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The *napF* and *narG* Nitrate Reductase Operons in *Escherichia coli* Are Differentially Expressed in Response to Submicromolar Concentrations of Nitrate but Not Nitrite

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***Escherichia coli* synthesizes two biochemically distinct nitrate reductase enzymes, a membrane-bound enzyme encoded by the *narGHJI* operon and a periplasmic cytochrome *c*-linked nitrate reductase encoded by the *napFDAGHBC* operon. To address why the cell makes these two enzymes, continuous cell culture techniques were used to examine *napF* and *narG* gene expression in response to different concentrations of nitrate and/or nitrite. Expression of the *napF-lacZ* and *narG-lacZ* reporter fusions in strains grown at different steady-state levels of nitrate revealed that the two nitrate reductase operons are differentially expressed in a complementary pattern. The *napF* operon apparently encodes a "low-substrate-induced" reductase that is maximally expressed only at low levels of nitrate. Expression is suppressed under high-nitrate conditions. In contrast, the *narGHJI* operon is only weakly expressed at low nitrate levels but is maximally expressed when nitrate is elevated. The *narGHJI* operon is therefore a "high-substrate-induced" operon that somehow provides a second and distinct role in nitrate metabolism by the cell. Interestingly, nitrite, the end product of each enzyme, had only a minor effect on the expression of either operon. Finally, nitrate, but not nitrite, was essential for repression of *napF* gene expression. These studies reveal that nitrate rather than nitrite is the primary signal that controls the expression of these two nitrate reductase operons in a differential and complementary fashion. In light of these findings, prior models for the roles of nitrate and nitrite in control of *narG* and *napF* expression must be reconsidered.**

Escherichia coli can respire anaerobically by using a number of alternative terminal electron acceptors, such as nitrate, nitrite, dimethyl sulfoxide, trimethylamine-*N*-oxide, and fumarate, in order to generate energy by electron transport-linked phosphorylation reactions (11, 12). To accomplish nitrate reduction, *E. coli* can synthesize three distinct nitrate reductase enzymes. The *napFDAGHBC* operon, located at 46.5 min on the chromosome and previously named *aeg-46.5* (6), encodes a periplasmic nitrate reductase enzyme homologous to the NapAB enzyme of *Alcaligenes eutrophus* (*Ralstonia eutropha*), *Rhodobacter capsulatus*, and *Thiosphaera pantotropha* (*Paracoccus denitrificans*) (1, 3, 15, 19, 20). The *narGHJI* operon, which has been the subject of intensive study for many years, encodes the major respiratory nitrate reductase located in the cytoplasmic membrane (11, 26). A third nitrate reductase, encoded by the *narZYWV* operon, is biochemically similar to the NarGHJI enzyme but is constitutively expressed at relatively low levels in the cell (2). The physiological rationale for why *E. coli* possesses three nitrate reductase operons and when two of these are expressed during anaerobic cell growth is not clear.

In this study we examined the patterns of *napF* and *narG* gene expression in response to different steady-state levels of nitrate by using *napF-lacZ* and *narG-lacZ* reporter fusions in anaerobic chemostat culture. The product of nitrate reduction, nitrite, was also tested for its ability to alter *napF* and *narG* expression. Finally, the levels of nitrate and nitrite in the chemostat vessel were measured to better understand the relationship between nitrate and nitrite consumption and *napF* and *narG* expression. The results of these continuous cell culture experiments reveal a differential and complementary pattern of nitrate reductase gene expression whereby the *napF* operon is expressed preferentially before the *narG* operon under limiting nitrate conditions.

MATERIALS AND METHODS

Bacteria, plasmids, and phages. All experiments were performed with *E. coli* MC4100 [F^- Δ(*argF-lac*)*U169 araD139* Δ(*argF-lac*)*U169 rpsL150 deoC1 relA1 flbB5301 rbsR ptsF25*] (21). The *lacZ* reporter fusions used to monitor expression of the two nitrate reductase operons were λHW2 (*napF-lacZ*) (this study) and λPC50 (*narG-lacZ*) (8). Since the respective fusions are integrated at the λ^{att} site on the chromosome, each strain is wild type for the *narGHJI* and *napF* operons. The *napF-lacZ* fusion was constructed by the generation of a DNA fragment containing the *napF* (*aeg-46.5*) control region from position −224 to position +169 relative to the start of transcription by using standard PCR protocols (17). The resulting DNA fragment was then inserted into plasmid pRS415 (22) to give the *napF-lacZ* operon fusion plasmid designated pHW2. The entire DNA insert in pHW2 was DNA sequenced to confirm the intended construction (18). The *napF-lacZ* fusion was then transferred onto λRS45 to generate λHW2. A high-titer lysate was then used to introduce the phage into MC4100 as previously described (22).

Cell growth. For routine cell growth and plasmid construction, cells were grown in Luria-Bertani liquid or solid medium. For batch cell culture, cells were grown in a glucose (40 mM) minimal medium (9). Where indicated, sodium nitrate (40 mM) or sodium nitrite (5 mM) was added to the growth medium after sterilization. Anaerobic growth was performed at 37°C in 10-ml anaerobic culture tubes fitted with butyl rubber stoppers (13). Cells grown overnight under identical conditions in the same medium were used for inoculation.

For continuous culture experiments, a Bioflo 3000 Bioreactor (New Brunswick Scientific, New Brunswick, N.J.) was fitted with a 2-liter glass vessel and operated at a 1-liter liquid working volume. A modified Vogel-Bonner medium supplemented with glucose (2.25 mM) was used to limit cell growth (i.e., carbon-limiting conditions) (25). During the experiments, the chemostat was maintained at 37°C and at a medium flow rate of 10 ml/min, which corresponds to a cell doubling time of 70 min. Anaerobic culture conditions were maintained by

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continuously sparging the vessel with oxygen-free nitrogen at a flow rate of 200 ml/min (25). To vary the concentration of nitrate or nitrite in the medium, sodium nitrate or sodium nitrite was added at the amount indicated after medium sterilization.

When the chemostat was shifted to a new nitrate (or nitrite) concentration, steady-state levels of gene expression were generally achieved within five reactor residence times. This was confirmed by monitoring the β-galactosidase activity of cells harvested from the reactor. To ensure that equilibrium was attained, the chemostat was maintained under the same conditions until the β-galactosidase values remained constant. The values obtained for each culture condition were independently determined at least twice, and there was less than 10% variation in the β-galactosidase activity.

Strain stability and purity were monitored by plating cell samples from the chemostat on 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) indicator plates each day to check for the loss of the *lacZ* fusion or for strain contamination (25). To ensure that no deleterious mutations had occurred in the *lacZ* fusion strains, cell samples were also periodically sampled and grown in batch culture to verify that the β-galactosidase level was identical to that of the wild-type strain originally used to inoculate the vessel. Finally, the chemostat was shifted periodically to the starting condition with no nitrate (or nitrite) added, and cell samples were taken for β-galactosidase assays to confirm strain stability after steady state was reached in the vessel.

Enzyme assays. β-Galactosidase assays were performed as described previously (9). One unit of β-galactosidase is defined as the hydrolysis of 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per min per mg of protein. Nitrate reductase activity was measured as previously described (14).

Nitrate and nitrite determination. To determine the concentrations of nitrate and nitrite in the culture medium, high-pressure liquid chromatography (HPLC) methods were used. The analytical conditions were as follows. A Waters 625 LC pump and 600E controller unit were used as the delivery system under ambient temperature conditions; the column was a Whatman Partisphere SAX cartridge (4.6 by 250 mm). A Shimadzu SPD-6AV UV-visible detector was used at a wavelength of 210 nm. The buffer was 50 mM phosphate (pH 3.0), and the elution rate was 1.0 ml/min. The sample injection volume was 50 μl. The sensitivity for nitrate detection was 0.03 μM, and that for nitrite was 0.04 μM.

Materials. ONPG was purchased from Sigma Chemical Co., St. Louis, Mo. X-Gal was obtained from International Biotechnologies, Inc., New Haven, Conn. Casamino Acids was from Difco Laboratories, Detroit, Mich. Nitrogen gas was supplied by Arco, Inc. All other chemicals used in this study were of reagent grade.

RESULTS AND DISCUSSION

Effect of nitrate concentration on *narG-lacZ* expression. To determine how *narGHJI* operon expression varies in response to different steady-state levels of nitrate, anaerobic continuous cell culture methods were employed with a strain containing a *narG-lacZ* reporter fusion (Fig. 1A). Gene expression was lowest in cells grown anaerobically in the absence of any added nitrate. When the concentration of nitrate in the added medium was 1 mM, expression was increased by twofold. Maximal *narG-lacZ* gene expression (i.e., a 90-fold increase) was not seen until the nitrate addition level was 8 mM. The cell has the potential to fine-tune *narG* gene expression over a wide range in response to nitrate availability. As the nitrate level was increased above 8 mM to 40 mM, gene expression remained relatively unchanged (Fig. 1A and data not shown). Interestingly, at between 3 and 4 mM nitrate added, a modest plateau in *narG-lacZ* gene expression was seen. This pattern was consistently reproduced in independent experiments (data not shown). It should be noted that since nitrate was continually being consumed by the cells in the chemostat growth vessel, the nitrate addition values shown in Fig. 1A are not the same as the actual level of nitrate present in the growth vessel and, thus, the level needed to induce *narG-lacZ* expression (see below).

The level of nitrate reductase activity in the *narG-lacZ* reporter strain was also examined over the same range of nitrate additions (Fig. 1B). Enzyme levels closely paralleled *narG-lacZ* expression (Fig. 1A). Nitrate reductase activity also exhibited a modest plateau from 2 to 4 mM nitrate added, and maximum enzyme activity was observed at above 7 mM nitrate. Since the *narG-lacZ* operon reporter fusion used in these studies was present on the chromosome at the *att* site for lambda integration, the wild-type *narGHJI* operon was preserved intact. The similar patterns of gene expression and enzyme activity suggest that the primary level of control of enzyme production occurs at the level of *narGHJI* transcription regulation.

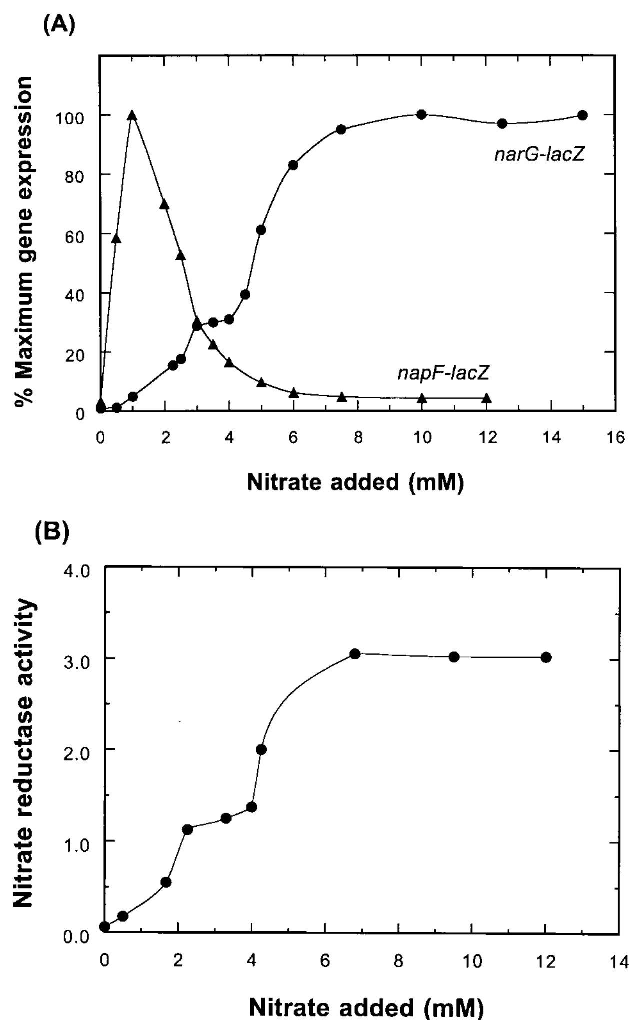

FIG. 1. Effect of nitrate on *narG-lacZ* and *napF-lacZ* expression during anaerobic cell growth. The amount of nitrate added via the medium addition to the chemostat vessel is indicated. When cells were shifted to a new condition, steady state was generally achieved within five residence times (see Materials and Methods). (A) Expression of the *narG-lacZ* fusion and the *napF-lacZ* fusion. The maximal levels of *narG-lacZ* and *napF-lacZ* expression were 16,000 and 5,800 U, respectively. (B) Effect of nitrate concentration on nitrate reductase enzyme activity in the *narG-lacZ* reporter strain. The chemostat was sampled after each steady state was achieved, and nitrate reductase activity was determined. The fermentor conditions were identical to those used for panel A.

Effect of nitrate concentration on *napF-lacZ* gene expression. The pattern of *napF-lacZ* expression seen in response to nitrate additions was strikingly different than that seen for *narG-lacZ* expression (Fig. 1A). Half-maximal *napF* gene expression was seen at 0.5 mM nitrate added, the condition where *narG-lacZ* expression was still minimal. Maximal *napF-lacZ* expression (ca. 30-fold induction) was achieved at 1 mM nitrate added. At higher levels of nitrate addition, *napF-lacZ* expression then declined to a near-basal level seen when no nitrate was added (Fig. 1A). At 4 mM nitrate added, *napF-lacZ* expression was less than 20% of the maximal expression level, conditions where *narG-lacZ* expression was not yet one-half of

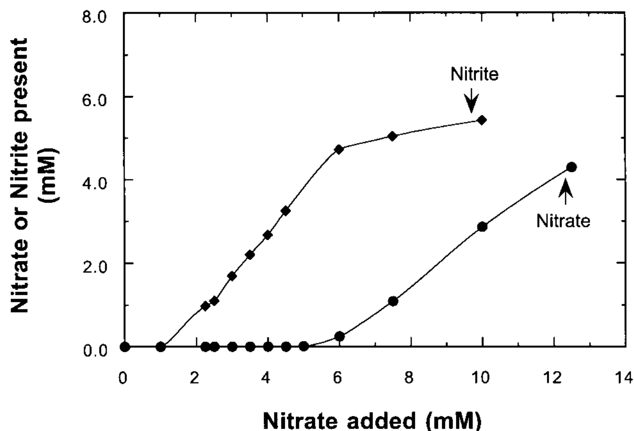

FIG. 2. Levels of nitrate remaining and nitrite accumulated in the chemostat vessel with different concentrations of nitrate added. Upon the shift to each new condition, steady state was generally achieved within five residence times. The vessel was then sampled, and the concentrations of nitrate and nitrite remaining in the growth medium were determined by HPLC (see Materials and Methods).

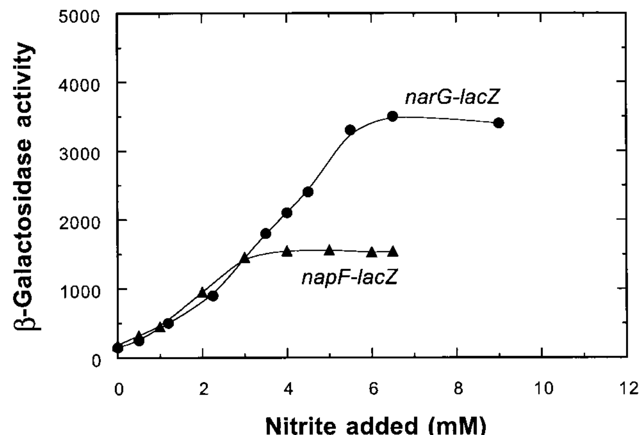

FIG. 3. Effect of nitrite on *narG-lacZ* and *napF-lacZ* expression during anaerobic cell growth. The amount of nitrite present in the medium addition was varied as described for Fig. 1A.

its fully induced level. These two nitrate reductase operons are differentially expressed in a complementary fashion in response to changing levels of nitrate addition. The *napF* operon is induced only within a low substrate concentration range, while the *narGHJI* operon is induced maximally only when considerably higher substrate levels are achieved. In the nitrate addition range of between 1 and 3 mM, both the NapF and NarG nitrate reductases appear to function simultaneously to consume nitrate, as evidenced by their overlapping patterns of gene expression.

Concentrations of nitrate and nitrite remaining in the culture medium. As noted above, the steady-state level of nitrate remaining in the chemostat vessel must be lower than the concentration of nitrate being added, since the cells are continually removing nitrate by reducing it to nitrite. HPLC methods were employed to measure the levels of nitrate and nitrite present in the vessel (Fig. 2). At nitrate addition levels of below 5.0 mM, no nitrate was detected in the vessel (where the detection limit was less than 0.03 μM nitrate [see Materials and Methods]). Above this addition level, the concentration of nitrate in the vessel was gradually increased in a linear fashion to 4.5 mM as the nitrate additions were increased to 12.5 mM. Within this range, nitrate was clearly present in excess of the cell's capacity to accumulate and reduce it.

Nitrite, the product of nitrate reduction, was also measured in the chemostat vessel (Fig. 2). Only when the nitrate addition level was above 1 mM was any nitrite detected (where the detection limit was 0.04 μM). As previously seen for nitrate accumulation, the nitrite concentration then increased in a linear fashion proportional to the nitrate additions until nitrite reached about 5 mM (i.e., when the nitrate was added at 6 mM). Thereafter, the nitrite level continued to accumulate but at a reduced rate. Over the latter range, nitrate was present in excess as evidenced by its steady-state accumulation in the vessel (e.g., to 4.5 mM). Four additional observations are noted. First, below the nitrate addition level of 6 mM, all of the added nitrate was consumed by the cells (Fig. 2). Correspondingly, under these conditions, all of the added nitrate was being reduced and excreted from the cells as nitrite except for the 1 mM nitrogen that was unaccounted for. This value was determined by subtracting the total amount of nitrate and nitrite remaining in the vessel at each steady-state condition from the amount of nitrate added. Second, nitrite accumulation occurred in the range where *narG-lacZ* gene expression, but not *napF-lacZ* expression, was increasing in response to nitrate additions (Fig. 1 and 2). Third, above the 6 mM nitrate addition level, where the cells exhibited fully induced levels of *narG-lacZ* expression, the amount of nitrite accumulated increased but at a much reduced rate. Finally, under the conditions used in this study (i.e., glucose added at 2.25 mM), the maximal capacity for nitrate consumption was about 2 mol of nitrate converted to nitrite per mol of glucose consumed.

The unaccounted or "missing" nitrogen (ca. 1 mM) is presumed to be further reduced to ammonia by one of the two *E. coli* nitrite reductase enzymes (7). It is conceivably either used for cell biosynthetic needs or excreted into the medium. Interestingly, maximal *napF-lacZ* expression occurred when no nitrite was being accumulated in the medium. This may be consistent with a role for NapAB in nitrate assimilation.

Effect of nitrite addition on *napF-lacZ* and *narG-lacZ* expression. To establish how *napF-lacZ* and *narG-lacZ* expression responds to addition of nitrite, the product of nitrate reduction by nitrate reductase, similar steady-state chemostat experiments were performed where nitrite was added in place of nitrate (Fig. 3). Three observations are readily apparent. First, nitrite additions did not lead to the maximal level of *napF-lacZ* expression seen when nitrate was added. Rather, nitrite caused a maximal level of induction that was about 25% of that seen for nitrate (i.e., 1,400 versus 5,800 U). To reach these respective maximum levels, a fourfold higher concentration of nitrite addition relative to nitrate addition was needed. For the maximal nitrite induction, the nitrite level remaining in the vessel was between 2 and 4 mM. As noted above, to give maximal gene expression with nitrate, the detectable level of nitrate in the vessel was below 0.03 μM. These findings suggest that nitrate is a stronger regulatory signal than nitrite by at least 2 to 3 orders of magnitude. Finally, *napF-lacZ* expression remained elevated when nitrite was present (Fig. 3). In contrast, when high levels of nitrate were present, *napF-lacZ* expression was reduced nearly to the basal level seen when no nitrate or nitrite was present (Fig. 1A). Nitrate, but not nitrite, is therefore required to repress *napF-lacZ* expression.

Nitrite addition also failed to elicit the same magnitude of *narG-lacZ* expression that nitrate addition did (Fig. 3). The maximal level of gene expression was only about 21% of that when nitrate was used (i.e., 3,500 versus 16,000 U). With nitrite

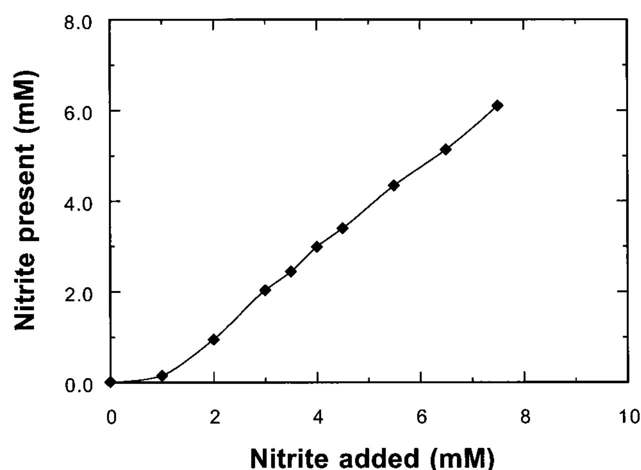

FIG. 4. Level of nitrite remaining in the chemostat vessel with different concentrations of nitrite added. Following the sampling of the vessel as described for Fig. 1B, the nitrite concentration was determined by HPLC (see Materials and Methods).

addition, gene expression slowly increased and reached the maximal level at 5 to 6 mM nitrite. These results indicate that nitrite is also inferior to nitrate as an inducer of *narG-lacZ* expression. The overall levels of gene induction caused by nitrite were only 7- and 18-fold for *napF-lacZ* and *narG-lacZ*, respectively. In contrast, nitrate caused 30- and an 90-fold inductions, respectively, of the two reporter fusions. Finally, as noted above, a significantly higher concentration of nitrite than nitrate was required to give the lower induction levels seen for nitrite. These data clearly demonstrate that nitrate is the more effective regulatory signal for controlling *narG* and *napF* gene expression.

We also examined whether the added nitrite accumulated in the vessel or was further metabolized by the cells (Fig. 4). When up to 1 mM nitrite was added, no nitrite accumulated in the vessel (i.e., accumulation was to less than 0.04 μM nitrite). At higher nitrite addition levels, nitrite accumulated, and its concentration increased proportionally to the nitrite additions. As some of the added nitrite was unaccounted for, the cells were reducing it further to ammonia for either cell biosynthetic needs or subsequent disposal.

Does nitrite antagonize *napF-lacZ* or *narG-lacZ* expression? To establish whether the cell can effectively discriminate between nitrate and nitrite as a signal for *napF-lacZ* expression, we examined the effect of nitrite-dependent induction in the presence of 0.5 or 1.0 mM nitrate (Fig. 5). Under these conditions, *napF-lacZ* expression was near maximal due to the added nitrate. When both anions were added to the vessel simultaneously, the nitrite addition at either an equimolar concentration or an eightfold molar excess relative to nitrate had little to no effect on *napF-lacZ* expression (Fig. 5A). Similar results were seen when nitrate was present at 1 mM (Fig. 5B). Therefore, nitrite is not a significant coinducer, corepressor, or antagonist of the cells' ability to recognize nitrate as a signal.

Similar studies were performed to examine whether nitrite can antagonize nitrate-dependent induction of *narG-lacZ* expression (Fig. 6). In the presence of 1.0 mM nitrate additions, *narG-lacZ* expression was induced to about 10% of its maximal level (see also Fig. 1A). However, the simultaneous addition of nitrite at either 1.0 or 5.0 mM had only a modest effect (ca. twofold) on further induction of *narG-lacZ* gene expression. Therefore, nitrite neither antagonizes the cells' ability to detect

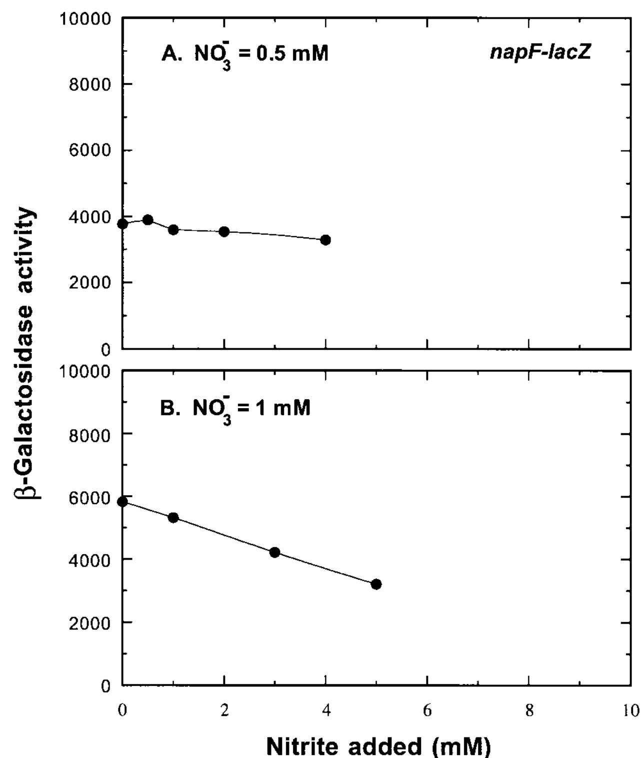

FIG. 5. Effect of various nitrite additions on *napF-lacZ* expression in the presence of 0.5 mM nitrate (A) and 1 mM nitrate (B). The medium addition contained a fixed amount of nitrate and the indicated amounts of nitrite. β-Galactosidase levels in cells adapted to the indicated oxyanion levels were monitored.

nitrate nor serves as a significant inducer molecule for *narG* gene expression. *E. coli* exhibits a considerable ability to discriminate between the two structurally related oxyanion molecules (discussed below).

Comparison of *napF-lacZ* and *narG-lacZ* gene expression in batch culture and continuous culture. Prior studies of *napF* and *narGHJI* operon expression in response to nitrate and nitrite additions had been done only with batch cell culture. However, it is evident from the chemostat studies described

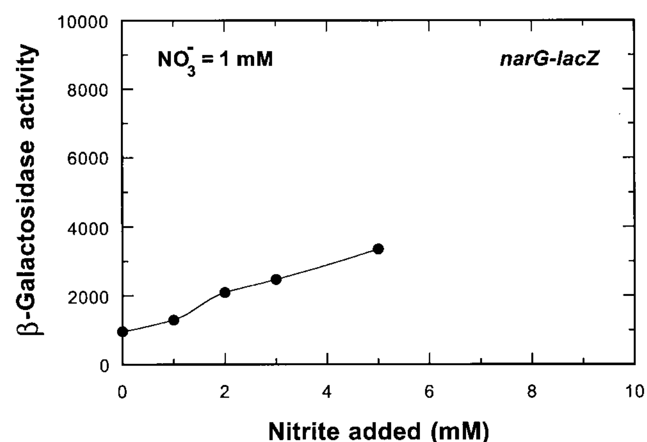

FIG. 6. Effect of increasing amounts of nitrite on *narG-lacZ* expression in the presence of 1 mM nitrate. The medium additions were as described for Fig. 5.

TABLE 1. Expression of *napF-lacZ* and *narG-lacZ* fusions during continuous culture and batch culture[a]

Fusion	β-Galactosidase υ (nmol of ONPG hydrolyzed/min/mg of protein)					
	Batch culture			Continuous culture		
	No addition	NO_2 added	NO_3 added	No addition	NO_2 added	NO_3 added
napF-lacZ	295	2,550	830	185	1,500	210
narG-lacZ	550	1,580	8,300	175	3,400	15,500

[a] Cultures were grown as described in Materials and Methods. For continuous cell culture experiments, a cell growth rate of 0.60 h^{-1} corresponds to a vessel dilution rate of 0.6 h^{-1}. The nitrate or nitrite addition level was 10 or 6 mM, respectively. For batch culture experiments, the cell growth rate was determined during anaerobic growth by measuring the optical density at 600 nm (see Materials and Methods) and corresponds to a cell generation time of 55 min. Nitrate or nitrite was present at 40 or 5 mM, respectively.

above that considerable regulatory information was not revealed by the batch culture studies. To directly compare the two methods, the same *napF-lacZ* and *narG-lacZ* reporter strains used in the continuous culture studies were also analyzed in batch culture (Table 1). For the *narG-lacZ* strain grown in the presence (40 mM) or absence of nitrate, the patterns of gene expression were similar, although the absolute levels of β-galactosidase were higher (by ca. twofold) during chemostat culture. This minor difference may be due to cell growth rate differences between the two methods. We previously demonstrated that *narG-lacZ* expression is mildly affected by cell growth rate (25).

For *napF* expression in batch culture, nitrite, not nitrate, was reported to be the primary inducer of *napF* gene expression (10). Under similar experimental conditions (Table 1), we also found that nitrate did not elicit as large a *napF-lacZ* induction as nitrite did. However, this conclusion is based on limited information obtained when nitrate and/or nitrite is present at very high concentrations in batch cultures. The batch culture experiments do not permit the effect of either low or intermediate levels of either oxyanion signal to be evaluated, because nitrate is rapidly consumed by the cells while nitrite is being accumulated (i.e., non-steady-state conditions). The continuous culture methods clearly demonstrate dramatically different patterns of *napF* and *narG* gene regulation in response to the two anions (Fig. 1 and 4). These studies have significant physiological importance because of the environmental habitats where nitrate and/or nitrite is limiting (e.g., soil, gut, or marine environments). The steady-state chemostat experiments reveal that low (micromolar) levels of nitrate but not nitrite can fully induce *napF* gene expression. Nitrate is also required for the subsequent repression of *napF* gene expression, while nitrite cannot cause such repression. Therefore, even at very high concentrations of nitrite (ca. 5 mM), it serves a minor regulatory role compared to nitrate (Fig. 1 and 4).

What nitrate level can *E. coli* sense? It is apparent that submicromolar to micromolar levels of nitrate are sufficient to induce both *narG-lacZ* and *napF-lacZ* gene expression. When the levels of nitrate remaining in the vessel (Fig. 2) were compared to the gene expression levels (Fig. 1A), induction of both reporter fusions was found to occur at a nitrate level below that detected by the analytical methods used here (below 0.03 μM nitrate). The *E. coli* nitrate two-component regulatory system, composed of the *narX*, *narQ*, *narL*, and *narP* gene products, must therefore be extremely sensitive to nitrate as an environmental signal. Nitrite, the product of nitrate reduction, appears to play a minor role in regulating the two nitrate reductase operons, since the effect elicited by millimolar levels of this anion were inferior to that elicited by micromolar levels of nitrate. Future studies to examine the roles of the two sensor-transmitter proteins, NarX and NarQ, and the two response regulators, NarL and NarP, should be informative concerning differences in the abilities of the two sensor-transmitter proteins and the two response regulator proteins to modulate nitrate-dependent induction of the *narGHJI* and the *napF* operons.

The *narGHJI* and *napF* operons are expressed in a complementary style. From the chemostat gene expression studies, we must conclude that the two nitrate reductases in *E. coli*, encoded by the *napF* and *narGHJI* operons, must serve quite different purposes in the cell. When the nitrate level is very low, the cell induces the "low-substrate" nitrate reductase encoded by *napF* operon to consume nitrate from the environment. The NapF nitrate reductase may therefore be predicted to have a higher (i.e., stronger) affinity for nitrate than does the *narGHJI*-encoded nitrate reductase. The V_{max} value might also be predicted to be lower than that of the NarG enzymes. Tests of these predictions must await the development of suitable assays for the biochemical purification and characterization of the NapAB enzyme.

Since the periplasmic NapAB enzyme is synthesized only at very low nitrate levels relative to the cytoplasmic membrane-bound NarG enzyme, it is interesting to speculate that nitrate reduction can still occur even when nitrate uptake into the cell is energetically unfavorable. Uptake of this anion would be needed to supply the substrate to the active site of NarG that is exposed to the cell cytoplasm. In contrast, no nitrate uptake is needed to supply the periplasmic NapAB enzyme.

When the nitrate level is further elevated, the alternative nitrate reductase, encoded by *narGHJI*, is then expressed and becomes the predominant enzyme in the cell. This can be termed the "high-substrate response" nitrate reductase. At the high nitrate level needed to fully induce *narG*, the NapAB enzyme is predicted from the gene expression data to be nearly absent in the cell (Fig. 1). Apparently, the two nitrate reductase enzymes have evolved to function in different ranges of nitrate availability in a complementary way to provide nitrate reduction. The complementary regulatory pattern for the *narG* and *napF* genes is analogous to the oxygen control of the dual cytochrome oxidase operons in *E. coli*, *cydAB* and *cyoABCDE*. These operons encode the high- and low-affinity cytochrome oxidases that are expressed under oxygen-limiting and oxygen-rich conditions, respectively (24).

Regulatory implications. From the chemostat studies it is apparent that the nitrate signal transduction system in *E. coli* is operative when the nitrate concentration in the culture medium (i.e., outside the cell) is in the submicromolar range (Fig. 2). This implies that one or both of the sensor proteins, NarX or NarQ, can detect this low concentration of nitrate in the environment. Greater than 100- to 1,000-fold higher levels of nitrite are needed to give lower levels of *narG* and *napF* induction (this study). Therefore, the prior model of Stewart and coworkers (10, 23) for nitrite- and nitrate-dependent gene control by the Nar regulon needs to be revised to account for the ability of the cell to discriminate between these two anions. The prior model for *napF* control states that nitrite is superior to nitrate as an inducer signal, because any nitrate present in the cell environment would lead to inactivation of NarL-phosphate via NarX cophosphatase activity. In contrast, nitrite presumably does not elicit this effect, so that nitrite is a better inducer of *napF* gene expression. However, the chemostat data invalidate these viewpoints. Studies to resolve the individual contributions of the NarL and NarP proteins at low substrate

levels to the activation and repression of *napF* gene expression are in progress.

The chemostat studies also demonstrate that *E. coli* has the ability to adjust the capacity for nitrate reduction by fine-tuning *narGHJI* expression over a wide dynamic range (ca. 90-fold). Under similar conditions, *napF* expression varied by about 30-fold (Fig. 1A). With this ability to control gene expression, the cell is thus presumably better able to conserve energy by not synthesizing unneeded nitrate reductase enzymes. Additionally, an alternative regulatory strategy whereby the cell uses an abrupt "switch" to turn on or turn off nitrate reductase gene expression is ruled out by the chemostat studies. We have previously proposed that both nitrate and nitrite are detected by NarX and NarQ by their respective periplasmic regions (4, 5). From the above chemostat data, NarX and NarQ must operate over a wide range of signal concentrations. In an accompanying in vitro study we demonstrate that NarX is able to detect nitrate over the range from about 5 to 500 μM. In contrast, nitrite is detected in the range from 500 μM to 30 mM as measured by anion-dependent stimulation of the NarX autokinase activity (16). Future studies to further elucidate the mechanism for this nitrate-nitrite sensing should provide valuable information for understanding the regulation of nitrate metabolism in *E. coli* as well as in many other types of microorganisms, including *A. eutrophus* (*R. eutropha*), *R. capsulatus*, *T. pantotropha* (*P. denitrificans*), and *Pseudomonas* (1, 15, 19, 20, 26).

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