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NarK Enhances Nitrate Uptake and Nitrite Excretion in *Escherichia coli*

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narK **mutants of *Escherichia coli* produce wild-type levels of nitrate reductase but, unlike the wild-type strain, do not accumulate nitrite when grown anaerobically on a glucose-nitrate medium. Comparison of the rates of nitrate and nitrite metabolism in cultures growing anaerobically on glucose-nitrate medium revealed that a *narK* mutant reduced nitrate at a rate only slightly slower than that in the NarK$^+$ parental strain. Although the specific activities of nitrate reductase and nitrite reductase were similar in the two strains, the parental strain accumulated nitrite in the medium in almost stoichiometric amounts before it was further reduced, while the *narK* mutant did not accumulate nitrite in the medium but apparently reduced it as rapidly as it was formed. Under conditions in which nitrite reductase was not produced, the *narK* mutant excreted the nitrite formed from nitrate into the medium; however, the rate of reduction of nitrate to nitrite was significantly slower than that of the parental strain or that which occurred when nitrite reductase was present. These results demonstrate that *E. coli* is capable of taking up nitrate and excreting nitrite in the absence of a functional NarK protein; however, in growing cells, a functional NarK promotes a more rapid rate of anaerobic nitrate reduction and the continuous excretion of the nitrite formed. Based on the kinetics of nitrate reduction and of nitrite reduction and excretion in growing cultures and in washed cell suspensions, it is proposed that the *narK* gene encodes a nitrate/nitrite antiporter which facilitates anaerobic nitrate respiration by coupling the excretion of nitrite to nitrate uptake. The failure of nitrate to suppress the reduction of trimethylamine *N*-oxide in *narK* mutants was not due to a change in the level of trimethylamine *N*-oxide reductase but apparently resulted from a relative decrease in the rate of anaerobic nitrate reduction caused by the loss of the antiporter system.**

Nitrate, which is utilized as a preferred electron acceptor for anaerobic respiration, suppresses the reduction of other potential electron acceptors such as trimethylamine *N*-oxide (TMNO) by *Escherichia coli*. Mutants designated as *narK* were originally identified by Stewart and MacGregor (20) among a group of Tn*10* insertion mutants of *E. coli* in which the reduction of TMNO was no longer suppressed by nitrate and which were closely linked to the *nar* region. Most of the mutants in this group produced little or no nitrate reductase and resulted from insertions in the structural (*narGHJI*) or regulatory (*narL*) genes for this enzyme. In contrast, the *narK* mutants produced normal levels of nitrate reductase, leading to the suggestion that a functional *narK* gene is required for nitrate-dependent repression of reductases for other potential electron acceptors. However, it has been found that *narK* mutations do not affect the normal repression of other terminal reductases by nitrate (7, 19), and the role of *narK* in nitrate metabolism and the basis for the phenotypic release of TMNO reduction from inhibition by nitrate has remained unclear.

During the characterization of the cloned *narGHJI* operon (16), we made the surprising observation that, in contrast to the wild-type parental strain, *narK* mutants failed to accumulate nitrite on plates when incubated with an overlay containing nitrate and formate (5), although extracts from the same strains contained normal levels of nitrate reductase. Furthermore, the ability to accumulate nitrite on plates was restored by transformation of the *narK* mutant with plasmids which contained the chromosomal region just upstream from the *narGHJI* operon (16). Noji et al. (12) have sequenced this region of the chromosome and found that the predicted *narK* gene product is a hydrophobic protein with likely multiple membrane-spanning regions. They postulated that the NarK protein is a nitrate transporter or a nitrate/nitrite antiporter.

We report here experiments which explored the possible roles of NarK in nitrate uptake and/or nitrite excretion. NarK is not absolutely required either for nitrate uptake or for nitrite excretion by *E. coli*; it does, however, appear to catalyze these processes in nitrate-respiring cells.

MATERIALS AND METHODS

Strains. All strains used in this study were derived from *E. coli* K-12 strain MC4100 (2). The *narK*::Tn*10* mutant RK5266 and its NarK$^+$ parent RK4353 (20) were kindly supplied by Valley Stewart. Strains JD1101 and JD1102 were *cysG*::Tn*5* derivatives of strains RK4353 and RK5266, respectively, constructed by P1 vir-mediated transduction of Kan^r from strain CBK103 [λ$^-$ *thyA36 cysG98*::Tn*5 polA1 deoC2* IN(*rrnD-rrnE*)*1*] obtained from the *E. coli* Genetic Stock Center (Yale University).

Plasmids pMV2 and pMV3 were derived by subcloning from plasmid pSR9 (16) and contain, respectively, an intact *narK* gene and a *narK* gene from which most of the *narK* open reading frame has been deleted.

Culture conditions and analyses. Inocula were prepared by growing cultures overnight in L broth (8) standing at 37°C. For the experimental cultures, 2 ml of the overnight culture was transferred to 50 ml of L broth plus 1% glucose and 100 mM potassium nitrate in a 200-ml-capacity tube fitted with a

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Klett tube side arm for observing culture turbidity and a three-way stopper for sparging and sampling. The culture was incubated in a 37°C water bath and sparged with a mixture of 95% N_2 and 5% CO_2. When indicated, the medium was buffered by including 100 mM potassium phosphate (pH 7.3) in the L broth or was supplemented with 100 mM TMNO.

Growth was monitored in the Klett tube by using a green filter. Samples (1.0 ml) were transferred to Eppendorf tubes on ice at 30-min intervals, and the cells were immediately removed by centrifugation for 10 min at top speed in an Eppendorf centrifuge in the cold room. The upper two-thirds of the supernatants were removed and stored at −15°C prior to analysis.

Nitrite was determined colorimetrically (17) in aliquots of each sample. Nitrate was determined from the level of nitrite present in the samples before and after the reduction of nitrate to nitrite with purified nitrate reductase. For this purpose, appropriate aliquots of each sample were incubated for 30 min at 37°C under argon in a 1.0-ml mixture including 100 mM potassium phosphate (pH 7.3), 1 mM methyl viologen reduced by the addition of sodium dithionite (MVH), and 1 U of nitrate reductase purified as described previously (18). It was established that purified nitrate reductase was not inhibited by the levels of TMNO that were present in some of the samples.

Enzyme activities. To determine the levels of enzymatic activities, we grew cultures as described above to approximately 80 Klett units. Cells were harvested by centrifugation, washed once with 50 mM potassium phosphate (pH 7.0), and finally suspended in the same buffer. Formate-linked nitrate reductase activity was assayed as previously described (15) except that samples for nitrite formation were taken from each incubation mixture at 5-, 10-, 15-, and 20-min intervals to establish a linear reaction rate. MVH-linked nitrate reductase activity was determined as previously described (17). TMNO reductase was assayed anaerobically by observing the oxidation of reduced benzyl viologen at 600 nm (7).

For the determination of NADH-linked nitrite reductase activity, cell suspensions were prepared as described above but finally suspended in 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA, 5 mM sodium ascorbate, and 2 mg of lysozyme per ml. The suspension was frozen at −15°C, and after thawing, cell debris and whole cells were removed from the crude extract by centrifugation at 15,000 × *g* for 15 min. Activity was determined as described by Coleman et al. (4).

Protein was determined by the procedure of Lowry et al. (9), and enzyme specific activities are expressed as units (micromoles per minute) per milligram of protein.

RESULTS

Since nitrate reductase is localized on the inner aspect of the cell membrane of *E. coli* (6, 11), the accumulation of nitrite in the medium of nitrate-respiring cells requires both the uptake of nitrate and the excretion of nitrite across the cell membrane. To explore the possible role of NarK in these processes, we compared the relative rates of the two processes in growing cultures of the wild-type parental strain RK4353 and the *narK* mutant RK5266 (Fig. 1). These results demonstrated that nitrate uptake was not absolutely dependent on a functional *narK* gene, since both strains removed nitrate from the medium. However, inactivation of the *narK* gene altered the relative rates of nitrate and nitrite reduction. In the parental strain, nitrate was reduced almost completely to nitrite before nitrite reduction commenced, while the *narK* mutant reduced nitrate somewhat more slowly and nitrite was reduced almost as rapidly as it was formed so that little accumulated in the medium until the culture reached the stationary phase. The *narK* mutant grew to a higher cell density and produced a significantly more acid medium, reaching a pH between 5.0 and 5.5, in comparison with the wild-type strain, which reached a pH of 5.5 to 6.0 (data not shown). Growth on buffered L broth increased the final cell yields but did not change the nitrite accumulation patterns or the relative cell yield differences between the two strains. The wild-type nitrite accumulation pattern was restored when RK5266 was transformed with plasmid pMV2, which carries the intact *narK* gene (Fig. 1C), but not when it was transformed with plasmid pMV3, which carries a partially deleted *narK* gene.

The levels of nitrate and nitrite reductase activities were very similar in RK4353 and the *narK* mutant RK5266 when grown on L broth plus glucose and nitrate (Table 1), indicating that the failure of the *narK* mutant to accumulate nitrite was not due to an increase in nitrite reductase relative to that of nitrate reductase. Nitrite reductase activity was determined in crude extracts with NADH as the electron donor (4) since preliminary experiments (not shown) established that when grown under these conditions neither strain expressed detectable levels of pyruvate- or formate-linked nitrite reductase activity (13) in either whole-cell suspensions or cell extracts. Nitrate reductase was measured both as MVH-linked activity, which measures the terminal reductase activity directly, and as formate-linked activity, which assesses the capacity to reduce nitrate with electrons derived from physiological donors through the membrane quinone pool. The specific activity for each was slightly higher in the *narK* mutant. Somewhat surprisingly, washed cell suspensions of the *narK* mutant accumulated nitrite in the assay medium when formate was the electron donor at essentially the same rate as the parental strain (Table 1), even though the growing culture of the mutant had accumulated no nitrite at the time of harvest. Addition of formate to the growing *narK* strain did not stimulate nitrite accumulation, demonstrating that the failure to accumulate nitrite during growth was not due to a limitation of an effective electron donor for nitrate reduction. Rather, it appears that the washed cell suspensions reduced nitrate but not nitrite when formate was the sole electron donor and that the accumulating nitrite was excreted into the medium.

Based on the activities shown in Table 1, the failure of the growing *narK* mutant to accumulate nitrite is not the result of a change in the ratio of nitrate reductase to nitrite reductase present in the cells but rather must result from an alteration in the relative flux through these two enzyme reactions caused by the *narK* lesion. A possible explanation for a change in the relative flux through these two reactions in growing *narK* mutant cultures is that a functional NarK protein is required for excretion of nitrite by actively growing *E. coli*. In this case, nitrite would be excreted by the wild-type strain as rapidly as it was formed, while in the *narK* mutant, nitrite would accumulate inside the cells and, perhaps by mass action, slow the rate of nitrate reduction to that corresponding to nitrite reduction. To test this hypothesis, we examined the accumulation of nitrite in *cysG* derivatives, which produce no detectable nitrite reductase activity (10). Figures 2A and B compare the growth and accumulation of nitrite in strains JD1101 (*cysG*) and JD1102 (*cysG narK*) growing on L broth plus glucose and nitrate. Both strains accumulated nitrite stoichiometrically in the

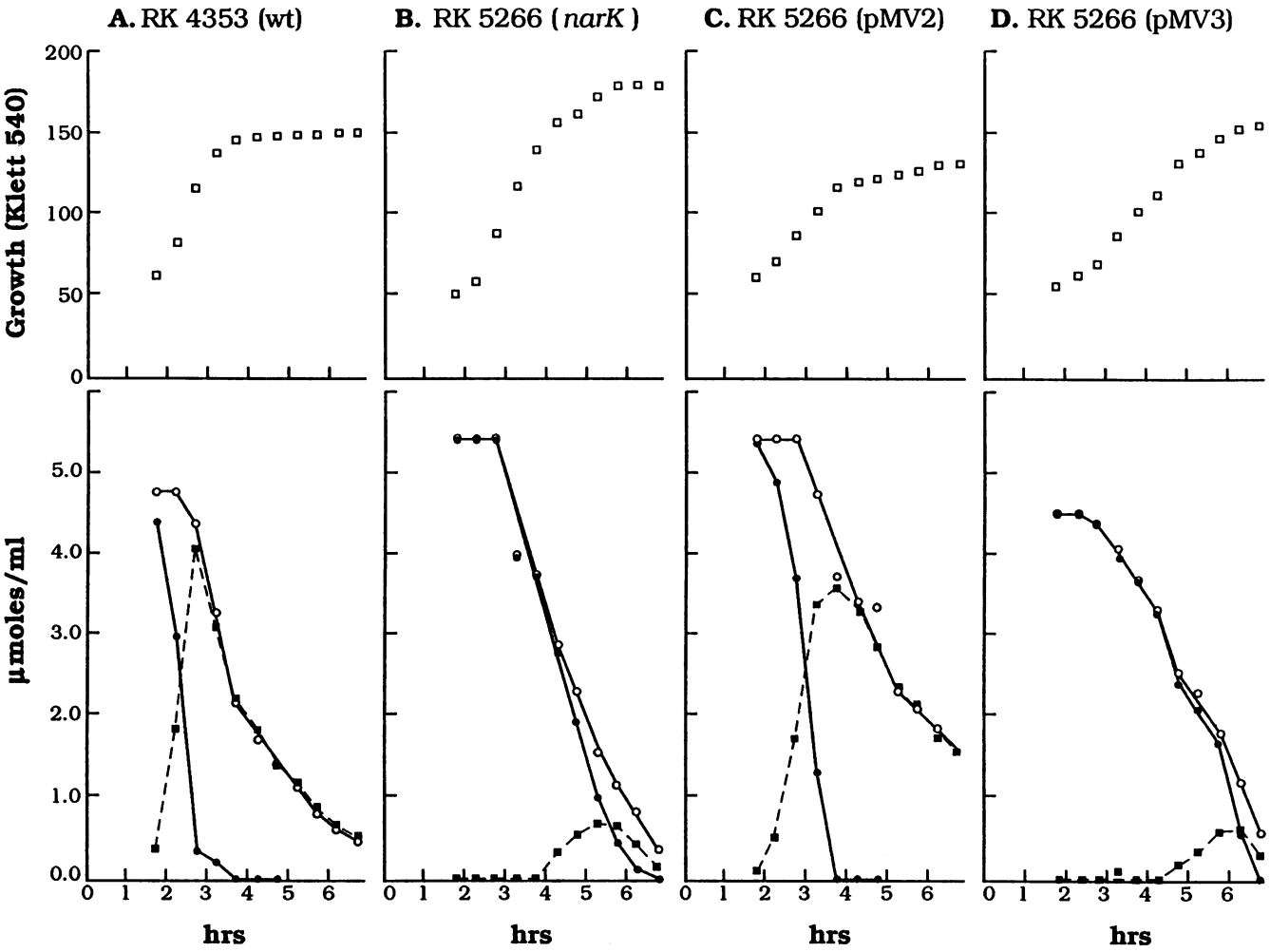

FIG. 1. Comparison of nitrate metabolism in the *narK* and parental strains. Cells were grown anaerobically in L broth plus 1% glucose and 5 mM potassium nitrate, and samples of culture medium were analyzed for nitrate and nitrite as described in Materials and Methods. Plasmid pMV2 contains an intact *narK* gene and promoter, while pMV3 has most of the *narK* open reading frame deleted. Symbols: ■, nitrite; ○, nitrite plus nitrate; ●, nitrate (calculated). wt, Wild type.

TABLE 1. Activity levels in the *narK* mutant and parental strains

Strain	Glucose in growth medium[a]	Nitrate reductase[b]		Nitrite reductase[c] (NADH)
		Formate	MVH	
RK4353	+	0.65	2.1	0.82
RK5266	+	0.91	2.7	0.86
RK4353	−	0.65	2.5	0.00
RK5266	−	0.41	3.7	0.00

[a] Cells were grown anaerobically on L broth containing 0.1 M potassium phosphate (pH 7.5) and 10 mM nitrate with 1% glucose as indicated. Cells were harvested at 70 to 75 Klett units.

[b] Nitrate reductase was determined in washed cell suspensions with formate or MVH as the electron donor and is expressed as micromoles of nitrite formed per minute per milligram of protein.

[c] NADH-nitrite reductase was determined in a crude extract and is expressed as micromoles of NADH oxidized per minute per milligram of protein.

medium, although there was a distinct lag in the *narK* culture before the maximum rate of nitrite appearance in the medium was reached. Figure 2C shows the magnitude of this lag relative to the increase in cell mass; once nitrite accumulation was initiated, the differential rate of nitrite accumulation in the medium was slightly slower for the *narK* strain.

The demonstration that washed cell suspensions of the *narK* mutant could reduce nitrate to nitrite with formate as the electron donor at essentially the same rate as the parental strain (Table 1) appeared to contradict the report by Noji et al. (12) that a cell suspension of a *narK* mutant could remove nitrate from the medium at only a very limited rate relative to the wild-type strain under very similar conditions. Although the levels of nitrate used by Noji et al. (12) in their determinations were not specified, a very high concentration of nitrate (100 mM) was used for the measurements reported in Table 1. We therefore investigated the reduction of nitrate to nitrite by the cell suspensions at limiting nitrate concentrations (Fig. 3). The wild-type strain RK4353 exhibited saturation kinetics for nitrate, with the half-maximal rate occurring at approximately 0.4 mM. The initial rates of nitrite formation with the mutant suspensions were significantly

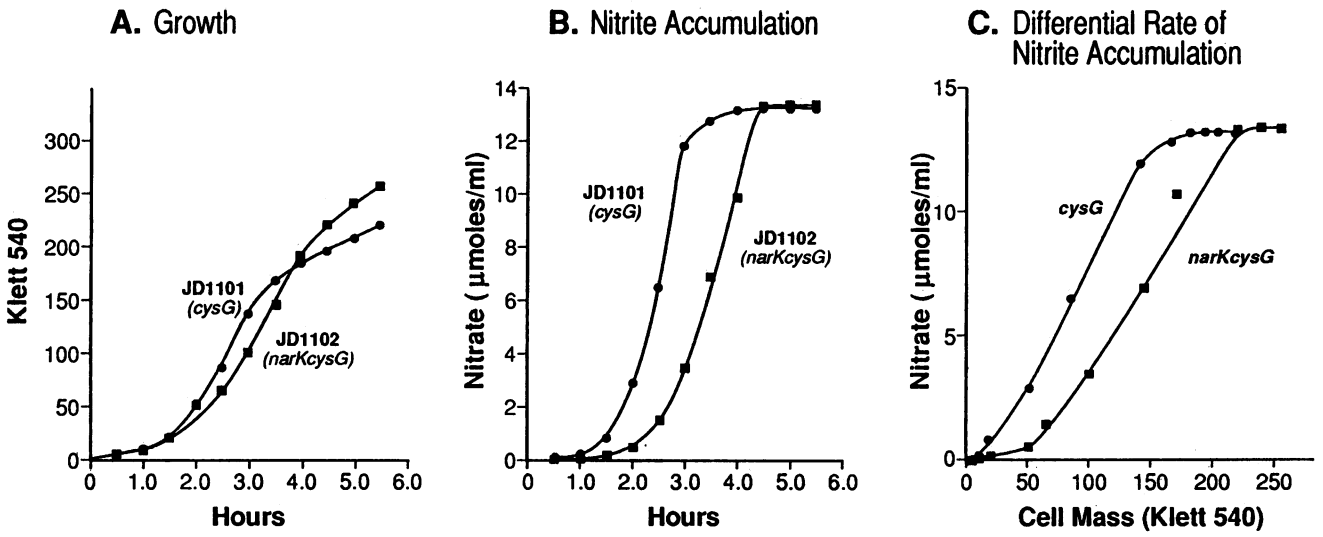

FIG. 2. Nitrite accumulation by *cysG* derivatives of *narK* and parental strains. Each strain was grown anaerobically in L broth containing 100 mM potassium phosphate (pH 7.3), 1% glucose, and 13 mM potassium nitrate. Growth and nitrite accumulation were determined as indicated in Materials and Methods.

reduced at concentrations of nitrate less than 10 mM, with the apparent half-maximal rate occurring at approximately 1.7 mM nitrate. Since nitrate reductase levels were essentially the same in the parental and mutant strains (Table 1), these results suggested that nitrate uptake became limiting in the mutant strain at low nitrate concentrations; however, at nitrate concentrations of 30 mM and greater, cell suspensions of the mutant appeared to catalyze nitrate reduction at a rate similar to that of the parental strain.

That a growing culture of the *narK* mutant is able to excrete nitrite as well as take up and reduce nitrate was also demonstrated when cultures were grown in the absence of glucose. The growth and nitrite accumulation patterns of the wild-type and the *narK* strains on buffered L broth plus nitrate with and without glucose are shown in Fig. 4. During growth on glucose, the wild-type strain RK4353 accumulated nitrite transiently in the medium as expected, while in the absence of glucose it grew to only about one-half the cell density of the glucose culture and nitrate was converted stoichiometrically to nitrite which accumulated in the medium (Fig. 4A). When glucose was added to the L broth culture midway through its growth phase, RK4353 grew to full cell density and the accumulated nitrite was completely reduced after nitrate was exhausted. As expected, during growth of RK5266 on glucose, no nitrite accumulated although nitrate disappeared from the medium at a rate similar to that of the wild-type cultures (Fig. 4B). In contrast, when the mutant strain was grown in the absence of glucose, nitrate was converted stoichiometrically to nitrite, which accumulated in the medium in this case, and when glucose was added to the L broth culture at midgrowth, the cultured cells resumed a more rapid growth rate and the accumulated nitrite was rapidly removed from the medium and presumably reduced to ammonia. For either strain, growth in the absence of glucose resulted in the complete cessation of nitrite reduction, and under these conditions, the *narK* mutant excreted the nitrite formed from nitrate reduction into the medium.

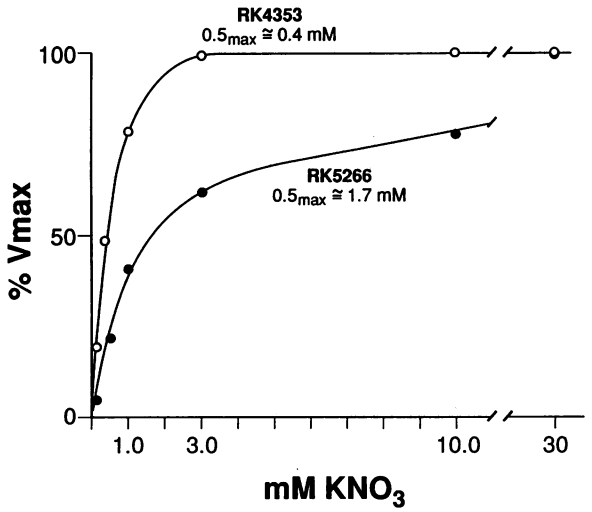

FIG. 3. Effect of nitrate concentration on the rate of nitrite formation by cell suspension. Washed cell suspensions were incubated with 50 mM formate as the electron donor and various levels of nitrate. Initial reaction rates of nitrite formation were determined as described in Materials and Methods. The rates were the same at 30 and 50 mM nitrate for each strain, and these values were designated V_{max}.

When grown without glucose, both strains produced slightly higher levels of MVH-nitrate reductase and both were completely devoid of NADH-nitrite reductase activity (Table 1). Thus, when nitrite reduction was suppressed, the *narK* mutant accumulated and excreted nitrite into the medium, and under these conditions, the rate of nitrate reduction was significantly diminished compared with that of the glucose-grown culture (Fig. 4B). Nitrate reductase levels were slightly higher in the cells grown without glucose (Table 1), suggesting that nitrate reduction is slowed by the accumulation of nitrite in the mutant. In the absence of glucose, the parental strain accumulated similar levels of nitrite without diminishing the rate of nitrate reduction, suggesting that the presence of NarK enhanced the rate of

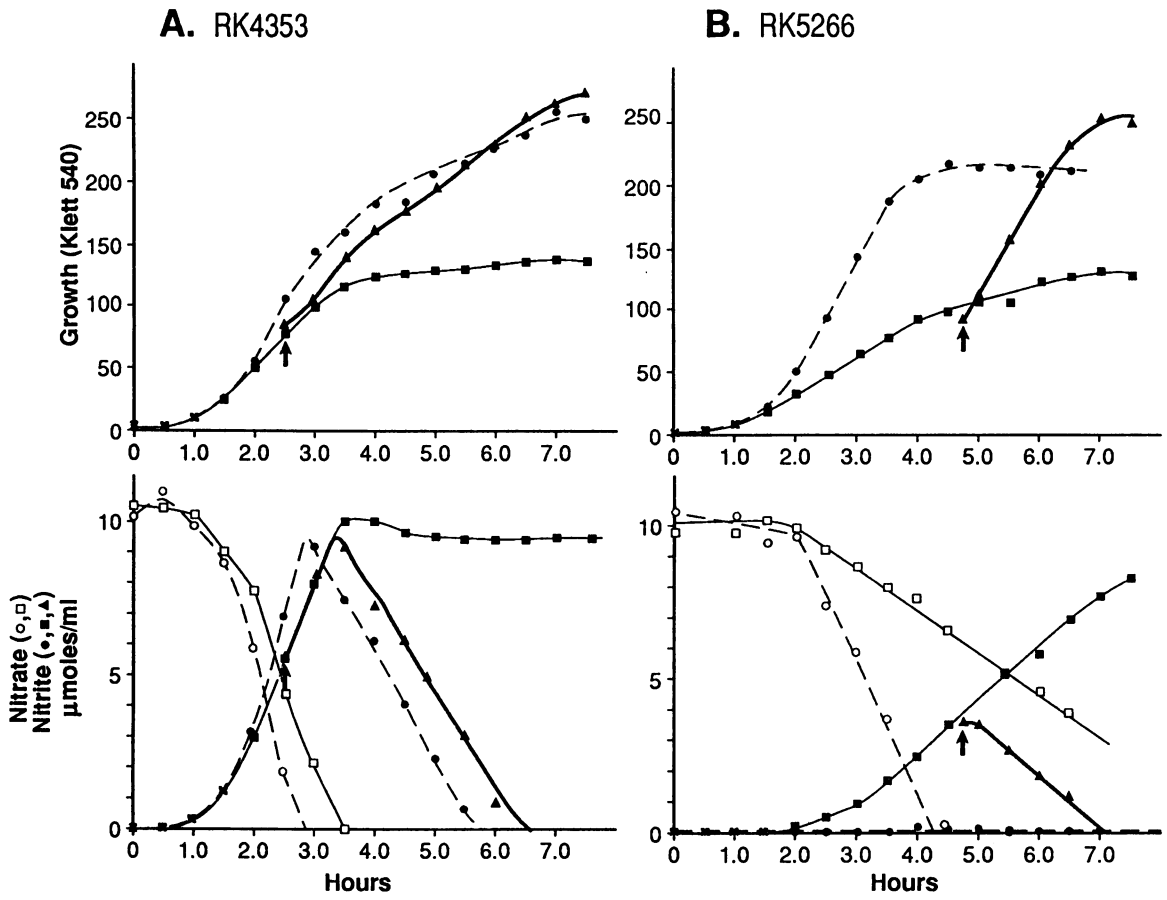

FIG. 4. Effect of glucose on nitrate metabolism in the *narK* mutant and parental strains. Strains were grown anaerobically in L broth containing 100 mM potassium phosphate (pH 7.2) and 10 mM potassium nitrate with or without 1% glucose. Glucose was added to the medium without glucose at the time indicated by the arrow. Nitrate and nitrite were determined as indicated in Materials and Methods. – – –, plus glucose; ——, no glucose; ——, glucose added at arrow.

nitrate reduction under these conditions. Apparently, nitrite can exit from the mutant cells but not rapidly enough to sustain an optimum rate of nitrate reduction.

The *narK* mutants were originally isolated by Stewart and MacGregor (20) based on their distinctive phenotype on MacConkey agar containing TMNO and nitrate. When wild-type *E. coli* was grown on MacConkey agar with TMNO, the colonies remained white due to the reduction of TMNO to trimethylamine, while the addition of nitrate caused the colonies to turn red, presumably because nitrate suppressed the reduction of TMNO. When the *narK* mutant was streaked on the same medium, nitrate did not suppress TMNO reduction and the colonies remained white. Figure 5 shows the general effects of nitrate on TMNO reduction by the *narK* mutant and its parental strain during growth on L broth containing nitrate and TMNO. While anaerobic growth of either strain in L broth with glucose and nitrate was accompanied by a decrease in the medium pH to 5.5 to 6.0 as nitrate was reduced to nitrite, when either strain was grown anaerobically in L broth with glucose and TMNO, the medium pH remained close to pH 7.0 as the TMNO was reduced to trimethylamine (data not shown). In the medium containing both TMNO and nitrate, the parental strain produced an increasingly acid pH and reduced nitrate to nitrite at a rate equivalent to that without TMNO (Fig. 5A). In contrast, the reduction of nitrate appeared to be completely inhibited during anaerobic growth of the *narK* mutant in this medium (Fig. 5B) and the pH of the medium remained near 7.0, indicating that TMNO was being reduced to trimethylamine at a significant rate. The growth rate and yield for the *narK* mutant were also dramatically increased in the presence of TMNO, while the growth pattern of the parental strain was similar to that which occurred on nitrate alone. The preferential utilization of nitrate as an electron acceptor for anaerobic respiration has been altered in the *narK* mutant so that nitrate reduction is inhibited and TMNO is apparently reduced to trimethylamine when both electron acceptors are present.

Table 2 shows the levels of TMNO reductase produced by the *narK* mutant and parental strains when grown anaerobically on TMNO with and without nitrate. As shown by others (7), TMNO alone induced the expression TMNO reductase about 10-fold in wild-type parental strain RK4353 and nitrate repressed the induced level as well as the uninduced level about 50%. For the *narK* mutant, the level of expression induced by TMNO was essentially the same as in the parental strain, but in the presence of nitrate, there was a substantial increase in the level of TMNO-induced activity compared with the parental strain. Furthermore, nitrate repression of TMNO reductase induction was re-

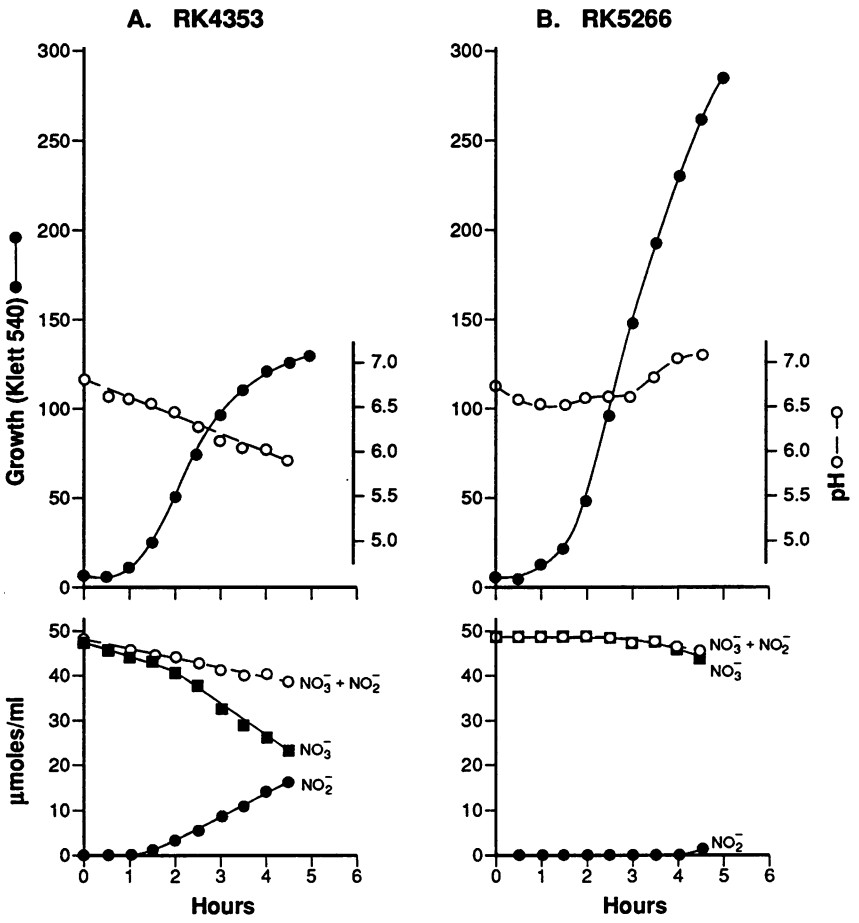

FIG. 5. Effect of TMNO on nitrate metabolism in the *narK* and parental strains. Strains were grown anaerobically in L broth containing 100 mM potassium phosphate (pH 7.3), 1% glucose, 50 mM potassium nitrate, and 50 mM TMNO. Growth, nitrate, nitrite, and pH of the medium were determined as indicated in Materials and Methods.

stored when RK5266 was transformed with plasmid pMV2 ($NarK^+$) but not with pMV3 ($NarK^-$).

There was no reciprocal effect of TMNO on nitrate induction since the activities of both formate- and MV-linked nitrate reductase induced by nitrate were similar in both the mutant and parental strains with or without TMNO in the medium (data not shown).

DISCUSSION

Boogerd et al. (1) proposed that two separate systems are involved in the uptake of nitrate by *Paracoccus denitrificans*, which catalyzes the reduction of nitrate to nitrite on the cytoplasmic side of the inner membrane and the reduction of nitrite on the periplasmic side of the inner membrane. One system, a nitrate/proton symporter, was postulated to be a high-affinity transporter which initiated nitrate reduction, while the second was postulated to be a nitrate/nitrite antiporter which helped sustain anaerobic nitrate respiration by delivering nitrite to its site of reduction. In studies on the kinetics of nitrate reduction in whole cells and vesicles of *P. denitrificans*, Parsonage et al. (14) found no evidence supporting either of these transport mechanisms. They concluded that nitrate uptake depended on a transport system or a specific pore, while nitrite diffused passively across the inner membrane in its protonated form. In a study using [^{13}N]nitrate, Thayer and Huffaker (21) demonstrated that *Klebsiella pneumoniae* expresses two assimilatory nitrate transport processes. One high-affinity system catalyzed the active transport of nitrate against a concentration gradient, but it was not possible to establish whether the second, low-affinity system represented active transport, facilitated diffusion, or passive permeability of nitrate.

For nitrate reduction to occur in *E. coli*, nitrate must cross the cell membrane in order to interact with nitrate reductase (6, 11). As shown here, the nitrite formed during the anaerobic respiration of nitrate is rapidly excreted into the medium, and when nitrate is exhausted, nitrite is taken up again and reduced to ammonia (3). Nitrite reduction in *E. coli* is catalyzed by an NADH-linked nitrite reductase located in the cytoplasm or a formate-linked nitrite reductase located in the periplasm (13). With the strains and growth conditions used in the studies presented here, the latter pathway appeared to be inoperative and therefore the excreted nitrite had to cross the cell membrane again to be reduced by NADH-linked nitrite reductase to ammonia.

On the basis of the results presented here, we conclude that *E. coli* is capable of taking up nitrate and excreting nitrite in the absence of a functional NarK protein. However, the presence of a functional NarK protein appears to alter the rates of both of these processes. At concentrations of nitrate of less than 10 mM, the initial rates of nitrite

TABLE 2. Induction and repression of TMNO reductase in *narK* and parental strains

Strain	Addition(s) to medium[a]	TMNO reductase (mmol/min/mg of protein)
RK4353	None	0.05
	Nitrate	0.02
	TMNO	0.48
	TMNO, nitrate	0.25
RK5266 (*narK*)	None	0.04
	Nitrate	0.03
	TMNO	0.49
	TMNO, nitrate	0.63
RK5266 (pMV2)	TMNO	0.48
	TMNO, nitrate	0.26
RK5266 (pMV3)	TMNO	0.58
	TMNO, nitrate	0.67

[a] Growth was in buffered L broth with 1% glucose plus 50 mM TMNO and 100 mM nitrate as indicated.

formation from nitrate by cell suspensions were significantly reduced in the *narK* mutant relative to those of the parental strain (Fig. 3), suggesting that the *narK* gene encodes a high-affinity uptake system which enhances nitrate uptake at low concentrations. In addition, at high concentrations of nitrate the capacity of the *narK* mutant to excrete nitrite during growth appeared to be significantly reduced. When growing on glucose, the *narK* mutant reduced nitrate at a rate which was only slightly slower than that of the parental strain, but when growing in the absence of glucose, the *narK* mutant catalyzed the reduction of nitrate at a significantly slower rate while the parental strain rate remained effectively unchanged (Fig. 4). Under the latter conditions, the two strains produced similar levels of nitrate reductase but no detectable nitrite reductase activity. The dramatic decrease in flux through the nitrate reductase reaction in the mutant but not the parental strain under these conditions suggests that the mutant but not the parental strain under these conditions suggests that the mutant cannot excrete nitrite as effectively as the parental strain, leading to accumulation of internal nitrite, which in turn slows nitrate reduction.

It is not clear whether the NarK-independent movement of nitrate and nitrite across the cell membranes in the *narK* mutant involves separate transport systems or passive diffusion. However, the excretion of nitrite appears to be inhibited during growth of the *narK* mutant on glucose so that nitrite accumulating within the cells slows nitrate uptake and/or reduction to a rate equal to that of nitrite removal by the nitrite reductase reaction. The inhibition of nitrite excretion in the *narK* mutant is circumvented when nitrite reductase is absent (either in the *cysG* derivative or during growth without glucose). The inhibition is also circumvented when the glucose-grown cells are harvested, washed, and resuspended in phosphate buffer with formate as the sole electron donor for nitrate reduction. While the basis of the inhibition of NarK-independent nitrite excretion is not known, it apparently involves some aspect of growing cells, such as an active transmembrane potential, which is dissipated when the cells are washed and resuspended and is overcome in some way when internal nitrite concentrations reach high levels in the nitrite reductase-deficient cells.

The contrasting behavior of the *narK* mutant and its parental strain can be explained by assuming that, as suggested by Noji et al. (12), the *narK* gene encodes a nitrate/nitrite antiporter which increases the capacity of the cells to move nitrate and nitrite across the cell membrane. This antiport system would facilitate anaerobic nitrate respiration by serving as a high-affinity nitrate uptake system when nitrate is being reduced to nitrite, by using the nitrate concentration gradient to help drive the product, nitrite, from the cell as rapidly as it is formed, and by helping to maintain electrogenic balance through the linkage of nitrate uptake to nitrite excretion. This would explain why the wild-type strain growing anaerobically on glucose plus nitrate converts the nitrate almost stoichiometrically to nitrite before reducing the nitrite further to ammonia. It would also explain why nitrate reduction occurs in the parental strain at essentially the same rate during anaerobic growth on L broth plus nitrate without glucose, while in the *narK* mutant the rate of nitrate reduction is severely slowed when nitrite reductase formation is repressed by glucose deprivation. In each case, the NarK antiporter in the parental strain would facilitate the uptake of nitrate and the expulsion of nitrite from the cell as rapidly as nitrate was reduced to nitrite by nitrate reductase.

The basis of the effects of nitrate on TMNO reduction, which was originally used to distinguish a spectrum of *nar* mutants from the parental strain, can be deduced from the results presented here. Stewart and MacGregor (20) showed that the inhibition of TMNO reduction by nitrate which occurs in the parental strain can be relieved by mutations which block or reduce the formation of active nitrate reductase (*narGHJI* and *narL* mutants) or by *narK* mutations. As shown here, nitrate represses the level of TMNO-induced TMNO reductase about 50%, while the *narK* mutation relieves this repression and permits an increased level of TMNO induction when nitrate is present. The 50% repression by nitrate of the parental strain would not appear to be sufficient to account for the apparent complete inhibition of TMNO reduction. Rather, TMNO and nitrate would appear to be competing as electron acceptors in anaerobic respiration. In the wild-type strain, nitrate is the preferred acceptor, presumably due to the high level of activity of the nitrate reduction pathway which is produced. The *narGHJI* and *narL* mutations reduce the level of active nitrate reductase (20) and permit the rapid reduction of TMNO. The effect of the *narK* mutation, as shown here, is to slow the rate of flux through the nitrate reduction pathway, permitting reduction of TMNO to take place with the excess electron pool generated by anaerobic respiration pathways.

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REFERENCES

1. **Boogerd, F., H. Van Verseveld, and A. Stouthammer.** 1983. Dissimilatory nitrate uptake in *Paracoccus denitrificans* via a $\Delta\mu_H^+$-dependent system and a nitrate-nitrite antiport system. Biochim. Biophys. Acta **723**:415–427.
2. **Casadaban, M.** 1976. Transpositions and fusions of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. J. Mol. Biol. **104**:541–555.
3. **Cole, J. A.** 1978. The rapid accumulation of large quantities of ammonia during nitrite reduction by *Escherichia coli*. FEMS Microbiol. Lett. **4**:327–329.
4. **Coleman, K. J., A. J. Cornish-Bowden, and J. A. Cole.** 1978. Purification and properties of nitrite reductase from *Escherichia coli* K12. Biochem. J. **175**:495–499.
5. **Glaser, J., and J. A. DeMoss.** 1971. Phenotypic restoration by

molybdate of nitrate reductase activity in *chlD* mutants of *Escherichia coli*. J. Bacteriol. **108**:854–860.
6. **Graham, A., and D. H. Boxer.** 1980. Arrangement of the respiratory nitrate reductase in the cytoplasmic membrane of *Escherichia coli*. FEBS Lett. **113**:15–20.
7. **Iuchi, S., and E. C. C. Lin.** 1987. The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine *N*-oxide reductase operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:3901–3905.
8. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P_1. Virology **1**:109–206.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. **MacDonald, H. M., and J. A. Cole.** 1985. Molecular cloning and functional analysis of the *cysG* and *nirB* genes of *Escherichia coli* K12, two closely-linked genes required for NADH-dependent nitrite reductase activity. Mol. Gen. Genet. **200**:328–334.
11. **MacGregor, C. H., and A. R. Christopher.** 1978. Assymetric distribution of nitrate reductase subunits in the cytoplasmic membrane of *Escherichia coli*. Arch. Biochem. Biophys. **185**: 204–213.
12. **Noji, S., T. Nohno, T. Saito, and S. Taniguchi.** 1989. The *narK* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-respiring cells. FEBS Lett. **252**:139–143.
13. **Page, L., L. Griffiths, and J. A. Cole.** 1990. Different physiological roles of two independent pathways for nitrite reduction to ammonia by enteric bacteria. Arch. Microbiol. **154**:349–354.
14. **Parsonage, D., A. Greenfield, and S. Ferguson.** 1985. The high affinity of *Paracoccus denitrificans* cells for nitrate as an electron acceptor. Analysis of possible mechanisms of nitrate and nitrite movement across the plasma membrane and the basis for inhibition by added nitrite of oxidase activity in permeabilized cells. Biochim. Biophys. Acta **807**:81–95.
15. **Ruiz-Herrera, J., M. K. Showe, and J. A. DeMoss.** 1969. Nitrate reductase complex of *Escherichia coli* K-12: isolation and characterization of mutants unable to reduce nitrate. J. Bacteriol. **97**:1291–1297.
16. **Rondeau, S. S., P.-Y. Hsu, and J. A. DeMoss.** 1984. Construction in vitro of a cloned *nar* operon from *Escherichia coli*. J. Bacteriol. **159**:159–166.
17. **Showe, M. K., and J. A. DeMoss.** 1968. Localization and regulation of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95**:1305–1313.
18. **Sodergren, E. J., P.-Y. Hsu, and J. A. DeMoss.** 1988. Roles of the *narJ* and *narI* gene products in the expression of nitrate reductase in *Escherichia coli*. J. Biol. Chem. **263**:16156–16162.
19. **Stewart, V., and B. L. Berg.** 1988. Influence of *nar* (nitrate reductase) genes in nitrate inhibition of formate-hydrogen lyase and fumarate reductase gene expression in *Escherichia coli* K-12. J. Bacteriol. **170**:4437–4444.
20. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–799.
21. **Thayer, J., and R. Huffaker.** 1982. Kinetic evaluation, using ^{13}N, reveals two assimilatory nitrate transport systems in *Klebsiella pneumoniae*. J. Bacteriol. **149**:198–202.