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Modification of Polypeptide Patterns During Nodule Development in the *Frankia-Alnus* Symbiosis

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Abstract

The soluble proteins from nodules (actinorhizae), roots of *Alnus glutinosa* (L.) Gaertn., and free-living *Frankia alni* subsp. *pommerii* isolate ACN1 AG cultures were analysed in order to investigate if any actinorhiza-specific proteins were induced by the inoculation of clonal plantlets (AG2) by *Frankia*. Comparisons of protein patterns from actinorhizae and non-infected roots, obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), showed that three polypeptides of molecular weights 36, 50 and 61 kD could be detected in actinorhizae but not in roots. Immunoblotting techniques using heterologous antisera "directed" against members of the nitrogenase enzyme complex identified the 36 and 61 kD polypeptides as the Fe protein and MoFe protein of the *Frankia* nitrogenase. Analysis of the protein extracts by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) revealed five additional nodule-specific polypeptides in developing actinorhizae.

Keywords: Actinorhizal, actinorhizins, *Alnus*, *Frankia*, gel electrophoresis, nitrogen fixation, nodule, symbiosis

1. Introduction

Actinorhizal plants have the ability to form a root symbiosis with the actinomycete *Frankia*, a filamentous prokaryote (reviewed in Schwintzer and Tjepkema, 1990; Simonet et al., 1990). This actinorhizal symbiosis is found

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among at least 25 genera of dicotyledonous plants belonging to 8 different families (Baker and Schwintzer, 1990). Among the wide variety of the plant genera associated with *Frankia*, the genus *Alnus* has been very much documented in terms of physiological and biochemical studies. Consequently, we have chosen *Alnus* as a model species for the study of actinorhiza development at the molecular level.

The establishment of the actinorhizal symbiosis is a complex process involving many developmental steps. Both plant and prokaryote undergo biochemical, physiological and molecular changes during this interaction (reviewed in Berry and Sunell, 1990; Huss-Danell, 1990). It is likely that these modifications involve several biochemical compounds which are synthesized by either or both symbiotic partners. In actinorhizal plants, these compounds have been previously described as actinorhizins (Tremblay et al., 1986). In the case of *Rhizobium*-legume symbiosis, the compounds involved in the proper establishment and maintenance of the nodules are plant proteins known as nodulins (van Kammen, 1984). It is important to mention, by contrast to the *Rhizobium*-legume symbiosis where the nodulins are plant proteins, that the term actinorhizin is applied to bacterial as well as plant proteins. In fact, according to the present terminology an actinorhizin is not necessarily a gene product (protein) but might be a specific compound shown to be essential for nodule (actinorhiza) development. It is clear that further progress in the elucidation of *Frankia*-actinorhizal interactions will require a more elaborate terminology to designate the plant "symbiotic proteins" like the nodulins.

Much progress in the elucidation of the *Rhizobium*-legume symbiosis has been achieved through the application of well established techniques used in molecular biology (reviewed in Appelbaum, 1990; Govers et al., 1987; Verma et al., 1986). Hence, the *Rhizobium*-legume symbiosis has emerged as the better understood plant-microbe symbiosis. In spite of several differences that are bound to arise in an actinorhizal plant host, the *Rhizobium*-legume symbiosis can serve as an excellent model for the study of actinorhizal symbiosis which has not yet been investigated at the molecular level.

Although the physiology of the actinorhizal symbiosis has received much attention during the past 10 years (reviewed in Huss-Danell, 1990), little is known on the molecular aspects of the interactions between actinorhizal plants and *Frankia*. In this paper we have evaluated SDS-PAGE and 2D-PAGE methods to investigate the presence and origin of actinorhiza-specific proteins which are expressed during the development of the actinorhizal nodules. Total protein extracted from *Alnus glutinosa* roots, infected root systems, actinorhizae and free-living cultures of *Frankia alni* subsp. *pommerii* have been compared using SDS-PAGE and 2D-PAGE techniques. Immunoblotting techniques were

used to identify two polypeptides, detected in actinorhizae, as members of the nitrogenase enzyme complex.

2. Materials and Methods

Plant materials, bacterial strains and chemicals

For the inoculation of *A. glutinosa* plantlets, the *Frankia alni* subsp. *pommerii* isolate ACN1 AG (ULQ 0102001007) was grown at 30° C in standard Qmod B liquid medium (Lalonde and Calvert, 1979) containing glucose (10 g/L) and α-lecithin (5 mg/L). The same isolate was also grown in a culture medium containing propionate as the only carbon and energy sources (described by Meesters, 1987). Nitrogen-fixing *Frankia* cultures were obtained by using medium without NH_4Cl ("P–N") and non-nitrogen fixing cultures were obtained by using medium containing 0.2 g NH_4Cl/l ("P+N") as described by Meesters (1987). Vesicles were observed only in the nitrogen-fixing *Frankia* cultures.

Ultrapure grade Tris, sodium dodecyl sulfate (SDS), glycine, and acrylamide were obtained from Bethesda Research Lab (Gaithersburg, MD). Immobilon polyvinylidine difluoride (PVDF) transfer membranes were obtained from Milipore Ltd. (Bedford, MA). Carrier Ampholine, Coomassie brilliant blue R-250 and tetramethylethylediamine (TEMED) were purchased from Bio-Rad (Richmond, CA). Unless otherwise stated, all other organic chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture and inoculation of A. glutinosa *plantlets*

A. glutinosa plantlets (clone AG2) were micropropagated and nodulated as described in Périnet and Lalonde (1983). Rooting of the shoots occurred after the transfer of individual shoots to media containing indolebutyric acid (IBA) at 1 μM for 5 to 7 days. The plantlets were then transferred to growth pouches containing sterile N-free Crone's solution (Lalonde and Calvert, 1979). After 3 weeks in the growth pouch, the *A. glutinosa* plantlets were inoculated with a *Frankia* suspension previously washed and homogenized by repeated vigorous passage through a 18 G needle. After 2 weeks, the ability of the inoculated plants to fix nitrogen was measured by acetylene reduction, as described in Simon et al. (1985).

Protein extraction

Freshly harvested roots or actinorhizae were frozen in liquid nitrogen and ground to fine powder using a mortar and pestle. *Frankia* cells were collected

by centrifugation at 8,000 g for 15 min. The proteins were extracted using a modified procedure of the method described in Damerval et al. (1986). Approximately 2 g of tissue or 0.5 ml of packed *Frankia* cells were homogenized in 10 ml of a 10% (W/V) trichloroacetic acid (TCA) acetone solution containing 0.07% (V/V) β-mercaptoethanol. After standing at $-20°$ C for 1 hr, the pellet was recovered by centrifugation for 15 min at 15,000 g and washed twice with 10 ml of 10% (W/V) TCA acetone solution. This material was dried under vacuum and dissolved in denaturing buffer (60 mM Tris/HCl), pH 6.8, 15% (V/V) glycerol, 4% (W/V) SDS, and 4% (V/V) β-mercaptoethanol) at a concentration of 20 mg/ml. The following buffer was used for 2-D PAGE analysis: 9.5 M urea, 50 mM Tris/HCl, pH 7.5, 0.5% (W/V) DTT, 1.25% (W/V) SDS, 1.0% (V/V) Triton, 3.0% (W/V) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 1.6% (V/V) Ampholine, pH 5–7, 0.4% (V/V) Ampholine, pH 3–10. Solubilisation of the protein material was facilitated by sonication (20–40 sec). PVP and Amberlite IRA-400 (Cl^-) anion exchanger resins were added (20 mg/ml of buffer for each) to the denaturing buffers to remove polyphenols. The supernatants containing the solubilized proteins were recovered by centrifugation at 13,000 g for 10 min.

Polyacrylamide gel electrophoresis

SDS-PAGE (Laemmli, 1970) was carried out using a Protean^{TM} II slab cell (Bio-Rad, Richmond, CA). The gel contained 12% (W/V) acrylamide, 0.3% (W/V) N,N′-methylene-bisacrylamide, 0.1% SDS (W/V), 0.375 M Tris/HCl, pH 8.8, 0.03% (W/V) ammonium persulfate and 0.14% (V/V) TEMED. Before one-dimensional analysis, protein samples were incubated for 10 min in denaturing buffer at 100° C. Samples were loaded onto a slab gel ($0.001 \times 15 \times 16$ cm) and separated using running buffer containing 0.025 M Tris, 0.192 M glycine, 0.1% SDS (W/V). SDS-PAGE gels were stained for 1 hr in 0.05% (W/V) Coomassie Brilliant blue R-250 dissolved in a solution containing 10% (V/V) acetic acid and 45% (V/V) methanol, then destained for 5 to 18 hr in the same solution without Coomassie blue.

Two-dimensional PAGE was based on the method of O'Farrell (1975). Isoelectric focusing (IEF) was performed in cylindrical polyacrylamide gels (0.001×15 cm) in the Protean^{TM} II 2-D cell. This gel, containing 8.5 M urea, 1.5% (W/V) CHAPS, 0.5% (V/V) nonidet P-40 (NP-40), 1.8% (V/V) carrier ampholytes pH 5–7, 0.4% (V/V) carrier ampholytes pH 3–10, 4% (W/V) acrylamide, 0.1% (W/V) N, N′-methylene-bisacrylamide, was polymerized using 0.015% (W/V) ammonium persulfate and 0.1% (V/V) TEMED (Hochstrasser et al., 1988). Thirty μl samples were loaded on the basic side of the gel. The

anolyte consisted of 0.06% (V/V) H_3PO_4, while the catholyte was made of 0.1 N NaOH. IEF was carried out at 400 V constant voltage for 12–15 hr followed by 2 hr at 800 V. Gels were extracted using a syringe with a gel extrusion needle and immediately transferred in the denaturing buffer (0.0625 M Tris/HCl, pH 6.8, 10% (V/V) glycerol, 5% (V/V) β-mercaptoethanol, 3% (W/V) SDS). After incubation for 10 min in the denaturing buffer, the extruded gel was placed on top of the slab gel (0.001×15×16 cm). The second-dimension separation conditions were the same as for the SDS-PAGE procedure described earlier. Polypeptides on 2D-PAGE gels were detected by silver staining (Bio-Rad, Richmond, CA).

Immunoblotting

Polypeptides were transferred from gels onto Immobilon™ PVDF membrane in a Bio-Rad Trans-Blot™apparatus. The membrane was immersed for 1 min in methanol prior to a 10 min incubation in transfer solution. The transfer was carried out at 60 V for 14 to 16 hr in a solution of 20 mM Tris, 150 mM glycine, 20% (V/V) methanol as described in Towbin et al. (1979). Membranes were then blocked for 2 hr at room temperature with gentle shaking in TBS buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl) containing 1% (W/V) bovine serum albumin (BSA). Membrane strips containing the molecular weight markers were stained in a mixture of 10% (V/V) acetic acid and 45% (V/V) methanol. Antiserum directed against total proteins from *Frankia* was prepared by the immunization of New Zealand white rabbits (Lalonde et al., 1975). The antiserum against purified *Rhodospirillum rubrum* dinitrogenase reductase (Fe protein) extract (kindly provided by Dr. Paul Ludden, University of Wisconsin-Madison) and the antiserum against purified MoFe protein (Burgess et al., 1980) of *Azotobacter vinelandii* dinitrogenase (kindly provided by Dr. Barbara Burgess, University of California at Irvine), were also raised in rabbits. Primary antisera were used at a 1:500 dilution in TBS buffer containing 1% (W/V) BSA; goat anti-rabbit immunoglobulin G conjugated to biotin (Sigma) was used at a 1:700 dilution. The first immunological reactions and the subsequent reactions using goat anti-rabbit antibody or avidin-horseradish peroxidase complex (ExtrAvidin™ Peroxidase system, Sigma, St. Louis, MO) were performed for 2 hr with gentle agitation at room temperature. After the first antibody reaction and between every subsequent reaction step, the membrane was rinsed twice for 15 min in TBS buffer containing 0.05% (V/V) Tween 20. The immunoblots were stained in 50 ml 0.03% (V/V) H_2O_2 and 0.05% (W/V) O-dianisidine in TBS.

3. Results

SDS-PAGE analysis of polypeptide patterns from Alnus *roots and actinorhizae*

The modified procedure used to extract proteins from roots, actinorhizae and free-living *Frankia* cultures generated enough material for several repeated analyses. As shown in Fig. 1, the polypeptide patterns of non-infected roots of *A. glutinosa* remain identical after 10 days of growth (lanes 1 and 2). The

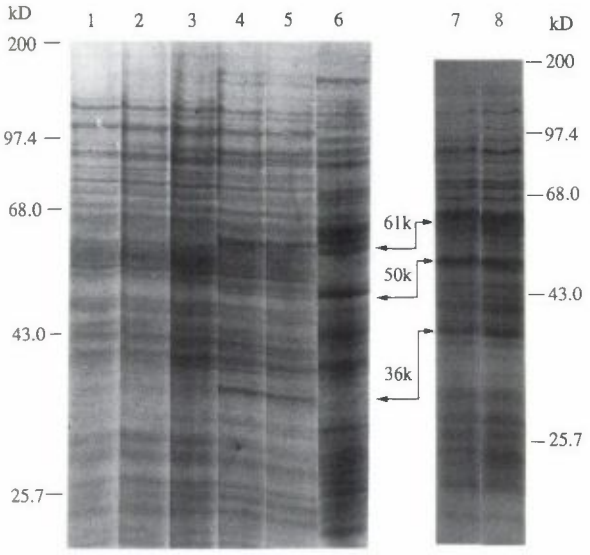


Figure 1. Changes in the patterns of proteins during the development of ***Alnus glutinosa*** actinorhizae. SDS-PAGE gels were stained with Coomassie blue. Non-infected roots of *A. glutinosa* at the time of inoculation (lane 1), non-infected roots 10 days after inoculation (lane 2), root systems bearing actinorhizae 14 days after inoculation (lane 3), excised actinorhizae 14 (lane 4) and 24 (lane 5) days after inoculation, free-living *Frankia Alni* grown in Qmod B medium (lane 6), or propionate medium "P+N" (lane 7, "P−N" (lane 8). The positions of molecular weight standards are given on the left side of the gel.

roots bearing actinorhizae did not produce any significant modification in the polypeptide patterns 14 days after inoculation (lane 3). However, when the actinorhizae were excised (14 or 24 days after inoculation) and analysed on SDS-gels, (lanes 4, 5), at least three distinct additional polypeptides in the 36 kD, 50 kD, and 61 kD regions were seen. Two of these polypeptides (36 kD and 61 kD) could not be detected in the extracts from non-nitrogen fixing *Frankia* cultures (lane 6 and 7) although a polypeptide in the 50 kD region was clearly visible in extracts from all *Frankia* cultures (lane 6, 7 and 8). Very

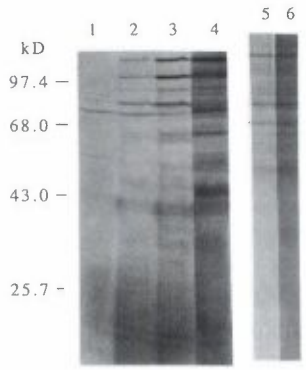
faint bands corresponding to 36 kD and 61 kD were visible in the extract from the nitrogen fixing *Frankia* culture (lane 8).

Identification of actinorhiza-specific proteins by immunoblotting

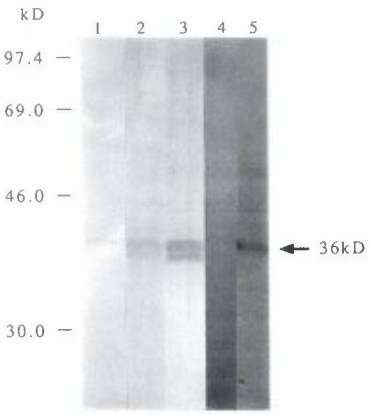
In a first attempt to assess the origin of the additional polypeptides, an antiserum directed against total soluble protein extracted from *Frankia* in culture was reacted with immobilon membranes bound with polypeptides from *Alnus* roots, actinorhizae, and free-living *Frankia*. The antiserum reacted with several polypeptides (Fig. 2,A) extracted from *Alnus* roots bearing actinorhizae (lane 2), excised actinorhizae (lane 3) and free-living *Frankia* in different culture conditions (lane 4, 5 and 6). However, the antiserum did not recognise any of the additional novel polypeptides found in the actinorhizae extracts. Some bands (lane 1) were also detected when polypeptides from non-infected *Alnus* roots were reacted with the antiserum and very similar bands were also obtained when normal rabbit preimmune antiserum was tested with *Alnus* root polypeptides (results not shown). The observation of these bands might be due to the presence of antibodies in the antisera that recognise conserved plant proteins present in *Alnus* roots.

Due to the fact that the additional 36 kD and 61 kD polypeptides (Fig. 1, lanes 4 and 5) had molecular weights similar to the constituents of the nitrogenase enzyme complex (Sprent and Sprent, 1990), immunoblots bearing polypeptides of *Alnus* roots, *Alnus* roots bearing actinorhizae, excised actinorhizae and free-living *Frankia* cultures were reacted with antisera raised against components of the nitrogenase enzyme complex from *R. rubrum* (Fe protein) and *A. vinelandii* (MoFe protein). The results (Fig. 2, panels B and C, lanes 2 and 3) indicated that the novel 36 kD and 61 kD polypeptides induced in the actinorhizae are recognised by heterologous antisera against proteins of the nitrogenase enzyme complex. The polypeptides of the nitrogenase complex were also found in extracts from nitrogen-fixing cultures of *Frankia* (Fig. 2, panels B and C, lane 5) but a larger amount of total *Frankia* proteins (30 μg) had to be loaded on the SDS-PAGE gels to enable a signal comparable to the actinorhizae extracts. Minor bands of lower molecular weight were also observed in extracts from actinorhizae on the two immunoblots (Fig. 2, panels B and C, lane 3). It is possible that these minor bands are degradation products due to the action of plant proteases because such minor bands of lower molecular weight were not visible in the nitrogen-fixing cultures of *Frankia* (Fig. 2,B and C, lane 5).

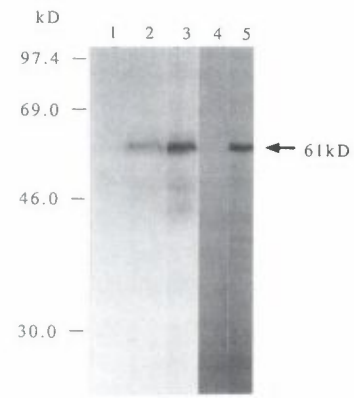
Figure 2. Immunoblot analysis after separation of proteins by SDS-PAGE.



A. (panelA) The membrane was reacted with an antiserum raised against proteins extracted from *Frankia* in culture. Non-infected roots of *A. glutinosa* at the time of inoculation (lane 1), root systems bearing actinorhizae 14 days after inoculation (lane 2), excised actinorhizae 14 days after inoculation (lane 3), free-living *Frankia alni* grown in Qmod B medium (lane 4), or propionate medium "P+N" (lane 5), "P–N" (lane 6).



B. (panel B) The membrane was reacted with an antiserum raised against the dinitrogenase reductase (Fe protein) of *R. rubrum*.



C. (panel C) The membrane was reacted with an antiserum raised against the dinitrogenase (MoFe protein) of *A. vinelandii*. Non-infected roots of *Alnus glutinosa* at the time of inoculation (1), root systems bearing actinorhizae 14 days after inoculation (lane 2), excised actinorhizae 14 days after inoculation (lane 3), free-living *Frankia alni* grown in propionate medium "P+N" (lane 4) or "P–N" (lane 5). The positions of molecular weight standards are given on the left side of the gel.

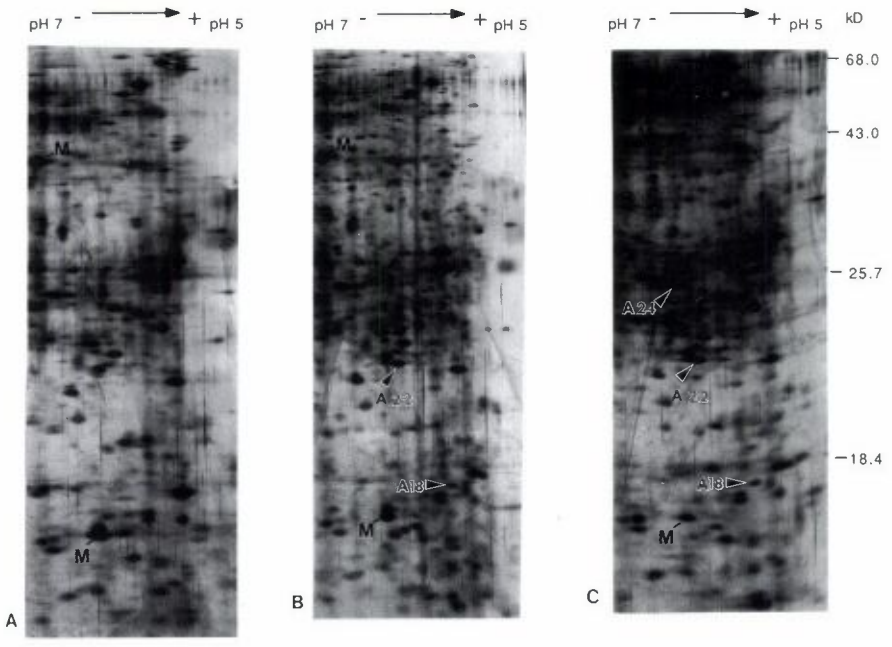


Figure 3. Silver stained 2D-PAGE of polypeptide from *Alnus glutinosa* roots and actinorhizae. Protein extracts from non-infected roots of *A. glutinosa* at the time of inoculation (panel A), excised actinorhizae 14 (panel B) and 24 days (panel C) after inoculation. Polypeptides A18, A22 and A24 are proteins specific to the actinorhiza (arrowhead). M indicates marker plant root proteins present on all gels. The positions of molecular weight standards are given on the right side of the gel.

Two dimensional SDS-PAGE analysis of polypeptides from Alnus *roots and actinorhizae*

An examination of the polypeptide patterns of *Alnus* roots and actinorhizae by 2D-PAGE revealed the presence of five actinorhiza-specific polypeptides. Despite some variation in the intensity of staining between successive experiments, the presence of actinorhiza-specific polypeptides was constant. Each protein extract was analysed by 2D-PAGE at least four times to confirm reproducibility. The polypeptides specific to the actinorhizae are shown by arrowheads. Figure 3 shows the different 2Dpolypeptide patterns of the acidic region of the gel, obtained with *Alnus* roots and actinorhizae excised 14 and 24 days after inoculation. Examination of two basic regions of the gels (Fig. 4) of *Alnus* roots and actinorhizae excised 14 days after inoculation shows at least one polypeptide specific to the actinorhizae in each region. The examination of a similar region of the gels, from extracts obtained 24 days after inoculation,

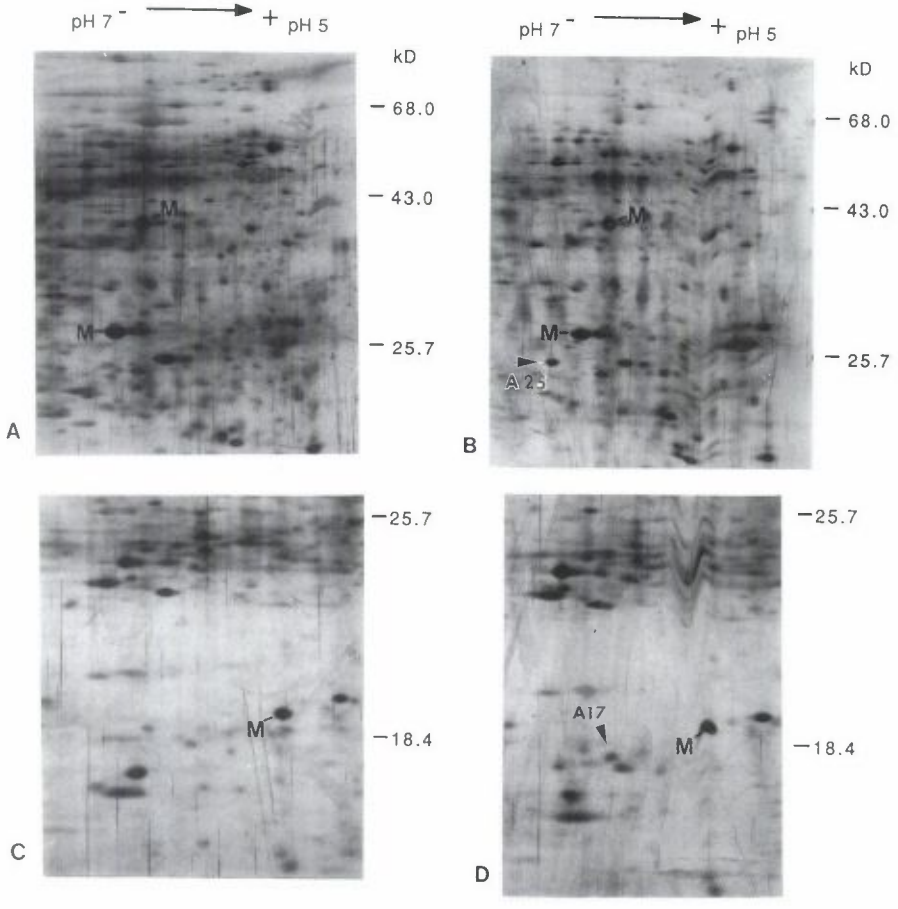


Figure 4. Silver stained 2D-PAGE of polypeptide from *Alnus glutinosa* roots and actinorhizae. Protein extracts from non-infected roots of *A. glutinosa* at the time of inoculation (panel A), excised actinorhizae 14 days after inoculation (panel B). Non-infected roots of *A. glutinosa* at the time of inoculation (panel C), excised actinorhizae 14 days after inoculation (panel D). Polypeptides A17 and A25 are proteins specific to the actinorhiza (arrowhead). M indicates marker plant root proteins present on all gels. The positions of molecular weight standards are given on the right side of the gel.

did not show the presence of any additional actinorhiza-specific polypeptides (data not shown). The five major spots that were specific to the actinorhizae had apparent molecular weights of 17 kD, 18 kD, 22 kD, 24 kD and 25 kD and indicated as A17, A18, A22, A24 and A25 respectively. In order to identify the origin of these actinorhiza-specific polypeptides, we also analysed protein extracts from free-living *Frankia* cultures on 2D-PaGE. We had to modify the protein extraction procedure for the *Frankia* cultures because of the poor resolution and the reproducibility obtained using the method for the plant material (data not shown). We used a protein extraction method described by Benson, Buchholz and Hanna (1984) for the *Frankia* cultures and were able to improve the quality of the 2D gels. However, variability in the polypeptide patterns was observed when cultures were grown under different conditions (Qmod B or propionate media) and it was not possible to correlate any *Frankia* polypeptides to the actinorhiza-specific polypeptides previously described (data not shown). An alternative approach to overcome these problems is described in the discussion.

4. Discussion

In this study we compared the patterns of polypeptide extracts from *Alnus* roots, actinorhizae and free-living *Frankia* cultures to determine if the development of actinorhizae is accompanied by specific protein synthesis. Comparisons of Coomassie blue stained one dimensional polyacrylamide gels showed 3 additional polypeptides (36, 50 and 61 kD) in the actinorhizae. An antiserum raised against total protein extracted from *Frankia* in culture permitted the recognition of several polypeptides common to the free-living *Frankia* and the actinorhizae and the actinorhizae but did not detect any of the three additional polypeptides previously described. Antisera raised against nitrogenase enzymes from other bacteria showed a strong affinity for the proteins at 36 kD and 61 kD in actinorhizae and in nitrogen fixing cultures of *Frankia*. A similar approach used to immunologically detect the nitrogenase enzymes of *Frankia* isolated form purified vesicles or whole cell proteins showed that these polypeptides had similar migration patterns in SDS-PAGE (Lundquist and Huss-Danell, 1991a,b; Meesters et al., 1985). Together, these observations strongly indicated that the 36 kD and 61 kD polypeptides are part of the nitrogenase enzyme complex of *Frankia*. Repeating of the same immunological techniques with an actinorhiza-specific antiserum might prove useful in highlighting other polypeptides present specifically in the actinorhiza. The identification of 30 different pea nodule-specific polypeptides was achieved

by western blotting analysis using a nodule-specific antiserum preparation (Bisseling et al. 1983).

The 50 kD polypeptide found in actinorhizae extracts is present in all extracts from *Frankia* cultures. Polypeptides with nearly the same molecular weight have also been found in protein extracts of different *Frankia* isolates cultivated in different ways (Fig. 2 in Benson and Hanna, 1982; Figs. 1 and 3 in Gardes and Lalonde, 1987; Fig. 1 in Faure-Raynaud et al., 1990). Thus the expression of this 50 kD polypeptide is not dependent of the culture medium used. Moreover, the fact that the 50 kD polypeptide is produced either in pure culture or as a microsymbiont suggests that it is an important structural protein or part of an essential enzyme system.

Among the wide variety of nodulins described in different *Rhizobium*-legume symbioses, leghemoglobin is one of the most abundant. In several species of actinorhizal plants, the presence of hemoglobin, on the basis of spectrophotometric measurements, has been reported (Tjepkema, 1983). We did not investigate the presence of a leghemoglobin-like protein in *Alnus* actinorhizae because previous studies using antisera against pea and soybean leghemoglobins did not show positive reactions, when tested on western blots from SDS-PAGE, with nodule protein extracts from *Alnus* (Simonet et al., 1990) or *Casuarina* (Flemming et al., 1987).

The use of 2D-PAGE enabled us to detect at least five polypeptides present in actinorhizae. All these polypeptides were identified by their low molecular weights (ranging from 15 kD to 25 kD). It could not be determined whether these polypeptides were produced by the host or the microsymbiont. Indeed, the very low amounts of proteins loaded on the 2-D polyacrylamide gels were not sufficient to allow the usual immunoblotting experiments as already described in the text. However, methods on direct sequencing of polypeptides after electroblotting and staining on PVDF membrane have been described (Matsudaira, 1987). This technology is available for direct N-terminal sequencing of polypeptide represented by as little as a single spot on a 2D-PAGE gel. Although this technology presently requires expensive equipment, it can increase the power and utility of 2D-PAGE in the analysis of polypeptides. Having a short amino acid sequence of the polypeptide of interest may provide some information on the nature (plant or bacterial) of this polypeptide. Also, comparing the terminal sequence to a protein databank may give some information on the possible function of this protein. Further, a synthetic polypeptide corresponding to the amino acid sequence could be used to obtain a specific polyclonal antiserum (Guy, 1989). This antiserum might be used with different immunological techniques to characterize the polypeptide of interest. Finally

and perhaps most important, a synthetic oligonucleotide sequence corresponding to the amino acid sequence could be used as a probe for direct screening for the corresponding gene in genomic or cDNA libraries of one or both symbiotic partners. This would represent a major advantage for the molecular study of the gene regulation in interactions like the *Frankia-Alnus* symbiosis.

Another approach to characterize the changes in induction and expression of protein synthesis during actinorhiza formation would be to do *in vitro* translations of the RNA isolated from *Alnus* roots and actinorhizae. Comparisons of the 2D-PAGE polypeptide patterns of the *in vitro* translated products would enable us to focus on the host polypeptides induced during the development of the actinorhizae. This method has been successfully used to study plant gene expression in legumes during nodule formation (Legocki and Verma, 1980; Govers et al., 1985; Gloudemans et al., 1989).

The use of some or all of these techniques may eventually lead to the identification of host plant specific genes that might be involved in the development of actinorhizal symbioses and a better understanding of the mechanisms of the actinorhiza development.

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