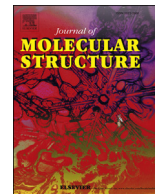




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FTIR spectroscopic study of biofilms formed by the rhizobacterium *Azospirillum brasilense* Sp245 and its mutant *Azospirillum brasilense* Sp245.1610

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ABSTRACT

Biofilms are spatially and metabolically structured communities of microorganisms, representing a mode of their existence which is ubiquitous in nature, with cells localised within an extracellular biopolymeric matrix, attached to each other, at an interface. For plant-growth-promoting rhizobacteria (PGPR), the formation of biofilms is of special importance due to their primary localisation at the surface of plant root systems. In this work, FTIR spectroscopy was used, for the first time for bacteria of the genus *Azospirillum*, to comparatively study 6-day-mature biofilms formed on the surface of ZnSe discs by the rhizobacterium *Azospirillum brasilense* Sp245 and its mutant *A. brasilense* Sp245.1610. The mutant strain, having an Omegon Km insertion in the gene of lipid metabolism *fabG1* on the plasmid AZOBR_p1, as compared to the wild-type strain Sp245 (see <http://dx.doi.org/10.1134/S1022795413110112>), had previously been shown to possess alterations in the synthesis of fatty acids, as well as in the amount of biomass and relative content of lipopolysaccharide antigens in mature biofilms formed at a hydrophilic or hydrophobic surface (see <http://dx.doi.org/10.1134/S002626171602017X>). The 6-day biofilm of the wild-type strain *A. brasilense* Sp245 was found to contain moderate amounts of poly-3-hydroxybutyrate (PHB, a reserve biopolyester) featured by its typical bands (e.g., the ester carbonyl stretching mode at 1732 cm⁻¹), while its mutant strain showed a diminished PHB content in the biofilm (the observed differences were also confirmed by analysing the second derivatives of the FTIR spectra). Thus it may be assumed that in this mutant strain, PHB synthesis is also affected which, in turn, can affect the formation and stability of biofilms.

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1. Introduction

The formation of biofilms is a natural process in microbial ecology which plays diverse important roles in the environment (see, e.g. Refs. [1–3] and references reported therein). For plant-growth-promoting rhizobacteria (PGPR), this feature is yet more important in view of their primary localisation at the surface of plant root systems. Among PGPR, bacteria of the genus *Azospirillum*, and particularly the ubiquitous species *A. brasilense*, are widely

studied because of their phytostimulating potential [4,5]. They can form the associations with plant roots and fix atmospheric nitrogen [5,6]. Our previous studies using Fourier transform infrared (FTIR) spectroscopy in various modes (see, e.g. Refs. [7–10]) showed this technique to be a sensitive and informative tool for monitoring molecular-level changes in bacteria (particularly on the examples of *A. brasilense*) induced by various environmental factors, including different stresses.

In natural conditions, the bacteria of the genus *Azospirillum* (as well as many other PGPR) exist mainly in the form of biofilms and are often under various stress conditions. Biofilms are spatially and metabolically structured communities of microorganisms, representing a mode of their existence which is ubiquitous in nature,

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with cells localised within an extracellular biopolymeric matrix, attached to each other, at an interface. Biofilms of various bacteria are currently under thorough investigation [1–3], as information on their structure, composition and modes of formation is of topical interest and importance. However, information on PGPR biofilms, including those of azospirilla [11–15], is scarce, and the application of FTIR spectroscopy may be helpful in elucidating possible molecular structural and compositional changes which accompany the transformation of planktonic cultures (i.e. cells in a cultural solution) to a biofilm.

In this communication, FTIR spectroscopy was used, for the first time for bacteria of the genus *Azospirillum*, to comparatively characterise 6-day-mature biofilms of *A. brasilense* wild-type strain Sp245 and its mutant strain Sp245.1610 (defective in mobility and capability of swimming [16]).

2. Materials and methods

2.1. Bacterial strains and growth conditions

Wild-type *A. brasilense* strain Sp245 [17] (from The Collection of Rhizosphere Microorganisms, [WDCM 1021], Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia) and its mutant leaky $\text{Fla}^-/\text{Mot}^-/\text{Laf}^-/\text{Swa}^-$ Sp245.1610 (*fabG1::Omegon-Km*) [16], which forms biofilms different from those of the wild-type strain [12], were used in this work.

Bacteria were cultivated in a liquid modified malate salt medium (MSM) [18] which contained the following salts ($\text{g}\cdot\text{l}^{-1}$): K_2HPO_4 , 3.0; KH_2PO_4 , 2.0; NH_4Cl , 1.0; NaCl , 0.1; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.02 (added as chelate with nitrilotriacetic acid); 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), pH 6.8–7.0. Kanamycin (Km) was added to the medium at a final concentration of 50 $\mu\text{g}/\text{ml}$ in all cases when the mutant strain was cultivated.

The inoculation cultures (100 ml in 250-ml Erlenmeyer flasks) were grown under aerobic conditions on a shaker (140 rpm) for up to 18 h. Cell growth was monitored by optical density A_{590} at $\lambda = 590$ nm (Spekol 221, Germany, optical path length 0.5 cm).

Biofilms of the cultures under study were obtained as follows (all procedures were carried out under sterile conditions). A clean ZnSe disc (CVD-ZnSe, “R’AIN Optics”, Dzerzhinsk, Russia; diameter 2.5 cm, thickness 0.2 cm) was placed on the bottom of a Petri dish (diameter 4 cm), in which 3 ml of the MSM medium with bacteria (*A. brasilense* strains inoculated up to the final optical density $A_{590} = 0.05$ to 0.1) were added. Then the Petri dishes were placed into a thermostat at 28 °C and incubated for 6 d.

2.2. Sample preparation and Fourier transform infrared (FTIR) spectroscopy

For FTIR spectroscopic measurements, the 6-day-mature biofilms that had formed on the surface of the ZnSe glass discs (see above) were carefully separated from the culture medium with the remaining planktonic culture and dried at 45 °C up to a constant weight. (It should be noted that this sample preparation procedure, without active washing, was necessary to obtain an intact biofilm. It was experimentally found that, under active washing in order to fully remove the culture medium components, the formed biofilm could be partly damaged or even removed by the water flow.) As a cell-free control, a minimal volume of the MSM medium was placed as a thin film on a clean flat ZnSe disc and dried.

Infrared spectroscopic measurements were performed on a Nicolet 6700 FTIR spectrometer (Thermo Electron Corporation, USA; DTGS detector; KBr beamsplitter). Spectra were collected in

the transmission mode with a total of 64 scans (resolution 2 cm^{-1}) against the ZnSe disc background and manipulated using the OMNIC (version 8.2.0.387) software supplied by the manufacturer of the spectrometer. All spectra were smoothed using the standard “automatic smooth” function of the 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. All the FTIR spectroscopic measurements were done in triplicate and were reproducible. The second derivatives of the FTIR spectra of the biofilms were calculated using the OMNIC software (the Savitsky–Golay method; number of points: 29; third-degree polynomial).

3. Results and discussion

For a successful functioning of a plant-bacterial association, the ability of rhizobacteria, including azospirilla, to form biofilms may be essential [13,19]. In the recent years, biofilms formed by agrobiotechnologically promising strains *A. brasilense* Sp7 and *A. brasilense* Sp245 have been under investigation; particularly, metabolic processes important for biofilm formation were studied using their mutant strains defective in flagellation, motility, etc. [11–15].

In 1985, a report was published on the use of FTIR spectroscopy in microbial ecology for the analysis of bacteria, bacteria-polymer mixtures and biofilms [20]. Currently, FTIR spectroscopy is a well-established tool in microbiology [21–23], including a few reported studies of biofilms formed by various bacteria [24–27]. This technique can be used to obtain information on the physico-chemical state of biofilms and on the relative composition of their macrocomponents (using an appropriate sample preparation procedure) virtually *in situ* without isolation and characterisation of the matrix constituents. This information complements that obtained by other (mainly biochemical) methods on the formation and structure of microbial biofilms.

Fig. 1(A–C) shows FTIR spectra (registered in the transmission mode) of the samples (as dried 6-day-mature biofilms formed on the surface of ZnSe discs) of *A. brasilense* Sp245 (Fig. 1A) and its mutant Sp245.1610 (Fig. 1B), as well as of the control sample of the MSM (Fig. 1C). Wavenumbers of the maxima for the main bands in the FTIR spectra are summarised in Table 1 together with their tentative assignment [8,9,21–31].

The presented FTIR spectra of the 6-day-mature biofilms formed by both *A. brasilense* Sp245 and its mutant *A. brasilense* Sp245.1610 show all the typical bands corresponding to bacterial macrocomponents (proteins, polysaccharides, lipids, etc.; see Fig. 1A and B and Table 1). The differences between the biofilms are small but clearly visible. First of all, in the spectrum of the wild-type strain (Fig. 1A) there is a well-resolved band at 1732 cm^{-1} typical of the $\nu(\text{C}=\text{O})$ mode in poly-3-hydroxybutyrate (PHB; reserve biopolyester that can be synthesised and accumulated by azospirilla as intracellular granules [8]). It should be noted that in *A. brasilense* the accumulation of noticeable amounts of PHB (detectable by FTIR spectroscopy) is induced mainly by nitrogen deficiency [7,8]. In this case, however, the MSM contained 1 g l^{-1} of NH_4Cl , which is commonly sufficient for this nitrogen-fixing bacterium to avoid trophic stress caused by lack of bound nitrogen (see, e.g. the data reported in Ref. [32]). The accumulation of PHB up to about 10% of dry cell biomass (estimated from the FTIR spectrum in Fig. 1A as reported in Ref. [7]) in this case is evidently related to the prolonged cultivation (6 d) of the biofilm when most of the nitrogen might have been consumed causing its deficiency and, consequently, PHB accumulation (note that carbon-containing nutrients in the culture medium available for this moderate PHB biosynthesis in the biofilm might well have been represented by bacterial exopolysaccharides

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