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Roles for Enteric *d*-Type Cytochrome Oxidase in N_2 Fixation and Microaerobiosis

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Escherichia coli strains that lacked the _d_-type cytochrome oxidase, the terminal oxidase with a high affinity for O_2, grew anaerobically as well as the wild type did and were not impaired in the ability to evolve H_2 from either glucose or formate. The anaerobic synthesis and activity of nitrogenase in transconjugants of these strains carrying _Klebsiella pneumoniae nif_ genes were also normal. However, the behavior towards O_2 of anaerobically grown bacteria lacking the _d_-type oxidase differed from that of the wild type in the following ways: the potential O_2 uptake was lower, H_2 evolution and nitrogenase activity supported by fermentation were more strongly inhibited by O_2, and microaerobic O_2-dependent nitrogenase activity in the absence of a fermentable carbon source did not occur. These results show that the _d_-type oxidase serves two functions in enteric bacteria—to conserve energy under microaerobic conditions and to protect anaerobic processes from inhibition by O_2.

A branched respiratory chain terminating in oxidases of differing affinities for O_2 is common among procaryotes (1, 28). In *Escherichia coli*, the low-affinity *o*-type oxidase (or cytochrome *bo*) is the principal oxidase under aerobic conditions and the high-affinity *d*-type oxidase (or cytochrome *bd*) (encoded by *cyd* genes) predominates under microaerobic or anaerobic conditions (1, 9, 34). Mutants of *E. coli* that lack either oxidase have no growth defects under the various laboratory conditions tested (3, 11), implying a possible redundancy of terminal oxidases (see reference 9). In strictly aerobic diazotrophs, a high-affinity terminal oxidase has been implicated in N_2 fixation as a means of supporting ATP production at low O_2 concentration so that the O_2-sensitive nitrogenes can function. However, in none of them has this requirement been unequivocally established, although three types of evidence supporting this hypothesis have been published. First, the biochemical and physiological analyses of microaerophilic N_2 fixation, such as that occurring in the legume symbiosis, show that the O_2 concentration surrounding the bacteroids is very low (2, 4, 5). Second, the inferior growth yield of an ascorbate-tetramethyl-*p*-phenylenediamine-oxidase-negative mutant of *Azotobacter vinelandii* during O_2-limited N_2-dependent growth suggests that the higher-affinity cytochrome a_1o branch of the respiratory chain is needed for energy conservation at low dissolved O_2 concentration (DOC) (25). Third, the correction of O_2-sensitive mutants of *Azotobacter chroococcum* to aerotolerance by the provision of tricarboxylic acid cycle intermediates suggests that a high electron pressure to maintain an adequate O_2 uptake at low DOC is required for nitrogen fixation in air (31–33).

In the facultative anaerobe *Klebsiella pneumoniae* the efficiency of N_2 fixation (milligrams of nitrogen fixed per gram of glucose consumed) is improved by providing small amounts of O_2 (16). The optimum DOC for nitrogenase activity (30 nM) is near the apparent K_m of the purified *d*-type oxidase complex (20 nM O_2) (20, 39a). The respiratory chain in *K. pneumoniae* (39a) is very similar to that of the better studied one in *E. coli* (1, 9, 13, 27, 28, 34, 40). The high-affinity *d*-type oxidase predominates under microaerobic or anaerobic conditions and is present under all conditions that permit expression of *nif* genes (39a), that is, under either anaerobiosis or microaerobiosis during N-limited growth (18). This fact, together with the extremely high affinity for O_2 demonstrated for the *d*-type oxidase, has led to the proposal that the role of this oxidase is to lower the O_2 concentration to allow nitrogenase synthesis and function and to provide a terminal oxidase to permit electron transport-coupled ATP synthesis which supports the increase in efficiency of N_2 fixation observed under microaerobic conditions (39a). It was also suggested that the *d*-type oxidase in *E. coli* might have an analogous role. The present paper tests these hypotheses.

Mutants lacking the *d*-type oxidase exist for *E. coli* but have not yet been isolated for *K. pneumoniae*. In order to investigate further the importance of the *d*-type oxidase in microaerobic nitrogenase activity, we have exploited the ability of *E. coli* to fix N_2, when carrying *K. pneumoniae nif* genes (30).

This characterization of an *E. coli* cytochrome *d* mutant has demonstrated, for the first time, two likely functions for the oxidase that are relevant not only to microaerobic diazotrophy but also to the metabolism of enteric bacteria. The two functions are to support energy-requiring processes under microaerobiosis and to protect anaerobic processes from O_2 inhibition.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

E. coli J62-1 and UNF3501 were transduced with the chloramphenicol-resistant bacteriophage P1 grown on *E. coli* G0103, which carries a kanamycin (Km) resistance cassette close to the partially deleted *cydAB* locus (R. B. Gennis,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or derivation
E. coli		
G0103	F^- *rpsL thi gal* Δ*cydAB* Km[r][a]	R. B. Gennis
J62-1	*pro his trp nal lac*	N. Datta
UNF3500	*pro his trp nal lac* Δ*cydAB* Km[r][a]	J62-1 × P1Cm[r] grown on G0103
JC5466	*trp his rpsE recA56*	N. Willetts
UNF3501	*trp his rpsE*	Rec^+ derivatives of JC5466 selected on methyl-methane-sulphonate
UNF3502	*trp his rpsE* Δ*cydAB* Km[r][a]	UNF3501 × P1Cm[r] grown on G0103
Plasmids		
pRD1	Km[r] Cb[r] Tc[r] Gnd^+ His^+ Nif^+ $ShiA^+$ Tra^+ IncP	7
pMF100	Gnd^+ His^+ Nif^+ $ShiA^+$ Tra^+ IncP	8
pNG2	Tc[r] *cydA*$^+$*B*$^+$	12

[a] The Km[r] cartridge is located at the *Bgl*II site 5′ to Δ*cydAB* (R. B. Gennis, personal communication).

personal communication). Clones resistant to kanamycin (25 $\mu g \cdot ml^{-1}$) were checked for failure to grow on freshly prepared nutrient agar containing $ZnSO_4$ (0.1 mM) and NaN_3 (0.1 mM) (NAAZ), which is a property of Cyd^- mutants (22; A. T. Smith, unpublished observations). Transformations of strains J62-1 and UNF3500 with pNG2 were performed by the method of Merrick et al. (26). Transformants were selected by resistance to tetracycline (5 $\mu g \cdot ml^{-1}$) on nutrient agar and were checked for growth on the NAAZ medium and for the presence of the desired plasmid by *Pvu*I and *Eco*RI restriction analysis (24). His^+ *E. coli* transconjugants were selected from matings with suitable *K. pneumoniae* strains carrying either pRD1 or pMF100 (6).

Growth conditions. Strains were maintained and grown for inocula either on nutrient agar or, for Nif^+ transconjugants, on minimal glucose medium (6) supplemented, when required, with tryptophan and proline (each at 25 $\mu g \cdot ml^{-1}$). Where necessary, tetracycline (5 $\mu g \cdot ml^{-1}$) was added. Anaerobic glucose-limited growth was achieved after 18 to 24 h of bubbling (about 50 ml $\cdot$ min^{-1}) with CO_2 (1%, vol/vol) in N_2 at 28°C in 15 to 50 ml or 1.5 liters of a modified NFDM medium (6) containing glucose (0.25%, wt/vol), twice the usual phosphate concentration, the trace elements of Poole et al. (29), nutrient broth (5%, vol/vol), vitamin-free Casamino Acids (800 $\mu g \cdot ml^{-1}$), and when required, tryptophan, proline, or histidine (all at 25 $\mu g \cdot ml^{-1}$). Exhaustion of glucose was detected by Clinistix (Miles Laboratories Ltd., United Kingdom), and growth was estimated by measurements of optical density (540 nm); protein concentration was estimated by using the BCA reagent (Pierce Chemical Co., United Kingdom) in microdilution plates (39). The protein concentration in crude extracts (see below) was measured with Coomassie blue G-250 (Pierce).

Assays. H_2 evolution and C_2H_2 reduction assays were performed in Suba-seal capped serum bottles (8 ml) containing the indicated substrates in 0.1 ml under Ar and for the latter assays, 10 kPa of C_2H_2. The desired atmospheric O_2 was achieved by injecting a volume of air. The assay, performed at 30°C with shaking (136 strokes of 3.8 cm $\cdot$ min^{-1}), was started by injecting a sample of culture (1.0 ml) that had been collected anaerobically and was terminated after either 30 or 60 min by injecting 0.1 ml of 30% (wt/vol) trichloroacetic acid. Gas samples (0.5 ml) were then taken for analysis by gas chromatography (20). For measurements of O_2 uptake, a sample of culture (3 ml) was stirred under air in a Clark-type O_2 electrode chamber (Rank Bros., United Kingdom) at 30°C until the O_2 concentration had reached approximately 180 μM when air was excluded. O_2 consumption supported by endogenous fermentation products was allowed to proceed until the O_2 concentrations had reached approximately 100 μM, when the indicated additions (in 0.3 ml) were made. Initial rates of O_2 uptake were calculated by assuming that the O_2 concentration in air-saturated saline phosphate buffer (6) was 240 μM.

Absorption spectra. Organisms harvested from 1.5-liter cultures were suspended in about 6 ml of N_2-sparged 50 mM Tris hydrochloride (pH 8) containing 1 mM EDTA, DNase (10 $\mu g \cdot ml^{-1}$), 0.5 mM phenylmethylsulfonyl fluoride, and dithiothreitol (0.1 mg $\cdot$ ml^{-1}). Bacteria were disrupted by two passages through a French pressure cell as described previously (19). After centrifugation at 3,670 × *g* for 15 min at 4°C, the supernatants, termed crude extracts, were stored at −20°C. Samples of the thawed crude extracts were either oxidized by addition of a crystal of ammonium persulphate or reduced by the addition of a crystal sodium dithionite, and the reduced-minus-oxidized difference absorption spectra were recorded at room temperature on a Unicam SP1800 ultraviolet spectrophotometer with a spectral band width of 0.9 nm, a light path of 10 mm, and a scan speed of 60 nm $\cdot$ min^{-1}.

RESULTS

***cydA*$^+$*B*$^+$ and *cydAB* strains used in this work.** An *E. coli* strain that lacks the structural genes for cytochrome *d* was obtained (from R. B. Gennis) in order to determine whether the *d*-type cytochrome oxidase has any role in microaerobic processes, such as nitrogenase activity. This strain (GO103) carries a Km[r] cassette about 1,500 base pairs away from a *cydAB* deletion (R. B. Gennis, personal communication). Because the phenotypes of this deletion mutation might be influenced by a variety of other characteristics associated with the respiratory chain, two *E. coli* strains, UNF3501 and J62-1, were transduced to Km[r] with P1 grown on GO103. These two *his* mutants were chosen because the *K. pneumoniae* Nif^+ plasmid pRD1 or pMF100 could be retained by *his* prototrophy, and in such transconjugants, good levels of N_2 fixation or nitrogenase activity have previously been found (30). The transductants were shown to have received the *cydAB* deletion because, unlike *cydA*$^+$*B*$^+$ strains, they failed to grow on NAAZ medium (see Materials and Methods). Furthermore, the introduction of pNG2 (12), which carries the *cydAB* genes, restored the ability to grow on this medium. The plasmid pNG2 carries not only the *cydAB* genes but also the adjacent region in which the Km[r] cassette is inserted in the chromosome (R. B. Gennis, personal communication). Therefore Km[r] *cydA*$^+$*B*$^+$ derivatives of J62-1 were sought by selecting for transductants on the

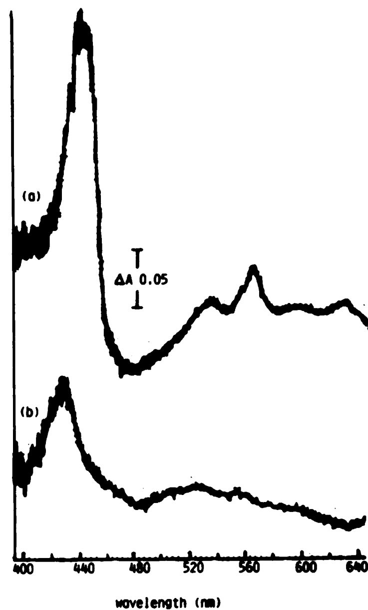

FIG. 1. Dithionite-reduced-minus-persulphate-oxidized difference absorption spectra of crude extracts of anaerobic glucose-limited growth of *E. coli* strains. The strains carried a Nif^+ plasmid and were UNF3501(pRD1) (Cyd^+), at 9.7 mg protein · ml^{-1} (a), and UNF3502(pRD1) (Cyd^-), at 8.3 mg protein · ml^{-1} (b).

NAAZ medium containing kanamycin. Only 2% of the Km^r transductants grew on the NAAZ medium. One of these Km^r *cydA*$^+$*B*$^+$ transductants was tested and showed the wild-type characteristics for O_2-dependent nitrogenase activity and O_2 inhibition of fermentative H_2 evolution and nitrogenase activity (see below). Thus, the *cydAB* deletion and not the Km^r insertion was responsible for the mutant phenotypes described below.

The presence of the *d*-type oxidase in haploid *cydA*$^+$*B*$^+$ strains was sometimes indicated by the green color of packed cells from anaerobic glucose-limited cultures. This green color was more pronounced in diploid strains carrying the Cyd^+ plasmid pNG2, as described previously (12), but was absent in the *cydAB* mutants. Spectroscopic examination of the *cydA*$^+$*B*$^+$ bacteria demonstrated the presence of the *d*-type cytochrome oxidase complex which contains cytochrome *b* plus cytochrome *d* (27, 28, 39a). The peak at 630 nm, indicative of cytochrome *d*, was present in *cydA*$^+$*B*$^+$ but not in *cydAB* strains (Fig. 1). There was also considerably more cytochrome *b* (peak around 565 nm) in the *cydA*$^+$*B*$^+$ strains than the *cydAB* mutants. Thus, during anaerobic glucose-limited growth the *d*-type cytochrome oxidase was synthesized in the wild-type strain but was, as expected, absent from the *cydAB* mutants.

Cyd^- strains show no defects in anaerobic processes (growth, H_2 evolution, and C_2H_2 reduction). Good anaerobic glucose-limited growth was obtained in the modified NFDM medium (see Materials and Methods) after about 18 h of incubation of all the *cydAB* and *cydA*$^+$*B*$^+$ strains listed in Tables 2 and 3. The yields, measured either by optical density (data not shown) or by estimates of protein content (Tables 2 and 3), were similar in wild-type and mutant strains.

The evolution of H_2 during glucose fermentation in *E. coli* occurs via pyruvate formate lyase and formate hydrogen lyase; therefore, in strains that did not carry *nif* genes, H_2 evolution from added glucose or formate was used as a measure of electron flux associated with fermentation. Samples of the glucose-limited cultures showed only low levels of H_2 evolution under anaerobiosis (Table 2). The addition of glucose or formate markedly stimulated H_2 evolution, and the rate with formate was approximately twice that with glucose (Table 2). Thus, electron flux associated with H_2 evolution during glucose fermentation was probably not limited by the activity of formate hydrogen lyase. These activities were similar in *cydA*$^+$*B*$^+$ strains and *cydaAB* mutants (Table 2).

C_2H_2 reduction by Nif^+ transconjugants was used as a measure of electron flux associated with nitrogenase activity or as a reporter of ATP production (nitrogenase activity requires 16 moles of ATP per mole of N_2 reduced [see reference 18]). Anaerobic samples of glucose-limited cultures showed little C_2H_2 reduction in the absence of added substrates (Table 3). Addition of glucose enhanced the anaerobic C_2H_2-reducing activities to similar extents in *cydA*$^+$*B*$^+$ and *cydAB* Nif^+ strains (Table 3).

Cyd^- strains show a lower potential for O_2 uptake after anaerobic growth. The potential for O_2 uptake by samples of the anaerobic glucose-limited cultures was measured polagraphically (see Materials and Methods), and the initial rates of O_2 consumption in the absence and in the presence of added glucose or formate are presented in Table 4. The O_2 consumption that occurred in all strains in the absence of an added substrate was presumed to be due to fermentation products in the growth medium. Formate usually stimulated O_2 uptake in *cydA*$^+$*B*$^+$ strains, but this never occurred in

TABLE 2. Anaerobic growth and hydrogen evolution by Cyd^+ and Cyd^- *E. coli* strains carrying, where indicated, the Cyd^+ plasmid pNG2

Strain (plasmid)	Cyd[a] phenotype	Growth (mg of protein · ml^{-1})	Anaerobic H_2-evolving activity (% of wild type with either glucose or formate) with[b]:		
			No additions	Glucose (13 mM)	Formate (46 mM)
J62-1	+	0.15	13 (9)[c]	100	100
J62-1(pNG2)	+	0.13	ND[d]	79	92
UNF3500	−	0.14	9 (5)	97 (11)	84 (25)
UNF3500(pNG2)	+	0.13	ND	108	95

[a] Ability to grow on NAAZ medium.
[b] Activities (micromoles of H_2 per milligram of protein per 30 minutes) of the wild-type J62-1 were with glucose 12.4 (2.2) and with formate 28.5 (7.6). The activity without addition is shown as the percentage of the activity with added glucose.
[c] The standard deviation is shown in parentheses.
[d] Not determined.

TABLE 3. Anaerobic growth and C_2H_2 reduction by Cyd+ and Cyd− *E. coli* strains carrying a Nif+ plasmid (either pRD1 or pMF100) and, where indicated, the Cyd+ plasmid pNG2

Strain (plasmids)	Cyd[a] phenotype	Growth (mg of protein · ml^{-1})	Anaerobic C_2H_2-reducing activity (% of wild type with glucose)[b]	
			No additions	Glucose added (13 mM)
J62-1(pMF100)	+	0.11	0.1 (0.1)[c]	100
J62-1(pMF100, pNG2)	+	0.14	0.4 (0.1)	90 (17)
UNF3500(pMF100)	−	0.12	0.4 (0.5)	100 (25)
UNF3500(pMF100, pNG2)	+	0.13	0.2 (0.1)	108 (17)
UNF3501(pRD1)	+	0.14	0.8 (0.2)	100
UNF3502(pRD1)	−	0.14	<0.1	79 (18)

[a] Ability to grow on NAAZ medium.
[b] Activities (micromoles of C_2H_4 produced per milligram of protein per hour) of the wild-type strains with added glucose were 2.3 (0.4) for J62-1(pMF100) and 2.6 (0.5) for UNF3501(pRD1).
[c] The standard deviation is shown in parentheses.

cydAB mutants. The addition of glucose increased the rate of O_2 uptake in all strains, but the increase was sometimes less marked in *cydAB* mutants than in *cydA+B+* strains. The most important observation recorded in Table 4 is that in all *cydAB* mutants, the potential for O_2 consumption was markedly less than in equivalent wild-type strains, and in all cases, introduction of *cydA+B+* genes (on pNG2) increased the potential O_2 uptake.

The rates of O_2 uptake reported in Table 4 were measured during a decrease in DOC from about 180 to 40 μM (Fig. 2). At DOCs below 40 μM, the rate declined in *cydAB* mutants, unlike that in *cydA+B+* strains (Fig. 2). This decline indicates that the affinities for O_2 of the processes consuming O_2 were lower in the *cydAB* mutants than in the *cydA+B+* strains, which is consistent with the absence of the high-affinity *d*-type cytochrome oxidase in the *cydAB* mutants. The ability of *cydAB* mutants to take up O_2 after anaerobic growth (Table 4) suggested that the *o*-type cytochrome oxidase was synthesized under anaerobiosis. This is consistent with earlier spectral evidence for *cydA+B+* strains of *E. coli* (13) and *Klebsiella aerogenes* (14). On the other hand, spectral identification of the *o*-type cytochrome oxidase in anaerobically grown *K. pneumoniae* is equivocal (39a). Alternatively, O_2 consumption in the *cydAB* mutants could have occurred by autooxidation of other redox proteins, such as the Fe protein of nitrogenase (41). However, the latter was not responsible in these experiments, since O_2 uptake occurred in *cydAB* mutants that lacked a Nif+ plasmid (Table 4).

Inhibition by O_2 of activities of anaerobic enzymes is greater in Cyd− strains. In aerobic diazotophs, respiration is considered to be important for keeping O_2 away from the O_2-sensitive enzyme nitrogenase (18, 35, 42). In the facultative anaerobe *K. pneumoniae*, glucose-supported nitrogenase activity is partially inhibited in the presence of low levels of O_2 (16). In order to determine whether the *d*-type cytochrome oxidase has any role in keeping O_2 away from nitrogenase, the effect of O_2 on nitrogenase activity was investigated in *cydAB* mutants and *cydA+B+* *E. coli* transconjugants carrying a Nif+ plasmid from *K. pneumoniae*. In the *cydA+B+* strains, a similar pattern of O_2 inhibition of glucose-supported nitrogenase activity was observed as had been seen in *K. pneumoniae* (16); about 50% inhibition occurred under 1.3 kPa of O_2 (Fig. 3). By contrast, this inhibition was much more marked in *cydAB* mutants; 99% inhibition occurred under 1.3 kPa of O_2 (Fig. 3). The introduction of pNG2 into one of these strains gave rise to *cydA+B+* transformants which showed the same inhibition by O_2 of nitrogenase activity as had been found in the

TABLE 4. O_2 uptake rates by Cyd+ and Cyd− *E. coli* strains carrying, where indicated, a Nif+ plasmid (either pMF100 or pRD1) and the Cyd+ plasmid pNG2

Strain (plasmids)	Cyd[a] phenotype	O_2 uptake (% of wild type without additions) with[b]:		
		No additions	Glucose (13 mM)	Formate (46 mM)
J62-1	+	100	359 (59)[c]	227 (51)
J62-1(pNG2)	+	84	397	292
UNF3500	−	85 (20)	118 (18)	74 (15)
UNF3500(pNG2)	+	119	247	161
J62-1(pMF100)	+	100	176 (28)	79 (29)
J62-1(pMF100, pNG2)	+	167 (48)	337	85
UNF3500(pMF100)	−	29 (7)	66 (4)	25 (6)
UNF3500(pMF100, pNG2)	+	126 (9)	181 (6)	99
UNF3501(pRD1)	+	100	681	698 (78)
UNF3502(pRD1)	−	91 (20)	162	79 (37)

[a] Ability to grow on NAAZ medium.
[b] O_2 uptake rates (nanomoles of O_2 per milligram of protein per minute) without additions in wild-type strains were 73 (31) for J62-1, 89 (12) for J62-1 (pMF100), and 20 (3) for UNF3501(pRD1).
[c] The standard deviation is shown in parentheses.

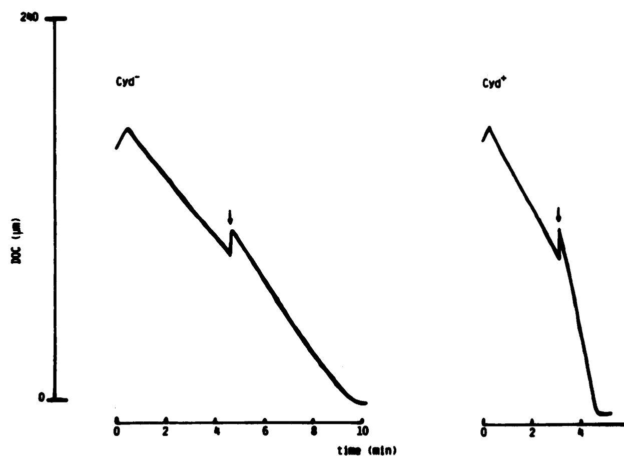

FIG. 2. Polographic O_2 electrode traces of samples from anaerobic glucose-limited cultures of *E. coli* strains J62-1 (Cyd+) and UNF3500 (Cyd−). The first decline in DOC was supported by endogenous substrates. The second decline occurred after the addition of glucose (13 mM), which is indicated by the arrow.

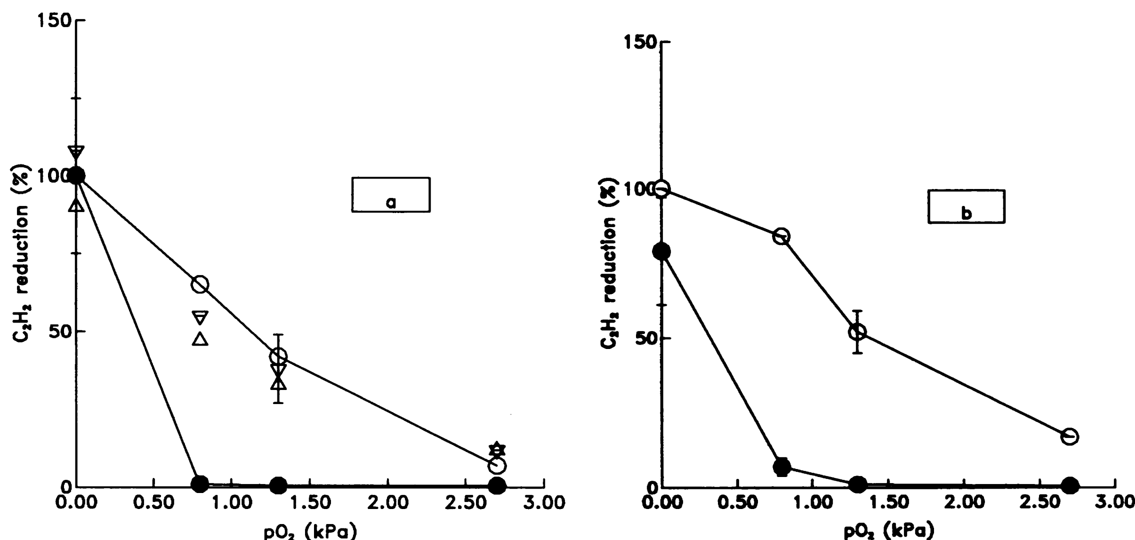

FIG. 3. Influence of O_2 on C_2H_2 reduction in samples from an anaerobic glucose-limited culture of Cyd^+ (open symbols) and Cyd^- (filled symbols) *E. coli* strains provided with glucose. (a) Strains carrying the Nif^+ plasmid pMF100 were J62-1 (○), J62-1 also carrying the Cyd^+ plasmid pNG2 (△), UNF3500 (●), and UNF3500(pNG2) (▽). (b) Strains carrying the Nif^+ plasmid pRD1 were UNF3501 (○) and UNF3502 (●). C_2H_2 reduction is shown as a percentage of the C_2H_2-reducing activity of the relevant wild type (see Table 3).

cydA$^+$*B*$^+$ strain from which the *cydAB* mutants had been derived (Fig. 3a). In these experiments, protein concentrations and shaking rates were all the same; the only factor affecting the DOC was the respiration rate. It can be concluded, therefore, that in vivo the *d*-type oxidase of *E. coli* is involved in preventing inhibition of nitrogenase by molecular O_2.

The production of H_2 during glucose fermentation by *E. coli* involves at least one O_2-sensitive enzyme complex, pyruvate formate lyase (23). In addition, the enzyme complex formate hydrogen lyase (36, 37) may be O_2 sensitive. Neither component of this complex has been purified, so their O_2 sensitivities are unknown. In order to determine whether the *d*-type cytochrome oxidase has a role in keeping O_2 away from these enzymes, the O_2 inhibition of H_2 evolution in *cydAB* mutants and *cydA*$^+$*B*$^+$ strains was investigated. The H_2 evolution supported by either glucose or formate in samples of anaerobic glucose-limited cultures of the *cydA*$^+$*B*$^+$ strain J62-1 was inhibited by O_2; about 55% inhibition occurred under 0.8 kPa of O_2 (Fig. 4). The inhibition was much greater in the *cydAB* mutants derived from J62-1; 95% inhibition occurred under 0.8 kPa of O_2 (Fig. 4). When *cydA*$^+$*B*$^+$ genes were reintroduced into this *cydAB* mutant with the plasmid pNG2, the level of inhibition became similar to that in the original *cydA*$^+$*B*$^+$ strain J62-1 (Fig. 4). The degree of inhibition by O_2 was similar, with either glucose or formate as substrate. Thus, in vivo the sensitivity to O_2 of formate hydrogen lyase activity was

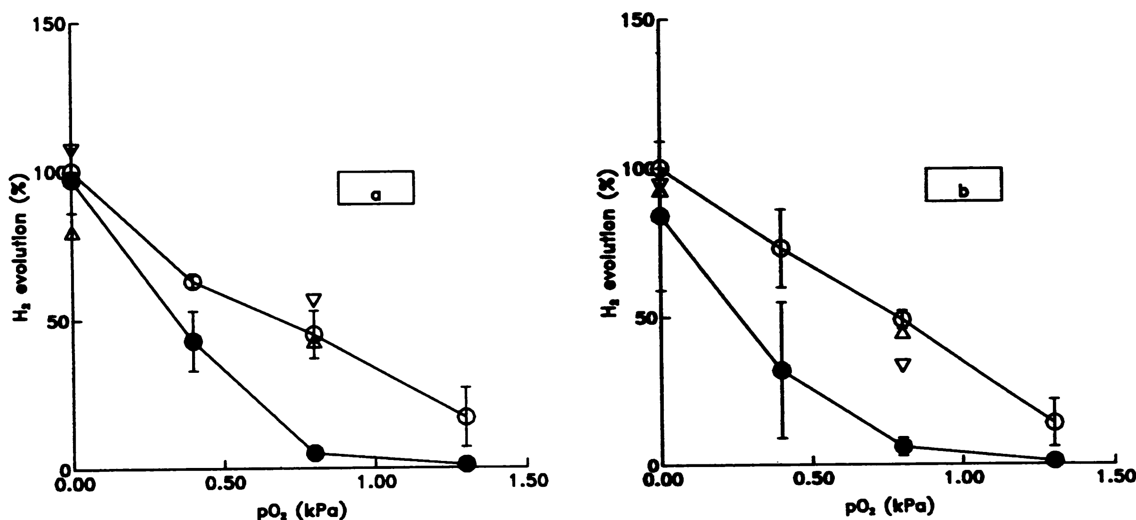

FIG. 4. Influence of O_2 on H_2 evolution in samples from anaerobic glucose-limited cultures of Cyd^+ (open symbols) and Cyd^- (filled symbols) *E. coli* strains with added glucose (a) or formate (b). H_2 evolution is shown as a percentage of the activity in the wild-type strain J62-1 under anaerobiosis (see Table 2). The strains were J62-1 (○), J62-1 carrying the Cyd^+ plasmid pNG2 (△), UNF3500 (●), and UNF3500(pNG2) (▽).

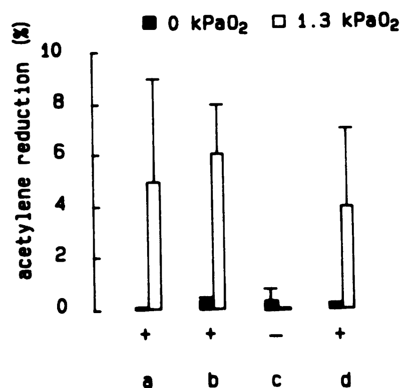

FIG. 5. Influence of O_2 on C_2H_2-reducing activity in unsupplemented samples from anaerobic glucose-limited cultures of transconjugants of Cyd$^+$ (+) and Cyd$^-$ (−) *E. coli* strains carrying the Nif$^+$ plasmid pMF100. The strains were J62-1(pMF100) (a); J62-1(pMF100), which also carried the Cyd$^+$ plasmid pNG2 (b); UNF3500(pMF100) (c); and UNF3500(pMF100, pNG2) (d). C_2H_2 reduction was measured under either anaerobiosis (■) or a pO_2 of 1.3 kPa (□) and is shown as a percentage of the wild-type J62-1(pMF100) with added glucose under anaerobiosis (see Table 3).

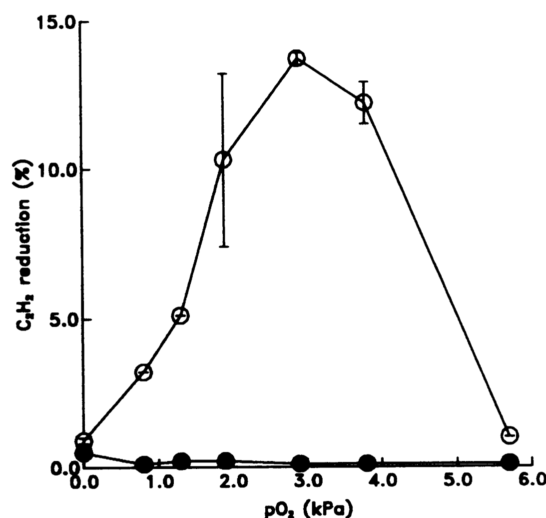

FIG. 6. Influence of atmospheric pO_2 on microaerobic C_2H_2-reducing activity supported by fermentation products in Cyd$^+$ (○) and Cyd$^-$ (●) *E. coli* Nif$^+$ transconjugants. Strains UNF3501 (pRD1) and UNF3502(pRD1) were assayed with lactate (46 mM) and formate (46 mM). The anaerobic glucose-supported activity of strain UNF3501(pRD1) (100%) is shown in Table 3.

similar to that of pyruvate formate lyase, which is known to be an O_2-sensitive enzyme (23). The results in this section thus confirm that the *d*-type cytochrome oxidase has a role in preventing inhibition of a number of O_2-sensitive enzymes.

Cyd$^-$ strains are defective in energy conservation under microaerobiosis. In the absence of added glucose, C_2H_2 reduction by samples of anaerobically grown glucose-limited *K. pneumoniae* is dependent upon the presence of low levels of O_2 (16). Such behavior is consistent with microaerobic respiratory activity providing ATP for nitrogenase activity (16). A comparison of O_2-dependent nitrogenase activity in *cydAB E. coli* transconjugants carrying *K. pneumoniae nif* genes with that in the isogenic *cydA$^+$B$^+$* transconjugant has revealed that the *d*-type cytochrome oxidase has a role in energy conservation under microaerobic conditions. In the absence of added glucose, C_2H_2 reduction by the anaerobic glucose-limited cultures of *cydA$^+$B$^+$* strains was significantly improved by providing low levels of O_2 (Fig. 5 and 6). This O_2-dependent activity was increased by providing fermentation products such as formate plus lactate (Table 5). The optimum atmospheric O_2 concentration for activity (about 3 kPa; Fig. 6) was similar to that found previously for *K. pneumoniae* (16). Strikingly, no O_2-dependent C_2H_2-reducing activity was found in cultures of *cydAB* mutants, even when potential fermentation products (lactate, formate, or pyruvate) were added (Table 5) or when the atmospheric O_2 concentration was varied (Fig. 6). The reintroduction of *cydAB* genes (with plasmid pNG2) restored O_2-dependent C_2H_2-reducing activity (Fig. 5). Therefore, we conclude that the *d*-type cytochrome oxidase is required to support microaerobic nitrogenase activity in *E. coli*.

DISCUSSION

A specific role for the *d*-type cytochrome oxidase of *E. coli* has not previously been described. The phenotypes described here of strains carrying a deletion in the *cydAB* genes strongly suggest that this high-affinity oxidase is important in facultative anaerobiosis for exploiting microaerobic conditions such as occur in some batch cultures and in oxygen-limited continuous culture.

The *cydAB* mutants, which were shown to lack the *d*-type cytochrome oxidase, grew anaerobically as well as wild-type bacteria did. They were shown to be unimpaired in their fermentative metabolism, as indicated by their ability to evolve H_2 from either glucose (by way of pyruvate formate lyase plus formate hydrogen lyase) or formate (by way of formate hydrogen lyase only). The anaerobic synthesis and activity of nitrogenase in transconjugants of *cydAB* mutants carrying *K. pneumoniae nif* genes were also normal. Although the anaerobic metabolism of the *cydAB* mutants was unaltered, their potential for aerobic respiration after growth in anaerobic conditions was markedly diminished. This diminished respiratory activity at low oxygen concentrations resulted in two independent effects; one is the failure of respiratory activity to remove inhibitory O_2, and the other is the diminished respiration-linked ATP production.

The increased sensitivity to O_2 of fermentation, as measured by H_2 production by way of pyruvate formate lyase and formate hydrogen lyase in *cydAB* mutants, as well as the increased sensitivity of in vivo nitrogenase activity confirm our earlier suggestion (39a) that a critical function of the high-affinity *d*-type cytochrome oxidase is respiratory protection in both *K. pneumoniae* and *E. coli*. In relation to inhibition by O_2 of nitrogenase activity and H_2 production by way of fermentation, it is worth mentioning that in wild-type bacteria containing the *d*-type oxidase, O_2 will have at least two possible inhibitory effects: one is due to the inhibition of specific enzymes, and the other may arise by competition for reducing equivalents resulting from an active respiratory electron transport chain (see reference 10).

It has been demonstrated that low levels of oxygen are required for C_2H_2 reduction by glucose-limited cultures of *E. coli cydA$^+$B$^+$* Nif$^+$ transconjugants (Table 5; Fig. 5 and 6), as was previously shown in *K. pneumoniae* (16). The results in the present paper confirm the suggestion (39a) that the ATP required for this process is provided by the activity of the high-affinity oxidase, which also lowers the DOC to sufficiently low levels to prevent inhibition of the nitrogenase. The DOC achieved by the *cydA$^+$B$^+$* strain under 3 kPa

TABLE 5. Effect of added substrates on microaerobic C_2H_2-reducing activity by transconjugants of Cyd^+ and Cyd^- *E. coli* strains carrying the Nif^+ plasmid pRD1

Strain (plasmid)	Cyd[a] phenotype	C_2H_2-reducing activity (%) with[b]:					
		No additions	Lactate	Formate	Lactate plus formate	Pyruvate	Pyruvate plus formate
UNF3501(pRD1)	+	4 (4)[c]	3.3 (1.5)	1.5 (1.0)	12 (5)	0.2	6.5 (0.5)
UNF3502(pRD1)	−	<0.1	<0.1	<0.1	<0.1	<0.1	0.2 (0.2)

[a] Ability to grow on NAAZ medium.
[b] C_2H_2-reducing activity was measured under a pO_2 of 1.3 kPa in samples from anaerobic glucose-limited cultures, with additions at either 92 mM when provided singly or 46 mM when provided in pairs, and is shown as a percentage of the wild-type [UNF3501(pRD1)] activity assayed with added glucose under anaerobiosis (See Table 3).
[c] The standard deviation is shown in parentheses.

of O_2 (Fig. 6) was probably about 30 nm, since this is the optimum DOC for nitrogenase activity in *K. pneumoniae* (20), which is very near the K_m value for O_2 for the purified *d*-type oxidase (39a). Further work in which either leghemoglobin or photobacteria are used to measure such low levels of dissolved O_2 (see reference 18) is necessary to confirm the optimum concentration for the respiration-supported N_2 fixation. The nature of the carbon substrate supporting this microaerobic respiration in *E. coli* has also to be elucidated. Preliminary results with *E. coli* (Table 5) and *K. pneumoniae* (A. Smith, unpublished observations) indicate that the fermentation products lactate and formate are likely candidates. Pyruvate, the source of electrons for nitrogenase in *K. pneumoniae* (19, 38) and presumably in *E. coli* Nif^+ transconjugants, is presumably generated by the activity of lactate dehydrogenase.

It is worth noting that microaerobic respiration benefits anabolic processes other than N_2 fixation, because growth yields in anaerobic glucose-limited chemostats of *E. coli* (15) and *K. pneumoniae* (17) are increased by providing small amounts of O_2.

In conclusion, our results strongly suggest that the high-affinity *d*-type cytochrome oxidase in enteric bacteria serves two functions: to conserve energy under microaerobic conditions and to protect anaerobic processes from inhibition by O_2. Such functions are essential for aerobic diazotrophy in both facultative anaerobes and obligately aerobic diazotrophs (35, 42) and are probably of benefit for the persistence of enteric bacteria in the gut, where fluctuating supplies of carbon sources and O_2 are common (see reference 21).

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