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Characterization of *nifH* Mutations of *Klebsiella pneumoniae*†

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Nucleotide changes in the *nifH* gene of *Klebsiella pneumoniae* were identified by DNA cloning and sequencing of six selected mutant strains. The strains were UN60, C-640-GC → TGC; UN116, C-67-TC → TTC; UN117, G-688-AG → AAG; UN1041, CG-302-C → CAC; UN1678, GC-713-C → GTC; and UN1795, G-439-AG → AAG. Their corresponding amino acid substitutions were UN60, Arg-214 → Cys; UN116, Leu-23 → Phe; UN117, Glu-230 → Lys; UN1041, Arg-101 → His; UN1678, Ala-238 → Val; and UN1795, Glu-147 → Lys. Results from Western and Northern blots of the mutant strains showed significant reductions in both steady-state levels of the accumulated Fe protein and *nifH* mRNA during derepression in the presence of serine. The relative specific activities of the nitrogenases in strains UN60, UN1041, and UN1795 were less than 2% of the wild type, whereas those in UN116, UN117, and UN1678 were between 28 and 40% of the wild type during enhanced derepression with serine. The residues of Arg-101 (UN1041), Glu-147 (UN1795), and Arg-214 (UN60) were invariant in sequences of a dozen diazotrophs that have been examined thus far. In UN1041, in which Arg-101 of the Fe protein was replaced by His, the Fe protein had a larger apparent molecular weight than that of the other strains on sodium dodecyl sulfate-gel electrophoresis, as detected with rabbit antiserum raised against the C-terminal peptide of the wild-type Fe protein. The reduced levels of *nifH* mRNA in point mutant strains suggests that *nifH* (the gene or gene product) may be involved in self-regulation. mRNA transcripts of different sizes were detected when a *nifH*-specific probe, CCKp2003, was used in the Northern blot hybridization.

Biological nitrogen fixation, the enzymatic reduction of atmospheric dinitrogen to ammonia, is dependent on the functioning of three polypeptides which constitute the nitrogenase enzyme (20), in addition to a number of ancillary proteins, enzymes, and other gene products. In *Klebsiella pneumoniae*, in which such a system has been best studied, there are approximately 20 identified genes that are associated with nitrogen fixation (2, 3). Some of these serve multiple roles in the process, and the regulatory interactions of the genes are not yet fully understood.

The nitrogenase enzyme complex per se consists of the Fe protein, which contains two identical subunits and a single 4 Fe-4 S cluster, and the MoFe protein, which has an $\alpha_2\beta_2$ structure and contains MoFeS clusters (32) plus FeS clusters. The smaller Fe protein binds ATP and serves as an electron donor to the MoFe protein, which binds reducible substrates including N_2 and C_2H_2. Mutations in any of the three polypeptides may lead to an enzyme with an altered efficiency in these reduction processes. There are about 55 discrete mutant strains with defects that map in the *nifH* gene, which encodes the Fe protein (25, 26; G. P. Roberts, personal communication). For a polypeptide of 298 amino acids, this should represent a reasonable saturation of essential residues. The mutants were selected as nonleaky and thus represent major changes of protein functionality.

Because classical methods of protein modification have proved to be difficult to use with nitrogenase (11), we began a program of sequencing all of the available mutants in order to relate their amino acid alterations to changes in function. A short report on the sequencing of three of the mutant genes has appeared already (4). In this report we provide results of studies of mRNA and polypeptide production as well as the sequences of three more mutants. One of these mutants, UN1041, with a substitution of His for Arg at position 101, proved to be particularly interesting because it is no longer a substrate for riboadenylation by the modifying enzyme from *Rhodospirillum rubrum* (22; R. Lowery and P. W. Ludden, personal communication). The mutants should also prove to be useful for interpreting the crystal structure of the Fe protein (24).

(This study was taken from a thesis submitted by C. L. Chang in partial fulfillment of the requirements for a Ph.D. degree [1988],)

MATERIALS AND METHODS

Chemical reagents. Unless otherwise noted, all chemicals were reagent grade and were obtained from commercial sources. Restriction endonucleases, *Eco*RI linkers, DNA polymerase I, phage T4 DNA kinase, T4 DNA polymerase, and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.); and acrylamide, ultrapure urea, and restriction endonucleases were purchased from International Biotechnologies, Inc. Antibiotics were obtain from Sigma Chemical Co. (St. Louis, Mo.), while tryptone and yeast extract were from Difco Laboratories (Detroit, Mich.). Glycogen, lysozyme, proteinase K, and DNase were purchased from Sigma. RNase T1, low-melting-point agarose, and RNA standards were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-galactopyranoside were purchased from Sigma and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), respectively. Calf intestinal alkaline phosphatase was from P-L Biochemicals, Inc. (Milwaukee, Wis.). Nitrocellulose paper was purchased from Schleicher & Schuell, Inc. (Keene, N.H.), and 3MM filter paper was from Whatman, Inc. (Clifton, N.J.). Film for autoradiography was XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

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Oligonucleotides were synthesized in the Plant Pathology Department at Kansas State University by using a DNA synthesizer (381A; Applied Biosystems). They were purified by high-pressure liquid chromatography on a PRP-1 column (The Hamilton Co., Reno, Nev.) prior to use. For sequencing reactions [α-^{35}S]ATP, at a specific activity above 1,200 Ci/mmol, was obtained from E. I. du Pont de Nemours & Co., Inc. (Wilmington, Del.). Peptides were synthesized as described previously (9, 35). Protein A for immunodetection was iodinated with ^{125}I as described previously (12).

Specific probe construction. To facilitate work with the *nifH* gene and to eliminate any possible ambiguities when examining restriction fragments or mRNA patterns, a probe specific for the structural portion of the *nifH* gene was constructed from pJC082 (10). The final probe contained bases −14 to +186 of the *nifH* structural gene. For use as a probe, primer extension of the single-stranded form of the phage was found to be convenient. There was no cross-hybridization of the M13mp19 vector with *K. pneumoniae* genomic DNA and the pBR329 vector or in Northern blot analyses. This clone, identified as CCKp2003, shares a two-thirds sequence identity with seven published sequences from other species (13, 18, 21, 23, 30, 31, 36) but only 53% identity with either *Clostridium pasteurianum* (5) or *Methanococcus voltae* (33) sequences.

Isolation of DNA and selection of clones for sequencing. *K. pneumoniae* strains were grown overnight in a medium containing combined nitrogen and were derepressed in the presence of serine (R. T. St. John, Ph.D. dissertation, University of Wisconsin-Madison, 1973). DNA was isolated by modification of the method of Marmur (16). Purified DNA was digested with *Eco*RI and *Bam*HI and then, following electrophoretic fractionation and excision of fragments around the 1.8-kilobase-pair region, was ligated into plasmid pBR329 (which was similarly digested and dephosphorylated to prevent self-ligation).

The cloned DNA was transformed into *Escherichia coli* DH1. Specifically, the single-stranded-labeled probe CCK p2003 was used for colony hybridization, to select those colonies with *nifH* fragments. This was verified by probing a Southern blot of isolated plasmids with CCKp2003.

The 1.8-kilobase-pair fragments containing *nifH* were subcloned into M13mp18 and M13mp19 in *E. coli* JM109. For sequencing, single-stranded DNA of the modified phage was isolated (17) and used in the sequencing procedure described by Sanger et al. (28).

Sequencing strategies. Because the sequence of the *nifH* gene has been determined previously, specific primer-directed sequencing (34) was the most efficient approach for sequencing the cloned DNA of the mutant organisms. Eight different primers, four in each orientation and each of which was 15 nucleotides long, were synthesized. These were chosen to avoid self-annealing and to give an even distribution throughout the coding region. In addition, universal primers (New England BioLabs) were used to sequence the end regions of the insert directly from the phage DNA.

To maximize sequence information in a single run, ^{35}S labeling was used and reaction products were separated on buffer gradient polyacrylamide gels (1). By running four mutants side by side for each termination reaction, it was possible to identify base changes up to at least 450 bases from the lower end of the gel in a single loading. Alterations were verified by sequencing both strands, with primers located as close as practical to the site of alteration. Only primers designated 2, 3, 5, 6, and 8 and the universal primer were needed.

The method of dideoxynucleotide chain termination described by Sanger et al. (28) was used, as described by Williams et al. (38). The sequencing gel was prepared with 6% polyacrylamide and 7.8 M urea and a gradient with buffer concentrations of (1) 0.5×, 1×, and 4×.

Derepression of *K. pneumoniae* nitrogenase. Nitrogenase was derepressed by growing the cultures under limited nitrogen and oxygen conditions. Two nitrogen sources were used during the derepression: L-serine (St. John, Ph.D. dissertation) and L-histidine (25). The growth media used were designated KN and K (St. John, Ph.D. dissertation). The K medium is a minimal salts medium, while KN is the same as K medium but it also contains 0.4 mg of ammonium N per ml.

(i) Serine method. A small volume of a mutant or wild-type strain was grown overnight on KN medium and then transferred to 30 or 100 ml of KN in a 250-ml flask with a side arm. The cell culture was allowed to grow to 100 Klett units (no. 64 filter). The cells were centrifuged and suspended in the same volume of K medium. For the 30-ml culture, a 30-ml centrifuge tube with a screw cap was used, and the cell culture was flushed with N_2 gas for 5 min before the cap was closed. This was used for both the initial screening of nitrogenase activity and for chromosomal DNA isolation. The 100-ml culture was grown in a 500-ml aspirator flask with the side arm topped with a rubber stopper from which samples could be drawn periodically for the time course study. This large culture was sparged continuously with N_2 at a low rate. L-Serine (2×10^{-4} M) was added to the culture 1 h after it was transferred to K medium. Cells were derepressed in the 30°C water bath for 8 h without shaking.

(ii) Histidine method. After growing overnight, a strain was subcultured to a 250-ml flask containing 30 ml of KN medium. When the Klett reading reached 40 U, 19 ml of the culture was centrifuged and the pellet was rinsed with K medium and then suspended in 30 ml of K medium containing 0.15% L-histidine. This resuspended culture (6 ml) was transferred into a 10-ml test tube that was capped with a stopper. The culture in the test tube was flushed with argon 3 times and grown at 30°C. Growth was monitored by the Klett reading method for 3 days. The pH of the cell culture was monitored with methyl red and bromothymol blue, which were added to K medium during the derepression.

Acetylene reduction assay in vivo. A total of 1 or 3 ml of the culture was transferred into a 6.5-ml serum vial that was evacuated and flushed with argon 3 times. Freshly made acetylene (0.5 ml; made from calcium carbide) was injected into each vial with a syringe, and the vials were incubated at 30°C with vigorous shaking for 30 min. The reduction reaction was terminated by adding 0.5 ml of 37% formaldehyde. If the culture was needed for protein isolation, no formaldehyde was added. Ethylene production was measured by gas chromatography (Aerograph series 1200; Varian Instruments), as described by Davis (7). The activity of the nitrogenase was expressed as nanomoles of ethylene produced per milliliter of culture per hour.

Gel electrophoresis and protein transfer to nitrocellulose. Cell cultures were centrifuged, and the pellet was suspended in the sodium dodecyl sulfate (SDS) sample loading buffer (19). The suspensions were boiled for 3 min, and then the cell debris was removed by centrifugation. The A_{280} was used to adjust the protein concentrations applied to the gel system to give comparable amounts of total protein. Electrophoresis was done as described by O'Farrell (19) for the second dimension of his system, with 0.4% SDS used in both stacking and separation gels. The molecular weight markers

TABLE 1. DNA and protein alterations in mutant strains[a]

Mutagen[b]	Mutant strain	Allele no.	*nifH* gene base change (position[c])	Fe Protein	
				Change (position[c])	Abbreviation
NTG	UN60	4060	CGC → TGC (640)	Arg → Cys (214)	R214C
NTG	UN116	4116	CTC → TTC (67)	Leu → Phe (23)	L23F
NTG	UN117	4117	GAG → AAG (688)	Glu → Lys (230)	E230K
DES	UN1041	4384	CGC → CAC (302)	Arg → His (101)	R101H
DES	UN1678	4714	GCC → GTC (713)	Ala → Val (238)	A238V
DES	UN1795	4764	GAG → AAG (439)	Glu → Lys (147)	E147K

[a] Mutagens, mutant strains, and allele numbers are as given by Roberts and Brill (25) and G. P. Roberts (personal communication).
[b] NTG, *N,N'*-nitrosoguanidine; DES, diethyl sulfate.
[c] Numbering of the positions is based on the sequence given by Scott et al. (30), with the ATG (formylmethionyl) initiation codon considered as position 1. Amino acid changes are shown by use of one-letter abbreviations, with position numbers indicated between wild-type and mutant residues.

used were bovine albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), and α-lactalbumin (14,000). Proteins were stained with Coomassie blue R250 or transferred to nitrocellulose with a transphor electrophoresis unit (TE series; Hoefer). The transfer buffer was as described by Towbin et al. (37). After transfer the proteins were stained with 0.1% amido black in 25% propanol–10% acetic acid for 5 min, to locate the protein standards that were run with each gel. Destaining was done with 25% propanol–10% acetic acid for 10 min.

Radioimmunodetection of Fe protein. A synthetic peptide containing the last 15 amino acids at the C terminus of the Fe protein was made, and antiserum against the synthetic peptide was raised (35). To remove low levels of nonspecific binding, the antiserum was treated by a preadsorption procedure before the immunodetection process was done. This antiserum identified full-length Fe protein translation products only and was not significantly reactive with *Azotobacter vinelandii* nitrogenase.

The nonderepressed wild-type strain was grown aerobically to the stationary phase in KN medium. A total of 5 ml of the culture was spun, and the cells were suspended in 250 μl of buffer (0.1 M NaCl, 10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). Lysozyme was added to a final concentration of 1 mg/ml, and the cell mixture was incubated at 37°C for 10 min. The cells were lysed within 5 min after the addition of SDS (1%). To this cell lysate, 750 μl of buffer B (50 mM NaCl, 2 mM EDTA, 10 mM Tris hydrochloride [pH 7.4]) was added. Two small pieces of nitrocellulose paper were incubated in the diluted cell lysate for 15 min at room temperature and then washed with buffer B. In a microcentrifuge tube, 0.9 ml of buffer B and 0.1 ml of antiserum were mixed, and the washed protein-bound nitrocellulose papers were added for 15 min. This treated antiserum was then used for the immunodetection method.

A protein blot was blocked overnight with 25 ml of buffer C (2% bovine serum albumin and 0.1% NaN_3 in buffer B) at room temperature. The preadsorbed antiserum was then added to buffer C, giving a final dilution of 1/250, and incubated with the blot for 4 h at room temperature. The protein blot was then washed with about 500 ml of buffer B. Specifically bound antibody was then detected by incubating the washed protein blot with ^{125}I-labeled protein A for 2 h at room temperature, and the blot was washed with a total of 500 ml of buffer B. This protein blot was then air dried and autoradiographed at −70°C overnight or an appropriate time interval.

A quantitative estimate of the protein accumulation during derepression was made by scanning densitometry of the autoradiograms. Equal amounts of total protein were applied to the gels, and the times of autoradiography were adjusted so that no bands were overexposed. A dilution series of wild-type protein was used to calibrate the method visually, and for each gel a wild-type sample was included. Scans of duplicate samples were reproducible to ±10% for wild-type protein and visually appeared to be proportional to the amount of protein added.

RNA isolation and hybridization. Total RNA was isolated from cultures that were derepressed for nitrogenase synthesis with serine at appropriate times up to 8 h. A rapid RNA isolation method was developed (C. L. Chang and L. C. Davis, submitted for publication) based on a rapid plasmid isolation technique. The isolated RNA was then subjected to electrophoresis in 1% agarose containing 2.2 M formaldehyde (15). The separated RNA was transferred to nitrocellulose as described previously (15). Hybridization was performed at 42°C in the presence of 50% deionized formamide. After hybridization and washing of the blots, autoradiography was done, and suitable exposure intervals were used to ensure that no bands were excessively dark. Densitometry of the developed autoradiograms (fiber optic scanner; Kontes) was used to estimate the quantity of mRNA that was present under the derepression conditions. Results are shown for equal amounts of total RNA, normalized to the wild type at 4 h. Density estimates varied by ±10% for replicates of the wild type exposed to yield the optimum film density.

RESULTS

For each of six mutant strains whose sequences are reported here, only a single base change was found in the *nifH* gene, including the region extending upstream to the *Eco*RI site. A summary of the base changes and the consequent amino acid substitutions is given in Table 1. Three of the mutations mapped into phage Mu deletion interval 2 of *nifH* defined by Roberts et al. (26), and three mapped into interval 3. Their locations indicate that the deletion intervals are both reasonably long and are located within the structural coding portion of the gene. Deletion interval 1 cannot extend into the coding region further than base 67, and interval 3 cannot end prior to base 713.

As expected, all six mutant strains showed lower in vivo acetylene reduction activities than that of the wild type. They were originally selected for failure to grow on N_2 and were thus expected to have steady-state acetylene reduction levels of less than 10 to 20% of the wild type (26; G. P. Roberts, personal communication). When followed during derepression with serine, two mutants accumulated significant activity compared with the wild type (Table 2). All of the mutants showed detectable amounts of the Fe protein by

TABLE 2. Time course of nitrogenase activity (serine method)

Strain (mutation)	Activity (nmol/ml of culture per h) at the following times (h):			
	3	4	6	8
Wild type	29	109	302	450
UN60 (R214C)	0	0	0	0.5
UN116 (L23F)	3	10	22	64
UN117 (E230K)	0	0	1	6
UN1041 (R101H)	0	0	0	0.5
UN1678 (A238V)	0	1	12	30
UN1795 (E147K)	0	0	0	0.5

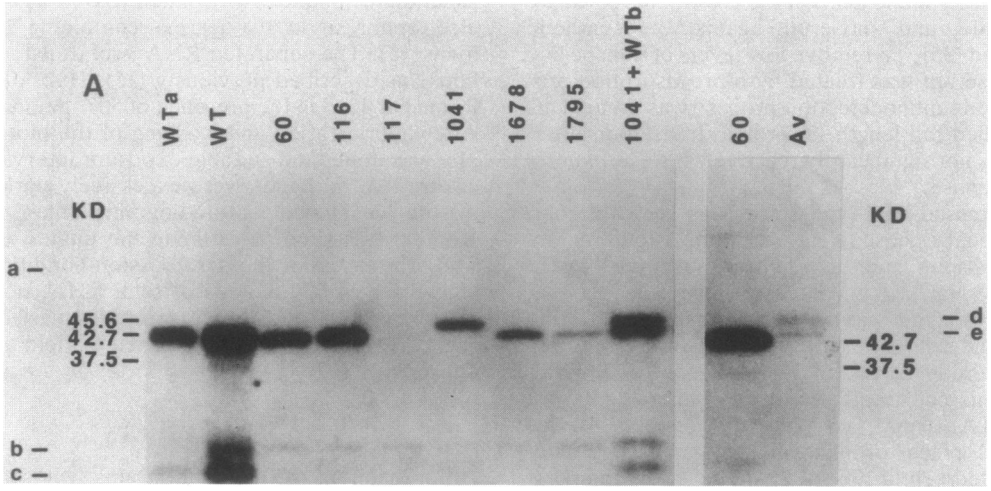
TABLE 3. Fe protein level during derepression (serine method)

Strain (mutation)	Fe protein level[a] at the following times (h):			
	3	4	6	8
Wild type	26	43	91	100
UN60 (R214C)	30	31	30	30
UN116 (L23F)	23	32	39	43
UN117 (E230K)	0.6	0.6	0.6	3
UN1041 (R101H)	17	22	23	26
UN1678 (A238V)	2	6	13	17
UN1795 (E147K)	4	5	7	7

[a] Levels are relative to that of the wild type at 8 h.

8 h under these conditions, but the levels varied widely (Fig. 1A and Table 3). When the data are compared as relative specific activity (Table 4), one can see two classes of mutants, those with a high relative specific activity and those with a low relative specific activity. The in vivo activities of strains UN60, UN1041, and UN1795 were not reliably measurable under these conditions. The amino acid substitutions in these three strains were at positions which are invariant in a dozen *nifH* genes that have been sequenced thus far (5, 13, 18, 21, 23, 29, 30, 31, 33, 36). The relatively high specific activities of UN117 and UN1678 were associated with slower protein accumulation than that in the wild type, whereas UN116 accumulated protein at the same relative rate but to a lower extent.

When the histidine derepression method was used, all mutants accumulated amounts of protein comparable to those of the wild type grown under identical conditions (Fig. 1B and Table 5). The in vivo activities were lower for the wild type under these conditions than with serine derepression but were higher for some of the mutant strains (Table 6). Under these conditions, strain UN1041 showed very definite activity, while strain UN1795 showed marginal activity at best and strain 1678, which was highly active by the serine derepression method, showed very little activity. Little



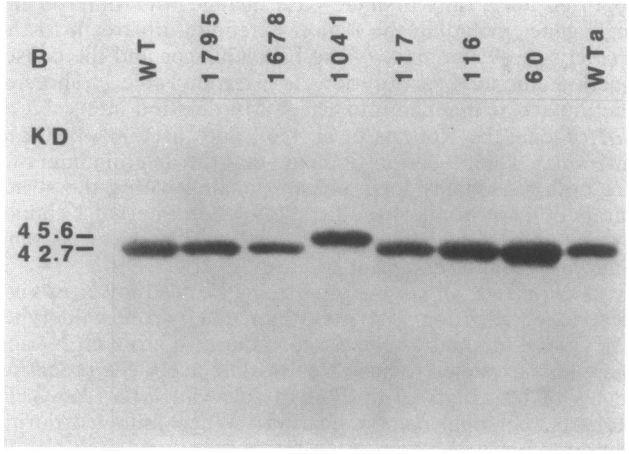


FIG. 1. Radioimmunoprecipitation analysis of Fe proteins from *K. pneumoniae* strains by using serine or histidine as the nitrogen source. (A) At 6 h of derepression with serine, 200 μg of total protein from each mutant strain was electroblotted onto nitrocellulose following SDS-gel electrophoresis. Antiserum against the C-terminal peptide of the Fe protein was used with the addition of radioiodinated protein A. In lane WTa, 25 μg of wild-type (WT) protein was used. In lane 1041 + WTb, 200 μg of protein from UN1041 and 50 μg from the wild type were coelectrophoresed. The minor band at 37.5 kilodaltons (KD) was observed for each *K. pneumoniae* strain when the amount of the major band was high enough during derepression. Bands at positions a, b, and c were observed for all *K. pneumoniae* strains under both derepressed and nonderepressed conditions. In lane Av, both purified Fe protein and MoFe protein of *A. vinelandii* were applied. Bands d and e, corresponding to the Fe protein, were detected. (B) At 2 days of derepression with histidine, 100 μg of total protein from each *K. pneumoniae* mutant strain was used for SDS-gel electrophoresis. Lane WTa contained 20 μg of total protein from the wild type (WT). After peptides were electroblotted to nitrocellulose, they were detected by using preadsorbed antiserum and radioiodinated protein A.

TABLE 4. Relative specific activity of nitrogenase

Strain (mutation)	Relative sp act at the following times (h):			
	3	4	6	8
Wild type	25	57	74	100
UN60 (R214C)				0.4
UN116 (L23F)	3	7	12	33
UN117 (E230K)			32	28
UN1041 (R101H)				0.4
UN1678 (A238V)		5	22	40
UN1795 (E147K)				2

growth occurred in any of the strains. On the basis of the Klett reading, there was less than one cell doubling in 3 days for any of the strains, including the wild type, under argon. Changes in cell size were not monitored.

Comparison of the mRNA levels specific for *nifH* during serine derepression (Fig. 2) revealed that the mutant strains showed different patterns of mRNA production, turnover, or both (Table 7). As described by Collins and Brill (6) for the wild type, the peak level of *nif*-specific mRNA was found at 4 h. Strains UN60, UN1041, and UN1795 showed similar patterns but reached lower levels of mRNA. Note that all three of these mutant strains failed to give significant in vivo activity. Strains UN116 and UN1678 appeared to keep nearly constant mRNA levels from 2 to 8 h and showed some in vivo activity. In strain UN117, the levels of mRNA were much lower at the early times than later on, which is consistent with its delayed appearance of activity and protein. The relative intensities of the mRNA bands of different sizes were generally constant over time and between mutants. It was necessary to use several different exposure times for the autoradiograms to verify this point, while results for only one exposure time are given in Fig. 2.

DISCUSSION

We chose to examine eight mutants of *K. pneumoniae* with defects in nitrogen fixation that map in the *nifH* gene. For one mutant (UN1788), the cloned DNA could not be removed from the cloning vector by *Eco*RI-*Bam*HI enzyme digestion. For another mutant (UN1658), no alteration of the DNA sequence was detected within the cloned *Eco*RI-*Bam*HI fragment. In each case cloning and sequencing were attempted twice.

For those six mutants that were sequenced successfully, only a single base change was found. They were initially chosen to map into different phage Mu deletion intervals (26). Three were induced by nitrosoguanidine and three were induced by diethyl sulfate. Three were C → T changes, and three were G → A changes; four were at the first position of a codon and two were at the second position. The amino acid substitutions included changes of charge and an increased bulk of amino acids. Those mutants with changes of amino acid side chain bulk showed relatively greater in vivo nitrogenase activities when tested by the serine derepression method than did those mutants with charge changes.

TABLE 5. Comparison of serine and histidine derepression methods

Strain (mutation)	Fe protein level at[a]:		Relative sp act at[b]:	
	8 h (serine)	2 day (histidine)	8 h (serine)	2 day (histidine)
Wild type	100	124	100	2
UN60 (R214C)	30	139	0.4	0.4
UN116 (L23F)	43	146	33	1
UN117 (E230K)	3	100	28	0.6
UN1041 (R101H)[c]	26	161	0.4	1
UN1678 (A238V)	17	93	40	0.1
UN1795 (E147K)	6	95	2	0.1

[a] Levels relative to that of the wild type at 8 h.
[b] Relative specific activity was calculated after Klett units were normalized.
[c] The apparent molecular weight of strain UN1041 was about 5% greater than those of the wild type or the other mutants.

TABLE 6. In vivo nitrogenase activity (histidine method)

Time (day)	nmol of C_2H_4/ml of culture per h for the following strains (mutations)						
	Wild type	UN60 (R214C)	UN116 (L23F)	UN117 (E230K)	UN1041 (R101H)	UN1678 (A238V)	UN1795 (E147K)
1[a]	9	0	1	0.2	0.05	0.1	0.05
1–2[b]	26	1	9	6	2	0.8	0.02
2[a]	7	1	3	1	4	0.3	0.3
2–3[b]	3	2	2	2	9	0.3	0.002

[a] In vivo acetylene reduction assay was carried out for 30 min at 1 and 2 days of derepression. Klett readings ranged from 39 to 64.
[b] In vivo acetylene reduction assay was carried out for 24 h for 1- to 2-day and 2- to 3-day cultures.

When the amount of Fe protein was measured by radioimmunodetection (Western blot) of the C-terminal region, the mutants showed different time courses during derepression with serine. One mutant (UN60) had as much Fe protein as the wild type at 3 h but showed no further increase in its Fe protein level. In this same mutant the mRNA level peaked at 4 h with one-fifth the amount of that in the wild type and declined thereafter. In mutant UN116, the protein and mRNA levels both increased slowly after 3 h but only reached a fraction of the wild-type level. Another mutant (UN1678) had the same pattern of mRNA but a much slower increase in Fe protein. Both showed increasing in vivo nitrogenase activities, but UN1678 had a longer lag before measurable activity was present. Mutant UN117 accumulated only low levels of mRNA and showed very low levels of Fe protein and activity.

The pattern of mRNA accumulation in UN1041 was very like that of UN60, but the Fe protein level increased somewhat more slowly. The in vivo activity was too low for a reliable measurement to be made during serine derepression. Strain UN1795 showed a very similar mRNA profile but did not accumulate either Fe protein or activity.

The presence of discrete multiple mRNA transcripts that react specifically with a probe that is very highly specific for the region encoding the N terminus of the *nifH* protein was unexpected. However, such multiple transcripts have been observed by Krol et al. (14) in earlier studies in which they used a quite different method of mRNA isolation but a less specific probe. A similar result has been obtained in the laboratory of G. P. Roberts (results were reported at the 7th International Congress on Nitrogen Fixation, 1988). Jones et al. (12) used a somewhat less specific probe that contained a portion of *nifD* and observed a similar phenomenon in *Azotobacter chroococcum*. In that system the interpretation was made complicated because there were multiple regions of the genome that also hybridized to *nifH* probes. They were not able to determine whether the multiple transcripts came from the different regions of DNA or whether there was processing or transcriptional attenuation of a single DNA region. The specific clone CCKp2003 may prove useful for studies of *nifH* gene expression in other species because

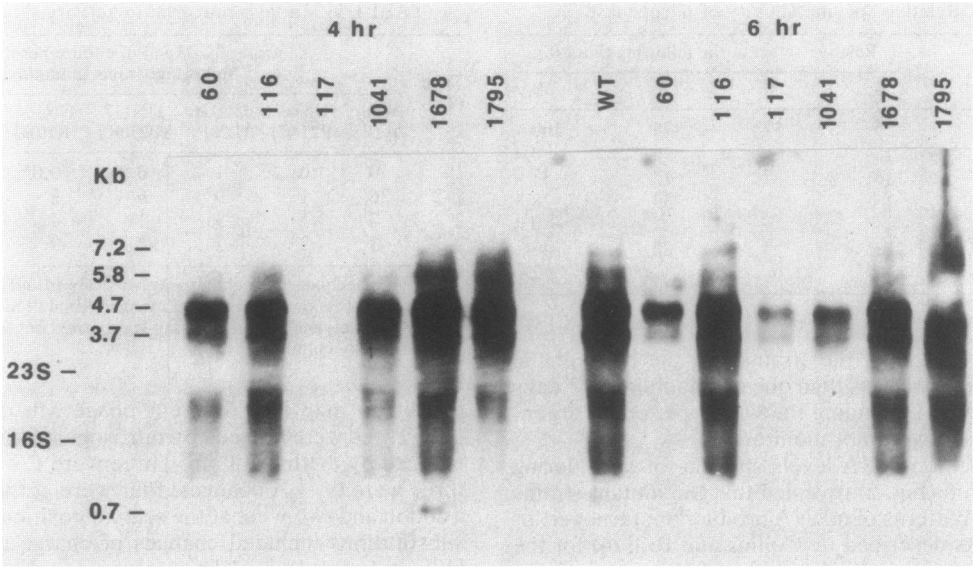


FIG. 2. Northern blot hybridization probed with ^{32}P-labeled CCKp2003. Total RNAs from each strain at 4 and 6 h of derepression with serine were blotted onto a nitrocellulose membrane after they were subjected to formaldehyde agarose gel electrophoresis. ^{32}P-labeled CCKp2003 was labeled by the primer extension method, and hybridization (10^6 cpm/ml) was performed at 42°C overnight in the presence of 50% formamide. Posthybridization wash was carried out with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C. The RNA size standards (from Bethesda Research Laboratories) were 9.5, 7.5, 4.4, 2.4, 1.3, and 0.3 kilobases. Abbreviations: Kb, kilobases; WT, wild type; 23S, 23S rRNA; 16S, 16S rRNA.

it contains the region of greatest similarity to other *nifH* genes, with no promoter region or *nifD* sequence present. The transcripts observed in other studies are probably all long enough to encode the *nifH* protein. The nature of the antibody preparation also ensures that only full-length products would be measured. We have not yet used specific probes to determine the endpoints of the observed mRNA species, so we do not know whether they possess a common starting point.

It is somewhat surprising that so many different patterns of protein and mRNA accumulation should be observed with only six mutants, each of which showed only a single base change within the coding portion of the *nifH* gene. The amount of serine N was not great (2×10^{-4} M), so it is possible that nitrogen starvation may have caused some general effects. The differential stability of mRNA based on single base changes within the coding region is not expected. The relative stability of proteins could be altered by single residue substitutions, but the results of histidine derepression and the earlier work of Roberts and Brill (25) argue against this. The results suggest that *nifH* mRNA or protein products have another function besides electron donation to MoFe protein.

Histidine is a poor N source for *K. pneumoniae*, requiring induction of the *hut* genes, but the amount of histidine supplied in the experiments reported here provided considerably more N than did the serine method. A level of 0.15% histidine yielded ~30 mM N. During the derepression, which was conducted under argon, nitrogenase proteins accumulated to levels equivalent to those obtained with the wild type in 8 h of derepression with serine. Roberts et al. (26) earlier examined nitrogenase protein synthesis by pulse-labeling histidine-derepressed cultures. For those strains that were examined, synthesis of nitrogenase proteins occurred after 1 day of derepression. Growth with histidine is, however, very slow. We did not directly measure the rates of synthesis and degradation for either mRNA or protein in our experiments, but we can say that the mutants examined here accumulated steady-state levels of *nifH* polypeptide that were comparable to wild-type levels. The differences in accumulation observed during serine derepression may reflect different rates of synthesis, if we assume that the protein steady-state levels observed under histidine derepression conditions reflect the similarity of the wild type and the various mutants. Roberts and Brill (25) have shown that under anaerobic conditions (helium) the structural proteins *nifH*, *nifD*, and *nifK* were not turned over in the wild type during 20 h of derepression in the presence of serine.

It seems unreasonable to postulate that the various mutants each carry a second defect in a separate protein- or mRNA-stabilizing system because all were mapped by Roberts et al. (26) as single point mutations by using a battery of phage Mu deletions and complementation. All are revertible. We therefore suggest that, in some manner, the functionality of the *nifH* gene controls mRNA synthesis or degradation, in order to account for the very different patterns observed during serine derepression.

It has been established that the Fe protein is required for synthesis of the FeMo cofactor of the MoFe protein (8, 27).

TABLE 7. Relative levels of *nifH* mRNA during derepression with serine

Time (h)	Relative levels (%) of *nifH* mRNA in the following strains (mutations)[a]						
	Wild type	UN60 (R214C)	UN116 (L23F)	UN117 (E230K)	UN1041 (R101H)	UN1678 (A238V)	UN1795 (E147K)
3	49	17	20	6	18	22	13
4	100	21	26	6	24	34	26
6	31	12	30	11	12	29	24
8	—[b]	5	30	20	5	30	6

[a] Amounts expressed relative to wild type at 4 h of derepression.
[b] —, No data were collected.

However, truncated gene products may be sufficient to carry out this process, and we do not know the effectiveness in FeMo cofactor synthesis for all of these mutants. Strain UN1041 is effective (R. Lowery and P. W. Ludden, personal communication), and strains UN60 and UN116 also appear to be effective, as determined from results of preliminary experiments (J. Imperial, personal communication). The appearance of in vivo nitrogenase activity requires synthesis of the FeMo cofactor. None of the mutant strains completely lacked in vivo activity, but a strain such as UN1795 which has a very low activity may be defective in this function; at least it produces no free FeMo cofactor (J. Imperial, personal communication). The in vitro activity of these mutants has not been quantitated, so we do not know in all cases that their defect is in the usually defined Fe protein functions. (They could be defective, for instance, in FeMoCo synthesis but normal in electron transfer.) Nor have nondenaturing gels been run to determine whether insertion of the FeS centers are altered in these mutants. Such experiments are in progress.

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