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The *nac* (Nitrogen Assimilation Control) Gene from *Escherichia coli*

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The nitrogen assimilation control gene, *nac*, was detected in *Escherichia coli* but not in *Salmonella typhimurium* by Southern blotting, using a probe from the *Klebsiella aerogenes nac* (nac_K) gene. The *E. coli nac* gene (nac_E) was isolated from a cosmid clone by complementation of a *nac* mutation in *K. aerogenes*. nac_E was fully functional in this complementation assay. DNA sequence analysis showed considerable divergence between nac_E and nac_K, with a predicted amino acid sequence identity of only 79% and most of the divergence in the C-terminal half of the protein sequence. The total predicted size of NAC_E is 305 amino acids, the same as for NAC_K. A null mutation, *nac-28*, was generated by reverse genetics. Mutants bearing *nac-28* have a variety of phenotypes related to nitrogen metabolism, including slower growth on cytosine, faster growth on arginine, and suppression of the failure of an Ntr-constitutive mutant to grow with serine as sole nitrogen source. In addition to a loss of nitrogen regulation of histidase formation, *nac-28* mutants also showed a loss of a weak repression of glutamate dehydrogenase formation. This repression was unexpected because it is balanced by a NAC-independent activation of glutamate dehydrogenase formation during nitrogen-limited growth. Attempts to purify NAC_E by using methods established for NAC_K failed, and NAC_E appears to be degraded with a half-life at 30°C as short as 15 min during inhibition of protein synthesis.

The nitrogen assimilation control protein (NAC) of *Klebsiella aerogenes* plays an important role in regulating the nitrogen metabolism of this enteric bacterium (4, 25, 40). NAC allows the coupling of operons transcribed by RNA polymerase carrying σ^{70} to the nitrogen regulatory (Ntr) system (26), which uses RNA polymerase carrying σ^{54}. In brief, nitrogen-limited growth leads to a starvation for glutamine. Through a complex cascade of events, glutamine starvation leads to phosphorylation (and activation) of the transcriptional regulator NtrC. Phosphorylated NtrC activates RNA polymerase carrying σ^{54} to transcribe a number of genes, one of which is *nac*, which codes for NAC. NAC in turn activates RNA polymerase carrying σ^{70} to transcribe a number of operons whose products can supply the cell with ammonium or glutamate from alternative organic sources (4). NAC also represses operons whose function is to assimilate ammonium when ammonium is present in abundance (4). The operons activated by NAC in *K. aerogenes* include *hutUH*, *putP*, and *ureDABCEFG*, which code for enzymes required for the catabolism of histidine, proline, and urea, respectively. The operons repressed by NAC include *gdhA* (glutamate dehydrogenase [GDH]), *gltBD* (glutamate synthase), and *nac* itself (4, 16, 25).

Using the *hutUH* operon as a model system, we have learned that NAC is both necessary and sufficient to activate transcription from a σ^{70}-dependent promoter, that no coeffector is needed for NAC's activity, and that a NAC-binding site placed at −64 relative to the start of transcription can activate RNA polymerase even at the *lacZ* promoter (19, 36, 40). NAC is a member of the LysR family of transcriptional regulators (41), which includes over 50 members. NAC is a typical member of this family, with two differences: NAC does not require a coeffector for its actions, and the *nac* gene is not divergently transcribed from an operon that it regulates.

Although NAC plays an important role in the nitrogen regulation of *K. aerogenes*, *Salmonella typhimurium* appears to lack a functional *nac* gene. The *hutUH* operon of *S. typhimurium* does not respond to nitrogen starvation unless it is moved to a *K. aerogenes* cytoplasm (34) or unless a nac^+ plasmid from *K. aerogenes* is present in *S. typhimurium* (6).

The existence of a *nac* gene in *Escherichia coli* K-12 has remained an open question. Many of the NAC-regulated operons of *K. aerogenes* either are absent from *E. coli* (*hut* and *ure*) or do not respond to nitrogen regulation (*put* and *gdh*) (45). When the *hutUH* operon from *K. aerogenes* (or *S. typhimurium*) was transferred to an *E. coli* cytoplasm, *hutUH* expression was nitrogen regulated but the degree of that regulation was much less than is seen in *K. aerogenes* (18). In other words, the fact that *hutUH* shows some nitrogen regulation in *E. coli* suggests the presence of an active *nac* gene, but the weakness of that regulation and the lack of NAC-regulated targets is suggestive of the absence of an active *nac* gene. The data presented here show that *S. typhimurium* does indeed lack a *nac* gene and that *E. coli* has a *nac* gene that is a fully functional analog of the *nac* gene from *K. aerogenes*.

MATERIALS AND METHODS

Strains and plasmids. All *K. aerogenes* and *E. coli* strains used in this study are derived from W70 and K-12 respectively, and are listed in Table 1.

Media and chemicals. Strains were grown in W4 salts (W salts adjusted to an initial pH of 7.4 [25]) supplemented with carbon and nitrogen sources at 0.4 and 0.2% (wt/vol), respectively, or in rich LB medium (29). TB medium (44) was used for plasmid isolation. Media for plasmid-bearing strains were supplemented with ampicillin (100 μg/ml), kanamycin sulfate (50 μg/ml), or tetracycline (25 μg/ml) as indicated. Glutamine was always Calbiochem A grade. IPTG (isopropyl-β-D-thiogalactopyranoside) was from Sigma Chemical Company. Sequencing reagents (Sequenase) were from United States Biochemicals, and [α-^{32}P]dATP at 3,000 Ci/mmol was from ICN Pharmaceuticals.

Genetic techniques. Recombinant DNA techniques were carried out essentially as described by Maniatis et al. (27). DNA was often purified by separating digested DNA fragments in agarose buffered with TAE (27) buffer and then recovering fragments by use of glass resin or by electroelution and precipitation with ethanol. Plasmid constructs were often passed through *E. coli* DH5α as a host, and in all cases, only secondary transformants were studied. Strains were made competent for transformation by treatment with calcium chloride (30). Transduction using phage P1*vir* was performed as described previously (17).

DNA sequence analysis. DNA nucleotide sequence was determined by the chain-terminating method, using modified T7 DNA polymerase (Sequenase) on double-stranded templates, with the following modifications to the protocol

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TABLE 1. Strains and plasmids used

Strain	Relevant characteristics	Source or reference
E. coli		
DPB271	F$^-$ λ$^-$ *recD1903*::Tn*10d*Tet	Stan Maloy
EB3048	*glnL302 hutC515*$_K$[a] *nac-10*	P1 · EB3044 × YMC15
EB3044	*recD1903*::Tn*10d*Tet *nac-10*	This study
EB3046[c]	*hutC515*$_K$ *nac-10*	P1 · EB3044 × YMC10
EB3095	*recD1903*::Tn*10d*Tet *nac-28*	This study
EB3099	*glnL302 hutC515*$_K$ *nac-28*	P1 · EB3095 × YMC15
EB3364	*nac-28*	P1 · EB3095 × W3110
EB3365	*hutC515*$_K$ *nac-28*	P1 · EB3099 × YMC10
EB4457	YMC10/pCB1056	Transformation
EB4458	YMC10/pCB1057	Transformation
EB3846	W3110/pPC36	21
EB4459	EB3365/pCB1056	Transformation
EB4460	EB3365/pCB1057	Transformation
W3110	Wild type	R. Matthews
YMC10	*hutC515*$_K$	2
YMC15	*glnL302*[b] *hutC515*$_K$	9
K. aerogenes		
KC 2914	KC2330/pTROY11	Transformation
KC2330	*hutC515 galK6*	This laboratory
KC2668	*hutC515* Δ[*bla*]*-2*	This laboratory
KC2725	*hutC515 nac203*::Tn*5-131*[d] Δ[*bla*]*-2*	This laboratory
KC2937	KC2914 (λp*gal*8)	Lysogen
KC2939	KC2941 (λ347 Km^r)[e]	Dilysogen
KC2941	KC2937 *nac-203*::Tn*5-131*	Transduction
KC2972	KC2941/pCB511	Transformation
KC3367	KC2941 (λ345 Km^r)[e]	Dilysogen
Plasmids		
pBGS9	High-copy-number vector, Km^r	43
pCB511	*E. coli nac* on a *Bam*HI-to-*Hin*dIII fragment subcloned in pGB2	This study (see Fig. 2)
pCB524	*E. coli nac* on a *Bam*HI-to-*Hin*dIII fragment subcloned in pBGS9	This study (see Fig. 2)
pCB529	Km^r cartridge from pWW97 cloned into *Bgl*II site of pCB511	This study (see Fig. 2)
pCB547	pCB529 *Sac*I-to-*Pvu*II deletion	This study (see Fig. 2)
pCB552	*nac* coding sequence in pJLA503	This study
pCB554	*nac-lacZ* fusion in pJLA503	This study
pCB577	5.1-kb *Bam*HI subclone carrying *E. coli nac*	This study
pCB1056	*E. coli nac* promoter driving *lacZ* in pRJ800	This study
pCB1057	*K. aerogenes nac* promoter driving *lacZ* in pRJ800	This study
pCJ5	2-kb *Bam*HI subclone carrying *K. aerogenes nac*	6
pGB2	Low-copy-number vector	11
pJLA503	λp_L expression vector	38
pRJ800	Promoterless *lacZ* reporter	Reid Johnson (33)
pRS415	Promoterless *lacZYA* reporter	42
pTROY11	Plasmid carrying λ receptor	Robert Ludwig (12)
pWW97	Km^r	31

[a] The subscript "*K*" indicates that the *hut* operons are from *K. aerogenes*. The *hutC515* mutation inactivates the *hut*-specific repressor.
[b] Mutation that makes the strains constitutive for the Ntr system.
[c] P1*vir*-mediated generalized transduction of strain EB1926 with phage grown on strain EB3044.
[d] Tn*5-131* is a derivative of Tn*5* with the transposase and Km^r genes replaced by a tetracycline resistance gene (13). Thus, Tn*5-131* is Tnp$^-$, Km^s, and Tc^r.
[e] λ347 and λ345 are members of the ordered genomic cosmid library of Kohara et al. (22). Km^r indicates that a Km^r gene has been added to the phage by the method of Henry and Cronan (20).

supplied by the manufacturer. Strand denaturation by NaOH was replaced by heat denaturation. The reactions mixture, containing 1 μg of plasmid DNA, 50 pmol of primer (usually 17- to 20-bp oligomer), and water to a final volume of 10 μl, was heated to 94°C for 3 min, followed by quick cooling on ice. Reaction buffer was added after denaturation, and subsequent steps followed the Sequenase protocol. Overlapping sequences were determined for both strands with pCB511 and pCB524 as templates. The sequencing strategy involved primer walking, in which new primers were synthesized by using sequence determined by the results of the previous sequence determination.

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 make cells permeable for proline oxidase assays. Assays for histidase (37), GDH (7), glutamate synthase (28), urease (25), and proline oxidase (37) have been described. Histidase, glutamate synthase, and GDH activities 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 total cell protein. Cell protein was measured by the method of Lowry et al. (24) 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.

Complementation of *K. aerogenes nac* with genomic clones from *E. coli*. Derivatives of several of the members of the Kohara *E. coli* genomic phage library (22) (miniset 345-348, generously provided by F. Neidhardt) that carried a kanamycin resistance (Km^r) gene were isolated as described by Henry and Cronan (20). To insert these derivatives, a *K. aerogenes* host strain required a preexisting λ prophage to provide sufficient homology for recombination. Since *K. aerogenes* lacks the λ receptor and an *att*λ site, plasmid pTROY11 was used to transform several stains to λ sensitivity as described previously (12). To provide a selectable marker for lysogeny, phage λp*gal*8 (14) (provided by K. McKenny) was used to transduce strain KC2330 to Gal$^+$. The details of this lysogeny will be

described elsewhere (35). The resulting λ*pgal*8-containing strains were used as recipients for transduction by derivatized (Km^r) Kohara phage λ clones.

Southern hybridizations. Chromosomal DNA from *E. coli*, *S. typhimurium*, and *K. aerogenes* was prepared from log-phase cultures by using a Puregene DNA isolation kit (Gentra Systems Inc.). Phage DNA from the Kohara miniset members was prepared as described by Chisholm (10). Restriction digests were performed with enzymes supplied by Boehringer Mannheim. DNA fragments were separated by electrophoresis on an 0.8% agarose gel buffered with Tris-borate-EDTA (TBE) and blotted as described by Maniatis et al. (27). Hybridization with probe prepared by random-primed labeling of the 920-bp *Afl*II fragment from pCJ5 (6) containing the *K. aerogenes nac* gene was performed as described previously (27), varying the hybridization and wash temperatures between 50 and 65°C.

Mutagenesis of *E. coli nac*. Using the cloned *nac* gene on plasmid pCB511, we introduced various disruptions into the coding sequence. Initially, a Km^r gene cartridge from plasmid pWW97 (31) was introduced as a *Bam*HI fragment into the compatible *Bgl*II site internal to *nac* (see Fig. 2). An amber stop codon linker (*Spe*I; New England Biolabs) was introduced into the *Sma*I site present in the cartridge's multiple cloning site (just 3′ to the now destroyed *Bgl*II site) to create plasmid pCB529. This plasmid produces a truncated NAC of 169 amino acids, 165 amino acids from NAC and 4 amino acids provided by the multiple cloning site from the *Bgl*II site to the stop codon. This construct was used to make the *nac-10* allele. A further and more complete disruption was made by deleting the region between the *Sac*I and *Pvu*II sites of this plasmid to delete the promoter and 5′ end (N terminus) of the *nac* gene. This construct, on plasmid pCB547, was *nac-28*. Replacement of the chromosomal *nac* gene with the disrupted gene was achieved following electroporation of linearized plasmid DNA into a *recD* strain of *E. coli* (32). These Km^r-tagged *nac* alleles were immediately transferred into stable host backgrounds by P1*vir*-mediated transduction of various *E. coli* host strains.

Mobility shift retardation assays. Mobility shift assays were performed with purified *K. aerogenes* NAC protein. Pure NAC protein was isolated as described by Goss and Bender (19). DNA targets were prepared by digestion and precipitation followed by resuspension in Tris-EDTA (TE). Radioactively labeled targets were prepared by filling in the overhanging 5′ ends with [α-^{32}P]dATP (ICN Pharmaceuticals) and the Klenow fragment of DNA polymerase I. Reaction mixtures contained 1 μl of DNA, 1 μl of poly(dI-dC) (50 ng/μl), 4 μl of double-distilled H_2O, and 1 μl NAC dilution in dilution buffer (50 mM $NaPO_4$ [pH 7], 125 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM β-mercaptoethanol, 50% glycerol, 1 mg of bovine serum albumin/μl). Reaction mixtures contained dilutions of NAC with 1 to 200 ng of protein/μl and were allowed to incubate with the DNA for 30 min before 1.5 μl of loading buffer (40 mM Tris [pH 8.4], 4 mM EDTA, 0.2% bromothymol blue, 0.2% xylene cyanol, 15% Ficoll) was added and the mixture was loaded for electrophoresis onto 4% acrylamide gels buffered with TE or 2% agarose gels with TBE.

Primer extension assays. Primer extension analysis was carried out as described by Ausubel et al. (1), with the following modifications: RNA was purified from log-phase cultures grown in rich broth (LB) or in W4 salts medium supplemented with 0.4% glucose and 0.2% L-arginine as the sole nitrogen source (ammonia limiting) or 0.2% ammonium sulfate plus 0.2% glutamine (ammonia excess). A template of 25 μg of total RNA was used in each reaction. A modified reverse transcriptase (Superscript II) was used in the extension reaction at 52°C. Reactions were loaded alongside DNA sequencing reactions on a 0.8% sequencing gel. The same primer was used for primer extension and for sequencing.

Expression of the *E. coli* NAC protein. Initial expression of the *E. coli* NAC protein was carried out by putting the *nac* gene under the control of a temperature-inducible promoter in the expression vector pJLA503 (38). This construct, pCB552, was made by PCR amplification of *nac* from pCB511. Plasmid pCB554 was made by cloning the β-galactosidase gene from pRS415 into the *Eco*RI and *Sal*I sites of pCB552 such that *lacZ* would be transcriptionally fused to *nac* and thus coexpressed when the promoter was induced by raising the temperature above 35°C.

SDS-PAGE analysis of expressed protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell extracts was carried out as described by Ausubel et al. (1), using a polyacrylamide concentration of either 10, 12.5, or 15%.

To determine the half-life of overexpressed NAC protein relative to β-galactosidase, *E. coli* DH5α bearing plasmid pCB554 was induced by shifting growth from 30 to 45°C for 40 min when the culture reached 50 Klett units (green filter). Cells were allowed to grow at this temperature for 1 h, at which time chloramphenicol and rifampin were added to 100 and 50 μg/ml, respectively, along with reduction of the growth temperature to 30°C. Samples (0.2 ml) were taken every 5 min from the culture flask and placed immediately on ice. Cells were collected by centrifugation at 4°C and resuspended in 20 μl of TE; 10 μl of each sample was loaded on an SDS-gel for PAGE.

Nucleotide sequence accession number. The DNA sequence has been deposited in GenBank with accession no. U56736.

RESULTS

***E. coli* carries a functional *nac* gene.** Interspecies complementation has suggested that *E. coli* expresses a functional NAC but *S. typhimurium* does not (6, 18). To test whether *E. coli* and *S. typhimurium* possess a homolog of the *K. aerogenes nac* gene, we looked for hybridization between the *K. aerogenes nac* gene and DNA from these organisms. DNA from *K. aerogenes* W70 (strain KC1043), *E. coli* K-12 (strain W3110), and *S. typhimurium* 15-59 (strain NE7) was digested with *Eco*RI or with *Bam*HI. After separation of the resulting fragments by agarose gel electrophoresis, a hybridization analysis by the method of Southern was carried out with an *Afl*II fragment from within the *K. aerogenes nac* gene as probe. Under stringent conditions (hybridization and washes at 65°C), this 920-bp probe hybridized to a single fragment of *K. aerogenes* DNA but did not hybridize to any DNA fragment from *E. coli* or *S. typhimurium*. When the stringency of the hybridization and washes was reduced (58°C), we detected a single fragment from *E. coli* DNA but no hybridization with *S. typhimurium* DNA (Fig. 1). The positions of the bands suggested that the *Eco*RI and *Bam*HI fragments from *E. coli* were about 11.6 and 5.1 kb in size. When the stringency of the hybridization and washes was reduced still further (50°C), hybridization to *S. typhimurium* DNA was detected but there were many fragments showing hybridization in the lanes containing DNA from each of the three organisms under these conditions (not shown). Thus, *E. coli* appears to have a locus with strong sequence similarity to *nac* from *K. aerogenes*; *S. typhimurium* appears to lack such a sequence.

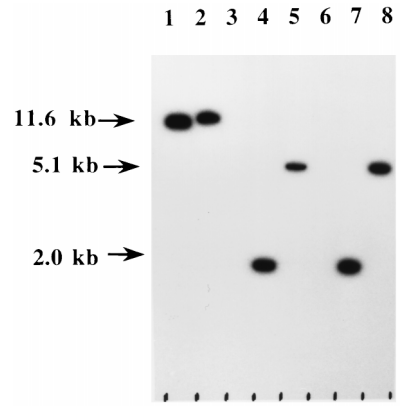

FIG. 1. Southern blot of digested chromosomal DNA probed with a 920-bp *Afl*II fragment (carrying the *K. aerogenes nac* gene). Lanes 1 to 3 and 4 to 6, *K. aerogenes*, *E. coli*, and *S. typhimurium*, respectively, digested with *Eco*RI (lanes 1 to 3) and *Bam*HI (lanes 4 to 6); lanes 7 and 8, *K. aerogenes* and *E. coli* plasmid clones (pCJ5 and pCB577, respectively) digested with *Bam*HI.

The genetic maps of *E. coli* and *K. aerogenes* are quite similar in all regions that have been compared (5), so it seemed likely that the *nac* gene of *E. coli* would lie in a region analogous to that of the *nac* gene of *K. aerogenes*. Several λ clones from the Kohara ordered set of genomic clones (22) were tested for complementation of a *nac* mutant of *K. aerogenes*. Four clones (345 through 348) believed to cover the *E. coli* chromosomal region analogous to that containing the *nac* gene in *K. aerogenes* were chosen and modified to encode kanamycin resistance as described by Henry and Cronan (20). These derivatized phage were then used to transduce a *nac*-deficient λ lysogen of *K. aerogenes* (strain KC2941) to kanamycin resistance. Transductants were assayed for histidase and GDH activities. Only the transductants derived from clones 346 and 347 showed NAC-dependent regulation of histidase and glutamate dehydrogenase formation. As shown in Table 2, the strain carrying the prophage derived from clone 347 showed the same activation of histidase, urease, and proline oxidase in response to nitrogen starvation as the wild type. That strain

TABLE 2. Regulation of enzyme formation in a *K. aerogenes nac* mutant carrying the *nac* gene from *E. coli*

Strain	Relevant genotype	Growth medium[b]	Sp activity (U/mg of protein)[a]				
			Histidase	GDH	Urease	Proline oxidase	Glutamate synthase
KC2668	Wild type	+N	33	361	11	9	193
		−N	357	52	1,380	47	70
KC2941	*nac-203*::Tn*5-131*	+N	33	379	10	12	260
		−N	51	401	242	17	245
KC2939	*nac-203*(λ347 Km^r)[c]	+N	39	369	15	11	216
		−N	319	94	1,212	32	125
KC3367	*nac-203*(λ345 Km^r)	+N	29	423	21	11	ND[e]
		−N	54	411	207	13	ND
KC2972	*nac-203*/pCB511[d]	+N	31	292	13	8	232
		−N	460	41	1,275	39	98

[a] Average of three independent cultures. Units of urease activity are calculated based on urea cleaved.
[b] Nitrogen-limiting medium (−N) is W4 salts supplemented with glucose (0.4%) and L-glutamine (0.2%) as sole nitrogen sources. Nitrogen-excess medium (+N) is further supplemented with ammonium sulfate (0.2%). Cultures were grown at 30°C with vigorous shaking to mid-log phase (50 Klett units). Media for cells used in urease assays were supplemented with 1 μM nickel sulfate. Media for cells used in proline oxidase assays were supplemented with 0.01% L-proline.
[c] λ347 carries the *E. coli nac* gene; λ345 does not.
[d] Plasmid pCB511 carries the intact *E. coli nac* gene.
[e] ND, not done.

also showed the same NAC-dependent repression of GDH and glutamate synthase as the wild type (compare KC2939 with KC2668). In contrast, a strain carrying the prophage derived from clone 345 was as defective as the *nac* mutant in activation and repression of enzyme formation in response to nitrogen starvation (compare KC3367 with KC2941). Thus, the *nac* gene from *E. coli* seems fully able to complement a *K. aerogenes nac* mutant.

The region of overlap between the two complementing clones (346 and 347) includes an *Eco*RI fragment of about 12 kb which in turn contains a *Bam*HI fragment of about 5.4 kb, in close agreement with the sizes predicted from the Southern blot in Fig. 1.

Isolation and characterization of the *E. coli nac* gene. The *E. coli nac* gene was isolated as a 5.4-kb *Bam*HI fragment from the Kohara miniset clone 347 in both a low-copy-number vector (pGB2) and a high-copy-number vector (pBGS9). High-copy-number plasmids carrying *nac* cause the cells that carry them to grow slowly, perhaps because of the high-level expression of the adjacent *asnV* gene (tRNA^{Asn}). A restriction map of this fragment is shown in Fig. 2.

The sequence of the *nac* gene and those of the two adjacent genes (*asnV* and *cbl*) were determined by the dideoxy sequencing method on both strands. The sequence of the *E. coli nac* promoter region is very different from the corresponding region from the *K. aerogenes nac* gene, except in those regions known to specify the binding of important regulatory proteins, including NAC, NtrC, and RNA polymerase (Fig. 3). The deduced protein sequence of *E. coli* NAC differs considerably from that of *K. aerogenes*, in contrast to the situation with most regulatory proteins, where strong similarity (often approaching 100%) exists. The N-terminal one-third is quite similar (greater than 90% identity), but the C-terminal two-thirds differs much more than expected (only about 75% identity). Thus, the overall deduced amino acid sequence identity is about 79%.

Like the *K. aerogenes nac* gene (41), the *E. coli nac* gene is preceded by a sequence resembling a σ^{54}-dependent promoter. The start of transcription was determined by primer extension analysis (Fig. 4) and confirms that the putative σ^{54}-dependent promoter is in fact the promoter of the *E. coli nac* gene.

Isolation of *nac* mutations. Two *nac* mutations were isolated by reverse genetics, taking advantage of a *Bgl*II site at the 165th codon of the *nac* coding sequence and a *Pvu*II site upstream of the *nac* promoter. The *nac-10* mutation is an insertion of a Km^r cassette from plasmid pWW97 into the *Bgl*II site of *nac*. Such a mutation leads to a truncated NAC with only the N-terminal 165 amino acids of NAC plus 4 amino acids encoded in the linker used to provide a stop codon. The *nac-28* mutation was derived from *nac-10* by deletion of the DNA from the *Pvu*II site upstream of the *nac* promoter to a *Sac*I site just inside the Km^r cassette. Thus, *nac-28* is a null allele for *nac* that deletes the *nac* promoter and the entire N-terminal half of the *nac* coding sequence. The *nac-28* mutation was crossed onto the *E. coli* chromosome as described in Materials and Methods, and replacement of the wild-type *nac* gene was confirmed by Southern blot analysis (not shown). The resulting mutant strains were tested for regulation of the *K. aerogenes hutUH* operon in these *E. coli* strains (Table 3).

Strains carrying *nac-28* were unable to derepress histidase synthesis whether *nac* expression was induced physiologically (by nitrogen-limited growth of strain EB3365) or genetically

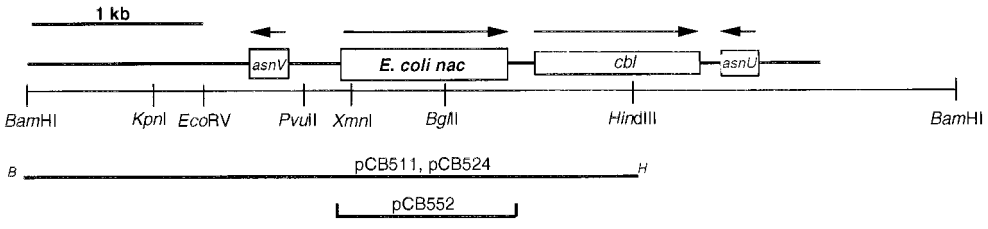

FIG. 2. *E. coli nac* region and clones.

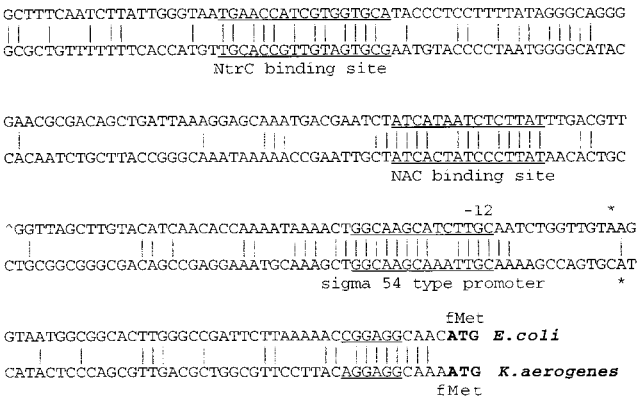

FIG. 3. Comparison of the promoter regions from the *nac* genes of *E. coli* (top) and *K. aerogenes* (bottom). |, identity; ^, space added for better alignment; *, start of transcription as determined by primer extension.

(by the *glnL302* mutation in strain EB3099, which makes the Ntr system constitutively active).

The *gdhA* genes (encoding GDH) from *E. coli* and *K. aerogenes* are quite similar. In particular, a sequence resembling a NAC-binding site is found at about −80 in the promoter regions of the *gdhA* genes from both organisms. However, expression of GDH activity in *E. coli* does not vary appreciably in response to nitrogen limitation, in contrast to the strong repression seen in *K. aerogenes*. Analysis of GDH expression in strains with the *nac-28* mutation reveals a subtle regulatory complexity. The *nac-28* mutants show about a twofold increase in GDH expression when the Ntr system is activated either by nitrogen limitation or by the *glnL302* mutation (strains EB3365 and EB3099 in Table 3). In contrast, the *nac*$^+$ strain YMC10 showed a slight but reproducible repression of GDH expression. Thus, there appears to be an approximately twofold repression of GDH expression in *E. coli* by NAC, and this is almost exactly balanced by a nearly twofold increase in GDH expression during nitrogen-limited growth by an unknown mechanism. There is considerable variation in the expression of GDH among different *E. coli* strain backgrounds; therefore, we compared a *nac-28* mutant of strain W3110 with its *nac*$^+$ parent and found essentially the same effect as seen with YMC10 (Table 3). Although the regulatory effects of NAC on GDH expression are small, they are physiologically significant. *gltD* mutants lack glutamate synthase activity, and their assimilation of ammonium is completely dependent on GDH. Strain EB3135 (*gltD nac-28*) grows faster than EB3134 (*gltD*) in glucose medium with ammonium as the sole nitrogen source (doubling times of 49 ± 8 and 65 ± 10 min for EB3135 and EB3134, respectively).

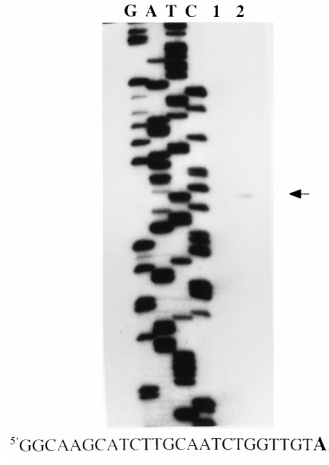

FIG. 4. Primer extension analysis of the *E. coli nac* promoter. RNA was extracted from YMC10 cells grown in nitrogen-excess medium containing ammonium sulfate (0.2%) and glutamine (0.2%) as nitrogen sources (lane 1) and from YMC10 cells grown in nitrogen-limiting medium containing arginine (0.2%) as the nitrogen source (lane 2). A sequencing ladder was generated by using the *nac* containing plasmid pCB524 as template. The same oligonucleotide (5′ GCTGGTTGTGCGATATGCA 3′) was used as a primer for primer extension and for the sequencing reactions; thus, the sequencing ladder represents the complement of the sequence shown. The sequence at the bottom is the *E. coli nac* promoter, with the start of transcription indicated by the bold A.

TABLE 3. Nitrogen regulation of enzyme formation in *E. coli nac* mutants

Strain	Relevant genotype	Growth medium[b]	Sp activity (U/mg of protein)[a]	
			Histidase	GDH
YMC10	$hutC515_K$	+N	98	175
		−N	320	145
EB3365	YMC10 *nac-28*	+N	96	118
		−N	108	257
YMC15	*glnL302* $hutC515_K$	+N	375	86
EB3099	YMC15 *nac-28*	+N	145	130
W3110	Wild type	+N	NA[c]	96
		−N	NA	84
EB3364	W3110 *nac-28*	+N	NA	91
		−N	NA	146

[a] Average of four independent cultures.
[b] Nitrogen-limiting (−N) and nitrogen-excess (+N) indicate growth at 37°C in W4 salts supplemented with glucose (0.4%) and either L-arginine (0.2%; −N) or ammonium sulfate (0.2%; +N).
[c] NA, not applicable.

Effect of *nac* mutations on growth rate of *E. coli*. In *K. aerogenes*, *nac* mutations have no phenotype except slower growth on substrates like histidine, proline, or urea that are catabolized by products of NAC-dependent operons (3). This was also seen in *E. coli* in that *nac* mutants grew significantly more slowly in glucose minimal medium with cytosine as the sole nitrogen source, with doubling times of 180 ± 5 and 235 ± 10 min for the *nac*$^+$ and *nac* strains, respectively. The *nac* mutant also grew somewhat more slowly when either serine or threonine was the sole nitrogen source. This was readily seen as a difference in colony size, but the doubling times of *nac* mutants on serine or threonine were only about 5 min (about 3%) longer than the *nac*$^+$ parent.

In contrast, when *nac* mutants of *E. coli* were grown on glucose-arginine medium (i.e., glucose minimal medium with L-arginine as the sole nitrogen source [Table 4]) the growth rate actually increased, with doubling times of 147 ± 3 and 134 ± 4 for the *nac*$^+$ and *nac* strains, respectively. The increase was always small but was clearly seen in all five of the paired growth experiments attempted. This difference was particularly large in strains which carry the *glnL302* allele, which leads to constitutive expression of the Ntr system and thus constitutive NAC expression. In addition to an increased growth rate on glucose-arginine medium, there is also a decrease in the growth lag when cells are transferred from nitrogen-rich medium (glucose minimal medium supplemented with 0.2% ammonium sulfate) to glucose-arginine. The growth rate advantage of the *nac* mutants was minimized when even small amounts of glutamate (0.02%) or aspartate (0.01%) were

TABLE 4. Growth rates of *E. coli* strains containing *nac* and *glnL* mutations[a]

Medium	Strain	Relevant genotype	Lag time (min)	Doubling time (min)
Glucose (0.4%)–L-arginine (0.2%)				
	YMC10	Wild type	245	147
	YMC15	*glnL302*	225	140
	EB3365	*nac-28*	196	134
	EB3099	*glnL302 nac-28*	152	109
Glucose (0.4%)–L-arginine (0.2%)–glutamate (0.02%)				
	YMC10	Wild type	198	135
	YMC15	*glnL302*	159	110
	EB3365	*nac-28*	193	133
	EB3099	*glnL302 nac-28*	110	106

[a] Growth rates were measured at 37°C in W4 medium supplemented as indicated. Lag times were calculated upon shift from log-phase growth on glucose (0.4%)-ammonia (0.2%) cultures to the indicated medium. Cells were washed twice in cold 1% KCl before inoculation into new medium. Growth rate was determined by monitoring the cell density of a vigorously shaking culture in sidearm flasks.

added to the glucose-arginine medium (Table 4). Another phenotype involved the ability of a *glnL302* strain to grow in glucose minimal medium with serine as the sole nitrogen source. Strains carrying the *glnL302* mutation failed to grow on glucose-serine medium. The reason for this failure is not known. Nonetheless, *nac* mutations allow the *glnL* strain to grow in glucose with serine as the sole nitrogen source (as do *glnE* mutations).

Effect of NAC on *nac* expression in *E. coli*. Transcription of the *K. aerogenes nac* gene is strongly regulated by the Ntr system in response to the nitrogen supply (25). It is also repressed by the binding of NAC to a site centered about 77 bp upstream from the start of transcription (6, 16). When the *K. aerogenes nac* promoter was cloned on a high-copy-number plasmid driving β-galactosidase expression, the *E. coli* Ntr system was fully functional in regulating transcription from the *K. aerogenes nac* promoter (Table 5, lines 1 and 2 or 3 and 4). The chromosomally encoded NAC exerted a weak repression on *nac*-driven β-galactosidase expression from this multicopy plasmid (Table 5, lines 2 and 4). When the *E. coli nac* promoter replaced that from *K. aerogenes*, a similar regulation in response to the nitrogen supply (Table 5, lines 5 and 6 or 7 and 8), as well as a similar repression by NAC (Table 5, lines 6 and 8), was seen. The *E. coli nac* promoter contains a sequence resembling the NAC-binding site from the *K. aerogenes nac* promoter, and this site is also in a very similar position (centered at −76). A gel mobility shift assay (Fig. 5) confirms that NAC binds to the *E. coli nac* promoter region, consistent with the argument that the *E. coli nac* gene is autogenously regulated, just like that from *K. aerogenes*.

Expression of *E. coli nac* coding sequence. The coding sequence of *E. coli nac* was expressed from a plasmid expression vector with a temperature-inducible phage λ p_L promoter (38). SDS-PAGE of expressed NAC showed a protein of 33 kDa as expected from the sequence. Curiously, no NAC protein was seen when strains bearing this construct were grown at 42°C. To visualize protein, the strains had to be grown at 46°C. Control experiments using this vector (38) suggested that an ample amount of protein should have been made at 42°C. To reconcile this discrepancy, a transcriptional fusion was made by placing the *lacZ* coding sequence immediately downstream of the *nac* coding sequence, creating an artificial operon with *nac* and *lacZ* cotranscribed. Expressed β-galactosidase was readily detected at the lower temperatures (37 and 42°C), suggesting that both genes were being adequately transcribed. It seemed likely that either the NAC transcript was being inefficiently translated or the NAC protein was being degraded. Therefore, we compared the stabilities of the NAC and β-galactosidase polypeptides. NAC and β-galactosidase were produced by growing strains at 46°C for 1 h and then shifting the temperature to 30°C along with the addition of chloramphenicol and rifampin to prevent further translation and transcription. The disappearance of the NAC and β-galactosidase polypeptides was monitored by SDS-PAGE (Fig. 6). The NAC bands exhibited a half-life of about 15 min following overexpression and growth at 30°C, in contrast to the more stable β-galactosidase protein (half-life of >35 min).

TABLE 5. Regulation of *nac* promoter expression by *E. coli nac*

Strain	Relevant genotype[a]	Growth medium[b]	β-Galactosidase sp act (U/mg)[c]
EB4458	Wild type/pCB1057	+N	487
		−N	13,074
EB4460	*nac-28*/pCB1057	+N	502
		−N	25,011
EB4457	Wild type/pCB1056	+N	449
		−N	16,901
EB4459	*nac-28*/pCB1056	+N	507
		−N	28,290

[a] Plasmid pCB1056 carried the *nac* promoter from *E. coli* fused to the promoterless *lacZ* gene of pRJ800; pCB1057 carries the *nac* promoter from *K. aerogenes* fused to the promoterless *lacZ* gene of pRJ800.

[b] Nitrogen-excess growth medium (+N) is W4 salts supplemented with glucose (0.4%), ammonium sulfate (0.2%), and L-arginine (0.2%). Nitrogen-limiting (−N) medium was supplemented with glucose (0.4%) and L-arginine (0.2%). Cultures were grown at 37°C assayed as described in Materials and Methods.

[c] Average of five independent determinations.

DISCUSSION

The data presented here show clearly that *E. coli* has a functional homolog of the *K. aerogenes nac* gene. DNA sequence analysis of *E. coli nac* (hereafter referred to as nac_E) predicts a protein with about 80% identity to the *K. aerogenes* NAC (hereafter referred to as NAC_K). Moreover, nac_E is fully able to complement a *K. aerogenes nac* mutant for activation of

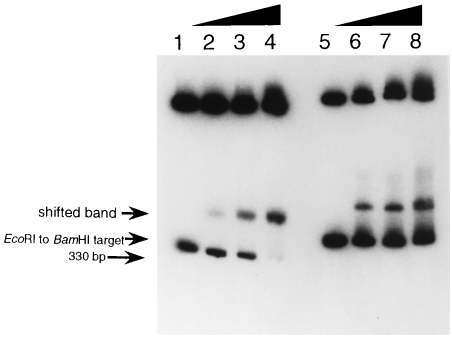

FIG. 5. Gel mobility shift assay showing the interaction of NAC with the *E. coli nac* region. Lanes: 1 to 4, 330-bp *Eco*RI-*Hin*dIII fragment containing the *K. aerogenes ureD* promoter incubated with 0, 0.35, 0.7, 1.7 pmol of purified *K. aerogenes* NAC, respectively; 5 to 8, 378-bp *Eco*RI-to-*Bam*HI fragment containing the *E. coli nac* promoter incubated with 0, 0.35, 0.7, and 1.7 pmol of *K. aerogenes* NAC, respectively.

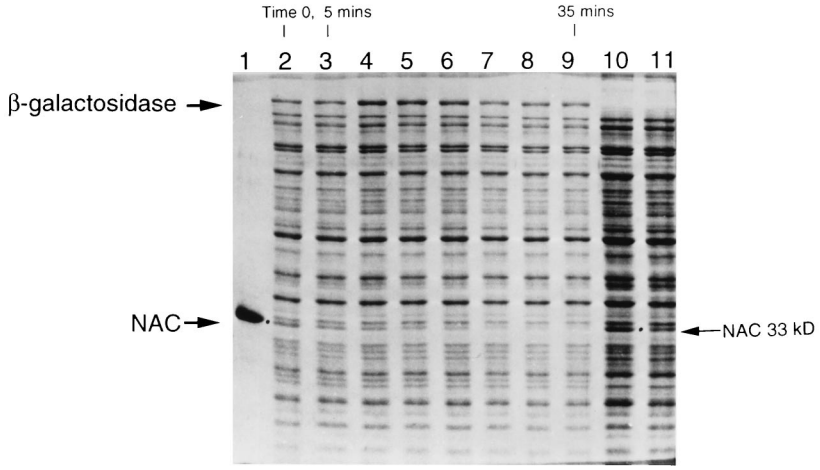

FIG. 6. Relative stabilities of *E. coli* NAC and β-galactosidase. *E. coli* NAC and β-galactosidase were cotranscribed from the temperature-inducible phage λp_L promoter in plasmid pCB554. Cells were grown to a density of approximately 50 Klett units, and expression was induced by growth at 45°C for 40 min. At time zero, the culture was shifted to 30°C and chloramphenicol and rifampin were added. Samples (0.2 ml) were removed at 5-min intervals and concentrated 10-fold, and 10 μl was applied to an SDS–12.5% polyacrylamide gel. Lanes: 1, 5 μg of *K. aerogenes* NAC as a standard; 2, sample at time zero; 3 to 9, samples at 5-min intervals; 10 and 11, *E. coli* NAC expressed from plasmid pCB552 (which transcribes NAC alone).

hut, *put*, and *ure* operon expression as well as for repression of *gdhA* expression and probably for autogenous repression of nac_K expression. On one hand, the existence of nac_E was not surprising given the close evolutionary relationship between *E. coli* and *K. aerogenes*. On the other hand, we have long known that the operons that NAC regulates in *K. aerogenes* are absent from *E. coli* (*hut* and *ure*) or are either unregulated or only slightly regulated by nitrogen in *E. coli* (*gdhA* and *put*). That raised the question of whether *E. coli* had a *nac* gene and, if so, what that gene did. We continue to find operons in *K. aerogenes* with a NAC-dependent regulation, most recently the *dadAB* operon which is involved in alanine catabolism (21). Again, *dad* is NAC regulated in *K. aerogenes* but not in *E. coli*. Nevertheless, it seems likely that as more NAC-regulated operons are discovered, some of them will be NAC regulated in *E. coli* as well.

Although NAC_E is a functional homolog of NAC_K, the degree of sequence divergence between NAC_E and NAC_K is surprising. Most regulatory proteins are highly conserved between these two bacteria. NtrC and Lrp are identical in the two strains, and catabolite gene activator protein and the α subunit of RNA polymerase differ by only one and two amino acids in these bacteria. Homologous LysR family members also tend to be highly conserved within the enteric bacteria. For example, the CysB proteins from *E. coli* and *S. typhimurium* are 95% identical in amino acid sequence (39). In fact, the entire LysR family shows surprising sequence conservation. NAC from *K. aerogenes* is 50% identical to OxyR (a regulator that senses oxidative stress) from *E. coli* (41). Thus, the >20% nonidentity between NAC_E and NAC_K is striking. It should be noted that there is strong sequence conservation in the amino-terminal one-third of the protein and that most of the sequence divergence is found in the carboxy-terminal two-thirds. The carboxy-terminal portion is generally thought to be important for interaction of LysR family proteins with their regulatory coeffectors (39). NAC_K appears to lack any coeffectors and to be a constitutively active transcriptional regulator (25). This may explain the lack of sequence conservation in this region. However, it must be noted that NAC_E and NAC_K both have exactly 305 amino acids and that their length is quite typical of all other LysR family members. Therefore, the carboxy-terminal domain(s) may play some role (such as stability) other than response to a coeffector signal. In support of this argument, we noted that the hydropathy plots of NAC_E and NAC_K are remarkably similar, despite the 25% divergence of the amino acid sequence in the carboxy-terminal region.

The physiological role for NAC_E remains unproved, though the data presented here suggest that NAC_E, like NAC_K, is involved in regulating the nitrogen metabolism of the cell. Several lines of evidence support this hypothesis. First, the *nac-28* mutation affects the growth rate of *E. coli* on a variety of nitrogen sources, including arginine (faster), cytosine (slower), and serine (allowing growth of Ntr-constitutive strains). Second, even though the strong repression of *gdhA* by NAC seen in *K. aerogenes* does not occur in *E. coli*, a small but significant effect is detectable. In the *nac-28* mutant, GDH expression increases about twofold in response to nitrogen limitation. In wild-type strains, this increase does not occur. In a *gltB* mutant, where glutamate formation from ammonia is completely dependent on GDH, a strain carrying *nac-28* grows faster with ammonium as nitrogen source than does a nac^+ strain, suggesting that this twofold effect on GDH expression is important. Third, the key regulatory sites in the nac_K promoter region (15, 16) are well conserved, both in sequence and in position, in the nac_E promoter region. These include the σ^{54}-dependent promoter and the NtrC-binding enhancer sequence. Thus, it is likely that nac_E, like nac_K, is transcribed as part of the Ntr response during nitrogen limitation.

The absence of any *nac*-like sequences in *S. typhimurium* is consistent with older observations that NAC-dependent operons cannot be expressed in response to nitrogen limitation (6, 8). The absence of a *nac* gene in *S. typhimurium* also raises questions about the evolutionary plasticity of this chromosomal region in the *Enterobacteriaceae*. The nac_K gene is preceded by *asnV*, the gene that codes for $tRNA^{Asn}$, and it is followed by an apparent operon composed of *cblA* (*orf2*), itself a member of the LysR family, and *orf3*. The function of this operon is unknown but may be related to sulfur metabolism. In *E. coli*, *nac* is again preceded by *asnV* and followed by *cblA*, but *orf3* is located upstream from *nac*, on the other side of *asnV*. In *S. typhimurium*, *nac* is not present. There is evidence that the *asnV* region may be where the large cluster of *cob* genes,

encoding the enzymes of cobalamine synthesis, lies in *S. typhimurium* (23). (*E. coli* lacks this *cob* cluster.) Similarly, the large cluster of *nif* genes (encoding the enzymes of nitrogen fixation) in *K. pneumoniae* appear to be linked to *nac*. (The *nif* genes are not present in *K. aerogenes*.) In short, this region has been subject to considerable insertion or deletion during the evolutionary divergence of the enteric bacteria. It is tempting to note that *asnV* and *asnU* flank nac_E and present an 86-bp direct repeat. If no essential genes lie between these (as is seen for *E. coli*), simple deletion of the intervening region by *asnV-asnU* recombination could lead to loss of *nac*, but such a speculation remains untested.

In summary, *E. coli* has a *nac* gene that is a functional homolog of the *nac* gene from *K. aerogenes*, and this *nac* gene is probably involved in the nitrogen regulation of *E. coli*. The strong sequence conservation within the first 100 or so amino acids suggests an important role for the domain(s) in this region, whereas the large number of differences in the last 200 or so amino acids suggests that the domain(s) in this region are quite plastic and can withstand considerable amino acid substitution without loss of function.

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