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Evidence for a Second Nitrate Reductase Activity That Is Distinct from the Respiratory Enzyme in *Salmonella typhimurium*

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Significant nitrate reductase activity was detected in mutants of *Salmonella typhimurium* which mapped at or near *chlC* and which were incapable of growth with nitrate as electron acceptor. The same mutants were sensitive to chlorate and performed sufficient nitrate reduction to permit anaerobic growth with nitrate as the sole nitrogen source in media containing glucose. The mutant nitrate-reducing protein did not migrate with the wild-type nitrate reductase in polyacrylamide electrophoretic gels. Studies of the electrophoretic mobility in gels of different polyacrylamide concentration revealed that the wild-type and mutant nitrate reductases differed significantly in both size and charge. The second enzyme also differed from the wild-type major enzyme in its response to repression by low pH and its lack of response to repression by glucose. The same mutants were found to be derepressed for nitrite reductase and for a cytochrome with a maximal reduced absorbance at 555 nm at 25°C. This cytochrome was not detected in preparations of the wild type grown under the same conditions. Extracts of these mutants contained normal amounts of the *b*-type cytochromes which, in the wild type, were associated with nitrate reductase and formate dehydrogenase, respectively, although they could not mediate the oxidation of these cytochromes with nitrate. They were capable of oxidizing the derepressed 555-nm peak cytochrome with nitrate. It is suggested that these mutants synthesize a nitrate-reducing enzyme which is distinct from the *chlC* gene product and which is repressed in the wild type during anaerobic growth with nitrate.

Both *Salmonella typhimurium* and *Escherichia coli* produce respiratory nitrate reductase, a molybdoenzyme which participates in the coupling of anaerobic nitrate reduction to oxidative phosphorylation (8, 18, 19). It is thought that the same enzyme can also serve in an assimilatory capacity, since these organisms can use nitrate as a nitrogen source under anaerobic, but not aerobic, growth conditions (7, 23). The nitrite reductase to which this assimilatory activity is presumably linked is also synthesized only during anaerobic growth (1, 7, 9).

The study of *E. coli* nitrate reductase has been greatly facilitated by the use of chlorate-resistant mutants. Chlorate is reduced to toxic chlorite by nitrate reductase, and spontaneous chlorate-resistant mutants characteristically lack nitrate reductase (26). Although most of these spontaneous mutants are pleiotropic, lacking all molybdoenzymes, some are defective specifically in nitrate reductase (14, 15). If mutagenized cultures are used for chlorate-resistant mutant selection, the percentage of isolates affected only in nitrate reductase can be as high as 20% (15). Nonpleiotropic nitrate reductase mutants of *E. coli* contain lesions in *chlC* (near *trp*), which appears to be the structural gene for nitrate reductase (11, 15, 22). *E. coli chlC* mutants have also been isolated from mutagenized cultures by means of a direct selection for fermentative acid accumulation in the presence of nitrate (29), for the failure to produce nitrite from nitrate (14, 29), or for the inability to perform nitrate respiration with lactate (15). Some of the *chlC* mutants isolated by these other methods are actually sensitive to chlorate (14).

For previously unexplained reasons, it is more difficult to obtain the nonpleiotropic nitrate reductase mutants in *Salmonella*. Selection for chlorate resistance typically yields only the pleiotropic molybdoenzyme mutants, including most of the classes described for *E. coli* (5, 32). Although direct isolation might seem to be the solution and has been used by Casse et al. (5) to obtain *Salmonella* mutants which map at *chlC*, we have never been able to isolate mutants comparable to the *E. coli chlC* strains on any medium designed for this purpose. On the contrary, in our experience, direct isolation of

strains of *S. typhimurium* which do not perform nitrate respiration using mutagenized cultures yields two classes of mutants: pleiotropic mutants affected in *chl* genes other than *chlC*, and partial nitrate reductase mutants which retain activity under certain growth conditions (E. L. Barrett, A. E. Davidson, and G. W. Chang, unpublished data). We have now characterized in more detail some of the partial nitrate reductase mutants obtained by direct isolation procedures. The results presented here indicate that *chlC* mutants of *S. typhimurium* characteristically retain a small but significant nitrate reductase activity which appears to be unrelated to the *chlC* gene product in the wild type. The existence of this second nitrate reductase activity may help to explain the difficulty in obtaining nitrate reductase mutants in *Salmonella*.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains of *S. typhimurium* used in these studies.

Growth media and incubation conditions. Nitrate broth consisted of nutrient broth (Difco Laboratories) supplemented with 0.085 M NaCl, 100 mM KNO_3, and, when indicated, 50 mM glucose. McConkey nitrate medium was described previously (2). Glucose minimal medium (VBC) used for transductions consisted of Vogel and Bonner medium E (34) supplemented with 1% glucose and, when necessary, amino acids (0.1 mM), adenosine (0.1 mM), or vitamins (0.01 mM). Nitrate minimal medium consisted of the basal medium of Gutnick et al. (16) supplemented with 10 mM KNO_3 as the sole nitrogen source and either glycerol, lactate, or glucose at 50 mM. Gas production was tested in triple sugar iron agar (Difco). All incubations were at 37°C. Anaerobic incubations were carried out in GasPak (BBL Microbiology Systems) anaerobic jars, using an atmosphere of 95% H_2 and 5% CO_2.

Mutant selection. 1-Methyl-3-nitro-1-nitrosoguanidine was used as a mutagen according to the methods of Roth (27). Freshly mutated cells were diluted and spread onto McConkey nitrate plates and incubated anaerobically for 24 h. Rare tiny colonies among the large vigorous wild-type colonies were picked and purified. Those isolates which produced gas from glucose but failed to produce nitrite from nitrate in glucose-containing nitrate broth were considered presumptive nitrate reductase mutants.

TABLE 1. Bacterial strains

Strain	Genotype	Source
LT2	Wild type	B. N. Ames
SL751	*flaA56 H1-iM10 ilvA454 pil proA46 purC7 purI1590 rha-461 rpsL166*	B. N. Ames
EB7	*chl-1127 trp*	Mutagenized culture of LT2
EB8	*chl-1128*	Mutagenized culture of LT2
EB9	As SL751, also *chl-1129*	Mutagenized culture of SL751
TC110	As SL751, also *chlA1110*	G. W. Chang
JL1238	*pyrC1502 cdd-9 cod-8 deo-A1 udp-11 tdk-1 thyA1391*	J. L. Ingraham

Genetic techniques. Transductions were performed according to the methods of Ely et al. (12), using phage P22 *int-4* (31). In all experiments, nitrate reductase was the unselected marker and was scored as very poor growth on McConkey nitrate plates.

Cell extracts. Cells were grown to an optical density at 650 nm of 0.18 in screw-cap bottles filled completely with nitrate broth. After harvesting, they were washed in 50 mM phosphate buffer, pH 7.2. To obtain frozen-thawed preparations, the washed cells were suspended in the same buffer to a concentration of about 7 to 8 mg of protein per ml and then frozen (−15°C) and thawed three times. To prepare sonicated cells, the washed cells were suspended in 0.6 M sucrose containing 100 μg of lysozyme and 400 μg of EDTA per ml, incubated for 15 min at 30°C, and then harvested by centrifugation at 10,000 × *g* for 10 min and sonicated.

Enzyme assays. Nitrate reductase was assayed by using either sonicated or frozen-thawed cell preparations. The method of Lester and DeMoss (19) was used for assays with benzyl viologen as the electron donor. In assays with methyl viologen as the donor, we used the procedure of Lowe and Evans (20) except that the reaction mixture was scaled up to 4 ml, and nitrite was determined at several time intervals rather than at 10 min only. Nitrate reductase with formate as the electron donor was assayed by the same procedure except that methyl viologen and the dithionite-bicarbonate reagent were replaced by 1 M NaCOOH (100 μl/4 ml), and the reaction was stopped by the addition of the sulfanilamide reagent to a 0.5-ml sample rather than by vigorous aeration.

Nitrite reductase activities with methyl viologen and formate as electron donors were assayed with sonicated cells and the same assay procedures as were used for nitrate reductase except that KNO_3 was replaced by KNO_2, and nitrite disappearance rather than appearance was measured.

Cytochrome scans. Frozen-thawed cell preparations were scanned in a Perkin-Elmer Coleman 575 scanning spectrophotometer to obtain absorption spectra. A slit width of 0.5 nm was used.

Polyacrylamide gel electrophoresis. For electrophoretic studies, deoxycholate (1 mg/mg of protein) was added to the sonicated cells, which were then stirred for 3 h on ice. The suspension was centrifuged at 22,000 × *g* for 20 min. The supernatant was layered on a 1.5 by 5-cm Sephadex G-25M column which had been equilibrated with 0.1 M Tris buffer, pH 7.2, and then eluted with the same buffer.

Slab gels used to locate nitrate and nitrite reductase activities consisted of a 4% polyacrylamide separating gel containing 0.4 M Tris, pH 8.8, and 0.1% Triton X-100 and a stacking gel of 2.5% polyacrylamide with 0.125 M Tris, pH 6.8, and 0.1% Triton X-100. Bromphenol blue was used as the tracking dye. Protein was stained with 0.1% Coomassie blue R-250 in a 5:1:5 solution of water, glacial acetic acid, and methanol.

Nitrate reductase was stained by the method of Lund and DeMoss (21). Nitrite reductase was stained with the same procedure except that KNO_2 was substituted for KNO_3. Tube gels used to determine the dependence of nitrate reductase mobility on polyacrylamide concentration (17) consisted of the same reagents as the slab gels except that the polyacrylamide concentration was varied. Nitrate reductase was located by means of activity stains (21).

RESULTS

Isolation and mapping of nitrate reductase mutants. Nitrate reductase mutants were isolated from nitrosoguanidine-mutagenized cultures of *S. typhimurium* LT2 or SL751 as tiny colonies on anaerobically incubated McConkey nitrate plates. Wild-type cells grow vigorously on this medium, whereas cells defective in nitrate respiration grow to a very limited extent at the expense of the trace amounts of glucose present. Formate dehydrogenase (FDH) mutants and pleiotropic chlorate-resistant mutants also form tiny to small colonies on this medium. However, colonies of these mutant classes are deep red, whereas colonies of nitrate reductase mutants which retain FDH activity are pale (2, 3). For these studies, we purified three such tiny pale colony isolates which produced gas from glucose, but not nitrite from nitrate in glucose-containing nitrate broth. The three (EB7, EB8, and EB9) were derived, respectively, from separate mutagenesis experiments.

chlC in *S. typhimurium* has been reported to be cotransducible with the gene for thymidine kinase (*tdk*), using phage P22 (5). Using the transductant selection procedures for Tdk^+ outlined by Beck et al. (4), we transduced *tdk* mutant JL1238 to Tdk^+, using phage P22 grown on each of our isolates. We then scored the Tdk^+ transductants for poor growth on McConkey nitrate. In all three, the nitrate reductase defect was 40 to 44% cotransduced with *tdk*. One of the mutants, EB7, was also Trp^-. We screened the Tdk^+ transductants obtained from crossing EB7 with JL1238 for Trp^- and found that *trp* in EB7 was 4% cotransduced with *tdk*, but that it was never coinherited with the nitrate reductase defect. These data are in agreement with the gene order *trp-tdk-chlC* previously reported (5).

Initial mutant characterization. In light of our previous inability to find *chlC* mutants among chlorate-resistant isolates, we were not surprised to find that EB7, EB8, and EB9 had all retained sensitivity to chlorate. However, their response to chlorate differed qualitatively as well as quantitatively from that of the wild type. Table 2 presents the effect of chlorate on the anaerobic growth of EB7, EB8, and EB9 in the presence and absence of nitrate along with the comparable results for wild-type LT2 and *chlA* mutant TC110, which is a typical chlorate-resistant mutant. The data indicate that although the nitrate reductase mutant isolates, as compared with the wild type, are less sensitive to chlorate in the absence of nitrate, they are more sensitive in its presence. Apparently, nitrate has a protective effect on the wild type, but no effect on the mutants.

We also found that EB7, EB8, and EB9, although incapable of anaerobic growth in glycerol- or lactate-containing nitrate minimal media (in which nitrate was the sole source of nitrogen), grew almost as well as the wild type in nitrate minimal medium with glucose. All grew as well as the wild type in aerobically incubated nitrate minimal medium which was supplemented with 10 mM NH_4Cl and either glycerol or lactate for carbon. Apparently, our isolates perform sufficient nitrate reduction for assimilatory purposes, even though they perform little or no nitrate respiration.

Further physiological characterization experiments revealed that although EB7, EB8, and EB9 all failed to produce nitrite from nitrate in glucose-containing nitrate broth, they did produce it in the same medium without glucose. Interestingly, the only other *Salmonella chlC* mutant for which nitrate reductase assays have been reported are those described by Casse et al. (5). The assay procedure cited by these authors (25) specifies the growth of the cells in a medium containing 0.2% glucose. When we looked at the glucose effect in more detail to see if it represented merely a quantitative difference from the wild type, we found that pH over the range 4.5 to 7.5, rather than glucose, was the negative effector for nitrite formation by the nitrate reductase mutants in nitrate broth. In contrast, specific nitrite formation by the wild type in this medium was negatively affected by glucose, but not by pH in this range (Fig. 1).

TABLE 2. Effect of chlorate on anaerobic growth

Strain	Final cell density in media with 0.1 mM $KClO_3$[a]	
	No addition	100 mM KNO_3
LT2	25	89
TC110	96	100
EB7	56	58
EB8	52	53
EB9	68	68

[a] Each optical density value at 650 nm was measured after 6 h of growth in anaerobically incubated nutrient broth containing 50 mM glucose and a 1% inoculum from a nutrient broth overnight culture of wild-type LT2, *chlA* mutant TC110, and nitrate reductase mutants EB7, EB8, and EB9, respectively. Data are expressed as percentage of density obtained without $KClO_3$.

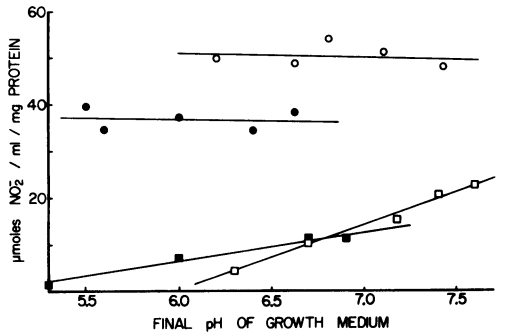

FIG. 1. Effect of glucose and pH on nitrite accumulation by wild-type LT2 and mutant EB8. Tubes of nitrate broth with or without 50 mM glucose and prepared with no buffer or with 50 mM phosphate buffer of pH 6.0, 6.5, 7.0, or 7.5 were inoculated with a 1% inoculum from an overnight culture in nutrient broth. After 16 h, cultures were analyzed for pH, for optical density at 650 nm, and for nitrite concentration. Protein was calculated as 50% of the dry weight (in milligrams) indicated by the optical density at 650 nm. Final pH is plotted against specific nitrite concentration. Except for strain EB8 grown in glucose-containing broth, the five data points in each set, left to right, were obtained from tubes with no buffer and with buffer pH 6.0, 6.5, 7.0, and 7.5, respectively. The plot for EB8 grown in glucose is similar except that there is no point representing the results obtained without buffer since the final pH in that case (4.5) was off the scale. LT2 grown with glucose (●); LT2 grown without glucose (○); EB8 grown with glucose (■); EB8 grown without glucose (□).

Apparently, the reason for the total lack of nitrite in cultures of EB7, EB8, and EB9 grown in unbuffered nitrate broth with 50 mM glucose is that the pH drops to about 4.5 during growth of these mutants in this medium.

Although the chlorate sensitivity and nitrate assimilation by EB7, EB8, and EB9 could be easily explained by a leaky *chlC* mutation, the different effects of pH and glucose were more suggestive of two separate enzymes. We selected strain EB8 for further studies directed toward distinguishing between these alternatives.

Nitrate and nitrite reductase activities. Preparations of EB8 and LT2 grown in nitrate broth without glucose were assayed for nitrate reductase by three different assay procedures. The results (Table 3) showed (i) that strain EB8 had much less activity than LT2 and (ii) that viologen dye oxidation in EB8 was not correlated with nitrite appearance as it was in LT2. These results could be explained by a somewhat weak nitrate reductase in EB8 functioning in the presence of a derepressed nitrite reductase and by the capacity of both benzyl and methyl viologen to reduce both nitrate and nitrite. Assay results for nitrite reductase in cells of EB8 and LT2 grown under the same conditions (also shown in Table 3) indicated that EB8 was indeed derepressed for nitrite reductase. The fact that formate was not an effective donor for nitrite reduction in these assays is probably related to the fact that sonicated cell preparations were used. Formate will reduce nitrite in whole cells, but not in sonicated preparations (D. L. Riggs and E. L. Barrett, unpublished data). The low levels of nitrite reductase activity in the wild type may be related to the observation that nitrate at concentrations greater than 40 mM represses and inhibits nitrite reductase in wild-type *E. coli* (1, 8).

Electrophoretic studies. Preparations of EB8 and LT2 grown in nitrate broth without glucose were subjected to electrophoresis in 4% polyacrylamide slab gels. Figure 2 shows gel tracks stained for protein and for nitrate and nitrite reductases. The respective nitrate reductase activities were associated with different proteins, and neither organism contained a protein corre-

TABLE 3. Specific nitrate and nitrite reductase activities of wild-type LT2 and nitrate reducate mutant EB8

Strain	Assay method				
	Nitrate reductase			Nitrite reductase, nitrite disappearance with given electron donor[c]	
	Benzyl viologen oxidation[a]	Nitrite appearance with given electron donor[b]		Methyl viologen	Formate
		Methyl viologen	Formate		
LT2	6,600	2,410	580	16	<1
EB8	1,300	0–20[d]	75	137	<1

[a] Activity expressed as nanomoles of dye oxidized per minute per milligram of protein. Note: Two moles of benzyl viologen is required to reduce 1 mol nitrate.

[b] Activity expressed as nanomoles of nitrite produced per minute per milligram of protein.

[c] Activity expressed as nanomoles of nitrite consumed per minute per milligram of protein.

[d] There was some variability in the results depending on the length of time before the reaction was stopped. This appeared to be due to an immediate production of nitrite at the onset of the reaction followed by its disappearance. Nitrite production by LT2, in contrast, was linear with time.

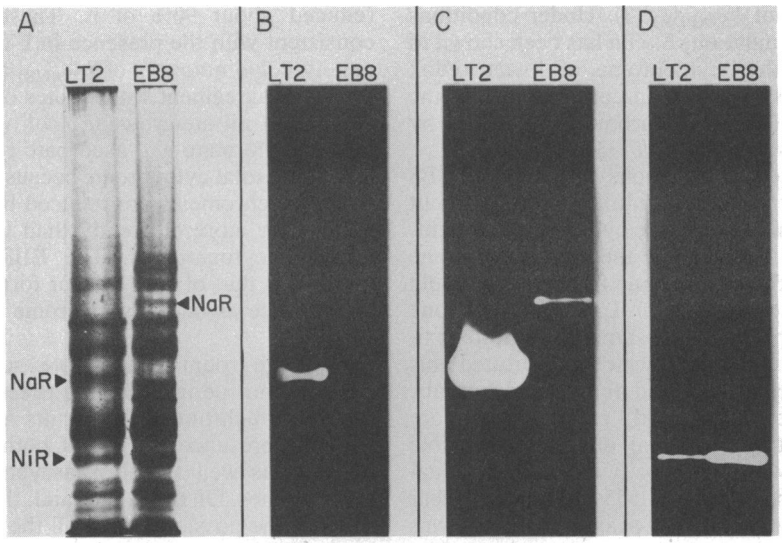

FIG. 2. Nitrate reductase (NaR) and nitrite reductase (NiR) activities in preparations of wild-type LT2 and mutant EB8 subjected to electrophoresis in 4% polyacrylamide gels. Nitrate reductase was stained by the procedures of Lund and DeMoss (21). Gels were incubated at 37°C in the activity stain reagents for 10 min (B) or 30 min (C). Nitrite reductase (D) was stained with the same procedures except that KNO_2 was substituted for KNO_3. These stains were incubated for 10 min. Protein (A) was stained with 0.1% Coomassie blue R-250 in a 5:1:5 solution of water, glacial acetic acid, and methanol.

sponding to the active band in the other. Mutant EB8 also appeared to make several other proteins which were not found in the wild-type preparation. The active nitrate reductase and the other proteins found in EB8 but not LT2 may represent altered forms of the *chlC* gene product, or they may represent proteins repressed in the wild type but not in the nitrate reductase mutant. The nitrite reductases in EB8 and LT2 were associated with protein bands of the same electrophoretic mobility, but the EB8 preparation was more active than the wild-type preparation (Fig. 2).

To assess the extent of physical difference between the active nitrate reductase proteins of EB8 and LT2, we determined their respective ratios of electrophoretic mobility to polyacrylamide concentration (Fig. 3). Proteins of different net charge but the same size give parallel lines in such a plot, whereas proteins of the same charge but different size give a family of lines which intersect at a point near 0% polyacrylamide (17). It can be seen that the respective nitrate reductase proteins of EB8 and LT2 are of very different size and charge.

Spectral analyses. The formate-nitrate reductase pathway in *E. coli*, and presumably *Salmonella*, is associated with two *b*-type cytochromes, each of which has a reduced absorbance maximum at 559 nm at 25°C (28, 30). One is associated with nitrate reductase [$b_{559(NR)}$], and the other is associated with FDH [$b_{559(FDH)}$]. The two cytochromes can be distinguished by their response to certain electron donors; ascorbate reduces only $b_{559(NR)}$, whereas formate can provide electrons for the sequential reduction of both cytochromes (28). *Salmonella* mutants which are defective in the FDH linked to nitrate reductase but which retain formate hydrogenlyase activity have been shown to produce

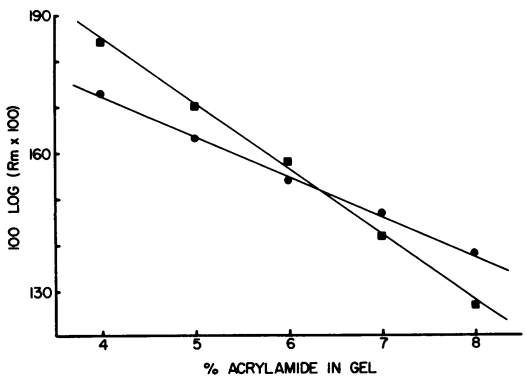

FIG. 3. Effect of different polyacrylamide gel concentration on the mobility of nitrate reductase in wild-type LT2 (■) and the nitrate-reducing protein in mutant EB8 (●). Tube gels of the indicated polyacrylamide concentrations were run simultaneously. The locations of the respective nitrate-reducing proteins were determined with activity stains by the procedure of Lund and DeMoss (21).

$b_{559(NR)}$ but not $b_{559(FDH)}$ (3). Under conditions of nitrate assimilation, *E. coli* has been shown to produce another cytochrome of lower redox referred to as c_{552} (13). In complex media, the production of this cytochrome is repressed by nitrate in wild-type *E. coli* (33).

We analyzed preparations of LT2 and EB8 grown anaerobically in nitrate broth without glucose for their respective cytochrome content. The scans in Fig. 4 show the total cytochrome content and the cytochrome fraction that could be oxidized with nitrate. LT2 had only one absorbance peak, at 559 nm, and nitrate oxidized the cytochromes which constituted this peak as efficiently as did ferricyanide or air. EB8, on the other hand, presented a more complex picture. Its total dithionite-reducible cytochrome was a composite including two major peaks, one at around 555 nm and one near 559 nm. Only the 555-nm-peak cytochrome was efficiently oxidized by nitrate. The scans in Fig. 5 represent the cytochrome fraction that could be reduced by ascorbate and by formate. It appears that ascorbate reduced about 40% of the 559-nm cytochrome in LT2 and that formate reduced about 90% of it. These results are consistent with the presence in LT2 of approximately equal amounts of $b_{559(NR)}$ and $b_{559(FDH)}$. This is in agreement with studies of cytochrome content in nitrate-grown *E. coli* (28). In EB8, however, formate and ascorbate reduced much less of the total cytochrome because the 559-nm-peak cytochromes were reduced by these compounds far more efficiently than were the 555-nm-peak cytochromes. The EB8 preparation resembled that of LT2 in that formate reduced about twice as much cytochrome as did ascorbate.

Although room temperature scans such as these cannot be used for the precise identification of cytochromes, the results are consistent with the presence in EB8 of both $b_{559(NR)}$ and $b_{559(FDH)}$ as well as other, as yet unidentified, cytochromes. On the other hand, the data do not rule out the possibility that all the cytochromes in the EB8 preparation are different from the wild-type cytochromes. What can be concluded is that if mutant EB8 produces the two cytochromes normally associated with the formate-nitrate reductase pathway, then it cannot oxi-

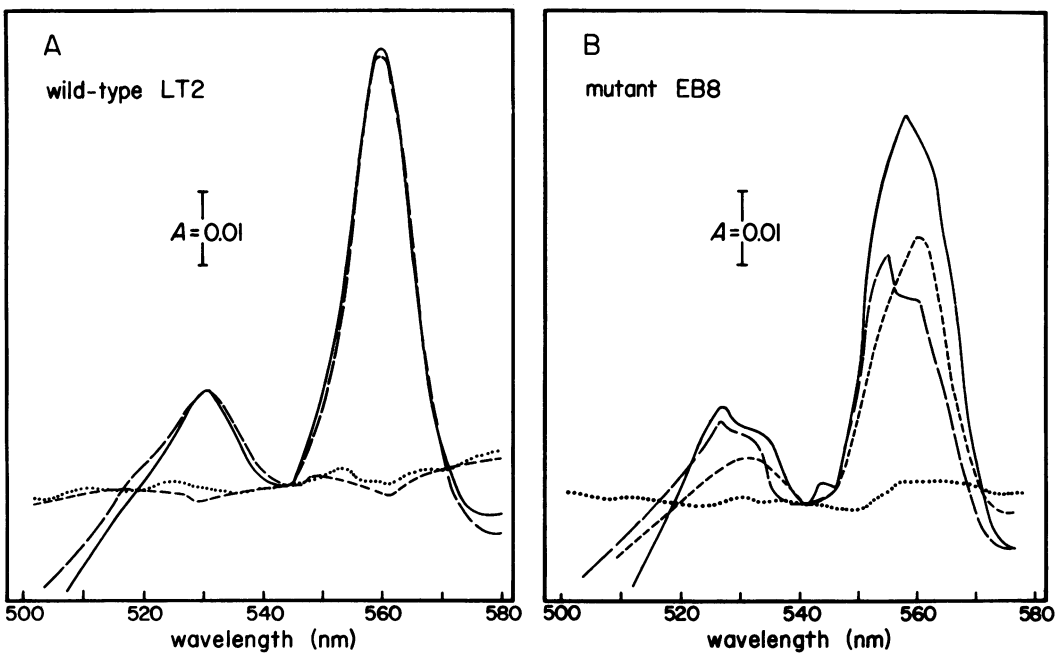

FIG. 4. Cytochrome content and cytochrome oxidized by nitrate in extracts of wild-type LT2 and mutant EB8 grown anaerobically with nitrate. Cells were grown to late exponential phase in nitrate broth without glucose and used as frozen-thawed suspensions of protein concentration 7 to 9 mg/ml for scanning at room temperature in a Perkin-Elmer Coleman 575 spectrophotometer. Total cytochrome, as determined by using a dithionite-reduced sample against a ferricyanide-oxidized reference (——); cytochrome oxidized by nitrate, as determined by using a dithionite-reduced sample against a nitrate-oxidized reference (— —); cytochrome which cannot be oxidized by nitrate, as determined by using a nitrate-oxidized sample against a ferricyanide-oxidized reference (– – –); cytochrome which cannot be oxidized by air or ferricyanide, as determined by using an air-oxidized sample against a ferricyanide-oxidized reference (. . . . . .).

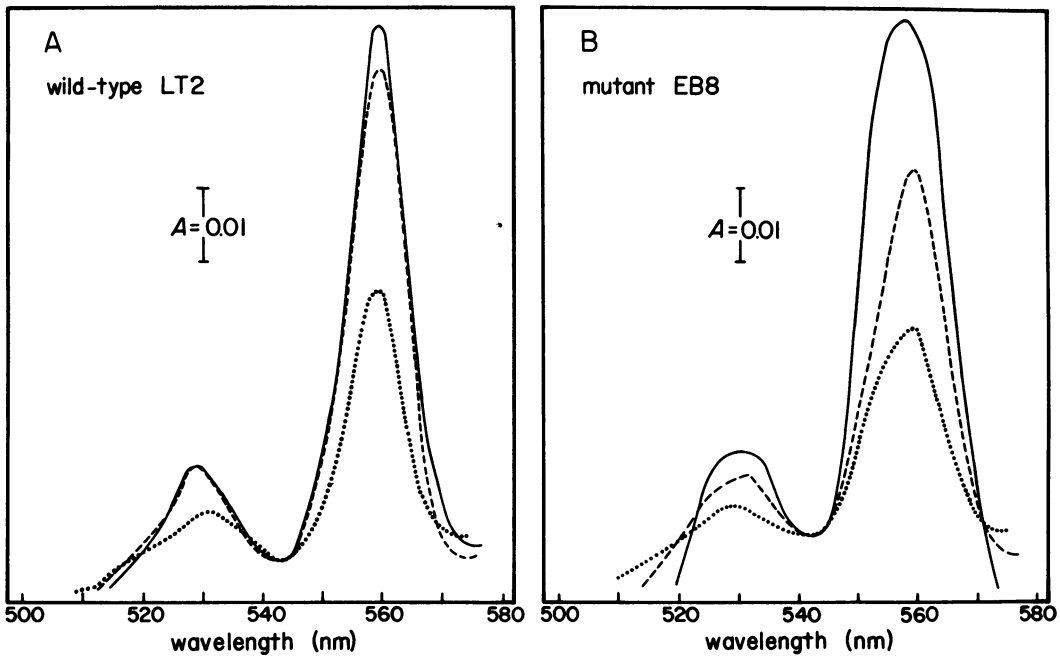

FIG. 5. Cytochromes reduced by formate and by ascorbate in extracts of wild-type LT2 and mutant EB8 grown anaerobically with nitrate. Cells were grown to late exponential phase in nitrate broth without glucose and used as frozen-thawed suspensions of protein concentration 7 to 9 mg/ml for scanning at room temperature in a Perkin-Elmer Coleman 575 spectrophotometer. Total cytochrome, as determined by using a dithionite-reduced sample against a ferricyanide-oxidized reference (——); cytochrome reduced by formate, as determined by using a formate-reduced sample against a ferricyanide-oxidized reference (– – – –); cytochrome reduced by ascorbate, as determined by using an ascorbate-reduced sample against a ferricyanide-oxidized reference (. . . . . .).

dize them with nitrate, and that it apparently contains cytochrome other than b_{559} which is not found in the wild-type grown under the same conditions and which can be oxidized by nitrate but not reduced by formate or ascorbate.

We also examined a preparation of mutant EB7 for cytochrome content to see if the results for EB8 were unique to this mutant or if they represented a common picture for nitrate reductase mutants of *Salmonella*. The scans of EB7 (not pictured) were comparable to those of EB8.

Nitrate reductase mutant reversions. We isolated a reversion mutant of EB8 which was capable of nitrate respiration and examined it for cytochrome content and nitrate reductase activity to see if the restoration of the ability to perform nitrate respiration coincided with the loss of 555-nm-peak cytochrome. The revertant was capable of growth on glycerol-nitrate minimal medium and had significant nitrate reductase activity, although this activity was less than that of the wild type. It no longer produced 555-nm cytochrome. Since the spontaneous revertant simultaneously gained the capacity for nitrate respiration and lost the characteristic 555-nm cytochrome, it is likely that the lesion in EB8, which resulted in the concomitant loss of the capacity for nitrate reduction and the production of an additional cytochrome, was the result of a single rather than a double mutation.

DISCUSSION

The results presented here show that certain mutants of *S. typhimurium* which cannot perform nitrate respiration and which map at or near *chlC* are capable of reducing nitrate with an enzyme distinct from the wild-type nitrate reductase. The mutant enzyme is sufficiently active to permit anaerobic growth with nitrate as the nitrogen source and to confer sensitivity to chlorate even in the presence of nitrate, which appears to protect wild-type cells from the effects of chlorate. The mutant enzyme was found to differ from the wild-type nitrate reductase in its response to repression by low pH and its lack of response to repression by glucose.

The electrophoretic studies suggested that the nitrate-reducing protein in at least one such mutant was unrelated in structure to the wild-type nitrate reductase. The same studies also revealed that the mutant synthesized several other proteins which were not found in the wild type grown under the same conditions. Although

some of the proteins produced by the mutant and not the wild type might be altered products of the *chlC* gene such as have been identified in *chlC* mutants of *E. coli* (22), they might also be proteins repressed by the wild type but not the *chlC* mutant during growth in nitrate broth. Nitrate in the growth medium has been shown to repress other anaerobic enzymes such as fumarate reductase and hydrogenase (35) and, in the hydrogen sulfide-producing bacteria, thiosulfate reductase and tetrathionate reductase (6, 10). Perhaps one of the anaerobic reductases synthesized by *Salmonella* is a molybdoenzyme with low affinity for nitrate and higher affinity for chlorate, and is derepressed in the mutants described here as a secondary result of the loss of *chlC* gene function. A regulatory role for the *chlC* gene product in the repression of other reductases by nitrate is suggested by the overproduction of certain anaerobic enzymes in nitrate reductase mutants of *E. coli* (33).

The results of the spectral studies indicate that although the nitrate reductase mutants may produce the anaerobic *b*-type cytochromes associated with the formate-nitrate reductase pathway in normal amounts, they cannot oxidize these cytochromes with nitrate. In this respect, they differ from the *E. coli* "*chlI*" mutant described by Orth et al. (24) which, like EB8, maps in the vicinity of *chlC* and which is defective in nitrate respiration, although it exhibits nitrate reductase activity with benzyl viologen. The latter mutant was reported to synthesize only low levels of anaerobic *b*-type cytochromes. The mutants described here also differ from previously characterized *chlA*, *chlB*, *chlC*, and *chlE* mutants of *E. coli* which have also been shown to produce low levels of cytochrome b_{559} (22).

Although preparations of EB8 could not oxidize cytochrome b_{559} with nitrate, they were able to use nitrate to oxidize a different cytochrome detected in the mutant preparations, but not in those of the wild type grown under the same conditions. The derepression of this cytochrome in the nitrate reductase mutants described here may be related to the derepression of nitrite reductase noted in the assay results. In wild-type *E. coli*, cytochrome c_{552} is coinduced with nitrite reductase (7, 9, 13), and in nitrate reductase mutants of *E. coli*, both are derepressed even in complex media (33).

The number of differences between the nitrate-reducing activity of mutants EB7, EB8, and EB9 and the wild-type nitrate reductase suggest that there may be two distinct enzymes in *S. typhimurium* which catalyze the anaerobic reduction of nitrate. Further studies are planned to determine the in vivo function of the nitrate reductase activity detected in the respiratory nitrate reductase mutants of *Salmonella*.

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