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Outer Membrane Protein Changes During Bacteroid Development Are Independent of Nitrogen Fixation and Differ Between Indeterminate and Determinate Nodulating Host Plants of *Rhizobium leguminosarum*

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The outer membrane of bacteroids contains largely decreased levels of protein antigen groups II and III in comparison with that of free-living rhizobia (R. A. de Maagd, R. de Rijk, I. H. M. Mulders, and B. J. J. Lugtenberg, J. Bacteriol. 171:1136-1142, 1989). Since we intend to study the molecular basis of the development of bacterium to bacteroid, we wanted to know whether these outer membrane protein differences are conserved in various plant–*Rhizobium* combinations. For this purpose we developed a faster assay in which cell lysates instead of isolated cell envelopes were used to analyze these outer membrane changes. With this method the previously described low levels of antigen groups II and III in isolated bacteroid cell envelopes were confirmed. Moreover the described decrease in antigen groups II and III was also found in bacteroids of *Rhizobium leguminosarum* bv. *viciae* with a mutated *nifA* or *nifK* gene as well as in the non-fixing pea mutant FN1 inoculated with the wild-type strain 248. This indicates that the decrease in the antigen levels is not restricted to effective nodules. The results also showed that the decrease in antigen group II not only occurs in bacteroids from pea, but also in bacteroids from vetch, broadbean, white clover, and common bean. Antigen group III, however, remained present in bacteroids from common bean. It is concluded that the changes in antigen group II are not restricted to a specific cross-inoculation group but represent a general phenomenon in the rhizobial bacteroid differentiation process. Of the tested plants, the decrease in antigen group III was not found in bacteroids from common bean and appeared to be restricted to bacteroids from indeterminate nodules. Therefore one should expect that at least two molecular mechanisms are responsible for these outer membrane protein changes and that elucidation of these mechanisms will contribute to our understanding of bacteroid development.

Additional keyword: cell surface.

Bacteria of the genus *Rhizobium* can infect leguminous plants, thereby inducing the formation of root nodules. After entering the nodule tissue through infection threads, bacteria are released into the plant cells. Here they undergo a number of changes resulting in the endosymbiotic nitrogen-fixing form, the bacteroid. Compared to free-living bacteria, bacteroids are often enlarged, branched, or club-shaped, and they are, individually or in groups, enveloped by a plant cell-derived peribacteroid membrane (Robertson *et al.* 1978; Sprent *et al.* 1980). Bacteroid cell walls are weaker and more sensitive to the action of lysozyme and several detergents (van Brussel *et al.* 1977) and bacteroids do not appear to synthesize an acidic exopolysaccharide (Latchford *et al.* 1991). The content of LPS, particularly of the higher molecular weight form LPS I, is decreased in the isolated LPS fraction (Brewin *et al.* 1986) and the cell envelope fraction (de Maagd *et al.* 1989a). As was shown both by chemical analysis (Planqué *et al.* 1979; Bhat and Carlson 1992) as well as by Western blotting of LPS (Brewin *et al.* 1986; Tao *et al.* 1992), the composition of LPS changes during bacteroid differentiation. Sindhu *et al.* (1990) showed that the LPSs of bacteroids from peas and beans differ, even after infection with bacteria that have the same chromosomal background. Using antibodies, it was shown that the protein composition of the cell envelope also changes, with some protein antigens remaining present while others almost completely disappear from pea nodule bacteroids (de Maagd *et al.* 1989a). Recently it was suggested that the expression of changes in the LPS of bacteroids is caused by the distinct physiological environment inside the plant cell, as compared to the free-living situation. C4-Dicarboxylic acids as carbon source, low oxygen pressure, and low pH are factors that can induce the same changes in the LPS of cultured bacteria when compared with bacteroids (Kannenberg and Brewin 1989; Sindhu *et al.* 1990; Tao *et al.* 1992; Wood *et al.* 1989). As part of our program to investigate the regulation of bacteroid development, we

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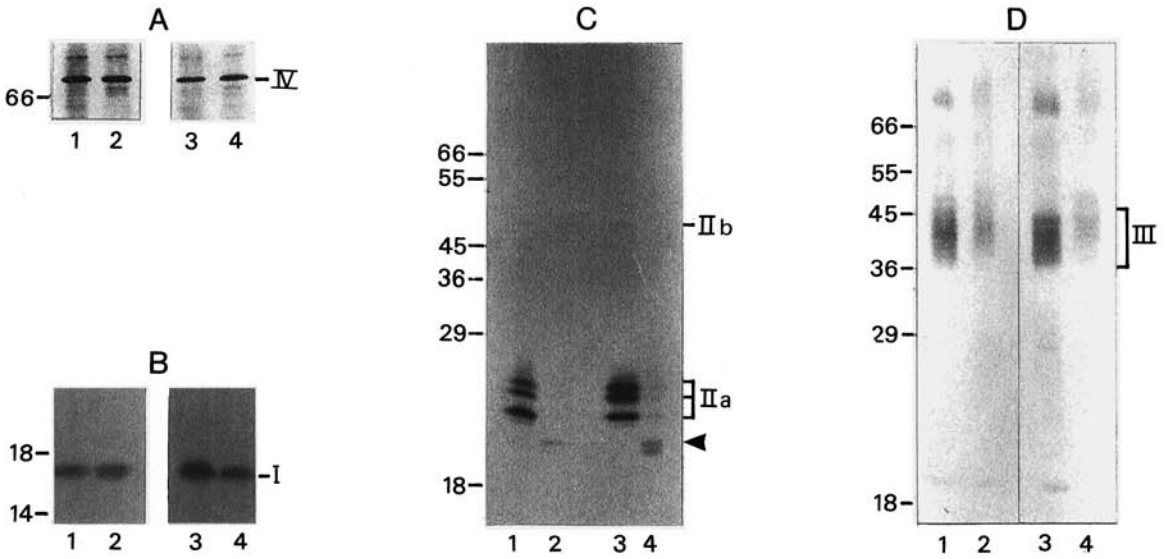

Fig. 1. Western blots (A–D) of cell envelopes (lanes 1 and 2) and cell lysates (lanes 3 and 4) of TY grown bacteria (lanes 1 and 3) and pea bacteroids (lanes 2 and 4) of *Rhizobium leguminosarum* bv. *viciae* strain 248. Western blots were incubated with rabbit antiserum raised against cell envelopes **(A)** and with monoclonal antibodies Mab40 **(B),** Mab8 **(C),** and Mab37 **(D).** The arrowhead points to the novel 19 kDa antigen detected in bacteroids by Mab8. For antigen groups IV and I (A and B, respectively) only the relevant part of the blot is shown.

focused on the changes in outer membrane proteins. Using antibodies against four of these proteins it was shown that two (antigen groups I and IV with M_r of 17 and 74 kDa, respectively) remained present in the bacteroid while the other two (antigen group II with M_r of 22, 24, 26, and 48 kDa and antigen group III with M_r ranging from 35 to 45 kDa) are present in strongly decreased amounts (de Maagd *et al.* 1989a). As part of a study on the regulation of these outer membrane protein changes during bacteroid development, we report here that these changes are common to all studied *Rhizobium*/host plant combinations that form indeterminate nodules. Analysis of bacteroids from common bean, which forms determinate nodules, show that the decrease in antigen group II is not coupled to a decrease in antigen group III. It is suggested that this may be due to a fundamental difference between determinate and indeterminate nodulating plants. Moreover, we present data indicating that active nitrogen fixation is not a prerequisite for the induction of the changes in the cell envelope of bacteria differentiating into bacteroids.

RESULTS

Detection of outer membrane protein antigens in unfractionated cell lysates.

The previously used method for studying bacteroid cell envelopes has two major disadvantages. Firstly, in contrast to pea nodules, many of the host plants for *Rhizobium* like vetch and clover yield only a small amount of bacteroid material per plant. Therefore, large numbers of plants are required to allow the isolation of sufficient, practical amounts of bacteroid cell envelopes. Secondly, the observed loss of antigens in bacteroid cell envelopes could be a result of preferential loss of these antigens during the bacteroid cell envelope isolation procedure. To establish whether detection of antigens in cell lysates can substitute for the use of isolated cell envelopes, we divided a bacteroid fraction of *R. l.* bv. *viciae* strain 248, isolated from pea nodules, in two parts. One part was used for cell envelope isolation and the other part was only sonicated and used without fractionation. The latter fraction is further referred to as cell lysate. Both preparations were also compared with cell envelopes and cell lysates from cultured bacteria. The effect of these methods on the levels of outer membrane protein antigens after differentiation from bacterium to bacteroid was compared. Figures 1A–D show the reaction of monoclonal and polyclonal antibodies recognizing the earlier defined antigen groups I–IV present in

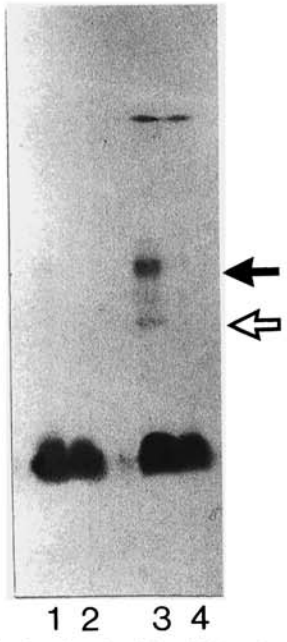

Fig. 2. Western blot incubated with rabbit antiserum raised against whole cells of *Rhizobium leguminosarum* bv. *viciae* strain 248. Lane 1, cell envelopes of TY grown bacteria; lane 2, cell envelopes of pea bacteroids; lane 3, cell lysate of TY grown bacteria; lane 4, cell lysate of bacteroids. Indicated are the major flagellar protein (closed arrow) and the RhiA protein (open arrow).

the cell envelope of free-living bacteria of *R. leguminosarum* bv. *viciae* strain 248. Two of these, groups II and III, have been shown to almost disappear from bacteroid cell envelopes, whereas the other two, groups I and IV, remain present (de Maagd *et al.* 1989a). Figure 1A shows the reaction of a polyclonal antiserum raised against cell envelope components (de Maagd *et al.* 1989a) with cell envelopes (lanes 1 and 2) and cell lysates (lanes 3 and 4) of

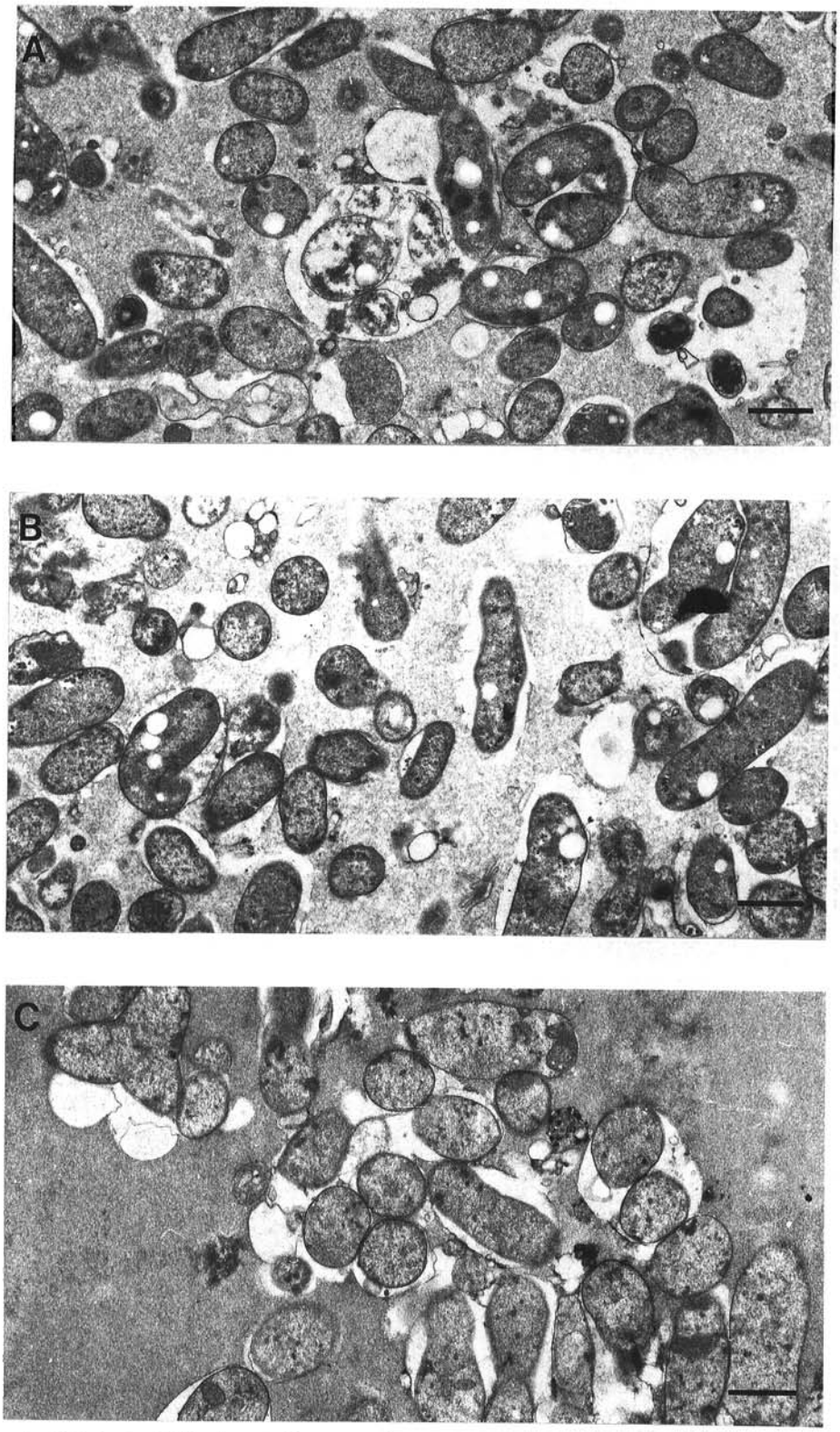

Fig. 3. Electron micrographs of the bacteroid fractions used to prepare lysates, isolated from pea root nodules 16 days **(A,B)** and 24 days **(C)** after inoculation with *Rhizobium leguminosarum* bv. *viciae* strains RBL1442 **(A;** *nifA*22::Tn*5*), RBL1433 **(B;** *nifK*13::Tn*5*) and 248 **(C).** Bar represents 1 μm.

bacteria (lanes 1 and 3) and bacteroids (lanes 2 and 4), respectively. This antiserum was used for the detection of antigen group IV. No difference in expression of this antigen between bacteria and bacteroids can be seen in Figure 1A, neither in isolated cell envelopes nor in cell lysates. As is shown in Figure 1B, no decrease in reaction of antigen group I with Mab40 was observed in bacteria (lanes 1 and 3) or bacteroids (lanes 2 and 4) in either cell envelopes (lanes 1 and 2) or in lysates (lanes 3 and 4). Figure 1C shows the reaction of antigen group II which is recognized by Mab8. This reaction was absent in bacteroid cell envelopes (Fig. 1C, lane 2) and severely decreased in bacteroid cell lysates (lane 4) as compared with the same fractions obtained from free-living bacteria (lanes 1 and 3, respectively). The reaction of Mab8 with cell envelopes and cell lysates of bacteroids (Fig. 1C, lanes 2 and 4, respectively) also revealed additional new bands. In bacteroid cell envelopes and cell lysates reaction with a protein of approximately 19 kDa was detectable. This epitope is in bacteroid cell lysates sometimes present as a double band. The decrease as described for antigen group II was also observed for antigen group III. Both the cell envelopes (Fig. 1D, lane 2) as well as the cell lysate (Fig. 1D, lane 4) of bacteroids showed a strongly decreased reaction in the region of 36–45 kDa with Mab37 when compared with cell envelopes and cell lysate of free-living bacteria (Fig. 1D, lanes 1 and 3, respectively). From these data it can be concluded that the use of cell lysates instead of cell envelopes results in detection of the same differences in the outer membrane protein antigens between bacteria and bacteroids. The observed loss of bacteroid outer membrane antigens belonging to antigen groups II and III is therefore not caused by the isolation procedure, but is due to changes in expression during symbiosis. These results enable us to use cell lysates instead of isolated cell envelopes to study plant/bacterium combinations which yield relatively small amounts of nodule material.

Bacteroid lysates do not contain RhiA protein and the flagellar subunit protein present in lysates of free-living bacteria.

The polyclonal antiserum raised against whole cells of *R. l.* bv. *viciae* strain 248 reacts with LPS (Fig. 2, lanes 1–4, lower band) and also with other antigens present in cell lysates. Two of these antigens have been characterized (Fig. 2). They are the main flagellar subunit protein of 35 kDa (closed arrow) and the 24 kDa cytosolic RhiA-protein (open arrow) (de Maagd *et al.* 1988b). These two antigens are absent in cell envelopes of bacteria and bacteroids as well as in bacteroid cell lysates (Fig. 2, lanes 1, 2, and 4, respectively) and are also not detectable in the peribacteroid space (data not shown). The absence of RhiA in bacteroids is in agreement with the results presented by Dibb *et al.* (1984). The absence of the major flagellar protein indicates that not only RhiA and antigen groups II and III are absent in bacteroids, but also that flagella are no longer present.

The observed changes in outer membrane antigens also occur in bacteroids of ineffective pea/*Rhizobium* combinations.

To investigate the possible role of nitrogen fixation in the decrease of expression of the above described antigen groups II and III during bacteroid development we used a non-fixing pea mutant as well as bacterial mutants. The bacterial mutants we used were strains RBL1442 and RBL1433 (*nifA*22::Tn*5* and *nifK*13::Tn*5*, respectively), both derivatives of *R. leguminosarum* bv. *viciae* strain 248. Strains containing mutated *nif* genes induce pale pink to green nodules due to a low level of leghemoglobin (Downie *et al.* 1983; Ma *et al.* 1982). A microscopic study of such nodules of 21 days or older revealed only uninfected plant cells and degenerated plant cells containing bacteria. These bacteria were probably released from degraded infection threads present in these cells (data not shown). To circumvent this problem of early senescence of the nodules, bacteroids were already isolated 16 days after infection with strains RBL1442 and RBL1433. An electron microscopic analysis of part of the fractions was carried out to check whether the fractions used for immunochemical studies indeed contain bacteroids. Figure 3A and B shows the bacteroid fractions isolated after infection with RBL1442 and RBL1433, respectively. Only very few bacteria are present and the bacteroids appear normally enlarged in morphology and electron density compared with bacteroids from pea plants 21 days after inoculation with wild type strain 248 (Fig. 3C). A small number of bacteroids still have a peribacteroid membrane as do bacteroids in the wild-type bacteroid fraction (Fig. 3C). The effects on bacteroid development by a non-fixing pea mutant, FN1, was investigated using bacteroids isolated from nodules 21 days after infection with the wild-type strain *R. l.* bv. *viciae* strain 248. The morphology of bacteroids isolated from nodules of FN1 resembles that of bacteroids isolated from effective pea nodules (Postma *et al.* 1990). Cell lysates of bacteroids of the non-fixing pea/*Rhizobium* combinations were compared with cell lysates of free-living bacteria and a cell lysate of wild-type nitrogen-fixing bacteroids. Silver-stained protein profiles of cell lysates of free-living bacteria (Fig. 4A, lanes 1 and 4) showed the same differences between free-living bacteria and bacteroids as mentioned previously. The detection of the outer membrane antigen groups I to IV is shown in Fig. 4B–E. In Western blots, both the reactions of antigen group IV with the polyclonal antiserum, as well as that of antigen group I with Mab40, were comparable between cell lysates of free-living bacteria (Fig. 4B and C, lanes 1 and 4), nitrogen fixing bacteroids (Fig. 4B and C, lane 6) and bacteroids from ineffective pea/*Rhizobium* combinations (Fig. 4B and C, lanes 2, 3, and 5). The reactions with the monoclonal antibodies recognizing antigen groups II and III (Mab8 and Mab37, respectively) are shown in Fig. 4D and E, respectively. Expression of antigen group II, recognized by Mab8, was again severely decreased in lysates of bacteroids from non-fixing nodules (Fig. 4D lanes 2, 3, and 5) and absent in the lysate of bacteroids isolated from normal, fixing nodules (Fig. 4D, lane 6). The 19-kDa bacteroid antigen was present in all three ineffective combinations, however, more prominent in bacteroids of strain RBL1433 than in bacteroids of the two other strains. Reaction of these cell lysates with Mab37, recognizing antigen group III, is shown in Figure 4E. The reaction of Mab37 was again severely decreased in all bacteroid lysates, both for bacteroids isolated from non-fixing nodules (Fig. 4E, lanes 2, 3, and 5) as well as for those of wild-type fixing nodules (Fig. 4E, lane 6) when compared with cell lysates of

free-living bacteria (Fig. 4E, lanes 1 and 4). Although the decrease is less prominent in bacteroids of RBL1433 than in bacteroids of the other two ineffective combinations. These results indicate that the decrease of antigen groups II and III during bacteroid development is independent of active nitrogen fixation.

Influence of the bacterial chromosomal background on the developmental regulation of outer membrane antigenic changes.

The influence of the chromosomal background of the bacteria on the expression of the antigens was studied in the following way. Cell lysate of bacteroids of *R. l.* bv. *viciae* strain 248 were compared with ANU845.pRL1JI, a cured. *R. l.* bv. *trifolii* ANU843 harboring pRL1JI, the Sym plasmid of strain 248. Both strains therefore nodulate pea and the strains differ only in the chromosomal background. As shown earlier (de Maagd *et al.* 1988a) free-living bacteria of strain ANU845. pRL1JI react with the monoclonal and polyclonal antibodies used in this study. Figure 5A and B shows that the antigen groups I and IV are still expressed in bacteroids of *R. l.* bv. *viciae* strain ANU845.pRL1JI (lane 7). Their expression in this strain is at a level comparable to that in bacteroids of the strain 248 (lane 2). The reaction with Mab8, the monoclonal antibody reacting with antigen group II, and with Mab37, reacting with group III, was severely decreased in the cell lysate of bacteroids of strain ANU845.pRL1JI (Fig. 5C and D, lane 7) as compared to cultured bacteria of this strain (lane 6). The appearance of an additional antigen, reacting with Mab8, a protein of 19 kDa, described previously, was also observed in bacteroids of strain ANU845.pRL1JI. From these results we conclude that bacteroids with a different chromosomal background are also severely decreased in antigen groups II and III.

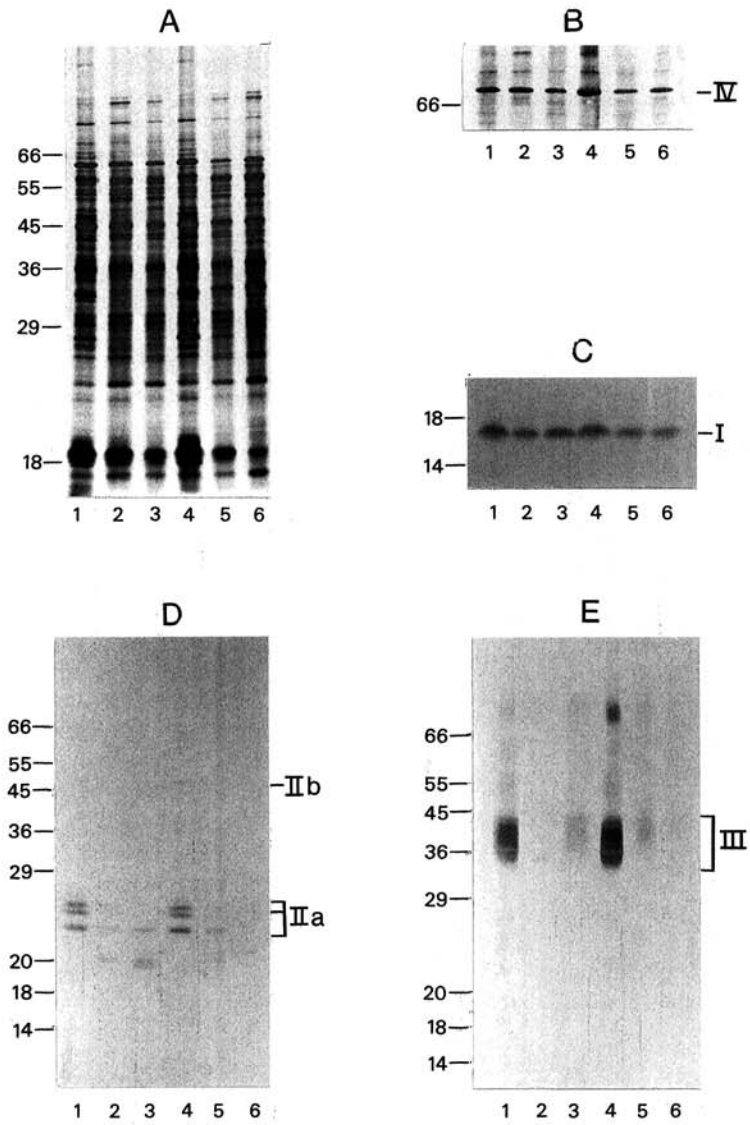

Fig. 4. Silver-stained gels **(A)** and Western blots **(B–E)** of cell lysates from TY grown bacteria (lanes 1, 4) and of bacteroids (lanes 2, 3, 5, 6). Reaction with antiserum raised against cell envelopes of strain 248 **(B)**, Mab40 **(C)**, Mab8 **(D)**, and Mab37 **(E)**. Lane 1, RBL1442 bacteria; lane 2, RBL1442 bacteroids; lane 3, RBL1433 bacteroids; lane 4, strain 248 bacteria; lane 5, strain 248 bacteroids isolated from non-fixing pea mutant FN1; lane 6, strain 248 bacteroids isolated from nitrogen-fixing wild type pea nodules.

Decrease of outer membrane proteins differs between indeterminate and determinate nodulating host plants.

To investigate whether the differences observed in pea-derived bacteroids also occur in bacteroids grown in other host plants, broadbean (*Vicia faba*) and common vetch (*Vicia sativa* ssp. *nigra*) were inoculated with *R. l.* bv. *viciae* strain 248. In addition, we also studied the bacteroids of white clover (*Trifolium repens*), a host plant from the clover cross-inoculation group and from common bean (*Phaseolus vulgaris*), a host plant of the bean cross-inoculation group. The latter group forms determinate-type nodules in contrast with the former host plants, all of which form indeterminate nodules. To use strains with identical chromosomal backgrounds we constructed two new strains. To obtain a strain that nodulates white clover, the Sym plasmid of *R. l.* bv. *trifolii* strain RCR5, pRtr5a, was introduced into the cured *R. l.* bv. *viciae* strain 248, resulting in strain RBL1233 (248^{c}pRtr5a). The strain that nodulates common bean was constructed in a similar way, introducing pSym9 of *R. l.* bv. *phaseoli* RCC3622 into the cured *R. l.* bv. *viciae* strain 248, resulting in strain RBL1577 (248^{c}pSym9). Figure 5A and B show antigen groups I and IV, respectively. Both appear to be present in pea bacteroids in equal amounts (Fig. 5A–B, lane 2) as compared with lysates of free-living bacteria of strain 248 (Fig. 5A–B, lane 1). These antigen groups are also present in equal amounts in bacteroids from the other four tested host plants, i.e., broadbean (lane 3), vetch (lane 4), clover (lane 5), and common bean (lane 9) as compared with the free-living form of the strains (lanes 1 and 8). The decrease in expression of the prominent outer membrane proteins of group II compared to the free-living *Rhizobium* bacteria was not only observed in bacteroids derived from pea nodules (Fig. 5C, lane 2) but also in bacteroids from other host plants, including white clover and common bean (Fig. 5C, lanes 2–5 and 9). The new antigen of group II, detected by Mab8 in bacteroids isolated from pea nodules, is indicated in Figure 5C with an arrowhead. This antigen is present in bacteroids of all tested indeterminate nodulating plants, but much less abundant in bacteroids derived from nodules of broadbean (Fig. 5C, lane 3). This antigen is absent in bacteroids isolated from nodules of common bean (Fig. 5C, lane 9). The decrease in antigen group III, as detected with Mab37, was observed in bacteroids isolated from pea, broadbean, vetch, and white clover (Fig. 5D, lanes 2–5) but not in common bean, the host plant of *R. l.* bv. *phaseoli* (Fig. 5D, lane 9). The bacteroid cell lysate of strain *R. l.* bv. *phaseoli* RBL1577 showed a slower mobility of the antigen group III in polyacrylamide gels as compared with the cell lysate of free-living RBL1577 bacteria (Fig. 5D, lanes 9 and 8, respectively).

The results presented here show that the strong decrease of antigen group II during bacteroid development of strain 248 occurs in different host plants of various cross-inoculation groups and is a general phenomenon in both determinate and indeterminate nodules. The decrease in antigen group III is only found in bacteroids derived from indeterminate nodules but not in bacteroids from common bean. These results indicate that the decrease in antigen group II is controlled by a different mechanism than the one responsible for the decrease in antigen group III, and that the latter control mechanism may only be present in indeterminate nodulating plants.

DISCUSSION

Use of cell lysates for detection of outer membrane antigens.

De Maagd *et al.* (1989a) described five antigen groups in the outer membrane of *R. l.* bv. *viciae* strain 248. Four of them are proteins, designated antigen groups I through IV, and the fifth is LPS I. After differentiation into bacteroids the antigen groups I and IV were still detectable at levels comparable to those in free-living bacteria and the levels of antigen groups II and III and LPS I were strongly decreased. By using a new method, in which cell lysates were used instead of isolated cell envelopes, the loss of antigen groups II and III was still observed (Fig. 1). In addition to the decrease of the antigen group II proteins, we also observed the appearance in bacteroids of a novel group II antigen. This antigen was detected with Mab8 (Fig. 1C, indicated with an arrowhead) and is located in the cell envelope (Fig. 1C, lane 2). The fact that it was not detected in previous studies is explained by a variation in the experimental procedure. Lysozyme treatment prior to cell envelope harvesting (De Maagd *et al.* 1988a) may cause the release of this putatively peptidoglycan-bound antigen as a soluble protein. Since in

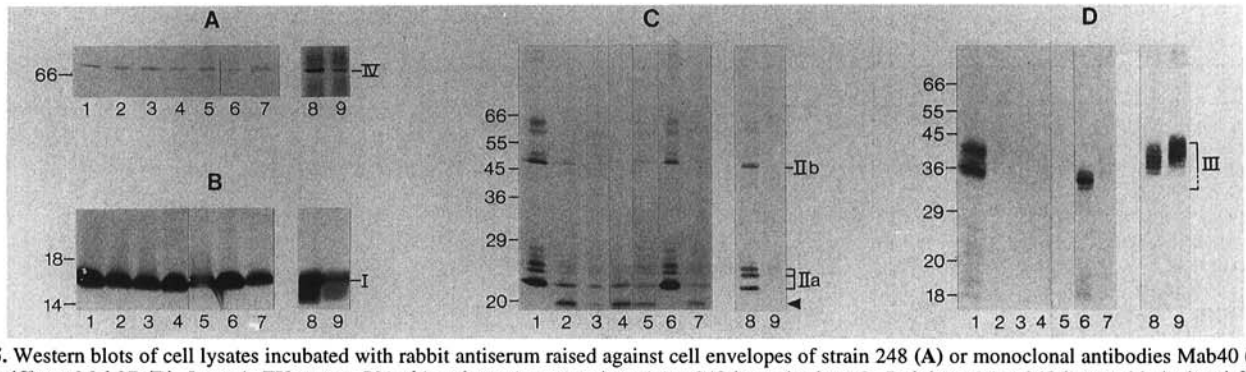

Fig. 5. Western blots of cell lysates incubated with rabbit antiserum raised against cell envelopes of strain 248 **(A)** or monoclonal antibodies Mab40 **(B)**, Mab8 **(C)**, or Mab37 **(D)**. Lane 1, TY grown *Rhizobium leguminosarum* bv. *viciae* 248 bacteria; lane 2, *R. l.* bv. *viciae* 248 bacteroids isolated from *Pisum sativum* nodules; lane 3, idem from *Vicia faba* nodules; lane 4, idem from *V. sativa* nodules; lane 5, *R. l.* bv. *trifolii* strain RBL1233 bacteroids isolated from *Trifolium repens* nodules; lane 6, TY grown *R. l.* bv. *viciae* ANU845.pRL1JI bacteria; lane 7, *R. l.* bv. *viciae* ANU845.pRL1JI bacteroids isolated from *Pisum sativum* nodules; lane 8, TY grown *R. l.* bv. *phaseoli* RBL1577 bacteria; lane 9, *R. l.* bv. *phaseoli* RBL1577 bacteroids isolated from *Phaseolus vulgaris* nodules. The arrowhead in Fig. 5C indicates the 19-kDa antigen recognized by Mab8. The results shown in lanes 8 and 9 are from another experiment.

the present study lysozyme treatment occurred after cell envelope harvesting, the antigen could be detected.

Also, the major flagellar protein was absent in bacteroids, whereas cell lysates of free-living bacteria do react with antibodies against this component. This indicates that bacteroids are devoid of flagella. From the observation that the major flagellar protein was also not detected in the peribacteroid space we conclude that the synthesis of flagella stops before the bacteria are released from the infection thread.

Ineffective plant/bacterium combinations.

So far the mentioned changes were only observed in effective pea/*Rhizobium* combinations. It is known that leguminous plants inoculated with compatible *Rhizobium* strains containing a mutation in one of their *nif* genes form ineffective nodules, but still allow bacteroid differentiation to some extent (Downie *et al.* 1983, Hirsch *et al.* 1983 and 1987, Ma *et al.* 1982). These reports also show that senescence of bacteroids from these ineffective strains starts earlier and that, depending on the stage of nodule development, the bacteroids differ from wild-type bacteroids in morphology. Isolating bacteroids from pea root nodules 16 days instead of 23 days after inoculation with either strain RBL1442 (*nifA*22::Tn5) or strain RBL1433 (*nifK*13::Tn5), resulted in apparently normal bacteroids (Fig. 3) and expression of nitrogenase component CII in the case of bacteroids of strain RBL1433 (data not shown). Bacteroids of strains RBL1442 and RBL1433 also showed the decrease in the antigen groups II and III. In the immunochemical studies, however, some variation in the decrease of these antigen groups II and III can be observed. This can either be caused by a reduction of the bacteroid/bacterium ratio isolated from the root nodules or it can indicate that there is a difference in regulation. Bacteroids isolated from root nodules of pea mutant FN1 after infection with wild-type strain 248 also showed the same decrease in antigen expression. We therefore conclude that the observed decreases in protein antigens of bacteroid outer membranes are not (group III) or at least not completely (group II) dependent on active nitrogen fixation, nor on the activity of the regulatory *nifA* gene.

Outer membrane changes are independent of the chromosomal background.

Because Mab8 and Mab37, recognizing antigen group II and III, respectively, also react with *R. leguminosarum* strains with different chromosomal backgrounds, their expression in bacteroids was examined using *R. l.* bv. *viciae* strain ANU845.pRL1JI. Maintaining the same Sym plasmid and host plant but using a chromosomal background different from strain 248 also has no influence on the decrease of reaction with antibodies against antigen groups II and III. In conclusion, our results indicate that a decrease in these outer membrane antigens during bacteroid development does not depend on the chromosomal background of the bacterium.

Decrease of antigen group III does not occur in common bean, a plant with determinate nodules.

Bacteroids in determinate nodules show some striking differences with bacteroids in indeterminate nodules, e.g., they are smaller and are not individually enveloped by a peribacteroid membrane but in groups (Sprent *et al.* 1980). Our results (Fig. 5) in addition show a difference at the outer membrane protein level. In contrast to bacteroids isolated from indeterminate nodulating plants like pea, broadbean, vetch, and clover, the antigen group III is still detectable in a cell lysate of bacteroids isolated from common bean, a determinate nodulating host plant. The multiple banding pattern of antigen group III in Western blots is caused by the attachment of murein residues from the peptidoglycan layer to the outer membrane protein after lysozyme treatment (de Maagd *et al.* 1989b). The shift in mobility between bacterial cell lysate and bacteroid cell lysate of strain RBL1577 (Fig. 5D, lanes 8 and 9, respectively) is consistent with the suggestion that the peptidoglycan layer in bacteroids differs in composition from the peptidoglycan layer in bacteria of the same strain (van Brussel *et al.* 1977). The antigen group II, however, is severely decreased in bacteroids of strain RBL1577, which contains the *R. l.* bv. *phaseoli* Sym plasmid pSym9. This indicates that decreases in antigen groups II and III are regulated by distinctive mechanisms.

Recently the cloning of a gene coding for an antigen of group III, *ropA*, was described (de Maagd *et al.* 1992), thereby allowing us to study its regulation at the transcriptional

Table 1. Strains and plasmids with their relevant characteristics

Strain	Characteristics	Source or reference
Rhizobium leguminosarum bv. *viciae*		
248	Wild-type bv. *viciae* containing pRL1JI	Josey *et al.* 1979
RBL1442	248, pRL1JI *nifA*22::Tn5	This study
RBL1433	248, pRL1JI *nifK*13::Tn5	This study
RBL1532	248 cured of pRL1JI, *rif*[a], *spc*[a]	de Maagd *et al.* 1988b
Rhizobium leguminosarum bv. *trifolii*		
RCR5	Wild-type bv. *trifolii* containing pRtr5a	Rothamsted Culture Collection, Harpenden, U.K.
RBL1233	RBL1532 containing pRtr5a::Tn*5*	This study
ANU843	Wild-type bv. *trifolii* containing pRtr843	Rolfe *et al.* 1980
ANU845	ANU843 cured of pRtr843, spec[a]	Rolfe *et al.* 1980
Rhizobium leguminosarum bv. *phaseoli*		
RCC3622	Wild-type bv. *phaseoli* containing pSym9	Rothamsted Culture Collection, Harpenden, U.K.
RBL1577	RBL1532 containing pSym9::Tn*5*	This study

[a] Antibiotic resistance: rif = rifampicin, spc = spectinomycin.

level. We are currently isolating *ropA* homologues from other *Rhizobium* strains as well as a gene coding for the protein of antigen group II. Upstream regions of these genes may identify regulatory sequences involved in their (down) regulation during bacteroid development.

MATERIALS AND METHODS

Strains and growth conditions.

Strains and plasmids used in this study are listed in Table 1. Bacteria were grown in liquid TY-medium (Beringer 1974), with antibiotics where appropriate, for 16 hr at 28° C on a rotary shaker. The antibiotics and concentrations used were (per milliliter): rifampicin (20 μg), spectinomycin (400 μg), streptomycin (500 μg), and tetracyclin (2 μg).

Growth of plants.

Plant seeds were obtained from Cebeco (Lelystad, The Netherlands), except for pea (*Pisum sativum* L.) mutant FN1 seeds which were a gift of W. J. Feenstra (State University of Groningen, The Netherlands) and common beans (*Phaseolus vulgaris*) which were purchased from the Leidsche Zaadhandel (Leiden, The Netherlands). All seeds were surface-sterilized by subsequent soakings in sulphuric acid and hypochlorite as described previously (van Brussel *et al.* 1982). White clover (*Trifolium repens*) and vetch (*Vicia sativa* ssp. *nigra*) seeds were pre-germinated on Jensen medium (Vincent 1970) with 1.0% agar and common bean between moist filter paper for 2 days at room temperature in sterile petri dishes. Seeds or seedlings were inoculated with the appropriate strains and kept in the dark for 3 days and subsequently grown at 20° C with a daily regime of 16 hr light and 8 hr darkness. Plants were grown in gravel and watered with nitrogen-free medium (Raggio and Raggio 1956), except for common beans which were grown in sterile vermiculite as described by Wacek and Brill (1976). Nodules were harvested 23 ± 2 days after inoculation. In experiments with wild-type pea plants inoculated with non-fixing bacterial mutants, bacteroids were isolated from nodules 16 days after infection.

Isolation of bacteroids.

Bacteroids with intact peribacteroid membranes (PBM) were isolated as described by Brewin *et al.* (1985). Peribacteroid membranes were removed by osmotic shock. For this purpose bacteroids were resuspended in isolation buffer without sucrose and polyvinylpolypyrrolidone (PVPP) and pelleted by centrifugation for 1 min at 10,000 *g*. Bacteroids were stored at –20° C when not used immediately.

Preparation of cell lysates.

For the preparation of lysates, bacteria grown as described previously, harvested by centrifugation for 8 min at 6000 *g*, were resuspended in 2 mM Tris.HCl, pH 7.8, and sonicated on ice with a Branson sonifier using a microtip (Branson Sonic Power Co., Danbury, CT) three to five times for 15 sec with intervals of 1 min. Subsequently, cell lysates were incubated with 1 mg of lysozyme per milliliter of lysate for 1 hr at 37° C. Bacteroids, isolated as described above, were treated in the same way to obtain bacteroid cell lysates.

Isolation of cell envelopes from cultured bacteria and bacteroids.

Cell envelopes were isolated as described previously (de Maagd *et al.* 1988a), except that cell envelope suspensions were treated with 1 mg of lysozyme per milliliter sample after the cell envelopes were pelleted.

Protein determination.

Protein concentrations of cell envelope suspensions and cell lysates were estimated as described by Markwell *et al.* (1978) using bovine serum albumin as a standard.

Preparation and characterization of polyclonal and monoclonal antibodies.

The characteristics of the monoclonal antibodies and the polyclonal antisera raised against cell envelopes have been described by de Maagd *et al.* (1989a). The characteristics of the polyclonal antisera against whole cells have been described by de Maagd *et al.* (1988b).

Gel electrophoresis and Western blotting.

Cell envelopes and lysates were treated with lysozyme (1 mg/ml sample) to release the covalently bound proteins from the peptidoglycan. Subsequently they were mixed with concentrated sample buffer (Lugtenberg *et al.* 1975), supplemented with 20 mM EDTA. Samples were routinely incubated for 15 min at 95° C prior to electrophoresis. After electrophoresis on 13% polyacrylamide gels as described previously (Lugtenberg *et al.* 1975), gels were either silver-stained (Blum *et al.* 1987) to visualize proteins or used for Western blotting. For immunodetection with polyclonal or monoclonal antibodies, cell constituents were transferred to nitrocellulose (Schleicher & Schull BA85, Dassel, Germany) by electroblotting. Protein antigens were detected as described earlier (de Maagd *et al.* 1988a) and visualized using nitrotetrazolium blue and 5-bromo-4-chloro-3-indolylphosphate as substrates (Ey and Ashman 1986).

Microscopy.

Bacteroid fractions were isolated as described above, resuspended in 5% (w/v) gelatin and spun down at 3,000 *g* for 5 min. The tip of the Eppendorf tube was cut off to remove the pellet which was then fixed for 16 hr in 2.5% (v/v) glutaraldehyde-2% (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), washed with cacodylate buffer, and post-fixed in 1% osmium tetroxide for 1 hr. After washing with doubly demineralized water, the pellet was dehydrated in an ethanol series first and subsequently in a propylene/Epon series and embedded in Epon (Serva, Heidelberg, Germany). Semithin and ultrathin sections were cut using a Reichert & Jung Ultracut-E microtome. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and viewed using a Philips 300 electron microscope operating at 60 kV.

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