MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 1, pp. 9-17, 1990

Changes in the *Rhizobium meliloti* Genome and the Ability to Detect Supercoiled Plasmids During Bacteroid Development

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Received 19 September 1988. Accepted 15 August 1989.

Medicago sativa **(alfalfa) roots were infected with** ***Rhizobium meliloti*** **strain Balsac. Nodule extracts were fractionated isopycnically on Percoll density gradients into nodule bacteria, transforming bacteria, and mature bacteroids. The average DNA content of mature bacteroids was found to be double that of free-living cells grown in liquid culture to stationary phase. This corresponded to the ratio of discrete nucleoid structures observed in stained cells of the two types. Probing blots of restricted total cellular DNA with plasmid-specific and other DNA probes, including** ***nod, nif, exo, dct,*** **and other** ***fix*** **gene regions, gave no indication of structural rearrangement or unequal amplification of the genome during bacteroid development. Supercoiled plasmids of Balsac were detected in Eckhardt gels and purified on ethidium bromide-CsCl density gradients from free-living cells but not from mature bacteroids. Transforming bacteria were intermediate in that plasmids other than megaplasmids were detected in gels. We conclude that plasmid supercoiling is not maintained in nitrogen-fixing bacteroids in the nodule or is readily lost upon their extraction, or both. We suggest that modification of the DNA superhelical structure may be important in bacteroid development and for the expression of symbiotic genes in** ***R. meliloti.***

Additional keywords: DNA rearrangement, nicking, topoisomerase.

Free-living bacteria of the genus *Rhizobium* infect the roots of specific legumes and stimulate the formation of nodules (reviewed by Rolfe and Gresshoff 1988). Some vegetative cells are always present in infection threads, but the majority eventually differentiate to form plant-dependent bacteroids. These specialized cells reduce atmospheric nitrogen to ammonia, the major portion of which is used by the plant (reviewed by Bergersen 1982; Guerinot and Chelm 1987). The symbiosis is tightly controlled by interacting plant and bacterial genes and signals (reviewed by Verma and Long 1983; Halverson and Stacey 1986; Watson 1989).

The development of bacteria to form nitrogen-fixing bacteroids involves complex morphological, physiological, and biochemical changes (reviewed by Newcomb 1981). In *R. meliloti* Dangeard there is an apparent loss of the ability of cells to divide such that bacteroids are nonculturable (Sutton and Paterson 1983; Cao *et al.* 1984; McRae *et al.* 1989). In previous work, changes in the cytoplasmic membrane were investigated; these changes may be associated with the inhibition of cell division (Miller and Tremblay 1983; Tremblay and Miller 1984). Here, we have looked directly for an underlying change in the DNA of differentiating cells that might initiate these and other irreversible changes during bacteroid development.

One possibility is a sequence rearrangement comparable to that reported for nitrogen-fixing heterocystic cyanobacteria (Haselkorn *et al.* 1987) and for other organisms (reviewed by Borst and Greaves 1987). Another possibility is the modification of the superhelical structure of DNA essential for normal cell function, comparable to the changes that accompany growth transitions of *Escherichia coli* (Migula) Castellani and Chalmers (Balke and Gralla 1987; Dorman *et al.* 1988). Such changes in DNA supercoiling are implicated in the regulation of asymbiotic nitrogen fixation in *Klebsiella pneumoniae* (Schroeter) Trevisan (Dimri and Das 1988; Dixon *et al.* 1988), *Rhodopseudomonas capsulata* (Molisch) van Niel (Kranz and Haselkorn 1986), and *Bradyrhizobium* sp. (Gober and Kashket 1989).

Previous studies of symbiotic rhizobia have shown no difference in the buoyant density, melting temperature, molecular weight, or kinetic complexity of DNA from free-living bacteria and bacteroids (Sutton 1974; Reijnders *et al.* 1975). However, differences in the amount of DNA per cell have been reported (Bisseling *et al.* 1977; Paau *et al.* 1978, 1979a, 1979b). In *R. meliloti*, the DNA was found to be higher in mature bacteroids, although no specific amplification or rearrangement of the nitrogenase genes was detected in strain 102F51 (Paau and Brill 1982).

This study of *R. meliloti* strain Balsac supports and expands upon these earlier reports. We obtained the same general fractionation of nodule extracts into nodule bacteria, transforming bacteria, and mature bacteroids as did Ching *et al.* (1977). However, isotonic Percoll gradients were used in place of sucrose gradients, allowing bacteroids to be obtained intact and undamaged (Reibach *et al.* 1981; McRae *et al.* 1989). The DNA content of nodule-fraction and free-living cells was estimated, and Southern blots of restriction fragments were compared by probing. Despite an apparent doubling of the DNA content per cell, no specific amplification or rearrangement of any plasmid or symbiotic gene region was detected that was associated with bacteroid development.

Supercoiled plasmid DNA from free-living cells of Balsac was purified on an ethidium bromide-CsCl density gradient;

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three plasmid bands were resolved in Eckhardt gels, including a possible multiple megaplasmid band (Banfalvi *et al.* 1985). Because supercoiled plasmids were not detected in mature bacteroids in these experiments, we have used gels to determine the stage of development at which they cease to be detectable. The timing was found to be consistent with the arrest of division of differentiating cells in the nodule and with the doubling of their DNA content.

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* strain Balsac was obtained from L. Bordeleau, Agriculture Canada, Ste. Foy Research Station, Quebec, and identified thereafter by IS*Rm1* (IS designating insertion sequence) probing (Wheatcroft and Watson 1988a). It is a natural isolate, formerly known as strain A2 and currently used commercially as a soil inoculant (Bordeleau *et al.* 1977). Its physiology and development in the nodule are well-documented (Bordeleau *et al.* 1977, 1981; Miller *et al.* 1988). Balsac(RP4) (RmRW880), which does not require tryptophan for growth (Trp^+) and is resistant to kanamycin (Km^r) and tetracycline (Tc^r), is an exconjugant resulting from the filter mating of Balsac and *Pseudomonas putida* (Trevisan) Migula (RP4) (PpRW246), which requires tryptophan for growth (Trp^-) and is Km^r and Tc^r (this laboratory). It was selected on M9 plates (Maniatis *et al.* 1982) containing Tc (10 μg/ml). *R. meliloti* strain 1021 (Meade *et al.* 1982) was obtained from S. R. Long, Stanford University, Stanford, CA.

R. meliloti cultures were grown at 30° C in yeast-mannitol broth (YM) (Vincent 1970). To stain nucleoids in both bacteroids and free-living bacteria, cells were suspended in 125 mM KCl, 50 mM sodium succinate, 0.1% (w/v) bovine serum albumin (BSA), 50 mM Tes-KOH (pH 8.0) containing ethidium bromide (25 μg/ml) or Hoechst 33258 (25 μg/ml). Unincorporated dye was removed from the cells by centrifugation and washing with fresh medium. Photomicrographs were taken of UV-fluorescent cells immobilized on detergent-cleaned slides coated with polylysine (M_r 800,000).

Bacteroid isolation and fractionation. Seedlings of *Medicago sativa* L. (alfalfa cv. Saranac) were inoculated with *R. meliloti* strain Balsac and grown in vermiculite supplied with nitrogen-free medium under controlled environmental conditions as previously described (Miller *et al.* 1988). Nodules (8–10 g fresh weight) harvested 7 wk after inoculation were surface-sterilized with 20% (v/v) household bleach (6% sodium hypochlorite) for 2 min, followed by 10 washes with sterile distilled water. The crude nodule extracts, containing bacteroids with high levels of respiration-supported nitrogenase activity, were obtained as previously described (Miller *et al.* 1988). Samples were then resuspended in 1 ml of 50 mM sodium succinate, 125 mM KCl, 50 mM Tes-KOH buffer (pH 7.0) and layered over 55% (v/v) sterile Percoll suspension (Sigma, St. Louis, MO).

After centrifuguation at 48,000 × *g* for 30 min, two distinct bands were obtained. Cells from each band were pelleted at 4,000 × *g* and washed in fresh medium to remove Percoll. The upper band contained mainly mature bacteroids and possessed more than 90% of the total nitrogenase activity of crude nodule extracts. The more dense fraction was recentrifuged in 70% Percoll, and cells from the two resulting bands were collected and washed free of Percoll as was done previously. The band of lower density contained transforming bacteria distinguishable by their cellular morphology (enlarged rods) and minimal nitrogenase activity. The most dense band contained mainly undifferentiated cells (nodule bacteria) having no nitrogenase activity and resembling free-living *R. meliloti* cells except for the reduced size of polyhydroxybutyrate inclusions (McRae *et al.* 1989).

A series of modifications was made to the procedure above in attempts to detect supercoiled plasmids in mature bacteroids. These included the following: 1) substitution with nonionic 0.4 M sorbitol for 125 mM KCl in the extraction medium; 2) anaerobic (argon) extraction; 3) extraction at O° C throughout; and 4) extraction in the presence of DNase inhibitor (diethylpyrocarbonate or EDTA).

DNA manipulations. With the exception of mature bacteroids, plasmid bands were visualized in *R. meliloti* cell samples separated by electrophoresis in horizontal agarose gels using a modification of the method of Eckhardt (1978). Routinely, the number of cells equivalent to 1 ml of YM culture of free-living cells at $OD_{620} = 0.2$ was harvested by centrifugation. The pellet was suspended in 0.5 ml of water or bacteroid extraction medium at 4° C. The cell suspension was then layered onto 1 ml of ice-cold 0.3% sodium sarcosinate. Following centrifugation, all traces of supernatant were removed from the pellet, which was resuspended in 40 μl of 10 mM Tris, 10 mM EDTA, 20% (w/v) Ficoll (M_r 400,000), or this solution was made isotonic for bacteroids by including 0.4 M sorbitol. Samples were then held on ice. An 0.8% (w/v) agarose gel (5-mm thick) in 89 mM Tris, 2.5 mM EDTA, 89 mM boric acid (Greene *et al.* 1974) was prepared by loading 25 μl of 10% (w/v) sodium dodecyl sulfate (SDS) containing xylene

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pBB100	Cloned *nodDABC/hsn* gene region (34 kilobase [kb]) of *nod* megaplasmid of *Rhizobium meliloti* JJ1c10, pRmeJJ1c10a	Selvaraj *et al.* 1987
pBB113	Cloned *nifHDK/fix* gene region (28 kb) of pRmeJJ1c10a	Selvaraj *et al.* 1987
pBB119	Cloned *dct* gene region (33 kb) of *exo* megaplasmid of JJ1c10, pRmeJJ1c10b	Watson *et al.* 1988
pBR322	4.4 kb	Bolivar *et al.* 1977
pRmT9	Cloned *exoA* gene region (20 kb)[a] of *exo* megaplasmid of *R. meliloti* 1021, pRmeSu47b	Finan *et al.* 1986
pRWRm10	Cloned *nodDABC* gene region (8.7 kb) of pRmeJJ1c10a	Wheatcroft and Watson 1987
pRWRm13	Cloned *Bgl*II-*Eco*RV IS*Rm1* fragment (0.9 kb) in pUC9	Wheatcroft and Watson 1987
pUC9	Cloning vector (2.7 kb)	Vieira and Messing 1982
RP4	Broad host range (60 kb)	Datta *et al.* 1971

[a]Size determined by S. T. Whitwill, Plant Research Centre, Agriculture Canada (personal communication); other sizes are given as published.

cyanole FF (1 mg/ml) into each origin slot and backtracking at 100 V for 15 min so that the dye migrated 1 cm behind the origin line. The gel was then flooded with buffer. Next, 10 μl of a lytic agent containing 10 mM Tris, 10 mM EDTA, boiled RNase Type A (0.4 mg/ml), lysozyme (1 mg/ml), bromphenol red (1 mg/ml) was mixed into each cell sample, of which 25 μl was loaded into the gel under the buffer layer. The polarity of the gel electrodes was reversed after the backtracking step for electrophoresis at 40 V for 30 min followed by 100 V for 3 hr. The gel was stained in ethidium bromide (0.5 μg/ml) for 20 min, destained for 1 hr in 1 L of water, and photographed on a short-wavelength UV-transilluminator.

A series of modifications was made to the procedure above in further attempts to visualize plasmid bands in gels of mature bacteroid samples. In each test, the concentration of cells extracted and loaded into the gel was varied by 0.5, 1, 2, and 4 times the optimum for resolution of plasmids from a sample of free-living cells. The tests comprised the following: 1) omission of sodium sarcosinate to avoid premature lysis of cells during washing, although plasmid resolution from free-living cells was then poor; 2) addition of proteinase K (0.4 mg/ml) to the lytic agent; 3) encapsulation of samples in 1% (w/v) agarose blocks for loading gels as described by Smith *et al.* (1988); 4) variation of the concentration of SDS used in backtracking, although plasmids were not detected in free-living cells using less than 1% (w/v), resolution was enchanced up to 10% (w/v); and 5) incorporation of ethidium bromide (0.05, 0.1, 0.5, and 1.0 μg/ml) or chloroquine (25 and 1,000 μg/ml) into gels to alter plasmid superhelical structure and mobility. After electrophoresis, chloroquine gels were rinsed for 24 hr with water and then stained with ethidium bromide.

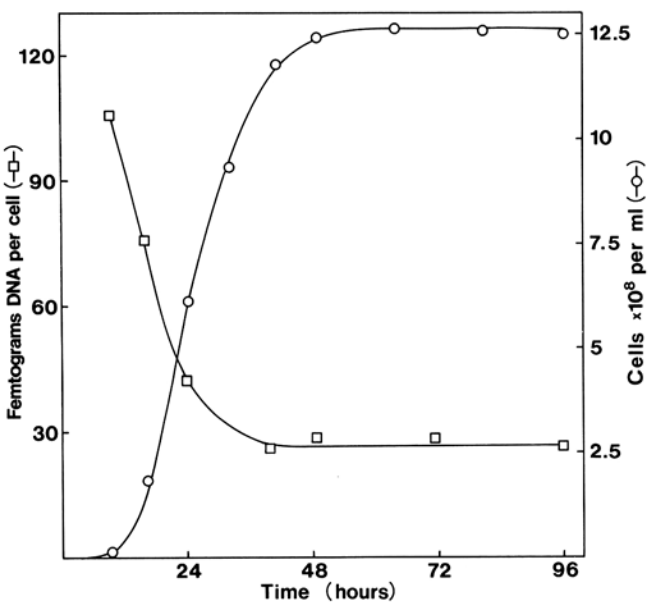

Fig. 1. Number (○) and DNA content (□) of free-living *Rhizobium meliloti* strain Balsac cells cultured in yeast-mannitol culture at 30° C. Cell counts were made using a Petroff-Hausser bacterial cell counter under a phase-contrast microscope. Cells were extracted and purified DNA was measured by UV absorption; the average DNA content of mature bacteroids was about twice that of free-living cells in stationary phase.

Plasmid DNA of Balsac free-living cells was visualized on an ethidium bromide-CsCl density gradient following extraction from 40 ml of a 24-hr-old YM culture. The equivalent number of cells of mature bacteroids was extracted identically for comparison. After harvest by centrifugation, cells were resuspended in 2 ml of ice-cold 0.4 M sorbitol, 50 mM sodium succinate, 0.1% (w/v) BSA, 50 mM Tes-KOH (pH 7.0) and transferred to a 27-ml Beckman tube suitable for density gradient centrifugation, then 0.5 ml of lysozyme (10 mg/ml) was added with gentle swirling. After 30 min of incubation on ice, 0.5 ml of 2% (w/v) sodium sarcosinate and 0.5 ml of ethidium bromide (10 mg/ml) were added, followed by the addition of 22.2 g CsCl in 18 ml of buffer containing 8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50 mM EDTA, 10 mM Tris-HCl (pH 7.0). The tubes were heat-sealed and inverted to mix the contents gently; centrifugation was then conducted to equilibrium at 42,000 rpm for 60 hr at 15° C.

Total cellular DNA was purified as described previously (Wheatcroft and Watson 1988a), except where cells were pretreated with DNase I (Boehringer Mannheim, Dorval, Quebec, Canada) in 5 mM $MgCl_2$ for 1 hr at 25° C followed by 15 min at 45° C to inactivate the enzyme. DNA yield was measured by UV absorption (Maniatis *et al.* 1982). Routinely, 2 μg of DNA was restricted with endonucleases according to the supplier's recommendations (Boehringer Mannheim), and restriction fragments were separated by electrophoresis in denaturing and nondenaturing gels as described by Ogden and Adams (1987). Plasmid DNA in Eckhardt gels was fragmented for blotting by exposure on a short-wavelength UV-transilluminator for 10 min. Blots were prepared as described by Southern (1975) using Biodyne membranes (Pall Ultrafine Filtration Corp., Glen Cove, NY). These were then irradiated with short-wavelength UV to cross-link the DNA to the membrane (Church and Gilbert 1984) and dried at 80° C for 2 hr under vacuum.

Plasmids used as probes are given in Table 1. DNA purification, nick translation, hybridization, and autoradiography were as described previously (Wheatcroft and Watson 1987). Membranes were reprobed successively and

Table 2. Estimated DNA content and distribution of nucleoids in cells of *Rhizobium meliloti* strain Balsac[a]

Cell type	Mean DNA content per cell[b,c] ($\times 10^{-15}$ g)	Number of cells[d] with 1 UV-fluorescent nucleoids[e]	2	3	4	Mean nucleoid content per cell
Free-living cells (stationary-phase culture)	28 ± 3 (6)	36	12	0	0 (48)	1.25 ± 0.4
Nodule bacteria	24 (2)					
Transforming bacteria	26 (2)					
Mature bacteroids	59 ± 5 (6)	1	30	18	4 (53)	2.47 ± 0.7

[a] Mean values are given ± standard errors where applicable.
[b] The number of replicates (separate experiments) is given in parentheses.
[c] Nodule fractions were treated with DNase I before extraction to remove extraneous plant DNA.
[d] Cells were stained with ethidium bromide; the totals counted in UV micrographs are given in parentheses.
[e] Unresolved doublets were counted as two.

were stripped after each round of autoradiography in 50% (v/v) formamide for 1 hr at 65° C.

RESULTS

DNA content of *R. meliloti* cells in culture and nodule fractions. The number and DNA content of free-living cells of *R. meliloti* strain Balsac grown in 1 L of YM at 30° C were estimated at intervals to 96 hr (Fig. 1). The mean amount of DNA extracted per cell was found to be inversely related to the cell density in culture, falling to its lowest level ($28 \pm 3 \times 10^{-15}$ g) in stationary-phase cells.

Similar estimates of DNA content were made for nodule bacteria, transforming bacteria, and mature bacteroids separated from crude nodule extracts by Percoll density gradient centrifugation (Table 2). The mean DNA content per cell of mature bacteroids ($59 \pm 5 \times 10^{-15}$ g) was found to be double that of the other *R. meliloti* cell types in the nodule, which gave results comparable to stationary-phase cells in culture.

Mature bacteroids and free-living cells were stained with dyes that bind DNA. UV-fluorescent nucleoids were observed with both ethidium bromide and Hoechst 33258 (Fig. 2). The mean nucleoid content of mature bacteroids was twice that of free-living stationary-phase cells, consistent with the ratio of DNA extracted (Table 2).

Detection of supercoiled plasmids in free-living and differentiating cells. Cells were lysed in the origin slots of horizontal agarose gels, and UV-fluorescent plasmid profiles were obtained after electrophoresis and staining with ethidium bromide. Free-living cells of Balsac were harvested during YM culture up to 96 hr and consistently gave three distinct plasmid bands (Fig. 3, lane 1). By probing Southern blots, it was shown that DNA of the megaplasmid band hybridized with the following symbiotic gene probes: pRmT9 (*exo*), pRWRm10 (*nod*), and pBB119 (*dct*) (Fig. 3, lanes 2–4). The probe pRWRm13, which is specific for the insertion sequence IS*Rm1*, showed that at least one of the two copies present in the Balsac genome (Wheatcroft and Watson 1988b) is present on a cryptic plasmid which is not a megaplasmid (Fig. 3, lane 5).

The plasmid profiles of Balsac cells from the three nodule fractions differed from one another. Nodule bacteria gave three bands equivalent to free-living bacteria (Fig. 4A, lanes

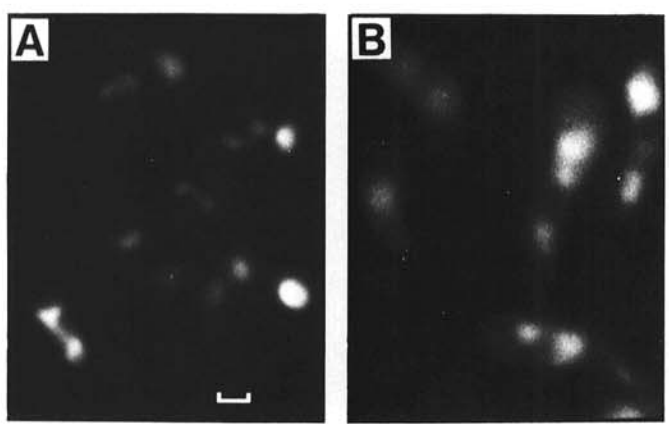

Fig. 2. Micrographs of *Rhizobium meliloti* strain Balsac cells stained with ethidium bromide at pH 8.0 to show cell size and UV-fluorescent nucleoids of condensed DNA. **A**, Free-living bacteria from yeast-mannitol culture in stationary phase. **B**, Mature bacteroids isolated from nodules 7 wk after plant inoculation. The ratio of increase of both the number of nucleoids, frequently as unresolved doublets in bacteroids not observed in electron micrographs (McRae *et al.* 1989), and the DNA content per cell was approximately two. Bacteroids, but not free-living cells, were also effectively stained with ethidium bromide at pH 7.0. Scale bar, 1 μm.

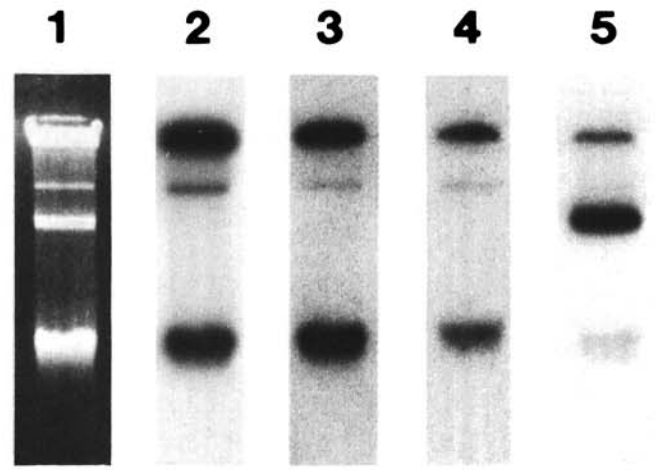

Fig. 3. Hybridization of DNA probes to plasmids of *Rhizobium meliloti* strain Balsac. Lane 1, ethidium bromide-stained agarose gel showing plasmids of free-living bacteria from a 24-hr-old yeast-mannitol culture separated by electrophoresis. Lanes 2–5, autoradiographs of a Southern blot probed or reprobed with the following plasmids: lane 2, pRmT9 (*exo*); lane 3, pRWRm10 (*nod*); lane 4, pBB119 (*dct*); and lane 5, pRWRm13 (IS*Rm1*).

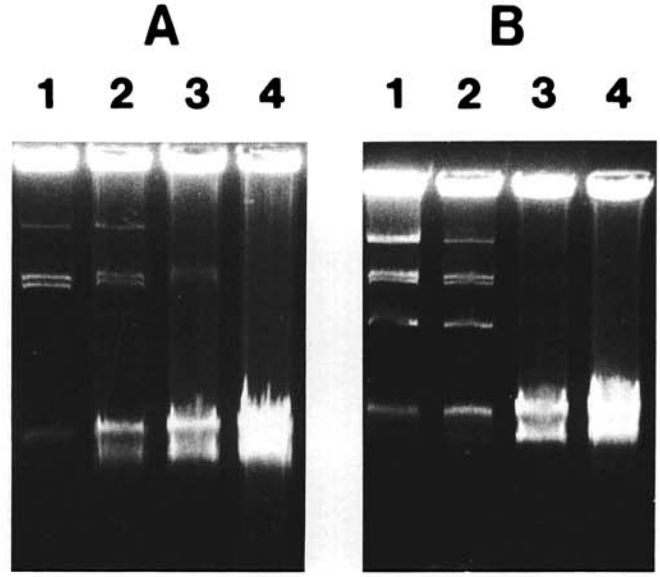

Fig. 4. *Rhizobium meliloti* plasmid detection in horizontal Eckhardt gels after staining with ethidium bromide. **A**, Balsac. **B**, Balsac(RP4). Lane 1, free-living bacteria from a 24-hr-old yeast-mannitol culture; lane 2, nodule bacteria isolated 7 wk after plant inoculation; lane 3, transforming bacteria showing loss of resolution of the megaplasmid band; and lane 4, mature bacteroids showing no plasmid bands. Each sample contained an equivalent number of cells. A progressive increase in the amount of sheared DNA is observed, some of which is plant DNA (bottom band). The presence of RP4 in the cells had no detectable effect on nitrogenase activity of isolated bacteroids, which was measured by the production of ethylene from acetylene.

1 and 2). Transforming bacteria did not give a distinct megaplasmid band although the other two plasmid bands were clearly seen (Fig. 4A, lane 3). However, no plasmid bands were ever detected from mature bacteroids of either Balsac (Fig. 4A, lane 4) or strain 1021, included for comparison. To verify that the apparent loss of plasmid bands associated with bacteroid development was a general phenomenon and not a property peculiar to indigenous plasmids of Balsac, a derivative (RmRW880) containing the promiscuous plasmid RP4 was tested. RP4, which is smaller than any plasmid of Balsac, was detected in ethidium bromide-stained gels in transforming bacteria but not in mature bacteroids (Fig. 4B, lanes 3 and 4).

A series of modifications to the methods (see Materials and Methods) was made in an extensive, yet unsuccessful, attempt to detect plasmid bands in mature bacteroid samples in gels. A test was made to determine whether a component of bacteroid preparations was responsible for this consistent failure. Samples of free-living bacteria and mature bacteroids were mixed and lysed together. The plasmid profile for free-living bacteria, including the megaplasmid band, was unaffected in the resultant gel, showing that bacteroid preparations did not themselves degrade plasmid DNA or make plasmids undetectable.

The possibility that plasmid DNA of bacteroids might be severely degraded during extraction was tested further. pBR322 DNA was added to Balsac bacteroid samples, which were then treated with DNase I. Restriction fragments of the total DNA extracted after inactivation of the DNase I were probed with pRWRm13, which contains cloning vector sequences homologous to pBR322. The complete degradation of pBR322 DNA was demonstrated with no apparent loss of Balsac IS*Rm1* markers. This result indicated that plasmid DNA was conserved in the bacteroids and that lysis had not occurred precociously.

We concluded from these results that if plasmids were present as separate entities in mature bacteroids of Balsac, they were in a form different from that of free-living cells such that they could not be resolved by gel electrophoresis. The possibility was therefore tested that bacteroid plasmids were intact, covalently closed molecules but with reduced superhelix density such that their entry into a gel would be prevented by their enlarged size. Gels containing ethidium bromide or chloroquine, both used to assay DNA supercoiling in much smaller plasmids, did not show any plasmid bands in mature bacteroid samples. This result indicated that the plasmids may not be maintained as intact, covalently closed molecules and thus might have contributed disproportionately to the amount of sheared DNA detected in gels of bacteroid samples (Fig. 4).

The hypothesis was tested that the plasmid DNA of bacteroids was extensively nicked in the component strands, yet had retained its integrity as a duplex still capable of being cleaved by restriction endonucleases. pRmT9 and pRWRm13 were used to probe for the plasmids of Balsac and strain 1021, respectively, comparing restricted DNA of bacteroids and free-living cells in samples denatured before electrophoresis or run under denaturing gel conditions (pH 12.5). No differences or evidence of degradation correlated with bacteroid development was detected indicating that bacteroid plasmid DNA was not extensively nicked at random (Fig. 5, lanes 8 and 9).

Comparison of symbiotic gene regions of bacteroid and free-living cells. The following hybridization experiments were conducted to seek evidence of plasmid integration into the chromosome or other reorganization during bacteroid development. Balsac DNA, purified from mature bacteroids and free-living cells, was digested with various restriction enzymes, blotted, and probed with the following megaplasmid probes: pRmT9, pBB100, pBB113, and pBB119. No gain, loss, or rearrangement of restriction fragments that hybridized was detected (Fig. 5, lanes 1–4). Moreover, when pRWRm13 was used to probe for sequences of the cryptic plasmid in Balsac (Fig. 3), and RP4 in RmRW880, no change was observed in autoradiograms either in the position or relative intensity of bands (Fig. 5, lanes 5 and 6). This was also true using pRWRm13 as a probe for the copies of IS*Rm1* dispersed throughout the megaplasmids and the chromosome of strain 1021 (Fig. 5, lane 7). Thus, no gross reorganization of plasmid structure, chromosome integration, specific gene amplification, or change in plasmid copy number relative to the chromosome was detected that was associated with the development of bacteroids.

CsCl density gradient analysis of the mature bacteroid nodule fraction. A test was made to determine whether plasmid DNA could be detected on ethidium bromide-CsCl density gradients of bacteroid preparations as an alternative to gel electrophoresis. The control of free-living Balsac cells gave a fluorescent band of supercoiled plasmid DNA; no band of equivalent density was seen in samples from mature bacteroids of Balsac or strain 1021, but a satellite band of DNA less dense than the main band was observed (Fig. 6).

The vulnerability of plasmid DNA supercoiling was demonstrated by subjecting the gradient samples of free-living cells to either shear forces, sonication, or UV treatment. When recentrifuged, the plasmid band was detected only in the untreated control; no new band, comparable to the satellite from bacteroids, appeared on the gradients of the treated samples.

The satellite band, which occurred consistently on the ethidium bromide-CsCl density gradients of mature bacteroid preparations but not of nodule bacteria, was extracted and used as a probe. It proved to consist of plant DNA with no detectable homology to the *R. meliloti* genome. Incubation with DNase I, before the extraction of the bacteroid DNA, eliminated only DNA of plant origin, demonstrating that it was located outside the bacteroid cell membranes.

DISCUSSION

Three distinct cell fractions of *R. meliloti* strain Balsac were obtained from crude nodule extracts separated by density on Percoll gradients. Each fraction was characterized by a predominant cell type. We noted a difference in that the supercoiled megaplasmid band which was present in the gel profiles of free-living and undifferentiated nodule bacteria was not visible in gels prepared from

transforming bacteria or mature bacteroids. The possibility of the complete loss of megaplasmid DNA from these cells was excluded by probing.

Symbiotic genes, shown to be carried on megaplasmids, were used as probes to test for specific amplification or rearrangement of restriction fragments in regions that are involved in nodulation and bacteroid development. Paau and Brill (1982) found no evidence for DNA rearrangement associated with bacteroid development in about 5 kilobases (kb) of the *nif* gene region of *R. meliloti* 102F51. Our results for Balsac were in agreement, following a more extensive (yet still far from complete) coverage of the pSym (*nod*) megaplasmid, including about 60 kb with homology to the *nod, hsn, nif*, and *fix* gene regions cloned from strain JJ1c10 (Selvaraj *et al.* 1987). In addition, we surveyed the regions with homology to *exo* and *dct* genes, which are also required for symbiosis and are located on a separate *exo* megaplasmid in other strains (Finan *et al.* 1986; Watson *et al.* 1988). We found no evidence of structural rearrangement or specific amplification involving any megaplasmid region, although integration into the chromosome would not necessarily have been detected with these probes.

Probing bacteroid DNA of strain 1021 for the insertion sequence IS*Rm1* gave a simultaneous assay of both the chromosome and megaplasmids, because each replicon contains multiple copies in this strain (Wheatcroft and Watson 1988b). No differences were observed in the patterns or relative intensity of hybridization bands in autoradiograms, indicating that there was no integration into the chromosome or change in copy number of symbiotic plasmids relative to the chromosome during bacteroid development. This was also shown for plasmids other than megaplasmids in Balsac and an RP4-containing derivative.

The lack of amplification of symbiotic plasmids relative to the chromosome is a significant new observation. With the lack of specific gene amplification, we clearly ruled out a major change in symbiotic gene dosage during bacteroid development, though in Balsac a modest general increase in the total DNA content of cells was observed. The latter was apparent only in the mature bacteroid fraction and may be explained by a final round of DNA synthesis when cells have ceased to divide in the nodule. This explanation is consistent with our observation of a doubling in the number of nucleoids per cell. It follows that the arrest of cell division in Balsac bacteroid development (McRae *et al.* 1989) is not caused by inhibition or failure of DNA replication before nucleoid partition. However, postreplicative DNA conformational change is not excluded and must be inferred to explain the absence

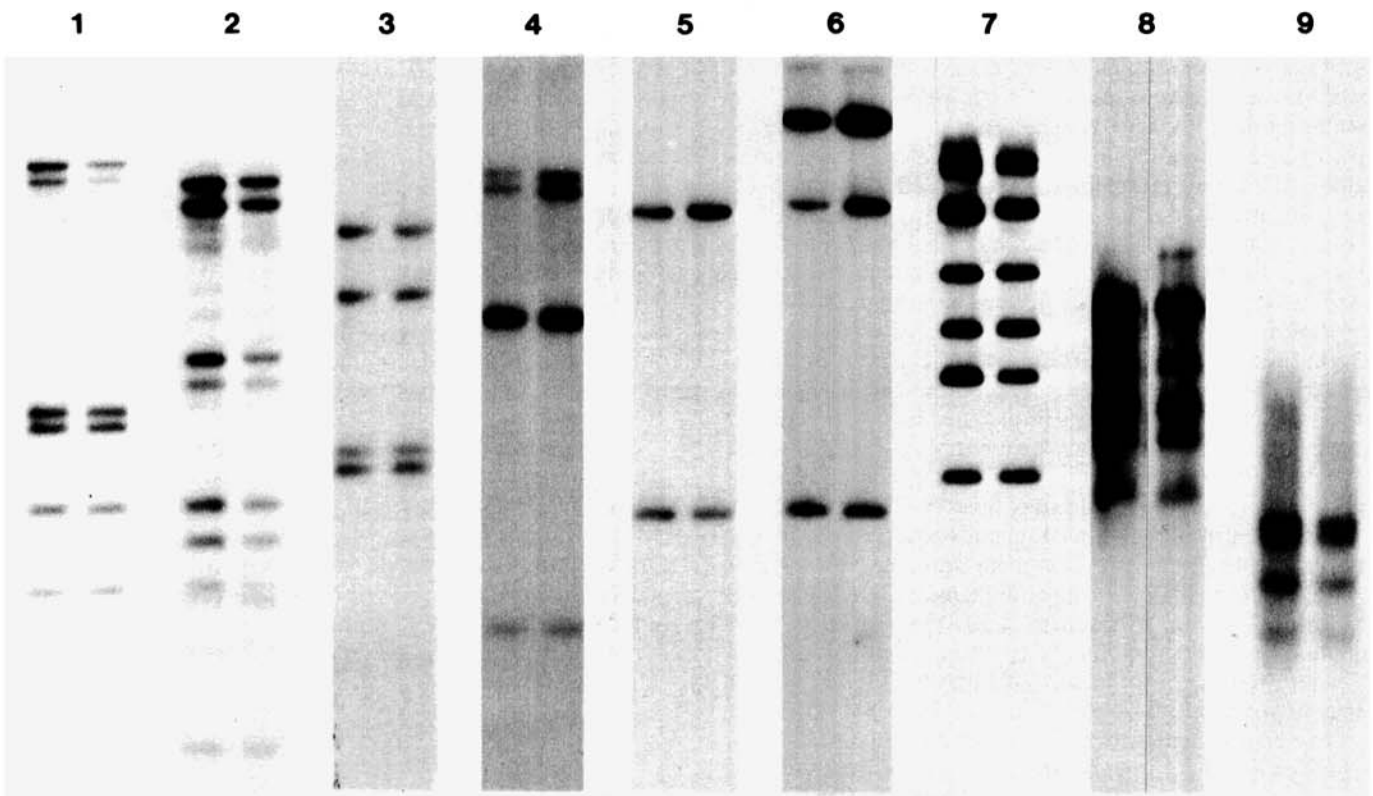

Fig. 5. Autoradiographs demonstrating the congruence of hybridization of various probes to DNA extracted at different stages of *Rhizobium meliloti* development. In each pair of lanes, a *Hin*dIII digest of 2 μg of total cellular DNA of free-living bacteria from a 24-hr-old yeast-mannitol culture is shown on the left, and that of mature bacteroids, isolated from nodules 7 wk after plant inoculation, is on the right. Pairs 1–5 and 9, Balsac; pair 6, Balsac(RP4); and pairs 7 and 8, strain 1021. The probes used were: pairs 1 and 9, pRmT9 (*exoA* gene region); pair 2, pBB100 (*nod/hsn* gene region); pair 3, pBB113 (*nif/fix* gene region); pair 4, pBB119 (*dct* gene region); and pairs 5, 6, 7, and 8, pRWRm13 (IS*Rm1*). Pair 8, before loading, the gel samples were denatured by boiling for 5 min and then rapidly chilled; pair 9, samples were run under denaturing conditions in an alkaline agarose gel (McDonell *et al.* 1977). The hybridization banding patterns were produced by restriction fragments ranging in size from 0.5 to 60 kilobases; in pairs 1–7, gel migration distances are directly comparable.

of detectable nucleoids in electron micrographs of mature bacteroids (Paau *et al.* 1978; McRae *et al.* 1989).

The timing of these changes is relevant because the loss of detectable supercoiled plasmids was more pronounced in mature bacteroids than in transforming bacteria such that even the smaller plasmids of Balsac were not detected. The presence of traces of these smaller plasmids in the gel profiles of transforming bacteria suggested that this cell type was genuinely a transitional stage in the development of the bacteroid and not an alternative terminal phenotype. Following an extraction procedure involving minimum manipulation, supercoiled plasmid DNA was detected in CsCl density gradients from free-living cells but not from mature bacteroids.

These results can be explained either by a conformational change of plasmids taking place in differentiating cells *in vivo* or as an artifact of their fractionation and extraction. Steps were taken to address the latter possibility and to minimize the loss of DNA supercoiling *in vitro*. In particular, experiments were designed to prevent uncontrolled cell lysis, because bacteroid membranes differ from those of free-living cells (Miller and Tremblay 1983; Tremblay and Miller 1984) and there are no comparable cell walls (Bal and Wong 1982). For example, the use of detergents was modified and different cell-support media were tried. In other experiments, the generation of oxygen-free radicals likely to cause DNA lesions was suppressed by using anaerobic conditions, and DNA degradation by nucleases was prevented by using inhibitors. We were able to show conclusively that precocious lysis of mature bacteroids did not occur and that their lysates contained no active antagonist to supercoiled DNA. Contrary to Paau and Brill (1982) who used strain 102F51, we were unable to detect supercoiled plasmids in mature bacteroids of Balsac or strain 1021 in any experiment.

It is significant that our ability to detect supercoiled plasmids depended not only on the nodule fraction under test but also on molecular size. The disappearance from gels of the larger plasmids first is most simply explained by the occasional occurrence of single-strand DNA nicks. As few as one nick is sufficient to relax a supercoiled plasmid molecule. This is sufficient to subvert its separation from relaxed or linear DNA on an ethidium bromide-CsCl density gradient (Radloff *et al.* 1967) and, if the molecule were an open circle of sufficient size, prevent its penetration into a gel and resolution by electrophoresis. Larger molecules like megaplasmids, by presenting a larger target are more likely to be affected, as was observed in transforming bacteria. In time, and in the absence of repair, smaller plasmids like RP4 would be expected to succumb, as was observed in mature bacteroids. A corresponding increase in the amount of sheared DNA was not unexpected, but this was obscured in gels and on gradients by a proportion of plant DNA that persisted in nodule fractions.

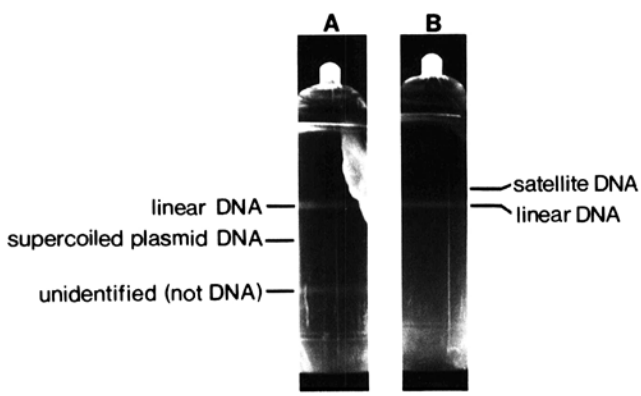

Fig. 6. Ethidium bromide-CsCl density gradients at equilibrium containing lysates prepared identically from *Rhizobium meliloti* strain Balsac cells photographed in UV light. **A**, Free-living bacteria from a 24-hr-old yeast-mannitol culture. **B**, Crude nodule extracts of mature bacteroids 7 wk after plant inoculation. The satellite band of less dense DNA of plant origin was eliminated by DNase I treatment of mature bacteroids before lysis but not by repeated washing of the cells with 125 mM KCl.

Bacteroid DNA was probed for evidence of the accumulation of unrepaired nicks, more than might be found in the DNA of actively dividing free-living cells presumed to be in a good state of repair. Because no difference in the hybridization patterns of denatured restriction fragments was found, we conclude that bacteroid DNA is not degenerate and single-strand nicking is either not extensive, soon repaired, or not at random. However, occasional or nonrandom nicking would be beyond the resolution of the denatured-fragment assay and sufficient to explain our results in the absence of DNA repair. It is pertinent that repair ligation is ATP-dependent and may be ATP-limited in mature bacteroids, because the availability of ATP for reactions other than the nitrogenase system is known to fall following bacteroid development. In fact, the ATP utilized in driving nitrogenase is so great that mature *R. meliloti* bacteroids have an energy charge (0.6) which is comparable to that of nonviable cells (Miller *et al.* 1988).

Direct attempts were made to determine whether plasmids in bacteroids were relaxed, covalently closed molecules or unrepaired nicked DNA. Gels containing ethidium bromide or chloroquine, which unwind the primary helix and impose positive supercoiling on covalently closed molecules (Keller 1975; Shure *et al.* 1977), did not reveal plasmid bands. However, such tests are usually made on much smaller molecules than the plasmids of Balsac or RP4, and perhaps the single nick per molecule sufficient to give a negative result was an unavoidable artifact in these experiments. It is of interest, nonetheless, that topoisomerase II (DNA gyrase), which with topoisomerase I controls the oxygen-regulated superhelical conformation of DNA in bacteria (Richardson *et al.* 1984; Yamamoto and Droffner 1985; Dorman *et al.* 1988), requires ATP to increase negative supercoiling. In the absence of available ATP, it cleaves DNA and assists topoisomerase I in the relaxation of supercoiling (Sugino *et al.* 1977; Gellert *et al.* 1977). An alternative interpretation of our results is, therefore, that the relaxation of plasmid supercoiling is a consequence of the ATP-limited activity of topoisomerases in the nodule.

Although the mechanism remains an open question and the possibility of an artifact of extraction cannot be ruled out completely, we conclude that plasmid supercoiling is

not maintained in *R. meliloti* bacteroids. It is perhaps no coincidence that these cells do not divide and are active in nitrogen fixation. The modification of DNA supercoiling is specifically implicated in the oxygen-regulated transcription of nitrogen-fixation genes in free-living diazotrophs (Kranz and Haselkorn 1986; Dimri and Das 1988; Dixon *et al.* 1988) and *Bradyrhizobium ex planta* (Gober and Kashket 1989). We are currently investigating the possibility that topoisomerases in *R. meliloti* might also be important for the expression of symbiotic genes in the oxygen-regulated environment within the nodule.

ACKNOWLEDGMENTS

We are most grateful to W. Berndt, S. Ireton, and A. Middleton for their technical help. We are also indebted to Y.-K. Chan, B. S. Cox, and R. J. Watson for their critical comments on this manuscript and to T. Finan and R. J. Watson for the gift of the probes.

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