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Mutations in NADH:Ubiquinone Oxidoreductase of *Escherichia coli* Affect Growth on Mixed Amino Acids

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We isolated and characterized mutants defective in *nuo*, encoding NADH dehydrogenase I, the multisubunit complex homologous to eucaryotic mitochondrial complex I. By Southern hybridization and/or sequence analysis, we characterized three distinct mutations: a polar insertion designated *nuoG*::Tn*10*-1, a nonpolar insertion designated *nuoF*::Km-1, and a large deletion designated Δ(*nuoFGHIJKL*)-1. Cells carrying any of these three mutations exhibited identical phenotypes. Each mutant exhibited reduced NADH oxidase activity, grew poorly on minimal salts medium containing acetate as the sole carbon source, and failed to produce the inner, L-aspartate chemotactic band on tryptone swarm plates. During exponential growth in tryptone broth, *nuo* mutants grew as rapidly as wild-type cells and excreted similar amounts of acetate into the medium. As they began the transition to stationary phase, in contrast to wild-type cells, the mutant cells abruptly slowed their growth and continued to excrete acetate. The growth defect was entirely suppressed by L-serine or D-pyruvate, partially suppressed by α-ketoglutarate or acetate, and not suppressed by L-aspartate or L-glutamate. We extended these studies, analyzing the sequential consumption of amino acids by both wild-type and *nuo* mutant cells growing in tryptone broth. During the lag and exponential phases, both wild-type and mutant cells consumed, in order, L-serine and L-aspartate. As they began the transition to stationary phase, both cell types consumed L-tryptophan. Whereas wild-type cells then consumed L-glutamate, glycine, L-threonine, and L-alanine, mutant cells utilized these amino acids poorly. We propose that cells defective for NADH dehydrogenase I exhibit all of these phenotypes, because large NADH/NAD$^+$ ratios inhibit certain tricarboxylic acid cycle enzymes, e.g., citrate synthase and malate dehydrogenase.

Membrane-bound NADH dehydrogenase (NADH dh [EC 1.6.99.3]) is the first enzyme complex in the electron transport chain, transferring electrons to ubiquinone, which then transfers electrons to either of two terminal oxidases (1). *Escherichia coli* possesses two membrane-bound NADH dhs, NADH dhI and NADH dhII (25). NADH dhI (NADH:ubiquinone oxidoreductase), a multisubunit complex homologous to eukaryotic mitochondrial complex I (48), couples its reaction with the production of a proton motive force (24, 25). It contains four iron-sulfur clusters (26) and exhibits both NADH oxidase and NADH dh activities, oxidizing either NADH or deamino NADH (dNADH [15]). Ferricyanide is its preferred electron acceptor in vitro (15, 25). NADH dhII consists of a single polypeptide (47 kDa) that contains flavin adenine dinucleotide but no iron (16). It exhibits both NADH oxidase and NADH dh activities (10), oxidizing NADH, but not dNADH. Ubiquinone seems to be its favored electron acceptor in vitro (15). This reaction does not lead to the formation of a proton motive force (25). Although the function of these two NADH dhs remains unclear, Calhoun and Gennis (6) propose that *E. coli* might regulate the amount of energy recovered from NADH oxidation by modulating the relative levels of these two NADH dhs.

The genetic loci *nuo* (NADH:ubiquinone oxidoreductase), which maps 51.5 and 51.8 min on the *E. coli* linkage map (33a), and *ndh* (NADH dehydrogenase), which maps at 25.1 min, encode NADH dhI and NADH dhII, respectively. Both loci were identified in related mutant strains, AN589 (52) and IY12 (6). Cells containing mutations in both *nuo* and *ndh*, e.g., those of strains AN589 and IY12, cannot grow on minimal mannitol medium and form small, slowly growing colonies on rich medium (6, 52). In contrast, cells containing a mutation in either locus, but not both, were indistinguishable from wild-type cells with respect to both phenotypes (6).

In the present study, we show that cells possessing mutations in the *nuo* locus exhibited a pleiotropic phenotype. Although they possessed normal acetate kinase (Ack [EC 2.7.2.1]) and phosphotransacetylase (Pta [EC 2.3.1.8]) activities, these cells grew poorly on minimal acetate medium. They also failed to produce the inner, L-aspartate band on tryptone swarm plates. When growing in tryptone broth, they abruptly and dramatically slowed their growth at the end of exponential phase. They also secreted large amounts of acetate into the medium and ineffectively consumed certain amino acids, i.e., L-glutamate, L-threonine, glycine, and L-alanine. We propose that cells defective for NADH dhI exhibit all of these phenotypes because large NADH/NAD$^+$ ratios inhibit enzymes, e.g., citrate synthase and malate dehydrogenase, shared by the tricarboxylic acid (TCA) cycle and the glyoxylate shunt.

MATERIALS AND METHODS

Chemicals. Enzymes, substrates, and restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, Ind.), bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, Ill.), radioactive isotopes were purchased from Amersham (Arlington Heights, Ill.), and reagents for high-performance liquid chromatography (HPLC) analysis were purchased from Beckman Instruments (Palo Alto, Calif.).

Bacterial strains and plasmids. Table 1 lists strains and bacteriophages. Figure 1 shows plasmids constructed for this

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TABLE 1. Bacterial strains and bacteriophage used in this study

Strain or bacteriophage	Relevant genotype[a]	Source or reference
Strain		
AJW508	CP762(pAJW104)	This study
AJW509	CP762(pAJW105)	This study
AJW510	CP762(pAJW110)	This study
AN589	*metB ndh-401 nuo*	52
BW13711	Δ*lacX74*	28
CP750	*thi-1 thr*(Am)-*1 leuB6 hisF*(Am)*159 rpsL136 lacY1*	38
CP758	CP750 *ackA*::Tn*phoA'*-9	38
CP760	CP750 *pta*::Tn*phoA'*-3	This study
CP762	CP750 *nuoG*::Tn*10*-1	This study
CP790	*nuoG*::Tn*10*-1 *polA*(Ts) *thi-1 thr*(Am)-*1 leuB6 hisF*(Am)*159 rpsL136 lacY1 xyl-5 ara-14 tonA31 tsx-78 rha zig*::Tn*10*	This study
CP811	*polA* (Ts) *thi-1 thr*(Am)-*1 leuB6 hisF*(Am)*159 rpsL136 xyl-5 ara-14 tonA31 tsx-78 rha zig*::Tn*10*	This study
CP875	CP750 Δ*lacX74* λ*lacY*	This study
CP909	CP875 *purF nuoG*::Tn*10*-1	This study
CP911	CP875 Δ(*ackA pta hisJ hisP dhu*) *zej-223*::Tn*10*	This study
CP938	CP875 Δ(*nuoFGHIJKL*)-1	This study
CP956	CP875 *nuoF*::Km-1	This study
FD500	*thi-1 thr*(Am)-*1 leuB6 hisF*(Am)*159 rpsL136 lacY1 xyl-5 ara-14 tonA31 tsx-78 eda50 mtl-1 nuoG*::Tn*10*-1	9
HCB433	*thi-1 thr*(Am)-*1 leuB6 hisF*(Am)*159 rpsL136 lacY1 xyl-5 ara-14 tonA31 tsx-78 eda50 mtl-1* Δ(*tsr*)*7021* Δ(*trg*)*100 zdb*::Tn*5*	50
W3110	Wild type	3
Bacteriophage		
λ403	Derived from λ2001; also designated E9B9	19
λ*tar*	c*I857*, derived from λgt-4	22
λ*vir*		Laboratory collection

[a] *nuoG*::Tn*10*-1, *zej-223*::Tn*10*, and *zig*::Tn*10* confer Tc[r]; *nuoF*::Km-1, *zdb*::Tn*5*, *ackA*::Tn*phoA'*-9, and *pta*::Tn*phoA'*-3 confer Km[r].

work. Generalized transductions and transformations were performed as described elsewhere (39).

All strains were derivatives of *E. coli* K-12. Strains were kindly provided by the following: F. Dailey (Harvard University), strain FD500; B. Wanner (Purdue University), strain BW13711; and B. Bachmann (Yale University), strain AN589.

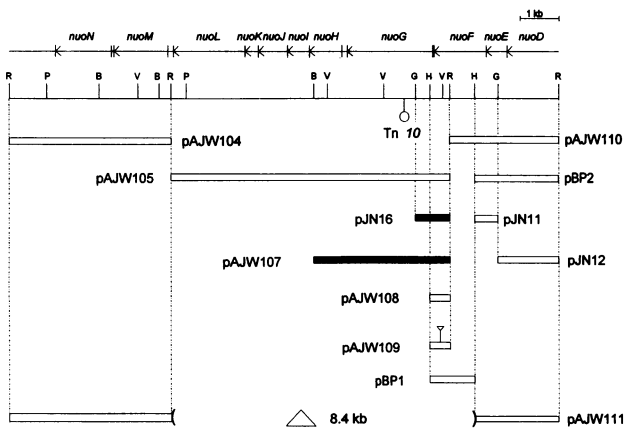

FIG. 1. Region of the chromosome, carried by the phage λ403, that contains the *nuo* locus. Arrows indicate the locations and transcriptional orientations of *nuo* genes. Endpoints of plasmids are indicated by the restriction sites (B, *Bam*HI; G, *Bgl*II; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; V, *Eco*RV). The open circle with vertical line represents the location in the chromosome of *nuoG*::Tn*10*-1; The open triangle with vertical line represents that of *nuoF*::Km-1 in the plasmid pAJW109. The cross-hatching represents fragments used as probes for Southern hybridization.

Strain CP762 was constructed by transducing the Tn*10* insertion (subsequently designated *nuoG*::Tn*10*-1) (Fig. 1) from strain FD500 into cells of strain CP750 and by selecting for tetracycline-resistant transductants. Strains CP938 and CP956 were constructed by introducing an *Eco*RI-*Hin*dIII deletion [designated Δ(*nuoFGHIJKL*)-1] and an insertion by the pUC4K kanamycin gene (designated *nuoF*::Km-1) into the chromosome by means of homologous recombination in the *polA*(Ts) *nuoG*::Tn*10*-1 and *polA*(Ts) *nuo*$^+$ host strains CP790 and CP811, respectively. Recombinants were selected as tetracycline sensitive or kanamycin resistant, respectively, and each allele was P1 transduced into the recipient strains CP909 and CP875, respectively. Recombinants and transductants were screened for their abilities to grow on minimal acetate medium and to produce the inner, L-aspartate band on tryptone swarm plates.

Homologous recombination between cells of strain CP762 and the phage λ403 was performed at 30°C as described elsewhere (30), with λ*tar* (22) as the source of *c*I repressor protein. Putative recombinants were identified as sensitive to both λ*tar* and λ*vir*.

Medium and growth conditions. Minimal medium consisted of M63 minimal salts (39), containing either D-glucose, D-pyruvate, glycerol, or acetate (25 mM each) and the amino acids required for growth (20 μg/ml each). Tryptone broth consisted of 1% tryptone and 0.5% NaCl (29). If necessary, 10 μg of tetracycline or kanamycin per ml was added. Cells were grown at 37°C, and the optical density at 610 nm (OD_{610}) was monitored.

Swarm assays. Cells were grown at 35°C to mid-exponential phase in tryptone broth. Tryptone swarm plates were 0.20 to 0.25% agar in the same broth. Antibiotics were not added. A 5-μl aliquot of the appropriate culture (10^6 to 10^7 cells) was

inoculated on the surface of a swarm plate near its center, and the plate was incubated at 35°C in a humid environment. Swarm plates were handled and measured as described elsewhere (49).

Southern blot analysis. DNA-DNA hybridization was performed as described elsewhere (12) by the method described by Southern (42). After the first hybridization, the nitrocellulose filter was washed for 20 min with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 95°C to remove the probe, and the filter was hybridized with a second probe.

Sequence analysis. Double-stranded DNA was purified with the Magic Miniprep Purification System (Promega Corp., Madison, Wis.) and denatured as described elsewhere (8). Single-stranded DNA was prepared by the method described by Messing (27) with the Magic M13 DNA Purification System (Promega). Both single- and double-stranded DNAs were sequenced by the chain termination method (35) with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio). Sequence analysis was performed with the software package (version 7.2 [1991]) of the Genetics Computer Group.

Determination of Ack and Pta activities. Cells were grown in 30 ml of tryptone broth and crude extracts prepared as described elsewhere (5). The activities of Ack and Pta were determined by using the coupled reaction described by Brown et al. (5) which links acetyl coenzyme A formation with NAD^+ reduction through citrate synthase and malate dehydrogenase. Specific activities were expressed in units per milligram (where units are micromoles per minute and $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$).

Determination of NADH oxidase and NADH-DCIP reductase activities. Cells were grown in 20 ml of tryptone broth and membrane fractions were prepared as described elsewhere (46). NADH oxidase and NADH-DCIP (2,6-dichlorophenolindophenol) dh activities were measured by monitoring the oxidation of NADH at 340 nm (10). The initial reaction velocity at 600 nm was determined, and specific activities were expressed in units per milligram (where units are micromoles per minute and $\varepsilon_{340} = 20.6\ mM^{-1}\ cm^{-1}$).

Protein concentration determination. A 10-μl volume of the crude extract was diluted to 100 μl with 100 mM NaOH, and the protein concentrations were determined by the bicinchoninic acid reagent method, with bovine serum albumin as a standard (41).

Determination of the extracellular concentration of acetate. Cells were grown in tryptone broth, aliquots were removed at appropriate intervals, the cells were removed by centrifugation, and the supernatant liquid (1 ml) was assayed for acetate in a coupled reaction as described elsewhere (4). The concentration of acetate was expressed in micromolar units.

Amino acid analysis. Cells were grown at 37°C in 200 ml of tryptone broth. At appropriate intervals, 1-ml samples were taken and centrifuged for 5 min at 24,000 × *g*. To remove proteins, 300 μl of the supernatant was treated with 100 μl of 5-sulfosalicyclic acid and centrifuged for 5 min. The supernatant was diluted 1:10 with Beckman sample dilution buffer containing 0.125 μM *S*-2-aminoethyl-L-cysteine as the internal standard. HPLC was performed with the Beckman System 6300 High Performance Analyzer (Palo Alto, Calif.) according to the manufacturer's instructions.

RESULTS

Isolation and initial characterization of mutations in the *nuo* locus. While investigating the role in bacterial chemotaxis of acetyl phosphate synthesis and degradation, we isolated a transposon insertion (subsequently identified as *nuoG*::Tn*10*-1) that caused cells, e.g., those of strain CP762, to grow more poorly on acetate as the sole carbon source (Table 2) than did cells of the wild-type strain (CP750). Although they grew even more poorly on acetate than did cells deficient for the acetate metabolism enzymes Ack (strain CP758), Pta (strain CP760), or both (strain CP911), cells of strain CP762 exhibited Ack and Pta activities that most closely resembled those of wild-type cells (Table 2). Similar results were obtained with cells of strains CP938 [Δ(*nuoFGHIJKL*)-1] and CP956 (*nuoF*::Km-1) and those of *nuoG*::Tn*10*-1 transductants of two unrelated genetic backgrounds, BW13711 and W3110. Cells of all strains tested grew equally well on D-glucose, D-pyruvate, or glycerol (data not shown).

TABLE 2. Phenotypes of wild-type strains and strains that are defective for growth on acetate

Strain	Relevant genotype	Growth on acetate (25 mM)		Sp act (U/mg)	
		On plates[a]	In liquid[b]	Ack	Pta
CP750	Wild type	+++	1.0	0.12	1.1
CP758	*ackA*	++	0.4	≤0.02	1.0
CP760	*pta*	++	0.4	0.17	≤0.02
CP911	*ackA pta*	++	ND	≤0.02	≤0.02
CP762	*nuoG*::T + n*10* − 1[c]	+	0.3	0.15	1.0

[a] +++, good growth; ++, moderate growth; +, poor growth. ND, not determined.
[b] In generations per hour.
[c] See Table 1.

Cells of strain CP762 (*nuoG*::Tn*10*-1) also exhibited an unusual chemotactic phenotype. When they were inoculated at the center of tryptone swarm plates and their swarms were compared (Fig. 2), cells of the wild-type strain (CP750) formed two concentric bands, indicating that such cells were chemotactic to both L-serine and L-aspartate. In contrast, cells of the mutant strain (CP762) formed only a single band that corresponded to and migrated about as rapidly (0.56 ± 0.01 cm/h) as the outermost, L-serine band produced by cells of strain CP750 (0.58 ± 0.01 cm/h). Similarly, cells of strains CP938 [Δ(*nuoFGHIJKL*)-1] and CP956 (*nuoF*::Km-1) and those of *nuoG*::Tn*10*-1 transductants of strain W3110 formed a single band (data not shown). Since cells of strain BW13711 swim poorly, we did not determine the effect of *nuoG*::Tn*10*-1 upon their chemotactic behavior.

We verified the location of *nuoG*::Tn*10*-1, mapping it to 51.6 min on the *E. coli* chromosome with the order *nuoG ackA pta purF* (data not shown). Since the *nuo* locus had been previously mapped to 51 min on the *E. coli* linkage map (6), we transduced the *nuo* mutation from strain AN589 into strain CP909, a *nuoG*::Tn*10*-1 *purF* derivative of CP750. We selected Pur^+ transductants and screened them for tetracycline sensitivity. Of 200 Pur^+ transductants, 40 (20%) were sensitive to tetracycline. All of these tetracycline-sensitive transductants grew poorly on minimal acetate medium and failed to form the inner band on tryptone swarm plates, which is a behavior similar to that of the *nuoG*::Tn*10*-1 parent strain CP909. On the basis of these results, we determined that the Tn*10* had inserted within the *nuo* locus.

To further define its location, we subcloned portions of λ403 from the Kohara library (19), which carries this region of the chromosome. The resultant plasmids, pAJW104, pAJW105, and pAJW110 (Fig. 1), were transformed into strain CP762 to yield the strains AJW508, AJW509, and AJW510, respectively. Although cells of all three strains grew poorly on minimal

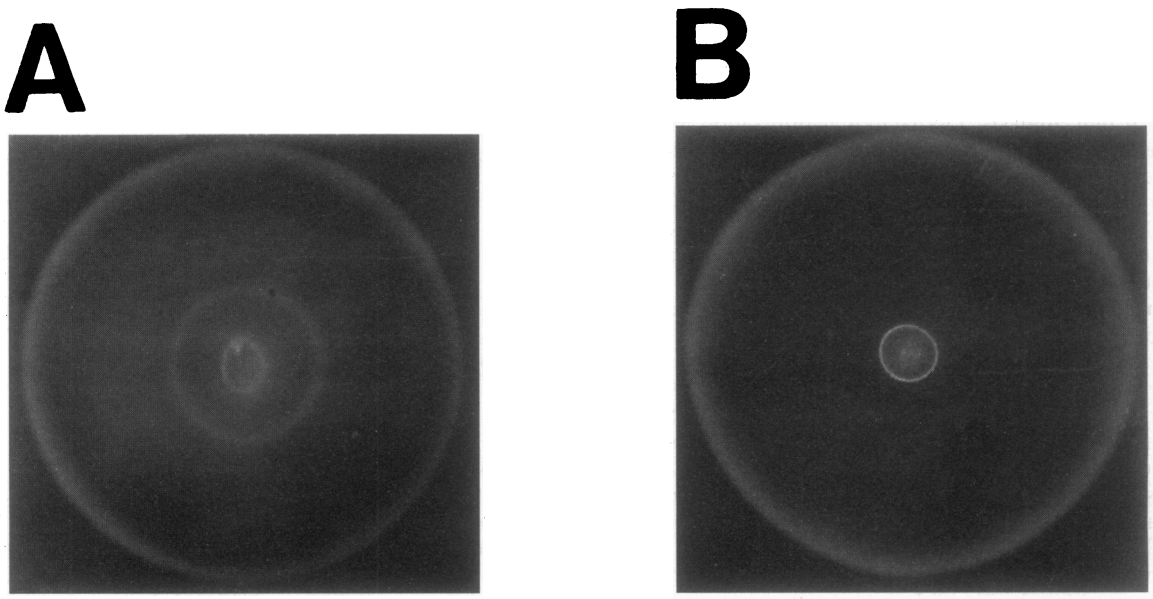

FIG. 2. Photographs of swarms produced by the wild-type strain CP750 (A) or the *nuoG*::Tn*10*-1 strain CP762 (B). Cells were inoculated in the center of a swarm plate containing 0.25% agar, and the plate was incubated at 35°C for 8 h.

acetate medium, papillae composed of faster-growing recombinants arose only from strain AJW509. Cells isolated from these papillae grew well on minimal acetate medium, formed two bands on swarm plates, and exhibited sensitivity to tetracycline, indicating that the Tn*10* had inserted within the region carried by plasmid pAJW105, i.e., from the 3′ end of *nuoF* to *nuoL*.

To continue our refinement, we performed Southern blot analysis (Fig. 3). The 3.6-kb *Bam*HI-*Eco*RI probe (Fig. 3A), derived from plasmid pAJW107 (Fig. 1), hybridized to 8-kb *Eco*RI (lane 1) and 9.8-kb *Bam*HI (lane 3) chromosomal fragments of strain CP750. In contrast, this probe hybridized to 9.8- and 7.5-kb *Eco*RI (lane 2) and 10.9- and 8.5-kb *Bam*HI (lane 4) chromosomal fragments of strain CP762. The 1.1-kb *Bgl*II-*Eco*RI probe (Fig. 3B), derived from plasmid pJN16 (Fig. 1), also hybridized to the 8-kb *Eco*RI (Fig. 3B, lane 1) and 9.8-kb *Bam*HI (lane 3) chromosomal fragments of CP750. In contrast to the 3.6-kb *Bam*HI-*Eco*RI probe, the 1.1-kb *Bgl*II-*Eco*RI probe hybridized only to the 7.5-kb *Eco*RI (lane 2) and the 10.9-kb *Bam*HI (lane 4) chromosomal fragments of strain CP762. As a positive control, we hybridized each probe (Fig. 3A and B, lanes 5) to the 8-kb *Eco*RI fragment of pAJW105 (Fig. 1). On the basis of these results and the restriction map of Tn*10* (17), we estimated that the insertion occurred about 0.2 kb to the left of the *Bgl*II site, i.e., within *nuoG* (Fig. 1). This conclusion has been confirmed by sequence analysis (data not shown).

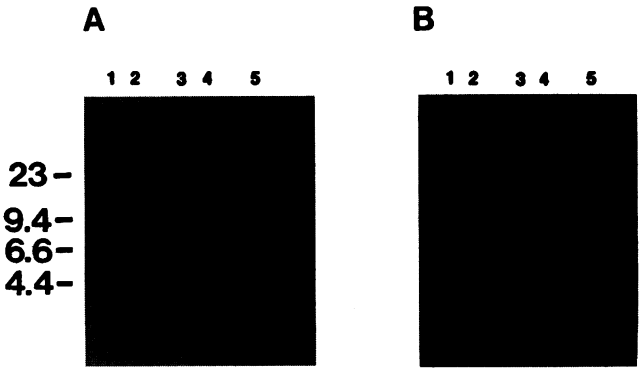

FIG. 3. Southern hybridization analysis demonstrating the location of the transposon *nuoG*::Tn*10*-1. Chromosomal DNA, isolated from the wild-type strain (CP750) or its *nuoG*::Tn*10*-1 derivative (CP762), was digested with *Eco*RI or *Bam*HI, hybridized with the 3.6-kb *Bam*HI-*Eco*RI fragment of pAJW107 (A), stripped, and rehybridized with the 1.1-kb *Bgl*II-*Eco*RI fragment of pJN16 (B). Lanes: 1, *Eco*RI digest of chromosomal DNA derived from strain CP750; 2, *Eco*RI digest of DNA derived from strain CP762; 3, *Bam*HI digest of DNA derived from strain CP750; 4, *Bam*HI digest of DNA derived from strain CP762; 5, *Eco*RI digest of pAJW105 as a positive control.

Molecular characterization of *nuo* mutants. For sequence analysis, restriction fragments (Fig. 1) of the Kohara clone λ403 were cloned into the expression vectors pT7/T3α-18 and pT7/T3α-19 and sequenced. The nucleotide sequence from the *Bgl*II restriction site in plasmid pAJW105 to the distal *Eco*RI site in pAJW110, consisting of 3,667 bp, revealed four open reading frames. According to the nomenclature of Weidner et al. (47), we identified these open reading frames as *nuoD*, *nuoE*, *nuoF*, and *nuoG*. Table 3 shows the differences between the sequence determined in this study and that obtained by Weidner et al. (bp 1841 to 5504 [47]). The only major difference involves the region bounded by bp 2411 and bp 2446, which contains 3 additional bp. This results in the addition of one amino acid to the predicted *nuoD* gene product and the improvement in its homology with the bovine mitochondrial 49-kDa subunit of NADH dhI (47, 48).

Mutations in *nuo* reduce NADH oxidase activity. We measured oxidase and DCIP reductase activities (Table 4) in the membrane fractions of cells of strains CP750 (wild-type) and CP762 (*nuoG*::Tn*10*-1), using both dNADH and NADH as substrates. Relative to those of wild-type cells, the NADH and dNADH oxidase activities of the mutant cells were reduced 36 and 84%, respectively. In contrast, NADH-DCIP and dNADH-DCIP reductase activities did not differ from those of the wild-type parent. Similar results were observed with

TABLE 3. Nucleotide sequence comparison[a]

Gene[b]	Location[c]	Nucleotide change	Amino acid change
nuoD	1972	T→G	No change
	1980–1988	GTCGCGCGG→CGTCGGCGC	RRAD→PSAH
	2004–2005	CT→TC	T→I
	2411–2412 and 2445–2446	CG→GCGC and CT→CGT	RRPAARLGSPAA→AHDLPRGWDRLLR
	2725–2726	CG→GC	G→R
	2785–2787	CAG→AGC	NR→KA
nuoE	3171–3172	AG→GA	V→I
nuoF	3682–3683	GT→TG	R→L
	3726–3727	CG→GC	R→A
	3797	G→C	No change

[a] Comparison of sequence determined by this study with that determined by Weidner et al. (47).
[b] No differences were observed in the 561 bp that we sequenced in *nuoG*.
[c] The locations are given according to Weidner et al.

nuoG::Tn*10*-1 transductants of strains HCB433 and CP875 (data not shown).

Mutations in *nuo* cause a growth defect in tryptone broth. We inoculated tryptone broth with wild-type cells (strain CP750) and with cells that were defective for *nuoG* (strain CP762), aerated the cultures at 37°C, monitored their growth rates by optical density, and measured the concentrations of acetate in their media (Fig. 4). Prior to the transition to stationary phase ($OD_{610} \leq 0.3$), wild-type and mutant cells grew at similar rates (2.0 versus 2.3 generations per h, respectively) and excreted similar amounts of acetate into the medium (660 ± 38 μM versus 760 ± 40 μM, respectively). Whereas wild-type cells continued relatively rapid growth beyond an OD_{610} of ~0.3 (0.6 generations per h), mutant cells abruptly and dramatically reduced their growth rate (0.16 generations per h). During this period, the concentration of acetate in the growth medium of wild-type cells declined to 124 ± 0 μM. In contrast, the acetate in the medium of mutant cells continued to accumulate, reaching a maximal concentration of 1,100 μM before declining rapidly to 450 ± 58 μM. As wild-type cells entered stationary phase, they once again accumulated acetate, reaching a maximal concentration of about 400 μM. Although the mutant cells did not enter a true stationary phase in these experiments, they did accumulate acetate during the equivalent time course, reaching a maximal concentration of about 800 μM. Thus, the inability of the mutant cells to continue rapid growth beyond an OD_{610} of about 0.3 seemed to correlate with a delay in the uptake and utilization of acetate from the medium.

To determine the nature of this defect, we grew mutant cells (strain CP762) in tryptone broth at 37°C to mid-exponential phase (OD_{610} of ~0.3), harvested those cells, resuspended them in either fresh medium or medium in which wild-type cells had been grown to a range of OD_{610}s, and measured their growth as a function of OD_{610} (Fig. 5). The mutant cells grew best in fresh medium and grew worst in medium harvested from wild-type cells entering stationary phase (OD_{610} = 0.8). Thus, the ability of the mutant cells to grow depended on the growth phase at which the wild-type culture medium had been harvested.

In an attempt to suppress this defect, we inoculated individual tryptone broth cultures with mutant cells (strain CP762), incubated them at 37°C to mid-exponential phase, added to each a different amino acid or TCA cycle intermediate, and monitored their growth by measuring the OD_{610}. Metabolites which can be converted directly to acetate and ATP through an acetyl phosphate intermediate, i.e., L-serine, D-pyruvate, D-lactate, D-malate, and glycerol, restored rapid growth to these mutant cells. Other compounds, i.e., fumarate, α-ketoglutarate, succinate, and acetate, exhibited only a weak effect on mutant cell growth. A third set of metabolites, i.e., citrate, L-aspartate, and L-glutamate, exerted little or no effect on the growth of mutant cells. Whereas the addition of L-serine resulted in a 250 to 500% increase in the extracellular acetate

TABLE 4. Oxidase and DCIP reductase activities of wild-type strains and a strain that is mutant for *nuoG*

Strain	Relevant Genotype	Sp act[a]			
		NADH oxidase	dNADH oxidase	NADH-DCIP reductase	dNADH-DCIP reductase
CP750	Wild type	140 ± 34	103 ± 30	28 ± 4	20 ± 3
CP762	*nuoG*::Tn*10*-1	90 ± 23	17 ± 10	23 ± 6	17 ± 3

[a] Activities are expressed in nanomoles per minute per milligram of protein (milliunits per milligram). Each mean ± standard error of the mean was calculated over 5 to 11 identical experiments.

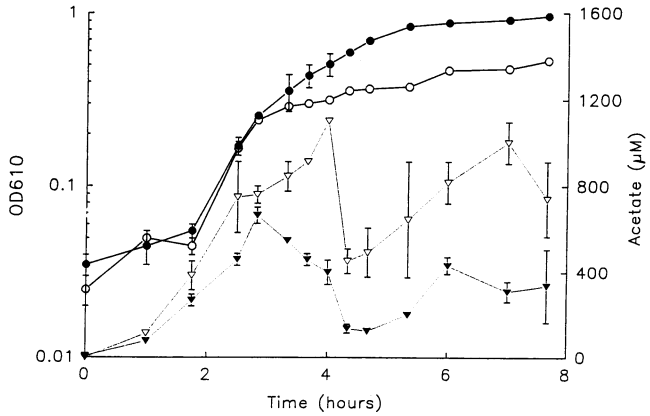

FIG. 4. The OD_{610}s and extracellular acetate concentrations of the wild-type strain (CP750 [● and ▼, respectively]) and the *nuoG*::Tn*10*-1 strain (CP762 [○ and ▽], respectively). Cells were grown in tryptone broth at 37°C.

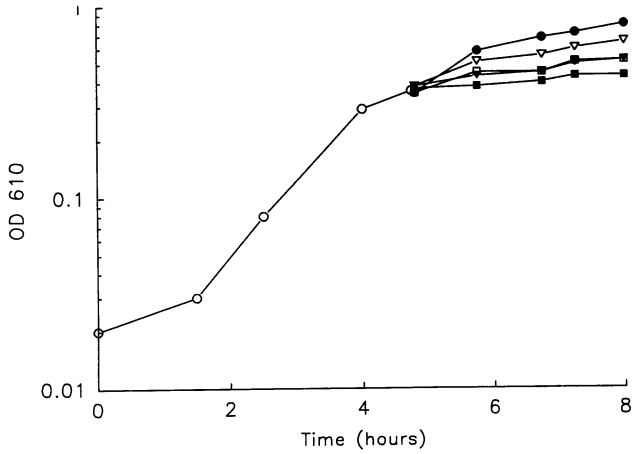

FIG. 5. Growth of the *nuoG*::Tn*10*-1 strain (CP762) at 37°C in tryptone broth (○). The cells were grown to an OD_{610} of about 0.30, harvested, washed, and resuspended with either fresh medium (●) or filter-sterilized medium in which wild-type cells (strain CP750) had been grown to OD_{610}s of 0.14 (▽), 0.38 (▼), 0.55 (□), or 0.80 (■).

concentrations of mutant cells, that of L-aspartate resulted in only a 25% increase (data not shown).

To explore the relationship between the production of acetate and the metabolism of amino acids, we grew cells of strains CP750 (wild type) and CP762 (*nuoG*::Tn*10*-1) in tryptone broth at 37°C and, at appropriate intervals, removed aliquots for amino acid analysis (Fig. 6). Initially, both wild-type (Fig. 6A) and mutant (Fig. 6B) cells consumed exclusively L-serine. Next, both consumed L-aspartate, followed by L-tryptophan. Whereas wild-type cells continued by consuming L-glutamate, glycine, L-threonine, and L-alanine efficiently, the mutant cells consumed L-glutamate slowly and glycine, L-threonine, and L-alanine poorly. Throughout the course of the experiment, both wild-type and mutant cells excreted similar amounts of ammonia (Fig. 6 [inset]).

DISCUSSION

In summary, we investigated mutants that grew poorly on acetate as the sole carbon source and did not form the inner, L-aspartate band on tryptone swarm plates. We localized the causative mutations to *nuo*, the locus which encodes the *E. coli* NADH dhI (6), and determined that they resulted in reduced dNADH and NADH oxidase activities. We sequenced a portion of the *nuo* locus and identified four putative open reading frames as *nuoD*, *nuoE*, *nuoF*, and *nuoG* (47).

We extended these studies, analyzing the sequential consumption of amino acids by both wild-type and *nuo* mutant cells growing in tryptone broth. We showed that the growth of wild-type cells can be divided into four distinct phases: (i) lag phase associated with the consumption of L-serine and the accumulation of acetate in the growth medium; (ii) exponential growth associated with the consumption of L-aspartate and continued accumulation of acetate; (iii) early transition to stationary phase associated with the consumption of L-tryptophan and the disappearance of acetate; and (iv) entry into stationary phase associated with the consumption of L-glutamate, glycine, L-threonine, and L-alanine and the renewed accumulation of acetate.

Like wild-type cells, *nuo* mutant cells grew rapidly during the first two phases, consuming L-serine and L-aspartate while producing acetate. Unlike wild-type cells, once these two amino acids had been consumed, the mutant cells dramatically slowed their growth. Like wild-type, the mutant cells next consumed L-tryptophan. However, in contrast to wild-type cells, they continued accumulating acetate. Next, the mutant cells utilized L-glutamate, albeit more slowly than wild type. Finally, the mutants consumed glycine, L-threonine, and L-alanine poorly.

We propose that cells deficient for NADH dhI grow slowly during the later phases of growth because, unlike wild-type cells, they cannot effectively oxidize NADH to NAD^+. In the absence of NADH dhI, they must rely on the less-efficient NADH dhII. Even if *nuo* mutant cells can express NADH dhII during the transition to stationary phase, they would not be expected to grow as rapidly as wild-type cells. As a consequence of a reduced growth rate, these cells should reduce the rates of their consumption of the amino acids L-glutamate, L-threonine, glycine, and L-alanine. Thus, when we plotted the extracellular concentrations of these amino acids versus OD_{610} rather than time, some of the differences between wild-type and mutant consumption disappeared (data not shown). However, all of the differences were not eliminated. Since NADH

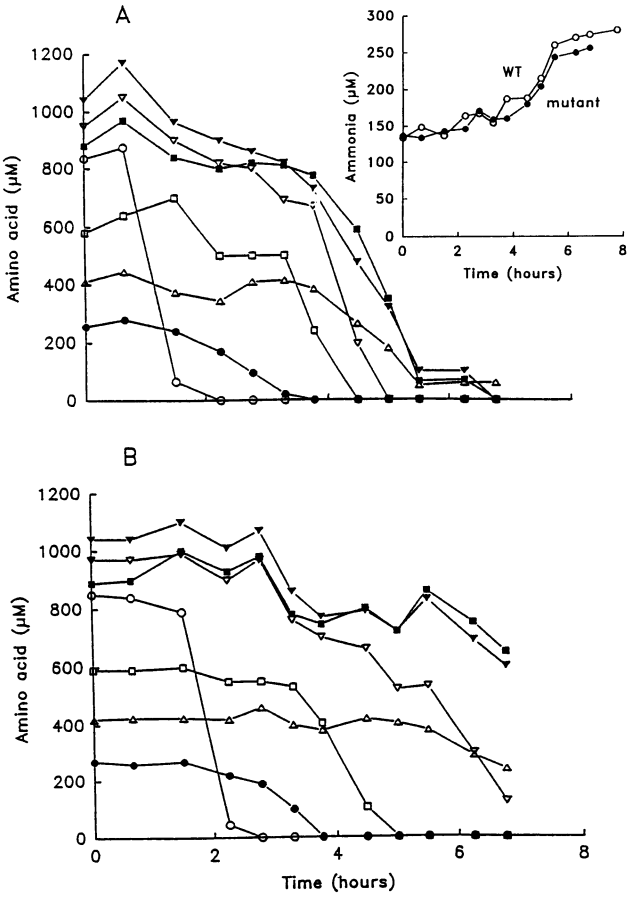

FIG. 6. Amino acid concentrations in the medium during growth in tryptone broth at 37°C of the wild-type strain (CP750) (A) and the *nuoG*::Tn*10*-1 strain (CP762) (B). ○, L-serine; ●, L-aspartate; □, L-tryptophan; ▽, L-glutamate; △, glycine; ▼, L-threonine; ■, L-alanine. (Inset) Concentrations of ammonia in the medium during growth of the wild-type strain (CP750 [○]) and the *nuoG*::Tn*10*-1 strain (CP762 [●]). Each point represents the mean of duplicate measurements from two independent experiments. The standard errors of the means were less than 20 μM.

dhI-deficient cells should accumulate NADH and since large NADH/NAD^+ ratios inhibit allosterically both citrate synthase (EC 4.1.3.7 [11]) and malate dh (EC 1.1.1.37 [36]), the mutant cells should experience difficulty in utilizing amino acids, e.g., L-glutamate, which require a functional TCA cycle for degradation (45). Since large NADH/NAD^+ ratios also affect the reaction equilibria of the enzymes threonine dh (EC 1.1.1.103 [7]) and alanine dh (EC 1.4.1.1 [40]), the mutant cells should also experience difficulty in utilizing L-threonine and L-alanine. The last two amino acids can be metabolized by means of one or more additional pathways. For example, the enzyme L-threonine aldolase (EC 2.1.2.1 [13]) can cleave L-threonine to glycine and acetaldehyde. This might explain why, in the case of the mutant cells, the extracellular glycine concentration failed to decrease even though metabolism of this amino acid does not require a functional TCA cycle (37). In addition to glycine and in contrast to L-glutamate, L-threonine, and L-alanine, some amino acids can be metabolized without the participation of the TCA cycle. For example, L-serine (31), L-aspartate (20, 21, 23, 34), and L-tryptophan (14, 51) can be converted directly to D-pyruvate and, thus, to acetate and ATP (18, 33, 44).

Several other facts support this hypothesis. (i) In contrast to NADH dhII, NADH dhI translocates protons (24, 25). It also possesses a smaller apparent K_m for NADH (5 to 15 μM) than does NADH dhII (50 μM [25]). Thus, NADH dhI oxidizes NADH more efficiently than does NADH dhII. (ii) Whereas cells induce total NADH dh activity at low oxygen tensions, they repress NADH dhII expression under those same conditions (43). Thus, it is likely that wild-type cells induce NADH dhI while repressing NADH dhII as they approach stationary phase. (iii) *nuo* mutants grow poorly on acetate as their sole carbon source and, when growing in tryptone broth, produce more acetate than wild-type cells. Since elevated levels of NADH inhibit both citrate synthase (11) and malate dh (36), the function of both the acetate-induced glyoxylate shunt and the TCA cycle should be diminished. A similar observation was made by Archer et al. (2), who isolated Tn*10d*-Tet insertion mutants in NADH dhI of *Salmonella typhimurium* by screening for a decrease in energy-dependent proteolysis after carbon starvation. These mutants were also found to be defective for growth on acetate as the sole carbon source. (iv) Metabolites that cells convert to D-pyruvate suppress the growth defect exhibited by *nuo* mutants growing in tryptone broth. Compounds metabolized through the TCA cycle or glyoxylate shunt either suppress the defect weakly or not at all. (v) Cells lacking a functional NADH dhI possess a competitive disadvantage in stationary-phase cultures (53). These experiments were performed in Luria broth, which consists of tryptone broth plus 0.5% yeast extract. This disadvantage appears to be due to the inefficient utilization of TCA cycle-dependent amino acids and derivatives as well as the inability to produce energy from the NADH formed.

It is surprising that *nuo* mutant cells do not produce an aspartate band on tryptone swarm plates, since they exhibit no difficulty in consuming L-aspartate. Lack of this band cannot be due to a defect in chemotaxis, however, since *nuo* mutant cells do produce the band when growing on glycerol as the carbon source. Also, *nuo* mutant cells missing the serine chemoreceptor, Tsr, form an aspartate band on tryptone swarm plates, albeit more slowly than their Nuo^+ parent. Under these conditions, cells that form the aspartate band consume L-aspartate and L-serine almost simultaneously (32). Thus, to form an aspartate band it appears that cells must consume L-aspartate efficiently as well as another amino acid or another carbon source.

Our results clearly indicate that NADH dhI mediates a critical metabolic switch. This raises interesting questions concerning the regulation of both the expression and the activity of NADH dhI. Do wild-type cells regulate NADH dhI as they make the transition from exponential growth to stationary phase? What mechanism(s) do cells use to perform such regulation? In response to which environmental cues do cells initiate this process? Now that the *nuo* locus of *E. coli* has been sequenced and the phenotypic consequences of mutation in that locus have been identified, these and other questions concerning the physiological role of NADH dhI can be addressed.

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