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Escherichia coli Mutants Lacking NADH Dehydrogenase I Have a Competitive Disadvantage in Stationary Phase

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We have previously characterized mutant strains of *Escherichia coli* that are able to take over stationary-phase cultures. Here we describe two insertion mutations that prevent such strains from expressing this phenotype. Both insertions were mapped to min 51, and sequence analysis revealed that both mutated genes encode proteins homologous to subunits of mitochondrial NADH dehydrogenase I. Crude extracts prepared from both mutant strains were able to oxidize NADH but lacked the enzymatic activity needed to oxidize deamino-NADH, a substrate specific for NADH dehydrogenase I. This is the first identification of genes encoding subunits of NADH dehydrogenase I in *E. coli*. The significance of the inability of these mutant strains to compete in stationary-phase cultures is discussed.

An important feature of aerobic respiration in *Escherichia coli* involves the oxidation of NADH coupled to the generation of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$). This reaction is carried out by the enzyme NADH dehydrogenase, a membrane-bound component of the respiratory chain. Immunochemical studies of membrane vesicles (15) indicated the presence of at least two distinct NADH dehydrogenases in *E. coli* cytoplasmic membranes. Matsushita et al. were able to distinguish between two distinct membrane-bound NADH dehydrogenases, NDH-1 and NDH-2, on the basis of substrate specificity, sensitivity to inhibitors, and ability to generate $\Delta\bar{\mu}_{H^+}$ (13). The enzyme NDH-1, which contains nonheme iron and can oxidize both NADH and reduced nicotinamide hypoxanthine dinucleotide (deamino-NADH, or d-NADH), can lead to the generation of $\Delta\bar{\mu}_{H^+}$ (13). NDH-2, on the other hand, utilizes only NADH, and its oxidation is not coupled to the generation of $\Delta\bar{\mu}_{H^+}$. Purified NDH-2 enzyme consists of a single polypeptide of 47 kDa containing flavin adenine dinucleotide as a cofactor (8), whereas NDH-1 contains flavin mononucleotide as a cofactor (5). The gene encoding NDH-2 (*ndh*) has been cloned and sequenced (31, 32). An initial description of genes potentially encoding NDH-1 subunits has been reported (26), and during the review process of this paper, a mutant lacking NDH-1 (*nuo*) was described (4).

During growth, electron transport chains result in the electrogenic translocation of protons across the cytoplasmic membrane to generate a proton motive force used to drive ATP synthesis and a number of other cellular processes (2). The physiological importance of electron transport chains during conditions of starvation or poor nutrient availability, however, are less well understood.

It is known that *E. coli* cells can remain viable during prolonged periods of starvation (20). We have recently reported that population changes take place in stationary-phase cultures as mutants with a growth advantage take over the population (34). We refer to this phenomenon as the GASP (growth advantage in stationary phase) phenotype. Certain down mutations in *rpoS*, the gene encoding the alternative sigma factor σ^S, which regulates expression of several genes in stationary phase (6, 22), can confer the GASP phenotype on cells (34).

We have carried out a mutational analysis to identify genes required for the expression of the GASP phenotype. Here we describe two of the mutants that lost the GASP phenotype because of insertions in genes whose predicted products are homologous to subunits of the mitochondrial NDH-1. In addition, the mutant strains lost the ability to oxidize d-NADH but not NADH. Thus, we have identified and characterized two genes, designated *nuoA* and *nuoB*, encoding subunits of NDH-1 from *E. coli*, and we have shown that cells lacking this enzyme have a competitive disadvantage in stationary-phase cultures.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study were ZK126 (= W3110 *tna2* Δ*lacU169* (23), ZK1141 (= ZK126 *rpos819 sga rpsL* [this study]), ZK820 (= ZK126 *rpoS819 gyrA* [34]), ZK1362 (= ZK1141 *nuoA*::mini-Tn*10*Cm [this study]), ZK1363 (= ZK1141 *nuoB*::mini-Tn*10*Cm [this study]), and MC1061 for routine transformations (28).

The media used have been described previously (14). M63 medium was supplemented with 1 μg of thiamine per ml and 1 mM $MgSO_4$. Glucose was added at a final concentration of 0.2%, mannitol was added at 0.2%, succinate was added at 0.4%, glycerol was added at 0.5%, and Casamino Acids were added at 0.5%. Antibiotics were used at the following concentrations: streptomycin at 20 μg/ml, nalidixic acid at 20 μg/ml, and chloramphenicol at 30 μg/ml.

Mixed-culture conditions. Cultures were grown in 3 ml of Luria broth (LB) in glass test tubes (18 by 150 mm) and kept aerated by rotation in a New Brunswick roller at 37°C. Mixes were done after 1 day of growth by transferring 3 μl of the culture to be placed as a minority into a culture of strain ZK820. The titers of cultures were determined by making dilutions in M63 salts and plating on LB plates containing the appropriate antibiotic.

Mutagenesis and screen. Random insertions of mini-Tn*10*Cm into the chromosome of ZK1141 were done as previously described (25) by using the λ vector NK1324 (9). Chloramphenicol-resistant (Cm^r) colonies harboring the

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mini-Tn*10*Cm element were picked onto LB-chloramphenicol plates to form a grid pattern of 48 colonies per plate and grown overnight. A 48-prong metal adapter was used to transfer cells from these colonies to microtiter dishes containing 100 μl of M63 salts per well. Portions (2.5 μl each) of the resulting cell suspensions were spotted onto a freshly made lawn of ZK820 cells on LB plates and incubated at 37°C for several days. The random-insertion mutants were monitored for growth on the lawn. ZK1141 spotted onto a freshly made lawn of ZK820 cells can grow over the lawn after several days of incubation at 37°C. This growth over the background lawn, characteristic of ZK1141, is absent in cells which do not express the GASP phenotype in mixed cultures with ZK820. Potential GASP$^-$ mutants were retested on plates, streaked for single colonies from the master plate, and tested as a minority in liquid mixed cultures with ZK820 as the majority cell population. From 5,500 colonies screened, five GASP$^-$ mutants were isolated. Two of these mutants, designated ZK1362 and ZK1363, are described in this paper.

Cloning, mapping, and sequencing. Chromosomal DNA was prepared from strains ZK1362 and ZK1363 as described elsewhere (3), digested to completion with *Pst*I (New England Biolabs), ligated to *Pst*I-digested pUC19, and used to transform competent MC1061. Plasmid DNA was purified from Cm^r transformants and analyzed by restriction enzyme digestions. Recombinant plasmids were either radioactively labelled by nick translation (12) or labelled with digoxigenin by following the manufacturer's protocol (Boehringer) and used as probes against filters containing Kohara's ordered λ library of the *E. coli* chromosome (10). Plasmid DNA was purified with Qiagen columns and sequenced with Sequenase (U.S. Biochemicals).

Preparation of cell extracts and NDH assays. Cells grown in 20 ml of LB at 37°C with aeration were harvested by centrifugation after 18 h and resuspended in 2 ml of buffer A (50 mM KPO_4 [pH 7.5], 5 mM $MgSO_4$) (13). Samples were sonicated and then centrifuged for 15 min at 15,000 × *g* in a microcentrifuge. Supernatants, containing 4 to 5 mg of total protein per ml, were used for assays after the addition of dithiothreitol to a final concentration of 1 mM. Assays were conducted with 50 μl of the extract in 0.5 ml of buffer A and started by the addition of 125 μM NADH or d-NADH. Oxidation of NADH and d-NADH at 25°C was measured spectrophotometrically at 340 nm.

RESULTS

Isolation of mutants unable to express the GASP phenotype. We have previously shown that the *rpoS819* allele can confer the GASP phenotype on otherwise wild-type cells (34). Furthermore, prolonged incubation of an *rpoS819* strain selects for mutants that can take over cultures of the parental strain harboring the *rpoS819* allele (34). The mutation responsible for the growth advantage over *rpoS819* cells has not been mapped but has been designated *sga* (stationary-phase growth advantage). Strain ZK1141 has the *rpoS819* allele and the *sga* mutation and thus can take over a culture of ZK820 (ZK126 *rpoS819 gyrA*) (Fig. 1A). To understand some of the functions required for expression of this phenotype in ZK1141, mutagenesis was carried out with the defective transposon mini-Tn*10*Cm (9). Cells were screened for the loss of the GASP phenotype by their inability to grow on a lawn of cells on LB plates (see Materials and Methods). In contrast to the parental strain (ZK1141), one of the insertion mutants obtained, ZK1362, died quickly when mixed as a minority with a 1-day-old ZK820 culture (Fig. 1B).

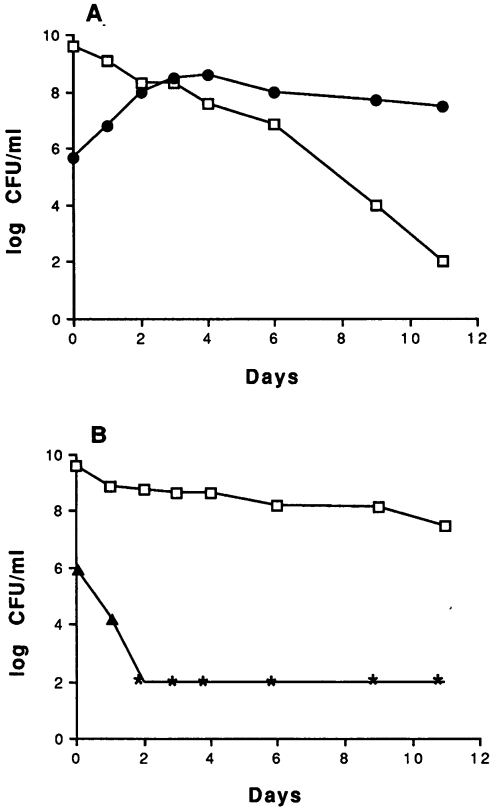

FIG. 1. Phenotypes of ZK1141 (A) and insertion mutant ZK1362 (B) in liquid mixed cultures. Strains ZK1141 (●) and ZK1362 (▲) were mixed as a minority with ZK820 (□) cultures. Viable cell counts, determined by plating dilutions of the cultures on appropriate media, are expressed as CFU per milliliter. Asterisks indicate concentrations lower than 10^2 CFU/ml.

Viability during prolonged incubation was determined to see whether this mutant differed from the parental strain in terms of its capacity to survive under conditions of starvation. Like the parental strain (Fig. 2A), ZK1362 remained viable during several days of incubation in LB and M63-glucose media with chloramphenicol to select for the presence of the transposon in the chromosome (Fig. 2B). The phenotype of the second mutant isolated, ZK1363, was the same as that of ZK1362 both in mixed cultures and during prolonged incubation in LB and M63-glucose media (not shown).

The mutant strains ZK1362 and ZK1363 were further characterized by determining their capacity to grow on M63 minimal medium supplemented with different carbon sources. Both the mutants and parental strains displayed indistinguishable doubling times on mannitol (1.7 h), glycerol (1.2 h), and Casamino Acids (3.0 h), and the cell yield (as determined by final optical density) was the same for both strains in each carbon source. However, in succinate-containing medium the growth rates differed greatly. While the parent ZK1141 displayed a doubling time of more than 10 h in succinate-containing medium, the mutants grew much better, with doubling times of about 6 h. Consistent with these rates, the mutants gave rise to visible colonies after 3 days on succinate minimal plates while ZK1141 did not. The reason for this phenotype remains unknown.

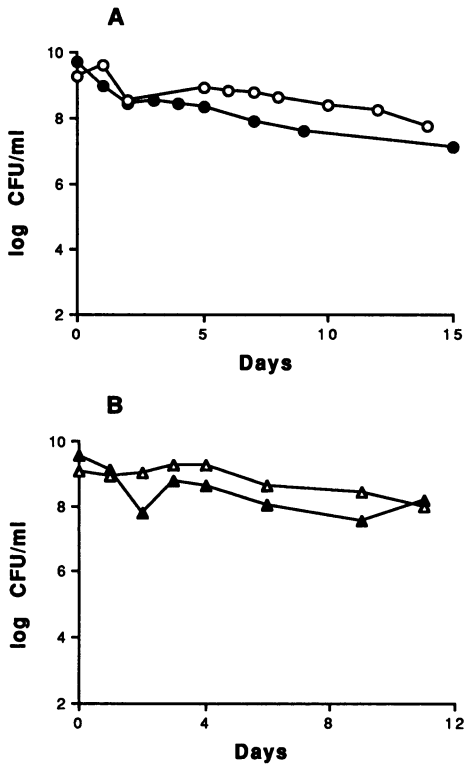

FIG. 2. Viability of strains in stationary phase. Strains ZK1141 (A) and ZK1362 (B) were grown in LB (closed symbols) or M63-glucose (open symbols), and the titers of cultures were determined on the indicated days. Viability is expressed as CFU per milliliter.

Cloning of the chromosomal fragments containing the mini-Tn*10*Cm element. *Pst*I fragments from each of these strains, containing the intact mini-Tn*10*Cm element and flanking chromosomal DNA sequences, were cloned in pUC19. These plasmids were then used as probes against Kohara's ordered λ library of the *E. coli* chromosome (10). The transposon insertions, approximately 10 kb apart from each other, mapped to adjacent *Pst*I fragments in min 51 of the *E. coli* chromosome (Fig. 3) (19). The DNA flanking the mini-Tn*10*Cm insertion element in ZK1362 was sequenced directly from the plasmid DNA and found to contain an open reading frame of 1,527 bp. The predicted gene product is extremely hydrophobic, containing 12 membrane-spanning domains as predicted by the method of von Heijne (24). When the predicted gene product of 509 amino acids was compared with the PIR protein sequence data base, it was found to have 35% identity over the entire protein sequence with subunit 4 of the wheat mitochondrial NDH-1 (Fig. 4A) (11). The partial sequence obtained so far from the disrupted locus in ZK1363 also predicted a peptide homologous to the NADH-binding subunit of NDH-1 from mitochondria (16, 17) and the bacterium *Paracoccus denitrificans* (Fig. 4B) (29).

NDH-1 and NDH-2 assays of the mutants. To test the possibility that strains ZK1362 and ZK1363 contained insertion mutations in genes encoding subunits of the enzyme NDH-1, cell extracts were prepared from both the parental strain, ZK1141, and the two mutants. Crude extracts from LB-grown cells were prepared by sonication and assayed for their capacity to oxidize both NADH and d-NADH. As shown in Fig. 5, the parental strain ZK1141 was capable of oxidizing both substrates, whereas the mutant strain ZK1362 was not. Extracts from the mutant contained an enzymatic activity capable of oxidizing NADH yet lacked the activity necessary for oxidation of d-NADH. The difference between the parental and mutant strains in their capacity to oxidize NADH (Fig. 5) is due to the fact that both NDH-1 and NDH-2 can utilize this substrate but only NDH-2 is present in the mutant strain. The results obtained for strain ZK1363 were virtually identical to those obtained for ZK1362 (results not shown).

DISCUSSION

Two insertion mutants derived from strain ZK1141 which no longer express the GASP phenotype in mixed cultures were isolated. Whereas ZK1141 can both grow and cause the death of the original majority population over the span of several days in stationary phase, both these mutants died

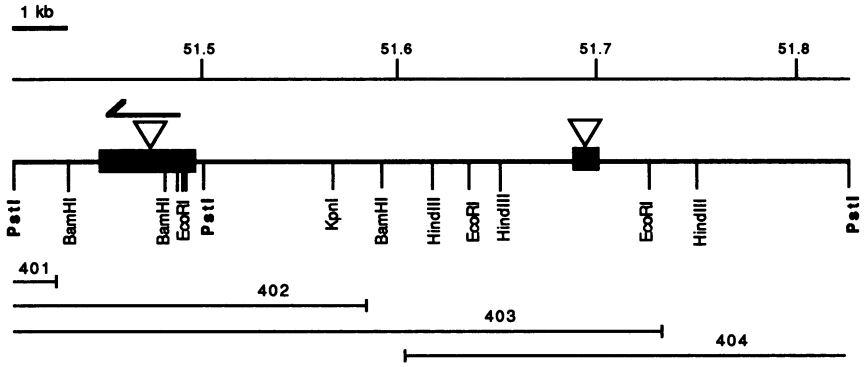

FIG. 3. Map locations of the disrupted genes in strains ZK1362 and ZK1363. *Pst*I fragments containing the mini-Tn*10*Cm insertion elements were predicted to be contiguous on the basis of restriction enzyme analysis of the cloned fragments and hybridization to Kohara phages (numbers shown below) carrying DNA fragments covering the region. Most of the restriction sites found in the cloned fragments matched the Kohara map. However, we found one fewer *Pst*I site. In addition, contrary to expectation, our clone containing open reading frame 2 did not hybridize to phage 404 from the Kohara collection. Triangles indicate the relative positions of the mini-Tn*10*Cm elements within the sequenced regions (shaded rectangles) identified in ZK1362 (rectangle 1) and ZK1363 (rectangle 2). The arrow indicates the predicted direction of transcription. The coordinates shown at the top give the positions, in minutes, of the region according to the published physical map of the *E. coli* genome (19).

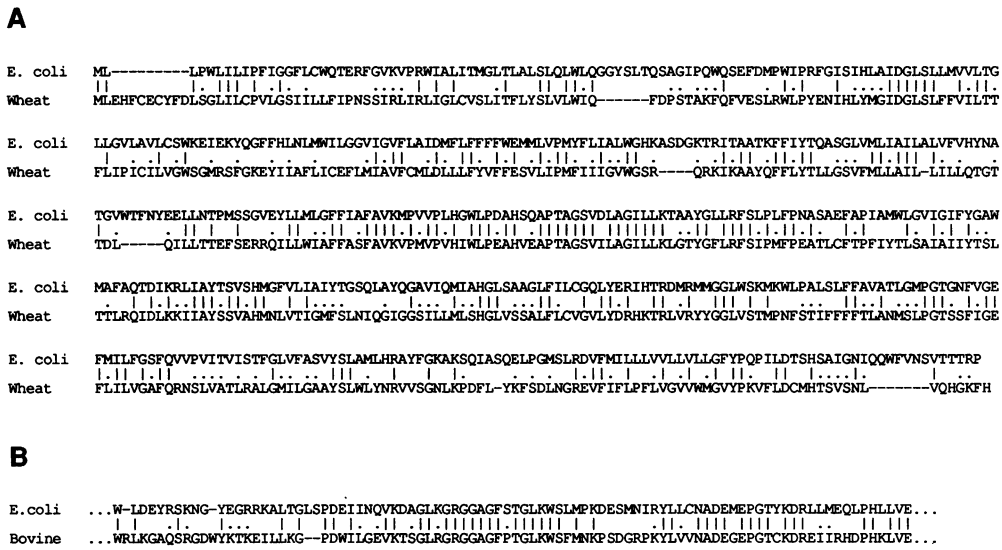

FIG. 4. Alignment of the predicted amino acid sequences of NuoA and wheat mitochondrial NDH-1 subunit 4 (11) (A) and NuoB and the bovine mitochondrial NADH binding subunit (17) (B). Alignments were obtained by the method of Altschul et al. (1). Vertical bars indicate identical amino acids, and dots indicate conserved changes. Dashes represent gaps in the sequence introduced to obtain the optimal alignment.

under these particular mixed-culture conditions, as shown in Fig. 1. Viability in stationary phase, however, seemed largely unaffected (Fig. 2). This suggests that these mutations do not affect viability in stationary phase per se but rather undermine the cell's capacity to compete against cells with a functional NDH-1 under the particular conditions imposed by starvation in LB medium.

Both these mutants contained chromosomal insertions on contiguous *Pst*I fragments located at min 51 of the *E. coli* physical map. Sequence analysis of the DNA flanking the insertion elements in both ZK1362 and ZK1363 revealed predicted protein products homologous to subunits of the energy-transducing NDH-1 complex in mitochondria and the bacterium *P. denitrificans* (11, 16, 17, 29). Identity is conserved throughout the entire predicted protein sequence in the case of ZK1362 and through most of the partial sequence (112 amino acids) obtained thus far for ZK1363. In addition, cell extracts prepared from both mutant strains lacked the enzymatic activity needed for oxidation of d-NADH, suggesting that the insertion mutations in strains ZK1362 and ZK1363 disrupt NDH-1 activity. The mitochondrial NDH-1 is a complex protein consisting of at least 30 different subunits encoded by both nuclear and mitochondrial genes (27). The similar, although smaller, energy-generating enzyme in *P. denitrificans* is composed of approximately 10 different polypeptides (30). The identification of two mutations mapping close to each other and having similar phenotypes indicates that the *E. coli* enzyme also consists of several polypeptides, as was suggested in a preliminary report by Weidner et al. (26). On the basis of protein sequence similarities, the mutated genes in strains ZK1362 and ZK1363 are likely to encode different subunits of NDH-1. Recently, a new locus encoding NDH-1, designated *nuo* (NADH-ubiquinone oxidoreductase) and located at min 49 (min 51 in the revised version) on the *E. coli* map, has recently been reported (4). The disrupted genes in strains ZK1362 and ZK1363 identified in this study are therefore designated *nuoA* and *nuoB*, respectively.

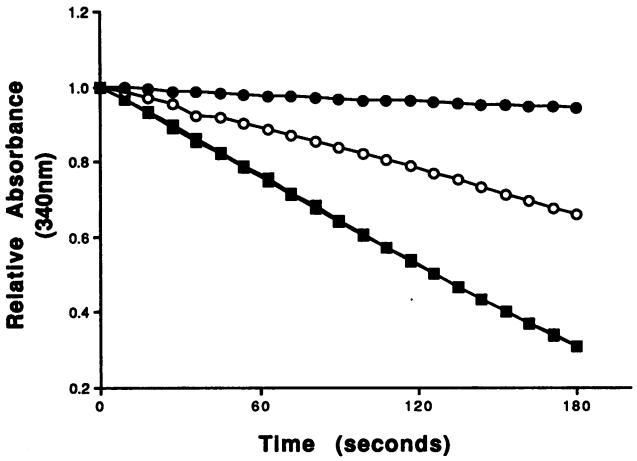

FIG. 5. NDH-1 and NDH-2 assays. Crude extracts prepared from strains ZK1141 (squares) and the mutant ZK1362 (circles) were assayed for the capacity to oxidize NADH (open symbols) and d-NADH (closed symbols). Results were normalized to 1 and are therefore given as relative absorbance. Note that in the case of ZK1141, the curves obtained with both substrates (open and closed squares) overlap one another.

The mutants isolated in this study are phenotypically distinct from cells bearing mutations in the *ndh* gene for NDH-2. Both of our *nuo* mutant strains grew well on M63-mannitol and LB, contrary to what has been reported for the previously identified *ndh* mutant (7, 33). It has recently been shown, however, that the strain used in these previous studies contained mutations in both NADH dehydrogenases and that mutations in either one of these enzymes do not significantly alter growth ability (4). Even though our mutant strains are apparently defective in what is likely to be the major enzyme in NADH oxidation, they showed no obvious difference in growth ability (growth rate or cell yield) from strains with a functional NDH-1 on any of the media tested, with one notable exception: both mutants

displayed a much higher growth rate than the parental strain on M63-succinate (6-h versus 10-h doubling times). It is unclear why mutations in NDH-1 result in faster growth on succinate-containing medium.

The *nuo* mutations described here result in a clear disadvantage for the cells in mixed stationary-phase cultures, reflecting the importance of electron transport chains in stationary-phase physiology. What might account for this competitive disadvantage? The efficiency of the respiratory chain may depend greatly on NDH-1 activity during stationary phase for two reasons. First, NDH-2 activity may be repressed in stationary phase when the oxygen tension is low, since it is known to be repressed by the global regulator Fnr under anaerobic conditions (21). Second, stationary-phase *E. coli* utilizes cytochrome *d*, which has a H^+/e^- ratio of only 1 and is less efficient than cytochrome *o*, whose H^+/e^- ratio is 2 (18). Thus, the lack of NDH-1 would necessarily mean that a lower amount of energy can be obtained from a given carbon source. Under conditions of poor nutrient availability in which cell growth of a minority population can still take place, such as those present in stationary-phase LB cultures, optimal utilization of scarce energy sources might confer a significant competitive advantage. On the other hand, during exponential growth when nutrients are plentiful, mutations in NDH-1 are apparently less critical, since no obvious difference in growth rate was observed between the mutant and parental strains. Further characterization of these mutants is necessary to understand more fully the role of NDH-1 both during growth and during starvation. Identification of the *E. coli* genes encoding the NDH-1 enzyme provides a powerful system for biochemical and genetic analysis of this key respiratory enzyme.

Nucleotide sequence accession numbers. The sequences of *nuoA* and *nuoB* have been submitted to GenBank under accession numbers L19568 and L19569, respectively.

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ADDENDUM IN PROOF

After consultation with K. Rudd, and to be consistent with accepted nomenclature, *nuoA* and *nuoB* will henceforth be designated *nuoF* and *nuoM*, respectively.

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