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Mutations Affecting the Cytochrome *d*-Containing Oxidase Complex of *Escherichia coli* K12: Identification and Mapping of a Fourth Locus, *cydD*

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A mutant of *Escherichia coli* K12 has been isolated affected in a gene, designated *cydD*, distinct from the three previously described loci involved in the synthesis of assembly of the cytochrome *bd* oxidase complex. The mutant, obtained by nitrosoguanidine mutagenesis, lacks the spectroscopically detectable components of this oxidase, namely cytochromes b_{558}, b_{595} and *d*. Cytochrome oxidase *o* is the sole CO-binding cytochrome in membranes of the mutant, but the soluble haemoprotein *b*-590 and catalase activity appear unaffected. Discrimination between Cyd^+ and Cyd^- strains is facilitated by the development of a defined low-phosphate medium that allows the inclusion of Zn^{2+} as well as azide, inhibitors of respiratory electron transfer particularly via cytochrome *o*. Mapping with F-prime factors and by P1 cotransductional frequencies shows the mutation to map near 19·3 min on the *E. coli* chromosome, distinct from *cydC*, which maps at 18·9 min. The gene order in this region was tested in a three-factor cross and demonstrates the order *zbj*::Tn*10*(YYC199)–*cydD*–*aroA*, consistent with cotransduction frequencies.

INTRODUCTION

Cytochrome *d* is the oxygen- and carbon monoxide-reactive component of a respiratory oxidase complex widely distributed in bacteria, particularly Gram-negative heterotrophs (for reviews, see Poole, 1983, 1988). Purification of the oxidase complex from membranes of *Escherichia coli* reveals the presence of two dissimilar subunits (Miller & Gennis, 1983; Kita *et al.*, 1984*a*; Finlayson & Ingledew, 1985). The larger subunit (I) binds a *b*-type haem with a typical low-spin spectral appearance and is thus equated with cytochrome b_{558} (Green *et al.*, 1986). The smaller subunit (II) is thought to bind two haem types; one of these is the distinctive chlorin haem of cytochrome *d* and the other is responsible for the absorbance (in reduced minus oxidized difference spectra) near 595 nm (Lorence *et al.*, 1986) previously, but erroneously, attributed to 'cytochrome a_1' (Keilin, 1966). Potentiometric resolution of the a_1-like signal and the quantification of haem types has led to the view that this component is a high-spin *b*-type haem with an unusually distinct α band near 595 nm ('cytochrome b_{595}'; Lorence *et al.*, 1986). The resolved spectrum is very similar to that of the soluble haemoprotein *b*-590 previously purified and characterized by Poole *et al.* (1986) and also shown to contain only high-spin *b*-type haem.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethylphenylenediamine.

The reaction of the oxidase complex with oxygen involves binding of the ligand to form a relatively stable oxygenated species with a distinctive absorption maximum at about 650 nm (Poole *et al.*, 1983*a*). The electron donor to haem *d* is the high-spin cytochrome b_{595}, which in turn accepts electrons from low-spin cytochrome b_{558} (Poole & Williams, 1987; Poole *et al.*, 1983*b*).

In *E. coli*, and most other bacteria in which the cytochrome *d*-containing oxidase complex is found, cytochrome *o* coexists as an alternative terminal oxidase so that isolation of cytochrome *d*-defective mutants requires a screen that distinguishes lesions in this complex from those in other oxidases and their associated respiratory chains. Green & Gennis (1983) isolated *E. coli* mutants unable to oxidize TMPD and which lacked all spectroscopically detectable features of the cytochrome oxidase *d* complex. Further localized mutagenesis in the 17 min region of the chromosome revealed two loci, *cydA* and *cydB* (Green *et al.*, 1984). The phenotype of *cydA* mutants is the absence of subunits I and II and of all three chromophores of the complex; *cydB* strains lack subunit II, cytochrome *d* and the high-spin haem *b*, but retain subunit I. A third locus, *cydC*, has been mapped at 18·9 min (Georgiou *et al.*, 1987); the gene product may be required for synthesis of the unique haem of cytochrome *d*.

This paper describes a mutant of *E. coli* in which components of the cytochrome *d* complex are scarcely detectable spectrophotometrically. The gene involved maps at a position distinct from previously described genes affecting expression of the cytochrome *d* oxidase complex and has been named *cydD*.

METHODS

Bacterial strains. The strains used in this work are derivatives of *E. coli* K12 and are described in Table 1.

Media and growth conditions. MG medium contained (g l^{-1}): Difco beef extract, 10; Oxoid peptone, 10; Difco yeast extract, 3; NaCl, 5. The pH was adjusted to 7·5, 20 g of Difco agar added and glucose (6 g) added after autoclaving. Tetracycline-resistant strains were routinely grown and selected on MG-Tet plates, containing tetracycline at 25 μg ml^{-1}. Succinate minimal medium contained (g l^{-1}): $Na_2HPO_4.12H_2O$, 10·96; KH_2PO_4, 2·7; $(NH_4)_2SO_4$, 1; sodium succinate, 5; and 10 ml trace elements solution. The trace elements solution (pH 7) was based on that of Poole & Haddock (1974) and contained (g l^{-1}): EDTA, disodium salt, 5; $FeCl_3$, 0·5; ZnO, 0·05; $CuCl_2$, 0·01; $CoCl_2.6H_2O$, 0·01; H_3BO_3, 0·01. After autoclaving, the medium was supplemented with 1 ml 1 M-Mg^{2+} and with histidine (0·28 mM), proline (1·4 mM) and thiamin (1·5 μM) for selection of *aroA* strains.

Discrimination between Cyd^+ and Cyd^- strains utilized either glucose minimal medium (Gibson *et al.*, 1977) supplemented with sodium azide at 20 μg ml^{-1} (Green *et al.*, 1984) or a low-phosphate minimal medium, supplemented with Zn^{2+} and azide (ZAB; see Results). ZAB medium contained (g l^{-1}): Tris, 14·5; NaCl, 4·68; KCl, 1·5; NH_4Cl, 1·0; glycerol, 5; Na_2SO_4, 0·043; $CaCl_2$, 0·03. The pH was adjusted to 7·5 with conc. HCl. Trace elements solution (10 ml of the composition described above) was added before autoclaving, after which the following (to the final concentrations shown) were added: $MgCl_2$ (1 mM), glycerol 3-phosphate (0·5 mM), $ZnSO_4$ (0·15 mM) and NaN_3 (0·15 mM). Where indicated, ZAB medium was supplemented with Casamino acids (Difco, 0·05%) to give ZABC. For strain AB1321 (Table 1), ZAB was supplemented with phenylalanine (200 μM, tryptophan (200 μM), tyrosine (200 μM), histidine (284 μM), thiamin (1·5 μM) and proline (1·4 mM).

Growth of AN2342 and AN2343 in fermenters was in liquid '56' medium (Gibson *et al.*, 1977) supplemented with trace elements solution (above, 10 ml l^{-1}), Luria broth (Miller, 1972; 50 ml l^{-1}) and glucose (0·6%, w/v). A starter culture (1 litre) was inoculated with cells recovered from two MG plates and grown in a 2-litre baffled flask with vigorous shaking at 37 °C. After 9 h, this culture was used to inoculate 10 litres of the same medium in a New Brunswick 14-litre fermenter vessel in a Labroferm FS-314 fermenter drive assembly. Stirring was at 300 r.p.m. with forced aeration at 10 litres of air min^{-1}. After a further 14 h, the stirring was reduced to 150 r.p.m. and the air flow to 2 litres min^{-1}. Cells were harvested 3·5 h after this 'step-down', which served to increase expression of the cytochrome *d* oxidase complex in the Cyd^+ strain.

Isolation of the cytochrome d-deficient mutant. Strain AN248 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine using conventional methods (Cox & Downie, 1979) and plated without selection onto MG medium. Single colonies were streaked onto MG medium and cells scraped from solid medium using a spatula; the thick paste was transferred to holders containing a sample chamber of 7 mm diameter and 1 mm pathlength bounded by Perspex windows. After freezing in liquid N_2, the sample was examined in a hand spectroscope (Beck, London), illuminated by the focussed beam of a 500 W microscope lamp. Mutants lacking the distinctive 630 nm band of reduced cytochrome *d* in the region of the spectrum were sought.

Genetic techniques. Matings with F' strains were carried out by cross-streaking on solid selective media using conventional techniques. Transductions were carried out using P1kc or P1vir as described by Silhavy *et al.* (1984). For linkage experiments using P1kc, multiplicities of infection (m.o.i.) of between 10 : 1 and 1 : 1 were used, while

Table 1. *E. coli strains*

Strain	Genotype	Source
AN248	*ilvC argH entA*	Laboratory stock
KLF26/181 (CGSC 4253)	F126; *pyrD34 thi-1 mtl-2 xyl-7 str-118 his-1 trp-1 gal-6 recA malA1*	B. Bachmann
YYC188	*zbi-927*::Tn*10 azi-14? (aroP–aceF)73 ton-54? lacZ608*(am) c*I857 poxB11 pfl-1 pps-4 rpsL10L thi-1* Hfr	B. Bachmann
YYC199	*zbj*::Tn*10 (aceEF) cps-L pps-4 pfl-1* Hfr	J. Cronan
RW1230 (GCSC 6392)	*zbj-1230*::Tn*10 (gpt–proA)62 hisG4 thi-1 lacY1 galK2 xyl-5 mtl-1 supE44* F^-	B. Bachmann
AB1321 (CGSC 1321)	*aroAZ (gpt–proA)62 lacY1 tsx-36 supE44? galKZ hisG4 xyl-5 mtl-10 thi-1*	B. Bachmann
KL701 (CGSC 4256)	F123; *pyrD trp his recA thi galK malA* (λ^R) *xyl mtl rspL* λ^-	B. Bachmann
ORF4/KL251 (CGSC 4282)	F254; *leuB proC purE trpE recA metE thi ara lacZ xyl mtl azi rpsL tonA tsx supE*	B. Bachmann
AB2829	*aroA*	J. Pittard
LCB273 (CGSC 6542)	*lacY gal pyrD trp*::Tn*5 mal thi* Val^R F106-3 *aroA273*::Tn*10*	B. Bachmann
AN1495	*argH pyrE entA recA*::Tn*10*	This laboratory
AN2342	F^-	P1(AN801) × AB2829; this study
AN2343	F^- *cydD*	P1(AN801) × AB2829; this study
AN2344	F^- *cydD* Str^r	Spontaneous Str^r derivative of AN2343; this study
AN2346	F^- *cydD recA*	P1(AN1495) × AN2344; this study
HW133	*zbj*::Tn*10*(YYC199) in AB1321	P1(YYC199) × AB1321; this study
AN801	*cydD*	This study

for strain construction the m.o.i. used was between 0·1 and 0·001. P1 lysates were prepared as described by Miller (1972).

Preparation of cell-free extracts and membranes. Cells (80–95 g) were disrupted in a Ribi fractionator and subcellular fractions prepared by differential centrifugation in buffers containing the protease inhibitors 6-aminohexanoic acid (40 mM) and *p*-aminobenzamidine (6 mM) as described by Poole & Williams (1987). S_1 refers to the supernatant fraction obtained after centrifuging a cell extract at 160000 *g* for 1 h. Fractions were stored at −20 °C until required.

Scanning spectrophotometry. Reduced (dithionite) *minus* oxidized (H_2O_2 or ammonium persulphate) difference spectra were recorded at room temperature or 77 K in an Aminco-Chance DW2 spectrophotometer. For 77 K spectra, cells were suspended in 1 M-sucrose (Wilson, 1967). CO difference spectra were obtained at room temperature using the split-beam mode of a Hitachi–Perkin Elmer model 557 spectrophotometer.

Enzyme assays. Catalase was assayed by the method of Aebi (1974). NADH oxidase activity was determined spectrophotometrically at 340 nm. The assay mixtures (2·5 ml) contained 60 mM-Tris/HCl (pH 7·5) and 1 μmol NADH; the reaction was initiated by adding sample to the test cuvette only. The ubiquinol oxidase assay was based on that of Kita *et al.* (1982). The assay contained, in volumes of 2·5 ml, 60 mM-Tris/HCl (pH 7·5) and 10 μl of a solution (23·2 mM) of ubiquinol-1, previously reduced with a few grains of sodium borohydride. After recording the rate of quinol autoxidation at 275 nm, the reaction was initiated by adding 10–20 μl sample to the test cuvette only. Protein was estimated by the method of Markwell *et al.* (1978).

Chemicals. Most reagents were from Ajax Chemicals (Sydney) or BDH. DL-α-Glycerophosphate (disodium salt) and NADH were from Sigma.

RESULTS

Isolation of the mutant

A mutant lacking cytochrome oxidase *d* was isolated by screening survivors of nitrosoguanidine mutagenesis for loss of spectroscopically detectable cytochrome *d*, identified by its characteristic absorption band at about 630 nm. Preliminary mapping experiments suggested that the gene affecting cytochrome *d* was highly cotransducible with *aroA* (approximately 20 min), and so strain AB2829 was transduced to Aro^+ with a P1 lysate prepared

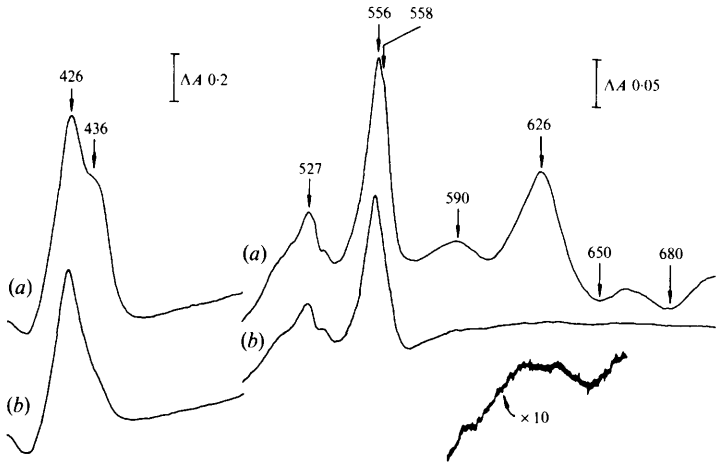

Fig. 1. Reduced (dithionite) minus oxidized (persulphate) difference spectra of washed membranes from (*a*) AN2342 (Cyd$^+$) and (*b*) AN2343 (Cyd$^-$). The scan rate was 0·5 nm s^{-1} and the spectral band width 2·0 nm (400–500 nm) or 1·25 nm (500–700 nm). The pathlength was 2 mm and the protein concentrations were 2·4 (*a*) and 3·0 (*b*) mg ml^{-1}. The bottom trace shows the 580–660 nm region of the AN2343 spectrum at 10-fold increased recorder gain.

on the oxidase mutant AN801. Transductants that were Aro$^+$ were screened for azide sensitivity so that isogenic mutant (*cydD*, AN2343) and parental (*cydD*$^+$, AN2342) strains could be isolated.

Spectral characterization of the mutant

Reduced minus oxidized difference spectra recorded at room temperature (not shown) or 77 K (Fig. 1) clearly revealed, in washed membranes from strain AN2342, after step-down to oxygen-limiting conditions, all components of the cytochrome *d*-containing oxidase complex (Fig. 1*a*). The most distinctive feature is the peak at 626 nm due to the ferrous form of the chlorin haem. The troughs at 650 and 680 nm are attributable to the oxy- (Poole *et al.*, 1983*a*, *b*) and peroxy- (Poole & Williams, 1988) forms, respectively, of this cytochrome. The band at 590 nm is d ıe to high-spin 'b_{595}' (Lorence *et al.*, 1986), as is much of the absorbance at about 436 nm (for surveys, see Poole, 1983, 1988). Cytochrome b_{558} is seen as a shoulder to the peak of cytochrome b_{556}, both of which will contribute to the Soret band at 426 nm and the partially overlapping β bands near 527 nm. In marked contrast, the corresponding spectrum of the mutant AN2343 (Fig. 1*b*), otherwise isogenic with AN2342, shows the components of the cytochrome *d*-containing complex to be present only at levels approaching the sensitivity limits of the technique. Only traces of the bands at 630 and 650 nm were detectable, although a shoulder at 440 nm and a band at 590, both weak, were seen. Using an absorption coefficient of 18·5 mM^{-1} cm^{-1} from $A_{630-650}$ (Kita *et al.*, 1984*a*) and a measured signal intensification at 77 K (with respect to room temperature) of 20, the cytochrome *d*-content in the parent membranes is 0·9 nmol mg^{-1} whilst in membranes from the mutant the estimated level is 0·01 nmol mg^{-1}. In room-temperature spectra, cytochrome *d* was undetectable in strain AN2343 (or membranes therefrom), whether grown in complex or defined medium, and with glucose or succinate as carbon source.

CO difference spectra (Fig. 2) also demonstrated the lack of detectable cytochrome *d* in mutant strain AN2343. The spectrum of membranes from strain AN2342 (Fig. 2*a*) showed a distinctive peak at 640 nm due to the ferrous cytochrome *d*–CO complex and a very broad and complex trough extending from about 570 to 620 nm with contributions from the bleached absorbance bands of the high-spin haem *b* and cytochrome *d*. The Soret region showed a trough at 442 nm, probably due to both cytochromes *b*-595 and *d*, and a signal attributable to cytochrome *o* comprising a peak at 412 nm (cytochrome *o*–CO complex) and a trough near

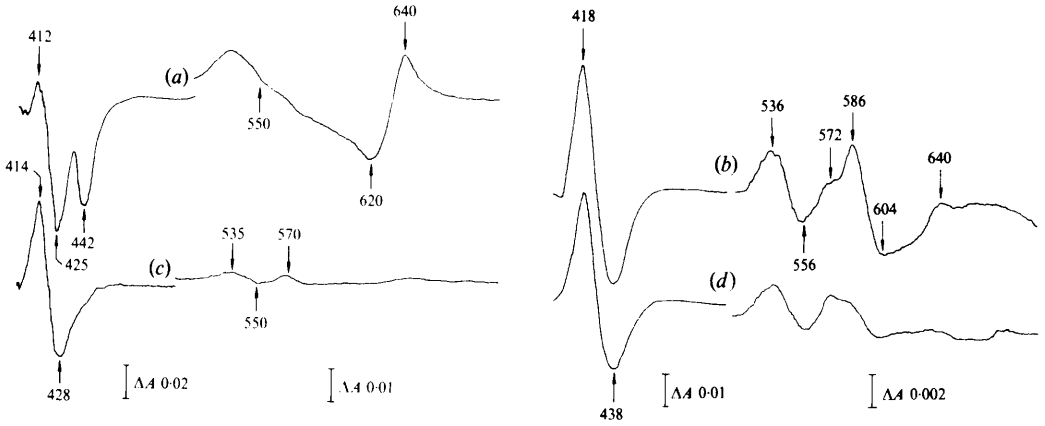

Fig. 2. CO difference spectra of washed membranes (*a*, *c*) and soluble fractions ('S_1'; *b*, *d*) from AN2342 (Cyd^+) (*a*, *b*) and AN2343 (Cyd^-) (*c*, *d*). Samples were reduced with dithionite and bubbled with a fine stream of CO for 2 min. Each spectrum is the computed difference between a CO-treated, reduced sample and the same sample before bubbling with gas. Reduced *minus* reduced baselines (not shown) were recorded; they were flat. Spectra were recorded at a scan rate of 2 nm s^{-1} and with a spectral band width of 2 nm. The path length was 10 mm and the protein concentrations 3·6 and 4·4 mg ml^{-1} for (*a*) and (*c*) respectively and 35 and 37 mg ml^{-1} for (*b*) and (*d*) respectively.

425 nm due to absorbance loss of the reduced cytochrome *o* at this wavelength by reaction with CO. The corresponding spectrum of AN2343 membranes (Fig. 2*c*) resembled that of 'pure' cytochrome *o* (Kita *et al.*, 1984*b*) or an inverted low-temperature photodissociation spectrum (Poole *et al.*, 1979), indicating that cytochrome *o* is the sole CO-binding cytochrome in these membranes.

The high-speed supernatant fractions from the two strains were more similar in their complement of CO-reactive haemoproteins. Both spectra (Fig. 2*b*, *d*) were dominated by Soret features that suggested that the presence of the soluble hydroperoxidase, haemoprotein *b*-590 (Poole *et al.*, 1986). A 586 nm band is evident in spectra (*b*) and (*d*) and is particularly strong in the case of the soluble fraction from the $cydD^+$ strain. The band is probably due in part to the α-peak of the CO complex of ferrous haemoprotein, being shifted from the position (578 nm) seen in the purified protein as a result of interference by the neighbouring α-band of a CO-binding cytochrome *c* (see below) and CO-binding by a small amount of cytochrome *d* (see 640 nm peak) in the extract of strain AN2342 (Fig. 2*b*). This latter component may have been washed from the membranes or, more likely, associated with small membrane fragments non-sedimentable under these conditions. Catalase activity was detectable in the soluble fractions of both strains (K_m 17 mM), but the activity in the mutant was about 40% of that (expressed as *k*, a first-order rate constant; Aebi, 1974) in the parent. This is in agreement with the attribution of the 586 nm peak and the 604 nm trough to a hydroperoxidase. Both spectra also exhibited a broad trough at 556 nm, with flanking peaks, that suggested some CO-binding cytochrome *c* or low-spin haem *b*.

Selective media for Cyd^+ and Cyd^- strains

The cytochrome *d*-terminated branch of the aerobic respiratory chain of *E. coli* is less sensitive to azide and cyanide than is the cytochrome *o*-mediated branch (Kita *et al.*, 1984*a*). Although azide sensitivity has been successfully used to isolate cytochrome *d*-deficient mutants, and azide resistance may indicate the presence of this oxidase (Green *et al.*, 1984; this work), other loci (*azi*; see Bachmann, 1983) not known to be related to respiratory metabolism are involved in the response to azide.

Biochemical studies of the purified *bd* and '$b_{562}o$' oxidase complexes have shown (Kita *et al.*, 1984*a*, *b*) that the latter is also more sensitive to inhibition by heavy metal ions, notably Zn^{2+}, Co^{2+} and Cd^{2+}. In an attempt to improve the selectivity of media based on respiratory inhibitors

Table 2. *Transductional mapping of the cydD locus*

Transduction	Selected marker	Non-selected marker	Cotransduction frequency (%)
AN2343 × P1(YYC188)	*zbi-927*::Tn*10*	*cydD*	28 (29/99)
AN2343 × P1(YYC199)	*zbj*::Tn*10*	*cydD*	88·5 (177/200)
AN2343 × P1(RW1230)	*zbj-1230*::Tn*10*	*cydD*	76 (114/150)
AN2343 × P1(LCB273)	*aroA*::Tn*10*	*cydD*	48 (92/191)
AB1321 × P1(YYC199)	*zbj*::Tn*10*	*aroA*	33·5 (67/200)
AB1321 × P1(RW1230)	*zbj-1230*::Tn*10*	*aroA*	86 (171/199)

we have included metal ions in various media and tested these with strains AN2342 (Cyd^+), AN2343 (Cyd^-) and AN2346 (Cyd^-RecA^-). Glucose minimal and MG media were used to prepare gradients (Carlton & Brown, 1981) of metal and azide concentrations across plates. Non-fermentable substrates in defined media afforded better discrimination between Cyd^- and Cyd^+ strains by azide than did glucose. The presence of Zn^{2+} (up to 10 mM) gave clearer discrimination between Cyd^+ and Cyd^- strains than did Co^{2+} or Cd^{2+}. The high metal concentrations required in these media are presumably due to chelation and/or precipitation of the ion (particularly by phosphate); thus, a medium low in phosphates and sulphates with glycerol as carbon source was developed based on that of Echols *et al.* (1961). In this medium, toxic concentrations of zinc for each strain were about 20-fold lower than in MG or phosphate-rich minimal medium whereas the effects of azide were altered little. Either zinc (~0·2 mM) or azide (0·15–0·45 mM) could be used separately for selection of Cyd^+ strains. However, AN2346 ($RecA^-$) appeared more resistant to Zn^{2+} than did AN2343 whilst the converse was true for azide resistance. The medium used subsequently contains both inhibitors and substantially inhibits growth of either $RecA^-Cyd^-$ or $RecA^+Cyd^-$ strains.

Mapping of the gene by mating and P1 transduction

The cytochrome *d*-deficient mutant was mated in turn with strains carrying various F-prime factors. Selection was on glucose-containing minimal medium plates supplemented with sodium azide (20 μg ml^{-1}) and counter-selection (against the donor) was by virtue of the prototrophic nature of the mutant background. One F-prime, F126 (Low, 1973), covering the region 17–29 min of the *E. coli* chromosome, complemented the mutant phenotype. The mutation was not complemented by F123 (27–30 min) or F254 (9–15 min).

The gene affecting cytochrome *d*, designated *cydD*, was highly cotransducible with *aroA* (approximately 20 min) but not with *pyrC* (23·4 min) or *ndh* (22·4 min); the cotransduction of *cydD* with four markers in the 18–20 min region of the chromosome was thus investigated. These were *zbi-927*::Tn*10* at 18·5 min, *zbj*::Tn*10*(YYC199) at 19·1min (Chang & Cronan, 1983), *zbj-1230*::Tn*10*, which is 88% cotransducible with *aroA* (B. Bachmann, personal communication), and *aroA*. The results of the cotransductional mapping are summarized in Table 2. The *cydD* locus was cotransduced with *zbj*::Tn*10*(YYC199) and *zbj-1230*::Tn*10* at frequencies of 88·5% and 76% respectively, suggesting that *cydD* lay between *zbj*::Tn*10*(YYC199) at 19·1 min and *zbj-1230*::Tn*10*. The rest of the cotransduction data are consistent with this result.

The gene order was tested in a three-factor cross in which a P1 lysate made on HW133 [*zbj*::Tn*10*(YYC199) *aroA*] was used to transduce AN2343 (*cydD*) to tetracycline resistance. Analysis of the distribution of unselected markers in the three-factor cross indicated that the least frequent recombinant type was Cyd^-Aro^-. No such transductants were found amongst 200 Tet^R strains screened, whereas the frequency of the other classes was as follows: Cyd^+Aro^-, 43% (86/200); Cyd^+Aro^+, 46·5% (93/200); Cyd^-Aro^+, 10·5% (21/200). If one assumes that the rarest recombination class is the result of a quadruple cross-over event, then the gene order is *zbj*::Tn*10*–*cydD*–*aroA*, as indicated in Fig. 3. This order is entirely consistent with the cotransduction frequencies (Table 2). The location of *cydD*, based on these data, is shown in Fig. 4.

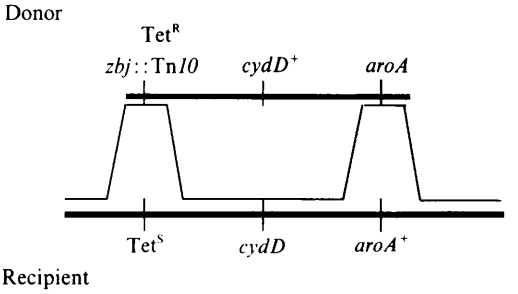

Fig. 3. Probable gene order in the *aroA* region assuming that the least frequent recombination class, Tet^{R}Cyd$^-$Aro$^-$, is due to a quadruple cross-over event.

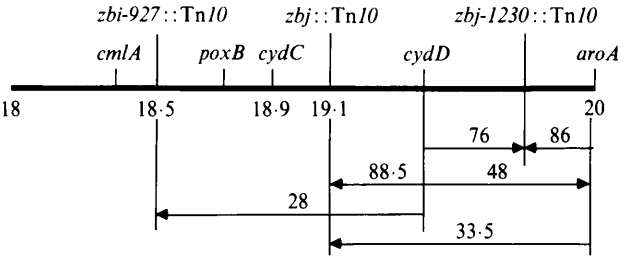

Fig. 4. Relevant portion of the *E. coli* genetic map. The linkages given are cotransduction frequencies (percentages) taken from experiments shown in Table 2. The arrows indicate the selected markers.

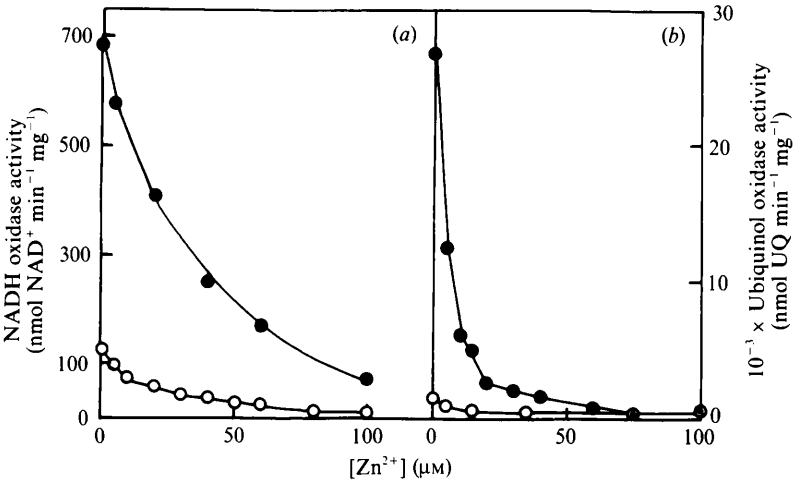

Fig. 5. Titration of NADH oxidase activity (*a*) or ubiquinol oxidase activity (*b*) with Zn^{2+} in washed membranes from AN2342 (*cydD*$^+$, ●) and AN2343 (*cydD*, ○). Activities are expressed as nmol NAD$^+$ or ubiquinone formed min^{-1} (mg membrane protein)$^{-1}$.

Oxidase activities of membranes

NADH oxidase activities and ubiquinol oxidase activities were lowered by 82% and 94% respectively in the *cydD* mutant (Fig. 5). Titration of both oxidase activities with Zn^{2+} showed that the high oxidase activities of AN2342 (*cydD*$^+$) and the residual cytochrome *o*-mediated respiration of AN2343 (*cydD*) were sensitive to the metal ion. At 40 μM-Zn^{2+}, ubiquinol oxidase activity of AN2342 membranes was diminished to the level of the residual activity in AN2343 membranes (Fig. 5*b*). NADH oxidase activity (Fig. 5*a*) appeared more resistant, such that

activity in AN2342 membranes was reduced to the uninhibited level in the mutant only at 80 μM-Zn^{2+}. The results suggest that the growth-inhibitory effects of Zn^{2+} reflect the residual rate of uninhibited respiration, rather than the fractional inhibition of respiration in Cyd^+ and Cyd^- strains.

DISCUSSION

The cytochrome *d*-containing respiratory oxidase complex of *E. coli* has long been known to be subject to marked regulatory effects. Although cytochrome *d* appears under oxygen-limiting growth conditions, this is but one of a number of 'inducing' conditions reported in *E. coli* and other bacteria (Poole, 1983, 1988; Ingledew & Poole, 1984) which include attainment of the late-exponential or stationary phases of growth, and growth on glucose, with cyanide, or under sulphate- or copper-limited conditions. No conditions have been found, however, under which the *cydD* mutant described here synthesizes components of the cytochrome *d* complex detectable in room-temperature spectra. In this respect the mutation differs from that reported by Johnson & Bragg (1985), in which control was exerted by growth substrate; the components of the complex were present during growth on glycerol or glucose, but not on succinate. Recently the *cyd* operon (*cydA*, *cydB*) has been shown to be transcriptionally regulated and induced when steady-state levels of oxygen fall. Other growth conditions, notably carbon source, also influence transcriptional activity (Georgiou *et al.*, 1988). Regulatory genes have not yet been identified, but the $cydD^+$ locus reported here may have such a function.

The new screening method described here for discrimination between Cyd^+ and Cyd^- strains exploits the reported sensitivity of cytochrome *o* not only to azide but also to heavy metal ions. Zinc ions are potent respiratory inhibitors in *E. coli* (Kita *et al.*, 1984*b*) and the usefulness of Zn^{2+} may reside in part in the further diminution of overall respiratory activity rather than to specific effects of cytochrome *o*. Nevertheless, a low-phosphate medium that incorporates zinc and azide has facilitated genetic analysis by P1 transduction, which demonstrates that the *cydD* locus is distinct from the map position reported for *cydC* by Georgiou *et al.* (1987).

Direct spectroscopic screening of nitrosoguanidine-mutagenized *E. coli* allowed the isolation of a mutant defective in the distinctive absorption band of cytochrome *d*. Other chromophores of the cytochrome *d*-containing oxidase complex were apparently absent, namely cytochrome b_{558} and the cytochrome a_1-like high spin *b*-type haemoprotein. Although the absence or presence in the membrane of the two oxidase subunits has yet to be determined, transformation of the *cydD* mutant with a multicopy plasmid (pNG2, kindly made available by Dr R. B. Gennis), bearing the structural genes for the oxidase complex, does not result in overexpression of cytochromes b_{595} or b_{558}. In contrast, overproduction of the subunits using the same plasmid in a *cydC* mutant (Georgiou *et al.*, 1987) results in overexpression of both *b*-type cytochromes and suggests the involvement of $cydC^+$ in haem *d* biosynthesis. Nevertheless, *cydC* and *cydD* appear closely linked, and complementation data should be obtained.

Further studies will be directed to determining whether *cydD* is a regulatory gene. This might be achieved by raising specific antibodies to the subunits of the complex, and probing membranes from the *cydD* mutant for presence of the subunits. The construction of a *cydA–lac* fusion in $cydD^+$ and *cydD* strains should allow the role of $cydD^+$ to be determined at various oxygen tensions in the medium and in the presence of respiratory inhibitors. The recent cloning of $cydD^+$ (unpublished results) will aid future studies of this locus and its function.

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