Cytochrome c_{550} expression in *Paracoccus denitrificans* strongly depends on growth condition: identification of promoter region for *cycA* by transcription start analysis

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The periplasmic cytochrome c_{550} content of *Paracoccus denitrificans* has been shown by immunological detection to be strongly dependent on the mode of growth. Cells grown under anaerobic, denitrifying conditions or methylotrophically in the presence of oxygen contained substantially more cytochrome c_{550} than cells grown aerobically on multicarbon substrates. A similar pattern was observed when expression of the *cycA* gene (encoding cytochrome c_{550}), was monitored using an *Escherichia coli* alkaline phosphatase gene (*phoA*) fusion as a reporter of *cycA* promoter activity. The increase in *cycA* expression observed during growth on C_1 substrates was substantially diminished if succinate was also present. These results reveal that expression of *cycA* is subject to multiple regulatory controls and suggest that cytochrome c_{550} has a general role in electron transfer to periplasmic reductases required for anaerobic denitrifying growth and from dehydrogenases required for aerobic growth on C_1 compounds. Two major transcriptional initiation start points for the *cycA* gene have been identified.

Keywords: *Paracoccus denitrificans*, cytochrome c_{550} , growth-condition-dependent gene expression

INTRODUCTION

The electron transport system of *Paracoccus denitrificans* contains a water-soluble and periplasmic c -type cytochrome designated cytochrome c_{550} . The structure of this protein, determined by X-ray crystallography, is very similar to that of mitochondrial cytochrome c (Timkovich & Dickerson, 1976; Benning *et al.*, 1994), thus placing cytochrome c_{550} as a member of the class I group of c -type cytochromes (Pettigrew & Moore, 1987), which, at least in mitochondria, function to shuttle electrons between the cytochrome bc_1 and cytochrome aa_3 oxidase complexes. By analogy, this is a function that has often been attributed to the cytochrome c_{550} of *P. denitrificans* (e.g. John & Whatley, 1975, 1977a, b; Page *et al.*, 1989), but several investigations on the electron transport system of this organism have rendered the role of the cytochrome

something of an enigma. For instance, a variety of studies have indicated that it is not a particularly efficient donor of electrons to cytochrome aa_3 and that electrons may be normally transferred to the latter from the cytochrome bc_1 complex by a membrane-bound cytochrome c_{552} (Smith & Davies, 1991; Steinrücke *et al.*, 1991; Turba *et al.*, 1995). However, cytochrome c_{550} is an effective donor of electrons *in vitro* to the enzymes of the anaerobic denitrifying pathway such as periplasmic nitrite and nitrous oxide reductases (Newton, 1969; Boogerd *et al.*, 1980; Matchova & Kucera, 1991). Cytochrome c_{550} is analogous to cytochrome c_2 which is undoubtedly a donor to nitrous oxide reductase in *Rhodobacter capsulatus* (Richardson *et al.*, 1991), and to the cytochrome c_{550} of *Bradyrhizobium japonicum*, which is required for respiration associated with reduction of nitrate to dinitrogen (Bott *et al.*, 1995). Cytochrome c_{550} could also play a role in the transfer of electrons from the periplasmic dehydrogenases for methanol and methylamine to the aa_3 cytochrome oxidase (Davidson & Kumar, 1989; Ferguson, 1987) to the cbb_3 oxidase (de Gier *et al.*, 1994) or perhaps to the membrane-bound cytochrome c_{552} (Ferguson,

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Table 1. Characteristics of strains and plasmids

Strain or plasmid	Characteristics	Source or reference*
<i>E. coli</i>		
JM101	<i>supE thi-1 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^q</i> ΔM15]	a
<i>P. denitrificans</i>		
NCIMB8944	Wild-type strain	b
PD1222	Rf ^R Sp ^R , restriction-deficient	c
Plasmids		
pEG400	Sp ^R Sm ^R , broad host-range	d
pRK2013	Km ^R , carries <i>tra</i> functions	e
pKPD1	pKK223-3 derivative carrying <i>cycA</i>	f
pEPD1R	pEG400 derivative carrying <i>cycA</i>	g
pEPD60	pEG400 derivative carrying <i>cycA-phoA</i> fusion	g

* a, Stratagene; b, NCIMB; c, N. Harms, Amsterdam (de Vries *et al.*, 1989); d, B. Ludwig, Frankfurt (Gerhus *et al.*, 1990); e, Figurski & Helinski (1979); f, Sambongi & Ferguson (1994); g, this study.

1987; Smith & Davies, 1991). Cytochrome c_{550} is similar to the cytochrome c_H of *Methylobacterium extorquens* for which there is good evidence for a role in carrying electrons between cytochrome c_L , the immediate acceptor from methanol dehydrogenase, and the oxidase (Goodwin & Anthony, 1995).

It is striking that there is no obligatory role for cytochrome c_{550} in *P. denitrificans*; a strain with the cytochrome c_{550} structural gene (*cycA*) interrupted was still capable of growth, albeit slightly retarded in some instances, under either aerobic heterotrophic (succinate as carbon source), denitrifying or methylotrophic conditions (van Spanning *et al.*, 1990). These somewhat unexpected findings do not necessarily mean that cytochrome c_{550} has no role in these electron transport reactions; its absence may be compensated by the activity of another protein. A role for pseudoazurin in this context has been suggested by Moir & Ferguson (1994) and Moir *et al.* (1993, 1995) for the anaerobic electron transport pathways to nitrite, nitric oxide and nitrous oxides. A possible functional relationship between cytochrome aa_3 and cytochrome c_{550} is also indicated by the finding that the gene for the latter is adjacent to and upstream of one of the copies of the gene for subunit I of cytochrome aa_3 (Raitio *et al.*, 1990; van Spanning *et al.*, 1990) and that absence of cytochrome c_{550} as a consequence of specific mutagenesis is paralleled by severely attenuated levels of the cytochrome aa_3 oxidase (van Spanning *et al.*, 1990, 1991).

If cytochrome c_{550} does have a physiological role, even a non-obligatory one, in shuttling electrons to periplasmic reductases and/or from periplasmic dehydrogenases, it might be expected that its synthesis would be elevated above a basal level following growth under conditions in which these types of enzymes are themselves induced. Whilst the expression of cytochrome c_{550} has often been regarded as constitutive (e.g. Bosma *et al.*, 1987; Davidson & Kumar, 1989), preliminary indications for such an elevation have been reported previously (Page *et al.*, 1989; Ferguson & Page, 1990), which are themselves consistent

with earlier evidence that levels of soluble c -type cytochromes in general are higher in anaerobically grown cells (Scholes & Smith, 1968; Sapshead & Wimpenny, 1972). In this paper a detailed study, using two different approaches to assess the extent of *cycA* expression under different growth conditions, is reported, together with the identification of transcriptional start points of *cycA* by the method of primer extension.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are described in Table 1. *P. denitrificans* strains were grown aerobically either in the medium of Burnell *et al.* (1975) containing one of sodium succinate (50 mM), glucose (60 mM) or choline (0.5%, w/v) or in the medium described by Alefounder & Ferguson (1981) containing methanol (0.5%, v/v), methylamine (0.4%, w/v), methanol (0.5%, v/v) plus succinate (50 mM), or methylamine (0.4%, w/v) plus succinate (50 mM). Media for anaerobic growth either contained 100 mM KNO₃ or were sparged with N₂O prior to inoculation and sparged again at intervals until the required cell density was attained. Cells were grown at 37 °C in all conditions. *Escherichia coli* strains were grown in LB broth at 37 °C. Antibiotics were used at the following concentrations (μg ml⁻¹): ampicillin, 50; rifampicin, 50; spectinomycin, 25 (for *E. coli*) or 50 (for *P. denitrificans*); streptomycin, 60.

Construction of reporter and control plasmids. The 1.9 kb *SalI-PstI* DNA fragment carrying the *cycA-phoA* fusion previously constructed was excised from pKPD60 (Sambongi *et al.*, 1996; Table 1) and cloned in pEG400 to generate pEPD60. The 1.0 kb *SalI-PstI* DNA fragment carrying the *cycA* gene was excised from pKPD1 (Sambongi & Ferguson, 1994; Table 1) and cloned in pEG400 to generate pEPD1R. Recombinant plasmids were mobilized into *P. denitrificans* PD1222 by triparental matings (Bagdasarian *et al.*, 1981) and exconjugants selected on L-agar containing rifampicin plus streptomycin.

Analytical methods. Total soluble extracts of the cells of *P. denitrificans* NCIMB8944 in mid-exponential growth phase were prepared using the freeze-thaw-lysozyme and sonication method of Berks *et al.* (1993). The resulting soluble extracts from three independent cultures were used for ELISA measurements as

described by Johnstone & Thorpe (1987) using antibody against *P. denitrificans* cytochrome c_{550} raised as described by Page & Ferguson (1990). Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Alkaline phosphatase in whole cells was determined as described by Brickman & Beckwith (1975). For measurement of the specific activity of alkaline phosphatase activity in total soluble extracts, these were diluted to 0.9 ml with 50 mM Tris/HCl (pH 8.0), prewarmed to 37 °C, and 0.1 ml *p*-nitrophenyl phosphate solution (0.4%, w/v, in the same buffer) added. The molar extinction coefficient at 420 nm of the nitrophenolate ion in 50 mM Tris/HCl (pH 8.0) was $13.1 \text{ mM}^{-1} \text{ cm}^{-1}$.

Analysis of *cycA* transcript by primer extension. Total RNA was isolated from *P. denitrificans* PD1222 as described by Aiba *et al.* (1981). The 5' terminus of the primer 5' GATGCTGATCTTCATCGCGTTTCCTCTTGG 3' was labelled with [γ - ^{32}P]ATP and 1.2×10^4 c.p.m. of the labelled primer annealed to 25–45 μg total RNA. Primer extension with murine reverse transcriptase was performed as described by Sambrook *et al.* (1989).

RESULTS

Immunological analyses of *cycA* expression

Quantification of cytochrome c_{550} in total soluble extracts of *P. denitrificans* NCIMB8944 grown under various conditions was achieved by ELISA. A marked growth-condition-dependent variation in the specific cellular content of cytochrome c_{550} was observed (Table 2). The highest levels of cytochrome c_{550} were found in extracts prepared from cells grown aerobically with methylamine as carbon source. In contrast, the cytochrome c_{550} contents of cells grown aerobically with either of two multi-carbon sources, succinate or glucose, were of the order of 100-fold lower (Table 2). Growth with a second C_1 substrate, methanol, also resulted in a high content of cytochrome c_{550} whilst choline, which is able to mimic methylamine or methanol in causing the induction of enzymes associated with C_1 metabolism in this organism (de Vries *et al.*, 1988; Page & Ferguson, 1993), resulted in intermediate, but nevertheless substantially enhanced compared with glucose- or succinate-grown cells, levels of cytochrome

Table 2. Determination by ELISA of cytochrome c_{550} content in cells of *P. denitrificans* grown under different conditions

Growth conditions	Cytochrome c_{550} content	
	ng (mg total soluble protein) $^{-1}$	%
Methylamine + O_2	300 ± 10	100
Methanol + O_2	120 ± 12	40
Succinate + NO_3^-	44 ± 2	15
Choline + O_2	46 ± 12	15
Glucose + O_2	2 ± 0.2	1
Succinate + O_2	3 ± 0.1	1

Table 3. Determination of the expression of the *P. denitrificans cycA* gene using alkaline phosphatase as a reporter for cells grown under different conditions

Growth conditions	Alkaline phosphatase activity	
	nmol min $^{-1}$ (mg total soluble protein) $^{-1}$ *	%
Methylamine + O_2	76 ± 1	100
Methanol + O_2	34 ± 5	45
Succinate + NO_3^-	20 ± 3	26
Choline + O_2	33 ± 3	43
Glucose + O_2	1 ± 0.5	1
Succinate + O_2	3 ± 2	4

* Activities are expressed as the difference between cells grown under a given set of conditions carrying pEPD60 (i.e. the *cycA-phoA* fusion) and those carrying pEPD1R (control plasmid) grown under the same conditions. The alkaline phosphatase activities found with the control pEPD1R were smaller than the errors for the activities with pEPD60 under the various conditions.

c_{550} formation. A comparable content of the cytochrome was found in cells that had been grown anaerobically with succinate as carbon source and nitrate as electron acceptor (Table 2). These observations were qualitatively confirmed by SDS-PAGE with immunoblotting and by rocket electroimmunoassay (data not shown).

Expression of a *CycA-PhoA* fusion protein in *P. denitrificans*

In a second series of experiments, a *cycA-phoA* gene fusion was used as a reporter of *cycA* expression in *P. denitrificans*. A cloned fragment of *P. denitrificans* DNA encompassing the 5' 243 bp of *cycA*, together with 235 bp of the upstream DNA, was fused in-frame to the alkaline phosphatase gene (*phoA*) from *E. coli* and the construct cloned in the broad host range vector pEG400 (see Methods). To render expression of the *CycA-PhoA* fusion protein dependent solely on *cycA* promoter function, the plasmid (pEPD60) was constructed such that the direction of transcription from the *cycA* promoter opposed that from the pEG400 *lac* promoter. A control plasmid was constructed by cloning the entire *cycA* structural gene and its putative promoter region in pEG400 in the same orientation to generate pEPD1R. Plasmids pEPD1R and pEPD60 were introduced into *P. denitrificans* PD1222, a restriction-deficient derivative of *P. denitrificans* NCIMB8944 exhibiting increased conjugational frequency (de Vries *et al.*, 1989), by conjugation. Table 3 shows that the level of alkaline phosphatase activity in strain PD1222(pEPD60) varied with growth conditions in very similar fashion to the immunologically detected cytochrome c_{550} protein (Table 3). These results indicate that the expression of cytochrome c_{550} was

Table 4. Attenuation by succinate of expression of the *cycA-phoA* gene fusion during methylotrophic growth of *P. denitrificans*

Alkaline phosphatase activities are expressed as a percentage of the activity expressed by cells grown aerobically with methylamine. Activities determined under a given set of conditions in cells carrying pEPD60 (i.e. the *phoA* fusion) have been corrected by subtraction of the activity measured in cells carrying pEPD1R (i.e. the control plasmid) grown under the same conditions.

Growth conditions	Alkaline phosphatase activity (%)
Methylamine + O ₂	100
Methylamine + Succinate + O ₂	8
Methanol + O ₂	55
Methanol + Succinate + O ₂	3
Succinate + O ₂	2

strongly regulated by the growth conditions. Similar differences in alkaline phosphatase activities between growth conditions were determined at various stages of growth in batch cultures. The close agreement between these data and those obtained using ELISA (Table 2), where the protein is expressed from the genome, suggests that the cloned 235 bases of DNA upstream of the plasmid-borne *cycA* mediate the whole range of regulatory effects that are observed *in vivo* in *P. denitrificans*. When *P. denitrificans* was grown aerobically with succinate plus methanol, or succinate plus methylamine, the activities of alkaline phosphatase were low and very similar to those in cells grown aerobically with succinate alone (Table 4). This is a clear indication of a catabolite repression-like mechanism controlling *cycA* expression.

It is clear from Tables 2 and 3 that cytochrome *c*₅₅₀ synthesis is enhanced by anaerobic growth conditions. In a recent study of the factors influencing the synthesis of proteins of denitrification in *Thiosphaera pantotropha*, an organism that is very closely related, but not necessarily identical, to *P. denitrificans* (Goodhew *et al.*, 1996; Ludwig *et al.*, 1993; Samyn *et al.*, 1994), it was found that anaerobic growth with nitrous oxide as electron acceptor was much less effective at inducing synthesis of pseudo-azurin than anaerobic growth with nitrate (Moir *et al.*, 1995). Comparison of alkaline phosphatase activity in cells grown anaerobically with succinate plus either nitrate or nitrous oxide showed that cytochrome *c*₅₅₀ expression was halved under the latter set of conditions.

Determination of the transcriptional start points of *cycA* mRNA by primer extension analysis

Primer extension analysis indicated the presence of two major transcripts, P1 and P2, in cells grown anaerobically with succinate plus nitrate; under these conditions the

Fig. 1. Primer extension analysis of transcripts generated from promoters for the *P. denitrificans cycA* gene. The same labelled oligonucleotide as that used for primer extension was used to generate the sequencing ladders in lanes A, G, C and T. The sequence shown is that for the coding strand. Bases representing the start of transcription are indicated by * for promoter P1 and ** for promoter P2. Lanes: 1, RNA (50 µg) isolated from cells grown anaerobically with succinate plus nitrate; 2, RNA (25 µg) isolated from cells grown aerobically with methanol and oxygen.

longer transcript, P1, was more abundant than P2 (Fig. 1). Transcript P1 started at the G residue located 53 bp upstream of the *cycA* translational initiation codon (Fig. 2). Located 37 and 11 bp, respectively, upstream from the deduced P1 transcription start are the DNA sequences 5' TCCTGT 3' and 5' GATAGC 3' (Fig. 2); these are very similar to the *P. denitrificans* promoter consensus sequences proposed by Steinrücke & Ludwig (1993). Two minor transcripts, either one or two bases longer than P1, were also detected. The shorter major transcript, P2, started at the G residue located 46 bp upstream of the *cycA* translational start codon (Fig. 1). Located 36 and 9 bp, respectively, upstream from the deduced P2 transcription start are the DNA sequences 5' TCTGCC 3' and 5' GTCAAT 3' which also resemble the proposed *P.*

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1  GTCGACATGG GCCTGCCGGT CGGCATGGAT GACCTGGACC TGGCCCTGCG
51  CCGCAGCTTC GCACGGAACT TTCCGCGCT CGCCGGCTGA TTTCGCCCT
    ORE/HAP1
101 GCGACATATT CTCCTCAGC CGTCTACGA CGTTTTCCT GATTTCTGCC
    P1 -10 P2 -10 *P1 *P2
151 CGCTTGGCAC ATGATAGCC TGTCAATCGG GAAGCGGAGA GGTCGGTTGC
201 CCGGACATGA GCGATACCCA AGAGGAAACG CGATGAAGAT CAGCATCTAT
    RBS M K I S I Y
251 GCCACTCTCG CGGCCATCAC CCTGCCCTG CCGCGTCGG CCCAGGATGG
    A T L A A I T L A L P A A A Q D A

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Fig. 2. Analysis of DNA sequences upstream of the transcriptional start of *P. denitrificans* *cycA*. The N-terminal region of *CycA* is shown beneath the DNA sequence; the putative *cycA* ribosome binding site (RBS) is underlined, as is a potential ORE or HAP1-like motif further upstream. The first nucleotides of the two transcripts P1 and P2 are indicated as *P1 and *P2, and the proposed -10 and -35 regions of the P1 and P2 promoters are boxed.

denitrificans consensus promoter sequence (Steinrücke & Ludwig, 1993). This consensus sequence is based on a limited data set but experimental study of the recently described succinate dehydrogenase operon indicates a promoter with sequence 5' TGTGAT-N₁₈-GATCAC 3' (Dickins *et al.*, 1995); this is broadly similar to the proposed *P. denitrificans* promoter consensus sequence, especially in the -10 region.

Similar evidence for the *cycA* promoters was obtained when RNA isolated from cells grown aerobically with methanol was analysed, except that under these growth conditions P2 was more abundant than P1 (Fig. 1). Low levels of *cycA* mRNA present in cells during aerobic heterotrophic growth have so far precluded analysis of *cycA* transcription start sites under these conditions.

DISCUSSION

The expression of *cycA* in *P. denitrificans* has previously been regarded as essentially constitutive, since even under aerobic growth conditions with succinate a significant level of *cycA* expression was observed by both haem staining after SDS-PAGE (Bosma *et al.*, 1987) and by measurement of β -galactosidase expression from a *cycA-lacZ* transcriptional fusion (van Spanning, 1991). The latter approach suggested that *cycA* expression during aerobic heterotrophic growth was only two- to fourfold lower than during methylotrophic or anaerobic heterotrophic growth. In contrast, the two independent quantitative methods used in the current work indicate that *cycA* expression is strongly induced (or derepressed) under methylotrophic and denitrifying growth conditions. The data obtained by the two methods were in close agreement, the only variations being the relatively higher figures indicated by the *phoA* fusion technique relative to ELISA for cytochrome c_{550} content following anaerobic growth or aerobic growth with choline (Tables 2 and 3). The disparity between our results and those of van Spanning (1991) may be due to differences in experimental approach; for example, it is widely appreciated that haem staining is hard to quantify (Goodhew

et al., 1986). However, it may also indicate that synthesis of the *CycA-PhoA* fusion protein is subject to regulation at both transcriptional and post-transcriptional levels. Whichever is the case, expression of alkaline phosphatase activity from the *cycA-phoA* translational fusion utilized in this work appears accurately to reflect expression of cytochrome c_{550} in *P. denitrificans*. This is reassuring because appearance of cytochrome c_{550} in the periplasm depends upon a number of post-translational events (see for example Page & Ferguson, 1995; Sambongi *et al.*, 1996) which are not required for the alkaline phosphatase activity. Additionally, the fact that the ELISA data were obtained as a consequence of expression from the chromosomal copy of *cycA*, whereas the alkaline phosphatase data depended upon expression from a plasmid, means that the agreement between the two can be taken as good evidence that the *P. denitrificans* DNA cloned in plasmid pEPD60 contains all the regulatory regions associated with *cycA*.

The finding that the expression of cytochrome c_{550} is markedly elevated under certain sets of growth conditions implies that this protein is functionally important under these conditions. In the case of denitrification, cytochrome c_{550} is probably one of two proteins, the other being pseudoazurin, that can transfer electrons between the cytochrome bc_1 complex and the reductases for nitrite, nitrous oxide and (possibly) nitric oxide (Moir & Ferguson, 1994). On the other hand, methylotrophic growth requires the transfer of electrons from the periplasmic dehydrogenases for methanol and methylamine to the *aa₃*- and *cbb₃*-type oxidases; it is known that *c*-type cytochromes are involved in this transfer (De Gier *et al.*, 1995). A role for cytochrome c_{550} as an acceptor from a specific *c*-type cytochrome associated with methanol oxidation has been proposed by Davidson & Kumar (1989).

Methylotrophic growth by *P. denitrificans* involves complete oxidation of methanol or methylamine via formaldehyde and formic acid to CO₂, followed by fixation of CO₂ via the Calvin Cycle. This means that the requirement for ATP is high and thus it is possible that a higher proportion of the electron flow from NADH proceeds via the cytochrome bc_1 complex and cytochrome c_{550} to cytochrome oxidases than when succinate or glucose is the carbon source. This may provide an additional reason for elevation of c_{550} levels under methylotrophic growth conditions. In the case of cells growing aerobically with succinate or glucose, the *ba₃*-type quinol oxidase that bypasses *c*-type cytochromes including the bc_1 complex (Parsonage *et al.*, 1986; Richter *et al.*, 1994) may be engaged, with the consequence that there is a much lower requirement for cytochrome c_{550} to act as a putative electron acceptor from the cytochrome bc_1 complex. However, this proposal is at odds with the conclusions of De Gier *et al.* (1995) that the formaldehyde and formate produced during growth on methanol or methylamine are predominantly oxidized via the quinol oxidase.

Our results indicate that expression of *cycA* is subject to a number of controls: (1) induction (or derepression)

<i>P. denitrificans</i> <i>mxoF</i> ORE	G A C A A - G G C G
<i>P. denitrificans</i> <i>cycA</i> ORE	G A A C T T T C C G
<i>S. cerevisiae</i> <i>cyc1</i> HAP1	G A T C T T T C C G
<i>S. cerevisiae</i> <i>cyc7</i> HAP1	G A T A A T A G C G

Fig. 3. Comparison of the proposed ORE sequence in the promoter region of the *P. denitrificans* *cycA* gene with the *S. cerevisiae* *cyc1* and *cyc7* HAP1 binding sites (sequences taken from Tzagoloff *et al.*, 1990) and with a proposed ORE upstream of the *P. denitrificans* *mxoF* gene (Steinrücke & Ludwig, 1993).

during the methylotrophic growth mode; (2) catabolite repression during growth on heterotrophic substrates; (3) induction (or derepression) under conditions of anaerobic growth. In respect of (1) and (2), the pattern of cytochrome c_{550} expression in *P. denitrificans* closely resembles that of the enzyme methanol dehydrogenase (MDH) and two c -type cytochromes, c_{551i} and c_{553i} , which are induced (or derepressed) during the growth of *P. denitrificans* on methanol, methylamine or choline but repressed if a multicarbon substrate supporting rapid growth is also present (de Vries *et al.*, 1988). MDH expression in *P. denitrificans* is proposed to be mediated by the product of methanol oxidation, formaldehyde, which is also released during the metabolism of methylamine and choline (de Vries *et al.*, 1988); thus it seems likely that formaldehyde is also the signal for the increased expression of cytochrome c_{550} observed during growth of *P. denitrificans* on methanol, methylamine or choline. Expression of MDH and cytochrome c_{551i} has recently been shown to be subject to regulation by the two-component regulatory system encoded by *mxoYX* (Harms *et al.*, 1993); however, the expression of cytochrome c_{553i} is not abolished in *mxoX* mutants (Harms *et al.*, 1993), indicating that another regulatory system is responsible for the increased expression of this cytochrome during methylotrophic growth. It would clearly be interesting to examine cytochrome c_{550} expression in *mxoX* mutant strains. It is also possible that MauR, which regulates expression of the genes for methylamine oxidation (van Spanning *et al.*, 1995a) is implicated in the control of *cycA* expression.

In respect of (3), the observation that cells grown with nitrous oxide as terminal electron acceptor contain lower levels of cytochrome c_{550} than cells grown with nitrate suggests that *cycA* expression is positively regulated not only by low oxygen tension (i.e. anaerobiosis) but also by nitrate. Most anaerobic respiratory components in *E. coli* exhibit this pattern of dual regulation (Stewart, 1988). No sequence resembling an FNR or NNR (de Boer *et al.*, 1994; van Spanning *et al.*, 1995b) consensus binding motif could be discerned upstream of *cycA*, and thus another basis for the enhancement of *cycA* expression under anaerobic, denitrifying conditions must be sought. Steinrücke & Ludwig (1993) have proposed an oxygen responsive element (ORE) as an anaerobic activator in bacteria of the α -subdivision of bacteria. A putative ORE sequence can be identified at 104 bp upstream of the P1 start (Fig. 3). A single ORE site was also detected

upstream of the *Rhodobacter sphaeroides* *cycA* gene (MacGregor & Donohue, 1991). Two *cycA* transcripts were detected in the latter organism, the longer (from P2) being more abundant in cells grown under photosynthetic anaerobic conditions, the shorter (from P1) more abundant in aerobically grown cells. The putative ORE lies upstream of both P1 and P2 and it has been proposed that binding of a regulatory protein at this site could simultaneously activate transcription of *R. sphaeroides* *cycA* from P1 and repress transcription from P2 (Steinrücke & Ludwig, 1993). As these authors have noted, the postulated ORE sequence is similar to the yeast HAP1 (haem activator protein 1) binding site, implying that *cycA* gene expression might be further regulated by haem availability as expression of iso-1- and iso-2-cytochromes c in *Saccharomyces cerevisiae* is stimulated by the haem-HAP1 complex (Kim & Guarente, 1989) (Fig. 3). It should be noted, however, that reduced haem availability does not appear substantially to reduce *cycA* expression in *P. denitrificans* (Page & Ferguson, 1990, 1994).

A number of regulatory systems controlling the switch from aerobic to anaerobic photosynthetic growth have recently been identified in *R. capsulatus* and *R. sphaeroides* (CrtJ/PpsR; RegA-RegB/PrrA-PrrB; SenC; reviewed by Bauer & Bird, 1996). Given the close phylogenetic relationship between *R. capsulatus*, *R. sphaeroides* and *P. denitrificans*, these or other proteins, e.g. ChrR in *R. sphaeroides* (Schilke & Donohue, 1995), may regulate *cycA* expression in *P. denitrificans*. Indeed, two of these systems (PrrA-PrrB, ChrR) have been implicated in the expression of cytochrome c_2 in *R. sphaeroides* (Eraso & Kaplan, 1994); cytochrome c_2 , like cytochrome c_{550} , has a dual role in that it transfers electrons from the cytochrome bc_1 complex to cytochrome oxidase under aerobic conditions and to the reaction centre during anaerobic photosynthetic growth.

Concluding remarks

In summary, the work presented here, together with a range of earlier studies, implicates cytochrome c_{550} of *P. denitrificans* as an electron carrier, which can if necessary be substituted by another protein, in the oxidation of methanol or methylamine. In the former case it could receive electrons from cytochrome c_{551i} and in the latter from amicyanin. Electrons would then be passed on to the aa_3 -type cytochrome oxidase or the recently described cbb_3 -type oxidase. There is no doubt that cytochrome c_{550} can donate to cytochrome aa_3 , albeit less well than some other cytochromes (Smith & Davies, 1991), and the fact that *cycA* is upstream of *ctaDII* and that interruption of *cycA* attenuates expression of cytochrome aa_3 strongly suggest that there is a functional link between cytochromes c_{550} and aa_3 . In the case of denitrification there is evidence, consistent with the pattern of gene expression, that cytochrome c_{550} can donate to the nitrite and nitrous oxide reductases, although it can be dispensed with as a consequence of substitution by pseudoazurin. Such a role implies that cytochrome c_{550} is reduced by the cytochrome bc_1 complex but direct evidence for this is not available. It follows from the above considerations that cytochrome c_{550} should catalyse electron transfer from the cytochrome

bc_1 complex to cytochrome aa_3 , but present data indicate that under at least some growth conditions *P. denitrificans* selects cytochrome c_{552} for this role. The complex regulation of the *cycA* gene now requires further study as does the possibility that the expression of cytochrome aa_3 oxidase may have to be regulated in a coordinated fashion when growth is on methanol or methylamine.

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