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A Mutant of *Paracoccus denitrificans* with Disrupted Genes Coding for Cytochrome c_{550} and Pseudoazurin Establishes These Two Proteins as the In Vivo Electron Donors to Cytochrome cd_1 Nitrite Reductase

Isobel V. Pearson,[1] M. Dudley Page,[1] Rob J. M. van Spanning,[2] and Stuart J. Ferguson[1]*

Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom,[1] and Department of Molecular Cell Physiology, Faculty of Biology, Biocentrum Amsterdam, Free University, 1081 HV Amsterdam, The Netherlands[2]

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In *Paracoccus denitrificans*, electrons pass from the membrane-bound cytochrome bc_1 complex to the periplasmic nitrite reductase, cytochrome cd_1. The periplasmic protein cytochrome c_{550} has often been implicated in this electron transfer, but its absence, as a consequence of mutation, has previously been shown to result in almost no attenuation in the ability of the nitrite reductase to function in intact cells. Here, the hypothesis that cytochrome c_{550} and pseudoazurin are alternative electron carriers from the cytochrome bc_1 complex to the nitrite reductase was tested by construction of mutants of *P. denitrificans* that are deficient in either pseudoazurin or both pseudoazurin and cytochrome c_{550}. The latter organism, but not the former (which is almost indistinguishable in this respect from the wild type), grows poorly under anaerobic conditions with nitrate as an added electron acceptor and accumulates nitrite in the medium. Growth under aerobic conditions with either succinate or methanol as the carbon source is not significantly affected in mutants lacking either pseudoazurin or cytochrome c_{550} or both these proteins. We concluded that pseudoazurin and cytochrome c_{550} are the alternative electron mediator proteins between the cytochrome bc_1 complex and the cytochrome cd_1-type nitrite reductase. We also concluded that expression of pseudoazurin is mainly controlled by the transcriptional activator FnrP.

The electron transport system of the facultative organism *Paracoccus denitrificans* is one of the best-characterized electron transport systems of bacteria (6, 15). Many of its components are closely related to their counterparts in the mitochondrial respiratory chain. There is a good understanding of how nonmitochondrial features, such as oxidases that are alternatives to cytochrome aa_3, along with the apparatus for catalyzing oxidation of C_1 compounds or reduction of nitrate through to nitrogen, are connected to the underlying mitochondrion-type respiratory chain (6, 32). A key component in such connections is often considered to be cytochrome c_{550} (analogous to cytochrome c_2 in photosynthetic bacteria). This protein has the same tertiary structure as mitochondrial cytochrome *c* and a similar reduction potential (32). Thus, it has been reasonable to propose that it is an electron acceptor from the cytochrome bc_1 complex and therefore the electron donor to periplasmic components of the electron transport system that receive electrons via this complex. One such component is the cytochrome cd_1-type nitrite reductase, with which cytochrome c_{550} has been shown to interact in vitro (27, 39, 42). However, if cytochrome c_{550} does indeed carry electrons from the cytochrome bc_1 complex to this enzyme, it cannot be the only component of the electron transfer system capable of this reaction. This is because a mutant of *P. denitrificans* that lacks the gene (*cycA*) encoding cytochrome c_{550} is still able to reduce nitrite; indeed, no respiratory activity of *P. denitrificans* is lost as a result of interruption of the *cycA* gene (44). The role of the cytochrome bc_1 complex in electron delivery to cytochrome cd_1 has been established through studies with both inhibitors (2) and mutants specifically deficient in this complex (33). In the case of nitrite reduction, it was proposed by Moir and Ferguson (30) that the absence of cytochrome c_{550} was compensated for by another protein that was able to act as an electron donor to cytochrome cd_1. On the basis of the finding that nitrite respiration was much more sensitive to the copper chelator diethyldithiocarbamate in the cytochrome c_{550}-deficient mutant than in the wild type (30) and because cytochrome cd_1 does not contain copper, it was suggested that the substitute protein might be pseudoazurin. The latter protein is a molecule with a single type I copper center per polypeptide chain and is a member of a family of Greek-key fold proteins that includes bacterial azurins and plant plastocyanins. However, another possible interpretation has been put forward on the basis of the observations made with diethyldithiocarbamate (24). A further indication of the importance of a copper protein in the absence of cytochrome c_{550} came from the observation that the mutant grew anaerobically much more poorly in growth media with low copper contents than did the wild-type organism (33, 45). Furthermore, the nitrite reductase exhibits activity in vitro with pseudoazurin as an electron donor (29). A critical and definitive test of the proposal that cytochrome c_{550} and pseudoazurin are alternative electron donors to cytochrome cd_1-type nitrite reductase would be provided by an examination of the properties of a mutant of *P. denitrificans* that has interruptions in both the cytochrome c_{550} (*cycA*) and pseudoazurin (*pazS*) genes. This is the subject of the present paper.

* Corresponding author. Mailing address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom. Phone: 44 1865 275240. Fax: 44 1865 275259. E-mail: stuart.ferguson@bioch.ox.ac.uk.

TABLE 1. Bacterial strains and plasmids used in this study

Organism or plasmid	Characteristics	Reference or source
Paracoccus denitrificans Pd1222	Sp^r Rif^r, restriction deficient	13
Paracoccus denitrificans Pd21.31	Pd1222 *cycA*	44
Paracoccus denitrificans Pd77.71	Pd1222 *nnr*	47
Paracoccus denitrificans Pd29.31	Pd1222 *fnrP*::Km^r	47
Paracoccus denitrificans Pd92.30	Pd1222 *fnrP*::Km^r *nnr*	47
Paracoccus denitrificans DPΩNOSR	Pd1222 *nosR*::Sm^r	This study
Paracoccus denitrificans IP1013	Pd1222 *pazS*::Km^r	This study
Paracoccus denitrificans IP1121	Pd21.31 *pazS*::Km^r	This study
Escherichia coli XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ [$proAB^+$ $lacI^q$ *lacZ*ΔM15 Tn*10*(tet^r)]	8
Escherichia coli GM2163	F′ *dam-13*::Tn*9 dcm-6 hsdR2 leuB6 his-4 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 rpsL136 tonA31 tsx-78 supE44* $McrA^-$ $McrB^-$	41
Escherichia coli S17-1	*pro thi* $hsdR^-$ M^+ *recA* Str^r with integrated RP4 2-(*tet*::Mu) (*kan*::Tn*7*)	41
Escherichia coli NM554	F^- *recA13 araD139* Δ(*ara-leu*) Δ(*lac*)*X74 galU galK hsdR2 mcrB1 rpsL*	38
pBluescript SK(+)	General cloning vector; Ap^r	Stratagene
pUC18	General cloning vector; Ap^r	Amersham Pharmacia Biotech
pUC4K	Km^r cassette vector; Ap^r Km^r	Amersham Pharmacia Biotech
pWE16	Cosmid cloning vector	Stratagene
pGRPd1	Mobilizable suicide vector; Ap^r Sp^r/Sm^r	44
pHP45Ω	Sp^r/Sm^r Ω cassette vector; Ap^r Sp^r/Sm^r	36
pAR0181	Mobilizable suicide vector; Km^r	35
pRK2013	Helper plasmid, carries *tra* genes; Km^r	16
pBK11.paz	1.9-kb *Eco*RI-*Sph*I fragment containing the 5′ region of the *P. denitrificans* pseudoazurin gene, cloned in pBK11[a]	R. J. M. van Spanning, Vrije Universiteit, Amsterdam, The Netherlands
pIP0724	*Eco*RI-*Sph*I fragment from pBK11.paz excised with *Eco*RI-*Hin*dIII and cloned in pBluescript SK(+)	This study
pDP0725	*Eco*RI-*Sph*I fragment from pHP45Ω cloned into *Mun*I-cut pIP0724	This study
pDP0726	*nosR*::Ω from pDP0725 ligated to *Eco*RI-cut pAR0181	This study
cIP0727A	Approximately 50 kb of genomic DNA from *P. denitrificans* DPΩNOS cloned in pWE16	This study
cIP0728	cIP027A cut with *Eco*RI and self-ligated	This study
pIP0729	*Xho*I-*Eco*RI fragment from cIP0728 cloned in pBluescript SK(+)	This study
pIP0730	pIP0729 cut with *Sma*I and self-ligated	This study
pIP0731	*Bam*HI-*Acc*65I fragment from pIP0729 cloned *Bam*HI-*Kpn*I in pUC18	This study
pIP0515	pIP0731 cut with *Bcl*I and ligated to the *Bam*HI-excised Km^r cassette from pUC4K	This study
pIP0516	*Bam*HI-*Eco*RI fragment from pIP0515 cloned in pGRPd1	This study

[a] See reference 21.

MATERIALS AND METHODS

Bacterial strains and growth. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were grown aerobically in Luria-Bertani medium at 37°C. *P. denitrificans* strains were routinely grown at 37°C in minimal succinate medium (9). Media used for anaerobic growth contained KNO_3 at a concentration of 100 mM unless specified otherwise. Tests for aerobic growth of *P. denitrificans* strains on methanol or methylamine were performed by using the media described by Alefounder and Ferguson (1). Antibiotics were added as required at the following final concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; rifampin, 40 μg ml^{-1}; spectinomycin, 25 μg ml^{-1}; and streptomycin, 25 μg ml^{-1}.

General DNA manipulations. General DNA manipulations were performed as described by Sambrook et al. (40). DNA sequencing was performed by the PNACL facility at the University of Leicester, Leicester, United Kingdom. Southern blotting was performed by using the digoxigenin labeling system (Boehringer Mannheim) according to the manufacturer's instructions.

Cloning and disruption of the *pazS* gene. The *P. denitrificans* DNA fragment cloned in pBK11.paz was excised with *Eco*RI plus *Hin*dIII and cloned in *Eco*RI-*Hin*dIII-digested pBluescript SK(+) to generate pIP0724 (Fig. 1). For construction of DPΩ*nosR*, pIP0724 was digested with *Mun*I and ligated to the Ω cassette isolated from *Eco*RI-cut pHP45Ω. The resulting plasmid, pDP0725, was digested with *Eco*RI, and the DNA fragment containing the Ω-disrupted *nosR* gene was cloned in pARO181. The resulting plasmid, pDP0726, was transferred to *P. denitrificans* by triparental conjugation by using the helper plasmid pRK2013 (14). Sm^r Km^s exconjugants were designated DPΩ*nosR*. DNA contiguous with the introduced Ω cassette in DPΩ*nosR* was isolated by cosmid cloning essentially as described by Viebrock and Zumft (48). DPΩ*nosR* genomic DNA was digested with *Sau*3A to obtain a fragment length of approximately 50 kb and was ligated to *Bam*HI-cut and dephosphorylated pWE16. Recombinant molecules were packaged into λ phage by using the Gigapack III XL system (Stratagene) and were transfected into *E. coli* NM554. Clones carrying the Ω cassette were selected as Ap^r Sp^r. One of the cosmids obtained, cIP0727A, was digested with *Eco*RI and self-ligated. Transformed *E. coli* cells were plated onto media containing spectinomycin in order to isolate clones carrying the Ω cassette. The

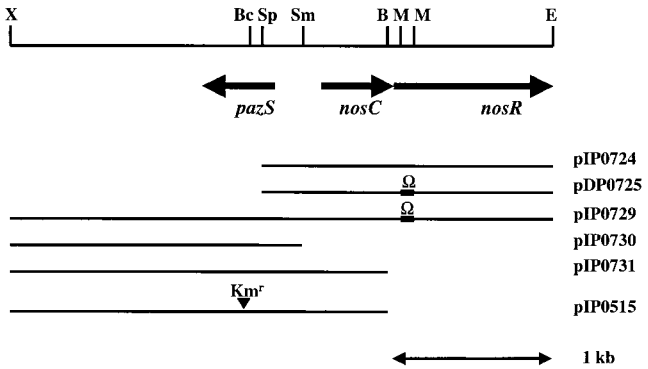

FIG. 1. Physical and restriction map of the *P. denitrificans* chromosomal DNA region containing the *pazS* gene. The map at the top is a restriction map. B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; M, *Mun*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I. The arrows indicate the positions of the *pazS* and *nosC* genes and the *nosR* 5′ region. The lines below the map are the *P. denitrificans* DNA fragments cloned in this study. Ω and Km^r indicate the positions of Ω (spectinomycin-streptomycin) and kanamycin resistance cassettes, respectively.

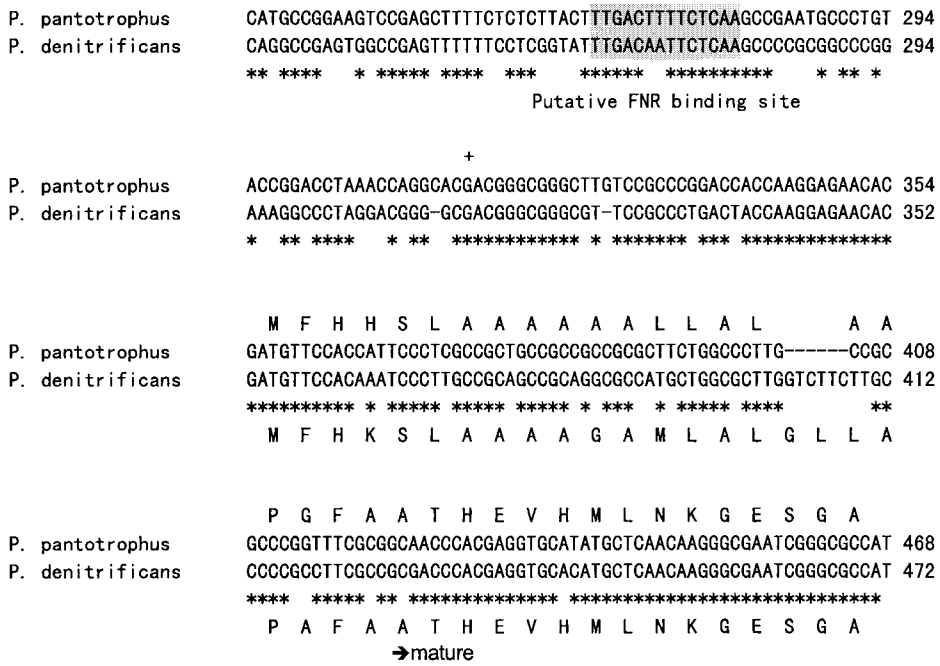

FIG. 2. Nucleotide sequences of the 5′ ends of the *pazS* genes of *P. denitrificans* and *P. pantotrophus* together with the amino acid sequences of the signal peptides and the N-terminal regions of the mature proteins. The plus sign indicates the experimentally determined transcriptional initiation site for *P. pantotrophus pazS*. The shaded area is a presumed binding site for the transcriptional activator FnrP. Asterisks indicate DNA sequence identity.

resulting cosmid, cIP0728, consisted of a single *Eco*RI fragment of *P. denitrificans* DNA (approximately 30 kb) cloned in pWE16. Cosmid cIP0728 was digested with *Eco*RI plus *Xho*I, and the resulting restriction fragments were isolated and ligated to *Eco*RI-*Xho*I-digested pBluescript SK(+). Transformed *E. coli* cells were again plated onto media containing spectinomycin. This procedure yielded plasmid pIP0729, which contained the complete *pazS* gene together with *nosC*, the 5′ region of *nosR*, and the Ω cassette. pIP0729 was then digested with *Sma*I and self-ligated to obtain pIP0730, which contained *pazS* plus approximately 100 bp of upstream DNA and 1.4 kb of downstream DNA (Fig. 1).

For *pazS* disruption, the *pazS*-containing *Bam*HI-*Acc*65I fragment from pIP0729 was isolated and cloned in *Bam*HI-*Kpn*I-digested pUC18. The resulting plasmid, pIP0731, was digested with *Bcl*I and ligated to the Km^r cassette isolated from *Bam*HI-cut pUC4K to produce pIP0515. This construct was digested with *Bam*HI plus *Eco*RI, and the DNA fragment containing the disrupted *pazS* gene was ligated to *Bam*HI-*Eco*RI-digested pGRPd1 to obtain pIP0516. This plasmid was transformed into *E. coli* S17-1 and transferred to *P. denitrificans* Pd1222 (wild type) and Pd2131 (*cycA*) by biparental mating (5).

Analytical methods. Preparation of total soluble protein fractions from *P. denitrificans* strains, determination of protein contents, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (34). Pseudoazurin was polymerized with glutaraldehyde as described by Hennig and Neupert (20), and antibodies were raised in sheep by the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland. Nitrite accumulation in the medium of growing cultures was determined as described by Nicholas and Nason (31).

Nucleotide sequence accession number. The *P. denitrificans pazS* sequence, together with approximately 1,500 flanking base pairs, has been deposited in the GenBank database under accession no. AF334183.

RESULTS

Cloning and sequencing of the *P. denitrificans* pseudoazurin structural gene, *pazS*. The starting material for the cloning of *pazS* from *P. denitrificans* was a DNA fragment (pBK11.paz) that contained (GenBank accession no. AJ010260) 135 bp of the 5′ end of *pazS* (Fig. 1). This fragment had been found unexpectedly, oriented in the opposite direction, adjacent to the *nosC* and *nosR* genes in a cluster of *nos* genes that are required for formation of nitrous oxide reductase (N. F. W. Saunders and R. J. M. van Spanning, unpublished observations). Because previous attempts to clone *pazS* had proved to be problematic, the strategy adopted in the present work was to generate a marked mutation in the chromosomal *nosR* gene and then to isolate DNA containing the marker and the flanking region containing the full-length *pazS* gene. The cosmid-based cloning procedure leading to plasmid pIP0730 is described in Materials and Methods.

The protein sequence translated from the *P. denitrificans pazS* structural gene sequence was in exact agreement with that obtained by direct protein sequencing (25). These gene and protein sequences are very similar to their counterparts in the closely related organism (37) *Paracoccus pantotrophus* (11, 25). However, immediately beyond the structural gene region the DNA sequences of *P. pantotrophus* and *P. denitrificans* had considerable differences. At the 5′ end, these differences not only were apparent in the noncoding region but also reflected much variation in the periplasmic targeting sequences, although this variation was conservative (Fig. 2). Nevertheless, in both organisms the *pazS* gene clearly had an anaerobox of the Fnr type centered at bp 80.5 upstream of the translational initiation point and most likely at a similar position, bp 41.5, relative to the transcriptional start point, if the latter was assumed to be identically positioned in *P. denitrificans* and *P. pantotrophus* (6, 25) (Fig. 2).

Two transcription factors, NNR and FnrP, bind differentially to anaeroboxes in *P. denitrificans* (46, 47). The synthesis of pseudoazurin by mutants of *P. denitrificans* deficient in either FnrP or NNR was therefore examined. The former protein,

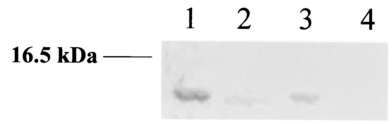

FIG. 3. Pseudoazurin expression in *P. dentrificans nnr* and *fnrP* mutant strains. Proteins were separated by SDS-PAGE, and pseudoazurin was detected by immunoblotting. Lane 1, Pd1222 (wild type); lane 2, Pd29.31 (*fnrP*::Km^r); lane 3, Pd77.71 (*nnr*); lane 4, Pd92.30 (*fnrP*::Km^r *nnr*). The lanes contained approximately 20 μg of total protein. The position of a 16.5-kDa molecular mass marker is indicated on the left.

along with anaerobic conditions, could be shown to be essential for significant expression of pseudoazurin, as judged by immunoblotting (Fig. 3), whereas the presence of NNR appeared to be less significant for *pazS* expression. The attenuated expression in the NNR mutant may be explained by the poor growth of the cells under anaerobic conditions, a consequence of their inability to reduce nitrite or nitric oxide (46).

Targeted disruption of *pazS* in *P. denitificans* Pd1222 (wild type) and Pd21.31 (*cycA*). The cloned *pazS* gene was disrupted by insertion of a kanamycin resistance cassette, and the disrupted gene was cloned in the mobilizable suicide vector pGRPd1. The resulting plasmid, pIP0516, was transferred by conjugation into *P. denitrificans* strains Pd1222 (wild type) and Pd21.31 (carrying an unmarked mutation in *cycA*). Kanamycin-resistant mutants of *P. denitrificans*, putatively carrying interruptions in either *pazS* alone or in *pazS* and *cycA*, were analyzed to identify mutants that had undergone the required double homologous recombination event leading to a genomic kanamycin-marked *pazS* gene. A single homologous recombination event would have resulted in both the marked *pazS* gene and the original intact *pazS* gene being present. There was no antibiotic resistance marker that could be used for this discrimination (the *Bam*HI/*Eco*RI component of pGRPd1 present in pIP0516 did not contain the spectinomycin-streptomycin resistance locus), and so the presence of a large region of DNA originating from the vector pGRPd1 was used as a probe to detect the unwanted cells that had undergone only a single recombination event rather than the required double recombination event.

*Pvu*II digests of genomic DNA from one in five putative pseudoazurin single mutants did not hybridize to the pGRPd1-specific probe, suggesting that each of these mutants was a product of a double recombination event. *Pvu*II was used because there are no restriction sites for this enzyme within either the cloned *pazS* DNA or the kanamycin resistance cassette. The *pazS* gene was used as a second probe for the appearance in the *P. denitrificans* genome of a *pazS* gene interrupted by the kanamycin resistance cartridge. This proved to be problematic as only very weak probes could be generated in the present study when *paz* alone was labeled. Thus, we labeled pIP0731, which is a pUC18-derived construct containing a *Bam*HI/*Acc*65I *pazS* fragment. Southern blotting with this probe showed that the putative mutants indeed had an interrupted *pazS* gene. One of these mutants, IP1013, was selected for further characterization. Immunoblotting (Fig. 4) confirmed the absence of the pseudoazurin protein from cells of this mutant after growth under anaerobic conditions.

Coincidentally, one in five putative mutants with mutations in the *pazS* gene that resulted from a double recombination event were again isolated following conjugation of pIP0516 into *P. denitrificans* Pd21.31 (the *cycA* mutant). One of these mutants, designated IP1121, for which Southern blotting clearly demonstrated the absence of the vector pGRPd1 but the presence of the kanamycin insertion in *pazS*, was selected for further study.

The absence of pseudoazurin from the double cytochrome c_{550} and pseudoazurin mutant, IP1121, was further confirmed by Western blotting. It is clear (Fig. 4) that whereas wild-type cells or the strain with the unmarked mutation for cytochrome c_{550} contained pseudoazurin, both types of *pazS* mutants lacked this protein. It was noticed that the absence of cytochrome c_{550} did not result in an increase in the amount of pseudoazurin in the *P. denitrificans* Pd21.31 (*cycA*) cells. As expected, cytochrome c_{550} was still expressed in the single *pazS* mutant, but strikingly, it was expressed at a higher level than in the wild-type cells (Fig. 4), suggesting there was some compensatory effect of the absence of pseudoazurin on expression of this cytochrome.

The construction of the *pazS cycA* double mutant was designed to show whether in vivo nitrite reductase activity was compromised. In this context it was important to show that loss of both pseudoazurin and cytochrome c_{550} from the cell had not caused a loss of cytochrome cd_1 from the cell. The Western blot in Fig. 5 shows that the latter cytochrome was still present after anaerobic growth, but at lower levels than those observed for the wild type or the *pazS* and *cycA* single mutants. The explanation for this is that maximal transcription of the *nirS* gene, coding for cytochrome cd_1-type nitrite reductase, re-

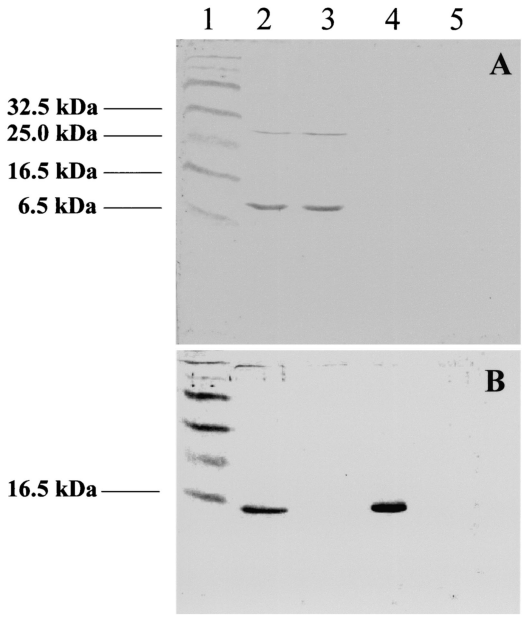

FIG. 4. Detection of pseudoazurin and cytochrome c_{550} in total soluble extracts from wild-type and mutant strains of *P. denitrificans*. Proteins were separated by SDS-PAGE, and pseudoazurin (A) or cytochrome c_{550} (B) was detected by immunoblotting. Lane 1, molecular weight markers; lane 2, Pd1222 (wild type); lane 3, Pd21.31 (*cycA*); lane 4, IP1013 (*pazS*::Km^r); lane 5, IP1121 (*cycA pazS*::Km^r). The lanes contained approximately 20 μg of total protein.

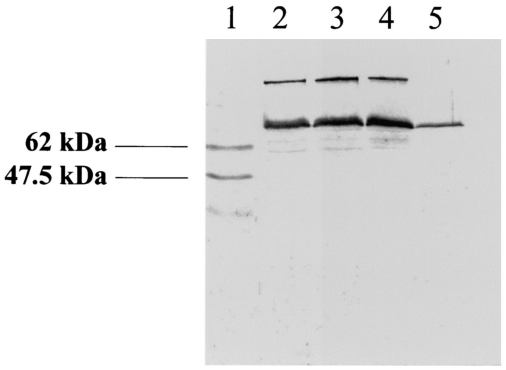

FIG. 5. Detection of cytochrome cd_1 in total soluble extracts from wild-type and mutant strains of *P. denitrificans*. Proteins were separated by SDS-PAGE, and cytochrome cd_1 was detected by immunoblotting. Lane 1, molecular weight markers; lane 2, Pd1222 (wild type); lane 3, Pd21.31 (*cycA*); lane 4, IP1013 (*pazS*::Km^r); lane 5, IP1121 (*cycA pazS*::Km^r). The lanes contained approximately 20 μg of total protein.

quires production of the enzyme's reaction product, nitric oxide, in order to activate the transcription factor, NNR, that stimulates the expression of *nirS*. (46). Clearly, in a mutant unable to form nitric oxide the amount of cytochrome cd_1-type nitrite reductase synthesized is considerably attenuated. Thus, the decrease in the amount of cytochrome cd_1-type nitrite reductase as a consequence of the absence of both cytochrome c_{550} and pseudodazurin is itself evidence that these molecules are the two physiological electron donors.

Growth characteristics. It was not thought that aerobic growth on a heterotrophic carbon and free energy source should require either cytochrome c_{550} or pseudoazurin, because cytochrome c_{552} is a protein that accepts electrons from the cytochrome bc_1 complex and delivers them to the cytochrome aa_3 oxidase (33), while cytochrome ba_3, which is a quinol oxidase, functions independent of either pseudoazurin or cytochrome c_{550} (15, 33). This expectation was confirmed by the finding that aerobic growth with succinate as a carbon and free energy source was not affected in any of the mutants. Thus, the stationary phase was reached after approximately 14 h with a turbidity (optical density at 650 nm [OD_{650}]) between 2.5 and 3 for both wild-type cells and cells having mutations in the gene(s) encoding either cytochrome c_{550} or pseudoazurin or both.

Aerobic growth on methanol or methylamine is more dependent on the cytochrome bc_1-dependent oxidase, cytochrome aa_3, than growth on succinate is. In *Methylotrophus methylophilus* a pseudoazurin has been implicated in electron transfer to oxidases from the methylamine dehydrogenase (4). However, growth of *P. denitrificans* on either of these two one-carbon sources was also not affected by the absence of both pseudoazurin and cytochrome c_{550}, implying that neither of these molecules has an obligatory role in this case, a conclusion for pseudoazurin that is consistent with the presence of the upstream anaerobox (Fig. 2).

Growth under anaerobic conditions with nitrate as an added electron acceptor and succinate as a carbon and free energy source gave a different result. The double mutant (*cycA pazS*) grew to a lower final turbidity than the other organisms; nitrite accumulated and remained in the medium (Fig. 6). When both the cell density and the nitrite concentration in the medium were measured under these conditions, it was clear that the nitrite concentration in wild-type and single mutant cultures rose and then declined to zero (Fig. 6); gas production resulting from denitrification proceeding beyond nitrite could clearly be observed in the medium. In contrast, the double mutant cells converted nitrate to nitrite almost quantitatively (Fig. 6), and production of gas did not occur. The formation of 25 mM nitrite correlated with cessation of cell growth, which occurred at an OD_{650} of 0.4, compared with an OD_{650} of 1.2 for the wild-type and single mutants (Fig. 6). This was expected, because once all the added nitrate had been reduced to nitrite, there was no useable electron acceptor available in such anaerobic cultures (i.e., the nitrite could not be used owing to the double mutation, which in turn meant that nitric and nitrous oxides could not be generated for respiration). All types of cells took approximately 12 h, after any lag phases, to reach the final OD_{650}, showing that the double mutant had a similar time course of growth but produced less biomass. These observations clearly imply that the double mutant was unable to reduce nitrite, in contrast to the wild-type strain and strains having a single mutation in either *cycA* or *pazS*.

The transient accumulation of nitrite (Fig. 6) in cultures of anaerobically grown wild-type *P. denitrificans* has been observed previously (7; Ferguson, unpublished observations) and is thought to reflect the later onset of nitrite reductase synthesis compared to the onset of nitrate reductase synthesis. The

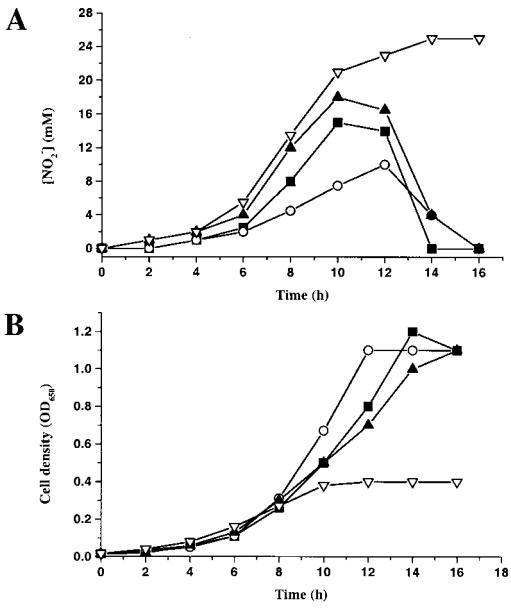

FIG. 6. Nitrite accumulation (A) and growth curves (B) for cultures of the *P. denitrificans* wild type and *cycA*, *pazS*, and *cycA pazS* mutants. The data are typical of data for three replicates for each strain; in each case variable lag phases following inoculation were omitted to permit comparisons between strains. For these experiments the concentration of nitrate initially present was lowered to 25 mM in order to facilitate measurement of the stoichiometry of nitrite accumulation; concentrations of accumulated nitrite higher than 25 mM are toxic. Symbols: ■, wild type; ▲, *pazS* mutant; ○, *cycA* mutant; ▽, *cycA pazS* mutant.

presence of nitrite at millimolar levels disrupts the pattern of growth away from the ideal behavior (33). In the wild-type and single mutant cells nitrite formation correlated with the early stage of growth following any lag phase, but why different strain-dependent nitrite concentrations were reached (Fig. 6) before the concentrations declined to zero is not clear. Thus, lower nitrite concentrations were attained in the media of the *cycA* mutant cells than in the media of either the wild type or the *pazS* mutant. This observation suggests that there may be subtly different consequences of the absence of either cytochrome c_{550} or pseudoazurin. The reason for this behavior is not clear but may be related to the requirement for nitric oxide formation to in turn stimulate the production of more nitrite reductase (see above). Thus, there may be a relationship between the rate of nitrite reduction in the early stages of growth and the subsequent formation of more nitrite reductase.

DISCUSSION

Whereas the absence of either pseudoazurin or cytochrome c_{550} has no major effect on anaerobic respiration of nitrite in *P. denitrificans*, this process is clearly severely compromised in the *P. denitrificans* mutant that lacks both these periplasmic electron transfer proteins. Thus, either cytochrome c_{550} or pseudoazurin is required for one, or most probably the only, step of the electron transfer pathway from the cytochrome bc_1 complex to cytochrome cd_1. This conclusion is in agreement with the recent report that addition of pseudoazurin stimulated the rate of electron transfer from succinate to nitrite in right-side-out vesicles, which must have retained externally bound cytochrome cd_1-type nitrite reductase, from cytochrome c_{550}-deficient *P. denitrificans* (23). A previous proposal (28) that one or more additional *c*-type cytochromes act as alternatives to cytochrome c_{550} in electron transfer to nitrite reductase is not supported by the present work. This conclusion is enhanced by the demonstration (33) that another *c*-type cytochrome, cytochrome c_{552}, functions only in electron transfer from the cytochrome bc_1 complex (or from amicyanin during growth on methylamine) to cytochrome aa_3 oxidase. Cells of a *P. denitrificans* mutant deficient in cytochrome c_{552} denitrify as well as cells carrying a disrupted cytochrome c_{550} gene, but the former, unlike the latter, do not require copper in the growth medium for denitrification to occur. The implication is again that only a copper-containing protein can substitute for cytochrome c_{550} in nitrite reduction during denitrification. As might be predicted on this basis, a double deletion mutant with deletion of both cytochrome c_{550} and cytochrome c_{552} also requires copper in the medium for anaerobic growth on succinate plus nitrate (33). Cytochrome c_{552} is expressed constitutively, and its globular heme-containing domain has a structure very similar to that of cytochrome $c_{550,}$ although it possesses an acidic patch and overall has a less positive charge (18). Nevertheless, although it shares a capability with cytochrome c_{550} and (as implied by the present work) pseudoazurin to interact with the cytochrome bc_1 complex, it seems to be specific as an electron donor to cytochrome aa_3 (33). The acidic patch or the transmembrane anchor of cytochrome c_{552} may prevent its productive interaction with cytochrome cd_1.

In an early in vitro study it was concluded that cytochrome c_{550}, but not pseudoazurin, is the electron donor to cytochrome cd_1-type nitrite reductase (26, 42). However, recent work with *P. pantotrophus* proteins has shown that pseudoazurin is an effective electron donor to *Paracoccus* cytochrome cd_1, provided that the latter molecule is first activated by reduction (3, 39). Presumably, this explains why Martinkus et al. (26) observed little activity with pseudoazurin, in contrast to Moir et al. (29), who in retrospect fortuitously activated cytochrome cd_1 before their assay. Since the presence of at least cytochrome c_{550} or pseudoazurin is required for in vivo nitrite respiration, any activator for this cytochrome cd_1 must be a different protein for which the midpoint potential is less than 60 mV and thus able to change the inactive oxidized protein into the active reduced state (3, 22, 39).

One conclusion of the present work is that not only must pseudoazurin and cytochrome c_{550} be alternative electron donors to cytochrome cd_1, but they must also be alternative acceptors from the cytochrome bc_1 complex. This conclusion leads to the question of how two such structurally distinct proteins as pseudoazurin and cytochrome c_{550} can interact with the same reductant and oxidant. In the case of interaction with cytochrome cd_1-type nitrite reductase, it has been proposed that pseudoazurin and cytochrome c_{550} each interact with a hydrophobic patch on the surface of the enzyme, with the reaction promoted by patches of positive charges on the cytochrome c_{550} or pseudoazurin and negative charges on cytochrome cd_1 (50). There is at least one precedent for the cytochrome bc_1 complex interacting with a copper protein. In the green sulfur bacterium *Chloroflexus aurantiacus* there is no evidence that there are water-soluble *c*-type cytochromes analogous to cytochrome c_{550}, and the cytochrome bc_1 complex is thought to use auracyanin, a copper protein related to pseudoazurin (43), as its electron acceptor. In thylakoids of both cyanobacteria and *Arabidopsis*, it is known that either cytochrome c_6 or plastocyanin, which is related to pseudoazurin, is an alternative molecule for transfer of electrons from the cytochrome *bf* complex to photosystem I (12, 17). Negatively charged patches on cytochrome c_6 or plastocyanin are thought to present a recognition feature to complementary charged regions on partner proteins. Thus, there are several good examples of an organism that is able to use a type I copper protein instead of a *c*-type cytochrome in an electron transfer step from membrane-bound electron transfer complexes that occur in both respiratory and photosynthetic systems.

It has been deduced that only cytochrome c_{551}, and not azurin, could act as an electron donor to the cytochrome cd_1-type nitrite reductase from *Pseudomonas aeruginosa* (49); this contrasts with the present work. There is much evidence which shows that azurin can function as an electron donor to the *P. aeruginosa* nitrite reductase in vitro, and thus the data are slightly surprising. The apparent failure of azurin to sustain substantial nitrite respiration in this organism in the absence of cytochrome c_{551} might be attributed to several factors. Among these factors could be a failure of azurin to accept electrons from the cytochrome bc_1 complex. An explanation based on the possibility of a failure to express cytochrome cd_1-type nitrite reductase in the absence of azurin was eliminated by Vijgenboom et al. (49). In fact, in *P. aeruginosa* it appears that denitrifying conditions do not promote the expression of azurin (49), and thus in this organism only one electron-carrying protein, cytochrome c_{551}, can act between the cytochrome

bc_1 complex and the nitrite reductase. The situation in *P. aeruginosa* has been further complicated by the subsequent finding that a second *c*-type cytochrome, NirC, can also act in vitro as a donor to cytochrome cd_1-type nitrite reductase (19), a result that contrasts with the data of Vijgenboom et al. (49).

The characteristic anaerobox sequence that is found upstream of the *pazS* gene in *P. denitrificans* could be a binding site for FnrP or NNR, two molecules which bind to similar sequences, although the context of these sequences must confer specificity (e.g., for NNR binding rather than FnrP binding upstream of *nirS* [47]) for one of the two transcription factors. The results in the present paper suggest that it is FnrP that is the more important of these transcription factors for activating transcription of *pazS.* Stimulation of pseudoazurin production by anaerobiosis signaled via FnrP rather than by NNR signaled by nitric oxide would be consistent with the finding (N. F. W. Saunders and S. J. Ferguson, unpublished data) that microanaerobic growth conditions stimulate production of not only the cytochrome *c* peroxidase, which is known to be under FnrP control (47), but also pseudoazurin. The −10 regions of the *pazS* genes of the two *Paracoccus* species have a CCTA sequence in common. The TA pair, in conjunction with the presence of an anaerobox at position −41.5, is diagnostic of the class II-type CAP family of promoters, in which the −10 region is likely to be dependent upon a σ^{70} factor (10).

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