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Anaerobic regulation of nitrogen-fixation genes in *Rhodopseudomonas capsulata*

(*lac* fusions/ammonia-constitutive mutants/DNA gyrase inhibitors)

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ABSTRACT **A *Rhodopseudomonas capsulata nifH::lacZ* gene fusion was used to isolate constitutive mutants of *R. capsulata*, unable to repress *nif* gene transcription anaerobically with every fixed-nitrogen source tested. When these *nif*^{c} strains were grown aerobically, *nif* gene transcription was repressed. These results indicate that the regulation of *nif* gene transcription by fixed nitrogen is different from the regulation by oxygen. Under anaerobic conditions, *nif* gene transcription in both *R. capsulata* and *Klebsiella pneumoniae* is specifically prevented by inhibitors of DNA gyrase [DNA topoisomerase type II (ATP-hydrolyzing), EC 5.99.1.3]. A recent study has shown that anaerobically grown *Salmonella typhimurium* have high DNA gyrase activity, whereas aerobically grown cells have high DNA topoisomerase type I (EC 5.99.1.2) activity and DNA that is more relaxed [Yamamoto, N. & Droffner, M. L. (1985) *Proc. Natl. Acad. Sci. USA* 82, 2077–2081]. In view of these results, we suggest that the control of *nif* gene transcription in response to oxygen is determined by the action of DNA gyrase and DNA topoisomerase I. Thus, although nitrogen control of *nif* gene expression requires the products of regulatory genes for which constitutive mutations can be isolated, oxygen appears instead to prevent the adoption of a DNA conformation necessary, directly or indirectly, for *nif* gene transcription.**

The organization and regulation of genes required for nitrogen fixation have been most extensively studied in the bacterium *Klebsiella pneumoniae* (for review, see ref. 1). In that organism, 17 *nif* genes are transcribed in seven or eight adjacent operons. Transcription of these *nif* genes is repressed by a number of effectors, including oxygen and a variety of fixed-nitrogen compounds (e.g., ammonia and glutamine). The response to limiting fixed nitrogen, under anaerobic conditions, occurs at two levels. First, the *ntrC* gene product, together with the *ntrA* gene product, activates transcription of the *nifLA* operon. This operon includes two regulatory genes, *nifA* and *nifL*, whose products are required for positive and negative control of *nif* structural gene transcription, respectively. The *ntrC* and *ntrA* gene products are also required for activation of a number of other operons whose functions control transport and degradation of amino acids used as a source of nitrogen. Mutation in either of the genes *ntrA* or *ntrC* results in an Ntr$^-$ phenotype—i.e., inability to use histidine, proline, or arginine as nitrogen source and a requirement for high external concentration of glutamine. Second, the *nifA* gene product, together with the *ntrA* gene product, activates transcription of all other *nif* operons.

At low concentrations of ammonia, the *nifLA* operon is still activated by *ntrC*/*ntrA*. Under these conditions the *nifL* gene product somehow inactivates the *nifA* gene product (2); transcription of other *nif* operons is thus repressed at low concentrations of fixed nitrogen. *Klebsiella* mutations in *nifL* (that are not polar on *nifA*) therefore show constitutive expression of *nif* structural genes at low concentrations of fixed nitrogen (2–4). These *nifL* mutants are also able to activate *nif* gene transcription in the presence of low concentrations of oxygen (2–4). Consequently, the *nifL* gene product has been implicated in both fixed-nitrogen and oxygen repression of *nif* gene transcription. An earlier study (5) suggested that a *nif*-specific gene (*nifL*?) was responsible for repression at low oxygen tensions and a more general mechanism was responsible for repression at higher oxygen concentrations (5). We will show that the latter suggestion is probably correct.

Although *Klebsiella nif* genetics has served as the model system for the organization and regulation of nitrogen-fixation genes, the analogies to other organisms are not exact. In *Rhodopseudomonas capsulata*, we have characterized an *ntrC*-homologous gene (called *nifR1*) as well as three other *nif*-specific genes (*nifR2-*, *-3*, and *-4*) required for activation of *R. capsulata nifH* transcription, but none of these genes affects Ntr functions (6). Like *Klebsiella nifA*, *R. capsulata nifR4* is linked to *nifHDK* and is itself repressed by ammonia (6).

We describe here the isolation of 13 *R. capsulata* mutants unable to repress *nif* gene transcription in response to fixed-nitrogen sources. All 13 *nif*-constitutive mutants will repress transcription of a *R. capsulata nifH::lacZ* fusion when oxygen is present. These results suggest that the mechanism of repression by oxygen differs from that of repression by fixed nitrogen. We further show that inhibitors of DNA gyrase [DNA topoisomerase type II (ATP-hydrolyzing), EC 5.99.1.3], the enzyme that negatively supercoils DNA, completely and selectively inhibit transcription of *nifHDK*. Recent studies (7) have shown that anaerobically grown cells of *Salmonella typhimurium* contain high gyrase activity and increased negatively supercoiled DNA compared to aerobically grown cells. We therefore suggest that anaerobiosis is required for transcription of the *nif* genes of *R. capsulata* because at least some of those genes must be in a negatively supercoiled conformation in order to be expressed and that oxygen repression of *nif* gene transcription is mediated at the level of DNA supercoiling. *nif* gene expression in *Klebsiella* is also prevented by an inhibitor of gyrase, indicating that negative supercoiling is required in that organism as well.

MATERIALS AND METHODS

Chemicals and Media. Coumermycin A, novobiocin, and *Staphylococcus aureus* protein A were purchased from Sigma. Coumermycin A was dissolved in dimethyl sulfoxide; in experiments with coumermycin, each culture contained an equivalent concentration of dimethyl sulfoxide. Novobiocin was dissolved in water. ^{3}H-labeled amino acids were purchased from New England Nuclear. Na^{125}I was purchased

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from Amersham. RCVB medium (with ammonia) and NF medium (without ammonia) have been described (8).

Bacteria and Plasmids. *R. capsulata* strains SB1003 (wild type) and mutant J61 have been described (9, 10). Construction of the *R. capsulata nifH::lacZ* fusion plasmid pRGK0 has been described (6). This *nifH::lacZ* fusion was recombined into the chromosome by conjugating in the incompatible plasmid pPH1 (which confers gentamicin resistance, Gm^r) and selecting for kanamycin-resistant (Km^r), Gm^r, Nif^- cells. This strain is denoted SB1003:*nifH::lacZ*. *R. capsulata* mutants that constitutively express *nif* genes (*nif*^{c} mutants) were obtained by plating *R. capsulata* containing the *nifH::lacZ* fusion [either on a plasmid (*nif*^{c} 19, 20, 21, 34, 46, 48, and 49) or in the chromosome (*nif*^{c} 51–56)] onto RCVB medium with lactose replacing malate. Strains 55 and 56 were spontaneous mutants. All other constitutive mutants were isolated from cells treated with ethyl methanesulfonate. *Klebsiella* UN4102 (Nif^+, His^-, Lac^-) (11) and plasmid pRPA8, containing the *R. capsulata nifHDK* operon, have been described (12).

Other Methods. β-Galactosidase and nitrogenase activity measurements (13, 14) and $NaDodSO_4$/polyacrylamide gel electrophoresis and immunoblotting (15) have been described. Antiserum to *Rhodospirillum rubrum* Fe protein (the *nifH* gene product) was kindly provided by P. Ludden (University of Wisconsin). Rabbit antiserum to partially purified *Anabaena* Mo–Fe protein (*nifD* and *nifK* gene products) was prepared in our laboratory by B. Mazur and J. Orr.

RESULTS

Characterization of *R. capsulata nif*-Constitutive (*nif*^{c}) Mutants. We previously reported the *in vivo* construction of an *R. capsulata nifH::lacZYA* gene fusion (6). When the plasmid containing this fusion, pRGK0, is present in wild-type *R. capsulata* SB1003, the strain can grow in medium with lactose as the only carbon source, under conditions that derepress the *nif* genes (Fig. 1). Wild-type *R. capsulata* without the plasmid or strain J61 with the plasmid are unable to grow under these conditions (Fig. 1). Strain J61 (10) is mutated in the *ntrC*-like gene required, directly or indirectly, for activation of the *nifH* promoter (6). Growth of wild-type *R. capsulata*(pRGK0) on lactose occurs only when fixed nitrogen is excluded from the medium, under anaerobic conditions.

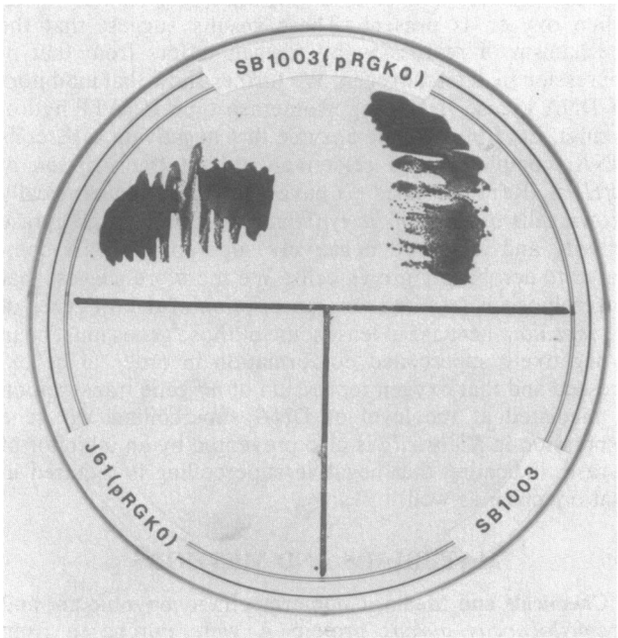

FIG. 1. *R. capsulata* wild type with the *nifH::lacZ* fusion plasmid [SB1003(pRGK0)], *nif* mutant J61(pRGK0), and SB1003 without the fusion plasmid, grown on ammonia-free medium with lactose as the sole carbon source. Only SB1003(pRGK0) can grow, due to derepression of *nifH* transcription, which drives the synthesis of both β-galactosidase and lactose permease.

R. capsulata nif^c mutants were isolated by including ammonia in the lactose minimal medium and selecting anaerobically in the light for growth. Originally, 50 *nif*^{c} mutants were isolated from SB1003(pRGK0). Seven strains expressing the highest β-galactosidase activity were studied further. These strains either reverted or lost pRGK0 at relatively high frequencies (0.1–1%). We therefore recombined the *nifH::lacZ* fusion into the chromosome and then selected new *nif*^{c} mutants (nos. 51–56). β-Galactosidase activities of extracts prepared from 13 *nif*^{c} strains (7 plasmid, 6 chromosome) grown anaerobically in medium containing ammonia are shown in Table 1. All 13 mutants were tested on a variety of fixed-nitrogen sources, including glutamine, proline, arginine, glutamate, and peptone-yeast extract. No nitrogen source tested was able to repress *nifH::lacZ* transcription under anaerobic conditions (data not shown).

Whole-cell protein extracts of all the *nif*^{c} mutants (grown anaerobically in medium containing ammonia) were examined by electrophoresis in $NaDodSO_4$/polyacrylamide gels. These gels were then immunoblotted with antibodies against the *nifD* and *nifK* gene products. Immunoblot analysis of *nif*^{c} mutants 51–56 is shown in Fig. 2; similar results were obtained with all of the other *nif*^{c} mutants (data not shown). The levels of the NifDK polypeptides are correlated with the β-galactosidase activities of the individual *nif*^{c} strains. Nitrogenase activities of *nif*^{c} mutants 51–56 (carrying pRPA8 to supply active *nifH, -D,* and *-K* genes) are high even in medium containing ammonia (Table 1). The latter results show that all the genes required for nitrogen fixation are transcribed and translated constitutively in the *nif*^{c} strains.

Oxygen Repression of the *nif*^{c} Mutants. *R. capsulata nif*^{c} mutants plated on X-Gal (5-bromo-4-chloro-3-indolyl β-D-

Table 1. β-Galactosidase and nitrogenase activities of *nif*^{c} strains

nif^c strain	β-Galactosidase		Nitrogenase
	+ NH_4, anaerobic	+ NH_4, aerobic	(+ NH_4, anaerobic)
19	89	3	—
20	514	23	—
21	154	21	—
34	177	12	—
46	1380	15	—
48	1540	14	—
49	690	20	—
51	1400	33	1.3*
52	1740	87	2.6
53	1420	16	2.4
54	1270	13	2.4
55	1440	97	3.9
56	1550	89	3.4

Cultures were grown in RCVB medium (8) to stationary phase and then induced anaerobically in the light for 12–24 hr. For aerobic growth, 25-ml cultures in 250-ml baffled flasks were shaken at 300 rpm. Culture OD_{550} at harvesting for aerobic cultures was approximately 0.7; even at this cell density, oxygen tensions were low due to endogenous oxidase activity. Nitrogenase activity of wild-type *R. capsulata* in ammonia-free medium ranges from 2 to 6 nmol of ethylene produced per min per mg of protein. β-Galactosidase activities of derepressed wild type with the *nifH::lacZ* fusion range from 500 to 5000 nmol of *o*-nitrophenol formed per min per mg of protein (depending on length of induction and culture density).
*pRPA8 (containing the *R. capsulata nifH, -D, -K* genes) was present in *nif*^{c} strains 51–56 for these inductions.

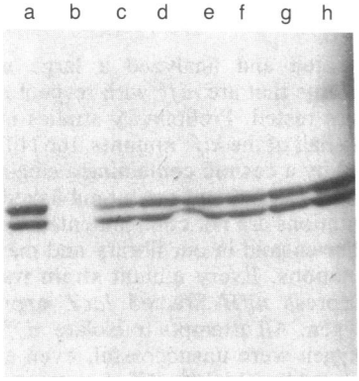

FIG. 2. Immunoblot of *nifDK* gene products from extracts of *nif*^{c} strains grown in medium containing ammonia. *nif*^{c} strains 51–56 (containing pRPA8, which supplies active *nifHDK* genes) were grown anaerobically in the light in RCVB containing ammonia. Extracts from sonicated cells were immunoblotted, using antiserum against the NifD and NifK proteins of *Anabaena* and ^{125}I-labeled protein A. Lane a: SB1003, grown without ammonia. Lane b: SB1003, grown with ammonia. Lanes c–h: *nif*^{c} mutants 51–56, grown with ammonia. Fifty micrograms of protein were loaded per lane.

galactoside) plates, with any fixed-nitrogen source, and incubated aerobically appear blue only in the anaerobic center of each colony. This result suggested that *nifH*::*lacZ* transcription is still repressed by oxygen. All 13 *nif*^{c} strains were grown aerobically in liquid medium containing ammonia. Under these conditions, less than 5% of the β-galactosidase activity of anaerobically induced cultures was observed (Table 1).

We attempted to isolate oxygen-constitutive mutants by a procedure analogous to that used for the *nif*^{c} mutants already described. When *R. capsulata* SB1003(pRGK0) was mutagenized and plated on lactose/ammonia minimal plates aerobically, no colonies grew. Sufficient cells were plated to yield 10^4 *nif*^{c} mutants anaerobically. In a second attempt, a mutant constitutive with respect to fixed nitrogen was remutagenized and plated on lactose/ammonia medium. Again, no colonies grew under aerobic incubation.

Effects of DNA Gyrase Inhibitors on *R. capsulata nifH*::*lacZ* Transcription. Recent studies with *S. typhimurium* have shown that anaerobically induced cultures possess high DNA gyrase activity and DNA with increased supercoiling; aerobically grown cells contained no measurable gyrase activity, high DNA topoisomerase type I activity, and DNA that is more relaxed (7). If supercoiling plays a role in oxygen repression of *nif* gene activation in *R. capsulata*, then inhibitors of gyrase activity should also inhibit transcription of the *R. capsulata nifH*::*lacZ* gene fusion.

Three experiments were performed to test this possibility. First, the *nif*^{c} mutant 55 was grown aerobically in medium with ammonia. These cells were then added to medium with increasing concentrations of the gyrase inhibitor novobiocin. After anaerobic induction in the light for 8 hr, β-galactosidase activities were measured (Table 2, Exp. 1). At sublethal concentrations of novobiocin, complete inhibition of *nifH*::*lacZ* transcription was observed. We noted in this experiment that the synthesis of the photosynthetic pigments was also repressed at the same concentrations of novobiocin that inhibited *nifH* transcription.

We next used *R. capsulata* SB1003:*nifH*::*lacZ*. In these experiments, aerobically grown cells were first transferred to medium containing ammonia and were then incubated anaerobically in the light to induce the photosynthetic complexes needed for ATP production. These cells were then washed and resuspended in ammonia-free medium with increasing concentrations of the gyrase inhibitors coumermycin (Exp. 2) or novobiocin (Exp. 3) and induced anaerobically in the light for 5 hr (Table 2). Again, sublethal concentrations of the gyrase inhibitors completely prevented *nifH* transcription, as judged by *lac* gene expression from the fusion.

Specific Effects of DNA Gyrase Inhibitors on *R. capsulata nifHDK* Gene Product Synthesis. In order to determine whether inhibition of *nifH* transcription by these drugs was specific, wild-type *R. capsulata* SB1003 was first grown aerobically in medium containing ammonia and was then grown anaerobically in the light for 8 hr and finally induced for 5 hr in ammonia-free medium in the light. During the final 5-hr induction period, portions of the culture were exposed to novobiocin and tritiated amino acids were added in order to label cellular proteins. Soluble proteins were then separated by polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 3*A*). Three polypeptides disappeared with increasing concentrations of novobiocin. These three migrated at the reported molecular weights of the *R. capsulata nifH*, *-D*, and *-K* gene products (M_r 33,500, 55,000, and 59,500; ref. 16). An identical gel was immunoblotted, using antibodies against the *nifHDK* gene products and ^{125}I-labeled protein A (Fig. 3*B*). This blot verifies that the three polypeptides that disappear when novobiocin is present are the NifH, -D, and -K proteins. Most of the other polypeptides synthesized are unaffected by novobiocin (Fig. 3*A*).

This experiment was repeated with the following difference: labeling of proteins with tritiated amino acids was begun 3 hr after the addition of novobiocin. For novobiocin concentrations of 10–50 μg/ml, the results were identical to those of Fig. 3: specific inhibition of the synthesis of the products of the *nifHDK* genes, enhancement of synthesis of a protein in the M_r 90,000 range, and no effect on the synthesis of most other proteins. At higher concentrations of novobiocin, there was general inhibition of all protein synthesis, although viability was not affected up to 150 μg/ml.

Effects of DNA Gyrase Inhibitors on *Klebsiella*. In the labeling experiment described above for *R. capsulata*, nitrogenase activities were measured after a 5-hr anaerobic induction. Every culture containing novobiocin (10–200 μg/ml) showed no nitrogenase activity, whereas the culture without novobiocin showed high activity (13.3 nmol of ethylene produced per min per ml of culture). *Klebsiella*

Table 2. Effects of DNA gyrase inhibitors on *R. capsulata nifH*::*lacZ* transcription

Exp.	Strain and condition	Drug, μg/ml	β-Galactosidase
1	*nif*^{c} 55 grown in RB aerobically,	0	1250
	then induced in RB	10	139
	anaerobically ± novobiocin	50	9
	for 8 hr	150	0
		500	0
2	SB1003:*nifH*::*lacZ* grown in RB	0	3340
	aerobically, then grown in RB	1	1570
	anaerobically in the light 8 hr,	5	956
	then induced in NF glutamate ±	25	493
	coumermycin for 5 hr	100	0
3	SB1003:*nifH*::*lacZ* grown in RB	0	3680
	aerobically, then in RB	10	267
	anaerobically in the light 8 hr,	50	0
	then induced in NF glutamate ±	150	0
	novobiocin for 5 hr	500	0

β-Galactosidase activity is expressed as nmol of *o*-nitrophenol formed per min per mg of protein. All drug concentrations used were sublethal (10^9 cells per ml), except novobiocin at 500 μg/ml (10^6 cells per ml).

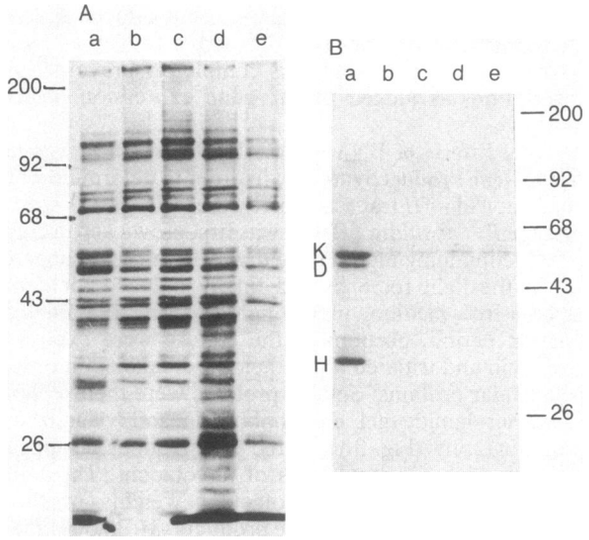

FIG. 3. Polyacrylamide gel profiles of proteins in extracts of *R. capsulata* SB1003 derepressed in the presence of increasing concentrations of novobiocin. Cells were grown aerobically until midlogarithmic phase of growth, then grown anaerobically in the light in RCVB, then derepressed in NF glutamate medium for 5 hr. ^{3}H-labeled amino acids and various concentrations of novobiocin were added during the derepression period. Lanes: a, no novobiocin; b, 10 μg/ml; c, 30 μg/ml; d, 90 μg/ml; e, 200 μg/ml. Cells were washed, sonicated, and spun in a microcentrifuge (Eppendorf) for 4 min. $NaDodSO_4$ sample buffer was added to each supernatant and 100,000 cpm were loaded in each lane. (*A*) Gel was treated with EN^{3}HANCE and autoradiographed to show total protein profiles. (*B*) Gel was immunoblotted, using antiserum to all three nitrogenase polypeptides followed by ^{125}I-labeled protein A, and autoradiographed. The positions of molecular weight standards are indicated at left and right. The NifH, NifD, and NifK polypeptides (M_r 33,000, 55,000, and 59,000, respectively) were synthesized only in the absence of novobiocin.

pneumoniae appeared to be very resistant to novobiocin (up to 200 μg/ml) but sensitive to coumermycin. *Klebsiella* was therefore grown anaerobically in medium containing ammonia for 7 hr and then induced anaerobically overnight in ammonia-free medium with various (sublethal) concentrations of coumermycin. Nitrogenase activities fell to zero (Table 3). An experiment similar to that shown in Fig. 3 was performed on *Klebsiella* UN4102. Total cell proteins were labeled metabolically with tritiated amino acids, and nitrogenase polypeptide synthesis was monitored using antiserum to the NifH, -D, and -K proteins. The results (not shown) were nearly identical to those shown for *R. capsulata* in Fig. 3. The NifH, -D, and -K proteins failed to appear with increasing concentrations of coumermycin, while most of the other polypeptides synthesized were unaffected.

Table 3. Effect of coumermycin on *Klebsiella* nitrogen fixation

Coumermycin, μg/ml	Nitrogenase*
0	0.14
1	0.054
25	0.0052
100	<0.00017
300	<0.00017

Klebsiella UN4102 (11) was grown in Luria–Bertani medium overnight, then for 7 hr anaerobically in KN medium (contains ammonia) (17), washed, and resuspended in K medium (contains no ammonia) with increasing concentrations of coumermycin. After 12 hr, nitrogenase was assayed by acetylene reduction. All drug concentrations used were sublethal (≈3 × 10^9 cells per ml).
*Expressed as nmol of ethylene produced per min per ml of culture.

DISCUSSION

We have isolated and analyzed a large number of *R. capsulata* mutants that are *nif*^{c} with respect to every fixed-nitrogen source tested. Preliminary studies indicate that in approximately half of the *nif*^{c} mutants, the Nif^c phenotype is complemented by a cosmid containing a single *R. capsulata* wild-type *Eco*RI DNA fragment (unpublished results). The other *nif*^{c} mutations are not complemented by this fragment or by any other cosmid in our library and may therefore be dominant mutations. Every mutant strain was still able to completely repress *nifH*-directed *lacZ* expression in response to oxygen. All attempts to isolate *nif*^{c} mutants with respect to oxygen were unsuccessful, even at cell concentrations that would yield 10^4 *nif*^{c} mutants with respect to fixed nitrogen. The mechanisms controlling *nif* transcription in response to fixed nitrogen are clearly different from those controlling oxygen repression.

Past studies have focused on the *nifL* gene product as being responsible for both low-ammonia and oxygen control of *nif* transcription in *Klebsiella* (e.g., refs. 2–4). Most of these studies used external oxygen concentrations that were borderline for *nif* expression (i.e., up to 60 μM O_2). Possibly, as suggested in an earlier study (5), the *nifL* gene product mediates this borderline effect. Our results on *R. capsulata* *nif*^{c} mutants are not consistent with a *nifL*-like gene product mediating both ammonia and oxygen effects. Instead, the inability to obtain *nif* mutants constitutive with respect to oxygen could be explained if DNA gyrase (i.e., supercoiling) were the controlling factor in response to oxygen.

The drugs novobiocin and coumermycin are specific inhibitors of DNA gyrase subunit B (18, 19). These inhibitors have been used to determine the effect of supercoiling on specific gene transcription in a number of systems (e.g., refs. 20–24; for review, see ref. 25). We have shown that both coumermycin and novobiocin completely block activation of *R. capsulata nifHDK* transcription. This is a specific effect on the *nifH* gene: a *nifH*::*lacZ* fusion was not activated and the NifH, -D, and -K proteins were missing from extracts of novobiocin-treated cultures. We have also found that both novobiocin and coumermycin specifically inhibit expression of the genes for photosynthetic reaction center proteins and dimethyl sulfoxide reductase (unpublished results). Like the *nif* gene products, these proteins are normally expressed only under anaerobiosis. Although it is possible that the drugs are directed against a target other than DNA gyrase, the direct link observed between DNA gyrase activity and anaerobiosis in other Gram-negative bacteria (7) argues against this possibility. In the light of our results and those obtained with *Salmonella* (7), we suggest that transcriptional control of *nif* genes in response to oxygen is due to a requirement for negatively supercoiled DNA. This appears to be the case for both *R. capsulata* and *Klebsiella*. We next address the question whether the requirement is a direct one on the *nif* structural genes or an indirect one mediated through the various regulatory genes.

Since the product of *nifA* is absolutely required for transcription of the other *nif* genes in *Klebsiella*, oxygen inhibition of *nifLA* transcription will clearly result in prevention of expression of the other genes. Although some previous reports have concluded that transcription of the *nifLA* operon in *Klebsiella* is insensitive to oxygen (5, 26, 27), recent data show that a chromosomal *nifL*::*lacZ* fusion is 90% repressed by air (S. Hill, personal communication). This result is in agreement with an earlier study using a *nifL*::*lacZ* fusion (83% repressed by aeration) and a *nifA*::*lacZ* fusion (94% repressed by aeration) (28).

We have recently found that *nifR4* transcription in *R. capsulata* is repressed by either aeration (at least 70%) or novobiocin (100%) (unpublished results). Like *nifA* in *Kleb-*

siella, nifR4 in *R. capsulata* is closely linked to *nifHDK* (29) and is repressed by fixed nitrogen (6). In *Klebsiella, nifHDK* transcription is insensitive to oxygen when *nifA* is expressed constitutively from the kanamycin- or tetracycline-resistance gene promoters (30). These results suggest that oxygen prevents expression of the positive regulatory genes *nifA* in *Klebsiella* and *nifR4* in *R. capsulata*. Our experiments with inhibitors of DNA gyrase indicate that decreasing the level of DNA supercoiling results in decreased expression of these regulatory genes, without whose products the other *nif* genes cannot be transcribed. There must be other genes in *R. capsulata*, perhaps regulatory genes, equally sensitive to the level of DNA supercoiling, because novobiocin also prevents the appearance of proteins required for photosynthesis, for dimethyl sulfoxide reduction, and for anerobic growth in general. It is important to point out that although DNA gyrase inhibitors have been shown to prevent the induction of the genes for nitrogen fixation, more work is needed to show direct relationships among *nif* gene expression, DNA gyrase activity, and chromosome topology in *R. capsulata* grown under defined oxygen tensions.

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