Rhizobium strains differ considerably in outer membrane permeability and polymyxin B resistance

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Six rhizobium (Rhizobium leguminosarum bv. Trifolii TA1, Sinorhizobium melloti 1021, Mesorhizobium huakuii IFO 152433, Ochrobactrum lupini LUP 211, Bradyrhizobium japonicum USDA110 and B. elkanii USDA 76) and two Escherichia coli strains (E. coli ATCC 25922 and E. coli HB 101) were compared in respect to polymyxin B and EDTA resistance, as well as bacterial outer membrane (OM) permeability to a fluorescent hydrophobic agent (N-phenyl-1-naphthylamine – NPN). TEM (Transmission Electron Microscopy) and a microbial test demonstrated that all the rhizobia were much more resistant to polymyxin B in comparison with E. coli strains. EDTA and polymyxin B enhance permeability of B. japonicum and O. lupini OM. Other rhizobia incorporated NPN independently of the presence of membrane-deteriorating agents; however, the level of fluorescence (measured as NPN absorption) was strain dependent.

Key words: rhizobium; outer membrane; lipopolysaccharide; N-phenyl-1-naphthylamine; NPN; polymyxin B

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INTRODUCTION

The cell walls of Gram-negative bacteria, including rhizobia, possess characteristic spatial organization which is composed of a cytoplasmic membrane (CM), a periplasmic space where the murein sacculus is located, and an outer membrane (OM). The latter bilayer (OM) mentioned is usually composed of three different types of molecules: phospholipids, peripheral and transmembrane proteins, and a complex glycolipid, called lipopolysaccharide (LPS). The OM has a highly asymmetric structure with phospholipids on the inner leaflet and LPS molecules furnishing the outer part of the bilayer. The OM is a selectively permeable barrier to external matter, and its impermeability to hydrophobic compounds is mainly due to the presence of LPS, occupying the entire outer part of the OM. When phospholipids are present in this leaflet (as observed in the case of certain mutants), the barrier is ineffective (Nikaido, 2003). To facilitate the diffusion of small (about 1500 Da) molecules across the wall, bacteria incorporate porins into the outer bilayer. Some polycationic compounds (e.g. aminoglycoside antibiotics), along with polycationic detergents (e.g. polymyxin B), can bind to lipid A (a hydrophobic anchor of the LPS) deteriorating the OM barrier and penetrate it in a self-promoted way (Vaara, 1992; Martínez de Tejada et al., 1995; Nikaido, 2003; Mares et al., 2009).

The lipopolysaccharide is typically composed of three domains: lipid A, which constitutes an OM outer leaflet, a short core oligosaccharide, and, very often, an O-specific polysaccharide (O-chain, O-PS). The structures of O-poly saccharides and core oligosaccharides obtained from rhizobial LPS, as well as their functions, are described in detail in review articles by De Castro and co-workers (2008) and Carlson and co-workers (2010).

Enterobacterial lipid A, toxic to humans, consists of a β-(1→6)-linked glucosaminyl disaccharide substituted on both sides by phosphate groups. Six fatty acid residues, which form two acyloxyacyl moieties, are linked in distinct positions to the sugar backbone (Zähringer et al., 1999; Raetz & Whitfield, 2002). Lipids A, the structures of which differ from enterobacterial ones, are not as unusual as it was thought previously. The rhizobial lipid A especially represents a highly structurally diversified group of molecules (De Castro et al., 2008). The backbone of the lipids A can be composed either of a glucosaminyl-(D-GlcN) (Rhizobium and Sinorhizobium genera) or a 2,3-diamino-2,3-dideoxyglucosyl-(D-GlcN3N) disaccharide (Mesorhizobium, Bradyrhizobium, and Azorhizobium genera). Moreover, in the glucosamine-containing lipid A of Rhizobium leguminosarum, the reducing residue is partly oxidized to 2-amino gluconate (Bhat et al., 1994). The sugar backbone of these lipids A can be decorated either by phosphate (Sinorhizobium and Mesorhizobium), uronic acid (Mesorhizobium and Bradyrhizobium), or mannose (Bradyrhizobium) (Gudlavaletti & Forsberg, 2003; Choma & Sowinski, 2004; Komaniecka et al., 2010; Komaniecka et al., 2014; Silipo et al., 2014). All amino groups of amino sugars, as well as the C-3 and C-3’ positions of D-GlcN, are substituted by 3-hydroxy fatty acids. The hydroxyl groups of these primary fatty acids can be further substituted by nonpolar or (β-1)-hydroxy long chain fatty acids (VLCFAs), forming acyloxyacyl moieties (Gil-Serrano et al., 1994; Russa et al., 1995; Que et al., 2000; Choma & Sowinski, 2004). Among VLCFAs, 27-octacosanoic acid is present in lipid A of all members of Rhizobiales, except for Azorhizobium caulinodans (Bhat et al., 1991a, Bhat et al., 1991b; Choma et al., 2012). Moreover, in bradyrhizobial LPSs, a number of VLCFAs were identified, including straight-, mono-, and dimethyl branched-chain fatty acids containing 26 up to 34 carbons (Choma & Komaniecka, 2011). In this
group of bacteria, primary fatty acids are substituted by at least two or even three VLCFAs. Additionally, in some bradyrhizobial strains, 3-hydroxybutyric acid can be linked to the (ω-1) hydroxyl of the VLCFAs. Also, hopanoid compounds seem to be inseparable elements of this class of lipids A (Komaniecka et al., 2010; Komaniecka et al., 2014; Silipo et al., 2014).

It has been hypothesized that the presence of VLCFAs in rhizobia, and especially the presence of hopanoids in Bradyrhizobium, lipids A are necessary for maintaining the stability of the bacterial OM during the endocytotic invasion process and also for survival of the bacteria within the symbiosomes (Silipo et al., 2014). The alkyl chains of these fatty acids are long enough to span the entire OM bilayer and if additionally they are covalently linked to the hopanoid residue, they cause an increase in the membrane stability and rigidity (De Castro et al., 2008; Carlson et al., 2010; Silipo et al., 2014).

The outer membrane plays a critical role in the interaction of the bacteria with the environment and in establishment of a symbiotic relationship with leguminous plants. Therefore, we decided to compare Bradyrhizobium and Rhizobium OMs examining their permeability using a fluorescent hydrophobic probe, N-phenyl-1-naphthylamine (NPN), and the OMs resistance to EDTA and polymyxin B.

N-phenyl-naphthylamine is a frequently used probe to study the structure and function of biological membranes (Loh et al., 1984). This probe is a hydrophobic fluorescent compound, which fluoresces weakly in aqueous solutions but strongly when transferred into a hydrophobic environment. Such conditions can be found within bacterial membranes. Polymyxin B is a mixture of nonribosomally synthesized basic lipopeptides produced by *Bacillus polymyxa*. These molecules contain a heptapeptide ring with a tripeptide tail to which different short fatty acids are attached through an amide bond. Polymyxin B has antibiotic properties and is active against Gram-negative bacteria. This selective activity is related to its high affinity for lipopolysaccharides, especially for the lipid A-inner core part (Orwa et al., 2001; Zavaski et al., 2007; Mares et al., 2009). The importance of electrostatic interactions between LPS acidic groups and six positively charged 2,4-diaminobutyric acid (DAB) residues as well as hydrophobic interactions of polymyxin B (acyl chain, Phe and Leu) with fatty acids from lipid A are emphasized in descriptions of LPS/polymyxin complexes (Mares et al., 2009). Given these properties, this antibiotic is a useful tool to characterize the integrity of Gram-negative bacteria outer membranes.

In our comparative study, we used six rhizobial strains belonging to five genera within the Rhizobiales order. The selected strains produced lipopolysaccharides with very different structures. Because the entire LPS structure can affect membrane properties, we used the rough and smooth type of *E. coli* as the reference strains.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. This table also includes abbreviations of bacterial names used to simplify the text. To provide the optimal growth conditions for bacterial cultures, rhizobial strains were grown in tryptone-yeast (TY) medium (tryptone (5 g), yeast extract (3 g), CaCl₂ (0.1 g) per liter of H₂O). Vincent's minimal medium (79CA) (Vincent, 1970), and modified Vincent's medium called LPC (Dr. T. Stepkowski, Warsaw Agriculture University, personal communication), respectively (see Table 1). The LPC medium consisted of mannitol (1.0 g), sodium succinate (1.0 g), yeast extract (0.4 g), MgSO₄ (0.2 g), KH₂PO₄ (0.1 g), CaCl₂×6H₂O (0.04 g), and NaCl (0.05 g) per liter of

### Table 1. Characterization of bacterial strains used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain</th>
<th>Host plant and geographic origin</th>
<th>Description</th>
<th>Source* and references</th>
<th>Abbreviation used in the text</th>
<th>Culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Escherichia coli</em> ATCC 25922 (DSM 1103, FDA strain Seattle 1946) Strain O6, Biotype 1</td>
<td>not applicable</td>
<td>Smooth type of <em>E. coli</em></td>
<td>ATCC, ZGM</td>
<td><em>Escherichia coli</em> ATCC</td>
<td>LB</td>
</tr>
<tr>
<td>2.</td>
<td><em>Escherichia coli</em> HB 101</td>
<td>not applicable</td>
<td>Rough variant of laboratory strain K12</td>
<td>laboratory strain ZGM</td>
<td><em>Escherichia coli</em> HB 101</td>
<td>LB</td>
</tr>
<tr>
<td>3.</td>
<td><em>Ochrobactrum lupini</em> LUP 21*</td>
<td><em>Lupinus albus</em>, Spain</td>
<td>Known structure of O-chain (Pac et al., 2015)</td>
<td>Spain (Trujillo et al., 2008)</td>
<td><em>Ochrobactrum lupini</em></td>
<td>LPC</td>
</tr>
<tr>
<td>7.</td>
<td><em>Bradyrhizobium japonicum</em> USDA 110 (Bradyrhizobium diaeocœnsis USDA 110)</td>
<td>Glycine max, USA</td>
<td>Known structure of lipid A (Komaniecka et al., 2014)</td>
<td>USDA (Jordan, 1982; Delamuta et al., 2013)</td>
<td><em>Bradyrhizobium japonicum</em></td>
<td>LPC</td>
</tr>
<tr>
<td>8.</td>
<td><em>Bradyrhizobium elkanii</em> USDA 76</td>
<td>Glycine max, USA</td>
<td>Known structure of lipid A (Komaniecka et al., 2010)</td>
<td>USDA (Kundall et al., 1992)</td>
<td><em>Bradyrhizobium elkanii</em></td>
<td>LPC</td>
</tr>
</tbody>
</table>

*ATCC, American Type Culture Collection; ZGM, Department of Genetics and Microbiology, Maria Curie-Skłodowska University in Lublin, Poland; IFO, Institute for Fermentation, Osaka, Japan; USDA – United States Department of Agriculture, Beltsville, Md.
Table 2. The influence of increasing concentrations of polymyxin B on the viability of bacterial strains (crude experimental data). All experiments were done in triplicate and are shown as a mean ± S.D.

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<td>0</td>
<td>880.0±19.1</td>
<td></td>
<td>1606.0±39.0</td>
<td>391.0±15.1</td>
<td>767.5±12.0</td>
<td>413.7±9.0</td>
<td>847.6±6.1</td>
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<td>5</td>
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<td>185.0±8.0</td>
<td>256.0±11.8</td>
<td>654.3±48.0</td>
<td>404.3±7.6</td>
<td>88.5±2.1</td>
<td>312.3±8.1</td>
<td>1480.7±15.5</td>
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<td>157.7±5.7</td>
<td>563.0±28.5</td>
<td>363.3±16.9</td>
<td>78.0±14.0</td>
<td>305.5±3.5</td>
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<td>25</td>
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<td>9.7±2.1</td>
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<td>319.0±9.0</td>
<td>79.3±11.9</td>
<td>285.7±10.1</td>
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<td>35.7±5.9</td>
<td>2.3±1.2</td>
<td>103.7±17.2</td>
<td>255.0±10.6</td>
<td>69.7±16.6</td>
<td>215.3±19.9</td>
<td>1550.7±98.1</td>
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<td>13.7±3.1</td>
<td>1.3±0.6</td>
<td>16.3±5.7</td>
<td>205.0±15.0</td>
<td>77.0±5.2</td>
<td>209.0±25.5</td>
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<td>0.0±0.0</td>
<td>3.3±1.5</td>
<td>165.3±7.2</td>
<td>62.3±3.1</td>
<td>208.3±15.5</td>
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<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>51.3±3.8</td>
<td>55.3±18.0</td>
<td>213.5±10.6</td>
<td>1578.7±96.8</td>
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<td>31.0±2.6</td>
<td>201.7±3.5</td>
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<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>16.0±1.7</td>
<td>153.3±1.2</td>
<td>1586.7±60.2</td>
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<tr>
<td>1000</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>3.7±2.1</td>
<td>126.7±8.1</td>
<td>1523.7±116.7</td>
</tr>
</tbody>
</table>

H₂O (pH 7.0–7.2). Rhizobia were cultivated at 28°C for 48 h. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook 1989) at 37°C for 18 h.

**SDS-PAGE.** Ticine SDS-PAGE (using 12.5% acrylamide) of lipopolysaccharide samples obtained from whole-cell lyases digested by Proteinase K was performed as described (Hitchcock & Brown, 1983; Lesse et al., 1990). Before analysis, bacterial cells were extensively washed with 0.9% saline to remove the surface capsular material. The gel was silver-stained (Tsai & Frash, 1982).

**Fluorimetry.** Exponentially growing cells were harvested and resuspended in 1 mM KCl–10 mM HEPES (pH 7.2) at an optical density (at 600 nm) of 0.50 and transferred immediately to 1-cm-diameter quartz cuvettes with four optically clear sides. After 50 seconds, N-phenyl-1-naphthylamine (Sigma, cat. no. 104043; NPN, 500 mM in acetone) was added to a final concentration of 10 mM. Polymyxin B (Sigma, cat. no. P1004) and EDTA (Sigma, cat. no. E5134) were added to the cuvettes either before or after NPN addition, at the following final concentrations: EDTA – 5 mM, polymyxin B – 100 U/ml, as described previously in Martínez de Tejada & co-workers (1995). Fluorescence was monitored at 25°C with a FluoroMax-2 spectrofluorometer (Instruments S.A., Inc., JOBIN YVON/SPEX Division, USA) set as follows: excitation 350 nm; emission 420 nm; slit width 5 nm. The results were expressed in RFU (relative fluorescence units).

**Sensitivity to polymyxin B assay.** Bacterial sensitivity was measured as the effect of increasing concentrations of polymyxin B on cell viability. The assay described by Riley & Robertson (1984) was used with some modifications (Martínez de Tejada et al., 1995). One mg of Polymyxin B is equivalent of 6000 units. The stock solution of polymyxin B (2000 U/ml) was prepared in sterile 10 mM PBS (pH 7.0), and serial dilutions were made directly in sterile 96-well tissue culture plates using the same buffer as a diluent (100 µl) (for final concentrations see Table 2). Bacteria were resuspended in the same buffer solution at approximately 4×10⁴ CFU/ml, 100 µl of this suspension was dispensed in duplicated series of wells (4×10⁴ CFU per well in 200 µl), and the plates were incubated for 1 h at 28°C or 37°C, depending on the bacteria cultivated. Viable counts were performed by spreading 100 µl from each well on LPC or LB agar plates, and the results were expressed as the percentage of surviving cells with respect to controls incubated without the antibiotics. Raw experimental data are collected in Table 2 as an average value from three independent experiments and are plotted in Fig. 4. The plots in Fig. 4 were smoothed using polynomial functions from Excel 2013 trend options.

**Transmission electron microscopy analysis of bacterial cells.** Bacterial cells were prepared for TEM microscopy using modified procedures described by Martínez de Tejada & co-workers (1995), Velasco & co-workers (2000), and Palusińska-Szysz & co-workers (2012). Briefly, after a 20 min. incubation in the presence of polymyxin B (20 µg/ml) at 28°C, the bacteri suspensions were centrifuged at 8000×g for 10 min. The resulting bacterial pellets were used for microscopic analyses. The pelleted bacterial cells exposed to polymyxin B, as well as the control samples, were fixed with PBS buffer (Sigma, cat. no. P4417; pH 7.4) containing 2.5% glutaraldehyde (GA). Prior to fixation, each sample was centrifuged at 1500×g for 10 min in 4°C. Bacterial pellets were washed with fresh fixative, fixed for 2 h at 4°C in PBS buffered GA (2.5%), and centrifuged (5000×g for 8 min, at 4°C). Next, the specimens were rinsed twice with PBS and post-fixed in a 1% osmium tetroxide solution.
in PBS (1.5 h, at 4°C). After another wash with PBS, the bacteria were stained en bloc in 0.5% uranyl acetate (30 min), dehydrated in a series of alcohol and acetone solutions, and embedded in the LR White resin. Ultrathin sections were cut with a diamond knife using an RMC MT-XL microtome (Boeckeler Instruments, Tucson, AZ, USA), collected on copper grids, and contrasted using uranyl acetate and Reynold’s liquid. The samples were observed under a LEO-Zeiss 912 AB electron microscope (Carl Zeiss Microscopy, Oberkochen, Germany).

RESULTS

Lipopolysaccharides, released from bacterial cells using a micro-method, were compared by SDS-PAGE (Fig. 1). Based on this analysis, the types of LPS (rough – R, semi smooth – S/R, or smooth – S) were assessed. A classical ladder-like pattern of LPS mass distribution was observed for the \textit{S. enterica} preparation. In this case, the ladder was stretched from the top to the bottom of the gel and the most abundant region was located in the upper part of the gel. Rhizobium LPS samples possessed more complex patterns. There were many irregu-

Figure 1. Silver-stained tricine SDS-PAGE gel of lipopolysaccharides from: lane 1, \textit{Rhizobium leguminosarum} bv. Trifolii TA1; lane 2, \textit{Escherichia coli} HB 101; lane 3, \textit{Escherichia coli} ATCC 25922; lane 4, \textit{Mesorhizobium huakuii} IFO15243\textsuperscript{3}; lane 5, \textit{Ochrobactrum lupini} LUP 21\textsuperscript{1}; lane 6, \textit{Bradyrhizobium japonicum} USDA 110; lane 7, \textit{Sinorhizobium mellotii} 1021; lane 8, \textit{Bradyrhizobium elkanii} USDA 76; lane 9, \textit{Salmonella enterica} sv. Typhimurium (Sigma, cat. no. L6511). The side panel shows approximate ranges of rough (R), semi smooth (S/R), and smooth (S) LPS forms.

Figure 2. Effects of polymyxin B and EDTA on the incorporation of NPN into the outer membranes of \textit{Sinorhizobium mellotii} 1021 (A), \textit{Mesorhizobium huakuii} IFO15243\textsuperscript{3} (B), \textit{Rhizobium leguminosarum} bv. Trifolii TA1 (C), \textit{Ochrobactrum lupini} LUP 21\textsuperscript{1} (D), \textit{Bradyrhizobium japonicum} USDA 110 (E), \textit{Bradyrhizobium elkanii} USDA 76 (F), \textit{Escherichia coli} ATCC 25922 (G), and \textit{Escherichia coli} HB 101 (H). Green line – incorporation of NPN in the presence of polymyxin B, red line – incorporation of NPN in the presence of EDTA, blue line - incorporation of NPN in the absence of membrane-deteriorating agents.
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larities in these profiles. High quantities of the low molecular mass material (a very intense staining) equivalent of high quantities of the rough fraction of lipopolysaccharides were observed in preparations from *R. leguminosarum*, *M. huakuii*, *O. lupini*, and *S. meliloti*, as well as *E. coli* HB101 and *E. coli* ATCC. Moderate intensity of the lipid A-core fraction was noticed for *B. elkanii* and *B. japonicum* strains. The smooth fractions of LPS located in the upper half of the PAGE gel were observed in the case of *B. elkanii*, *S. meliloti*, *O. lupini*, *M. huakuii*, and *E. coli* ATCC. Lipopolysaccharides from *R. leguminosarum*, *S. meliloti*, *M. huakuii*, and *E. coli* ATCC possessed intense stained bands in the middle part of the gel. These intermediate fractions represent incomplete LPS (S/R-LPS) with one or only few repeating units attached and are common for rhizobia (Carlson, 1984; Pac et al., 2015).

In the analysis of the SDS-PAGE gel, we took into consideration the fact that the intensity of silver staining of a separated material strongly depends on the susceptibility of its structure to oxidation with sodium periodate (a reagent opening saccharide rings between vicinal diols and leaving two aldehyde groups).

All bacterial strains were tested in terms of their capacity for NPN incorporation into the cell membranes under different experimental conditions (Fig. 2). Generally, NPN readily partitioned into cell envelopes of all tested bacteria, reaching an equilibrium at 50 seconds after mixing this agent with the microorganism suspension. *S. meliloti* and *R. leguminosarum* cells were the only exceptions. In the case of *S. meliloti*, a plateau was observed after 150 seconds and, when *R. leguminosarum* cells were analyzed, the fluorescence grew exponentially along the entire observation time. Presumably, the spatial structures of the O-polysaccharides are responsible for these effects. However, in both cases, partition equilibrium was reached considerably faster when EDTA or polymyxin B was added (Fig. 2A, C). Both *E. coli* strains, *B. japonicum*, *B. elkanii*, and *O. lupini* hardly took up any NPN, giving a low level of emission at 450 nm (~200 RFU). High fluorescence (~1000 RFU) was observed when *S. meliloti*, *M. huakuii*, or *R. leguminosarum* cells were treated with the NPN-containing solution. EDTA and polymyxin B substantially facilitated transfer of NPN molecules to the hydrophobic environment of membranes and caused an increase in the fluorescence of *B. japonicum*, *O. lupini*, and *E. coli* ATCC suspensions, when compared with the untreated bacteria (Fig. 2D, E, G). The interaction of the membranes (OM and CM) with both deteriorating agents resulted in elevation of the fluorescence intensity (Fig. 2D, E, G). However, *B. japonicum* OM was more susceptible to the EDTA action (Fig. 2E), contrary to *E. coli ATCC* OM (Fig. 2G), which was more sensitive to polymyxin B. Only the *O. lupini* envelope was found to be susceptible to both agents in the same manner (Fig. 2D). The permeability of the cell membranes from *S. meliloti*, *M. huakuii*, *B. elkanii*, and *E. coli* HB101 measured by the fluorescence effect was independent of the presence of EDTA or polymyxin B (Fig. 2A, B, F, H). Moreover, in a longer period of observation (about 150 seconds), the same conclusion was true for *R. leguminosarum* (Fig. 2C).
The joint action of EDTA and polymyxin B was observed in experiments where, after addition of the NPN to bacterial suspension, the reaction mixture (in a cuvette) was supplemented with EDTA and then with polymyxin B at 150 and 300 seconds, respectively, in the course of an experiment (Fig. 3). The orange colored line shows the course of the experiment when *E. coli* ATCC cells were used. Addition of EDTA to the suspension elevated fluorescence (in about 10 seconds). Subsequent addition of polymyxin B caused further fluorescence intensification. The same shape of experimental plots, but not as spectacular, was observed when *O. lupini* and *B. japonicum* cells were studied (data not shown).

In a reverse experiment, the increase of fluorescence was observed only after addition of polymyxin B (data not shown). This indicates that polymyxin B can fully destroy the permeability barrier of the outer membrane on its own and further EDTA addition is unnecessary.

The permeability of *B. elkanii*, *M. huakuii*, and *E. coli* HB101 cell membranes to NPN was independent of EDTA and polymyxin B presence. The experiment with the *B. elkanii* strain shows only a weak effect of dilution of the cell suspension by the added agents, manifested by a slight reduction in fluorescence intensity (Fig. 3, green plot). As mentioned above, the cells of *R. leguminosarum* and *S. meliloti* incorporated NPN slowly and that could be the reason why the action of EDTA together with polymyxin B was merely visible (data not shown).

The influence of the increasing concentrations of polymyxin B (from 0 to 1000 U/ml) on the viability of the eight investigated strains is illustrated in Fig. 4 and Table 2. This assay indicated that *B. japonicum* was completely resistant to the antibiotic within the range of the concentrations used. Also, *B. elkanii* exhibited elevated tolerance to polymyxin B. About 40% of *B. elkanii* cells survived a one-hour incubation in the most concentrated (1000 U/ml) solution of the antibiotic. *O. lupini* can be classified as polymyxin B-resistant bacteria, but the concentration of 1000 U/ml was bactericidal to them. The effect of polymyxin B on the viability of *R. leguminosarum*, *S. meliloti*, and *M. huakuii* is illustrated in Fig. 4 by the sigmoidal-shape curves. *E. coli* strains were the most sensitive to this agent. Moreover, *E. coli* HB 101, the R-type strain, was found to be the least resistant strain among the investigated bacteria. The estimated bactericidal concentration of the lipopeptide to the HB 101 strain was lower than 5 U/ml (Table 2).

To demonstrate how polymyxin B affects the rhizobial cells, transmission electron microscopy was used. All cells had a typical size of about 1.2±1.6 µm in length.
and 0.4±0.6 µm in diameter. The TEM micrographs of control cells (E. coli HB101 – Fig. 5A and E. coli ATCC – Fig. 5C), not treated with the antibiotic, revealed bacterial envelopes surrounded by well discernible outer and cytoplasmic membranes. The OM was always slightly waved. The cytosol of these bacteria was intensely electron-dense with local brighter areas of intracellular DNA concentration. E. coli cells treated with polymyxin B changed dramatically with respect to their wall structure. The peptide antibiotic induced extensive blebbing in the rough- and smooth-type E. coli outer membranes (Fig. 5B and Fig. 5D). This process is well documented in Fig. 5D, where impressive blebbing of E. coli ATCC is presented. This phenomenon was limited only to enterobacterial cells used in our experiment. None of the rhizobium bacteria reacted in a similar manner in response to the presence of polymyxin B in the growth medium. No distortion and/or loss of cell membranes were observed. Moreover, the rhizobial cells exposed to the peptide antibiotic were undistinguishable from the same bacteria not treated with polymyxin B (used as a control). For instance, in both TEM micrographs of M. huakuii, the membranes were found intact; however, electron-lucent zones were visible (Fig. 5K and Fig. 5L). Presumably, these places represent empty spaces where PHB granules were located and removed during the dehydration procedure. Some cells presented in Fig. 5 contained black spots (very electron-dense dots) probably representing phosphate granules. They were observed in a series of micrographs (Fig. 5F, 5G, 5H, 5I, 5J, 5L, 5N). Also, in these cases there was no relation between the polymyxin B treated cells and the presence of the listed specific morphological structures. Thus, we can conclude that the tested rhizobia were much more resistant to polymyxin B than the enterobacteria represented in this study by two E. coli strains.

**DISCUSSION**

The results of our experiments clearly indicate that the integrity of the rhizobium outer membranes is higher than those of enterobacterial cells. Moreover, rhizobia are covered with envelopes differing in their physico-chemical properties.

After treatment of the reference E. coli strains with polymyxin B, distinct signs of damage to the cell envelope were detected in the TEM micrographs. Among others, blisters and huge amounts of protruding bubbles were observed. Appearance of such symptoms of bacterial envelope destabilization by cationic peptides has been described previously (Schnider & Teuber, 1975; Martinez de Tejada et al., 1995; Velasco et al., 2000; Hartmann et al., 2010). For example, outer membrane blebbing was observed in the case of P. aeruginosa cells incubated in a medium containing EDTA, polymyxin B, gentamicin, or cationic peptides isolated from macrophages (Sawyer et al., 1988). It is postulated that positively charged polymyxin B molecules can displace Ca\(^{2+}\) and Mg\(^{2+}\) ions from their sites in the lipopolysaccharide layer and in this way destabilize the OM and then the OM, providing further stability to this structure. All strains with undamaged membranes observed in the TEM experiments were resistant to polymyxin B (Fig. 4). Similar results were obtained in the case of Brucella spp. (Martinez de Tejada et al., 1995). In contrast to Brucella, the simultaneously tested enterobacteria (represented therein by E. coli and Y. enterocolitica) were sensitive to polymyxin B. In our experiments, E. coli HB101 with the rough-type LPS was much more susceptible to polymyxin B than the E. coli ATCC containing complete LPS. This phenomenon was described earlier in the work of Martinez de Tejada & co-workers (1995). No similar correlations were observed in the case of rhizobia. On the contrary, B. elkanii which secreted a moderate amount of the S-form LPS was much more resistant to polymyxin B than M. huakuii, S. meliloti, and O. lupini bacteria producing a higher than B. elkanii and similar (among these bacteria) quantity of smooth LPS. We have shown that B. japonicum, producing smooth LPS, was fully resistant to the antibiotic within the range up to 1000 U/ml. This observation allows a conclusion that, although O-chains play a vital role in bacterial response to polymyxin B, the structure of lipid A as well as the negative net charge in its vicinity seems to be more important. All rhizobia produce completely chemically different types of lipids A from those produced by enterobacteria. Rhizobial lipids A differ in the content and quality of fatty acids and negatively charged groups decorating the core oligosaccharide and the lipid A sugar backbone (Carlson et al., 2010). In the case of rhizobia, intracellular symbionts, and intracellular pathogens, such as Brucella and Legionella, lipid A is highly hydrophobic, mainly because of the presence of the VLCFAs, which can span the OM, providing further stability to this structure. All rhizobia studied to date possess at least one residue of VLCFA within their lipid A and (with the evident exception of Azorhizobium) their negatively charged groups are usually weak acid sugars (i.e., uronic acids and 2-amino-2-deoxyglucuronate). Presumably, carboxylic groups weakly interact with γ-amino groups of DAB of polymyxin B. Moreover, it can be postulated that the lipophilic part of this antibiotic hardly intercalates into the rigid membrane stabilized by VLCFA or VLCFA connected with hopanoids (Slipo et al., 2014).

Based on the physico-chemical properties of bacterial OM discussed above and the known structures of their main components – lipids A, it is possible to divide bacteria into two groups. The first one comprises bacteria with an enterobacterial type of lipid A. Generally, they have OMs highly sensitive to deteriorating agents. The second group comprises bacteria that can synthetize lipid A modi-
fied with VLCFAs. This group includes all rhizobia (with the exception of *Agrobacterium*), as well as pathogens such as *Brucella*, *Ochrobactrum*, or *Legionella* species. This group contains a subgroup of bacteria that are extremely resistant to polycationic antibiotics (in our investigations represented by *B. elkanii* and *B. japonicum*). These bacteria are able to produce lipid A with a highly expanded hydrophobic part and weak anionic decorations.

It was clear from the NPN fluorescence tests that the surface of the rhizobial cells were being very moderately modified by membrane-destroying agents to allow uptake of this hydrophobic probe. However, exposure of *O. lupini* and *B. japonicum* bacteria to polymyxin B or EDTA caused a distinct elevation of NPN fluorescence; therefore, it can be assumed that in both cases displacement of divalent-cations from their binding sites on the outer leaflet did take place and resulted in loosening of the LPS-made hydrophobic barrier. The same conclusion seems to be ineligible regarding other rhizobia where the level of probe fluorescence was irrespectable of the membrane-destroying agents.

Another issue is the capacity of OM to absorb NPN. The double-lipid layer of OM of Gram-negative bacteria has an approximately similar volume (hydrophobic space), thus one can expect that the maximum uptake of NPN by bacteria should be comparable. This hypothesis was not proven true by the experiments presented here (see Fig. 2). The amount of incorporated NPN seems to be strain dependent, and in some cases can be modified by external agents (see also: Loh et al., 1984; Martínez de Tejada et al., 1995; Velasco et al., 2000).

Given all the experimental data, it can be postulated that the integrity of the rhizobium OM is considerably higher than that of the enterobacterial OM. Moreover, electrostatic interactions of divalent-cations with LPS molecules seem to be as important as the hydrophobic forces among the membrane-forming fatty acids. A particular role in maintaining stability is ascribed to VLCFAs and hopanoids, being independent components of OM as well as integral elements of bradyrhizobial lipids A.

Despite the above conclusions, it is necessary to emphasize that there is no direct and simple answer to the question why rhizobial cells differ in their sensitivity/resistance to polymyxin B and why their cell envelopes differ so much in response to a hydrophobic probe (NPN). The answer must cover many aspects of individual membrane components (OM proteins, lipid anchored polysaccharides, the presence of cations, and so on) as well as entire individual LPS structures. It should be highlighted that completely different OM structures and different resistance to deteriorating agents do not affect the abilities of rhizobia to penetrate legume tissues and colonize the developed nodules. However, the method used here is simple and effective in showing differences among bacterial OMs.

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