JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 5077–5085
0021-9193/94/$04.00+0

Vol. 176, No. 16

Identification and Structure of the *nasR* Gene Encoding a Nitrate- and Nitrite-Responsive Positive Regulator of *nasFEDCBA* (Nitrate Assimilation) Operon Expression in *Klebsiella pneumoniae* M5al

BARRY S. GOLDMAN,[1]† JANINE T. LIN,[1] AND VALLEY STEWART[1,2]*

Sections of Microbiology[1] and Genetics & Development,[2] Cornell University, Ithaca, New York 14853-8101

Received 2 February 1994/Accepted 8 June 1994

***Klebsiella pneumoniae* can use nitrate and nitrite as sole nitrogen sources through the nitrate assimilatory pathway. The structural genes for assimilatory nitrate and nitrite reductases together with genes necessary for nitrate transport form an operon, *nasFEDCBA*. Expression of the *nasF* operon is regulated both by general nitrogen control and also by nitrate or nitrite induction. We have identified a gene, *nasR*, that is necessary for nitrate and nitrite induction. The *nasR* gene, located immediately upstream of the *nasFEDCBA* operon, encodes a 44-kDa protein. The NasR protein shares carboxyl-terminal sequence similarity with the AmiR protein of *Pseudomonas aeruginosa*, the positive regulator of *amiE* (aliphatic amidase) gene expression. In addition, we present evidence that the *nasF* operon is not autogenously regulated.**

Klebsiella pneumoniae, a member of the family *Enterobacteriaceae*, can use both nitrate (NO_3^-) and nitrite (NO_2^-) as sole nitrogen sources. Nitrate is converted to nitrite by assimilatory nitrate reductase, and nitrite is further reduced to ammonium by assimilatory nitrite reductase. Ammonium is then incorporated into central metabolism by the action of glutamine synthetase and glutamate synthase (40). Assimilatory nitrate and nitrite reductases require molybdenum cofactor and siroheme, respectively, for activity.

The genetics of nitrate assimilation has been extensively studied in fungi and plants (reviewed in references 12 and 36). By contrast, genetic analysis of nitrate assimilation in procaryotes has only recently begun in earnest. Mutants specifically defective in nitrate assimilation have been isolated in *Pseudomonas aeruginosa* (23), *K. pneumoniae* M5al (9), *K. aerogenes* W70 (4), *Synechococcus* sp. strain PCC 7942 (formerly *Anacystis nidulans* R2) (31, 42), and *Azotobacter vinelandii* (44). In *K. pneumoniae* the structural genes for nitrate assimilation form an operon, *nasFEDCBA* (27, 28). The *nasFED* genes apparently encode a nitrate transport system similar to that characterized in *Synechococcus* spp. (42). The *nasC* and *nasA* genes encode two subunits of assimilatory nitrate reductase, and the *nasB* gene encodes assimilatory nitrite reductase.

Synthesis of assimilatory nitrate reductase in *Klebsiella* spp. is inhibited during growth in ammonium-containing medium (52). Enzyme formation during ammonium-limited growth requires the general nitrogen control (Ntr) system (9, 32). Ntr regulation has been extensively studied in enteric bacteria (reviewed in references 34, 37, and 41). This regulation requires the products of several genes, including *ntrA* (also termed *rpoN*), *ntrB*, and *ntrC*. The NtrA protein is an alternate sigma factor (σ^N; also termed σ^{54}) that directs core RNA polymerase transcription initiation at −12/−24 promoters (reviewed in reference 38). Transcription initiation at nitrogen-regulated σ^N promoters requires the *ntrC* gene product. The NtrC protein is itself regulated by the NtrB protein, which acts to phosphorylate the NtrC protein when the availability of nitrogen is limiting. The phosphorylated NtrC protein activates transcription.

The expression of some Ntr-controlled operons in *Klebsiella* spp. is not directly controlled by the NtrC and σ^N proteins. Rather, expression of these operons is activated by the Nac protein (reviewed in reference 3). The *nac* gene itself is under the control of an NtrC-regulated σ^N promoter (32). Thus, Ntr-controlled genes fall into two categories: those that are directly activated by the NtrC and σ^N proteins (e.g., *glnA* and *nifL*) and those that are directly activated by the Nac protein (e.g., *hut* and *put*). Assimilatory nitrate reductase formation is apparently independent of the Nac protein in *K. aerogenes* W70 (32), suggesting that *nasF* operon expression is under the direct control of the NtrC and σ^N proteins.

Synthesis of assimilatory nitrate and nitrite reductases in *Klebsiella* spp. is subject to a second, pathway-specific level of regulation: induction by nitrate or nitrite (4, 9, 50). Thus, *nasF* operon expression is unique in that pathway-specific regulation (nitrate induction) is superimposed upon NtrC-mediated Ntr control.

We report here our isolation of transposon insertion mutations in which the *nasF* operon is no longer induced by nitrate or nitrite but is still under Ntr control. These mutations define the *nasR* gene, which is located in an ~2-kb region upstream of the *nasF* operon in *K. pneumoniae*. Insertions in the *nasR* gene eliminated nitrate and nitrite induction of *nasF* operon expression. The *nasR* gene encodes a predicted protein of approximately 44 kDa, and a protein of this mass was synthesized in *Escherichia coli* strains carrying *nasR*$^+$-containing plasmids. The NasR protein shares carboxyl-terminal sequence similarity with the *P. aeruginosa* AmiR protein, which is a positive regulatory element for aliphatic amidase (*amiE*) gene expres-

* Corresponding author. Mailing address: Section of Microbiology, Cornell University, Wing Hall, Ithaca, NY 14853-8101. Phone: (607) 255-2416. Fax: (607) 255-3904. Electronic mail address: vjs2@cornell.edu.

† Present address: Department of Biology, Washington University, St. Louis, MO 63130-4849.

TABLE 1. Strains and Plasmids

Strain or plasmid	Genotype	Source or reference
K. pneumoniae		
VJSK009	*hsdR1* Gal^s	9
VJSK014	*hsdR1 Δlac-2001* Gal^r Tn*7*	9
VJSK017	As VJSK014 but *moa-106*::MudJ (Lac$^-$)	19
VJSK051	As VJSK014 but *nasD111*::MudJ	9
VJSK056	As VJSK014 but *nasB116*::MudJ	9
VJSK092	*hsdR1 ntrB4*	9
VJSK581	As VJSK092 but *nasD111*::MudJ	Laboratory collection
VJSK582	As VJSK581 but *lacZ*::Tn*10*d(Tc)	Laboratory collection
VJSK587	As VJSK009 but *nasR126*::Tn*5*d(Sp)	This work
VJSK1188	As VJSK009 but *nasR130*::Tn*10*d(Cm)	This work
VJSK1190	As VJSK009 but *nasR131*::Tn*10*d(Cm)	This work
VJSK1198	As VJSK009 but *nasR129*::Ω-Sp	This work
VJSK1311	As VJSK009 but *zxx-103*::*cat*	This work
E. coli K-12		
RK4353	*araD139* Δ(*argF-lac*)*U169 deoC1 flhD5301 gyrA219 non-9 ptsF25 relA1 rpsL150*	27
S17-1 λ*pir*	*hsdR supE44 endA thi pro recA* RP4 2-Tc::Mu-Km::Tn*7* λ*pir*	V. de Lorenzo
VJS482	Δ(*argF-lac*)*U169 gal hsdR metB1 recA56 supE44 supF58 trpR*	27
VJS2236	As VJS482 but pGP1-2	27
Plasmids		
pACYC184	Cm^r Tc^r; *ori* P15A	11
pCAT19	Ap^r Cm^r	18
pGEM-7Zf(+)	Ap^r; T7 ϕ10 promoter	Promega
pGP1-2	Km^r; T7 gene *1* (RNA polymerase); *ori* P15A	51
pHG329	Ap^r; Δ(*lacZ*)*M15*; pUC9 polylinker	48
pJP5603	Km^r; *ori* R6K; *mob* RP4 (suicide vector)	43
pRS415	Ap^r; *lacZYA* (operon fusion vector)	47
pUT-Sm/Sp	Ap^r Sp^r; *ori* R6K; *mob* RP4 (suicide vector)	14
pVJS1340	Ap^r; Φ(*nasC-lacZ*) in *Bam*HI site of pRS415	This work
pVJS1344	Cm^r; *nasR nasFEDCBA narXL*; ~17 kb in *Hin*dIII site of pACYC184	This work
pVJS1367	Ap^r; Φ(*nasF-lacZ*) in *Bam*HI site of pRS415	This work
pVJS1405	Km^r; *nasR*; ~1.7 kb in *Hin*dIII and *Sac*I sites of pJP5603	This work
pVJS1411	Ap^r; *nasR*; ~7.8 kb in *Eco*RI and *Bam*HI sites of pHG329	This work
pVJS1417	Ap^r; *nasR*; ~1.7 kb in *Hin*dIII and *Sac*I sites of pGEM-7Zf(+)	This work
pVJS1429	As pVJS1417 but −1 frameshift in *nasR* (*Pst*I site)	This work
pVJS1430	As pVJS1417 but +1 frameshift in *nasR* (*Sal*I site)	This work

sion (30). Evidence suggests that the AmiR protein acts as a transcription antiterminator (16).

MATERIALS AND METHODS

Strains. Strains and plasmids are listed in Table 1. MudJ (Mu dI1734) and MudK (Mu dII1734) are bacteriophage transposons which encode kanamycin resistance (Km^r). Transposons MudJ and MudK are used for isolating *lacZ* operon and gene fusions, respectively (10). The Tn*5*d(Sp) and Tn*10*d(Cm) elements are transposition-deficient derivatives of transposons Tn*5* and Tn*10* (14, 25). The *nasD111*::MudJ, *nasB116*::MudJ, and *moa-106*::MudJ insertions were previously isolated and characterized (9, 19, 28). Standard procedures were used for DNA manipulations (13).

Plasmid pRS415 (47) was used for construction of the plasmid-borne operon (transcriptional) fusions (Fig. 1). The Φ(*nasC-lacZ*) operon fusion plasmid (pVJS1340) includes *nas* DNA from a *Bam*HI site at the end of MudK insertion 73 (Fig. 1) to a *Bam*HI site within the *nasC* gene. The Φ(*nasF-lacZ*) operon fusion plasmid (pVJS1367) includes *nas* DNA from an *Eco*RI site that was introduced immediately upstream of the *nasF* operon control region (29) to a *Bam*HI site at the end of MudK insertion 34 (Fig. 1).

Media. Defined, complex, and indicator media for routine genetic manipulations were used as described previously (13, 39). Nitrogen-free medium contained 0.2% glucose, 1% sodium citrate, 7.4% sodium phosphate (pH 8), and 1 mM $MgSO_4$ (27). This medium was supplemented with additional nitrogen sources (10 mM $NaNO_3$, 5 mM $NaNO_2$, or 10 mM NH_4Cl) as indicated. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) or triphenyl tetrazolium chloride (TTC; 0.0025%) was used to score the Lac phenotypes of MudJ insertions. Selection for *K. pneumoniae* transformants carrying *bla*-containing plasmids was accomplished with a 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. pneumoniae* and *E. coli* transformants, respectively. Kanamycin was used at 100 and 75 μg/ml for selecting *K. pneumoniae* and *E. coli* transformants, respectively. Spectinomycin was used at 25 μg/ml, and tetracycline was used at 20 μg/ml. Chloramphenicol and spectinomycin were used at 200 μg/ml for selecting for Tn*10*d(Cm) and Tn*5*d(Sp) insertions into plasmids. Agar and dehydrated media were from Difco Laboratories (Detroit, Mich.). Other components were from Sigma Chemical Co. (St. Louis, Mo.).

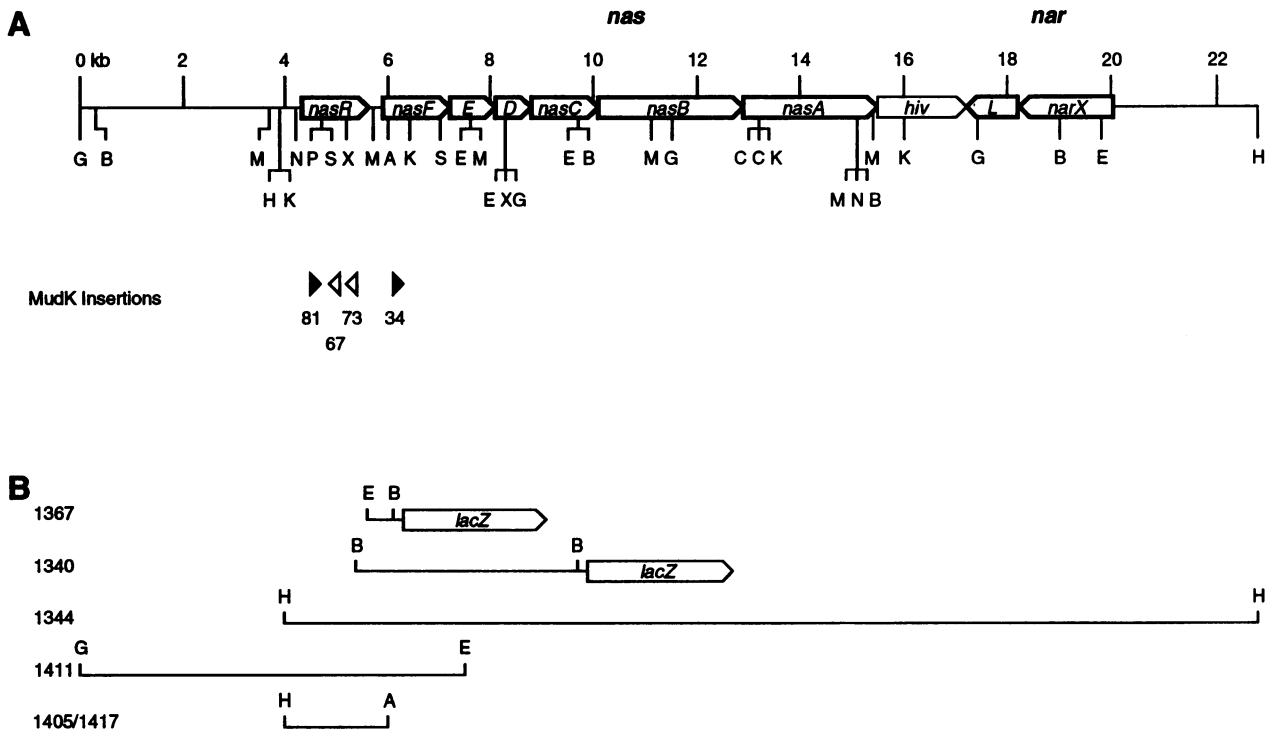

FIG. 1. Physical map of the *nasRFEDCBA—narLX* region and subclones used for constructing insertions and for complementation analysis. Modified from reference 28. (A) Restriction map of the *nasRFEDCBA—narLX* region. Restriction site abbreviations: A, *Sac*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; M, *Sma*I; N, *Nru*I; P, *Pst*I; S, *Sal*I; X, *Xho*I. Additional sites for some of these enzymes may be located in the areas flanking the *nasRFEDCBA* coding regions. Additional *Pst*I and *Sal*I sites within the *nasRFEDCBA* coding regions are not shown. The locations and orientations of four representative MudK insertions in the *nasR-F* region are depicted by triangles, identified by their isolation numbers. Lac^+ insertions are solid, and Lac^- insertions are open. The point of each triangle indicates the orientation of the *lacZ* gene with respect to the *nas* genes. (B) Subclones used for β-galactosidase assays (pVJS1367 and pVJS1340), *nas* gene expression in *E. coli* (pVJS1344), Tn*10*d(Cm) mutagenesis (pVJS1411), in vivo protein expression (pVJS1417), and *nasR* allele replacement (pVJS1405).

Tn*5*d(Sp) and Tn*10*d(Cm) insertion mutagenesis. The Tn*5*d(Sp) insertion contained on the plasmid pUT-Sm/Sp (14) was carried in a λ*pir* derivative of the mobilizing strain S17-1 (46). Fresh single colonies of this donor strain were cross-streaked with strain VJSK582 (*ntrB4 nasD*::MudJ) on nutrient agar without ampicillin or kanamycin, and the plates were incubated overnight at 37°C. The resulting patches were dispersed into 1 ml of TY broth, diluted 1,000-fold, and plated onto nutrient agar containing spectinomycin, kanamycin, TTC, lactose (1%), and nitrate (10 mM). Plates were incubated overnight at 37°C.

Tn*10*d(Cm) transposon insertions were generated in plasmid pVJS1411 as described previously (25). Plasmid pVJS1411 contains the leftmost 8-kb *Eco*RI-*Bgl*II fragment of pVJS401 (9) cloned into the vector pHG329 (48). We generated and localized 124 Tn*10*d(Cm) insertions in this plasmid.

DNA sequencing. The *nasR* sequence was determined from double-stranded templates by the dideoxynucleotide chain termination method (45) with modified T7 DNA polymerase (51) and [α-^{35}S]dATP labeling (7). DNA for sequencing was prepared as described previously (26). Plasmids containing MudK insertions in different positions were primed with oligonucleotides complementary to the ends of transposon MudK as described previously (5). Gaps in the resulting sequence were covered by sequencing from oligonucleotide primers complementary to internal sequences. Oligonucleotides were synthesized at the Synthesis Facility of the Cornell University Biotechnology Program or at Integrated DNA Technologies, Inc. (Coralville, Iowa). T7 DNA polymerase (Sequenase 2.0) and sequencing reagents were from United States Biochemical Corp. (Cleveland, Ohio), and [α-^{35}S]dATP was from Amersham Corp. (Arlington Heights, Ill.). DNA sequences were analyzed with the DNA Inspector IIe program (Textco, West Lebanon, N.H.) and with the sequence analysis software package assembled by the Genetics Computer Group (15). Database searches were performed with the BLAST programs (1).

Detection of plasmid-encoded proteins. Plasmid-encoded proteins were detected with an in vivo T7 expression system (51) as previously described (6). We cloned the *nasR* region into pGEM-7Zf(+), which contains the bacteriophage T7 φ10 promoter. Frameshift mutations in the *nasR* gene were generated by cutting plasmid pVJS1417 with either *Sal*I or *Pst*I and then treating with T4 DNA polymerase and nucleotides. DNA cut with *Sal*I leaves a 5′ overhang, and treatment with T4 DNA polymerase and ligase fills in the sequence, generating a +1 frameshift mutation. DNA cut with *Pst*I leaves a 3′ overhang, and treatment with T4 DNA polymerase and ligase chews back four bases, generating a −1 frameshift. The plasmids were transformed into strain VJS2236, which carries the gene for T7 RNA polymerase (gene *1*) under the control of a heat-inducible promoter on plasmid pGP1-2. After heat induction of T7 RNA polymerase synthesis, rifampicin was added to inhibit host RNA polymerase. The subsequent addition of [^{35}S]methionine resulted in selective labeling of plasmid-en-

coded gene products. Samples were electrophoresed on Laemmli gels, dried, and exposed to X-ray film.

Analysis of insertion mutations. To determine the insertion sites of the chromosomal *nasR*::Tn*5*d(Sp) insertions, we recombined them from the chromosome onto plasmid pVJS1411 by selecting for resistance to high levels of spectinomycin (200 μg/ml). The three insertions were subsequently characterized by sequence analysis using primers generated from sequences internal to the Tn*5*d(Sp) element (14).

The locations of the plasmid-borne *nasR*::Tn*10*d(Cm) insertions were determined by restriction analysis, and six were subsequently characterized by sequence analysis using primers generated from sequences internal to the Tn*10*d(Cm) insertion (25). The *nasR*::Tn*10*d(Cm) insertions were subcloned on a *Sma*I fragment into the suicide vector pJP5603 (43).

The Ω-Sp insertion in the *Xho*I site was generated by subcloning a *Sal*I fragment containing the Ω-Sp element from Tn*5*d(Sp) (14, 17) into plasmid pVJS1405, which contains the *nasR* region in the suicide vector pJP5603. The *cat* insertion in the *Nru*I site was generated by subcloning a *Sma*I fragment containing the *cat* gene from pCAT19 (18) into an *Nru*I partial digest of plasmid pVJS1405.

All insertions were recombined from the suicide vectors into the chromosome by transconjugation as previously described (27). The double recombination event generating the allelic replacement was determined after selection on defined medium containing the appropriate antibiotic (see Fig. 2B).

The authenticity of the *nasR* chromosomal insertions was confirmed by determining linkage to *nasB*::MudJ or *nasD*::MudJ insertions by bacteriophage P1 *kc*-mediated transduction (39). The insertions were backcrossed to Nas$^+$ *K. pneumoniae* strains, and the resulting recombinants were tested for their Nas phenotypes by streaking on appropriate media as described previously (27).

Growth of mixed cultures. Wild-type, *nas*::MudJ and *moa*::MudJ strains were pregrown separately as previously described (9). These starter cultures were mixed in wild-type-to-*nas*::MudJ or *moa*::MudJ-to-*nas*::MudJ ratios of 1:10, 1:4, and 1:2 in separate flasks containing glucose-MOPS (morpholinepropanesulfonic acid) medium, and cultures were aerated and harvested as described previously (9). A portion of each harvested culture was plated onto indicator and antibiotic media to determine the actual percentages of each parental type in the cultures. This measured percentage was used as a correction factor for reporting β-galactosidase specific activities. For example, with measured *nas*::MudJ:wild-type ratios of 50:50 and 75:25, the specific activities were multiplied by 2 and by 1.33, respectively, to allow direct comparison of values from different cultures.

Nucleotide sequence accession number. The DNA sequence reported in this article has been deposited in the GenBank nucleotide sequence database under accession number L27824.

RESULTS

Isolation of *nasR*::Tn*5*d(Sp) insertion mutants. Expression of the *nasF* operon is subject to two levels of control: activation by general nitrogen regulation (Ntr) and further induction by either nitrate or nitrite (9, 32). We sought to separate these two effects genetically by isolating mutants that retained Ntr control but that were defective for nitrate or nitrite induction. We performed Tn*5*d(Sp) mutagenesis (14) on strain VJSK582, which contains a Φ(*nasD-lacZ*) operon fusion, as described in Materials and Methods. We screened about 20,000 Sp^r Km^r exconjugants for the Lac$^-$ phenotype on indicator medium containing nitrate. Three mutants were altered for regulation not only of the original Φ(*nasD-lacZ*) fusion, but also of a plasmid-borne Φ(*nasC-lacZ*) fusion. These three mutations [*nasR126*::Tn*5*d(Sp), *nasR127*::Tn*5*d(Sp), and *nasR128*::Tn*5*d (Sp)] thus acted in *trans*, and were used for further analysis.

TABLE 2. Φ(*nasC-lacZ*) expression in *E. coli* RK4353

Plasmids	Genotype	β-Galactosidase sp act[a] with:		
		NH_4^+, Gln	Gln	NO_3^-, Gln
pVJS1340; pACYC184	Φ(*nasC-lacZ*)	50	510	600
pVJS1340; pVJS1344	Φ(*nasC-lacZ*); *nasR*$^+$	100	1,900	7,100

[a] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown with aeration in the indicated media as described in Materials and Methods.

The three *nasR*::Tn*5*d(Sp) insertions were transduced into the wild-type strain, and each was 100% linked to the Lac$^-$ phenotype. In an otherwise wild-type background, each *nasR*::Tn*5*d(Sp) mutant was defective for growth with either nitrate or nitrite as the sole nitrogen source, but each grew well with arginine, glutamine, or ammonium as the sole nitrogen source. The *nasR*::Tn*5*d(Sp) mutants were thus specifically altered for *nasF* operon regulation and were not defective in general Ntr regulation.

Localization of the *nasR* gene. The *nasR* gene was localized to the chromosome in two ways. First, we found that the all three *nasR*::Tn*5*d(Sp) insertions were about 85% linked to the *nasD*::MudJ insertion, as judged by phage P1-mediated general transduction. The *nasR* insertions were further localized by complementation of the NasR$^-$ phenotype. Φ(*nasD-lacZ*) strains are normally Lac$^+$ on indicator medium, but introduction of a *nasR*::Tn*5*d(Sp) insertion confers a Lac$^-$ phenotype. Introduction of a plasmid carrying an ~2-kb *Hin*dIII-*Sac*I fragment upstream of the *nasF* gene (Fig. 1) conferred a Lac$^+$ phenotype. This indicates that the *nasR* gene lies within this ~2-kb region.

To determine whether this upstream region is involved in nitrate regulation, we sought to reconstitute *nasF* operon expression in *E. coli*. This was done by transforming two plasmids into *E. coli* RK4353. One plasmid (pVJS1340) contains a Φ(*nasC-lacZ*) operon fusion. In *E. coli*, Φ(*nasC-lacZ*) expression was induced about 10-fold by growth on a limiting nitrogen source but was not further induced by nitrate (Table 2). The other plasmid (pVJS1344) contains the entire *nas* region including ~2 kb upstream of the *nasF* operon in plasmid pACYC184 (11). Inclusion of this plasmid raised the basal level of Φ(*nasC-lacZ*) expression, but more importantly, it conferred about fourfold induction by nitrate. This observation confirms that the *nasR* region is required for nitrate regulation of *nasF* operon expression.

Insertion mutagenesis of the *nasR* region. To localize the gene(s) whose product mediates nitrate induction of the *nasF* operon, the DNA containing the *nasR* region was subjected to insertion mutagenesis. The plasmid pVJS1411 was mutagenized with transposon Tn*10*d(Cm). Plasmid preparations were made on four pools of the Cm^r colonies generated from this mutagenesis (average, 10,000 colonies per pool). The DNA from these pools was transformed into a *nasR*::Tn*5*d(Sp) Φ(*nasD-lacZ*) strain (VJSK587). Normally, strain VJSK587 is Lac$^-$ on indicator plates, but complementation by the *nasR*$^+$ gene, on pVJS1411, conferred a Lac$^+$ phenotype. Insertions within nitrate regulatory genes would abolish this complementation.

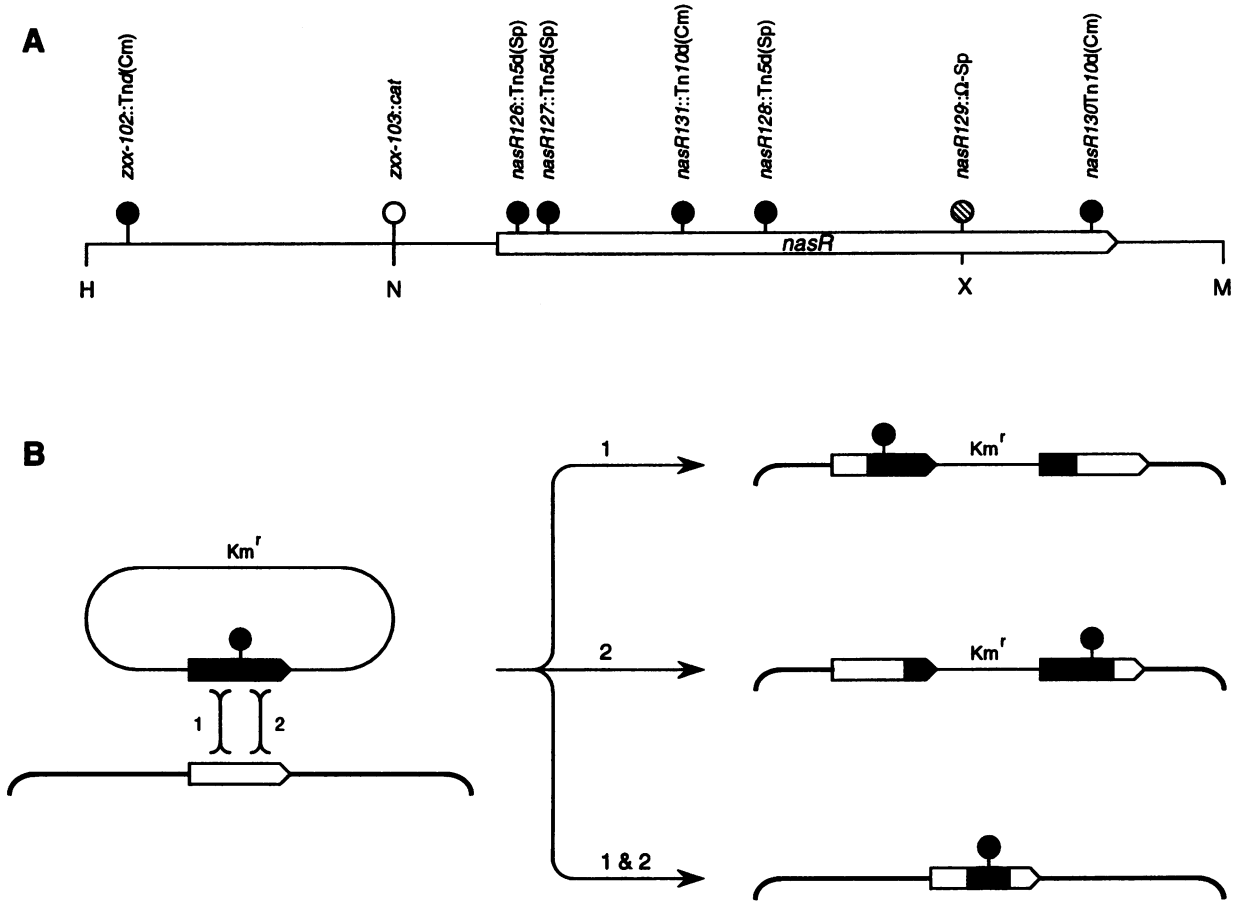

FIG. 2. Insertions in the *nasR* region and strategy for transfer to the chromosome. (A) Locations of representative insertions. Each insertion shown was localized by DNA sequence analysis and phenotypically tested after alleleic replacement. (B) Strategy for transferring insertions to the chromosome from the suicide vector pJP5603 (see text for details). Homologous recombination at positions 1 or 2 result in integration of the suicide vector and generation of a merodiploid *nasR*$^+$/*nasR* tandem duplication. Homologous recombination involving both positions 1 and 2 results in a haploid *nasR* strain (via allelic replacement).

Restriction analysis of 49 Lac$^-$ Cm^r insertions revealed that all were clustered between the *Nru*I and *Sma*I sites (Fig. 2), while all 75 Lac$^+$ Cm^r insertions characterized were outside of this region. One of these insertions, *zxx-102*::Tn*10*d(Cm), was about 450 bp upstream of the *nasR* initiation codon (Fig. 2).

We also constructed two insertions in the *nasR* region by molecular cloning (Fig. 2): an Ω-Sp insertion in the *Xho*I site (within the *nasR* coding region; see below) and a *cat* insertion in the *Nru*I site (approximately 90 bp upstream of the *nasR* coding region).

Phenotypes of the *nasR* insertion mutants. To determine the phenotypes conferred by these insertions, several were exchanged into the *K. pneumoniae* chromosome as described in Materials and Methods. Strains carrying all of the Lac$^-$ Tn*10*d(Cm) insertions tested, as well as the insertion in the *Xho*I site, were phenotypically Nas$^-$. By contrast, the strain carrying the insertion in the *Nru*I site was phenotypically Nas$^+$.

We further examined four insertions [one of the original *nasR*::Tn*5*d(Sp) insertions, the Ω-Sp insertion in the *Xho*I site, one of the *nasR*::Tn*10*d(Cm) insertions, and the *cat* insertion in the *Nru*I site] for their effects on *nasF* operon expression. These experiments utilized a plasmid-borne Φ(*nasF-lacZ*) operon fusion. This plasmid was introduced into each of the mutants, and cultures were grown and assayed for β-galactosidase activity (Table 3). Each of the three *nasR* insertions conferred a similar phenotype: each abolished nitrate and nitrite induction, and each also lowered the level of expression during nitrogen-limited growth. By contrast, the strain carrying the insertion in the *Nru*I site upstream of the *nasR* gene behaved essentially as the wild type.

To confirm that these phenotypes were recessive to the *nasR*$^+$ gene, we used mutant derivatives of the *nasR*$^+$ suicide plasmid pVJS1405 to generate tandem duplications containing both mutant and wild-type copies of the *nasR* gene. These suicide plasmids can experience either of two fates (Fig. 2B). One fate is a double recombination event which replaces the endogenous wild-type sequence with the plasmid-borne insertion. This double recombination event occurred about 2% of the time. The second fate is a single recombination event which generates a tandem duplication. These latter strains acquired a Km^r phenotype along with the selected Cm^r or Sp^r phenotype, indicating that the suicide plasmid containing the insertion had integrated into the chromosome. These tandem duplication strains were recovered in the same experiments that generated the haploid *nasR* insertion strains, described above. The results for three such pairs of strains are shown in Table 3. Expression of the Φ(*nasF-lacZ*) fusion in the merodiploid tandem duplication strains was indistinguishable from that of the haploid

TABLE 3. Φ(*nasF-lacZ*) expression in *nasR* haploid and *nasR*/*nasR*$^+$ merodiploid strains

Stain	Genotype	β-Galactosidase sp act[a] with:			
		NH_4^+, Gln	Gln	NO_3^-, Gln	NO_2^-, Gln
VJSK1251	Φ(*nasF-lacZ*); *nasR*$^+$	90	2,500	10,000	14,000
VJSK1183	Φ(*nasF-lacZ*); *nasR126*::Tn*5*d(Sp)	110	450	220	320
VJSK1185	Φ(*nasF-lacZ*); *nasR126*::Tn*5*d(Sp)/*nasR*$^+$ [b]	100	2,200	10,000	16,000
VJSK1306	Φ(*nasF-lacZ*); *nasR129*::Ω-Sp	110	450	270	240
VJSK1307	Φ(*nasF-lacZ*); *nasR129*::Ω-Sp/*nasR*$^+$	110	4,000	14,000	14,000
VJSK1200	Φ(*nasF-lacZ*); *nasR130*::Tn*10*d(Cm)	70	550	160	190
VJSK1301	Φ(*nasF-lacZ*); *nasR130*::Tn*10*d(Cm)/*nasR*$^+$	110	2,100	10,000	14,000
VJSK1314	Φ(*nasF-lacZ*); *zxx-103*::*cat*(*nasR*$^+$)	120	780	8,700	16,000

[a] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown with aeration in the indicated media as described in Materials and Methods.
[b] Merodiploid strains carry tandem duplications of the *nasR* region (see text and Fig. 2B).

nasR$^+$ strain, indicating that each of the *nasR* insertions is recessive, as expected. Unfortunately, we were unable to recover haploid segregants of these strains (20), presumably because of the small region available for homologous recombination.

Detection of plasmid-encoded proteins. We used an in vivo T7 expression system (51) to detect the protein(s) encoded by the *nasR* region. We cloned the *Hin*dIII-*Sac*I fragment from pVJS961 (28) into a T7 promoter-carrying vector, pGEM7Zf(+), such that the *nasR* region was transcribed from the T7 promoter (pVJS1417). Two polypeptide bands with mobilities corresponding to approximately 47 and 46 kDa were observed (Fig. 3). This compares with the predicted molecular mass for the presumed NasR protein, 44 kDa (see below). In addition, two derivatives of this plasmid were used. One contained a 4-base insertion in the *nasR* gene generated by filling in the *Sal*I site (pVJS1430), and the other contained a 4-base deletion in the *nasR* gene generated by chewing back the *Pst*I site (pVJS1429). Neither of the two bands was visible in preparations from strains carrying the frameshift mutations (Fig. 3). When the *nasR* gene was inserted in the opposite orientation, no protein products were observed (data not shown). The amino-terminal portion of the NasF protein was visible on some gels, and its production was not affected by either frameshift mutation (data not shown).

DNA sequence of the *nasR* gene. The DNA sequence of the *nasR* region was determined on both strands. The *nasR* gene is in the same orientation as the *nasF* operon (Fig. 1). The presumed translational start site for the *nasR* gene is 5′-AG AGGGGTATGAATAATATGGC-3′ (the presumed Shine-Dalgarno region and two possible initiation codons are underlined). Conceptual translation from the first ATG codon yields a 393-residue protein with a molecular mass of approximately 44 kDa (Fig. 4).

The positions of six insertions which generated a NasR$^-$ phenotype were determined by sequence analysis as described in Materials and Methods. All six were in the *nasR* gene and are shown in Fig. 2. The positions of several of the NasR$^+$ insertions were also determined, and two are shown in Fig. 2 [*zxx-102*::Tn*10*d(Cm) and *zxx-103*::*cat*]. Finally, one of the Tn*10*d(Cm) insertions (~2.7 kb upstream of the *nasR* gene) was in a gene whose product is about 90% identical to the *chaA* gene of *E. coli* (data not shown).

Additional regulatory elements? Two other genes that affect

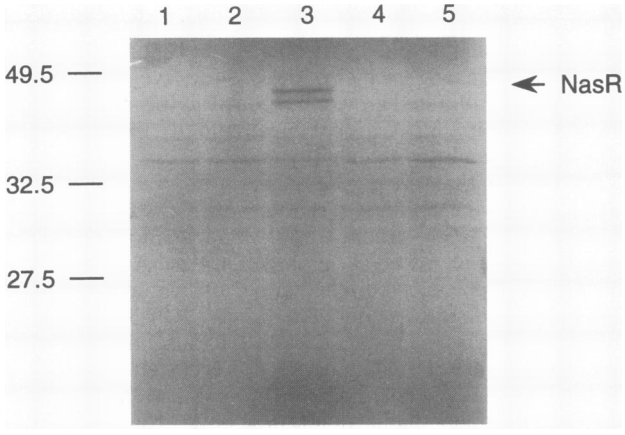

FIG. 3. The *nasR*-encoded polypeptides. Polypeptides encoded by the *nasR*-containing plasmid were detected by the in vivo T7 expression system (see text for details). Samples were electrophoresed on a 12% polyacrylamide Laemmli gel. Lanes: 1, no vector; 2, pGEM7Zf (+) (vector); 3, pVJS1417 (*nasR*$^+$); 4, pVJS1429 (−1 frameshift); pVJS 1430 (+1 frameshift). The migration of prestained molecular markers is indicated: bean trypsin inhibitor, 27.5 kDa; carbonic anhydrase, 32.5 kDa; ovalbumin, 49.5 kDa.

```
NasR   MNNMAGNTPEVVDWFRRARRLQKQQLHQLAQQGTLAGQISALVHMLQCER   50
NasR   RSNIWLCSGGRLYAAECRAGAALVDEQLTRFYAALEPARDAASSALCWRI  100
NasR   ACAVWYLPQLAALRKACATGRLPPKRPPGSLAVIRHLLNRTAAQRQHRRS  150
NasR   ANRRPHGCALQLYAGKRAGRTGARAGRAGFARGQFSDELRQQLVDRIDGQ  200
NasR   QPCFDSFQALAQPPQTALFAEQCQASLEIEQLRRVACTRQPPADEGETAL  250
NasR   RWFCAQTQRLEQLRGVEELLIVDLLNAADALLEGEEPEAHVPPADWQEDS  300
NasR   IALRLDKQLLPLVRQQAHELQQLSGQLASLKDALEERKLIEKAKSVLMTY  350
AmiR   ···HRVLPVLVSARRISEEMAKLKQKTEQLQDRIAGQARINQAKVLLMQR  159
NasR   QGMQEEQAWQALRKMAMDKNQRMVEIARALLTVKALWRVTPKE*        393
AmiR   HGWDEREAHQHLSREAMKRREPILKIAQELLGNEPSA*              196
```

FIG. 4. Amino acid sequence of the deduced NasR polypeptide. Amino acids are indicated by the standard single-letter code. Alignment with the deduced carboxyl-terminal portion of the AmiR polypeptide is also shown. Identical residues are indicated with vertical lines, and analogous residues are indicated with colons. Analogous residues are Arg-Lys, Asp-Glu, Asn-Gln, Ile-Val, Leu-Met, Phe-Tyr, and Ser-Thr.

TABLE 4. Φ(*nasB-lacZ*) expression in mixed cultures

Strain(s)	Genotype	Ratio[a]	β-Galactosidase sp act[b] with:			
			NH_4^+, Gln	Gln	NO_3^-, Gln	NO_2^-, Gln
VJSK056	*nasB*::MudJ	—	<1	710	2,100	1,400
VJSK056; VJSK014	*nasB*::MudJ; *nas*$^+$	90:10	<1	360	1,900	1,200
VJSK056; VJSK014	*nasB*::MudJ; *nas*$^+$	75:25	<1	200	2,200	1,300
VJSK056; VJSK014	*nasB*::MudJ; *nas*$^+$	50:50	<1	150	2,200	1,500
VJSK056; VJSK017	*nasB*::MudJ; *moa*::MudJ (Lac$^-$)	90:10	<1	660	1,900	1,200
VJSK056; VJSK017	*nasB*::MudJ; *moa*::MudJ (Lac$^-$)	75:25	<1	800	2,000	1,400
VJSK056; VJSK017	*nasB*::MudJ; *moa*::MudJ (Lac$^-$)	50:50	<1	670	2,000	1,300

[a] Approximate ratio of the two strains in the cultures. The exact ratio at harvest for each culture was determined by plating as described in Materials and Methods. Specific activity values shown are corrected for this measured ratio.

[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown with aeration in the indicated media as described in Materials and Methods.

either nitrate regulation (the *narL* gene) or Ntr control (the *nac* gene) were tested for their effects on *nasF* operon expression. The *narL* gene encodes a response regulator that is required for nitrate induction of respiratory nitrate reductase synthesis (reviewed in reference 49). The *nac* gene encodes a positive regulator that is required for the expression of a subset of genes under Ntr control (see introduction; reviewed in reference 3). Previous examination of nitrate reductase activities in mutant strains led to the conclusion that neither the *narL* nor the *nac* genes are involved in assimilatory nitrate reductase formation in *Klebsiella* spp. (32, 50).

Measurements of assimilatory nitrate reductase activity is potentially complicated by the presence of respiratory nitrate reductase in cultures that are not fully aerated (50, 52). Therefore, the Φ(*nasC-lacZ*)-containing plasmid (pVJS1340) was introduced into a *K. pneumoniae narL*::Km strain (50) and also into a *K. aerogenes nac*::Tn*5* strain (32). *nasF* operon expression was unaffected by either mutation (data not shown), thereby confirming previous observations (32, 50).

Mixed cultures. Haploid Nas$^-$ Φ(*nas-lacZ*) insertion strains synthesize β-galactosidase irrespective of the presence of nitrate or nitrite in the culture medium, whereas diploid *nas*$^+$/Φ(*nas-lacZ*) strains exhibit normal nitrate induction (9). To determine whether this haploid constitutivity is due to intracellular or extracellular factors, we performed experiments with mixtures of wild-type and haploid Nas$^-$ Φ(*nas-lacZ*) insertion strains. If the apparent constitutivity is due to the presence of contaminating amounts of nitrate (or another inducer) in the culture medium (9, 21), then the included wild-type cells would be expected to metabolize this inducer, thereby restoring normal nitrate induction in the haploid Nas$^-$ Φ(*nas-lacZ*) insertion cells. Conversely, if the constitutivity is directly caused by loss of *nasF* operon gene products in the haploid strain, then inclusion of the wild-type cells would be expected to have no effect on Φ(*nas-lacZ*) expression. As a control, we separately included cells of a chlorate-resistant strain (*moa*::MudJ) that cannot synthesize molybdenum cofactor and thus cannot metabolize nitrate.

Results are shown in Table 4. The Φ(*nasB-lacZ*) haploid strain synthesized relatively high levels of β-galactosidase in the absence of added inducer when cultured alone. Inclusion of increasing amounts of wild-type cells during growth progressively depressed this constitutive expression. By contrast, inclusion of the molybdenum cofactor mutant cells had no effect. This experiment argues that the apparent constitutivity is due to extracellular factors that can be ameliorated by inclusion of phenotypically Nas$^+$ cells, but not by phenotypically Nas$^-$ cells.

DISCUSSION

Synthesis of nitrate and nitrite assimilatory enzymes in *Klebsiella* spp. is subject to dual control by general nitrogen regulation (Ntr) and by nitrate and/or nitrite induction (4, 9, 52). Evidence suggests that Ntr control of *nasF* operon expression is mediated directly by the NtrC protein. First, expression of a Φ(*nasC-lacZ*) operon fusion plasmid was not altered in a *nac* mutant of *K. aerogenes* (this work), supporting previous conclusions that the Nac protein plays no role in assimilatory nitrate reductase formation (32). Second, molecular genetic analysis of the *nasF* operon regulatory region has revealed the presence of characteristic upstream NtrC protein binding sites (29). Thus, the *nasF* operon is unique in that a pathway-specific regulatory mechanism, induction by nitrate and nitrite, is integrated with NtrC protein control. We wish to understand how these dual regulatory circuits are integrated.

Identification of the *nasR* gene. We isolated insertion mutations that abolish nitrate and nitrite induction of *nasF* operon expression. The three insertions were tightly linked to each other and to the *nasF* operon, and none influenced growth on alternate nitrogen sources such as arginine. Thus, these insertions did not affect general Ntr control. The phenotypes of these *nasR* insertion mutants were complemented by the region immediately upstream of the *nasF* operon (Fig. 1; Table 2 and 3). This indicates that these insertions are null alleles and therefore that the NasR protein is a positive regulator of *nasF* operon expression. All three insertions were physically localized to this upstream region, and additional insertions in this region, when transferred to the chromosome, also conferred the NasR$^-$ phenotype (Fig. 2). Finally, DNA sequence analysis identified a single gene, *nasR*, which encodes a protein of approximately 44 kDa (Fig. 3 and 4).

The *nasR*$^+$-containing plasmids directed the synthesis of two polypeptides with molecular masses of approximately 47 and 46 kDa, and the DNA fragment used does not have the capacity to encode two separate polypeptides of this size. Additionally, synthesis of both proteins was abolished by either +1 or −1 frameshift mutations within the *nasR* coding region (Fig. 3). Thus, one polypeptide likely represents a breakdown product or posttranslational modification of the other. Alternatively, it is possible that both of the potential initiation codons were recognized, although the two polypeptides would differ by only three amino-terminal residues (Met-Asn-Asn).

Nitrate and nitrite induction of *nasF* operon expression was abolished in strains carrying *nasR* insertions (Table 3). However, when cultures were grown on a neutral nitrogen source (glutamine), the level of *nasF* operon expression was also

decreased relative to that of the wild type, although significant Ntr control was still apparent (Table 3). Wild-type and mutant cultures exhibited comparable levels of *nasF* operon expression when grown with ammonium. Thus, Ntr control appeared to be somewhat defective in the *nasR* mutants. However, we believe that this was an indirect effect and that the NasR protein has some activation function even in the absence of added nitrate or nitrite.

The mode of NasR protein action is unknown. The predicted carboxyl terminus shares similarity with the predicted carboxyl terminus of the AmiR protein from *P. aeruginosa* (Fig. 4; reference 30), but no other similarities to the AmiR protein or to other known proteins were noted. The AmiR protein is thought to mediate transcription antitermination control of *amiE* (aliphatic amidase) gene expression (16).

Evidence against autogenous regulation of *nasF* operon expression. Experiments with the ascomycetes *Emericella* (*Aspergillus*) *nidulans* and *Neurospora crassa* have led to the proposal that nitrate reductase autogenously regulates its own synthesis in these organisms (reviewed in references 12, 35, and 36). This idea is based on the observation that nitrate reductase cross-reacting material and mRNA are synthesized constitutively in certain nitrate reductase structural gene mutants. Analogous observations, that Φ(*nas-lacZ*) operon fusion expression is constitutive in Nas$^-$ strains, have been made with *K. pneumoniae* and *A. vinelandii* (9, 44). The autogenous regulation hypothesis explains these observations as the loss of negative control, mediated by nitrate reductase itself. However, our identification of the *nasR* positive regulatory gene complicates this hypothesis, as did the identification of the pathway-specific positive regulatory genes *nirA* and *nit-4* in *E. nidulans* and *N. crassa*, respectively (reviewed in references 12, 35, and 36).

An alternate explanation for these observations can be formulated (9, 21; reviewed in reference 35). Null mutants devoid of nitrate reductase activity would be unable to metabolize trace amounts of nitrate (or similar compounds) that may contaminate culture media, and therefore the apparent constitutivity of nitrate reductase gene expression could actually be due to gratuitous induction by this low level of nitrate. Wild-type organisms would metabolize this contaminating nitrate and thereby exhibit normal regulation.

A prediction of this hypothesis (suggested to us by R. Kadner) is that a coculture of a Nas$^-$ Φ(*nas-lacZ*) strain and a Nas$^+$ Lac$^-$ strain would exhibit inducible β-galactosidase activity. Indeed, we observed exactly this result: inclusion of a wild-type strain in a culture of a Nas$^-$ Φ(*nasB-lacZ*) strain conferred essentially normal nitrate induction (Table 4). Coculture with a chlorate-resistant (Mol$^-$) mutant, devoid of nitrate reductase activity, had no effect on the constitutive expression. This experiment strongly suggests that the observed constitutivity is due to an external, physiological effect rather than an internal, genetic phenomenon (see also reference 9).

Genetic organization of the *nas-nar* region in *K. pneumoniae*. The *E. coli* K-12 genetic map exhibits a gene order of *chaA—narL—narGHJI—tonB—trp* (2, 22), and the *nas* locus is immediately upstream (left) of the *narL* gene in *K. pneumoniae* M5al (27). (To our knowledge, *E. coli* K-12 does not contain any sequences related to the *nasF* operon.) However, *nas* and *nar* insertions are not transductionally linked to the *trp* operon in *K. pneumoniae* (24), although the *nar* locus is about 50% linked to the *trp* operon in *E. coli*. Likewise, the *tonB* genes of *E. coli* and *K. aerogenes* are in different genetic contexts (8). However, in characterizing the insertion sites of Tn*10*d(Cm) insertions, we observed that one insertion approximately 2.7 kb upstream of the *nasR* gene lies in a coding region with over 90% predicted amino acid identity to the *chaA* gene of *E. coli*. Thus, at least with respect to the *nar* region, the two organisms share both similarities and differences in gene organization. Further characterization of the *K. pneumoniae* genetic map thus may be useful in understanding enterobacterial genome organization and evolution.

ACKNOWLEDGMENTS

We thank B. Bartolomé, D. Bauer, R. Bender, V. de Lorenzo, J. Frey, W. C. Fuqua, J. Pemberton, R. Simon, G. S. A. B. Stewart, and K. Timmis for gifts of plasmids and strains. We thank A. Darwin, R. Kadner, R. S. Rabin, and S. Williams for thoughtful discussions.

This study was supported by U.S. Department of Energy grant 91ER20027 from the Division of Energy Biosciences.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54:**130–197.
3. **Bender, R. A.** 1991. The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. Mol. Microbiol. **5:**2575–2580.
4. **Bender, R. A., and B. Friedrich.** 1990. Regulation of assimilatory nitrate reductase formation in *Klebsiella aerogenes* W70. J. Bacteriol. **172:**7256–7259.
5. **Berg, B. L., J. Li, J. Heider, and V. Stewart.** 1991. Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. I. Nucleotide sequence of the *fdnGHI* operon and evidence that opal (UGA) encodes selenocysteine. J. Biol. Chem. **266:**22380–22385.
6. **Berg, B. L., and V. Stewart.** 1990. Structural genes for nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. Genetics **125:**691–702.
7. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80:**3963–3965.
8. **Bruske, A. K., M. Anton, and K. J. Heller.** 1993. Cloning and sequencing of the *Klebsiella pneumoniae tonB* gene and characterization of *Escherichia coli-K. pneumoniae* TonB hybrid proteins. Gene **131:**9–16.
9. **Cali, B. M., J. L. Micca, and V. Stewart.** 1989. Genetic regulation of nitrate assimilation in *Klebsiella pneumoniae* M5al. J. Bacteriol. **171:**2666–2672.
10. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158:**488–495.
11. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
12. **Crawford, N. M., and H. N. Arst.** 1993. The molecular genetics of nitrate assimilation in fungi and plants. Annu. Rev. Genet. **27:**115–146.
13. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. A manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
16. **Drew, R., and N. Lowe.** 1989. Positive control of *Pseudomonas aeruginosa* amidase synthesis is mediated by a transcription antitermination mechanism. J. Gen. Microbiol. **135:**817–823.
17. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. Gene **52:**147–154.
18. **Fuqua, W. C.** 1992. An improved chloramphenicol resistance gene

cassette for site-directed marker replacement mutagenesis. BioTechniques **12**:223–225.
19. **Garzón, A., J. Li, A. Flores, J. Casadesus, and V. Stewart.** 1992. Molybdenum cofactor (chlorate-resistant) mutants of *Klebsiella pneumoniae* M5al can use hypoxanthine as the sole nitrogen source. J. Bacteriol. **174**:6298–6302.
20. **Hughes, K. T., and J. R. Roth.** 1985. Directed formation of deletions and duplications using Mu*d* (Ap, *lac*). Genetics **109**:263–282.
21. **Iuchi, S., and E. C. C. Lin.** 1987. Molybdenum effector of fumarate reductase repression and nitrate reductase induction in *Escherichia coli*. J. Bacteriol. **169**:3720–3725.
22. **Ivey, D. M., A. A. Guffanti, J. Zamsky, E. Pinner, R. Karpel, E. Padan, S. Schuldiner, and T. A. Krulwich.** 1993. Cloning and characterization of a putative Ca^{2+}/H^{+} antiporter gene from *Escherichia coli* upon functional complementation of Na^{+}/H^{+} antiporter-deficient strains by the overexpressed gene. J. Biol. Chem. **268**:11296–11303.
23. **Jeter, R. M., S. R. Sias, and J. L. Ingraham.** 1984. Chromosomal location and function of genes affecting *Pseudomonas aeruginosa* nitrate assimilation. J. Bacteriol. **157**:673–677.
24. **Kisloff, L. E., B. S. Goldman, and V. Stewart.** Unpublished results.
25. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons, with emphasis on Tn*10*. Methods Enzymol. **204**:139–180.
26. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase™. BioTechniques **6**:544–547.
27. **Lin, J. T., B. S. Goldman, and V. Stewart.** 1993. Structures of genes *nasA* and *nasB* encoding assimilatory nitrate and nitrite reductases in *Klebsiella pneumoniae* M5al. J. Bacteriol. **175**:2370–2378.
28. **Lin, J. T., B. S. Goldman, and V. Stewart.** 1994. The *nasFEDCBA* operon for nitrate and nitrite assimilation in *Klebsiella pneumoniae* M5al. J. Bacteriol. **176**:2551–2559.
29. **Lin, J. T., and V. Stewart.** Unpublished results.
30. **Lowe, N., P. M. Rice, and R. E. Drew.** 1989. Nucleotide sequence of the aliphatic amidase regulator gene (*amiR*) of *Pseudomonas aeruginosa*. FEBS Lett. **246**:39–43.
31. **Luque, I., A. Herrero, E. Flores, and F. Madueno.** 1992. Clustering of genes involved in nitrate assimilation in the cyanobacterium *Synechococcus*. Mol. Gen. Genet. **232**:7–11.
32. **Macaluso, A., E. A. Best, and R. A. Bender.** 1990. Role of the *nac* gene product in the nitrogen regulation of some NTR-regulated operons of *Klebsiella aerogenes*. J. Bacteriol. **172**:7249–7255.
33. **MacNeil, D., J. Zhu, and W. J. Brill.** 1981. Regulation of nitrogen fixation in *Klebsiella pneumoniae*: isolation and characterization of strains with *nif-lac* fusions. J. Bacteriol. **145**:348–357.
34. **Magasanik, B., and F. C. Neidhardt.** 1987. Regulation of carbon and nitrogen utilization, p. 1318–1325. *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. American Society for Microbiology, Washington, D.C.
35. **Maloy, S., and V. Stewart.** 1993. Autogeneous regulation of gene expression. J. Bacteriol. **175**:307–316.
36. **Marzluf, G. A.** 1993. Regulation of sulfur and nitrogen metabolism in filamentous fungi. Annu. Rev. Microbiol. **47**:31–55.
37. **Merrick, M. J.** 1988. Regulation of nitrogen assimilation by bacteria. Symp. Soc. Gen. Microbiol. **42**:331–361.
38. **Merrick, M. J.** 1993. In a class of its own—the RNA polymerase sigma factor σ^{54} (σ^{N}). Mol. Microbiol. **10**:903–909.
39. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Nagatani, H., M. Shimizu, and R. C. Valentine.** 1971. The mechanism of ammonia assimilation in nitrogen fixing bacteria. Arch. Mikrobiol. **79**:164–175.
41. **North, A. K., K. E. Klose, K. M. Stedman, and S. Kustu.** 1993. Prokaryotic enhancer-binding proteins reflect eukaryote-like modularity: the puzzle of nitrogen regulatory protein C. J. Bacteriol. **175**:4267–4273.
42. **Omata, T., X. Andriesse, and A. Hirano.** 1993. Identification and characterization of a gene cluster involved in nitrate transport in the cyanobacterium *Synechococcus* PCC7942. Mol. Gen. Genet. **236**:193–202.
43. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118**:145–146.
44. **Ramos, F., G. Blanco, J. C. Gutierrez, F. Luque, and M. Tortolero.** 1993. Identification of an operon involved in the assimilatory nitrate-reducing system of *Azotobacter vinelandii*. Mol. Microbiol. **8**:1145–1153.
45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
46. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. BioTechnology **1**:784–791.
47. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53**:85–96.
48. **Stewart, G. S. A. B., S. Lubinsky-Mink, C. G. Jackson, A. Kassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15**:172–181.
49. **Stewart, V.** 1993. Nitrate regulation of anaerobic gene expression in *Escherichia coli*. Mol. Microbiol. **9**:425–434.
50. **Stewart, V., and B. M. Cali.** 1990. Genetic evidence that NarL function is not required for nitrate regulation of nitrate assimilation in *Klebsiella pneumoniae* M5al. J. Bacteriol. **172**:4482–4488.
51. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
52. **van't Riet, J., A. H. Stouthamer, and R. J. Planta.** 1968. Regulation of nitrate assimilation and nitrate respiration in *Aerobacter aerogenes*. J. Bacteriol. **96**:1455–1464.