (WGA). Our interest in this question was caused by failure to reveal any clearly detectable cell response of aerobically grown *A. brasilense* Sp245 to WGA, a molecular signal of wheat plant to the bacterium, in contrast to the multiple effects of WGA found under *microaerobic* conditions [4, 7].

Materials and Methods

Bacterial Strain, Growth Conditions and Preparation of Cell Biomass

A. brasilense strain Sp245 (from the Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia), used in this work, had been originally isolated from surface-sterilised wheat roots in the early 1980s in the wheat region of South Brazil [10]. This facultative endophyte was found at high densities in the interior of root hairs, in the root cylinder and in the intercellular spaces of wheat-root epidermal cells [9, 53]. This is a widely studied strain well known for its phytostimulating capabilities [10, 11, 21, 22]; moreover, it exhibits relatively high heavy-metal tolerance [37, 62] and even some crude-oil-degrading potential [43].

In this study, *A. brasilense* Sp245 was cultivated in a malate salt medium (MSM) containing (g l^{-1}): K_2HPO_4 3.0, KH_2PO_4 2.0, NH_4Cl 3.0, NaCl 0.1, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 0.1, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, CaCl_2 0.02, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.002, sodium malate 5.0 (obtained by mixing 3.76 g of malic acid with 2.24 g NaOH per litre), yeast extract 0.1 (pH 6.9). In all cases (if not indicated otherwise), the bacteria were grown with stirring (160 rpm) at 32 °C for 66 h. The culture volume was 100 ml in 250-ml Erlenmeyer flasks when the bacteria were grown to estimate their CFU, and 500 ml in 1,000-ml Erlenmeyer flasks when the bacteria were grown to estimate their final cell-biomass quantity dried to a constant weight (also used

for spectroscopic measurements; see below). For combined stress conditions, NH_4Cl was excluded from the MSM, with stirring being applied during cultivation. In the case of bacterial growth in the presence of WGA (“Lektinotest”, Ukraine), the protein was added together with inoculum to a final concentration of 200 ng ml^{-1} .

The starting cell density of *A. brasilense* Sp245 cultures was 2.0×10^7 colony-forming units (CFU) per ml in all cases. Under nitrogen deficiency, after 66 h of incubation, the culture grew up to ca. $6.7 \times 10^8 \text{ CFU} \cdot \text{ml}^{-1}$, while under similar conditions, in the NH_4^+ -containing medium, the culture reached ca. $5 \times 10^9 \text{ CFU} \cdot \text{ml}^{-1}$ by the stationary growth phase. For 500 ml of NH_4^+ -free culture medium, the final weight of dried cell biomass was 71 mg, while for the NH_4^+ -containing medium it reached 350 mg. Note that for strain Sp245 and some other *A. brasilense* strains, growth under aeration (on a shaker) in liquid cultures is known to be accompanied by an enhanced production of slime comprising extracellular polysaccharides (EPS) [31, 40, 66]. The culture grown aerobically without ammonium visually looked more slimed than that grown in the NH_4^+ -containing medium, which might account for the disproportion between the aforementioned CFU and dry-weight data. Nevertheless, the EPS, which are known to comprise a few percent of dry cell biomass [40], in our experiments were not isolated or analysed. The presence of WGA did not influence growth of the bacterium in the NH_4^+ -containing medium, although under nitrogen deficiency, some increase in the biomass in the presence of WGA was observed (71 ± 1 mg of dry biomass without WGA and 89 ± 6 mg in the presence of WGA per 500 ml of culture), with some parallel increase in CFU: $(6.7 \pm 0.7) \times 10^8$ and $(8.6 \pm 1.6) \times 10^8 \text{ CFU} \cdot \text{ml}^{-1}$, respectively.

Spectroscopic Measurements

For DRIFT spectroscopic measurements, biomass of bacterial cells grown under appropriate conditions was separated from the culture liquid, washed three times with aqueous 0.9% NaCl, centrifuged to obtain a dense residue, dried at 105 °C for 1 h and powdered in an agate mortar. Samples were prepared by placing 0.2 to 0.5 mg of the resulting dry biomass in a Micro sampling cup (Spectra-Tech Inc., USA), lightly pressing the surface of the powdered sample with a flat glass spatula and mounting the sampling cup onto the DRIFT accessory sample holder of the FTIR spectrometer (Nicolet, model Magna IR 750; USA; DTGS detector; Nichrome source; KBr beamsplitter). Spectra were collected with a total of 100 scans (resolution 4 cm^{-1}) against a KBr background and manipulated using the OMNIC (version 3.1) software supplied by the manufacturer of the spectrometer. All spectra were smoothed using the standard “automatic smooth” function of the above software, which

uses the Savitsky–Golay algorithm (95-point moving second-degree polynomial), and then the baseline was corrected using the “automatic baseline correct” function. Other details of the DRIFT spectroscopic methodology were reported elsewhere [36, 37, 47, 54].

All the DRIFT spectroscopic measurements were repeated two or three times using samples of bacterial biomass newly grown under appropriate conditions, with all the effects described being reproducible (representative spectra or spectral regions are shown in Figs. 1, 2, 3, 4). It should also be specially noted that using pure dried biomass in DRIFT spectroscopic measurements, without mixing it with KBr (which is a common practice in FTIR spectroscopy), made it possible to avoid any possible effects of the KBr matrix on the spectroscopic images of polar functional groups involved in H-bonding (e.g., polyester or protein O- or N-containing moieties).

Results and Their Comparative Analysis

For *A. brasilense* Sp245 grown in the whole medium with stirring, a DRIFT spectrum is presented in Fig. 1. Although *Azospirillum* is known to have a narrow oxygen concentration optimum of 3 to 5 μM [71], which is 1.2% to 2% of oxygen solubility in air-saturated water under normal conditions (0.25 mM; [14]), and avoids microenvironments with elevated oxygen concentrations [72], under the conditions applied, the bacteria seemed not to be under oxygen stress. Indeed, while stirring for at least the first several days, the *A. brasilense* Sp245 culture grew up to somewhat higher optical densities (D_{595} ; at 595 nm) as compared to that for the culture grown in the same medium without stirring ($D_{595}=0.92\pm 0.01$ and 0.81 ± 0.11 for the 70-

h cultures, respectively). Thus, the spectrum in Fig. 1 may be regarded as a control (corresponding to unstressed cells).

The overall shape of this spectrum of *Azospirillum* cells grown without stress (see Fig. 1) is generally typical, being close to those of other bacteria (see, e.g. [28, 41, 47, 54, 55]). Thus, the spectrum comprises a strong broad composite absorption band around $3,500\text{--}3,000\text{ cm}^{-1}$ (largely stretching vibrations of O–H groups in various compounds, including traces of bound water, as well as weaker stretching modes of N–H moieties), a superimposed much less intensive absorption region at about $3,000\text{--}2,800\text{ cm}^{-1}$ (C–H stretch), as well as a number of bands below $1,800\text{ cm}^{-1}$ often referred to as the fingerprint region (for example, carbonyl stretching vibrations in various functional groups, such as carboxyl, ester, peptide –CONH– moieties, within $1,800\text{--}1,600\text{ cm}^{-1}$; different deformational modes of –CH₃ and –CH₂– groups around $1,470\text{--}1,350\text{ cm}^{-1}$; different phosphate and polysaccharide stretching modes within ca. $1,240\text{--}900\text{ cm}^{-1}$; the “true fingerprint region” below 900 cm^{-1} featuring a complicated superposition of various aromatic ring vibrations of amino acid residues, nucleotides, etc. [47]).

A spectrum of *A. brasilense* Sp245 cells grown under nutritional stress (in the ammonium-free medium) is shown in Fig. 2. *Azospirillum* are known to readily adapt to a lack of bound nitrogen in the medium by the synthesis of nitrogenase and thereafter fixing molecular nitrogen. Bacterial nitrogenase is well known to be sensitive to O₂, in contact with which the enzyme is irreversibly inhibited, thus blocking the nitrogen-fixation process. In the present experiments, despite the intensive aeration (stirring) that evidently blocks nitrogen fixation, the bacteria grew well: after 66 h of incubation, the number of cells increased from 2×10^7 up to 6.7×10^8 CFU·ml⁻¹, i.e. by 1.5 orders of

Figure 1 FTIR spectrum (in the diffuse reflectance mode) of dry cells of *A. brasilense* Sp245 grown under stirring in whole malate salt medium (MSM; supplemented with 3.0 g l^{-1} NH₄Cl)

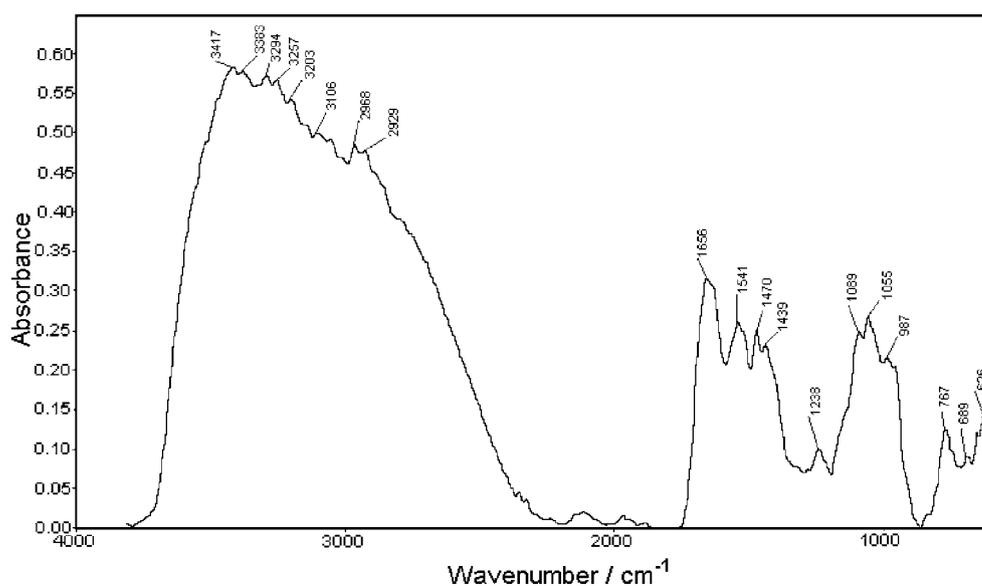
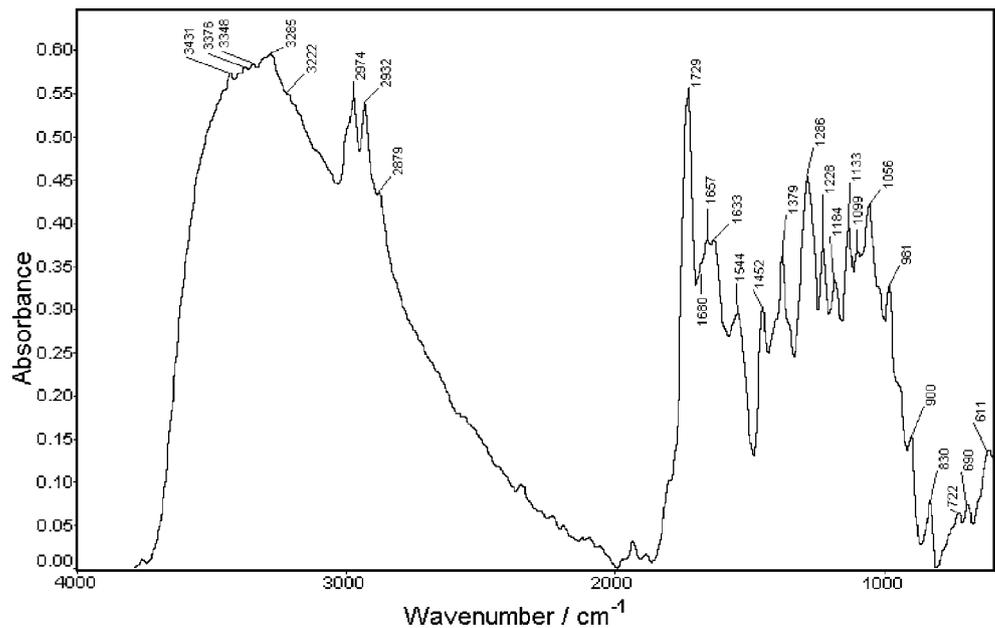


Figure 2 FTIR spectrum (in the diffuse reflectance mode) of dry cells of *A. brasilense* Sp245 grown under stirring in the malate salt medium from which NH_4Cl had been excluded



magnitude. Note that under the conditions applied, hindered physical contact between cells was an additional stress factor for *Azospirillum*, along with a high C:N ratio.

The data of Fig. 2 show that the spectrum of cells under the aforementioned combined stress notably differs from that of the control (see Fig. 1). This is in good agreement with the reported data on significant alterations in *A. brasilense* metabolism in going from optimal to unfavourable conditions

related to nitrogen deficiency with a high C:N ratio in the culture medium [50].

One of the notable features of the spectrum in Fig. 2 is the appearance of prominent bands characteristic of poly-3-hydroxybutyrate (PHB). Accumulation of this biopolymer

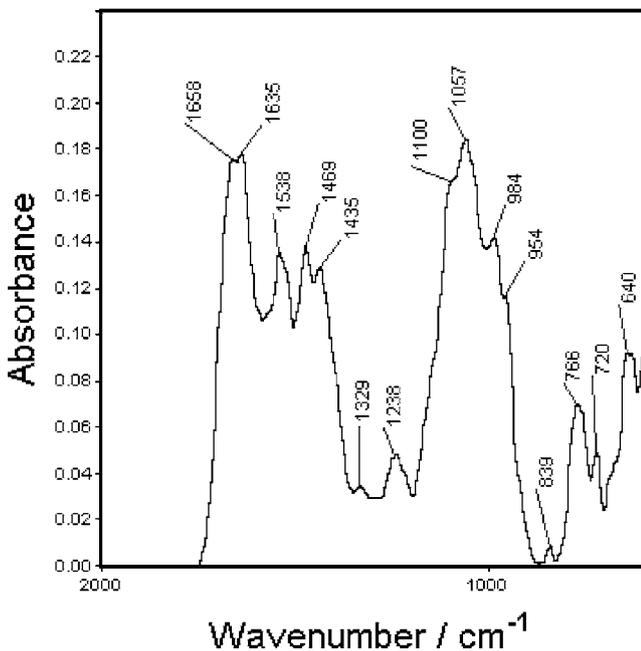


Figure 3 FTIR spectrum (in the diffuse reflectance mode; fingerprint region) of dry cells of *A. brasilense* Sp245 grown under stirring in whole malate salt medium (MSM; supplemented with $3.0 \text{ g l}^{-1} \text{ NH}_4\text{Cl}$) in the presence of wheat germ agglutinin (200 ng ml^{-1})

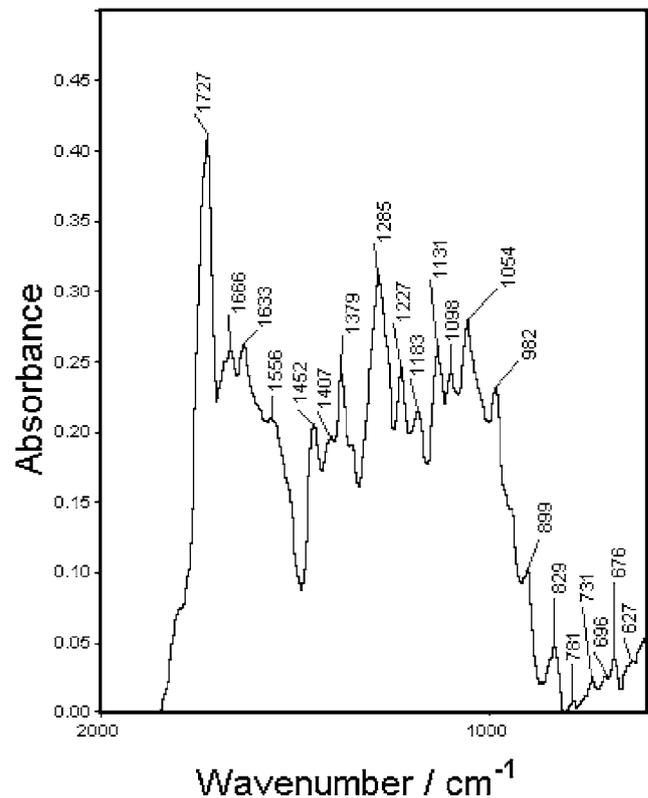


Figure 4 FTIR spectrum (in the diffuse reflectance mode; fingerprint region) of dry cells of *A. brasilense* Sp245 grown under stirring in the malate salt medium from which NH_4Cl had been excluded, in the presence of wheat germ agglutinin (200 ng ml^{-1})

in microbial cells under nutritional stress conditions was reported both for azospirilla [11, 29, 50] and for some other bacteria [27, 30, 38]. The main IR absorption bands characteristic of PHB are those with maxima at around $1,730\text{ cm}^{-1}$ (stretching vibrations of the carbonyl group $\nu(\text{C}=\text{O})$ of the ester moieties), $1,450\text{--}1,452$ and $1,379\text{ cm}^{-1}$ (antisymmetric and symmetric bending vibrations of methyl ($\text{CH}_3\text{--}$) and methylene ($\text{--CH}_2\text{--}$) groups, respectively), as well as at about $1,285$, $1,135$ and $1,055\text{ cm}^{-1}$ (polyester C--O--C/C--O fragment vibrations), etc. [27, 47, 51]. In particular, Hong et al. [27] showed that pure bacterial PHB is characterised by the $\nu(\text{C}=\text{O})$ band with a maximum not exceeding $1,730\text{ cm}^{-1}$, depending on the degree of PHB crystallinity [51], in contrast to other microbial polyhydroxyalkanoates (PHA) that exhibit $\nu(\text{C}=\text{O})$ absorption bands with maxima at about $1,732\text{--}1,744\text{ cm}^{-1}$ (at room temperature). PHB accumulation in the cells grown under tough stress with a high C:N ratio is manifested also by a noticeable increase in the intensity of stretching vibrations of methyl and methylene groups around $3,000\text{--}2,800\text{ cm}^{-1}$ (see Fig. 2), as compared to that for control cells (see Fig. 1).

The second significant alteration in the spectrum of stressed cells concerns the proteinaceous cellular constituents of the bacterium. It involves changes in the amide I region of vibrations comprising different spectroscopic components within 1620 to 1690 cm^{-1} depending on the conformations of the polypeptide chains. These differences allow FTIR spectroscopy to be used to characterise various types of the secondary structure of proteins [3, 13, 17, 45], in addition to other techniques, such as circular dichroism spectroscopy in the UV region in aqueous solutions (see, e.g. [8, 59] and references therein). Thus, in the control sample (Fig. 1), this region is represented by a broad peak with a single maximum at $1,656\text{ cm}^{-1}$, whereas in the stressed cells, the amide I band is split, exhibiting two resolved maxima ($1,657$ and $1,633\text{ cm}^{-1}$, see Fig. 2) with a shoulder at about $1,680\text{ cm}^{-1}$.

Cellular proteins are known to comprise components with different secondary structure which is represented by α -helix regions, various types of β -structure, as well as unordered polypeptide chain fragments (random coil). The main contribution to the IR absorption in the amide I region is made by stretching vibrations of the carbonylic group, $\nu(\text{C}=\text{O})$, of peptidic bonds in proteins [17]. The appearance of different bands within the amide I region ($1,620\text{--}1,690\text{ cm}^{-1}$) is due to different types of H-bonding between the carbonyl ($\text{C}=\text{O}$) and amide (N--H) moieties in peptide fragments (see, e.g. [23]) with different $\text{C}=\text{O}$ bonding energies, which influences the vibration frequency of this group in protein macromolecules with different types of secondary structure. The α -helix type of protein secondary structure is featured by a single relatively narrow band with a maximum at about $1,656\text{--}1,658\text{ cm}^{-1}$; different types of

β -sheets (differing in the strength of interaction between the chains—from weak to strong) exhibit bands with a maximum from $1,637$ to $1,623\text{ cm}^{-1}$, respectively, usually with an accompanying band at $1,680\text{--}1,690\text{ cm}^{-1}$ (β -antiparallel); β -turns give a band at $1,660\text{--}1,680\text{ cm}^{-1}$; random coil is commonly characterised by a relatively broad band around $1,645\text{--}1,648\text{ cm}^{-1}$ [13, 17, 45].

In view of the aforementioned, comparing the spectra in Figs. 1 and 2, it can be concluded that the *A. brasilense* Sp245 cells grown under the combined stress, as compared with those in the control (see Fig. 1), are characterised by a redistribution of the secondary structure components of cellular proteins; namely, by a relative increase in the β -sheet fragments in the overall pool of cellular proteins.

In the two other cases represented by Figs. 3 and 4, where *Azospirillum* cells were similarly grown either under optimal conditions (Fig. 3) or under the combined stress (Fig. 4), in contrast to the variants described above (see Figs. 1 and 2), the medium also contained plant lectin (WGA) at a low concentration of $5.6 \times 10^{-9}\text{ M}$. This lectin, acting as a molecular signal of the host plant for *A. brasilense* Sp245, induces a pleiotropic cellular response in the bacterium [5, 6], including an enhanced synthesis of intracellular proteins and a change in their composition [7]. However, earlier, we had failed to register any pronounced cellular response to WGA in *A. brasilense* Sp245 grown under aeration (note that regulation of the process of metabolic activation in the bacterium by the lectin is not yet well studied, and this fact remains to be understood). Thus, comparison was made between the spectra in Figs. 1 and 3 with the aim of revealing possible distinctions induced by WGA in *Azospirillum* cells grown under aeration.

From comparing Figs. 1 and 3, it can be seen that nanomolar concentrations of WGA in the bacterial growth medium induced splitting of the amide I band. Comparing the latter change in the amide I region induced by WGA (see Fig. 3) with that caused by the lack of ammonium in the medium (see Fig. 2), the newly appearing lower-wavelength bands were virtually similar in both cases ($1,633$ and $1,635\text{ cm}^{-1}$ in Figs. 2 and 3, respectively). As was noted above, the band at $1,657\text{ cm}^{-1}$ refers to α -helix protein regions, while the appearance of a maximum at about $1,635\text{ cm}^{-1}$ represents an increase in β -sheets [13, 17, 45]. In general, the WGA-induced changes in the region of amide I vibrations under optimal bacterial growth conditions reflect a decrease in the proportion of α -helix regions with a concomitant increase in those containing β -structures.

The final variant analysed in this work includes stressed cells grown in the presence of nanomolar WGA in the medium (Fig. 4). As can be seen from comparing it with those in Figs. 1 and 2, the presence of WGA did not preclude PHB accumulation by the stressed bacterium: all

the major peaks characteristic of PHB (at 1,727, 1,452, 1,379, 1,285, 1,131 and 1,054 cm^{-1}) are clearly seen. Note also that the splitting of the amide I band (exhibiting two maxima at 1,666 and 1,633 cm^{-1}) for the variant of stressed cells grown with WGA (see Fig. 4) is similar to that induced by WGA in cells grown under optimal conditions (appearance of an additional peak at 1,635 cm^{-1} , see Fig. 3), featuring a similarly increased proportion of β -structures in cellular polypeptides.

Discussion

For the rhizobacterium *A. brasilense*, the optimal nutritional range of C:N ratios corresponds to the presence of malate (ca. 3 to 5 g l^{-1} of its sodium salt) and ammonium (ca. 0.5 to 3 g l^{-1} of NH_4Cl) as preferred carbon and nitrogen sources, respectively [11, 22]. Within the above ranges, the growth rate of the bacterium practically does not change in liquid medium at 30 to 32 °C under aeration. It is also well known that under nitrogen-fixation conditions (bound-nitrogen deficiency in the medium), *Azospirillum* grows more slowly than in whole nutritionally rich media, which is largely related to the fact that N_2 fixation is a very energy-demanding process [11].

The comparative DRIFT spectroscopic study of *A. brasilense* Sp245 cells performed in the present work has allowed several important points to be revealed. First, it should be noted that, despite some specific features of *Azospirillum* in its physiology and molecular genetics [7, 11, 50, 60, 61, 63] which distinguish it from other members of *Alphaproteobacteria*, the spectrum of strain Sp245 grown under optimal conditions (see Fig. 1) was generally similar to those of many other bacteria [28, 47, 54].

In this work, the effects of two factors on *A. brasilense* Sp245 were studied: combined stress conditions on one hand, and the presence of wheat lectin, a molecular signal of the host plant for this bacterium [7], on the other hand. Both of these factors were applied either separately (see Figs. 2 and 3) or jointly (see Fig. 4). As follows from the data presented above, both factors influenced the major cellular organic composition of the bacterium.

One of the most expressed changes observed only in the case of the combined stress (both in the presence and in the absence of WGA) was PHB accumulation in stressed cells. This fact, in agreement with literature data for azospirilla grown under stress conditions [30, 50], is related most probably to an unfavourably high C:N ratio in the growth medium. Besides that, a retarded physical contact between bacterial cells (as in this work the bacterium was grown under forced stirring) is known to be another unfavourable factor that might hinder bacterial adaptation to stress [65]. Thus it cannot be excluded that this factor, along with the

deficiency of bound nitrogen, contributed to the stress-induced PHB accumulation in cells.

A. brasilense (strain Cd) was reported to produce small amounts of PHB (21 mg/g of dry weight) when grown in a synthetic medium containing succinate and KNO_3 , but when transferred into a medium with a high C:N ratio and with fructose instead of succinate, that strain produced 30 times more PHB [50]. In strain Sp245, from its DRIFT spectrum (see Fig. 1) virtually no PHB (or very little, below the sensitivity of the technique) could be observed in the malate medium under optimal conditions. On the contrary, the spectra in Figs. 2 and 4 clearly demonstrate PHB production by stressed Sp245 cells. Here it can be assumed, however, that Sp245 produced less PHB than strain Cd, as under the conditions of our experiments *A. brasilense* Sp245 had to produce also significant amounts of extracellular polymers to protect itself from excess of oxygen [31, 40]. The second reason for a possibly lower PHB production level in Sp245, as compared to strain Cd, could be the lack of bound nitrogen in the medium. Under these conditions of a combined stress and in the presence of WGA as a signal of the presence of the host plant [7], the only source of bound nitrogen for *A. brasilense* Sp245 could be dinitrogen fixation. As mentioned above, this process is well known to be highly energy-demanding [11] and is accompanied by an enhanced respiration of the culture. As can be seen from Figs. 3 and 4, WGA at the concentration applied did not influence PHB production by *A. brasilense* Sp245 cells, in agreement with our earlier data on the effects of this regulatory protein on *Azospirillum* metabolism [7].

It can also be concluded that the results obtained demonstrate the capability of detecting PHB production nondestructively in bacterial cells using DRIFT spectroscopy of pure dry biomass (not mixed and/or pressed with KBr) without PHB extraction from the cells for analysis [50], a result which has never been reported for azospirilla (note that in our earlier report, where DRIFT spectroscopy was applied to *A. brasilense* Sp245 cells [36], the latter did not produce and accumulate any noticeable quantities of PHB).

The second important finding, observed under the influence of each of the two factors studied, was the splitting of the amide I band reflecting certain conformational changes in proteinaceous cellular constituents. While the overall protein content in *A. brasilense* has been reported earlier to be decreased ca. six fold in response to stress caused by an unfavourable C:N ratio [50] or, in *A. lipoferum*, starvation has been shown to induce changes in the content of several cell-surface proteins [18], nothing is known about conformational structural changes in *Azospirillum* cellular proteins induced by external factors. The conformational changes in protein constituents observed

herein were registered both under stress and in response to WGA, a communicative factor of the host plant.

Comparison of the data obtained in this study with those from other laboratories (see below) allows the changes observed to be related to changes in surface proteins (glycoproteins) in *A. brasilense* Sp245. It is common knowledge that bacteria can rapidly and adequately alter their cell surface, thus adapting to the changing conditions of the environment. In this process of adaptation of bacteria to environmental stresses, a substantial role is known to be played by cell-surface biopolymers including proteins, glycoproteins, glycoproteolipids, as well as other macromolecular metabolites excreted by the cell into the environment.

For azospirilla, a number of cell-surface biopolymers have already been characterised, many of which were shown to be excreted into the environment and to be of importance for the strategy of bacterial survival. Such biopolymers, both found extracellularly and detected in the growth medium, included lectin (hemagglutinin), proteolytic and pectolytic enzymes [11, 19, 48, 49], lipopolysaccharide–protein (LPPC) and polysaccharide–lipid (PSLC) complexes [39, 40, 58, 67]. Besides these extracellular biopolymers, the major outer membrane protein (MOMP) in *A. brasilense* has also been characterised [16]. The MOMP gene, *omaA*, has been sequenced, and analysis of the deduced amino acid sequence showed a significant homology of MOMP to certain bacterial porins [15].

For all the aforementioned bacterial cell-surface biopolymers, there is experimental evidence for their involvement in host-plant colonisation [16, 19, 39, 48, 49, 58, 67]. Moreover, at least three of the biopolymers—hemagglutinin, LPPC and PSLC—are involved in *A. brasilense* stress resistance [39, 49]. It is conceivable that host-plant colonisation is one of bacterial strategies helping the bacteria to survive certain stresses, as plants are well known to maintain homeostasis (optimal levels of temperature, acidity, etc.), besides their “nutritional support” for the bacteria.

In view of the aforementioned, the data obtained herein allow us to assume that the stress conditions applied and/or the presence of WGA induce bacterial biosynthesis of one or two cell-surface (glyco)proteins—hemagglutinin and (or) porin. This assumption is based on the high content of β -structures in these two microbial proteins. For instance, the content of the β -sheet structure in hemagglutinin from *Clostridium botulinum* is 74% to 77% [56]; in that from *Mycococcus xanthus* around 50% [20]; β -sheets are the major secondary structure of *Physarum polycephalum* hemagglutinin [42]. Moreover, while lectins are generally regarded as proteins rich in β -sheets [20, 42, 56, 70], a group of lectins were reported to consist merely of β -structures [70]. Bacterial porins are also rich in β -structures [1]; for instance, the two known *E. coli* porins, maltoporin

and OmpA, have similar structures containing 50% to 60% β -strands, about 20% β -turns and less than 15% α -helices [64].

It has also to be noted that the amount of WGA bound to the bacterial cells could not contribute by itself to the observed spectroscopic effects (i.e. to the splitting of the amide I band) for the following reasons. First, the overall WGA concentration applied ($0.2 \mu\text{g ml}^{-1}$) was too low to give any noticeable contribution to the spectra of the resulting bacterial biomass; second, the amide I band splitting was observed for stressed bacterial cells also without WGA (see Fig. 2). Thus it can be inferred that low quantities of WGA induced additional synthesis of hemagglutinin and/or porin and hence its possible enhanced exposure at the bacterial surface. Interestingly, for *A. brasilense* Sp245 growing under nitrogen-fixing conditions, evidence has been obtained for WGA-induced synthesis of bacterial lectin which in this strain is ca. 42-kDa surface glycoprotein (L.P. Antonyuk, paper in preparation).

As mentioned above, for *Azospirillum*, WGA is a signal of the presence of the host plant. At the sites of localisation of azospirilla (wheat-root tips), WGA is not only excreted into the medium but is present also at the root surface and can thus serve as a potential binding site for bacterial cells in the course of their attachment to the root. The results obtained herein suggest that *Azospirillum* responds to the presence of the plant by “getting ready” for its colonisation, viz by an enhanced synthesis of biopolymers involved in colonisation (note that in *A. brasilense* both MOMP and hemagglutinin are adhesins [16, 48]).

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