Volume 13 Number 21 1985 Nucleic Acids Research

Site-directed mutagenesis of the *Klebsiella pneumoniae nifL* and *nifH* promoters and *in vivo* analysis of promoter activity

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Received 15 August 1985; Revised and Accepted 7 October 1985

Abstract

The role of conserved nucleotides in nitrogen-fixation promoter function has been examined using both oligonucleotide and chemical mutagenesis to introduce base changes in the Klebsiella pneumoniae nifL and nifH promoters. Among ten mutations analysed, including six spontaneous mutations, base changes at -12, -13, -14, and -26, located in previously identified conserved sequences, perturbed the activity of the promoters, demonstrating that these sequences are required for transcription. Not all base changes produced similar strong promoter down phenotypes when the nifL and nifH promoters were compared: activation of the nifH promoter by the nifA gene product was less sensitive to base changes in conserved nucleotides than was activation of the equivalently altered nifL promoter by the nifA or ntrC products. We have found that the nifH promoter can be weakly activated by the ntrC product; this activation shows the same down response to base changes seen with ntrC activation of the nifL promoter. We present evidence that the efficient activation of the nifH promoter by nifA (but not ntrC) can be attributed to specific upstream sequences present in the nifH promoter.

INTRODUCTION

The nitrogen fixation (nif) gene cluster of Klebsiella pneumoniae consists of 17 contiguous genes organised as 8 transcriptional units (1,2). Expression of nif at the transcriptional level is positively regulated by the nifA gene product and gene products of the nitrogen regulation (ntr) system (3,4,5,6). The ntr system provides external regulation of nif by controlling expression of the nifLA operon which encodes the nif-specific activator nifA (7,8). Under conditions of nitrogen limitation the ntrC gene product, in concert with the ntrA gene product (9,10), positively activates transcription of the nifLA operon. The nifA gene product then promotes

Table 1
NtrA dependent promoters

Klebsiella pneumoniae nif promoters	-24	-12	Reference
nifF	C T G G C A C A G C C T T C G C T		(11)
ORF	C T G G C A C A G G C T G C G C A		(12)
nifL	A G G G C G C A C G G T T T G C A		(7)
nifB	C T G G T A C A G C A T T T G C A		(11)
nifM	C T G G C C G G A A A T T T G C A		(11)
nifU	C T G G T A T C G C A A T T G C T		(11)
nifE	C T G G A G C G C G A A T T G C A		(11)
nifH	C T G G T A T G T T C C C T G C A		(13)
Consensus	C T G G C A C N_5 T T G C A		
K. pneumoniae glnA(RNA1)	T T G G C A C A G A T T T C G C T		(14)
S. typhimurium argTr	A T G G C A T A A G A C C T G C A		(15)

transcription of all other nif operons (8, for review see 1 and references therein). Other genes involved in nitrogen assimilation, for example glnA (glutamine synthetase), put (proline utilisation), aut (arginine utilisation) and hut (histidine utilisation), require the ntrA and ntrC gene products for expression (8). The nifA and ntrC gene products are similar in several respects (8,9). Both require the ntrA gene product for their activator roles, and the nifA gene product can substitute for ntrC in the activation of the glnA, put, aut and hut genes (8). In addition the nifA gene product can autogenously activate the nifLA operon (7) and in this communication we show that ntrC can also activate the K. pneumoniae nifHDK operon, albeit far less efficiently than nifA.

The functional similarity of ntrC and nifA is reflected in sequence homology among the promoters which they regulate. The K. pneumoniae nif promoters have all been sequenced (7,8,11,12, 13), and can be compared to the ntr-regulated glnA promoter (14) and the sequences present in the promoters of the D-histidine

utilisation (dhuA) and arginine transport (argTr) systems of Salmonella typhimurium (15). These promoters lack the conserved -10 and -35 regions found in typical Escherichia coli promoters (16) but contain conserved sequences at -24 and -12 with respect to the transcription start, where this has been determined (see Table 1). An invariant GG dinucleotide is found within the consensus sequence CTGGCAC from -27 to -20. The consensus sequence TTGCA at -15 to -11 contains the invariant dinucleotide, GC. In order that these sequences can be recognised as promoters by RNA polymerase it has been suggested that the transcriptional specificity of polymerase may be modified, possibly by ntrA acting as an alternative sigma factor (17,18).

In this study we have examined the role the consensus sequence plays in determining the activities of the nifL and nifH promoters. We show that the nifH promoter can be activated by ntrC and that the consensus sequence is required for this, but that nifA activation requires in addition an element as far upstream as -136.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Tables 2 and 2a.

Media

Complete medium was LB (25) and nitrogen-free medium (NFDM) and minimal medium were as previously described (26). Antibiotics used were kanamycin (Km, 30 μg/ml), chloramphenicol (Cm, 15 μg/ml), carbenicillin (Cb, 100 μg/ml for E. coli strains) or carbenicillin plus ampicillin (200 μg/ml each for K. pneumoniae strains) and tetracycline (Tc, 10 μg/ml). X-gal (5-bromo-4-chloro-3-indoylyl-β-D-galactosidase) was used as a final concentration of 20 μg/ml to detect lac^+ colonies (27).

Cloning and sequencing

All enzymes were obtained from commercial sources and used according to the manufacturer's instructions. Dideoxy sequencing was performed using ^{35}S-α-dATP as the label (28). Fragments of interest were cloned into the M13 mp8 and mp9 vectors for sequencing (29). Transformation was as described except that 50 mM $CaCl_2$ was used (30).

Table 2

Bacterial strains and plasmids

Genotype or phenotype		Source or reference
Klebsiella pneumoniae:		
UNF926	Δ(his-nif)2632 Δlac2001 recA hsdRI sbl300::Tnl0	This laboratory
UNF931	hisD2 Δlac2001 recA hsdRI sbl300::Tnl0	This laboratory
Escherichia coli K12:		
ET8894	Δ(rhaA glnA ntrB ntrC) 1703 rbs gyrA hutC lacZ::IS1 Mucts62	(19)
MC1061	ara D139 Δ(ara,leu)7697 Δlac 74 galU galK strA r^-m^+	(20)
Plasmids:		
pSA30	nifHDKY, Tc^R EcoRI insert in pACYC184	(21)
pSB1	C to A transversion in nifH promoter at -12, Tc^R pSA30 derivative	
pSB5	C to T transition in nifH promoter at -12, Tc^R " "	
pSB30	T to C transition in nifH promoter at -14, Tc^R " "	(22)
pSB75	G to T transversion in nifH promoter at -136, Tc^R " "	
pSB82	112bp internal deletion in nifH promoter, -72, -184, Tc^R " "	
pVW16	nifH promoter, Cb^R.EcoRI-BglII insert in pBR322	(23)
pMC1403	Translational fusion vector, Cb^R. pBR322 derivative	(24)
pMD1405	Translational fusion vector, Cb^R. pBR322 derivative	M. Drummond
pMC71A	$nifA^C$, Cm^R. SalI insertion pACYC184	(4)
pMM14	$ntrC^C$, Km^R. SmaI insert in pACYC177	(9)
pRD554	nifL-lacZ fusion, Cb^R. pMC1403 derivative	(7)

Table 2a

nif-lac fusions used in this study

Plasmid	Genotype	Method of construction
nifL-lac		
pRD554	wild-type	Described in reference 7
pHB5	G→A at -13	Hydroxylamine mutagenesis of pRD554
pHB8	C→T at -12	" " " "
nifH-lac		
pMB1	wild-type	pnifH fragment from pVW16 carrying 17aa's cloned into pMC1403
pMB2	G→A at -13	oligonucleotide mutagenesis of pMB1
pMB3	T→A at -18	" " " "
pMB4	T→C at -26	" " " "
pMB3012	wild-type	EcoRI-Sau3A pnifH fragment from pSA30 cloned into pMC1403
pMB12	C→A at -12	" " " " from pSB1 " " "
pMB52	C→T at -12	" " " " from pSB5 " " "
pMB302	T→C at -14	" " " " from pSB30 " " "
pMB752	G→T at -136	" " " " from pSB75 " " "
pMB822	112bp Δ	" " " " from pSB82 " " "

Wild-type nifH-lac fusions pMB1 and pMB3012 were constructed by cloning pnifH fragments into EcoRI-SmaI digested pMC1403, and differ only by the presence of 17(pMB1) or 36(pMB3012) amino acids of nifH. Blunt ends were generated by filling in the Sau3A site (pMB3012) or by Bal31 nuclease treatment (pMB1) before excising the promoter fragment by EcoRI digestion for ligation into pMC1403.

Plasmid construction

The nifH-lacZ translational fusion plasmid pMB1 was constructed by digesting the nifH carrying plasmid pVW16 with SstII, followed by treatment with Bal31 nuclease and EcoRI digestion. The shortened nifH fragment was ligated into the translational fusion expression vector pMC1403 (24) cut with EcoRI and SmaI. The ligated material was then used to transform E. coli MC1061/pMC71A and an inframe nifH-lacZ fusion chosen on the basis of a lac^+ phenotype on EMB MacConkey agar. The DNA was sequenced to determine the fusion junction. Plasmid pMB1 carries 17 amino acids of nifH (31) before the junction with the lacZ gene (see Table 2a).

Oligonucleotide synthesis and purification

Oligonucleotides were synthesised on an Applied Biosystems automated DNA synthesiser using dimethoxytritrylnucleoside phosphoramidites. The fully deprotected oligonucleotide was purified by ion-exchange HPLC followed by reverse phase HPLC (32). The oligonucleotide used to construct the G to A transition at -13 in the nifH promoter was purchased from CellTech.

Oligonucleotide-directed mutagenesis

Single primer mutagenesis of the nifH promoter, cloned as an EcoRI-BamHI fragment from pMB1 into M13 mp8, was performed essentially as described (33). Oligonucleotides kinased with γ-^{32}P-ATP were used in the mutagenesis (34). After the extension and ligation reaction, covalently closed circular DNA was enriched by agarose gel electrophoresis. This material was treated briefly with S1 nuclease (35) and used to transform E. coli 71-18. From the resulting plaques, phage was prepared and screened by dot blot hybridisation. Following plaque purification and DNA sequencing to confirm the presence of the mutation, replicative form (RF) DNA was prepared, restricted with EcoRI and BamHI and the fragment carrying the nifH promoter cloned into the expression vector pMC1403. For the construction of the G to A transition, plaques obtained from the transformation of E. coli 71-18 with covalently closed circular DNA were pooled and RF DNA prepared. The EcoRI-BamHI restriction fragment carrying the nifH promoter was purified from this DNA preparation and cloned into pMC1403. Following

transformation of E. coli MC1061/pMC71A, colonies displaying a lac$^-$ phenotype on EMB MacConkey agar were chosen, small scale plasmid DNA preparations made (36) and screened by dot blot hybridisation for the mutation. Four plasmid preparations which gave a strong hybridisation signal were shown to carry the G to A transition by DNA sequencing; no other mutations were detected. One such plasmid was chosen for further study and was designated pMB2.

Hydroxylamine mutagenesis of the nifLA promoter

A 1.6 kb PstI-HpaI fragment which carries the nifLA promoter was purified from pRD554 (7) and subjected to hydroxylamine mutagenesis. The fragment (60-100 ng) was incubated in a 20 µl reaction containing 0.5 M hydroxylamine hydrochloride, 0.1 M sodium phosphate buffer pH 6.0 and 1 mM Na_2EDTA for 30 mins at 75°C. DNA was ethanol precipitated, washed with 80% ethanol, resuspended in 20 µl H_2O and then passed through a Sephadex G-50 minicolumn equilibrated with 5 mM Tris, 0.25 mM Na_2EDTA pH 8.0. Since hydroxylamine preferentially mutagenises single-stranded DNA, the fragment was then digested with EcoRI and BamHI to release a 360 bp fragment with cloneable ends which was then ligated into EcoRI-BamHI digested, phosphatase-treated pMC1403. Mutants were screened among transformants of strain MC1061 on NFDM plates containing the appropriate supplements and the chromogenic substrate X-gal. Colonies which gave a lighter blue colour than the wild-type were retained for further analysis.

Construction of inframe lacZ translational fusions from pSA30 and pSB plasmids

Plasmid pSA30 and those plasmids derived from it (pSB1, 5, 30, 75, 82) carrying nifH promoter mutations were all digested with EcoRI and BglII and the resulting nifH promoter fragment cloned into M13 mp8 cut with EcoRI and BamHI. RF DNA was prepared from these clones, digested with EcoRI and PstI and this material ligated into the lacZ translational fusion expression vector pMD1405 (kindly provided by M. Drummond) previously cut with EcoRI and PstI, to yield inframe nifH'-lacZ fusions. pMD1405 is a derivative of pMC1403 in which the SalI and PstI sites have been removed and the cloning nest from M13 mp10 has been introduced (M. Drummond, unpublished). The above

nifH'-lacZ fusions carry 146 amino acids of nifH before the fusion junction with lacZ. Derivatives of these carrying less of the nifH coding sequence were constructed in the following manner. Each nifH'-lacZ fusion plasmid was digested with ScaI and SstII and the fragment carrying the nifH promoter isolated. This was digested with Sau3A, filled in with the Klenow fragment of DNA polymerase, digested with EcoRI and ligated into pMC1403 cut with EcoRI and SmaI to yield inframe nifH'-lacZ fusions carrying 36 amino acids of nifH. Activities of the latter nifH'-lacZ fusions are reported here and are very similar to those obtained with the fusions carrying the longer nifH coding sequence (See Table 2a).

β-galactosidase assays

Bacteria were grown anaerobically at 28°C in NFDM supplemented with appropriate antibiotics to maintain plasmid selection. K. pneumoniae strains were grown with 100 μg/ml aspartic acid (-N) or 2 mg/ml NH_4SO_4 (+N). Histidine (50 μg/ml) was added where appropriate. All E. coli ET8894 derivatives were grown in the presence of 200 μg/ml L-glutamine. β-galactosidase activities were assayed as described (27) for nifL-lacZ fusions and by a modified lysis procedure for nifH-lacZ fusions (37).

Acetylene reduction assays

Whole cell nitrogenase assays (26) were done on cells grown in NFDM after 20 hours derepression. Reduction of acetylene to ethylene was measured after a one hour incubation.

RESULTS

Mutations in the nifL and nifH promoter

Using translational nif-lacZ fusions we have examined the behaviour of seven point mutations and one deletion in the nifH promoter and two point mutations in the nifL promoter (see Figure 1). The influence of C to T and G to A transitions at -12 and -13 respectively in both the nifL and nifH promoters has been compared. These mutations in the nifL promoter (pHB5, pHB8) were obtained by hydroxylamine mutagenesis of the nifL-lacZ fusion plasmid pRD554. Plasmids pHB8 and pHB5 contain no other base changes. The analogous G to A transition in the nifH

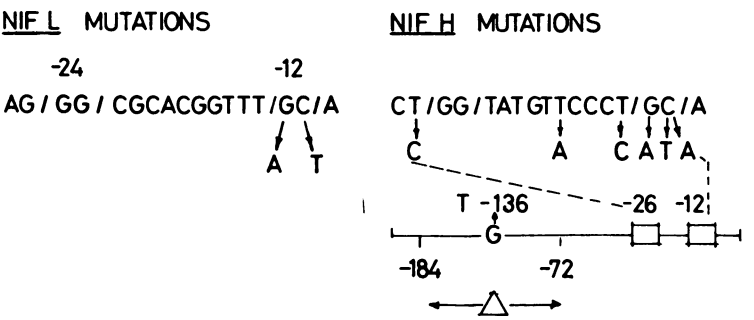

Figure 1

Mutations in the nifL and nifH promoters analysed in this work.

promoter (pMB2) was constructed by oligonucleotide-directed mutagenesis.

Multiple copies of the nifH promoter prevent the synthesis of active nitrogenase from chromosomal nif genes (23,38). Brown and Ausubel (22) exploited this observation to isolate spontaneous mutations in the multicopy nifHDK plasmid pSA30 which relieved nif inhibition. Several of these mutations were located in the nifH promoter, however no direct assay of the transcriptional or translational activity of these mutant promoters was performed. One spontaneous nifH promoter mutation was a C to T transition at -12. In order to assay the influence of this mutation upon promoter activity, and therefore compare it to the transitions in the nifL promoter and the nifH G to A -13 transition, a nifH-lacZ fusion (pMB52) and its appropriate wild-type control (pMB3012) were constructed. Thus transitions in the invariant GC dinucleotide at -12 were compared between the nifL and nifH promoters (See Table 2a).

To directly assay expression from the other spontaneous nifH promoter mutants a set of nifH-lacZ fusions were constructed from these, enabling the effect of mutations at -12 (pMB12), -14 (pMB302), -136 (pMB752) and the 112 bp internal deletion (pMB822) upon nifH promoter activity to be examined quantitatively. Two oligonucleotide-directed mutations were made at -18 (a T to A transversion, pMB3), and at -26 (a T to C transition, pMB4). We examined the activities of the mutant nifL and nifH promoters in

Table 3

Comparison of the influence of transitions at -12 and -13 on *nifL* and *nifH* promoter activities

		β-galactosidase activities in:							
Plasmid	Genotype	(1) ET8894	(2) UNF596 (*ntrC*^{C})	(3) UNF597 (*nifA*^{C})	(4) UNF926 -N	(4) UNF926 +N	(5) UNF2247 (*nifA*^{C})	(6) UNF931 -N	(6) UNF931 +N
pRD554	*nifL* wt.	155(±8)[ii]	2800(±139)[ii]	2200(±68)[ii]	8,400(±300)[ii]	161(±12)[ii]	-	7,200(±470)[ii]	161(±7)[ii]
pHB8	*nifL* C→T-12	100(±20)	162(±15)	146(±30)	566(±150)	88(±12)	-	494(±175)	92(±10)
pHB5	*nifL* G→A-13	151(±6)	88(±13)	159(±13)	277(±42)	91(±12)	-	244(±40)	92(±10)
pMB3012	*nifH* wt.	13(±3)[ii]	208(±5)[ii]	23,600(±4%)[i]	652(±100)[ii]	24(±8)[ii]	31,200(±23%)[i]	24,400(±1%)[i]	28(±11)[ii]
pMB52	*nifH* C→T-12	9(±1)	7(±1)	22,900(±4%)	47(±18)	24(±3)	47,500(±22%)	47,000(±17%)	18(±1)
pMB1	*nifH* wt.	15(±3)	337(±7)	21,300(±6%)	1000(±300)	14(±5)	146,200(±12%)	24,700(±16%)	20(±2)
pMB2	*nifH* G→A-13	15(±1)	28(±2)	116(±14%)	39(±2)	10(±1)	395(±9%)	1,900(±8%)	19(±2)

Relevant genotypes are: (1) ET 8894 = *glnA ntrBC*$^{\Delta}$; (2) ET8894/pMM14 = *glnA ntrBC*$^{\Delta}$*ntrC*^{C};
(3) ET8894/pMC71A = *glnA ntrBC*$^{\Delta}$*nifA*^{C}; (4) UNF926 = *ntr*$^{+}$*nif*$^{\Delta}$;
(5) UNF926/pMC71A = *ntr*$^{+}$*nif*$^{\Delta}$*nifA*^{C}; (6) UNF931 = *ntr*$^{+}$*nif*$^{+}$

(i) Standard deviations as a percentage of the mean.

(ii) Standard deviations.

β-galactosidase assays were performed after 17-21 hours growth in NFDM.

E. coli and *K. pneumoniae* in the presence and absence of the activator gene products (*nifA* or *ntrC*) provided either by a regulated chromosomal copy of the activator gene or constitutively from a plasmid in *trans*.

Transitions at -12 and -13 in the *nifL* and *nifH* promoters

Many *ntr* regulated promoters and all *K. pneumoniae* *nif* promoters contain an invariant GC dinucleotide at -12 (1). Transitions in these bases clearly perturb the function of the *nifL* and *nifH* promoters (Table 3). The G to A transition at -13 resulted in a strong down phenotype for both the *nifL* and *nifH* promoters. The *nifH* promoter was activated by *ntrC* (Table 3, columns 2 and 4), although weakly when compared to *nifA* (Table 3, columns 3 and 6), and the G to A transition in both promoters behaved as a strong down mutation with respect to both *nifA*- and *ntrC*-mediated activation, indicating an equivalent role for G-13 in both the *nifL* and *nifH* promoters. An interesting feature of the G to A transition in the *nifH* promoter was the *ca*. 5-fold increase in activity in a *nif*$^{+}$ background not seen with the mutant *nifL* promoter. It is plausible that a *nif* specific gene product may partially suppress the down phenotype.

Table 3 shows further differences between the *nifL* and *nifH* promoters as regards the C to T transition at -12. For the *nifL*

Table 4

Comparison of mutant nifH promoter activities

Plasmid	Genotype	β-galactosidase activities in: (1) ET8894	(2) UNF596 (ntrC^{C})	(3) UNF597 (nifA^{C})	(4) UNF926 -N	(4) UNF926 +N	(5) UNF2247 (nifA^{C})	(6) UNF931 -N	(6) UNF931 +N
pMB1	nifH wt.	15(±3)[ii]	337(±7)[ii]	21,300(±6%)[i]	1000(±401)[ii]	14(±5)[ii]	145,200(±12%)[i]	24,700(±16%)[i]	20(±2)[ii]
pMB3	T→A-18	14(±2)	75(±4)	17,200(±6%)	264(±32)	7(±1)	104,800(±5%)	25,300(±17%)	20(±1)
pMB4	T→C-26	14(±3)	65(±2)	17,500(±13%)	180(±14)	19(±1)	136,700(±11%)	18,100(±21%)	23(±2)
pMB3012	nifH wt.	13(±3)	208(±5)	23,600(±4%)	652(±100)	24(±8)	31,200(±23%)	24,400(±1%)	28(±11)
pMB12	C→A-12	14(±2)	27(±4)	31,600(±6%)	99(±38)	48(±25)	72,500(±15%)	29,000(±28%)	34(±2)
pMB302	T→C-14	13(±3)	23(±2)	21,300(±7%)	53(±20)	40(±16)	49,100(±2%)	34,800(±31%)	30(±4)
pMB752	G→T-136	14(±3)	178(±5)	27,300(±7%)	780(±183)	33(±13)	69,300(±9%)	20,400(±16%)	36(±6)
pMB822	112 bpΔ	15(±4)	127(±9)	400(±4%)	730(±76)	45(±22)	2,700(±13%)	1,100(±3%)	32(±2)

Relevant genotypes are: (1) ET8894 = glnA ntrBC$^{\Delta}$; (2) ET8894/pMM14 = glnA ntrBC$^{\Delta}$ntrC^{C}; (3) ET8894/pMC71A = glnA ntrBC$^{\Delta}$nifA^{C}; (4) UNF926 = ntr^{+}nif^{-}; (5) UNF926/pMC71A = ntr^{+} nif$^{\Delta}$ nifA^{C}; (6) UNF931 = ntr^{+} nif^{+}

(i) Standard deviations as a percentage of the mean.

(ii) Standard deviations.

promoter, this transition resulted in a promoter down phenotype virtually identical to that found with the G to A transition. For the nifH promoter this transition had a silent phenotype, irrespective of the genetic background, when nifA activation was examined (Table 3, columns 3, 5 and 6). Thus the presence of a C residue at -12 is necessary for transcriptional activation of the nifL promoter, but plays a lesser role in nifA activation of the nifH promoter. Only when ntrC activation of the nifH promoter was examined did the C to T transition display a down phenotype (Table 3, columns 2 and 4); the severity of this mutation being comparable to that of the G to A transition. Thus the C residue at -12 is necessary for ntrC activation of both the nifL and nifH promoters but is not required for efficient activation of the nifH promoter by nifA.

Point mutations in the nifH promoter at -12, -14, -18 and -26

Surprisingly, mutations changing the invariant C at -12 (pMB12), T at -26 (pMB4) and semi-invariant T at -14 (pMB302) were silent with respect to nifA-mediated activation (Table 4). Changing the non-conserved T residue at -18 to A (pMB3) was without effect when nifA activation was examined. Preculturing UNF931 harbouring the mutant nifH promoter plasmids in the presence of ammonia and then derepressing for short periods (3

Table 5

Nitrogenase assays in K. pneumoniae strains carrying multiple copies of mutant nifH promoters.

Plasmid	Mutation	Acetylene reduction activity[a]
pMC1403	-	100% (±10)
pMB1	wild-type	1% (±0.1)
pMB3012	wild-type	0.2% (±0.1)
pMB52	C→T-12	19% (±5)
pMB12	C→A-12	20% (±4)
pMB2	G→A-13	96% (±2)
pMB302	T→C-14	17% (±3)
pMB3	T→A-18	6% (±3)
pMB4	T→C-26	2% (±0.5)
pMB752	G→T-136	68% (±15)
pMB822	112 bp Δ	90% (±6)

[a] Values are expressed as a percentage of the pMC1403 control culture included in each set of assays. All assays were done on duplicate cultures. Relative errors between duplicate cultures are presented.

and 6 hours), conditions under which nifA is likely to limit expression, did not reveal a promoter down phenotype for any of the mutations at -12, -14 or -18. However, the T to C transition at -26 resulted in moderate (ca. 50%) reduction in expression in short term derepression assays (data not shown). Although mutations in several of the invariant or semi-invariant nucleotides of the nifH promoter did not result in a strong promoter down phenotype when nifA activation was assayed (Table 4, columns 3, 5 and 6) each had a down phenotype when ntrC activation was examined (Table 4, columns 2 and 4). In the case of mutations in invariant or semi-invariant nucleotides (-12, -14, and -26) the down phenotype was more severe than with the mutation at -18 which is in a non-conserved nucleotide. Thus the results parallel those obtained with the C to T transition at -12: conserved nucleotides are necessary for ntrC activation but not always for nifA activation of the nifH promoter.

Mutations upstream of the -24 consensus sequence affect activation of the nifH promoter

In addition to the point mutations isolated in the -12 region of the nifH promoter, a G to T transversion at -136 and a 112 base pair internal deletion starting at position -72 were

Table 6

Classes of nifH mutant promoters

Class	Mutations	Activation		Multicopy inhibition
		nifA	ntrC	
0	wild-type	+++	+	+
I	G→A-13	-	-	-
II	C→T-12	+++	-	-
	C→A-12	+++	-	-
	T→C-14	+++	-	-
III	T→A-18	+++	±	±
	T→C-26	+++	±	±
IV	G→T-136	+++	+	-
V	112bpΔ	-	+	-

isolated (22). The influence of these mutations upon nifH promoter function is shown in Table 4. The point mutation at -136 (pMB752) was silent whether the promoter was activated by ntrC or nifA; short term derepression assays of nifA activation did not reveal a promoter down phenotype. The internal deletion (pMB822) reduced nifA-mediated activation to less than 5% of the wild-type (Table 4, columns 3, 5 and 6), but was without effect when activation by ntrC was examined (Table 4, columns 2 and 4). Thus the sequences delineated by the internal deletion appear to play a critical role in nifA activation of the nifH promoter but are not essential for ntrC activation.

Multicopy inhibition by mutant nifH promoters

The degree of inhibition of nif expression by multiple copies of the nifH promoter affords a second assay for nifH promoter activity. Table 5 summarises the results of the multicopy inhibition assays with the mutant nifH promoters. The spontaneous mutations used in this study were isolated as derivatives of pSA30 (22). Since the sub-clones used here contain only the nifH promoter and short regions of nifH coding sequence the results in Table 5 confirm that the relief of multicopy inhibition previously reported (22) was a result solely

of mutations in the nifH promoter sequence. With the exception of the T to C transition at -26, all mutations in invariant or semi-invariant nucleotides relieved multicopy inhibition by the nifH promoter, but to different extents. The T to A transversion at -18, a change in a non-conserved nucleotide, did not significantly relieve multicopy inhibition. It is worth noting that mutations which relieve multicopy inhibition do not always result in a promoter down phenotype.

DISCUSSION

The nif promoters of K. pneumoniae are a new special class of promoters (11), the usual features of prokaryotic promoters being absent (39). The nifL and nifH promoters are among the best studied and although both conform to the consensus sequence for ntr-regulated promoters, they differ in a number of respects. Firstly, the nifL promoter can be activated equivalently by either ntrC or nifA (7) whereas the nifH promoter was thought to be activated only by nifA (9,10,40). As shown in this paper, the nifH promoter can be activated by ntrC, but only weakly. Secondly, the nifH promoter shows the multicopy inhibition effect whereas the nifL promoter sequence does not. Lastly, as shown in this paper, upstream sequences are important for nifA (but not ntrC) activation of the nifH promoter but do not play the same role in activation of the nifL promoter; activation of the nifL promoter by nifA or ntrC is not differentially affected by deletions extending upstream (7).

Therefore, although both promoters share sequence homology (11) they clearly differ in their interaction with the activators of transcription. The non-equivalence of the C to T transition in the nifL and nifH promoters emphasises the difference which exists between the nifL and nifH promoters. On the basis of nifA or ntrC activation and multicopy inhibition we can classify the promoter mutants studied into five groups (Table 6).

The two mutations examined in the nifL promoter are class I type mutations, being strong promoter down mutations for both ntrC and nifA mediated activation. Only the G to A transition at -13 in the nifH promoter is a class I mutation. Class II mutations can be defined as those which are relatively silent

with respect to nifA activation but are strong down mutations when ntrC activation is examined. This class of mutation also partially relieve multicopy inhibition by the nifH promoter. Included in this class are a C to T transition (a class I mutation in the nifL promoter) and a C to A transversion at -12 and at -14, a T to A transversion. Although all these mutations occur in conserved nucleotides, they do not (unlike the G to A transition) have a severe effect upon promoter activity if nifA activation alone is considered. Similar results were obtained by Ow et al. (40) who used deletion loop bisulphite mutagenesis to obtain mutations at -12 and -13 in the nifH promoter. Although it is not certain that other mutations were absent from their clones (the nifH promoter fragment was not sequenced in its entirety), they also found the G to A transition at -13 to be a much stronger down mutation than the C to T transition at -12 when nifA activation was examined. In their hands the C to T transition was a moderate down mutation which may be attributed to differences which exist between their assay conditions and ours (8,41) or indeed the presence of other mutations. With respect to mutations at -12, it is worth noting that the Rhizobium trifolii (42) and Rhizobium phaseoli nifH promoter sequences (43) differ from the usual nif or ntr consensus by the presence of an A residue at -12 rather than a C. Therefore a C residue at -12 may not be essential for nifH promoter function. The results obtained with class I and II mutations are consistent with the TGCA sequence mediating transcriptional activation of nif promoters. However the failure of the C to T transition or C to A transversion in the nifH promoter at -12 to produce a strong down phenotype implies that this promoter differs from the nifL promoter in its requirement of the TGCA sequence for activation by nifA.

Class III mutations represent an intermediate type of mutation, the only phenotype being a moderate down effect upon ntrC activation. That nifA activation or multicopy inhibition are unaffected is consistent with the class III mutation occurring in a non-conserved nucleotide (T-18) or in nucleotides not involved in activator specificity (T-26).

Class IV mutations affect only the multicopy inhibition

displayed by the nifH promoter. Activation by ntrC or nifA is not altered. The class IV mutation occurs a considerable distance from the start of transcription at position -136 and may occur in a sequence which interacts with nifA. Assuming that multicopy inhibition is due to the titration of activators (nifA, ntrA or both) then the mutation at -136 (and at -12 and -14, Table 5) must increase the availability of activators, but not to such an extent that the mutated promoter cannot interact with these to facilitate its own transcriptional activation.

Class V mutations result in a severe promoter down phenotype (with respect to nifA activation) and relieve the multicopy inhibition of the nifH promoter. They differ from class I mutations only in that ntrC activation is not affected. The class V mutation is an upstream deletion, covering the region at -136 which may be a site at which nifA interacts with the nifH promoter (see above), perhaps explaining the differential effect upon ntrC and nifA activation.

Results presented here show that the nifH promoter is weakly activated by ntrC, contrasting with the nifL promoter. This may be related in part to differences in nucleotide sequence which exist between the nifL and nifH promoters in the -26 and -12 regions (11). Recently C to T transitions in the nifH promoter at -17 were shown to render this promoter activatable by ntrC, but to a level of only ca. 10% that seen with nifA (40). This is consistent with the suggestion that the failure of ntrC to activate the K. pneumoniae nifH promoter efficiently resides in a sequence difference between the -11 to -17 region of the nifH promoter CCCTGCA and a consensus for ntrC-activated promoters TTTTGCA (41). Activator specificity has also been ascribed to sequence differences which exist in the -24 region between ntrC and nifA activatable promoters (11). However the silent nature of the T to C transition at -26 constructed by us in the nifH promoter indicates that this nucleotide is not critical for nifA activation and has only a moderate down effect upon ntrC activation. Only in the case of a G to A transition at -24 has it been shown that these sequences are necessary for nifA activation; however the effect upon ntrC activation was not examined (40). As mentioned earlier, efficient activation of

the nifH promoter by nifA requires upstream sequences, the inefficient activation by ntrC may reflect a lack of productive interaction of ntrC with upstream elements in the nifH promoter.

Activation, albeit weakly, of the upstream deleted nifH promoter by nifA and ntrC implies that sufficient information is present in the -24 and -12 regions for transcription to be initiated. Similar conclusions were reached with the nifL promoter (7). Clearly upstream sequences enhance the activation of both promoters; however the mechanism of transcriptional activation by nifA or ntrC in concert with ntrA is not yet well understood. Mutations which reduce the expression of positively activated promoters can affect (i) activator binding sites, (ii) RNA polymerase binding sites, or (iii) sites at which the activator(s) and RNA polymerase interact. Should such sites overlap, but correspond to different sequences they may be difficult to distinguish (44). In the nifH promoter only one base change (the G to A transition at -13) is a promoter down mutation for both nifA and ntrC activation, as it is in the nifL promoter. It is possible that this residue is essential for some common step of the activation process, perhaps an interaction with RNA polymerase or with ntrA.

In conclusion, results presented in this paper illustrate that the regions of nucleotide conservation identified amongst the K. pneumoniae nif promoters are important for promoter activity and function. These sequences are also found amongst promoters positively activated by ntrC (for example the glnA promoter, 14) and nif promoters from other organisms (2,45). It therefore seems likely that these sequences are functionally important in these promoters. Lastly, results with the nifH promoter have shown upstream sequences are essential for nifA activation of this promoter. Upstream sequences appear to be important for the function of a number of other K. pneumoniae nif promoters (M. Buck. S. Miller, M. Drummond, R.A. Dixon, manuscript in preparation). The mutations described in this paper will be valuable in analysing in vitro the interaction of nif promoters with those factors necessary for transcriptional activation.

ACKNOWLEDGEMENTS

The authors wish to thank M. Drummond for making available pMD1405 and for his comments on the manuscript, M. Merrick for valuable discussions and John Postgate for his support and reading of the manuscript. Our thanks go also to W. Cannon and J. Chown for skilled technical assistance and Beryl Scutt for typing the manuscript. H.K. was recipient of a Commonwealth Fellowship. The pSB plasmids used in this work were kindly provided by F. Ausubel.

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