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Role of the *nac* Gene Product in the Nitrogen Regulation of Some NTR-Regulated Operons of *Klebsiella aerogenes*

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A positive, genetic selection against the activity of the nitrogen regulatory (NTR) system was used to isolate insertion mutations affecting nitrogen regulation in *Klebsiella aerogenes*. Two classes of mutation were obtained: those affecting the NTR system itself and leading to the loss of almost all nitrogen regulation, and those affecting the *nac* locus and leading to a loss of nitrogen regulation of a family of nitrogen-regulated enzymes. The set of these *nac*-dependent enzymes included histidase, glutamate dehydrogenase, glutamate synthase, proline oxidase, and urease. The enzymes shown to be *nac* independent included glutamine synthetase, asparaginase, tryptophan permease, nitrate reductase, the product of the *nifLA* operon, and perhaps nitrite reductase. The expression of the *nac* gene was itself highly nitrogen regulated, and this regulation was mediated by the NTR system. The loss of nitrogen regulation was found in each of the four insertion mutants studied, showing that loss of nitrogen regulation resulted from the absence of *nac* function rather than from an altered form of the *nac* gene product. Thus we propose two classes of nitrogen-regulated operons: in class I, the NTR system directly activates expression of the operon; in class II, the NTR system activates *nac* expression and the product(s) of the *nac* locus activates expression of the operon.

The enteric bacterium *Klebsiella aerogenes* is capable of using a large number of compounds as its sole source of nitrogen. The formation of the enzymes needed to metabolize these compounds is in most, if not all, cases regulated by the availability of the preferred nitrogen source, ammonium (22). The formation of the enzymes needed for the conversion of poor nitrogen sources to either ammonia or glutamate is increased when ammonia is absent. Conversely, the formation of the two enzymes ultimately responsible for assimilating ammonia into glutamate (glutamate dehydrogenase and glutamate synthase) is repressed when ammonia is absent. As early as 1973, Prival et al. (33) recognized a role for the *glnA* locus in the genetic control of nitrogen-regulation in *K. aerogenes*. After a false start suggesting that the product of the *glnA* gene (glutamine synthetase) was itself the activator of transcription for nitrogen-regulated genes (23), it eventually became clear that the glutamine synthetase encoded by the *glnA* locus was physiologically important for nitrogen regulation because it synthesized the internal signal of nitrogen excess (glutamine or a product thereof) from ammonia, but that *glnA* was not directly involved in the genetic control of nitrogen regulation. The work from several groups (for reviews, see references 19, 22, and 24) has established that the products of two genes downstream of *glnA* in a complex *glnAntrBntrC* operon are the key regulators in the nitrogen regulatory system, usually called the NTR system.

In the NTR system a protein phosphotransferase (NTRB, encoded by *ntrB*) phosphorylates or (in response to signals passed by the products of the *glnB* and *glnD* genes) dephosphorylates an enhancer-binding protein, NTRC (26). In its phosphorylated form, the enhancer-binding NTRC protein can cause the conversion of promoter-bound RNA polymerase containing the novel sigma subunit sigma-54, encoded by *rpoN* (instead of the normal sigma-70), from a closed complex to an open complex—at least in the case of the *glnA* promoter (32). Thus NTRC can activate transcription directly.

This transcriptional activation was directly demonstrated in vitro for the *Escherichia coli glnA* promoter (18, 32) as well as for the *nifL* promoter derived from *Klebsiella pneumoniae* M5a1 (1). In addition, mutants lacking either the NTR system or sigma-54 were defective in nitrogen regulation of every nitrogen-regulated operon tested (including *glnA*, *hutUH*, *gdh*, and *nifLA* of *Klebsiella* spp.). It was therefore attractive to postulate that the NTR system was directly responsible for all nitrogen regulation in *K. aerogenes*. However, Nieuwkoop et al. (25) showed that nitrogen-regulated transcription of the histidine utilization (*hutUH*) operon initiated from a sigma-70-specific promoter, not a sigma-54-specific promoter, and Bender et al. (6) described a mutation (*nac-1*) that resulted in the loss of nitrogen regulation for a family of nitrogen-regulated operons (including *hut* and *gdh*). However, *nac-1* did not affect the regulation of *glnA*, which is known to be under direct NTR system control. These results suggested that for some nitrogen-regulated operons the nitrogen regulation might be indirect, involving NAC, the product of the *nac* gene.

The goals of the research described here were to describe the *nac* locus in more detail, to identify which nitrogen-regulated systems were under indirect (NAC) control, and to formulate a working model for the role of NAC in the indirect regulation of nitrogen-regulated enzymes by the NTR system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Growth media

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TABLE 1. *K. aerogenes* strains

Strain	Genotype	Source or reference
KC895	*gltB200 ntr-45* (PW52)	MK189 (9)[a]
KB958	*nac-1 nal-1 hutC515*(pPN100, RP4)	6
KC1043	*hutC515*	28
KC1047	Δ(*gal-hut-bio-chl*)*401*	3
KC1473	*gltB200 ntr-45 nac-112*::Mu dX (PW52)	This work
KC1570	*rpoN5018 hutC515*	4
KC1604	*gltB200 ntr-45 ntrC200*::Tn*5* *nac-112*::Mu dX(PW52)	This work
KC1611	*nac-112*::Mu dX *hutC515*	This work
KC1665	*nac-101*::Mu dX *hutC515*	This work
KC1786	*nac-203*::Tn*5* *hutC515*	This work
KC1833	*gltB200 ntr-45 nac-204*::λ *plac* Mu 53(pTROY11,PW52)	This work
KC1839	*nac-204*::λ *plac* Mu 53 *hutC515*	P1 · KC1833 × KC1043[b]
KC1856	*rpoN5018 nac-204*::λ *plac* Mu 53 *hutC515*	P1 · KC1839 × KC1570
KC2005	*ntrC5*::Tn*5-131 ntr-45 hutC515*	This work
KC2298	*ntrC5*::Tn*5-131 ntr-45 nac-204*::λ *plac* Mu 53 *hutC515*	This work
KC2562	*rpoN5018 hutC515*	P1 · KC1570 × KC2501[c]

[a] Lysogenic for phage PW52.
[b] Generalized transduction of strain KC1043 with phage grown on strain KC1833.
[c] KC2501 is *argG2 gltB702*::Tn*5* derived by multiple transductions from KC1043.

and culture conditions were as described previously (5), except that the pH of the minimal salts medium was raised to 7.4 with KOH and the concentration of L-glutamine (Calbiochem grade A, freshly made) was reduced to 0.04 or 0.025% (wt/vol) in some experiments to guarantee nitrogen limitation. Carbon sources were provided at 0.4%, and nitrogen sources were provided at 0.2%. When more than one nitrogen source was present, each was provided at 0.2% except for potassium nitrite, which was used at 1 mM because of the toxicity of nitrite. In experiments where urease was measured, the growth medium was supplemented with 1 μM $NiSO_4$. In experiments where nitrate or nitrite reductase was measured, the trace metal solution SL6 (31) was included as a source of molybdenum.

Enzyme assays. All assays were performed with cells that had been washed in 1% KCl and suspended at a concentration that contained 1 to 1.5 mg of protein per ml. Most assays were performed with cells permeabilized by hexadecyltrimethylammonium bromide. Toluene was used to permeabilize cells for β-galactosidase assays, and a freeze-thaw procedure was used for nitrate reductase and nitrite reductase (20).

Assays for histidase (34), glutamate dehydrogenase (9), glutamine synthetase (5), β-galactosidase (3), glutamate synthase (9), and proline oxidase (33) were essentially as described previously. Urease was measured as described previously (13), except that hexadecyltrimethylammonium bromide (20 μg/ml) was included to permeabilize the cells and the concentration of urea was increased from 5 mM to 100 mM. Assimilatory nitrate reductase and nitrite reductase were assayed essentially as described previously (20), except that the reaction was initiated by the addition of the broken (i.e., frozen and thawed) cells and the nitrate-dependent (or nitrite-dependent) oxidation of benzyl viologen was measured during the first few seconds of the reaction. The nitrate reductase values obtained in this way varied by as much as twofold from the mean in replicate experiments with identically grown cultures. For example, the value 2.0 reported in Table 5, line 1, is the mean of five values ranging from 1.1 to 3.7. Thus, small differences are not reliable. Variations of threefold or greater were reproducible in replicate experiments and in experiments with similar strains. No effort was made to inhibit nitrite reductase during the nitrate reductase assay.

TABLE 2. EOP of Mu h^+ and Mu *h*P1 on *K. aerogenes* and *E. coli*

Phage (host)	PFU/ml when plated on:	
	KC1043	W3110r
Mu *h*P1 (*K. aerogenes*)	9×10^9	7×10^8
Mu *h*P1 (*E. coli*)	6×10^8	8×10^9
Mu h^+ (*E. coli*)	8×10^7	7×10^9

L-Asparaginase was measured by the γ-glutamyltransferase reaction in the absence of $KAsO_4$ and ADP (36); this value was subtracted from the $KAsO_4$ plus ADP-supplemented reaction value to give glutamine synthetase values.

Histidase, glutamate dehydrogenase, glutamine synthetase, and asparaginase were measured at 37°C for historical reasons; all other enzymatic activities were measured at 30°C. Specific activities are reported as nanomoles of product formed or substrate consumed per minute per milligram of cell protein. Nitrate and nitrite reductase activities are reported as nanomoles of benzyl viologen oxidized per minute per milligram of protein. Cell protein was measured by the method of Lowry et al. (21) with bovine serum albumin as the standard, except that whole cells suspended in 1% KCl were added directly to the test mixture without prior disruption (5).

Tryptophan permease was measured by a qualitative test based on that described earlier (30). The test measured the ability of the constitutively produced tryptophan transaminase to convert exogenous tryptophan to indolyl pyruvate, which can be detected by its strong absorbance in the near-UV range. In the absence of a detergent, the transaminase can act on the exogenous tryptophan only when a tryptophan permease is present (30). For the data reported here, a single colony of the test strain was inoculated into 5 ml of growth medium containing 0.2% (wt/vol) tryptophan in addition to the carbon and nitrogen sources indicated in the tables. After 16 h of aerated incubation at 30°C, the cells were removed by centrifugation and the A_{340} of the culture fluid was determined. An A_{340} of >4.0 was interpreted as positive for tryptophan permease, and an A_{340} of <0.4 was interpreted as negative.

Mutagenesis with phage Mu dX. *K. aerogenes* W70 is resistant to the coliphages P1, λ, and Mu 1. P1-sensitive variants are easily obtained (15), and we hoped that these might also be sensitive to Mu *h*P1, which has the host range of P1. P1-sensitive strains of *K. aerogenes* such as KC1043 do, indeed, plate Mu *h*P1 grown on *E. coli* with an efficiency of plating (EOP) of about 0.1 (Table 2). Restriction-negative *E. coli* strains such as W3110r plate Mu *h*P1 grown on *K. aerogenes* with a similar EOP (about 0.1). Restriction-proficient *E. coli* strains plate Mu *h*P1 grown on *K. aerogenes* with an EOP of about 10^{-4}. Both *K. aerogenes* KC1043 and *E. coli* W3110r are thought to lack restriction systems, so this 10-fold reduction in the EOP of Mu *h*P1 may reflect the inversion of the G region of phage Mu (37), but we have not examined this directly. We were surprised to find

TABLE 3. EOP and transducing ability of Mu h^+ Mu dX lysates grown on *E. coli*

Indicator	PFU/ml	Ap^r Cm^r CFU/ml
W3110	2.1×10^{10}	1.3×10^7
KC1043	4×10^7	5.7×10^4
KC1043/W3110 ratio	2×10^{-3}	4×10^{-3}

that P1-sensitive strains of *K. aerogenes* also plate Mu with the normal host range of Mu, but the EOP is about 10^{-2} or 10^{-3} (Table 2). Mu h^+ makes small, irregular plaques on *K. aerogenes*, and we worried that the EOP might underestimate the actual infectivity of Mu on *K. aerogenes*. We therefore took advantage of the defective transducing phage Mu d1 *lac(amp)B*::Tn*9* (hereafter called Mu dX), whose adsorption apparatus is provided by a helper Mu h^+ in this case (2). The same lysate induced from the double lysogen CAG5050 (Mu h^+, Mu dX) was titered both for plaque-forming units (reflecting Mu h^+) and for drug-resistant colony-forming units (reflecting the Ap^r Cm^r conferred by Mu dX) on both *E. coli* and *K. aerogenes*. Both plaque-forming units and colony-forming units were several hundredfold less frequent on *K. aerogenes* than on *E. coli* (Table 3). Thus either Mu *h*P1 or Mu h^+ can be used as a helper phage to generate Mu dX particles for mutagenesis experiments with P1-sensitive strains of *K. aerogenes*. We used Mu h^+ for the isolates described here, with the procedures described previously for mutagenesis of *E. coli* (2). The details for the selection of nitrogen regulation mutants are described in Results.

Mutagenesis with Tn*5* and λ p*lac* Mu 53. The P1-sensitive variants of *K. aerogenes* W70 remained insensitive to coliphage λ. We therefore transformed several strains to λ sensitivity with plasmid pTROY9 or pTROY11 as described previously (10). Such λ-sensitive transformants plated λ at an EOP of <1 with barely visible, irregular plaques. The λ-sensitive strains were mutagenized with the phage λ O^-P^-::Tn*5* essentially as described previously (7). Mutagenesis of λ-sensitive strains with λ p*lac* Mu 53 used a mixture of λ p*lac* Mu 53 phage and λ p Mu 507 as described previously (8).

Replacement of Tn*5* with Tn*5-131*. In some experiments we needed to convert the selectable kanamycin resistance marker associated with the wild-type Tn*5* to the tetracycline resistance marker of Tn*5-131* (11). Since Tn*5-131* is also transposase deficient (11), this replacement resulted in a genetically more stable mutation as well. To achieve this replacement, P1 *vir* grown on *E. coli* CE78 *lacZ*::Tn*5-131* (11) was used to transduce *K. aerogenes* Tn*5* strains to tetracycline resistance with a corresponding loss of kanamycin resistance. The only homology available is at the ends of the Tn*5* elements and results in the conversion of a Tn*5* insertion to a Tn*5-131* insertion at the same site. Since the site of the insertion is unchanged, we retain the same allele number, changing only the transposon designation.

RESULTS

Isolation and characterization of mutations affecting nitrogen regulation. *K. aerogenes* strains that carry the *ntr-45* mutation (thought to lie in *ntrB* [also called *glnL*]) express the NTR system constitutively (9). Such strains produce very low levels of glutamate dehydrogenase, one of the two enzymes capable of de novo synthesis of L-glutamate (9). When such strains also carry a *gltB* mutation, they lack glutamate synthase, the other enzyme capable of de novo glutamate synthesis. Thus *gltB200 ntr-45* strains are auxotrophic for glutamate (9). Spontaneous variants of a *gltB200 ntr-45* strain able to grow on glucose minimal medium with no added glutamate arose at frequencies of 10^{-5} to 10^{-7} (6) and fell into two broad categories: (i) those affecting glutamate synthesis specifically (either because of reversion at the *gltB* locus or because of mutation at the *gdh* locus, which is required for glutamate dehydrogenase formation) and (ii) those affecting nitrogen regulation in general. The latter group contained two specific classes of mutation in about equal numbers: *ntrC* (*glnG*) mutations, which destroy the activity of NTRC, and *nac* mutations, which affect the regulation of some but not all NTR-regulated genes (6; Marie Quinto and R. Bender, unpublished observation). We used three different transposable elements to generate mutants affected in nitrogen regulation: Tn*5*, Mu dX, and λ p*lac* Mu 53. No glutamate-specific mutants were expected, since $gltB^+$ revertants and *gdh* promoter-enhancement mutations would not be likely consequences of insertion sequence mutagenesis; none was found. Both classes of nitrogen regulatory mutations were found: those affecting *ntrC* and those affecting *nac*.

Insertions affecting *ntrC*. Insertion mutagenesis with either P1::Tn*5* as a vector (35) or λ::Tn*5* as a vector (Materials and Methods) gave a class of Tn*5*-induced mutations that were classified as *ntrC*::Tn*5* based on several criteria: (i) they were tightly linked to *glnA* by P1-mediated generalized transduction (>90% cotransduction); (ii) they grew poorly on glucose minimal medium with either histidine or proline as the sole nitrogen source; (iii) all of the *ntrC*::Tn*5* alleles tested relieved the glutamine auxotrophy imposed by the *rpoN5018* (formerly *glnF5018*) mutation (4); and (iv) the levels of glutamine synthetase in cells carrying *ntrC*::Tn*5* alleles were less derepressable in response to nitrogen starvation than were those in the wild type (see below). However, two features of these *ntrC* alleles should be noted: (i) we cannot distinguish an insertion in *ntrC* itself from an insertion in *ntrB* that is polar on *ntrC*, and (ii) even though the *ntrC* phenotype is completely epistatic to *ntr-45*, the *ntr-45* mutation is presumed to be present in all of these mutants. Thus, although we should properly list these mutations as *ntrBC*::Tn*5* *ntr-45*, for simplicity we call them *ntrC*::Tn*5* throughout this discussion. For technical reasons, we converted the kanamycin resistance of Tn*5* to the tetracycline resistance of Tn*5-131* in some cases as described in Materials and Methods. The *gltB200 ntr-45* strain was also mutagenized with Mu dX as described in Materials and Methods. About 50% of the prototrophic colonies arising from the Mu dX selection were blue when plated on media containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Fourteen mutants that gave blue colonies in the presence of X-gal were selected for further characterization; 11 of these 14 mutations were classified as *ntrC*::Mu dX alleles by the same criteria used for the Tn*5*-induced mutations described above, except that they were not tested for suppression of the *rpoN*-induced glutamine requirement.

Insertions affecting *nac*. Mutations resulting from insertion of Mu dX, Tn*5*, or λ p*lac* Mu 53 were assigned to the *nac* locus by the following criteria: (i) they were linked to *zzz-277*::Tn*5* (generously provided by A. Ninfa) by P1-mediated transduction with cotransduction frequencies of about 70 to 75%, similar to the cotransduction frequencies of *nac-1* to this Tn*5* insertion; (ii) they grew almost as fast as the wild type with histidine as the sole nitrogen source and somewhat more slowly than the wild type with proline or

TABLE 4. Specific activities of nitrogen-regulated enzymes in the presence and absence of ammonia[a]

Strain	Relevant genotype	Sp act (nmol of product formed per min per mg of protein)													
		Glutamine synthetase		Histidase		Glutamate dehydrogenase		Asparaginase		Proline oxidase[b]		Urease		Tryptophan permease	
		−N	+N	−N	+N	−N	+N	−N	+N	−N	+N	−N	+N	−N	+N
KC1043	*nac*$^+$	2,150	260	382	43	45	370	180	<10	48	13	1,180	32	+	−
KB958	*nac-1*	2,260	250	46	34	510	410	196	<10	14	11	80	13	+	−
KC1611	*nac-112*::Mu dX	2,050	190	122	38	260	340	195	<10	30	12	810	21	+	−
KC1665	*nac-101*::Mu dX	2,110	190	40	29	420	380	220	<10	18	12	110	30	+	−
KC1786	*nac-203*::Tn*5*	2,260	230	47	34	460	360	300	<10	16	12	410	19	+	−
KC1839	*nac-204*::λ *plac* Mu	2,430	260	69	38	460	360	291	<10	18	13	220	51	+	−

[a] Specific activities were determined as described in Materials and Methods. Tryptophan permease was measured qualitatively as described in Materials and Methods, with an A_{340} of >4.0 scored as positive (+) and an A_{340} of <0.4 scored as negative (−). Cells were grown in glucose minimal medium (5) in the presence (+N) or absence (−N) of 0.2% (wt/vol) ammonium sulfate. Cultures also contained growth-rate-limiting concentrations of glutamine. All cultures with ammonium sulfate contained 0.2% glutamine except those assayed for glutamate dehydrogenase, which contained 0.025% glutamine. The cultures without ammonium sulfate assayed for glutamine synthetase, histidase, asparaginase, and urease contained 0.04% glutamine; those assayed for proline oxidase and tryptophan permease contained 0.2% glutamine; and those assayed for glutamate dehydrogenase contained 0.025% glutamine. Glutamine concentrations of 0.1% or less result in slightly higher derepression of glutamine synthetase than does 0.2% (Tables 4 and 5), but no other differences (data not shown; Tables 4 and 5).

[b] Proline was included in the culture media at 0.1% to allow induction.

urocanate as the nitrogen source; (iii) the regulation of glutamine synthetase formation in these mutants was indistinguishable from that of the wild type (Table 4). Like the *ntrC5*::Tn*5* allele, the *nac-203*::Tn*5* allele has been converted to an *nac203*::Tn*5-131* form.

Effect of *nac* mutations on nitrogen regulation. The original *nac-1* mutant showed normal regulation of glutamine synthetase formation but was unable to derepress histidase formation or to repress glutamate dehydrogenase formation in response to nitrogen starvation (6). This was also true of each of the four *nac* insertion mutants (Table 4). The only exception was that the histidase in strain KC1611 (*nac-112*::Mu dX) was noticeably derepressed by nitrogen starvation, although still less than that of the wild type. The regulation of several other nitrogen regulated enzymes is also shown in Table 4. The nitrogen regulation of degradative L-asparaginase and of tryptophan permease were unaffected by *nac*. The nitrogen regulation of proline oxidase was like that of histidase: the *nac* strains did not derepress proline oxidase formation in response to nitrogen starvation (except KC1611, in which derepression was incomplete). The nitrogen regulation of urease was more complicated. All *nac* strains were defective in derepressing urease in response to nitrogen starvation, but the defects varied in degree. The leaky *nac* strain KC1611 showed nearly full derepression, whereas both KB958 (*nac-1*) and KC1665 (*nac-101*) were almost completely defective in urease derepression. Strains KC1786 (*nac-203*) and KC1839 (*nac-204*) showed an intermediate level of derepression. Thus all four insertions resembled the original *nac-1* mutation in that all of the *nac* mutations led to defects in the nitrogen regulation of histidase, glutamate dehydrogenase, proline oxidase, and urease and none of the *nac* mutations affected the nitrogen regulation of glutamine synthetase, L-asparaginase, or tryptophan permease. We therefore chose a representative *nac* insertion (*nac-203*) for a comparison with mutations affecting the NTR system.

Effect of NTR mutations on nitrogen regulation. Three mutants affected in the NTR regulatory system of *K. aerogenes* were compared with a *nac* mutant and with the wild type: KC2562 (*rpoN5018*) is defective in *rpoN* (*ntrA glnF*), which encodes the sigma-54 subunit of RNA polymerase (4, 17); KC2005 (*ntrC5*::Tn*5-131*) is defective in *ntrC* (*glnG*), which encodes NTRC, the enhancer-binding protein that is necessary for the activation of NTR-specific transcription (27, 35); KC895 (*ntr-45*) is thought to have an altered *ntrB* (*glnL*), the regulated phosphotransferase, and causes an NTR-constitutive phenotype (9, 26). Each of the NTR mutants displayed the expected defects in the regulation of glutamine synthetase expression (Table 5): KC895 (*ntr-45*) had constitutively high levels of glutamine synthetase even in the presence of ammonia, KC2562 (*rpoN*) had very low levels of glutamine synthetase that could not be derepressed by nitrogen limitation, and KC2005 (*ntrC*) had intermediate levels of glutamine synthetase that could be derepressed about fourfold by nitrogen limitation. These data confirmed (i) that there was nothing unexpected about the phenotypes of the particular alleles or strains used here as typical NTR mutants, and (ii) that the phenotype of the *ntrBC*::Tn*5 ntr-45* strain was indeed that expected of an *ntrC*::Tn*5* strain.

Every nitrogen-regulated enzyme tested required the NTR system for its nitrogen regulation (22; Tables 4 and 5). In every case, the *ntr-45* strain (KC895) was resistant to ammonia repression, and every enzyme tested in these experiments remained fully or almost fully derepressed in the presence of excess ammonia (glutamate dehydrogenase remained fully repressed). Likewise, the *ntrC* and *rpoN* mutations led to either a complete block of derepression in response to nitrogen starvation (histidase, asparaginase, proline oxidase, nitrite reductase, *nif-lac*) or a much reduced response (urease, nitrate reductase).

Effect of NTR mutations on *nac* expression. Three of the four insertion mutations in *nac* resulted in a fusion of the *lacZ* gene to the *nac* promoter, allowing us to use β-galactosidase as a reporter for *nac* gene expression. In each fusion, the synthesis of β-galactosidase responded strongly to the nitrogen source, with a 100- to 1,000-fold derepression in response to nitrogen starvation (Table 6). The nitrogen regulation of two of these *nac-lacZ* fusions was studied in detail to determine the role of the NTR system. Both the *rpoN* function and the *ntrC* function were necessary for derepression of the *nac-lacZ* fusion in response to nitrogen starvation (Table 6). Moreover, the *ntr-45* mutation, which makes the NTR system largely resistant to repression by ammonia, made the *nac-lacZ* fusion largely resistant to ammonia (Table 6). Thus, *nac-lacZ* expression was strongly nitrogen regulated, and the NTR system was required for this regulation.

TABLE 5. Nitrogen regulation in NTR mutants[a]

Strain	Relevant genotype	Sp act (nmol of product formed per min per mg of protein)													
		Glutamine synthetase		Asparaginase		Urease		Nitrate reductase[b]		Nitrite reductase[b]		β-Galactosidase (*nifL*)[c]		Tryptophan permease	
		−N	+N	−N	+N	−N	+N	−N	+N	−N	+N	−N	+N	−N	+N
KC1043	+	1,440	180	180	<10	1,240	50	2.0	<0.01	3.3	<0.01	47	2	+	−
KC895	*ntr-45* (NTR^{const})	1,850	1,710	300	310	1,070	1,040	1.7	1.6	2.6	2.3	NT[d]	NT	+	+
KC2005	*ntrC5*::Tn*5-131* (NTR$^-$)	550	130	<10	<10	270	40	0.7	NT	<0.01	NT	NT	NT	−	−
KC2562	*rpoN5018* (sigma-54$^-$)	<14	0	<10	<10	300	70	0.5	NT	<0.01	NT	NT	NT	−	−
KC1786	*nac-203*::Tn*5*	2,260	230	300	<10	410	19	2.0	0.2[e]	0.7	0.2[e]	106	1	+	−

[a] Cells were grown and enzyme activities were measured as for Table 4, except for the growth-rate-limiting concentrations of glutamine present. Cultures assayed for glutamine synthetase, asparaginase, urease, nitrate reductase, and nitrite reductase grew in the presence of 0.04% (wt/vol) glutamine; those assayed for β-galactosidase contained 0.025% glutamine; and those assayed for tryptophan permease contained 0.2% glutamine. The +N cultures all contained 0.2% glutamine except those assayed for β-galactosidase, which contained 0.025% glutamine. The glutamine synthetase, asparaginase, and urease values for strain KC1786 are repeated from Table 4.

[b] Potassium nitrate was present in these culture to allow induction.

[c] For these assays, the cells carried a *nifL-lacZ* operon fusion on plasmid pDO531 (29).

[d] NT, Not tested.

[e] We do not know whether these low values differ significantly from the undectable levels in strain KC1043. If not, they may represent slight induction of the dissimilatory nitrate reductase system during harvesting.

DISCUSSION

K. aerogenes is capable of using a large number of nitrogen-containing compounds as the sole nitrogen source. In most, if not all, cases, the expression of the genes encoding these metabolic activities is regulated by the quality of the nitrogen source. In every case tested, this nitrogen regulation was controlled by the quality of the nitrogen source. In every case tested, this nitrogen regulation required NTRC and sigma-54. Mutants lacking either the NTR system or sigma-54 could not derepress the formation of enzymes needed to catabolize poor nitrogen sources, nor could they repress the formation of enzymes needed for the synthesis of glutamate from 2-oxoglutarate. The *K. aerogenes* NTRC mutants differ from those of *E. coli* or *Salmonella typhimurium* in that *ntrC* (*glnG*) mutants retain some ability to respond to nitrogen starvation. This is particularly evident for glutamine synthetase; our data (Table 5) confirmed the observation that glutamine synthetase is derepressed about fourfold in all *ntrC* mutants of *K. aerogenes* thus far isolated (14, 16). The partial derepression of urease in *ntrC* mutants similarly shows that there must be some other effector that can partially replace or mimic NTR in these mutants. The partial derepression of urease in the sigma-54 mutant (Table 5) suggests that if such a system exists, it may be sigma-54 independent, but it is also possible that the *rpoN5018* mutation (induced by nitrous acid) leaves residual sigma-54 activity.

TABLE 6. Nitrogen regulation of β-galactosidase formation from *nac-lacZ* fusions in wild-type and NTR-defective strains[a]

Strain	*nac-lacZ* fusion	NTR genotype (phenotype)	β-Galactosidase (*nac-lacZ*) activity[b]	
			−N	+N
KC1043	None	+	<1	<1
KC1665	*nac-101*::Mu dX	+	730	4
KC1611	*nac-112*::Mu dX	+	2,000	7
KC1473	*nac-112*::Mu dX	*ntr-45* (NTR^{const})	1,900	1,100
KC1604	*nac-112*::Mu dX	*ntrC200*::Tn*5* (NTR$^-$)	28	24
KC1839	*nac-204*::λ *plac* Mu 53	+	7,500	4
KC1833	*nac-204*::λ *plac* Mu 53	*ntr-45* (NTR^{const})	4,800	920
KC1856	*nac-204*::λ *plac* Mu 53	*rpoN5018* (sigma-54$^-$)	12	6
KC2198	*nac-204*::λ *plac* Mu 53	*ntrC5*::Tn*5-131* (NTR$^-$)	24	22

[a] Cells were grown in glucose minimal medium, and enzyme activities were determined as described in footnote *a* of Table 4. All cultures contained 0.025% (wt/vol) glutamine except strains KC1611 and KC1473, for which 0.2% glutamine was used. The +N cultures contained 0.2% ammonium sulfate as excess nitrogen source, which the −N cultures did not contain.

[b] Nanomoles of product formed per minute per milligram of protein.

Although every nitrogen-regulated system required the NTR system for its regulation by nitrogen, some systems also required the product of the *nac* locus. Clearly histidase (the first enzyme of histidine utilization), proline oxidase, and urease could not be fully derepressed by nitrogen starvation in *nac* mutants (Table 4). Nor could glutamate dehydrogenase (Table 4) or glutamate synthase (6) be repressed by nitrogen starvation in the *nac* mutants. This defect was seen whether we used 0.2% glutamine (with which growth is only slightly retarded) or 0.025% glutamine (with which growth is severely retarded) as the limiting nitrogen source. Thus the defect is unlikely to result from physiological changes that alter the ability of glutamine to serve as a growth-rate-limiting source of nitrogen.

Both NAC and NTR are required for nitrogen regulation of the NAC family, so it is unlikely that NAC represents a form of nitrogen regulation that is independent from the NTR system. This leaves two models for the nitrogen regulation of genes in the NAC family: coordinate, in which NAC and NTR act in concert, and sequential, in which NTR regulates NAC expression and NAC regulates expression of the NAC family. Unpublished data from this laboratory (Anthony Schwacha and R. A. Bender) show that when *nac* was expressed from an isopropyl-β-D-thiogalactopyranoside-inducible promoter instead of its own promoter, *hut* activation became dependent on isopropyl-β-D-thiogalactopyranoside and completely independent of the NTR system and the nitrogen supply. Thus we propose the working model for nitrogen regulation of *nac*-dependent operons illustrated in Fig. 1. In this model, NTRC phosphate activates sigma-54-dependent expression of *nac*, and the NAC thus produced acts alone to activate sigma-70-dependent expression of nitrogen-regulated operons such as *hutUH*.

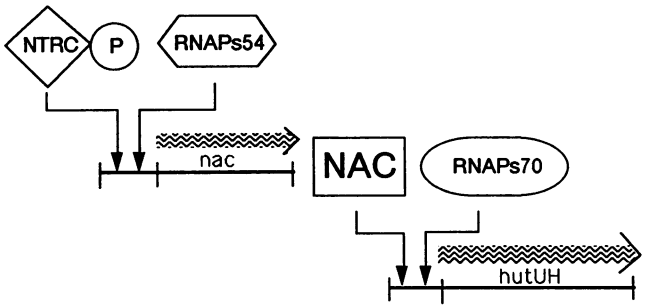

FIG. 1. Model for the role of NAC in the nitrogen regulation of *hut* expression. In this model, NTRC phosphate, the transcriptional activator produced in abundance by the NTR system, activated RNA polymerase containing the sigma-54 subunit (RNAPs54) to transcribe *nac*. The NAC thus produced activates RNA polymerase containing the normal sigma-70 subunit (RNAPs70) to transcribe *hut*.

We further propose that NAC blocks the sigma-70-dependent expression of nitrogen-repressible operons such as *gdh*. Although the NAC-dependent nitrogen regulation of *hut* stands in contrast to the well-characterized nitrogen regulation of *glnA* and *nifLA*, in which NTRC phosphate directly activates the target operon, one element of similarity remains. The nitrogen regulation of *nac* expression, like that of *glnA* and *nifLA*, is postulated to be direct.

One question raised by the data presented here is why the cell needs an indirect as well as a direct form of NTR-mediated nitrogen regulation. There is no obvious physiological feature that distinguishes the two types of regulation. Ammonia-assimilating enzymes fall into both groups (glutamine synthetase formation is regulated directly by NTR, glutamate dehydrogenase requires NAC). Amino acid utilization systems fall into both groups (asparaginase and tryptophan permease, direct; histidase and proline oxidase, via NAC). Systems that yield only ammonia fall into both groups (tryptophan permease and nitrite reductase, direct; urease, via NAC). However, several of the systems regulated by NAC are also regulated by other regulatory mechanisms (e.g., the CAP-cyclic AMP-mediated activation of *hut* or the glutamate-mediated repression of *gdh*), whereas those directly regulated by NTR have only NTR-mediated regulation (22). Moreover, it appears that the *hutUH* mRNA made under nitrogen starvation initiates from a sigma-70-dependent promoter (25). The two directly NTR-regulated systems (*glnA* and *nifL*) use sigma-54-dependent promoters (22). Perhaps the role of NAC is to connect the NTR system to the regulation of sigma-70-dependent operons. If these operons originally had other functions and were later recruited for nitrogen utilization, then it might have been difficult to endow each with a sigma-54 type promoter without destroying the other regulatory signals present in the recruited operons. In general, sigma-54-dependent promoters activated directly by NTR show very large derepression ratios with almost no expression in the absence of the activator. NAC-dependent promoters can also show large derepression ratios (e.g., urease), but many have rather lower derepression ratios and have substantial background transcription. Transcription for sigma-70-dependent promoters in the absence of positive regulators is quite common, but there is no case reported of a sigma-54-dependent promoter that can function at all without its cognate positive activators (32). Thus it may be important for the cell to connect the very tight NTR regulation of sigma-54-dependent promoters to the rather more flexible NAC regulation of sigma-70-dependent promoters.

It seemed surprising that NTR mutants grow poorly with histidine as the sole nitrogen source but NAC mutants (equally defective in activating histidase formation) grow quite well. The inability of NTR mutants to grow with histidine as the sole nitrogen source probably reflects a glutamine auxotrophy brought on by the inability of the *ntrC* (*glnG*) mutant to derepress glutamine synthetase formation adequately under conditions where the supply of ammonia is low. This difference between NTR and NAC phenotypes is seen for compounds that are capable of yielding free ammonia (histidine and urea) but not for those that yield only glutamate (urocanate and proline). Since both histidine and urocanate are degraded by the *hut* pathway, with urocanate as the first intermediate in histidine degradation, the argument seems even stronger. Moreover, when the *gltB200* and *ntr-45* mutations are present in the NAC-defective strains, blocking the assimilation of the low levels of ammonia released by the nonderepressed histidase, then the NAC-defective strains grow as poorly as the NTR-defective strains even on ammonia-generating nitrogen sources (data not shown). In other words, the ammonia released by the action of low levels of histidase or urease seems sufficient to allow nearly normal growth if glutamine synthetase levels can be fully derepressed (*nac* strains) but not if derepression of glutamine synthetase is restricted (*ntrC* strains). Thus the growth defect of *ntrC* strains on poor nitrogen sources appears to be a glutamine auxotrophy. Apparently the release of ammonia from glutamate (whether generated from histidine or from proline) is insufficient to allow growth of either *nac* or *ntrC* strains. Since we do not know the pathway by which ammonia is generated from glutamate, we can only postulate that this unknown pathway must be tightly regulated.

In summary, we have identified a number of systems in which NTR-mediated nitrogen regulation is indirect, requiring the product of the *nac* locus. This regulation includes activation of histidase (histidine utilization), proline oxidase (proline utilization), and urease (urea utilization) as well as the repression of glutamate dehydrogenase (ammonia assimilation in the presence of high concentrations of ammonia). Glutamate synthetase (the major glutamine-degrading enzyme of the cell) was previously shown to require NAC for its repression by ammonia. For at least two NAC-independent operons, *glnA* and *nifLA*, the nitrogen regulation has been shown to be exerted directly by the NTR system. It will be interesting to discover whether the remaining NAC-independent operons (asparaginase, nitrate reductase, nitrite reductase, and tryptophan permease) are also under direct NTR control.

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