APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1993, p. 3385–3392
0099-2240/93/103385-08$02.00/0

Vol. 59, No. 10

Production and Excretion of Nod Metabolites by *Rhizobium leguminosarum* bv. trifolii Are Disrupted by the Same Environmental Factors That Reduce Nodulation in the Field

IAN A. McKAY* AND MICHAEL A. DJORDJEVIC

Plant-Microbe Interaction Group, Research School of Biological Sciences, Institute of Advanced Studies, The Australian National University, P.O. Box 475, Canberra City, ACT 2601, Australia

Received 19 May 1993/Accepted 26 July 1993

Lipooligosaccharides (Nod metabolites) have been shown to be essential for the successful nodulation of legumes. In strains of *Rhizobium leguminosarum* bv. trifolii, Nod metabolites were detected predominantly within the cell and to a lesser extent in the periplasmic space and the growth medium. The production, and in particular the excretion, of Nod metabolites was restricted by a range of environmental conditions which are associated with poor nodulation in the field. Lowering the medium pH from 7.0 to 5.0, reducing the phosphate concentration from 1 mM to 5 μM KH_2PO_4, and lowering the incubation temperature from 28 to 18°C affected the number and relative concentrations of the Nod metabolites made. The form and concentration of the nitrogen source affected the relative concentrations of the Nod metabolites produced and excreted. KNO_3 concentrations of >10 mM did not affect cell growth rate but substantially reduced the number of Nod metabolites released. Environmental stresses differentially altered Nod metabolite production and excretion in the same strain carrying different introduced *nod* regions. Strain ANU845(pWLH1) produced and excreted comparatively fewer Nod metabolites at pH 5.0 and at 18°C than strain ANU845(pRI4003). The excretion but not the production of Nod metabolites by strain ANU845(pRtO32) was dependent on the presence of both *nodI* and *nodJ*. Tn*5*-induced *nodI* and *nodJ* mutants did not accumulate any metabolites either outside the cell or within the outer membrane or periplasmic space. Recognition that Nod metabolite accumulation is a complex system of production and excretion, with each component responding differently to changes in environmental conditions, has many consequences, both at the molecular level and in the field. The ability of different strains to produce and release Nod metabolites is likely to be a major determinant of nodule occupancy and should be considered when screening strains suitable for adverse environments.

Successful nodulation of legumes by rhizobia results from a series of complex plant-microbe interactions, which involve the production of specific plant flavonoid compounds as signal molecules (5, 25, 30, 33). Flavonoids are exuded from roots and cause the induction of nodulation (*nod*) genes in compatible rhizobia (3, 25). The expression of these genes can lead to the production and excretion of lipooligosaccharides, which have the ability to elicit a number of plant responses, including root hair curling, initiation of cortical cell division (35, 39), induction of early nodulation (ENOD) genes (23, 31), preinfection thread formation, increases in the number of root hairs (40), and thick and short roots (41). While the common *nod* genes (*nodABC*) are thought to be required for the synthesis of Nod metabolites, other genes, namely *nodIJ* (which have homology with known active transport proteins), have been implicated in the transport of metabolites across the inner membrane (7), although no conclusive data have been presented to date. In this regard, Spaink et al. (34) have shown that Nod metabolites do not accumulate in the cell and that mutations of *nodI* or *nodJ* on the symbiosis plasmid do not affect the excretion of Nod metabolites.

Nodulation compatibility between a particular legume cultivar and a rhizobial strain is determined by the presence of the appropriate genes carried by both the plant and the microbe. However, while combinations of rhizobia and plants can be compatible, nodulation failure can still occur in the field (reviewed in reference 29). Poor nodulation, which can lead to substantial loss of yield, has been attributed to a range of environmental conditions, including unfavorable soil pH (reviewed in references 2 and 22), high salinity (32, 45), the presence of ions such as aluminum (15, 44) and nitrate (reviewed in reference 37), and deficiencies in essential elements, including calcium (21, 24, 28) and phosphorus (11, 24, 28). Soil temperature is also an important environmental variable which affects legume nodulation. In addition to the reduction of nodulation at temperature extremes (reviewed in references 9 and 36), there are also specific temperature-sensitive legume-rhizobium combinations. For example, *Rhizobium leguminosarum* bv. trifolii TA1 forms nodules with *Trifolium subterraneum* cv. Woogenellup in the laboratory at growth temperatures above 25°C but not below 22°C, although it nodulates a range of other cultivars at the lower temperature (17, 18). In the field, strain TA1 is renowned for its failure to nodulate cv. Woogenellup (8).

Nodulation failure resulting from unfavorable environmental conditions has been attributed to a variety of mechanisms. Inability to nodulate is often attributed to a breakdown of the early events of nodulation, such as stimulation of root hair curling and formation of infection threads, and can occur even when the growth of the individual partners is not unduly affected by the limiting condition. Factors that have been proposed to restrict these early events of nodulation include salinity (32, 45) and low levels of calcium (26, 27) or phosphorus (1, 11, 16). Molecular techniques have shown how changes in environmental conditions can affect the production of signal molecules by legumes. The exuda-

* Corresponding author.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Reference
R. leguminosarum bv. trifolii		
TA1	Wild-type strain (acid sensitive)	17, 18
ANU794	Sm^r derivative of TA1	17, 18
ANU843	Wild-type strain	4
ANU845	ANU843, pSym cured	4
Plasmids		
pGMI515	RP4-prime containing 27.1 kb of *R. meliloti* DNA, including *nodFEGHPQ*	38
pRI4003	pRI40 containing 32 kb of *R. leguminosarum* bv. trifolii DNA, including *nodDABC-IJTFERLMNX* and sufficient *fix* and *nif* genes to confer nitrogen fixation	14
pWLH1	Contains 32 kb of DNA from strain TA1 *nod/nif* region cloned into pLAFR3, Tc^r	18
pRtO32::A6	pRtO32 containing Tn*5* inserted into a vector location	4
pRtO32::B15	pRtO32 *nodI*::Tn*5* Km^r	4
pRtO32::H2	pRtO32 *nodJ*::Tn*5* Km^r	4
pRtO32::M114	pRtO32 *nodA*::Tn*5* Km^r	19

[a] Sm^r, streptomycin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

tion from subclover roots of flavonoid compounds required for *nod* gene induction in *R. leguminosarum* bv. trifolii was reduced when the plants were grown at a pH of <5 (26). Low pH and increased aluminum concentrations were also shown to restrict *nod* gene induction in *R. leguminosarum* bv. trifolii, although this effect could be partially reversed by addition of calcium (27). The presence of combined nitrogen also limits the nodulation of legumes (37). While nitrogen (as ammonia) has been shown to limit the induction of the *nodABC* genes (6), the level which caused depressed induction [30 mM $(NH_4)_2SO_4$] is higher than would be found in most field situations. Dusha et al. (6) also found no effect of KNO_3 (up to 70 mM) on the expression of *nodABC* genes.

The increasing use of molecular techniques enables us to relate a number of field-based observations to precise effects upon plant-microbe interactions. In this study, we have examined Nod metabolite production and excretion in relation to a range of environmental conditions which have often been reported as delaying, limiting, or preventing the nodulation of otherwise compatible legumes and rhizobia. In particular, we have shown that the successful release of Nod metabolites is a two-component system of production and excretion (which requires the presence of *nodIJ*), with both components affected by changes in environmental conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Media. Cells were grown in BIII medium (12) or acid stress medium (ASM) (27) as indicated. Variations in pH, mineral composition, and growth temperature are described in the text. Seeds were germinated and grown on modified Fahraeus (F) medium (43).

Radioactive labelling of Nod metabolites. Cells were grown as batch cultures in 10-ml Falcon tubes by a modification of a described method (34). Inocula were grown to an A_{600} of 0.1 to 0.2 and diluted to an A_{600} of 0.01. Radioactively labelled [1-^{14}C]acetate (10 mCi; specific activity, 50 to 60 mCi/mmol; obtained from Amersham Australia) was added to 0.5 ml of culture. Cells were allowed to grow to an A_{600} of 0.2 to 0.4 in the presence or absence of 2 mM 7,4-dihydroxyflavone (DHF) on an orbital shaker at 200 rpm. Changes in the growth temperature from 28 to 18°C are described in the text.

Analytical methods. Nod metabolites were extracted in water-saturated *n*-butanol as described by Spaink et al. (34) with the following modifications. After cell centrifugation, the supernatant fraction (containing the excreted Nod metabolites) was separated from the cell pellet (containing cell-associated Nod metabolites), and each was extracted separately. Also, the butanol was removed by evaporation overnight in a fume cabinet, and the dry matter was resuspended in 30 μl of water-saturated *n*-butanol. Samples were stored at 4°C. To determine whether Nod metabolites were associated with the outer membrane and the periplasmic space, selected pellets (as indicated in the text) were treated with lysozyme-EDTA (10). After centrifugation (8,000 × *g* for 5 min), the supernatant and pellet fractions were extracted in butanol as above. Nod metabolites (4 μl for pellets and 6 μl for supernatants) were separated by thin-layer chromatography (TLC) with C_{18} reverse-phase plates (Sigma) with acetonitrile-water (49:51) as the solvent system. Radioactivity was visualized with a PhosphorImager (Molecular Dynamics) after exposure for 24 to 72 h.

Bioassay of butanol-extractable metabolites. Cells were grown without [^{14}C]acetate in the presence and absence of 2 μM DHF in 50-ml cultures in 250-ml conical flasks. The cultures were incubated at 28°C and shaken at 200 rpm on an orbital shaker. Nod metabolites were extracted from the growth medium with redistilled, water-saturated *n*-butanol (50 ml) as described above. After concentration to 10 ml, 100 μl of the butanol extract was added to sterile petri dishes. When the butanol had evaporated, 40 ml of Fahraeus medium containing 1% agar was added. Surface-sterilized *T. subterraneum* seeds were allowed to germinate at 22°C for 24 h before being placed on the medium containing the butanol-extracted metabolites and observed for root hair curling at 2-h intervals. As a negative control, 100 μl of *n*-butanol without Nod metabolites was added to plates to assess any effects on plant growth and root development.

RESULTS

Effect of butanol-extractable metabolites on plant roots. By using the standard procedures for extracting ^{14}C-Nod metabolites (Materials and Methods), unlabelled metabolites were extracted from the growth medium to determine their biological activity on the roots of *T. subterraneum* cv. Karridale and Woogenellup. The addition of 100 μl of *n*-butanol which did not contain Nod metabolites had no

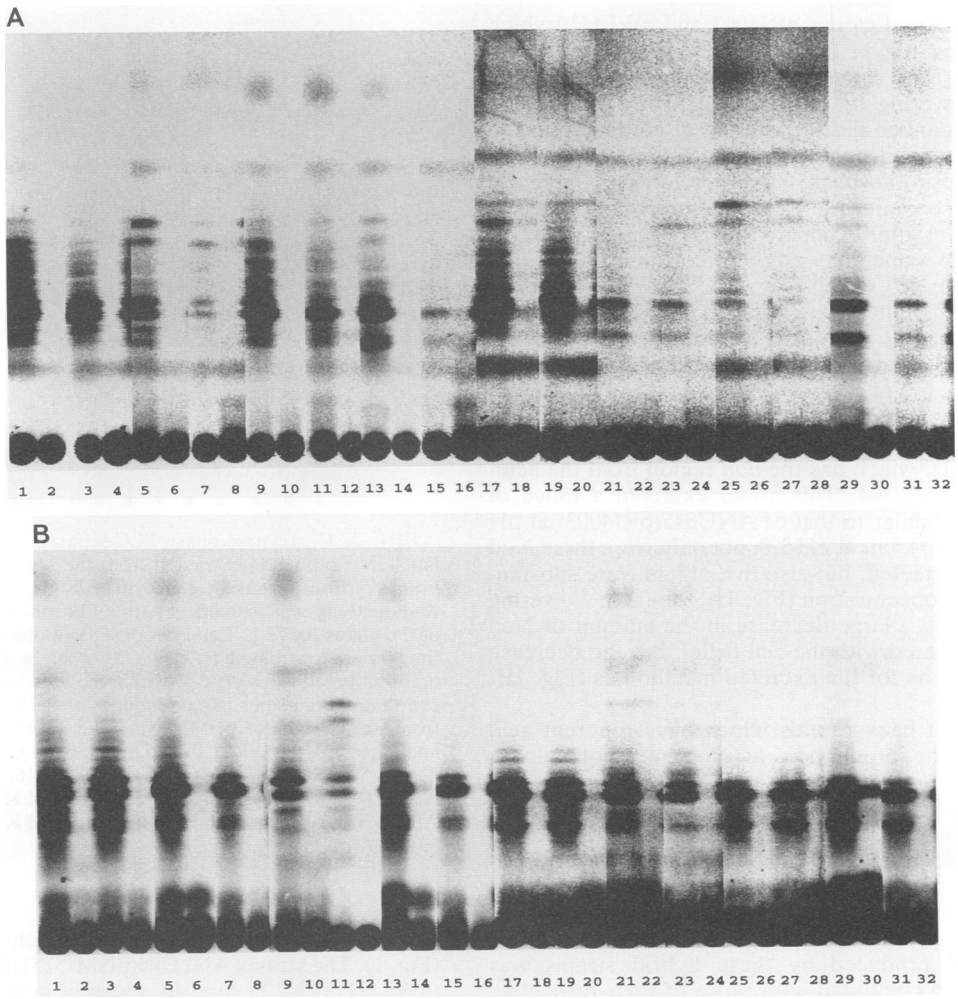

FIG. 1. C_{18} reverse-phase TLC separation of excreted ^{14}C-radiolabelled compounds extracted from the medium (A) and cell pellets (B) of strains of *R. leguminosarum* bv. trifolii after growth under various conditions. Radioactivity was visualized with a PhosphorImager after exposure of the plates for 72 h. Lanes from separations performed at different times were combined by using Micrografx Designer. Samples in odd-numbered lanes were grown in the presence of DHF; samples in even-numbered lanes had no inducer present. Lanes 1 to 16, extracts from strain ANU845(pRI4003): 1 and 2, 28°C, pH 7.0, 1 mM KH_2PO_4; 3 and 4, 28°C, pH 7.0, 5 μM KH_2PO_4; 5 and 6, 28°C, pH 5.0, 1 mM KH_2PO_4; 7 and 8, 28°C, pH 5.0, 5 μM KH_2PO_4; 9 and 10, 18°C, pH 7.0, 1 mM KH_2PO_4; 11 and 12, 18°C, pH 7.0, 5 μM KH_2PO_4; 13 and 14, 18°C, pH 5.0, 1 mM KH_2PO_4; 15 and 16, 18°C, pH 5.0, 5 μM KH_2PO_4. Lanes 17 to 32, extracts from strain ANU845(pWLH1): 17 and 18, 28°C, pH 7.0, 1 mM KH_2PO_4; 19 and 20, 28°C, pH 7.0, 5 μM KH_2PO_4; 21 and 22, 28°C, pH 5.0, 1 mM KH_2PO_4, 23 and 24, 28°C, pH 5.0, 5 μM KH_2PO_4; 25 and 26, 18°C, pH 7.0, 1 mM KH_2PO_4; 27 and 28, 18°C, pH 7.0, 5 μM KH_2PO_4; 29 and 30, 18°C, pH 5.0, 1 mM KH_2PO_4; 31 and 32, 18°C, pH 5.0, 5 μM KH_2PO_4.

effect on the plant roots. For both cultivars, root hair distortion was evident 4 h after the addition of DHF-induced Nod metabolites from strains ANU845(pRI4003) and ANU845(pWLH1). No root hair curling was associated with butanol extracts taken from the medium of strains grown in the absence of DHF. In contrast, butanol-extracted material from strain ANU845 (Sym plasmid cured) or strain ANU845(pRtO32::M114) (mutated for *nodA*) had no effects on the root hairs of either cultivar, irrespective of the addition of DHF to the culture. When grown in the presence of [^{14}C]acetate, neither ANU845 nor ANU845(pRtO32::M114) produced any DHF-inducible Nod metabolites, either associated with the cell pellet or excreted into the medium (data not shown).

Influence of pH on the production and excretion of Nod metabolites. The production and excretion of Nod metabolites by strains of *R. leguminosarum* bv. trifolii grown at 28°C after exposure to the inducer DHF are shown in Fig. 1. In all cases, cells were grown in ASM and buffered with 70 mM MES (morpholineethanesulfonic acid)-NaOH (which was shown to have no effect on cell growth rates [results not shown]). The maximum change in pH as a result of cell growth (A_{600} = 1.4) was an increase of 0.1 pH unit at pH 5.0 (results not shown). To minimize any change in pH during growth, cells were harvested at an A_{600} of 0.2 to 0.4. The growth rates of cells were shown to vary as a result of different treatments, for example, the growth temperature (results not included). Growth was monitored throughout to ensure that differences in Nod metabolite production between strains were not a just a function of different growth rates.

Strain ANU845 was used as a common background for the

introduction of cosmid clones pRI4003 and pWLH1, which contained the *nod* region from a moderately acid-tolerant strain (ANU843) and an acid-sensitive strain (ANU794), respectively. Lowering the pH of the growth medium affected both the number and the amount of Nod metabolites excreted and produced by each strain. At 28°C and in the presence of excess phosphate (1 mM KH_2PO_4), ANU845 (pRI4003), which contains the ANU843 *nod* region, released 10 Nod metabolites after exposure to DHF (Fig. 1A, lane 1). When the other parameters were kept constant but the pH was reduced from 7.0 to 5.0 (Fig. 1A, lane 5), six of the excreted Nod metabolites were reduced in intensity and one was not detected at all. Similarly, both the number and the intensity of cell pellet-associated Nod metabolites were reduced when the pH of the growth medium was reduced to pH 5.0. The effect of lowering the pH on Nod metabolite release under these conditions was greater in strain ANU845(pWLH1), which has the *nod* region from the acid-sensitive strain ANU794. This strain excreted a range of Nod metabolites similar to that of ANU845(pRI4003) at pH 7.0 (Fig. 1A, lane 17), but at pH 5.0, not only were three Nod metabolites not detected, but also five others were substantially reduced in concentration (Fig. 1A, lane 21). Lowering the pH also caused a large decrease in the amount of Nod metabolites associated with the cell pellet, but the decrease was not as severe as for the excreted metabolites (Fig. 1B, lanes 19 and 23).

The influence of backgrounds which have different acid sensitivities on the production of Nod metabolites was examined with strains ANU843 and ANU794, containing plasmid pGMI515. This plasmid contains the host range nodulation genes from *Rhizobium meliloti*, including *nodPQH*, which collectively add an SO_3 group to the lipooligosaccharides, enhance the amount of Nod metabolites made by each strain, and change the mobility pattern of the Nod metabolites produced. As expected, the selection of Nod metabolites produced by these hybrid strains was completely altered compared with those of their respective parental strains. Studies with ^{35}S radiolabel showed that all the Nod metabolites were sulfonated, unlike the Nod metabolites from strains ANU843 and ANU794, which did not contain any sulfonated groups. This indicated that the *R. meliloti* host range genes that mediate sulfonation (*nodPQH*) were dominant over the resident host range genes of *R. leguminosarum* bv. trifolii. At 28°C and in the presence of 1 mM KH_2PO_4, the faster-migrating sulfonated Nod metabolites characteristic of *R. meliloti* are clearly evident at pH 7.0, in both the ANU843 (Fig. 2, lane 1) and the acid-sensitive ANU794 (Fig. 2, lane 9) backgrounds. At pH 5.0, both ANU794(pGMI515) and ANU843(pGMI515) showed a reduced number and intensity of Nod metabolites, but the metabolites were more severely reduced in strain ANU794 (pGMI515).

Phosphate is a key parameter in the production and excretion of Nod metabolites. The production and excretion of Nod metabolites are clearly sensitive to the phosphate concentration in the growth medium. Reduction of the phosphate concentration from 1 mM to 5 μM KH_2PO_4 (a level not unusual in Australian soils) resulted in no changes in the number or the intensity of DHF-inducible Nod metabolites associated with the pellets of strains ANU845(pRI4003) and ANU845(pWLH1) at pH 7.0 (Fig. 1B). However, the lower phosphate concentration resulted in significant reduction in the concentration of over half of the excreted Nod metabolites by both strains. A low-pH, low-phosphate growth medium caused a marked reduction in the production, and in particular the excretion, of Nod metabolites by all strains (Fig. 1). The strains ANU794(pGMI515) (Fig. 2) and ANU845 (pWLH1) (Fig. 1) showed a loss of more than 95% of their Nod metabolites. A low phosphate concentration also exacerbated the loss of Nod metabolites caused by a reduction in the growth temperature from 28 to 18°C, as described below.

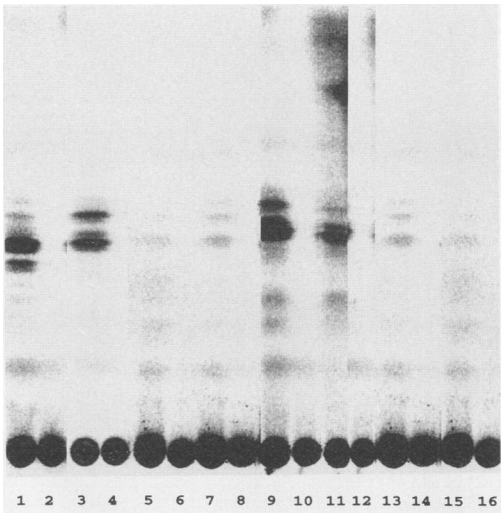

FIG. 2. C_{18} reverse-phase TLC separation of excreted ^{14}C-radiolabelled compounds extracted from the medium of *R. leguminosarum* bv. trifolii strains after growth at 28°C under various conditions. Radioactivity was visualized with a PhosphorImager after exposure of the plates for 72 h. Lanes from separations performed at different times were combined by using Micrografx Designer. Samples in odd-numbered lanes were grown in the presence of DHF; samples in even-numbered lanes had no inducer present. Lanes 1 to 8, extracts from strain ANU843(pGMI515): 1 and 2, pH 7.0, 1 mM KH_2PO_4; 3 and 4, pH 7.0, 5 μM KH_2PO_4; 5 and 6, pH 5.0, 1 mM KH_2PO_4; 7 and 8, pH 5.0, 5 μM KH_2PO_4. Lanes 9 to 16, extracts from strain ANU794(pGMI515): 9 and 10, pH 7.0, 1 mM KH_2PO_4; 11 and 12, pH 7.0, 5 μM KH_2PO_4; 13 and 14, pH 5.0, 1 mM KH_2PO_4; 15 and 16, pH 5.0, 5 μM KH_2PO_4.

Influence of temperature on the production and excretion of Nod metabolites. Cells were grown in ASM as described above, but the growth temperature for both the inocula and the production of Nod metabolites was reduced from 28 to 18°C. Whereas ANU845(pRI4003) produced and excreted a full range of Nod metabolites at 18°C when the pH was 7.0 and 1 mM KH_2PO_4 was present, the lower temperature had significant effects on metabolite production, and in particular excretion, with all other treatments (Fig. 1). Although the intensities of some of the metabolites produced by ANU845(pRI4003) at 18°C were reduced when the strain was grown at pH 5.0, the major effect was observed when the phosphate concentration was reduced to 5 μM. At pH 7.0 and even more significantly at pH 5.0, there were major reductions in Nod metabolite production. At the lower temperature and pH and when the phosphate concentration was not limiting, there was a relative increase in slower-migrating Nod metabolites (Fig. 1A and B, lanes 13 and 29 compared with lanes 1 and 17).

Strain ANU845(pWLH1) has the same temperature-sensitive nodulation phenotype with *T. subterraneum* cv. Woogenellup as the parental strain from which the *nod* genes were derived (18). In contrast to strain ANU845(pRI4003), strain ANU845(pWLH1) showed a greater reduction in the excretion of Nod metabolites when grown at 18°C at both

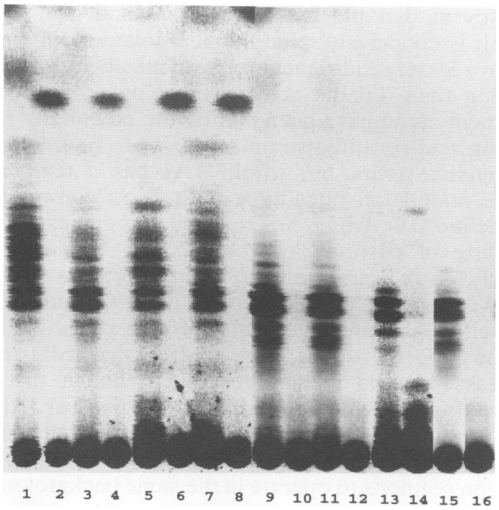

FIG. 3. C_{18} reverse-phase TLC separation of ^{14}C-radiolabelled compounds extracted from the medium and cell pellets of *R. leguminosarum* bv. trifolii ANU845(pRI4003) after growth at 28°C in the presence of KNO_3 or $(NH_4)_2SO_4$ at 10 or 50 mM. Radioactivity was visualized with a PhosphorImager after exposure of the plates for 72 h. Lanes from separations performed at different times were combined by using Micrografx Designer. Samples in odd-numbered lanes were grown in the presence of DHF; samples in even-numbered lanes had no inducer present. Lanes 1 to 8, samples extracted from medium: 1 and 2, 10 mM KNO_3; 3 and 4, 50 mM KNO_3; 5 and 6, 10 mM $(NH_4)_2SO_4$; 7 and 8, 50 mM $(NH_4)_2SO_4$. Lanes 9 to 16, samples extracted from cell pellet: 9 and 10, 10 mM KNO_3; 11 and 12, 50 mM KNO_3; 13 and 14, 10 mM $(NH_4)_2SO_4$; 15 and 16, 50 mM $(NH_4)_2SO_4$.

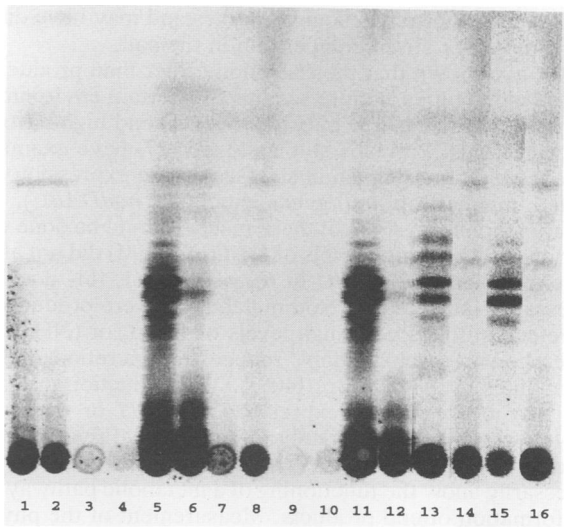

FIG. 4. C_{18} reverse-phase TLC separation of ^{14}C-radiolabelled compounds extracted from the medium, lysozyme fraction, and cell pellets of strains of *R. leguminosarum* bv. trifolii which lack *nodI* or *nodJ* after growth at 28°C. Radioactivity was visualized with a PhosphorImager after exposure of the plates for 72 h. Lanes from separations performed at different times were combined by using Micrografx Designer. Samples in odd-numbered lanes were grown in the presence of DHF; samples in even-numbered lanes had no inducer present. Lanes 1 to 6, extracts from strain ANU845 (pRtO32::B15), which lacks *nodI*: 1 and 2, medium fraction; 3 and 4, lysozyme-extractable fraction; 5 and 6, cell pellet fraction. Lanes 7 to 12, extracts from strain ANU845(pRtO32::H2), which lacks *nodJ*: 7 and 8, medium fraction; 9 and 10, lysozyme-extractable fraction; 11 and 12, pellet fraction. Lanes 13 to 16, extracts from the parental strain ANU845(pRtO32::A6): 13 and 14, medium fraction; 15 and 16, lysozyme-extractable fraction.

phosphate levels and at both pH 5.0 and 7.0. Cell-associated Nod metabolites were also reduced, but the biggest loss occurred in the released metabolites (Fig. 1).

Form and concentration of the nitrogen source affect the production and excretion of Nod metabolites. To assess the effect of different nitrogen sources on Nod metabolite production, strain ANU845(pRI4003) was grown at 28°C in ASM plus 1 mM KH_2PO_4, with KNO_3 or $(NH_4)_2SO_4$ replacing sodium glutamate as the sole nitrogen source. Although high concentrations (50 mM) of KNO_3 caused substantial reductions in excreted Nod metabolites, production of these compounds (albeit reduced) was still evident when the metabolites associated with the cell pellet were examined (Fig. 3). Addition of KNO_3 to the growth medium at a lower concentration (10 mM) resulted in a full range of excreted Nod metabolites but with distinct changes in their relative concentrations. In contrast, growth in the presence of 10 or 50 mM $(NH_4)_2SO_4$ allowed excretion of the full range of Nod metabolites.

Role of *nodIJ* on the excretion of Nod metabolites. The essential role of *nodIJ* in the excretion of Nod metabolites is clearly shown in Fig. 4. When *R. leguminosarum* bv. trifolii strains which lack either *nodI* [ANU845(pRtO32::B15)] or *nodJ* [ANU845(pRtO32::H2)] were grown at 28°C in BIII medium, they showed no release of Nod metabolites. Furthermore, treatment of the cell pellets with lysozyme revealed that no Nod metabolites were associated with the outer membrane or periplasmic space of strains lacking *nodI* or *nodJ*, although a range of metabolites did accumulate within the cell. The control strain ANU845(pRtO32::A6), which has the complete *nod* region, had Nod metabolites associated with the cell, the outer membrane, and the periplasmic space and also released Nod metabolites into the growth medium (Fig. 4). The relative ratios of major ^{14}C-labelled Nod metabolites that accumulated in the cell pellet, the periplasmic space, and the growth medium were calculated. When ANU845(pRI4003) was grown at 28°C and pH 7.0 with 1 mM KH_2PO_4, approximately 50% of the major Nod metabolites accumulated in the cell, 30% in the growth medium, and 20% in the outer membrane and periplasmic space.

DISCUSSION

The failure of otherwise compatible rhizobia and legumes to form nodules as a result of certain environmental conditions is an ongoing problem of great commercial and academic interest. The data we have provided in this article point to how specific environmental conditions affect two of the essential steps of nodulation—the production and excretion of Nod metabolites. In Australia, where large areas of broad-acre cropping are based on legume rotation, the soils are often low in nutrients (particularly phosphate) as well as being of low pH. We have shown that conditions of lowered phosphate level, lowered pH, and lowered temperature all reduce Nod metabolite production and excretion, with certain combinations of these treatments causing the greatest reductions. In addition to providing explanations of long-standing field and glasshouse observations, the results we have presented have implications for the interpretation of

genetic and other physiological studies and may have direct implications for strain competition in the soil.

We have shown that the excretion rather than production of Nod metabolites is more sensitive to certain environmental stresses, such as low phosphate levels and high nitrogen concentrations. Previous studies (6, 26, 27) have examined the effects of environmental stresses on the expression of a limited number of *nod* genes (such as *nodDABC*), but extrapolation from some of these results should be done with caution. Whereas high levels of KNO_3 (70 mM) did not affect the expression of *nodABC* in *R. meliloti* (6), this does not necessarily indicate that Nod metabolites were produced or excreted. In this study, high levels of KNO_3 or $(NH_4)_2SO_4$ were shown to substantially reduce the excretion of Nod metabolites by ANU845(pRI4003). This reduction in excretion may be related to expression of *nodIJ* or effects on enzyme regulation and membrane integrity. Environmental conditions that permit the expression of a gene do not necessarily allow the functioning of a metabolic pathway and the formation of end products. Measurement of the physiologically active products (lipooligosaccharide Nod metabolites) may well have more value when trying to relate field-based observations to precise molecular events.

In this study, we have clearly shown that the function of the *nodI* and *nodJ* products is to excrete Nod metabolites. No Nod metabolites were found to be excreted into the medium or into the periplasmic space in these mutants. This is consistent with the poor infectivity of these mutants and with the delayed and low-nodulation phenotypes that they exhibit on all clovers (4, 13). In other species in which mutations in *nodIJ* do not lead to severe phenotypes, the function of NodI and NodJ must be complemented by other "housekeeping" genes. Since a significant proportion of the Nod metabolites (~20%) of nodulating strains are located in the periplasmic space, it is highly likely, given the low solubility of these Nod metabolites, that they are associated with the outer membrane. For similar reasons, there is also a high probability that the Nod metabolites form micelles when excreted into the growth medium.

Changes in nodule occupancy by different rhizobial serogroups as a result of environmental change provide a practical insight into plant-microbe interactions which can be related to the production and excretion of Nod metabolites. Previous studies (1, 11, 20) have shown how nodule occupancy by different serogroups of rhizobia can be dramatically changed as a result of changes in the phosphate concentration, growth temperature, or pH. We have shown that strains show clear differences in their abilities to produce and excrete Nod metabolites at a low temperature, low pH, and low phosphate level. These observed changes in nodule occupancy as a result of environmental change may well relate to differences in the abilities of strains to produce and excrete Nod metabolites and hence to be competitive. In contrast, Zaroug and Munns (46) found that phosphate addition had no effect on the nodulation of *Lablab purpureus* by two strains of *Bradyrhizobium*, CB756 and CB1024. Although CB1024 had a higher phosphate requirement for growth in culture than CB756 (46), our results suggest that growth per se is not necessarily the determinant of Nod metabolite production or, by extrapolation, of successful nodulation. Vargas and Graham (42) demonstrated the role of the plant in determining nodule occupancy by strains of *Rhizobium phaseoli* with different acid sensitivities. An acid-sensitive cultivar of *Phaseolus vulgaris* was principally nodulated by an acid-tolerant strain of *R. phaseoli* at pH 4.5. In contrast, an acid-tolerant cultivar showed equal nodule occupancy at that pH by acid-tolerant and acid-sensitive strains. It is conceivable that the acid-tolerant cultivar may have had a lesser requirement for Nod metabolites to initiate nodulation than did the acid-sensitive one. As such, this requirement could have been met by the potentially poorer-producing acid-sensitive strain.

R. leguminosarum bv. trifolii TA1 has a temperature-sensitive nodulation phenotype with *T. subterraneum* cv. Woogenellup (18), which has resulted in significant nodulation failure of pastures in Australia (8). Strain ANU845 (pWLH1) has the same nodulation phenotype as strain TA1 on cv. Woogenellup (18). Moreover, it is apparent from Fig. 1 that, although extensive signal production is evident at 28°C, lowering the temperature to 18°C markedly reduced the release of Nod metabolites, even though this strain maintained a growth rate similar to that of ANU845 (pRI4003) at 18°C. It is clear from this result that there is a qualitative and a quantitative difference in the ability of the nodulation regulons to operate in the same background. This may imply a direct effect of environmental conditions on transcription, translation, or posttranslation events that affect the production and excretion of Nod metabolites. Since there is such a substantial difference in the Nod metabolites produced by the same strain under different conditions, caution should be exercised in interpreting minor variations in Nod metabolite patterns between related strains. The variation in the production and excretion of the Nod metabolites of a particular strain at 28°C in laboratory media may bear little relationship to the pattern produced in the field. Indeed, if bacterial membrane fatty acid moieties are used in Nod metabolite production, as has been hypothesized (33), the degree of saturation of the fatty acids may vary, and hence the structure of the acyl moiety of the Nod metabolites produced at 18°C may differ from that of those produced at 28°C. The conditions used to grow strains in this study are more relevant to field situations than those commonly used to grow strains for structural determinations of the Nod metabolites.

Interestingly, the general loss of Nod metabolite production and excretion which occurred when cells were grown at pH 5.0 rather than pH 7.0 was much less pronounced when the growth temperature was simultaneously reduced to 18°C with a phosphate concentration of 1 mM. Under these conditions, some of the more slowly migrating metabolites increased in intensity while the faster-migrating metabolites were absent. It is conceivable that this reflects changes in membrane structure and function under these conditions or structural variations in the Nod metabolites. Much work needs to be done in this area of environmental impact on plant-microbe interactions. Other environmental stresses, such as high levels of Al and salinity, may well affect nodulation by reducing the production and excretion of Nod metabolites. It is not known which of the 10 Nod metabolites excreted by *R. leguminosarum* bv. trifolii are critical for initiation of plant responses. Identification of these metabolites may allow a convenient method of screening suitable strains for effective nodulation in adverse environments.

ACKNOWLEDGMENTS

We thank Barry Rolfe for thoughtful discussions and comments and David Loschke for help with the generation of the figures. Jan McIver is thanked for providing strains.

REFERENCES

1. **Almendras, A. S., and P. J. Bottomley.** 1987. Influence of lime and phosphate on nodulation of soil-grown *Trifolium subterra-*

neum L. by indigenous *Rhizobium trifolii*. Appl. Environ. Microbiol. **53**:2090–2097.
2. **Coventry, D. R., and J. Evans.** 1989. Symbiotic nitrogen fixation and soil acidity, p. 103–137. *In* A. D. Robson (ed.), Soil acidity and plant growth. Academic Press, New York.
3. **Djordjevic, M. A., J. W. Redmond, M. Batley, and B. G. Rolfe.** 1987. Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. EMBO J. **6**:1173–1179.
4. **Djordjevic, M. A., P. R. Schofield, and B. G. Rolfe.** 1985. Tn*5* mutagenesis of *Rhizobium trifolii* host-specific nodulation genes results in mutants with altered host range ability. Mol. Gen. Genet. **200**:463–471.
5. **Djordjevic, M. A., and J. J. Weinman.** 1991. Factors determining host recognition in the clover-*Rhizobium* symbiosis. Aust. J. Plant Physiol. **18**:543–557.
6. **Dusha, I., A. Bakos, A. Kondorosi, F. J. de Bruijn, and J. Schell.** 1989. The *Rhizobium meliloti* early nodulation genes (*nodABC*) are nitrogen-regulated: isolation of a mutant strain with efficient nodulation capacity on alfalfa in the presence of ammonium. Mol. Gen. Genet. **219**:89–97.
7. **Evans, I. J., and J. A. Downie.** 1986. The *nodI* product of *Rhizobium leguminosarum* is closely related to ATP-binding bacterial transport proteins: nucleotide sequence analysis of the *nodI* and *nodJ* genes. Gene **43**:95–101.
8. **Gibson, A. H.** 1968. Nodulation failure in *Trifolium subterraneum* L. cv. Woogenellup (Syn Marrar). Aust. J. Agric. Sci. **19**:907–918.
9. **Gibson, A. H.** 1977. The influence of the environment and managerial practices on the legume-*Rhizobium* symbiosis, p. 393–450. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation. Wiley, New York.
10. **Glenn, A. R., and M. J. Dilworth.** 1979. An examination of *Rhizobium leguminosarum* for the production of extracellular and periplasmic proteins. J. Gen. Microbiol. **112**:405–409.
11. **Hicks, P. M., and T. E. Loynachan.** 1987. Phosphorus fertilisation reduces vesicular-arbuscular mycorrhizal infection and changes nodule occupancy of field-grown soybeans. Agronomy **79**:841–844.
12. **Hollingsworth, R. I., M. Abe, J. E. Sherwood, and F. B. Dazzo.** 1984. Bacteriophage-induced acidic heteropolysaccharide lyases that convert the acidic heteropolysaccharides of *Rhizobium trifolii* into oligosaccharide units. J. Bacteriol. **160**:510–516.
13. **Huang, S. Z., M. A. Djordjevic, and B. G. Rolfe.** 1988. Characterisation of aberrant infection events induced on *Trifolium subterraneum* by *Rhizobium trifolii* region II mutants. J. Plant Physiol. **133**:16–24.
14. **Innes, R. W., M. A. Hirose, and P. L. Kuempel.** 1988. Induction of nitrogen-fixing nodules on clover roots requires only 32 kilobase pairs from the *Rhizobium trifolii* symbiosis plasmid. J. Bacteriol. **170**:3793–3802.
15. **Kim, M. M., C. J. Asher, D. G. Edwards, and R. A. Date.** 1985. Aluminium toxicity: effects on growth and nodulation of subterranean clover, p. 501–503. Proc. 15th Int. Grasslands Congress.
16. **Leung, K., and P. J. Bottomley.** 1987. Influence of phosphate on the growth and nodulation characteristics of *Rhizobium trifolii*. Appl. Environ. Microbiol. **53**:2098–2105.
17. **Lewis-Henderson, W. R., and M. A. Djordjevic.** 1991. A cultivar-specific interaction between *Rhizobium leguminosarum* bv. trifolii and subterranean clover is conditioned by *nodM*, other bacterial cultivar specificity genes, and a single recessive host gene. J. Bacteriol. **173**:2791–2799.
18. **Lewis-Henderson, W. R., and M. A. Djordjevic.** 1991. *nodT*, a positively-acting cultivar specificity determinant controlling nodulation of *Trifolium subterraneum* by *Rhizobium leguminosarum* biovar *trifolii*. Plant Mol. Biol. **16**:515–526.
19. **McIver, J., M. A. Djordjevic, J. J. Weinman, G. L. Bender, and B. G. Rolfe.** 1989. Extension of host range in *Rhizobium leguminosarum* bv. *trifolii* caused by point mutations in *nodD* that result in alterations in regulatory function and recognition of inducer molecules. Mol. Plant-Microbe Interact. **2**:97–106.
20. **Mullen, M. D., D. W. Israel, and A. G. Wollum.** 1988. Effects of *Bradyrhizobium japonicum* and soybean [*Glycine max* (L.) Merr.] phosphorus nutrition on nodulation and dinitrogen fixation. Appl. Environ. Microbiol. **54**:2387–2392.
21. **Munns, D. N.** 1977. Mineral nutrition and the legume symbiosis, p. 353–391. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation. Wiley, New York.
22. **Munns, D. N.** 1986. Acid tolerance in legumes and rhizobia. Adv. Plant Nutr. **2**:63–91.
23. **Nap, J.-P., and T. Bisseling.** 1990. Developmental biology of a plant-prokaryote symbiosis: the legume root nodule. Science **250**:948–954.
24. **O'Hara, G. W., N. Boonkerd, and M. J. Dilworth.** 1988. Mineral constraints to nitrogen fixation. Plant Soil **108**:91–110.
25. **Redmond, J. R., M. Batley, M. A. Djordjevic, R. W. Innes, P. L. Keumpel, and B. G. Rolfe.** 1986. Flavones induce expression of *nod* genes in *Rhizobium*. Nature (London) **323**:632–635.
26. **Richardson, A. E., M. A. Djordjevic, R. J. Simpson, and B. G. Rolfe.** 1988. The effects of pH, Ca and Al on the exudate from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. Plant Soil **109**:37–47.
27. **Richardson, A. E., R. Simpson, M. A. Djordjevic, and B. G. Rolfe.** 1988. Expression and induction of nodulation genes in *Rhizobium trifolii* at low pH and in the presence of Ca and Al. Appl. Environ. Microbiol. **54**:2541–2548.
28. **Robson, A. D.** 1983. Mineral nutrition, p. 36–55. *In* W. J. Broughton (ed.), Nitrogen fixation, vol. 3: legumes. Clarendon Press, Oxford.
29. **Robson, A. D., and P. J. Bottomley.** 1991. Limitations in the use of legumes in agriculture and forestry, p. 320–349. *In* M. J. Dilworth and A. R. Glenn (ed.), Biology and biochemistry of nitrogen fixation. Elsevier, Amsterdam.
30. **Rolfe, B. G., M. Batley, J. W. Redmond, A. E. Richardson, R. J. Simpson, B. J. Bassam, C. L. Sargent, J. J. Weinman, M. A. Djordjevic, and F. B. Dazzo.** 1988. Phenolic compounds secreted by legumes, p. 405–409. *In* H. Bothe, F. J. de Bruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Proceedings of the 7th International Congress on Nitrogen Fixation. Gustav Fischer, Stuttgart, Germany.
31. **Scheres, B., C. van de Wiel, A. Zalensky, B. Horvath, H. P. Spaink, H. van Eck, F. Zwartkruis, A. M. Wolters, T. Gloudermans, A. van Kammen, and T. Bisseling.** 1990. The Enod12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. Cell **62**:281–294.
32. **Singleton, P. W., and B. B. Bohlool.** 1984. Effect of salinity on nodule formation by soybean. Plant Physiol. **74**:72–76.
33. **Spaink, H. P.** 1992. Rhizobial lipo-oligosaccharides: questions and answers. Plant Mol. Biol. **20**:977–986.
34. **Spaink, H. P., A. Aarts, G. Stacey, G. V. Blomberg, B. J. J. Lugtenberg, and E. P. Kennedy.** 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin layer chromatography. Mol. Plant-Microbe Interact. **5**:72–80.
35. **Spaink, H. P., D. M. Sheeley, A. A. N. van Brussel, J. Glushka, W. S. York, T. Tak, O. Geiger, E. P. Kennedy, V. N. Reinhold, and B. J. J. Lugtenberg.** 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature (London) **354**:125–130.
36. **Sprent, J. L., and F. R. Minchin.** 1983. Environmental effects on the physiology of nodulation and nitrogen fixation, p. 269–318. *In* D. G. Jones and D. R. Davies (ed.), Temperate legumes. Pitman, London.
37. **Streeter, J.** 1988. Inhibition of legume formation and N_2 fixation by nitrate. Crit. Rev. Plant Sci. **7**:1–24.
38. **Truchet, G., F. Debelle, J. Vasse, B. Terzaghi, A.-M. Garnerone, C. Rosenberg, J. Batut, F. Maillet, and J. Denarie.** 1985. Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol. **164**:1200–1210.
39. **Truchet, G., P. Roche, P. Lerouge, J. Vasse, S. Camut, F. de Billy, J.-C. Prome, and J. Denarie.** 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature (London) **351**:670–673.
40. **van Brussel, A. A. N., R. Bakhuizen, P. C. van Spronsen, H. P. Spaink, T. Tak, B. J. J. Lugtenberg, and J. W. Kijne.** 1992.

Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. Science **257**:70–72.

41. **van Brussel, A. A. N., S. A. J. Zaat, H. C. J. Canter-Cremers, C. A. Wijffelman, E. Pees, T. Tak, and B. J. J. Lugtenberg.** 1986. Role of plant root exudate and Sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of Tsr factor, which causes thick and short roots on common vetch. J. Bacteriol. **165**:517–522.
42. **Vargas, A. T., and P. H. Graham.** 1989. Cultivar and pH effects on competition for nodule sites between isolates of *Rhizobium* in beans. Plant Soil **117**:195–200.
43. **Vincent, J. M.** 1986. A manual for the practical study of root-nodule bacteria. IBP Handbook No. 15. Blackwell Publishers, Oxford.
44. **Wood, M., J. E. Cooper, and A. J. Holding.** 1984. Aluminium toxicity and nodulation of Trifolium repens. Plant Soil **78**:381–391.
45. **Zahran, H. H., and J. I. Sprent.** 1986. Effect of sodium chloride and polyethylene glycol on root hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta (Berlin) **167**:303–309.
46. **Zaroug, M. G., and D. N. Munns.** 1979. Nodulation and growth of *Lablab purpureus* (*Dolichos Lablab*) in relation to *Rhizobium* strain, liming and phosphorus. Plant Soil **53**:329–339.