Properties of a *Paracoccus denitrificans* mutant deleted in cytochrome c_{550} indicate that a copper protein can substitute for this cytochrome in electron transport to nitrite, nitric oxide and nitrous oxide

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Disruption of the gene for the periplasmic cytochrome c_{550} in *Paracoccus denitrificans* did not result in substantial alteration of rates of electron flow from physiological substrates to the reductases for nitrite, nitric oxide and nitrous oxide, thus confirming and extending earlier findings. In wild-type cells the chelating agent diethyldithiocarbamate (DDC) caused a partial inhibition of these electron transport processes. When the same concentrations of this inhibitor were added to a mutant of *P. denitrificans* in which the gene for cytochrome c_{550} was specifically disrupted an almost complete inhibition of these reactions was observed. It is known from previous work with the closely related organism *Thiosphaera pantotropha* that DDC effectively and rapidly removes copper from the periplasmic pseudoazurin that is also found in *P. denitrificans*. Therefore, it is concluded that in the absence of cytochrome c_{550} , a copper protein, probably pseudoazurin, acts as the electron carrier between the cytochrome bc_1 complex and the reductases for nitrite, nitric oxide and nitrous oxide. Cells with the gene for cytochrome c_{550} deleted continued to give a positive Nadi test.

Keywords: *Paracoccus denitrificans*, cytochrome c_{550} , pseudoazurin, electron transport, nitrite/nitric oxide/nitrous oxide

INTRODUCTION

In denitrifying bacteria nitrate can be reduced via nitrite, nitric oxide and nitrous oxide to dinitrogen gas by four distinct reductases (Zumft, 1992). The reductases for nitrite, nitric oxide and nitrous oxide are linked to the electron transport system after the cytochrome bc_1 complex, as evidenced by the sensitivity of these reactions to antimycin and myxothiazol which act on this complex (Alefounder *et al.*, 1981; Alefounder & Ferguson, 1982; Parsonage *et al.*, 1986; Carr *et al.*, 1989; Page *et al.*, 1989). In *Paracoccus denitrificans*, nitrite reduction is catalysed by a cytochrome cd_1 -type nitrite reductase in the periplasm (Lam & Nicholas, 1969; Newton, 1969; Alefounder & Ferguson, 1980); the nitric oxide reductase is a membrane-bound haem-containing enzyme (Carr *et al.*, 1989; Carr & Ferguson, 1990; Dermastia *et al.*, 1991) and the nitrous

oxide reductase is a periplasmic protein containing copper (Boogerd *et al.*, 1981; Alefounder *et al.*, 1983; Snyder & Hollocher, 1987). The pathways of electrons from the cytochrome bc_1 complex to the nitrite, nitric oxide and nitrous oxide reductases have not been elucidated.

Cytochrome c_{550} has been thought to act as an electron donor to the nitrite reductase in *P. denitrificans* because ferrocyclochrome c_{550} can be reconstituted with cd_1 and nitrite to give enzyme activity (Timkovich *et al.*, 1982; Matchova & Kucera, 1990). However, deletion of the gene for c_{550} did not lead to a build-up of nitrite when this organism was grown anaerobically (van Spanning *et al.*, 1990). Another possible electron donor is the small copper protein azurin (Martinkus *et al.*, 1980), although in a reconstituted system it had a high K_m value for interaction with cytochrome cd_1 of *P. denitrificans*. Azurin from *P. denitrificans* has since been classified as a pseudoazurin (Ambler & Tobari, 1985). Pseudoazurin from the closely related organism *Thiosphaera pantotropha* (Stouthamer,

Abbreviation: DDC, diethyldithiocarbamate.

Table 1. Plasmids and strains

Plasmid or strain	Relevant characteristics	Source or reference
pUCC5502	Ampicillin resistance, <i>cycA</i>	van Spanning <i>et al.</i> (1990)
pSUP202	Ampicillin resistance, recognition site for mobilization	Simon <i>et al.</i> (1983)
pUC4K	Ampicillin and kanamycin resistance	Pharmacia
pSUPC550	pSUP202 carrying <i>cycA</i>	This study
pSUPC550KAN	pSUPC550 carrying <i>cycA</i> interrupted with kanamycin resistance cassette	This study
<i>E. coli</i> JM83	Recipient for plasmid DNA	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> S17-1	Recipient for plasmid DNA and donor for conjugations	Simon <i>et al.</i> (1983)
<i>P. denitrificans</i> 1222	Restriction deficient strain used as recipient in conjugations	De Vries <i>et al.</i> (1989)
<i>P. denitrificans</i> C010	Kanamycin resistant; <i>cycA</i> gene is non-functional	This study

1992) is able to donate electrons to the cytochrome *cd*₁ nitrite reductase of this organism *in vitro* (Moir *et al.*, 1993). Evidence for its activity as an electron donor *in vivo* is that the copper chelator diethyldithiocarbamate (DDC), which very effectively removes copper from pseudoazurin, inhibits nitrite reduction to some extent in whole cells (Moir *et al.*, 1993).

Little is known about the pathways of electrons to nitric oxide reductase in *P. denitrificans* because it has not yet been possible to reconstitute this membrane-bound enzyme with electron donors.

Boogerd *et al.* (1980), Matchova & Kucera (1990) and Richardson *et al.* (1991) reported that addition of nitrous oxide to the periplasm of anaerobically grown *P. denitrificans* caused the oxidation of a *c*-type cytochrome. A likely candidate is cytochrome *c*₅₅₀. In apparent contradiction is the finding that the *c*₅₅₀ deletion mutant of van Spanning *et al.* (1990) denitrifies without the accumulation of nitrous oxide. An alternative electron donor in the form of pseudoazurin in *P. denitrificans* warrants consideration because pseudoazurin from *T. pantotropha* has been found to donate electrons to the nitrous oxide reductase of this organism *in vitro* (Berks *et al.*, 1993).

The continuation of electron flow via the denitrification pathway of *P. denitrificans* in the absence of cytochrome *c*₅₅₀ could mean that, contrary to the common assumption (e.g. Ferguson, 1987), this cytochrome is not involved in these electron transport pathways. Alternatively, the observations could be explained by the substitution of another protein for the absent cytochrome *c*₅₅₀. There is precedent in the case of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* where deletion of the gene for cytochrome *c*₂ does not prevent light-driven cyclic electron transport because another *c*-type cytochrome can substitute (Jones *et al.*, 1990; Jenney & Daldal, 1993; Rott *et al.*, 1993). Our observations concerning the possible role of pseudoazurin as an electron donor to nitrite and nitrous oxide reductases (Moir *et al.*, 1993; Berks *et al.*, 1993) led

us to consider whether cytochrome *c*₅₅₀ may be substituted by pseudoazurin in *P. denitrificans*. In this paper we report the generation of a cytochrome *c*₅₅₀ deletion mutant of *P. denitrificans* that has allowed us to test this hypothesis.

METHODS

Preparation of a *c*₅₅₀ deletion mutant. The cytochrome *c*₅₅₀ deletion mutant of *P. denitrificans* prepared by van Spanning *et al.* (1990) was not available; therefore, we constructed an equivalent strain by a similar method, but using different plasmid constructs. Table 1 lists the plasmids and strains used and constructed. Genetic manipulations were carried out as described by Sambrook *et al.* (1989). DNA was isolated from agarose gels using GeneClean II (BIO 101). Plasmids pUCC5502 [which contains a 1 kb *Sal*I/*Pst*I fragment of the gene for *c*₅₅₀ from *P. denitrificans* (*cycA*) within plasmid pUC19] and pSUP202 were digested with both *Sal*I and *Sph*I and separated on agarose gels. The 6.5 to 7 kb fragment of pSUP202 was isolated with GeneClean II. The digestion of pUCC5502 gave 2.7 and 1.0 kb fragments. The 1.0 kb fragment contained *cycA* and was isolated with GeneClean II. The GeneClean fragments were ligated using T4 ligase and were transformed into *Escherichia coli* strain JM83. The plasmid thus obtained was pSUPC550. pUC4K was cut with *Sal*I to give fragments of 2.6 and 1.3 kb. These fragments were the pUC4 plasmid and the kanamycin resistance gene respectively. The 1.3 kb *Sal*I fragment and a 8 kb *Xho*I fragment of pSUPC550 were isolated from gels by GeneClean II. *Xho*I and *Sal*I give compatible cohesive ends and therefore by ligation a plasmid was obtained which consisted of pSUP202 containing *cycA* which itself contained the kanamycin resistance cassette. This is plasmid pSUPC550KAN. This was transformed into *E. coli* strain S17-1. One of the resulting transformants was used for mating *cycA* containing the kanamycin resistance cassette into *P. denitrificans* 1222. *P. denitrificans* 1222 and *E. coli* S17-1 (containing pSUPC550KAN) were grown aerobically in 30 ml of LB medium into mid-exponential phase, harvested in a bench-top centrifuge and washed with LB. The pellets were each resuspended in 1 ml of LB and a 1:1 mixture of the two bacteria was incubated on a 0.22 µm nitrocellulose filter on an LB plate for 24 h at 30 °C. The mating mix contained approximately 5 × 10⁹ cells of *P. denitrificans* and was resuspended into 1 ml. A subsample of 100 µl was plated on LB agar containing 100 µg ml⁻¹ of each of spectinomycin, rifampicin and

Fig. 1

Fig. 2

Fig. 1. SDS-PAGE and subsequent haem staining of extracts from *P. denitrificans* 1222 and C010. Periplasmic extracts from *P. denitrificans* 1222 and C010 were run on a 12% (w/v, acrylamide) SDS-PAGE. The gel was stained for haem. Lane 1 contains 100 µg of periplasmic extract from *P. denitrificans* 1222. Lane 2 contains 100 µg of periplasmic extract from *P. denitrificans* C010. The numbers to the left of the gel are the molecular masses of some of the haem staining bands.

Fig. 2. Column profiles of extracts of *P. denitrificans* 1222 and *P. denitrificans* C010 run on ion exchange. Periplasmic extracts of *P. denitrificans* 1222 and C010 were each made from 1 litre of cells, anaerobically grown to the same optical density. These extracts were loaded onto a 1.7 × 15 cm DEAE-Sepharose CL-6B column and proteins were eluted with a gradient of 0–300 mM NaCl in 100 mM Tris/HCl (pH 8). The elution of cytochromes was followed by measuring the absorbance at 410 nm (A_{410}). ○, A_{410} profile of *P. denitrificans* 1222 periplasm; ●, A_{410} profile of *P. denitrificans* C010 periplasm. ---, NaCl concn. The cytochrome with a peak in fractions 22 and 23 had the spectrum of cytochrome c_{550} . This cytochrome is absent from *P. denitrificans* C010 periplasm.

kanamycin to select for *P. denitrificans* which had obtained kanamycin resistance.

Analysis of mutants. Mutants of *P. denitrificans* were grown anaerobically at 32 °C in minimal medium containing acetate and nitrate, previously described for growth of *T. pantotropha* (Robertson & Kuenen, 1983) with kanamycin at a concentration of 100 µg ml⁻¹. Wild-type *P. denitrificans* 1222 was grown in the absence of the antibiotic. Chromosomal DNA was prepared as

described by Harms *et al.* (1987). Southern blot analysis of mutant *P. denitrificans* was carried out using the *SalI/PstI* restriction cut fragment of *cycA* as a probe. The probe was labelled for 24 h using the digoxigenin labelling system from Boehringer-Mannheim. Blot development was carried out according to the manufacturer's instructions.

Periplasmic extracts of *P. denitrificans* were made as described for *T. pantotropha* (Moir *et al.*, 1993). Extracts were separated by

electrophoresis on SDS/PAGE and the gels stained for haem (Goodhew *et al.*, 1986). Periplasmic proteins were fractionated by ion exchange using a DEAE-Sephacrose CL-6B column. Extracts of wild-type and mutant *P. denitrificans* were assayed *in vitro* for nitrite reductase activity using reduced methyl viologen as an electron donor (Moir *et al.*, 1993). *Rhodobacter sphaeroides* ssp. *denitrificans* was obtained from Dr A. G. McEwan (Univ. Queensland, Brisbane, Australia); periplasmic extracts were made by the same method as above.

Assays of nitrite reduction by whole cells were carried out as previously described (Moir *et al.*, 1993). Nitrous oxide reduction and nitric oxide reduction were measured using a modified Clark-type electrode as described previously (Alefounder & Ferguson, 1982; Carr *et al.*, 1989). Nitrate reduction by whole cells was assayed by measuring nitrite accumulation after adding nitrate to cells treated with enough myxothiazol to completely block nitrite reduction. DDC solutions were freshly made just before carrying out assays to determine the inhibitory effect of this copper chelator.

Nadi tests were carried out on wild-type and mutant *P. denitrificans* streaked on minimal medium agar plates as previously described (Marrs & Gest, 1973).

RESULTS

Plating of 100 µl of the *P. denitrificans*/*E. coli* mating mixture gave approximately 5000 kanamycin resistant colonies indicating a mating frequency of 10^{-5} . Transconjugants were picked and streaked onto another LB plate containing spectinomycin, rifampicin and kanamycin. Subsamples of these streaks were grown anaerobically in 20 ml of minimal medium containing 100 µg kanamycin ml⁻¹.

Of the periplasmic extracts from 25 randomly picked

Fig. 3. Southern blot analysis of *P. denitrificans* 1222 and C010. Samples (50 µg) of chromosomal DNA prepared from *P. denitrificans* 1222 or C010 were digested with *SalI* or *HindIII* and run on a 0.7% agarose gel before transfer onto nitrocellulose and probing with the *cycA* gene. Lanes 1 and 2, *HindIII* digests of DNA from *P. denitrificans* C010 and *P. denitrificans* 1222, respectively. Lanes 3 and 4, *SalI* digests of DNA from *P. denitrificans* C010 and *P. denitrificans* 1222, respectively. To the right of the Southern blot are indicated the sizes of DNA standard markers.

Fig. 4. Effect of the inhibitor DDC on nitrite reduction in *P. denitrificans* 1222 and *P. denitrificans* C010. Anaerobically grown *P. denitrificans* was harvested, resuspended in 200 mM sucrose, 10 mM potassium acetate, 10 mM HEPES/NaOH (pH 7.3) and allowed to become anaerobic in the chamber of a Clark-type oxygen electrode, at 30 °C. Nitrite was added (and re-added repeatedly when it had been consumed) to an initial concentration of 0.25 mM and its rate of disappearance was followed. In both (a) and (b) 1 mM DDC was added after 10 min (vertical line). In (a) 1.5 mg protein of *P. denitrificans* 1222 was resuspended in 3 ml of buffer. In (b) 1.5 mg protein of *P. denitrificans* C010 was resuspended in 3 ml of buffer.

colonies, three showed the absence of a cytochrome with molecular mass 17 kDa. Fig. 1 shows the haem stain of one of these transconjugants, named *P. denitrificans* C010. To be convinced of the absence of ϵ_{550} from this transconjugant, *P. denitrificans* C010 was grown anaerobically in 1 litre of minimal medium. The periplasmic extract from this was loaded onto an anion exchange column and a salt gradient was run to elute the periplasmic proteins. A comparison of the elution profile of *P. denitrificans* strain C010 with that of wild-type *P. denitrificans* 1222 (Fig. 2) shows the absence of a peak with an absorbance at 410 nm in strain C010. The spectrum of the dithionite reduced form of this peak from the wild-type shows a cytochrome with the same spectral features as ϵ_{550} (results not shown). The other cytochrome peaks do not have a ϵ_{550} -like spectrum and so it is clear that ϵ_{550} is not produced in *P. denitrificans* C010. This has been further confirmed by failure of immunoblotting to detect cyto-

Fig. 5. Effect of the inhibitor DDC on nitric oxide reduction in *P. denitrificans* 1222 and *P. denitrificans* C010. Anaerobically grown *P. denitrificans* was harvested and resuspended in 200 mM sucrose, 10 mM potassium acetate, 10 mM HEPES (pH 7.3) and allowed to become anaerobic in the chamber of an NO electrode, at 30 °C. The rate of NO disappearance was followed after adding 200 nmol NO from an anaerobic saturated solution of the gas in water. The rate of NO reduction by 0.375 mg protein of *P. denitrificans* 1222 was followed in (a). The rate of NO reduction by 0.325 mg protein of *P. denitrificans* C010 was followed in (c). Subsequently, the cells were incubated for 40 min with 1 mM DDC before the rates of nitric oxide reduction were remeasured (b, d).

chrome c_{550} (Y. Sambongi & S. J. Ferguson, unpublished results).

P. denitrificans C010 was analysed by Southern blot hybridization to determine whether the kanamycin resistance cassette had recombined within the *cycA* gene. Chromosomal DNA was prepared from *P. denitrificans* 1222 and *P. denitrificans* C010, digested, separated in agarose gels, and blotted onto nitrocellulose. The blot was probed with the *cycA* gene (Fig. 3). The results indicate that the 1.3 kb kanamycin resistance cassette is inserted in the *cycA* gene in *P. denitrificans* C010 because only in the latter does *Hind*III digestion give two fragments containing regions of *cycA* (Fig. 3, compare lanes 1 and 2). Furthermore, comparison of lanes 3 and 4 in Fig. 3 shows an insertion of approximately 1.5 kb in the region of *cycA* in *P. denitrificans* C010.

P. denitrificans C010 grew at the same rate as *P. denitrificans* 1222 in anaerobic batch culture. After anaerobic growth into late exponential phase, the rates of reduction of nitrite, nitric oxide and nitrous oxide were measured to be similar *in vivo* in *P. denitrificans* 1222 and *P. denitrificans* C010 using acetate as an energy source. Van Spanning *et al.* (1990) did not directly measure the rates of the denitrification reactions; they simply showed that the concentrations of nitrite and nitrous oxide did not build-up during anaerobic growth of the c_{550} deletion mutant.

The copper chelating agent DDC had a dramatically different effect on nitrite reduction in *P. denitrificans* 1222 and *P. denitrificans* C010. After 40 min incubation (Fig. 4), nitrite reduction by *P. denitrificans* 1222 was maximally inhibited by 40–50%; in contrast the rate of nitrite reduction was inhibited 99% in *P. denitrificans* C010. Similarly, nitric oxide reduction was inhibited 30% by 1 mM DDC in *P. denitrificans* 1222 and by at least 90% in *P. denitrificans* C010. The records from the nitric oxide electrode are shown in Fig. 5.

Inhibition of nitrous oxide reduction by DDC was difficult to measure with the nitrous oxide electrode because incubation with DDC itself led to a decrease in the response of the instrument. Also, since the nitrous oxide

reductase itself is a copper enzyme, the concentration of DDC used to inhibit electron transport to the enzyme, but not the enzyme itself, had to be carefully chosen. Nitrous oxide reduction by *P. denitrificans* 1222 was inhibited 50% by DDC at a concentration of either 100 μ M or 1 mM after incubation with the inhibitor for 40 min. Nitrous oxide reduction in *P. denitrificans* C010 was inhibited 100% by 30 μ M DDC. The records from the nitrous oxide electrode are shown in Fig. 6.

Although the effects of DDC are clearly altered upon the deletion of cytochrome c_{550} , it was important to test whether an electron transport reaction known not to involve cytochrome c_{550} , e.g. nitrate reductase (Parsonage *et al.*, 1986; Page *et al.*, 1989), was affected by DDC. To test this, *P. denitrificans* 1222 or C010 grown into late exponential phase was harvested and 1 mg protein was resuspended in 200 mM sucrose, 10 mM potassium acetate, 10 mM HEPES/NaOH (pH 7.3), allowed to become anaerobic in the chamber of a Clark-type oxygen electrode and treated with 100 nmol myxothiazol to completely block nitrite reduction. Nitrate was added to 100 μ M and the rates of nitrate reduction (measured as nitrite accumulation) by *P. denitrificans* 1222 and *P. denitrificans* C010 *in vivo* were found to be unaffected by a 40 min incubation with 1 mM DDC.

DDC is known to inhibit the copper-type of nitrite reductase. *P. denitrificans* is recognized to contain only the cytochrome cd_1 nitrite reductase, but it was possible that a copper nitrite reductase was formed in the absence of cytochrome c_{550} . However, when nitrite reductase was assayed *in vitro*, using reduced methyl viologen in extracts of *P. denitrificans* 1222 and *P. denitrificans* C010, the activity was found to be insensitive to DDC whereas the nitrite reductase activity of periplasmic extracts from *R. sphaeroides* ssp. *denitrificans*, which contains a copper nitrite reductase (Sawada *et al.*, 1978), was completely inhibited by DDC.

The presence of a c -type cytochrome dependent oxidase activity in bacteria can be assessed by the Nadi test (Marrs & Gest, 1973). Applying this test to *P. denitrificans* 1222 and *P. denitrificans* C010 showed that they gave equivalent

Fig. 6. Effect of the inhibitor DDC on nitrous oxide reduction in *P. denitrificans* 1222 and *P. denitrificans* C010. A 3 ml solution of 200 mM sucrose, 10 mM potassium acetate, 10 mM HEPES (pH 7.3) was made anaerobic in the chamber of a N_2O electrode at 30 °C by the addition of 16 mM D-glucose, 12 units glucose oxidase and 150 units catalase; N_2O was added and its disappearance followed. In trace (a) 0.22 mg protein of *P. denitrificans* 1222 was added as marked by the arrow and its N_2O reduction rate was followed. Subsequently, the cells were incubated for 40 min with 1 mM DDC and the rate of nitrous oxide reduction was remeasured (b). In trace (c) 0.20 mg protein of *P. denitrificans* C010 was added as marked by the arrow and the rate of N_2O disappearance was followed. Subsequently, the cells were incubated for 40 min with 30 μ M DDC and the rate of nitrous oxide reduction was remeasured (d). The drift rate in (c) was used as a comparison to the 'rate' in (d).

amounts of staining. This indicated that the absence of c_{550} in *P. denitrificans* C010 does not eliminate the pathway of electrons from the dimethylphenylenediamine dye at the level of c -type cytochromes to the oxidase.

DISCUSSION

DDC is a chelator with selectivity for copper (De Silva, 1974) and we have previously shown that such chelating power readily removes copper from pseudoazurin of *T. pantotropha*, an organism that is very closely related to *P. denitrificans* (Moir *et al.*, 1993). Thus, the great sensitivity to DDC of electron transport to the reductases for nitrite, nitric oxide and nitrous oxide in the cytochrome c_{550} mutant relative to the wild-type is evidence for the roles of both cytochrome c_{550} and pseudoazurin in those electron transport pathways. The supporting biochemical evidence for a role of pseudoazurin as electron donor to nitrite and nitrous oxide reductases was given in the Introduction. The finding of Shapleigh & Payne (1985) that the activity of cytochrome cd_1 nitrite reductase was unaffected by 10 mM DDC indicates that DDC cannot extract iron from typical haem centres. One or more secondary, and possibly non-specific, effects of DDC would be expected to have the same effects on wild-type and mutant cells, contrary to what is observed. Furthermore, electron transport to nitrate reductase, a reaction that is known not to involve components of the electron transport system on the high potential side of the cytochrome bc_1 complex was not affected by DDC. The unlikely possibility that inhibition of nitrite reductase was a consequence of the induction of a copper-type enzyme in the mutant is ruled out by lack of inhibition *in vitro* by DDC of nitrite reductase activity from the mutant and is consistent with failure of antibodies to the copper-type enzyme to react with extracts of *P. denitrificans* (Coyne *et al.*, 1989). Plausible, but very unlikely, alternative explanations for the inhibition by DDC of electron transport to

the three reductases in *P. denitrificans* C010 are that the organism makes respiratory copper proteins other than pseudoazurin and that these are affected by DDC, or that there is synthesis of a novel iron (or even other metalloprotein) that is sensitive to DDC. However, there are no indications for the existence of such hypothetical novel proteins, except for amicyanin, which is specifically induced during growth on methylamine (Husain & Davidson, 1985; van Spanning *et al.*, 1991).

The target proposed for DDC in this work was originally designated an azurin by Martinkus *et al.* (1980), but is now classified as a pseudoazurin (Ambler & Tobari, 1985). Reconstitution of pseudoazurin with the cd_1 -type nitrite reductase to give activity in *P. denitrificans* (Martinkus *et al.*, 1980), and in the closely related bacterium *T. pantotropha* (Moir *et al.*, 1993), supports the role for this protein in electron transport *in vivo* as indicated by the present work. Martinkus *et al.* (1980) reported that the yield of pseudoazurin was very low from *P. denitrificans* whereas we find a high yield of around 1 mg pseudoazurin l^{-1} of anaerobically grown cells from both *P. denitrificans* 1222 and C010 (unpublished data) and *T. pantotropha* (Moir *et al.*, 1993). Although the K_m in the reconstitution is high compared to that for c_{550} (Matchova & Kucera, 1990) we have previously argued that this can be disregarded since the molarity of the protein in the periplasm is probably far higher than its K_m (Moir *et al.*, 1993). Pseudoazurin can also reconstitute with nitrous oxide reductase *in vitro* (Berks *et al.*, 1993).

The partial inhibition of the N -oxide reductase reactions *in vivo* by DDC in *P. denitrificans* 1222 indicates the probability that the capacity of c_{550} for electrons is not sufficient to account for the whole of the flux of electrons from the cytochrome bc_1 complex to the terminal electron acceptors. Hence, a copper protein in addition to cytochrome c_{550} is necessary to carry the total flux of electrons in wild-type *P. denitrificans* 1222. An alternative

explanation of the partial inhibition by DDC is that the copper protein which is involved in electron transport acts as an inhibitor after it has been depleted of copper.

Cytochrome c_{550} and pseudoazurin are proposed to act as alternative electron carriers between the cytochrome bc_1 complex and the terminal electron acceptors in *P. denitrificans*. The demonstration of two electron donors both interacting with the same electron acceptor has previously been demonstrated in certain photosynthetic cyanobacteria (Sandmann *et al.*, 1983; Ho & Krogmann, 1984) in which plastocyanin and cytochrome c_{553} both donate electrons to the photosystem I reaction centre. A further example may be an obligate methylotroph where azurin has been suggested to replace c -type cytochrome for some electron-transport functions (Auton & Anthony, 1989a, b). In each case the relative availability of copper and iron influences the pattern of redox protein production.

The finding that nitric oxide reductase accepts electrons from both c_{550} and the copper protein is of particular interest since it indicates that electrons from the membrane-bound bc_1 complex are carried via periplasmic proteins before returning to the membrane-bound nitric oxide reductase. Cytochrome c_{550} and pseudoazurin seemingly act as a pool carrying electrons from the bc_1 complex to the three N-oxide reductases which are responsible for the reduction of nitrite to dinitrogen. Many electron carrying proteins have previously been found to be specific for single electron acceptors, e.g. cytochrome c_{552} which donates electrons to the periplasmic nitrate reductase of *R. capsulatus* (Richardson *et al.*, 1990) and cytochrome c_{556} which donates electrons to dimethylsulphoxide reductase of *R. capsulatus* (McEwan *et al.*, 1989). Similarly, cytochrome c_{5511} and amicyanin act as specific electron acceptors from methanol dehydrogenase and methylamine dehydrogenase, respectively (van Spanning *et al.*, 1991).

The Nadi test is used to indicate the existence of a functioning pathway from c -type cytochromes to the aa_3 oxidase (Marrs & Gest, 1973). An alternative quinol oxidase will not give a positive reaction to the Nadi test (Willison & John, 1979). The demonstration that *P. denitrificans* C010 gives the same reaction to the Nadi test as *P. denitrificans* 1222 indicates that the presence of c_{550} is not necessary for electrons to be transferred from the level of cytochrome c to the aa_3 oxidase (or an alternative cytochrome oxidase). In related work it was shown that a Nadi negative phenotype in *Bradyrhizobium japonicum* correlated with the absence of a specific membrane-bound c -type cytochrome against a background of retention of the soluble c -type cytochromes (Bott *et al.*, 1990). All these observations are in agreement with Berry & Trumpower (1985) who isolated a membrane super-complex of bc_1 , cytochrome c_{552} and aa_3 oxidase in *P. denitrificans*. Antibodies raised to c_{552} were found to inhibit electron transport from bc_1 to aa_3 oxidase in membrane vesicles (Steinrucke *et al.*, 1991). All these findings contrast with the view of van Spanning *et al.* (1990) who found aerobic growth yields to be lower in the c_{550} deletion mutant than in the wild-type and, hence, proposed that the c_{550} deletion

mutant does not use aa_3 but rather an alternative oxidase which does not conserve as much energy.

Evidence indicates that there are differences in the electron transport pathways of denitrification in other bacteria. *Rhodobacter capsulatus* is not capable of complete denitrification (it has no nitrite reductase) but it has been found that the nitrous oxide reductase of this organism is not functional in the absence of cytochrome c_2 (Richardson *et al.*, 1991). This cytochrome is structurally related to c_{550} in *P. denitrificans*. Pseudoazurin has not been reported for *R. capsulatus* and we suggest that this is probably why cytochrome c_2 is obligatory for the pathway of electrons to nitrous oxide reductase. In *R. capsulatus*, c_2 is not obligatory for the reduction of nitric oxide (Bell *et al.*, 1992). In this case an alternative redox protein substitutes for c_2 , possibly the cytochrome species identified by Jones *et al.* (1990) or Jenney & Daldal (1993) to substitute for the latter in cyclic electron flow. In *Pseudomonas aeruginosa*, azurin and cytochrome c_{551} have been found to act as electron donors to the cd_1 nitrite reductase *in vitro* (Horio *et al.*, 1961; Parr *et al.*, 1977) and are presumed to do so *in vivo* also. In *Pseudomonas stutzeri*, which contains no azurin or pseudoazurin, deletion of a gene coding for a c -type tetrahaem protein (*nirT*) leads to the complete removal of *in vivo* nitrite reductase activity, but does not affect *in vitro* activity, indicating that this protein may act as an obligatory electron donor *in vivo* to the nitrite reductase (Jungst *et al.*, 1991). Denitrification in *Ps. stutzeri* clearly differs in some respects from that in *P. denitrificans*, even though they contain similar terminal electron acceptors. This comparison reinforces the need to continue the study of the denitrification electron transport pathway in a variety of organisms.

A recent report (Matchova *et al.*, 1993) indicated that in the absence of cytochrome c_{550} a 40 kDa c -type cytochrome participates in the pathway of electron transfer to nitrite reductase in *P. denitrificans*. This conclusion is not easy to reconcile with the recognition that the 40 kDa cytochrome is most probably a cytochrome c peroxidase. An extract containing cytochrome c peroxidase was used to reconstitute nitrite reductase activity. Since the extract was only partially purified it would be inappropriate to interpret this result to mean that cytochrome c peroxidase is an electron donor to the cd_1 nitrite reductase.

Matchova *et al.* (1993) report that on transition from aerobic growth to anaerobic growth there is a longer lag before nitrite reduction with physiological electron donors in the cytochrome c_{550} deficient mutant compared with the wild-type *P. denitrificans*. This observation can be interpreted in terms of the expression of pseudoazurin and c_{550} . Cytochrome c_{550} is expressed under aerobic and anaerobic conditions (Bosma *et al.*, 1987), whereas pseudoazurin, like azurin (Hoitink *et al.*, 1990), is expressed strictly under anaerobic conditions in *Alcaligenes faecalis* S-6 (Kakutani *et al.*, 1981) and *T. pantotrophica* (J. W. B. Moir, D. J. Richardson and S. J. Ferguson, unpublished data). A longer lag before nitrite reduction starts in the c_{550} deletion mutant relative to the wild-type could result from a delay in synthesis of pseudoazurin on switching to anaerobic conditions. Therefore, the

observations of Matchova *et al.* (1993) can be accommodated within the electron transport pathway proposed in this paper.

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