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

Raphael Stoll,† M. Dudley Page, Yoshihiro Sambongi; and Stuart J. Ferguson

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 methylo trophically 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 \(C\), 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 \(C\), 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 \(c_{552}\) 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 \(bb\_3\) oxidase (de Gier et al., 1994) or perhaps to the membrane-bound cytochrome \(c_{552}\) (Ferguson,
Table 1. Characteristics of strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM101</td>
<td>supE thi-1 Δ(lac-pro.AB) [F' traD36 pro.AB lacPZAM15]</td>
<td>a</td>
</tr>
<tr>
<td>P. denitriificans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCIMB8944</td>
<td>Wild-type strain</td>
<td>b</td>
</tr>
<tr>
<td>PD1222</td>
<td>$R^+$ Sp$^+$, restriction-deficient</td>
<td>c</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEG400</td>
<td>$Sp^+$ Sm$^+$, broad host-range</td>
<td>d</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km$^+$, carries tra functions</td>
<td>e</td>
</tr>
<tr>
<td>pKPD1</td>
<td>pKK223-3 derivative carrying gcA</td>
<td>f</td>
</tr>
<tr>
<td>pEPD1R</td>
<td>pEG400 derivative carrying gcA</td>
<td>g</td>
</tr>
<tr>
<td>pEPD60</td>
<td>pEG400 derivative carrying gcA-phoA fusion</td>
<td>g</td>
</tr>
</tbody>
</table>

*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 *Methylabacterium extorquens* for which there is good evidence for a role in carrying electrons between cytochrome $c_L$, the immediate acceptor for 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 (gcA) interrupted was still capable of growth, albeit slightly retarded in some instances, under either aerobic heterotrophic (sucinate 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 gcA expression under different growth conditions, is reported, together with the identification of transcriptional start points of gcA 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 Akefouder & Ferguson (1981) containing methanol (0.5 %, v/v), methylamine (0.4 %, w/v), methanol (0.5 %, v/v) plus succinate (50 mM), or methyamine (0.4 %, w/v) plus succinate (50 mM). Media for anaerobic growth either contained 100 mM KNO$_3$ or were sparged with N$_2$O 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 gcA-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 gcA 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 c₅₅₀ 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) is 1.31 mM⁻¹ cm⁻³.

**RESULTS**

**Immunological analyses of cycA expression**

Quantification of cytochrome c₅₅₀ 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 c₅₅₀ was observed (Table 2). The highest levels of cytochrome c₅₅₀ were found in extracts prepared from cells grown aerobically with methanol as carbon source. In contrast, the cytochrome c₅₅₀ 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 C₄ substrate, methanol, also resulted in a high content of cytochrome c₅₅₀ whilst choline, which is able to mimic methanoline or methanol in causing the induction of enzymes associated with C₄ 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 c₅₅₀ 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 conjugalational 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 c₅₅₀ protein (Table 3). These results indicate that the expression of cytochrome c₅₅₀ was

**Table 2. Determination by ELISA of cytochrome c₅₅₀ content in cells of P. denitrificans grown under different conditions**

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Cytochrome c₅₅₀ content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng (mg total soluble protein) %</td>
</tr>
<tr>
<td>Methyamine + O₂</td>
<td>300 ± 10</td>
</tr>
<tr>
<td>Methanol + O₂</td>
<td>120 ± 12</td>
</tr>
<tr>
<td>Succinate + NO₂</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>Choline + O₂</td>
<td>46 ± 12</td>
</tr>
<tr>
<td>Glucose + O₂</td>
<td>2 ± 0·2</td>
</tr>
<tr>
<td>Succinate + O₂</td>
<td>3 ± 0·1</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Alkaline phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol min⁻¹ mg total soluble protein⁻¹</td>
</tr>
<tr>
<td>Methyamine + O₂</td>
<td>76 ± 1</td>
</tr>
<tr>
<td>Methanol + O₂</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>Succinate + NO₂</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Choline + O₂</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>Glucose + O₂</td>
<td>1 ± 0·5</td>
</tr>
<tr>
<td>Succinate + O₂</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

*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.*
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 pEPD6O (i.e. the pboA fusion) have been corrected by subtraction of the activity measured in cells carrying pEPD1R (i.e. the control plasmid) grown under the same conditions.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Alkaline phosphatase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylamine + O₂</td>
<td>100</td>
</tr>
<tr>
<td>Methylamine + Succinate + O₂</td>
<td>8</td>
</tr>
<tr>
<td>Methanol + O₂</td>
<td>55</td>
</tr>
<tr>
<td>Methanol + Succinate + O₂</td>
<td>3</td>
</tr>
<tr>
<td>Succinate + O₂</td>
<td>2</td>
</tr>
</tbody>
</table>

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 ε₅₅₀ 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 pseudoazurin 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 ε₅₅₀ 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 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.
denitrificans consensus promoter sequence (Steinrücke & Ludwig, 1993). This consensus sequence is based on a limited dataset but experimental study of the recently described sucinate dehydrogenase operon indicates a promoter with sequence 5'-TGTGAT-N6-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 methylothetic 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 pboA fusion technique relative to ELISA for cytochrome c550 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 c550 in P. denitrificans. This is reassuring because appearance of cytochrome c550 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 c550 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 c550 is probably one of two proteins, the other being pseudooxazurin, that can transfer electrons between the cytochrome bc1 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 aao 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 c550 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 bc1 complex and cytochrome c550 to cytochrome oxidases than when succinate or glucose is the carbon source. This may provide an additional reason for elevation of c550 levels under methylotrophic growth conditions. In the case of cells growing aerobically with succinate or glucose, the aao3-type quinol oxidase that bypasses c-type cytochromes including the bc1 complex (Parsonage et al., 1986; Richter et al., 1994) may be engaged, with the consequence that there is a much lower requirement for cytochrome c550 to act as a putative electron acceptor from the cytochrome bc1 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)
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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 \( c_{so} \) expression in \textit{P. denitrificans} closely resembles that of the enzyme methanol dehydrogenase (MDH) and two \( c \)-type cytochromes, \( c_{5s11} \) and \( c_{5351} \), which are induced (or derepressed) during the growth of \textit{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 \textit{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 \( c_{so} \) observed during growth of \textit{P. denitrificans} on methanol, methylamine or choline. Expression of MDH and cytochrome \( c_{5s31} \) has recently been shown to be subject to regulation by the two-component regulatory system encoded by \textit{mxaYX} (Harms et al., 1993); however, the expression of cytochrome \( c_{5351} \) is not abolished in \textit{mxaX} 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 \( c_{558} \) expression in \textit{mxaX} 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 \( cytA \) 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 \( cytA \) expression is positively regulated not only by low oxygen tension (i.e. anaerobiosis) but also by nitrate. Most anaerobic respiratory components in \textit{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 \( cytA \), and thus another basis for the enhancement of \( cytA \) expression under anaerobic, denitrifying conditions must be sought. Steinrucke & Ludwig (1993) have proposed an oxygen responsive element (ORE) as an anaerobic activator in bacteria of the \( \alpha \)-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 \textit{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 \textit{R. sphaeroides} \( cycA \) from \( P1 \) and repress transcription from \( P2 \) (Steinrucke & 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 \( cytA \) gene expression might be further regulated by haem availability as expression of iso-1 and iso-2-cytochromes \( e \) in \textit{Saccharomyces cerevisiae} is stimulated by the haem-HAP1 complex (Kim & Guarante, 1989) (Fig. 3). It should be noted, however, that reduced haem availability does not appear substantially to reduce \( cytA \) expression in \textit{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 \textit{R. capsulatus} and \textit{R. sphaeroides} (CrtJ/PpsR; RegA-RegB/PtrA-PrrB; SenC; reviewed by Bauer & Bird, 1996). Given the close phylogenetic relationship between \textit{R. capsulatus}, \textit{R. sphaeroides} and \textit{P. denitrificans}, these or other proteins, e.g. ChrR in \textit{R. sphaeroides} (Schilke & Donohue, 1995), may regulate \( cytA \) expression in \textit{P. denitrificans}. Indeed, two of these systems (PrrA-PrrB, ChrR) have been implicated in the expression of cytochrome \( e_{550} \) in \textit{R. sphaeroides} (Eraso & Kaplan, 1994); cytochrome \( e_{550} \), like cytochrome \( e_{550} \), 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 \( e_{550} \) of \textit{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 \( f_{551} \) and in the latter from amicyanin. Electrons would then be passed on to the \( aa_{3} \)-type cytochrome oxidase or the recently described \( chb_{1} \)-type oxidase. There is no doubt that cytochrome \( e_{550} \) can donate to cytochrome \( aa_{3} \), albeit less well than some other cytochromes (Smith & Davies, 1991), and the fact that \( cytA \) is upstream of \textit{ctuDII} and that interruption of \( cytA \) attenuates expression of cytochrome \( aa_{3} \) strongly suggest that there is a functional link between cytochromes \( e_{550} \) and \( aa_{3} \). In the case of denitrification there is evidence, consistent with the pattern of gene expression, that cytochrome \( e_{550} \) can donate to the nitrite and nitrous oxide reductases, although it can be dispensed with as a consequence of substitution by pseudooazurin. Such a role implies that cytochrome \( e_{550} \) is reduced by the cytochrome \( bc_{1} \) complex but direct evidence for this is not available. It follows from the above considerations that cytochrome \( e_{550} \) should catalyse electron transfer from the cytochrome...
be₄ complex to cytochrome aa₃, but present data indicate that under at least some growth conditions P. denitrificans selects cytochrome c₅₅₀ for this role. The complex regulation of the cyaA gene now requires further study as does the possibility that the expression of cytochrome aa₃ oxidase may have to be regulated in a coordinated fashion when growth is on methanol or methylamine.

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