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Bacterial Catalysis of Nitrosation: Involvement of the *nar* Operon of *Escherichia coli*

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We have developed a rapid and sensitive fluorimetric method, based on the formation of a fluorescent product from nitrosation of 2,3-diaminonaphthalene, for measuring the ability of bacteria to catalyze nitrosation of amines. We have shown in *Escherichia coli* that nitrosation can be induced under anaerobic conditions by nitrite and nitrate, that formate is the most efficient electron donor for this reaction, and that nitrosation may be catalyzed by nitrate reductase (EC 1.7.99.4). The *narG* mutants defective in nitrate reductase do not catalyze nitrosation, and the *fnr* gene is essential for nitrosation. Induction by nitrite or nitrate of nitrosation, N_2O production, and nitrate reductase activity all require the *narL* gene.

The concept that bacteria might participate in nitrosation (i.e., formation of an NNO bond to yield the *N*-nitroso derivative of amines) has been considered to some extent in most hypotheses concerning the possible role of endogenous nitrosation in cancer etiology (2, 4, 5, 16, 20, 22, 29). As recently as 1981, it was felt that the major contribution of bacteria to nitrosation was the reduction of nitrate to nitrite, resulting in an increase in the concentrations of nitrosating species (21). More recently, however, several groups have shown that bacteria can in fact participate directly in the nitrosation of amines, and this area has consequently gained renewed interest (4, 5, 16, 17, 18).

A number of bacterial genera, including *Neisseria*, *Pseudomonas*, *Escherichia*, *Klebsiella*, *Proteus*, *Alcaligenes*, and *Bacillus*, have been investigated from several experimental perspectives and reported to have some ability to catalyze nitrosation (4, 5, 8, 16, 17, 19, 20, 29). This catalysis is generally believed to be an anaerobic process which occurs in intact resting cells. It should probably be noted that, at this stage of research, it is not clear that all investigators have been examining the same catalytic activity.

Typically, these studies have been concerned with the identification of bacterial strains (mostly clinical isolates) capable of nitrosation, rather than characterization of the reaction itself. We have thus begun a detailed investigation of this activity in *Escherichia coli*, which is well characterized from both biochemical and genetic perspectives. To facilitate the study of a number of bacterial strains under a wide range of reaction conditions, we have developed a new assay. This assay is based on the formation of a fluorescent product upon nitrosation of 2,3-diaminonaphthalene (31) as an alternative to the gas chromatographic-thermal energy analysis method (11) for following nitrosation of secondary amines.

We have attempted to characterize the bacterial catalysis of nitrosation from a biochemical point of view by examining the effects of nitrite, nitrate, and electron donors on this process.

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Under anaerobic conditions, nitrate becomes the most favored electron acceptor which can induce nitrate reductase and repress the expression of fumarate and trimethylamine oxide reductases (14, 15). Nitrate reductase is encoded by the *narC*, *narH*, and *narI* genes, which are organized into an operon at min 27 of the *E. coli* chromosomal map (3, 9, 28). The operon is positively controlled by an activator protein encoded by the closely linked *narL* gene and responds to nitrate as an inducer (15, 27). The *narC* operon is inducible only anaerobically, and its expression also depends on the pleiotropic activator protein encoded by *fnr* (6, 27). This report will establish the involvement of nitrate reductase in nitrosation, as well as in N_2O production, and demonstrate the induction of the formate-nitrate oxidoreductase activity by nitrite through the *narL* gene.

MATERIALS AND METHODS

Chemicals. 2,3-Diaminonaphthalene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) could be used without further purification, but the background fluorescence was reduced by recrystallization. The necessity for recrystallization may vary among different lots of this chemical. The identity of the triazole as the nitrosation product was confirmed by isolation and crystallization of the compound (31) followed by mass spectrometry (model 5987; Hewlett-Packard Co., Palo Alto, Calif.). $Na^{15}NO_2$ was purchased from Merck & Co. Isotopes, St. Louis, Mo. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. LB growth medium was prepared as described previously by Miller (18).

Bacterial strains and growth conditions. *E. coli* K-12 strains are listed in Table 1.

Cells were grown overnight (15 to 17 h) at 37°C, without shaking, in 250-ml flasks filled to the top with LB medium. In some experiments, sodium nitrite (10 mM) or potassium nitrate (100 mM) was added to the medium as noted. The cells were then collected by centrifugation, washed twice with phosphate-buffered saline (PBS) (NaCl [8 g/liter], KCl [0.2 g/liter], Na_2HPO_4 [4.6 g/liter], KH_2PO_4 [0.8 g/liter]) at pH 7.3, and finally resuspended in PBS to yield the desired cell densities. Cells grown and harvested in the logarithmic phase exhibited the same nitrosation activity as did stationary cells; thus, for convenience, cells were grown overnight.

TABLE 1. *E. coli* K-12 strains

Strain	Description	Source
RF1005	*lacY hsdR supE44 supF* Thy⁻ *met*	D. Botstein
RF1595	RF1005 *narC205*::Tn*10*	This study
RF1625	RF1625 *narL215*::Tn*10*	This study
RK4353	*araD139 gyrA* Δ*lacU169 non rspL*	V. Stewart
RK5265	RK4353 *narC202*::Tn*10*	V. Stewart
RK5278	RK4353 *narL215*::Tn*10*	V. Stewart
RK5297	RK4353 *fnr250*	V. Stewart

For protein determinations, cell suspensions were heated at 90°C in 1 N NaOH for 10 min, cooled, and neutralized with HCl. Protein standards (bovine serum albumin) were treated similarly, and protein was measured via the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Transductions were mediated by bacteriophage P1Cm *clr-100* (24). Bacteriophage P1Cm *clr-100* lysates and lysogens were prepared, and transductions were performed according to the method of Miller (18). The construction of strain RF1595 was done by transduction of the *narC205*::Tn*10* marker to strain RF1005. The tetracycline-resistant colonies (15 μg/ml) were tested for nitrate reductase activity by the overlay procedure of Glaser and DeMoss (13). Activities in liquid cultures were measured by the same procedure except agar was omitted from all solutions, and changes in absorbance were monitored spectrophotometrically at 540 nm.

Nitrosation assay. The analysis is based on the formation of a fluorescent triazole (1-[H]-naphtho[2,3-d]triazole) following nitrosation of 2,3-diaminonaphthalene (Fig. 1). Nitrosation of 2,3-diaminonaphthalene is faster than is nitrosation of morpholine (the amine most typically used in previous work) at physiological pH 7.3 (Fig. 2). This reagent, then, not only provides a relatively easy and rapid method for assessing rates of nitrosation but also has allowed experiments at lower cell densities and with lower nitrite and amine concentrations than would have been practical with morpholine.

Reaction mixtures consisting of cells, nitrite, amine, and, in some cases, reducing donor were incubated at 37°C. Small (0.6-ml) closed Eppendorf tubes were used for anaerobic incubations without further control of anaerobiosis. For aerobic incubations, 10-ml open rotating tubes were used. The mixtures were made up to final volumes of 0.6 ml containing washed cells to yield 0.5 to 1.5 mg of protein per ml, sodium nitrite in PBS to yield final concentrations of 0.5 to 35 mM, 2,3-diaminonaphthalene to yield 0.2 mM, and, in some experiments, a reducing donor (formate, glucose, or ethanol at final concentrations of 20 mM and DL-lactate at a final concentration of 40 mM, as noted). After incubation for 15 to 60 min, the cells were collected by centrifugation and the supernatant was diluted 10-fold in PBS. This solution was analyzed directly in a spectrofluorometer (model 450; Sequoia-Turner Corp., Mountain View, Calif.) Excitation at 375 nm and observation at 450 nm gave adequate sensitivity

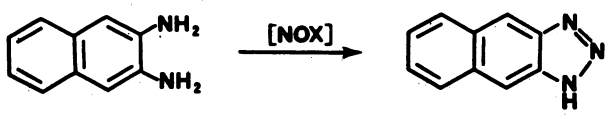

FIG. 1. Formation of 1-[H]-naphtho[2,3-d]triazole from nitrosation of 2,3-diaminonaphthalene. [NOX] is a general representation for a variety of related nitrosating agents.

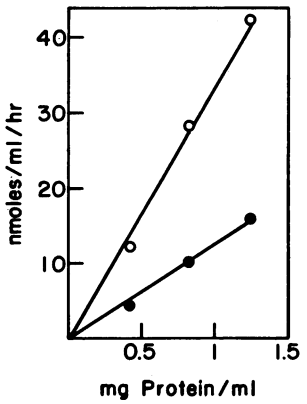

FIG. 2. Nitrosation of 2,3-diaminonaphthalene and morpholine catalyzed by RF1005. Bacteria were grown in LB medium and prepared and assayed anaerobically in the presence of 30 mM $NaNO_2$ as described in Materials and Methods. Symbols: ○, nanomoles per milliliter per hour of 1-[H]-naphtho[2,3-d]triazole; ●, nanomoles per milliliter per hour of *N*-nitrosomorpholine.

and minimum interference from other components of the complex mixtures.

Controls consisted of cells plus diaminonaphthalene without nitrite and diaminonaphthalene plus nitrite without cells (with and without reducing donors); all values reported in the tables and figures are corrected for control reactions. The rate of chemical nitrosation at pH 7.3 was about 2 nmoles/ml per h in the presence of 35 mM nitrite. The pH was monitored at the completion of the incubation and did not change sufficiently to significantly affect the outcome. Below pH 6, biological nitrosation of diaminonaphthalene was indistinguishable from chemical nitrosation; at pH 7.3, biological nitrosation was typically 10 to 100 times faster than chemical nitrosation was.

N_2O production. Production of N_2O was measured in the gas phase of vacuum-sealed tubes inoculated with cells, 20 mM sodium formate, and 20 mM $Na^{15}NO_2$ in PBS buffer. Gas samples were kept frozen until ready for analysis. The headspace above the frozen aqueous solution was sampled and analyzed for nitrous oxide. Fifty microliters of headspace was injected into a model 5970 gas chromatograph-mass spectrometer (Hewlett-Packard) with a 10-by-0.32-mm porous layer open tubular capillary column coated with Molsieve 5A (Chrompack, Inc., Bridgewater, N.J.). The initial column temperature was set to 175°C and increased 15°C/min after injection. The injection temperature was 220°C and the gas chromatograph transfer line temperature was set to 220°C. The carrier gas was helium, with a flow rate of 1 ml/min and a split ratio of 20:1. Selected ion monitoring of m/z = 44, 45, and 46 was used to monitor the three different permutations of nitrous oxide isotopes, namely, unlabeled, singly labeled (^{15}N), or doubly labeled ($^{15}N_2$). The area under the curve for different concentrations of a reference gas of nitrous oxide was used to construct a standard curve and to calculate the amount of nitrous oxide in each sample.

Nitrosation of morpholine. Incubations were carried out as described above for 2,3-diaminonaphthalene except that the concentration of morpholine was 15 mM, the concentration of sodium nitrite was 30 mM, and the reaction time was 60 min. After incubation, the reaction was quenched with NaOH. Methylene chloride (0.2 ml) was added and the mixture was vortexed and then centrifuged to separate the

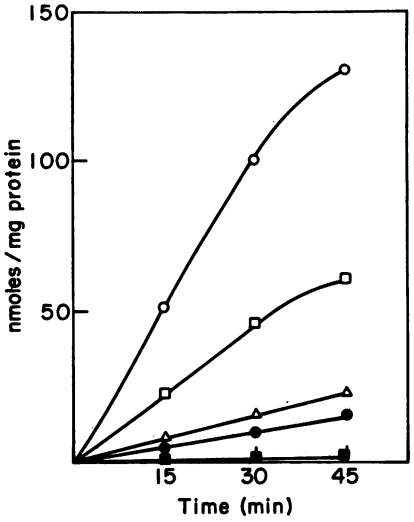

FIG. 3. Nitrosation of 2,3-diaminonaphthalene catalyzed by RF1005. Bacteria were grown in LB medium (△), LB plus 10 mM $NaNO_2$ (□), and LB plus 100 mM KNO_3 (○) as described in Materials and Methods. Closed symbols represent nanomoles per milligram of protein of 1-[H]-naphtho[2,3-d]triazole formed in the presence of 25 mM nitrite; open symbols represent nanomoles per milligram of protein of 1-[H]-naphtho[2,3-d]triazole formed in the presence of 25 mM nitrite and 20 mM formate. Assays were performed under anaerobic conditions as described in Materials and Methods.

layers. The methylene chloride layer was then analyzed by gas chromatography with a Thermal Energy Analyzer used as the detector (Thermo Electron Corp. Waltham, Mass.) (11).

RESULTS

Nitrosation of 2,3-diaminonapthalene versus nitrosation of morpholine. In most of the previous work, bacterial catalysis of nitrosation was measured by using morpholine as the nitrosatable amine (5, 16, 19). Figure 2 compares the ability of *E. coli* RF1005 to catalyze nitrosation of morpholine and 2,3-diaminonapthalene. Nitrosation in both cases is proportional to cell density. Despite a 75-fold-higher concentration, morpholine was nitrosated more slowly than was 2,3-diaminonaphthalene; this slower rate might have been due to the differences in pK_as for the two compounds. The pK_a for morpholine is 8.7, whereas that for 2,3-diaminonaphthalene, by analogy with aniline and 1,2-diaminobenzene, is approximately 4 (10, 16, 30).

Biochemical characterization. Nitrate is known to repress the synthesis of many proteins induced under anaerobic conditions (26). We consequently studied the effects of nitrate in the growth medium on bacterial catalysis of nitrosation. Additionally, since nitrite is a substrate for this reaction and might also induce nitrosation, we studied the effects of cell growth in the presence of nitrite. Finally, based on the knowledge that many anaerobic reactions involve oxidoreductase activity, i.e., that an electron donor might be required, we carried out experiments with a variety of potential donors present during the assay incubations. A series of such experiments with nitrate, nitrite, or formate as the electron donor is summarized in Fig. 3 and 4.

Figure 3 demonstrates that cells grown in the presence of nitrite or nitrate are better nitrosation cataylsts than are cells

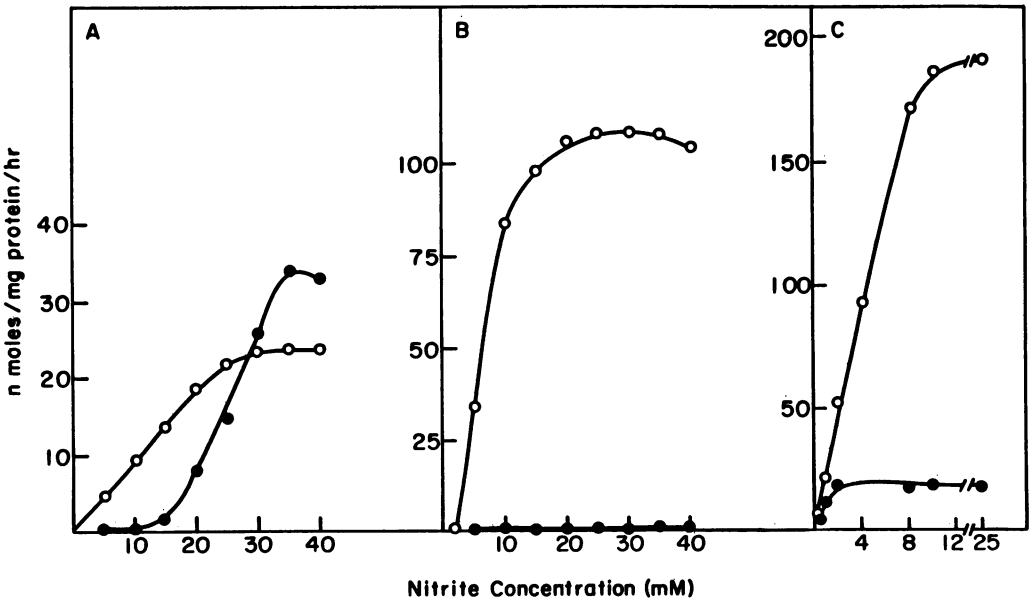

FIG. 4. Effect of nitrite concentration on catalysis of nitrosation of 2,3-diaminonaphthalene by RF1005. Cells were grown and assayed anaerobically as described in Materials and Methods. Symbols: ●, nanomoles per milligram of protein per hour of 1-[H]-naphtho[2,3-d]triazole formed in the absence of external donor; ○, nanomoles per milligram of protein per hour of 1-[H]-naphtho[2,3-d]triazole formed in the presence of 20 mM formate. (A) Cells grown in LB medium; (B) cells grown in LB medium plus 10 mM $NaNO_2$; (C) cells grown in LB medium plus 100 mM KNO_3.

TABLE 2. Nitrate induced nitrosation activity in wild-type, *nar*, and *fnr* mutants[a]

Strain	Relevant genotype	2,3-Naphthotriazole[b] (nmol/mg of protein per h)
RK4353	wild type	79
RK5265	*narG*	<0.5
RK5278	*narL*	3
RK5297	*fnr*	<0.5

[a] Cells were grown in LB plus 100 mM KNO_3 and assayed anaerobically in the presence of 25 mM $NaNO_2$ and 20 mM formate as described in Materials and Methods.
[b] 1-[H]-naphtho[2,3-d]triazole.

grown in their absence, provided that formate is present in the assay mixture.

Figure 4 illustrates the effect of nitrite concentration in the assay mixture on the catalytic ability of cells grown in LB medium (panel A), LB medium plus nitrite (panel B), and LB medium plus nitrate (panel C). The highest activities were observed with cells grown in the presence of nitrate and assayed in the presence of formate. Under aerobic conditions, these cells catalyzed nitrosation as efficiently as under anaerobic conditions (data not shown), i.e., the nitrosation activity was not inhibited by oxygen.

A set of experiments with other electron donors, i.e., lactate, glucose, and ethanol is summarized in Fig. 5. The highest activities were observed with formate and lactate.

Cells grown in the presence of nitrate may have any or a combination of several biochemical consequences which might be related to the catalysis of nitrosation. These consequences include induction of a formate-nitrate oxidoreductase, which may catalyze nitrosation and repression of fumarate and trimethylamine *N*-oxide reductases, which may compete with the nitrosation reaction.

To test for the possibility of nitrosation catalysis by nitrate reductase, we measured the formate-nitrate oxidoreductase activity of RF1005 grown in LB medium and in LB medium with either nitrite or nitrate. The respective activities (see Table 3) suggest that nitrite, as well as nitrate, can induce both nitrosation catalysis and formate-nitrate oxidoreductase activity and thus suggest that nitrate reductase may indeed be involved with nitrosation.

Genetic characterization. Calmels and co-workers (4) reported that a chlorate-resistant mutant of *E. coli* did not catalyze nitrosation. The specific gene was not identified and, moreover, nitrosation was measured in the absence of an electron donor, a fact that by itself may explain the lack of nitrosation activity in the mutant.

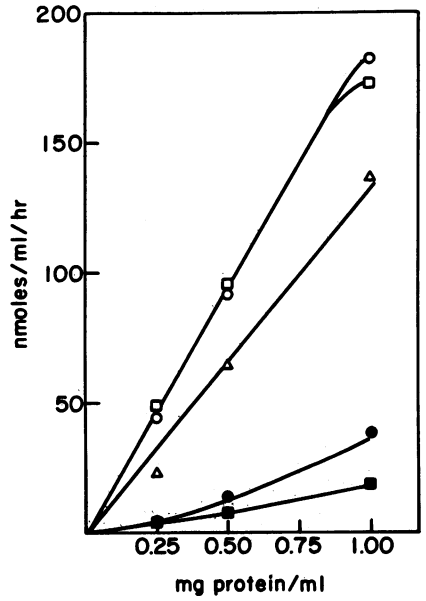

FIG. 5. Effect of electron donors on nitrosation of 2,3-naphthalene-diamine by RF1005. Cells were grown in LB medium plus 100 mM KNO_3 and assayed anaerobically in the presence of 10 mM $NaNO_2$ as described in Materials and Methods. Symbols: ■, nanomoles per milliliter of 1-[H]-naphtho[2,3-d]triazole formed in the absence of external donor; ●, 1-[H]-naphtho[2,3-d]triazole formed in the presence of 20 mM ethanol; △, reactions assayed in the presence of 20 mM glucose; □, reactions assayed in the presence of 40 mM DL-lactate; ○, reactions assayed in the presence of 20 mM formate.

We consequently tested the involvement of *fnr* and *nar* genes in nitrosation. The results (summarized in Table 2) show that mutations in *fnr* and *narG* genes destroy the ability of cells to catalyze nitrosation, whereas mutation in the *narL* gene results in decreased nitrosation activity.

Strain RK4353 did not exhibit nitrite-induced catalytic activity (see Discussion). To test whether this activity was

TABLE 3. Effects of nitrite and nitrate on nitrate reductase, N_2O production, and nitrosation activities in *E. coli*[a]

Strain	Growth conditions[b]	Nitrate reductase (μmol/mg of protein per min)	N_2O production (nmol/mg of protein per h)	Nitrosation of 2,3-diaminonaphthalene (nmol/mg of protein per h)
RF1005	A	0.03	14	12
	B	0.15	48	63
	C	1.12	95	182
RF1625	A	0.03	7	3
	B	0.03	12	2
	C	0.04	15	5
RF1595	A	<0.01	ND[c]	ND
	B	<0.01	ND	ND
	C	<0.01	ND	ND

[a] Cells were grown as described in Materials and Methods; nitrosation and N_2O production were assayed anaerobically in the presence of 25 mM $NaNO_2$ with formate (20 mM) as the electron donor.
[b] Abbreviations: A, cells grown in LB medium; B, cells grown in LB medium plus 10 mM $NaNO_2$; C, cells grown in LB medium plus 100 mM KNO_3.
[c] ND, Not detected.

also *nar* dependent, we constructed a *narG* mutant and a *narL* mutant in the RF1005 background. Since it has been suggested that nitrate reductase may be involved in N_2O production in *E. coli* (25), we have characterized these mutants for N_2O production as well. Table 3 summarizes the effects of nitrite and nitrate on nitrate reductase, N_2O production, and nitrosation activity in the *E. coli* parent 1005 and its mutants *narG* and *narL*. The *narG* mutant was defective in all three activities under all growth conditions, whereas the *narL* mutant exhibited residual activity which could not be induced by either nitrite or nitrate.

DISCUSSION

We have described an efficient method for measuring the ability of bacteria to catalyze nitrosation and have used this method to begin a comprehensive investigation of the biochemical and genetic factors which affect this ability.

This study demonstrates nitrosation catalysis by bacteria with physiological concentrations of nitrite (1 mM or less). We have shown that the ability of cells to catalyze nitrosation reactions can be induced anaerobically by nitrite and nitrate. Not all *E. coli* strains, however, show the nitrite-induced activity (e.g., RK4353). This lack of activity might be related to genetic or phenotypic differences between the strains. It has been noted that *E. coli* strains vary with regard to nitrite reductase activity (21). This activity will compete for nitrite with the nitrosation reaction and thus indirectly affect the nitrite-induced nitrosation.

The data suggest that the major nitrite reductase (EC 1.6.6.4) is not involved in catalysis of nitrosation, since chlorate-resistant mutants have no nitrosation activity but are partially constitutive for nitrite reductase (7). The formate-nitrite oxidoreductase (a minor nitrite reductase in *E. coli* strains) is probably also not involved directly since, in contrast to nitrosation, this activity is inhibited by oxygen (1).

This report further substantiates findings that nitrite may serve as a substrate for nitrate reductase in the production of nitrous oxide. It is possible that this process may be related to nitrosation catalysis and that both may be catalyzed by or require nitrate reductase. A relationship between nitrosation and denitrification was suggested by Garber and Hollocher (12) in denitrifying bacteria which catalyze these reactions via nitrite reductase and by Leach et al. (17), who observed higher nitrosation activities with denitrifiers than with non-denitrifiers.

The rates of nitrosation and N_2O production in *E. coli* are about 3 orders of magnitude lower than the rate of nitrate reduction. This finding suggests that if indeed nitrate reductase does catalyze these reactions, it is most likely a fortuitous activity of this enzyme.

In summary, we have established the involvement of the *nar* operon, suggesting a possible role for nitrate reductase in nitrosation catalysis. Full induction of this activity requires anaerobic growth in the presence of nitrate, along with an electron donor to drive the reaction. The potential for bacterial nitrosation catalysis may consequently have been underestimated, and many strains which have been reported to have low or no catalytic ability may in fact be able to catalyze nitrosation if grown and assayed under the appropriate conditions. Our data suggest that nitrite, as well as nitrate, regulates nitrate reductase activity via the *narL* gene and support the hypothesis that this enzyme can act on nitrite, catalyzing both nitrosation and denitrification. The induction by nitrite, as well as the requirement of the *narL* gene, further attests that both denitrification and nitrosation are involved in the complex nitrate and nitrite *E. coli* metabolism under anaerobic conditions.

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