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Mutations Affecting Regulation of the *Klebsiella pneumoniae nifH* (Nitrogenase Reductase) Promoter

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Starting with plasmid pSA30 which contains an intact *nifHDKY* operon, we selected mutants that no longer inhibited nitrogen fixation in *Klebsiella pneumoniae*. Three categories of mutants were found among eight mutant plasmids examined in detail. Three mutant plasmids carried a single-base-pair (bp) change at position −12 or −14 relative to the *nifH* transcription start site. These mutations were located in a previously described consensus sequence found in the −10 to −15 region of *nif* promoters. Four of the mutant plasmids contain lesions considerably upstream from the start point of transcription. Two of these upstream mutations are identical 112-bp deletions of nucleotides −72 to −184, and two others are a single-bp change at position −136. The final plasmid does not contain a mutation within a 525-bp region which includes 289 bp upstream from the *nifH* ATG initiation codon and which extends 69 codons into the *nifH* gene. All eight of the mutant pSA30 plasmids failed to complement a chromosomal *nifH* mutation, suggesting that the mutations that block inhibition of *nif* expression also prevent transcription of the *nifHDKY* operon.

A cluster of 17 contiguous nitrogen fixation (*nif*) genes, grouped in seven or eight operons, has been identified in *Klebsiella pneumoniae* (Fig. 1; reference 2 and reviewed in references 1 and 33). These *nif* genes are subject to two levels of positive regulation and at least one level of negative regulation in response to ammonia and oxygen. The first level of positive control is mediated by a general, centralized control system (the *ntr* system) which regulates the expression of a variety of nitrogen assimilatory genes in enteric bacteria (for a recent review, see reference 22). Under conditions of limiting ammonia, the products of *ntrA*(*glnF*) and *ntrC*(*glnG*) act in concert to activate transcription of genes of the *nifLA* operon (5, 12, 13, 21, 25, 27; F. J. de Bruijn and F. M. Ausubel, Mol. Gen. Genet., in press) as well as other genes and gene systems involved in nitrogen assimilation, such as *glnA* (glutamine synthetase), *hut* (histidine utilization), *aut* (arginine utilization), and *put* (proline utilization) (Fig. 1; reference 22).

The second level of positive control is mediated by the *nifA* product, which acts as a *nif*-specific transcriptional regulator and is required for expression of all the *nif* operons, except its own (6, 14, 34, 35). Negative regulation is mediated by the *nifL* product which responds to an increase in ammonia or oxygen levels once the *nif* genes have been derepressed (7, 18, 26).

The *nifA* gene product appears to be similar in several respects to the *ntrC*(*glnG*) product. First, just as both *ntrC*(*glnG*) and *ntrA*(*glnF*) products are required to activate transcription of the *nifLA* operon, the *nifA* product requires the *ntrA*(*glnF*) product to activate the *nifHDKY* operon and, presumably, the remaining six *nif* operons as well (25, 28; Fig. 1). Moreover, the *nifA* product is capable of substituting for the *ntrC*(*glnG*) product in the activation of nitrogen assimilatory genes such as *glnA*, *put*, *aut*, and *hut*; this activation also requires the *ntrA*(*glnF*) product (25, 27). The *nifA* product also autogenously activates the *nifLA* operon (15, 27). Interestingly, the *nifA* product was also shown to activate the *Azotobacter vinelandii* and *Azotobacter chroococcum nifHDK* genes (20), and both the *K. pneumoniae nifA* and the *Escherichia coli ntrC* products were shown to be capable of activating the *Rhizobium meliloti nifHDK* operon (35, 36). In contrast, the *ntrC*(*glnG*) product could not be a substitute for the *nifA* product in the activation of *K. pneumoniae nif* operons other than those in the *nifLA* operon (25, 36).

The ability of the *nifA* product to be a substitute for the *ntrC*(*glnG*) product in the activation of the *nifL*, *glnA*, *aut*, and *put* genes led to the discovery of two consensus sequences located in the promoter regions of *nifA*/*ntrC*-activated genes (29). In the case of the *K. pneumoniae nifL* and *R. meliloti nifH* genes, which can be activated either by *nifA* or *ntrC*, the consensus sequence is TTTTGCA and is located in the −10 to −15 region. In contrast, the *K. pneumoniae nif* promoters that can only be activated by *nifA*, contain a subset of the heptameric sequence, TTGCA, at the same position (2, 35). In the case of the *nifHDKY* promoter, the TTGCA consensus sequence is represented by CTGCA.

In the experiments described in this paper, DNA sequences involved in the expression of the *K. pneumoniae nifH* gene were identified by exploiting the observation that multicopy plasmids carrying the *nifH* promoter region inhibit the derepression of chromosomal *nif* genes (8, 32; G. Riedel, Ph.D. thesis, Harvard University, Cambridge, Mass., 1980, V. Sundaresan, unpublished data). Because this same region responds to *nifA* + *ntrA* activation (6, 35) and because *nifH* transcription starts within this region (35), it was postulated that a positive activator, such as the *nifA* product, is present in limiting quantities and that the presence of the *nifH* promoter in a multicopy state leads to the titration of the activator (7; G. Riedel, Ph.D. thesis).

Based on this model, we expected that mutant derivatives of plasmids carrying the *nifH* promoter containing alterations in the presumptive activator binding site might fail to cause *nif* inhibition. Indeed, as shown here, three mutations which resulted in a loss of *nif* inhibitory activity were single-base-pair (bp) substitutions in the −10 to −15 CTGCA sequence of the *nifH* promoter. Plasmids carrying these mutations failed to complement a chromosomal *nifH* muta-

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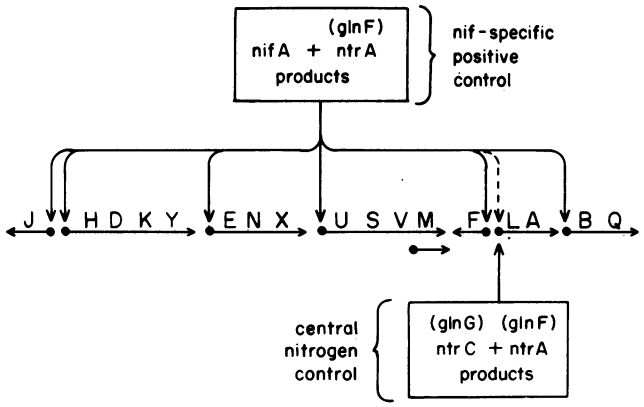

FIG. 1. *nif* regulation in *K. pneumoniae*. The letters *J, H, D, K, Y, E, N, X, U, S, V, M, F, L, A, B,* and *Q* represent the 17 genes of the *nif* regulon. These genes are arranged in seven or eight operons as indicated by the arrows just below the gene designations (2). The products of the *ntrC*(*glnG*) and *ntrA*(*glnF*) genes activate the *nifLA* operon, and the products of *nifA* and *ntrA*(*glnF*) in turn activate the other *nif* operons. See the text for details and references.

tion. These results indicated that the CTGCA sequence is required for *nifHDKY* transcription and suggested that the CTGCA sequence is a major structural component of the *nifH* promoter. In addition to the mutations in the −10 to −15 region, several mutations which abolished *nifH* inhibition were located considerably upstream of the start point of transcription. Two of these upstream mutations were identical 112-bp deletions of nucleotides −72 to −184, and two others contained a single-bp substitution at position −136. The significance of these upstream mutations with respect to *nifHDKY* transcription is not clear.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in and constructed for this study are listed in Table 1.

Media and anaerobic growth of bacteria. Rich medium (L broth [8]) was solidified with 1.5% Bacto-agar (Difco Laboratories, Detroit, Mich.). Nitrogen-free medium (NFDM [11] was supplemented with Casamino acids (100 μg/ml) and solidified with 1.5% Serva ultrapure agar (type 11396, Accurate Chemical and Scientific Corp., Hicksville, N.Y.). When appropriate, histidine, tetracycline (Tc) and chloramphenicol were added to LB or NFDM at concentrations of 20, 10, and 50 μg/ml, respectively. Solid NFDM medium was incubated for 6 days at 32°C in anaerobic chambers (Becton, Dickinson, and Co., Cockeysville, Md.).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype	Source or reference
E. coli HB101	F⁻*hsdS20* (r_B^- m_B^-) *recA13 ara-14 lacY proA2 supE44 galK2 rpsL20 xyl-5 mtl-1*	4
K. pneumoniae		
KP5525	*recA56*	32
UNF698	*hisD2 nifH recA56 srl*::Tn*10*	F. Cannon
KP5805	*hisD2 nifH recA56 srl*	This paper
Plasmids		
pACYC184	Tc^r Cm^r	10
pSA30	pACYC184 + 6.0 kb *Eco*RI fragment, Tc^r *nifHDKY*	19

Strain construction. Plasmids were introduced into *K. pneumoniae* strains by the $CaCl_2$-heat shock transformation procedure of Mandel and Higa (23). A Tc-sensitive derivative of UNF698 (strain KP5805) was constructed as follows. A saturated culture of UNF698 grown in LB was diluted to a density of 5×10^8 cells per ml in 5 ml of LB + Tc medium and then incubated at 32°C until two doublings had occurred. Penicillin (10,000 U/ml; PenK, Sigma) was added, and the culture was incubated at 32°C for 3 to 8 h. Penicillinase (0.2 ml/5 ml; Bacto-penase; Difco) was added, and after 30 min, the cells were collected on a filter (0.45-μm pore size). The filter was vortexed in 5 ml of LB medium to suspend the cells. This series of steps was repeated 10 times. Finally, the culture was plated on LB solid medium, and individual colonies were tested for a Tc^s phenotype.

Enzyme assays. Nitrogenase assays in anaerobic whole-cell liquid cultures (grown in NFDM) were carried out as described previously (32).

DNA biochemistry. Supercoiled plasmid DNA used for DNA sequencing was isolated by the cleared lysate procedure and purified by CsCl-ethidium bromide equilibrium centrifugation (11). Small-scale preparations of plasmid DNA were prepared from 5 ml of a saturated LB-grown culture by the boiling method of Holmes and Quigley (19). Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, Md., and used according to the manufacturer's instructions. Conditions used for horizontal agarose gel electrophoresis have been described previously (31).

DNA sequencing. The *nifH* promoter is contained within a

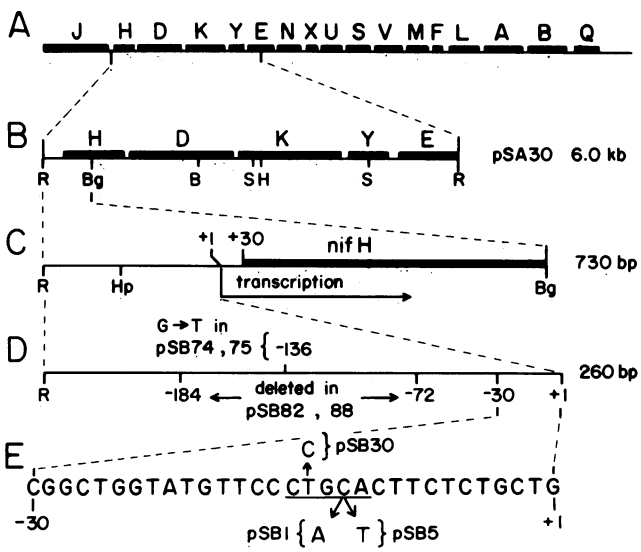

FIG. 2. Location of mutations in the *nifH* promoter region. (A) The 24-kilobase-pair *K. pneumoniae nif* regulon showing the positions of each *nif* gene (30, 31). (B) The 6.0 kilobase-pair *Eco*RI fragment containing the *nifHDKY* operon present in pSA30 (10). Symbols: R, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI; S, *Sal*I; and H, *Hin*dIII. (C) A 730 bp *Eco*RI-*Bgl*II fragment containing the *nifH* promoter indicating the start point of transcription (35). Hp, *Hpa*I. (D) A 260-bp region extending upstream from the start point of transcription showing the locations of the mutations in plasmids pSB74, pSB75, pSB82, and pSB88. The G-to-T mutation is in the coding strand. (E) A 30-bp region of the coding strand of the *nifH* promoter extending upstream from the start point of transcription showing the location of mutations in plasmids pSB1, pSB5, and pSB30. The CTGCA sequence, representing the TTGCA consensus sequence at positions −10 to −15, is underlined.

730-bp *Eco*RI-*Bgl*II fragment as illustrated in Fig. 2C. Supercoiled pSA30 DNA, purified by CsCl-ethidium bromide centrifugation, was digested with *Eco*RI + *Bgl*II, and the 730-bp *Eco*RI-*Bgl*II fragment, visualized by methylene blue (0.01%) staining, was electro-eluted from a 6% acrylamide gel. The purified fragment was end-labeled with the Klenow fragment of DNA polymerase I as described previously (24). Alternatively, the *Eco*RI-*Bgl*II fragment was digested with *Hpa*II (Fig. 2C) to provide a shorter fragment (620 bp) for sequencing. Labeled fragments were denatured, and the single strands were separated on a 6% acrylamide gel as described previously (24). Sequencing reactions and electrophoresis were carried out as described by Maxam and Gilbert (24).

RESULTS

Strategy and selection of mutations which affect *nifHDKY* transcription. Wild-type *K. pneumoniae* strains containing pSA30 express only 1 to 3% of the nitrogenase activity of control strains and do not form colonies on nitrogen-free plates when forced to utilize N_2 as the sole source of nitrogen (Table 2 and reference 32). It should be possible, therefore, to select directly for mutations in the site(s) where activation of the *nifHDKY* operon occurs, simply by selecting for *K. pneumoniae* cells that carry pSA30 and are capable of growth on N_2 as the sole nitrogen source. Such strains might contain plasmid-borne mutations in the activator binding site(s); such mutations might prevent the plasmid from competing effectively for the limiting quantity of positive activators. Alternatively, mutant strains might overproduce the *nifA* products, the *ntrA* products, or both or contain plasmid copy number mutations. We chose a *recA K. pneumoniae* host, Kp5525, carrying pSA30 because we wanted to avoid the possibility that a plasmid-plasmid or a plasmid-chromosome recombination might interfere with the selection and characterization of potential mutants.

Approximately 10^7 KP5525(pSA30) cells from a washed, saturated, LB culture were plated per petri plate on NFDM + Tc medium and incubated for 3 to 5 days at 30°C in anaerobic chambers (see above). In 10 independent experiments, colonies appeared at a frequency of less than 10^{-7} to 10^{-9}. A total of 88 colonies from two experiments were purified on NFDM + Tc medium for further study. Each of the 88 clones was tested for nitrogenase activity by the liquid whole-cell acetylene reduction method. Of the 88 presumptive mutant clones, 85 had nitrogenase levels significantly higher (fivefold) than did KP5525(pSA30), and these 85 clones were studied further.

TABLE 2. Nitrogenase assays in *K. pneumoniae* strains containing mutant pSA30 plasmids

Plasmid	Acetylene reduction activity[a] in:	
	KP5525	KP5805
pACYC184	100 ± 60	2
pSA30	3 ± 2.4	<1
pSB1	97 ± 47	3
pSB5	68 ± 31	3
pSB30	42 ± 19	<1
pSB60	73 ± 50	<1
pSB74	105 ± 77	4
pSB75	71 ± 53	2
pSB82	44 ± 17	<1
pSB88	25 ± 14	<1

[a] Expressed as percent (± the standard deviation) of KP5525(pACYC184) activity. The 100% level represents the production of 0.2 nmoles of ethylene per h per 10^9 cells. As indicated by the large standard deviations, there was considerable variation in the nitrogenase activity in any particular strain on different days. The reason for this variability is unknown.

Location of mutations. Plasmid DNA was isolated from each of the 85 mutant clones by a small-scale plasmid isolation procedure (see above). Each of the 85 plasmid DNA preparations was then used to retransform strain KP5525, selecting the Tc^r marker on pSA30, and each transformant was tested for acetylene-reducing activity. Of the 85 transformants, 80 exhibited a significant increase (fivefold or greater) in nitrogenase activity compared with KP5525(pSA30), indicating that in these 80 cases, the lesion responsible for the lack of *nif* inhibitory phenotype in the original Nif^+ revertant clones was located on pSA30.

Characterization of pSA30 mutants. Each of the 80 pSA30 presumptive mutant plasmid DNA preparations was examined for large deletions by cleaving each DNA sample with *Eco*RI + *Hin*dIII (Fig. 2B) and examining the digestion products on 1.0% agarose gels. Four of the 80 plasmids contained a deletion of at least 100 bp in the *Eco*RI-*Hin*dIII fragment which contains the *nifH* promoter. Two of these deletion mutants, plus six others which did not contain a detectable deletion but which consistently exhibited the highest levels of acetylene reduction activity among the presumptive mutants, were chosen for further analysis. Table 2 summarizes the acetylene reduction activity of these eight mutant plasmids.

The eight mutant plasmids (Table 2) were then tested for their ability to complement the chromosomal *nifH* mutation in the *recA* strain KP5805. A mutation on pSA30 which resulted in a significant reduction in the affinity of pSA30 for the *nifA*, *ntrA*, or *nifA* + *ntrA* products might also result in a major loss of promoter function. Therefore, such a mutant plasmid should not complement a chromosomal *nifH* mutation, despite the fact that pSA30 carries the entire *nifHDKY* operon. As expected, none of the eight mutants complemented the *nifH* mutation in strain KP5805 (Table 2).

DNA sequence analysis of mutant plasmids. The DNA sequences of the *nifH* promoter regions of the eight mutant plasmids listed in Table 2 were determined by the Maxam and Gilbert chemical sequencing method. The results are shown in Fig. 2D and E. The two plasmids containing detectable deletions (pSB82 and pSB88) contained an identical 112-bp deletion and are probably siblings since they originated from the same mutant selection experiment. Similarly, plasmids pSB74 and pSB75 contained the same single-bp change at position −136 and are also probably siblings. Plasmids pSB1, pSB5, and pSB30, contained single-bp changes in the −10 to −15 region in the CTGCA sequence (2, 29, 36). Plasmid pSB60 did not contain a mutation within the 525-bp region starting at the *Eco*RI site upstream from the *nifH* promoter and extending 69 codons into the *nifH* gene.

DISCUSSION

The structures of seven *K. pneumoniae nif* promoters and the *R. meliloti nifH* promoter were compared in (Fig. 3A). Because all of these promoters share the consensus sequence TTGCA in the −10 to −15 region, Beynon et al. (2) have suggested that this homology is equivalent to the −10 region of the standard *E. coli* promoter and plays an important role in the formation of the RNA polymerase initiation complex. The results reported here confirm the importance of the −10 region in *nifH* transcription. Three of eight

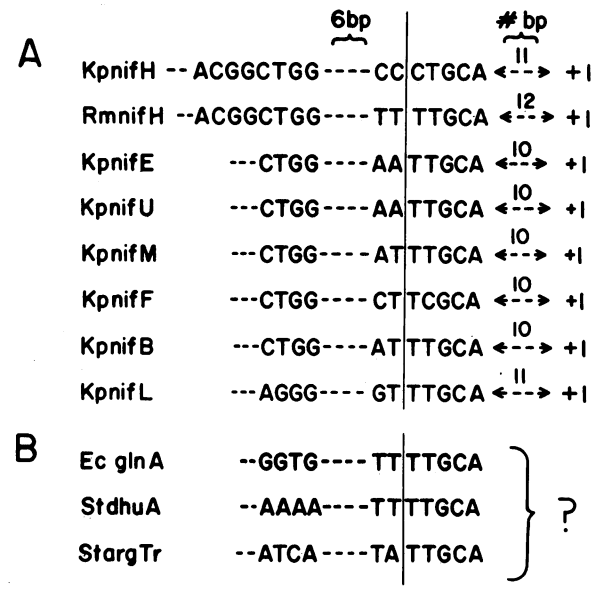

FIG. 3. Comparison of several promoters involved in nitrogen assimilation. (A) Sequences of the *K. pneumoniae nifH* (35), *nifL* (15, 29), *nifE*, *U*, *M*, *F*, *B* (2), and the *R. meliloti nifH* (35) promoters. (B) Sequences in the *E. coli glnA* (29), the *Salmonella typhimurium argTr* (arginine transport) (17), and the *S. typhimurium dhuA* (histidine transport) (17) promoter regions. The start points of transcription have not been determined for these latter genes. The vertical line marks the upstream boundary of the 5-bp consensus sequence TTGCA.

mutant plasmids that failed to inhibit *nif* expression contain single-bp changes in the −10 CTGCA sequence in the *nifH* promoter (Fig. 2E, underlining).

It has been proposed that the *nif* inhibitory phenotype is caused by the titration of limiting transcriptional activators (*nifA* products, *ntrA* products, or both) by multiple copies of the *nifH* promoter region (7); G. Riedel, Ph.D. thesis). If this hypothesis is correct, then the isolation of mutations in the CTGCA sequence is consistent with the idea that the action of the transcriptional activators is mediated, at least in part, through the −10 to −15 CTGCA sequence (2). Because the −10 region of *E. coli* promoters is required for initiation by RNA polymerase and because the *E. coli* consensus −10 region has no homology to CTGCA (see reference 16 for a recent compilation of *E. coli* promoter sequences), it is possible that the *nifA* product, in concert with the *ntrA* product, interacts with and modifies RNA polymerase so that it can recognize the CTGCA sequence. According to this model, the limiting factor would be a modified RNA polymerase, complexed with the *nifA* product, the *ntrA* product, or both.

The significance of the upstream mutations in plasmids pSB74, pSB75, pSB82, and pSB88 with respect to *nifH* promoter function is not clear at this time. Drummond et al. (15) observed a somewhat similar phenomenon in an analysis of the *K. pneumoniae nifL* promoter. They found that deletions starting at ca. −150 and extending upstream affected promoter activity, although not so dramatically as in the case reported here. One possibility is that these upstream mutations (defined by the single-base-pair change at position −136 in plasmids pSB74 and pSB75) are located in an essential binding site for a regulatory protein. Binding by the regulatory protein at this site would be required for an RNA polymerase-*nifA* product complex to bind to the −10 to −15 sequence, as suggested by T. Hunt and B. Magasanik (personal communication).

Although the mutation in an eighth plasmid, pSB60, drastically reduced the inhibitory phenotype of pSA30, we did not detect a mutation in pSB60 in the *nifH* promoter region. As was the case of the other mutant plasmids, there was no apparent change in the copy number of the plasmid. We have no explanation for the mutant phenotype of pSB60 at the present time. Because this plasmid contained a mutation outside of the *nifH* promoter region, it was formally possible that the other mutant plasmids also contained mutations outside of the sequenced region. We thought that this possibility was unlikely because all of the mutant plasmids were obtained spontaneously without mutagenesis.

All eight of the mutant plasmids which failed to inhibit *nif* expression also failed to complement a chromosomal *nifH* mutant, suggesting that these mutations blocked transcription of the *nifHDKY* operon. This result is consistent with the interpretation that the mutations define important structural components of the *nifH* promoter. Nevertheless, this interpretation could only be considered tentative, owing to the fact that it was not possible to perform the most suitable positive control for the complementation experiments. This would require a plasmid containing the *nifHDKY* operon under the control of a strong constitutive promoter; such a plasmid is currently not available. On the other hand, both Riedel (Ph.D. thesis) and Buchanan-Wollaston et al. (7) have shown that in a cell containing a chromosomal *nifH* promoter and a multicopy plasmid carrying the *nifH* promoter region, the vast majority of *nifH* transcription occurs from the plasmid promoters. These latter experiments support the conclusion that the mutations we characterized blocked *nifH* transcription since a wild-type plasmid-borne *nifH* promoter was actively transcribed.

Bitoun et al. (3) have recently reported the isolation of a mutation at position −7 in the *K. pneumoniae nifH* promoter region which results in the partial constitutive expression of a *nifH-lacZ* fusion. At this time, it is difficult to interpret the significance of this result in the light of the promoter region mutations which we have reported in this paper.

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