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Regulation and Characterization of Protein Products Coded by the *nif* (Nitrogen Fixation) Genes of *Klebsiella pneumoniae*

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Two hundred and thirty-five Nif⁻ strains of *Klebsiella pneumoniae* were characterized by two-dimensional polyacrylamide gel electrophoresis. Forty-two of these strains were tested further by in vitro acetylene reduction assays. By these techniques, nine *nif*-coded polypeptides were identified, and eight of these were assigned to specific *nif* genes. Nitrogenase component I required *nifK* and *nifD*, which coded for the β and α subunits, and *nifB*, *-E*, and *-N* were required for the iron-molybdenum cofactor, which is a part of the active site of nitrogenase. *nifH* coded for the structural protein of component II, and *nifM* and *nifS* products seemed to be necessary for the synthesis of an active component II. There were two genes, *nifF* and *nifJ*, that were required for N_2 fixation in vivo but not for N_2 fixation in vitro. There were at least two cases (*nifE* and *nifN*, *nifK* and *nifD*) of two proteins that seemed to require each other for stability in vivo. Regulation of N_2 fixation is apparently complex, and this is reflected by the assignment of regulatory functions to the gene products of *nifA*, *nifL*, *nifK*, *nifD*, *nifH*, and *nifJ*.

Two proteins are required for N_2 fixation in vitro. Nitrogenase (component I) is a tetramer ($\alpha_2\beta_2$) of two polypeptides of 54,000 daltons (54K; β subunit) and 57,000 daltons (α subunit) (8). Nitrogenase reductase (component II) is a dimer of identical polypeptides of 34,000 daltons (8). Component I contains a cofactor of Fe, Mo, and S (FeMo-co) at its active site (16). This cofactor has the ability to restore in vitro the activity of defective nitrogenase proteins from certain mutant strains (13).

Expression of the Nif system in *Klebsiella pneumoniae* requires the enzyme glutamine synthetase (18, 19), Mo (2), and anaerobic conditions (14). Some evidence exists that NH_4^+ prevents Nif function by action at the level of transcription (20), although apparently not at the same site of action as rifampin (3), that is, at RNA initiation.

Several previous publications have reported biochemical characterizations of a number of *nif* mutations in terms of component activity and presence of cross-reactive material for components I and II. These experiments were performed in an attempt both to identify the genes encoding component I and component II structural proteins and to determine the functions of the gene products of other *nif* genes.

St. John et al. (13) tentatively identified *nifD*, *-H*, *-B*, and *-F* as the genes for component I, component II, FeMo-co synthesis, and an electron transport factor, respectively. Dixon et al. (4) and Kennedy (7) added *nifA*, *-K*, and *-E*. The *nifA* product appeared to be be involved in regulation, the *nifK* product was thought to encode the other nitrogenase polypeptide, and the *nifE* product function was unclear. Conclusive identification of the structural genes for components I and II was lacking, as was the physical characterization of other *nif*-associated proteins. A number of other loci have been reported, but in each case the report has been based on only a single mutant strain which was not well characterized biochemically. They include *nifG* (regulatory; 13), *nifL* (regulatory; 7) *nifT* (regulatory; 1), and *nifJ* (unknown; 7).

The presence of amber mutations in 12 of the 14 *nif* genes (the exceptions are *nifK* and *nifH*) indicates that protein products are coded by these genes (11). This report presents two-dimensional polyacrylamide gel electrophoresis analysis of Nif⁻ strains (12). A representative selection of these strains has been further characterized by in vitro nitrogenase assays as measured by acetylene reduction (17). These techniques, coupled with the availability of a large number of genetically well-characterized strains with mutations throughout the *nif* operons, have allowed us to conclusively demonstrate the structural genes for components I and II as well as to determine the physical characteristics and probable biochemical function for many of the protein products of the *nif* genes. These techniques have also allowed us to assign regulatory

and catalytic functions to the products of most of the *nif* genes.

MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* strain M5a1 (UN) was obtained from P. W. Wilson. All strains used in this paper have been described by MacNeil et al. (11).

Chemicals and isotopes. Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), and Coomassie brilliant blue R-250 were obtained from Biorad Inc. (Richmond, Calif.). Ultrapure urea was purchased from Schwarz/Mann Co. (Orangeburg, N.Y.), and the carrier ampholytes for isoelectric focusing were purchased from LKB Instruments, Inc. (Rockville, Md.). Radioactive amino acids were obtained from the Radiochemical Centre (Amersham, England).

Growth of strains. Cell pellets for use in gel electrophoresis were prepared by inoculating 10 ml of K medium (10), containing 0.15% L-histidine as the N source and 2% sucrose, with 0.2 ml of washed, stationary-phase, NH_4^+-grown cells. The test tubes were stoppered, and the gas phase was evacuated and replaced with a helium atmosphere. After 48 h of incubation at 30°C, the cells were centrifuged, the supernatant solution was removed by syringe, and the pellet was frozen anaerobically. When samples for in vitro nitrogenase assays were desired, the same procedure was followed with all volumes 40-fold greater.

In vivo nitrogenase assays. Cells were derepressed as described above, and 0.5 ml was removed to a 10-ml vial containing an He atmosphere. A 0.5-ml amount of 1 atm acetylene was injected, the sample was shaken vigorously at 30°C for 15 min, and the assay was terminated by addition of 0.1 ml of 30% trichloroacetic acid.

In vitro nitrogenase assays. The crude extracts used in all in vitro assays were prepared by breaking cell pellets obtained as described above in 3 ml of 25 mM tris(hydroxymethyl)aminomethane-hydrochloride, 1 mM $MgCl_2$, and 0.3 mg of $Na_2S_2O_4$, pH 7.5, per ml using a French pressure cell operated at 18,000 lb/in^2. The extracts then were centrifuged at 3,000 × *g* for 10 min, and the supernatant solution was assayed the same day. All operations were performed under a N_2 atmosphere. The amount of protein in the extracts was determined by the biuret method (6).

In vitro nitrogenase assays using acetylene reduction were performed with crude extracts both in the presence and absence of added purified nitrogenase components as described previously (15, 17).

In vitro FeMo-co acceptance assays. The nitrogenase present in extracts of certain mutant strains can be activated by addition of purified FeMo-co (16). A strain so activatable would presumably be Nif$^-$ by virtue of a lesion affecting FeMo-co synthesis and contain an otherwise active component I protein.

Extracts were prepared as described above, and assays were performed by the method of Shah and Brill (16) except that an excess of partially purified component II was often added to the assay. This insured that the observed amount of acetylene reduction was not limited by a lack of active component II in the extract and was therefore an accurate reflection of the amount of activatable component I present.

Two-dimensional polyacrylamide gel electrophoresis. The techniques used in performing two-dimensional polyacrylamide gel electrophoresis were previously described (12) with the following modifications. A 10-ml culture was grown as described above, the cells were pelleted by centrifugation, the supernatant solution was removed, and 250 μl of the "lysis buffer" was added. The samples were then subjected to five cycles of freezing (using a dry ice-ethanol bath) and thawing. The sample was centrifuged at 3,000 × *g* for 10 min, and 60 to 100 μl of the supernatant solution was loaded onto the isoelectric-focusing tube gel. The slab gels used in the second (SDS) dimension were 1.5 mm thick, and the height of the stacking gel was 1 cm. The gels were run at 15 mA/gel until the tracking dye reached the running gel, whereupon the current was increased to 35 mA/gel. The power supply was set so that voltage became limiting at a value of 110 V. In the isoelectric-focusing dimension, Triton X-100 was used instead of Nonidet P-40.

Radioactive labeling. Cells were labeled with either ^{14}C uniformly-labeled amino acids, [^{35}S]methionine, or [^3H]leucine. Depending on the experiment, label was added at the start of derepression (when the cells are placed in NH_4^+-deficient media) or after 24 h of growth in the histidine-containing medium described above. When a pulse of labeling was desired, 2 to 20 μCi of labeled amino acids was added anaerobically to a 10-ml derepressing culture. The culture was incubated for 5 min at 30°C, 0.1 ml of unlabeled amino acid (at a 1% concentration) was added anaerobically, and incubation was continued for an additional 5 min. The culture then was chilled and centrifuged at 5,000 × *g* for 5 min, and the cell pellet was frozen in a dry ice-ethanol bath and stored at −20°C.

Autoradiography, fluorography, and staining of gels. Nonradioactive gels were stained for 30 to 60 min in 50% trichloroacetic acid containing 0.2% Coomassie brilliant blue R-250, destained partially with several washes of 7% acetic acid, and destained to completion with 7% acetic acid in 30% ethanol. The gels were then equilibrated with 7% acetic acid in 2% glycerol and dried onto Whatman no. 1 filter paper with a Hoffer slab gel dryer.

Gels which contained ^{14}C- or ^{35}S-labeled amino acids were first fixed for 15 min in 50% trichloroacetic acid, rinsed in distilled water, dried as above, and placed directly in contact with Kodak X-OMat X-ray film (Eastman Kodak, Rochester, N.Y.).

When ^3H-labeled amino acids were used, the technique of fluorography was used (9). The only modifications to the published method were the use of five rinses with dimethyl sulfoxide to remove water and the equilibration of the gels in a dimethyl sulfoxide solution saturated with 2,5-diphenyloxazole. All autoradiography and fluorography was performed with incubation at −70°C.

RESULTS AND DISCUSSION

Identification and characterization of gene products. The *nif* proteins which were visualized upon gel electrophoresis are noted in Fig. 1a. The pattern shown is that of all major

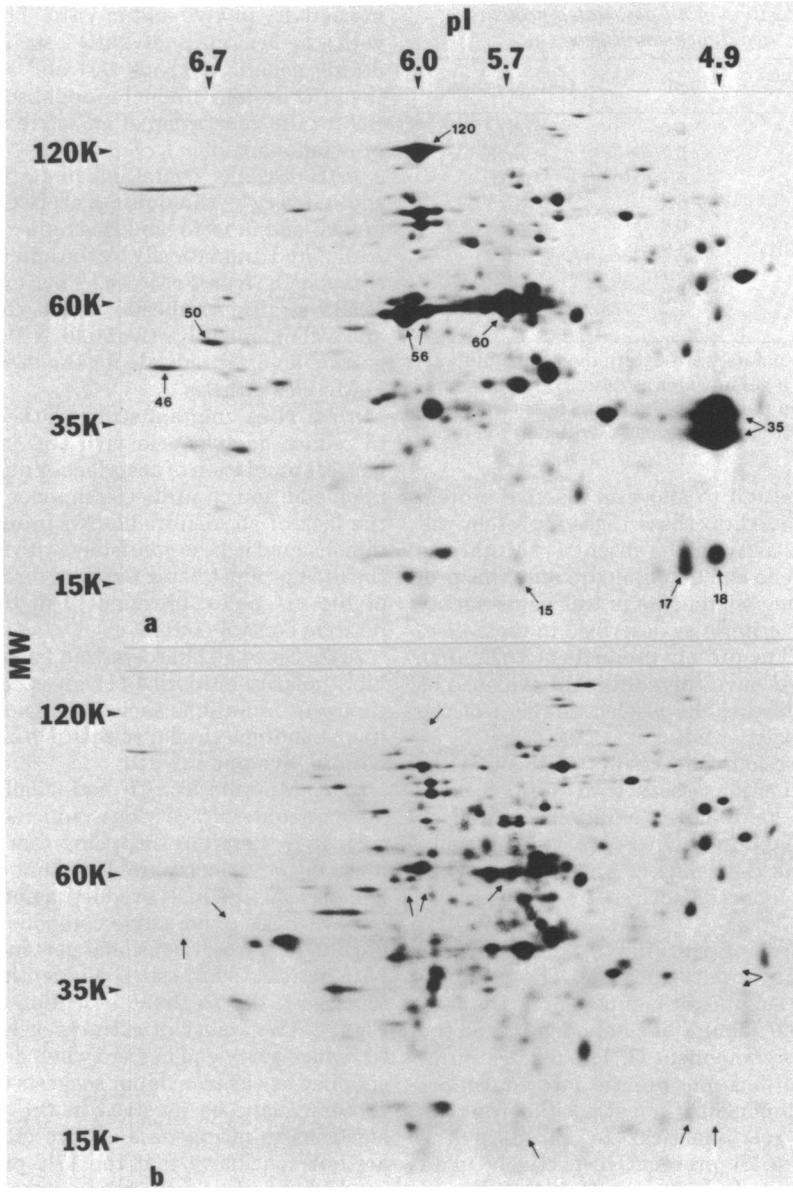

FIG. 1. *nif-associated proteins. (a) UN (nif⁺); (b) UN1234 (nif-4553, a total nif deletion). nif-associated proteins are indicated by arrows and molecular weight (MW). Autoradiography on [^{35}S]methionine-pulsed extracts was used.*

cell proteins from a derepressed Nif⁺ strain. For comparison, Fig. 1b shows the electrophoretic pattern of an identical protein extract from a Nif⁻ strain containing a total deletion of the *nif* genes. Table 1 lists the *nif*-coded proteins, their apparent molecular weight, isoelectric point, and the *nif* gene by which they are encoded. All of these *nif*-coded proteins were absent when the cells were grown in the presence of excess NH_4^+.

Our criteria for associating a protein product with a given gene are the following. (i) Deletions extending into the gene should eliminate the protein as well as the activity. (ii) Insertion mutations, shown genetically to lie within a given gene, should eliminate both the gene product and its activity. (iii) A fraction of point mutations within a gene should eliminate both the protein and the activity. (iv) Some mutations

TABLE 1. *Position of nif-associated proteins on two-dimensional gels*

Protein designation (approx mol wt)	pI[a]	Gene assignment
120K	6.0	*nifJ*
60K	5.7	*nifK*
56K	6.1 (6.0)[b]	*nifD*
50K	6.7	*nifN*
46K	6.8	*nifE*
35K (39K)[b]	4.9	*nifH*
18K	5.1	*nifS*
17K	5.0	*nifF*
15K	5.7	?

[a] Isoelectric points were determined by equilibrating sections of a gel in water as described (12).

[b] Numbers in parentheses refer to minor spots associated with these proteins as described in the text.

might be expected to allow an inactive protein to be synthesized; in these cases a protein will be seen but activity will be absent. (v) Mutations in no other gene should eliminate only this protein. The gene assignments in this paper satisfy most of these criteria as described in the following sections. The protein products of *nifK, nifD, nifH,* and *nifJ* have the additional evidence of mutations affecting the isoelectric point of the appropriate polypeptide.

Table 2 presents the results of the in vitro acetylene reduction assays with purified components and FeMo-co as additions as well as acetylene reduction in vivo with growing cells. Table 3 shows the range of phenotypes as observed with nonradioactive, two-dimensional gels.

Assignments of proteins to the structural genes of components I and II. The 56K protein is the α subunit of component I, the 60K protein is the β subunit of component I, and the 35K protein is component II. The data confirming these identifications are: (i) purified component I and component II ran at positions on two-dimensional gels identical to those of the 56K–60K and 35K proteins, respectively (data not shown); (ii) the presence of 56K–60K and 35K proteins in gels correlated well with the presence of cross-reactive material corresponding to component I and component II (14; R. T. St. John, Ph.D. thesis, University of Wisconsin, Madison, 1973); (iii) the presence of 56K–60K and 35K proteins in gels correlated with component I and component II activity, respectively, in in vitro assays described in this report. For the remainder of this paper, the 56K and 60K proteins, together, will be referred to as component I, and the 35K protein will be referred to as component II.

nifQ. All strains tested containing *nifQ* mutations showed a high level of acetylene-reducing activity in vivo and in vitro. This correlates with the leakiness of NifQ$^-$ strains observed during genetic analysis (11) and suggests that the *nifQ* protein product is not absolutely essential for the generation of an active component I or component II.

nifB. Strains containing mutations in *nifB* showed very low amounts of acetylene reduction in vivo and in vitro, and this reduction could be stimulated dramatically by the addition of either component I or FeMo-co. This indicates that NifB$^-$ strains synthesize active component II and a component I activatable by FeMo-co, suggesting an essential role for the *nifB* product in FeMo-co synthesis.

nifA. NifA$^-$ mutant strains lacked the ability to reduce acetylene in vivo and in vitro. The lack of stimulation of acetylene-reducing activity upon addition of purified components, as well as the lack of all identifiable Nif proteins on two-dimensional gels, is consistent with the model of the *nifA* product being required for derepression of the *nif* genes, presumably in the role of a positive control factor.

nifL. Since all characterized NifL$^-$ strains exhibit polarity onto *nifA* (11), it is not surprising that *nifL* mutations showed a pleiotropic-negative phenotype similar to that of *nifA* mutations both in vivo and in vitro.

nifF. Mutants in *nifF* had small but significant amounts of acetylene-reducing activity in vivo. It is therefore surprising that their activity in vitro was comparable to that of wild type. Apparently, the *nifF* product is not involved in the synthesis of an active component I or component II. Two-dimensional gels indicated that NifF$^-$ strains synthesized all identified Nif proteins with the frequent exception of 17K (see Fig. 2). The ability of extracts of NifF$^-$ strains to reduce acetylene in vitro when dithionite was supplied as electron donor suggests that the 17K protein might be involved in the transport of electrons to nitrogenase in vivo (13). The data are not conclusive that the 17K protein is the product of the *nifF* gene, since no charge changes in that protein were observed. The possibility remains that the 17K protein is merely induced to higher levels of synthesis by the product of the *nifF* gene.

nifM. NifM$^-$ strains appeared to have low levels of acetylene-reducing activity in vivo. In vitro, the addition of component II greatly stimulated acetylene reduction. Two-dimensional gels showed normal amounts of component I and low levels of component II (especially the 39,000-dalton spot) when compared with wild type. The presence of significant amounts of inactive component II even in *nifM* Mu insertions argues that *nifM* is not the structural gene

TABLE 2. *Component activities of selected mutant strains*

Allele	Strain	Sp act[a]					
		In vivo	In vitro				
			No addition	+ Component I	+ Component II	+ FeMo-co	+ FeMo-co and component II[b]
	Wild type	320	410	400	490	400	480
nifQ4969	UN2138	91	57	88	52	ND	54
nifQ4970	UN2139	73	78	117	93	ND	60
nifB4106	UN106	1	4	27	5	ND	114
nifB4408[c]	UN1088	1	0	42	0	ND	71
nifB4691	UN1655	1	0	90	0	57	83
nifA4477[c]	UN1158	0	0	0	0	0	0
nifA4739	UN1770	1	0	0	0	0	ND
nifA4790	UN1821	2	0	0	0	0	ND
nifL4373	UN1030	0	0	0	0	0	0
nifL4478[c]	UN1159	0	0	0	0	ND	0
nifF4445[c]	UN1126	11	242	251	389	ND	220
nifF4692	UN1656	9	258	103	277	238	ND
nifF4758	UN1789	8	96	44	132	130	ND
nifM4093	UN93	2	10	4	143	3	ND
nifM4425[c]	UN1105	12	0	0	43	ND	39
nifM4733	UN1697	10	1	1	54	ND	ND
nifV4092	UN92	210	315	298	315	281	ND
nifV4423[c]	UN1103	24	17	90	35	15	ND
nifV4944	UN1990	40	127	242	160	97	ND
nifV4945	UN1991	61	181	251	203	151	ND
nifS4282	UN282	9	4	5	21	ND	11
nifS4389	UN1046	7	2	4	12	0	9
nifS4882	UN1925	6	1	2	24	0	22
nifN4406[c]	UN1086	12	0	36	2	ND	92
nifN4687	UN1651	3	0	32	0	38	ND
nifN4753	UN1784	5	1	49	0	ND	132
nifE4432[c]	UN1112	7	1	7	4	ND	143
nifE4669	UN1618	2	0	6	3	2	41
nifE4701	UN1665	0	0	54	0	ND	23
nifK4454[c]	UN1135	0	0	7	0	ND	0
nifK4466[c]	UN1147	0	0	20	0	ND	0
nifK4671	UN1635	1	0	33	0	0	ND
nifK4768	UN1799	0	0	71	0	ND	0
nifD4481[c]	UN1162	0	0	0	0	ND	0
nifD4685	UN1649	6	7	78	9	ND	6
nifD4697	UN1661	1	0	23	0	0	ND
nifH4677	UN1641	27	0	0	164	0	0
nifH4726	UN1690	2	0	0	14	ND	ND
nifH4764	UN1795	0	0	0	6	ND	0
nifH5118[c]	UN2545	0	0	0	0	ND	0
nifJ4456[c]	UN1137	12	51	90	76	80	ND
nifJ4727	UN1691	9	80	129	85	ND	64
nifJ4803	UN1834	5	12	31	28	ND	ND

[a] Specific activities are expressed as nanomoles of acetylene reduced per minute per milligram of protein. ND, Not determined.

[b] FeMo-co acceptance assays were performed either in the presence or absence of added component II. The latter assay often gives a lower estimate of the amount of FeMo-co-activatable component I because of limiting component II. For this reason, the assays performed with added component II are a more reliable estimate of activatable component I.

[c] These strains contain Mu insertions in *nif*.

for component II but rather is involved in a processing step necessary to generate an active component II. Either this processing step performed by the *nifM* product fails to alter the charge of component II or the modification is labile under the conditions used in electrophoresis, since component II from NifM$^-$ strains was identical in electrophoretic behavior to that

TABLE 3. *Levels of nif-associated proteins in unlabeled extracts*

Gene affected	Relative abundance[a]											Strain (allele)[e]
	No. examined		60K[b]	56K[b]	35K[c]	120K	46K	50K	18K[d]	17K[d]	15K[d]	
	Mu insertions	Point mutations										
None			++	++	++	++	++	++	++	++	++	
Q		2	++	++	++	++	++	++	++	++	++	UN2138 (*nifQ4969*), UN2139 (*nifQ4970*)
B	4	14	+	+	++	++	++	++	++	++	++	UN1655 (*nifB4691*), UN1088 (*nifB4408*), UN106 (*nifB4106*)
A	5	23	−	−	−	−	−	−	−	−	−	UN1158 (*nifA4477*), UN1770 (*nifA4739*), UN1821 (*nifA4790*)
L	1		−	−	−	−	−	−	−	−	−	UN1159 (*nifL4478*)
F	2	8	++	++	++	++	++	++	++	−	++	UN1656 (*nifF4692*), UN1126 (*nifF4445*)
F	1	3	++	++	++	++	++	++	++	+	++	UN1789 (*nifF4758*), UN31 (*nifF4031*), UN1110 (*nifF4430*), UN1777 (*nifF4746*)
M		3	++	++	+	++	++	++	−	++	+	UN1654 (*nifM4690*)
M	1	6	++	++	+	++	++	++	−	++	−	UN93 (*nifM4093*), UN1105 (*nifM4425*), UN1697 (*nifM4733*)
V	1	1	++	++	++	++	++	++	++	++	++	UN1986 (*nifV4940*), UN1116 (*nifV4436*)
V	2	4	++	++	+	+	++	++	+	++	−	UN1153 (*nifV4472*), UN1103 (*nifV4423*), UN92 (*nifV4012*), UN1990 (*nifV4944*), UN1991 (*nifV4945*), UN1140 (*nifV4459*)
S	4	2	−	−	+	+	−	−	−	++	−	UN1145 (*nifS4464*), UN1097 (*nifS4417*), UN1098 (*nifS4418*), UN1138 (*nifS4457*), UN2001 (*nifS4955*)
S		3	+	+	+	−	−	−	−	++	−	UN282 (*nifS4282*), UN2007 (*nifS4961*), UN2013 (*nifS4967*)
N	9	8	++	++	++	++	−	−	++	++	+	UN1784 (*nifN4753*), UN1651 (*nifN4687*), UN1086 (*nifN4406*)
E	8	8	++	++	++	++	−	−	+	++	+	UN1618 (*nifE4669*), UN1112 (*nifE4432*), UN1665 (*nifE4701*)
K		5	+	+	++	++	++	++	++	++	−	UN12 (*nifK4012*), UN72 (*nifK4072*), UN178 (*nifK4178*), UN194 (*nifK4194*), UN238 (*nifK4238*)
K	2	8	−	−	+	+	+	+	−	+	−	UN1782 (*nifK4751*), UN1635 (*nifK4671*), UN1147 (*nifK4466*), UN1799 (*nifK4768*), UN1791 (*nifK4760*)
K		7	−	−	++	++	++	++	++	++	−	UN115 (*nifK4115*), UN195 (*nifK4195*), UN1696 (*nifK4732*), UN1782 (*nifK4751*), UN1790 (*nifK4759*)
D		12	++	++	++	++	++	++	++	++	+	UN1661 (*nifD4697*), UN1649 (*nifD4685*), UN1772 (*nifD4741*)
D	7		−	−	w	w	w	w	−	+	−	UN1162 (*nifD4481*)
D		3	−	−	++	++	++	++	+	+	−	UN1673 (*nifD4709*), UN1687 (*nifD4723*), UN1662 (*nifD4698*)
H	3		−	−	−	−	+	+	−	−	−	UN2545 (*nifH5118*), UN2546 (*nifH5119*), UN2558 (*nifH5131*)
H		6	++	++	++	++	++	++	+	++	−	UN60 (*nifH4060*), UN67 (*nifH4067*), UN1044 (*nifH4387*), UN116 (*nifH4116*), UN85 (*nifH4085*)
H		3	−	−	++	w	−	−	−	−	−	UN1678 (*nifH4714*), UN1795 (*nifH4764*), UN111 (*nifH4111*)
H		13	+	+	+	+	+	+	+	−	−	UN84 (*nifH4084*), UN1684 (*nifH4720*), UN1641 (*nifH4677*), UN1041 (*nifH4384*)
J		20	++	++	++	++	++	++	++	−	++	UN81 (*nifJ4081*), UN68 (*nifJ4068*), UN1666 (*nifJ4702*), UN1769 (*nifJ4738*)
J	7	16	++	++	++	−	++	++	++	−	++	UN1691 (*nifJ4727*), UN1137 (*nifJ4456*), UN1834 (*nifJ4803*)

[a] A wild-type level is arbitrarily set at ++ for each protein, so that amounts of protein indicated are relative. −, No spot was visible; w, the spot was weakly visible.

[b] Component I.

[c] Component II.

[d] The 18K, 17K, and 15K proteins are often difficult to score so that a significantly diminished amount would be scored "−" with some frequency.

[e] Strains presented were selected as representative and typically include those strains appearing in Table 2. Strains UN1086-UN1164, UN2545, UN2546, and UN2558 contain Mu insertions in *nif*, whereas all other strains contain *nif* point mutations.

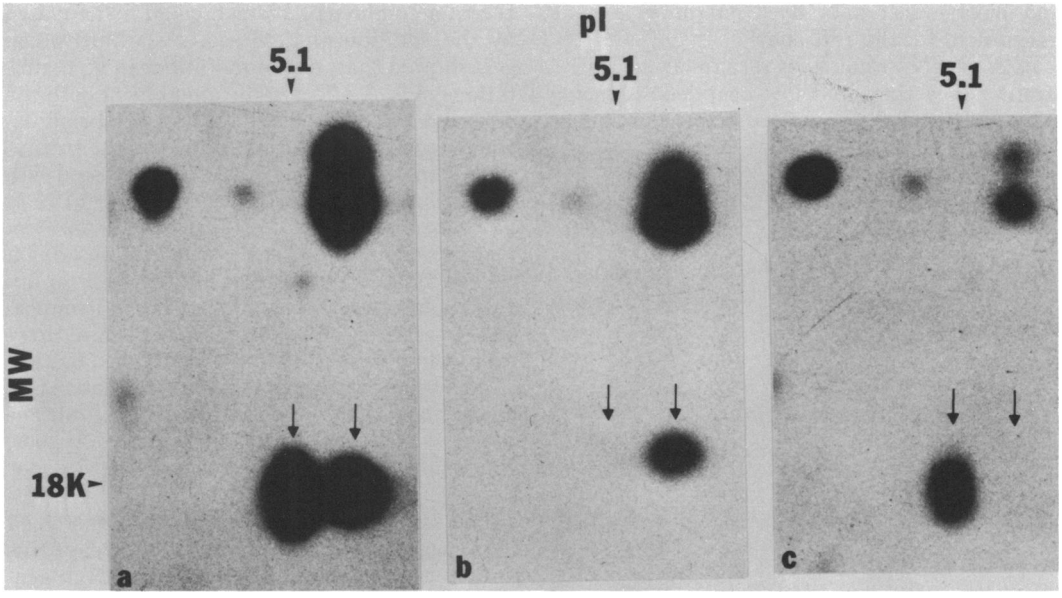

FIG. 2. *17K and 18K. (a) The position of the 17K and 18K proteins from the wild type. (b) The absence of the 17K protein in UN1126 (nifF4445). (c) The absence of the 18K protein in UN1145 (nifS4464). Arrows indicate the positions of the 17K and 18K proteins from the wild type. Extracts were pulsed with [^{3}H]leucine. MW, Molecular weight.*

observed in NifM$^+$ strains. The identification of a protein product of *nifM* is discussed below in the section of *nifS*.

nifV. Like NifQ$^-$ strains, NifV$^-$ strains had rather high levels of acetylene-reducing activity both in vivo and in vitro. The *nifV* product is apparently not essential for the synthesis of active component I or component II, nor does it seem to be involved in the control of synthesis of other observable Nif proteins as evidenced by the gel data.

nifS. NifS$^-$ strains showed the anomalous behavior of leakiness of plates lacking fixed N sources (11), while lacking significant amounts of acetylene-reducing activity when extracts were prepared. The apparent regulatory effect (see in Table 3) on the synthesis of Nif proteins might be direct, by affecting transcription, or indirect. Since it seems possible that component I and component II might have some involvement in derepression of several of the Nif proteins (see below), an indirect effect on derepression might be seen in mutations which failed to produce components capable of derepression. The results of in vitro assays suggest that NifS$^-$ strains containing point mutations have low levels of active component I but lack active component II. Since two-dimensional gels of NifS$^-$ strains revealed low amounts of protein for both component I and component II, it appears possible that inactive component II is being synthesized in these strains. The phenotype of NifS$^-$ strains might be explained if *nifS* encoded a protein which was involved in component II processing so that a component II was synthesized which was inactive and relatively unstable, thus affecting the derepression of the rest of the system.

The identification of the protein products of *nifM, nifV,* and *nifS* is complicated by the polarity of transcription, the relatively low amounts of the proteins in question, the lack of charge altered proteins, and a possible interaction among the products of these genes. While the 18K protein was apparently missing in unlabeled extracts from a variety of strains, in pulse-labeled extracts the spot was present in all but *nifS* Mu-insertion mutations (see Fig. 2) and the pleiotropic *nifA* and *nifL* mutations. This spot was weak in UN2007, a strain with the *nifS4961* point mutation. This is suggestive, but not conclusive, evidence that the 18K protein is the product of *nifS*. The 15K spot was similarly difficult to score in unlabeled extracts. Unfortunately, while it was absent in UN1105 (*nifM4425,* a Mu insertion; see Fig. 3), it was present in UN1145 (*nifS4464,* a Mu insertion) and AN1986 (*nifV4940,* a frameshift mutation), both of which have been shown genetically to be polar onto *nifM* (11). This inconsistency between genetic

and biochemical data does not allow a gene assignment for the 15K spot.

nifN. NifN$^-$ strains were similar to NifB$^-$ strains since they had low acetylene-reducing activity in vivo and possessed active component II and a component I which could be activated by the addition of FeMo-co. Two-dimensional gels showed that, unlike mutations in *nifB*, mutations in *nifN* affected the amounts of both the 46K and 50K proteins. By using unlabeled extracts (and therefore examining only the steady-state levels of proteins), all 33 strains tested with point mutations and Mu insertions in *nifN* or *nifE* eliminated both the 46K and 50K proteins. Unfortunately, the absence of mutations which caused only a single spot of the pair or an electrophoretically altered spot made it impossible to assign proteins to structural genes. However, when several NifN$^-$ and NifE$^-$ mutant strains were derepressed, pulsed with label, and quickly harvested, it was observed that mutant strains with lesions in *nifN* lacked the 50K spot, whereas *nifE* mutant strains lacked the 46K spot (Fig. 4).

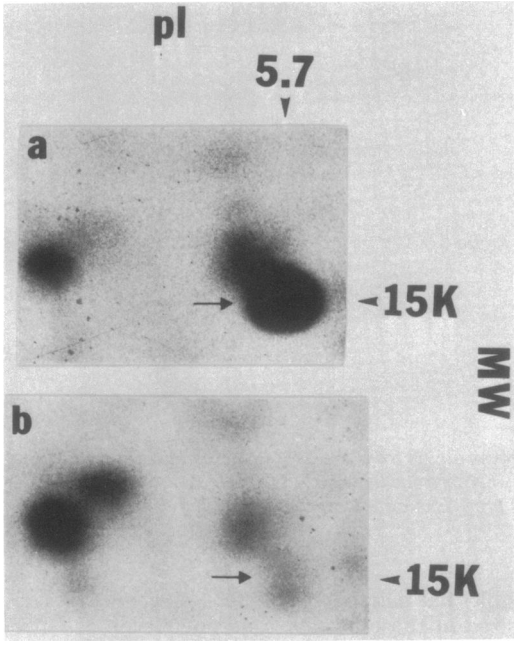

FIG. 3. *15K. (a) The position of the 15K protein from the wild type. (b) The absence of the 15K protein in UN1105 (nifM4425). Arrows indicate the position of the 15K protein from the wild type. Extracts were pulsed with [^{3}H]leucine. MW, Molecular weight.*

The absence of the 46K protein in unlabeled cells suggests that the steady-state level of this protein is low in nifN$^-$ strains even though synthesis is apparently occurring. This suggests that the 46K protein (the product of the *nifE* gene) is unstable in the absence of the *nifN* gene product, the 50K protein, and vice versa. The above information is consistent with *nifN* encoding the 50K protein and *nifE* coding for the 46K protein. The in vitro assays suggest that the *nifN* product is an essential step in FeMo-co synthesis.

nifE. NifE$^-$ strains were identical to NifN$^-$ strains both in vivo and in vitro except that they seemed to have a consistently lower amount of active component II. As described above, *nifE*

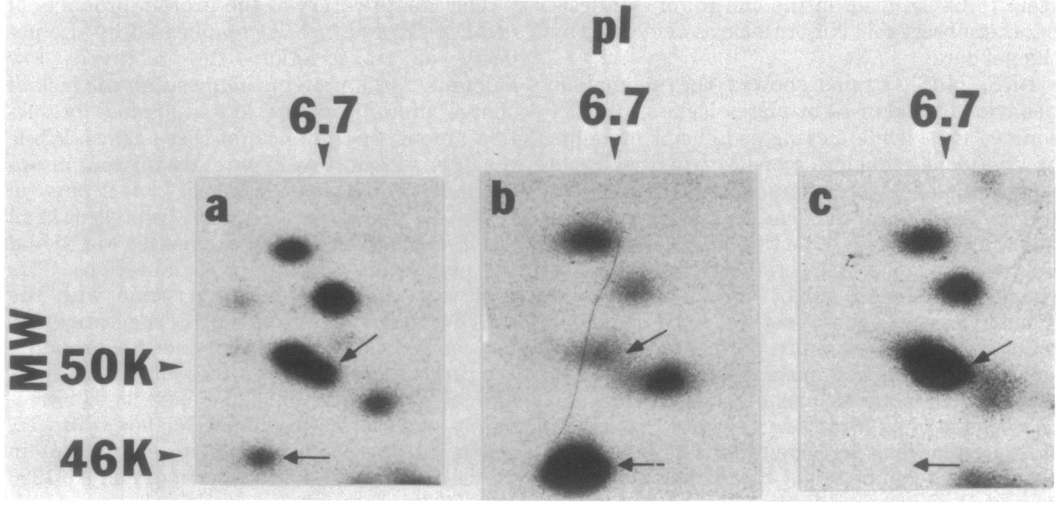

FIG. 4. *46K and 50K. (a) The 46K and 50K proteins from the wild type; (b) The absence of the 50K protein in UN1091 (nifN4411); (c) Absence of the 46K protein in UN1618 (nifE4669). Arrows indicate the positions of both proteins from the wild type. Extracts were pulsed with [^{3}H]leucine.*

appeared to have 46K protein as its product, and the in vitro assays are strongly suggestive of an essential involvement of the *nifE* protein in FeMo-co synthesis.

nifK. NifK$^-$ strains possessed negligible amounts of acetylene-reducing activity in vivo, and their extracts were activated only by the addition of active component I. Since most of the NifK$^-$ strains lacked both subunits of component I (60K and 56K protein) on gels and since an NifK$^-$ strain, UN72 (*nifK4072*), was characterized which showed an electrophoretically altered 60K protein (Fig. 5), *nifK* is apparently the structural gene for the protein, the acidic subunit of component I. Mutations in *nifK* typically caused a reduction in the levels of many of the observable Nif proteins, suggesting some involvement of its protein product in the mechanism for full derepression of the *nif* genes. The lack of the 56K protein in unlabeled extracts of NifK$^-$ strains lacking the 60K protein suggests that the 56K subunit of component I might be unstable in vivo in the absence of the 60K subunit. When UN1135 (*nifK4454*), a strain containing a Mu insertion of *nifK*, was pulse-labeled, only the 56K subunit of component I was visible along with the other identified Nif proteins. This suggests that the lack of both component I proteins in *nifK* (and *nifD*) mutants is due to instability of each component in the absence of the other.

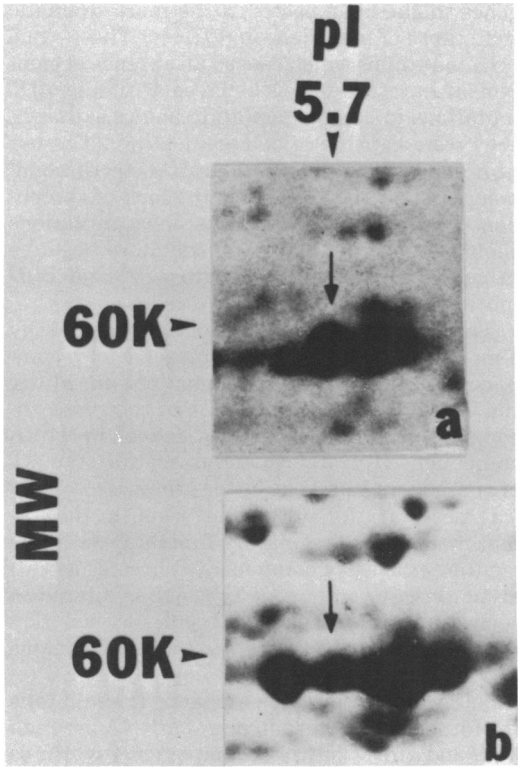

FIG. 5. *60K. (a) The 60K protein from the wild type. (b) A charge-altered 60K protein from UN72 (nifK4072). The altered 60K polypeptide is visible to the left of the normal position. The polypeptide which remains at the wild-type position is apparently not nif associated (Fig. 1). The arrows indicate the position of the 60K protein from the wild type. Unlabeled extracts were used. MW, Molecular weight.*

nifD. NifD$^-$ strains are similar to those of NifK$^-$ with the exception that Mu insertions in *nifD* result in even less component II on gels and in in vitro assays. This is consistent with the curious complementation pattern seen in these strains, that is, negative for *nifK, nifD,* and *nifH* in complementation analyses when growth on N_2 is assayed (11). Strains containing a Mu insertion in *nifD* are therefore only poorly activated by the addition of component I in in vitro assays since they are rather low in component II. Extracts of strains containing point mutations in *nifD* do not have this difficulty and are complemented well by component II addition.

The 56K protein appeared in gels as two spots of molecular weight 56,000 and pI of 6.1 and 6.0. The pair was observed consistently and is unlikely to be an artifact of the electrophoretic system, although the reason for two spots is unclear. The more basic of the pair was present in a significantly higher level (Fig. 1). We feel that they are both forms of the same protein encoded by *nifD* for the following reasons. All seven strains tested with Mu insertions and 3 of 15 strains with point mutations in *nifD* lacked both spots and 9 strains with point mutations in *nifD* showed an alteration in the isoelectric point of both spots in an identical fashion (examples are given in Fig. 6). The existence of a number of NifD$^-$ strains with charge alterations in the 56K subunit is conclusive evidence that *nifD* codes for the basic subunit of component I (56K). As is the case of NifK$^-$ strains, the diminished amount of Nif proteins both on gels and assays suggests that component I might have some involvement in the derepression mechanism.

nifH. NifH$^-$ strains had low amounts of acetylene-reducing activity in vivo and a variety of phenotypes on gels. They lacked activity for component II. The criteria for identification of the 35K protein as the *nifH* product are the following. The 35K protein appears on gels as two protein spots of apparent molecular weight of 35,000 and 39,000, pI = 4.9. The reason for two spots is unknown, but all tested strains had the two spots in a comparable ratio (with the exception of NifM$^-$ mutants mentioned above). Both spots were present in pulsed gels, and both

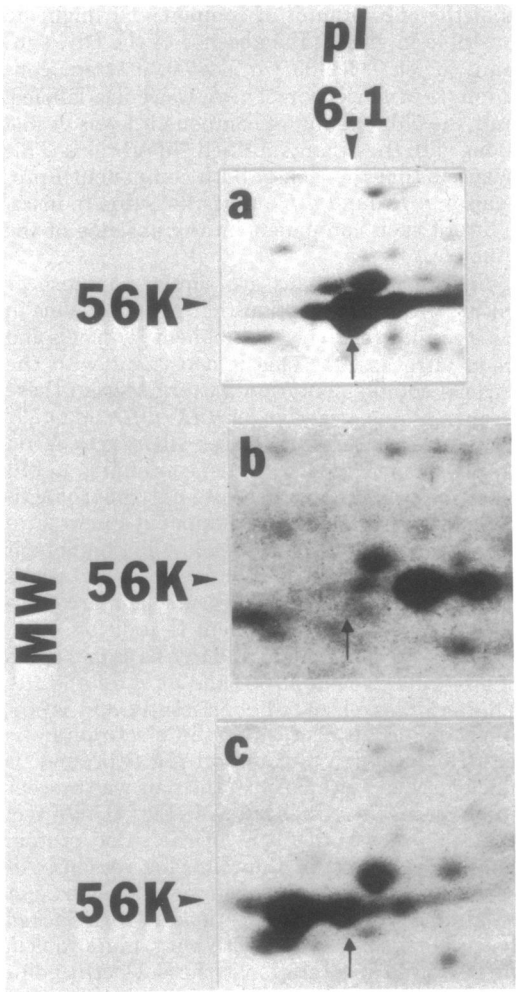

FIG. 6. *56K. (a) The 56K protein from the wild type. (b and c) Charge-altered 56K proteins from UN79 (nifD4079) and UN1683 (nifD4719), respectively. The arrows indicate the position of the 56K protein from the wild type. Unlabeled extracts were used. MW, Molecular weight.*

were similarly affected in their electrophoretic behavior by mutations in nifH. Thirteen tested NifH$^-$ mutants had both of these spots at a position significantly different from the wild-type position. Two examples are shown in Fig. 7. Eleven out of 13 of these strains had an alteration in the SDS dimension. As in the case of 56K, the reason for two spots is unclear.

A large number of point mutations in *nifH* failed to allow high levels of any observable Nif protein to be seen in steady-state conditions. It is unlikely that this was due solely to transcriptional polarity for the following reasons. (i) No mutant strains were characterized which lacked detectable component II on gels yet contained more than a trace amount of component I protein. (ii) Some NifH$^-$ strains which contained normal amounts of inactive component II of apparently the correct molecular weight had dramatically reduced amounts of component I protein of gels. For such a large "polarity" effect to be due to transcriptional polarity, one would expect a nonsense or frameshift mutation in the promoter-proximal portion of the *nifH* gene, yet this cannot be the case where large amounts of normal-size component II is visible. (iii) *nifH* mutations cannot be transcriptionally polar onto both *nifK* and *nifD* as well as *nifJ,* since it lies in the middle of these genes. (iv) Many *nifH* mutations caused a lack of the 46K and 50K proteins (products of *nifN* and *nifE*) which was more dramatic than that observed in *nifD* and *nifK* mutations, though these latter genes are located immediately adjacent to *nifN* and *nifE* and transcriptional effects of mutations in these genes might be expected to be more dramatic than those of mutations in *nifH.* (v) These same *nifH* mutations which caused an absence of component I protein on gels behaved as simple *nifH* mutations in complementation analysis; that is, they were capable of complementing all tested *nifK* and *nifD* point mutations. If transcriptional polarity were the reason for the lack of component I protein in these strains, these mutations should be *cis* dominant and therefore negative in complementation tests with *nifK* and *nifD* mutation.

The above observations can be explained by a model which demands the presence of a component II with "regulatory function" for either the transcription or translation of the genes encoding component I or by a model in which component II protein is necessary for stability of component I and the latter protein is in some way necessary for full derepression. In the former model, this "regulatory function" would be another activity of component II besides its catalytic one. A component II which is altered in such a way that it has lost regulatory function might still be visible on gels, but would be unable to make normal amounts of component I. Presumably this action of component II could take the form of an antiterminator of mRNA between *nifH* and *nifD.* Control of component I synthesis by component II could be a useful mechanism by which the correct ratio of the two components is produced. It is known that an excess of component I will inhibit nitrogen fixation (5). The latter model gains some support from the results of gels performed by using extracts of UN1041 (*nifH4384*) and UN1657 (*nifH4693*). With un-

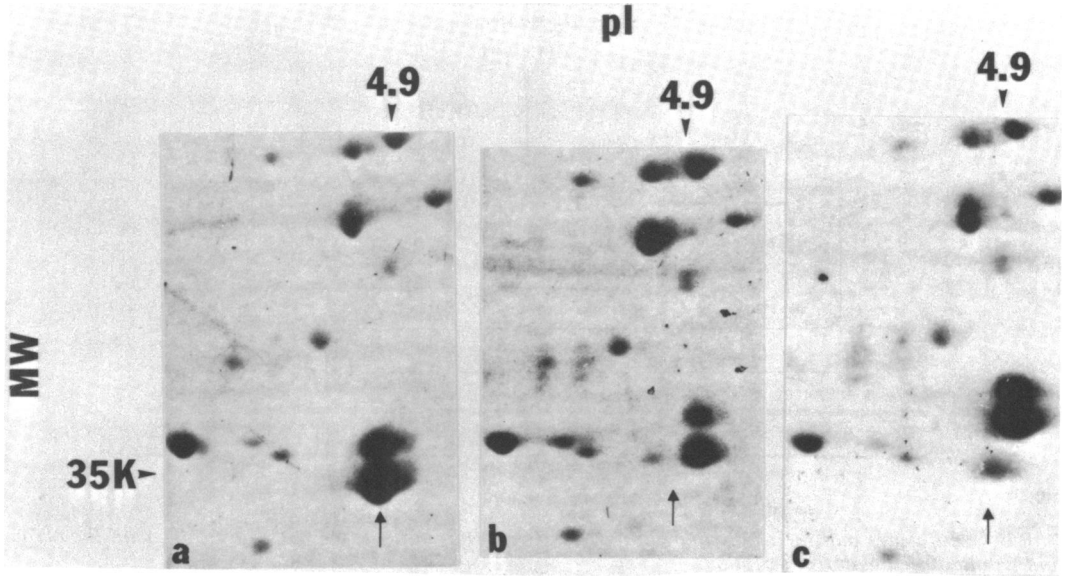

FIG. 7. *35K. (a) The 35K protein from the wild type. (b and c) Altered 35K proteins in UN215 (nifH4215) and UN1 (nifH4001), respectively. In both cases, the alterations affect both the charge and the apparent molecular weight (MW). The arrows indicate the position of the 35K protein spots from the wild type. Unlabeled extracts were used.*

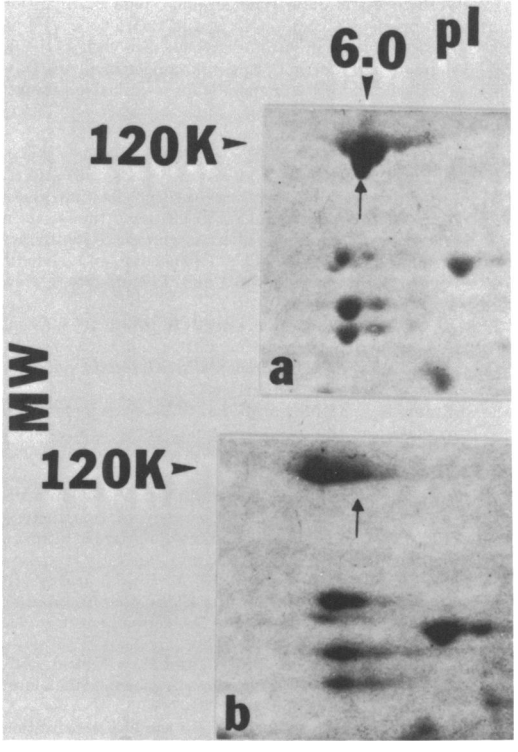

FIG. 8. *120K. (a) The 120K protein from the wild type. (b) A charge-altered 120K protein with strain UN88 (nifJ4088). The arrows indicate the position of the 120K protein from the wild type. Unlabeled extracts were used. MW, Molecular weight.*

labeled extracts, these strains showed no visible component I, whereas pulse-labeled extracts appeared to contain component I, suggesting that stability is the problem with component I in those *nifH* strains.

nifJ. NifJ$^-$ strains, like NifF$^-$ strains, have low but detectable levels of acetylene-reducing activity in vivo and very high activity in vitro. The identification of the 120K protein as a product of the *nifJ* gene is based on the following. All seven tested strains with Mu insertions in *nifJ* and 16 of 36 tested strains with point mutations in *nifJ* lacked the 120K protein. More conclusively, eight strains carrying point mutations in *nifJ* had a 120K protein of altered pI (Fig. 8). Mutations in no other gene resulted either in an alteration of the electrophoretic mobility of the 120K protein or in the exclusive absence of the 120K protein. The data indicate strongly that *nifJ* encodes the 120K protein. The failure of the addition of component I or component II to stimulate activity along with the high level of activity without component addition suggests that the *nifJ* product (120K) is not essential for the generation of active component I or component II. Since the *nifJ* product is not necessary for acetylene reduction in vitro,

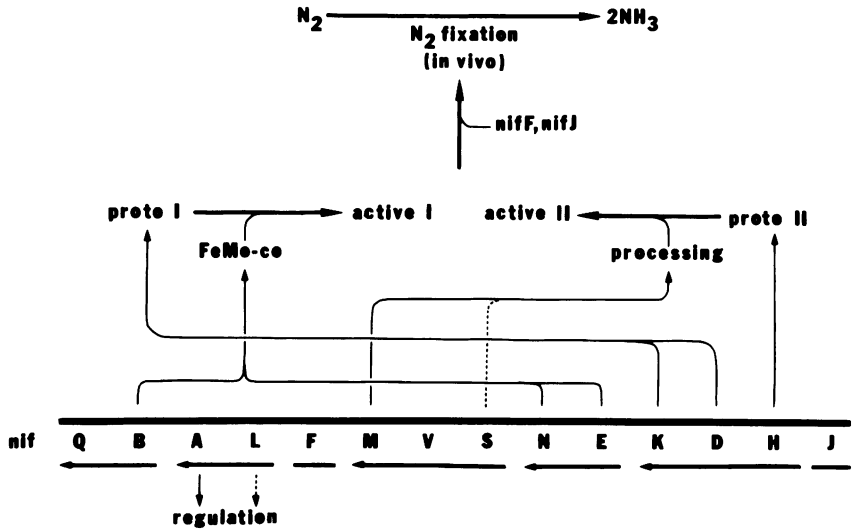

FIG. 9. *nif genes and their functions. Lines below the genes indicate mRNA transcripts, with the arrows indicating direction of transcription.*

is not required for the formation of active component I and component II, and is present at high levels in the cell (see Fig. 1), it is an element required for function of N_2 fixation in vivo. Virtually all NifJ$^-$ strains lacked significant amounts of the 17K protein, so that 120K might act to regulate either the synthesis or stability of the 17K protein.

In summary, the major points of this paper are the following. (i) The structural genes for components I and II have been conclusively identified. (ii) Nine *nif*-specific polypeptides have been identified, eight of which can be assigned to a *nif* gene and all of which are NH_4^+ repressed. (iii) There are at least three genes (*nifB, nifE, nifN*) whose products are involved in FeMo-co synthesis. (iv) The *nifM* gene product (and perhaps that of *nifS*) is involved in the formation of an active component II. (v) At least two cases (*nifE-nifN, nifK-nifD*) exist where complexes of nonidentical polypeptides are necessary for the stability of these polypeptides. (vi) Regulatory functions have tentatively been assigned to *nifA, infL,* (*nifK-nifD*), *nifH,* and *nifJ.* (vii) The products of *nifJ* and *nifF* were required for acetylene-reducing activity in vivo, but not in vitro. (viii) The patterns of the *nif*-coded proteins in mutant strains are consistent with the genetically determined polarity of the polycistronic messages (11). The results are summarized in Fig. 9.

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