JOURNAL OF BACTERIOLOGY, Dec. 1999, p. 7274–7284 Vol. 181, No. 23
0021-9193/99/$04.00+0


# General Nitrogen Regulation of Nitrate Assimilation Regulatory Gene *nasR* Expression in *Klebsiella oxytoca* M5al

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Received 28 July 1999/Accepted 22 September 1999

***Klebsiella oxytoca*** **can assimilate nitrate and nitrite by using enzymes encoded by the *nasFEDCBA* operon. Expression of the *nasF* operon is controlled by general nitrogen regulation (Ntr) via the NtrC transcription activator and by pathway-specific nitrate and nitrite induction via the NasR transcription antiterminator. This paper reports our analysis of *nasR* gene expression. We constructed strains bearing single-copy Φ(*nasR-lacZ*) operon fusions within the chromosomal *rhaBAD-rhaSR* locus. The expression of Δ*rhaBS*::[Φ(*nasR-lacZ*)] operon fusions was induced about 10-fold during nitrogen-limited growth. Induction was reduced in both *ntrC* and *rpoN* null mutants, indicating that Ntr control of *nasR* gene expression requires the NtrC and $\sigma^N$ ($\sigma^{54}$) proteins. Sequence inspection of the *nasR* control region reveals an apparent $\sigma^N$-dependent promoter but no apparent NtrC protein binding sites. Analysis of site-specific mutations coupled with primer extension analysis authenticated the $\sigma^N$-dependent *nasR* promoter. Fusion constructs with only about 70 nucleotides (nt) upstream of the transcription initiation site exhibited patterns of β-galactosidase expression indistinguishable from Φ(*nasR-lacZ*) constructs with about 470 nt upstream. Expression was independent of the Nac protein, implying that NtrC is a direct activator of *nasR* transcription. Together, these results indicate that *nasR* gene expression does not require specific upstream NtrC-binding sequences, as previously noted for *argT* gene expression in *Salmonella typhimurium* (G. Schmitz, K. Nikaido, and G. F.-L. Ames, Mol. Gen. Genet. 215:107–117, 1988).**

*Klebsiella* spp., members of the family *Enterobacteriaceae*, can use nitrate ($NO_3^-$) and nitrite ($NO_2^-$) as sole nitrogen sources during aerobic growth. Nitrate and nitrite are reduced to ammonium by assimilatory nitrate and nitrite reductases, respectively (reviewed in reference 31). The resulting ammonium is incorporated into central metabolism through the action of glutamine synthetase and glutamate synthase (reviewed in reference 49).

Molecular genetic analysis of *K. oxytoca* (*pneumoniae*) M5al has identified the *nasFEDCBA* operon required for nitrate and nitrite assimilation. The *nasFED* genes encode a periplasmic binding protein-dependent nitrate and nitrite transporter (64). The *nasCA* genes encode assimilatory nitrate reductase, and the *nasB* gene encodes assimilatory nitrite reductase (28, 29). The *nasR* gene, located immediately upstream of *nasF*, encodes a nitrate- and nitrite-responsive positive regulator for *nasF* operon expression (19). Expression of the *nasF* operon is controlled by general nitrogen regulation (Ntr) via the NtrC transcription activator (reviewed in reference 31) and by pathway-specific nitrate and nitrite induction via the NasR transcription antiterminator (8, 9, 30). The regulation of *nasR* gene expression has not previously been examined.

Ntr control in enterobacteria has been extensively studied (reviewed in references 34, 38, 44, and 48). Genes required for Ntr control include *rpoN* (also called *ntrA* and *glnF*), which encodes the sigma factor $\sigma^N$ ($\sigma^{54}$); *ntrC* (also called *glnG*), which encodes the enhancer binding transcription activator NtrC; and *ntrB* (also called *glnL*), which encodes the protein kinase/phosphoprotein phosphatase (NtrB) that controls NtrC activity. Promoters recognized by $\sigma^N$-RNA polymerase ($E\sigma^N$) contain GG and GC at 24 and 12 bp, respectively, upstream of the transcription initiation site (reviewed in reference 36). The activity of the NtrB protein is controlled in response to internal nitrogen such that the phosphorylation state of the NtrC protein is elevated during nitrogen-limited growth (22, 24). Phosphorylation stimulates transcription activation by NtrC (reviewed in references 34 and 48).

The DNA binding sites for phospho-NtrC are located 100 bp or more upstream of the transcription initiation site and thereby constitute upstream activation sequences (UAS) or enhancers. The best-studied examples are those for the *Escherichia coli* and *Salmonella typhimurium glnA-ntrBC* operon, encoding glutamine synthetase along with NtrB and NtrC, and the *K. oxytoca* (*pneumoniae*) *nifLA* operon, encoding the regulators of dinitrogen fixation (*nif*) gene expression (41, 45, 50, 63). Formation of a DNA loop facilitates contact between upstream-bound phospho-NtrC and $E\sigma^N$ to activate transcription initiation (reviewed in references 34 and 48). Binding to the UAS or enhancer increases the local concentration of phospho-NtrC. Nevertheless, NtrC can significantly stimulate transcription, even for constructs in which the NtrC binding sites have been deleted (14, 46, 50, 53). Indeed, no upstream binding sites have been identified for NtrC-dependent activation of the *S. typhimurium argT* gene, encoding the periplasmic lysine-arginine-ornithine binding protein (52).

In enterobacteria, some Ntr-regulated operons are controlled only indirectly by phospho-NtrC. Rather, the Nac protein activates the expression of $\sigma^{70}$-dependent operons such as

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*hut* (histidine utilization) and *put* (proline utilization) (10, 23, 32). Expression of the *nac* gene itself is activated by phospho-NtrC during nitrogen-limited growth (18, 54). However, *nasF* operon expression is independent of *nac*$^+$ (19, 32).

In this paper, we describe a system for integrating single-copy constructs into the chromosomal *rhaBAD-rhaSR* locus of *K. oxytoca* M5al and illustrate its use in the analysis of Φ(*nasR-lacZ*) operon fusions. We demonstrate that *nasR* gene expression is subject to Ntr control, requires a $\sigma^N$-dependent promoter, and is independent of *nac*$^+$. Deletion analysis revealed that the regulatory elements for *nasR* expression lie within 71 bp of the transcription initiation site.

## MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used in this work are listed in Table 1. *Aerobacter aerogenes* M5al was reclassified as *K. pneumoniae* (61). However, its phenotypic properties (such as a positive reaction in the indole test) place this strain in the species *K. oxytoca*. Genetic crosses were performed by bacteriophage P1 *kc*-mediated transduction (39, 60). Standard methods were used for restriction endonuclease digestion, ligation, and transformation of DNA (35).

**Culture media.** Defined, complex, and indicator media for genetic manipulations were used as described previously (35). Nitrogen-free medium contained 0.2% (wt/vol) glucose, 1% (wt/vol) sodium citrate, 0.74% (wt/vol) sodium phosphate (pH 8), and 1 mM $MgSO_4$ (28). This medium was supplemented with additional nitrogen sources (5 mM $NaNO_3$, $NaNO_2$, or $NH_4Cl$) as indicated to test nitrogen utilization phenotypes. Alanyl-glutamine (5 mM) was added to all solid media used for cultivation of *rpoN* (Gln$^-$) and *ntrC* strains (11).

Selection for *Klebsiella* transformants carrying *bla*-containing plasmids was accomplished with combination of carbenicillin and ampicillin at 800 and 60 μg/ml, respectively (33). *E. coli* transformants were selected on 200 μg of ampicillin per ml. Chloramphenicol was used at 50 and 25 μg/ml for selecting *K. oxytoca* and *E. coli* transformants, respectively. Kanamycin was used at 100 and 75 μg/ml for selecting *K. oxytoca* and *E. coli* transformants, respectively. Tetracycline was used at 20 μg/ml for selecting *E. coli* transformants. Streptomycin was used at 500 μg/ml for selecting *K. oxytoca* Sm$^r$ segregants.

Defined medium to grow cultures for β-galactosidase assays was buffered with 3-(*N*-morpholino) propanesulfonic acid (MOPS) as previously described (58). The initial pH of this medium was adjusted with NaOH to 8.0. Glucose (40 mM) was used as the sole carbon source. The nitrogen sources $NaNO_3$, $NaNO_2$, $NH_4Cl$, and L-glutamine were each added to 5 mM as indicated. Arginine, hypoxanthine, thiamine, and uracil were added to stimulate the growth of *rpoN* strains (26). For induction of *tac-nac* expression, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added very early in the exponential phase (about 15 Klett units [54]).

**Culture conditions.** Cultures for β-galactosidase assays were grown at 30°C to minimize deamidation of glutamine (4). Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a no. 66 (red) filter. Cultures were aerated at 240 rpm in 10 ml of medium in 125-ml sidearm flasks. Cultures in the mid-exponential phase (about 40 Klett units) were harvested, chilled on ice, and washed with saline. Cell pellets were stored overnight at −20°C, prior to assay for β-galactosidase activity.

**β-Galactosidase assays.** β-Galactosidase assays were done at room temperature, approximately 21°C. Cell pellets were suspended in 4 ml of Z buffer (39) and stored on ice. β-Galactosidase activity was measured in $CHCl_3$-sodium dodecyl sulfate-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in terms of cell density (absorbance at 600 nm), using the formula of Miller (39). All reported values are averages from at least three independent experiments.

**DNA sequencing.** Double-stranded templates were sequenced on a model 373A stretch DNA sequencer by using dye terminator chemistry and AmpliTaq-FS DNA polymerase (Perkin Elmer/Applied Biosystems Division, Foster City, Calif.). Templates were prepared by using QIAprep spin plasmid kits (Qiagen Inc., Chatsworth, Calif.). DNA sequences were analyzed with programs from DNASTAR Inc. (Madison, Wis.), and database searches were performed with the BLAST programs (1) accessed through the National Center for Biotechnology Information.

**DNA oligonucleotides.** In the following sequence, nucleotide substitutions for site-specific mutagenesis are underlined, added nucleotide tails are in lower case, and introduced restriction endonuclease sites are in boldface. Sequences are presented from 5′ to 3′. The nucleotide sequences of oligonucleotides used for site-specific mutagenesis were −24G→A, TCCTTCTATAAGACACGGTTAT TGC; −13/−12GC→AT, TCCTTCTATAAGGCACGGTTATTATTTGGCTG AAGTATAAAGCGTTAA; −29/−28 AT→GC, TAAGGACTCCTTCTGCAA GGCACGGTTATT; +36/+38ATG→TAA, GGAGAGGGGTATGAATAAT TAAGCGGGCAATACGCCTGAGGT. The nucleotide sequences of oligonucleotides used for construction of *lacZ* fusions were RZF1 (fusion starting from −469), ttagtc**ggatcc**GCTTTCAGCTGGCATTGT; RZF2 (fusion starting from −71), taact**ggatcc**CCGTTCGCGATAATCACAAT; RZF3 (fusion ending at +71), tggctg**tctaga**AAACCAGTCGACCACCTCAG; RZF4 (fusion starting from −14), taact**ggatcc**TGCTTGGCTGAAGTATAAA; and RZF5 (fusion ending at +362), tggctg**tctaga**CGCAAGCTGGGGCAGATA. The nucleotide sequences of oligonucleotides used for colony PCR analysis were C1, CCTGAC GCGGGCGCATTTACA; B2, GTCCGGCTCATCGCTGTTCACG; RB1, AA TTTCATTTTCAGGATTAGG; and LZ1, GCGAATGACCTTGAGTTTGTC. The nucleotide sequences of oligonucleotides used for loop deletion mutagenesis were *nasC* Δ(95–343), GAAACGGTTACCGCGGTGGAT**ATGCAT**CCGCTG GACCGCCACTACCGT; and *nasB* Δ(86–713), CGCCTGAGCGAATCGGTC GCCAGC**ATGCAT**GCCGACCTGTTCGCCAGCGAT. The nucleotide sequence of the oligonucleotide used for primer extension was TGCCTGCAGGTCGAC TCTAGAAAACCAGT.

**Site-specific mutagenesis.** Oligonucleotide-directed site-specific mutagenesis was performed by ampicillin selection as described previously (27, 35). The DNA sequence of each mutant insert was determined to confirm the mutational alterations and to ensure that no spurious changes were introduced.

**PCR.** PCR-generated DNA fragments from the *nasR* control region were amplified (51) from plasmid pVJS2502. *Bam*HI and *Xba*I sites were introduced into the upstream and downstream primers, respectively, for subsequent cloning to construct Φ(*nasR-lacZ*) fusions. Different pairs of primers resulted in different fusions; for example, primers RZF1 and RZF3 were used to construct the Φ(*nasR-lacZ*) {−469/+71} fusion, whereas primers RZF2 and RZF3 were used to construct the Φ(*nasR-lacZ*) {−71/+71} fusion (Fig. 1). (Fusions are designated by their upstream and downstream junctions relative to the transcription initiation site [Fig. 1].)

PCR was performed by using Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.). The final PCR mixture (100 μl) contained 4 ng of plasmid DNA, 1× ThermoPol reaction buffer, 200 μM deoxynucleoside triphosphates, 1 mM each oligonucleotide primer, and 2.5 U of Vent DNA polymerase. The program of reactions was exactly as described previously (64). PCR products were purified with the Wizard PCR Preps DNA purification system (Promega Corp., Madison, Wis.) and then cloned into plasmid pALTER-1. The DNA sequence of the entire insert was determined to confirm that no spurious changes were introduced.

**Electrotransformation.** A *K. oxytoca* culture (50 ml) was grown at 37°C in a 250-ml sidearm flask and harvested in the early exponential phase (about 25 to 30 Klett units). The cell pellet was washed three times with 1 M glycerol and then resuspended in 200 μl of 1 M glycerol. A 2- to 5-μl volume of plasmid DNA (100 ng/μl) was mixed with 40 μl of glycerol-washed cells in a 1.5-ml microcentrifuge tube and incubated on ice for 5 min. This mixture was pipetted into a precooled 0.2-cm electroporation cuvette and electroporated at 2.5 kV with an *E. coli* Pulser electrotransformation apparatus (Bio-Rad, Hercules, Calif.). After the pulse, 1 ml of TY broth (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl) was immediately added to the cuvette, and the cell suspension was quickly transferred into a 1.5-ml microcentrifuge tube and incubated at 30°C for 1 h prior to plating on selective medium.

**Construction of plasmid-borne Φ(*nasR-lacZ*) and Φ(*nasF-lacZ*) operon fusions.** Plasmid pRS415 (56), with the pBR322 replication origin, served as the vector. The downstream junction was the *Bam*HI site near the end of MudK #7 (29), which is inserted into codon 304 of *nasR*. The upstream cloning site was the *Hin*dIII site distal to *ychN*, located 471 nucleotides (nt) upstream of the *nasR* transcription initiation site (Fig. 1). Recloning into a polylinker plasmid placed this *Hin*dIII site adjacent to an *Eco*RI site, which was used for cloning into plasmid pRS415. This formed the Φ(*nasR-lacZ*) {−471/+910} operon fusion (Fig. 1). A second plasmid was constructed by joining the *Nru*I site, just upstream of *nasR*, to the *Sma*I cloning site in plasmid pRS415. This formed the Φ(*nasR-lacZ*) {−64/+911} operon fusion.

The Φ(*nasF-lacZ*) fusions (30) were constructed similarly, except that the downstream junction was the *Bam*HI site near the end of MudK #34, which is inserted into codon 53 of *nasF*.

**Construction of plasmid-borne Φ(*nasR′-′lacZ*) gene (translational) fusions.** Plasmid pNM481 (42), with the pBR322 replication origin, served as the vector. PCR-generated *nasR* inserts, described above, were cloned into plasmid pALTER-1 for sequence analysis and for site-specific mutagenesis. This cloning placed the downstream *Xba*I site next to the polylinker *Hin*dIII site. Therefore, these inserts were cloned as *Bam*HI-*Hin*dIII fragments into plasmid pNM481.

**Construction of the *rha* integration plasmid.** The vector pVJS2354, used for integrating *lacZ* operon fusion constructs into the chromosomal *rhaBAD-rhaSR* locus, is based on the allelic-exchange vector pKAS46 (57). Plasmid pKAS46 contains the R6K replication origin and therefore requires the π protein (product of the *pir* gene) for replication. It also contains the *rpsL*$^+$ gene, allowing for the selection (in an *rpsL* strain background) of plasmid-free segregants as Sm$^r$ colonies (57). We have previously used plasmid pKAS46 to construct several allelic-replacement strains of *K. oxytoca* (64).

Construction of plasmid pVJS2354 proceeded in several steps. First, we cloned and identified the *rha* (rhamnose utilization) locus of *K. oxytoca* M5al. The in vivo cloning method of Groisman and Casadaban (21) was used essentially as described previously (28, 35). An *E. coli rha*::Tn*10 hsdR* recipient was used to identify Rha$^+$ clones. Next, we identified an approximately 7-kb *Hin*dIII subclone that complemented *E. coli rhaB*, *rhaA*, *rhaD*, *rhaS*, and *rhaR* mutants

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Genotype | Reference or source |
|---|---|---|
| *K. oxytoca* M5al strains | | |
| UNF1801 | *hsdR1 hisD2 ntrC36*::Tn*7* | 17 |
| UNF1831 | *hsdR1 hisD2 ntrC50*::Tn*5* | 17 |
| UNF2651 | *hsdR1 hisD2 rpoN*::Km *lac-2002* | 37 |
| VJSK838 | *hsdR1 lacZ101*::Tn*10*d(Tc) | Laboratory collection |
| VJSK1018 | *hsdR1 nasD124*::Ω-Cm | 29 |
| VJSK1190 | *hsdR1 nasR131*::Tn*10*d(Cm) | 19 |
| VJSK1603 | *hsdR1 lacZ101*::Tn*10*d(Tc) *ntrC36*::Tn*7* | Laboratory collection |
| VJSK2216 | *hsdR1 lacZ101*::Tn*10*d(Tc) Δ(*narKG*)*302 rpsL* | 64 |
| Derivatives of strain VJSK2216 | | |
| VJSK2212 | Δ(*nasFED*)*137* | 64 |
| VJSK2215 | Δ(*nasCB*)*139* | This work |
| VJSK2504 | Δ*rhaBS*::*lacZ* | This work |
| VJSK2506 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−71/+71}] | This work |
| VJSK2507 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] | This work |
| VJSK2512 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] Δ(*nasFED*)*137* | This work |
| VJSK2516 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] Δ(*nasCB*)*139* | This work |
| VJSK2517 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] *nasR131*::Tn*10*d(Cm) | This work |
| VJSK2520 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] *rpoN*::Km | This work |
| VJSK2521 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] *ntrC50*::Tn*5* | This work |
| VJSK2529 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] *nasD124*::Ω-Cm | This work |
| VJSK2531 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] (−24G→A) | This work |
| VJSK2541 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−14/+362}] | This work |
| VJSK2548 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−71/+362}] | This work |
| VJSK2588 | Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] (−13,−12GC→AT) | This work |
| *K. aerogenes* W70 strains | | |
| KC1043 | *hutC515* | 54 |
| KC2000 | *hutC515 nac-203*::Tn*5-131* | 54 |
| KC2473 | *hutC515 nac-306*::Tn*5-tac1* | 54 |
| KC2582 | *hutC515 nac-306*::Tn*5-tac1 ntrB45 ntrC5* | 54 |
| Plasmids | | |
| pALTER-1 | $Ap^s$ $Tc^r$ (for site-specific mutagenesis) | 27 |
| pKAS46 | $Ap^r$ $Km^r$, *ori* R6K *rpsL*$^+$ (suicide vector) | 57 |
| pNM481 | $Ap^r$, *'lacZ lacY*$^+$ (for gene fusions) | 42 |
| pRS415 | $Ap^r$, *lacZ*$^+$ *lacY*$^+$ (for operon fusions) | 56 |
| pV16 | $Ap^r$, Φ(*hutU-lacZ*) | 47 |
| pVJS1370 | As pRS415 but Φ(*nasF-lacZ*) {−38/+277} | 30 |
| pVJS1376 | As pRS415 but Φ(*nasF-lacZ*) {−141/+277} | 30 |
| pVJS2009 | As pRS415 but Φ(*nasR-lacZ*) {−471/+910} | This work |
| pVJS2010 | As pRS415 but Φ(*nasR-lacZ*) {−64/+910} | This work |
| pVJS2354 | As pKAS46 but Δ*rhaBS*::*'lacZ lacY*$^+$ | This work |
| pVJS2502 | $Tc^r$, *nasRFED*; ~5.7-kb insert in pALTER-1 (for gene fusions) | 64 |
| pVJS2520 | $Tc^r$, *nasCBA*; ~7.8-kb insert in pALTER-1 | 64 |
| pVJS2558 | $Ap^r$ $Km^r$, Δ(*nasCB*); *Eco*RI-*Kpn*I fragment in pKAS46 | This work |
| pVJS2595 | $Tc^r$, {−469 to +71} of *nasR* in pALTER-1 | This work |
| pVJS2596 | As pVJS2354 but Φ(*nasR-lacZ*) {−71/+71} | This work |
| pVJS2597 | As pVJS2354 but Φ(*nasR-lacZ*) {−469 to +71} | This work |
| pVJS3002 | As pVJS2597 but −24G→A [Φ(*nasR-lacZ*)*6*] | This work |
| pVJS3004 | $Ap^r$, {−469 to +71} of *nasR* in pNM481 [Φ(*nasR'-lacZ*$^+$)*11*] | This work |
| pVJS3006 | As pVJS2354 but Φ(*nasR-lacZ*) {−71 to +362} | This work |
| pVJS3009 | As pVJS2354 but Φ(*nasR-lacZ*) {−14 to +362} | This work |
| pVJS3029 | As pVJS3004 but −29,−28AT→GC [Φ(*nasR'-lacZ*$^+$)*13*] | This work |
| pVJS3030 | As pVJS3004 but +36 to +38ATG→TAA [Φ(*nasR'-lacZ*$^+$)*12*] | This work |
| pVJS3033 | As pVJS2597 but −13,−12GC→AT [Φ(*nasR-lacZ*)*5*] | This work |

(kindly provided by Susan Egan, University of Kansas, Lawrence, Kans.). End sequencing of this and further subclones was used to localize the individual *rha* genes by comparison to the *E. coli rha* sequence (43). Next, an approximately 5-kb *Bam*HI fragment containing *rhaBA-rhaSR-rhaT* was made blunt ended and cloned into *Eco*RI-*Eco*RV-digested, blunt-ended plasmid pKAS46. A *rha*-internal *Eco*RI fragment of approximately 2.1 kb was deleted, removing all of *rhaB* and most of *rhaS*. The two *Bam*HI sites in the pKAS46 backbone were made blunt ended and religated, removing the 1.7-kb *mob* site (derived from plasmid RP4). Finally, a 5.1-kb PCR fragment containing the molecular cloning site, the RNase III-processing site, and the promoterless *lacZY* operon from vector λTXF97 (59) was cloned into the *Eco*RI site, resulting in plasmid pVJS2354.

**Construction of chromosomal Δ*rhaBS*::[Φ(*nasR-lacZ*)] operon fusions.** Derivatives of plasmid pVJS2354 containing different *nasR* upstream regions were constructed by cloning the PCR-generated *nasR* inserts described above. Plasmids were electrotransformed into strain VJSK2216 or derivatives with selection for $Km^r$. Integrants ($Km^r$ $Sm^s$ $Rha^+$) were streaked on MacConkey-rhamnose-streptomycin agar, and segregants ($Km^s$ $Sm^r$ $Rha^-$) were colony purified. The veracity of the resulting Δ*rhaBS*::[Φ(*nasR-lacZ*)] allelic replacements was confirmed by colony PCR analysis with primers RB1 (*rhaB-rhaA* intergenic region) and LZ1 (*lacZ*) as previously described (64).

**Construction of the Δ(*nasCB*) deletion mutant.** Loop deletion mutagenesis and allelic exchange of the Δ(*nasCB*)*139* deletion into the chromosome of *K.*

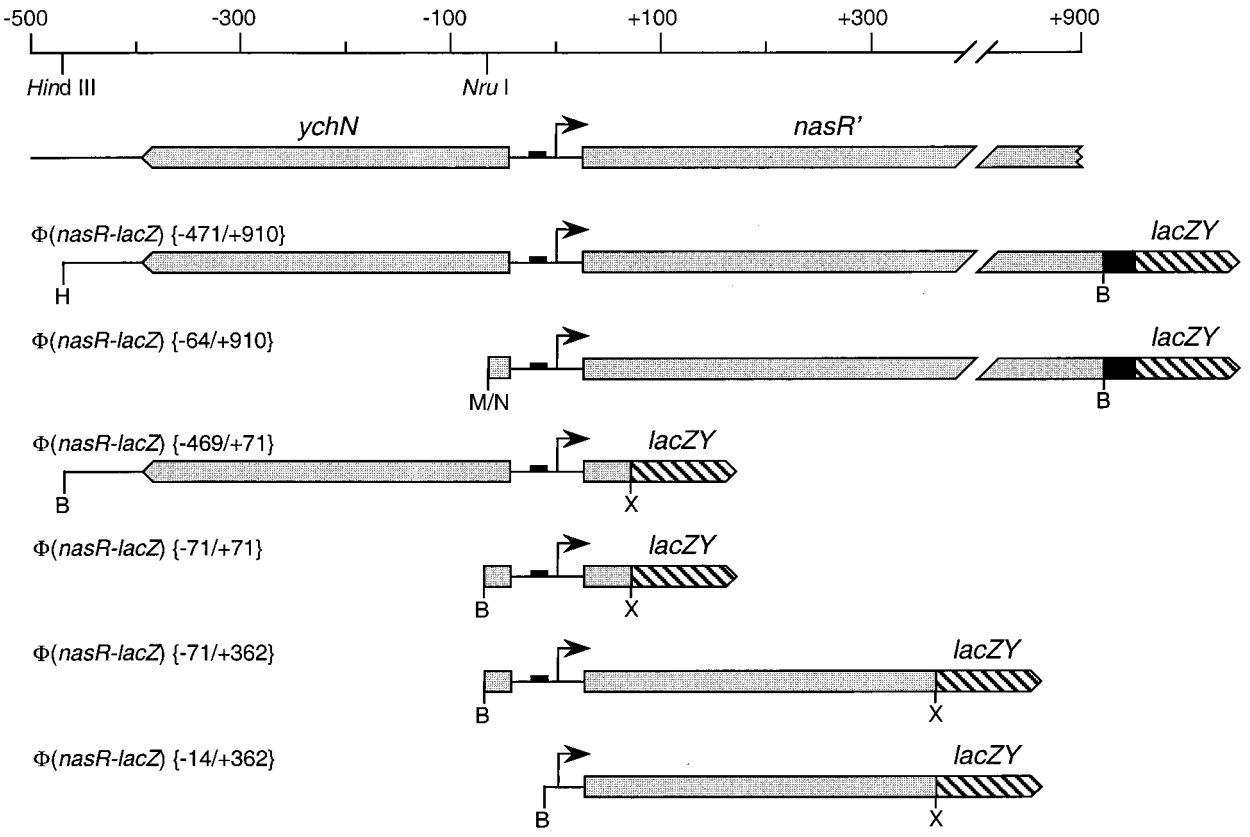


FIG. 1. Φ(*nasR-lacZ*) fusion constructs (drawn to scale). The $\sigma^N$-dependent promoter is indicated by a small solid box, and the transcription initiation site is indicated by an arrow. Upstream and downstream endpoints for each construct are indicated in brackets. The *lacZY* genes are not to scale. Restriction sites: B, *Bam*HI; H, *Hin*dIII; M/N, *Sma*I-*Nru*I fusion; X, *Xba*I.

*oxytoca* were performed essentially as described previously (64). The deletion Δ(*nasCB*)*139* was constructed on plasmid pVJS2520 by adding two oligonucleotides (for Δ*nasC*, deleting codons 95 through 343, and for Δ*nasB*, deleting codons 86 through 713) to a single mutagenesis reaction. The double-deletion Δ*nasC* and Δ*nasB* was isolated and subjected to *Nsi*I reduction to generate Δ(*nasCB*)*139*. All deletions are in frame. Oligonucleotide primers C1 and B2 were used for colony PCR to confirm the veracity of the allelic exchanges as described previously (64).

**Primer extension.** Total RNA was isolated from *K. oxytoca* VJSK2507 by using the RNeasy method (Qiagen Inc.). Cultures were grown in 20 ml of 10 mM MOPS-glutamine medium in a 250-ml sidearm flask. The cultures were harvested in the latter part of the exponential phase (approximately 60 Klett units).

To determine the 5′ end of *nasR* RNA, a 29-bp oligonucleotide complementary to the fusion junction was first end labeled with [γ-$^{32}$P]ATP (Amersham Life Science Inc., Arlington Heights, Ill.) by using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass.) and then annealed to approximately 40 μg of isolated RNA. Primer extension reactions with avian myeloblastosis virus reverse transcriptase (Promega Corp.) were performed by the method of Kingston (25). The DNA sequence ladder was generated by using plasmid pVJS2595 as the DNA template and the same oligonucleotide as primer.

**Nucleotide sequence accession number.** The DNA sequence reported in this paper has been deposited in the GenBank nucleotide sequence database under accession no. L27824.

## RESULTS

**The *nasR* upstream sequence.** We previously reported the sequence of the *nasR* structural gene (19). For this study, the DNA sequence of the *nasR* upstream region was determined on both strands. A 354-bp hypothetical *ychN* gene lies in the opposite orientation to the *nasR* gene (Fig. 2). The *ychN* initiation codon is located 68 bp upstream of the assigned *nasR* initiation codon (see below). The deduced amino acid sequence of the *K. oxytoca* YchN protein is 88% identical to that of the *E. coli* YchN protein (5). The function of the YchN protein is unknown.

A presumptive $\sigma^N$-dependent promoter lies just upstream of the *nasR* initiation codon (Fig. 2). However, potential sites for binding phospho-NtrC protein (half-site consensus TGCAC CA; reviewed in reference 34) are not evident (Fig. 2). Below we present experimental evidence that confirms the identity of this promoter and that also permits us to assign the transcription initiation site to nucleotide position +1 (Fig. 2). All nucleotide coordinates described in this paper are therefore in relation to position +1.

**Analysis of plasmid-borne Φ(*nasR-lacZ*) operon fusions.** Finding the presumptive $\sigma^N$-dependent promoter sequence suggested that *nasR* gene expression is controlled by Ntr. To evaluate this possibility, we constructed the Φ(*nasR-lacZ*) {−471/+910} operon (transcriptional) fusion containing DNA from 471 nt upstream to 910 nt downstream of the transcription initiation site (Fig. 1; see Materials and Methods). We also constructed a derivative containing DNA from only 64 nt upstream to 910 nt downstream, with the goal of determining if phospho-NtrC binding sites lie upstream of position −64. Both plasmids were introduced into *ntr*$^+$, *ntrC* null, and *rpoN* null strains of *K. oxytoca*. Cultures were grown in defined medium supplemented with the nitrogen sources glutamine (nitrogen limiting) or glutamine plus ammonium (nitrogen excess), and assayed for β-galactosidase specific activity.

Expression of the Φ(*nasR-lacZ*) {−471/+910} operon fusion was induced between 5- and 20-fold by nitrogen limitation, and this induction required *ntrC*$^+$ (Table 2) (15). Surprisingly,

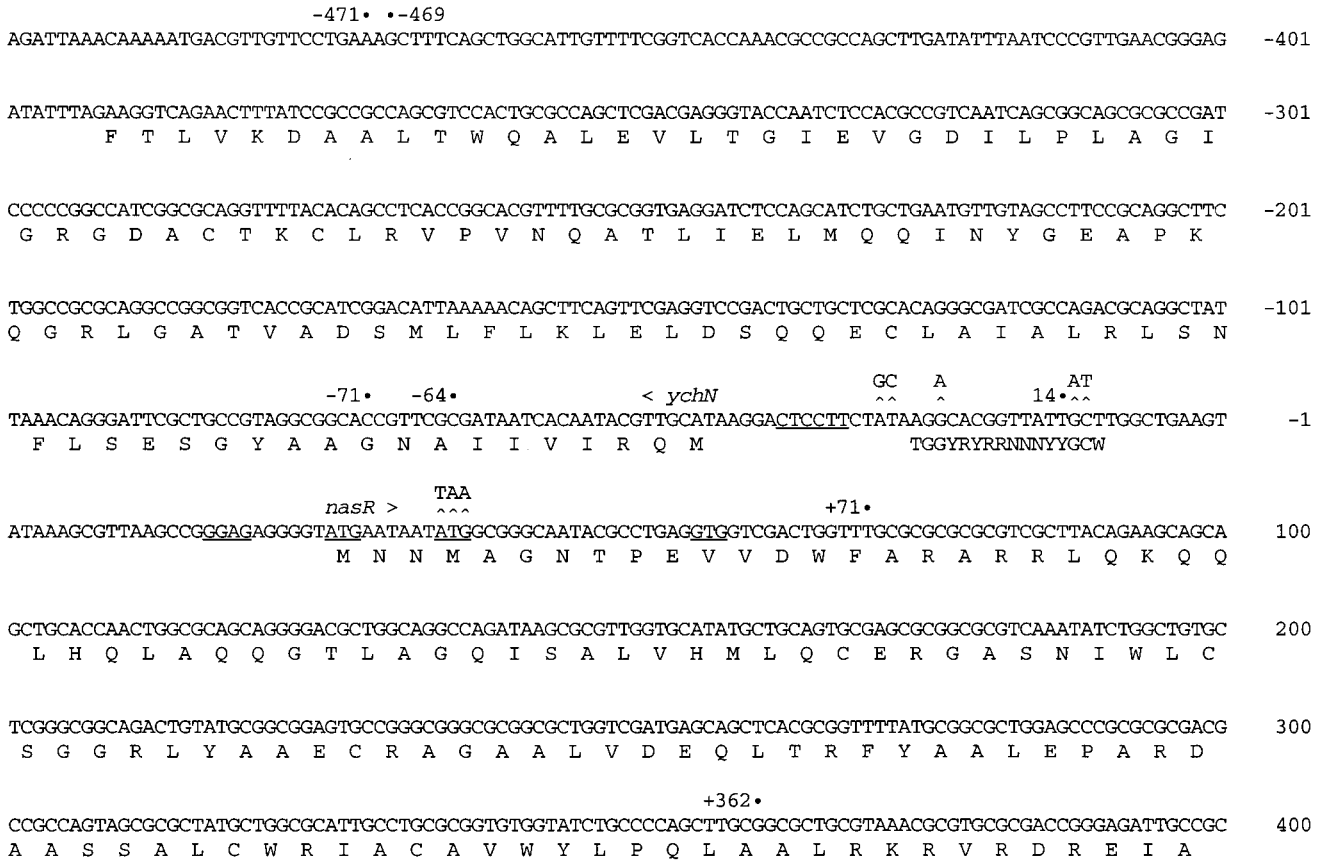


FIG. 2. Nucleotide sequence around the *nasR* control region. Numbering is with respect to the transcription initiation site (+1). A $\sigma^N$ promoter consensus sequence (34) is shown below the *nasR* promoter sequence (R = purine, Y = pyrimidine, and W = A or T). The deletion endpoints of *lacZ* fusion constructs are indicated (see also Fig. 1). Site-specific mutational changes are shown above the sequence. The Shine-Dalgarno regions for the *nasR* and *ychN* genes are underlined. Hypothetical *nasR* initiation codons (encoding Met-1, Met-4, and Val-11) are also underlined; see the text for details. The *ychN* gene is in the opposite orientation from the *nasR* gene. Deduced amino acid sequences are shown in standard single-letter code.

expression of the Φ(*nasR-lacZ*) {−64/+910} fusion was indistinguishable from expression of the Φ(*nasR-lacZ*) {−471/+910} fusion. Expression of both fusions required *ntrC*$^+$ (Table 2) and *rpoN*$^+$ (15), as expected.

As a control, we also measured β-galactosidase specific activity expressed from two previously characterized plasmid-borne Φ(*nasF-lacZ*) operon fusions (30). The Φ(*nasF-lacZ*) {−141/+277} fusion contains the upstream phospho-NtrC binding sites, whereas these sites are deleted from the Φ(*nasF-lacZ*) {−38/+277} fusion. As expected, the construct containing 141 bp upstream of the transcription initiation site directed an approximately 10-fold induction in β-galactosidase activity in response to nitrogen limitation whereas the construct containing only 38 bp upstream directed only a 3-fold increase. Again, induction required both *ntrC*$^+$ and *rpoN*$^+$ (15).

TABLE 2. Expression of plasmid-borne Φ(*nasR-lacZ*) operon fusions

| Plasmid[a] | Genotype[b] | β-Galactosidase sp act[c] with: | | | |
|---|---|---|---|---|---|
| | | *ntrC*$^+$ | | *ntrC*::Tn*7* | |
| | | $NH_4^+$, Gln | Gln | $NH_4^+$, Gln | Gln |
| pVJS2009 | Φ(*nasR-lacZ*) {−471/+910} | 200 | 3,590 | 260 | 420 |
| pVJS2010 | Φ(*nasR-lacZ*) {−64/+910} | 190 | 4,200 | 210 | 550 |

[a] Plasmids in strain VJSK838 (*ntrC*$^+$) or VJSK1603 (*ntrC36*::Tn*7*).
[b] See Fig. 1 for structures of Φ(*nasR-lacZ*) operon fusions.
[c] Determined as described in Materials and Methods and expressed in arbitary (Miller) units. Cultures were aerated in media containing the indicated nitrogen sources as described in Materials and Methods.

These results indicated that *nasR* gene expression is subject to Ntr control and that sequences upstream of position −64 are dispensable for this regulation. However, β-galactosidase activities measured from plasmid-borne fusions exhibited significant differences in independent experiments, perhaps due in part to variations in plasmid copy number. Therefore, we elected to conduct further analysis with chromosomal monocopy Φ(*nasR-lacZ*) operon fusion constructs.

**System for construction of monocopy *lacZ* operon fusions.** Our laboratory routinely uses bacteriophage λ specialized transduction for constructing single-copy *lacZ* operon fusions in *Escherichia coli* (56). However, we have been unsuccessful in our efforts to adapt bacteriophage λ for use with *K. oxytoca* M5al. We therefore elected to follow the strategy of Shimotsu and Henner (55), who used the *amyE* gene of *Bacillus subtilis* as a target for integration, via homologous recombination, of fusion constructs into the chromosome. Two considerations guided our scheme. The first was to use as the target a locus involved in sugar catabolism, so that we could readily screen colonies arising from allelic replacements by virtue of their fermentation-negative phenotype. The second was to embed

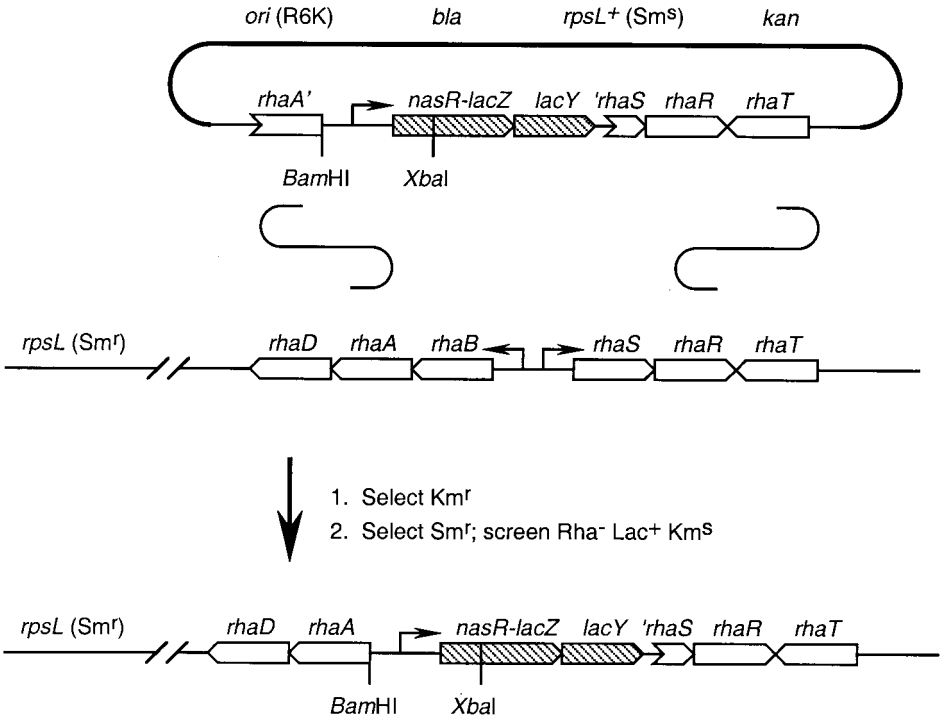


FIG. 3. Strategy for single-copy transplantation of Φ(*nasR-lacZ*) fusion constructs into the *K. oxytoca* chromosome. See Materials and Methods for details. The Φ(*nasR-lacZ*) operon fusion constructs, derivatives of plasmid pVJS2354, span the divergent *rhaB* and *rhaS* genes. Conventional integration-excision allelic exchange (not depicted) results in transfer of the construct to the chromosome, replacing the resident *rha*$^+$ locus.

the fusion constructs between divergently transcribed genes to help insulate them from exogenous promoters (16). The *ara* and *rha* operons of enterobacteria satisfy both of these criteria. *K. oxytoca* M5al is Ara$^-$ Rha$^+$, and so we cloned and analyzed its *rhaBAD-rhaSR-rhaT* locus as described in Materials and Methods.

The suicide integration vector pVJS2354 contains a promoterless *lacZY* operon (59) between the divergent *rhaA* and *rhaR* genes (Fig. 3). It also carries the *rpsL*$^+$ (Sm$^s$) allele, in addition to Km$^r$ and Ap$^r$ markers. In the absence of the *pir* gene encoding the replication protein π, Km$^r$ transformants arise only from integration of the plasmid into the chromosome. The *rha* locus homology ensures that most integrations will occur at *rha* (Fig. 3). Integration by recombination within *rhaR* yields an intact *rhaBAD-rhaSR* locus (Rha$^+$ phenotype), whereas integration by recombination within *rhaA* serves to separate *rhaD* from the upstream *rhaB* promoter (43). Thus, integration within *rhaA* is expected to yield the Rha$^-$ phenotype (Fig. 3). In practice, however, we have observed that virtually all integrants are Rha$^+$.

Segregants lose the integrated plasmid by homologous recombination between the tandemly repeated *rha* sequences. They were selected as Sm$^r$ colonies, because loss of the dominant *rpsL*$^+$ (Sm$^s$) allele unmasked the phenotype conferred by the chromosomal *rpsL* (Sm$^r$) allele (57). Authentic segregants were also Km$^s$ (Fig. 3). The desired strains, in which the Φ(*nasR-lacZ*) construct had replaced the *rhaB* and *rhaS* genes, were thus readily identified as Sm$^r$ Rha$^-$ colonies (see Materials and Methods). Candidates were tested for the Lac$^+$ and Km$^s$ phenotype, and then two independent isolates were subjected to colony PCR analysis to verify the structure of the Δ*rhaBS*::[Φ(*nasR-lacZ*)] allelic replacement as described in Materials and Methods (data not shown).

**Analysis of monocopy Δ*rhaBS*::[Φ(*nasR-lacZ*)] operon fusions.** We constructed four different Δ*rhaBS*::Φ[(*nasR-lacZ*)] operon fusions (Fig. 1), with different amounts of upstream and downstream sequence, in order to localize the *nasR* regulatory elements. We monitored the expression of these fusions by measuring β-galactosidase activities from cultures grown in defined medium with limiting or excess nitrogen. The results are shown in Table 3.

Three Φ(*nasR-lacZ*) constructs (−469/+71, −71/+71, and −71/+362) exhibited indistinguishable patterns of β-galactosidase synthesis: each was induced about 10-fold by nitrogen limitation (Table 3). This delimits the region extending from positions −71 to +71 as containing all necessary information

TABLE 3. Expression of chromosomal Δ*rhaBS*::[Φ(*nasR-lacZ*)] operon fusions

| Strain[a] | Genotype[b] | β-Galactosidase sp act[c] with: | | Ratio of sp act values |
|---|---|---|---|---|
| | | $NH_4^+$, Gln | Gln | |
| VJSK2504 | *lacZ*[d] | 5 | 9 | 1.8 |
| VJSK2507 | Φ(*nasR-lacZ*) {−469/+71} | 7 | 67 | 9.6 |
| VJSK2506 | Φ(*nasR-lacZ*) {−71/+71} | 6 | 65 | 11 |
| VJSK2548 | Φ(*nasR-lacZ*) {−71/+362} | 17 | 150 | 8.8 |
| VJSK2541 | Φ(*nasR-lacZ*) {−14/+362} | 16 | 29 | 1.8 |

[a] Derivatives of strain VJSK2216 (Table 1).
[b] See Fig. 1 for structures of Φ(*nasR-lacZ*) operon fusions.
[c] Determined as described in Materials and Methods and expressed in arbitary (Miller) units. Cultures were aerated in media containing the indicated nitrogen sources as described in Materials and Methods.
[d] Allelic replacement with plasmid pVJS2354 only, with no cloned insert, to monitor background *lacZ* expression.

TABLE 4. Effects of regulatory mutations on expression of a chromosomal Φ(*nasR-lacZ*) operon fusion

| Strain[a] | Genotype[b] | β-Galactosidase sp act[c] with: | | Ratio of sp act values |
|---|---|---|---|---|
| | | $NH_4^+$, Gln | Gln | |
| VJSK2507 | Wild type | 7 | 67 | 9.6 |
| VJSK2520 | *rpoN*::Km | 15 | 22 | 1.5 |
| VJSK2521 | *ntrC*::Tn*5* | 11 | 21 | 1.9 |
| VJSK2588 | −13/−12GC→AT | 13 | 22 | 1.7 |
| VJSK2531 | −24G→A | 8 | 14 | 1.8 |

[a] Derivatives of strain VJSK2216 (Table 1).
[b] All strains carry Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] (Fig. 1).
[c] Determined as described in Materials and Methods and expressed in arbitary (Miller) units. Cultures were aerated in media containing the indicated nitrogen sources as described in Materials and Methods.

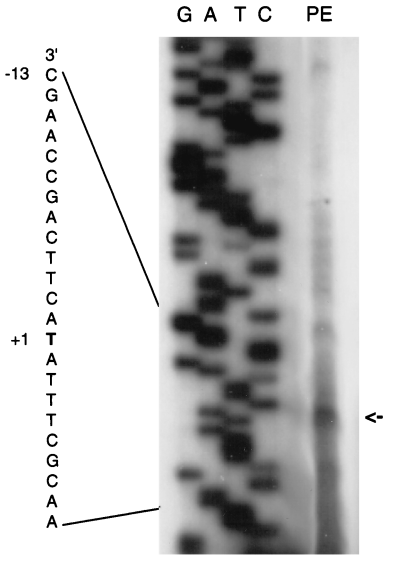


FIG. 4. Primer extension analysis of *nasR* mRNA (see Materials and Methods for details). Total RNA was isolated from *K. oxytoca* VJSK2507. The assigned transcription start site (+1) and part of the $\sigma^N$-dependent promoter sequence (−13) are labeled. The lanes marked G, A, T, and C show the corresponding dideoxynucleotide chain termination sequencing reactions. The lane marked PE shows the primer extension product, indicated by an arrow.

for Ntr-controlled *nasR* expression. The fourth Φ(*nasR-lacZ*) construct (−14/+362) was expressed at a low unregulated level, indicating that the promoter is deleted from this cloned insert, as expected (see also below). Both $ntrC^+$ and $rpoN^+$ were required for Ntr-regulated expression of the monocopy constructs, Φ(*nasR-lacZ*) {−469/+71} (Table 4) and Φ(*nasR-lacZ*) {−71/+71} (data not shown), congruent with results from the analogous plasmid-borne fusions (Table 2).

**Transcription initiation.** To confirm the −24/−12 element as the $\sigma^N$-dependent *nasR* promoter, we constructed two different site-specific mutants: one with G at position −24 changed to A, and one with GC at positions −13 and −12 changed to AT (Fig. 2). We constructed Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] operon fusions bearing these alterations and monitored their expression by measuring β-galactosidase activities from cultures grown in defined medium with limiting or excess nitrogen. The results are shown in Table 4. The changes at −24 and at −13/−12 both virtually abolished expression. These results establish the −24/−12 sequence identified by sequence inspection as the *nasR* promoter.

We sought to evaluate this assignment independently by using primer extension to determine the 5′ end of *nasR* mRNA. We isolated total RNA from strain VJSK2507, which contains the Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] operon fusion. Cultures were grown aerobically in defined medium with limiting nitrogen. The products of reverse transcriptase extension from a primer complementary to sequence spanning the *nasR-lacZ* fusion junction were resolved by gel electrophoresis and visualized by radioautography (Fig. 4). The significant background resulted from the overexposure required to detect the signal from a single copy of a weakly expressed gene. Nevertheless, the predominant band corresponded to a 92-bp cDNA (Fig. 4). This band aligns with a T in the sequence ladder that corresponds to the A residue at position +1 in the *nasR* coding strand, appropriately spaced from the −24/−12 promoter (Fig. 2; see above).

**Nac protein is not required for *nasR* gene expression.** Work with *K. aerogenes* has demonstrated that expression of certain nitrogen-regulated operons, such as *hut* and *put*, requires the *nac* gene product in addition to a functional Ntr system (reviewed in reference 3). The *nac* gene, which is under positive regulation by Ntr, encodes a LysR family regulator that couples Ntr regulation to target $\sigma^{70}$-dependent operons. Although the results summarized above establish that the *nasR* gene is expressed from a $\sigma^N$-dependent promoter, the absence of essential regulatory sites upstream of position −71 (i.e., an enhancer) directed us to evaluate an alternative hypothesis, namely, that the Nac protein mediates Ntr control of *nasR* gene expression.

We do not have a *nac* mutant of *K. oxytoca* M5al, and so we used strains of the close relative *K. aerogenes* W70. The *K. aerogenes nasF* operon control region is very similar to that of *K. oxytoca* (9), and a *K. oxytoca* Φ(*nasC-lacZ*) operon fusion is expressed normally in *K. aerogenes* (19), demonstrating that *K. aerogenes* contains $nasR^+$. Thus, *K. aerogenes* provides an appropriate surrogate strain for examining the Nac dependence of *K. oxytoca nasR* gene expression.

Plasmid pVJS3004, carrying the Φ(*nasR-lacZ*) {−469/+71} gene (translational) fusion, was used to monitor *nasR* gene expression. Plasmid pV16, which contains a Φ(*hutU-lacZ*) operon fusion, provided a control for Nac function (47). We monitored the expression of these fusions by measuring β-galactosidase activities from cultures grown in defined medium with limiting or excess nitrogen. The results are shown in Table 5.

In the $nac^+$ *K. aerogenes* strain KC1043, both *nasR* and *hutU* gene expression were strongly induced by nitrogen limitation (Table 5). In the *nac* null strain KC2000 grown with limiting nitrogen, *nasR* gene expression was slightly increased whereas *hutU* expression was significantly decreased as expected. This result demonstrates that the Nac protein is not essential for activating *nasR* gene expression. Further analysis was performed with a *nac*::Tn*5 tac* strain, in which *nac* gene expression is controlled by the IPTG-inducible *tac* promoter (54). In this strain cultured with IPTG, Φ(*hutU-lacZ*) expression was indifferent to the nitrogen source whereas Φ(*nasR-lacZ*) expression remained nitrogen responsive (Table 5). Finally, an *ntrC* null allele abolished Φ(*nasR-lacZ*) expression in the *nac*::Tn*5 tac* strain, whereas Φ(*hutU-lacZ*) expression was little affected. These results establish that nitrogen-regulated *nasR* gene expression is independent of the Nac protein.

Mutational analysis indicates that the sequence ATA-$N_9$-TAT, centered at −64, is an important element of Nac DNA binding sites (10, 20, 47). That sequence is also present in the

TABLE 5. Effects of *nac* on expression of plasmid-borne Φ(*nasR-lacZ*) and Φ(*hutU-lacZ*) operon fusions

| Genotype[a] | Fusion[b] | β-Galactosidase sp act[c] with: | | | |
|---|---|---|---|---|---|
| | | No IPTG | | IPTG added | |
| | | $NH_4^+$, Gln | Gln | $NH_4^+$, Gln | Gln |
| *nac*$^+$ | *nasR* | <1 | 230 | —[d] | — |
| | *nasR* −29/−28AT→GC | 4 | 290 | — | — |
| | *hutU* | 260 | 9,510 | — | — |
| *nac*::Tn*5* | *nasR* | <1 | 670 | — | — |
| | *nasR* −29/−28AT→GC | <1 | 460 | — | — |
| | *hutU* | 240 | 1,340 | — | — |
| *tac-nac*$^+$ | *nasR* | <1 | 230 | 8 | 140 |
| | *nasR* −29/−28AT→GC | <1 | 300 | 9 | 150 |
| | *hutU* | 340 | 3,270 | 6,830 | 6,230 |
| *tac-nac*$^+$ *ntrC* | *nasR* | <1 | <1 | 6 | 3 |
| | *nasR* −29/−28AT→GC | <1 | <1 | 8 | 9 |
| | *hutU* | 460 | 1,090 | 3,610 | 3,250 |

[a] *K. aerogenes* W70 strains (Table 1).
[b] Φ(*nasR-lacZ*) {−469/+71} gene fusion on plasmids pVJS3004 (wild type) and pVJS3029 (−29/−28AT→G), and Φ(*hutU-lacZ*) operon fusion on plasmid pV16 (Table 1).
[c] Determined as described in Materials and Methods and expressed in arbitary (Miller) units. Cultures were aerated in media containing the indicated nitrogen sources as described in Materials and Methods.
[d] —, not determined.

TABLE 6. Effects of nitrate and nitrite on expression of a chromosomal Φ(*nasR-lacZ*) operon fusion

| Strain[a] | Genotype[b] | β-Galactosidase sp act[c] with: | | | |
|---|---|---|---|---|---|
| | | Gln | $NO_3^-$, Gln | $NO_2^-$, Gln | $NH_4^+$, Gln |
| VJSK2507 | *nas*$^+$ | 67 | 22 | 28 | 7 |
| VJSK2517 | *nasR131*::Tn*10*d(Cm) | 77 | 140 | 96 | 13 |
| VJSK2512 | Δ(*nasFED*)*135* | 70 | 140 | 32 | 8 |
| VJSK2516 | Δ(*nasCB*)*139* | 61 | 135 | 106 | 5 |
| VJSK2529 | *nasD124*::Ω-Cm | 67 | 150 | 101 | 6 |

[a] Derivatives of strain VJSK2216 (Table 1).
[b] All strains carry Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] (Fig. 1).
[c] Determined as described in Materials and Methods and expressed in arbitary (Miller) units. Cultures were aerated in media containing the indicated nitrogen sources as described in Materials and Methods.

*nasR* control region, centered at −22 and thereby overlapping the promoter sequence (Fig. 2). We therefore constructed a site-specific mutant changing AT at positions −29 and −28 to GC, thereby significantly damaging the upstream ATA element while leaving the promoter elements intact (Fig. 2). However, this double change had no discernible effect on Φ(*nasR-lacZ*) expression (Table 5). This result reinforces the above conclusion, i.e., that *nasR* gene expression is Nac independent.

**Translation initiation.** Previous sequence analysis revealed the presumed *nasR* translation initiation region, as indicated in Fig. 2, where the presumed Shine-Dalgarno region and two potential initiation codons (encoding Met-1 and Met-4, as shown) are underlined (19). However, we had no direct evidence to show that *nasR* translation initiates at one of these ATG codons rather than further downstream (for example, the underlined GTG codon for Val-11). We wished to more precisely localize the start of *nasR* translation, in order to provide a context for evaluating transcriptional regulatory signals.

To determine if either ATG codon serves in initiating translation, we first constructed a plasmid-borne Φ(*nasR′-′lacZ*) {−469/+71} gene (translational) fusion (Fig. 1; see Materials and Methods), in which the synthesis of the active NasR-LacZ fusion protein is directed by *nasR* translation initiation signals. The cloned insert results in the in-frame fusion of the *nasR* codon for Phe-15 to *lacZ* codon 9. (Ten additional codons derived from polylinker sequence were introduced at the fusion junction.) We also constructed a site-specific mutant in which the second ATG codon, at nucleotides 36 to 38, was changed to ochre (TAA; Fig. 2). Both plasmids were introduced into *K. oxytoca*, and β-galactosidase specific activity was measured after growth in defined medium with limiting nitrogen. Whereas the wild-type fusion expressed about 230 Miller units, activity from the ochre fusion was negligible (about 1.5 Miller units).

The high activity expressed from the wild-type plasmid establishes the *nasR* translation initiation site as being located upstream of nucleotides 69 to 71 (codon 15). The very low activity expressed from the ochre (nt 36 to 38) mutant shows that translation initiates either at this second ATG or further upstream. Based on spacing from the Shine-Dalgarno sequence, we assign the first ATG (+27 to +29) as the likely *nasR* initiation codon. Irrespective of which ATG is the initiator, the short untranslated leader region seemingly presents an insufficient target for either antitermination or translational autoregulation by NasR protein.

**Effects of nitrate and nitrite on *nasR* gene expression.** The NasR protein is a nitrate- and nitrite-responsive regulator of *nasF* operon transcription antitermination. To determine whether *nasR* gene expression is autoregulated, we examined the effects of nitrate and nitrite on expression of the Δ*rhaBS*::[Φ(*nasR-lacZ*) {−469/+71}] operon fusion. Indeed, *nasR* gene expression was decreased by about threefold during growth with nitrate and nitrite (Table 6). A *nasR* null allele abolished this nitrate- and nitrite-dependent inhibition while having no effect on Ntr-dependent ammonium inhibition (Table 6). On their face, these results could suggest that the NasR protein is a weak negative regulator of *nasR* gene expression.

An alternative hypothesis is that the decreased *nasR* gene expression was due to assimilation of nitrate and nitrite into ammonium, thereby diminishing the magnitude of Ntr activation. To test this idea, we introduced mutational alterations that block various steps of nitrate assimilation. The Δ(*nasFED*) deletion eliminates the nitrate and nitrite uptake system, without affecting the expression of *nasCBA* genes encoding assimilatory nitrate and nitrite reductases (64). The strain carrying this deletion exhibited nitrate-insensitive *nasR* gene expression, although nitrite inhibition was unaffected (Table 6). In fact, nitrite is efficiently transported by a second, uncharacterized pathway in *nasFED* mutants (under the growth conditions used [64]), and so this result indicates that nitrate must be transported to exert its inhibitory effect on *nasR* gene expression.

The Δ(*nasCB*) deletion eliminates assimilatory nitrate and nitrite reductase activities while leaving the *nasFED*-encoded transport system intact. The strain carrying this deletion exhibited nitrate- and nitrite-insensitive *nasR* gene expression (Table 6), indicating that nitrate and nitrite must be converted to ammonium to exert their inhibitory effect on *nasR* gene expression. Finally, the *nasD*::Ω insertion eliminates both nitrate transport (through inactivation of the *nasD* gene [64]) as well as assimilatory nitrate and nitrite reductase activities (through strong polarity on *nasCBA* expression [29]). Again, nitrate- and nitrite-dependent inhibition of *nasR* gene expression was abol-

ished in this strain. Together, these results indicate not that NasR is a negative regulator of *nasR* gene expression but, rather, that nitrate and nitrite conversion to ammonium influences Ntr regulation sufficiently to measurably decrease *nasR* gene expression. [We do not understand why nitrate caused an approximately twofold increase in Φ(*nasR-lacZ*) expression in the various *nas* mutants (Table 6).]

## DISCUSSION

*K. oxytoca* (*pneumoniae*) M5al is a genetically amenable enterobacterium that has been widely used for studies of dinitrogen fixation and Ntr (60). Previous studies have shown that *nasF* (nitrate assimilation) operon expression is subject to dual regulation: phospho-NtrC-dependent activation of a $\sigma^N$-dependent promoter, and NasR-dependent nitrate- and nitrite-responsive transcription antitermination (8, 9, 30). We report here our analysis of *nasR* gene regulation. We found that *nasR* gene expression was modulated about 10-fold by Ntr. This regulation required a $\sigma^N$-dependent promoter and the *ntrC*$^+$ gene and was independent of the *nac*$^+$ gene. However, deletion of upstream sequences had no discernible effect on *ntrC*$^+$-dependent stimulation of Φ(*nasR-lacZ*) expression. Thus, like the *S. typhimurium argT* promoter (2, 52), the *nasR* promoter may not require an upstream binding site for full-level NtrC activation.

**The *nasR* promoter.** Sequence inspection identified the critical elements of a $\sigma^N$-dependent promoter — GG and GC — upstream of the *nasR* coding region (Fig. 2). Site-specific changes at −24 and at −12 plus −13 both virtually abolished Φ(*nasR-lacZ*) expression, as did introduction of an *rpoN* null allele (Table 4; *rpoN* encodes $\sigma^N$). A deletion to −14 likewise eliminated Φ(*nasR-lacZ*) expression (Table 3), demonstrating that no other promoters lie between −14 and +362 (Fig. 1). Primer extension analysis provided independent support for these conclusions (Fig. 4). Therefore, *nasR* gene expression is controlled by a single $\sigma^N$-dependent promoter.

Although the −24 GG and −12 GC elements are critical for $\sigma^N$-dependent promoter function, nucleotides surrounding the −12 sequence also contribute to promoter function and activation (reviewed in references 34 and 36). One example comes from study of the NifA-activated *K. oxytoca nifH* promoter. Transcription from a mutant promoter, changed from the wild-type CCCTGC to TTTTGC, is much less dependent on NifA binding to the UAS, perhaps due to increased affinity for $\sigma^N$ (6, 7). Note that the corresponding region of the *nasR* promoter, TATTGC, is also T rich (Fig. 2). Nevertheless, it is not yet possible to predict reliably the $\sigma^N$-dependent promoter function from sequence inspection alone (62).

**The Nac protein does not control *nasR* expression.** One mechanism for controlling nitrogen-regulated gene expression involves the Nac protein, which is known to activate only $\sigma^{70}$-dependent promoters (3). However, Φ(*nasR-lacZ*) expression was indifferent to *nac*$^+$ in *K. aerogenes* W70 (Table 5). Site-specific mutational analysis further demonstrated that a sequence (ATA-$N_9$-TAT [Fig. 2]) that might be construed as a Nac protein binding site (10, 20, 47) was irrelevant for *nasR* gene control (Table 5). Therefore, Ntr control of *nasR* gene expression does not involve the Nac protein.

**How does phospho-NtrC protein activate *nasR* gene expression?** The only other known mechanism for nitrogen-regulated gene expression in enterobacteria involves phospho-NtrC protein, which activates transcription initiation at $\sigma^N$-dependent promoters (reviewed in references 34, 38, 44, and 48). For most of these, including the well-studied *glnA*, *nifL*, and *glnH* promoters, activation is stimulated by phospho-NtrC binding to upstream sites, the proximal boundaries of which are located 70 to 100 bp upstream of the −24 GG element.

Characterized NtrC binding sites consist of 17-bp sequences, containing an inverted pair of 7-bp half-sites, that are similar to the consensus sequence TGCACCA-$N_3$-TGGTGCA (reviewed in reference 34). The *glnA*, *nifL*, and *glnH* upstream control regions each contain at least two such 17-mers, and only one of the two *nifL* 17-mers has as many as 6 of 14 mismatches from this consensus. Inspection of the *nasR* upstream region to position −300 failed to reveal even a single 17-mer with fewer than 7 of 14 mismatches (Fig. 2). Furthermore, deletions to positions −64 and −71 had no influence on Φ(*nasR-lacZ*) expression (Tables 2 and 3). Thus, any upstream NtrC binding site must have its distal boundary no more than 39 bp upstream of the −24 GG element (Fig. 2); the proximal boundary of such a 17-mer would be at position −47. Such a location is unprecedented. Additionally, any NtrC binding sites in this region would overlap with the beginning of the *ychN* gene (Fig. 2), which, unlike the *nasRFEDCBA* region, is conserved in *E. coli* K-12 (5). Finally, any downstream NtrC binding site must have its distal boundary upstream of +71. Nonetheless, *nasR* gene expression was fully dependent upon *ntrC*$^+$, even in fusion constructs with only 64 or 71 nt upstream of the transcription initiation site (Table 2 and data not shown). (The residual twofold nitrogen regulation in *ntrC* null strains is an unexplained peculiarity of *Klebsiella* spp. [32].)

These observations mimic those made by Schmitz et al., who found that Ntr control of *argT* gene expression operates through a $\sigma^N$-dependent promoter (52). Further analysis demonstrated that sequences within 44 bp of the transcription initiation site are sufficient to confer essentially wild-type Ntr control — about fivefold — on expression of Φ(*argT-galK*) operon fusions. Furthermore, NtrC protein failed to protect *argT* control region DNA from DNase I digestion under conditions where the *glnA*, *ntrBC*, and *dhuA* NtrC binding sites were well protected (2). Together, these results indicate that *argT* gene expression is independent of upstream or downstream NtrC binding sites.

Although stimulatory, upstream binding sites are not essential for phospho-NtrC activation of the *glnA* or *nifLA* promoters (53). Expression of single- or low-copy Φ(*glnA-lacZ*) operon fusion constructs during nitrogen-limited growth is reduced by 10-fold or less upon deletion of the upstream binding sites, whereas introduction of an *ntrC* null allele reduces expression by about 100-fold (12, 50). Likewise, Φ(*nifL-lacZ*) expression is reduced but not eliminated upon deletion of the upstream binding sites (14, 40). Thus, significant phospho-NtrC-dependent transcription activation can occur even without specific DNA binding sites (46).

Schneider et al. suggested that "minimal" Ntr-controlled promoters with no NtrC binding sites (such as the *argT* promoter) may allow differentially regulated gene expression, perhaps in response to different nitrogen sources (53). Presumably, phospho-NtrC activation of these promoters results from nonspecific binding to DNA (46). It would be tempting to speculate that the nucleotide sequences of these minimal promoters provide sufficient activation in the absence of activator binding sites (see above). However, the *S. typhimurium argT* and *K. oxytoca nasR* promoters exhibit little sequence similarity aside from the conserved −24 and −12 elements. Evaluating this and other ideas will require further investigation.

**Ntr control of nitrate assimilation.** Manifestation of the nitrate assimilation phenotype involves at least two targets for Ntr control (Fig. 5). The first target is expression of the *nasF* structural gene operon, which is controlled by phospho-NtrC binding to a conventional upstream sequence (30). The second

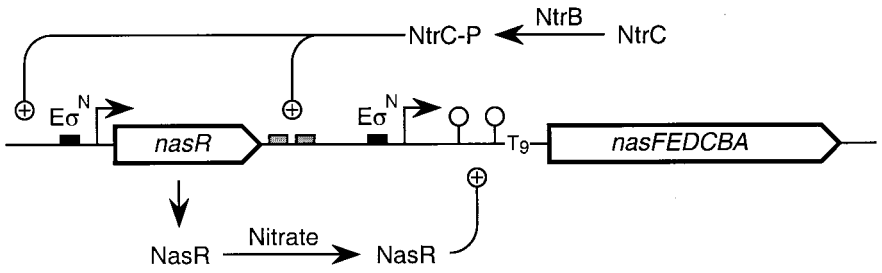


FIG. 5. Control of nitrate assimilation gene expression (not to scale). The promoters for the *nasR* gene and the *nasF* operon, indicated by solid rectangles, are recognized by $\sigma^N$-RNA polymerase ($E\sigma^N$). Transcription initiation sites are indicated by arrows. Phospho-NtrC protein binding sites upstream of the *nasF* operon are indicated by shaded rectangles. NtrC is phosphorylated by NtrB protein in response to nitrogen limitation. The *nasF* operon leader region contains two RNA stem-loop structures, the distal one of which is a factor-independent terminator that includes a polythymidine tract ($T_9$). NasR protein binds to leader RNA to effect transcription antitermination.

target is expression of the *nasR* regulatory gene, also apparently controlled by phospho-NtrC albeit in an unconventional manner. Thus, growth under nitrogen-sufficient conditions serves to dampen *nasF* operon expression both directly (by decreasing Ntr activation of the *nasF* promoter) and indirectly (by decreasing synthesis of the NasR regulatory protein) (Fig. 5).

Why should *nasR* gene expression be controlled by Ntr? One idea is that elevation of NasR protein levels during nitrogen-limited growth serves to sensitize the response of the organism to even relatively low levels of nitrate, which is a preferred alternative nitrogen source. A second idea derives from the notion that NasR protein can effect a significant level of transcription antitermination in the *nasF* operon leader region even in the absence of nitrate or nitrite (9). For example, introduction of a *nasR* null allele results in a fivefold decrease of *nasF* operon expression during nitrogen-limited growth in the absence of nitrate or nitrite (19). Thus, Ntr control of NasR synthesis might provide an additional means of further reducing the basal level of *nasF* operon expression.

The control of nitrate assimilation (Fig. 5) shares some general features with control of dinitrogen fixation in *K. oxytoca* M5al. In both cases, expression of pathway-specific regulatory genes (*nasR* and *nifLA*, respectively) is directly controlled by Ntr. The activities of these regulatory proteins are themselves controlled by physiological cues (nitrate and nitrite for NasR; oxygen and fixed nitrogen for NifL-NifA). However, the correspondence is inexact. Expression of the *nif* structural genes is directly activated by the NifA protein and is not known to be regulated by phospho-NtrC under ordinary physiological conditions (reviewed in reference 13). By contrast, expression of the *nasF* structural gene operon appears to be subject directly to dual control by both NasR and phospho-NtrC (Fig. 5).

**Effect of nitrate and nitrite on *nasR* gene expression.** Both nitrate and nitrite decreased Φ(*nasR-lacZ*) expression only in *nasR*$^+$ strains (Table 6). However, this diminution required both the transport and reduction of nitrate and nitrite. Thus, the observed inhibition of Φ(*nasR-lacZ*) expression is due to decreased Ntr activation in response to formation of ammonium from nitrate. Furthermore, the short distance (26 bp) between the transcription initiation site and the probable *nasR* initiation codon is insufficient to contain a regulated transcription terminator, which is the target of NasR action in the *nasF* operon. Thus, we conclude that *nasR* gene expression is not subject to transcriptional autoregulation.

## ACKNOWLEDGMENTS

Catherine Dunn (University of California, Davis) performed experiments to confirm and extend the results summarized in Table 2. Robert Bender, Alexander Ninfa, and an anonymous reviewer provided invaluable constructive critique of an early version of the manuscript. We are obliged to Mike Merrick for sharing many useful *K. oxytoca* M5al strains. We thank Karen Skorupski for providing her allelic exchange vector in advance of publication, Thomas Linn for the λTXF97 transcriptional fusion vector, Brian Janes and Robert Bender for the *K. aerogenes nac* strains, and Susan Egan for *E. coli rha* mutants. Automated DNA sequence analyses were performed by the Cornell University Biotechnology Program central services group.

This study was supported by U.S. Department of Energy grant 91ER20027 from the Division of Energy Biosciences and by Hatch project CA-D*-MIC-6572-H from the California Agricultural Experiment Station.

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