JOURNAL OF BACTERIOLOGY, Mar. 1975, p. 1111-1116

Vol. 121, No. 3
Printed in U.S.A.

Anaerobic Cytochrome b_1 in *Escherichia coli*: Association with and Regulation of Nitrate Reductase

C. H. MACGREGOR

Department of Microbiology, University of Virginia Medical School, Charlottesville, Virginia 22901

Received for publication 25 November 1974

Nitrate reductase solubilized from the membrane of *Escherichia coli* by alkaline heat treatment was purified to homogeneity and used to prepare specific antibody. Nitrate reductase, precipitated by this antibody from Triton extracts of the membrane, contained a third subunit, not present in the purified enzyme used to prepare the antibody. This third subunit was identified as the cytochrome b_1 apoprotein. This cytochrome is bound to nitrate reductase from wild-type *E. coli* in a ratio of 2 mol of cytochrome per mol of enzyme complex. In mutants unable to synthesize heme, this cytochrome b_1 apoprotein is not bound to nitrate reductase. In these same mutants, the enzyme is overproduced and accumulates in the cytoplasm. The absence of cytochrome also affects the stability of the membrane-bound form of the enzyme.

The nitrate reductase enzyme of *Escherichia coli* is a membrane-bound enzyme which functions anaerobically as a terminal electron acceptor. Under usual physiological circumstances the electron donor is formate, which is oxidized by the enzyme formate dehydrogenase (9). Cytochrome b_1 appears to be an intermediate in the transfer of electrons from formate dehydrogenase to nitrate reductase (9), and the amount of membrane-bound cytochrome b_1 increases several-fold when nitrate is added to cultures growing anaerobically (6).

The question arises as to how this cytochrome b_1 is related to or coupled to the enzymes formate dehydrogenase and nitrate reductase. Is the cytochrome b_1 a separate component of the system, free to diffuse in the membrane and interact at random with the enzymes in a more rigid complex? Is the cytochrome b_1 distinct and separable from the donor and acceptor enzymes, or do each of the enzymes have their own cytochrome b_1 as tightly bound subunits?

Formate dehydrogenase which has been solubilized and purified to the point where it is free of nitrate reductase activity has been shown to contain functional cytochrome b_1 (13). The enzyme nitrate reductase, which has been purified to homogeneity, does not contain cytochrome b_1 (8, 10). However, this purification involved heating at alkaline pH to solubilize the enzyme. As was shown in the previous paper in this series (4), this procedure results both in limited proteolysis of the enzyme and in the complete loss of a small subunit (subunit C) which is present when the enzyme is solubilized without heating using the detergent Triton X-100.

This paper presents evidence that the Triton-solubilized enzyme contains bound cytochrome b_1, which is present in the ratio of 2 mol of cytochrome per mol of molybdenum, and that subunit C is the apoprotein of this cytochrome. Furthermore, this cytochrome appears to play a role in the regulation of synthesis of nitrate reductase and in the in situ proteolysis of nitrate reductase.

MATERIALS AND METHODS

Strains. Unless otherwise indicated, all experiments were performed using *E. coli* RK7 (8). For studies with *hemA* mutants, strains AN344 (*pro*$^-$, *leu*$^-$, and *hemA*$^-$) and the isogenic strain AN345 (*pro*$^-$, *leu*$^-$, *hemA*$^+$) were used. Strains AN344 and AN345 were provided by Graeme Cox.

Growth medium. Strain RK7 was grown anaerobically as previously described (4). For growth of strains AN344 and AN345, this basic medium was supplemented with a mixture of amino acids and vitamins (5). δ-Amino levulinic acid was added at a final concentration of 20 μg/ml.

Cell fractionation. Cells were broken in a French pressure cell as previously described (4). The crude extract was centrifuged at 200,000 × *g* for 45 min. The supernatant fraction resulting from this centrifugation was used to obtain the cytoplasmic fraction. This supernatant was incubated at 32 C for 2 h. After this incubation, the small membrane fragments contained in the supernatant had aggregated and could be removed by centrifugation (7). This final soluble fraction is referred to as cytoplasm. Isolation of the membrane fraction has been described elsewhere (4).

Difference spectra. All difference spectra were

measured using an Aminco DW-2 spectrophotometer. In each case, the sample was divided in half and the dithionite-reduced sample was read against an air-oxidized reference.

All other methods have been described in the preceding paper (4).

RESULTS

Nitrate reductase purified by heat solubilization contains two different subunits with molecular weights of 142,000 (A) and 58,000 (B). When specific antibody was used to precipitate the enzyme from Triton-solubilized membrane, the protein profile of this precipitate on SDS (sodium dodecyl sulfate) gels showed that a third protein was present. This protein, termed subunit C, had a molecular weight of 19,500 and was present in a ratio of 2 mol of subunit C to 1 mol of subunit B to 1 mol of subunit A (4). One explanation for the existence of this protein in Triton-solubilized nitrate reductase might be that it is the cytochrome b_1 apoprotein, that it is normally associated with nitrate reductase, and that it is removed from the enzyme during the heat extraction process. This possibility is strengthened by the fact that the reduction of nitrate to nitrite involves the transfer of two electrons; therefore 2 mol of cytochrome would need to be present per mol of enzyme for this transfer to occur in one step.

This possibility was tested in the following way. Membrane protein was solubilized with Triton X-100. To one part of the Triton extract, rabbit anti-nitrate reductase was added, followed by goat anti-rabbit gamma globulin. To another part of the extract, preimmune rabbit serum was added, followed by the same goat serum. The resulting precipitates were washed and their spectra were determined. Figure 1 shows the difference spectrum of each precipitate. The characteristic cytochrome b_1 peak at 558 nm was found in the anti-nitrate reductase precipitate but not in the preimmune precipitate. This figure also shows the difference spectrum of nitrate reductase precipitated from a heat-solubilized extract. Although this sample contained more total nitrate reductase protein then the Triton-solubilized material, only a very small peak at 558 nm was observed. It can be calculated that the material precipitated from the crude heat-solubilized membrane contained 9% of the cytochrome b_1 found in a corresponding amount of material precipitated from the Triton extract. The membrane protein which remains insoluble after heat extraction also contains very little cytochrome b_1. Since the cytochrome b_1 is virtually absent both from the soluble and from the insoluble fractions after heat extraction, it must be heat labile. The purified, heat-extracted protein which was used to immunize rabbits to obtain the antiserum did not contain any cytochrome b_1, since there were no characteristic absorption peaks for cytochrome b_1 detectable in difference spectra of the purified enzyme and since there was no subunit C detected in SDS gels of the purified enzyme. Therefore, the cytochrome b_1 which is precipitated by the antiserum must either be precipitated nonspecifically or it must be bound to the enzyme.

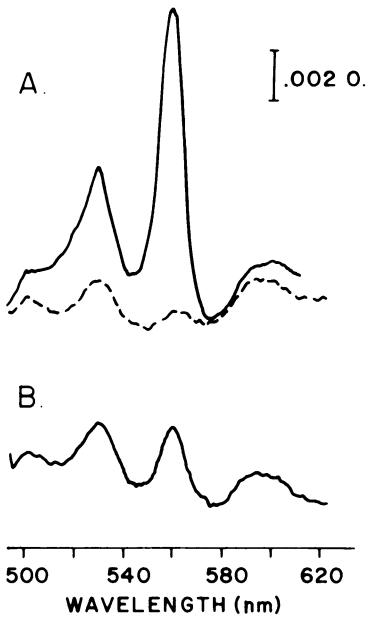

FIG. 1. *Difference spectra of antibody-precipitated nitrate reductase. (A) Equal amounts of rabbit anti-nitrate reductase (solid line) or preimmune rabbit serum (dotted line) were added to identical samples of Triton-solubilized membrane (4). These were then precipitated with goat anti-rabbit gamma globulin. The resulting precipitates were washed and suspended in 0.15 M NaCl. Each suspension was divided between two cuvettes. A small amount of sodium dithionite was added to one cuvette and the difference spectrum was measured with an Aminco spectrophotometer from 500 to 600 nm at 0.02 optical density full scale. (B) Anti-nitrate reductase serum and goat serum were added as in (A) to membrane preparations solubilized by heating at 60 C at pH 8.3. The antibody precipitate was treated as in (A) and similarly scanned. (B) contained 2.6 times the amount of protein as (A).*

Proof that the precipitation of cytochrome results from antigen-antibody combination is provided in part by the preimmune serum control experiment shown in Fig. 1, and by the

results given in Tables 1 and 2. In the experiment shown in Table 1, various concentrations of rabbit antiserum were added to a fixed amount of Triton-solubilized extract. The immune precipitates were removed by centrifugation, and difference spectra of both the precipitates and the supernatants were measured. The removal of nitrate reductase activity by immune precipitation was also determined by measuring the activity remaining in the supernatant after precipitation. If the cytochrome b_1 (or at least a portion of it) is bound to the enzyme, then a simultaneous removal of cytochrome and enzyme activity should occur and Table 1 shows that this does in fact occur. However, when only 1% of the initial nitrate reductase activity remains in the supernatant, there is still 35% of the initial cytochrome b_1 present in the supernatant. Thus, not all of the cytochrome b_1 in the Triton extract is bound to nitrate reductase, as might be expected.

TABLE 1. *Co-precipitation of nitrate reductase and cytochrome b_1 by anti-nitrate reductase serum*[a]

Dilution of anti-NR serum added	Supernatant		Precipitate (total cytochrome b_1)
	Total cytochrome b_1	Total NR (mU)	
No serum	4.72	1,848	0
1:10	4.48	1,568	0.16
1:5	4.28	1,400	0.32
1:2	3.28	632	1.52
Undiluted	1.64	22	3.20

[a] Equal amounts of 0.15 M NaCl, anti-nitrate reductase (NR) serum diluted with 0.15 M NaCl, or the concentrated antiserum were added to identical samples of a Triton extract. The immune precipitates were washed once (4) and suspended in 0.15 M NaCl. Cytochrome b_1 was measured from the difference spectrum as shown in Fig. 1A, and is expressed as the difference in optical density at 558 nm $\times 10^2$.

TABLE 2. *Co-precipitation of Triton-solubilized nitrate reductase and cytochrome b_1 from mixtures of Triton- and heat-solubilized extracts*[a]

NR (mU)		Total protein in precipitate (mg)	TS protein in precipitate (% of total)	Cytochrome b_1/mg total protein in precipitate
HS	TS			
0	8,640	0.358	100	1.56
3,450	5,180	0.558	48	0.54
5,180	3,450	0.884	35	0.32
6,910	1,720	0.992	22	0.22
8,640	0	0.970	0	0.18

[a] Membrane preparations were isolated from identical cultures labeled with [^{3}H]leucine or [^{14}C]leucine. The ^{3}H-labeled membrane was extracted with Triton X-100, and the ^{14}C-labeled membrane was extracted by heating at 60 C. The heat-solubilized (HS) extract was concentrated by pressure filtration to give the same nitrate reductase (NR) activity per milliliter as the Triton extract. Samples of the same volume were prepared so that the ratio of HS enzyme activity to Triton-solubilized (TS) enzyme activity were 0:100, 40:60, 60:40, 80:20, and 100:0. To each of these was added a constant amount of rabbit anti-NR sufficient to precipitate greater than 99% of the NR activity. Cytochrome b_1 in the washed immune precipitates was measured and is expressed as in Table 1. The amount of TS and HS protein in the washed immune precipitates was calculated from the ^{3}H and ^{14}C counts. In this experiment, the specific activity of NR was somewhat lower in the HS preparation than in the TS preparation; hence, the total amount of protein was greater in the samples with greater amounts of HS extract.

In the experiment shown in Table 2, a Triton extract and a heat extract were mixed in various proportions. To these mixtures, a fixed amount of antibody was added. The amount of cytochrome b_1 and the amount of protein derived from the Triton extract and the heat extract were measured in the immune precipitates. As the amount of Triton-solubilized protein in the precipitates decreased, there was a corresponding decrease in the amount of cytochrome b_1 in the precipitates, even though the total amount of protein in the precipitates increased. This also shows that the precipitation of the cytochrome b_1 is not due to nonspecific binding to the immune precipitate.

It was possible to calculate the extinction coefficient of the cytochrome b_1 from the data shown in the first line of Table 2, since both the difference spectrum and the amount of protein in the immune precipitate were determined, and since the molecular weights and molar ratios of the subunits present in the enzyme which is immune precipitated from Triton extracts are known (4). A millimolar extinction coefficient of 19.5 was calculated for dithionite-reduced versus air-oxidized cytochrome b_1 at a wavelength of 558 nm and a light path of 1 cm. This calculation is based on molecular weight of 19,500 for the apoprotein. This extinction coefficient is in agreement with other extinction coefficients which have been previously reported for cytochrome b_1 (1, 2), and this agreement provides further proof that subunit C is in fact cytochrome b_1.

Evidence that the 19,500-molecular-weight protein is actually cytochrome b_1 was also provided by examining the nitrate reductase protein found in mutants unable to synthesize heme without the addition of δ-amino levulinic acid (δALA). Strain AN344 (*hemA*$^-$) was grown

anaerobically in the presence of nitrate either with or without δALA. When the cytoplasm and Triton extracts of the membrane fraction of this mutant grown without δALA were examined for the presence of cytochromes by difference spectrum, no trace of any cytochrome was found, even at the most sensitive setting. When in vivo nitrate reductase activity was determined by measuring the amount of nitrite present in the culture fluid (8), no significant nitrate reductase activity could be found (approximately 0.1% of the wild-type level). Strain AN344 grown with δALA, however, had higher amounts of cytochrome than an isogenic *hemA*$^+$ strain but comparable levels of in vivo nitrate reductase activity. Enzyme assays using an artificial electron donor (methyl viologen) provided contrasting results to those just mentioned. The mutant AN344 grown without δALA had 3.6 times the total nitrate reductase activity (membrane plus cytoplasm) than that measured in the same strain grown with δALA. Obviously, the unsupplemented mutant was able to make enzyme which functions in the methyl viologen assay but not in the organism itself. If a cytochrome were necessary to couple nitrate reductase to its electron donor, it would follow that any enzyme synthesized in the mutant which could not make heme (without δALA) would not be able to function in vivo but might still appear active when assayed with an artificial electron donor.

Assuming that the 19,500-molecular-weight subunit found in the Triton-solubilized nitrate reductase is the cytochrome b_1 apoprotein, it might be absent in enzyme from AN344 grown without δALA but present in enzyme from AN344 grown with δALA. Figure 2 shows that this is the case. Antibody was used to precipitate nitrate reductase from Triton-solubilized membrane fractions of AN344 grown with and without δALA. The antibody-precipitated enzyme from the membrane of AN344 grown with δALA was composed of subunits of 142,000 (A), 60,000 (B), and 19,500 (C) molecular weights in a ratio of 1:1:2, as determined on SDS gels (Fig. 2A). An isogenic *hemA*$^+$ strain without δALA had an identical gel pattern. The enzyme precipitated from the Triton-solubilized membrane proteins of AN344 grown without δALA contained none of the 19,500-molecular-weight (C) protein. Moreover, in place of the 60,000-molecular-weight (B) subunit, only very small peaks were present with molecular weights ranging from 55,000 to 25,000. The 142,000-molecular-weight (A) subunit was present (Fig. 2A). A comparison of enzyme activity (Table 3) in the Triton-solubilized membranes shows that the methyl viologen enzyme activity is slightly higher in the unsupplemented mutant. However, if total antibody-precipitable protein is compared, more nitrate reductase protein is

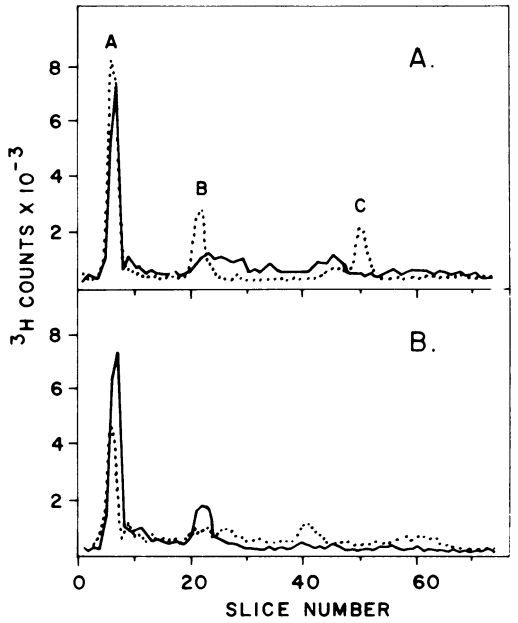

FIG. 2. *Protein profile on SDS gels of ^{3}H-labeled material precipitated by rabbit anti-nitrate reductase from Triton-solubilized membrane (A) and cytoplasmic (B) fractions of strain AN344. In each case the solid line represents AN344 grown without δALA and the dotted line represents AN344 grown with δALA. A, B, and C identify the three peptide peaks found in nitrate reductase solubilized by Triton from the membrane of wild-type E. coli (4). Methods for Triton extraction, precipitation with antisera, and gel procedures are described elsewhere (4).*

TABLE 3. *Distribution of nitrate reductase activity and immune-precipitable nitrate reductase protein in a hemA mutant grown with and without δALA*[a]

Supplement	Total nitrate reductase protein (mg)		Total nitrate reductase activity (mU)	
	TS membrane	Cytoplasm	TS membrane	Cytoplasm
none	0.78	2.00	24,000	117,000
δALA	1.30	0.60	20,000	19,000

[a] Identical cultures of strain AN344 were grown with and without δALA. Both cultures were grown with [^{3}H]leucine to permit determination of protein in immune precipitates from the cytoplasm and from Triton extracts of the membrane fractions. Enzyme activity was measured in each fraction before and after immune precipitation, and in all cases greater than 99% of the activity was removed by immune precipitation. TS, Triton solubilized.

found in the membrane from the supplemented mutant. A similar comparison of enzyme activities in the cytoplasm fractions from this same experiment demonstrated that there was six times the assayable activity in the cytoplasm from the strain grown without δALA as compared to that in the supplemented strain but only 3.3 times the amount of precipitable protein. The excessive amount of nitrate reductase seen in this *hemA*$^-$ mutant grown without δALA, then, is not bound to the membrane but located in the cytoplasm. An SDS gel comparison of the nitrate reductase precipitated from each cytoplasmic fraction is shown in Fig. 2B. The δALA-supplemented mutant has the same gel pattern seen in the isogenic *hemA*$^+$ strain and in wild-type RK7 (4), i.e., only the A subunit is distinguishable. However, the nitrate reductase in the cytoplasm of the unsupplemented mutant is composed of both the A and B subunit. In this case, the ratio of subunit A to subunit B is greater than 1:1. In neither the cytoplasm nor the membrane fraction of the mutant grown without δALA is there any 19,500-molecular-weight protein (subunit C) bound to the nitrate reductase.

DISCUSSION

Two kinds of experiments have been used to prove that Triton-solubilized nitrate reductase contains bound cytochrome b_1 and that the 19,500-molecular-weight subunit C is actually the cytochrome b_1 apoprotein. One series of experiments involved the spectral measurement of cytochrome b_1 in antibody precipitates. These experiments demonstrated that cytochrome b_1 co-precipitates with nitrate reductase from Triton-extracted membrane. The following evidence has been given: (i) anti-nitrate reductase serum precipitates cytochrome b_1 but preimmune serum does not. (ii) The precipitation of cytochrome b_1 from Triton extracts follows the precipitation of nitrate reductase from these extracts. (iii) Antibody precipitates from mixtures of Triton- and heat-solubilized membrane proteins contain decreasing amounts of cytochrome b_1 as the ratio of Triton-solubilized enzyme to heat-solubilized enzyme in the precipitate decreases.

A second series of experiments involved the use of a *hemA* mutant. Grown under conditions where it synthesizes no heme, this mutant still makes nitrate reductase but the antibody-precipitated enzyme contains no subunit C.

Difference spectra revealed the presence of only very small amounts of cytochrome b_1 in the enzyme precipitated from crude heat-solubilized membrane or in the crude heat extract itself. (The cytochrome was completely removed from the enzyme during purification after alkaline heat extraction (8).) Equally small amounts of cytochrome were found in the material which remained bound to the membrane after heating. It has been shown previously (4) that when the membrane is heated in the presence of the protease inhibitor *p*-aminobenzamidine, nitrate reductase is not degraded and the activity remains in the membrane. Subunits A and B also remain in the membrane but subunit C is lost. Thus, the loss of the difference peak at 558 nm after heating corresponds to the loss of the C subunit from the enzyme after heating.

The fact that total removal of nitrate reductase from Triton extracts results in the removal of only 65% of the cytochrome b_1 is probably due to the remainder of the cytochrome b_1 being associated with other enzymes, such as formate dehydrogenase (3).

Cytochrome b_1 must also be involved in the regulation of nitrate reductase. This regulation is in two directions: amount of enzyme produced and attachment of the enzyme to the membrane. Measurements of the amount of antibody-precipitable nitrate reductase in the cytoplasm and Triton-soluble membrane fractions indicated that there is at least a 1.6-fold increase in the total amount of enzyme protein synthesized in mutants lacking functional cytochrome b_1, compared to those with functional cytochrome b_1. The protein profile on SDS gels of membrane-bound nitrate reductase from the mutant lacking functional cytochrome b_1 indicates that when the cytochrome is not bound to the enzyme, the enzyme appears to be degraded. This is evidenced by the loss of the B subunit (Fig. 2A) and by the reduction of the total amount of membrane-bound nitrate reductase (Table 3). In contrast, the cytoplasm of the mutants without functional heme contains enzyme with the B subunit intact. However, there is about 25% more A subunit present than B subunit. My interpretation of these data is the following: if all the nitrate reductase synthesized in the unsupplemented *hemA* mutant were inserted into the membrane, the membrane would be 32% nitrate reductase. Such a membrane could probably not be made. Therefore much of the nitrate reductase synthesized under these conditions is never inserted into the membrane and, consequently, appears undegraded. The excess A subunit present is that released from the membrane. These observations support the previous conclusion (4) that

the B subunit is responsible for the attachment of the enzyme to the membrane.

It should be noted that Haddock and Schairer (1) have shown that a similar *hemA* mutant is able to synthesize the cytochrome apoproteins when grown without δALA, although they were not able to demonstrate whether these apoproteins were inserted into the membrane or remained cytoplasmic. This may also be the case in this mutant. The apoprotein without heme may influence the regulation of nitrate reductase in a different way than does the intact cytochrome. What is obvious in this mutant is that the cytochrome b_1 apoprotein, present or not, is not attached to the nitrate reductase in the same way as in the wild type.

It has been demonstrated previously that increased amounts of nitrate reductase can be measured by methyl viologen assay after heating the membrane-bound enzyme (4). A difference in assayable enzyme activity can also be seen in the different forms of nitrate reductase found in the *hemA* mutant. When milligrams of enzyme protein is compared with units of enzyme activity in Table 3, it becomes obvious that the relationship between protein and activity differs. Further, it can be demonstrated in the wild type that cytoplasmic nitrate reductase has a higher specific activity (based on milligrams of antibody-precipitable protein) than that of membrane-bound enzyme. The cytoplasmic enzyme contains only subunit A, whereas the membrane-bound form contains all three subunits. If specific activity in the membrane fraction is calculated on only the amount of A protein present, the activity increases to that of the cytoplasmic nitrate reductase. These observations indicate that the only part of the enzyme necessary for methyl viologen-nitrate reductase activity is subunit A. Thus the methyl viologen assay is not an accurate measure of the total amount of nitrate reductase protein present, nor is the specific activity based on this assay a proper measure of the purity of the enzyme.

Finally, one might gain insight into the functioning of anaerobic electron transport, using the information we have accumulated about nitrate reductase. Since the enzyme complex appears to always have cytochrome b_1 bound to it (that is, the enzyme which is functional in vivo) and the cytochrome is always present in a fixed ratio of cytochrome to enzyme, and since there is evidence that formate dehydrogenase and cytochrome b_1 have a similar relationship, the transfer of electrons from formate dehydrogenase to nitrate reductase might be best pictured as occurring as a result of the collision of individual enzyme-cytochrome complexes. The classical concept of a chain of cytochromes and enzymes fixed in the membrane is likely not to apply in this case.

ACKNOWLEDGMENTS

I thank Gary Norton and Montserrat Salsas for their skilled assistance.

This research was supported by research grant GB-25273 from the National Science Foundation, and by Public Health Service research grant GM-18006 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Haddock, B. A., and H. U. Schairer. 1973. Electron transport chain in *E. coli*. Eur. J. Biochem. **35**:34-45.
2. Hager, L., and E. Itagaki. 1967. The preparation of bacterial cytochrome b_1, p. 367-372. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 10. Academic Press Inc., New York.
3. Linnane, A. W., and C. W. Wrigley. 1963. Fragmentation of the electron transport chain of *Escherichia coli*. Biochim. Biophys. Acta **77**:408-418.
4. MacGregor, C. H. 1975. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. **121**:1102-1110.
5. MacGregor, C. H. 1975. Synthesis of nitrate reductase components in chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **121**:1111-1121.
6. MacGregor, C. H., and C. A. Schnaitman. 1971. Alterations in the cytoplasmic membrane proteins of various chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **108**:564-570.
7. MacGregor, C. H., and C. A. Schnaitman. 1973. Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **114**:1164-1176.
8. MacGregor, C. H., C. A. Schnaitman, D. E. Normansell, and M. G. Hodgins. 1974. Purification and properties of nitrate reductase from *Escherichia coli* K-12. J. Biol. Chem. **249**:5321-5327.
9. Ruiz-Herrera, J., and J. A. DeMoss. 1969. Nitrate reductase complex of *Escherichia coli* K-12: participation of specific formate dehydrogenase and cytochrome b_1 components in nitrate reductase. J. Bacteriol. **99**:720-729.
10. Taniguchi, S., and E. Itagaki. 1960. Nitrate reductase of nitrate respiration type from *E. coli*. Biochim. Biophys. Acta **44**:263-279.