The *Paracoccus denitrificans* *ccmA*, *B* and *C* genes: cloning and sequencing, and analysis of the potential of their products to form a haem or apo- *c*-type cytochrome transporter

M. Dudley Page,^{1,2} David A. Pearce,^{1†} Hilary A. C. Norris¹
and Stuart J. Ferguson^{1,2}

Author for correspondence: Stuart J. Ferguson. Tel. +44 1865 275240. Fax +44 1865 275259.
e-mail: ferguson@bioch.ox.ac.uk

¹ Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

² Oxford Centre for Molecular Sciences, New Chemistry Laboratory, South Parks Road, Oxford OX1 3QT, UK

Two *c*-type cytochrome deficient mutants of *Paracoccus denitrificans*, HN49 and HN53, were isolated by Tn5 mutagenesis and screening for failure to oxidize dimethylphenylenediamine (the Nadi test). Both were completely deficient in *c*-type cytochromes. Genomic DNA flanking the site of Tn5 insertion in HN53 was cloned by marker rescue and a 3.1 kb region sequenced. Three of the genes, designated *ccmA*, *ccmB* and *ccmC*, present in this region are proposed to encode the components of a membrane transporter of the ABC (ATP-binding cassette) superfamily, which is similar to a group of transporters postulated to translocate either haem or apocytochromes *c*. The Tn5 elements in HN49 and HN53 were shown to be inserted in *ccmB* and *ccmA*, respectively. Sequence analysis suggested that both CcmB and CcmC have the potential to interact with CcmA and thus that the three gene products probably associate to form a complex with (CcmA)₂-CcmB-CcmC stoichiometry; it also indicated a lack of similarity between CcmB and CcmC and the membrane-integral components of transporters mediating uptake of haem or other iron complexes. Supplementation of growth media with haem did not stimulate *c*-type cytochrome formation in HN49 or HN53, although it elevated levels of soluble haemoproteins and membrane-bound cytochromes *b*, suggesting that exogenous haem can traverse both outer and inner membranes of *P. denitrificans*. HN49 and HN53 accumulated apocytochrome *c*₅₅₀ to much lower levels than other *c*-type cytochrome deficient mutants of *P. denitrificans* but expression and translocation of an apocytochrome *c*₅₅₀-alkaline phosphatase fusion protein and apocytochrome *cd*₁ were unaffected in HN53. The results suggest that the substrate for the putative CcmABC-transporter is probably neither haem nor *c*-type apocytochromes.

Keywords: *Paracoccus denitrificans*, *c*-type cytochrome biogenesis, apocytochrome, putative ABC-transporter, haem

INTRODUCTION

Cytochromes *c* are distinguished from cytochromes of

[†] **Present address:** Department of Biochemistry, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, USA.

Abbreviations: ABC, ATP-binding cassette; PBT, periplasmic-binding-protein-dependent transporter.

The GenBank accession number for the sequence reported in this paper is Z71971.

other classes by covalent attachment of the haem moiety to the cytochrome polypeptide via thioether links between the two haem vinyl groups and the thiol groups of the two cysteine residues in the conserved motif Cys-X-Y-Cys-His. How this important post-translational modification is achieved in either mitochondria or bacteria is currently unknown. A number of genes required for *c*-type cytochrome maturation in Gram-negative bacteria have recently been described (reviewed by Thöny-Meyer *et al.*, 1994; Crooke & Cole, 1995). Of these, the *Bradyrhizobium japonicum* *cycVW* genes

(Ramseier *et al.*, 1991) and the homologous *helAB* genes in *Rhodobacter capsulatus* (Beckman *et al.*, 1992) appear to code for an ATP-driven membrane transporter of the ABC (ATP-binding cassette) superfamily (Ames *et al.*, 1992; Higgins, 1992; Reizer *et al.*, 1992). Homologous genes (designated *ccmAB*) have subsequently been identified in *Escherichia coli* (Richterich *et al.*, 1993; Thöny-Meyer *et al.*, 1995; Grove *et al.*, 1996), *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Rhizobium etli* (Aguilar & Soberon, 1996). The *cycV/helA/ccmA* genes code for the ATP-binding subunit of the transporter, while *cycW/helB/ccmB* code for an integral membrane component, but the function, subunit composition and stoichiometry of the transporter are uncertain. The *R. capsulatus helC* and *E. coli* and *H. influenzae ccmC* genes and the homologous ORF *orf263* in *B. japonicum* also code for integral membrane proteins which may be components of the putative transporter, but the situation is unclear because whilst *helC* is essential for *c*-type cytochrome biogenesis (Beckman *et al.*, 1992), *orf263* reportedly is not (Ramseier *et al.*, 1991). No gene corresponding to *ccmC* has yet been reported in *R. etli*. The substrate of the putative CcmABC/HelABC/CycVW($\pm$ *orf263* gene product) transporter is also unknown. It has been suggested that this may be haem (Ramseier *et al.*, 1991; Beckman *et al.*, 1992); if *c*-type cytochrome maturation occurs in the periplasm, as is suggested by an increasing body of evidence (Page & Ferguson, 1989, 1990; Sambongi & Ferguson, 1994a; Thöny-Meyer *et al.*, 1996), then provision must be made for the translocation of haem from the cytoplasm to the periplasm prior to its reaction with apocytochromes *c*. The finding that the *ccmABC/helABC/cycVW-orf263* loci, unlike those of the bacterial periplasmic-binding-protein-dependent transporters, do not contain a gene coding for a periplasmic component has been taken as evidence that the putative transporter may be an exporter rather than an importer (Beckman *et al.*, 1992). Some support for the idea that haem translocation to the periplasm is required for assembly of certain cytochrome components in bacteria has come from study of the *E. coli cydDC* genes, which code for the subunits of a heterodimeric ABC-transporter required for assembly of the *bd*-type cytochrome oxidase and which, it has been suggested, may transport the *d*-type haem to the periplasmic face of the membrane prior to its association with the oxidase apoprotein (Poole *et al.*, 1993). Alternatively, the putative CcmABC/HelABC/CycVW($\pm$ *orf263* gene product) transporter might serve to translocate apocytochromes *c* (Ramseier *et al.*, 1991; Beckman *et al.*, 1992), or even both the latter plus haem (Thöny-Meyer *et al.*, 1994).

The first reports of the accumulation of apocytochromes *c* in the periplasm of a Gram-negative bacterium were for a mutant of *Paracoccus denitrificans* (Page & Ferguson, 1989, 1990). Subsequently, apocytochrome *c*₅₅₀ has been detected in a number of other mutants of this organism, including those deficient in 5-amino-laevulinate synthase (DP104; Page & Ferguson, 1994)

and *cycH* (DP108; Page & Ferguson, 1995). Apocytochromes *c* have not been readily detected in other bacteria in which *c*-type cytochrome biogenesis has been studied by the disruption of genes required for this process. It is possible that the stability of such apocytochromes varies with the locus of mutations preventing the formation of holo- *c*-type cytochromes. Any such differential stability could provide important clues as to the roles of the gene products identified as participating in *c*-type cytochrome biogenesis. Given the background of work on *c*-type cytochrome biogenesis in *P. denitrificans* (Page & Ferguson, 1989, 1990, 1994, 1995), and the vast amount of information available on *c*-type cytochromes in this organism (Steinrücke & Ludwig, 1993), it is important that other genes required for *c*-type cytochrome biosynthesis in this organism be identified and characterized alongside studies of the consequences of their inactivation. We now report the isolation of mutants of *P. denitrificans* with Tn5 insertions in *ccmA* or *ccmB* and the cloning and sequencing of the *P. denitrificans ccmABC* gene region. The availability of these additional sequences has permitted us to make certain new deductions regarding the probable organization of the putative CcmABC/HelABC/CycVW($\pm$ *orf263* gene product) transporter. Furthermore, the two mutants proved to have a novel phenotype in respect of apocytochrome *c*₅₅₀ accumulation.

METHODS

Bacterial strains, plasmids and growth conditions. The following strains and plasmids were used in this study: *P. denitrificans* PD1222 (restriction deficient; de Vries *et al.*, 1989) and DP108 (*c*-type cytochrome deficient; Page & Ferguson, 1995); *E. coli* JM83 [*ara* Δ (*lac-proAB*) *rspL* ϕ 80 *lacZ*AM15] was used for maintenance and propagation of plasmids; NM554 [*recA1 araD139* Δ (*ara-leu*)7696 Δ (*lac*)7A *galU galK hsdR strA* (Str^r) *mcrA mcrB*] for cosmid cloning; S17-1 (C600::RP-4 2-Tc::Mu-Km::Tn7 *hsdR hsdM⁺ recA*) for mobilization of pSUP202::Tn5; pUC18 (Yanisch-Perron *et al.*, 1985); pBluescriptII SK(+) and pWE16 (Stratagene); pEPD60 (Stoll *et al.*, 1996); pKN2 (which comprises the internal *Hind*III fragment of Tn5 cloned in pBR322; Sockett & Armitage, 1991); pRK2013 (Ditta *et al.*, 1980); pSUP202 and pSUP202::Tn5 (Simon *et al.*, 1983).

P. denitrificans strains were grown aerobically in the minimal medium of Burnell *et al.* (1975) containing either sodium succinate (50 mM) or choline (0.5%, w/v). Cultures were harvested in either late (OD₆₅₀ 1.2, equivalent to 0.6–0.8 mg dry weight of cells per ml) or early (OD₆₅₀ 0.6, equivalent to 0.3–0.4 mg dry weight of cells per ml) exponential phase, as indicated in the text. Media for anaerobic growth contained 100 mM KNO₃ or were periodically sparged with N₂O. The ability of exconjugants to utilize methanol was assessed using the medium of Alefounder & Ferguson (1981). *E. coli* strains were grown in LB (Luria–Bertani medium). Antibiotics were added to the following concentrations (in μ g ml⁻¹): ampicillin, 50; kanamycin and spectinomycin, 25 (for *E. coli*) or 100 (for *P. denitrificans*); streptomycin, 60; rifampicin, 100. Stock solutions of haemin were prepared as described by Karim *et al.* (1993).

Tn5 mutagenesis and screening of exconjugants. *P.*

denitrificans PD1222 was mutagenized by conjugation with *E. coli* S17-1(pSUP202::Tn5) as described by Bagdasarian *et al.* (1981). After mating for 12–22 h at 37 °C, cells were plated onto nutrient agar containing kanamycin, spectinomycin and rifampicin. When exconjugants appeared the plates were flooded with the Nadi reagent; colonies which did not exhibit a dark blue colouration after 15 s were picked and immediately inoculated into 2 ml LB for growth.

Analytical methods. Induction of apocytochrome *cd*₁ synthesis in DP108 and HN53, preparation of total soluble and periplasmic protein fractions and solubilized membranes from *P. denitrificans* strains, SDS-PAGE, nondenaturing PAGE, staining for haem, determination of protein, the Nadi test, production of antibodies to apocytochrome *c*₅₅₀, immunoblotting of proteins separated by SDS or nondenaturing PAGE and analysis of apocytochrome *c*₅₅₀ and apocytochrome *cd*₁ localization were as described previously (Page & Ferguson, 1989, 1994). Periplasmic fractions were concentrated where necessary using the Amicon Centricon system (YM3 membrane) after dialysis against 20 mM Tris/HCl pH 7.5 containing 1 mM EDTA plus 1 mM [4-(2-aminoethyl)-benzenesulfonyl fluoride].HCl and centrifugation. Mouse anti-nitrite reductase serum was a gift from Dr A. de Boer (Vrije Universiteit, Amsterdam); in this case a horse anti-(mouse IgG)-alkaline phosphatase conjugate was used as the secondary antibody in immunoblotting. Alkaline phosphatase activities were determined as described by Stoll *et al.* (1996). Spectra were recorded on Aminco DW2000 or Perkin Elmer Lambda2 spectrophotometers.

DNA manipulations, sequencing and analysis. General DNA manipulations were as described by Sambrook *et al.* (1989). The DNA region around the site of Tn5 integration in HN53 was sequenced following subcloning. DNA fragments generated by cutting with *Apa*I, *Bgl*II, *Eco*RV, *Msc*I, *Nco*I, *Taq*I and *Xho*I were cloned in pUC18 or pBluescript and sequenced using Sequenase version 2.0 and M13 universal primers. Gaps in the sequence thus obtained were bridged using custom primers. Tn5-flanking DNA fragments cloned from HN49 and HN53 were sequenced using the primer 5'-CGTTCAGGACGCTAC-3', complementary to bases 17–34 within the Tn5 inverted repeat (Auerswald *et al.*, 1981). Sequence analysis made use of the University of Wisconsin Genetics Computer Group software package releases 7.0 and 8.0 (Devereux *et al.*, 1984). Database searches were made using the program BLAST (Altschul *et al.*, 1990). Membrane-spanning α -helices were assigned for multiple sequences by the statistical method of Persson & Argos (1994). These assignments were in agreement with assignments for individual sequences made using the statistical/topology analysis of Jones *et al.* (1994). In all cases a minimum transmembrane helix length of 21 amino acids was imposed. The orientation of transmembrane α -helices was assigned for individual sequences using the algorithm of Jones *et al.* (1994); in all cases the result agreed with that obtained by simple assignment of topology using the 'inside positive' rule of von Heijne (1992). There were no discrepancies in the predicted topology of the same protein from different species.

Plasmid pEPD60 was transferred to PD1222, DP108 or HN53 in triparental conjugations using pRK2013 as helper plasmid (Ditta *et al.*, 1980). After mating, cells were plated onto minimal succinate agar containing streptomycin and rifampicin (for PD1222) or streptomycin, rifampicin and kanamycin (for DP108 and HN53). In the case of DP108 and HN53, exconjugants were rechecked for the Nadi-negative phenotype.

RESULTS

Isolation and preliminary characterization of mutants HN49 and HN53

Approximately 200 000 kanamycin-resistant exconjugants from three independent matings between *E. coli* S17-1(pSUP202::Tn5) and *P. denitrificans* PD1222 were screened for the ability to oxidize dimethylphenylenediamine (the Nadi reaction; Marrs & Gest, 1973) and for the ability to grow aerobically with methanol. Of 63 Nadi-negative exconjugants, 9 were also unable to utilize methanol. This paper describes the properties of two of these mutants, designated HN49 and HN53, selected for study on the basis of their failure to accumulate the cytochrome *c*₅₅₀ polypeptide (see below). Both HN49 and HN53 were also incapable of aerobic growth on methylamine, or of anaerobic growth with either nitrate or nitrous oxide as terminal electron acceptor. In spite of these defects in electron transport,

Fig. 1. Visible absorption spectra of total soluble extracts (a) and dodecylmaltoside-solubilized membranes (b) from *P. denitrificans* PD1222, HN49 and HN53. Strains were grown with choline as carbon source to maximize expression of polypeptides for *c*-type cytochromes. Total soluble extracts were adjusted to 15 mg protein ml⁻¹. Membrane samples contained 10 mg dodecylmaltoside-solubilized protein ml⁻¹. All samples were reduced with sodium dithionite and spectra measured against a buffer reference.

Fig. 2. *P. denitrificans* mutants HN49 and HN53 accumulate only low levels of apocytochrome c_{550} , but these levels are higher in cells grown to early, rather than late, exponential phase. Total soluble extracts were analysed by SDS-PAGE and immunoblotting with antisera raised against the chemically prepared apocytochrome. Lanes 1, 2, 3 and 4, PD1222, DP108, HN49 and HN53, respectively, all grown to late exponential phase (OD_{650} 1.1–1.2); lanes 5 and 6, HN49 and HN53, respectively, grown to early exponential phase (OD_{650} 0.6–0.7). Lanes contained approximately 20 μ g total protein.

both mutants grew aerobically on either succinate minimal media or LB at rates very similar to those of the parental strain PD1222. In these respects they closely resemble the *c*-type cytochrome deficient mutants of *P. denitrificans* described previously (Willison & John, 1979; van Verseveld *et al.*, 1981; Harms *et al.*, 1985; Page & Ferguson, 1994, 1995).

Mutants HN49 and HN53 are pleiotropically deficient in *c*-type cytochromes

Spectroscopic analysis of both total soluble and membrane fractions prepared from HN49 and HN53 grown on choline, a gratuitous inducer of *c*-type cytochromes associated with methylotrophic growth (Harms & van Spanning, 1991), indicated that both mutants were devoid of *c*-type cytochromes (Fig. 1a, b). However, membranes from both strains contained *a*- and *b*-type cytochromes at levels equivalent to those observed in PD1222 (Fig. 1b). The assembly of a residual level of membrane-bound *c*-type cytochromes in the *cycH* mutant DP108 was previously inferred from the presence of a shoulder at 550 nm to the *b*-type cytochrome 560 nm absorbance peak (Page & Ferguson, 1995); this shoulder was absent from spectra recorded from HN49 or HN53 membranes. The cytochrome c_1 and c_{552} polypeptides were absent from membranes of HN49 and HN53 as judged by SDS-PAGE and immunoblotting (data not shown). The complete absence of cytochrome c_1 from HN49 and HN53 was also indicated by the failure of the two mutants to grow anaerobically with N_2O as terminal electron acceptor; it has been demonstrated that the cytochrome bc_1 complex is required for N_2O respiration by *P. denitrificans* (Moir, 1993). SDS-PAGE and haem staining analyses showed that none of the four major soluble, or three major membrane-bound *c*-type cytochromes, synthesized by choline-grown PD1222 (Harms & van Spanning, 1991; Page & Ferguson, 1994, 1995) were detectable in total soluble or membrane fractions of choline-grown HN49 or HN53 (data not shown). Thus the genes disrupted in HN49 and HN53

are absolutely required for the biogenesis of all *c*-type cytochromes in *P. denitrificans*.

Mutants HN49 and HN53 accumulate only trace amounts of apocytochrome c_{550}

All the previously described *c*-type cytochrome deficient mutants of *P. denitrificans* (HUUG25, PD1219, DP104 and DP108) accumulated apocytochrome c_{550} , which was readily detected by SDS-PAGE or nondenaturing PAGE followed by immunoblotting of total soluble or periplasmic extracts (Page & Ferguson, 1990, 1994, 1995). In contrast, the cytochrome c_{550} polypeptide could not be detected in periplasmic fractions from mutants HN49 and HN53 and was present only in trace amounts in total soluble extracts as judged by SDS-PAGE and immunoblotting (Fig. 2). This was the case when cells were grown to late exponential phase, as for previous analyses of apocytochrome c_{550} accumulation (Page & Ferguson, 1990, 1994, 1995). When cells were harvested in early exponential phase, however, detectable (but still low) levels of the cytochrome c_{550} polypeptide were observed (Fig. 2). Harvesting of DP108 in early exponential phase also resulted in the detection of an elevated level of apocytochrome c_{550} in this mutant. On SDS-PAGE the cytochrome c_{550} polypeptide in total soluble extracts of HN49 and HN53 was indistinguishable from the holocytochrome in extracts of the wild-type strain PD1222, indicating proteolytic removal of the signal sequence and thus translocation to the periplasm; a periplasmic location for the cytochrome c_{550} polypeptide in the two mutant strains was confirmed by fractionation of cells into periplasm, cytoplasm and membranes followed by SDS-PAGE and immunoblotting (data not shown). That the immunoreactive polypeptide was apocytochrome c_{550} rather than the holocytochrome was confirmed by nondenaturing PAGE and immunoblotting after concentrating periplasmic fractions from cells grown to early exponential phase (data not shown). These results indicate that apocytochrome c_{550} is expressed and translocated to the periplasm in both mutants but that its synthesis, efficiency of translocation or stability, either alone or in combination, are reduced in HN49 and HN53 compared to other *c*-type cytochrome deficient mutants of *P. denitrificans* we have analysed (Page & Ferguson, 1990, 1994, 1995).

Cloning and analysis of transposon-flanking DNA from HN53 and HN49

Hybridization of HN53 chromosomal DNA with the internal *Hind*III fragment from Tn5 indicated the presence of a single copy of the transposon in the HN53 genome; failure to hybridize with pSUP202 indicated the absence of cointegrated vector DNA. Using the kanamycin-resistance determinant of Tn5 as a selectable marker, HN53 genomic DNA contiguous with the 5' end of the Tn5 element was cloned in pUC18 as a 4.2 kb *Sal*I fragment comprising the 5' region of Tn5 plus 1.4 kb of HN53 genomic DNA.

Fig. 3. Map of the *P. denitrificans* *ccmABC* gene region. Arrows indicate the predicted direction of transcription of the assigned ORFs. The sites of Tn5 integration in mutants HN49 and HN53 are as indicated. The structure of the *orf5* region is shown beneath the map. Restriction sites: A, *Apal*; B, *BamHI*; Bg, *BglII*; M, *MscI*; N, *NcoI*; RV, *EcoRV*; S, *SalI*; X, *XhoI*. *, Site not unique.

HN53 genomic DNA contiguous with the 3' end of the Tn5 element was recovered by cosmid cloning using the kanamycin-resistance determinant of Tn5 as a selectable marker (Viebrock & Zumft, 1987). Cosmids carrying the Tn5 element were digested with *SalI* and the two restriction fragments hybridizing with the pKN2 insert were isolated and cloned. One of these was the 4.2 kb *SalI* fragment described above; the other was a 6.7 kb *SalI* fragment comprising the 3' end of Tn5 plus 3.7 kb of HN53 genomic DNA.

When Tn5 DNA was hybridized to *SalI*-digested HN49 chromosomal DNA four hybridizing DNA fragments were observed, indicating the presence of two copies of Tn5 in the HN49 genome. Cointegrated vector DNA was absent. HN49 genomic DNA contiguous with the 5' end of one of the Tn5 elements was cloned in pUC18 as a 4.4 kb *SalI* fragment comprising the 5' region of Tn5 plus 1.6 kb of HN49 genomic DNA.

All of the cloned genomic DNA upstream of the Tn5 element in HN53 and 1673 bp of DNA downstream of it was sequenced; this DNA region contained four complete and two partial large ORFs (Fig. 3) with a codon usage typical for genes in *P. denitrificans* (Steinrücke & Ludwig, 1993).

The predicted protein products of the second, third, and fourth complete large ORFs identified exhibited extensive homology to Hel A, B, and C of *Rhodobacter capsulatus* (Beckman *et al.*, 1992), the *cycVW-orf263* gene products of *B. japonicum* (Ramseier *et al.*, 1991) and CcmA, B and C of *E. coli* (Richterich *et al.*, 1993; Thöny-Meyer *et al.*, 1995; Grove *et al.*, 1996), and they were termed CcmA, CcmB, and CcmC in accordance with the *E. coli* nomenclature. *ccmA* and *ccmB* were separated by a short ORF, *orf5* (shown in Fig. 3), while *ccmB* and *ccmC* were separated by an untranslated region of 52 bp. The predicted product of the first complete ORF (*orf117*) was similar to that of the *orf124* immediately upstream of *helA* in *R. capsulatus* (Beckman *et al.*, 1992), while the predicted product of

the 5' partial ORF was similar to the C-terminal regions of the predicted products of the *E. coli* *secF* gene (Gardel *et al.*, 1990) and a *secF*-like gene located immediately upstream of *orf124* in *R. capsulatus* (Beckman *et al.*, 1992). The predicted product of the 3' partial ORF, termed *ccmD*, exhibited similarity to the N-terminal regions of *R. capsulatus* HelD, *B. japonicum* CycX and *E. coli* CcmD (Beckman & Kranz, 1993; Ramseier *et al.*, 1991; Richterich *et al.*, 1993). The start codons of *ccmB*, *ccmC*, *ccmD*, *orf117* and *orf5* were all preceded by potential ribosome-binding sites at distances of 5–7 bp, but a strong ribosome-binding site was not observed upstream of *ccmA*, a feature which has also been noted for *helA* in *R. capsulatus* (Beckman *et al.*, 1992).

The organization of the *P. denitrificans* *ccmABC* gene region thus closely resembles that reported for the corresponding region in *Rhodobacter capsulatus* (Beckman & Kranz, 1993), consistent with the close evolutionary relationship between these two organisms. An interesting feature of the *P. denitrificans* *ccmABC* region is the small ORF, *orf5*, located between *ccmA* and *ccmB*. The start codon of *orf5* overlapped the stop codon of *ccmA*, and its stop codon overlapped the start codon of *ccmB*, suggesting that the three genes may be translationally coupled (Kozak, 1983). Thöny-Meyer *et al.* (1994) have noted that there are ORFs between the corresponding genes *cycV* and *cycW* in *B. japonicum* (*orf105*) and *helA* and *helB* in *R. capsulatus* (*orf41*); they have suggested that, since there is no homology between the predicted products of *orf105* and *orf41*, these may serve to translationally couple the two pairs of genes. In *Rhizobium etli*, *ccmA* and *ccmB* are also separated by an ORF, *orf80* (Aguilar & Soberon, 1996), which shows no similarity to either *orf105* or *orf41*. In all three organisms, as in *P. denitrificans*, the start and stop codons of the intervening ORF overlap the stop and start codons of the preceding and succeeding genes. In *E. coli* the start codon of *ccmB* overlaps directly with the stop codon of *ccmA* so that the two genes may be translationally coupled without there being an intervening ORF.

Sequencing indicated that the Tn5 element present in HN53 was located in the 3' region of *ccmA* and that one of the Tn5 elements present in HN49 was located in the 5' region of *ccmB*. Genomic DNA contiguous with the second Tn5 element in HN49 was not recovered, so that its position in the *P. denitrificans* genome is currently unknown. For this reason, HN49 was excluded from some of the analyses described below.

The predicted CcmA, CcmB and CcmC gene products; sequence analysis

The *P. denitrificans* *ccmA* gene encodes a predicted protein of 211 amino acids which is highly similar to *Rhodobacter capsulatus* HelA, *B. japonicum* CycV, and CcmA in *E. coli*, *H. influenzae* or *Rhizobium etli* and to the ATP-binding subunits or domains of many other ABC membrane transporters. *P. denitrificans* CcmA contained the conserved Walker A and B motifs that

Fig. 4. For legend see facing page.

have been identified in all nucleotide-binding proteins (Walker *et al.*, 1982), together with the invariant glycine residue located just upstream of the Walker A motif (Higgins *et al.*, 1986) (Fig. 4a).

The *P. denitrificans* *ccmB* and *ccmC* genes encode predicted integral membrane proteins of 215 and 242 amino acids, respectively, both with six membrane-spanning helices oriented such that their N- and C-

terminal regions are cytoplasmic (Fig. 4); this is typical for the membrane-integral components of ABC-transporters (Higgins, 1992), but has not been deduced previously for these components of the putative CcmABC/HelABC/CycVW ($\pm$ *orf263* gene product) transporter. CcmB and CcmC are highly homologous to the predicted protein products of *helB* and *helC* in *Rhodobacter capsulatus*, *cycW* and *orf263* in *B. japonicum*, together with *ccmB* and *ccmC* in both

Fig. 4. (a) Deduced amino acid sequence of *P. denitrificans* CcmA (pdccma). Residues forming the predicted ATP-binding site are shown in white on black. (b, c) Comparisons (b) of the deduced amino acid sequence of *P. denitrificans* CcmB (pdccmb) with those of *Rhodobacter capsulatus* HelB (rchelb), *Bradyrhizobium japonicum* CycW (bjcycw), *Rhizobium etli* CcmB (reccmb), *E. coli* CcmB (ecccmc) and the predicted product of the *Haemophilus influenzae* ORF HI1090 (hiccmc), and (c) of the deduced amino acid sequence of *P. denitrificans* CcmC (pdccmc) with those of *R. capsulatus* HelC (rchelc) and the predicted *B. japonicum* orf263 (bjo263), *H. influenzae* ORF HI1091 (hicmc) and *Pseudomonas fluorescens* cyt1 (pfcty1) gene products. Residues conserved in four or more sequences are boxed. Predicted membrane-spanning α -helices in CcmB/HelB/CycW and CcmC/HelC/the predicted orf263 and cyt1 gene products are indicated by the barrel-like symbols above the sequences. The predicted orientations of these α -helices with respect to the inner (i.e. cytoplasmic) and outer (i.e. periplasmic) faces of the cytoplasmic membrane are indicated by the letters *i* and *o* at the helix ends. Conserved residues, or residues in the ILV (i.e. small hydrophobic sidechain) conservation group, proposed to form, or to contribute to, a motif mediating the interaction of CcmB/HelB/CycW and CcmC/HelC/the predicted orf263 and cyt1 gene products with the cognate CcmA/HelA/CycV components (see text), are shown in white on black. Substitutions of T (bjcycw) or A (reccmb, bjo263, pfcty1) for the glycine residue usually invariant in this motif are shaded. Changes to *P. fluorescens* cyt1, thus correcting two frameshifts to restore obvious homology with the other gene products shown in (c), were made by deleting a G residue at position 233 and inserting one at position 391; the cyt1 start codon was also reassigned (from ATG at positions 144–146 to ATG at positions 107–109; numbering from Gaballa *et al.*, 1995) to extend the N-terminal region of Cyt1.

E. coli and *H. influenzae*. Additionally, CcmB is homologous to the predicted *Rhizobium etli* CcmB (Aguilar & Soberon, 1996) and CcmC to the predicted

product of the *Pseudomonas fluorescens* cyt1 gene (Gaballa *et al.*, 1995). CcmB and CcmC also exhibit extensive similarity to the predicted products of ORFs in

the mitochondrial and chloroplast genomes of a number of eukaryotes, for example ORF277 and ORF228 in the mitochondrial genome of *Marchantia polymorpha* (Oda *et al.*, 1992) and ORF206 and ORF250 in the chloroplast genome of *Oenothera berteriana* (Schuster, 1994; Jekabsons & Schuster, 1995). The tryptophan-rich motif identified in the *ccmC/helC/orf263* gene products (Thöny-Meyer *et al.*, 1994) is completely conserved in CcmC of *P. denitrificans*.

The integral membrane components of bacterial periplasmic-binding-protein-dependent transporters (PBTs) contain a short conserved motif, EAA-X₃-G-X₉-IXLP, proposed to mediate their interaction with the more hydrophilic subunit(s) that catalyse ATP hydrolysis (Dassa & Hofnung, 1985). A statistical analysis of 45 PBT sequences led Köster & Böhm (1992) to define a longer consensus motif, RL(E/L)-LP-(E/D)L(E/D)(E/D)AARALG(A/L)(S/N)PW(R/Q)TFF(K/R)(L/V)-LLPLA. This motif has not hitherto been identified in either the predicted *ccmB/helB/cycW* or the *ccmC/helC/orf263* gene products; however, elements of it are clearly conserved in both groups of proteins (Fig. 4). In the CcmB/HelB/CycW group of proteins the conserved motif L-X₆-GAA-X₃-GL-X₉-LVLPL is found, while the predicted *ccmC, helC, orf263* and *cyt1* gene products contain the conserved motif L-X₆-AA(R/K)A-G-X₇-(I/V)XLP(I/L) plus acidic residues in positions -7 or -9 relative to the conserved glycine residue. This usually invariant glycine residue is replaced by T in CycW and by A in the predicted *orf263, cyt1* and *Rhizobium etli ccmB* gene products, but substitution of this residue, e.g. by alanine, by directed mutagenesis is tolerated in FhuB of *E. coli* (Köster & Böhm, 1992). Furthermore, the present new analyses of transmembrane topology suggest that in both groups of proteins these conserved motifs span a cytoplasmic loop between the fourth and fifth predicted transmembrane helices, as shown for other PBT membrane-integral subunits (Dassa & Hofnung, 1985). This analysis suggests that in *P. denitrificans* both CcmB and CcmC have the potential to interact with CcmA, and that the same is true for the corresponding gene products in other bacteria. Since a typical prokaryotic ABC-transporter is formed of four membrane-associated protein domains, two membrane-integral and two ATP-hydrolysing (Higgins, 1992), the putative transporter formed by CcmA, CcmB and CcmC of *P. denitrificans* most likely has the subunit structure (CcmA)₂-CcmB-CcmC, similar to that of (for example) the histidine transporter ([HisP]₂-HisQ-HisM) of *Salmonella typhimurium* (Ames & Lecar, 1992) and the *E. coli* maltose transporter (MalF-MalG-[MalK]₂) (Dassa *et al.*, 1993). It has been suggested previously that HelA, HelB and HelC may form a complex with this structure in *R. capsulatus* (Kranz & Beckman, 1995) but this is the first time to our knowledge that any evidence for a particular subunit organisation of the putative transporter has been presented.

While the suggestion that the substrate for the putative transporter formed by the *ccmABC/helABC/cycVW-*

(*± orf263*) gene products may be haem (Ramseier *et al.*, 1991; Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994) has been widely accepted, no experimental evidence supporting this assignment has been presented. An ABC-type transporter, *hemUV*, mediating haem import in *Yersinia enterocolitica* has recently been described; the *hemV* gene product is predicted to be the ATP-hydrolysing component of this transporter, while the product of the *hemU* gene is predicted to be an integral membrane protein with six transmembrane helices (Stojiljkovic & Hantke, 1994). The substrate specificity of ABC transporters is determined by the sequence(s) of the membrane-integral component(s) (Hekstra & Tommassen, 1993; Higgins, 1992). Comparison of the sequences of the *P. denitrificans* CcmB and CcmC and homologous gene products with the predicted product of *Y. enterocolitica hemU* indicated very limited homology between either the predicted *ccmB/helB/cycW* gene products and HemU or between HemU and the predicted *ccmC/helC/orf263* gene products. HemU is, in contrast, clearly homologous to the integral membrane subunits of ABC-transporters implicated in the uptake of ferrisiderophores, ferric dicitrate and vitamin B₁₂, the corrin ring of which resembles the porphyrin ring of haem (Stojiljkovic & Hantke, 1994). These proteins exhibit extensive sequence homology over their whole lengths and certain highly conserved motifs (e.g. -RLPR-, -RNP- and -F(I/V)GL-) have been identified (Staudenmaier *et al.*, 1989). None of these motifs could be identified in the *ccmB/helB/cycW* or *ccmC/helC/orf263* gene products. The only motif shared between the CcmB/CcmC group and the iron complex transporters is that proposed to mediate interaction with the ATP-binding subunits as discussed above. A similar concentration of positive charges at the C-termini of the CcmC group and the iron-complex transporters (Staudenmaier *et al.*, 1989) may be related to membrane insertion (von Heijne, 1992). The outer-membrane haem receptor, HutA, of *Vibrio cholerae* has been reported to have a haem-binding pocket similar to that observed in the horse haemoglobin ζ chain (Henderson & Payne, 1994); conserved residues which might form a similar feature could not be identified in CcmB or CcmC. The possibility that CcmB or CcmC, or both, contain a novel motif associated with haem binding and/or translocation cannot be ruled out; indeed, it has been suggested that the tryptophan-rich regions of the *ccmC/helC/orf263* gene products may bind haem (Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994). However, analyses of the topology of this group of proteins place this motif in a periplasmic loop rather than in a membrane-spanning region (Fig. 4; Thöny-Meyer *et al.*, 1994), suggesting that while it may bind haem it is unlikely to contribute directly to its transport across the cytoplasmic membrane. Thus a role for the putative CcmABC transporter in haem transport cannot be inferred from the predicted amino acid sequences of CcmB or CcmC. We note also that there is little similarity between CcmB and CcmC and the CydC and CydD gene products suggested to mediate export of haem *d* and possibly protohaem in *E. coli* (Poole *et al.*,

Fig. 5. Visible absorption spectra of total soluble (a) and membrane (b) fractions from *P. denitrificans* mutants HN49 and HN53 grown in the presence and absence of haemin (4.5 μ M). Strains were grown with choline as carbon and energy source to maximize *c*-type cytochrome expression. Traces A and B, HN49 and HN53, respectively, grown without added haemin; traces C and D, HN49 and HN53, respectively, grown with added haemin. Total soluble extracts were adjusted to 15 mg protein ml⁻¹. Membrane samples contained 10 mg dodecyl-maltoside-solubilized protein ml⁻¹. All samples were reduced with sodium dithionite and spectra measured against a buffer reference.

1993, 1994) and that these latter gene products show only extremely limited similarity to HemU or the membrane-integral components of other iron-complex transporters.

Exogenous haem does not stimulate *c*-type cytochrome synthesis in HN49 or HN53

If the *ccmABC* genes code for an outwardly directed haem transporter, it might be possible to restore *c*-type cytochrome biosynthesis in HN49 and HN53 by the external addition of haem, provided the latter is able to enter the periplasm and interact with the *c*-type cytochrome biosynthetic apparatus. Mutants HN49 and HN53 were grown aerobically with choline (to maximize expression of *c*-type cytochrome polypeptides), in the presence and absence of added haemin (4.5 μ M), and total soluble and cytoplasmic membrane fractions were prepared and analysed by spectroscopy. No absorbance at 550 nm, which would indicate the formation of *c*-type cytochromes, was observed in either soluble or membrane fractions from M49 or M53 grown

with added haemin (Fig. 5a, b). However, total soluble fractions of HN53 and HN49 grown in the presence of haemin exhibited increased (two- to threefold) absorbance maxima at 560 and 535 nm, indicating increased levels of soluble haemoproteins compared to total soluble fractions from cells grown in the absence of haemin (Fig. 5a). Cytoplasmic membranes from HN49 and HN53 grown in the presence of haemin also exhibited increased (two- to threefold) absorbance maxima at 560 and 535 nm, indicating increased levels of *b*-type cytochromes compared to membranes from cells grown without added haem (Fig. 5b). In all cases the cells were grown in iron-sufficient media, so it is unlikely that the increased cytochrome content of the cells grown in the presence of haem is the result of an increase in the amount of iron available for cytochrome formation. The increased levels of haemoproteins observed may result from association of haem with preformed apohaemoproteins (as reported for *E. coli*; Haddock & Schairer, 1973) or from increased apohaemoprotein expression followed by association of these apohaemoproteins with free haem. Whichever is the case, these results indicate that haemin added to growth media is able to cross both outer and inner membranes of *P. denitrificans*. This conclusion is supported by the observation that membranes from both HN49 and HN53 grown in the presence of haem contained reduced levels of cytochrome *aa*₃ as judged by a reduction in the characteristic absorbance peak at 604 nm. This may be due either to inhibition by haem of the enzymes converting haem to haem *a* or to the *aa*₃ oxidase being repressed.

While elimination of a role for CcmA or CcmB in haem transport would be consistent with the lack of sequence similarity between CcmB and CcmC and the membrane-integral components of iron-complex transporters (see above), failure of complementation by exogenous haem of a mutant putatively disrupted in haem export could be due to haem ligation to apocytochromes *c* occurring only within a protein complex interacting with and occluding a hypothetical outwardly directed haem transporter channel; in this case the active site might not be accessible to exogenous haem.

Dumont *et al.* (1994) have reported that yeast mitochondrial apocytochrome *c* can bind haem non-covalently and fold to a more compact (and hence possibly protease-resistant) form. This observation, while in conflict with previous reports (Fisher *et al.*, 1973), suggests a mechanism whereby disruption of haem export to the periplasm in *P. denitrificans* could lead to an increased rate of degradation of periplasmic apocytochrome *c*₅₅₀ and thus to the reduced levels of apocytochrome *c*₅₅₀ observed in HN49 and HN53. Mutants HN49 and HN53 were therefore grown aerobically in minimal succinate media in the presence and absence of haemin (4.5 μ M) and total soluble fractions were prepared and analysed by SDS-PAGE and immunoblotting. No effect of haemin on apocytochrome *c*₅₅₀ levels was observed (data not shown).

Strain	Alkaline phosphatase specific activity (nmol <i>o</i> -NPP hydrolysed min ⁻¹ mg ⁻¹)	
	Cells grown with succinate	Cells grown with choline
PD1222(pEPD60)	3 ± 2	29 ± 3
DP108(pEPD60)	4 ± 2	34 ± 3
HN53(pEPD60)	4 ± 2	28 ± 4

Figure 1 consists of two Western blot panels, (a) and (b). Panel (a) shows three lanes labeled 1, 2, and 3. A horizontal arrow on the left points to a band at 51 kDa. Panel (b) shows two lanes labeled 1 and 2. A horizontal arrow on the right points to a band at 65 kDa.

We have previously reported that a *c*-type cytochrome deficient mutant of *P. denitrificans*, HUUG25, obtained by chemical mutagenesis and of unknown genotype, accumulates apocytochrome *cd*₁ (apocytocchrome reductase) when incubated anaerobically with nitrate after a period of aerobic growth (Page & Ferguson, 1989). In a similar experiment, HN53 and the *cycH* mutant DP108 were grown aerobically to mid-exponential phase and then incubated anaerobically with nitrate. Under these conditions both DP108 and HN53 accumulated apocytochrome *cd*₁ as judged by SDS-PAGE and immunoblotting of total soluble extracts. Comparison of the intensity of bands produced on immunoblotting suggested that the two mutants accumulated similar

amounts of apocytochrome *cd*₁ when induced under similar conditions (Fig. 6b). The molecular masses of the cytochrome *cd*₁ polypeptides accumulated by DP108 and HN53 were identical to that of the holocytochrome purified from wild-type cells, indicating that apocytochrome *cd*₁ was translocated to the periplasm in both DP108 and HN53; this was confirmed by fractionation of cells followed by SDS-PAGE and immunoblotting (data not shown). Total soluble extracts from DP108 and HN53 grown aerobically to mid-exponential phase, but then harvested without exposure either to anaerobic conditions or to nitrate, contained no material reacting with antisera raised to cytochrome *cd*₁.

DISCUSSION

The present work extends knowledge of the *ccmA*, *ccmB* and *ccmC* gene products by presenting evidence that (i) they form a complex with organization (CcmA)₂-CcmB-CcmC, and (ii) that, contrary to previous suggestions (see Introduction), they transport neither *c*-type apocytochromes nor haem.

The failures of HN49 and HN53 to accumulate apocytochrome *c*₅₅₀ were initially thought to implicate *ccmA* and *ccmB* in apocytochrome translocation. This was unexpected because the majority of bacterial *c*-type cytochromes studied to date are synthesized as precursors with conventional N-terminal signal peptides, and, as a consequence, are almost certain to be translocated in a *sec*-dependent manner rather than by a specific transporter (Kranz & Beckman, 1995). The results obtained here with an apocytochrome *c*₅₅₀-alkaline phosphatase fusion protein and with apocytochrome *cd*₁ support this latter view and strongly suggest that the putative CcmABC-transporter is not a *c*-type apocytochrome exporter. Moreover, these results would appear to rule out a general protein translocation defect in HN53, the possibility of which was raised by the proximity of a homologue of the *E. coli* *secF* gene to *ccmA*.

The putative CcmABC-transporter might translocate some component other than haem required for *c*-type cytochrome biogenesis (Kranz & Beckman, 1995); this could be any of the wide range of compounds transported by this class of membrane pump (Fath & Kolter, 1993). A possible candidate might be a low molecular mass thiol or oxidized thiol compound; the thiol-disulphide redox balance in the periplasm has been shown to influence *c*-type cytochrome biogenesis in *E. coli* (Crooke & Cole, 1995; Sambongi & Ferguson, 1994b; and unpublished data).

Why HN49 and HN53 fail to accumulate apocytochrome *c*₅₅₀ to the levels observed in other *c*-type cytochrome deficient mutants is unclear. The similar levels of expression of the *cycA-phoA* fusion in PD1222 and HN53, and the observation that its expression is increased to a similar extent in choline-grown PD1222 and HN53, suggest that the expression and regulation of *cycA* in HN53 are probably normal. Possibly the

simplest interpretation of the available data is that apocytochrome *c*₅₅₀ is translocated to the periplasm in HN49 and HN53 but is then degraded rapidly. We have previously inferred that the apocytochrome *c*₅₅₀ accumulated by *c*-type cytochrome deficient mutants of *P. denitrificans* is subject to a constant rate of proteolytic degradation (Page & Ferguson, 1994). The absence of CcmA and/or CcmB, CcmC, CcmD or the product of the *cycY/helX/tlpB* homologue presumed, by analogy with other organisms, to lie downstream of *ccmD*, evidently promotes this process to a greater extent than absence of CycH. Apocytochrome *c*₅₅₀ is thought to be susceptible to proteolysis due to its extended conformation (Page & Ferguson, 1990, 1994). In contrast, apocytochrome *cd*₁ (apocytocytide reductase) retains a compact conformation in spite of the absence of both *c* and *d*₁ haems (Page & Ferguson, 1989) and is therefore not degraded, while the N-terminal portion of apocytochrome *c*₅₅₀ present in the apocytochrome *c*₅₅₀-alkaline phosphatase fusion protein may well be stabilized by interaction with its the folded alkaline phosphatase domain.

The role of the putative CcmABC complex has so far been discussed solely in terms of a transporter, but recent developments in the ABC-transporter field and the observation that disruption of the *Saccharomyces cerevisiae* mitochondrial ABC-transporter ATM1 results in a number of defects including, but not restricted to, loss of *c*-type cytochromes (Leighton & Schatz, 1995), suggest that the other possible functions for CcmABC ought to be considered. It is possible that the putative CcmABC transporter is not an ATP-driven membrane pump but instead is a passive channel, as has been shown for the cystic fibrosis transmembrane conductance regulator (CFTR), which has the appearance of an ABC-transporter but which is a chloride ion channel (reviewed by Ames & Lecar, 1992). The putative CcmABC-transporter could also be bifunctional, regulating the activity of other membrane protein(s) independently of its own transporter/channel activity. This property is exhibited by the human multidrug resistance P-glycoprotein, which regulates a chloride ion channel, by CFTR, which regulates two chloride ion channels and a sodium ion channel, and in *Salmonella typhimurium*, where SapABCDF, an ATP-driven peptide transporter, regulates the activity of SapJ, a potassium ion channel (reviewed by Higgins, 1995). Were this to be the case for CcmABC, the function regulated would obviously be one required for *c*-type cytochrome maturation but the species carried by the transporter/channel itself might not be directly required in the process of *c*-type cytochrome assembly. ABC-transporters also have the capacity for signal transduction (Petronilli & Ames, 1991).

ACKNOWLEDGEMENTS

We gratefully thank N. Karpovich-Tate for technical assistance, A. de Boer and R. J. M. van Spanning for the gift of antiserum to nitrite reductase, B. Ludwig for antibodies to cytochromes *c*₁ and *c*₅₅₂, V. Cooper (OCMS) for oligo-

nucleotide synthesis, and B. C. Berks and N. F. W. Saunders for advice on sequence analysis. This work was supported by BBSRC grant GR/H70348 to S. J. F. H.A.C.N. thanks the SERC for a research studentship. This is a contribution from the Oxford Centre for Molecular Sciences, which is supported by the BBSRC, EPSRC and MRC.

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Received 7 August 1996; revised 12 September 1996;

accepted 16 September 1996.