The *nifA* Gene of *Rhizobium meliloti* Is Oxygen Regulated

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Experiments using plasmid-borne gene fusions and direct RNA measurements have revealed that expression from the *nifA* gene is induced in *Rhizobium meliloti* when the external oxygen concentration is reduced to microaerobic levels. Induction occurs in the absence of alfalfa and in the presence of fixed nitrogen and does not require *ntrC*. The production of functional *nifA* gene product (NifA) can be demonstrated by its ability to activate the nitrogenase promoter P1. Aerobic induction of *nifA* can also occur during nitrogen starvation at low pH, but in this case induction is dependent on *ntrC* and does not lead to P1 activation. The data indicate that reduced oxygen tension is potentially a major trigger for symbiotic activation of nitrogen fixation in *Rhizobium* species.

A positively acting regulatory gene (*nifA*) that controls the expression of nitrogenase and associated nitrogen fixation (*nif*) genes in legume root nodules has been identified in both *Rhizobium meliloti* (31, 32) and *Rhizobium japonicum* (15). Rhizobium *nifA* is structurally and functionally related to the corresponding *nifA* gene in the free-living nitrogen-fixing bacterium *Klebsiella pneumoniae* that is required for transcription from six sequence-related *nif* promoters (6, 7). Target promoters for the *nifA* gene product, NifA, are similar in both *Rhizobium* spp. and *K. pneumoniae* (2).

Since NifA is the central regulatory element for *nif* gene expression, nitrogen fixation decisions are made through controls exerted on either NifA functionality or *nifA* expression. In *K. pneumoniae*, nitrogen fixation is induced in response to nitrogen starvation during anaerobiosis; its purpose is to provide fixed nitrogen for cell growth. Both fixed nitrogen and oxygen are negative effectors of NifA activity through the action of the *nifL* gene product, which is made together with NifA from the *nifLA* operon (25). Regulation of *nifLA* expression in response to fixed nitrogen is well documented in *K. pneumoniae* and is known to be mediated by a second positively acting regulatory protein called NtrC (14, 28). Both NifA and NtrC require a unique sigma factor (RpoN) for activity (17, 18), and target promoters for NtrC activation (e.g., *nifLA*) have structures similar to those of *nif* promoters (6). Inhibition of *nifLA* expression by oxygen has also been reported (13, 21), but other studies have found little or no such regulation (8, 10).

Since *Rhizobium* species are obligate aerobes and since most of the nitrogen fixed during symbiosis is exported to the plant, it has been anticipated that control of *nif* gene expression in these bacteria might be different than that in *K. pneumoniae*. This is particularly true for fast-growing *Rhizobium* species, all of which are unable to carry out nitrogen fixation in the absence of their legume host, and has led to speculation that *nif* induction might depend upon a unique symbiotic signal from the plant or upon some particular aspect of nodule physiology. Recently it has been shown that the *ntrC* gene of *R. meliloti* is not required for symbiotic nitrogen fixation with alfalfa (30), confirming that regulation is indeed different in *Rhizobium* spp. than in *K. pneumoniae*. Here we report that the *R. meliloti nifA* gene can be induced asymbiotically to levels exceeding those in nodule bacte-

roids when proper microaerobic conditions are achieved and that in a wild-type strain of *R. meliloti*, such induction leads to the NifA-mediated activation of the nitrogenase promoter P1. If the same induction mechanism is operative during symbiosis, as seems likely, then bacterial sensing of the free oxygen concentration may be a fundamental aspect of *Rhizobium nif* gene regulation.

MATERIALS AND METHODS

Bacterial strains. Wild-type *R. meliloti* 102F34 (5) and 1021 (23) have previously been described. Strain N266 is a *nifA*::Tn5 mutant of F34 (11). Strain 5001 is an *ntrC*::Tn5 mutant of 1021 (30). Plasmid pMB210 (4) is a translational fusion of the *nifH* gene and P1 promoter to codon 8 of *lacZ* in the broad-host-range vector pGD926 (12). Plasmid pMB211 (4) is the equivalent translational fusion of the P2 promoter and the *fixA* gene in pGD926.

Media. TY medium contains 6 g of tryptone, 3 g of yeast extract, and 4.5 mM CaCl₂ per liter. Nitrogen-free medium contains 7.4 mM KH_2PO_4, 5.8 mM K_2HPO_4, 2.5 mM $MgSO_4$, 6.7 mM $CaCl_2$, and 10 mM sodium succinate at a final (unadjusted) pH of 6.75 to 6.8. Lower pH values for this medium were obtained by titrating with HCl.

STA. One milliliter of cell suspension was placed in a Vacutainer tube (100 by 16 mm; Becton Dickinson) and tightly stoppered for stoppered-tube assay (STA). After flushing for 10 min with nitrogen, air was readmitted to give the appropriate final oxygen concentration. Cultures were shaken at 30°C overnight or for shorter periods as required. β-Galactosidase assays were performed as described by Miller (26).

RNA isolation and dot blot hybridization. The method of Zhu and Kaplan (33) was used with minor modifications to isolate total RNA from *R. meliloti*. Changes include: (i) the omission of sodium azide during cell harvesting; and (ii) lysis with 500 μg of predigested pronase per ml and 1% sodium dodecyl sulfate in 50 mM Tris-20 mM EDTA, pH 8.0, at 50°C for 10 min.

After treatment with formaldehyde, RNA was loaded onto nitrocellulose, as described by Meinkoth and Wahl (24). Hybridizations were carried out in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, and 200 μg of heparin per ml for 18 h at 37°C.
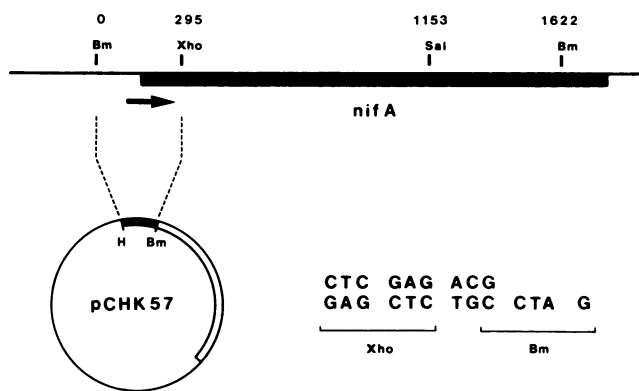
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FIG. 1. Construction of the nifA::lacZ fusion plasmid pCHK57. The nifA region of R. meliloti with coordinates taken from Buikema et al. (7) is indicated at the top of the figure; the solid bar delineates the nifA coding region, and the arrow shows the transcriptional start site. The 295-bp BamHI-to-XhoI fragment was introduced into pGD926 (12) as described in the text, resulting in a translational fusion of nifA (solid bar on circle) to lacZ (open bar on circle) at codon 8. The nifA sequence at the lacZ fusion junction is shown.

RESULTS

Construction of a nifA::lacZ gene fusion on a broad-host-range plasmid vector. A small fragment of DNA carrying the nifA promoter region of R. meliloti F34 was cloned into the β-galactosidase translational fusion plasmid pGD926 (12) (Fig. 1). To establish the correct reading frame at codon 8 of lacZ, a 295-base-pair (bp) BamHI-to-XhoI fragment from nifA was filled in by using a Klenow fragment and cloned into the HincII site of pUC9, selecting for the orientation that recreated the XhoI site of nifA immediately adjacent to the BamHI site of the polylinker region. After transfer of the HindIII-to-BamHI fragment to pGD926, the resulting plasmid pCHK57 contained codon 22 of nifA fused to lacZ, based on the translational start assigned by Buikema et al. (7) for R. meliloti 1021. The restriction map for strain F34 is identical to that for strain 1021 in the nifA region, and the entire HindIII-to-BamHI fragment of pCHK57 was sequenced to confirm that it contained the expected nifA promoter region (nifAp), leader, 5' protein-coding sequence, and fusion junction. The region upstream of the RNA start site is identical in strains F34 and 1021 for 92 bp, and the 295-bp fragment shares 98% homology with the corresponding sequence reported by Buikema et al. (7).

Symbiotic properties of pCHK57. Table 1 shows the symbiotic activity of pCHK57 in bacteroids from 3-week-old alfalfa root nodules induced by either the wild-type strain F34 or its nifA::Tn5 mutant N266 (11). For comparison, the activity of equivalent translational fusions of either the nifHDK (nitrogenase) promoter P1 (pMB210) or the fixABC promoter P2 (pMB211) is also shown. Both P1 and P2 required NifA for symbiotic activity and, as expected, showed substantial activity in F34 but essentially no activity in N266. nifAp, on the other hand, was expressed to the same extent in both hosts, showing that NifA is not required for symbiotic activation of its own promoter.

Induction of nifAp and P1 in an STA. A simple assay was used to study the effects of oxygen availability on promoter activity in R. meliloti over a range of oxygen concentrations. Small samples of logarithmically growing cells were transferred to stoppered tubes and flushed extensively with

nitrogen gas (N₂). Measured amounts of air were then introduced with a syringe, and the samples were shaken at 30°C for various lengths of time.

Figure 2 shows the kinetics of cell growth and resultant nifA promoter induction for cells in TY medium when placed under STA conditions with an initial air input equivalent to 1% oxygen. Panel A shows data for strain F34, and panels B and C show data for strain 1021 and its ntrC::Tn5 derivative strain 5001, respectively. It can be seen that growth rapidly slowed and then essentially ceased within 6 h at this particular combination of input oxygen and culture density. nifA promoter activity, as represented by the accumulation of β-galactosidase activity from pCHK57, rapidly increased during the same period. β-Galactosidase activity for F34 at the end of the experiment was in excess of that seen for nodule bacteroids (Table 1). The absence of a requirement for NtrC is consistent with the fact that induction was occurring in the presence of abundant fixed nitrogen sources (Fig. 2B and C). Microaerobic induction of nifA also occurred in the presence of ammonium ions (data not shown).

The production of functionally active NifA from genomic nifA under microaerobic conditions is detailed in Fig. 3. STA conditions were used to induce nifAp and P1 over a range of input oxygen concentrations both in F34 and in the nifA mutant N266. Incubations were for 18 to 20 h. In Fig. 3, panels A and B show induction in TY medium; panels C and D show induction after transfer from TY medium to nitrogen-free medium containing succinate as the sole carbon source.

Induction of nifAp was similar in both F34 and N266 in TY medium, with a peak occurring around 1% input oxygen (Fig. 3A). As is true for symbiotic induction of nifAp (Table 1), microaerobic induction of nifAp did not require functional NifA. At the input oxygen concentration with maximal nifAp expression, induction of P1 could also be demonstrated in F34 but not in N266 (Fig. 3B). NifA is thus absolutely required for P1 (nifHDK) activity during microaerobiosis. Since P1 activation in F34 was in rich medium in this experiment, it is obvious that NifA function is also not repressed by fixed nitrogen.

The response of nifAp to microaerobiosis in nitrogen-free medium was generally similar to that seen in rich medium (Fig. 3C), whereas the response of P1 in nitrogen-free medium differed substantially (Fig. 3D). The elevated level of background induction seen for P1 in both F34 and N266 at input oxygen concentrations greater than 1% is most striking (Fig. 3D). Aerobic induction of P1 under nitrogen starvation conditions in R. meliloti has previously been reported and been shown to be due to the ntrC gene product (30). As is

TABLE 1. Promoter activities in bacteroids[a]

Plasmid	Promoter	β-Gal U[b]	
		F34[c]	N266[d]
pCHK57	nifAp	1,400	1,420
pMB210	P1	11,460	77
pMB211	P2	9,420	28

[a] Nodules (20 to 40) were picked from duplicate groups of 12 to 15 alfalfa plants grown for 3 weeks in nitrogen-free medium. The nodules were crushed, and the β-galactosidase (β-Gal) activity of bacteroids was determined as previously described (4). The level of background expression in aerobically growing free-living cells for pCHK57 was comparable to that for pMB210 (approximately 40 U).

[b] Units as defined by Miller (26).
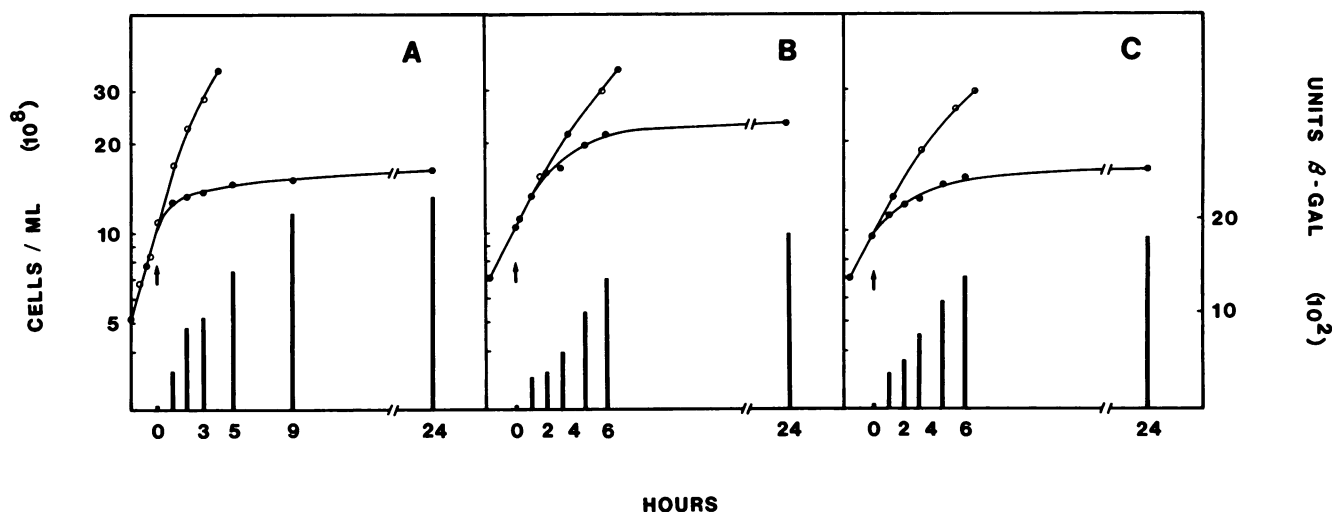
[c] Wild-type R. meliloti.

[d] nifA::Tn5 mutant of F34.

FIG. 2. Induction kinetics of *nifAp* in an STA. Cell density is indicated on the left ordinate, and β-galactosidase activity is on the right. The length of time after transfer to STA conditions with 1% oxygen (indicated by an arrow) is shown on the abscissa. Symbols: O, aerobically grown culture; ●, STA culture. The vertical bars indicate the β-galactosidase activity. Basal activity was approximately 50 U in each case. (A) F34(pCHK57); (B) 1021(pCHK57); (C) 5001(pCHK57).

true for enteric bacteria, NtrC expression in *R. meliloti* is induced by the removal of fixed nitrogen sources (30). The observation that NifA-specific activation of P1 occurred at input oxygen concentrations of 0.3 to 0.5% [F34(pMB210)] whereas activation attributable to NtrC [N266(pMB210)] decreased in this oxygen range is of particular interest (Fig. 3D).

Promoter P2 was found to be activated by microaerobiosis under STA conditions in a manner analogous to that shown for P1 (data not shown).

Induction of *nifAp* and P1 under constant low oxygen supply. In the STA system, each data point represents an average level of expression over the range of continually declining oxygen concentrations experienced by the culture. An alternative way to induce microaerobiosis is to provide cells with a constant supply of an artificially prepared gas mixture containing a low concentration of oxygen. This procedure does not maintain a constant dissolved oxygen tension, since oxygen is limiting for growth and the rate of oxygen consumption will increase with increasing cell density. Nevertheless, it does provide a means of lengthening the period of time during which induction can occur.

Figure 4 shows the results obtained when logarithmically growing cultures of F34(pCHK57) and F34(pMB210) in TY medium were switched from air to a mixture of 0.83% oxygen and 99.17% nitrogen. A constant aeration rate was maintained with bubbler tubes. Under low oxygen, cell growth showed continual slowing, as expected (Fig. 4A). Promoter induction showed several interesting features (Fig. 4B). First, both fusion plasmids showed multiple stages of induction and quiescence. This was not the result of sampling error, and we can offer no explanation for its cause. It should be noted that Jones et al. (19) reported multiphasic growth of slow-growing *Rhizobium* species, such as *R. japonicum*, after treatment with cyclic GMP (cGMP). In the same study, cGMP was found to have no effect on fast-growing rhizobia. We found that neither cGMP nor cAMP had any significant effect on *nifAp* induction in *R. meliloti* (G. Ditta, unpublished observations). Second, P1 induction lagged behind that of *nifAp* by approximately 45 to 60 min under these conditions. Despite the multiphasic induction

pattern eventually displayed by both promoters, this initial delay in P1 induction was very reproducible. The simplest interpretation is that a threshold level of NifA has to be synthesized before P1 can be activated. Third, after the initial delay, β-galactosidase accumulation from the P1 fusion plasmid approximated that from the *nifAp* fusion plasmid. By comparison with Table 1, it can be seen that the amount of β-galactosidase induced from pCHK57 under these conditions was at least twofold greater than that seen in nodule bacteroids, whereas that from pMB210 was roughly 25% of bacteroid levels. Relative to the basal level of β-galactosidase seen for each plasmid during logarithmic growth before exposure to low oxygen, there was a 74-fold increase for pCHK57 and a 59-fold increase for pMB210.

Analysis of *nifAp* induction by RNA dot blots. To confirm the gene fusion data, steady-state RNA levels were analyzed for a culture of F34(pCHK57) both before and after exposure to 0.83% oxygen for 180 min in TY medium. During this time, β-galactosidase activity increased 23-fold. Figure 5 shows dot blots of total bacterial RNA hybridized with three different ^{32}P-labeled nick-translated DNA probes. A 3-kilobase DNA fragment internal to the *lacZ* gene was used to identify RNA transcripts originating from pCHK57 (Fig. 5A). The results show an 11- to 12-fold increase in *lacZ* mRNA after microaerobic induction. In panel B, the 1,327-bp *Xho*I-to-*Bam*HI fragment internal to *nifA* but not present on pCHK57 (Fig. 1) was used to identify mRNA derived from the genomic copy of *nifA*. The same relative increase in mRNA levels was observed as for the fusion plasmid. Lastly, total genomic DNA from F34 was used as a probe in Fig. 5C to show that equivalent amounts of RNA were spotted in each case. The data show that increased levels of *nifA* mRNA are present during microaerobiosis. Results identical to those in Fig. 5B were obtained when RNA was prepared from F34 not carrying pCHK57 (data not shown).

Approximately half the *nifA* transcription in alfalfa nodules is derived from the upstream promoter P2, raising the possibility that autoactivation of *nifA* can occur through P2 (20a). The data in Fig. 5 do not support such a mechanism during microaerobiosis insofar as the same relative induction
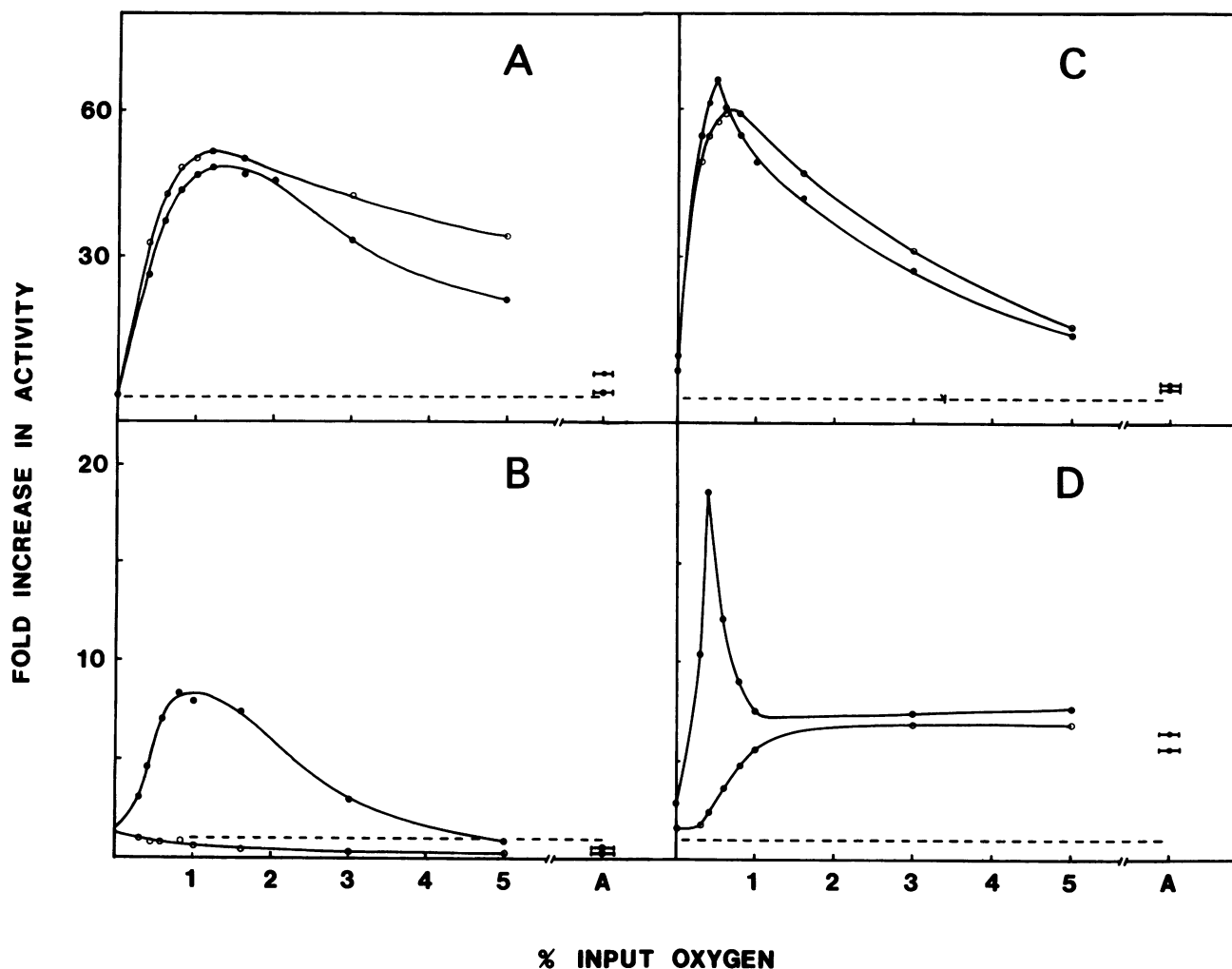
FIG. 3. Promoter induction as a function of oxygen concentration. STA conditions were used with an 18-h incubation time. The ordinate shows the ratio of β-galactosidase activity at the end of the experiment to the activity at the beginning. The dotted line (ratio = 1) indicates no induction. The average basal levels for the two promoters studied, *nifAp* (pCHK57) and P1 (pMB210), were approximately 40 and 30 U, respectively, in panels A and B (taken in TY medium), and 60 and 40 U, respectively, in panels C and D (determined after the final wash in nitrogen-free medium). The abscissa shows the initial oxygen concentration in the tube. Complete access to atmospheric levels of oxygen in an unstoppered tube is indicated by A on the abscissa. Symbols: ●, F34; ○, N266. (A) Activity of pCHK57 in TY medium; (B) activity of pMB210 in TY medium; (C) activity of pCHK57 in nitrogen-free medium; (D) activity of pMB210 in nitrogen-free medium. All data are the average of two experiments.

is seen for the plasmid-borne copy of *nifAp* (lacking P2) as for the genomic copy (plus P2). This is in agreement with our data showing that induction of pCHK57 did not require functional *nifA* gene product (Fig. 3A).

Effect of energy inhibitors on *nifAp* activity during aerobic growth. Since rhizobia are obligate aerobes, it is conceivable that microaerobic expression of *nifA* is in some way a cellular response to reduced energy availability. We therefore treated aerobically growing cultures of F34(pCHK57) with either sodium azide or 2,4-dinitrophenol to cause various degrees of energy starvation. Early-log-phase cells in TY medium were exposed to various concentrations of either inhibitor for 9 h, at which time the relative cell growth and β-galactosidase activity were measured. Over a wide range of growth inhibition, there was no significant induction of expression from pCHK57 (data not shown).

NtrC-mediated induction of *nifA* under nitrogen-limiting conditions. There was little aerobic induction of *nifA* expres-

sion in F34 under nitrogen-limiting conditions (Fig. 3). However, we observed that by substantially lowering the pH during nitrogen starvation, *nifA* expression could be induced by an *ntrC*-dependent mechanism. Figure 6 documents this phenomenon for *R. meliloti* F34 and 1021 and the *ntrC* mutant 5001. In all cases, logarithmically growing cells in TY medium were washed twice and resuspended in nitrogen-free medium containing succinate as the carbon source. Equivalent results were obtained using mannitol as the carbon source or using cells pregrown in TY plus succinate. Since there was significant variation from experiment to experiment in this study, it is likely that not all parameters affecting such induction have been identified.

The predominant variable affecting expression from pCHK57 was pH (Fig. 6A and B). Below pH 6, both F34 and 1021 showed substantial increases in *nifAp* activity, although strain 1021 was much more responsive at all pH values than was F34. Induction required NtrC (Fig. 6C). In both F34 and

1021, the P1 promoter was unaffected by pH and displayed only the previously described level of induction in nitrogen-free medium (Fig. 3) which is attributable to NtrC (30).

DISCUSSION

These experiments show that microaerobiosis is sufficient to induce *nifA* expression during vegetative growth in the fast-growing *Rhizobium* species *R. meliloti*. When the free oxygen concentration is reduced to appropriate levels, transcription of *nifA* is induced to levels as great as or greater than those seen in alfalfa nodules. Promoters in *R. meliloti* that are targets of NifA activation (P1 and P2) can correspondingly be induced in a *nifA*$^+$ host but not in a *nifA* mutant. Both *nifA* expression and NifA function are independent of fixed nitrogen availability under such conditions.

In legume root nodules, the free oxygen concentration is kept very low through the action of leghemoglobin, a symbiotically specific plant protein that mediates oxygen delivery to bacteroid cytochromes (1). These conditions simultaneously protect nitrogenase from irreversible oxygen denaturation while providing an adequate supply of oxygen for bacterial respiration and energy generation. Data presented here make it seem likely that the reduced oxygen tension in nodules also serves to activate transcription of nitrogenase and other *nif* genes by inducing *nifA*. While this does not rule out the possibility of other types of regulation also acting at *nifA* during symbiosis, regulation by oxygen is reasonable since it would coordinate overall *nif* gene expres-

FIG. 5. Hybridization analysis of *nifA* expression during microaerobiosis. Total RNA was prepared before and after 3 h of induction of F34(pCHK57) by 0.83% oxygen as shown in Fig. 4 and transferred to nitrocellulose for hybridization with nick-translated DNA probes. Amounts of RNA loaded per spot are given at the right. Abbreviations: atm, aerobically grown cells; 0.8%, microaerobically induced cells. (A) Probe is a 3.0-kilobase *Bam*HI-to-*Eco*RI fragment from pMC931 (Casadaban et al. [9]) internal to *lacZ*. (B) Probe is the 1,327-bp *Xho*I-to-*Bam*HI fragment internal to *nifA* (Fig. 1). (C) Probe is total F34 DNA.

sion with development of the appropriate respiratory physiology to support nitrogen fixation.

For those strains of slow-growing rhizobia known to be capable of asymbiotic nitrogen fixation, microaerobiosis has long been recognized as essential for induction (3, 20). In the absence of leghemoglobin and the stable physiological environment of the legume root nodule, artificial maintenance of an appropriate low free-oxygen concentration is necessary to balance energy demands against nitrogenase sensitivity. It is highly likely, in view of the present study, that reduced oxygen tension is also a critical variable directly affecting *nif* gene expression in such circumstances.

Neither the mechanism nor the gene(s) involved in transducing the low oxygen signal to the *R. meliloti nifA* promoter has been identified. Induction could involve either derepression or direct activation. Energy limitation by treatment with sublethal levels of sodium azide or 2,4-dinitrophenol did not suffice to induce *nifA* expression during aerobic growth. In both *K. pneumoniae* and *Rhodopseudomonas capsulata*, a *nif* regulatory mechanism has recently been demonstrated that is specifically linked to anaerobiosis and appears to involve DNA conformational changes induced by DNA gyrase (22). It is not known whether this mode of regulation directly involves a *nifA*-like promoter element. Also of relevance are two reports showing modest (two- to fourfold) inhibition of nitrogen starvation-mediated induction of *nifLA* by oxygen in *K. pneumoniae* (13, 21). The significance of this finding is unclear, however, since other workers have shown that there is little or no effect of oxygen on *nifLA* transcription once induction has been initiated (8, 10).

The fact that microaerobic induction of *nifA* expression occurs in the presence of fixed nitrogen sources and requires neither NtrC nor NifA is in line with several previous observations. First, rhizobia fix nitrogen primarily for export to their symbiotic host and not to support vegetative growth. It is therefore likely that nodule bacteroids function in a nitrogen-replete environment. Asymbiotic nitrogen fixation by *Bradyrhizobium japonicum* has been shown to occur under conditions where ammonium ions are exported into
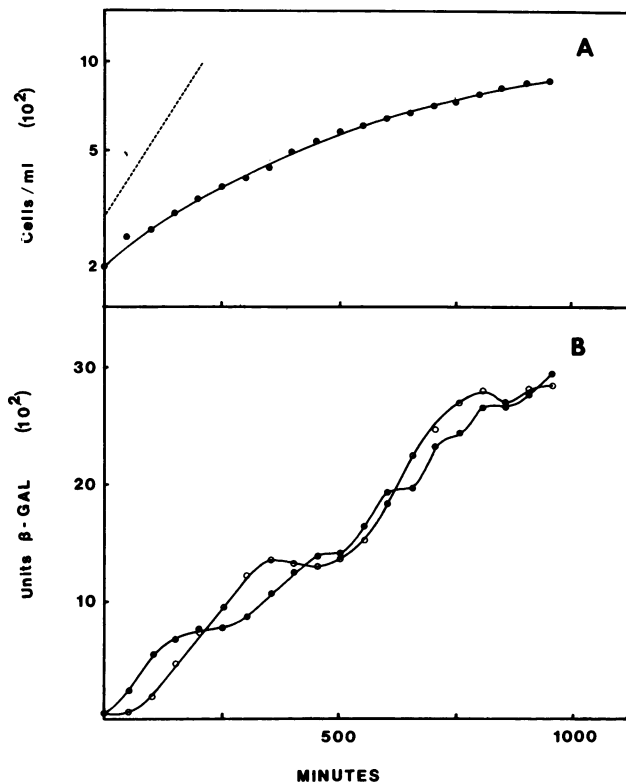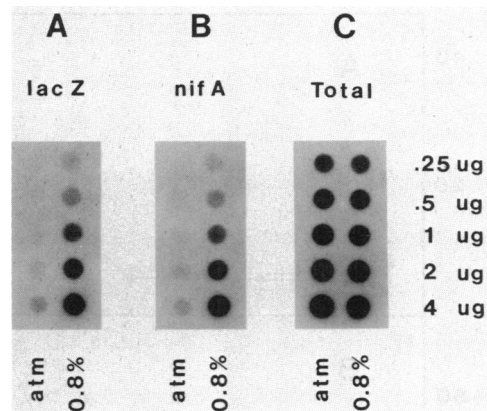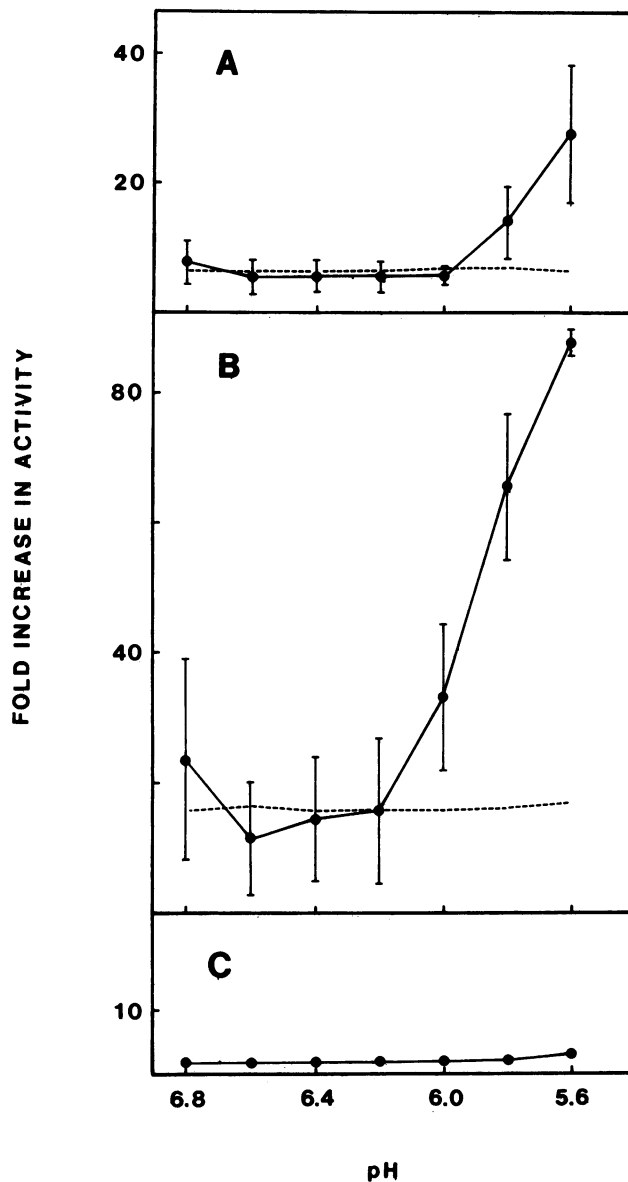

FIG. 4. Induction of *nifAp* and P1 under constant low oxygen supply. Early-log-phase cultures in bubbler tubes in TY medium were shifted to a gas mixture containing 0.83% oxygen and 99.17% nitrogen at the beginning of the experiment. Symbols: ●, F34(pCHK57); ○, F34(pMB210). (A) Growth curve for F34(pCHK57). The dotted line shows theoretical doubling time during normal aerobic growth. (B) β-Galactosidase induction.

FIG. 6. Effect of low pH on nifAp induction. Cells were pregrown in TY medium, washed twice with nitrogen-free medium of the appropriate pH, resuspended in the same, and shaken overnight at 30°C. The ordinate shows the ratio of β-galactosidase activity at the beginning of the experiment to that at the end. The basal level in this experiment corresponded to the starting activity in TY. The abscissa shows the pH of incubation. The dotted line shows the relative activation of pMB210. In panel C, no detectable activation of pMB210 was observed. The error bars show the standard deviation of three experiments. (A) F34(pCHK57); (B) 1021(pCHK57); (C) 5001(pCHK57).

the medium (27) and to actually require small amounts of organic nitrogen for induction (3, 16). Second, the ntrC gene of R. meliloti has specifically been shown to be unnecessary for symbiotic nitrogen fixation (30). Third, the DNA sequence of the R. meliloti nifA promoter region has no homology to promoters known to be regulated by either NtrC or NifA (7).

Since nifA expression is uncoupled from nitrogen regulation during microaerobiosis, it was not surprising that we

also observed NifA function to be similarly independent of fixed nitrogen availability. During microaerobiosis in rich medium, the nitrogenase promoter P1 was readily activated by NifA, whereas in nitrogen-free medium, both NifA and NtrC activated P1 but at different oxygen concentrations. NifA functioned at the lowest oxygen concentrations, and NtrC was operative at all higher oxygen concentrations. Aerobic induction of P1 by NtrC has been reported in both Escherichia coli (5) and R. meliloti (30), and certain DNA sequence features of P1 responsible for this capability are known (29). The physiological significance of such activation is unclear, however, in light of the dispensability of NtrC for symbiosis (30). The simplest interpretation for the failure of presumptive NtrC-mediated activation of P1 at low oxygen concentrations (Fig. 3D) is that NtrC is not made under these conditions, despite nitrogen limitation. An alternative possibility is that structural features of P1 discriminate between utilization by either activator, depending on the oxygen concentration.

Even though NtrC is not required for microaerobic induction of nifA under normal physiological conditions, we have nevertheless found that at low pH values, aerobic nitrogen starvation can lead to substantial activation of nifA by an NtrC-dependent mechanism. While we do not understand the significance of this phenomenon, the unusually low pH required for maximal activation, the lack of any effect of nifA induction on P1, the presence of atmospheric oxygen levels during induction, and the aforementioned absence of a symbiotic requirement for NtrC (30) all argue against an important symbiotic role. Further, since the nifA promoter region lacks homology to consensus nif promoter sequences, it is unlikely that NtrC is acting directly at nifAp. Despite the possibility that this mode of nifA activation may be largely artifactual, it is important to consider in light of the strong precedent for NtrC control of nifA in K. pneumoniae. Also of consequence is the fact that experimental attempts to demonstrate plant-derived signal compounds for nifA activation may inadvertently involve medium shifts toward low pH, since plant media and disrupted plant tissue are acidic.

Lastly, it is worth noting that we have been unsuccessful at simplistic attempts to induce asymbiotic nitrogen fixation in R. meliloti by microaerobiosis. This is not surprising, since nitrogen fixation is a complex enzymatic process and more stringent demands may exist for successful interaction of the pertinent gene products than for their production. Continued investigation of nif gene regulation in R. meliloti under microaerobic conditions plus additional studies on nif expression in slow-growing rhizobia should provide insight into the essential genes and conditions for nitrogen fixation.

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