Electron transfer to nitrite reductase of *Rhodobacter sphaeroides* 2.4.3: examination of cytochromes c_2 and c_Y

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Received 15 September 2005

Revised 9 January 2006

Accepted 18 January 2006

The role of cytochrome c_2 , encoded by *cycA*, and cytochrome c_Y , encoded by *cycY*, in electron transfer to the nitrite reductase of *Rhodobacter sphaeroides* 2.4.3 was investigated using both *in vivo* and *in vitro* approaches. Both *cycA* and *cycY* were isolated, sequenced and insertionally inactivated in strain 2.4.3. Deletion of either gene alone had no apparent effect on the ability of *R. sphaeroides* to reduce nitrite. In a *cycA*–*cycY* double mutant, nitrite reduction was largely inhibited. However, the expression of the nitrite reductase gene *nirK* from a heterologous promoter substantially restored nitrite reductase activity in the double mutant. Using purified protein, a turnover number of 5 s^{-1} was observed for the oxidation of cytochrome c_2 by nitrite reductase. In contrast, oxidation of c_Y only resulted in a turnover of $\sim 0.1\text{ s}^{-1}$. The turnover experiments indicate that c_2 is a major electron donor to nitrite reductase but c_Y is probably not. Taken together, these results suggest that there is likely an unidentified electron donor, in addition to c_2 , that transfers electrons to nitrite reductase, and that the decreased nitrite reductase activity observed in the *cycA*–*cycY* double mutant probably results from a change in *nirK* expression.

INTRODUCTION

Rhodobacter sphaeroides 2.4.3 is a purple nonsulfur photosynthetic bacterium capable of complete denitrification (Michalski & Nicholas, 1988). Denitrification involves the sequential reduction of nitrogen oxides from nitrate to nitrite, nitric oxide, nitrous oxide and dinitrogen gas. The key step in denitrification, separating it from other nitrogen oxide reductive processes, is the one-electron reduction of nitrite to nitric oxide by nitrite reductase (Nir) (Zumft, 1997). Denitrifying strains of *R. sphaeroides* utilize a copper-containing Nir (Michalski & Nicholas, 1985; Tosques *et al.*, 1997), a trimeric protein with each monomer binding a type 1 copper and a type 2 copper (Godden *et al.*, 1991). The electron transport chain of *Rhodobacter* spp. has been well studied, and it has been demonstrated that, during denitrification, electrons destined for Nir flow from the quinone pool to the bc_1 complex (Urata & Satoh, 1984). Electron transfer from the bc_1 complex to Nir involves small metalloproteins that function as electron shuttles. Previous *in vitro* studies suggest that cytochrome c_2 may shuttle electrons to Nir, but genetic evidence for this is lacking (Urata & Satoh, 1984).

Work with other denitrifiers suggests that multiple proteins shuttle electrons to Nir. In *Paracoccus denitrificans*, *in vitro* studies demonstrate that cytochrome c_{550} can shuttle electrons to Nir, but disruption of the gene encoding this protein has no effect on Nir activity (Richter *et al.*, 2002; Van Spanning *et al.*, 1990). Subsequently, it was shown that a copper-containing protein, pseudoazurin, can also donate electrons to Nir of *P. denitrificans* (Moir & Ferguson, 1994). Mutants lacking both c_{550} and pseudoazurin grow poorly under denitrification conditions (Pearson *et al.*, 2003). Pseudoazurin also has been shown to donate electrons to the cd_1 -type Nir of *Thiosphaera pantotropha* (Moir *et al.*, 1993). *In vitro* studies using proteins from *Pseudomonas aeruginosa* demonstrate that both cytochrome c_{551} and the copper-containing protein azurin can transfer electrons to cd_1 Nir (Parr *et al.*, 1977; Wharton *et al.*, 1973). Inactivation of the genes encoding c_{551} and azurin reveals that c_{551} is involved in electron transfer to Nir but that azurin is not involved (Vijgenboom *et al.*, 1997).

In *R. sphaeroides*, cytochrome c_2 is encoded by *cycA*. *cycA* is transcribed under all growth conditions consistent with its role in electron transport during both oxic and anoxic growth (Donohue *et al.*, 1986). Deletion of the *cycA* gene in *R. sphaeroides* results in the strain being unable to grow photosynthetically, suggesting that cytochrome c_2 is the sole electron donor to the reaction centre in this strain (Donohue *et al.*, 1988). In the related bacterium *Rhodococcus capsulatus*, a *cycA* mutant can still grow photosynthetically

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Abbreviations: HT, histidine tag; Ni-NTA, nickel-nitriloacetic acid; Nir, nitrite reductase.

(Daldal *et al.*, 1986). Subsequent work demonstrates that *R. capsulatus* contains a membrane-anchored cytochrome *c_Y*, which is encoded by the *cycY* gene, and that in the absence of cytochrome *c₂*, *c_Y* can mediate electron flow to the photosynthetic reaction centre (Jenney & Daldal, 1993). *R. sphaeroides* 2.4.1, a non-denitrifying strain, also encodes a cytochrome *c_Y* that can transfer electrons to terminal oxidases, but it is unable to support the transfer of electrons to the reaction centre (Daldal *et al.*, 2001; Myllykallio *et al.*, 1999). The facts that *c_Y* has similar redox characteristics and significant substrate overlap with *c₂*, and that it is highly expressed under both oxic and anoxic conditions (Pappas *et al.*, 2004), suggests the possibility that *c_Y* can transfer electrons to nitrogen oxide reductases under denitrification conditions. The work described in this paper was designed

to better define the role of cytochromes *c₂* and *c_Y* in electron transfer to the Nir of *R. sphaeroides* 2.4.3.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* strain DH5 α was used as the maintenance strain for plasmids, while *E. coli* S-17-1 was the donor for conjugal matings (Simon *et al.*, 1983). *R. sphaeroides* 2.4.3 (ATCC 17025) is the wild-type denitrifying strain and 11.10 is a Nir-deficient mutant of strain 2.4.3 (Tosques *et al.*, 1997). pRK415 (Keen *et al.*, 1988) is a broad-host-range plasmid and pWLNir is a pRK derivative that contains *nirK* expressed from a ribosomal promoter (Table 1) (Laratta *et al.*, 2002).

E. coli strains were grown in LB medium (Maniatis *et al.*, 1982) and, when necessary, supplemented with antibiotics at the following

Table 1. Bacterial stains and plasmids used in this study

Abbreviations: Tc, tetracycline; Km, kanamycin; Sm/Sp, streptomycin/spectinomycin.

Strains/plasmids	Genotype or description	Reference or source
Strains		
DH5 α F'	Host for <i>E. coli</i> cloning: F' Φ 80 <i>lacZ</i> Δ m15 <i>redA endA1 gyrA96 thi-1</i> <i>hsdR17</i> (r _k m _v) <i>supE44 relA1 deoR</i> (Δ <i>lacZYA-argF</i>)U169	
S17-1	For conjugal transfer of plasmids from <i>E. coli</i> into <i>R. sphaeroides</i> : <i>recA thi pro</i> <i>hsdRM+</i> RP4; 2-Tc:Mu:Km:TnZ	
2.4.3	Wild-type denitrifying strain of <i>R. sphaeroides</i>	ATCC 17025
11.10	<i>nirK::aph</i> ; 2.4.3 derivative; Km ^r	Tosques <i>et al.</i> (1997)
CYT-2	<i>cycA::aph</i> ; 2.4.3 derivative; Km ^r	This work
CYT-Y	<i>cycY::spe/str</i> ; 2.4.3 derivative; Ω Sm ^r /Sp ^r	This work
CYT-2/Y	<i>cycA::aph cycY::spe/str</i> ; 2.4.3 derivative; Km ^r , Ω Sm ^r /Sp ^r	This work
Plasmids		
pUC19	Used for cloning in <i>E. coli</i>	Yanisch-Perron <i>et al.</i> (1985)
pUC4K	Source of <i>aph</i> gene; used as restriction site mobilizing element	Pharmacia
pHP45	Source of <i>spe/str</i> cassette	Prentki & Krisch (1984)
pRK415	Broad-host-range plasmid (Tc ^r)	Keen <i>et al.</i> (1988)
pSUP202	Mobilizable suicide vector	Simon <i>et al.</i> (1983)
pYSW35	pRK415 carrying the <i>PrrnB</i> ribosomal promoter (Tc ^r)	Y. Wang & J. P. Shapleigh
pUC19-cycA1	pUC19 with a ~1.5 kb fragment containing the <i>cycA</i> gene from 2.4.3	This work
pUC19-cycA2	pUC19 with a ~1.0 kb fragment containing the <i>cycA</i> gene from 2.4.3	This work
pUC19-cycAX	pUC19 with a 995 bp <i>cycA</i> fragment containing an <i>XhoI</i> restriction site	This work
pSUP-cycAX	pSUP202 with an <i>EcoRI-PstI</i> -containing <i>cycA</i> fragment from pUC19-cycAX	This work
pCYCA	pSUP202 containing <i>cycA::aph</i> ; used for allelic exchange of chromosomal <i>cycA</i>	This work
pUC19-cycY	pUC19 with a ~1.0 kb fragment containing the <i>cycY</i> gene from 2.4.3	This work
pUC19-cycY Ω	pUC19-cycY with an Ω <i>spe/str</i> cassette cloned into <i>cycY</i>	This work
pCYCY	pSUP202 containing <i>cycY::Ωspe/str</i> ; used for allelic exchange of chromosomal <i>cycY</i>	This work
pWLNIR	Derivative of pYSW35 with <i>PrrnB'</i> - <i>nirK</i> fusion (Tc ^r)	Laratta <i>et al.</i> (2002)
pC2P404.1	Plasmid carrying the <i>cycA</i> gene from <i>R. sphaeroides</i> 2.4.1(Tc ^r)	Donohue <i>et al.</i> (1986)
pCytY	pRK415 carrying the <i>cycY</i> gene (Tc ^r)	This work
pWL200	2.4.3 <i>cycA</i> gene ligated into <i>Bam</i> HI- <i>Eco</i> RI digested pYSW35 to construct a <i>PrrnB'</i> - <i>cycA</i> fusion	This work
pWL202a	pUC19 containing a 0.6 kb <i>cycA</i> fragment fused in-frame to a 0.5 kb <i>cycY</i> fragment	This work
pWL202b	pYSW35 with a 0.65 kb <i>cycA'</i> - <i>cycY</i> fusion cloned downstream of the <i>PrrnB</i> promoter (Tc ^r)	This work
pWL202his	pRK415 with a <i>PrrnB'</i> - <i>cycA'</i> - <i>cycY</i> fusion encoding a C-terminal HT (Tc ^r)	This work

concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; tetracycline, 10 $\mu\text{g ml}^{-1}$; streptomycin/spectinomycin, 25 $\mu\text{g ml}^{-1}$ each; kanamycin, 25 $\mu\text{g ml}^{-1}$. *Rhodobacter* strains were grown in Sistrom's minimal medium at 30 °C (Leuking *et al.*, 1978). When appropriate, antibiotics were added to *Rhodobacter* cultures at the following concentrations: tetracycline, 1 $\mu\text{g ml}^{-1}$; kanamycin, 25 $\mu\text{g ml}^{-1}$; streptomycin/spectinomycin, 50 $\mu\text{g ml}^{-1}$ each. For photosynthetic growth, cells were cultured over incandescent light in a jar made anoxic using a Difco anaerobic system. For denitrifying growth, cultures were grown under oxygen-limiting conditions in the presence of 12 mM nitrate, while other culture conditions were as described previously (Tosques *et al.*, 1996, 1997).

Cloning and sequencing of the *cycA* and *cycY* genes.

Attempts to isolate *cycA* from strain 2.4.3 using oligonucleotide primers designed from internal portions of *cycA* from the related *R. sphaeroides* 2.4.1 were unsuccessful. However, the genes flanking *cycA*, as well as *cycY*, in strain 2.4.1 encode products that have high sequence similarity to genes in related α -proteobacteria, making them suitable targets for amplification (Mackenzie *et al.*, 2001). Assuming synteny between the strain 2.4.1 and 2.4.3 genomes, primers were designed to target conserved regions in genes flanking *cycA* and *cycY*. *cycY* was amplified with a primer targeting *pheA*, 5'-GCGGTACCTCGATGTCGGCATAG-3', and a primer targeting RSP0704, 5'-GGGAATTCGAAATCGGCCGATATT-3'. The fragment was amplified using Vent polymerase (New England Biolabs). All the amplified fragments generated in this study were confirmed by sequencing. The ~1.0 kb amplification product was digested with *EcoRI/KpnI*, cloned into pUC19 and sequenced (Table 1). The underlined regions in all listed primers are restriction sites added to facilitate cloning.

A primer targeting RSP0297, 5'-GCGAATTCGCTTCTGGACGAGCC-3', which is downstream of *cycA*, and a primer for RSP0295, 5'-GCGGTACCGTCTCGGCCAAGCTGAA-3', were used to amplify an ~1.5 kb fragment containing *cycA*. The amplicon was digested with *EcoRI/KpnI*, cloned into pUC19 and sequenced from both ends (Table 1). Due to its length, the fragment could not be fully sequenced, and a second set of primers was used to amplify a smaller internal fragment. The sequences for the primers used in the second round of amplification were 5'-GCGAGGTACCGTTCTTGAACATTGG-3' and 5'-GCAGAATTCGAACGCGATCAGTTGGT-3', and the ~1.0 kb amplification product was digested with *EcoRI/KpnI*, cloned into pUC19 and sequenced.

Construction of plasmids and strains. To insertionally inactivate *cycY*, pUC19-*cycY* was partially digested with *Bam*HI, which cuts a single site within the *cycY* ORF, and a *Bam*HI fragment carrying the Ω spd/str cassette from pHP45 (Prentki & Krisch, 1984) was ligated into that site. The resulting plasmid was digested with *EcoRI/PstI* and the fragment containing the disrupted *cycY* gene was cloned into pSUP202 (Simon *et al.*, 1983) to generate pCYCY. This was mobilized into strain 2.4.3 and an exconjugant with appropriate antibiotic resistance was selected and designated CYT-Y (Table 1). Plasmid pCytY, used for complementation experiments, was constructed by digesting pUC19-*cycY* with *EcoRI/KpnI*, isolating the 1.0 kb fragment containing *cycY* and ligating it into pRK415, digested with the same enzymes (Table 1).

To inactivate *cycA*, primers with the sequences 5'-GCAGGTACCGT-TCTTGAACATTGGTTC-3' and 5'-GCACTCGAGTCTATGGGTAG-ATGATTGC-3' were used to amplify a 510 bp fragment upstream of *cycA*, while a 485 bp downstream fragment was amplified using primers with the sequences 5'-GCACTCGAGAAGACCGGCCGAA-CCTC-3' and 5'-GCCGAATTCGCCGACCGCTTCTATG-3'. The upstream fragment was digested with *KpnI/XhoI*, while the downstream fragment was digested with *XhoI/EcoRI*. These fragments were cloned into pUC19 (Yanisch-Perron *et al.*, 1985) using a three-piece ligation,

creating pUC19-*cycAX*, which contained a single, internal *XhoI* site (Table 1). The cloned fragment was missing the first 165 bases of the *cycA* ORF. This truncated *cycA* ORF was ligated into pSUP202 (Simon *et al.*, 1983) and the resulting pSUP-*cycAX* digested with *XhoI*, and an *aph* carrying *SalI* fragment from pUC4K (Pharmacia) was cloned into the *cycA* gene, resulting in pCYCA (Table 1). This plasmid was used for conjugal matings with strain 2.4.3 to construct a *CycA*-deficient strain, and CYT-Y to generate a strain lacking both cytochromes. The resulting strains were designated CYT-2 and CYT-2/Y (Table 1).

Construction of expression vectors for cytochromes c_2 and c_Y .

To facilitate protein purification, *cycA* and *cycY* were fused downstream of the *PrrnB* ribosomal promoter, a strong, constitutive promoter that was used to overproduce the products of each gene. For the *PrrnB*-*cycA* fusion, primers with the sequences 5'-GCAAG-ATCTTATCGCAATCATCTACCC-3' (designated C2U) and 5'-CG-AGAATTCATGTGCTCAAGCGC-3' amplified the *cycA* ORF. After digestion, the 665 bp amplicon was ligated into *Bam*HI/*EcoRI*-digested pYSW35 (Y. Wang & J. P. Shapleigh, unpublished), which carries the *PrrnB* promoter, yielding pWL200.

Cytochrome c_Y is membrane-bound, which makes its purification and use in turnover experiments problematic. Therefore, a clone encoding a hybrid protein was constructed, such that the membrane-spanning portion of c_Y was replaced with the signal sequence of cytochrome c_2 . Amplification of the *cycA* portion of the construct used primers with the sequences 5'-GCTAAGCTTGAAATGGATGATGCCC-3' and 5'-GCACTGCAGGTTGAAGGCTTCTCACC-3', resulting in production of a 0.6 kb fragment with *Hind*III and *PstI* sites. Amplification of the *cycY* portion used primers with the sequences 5'-GCACTGCA-GGCCTATGTGATCG-3' and 5'-CGAGAATTCAGTTGGGATTGCGTG-3' (designated CYE), the former annealing downstream of the region encoding the c_Y membrane-spanning domain. This amplification produced a 0.5 kb fragment with *PstI* and *EcoRI* sites. After digestion with appropriate enzymes, these two fragments were ligated into pUC19, which had been digested with *EcoRI* and *Hind*III. The resulting pWL202a contained 102 bp from the 5' end of *cycA* fused upstream and in-frame with the 396 bp of the 3' end of *cycY*. A second round of amplification, using pWL202a as a template and C2U and CYE as primers, resulted in production of a 0.65 kb fragment with an upstream *Bgl*II site and a downstream *EcoRI* site. After digestion, this fragment was ligated into *Bam*HI/*EcoRI*-digested pYSW35, yielding pWL202b. Due to problems encountered during purification of the soluble c_Y , the construct was further modified to include a histidine tag (HT). Two new primers, annealing to the pWL202b *PrrnB*-*cycY* construct, were designed such that the downstream primer would encode six histidine residues lying immediately upstream of the translation stop codon. The primer sequences were 5'-CAATATAGG-GAAAGCTTGCAT-3' and 5'-CCGGAATTCCTAGTGGTGATGGTG-GTGATGCTGAAGCGAGGCGAGATA-3', and allowed amplification of a 650 bp fragment containing the *cycA*-*cycY* fusion, as well as the *PrrnB* promoter region. After digestion with *EcoRI* and *Hind*III, this product was ligated into pRK415 to generate pWL202his. The protein produced by this fusion contained the first 34 residues of cytochrome c_2 , including the export signal sequence, fused in-frame to residues 44–131 of cytochrome c_Y .

Absorbance spectrum of crude extracts. For the analysis of mutants and complemented strains, cells were grown microaerobically in 100 ml cultures overnight at 30 °C, harvested, washed, and resuspended in 10 ml phosphate buffer (pH 7.4). Cells were lysed by two passages through a French pressure cell at 125 000 MPa. Cell-free extracts were prepared by centrifugation for 5 min at 23 000 g. The total protein concentration of the crude extracts was determined using the BCA Assay kit (Pierce) and samples were normalized to

1.8 mg ml⁻¹ prior to spectrophotometry. Dithionite-reduced minus air-oxidized absorbance spectra were obtained at wavelengths of 400–700 nm using a Beckman DU 640 spectrophotometer.

Protein purification. Nir was purified as previously described (Olesen *et al.*, 1998). For cytochrome *c*₂ purification, cells carrying pWL200 were cultured for 18 h under oxic conditions in 1 l Siström's medium, harvested, resuspended in 20 mM phosphate buffer (pH 7.4) and disrupted by two passages through a French pressure cell at 120 MPa. Crude extracts were prepared by centrifugation at 23 000 g for 20 min to remove cell debris, followed by a second centrifugation at 160 000 g for 3 h to remove membranes. The supernatant was loaded onto a column containing DEAE cellulose (Sigma) fast-flow anion exchanger. The extract was washed with ~10 column volumes of resuspension buffer followed by stepwise washes with 5–10 volumes of resuspension buffer containing 40 mM NaCl and then 80 mM NaCl. Cytochrome *c*₂ was eluted after addition of phosphate buffer 120 mM in NaCl.

For cytochrome *c*_Y-HT purification, cells were cultured for 18 h under oxic conditions in 1 l Siström's medium, disrupted, and a high-speed supernatant prepared, as described for purification of *c*₂. Purification of the His-tagged protein was achieved using nickel-nitriloacetic acid (Ni-NTA) agarose, as previously described (Bartnikas *et al.*, 2002).

Western immunoblot analysis. The expression and purification of Nir have been described previously (Olesen *et al.*, 1998). To further purify Nir for antibody production, the protein was resolved on a 12% SDS-polyacrylamide gel, which was stained with Coomassie blue in water. The band corresponding to the expected size of Nir was isolated, pulverized and sent to the Center for Research Animal Resources, Cornell Veterinary School, Ithaca, NY, USA, where it was injected into a rabbit for production of anti-Nir antibodies.

Cultures used in Western blotting were grown overnight to mid-exponential phase in the presence of nitrate. Crude extracts for immunoblotting were prepared by harvesting overnight cultures, resuspending in 3.0 ml of 50 mM MOPS buffer (pH 7.4) and disrupting using a French pressure cell. Immediately before cell disruption, protease inhibitors were added at the following final concentrations: 2 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ pepstatin and 1 mM PMSF. Cell debris was removed by centrifugation at 12 000 g for 15 min. Total protein concentration of cell extracts was determined using the BCA-200 Protein Assay kit (Pierce). Sample extracts were resolved using SDS-PAGE. Proteins were transferred to PVDF membranes (BioTrace, 0.45 µm pore-size, Pall Gelman Sciences). The membrane was exposed overnight to antibody in PBS with 3% dry milk, using anti-Nir antiserum (1:1600). This was followed by a 1.5 h exposure to goat anti-rabbit IgG (Fc)-alkaline phosphatase conjugate (1:5000) (Promega). Immunoblots were visualized using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche).

Cytochrome oxidation. Prior to use, all cytochrome *c* preparations were reduced with ascorbate. Excess ascorbate was removed using a PD-10 desalting column (Amersham Biosciences). After reduction, the cytochrome preparations were adjusted to a final absorbance at 550 nm of 0.12–0.15, which gave a final concentration of ~5–8 µM. Cytochromes were prepared in 50 mM MES at pH 6.0, which is the optimal pH for Nir activity (Olesen *et al.*, 1998). Reduced cytochrome (0.5 ml) was added to a cuvette and placed in the spectrophotometer. Oxidation of the cytochrome was monitored by a decrease in absorbance at 550 nm for *c*₂ or at 551 nm for *c*_Y, after manual addition of 50 µl of 50 mM MES containing one of the following: varying concentrations of Nir and 200 µM nitrite; 20 nM Nir; or 200 µM nitrite. Changes in absorbance were recorded using a Beckman DU 640 spectrophotometer. For assays using cytochrome oxidase, the reactions were run in 25 mM HEPES (pH 6.5), 50 mM

KCl and 0.1% dodecylmaltoside. Each reaction used 150 nM purified oxidase. For turnover calculation, a $\Delta\epsilon_{(\text{Red-Ox})550}$ value of $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was used (Jensen *et al.*, 1981).

Enzymic assays. To estimate Nir activity, cells were grown under denitrifying conditions, typically to an optical density at 600 nm of ~0.7–0.8. For most experiments 500 µl of culture was removed, washed twice in an equal volume of phosphate buffer (pH 7.4) and resuspended in 500 µl phosphate buffer (pH 7.4). Then, 36 nmol sodium nitrite was added to each tube and the cells were incubated at 30 °C. In a modification of a previously described protocol for quantifying nitrite accumulation (Stewart & Parales, 1988), a colorimetric assay was used to measure the decrease in nitrite concentration over time, which correlates to Nir activity. Nitrite concentration in this assay is proportional to the *A*₅₄₀ of a sample. Thus, by subtracting the *A*₅₄₀ obtained during a test reaction from the *A*₅₄₀ of a standard containing 36 nmol sodium nitrite in the absence of cells, we were able to quantify the amount of nitrite reduced. Activity was calculated by using the formula units, $100 A_{540}/t \text{ OD}_{600}$, where *A*₅₄₀ represents nitrite consumed during the course of incubation (*A*_{540standard} – *A*_{540test}), *t* is the time of incubation in min and OD₆₀₀ is the density of cells in the culture. For experiments with antimycin A (Sigma), cells were preincubated for 10 min with 7.5 µM antimycin A prior to measurement of Nir activity. Antimycin A stock solutions were made fresh prior to use.

RESULTS

Sequence analysis

Based on DNA sequence analysis, the *cycA* gene from strain 2.4.3 was predicted to encode a protein 155 aa in length (Fig. 1A). Sequence data also indicated that the organization of genes immediately flanking *cycA* in strain 2.4.3 was the same as in the non-denitrifying strain *R. sphaeroides* 2.4.1, indicating that this gene is monocistronic. The predicted protein was slightly larger than its orthologue in strain 2.4.1 due to a 12 aa extension at the C terminus. Such extensions are not typical of putative cytochrome *c*₂ orthologues, but cytochrome *c*₅₅₀ from *P. denitrificans* contains an extension that has sequence similarity to the extension in *c*₂ (Van Spanning *et al.*, 1990). The deduced cytochrome *c*₂ sequences from the 2.4.1 and 2.4.3 strains have 71% identity (Fig. 1A). Based on their precursor polypeptide sequences, cytochrome *c*₂ from both 2.4.1 and 2.4.3 had a CxxCH haem-binding motif beginning at residue 36. A number of conserved lysine residues (Lys31, Lys116, Lys118, Lys120 and Lys146) found in strain 2.4.1 cytochrome *c*₂ are believed to make critical interactions with redox partners (Brandner *et al.*, 1991; Hall *et al.*, 1989). The deduced sequence of *c*₂ from strain 2.4.3 had arginine at positions 118 and 146 and lysine at the remaining three positions (Fig. 1A).

Sequence analysis of *cycY* revealed a 525 bp ORF encoding a protein with a predicted length of 175 aa. The predicted sequence from strain 2.4.3 had 84% amino acid identity with the strain 2.4.1 orthologue and was one amino acid longer (Fig. 1B). Interestingly, the predicted N-terminal membrane anchor for the strain 2.4.3 protein was identical to the strain 2.4.1 orthologue, with the exception of a Ser to Thr substitution at position 29 (Fig. 1B). It has been suggested that four lysines, residues 81, 85, 145 and 160, of

(A) Signal sequence					#	##
2.4.3	MKFEIKALAA	IAALAAAPAL	AQEGDAAAGE	KAFNQCCQCH	-40	
2.4.1	<u>MKFOYKALAA</u>	<u>IAAFALPAL</u>	<u>AQEGDPEAGA</u>	KAFNQCCQCH	-40	
	**	*****	*** ** *	***** *	*****	
2.4.3	VIVDDAGTTI	AGRNAKTGPN	LYGVVGRQAG	TREDFKGYGK	-80	
2.4.1	VIVDESSTTI	AGRNAKTGPN	LYGVVGRTAG	TQADFKEGYGE	-80	
	***	*****	*****	*** ** *	*****	
2.4.3	DLVAAGESGL	VWNEEQFTAY	TKDPTAFLKE	YTDNSKARGK	-120	
2.4.1	GMKEAGAKGL	AWDEEHFYGY	VQDPTKFLKE	YTGDAKAKGK	-120	
	**	**	** ** *	*** ** *	** ** *	
2.4.3	MTYKVRKEED	AHNIWAYL--	VSVSPAEEA	PAEAATN	-155	
2.4.1	MTFKLKKEAD	AHNIWAYLQQ	VAVRP		-145	
	**	**	*****	** *		
(B) Membrane anchor						
2.4.3	MPDITMTLTKA	VGAGCGALLV	FLGSGWAATG	LYTTGGGHGE	-40	
2.4.1	MPDITMTLTKA	VGAGCGALLV	FLGSGWAASG	LYTFGEHGE	-40	
	*****	*****	*****	*** *	*****	
2.4.3	DAVQAYVIET	GGGGAEEEP	DAEAVPFAEL	VAAADPAAGQ	-80	
2.4.1	DVTQAYVIDT	GASAAEE-P	AAEAVPFPPEL	VAAADAAAGH	-79	
	**	***** *	***** *	***** *	***** *	
	•	•	•	•		
2.4.3	KVYAKCAACH	KLDGSDATGP	HLGDIIDRAV	AGVDGFAYSD	-120	
2.4.1	KVFAKCAACH	KVDGSDATGP	HLNGVVDRAV	ASVGGFAYSD	-119	
	**	***** *	*** ** *	** ** *	*****	
2.4.3	AMVAHAADAP	AWTVEELGAF	VQNPRSHVPG	TKMTFAGLFPK	-160	
2.4.1	AMVAHAADAP	TWTLEELGAF	VENPKGHVPG	TKMTFAGLFPK	-159	
	*****	**	*****	** ** *	*****	
2.4.3	EQDRADVVA	YLASLQ			-175	
2.4.1	EQDRANVVA	YLASLQ			-174	
	*****	**	*****			

Fig. 1. Alignment of amino acid sequences of cytochrome c_2 and c_Y from *R. sphaeroides* strains 2.4.3 and 2.4.1. Asterisks underneath residues indicate conserved residues, residues postulated to be involved in interaction with redox partners (Brandner *et al.*, 1991; Myllykallio *et al.*, 1999) are overscored with a black dot, and the CxxCH haem-binding motifs are overscored with #. (A) Cytochrome c_2 ; the underlined region represents the 21 aa signal sequence (Brandner *et al.*, 1991). (B) Cytochrome c_Y ; the underlined residues represent the predicted membrane anchor region (Myllykallio *et al.*, 1997, 1999).

the strain 2.4.1 cytochrome c_Y correspond to those in equine cytochrome c that are believed to interact with redox partners (Myllykallio *et al.*, 1999). Three of these lysines were conserved in the strain 2.4.3 sequence, while there was a conservative substitution to arginine at the position corresponding to residue 145 of the strain 2.4.1 sequence (Fig. 1B).

Disruption of *cycA* and *cycY*

To investigate whether cytochrome c_2 or c_Y are electron donors to Nir, the *cycA* and *cycY* genes were disrupted in strain 2.4.3. For *cycY*, chromosomal disruption was accomplished using allelic exchange by selecting for resistance to streptomycin/spectinomycin. The resulting strain was designated CYT-Y (Table 1). The same strategy was employed for construction of a *cycA* mutant and a *cycA*-*cycY* double mutant, except that resistance to kanamycin was selected. In the latter case, the plasmid used to disrupt *cycA* was mobilized into strain CYT-Y. The c_2 -deficient strain was designated CYT-2 and the double mutant CYT-2/Y. Absorption

spectra of extracts from the various strains showed that loss of c_Y only slightly reduced the amplitude of the peak in the 550 nm region of the spectrum, where the α band of c -type cytochromes has maximal absorption (data not shown). However, the loss of c_2 caused a significant decrease in the absorbance maximum in the 550 nm region. The strain devoid of both cytochromes showed the greatest decrease in the 550 nm region, greater than would be predicted by the changes observed in the single mutants.

The phenotypes of strains CYT-Y and CYT-2 were similar to that of the wild-type strain, with the exception that CYT-2 was unable to grow photosynthetically (Fig. 2, segment B). By comparison, when CYT-2/Y cells were grown aerobically they had a much darker pigmentation and an approximately twofold slower growth rate than wild-type cells grown under the same conditions. As expected, the double mutant was unable to grow photosynthetically due to a loss of c_2 (Fig. 2, segment D). The slow aerobic growth of strain CYT-2/Y suggests impairment in the aerobic respiratory chain, most likely a restriction in flow of electrons from the bc_1 complex to the cytochrome c oxidases. The change in pigmentation of these cells is consistent with previous observations in *R. sphaeroides* 2.4.1 demonstrating that disruption of electron flow, because of the loss of these two cytochromes, results in the aerobic expression of light-harvesting complexes (Mouncey *et al.*, 2000; Oh & Kaplan, 1999, 2001).

To assess the effect of the various mutations on Nir activity, nitrite accumulation was measured during growth in nitrate-supplemented media under limiting oxygen. Neither the wild-type strain nor either of the single mutants was observed to accumulate nitrite. The double mutant, strain CYT-2/Y, accumulated large amounts of nitrite (Table 2). To verify that the observed phenotypes were associated with a loss of Nir activity, relative *in vivo* activity was measured in

Fig. 2. Comparison of growth of wild-type and various mutant strains after incubation for 7 days under photosynthetic conditions. Sectors: A, wild-type strain 2.4.3; B, strain CYT-2; C, CYT-2 containing pWL202his; D, CYT-2/Y; E, strain CYT-2/Y containing pWL202his.

the wild-type and the mutant strains when grown in nitrate-supplemented medium under limiting oxygen. The wild-type strain had ~10 units of activity, as did both CYT-Y and CYT-2. Strain CYT-2/Y, however, had only 0.5 units of activity (Table 2).

To confirm that the phenotypic changes in CYT-2/Y arose from the loss of both cytochromes and was not due to polarity effects, the strain was complemented with either *cycA* or *cycY*. Complementation of CYT-2/Y with *cycA* from strain 2.4.1 (Donohue *et al.*, 1986) resulted in wild-type growth rates under oxic conditions and restored both photosynthetic growth and nitrite utilization (data not shown). Complementation with *cycY* from strain 2.4.3 also restored near wild-type growth rates under oxic conditions and nitrite utilization but did not restore the ability of this strain to grow photosynthetically.

The decrease in Nir activity seen in CYT-2/Y would be expected to be accompanied by a decrease in Nir levels, since *nirK* expression requires Nir turnover (Tosques *et al.*, 1997). To confirm that Nir levels decrease in strain CYT-2/Y, Nir levels in strain 2.4.3 and the double mutant were compared by Western blotting. Both strains were grown microaerobically in nitrate-supplemented media. As shown in Fig. 3, the Nir concentrations in strain CYT-2/Y were between eight and 16-fold lower than the wild-type strain under these growth conditions.

Heterologous expression of *nirK*

If c_2 and c_Y are the principal electron donors to Nir it would be predicted that expression of *nirK* using a heterologous, NO-independent promoter would not restore Nir activity in CYT-2/Y. To test this, pWLNIR, which contains *nirK* under the control of the *PrnB* ribosomal promoter, was moved into CYT-2/Y. As a control, pWLNIR was also moved into strain 11.10 (Tosques *et al.*, 1997), which carries a disruption in *nirK* (Table 1). pWLNIR restored the ability of strain 11.10 to utilize nitrite (not shown), and the *in vivo* Nir activity of this strain was not significantly different from that of the wild-type strain (Table 3). This indicates that use of

Fig. 3. Nir levels in strain CYT-2/Y in comparison to 2.4.3. Both strains were cultured microaerobically in Sistrom's medium supplemented with nitrate. The total protein concentration of crude extracts was normalized. Crude extracts of the wild-type strain are in the lanes above the bracket and diluted as indicated. Lane C is the crude extract from CYT-2/Y.

the ribosomal promoter to regulate *nirK* expression does not lead to anomalously high levels of Nir activity. The presence of pWLNIR in CYT-2/Y resulted in a Nir activity that was about 10-fold higher than that of CYT-2/Y without this plasmid, and half that of the wild-type strain (Table 3). To eliminate the possibility that pWLNIR caused a large overexpression of Nir that resulted in the activity observed in CYT-2/Y, a Western blot was run to determine the level of Nir in strains carrying pWLNIR. As can be seen in Fig. 4, the level of Nir in CYT-2/Y with pWLNIR was not significantly higher than that in wild-type strain 2.4.3. This is consistent with previous results based on activity assays (Laratta *et al.*, 2002). Therefore, it is unlikely that overexpression from pWLNIR was the cause of the activity in the CYT-2/Y strain with pWLNIR. This suggests that loss of c_2 and c_Y does not preclude electron transfer to Nir.

The nature of the component transferring electrons to Nir in the absence of cytochromes c_2 and c_Y is unclear. Since pseudoazurin is an unlikely electron donor (Jain & Shapleigh,

Table 2. Effect of cytochrome mutations on Nir activity of strains cultured microaerobically in media supplemented with nitrate

Each measurement represents the mean of at least three separate experiments.

Strain genotype	Nitrite accumulation*	Nir activity†
Wild-type	—	10.6 ± 0.3
<i>cycA</i>	—	9.7 ± 0.4
<i>cycY</i>	—	10.7 ± 0.7
<i>cycA-cycY</i>	+++	0.3 ± 0.2

*Nitrite levels were measured after cells had grown for 24 h.

†Whole cell Nir activity, as previously described (Laratta *et al.*, 2002).

Table 3. Nir activity in the presence or absence of antimycin A, an inhibitor of the bc_1 complex

Strain genotype*	Antimycin A	Nir activity†
Wild-type	—	10.6 ± 0.3
Wild-type	+	0.6 ± 0.2
<i>nirK</i> (11.10) pWLNIR	ND‡	10.0 ± 0.2
<i>cycA-cycY</i> pWLNIR	—	4.9 ± 0.6
<i>cycA-cycY</i> pWLNIR	+	0.5 ± 0.1

*pWLNIR carries *nirK* expressed from a ribosomal promoter, eliminating regulatory effects due to redox status and nitric oxide production.

†Whole cell Nir activity, as previously described (Laratta *et al.*, 2002).

‡Assay not performed.

Fig. 4. Nir levels in strains containing pWLNIR in comparison to 2.4.3. Cells were grown microaerobically in the presence of nitrate, lysed and the total protein concentration of crude extracts normalized. Lane 1, wild-type strain 2.4.3; lane 2, strain 2.4.3 + pWLNIR; lane 3, strain CYT-2/Y + pWLNIR. The numbers to the left of the first lane represent the size, in kDa, of the standards. Samples were resolved on a 12% denaturing acrylamide gel, transferred to a PVDF membrane, exposed to anti-Nir antiserum (1:1600), and then alkaline phosphatase (AP)-conjugated goat anti-rabbit antibody (1:5000) (Promega). Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche) were used for visualization.

2001), another c -type cytochrome was probably the donor to Nir in the double mutant. If so, this protein would likely receive electrons from the bc_1 complex. To test this, Nir activity assays were carried out in the CYT-2/Y strain containing pWLNIR in the presence of antimycin A, which inhibits electron transfer through the bc_1 complex. Under these conditions there was no detectable Nir activity (Table 3), demonstrating that the protein that transferred electrons to Nir did so after receiving electrons from the bc_1 complex.

Cytochrome oxidation by Nir

Experiments with pWLNIR in CYT-2/Y indicate that there is a protein other than c_2 or c_Y in strain 2.4.3 capable of transferring electrons to Nir. The somewhat diminished Nir activity in this strain, relative to wild-type, suggests that c_2 or c_Y is also a source of electrons for Nir. Therefore, an *in vitro* approach, using purified c_2 and a purified soluble form of c_Y , was utilized to determine more directly the relative abilities of each cytochrome to reduce Nir. To facilitate protein purification, *cycA* and a *cycA*–*cycY* fusion were placed under the regulation of the *PrnB* promoter. Unexpectedly, the expression of the soluble c_Y restored the ability of the c_2 -deficient strain to grow photosynthetically (Fig. 2, segment C). This soluble form of c_Y also restored photosynthetic growth to CYT-2/Y, indicating that electron transfer to the reaction centre did not require a membrane-bound copy of c_Y , although growth was enhanced if the native protein was present (Fig. 2, segment E). These results indicate that the soluble c_Y is functionally equivalent to c_2 when the cells are growing photosynthetically.

Purification of c_2 was achieved using previously published protocols (Meyer & Cusanovich, 1985). The isolated protein was highly purified, as judged by both Coomassie blue

staining (data not shown) and haem staining, which showed only a single c -type cytochrome (Fig. 5C). Absorption spectra of the reduced, purified protein showed absorbance maxima at 415.5 nm in the Soret region and at 550 nm in the α region of the spectrum (Fig. 5A). Reduced, purified c_2 was oxidized in the presence of Nir and nitrite (Fig. 6A). The addition of either Nir or nitrite alone had no effect on cytochrome oxidation, demonstrating that both were necessary for electron transfer from the reduced cytochrome.

The soluble form of c_Y was purified by fusing an HT to the haem-binding domain. Purification using Ni-NTA yielded a

Fig. 5. Characterization of purified cytochrome c_2 and c_Y . (A) As-purified (dashed line) and dithionite-reduced (solid line) spectra of purified cytochrome c_2 . (B) As-purified (dashed line) and dithionite-reduced (solid line) spectra of purified cytochrome c_Y . (C) Haem stain of purified protein after SDS-PAGE on a 15% gel. Lane 1, purified His-tagged c_Y ; lane 2, purified c_2 ; lane 3, horse heart cytochrome c as a haem control. Values to the left of the gel are the molecular masses, in kDa, of the two protein standards closest in size to the cytochromes. The horse heart haem control had a molecular mass of ~ 12.4 kDa.

Fig. 6. Progress curves of oxidation of *c*-type cytochromes in the presence of Nir. (A) Changes in absorbance at 550 nm of purified c_2 from *R. sphaeroides* 2.4.3 after various additions. (B) Changes in absorbance at 551 nm of purified His-tagged cytochrome c_Y . Cytochromes were prepared in 50 mM MES buffer (pH 6.0), reduced with ascorbic acid and run over a PD-10 desalting column (Amersham Biosciences). Reduced cytochrome (0.5 ml), adjusted to have a starting absorbance of ~ 0.12 , was added to a cuvette and placed in a Beckman DU-640 spectrophotometer. Kinetics were monitored after addition of 50 μ l of 50 mM MES buffer containing: (A) 20 nM Nir and 200 μ M nitrite (■); 20 nM Nir (▲); 200 μ M nitrite (◆); (B) 20 nM Nir and 200 μ M nitrite (■); 20 nM Nir (▲); 150 nM Nir and 200 μ M nitrite (◆); 1 μ M Nir and 200 μ M nitrite (▼).

soluble cytochrome with maxima at 416 nm in the Soret and 551 nm in the α region, consistent with spectra of the purified, intact protein (Fig. 5B) (Myllykallio *et al.*, 1997). Both Coomassie blue (data not shown) and haem staining revealed that the purified material consisted of three *c*-type cytochromes of similar size (Fig. 5C). A similar pattern of bands was observed after partial purification of a soluble c_Y lacking the HT (data not shown). The extra bands were most likely forms of c_Y , since no haem-staining material of this size was purified from cells lacking the His-tagged chimera. The purification of multiple bands suggests that removal of the signal sequence occurred at more than one point along the primary sequence as the c_2 - c_Y fusion was exported.

In contrast to experiments with c_2 , no oxidation of the purified c_Y was observed in the presence of 20 nM Nir and nitrite (Fig. 6B). Similar results were obtained using the truncated c_Y lacking the HT (data not shown). If the Nir concentration was increased to 150 nM, a moderate level of c_Y oxidation could be detected (Fig. 6B). Oxidation of c_2 in the presence of 150 nM Nir was nearly complete in the interval between addition of Nir and commencement of absorbance measurements (data not shown). Further increasing Nir to 1.0 μ M resulted in a rate of c_Y oxidation that was similar to that observed for c_2 in 20 nM Nir (Fig. 6B). In an additional test, c_Y was found to be oxidized by purified aa_3 -type cytochrome oxidase from *R. sphaeroides* 2.4.1. In this case, >100 nM oxidase had to be present to obtain a detectable rate of oxidation (data not shown).

DISCUSSION

In this study, the genes encoding cytochromes c_2 and c_Y were disrupted to assess their involvement in shuttling electrons to Nir during denitrification. Disruption of either the *cycA* or *cycY* gene alone had no discernible effect on Nir activity, though disruption of the former did prevent photosynthetic growth. On the other hand, when both genes were disrupted, Nir activity was impaired. Importantly, complementation of the double mutant with either the *cycA* or *cycY* gene restored Nir activity. Complementation with *cycA* also restored photosynthetic growth. These complementation experiments demonstrate that the phenotypic effects observed were due directly to the disruption of the respective gene rather than polar effects of the mutations.

The simplest interpretation of the loss of Nir activity in CYT-2/Y is that cytochrome c_2 and cytochrome c_Y are the principal sources of electrons for Nir. This explanation is unsatisfactory, however, since it does not account for the significant levels of Nir activity observed in CYT-2/Y when *nirK* was expressed from a heterologous promoter (Table 3). The protein(s) donating electrons to Nir in CYT-2/Y with pWLNIR should also be present in CYT-2/Y, since the only difference between the two strains is in the regulation of *nirK*. This indicates that electron flow to Nir does not limit Nir activity in CYT-2/Y. An alternative explanation, consistent with these observed results, is that the decrease in Nir activity observed in CYT-2/Y is due to a change in the expression of *nirK*. Investigation into the regulation of *nirK* has found that inactivation of one of the genes encoding the *cbb_3* oxidase leads to a significant decrease in its expression (Laratta *et al.*, 2002). Work with *R. sphaeroides* 2.4.1, which lacks *nirK*, has shown that a decrease in activity of *cbb_3* oxidase has a significant impact on the expression of numerous genes (Oh & Kaplan, 2000). These changes were observed either when genes encoding the *cbb_3* oxidase were inactivated or when the genes encoding both c_2 and c_Y were inactivated (Oh & Kaplan, 2000). It is proposed that loss of both c_2 and c_Y limited turnover of *cbb_3* making the turnover of this oxidase the key factor in the observed regulatory anomalies. If this is true, it is not unreasonable to

speculate that the decrease in Nir activity in CYT-2/Y is due to changes in *nirK* expression as a consequence of a decrease in *cbb₃* activity. How inactivation of the *cbb₃* oxidase might lead to changes in gene expression is unclear, but it may involve the two-component global regulatory system PrrBA (Oh *et al.*, 2004).

Turnover of Nir with truncated c_Y gave a turnover number of $\sim 0.1 \text{ s}^{-1}$. Previous work with other truncated cytochromes has shown that removal of the membrane anchor does not have a significant impact on a protein's catalytic capacity. In particular, truncation of the membrane-bound c_{552} of *P. denitrificans*, a homologue of c_Y , does not significantly affect its ability to transfer electrons to the *aa₃*-type oxidase (Drosou *et al.*, 2002). Under nearly identical conditions, turnover of Nir with c_2 was $\sim 5 \text{ s}^{-1}$. While these values likely underestimate the true turnover rates, due to the difficulty in obtaining accurate initial rates of cytochrome oxidation, it is unlikely that activity is significantly underestimated. Previous reports have noted slow turnover with c_2 as a donor, and the fastest turnover rates, $\sim 50 \text{ s}^{-1}$, are obtained using a yeast isocytochrome (Zhao *et al.*, 2002).

The identity of additional protein(s) transferring electrons to Nir in CYT-2/Y is unclear. In other denitrifiers, copper-containing proteins such as azurin or pseudoazurin have been found to donate electrons to Nir. *R. sphaeroides* encodes a pseudoazurin-like protein, but this protein is an unlikely electron donor to Nir, since it is missing three of the four residues critical for copper ligation and is unable to bind copper (Jain & Shapleigh, 2001). A nitrate-inducible *c*-type cytochrome has been identified in *R. sphaeroides* f. sp. *denitrificans*, suggesting that there might be additional *c*-type cytochromes involved in denitrification (Rott *et al.*, 1992). However, no additional work has been done to characterize this cytochrome.

The ability of soluble c_Y to restore photosynthetic growth to strains CYT2 and CYT2/Y (Fig. 2, segments C and E) was unexpected. Previous work comparing the c_Y cytochrome from *R. capsulatus* with the one from *R. sphaeroides* suggests that only the *R. capsulatus* protein can transfer electrons to the photosynthetic reaction centre (Myllykallio *et al.*, 1999). Even a chimeric cytochrome, consisting of the *R. capsulatus* anchor-linker domain fused to the *R. sphaeroides* cytochrome c_Y domain, did not support photosynthetic growth in a cytochrome c_2 -deficient *R. sphaeroides* strain, a fact that suggests that the *R. sphaeroides* cytochrome c_Y might have a low binding affinity for the photosynthetic reaction centre (Myllykallio *et al.*, 1999). Both versions of the soluble c_Y produced in this work were expressed from a strong ribosomal promoter, making it possible that the production of an excess of cytochrome c_Y , in the absence of intrinsic physical constraints relating to membrane anchoring, could overcome issues of low binding affinity, allowing sufficient electron transfer to the reaction centre to restore photosynthetic growth.

In conclusion, the work presented here better defines the roles of both cytochrome c_2 and cytochrome c_Y in electron transfer during denitrification. While genetic studies suggest that either electron carrier is equally effective at donating electrons to Nir, this conclusion is not supported by *in vitro* assays of cytochrome oxidation in the presence of Nir and nitrite. This result demonstrates that care must be taken in interpreting the results of other studies that use only genetic approaches for identifying electron donors to Nir.

ACKNOWLEDGEMENTS

We wish to thank Sam Kaplan and Tim Donohue for generous gifts of plasmids and strains. We also thank Jon Hosler for purified oxidase and helpful discussions, and Charles Scholes for concentrated Nir. This work was supported by the Department of Energy (95ER20206).

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