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NOTES

Regulation of Assimilatory Nitrate Reductase Formation in *Klebsiella aerogenes* W70

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***Klebsiella aerogenes* W70 could grow aerobically with nitrate or nitrite as the sole nitrogen source. The assimilatory nitrate reductase and nitrite reductase responsible for this ability required the presence of either nitrate or nitrite as an inducer, and both enzymes were repressed by ammonia. The repression by ammonia, which required the NTR (nitrogen regulatory) system (A. Macaluso, E. A. Best, and R. A. Bender, J. Bacteriol. 172:7249–7255, 1990), did not act solely at the level of inducer exclusion, since strains in which the expression of assimilatory nitrate reductase and nitrite reductase was independent of the inducer were also susceptible to repression by ammonia. Insertion mutations in two distinct genes, neither of which affected the NTR system, resulted in the loss of both assimilatory nitrate reductase and nitrite reductase. One of these mutants reverted to the wild type, but the other yielded pseudorevertants at high frequency that were independent of inducer but still responded to ammonia repression.**

Klebsiella aerogenes W70 can use nitrate as the sole source of nitrogen under aerobic or anaerobic conditions. *K. aerogenes* can also use nitrate as the terminal electron acceptor in anaerobic respiration. Van 'T Reit et al. (9) compared the nitrate reductases found in *K. aerogenes* S45 under assimilatory and respiratory conditions and identified several important differences. (i) The assimilatory nitrate reductase (NAS) was repressed by ammonia; the respiratory nitrate reductase (NAR) was not. (ii) NAR was repressed by oxygen; NAS was not. (iii) NAR activity was accompanied by the accumulation of nitrite in the medium; NAS activity was not. (iv) NAS was relatively unstable in disrupted cells; NAR was not. (v) NAR (which could accumulate during anaerobic growth in the presence of ammonia) did not allow aerobic growth on nitrate as the sole nitrogen source; NAS (which could accumulate during anaerobic growth in the absence of ammonia) did. Thus it appeared that NAS and NAR were quite distinct activities. NAS and its companion, assimilatory nitrite reductase (NIS), are poorly characterized in *K. aerogenes*, partly because the related bacteria *Escherichia coli* and *Salmonella typhimurium* both lack an aerobic NAS and NIS but mostly because the instability of the NAS made purification and even reliable enzyme activity measurements difficult. We have not solved the instability problem, but the extreme instability of NAS in the reaction mixture was partially overcome by initiating the reaction with broken cells and monitoring the oxidation of benzyl viologen during the first minute or less as described in the accompanying report (5). This allowed us to assay the enzyme reproducibly and begin its genetic analysis.

Physiological studies with *K. aerogenes* showed that NAS is a highly regulated enzyme. NAS was shown to be present when nitrate was the sole nitrogen source but not when ammonia was the sole nitrogen source (9). When both nitrate and ammonia were present, NAS was absent, indicating that ammonia played a repressing role (9). The repression of NAS by ammonia required the general nitrogen regulatory (NTR) system of *K. aerogenes* but not the more limited nitrogen assimilation control system (5). This left open three questions about the regulation of NAS formation in *K. aerogenes*. (i) Is an inducer (e.g., nitrate) required for induction, or is the absence of ammonia sufficient for induction? (ii) If an inducer is required, is it nitrate or some metabolic product of nitrate? (iii) Does the ammonia repression of NAS accumulation act at the level of NAS formation or at the level of inducer exclusion?

Induction of NAS and NIS by nitrate and nitrite. In the absence of ammonia, the NAS and NIS of *K. aerogenes* KC1043 (a derivative of W70; Table 1) were undetectable unless either nitrate or nitrite was present in the medium (Table 2). Use of a poor (growth-rate-limiting) nitrogen source such as histidine (Table 2) or glutamine (Table 3) was not sufficient to induce formation of NAS or NIS. Since NAS and NIS were induced when nitrate was present along with other poor nitrogen sources such as histidine or glutamine (Table 2), we concluded that the formation of NAS and NIS was induced by the presence of nitrate rather than by the absence of the alternative nitrogen sources.

In *K. aerogenes*, many catabolic systems are induced by the first intermediate in the pathway rather than by the initial substrate. For example, the histidine utilization system uses urocanate as the physiological inducer (6), and the lactose operon is induced by galactose (1). We therefore tested the ability of low concentrations of nitrite, the first intermediate in nitrate assimilation, to induce NAS and NIS. A low concentration of nitrite (1 mM) was chosen to avoid the toxicity associated with nitrite, and glutamine was provided as an additional nitrogen source to allow a full growth yield of the culture. Even at 1 mM, nitrite caused substantial

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TABLE 1. *K. aerogenes* strains

Strain	Genotype	Source or reference
KC1043	*hutC515*	5
KC1047	Δ(*gal-hut-bio-chl*)	5
KC1419	*hutC515*(pTROY11)	This work
KC1953	*nas-1*::Tn*5 hutC515* (pTROY11)	λ::Tn*5* × KC1419[a]
KC1954	*ntrC*::Tn*5 hutC515* (pTROY11)	λ::Tn*5* × KC1419
KC1955	*nas-3*::Tn*5 zxx-4*::Tn*5 hutC515*(pTROY11)	λ::Tn*5* × KC1419
KC2005	*ntrBC5*::Tn*5-131 ntr-45 hutC515*	5
KC2569	*nas-3*::Tn*5-131 hutC515*	This work
N103	*nas-3*::Tn*5 hutC515*	P1(KC1955) × KC1043[b]
1R2	*nas*$^+$ *hutC515*	KC1953→Nas$^+$[c]
3R2	*nas-3*::Tn*5 rev-32 zxx-4*::Tn*5*	KC1955→Nas$^+$
3R1	*nas-3*::Tn*5 rev-31*	N103→Nas$^+$

[a] Mutagenesis of strain KC1419 with λ::Tn*5* as described in the accompanying report (5).
[b] Transduction of strain KC1043 by P1 grown on strain KC1955.
[c] Spontaneous reversion of strain KC1953 to Nas$^+$ (ability to grow with nitrate as the sole nitrogen source).

induction of both NAS and NIS (Table 2). The reduced levels of NAS and NIS found when 1 mM nitrite was used as the inducer might have resulted from complete metabolism of the nitrite, leading to premature exhaustion of the inducer and a reduced final yield of NAS and NIS. Thus, we repeated the experiment with 1 mM nitrite in the initial culture fluid, but we added an additional 1 mM nitrite to the culture one generation before harvesting the cells. Under these conditions, induction by nitrite was as efficient as induction by nitrate (Table 2).

Since nitrite induced NAS and NIS formation, it was important to determine whether nitrate was itself an inducer or whether nitrate was metabolized to nitrite, which in turn served as the physiological inducer. To test this, we analyzed induction patterns of NIS in strain KC1047, which carries a deletion of the *gal-chl* region and is thus unable to form the molybdenum cofactor needed for active NAS (and NAR) (4). As expected, strain KC1047 was devoid of NAS activity under all conditions tested (Table 2). In contrast, KC1047 had fully induced levels of NIS activity even when nitrate was used as the inducer (Table 2). Thus nitrate served as inducer in its own right without metabolic conversion to nitrite, which also served as inducer (Table 2).

TABLE 2. Regulation of NAS and NIS formation by nitrate, nitrite, and ammonia[a]

Strain and growth medium	Sp act			
	NAS		NIS	
	−N	+N	−N	+N
KC1043 (wild type)				
G H	<0.01	ND[b]	<0.01	ND
G H NO_3	1.7	ND	3.4	ND
G NO_3	4.6	<0.01	6.5	<0.01
G Gln NO_3	2.0	<0.01	3.3	<0.01
G Gln (NO_2)	0.9	<0.01	1.7	<0.01
G Gln (NO_2)+(NO_2)	3.0	ND	3.3	ND
KC1047 (*chl*)				
G Gln NO_3	<0.01	ND	2.4	ND
G Gln (NO_2)	<0.01	ND	2.4	ND
G Gln (NO_2)+(NO_2)	<0.01	ND	2.8	ND

[a] Cells were grown in glucose (G) minimal medium in the presence (+N) or absence (−N) of 0.2% (wt/vol) ammonium sulfate plus the nitrogen sources indicated: H, 0.2% (wt/vol) histidine, NO_3, 0.2% KNO_3; Gln, 0.04% glutamine; (NO_2), 1 mM KNO_2; (NO_2)+(NO_2), 1 mM KNO_2 present at inoculation and another 1 mM KNO_2 added when cells reached 50 Klett units (1 doubling before harvesting). NAS and NIS specific activities are defined as nanomoles of nitrate- and nitrite-specific benzyl viologen oxidation per minute per milligram of cell protein.
[b] ND, Not determined.

TABLE 3. Regulation of NAS and NIS in *nas* mutants and revertants[a]

Strain and growth medium	Sp act			
	Nitrate reductase		Nitrite reductase	
	−N	+N	−N	+N
KC1043 (*nas*$^+$)				
G Gln	<0.01	ND	<0.01	ND
G Gln NO_3	2.0	<0.01	3.3	<0.01
G NO_3 (anaerobic)	ND	4.1	ND	1.8
NO1 (*nas-1*)				
G Gln NO_3	0.3	ND	0.3	ND
G NO_3 (anaerobic)	ND	3.1	ND	1.5
NO3 (*nas-3*)				
G Gln NO_3	0.3	0.02	0.1	<0.02
G NO_3 (anaerobic)	ND	2.1	ND	0.5
1R2 (*nas-1*→*nas*$^+$)				
G Gln	<0.01	ND	<0.01	ND
G Gln NO_3	1.9	0.06	4.2	<0.01
3R2 (*nas-3 rev-32 zxx-4*)				
G Gln	1.8	ND	2.2	ND
G Gln NO_3	2.1	<0.05	3.0	0.6
3R1 (*nas-3 rev-31*)				
G Gln	3.3	ND	3.0	ND
G Gln NO_3	3.0	<0.1	3.5	<0.1

[a] Growth and assay conditions, abbreviations, and specific activities were as described in the footnotes of Table 2, and growth was aerobic unless otherwise indicated. Anaerobic cultures were grown in tightly capped bottles with little or no air above the culture fluid (3). All values presented are the averages of three to six separate cultures except those reported for strain 3R1.

Genetic analysis of NAS and NIS. A brute-force approach was used to identify Tn*5* insertion mutants defective in NAS expression. Several thousand Tn*5* insertion mutants were isolated (5) and screened for their ability to use nitrate as the sole nitrogen source. Three such mutants were identified in the first screen and were characterized further. One of these had a Tn*5* insertion in the *ntrBC* (also called *glnLG*) operon of the NTR system as shown below and was defective in its ability to use many poor nitrogen sources such as histidine (data not shown). The two other mutants appeared to be specifically defective in nitrate assimilation and are similar to each other in that (i) the growth phenotype (Nas$^-$) is tight, (ii) both make 10-fold less nitrate or nitrite reductase than does the wild type aerobically (Table 3), and (iii) both make nitrate reductase and nitrite reductase at normal levels anaerobically (Table 3).

Strain KC1953 carried a single Tn*5* insertion responsible for the Nas$^-$ phenotype (*nas-1*::Tn*5*) (Table 4). Strain KC1955 apparently carried two Tn*5* insertions (*nas-3*::Tn*5* and *zxx-4*::Tn*5*), only one of which was responsible for the Nas$^-$ phenotype. When phage P1 grown on a *nas*$^+$ strain was used to transduce KC1953 (*nas-1*::Tn*5*) to Nas$^+$ (growth

TABLE 4. Genetic analysis of *nas* mutant strains[a]

Donor	Recipient	Selection	Scoring
KC1043 (wild type)	NC1953 (*nas-1*::Tn*5*)	Nas^+	37/37 Km^s
KC1043 (wild type)	KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*)	Nas^+	12/23 Km^s
KC1043 (*nas-1*::Tn*5*)	KC1043 (wild type)	Km^r	34/35 Nas^-
KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*)	KC1043 (wild type)	Km^r	12/21 Nas^-[b]
KC1043 (wild type)	N103 (*nas-3*::Tn*5*)	Nas^+	29/30 Km^s
KC1953 (*nas-1*::Tn*5*)	N103 (*nas-3*::Tn*5*)	Nas^+	32/33 Km^s
KC2569 (*nas-3*::Tn*5-131*)	N103 (*nas-3*::Tn*5*)	Tc^r	8/8 Km^s
KC2569 (*nas-3*::Tn*5-131*)	KC1953 (*nas-1*::Tn*5*)	Tc^r	0/8 Km^s
KC2569 (*nas-3*::Tn*5-131*)	KC1954 (*glnG2*::Tn*5*)	Tc^r	0/8 Km^s
KC2005 (*ntrC5*::Tn*5-131*)	N103 (*nas-3*::Tn*5*)	Tc^r	0/8 Km^s
KC2005 (*ntrC5*::Tn*5-131*)	KC1953 (*nas-1*::Tn*5*)	Tc^r	0/8 Km^s
KC2005 (*ntrC5*::Tn*5-131*)	KC1954 (*ntrC2*::Tn*5*)	Tc^r	8/8 Km^s
3R2 (KC1955 *rev-32*)	KC1043 (wild type)	Km^r	0/108 Nas^-[c]
3R2 (KC1955 *rev-32*)	KC1953 (*nas-1*::Tn*5*)	Nas^+	10/16 Km^s
3R2 (KC1955 *rev-32*)	KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*)	Nas^+	0/17 Km^s
3R2 (KC1955 *rev-32*)	N103 (*nas-3*::Tn*5*)	Nas^+	1/40 Km^s

[a] Phage P1 grown on the donor strain was used to transduce the recipient strain to the phenotype listed under the Selection column. Several transductants were purified by single-colony isolation and were scored for a second phenotype with the results indicated under the Scoring column. For example, in line 2, 12 of the 23 Nas^+ transductants tested were Km^s. Phenotypes: Nas^+ and Nas^-, ability and inability to use nitrate as the sole source of nitrogen; Km^r and Km^s, resistance and sensitivity to 50 μg of kanamycin per ml; Tc^r, resistance to 30 μg of tetracycline per ml.

[b] One Km^r Nas^- colony was saved as strain N103.

[c] Eight colonies were tested for inducibility of NAS formation; four were inducible, four were constitutive (see the text).

with nitrate as the sole nitrogen source), all of the Nas^+ transductants lost the Km^r associated with Tn*5* (Table 4). Conversely, when phage grown on KC1953 (*nas-1*::Tn*5*) were used to transduce the wild type to Km^r, virtually all of the Km^r transductants became Nas^- (Table 4). The rare exceptions were assumed to represent the instability of Tn*5* sometimes seen during P1-mediated transduction of *K. aerogenes* (8). Thus KC1953 carried a single Tn*5* insertion, *nas-1*::Tn*5*.

In contrast, when phage grown on the wild type were used to transduce KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*) to Nas^+, a substantial number of the Nas^+ transductants remained Km^r (Table 4), suggesting that a Tn*5* insertion not related to *nas* might be present (and perhaps even linked to *nas-3*). When phage grown on KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*) were used to transduce the wild type to Km^r, about half of the Km^r transductants were Nas^+ and half were Nas^- (Table 4), suggesting either a single Tn*5* insertion 50% linked to *nas-3* or two insertions (*nas-3*::Tn*5* and *zxx-4*::Tn*5*). One of the Km^r Nas^- transductants (strain N103) resulting from this cross (Table 4) was therefore tested. Phage grown on the wild type were used to transduce N103 (*nas-3*::Tn*5*) to Nas^+, and virtually all of the Nas^+ transductants became Km^s, in marked contrast to the analogous cross with KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*) as the recipient (Table 4). The phenotype of N103 (*nas-3*::Tn*5*) was indistinguishable from that of KC1955 (*nas-3*::Tn*5* *zxx-4*::Tn*5*), and the phenotype of the Nas^+ Km^r strains (presumed to be *zxx-4*::Tn*5*) was indistinguishable from the wild type with respect to the Nas and NTR phenotypes (data not shown). Thus, we assumed that *nas-3*::Tn*5* was entirely responsible for the Nas^- phenotype and that *zxx-4*::Tn*5* was unrelated to Nas.

The *nas-1* and *nas-3* mutations appeared to define different loci. Phage grown on KC1953 (*nas-1*::Tn*5*) were able to transduce strain N103 (*nas-3*::Tn*5*) to Nas^+ at appreciable frequencies, and the resulting Nas^+ transductants were Km^s (Table 4). To confirm that *nas-1* and *nas-3* are nonallelic, we converted the *nas-3*::Tn*5* (Km^r) allele to a tetracycline resistance form, *nas-3*::Tn*5-131*, as described in the accompanying report (5). Phage grown on KC2569 (*nas-3*::Tn*5-131*) were used to transduce various Km^r test strains to Tc^r. If the mutations were allelic, then the Tc^r allele should replace the Km^r allele by homologous recombination; if they were not allelic, then the Tc^r allele should be gained by transduction without loss of the Km^r insertion. The data in Table 4 show clearly that *nas-3*::Tn*5-131* replaced *nas-3*::Tn*5* but not *nas-1*::Tn*5* or a control marker, *ntrC2*::Tn*5*. Thus *nas-3* and *nas-1* define separate genetic loci. The data in Table 4 confirm that the mutation thought to lie in the *ntrBC* (*glnLG*) locus (see above) was, indeed, at the same site as a well-defined *ntrBC* mutation and that neither *nas-1* nor *nas-3* lay near *ntrBC*.

Analysis of revertants. In addition to their differences in genetic linkage, the *nas-1* and *nas-3* mutants also differed in their patterns of reversion. Broth cultures of the *nas-1*::Tn*5* mutant contained few Nas^+ revertants (10^{-8} to 10^{-9}), and these Nas^+ revertants were kanamycin sensitive, suggesting loss of the Tn*5* element. The Nas^+ revertants of *nas-1*::Tn*5* strains (such as strain 1R2) regained wild-type regulation of NAS and NIS formation, suggesting that they may be true *nas*$^+$ revertants (Table 3). In contrast, broth cultures of the *nas-3*::Tn*5* mutant strains KC1955 and N103 contained many Nas^+ revertants (ca. 10^{-7}), and these Nas^+ revertants remained Km^r (even when the *zxx-4*::Tn*5* was not present in the strain), suggesting pseudoreversion rather than true reversion of the mutation. Moreover, the Nas^+ revertants of *nas-3*::Tn*5* such as strains 3R2 and 3R1 no longer required nitrate or nitrite for induction of NAS or NIS (Table 3).

If the Nas^+ derivatives of *nas-3*::Tn*5* strains were indeed pseudorevertants, it ought to be possible to recover the original *nas-3* mutation from the pseudorevertants by transduction with the Km^r associated with Tn*5*. Phage P1 grown on strain 3R2 were used to transduce wild-type strain KC1043 to Km^r. Surprisingly, all of the Km^r transductants were Nas^+ (Table 4). Eight of these Nas^+ Km^r transductants were tested for NAS activity after growth in the absence of inducer. Four of the eight produced NAS constitutively (like the 3R2 donor), and four did not (data not shown). In light of the fact that strain KC1955 (and presumably its Nas^+ revertant, 3R2) carried two Tn*5* insertions (*nas-3*::Tn*5* and *zxx-4*::Tn*5*), we assumed that the wild-type (Nas^+, regulated) transductants carried *zxx-4*::Tn*5* and that the constitutive transductants carried both the *nas-3*::Tn*5* and *rev-32*, the mutation responsible for the pseudoreversion. Since no Km^r Nas^- transductants were obtained from the 3R2 donor, and since strain N103 (*nas-3*::Tn*5*) also gave constitutive Km^r revertants (Table 3), we suggest that the original *nas-3*::Tn*5* insertion mutation is obligately involved in the constitutive phenotype of the revertants (see below). Moreover, the *rev-32* allele postulated to be responsible for the pseudoreversion must be tightly linked to the original *nas-3*::Tn*5* mutation.

Although we do not understand the nature of the *rev-32* mutation that suppresses the Nas^- phenotype associated

with *nas-3*::Tn*5*, we note that *rev-32* may also suppress *nas-1*. When phage grown on strain 3R2 (*nas-3*::Tn*5 rev-32 zxx-4*::Tn*5*) were used to transduce strain KC1953 (*nas-1*::Tn*5*), Nas^+ transductants were readily recovered (Table 4), as expected, since *nas-3* and *nas-1* are unlinked and strain 3R2 is *nas-1*$^+$. However, about half the Nas^+ transductants remained Km^r. Thus, either *zxx-4*::Tn*5* was 50% linked to the *nas-1*$^+$ allele in strain 3R2 or the *nas-3*::Tn*5 rev-32* combination was able to suppress the Nas^- phenotype associated with *nas-1*, yielding Nas^+ Km^r transductants. We have not pursued this observation further.

NTR regulation of NAS and NIS. The isolation of strains such as 3R2 and 3R1 that produced NAS and NIS constitutively in the absence of inducer allowed us to determine whether the NTR-mediated ammonia repression of NAS and NIS is mediated solely at the level of inducer exclusion or at some other level, perhaps gene expression. The data in Table 3 show clearly that ammonia repressed NAS and NIS formation in the constitutive strains. Thus NTR-mediated ammonia repression did not act solely at the level of inducer exclusion.

Cali et al. have recently considered the genetic regulation of NAS in *Klebsiella pneumoniae* (*oxytoca*) M5a1 (2). They overcame the instability of NAS in the assay mixture by isolating fusions of the *lacZ* gene to *nas* promoters and assaying β-galactosidase instead of NAS. Surprisingly, all of their *nas-lac* fusions showed constitutive production of β-galactosidase (no inducer was required). Nevertheless, when complemented by a wild-type *nas* gene, the fusions were inducible either with nitrate or with nitrite. Thus in both *K. aerogenes* and *K. pneumoniae* nitrate and nitrite appear to be equally good inducers of NAS. The fact that all of the *K. pneumoniae nas-lac* fusions were constitutive may reflect the same property that resulted in constitutive pseudorevertants of the *nas-3* allele of *K. aerogenes*. The significance of this constitutivity remains unknown. Cali et al. showed clearly that the *K. pneumoniae nas-lac* fusions were regulated by the NTR system at the level of gene expression (2). Macaluso et al. (5) showed that NAS formation in *K. aerogenes* similarly required the NTR system (but not the nitrogen assimilation control system) for nitrogen regulation. (Regulation of NIS formation also required the NTR system and may have been influenced by the nitrogen assimilation control system [5].) One final similarity between the regulation of NAS in the two organisms is the observation that all of the Nas^- mutants obtained were also Nis^- (unable to use nitrite as the sole source of nitrogen), even though the selection required only the loss of one or the other. Cali et al. argue that this may reflect an operon structure with *nis* genes downstream of *nas* genes (2). Whether an operon structure exists or whether there is some aspect of regulation that we do not yet understand is unclear.

One clear difference between the *nas* mutants of *K. aerogenes* and *K. pneumoniae* is that there was only a single locus identified by the 18 *K. pneumoniae* mutants isolated (2); the two nitrate-specific *K. aerogenes* mutants clearly define two distinct loci. Whether either of these corresponds to the locus defined by Cali et al. (2) is unknown and must await either mapping of the loci in the two organisms or cross-complementation with cloned genes.

Finally, we note that both the *K. aerogenes nas* mutants and the *K. pneumoniae nas* mutants retained NAR activity. These findings, coupled with the many differences identified by van 'T Reit et al. (9) listed above, lead to the conclusion that the structural genes for NAS and NAR are likely to be different or at least that NAS and NAR are expressed from different promoters.

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