Identification of the contiguous *Paracoccus denitrificans* *ccmF* and *ccmH* genes: disruption of *ccmF*, encoding a putative transporter, results in formation of an unstable apocytochrome *c* and deficiency in siderophore production

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Apocytochrome *c*₅₅₀ was detected in the periplasm of a new mutant of *Paracoccus denitrificans*, HN48, that is pleiotropically lacking *c*-type cytochromes, produces reduced levels of siderophores and carries a Tn5 insertion in the *ccmF* gene for which sequence data, along with that for the contiguous *ccmH*, are reported. A counterpart to the *ccmF* gene was found in an archaeobacterium but could not be located in the yeast genome, whereas mitochondrial haem lyases in the latter were not present in an archaeobacterial or in eubacterial genomes. A topological analysis for CcmF is presented which indicates at least eleven transmembrane helices, suggesting a role as a transporter; evidence against the substrate being haem is presented but sequence similarity with *Escherichia coli* γ -aminobutyric acid transporter was identified. Analysis by pulse-chase methodology has shown that, in this and another cytochrome-*c*-deficient mutant, the apo form of *P. denitrificans* cytochrome *c*₅₅₀ is much less stable than the holo form, directly demonstrating the presence of a periplasmic degradation system in *P. denitrificans* that removes non-functional proteins. A variety of phenotypes are observed for *P. denitrificans* mutated in different *ccm* genes, thus indicating that the stability of the *ccm* gene products does not require assembly of a complex of all the Ccm proteins.

Keywords: *Paracoccus denitrificans*, *c*-type cytochrome biogenesis, apocytochrome, periplasmic proteolysis, *ccmF*

INTRODUCTION

Cytochromes *c* play a variety of important roles in the electron transport processes of mitochondria, bacteria and thylakoids. In bacteria these cytochromes, distinguished by the presence of two thioether bonds formed between the thiol groups of two cysteine residues, located in the characteristic sequence motif

CXXCH and the two vinyl groups of protohaem IX, are often water-soluble proteins located in the periplasm. Alternatively, they are proteins with the globular haem-binding domain exposed on the external surface of the cytoplasmic membrane. The assembly pathway for these cytochromes is not understood, but in several bacterial species a number of necessary genes (Thöny-Meyer *et al.*, 1994) have been identified and a variety of evidence indicates that haem is added to the polypeptide in the periplasm of Gram-negative organisms (e.g. Thöny-Meyer *et al.*, 1994; Sambongi & Ferguson, 1994). Early studies showed that in *Paracoccus denitrificans* an apo form of a *c*-type cytochrome can be found in the periplasm of chemically derived mutants pleiotropically

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deficient in *c*-type cytochromes (Page & Ferguson, 1989, 1990). Subsequently, genes have been identified in this organism for CcmA, CcmB and CcmC (Page *et al.*, 1997a) that together comprise an ABC transporter, which has been postulated to export haem to the periplasm (Ramseier *et al.*, 1991; Beckman *et al.*, 1992), although there are reasons to suppose that haem may not be its substrate (Page *et al.*, 1997a; Throne-Holst *et al.*, 1997), CcmG, which is a periplasmic disulphide reductase (Page & Ferguson, 1997), and CycH which is not absolutely required for *c*-type cytochrome biogenesis but clearly increases the efficiency of this process manyfold (Page & Ferguson, 1995). When the genes are individually disrupted in *P. denitrificans* variations in the phenotype are observed which should provide clues as to the assembly pathway for *c*-type cytochromes. Thus, whereas disruption of *cycH* results in pleiotropic cytochrome-*c* deficiency and apocytochrome c_{550} readily found in the periplasm, disruption of *ccmG* additionally causes loss of cytochrome aa_3 oxidase and a sensitivity to growth inhibition by disulphide compounds or rich growth media (Page & Ferguson, 1997). On the other hand, disruption of expression of *ccmAB* results in unusually low levels of apocytochrome *c* in the periplasm, but retention of cytochrome aa_3 and no growth sensitivity to rich media (Page *et al.*, 1997a).

Two genes required for biogenesis of *c*-type cytochromes, first described for *Rhodobacter capsulatus* (Beckman *et al.*, 1992) and named *ccl1* and *ccl2*, have not hitherto been identified in *P. denitrificans*. We now report the sequencing of these genes, which we have named, according to the nomenclature used for *Escherichia coli*, as *ccmF* (which in *E. coli* is very closely related to *ccl1*) and *ccmH* [justified because the 5' end of *ccmH* in *E. coli* is very similar to *ccl2* whilst the 3' end of *ccmH* in *E. coli* is not needed for *c*-type cytochrome biogenesis (Grove *et al.*, 1996)], together with the consequences of disruption of *ccmF*. Disruption of the *ccmF*-homologous genes (*cycK*) has also been reported for several species of rhizobia, with the outcome that in addition to loss of all *c*-type cytochromes there is variation between either concomitant complete loss of cytochrome aa_3 oxidase (Ritz *et al.*, 1995) or variable attenuation of this cytochrome (Delgado *et al.*, 1995; Kereszt *et al.*, 1995). In none of the rhizobial species was it possible to detect apocytochromes *c*, as was also the case for *R. capsulatus*, an organism that does not synthesize an aa_3 oxidase, but in which inactivation of the *ccmF* gene causes excretion of pigments (Biel & Biel, 1990; Beckman *et al.*, 1992). In view of this background of variability in phenotype following disruption of either *ccmF* and *ccmH* in rhizobial species or different *c*-type cytochrome biogenesis genes of *P. denitrificans*, it has become important to identify the *ccmF* and *ccmH* genes in the latter organism and determine the phenotypic consequence of their disruption. A further impetus to this work comes from the recent reports that disruption of expression of the *cycK* genes in one species of *Rhizobium* results additionally in loss of siderophore synthesis (Yeoman *et al.*, 1997), whilst in *Pseudomonas*

fluorescens siderophore synthesis is compromised by inactivation of the *ccmC* gene (Gaballa *et al.*, 1996). Thus this paper describes the *ccmF* and *ccmH* genes for *P. denitrificans* along with the phenotype that results from *ccmF* disruption and analysis of the stability of holo and apo forms of cytochrome c_{550} in this organism. The availability of several CcmF sequences has permitted a firmer topological prediction for this protein than has been possible previously.

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* XL1-Blue (Stratagene; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^q ZAM15 Tn10(Tet^r)*]) was used for maintenance and propagation of plasmids; and S17-1 (C600::RP-4 2-Tc::Mu-Km::Tn7 *hsdR⁻ hsdM⁺ recA*) for mobilization of pSUP202::Tn5; pUC18 (Yanisch-Perron *et al.*, 1985); pBluescript II SK(+) and pCR-Script Amp SK(+) (Stratagene); 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 late exponential phase (OD₆₅₀ 1.2, equivalent to 0.6–0.8 mg dry weight cells per ml), as indicated in the text. Media for anaerobic growth contained 100 mM KNO₃. The ability of exconjugants to utilize methanol was assessed using the medium of Alefounder & Ferguson (1981). *E. coli* strains were grown in LB. Antibiotics were added to the following concentrations (in µg/ml): ampicillin, 50; kanamycin, 25 (for *E. coli*) or 100 (for *P. denitrificans*); spectinomycin, 100; rifampicin, 100.

Isolation of mutant HN48. Tn5 mutagenesis of PD1222 and screening of exconjugants was performed as described by Page *et al.* (1997a). Apocytochrome c_{550} accumulation in *P. denitrificans* mutants was assessed by the method of Page & Ferguson (1995).

Preparation of bacterial extracts and subcellular fractions. Total soluble, cytoplasmic and periplasmic protein fractions, together with solubilized membranes from *P. denitrificans* strains, were prepared as described previously (Page & Ferguson, 1989; Page *et al.*, 1997a).

DNA sequence determination and analysis. General DNA manipulations were as described by Sambrook *et al.* (1989). Using transposon-encoded kanamycin resistance as a selectable marker, HN48 DNA contiguous with the 5' end of the Tn5 element in HN48 was cloned in pUC18 as a 3.6 kb *SalI* fragment containing the 5' end of Tn5 plus 0.8 kb of flanking DNA. This region was subcloned as a 1.0 kb *HpaI*–*SalI* fragment containing 0.2 kb Tn5 DNA and completely sequenced using Sequenase version 2.0 and custom primers. The remainder of the *ccmFH* gene region was retrieved by PCR amplification from PD1222 genomic DNA. In a first round of amplification, primers F1 (5' AAAAGGCCTTTA-CCCCTTC 3') and R1 (5' GCCGACGGTCAGGAAGATC-CA 3'), based on the Tn5-flanking DNA sequence, and the degenerate primers F2 (5' CCGCATGAT[C/T/G/A]GT[C/G/GA][G/A]AC[C/G]GG 3' and R3 (5' TCCCTA[G/C]TC[G/A]TA[C/G/T/A]AG[C/T/G/A]AG[C/A]AG 3'), based on knowledge of the corresponding gene and protein sequences in *R. capsulatus* and *P. denitrificans* codon usage,

Fig. 1. Organization of the *ccmF* and *ccmH* genes in *P. denitrificans* showing locations of sequences corresponding to primers used in sequencing and the site of Tn5 insertion. The primers F1–4 and R1–4 are described in Methods. The Tn5 was inserted before base 1352 in *ccmF*, where base 1 refers to the A of the ATG start codon for CcmF. The *ccmF* and *ccmH* sequences are separated by the termination codon for *ccmF* plus three other bases, and a putative ribosome-binding site can be located starting 10 base pairs upstream of the ATG start for *ccmH*. This sequence also codes for the C-terminal amino acids of *ccmF*. Neither plausible putative promoter nor terminator sequences can be recognized upstream of *ccmH*.

were used (Fig. 1). Overlapping fragments for the remainder of *ccmF* of *P. denitrificans* were then amplified using primers F3 (5' GAGGTTCGACGGGTTACA 3') and R4 (5' AG[C/G/T/A][C/T]TG[C/T]GA[A/G]AT[C/T]TG[C/A] 3'), based on the sequence data obtained. The *ccmH* sequence was derived from amplification products generated using primers R2 (5' [G/C]GT[C/G/A/T]GC[G/C/A/T]AC[C/A/G/T]-AGGTT[G/C/A/T]CC[G/C/A/T]CC 3'), R4 and F4 (5'-CCCACGAGGTCTTCTCC-3'). Template DNA was prepared by lysing single colonies of cells by boiling in 50 µl water for 15 min followed by removal of cell debris by centrifugation at 15000 *g* for 1 min. A 10 µl aliquot of the supernatant supplied template DNA for one PCR reaction. PCR-amplified DNA fragments were cloned in PCR-Script Amp SK(+) and several clones of each sequenced. Sequence analysis made use of the University of Wisconsin Genetics Computer Group software package releases 7.0 and 8.0 (Devereux *et al.*, 1984). Membrane-spanning α -helices were assessed for multiple sequences by the statistical method of Persson & Argos (1994). Assessments for individual sequences were made using the statistical/topology method of Jones *et al.* (1994). In all cases a minimum transmembrane helix length of 21 amino acids was imposed. The probable transmembrane orientation of individual helices was assessed using the algorithm of Jones *et al.* (1994) and the probable topology of CcmH analysed using the 'inside positive' rule of von Heijne (1992). Database searches were made using the program BLAST (Altschul *et al.*, 1990).

Pulse-chase procedure. Pulse-chase measurement of cellular degradation of apo- and holocytochrome c_{550} used a method similar to that of Pearce & Sherman (1995). Cells were grown aerobically to mid-exponential phase in *P. denitrificans* minimal media containing 0.6% (w/v) Bacto-peptone (Difco) and 0.2% (w/v) yeast extract (Difco), harvested by centrifugation at 10000 *g* for 5 min and twice washed by suspension in sterile distilled water and recentrifugation. Cells were then resuspended to their original density at the time of harvesting in *P. denitrificans* minimal growth medium, and incubated for 20 min at 37 °C with shaking. [35 S]methionine [1300 Ci mmol $^{-1}$ (48 MBq mmol $^{-1}$); Amersham] was added to a final concentration of 125 µCi ml $^{-1}$ and the culture was incubated for 5 min at 37 °C. Samples were then processed essentially as described by Pearce & Sherman (1995) except that anti-cytochrome c_{550} (*P. denitrificans*) serum, which recognizes both apo and holo forms of the cytochrome, was used.

Analytical methods. Siderophore biosynthesis/secretion was assessed on chrome azurol sulfonate (CAS) agar plates

prepared as described by Schwyn & Neilands (1987), except that the MM9-based growth medium was replaced by the minimal medium of Burnell *et al.* (1975) containing sodium acetate (20 mM) as sole carbon and energy source. Growth of HN48 in the presence of haemin and subsequent analyses were as described by Page *et al.* (1997a). The possible intracellular accumulation of porphyrins was assessed as described by Yeoman *et al.* (1997), and possible porphyrin excretion by the method of Biel & Biel (1990). SDS-PAGE gels were stained for haem as described previously (Page *et al.*, 1997a).

RESULTS

Isolation and initial characterization of mutant HN48

A Tn5 mutant of *P. denitrificans*, designated HN48, was isolated in the same round of mutagenesis and screening which led to the isolation of mutants HN49 and HN53 (Page *et al.*, 1997a). *P. denitrificans* HN48 had a phenotype similar to the previously described mutants DP108, HN49 and HN53 (Page & Ferguson, 1995; Page *et al.*, 1997a), in that not only were *c*-type cytochromes not detectable by haem staining (Fig. 2) but also spectroscopic analysis of soluble fractions (not shown) and cytoplasmic membranes (Fig. 3) showed their absence. Cytochrome aa_3 was clearly present in the mutant as judged by the absorbance at 604 nm (Fig. 3) and, unlike a *ccmG* mutant (Page & Ferguson, 1997), *P. denitrificans* HN48 grew normally on rich media. Colonies on plates had a fish-eye character with a darker inner zone. This is different from other mutants of *P. denitrificans* that are pleiotropically deficient in *c*-type cytochromes. Apocytochrome c_{550} was readily detected by immunoblotting (Fig. 4). This material was, just as for the other mutants (Page & Ferguson, 1990, 1995), located in the periplasm and had the same molecular mass as the holoprotein; therefore, it is deduced that the signal sequence was removed. In common with mutants of *P. denitrificans* defective in the expression of the *ccmAB* genes (Page *et al.*, 1997a), inclusion of haem in the growth medium did not restore *c*-type cytochrome biogenesis to mutant HN48. Evidence that exogenous haem gains access to the haem pool of *P. denitrificans* has been presented previously (Page *et al.*, 1997a), although it cannot be completely excluded that haem destined for *c*-type cytochromes is sequestered within a multiprotein com-

Fig. 2. Haem staining of membrane and periplasmic fractions from wild-type, 1222 and HN48 *P. denitrificans* each grown on choline. Lanes: 1, purified cytochrome c_{550} ; 2, periplasm of *P. denitrificans* 1222; 3, periplasm of HN48; 4, solubilized membranes from *P. denitrificans* 1222; 5, solubilized membranes from mutant HN48. Lanes 2–5 contained approximately 100 μ g protein. The three indicated proteins have the following approximate molecular masses: cytochrome c_{550} , 14 kDa; cytochrome c_{552} , 24 kDa; cytochrome c_1 , 62 kDa.

Fig. 3. Visible absorption spectra of PD1222 (A) and HN48 (B) membrane fractions, 10 mg protein ml^{-1} of dodecylmaltoside-solubilized material, from *P. denitrificans* grown aerobically with choline as carbon source. Both samples were reduced with sodium dithionite and spectra measured against a buffer reference.

plex and thus unable to equilibrate freely with exogenous haem.

P. denitrificans synthesizes several catecholate siderophores (Tait, 1975). Wild-type cells produced a clear surrounding zone in a CAS plate assay. Mutant HN48 produced a smaller (about twofold) cleared zone,

Fig. 4. Detection of [^{35}S]methionine-labelled cytochrome c_{550} at various times following addition of a chase of unlabelled methionine to cells of *P. denitrificans*, immunoprecipitation and autoradiography of material separated by SDS-PAGE. (a) HN48; (b) DP108; (c) PD1222.

indicative of attenuated biosynthesis or secretion of one or more siderophores by this strain. Siderophore biosynthesis/secretion was unaffected in the *c*-type-cytochrome-deficient mutant DP108, which lacks a functional *cycH* gene, indicating that *c*-type cytochrome deficiency *per se* does not cause defective siderophore production in *P. denitrificans*. Excreted protoporphyrins could not be detected in supernatants from *P. denitrificans* HN48, in contrast to a mutant of *R. capsulatus* with comparable genotype (Biel & Biel, 1990), neither were porphyrins accumulated intracellularly, as described for a *cycK* mutant of *Rhizobium leguminosarum* (Yeoman *et al.*, 1997).

P. denitrificans HN48 proved to have Tn5 inserted in the *ccmF* gene, which permitted sequencing of this and the neighbouring *ccmH* gene (Fig. 1) (see Methods). CcmH showed considerable sequence similarity to its counterparts in other bacteria, especially Ccl2 in *R. capsulatus* but also the N-terminal end of the *E. coli* CcmH protein (cf. Introduction). In particular, the motif LRCXXCQ, common to all CcmH proteins identified to date, was present. *P. denitrificans* CcmH shares with *R. capsulatus* Ccl2 the CPV motif, which has been suggested, by virtue of its resemblance to motifs in *Saccharomyces cerevisiae* HAP1 and several haem lyases, to bind haem (Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994). This motif is not, however, conserved in other CcmH-like sequences, where it is replaced by CMV, CPQ or CPK (e.g. Kereszt *et al.*, 1995; Fleischmann *et al.*, 1995; Gaballa *et al.*, 1996).

The sequence of *P. denitrificans* CcmF is shown in Fig. 5(a) along with a number of related sequences, including homologues known as NrfE which occur in *Haemophilus influenzae* and *E. coli*; in the latter a role for NrfE in the biogenesis of a nitrite respiration system has been implicated (Grove *et al.*, 1996). We have also identified for the first time (Fig. 5a) a *ccmF* homologue in an archaeobacterium, *Archaeoglobus fulgidus*, the genome of which has recently been sequenced (accessible on the World Wide Web at <http://www.tigr.org>). Although

Fig. 5. For legend see page 473.

the sequence in *A. fulgidus* does not extend over the full length of the other CcmF sequences, it is notable that the characteristic tryptophan-rich domain is present (Fig. 5a). With the number of sequences shown in Fig. 5(a) it is possible to draw some firmer conclusions as to the organization of this protein than was possible in the

only other such analysis (Kereszt *et al.*, 1995), which suggested that the CcmF proteins contain 12 membrane-spanning α -helices. Our analysis of the *P. denitrificans* and other CcmF protein sequences indicated that while a topological model of the N-terminal halves (residues 1–330 approx.) of the proteins could be derived without

Fig. 5. For legend see facing page.

difficulty, this was not the case for the C-terminal halves (residues 330–650 approx.). The N-terminal regions of the CcmF proteins were predicted to contain 11 membrane-spanning α -helices. The predicted orientation of transmembrane helices for individual sequences agreed with that obtained by simple assignment of topology using the ‘inside positive’ rule of von Heijne (1992) and

there was no disparity between predictions for individual sequences. The topology shown in Fig. 5(a) is consistent with the expression of alkaline phosphatase activity from a Ccl1-alkaline phosphatase fusion constructed by Beckman *et al.* (1992); the fusion junction would lie in the predicted external loop between helices 10 and 11. The tryptophan-rich region postulated to be

(b)

ecgabp	-----	-MCOSSOPHE	LGG	GLKSRHV	TML	SIAGVIG	ASL	FVGS	MA	IAEAC	-----	44											
pfccmf	PFSRMLPQIP	ADCHDLNPLL	ODI	GL-IVHP	PM	LYM-GYVG	FS	VAF	AF	ALLG	ORLDAA	208											
rcgcc1	PFLRLLEEA-P	FNCRDMLNPLL	ODP	GL-AFHP	PFL	LYL-GYVG	LS	MAFS	F	ALIE	ORVDA	202											
bjcyck	PFLRLIANP-P	IECRDLNPNVL	ODI	GL-AVHP	PM	LYL-GYVG	FS	SFS	F	ALME	ORIDAA	202											
rlcyck	PFLRLDPA-P	AECDRLNPNVL	QDV	GL-AIHP	PFL	LYL-GYCR	LL	RCFS	F	ALLE	ORIDAA	202											
rmcyck	PFARLVPA-P	GECDRLNPNVL	QDI	GL-AIHP	PFL	LYL-GYVG	FS	VCF	S	ALIE	ORIDAA	202											
ecgabp	-----	PAVL	LAYL	FAGLLV	VM	MRLAEM	AVATPDT	GSF	STYADKAIGR	WACY	ITGLWLY	98											
pfccmf	WARWSRPWTIL	VWAF	LGIGI	TL	-----	GS	-----	GS	-----	W	WAY	YELG	GG	243									
rcgcc1	WARWVRPWTIL	AAWIF	LTIGI	AL	-----	GS	-----	GS	-----	W	WAY	YELG	GG	237									
bjcyck	WALWVRPWTIL	VAWIF	LTIGI	AM	-----	GS	-----	GS	-----	Y	WAY	YELG	GG	237									
rlcyck	WARWVRPWTIL	AAWIF	LTIGI	AM	-----	GS	-----	GS	S	-----	Y	WAY	YELG	GG	239								
rmcyck	WARWVRPWTIL	AAWIF	LTIGI	AM	-----	GS	-----	GS	-----	Y	WAY	YELG	GG	237									
ecgabp	WWFVWLVIPL	ENAN	-----	I	AAMI	LHS	-----	W	VGP	ITWL	FS	TVI	-----	TLALT	138								
pfccmf	WWFWD	-----	PV	ENAS	FMPWL	V	GTALL	HS	LA	TEKRG	VFKS	TVLL	LA	TA	AFS	300							
rcgcc1	WWFWD	-----	PV	ENAS	LMPWL	L	AAALL	HS	AI	VEKRE	ALKS	TILL	LA	TA	AFS	294							
bjcyck	WWFWD	-----	PV	ENAS	LMPWL	A	GTALL	HS	AI	MEKRN	ALKV	TILL	LA	TA	AFS	294							
rlcyck	WWFWD	-----	PV	ENAS	FMPWL	A	GTALL	HS	AI	MEKRE	ALKV	TVLL	LA	TA	AFS	296							
rmcyck	WWFWD	-----	PV	ENAS	FMPWL	A	GTALL	HS	AI	MEKRE	ALKV	TVLL	LA	TA	AFS	294							
ecgabp	GSN	DL	SV	KNY	-----	GE	FE	FWL	ALCKVIA	FL	AFIFL	CAV	AIS	GFPY	PAE	VSG	-----	185					
pfccmf	SGV	LT	SV	HAF	ASD	PER	GV	-----	ILI	FL	LFV	GC	TLF	V	RA	PV	K	SO	VGF	NLWS	351		
rcgcc1	SGV	LT	SV	HAF	ASD	PER	GV	-----	ILF	FL	AF	FT	GC	TL	Y	A	R	A	S	E	M	345	
bjcyck	SGV	LT	SV	HAF	ATD	PT	RG	-----	ILL	FL	CL	F	IG	CS	SL	F	A	G	R	A	T	S	345
rlcyck	SGV	LT	SV	HAF	ASD	PS	RG	-----	ILC	FL	LI	F	IG	CA	SL	F	A	F	R	A	P	R	347
rmcyck	SGV	LT	SV	HAF	ATD	PT	RG	-----	ILA	FL	LI	V	F	IG	CA	SL	F	A	F	R	A	S	345

Fig. 5. (a) Comparison of the deduced amino acid sequence of *P. denitrificans* CcmF (pdccmf) with those of *Rhodobacter capsulatus* Ccl1 (rcgcc1), *Rhizobium leguminosarum* CycK (rlcyck), *Rhizobium meliloti* CycK (rmcyck), *Bradyrhizobium japonicum* CycK (bjcyck), *Escherichia coli* CcmF (ecccmf), *Haemophilus influenzae* CcmF (hiccmf1), *Pseudomonas fluorescens* CcmF (also known as CytD) (pfccmf), *Chromatium vinosum* CcmF (cvccmf; partial sequence), *E. coli* NrfE (ecnrf); a second CcmF-like protein, presumed from its location to be a NrfE homologue, from *H. influenzae* (hiccmf2), and a CcmF-like protein from *Archaeoglobus fulgidus* (afccmf; partial sequence). Residues conserved in seven or more sequences are shaded. Strongly predicted membrane-spanning α -helices are indicated by thick lines above the sequences. The orientation of these 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. Further potential membrane-spanning α -helices are indicated by thin lines above the sequences. (b) Comparison of the deduced amino acid sequences of the *Escherichia coli* GABA permease (ecgabp) with those of *Pseudomonas fluorescens* CcmF (CytD) (pfccmf), *Rhodobacter capsulatus* Ccl1 (rcgcc1), *Bradyrhizobium japonicum* CycK (bjcyck), *Rhizobium leguminosarum* CycK (rlcyck) and *Rhizobium meliloti* CycK (rmcyck). The regions shown are the N-terminal half of the *E. coli* GabP and the central regions of the Ccl1/CycK gene proteins. Residues conserved in all six sequences are shown in white on black; those conserved between *E. coli* GabP and four of the five Ccl1/CycK sequences are boxed.

involved in haem binding (Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994) is predicted to be periplasmically oriented. In the C-terminal regions of the proteins, however, discrepancies between transmembrane helix assignments for individual sequences and between topologies as predicted by the method of Jones *et al.* (1994) and those predicted by the 'positive inside' rule were encountered. In particular, the potential helix 13 was predicted in only 6 out of 9 sequences considered, the region containing potential helices 12 and 13 is absent from NrfE, and the hypothetical hydrophilic region between potential helices 14 and 15 appears to contain a higher concentration of positively charged residues than would be consistent with a periplasmic orientation. Given that the C-termini of the proteins are in all cases strongly predicted to be cytoplasmically oriented, our topological analysis suggests that the C-terminal half of CcmF may contain no membrane-spanning α -helices and may instead form a cytoplasmically oriented, predominantly hydrophilic domain.

A characteristic of the CcmF sequence is the tryptophan-rich motifs. It has often been assumed that these are

concerned with the binding of haem (e.g. Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994). However, as shown in Fig. 5(b), we have identified significant sequence similarity, particularly in the unusual tryptophan-rich region, between CcmF proteins and the γ -aminobutyric acid transporters of *E. coli* (Niegemann *et al.*, 1993) and *Mycobacterium tuberculosis* (D. M. Brown & C. M. Churcher, GenBank accession number Z97831). A similar set of helices is predicted for this transporter, although in contrast to CcmF the tryptophan-rich region is predicted to face the cytoplasm.

In vivo degradation of apocytochrome c_{550}

HN48 is one of several mutants of *P. denitrificans* in which the apo form of cytochrome c_{550} has been detected in the periplasm. In cytochrome-*c*-deficient mutants of other bacterial species the apo form of a *c*-type cytochrome has largely escaped detection, whilst it is well established that the apo form of yeast mitochondrial cytochrome *c* is highly susceptible to proteolytic degradation (Pearce & Sherman, 1995). In this context it was important to determine the stability of the

apo form of cytochrome c_{550} in *P. denitrificans*. This was assessed by a pulse-chase procedure. The apocytochrome is degraded with a half-time of approximately 30 min (Fig. 4) in both HN48 and also a second cytochrome-*c*-deficient mutant, DP108, which has an inactivated *cycH* gene (Page & Ferguson, 1995). The low levels (Page *et al.*, 1997a) of apocytochrome *c* found in mutants disrupted in the *ccmAB* genes prevented them being analysed in this way. In contrast, mature periplasmic holocytochrome c_{550} was not degraded for up to 5 h (Fig. 4).

DISCUSSION

The present work shows that *P. denitrificans* contains two genes, *ccmF* and *ccmH*, of which at least the former is essential for the synthesis of *c*-type cytochromes and normal production of siderophores. Although *ccmF* and *ccmH* are separated by only six non-coding bases and there is no obvious promoter upstream of the *ccmH* sequence, suggesting organization as an operon as very recently established for *R. capsulatus* (Gabbert *et al.*, 1997), we cannot exclude the possibility that CcmH is expressed to some extent in mutant HN48 despite insertion of Tn5 within *ccmF*. However, CcmH has been shown to be required for *c*-type cytochrome biogenesis in other organisms (Gabbert *et al.*, 1997) and thus a similar function is indicated in *P. denitrificans*. The *ccmF*, and probably *ccmH*, genes are not required for the synthesis of cytochrome aa_3 oxidase and thus the phenotype of the new mutant, HN48, is clearly different from that of DP307, which is deficient in synthesis of CcmG, a protein that shares in common with CcmH a CXXC motif. Recently Goldman *et al.* (1996) have shown that the latter motif is redox-active in CcmH and indeed can oxidize the corresponding motif in CcmG *in vitro*. If this redox reaction can occur *in vivo*, it is clear from the present work that it is not directly needed for the synthesis of cytochrome aa_3 oxidase. However, reduction of CcmH by CcmG would suggest that the CXXC group of CcmH is not such a powerful reductant as that in CcmG; this can account for the sensitivity of the mutant in the latter (DP 307), but not in the former (HN48), to rich growth media. The observations that in some rhizobial species cytochrome aa_3 synthesis is prevented by inactivation of CcmF and CcmH whereas in others it is not, indicates, when taken in conjunction with the present results, that CcmF and CcmH are not required for the oxidase assembly. The instances of aa_3 oxidase deficiency alongside *c*-type cytochrome absence are thus attributed to secondary effects of the lack of the latter rather than of a primary requirement for *ccmF* and *ccmH* in aa_3 oxidase assembly.

The finding that siderophore synthesis is compromised in HN48, but not in DP108, indicates that *c*-type cytochromes are not directly needed to complete the synthesis of this class of molecule, at least in *P. denitrificans*. This difference in phenotype, the variation in the level of apocytochrome c_{550} found in different mutants of *P. denitrificans* and absence of cytochrome

aa_3 oxidase only in the CcmG mutant (Page & Ferguson, 1997), together with the unusual fish-eye appearance of the HN48 mutant, also all imply that the absence of any one *ccm* gene product needed for *c*-type cytochrome biogenesis does not mean that all the other *ccm* gene products required are absent from the cells. In such a case, which implies a multipolypeptide complex of the Ccm proteins which would be unstable in the absence of any one component, an identical phenotype for all mutants would be predicted.

The present findings, and those of others (Gaballa *et al.*, 1996), do imply that some of the gene products required for *c*-type cytochrome biogenesis also contribute to other processes. There is an apparent conflict between the present work and that of Yeoman *et al.* (1997) in that the latter group reported that both *cycH* and *cycKL* (*ccmFH*) are required for siderophore synthesis. However, unlike in *R. leguminosarum*, the *cycH* and *ccmFH* genes are separated on the chromosome of *P. denitrificans* (H. A. C. Norris & S. J. Ferguson, unpublished results) and thus it is possible that the effects attributed to *cycH* in rhizobia are actually secondary consequences of disturbing the expression of the contiguous *cycKL* genes, although it was shown by Delgado *et al.* (1995) that a mutation in *cycH* was not fully polar on the downstream genes *cycK* and *cycL*.

Recently, Thöny-Meyer *et al.* (1996) reported on the stability of a *c*-type cytochrome from *Bradyrhizobium japonicum* when expressed in *E. coli*. They found that processed periplasmic forms of both the holoprotein and variants that lacked a haem attachment site were equally short-lived, contrary to the expectation that the latter apo forms should be less stable. In contrast, the present work shows that, at least for the homologous expression system studied here, the holoprotein is stable for many hours, whilst the apoproteins produced as a consequence of mutations in the *c*-type cytochrome biogenesis apparatus are much less stable. Cytochrome-*c*-deficient mutants of *P. denitrificans* are notable compared with those from other bacteria in that they contain detectable apocytochrome c_{550} . Only two mutants, disrupted in *ccmA* and *ccmB*, have noticeably less apoprotein, suggesting that for some reason the apocytochrome is less protected from proteolytic degradation than in the other mutants. In bacteria other than *P. denitrificans* the stability of apocytochromes *c* is presumably less, thus accounting for the absence of detectable amounts of this protein in various mutants.

The degradation of the apo form of cytochrome c_{550} in two distinct mutants, HN48 and DP108, of *P. denitrificans* is a demonstration of the degradation of a protein within the bacterial periplasm. The sequence of the *degP* (*htrA*) gene in both *E. coli* and *H. influenzae* clearly indicates that it encodes a periplasmic protease and its activity has been demonstrated by the lack of degradation of alkaline phosphatase fusion proteins in the periplasm of *degP*::Tn5 mutants (Lipinska *et al.*, 1990; Danese *et al.*, 1995). Whether a related or distinct protein is responsible for this activity in *P. denitrificans*

remains to be determined but we have here the first evidence for degradation of proteins within the *P. denitrificans* periplasm.

The functions of the *ccmFH* gene products are enigmatic. Homologues of CcmF are found in archaeobacteria (as shown here for the first time), on some chloroplast genomes (e.g. *Chlamydomonas reinhardtii*; Xie & Merchant, 1996) and on the mitochondrial genome in some eukaryotes, including in a protozoon, *Reclinomonas americana* (Lang *et al.*, 1997), that is argued to be a species very close to a putative endosymbiont precursor of the eukaryotic cell. In the latter, the *ccmF* gene is contiguous with *ccmABC* homologues, suggesting a coordinated role in some aspect of mitochondrial activity. However, according to our analysis, *ccmF* and *ccmH* are absent from the yeast genome, which explains why they or closely related proteins have not been found in extensive screens of respiratory-deficient mutants of yeast. On the other hand the *CYC3* and *CYT1* genes encoding mitochondrial haem lyases in yeast and *Neurospora* are, according to our analysis, absent from the *E. coli*, *H. influenzae* and *A. fulgidus* genomes, thus emphasizing the difference between *c*-type cytochrome biogenesis in eubacteria or archaeobacteria and mitochondria. It appears that *ccmF* appeared early in evolution and has been lost from yeast.

The large number of transmembrane α -helices that are predicted for CcmF suggests that this protein might be transporting something across the membrane, as does the newly identified similarity with a γ -aminobutyric acid transporter. However, the observations in the present paper argue against it being haem. This view, taken together with a similar earlier conclusion about the CcmABC system in this organism (Page & Ferguson, 1997), raises the question as to whether haem itself is translocated across the membrane, and if not, what are the substrates for CcmABC and CcmFH? Alternatively, the predicted transmembrane domain of CcmF might be a receptor for a signalling molecule. Whatever the role of CcmF, it seems improbable that the large number of helices serves only to anchor the protein to the cytoplasmic membrane.

E. coli and *H. influenzae* have not only *ccmF* and *ccmH* genes but also *nrfE* and *nrfF* genes, which have significant sequence similarity to *ccmF* and *ccmH*, respectively (shown in Fig. 4a for *ccmF*). The *nrf* genes are part of an operon that encodes a respiratory nitrite reductase system which includes a cytochrome c_{552} encoded by *nrfA*. NrfE is required for the activity of the nitrite reductase, but not for the covalent attachment of haem into cytochrome c_{552} or other *c*-type cytochromes, whereas NrfF is not required for any of these processes (Grove *et al.*, 1996). Presumably, therefore, the NrfE protein plays a role in correctly assembling some part, possibly a *c*-type haem bound to the CXXCK motif (Berks *et al.*, 1995; Grove *et al.*, 1996), of the cytochrome c_{552} protein. This suggests a protein recognition specificity difference between CcmF and NrfE, with the latter recognizing a specific feature in NrfA, the structural gene for nitrite reductase. Yet the similarity of the

proteins is very great (Fig. 5a). Assuming that the activity of this protein is at the periplasmic surface, there is a small number of significant changes present in the predicted periplasmic loop regions of both NrfE proteins (e.g. histidine instead of asparagine in loop 1, asparagine instead of proline in loop 5 and methionine instead of leucine in loop 6), that could be suggested to be responsible for the proposed specificity difference. Alternatively, given the proposition that a protein with multiple transmembrane α -helices is very probably involved in a transport function, the substrate translocated could be different for NrfE compared with CcmF. In this context it is notable that some slight differences between CcmF and NrfE can be identified in the predicted transmembrane helical regions (Fig. 5a). Difference in function between NrfE and CcmF must be determined by small changes in structure.

Homologues of *ccmF* have been widely reported in the mitochondrial or chloroplast genomes of plants and algae and in one case shown to be necessary for *c*-type cytochrome biogenesis in thylakoids of *C. reinhardtii* (Xie & Merchant, 1996). Although there is no doubt that these sequences in plants are for proteins related to those in bacteria, their sequences do not align well in a topological sense with those from bacteria. When analysed alone they also are predicted to be integral membrane proteins with (probably) eight transmembrane α -helices. If we assume that these chloroplast-encoded gene products are located in the thylakoid membrane then the loop regions that are predicted to face the periplasm in bacteria have close counterparts predicted to protrude into the thylakoid lumen. Our analysis of the mitochondrially coded homologues does not lead to any clear comparable prediction.

NOTES ADDED IN PROOF

1. Further scrutiny of the released parts of the *A. fulgidus* genome shows that also present are *ccdA* and *ccmC* genes which are likely to be involved in *c*-type cytochrome biogenesis in addition to the *ccmF* that is identified in Fig. 5(a). This contrasts with *Helicobacter pylori* in which *ccdA* is accompanied only by one other identifiable gene required for cytochrome *c* biogenesis which is *ycf5* (Page *et al.*, 1997b). The latter has similarity to *ccmF* but is more closely related to a chloroplast gene known both as *ycf5* and *ccsA* (Xie & Merchant, 1996).

2. Subsequent to our analysis of the released parts of the *A. fulgidus* genome, the full sequence has been published by Klenk *et al.* (1997).

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