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The *Klebsiella pneumoniae* cytochrome *bd′* terminal oxidase complex and its role in microaerobic nitrogen fixation

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Cytochrome *bd′* has been implicated in having an important role in microaerobic nitrogen fixation in the enteric bacterium *Klebsiella pneumoniae*, where it is expressed under all conditions that permit diazotrophy. In this paper the sequence of the genes encoding this terminal oxidase (*cydAB*) of *Klebsiella pneumoniae* and the characterization of a *cyd* mutant are reported. The deduced amino acid sequences support the proposal that His 19, His 186 and Met 393 provide three of the four axial ligands to the Fe of the three haems in the oxidase complex. The nitrogen-fixing ability of the mutant was severely impaired in the presence of low concentrations of oxygen compared with the wild-type bacterium. Only the wild-type organism was capable of microaerobic nitrogenase activity supported by fermentation products. It is proposed that formate dehydrogenase-O may be involved in supplying electrons to a respiratory chain terminated by the *bd*-type oxidase, which would remove inhibitory oxygen and supply ATP for nitrogenase activity.

Keywords: *Klebsiella pneumoniae*, *cydAB*, cytochrome *bd′*, nitrogen fixation, formate dehydrogenase-O

INTRODUCTION

The enzymes responsible for N_2 fixation, the nitrogenases, are very sensitive to O_2. To reduce N_2 to $2NH_3$ they require a source of low-potential reductant (between -400 and -500 mV at pH 7) as well as a minimum of 16 moles of ATP per mole of N_2 fixed (see Hill, 1992). The ability to fix N_2 is widely distributed amongst obligate anaerobic, facultative anaerobic and obligate aerobic prokaryotes (Young, 1992), where the most commonly occurring nitrogenases are Mo-dependent enzymes (Bishop & Premakumar, 1992). Clearly, the capacity to carry out an aerobic catabolism should help satisfy the high ATP requirement, but it could be detrimental as a result of inactivation of nitrogenase by O_2. A range of strategies in different bacteria have been adopted to cope with this apparent paradox (Sprent & Sprent, 1990). For example, the obligately aerobic *Azotobacter vinelandii* can fix N_2 in air-saturated media (225 μM O_2) (Poste *et al*., 1983). It has an extremely active cytochrome *bd*-type terminal oxidase, which plays an important part in removing unwanted O_2, as has been shown by a mutant lacking this oxidase becoming an obligate microaerophile when fixing N_2 (Kelly *et al*., 1990). By contrast, the facultative anaerobe *Klebsiella pneumoniae* fixes N_2 anaerobically using a fermentative catabolism (Hill, 1976b), with no N_2 fixation occurring in aerobic conditions. However, microaerobic respiration can benefit diazotrophy; the efficiency of N_2 fixation (moles N_2 fixed per mole glucose consumed) in glucose-limited chemostats is increased by providing low levels of O_2 (Hill, 1976a, b). Nitrogenase activity in such populations is dependent upon O_2 (Hill, 1976a) and is maximal at a dissolved O_2 concentration of 30 nM (Kavanagh & Hill, 1990). Thus, microaerobic catabolic activity supported by fermentation products appears to provide both ATP and electrons for nitrogenase activity in *K. pneumoniae*. The source of electrons for nitrogenase activity in *K. pneumoniae* is the *nif*-specific pyruvate:flavodoxin oxidoreductase (Hill & Kavanagh, 1980; Shah *et al*., 1983) an enzyme that is very O_2 sensitive (Shah *et al*., 1983). Thus the function of microaerobic respiratory

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activity under these conditions is probably to prevent O_2 from inhibiting nitrogenase and the pyruvate: flavodoxin oxidoreductase as well as to provide ATP.

K. pneumoniae, like *Escherichia coli*, has a branched respiratory chain terminating in two oxidases of the cytochrome *bo*- and *bd*-types (Smith *et al*., 1990; Anraku & Gennis, 1987). Whether *K. pneumoniae* carries the *app* locus, which in *E. coli* encodes a second *bd*-type oxidase (Dassa *et al*., 1991; Sturr *et al*., 1996), is unknown. In both organisms the low-affinity cytochrome *bo′* is the principal oxidase under aerobic conditions, whereas under microaerobic or anaerobic conditions the high-affinity cytochrome *bd′* predominates (Smith *et al*., 1990; Tseng *et al*., 1996); the latter conditions are those that permit diazotrophy in *K. pneumoniae* (Smith *et al*., 1990). In *A. vinelandii*, by contrast, conditions of high O_2 input lead to enhanced synthesis of the cytochrome *bd′* (D'mello *et al*., 1997, and references therein). The cytochrome *bd′* from *E. coli* (Kita *et al*., 1984), *K. pneumoniae* (Smith *et al*., 1990) and *A. vinelandii* (Kolonay *et al*., 1994; Jünemann *et al*., 1995) have been purified. They are membrane-bound dimeric ubiquinol oxidases containing haems B and D. In general their properties are similar, except that the values for k_{cat} and the apparent K_m values for O_2 differ, being lower for the *E. coli* and *K. pneumoniae* enzymes than for the *A. vinelandii* enzyme. The apparent K_m values for *E. coli* (8 nM) (D'mello *et al*., 1996) and *K. pneumoniae* (20 nM) (Smith *et al*., 1990) are much lower than that for *A. vinelandii* (4·5 μM) (D'mello *et al*., 1994; Kolonay *et al*., 1994).

The molecular and genetic characterization of the *E. coli* cytochrome *bd′* has been extensive. Subunit I (cytochrome b_{558}) is encoded by *cydA*, and subunit II (which contains haem b_{595} and haem *d*) is encoded by *cydB* (Green *et al*., 1988). The *cydAB* genes are co-transcribed from a promoter upstream of *cydA* and are separated by 18 bp (Fang & Gennis, 1993). In subunit I the axial ligands of haem b_{558} are His 186 and Met 393 (Fang *et al*., 1989; Kaysser *et al*., 1995); this haem serves to transfer electrons from ubiquinol to the oxidase. In addition, His 19 in subunit I is probably an axial ligand for haem b_{595} of subunit II (Sun *et al*., 1996). Subunit I also contains a hydrophobic stretch of 11 amino acids, termed the Q-loop, which resides on the periplasmic side of the inner membrane and probably forms part of the binding site for ubiquinol (Dueweke & Gennis, 1991). The *A. vinelandii bd*-type oxidase is also encoded by two co-transcribed genes (Moshiri *et al*., 1991b). The deduced amino acid sequences of these show considerable homology with those of *E. coli cydAB* (68% for subunit I and 83% for subunit II) (Moshiri *et al*., 1991a). In the *A. vinelandii* subunit I His 22 and His 188 probably correspond to the axial ligands His 19 and His 186 in the subunit I of *E. coli*. Although *E. coli* does not fix N_2 it can do so when carrying the *K. pneumoniae nif* gene cluster on a plasmid. Wild-type transconjugants fix N_2 under anaerobic conditions (Postgate *et al*., 1987). Moreover, O_2-dependent nitrogenase activity occurs under the similar microaerobic conditions (30 nM O_2) as in *K. pneumoniae* (Hill *et al*., 1984, 1994). However, an *E. coli cyd* mutant harbouring the *nif* plasmid showed no microaerobic nitrogenase activity although the anaerobic activity was unimpaired (Hill *et al*., 1990). Thus in the heterologous expression host *E. coli*, cytochrome *bd′* appears to be needed for microaerobic nitrogenase activity. This paper describes the construction of an insertion mutation in *cydA* of the *K. pneumoniae* chromosome, which resulted in a strain being unable to make the cytochrome *bd′*, to assess the roles of this oxidase in microaeroic nitrogenase activity. A preliminary summary of some of this work has been published elsewhere (Juty *et al*., 1995).

METHODS

Media and growth conditions. Bacteria were grown at 28 °C on Luria–Bertiani (LB) medium containing double-concentration yeast extract and Tryptone, nutrient broth (NB) (Difco) medium, minimal glucose medium (Cannon, 1984) and nitrogen-free Davis and Mingioli medium (NFDM medium) (Cannon, 1984), supplemented when required with histidine (at 25 μg ml^{-1}) and NH_4Cl (15 mM). Where indicated, plates were incubated anaerobically in a Gas Pak system (Becton Dickinson). Antibiotics for *E. coli* and for *K. pnuemoniae* were added at the following concentrations (μg ml^{-1}) respectively: chloramphenicol, 15 and 40; ampicillin, 100 and 200; carbenicillin, 150 and 200–300; tetracycline, 15 and 15; streptomycin, 250 and 250 for high copy-number plasmid selection, 40 and 40 (with chloramphenicol) for suicide vector selection (see below). For chromosomal selection, streptomycin and spectinomycin for *K. pneumoniae* were added at 15 μg ml^{-1} each. NAAZ medium was a freshly poured nutrient agar plate containing 0·16 mM NaN_3 and 0·24 mM $ZnSO_4$ (Hill *et al*., 1990). Anaerobic glucose-limited growth was achieved after 12–16 h of bubbling with N_2 (about 50 ml min^{-1}) at 28 °C in 100 ml NFDM medium (Cannon, 1984), containing glucose (0·25%, w/v), twice the normal phosphate concentration, the trace elements of Poole *et al*. (1979), vitamin-free Casamino acids (800 μg ml^{-1}) and histidine (25 μg ml^{-1}). Exhaustion of glucose was detected by Clinistix (Miles Laboratories) and growth was estimated either by eye for solid media or by OD_{540}; protein concentration was measured by using the BCA reagent (Pierce) in microdilution plates (Smith *et al*., 1990).

DNA manipulations. Bacterial strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* chromosomal DNA was purified essentially as described by Robson *et al*. (1984). A gene library of *K. pneumoniae* (M5aI) was prepared and screened using the *cydAB* genes of *E. coli* by the procedure described previously (Moshiri *et al*., 1991a). The Wizard miniprep DNA purification system (Promega) was used for small-scale plasmid isolation from *E. coli* strain JM109. Large-scale plasmid preparation was by the alkaline lysis method, DNA manipulations, transformation of *E. coli*, and agarose gel electrophoresis were done as described by Sambrook *et al*. (1989). Probes for Southern blotting were labelled with [α-^{32}P]dCTP and random hexamers using the Prime-a-Gene kit (Promega). Single- and double-stranded DNA was sequenced using the Sequenase version 2.0 kit (USB) with the 7-deaza-dGTP nucleotide mixtures, using specific custom-made primers. Separation of sequence transcripts by electrophoresis employed a modified acrylamide, Long Ranger (ATandT Biochem). Oligonucleotide primers were synthesized, with the trityl group attached, using a model 391 PCR-MATE DNA

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source/reference
Strains		
E. coli		
JM109	*endA recA1 hsdR17* Δ(*lac–proAB*) [F′ *recA13 proAB lacI*^{q}*Z*ΔMI5]	
K. pneumoniae		
M5a1	Wild-type	
UNF122	*hisD2* Δ*lac2002 hsdR1 nif*$^+$ *rec*$^+$	R. Dixon
UNF3504	*hisD2* Δ*lac2002 hsdR1 nif*$^+$ *rec*$^+$ *cydA1*::Ω	This work
Plasmids		
pHP45	Ω-fragment-containing plasmid	Prentki & Kirsch (1984)
pKAB	*K. pneumoniae cydAB* cloned on *Hin*dIII–*Xho*I fragment in pBluescript	This work
pMM46	Suicide vector with temperature-sensitive replication origin	Paul & Merrick (1989)
pNG2	*E. coli cydAB*	Green *et al.* (1984)
pNIKA1	1·6 kb PCR product carrying Kp *cydA* in *Hin*cII site of pTZ19	This work
pNJ3	*Sac*I–*Sal*I *cydA*::Ω fragment from pNSJ2 cloned in the *Hin*cII site of pMM46	This work
pNSJ2	Ω fragment from pHP45 in *Bam*HI site of pTRJ1	This work
pTRJ1	PstI–*Xba*I *cydA* fragment from pNIKA1 cloned in pBluescript	This work
pTZ19	Cb^r	Stratagene

synthesizer on a 40 nmol scale. The synthetic DNA was purified using oligonucleotide purification cartridges (Applied Biosystems). DNA fragments were cloned into M13mp18 and M13mp19, to facilitate sequencing of both strands. The sequence was also confirmed in both directions by automated DNA sequencing on a Li-Cor model 4000 automated DNA sequencer, using IRD41 fluorescently labelled primers (MWG-BIOTECH) and a thermocycler (Hybaid). DNA and protein sequences were analysed using PC/Gene (IntelliGenetics) and compared with sequences in the GenBank, SWISS-PROT and Prosite databases using the FASTA and BLAST programmes (Altschul *et al.*, 1990; Pearson & Lipman, 1988).

PCR procedures. PCR amplification reactions consisted of 100 pmol of each primer, 200 μM dNTPs, 10 mM KCl, 2 mM Tris/HCl, pH 8·5, 0·25 mM $MgCl_2$, 0·04 mg gelatin ml^{-1}, 2·5 units *Taq* DNA polymerase and approximately 1 ng template DNA (in 100 μl final volume), using a Hybaid thermocycler. Reactions used 30 cycles each consisting of 1 min at 93 °C, 1 min at 55 °C and 5 min at 72 °C. Templates for the reaction were chromosomal DNA (1 ng) from *K. pneumoniae* strains UNF122 or M5aI. Primers (based on the *E. coli* sequence) were designed to be complementary to the 5′ start of the *cydA* gene on one strand (GGGGTACCCGGGAGCAAGGAGTCATGATGTTAGATATAGTC) and to the 3′ end of the *cydA* gene on the complementary strand (CGGGATCCCCGGGTACTTCATAATCGATCAT). Restriction sites to facilitate cloning were included in each primer: *Kpn*I and *Sma*I for the first primer and *Sma*I and *Bam*HI for the second.

Hydrogen evolution and nitrogenase assays. Measurements of H_2 evolution and C_2H_2 reduction (as a measure of nitrogenase activity) were made in Suba-seal capped serum bottles as described previously (Hill *et al.*, 1990), using cell suspensions (0·08 mg protein ml^{-1}) in 0·5 ml saline phosphate buffer (Cannon, 1984) that had been anaerobically harvested, washed and re-suspended in the same buffer. Incubations were at 30 °C, with 50 mM substrate, in a shaking water-bath, and were terminated after 15 min incubation by injecting 0·2 ml 30% (w/v) trichloroacetic acid. Gas samples (0·5 ml) were analysed by gas chromatography (Hill *et al.*, 1990). Respiration was measured using the same cell suspensions (0·03 mg protein ml^{-1}) in 2·5 ml saline phosphate buffer in a Clark-type O_2 electrode chamber (Rank Bros) at 30 °C with 50 mM substrates. Rates of O_2 uptake were calculated assuming that the O_2 concentration in air-saturated saline phosphate buffer is 240 μM; no O_2 consumption occurred in the absence of added susbtrate. All experiments were performed at least twice.

Spectrophotometry. Spectra of washed cell suspensions (0·5–1·5 mg protein ml^{-1}) were recorded using a Shimadzu UV-3000 dual-wavelength spectrophotometer. A 2 nm band width, a 10 mm light path and a scan speed of 100 nm min^{-1} were used to obtain the sodium-dithionite-reduced-*minus*-oxidized difference spectra at room temperature.

RESULTS AND DISCUSSION

Cloning of the *cydAB* region of *K. pneumoniae*

Cytochrome *bd′* from *K. pneumoniae* is very similar to that of *E. coli*, so the sequence of the *E. coli cydAB* genes could be used in strategies for cloning the genes from *K. pneumoniae*. PCR was used to generate a DNA fragment of about 1·6 kb, a size equal to the *E. coli cydA* gene, using *K. pneumoniae* M5a1 (wild-type) chromosomal

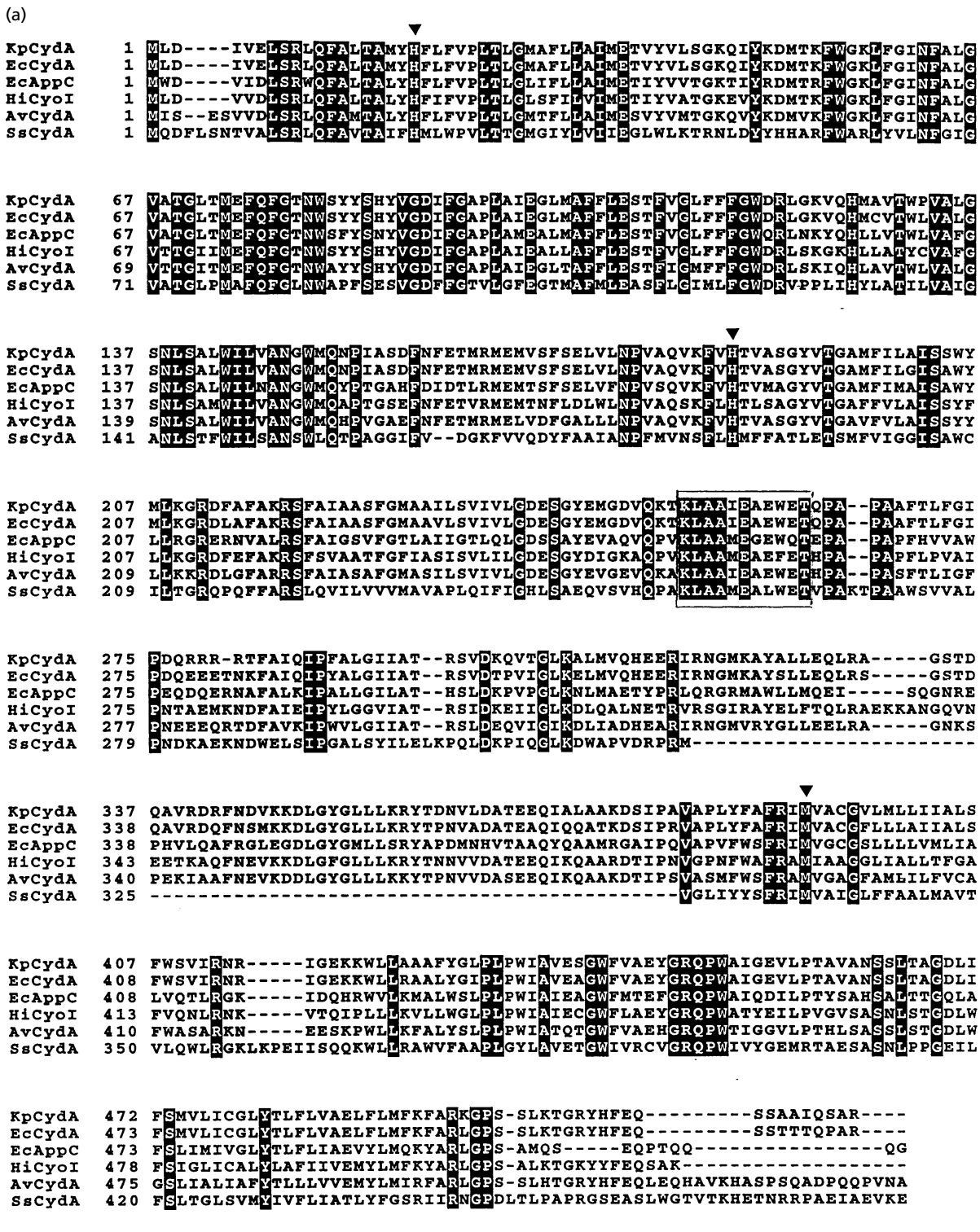

Fig. 1. For legend see facing page.

DNA as template and primers designed to be complementary to the 5′ (including the Shine–Dalgarno sequence) and 3′ ends of the coding region of the *E. coli cydA* gene. Using the *Sma*I sites incorporated in the primers, the *K. pneumoniae* PCR product was cloned into the *Hin*cII site of pTZ19 to yield pNIKA1. Subsequently the presumptive *K. pneumoniae cydA* gene was excised from pNIKA1 with *Pst*I and *Xba*I, and cloned into pBluescript SK+ to yield pTRJ1. It subsequently proved impossible to clone *cydB* separately

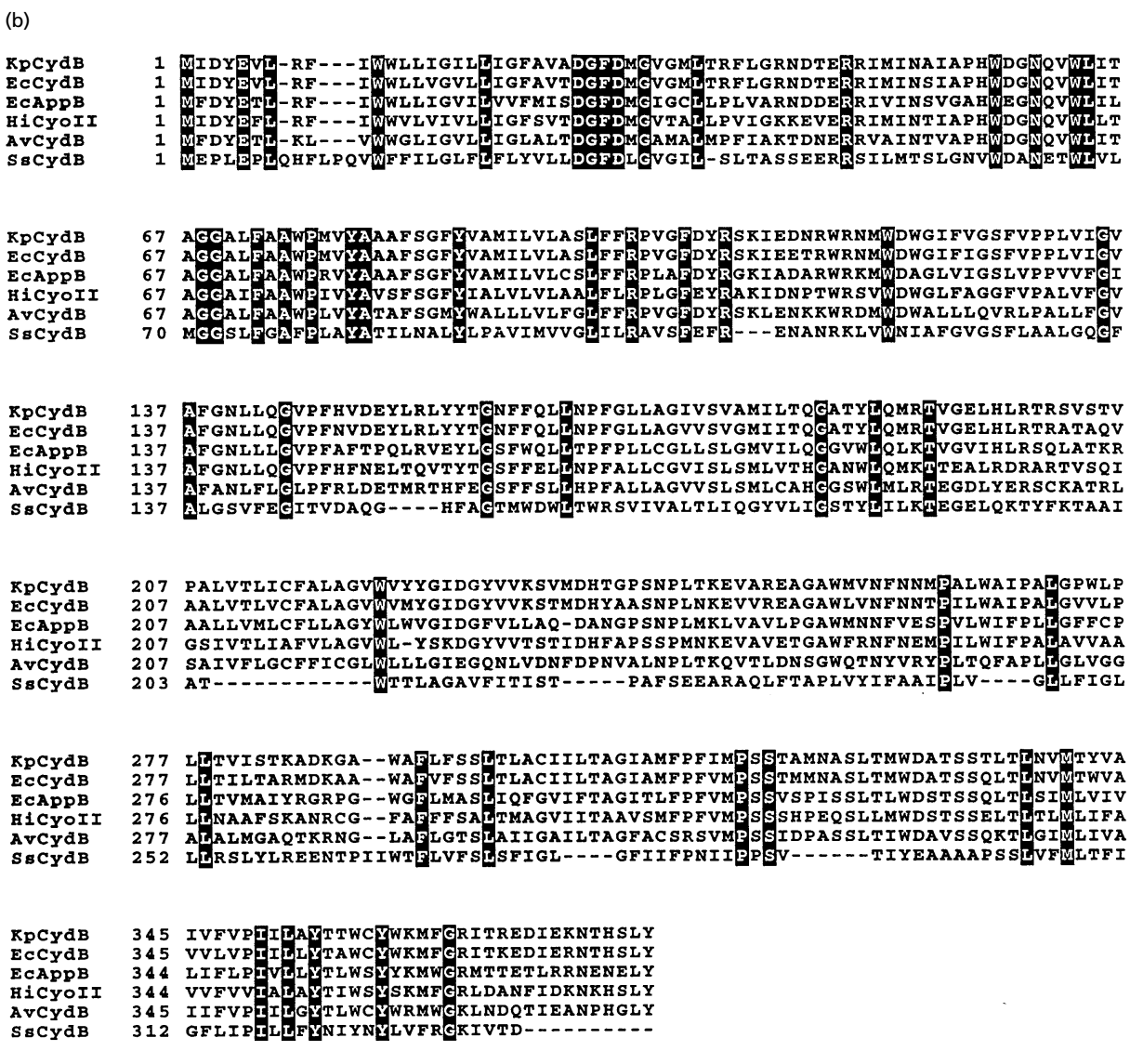

Fig. 1. Alignment of the amino acid sequences of CydA (a) and CydB (b) from various bacteria. Alignment was done using CLUSTAL V; positions with identical residues in all six proteins are shown in black boxes. The sequences presented are: CydAB from *K. pneumoniae*, CydAB from *E. coli*, AppCB from *E. coli*, CyoI and CyoII from *H. influenzae*, CydAB from *A. vinelandii* and CydAB from *Synechocystis* sp. In (a) the proposed ligands to the Fe of the various haem groups in the oxidase complex are indicated by arrows and the 11 amino acid stretch which is proposed to constitute part of the ubiquinol binding site is boxed.

and so the whole *cydAB* region was cloned using a gene library of *K. peumoniae* strain M5a1. Probing with the *cydAB* DNA of *E. coli*, a single *Hin*dIII–*Xho*I fragment of 5 kb was identified and cloned into pBluescriptSK + to yield pKAB. Sequencing from the *Xho*I site at the end of the insert revealed a region with close homology to the terminus of the *E. coli cydB* gene.

Sequence analysis of the *cydAB* region

Using plasmids pNIKA1 and pKAB the sequence of the entire *cyd* region (2747 bp) was determined and has been deposited with EMBL (accession no. Y10012). The sequence encodes two polypeptides (CydA and CydB) which, as expected, have high sequence identity to subunits I (CydA, 92% identity) and II (CydB, 87% identity) of cytochrome *bd′* from *E. coli* (Green *et al.*, 1988). BLAST searches identified four other pairs of proteins showing significant homology: AppC and AppB, which encode the equivalent subunits in a proposed third terminal oxidase in *E. coli* (Dassa *et al.*, 1991); CydA and Cyd B from *Azotobacter vinelandii* (Moshiri *et al.*, 1991a); CyoA and CyoB, which are subunits of an oxidase from *Haemophilus influenzae* (Fleischmann *et al.*, 1995); and proposed CydA and CydB subunits from *Synechocystis* sp. (Kaneko *et al.*, 1996). Alignments of all these amino acid sequences are presented in Fig. 1.

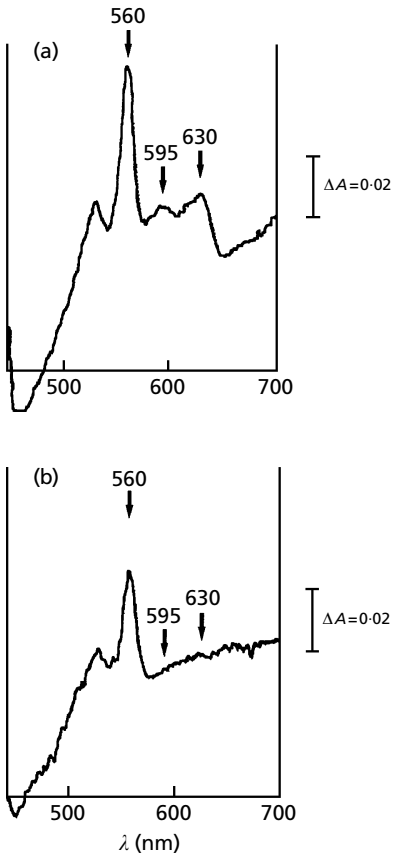

Fig. 2. Dithionite-reduced-*minus*-oxidized difference spectra of whole cells of *K. pneumoniae*, strains (a) UNF122 (*cyd*$^+$) and (b) UNF3504 (*cyd*). Cells were grown in air to stationary phase (in LB medium), harvested, washed and resuspended (see Methods). They were reduced with dithionite or oxidized with O_2. The *cyd*$^+$ strain (UNF122) showed the presence of cytochrome *bd*′ as seen by the characteristic absorbance at 560 nm, 595 nm and 630 nm. The *cyd* strain (UNF3504) showed no haem b_{595} or haem *d* (630 nm) by these criteria. The peak at 560 nm is due to the presence of other *b*-type haems. The protein concentrations (mg ml^{-1}) were 0·77 for UNF3504 and 1·03 for UNF122. Spectra were measured at room temperature.

Conservation in all five sequences supports conclusions from previous alignments of CydA and CydB of *E. coli* and *A. vinelandii* (Moshiri *et al.*, 1991a; Kaysser *et al.*, 1995) and from site-directed mutagenesis (Fang *et al.*, 1989; Kaysser *et al.*, 1995) which indicated that in CydA His 19, His 186 and Met 393 (marked in Fig.1a) provide three of the four axial ligands to the Fe of the three haems in the oxidase complex (numbers refer to *E. coli* residues). In *E. coli* an 11 amino acid stretch (Lys 254 to Thr 263) contains the epitope for two inhibitory monoclonal antibodies and has been proposed to form part of a functional domain in which ubiquinol is oxidized (Dueweke & Gennis, 1990). In *K. pneumoniae* this region in CydA is also highly conserved. One other sequence encoding a potential CydA protein was identified as part of a 4·5 kb genomic fragment from *Halobacterium salinarium* (EMBL accession no. 1070344). This sequence contains a truncated open reading frame that encodes the first 160 amino acids of a protein with 33 % identity to *E. coli* CydA.

Analysis of the multiple alignment of CydA sequences shown in Fig. 1(a) for predicted transmembrane helices using PHDhtm (Rost *et al.*, 1995) identified seven distinct transmembrane domains which are entirely consistent with the previously proposed topological model for subunit I (see Kaysser *et al.*, 1995). In this model the interhelical periplasmic domain (Gly 238 to Ile 392), called the Q-loop, between helices E and F has been proposed to constitute a functional domain near the periplasmic surface of the membrane and close to haem b_{558} (Dueweke & Gennis, 1990; Kaysser *et al.*, 1995). Whilst the role of the proteins encoded by the *Synechocystis* sp. *cydAB* genes is only inferred by homology it is of interest to note that the *Synechocystis* sp. CydA protein lacks almost half this domain (equivalent to residues Arg 319 to Arg 382 in *E. coli* CydA, see Fig. 1a).

Construction of a chromosomal mutation in *cyd* by *in vitro* mutagenesis

The plasmid pTRJ1 was used as a vehicle to insert the omega fragment (Ω) into the *K. pneumoniae* chromosomal *cydA* gene. This fragment, encoding resistance to streptomycin and spectinomycin, is flanked by signals for termination of transcription and translation (Prentki & Kirsch, 1984). It was released from pHP45 by digestion with *Bam*HI and cloned into the *Bam*HI site in the *cydA* insert of pTRJ1 to yield pNSJ2. Subsequently, the *Sac*I–*Sal*I *cydA*::Ω fragment of pNSJ2 was blunt-ended and cloned into the *Hin*cII site of the temperature-sensitive suicide vector pMM46 (Paul & Merrick, 1989) to yield pNJ3. This plasmid carries a temperature-sensitive origin of replication and the resistance gene for chloramphenicol as well as the Ω fragment. Transformants of UNF122 with pNJ3 were initially selected for resistance to chloramphenicol, streptomycin and spectinomycin, and were then sequentially subcultured (six times) at 41 °C in LB medium containing streptomycin and spectinomycin to prevent selection of spontaneously resistant colonies. Between subcultures, clones were checked for sensitivity to chloramphenicol (at 30 °C) and one clone (strain UNF3504) that was resistant to streptomycin and spectinomycin but sensitive to chloramphenicol was selected. To confirm that a double cross-over event had occurred (insertion of the mutant plasmid DNA into the *K. pneumoniae* chromosome) UNF3504 was compared with the wild-type strain (UNF122) by Southern blot analysis using the *cydA* PCR fragment as a probe. Using genomic digests with *Asp*718, *Eco*RI or *Pst*I a single hybridizing band which was 2 kb larger in UNF3504 than in UNF122 was identified in each case (data not shown). The *cydA*::Ω mutant was also screened for the ability to grow on NAAZ medium (which contains azide and thus inhibits the *o*-type oxidase). As predicted this medium did not support growth of the *cydA*::Ω mutant because it lacks the alternative oxidase.

Table 2. Anaerobic activities and potential respiratory activities of *K. pneumoniae* Cyd$^+$ and Cyd$^-$ strains

All activities were measured on glucose-depleted, anaerobically grown washed cells supplied with 50 mM substrates. Results are mean ± SE. Figures in parentheses are numbers of replicates.

Strain	Cyd phenotype	Anaerobic activities				Potential respiratory activities‡ with:	
		Nitrogenase* with:		H_2 evolution† with:			
		Glucose	Glucose + formate	Glucose	Formate	Glucose	Formate
UNF122	+	83 ± 7 (4)	87 ± 6 (4)	334 ± 14 (2)	638 ± 51 (2)	95 ± 5 (5)	72 ± 15 (3)
UNF3504	−	67 ± 6 (4)	75 ± 10 (4)	92 ± 6 (2)	229 ± 28 (2)	37 ± 3 (5)	39 ± 6 (3)

* Measured as C_2H_2 reduction: nmol C_4H_4 produced min^{-1} (mg protein)$^{-1}$.

† nmol H_2 evolved min^{-1} (mg protein)$^{-1}$.

‡ nmol O_2 consumed min^{-1} (mg protein)$^{-1}$.

Confirmation that strain UNF3504 lacks cytochrome *bd′*

The failure of UNF3504 to grow on the medium containing zinc azide is consistent with the lack of an active cytochrome *bd′* and this has been confirmed by studies of cell suspensions of the mutant. Packed cells of the wild-type strain UNF122, either grown in Luria Broth to late stationary phase, or grown anaerobically in glucose-depleted cultures, were green in colour indicating the presence of the *bd*-type oxidase. This green colouration was absent in the packed cells of strain UNF3504, which were light pink in colour, indicating that the mutant lacks the oxidase. This was consistent with the reappearance of the green colour in strain UNF3504 when transformed with either the *K. pneumoniae cyd*$^+$ plasmid (pKAB) or the *E. coli cyd*$^+$ plasmid (pNG2). Spectroscopic examination of the *cyd*$^+$ bacteria demonstrated the presence of cytochrome *bd′* as shown by the peak at 630 nm which was absent from strain UNF3504 (Fig. 2).

Activities of anaerobically grown washed cells

The *cydA* mutant was able to grow under anaerobic conditions, either diazotophically or with excess NH_4^+, at similar growth rates to the wild-type strain (UNF122); growth was measured under the following conditions: on solid NFDM medium with or without an excess of NH_4Cl in a gas pack anaerobic jar, in liquid NFDM medium containing glucose (excess or limiting) and bubbled with N_2 (see Methods).

When cells harvested from the anaerobic glucose-depleted cultures were assayed anaerobically the nitrogenase activities of the *cyd*$^+$ and *cyd* strains were not markedly different (Table 2). The rate of hydrogen evolution supported by glucose gives a measure of the total electron flux arising from glucose fermentation, whereas that supported by formate indicates the potential electron flow through the formate hydrogen lyase complex. In both strains, the rate of H_2 evolution from formate was about twice that from glucose (Table 2); in the *cyd* strain rates with both substrates were about a third of that measured in the wild-type (Table 2). Because the anaerobic growth of the mutant is not impaired, these lower rates of H_2 evolution in the mutant may indicate that the *bd*-type oxidase has a role in protecting O_2-sensitive processes during harvesting and washing procedures. Oxygen-sensitive fermentation enzymes include pyruvate formate-lyase, an important component of the fermentation pathway, and formate hydrogenlyase. Recently, an additional role for the *E. coli* oxidase has been proposed, to prevent the build-up of diffusible oxygen radicals (Goldman *et al.*, 1996). Such a role in *K. pneumoniae* may account for the poorer anaerobic H_2-evolving activity in the *cyd* mutant (UNF3504) compared to that of the wild-type (UNF122) (see Table 2), if such radicals were produced during the harvesting and washing procedures. Potential respiration rates with either glucose or formate as substrate were lower in the *cyd* strain UNF3504 than in wild-type UNF122 (Table 2). This difference is consistent with strain UNF3504 failing to make one of the two terminal oxidases, which was confirmed by spectroscopic examination of these anaerobically grown bacteria (data not shown).

Role of the cytochrome *bd′* in removal of inhibitory oxygen

To determine the effect of microaerobiosis on nitrogense activity in the glucose-depleted cells, nitrogenase activity was measured in the presence of very low levels of O_2. Activity supported by glucose was markedly more inhibited by O_2 in the *cyd* strain than the wild-type (Fig. 3a). For example, at 1·24 kPa O_2 there was only 60 % in the wild-type but complete inhibition in the mutant lacking cytochrome *bd′*. For nitrogenase activity low-potential electrons are required, these being supplied by the activity of pyruvate:flavodoxin oxidoreductase. For this reason, although formate is a potential electron donor for the respiratory chain (Table 2), it does not support nitrogenase activity. However, when formate was included together with glucose, at 1·24 kPa O_2, a

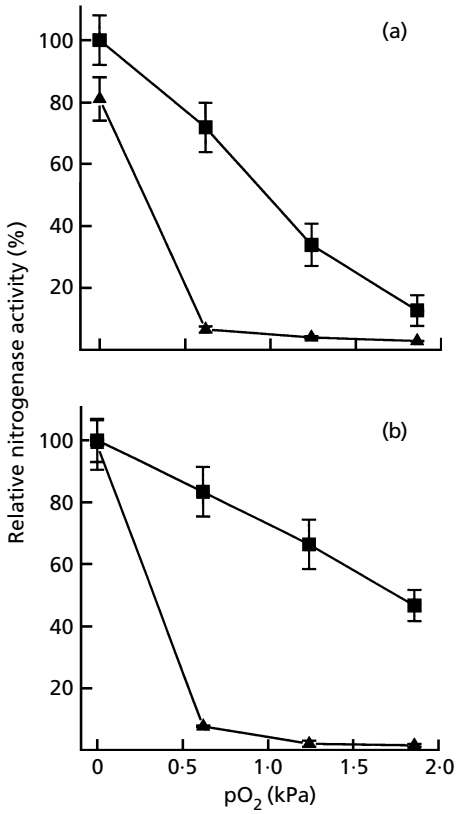

Fig. 3. Effect of O_2 on nitrogenase activity in *K. pneumoniae* strains, UNF122 (*cyd*$^+$, ■) and UNF3504 (*cyd*, ▲). (a) Nitrogenase activity supported by glucose alone. (b) Nitrogenase activity supported by glucose plus formate. Activity is shown as the percentage of the activity of the *cyd*$^+$ strain, under anaerobiosis (see Table 2).

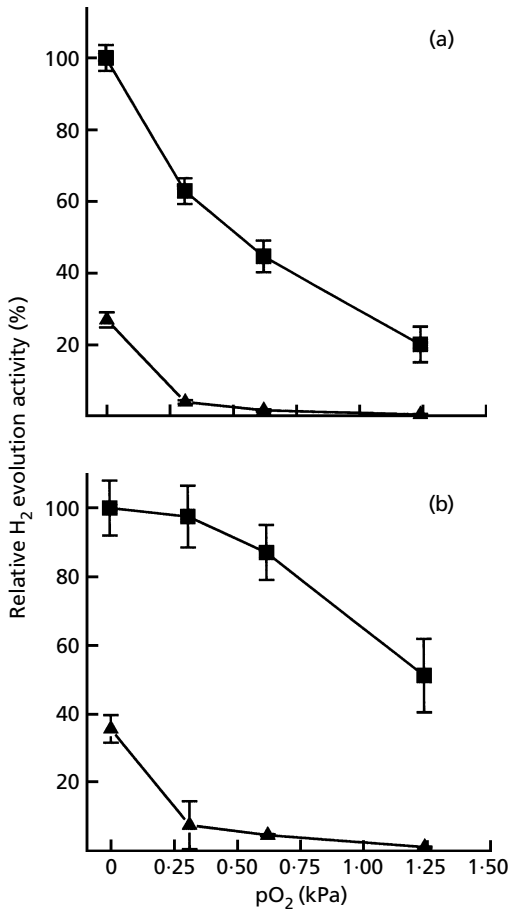

Fig. 4. Effect of O_2 on H_2-evolving activity in *K. pneumoniae* strains, UNF122 (*cyd*$^+$, ■) and UNF3504 (*cyd*, ▲). (a) H_2-evolving activity supported by glucose alone. (b) H_2-evolving activity supported by glucose plus formate. Activity is shown as the percentage of the activity of the *cyd*$^+$ strain, under anaerobiosis (see Table 2).

twofold stimulation of nitrogenase activity was seen in the wild-type organism but not in the *cyd* mutant (Fig. 3). Thus, electrons derived from a formate dehydrogenase under these microaerobic conditions probably pass to the branch of the respiratory chain terminating in the *bd*-type oxidase. By analogy with studies using *E. coli*, formate dehydrogenase-O is likely to be the enzyme involved (Sawers, 1994; Bock & Sawers, 1996). In all these experiments, the only factor affecting the dissolved O_2 is the rate of respiration. This was lower in the *cyd* strain than in the wild-type using either glucose or formate as substrate (Table 2). Therefore we conclude that *in vivo* the *bd*-type oxidase of *K. pneumoniae* is involved in preventing inhibition of nitrogenase activity by removing molecular O_2.

H_2 evolution from either glucose or formate was measured as an indication of flux through the fermentative pathways (Fig. 4). The proportion of H_2 evolution expected to arise from nitrogenase activity under these assay conditions is small (Hill, 1976b). Some enzymes in these fermentative pathways are oxygen-sensitive as shown by the inhibition of hydrogen evolution by low concentrations of oxygen (Fig. 4a). In the wild-type organism, O_2 inhibition occurred with both glucose and formate but it was considerably less pronounced with formate. By contrast, in the *cyd* strain inhibition by oxygen was far more severe and was the same with both substrates. This marked difference in the inhibition by O_2 of H_2 evolution in the mutant compared with the wild-type suggests that the *bd*-type oxidase has a role in protecting O_2-labile enzymes such as formate hydrogenlyase and pyruvate formate-lyase as well as nitrogenase. The particular effectiveness of formate in protecting against oxygen inhibition is consistent with the presence of an electron transport chain in which formate dehydrogenase-O is coupled to the *bd*-type oxidase.

Comparison of the data in Figs 3(a) and 4(a) indicates that the enzymes involved in H_2 evolution are apparently more sensitive to O_2 than is nitrogenase, consistent with a previous observation (Hill *et al.*, 1984). Differences in the degree of exposure to O_2 due to the location of these enzymes may be the reason; the formate hydrogenlyase complex is membrane bound (Sawers, 1994) whereas the nitrogenase is soluble within the cytoplasm. Further,

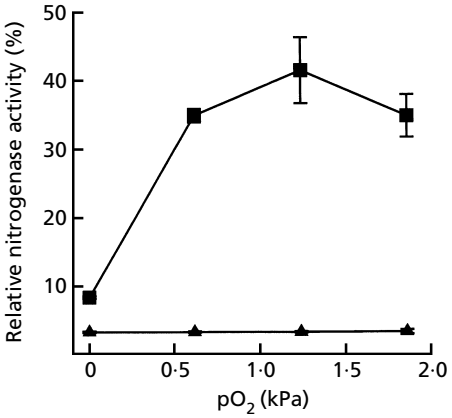

Fig. 5. Effect of O_2 on nitrogenase activity in *K. pneumoniae* strains, UNF122 (*cyd*$^+$, ■) and UNF3504 (*cyd*, ▲), supported by formate plus pyruvate. Activity is shown as the percentage of the activity of the *cyd*$^+$ strain, under anaerobiosis (see Table 2).

nitrogenase can reduce low concentrations of O_2 to water without loss of C_2H_2-reducing activity; this process could have an autoprotective function (Thorneley & Ashby, 1989).

Role of the *bd*-type oxidase in energy conservation

In the absence of added glucose, nitrogenase activity in samples from anaerobic glucose-limited chemostats of *K. pneumoniae* is dependent upon the presence of low levels of O_2 (Hill, 1976a). Such behaviour is consistent with fermentation products supporting microaerobic respiration to provide the necessary ATP. The required low-potential electrons arise from the activity of a *nif*-specific pyruvate:flavodoxin oxidoreductase, the pyruvate being a glycolytic product. Consistent with this dual requirement, no O_2-dependent nitrogenase activity was observed in the presence of either formate or pyruvate in wild-type or mutant strains lacking cytochrome *bd′* (data not shown). Thus in this situation the pyruvate can only provide electrons for nitrogenase and formate oxidation can provide the necessary ATP. By contrast, providing formate together with pyruvate resulted in significant O_2-dependent nitrogenase activity in the wild-type strain (Fig. 5). In the *cyd* strain, however, no O_2-dependent nitrogenase activity was observed with pyruvate plus formate (Fig. 5). We conclude, therefore, that in *K. pneumoniae* the *bd*-type oxidase is essential for O_2-dependent nitrogenase activity in which formate dehydrogenase-O may play a part in energy conservation.

Conclusions

The homology of the deduced amino acid sequences of the subunits of the cytochrome *bd′* (CydA and CydB) of *K. pneumoniae* with those of *E. coli* is high, which is not surprising in view of the similarity of these oxidases (Smith *et al.*, 1990; Kita *et al.*, 1984) and their occurrence under anaerobic and microaerobic conditions (Smith *et al.*, 1990; Tseng *et al.*, 1996). The alignments of the CydA and CydB sequences with other similar pairs of proteins – CydA and CydB from *E. coli*, *A. vinelandii* and *Synechocystis* sp., *E. coli* AppC and AppB, and *H. influenzae* CyoA and CyoB (see Fig. 1) – indicates that they all possess the same presumptive three axial ligands for haem binding in subunit I, and a region of high homology that has been proposed as an ubiquinol-binding site. The latter sequence in *K. pneumoniae*, *E. coli* and *A. vinelandii* is precisely conserved, so the architecture of this site is unlikely to be responsible for the markedly higher k_{cat} of the *A. vinelandii* enzyme (Jünemann *et al.*, 1995).

This work has identified two roles for the *bd*-type oxidase of *K. pneumoniae*. These are the conservation of energy under microaerobic conditions and the protection of anaerobic processes from inhibition by O_2. Furthermore, the work has revealed a probable specific function for formate dehydrogenase-O in microaerobic catabolism. Formate oxidation, by way of this enzyme, is apparently able to provide electrons for an electron transport chain terminating in the high-affinity *bd*-type oxidase, which plays a part in energy conservation (ATP synthesis) and the removal of inhibitory O_2.

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