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Identification of the *cydC* Locus Required for Expression of the Functional Form of the Cytochrome *d* Terminal Oxidase Complex in *Escherichia coli*

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The aerobic respiratory chain of *Escherichia coli* contains two terminal oxidases which are differentially regulated. The cytochrome *o* complex predominates under growth conditions of high aeration, whereas the cytochrome *d* complex predominates when the oxygen tension is low. Either terminal oxidase will support aerobic growth. The goal of the work presented in this paper was to identify genes required for the expression of the functional form of the cytochrome *d* complex, other than the genes encoding the polypeptide components of the oxidase complex (*cyd* locus). A strain lacking the cytochrome *o* complex (*cyo* mutant strain) was mutagenized by using a lambda-Mu hybrid hopper bacteriophage, λ p*lac*Mu53, which inserts randomly into the chromosome and carries a kanamycin resistance marker. Strains were isolated and examined which were unable to grow aerobically, i.e., which lacked functional cytochrome *d* complex, and which could not be complemented by introduction of the *cyd* gene on F-prime episomes. One strain was selected for characterization. The phage insert was mapped to min 18.9 on the genetic linkage map, defining a new genetic locus, *cydC*. Evidence described in the text suggests that the gene product is probably required for the synthesis of the unique heme *d* component of the cytochrome *d* complex.

The aerobic respiratory chain of *Escherichia coli* is branched, containing numerous dehydrogenases, which reduce ubiquinone in the cytoplasmic membrane, and two terminal oxidases, each of which is capable of oxidizing ubiquinol and reducing molecular oxygen to water (see reference 17 for a review). The two terminal oxidases are differentially regulated, with oxygen tension being one of the important factors determining their expression. Under high-aeration growth conditions, the cytochrome *o* terminal oxidase complex predominates, whereas under low-aeration conditions, the alternate cytochrome *d* complex predominates (20). Both oxidase complexes have been purified to homogeneity, spectroscopically characterized, and functionally reconstituted in phospholipid vesicles (7, 18, 19, 25, 28–30, 33). The genes encoding the polypeptide components of each oxidase have also been identified, mapped, and cloned (1, 12, 13). The *cyd* locus, encoding the cytochrome *o* complex, maps near min 10, and the *cyd* locus, encoding the cytochrome *d* complex, is located near min 16.5 on the genetic linkage map. The *cyd* locus contains two genes, *cydA* and *cydB*, which encode the two polypeptide subunits of the cytochrome *d* terminal oxidase complex.

The emerging picture of the organization of the respiratory chain of *E. coli* is remarkably simple. Dehydrogenases, such as succinate dehydrogenase, which are located on the inner surface of the cytoplasmic membrane, reduce ubiquinone-8 (i.e., 8 isoprene units in the side chain) in the bilayer. The ubiquinol-8 diffuses through the membrane and is reoxidized by either of the two terminal oxidase complexes. Electron flow through either of the oxidases, from ubiquinol to oxygen, is coupled to the generation of the proton motive force (7, 29, 33). Strains which lack either of the oxidases (*cyo* or *cyd* mutants) are not impaired in their ability to grow aerobically, but the absence of both oxidases (*cyo cyd*) results in strains which cannot grow aerobically on nonfermentable substrates such as lactate or succinate.

The cytochrome *d* complex has been best characterized and found to contain two polypeptide subunits (19, 32) and four heme groups (26). There are two *d*-type hemes, which are actually chlorins and are apparently unique to this enzyme (36). These prosthetic groups are responsible for binding oxygen and have an absorbance peak near 628 nm in the reduced-minus-oxidized spectrum. The structure of this chlorin suggests that it may be derived from protoporphyrin IX by a reductive dihydroxylation of one of the pyrrole rings.

The purpose of the work described in this paper was to locate genes other than *cydA* and *cydB* that are required for the expression of the functional form of the cytochrome *d* complex. This was done by mutagenizing a strain lacking cytochrome *o* (*cyo*) and screening for mutants incapable of aerobic growth, other than mutants with mutations in *cyd*. One might expect three potential classes: (i) genes encoding other required but unknown components of the electron transport chain; (ii) regulatory genes required for expression or assembly of the cytochrome complex; and (iii) genes required for the biosynthesis of the heme *d* prosthetic group. One mutant strain was characterized, and the results suggest that it is required for the synthesis of heme *d*.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. Descriptions of the various bacterial strains and phages used are listed in Table 1. Transductions were done with phage P1 *cml* or P1 *kc*.

Media and growth conditions. Several media were used in this work. To grow cells for isolating membranes and measuring the amount of cytochrome *d* spectroscopically, a minimal lactate medium was used. This consists of minimal A medium of Miller (31) with 0.3% sodium DL-lactate instead of sodium citrate plus 0.5 mg of ferrous sulfate per liter. Rich medium was used for growth on plates. For aerobic growth,

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TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Sex	Genotype	Reference or source[a]
E. coli			
RG136	F8	*cydA cyo zba*::Tn*10 thi recA*	This laboratory
MC4100	F⁻	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi*	35
RG139	F⁻	*cyo* Δ(*argF-lac*)*U169 zba*::Tn*10*	P1(RG136) × MC4100, this work
CG01	F⁻	RG139 ϕ(*cydC-lacZ*) Kan^r	This work
JW380	F⁻	*zch-506*::Tn*10* λ⁻	CGSC 6094
SHSP19T	F⁻	*hemA8 met lac* Str^r *zch-506*::Tn*10*	P1(JW380) × SHSP19 (34), this laboratory
GO100	F⁻	*cyo thi gal*	This laboratory
JC10240	Hfr	*thr-300 recA56 srl-300*::Tn*10 relA1 ilv-318 spoT1 thi-1 rpsE2300* λ⁻	CGSC 6074
CG01/λRG148	F⁻	*c*I857	This work
CG21	F⁻	G0100 ϕ(*cydC-lacZ*) Kan^r	P1(CG01/λ RG148) × G0100, this work
CG02	F⁻	RG139 ϕ(*cydC-lacZ*) Kan^r	P1(CG01/λ RG148)× RG139, this work
CG03	F⁻	CG21 *recA56 srl-300*::Tn*10*	P1(JC10240) × CG21, this work
CG04	F⁻	MC4100 ϕ(*cydC-lacZ*)	P1(CG01/λ RG148) × MC4100, this work
CG03/F13	F13	F13 carries *cyo*	This work
CG03/F106	F106	F106 carries *cydC*	This work
GR70N	F⁻	*gal thi* Str^r	This laboratory
CG05	F⁻	GR70N ϕ(*cydC-lacZ*) Kan^r	P1(CG01/λ RG148) × GR70N, this work
W3208	F8	*relA1 spoT1 metB1*	CGSC 5549
KL715	F147	*pyrD34 trp45 tyrA2 recA1 thyA33 thi-1 galK99 malT1 xyl-7 mtl-2 rpsL118* λ⁻	CGSC 4323
GR84N	F⁻	*nadA50 thi rpsL cydA2 recA*	13
ND18	F100-12	*thr-24 galT23 trp-56 recA41 rpsL178 tyr-7 phe-4 aro-14* λ⁻	CGSC 5204
KL725	F106	*galK35 pyrD34 trp-45 his-68 recA1 rpsL118 malA1 xyl-7 mtl-2* λ⁻	CGSC 4309
KL703	F126	*pyrD34 trp-45 his-68 recA1 thi-1 galK35 malA1 xyl-7 mtl-2 rpsL118* λ⁻	CGSC 4253
KL701	F123	*pyrD34 trp-45 his-68 recA1 thi-1 galK35 malA1 xyl-7 mtl-2 rpsL118* λ⁻	CGSC 4256
W3747	F13	*metB1 relA1* and deletion corresponding to F13	CGSC 5218
MA1003	Hfr	*pyrD67 thi-1 relA1 lacZ43 spoT1* λ⁻	CGSC 5876
AB2829	F⁺	*aroA354 supE42* λ⁻	CGSC 2829
G19	F⁻	*putA purE gltA his lac strA*	CGSC 5757
RE103	F⁻	*cmlA1 proA23 trp-30 his-51 lac-28 strA101*	CGSC 4698
YYC199	Hfr	*zbj*::Tn*10* Δ(*aceEF*) *rpsL pps-4 pfl-1*	8, 9
YYC201	Hfr	*zbi*::Tn*10* Δ(*aceEF*) *rpsL pps-4 pfl-1*	8, 9
YYC202	Hfr	*zbi*::Tn*10 poxB1* Δ(*aceEF*) *rpsL pps-4 pfl-1*	8, 9
Phages			
λ p*lac*Mu53		*imm* λ '*trp*' *lacZ*$^+$ *lacY*$^+$ *lacA*' '*uvrD*' *Xho*::Kan Mu[*cIts62 ner*$^+$ *A*$^+$ 'S]	Weinstock
λ pMu507		*c*I857 *S*am7 Mu[*cIts62 ner*$^+$ *A*$^+$ *B*$^+$]	Weinstock
λ RG148		λ SE6 carrying *cyo c*I857	11; this laboratory
Plasmids			
pNG2		pBR322 Δ(*Eco*RI-*Pvu*I) carrying *cyd*	13
pNG9		pNG2 with *Bst*EII deletion in subunit II of the *cyd* gene	This laboratory

[a] Numbers indicate references. CGSC denotes the *Escherichia coli* Genetic Stock Center, Yale University School of Medicine. Weinstock indicates the strain was in the Hopper Kit provided by G. M. Weinstock, Laboratory of Genetics and Recombinant DNA, Frederic Cancer Research Facility, Frederick, Md.

the plates contained either standard LB (containing, per liter, 15 g of agar, 10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract) or a rich medium containing a mixture of 0.3% sodium succinate and 0.3% DL-lactate plus (per liter) 15 g of agar, 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract. For anaerobic growth, a similar medium was used except that 0.3% glycerol was used in place of the succinate-lactate mixture and 0.5% potassium nitrate was included. Antibiotics were included at the following concentrations when indicated: kanamycin, 50 μg/ml; tetracycline, 20 μg/ml; and ampicillin, 40 μg/ml. The heme-deficient strain SHSP19T was grown semianaerobically in a narrow-mouth, closed bottle which was not shaken, with LB plus 0.3% (wt/vol) glucose and tetracycline. This strain was grown aerobically with LB supplemented with 0.3% sodium DL-lactate, tetracycline, and 50 μg of δ-aminolevulinic acid per ml. The Lac$^+$ phenotype was scored by using either lactose-MacConkey indicator plates or plates which contained the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (4, 31).

Mutagenesis by creating LacZ operon fusions with the *cydC* locus. Insertions of λ p*lac*Mu53 in the *cydC* locus were created by using a modification of the method of Bremer et al. (5). *E. coli* RG139 was grown overnight in 5 ml of LB supplemented with 5 mM $MgSO_4$, 5 mM $CaCl_2$, and 0.5% (wt/vol) maltose and then infected with λ p*lac*Mu53 at a

multiplicity of 0.1 and with the helper phage λ pMu507 at a multiplicity of 0.5. Following adsorption for 30 min at 30°C, the cells were washed three times with 10 ml of LB. They were then suspended in 1 ml of LB, plated on 10 plates containing glycerol-nitrate medium with kanamycin, and grown anaerobically at 37°C for 1 to 2 days. At this stage, the cells grew confluently. The colonies from the 10 plates were pooled, suspended in 10 ml of LB plus kanamycin, and used to inoculate 500 ml of the same medium. The inoculum was grown aerobically at 37°C by shaking at 200 rpm until the cells reached early log phase. Then ampicillin was added to the inoculum, and the shaking was continued until cell lysis, which was monitored by measuring the decrease in cell density with Klett spectrophotometer. When the cell density reached its minimum level (after 1 to 2 h), the cells were washed with LB plus kanamycin to remove the ampicillin and suspended in 5 ml of the same medium. Serial dilutions of 10^{-1} to 10^{-5} of the cell suspension were plated on glycerol-nitrate-kanamycin plates and grown anaerobically at 37°C for 1 to 2 days.

Colonies that grew anaerobically but not aerobially were selected. Their frequency was about 2 to 5%. These should include mutants with mutations in the *cyd* locus as well as in other genes required for this branch of the respiratory chain to function. The selected colonies were tested by complementing with λ RG148, which carries the *cyo* locus, as well as with the plasmid pNG2, which carries the *cyd* locus. Any mutation which is in a gene required specifically for the function of the cytochrome *d* branch of the respiratory chain should be complemented by the phage carrying *cyo*, since this will restore the function of the branch terminating in the cytochrome *o* complex. Of these strains, three were found which could not be complemented by pNG2, implying that a gene outside the structural locus for cytochrome *d* was mutated. One of these strains, CG01, was further characterized.

Immunological and spectroscopic procedures. The monoclonal antibody to subunit I and the polyclonal antibody to subunit II of the cytochrome *d* terminal oxidase used in this work have been described previously (21, 23). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western immunoblotting (6), samples containing 15 μg of membrane protein or 0.8 μg of the pure cytochrome *d* protein were used. Procedures for membrane preparation, protein determination, and difference spectroscopy have been described previously (13, 22). Plasmid transformations were carried out by the method of Maniatis et al. (27).

Genetic procedures. Conjugational transfer experiments and P1 transductions were carried out by the method of Miller (3).

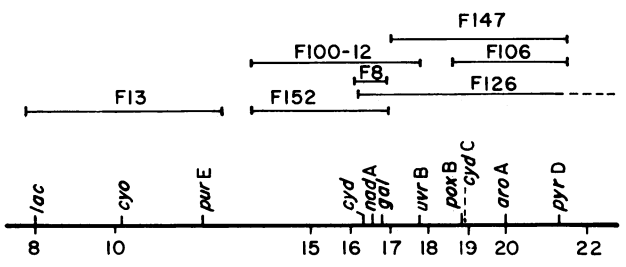

FIG. 1. Genetic map of *E. coli* showing relative positions of genetic loci and regions covered by F-primes. Gene locations are based on the 100-min *E. coli* linkage map. The distances of *cyo* and *cyd* have been determined previously (1, 12). The proposed map position of *cydC* is indicated.

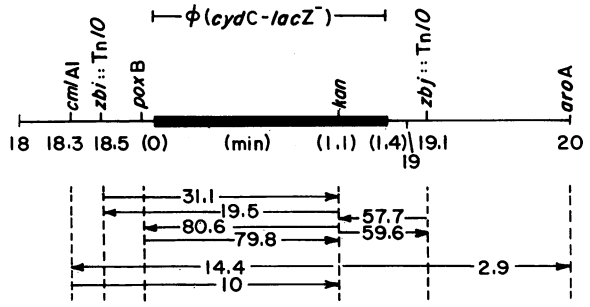

FIG. 2. Relevant portion of the *E. coli* genetic map containing the operon fusion of the *cydC* gene and *lacZ*. The linkages given are percent average cotransduction frequencies taken from Table 2. The map is a modification of that of Bachmann and co-workers (2, 3); the *poxB* position is from Chang (9).

In vitro transcription and translation. The in vitro transcription-translation system was prepared in a manner similar to that described by Chory and Kaplan (10), with cell extracts from *E. coli* CG05 and GR70N.

RESULTS

The mutation in strain CG01 was selected for further characterization because it was well behaved, that is, the phenotypic loss of cytochrome *d* cotransduced 100% with the kanamycin marker in the insert with several recipient strains. The mutant allele was moved into a new background which was *recA*. The resulting strain, CG03, which cannot grow aerobically on nonfermentable substrates, was used for the approximate *cydC* mapping by F-prime conjugation (Table 1). A set of F-primes covering the entire chromosome was tested. The strain was complemented for the ability to grow aerobically by F13, F147, F106, and F126. The F-primes that carry the *cyd* gene (i.e., F152, F8, and F100-12) did not complement for aerobic growth. F13 carries the *cyo* locus, and F147, F106, and F126 define a new genetic locus, *cydC* (Fig. 1).

Fine mapping was done by P1 cotransduction with the Kan^r marker on λ p*lac*Mu53. Cotransduction frequencies were obtained with two nearby positions: *zbj*::Tn*10* (60%) and *poxB* (80%). The contransduction frequency between *poxB* and *zbj*::Tn*10* was 45%, suggesting, but not proving, the following gene order: *zbi*::Tn*10* *poxB* *cydC* *zbj*::Tn*10* (Fig. 2; Table 2). The *cydC* locus is located at 18.9 min on the basis of the genetic linkage formula of Wu (37), $F = 1 - d/L)^3$, where F is the cotransduction frequency, d is the distance between two markers on the *E. coli* chromosome, and L is 2.3 min, the size of the chromosomal fragment length packaged by P1.

The *cydC* fusion with λ p*lac*Mu was moved by P1 transduction to MC4100, which carries a *lac* deletion. This strain (CG04) did not express *lacZ*, suggesting that the fusion between *cydC* and λ p*lac*Mu is oriented in the opposite direction from that required for transcriptional fusion.

Strain CG03/F13 was constructed by moving the *cyo* locus into CG03 by conjugation with F13. This strain can be grown aerobically, facilitating the further examination of the *cydC* phenotype. Reduced-minus-oxidized spectra were taken of membrane suspensions of CG03/F13 and show that cytochrome *d* was absent by spectroscopic criteria (Fig. 3). The diagnostic peak for cytochrome *d* at 628 nm was absent for

TABLE 2. Cotransductional mapping of the *cydC* locus

P1 donor and relevant markers	Recipient	Marker selected	% Cotransduction (no. transduced/ total no.)
CG05, Kan	AB2829	*aroA*$^+$	2.9 (6/208)
CG05, Kan	G19	*putA*$^+$	0.0 (0/156)
CG05, Kan	MA1003	*pyrD*$^+$	0.0 (0/104)
CG05, Kan	RE103	*cmlA*	14.4 (30/208)
RE103, *cmlA*$^+$	CG05	Kan^s	10.0 (21/208)
YYC199, *zbj*::Tn*10*	CG05	Kan^s	57.7 (120/208)
CG03/F13, Kan	YYC199	Tet^s	59.6 (124/208)
YYC201, *zbi*::Tn*10*	CG05	Kan^s	31.1 (81/260)
CG03/F13, Kan	YYC201	Tet^s	19.5 (41/210)
YYC202, *zbi*::Tn*10*	CG05	Kan^r Tet^r *poxB*$^+$	80.6 (75/93)
		Kan^r Tet^r *poxB*	19.4 (18/93)
CG03/F13, *Xho*::*Kan*	YYC202	Kan^r Tet^s *poxB*$^+$	79.8 (67/84)
		Kan^r Tet^s *poxB*	20.2 (17/84)

this strain. Reintroduction of the wild-type *cydC* on the F106 episome into strain CG03 (*cydC*) restored the spectrum to that of the wild-type strain. Transformation of strain CG03 (*cydC*) with a multicopy plasmid carrying the *cyd* gene resulted in overproduction of the cytochrome b_{558} and b_{595} spectroscopic components of the cytochrome *d* complex, but the peak at 628 nm (heme *d*) was still absent (Fig. 3, spectrum G).

Figure 4 shows the results of in vitro transcription-translation of the *cyd* gene with extracts prepared from a *cydC* mutant (CG05) and an isogenic strain which is *cydC*$^+$ (GR70N). Both extracts were equally capable of catalyzing the in vitro synthesis of the two subunits encoded on *cyd*. The results show that the *cydC* gene product is not required for in vitro transcription and translation. Western immunoblotting was used to study the effect of the *cydC* and *hemA* gene products on the membrane incorporation of subunits I and II of the oxidase (Fig. 5; Table 3). The levels of subunit

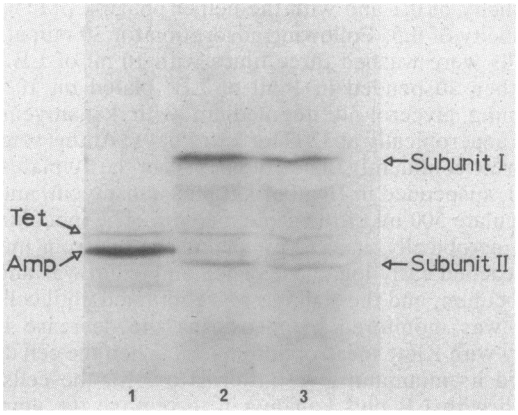

FIG. 4. Autoradiogram of in vitro transcription-translation products. Lane 1 shows the *tet* and *amp* gene products of pBR322, and lane 2 shows the *cyd* gene products from pNG2, with, in both cases, cell extract of the *cydC* mutant CG05. Lane 3 shows the gene products of pNG2, with a cell extract of strain GR70N (isogenic to CG05).

I in the membranes of the *cydC* mutant CG03/F13 (Fig. 5, lane 2) were very low compared with those of the isogenic *cydC*$^+$ strain G0100 (lane 4). No incorporation of subunit I was observed in the membranes of the *hemA* mutant SHSP19T when grown in the absence of δ-aminolevulinic acid (lane 5). Subunit I was incorporated in the membrane of the *hemA* mutant SHSP19T when it was grown either aerobically (lane 6) or semianaerobically in the presence of δ-aminolevulinic acid. Similar results were obtained for subunit II (data not shown). We also examined the incorporation of both subunits I and II into the membrane in strain CG03/pNG2, which carries a *cydC* mutation and contains a multicopy plasmid carrying *cyd*. Both subunits of the oxidase were observed in the membrane, but the amount of subunit II was significantly lower than that observed in the control strain GR84N/pNG2, which is *cydC*$^+$ (Table 3).

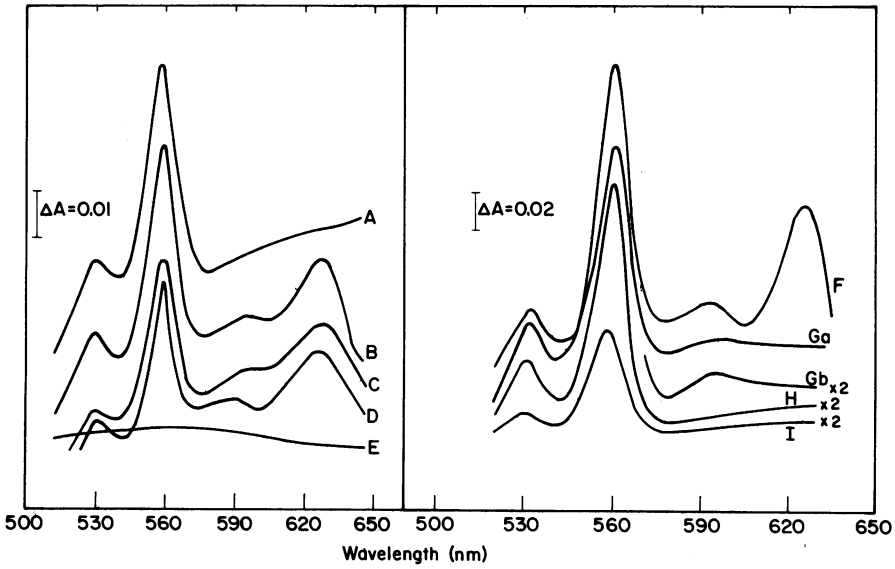

FIG. 3. Room-temperature reduced-minus-oxidized difference spectra of membrane preparations. Protein concentrations in membranes for spectra A to E and F to I were 3 to 5 mg/ml and 8 to 10 mg/ml, respectively. Spectra: A, CG03/F13; B, CG03/F106; C, GO100 (isogenic to CG03); D, SHSP19T grown aerobically with δ-aminolevulinic acid; E, SHSP19T grown semianaerobically without δ-aminolevulinic acid; F, GR84N/pNG2; G, CG05/pNG2; H, GR84N/pNG9; I, GR84N. Growth conditions were those stated in Materials and Methods.

DISCUSSION

In this paper, lambda hopper phages were used to create null mutations in genes required for the expression of functional cytochrome *d* terminal oxidase. The mutagenesis was performed in a *cyo* genetic background so that inserts in any gene of interest would result in the loss of ability to grow aerobically on nonfermentable substrates. One such mutant was characterized. Several other strains which were obtained appeared to have multiple insertions which were required for the "aerobic-minus" phenotype, and these were not investigated further.

This work was intended to further elucidate the genetic organization of the aerobic respiratory chain of *E. coli*. It is known that when oxygen tension drops, the expression of the cytochrome *d* complex is due to enhanced transcription of *cyd* (C. D. Georgiou and R. B. Gennis, unpublished results). Nothing is known about the regulatory apparatus. It is also known that the cytochrome *d* complex contains a unique heme *d*, but the enzyme(s) and corresponding genes involved in the heme *d* biosynthesis are not known. Finally, there may be components in the membrane, other than those already characterized, which are required for optimal electron transfer (see, for example, reference 28). The genetic screening procedure used in this work was designed to locate genes whose expression is required for electron transfer from succinate and lactate to oxygen via the cytochrome *d* complex.

The new locus, which is called *cydC*, may not be directly involved in the regulation of *cyd*, although further work is required to demonstrate this more definitively. An extract from a *cydC* mutant was capable of catalyzing in vitro transcription-translation of the two polypeptide subunits of the oxidase encoded on the *cyd* locus on a plasmid. It would appear, therefore, that any positive regulatory factors required for expression of *cyd* are present in the mixture. However, it is noted that in vitro transcription-translation could be artifactual and may not accurately reflect the situation in the cell. A possible regulatory role for *cydC* should be considered an open question.

Immunoblotting membranes from a *cydC* strain, following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, revealed that low levels of both subunits of the cytochrome *d* complex are present in a strain lacking any functional enzyme. Spectroscopically, the heme *d* component of the oxidase is clearly absent from these membranes. Examination of a strain which carried a *cydC* mutation and which contained a multicopy plasmid encoding the cytochrome subunits demonstrated that the cytochrome b_{558} and b_{595} spectroscopic components of the enzyme are overproduced, as expected, but that the heme *d* component is absent. It has been previously shown that subunit I of the oxidase is the cytochrome b_{558} component of the enzyme and that it can be synthesized and inserted into the membrane in the absence of the second subunit (14, 15).

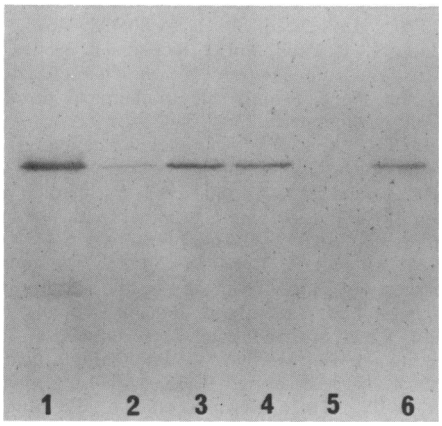

FIG. 5. Western immunoblotting of membranes from some of the strains whose spectra are shown in Fig. 3, with a monoclonal antibody against subunit I. Lanes: 1, pure cytochrome *d* complex; 2, CG03/F13; 3, CG03/F106; 4, GO100; 5, SHSP19T grown semianaerobically without δ-aminolevulinic acid; 6, SHSP19T grown aerobically with δ-aminolevulinic acid.

TABLE 3. Western immunoblotting of membrane preparation from a *cydC* mutant transformed with the multicopy plasmid pNG2 carrying the *cyd* gene

Strain(plasmid)	Subunit I[a]	Subunit II[a]
CG05(pNG2)	+	+
GR84N(pNG2)	+	+
GR84N(pNG9)	+	−
GR84N	−	−

[a] The amount of subunit II in the membranes of the *cydC* mutant CG05(pNG2) was approximately one-half of that in strain GR84N(pNG2). The levels of subunit I were the same in both strains as well as in GR84N carrying the multicopy plasmid pNG9, which contains subunit I and a deletion in subunit II.

The most likely interpretation of the data is that *cydC* encodes an enzyme required for the synthesis of heme *d*. The structure of this chlorin indicates that it can be derived relatively easily from protoporphyrin IX by a reductive dihydroxylation of one of the pyrrole rings (36). The oxidase subunits in the membrane may be destabilized in the absence of heme *d*, resulting in the reduced levels of the enzyme in the membrane. Alternatively, heme *d* may be required for the normal expression of *cyd* in vivo. When the subunits are overproduced by use of a multicopy plasmid, one can clearly see the other spectroscopic features diagnostic of the enzyme, including the small peak at 595 nm, indicative of the cytochrome b_{595} or a_1 component (26). This is the first time that this spectroscopic feature has been observed in *E. coli* in the absence of the cytochrome *d* component.

It has previously been reported that in a strain which does not synthesize any heme (*hemA*), the apocytochromes are assembled in the membrane (16, 24). It is surprising, therefore, that the specific lack of heme *d* results in low levels of the cytochrome *d* subunits in the membrane. For this reason, the earlier results were reexamined, specifically to see whether the subunits of cytochrome *d* are present in the membrane of a *hemA* strain grown under conditions of no heme synthesis. The results showed no evidence for the presence of subunits I or II in these membranes by immunoblotting following sodium dodecyl sulfate-polyacrylamide gel electrophoresis to resolve the membrane proteins. When the *hemA* strain was grown in the presence of δ-aminolevulinic acid, to allow heme biosynthesis, both subunits were clearly present in the membranes. It appears, therefore, that the subunits of the cytochrome *d* complex are not present when protoheme IX biosynthesis is blocked. Apparently, in the absence of heme *d*, the subunits are present, but in much reduced amount. The coupling between heme biosynthesis and the expression of the genes encoding the cytochrome of *E. coli* will be the subject of further investigation.

In summary, we have mapped a new genetic locus, *cydC*,

which may encode an enzyme required for converting protoporphyrin IX to heme *d*, the chlorin uniquely found in the cytochrome *d* terminal oxidase complex. This will be tested by cloning the gene and overproducing and purifying the gene product.

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