JOURNAL OF BACTERIOLOGY, May 1984, p. 621–627
0021-9193/84/050621-07$02.00/0

Vol. 158, No. 2

Identification of a Rhizosphere Protein Encoded by the Symbiotic Plasmid of *Rhizobium leguminosarum*

NICHOLAS J. DIBB,† J. ALLAN DOWNIE, AND NICHOLAS J. BREWIN*
John Innes Institute, Norwich NR4 7UH, United Kingdom

Received 9 December 1983/Accepted 25 February 1984

A protein was identified which was made by wild-type strains of *Rhizobium leguminosarum* but not by nodulation-deficient derivatives which had deletions of their symbiotic plasmids. The protein, which had a subunit molecular weight of ca. 24,000 (24K), was found to be present in large amounts within bacteria that had been reisolated from the surface of inoculated pea roots but was not detected in bacteroids isolated from nodules. The protein could also be induced during growth of *R. leguminosarum* on nutrient medium and was purified from the cytoplasmic fraction of broken cells. Antiserum raised against the purified protein was used to screen transposon-induced mutants of *R. leguminosarum*, and four independent mutants were isolated which lacked the protein. The sites of the Tn*5* insertions were found to map between the nitrogenase and nodulation genes on symbiotic plasmid pRL1JI, ca. 5 kilobases from the nitrogenase genes and 13 kilobases from the nodulation genes. Genetic determinants for the 24K protein were found to be closely linked to plasmid-borne nodulation genes for all strains of *R. leguminosarum* tested. However, the mutants which lacked the 24K protein still formed normal nitrogen-fixing nodules on peas, and the function of the protein is unknown.

Transposon Tn*5* mutagenesis has been used to identify and locate genes essential for nodulation (*nod*) and nitrogen fixation (*fix*) on the symbiotic plasmid pRL1JI from *Rhizobium leguminosarum*, (9, 10, 19). Mutations affecting nitrogen fixation were found to be clustered in two groups spaced ca. 30 kilobases (kb) apart. Between these two regions of *fix* genes was a cluster of *nod* genes (6 to 10 kb), but no mutations affecting symbiotic functions were identified in the regions of DNA immediately on either side of the *nod* region.

We report here that one of the functions of the 20-kb region of DNA between *nifKDH* and the *nod* genes is to code for a protein with a monomer subunit molecular weight of 24,000 (24K) that was a major component of rhizobial cells released from the surface of pea roots by a saline wash. Immunologically cross-reactive material of similar molecular weight (24K) was identified in free-living cultures of all strains of *R. leguminosarum* examined, and it appeared to be a characteristic of the symbiotic plasmids of this particular *Rhizobium* species. Some of the properties of this 24K protein have been investigated, and a genetic location has been established for the locus that determines the synthesis of this 24K polypeptide.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Tables 1 and 2.

Culture conditions. Culture conditions were as described previously (2, 18). All plant tests were conducted with peas (variety, Wisconsin Perfection). Seeds were surface sterilized with sodium hypochlorite (10% [wt/vol] for 15 min) and germinated in sterilized Erlenmeyer flasks containing Fahraeus medium in 0.5% (wt/vol) agar (4).

Preparation of protein samples for electrophoresis. (i) Free-living bacteria. Strains grown for 3 to 4 days on TY slants (2) were washed off with distilled water and suspended in sample buffer (17) at a protein concentration of 2 mg/ml. Cells were lysed by incubation at 90°C for 20 to 30 min, and material was stored at −20°C.

(ii) Rhizosphere bacteria. Sterile pea seeds were inoculated with less than 10^5 rhizobia and then grown under axenic conditions for 3 to 4 weeks (4). A single root system was shaken for ca. 15 s in 10 ml of 0.9% (wt/vol) NaCl in 0.025 M *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES buffer) and adjusted to pH 7.2 with KOH. Bacteria from 5 to 10 root systems were pooled, filtered through Miracloth (Calbiochem-Behring), centrifuged at 12,000 × *g* for 10 min, resuspended in 5 ml of TES buffer, filtered through cotton wool, and centrifuged for 10 min at room temperature. The rhizosphere bacteria were washed in 1 ml of TES buffer, centrifuged in a microcentrifuge for 1 min, resuspended, and lysed in sample buffer at a protein concentration of 2 mg/ml. Approximately 10^8 bacteria were recovered from one root system. (Single colonies, reisolated after dilution and plating of the rhizosphere bacteria, always had the same genetic markers as the inoculant strain.) Bacteria isolated from 5 to 10 roots yielded ca. 50 μg of protein.

(iii) Bacteroids. Bacteroids were isolated as previously described (19) and lysed in sample buffer at a protein concentration of 2 mg/ml.

Electrophoresis. Polypeptides were separated as described by Laemmli (17) and stained with Coomassie blue (12). The ratio of acrylamide to bisacrylamide was 30:0.8, and the concentration of acrylamide in the stacking and running gels was 5 and 12%, respectively.

Rhizobium plasmids were identified on agarose gels by the procedure of Eckhardt (11).

Antiserum. A large New Zealand white rabbit was immunized by subcutaneous injections of purified 24K protein to obtain reactive antiserum (anti-24K antibody).

Protein blotting to nitrocellulose and immunochemical detection. Polypeptides in sodium dodecyl sulfate (SDS)-polyacrylamide gels were blotted to nitrocellulose (5) and treated by indirect immunochemical staining (20). The blot was first incubated with anti-24K antiserum at a dilution of 1/1,000 (vol/vol) and subsequently incubated with a 1/2,000 dilution

* Corresponding author.
† Present address: Department of Molecular Biology, University of California, Los Angeles, CA 90024.

TABLE 1. Derivatives of *R. leguminosarum* 897 and 3855

Strain	Derivation and relevant genotype	Relevant phenotype	Reference
897	300 *phe trp str*	Nod$^+$ Fix$^+$ 24K$^+$	15
6007	897 Δ(*nod-fix*) *6007*	Nod$^-$ Fix$^-$ 24K$^-$	15
6015	6007 *rif*	Nod$^-$ Fix$^-$ 24K$^-$	15
T83K3	6015 pRL1JI::Tn*5*[a]	Nod$^+$ Fix$^+$ 24K$^+$	15
D1	6015 pIJ1208	Nod$^+$ Fix$^+$ 24K$^-$	This paper
D2	6015 pIJ1209	Nod$^+$ Fix$^+$ 24K$^-$	This paper
D3	6015 pIJ1210	Nod$^+$ Fix$^+$ 24K$^-$	This paper
D4	6015 pIJ1211	Nod$^+$ Fix$^+$ 24K$^-$	This paper
3855	128C53 *str*	Nod$^+$ Fix$^+$ 24K$^+$	7
B151	3855 cured of pRL6JI	Nod$^-$ Fix$^-$ 24K$^-$	7

[a] Plasmid pRL1JI::Tn*5*-pJB5JI (15).

of goat antirabbit antibody coupled to peroxidase (Miles-Yeda). Cross-reacting proteins were stained (13) by peroxidase-catalyzed oxidation of 4-chloro-1-naphthol (Sigma Chemical Co.).

Purification of the 24K protein. Strain 6007 *rif* pRL1JI was inoculated into 10 liters of liquid TY medium and grown at 28°C in an orbital shaker until stationary phase was reached, normally after 3 to 4 days. The cells were centrifuged at 4°C, suspended in 100 ml of 0.025 M TES buffer, and passaged through a French press at 4°C at a pressure of 10^8 Pa. The homogenate was centrifuged at 100,000 × *g* for 90 min at 4°C, and the supernatant was recentrifuged at 100,000 × *g* for 2 h at 4°C. This supernatant (the crude soluble extract) contained ca. 600 mg of total protein in a volume of 10 ml.

Finely ground ammonium sulfate was added with stirring at room temperature to 40% of saturation, and precipitate was removed by centrifugation at 25,000 × *g* for 10 min at room temperature. The ammonium sulfate was increased to 55% of saturation, and the precipitate was collected as described above. The pellet (containing ca. 90 mg of protein) was dissolved in 2 ml of 0.025 M imidazole buffer (pH 7.4), dialyzed overnight against 1 liter of the same solution at 4°C, and applied to a chromatofocusing column (1 by 30 cm) in 0.025 M imidazole (pH 7.4). The column was eluted with polybuffer 74-hydrochloride (pH 4.0) (Pharmacia Fine Chemicals, Inc.) at a flow rate of 20 ml/h, and 3-ml fractions were collected. Samples from each fraction were analyzed by slab gel electrophoresis, and those fractions containing the 24K protein were pooled and precipitated in 80% saturated ammonium sulfate. The pellet was suspended in 2 ml of 0.025 M Tris-hydrochloride (pH 8.0) at a protein concentration of ca. 10 mg/ml, dialyzed against this buffer, and applied to a Sephacryl S200 column (2 by 84 cm). This was eluted with the same buffer at a flow rate of 25 ml/h at 4°C. Fractions (3 ml) were collected, and those fractions corresponding to the major peak were pooled and dialyzed against water, freeze-dried, and stored at −20°C. Usually, ca. 20 mg of purified 24K protein was obtained from 600 mg of protein present in the crude soluble extract.

Molecular weight estimation. An estimate of the molecular weight of the purified protein was obtained by gel filtration (1), using a Sephacryl S75 column (2 by 60 cm) that had been calibrated with the following proteins of known molecular weight: bovine serum albumin (68K), ovalbumin (45K), chymotrypsinogen (25K), and ribonuclease A (14K).

RESULTS

Identification of a class of 24K proteins. Strain 6007 is a non-nodulating mutant of *R. leguminosarum*, obtained after UV treatment of wild-type strain 897 (15). The mutant phenotype is due to a deletion of ca. 50 kb of DNA from pRL10JI, the symbiotic plasmid that encodes the structural genes for nitrogenase as well as genes required for nodule formation (14).

Protein samples prepared from free-living cultures of strains 897 and 6007 were compared by SDS-polyacrylamide

TABLE 2. Plasmids of *R. leguminosarum*

Plasmid	Phenotype[a]	24K phenotype[b]	Origin	Source
Symbiotic				
pRL10JI	Nod$^+$ Fix$^+$	+	*R. leguminosarum* 897	14
pRL6JI	Nod$^+$ Fix$^+$ Hup$^+$	+	*R. leguminosarum* 128C53	7
pRL1JI	Nod$^+$ Fix$^+$ Tra$^+$ Bac$^+$	+	*R. leguminosarum* 248	15
pRL5JI	Nod$^+$ Fix$^+$ Tra$^+$	+	*R. leguminosarum* TOM	6
pRL14JI	Nod$^+$ Fix$^+$ Tra$^+$	+	*R. leguminosarum* 257	Field isolate, U.K.
pRL15JI	Nod$^+$ Fix$^+$ Tra$^+$	+	*R. leguminosarum* 279	Field isolate, U.K.
pRL16JI	Nod$^+$ Fix$^+$ Tra$^+$	+	*R. leguminosarum* 297	Field isolate, U.K.
pRL17JI	Nod$^+$ Fix$^+$ Tra$^+$	+	*R. leguminosarum* 304	Field isolate, U.K.
Nonsymbiotic				
pIJ1000	Nod$^-$ Fix$^-$	ND	pRL10JI with 50-kb deletion, Δ*nod 6007*	14
pRL3JI	Tra$^+$ Bac$^+$	ND	*R. leguminosarum* 306	7
pRL4JI	Tra$^+$ Bac$^+$	ND	*R. leguminosarum* 309	7
24K$^-$ mutants of pRL1JI (*rhi*$^-$)[c]				
pIJ1208	Nod$^+$ Fix$^+$ Bac$^+$ Kan^r	Trace	pRL1JI *rhi-1*	Strain D1
pIJ1209	Nod$^+$ Fix$^+$ Bac$^+$ Kan^r	22K	pRL1JI *rhi-2*	Strain D2
pIJ1210	Nod$^+$ Fix$^+$ Bac$^+$ Kan^r	ND	pRL1JI *rhi-3*	Strain D3
pIJ1211	Nod$^+$ Fix$^+$ Bac$^+$ Kan^r	ND	pRL1JI *rhi-4*	Strain D4

[a] Nod, Nodulation ability; Fix, nitrogen fixation; Hup, hydrogen uptake; Bac, bacteriocin production; Kan^r, resistant to kanamycin (60 μg/ml); 24K, 24K protein production; Tra, transmissible by conjugation.

[b] Synthesis of 24K polypeptide was tested in the genetic background of either 6007 or B151 (both of which are Nod$^-$ Fix$^-$ 24K$^-$); ND, not detectable.

[c] *rhi*, Allele for production of 24K protein.

gel electrophoresis (Fig. 1). It was found that strain 6007 lacked a major 24K polypeptide band, which was present in strain 897. This polypeptide band was regained after the introduction of plasmid pRL1JI into strain 6007 (Fig. 1, lane C). (As had been shown previously [15], plasmid pRL1JI also restored nodulation and nitrogen fixation functions to strain 6007.) Thus it was established that a 24K polypeptide was determined by the two symbiotic plasmids pRL1JI and pRL10JI. Similarly, *R. leguminosarum* 128C53 was found to contain a major 24K polypeptide band that was missing from the derivative strain B151, which had been cured of its symbiotic plasmid pRL6JI (7).

Twelve other field isolates of *R. leguminosarum*, originating from diverse regions of Europe and America, were also examined for the production of a major 24K polypeptide; free-living cultures of each of these strains were examined by SDS-polyacrylamide gel electrophoresis, and in all cases a prominent 24K polypeptide band was observed. In four of these *R. leguminosarum* strains the symbiotic plasmids were self-transmissible and could be transferred by conjugation (6) to the Nod^- recipient strains 6007 *rif* or B151. (Each of these four symbiotic plasmids had a different molecular weight within the range 150K to 220K.) In each case, transfer of plasmid-linked symbiotic determinants (*nod* and *fix*) was accompanied by cotransfer of determinants for production of a polypeptide of ca. 24K (subsequently referred to as the 24K protein). It is interesting to note that two other transmissible plasmids from *R. leguminosarum*, plasmids pRL3JI and pRL4JI, did not confer the ability to synthesize the 24K protein (Table 2); these two plasmids are known to carry determinants for bacteriocin production, but neither plasmid carries the symbiotic determinants that are associated with pRL1JI, a symbiotic plasmid of the same incompatibility group (7).

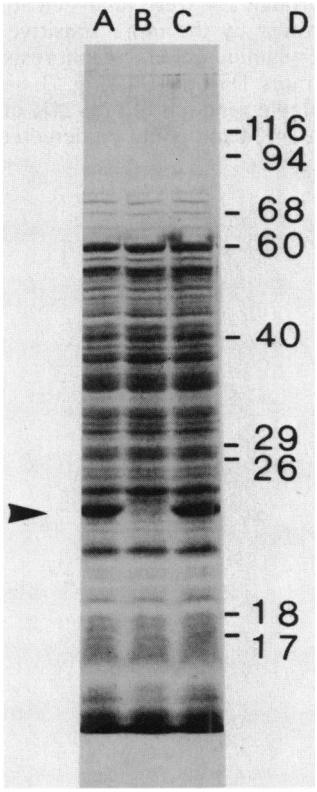

FIG. 1. Comparison by SDS-polyacrylamide gel electrophoresis of total proteins from free-living cultures of *R. leguminosarum* strains. Lanes: A, 897; B, 6007 (i.e., 897 Δ*nod fix*); C, 6007 pRL1JI (Nod^+ Fix^+). D shows molecular weight standards (in thousands). Protein samples (50 μg) from each strain were electrophoresed on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. An arrow indicates the position of a 24K polypeptide that was absent from strain 6007.

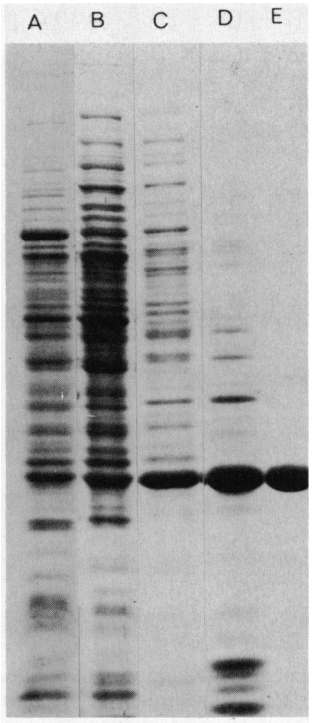

FIG. 2. Successive steps in the purification of the 24K protein from *R. leguminosarum*. Lanes: A, total protein; B, crude soluble extract; C, ammonium sulfate precipitation; D, chromatofocusing; E, gel filtration. Samples from the fractions obtained after each purification step were analyzed by SDS-polyacrylamide gel electrophoresis (20 μg of protein per channel) and stained with Coomassie blue.

Purification of the 24K protein. The 24K protein was purified from *R. leguminosarum* 6007 *rif* containing the symbiotic plasmid pRL1JI. The assay for the protein was its mobility on SDS-polyacrylamide gels.

The protein was not found in the extracellular fraction, and after differential centrifugation of the cell homogenate (160,000 × *g* for 90 min at 4°C), the 24K protein was in the soluble fraction but not in the pelleted membrane fraction. The 24K protein was then purified from the soluble fraction by ammonium sulfate fractionation, chromatofocusing, and gel filtration (Fig. 2). Most of the 24K protein was precipitated by a 45 to 55% ammonium sulfate cut. The 24K protein had an isoelectric point of 5.2, as determined by chromatofocusing. Gel filtration showed that the protein had a molecular weight of ca. 45K, suggesting that it was a dimer consisting of two identically sized 24K subunits. Treatment with Schiff reagent and periodate did not reveal the presence of carbohydrate residues associated with the purified protein.

Antiserum to the 24K protein. To examine the specificity of antiserum raised against the purified protein, samples from various strains were run on an SDS-polyacrylamide

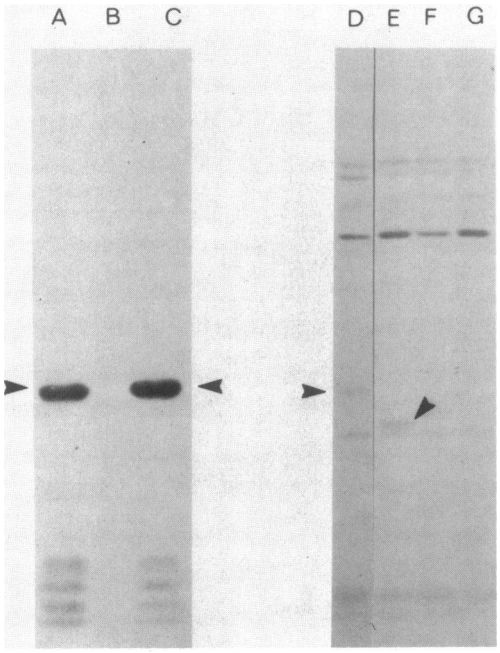

FIG. 3. Binding of anti-24K antibody to purified 24K protein and to protein samples (50 μg of protein per channel) prepared from various mutant strains of *R. leguminosarum* defective for 24K protein. After electrophoresis, the protein gel was blotted to nitrocellulose, treated with anti-24K antibody by the method of Towbin et al. (20), and stained with 4-chloro-1-naphthol and hydrogen peroxide after treatment with peroxidase-conjugated goat antirabbit antibody. Lanes: A, purified 24K polypeptide (1 μg); B, 6007 (deletion mutant); C, 6007 *rif* pRL1JI (wild-type); D, D1 (Tn*5* mutant); E, D2 (Tn*5* mutant); F, D3 (Tn*5* mutant); G, D4 (Tn*5* mutant). Arrows indicate the position of the 24K protein and the position of a novel, faintly staining 22K polypeptide band present in lane E. Note that lanes D, E, F, and G were stained for a longer time with 4-chloro-1-naphthol and hydrogen peroxide to identify minor bands. (This treatment also resulted in the nonspecific staining of other bands, particularly in the 50K to 60K size range.)

gel, transferred to nitrocellulose (5), treated with anti-24K antiserum, and stained by the immunochemical technique of Towbin et al. (20). Both the purified protein and the extract from 6007 *rif* pRL1JI showed a band that stained strongly in the 24K region (Fig. 3, lanes A and C). This band was absent from extracts of strain 6007 *rif* (Fig. 3, lane B). These results showed that a specific antiserum had been raised to the 24K protein. (The weakly staining polypeptide bands of lower molecular weight, seen in lanes A and C of Fig. 3 but absent from lane B, were presumed to be degradation products of the 24K protein.)

Expression of the 24K protein in the rhizosphere. By using the anti-24K protein antiserum as a molecular probe, it was possible to examine the occurrence of the 24K protein in free-living bacteria, rhizosphere bacteria, and bacteroids isolated from within root nodules. Proteins were examined after SDS gel electrophoresis and transfer to nitrocellulose (Fig. 4). For free-living rhizobia, the 24K protein accumulated in cells grown to stationary phase. Rhizosphere bacteria (Fig. 4, lane B) clearly made the 24K protein, which constituted a major band on Coomassie blue-stained protein gels (data not shown). The 24K protein appeared to be absent from bacteroids and extracts from nodules.

Distribution of the 24K protein in other bacterial species. The 24K proteins determined by the plasmids listed in Table 2 were shown, by gel blotting and immunochemical staining, to cross-react with anti-24K antibody. The antibody was also used to test for the presence of a cross-reacting antigen in other groups of soil microorganisms. Although all 10 field isolates of *R. leguminosarum* cross-reacted with the antibody, none of the five *Rhizobium phaseoli* isolates and only one (B079) of the five *Rhizobium trifolii* isolates was found to cross-react. (Strain B079 did not nodulate peas.) Strains of *Rhizobium japonicum* (SR), *Rhizobium meliloti* (102F28), *Agrobacterium tumefaciens* (C58 and AChL5), *Alcaligenes eutrophus* (type strain), and *Escherichia coli* (803) did not cross-react with anti-24K antiserum. Thus, the 24K protein appears to be largely confined to *R. leguminosarum*. It is, however, possible that functionally analogous proteins, which did not cross-react with anti-24K antibody, were made by other species of bacteria.

Isolation of mutants lacking the 24K protein. Antiserum raised to the purified protein was used to screen for mutants defective in the production of the 24K protein after transposon Tn*5* mutagenesis of plasmid pRL1JI as described by Ma et al. (19). By using an in situ immunoassay, 2,000 derivatives of strain 6007 *rif* pRL1JI were screened for the 24K protein (Fig. 5). Each derivative carried pRL1JI with a Tn*5* insertion, and nine of these were found to lack the 24K protein. Plasmid samples from these were analyzed by gel electrophoresis (11), and five were found to have large deletions of plasmid pRL1JI::Tn*5* (data not shown). The four nondeletion mutants were named D1 through D4.

Mutants D1 through D4 were analyzed for possible low levels of 24K protein by the more sensitive immunoassay after SDS-polyacrylamide gel electrophoresis (Fig. 3, lanes D, E, F, G). Strains D3 and D4 (Fig. 3, lanes F and G) showed no detectable reaction to anti-24K antibody. However, a very faint 24K band could be detected for strain D1

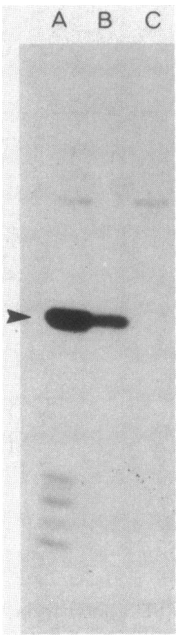

FIG. 4. Immunochemical detection of the 24K protein (arrow) from cultures of strain 6007 *rif* pRL1JI at free-living (A), rhizosphere (B), and bacteroid (C) stages. Total protein (50 μg) prepared from free-living and bacteroid stages and ca. 20 μg of protein prepared from rhizobia isolated from the rhizosphere were analyzed as described in the legend to Fig. 3.

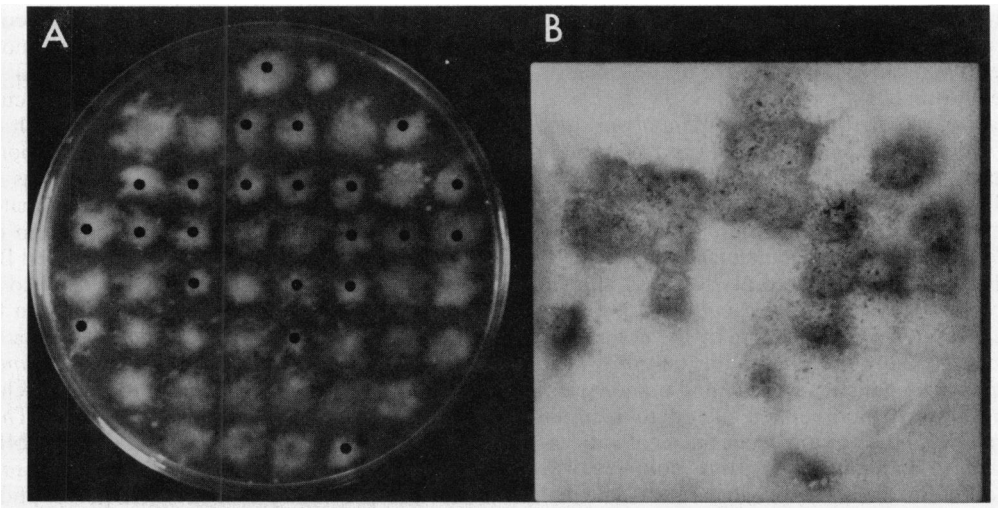

FIG. 5. Immunochemical screening technique that was used to identify colonies of *R. leguminosarum* defective in 24K protein. (In this example, the marked colonies in A were known to be wild type). Colonies were grown on a TY master plate for 3 to 4 days and replica plated onto Whatman no. 1 paper. The replica was placed, colonies facing downwards, onto a sheet of nitrocellulose which was on top of two sheets of Whatman 3MM paper soaked in 2% SDS. The papers were left overnight in a sealed box at room temperature, and the nitrocellulose blot (B) was then treated with anti-24K antibody as described in the legend to Fig. 3.

(Fig. 3, lane D), indicating that this mutant still made a low level of the 24K protein (ca. 1/5,000 of a wild-type strain, as estimated by serial dilution). In mutant strain D2, a novel 22K band was found to react weakly with antiserum to 24K protein (Fig. 3, lane E), indicating that strain D2 contained a small amount of a truncated protein that could have arisen by insertion of Tn*5* into the structural gene.

Mapping of the gene encoding the 24K rhizosphere protein. In a previous study (10), four overlapping DNA fragments were cloned from the region of pRL1JI involved in nodulation and nitrogen fixation. Derivatives of 6007 *rif* carrying three of these clones (plasmids pIJ1086, pIJ1088, and pIJ1089) contained very large amounts of the 24K protein, whereas the protein was absent from the strain carrying the fourth clone, plasmid pIJ1085.

One of the clones (plasmid pIJ1089), which had been found to carry determinants for 24K protein as well as nodulation determinants, was introduced into each of the mutants D1, D2, D3, and D4 to facilitate the mapping of the sites of Tn*5* insertions. The strategy was to select for recombinants in which Tn*5* had been transferred to the homologous site on pIJ1089 by homologous recombination. Plasmid DNA was prepared from each strain and used to transform *E. coli* 803, with selection being made for both tetracycline resistance (pIJ1089) and kanamycin resistance (Tn*5*). Restriction endonuclease analysis of the plasmids isolated from the transformants showed that in each case the plasmid had increased in size by 5.7 kb, the size of Tn*5* (16). The sites of the Tn*5* insertions (shown in Fig. 6) were mapped with *Eco*RI and *Hin*dIII. The four Tn*5* insertions were located within a 5-kb *Hin*dIII fragment and lay ca. 5 kb from the nitrogenase structural genes and ca. 13 kb from the *nod* genes.

The derivatives of pIJ1089 that had acquired Tn*5* by homogenotization from mutants D1, D2, D3, and D4 were each conjugated back from *E. coli* to *R. leguminosarum* 6007 *rif*, selecting for resistance to tetracycline and kanamycin. The transconjugants each had the same phenotype as the original mutant (D1 through D4), showing that the transposon insertion was responsible for the mutation affecting the 24K protein.

Plant tests. The mutants D1 through D4 were tested for their ability to form nitrogen-fixing nodules on the roots of pea plants. Strains D1 through D4 formed root nodules that reduced acetylene at normal rates, which proved that the 24K protein of strain 6007 *rif* pRL1JI was not essential for nodulation under the conditions tested. (Cultures established from bacteria that had been recovered from within surface-sterilized nodules still lacked the 24K protein.)

The mutants (which were all resistant to kanamycin) were also tested for their ability to form nodules in competition with the kanamycin-sensitive parent strain, 6007 *rif* pRL1JI, which showed normal levels of 24K protein production. Peas were co-inoculated with equal mixtures of 6007 *rif* pRL1JI and each of the mutant strains as described previously (8). When bacteria were reisolated from surface-sterilized nod-

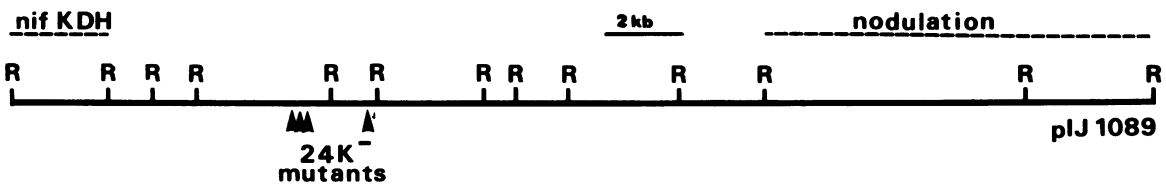

FIG. 6. Map of the symbiotic plasmid pRL1JI, showing the positions of four Tn*5* insertions (arrows) that affect synthesis of 24K protein. The allele numbers for these *rhi* mutants are, from left to right, *rhi-4*::Tn*5*, *rhi-2*::Tn*5*, *rhi-3*::Tn*5*, *rhi-1*::Tn*5*.

ules, the proportion of nodules containing kanamycin-resistant bacteria was ca. 50%, indicating that the mutant strains were at no competitive disadvantage relative to the kanamycin-sensitive parent strain.

A similar series of competition experiments was also conducted to test whether the production of the 24K protein conferred any growth advantage during repeated subculture on agar slants. Mixtures of each of the kanamycin-resistant mutants together with the parental strain 6007*rif* pRL1JI were co-inoculated onto agar slants containing complete (TY) medium. The cultures were incubated at 28°C and subcultured every 7 days for 5 weeks. (These culture conditions give very strong expression of 24K protein by the nonmutant strains.) At the end of the experiment, the cultures were serially diluted, and single colonies were plated on nonselective medium. These were then replica plated to medium containing kanamycin, and in every case the final ratio of kanamycin-resistant to kanamycin-sensitive colonies was not significantly different from the original ratio (data not shown). This experiment showed that the strain producing 24K protein had not been at a growth advantage during the duration of the experiment.

DISCUSSION

The 24K proteins have a number of interesting properties. First, they are characteristic of *R. leguminosarum*; all tested strains of diverse origin (Turkey, Denmark, England, and United States) made the protein, whereas it was found in none of five strains of *R. phaseoli* tested and in only one of six strains of *R. trifolii* (both of which are closely related to *R. leguminosarum*). It was not identified in any of a range of other soil bacteria that were tested. Second, the 24K proteins are encoded only by those plasmids of *R. leguminosarum* known to carry nodulation and nitrogen fixation genes (Table 2). Third, the 24K protein accumulates in stationary-phase cultures of free-living rhizobia and is one of the major protein components present in rhizobia isolated from the surface of pea roots by a saline wash; however, it is absent from bacteroids isolated from root nodules, and thus the control of gene expression for 24K protein production raises some interesting questions. Two of the mutants isolated in this study (D1 and D2) showed reduced levels of gene expression in free-living cultures.

We have tested a number of possible roles for the 24K protein in bacteroids, rhizosphere bacteria, and free-living bacteria. For bacteroids, transposon-induced mutants that lack the 24K protein had normal levels of nitrogenase activity within pea root nodules. (This was not surprising because, even in wild-type organisms, the 24K protein was not present in bacteroids.) Although rhizosphere bacteria did synthesize the 24K protein, two lines of evidence indicated that it did not have an essential role in the initiation of root nodules: first, mutants of the symbiotic plasmid pRL1JI that were defective in the synthesis of 24K polypeptide were not defective in nodulation ability; second, it was shown that a clone, termed pIJ1085 (9), that contained 10 kb of DNA from the nodulation region of the symbiotic plasmid pRL1JI was able to restore nodulation ability to strains of *R. leguminosarum* previously cured of their symbiotic plasmids, despite the fact that pIJ1085 did not carry the determinants necessary to synthesize the 24K protein.

For free-living bacteria, none of the known plasmid-determined functions, such as bacteriocin production and resistance, plasmid transfer, and incompatibility (3), was lost in any of the mutants that lacked the 24K protein. Furthermore, the nonsymbiotic *Rhizobium* plasmids listed in Table 2 expressed all of these functions but did not code for the 24K protein. Synthesis of the 24K protein could not be correlated with growth on any particular substrate (G. M. Sorensen, unpublished data). It could be expressed in cultures growing on minimal medium (Y) supplemented with glutamate and succinate or on a wide range of other carbon and nitrogen sources that were tested. In general, it was noted that the quantities of 24K protein were much greater in cultures grown on solid medium rather than in liquid medium.

For pRL1JI, the symbiotic plasmid from a British strain of *R. leguminosarum*, the 24K gene(s) mapped 5 kb from the nitrogenase structural genes and 13 kb from the nodulation region. Similarly for pRL5JI, the symbiotic plasmid from TOM, a Turkish strain of *R. leguminosarum*, the determinants for the 24K protein were found to be closely linked to the nodulation genes (unpublished data). This highly conserved linkage between determinants for 24K polypeptide and symbiotic functions on *R. leguminosarum* plasmids raises the possibility that the 24K polypeptide may have a nonessential symbiotic function in the rhizosphere under field conditions, for example, in enhancing *Rhizobium* competitiveness, survival, or plasmid stability. However, with the existing mutants and cured strains of *R. leguminosarum* that lack the 24K protein, no such role has yet been identified. In view of the fact that the protein is synthesized in the rhizosphere but has no known biochemical function, we tentatively propose to designate the genetic determinants for production of this plasmid-determined 24K protein of *R. leguminosarum* by the acronym *rhi*.

ACKNOWLEDGMENTS

Rod Casey and Andy Johnston are thanked for helpful discussions throughout the course of this work. We are grateful to Rod Townsend for help with the immunochemical techniques and to C. Knight for assistance with the mapping. The manuscript was scrutinized by A. W. B. Johnston, J. P. W. Young, and S. A. Kagan.

N.J.D. was supported by a postgraduate grant from the Agricultural Research Council.

LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. **91**:222–253.
2. **Beringer J. E.** 1974. R factor transfer in *Rhizobium*. J. Gen. Microbiol. **84**:188–198.
3. **Beringer, J. E., N. J. Brewin, and A. W. B. Johnston.** 1980. The genetic analysis of *Rhizobium* in relation to symbiotic nitrogen fixation. Heredity **45**:161–186.
4. **Beynon, J. L., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium trifolii*. J. Gen. Microbiol. **120**:421–429.
5. **Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub.** 1980. The detection of DNA-binding proteins by protein blotting. Nucleic Acids Res. **8**:1–18.
6. **Brewin, N. J., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmid mediated transfer of host range specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120**:413–420.
7. **Brewin, N. J., E. A. Wood, A. W. B. Johnston, N. J. Dibb, and G. Hombrecher.** 1982. Recombinant nodulation plasmids in *Rhizobium leguminosarum*. J. Gen. Microbiol. **128**:1817–1827.
8. **Brewin, N. J., E. A. Wood, and J. P. W. Young.** 1983. Contribution of the symbiotic plasmid to the competitiveness of *Rhizobium leguminosarum*. J. Gen. Microbiol. **129**:2973–2977.
9. **Downie, J. A., G. Hombrecher, Q.-S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston.** 1983. Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. Mol. Gen. Genet. **190**:359–365.
10. **Downie, J. A., Q.-S. Ma, C. D. Knight, G. Hombrecher, and A. W. B. Johnston.** 1983. Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between

the nitrogenase genes and a *nif*A-like gene. EMBO J. **2**:947–952.
11. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
12. **Fairbanks, G., T. L. Steck, and D. H. F. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2616.
13. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
14. **Hombrecher, G., N. J. Brewin, and A. W. B. Johnston.** 1981. Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. Mol. Gen. Genet. **182**:133–136.
15. **Johnston, A. W. B., J. L. Beynon, A. V. Buchanan-Wollaston, S. M. Setchell, P. R. Hirsch, and J. E. Beringer.** 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature (London) **276**:635–636.
16. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lamb, J. W., G. Hombrecher, and A. W. B. Johnston.** 1982. Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. Mol. Gen. Genet. **186**:449–452.
19. **Ma, Q.-S., A. W. B. Johnston, G. Hombrecher, and J. A. Downie.** 1982. Molecular genetics of mutants of *Rhizobium leguminosarum* which fail to fix nitrogen. Mol. Gen. Genet. **187**:166–171.
20. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.