The Product of the *Klebsiella pneumoniae* *nifX* Gene Is a Negative Regulator of the Nitrogen Fixation (*nif*) Regulon

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An insertional mutation was made in the *nifX* gene of *Klebsiella pneumoniae*. This mutation had little effect on the nitrogenase activity of the strain, as measured by acetylene reduction. However, on the addition of NH_4^+ or O_2 (conditions which block *nif* protein synthesis by transcriptional and posttranscriptional mechanisms), the *NifX*[−] mutant synthesized nitrogenase proteins longer and had more accumulated *nifHDKTY* mRNA than did the wild-type *K. pneumoniae* at all time points tested. Conversely, overexpression of the wild-type *nifX* region blocked *nif* protein synthesis, protein accumulation, and *nifHDKTY* mRNA accumulation. These complementary results indicate that a product of the *nifX* region has a role in the negative regulation of *nif* regulation in response to NH_4^+ and O_2 .

Nitrogenase is found in a number of procaryotic organisms and carries out the reduction of atmospheric N_2 to ammonia. In *Klebsiella pneumoniae*, the genes encoding the proteins involved in nitrogen fixation are located on a contiguous 24-kilobase fragment containing at least seven different operons and 20 different genes (19, 24, 31). The functions of most of the gene products are known. The *nifH*, *nifD*, and *nifK* genes encode the proteins that make up the structural components of nitrogenase (30). The gene products of *nifF* and *nifJ* are involved in the electron transport to the structural components (12, 22). The *nifQ*, *nifB*, *nifM*, *nifV*, *nifN*, and *nifE* gene products are involved in the processing of, or the synthesis of cofactors for, the structural components (13, 14, 30). The gene products of the *nifLA* operon are involved in the transcriptional and posttranscriptional regulation of the rest of the *nif* regulon (19, 30).

The *nifA* protein, in cooperation with the sigma factor encoded by *rpoN*, activates the transcription of all the other nitrogenase promoters (8, 18). Transcription of the *nifLA* operon is itself activated through the action of *rpoN*, *ntrB*, and *ntrC* gene products on NH_4^+ starvation (9, 25).

The *nifL* gene product is involved in the negative regulation of *nif* expression. On the addition of NH_4^+ or O_2 to wild-type *K. pneumoniae* cells derepressed for nitrogenase, the synthesis of new *nif* mRNA rapidly halts and the existing *nif* mRNAs become destabilized. Under these same conditions, *NifL*[−] mutants neither destabilize their *nif* mRNAs nor halt the synthesis of new *nif* mRNAs (5, 6). The exact mechanism by which the *nifL* gene product brings about these two effects is unknown.

In addition to the genes noted above, there are seven other genes in the *nif* region, the functions of whose products are poorly defined: *nifZ*, *nifW*, *nifU*, *nifS*, *nifX*, *nifY*, and *nifT*. In the case of *nifW* and *nifZ*, there is some evidence that their products may be involved in the processing of one of the structural components of nitrogenase (26). The functions of the other gene products are unknown, but the conservation of similar sequences in other diazotrophs suggests that they do play a role in nitrogen fixation. The *nifX* gene was

first identified by Pühler and Klipp (28) as a region of *nif* DNA which, when cloned behind a high-level promoter, synthesized a polypeptide of 18 kilodaltons (kDa). An open reading frame for this polypeptide was later identified when the *nif* regulon was sequenced (1).

In order to investigate the function of the *nifX* product, we constructed an insertional mutation in the chromosomal copy of *nifX* in *K. pneumoniae* and an expression vector that was capable of overproducing the *nifX* region on induction with isopropyl- β -D-thiogalactopyranoside (IPTG). In this report we present evidence that the *nifX* region is involved in the negative regulation of *nif* expression.

MATERIALS AND METHODS

Media and reagents. The recipe for the minimal medium used in this study is the same as that described previously (15), but we also added 2.0% sucrose as a carbon source. *Escherichia coli* was grown in minimal medium with 2.0% glucose–0.22% ammonium acetate–1 mM thiamine hydrochloride.

The following antibiotics were used at the indicated concentrations: kanamycin sulfate, 100 $\mu\text{g}/\text{ml}$; carbenicillin (disodium salt)–ampicillin (sodium salt), 800 and 60 $\mu\text{g}/\text{ml}$, respectively.

All chemicals, enzymes, and gases were of analytical grade or higher and were obtained from Sigma Chemical Co. (St. Louis, Mo.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Bio-Rad Laboratories (Richmond, Calif.), Promega Corp. (Madison, Wis.), or Pharmacia, Inc. (Piscataway, N.J.). The *trans*- ^{35}S label (a mixture of several ^{35}S -labeled amino acids) was obtained from ICN Biomedical, Inc. (Costa Mesa, Calif.).

Bacterial strains. The wild-type strain of *K. pneumoniae* (UN) was described previously (16). The *nifX* mutation (*nifX6289::aphA*) was first constructed on a plasmid by ligating the Kan^r cassette (the *aphA* gene) from pUC-4K (32) into the *nifX* gene in pMG103, which was itself constructed from the plasmids pKK223-3 (4) and pJC212 (J. J. Collins, Ph.D. thesis, University of Wisconsin, Madison, 1984), as shown in Fig. 1. Reciprocal recombination of the mutated *nifX* gene into the chromosome was enriched for by P1

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FIG. 1. Construction of the *nifX* insertion mutation (*nifX*6289::aphA). The region containing *nifX* was cut from pJC212 (a) by *Sal*I digestion and then ligated into the large fragment of *Sal*I-digested pKK223-3 (b), resulting in the plasmid pMG103 (c). The *Kan^r* cassette was cut from pUC-4K (d) by *Bam*HI digestion and then ligated into pMG103 that was partially digested with *Bam*HI, resulting in the plasmid pMG105 (e). The *nifX* insertion mutation was moved onto the *K. pneumoniae* chromosome by reciprocal recombination. The origin of replication and the ampicillin resistance marker were left off of all plasmids for clarity.

transduction (33) of *Kan^r* from UN containing the plasmid to a plasmidfree UN; inefficient packaging of the plasmid enriches for transductants carrying the chromosomally integrated region. Plasmidfree insertion mutants were identified by their kanamycin-resistant, ampicillin-sensitive phenotype and confirmed by Southern blot analysis. The strain with the *nifX* insertion was designated UN5359.

The *nifX* expression vector, pNF101, was constructed by ligating the *nifX* gene from pMG103 downstream of the P_{TAC} promoter of pKK223-3, as shown in Fig. 2. pNF101 was transformed into UN729 (a *rfb-4001*-containing derivative of UN), creating UN5373, and into *E. coli* CHS26 (20), creating UQ747. The expression vector without the insert, pKK223-3, was also transformed into UN729, creating the strain UN5366. Both strains also contained an F' plasmid that has the *lacI^q* gene, allowing regulation of P_{TAC} .

Derepression and assay of nitrogenase. *K. pneumoniae* cultures were grown overnight to the stationary phase at 30°C in minimal medium with sucrose plus NH_4^+ (as 0.22% ammonium acetate). For derepression, *K. pneumoniae* cultures were pelleted by centrifugation ($6,000 \times g$ for 10 min),

FIG. 2. Construction of the *nifX* expression vector (pNF101). The *nifX*-containing region was excised from pMG103 (a) by digestion with *Sal*I and *Eco*RI. The *nifX*-containing fragment was then ligated into the multicloning site (MCS) of pKK223-3 (b), which was digested to completion with *Eco*RI and partially digested with *Sal*I, resulting in the *nifX* expression vector pNF101 (c). The origin of replication and the ampicillin resistance marker were left off of all plasmids for clarity.

suspended in minimal medium with sucrose plus serine (150 mg/liter) to 3×10^8 cells per ml, and incubated at 30°C for 4 h.

The effects of the addition of NH_4^+ or O_2 to derepressed cultures were examined by adding ammonium acetate to 0.22% or by shaking them vigorously in air in a baffled flask of ≥ 10 sample volumes.

Derepressed strains were assayed for in vivo nitrogenase activity essentially as described by Roberts et al. (30), except that 1-ml portions of culture were used for each assay and the assays were terminated by the addition of 0.1 ml of NaOH.

Southern blot analysis. Chromosomal DNA was isolated as described by Perbal (27). Digested DNA samples were separated on a 1.2% agarose TBE gel and blotted as described by Maniatis et al. (17). The 709-base-pair (bp) *Eco*RI-*Ssp*I restriction fragment containing the *nifX* gene was labeled with ^{32}P by random hexamer labeling as described by Feinberg and Vogelstein (10) and was used as a probe. Hybridizations and washes were done essentially as described previously (2), except that washes were modified as follows: twice in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 15 min each time, twice in $2 \times$ SSC and 0.5% sodium dodecyl sulfate at 42°C for 30 min each time, and twice in $1 \times$ SSC and 0.1% sodium dodecyl sulfate at 65°C for 30 min each time.

Northern blot analysis. For the isolation of total RNA from derepressed cells, 1-ml portions of cell cultures were pelleted in a microfuge for 30 s and the cell pellets were suspended in 300 μ l of RNA solution A (10 mM MOPS [morpholinepropanesulfonic acid; pH 7.3], 10 mM KCl, 5

mM $MgCl_2$); and then 300 μ l of RNA solution B (20 mM MOPS [pH 7.5], 40 mM EDTA, 200 mM NaCl, 1.0% [wt/vol] sodium dodecyl sulfate) was added to each cell suspension. The mixture was incubated at 100°C for 2 min to lyse the cells. The RNA was isolated from the lysed cells by extracting them twice with an equal volume of unbuffered phenol, once with TE-equilibrated phenol-chloroform (50:50, by vol), and once with TE-equilibrated chloroform. The RNA was then precipitated by the addition of sodium acetate to 0.3 M and three volumes of 100% ethanol. RNA samples were suspended and separated on a 1.5% agarose formaldehyde gel and blotted as described in a manual (GeneScreen Hybridization Transfer Membrane: Instruction Manual, Dupont, NEN Research Products, Boston, Mass.), except that capillary blotting took place in 10 $\times$ SSC. TE and 10 $\times$ SSC buffers were prepared as described by Maniatis et al. (17).

RNA solutions A and B, gel buffers, and 10 $\times$ SSC were treated with diethyl pyrocarbonate before use, as described by Maniatis et al. (17). The 1,410-bp *Bam*HI-*Sal*II restriction fragment containing parts of the *nifDK* genes was labeled with ^{32}P by random hexamer labeling (10) and was used as a probe. Hybridizations and washes were done as described above for Southern blot analysis.

Protein analysis. Cells were labeled for two-dimensional gel analysis by the following technique. Portions (1.5 ml) of the cell culture were added to 5-ml stoppered, degassed vials containing 50 μ Ci of *trans*- ^{35}S label and incubated for 3 min at 30°C. Cells were then quick-frozen in a dry ice-ethanol bath, thawed at 0°C, and pelleted in a microfuge at 10,000 $\times$ g for 30 s.

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed as described previously (11, 23) and modified by Roberts and Brill (29).

For two-dimensional analysis of the effects of pNF101-encoded products on *nif* protein synthesis, the P_{TAC} promoter of pNF101 was induced by the addition of 5 mM IPTG to minimal medium with sucrose plus serine at the start of *nif* derepression.

RESULTS

Southern blot analysis of the *nifX* insertion. In order to examine the function of the *nifX* gene product, we constructed a *K. pneumoniae* strain with a Kan^r cassette inserted into the *nifX* gene. Southern blot analysis confirmed that the Kan^r cartridge from pUC-4K was successfully recombined into the *nifX* gene on the chromosome (Fig. 3). Since there was no wild-type band in the mutant, we conclude that there is no remaining wild-type copy of the *nifX* gene in our mutant. The upper-molecular-weight bands were due to homology elsewhere in the chromosome, since these bands appeared in Southern hybridization experiments with strains containing deletions of the entire *nif* region (data not shown).

Effect of a *nifX* insertion on nitrogenase activity. The insertion in the *nifX* gene had little effect on the nitrogenase activity of *K. pneumoniae*. Both UN and UN5359 (the *nifX* insertion mutant) began derepressing at the same time, and their activities increased to similar peak activities at approximately the same rate (Fig. 4). When grown on N_2 in liquid culture, UN5359 had a doubling time of 5 h, while UN had a doubling time of 4 h. Since the *nifX*6289::*aphA* mutation had little effect on the nitrogenase activity of *K. pneumoniae* under derepressing conditions, we investigated the possibility that the *nifX* protein has a role in the negative regulation of *nif*.

FIG. 3. Southern blot analysis of chromosomal DNA from UN (lane 1) and UN5359 (lane 2). DNA isolated from both strains was digested with *Sal*II, separated on a 1.2% agarose gel, and blotted to a nylon membrane. The membrane was hybridized with a ^{32}P -labeled probe starting from the *Eco*RI site in *nifN* and ending at the *Sph*I site 90 bp after the end of *nifX*. The arrows, from top to bottom, indicate the positions of 1,961, 1,377, and 584 bp, respectively.

Effect of NH_4^+ addition on protein synthesis and accumulated mRNA. On the addition of NH_4^+ , wild-type *K. pneumoniae* rapidly halts the synthesis of new nitrogenase proteins by stopping the synthesis of *nif* mRNAs and destabilizing existing *nif* mRNAs (5, 6). In order to determine whether the *nifX* mutation had any effect on the ability of *K. pneumoniae* to respond to NH_4^+ addition, we derepressed UN and UN5359 for *nif* expression, and then NH_4^+ was added to each strain. Protein synthesis in both strains, before and at several time points after NH_4^+ addition, was examined by pulse-labeling, followed by two-dimensional PAGE analysis. The results indicate that while both UN and UN5359 rapidly halt the synthesis of new *nif* proteins, UN5359 is slower in its response to the addition of NH_4^+ than is UN. For example, 45 min after the addition of NH_4^+ , UN5359 appeared to be synthesizing more component I and

FIG. 4. Acetylene reduction activities of strains UN, UN5359, and UN5373 during derepression. The wild type and UN5359 were derepressed for *nif* normally, while UN5373 was derepressed both in the presence and absence of IPTG. Acetylene reduction activities of all strains were followed for 9 h with assays every 30 min. Symbols: □, UN; ■, UN5359; △, UN5373 with IPTG; ▲, UN5373 without IPTG. OD₆₀₀, Optical density at 600 nm.

FIG. 5. Two-dimensional analysis of the effects of NH_4^+ addition on protein synthesis. The newly synthesized proteins of UN and UN5359 were labeled with *trans*- ^{35}S label and separated by two-dimensional PAGE. (a and b) The proteins being synthesized before the addition of NH_4^+ ; (c and d) the proteins being synthesized 45 min after the addition of NH_4^+ . Panels a and c are the results with UN, and panels b and d are the results with UN5359. Component I proteins, NifK and NifD, are highlighted by boxes. The component II protein, NifH, is circled.

II proteins than UN did (Fig. 5c and d) when compared with the other proteins found on these gels.

In order to determine the cause of this observed difference in response to NH_4^+ , we performed Northern blot analysis on the mRNA from both strains, before and after the addition of NH_4^+ (Fig. 6, left side). The results indicate that UN5359 has higher amounts of accumulated *nifHDKTY* mRNA than does the wild-type strain at all tested time points after the addition of NH_4^+ . These results suggest that the *nifX* region may be involved in the negative regulation of *nif* mRNA synthesis or stability on NH_4^+ addition.

Effect of O_2 addition on protein synthesis and accumulated mRNA. Since *K. pneumoniae* responds in a similar fashion to O_2 addition as it does to the addition of NH_4^+ , the response of UN and UN5359 to the addition of O_2 was also examined at several time points. The results indicate that UN5359 again continued to synthesize nitrogenase proteins

FIG. 6. Northern blot analysis of the effects of NH_4^+ or O_2 addition on accumulated *nifHDKTY* mRNA. Total RNA was isolated from the wild type and UN5359 before and at several time points after the addition of NH_4^+ . The Northern blot was hybridized with a ^{32}P -labeled DNA probe of the *Bam*HI-*Sal*I fragment containing the downstream end of the *nifD* gene and the upstream end of the *nifK* gene. The center pair of lanes shows the pattern for UN and UN5359 before any addition. The effects of various periods of NH_4^+ exposure are shown on the left side, while the effects of O_2 exposure are shown on the right side. The positions of the arrows indicate the positions of the rRNA bands (~3,000 and ~1,500 bp).

FIG. 7. Two-dimensional analysis of the effects of O_2 addition on protein synthesis. The newly synthesized proteins of strains UN and UN5359 were labeled with *trans*- ^{35}S label and separated by two-dimensional PAGE. (a and b) The proteins being synthesized before the addition of O_2 ; (c and d) the proteins being synthesized 30 min after the addition of O_2 . Panels a and c are the results with UN, and panels b and d are the results with UN5359. Component I proteins, NifK and NifD, are highlighted by boxes. The component II protein, NifH, is circled.

longer than did UN following exposure to O_2 . As can be seen (Fig. 7), 30 min after the addition of O_2 , UN5359 continued to synthesize significantly more component I and II proteins than did UN. The accumulated *nifHDKTY* mRNA was examined by Northern blot analysis, and the results indicated that UN5359 again had elevated levels of accumulated *nifHDKTY* mRNA, relative to that of UN, at all tested time points after the addition of O_2 (Fig. 6, right side).

Overexpression of the *nifX* region. In the original identification of *nifX* as a coding region, Pühler and Klipp (28) reported the existence of an 18-kDa protein encoded between the end of the *nifEN* operon and the start of the *nifUSVWZM* operon. We cloned the region containing the *nifX* gene behind the IPTG-inducible P_{TAC} promoter in the expression vector pKK223-3, generating the plasmid pNF101. When this promoter was induced, it caused the synthesis of an 18-kDa protein in both *E. coli* and *K. pneumoniae*. Two-dimensional analysis of the protein products from the in vitro translation of the purified plasmid (pNF101) further demonstrated that the 18-kDa protein was encoded by the plasmid and that the protein also had a very basic isoelectric focusing point, as predicted from the *nifX* sequence (data not shown). From these results we conclude that the protein being visualized is the *nifX* gene product.

Effect of overexpression of the *nifX* region on nitrogenase activity. When *nifX* product synthesis was induced at the start of *nif* derepression in UN5373 (a *nif* $^+$ *K. pneumoniae* strain containing the *nifX* expression vector pNF101), there was a complete inhibition of nitrogenase activity, as measured by acetylene reduction (Fig. 4). Even in the absence of IPTG, there was a decrease in peak acetylene reduction activity, probably because of low-level transcription initiation from the P_{TAC} promoter. Control experiments with the expression vector pKK223-3, which lacked the *nifX* insert, showed no effect on acetylene reduction activity, with or without IPTG addition (data not shown).

Effect of overexpression of the *nifX* region on protein synthesis and on accumulated mRNA. In order to determine whether the prevention of acetylene reduction activity by

FIG. 8. Effect of overexpression of the *nifX* gene product on *nif* protein synthesis. Strains UN5373 and UN5366 (*K. pneumoniae* UN729 containing the expression vector [pKK223-3] that lacks the *nifX* insert) were derepressed for *nif* in the presence of IPTG for 4 h. Newly synthesized proteins were labeled with ^{35}S and then separated by two-dimensional PAGE. (a) The proteins being synthesized by UN5366; (b) the proteins being synthesized by UN5373. Component I proteins, NifK and NifD, are highlighted by boxes. The component II protein, NifH, is circled.

overexpression of the *nifX* region resulted from blockage of the translation of *nif* products, we examined protein synthesis in UN5373. Two-dimensional PAGE analysis of the strain containing pNF101 revealed that when expression of the *nifX* region was induced at the start of derepression, there was complete elimination of the synthesis of nitrogenase proteins (Fig. 8).

We also examined the effect of overexpression of the *nifX* region on accumulated *nifHDKTY* mRNA by Northern blot analysis. The results indicate that no *nifHDKTY* mRNA was accumulated when the *nifX* region was induced at the start of *nif* derepression (Fig. 9).

DISCUSSION

In order to determine the role of the *nifX* gene product in nitrogen fixation, we took two different approaches in this study. One approach was to build a *K. pneumoniae* strain (UN5373) that contained a plasmid (pNF101) that was capable of overproducing the *nifX* gene product upon induction with IPTG. Another approach was to construct a *K. pneumoniae* strain (UN5359) which could not make the *nifX* gene product, and to that end an insertional mutation in *nifX* was constructed by using the Kan^r (*aphA*) cassette marker from plasmid pUC-4K. Southern blot analysis demonstrated that

FIG. 9. Effect of overexpression of the *nifX* gene product on accumulated *nifHDKTY* mRNA. Strain UN5373 was derepressed for *nif* in the presence of either 0.0 mM (lane 1) or 5.0 mM (lane 2) IPTG for 4 h. Total RNA was isolated from both samples. The Northern blot was hybridized with a ^{32}P -labeled DNA probe of the *Bam*HI-*Sal*I fragment containing the downstream end of the *nifD* gene and the upstream end of the *nifK* gene. The positions of the arrows indicate the positions of the rRNA bands ($\sim 3,000$ and $\sim 1,500$ bp). The band at the bottom of lane 2 is an artifact and was not reproducible.

the *nifX6289::aphA* mutation replaced the wild-type *nifX* gene in the chromosome. It also revealed several high-molecular-weight bands which hybridized with the labeled DNA probe. These high-molecular-weight bands appeared in the Southern blot analysis experiments, regardless of the amount of restriction enzyme added or the duration of incubation, suggesting that there are regions elsewhere in the chromosome that have some homology to the probe region. These hybridizing regions are not in the immediate vicinity of *nif* since deletions of the entire *his-nif* region show an identical hybridization pattern (data not shown).

Initial experiments on UN5359 revealed that the mutation had little effect on nitrogenase activity. This lack of effect suggested the possibility that the *nifX* gene product has a role in the negative regulation of *nif*. Therefore, the response of the mutant to the addition of NH_4^+ (a compound known to repress *nif*) was investigated. The experiments revealed that UN5359 continued to synthesize nitrogenase proteins longer than did the wild-type strain, following the addition of NH_4^+ . Further examination by Northern blot analysis revealed that there was a corresponding delay in the loss of accumulated *nifHDKTY* mRNA by the insertional mutant after NH_4^+ addition. The effects of the addition of O_2 on these two strains were also examined, with the results being similar to those seen with the addition of NH_4^+ . The mutant again showed a delay, following O_2 addition, in the loss of both *nif* protein synthesis and *nifHDKTY* mRNA accumulation. These results are consistent with the involvement of the *nifX* gene product in the negative regulation of *nif* at either transcriptional or posttranscriptional levels.

In order to examine whether the *nifX* gene product was indeed a negative regulator of nitrogenase expression, we constructed an expression vector that was capable of overproducing the *nifX* gene product in response to IPTG. The overproduction of the *nifX* gene product throughout *nif* derepression inhibited the appearance of nitrogenase activity, the accumulation of *nifHDKTY* mRNA, and the synthesis of nitrogenase proteins.

These results strongly suggest a negative regulatory role for the *nifX* protein. Since both the overexpression of *nifX* and the complementary experiment (the examination of a strain defective in *nifX*) indicates that the level of accumulated *nifHDKTY* mRNA is affected, we believe that the negative regulation must take place by either halting de novo synthesis of *nif* mRNA or by destabilizing the existing mRNAs directly or indirectly. Collins et al. (5, 6) have previously demonstrated that a functional *nifL* gene product is essential for the proper negative regulation of the *nif* regulon on the addition of either NH_4^+ or O_2 , and we suspect that the *nifX* gene product may also be involved in this regulation. Finally, UN5359, while slower in its response to the addition of NH_4^+ or O_2 , still exhibited some degree of negative regulation (unlike a *NifL*⁻ mutant). This result suggests that the *nifX* mutant may be defective in one, part of one, or part of both of the levels of *nif*-specific negative regulation, namely, the destabilization of mRNA or the halting of new mRNA synthesis.

It is of interest to know the relation of the *nifX* gene product to that of the characterized *nif*-specific system regulator, the *nifL* gene product. To that end, we constructed UN5363, a *nifL* mutant strain containing the *nifX* expression vector pNF101. While examining this strain, we found that induction of a high level of *nifX* expression resulted in no *nif* expression. However, when the level of *nifX* expression was tuned down with decreasing amounts of the inducer IPTG, we found that higher levels of *nifX*

expression were necessary to completely repress *nif* in the *nifL* mutant background than were needed to repress *nif* in the wild-type background.

The sequence of the *nifX* gene product has been examined and found to share a degree of sequence similarity with the *nifB* gene product (21). When we compared the similarities of the *nifX* and *nifB* gene products, using the GAP program from the Genetics Computer Group Sequence Analysis Software Package (7), we saw some similarity between these two proteins; however, we also saw a comparable degree of sequence similarity between the sequences of the *nifX* and *nifA* gene products. The significance, if any, of these results is unclear at this time.

Brigle et al. (3) also examined the role of the *nifX* gene product on nitrogen fixation by constructing several insertion and deletion mutations in the *nifX* gene of *Azotobacter vinelandii*. These mutations had no effect on the ability of *A. vinelandii* to grow on N₂ (3), similar to the results that we saw with our *K. pneumoniae nifX* insertion mutant. Brigle et al. (3) also indicated that their NifX⁻ mutants in *A. vinelandii* accumulated more MoFe protein than did their wild-type strain. These results support a role for the *nifX* gene product as a negative regulator since a strain defective for a negative regulator of nitrogenase may have a somewhat elevated accumulation of nitrogenase proteins under some conditions.

In conclusion, while we do not know the mechanism of action of the *nifX* gene product, our data demonstrate that the *nifX* gene product is involved in the negative regulation of the *nif* regulon.

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