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Importance of *cis* Determinants and Nitrogenase Activity in Regulated Stability of the *Klebsiella pneumoniae* Nitrogenase Structural Gene mRNA

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The *Klebsiella pneumoniae* nitrogen fixation (*nif*) mRNAs are unusually stable, with half-lives of 20 to 30 min under conditions favorable to nitrogen fixation (limiting nitrogen, anaerobiosis, temperatures of 30°C). Addition of O_2 or fixed nitrogen or temperature increases to 37°C or more result in the dramatic destabilization of the *nif* mRNAs, decreasing the half-lives by a factor of 3 to 5. A plasmid expression system, independent of *nif* transcriptional regulation, was used to define *cis* determinants required for the regulated stability of the 5.2-kb *nifHDKTY* mRNA and to test the model suggested by earlier work that NifA is required in *trans* to stabilize *nif* mRNA under *nif*-derepressing conditions. O_2 regulation of *nifHDKTY* mRNA stability is impaired in a plasmid containing a deletion of a 499-bp region of *nifH*, indicating that a site(s) required for the O_2-regulated stability of the mRNA is located within this region. The simple model suggested from earlier work that NifA is required for stabilizing *nif* mRNA under conditions favorable for nitrogen fixation was disproved, and in its place, a more complicated model involving the sensing of nitrogenase activity as a component of the system regulating mRNA stability is proposed. Analysis of *nifY* mutants and overexpression suggests a possible involvement of the protein in this sensing process.

In *Klebsiella pneumoniae*, a series of highly regulated events occur before the bacterium dedicates itself to the energy-intensive process of fixing nitrogen (reviewed in references 18 and 41). The nitrogen regulatory (*ntr*) system is responsible for the transcriptional regulation involved in the nitrogen regulatory cascade. Under conditions of fixed-nitrogen limitation, anaerobiosis, and temperatures at or below 30°C, the nitrogen fixation (*nif*) system is turned on by the transcriptional activator NtrC. NtrC, in conjunction with σ^{54} and RNA polymerase, is responsible for activating expression of the *nifLA* genes. Using an elegant regulatory scheme, the *nifLA* gene products are responsible for activating transcription of *nif* genes under conditions favorable for nitrogen fixation and for shutting down *nif* expression, at both the transcriptional as well as posttranscriptional levels, when conditions become unfavorable (11, 14, 15, 32). NifA is responsible for activating the σ^{54}-dependent expression of the other *nif* operons by binding to an upstream activating sequence (UAS) (44), while NifL interferes with that activation (4, 25, 26, 48). *glnK*, also under NtrC transcriptional control, is required to relieve NifL inhibition under N-limiting conditions (24). NifL and NifA are unusual members of a two-component regulatory system in that inhibition of NifA by NifL is not mediated by the typical phosphorylation reaction (3, 36). Instead, stoichiometric levels of NifL are required for the inhibition of NifA activity, implying protein-protein interaction may be involved.

While NifL is necessary for the inhibition of *nif* mRNA synthesis in response to fixed nitrogen and O_2, the protein is not necessary for the shutdown of synthesis in response to temperatures of 37°C or above (14). High temperature inactivates NifA in vitro (36), and it is thought that the temperature sensitivity of NifA is responsible for cessation of *nif* gene expression at increased temperatures.

Several reports demonstrated that *nif* mRNA stability is dramatically regulated in response to the same stimuli that regulate *nif* transcription (29, 31, 32). Research by Collins and coworkers (15) supported these claims and further demonstrated *nif* specificity for the regulation. By using pulse-labeling, filter hybridization, and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques, they determined that *nif* mRNAs under NifLA control decay with half-lives ($t_{1/2}$s) of 20 to 30 min under *nif*-derepressing conditions. They also showed that *nif* mRNA (except *nifLA*) is rapidly destabilized, decaying with $t_{1/2}$s of 4 to 6 min upon addition of fixed nitrogen or O_2 or by temperatures at or above 37°C, and that functional inactivation of the mRNAs approximated chemical decay. Furthermore, they demonstrated that NifL is necessary for destabilization of the mRNA upon addition of O_2 and fixed nitrogen, but is not required for the temperature effect.

Since NifL interferes with activation of *nif* transcription by NifA, a simple model was proposed (15) that predicted NifL also functions at the posttranscriptional level by inhibiting NifA activity. This model posited that NifA is directly or indirectly responsible for stabilizing *nif* mRNA under *nif*-derepressing conditions. The temperature-sensitive NifA would be unable to activate synthesis or stabilize *nif* mRNAs at or above 37°C, which would provide an explanation for the rapid destabilization of *nif* mRNA in *nifL* mutants at high temperatures.

While intriguing, this model has been difficult to test, because the overlapping roles that NifL and NifA would perform in transcription and posttranscriptional regulation make it difficult to distinguish effects due explicitly to posttranscriptional

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regulation. Additionally, *nif* mRNA stability cannot be examined in a *nifA* strain, because, with the exception of *nifLA*, no *nif* mRNA is expressed in such a mutant. In this work, we employed a *nifHDKTY* expression plasmid, pUX40, that separates *nif* transcription from posttranscriptional regulation without changing the wild-type mRNA sequence. We report here our studies defining the *cis* determinants required for the unusual anaerobic stability and for O_2 regulation of stability of the *nifHDKTY* mRNA in *K. pneumoniae*. We additionally used pUX40 to test and disprove the model that NifA is sufficient to stabilize *nif* mRNA under *nif*-derepressing conditions. The data instead suggest that regulation of *nif* mRNA stability involves a complex interaction of a number of different *nif* proteins and that nitrogenase activity is a key factor in determining stability.

MATERIALS AND METHODS

Media and reagents. The recipe for the minimal medium used for growth and derepression of strains for nitrogenase function was described previously (22).

The following antibiotics were used at the indicated concentrations: kanamycin sulfate, 50 μg/ml; chloramphenicol, 25 μg/ml; ampicillin (sodium salt), 50 μg/ml; tetracycline, 4 μg/ml; and carbenicillin (disodium salt)-ampicillin (sodium salt), 150 μg/ml each.

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.), New England Biolabs (Beverly, Mass.), or Pharmacia, Inc. (Piscataway, N.J.). The [α-^{32}P]dATP was obtained from Amersham Life Science, Inc. (Arlington Heights, Ill.).

Bacterial strains and plasmids. The relevant strains and plasmids used in this study are listed in Table 1 and described below.

Construction of pUX40. pUX40, a plasmid expressing *nifHDKTY* from a *nif*-independent promoter, was constructed as follows. By oligonucleotide synthesis, the promoter $P_{A1/04}$ (35) was fused to the first 15 bases at the 5′ start of *nifH*. PCR was performed to amplify a 724-bp partial *nifH* fragment fused to the $P_{A1/04}$ promoter, followed by ligation into pUX32 (a plasmid containing the wild-type *nifHDKTY* operon, which was itself constructed by standard cloning techniques [Table 1]) to construct pUX40. The details are as follows. Two oligonucleotides were synthesized (Department of Biochemistry, University of Wisconsin—Madison) and used in the PCR to construct and amplify the 724-bp fragment: (i) the 82-mer 5′-CAGGCGAGCTCTTTAAATAGTTTTTCTCACAACTGAACAC TCGCCTATTGTTACTATGAATCTAAGCCGTTTGTGAGTTGT-3′ (the −35 and −10 regions of the promoter are underlined), identical to the sense strand and consisting of the $P_{A1/04}$ promoter and the first 15 nucleotides (nt) of the *nifH* transcript; and (ii) the 15-mer 5′-GATCATCTGGGTACC-3′, complementary to the sense strand and hybridizing 627 nt downstream from the start of the *nifH* transcript. A 536-bp partial DNA fragment containing the $P_{A1/04}$/*nifH* fusion was isolated and ligated into the *Eco*RV and *Bgl*II sites of pUX32 by standard cloning techniques to construct pUX40. The construct was confirmed by sequencing with the Sequenase kit (Bio-Rad; Hercules, Calif.). The sequence of the pUX40 promoter controlling *nifHDKTY* expression is identical to that of the 82-mer through the promoter region. The initiation site of the mRNA expressed from pUX40 was determined to be that of the wild-type *nifHDKTY* mRNA (7) by primer extension analysis (data not shown).

Construction of pUX40 deletion derivatives. Various deletions were made in the *nifHDKTY* genes carried on the plasmid pUX40 by either conventional restriction analysis, as with pUX201, pUX202, pUX203, and pUX215, or by use of exonuclease III (Exo III), as in the case of pUX214 (see Fig. 3). All of the deletion plasmids were derived from pUX200, which was constructed from pUX40 by partial digestion with *Ahd*I, incubation with Klenow fragment to remove the single-base 3′ overhang, and ligation, resulting in the removal of the plasmid *Ahd*I site. Klenow fragment was employed to fill in the ends where required. *Nru*I and *Sma*I were used to construct both pUX201 and pUX202. The deletion in pUX201 extends from the *Nru*I site at *nif* nt 5704 (which refers to the nucleotide position by the numbering convention in reference 2) in *nifD* to the *Sma*I site at *nif* nt 8956 in *nifY* and removes 3,252 bp. The deletion was designated Δ*nifD-Y6298*. The deletion in pUX202 extends from the *Nru*I site at *nif* nt 8233 in *nifT* to the *Sma*I site at *nif* nt 8956 in *nifY*, removing 723 bp, and was designated Δ*nifTY6299*. pUX203 was made by removing 2,562 bp, between *nif* nt 5249 in *nifD* and *nif* nt 7811 in *nifK*, with a single enzyme, *Bsi*WI, creating Δ*nifDK6292*. pUX215 was constructed by using the *Ahd*I site at *nif* nt 4184 in *nifH* and the *Hpa*I site at *nif* nt 8140 at the end of *nifK*. The deletion extends from *nif* nt 4183 to nt 8140, removes 3957 bp, and was designated Δ*nifH-K6301*. pUX214 was constructed by using Exo III digestion as follows: pUX200 was digested with *Kpn*I, which has a unique site at nt 4794, and *Bgl*II, which has a unique site at nt 4617, both in *nifH*. While the former site should be resistant to Exo III digestion and the latter should be susceptible, sequence analysis of several clones revealed that Exo III digestion proceeded into each end. The deletion in pUX214 extends from *nif* nt 4303 to nt 4802 in *nifH*, removes 499 bp, and was designated Δ*nifH6300*.

The constructs were confirmed by restriction enzyme and, in cases where more than one enzyme was used to make the deletion, DNA sequence analysis. All deletions are in frame with the following exceptions: pUX201, in which case the 3′ end of the deletion falls near the end of the *nifHDKTY* transcript and the new reading frame contains a stop codon 35 nt from the end of the *nifY* gene; and pUX203, in which a stop codon is introduced 316 nt from the end of *nifK*.

Construction of strain UN5442. Δ*nifDK6292* was constructed in the plasmid pUX50 by digestion of pUX32 with *Bsi*WI to remove a 2.6-kb fragment internal to the *nifDK* genes, followed by intramolecular ligation. A 3.8-kb *Eco*RI fragment encompassing Δ*nifDK6292* was cloned into the plasmid pJR6, which had been partially digested with *Eco*RI to construct the plasmid pUX50. Taking advantage of the inability of pJR6 (an R6K vector derivative (43) to replicate in the absence of the *pir* gene product, the deletion was subsequently moved into the *K. pneumoniae* wild-type strain (UN) chromosome by homologous recombination. Plasmid-free deletion mutants were identified by their antibiotic-sensi-

TABLE 1. *K. pneumoniae* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
UN	Wild type for nitrogen fixation (*nif*$^+$)	P. W. Wilson
UN1696	*nifK4732*	37
UN1795	*nifH4764*	37
UN5360	*nifY6290::aphA*	This report
UN5397	*nif*$^+$/pNF107/F′*lacI*^{Q}	This report
UN5406	*nifL6302*/pNF107/F′*lacI*^{Q}	This report
UN5435	Δ*nifDK6292*	This report
UN5442	Δ*nifDK6292*/pUX40/F′*lacI*^{Q}	This report
UN5443	*nif-5000* (Δ*nifJ-A*)[b]/pUX40/F′*lacI*^{Q}	This report
UN5445	*nif-5000* (Δ*nifJ-A*)[b]/pUX40/pVL15/F′*lacI*^{Q}	This report
UN5448	*nif-4932* (Δ*nifD-Q*)/pUX40/F′*lacI*^{Q}	This report
UN5450	*nif-4932* (Δ*nifD-Q*)/pUX40/pVL15/F′*lacI*^{Q}	This report
UN5451	*inf-4934* (Δ*nifD-M*)/pUX40/F′*lacI*^{Q}	This report
UN5458	*nif-2635* (Δ*nifJ-A*)[b]/pUX40/F′*lacI*^{Q}	This report
UN5459	*nif-2635* (Δ*nifJ-A*)[b]/pUX40/pUXA1/F′*lacI*^{Q}	This report
UN5460	*nif*$^+$/pUX201/F′*lacI*^{Q}	This report
UN5461	*nif*$^+$/pUX202/F′*lacI*^{Q}	This report
UN5469	*nif*$^+$/pUX214/F′*lacI*^{Q}	This report
UN5470	*nif*$^+$/pUX215/F′*lacI*^{Q}	This report
UN5479	*nif*$^+$/pUX203/F′*lacI*^{Q}	This report
Plasmids		
pACYC184	Low-copy, parent plasmid	12
pJR6	Derivative of R6K, requires *pir* gene product to replicate	This report
pNF102	Derivative of pBR322 expressing *nifTY* from the P_{tac} promoter	This report
pNF107	pBR322 derivative expressing *nifY* under P_{tac} control	This report
pVL15	pBR322 derivative expressing *nifA* under P_{tac} control	23
pUXA1	pSC101 derivative expressing *nifA* under P_{lac} control	This report
pUX32	Derivative of pUX25 containing *nifJ′HDKTY*	This report
pUX40	*nifHDKTY* under $P_{A1/04}$ control (derivative of pUX32)	This report
pUX50	Δ*nifDK6292* (derivative of R6K)	This report
pUX200	nifHDKTY under $P_{A1/04}$ control (derivative of pUX40)	This report
pUX201	Δ*nifD-Y6298* under $P_{A1/04}$ control (derivative of pUX200)	This report
pUX202	Δ*nifTY6299* under $P_{A1/04}$ control (derivative of pUX200)	This report
pUX203	Δ*nifDK6292* under $P_{A1/04}$ control (derivative of pUX200)	This report
pUX214	Δ*nifH6300* under $P_{A1/04}$ control (derivative of pUX200)	This report
pUX215	Δ*nifH-K6301* under $P_{A1/04}$ control (derivative of pUX200)	This report

[a] For each strain, the *nif* allele is listed, followed by plasmids; chromosomal allele numbers and plasmids are separated by a "/".

[b] The deletion was not mapped physically, and the end falls either within *nifL* or *nifA*. The operon promoter is absent in either case, so the deletion eliminates expression through *nifA*.

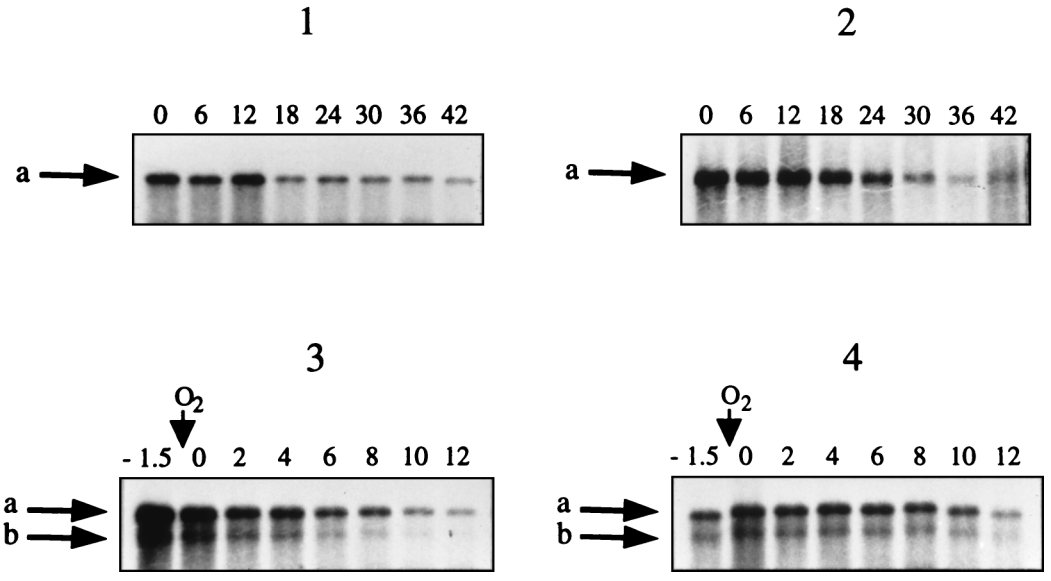


FIG. 1. Anaerobic stability and O_2 regulation of stability of the chromosomal *nifHDKTY* mRNA in the wild-type background and the pUX40 *nifHDKTY* mRNA in the Δ*nifDK* background. Representative Northern blots of RNA isolated after rifampin addition from chromosomal (UN) (panel 1) and Δ*nifDK*/pUX40 (UN5442) (panel 2) anaerobic cells derepressed for *nif* expression and from chromosomal (panel 3) and Δ*nifDK*/pUX40 (panel 4) cells treated with O_2. Sampling times are indicated in minutes above the lanes. The time of O_2 addition relative to sampling is indicated with a downward arrow above the indicated sampling times. A *nifTY*-specific probe was used in panels 1 and 2, and a *nifKTY*-specific probe was used in panels 3 and 4. Arrows a indicate *nifHDKTY* mRNA, and arrows b indicate *nifHDK* mRNA.

tive, Nif$^-$ phenotype and confirmed by Southern blot analysis (data not shown). The strain with Δ*nifDK6292* was designated UN5435. An F′ plasmid with the *lacI*^{Q} gene was moved by conjugation into strain UN5435 (as well as into all other strains used to harbor pUX40) to allow regulation of $P_{A1/04}$, constructing strain UN5439. pUX40 was subsequently transformed into UN5439 to construct strain UN5442.

Construction of Δ*nif* strains harboring pUX40. pUX40 was transformed into the following recipients to create strains in Table 1: UN5408 (Δ*nifJ-A*), to construct UN5443; UN5446 (Δ*nifD-Q*), to construct UN5448; UN5447 (Δ*nifD-M*), to construct UN5451; and UN5457 (Δ*nifJ-A*), to construct UN5458. UN5408, UN5446, UN5447, and UN5457 were constructed by the introduction of an F′ with *lacI*^{Q} into UN2408 (37), UN1978 (37), UN1980 (37), and UN5456 (40), respectively. UN2408, UN1978, and UN1980 are Mu-induced deletion strains; UN5456 was derived by deletion of Tn*10*.

UN5445 and UN5450 were constructed by transforming pVL15 (23) into UN5443 and UN5448, and UN5459 was constructed by transforming pUXA1 into UN5458 (Table 1). pVL15 is a pBR322 plasmid derivative expressing *nifA* from the *tac* promoter. pUXA1 expresses *nifA* from the *lac* promoter and was constructed by cloning a 3.0-kb *Sal*I fragment from pSB2001 (9) containing *nifA* into the *Sal*I site in pCL1920 (52). Expression of active NifA from pUXA1 was confirmed by complementation of *nifA* mutant strains and was observed to be similar to that of the wild-type strain, as determined by acetylene reduction analysis (data not shown).

Construction of *nifY* strains. Construction of the *nifY* and *nifY*-overexpressing strains listed in Table 1 was performed as follows. UN5360 was constructed by ligating the Kan^r cassette from pUC-4K (42, 46) into the *Sal*I site of the *nifY* gene located on a plasmid and then using reciprocal recombination, as described by Gosink et al. (22), to move this mutation, *nifY6290::aph*, into the *K. pneumoniae* chromosome. The mutation was confirmed by Southern blot analysis (data not shown).

The *nifY* expression vector, pNF107, was constructed by cloning the 925-bp *Nru*I-*Ssp*I fragment containing *nifY* downstream of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{tac} promoter of pKK223-3 (10). UN5397 was constructed by transforming pNF107 into UN5350, a wild-type strain of *K. pneumoniae* containing an F′ with *lacI*^{Q}. UN5406 was constructed by transforming pNF107 into UN5361 (*nifL* strain UN4357 (37) containing an F′ with *lacI*^{Q}). Upon induction of pNF107, a protein is synthesized in vivo that corresponded to the wild-type NifY as determined by mobility on one- and two-dimensional SDS-PAGE gels (data not shown).

Derepression of nitrogenase. Cell growth, *nif* derepression, assay for nitrogenase function, and the procedure for the addition of O_2 to derepressed cultures of *K. pneumoniae* have been described previously (22). When appropriate, the expression of *nif* mRNA from plasmids was induced with 150 μM IPTG before sampling, for times ranging between 7 and 12 min, depending upon the size of the mRNA expressed.

Isolation and analysis of RNA. Isolation of total RNA from derepressed cells and Northern blotting were carried out as described previously (22), except for the following changes. (i) Unbuffered phenol was used in place of Tris-EDTA-equilibrated phenol. (ii) After the initial ethanol precipitation, samples were treated with RNase-free DNase (Promega Corp.) for 10 min at 37°C. This digestion was stopped by the addition of 25 mM EDTA and phenol-chloroform extraction, followed by an ethanol precipitation. (iii) Samples were separated by loading 5 to 10 μg of RNA per lane onto 1 to 1.5% agarose, 0.2 M formaldehyde gels. (iv) Nytran Plus positively-charged nylon membranes (0.45 μM pore size) from Schleicher & Schuell (Keene, N.H.) were used for immobilization of RNA for hybridization.

A 0.87-kb *Nru*I-*Sac*II *nifTY* fragment and a 1.8-kb *Hinc*II *nifKTY* fragment were labeled with [α-^{32}P]dATP by random hexamer labeling (20) and used separately as probes in Northern analyses. Hybridization and washes were done as described previously (22).

Determination of RNA $t_{1/2}$. Cultures that had been derepressed for *nif* function and induced with 150 μM IPTG when appropriate (to express the cloned *nif* region) were treated for 3 min with rifampin at 200 mg/liter. Samples of 2 ml were withdrawn at intervals thereafter and centrifuged at 15,000 × *g* for 20 s. The RNA was then extracted and analyzed by Northern blotting as described above. Radioactivity contained in RNA bands hybridizing to the ^{32}P-labeled *nif* DNA probes was quantified by using the Ambis radioanalytic imaging device and Quant Probe software, version 3.0, from Ambis, Inc. (San Diego, Calif.), or the Molecular Dynamics PhosphorImager, model 445Si (Sunnyvale, Calif.). Least-squares (19) and DFFITS sensitivity (6a, 16) analyses were performed to obtain the $t_{1/2}$ of mRNA decay for each experiment, and $t_{1/2}$ errors were estimated from the standard error of the slope of each regression line. Comparisons between experiments and among strains were performed using a *t* test at the 5% significance level or, where noted, analysis of covariance (19). Data points that were <10% of the t_0 point were not included in analyses.

Detection of different *nif* mRNA species hybridizing to probes from the *nifHDKTY* region. Under *nif*-derepressing conditions, several mRNA species that hybridize to a *nifH* DNA probe accumulate in UN (wild type) (13, 22). Based on hybridization patterns of the mRNAs on Northern blots, we have noted two major species of approximately 5,000 and 4,500 nt in length that correspond to *nifHDKTY* and *nifHDK*, respectively (Fig. 1) (22). Preliminary evidence demonstrated that the decay rates for these two mRNAs were similar under both stabilizing and destabilizing conditions (plus O_2) (data not shown). We decided to focus our studies on the larger, 5,000-nt *nifHDKTY* mRNA, because it has not been determined whether the 3′ end of the shorter mRNA species arises due to transcription termination or mRNA processing. Therefore, an 874-bp *Nru*I-*Sac*II *nifTY* fragment was used for the selective detection of full-length *nifHDKTY* mRNA, with the exception of the Northern blots shown in panels 3 and 4 of Fig. 1, for which a 1.8-kb *Hinc*II *nifKTY* fragment was used for detection of *nif* mRNA.

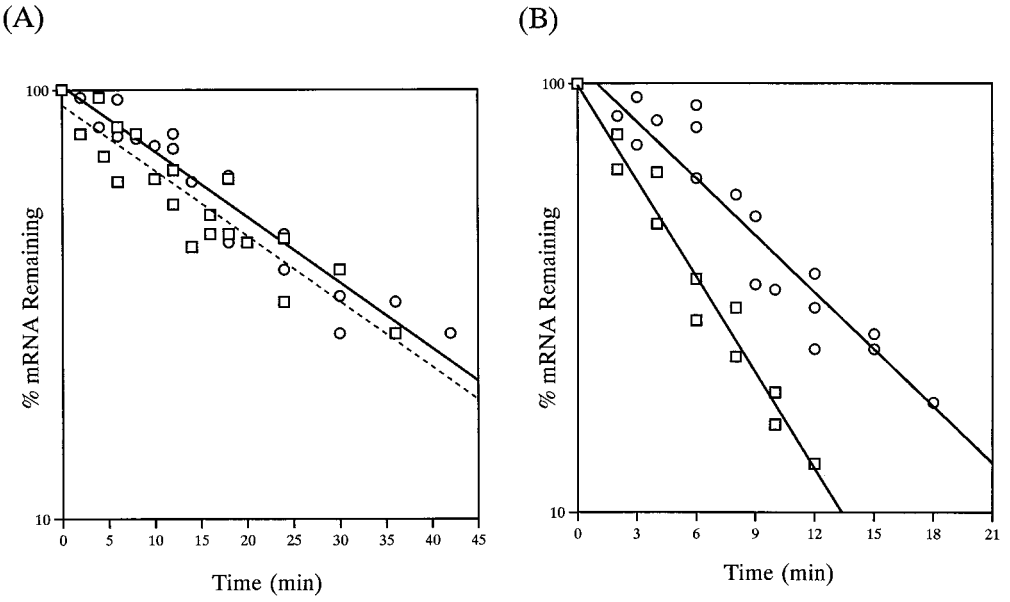


FIG. 2. Semilogarithmic plots of anaerobic and aerobic decay of pUX40 and chromosomal *nifHDKTY* mRNA. (A) RNA was isolated from anaerobic cells derepressed for *nif* expression. Time zero represents the first sample isolated after rifampin addition. □, chromosomal *nifHDKTY* mRNA (UN); ○, pUX40 *nifHDKTY* mRNA (UN5442). (B) RNA was isolated from *nif*-derepressed cells treated with rifampin and O_2. Time zero represents the first sample isolated after O_2 addition. □, chromosomal *nifHDKTY* mRNA (UN); ○, pUX40 *nifHDKTY* mRNA (UN5442). The data are from three independent experiments performed with chromosomal and pUX40 mRNAs under anaerobic conditions and pUX40 after O_2 addition and two independent experiments performed with chromosomal *nifHDKTY* mRNA after O_2 addition. (Chromosomal *nifHDKTY* mRNA was also examined after O_2 addition without rifampin [see text].) The data from each set of experiments were collectively graphed, and least-squares analysis was used to determine the slope of the line and the $t_{1/2}$ for the combined data (see Materials and Methods).

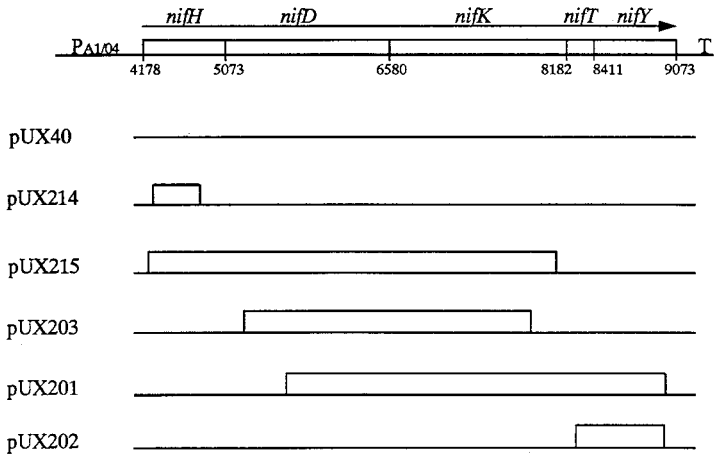
RESULTS AND DISCUSSION

Construction and characterization of pUX40, which allows controlled expression of *nifHDKTY* mRNA independent of *nif* regulation. Involvement of the *nifLA* gene products in both transcription and posttranscriptional regulation of *nif* gene expression made it essential to express the *nifHDKTY* mRNA from a *nif*-independent promoter to separate these two effects. We replaced the *nifH* promoter and NifA UAS in the *nifHDKTY* operon with $P_{A1/04}$, the modified P_{A1} promoter which was originally derived from phage T7 (35), designating this construct pUX40. The *nifHDKTY* mRNA expressed from pUX40 is identical in sequence to the wild-type mRNA, as confirmed by primer extension analysis (data not shown).

Strain UN5442 contains pUX40 and a 2.6-kb deletion in the chromosomal *nifDK* genes. The Δ*nifDK* background allowed us to distinguish the pUX40-expressed *nifHDKTY* mRNA from the mRNA expressed from the chromosome on Northern blots. The addition of 150 μM IPTG for 12 min to strain UN5442 (Δ*nifDK*/pUX40) provided approximately the same accumulation of *nifHDKTY* mRNA as in the wild-type strain, UN, under *nif*-derepressing conditions, which allowed the experiments to be performed at physiologically relevant levels of mRNA. With IPTG induction, UN5442 became phenotypically Nif^+, and levels of nitrogenase activity (as measured by acetylene reduction) were very similar to those of the wild-type strain (data not shown).

pUX40 and chromosomal *nifHDKTY* mRNA demonstrate similar anaerobic stability and O_2-induced decay kinetics. Rates of decay of pUX40 and chromosomal *nifHDKTY* mRNA were compared in the presence and absence of O_2. The response to O_2 was examined by transferring *nif*-derepressed cultures from anaerobic vials to baffled flasks of ≥10 sample volumes that were being shaken at 450 rpm. Figure 1 shows Northern blots of chromosomal and pUX40 *nifHDKTY* mRNA, isolated after the addition of rifampin, under anaerobic conditions (panels 1 and 2) or after O_2 addition (panels 3 and 4). Like the chromosomal *nifHDKTY* mRNA, the pUX40 *nifHDKTY* mRNA is stable under anaerobic conditions and is destabilized upon O_2 addition. Under anaerobic conditions, the $t_{1/2}$ of the pUX40 *nifHDKTY* mRNA was 20 ± 1.3 min, compared to 20 ± 1.7 min for the chromosomal *nifHDKTY* mRNA (Fig. 2A). Upon addition of O_2, the $t_{1/2}$ of the pUX40 *nifHDKTY* mRNA was 6.8 ± 0.5, compared to 4.0 ± 0.2 min in the wild type (Fig. 2B). Thus, the pUX40 *nifHDKTY* mRNA is comparable to the chromosomal mRNA in its anaerobic stability and is regulated by O_2 in a significant ($P < 0.005$) and dramatic manner, although the magnitude of the O_2 regulation is not as striking as that for chromosomal mRNA. These data indicate that the pUX40 mRNA is a reasonable model for determining *cis* sites and *trans*-acting factors required for anaerobic stability and O_2 regulation of stability. O_2 addition experiments with the chromosomal *nifHDKTY* mRNA were also performed in the absence of rifampin, and it was found that O_2 regulation of stability occurs similarly in the presence or absence of rifampin (data not shown).

Deletion analysis implicates multiple regions as being important for anaerobic stability of *nifHDKTY* mRNA. A series of deletion constructs were made starting with pUX40 (Table 1 and Fig. 3) to test the requirement of *cis*-acting sequences for the anaerobic stability of *nifHDKTY* mRNA. These constructs were examined under N-limiting, anaerobic conditions in a nif^+ background. We quantified the decay rate of the chromosomal *nifHDKTY* mRNA as an internal standard in strains expressing the deletion mRNAs and observed that the chromosomal mRNA decayed similarly, regardless of the presence of the deletion plasmids (data not shown), indicating that the plasmids were not perturbing the analyses. Only data from those experiments in which the decay rate of the chromosomal *nifHDKTY* mRNA was the same as in the control experiments (Fig. 2) at a 5% significance level were considered (data not



	T1/2 -O2	T1/2 +O2	p value -/+ O2
pUX40	20 +/- 1.3	6.8 +/- 0.50	
pUX214	24 +/- 2.5	14 +/- 1.9	< 0.100/0.001
pUX215	6.5 +/- 0.51	6.0 +/- 0.47	< 0.001/0.400
pUX203	16 +/- 1.4	8.0 +/- 0.91	< 0.100/0.400
pUX201	10 +/- 0.53	5.8 +/- 0.48	< 0.001/0.200
pUX202	14 +/- 1.2	5.6 +/- 0.97	< 0.025/0.400

FIG. 3. Map of deletion derivatives of pUX40 and the summary of measured $t_{1/2}$s under anaerobic and aerobic conditions. A map of the intact operon is shown above. The gene designations and direction of transcription are shown above the operon; T designates the terminator. The nucleotide positions at the start of each gene (2) are given below the line. pUX40 contains the intact *nifHDKTY* operon, and the deletion plasmids are represented below, with the deletions depicted as boxes. Data corresponding to the results in the table are shown graphically for the deletions in Fig. 4; the data for pUX40 are those shown in Fig. 2. The $t_{1/2}$ value ($T_{1/2}$) was calculated from the slopes of the combined regression analyses from two independent experiments for each deletion mRNA under anaerobic and aerobic conditions, with the chromosomal *nifHDKTY* mRNA used as an internal control (see text).

shown). Ninety-six percent of the experiments analyzed met this criterion.

The results from experiments examining the anaerobic stability of the deletion mRNAs are summarized in Fig. 3 and displayed graphically in Fig. 4A. The largest reductions in anaerobic stability were seen with two of the deletion mRNAs, pUX201 and pUX215, although more minor reductions were also seen with pUX202 and pUX203. Examination of the extent of the deletions and their effects leads to the conclusion that no single region is responsible for the great anaerobic stability of the normal *nifHDKTY* mRNA, and the data cannot support identification of a specific critical region. Although the size of the mRNA might have an effect on anaerobic stability, this seems unlikely, because the deletion in pUX203 is 3.5 times larger than that in pUX202, yet the two mRNAs decay with similar $t_{1/2}$s.

Sites involved in O_2 regulation of *nifHDKTY* mRNA stability. Results from experiments examining O_2 regulation of stability of the deletion mRNAs in the *nif*$^+$ background are summarized in Fig. 3 and displayed graphically in Fig. 4B. All of the deletion mRNAs except that expressed from pUX214 (Δ*nifH*) decay with a $t_{1/2}$ similar to that of the pUX40 *nifHDKTY* mRNA upon O_2 addition. The fact that the pUX214 mRNA is twice as stable as the pUX40 mRNA upon exposure to O_2

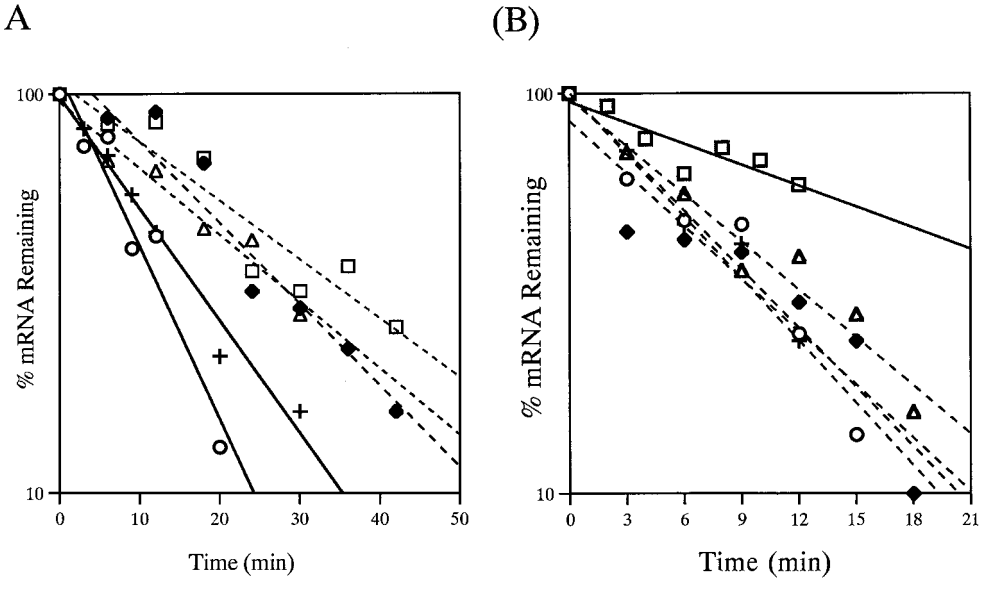


FIG. 4. Anaerobic and aerobic stability of the pUX40-derived deletion mRNAs. Semilogarithmic plots are shown of the data from a representative time-course experiment (summarized in Fig. 3). The point at time zero is the first sample after rifampin addition and, in O_2-treated cells, also the first sample after O_2 addition. (A) Anaerobic, *nif*-derepressing conditions. (B) O_2 treatment. Strains whose mRNA decay was significantly different ($P < 0.001$) from that of the control (pUX40 *nifHDKTY* mRNA) are shown by solid lines, while those with mRNA decay that was not significantly different from that of the control are shown by dashed lines. □, pUX214 (UN5469); ○, pUX215 (UN5470); △, pUX203 (UN5479); +, pUX201 (UN5460); ◆, pUX202 (UN5461).

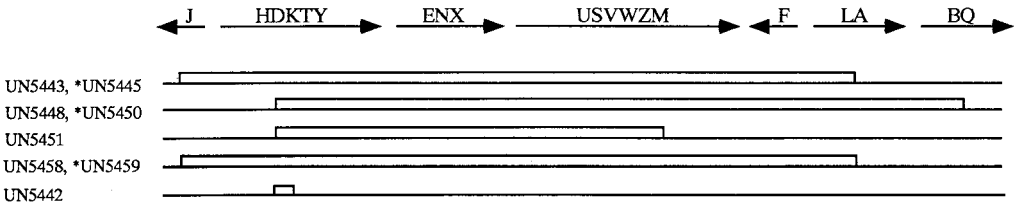


FIG. 5. Schematic of chromosomal *nif* operon deletions. The *nif* genes and direction of transcription are shown above; the strains used in this report, with deletions depicted as boxes, are shown below. As described in the text (and in Table 1), a *nifA* expression system was introduced into some deletion backgrounds, and these strains are indicated with an asterisk.

indicates that some portion of the 499-bp region of *nifH* deleted in pUX214 is essential for normal O_2 regulation of stability and suggests that a site required for the rate-limiting step in O_2 regulation has been deleted. The pUX215 mRNA is no longer stable under anaerobic conditions, and it is our hypothesis that in addition to losing the site required for O_2 regulation, the mRNA is also missing other region(s) required for its normal regulation.

The location of an O_2-destabilizing determinant within the coding region of *nifH* (deleted in pUX214), is somewhat unexpected, given that a majority of decay determinants characterized thus far that are required for the regulation of a given mRNA have been located in the 5′ untranslated region (UTR) (5, 6, 38, 39, 45). However, regulation requiring portions of the coding region of the mRNA, and often involving cleavage within that region, has been noted in other systems (21, 30, 33, 34). The fact that most of the other deletion mRNAs appear to be normally regulated in response to O_2 suggests that the majority of the *nifHDKTY* mRNA is not required for O_2 regulation of stability. At present, the facts are consistent with a model for a rate-limiting cleavage event within *nifH*, although more complicated models cannot be ruled out.

***nifHDKTY* mRNA is unstable in large Δ*nif* backgrounds under *nif*-derepressing conditions.** The pUX40 system also allowed us to ask if there is a requirement for *nif*-encoded *trans*-acting factors for the enhanced stability of the *nifHDKTY* mRNA under *nif*-derepressing conditions. The rates of decay of pUX40 *nifHDKTY* mRNA when expressed in several different *K. pneumoniae* strains that have deletions of different parts of the chromosomal *nif* regulon (Fig. 5) were compared. In the control strain, UN5442, the pUX40 *nifHDKTY* mRNA was characterized in the Δ*nifDK* background, which allowed it to be distinguished from the chromosomal *nifHDKTY* mRNA.

The only *nif* genes expressed in the strains with the largest *nif* deletions (Fig. 5, strains UN5443, UN5448, and UN5458) are the pUX40-encoded *nifHDKTY* genes (upon IPTG induction). Although these strains retain copies of one or more chromosomally encoded *nif* genes, the *nif*-specific transcriptional activator, NifA, is absent. In contrast, in the presence of IPTG, UN5442 (Δ*nifDK*/pUX40) expresses all of the *nif* genes under *nif*-derepressing conditions. In UN5442, pUX40 *nifHDKTY* mRNA decays with a $t_{1/2}$ of 20 ± 1.3 min (Fig. 2). A representative Northern blot analysis of UN5443 (Δ*J-Anif*/pUX40) is shown in Fig. 6A, panel 1. Analysis of covariance (19) showed that the rates of decay of pUX40 *nifHDKTY* mRNA were not significantly different in UN5443 (Δ*nifJ-A*/pUX40), UN5448 (Δ*nifD-Q*/pUX40), or UN5458 (Δ*nifJ-A*/pUX40). The data from experiments done with the three strains were thus combined for regression analysis (Fig. 6B), and the calculated $t_{1/2}$ was 8.8 ± 1.0 min. Not only is pUX40 *nifHDKTY* mRNA significantly less stable under anaerobic conditions in the absence of the normal complement of *nif* proteins ($P < 0.001$), but its decay approximates that of the O_2-destabilized pUX40 mRNA in UN5442 ($t_{1/2}$ = 6.8 ± 0.50 min). These results demonstrate that a *nif* factor or factors are required for the exceptional stability of the *nif* mRNA under *nif*-derepressing conditions.

NifA is not sufficient to stabilize the *nifHDKTY* mRNA under *nif*-derepressing conditions. Having established the requirement for a *nif* gene product(s) in stabilizing the *nifHDKTY* mRNA under *nif*-derepressing conditions, we tested the model

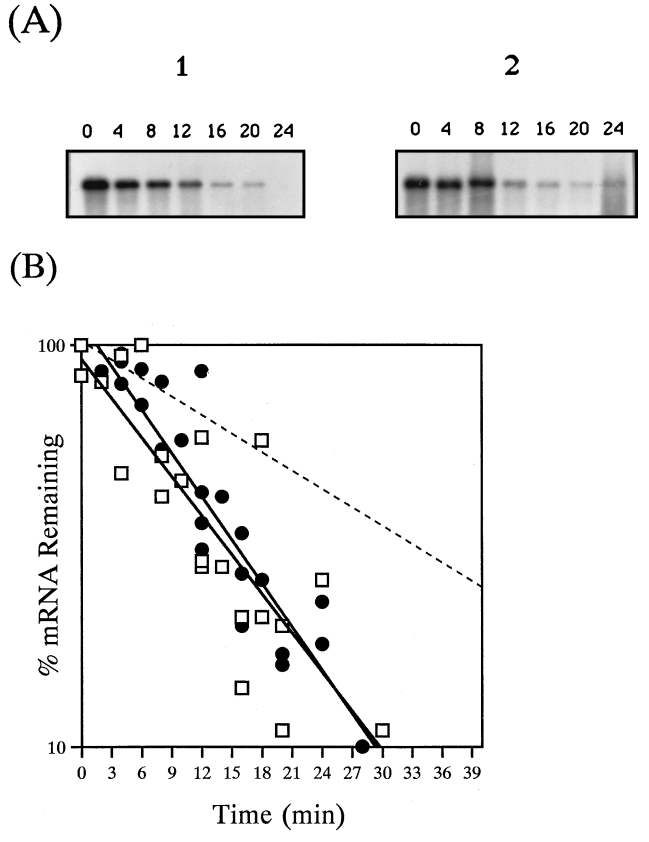


FIG. 6. Effect of NifA on pUX40 *nifHDKTY* mRNA stability in various Δ*nif* backgrounds under anaerobic, *nif*-derepressing conditions. Two sets of strains are shown: those with large chromosomal deletions that fail to express *nifA* (UN5443, UN5448, and UN5458) and another set in which *nifA* is either not deleted or is expressed from a plasmid (see text). (A) Representative Northern blots of pUX40 *nifHDKTY* mRNA decay in the Δ*J-Anif* background in the absence (panel 1) or presence (panel 2) of NifA. The numbers above the lanes indicate the time in minutes after rifampin treatment. (B) Semilogarithmic plot of pUX40 *nifHDKTY* mRNA decay in various Δ*nif* backgrounds in the absence (□) or presence (●) of NifA. The dashed line represents decay of pUX40 *nifHDKTY* mRNA in the presence of all of the *nif* proteins (strain UN5442) and has been reproduced from Fig. 2 for comparison; data points for this control are omitted for clarity.

suggested earlier (15) that NifA is that factor. The medium-copy *nifA* expression plasmid pVL15 (23) was introduced into strains UN5443 (Δ*nifJ-A*/pUX40) and UN5448 (Δ*nifD-Q*/pUX40) to construct UN5445 and UN5450, respectively, and the low-copy *nifA* expression plasmid pUXA1 was introduced into UN5458 (Δ*nifJ-A*/pUX40) to construct UN5459 (Table 1 and Fig. 5). In the presence of IPTG, these strains express both *nifHDKTY* and *nifA* from compatible plasmids. UN5451 (Δ*nifD-M*/pUX40) was also included in our analysis, because *nifA* is expressed from the chromosome in this strain, and this would avoid any problems that might arise due to concomitant expression from the two-plasmid system. A representative Northern blot analysis of UN5445 (Δ*nifJ-A*/pUX40/pVL15) is shown in Fig. 6A, panel 2. The rates of decay of pUX40 *nifHDKTY* mRNA were not significantly different in UN5445 (Δ*nifJ-A*/UX40/pVL15), UN5450 (Δ*nifD-Q*/pUX40/pVL15), UN5451 (Δ*nifD-M*/pUX40), UN5459 (Δ*nifJ-A*/pUX40/pUXA1). A $t_{1/2}$ of 8.3 ± 0.56 min, similar to what was observed in the absence of *nifA* expression, was obtained from analysis of covariance (19) of experiments with each of these strains and combined regression analysis (Fig. 6B). These results disprove the simple model that NifA is sufficient to stabilize *nif* mRNA and therefore that the *nifLA* gene products are solely responsible for posttranscriptional control of *nif* mRNA.

The results from experiments expressing *nifA* in addition to *nifHDKTY* from pUX40 in the deletion strains also allowed us to draw some conclusions concerning the ability of other *nif* factors to stabilize *nifHDKTY* mRNA. In addition to the genes expressed from the plasmid, any *nif* genes not deleted from the chromosome in these strains would still be expressed under NifA control. Thus, expression of *nifA* also results in the expression of *nifBQ* in UN5445 (Δ*nifJ-A*/pUX40/pVL15) and UN5459 (Δ*nifJ-A*/pUX40/pUXA1); *nifJ* and *nifH* in UN5450 (Δ*nifD-Q*/pUX40/pVL15); and *nifJ*, *nifH*, *nifF*, and *nifBQ* in UN5451 (Δ*nifD-M*/pUX40) (Fig. 5). We conclude that there was no increase in the stability of *nifHDKTY* mRNA expressed in the presence of NifA and any of the other *nif* proteins expressed in conjunction with NifA in these strains, compared to that of *nifHDKTY* mRNA expressed in their absence.

Nitrogenase activity regulates stability of *nifHDKTY* mRNA. To discover which *nif* factors are required for stability of *nif* mRNA, we examined chromosomal *nifHDKTY* mRNA stability in seven strains with point mutations in individual *nif* genes. Strains with point mutations in the following genes (with the roles of their protein products noted) were examined: *nifJ* and *nifF*, electron transport to nitrogenase (17); *nifE*, biosynthesis of FeMo-co (49, 50); *nifK*, one of the dinitrogenase structural gene subunits; and *nifH*, dinitrogenase reductase, a subunit of nitrogenase that is required for electron transport to dinitrogenase (17), FeMo-co biosynthesis (47, 49), and insertion of FeMo-co into apodinitrogenase (1). Surprisingly, the stability of *nifHDKTY* mRNA was increased in these mutants (14 to 22%) relative to that in the wild type. Results from Northern analyses performed with UN1795 (*nifH* mutant) (13, 37) and UN1696 (*nifK* mutant) (37) are shown in Fig. 7. While *nifHDKTY* mRNA expressed in UN (wild type) decays with a $t_{1/2}$ of 20 ± 1.3 min, the $t_{1/2}$s of *nifHDKTY* mRNA were 31 ± 5.0 min ($P < 0.025$) when expressed in UN1795 (*nifH*) and 29 ± 4.5 min ($P < 0.05$) when expressed in UN1696 (*nifK* mutant). A strain harboring a Mu insertion in *nifU* was also examined and found to behave in a manner similar to that of the point mutant strains.

The finding that point mutations in a number of *nif* genes with disparate functions have a similar effect on *nifHDKTY* mRNA stability (stability is increased over that of the wild type) suggests that these strains are perturbed in some common function. This stabilization is statistically significant and is particularly surprising given that this mRNA is already so stable in the wild-type background. Any model of the regulated stability must therefore take this effect into account. Each of these strains is affected in at least one of the several requirements for an active nitrogenase: electron transport, FeMo-co biosynthesis, FeMo-co insertion, or nitrogenase enzymatic function. Therefore, a common property shared among these different strains is the absence of nitrogenase activity in the presence of the otherwise complete *nif* system. This suggests that nitrogenase activity is a component of the system that regulates *nif* mRNA stability. Our data also indicate that a *nif* factor(s) is required to stabilize *nif* mRNA, and thus it follows that nitrogenase activity may be sensed as a signal for stabilization. The physiological relevance of such a mechanism would be to increase gene expression through more stable mRNA when nitrogenase activity is low and to decrease gene expression when high levels of activity are achieved. Consistent with this is the observation that the accumulation of nitrogenase activity plateaus several hours after the initial derepression of *nif* genes (data not shown).

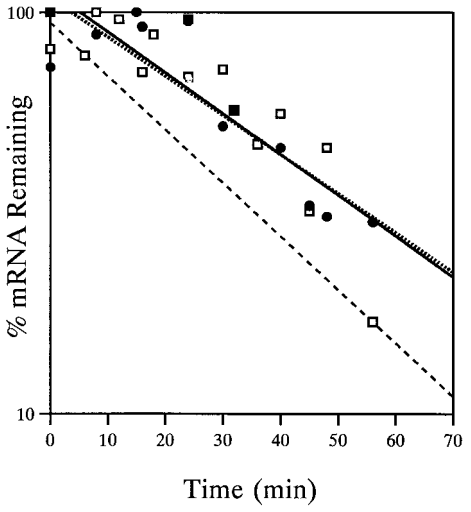


FIG. 7. Stability of the chromosomal *nifHDKTY* mRNA in a *nifK* and a *nifH* point mutant strain under anaerobic, *nif*-derepressing conditions. Semilogarithmic plot of *nifHDKTY* mRNA decay in UN1696 (*nifK*) (□) and UN1795 (*nifH*) (●). The results from two experiments for each mutant, which were determined to be the same at a 5% significance level, are plotted. Decay of *nifHDKTY* mRNA in UN (wild type) under the same conditions is reproduced from Fig. 2 for comparison (dashed line), but data points for this control are omitted for clarity.

It is paradoxical that large deletions of *nif* genes have the opposite effect on *nif* mRNA stability from point mutations. It cannot simply be a question of "tightness" of the mutation, for example, because UN1696 (*nifK*) has essentially no detectable nitrogenase activity. The paradox can be explained by postulating that even though the large deletions, like the point mutations, lack nitrogenase activity, they are missing the factor or factors essential for stabilizing the mRNA. Our failure to detect a single factor required for stabilization of the mRNA suggests that this effect is achieved by the complex interaction of a number of gene products.

Our working hypothesis posits that if there is a decrease in nitrogenase activity in the presence of a mostly complete contingent of *nif* proteins, *nif* mRNA turnover is reduced and gene expression increases. However, if both nitrogenase activity and the factor(s) required for stability of *nif* mRNA are absent, *nif* mRNA is destabilized. Since the hypothesis posits that nitro-

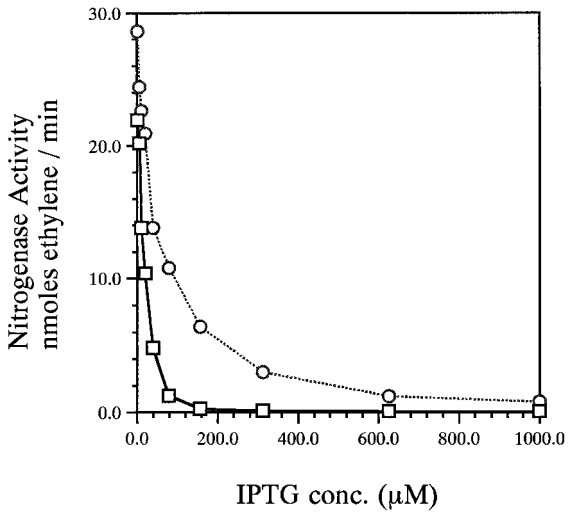


FIG. 8. Effect of overproduction of NifY on nitrogenase activity in the wild type and a *nifL* mutant. Strains were derepressed for *nif* function in the presence of IPTG at the concentrations shown for 4 h. Nitrogenase activity was measured as described in Materials and Methods for strains overexpressing *nifY* in the UN5397 (*nif*$^+$; □, solid line) and UN5406 (*nifL*; ○, dotted line) backgrounds.

genase activity indirectly regulates *nif* mRNA stability, then some mechanism must exist for sensing the status of nitrogenase. We considered that NifY, which is expressed from the *nifHDKTY* operon, might exhibit regulatory effects on *nifHDKTY* mRNA accumulation. A previous report from this laboratory established that NifY functions in nitrogenase maturation (28).

Construction of a *nifY* strain and its effect on nitrogenase activity. Upon *nif* derepression, UN5360, a *K. pneumoniae* strain with the Kan^r (*aphA*) cassette from pUC-4K (42, 46) incorporated into the *Sal*I site of *nifY*, possessed 50 to 70% of wild-type nitrogenase activity (as measured by acetylene reduction) and was phenotypically Nif$^+$ (data not shown). The Nif$^+$ phenotype of the *nifY* strain was surprising, given its apparent role in nitrogenase maturation (27), and it is our working model that another protein is able to substitute for NifY in its absence and fulfill its function, albeit less well.

Effect of the overexpression of *nifY* on nitrogenase activity and mRNA accumulation. UN5397, containing the NifY expression vector pNF107, was derepressed for *nif* expression in the presence of IPTG (to overexpress NifY) and monitored for acetylene reduction activity. An isogenic strain containing the parent plasmid without *nifY*, pKK223-3 (10), was examined as a control. The addition of 1.0 mM IPTG to UN5397 (*nif*$^+$/pNF107) completely blocked the appearance of acetylene reduction activity (Fig. 8), and Northern blot analysis revealed an absence of chromosomal *nifHDKTY* mRNA accumulation under those conditions (data not shown). In contrast, *nifLA* mRNA accumulation was not reduced in UN5397 compared to that of the wild type, indicating that the failure of UN5397 to accumulate *nifHDKTY* mRNA was not due to a reduction or absence of expression of the *nifLA* regulatory genes, which are under *ntr* control (data not shown). Examination of NifY overexpression in UN5406, a *nifL* mutant, demonstrated that levels of NifY expression capable of completely repressing nitrogenase activity in the wild-type strain did not do so in the *nifL* background (Fig. 8). These results suggest that NifY overexpression achieves its effect through interaction with the NifLA regulatory proteins.

Deletion of *nifY* from pUX40 increases the stability of the *nifHDKTY* mRNA in the Δ*nif* background under *nif*-derepressing conditions. Given the regulatory effects observed with *nifY* overexpression, the instability of pUX40 *nifHDKTY* mRNA expressed in the large *nif* deletion strains (Fig. 6) might be the result of expressing NifY (from pUX40) in the absence of a functional nitrogenase and/or stabilizing factor. That possibility is suggested by the following. The pUX202 (Δ*nifTY*) mRNA is 2 to 3 times more stable than the pUX40 *nifHDKTY* mRNA in the Δ*nif* background (data not shown), even though it did not have enhanced stability in the *nif*$^+$ background (Fig. 3). In contrast, decay of the pUX214 (Δ*nifH*) mRNA in the Δ*nif* background was similar to that of pUX40 (data not shown). This is most easily rationalized by the synthesis of NifT and/or NifY causing an instability of the mRNA under these, admittedly perturbed, conditions. The effect seen is likely due to the absence of NifY, rather than NifT, as we previously reported that the absence and overexpression of NifT alone had no discernible effect on the regulation, accumulation, and maturation of nitrogenase in *K. pneumoniae* (51).

Working hypothesis. Our results demonstrate that the O_2-regulated stability of *nifHDKTY* mRNA is controlled by a relatively small region and, furthermore, that there is no simple *cis* determinant for the unusual anaerobic stability of the mRNA in vivo. We have also demonstrated that there is a *nif* factor or factors required for the stability of the *nifHDKTY* mRNA under *nif*-derepressing conditions. We have disproved the model that NifA is sufficient for stabilizing *nif* mRNA and that the *nifLA* gene products are sufficient to achieve both transcriptional and posttranscriptional control. Our results strongly suggest that regulation of *nif* mRNA stability is not achieved through any simple mechanism.

The fact that the elimination of NifY, a protein involved in maturation of nitrogenase, from the pUX40 *nifHDKTY* mRNA increased the stability of that mRNA in the large *nif* deletion background, in addition to other regulatory effects discovered with NifY overexpression, is consistent with this hypothesis. NifY has been shown to associate with apodinitrogenase and dissociate upon insertion of the active site, FeMo-co (28), the final step in nitrogenase maturation. As part of the maturation process, NifY may have a role in sensing and signaling the status of nitrogenase. The purpose of sensing the status of nitrogenase in the wild type could be to serve as a feedback mechanism to regulate nitrogenase production to match the availability of various components required for nitrogen fixation, such as metals and reducing power.

A number of other observations are consistent with a role for the nitrogenase proteins themselves in the regulation of *nif* expression. Roberts et al. (37) reported that mutations in *nifH*, *nifD*, and *nifK* (encoding the nitrogenase component proteins) typically resulted in a reduction in the levels of many other Nif proteins. Chang et al. also noted differences in the accumulation of steady-state levels of *nifHDKTY* mRNA during *nif* derepression in a number of different *nifH* mutants compared to the wild-type strain (13). Also consistent is the evidence that *nifH* is required for the expression of the alternate nitrogenase transcriptional activator AnfA in *Azotobacter vinelandii* (8).

The fact that a non-*nif* protein, *glnK*, under NtrC control has been shown to relieve NifL inhibition of NifA activity under N-limiting conditions (24) is not inconsistent with our hypothesis of NifY sensing of nitrogenase status. NifY could interfere with the relief of inhibition by *glnK* under certain conditions, or there may be independent pathways for mRNA destabilization. In fact, the data for destabilization of *nif* mRNA in a *nifL* strain (15) demonstrate that destabilization still occurs in the presence of NH_4^+, albeit not to the same degree as in the

wild-type strain. This suggests other factors (in addition to NifL) are involved in *nif* mRNA destabilization under those conditions.

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