Identification of proteins involved in formaldehyde metabolism by *Rhodobacter sphaeroides*

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Formaldehyde is an intermediate formed during the metabolism of methanol or other methylated compounds. Many Gram-negative bacteria generate formaldehyde from methanol via a periplasmic pyrroloquinoline quinone (PQQ)-dependent dehydrogenase in which the α subunit of an $\alpha_2\beta_2$ tetramer has catalytic activity. The genome of the facultative formaldehyde-oxidizing bacterium *Rhodobacter sphaeroides* encodes XoxF, a homologue of the catalytic subunit of a proposed PQQ-containing dehydrogenase of *Paracoccus denitrificans*. *R. sphaeroides* xoxF is part of a gene cluster that encodes periplasmic c-type cytochromes, including CycI, isocytochrome c_2 and CycB (a cyt c_{553i} homologue), as well as *adhI*, a glutathione-dependent formaldehyde dehydrogenase (GSH-FDH), and *gfa*, a homologue of a glutathione-formaldehyde activating enzyme (Gfa). To test the roles of XoxF, CycB and Gfa in formaldehyde metabolism by *R. sphaeroides*, we monitored photosynthetic growth with methanol as a source of formaldehyde and whole-cell methanol-dependent oxygen uptake. Our data show that *R. sphaeroides* cells lacking XoxF or CycB do not exhibit methanol-dependent oxygen uptake and lack the capacity to utilize methanol as a sole photosynthetic carbon source. These results suggest that both proteins are required for formaldehyde metabolism. *R. sphaeroides* Gfa is not essential to activate formaldehyde, as cells lacking *gfa* are capable of both methanol-dependent oxygen uptake and growth with methanol as a photosynthetic carbon source.

Received 15 July 2007

Revised 15 October 2007

Accepted 20 October 2007

INTRODUCTION

Formaldehyde is produced at significant levels by abiotic and biological processes (Auerbach *et al.*, 1977; Levy, 1971). Because this compound can inactivate many cellular components, cells have systems to metabolize formaldehyde (Auerbach *et al.*, 1977; Barber & Donohue, 1998). We are studying proteins involved in formaldehyde metabolism by *Rhodobacter sphaeroides*, a bacterium with an inducible system for the metabolism of this compound (Barber & Donohue, 1998; Hickman *et al.*, 2004).

The study of formaldehyde metabolism in purple non-sulfur photosynthetic bacteria such as *R. sphaeroides* began

when Quayle and co-workers isolated species capable of photosynthetic growth on medium containing methanol and bicarbonate (Quayle & Pfennig, 1975; Sahm *et al.*, 1976). In media supplemented with methanol or methylated compounds such as choline, *R. sphaeroides* utilizes a GSH-dependent pathway to metabolize formaldehyde (Barber *et al.*, 1996; Barber & Donohue, 1998). Subsequent studies have shown that *R. sphaeroides* is not able to grow on methylamine (Barber *et al.*, 1996; Barber & Donohue, 1998), and that the genome of this bacterium lacks genes needed for the metabolism of this and other one-carbon (C_1) methyl donors (Mackenzie *et al.*, 2001).

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Abbreviations: Ap, ampicillin; cyt, cytochrome; GS-CH₂OH, S-hydroxymethylGSH; GSH-FDH, glutathione-dependent formaldehyde dehydrogenase; isocyt, isocytochrome; PQQ, pyrroloquinoline quinone; RC, reaction centre; Tc, tetracycline; Tp, trimethoprim.

In *R. sphaeroides*, formaldehyde (CH₂O) is added to the thiol of glutathione to form S-hydroxymethylGSH (GS-CH₂OH), a reaction which can either occur spontaneously or be facilitated by a glutathione/formaldehyde-activating enzyme (Gfa) (Goenrich *et al.*, 2002). GS-CH₂OH is oxidized to S-formylGSH (GS-CHO) by a GSH-dependent

formaldehyde dehydrogenase (GSH-FDH), then converted to formate (HCOO^-) by S-formylGSH hydrolase (FGH). Finally, formate is oxidized by formate dehydrogenase (FDH) to carbon dioxide (CO_2). The *R. sphaeroides* GSH-FDH structural gene (*adhI*) is part of a potential formaldehyde metabolism gene cluster (*gfa-adhI-cycI-xoxF-cycB*-RSP2580-RSP2581) (Mackenzie *et al.*, 2001) that encodes proteins with high amino acid identity to those involved in C_1 metabolism by facultative methylotrophs (Fig. 1a) (Chistoserdova, 1996; Harms & van Spanning, 1991; Harms *et al.*, 1996; Ras *et al.*, 1991). In contrast, *R. sphaeroides* genes encoding FGH and FDH homologues map elsewhere in the genome (Mackenzie *et al.*, 2001).

The periplasmic oxidation of methanol to formaldehyde is often catalysed by proteins that contain pyrroloquinoline quinone (PQQ) as a prosthetic group (Anthony & Williams, 2003; Goodwin & Anthony, 1998). In such PQQ-dependent methanol dehydrogenases, the α subunit (MxaF) has catalytic activity while the role of the β subunit (MxaI) is not known (Anthony & Williams, 2003;

Goodwin & Anthony, 1998). *R. sphaeroides* XoxF has amino acid sequence similarity to MxaF and XoxF of *Paracoccus denitrificans*. *P. denitrificans* XoxF has been implicated in formaldehyde formation from methanol, but an XoxF mutant of this bacterium is able to grow with methanol as a sole carbon source, albeit to a reduced extent, presumably because cells also contain MxaFI (Harms *et al.*, 1996; Ras *et al.*, 1991). In *Methylobacterium extorquens*, the reduction of PQQ is followed by successive one-electron transfers to periplasmic *c*-type cytochromes which bring reducing power to membrane-bound proteins (Anthony, 1992; Goodwin & Anthony, 1998).

This work takes advantage of features of *R. sphaeroides* to investigate proteins needed for formaldehyde metabolism. *R. sphaeroides* is able to co-metabolize methanol and other sources of formaldehyde to CO_2 in both the presence and the absence of oxygen. *R. sphaeroides* does not assimilate the CO_2 produced during aerobic respiration, as Calvin cycle enzyme activity is not present (Barber & Donohue, 1998). Under photosynthetic conditions (anaerobic plus

Fig. 1. Succinate and methanol oxidation by *R. sphaeroides*. (a) Shown is the direction of gene transcription (arrowhead) and the intergenic distances within this region of the *R. sphaeroides* genome. Trimethoprim (Tp^R) cartridge insertion is indicated by $\blacktriangle$; the extent of wild-type DNA in cosmid pUI8747 is shown above the map. Known operons are shown by a dashed arrow (Barber *et al.*, 1996; Rott *et al.*, 1993), whereas dotted arrows show potential operons (Y. Dufour, personal communication). (b) Periplasmic oxidation of methanol to formaldehyde results in the concomitant transfer of electrons to *CycB* and to isocyt *c*₂, and the reduction of O_2 by a terminal cytochrome oxidase. Alternatively, under photosynthetic conditions, electrons can be transferred to RC complexes in the membrane. (c) During succinate-dependent respiration, succinate dehydrogenase (SDH) reduces ubiquinone (UQH₂) via a terminal quinol oxidase. Alternatively, electrons from quinone can pass through *cyt bc*₁, then to periplasmic cytochromes such as isocyt *c*₂, and finally onto membrane-bound cytochrome oxidase to reduce O_2 .

light), *R. sphaeroides* can use methanol as a sole carbon source because it assimilates CO₂ via the Calvin cycle (Barber & Donohue, 1998). Since the *R. sphaeroides* genome lacks *mxoFI* (MxaFI), the contribution of XoxF to formaldehyde metabolism can be easily studied. In addition, *R. sphaeroides* encodes isocytochrome (isocyt) *c*₂ and CycB, a homologue of soluble cytochrome *c* proteins of *M. extorquens* (MxaG') and *P. denitrificans* (cyt *c*_{553i}), which could carry electrons from a periplasmic dehydrogenase to the membrane (Anthony, 1992; Harms & van Spanning, 1991; Ras *et al.*, 1991; Rott *et al.*, 1992, 1993). Furthermore, *R. sphaeroides* Gfa is a homologue of a *P. denitrificans* protein (Goenrich *et al.*, 2002b; Neculai *et al.*, 2005) which forms the GSH-FDH substrate (Barber *et al.*, 1996; Barber & Donohue, 1998). To analyse the requirements for formaldehyde metabolism by *R. sphaeroides*, we used growth in the presence of methanol and whole-cell O₂

uptake with wild-type cells as well as mutants lacking XoxF, PQQ, one or more *c*-type cytochromes, or Gfa.

METHODS

Bacterial strains and growth conditions. *R. sphaeroides* strains (Table 1) were grown in Sistrom's minimal medium (Sistrom, 1960) containing 35 mM succinate at 30 °C. For testing aerobic methanol metabolism, the medium was supplemented with 100 mM methanol. Monitoring photosynthetic growth by *R. sphaeroides* utilized either a succinate-based medium or one lacking succinate and amino acids but containing 100 mM methanol and 40 mM sodium bicarbonate. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani medium. *E. coli* strain DH5α was used as a plasmid host and S17-1 was used for conjugation of plasmids into *R. sphaeroides* (Davis *et al.*, 1988). When necessary, the medium was supplemented with 30 µg ml⁻¹ trimethoprim (Tp) or 1 µg ml⁻¹ tetracycline (Tc) for *R. sphaeroides*, and 100 µg ml⁻¹ ampicillin (Ap), 10 µg Tc ml⁻¹ or 100 µg Tp ml⁻¹ for *E. coli*.

Table 1. Plasmids and strains

Plasmid or strain	Relevant phenotype or genotype	Reference or source
Plasmids		
pUC19	Ap ^R	Messing (1979)
pSUP202	Ap ^R , Tc ^R , Cm ^R ; Mob ⁺ ; pBR322 derivative	Simon <i>et al.</i> (1983)
Litmus 28i	Multi-purpose cloning vector	New England BioLabs
pLA2917	Kn ^R , Tc ^R ; RK2 derivative, <i>cos</i>	Allen & Hanson (1985)
p34S-Tp	Source of <i>dhfRII</i> cassette; Tp ^R	Dennis & Zylstra (1998)
pWT-P1	6.7 kb <i>gfa-adhI-cycI-xoxF-cycB-xoxJ-rsp2581</i> <i>PstI</i> fragment cloned into pUC19	Witthuhn, This study
pXoxF2	1958 bp <i>EcoRI</i> fragment containing <i>xoxF</i>	This study
pTP16	589 bp deletion and Tp ^R insertion at <i>xoxF</i> codon 260	This study
pTP16-12	1.9 kb <i>EcoRI</i> fragment containing $\Delta xoxF::Tp$ from pTP16 cloned into pSUP202	This study
pSW502	1.2 kb <i>PstI-EcoRI</i> fragment containing <i>cycB</i>	This study
pSW517	120 bp deletion and Tp ^R insertion at <i>cycB</i> codon 71	This study
pSW521	1.7 kb <i>PstI-EcoRI</i> fragment containing $\Delta cycB::Tp$ from pSW517 cloned into pSUP202	This study
pSW161	1.2 kb <i>EcoRI</i> fragment containing <i>gfa</i>	This study
pSW161-4	147 bp deletion and Tp ^R insertion at <i>gfa</i> codon 74	This study
pSW170	1.1 kb <i>EcoRI</i> fragment containing $\Delta gfa::Tp$ from pSW161-4 cloned into pSUP202	This study
pUI8747	Tc ^R ; <i>gfa-adhI-cycI-xoxF-cycB-xoxJ</i> DNA in pLA2917	Rott <i>et al.</i> (1993)
Strains		
<i>R. sphaeroides</i>		
Ga	<i>crtD</i>	Laboratory strain
BOX9	<i>crtD</i> $\Delta adhI::\Omega$ Sp ^R <i>box-9::Tn5Tp^R</i>	Ozcan (1996)
CCMA1	<i>ccmA1::\Omega</i>	Cox <i>et al.</i> (2001)
CYCI1	<i>cycI::\Omega</i>	Rott <i>et al.</i> (1993)
CYCAI	<i>cycA cycI::\Omega</i>	Rott <i>et al.</i> (1993)
TP39	<i>crtD cycA spd-7 Tn5::I</i> , 2300	Rott <i>et al.</i> (1993)
BC1	<i>fbfBC::\Omega Tp^R</i>	Mouncey <i>et al.</i> (2000)
TP19	<i>crtD</i> $\Delta xoxF::Tp$	This study
SW426	<i>crtD</i> TP19 with pUI8747	This study
SW531	<i>crtD</i> $\Delta cycB::Tp$	This study
SW538	<i>crtD</i> SW531 with pUI8747	This study
SW102	<i>crtD</i> $\Delta gfa::Tp$	This study
<i>E. coli</i>		
DH5α	<i>supE44</i> $\Delta lacU169$ (Φ80 <i>lacZ</i> ΔM15) <i>hsdR178 recA1 endA1 gyrA96 thi-1 relA1</i>	Bethesda Laboratories Research (1986)
S17-1	C600::RP-4 2-(Tc::Mu) (Kn::Tn7) <i>thi pro hsdR hsdM⁺ recA</i>	Simon <i>et al.</i> (1983)

Molecular methods and plasmid construction. Standard methods were used for preparation of plasmid DNA from *E. coli*. Modification enzymes, restriction endonucleases and *Taq* or *Pfu* polymerase were used as instructed by the manufacturers. Genomic DNA from wild-type and mutant strains of *R. sphaeroides* was isolated (Chen & Kuo, 1993) and amplified with *Pfu* polymerase to facilitate cloning into *EcoRV*-digested Litmus 28i. Candidate genes were analysed by DNA sequence analysis using the Big Dye Cycle Sequencing protocol (Perkin Elmer). Reaction products were applied to Sephadex G-50 columns and analysed at the University of Wisconsin-Madison DNA Sequencing Facility. Primer sequences used are available from the authors upon request.

Creation of *R. sphaeroides* mutants. Primers specific to the *xoxF* upstream and downstream regions, both containing an *EcoRI* restriction endonuclease site, were used to amplify DNA from pWT-P1. These restriction endonuclease sites facilitated cloning of the 1958 bp PCR product in *EcoRI*-digested pUC19 (pXoxF2). Divergent primers were used with pXoxF2 to create a 589 bp deletion flanked by 780 bp of *xoxF* upstream DNA and 520 bp downstream DNA in pUC19. To degrade parental DNA, *DpnI* was added to the PCR product and incubated for 6 h at 37 °C. The p34S-Tp *SmaI*-digested Tp^R cartridge (Dennis & Zylstra, 1998) was ligated into the pXoxF2 PCR product and transformed into *E. coli* DH5 α (pTP16). The *EcoRI* insert of pTP16 was isolated and cloned into *EcoRI*-digested pSUP202 (pTP16-12). After mobilization of pTP16-12 into *R. sphaeroides*, Tp^R isolates were screened for Tc^S, to monitor the recombination of $\Delta xoxF::dhfRII$ by an even number of crossover events (strain TP19).

Upstream and downstream *cycB* primers with *PstI* restriction endonuclease sites were used to amplify DNA from pWT-P1. The 1210 bp PCR product contained an internal *EcoRI* site producing a *PstI*–*EcoRI* fragment that was cloned into a similarly digested pUC19 (pSW502). Divergent primers with *XbaI* restriction endonuclease sites were used with pSW502, creating a 120 bp deletion with 524 and 533 bp of upstream and downstream sequence, respectively, in pUC19. This PCR product was treated with *DpnI*. Following digestion of p34S-Tp with *XbaI*, a Tp^R cartridge was inserted into pSW502 (digested with *XbaI*) and transformed into DH5 α (pSW517). The *EcoRI*–*PstI* *cycB*-containing fragment of pSW517 was cloned into pSUP202 to produce pSW521. Following conjugal transfer of pSW521 into *R. sphaeroides*, strains exhibiting a Tp^R Tc^S phenotype were analysed to confirm *cycB::dhfRII* gene incorporation into the genome by an even number of crossover events.

A 1.2 kb region that included *gfa* was amplified with primers containing *EcoRI* sites and cloned into *EcoRI*-digested Litmus 28i (pSW161). Using divergent primers with *SphI* restriction sites, a 147 bp deletion was created in pSW161 and the PCR product was treated with *DpnI*. Following *SphI* endonuclease treatment of p34S-Tp, the Tp^R cartridge was ligated into pSW161 (pSW161-4). The *gfa*-containing *EcoRI* fragment of pSW161-4 was cloned into pSUP202 to produce pSW170. After mobilization of pSW170 into *R. sphaeroides*, Tp^R strains were screened for Tc^S to score recombination of the $\Delta gfa::dhfRII$ allele into the genome by an even number of crossover events.

Cosmid pUI8747 (Rott *et al.*, 1993), which contains wild-type *R. sphaeroides* DNA, was mobilized into TP19 (*XoxF* mutant) or SW531 (*CycB* mutant) to test for complementation.

Photosynthetic methanol utilization. To test for photosynthetic methanol utilization, aerobically grown cells were harvested and washed with Sistrom's medium lacking a carbon source. Following dilution into Sistrom's succinate media ($\sim 1.0 \times 10^8$ c.f.u. ml⁻¹), cells were placed in filled screw-capped test tubes and exposed to an incandescent light source (10 W m⁻²; measured with a

Yellow-Springs-Kettering model 6.5-A radiometer). After 3 days, cells were harvested, washed twice in media lacking a carbon source and diluted ($\sim 1.0 \times 10^8$ c.f.u. ml⁻¹) in media containing 100 mM methanol and 40 mM sodium bicarbonate. These tubes were exposed to light and monitored for up to 7 days to score photosynthetic growth.

Whole-cell O₂ uptake. Whole-cell O₂ uptake was monitored using a YSI model 5300A meter equipped with a Clark-type polarographic O₂ probe. For these assays, aerobically grown cultures ($\sim 2.5 \times 10^8$ c.f.u. ml⁻¹) were exposed to 100 mM methanol for 3 h to induce expression of genes required for methanol metabolism (Hickman *et al.*, 2004). These cells were harvested by centrifugation (10 min at 5000 g) and washed twice in medium lacking succinate. Then, $\sim 5.0 \times 10^8$ c.f.u. ml⁻¹ of washed cells were added to the sample chamber for O₂-uptake assays. O₂-consumption rates were determined in the absence of added carbon sources (endogenous rate), in the presence of 35 mM succinate (succinate-dependent respiration) or in the presence of 25–100 mM methanol (methanol-dependent rate). For each assay, measurements were recorded every 15 s over a 240 s period. By plotting O₂ consumption as a function of time, an endogenous, succinate-, or methanol-dependent rate of O₂ uptake was calculated [nmol O₂ consumed min⁻¹ (mg protein)⁻¹].

Computational prediction of potential operons. To predict co-transcribed ORFs (Fig. 1a), Pearson's correlation coefficients were calculated based on RNA abundance measurements from public-domain *R. sphaeroides* global gene expression datasets (Gene Expression Omnibus; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL162>) (Y. Dufour, personal communication). Statistical probabilities for co-transcription of ORFs were obtained from available models (www.microbesonline.org) (Bockhorst *et al.*, 2003; Price *et al.*, 2005).

RESULTS

Genes potentially involved in *R. sphaeroides* formaldehyde metabolism

Previous studies have demonstrated that the structural genes for GSH-FDH and isocyt c₂ (*adhI*–*cycI*) constitute an inducible operon (Fig. 1a) (Barber *et al.*, 1996; Rott *et al.*, 1993) which is controlled by the presence of formaldehyde or of the metabolic sources of this compound such as methanol (Barber & Donohue, 1998; Hickman *et al.*, 2004). Other products of potential ORFs in this region of the *R. sphaeroides* genome encode homologues of proteins known or predicted to be involved in the formaldehyde metabolism of other microbes (Fig. 1a).

One of these genes, *gfa* (Fig. 1a), encodes a protein with 73 % amino acid identity to *P. denitrificans* Gfa, and stimulates the formation of GS-CH₂OH from formaldehyde and GSH, the preferred substrate for GSH-FDH (Goenrich *et al.*, 2002; Neculai *et al.*, 2005). RSP2578 has 80 % amino acid sequence identity to XoxF, a putative PQQ-containing dehydrogenase of *P. denitrificans* (Harms & van Spanning, 1991; Harms *et al.*, 1996), 75 % amino acid identity to the *M. extorquens* XoxF homologue (MxaF') (Chistoserdova & Lidstrom, 1997) and 49 % amino acid identity to the catalytic (α) subunit of methanol dehydrogenase, MxaF. Based on the amino acid identity of

RSP2579 (64 %) with the mono-haem cyt c_{553i} (*cycB*) of *P. denitrificans* (Harms *et al.*, 1996; Ras *et al.*, 1991), we have designated this ORF as *R. sphaeroides* CycB (predicted pI of 4.2). A haem-staining protein of a molecular mass predicted for the mature CycB protein (~18 kDa after cleavage of predicted signal peptide) has been observed previously in *R. sphaeroides* Spd mutants, which have increased expression of some genes in this cluster (Rott *et al.*, 1992). RSP2580 has 63 % amino acid identity to XoxJ of *P. denitrificans* (Harms *et al.*, 1996) and resembles bacterial periplasmic solute binding proteins (Saier, 1994, 2000). Finally, RSP2581 has 62 % amino acid sequence identity to *P. denitrificans* XoxI. RSP2581 is predicted to have a rhodanese-like fold (Bordo & Bork, 2002; Spallarossa *et al.*, 2003, 2004), a pI of ~4.8, and a molecular mass of 19 kDa (www.expasy.org). While similar clusters of related genes are found in genomes from several bacteria, the contribution of *xoxF-cycB-RSP2580-RSP2581* to formaldehyde metabolism remains unclear. To test the role of these gene products in formaldehyde metabolism, we used enzymic and phenotypic assays to monitor the function of this pathway in wild-type and mutant cells.

Requirements for *R. sphaeroides* formaldehyde metabolism

One test of *R. sphaeroides* formaldehyde metabolism was a whole-cell O_2 -uptake assay. We used this assay because earlier attempts to monitor methanol oxidation by other methods in cell extracts of purple non-sulfur bacteria have been unsuccessful for unknown reasons (Hickman, 2003; Quayle & Pfennig, 1975; Sahm *et al.*, 1976).

To enhance our ability to assay methanol-dependent O_2 uptake, cells were grown in the presence of methanol for

one doubling prior to analysis. These growth conditions are known to increase the expression of genes required for the metabolism of this carbon source (Hickman *et al.*, 2004) and expression of genes involved in formaldehyde oxidation (Barber & Donohue, 1998). This regimen was preferred over growing cells in the presence of methanol for longer periods because we intended to assay both wild-type and mutant cells that might have contained defects in the formation or metabolism of formaldehyde. Following exposure to methanol, cells were harvested and suspended in medium lacking any carbon source to allow determination of a background (endogenous) O_2 -uptake rate. O_2 -uptake activity was then monitored after the addition of methanol or succinate (Fig. 2). The endogenous rate of O_2 uptake (O_2 consumed per unit time) was subtracted from the rate determined in the presence of succinate or methanol (Fig. 2).

R. sphaeroides Ga was used as the parent strain because many of the mutants previously analysed for defects in formaldehyde metabolism have been generated in this background (Barber, 1997; Hickman *et al.*, 2002; Ozcan, 1996; Rott *et al.*, 1993). After subtracting the endogenous rate of O_2 uptake, a significant and reproducible rate of methanol-dependent O_2 uptake was measured in strain Ga [$\sim 5.0 \text{ nmol } O_2 \text{ min}^{-1} (\text{mg protein})^{-1}$; Table 2]. For comparison, the rate of whole-cell O_2 uptake was $\sim 15.8 \text{ nmol } O_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ when succinate was provided as the electron donor (Table 2). These data indicate that the whole-cell O_2 uptake assay is able to report on the use of methanol or succinate as an electron donor. It is not surprising that the rates of O_2 uptake are higher with succinate as an electron donor than with methanol, since it is known that wild-type cells have a shorter doubling time in minimal medium that contain

Fig. 2. Whole-cell O_2 uptake by wild-type cells. Plotted is the percentage O_2 concentration as a function of time for each carbon source tested. (◆) Data used to calculate the endogenous rate of O_2 uptake (O_2 consumption without the addition of any carbon source); (■) samples containing methanol; (▲) samples containing succinate.

Table 2. Methanol metabolism in PQQ and c-type cyt mutants

Strain	Relevant genotype	O ₂ uptake*		PS growth†		
		Succinate	MeOH	Succinate	Succinate + MeOH	MeOH
Ga	<i>crtD</i>	15.8 ± 1.0	5.0 ± 0.7	+	+	+
BOX9	<i>crtD</i> Δ <i>adhI</i> :: Ω Sp ^R <i>box-9</i> ::Tn5Tp ^R	13.4 ± 2.0	≤ 0.2	+	+	–
CCMA1	<i>ccmA1</i> :: Ω Sp	9.7 ± 0.6	≤ 0.6	–	–	–
CYCI1	<i>cycI</i> :: Ω	17.0 ± 0.4	2.2 ± 0.3	+	+	–
CYCAI	<i>cycA cycI</i> :: Ω	14.1 ± 1.0	≤ 0.4	+	+	–

*Values shown for O₂ consumption have been corrected by subtracting the endogenous rate of O₂ uptake [~ 2 nmol O₂ min^{–1} (mg protein)^{–1}].

†Photosynthetic (PS) growth of bacteria in liquid medium with succinate, succinate and methanol, or only methanol; (+) growth, (–) no growth.

this compound as a sole carbon source (Barber *et al.*, 1996; Barber & Donohue, 1998).

To further verify the utility of this assay, we monitored whole-cell O₂ uptake in mutants with known or predicted defects in formaldehyde metabolism. Based on what is known about the formaldehyde metabolism of other Gram-negative bacteria, we predict that *R. sphaeroides* would require a PQQ-containing enzyme to generate formaldehyde from methanol. To test this hypothesis, BOX9, a mutant with a loss-of-function mutation in PQQ synthesis, was assayed for methanol oxidation by the whole-cell O₂-uptake assay. The rate of O₂ consumption in the presence of succinate by *R. sphaeroides* BOX9 was comparable to that of wild-type cells (Table 2). This was not surprising, since PQQ is not required for electron transport from succinate to O₂ (Fig. 1c). In contrast, when methanol was used as an electron donor there was no detectable O₂ consumption in the BOX9 strain over background. Results of control experiments indicate that cells lacking AdhI have a rate of methanol-dependent O₂ uptake comparable to that of wild-type cells (data not shown). Thus, the deficiency in methanol-dependent O₂ uptake exhibited by BOX9 demonstrates the requirement for the PQQ prosthetic group for the conversion of methanol to formaldehyde by *R. sphaeroides* (Table 2).

Methanol metabolism by Gram-negative bacteria often involves periplasmic c-type cytochromes to transfer electrons from a PQQ-dependent dehydrogenase to membrane-bound enzymes of the respiratory chain (Anthony, 1982, 1992) (Fig. 1b). As predicted, in CCMA1 cells, which contain a mutation that blocks assembly of many periplasmic and membrane-associated cyt c proteins, there is no detectable methanol-dependent O₂ uptake compared to background (Table 2). In contrast, succinate-dependent O₂ uptake is not abolished in CCMA1 cells (Table 2), presumably because *R. sphaeroides* contains a quinol oxidase which allows cyt c-independent electron transfer from succinate via quinol to O₂ (Fig. 1c). These results demonstrate that methanol-dependent respiration in *R. sphaeroides* requires one or more cyt c proteins, presumably to transfer electrons from a periplasmic PQQ-dependent enzyme to O₂. Indeed, a reduced

methanol-dependent O₂-uptake rate [~ 2.2 nmol O₂ min^{–1} (mg protein)^{–1}] is observed in strain CYCI1 (lacks CycI; Table 2), suggesting a potential role for isocyt c₂ in electron transfer during formaldehyde generation by this periplasmic protein. On the other hand, no detectable methanol-dependent O₂ uptake was measured in CYCAI, which has mutations that inactivate both *cycA* and the gene that encodes its isoform, *cycI* (Table 2).

Additional proteins required for *R. sphaeroides*

Using the O₂-uptake assay, we also analysed the properties of *R. sphaeroides* cells containing defined mutations in *xoxF*, *cycB* or *gfa* (Fig. 1a). Based on the direction of transcription of *xoxF* and *gfa* as well as their predicted positions in potential operons, we do not expect the properties of these mutants to be complicated by polarity on downstream genes (Fig. 1a).

Methanol-dependent O₂ uptake of *R. sphaeroides* cells that lacked XoxF (TP19) was not detectable over background (Table 3). As expected, cells lacking XoxF exhibited normal rates of O₂ uptake when using succinate as an electron donor (Table 3). Also, the ability to complement the defect of TP19 with cosmid pUI8747 (strain SW426) demonstrates that XoxF is required for methanol-dependent O₂ uptake by *R. sphaeroides* (Table 3). Additionally, cells that lacked CycB (SW531) were incapable of methanol-dependent O₂ consumption (Table 3). When SW531 cells containing pUI8747 (strain SW538) were analysed, rates of methanol-dependent O₂ uptake were restored (Table 3) to levels observed in wild-type cells. This suggests that CycB is also required for methanol-dependent O₂ uptake by *R. sphaeroides*, presumably since it carries electrons from the enzyme that generates formaldehyde to the respiratory chain (Fig. 1b). Thus, it appears that inhibiting electron transfer from the site of formaldehyde formation is sufficient to prevent methanol-dependent O₂ uptake. In the case of both TP19 and SW531, the addition of ascorbate was able to support O₂ uptake (data not shown). Ascorbate donates electrons directly to cytochrome oxidases; therefore, both of these strains contain a functional terminal cytochrome oxidase(s) (Fig. 1b). Thus, the failure of TP19 and SW531 to exhibit detectable

Table 3. Properties of methanol metabolism mutants

Strain	Relevant genotype	O ₂ uptake*		PS growth†		
		Succinate	MeOH	Succinate	Succinate + MeOH	MeOH
Ga	<i>crtD</i>	15.8 ± 1.0	5.0 ± 0.7	+	+	+
TP19	<i>crtD</i> Δ <i>xoxF</i> ::Tp	21.5 ± 1.0	≤ 0.2	+	+	–
SW426	<i>crtD</i> TP19 with cosmid pUI8747	12.6 ± 0.5	6.2 ± 1.0	+	+	+
SW531	<i>crtD</i> Δ <i>cycB</i> ::Tp	16.1 ± 2.0	≤ 0.2	+	+	–
SW538	<i>crtD</i> SW531 with cosmid pUI8747	13.0 ± 0.5	6.6 ± 0.7	+	+	+
SW102	<i>crtD</i> Δ <i>gfa</i> ::Tp	18.0 ± 1.4	3.6 ± 0.9	+	+	+

*Values shown for O₂ consumption have been corrected by subtracting the endogenous rate of O₂ uptake [~ 2 nmol O₂ min^{–1} (mg protein)^{–1}].

†Photosynthetic (PS) growth of bacteria in liquid medium with succinate, succinate and methanol, or only methanol; (+) growth, (–) no growth.

methanol-dependent O₂ uptake is not due to lack of this terminal enzyme in the respiratory chain.

To analyse the role of *R. sphaeroides* Gfa in formaldehyde metabolism (Goenrich *et al.*, 2002) we tested O₂ uptake by cells that lacked Gfa (SW102). Cells lacking Gfa (SW102) were capable of using succinate or methanol as an electron donor to support O₂ uptake; however, the rate of methanol-dependent O₂ uptake was slightly lower than that of wild-type cells (Table 3). Based on the observed activity of SW102, we conclude that loss of Gfa is not sufficient to prevent methanol-dependent O₂ uptake by *R. sphaeroides*.

Methanol metabolism during photosynthetic growth

R. sphaeroides requires a GSH-FDH-dependent pathway to use methanol as a sole photosynthetic carbon source. Under these conditions, formaldehyde is oxidized to CO₂ and subsequently assimilated by the Calvin cycle (Barber & Donohue, 1998). To further test the role of these proteins in formaldehyde metabolism, we also analysed the ability of wild-type and mutant strains to grow photosynthetically using either succinate or methanol as a carbon source. Based on the pivotal role of *c*-type cytochromes in photosynthetic and methanol-dependent electron transport (Davis *et al.*, 1988; Rott *et al.*, 1993; Zannoni *et al.*, 1976), it is not surprising that CCMA1, which lacks all *c*-type cytochromes, is photosynthetically incompetent when using either succinate or methanol as a carbon source (Table 2). Also, it is not surprising that cells lacking isocyt *c*₂ (CYC11) are able to grow photosynthetically in media containing either succinate or methanol (Table 2), since previous results have shown that cyt *c*₂ can support photosynthetic electron transport under both conditions (Fig. 1b) (Davis *et al.*, 1988; Rott *et al.*, 1992, 1993).

Both PQQ (BOX9) and XoxF (TP19) mutants grew photosynthetically in the presence of succinate. However, BOX9 and TP19 were photosynthetically incompetent when methanol was the sole carbon source (Tables 2 and 3). Presumably, the defect in using methanol during

photosynthetic growth for both the PQQ and XoxF mutants is a consequence of the requirement for a PQQ-dependent enzyme such as XoxF. In addition, the CycB mutant (SW531) was able to grow photosynthetically in the presence of succinate but not when methanol was used as the sole carbon source (Table 3). Based on this we predict that CycB is needed to bring electrons from the PQQ-dependent dehydrogenase XoxF to the photosynthetic electron transport chain (Fig. 1b). As expected, the photosynthetic growth defects in the presence of methanol of the XoxF and CycB mutants were each complemented by a cosmid (pUI8747, Fig. 1a) that carries this entire region of the genome (Table 3). Finally, photosynthetic growth of cells that lack XoxF, CycB or PQQ occurred when the media contained both succinate and methanol (Tables 2 and 3), indicating that these mutants are not simply sensitive to methanol. Rather, it appears that the loss of PQQ, XoxF or CycB causes the growth defect by blocking the pathway needed to generate formaldehyde from methanol under photosynthetic conditions.

In contrast, cells lacking Gfa (SW102) grew photosynthetically when using either succinate or methanol (Table 3). These data, plus the ability to measure methanol-dependent O₂ uptake in SW102 cells, support the notion that Gfa is not absolutely required for formaldehyde activation and its subsequent metabolism by GSH-FDH in *R. sphaeroides*.

DISCUSSION

In this work, we analysed products from a region of the *R. sphaeroides* genome, *gfa-adhI-cycI-xoxF-cycB*-RSP2580-RSP2581, which encodes proteins implicated in the metabolism of formaldehyde (Fig. 1). To address the function of these gene products in *R. sphaeroides*, mutants lacking the PQQ-dependent dehydrogenase (XoxF), a homologue of the periplasmic cyt *c*_{553i} (CycB) or the glutathione formaldehyde activating enzyme (Gfa), were monitored for methanol-dependent O₂ uptake or photosynthetic growth using methanol as a carbon source (Table 3). We used methanol as a carbon source for these studies, as previous work has shown that growth under these conditions requires the

activity of GSH-FDH to metabolize the formaldehyde that is produced (Barber *et al.*, 1996; Hickman *et al.*, 2002). Also, by analysing strains that lacked periplasmic *c*-type cytochromes (CYCII, CYCAI and CCMA1) or possessed a defect in PQQ synthesis, we were able to study other requirements for formaldehyde metabolism (Table 2). Our results show that formaldehyde generation from methanol by *R. sphaeroides* requires XoxF, PQQ and periplasmic *c*-type cytochromes, including CycB. However, *R. sphaeroides* does not require Gfa for the metabolism of formaldehyde.

Whole-cell methanol-dependent O₂ uptake and the use of methanol as a sole photosynthetic carbon source require either XoxF or PQQ biosynthesis (Table 3). Because *R. sphaeroides* BOX9 contains a lesion in PqqE, this finding suggests that XoxF requires PQQ for formaldehyde metabolism (Ozcan, 1996). The properties of the *R. sphaeroides* XoxF mutant (TP19) and the absence of a gene related to *mxal* predict that XoxF might function as an α_2 homodimer. In *R. sphaeroides* and other bacteria that contain homologues of XoxF (Fig. 3), these proteins lack a C-terminal domain of MxaF which interacts with MxaI (Ghosh *et al.*, 1995). This also suggests that active XoxF might not need a β subunit to form an active enzyme. Phylogenetic and bioinformatic analysis found high amino acid identity among other XoxF homologues (Fig. 3), even though the genomes of *P. denitrificans*, *M. extorquens* and *Rhodopseudomonas palustris* BisA53 contain both *mxal* and a homologue of *xoxF*.

Interestingly, *R. sphaeroides* lacks detectable activity using the dye-linked assay that is commonly employed with $\alpha_2\beta_2$

methanol dehydrogenases (Fig. 3) (Hickman, 2003; Quayle & Pfennig, 1975; Sahm *et al.*, 1976). The inability to detect dye-linked methanol dehydrogenase activity in extracts may be due to the absence of a β subunit which can stabilize the enzyme (Ghosh *et al.*, 1995).

It is accepted that periplasmic *c*-type cytochromes are involved in electron transfer to the membrane following the oxidation of methanol (Anthony, 1992). Since *R. sphaeroides* possesses well-characterized periplasmic electron transport carriers, we also tested the role of these proteins in methanol metabolism. One ORF in the proposed *R. sphaeroides* formaldehyde metabolism gene cluster encodes a *c*-type cyt with high amino acid identity to cyt *c*_{553i}, and mutational analysis of *cycB* showed that the protein is required for growth and O₂ uptake when *R. sphaeroides* is placed in formaldehyde-generating conditions. Although cyt *c*_{553i} homologues of *P. denitrificans* or *M. extorquens* (MxaG') have been suggested to be involved in C₁ metabolism, earlier biochemical or genetic attempts to demonstrate a role in this pathway have been unsuccessful (Chistoserdova & Lidstrom, 1997; Harms *et al.*, 1996; Ras *et al.*, 1991). Also, studies in *P. denitrificans* have demonstrated that the primary electron acceptor of MxaFI is cyt *c*_{551i} and not cyt *c*_{553i}, while in *M. extorquens* cyt *c*_L directly accepts electrons from methanol dehydrogenase (Anthony, 1992; Long & Anthony, 1991). While we cannot distinguish whether CycB is a direct electron acceptor from XoxF or an intermediate carrier of electrons to the membrane, we have shown that this protein is required by *R. sphaeroides* when methanol is oxidized to formaldehyde. The intergenic distance between *R. sphaeroides cycB* and adjacent

Fig. 3. Phylogenetic analysis of XoxF orthologues. Amino acid sequence identities among α -proteobacteria XoxF homologues are represented. The phylogenetic tree shows all three wild-type strains of *R. sphaeroides* grouping in one of two clades based on evolutionary distance. This analysis was generated with MEGA (Kumar *et al.*, 2004), and bootstrap values above 50 % from 500 replicates are shown. Scale bar, 0.02 substitutions per site.

ORFs (RSP2580 and RSP2581) combined with computational analysis of global gene-expression studies suggests that these three genes might be co-transcribed (Y. Dufour, personal communication). Thus, our findings could also reflect the effect of the *cycB* mutation on the expression of RSP2580 and RSP2581. If this was the case, then it would also provide the first evidence for a specific role of these uncharacterized proteins in formaldehyde metabolism.

Our data predict that one or more class I *c*-type cytochromes, isocyt *c*₂ or cyt *c*₂ is involved in electron transport from the periplasmic site of formaldehyde formation to the membrane. We propose that one or both of the class I *c*-type cytochromes transfers electrons to the membrane, especially since previous studies have shown that each cyt *c*₂ isoform can transfer electrons to reaction centre (RC) complexes (Fig. 1b) (Rott *et al.*, 1993).

Purified Gfa of *P. denitrificans* catalyses the condensation of formaldehyde and glutathione to generate the hydroxymethylGSH adduct used as a substrate by GSH-FDH (Goenrich *et al.*, 2002). We conclude that *R. sphaeroides* does not absolutely require Gfa for formaldehyde activation because cells lacking Gfa (SW102) were capable of electron transfer from methanol to O₂ and able to use methanol as a sole photosynthetic carbon source. Cells that lack GSH-FDH accumulate formaldehyde and are killed by methanol (Barber *et al.*, 1996; Rott *et al.*, 1993). Thus, the properties of SW102 suggest that formaldehyde can spontaneously condense with GSH at a sufficient rate *in vivo* to prevent accumulation of formaldehyde. In *M. extorquens* the formaldehyde activating enzyme (Fae) catalyses the condensation of formaldehyde and tetrahydromethanopterin (Vorholt *et al.*, 2000). Unlike Gfa of *R. sphaeroides*, Fae is necessary for growth on methanol (Vorholt *et al.*, 2000).

In summary, these experiments have identified additional proteins that are required by cells to generate formaldehyde from methylated compounds and to participate in other steps of the metabolism of this compound. Our results indicate that formaldehyde metabolism by *R. sphaeroides* requires XoxF, PQQ and periplasmic *c*-type cytochromes, including CycB, isocyt *c*₂ or cyt *c*₂. However, Gfa is not absolutely required by *R. sphaeroides* to condense formaldehyde with glutathione. Based on these findings, we predict that the PQQ-dependent dehydrogenase XoxF oxidizes methanol to formaldehyde, which stimulates electron transfer to CycB and isocyt *c*₂, and then onto membrane-associated electron acceptors (Fig. 1b). These findings make specific predictions for further analysis of this pathway in *R. sphaeroides* and other bacteria which contain a similar putative formaldehyde gene cluster.

ACKNOWLEDGEMENTS

This study was supported by grants GM020609-01 (National Institute of General Medical Sciences) to S.M.W. and DE-FG02-05ER15653 (Department of Energy) to T.J.D.

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Edited by: J. A. Vorholt