JOURNAL OF BACTERIOLOGY, Mar. 1975, p. 759-765
Vol. 121, No. 3
Copyright © 1975 American Society for Microbiology
Printed in U.S.A.

Biochemistry and Genetics of *Klebsiella pneumoniae* Mutant Strains Unable to Fix N_2

RICHARD T. ST. JOHN,[1] H. MARK JOHNSTON,[2] C. SEIDMAN, D. GARFINKEL, JOYCE K. GORDON, VINOD K. SHAH AND WINSTON J. BRILL*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 22 November 1974

Selected mutant strains of *Klebsiella pneumoniae* that are unable to fix nitrogen have been characterized according to nitrogenase component activity as well as antigenic cross-reacting material. The lesions in these strains have been mapped by transduction, and the results indicate that there are at least five genes specifically responsible for nitrogen fixation in vivo. Besides genes that specify the structure of the two nitrogenase components, there is a gene for a factor that is required for component I activity and a gene that codes for a factor possibly involved in electron transport to component II. A mutation in another site does not allow the organism to produce either of the nitrogenase components. All of these genes are co-transducible with the gene that specifies the structure of histidinol dehydrogenase.

Several laboratories have isolated mutant strains of N_2-fixing bacteria to understand more about the important process of converting atmospheric N_2 to NH_4^+ (8, 10, 21, 24, 26). *Azotobacter vinelandii* and *Klebsiella pneumoniae* mutant strains have been obtained with lesions in structural genes (2, 21, 26) and in genes that have a role in control of nitrogenase synthesis (11, 22). Derepressed mutant strains (i.e., those that produce nitrogenase in the presence of excess NH_4^+) have been isolated by reverting certain mutant strains that do not produce either of the two nitrogenase component proteins (2, 11). A strain of *A. vinelandii* was isolated that produces very high levels of component II, but no component I (22). Experiments with electron paramagnetic resonance spectroscopy of whole cells of mutant strains indicate (21) that the $g = 3.65$ signal is caused by an active site in component I (g is the spectroscopic splitting value).

An interesting form of regulation involving genes for N metabolism came from studies of glutamine synthetase (EC 6.3.1.2), an enzyme that plays a role in transcription of genes involved with proline or histidine degradation in *Klebsiella aerogenes* (17, 31). Use of glutamate analogues that inhibit glutamine synthetase and glutamate synthase alters the normal control of nitrogenase synthesis (12). Streicher et al. (28) and Tubb (29) have shown that glutamine synthetase is necessary for expression of the *nif* genes in *K. pneumoniae*. Studies on the regulation of N_2 fixation in *K. pneumoniae* indicate that molybdenum is required for the synthesis of both protein components (3) and that nitrogenase synthesis is repressed by aeration (19). Tubb and Postgate (30) showed that NH_4^+ in the medium prevents transcription of the *nif* genes. The designation of genes specifically involved with N_2 fixation is *nif; his*, histidine biosynthesis; *gnd*, gluconate-6-phosphate dehydrogenase; *rfb*, cell wall biosynthesis. Nif is the phenotype designation for ability to fix N_2; His is the phenotype designation for the ability to synthesize histidine.

Genetic analysis of mutant strains should be very useful for understanding N_2 fixation. Sen and Sen (20) reported that transformation can be used with *A. vinelandii*, but we have been unable to confirm their results. An important breakthrough was the discovery by Streicher et al. (26) that phage P1 is capable of generalized transduction in *K. pneumoniae*. This phage commonly is used for transduction analyses in *Escherichia coli*. Nitrogenase genes are co-transducible with the genes required for the structure of the enzymes of histidine biosynthesis. This was confirmed by Dixon and Postgate (8) by means of conjugation mediated by a drug resistance transfor factor. Streicher et al. (26, 27) showed that most *nif* mutations are linked in several clusters near the *his* operon. They also described several *nif* mutations unlinked to *his*.

[1] Present address: Department of Biochemistry, Brandeis Univ., Waltham, Mass. 02154.

[2] Present address: Department of Molecular Biology, Univ. of California, Berkeley, Calif. 94720.

By deletion analysis, Cannon et al. (5) indicated that the order of genes in a N_2-fixing *E. coli-K. pneumoniae* hybrid strain is *rfb nif gnd his*. On the other hand, Shanmugam et al. (23) determined that the order in *K. pneumoniae* is *rfb gnd his nif*.

We have isolated several hundred mutant strains of *K. pneumoniae* and have classified them according to techniques that we first established with *A. vinelandii* (4, 21). Properties examined in these strains include component activity, antigenic cross-reacting material, and Fe-stain reaction on polyacrylamide gels. The work reported here involves genetic mapping of selected mutant strains that are unable to grow with N_2 as the sole N source.

MATERIALS AND METHODS

Organisms used and media. The organism used is *K. pneumoniae* M5al obtained from P. W. Wilson. The medium described by Yoch and Pengra (33) was used as the basal medium. When excess NH_4^+ was required, 400 μg of N per ml was added as ammonium acetate. When L-histidine or L-histidinol was required, 10 μg/ml was added. Phage Plkc, obtained from W. H. McClain, was used for transduction. Mutant strains were isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (1) and penicillin selection (18). The assignment *hisD* to strains is based on the fact that these mutations allow the organism to grow in the presence of L-histidine but not in the presence of L-histidinol. Genetic nomenclature follows the suggestions of Demerec et al. (7). Chemicals used were of analytical grade, available commercially.

Growth of the organisms and assays. Mutant strains were derepressed by the same method used for derepression of the wild type (3). Preparation of extracts and assays for component activity by acetylene reduction and for component cross-reacting material have been described (3, 21). Acid treatment of component I to produce the activating factor and incubation of extracts with the activating factor has been described (14).

Transduction. Modifications of techniques for genetic analysis with phage P1 and selection and scoring of Nif$^+$ or His$^+$ transductants have been described previously (26, 32). Selection and scoring of Nif$^+$ recombinants was done on plates containing basal medium and purified agar (Difco) containing no fixed N source.

RESULTS

Phenotypes of Nif mutants. Mutant strains that are analyzed in this report are described in Table 1. These strains grew as well as the wild type in media containing excess NH_4^+, but were unable to grow with N_2 as the sole N source. All strains were revertible, suggesting that the lesion in the *nif* gene is a single mutation. Mutations *nif-4026*, *4106*, *4109*, and *4113* yielded the phenotype of inactive component I and active component II ($I^- II^+$). Antigenically-detectable component I still is produced by strains UN106, UN109, and UN113. The mutations of strains UN83 and UN116 caused the phenotype for activity to be $I^+ II^-$. Mutant strain UN116 synthesized an inactive component II, whereas component II from strain UN83 was not recognizable by antiserum against component II. A pleiotropic-negative phenotype was seen with strain UN179. Strain UN66 exhibited activity for both protein components when assayed by acetylene reduction or N_2 fixation in vitro, but was unable to grow in media in which N_2 was the only N source.

Activating factor. Nagatani et al. (14) have shown previously that a mutant strain of *A. vinelandii* that has the $I^- II^+$ activity phenotype and produces inactive component I is activated in vitro by acid-treated component I. It seems that there is a common factor in many molybdoproteins and that this factor is liberated upon acid treatment of a molybdoprotein such as component I (15). We tested many mutant strains of *K. pneumoniae* producing inactive component I for activation in vitro by acid-treated component I. Extracts from strains UN106 and UN109 were activated to produce 5 to 10% of the activity found in fully derepressed wild type (Table 2). Acid-treated component I

TABLE 1. *Genotypes and phenotypes of mutant strains*

Strain	Relevant genotype	Relevant Nif phenotype		Origin
		Activity	CRM[a]	
UN	Wild-type	$I^+ II^+$	$I^+ II^+$	
UN26	*nif-4026*	$I^- II^+$	$I^- II^+$	UN
UN66	*nif-4066*	$I^+ II^+$	$I^+ II^+$	UN
UN83	*nif-4083*	$I^+ II^-$	$I^+ II^-$	UN
UN106	*nif-4106*	$I^- II^+$	$I^+ II^+$	UN
UN109	*nif-4109*	$I^- II^+$	$I^+ II^+$	UN
UN113	*nif-4113*	$I^- II^+$	$I^+ II^+$	UN
UN116	*nif-4116*	$I^+ II^-$	$I^+ II^+$	UN
UN142	*nif-4083, hisD4003*	$I^+ II^-$	$I^+ II^+$	UN83
UN150	*nif-4106, hisD4006*	$I^- II^+$	$I^+ II^+$	UN106
UN179	*nif-4179*	$I^- II^-$	$I^- II^-$	UN
UN316	*nif-4113, hisD4019*	$I^- II^+$	$I^+ II^+$	UN113
UN318	*nif-4026, hisD4021*	$I^- II^+$	$I^- II^+$	UN26
UN328	*nif-4109, hisD4027*	$I^- II^+$	$I^+ II^+$	UN109
UN364	*nif-4116, hisD4033*	$I^+ II^-$	$I^+ II^+$	UN116
UN582	*nif-4179, hisD4113*	$I^- II^-$	$I^- II^-$	UN179
UN587	*nif-4066, his-4111*	$I^+ II^+$	$I^+ II^+$	UN66

[a] Cross-reacting material (CRM) is based on Ouchterlony plate technique with antiserum prepared against purified components. A positive reaction represents more than 10% of the reaction given by extracts in derepressed wild-type cells.

TABLE 2. *Activation of inactive component I by acid-treated component I*

Source of extract[a]	Acid-treated addition to extract[b]	Sp act[c]
UN	None	40.09
UN106	None	0.00
UN106	Component I	2.39
UN109	None	0.00
UN109	Component I	3.95
UN113	None	0.00
UN113	Component I	0.00
UN109	Extract of UN on N_2	4.21
UN109	Extract of UN on NH_4^+	0.00
UN109	Extract of derepressed UN83	1.55
UN109	Extract of derepressed UN179	0.00

[a] Extracts (0.2 ml) contained approximately 12 mg of protein per ml.

[b] Preparation of acid-treated component I and preincubation of extracts with acid-treated component I before addition of C_2H_2 to the assay vial is the same as previously described (14). Component I, before acid treatment, contained 25 μg of protein. Crude extracts, before acid treatment, contained 150 μg of protein.

[c] Shown as nanomoles of C_2H_2 reduced per minute per milligram of protein.

has no activity (14). Observed activity upon addition of acid-treated component I to mutant strains UN106 and UN109 seems to be lower than wild-type activity, but there are several possible explanations for this lower activity. Perhaps inactive component I in these strains is more unstable than active component I in wild type. A second possibility is that there is incomplete exchange of activating factor between acid-treated component I and inactive component I of the mutant strains. Complete activation also was not found in a similar mutant strain of *A. vinelandii* (14). Mutant strain UN113 that also produced inactive component I could not be activated in vitro upon addition of acid-treated component I.

Mutant strains lacking the activating factor were useful for determining conditions that allow synthesis of activating factor. Nitrogenase was completely repressed in media containing excess NH_4^+ and we determined that the activating factor also is not detected during repression of nitrogenase synthesis. Acid-treated crude extracts from N_2- or NH_4^+-grown cells were added to a crude extract of strain UN109 and Table 2 indicates that NH_4^+-grown cells do not produce the activating factor, therefore the activating factor is not present when cells do not fix N_2. Also, the activating factor was not made by mutant strain UN179 which does not produce either of the nitrogenase components. Strain UN83 extract, when acid treated, allowed activation of strain UN109 extract.

Electron transport factor. Strain UN66 had an interesting phenotype in that it had very low acetylene-reducing activity in vivo (compared to the wild type), but had high activity in vitro (Table 3). A possible explanation for this phenotype is that the mutation is in a site that allows the 2-electron transfer necessary for acetylene-reducing activity but not the 6-electron transfer required for N_2 fixation. However, nitrogen fixation did occur in vitro in strain UN66 (Table 3). A major difference between the assays in vivo and in vitro is that dithionite is used as the electron donor in vitro, thereby bypassing the natural electron donating system. This is a good indication that the lesion causing the phenotype in strain UN66 is in a gene that codes for some component of the electron donating pathway. Presently, we are using mutant strains such as UN66 to attempt to purify the factors responsible for electron transport.

Co-transduction of nif mutations. His^- strains were obtained by mutagenizing Nif^- mutant strains and screening for histidine requirement after penicillin selection. The Nif phenotype in these Nif^- His^- strains was identical to the Nif phenotype in the original mutant strain (Table 1). Linkage of the various *nif* mutations to *his* was determined by transducing phage grown on the wild type into the Nif^- His^- recipients. His^+ recombinants were selected and replica plated onto N-free plates so that the percentage of His^+ Nif^+ recombinants could be calculated (Table 4). Controls were performed to check for spontaneous reversion, and crosses were performed in which a given *nif* mutation was crossed into itself to determine whether transduction would increase mutation rate. Spontaneous reversion did not interfere with the crosses.

TABLE 3. *N_2-fixing and C_2H_2-reducing activities of the wild type and strain UN66*

Strain	Sp act		
	In vivo	In vitro	
	C_2H_2 reduced/min per 10^9 cells	C_2H_2 reduced/min per mg of protein	N_2 reduced/min per mg of protein
UN	2.43	30.6	8.32
UN66	0.16	13.8	3.64

By analysis of recombination frequencies, we determined that all of these *his* mutations are more than 70% co-transducible with each other. Most of the strains with the *his* mutations were unable to utilize histidinol as the source of histidine (the exception is *his-4111*), therefore we shall assume that at least one of the *his* mutations is in the gene *hisD* that specifies histidinol dehydrogenase. It is possible that some of these *his* mutations are in genes other than *hisD*, and that a polar effect causes the strain to be unable to utilize histidinol. We can assume, however, that the *nif* mutations described in this paper are co-transducible with *hisD*.

A preliminary assignment of mutation order can be made with these data if we assume that all of these *nif* mutations are on one side of the *hisD* locus. This is a reasonable assumption because Shanmugam et al. (23) showed that deletions from the *rfb* locus that extend into, but not through, the *his* operon(s) still allow the organism to grow on N_2. It seems (Table 4) that *nif-4106* is closest to *hisD*, with *nif-4066* in between *nif-4106* and the cluster containing *nif-4026*, *nif-4083*, *nif-4113*, *nif-4179*, and *nif-4116*. Unfortunately, the co-transduction technique was not satisfactory for ordering closely linked mutations. The frequency of co-transduction was dependent on the exact location of the *his* mutations.

TABLE 4. *Percentage co-transduction of nif mutations to his*

Recipient strain (genotype)	No. of His^+ recombinants analyzed	Co-transduction (%)
UN142 (*nif-4083, hisD4003*)	1,030	21
UN150 (*nif-4106, hisD4006*)	354	81
UN316 (*nif-4113, hisD4019*)	1,387	25
UN318 (*nif-4026, hisD4021*)	653	25
UN328 (*nif-4109, hisD4027*)	525	42
UN364 (*nif-4116, hisD4033*)	1,365	27
UN582 (*nif-4179, hisD4113*)	1,088	41
UN587 (*nif-4066, his-4111*)	540	72

Order of nif mutations. More detailed analysis of the mutation order could be obtained from reciprocal crosses in which phage from a Nif^- strain are introduced into a Nif^-His^- strain. His^+ recombinants were selected and analyzed for the Nif phenotype. Such crosses are described in Table 5. Cross number 1 had strain UN106 (*nif-4106*) as the donor and strain UN318 (*nif-4026*, *hisD4021*) as the recipient. The percentage of recombinants with Nif^+His^+ phenotype was 4.7%. The reciprocal cross (cross number 2) with strain UN26 (*nif-4026*) as the donor and strain UN150 (*nif-4106*, *hisD4006*) as recipient yielded 50% Nif^+His^+ recombinants. These data strongly support the order: *hisD nif-4106 nif-4026*, the 4.7% presumably representing quadruple crossovers. Reciprocal crosses 3 to 10 likewise show that mutations *nif-4083*, *4113*, *4116*, and *4066* are on the *his* distal side of *nif-4106*. Reciprocal crosses 11 to 14 showed that *nif-4066* is between *hisD* and *nif-4083*, as well as between *hisD* and *nif-4026*. The mutation *nif-4113* was between *hisD* and *nif-4179* (crosses 15 and 16). The high number of

TABLE 5. *Order of nif mutations by transduction analysis*

Number	Strains		No. of His^+ transductants analyzed	Nif^+ His^+ recombinants (%)	Order of mutations
	Donor	Recipient			
1	UN106	UN318	1,090	4.7	*hisD nif-4106 nif-4026*
2	UN26	UN150	117	50	
3	UN106	UN142	1,369	3.5	*hisD nif-4106 nif-4083*
4	UN83	UN150	187	49	
5	UN106	UN316	1,054	5	*hisD nif-4106 nif-4113*
6	UN113	UN150	93	34	
7	UN106	UN364	970	1.2	*hisD nif-4106 nif-4116*
8	UN116	UN150	950	37	
9	UN106	UN587	225	1	*hisD nif-4106 nif-4066*
10	UN66	UN150	116	9	
11	UN66	UN142	392	2.3	*hisD nif-4066 nif-4083*
12	UN83	UN587	261	19	
13	UN66	UN318	116	9	*hisD nif-4066 nif-4026*
14	UN26	UN587	354	34	
15	UN113	UN582	495	1	*hisD nif-4113 nif-4179*
16	UN179	UN316	393	40	
17	UN109	UN150	178	0.0	*hisD (nif-4106, nif-4109)*
18	UN106	UN328	1,028	0.0	

Nif+His+ recombinants in cross 16 indicates that *nif-4179* is relatively distant from *nif-4113*. Finally, *nif-4106* and *nif-4109* are so close to each other that no Nif+ recombinants were seen when one was tranduced into the other (crosses 17 and 18).

Confirmation of the order *hisD nif-4106 nif-4066 nif-4083* came from crosses in which three recipient strains were used in crosses in which strains UN, UN66, UN83, and UN106 were used as donors (Table 6). The number of Nif+ recombinants was determined and the percentage of Nif+ recombinants was calculated using the number of Nif+ recombinants in a cross of the wild type into a given recipient as 100%. The crosses with strain UN66 as recipient show that *nif-4106* is closer to *nif-4066* than to *nif-4083*. In crosses with strain UN83 as the recipient, it seems that *nif-4066* is closer than *nif-4106* to *nif-4083*. The mutation *nif-4066* is much closer to *nif-4106* than is *nif-4083* as determined by the crosses with UN106 as the recipient. These crosses support the order: *nif-4106 nif-4066 nif-4083*.

TABLE 6. *Determination of the order of nif-4106, nif-4066, and nif-4083 by frequency of Nif+ recombinants*

Donor strain	Recipient strain	No. of Nif+ recombinants (per 10^6 PFU[a])	Nif+ recombinants (%)[b]
UN	UN66	1.35	100
UN66	UN66	0	0
UN83	UN66	0.62	46
UN106	UN66	0.14	10
UN	UN83	1.27	100
UN66	UN83	0.45	35
UN83	UN83	0	0
UN106	UN83	0.64	50
UN	UN106	1.22	100
UN66	UN106	0.05	4
UN83	UN106	0.33	27
UN106	UN106	0	0

[a] PFU, Plaque-forming units.
[b] Normalized to the number of recombinants obtained with UN as the donor for each set of crosses with a given recipient.

The mutations *nif-4026*, *4113*, *4116*, and *4083* are closely linked (Table 4). The order of these mutations was obtained by reciprocal crosses (Table 7). The difference between percent wild-type recombinants in the reciprocal crosses is a difference between two small numbers so that the data are not as convincing as the data from reciprocal crosses in Table 5. For instance, no order can be obtained from crosses 3, 4, 11, and 12. The other crosses support the order: *hisD nif-4026 nif-4113 nif-4083 nif-4116*. Figure 1 summarizes the data obtained from these crosses.

DISCUSSION

It is important to know the function of genes responsible for N_2 fixation. Some genes should be responsible for control of nitrogenase synthesis, whereas others should specify the structure of the two nitrogenase components. Studies with these mutant strains have given us unexpected insight into other factors that are specifically responsible for N_2 fixation in vivo. We have shown that strain UN66 may lack a specific electron transport factor required for N_2 fixation. Strain UN106 requires a factor obtained by acid-treating component I. This factor may be the same as the factor described by Nason et al. (15) that seems to be common to all molybdoproteins so far examined. Nagatani et al. (14) have shown that the activating factor from *A. vinelandii* or *K. pneumoniae* compo-

TABLE 7. *Order of closely linked nif mutations by transduction analysis*

Cross no.	Strains: Donor	Strains: Recipient	No. of His+ transductants analyzed	Nif+ His+ recombinants (%)	Order of mutations
1	UN26	UN316	5.5×10^4	0.0	*hisD nif-4026 nif-4113*
2	UN113	UN318	13.4×10^4	0.6	
3	UN113	UN142	7.9×10^4	0.009	*hisD (nif-4113, nif-4083)*
4	UN83	UN316	3.3×10^4	0.018	
5	UN83	UN364	1.1×10^5	0.00	*hisD nif-4083 nif-4116*
6	UN116	UN142	0.7×10^5	0.09	
7	UN26	UN142	2.4×10^3	0.0	*hisD nif-4026 nif-4083*
8	UN83	UN318	31×10^3	2.0	
9	UN26	UN364	1.8×10^3	0.1	*hisD nif-4026 nif-4116*
10	UN116	UN318	2.3×10^3	1.9	
11	UN113	UN364	1.4×10^5	0.00	*hisD (nif-4113, nif-4116)*
12	UN116	UN316	9.7×10^4	0.00	

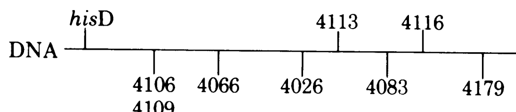

FIG. 1. *Order of the nif mutations.*

nent I will activate component I from a mutant strain of *A. vinelandii*. The activating factor also will activate component I from *A. vinelandii* derepressed in medium containing W instead of Mo (13).

Mutations *nif-4113* and *nif-4116* probably lie in genes specifying the structure of components I and II, respectively, because cross-reacting material is produced. Strains UN106 and UN109 that have the same phenotype as strain UN113 produce inactive component I that is activatable by acid-treated component I, however, component I from strain UN113 cannot be activated in vitro. Mutations in strains UN26 and UN83, which produce no detectable inactive components are very closely linked to *nif-4113* and *nif-4116*. It seems, therefore, that the genes specifying components I and II are closely linked, and this preliminary genetic data indicates that the component II gene(s) is distal to *his* with respect to component I gene(s). More detailed genetic analysis is required to confirm this order.

Mutant strain UN179 does not produce either of the components. The mutation does not seem to be in the nitrogenase structural genes because it is quite distant from the mutations causing the $I^- II^+$ or the $I^+ II^-$ phenotypes. Strain UN179 does not produce activating factor either, therefore *nif-4179* might be in a gene required for control of nitrogenase synthesis. If *nif-4179* is in a promoter of transcription, it is possible that the entire *nif* region is one operon. The site in which *nif-4179* lies might be the target of activation by glutamine synthetase (12, 28, 29). We are examining properties of strains such as UN179 in more detail to gain further insight into the control of *nif* genes.

A scheme incorporating the genetic and biochemical data is represented in Fig. 2. Other mapping experiments with many different mutant strains also support this gene order. We suggest that at least five genes are required for N_2 fixation. The mutation *nif-4106* is in the gene *nif*B that is essential for production of the functional activating factor. Active electron transport factor is specified by *nif*F, whereas *nif*D codes for component I and *nif*H codes for component II. A site required for synthesis of both components and the activating factor is *nif*G. This scheme does not assume that all of the *nif* genes have been identified. For instance, there may be several genes required for production of the activating factor. Also, there may be two genes that specify the structure of component I, one gene for each subunit. Complementation tests with episomes or with the P1 merodiploids produced by the method of Stodolsky (25) could be useful for determining the number of genes involved. It is quite possible that there are other gene products specifically related to N_2 fixation such as factors required for Mo, Fe, or S uptake and metabolism. Nothing is known about the number of operons involved or about their polarity. Suppression of nonsense mutations should be useful for answering these types of questions. Deletion analyses within the *nif* region could be important for more detailed mapping. Shanmugam et al. (23) showed that the operator of the *his* genes is located proximal to the *nif* genes. Chang et al. (6) have reported a technique for obtaining strains of *Salmonella typhimurium* with mutations in the operator for the histidine biosynthetic enzymes. Perhaps this technique can be applied to obtain mutant strains of *K. pneumoniae* with deletions beginning in the *his* operator and extending within the *nif* region. Presently, we are pursuing these approaches to gain a more detailed insight into the genetics and regulation of N_2 fixation.

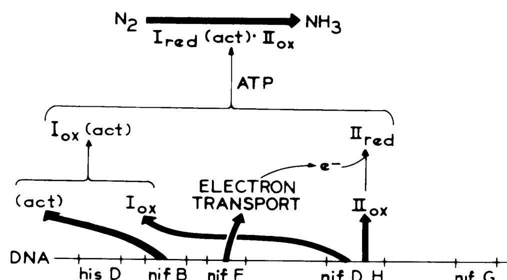

FIG. 2. *Location and function of nif genes. The mechanism of N_2 fixation is based on reference 16. The designation (act) represents the activating factor.*

ACKNOWLEDGMENTS

We thank M. Bachhuber for technical help and H. H. Nagatani for helpful advice.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by National Science Foundation grants GB36787 and GB42424X. R. T. St. John was supported by Public Health Services training grant 5-T01-GM-00686 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.

2. Brill, W. J. 1975. Nif⁻ mutants of free-living bacteria. *In* W. E. Newton (ed.), Proceedings of the International Conference on Nitrogen Fixation (in press). Washington State University Press, Pullman.
3. Brill, W. J., A. L. Steiner, and V. K. Shah. 1974. Effect of molybdenum starvation and tungsten on the synthesis of nitrogenase components in *Klebsiella pneumoniae*. J. Bacteriol. **118**:986-989.
4. Brill, W. J., J. Westphal, M. Stieghorst, L. C. Davis, and V. K. Shah. 1974. Detection of nitrogenase components and other non-heme iron proteins in polyacrylamide gels. Anal. Biochem. **60**:237-241.
5. Cannon, F. C., R. A. Dixon, J. R. Postgate, and S. B. Primrose. 1974. Chromosomal integration of *Klebsiella* nitrogen fixation genes in *Escherichia coli*. J. Gen. Microbiol. **80**:227-239.
6. Chang, G. W., D. Straus, and B. N. Ames. 1971. Enriched selection of dominant mutations: histidine operator mutations. J. Bacteriol. **107**:578-579.
7. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**:61-76.
8. Dixon, R. A., and J. R. Postgate. 1971. Transfer of nitrogen fixation genes by conjugation in *Klebsiella pneumoniae*. Nature (London) **234**:47-48.
9. Dixon, R. A., and J. R. Postgate. 1972. Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli*. Nature (London) **237**:102-103.
10. Fisher, R. J., and W. J. Brill. 1969. Mutants of *Azotobacter vinelandii* unable to fix nitrogen. Biochim. Biophys. Acta **184**:99-105.
11. Gordon, J. K., and W. J. Brill. 1972. Mutants that produce nitrogenase in the presence of ammonia. Proc. Nat. Acad. Sci. U.S.A. **69**:3501-3503.
12. Gordon, J. K., and W. J. Brill. 1974. Derepression of nitrogenase synthesis in the presence of excess NH_4^+. Biochem. Biophys. Res. Commun. **59**:967-971.
13. Nagatani, H. H., and W. J. Brill. 1974. Nitrogenase V. The effect of Mo, W and V on the synthesis of nitrogenase components in *Azotobacter vinelandii*. Biochim. Biophys. Acta **362**:160-166.
14. Nagatani, H. H., V. K. Shah, and W. J. Brill. 1974. Activation of inactive nitrogenase by acid-treated component I. J. Bacteriol. **120**:697-701.
15. Nason, A., K. Y. Lee, S. S. Pan, P. A. Ketchum, A. Lamberti, and J. DeVries. 1971. In vitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductase from a Neurospora mutant and a component of molybdenum-enzymes. Proc. Nat. Acad. Sci. U.S.A. **68**:3242-3246.
16. Orme-Johnson, W. H., W. D. Hamilton, T. L. Ljones, M.-Y. W. Tso, R. H. Burris, V. K. Shah, and W. J. Brill. 1972. Electron paramagnetic resonance of nitrogenase and nitrogenase components from *Clostridium pasteurianum* W5 and *Azotobacter vinelandii* OP. Proc. Nat. Acad. Sci. U.S.A. **69**:3142-3145.
17. Prival, M. J., J. E. Brenchley, and B. Magasanik. 1973. Glutamine synthetase and the regulation of histidase formation in *Klebsiella aerogenes*. J. Biol. Chem. **248**:4334-4344.
18. Roth, J. J. 1970. UGA Nonsense mutations in *Salmonella typhimurium*. J. Bacteriol. **102**:467-475.
19. St. John, R. T., V. K. Shah, and W. J. Brill. 1974. Regulation of nitrogenase synthesis by oxygen in *Klebsiella pneumoniae*. J. Bacteriol. **119**:266-269.
20. Sen, M. and S. P. Sen. 1965. Interspecific transformation in *Azotobacter*. J. Gen. Microbiol. **41**:1-6.
21. Shah, V. K., L. C. Davis, J. K. Gordon, W. H. Orme-Johnson, and W. J. Brill. 1973. Nitrogenase III. Nitrogenaseless mutants of *Azotobacter vinelandii*: activities, cross-reactions and EPR spectra. Biochim. Biophys. Acta **292**:246-255.
22. Shah, V. K., L. C. Davis, M. Stieghorst, and W. J. Brill. 1974. Mutant of *Azotobacter vinelandii* that hyperproduces nitrogenase component II. J. Bacteriol. **117**:917-919.
23. Shanmugam, K. T., A. S. Loo, and R. C. Valentine. 1974. Deletion mutants of nitrogen fixation in *Klebsiella pneumoniae*: mapping of a cluster of *nif* genes essential for nitrogenase activity. Biochim. Biophys. Acta **338**:545-553.
24. Sorger, G. J., and D. Trofimenkoff. 1970. Nitrogenaseless mutants of *Azotobacter vinelandii*. Proc. Nat. Acad. Sci. U.S.A. **65**:74-80.
25. Stodolsky, M. 1973. Bacteriophage P1 derivatives with bacterial genes: a heterozygote enrichment method for the selection of P1*dpro* lysogens. Virology **53**:471-475.
26. Streicher, S., E. Gurney, and R. C. Valentine. 1971. Transduction of the nitrogen-fixation genes in *Klebsiella pneumoniae*. Proc. Nat. Acad. Sci. U.S.A. **68**:1174-1177.
27. Streicher, S., E. G. Gurney, and R. C. Valentine. 1972. The nitrogen fixation genes. Nature (London) **239**:495-499.
28. Streicher, S. L., K. T. Shanmugam, F. Ausubel, C. Morandi, and R. B. Goldberg. 1974. Regulation of nitrogen fixation in *Klebsiella pneumoniae*: evidence for a role of glutamine synthetase as a regulator of nitrogenase synthesis. J. Bacteriol. **120**:815-821.
29. Tubb, R. S. 1974. Glutamine synthetase and ammonium regulation of nitrogenase synthesis in *Klebsiella*. Nature (London) **251**:481-485.
30. Tubb, R. S., and J. R. Postgate. 1973. Control of nitrogenase synthesis in *Klebsiella pneumoniae*. J. Gen. Microbiol. **79**:103-117.
31. Tyler, B., A. B. DeLeo, and B. Magasanik. 1974. Activation of transcription of *hut* DNA by glutamine synthetase. Proc. Nat. Acad. Sci. U.S.A. **71**:225-229.
32. Wolf, B., A. Newman, and D. A. Glaser. 1968. On the origin and direction of replication of the *Escherichia coli* K12 chromosome. J. Mol. Biol. **32**:611-629.
33. Yoch, D. C., and R. M. Pengra. 1966. Effect of amino acids on the nitrogenase system of *Klebsiella pneumoniae*. J. Bacteriol. **92**:618-622.