JOURNAL OF BACTERIOLOGY, Feb. 1998, p. 571–577 Vol. 180, No. 3
0021-9193/98/$04.00+0

Genetic Analysis, Using P22 Challenge Phage, of the Nitrogen Activator Protein DNA-Binding Site in the *Klebsiella aerogenes put* Operon

LI-MEI CHEN,[1]† THOMAS J. GOSS,[2] ROBERT A. BENDER,[2] SIMON SWIFT,[3] AND STANLEY MALOY[1]*

Department of Biology, University of Michigan, Ann Arbor, Michigan 48109-1048[2]; Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, United Kingdom[3]; and Department of Microbiology, University of Illinois, Urbana, Illinois 61801[1]

Received 11 July 1997/Accepted 24 November 1997

The *nac* gene product is a LysR regulatory protein required for nitrogen regulation of several operons from *Klebsiella aerogenes* and *Escherichia coli*. We used P22 challenge phage carrying the *put* control region from *K. aerogenes* to identify the nucleotide residues important for nitrogen assimilation control protein (NAC) binding in vivo. Mutations in an asymmetric 30-bp region prevented DNA binding by NAC. Gel retardation experiments confirmed that NAC specifically binds to this sequence in vitro, but NAC does not bind to the corresponding region from the *put* operon of *Salmonella typhimurium*, which is not regulated by NAC.

P22 challenge phages provide robust genetic tools for identifying nucleotides within a DNA site that play a critical role in specific DNA-protein interactions (reviewed in references 15 and 16). This approach is based upon the regulation of the lysis-lysogeny decision of phage P22 *mnt*::Kn*9 arc-1605*(Am). Under appropriate conditions, the decision between lysis and lysogeny is regulated by the P22 *ant* (antirepressor) gene product: expression of *ant* results in lytic growth of the phage and death of the cell, while repression of *ant* results in the survival of kanamycin-resistant (Kan^r) lysogens. To construct a challenge phage, the operator that controls *ant* expression is replaced by a DNA fragment that contains a site recognized by a specific DNA-binding protein. This places *ant* expression under the control of the cognate DNA-binding protein. If the site is occupied by the DNA-binding protein, *ant* expression is repressed and the phage carrying the site can be recovered from the resulting Kan^r lysogens. If a mutation in the site prevents the DNA-protein interaction, *ant* is expressed constitutively and the phage carrying the mutant site can be recovered from the resulting clear plaques. Thus, P22 challenge phage provide strong selections for identifying specific DNA sequences recognized by a DNA-binding protein and for mutations in the sequence that disrupt the DNA-protein interaction. In this study, we used challenge phage to characterize the DNA-binding site of the nitrogen assimilation control (NAC) protein from *Klebsiella aerogenes*.

NAC is a DNA-binding protein of the LysR family (reviewed in reference 1). NAC functions to couple the nitrogen-sensing mechanism of the nitrogen regulatory system (Ntr), which depends on RNA polymerase charged with σ54, to the expression of a variety of operons which are transcribed by RNA polymerase charged with σ70. When cells are starved for ammonium, the Ntr system activates *nac* expression and NAC accumulates and activates the expression of operons required for the catabolism of alternative nitrogen sources like proline (*put*), histidine (*hut*), and urea (*ure*). When ammonium is abundant, the Ntr system does not activate *nac* expression, so the NAC-dependent operons are expressed at low levels. Although the *nac* gene is present and functional in both *K. aerogenes* and *Escherichia coli*, *nac* is absent from *Salmonella typhimurium* (3). As a result, even if the NAC-binding site remains intact, there is no NAC-dependent nitrogen regulation in *S. typhimurium* unless NAC is supplied from another source.

The *put* genes of *K. aerogenes*, *S. typhimurium*, and many other bacteria allow growth on proline as a sole carbon and nitrogen source (4, 13, 26). Two genes are required for proline utilization: *putP* encodes a proline transport protein, and *putA* encodes a multifunctional enzyme that degrades proline to glutamate. In both *K. aerogenes* and *S. typhimurium*, expression of the *put* operon is derepressed by growth in the presence of exogenous proline and is subject to catabolite repression in the presence of glucose. However, nitrogen regulation of *put* differs in these closely related bacteria (4, 21). In *K. aerogenes* the *put* operon (put_K) is activated in response to nitrogen starvation in a NAC-dependent manner. In contrast, in *S. typhimurium* the *put* operon (put_S) is not activated in response to nitrogen starvation, even if a functional copy of the *nac* gene is provided in *trans* (3).

The differences in NAC regulation of the *put* operon could be due to differences in the DNA-binding sites for NAC in the *put* control regions of *K. aerogenes* and *S. typhimurium*. Therefore, we sought to answer the following questions: what is the DNA-binding site for NAC in put_K and does put_S lack this site? When these experiments were begun, the nature of the NAC-binding site was unknown, but we assumed that the NAC-binding site would be located within the put_K regulatory region between the divergent *putA* and *putP* genes. Based upon this assumption, we used challenge phage to identify a small fragment from put_K that contained a NAC-binding site. We then mutagenized this fragment to identify nucleotides that are essential for NAC binding in vivo. As expected, many of the nucleotides essential for NAC binding in put_K are absent from the sequence of put_S, explaining the failure of a functional *nac* gene to confer nitrogen regulation on put_S.

* Corresponding author. Mailing address: Department of Microbiology, University of Illinois, B103 Chemical and Life Sciences Laboratory, 601 S. Goodwin Ave., Urbana, IL 61801. Phone: (217) 333-3122. Fax: (217) 244-6697. E-mail: s-maloy@uiuc.edu.

† Present address: Department of Microbiology, SUNY, Stony Brook, N.Y.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Reference(s) or source
Strain		
S. typhimurium		
MS1582	*leuA414*(Am) Fels$^-$ *supE40 ataA*::(P22 *sieA44 16-amH1455 tpfr49*)	9
MST1762	MS1868/pMS421	P. Youderian
MS1868	*leuA414*(Am) Fels$^-$ *hsdSB*	9, 27
MS1883	*leuA414*(Am) Fels$^-$ *hsdSB supE40*	9
TH564	*leuA414*(Am) Fels$^-$ *supE40 ataA*::(P22 *sieA44 Ap7* [Tn*1*] *tpfr184* [*mnt-al*] 9$^-$ *att*$^+$)	12
MST2778	MS1868/pPC36	This study
MST2779	TH564/pPC36	This study
MST2780	TH564/pGW1700/pPC36	This study
E. coli DH1	*thi-1 supE44 hsdR17*(r$^-$ m$^+$) *endA1 recA1 relA gyr-96*	11
Plasmid		
pEC205.1	P_{tac}-*nac*$^+$ (pBR322)	24
pGW1700	Tet^r Amp^r *mucAB* (pBR322)	20
pMS421	Str^r Spc^r *lacI*^{q} (pSC101)	9
pKC7	Kan^r put_K^+ (*K. aerogenes*)	4
pKC9	Amp^r (subcloned pKC7 *Hin*dIII[283]-*Hin*dIII[621] into pTZ18U *Hin*dIII)	4
pPC6	Amp^r put_S^+ (*S. typhimurium*)	10
pPC36	Spc^r Str^r *nac*$^+$ (pMS421)	This study
pPC37	Tet^r Amp^r (subcloned pKC9 *Dra*I[381]-*Sma*I[436] into pPY190 *Sma*I)	This study
pPC38	Tet^r Amp^r (subcloned pKC9 *Sma*I[436]-*Dra*I[381] into pPY190 *Sma*I)	This study
pPC39	Tet^r Amp^r (subcloned pKC9 *Sma*I[436]-*Hin*cII[406] into pPY190 *Sma*I)	This study
pPC40	Tet^r Amp^r (subcloned pKC9 *Hin*cII[406]-*Sma*I[436] into pPY190 *Sma*I)	This study
pPC41	Amp^r (subcloned pKC7 between put_K oligonucleotide 1 and put_K oligonucleotide 6 into pTZ18U *Sma*I)	This study
pPC42	Amp^r (subcloned pKC9 *Hin*dIII[283]-*Sma*I[436] into pTZ18U *Sma*I)	This study
pPC43	Amp^r (subcloned pKC9 *Sma*I[436]-*Hin*dIII[621] into pTZ18U *Sma*I)	This study
pPC44	Amp^r (subcloned pKC9 *Hin*dIII[283]-*Hin*cII[406] into pTZ18U *Sma*I)	This study
pPC45	Amp^r (subcloned pKC9 *Hin*cII[406]-*Hin*dIII[283] into pTZ18U *Sma*I)	This study
pPC46	Amp^r (subcloned pKC9 *Hin*dIII[283]-*Dra*I[381] into pTZ18U *Sma*I)	This study
pPC47	Amp^r (subcloned pKC9 *Dra*I[381]-*Hin*dIII[283] into pTZ18U *Sma*I)	This study
pPY190	Amp^r Tet^r P22 *mnt* P_{ant} *Sma*I-*Xma*I *ant′* (pBR322)	2
pTZ18U	Amp^r	18

[a] Genetic nomenclature for bacterial genes is as described in Sanderson and Roth (22). Numbers in brackets indicate the map distances in base pairs of the restriction sites from the transcriptional start site of the *putP* gene.

MATERIALS AND METHODS

Bacterial strains and growth media. The strains used in this study are listed in Table 1. Nutrient broth (NB) (Difco) with 0.5% NaCl was used for rich medium. When needed, antibiotics were added in the following concentrations: sodium ampicillin, 50 μg/ml; kanamycin sulfate (KAN), 40 μg/ml; spectinomycin (SPC), 100 μg/ml; and tetracycline HCl (TET), 10 μg/ml. Dioxane-free isopropyl-β-D-thiogalactopyranoside (IPTG) was added at the concentrations indicated. Phage lysates were prepared in phage broth, NB supplemented with 1× E salts and 0.2% glucose (6).

Plasmids. The plasmids used in this study are listed in Table 1. The plasmid pPC41, which carries the entire put_K regulatory region, was constructed by PCR amplification of the put_K regulatory region from pKC7 and cloning of the amplified DNA (approximately 470 bp) into the *Hin*cII site on pTZ18U. PCR amplification was done as described by Ostrovsky de Spicer et al. (19). Plasmids carrying subfragments of the put_K regulatory region were constructed by purifying restriction fragments from pPC41 by electroelution from 10% polyacrylamide gels (14) and then subcloning the DNA fragments into the multiple cloning site on plasmid pTZ18U.

Small fragments from the *put* regulatory region were subsequently subcloned into the *Sma*I site on pPY190 for construction of challenge phage. pPY190 is a derivative of pBR322 that carries a P_{ant}-*arc-ant′* fragment from P22 with a unique *Sma*I site substituted for the *mnt* operator between P_{ant} and *arc* (2). The DNA sequences of potential clones were determined to confirm the identity of each of the cloned fragments and the orientation of the inserts.

The *K. aerogenes nac* gene was subcloned into pMS421 to construct a *nac* expression vector for the challenge phage experiments. A 2.8-kb *Hin*dIII fragment from plasmid pEC205.1 (24) containing a Tn*5 tac-nac* fusion (5) was eluted from a 0.8% agarose gel and subcloned into the *Hin*dIII site of pMS421.

Construction of challenge phage. Challenge phage with potential *nac*-binding sites were isolated by in vivo recombination between the pPY190 clones carrying put_K DNA fragments and P22 *mnt*::Kn*9 arc-1605*(Am) phage (15, 16). *S. typhimurium* strains carrying the pPY190 derivatives were grown overnight in NB containing TET (NB + TET), and then 0.1 ml of each overnight culture was infected with 0.1 ml of the phage (10^{10} PFU/ml), 5 ml of phage broth was added, and the cultures were incubated at 37°C. After 3 h the cultures were centrifuged, and the supernatant was collected and treated with chloroform. The resulting lysates were plated on strain MS1582. MS1582 contains an *mnt*$^+$ prophage that prevents the parental P22 *mnt*::Kn*9* O_{mnt}^+ *arc-1605*(Am) phage from growing lytically, so only recombinants or O_{mnt} mutants form plaques on MS1582. The clear plaques were purified, and the phage DNA was isolated from the concentrated lysate as described by Silhavy et al. (25). The desired recombinants were distinguished from parental phage by a restriction fragment linked polymorphism which results from the substitution. Restriction fragment length polymorphism mapping was done on PCR fragments from the O_{mnt} region (15), and the DNA sequence of this region was determined for each of the recombinants.

Challenge phage assays. Challenge phage assays were done as previously described (15, 16). Strain MST2778 was grown to mid-exponential phase in NB + SPC, and then 1-ml aliquots were subcultured into flasks containing 4 ml of NB + SPC with different concentrations of IPTG to induce NAC expression. After being incubated for 1 h at 37°C, the cultures were infected with challenge phage at a multiplicity of infection of 25. Phage were allowed to adsorb to the cells for 1 h at room temperature, and then serial dilutions of the infected cells were plated on NB + SPC + KAN plates containing the same concentration of IPTG used for preinduction. The plates were incubated at 37°C for 3 days, and then the number of Kan^r lysogens was counted.

Isolation of *nac* challenge phage mutants. Challenge phage with mutations in the NAC-binding site were obtained either (i) by error-prone repair after UV irradiation in a strain which expresses the *mucAB* gene products from the plasmid pGW1700 (20) or (ii) by isolating the plasmid from a *mutS* strain (15). MST2780 [pPC36 (P_{tac} *nac*) and pGW1700 (*mucA*$^+$*B*$^+$)] was grown to mid-exponential phase in NB + SPC + TET and then diluted 1:3 into NB + SPC + TET + IPTG (0.8 mM final concentration of IPTG). The put_K challenge phage (10^9 PFU/ml) were irradiated with 12,000 μJ/cm^2 of UV light. The UV-irradiated phage were then diluted 10^{-1}-, 10^{-2}-, and 10^{-3}-fold, and 100 μl of each dilution was added to 200 μl of the MST2780 culture. After phage adsorption, 3 ml of top agar was added, and the mixtures were plated on NB + SPC + 0.8 mM IPTG medium and incubated at 37°C. Mutant phage that formed clear plaques were picked and repurified.

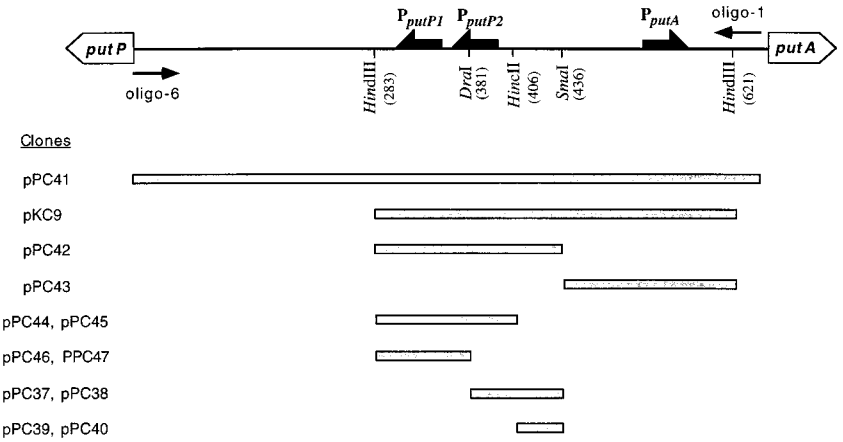

FIG. 1. Physical map of the *K. aerogenes put* regulatory region and subcloned DNA fragments. The positions of relevant restriction sites are indicated with the distances in base pairs from the *putP* transcriptional start site shown in parentheses. Subcloned regions used for construction of challenge phage are shown as shaded bars directly below the corresponding region of the restriction map.

PCR amplification of the P_{ant} region from challenge phage. The P_{ant} region from each challenge phage was amplified by PCR, and the resulting DNA fragments were used for both gel retardation assays (symmetric PCR products) and DNA sequence analysis (asymmetric PCR products). Approximately 500 ng of phage DNA was used for each PCR. The two primers used for symmetric PCR were the O_{mnt} primer (5′-CGGCATTTTGCTCATTCC-3′), complementary to the sequence upstream of the operator region of the *mnt* gene, and the anti-O_{mnt} primer (5′-GATCATCTCTAGCCATGC-3′), complementary to the sequence upstream of the −35 region of P_{ant} on the opposite strand (2). For asymmetric PCR, 5 μl of amplified double-stranded DNA from the symmetric reaction was further amplified by using only one primer (17).

Gel retardation assays. The *K. aerogenes* NAC protein was overproduced from plasmid pEC205.1 and purified as previously described (8, 24). Stock solutions containing purified NAC in storage buffer (50% glycerol, 125 mM NaCl, 50 mM NaH_2PO_4 [pH 7.0], 1.25 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol) were diluted in the same buffer supplemented with bovine serum albumin to a concentration of 1.0 mg/ml prior to use in gel retardation assays. To assay the relative affinities of NAC for various PCR-amplified DNA fragments, a 215-bp DNA fragment carrying a portion of the wild-type put_K control region, generated by digestion of plasmid pPC47 with *Hinc*II and *Bam*HI, was used as an internal standard. Gel retardation assays were performed essentially as follows (7). One-microliter aliquots of storage buffer with either bovine serum albumin or NAC were added to a 9-μl solution containing 0.1 pmol of digested pPC47 DNA plus 0.1 pmol of PCR-amplified experimental DNA. The protein-DNA solutions were incubated for 5 min at room temperature prior to the addition of 1.5 μl of gel-loading solution (40 mM Tris HCl [pH 8.4], 4 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol, and 25% glycerol), loaded onto a 4% polyacrylamide gel buffered with Tris-EDTA (pH 8.4), and subjected to electrophoresis at 200 volts for 2 h at 4°C. The extent of migration of the DNA fragments through the gel was determined by the UV fluorescence of ethidium bromide-stained gels.

DNA sequencing. The DNA sequences of *nac* challenge phage were determined from the single-stranded DNA obtained from asymmetric PCRs. The PCR products were purified with Geneclean (Bio 101, La Jolla, Calif.), and the purified DNA was resuspended in 20 μl of H_2O. The DNA sequence was then determined by dideoxy sequencing by using Sequenase (United States Biochemicals, Cleveland, Ohio).

RESULTS

NAC binds to specific fragments of the *K. aerogenes put* regulatory region in vivo. To characterize NAC binding to the put_K regulatory region in vivo, we used P22 challenge phage as a reporter for the protein-DNA interaction (2, 15, 16). To construct NAC challenge phage, the O_{mnt} site which regulates *ant* expression was replaced by a fragment expected to contain the NAC-binding site from put_K. Phage which carry the NAC-binding site place *ant* expression under the control of NAC. Thus, if the substituted operator site is occupied by NAC protein, transcription of the *ant* gene will be repressed, and the challenge phage will form Kan^r lysogens. The frequency of lysogeny is determined by the relative affinity of NAC for the DNA-binding site in vivo.

Because the precise NAC-binding site within the put_K DNA fragment was not known, we blindly tested challenge phage with restriction fragments from the put_K regulatory region derived from the plasmids shown in Fig. 1. Two of the restriction fragments showed positive results: pPC37 and pPC38 contain a 55-bp *Sma*I-*Dra*I fragment in opposite orientations, and pPC39 and pPC40 contain a 30-bp *Sma*I-*Hinc*II fragment in opposite orientations. These plasmids were crossed onto P22 *mnt*::Kn*9 arc*(Am) phage to construct the challenge phages SD2-1, SD1-1, SH4-1, and SH26-10 (Fig. 2). NAC binding to these challenge phages was tested by infection of MST2778 cultures that were preinduced with varying concentrations of IPTG to express different amounts of NAC (Fig. 3). When strain MST2778 was induced by a low concentration (0.01 mM) of IPTG, and infected by phages SD2-1 and SH4-1, the frequency of lysogeny was less than 10^{-5}%. As the IPTG concentration was increased to 0.1 mM, the frequency of lysogeny increased in proportion to the IPTG concentration. The maximal frequency of lysogeny reached 1% when the cells were induced with 0.8 mM IPTG. As a negative control, strain MST1762 (which is isogenic to MST2778 except that the plasmid pMS421 lacks the *nac* gene) was tested under identical conditions. The frequency of lysogeny in this NAC^- strain was less than 10^{-7}% under each of the conditions, indicating *nac* expression from plasmid pPC36 was required for efficient lysogeny of the challenge phage.

Phage SD1-1 carries the same insert as phage SD2-1 but in the opposite orientation. In contrast to phage SD2-1, when phage SD1-1 was used no lysogens were obtained at any of the IPTG concentrations. An explanation for this result could be that the binding site for NAC is close to one end of the inserted sequence: when the fragment is oriented as in SD2-1 the binding site is sufficiently close to the −10 region of the *ant* promoter that NAC binding occludes RNA polymerase binding to P_{ant} and thus represses *ant* transcription; in contrast, when the fragment is oriented as in SD1-1 the binding site is too far from the *ant* promoter to interfer with RNA polymerase binding. Although the insert in SH26-10 is in the same orientation as SD1-1, the smaller size of the insert in SH26-10 would place the NAC-binding site close to the *ant* promoter. Nevertheless, because NAC efficiently represses *ant* transcription in phages

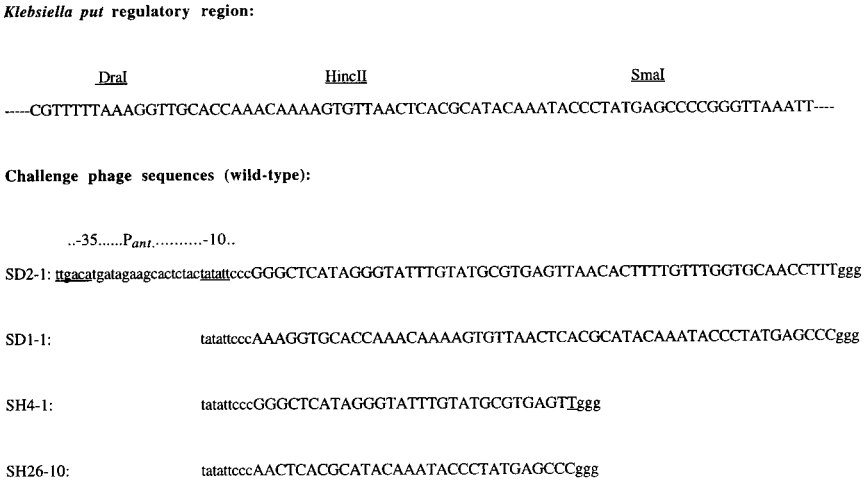

FIG. 2. DNA sequences of the *K. aerogenes put* regulatory region and challenge phages. The wild-type *K. aerogenes put* regulatory region and DNA sequences from the wild-type *K. aerogenes put* regulatory region present in challenge phages SD1-1, SD2-1, SH4-1, and SH26-10 are shown. The sequence of the *put* DNA is shown in capital letters. The adjacent sequences in the challenge phage are shown in lowercase letters. Note that the substitution is inverted in challenge phage SD2-1 versus SD1-1 and SH4-1 versus SH26-10. Underlined nucleotides represent the −10 and −35 region of P_{ant}.

SH4-1, SD2-1, and SH26-10, the region between the *Sma*I and *Hin*cII sites must contain the NAC-binding site.

NAC binds to the *put* regulatory region from *K. aerogenes* but not *S. typhimurium* in vitro. Gel retardation assays with purified NAC protein confirmed that NAC binds to a 335-bp *Hin*dIII fragment from pKC7 which contains most of the put_K control region (Fig. 1). Gel retardation assays with restriction digests of subclones derived from pKC7 indicated that a 30-bp *Hin*cII-*Sma*I fragment is necessary and sufficient for the interaction between NAC and the put_K control region in vitro. In contrast, NAC did not retard a DNA fragment containing the corresponding region from the *S. typhimurium put* operon (data not shown).

Isolation of challenge phage mutants that prevent NAC binding in vivo. To identify the nucleotide residues recognized by NAC, we isolated challenge phage mutants defective for NAC binding in vivo. In order to obtain a wide variety of mutations, the phage were irradiated with UV light and then grown in a strain carrying plasmid pGW1700, which enhances error-prone repair. Upon infection of cells which express high levels of NAC, the parent challenge phage formed lysogens, but challenge phage with mutations that prevent NAC binding grew lytically. The typical frequency of clear plaque mutations was approximately 10^{-5} per PFU of infecting phage. The clear plaques were purified and screened for challenge phage with mutations in the NAC-binding site.

Seventy-two clear plaque mutants were isolated from phage SH4-1, and the DNA sequences of the P_{ant} region on the mutant phage were determined (Table 2). The majority of the mutants had single base pair substitutions. Two of the mutations changed the −10 region of P_{ant} and probably increased the transcription of the *ant* gene by improving the promoter (27).

Fifty-seven of the mutants had single base pair substitutions within the put_K insert (Table 2). Challenge phage assays were performed for each of these mutants to determine the relative affinity of NAC for the mutant site in vivo. Compared to the parent phage SH4-1, the efficiency of lysogeny was severely decreased for each of the mutants. The frequency of lysogeny of these mutant challenge phage did not increase even when the IPTG concentration was increased to 5 mM, indicating that the mutations decrease the binding affinity so severely that high concentrations of NAC cannot overcome the mutant phenotype in vivo. These results indicate that the mutations affect nucleotides that are essential for the NAC-DNA interaction in vivo.

Another class of mutants had single base pair deletions or multiple nucleotide changes (Table 2). Most of the mutations that changed multiple nucleotides altered the same sequences in the put_K fragment as the single base mutations. As expected, the in vivo binding assays for each of these mutants revealed that the NAC-DNA interaction was severely defective. Typical results are shown in Fig. 3.

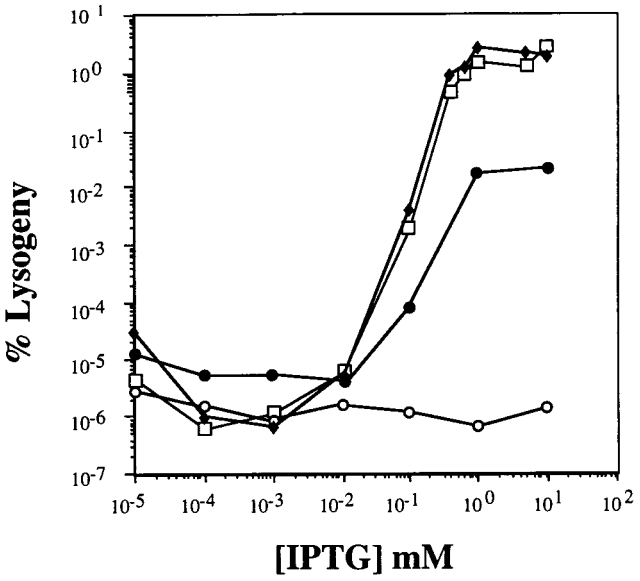

FIG. 3. Results of challenge phage assays with wild-type SH2-1 and SH4-1 and mutant derivatives of SH4-1 shown as follows: wild-type SH2-1, ◆; wild-type SH4-1, □; 6G mutant, ●; 8C mutant, ○. The percent lysogeny of the negative control which lacks the *nac* gene (MST1762) was less than 10^{-7} at all IPTG concentrations tested. The percent lysogeny indicates the number of Kan^r lysogens formed per viable cell infected. NAC is expressed from the P_{tac} promoter under the control of $LacI^q$, and thus the intracellular concentration of NAC increases as the concentration of IPTG is increased.

TABLE 2. Challenge phage mutations that decrease NAC binding

Mutation(s)	No. isolated[a]	DNA sequence[b]	In vitro DNA binding[c]
Wild type		GGGCTCATAG GGTATTTGTA TGCGTGAGTT	+
5A	2	GGGC**A**CATAG GGTATTTGTA TGCGTGAGTT	±
6T	3	GGGCT**T**ATAG GGTATTTGTA TGCGTGAGTT	±
6G	2	GGGCT**G**ATAG GGTATTTGTA TGCGTGAGTT	±
7G	1	GGGCTC**G**TAG GGTATTTGTA TGCGTGAGTT	ND
7T	1	GGGCTC**T**TAG GGTATTTGTA TGCGTGAGTT	−
8C	6	GGGCTCA**C**AG GGTATTTGTA TGCGTGAGTT	−
8A	10	GGGCTCA**A**AG GGTATTTGTA TGCGTGAGTT	−
8G	1	GGGCTCA**G**AG GGTATTTGTA TGCGTGAGTT	−
9G	8	GGGCTCAT**G**G GGTATTTGTA TGCGTGAGTT	−
11A	2	GGGCTCATAG **A**GTATTTGTA TGCGTGAGTT	±
12T	1	GGGCTCATAG G**T**TATTTGTA TGCGTGAGTT	±
14G	2	GGGCTCATAG GGT**G**TTTGTA TGCGTGAGTT	ND
15C	2	GGGCTCATAG GGTA**C**TTGTA TGCGTGAGTT	±
16G	1	GGGCTCATAG GGTAT**G**TGTA TGCGTGAGTT	±
19C	1	GGGCTCATAG GGTATTTG**C**A TGCGTGAGTT	ND
19A	2	GGGCTCATAG GGTATTTG**A**A TGCGTGAGTT	±
20T	4	GGGCTCATAG GGTATTTGT**T** TGCGTGAGTT	−
20G	2	GGGCTCATAG GGTATTTGT**G** TGCGTGAGTT	ND
21G	1	GGGCTCATAG GGTATTTGTA **G**GCGTGAGTT	−
21A	2	GGGCTCATAG GGTATTTGTA **A**GCGTGAGTT	±
35C	3	GGGCTCATAG GGTATTTGTA TGCG**C**GAGTT	±
12Δ	1	GGGCTCATAG G**-**TATTTGTA TGCGTGAGTT	−
13Δ	1	GGGCTCATAG GG**-**ATTTGTA TGCGTGAGTT	ND
19Δ	1	GGGCTCATAG GGTATTTG**-**A TGCGTGAGTT	−
8C, 9Δ	1	GGGCTCA**C-**G GGTATTTGTA TGCGTGAGTT	ND
9Δ, 14T	1	GGGCTCAT**-**G GGT**T**TTTGTA TGCGTGAGTT	−
13A, 14G, 15T	1	GGGCTCATAG GG**AGT**TTTGT ATGCGTGAGTT	ND
19A, 20T	1	GGGCTCATAG GGTATTTG**AT** TGCGTGAGTT	ND
19G, 20T	1	GGGCTCATAG GGTATTTG**GT** TGCGTGAGTT	ND

[a] Number of independent mutants with the same nucleotide changes.
[b] Nucleotide changes are shown in bold and underlined.
[c] In vitro binding indicates Relative binding affinity of NAC based upon gel retardation assays as shown in Figure 4. +, efficient binding; ±, weak binding; −, no binding detected. ND, not determined.

Challenge phage mutants differ in their ability to interact with NAC in vitro. Gel retardation assays were done to determine the influence of the challenge phage mutations on the interaction of NAC with wild-type and mutant DNA sites in vitro. Typical results are shown in Fig. 4. In the presence of either limiting or excess NAC, the interaction between NAC and the 150-bp SH4-1 fragment amplified from a wild-type put_K challenge phage was similar to that observed with the 215-bp *Hin*cII-*Bam*HI control fragment carried by pPC47. Likewise, NAC binding to a derivative of SH4-1 with a P_{ant} promoter mutation (90-32) was indistinguishable from binding to the wild-type sequence. In contrast, the interactions of NAC with fragments amplified from the mutant, NAC-insensitive challenge phages were either moderately reduced (e.g., 90-40 [6G mutation]) or severely reduced (e.g., 90-2 [8C mutation]) relative to the wild-type control. The gel retardation results are summarized in Table 2.

DISCUSSION

Expression of the *put* operon is regulated by nitrogen starvation in *K. aerogenes* but not *S. typhimurium*. Previous studies suggested that this lack of nitrogen regulation in *S. typhimurium* is due to the absence of both the *trans*-acting factor (NAC) and a *cis*-acting site in the *put* regulatory region (3, 4). To understand how NAC regulates expression of the *put* operon in *K. aerogenes*, we used challenge phage to genetically identify the DNA sequence from the *K. aerogenes put* regulatory region which is critical for NAC binding. The specific nucleotides required for NAC binding were then identified by isolating base substitution mutants that prevent NAC binding in vivo. Binding of NAC to the *K. aerogenes put* regulatory region was also assayed by gel retardation in vitro. Purified NAC protein specifically retarded DNA from the *K. aerogenes put* control region in vitro but not from the *put* control region from *S. typhimurium*.

Within the *K. aerogenes put* control region, NAC recognized a 55-bp *Sma*I-*Dra*I fragment. The *Sma*I-*Dra*I fragment was cloned onto the challenge phage in both orientations. Kan^r lysogens were only obtained when the *Sma*I site was closest to the *ant* promoter (challenge phage SH2-1), indicating that NAC binds at or near this *Sma*I site, and interaction is strong enough to interfere with RNA polymerase binding to P_{ant}. To narrow down the position of the NAC-binding site, the 30-bp *Hin*cII-*Sma*I half of this fragment was used to construct challenge phage SH4-1 and was tested for NAC binding in vivo. This 30-bp fragment alone was sufficient for efficient NAC binding.

To identify the specific nucleotides critical for NAC binding, we selected for mutations on challenge phage SH4-1 that prevented NAC binding. Fifty-seven mutants were isolated with single base substitutions in the *put* insert that decreased the NAC-binding affinity in vivo (Table 2). These mutations identified base substitutions in 15 different nucleotides within the DNA-binding site. Each of these mutant DNAs decreased the maximal frequency of Kan^r lysogens to 1% of that of the wild-type put_K challenge phage. Several of the mutants (5A, 6T, 6G, 11A, 15C, 19A, and 25C mutations) allowed weak NAC binding, producing tiny Kan^r colonies at high concentrations of IPTG. NAC binding to the rest of the mutants was severely disrupted, and hence no Kan^r lysogens were formed at any concentration of IPTG.

The relative binding of NAC to each of the mutant binding sites was measured by challenge phage assays in vivo (Fig. 3) and gel retardation assays in vitro (Fig. 4). Based on the results, the mutants fell into two groups (Table 2). One group of mutants severely disrupted NAC binding in vivo, and thus no Kan^r lysogens were formed. Gel retardation assays confirmed

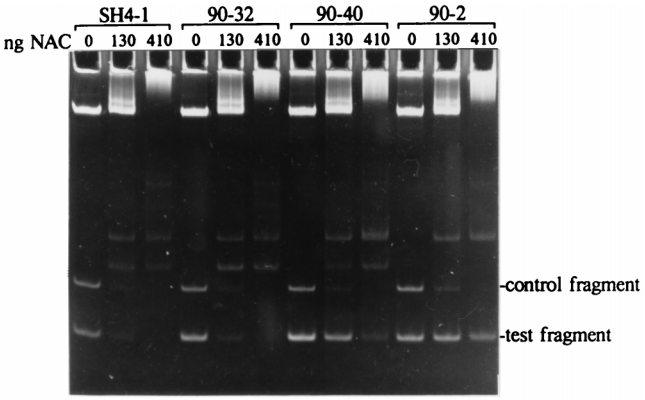

FIG. 4. Gel retardation assay of the interactions between NAC and DNA fragments derived from the *putAP* control region. The indicated amounts of NAC were incubated with a mixture of digested pPC47 and DNA amplified from challenge phages SH4-1 (wild-type), 90-32 (P_{ant} promoter mutation), 90-40 (6G mutation), and 90-2 (8C mutation). Digestion of pPC47 with *Hin*cII and *Bam*HI yielded a 215-bp control fragment containing nucleotides 406 to 621 of the *putAP* control region and a larger fragment containing the vector pTZ18U plus nucleotides 381 to 405 of the *putAP* control region. The migration position of the unretarded 215-bp control and 150-bp test fragments are indicated at the right.

TABLE 3. Sequence alignment of NAC-binding sites

Nucleotide importance[a]	Gene(s)	Nucleotide sequence
Very important	*putP*	.ATA.....AT...T
	hutU	.ATAA.....AT....
Moderately important	*putP*	CATA.G.T.T...TAT...T
	hutU	TATAA.A.....GTAT....
Consensus	*putP*, *hutU*, *ureD*	.ATA......T.GTAT....

[a] Nucleotides indicated as very important or moderately important reduce NAC binding severely or moderately, respectively, if mutated. The consensus sequence is for the 20-bp region from each of the NAC-binding sites of the indicated genes.

that NAC did not bind to these mutants in vitro. The second group of mutants decreased NAC binding, but Kan^r lysogens were obtained at a low frequency. Gel retardation assays confirmed that NAC could bind to these mutant sites but a higher concentration of NAC was required than for binding to the wild-type site. By comparing the in vivo and in vitro results, we concluded that the six nucleotides $ATAN_{10}ATN_3T$ are critical determinants for the interaction between NAC and DNA. Base substitutions at any of these nucleotides decreased NAC binding both in vivo and in vitro. Substitutions at five additional positions only decreased NAC binding in vivo, and thus these residues may play a role in the NAC-DNA interaction. Including all of these nucleotides would extend the NAC-binding site to $CATANGNTNTN_3TNTN_3T$.

Differences between the frequencies of lysogeny of challenge phages SD2-1 and SH4-1 suggested that the flanking sequence between *Hin*cII and *Dra*I might facilitate the interaction of NAC with the operator site in vivo. If this region plays an important role in NAC binding, we would expect that mutations in the critical nucleotides would decrease NAC binding. Therefore, we also selected for mutations that decrease NAC binding to phage SH2-1. Fifty-one challenge phage SD2-1 mutants were isolated that decreased NAC binding in vivo. All of these mutations were located within the *Sma*I-*Hin*cII fragment present on SH4-1. Furthermore, each of the single base substitutions obtained from challenge phage SD2-1 had also been obtained from phage SH4-1. The observation that none of these mutations were located within the *Dra*I-*Hin*cII region suggests that these sequences do not directly interact with NAC.

The NAC-binding site in put_K is between the divergent *putA* and *putP1* promoters, which are separated by about 125 bp (4). The site is located about 20 bp upstream of the *putP1* promoter, overlapping a putative cyclic AMP receptor protein-binding site, and about 90 bp upstream of the *putA* promoter. Previous primer extension analysis showed transcription from the *putP1* promoter increases during nitrogen starvation (4). Only two of the seven critical nucleotides in the NAC-binding site are present in the *put* control region from *S. typhimurium* (CATCTGGATTTATTTTCCTCTGCGGTAGTT). This DNA sequence divergence explains why the *put* operon from *S. typhimurium* is not activated by nitrogen starvation even when NAC is provided in *trans.*

In the accompanying paper by Pomposiello, Janes, and Bender (20a), the NAC-binding site in the *hutU* promoter region from *K. aerogenes* was mutagenized by using synthetic oligonucleotides. The results of that study also define nucleotides required for binding and transcriptional activation by NAC. The site-directed mutants that affect NAC binding to the P_{hutU} site are very analogous to the challenge phage mutants that affect NAC binding to the P_{put} site. The nucleotides identified as very important (the mutation of which results in a severe defect in NAC binding and activation) and the nucleotides that are moderately important (mutation results in a less severe defect) are similar in both the *put* and *hut* sites. An alignment of three sites known to bind NAC and to activate transcription (from P_{hutU}, P_{put}, and P_{ureD}) yields similar consensus patterns of nucleotides (Table 3).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM34715 (S.M.) and GM47156 (R.B.) from the National Institute of General Medical Sciences.

We thank Kelly Hughes, Graham Walker, and Phil Youderian for generously providing some of the strains used in this study and Jeff Gardner for helpful comments on the manuscript.

REFERENCES

1. **Bender, R. A.** 1991. The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. Mol. Microbiol. **5:**2575–2580.
2. **Benson, N., P. Sugiono, S. Bass, L. Mendelman, and P. Youderian.** 1986. General selection for specific DNA-binding activities. Genetics **114:**1–14.
3. **Best, E., and R. Bender.** 1990. Cloning of the *Klebsiella aerogenes nac* gene, which encodes a factor required for nitrogen regulation of the histidine utilization (*hut*) operons in *Salmonella typhimurium*. J. Bacteriol. **172:**7043–7048.
4. **Chen, L.-M., and S. Maloy.** 1991. Regulation of proline utilization in enteric bacteria: cloning and characterization of the *Klebsiella put* control region. J. Bacteriol. **173:**783–790.
5. **Chow, W., and D. Berg.** 1988. Tn5tac1, a derivative of transposon Tn*5* that generates conditional mutants. Proc. Natl. Acad. Sci. USA **85:**6468–6472.
6. **Davis, R., D. Botstein, and J. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Garner, M., and A. Rezin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: applications to components of the *E. coli* lactose operon regulatory system. Nucleic Acids Res. **9:**3047–3060.
8. **Goss, T. J., and R. A. Bender.** 1995. The nitrogen assimilation control protein, NAC, is a DNA binding transcription activator in *Klebsiella aerogenes*. J. Bacteriol. **177:**3546–3555.
9. **Graña, D., P. Youderian, and M. Susskind.** 1985. The effects of mutations in the *ant* promoter of *Salmonella* phage P22. Genetics **110:**1–6.
10. **Hahn, D., R. Myers, C. Kent, and S. Maloy.** 1988. Regulation of proline utilization in *Salmonella typhimurium*: molecular characterization of the *put* operon and DNA sequence of the *put* control region. Mol. Gen. Genet. **213:**125–133.
11. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
12. **Hughes, K. T., P. Youderian, and M. Simon.** 1988. Phase variation in *Salmonella*: analysis of Hin recombinase and *hix* recombination site interaction in vivo. Genes Dev. **2:**937–948.
13. **Maloy, S.** 1987. The proline utilization operon p. 1513–1519. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol 2. American Society for Microbiology, Washington, D.C.
14. **Maloy, S.** 1989. Experimental techniques in bacterial genetics. Jones and Bartlett, Boston, Mass.
15. **Maloy, S., V. Stewart, and R. Taylor.** 1996. Genetic analysis of pathogenic bacteria: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Maloy, S., and P. Youderian.** 1993. Challenge phage: a genetic tool kit for dissecting DNA-protein interactions in vivo. Methods Mol. Genet. **3:**205–233.
17. **McCabe, P.** 1990. Production of single-stranded DNA by asymmetric PCR, p. 76–83. *In* M. A. Innis, D. H. Gelfand, J. J. Snibsky, and T. J. White (ed.), PCR protocols: a guide to methods and Applications. Academic Press, New York, N.Y.
18. **Mead, D., E. Szczesna-Sorupa, and B. Kemper.** 1986. Single-stranded DNA 'Blue' T7 promoter plasmids: a versitile tandem promoter system for cloning and protein engineering. Protein Eng. **1:**67–74.
19. **Ostrovsky de Spicer, P., K. O'Brien, and S. Maloy.** 1991. Regulation of proline utilization in *Salmonella typhimurium*: a membrane-associated dehydrogenase binds DNA in vitro. J. Bacteriol. **173:**211–219.
20. **Perry, K., and G. Walker.** 1982. Identification of plasmid (pKM101)-coded proteins involved in mutagenesis and UV resistance. Nature **300:**278–281.

20a. **Pomposiello, P. J., B. K. Janes, and R. A. Bender.** Two roles for the DNA recognition site of *Klebsiella aerogenes* Nitrogen assimilation control protein. J. Bacteriol. **180:**578–585.

21. **Prival, M., and B. Magasanik.** 1971. Resistance to catabolite repression of histidase and proline oxidase during nitrogen-limited growth of *Klebsiella aerogenes*. J. Biol. Chem. **246:**6288–6296.
22. **Sanderson, K., and J. Roth.** 1988. Linkage map of *Salmonella typhimurium*, edition VII. Microbiol. Rev. **52:**485–532.
23. **Schwacha, A., and R. A. Bender.** 1993. 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. J. Bacteriol. **175:**2116–2124.
24. **Schwacha, A., and R. A. Bender.** 1993. The *nac* (nitrogen assimilation control) gene from *Klebsiella aerogenes*. J. Bacteriol. **175:**2107–2115.
25. **Silhavy, T., L. Enquist, and M. Berman.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Wood, J.** 1981. Genetics of L-proline utilization in *Escherichia coli*. J. Bacteriol. **146:**895–901.
27. **Youderian, P., A. Vershone, S. Bouvier, R. Sauer, and M. Susskind.** 1983. Changing the DNA-binding specificity of a repressor. Cell **35:**777–783.