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-0 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-0

**INTRODUCTION**

The enzymes responsible for *N₂* fixation, the nitrogenases, are very sensitive to *O₂*. To reduce *N₂* to 2NH₃ 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 *N₂* fixed (see Hill, 1992). The ability to fix *N₂* 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 *O₂*. 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 *N₂* in air-saturated media (225 μM *O₂*) (Poste et al., 1983). It has an extremely active cytochrome *bd*-type terminal oxidase, which plays an important part in removing unwanted *O₂*, as has been shown by a mutant lacking this oxidase becoming an obligate microaerophile when fixing *N₂* (Kelly et al., 1990). By contrast, the facultative anaerobe *Klebsiella pneumoniae* fixes *N₂* anaerobically using a fermentative catabolism (Hill, 1976b), with no *N₂* fixation occurring in aerobic conditions. However, microaerobic respiration can benefit diazotrophy; the efficiency of *N₂* fixation (moles *N₂* fixed per mole glucose consumed) in glucose-limited chemostats is increased by providing low levels of *O₂* (Hill, 1976a, b). Nitrogenase activity in such populations is dependent upon *O₂* (Hill, 1976a) and is maximal at a dissolved *O₂* 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 *O₂* sensitive (Shah et al., 1983). Thus the function of microaerobic respiratory
activity under these conditions is probably to prevent O₂ 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 daziotrophy in *K. pneumoniae* (Smith et al., 1990). In *A. vinelandii*, by contrast, conditions of high O₂ 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* (Koloney et al., 1994; Jüinemann 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 ΔG° and the apparent kₐₚ values for O₂ differ, being lower for the *E. coli* and *K. pneumoniae* enzymes than for the *A. vinelandii* enzyme. The apparent kₐₚ 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; Koloney et al., 1994).

The molecular and genetic characterization of the *E. coli* cytochrome *bd* has been extensive. Subunit I (*cytochrome b₅₉₅*) is encoded by *cydA*, and subunit II (which contains haem b₅₉₅ 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 b₅₉₅ 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 159 in subunit I is probably an axial ligand for haem b₅₉₅ 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 N₂ it can do so when carrying the *K. pneumoniae* *nif* gene cluster on a plasmid. Wild-type transconjugants fix N₂ under anaerobic conditions (Postgate et al., 1987). Moreover, O₂-dependent nitrogenase activity occurs under the similar microaerobic conditions (30 nM O₂) 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 microaerobic 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–Bertani (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 NH₄Cl (15 mM). Where indicated, plates were incubated anaerobically in a Gas Pak system (Becton Dickinson). Antibiotics for *E. coli* and for *K. pneumoniae* 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 Na₃ and 0·24 mM ZnSO₄ (Hill et al., 1990). Anaerobic glucose-limited growth was achieved after 12–16 h of bubbling with N₂ (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 OD₅₄₀ 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-³²P]dCTP and random hexamers using the Prime-a-Gen 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 employing a modified form of the Lerner, Rager and Rutter (ATandT Biochem). Oligonucleotide primers were synthesized, with the trityl group attached, using a model 391 PCR-MATE DNA.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em></td>
<td></td>
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<tr>
<td>JM109</td>
<td>*endA recA11 hsdR17 Δ[lac-proAB] [F’ recA13 proAB lacZΔM15]</td>
<td></td>
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<tr>
<td><strong>K. pneumoniae</strong></td>
<td></td>
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<tr>
<td>M5a1</td>
<td>Wild-type</td>
<td>R. Dixon</td>
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<tr>
<td>UNF122</td>
<td>*hisD2 Δlac2002 hsdR1 nif’ rec’</td>
<td>This work</td>
</tr>
<tr>
<td>UNF3504</td>
<td>*hisD2 Δlac2002 hsdR1 nif’ rec’ cydA1::Ω</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pH45</td>
<td>Ω-fragment-containing plasmid</td>
<td>Prentki &amp; Kirsch (1984)</td>
</tr>
<tr>
<td>pKAB</td>
<td>*K. pneumoniae cydAB cloned on HindIII-XhoI fragment in pBluescript</td>
<td>This work</td>
</tr>
<tr>
<td>pMM46</td>
<td>Suicide vector with temperature-sensitive replication origin</td>
<td>Paul &amp; Merrick (1989)</td>
</tr>
<tr>
<td>pNG2</td>
<td>*E. coli cydAB</td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td>pNIKA1</td>
<td>1.6 kb PCR product carrying Kp cydA in HindIII site of pTZ19</td>
<td>This work</td>
</tr>
<tr>
<td>pNJ3</td>
<td>Sacl-Sall cydA::Ω fragment from pNSJ2 cloned in the HindIII site of pMM46</td>
<td>This work</td>
</tr>
<tr>
<td>pNSJ2</td>
<td>Ω fragment from pH45 in BamHI site of pTRJ1</td>
<td>This work</td>
</tr>
<tr>
<td>pTRJ1</td>
<td>PsI-XbaI cydA fragment from pNIKA1 cloned in pBluescript</td>
<td>This work</td>
</tr>
<tr>
<td>pTZ19</td>
<td>Cb’</td>
<td>Stratagene</td>
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</table>

synthetizer 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 by PC/Gene (IntelliGenetics) and compared with sequences in the GenBank, SWISS-PROT and Prosise 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 MgCl₂, 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 95 °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 M5a1. Primers (based on the *E. coli* sequence) were designed to be complementary to the 5' end of the cydA gene on one strand (GGGTACCCGGGAGCAAGGACTCATGATTTAGATATCGATC) and to the 3' end of the cydA gene on the complementary strand (GGGGATCCGGGGATCTTCTCAATCGATC). Restriction sites to facilitate cloning were included in each primer: KpnI and SmaI for the first primer and SmaI and BamHI for the second.

**Hydrogen evolution and nitrogenase assays.** Measurements of H₂ evolution and C₃H₄ 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 O₂ electrode chamber (Rank Bros) at 30 °C with 50 mM substrates. Rates of O₂ uptake were calculated assuming that the O₂ concentration in air-saturated saline phosphate buffer is 240 μM; no O₂ consumption occurred in the absence of added substrate. 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 nm 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
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 *Smal* sites incorporated in the primers, the *K. pneumoniae* PCR product was cloned into the *HincII* site of pTZ19 to yield pNIKA1. Subsequently the presumptive *K. pneumoniae* cydA gene was excised from pNIKA1 with *PstI* and *XbaI*, and cloned into pBluescript SK+ to yield pTRJ1. It subsequently proved impossible to clone cydB separately.
and so the whole cydAB region was cloned using a gene library of K. pneumoniae strain Ms5A1. Probing with the cydAB DNA of E. coli, a single HindIII-XhoI fragment of 5 kb was identified and cloned into pBluescriptSK+ to yield pKAB. Sequencing from the XhoI 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 pNIKAl 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 I1 (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 CydB from Azotobacter vinelandii (Fleischmann et al., 1995); CyoA and CyoB, which are subunits of an oxidase from Haemophilus influenzae (Kaneko et al., 1996). Alignments of all these amino acid sequences are presented in Fig. 1.

**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.
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 b566 (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 BamHI and cloned into the BamHI site in the cydA insert of pTRJ1 to yield pNSJ2. Subsequently, the *Sal*I–*Sal*I cydA::Ω fragment of pNSJ2 was blunt-ended and cloned into the *Hinc*II 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*I, *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.
Role of cytochrome bd' in N₂-fixing K. pneumoniae

**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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cyd phenotype</th>
<th>Anaerobic activities</th>
<th>Potential respiratory activities</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Nitrogenase* with:</td>
<td>H₂ evolution† with:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td>Glucose + formate</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>UNF122</td>
<td>+</td>
<td>83 ± 7 (4)</td>
<td>87 ± 6 (4)</td>
</tr>
<tr>
<td>UNF3504</td>
<td>-</td>
<td>67 ± 6 (4)</td>
<td>75 ± 10 (4)</td>
</tr>
</tbody>
</table>

* Measured as C₄H₄ reduction: nmol C₄H₄ produced min⁻¹ (mg protein)⁻¹.
† nmol H₂ evolved min⁻¹ (mg protein)⁻¹.
‡ nmol O₂ 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 cyd⁻ mutant was able to grow under anaerobic conditions, either diazotopically or with excess NH₄⁺, 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 NH₄Cl in a gas pack anaerobic jar, in liquid NFDM medium containing glucose (excess or limiting) and bubbled with N₂ (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 H₂ 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 H₂ evolution in the mutant may indicate that the bd-type oxidase has a role in protecting O₂-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 H₂-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 nitrogenase activity in the glucose-depleted cells, nitrogenase activity was measured in the presence of very low levels of O₂. Activity supported by glucose was markedly more inhibited by O₂ in the cyd strain than the wild-type (Fig. 3a). For example, at 1-24 kPa O₂ 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 O₂, a
Fig. 3. Effect of O₂ 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).

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 O₂ 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 O₂.

H₂ evolution from either glucose or formate was measured as an indication of flux through the fermentative pathways (Fig. 4). The proportion of H₂ 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, O₂ 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 O₂ of H₂ evolution in the mutant compared with the wild-type suggests that the bd-type oxidase has a role in protecting O₂-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 H₂ evolution are apparently more sensitive to O₂ than is nitrogenase, consistent with a previous observation (Hill et al., 1984). Differences in the degree of exposure to O₂ 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,
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 O₂ (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 O₂-dependent nitrogenase activity was observed in the presence of either formate or pyruvate in wild-type or mutant strains lacking cytochrome 6d' (data not shown). Thus in this situation the pyruvate can only provide electrons for nitrogenase and respiration to provide the necessary ATP. The required levels of O₂ (Hill, 1976a). Such behaviour is consistent with the aerobic respiratory chain of Escherichia coli (Thorneley & Ashby, 1989).

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 O₂.

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; 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_\text{cat} of the A. vinelandii enzyme (Jüenemann et al., 1995).

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