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 c550 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 archaeabacterium but could not be located in the yeast genome, whereas mitochondrial haem lyases in the latter were not present in an archaeobacterial or in euabacterial 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 c550 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 (Thony-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. Thony-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...
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 accumulation in the periplasm, disruption of ccmG additionally causes loss of cytochrome aa₃ 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 aa₃ 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 aa₃ 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 aa₃ 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

**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 lac14ZAM15 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lac14ZAM15]) was used for maintenance and propagation of plasmids, and S17-1 (G600::RP-4-2-Tc::Mut-Km::Tn7 hsdR-EsrdM RhlR RecA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lac14ZAM15]) for mobilization of pSUP202::Tn5; pUC18 (Yanisch-Perron et al., 1985); pBruescript 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 glucose (100 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 c₅₅₀ 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 NalI 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 ccmF-homologous genes (cycK) was also 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 aa₃ 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 aa₃ 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 holohaperoxidase and apocytochrome c₅₅₀ in this organism. The availability of several CcmF sequences has permitted a firmer topological prediction for this protein than has been possible previously.
were used (Fig. 1). Overlapping fragments for the remainder of ccmF of P. denitrificans were then amplified using primers F3 (5' GAGGTCGCAGGGTTACA 3') and R4 (5' AG(C/G/T/A)ATC/TGTG/GA[A/G/A]ATC/TGTG/C/A 3'), based on the sequence data obtained. The ccmH sequence was derived from amplification products generated using primers R2 (5' GC/GTGTC/GC/A/TGC/G/C/A/TAC/C/G/A/T-AGGTG/C/A/TCC/G/C/A/TCC 3'), R4 and F4 (5' CCCCAGCAGGTCTTCTCC-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 was used 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 x-helices were assessed for multiple sequences by the statistical method of Persson et al. (1994) and 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 c550 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. [35S]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 c550 (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 aa₃ was clearly present in the mutant as judged by the absorbance at 604 nm (Fig. 3) and, unlike a ccmF 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 c550 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 completely excluded that haem destined for c-type cytochromes is sequestered within a multiprotein com-

![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.](image-url)
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 pg protein. The three indicated proteins have the following approximate molecular masses: cytochrome c_{550}, 14 kDa; cytochrome c_{552}, 24 kDa; cytochrome c_{552}, 62 kDa.

Fig. 3. Visible absorption spectra of PD1222 (A) and HN48 (B) membrane fractions, 10 mg protein ml^{-1} of dodecyl maltoside-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.

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.

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, 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 cych 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 Cc12 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 Cc12 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., 1993; 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 archaebacterium, Archaeoglobus fulgidus, the genome of which has recently been sequenced (accessible on the World Wide Web at http://www.tigr.org). Although...
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. 5a 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 \( \alpha \)-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
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 CcmF–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
involved in haem binding (Beckman et al., 1992; Thony-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 a-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; Thony-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 c₅₅₀

HN48 is one of several mutants of P. denitrificans in which the apo form of cytochrome c₅₅₀ 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 $a_{a3}$ 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 $a_{a3}$ 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 $a_{a3}$ oxidase 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 $a_{a3}$ 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 $a_{a3}$ 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 $a_{a3}$ 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-ccmF-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 \textit{P. denitrificans} periplasm.

The functions of the \textit{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. \textit{Chlamydomonas reinhardtii}; Xie & Merchant, 1996) and on the mitochrondrial genome in some eukaryotes, including in a protozoon, \textit{Reclinomonas americana} (Lang \textit{et al.}, 1997), that is argued to be a species very close to a putative endosymbiotic precursor of the eukaryotic cell. In the latter, the \textit{ccmF} gene is contiguous with \textit{ccmABC} homologues, suggesting a coordinated role in some aspect of mitochondrial activity. However, according to our analysis, \textit{ccmF} and \textit{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 \textit{Neurospora} are, according to our analysis, absent from the \textit{E. coli}, \textit{H. influenzae} and \textit{A. fulgidus} genomes, thus emphasizing the difference between \textit{c}-type cytochrome biogenesis in eubacteria or archaeobacteria and mitochondria. It appears that \textit{ccmF} appeared early in evolution and has been lost from yeast.

The large number of transmembrane \(\alpha\)-helices that are predicted for CcmF suggests that this protein might be transporting something across the membrane, as does the newly identified similarity with \(\gamma\)-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.

\textit{E. coli} and \textit{H. influenzae} have not only \textit{ccmF} and \textit{ccmH} genes but also \textit{nrfE} and \textit{nrfF} genes, which have significant sequence similarity to \textit{ccmF} and \textit{ccmH}, respectively (shown in Fig. 4a for \textit{ccmF}). The \textit{nrf} genes are part of an operon that encodes a respiratory nitrite reductase system which includes a cytochrome \(c_{552}\) encoded by \textit{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 \textit{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 \textit{et al.}, 1995; Grove \textit{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 \(\alpha\)-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 \textit{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 \textit{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 \(\alpha\)-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 \textit{A. fulgidus} genome shows that also present are \textit{ccdA} and \textit{ccmC} genes which are likely to be involved in \(c\)-type cytochrome biogenesis in addition to the \textit{ccmF} that is identified in Fig. 5(a). This contrasts with \textit{Helicobacter pylori} in which \textit{ccdA} is accompanied only by one other identifiable gene required for cytochrome \(c\) biogenesis which is \textit{ycf5} (Page \textit{et al.}, 1997b). The latter has similarity to \textit{ccmF} but is more closely related to a chloroplast gene known both as \textit{ycf5} and \textit{cesA} (Xie \& Merchant, 1996).

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

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