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The Product of the *Klebsiella aerogenes nac* (Nitrogen Assimilation Control) Gene Is Sufficient for Activation of the *hut* Operons and Repression of the *gdh* Operon

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In *Klebsiella aerogenes*, the formation of a large number of enzymes responds to the quality and quantity of the nitrogen source provided in the growth medium, and this regulation requires the action of the nitrogen regulatory (NTR) system in every case known. Nitrogen regulation of several operons requires not only the NTR system, but also NAC, the product of the *nac* gene, raising the question of whether the role of NAC is to activate operons directly or by modifying the specificity of the NTR system. We isolated an insertion of the transposon Tn*5tac1* which puts *nac* gene expression under the control of the IPTG-inducible *tac* promoter rather than the nitrogen-responsive *nac* promoter. When IPTG was present, cells carrying the *tac-nac* fusion activated NAC-dependent operons and repressed NAC-repressible operons independent of the nitrogen supply and even in the absence of an active NTR system. Thus, NAC is sufficient to regulate operons like *hut* (encoding histidase) and *gdh* (encoding glutamate dehydrogenase), confirming the model that the NTR system activates *nac* expression and NAC activates *hut* and represses *gdh*. Activation of urease formation occurred at a lower level of NAC than that required for glutamate dehydrogenase repression, and activation of histidase formation required still more NAC.

The enteric bacterium *Klebsiella aerogenes* grows well with a variety of organic and inorganic compounds as the sole nitrogen (N) source. The preferred N source is ammonium, and other N sources such as amino acids, nitrate, or urea are growth rate limiting. In every case studied, the enzyme systems responsible for the utilization of the various N sources are regulated by the quality and quantity of the N source (3). Thus, the histidine utilization (Hut) system, which provides ammonium and glutamate from histidine, is derepressed in the absence of ammonium, but not in its presence (28, 34). Conversely, glutamate dehydrogenase (GDH), which is responsible for assimilating ammonium when ammonium is abundant, is repressed in the absence of ammonium but not in its presence (9). Many of the N-regulated operons are subject to other controls (e.g., *hut* can also be activated by catabolite activator protein-(CAP) with cyclic AMP (cAMP), but these controls are independent of N regulation (34) and need not be considered here. In every case tested, N regulation requires the action of the nitrogen regulatory (NTR) system (26). The key element of the NTR system is an enhancer-binding, transcriptional activator (NTRC, also called NR_I) which must be phosphorylated to activate transcription (31). The remaining proteins of the NTR system regulate the phosphorylation (activation) and dephosphorylation (inactivation) of NTRC (31). Phosphorylated NTRC activates transcription from a set of novel promoters that are recognized by a minor form of RNA polymerase that contains the sigma factor σ^{54} rather than the usual σ^{70} (20, 22).

Although the NTR system and σ^{54} are necessary for regulation of every N-regulated system studied, the NTR system and σ^{54} are not sufficient in all cases (7). Many of the systems studied thus far also require NAC, the product of the *nac* gene (26). Mutants lacking an active *nac* gene have lost NTR-mediated N regulation of Hut, GDH, proline utilization (Put), urease (Ure), and glutamate synthase (26). However, *nac* mutants still show normal N regulation of glutamine synthetase (the paradigm NTR-regulated enzyme) as well as of the assimilatory nitrate reductase, the *nifL* operon, a catabolic asparaginase, and tryptophan permease. Two of the NAC-independent operons in *Klebsiella* species (*glnA* and *nifL*) have been shown to be transcribed by the σ^{54}-bearing RNA polymerase (1). Two of the NAC-dependent operons (*hut* and *put*) have been shown to be transcribed by the more common σ^{70}-bearing RNA polymerase (10, 29). Thus, we have suggested that NAC serves as a coupler between σ^{70}-dependent operons and the σ^{54}-dependent NTR system (3).

NAC is clearly necessary for N regulation of several operons, but whether NAC is sufficient for activation of Hut or repression of GDH remained an open question. Operon fusions that placed β-galactosidase formation under the control of the *nac* promoter showed that the transcription of the *nac* gene is strongly regulated by the N source and that this regulation is mediated by the NTR system (8, 26). Thus, two types of model could explain the role of NAC in N regulation. The simplest model is that the NTR system activates the expression of the *nac* gene and the NAC thus produced is sufficient to activate Hut and GDH. However, the data were also consistent with a model in which NAC interacts with the NTR system to modify its specificity to include operons like *hut* and *gdh*. To distinguish between these possibilities, we needed a way to produce NAC in the cell in the absence of the components of the NTR system. To this end, we took advantage of the transposon Tn*5tac1*, which carries a strong, regulated, outward-facing *tac* promoter (11). Insertions of Tn*5tac1* place an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter into the chromosome with a linked Km^r to allow selection. The presence of a $lacI^q$ gene within the transposon ensures an

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TABLE 1. List of strains

Strain	Genotype	Source or reference
KC1043	Wild type[a]	3
KB1567	*his-401 nal-1 zed-1*::Tn*10*	A. Ninfa[b]
KC1611	*nac-112*::MudX	26
KC1786	*nac-203*::Tn*5*	26
KC2000	*nac-203*::Tn*5-131*	8
KC2001	*gltB200 ntrC5*::Tn*5-131 ntrB45*(pTROY11)	26
KC2005	*ntrC5*::Tn*5-131 ntrB45*	P1 (KC2001) × KC1043
KC2159	*zed-2*::Tn*5*	A. Ninfa[b]
KC2174	*zed-2*::Tn*5-131*	This work[c]
KC2340	*his-33*::Tn*5mob*	λ::Tn*5mob* × KC1043
KC2472	*nac-305*::Tn*5tac1*	This work
KC2473	*nac-306*::Tn*5tac1*	This work
KC2474	*nac-308*::Tn*5tac1*	This work
KC2515	*nac-112*::MudX *zed-1*::Tn*10*	P1 (KB2521) × KC1611
KB2521	*nac-306*::Tn*5tac1 zed-1*::Tn*10 his-401 nal-1*	P1 (KC2473) × KB1567
KC2562	*rpoN5018*	26
KC2582	*ntrC5*::Tn*5-131 ntrB45 nac-306*::Tn*5tac1*	P1 (KC2473) × KC2005
KC2638	*rpoN5018 nac-306*::Tn*5tac1*	P1 (KC2473) × KC2562
KC2661	*glnD17*	14[b]
KC2662	*glnD66*	14[b]
KC2733	*glnB3*	33[b]
KC2734	*glnD17 nac-306*::Tn*5tac1*	P1 (KC2473) × KC2661
KC2735	*glnD66 nac-306*::Tn*5tac1*	P1 (KC2473) × KC2662
KC2738	*ntrC2*::Tn*5-131*	4[c]
KC2739	*ntrC2*::Tn*5-131 nac-306*::Tn*5tac1*	P1 (KC2738) × KC2473
KC2740	*glnB3 nac-306*::Tn*5tac1*	P1 (KC2473) × KC2733

[a] Strain MK1043 is derived from MK9000 (2) and differs from the wild-type isolate in carrying the *hutC515* mutation, being sensitive to phage P1, and being cured of the drug resistance plasmid, pPN100. All strains in this table except those with a KB prefix are derived from KC1043.
[b] Constructed by multiple transductions from strains provided by these sources.
[c] Replacement of the kanamycin-resistant Tn*5* element with the tetracycline-resistant Tn*5-131* element as described previously (26).

adequate supply of *lac* repressor to reduce expression from the *tac* promoter in the absence of IPTG. We demonstrate here that when *nac* was expressed from the IPTG-inducible promoter, the presence of NAC was indeed sufficient to activate Hut expression and repress GDH and that neither the components of the NTR system nor any physiological signals generated by N starvation were required if NAC was formed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains (described in Table 1) are derived from *K. aerogenes* MK9000 (39) and all carry the *hutC515* allele. Transposon Tn*5tac1* (11) was obtained on a lambda vector from D. Berg. Plasmid pTROY11 (12) was obtained from R. Ludwig. Minimal medium for growth of cultures was W4 salts (26) with glucose (0.4%, wt/vol) as the sole carbon source and glutamine (0.1%, wt/vol, Calbiochem grade A, freshly made) as the growth rate-limiting nitrogen source. Ammonium sulfate was added to 0.2% (wt/vol) where indicated. When present, IPTG was added at a final concentration of 1 mM unless otherwise indicated. Solid medium contained 1.5% (wt/vol) agar and W salts (5) instead of W4 salts. Histidine and proline, when present, were added at 0.2% (wt/vol); kanamycin sulfate and tetracycline were added at 50 and 40 μg/ml, respectively. Otherwise, growth conditions were as described previously (26).

Genetic methods. Transposon mutagenesis with λ::Tn*5tac1* (10) relied on the use of the plasmid pTROY11 (12) to provide the *K. aerogenes* strains with lambda receptor protein. Generalized transduction with bacteriophage P1*vir* was as described by Miller (27).

The *glnD17* and *glnD66* alleles were transduced into isogenic backgrounds by genetic linkage to *pan-70*::Tn*10*. This allele was isolated from a random pool of Tn*10* insertions in *K. aerogenes* with a λ561, lambda::Tn*10* vector, kindly provided by N. Kleckner. Transduction with P1*vir* was used to move the *pan-70*::Tn*10* mutation into the *glnD17* mutant MK9264 (a *pro metD* derivative of MK9159 [15]) and the *glnD66* mutant MK9599 (15) by selection for tetracycline resistance and scoring for a pantothenic acid requirement and inability to utilize proline as the sole nitrogen source. P1*vir* was grown on these transductants, and the lysate was used to transduce the wild-type strain KC1043 to tetracycline resistance, again scoring for the pantothenic acid requirement and the inability to use proline as the sole nitrogen source. The *pan-70*::Tn*10* allele was removed from constructs by transduction with a wild-type donor and selection for prototrophy for pantothenic acid. The retention of loss of the linked *glnD* was monitored by ability to use proline as the sole nitrogen source. One transductant from each cross was saved as strain KC2661 (*glnD17*) and KC2662 (*glnD66*). Similarly, the *glnB3* allele from MK9042 (a P1-sensitive *ilv* derivative of MK93 [9]) was used to transduce a *nadB92*::Tn*5tac1* mutant to nicotinamide prototrophy, scoring for kanamycin sensitivity and gain of a glutamine requirement. This resulted in strain KC2733. The *nadB92*::Tn*5tac1* mutation was characterized by its requirement for nicotinamide and by its cotransduction linkage with *tyrA* and *glnB*.

Mutagenesis with λ::Tn*5mob* to generate the *his-33*::Tn*5mob* allele was performed as described above. Histidine auxotrophs were isolated by replica plating onto minimal medium with and without histidine after several rounds of cycloserine enrichment. Transposon Tn*5mob* (Km^r) on plas-

mid pSUP5011 was obtained from B. Friedrich (37). This transposon was transferred to bacteriophage λ by infecting an *Escherichia coli* strain containing pSUP5011 with λ::Tn*5-132* (from D. Berg). The resulting phage lysate was plated at 30°C, and the lysogens in the turbid plaques were tested for Km^r (indicating Tn*5mob*) and Tc^r (indicating Tn*5-132*). One plaque in about 700 yielded lysogens that had replaced Tn*5-132* with Tn*5mob*. The construction was confirmed by retransfer of the transposon onto plasmids. The presence of the transposon was demonstrated by restriction analysis, and the activity of the *mob* site was confirmed by showing the ability of plasmid pVS1 (an RP4 derivative) to mobilize plasmids containing the transposon in conjugal crosses.

Enzyme assays. Cultures were grown at 37°C to a density of about 50 Klett units (filter 54). Assays for histidase, β-galactosidase, GDH, and glutamine synthetase were performed on detergent-treated whole cells as described previously (26). The protein content of whole cells was measured by the method of Lowry et al. (25) with bovine serum albumin as the standard. Specific activities are expressed as nanomoles of product formed or substrate consumed per minute per milligram of cell protein at 37°C except for β-galactosidase, which was assayed at 30°C (2).

RESULTS

Rationale for selection of *tac-nac* fusions. Mutants of *K. aerogenes* that cannot produce active NTRC (NR_I) product are unable to use either histidine or proline as their sole N source (18). However, such *ntrC* (*glnG*) mutants can use histidine as their sole N source if the *hutUH* operons are expressed at high levels, e.g., because of a mutational improvement in *hutUp*, the *hutUH* promoter (30). If NAC is the only gene product required for activation of *hutUH* expression, then insertion of an IPTG-inducible *tac* promoter upstream of the *nac* gene should cause IPTG-dependent expression of NAC and NAC-dependent derepression of *hutUH*. Such strains should grow with histidine as their sole N source (despite the *ntrC* mutation) in the presence of IPTG. Even if NAC were not sufficient for *hutUH* derepression, the selection should still yield mutants with the IPTG-inducible *tac* promoter in front of the *hutUH* operon.

Strain KC2001 is NTRC deficient either because of a Tn*5* insertion in *ntrC* or one in *ntrB* (*glnL*) that is polar on *ntrC* (26). This Tn*5* mutation was converted to Tn*5-131* as described previously (26) for two reasons: (i) Tn*5-131* is deficient in both transposition and excision, thus reducing the background of Ntr$^+$ revertants, and (ii) Tn*5-131* encodes tetracycline resistance (Tc^r), allowing the use of a kanamycin resistance (Km^r) marker in the selection. The *tac* promoter was carried on the transposon Tn*5tac1* (11), which carries a Km^r gene and the *lacI*^{q} (repressor) gene as well as a *tac* promoter that transcribes out from one end of the transposon. We used λ::Tn*5tac1* to mutagenize strain KC2001. This strain carries the plasmid pTROY11 (12) to confer λ sensitivity on *K. aerogenes*. In the presence of IPTG, five independent mutants able to use histidine as the sole N source and three more able to use proline as the sole N source were isolated.

Four of the insertions (*zzh-1*, *zzh-2*, *zzh-3*, *zzh-4*) appeared to be specific for *hut* expression since IPTG did not affect growth on proline. Two of these, *zzh-1* and *zzh-2*, were tested for linkage to *nac* and *hut* by P1-mediated transduction. As expected, no linkage to *nac* was detected, but surprisingly, no linkage to *hut* was detected (data not shown). These four *zzh*::Tn*5tac1* insertions were not studied further. The insertion *zzp-9* allowed growth on proline even in the absence of IPTG and was also not studied further. The three remaining insertions had the phenotype expected of an IPTG-inducible promoter driving *nac* expression. All three allowed the *ntrC* strain to grow with either histidine or proline as the N source, but only in the presence of IPTG (Table 2). For further study, these three insertions were backcrossed to a wild-type background (KC1043) by P1 transduction.

TABLE 2. Growth of regulatory mutants resulting from Tn*5tac1* mutagenesis

Mutation	Selection[a] (+IPTG)	Growth on[a]: GH + IPTG + Km	GH + Km	GP + IPTG + Km	GP + Km
zzh-1	GH	+	−	±	±
zzh-2	GH	+	−	±	±
zzh-3	GH	+	−	±	±
zzh-4	GH	+	−	±	±
nac-308	GH	+	−	++	±
zzp-9	GP	+	−	++	++
nac-305	GP	+	−	++	±
nac-306	GP	+	−	++	±

[a] Abbreviations for growth medium: GH and GP, glucose minimal medium with histidine and proline, respectively, as the sole nitrogen source; kanamycin sulfate (50 μg/ml).

Mapping the *nac*::Tn*5tac1* mutations. All three *nac*::Tn*5tac1* insertions were tightly linked to *nac-203*::Tn*5-131* (Table 3, crosses 1 to 3). In each case, phage P1 grown on strains carrying *nac*::Tn*5tac1* insertions was used to transduce KC2000 to Km^r. The loss of the Tc^r associated with Tn*5-131* indicated cotransduction of the markers. Only one Km^r Tc^r transductant was detected, and it proved to be a result of a transposition of Tn*5tac1* to a novel site; backcrosses showed that the Km^r and Tc^r characters were unlinked in this transductant (data not shown). A similar analysis showed that the three *nac*::Tn*5tac1* insertions were also tightly linked to *nac-112*::Mud(*lac*Ap)1. Again, no recombination between any of the three Tn*5tac1* insertions and the *nac-112* mutation was detected (Table 3, crosses 4 to 6). As expected, *nac-203* and *nac-112* were tightly linked (cross 7).

We next took advantage of a Tn*10* insertion (*zed-1*::Tn*10*) and a Tn*5* insertion (*zed-2*::Tn*5*, which we converted to a Tc^r Tn*5-131* derivative, *zed-2*::Tn*5-131*), generously provided by Alex Ninfa of Wayne State University and known to be linked to *nac*. The *nac* locus was about 18% linked to *zed-1*::Tn*10* (Table 3, crosses 8, 9, 10, 18b, and 19b) and about 60% linked to *zed-2*::Tn*5-131* (crosses 11, 12, and 13). The *zed-1*::Tn*10* and *zed-2*::Tn*5-131* insertions were about 7% linked to each other (Table 3, cross 14). Thus, the most likely order of the markers is *zed-2–nac–zed-1*. The *zed-1* locus was about 5% linked to *his* (crosses 15, 16b, and 18a), but neither *nac* (crosses 16a and 19a) nor *zed-2* (cross 17) was linked to *his*. Thus, the most likely gene order is *zed-2–nac–zed-1–his*.

***nac* expression overcomes repression by ammonium.** In *K. aerogenes*, all enzymes whose expression is regulated by the quality and quantity of the nitrogen source require the NTR system for their regulation (3). However, these enzymes fall into three classes with respect to NAC: those (like histidase, a reporter enzyme for the *hutUH* operon) that require NAC for activation of their expression, those (like GDH) that require NAC for their repression, and those (like glutamine

TABLE 3. Genetic mapping of the *nac* locus

Cross	Donor[a] (character selected)	Recipient (character replaced)	Frequency	%
1	*nac-305* (Km^r)	*nac-203* (Tc^r)	10/10	100
2	*nac-306* (Km^r)	*nac-203* (Tc^r)	29/29	100
3	*nac-308* (Km^r)	*nac-203* (Tc^r)	41/42[b]	100
4	*nac-305* (Km^r)	*nac-112* (Cm^r)	72/72	100
5	*nac-306* (Km^r)	*nac-112* (Cm^r)	69/69	100
6	*nac-308* (Km^r)	*nac-112* (Cm^r)	136/136	100
7	*nac-203* (Tc^r)	*nac-112* (Cm^r)	103/106[b]	100
8	*nac-305* (Km^r)	*zed-1*::Tn*10* (Tc^r)	14/84	17
9	*nac-306* (Km^r)	*zed-1*::Tn*10* (Tc^r)	19/91	21
10	*nac-308* (Km^r)	*zed-1*::Tn*10* (Tc^r)	16/93	17
11	*nac-306* (Km^r)	*zed-2*::Tn*5-131* (Tc^r)	22/42	52
12	*nac-308* (Km^r)	*zed-2*::Tn*5-131* (Tc^r)	24/33	73
13	*zed-2*::Tn*5-131* (Tc^r)	*nac-112* (Cm^r)	31/56	55
14	*zed-1*::Tn*10* (Tc^r)	*zed-2*::Tn*5* (Km^r)	6/81	7
15	*zed-1*::Tn*10* (Tc^r)	*his*::Tn*5mob* (Km^r)	2/74	3
16a	*his*::Tn*5mob* (Km^r)	*nac-112* (Cm^r)	0/259	<0.5
16b	*his*::Tn*5mob* (Km^r)	*zed-1*::Tn*10* (Tc^r)	14/259	5
17	*zed-2*::Tn*5-131* (Tc^r)	*his*::Tn*5mob* (Km^r)	0/72	<1
18a	*zed-1*::Tn*10* (Tc^r) (also *nac-306* [Km^r] and *his*)	His$^+$	2/85	2
18b	*zed-1*::Tn*10* (Tc^r) (also *nac-306* [Km^r] and *his*)	*nac*$^+$ (Km^s)	13/85	15
19a	*nac-306* (Km^r) (also *his* and *zed-1*::Tn*10* [Tc^r])	His$^+$	0/78	<2
19b	*nac-306* (Km^r) (also *his* and *zed-1*::Tn*10* [Tc^r])	*zed*$^+$ (Tc^s)	6/78	8

[a] Phage P1*vir* was propagated on the donor strain and used to transduce the recipient strain to the phenotype indicated. Progeny of each cross were purified by single-colony isolation and scored for the phenotypes indicated. Frequencies are the percentage of the total transductants that have lost the recipient character.
[b] The transductants resistant to both antibiotics were tested by a backcross against wild type. In none of the cases (four in all) were the two resistances linked, indicating that either the transposon in the donor or that in the recipient had transposed during the transduction. Thus, the linkage remains 100%.

synthetase) whose regulation is independent of NAC (3). Table 4 shows that, in the wild type, histidase and glutamine synthetase formation were activated about 10-fold in response to N starvation and GDH formation was repressed (strain KC1043). The *nac-203*::Tn*5* insertion eliminated the response of histidase and GDH, but left the regulation of glutamine synthetase intact (strain KC1786). The *nac-306* (strain KC2743) and *nac-308* (data not shown) insertions resembled *nac-203* in that regulation of histidase and GDH in response to N starvation was abolished and regulation of glutamine synthetase formation was normal. These strains thus displayed a Nac$^-$ phenotype in the absence of IPTG. Strain KC2472 (*nac-305*) had a phenotype intermediate between Nac$^+$ and Nac$^-$. Histidase derepression was impaired, but not abolished, and GDH repression was almost normal. The *nac-308* insertion responded in exactly the same way as *nac-306* in all cases tested, so only the data for *nac-306* are shown.

When IPTG was added to either of the Tn*5tac1* insertions, histidase was derepressed to very high levels and GDH was repressed to very low levels (Table 4, strains KC2473 and KC2472). This activation of histidase formation and repression of GDH occurred even though ammonium was present and was specific for the NAC-dependent systems. Glutamine synthetase regulation was only slightly affected by IPTG in these strains. Thus, when the *nac* gene was expressed from the *tac* promoter, histidase and GDH levels responded to the presence of IPTG rather than to the presence or absence of ammonium. Note that the *K. aerogenes lac* operon expression was induced to higher levels in strain KC1043 than in either of the Tn*5tac1* insertions. This may reflect the presence of an *E. coli lacI*^{q} gene (encoded on Tn*5tac1*) in addition to the *K. aerogenes lacI* gene.

Role of the NTR system in NAC-mediated regulation. Since production of NAC from the *tac* promoter was sufficient to overcome the physiological signal generated by adding ammonium to the medium, we next tested whether any elements of the NTR system were necessary for NAC-mediated activation of histidase expression or repression of GDH. To test the role of NTR, we constructed a set of isogenic strains each of which had a different mutation in the NTR system and a parallel set, identical to the first except that the *nac-306*::Tn*5tac1* insertion was also present.

NTRC and sigma 54. The *ntrC* mutant KC2005 showed little or no derepression of histidase or repression of GDH in response to N starvation, and of course, IPTG had no effect. Glutamine synthetase showed the typical two- to fourfold derepression seen in *K. aerogenes ntrC* strains (18, 26). The *nac-306* insertion did not change the response to N starvation in the absence of IPTG, but addition of IPTG caused both the activation of histidase expression and the repression of GDH. In addition to the *ntrC* allele, strain KC2005 also carried the *ntrB45* allele, which would cause the NTR system to be constitutively active. However, the phenotype of the *ntrC* insertion should be completely epistatic to *ntrB45* (26). As a control, another *ntrC* mutant that did not carry the *ntrB45* mutation, KC2738, was derived from KC1954 (4) and tested. KC2738 also failed to regulate histidase or GDH formation in response to N starvation. Addition of the *nac-306* insertion (KC2739) resulted in IPTG-dependent activation of histidase expression and repression of GDH expression, even in the presence of excess ammonium. Thus, the NTRC product was not necessary for NAC-dependent activation of histidase expression and repression of GDH.

The *rpoN* gene encodes the σ^{54} factor required for recognition of NTR-dependent promoters by core RNA polymerase (20). Strain KC2562 (*rpoN*) lacked regulation of histidase and GDH formation and was unable to produce glutamine synthetase at detectable levels. IPTG-dependent production of NAC from the *nac-306* insertion (KC2638) activated histidase expression and repressed GDH, but

TABLE 4. Regulation of enzyme formation in strains carrying *nac*::Tn*5tac1* insertions

Strain	Relevant genotype	Medium[a]	Sp act (nmol/min/mg of protein)			
			Histidase	GDH	Glutamine synthetase	β-Galactosidase
KC1043	Wild type	−N	377	23	2,366	ND[b]
		+N	31	433	204	ND
		+N + IPTG	53	269	209	148
KC1786	*nac-203*::Tn*5*	−N	47	460	2,260	ND
		+N	34	360	230	ND
KC2473	*nac-306*::Tn*5tac1*	−N	68	327	2,586	ND
		+N	33	396	193	ND
		+N + IPTG	723	4	340	22
KC2472	*nac-305*::Tn*5tac1*	−N	131	60	2,530	ND
		+N	30	378	136	ND
		+N + IPTG	598	11	416	35
KC2005	*ntrC5*	−N	109	273	520	1.5
		+N	54	284	236	1.0
		−N + IPTG	98	245	562	154
		+N + IPTG	51	290	263	116
KC2582	*ntrC5 nac-306*	−N	109	222	624	2.1
		+N	44	226	258	1.3
		−N + IPTG	841	7	316	28
		+N + IPTG	810	9	284	33
KC2738	*ntrC2*	−N	26	279	350	ND
		+N	17	179	137	0.6
		+N + IPTG	23	158	144	72
KC2739	*ntrC2 nac-306*	+N	63	200	157	1.8
		+N + IPTG	577	42	171	29
KC2562	*rpoN5018*	−N	135	307	<40	0.8
		+N	90	284	<40	1.2
		+N + IPTG	92	266	<40	114
KC2638	*rpoN5018 nac-306*	−N	109	239	<40	1.6
		+N	57	255	<40	1.3
		+N + IPTG	879	<2	<40	39
KC2733	*glnB3*	−N	70	215	228	ND
		+N	36	136	34	0.9
		+N + IPTG	42	132	37	112
KC2740	*glnB3 nac-306*	+N	51	130	61	1.2
		+N + IPTG	802	18	200	24
KC2661	*glnD17*	−N	137	90	1,126	ND
		+N	47	121	57	0.7
		+N + IPTG	52	117	42	103
KC2734	*glnD17 nac-306*	+N	108	102	106	0.6
		+N + IPTG	803	8	366	21
KC2662	*glnD66*	−N	129	82	1,167	ND
		+N	51	122	37	0.5
		+N + IPTG	53	130	45	102
KC2735	*glnD66 nac-306*	+N	102	96	76	0.6
		+N + IPTG	670	5	432	23

[a] Strains indicated were grown in glucose minimal medium with glutamine as the growth rate-limiting nitrogen source as described in Materials and Methods in the presence (+N) or absence (−N) of 0.2% (wt/vol) ammonium sulfate, and in the presence (+IPTG) or absence of 1 mM IPTG. Cells were harvested in the mid-log phase and assayed for the enzymes indicated.

[b] ND, not determined.

did not affect the NAC-independent glutamine synthetase operon. Thus, σ^{54} was not needed for NAC-dependent activation of histidase expression and repression of GDH.

***glnB* and *glnD*.** The *glnB* gene encodes a small protein, P_{II} (15, 16), that plays a key role in regulating the NTR system (31). When unmodified, P_{II} causes the NTRB protein (NR_{II}) to dephosphorylate NTRC (and thus inactivate it). When modified by the covalent attachment of UMP residues in response to nitrogen limitation, P_{II}-UMP does not signal NTRB to dephosphorylate NTRC and the active NTRC-phosphate accumulates (31). The *glnB3* mutation (by analogy to similar mutations in *K. pneumoniae*) probably prevents uridylylation of P_{II} and thus continuously signals dephosphorylation and inactivation of NTRC (21). As expected, strain KC2733 (*glnB3*) is defective in the activation of histidase expression and repression of GDH. Some regulation of glutamine synthetase was seen, but the absolute levels were almost 10-fold lower than wild-type levels. The *nac-306* insertion caused IPTG-dependent activation of histidase expression and repression of GDH, showing that modification of P_{II} was not necessary for NAC function at *hut* or *gdh*. Surprisingly, induction of *nac* expression from the *tac* promoter resulted in derepression of glutamine synthetase in the presence of ammonium to levels characteristic of the N-starved *glnB3* strain.

The *glnD* gene encodes a uridylyltransferase that modifies P_{II} and also removes the modification (15). The *glnD* product is allosterically responsive to the concentrations of glutamine and α-ketoglutarate (19). Since high intracellular glutamine concentrations signal N excess and low glutamine concentrations signal N starvation (6), the *glnD* product is thought to be the ultimate sensor of the N status of the cell (35). The two *glnD* mutants KC2661 (*glnD17*) and KC2662 (*glnD66*) were both partially defective in N regulation of histidase and GDH formation. Somewhat surprisingly, the regulation of glutamine synthetase formation appeared almost normal in both *glnD* strains. IPTG induction of *nac* expression from the *tac* promoter (strains KC2734 and KC2735) activated histidase expression and repressed GDH, showing that the uridylyltransferase was not necessary for NAC-mediated activation of histidase expression and repression of GDH. Unexpectedly, IPTG-mediated induction of *nac* expression caused an increase in glutamine synthetase levels in the presence of ammonium in both strains KC2734 and KC2735, suggesting an interaction between NAC or some NAC-controlled function and the regulation of glutamine synthetase formation seen in *glnB* and *glnD* strains, perhaps independent of the residual regulation seen in the *ntrC* strains.

Differential sensitivities of NAC-dependent systems. The *tac* promoter used to drive *nac* expression was under the control of two repressors, the *E. coli lacI*^{q} product carried on Tn*5tac1* and the *K. aerogenes lacI* product. Both repressors are inactivated by IPTG (2), so we used a variety of concentrations of IPTG to produce a variety of NAC concentrations in the cell. Figure 1 shows clearly that 1 mM IPTG (the concentration used in all the assays in this work) was sufficient for full derepression of histidase formation and repression of GDH. At 0.1 mM IPTG, GDH was almost fully repressed, but histidase showed little derepression. Thus, we conclude that activation of the *hutUH* operon required a higher concentration of NAC than did repression of the *gdh* operon. A third NAC-regulated enzyme, urease (26), was also tested and found to be very sensitive to activation by NAC. At the lowest concentration of IPTG tested, urease derepression was 100% that seen in the wild type and did not increase with increasing IPTG addition.

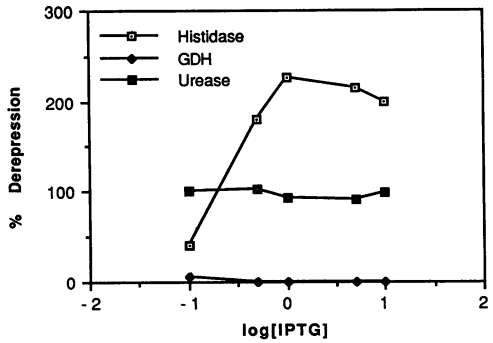

FIG. 1. Differential sensitivity of histidase, urease, and GDH formation to NAC concentration. Strain KC2473 with *nac* gene expression under control of the *tac* promoter was grown at 37°C in glucose minimal medium in the presence of excess ammonium sulfate (0.2%, wt/vol) and various concentrations of IPTG. For histidase and urease, 100% derepression was defined as 382 and 1,180 U/mg, the levels seen in the wild type grown with limiting nitrogen (26). For GDH, 100% was defined as 370 U/mg, the level seen in the wild type grown with excess nitrogen.

In the absence of IPTG, strain KC2743 showed little or no NAC-dependent activation of histidase formation or repression of GDH formation (compare KC2743 with KC1043 in Table 4). Moreover, nitrogen limitation had little effect on histidase or GDH formation in the absence of IPTG (compare −N with +N in Table 4). However, urease formation was significant in strain KC2743, even in the absence of IPTG (Table 5). Under conditions of nitrogen excess, urease was about 20% derepressed (about 10-fold more than seen in a *nac*-defective strain, KC1786). Under conditions of nitrogen limitation, the amount of urease was only about twofold higher. The failure of urease to increase dramatically in response to nitrogen limitation did not reflect a failure to activate the NTR system. Both glutamine synthetase and asparaginase (both NTR-dependent, NAC-independent operons) were derepressed to normal levels by nitrogen limitation in KC2473 (see KC1043, Tables 4 and 5).

DISCUSSION

The *nac-306*::Tn*5tac1* mutation had two distinct phenotypes. In the absence of IPTG, strains carrying *nac-306* are

TABLE 5. Urease regulation in KC2473 by NAC but not by nitrogen limitation

Strain[a]	Relevant genotype	Sp act (nmol/min/mg of protein)			
		Urease		Asparaginase	
		+N	−N	+N	−N
KC1043[b]	Wild type	32	1,180	<10	180
KC1786[b]	*nac-203*::Tn*5*	19	410	<10	300
KC2473	*nac-306*	240	520	<10	270
KC2473 (+IPTG)	*nac-306*	1,400	1,400	<10	140

[a] Strains were grown in glucose minimal medium with glutamine as the sole (growth rate-limiting) nitrogen source (−N) or with ammonium sulfate and glutamine both present (+N). IPTG was absent except where indicated (where it was added to 1 mM).

[b] The values for these strains have been reported previously (26) and are repeated here for the purpose of comparison.

Nac$^-$ in that *hut* expression could not be derepressed nor could *gdh* expression be repressed in response to N starvation. The partial response of the *ure* operon in a *nac-306* strain is discussed below. In the presence of IPTG, strains carrying *nac-306* were Nac constitutive in that *hut* expression was high and *gdh* expression was low whether N was limiting or abundant. The fact that NAC function was induced by IPTG suggests that the *nac* coding sequence was not disrupted by *nac-306* and the lack of response of NAC to the N status of the cell suggests that *nac-306* strongly interfered with NTR-mediated expression of *nac*. Indeed, DNA sequence analysis of the *nac* gene region has shown that *nac-306* is an insertion of Tn*5tac1* into the 56 bp between the *nac* open reading frame and the σ^{54}-dependent *nac* promoter (13, 36). Thus, it appears that transcription initiating at the natural *nac* promoter terminates somewhere within the Tn*5tac1* element and only transcription from the *tac* promoter actually enters *nac*. DNA sequence analysis showed that *nac-306* and *nac-308* were insertions of Tn*5tac1* into exactly the same site, explaining their identical phenotypes.

The *nac-305* insertion also resulted in full activation of histidase formation and repression of GDH in the presence of IPTG. However, in contrast to *nac-306*, *nac-305* did not abolish NTR-mediated regulation of Hut and GDH. DNA sequence analysis showed that *nac-305* lies farther upstream than *nac-306*, in fact, upstream from the *nac* promoter. Thus, both the *nac* promoter and the *tac* promoter are capable of driving *nac* transcription. To simplify the interpretation of the results, only the *nac-306* insertion (in which *nac* transcription comes from only the *tac* promoter) was studied in the combinations with *ntr* mutations.

The key observation reported here is that IPTG-mediated NAC production was effective in activating *hut* expression and repressing *gdh* expression even in the absence of NTRC and σ^{54}, the key elements of the NTR system. Thus, the role of the NTR system in the N regulation of *hut* and *gdh* is limited to the activation of *nac* expression. Once NAC is produced, the NTR system plays no further essential role.

The fact that IPTG-induced NAC could activate *hut* and repress *gdh*, even when the cells were grown with excess ammonium, seems to suggest that no cofactor or modification of NAC was required for regulation. Two objections to this conclusion might be raised. One might argue that the IPTG induction of NAC formation alters the physiology of the cell to make ammonium a poor medium for N excess. However, the production of glutamine synthetase, the classical reporter enzyme for the response to N regulation, and of asparaginase are just as repressed by ammonium in *nac-306* mutants as in *nac*$^+$ strains. This shows that the cells recognized the N excess but still activated *hut* if NAC formation was induced by IPTG. Alternatively, one might argue that active and inactive forms of NAC exist in some equilibrium and that normal regulation would shift more of the inactive form to the active form. Then overproduction of total NAC might result in a parallel increase of both active and inactive forms of NAC, with enough active form thus made to act at *hut*, *ure*, and *gdh*. The partial activation of *ure* in a *nac-306* strain even in the absence of IPTG provides evidence against this model. The repression of the *tac* promoter by the *lacI* product is known to be somewhat leaky (24). In the absence of IPTG, the *tac* promoter apparently provides enough NAC to activate *ure* expression to about 20% of the fully activated level. More active NAC, resulting from IPTG induction, gives more urease, showing a limitation for active NAC. However, in the absence of IPTG, N starvation increased *ure* expression only about twofold in a *nac-306* strain (Table 5), even though the derepression of glutamine synthetase and asparaginase shows that the cell was responding to the N starvation. In fact, even the slight increase in urease formation under N limitation may be NAC independent, since all *nac* mutants show a partial derepression of urease formation by N limitation (Table 5, strain KC1786 [26]). Thus, arguments involving a cofactor or modification that regulates NAC activity seem unlikely. In short, transcription of *nac* and translation of NAC seem sufficient to activate and repress NAC-dependent operons.

The existence of NAC as an intermediary between transcriptional regulation by the NTR system and transcription of *hut* and *gdh* raises the question of utility (3). If NTR can activate transcription and no regulation intervenes at the level of NAC, what advantage is there to having NAC over using NTR directly? The differential sensitivities of *ure*, *gdh*, and *hut* expression to NAC levels may provide an answer. The lowest levels of NAC (produced by the *nac-306* strain in the absence of IPTG) were enough to give substantial activation of *ure*. IPTG at 0.1 mM allowed enough NAC formation to repress *gdh* almost fully, but not to activate *hut*. IPTG at 1 mM provided enough NAC to activate *hut*. Thus, if both urea and histidine were present when N limitation was imposed, NAC accumulation would first lead to urease formation. The ammonia generated from urea cleavage might well be enough to prevent further NAC accumulation and thus spare the histidine for use as an amino acid rather than as a N source. Overproduction of NAC by IPTG induction of *nac-306* resulted in a higher level of histidase formation than was seen under standard N-limited growth conditions. Thus, we expect that in wild-type strains grown under N-limiting conditions, the *ure* system was more than saturated for NAC, but the *hut* system was poised on the shoulder of the activation curve. This may explain the observation of Friedrich and Magasanik (17) that addition of ammonium to an N-limited culture caused the rate of histidase accumulation to decline almost immediately, but the rate of urease accumulation declined only after a considerable lag.

The data in Table 4 show clearly that IPTG induction of NAC production in *nac-306* strains resulted in the activation of *hut* and repression of *gdh* regardless of the nitrogen source or presence of the NTR system. Moreover, at least in an NTR$^+$ background, IPTG induction of *nac-306* had little effect on the NTR-mediated regulation of glutamine synthetase formation. However, several features of Table 4 are rather more subtle and require comment. All the stains in Table 4 were isogenic to each other except for the mutations indicated. Thus, we are able to make direct comparisons without having to worry about the effects of strain background.

The first effect to note is that the *ntrC* strains showed a two- to threefold derepression of glutamine synthetase formation in response to N limitation. This residual regulation has been seen in every *ntrC* mutant of *K. aerogenes* (and *K. pneumoniae*) described so far, but is never seen in *E. coli* or *Salmonella typhimurium ntrC* mutants (23, 32). Note that this is not an example of phosphorylation of the activator by an inappropriate phosphate donor, a phenomenon generally called crosstalk (38). On the contrary, this is a N-responsive regulation mediated by an element other than NTRC. Whether this reflects regulation at the σ^{54}-dependent *glnAp2* promoter or the σ^{70}-dependent *glnAp1* promoter cannot be proved from the data in Table 4, but since *rpoN ntrC* double mutants show the same residual regulation as the single *ntrC*

mutants (18), it is more likely to be from *glnAp1*. In any event, the phenomenon is specific for *Klebsiella* species, and the residual derepression of glutamine synthetase formation in *ntrC* strains appears to be blocked by IPTG-induced NAC accumulation (KC2582, −N +IPTG).

A second effect, an interaction between NAC and glutamine synthetase regulation, is seen in the *glnB* and *glnD* strains. In both cases, IPTG-induced NAC production resulted in levels of glutamine synthetase that were elevated to a level characteristic of *ntrC* strains. Since *glnB* and especially *glnD* strains already showed considerable response to the N status of the cell, it is possible that NAC-mediated regulation of NAC-dependent operons may have altered the cell's metabolism in ways that depleted the intracellular glutamine pools, leading to excess derepression of glutamine synthetase. However, we cannot exclude a more direct interaction between NAC and glutamine synthetase regulation in *glnB* and *glnD* mutants. In particular, our data do not exclude a model suggested by Boris Magasanik (26a) in which NAC might be able to compete with NTRC but not with NTRC-phosphate for binding in the *glnA* regulatory region. Then NAC would have no effect on transcription from the σ^{54}-dependent, NTRC-phosphate-dependent *glnAp2*, but would alleviate the repression of *glnAp1* by NTRC (the major form of NTRC in *glnB* and *glnD* strains) but not by NTRC-phosphate (the major form of NTRC in *rpoN* strains grown in excess N). The NAC-dependent increase in glutamine synthetase levels seen in *glnB*, *glnD*, and even *gln*$^+$ strains grown with excess N would all be consistent with this model, as would the observation that NTRC-phosphate binds more effectively to its target than does NTRC (13, 40).

Finally, we note that there appeared to be a slight derepression of histidase formation in response to N limitation in every strain, however defective in NTR or NAC. We have seen this previously with a variety of *nac* mutants as well as with *ntr* mutants (26), and the pattern is repeated in Table 4. This cannot have been the result of *hut* activation by the CAP-cAMP system (34) since N limitation would have increased catabolite repression (lowering cAMP levels), not relieved it. This effect seems qualitatively different from the residual urease regulation seen in *ntr* and *nac* mutant strains. The residual urease regulation is highly variable and typically shows a 4- to 10-fold derepression (26).

The relationships among these residual forms of N regulation in *K. aerogenes* are unknown, but the data presented here allow us to propose a clear model for the principal N regulation of *hut* and *gdh*. The σ^{54}-dependent NTR system is entirely responsible for regulating *nac* expression in response to N limitation. The NAC thus produced then operates independently to activate (or repress) the σ^{70}-dependent target operons like *hut* and *gdh* and probably *ure*, *put*, and *gltB* as well.

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