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Deletion Analysis of the *Klebsiella pneumoniae* Nitrogenase Promoter: Importance of Spacing between Conserved Sequences around Positions −12 and −24 for Activation by the *nifA* and *ntrC* (*glnG*) Products

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The nitrogen fixation promoters of *Klebsiella pneumoniae* are atypical procaryotic promoters lacking the usual −10 and −35 elements, requiring instead conserved sequences around −12 and −24 for transcriptional activation. By constructing a set of five deletions between the −12 and −24 elements in the *nifH* promoter, the spacing between the conserved GC and GG motifs at −12 and −24, respectively, has been reduced from the wild-type 10 bases to 9, 8, 6, 5, and 4 bases. The deletion of a single nonconserved nucleotide was sufficient to eliminate transcriptional activation by either *nifA* or *ntrC* (*glnG*). All deletions relieved the multicopy inhibition of chromosomal *nif* expression normally shown by the *nifH* promoter. These results demonstrate a stringent requirement for the 10-base spacing found in *ntr*-activated promoters. In addition, specific sequences around the invariant GG at −24 were shown to be necessary for activation by either *nifA* or *ntrC*, with a minimal requirement for nucleotides through to position −27 for this activation.

Two features of *Escherichia coli* promoters are known to be important for transcriptional activity: (i) the two hexanucleotide sequences centered around positions −35 and −10 and (ii) the distance between these two sequences (36). The most frequently occurring sequences at −35 and −10 are TTGACA and TATAAT, respectively, referred to as the −35,−10 consensus sequence. Point mutations in these sequences often lead to a promoter down phenotype. Promoters which are relatively active in transcription tend to conform to the consensus sequence, whereas weaker promoters tend to deviate from the consensus sequence. The strength of a promoter conforming to the −35,−10 consensus sequence is also markedly influenced by the spacing between the regions of sequence conservation. Naturally occurring *E. coli* promoters typically display a spacing of 17 ± 1 base pairs (bp) (20). Deletions which reduce this spacing have been shown to reduce the activity of promoters (2, 4, 27, 34), whereas the efficiency of the *ampC* promoter was improved by increasing the spacing to 17 bp, but was reduced by further increasing the spacing to 18 bp (21). Increasing the spacing in the *lac* promoter from 18 to 20 bp similarly reduced expression (24; see also reference 32). Thus, a considerable body of evidence suggests an optimal spacing of 17 bp, but it should be recalled that the strong rRNA promoters have a 16-bp spacing (20), indicating that additional DNA sequences and proteins other than RNA polymerase can modulate promoter activity.

Recently a new class of procaryotic promoter was identified. These promoters lack the usual consensus sequences and instead show sequence conservation around positions −12 and −24 (Table 1). Included in this new promoter class are the nitrogen fixation (*nif*) promoters of *Klebsiella pneumoniae* (5).Transcription from the *nif* promoters is positively activated and requires that the *ntrA* (*glnF*) gene product act in concert with either the *nif*-specific activator *nifA* or the functionally related *ntrC* (*glnG*) gene product (17). The glutamine synthetase (*glnA*) promoter (RNA1) is also regulated by the *ntr* system [as is the expression of a number of other genes involved in the utilization of poor nitrogen sources, including *put* (proline), *aut* (arginine), and *hut* (histidine) utilization, respectively (28)], and the sequence of this promoter shows the typical −12,−24 sequence conservation of an *ntr*-regulated promoter (18), as do the *nif* promoters of many rhizobia (1, 3).

Analysis of point mutations has shown that nucleotides recognized as conserved around positions −12 and −24 are necessary for activation of the *nifH* and *nifL* promoters (7, 10, 29). Thus, the sequences at −12 and −24 constitute functionally important elements of an *ntr*-regulated promoter. In addition to these elements, efficient expression of certain *K. pneumoniae nif* promoters requires specific sequences (characterized by a TGT N_{10} ACA motif) located more than 100 bp upstream of the transcription start (see Fig. 1) (11). The spacing between the −12 and −24 elements is invariant in all *ntr*-regulated promoters thus far sequenced, in contrast to the variation in spacing found in naturally occurring promoters of the −10,−35 form. In this study the influence of spacer length between the −12 and −24 elements in *nifH* promoter function is examined. Since the *nifH* promoter is activated by *ntrC*, albeit weakly with respect to *nifA* activation, and since this activation is independent of the upstream element required for efficient *nifA* activation of

TABLE 1. *ntrA*-activated *K. pneumoniae* promoters

Promoter	Promoter sequence (−24 … −12)	Reference
nifF	C T GG C A C A G C C T T C GC T	5
ORF	C T GG C A C A G G C T G C GC A	33
nifL	A G GG C G C A C G G T T T GC A	19
nifB	C T GG T A C A G C A T T T GC A	5
nifM	C T GG C C G G A A A T T T GC A	5
nifU	C T GG T A T C G C A A T T GC T	5
nifE	C T GG A G C G C G A A T T GC A	5
nifH	C T GG T A T G T T C C C T GC A	35
Consensus	C T GG C A C N_5 T T GC A	
glnA, RNA1	T T GG C A C A G A T T T C GC T	18

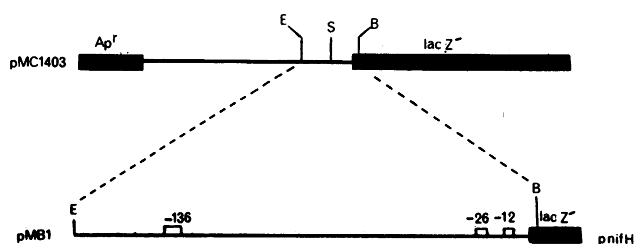

FIG. 1. The *K. pneumoniae nifH* promoter cloned into pMC1403. Relevant restriction sites are E, *Eco*RI; S, *Sma*I; and B, *Bam*HI. Boxes at −12 and −26 refer to the downstream promoter elements shown in Table 1, and the box at −136 refers to the upstream TGT-ACA promoter element. The *Pst*I site used in cloning promoter fragments for sequencing is upstream of the *Eco*RI site in the β-lactamase gene. Plasmid pMB1 and its derivatives carry 17 amino acids of *nifH* before the in-frame junction with *lacZ'* at the *Sma*I site in pMC1403 (10).

this promoter (10, 11), it was possible to distinguish between effects which result from changing the spacing between sequences around position −136 (Fig. 1) and the −12 element of the promoter (to which *nifA* activation may be sensitive) on the one hand, and those effects which result from changes in spacer length between the two downstream elements on the other (to which *ntrC* and *nifA* activation are both shown to be sensitive).

MATERIALS AND METHODS

Bacterial strains and plasmids. These are listed in Table 2. UNF932 is a spontaneous his^+ revertant of UNF931 (10).

Media. Complete medium was L broth (22). Nitrogen-free medium (NFDM) and minimal medium were as described previously (16). Plasmid selection was maintained with the appropriate antibiotic supplement: kanamycin (Km, 30 μg/ml), chloramphenicol (Cm, 15 μg/ml), carbenicillin (Cb, 100 μg/ml for *E. coli*) or carbenicillin plus ampicillin (200 μg/ml each for *K. pneumoniae*). L-Histidine (50 μg/ml), L-aspartate (100 μg ml), L-leucine (25 μg/ml), L-glutamine (200 μg/ml), vitamin B_1 (2.5 μg/ml), and NH_4SO_4 (2 mg/ml) were added where appropriate. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20 μg/ml) was used to detect Lac^+ colonies.

Cloning and sequencing. All enzymes were obtained from commercial sources and used according to the manufacturers' instructions. For sequencing, fragments were cloned into M13 mp9 (26) and dideoxy sequencing reactions were performed using [α-^{35}S]dATP as the label (6). Transformation was as described, except 50 mM $CaCl_2$ was used (14).

Plasmid construction. The spacer mutation plasmids described were derivatives of pMB3, a *nifH-lacZ* translational fusion in the vector pMC1403 (12). Plasmid pMB3 carries a silent point mutation at −18 which creates a unique *Rsa*I restriction site within the *nifH* promoter fragment (10) and is itself a derivative of the *nifH-lacZ* translational fusion plasmid pMB1 which carries the wild-type promoter sequence (Fig. 1 and Table 1). Plasmids pMB340 and pMB355, 1- and 2-bp deletion derivatives, respectively, of pMB3, were isolated in the following manner. The *Eco*RI-*Bam*HI *nifH* promoter fragment (Fig. 1) from pMB3 was isolated, digested with *Rsa*I (Bethesda Research Laboratories, Inc.) and religated into *Eco*RI-*Bam*HI-digested pMC1403 vector DNA using T4 DNA ligase (Bethesda Research Laboratories). The ligated DNA was used to transform *E. coli* MC1061, carbenicillin resistance was selected for, and cells were passaged twice in L broth plus carbenicillin from a 1:200 inoculum. Plasmid DNA was prepared from the final cell culture, and the cycle of restriction with *Rsa*I, ligation into pMC1403, transformation, and growth in the presence of carbenicillin was repeated twice more. Cells were finally plated on minimal medium with aspartate as the nitrogen source (to permit *ntrC* activation of the *nifH* promoter), and colonies displaying a Lac^+ phenotype different from that of pMB3 were chosen, checked for the *nifH* promoter fragment by restriction analysis, and then screened by dot-blot hybridization to determine the presence of a deletion (see below).

As a result of the deletion creating pMB355, a unique *Acc*I restriction site was created in the *nifH* promoter between the −12 and −24 elements. Plasmid pMB303 was generated by digesting pMB355 with *Acc*I, filling in the site using the Klenow fragment of DNA polymerase in the presence of all four deoxynucleotide triphosphates, restricting with *Eco*RI-*Bam*HI, and ligating this material into *Eco*RI-*Bam*HI-digested pMC1403. Plasmids pMB322, −323, −324, and −325 were generated in a similar manner except that *Acc*I-digested pMB355 was treated with S1 nuclease under conditions in which either its single- or double-stranded exonuclease activity was present (37). The nucleotide sequence of the promoter region of each plasmid was determined by cloning the *Pst*I-*Bam*HI fragment carrying the *nifH* promoter into M13 mp9 for DNA sequencing. The sequences delineating the deletion intervals generated in the *nifH* promoter are given in Table 3.

Plasmids pMB41 and pSM11 were constructed by digesting the *Eco*RI-*Bam*HI *nifH* promoter fragments from pMB4 and pMB3 with *Hae*III and *Rsa*I, respectively, and ligating this material into *Sma*I-*Bam*HI digested pMC1403 to yield *nifH'-lacZ* fusions which are subclones of the *nifH* promoter retaining nucleotides through to −27 (pMB41) and −18 (pSMM11), respectively. The *nifH* promoter fragment of pMB4 differs from the wild-type promoter sequence by the presence of a cytosine residue at −26 rather than thymine

TABLE 2. Bacterial strains and plasmids

Strains and plasmids	Characteristics	Reference
K. pneumoniae		
UNF926	Δ(*his-nif*)*2632* Δ*lac-2001 recA hsdR1 sbl-300*::Tn*10*	10
UNF932	Δ*lac-2001 recA hsdR1 sbl-300*::Tn*10*	This study
E. coli		
MC1061	*araD139* Δ(*ara leu*)*7697* Δ*lac-74 galU galK rpsL hsdR hsdM*$^+$	13
ET8000	*rbs gyrA hutCK*^{c} *lacZ*::IS*1* Mu cts62	23
ET8894	Δ(*rhaA glnA ntrB ntrC*)*1703 rbs gyrA hutCK*^{c} *lacZ*::IS*1* Mu cts62	23
ET8045	*ntrA208*::Tn*10 rbs gyrA hutCK*^{c} *lacZ*::IS*1* Mu cts62	23
Plasmids		
pMC1403	Translational fusion vector, Cb^r; pBR322 derivative	12
pMC71A	*nifA*^{c} *Cm*^{r}, *Sal*I insert in pACYC184	8
pMM14	*ntrC*^{c} *Km*^{r}, *Sma*I insert in pACYC177	25
pMB1	*nifH-lacZ'* translational fusion in pMC1403	10
pMB3	T to A transversion at position −18 in pMB1	10
pMB4	T to C transition at −26 in pMB1	10

TABLE 3. Spacer mutations in the *nifH* promoter

Plasmid	Relevant promoter sequence[a]	Method of construction
	−24 −12	
pMB1	ACGGCT GGT ATG TT CCCT GCACTT	Wild type
pMB3	ACGGCT GGT ATG TA*CCCT GCACTT	Oligonucleotide mutagenesis (10)
pMB340	ACGGCT GGT ATG T- CCCT GCACTT	Spontaneous deletion
pMB355	ACGGCT GGT AT- -A*CCCT GCACTT	Spontaneous deletion
pMB323	ACGGCT GGT--- -A*CCCT GCACTT	S1 nuclease digestion
pMB325	ACGGCT GGT--- -- CCCT GCACTT	S1 nuclease digestion
pMB324	ACGGCT GG---- -- CCCT GCACTT	S1 nuclease digestion
	−24 −10	
pMB322	ACGGCT GGT————Δ————CTT	S1 nuclease digestion
pMB303	ACGGCT GGT ATA*TA*CCCT GCACTT	Klenow fill-in
	−24 −12	
pMB4	ACGGCC*GGT ATG TT CCCT GCACTT	Oligonucleotide mutagenesis (10)
pMB41	CC*GGT ATG TT CCCT GCACTT	*Hae*III restriction
pSMM11	A*CCCT GCACTT	*Rsa*I restriction

[a] Each mutant *nifH* promoter sequence is aligned below the wild-type sequence, where +1 denotes the transcription start. Asterisks indicate single base pair changes present with respect to the wild-type sequence. Nucleotides recognized as conserved among *K. pneumoniae nif* promoters are underlined in the wild-type sequence.

(Table 3), thus creating the *Hae*III site used in the construction of pMB41 (10).

Dot-blot hybridization. Spontaneous deletions in the *nifH* promoter resulting from *Rsa*I restriction and religation were screened for by hybridizing plasmid DNA preparations of putative deletion mutants with the 5′-^{32}P-labeled oligonucleotide: 5′-TGCAGGGTACATACCAG (ca. 5×10^6 cpm per hybridization). This oligonucleotide is fully complementary to the *nifH* promoter fragment in pMB3 and has a single mismatch with pMB1 at −18 (10). Hybridization products were washed at increasing temperatures, and DNA preparations showing a weaker signal than either plasmids pMB3 or pMB1 were chosen for further analysis by DNA sequencing.

β-Galactosidase assays. Bacteria were grown anaerobically at 28°C in NFDM. *K. pneumoniae* strains and *E. coli* ET8000 were grown with aspartic acid (−N) or NH_4SO_4 (+N). All *E. coli* ET8894 derivatives and *E. coli* ET8045 were grown in the presence of L-glutamine. Promoter activity was measured as β-galactosidase units determined by the method of Putnam and Koch (30). Activation by *nifA* was determined in UNF932 (*nif*$^+$ *ntr*$^+$) and activation by *ntrC* in UNF926 [Δ(*his-nif*) *ntr*$^+$]. All β-galactosidase assays were performed at least twice on duplicate cultures.

Acetylene reduction assays. Whole-cell nitrogenase assays (16) were done on cells grown in NFDM after 20 h of derepression. Reduction of acetylene to ethylene was measured after 1 h of incubation.

RESULTS

Spacer mutations in the *nifH* promoter. The *nifH* promoter clone pMB3 carries a point mutation at −18 which is silent with respect to *nifA* activation, distinguishes it from the wild-type promoter (pMB1), and creates a unique *Rsa*I restriction site within the *nifH* promoter fragment. Restriction of the *nifH* promoter fragment from pMB3 with *Rsa*I and subsequent religation resulted in 30 of the 38 promoter clones screened displaying a hybridization signal weaker than that displayed by plasmid pMB1, which has a single base pair mismatch to the oligonucleotide used in the screening. This was interpreted as a destabilization of the plasmid DNA-oligonucleotide duplex resulting from the deletion of bases within the region of complementarity between the oligonucleotide and the *nifH* promoter fragment. This presumably reflects low levels of exonuclease present in the *Rsa*I enzyme or T4 DNA ligase preparation. DNA sequencing revealed in pMB340 the loss of the nucleotide at −18 (a nonconserved base which distinguishes pMB3 from the wild-type *nifH-lacZ* promoter clone pMB1) and in pMB355 the deletion of nucleotides at −20 and −21 (Table 3). Plasmid pMB303 was derived from pMB355 by filling in the *Acc*I site and differs from pMB3 by the presence of an adenine residue at −20 rather than the guanine residue found in this position in pMB3 and in the wild-type *nifH* promoter (Table 3). Plasmid pMB303 therefore permitted an assessment of the role of the base at −20 in *nifH* promoter function. Treatment of plasmid pMB355 with S1 nuclease generated a set of deletions between the −12 and −24 promoter elements by removing bases from −19 through −22 (pMB323), from −18 through −22 (pMB325), and from −18 through −23 (pMB324). In addition, an extensive deletion removing bases −11 through −22 was also isolated (pMB322). Thus, 1-, 2-, 4-, and 5-, and 6-bp deletions between the −12 −24 *nifH* promoter elements were obtained (Table 3).

Activities of mutant *nifH* promoters. Transcriptional activation in vivo by the *ntrC* gene product was measured in *K. pneumoniae* UNF926 and activation by *nifA* in *K. pneumoniae* UNF932. In addition, multicopy inhibition by the mutant promoters was assessed in UNF932. Multiple copies of the *nifH* promoter are known to prevent chromosomal *nif* expression; thus when a *nif*$^+$ strain harbors a multicopy plasmid bearing the *nifH* promoter, a Nif$^-$ phenotype is the result (9). It has been suggested that this is due to titration of activator (31) (i.e., *nifA* or *ntrA* products), and mutations which relieve inhibition are known to map in both the upstream and downstream promoter elements recognized as necessary for transcriptional activation of *nif* promoters (7, 10, 11, 29).

(i) Activation by *nifA*. Each internal deletion of the *nifH* promoter resulted in a strong promoter down phenotype when *nifA* activation was assayed (Table 4). Deletion of a single base (pMB340) produced as strong a down phenotype as larger deletions, indicating a strict requirement for a 10-bp spacing between the invariant GG (−24) and GC (−12) dinucleotides characteristic of *nif* and other *ntr*-activated promoters. The activity of pMB340 was found to be comparable to pMB322 which had been deleted through the −12

TABLE 4. Activation of deleted p*nifH* by *nifA* or *ntrC*

Plasmid	Deletion (bp)	β-Galactoside (U)[a]				Multicopy inhibition (% nitrogenase activity)
		nifA activation in UNF932		*ntrC* activation in UNF926		
		$+NH_4^+$	$-NH_4^+$	$+NH_4^+$	$-NH_4^+$	
pMB1 (wild type)	0	20	24,700	14	800	1
pMB3 (T → A, −18)	0	19	25,300	7	260	6
pMB340	1	1	50	2	9	76
pMB355	2	99	930	115	738	102
pMB323	4	109	70	285	558	80
pMB325	5	3	15	4	12	93
pMB324	6	5	14	9	39	86
pMB322	12	3	10	5	11	116
pMB303	0	6	12,100	23	186	7

[a] Activation by *nifA* (in UNF932) or by *ntrC* (in UNF926) was assayed after 20 h of growth in NFDM. Nitrogenase activity of UNF932 harboring pMB plasmids is expressed as a percentage of UNF932 harboring the vector pMC1403.

sequence (Table 3), confirming the severe promoter down effect of the single base deletion. Plasmid pMB303 differs from the parent plasmid pMB3 by the presence of an adenine rather than a guanine residue at −20, but it is still efficiently activated by *nifA*. Examination of the nucleotides present at position −20 in other *K. pneumoniae nif* promoters (Table 1) indicates this is a conservative substitution, as only the *nifU* promoter has a pyrimidine at −20. Plasmid pMB323 is of interest, since a GG dinucleotide is present in this deletion at position −24 by virtue of the 4-bp deletion moving sequence: ACGGTC downstream. However, the level of activation of pMB323 by *nifA* is very low and is below that of deletions which lack the upstream activator sequence necessary for the efficient expression of *nifH* (10, 11). The derepressed activity of pMB323 in UNF932 can be accounted for by the low level of constitutive expression from this plasmid and is described in more detail below together with pMB355.

(ii) Activation by *ntrC*. Results obtained with *ntrC* activation of the *nifH* promoter parallel those obtained for *nifA* activation as the single base pair deletion (pMB340) eliminates activation (Table 4). The behavior of plasmids pMB355 and pMB323, which both display some constitutive expression, is described below. Plasmid pMB303 is activated by *ntrC*, but to a slightly lesser extent than is the parent plasmid pMB3. The down phenotype of pMB303 with respect to *ntrC* activation can be largely attributed to the base change at −18 (compare plasmids pMB1 and pMB3) rather than the base change at −20 (compare plasmids pMB3 and pMB303). This agrees well with previous findings which demonstrated *ntrC* activation of *pnifH* was more sensitive to certain base changes than was *nifA* activation (10).

(iii) Multicopy inhibition by mutant p*nifH*. All deletions which changed the spacing of the −12 and −24 elements relieved the inhibition of chromosomal *nif* expression seen with pMB3 (Table 4). This is fully consistent with the finding that each deletion strongly decreases *nifA*-mediated transcription and presumably increases the availability of activator allowing expression of chromosomal *nif*. The efficient expression from pMB303 presumably reflects activator titration which results in the inhibition of chromosomal *nif* expression shown by this plasmid.

Activation by *ntrC* requires only the downstream consensus sequence. Upstream deletions in the *nifH* promoter have been shown to diminish activation by *nifA* but not *ntrC* (10). However, the possibility that nucleotides downstream of position −72 were involved in *ntrC* activation was not discounted (10, 11). Clearly, the results of *ntrC* activation given in Table 4 could be interpreted to mean that the deletions internal to the −12,−24 elements change not only the spatial relationship between these elements, but also the relationship between the −12 element and sequences downstream of position −72 which could be necessary for *ntrC* activation. Evidence that sequences between −72 and −27 are silent with respect to *ntrC* activation is given in Table 5. Deletion of nucleotides upstream of position −27 (pMB41) did not significantly alter expression in response to *ntrC* activation relative to the control *nifH* promoter clone pMB4. In addition, activation by *ntrC* was shown to be dependent upon the presence of the pMB24 element, as deletion of this sequence (pSMM11) reduced expression to the basal unactivated level. Results obtained with pMB41 clearly demonstrate that sequences at −35 are not necessary for activation by *ntrC* and that only the recognized downstream elements are needed.

Analysis of constitutive expression from pMB355 and pMB323. Both plasmids pMB355 and pMB323 showed a low level of constitutive expression in the presence of NH_4^+ in strains UNF932 and UNF926 (Table 4). In the case of pMB323 this was adequate to account for the level of activity in derepressing conditions in UNF932, indicating this mutant promoter was not activated by *nifA*. Plasmid pMB355 differed in this respect, since an apparent activation by *nifA* and *ntrC* was observed in *K. pneumoniae* above the level of constitutive expression (Table 4). Therefore, expression from plasmids pMB323 and pMB355 was further examined in several defined genetic backgrounds distinguished by the presence or absence of *nifA*, *ntrC*, and *ntrA* (Table 6). As with *K. pneumoniae* UNF926, results with the 2-bp deletion (pMB355) in *E. coli* ET8000 clearly showed that growth under nitrogen limitation was required to obtain an increase

TABLE 5. Activation of upstream-deleted p*nifH* by *ntrC*[a]

Plasmid	Deletion endpoint	β-Galactosidase (U)	
		$+NH_4^+$	$-NH_4^+$
pMB1 (wild type)	–	14	800
pMB4 (T → C, −26)	–	19	200
pMB41	−27	30	300
pSMM11	−18	30	60

[a] Activation by *ntrC* in UNF926 was assayed as described in Table 4. The slightly lower activity of pMB4 with respect to pMB1 and pMB3 can be attributed to the T → C transition at −26 in pMB4 (10).

TABLE 6. Constitutive promoter activities of pMB355 and pMB323[a]

Plasmid	Promoter activity (β-galactosidase [U]) in strain:					
	ET8000 *ntr*$^+$		ET8894 *ntrBC*$^-$	ET8894(pMC71A)	ET8894(pMM14)	ET8045 *ntrA*$^-$
	$+NH_4^+$	$-NH_4^+$	$-NH_4^+$	*nifA*^{c} $-NH_4^+$	*ntrC*^{c} $-NH_4^+$	$-NH_4^+$
pMB355	290	496	680	270	455	625
pMB323	117	131	131	40	86	25

[a] The dependence upon the *ntrC*, *ntrA*, and *nifA* gene products for expression was examined in *E. coli*, growth and assays of activity being performed as described in Materials and Methods.

in expression with respect to an ammonia-replete culture. However, the presence of *nifA* or *ntrC* (expressed constitutively) did not activate this mutant promoter, in contrast to results obtained with wild-type *nif* promoters (10). The 4-bp deletion pMB323 showed no increase in expression in nitrogen-limited *E. coli* ET8000 or in response to *nifA* or *ntrC*. For both pMB323 and pMB325, constitutive expression was shown to be *ntrA* independent as demonstrated by assays in *E. coli* ET8045. In conclusion, plasmids pMB355 and pMB323 do not carry functional *nifH* promoters.

DISCUSSION

Down phenotype of spacer mutants. The *K. pneumoniae nif* promoters are characterized by a 10-bp spacing between the conserved dinucleotides GG at −24 and GC at −12. In this study results are presented which demonstrate that this spatial relationship is an absolute requirement for activation of the *nifH* promoter by *ntrC* or *nifA*. This is exemplified by plasmid pMB340, in which the *nifH* promoter is deleted for the nucleotide at −18, a nonconserved base among *K. pneumoniae nif* promoters (Table 1). This mutant promoter shows very little transcriptional activity. It therefore seems likely that the origin of the promoter down phenotype is due to the change in the spatial relationship between the −24 and −12 promoter elements rather than the loss of a base (at −18) which interacts directly with proteins necessary for transcription. As mentioned earlier, the *nifH* promoter requires sequences upstream for *nifA* but not *ntrC* activation, the latter observation being extended in this study. Two points support the argument that the down phenotype of pMB340 with respect to *nifA* activation is due to the altered relationship between −24 and −12 sequences rather than the relationship between the −12 element and upstream sequences. First, this mutant promoter shows a 20-fold lower activity in response to *nifA* activation than does a *nifH* promoter deleted for the upstream *nifA*-specific activator sequence (10, 11). Second, two mutant *nifH* promoters have been constructed in which the position of the upstream activator sequence differs by a single base pair. Both of these mutants were activated to equal extents by *nifA* and to a level comparable to that of the wild-type *nifH* promoter in which the upstream sequence is located at −136 rather than at −155 or −156, as it is in the two upstream sequence spacer mutants considered above. Interpretation of the down phenotype of pMB340 with respect to *ntrC* activation does not require consideration of sequences upstream of position −27, since deletions extending to this position were shown not to affect activation by *ntrC*. Similar arguments can be applied to the interpretation of results obtained with all other deletion mutants, although the two deletions (pMB355 and pMB323) resulting in a low constitutive level of expression are considered in more detail below. It has been shown recently that mutations in nucleotides at −15 and −17 lead to an increased activation of the *nifH* promoter by *ntrC* (29). These bases are retained in the spacer mutants analyzed; therefore their down phenotype with respect to *ntrC* activation cannot be accounted for by the deletion of bases which may interact with *ntrC*.

Expression from plasmids pMB355 and pMB323. Expression from plasmids pMB323 and pMB355 was seen to be *ntr* independent, but to increase slightly and reproducibly when bacteria were grown under nitrogen limitation. It is possible that physiological changes associated with nitrogen-limited growth give rise to this apparent increase in expression. The constitutive expression observed with pMB355 may be due to the sequence AAGAAT-17 bp-TGGTAT created by the 2-bp deletion acting as a conventional promoter. This sequence may also be responsible for constitutive expression from pMB323.

Relief of multicopy inhibition. Each deletion mutation analyzed here relieved the multicopy inhibition of chromosomal *nif* displayed by the wild-type *nifH* promoter. Since the upstream and downstream elements act in *cis* to elicit the multicopy effect (11), changing the structure of any one promoter element results in a loss of inhibition. For the mutations described here it is probably the change in structure of the downstream promoter element which is responsible for the relief of inhibition. The molecular basis for the *cis* action of the promoter elements is not yet well understood, but it is possible that proteins which interact with the −12,−24 sequences (possibly RNA polymerase and *ntrA*) are required to be present so that activator can be titrated out by the upstream sequences (11). Presumably, this protein interaction at the downstream element is defective or absent in the spacer mutants. Alternatively, the promoter may need to be transcriptionally active to titrate out activator.

Possible basis of the down phenotype. Activation of transcription of *nif* promoters requires an activator (*nifA* or *ntrC*), *ntrA*, and RNA polymerase (17). Altering the optimal 10-bp spatial arrangement of the *nifH* promoter results in an equivalent down phenotype for both *nifA* and *ntrC* activation, indicating that some common step in activation or transcription initiation is changed in the deletion mutants. It is possible that the deletions change an interaction of RNA polymerase or activator with the entire spacer region sequence. However, as mentioned above, a point mutation at −18 in the spacer region is relatively silent and there is little conservation of sequences in this region, indicating that these nucleotides may not have a major role per se in directly interacting with the transcription apparatus. It is more likely that the deletion of bases in the spacer region disrupts a protein-DNA interaction which involves the simultaneous recognition of bases around the −12 and −24 promoter elements by complementary hydrogen bond donors and

acceptors, the complementarity being lost by the closer proximity of these sequences in the deletions (36).

The fact that the down phenotype of the internal deletion mutants is much more severe than that of the mutant *nifH* promoters which lack the proposed upstream *nifA* binding site (11) could be interpreted to mean that a protein-DNA interaction other than the one involving *nifA* is altered in these mutants. Should *ntrC* and *nifA* interact with RNA polymerase to activate transcription, then the down phenotype of the internal deletions could be explained by the disruption of an RNA polymerase-DNA interaction at the downstream promoter element. It has been proposed that *ntrA* may be an alternative sigma factor, facilitating recognition of the atypical *ntr*-activated promoters (15). If this is so then the spacer mutations may disrupt this aspect of RNA polymerase interaction with the *nifH* promoter if *ntrA* normally contacts both the −12 and −24 elements. Elucidation of the mechanism of transcriptional activation by *ntrA* acting in concert with either *nifA* or *ntrC* will no doubt clarify which of the alternatives discussed above accounts for the phenotype of the deletions described here.

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