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Isolation and Characterization of an *Escherichia coli* Mutant Lacking the Cytochrome *o* Terminal Oxidase

DOUGLAS C.-T. AU, ROBERT M. LORENCE, AND ROBERT B. GENNIS*
Departments of Chemistry and Biochemistry, University of Illinois, Urbana, Illinois 61801

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A respiration-deficient mutant of *Escherichia coli* has been isolated which is unable to grow aerobically on nonfermentable substrates such as succinate and lactate. Spectroscopic and immunological studies showed that this mutant lacks the cytochrome *o* terminal oxidase of the high aeration branch of the aerobic electron transport chain. This strain carries a mutation in a gene designated *cyo* which is cotransducible with the *acrA* locus. Mutations in *cyo* were obtained by mutagenizing a strain that was *cyd* and, thus, was lacking the cytochrome *d* terminal oxidase. Strain RG99, which carries both the *cyd*$^-$ and *cyo*$^-$ alleles, grows normally under anaerobic conditions in the presence of nitrate. Introduction of the *cyd*$^+$ allele into the strain restores the respiration function of the strain, indicating that the cytochrome *o* branch of the respiratory chain is dispensable under normal laboratory growth conditions.

The cytochromes present in the aerobic respiratory chain of *Escherichia coli* (5) have been characterized by spectroscopic (24, 27–29), electrochemical (7–9, 15, 19, 25, 26, 30), immunochemical (16–18), and biochemical methods (10–14, 23) and, more recently, by genetic approaches (6). When the cells are grown under high aeration to early log phase, the cytochrome *o* terminal oxidase complex and cytochrome b_{556} are present in the cytoplasmic membrane. The cytochrome *o* complex consists of cytochrome b_{555} and cytochrome b_{562} (10, 11, 18, 21). When the cells are grown under oxygen-limited conditions, the cytochrome *d* terminal oxidase complex, consisting of cytochromes b_{558}, a_1 and *d* (23), is induced. Both the cytochrome *o* and cytochrome *d* terminal oxidase complexes have been purified to homogeneity and shown to be coupling sites in in vitro reconstitution experiments (10–12, 14, 21, 23).

An *E. coli* mutant strain that lacks the cytochrome *d* complex was isolated recently (6). This strain was shown by spectroscopic and immunological criteria to be missing cytochromes b_{558}, a_1, and *d* (6, 16). The cytochrome composition of this mutant strain is similar to that of a wild-type strain grown under conditions of high aeration. Having obtained *cyd*$^-$ strains lacking the low-aeration branch of the respiratory chain made it possible to relatively easily obtain mutants deficient in other components required for aerobic respiration. Strains that are *cyd*$^-$ rely entirely on the high-aeration branch of the respiratory chain to grow aerobically on nonfermentable substrates. A mutation in any essential component of this branch will render this strain unable to grow aerobically and can easily be selected.

In the present paper we describe the isolation and characterization of a mutant strain of *E. coli* lacking the cytochrome *o* terminal oxidase, and we describe the genetic mapping of a locus affecting the synthesis of this terminal oxidase.

MATERIALS AND METHODS

Bacterial and phage strains. The properties of the various bacterial strains used are given in Table 1. Transductions were done with phage P1 cml or P1 kc.

Media and growth conditions. The minimal medium used throughout this work was the minimal A medium of Miller (22), except that no sodium citrate was added, and ferrous sulfate was added to a final concentration of 0.5 mg/liter. The rich agar medium used for aerobic growth contained the following (in grams per liter); agar, 15; tryptone, 10; NaCl, 5; and yeast extract, 1. The rich agar medium was supplemented with 0.3% sodium succinate and 0.3% sodium D,L-lactate. For anaerobic growth, the same agar medium was used, except sodium succinate and sodium D,L-lactate were replaced by 0.3% glycerol and 0.5% potassium nitrate. In some experiments, tetracycline was added to the above media to a final concentration of 20 μg/ml.

Aerobic cultures were grown in the medium described previously (2), except glucose was substituted by 0.3% sodium D,L-lactate. Anaerobic cultures were grown in LB medium (22) supplemented with 0.3% glucose. Strictly anaerobic conditions were maintained in an Oxoid anaerobic jar that generates an atmosphere of hydrogen gas.

Materials. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine, sodium D,L-lactate, NADH, lithium D,L-lactate, and tetracycline-hydrochloride were purchased from Sigma Chemical Co. Sodium succinate was a product of Mallinckrodt, Inc. Pure cytochrome *o* was kindly provided by Kimberly Carter, University of Illinois. Monospecific antibodies toward cytochrome *o* have been described previously (18).

Mutagenesis and isolation of mutants. Strain RG98 was grown in LB medium to the log phase and then mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine for 15 min at pH 6 as described by Adelberg et al. (1). After mutagenesis, the cells were diluted and grown anaerobically in 100 ml of rich medium containing 0.3% sodium D,L-lactate and 1% potassium nitrate to the log phase. The mutagenized cells were centrifuged at 8,000 rpm for 20 min and suspended in 100 ml of rich medium containing 0.3% sodium succinate and 0.3% sodium D,L-lactate. The culture was incubated at 37°C with shaking for 1 h, and ampicillin was added to a final concentration of 40 μg/ml. The culture was then incubated at 37°C for about 6 h until lysis was complete. The cells were centrifuged at 8,000 rpm for 20 min and plated on rich agar medium containing 0.3% glycerol and 0.5% potassium nitrate. The plates were then incubated at 37°C anaerobically

* Corresponding author.

in an Oxoid anaerobic jar for 2 to 3 days. Colonies appearing on the anaerobic plates were picked and screened for the inability to form colonies on rich agar medium containing 0.3% sodium succinate and 0.3% sodium D,L-lactate.

Genetic methods. P1 transductions and conjugational transfer experiments were carried out as described previously (6).

Measurement of growth. Cultures of 100 ml in a 250-ml sidearm flask were incubated at 37°C in a New Brunswick incubator shaker. Turbidity was measured at intervals in a Klett-Summerson colorimeter with a green filter.

Screening of other genetic markers. The temperature-sensitive *adk* marker was scored by a failure to grow at 42°C. the *acrA* strains were scored by an inability to grow on rich agar medium containing 50 μg of methylene blue per ml. The *purE*::Tn*10* and *phoR*::Tn*10* markers were scored by the ability to grow on plates supplemented with 20 μg of tetracycline per ml.

Other methods. Procedures for selection of Tc^s mutants from strains carrying Tn*10*, membrane preparation, spectrophotometric measurements, potentiometric titrations, oxygen uptake measurements, rocket immunoelectrophoresis analysis of cytochrome *o*, and protein determination have been described previously (2, 4, 18–20).

TABLE 1. Bacterial strains

Strain	Sex	Relevant markers	Source or reference
AN386	F$^-$	*menA rpsL gal*	Laboratory collection
GR57N	F$^-$	*cydA thi rha lacZ nadA*::Tn*10 recA*	This laboratory
GR75N	F$^-$	*cydA thi rpsL nadA*::Tn*10*	This laboratory
RG98	F$^-$	*nadA*::Tn*10*, *cydA* of AN386	P1(GR75N) × AN386
RG99	F$^-$	*cyo* of RG98	This work
RG100	F$^-$	Tc^s mutant from RG99	This work
RG101	F$^-$	*nadA*::Tn*10*, *cydA*$^+$ of RG100	P1(NK6033) × RG100
RG102	Hfr	*nadA*::Tn*10*, *cydA* of CV2	P1(GR75N) × CV2
RG103	F$^-$	*nadA*::Tn*10*, *cydA* of N43	P1(GR75N) × N43
RG104	F$^-$	*srl*::Tn*10*, *recA* of RG100	P1(JC10240) × RG100
χ573	F254	Δ(*lac-lip*) *serA supE* T3^r	CGSC[a] 6350
W3747	F13	*metB relA*, deletion corresponding to F13	CGSC 5218
F597/EB54	F597	*proB trpA trpR his recA metE lacI lacZ azi rpsL nal A*	CGSC 5886
χ790	F210	*his* T3^r	R. Curtiss III
NK6033	Hfr	DE5 *nadA*::Tn*10 relA spoT thi*	CGSC 6180
CV2	Hfr	*adk glpD glpR phoA relA spoT pit tonA* T2^r *fadL* (λ)	CGSC 4682
N43	F$^-$	*ara lac galK malA xyl mtl acrA rpsL* λ^r *supE44*?	CGSC 5583
NK6051	Hfr	Δ(*gpt-lac*) *purE*::Tn*10 relA spoT thi*	CGSC 6186
K797	F$^-$	*phoR*::Tn*10 purE aroA his ilv metB lacY xyl rpsL cycA cycB*? *tsx tonA*?	CGSC 6456
JC10240	Hfr	*thr recA srl*::Tn*10 relA ilv spoT thi rpsE*	CGSC 6074

[a] CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine.

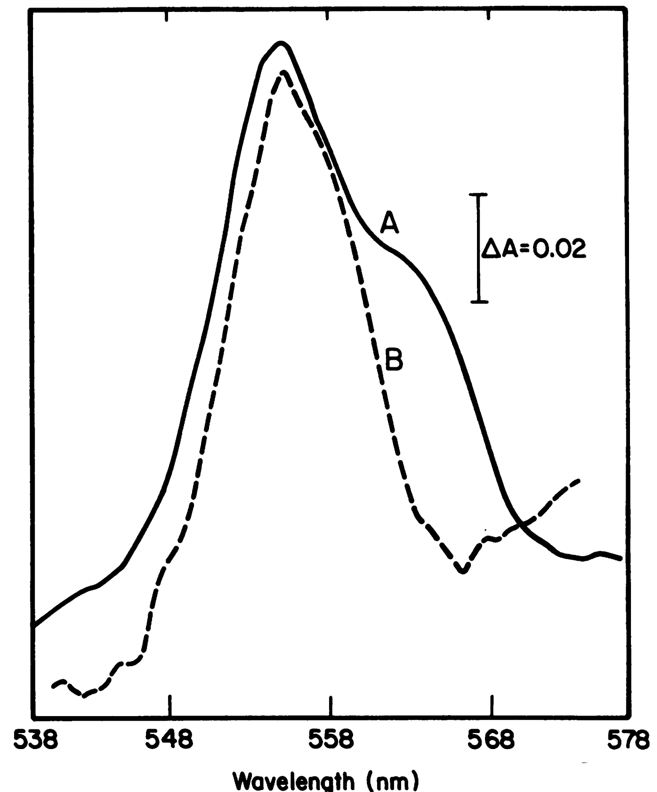

FIG. 1. Low-temperature reduced-minus-oxidized difference spectra of membranes from strains (A) AN386 (*cyo*$^+$ *cyd*$^+$) and (B) RG101 (*cyo*$^-$ *cyd*$^+$). Cells were grown under high aeration on lactate-rich medium to the early exponential phase. Membrane protein concentrations were 1.8 and 2.4 mg/ml, respectively.

RESULTS

Rationale of mutant selection procedure. Strain RG98 *men cyd* was treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and mutants were selected on agar medium as described above. This selection procedure is designed to avoid obtaining mutants in the H$^+$-ATPase or in heme or quinone biosynthesis. This is due to the fact that the cells must have heme, H$^+$-ATPase, and ubiquinone to grow anaerobically on nitrate. Dehydrogenase mutants were eliminated by an ampicillin treatment, which was performed under aerobic conditions in the presence of both succinate and lactate. After the ampicillin treatment, approximately 500 colonies were picked from nitrate anaerobic plates and screened for the inability to grow on rich agar medium containing succinate and lactate. Four individually isolated respiration-deficient mutants were obtained, and one of these strains, RG99, was chosen for further analysis.

Genetic analysis of the mutant. Strain RG100, a tetracycline-sensitive derivative of RG99, was made *recA* by transducing to tetracycline resistance with a P1 lysate grown on JC10240. Mating of RG104, the resulting *recA* strain, with a set of F′ strains and selection for aerobic growth indicated that the mutation was present in a region covered by the episomes F13 and F254. To determine whether these episomes carried the structural gene for the cytochrome *o* terminal oxidase, strain GR57N was allowed to mate with χ573 and W3747 and selected for Lac$^+$ merodiploids on lactose minimal plates. Tetracycline was used to select against the donor strains. The resulting Lac$^+$ merodiploid strains and the recipient strain GR57N were grown in a

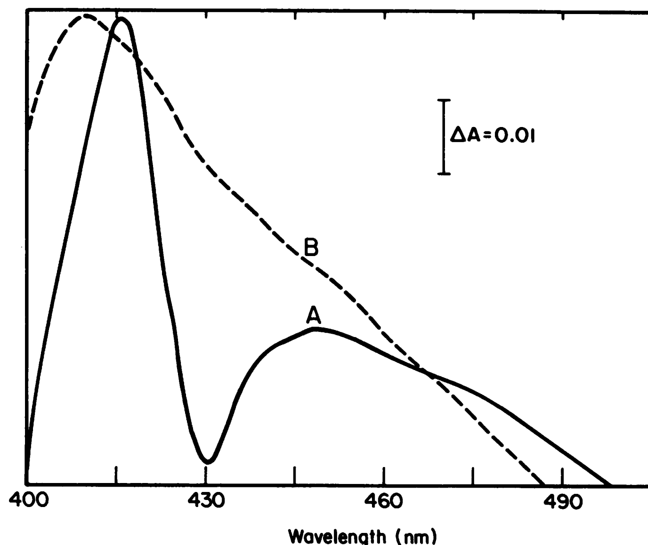

FIG. 2. Room-temperature carbon monoxide reduced-minus-reduced difference spectra of strains (A) AN386 (approximately 5 mg of membrane protein per ml) and (B) RG101 (approximately 7.5 mg of membrane protein per ml).

liquid medium containing lactate. The membrane preparations of both merodiploid strains were found, by spectroscopic measurements, to contain twice as much cytochrome *o* as in GR57N. These data suggest that the episomes F13 and F254 carry the structural gene for the cytochrome *o* terminal oxidase. The failure of two other F-primes (F210 and F597) to complement the mutation suggests that the mutation is located between 9.5 to 11.5 min of the *E. coli* linkage map (3).

Introduction of the cyd^+ allele into strain RG100 restored the respiration function of the mutant. This was done by transducing RG100 to tetracycline resistance anaerobically with a P1 lysate grown on NK6033, which has Tn*10* inserted near the *cyd* gene. More than 50% of the Tc^r transductants were able to form colonies on aerobic plates containing succinate and lactate.

Spectroscopic and potentiometric analyses of the mutant. Figure 1 shows the low-temperature (77 K) dithionite-reduced-minus-ferricyanide-oxidized spectra of membranes from AN386 and RG101 grown under high aeration to the early exponential phase. The spectrum of RG101 did not show the cytochrome b_{562} component of the cytochrome *o* terminal oxidase which was obviously present in the spectrum of the parent strain AN386. The room temperature CO reduced-minus-reduced difference spectra of membranes from AN386 and RG101 are shown in Fig. 2. The characteristic Soret absorption band of the cytochrome *o* terminal oxidase at 415 nm is absent in the spectrum of membranes from RG101. These two pieces of spectroscopic data demonstrate that strain RG101 is lacking the cytochrome *o* terminal oxidase.

Potentiometric analysis was performed in the presence of carbon monoxide to better resolve the *b*-type cytochromes of the two strains (Fig. 3). The *b*-type cytochromes of strain AN386 were resolved into three components, cytochromes b_{556}, b_{558}, and *o*. The lack of cytochrome *o* in strain RG101 was evident in the potentiometric titration of the strain (Fig. 3).

Immunological characterization of the mutant. With monospecific antibodies toward cytochrome *o*, rocket immunoelectrophoresis was performed to assay the cytochrome *o* terminal oxidase in membranes of the two isogenic strains. Analysis of the detergent-solubilized membrane extract from AN386 showed a rocket arc that stained for protein (Fig. 4, wells 7 and 9) and also stained for heme (data not shown). No heme-staining rocket arc was apparent when the same antibody was challenged with a detergent extract from strain RG101, a strain which lacks cytochrome *o* by spectroscopic criteria. However, the presence of a small amount of cross-reactive material was indicated by a slight increase in rocket heights when a detergent extract of membranes from strain RG101 was added to pure cytochrome *o* or to the membrane extract of AN386. These data showed that the mutated *cyo* gene in strain RG101 causes a dramatic change in the activity and in the amount of cytochrome *o* in the cells.

Physiology of the mutant. The growth rates (data not shown) and the rates of oxygen uptake (Table 2) with different substrates were compared for strains AN386 and RG101. Very little, if any, difference was observed. This indicates that the low-aeration branch of the electron transport chain is sufficient to allow the *cyo* strain to grow aerobically under normal laboratory growth conditions.

Mapping of the *cyo* gene by cotransduction. To further dissect the chromosomal segment between 9.5 to 11.5 min of the *E. coli* linkage map (3), the following P1 transductions were performed. The generalized transducing phage P1 kc was grown on strains NK6051 and K797, and the resulting lysates were used to transduce strain RG100 to tetracycline resistance under anaerobic conditions. The Tc^r transduc-

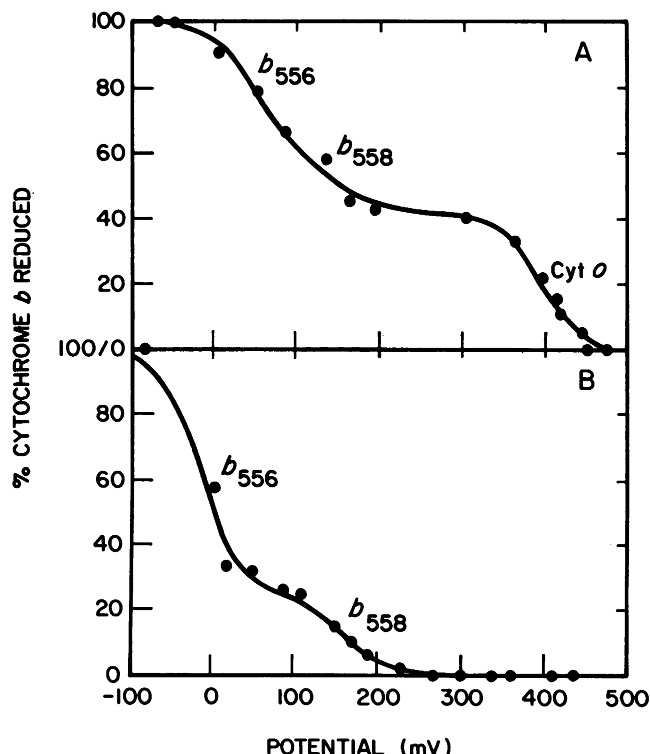

FIG. 3. Characterization by potentiometry, performed in the presence of carbon monoxide, of the membrane-bound *b*-type cytochromes of strains (A) AN386 and (B) RG101. Percent reduction of the *b*-type cytochromes was determined by measuring absorbancy at 560 nm minus that at 579 nm.

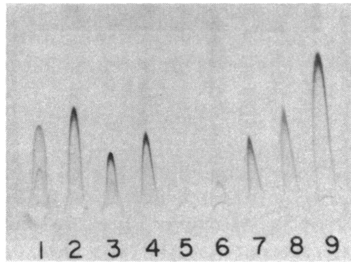

FIG. 4. Rocket immunoelectrophoresis assay of the cytochrome *o* terminal oxidase. In this assay, 75 μl of anti-cytochrome *o* per 12 ml of 1% agarose were used. Membranes were solubilized in 1% Zwittergent–1% Triton X-100. The samples used were as follows: well 1, 12 μl of purified cytochrome *o* (0.46 mg/ml); well 2, 6 μl of purified cytochrome *o* plus 1 μl of membrane extract from AN386 (12 mg of protein per ml) plus 5 μl of 0.05 M phosphate buffer (pH 7.0); well 3, 6 μl of purified cytochrome *o* plus 6 μl of buffer; well 4, 6 μl of purified cytochrome *o* plus 1 μl of membrane extract from RG101 (12 mg of protein per ml) plus 5 μl of buffer; well 5, 1 μl of membrane extract from RG101 plus 11 μl of buffer; well 6, 3 μl of membrane extract from RG101 plus 9 μl of buffer; well 7, 1 μl of membrane extract from AN386 plus 11 μl of buffer; well 8, 1 μl of membrane extract from AN386 plus 1 μl of membrane extract from RG101 plus 10 μl of buffer; well 9, 3 μl of membrane extract from AN386 plus 9 μl of buffer. The plate was stained for protein with Coomassie brilliant blue R250.

tants were then screened for the presence of the *cyo*$^+$ allele. Cotransduction of the *phoR*::Tn*10* allele with the *cyo*$^+$ allele occurred at a frequency of 7% (12 colonies out of 168 colonies tested), but no cotransduction between the *purE*::Tn*10* allele and the *cyo*$^+$ allele could be observed (0 out of 150). The P1 lysate prepared on K797 was also used to transduce strain N43 to tetracycline resistance. Ninety-four Tc^r transductants were obtained, but none of them was *acrA*$^+$. Strain RG102 carries mutations in the *adk* and *cyd* genes, and the P1 cml lysate prepared on strain RG101 was used to transduce *adk*$^+$ into strain RG102 under anaerobic conditions. The transductants obtained were then tested for the presence of the *cyo*$^-$ allele. Cotransduction of the *adk*$^+$ allele with the *cyo*$^-$ allele occurred at a frequency of 10% (16 out of 168). The P1 cml lysate grown on strain RG101 was used to transduce *acrA*$^+$ into strain RG103, which carries mutations in the *acrA* and *cyd* genes. The transductants obtained were again tested for the presence of the *cyo*$^-$ allele. Cotransduction of the *acrA*$^+$ allele with the *cyo*$^-$ allele occurred at a frequency of 44% (82 out of 187). Strain CV2 was transduced to *adk*$^+$ with P1 kc lysate grown on RG103, and the *adk*$^+$ transductants were picked and screened for the presence of the *acrA*$^-$ allele. Cotransduction of the *adk*$^+$ allele with the *acrA*$^-$ allele occurred at a frequency of 80% (120 out of 150). The frequency of cotransduction between the various markers described above is summarized

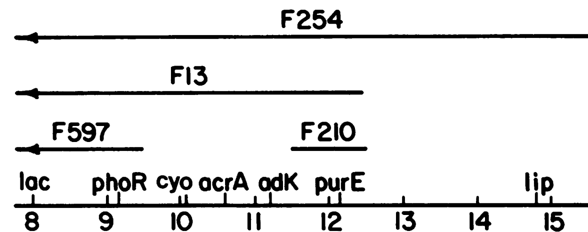

FIG. 5. Genetic map of *E. coli* showing relative positions of genetic loci and regions covered by F-primes. Gene locations (in minutes) are based on the 100-min *E. coli* linkage map. The map distance of *cyo* was calculated by the equation of Wu (31) from the cotransduction frequencies shown in Table 3.

in Fig. 5 and Table 3, establishing the gene order *phoR-cyo-acrA-adk-purE*.

DISCUSSION

The experiments reported in this paper show that it is possible to isolate viable cytochrome *o*-deficient mutants from *E. coli*. This study clearly demonstrates that the *cyo* mutation in a cytochrome *d*-deficient strain results in a respiration-deficient phenotype. This mutation also causes the lack of spectroscopically detectable level of the cytochrome *o* terminal oxidase. The physiological role played by the cytochrome *o*-containing branch is not clear. Reintroduction of the *cyd*$^+$ gene into the mutant restores the capability of the strain to grow aerobically under normal laboratory conditions. Membranes from strains that are *cyo*$^-$ *cyd*$^+$ and *cyo*$^+$ *cyd*$^+$ have similar oxygen uptake rates with three different substrates (Table 2). Further experiments need to be performed to better define the unique functions of these two terminal oxidases. Under normal laboratory growth conditions the two oxidases appear to be redundant. Possibly the presence of cytochrome *d* confers a growth advantage when cells are grown under conditions of very low aeration or oxygen stress. Experiments to test this possibility by strain competition will be carried out.

The nature of the lesion in the cytochrome *o*-deficient mutant (RG99) is not known. This mutant reverts to an aerobic growth phenotype at a frequency of about 10^{-6}. All of the revertants observed so far (about 20) reverted back to *cyd*$^+$, but not *cyo*$^+$. This indicates either that the lesion in the *cyo* gene does not revert or that it reverts at a frequency much lower than that of the *cyd* gene. Immunological analysis of the membrane extract from strain RG101 showed that this strain possesses a small amount of defective cytochrome *o* terminal oxidase. One possibility is that the lesion in the *cyo* gene results in a defective protein that is unable to bind to heme. These results suggest that *cyo* is the structural gene for cytochrome *o*, but further studies will be required to prove this. It should be noted that it has already been demonstrated that *cyd* is the structural gene for the cyto-

TABLE 2. Oxygen utilization of *E. coli* membrane preparations

Substrate	Oxygen utilization in prepns from strains[a]:	
	AN386 (*cyo*$^+$ *cyd*$^+$)	RG101 (*cyo*$^-$ *cyd*$^+$)
Succinate	141	102
D,L-Lactate	56	61
NADH	357	317

[a] Measured as nanomoles of molecular oxygen per minute per milligram of membrane protein.

TABLE 3. Cotransduction frequencies of various markers

Donor	Recipient	Selected marker	Unselected marker	% Cotransduction
NK6051	RG100	*purE*::Tn*10*	*cyo*$^+$	(0/150) 0%
K797	RG100	*phoR*::Tn*10*	*cyo*$^+$	(12/168) 7%
K797	N43	*phoR*::Tn*10*	*acrA*$^+$	(0/94) 0%
RG101	RG102	*adk*$^+$	*cyo*$^-$	(16/168) 10%
RG101	RG103	*acrA*$^+$	*cyo*$^+$	(82/187) 44%
RG103	CV2	*adk*$^+$	*acrA*$^-$	(120/150) 80%

chrome *d* complex (Green, Kranz, and Gennis, unpublished data). Inasmuch as *cyo* is cotransducible with *phoR*, *adk*, and *acrA* at frequencies of 7, 10, and 44%, respectively, but *cyo* and *purE* are not cotransducible, *cyo* must be situated between *phoR* and *acrA*. Wu (31) has derived the following equation to relate cotransduction frequency with phage P1 to the distance between two markers on the E. coli chromosome: $F = (1 - d/L)^3$, where F is the cotransduction frequency, d is the distance between the two markers, and L is the length of the transduced DNA fragment, which is approximately 2 min (3). From this formula, *cyo* is 1.15 min clockwise from *phoR* and 1.05 min counterclockwise from *adk*. According to the current linkage map (3), *phoR* and *adk* are located at min 9.1 and 11.2, respectively, which means that *cyo* is approximately at min 10.2.

In future studies, the other respiration-deficient mutants isolated will be examined. It is hoped that mutations in other essential components of the respiratory chain can be identified. The continuing study of both the *cyd* and *cyo* mutants should help to elucidate the structure and function of the *E. coli* aerobic respiratory system.

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