Expression of large plasmids in the endosymbiotic form of *Rhizobium leguminosarum*

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ABSTRACT

Isolated plasmid DNA from *Rhizobium leguminosarum* was hybridised with cellular RNA from broth-cultured bacteria and endosymbiotic bacteroids. From these hybridisation experiments it is concluded that plasmid genes are strongly expressed in bacteroids and only weakly or not at all in bacteria. From the hybridisation of plasmid DNA with the cloned structural *nif* genes of *Klebsiella pneumoniae* it is shown that at least part of the *nif* genes are located on a plasmid.

INTRODUCTION

Upon the transition of free living *Rhizobium leguminosarum* into nitrogen fixing bacteroids in root nodules, marked changes occur in the morphology, DNA content and the patterns of protein synthesis in the cells (1, 2). Since the presence of large plasmids (M.W. 100 - 250 x 10⁶) in different rhizobium species has been demonstrated by several authors (3 - 6), these plasmids have been attributed a key role in the infectivity of the *Rhizobium* bacteria, the induction of root nodules and the achievement of nitrogen fixation. Evidence for such a role of plasmids has been found in genetic experiments (7, 8), demonstrating that genes involved in host specific nodulation are located on a large plasmid, and in biochemical experiments (9, 10) which indicate that the structural genes for nitrogenase are at least partly plasmid borne.

This paper describes experiments to examine the extent to which plasmid genes are transcribed in free-living rhizobia or in bacteroids. For that purpose RNA from bacteria or bacteroids was hybridized to restriction enzyme-digested plasmid DNA using the Southern blotting technique (11). The results show that the large plasmids are actively transcribed in the bacteroid form, whereas transcription of plasmid genes remains undetectable in broth-cultured bacteria. The location of *nif* genes on the rhizobium plasmids was demonstrated by using cloned *Klebsiella pneumoniae nif*-sequences (12) as a probe.

MATERIALS AND METHODS

Cultivation of Rhizobium bacteria and bacteroids. *Rhizobium leguminosarum* (strain PRE) was grown in TY medium (5 g bactotryptone, Difco, Detroit Michigan U.S.A., 3 g yeast extract, Difco, 10 mg uracil in 1 l, plus separately sterilized 10 ml 13% $\text{Ca}(\text{NO}_3)_2$) at 30°C to $A_{660} = 0.15 - 0.20$. The bacteria were pelleted by centrifugation for 10 min at 16,000 $\times g$.

Pea plants were grown in gravel as described by Bisseling *et al.* (13). Nodules from 20 day old plants were picked and immediately frozen in liquid nitrogen; 25 g of nodules were crushed in a cold mortar in 50 ml of cold 0.15 M sucrose, 25 mM Tris-HCl pH 7.4, 100 mg/l chloramphenicol (Sigma, St. Louis, MO, U.S.A.), and filtered through Miracloth. Bacteroids were pelleted by centrifugation for 7 min at 27,000 $\times g$ prior to RNA isolation, or purified by sucrose gradient centrifugation (2) for DNA isolation.

DNA isolation. Cell pellets from 1 l cultures were suspended in 30 ml of TE buffer (0.05 M Tris-HCl, 0.02 M EDTA pH 8.0). After addition of SDS (SDS: sodium dodecylsulfate) to 1% (w/v) (from 20% stock) and proteinase K (Merck, Darmstadt F.R.G., preincubated for 1 h at 37°C) to 10 $\mu\text{g/ml}$ (from 200 $\mu\text{g/ml}$ stock), the suspension was incubated for 20 min at room temperature. The resulting lysate was extracted 3 - 4 times with phenol/cresol (phenol: m-cresol: hydroxyquinoline = 1000 : 140 : 4 (w/w/w)), until the aqueous layer was clear. After extraction of the aqueous layer with 1 vol of chloroform the solution was made up to 0.2 M sodium acetate. Nucleic acids were precipitated overnight at -20°C after adding 2 vols of ethanol. After centrifugation the pellet was dissolved in 5 ml 0.1 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate pH 7.0). RNA was digested with 100 $\mu\text{g/ml}$ RNase A (Sigma) (preincubated for 10 min at 80°C) and 250 units/ml RNase T1 (Sigma) for 30 min at 37°C. After incubation with 10 $\mu\text{g/ml}$ proteinase K (30 min at 37°C in 1% SDS) and phenol/cresol and chloroform extraction the DNA was ethanol-precipitated.

Isolation of large plasmid DNA. The procedure used was a modification of the procedure of Hansen and Ohlsen (14). Bacteria were grown and lysed as above. Cells from 1 l cultures were suspended in 900 ml TE to give 1 l lysate. The pH of the lysate was increased to 12.3 by addition of 3 M NaOH with gently stirring. After 20 min the pH was lowered to 8.5 with 2 M Tris-HCl pH 7.0 and 5 M NaCl was added to a final concentration of 1 M. After 5 h at 0°C precipitated material was removed by centrifugation (15 min at 16,000 $\times g$). Floating material was removed with a strip of Whatmann 3 MM paper, and 50% (w/v) polyethylene glycol (PEG) was added to the remaining supernatant to a final concentration of 10% (w/v). After leaving overnight at 0°C crude plasmid DNA was pelleted

(10 min at 16,000 xg) and dissolved in 8.5 ml TE + 0.5% diethylpyrocarbonate (DEP). 9 g CsCl and 0.5 ml ethidium bromide (10 mg/ml) were added and equilibrium centrifugation was performed in a 75 Ti rotor (30,000 rpm, 64 h, 20°C) in a Spinco centrifuge. DNA bands were visualized with long wavelength UV light. The lower band was sucked into a syringe after puncturing the tube. The DNA sample was diluted with 1 vol of water and precipitated with 2 vols of ethanol.

Isolation of plasmid pSA 30 (15). All manipulations with bacteria containing recombinant DNA were performed under the guidelines of the Dutch Committee in charge of the control on genetic manipulation. *E.coli* K 12 strain GTM-4, containing plasmid pSA 30, was grown in a medium containing 1% bacto-tryptone, 1% NaCl, 0.5% yeast extract and 0.1% glucose (w/v) at 37°C in a shaking incubator. Chloramphenicol (170 mg/l) was added and incubation was continued for 16 h at 37°C. The cells were pelleted, suspended in 50 ml TE buffer and lysed by addition of SDS to a final concentration of 1% (w/v). The lysate was made up to 1 M NaCl (from 5 M stock solution) and incubated at 4°C for 5 h. Precipitated material was removed by centrifugation (15 min at 16,000 xg), the supernatant was extracted once with phenol/cresol and plasmid was precipitated at -20°C overnight by the addition of 1 vol of ethanol. The plasmid was further purified by CsCl equilibrium centrifugation as described above.

Restriction enzyme digestion and gel electrophoresis. 1 - 5 μ g of DNA was incubated in 20 μ l of 90 mM Tris-HCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol pH 7.9 and 6 units of EcoRI or HindIII (both from Boehringer, Mannheim FRG). After a 2 h incubation at 37°C ficoll (5% w/v final concentration) was added and electrophoresis was performed in a horizontal 0.7% agarose (Miles Laboratories, England) gel (autoclaved before pouring) buffered with 40 mM Tris-acetate, 10 mM EDTA pH 7.7 (16). After electrophoresis (2.5V/cm) for 16 h the gel was stained in electrophoresis buffer containing 0.5 μ g/ml ethidium bromide. The DNA fragments were visualised with long wavelength UV light and photographed. If restriction fragments were to be isolated from a gel, electrophoresis buffer without EDTA was used. The DNA was freeze-squeezed from frozen gel slices (17) and used directly for nick translation.

Southern blotting. DNA bands were transferred from the agarose gel to a nitrocellulose filter essentially according to Southern (11). After blotting overnight at 4°C the slots were marked on the nitrocellulose filter; the filter was washed in 3 x SSC and baked for 2 h at 80°C.

RNA isolation. For RNA isolation, bacteria were cultured to $A_{660} =$

0.15. Chloramphenicol (75 mg/l) and NaN_3 (final conc. 10 mM) were then added and the culture was poured onto ice. The cells were collected by centrifugation at 16,000 $\times g$ for 10 min. To isolate the bacteroid RNA the sucrose gradient centrifugation step was omitted. Lysis was performed as described for DNA isolation, except for the proteinase K treatment. The lysate was immediately extracted with phenol/cresol. After ethanol precipitation the nucleic acids were dissolved in 10 mM Tris-HCl, 10 mM MgCl_2 pH 7.6. DNA was digested with 50 $\mu\text{g/ml}$ DNase I (Sigma) for 30 min at 37°C. After phenol/cresol extraction, RNA was precipitated with ethanol.

In vitro labelling of RNA. The method used was essentially that of Goldbach *et al.* (18). Specific activities obtained were 2 - 10 $\times 10^6$ dpm/ μg RNA.

DNA-RNA hybridisations. Southern blots were preincubated for at least 6 h in 3 x SSC, 0.1 % SDS, 100 $\mu\text{g/ml}$ yeast RNA at 63°C. The filters were sealed in a plastic bag with 10^7 dpm (0.5 - 2 μg) *in vitro* labelled RNA in a volume of 1 ml 3 x SSC, 0.1% SDS, 100 $\mu\text{g/ml}$ yeast RNA, 50 $\mu\text{g/ml}$ rhizobium ribosomal RNA and hybridised for 38 - 40 h at 63°C. The filters were washed three times for 2 h in 50 ml of 3 x SSC at 63°C, dried and autoradiographed. In some cases a Fast Tungstate intensifying screen (Ilford, England) was used.

In vitro labelling of DNA. Nick translation of DNA was essentially according to Rigby *et al.* (19). Specific activities obtained ranged from 2 - 5 $\times 10^7$ dpm/ μg DNA.

DNA-DNA hybridisation. Southern blots were preincubated at 63°C for 30 min in 3 x SSC, then for 3 h in 3 x SSC, 0.2% (w/v) ficoll, 0.2% (w/v) bovine serum albumin, 0.2% (w/v) polyvinylpyrrolidone (Denhardt's solution, ref. 20) and finally for 1 h in Denhardt's solution supplemented with 0.1% (w/v) SDS, 50 $\mu\text{g/ml}$ low molecular weight denatured calf thymus DNA and 10 $\mu\text{g/ml}$ poly (A). Nick translated DNA was denatured in 0.1 x SSC at 100°C for 10 min. Hybridisation was performed in 1 ml of final incubation buffer supplemented with 0.2 - 1 $\times 10^6$ dpm denatured DNA, in a sealed plastic bag at 63°C for 40 h. After hybridisation the filters were washed three times for 2 h in 3 x SSC at 63°C, dried and autoradiographed.

RESULTS

To study the expression of large plasmids in *Rhizobium*, plasmid DNA was purified from broth-cultured *R. leguminosarum*, digested with EcoRI restriction endonuclease and subjected to agarose gel electrophoresis. The DNA fragment pattern was blotted onto nitrocellulose filters and hybridised with *in vitro*

labelled ^{32}P -RNA from both broth-cultured rhizobium bacteria and endosymbiotic bacteroids. The autoradiograms of these blots are shown in fig. 1, lanes 1 and 4. It can be seen that bacteroids (lane 4) contain RNA molecules which hybridise with plasmid DNA, whereas in free-living bacteria such RNA is not detectable (lane 1). The results indicate that plasmid DNA is actively transcribed in bacteroids but not in free-living bacteria. It has been verified that the bacteroid RNAs hybridisable to the DNA fragments are indeed plasmid transcripts and not transcripts from chromosomal DNA, which might contaminate the plasmid preparation used. For what purpose *EcoRI* digests of total DNA from rhizobium bacteroids were separated in an agarose gel, transferred to nitrocellulose filters and hybridised with ^{32}P -RNA from both bacteria and bacteroids (fig. 1, lanes 2 and 3). The hybridisation pattern of bacteroid RNA to plasmid DNA (lane 4) is markedly different from that of bacteroid RNA to total DNA (lane 3). This indicates that the plasmid DNA is (almost) free of chromosomal DNA. Thus the RNA molecules hybridised in lane 4 must be trans-

Fig. 1: Hybridisation of *Rhizobium leguminosarum* plasmid DNA and total DNA with RNA from free-living and endosymbiotic cells. Plasmid DNA (lanes 1, 4 and 5; 5 μg) and total DNA (lanes 2 and 3; 1 μg) were digested with *EcoRI*, separated in an agarose gel and blotted on a nitrocellulose filter. Hybridisations were carried out with 10^7 dpm *in vitro* labelled RNA from broth-cultured bacteria (lanes 1 and 2; 0.5 μg RNA) or endosymbiotic bacteroids (lanes 3 and 4; 2 μg RNA). Lane 5 shows the pattern of the ethidium bromide-stained restriction fragments of the plasmid DNA, prior to the blotting. Lane 6 contains DNA, digested with *HindIII*, as a marker.

cripts of plasmid DNA. In contrast to this the hybridisation of bacterial RNA to plasmid DNA develops a hybridisation pattern identical to bacterial RNA hybridised to total DNA, if exposed for a much longer period (results not shown). This must be a result of a minor contamination of plasmid DNA with chromosomal DNA. From the intensity of the hybridisation we estimate that the plasmid preparation used contains 2 - 4% chromosomal DNA. The differences in the hybridisation patterns of bacterial or bacteroid RNA to total DNA (cf. lanes 2 and 3) are probably due to dissimilarities in gene expression in bacteria and bacteroids; *e.g.* the fragment of M.W. 2.5×10^6 which is strongly expressed in bacteroids but not in bacteria possibly contains a gene which is functional during the symbiosis. The amount of plasmid DNA in the total DNA of *Rhizobium* has been estimated to be 3 - 4% of the total DNA (3). This amount is apparently too small to be able to detect plasmid specific hybridisation with bacteroid RNA (Fig. 1, lane 3).

The degree of expression of plasmid DNA in endosymbiotic bacteroids is further illustrated by the stained *Eco*RI fragment pattern of the plasmid shown in lane 5. To understand the pattern it is important to know that the plasmid preparation is a mixture of two plasmids. If the crude plasmid preparation (after the PEG precipitation) is separated in an agarose gel (ref. 5) two plasmid bands are visible of molecular weights of approximately 210 and 240×10^6

(Fig. 2). Because of the large size of both plasmids an *Eco*RI digestion of purified plasmid will result in a relatively large number of fragments of similar size, which in an agarose gel will migrate to similar positions. As a result most bands in fig. 1, lane 5 will be composed of more than one fragment. Comparison of the hybridisation pattern (lane 4) and the stained fragment pattern (lane 5) demonstrates that the RNA used in lane 4 hybridises with many DNA fragments. The reason for the difference in the amount of hybridisation of the various DNA fragments may be caused by a dissimilarity in the expression of the plasmid genes, or alternatively that the respective mRNAs are stable to different degrees.

Hybridisation of Rhizobium RNA and DNA with pSA 30. Plasmid pSA 30 (12) was used as a probe to detect the presence of *nif* specific mRNA in RNA prepared from broth-cultured rhizobia and bacteroids and subsequently to identify the rhizobium plasmid restriction fragment from which this RNA has been transcribed; pSA 30 contains the structural genes for nitrogenase of *Klebsiella pneumoniae* (12). The experiment shown in Fig. 3 indicates that the *Eco*RI fragment of pSA 30 which contains the *nif* genes hybridises with 32 P-RNA from

Fig. 2: Evidence for two plasmids in *Rhizobium leguminosarum* strain PRE. A plasmid preparation (after the PEG precipitation) was separated in an agarose gel and stained with ethidium bromide; the UV pattern is shown in lane 2. The arrows indicate the two *Rhizobium* plasmids (estimated molecular weights approximately 210 and 240×10^6 , based on renaturation kinetics, data not shown). Lane 1 contains a plasmid preparation from *Agrobacterium tumefaciens*. The arrow indicates the Ti plasmid of M.W. 120×10^6 , the upper band is a cryptic plasmid of M.W. $>214 \times 10^6$ (ref. 5).

rhizobium bacteroids, but there is no hybridisation detectable with RNA from broth-cultured bacteria (cf. lanes 1 and 2). This result demonstrates that there is *nif* specific mRNA in the bacteroid RNA preparation. As expected such RNA is absent in RNA from broth-cultured bacteria because the free-living bacteria do not synthesize nitrogenase (21).

Plasmid pSA 30 was furthermore used to identify the *rhizobium* DNA fragment from which the nitrogenase mRNA is transcribed. For that purpose purified plasmid DNA and total DNA of *Rhizobium leguminosarum* were digested with EcoRI, electrophoresed and blotted onto nitrocellulose, similarly to the method described above. However DNA-DNA hybridisation was now performed using ^{32}P -labelled pSA 30. The results of the hybridisation are shown in the autoradiograms presented in Fig. 4. It is clear that only one EcoRI fragment of the *rhizobium* plasmids shows hybridisation with pSA 30 (Fig. 4, lane 3). The fragment pattern of total DNA shows a faint hybridisation band in a similar position, caused by the 3 - 4% of plasmid DNA in the preparation (lane 1). To determine which part of pSA 30 hybridises with the *rhizobium* plasmid fragment, pSA 30 was digested with EcoRI and HindIII. This results in two vector fragments, a fragment with gene K and a fragment with the genes D and H (12).

Fig. 3: Hybridisation of rhizobium RNA to *Klebsiella pneumoniae* *nif* genes. Cloned *nif* genes of *K. pneumoniae* were digested with EcoRI, separated in an agarose gel, blotted on a nitrocellulose filter and hybridised to *in vitro* labelled RNA from rhizobium bacteria (lane 1) and bacteroids (lane 2). The arrow indicates the *nif* fragment (M.W. 4.6×10^6) which hybridises with bacteroid RNA.

These fragments were separated in an agarose gel, isolated from the gel (17) and labelled *in vitro* (19). Only fragment D + H appeared to hybridise with the plasmid fragment (Fig. 4, lane 2); gene K or the vector failed to hybridise (results not shown). This confirms the results of Ruvkun and Ausubel (22), who also found that only fragment D + H is homologous to rhizobium DNA. Thus, at least a part of gene D, coding for one of the subunits of component I, or gene H, coding for the subunit or component II, or both, are located on a rhizobium plasmid. Fig. 4, lane 4 (identical to fig 1, lane 4) shows that this fragment is relatively strongly expressed in nitrogen-fixing bacteroids.

DISCUSSION

In this paper evidence has been presented that in endosymbiotic *Rhizobium leguminosarum*, large plasmids are extensively transcribed. As a large number of EcoRI fragments of the plasmids hybridise to total bacteroid RNA a substan-

Fig. 4: Detection of *nif* genes in *Rhizobium*. Plasmid DNA (lanes 2 and 3; 1 μ g and 4; 5 μ g) and total DNA (lanes 1 and 5; 1 μ g) from rhizobium were digested with EcoRI, separated in an agarose gel and blotted on a nitrocellulose filter. Hybridisations were performed with *in vitro* labelled preparations of pSA 30 (lanes 1 and 3; 0.2×10^6 dpm, 4 - 10 ng DNA), fragment D + H (lane 2; 0.2×10^6 dpm, 4 - 10 ng DNA), and rhizobium bacteroid RNA (lanes 4 and 5; 10^7 dpm, 2 μ g RNA). The arrow indicates the position of the *nif* genes (approx. M.W. 4.3×10^6).

tial part of these plasmids seems to code for proteins playing a role during the symbiosis. The results presented substantiate the conclusions of other authors (7 - 10) that plasmids in *Rhizobium* must play an important role in the process of infection and nitrogen fixation. We were not able to detect transcription of plasmid DNA in broth-cultured rhizobia, suggesting that under these conditions plasmid genes are not expressed or only so to a limited extent. A plasmid function which is reported to be expressed in bacteria is the production of bacteriocin (23). The amount of mRNA transcribed from these genes is possibly too low to be detected above the background. We conclude that the large rhizobium plasmids contain at least part of the genes which are functional during the differentiation of bacteria into bacteroids and which are necessary for the infection process in legumes. Genetic and

biochemical analysis of plasmid borne genes will therefore be of great importance for the understanding of these phenomena.

Preparations of total DNA, consisting of about 96% chromosomal DNA, were found to be expressed in free-living as well as in endosymbiotic rhizobia, although the restriction fragments showed different degrees of hybridisation. We suppose that in both broth-cultured and endosymbiotic rhizobia similar genes involved in metabolic processes are expressed, although probably at different rates as a result of the different conditions to which the bacteria or bacteroids are subjected. From the difference in the hybridisation patterns of lanes 2 and 3 it is not possible to conclude whether or not chromosomal genes are exclusively expressed during the symbiosis, especially in this case (bacteroid RNA hybridised to bacteroid DNA) because some hybridisation of contaminating plant material cannot be excluded. Experiments are in progress to decide whether the hybridisation at M.W. 2.5×10^6 (Fig. 1, lane 3) originates from *Rhizobium* genes.

The presence of *nif* specific mRNA in preparations from endosymbiotic rhizobia was shown by hybridisation to the cloned structural *nif* genes from *Klebsiella pneumoniae* (Fig. 3). This mRNA is transcribed from a plasmid, as is shown by the hybridisation of *K. pneumoniae nif* DNA to rhizobium DNA (Fig. 4). Not all the *nif* genes are homologous; only the EcoRI + HindIII fragment D + H, containing the *K. pneumoniae* genes coding for one of the component I subunits plus component II, hybridises (lane 2), as also described by Ruvkun and Ausubel (22). It cannot be ruled out however that only a part of the genes D or H is sufficient homologous to the corresponding gene in *Rhizobium* to hybridise.

Our results indicate that in *Rhizobium* not only part of the genes for nitrogenase are localised on a plasmid, but many other genes involved in symbiosis and nitrogen fixation.

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