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Mutational Analysis of the *Klebsiella pneumoniae* Nitrogenase Promoter: Sequences Essential for Positive Control by *nifA* and *ntrC* (*glnG*) Products

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***ntr* (nitrogen regulated) and *nif* (nitrogen fixation) promoters are structurally similar to each other but bear no resemblance to canonic *Escherichia coli* promoters. *ntr* promoters are normally activated by the *ntrC* (*glnG*) product, but they can also be activated by the *ntrC*-related *Klebsiella pneumoniae nifA* product. In contrast, *nif* promoters of *K. pneumoniae* such as the nitrogenase (*nifH*) promoter can only be *nifA* activated. In this paper, we report the isolation and characterization of 28 mutants of the *K. pneumoniae nifH* promoter. Class A mutants no longer respond to *nifA*-mediated transcription, and class B mutants can now respond to *ntrC*-mediated activation. These two classes of mutants define sequences important to *nifA*- and *ntrC*-mediated transcription. Most surprising is that a single base change is sufficient to convert a *nifA*-activated promoter into an *ntrC*-activated one.**

In enteric bacteria, a large set of nitrogen assimilation genes is activated in response to deprivation of fixed nitrogen (for a review, see reference 22). These genes are, for example, those involved in the transport and catabolism of certain amino acids (20, 22). In *Klebsiella pneumoniae*, an additional nitrogen assimilatory pathway is available: the direct reduction of dinitrogen. Activation of these N-assimilatory pathways requires two regulatory products: *ntrA*, also known as *glnF* (11, 12), and *ntrC*, also known as *glnG* (23, 19, 29). As for the *nif* (nitrogen fixation) genes of *K. pneumoniae*, *ntrA* and *ntrC* products act at the *nifLA* promoter (10, 27). The *nifA* product in turn activates all other *nif* genes (6, 9, 33, 34, 38).

Similarities between *nifA* and *ntrC* have been observed (24, 27). (i) The *nifA* product can activate *ntrC*-regulated promoters. (ii) The *nifA* product, like the *ntrC* product, requires the *ntrA* product as co-activator. (iii) The *nifA* and *ntrC* proteins are similar in size and charge. (iv) Both genes are the downstream gene of a two-gene regulatory operon. It has been proposed that the chromosomal duplication event that is believed to have occurred during the evolution of enteric bacteria (32) had at one point provided an additional copy of *ntrC*, from which evolved a *nif*-specific regulator (27). The rationale for a *nif*-specific regulator is the advantage of an additional level of stringent control over this most ATP-expensive pathway for obtaining fixed nitrogen. In addition, because the *nifA* product has the capacity to activate other nitrogen metabolism genes, it may act as a general regulator of nitrogen metabolism genes during active nitrogen fixation. Since *K. pneumoniae nif* promoters, aside from *nifLA*, cannot be activated by the *ntrC* and *ntrA* (9, 21), but only by the *nifA* and *ntrA* products, they will henceforth be referred to as *nifA*-activated promoters, in contrast to *ntrC*-activated promoters that can be activated via either *ntrC* or *nifA*.

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The ancestral relationship between the *nifA* product of *K. pneumoniae* and the *ntrC* product of enteric bacteria is further reflected by *nifA*-like or *ntrC*-like *nif* regulatory systems in other nitrogen-fixing bacteria. In *Azotobacter vinelandii*, an aerobic diazotroph, the *K. pneumoniae nifA* product can complement *nif* regulatory mutants, suggesting a *nifA*-like product in the wild-type host (18). However, whereas the *K. pneumoniae nifLA* and *nifF* promoters are expressed when introduced into this host, the *K. pneumoniae nifH* (nitrogenase) promoter is not (35). In this respect, the *Azotobacter nif* gene activator bears a resemblance to the *ntrC* product of enteric bacteria. An analogous case is found with *Rhizobium meliloti*, a symbiotic nitrogen-fixing species. When the *R. meliloti nifH* (nitrogenase) promoter is introduced into an *Escherichia coli ntrA*$^+$ host, it can be activated by either *ntrC* or the *K. pneumoniae nifA* product (40, 41); conversely, when the *K. pneumoniae nifH* promoter is introduced with *R. meliloti*, it is not expressed in the plant root nodule (42). Thus, whereas a strict requirement for the *K. pneumoniae nifA* product appears to be the case for the *K. pneumoniae nifH* promoter, the *R. meliloti nifH* promoter can respond to a number of *nifA*/*ntrC*-like products; and at least in *E. coli*, it behaves as a typical *ntrC*-activated promoter.

Comparison of the nucleotide sequences of *ntrC*-regulated promoters, which can also be activated by the *nifA* product, revealed a consensus heptameric sequence of TTTTGCA at the −15 region (28). This sequence is also found in several other *R. meliloti nif* promoters (3) that are presumably regulated in a fashion similar to that of the *R. meliloti nifH* promoter (42). In contrast, promoters that are activated by the *nifA* product, but not by the *ntrC* product, share the consensus sequence CTGG-8bp-TTGCA between −26 and −10 (4, 40; reviewed in reference 1). Because both *nifA*-and *ntrC*-activated promoter sequences differ from the canonical *E. coli* −35 (TTGACA) and −10 (TATAAT) promoter sequences (14, 36), it is tempting to speculate that transcriptional control in these promoters differs from that in those previously studied. Thus, seeking the molecular basis of *ntrC*- and *nifA*-mediated gene activation is not only of key

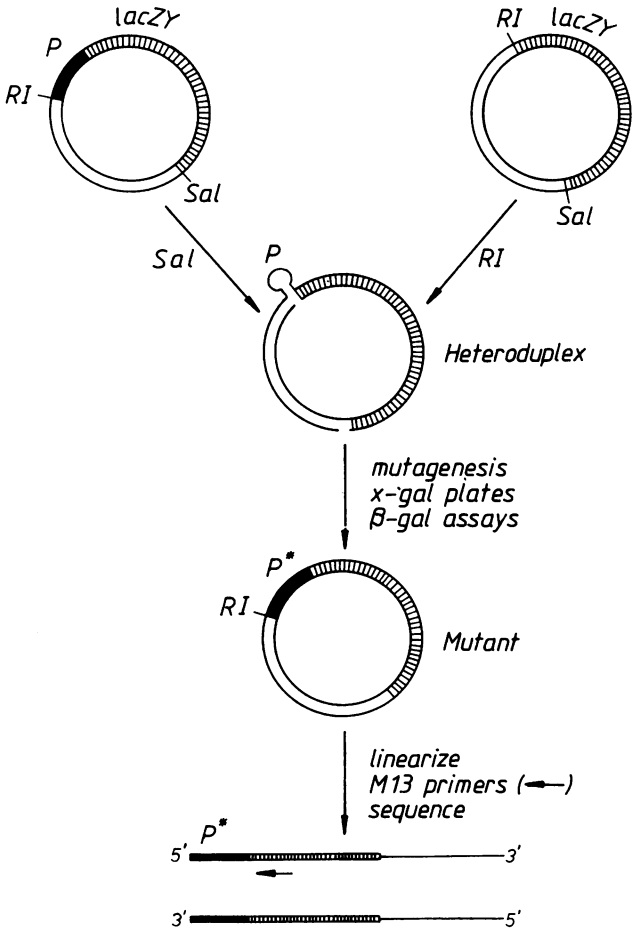

FIG. 1. Strategy for identifing *cis*-regulatory sequences. The *nifH* promoter-*lacZ* fusion plasmid pVSA2 (left) and the parental promoterless plasmid pMC1403 (right) were used for heteroduplex formation. After mutagenesis, promoter mutants were assayed for gene expression and subjected to sequence analysis, using M13 primers.

importance in understanding how a large class of nitrogen fixation and nitrogen metabolism genes is regulated, but in addition, a potentially novel mode of gene regulation may be uncovered.

In this paper, we report on the genetic and sequence characterization of 28 mutants obtained via site-directed chemical mutagenesis of the *K. pneumoniae nifH* promoter. Class A mutants fail to respond to *nifA*-mediated activation, and class B mutants can now be activated by the *ntrC* product. Mutants of classes A and B, respectively, define specific nucleotide base pairs important to *nifA*- and *ntrC*-mediated transcription. Most interesting of all, we found that a single base change can alter the specificity between *nifA*- and *ntrC*-mediated gene activation. Our experimental approach, involving a single promoter-*lacZ* fusion plasmid for mutagenesis, mutant isolation, assay for gene expression level, and, finally, DNA sequencing, demonstrates the relative ease of identifying *cis*-regulatory signals.

MATERIALS AND METHODS

Preparation of DNA. Plasmid DNA was prepared by the alkaline lysis procedure essentially as described previously (16), except that additional steps were included: (i) phenol-$CHCl_3$ extraction, (ii) $CHCl_3$ extraction, (iii) ethanol precipitation, and finally, (iv) Sephadex G-50 spun column chromatography. Total DNA was prepared by a simplification of the Marmur procedure as described previously (26).

Site-directed mutagenesis. The strategy of sodium bisulfite mutagenesis within deletion loops of DNA heteroduplexes was adapted as described previously (17, 30). Heteroduplexes were formed between (i) pVSA2 plasmid DNA (containing the *nifH* promoter) (41) linearized at the *Sal*I site and (ii) its parental promoterless pMC1403 DNA (8) linearized by *Eco*RI (Fig. 1). pVSA2-pMC1403 heteroduplex formation exposes to sodium bisulfite a 294 base pair promoter-containing sequence. Plasmid DNA was denatured in 0.1 M NaOH for 15 min at 25°C and renatured by the addition of 0.17 volume of 1 M Tris-hydrochloride (pH 7.5) and 0.9 volume of 1 M HCl for 3 h at 60°C. Renatured DNA was precipitated by ethanol, and mutagenesis was carried out as described previously (13). The DNA was incubated in 1.5 M sodium bisulfite (pH 6.1)–4.6 mM hydroquione (1.5 or 2.5 h at 37°C, in the dark) and immediately dialyzed in two changes of 5 mM KPO_4 (pH 6.8)–3 mM hydroquinone (16°C, 6 h), two changes of 0.2 M Tris-chloride (pH 9.2)–50 mM NaCl–2 mM disodium EDTA (37°C, 16 h), and finally in TE (10 mM Tris-hydrochloride (pH 8)–1 mM disodium EDTA) at 16°C for 4 h. The DNA was precipitated by ethanol, suspended into 200 μl of TE, and partitioned into 16 aliquots for separate transformations into BD1528 (*E. coli thyA met nadBF gal supE supF hsdR ungI*) (30). Plasmid DNA from Ap^r transformants of BD1528 was used to retransform strains of the appropriate genetic background for the selection of *nifH* promoter mutants.

Isolation of mutants. (i) Class A mutants. YMC9 (*E. coli thi endA hsdR Δlac U169*) (2) harboring pD0504, a Tc^r ColE1 compatible and low-copy-number plasmid carrying *nifLΔA*$^+$ (28) was transformed with mutagenized pVSA2 DNA. Pale blue colonies were visualized on indicator plates: KG agar medium supplemented with limiting ammonia [0.2 mg of $(NH_4)_2SO_4$, 20 μg of X-gal 100 μg of ampicillin, and 20 μg of tetracycline per ml]. KG is the nitrogen-free K medium (34) but with 0.4% glucose instead of sucrose. Beta-galactosidase and Nif inhibition (acetylene reduction) assays were performed as described previously (27, 31), except that KG medium was used in place or NFDM. For beta-galactosidase assays, cells were *ntr*-derepressed (starved for fixed nitrogen) aerobically.

(ii) Class B mutants. YMC9 transformed with mutagenized pVSA2 DNA was screened for the formation of dark blue colonies on indicator plates with limiting ammonia [0.2 mg of $(NH_4)_2SO_4$ per ml], but light blue colonies formed on indicator plates with high ammonia [2mg of $(NH_4)_2SO_4$ per ml]. Some mutants were unaffected by the ammonia concentration (400 series). These mutants were further examined in host YMC12 (YMC9 but *hutC ntrC*::Tn*5*; 2). Beta-galactosidase levels were determined as described above.

DNA sequencing. We adapted the use of double-stranded plasmid DNA as template (39) for the chain-termination sequencing method (37). pVSA2-derived plasmids were linearized with *Eco*RI. Two exceptions were mutants 317 and 319, which were linearized at the *Sal*I site because they became resistant to *Eco*RI cleavage. Presumably they had acquired a base change(s) at the *Eco*RI site. M13 primers (15-mer; New England BioLabs) were used for primed synthesis of the antisense strand beginning from the 12th codon of *lacZ*, which corresponds to +50 for pVSA2 derivatives, where +1 is the start of transcription (40).

Linearized double-stranded DNA template (0.3 pmol) and M13 primer (6 pmol) were boiled for 3 min in a sealed capillary tube and quenched immediately on ice for 3 min (39). Reactions were carried out by previous procedures (37), using dideoxy- to deoxynucleotide ratios of 100 to 1 and 1 μCi of label per reaction. Samples were run on 8% gels, and X-ray films were exposed at −70°C for 1 to 3 days with an intensifying screen.

RESULTS

Specific strategy. To facilitate the detection of mutant promoters, we employed the *nifH* promoter-*lacZ* fusion plasmid pVSA2 (41). An adaptation of the sodium bisulfite deletion loop mutagenesis procedure (17, 30) was used. pVSA2 and its parental plasmid pMC1403, which contains only the *lacZ* gene but is devoid of the *nifH* promoter (8), were linearized with restriction enzymes (Fig. 1). After denaturation and renaturation, the DNA molecules were treated with sodium bisulfite that preferentially modified the cytosine residues for the single-stranded promoter region.

The in vitro-modified DNA molecules were transformed into an *E. coli ung*$^-$ host. Due to a defective uracil N-glycosylase gene, this host allows for the replication of molecules containing uridine residues. Because linear homoduplexes transform poorly, transformants would arise predominantly from heteroduplex molecules. Upon subsequent replication, sodium bisulfite-modified molecules would produce mutant progeny molecules with G-C to A-T transitions. Plasmid DNA prepared from these primary transformants was then used to retransform strains of the appropriate genetic background. Promoter mutants were detected on agar plates containing the appropriate medium and the chromogenic compound X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside). Gene expression level was determined by assaying for beta-galactosidase.

To determine the nucleotide base changes in a large collection of mutants, we adapted the technique first introduced by Smith et al. (39), in which double-stranded plasmid DNA and oligonucleotides are used for template and primers, respectively, for the chain-termination sequencing method (37). Due to the proximity of the promoter to the *lacZ* gene, commercially available M13 primers were used for primed synthesis of the promoter region.

Class A: mutants that are insensitive to *nifA*-mediated activation. pVSA2 carries the *nifH* promoter of *K. pneumoniae* fused to the *lacZ* gene of *E. coli* as described previously (41). This promoter can be positively regulated by the *K. pneumoniae nifA* product but not by the *ntrC* product. The source of *nifA* product can be provided by the ColE1-compatible, low-copy-number plasmid pD0504, which carries *nifLΔA*$^+$ (28). Part of the *nifL* structural gene on this plasmid had been deleted; this gene encodes a *nif*-specific repressor that mediates repression by oxygen and ammonia (7, 15, 25). Activation of the *nifLΔA*$^+$ transcription unit is dependent on *ntrC* and *ntrA* products when the host cell is starved for fixed nitrogen. Hence, YMC9 harboring both pD0504 and pVSA2 forms blue colonies on X-gal plates that contain low concentrations of ammonia.

To facilitate the isolation of independently derived mutants, separate aliquots of mutagenized pVSA2 DNA were independently introduced into YMC9-pD0504. Light blue transformants were visually selected on indicator plates. For confirmation that presumptive mutant clones were of pVSA2 and not of pMC1403 origin, plasmid DNA from presumptive mutant clones were subjected to restriction enzyme analysis. Then plasmid DNA from these clones was used to retransform YMC9-pD0504. The secondary transformants were then assayed for beta-galactosidase after growth in liquid medium with ammonia, followed by a 10-h incubation period in medium with or without ammonia (to derepress the *ntr* system). Nineteen independent mutants were isolated and assigned allele numbers of the 200 series. Table 1 shows the basal levels of activity when mutants were incubated in ammonia-rich medium (in which the *nifLΔA*$^+$ transcription unit is not activated by the *ntr* system). No significant differences were found between mutant and wild-type promoters. In contrast, when the *nifA* product was expressed in ammonia-limiting medium, none of these mutants expressed more than a third of the pVSA2 level of activity. For confirmation that a low level of expression was not due to a copy number change, an equivalent amount of total DNA prepared from each plasmid-containing host strain was cleaved by *Eco*RI and fractionated in an agarose gel. The amounts of chromosomal versus plasmid DNA were qualitatively determined (data not shown). We found no apparent change of copy number in our collection of mutants.

To determine the nucleotide base change(s), mutant plasmid DNA was linearized at the *Eco*RI site that lies ca. 220 base pairs 5′ upstream of the promoter, which in turn lies some 60 base pairs away from the 12th codon of *lacZ*. Commercially available M13 primers were used for primed synthesis of the antisense strand of the promoter. In this fashion, we determined the nucleotide sequence from −100

TABLE 1. Expression of class A mutants[a]

Allele	YMC9-pD0504 (*ntrC*$^+$/*nifLΔA*$^+$) β-galactosidase units			KP5614 (wild type) nitrogenase activity
	+N	−N	% −N	
203	9	218	12	++
221	13	327	17	+
228	12	339	18	+
201	44	530	28	+/−
213	13	212	14	+
218	3	343	18	++
230	7	446	23	++
231	7	286	15	++
235	8	250	13	+
237	5	139	7	++
209	15	19	1	++
222	8	10	1	++
207	8	14	1	++
234	1	2	<1	++
215	12	308	16	++
217	1	3	<1	−
224	3	135	7	+/−
202	1	26	1	−
206	6	260	14	++
pVSA2 wild type	21	1,880	100	−
pMC1403	<1	<1	<1	++

[a] Beta-galactosidase units shown are mean values from at least three independent experiments. The presence or absence of NH_3 in the medium is indicated by +N or −N, respectively. For comparison, beta-galactosidase activity under N-limiting derepression is also shown as % of wild-type activity. Nitrogenase activity (acetylene reduction) is qualitatively expressed as −, +/−, +, or ++, indicating ca. <15, <30, ≥30, or ≥50%, respectively, of the activity found in KP5614 harboring the control plasmid pMC1403. − and +/− signs can be interpreted as having Nif inhibitory effects.

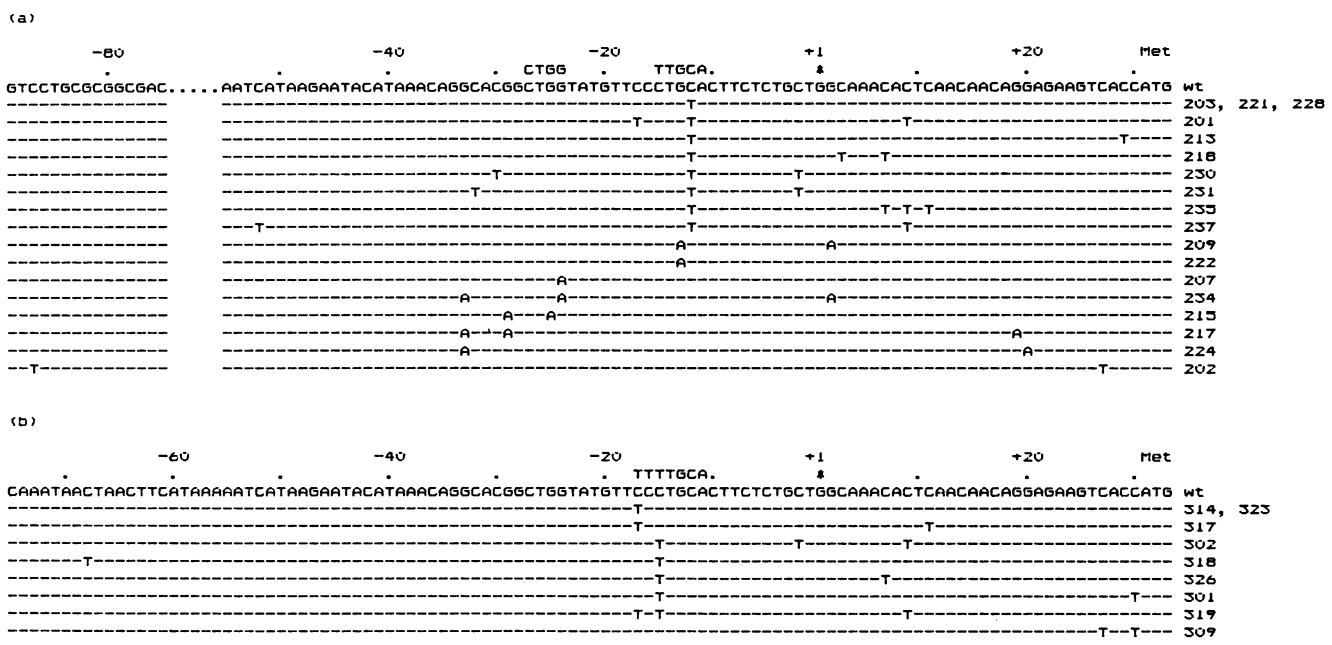

FIG. 2. (a) Nucleotide sequences of *K. pneumoniae nifH* promoter mutants (class A) that do not respond to *nifA*-mediated activation. The consensus *nifA* regulatory sequence CTGG-8bp-TTGCA (4) is shown above the wild-type (wt) promoter sequence (40). Base changes found in mutant promoters are shown below the wt sequence in the order given in Table 1. The regions from −100 to −90, −74 to −56, and +34 to +40 are not shown since no mutations were found there. (b) Nucleotide sequence of *K. pneumoniae nifH* promoter (class B) mutants that respond to *ntrC*-mediated transcription. The consensus sequence TTTTGCA (28) is shown above the wt promoter sequence. Base transitions of class B mutants are shown below the wt sequence in the order given in Table 2. No mutations were found between regions −100 to −76 and +34 to +40.

to +40, where +1 and +31 are the start points of transcription and translation, respectively (40). From the sequence data, we divided the class A mutants into six subclasses. Class A1 is represented by 10 mutants that share in common a C to T base change at −12 (Fig. 2a). Although seven of these mutants also have base changes elsewhere, only a single base alteration at −12 was found in the remaining three mutants (203, 221, and 228). These three mutants were independently isolated by the criteria that they were derived from different aliquots of mutagenized DNA. As a group, they showed expression of beta-galactosidase that varied from 7 to 28% of the wild-type level (Table 1), whereas the three single point mutants gave levels from 12 to 18%.

Class A2 mutants (209, 222) have in common a G to A transition at −13; mutant 222 is a single point mutant. Class A3 is represented by mutants 207 and 234 that have in common a G to A base change at −24; and mutant 207 is a single point mutant. Both classes A2 and A3 mutants express beta-galactosidase at ca. 1% of the wild-type level. Class A4 is represented by a single mutant (215) that has G to A transitions at −25 and −29 and whose expression level is 16%. Class A5 mutants 217 and 224 have base alterations at the ribosome binding site.

Mutants 202 and 206 are classified into the last subclass. Mutant 202 has C to T transitions at position −87 and +29. Whether and how mutations at these positions can affect transcription is not known. With mutant 206, no base changes were found between −100 and +40. One possible explanation for this anomaly is that another *cis*-regulatory locus lies outside the −100 to +40 region and that with both mutants, a base alteration(s) occurred in that hypothetical second *cis*-regulatory site.

It is known that the *nifH* promoter on a multicopy plasmid inhibits the N_2-dependent growth of *K. pneumoniae*, presumably by titrating away *nifA* products essential for the transcription of chromosomal *nif* genes (7, 31). In principle, one should be able to quantitate the level of "Nif inhibition" by measuring nitrogenase activity (acetylene reduction), and thus correlate promoter strength with the level of Nif inhibition. However, we found that this was not possible as data obtained from Nif inhibition assays were highly variable. Nevertheless, we were able to qualitatively determine whether the mutant promoters exert Nif inhibitory effects. When class A5 mutants (ribosome binding site mutants) were introduced into wild-type *K. pneumoniae* KP5614, they retained Nif inhibitory effects (Table 1), consistent with the assumption that only translation, not transcription, was affected. Nearly all other class A mutants did not exhibit significant Nif inhibitory effects; this is consistent with the conclusion with *nifA*-mediated transcription has been reduced by specific base changes.

Class B: promoter mutants that respond to nitrogen (*ntrC*-mediated) control. Under conditions of nitrogen limitation, the *ntrC* and *ntrA* products can activate a number of nitrogen-regulated promoters, but not the *K. pneumoniae nifH* promoter. This promoter, like all *K. pneumoniae nif* promoters except the *nifLA* promoter, responds only to *nifA* plus *ntrA* products. The functional similarity between the *nifA* and *ntrC* products prompted us to select for *K. pneumoniae nifH* promoter mutants that could be activated by the *ntrC* product. Aliquots of mutagenized pVSA2 plasmid DNA were independently introduced into YMC9. Due to the absence of the *nifA* product, YMC9 harboring wild-type pVSA2 forms very pale blue colonies on indicator plates (limiting ammonia plates with X-gal). Dark blue transformants, however, would indicate that either (i) a mutation

rendered the promoter sensitive to *ntrC*-mediated activation, or (ii) transcription is directed by a newly formed constitutive promoter.

Plasmid DNA prepared from blue colonies was analyzed by restriction enzymes before re-transformation into YMC9. Beta-galactosidase assays were determined for presumptive mutants derepressed in medium with or without ammonia. Clones that expressed beta-galactosidase at a significant level in medium containing either high or low concentrations of ammonia were classified as presumably having acquired a newly formed constitutive promoter. Of this latter group (400 series) we chose two clones for further analysis (see below).

Nine class B mutants (300 series) responded to activation when incubated in ammonia-limiting medium but had relatively low activities when incubated in ammonia-rich medium (Table 2). Furthermore, these class B mutants were unable to express beta-galactosidase at an appreciable level when introduced into the *ntrC*$^-$ host YMC12. In this respect, they resembled the activity of the *ntrC*-activated *R. meliloti nifH-lacZ* fusion plasmid pVSP9 (Table 2). In contrast, the two 400 series mutants were unaffected by the absence of a functional *ntrC* product, supporting the conclusion that they had acquired an *ntrC*-independent constitutive promoter.

Class B mutants were further examined in host YMC12-pST1021 to see whether the mutations affected *nifA*-mediated transcription. pST1021 expresses the *nifA* product from the *tet* promoter of pACYC184 (43). We found no significant difference in *nifA*-mediated expression between class B mutants and parental pVSA2 (data not shown).

As described for class A mutants, the promoter regions (−100 to +40) of the class B mutants were sequenced with the aid of M13 primers. These mutants were divided into four subclasses (Fig. 2b). Class B1 mutants (314, 323, 317) have in common a C to T transition at position −17, among which mutants 314 and 323 are independently derived single point mutants. When assayed for the expression of beta-galactosidase, these mutants averaged 123 units of activity during N-limitation (Table 2). Class B2 is represented by four mutants with a common C to T transition at −15. With the exception of mutant 301, which has an additional base change at position +30, this class averaged 256 units of activity. Class B3 is represented by mutant 319, which gave a high level of expression that even surpassed the level given by the similarly constructed but nonisogenic plasmid pVSP9, which contains the *R. meliloti nifH-lacZ* fusion.

Class B4 is represented by a single mutant (309) that showed mild activation by the *ntrC* product (Table 2). This mutant has C to T transitions at +27 and +30; mutant 301 also contains the same base change at +30. Mutant 301 deviates from other class B2 mutants in that this additional base change resulted in elevated expression. It therefore seems possible that the sequence around +30 is important to nitrogen regulation. The fact that this region is downstream from the point of transcription initiation raises the possibility of posttranscriptional control. For example, the base change at +30 could have conferred a more stable mRNA under N-limiting growth that would result, as in the case of mutant 309, in an increased level of translation from the same basal level of transcription.

TABLE 2. Expression of class B mutants

Allele	β-Galactosidase units[a]			
	YMC9 (*ntrC*$^+$)		YMC12 (*ntrC*$^-$)	
	+N	−N	+N	−N
314	13	131	14	21
323	11	119	12	16
317	9	120	10	14
302	4	317	2	1
318	7	192	9	11
326	6	258	7	8
301	20	533	13	19
319	19	559	13	20
309	14	58	17	21
406	78	73	94	103
412	70	75	68	83
pVSA2 wild type	8	16	7	11
pVSP9 wild type	46	347	64	61
pMC1403	<1	<1	<1	<1

[a] All values are the means from three independent experiments. The presence or absence of NH_3 in the medium is indicated by +N or −N, respectively.

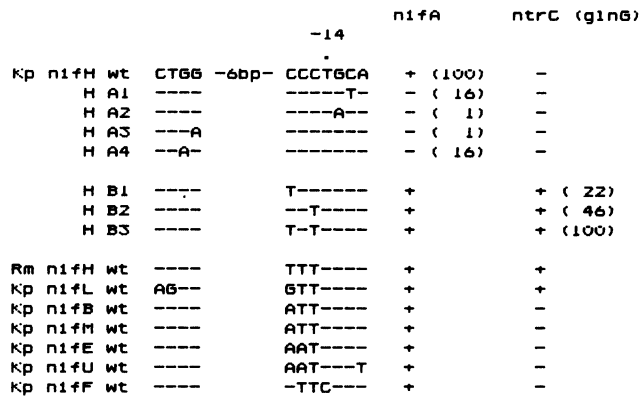

FIG. 3. Comparison of sequences essential for *nifA*- and *ntrC*-mediated positive control. + and − signs signify, respectively, activation and lack of activation by the *nifA* and *ntrC* products. For isogenic promoters, the approximate promoter strength is given in parentheses relative to the *Kp nifH* wild-type (wt) and class B3 promoters. Base differences from the reference promoter (Kp *nifH* wt) are indicated by respective nucleotide bases. Abbreviations: Kp, *K. pneumoniae*; Rm, *R. meliloti*; A1 to A4 and B1 to B3, the various mutant classes. Sequences were compiled from references 4, 28, and 40 and this paper. −14, relative to transcription, is shown above for the reference promoter and corresponds to −13 or −15 for other promoters.

DISCUSSION

Sequences affecting *nifA*-mediated transcription. It was first noted by Sundaresan et al. (40) that the sequence ACGGCTGG-9bp-TGCAC is found between positions −31 and −11 in the *nifH* promoters of both *K. pneumoniae* and *R. meliloti*. Since both promoters can be activated by the *nifA* product, this sequence was hypothesized to be involved in *nifA* regulation. In a more recent study, Beynon et al. (4) compared the primary structures of five additional *nifA*-activated promoters and identified a promoter consensus sequence which is a subset (CTGG-8bp-TTGCA) of the above sequence. Because the *K. pneumoniae nifH* promoter contains CTGCA instead of TTGCA, and because it is among

the most actively transcribed *nifA*-regulated promoters, it is likely that the C substitution does not negatively affect promoter strength. In this study, we describe four types of base changes in the consensus sequence that reduced transcription (Fig. 3). Of the five possible types of base transitions that can be obtained via sodium bisulfite mutagenesis of the consensus sequence, we only failed to find the change from CTGG to TTGG among our 19 mutants. It is possible that a C to T transition at this position has little, if any, effect on *nifA*-mediated transcription.

Based on the expression of beta-galactosidase, it is possible to tentatively assign promoter strength values to the various subclasses of mutants (Fig. 3). Recently, several *K. pneumoniae nifH* promoter mutants with base alterations at the CTGCA sequence were also reported by Brown and Ausubel (5). Isolation of these mutants was based on the inability of the mutant promoters to inhibit *nif*-dependent growth. Presumably, multicopies of these mutant promoters failed to titrate *nifA* activators away from chromosomal *nif* genes (7, 31). Although data on promoter strength were not available, these mutants are in good agreement with our results.

Our class A6 mutants are difficult to interpret. Similarly, Brown and Ausubel (5) also reported two mutants that exhibit reduced *nif*-inhibitory effects but do not contain base alterations in the −100 to +40 region. Instead, one mutant has a base change at −136 and the other has a deletion covering the −136 area. It is possible that there is another positive regulatory site around −136, perhaps an *ntrA* regulatory site. In light of these results, our data on the class A mutants cannot rule out the possibility that other regulatory sequences have been mutated in addition to those found within the −100 to +40 region.

Sequences affecting nitrogen (*ntrC*-mediated) control. The consensus heptameric sequence (TTTTGCA) for *ntrC*-activated promoters was identified by comparing six nitrogen-regulated promoters (28). However, the actual start points of transcription had not been defined for three of them. To establish genetic confirmation, one approach would be to mutate this sequence in an *ntrC*-activated promoter and determine its effect on transcription. An alternative strategy, which we report in this study, is to take advantage of the structural similarity between *nifA*- and *ntrC*-activated promoters to change a *nifA*-activated promoter into an *ntrC*-activated one.

Results from this latter approach are summarized in Fig. 3. Based on the previous sequence comparison study (28), we expected that at least two C to T transitions in the *K. pneumoniae nifH* CCCTGCA sequence would be required to obtain *ntrC*-mediated activation. Mutant 319 is such a case. Surprisingly, however, a single transition at the first or third position of the heptamer also allows *ntrC*-mediated transcription, with the change at the third position giving stronger *ntrC*-specific activation.

Class B2 mutants, which bear the CCTTGCA heptamer, were highly unexpected because the TTGCA sequence is also found in four other *K. pneumoniae nif* promoters (*nifB*, *M*, *E*, and *U*) that are activated by the *nifA* product but not by the *ntrC* product (Fig. 3). The difference between the class B2 mutants from these four promoters appears to be the nucleotide base at the first position of the heptameric sequence. It is possible that *ntrC*-mediated activation can tolerate T, C, or G (as in the *nifL* promoter), but not an A at this position. The first position of the *nifF* heptamer is also a C, but in this case, the lack of *ntrC*-mediated transcription may be due to the imperfect TGCA subset of the heptameric sequence (Fig. 3). In this regard, we point to the sequence TCCTGTA found in class A mutant 201. The C to T change at the first position of the heptamer should have conferred *ntrC*-mediated expression, but significant activity was not detected in YMC9 under N-limiting conditions (data not shown). It appears that the C to T transition at the 6th position of the heptamer affects both nifA- and ntrC-mediated activation. Finally, we were most surprised by mutants 309 and 301, which indicate the possibility of nitrogen control at the +30 region.

Conclusions. It appears that the *nif* genes of taxonomically unrelated bacteria are regulated by *nifA–ntrC*-like activators (reviewed in reference 1). How closely related these regulatory proteins are to the *K. pneumoniae nifA* or the *E. coli ntrC* product is of fundamental interest, both in terms of understanding the evolutionary divergence of gene regulatory elements and in understanding the molecular basis of nitrogen regulation. A possible approach to answering this question is to test mutant promoter activation with heterologous "*nifA*" products. As a first step, we have identified the class B mutants. Efforts are underway to investigate their promoter strengths in heterologous hosts.

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