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Recognition of Individual Strains of Fast-Growing Rhizobia by Using Profiles of Membrane Proteins and Lipopolysaccharides

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Membrane protein and lipopolysaccharide profiles of *Rhizobium leguminosarum* (biovars viciae, trifolii, and phaseoli), *R. meliloti*, and *Agrobacterium tumefaciens* strains were analyzed and compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Differences in one or both profiles allowed us to distinguish all 18 *R. leguminosarum* strains tested in this study from each other.

Rhizobia are gram-negative bacteria which form nitrogen-fixing root nodules on plants of the family Leguminosae. Bacteria present in these nodules in the form of bacteroids are responsible for the fixation of atmospheric nitrogen (27).

One of the objectives of research on *Rhizobium*-legume symbiosis is the construction of improved strains by genetic engineering. Risk assessment in field tests requires the determination of the fate of the modified microorganism, for which methods are needed that allow it to be distinguished from other strains. Methods for identifying rhizobial strains include serological techniques (10), introduction of antibiotic resistance markers, and analysis of total cell protein profiles (15). All of these techniques have practical drawbacks. Antisera to rhizobial cells are cross-reactive with many strains within the same species, and preabsorption (22), purification of strain-specific antigens (4), or production of strain-specific monoclonal antibodies (28) is required. An antibiotic resistance marker may interfere with symbiotic functions (17) or be exchanged between strains (2). Comparison of outer membrane protein and/or lipopolysaccharide (LPS) profiles has been used for strain identification in other species (7, 20, 23), e.g., to follow their fate (8).

In the present study we have analyzed and compared the cell surface proteins and LPSs of various *Rhizobium leguminosarum* strains by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to assess the value of these profiles in the identification of single strains.

For comparison, we used *R. leguminosarum* biovar viciae 248 (14), RBL1 (26), PRE (18), TOM (18), and 128C53k (Nitragin Co., Milwaukee, Wis.) and RCC1012, RCC1016, RCC1044, RCC1055, and RCC1056 (all from Rothamsted Culture Collection, Harpenden, United Kingdom); biovar trifolii RBL5020 (11), ANU843 (24), and 162S33 (Nitragin) and 0403 (F. B. Dazzo, Michigan State University, East Lansing); and biovar phaseoli 1233 (12), 127K17, 127K80e, and 127K85 (all from Nitragin). We also used *R. meliloti* LPR2 (Rothamsted), 1021 (21), and 102F28, 102F34, 102F51, and 102SR103 (all from S. R. Long, Stanford University, Stanford, Calif.) and *Agrobacterium tumefaciens* LBA201 (11) and LBA4301 (16). Strains were grown in tryptone-yeast extract medium (1) to an A_{620} of 0.2 to 0.5.

Isolation of membrane fractions. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C, washed once in phosphate-buffered physiological saline (10 mM sodium dihydrogen phosphate-hydrogen phosphate and 0.9% sodium chloride [pH 7.4]), and suspended in 5 ml of 50 mM Tris hydrochloride (pH 8.5). All subsequent procedures were carried out at 0 to 4°C. The cells were disrupted by sonication in three to five bursts of 30 s each with 15-s intervals by using a Sonifier (Branson Sonic Power Co., Danbury, Conn.) with water-jacket cooling. The remaining undisrupted cells and large fragments were removed by centrifugation at 900 × *g* for 20 min. After the addition of 0.2 mg of lysozyme per ml of supernatant and incubation for 30 min at room temperature, 2 M KCl was added to a final concentration of 0.2 M and membranes were pelleted by centrifugation for 60 min at 12,000 × *g*. The membrane pellet was suspended in a small volume of 2 mM Tris hydrochloride (pH 7.8).

Analysis of polypeptide patterns. SDS-polyacrylamide gel electrophoresis was performed as described previously (19). Samples were prepared by mixing suspensions of cell envelopes with concentrated sample buffer (19). Samples of cell envelopes were supplemented with 20 mM EDTA. Proteins were separated on 11% polyacrylamide gels and stained with fast green FCF. All samples were routinely heated for 10 min at 95°C prior to electrophoresis. The membrane protein profiles of the listed strains are shown in Fig. 1. Prominent outer membrane protein bands of strain 248, which were identified previously (6), could easily be identified in the protein patterns of its unseparated cell envelopes (Fig. 1, lane 1). The prominent outer membrane proteins were divided into numbered groups (I to IV in Fig. 1) according to their reactions in Western blots (immunoblots) with polyclonal and monoclonal antibodies raised against antigens of strain 248 (R. A. de Maagd, R. de Rijk, I. H. M. Mulders, and B. J. J. Lugtenberg, submitted for publication).

Comparison of the membrane protein patterns of the 18 *R. leguminosarum* strains representing three biovars (Fig. 1, lanes 1 to 18) revealed a number of similarities as well as some differences. The protein bands of groups I to IV appeared to be present also in *R. leguminosarum* strains other than strain 248 in similar positions. Homology with the corresponding proteins of strain 248 was confirmed by their cross-reactions with the polyclonal and monoclonal antibodies raised against outer membrane antigens of strain 248 (data not shown).

The similarities in the membrane protein patterns of the *R. leguminosarum* strains of all three biovars and the immunological cross-reactions in Western blots revealed their close relationship. When strains were compared within each separate biovar (viciae, trifolii, and phaseoli), no apparent

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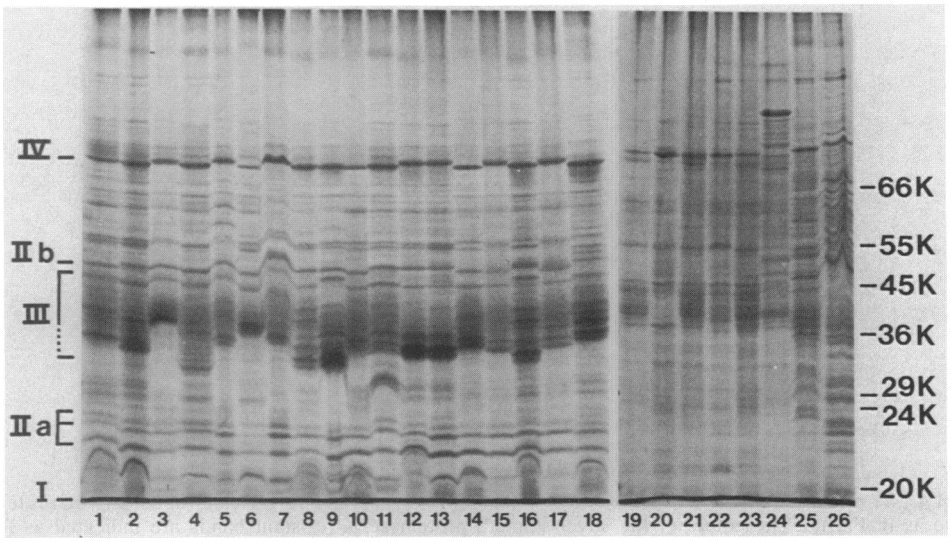

FIG. 1. SDS-polyacrylamide gel electrophoresis profiles of membrane proteins of the *Rhizobium* and *Agrobacterium* strains used in this study. Lanes 1 to 10 (lanes in parentheses) represent *R. leguminosarum* biovar viciae 248 (1), RBL1 (2), PRE (3), TOM (4), 128C53k (5), RCC1012 (6), RCC1016 (7), RCC1044 (8), RCC1055 (9), and RCC1056 (10). Lanes 11 to 14 represent *R. leguminosarum* biovar trifolii RBL5020 (11), ANU843 (12), 162S33 (13), and 0403 (14). Lanes 15 to 18 represent *R. leguminosarum* biovar phaseoli 1233 (15), 127K17 (16), 127K80e (17), and 127K85 (18). Lanes 19 to 23 represent *R. meliloti* LPR2 (19), 1021 (20), 102F28 (21), 102F34 (22), 102F51 (23), and 102SR103 (24). Lanes 25 and 26 represent *A. tumefaciens* LBA201 (25) and LBA4301 (26). Positions of molecular-weight (in thousands)-standard proteins are indicated on the right. Roman numerals I to IV on the left indicate the positions of the antigen groups recognized by a rabbit antiserum and monoclonal antibodies to outer membrane antigens of strain 248.

biovar-specific features of membrane protein patterns were observed. However, the membrane protein patterns could be used for strain identification. The profiles of group III proteins were particularly well suited to the recognition of individual strains of the species *R. leguminosarum*. Each of the 18 strains used in this study, with the exception of the four named below, could be distinguished from each other. In cases in which membrane protein profiles looked very much alike, such as for ANU843 and 162S33 (Fig. 1, lanes 12 and 13) or 1233 and 127K80e (Fig. 1, lanes 15 and 17), differences in LPS profiles allowed distinction (see below). Membrane protein profiles of *R. meliloti* (Fig. 1, lanes 19 to 24) and *A. tumefaciens* (Fig. 1, lanes 25 and 26) strains were clearly different from those of the *R. leguminosarum* strains. In these strains, the group II protein bands were absent and the profile of the group of proteins with electrophoretic mobilities approximately equal to those of the group III proteins had a different appearance. These differences were also reflected by the fact that these proteins cross-reacted only partially in immunoblots with the rabbit antiserum and monoclonal antibodies raised against outer membrane antigens of strain 248 (data not shown). These results confirm the already established distinctions between *R. leguminosarum* and *R. meliloti* as well as between *Rhizobium* and *Agrobacterium* spp. Moreover, our results provide an explanation for these differences at the molecular level.

Analysis of LPS profiles. To compare LPS profiles, we used the well-established, simple method of SDS-polyacrylamide gel electrophoresis of SDS-solubilized, proteinase K-digested cell envelope constituents. Electrophoresis samples of cell envelopes were heated for 10 min at 95°C, cooled to 60°C, incubated for 60 min at 60°C with 0.2 mg of proteinase K per ml, and diluted 15-fold with sample buffer without β-mercaptoethanol. After electrophoresis, LPS was visualized by silver staining (25). All gels contained 0.2% SDS. Comparison of such profiles with those of *Rhizobium* LPS isolated by others (3) and with those of LPS purified by hot phenol water extraction of cells of strains 248, RBL1, and RBL5020 by us (data not shown) confirmed that the profiles of the proteinase K-resistant cell envelope constituents represented LPS. The electrophoretic profiles of the LPSs of the analyzed strains are shown in Fig. 2. In similar profiles of *Escherichia coli* and *Salmonella typhimurium*, a high degree of heterogeneity in LPSs has been interpreted as a variation in the number of O-antigen chains substituted in a common core molecule (9). This heterogeneity occurred, although to only a limited degree, in a few of the *R. leguminosarum* strains analyzed here, i.e., strains PRE, TOM, 128C53, RCC1016, RCC1055, RBL5020, 1233, and 127K80e (Fig. 2, lanes 3, 4, 5, 7, 9, 11, 15, and 17). For these strains the general LPS structure may well be analogous to that in the other gram-negative bacteria mentioned in that the black-stained band with the highest mobility observed in all strains may represent the unsubstituted core LPS. In contrast to the above-mentioned *R. leguminosarum* strains, which showed heterogeneity in LPS chain length, other strains yielded, apart from the high-mobility band, only one orange-yellow-stained band, i.e., strains 248, RCC1044, ANU843, and 162S33 (Fig. 2, lanes 1, 8, 12, and 13). Other strains contained, in addition to one intense orange-yellow-stained band present in the above-mentioned strains, a number of black-stained bands of lower electrophoretic mobilities which did not show the regular spacing expected from LPSs with gradually increasing numbers of O-antigen subunits, i.e., strains RBL1, RCC1012, RCC1056, 0403, 127K17, and 127K85 (Fig. 2, lanes 2, 6, 10, 14, 16, and 18). The results of Carlson et al. (5) prompted us to investigate whether the observed multiple bands in our strains could be caused by an artifact. Carlson et al. (5) observed that *Rhizobium* ANU843 LPS yielded multiple bands upon electrophoresis in 0.1% SDS, whereas an increase in the SDS concentration to 0.5% resulted in only one band. These authors interpreted the multiple bands as various artificial aggregation forms of only one molecular LPS species. We

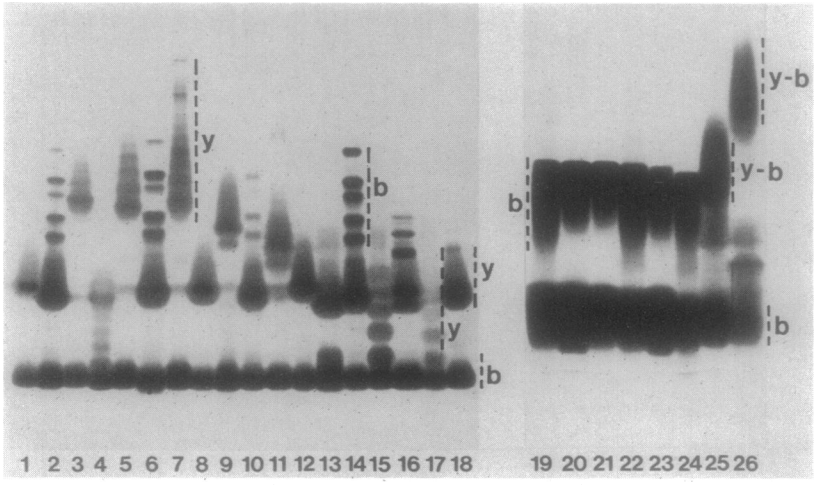

FIG. 2. Profiles of LPSs of the strains used in this study after SDS-polyacrylamide gel electrophoresis of proteinase K-treated cell envelopes. Lanes are as in Fig. 1. The colors of the silver-stained, proteinase K-resistant bands are indicated as follows: b, black; y, orange-yellow to brown; y-b, smears containing both orange-yellow and black regions.

conclude that such artifacts did not influence our results since (i) the LPS of strain ANU843 had only one intense orange-yellow-stained band and (ii) an increase in the SDS concentration from 0.2 to 0.5% did not cause any noticeable changes in LPS profiles.

No biovar specificity could be detected in the types of LPS profiles of the *R. leguminosarum* strains. Many different profiles occurred in a group of strains belonging to the same biovar, e.g., biovar viciae (Fig. 2, lanes 1 to 10). However, as with the membrane protein patterns, the differences in LPS profiles among strains made the analysis of LPS profiles a very useful tool for distinguishing between individual strains of the species *R. leguminosarum*. In examples of almost identical membrane protein profiles (strains ANU843 and 162S33 and strains 1233 and 127K80e [Fig. 1]), differences in LPS profiles allowed distinction (Fig. 2, lanes 12 and 13 and lanes 15 and 17). Moreover, in case of indistinguishable LPS profiles, as with strains 248, RCC1044, and ANU843 (Fig. 2, lanes 1, 8, and 12), differences in membrane protein profiles (Fig. 1) still allowed distinction between these strains. All other *R. leguminosarum* strains used in this study had LPS profiles that were clearly different from those of the other strains.

R. meliloti LPS profiles differed very little from strain to strain. They could easily be distinguished from those of any of the *R. leguminosarum* strains since they contained only one black-stained band of high mobility and a broad black-stained band of lower mobility (Fig. 2, lanes 19 to 24). It was therefore not possible to distinguish *R. meliloti* strains by their LPS profiles. The two *A. tumefaciens* strains had LPS profiles that were clearly distinct from those of both *Rhizobium* species. They contained, in addition to a common black-stained band of high mobility, a brown-black-stained smear consisting of closely spaced bands which were distinguishable by eye but very difficult to distinguish on a photograph (Fig. 2, lanes 25 and 26).

Major conclusions. In this study we have shown that the membrane protein profiles (Fig. 1) and LPS profiles (Fig. 2) of *R. leguminosarum* strains are easily distinguishable from those of *R. meliloti* and *A. tumefaciens* strains. These results are compatible with the already-known biochemical and morphological differences on which this species separation are based (13) and with serological differences (10). The membrane protein and LPS profiles of strains of all three biovars of *R. leguminosarum* showed a large number of similarities, and no biovar-specific features were observed. The similarities in electrophoretic profiles and cross-reactions with antibodies were consistent with the strong serological cross-reactions observed earlier between strains of different biovars (10). A major conclusion drawn from the present work is that the differences in electrophoretic profiles of the membrane proteins, particularly of the group III proteins, and of the LPSs of *R. leguminosarum* strains can be used for strain identification. These techniques, possibly in combination with other techniques or tools, can be used to distinguish strains from soil or nodule samples or to follow the fate of an introduced strain in field experiments for risk assessment.

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