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

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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 ~0.1 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.

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.
(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 $c_Y$, $c_Y$ 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 $c$, 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 $c$ 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 DH5x 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 concentrations: Tc, tetracycline; Km, kanamycin; Sm/Sp, streptomycin/spectinomycin.

### Table 1. Bacterial stains and plasmids used in this study

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

<table>
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<th>Strains/plasmids</th>
<th>Genotype or description</th>
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<td>S17-1</td>
<td>For conjugal transfer of plasmids from <em>E. coli</em> into <em>R. sphaeroides</em>: recA thi pro hsdRM+ RP4:2-Tc: Mu: Km: Tn7</td>
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<td>2.4.3</td>
<td>Wild-type denitrifying strain of <em>R. sphaeroides</em></td>
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<td>Tosques et al. (1997)</td>
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<td>CYT-2</td>
<td>cycA:: aph; 2.4.3 derivative; Km'</td>
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<td>cycY:: spe/str; 2.4.3 derivative; ΩSm'/Sp'</td>
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<td>Used for cloning in <em>E. coli</em></td>
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<td>Source of aph gene; used as restriction site mobilizing element</td>
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<td>pH45</td>
<td>Source of spe/str cassette</td>
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<td>Broad-host-range plasmid (Tc')</td>
<td>Simon et al. (1983)</td>
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<td>pSUP202</td>
<td>Mobilizable suicide vector</td>
<td>F. Wang &amp; J. P. Shapleigh</td>
</tr>
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<td>pYSW35</td>
<td>pRK415 carrying the PrmB ribosomal promoter (Tc')</td>
<td>Y. Wang &amp; J. P. Shapleigh</td>
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<td>Derivative of pYSW35 with PrmB--nirK fusion (Tc')</td>
<td>Laratta et al. (2002)</td>
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<td>pCP2P04.1</td>
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<td>pRK415 with a PrmB'–cycA'--cycY fusion encoding a C-terminal HT (Tc')</td>
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concentrations: ampicillin, 100 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; streptomycin/spectinomycin, 25 µg ml⁻¹; kanamycin, 25 µg ml⁻¹. *Rhodobacter* strains were grown in Siström's minimal medium at 30°C (Leuking et al., 1978). When appropriate, antibiotics were added to *Rhodobacter* cultures at the following concentrations: tetracycline, 1 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; streptomycin/spectinomycin, 50 µg ml⁻¹ each. For photosynthetic growth, cultures were cultured over indigence 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'-GGGTACCTCGATGTCGGCATAG-3', and a second set of primers was used to amplify a smaller internal fragment (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'-GGGGATCCCTGCTTCTGGACGAGG-3' and 5'-GGGAATTCGAAATCGGCCGGATATT-3', respectively. Amplification of the *cycY* fragment was digested with *EcoRI* and *KpnI*, cloned into pUC19 and sequenced (Table 1). The cloned fragment was missing the first 165 bases of the *cycY* ORF. This truncated *cycY* ORF was ligated into pSU202 (Simon et al., 1983) and the resulting pSU1-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 *cycA* and *cycY*.** To facilitate protein purification, *cycA* and *cycY* were fused downstream of the *PrnrB* ribosomal promoter, a strong, constitutive promoter that was used to overproduce the products of each gene. For the *PrnrB-cycA* fusion, primers with the sequences 5'-GCAAGATATCCATGTCAGCTGAAAGCTGAA-3' and 5'-CGAGAAATGTATGTCAGCTGAAAGCTGAA-3' were used to amplify fragments of *cycA* and *cycY*. Amplification of the *cycA* portion of the construct used primers with the sequences 5'-GCTAAGCTTGGAAATGAGGATGCC-3' and 5'-GCACTGCAAGTGGAAATGAGGATGCC-3', resulting in production of a 0.6 kb fragment with HinIII and *PstI* sites. Amplification of the *cycY* portion used primers with the sequences 5'-GCAAGATATCCATGTCAGCTGAAAGCTGAA-3' and 5'-CGAGAAATGTATGTCAGCTGAAAGCTGAA-3' (designated CYE), the former annealing downstream of the region encoding the *cycY* 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 *HindIII*. The resulting plasmid was ligated into pUC19 and sequenced (Table 1). To inactivate *cycY*, pUC19-cycA was partially digested with *BamHI*, which carries the *BamHI* site and a downstream fragment, and a primer for RSP0295, 5'-GGTACCTCGATGTCGGCATAG-3', annealing to the pWL202b *rrnB-P* promoter, a strong, constitutive promoter from *R. sphaeroides*. For the analysis of the *cycA* and *cycY* expression vectors, the construction of plasmids and strains.

**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 mg ml⁻¹. The *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 mg ml⁻¹.
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 c2 purification, cells carrying pWL200 were cultured for 18 h under oxic conditions in 1 l Sistrom’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 c2 was eluted after addition of phosphate buffer 120 mM in NaCl.

For cytochrome c5-HT purification, cells were cultured for 18 h under oxic conditions in 1 l Sistrom’s medium, harvested, and a high-speed supernatant prepared, as described for purification of c2. Purification of the His-tagged protein was achieved using nickel-nitriolate 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 (1:16 000). 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 c5 or at 551 nm for c2, after manual addition of 50 μl of 50 mM MES containing one of the following: varying concentrations of Nir and 200 μM nitrite; 20 mM 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 mM purified oxidase. For turnover calculation, a ΔA/Δt (Red-Orn)/550 value of 2·1 × 104 M−1 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 A540 of a sample. Thus, by subtracting the A540 obtained during a test reaction from the A540 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 A540/OD600 where A540 represents nitrite consumed during the course of incubation (A540standard − A540test), 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 c2 orthologues, but cytochrome c550 from P. denitrificans contains an extension that has sequence similarity to the extension in c2 (Van Spanning et al., 1990). The deduced cytochrome c2 sequences from the 2.4.1 and 2.4.3 strains have 71 % identity (Fig. 1A). Based on their precursor polypeptide sequences, cytochrome c2 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 c2 are believed to make critical interactions with redox partners (Brandner et al., 1991; Hall et al., 1989). The deduced sequence of c2 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

### References

Role of c2 and cY in nitrite reduction

Disruption of cycA and cycY

To investigate whether cytochrome c2 or cY 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 cycA-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 cY 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 c2 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 c2 (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 bc1 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

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Fig. 1. Alignment of amino acid sequences of cytochrome c2 and cY 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 c2; the underlined region represents the 21 aa signal sequence (Brandner et al., 1991). (B) Cytochrome cY; the underlined residues represent the predicted membrane anchor region (Myllykallio et al., 1997, 1999).

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.
Complementation with tosynthetic growth and nitrite utilization (data not shown). 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 c2 and cY 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 PrrnB 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 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 c2 and cY does not preclude electron transfer to Nir.

The nature of the component transferring electrons to Nir in the absence of cytochromes c2 and cY is unclear. Since pseudoazurin is an unlikely electron donor (Jain & Shapleigh, 1992), its absence in CYT-2/Y suggests an alternative mediator.

**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.

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Nitrite accumulation*</th>
<th>Nir activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>–</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>cycA</td>
<td>–</td>
<td>9.7 ± 0.4</td>
</tr>
<tr>
<td>cycY</td>
<td>–</td>
<td>10.7 ± 0.7</td>
</tr>
<tr>
<td>cycA–cycY</td>
<td>+ + +</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

*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 bc1 complex**

<table>
<thead>
<tr>
<th>Strain genotype*</th>
<th>Antimycin A</th>
<th>Nir activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>–</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>nirK (11.10)</td>
<td>ND $</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>cycA–cycY pWLNIR</td>
<td>–</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>cycA–cycY pWLNIR</td>
<td>+</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

*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.
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 PrrnB 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$_Y$ 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 $\alpha$ 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
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 cyY lacking the HT (data not shown). The extra bands were most likely forms of cyY, 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 c2-cY fusion was exported.

In contrast to experiments with c2, no oxidation of the purified cY was observed in the presence of 20 nM Nir and nitrite (Fig. 6B). Similar results were obtained using the truncated cY lacking the HT (data not shown). If the Nir concentration was increased to 150 nM, a moderate level of cY oxidation could be detected (Fig. 6B). Oxidation of c2 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 μM resulted in a rate of cY oxidation that was similar to that observed for c2 in 20 nM Nir (Fig. 6B). In an additional test, cY was found to be oxidized by purified aa2-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 c2 and cY 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 c2 and cytochrome cY 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 cbb3 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 cbb3 oxidase has a significant impact on the expression of numerous genes (Oh & Kaplan, 2000). These changes were observed either when genes encoding the cbb3 oxidase were inactivated or when the genes encoding both c2 and cY were inactivated (Oh & Kaplan, 2000). It is proposed that loss of both c2 and cY limited turnover of cbb3 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 cY 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 c552 of P. denitrificans, a homologue of cY, 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 c2 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 c2 as a donor, and the fastest turnover rates, $\sim 50 \text{ s}^{-1}$, are obtained using a yeast iso cytochrome (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 cY to restore photosynthetic growth to strains CYT2 and CYT2/Y (Fig. 2, segments C and E) was unexpected. Previous work comparing the cY 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 (Myllýkallio et al., 1999). Even a chimeric cytochrome, consisting of the R. capsulatus anchor-linker domain fused to the R. sphaeroides cytochrome cY domain, did not support photosynthetic growth in a cytochrome c2-deficient R. sphaeroides strain, a fact that suggests that the R. sphaeroides cytochrome cY might have a low binding affinity for the photosynthetic reaction centre (Myllykallio et al., 1999). Both versions of the soluble cY produced in this work were expressed from a strong ribosomal promoter, making it possible that the production of an excess of cytochrome cY, 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 c2 and cytochrome cY 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.

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