Mutational analysis of the dimethylsulfoxide respiratory (*dor*) operon of *Rhodobacter capsulatus*

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Four genes, *dorC*, *dorD*, *dorB* and *dorR* of the DMSO respiratory gene cluster of *Rhodobacter capsulatus* have been identified and sequenced. *dorC* encodes a pentahaem c-type cytochrome of the NirT class and the derived DorC protein sequence shows highest similarity to TorC from the *Escherichia coli* trimethylamine-*N*-oxide (TMAO) respiratory system. Mutagenesis of *dorC* resulted in the loss of a 46 kDa haem-staining polypeptide from membranes of *R. capsulatus*. *dorD* encodes a protein with highest sequence similarity to TorD from the *E. coli* TMAO respiratory system. DMSO reductase polypeptide (DorA) could not be detected in cell-free extracts of a *dorD* mutant and it is suggested that DorD has a role in stabilizing the DorA apo-protein prior to insertion of the pterin molybdenum cofactor. *dorB* encodes a protein with highest sequence similarity to NapD of *Paracoccus denitrificans*. Mutagenesis of *dorB* reduced the activity of DMSO reductase and led to the accumulation of a larger form of the enzyme that is presumed to represent a cytoplasmic precursor polypeptide. It is suggested that DorB has a role in the biogenesis of DMSO reductase prior to its secretion into the periplasm. *dorR* is transcribed in the opposite direction to *dorC*. The derived amino acid sequence of DorR indicates that it is a response regulator and mutation of *dorR* shows that it is essential for expression of the *dorCDA* operon. Expression of a chromosomal *dorA::lacZ* fusion was also dependent on the transcriptional regulator Fnr. The intergenic region between *dorR* and *dorC* contains four putative binding sites for DorR but no binding site for Fnr was identified.

Keywords: dimethylsulfoxide respiration, *Rhodobacter capsulatus*

INTRODUCTION

The ability to use DMSO and trimethylamine-*N*-oxide (TMAO) as electron acceptors is widespread among purple non-sulfur phototrophic bacteria (Zannoni, 1995). In *Rhodobacter capsulatus* DMSO reductase is located in the periplasm and electron transfer to this terminal reductase has been shown to involve cytochromes (McEwan *et al.*, 1989). Analysis of transposon mutants has shown that a single oxidoreductase is responsible for the reduction of both TMAO and DMSO

(Kelly *et al.*, 1988). Since the apparent second order rate constant (k_{cat}/K_m) of the enzyme is greater towards DMSO than TMAO (Sato & Kurihara, 1987) the enzyme is described as a DMSO reductase. The genes encoding DMSO reductase from *R. capsulatus* (Shaw *et al.*, 1996; Knaeblein *et al.*, 1996) and *Rhodobacter sphaeroides* (Yamamoto *et al.*, 1995) have been sequenced. Sequence analysis reveals that the *R. capsulatus* DMSO reductase (DorA) is more closely related to the TMAO reductase (TorA) of *Escherichia coli* (48% identical) than to DMSO reductase (DmsA) of *E. coli* (26% identity). For this reason we refer to genes encoding DMSO respiratory components in *Rhodobacter* as *dor* genes (Shaw *et al.*, 1996).

DMSO/TMAO reductases are members of the molybdenum oxotransferase family of enzymes (Kisker *et*

Abbreviations: Moco, pterin molybdenum cofactor; TMAO, trimethylamine-*N*-oxide; Gm, gentamicin; Tc, tetracycline.

The GenBank accession number for the sequence reported in this paper is U49506.

al., 1997). These enzymes catalyse the transfer of an oxygen atom to or from a substrate in a reaction which is linked to a two-electron transfer process. The unifying feature of these enzymes is the presence of a pterin molybdenum cofactor (Moco) at the active site. Oxomolybdenum enzyme research has rapidly expanded with the publication of the crystal structures of Moco-containing enzymes, including DMSO reductase from *R. sphaeroides* (Schindelin *et al.*, 1996) and *R. capsulatus* (Schneider *et al.*, 1996; McAlpine *et al.*, 1997). The simplicity of DMSO reductase has made it an excellent system for spectroscopic investigations of oxomolybdenum enzymes (Bennett *et al.*, 1994) and recent advances have opened the way for studies of structure-function relationships within the enzyme. As part of our efforts to understand the structure and mechanism of DMSO reductase from *R. capsulatus* we have cloned and sequenced the genes which flank *dorA*. It was expected that this would identify proteins involved in electron transport to DMSO reductase or proteins involved in the expression of DMSO reductase. In this paper we report the mutagenesis of three genes of the *dor* operon and the phenotypic characterization of the resulting mutants.

Recently, the sequence of the *dor* gene cluster of *R. sphaeroides* has been reported and mutational analysis of *dor* genes carried out (Mouncey *et al.*, 1997; Mouncey & Kaplan, 1998). This work identified a two-component sensor kinase/response regulator, DorS and DorR, that is required for the DMSO-dependent induction of the *dor* operon (Mouncey & Kaplan, 1998). In this paper we also describe aspects of the regulation of *dor* operon expression in *R. capsulatus*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

DNA sequencing and library screening. Plasmid pALS2 (Shaw *et al.*, 1996), which contains the complete *dorA* sequence, also contains the complete *dorD* sequence and a C-terminal portion of *dorC*. The partial sequence data from *dorC* were used to design a 30 bp oligonucleotide (5'-TGCGGCACCTGCCACGTGCTGCCCCATACC-3') which corresponds to a conserved haem-c binding site (C-X-X-C-H). This probe was labelled at the 3' end with fluorescein-11-dUTP using an Amersham 3' oligonucleotide labelling kit according to the manufacturer's instructions. The labelled probe was used to screen a *R. capsulatus* λ -ZAPII expression library (Stratagene), prepared as described by Shaw *et al.* (1996), using Amersham ECL detection reagents and Hybond-N+ membranes as described by the manufacturer, except that the membranes were blocked in 5% (w/v) non-fat milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 150 mM NaCl). This screening identified several clones that hybridized with the *dorC* probe. These clones were purified and the phage excised to form phagemids harboured in *E. coli* XL-1 Blue MRF' (Stratagene) according to protocols supplied by Stratagene.

Phagemids pALS3 and pALS4 were selected and initial sequencing was performed using primers designed from

sequence data derived from pALS2 and the T3 sequencing primer. Additional primers were made as more sequence was determined. Phagemid DNA was prepared using Qiagen columns according to the manufacturer's instructions. Automated dye terminator sequencing was performed with a Perkin Elmer sequencing premix as described previously (Shaw *et al.*, 1996). The reaction products were analysed on an Applied Biosystems 373A automated sequencer. All sequencing was performed on both strands and sequencing reactions were performed in triplicate. All sequence alignments, manipulations and analyses were performed using programs in the Wisconsin GCG software package version 8.1-UNIX.

Construction of *dorA*, *dorC*, *dorD*, *dorB* and *dorR* mutants.

All DNA cloning was performed according to standard methods (Sambrook *et al.*, 1989) using *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) for routine cloning. A *dorA* mutant was constructed using a 3451 bp *Pst*I-*Bam*HI fragment of pALS2 (Shaw *et al.*, 1996) containing the complete *dorA* gene which was cloned into *Pst*I/*Bam*HI-digested pSVB25 (Pharmacia). A 6.5 kb *Bgl*II-*Hind*III fragment from pWKR459II containing *mob* and tetracycline (Tc) resistance regions (Simon *et al.*, 1983) was cloned into this plasmid digested with *Bam*HI and *Hind*III. The resulting construct was cut with *Eco*RI, removing a 1515 bp internal fragment of *dorA*. A 2.6 kb Gentamicin (Gm) resistance interposon was cloned from *Eco*RI-digested R440 (Hirsch & Beringer, 1984) into the *Eco*RI-digested *dorA* gene in pSVB25. The orientation of the Gm resistance interposon was determined and two plasmids, pALS41 and pALS42, were selected for further cloning, each containing the Gm resistance interposon in opposite orientations. pALS41 contains a Gm resistance interposon that is transcribed in the same direction as *dorA*. For mutagenesis of *dorA*, pALS41 was mobilized from *E. coli* S17-1 into *R. capsulatus* by conjugation. Colonies where a double cross-over had occurred were identified as exhibiting resistance to Gm and sensitivity to Tc. In this way the wild-type chromosomal copy of *dorA* was exchanged for a *dorA* deletion-insertion mutation. For mutagenesis of *dorC* a 2.8 kb *Eco*RI fragment was cloned from pALS3 into *Eco*RI-digested pK18 (Pridmore, 1987). A 2.6 kb Gm resistance interposon (Hirsch & Beringer, 1984) was cloned into the *Cl*aI site in *dorC* and constructs were screened to determine the orientation of the Gm resistance cassette. Two constructs, each containing Gm resistance interposons in opposite orientations and thus opposite directions of transcription with respect to *dorC* were chosen. A 5.4 kb *Eco*RI fragment, containing *dorC* with the Gm resistance cassette inserted, was cloned from pK18 into *Eco*RI-digested pSUP202 (Labes *et al.*, 1990). The resulting constructs, pALS71 and pALS72, each containing the Gm resistance cassette in opposite orientations, were transformed into *E. coli* S17-1.

The *dorD* mutation was made by first cloning a 2.2 kb *Bam*HI-*Sal*I fragment from pALS2 into *Bam*HI/*Sal*I-digested pBlue-script KS(+) (Stratagene). This construct was digested with *Pst*I which cuts *dorD*. The 5' overhangs generated by *Pst*I digestion were filled with T4 DNA polymerase (New England Biolabs). A Gm resistance interposon (Hirsch & Beringer, 1984) was cut with *Eco*RI and the 3' overhangs removed with T4 DNA polymerase (New England Biolabs) to form blunt ends. The two fragments were ligated to give plasmids that contained a Gm resistance interposon inserted into *dorD*. Again, these plasmids were screened to obtain two constructs, each with opposite orientations of the Gm resistance interposon with respect to *dorD*. These two plasmids were linearized with *Bam*HI and cloned into *Bam*HI-digested

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype and properties*	Reference or source
<i>R. capsulatus</i>		
37b4	Wild-type	Kaufmann <i>et al.</i> (1984)
DOR-R1	<i>dorR</i> ::Gm	This work
RGK295	Δ <i>fnr</i> :: Ω Kn	Zeilstra-Ryalls <i>et al.</i> (1997)
<i>E. coli</i>		
XL-1 Blue MRF'	Host strain for pBK phagemid vectors	Stratagene
S17-1	RP4-2 (Tc::Mu) (Km::Tn7); integrated into chromosome	Simon <i>et al.</i> (1983)
Plasmids		
pALS3	pBK-CMV phagemid vector containing 4.8 kb <i>R. capsulatus</i> genomic DNA; Km ^r	This study
pALS4	pBK-CMV phagemid vector containing 5.1 kb <i>R. capsulatus</i> genomic DNA; Km ^r	This study
pUC8	Ap ^r <i>lacZ</i> α	Vieira & Messing (1982)
pWKR459II	pK18::mob (RP4), Tc ^r	W. Klipp
pALS48	Mobilizable plasmid carrying <i>dorR</i> ::Gm	This study
pJK1	5 kb <i>Bam</i> HI DNA fragment containing the <i>R. capsulatus</i> <i>dorA</i> gene in pBluescript SK(–)	Knaeblein <i>et al.</i> (1996)
pML5(B+)	Mobilizable, promoterless <i>lacZYA</i> transcriptional fusion vector, Tc ^r	Labes <i>et al.</i> (1990)
pSL111	Mobilizable plasmid carrying a <i>dorA</i> :: <i>lacZYA</i> transcriptional fusion	This study

* Ap, ampicillin; Km, kanamycin.

pSUP202-Spec (Labes *et al.*, 1990). These plasmids, pALS81 and pALS82, were then transformed into *E. coli* S17-1.

A mutation in *dorB* was generated by cloning a 1.8 kb *Pst*I–*Eco*RI DNA fragment from pALS2 into the *Pst*I and *Eco*RI sites of pSVB30 (Pharmacia). This construct was then digested with *Bal*I that cuts only *dorB*. A Gm resistance interposon with blunt ends was ligated into the *Bal*I site and constructs were screened to identify plasmids with the Gm resistance interposon inserted in both orientations. The two plasmids were then cut with *Pst*I and an 8.4 kb *Pst*I DNA fragment from pWKR459II, containing *mob* and Tc resistance genes, was cloned into this site generating plasmids pALS91 and pALS92.

For construction of a *dorR* mutant, a 1294 bp *Pst*I–*Eco*RI fragment from pALS4 was cloned into pUC8, a 942 bp *Eco*0109I fragment containing part of *dorC* and pUC8 was removed and the plasmid recircularized. This created a plasmid in which *dorR* contained the only *Sal*I site and this enabled a 2.6 kb Gm resistance interposon to be inserted into *dorR*. Constructs were screened to identify a plasmid in which the Gm interposon had been inserted in a direction that was opposite to the direction of transcription of *dorR*. The resulting plasmid was digested with *Eco*RI and an 8.8 kb *Eco*RI fragment from plasmid pWKR459II containing the RP4:*mob* locus and the Tc resistance gene was cloned into this site creating plasmid pALS48. After transformation into *E. coli* S17-1 (Simon *et al.*, 1983), pALS48 was transferred to *R. capsulatus* as described by Masepohl *et al.* (1988). Colonies where a double cross-over had occurred were identified as exhibiting resistance to Gm and sensitivity to Tc.

Bacterial matings with *R. capsulatus* 37b4 were performed according to the filter mating method as described by

Masepohl *et al.* (1988). Mutants were selected by growing the cells on RCV medium (Weaver *et al.*, 1975) supplemented with Gm at 4 μ g ml^{–1}. Double cross-over events were then selected for by isolating cells that were Gm-resistant but sensitive to spectinomycin at a concentration of 25 μ g ml^{–1} (*dorD* mutants) or Tc at 1 μ g ml^{–1} (*dorC* mutants and *dorB* mutants) in RCV medium. *E. coli* was selected against by the inclusion of tellurite at 20 μ g ml^{–1}. The *dorC* mutants are designated mutant C1, in which the Gm resistance gene is transcribed in the opposite direction to *dorC*, and mutant C2, in which the Gm resistance gene is transcribed in the same direction as *dorC*. The *dorD* mutants are designated mutant D1, in which the Gm resistance gene is transcribed in the opposite direction to *dorD*, and mutant D2, in which the Gm resistance gene is transcribed in the same direction as *dorD*. The *dorB* mutants are designated mutant B1, in which the Gm resistance gene is transcribed in the opposite direction to *dorB*, and mutant B2, in which the Gm resistance gene is transcribed in the same direction as *dorB*.

Construction of chromosomal *dorA*::*lacZ* fusions. A *Bam*HI–*Hind*III fragment containing *dorA* from plasmid pJK1 (Knaeblein *et al.*, 1996) was cloned into pSUP401 (Simon *et al.*, 1983) generating pSL109. The *lacZYA* genes from pML5(B+) (Labes *et al.*, 1990) were cloned into the *Bam*HI site of pSL109 to produce pSL111. This plasmid was transferred from *E. coli* S17-1 to *R. capsulatus* as described above and exconjugants with a *dorA*::*lacZ* fusion integrated into the chromosome via a single cross-over were identified by selection for Tc resistance.

Cell growth and β -galactosidase assays. *R. capsulatus* was grown chemoheterotrophically on RCV medium with oxygen as electron acceptor. The cells were shaken vigorously (250

r.p.m.) in a total volume that was less than 10% of the flask volume. For measurement of $\phi[dorA::lacZ]$ expression under aerobic conditions a 2% inoculum of cells was transferred to 20 ml RCV medium in a 250 ml flask and the cells were shaken for a further 7 h before harvesting. For measurements of anaerobic induction of *dor* expression, aerobically grown cells were inoculated (7.5% inoculum) into 220 ml RCV medium in a 250 ml flask. The cells were incubated for 15 h in the dark without shaking. DMSO or TMAO were added to a final concentration of 30 mM. β -Galactosidase assays were performed using the method of Miller (1972). All experiments were performed in triplicate and the results presented represent the mean of three independent experiments.

Western blots, haem stain and enzyme assay. Cells from cultures grown photoheterotrophically for 24 h were harvested by centrifugation at 10000 g in a Beckman JA20 rotor for 20 min and resuspended in 50 mM Tris/HCl, pH 8.0. Crude cell extracts were prepared by passage of the cells twice through a French Pressure cell at 11000 p.s.i. Insoluble matter was removed by centrifugation at 10000 g in a Beckman JA20 rotor for 20 min. The resulting supernatant was used as a crude extract. Protein concentration was determined by the bicinchoninic acid method (Sigma) using bovine serum albumin as standard. DMSO reductase activity was determined as described previously (McEwan *et al.*, 1985). Western blotting of cell extracts and development of blots using anti-DMSO reductase antibody was performed as described by Hatton *et al.* (1994). Staining of proteins separated by SDS-PAGE for haem-dependent peroxidase activity was performed as described by Vargas *et al.* (1993). ECL reagents and 'rainbow' molecular mass markers were obtained from Amersham.

RESULTS

Nucleotide sequence and organization of the *dor* operon

The cloning of *dorA* from two distinct libraries of *R. capsulatus* strain 37b4 DNA has already been reported, the nucleotide sequences have been deposited in the GenBank database (U49506 and X95407) and the primary structure of DMSO reductase has been analysed (Shaw *et al.*, 1996; Knaeblein *et al.*, 1996). Analysis of the nucleotide sequence 5' to *dorA* identified two ORFs that we have named *dorD* and *dorC*, for reasons explained below (Fig. 1). The translation termination

codon of *dorD* overlaps with the translation initiation codon of *dorA* and the start codon for translation of *dorD* also overlaps the stop codon of *dorC* (Fig. 1). Another ORF, which encodes a response regulator in a two-component signal transduction system (designated *dorR*), was identified approximately 180 bp upstream of the *dorC* start codon (Fig. 1). *dorR* is transcribed in the opposite direction to *dorCDA* (Fig. 1). The 180 bp region which lies between *dorR* and *dorC* represents a potential region for regulation of the expression of the *dor* operon. Inspection of the nucleotide sequence of this region is consistent with this view since this DNA contains four Tor boxes (data not shown) which have been shown to bind the response regulator TorR in the *tor* operon of *E. coli* (Simon *et al.*, 1995). Analysis of DNA 3' to *dorA* identified a stable double stem-loop structure which may act as a transcription terminator (Fig. 1). Another ORF was identified downstream of this putative terminator which we have designated *dorB* (Fig. 1). *dorB* is located between *dorA* and a gene cluster encoding enzymes involved in Moco biosynthesis (Solomon *et al.*, 1999).

Analysis of DorC, DorD, DorB and DorR

The putative DorC apoprotein has a molecular mass of 39690 Da and a calculated pI of 10.52. The protein also contains five motifs for the binding of *c*-type haems (Fig. 2). A possible signal peptidase cleavage site was identified between amino acids 24 and 25 using the method of Von Heijne (1986) which suggests that the protein might be synthesized as a pre-protein. Hydropathy plots did not indicate that DorC possessed any hydrophobic regions of sufficient size to be membrane-spanning helices. DorC has a hydrophobic N-terminal sequence that could act as a membrane anchor. Amino acid sequence comparisons revealed that DorC has highest sequence similarity to TorC (33.5% identity, 48.5% similarity). TorC has been shown to be a member of the NirT family of multi-haem *c*-type cytochromes, most of which possess four haem *c* prosthetic groups (Berks *et al.*, 1995a). The sequence alignment (Fig. 2) shows that four of the *c*-type-haem-binding motifs of DorC and

Fig. 1. Organization of the *dor* operon of *R. capsulatus*. B, *Bam*HI; C, *Cl*AI; E, *Eco*RI; P, *Pst*I; S, *Sma*I. The shaded region represents the putative *dor* regulatory sequences. Overlapping start and stop codons are indicated as a solid overline and a dashed underline, respectively.

Fig. 3. Sequence alignment of *R. capsulatus* DorB and *Paracoccus denitrificans* NapD (Berks et al., 1995b). Asterisks denote amino acids that are identical in all sequences and dots denote conservative changes.

Fig. 4. Sequence alignment of DorR with related bacterial response regulators. Asterisks denote amino acids that are identical in all sequences and dots denote conservative changes. The three helices of the DNA-binding helix-turn-helix of OmpR and *E. coli* VirG are known (Suzuki & Brenner, 1995) and are highlighted in bold italics and underlined. The corresponding regions of the other proteins would be expected to form functional helix-turn-helix DNA-binding domains and have been highlighted in bold italics only. *A. tumf*, *Agrobacterium tumefaciens*; *Y. entl*, *Yersinia enterocolitica*.

1995a). DorB has 37.5% similarity and 27.7% identity to NapD (Fig. 3).

Fig. 4. shows an alignment of DorR with response regulators of the OmpR subclass. This includes DorR from *R. sphaeroides* and TorR from *E. coli*, response regulators involved in the induction of DMSO respiration and TMAO respiration, respectively. By analogy with TorR it is expected that in *R. capsulatus* DorR the phosphoryl acceptor in the N-terminal receiver domain is Asp-55. Part of an ORF was identified 5' of *dorR* which appears to be transcribed in the opposite direction to *dorR*. The derived amino acid sequence of part of this gene indicates that it encodes a sensor histidine kinase. This ORF has highest sequence similarity to *E. coli* TorS and *R. sphaeroides* DorS (data not shown). These are complex sensors which possess both classical and alternative transmitter domains, separated by a receiver domain which is homologous to the

domain found in response regulators (Parkinson, 1993). Although the sequence information for *R. capsulatus* DorS is incomplete it also appears to be a complex sensor; the aspartyl residue of the receiver domain which acts as a phosphoryl receiver is conserved while a conserved histidine is present in the alternative transmitter domain.

A *dorC* mutation abolishes expression of a 46 kDa DMSO-inducible cytochrome

dorC of *R. capsulatus* was inactivated *in vitro* by insertion of a Gm resistance cassette. This mutated *dorC* was exchanged for the wild-type copy of *dorC* by site-specific recombination, as described in Methods. Two mutants were constructed in which the Gm cassette was transcribed in a direction opposite to that of the *dor* operon (mutant *dorC1*) and in which transcription of

Fig. 5. Analysis of polypeptides from membranes of *R. capsulatus* for haem-dependent peroxidase activity. Polypeptides were separated using SDS-PAGE, 50 µg protein per lane. Lanes: 1, strain 37b4 (wild-type) grown in absence of DMSO; 2, strain 37b4 grown in presence of DMSO; 3 and 4, *dorC2* grown in the absence and presence of DMSO, respectively; 5 and 6, *dorC1* grown in the absence and presence of DMSO, respectively.

the Gm cassette is in the same direction as *dorC* (mutant *dorC2*). The presence of a DMSO-inducible *c*-type cytochrome in membranes of *R. capsulatus* and *R. sphaeroides* has been recognized for many years and it can be observed as a polypeptide that stains for haem-dependent peroxidase activity. Fig. 5 shows a SDS-PAGE gel of cell extracts from wild-type *R. capsulatus* and the *dorC* mutants stained for haem-dependent peroxidase activity. In extracts from wild-type cells a DMSO-inducible haem-staining polypeptide was observed with a molecular mass of about 46 kDa (Fig. 5, lane 2). This haem-staining polypeptide was absent from mutants *dorC1* and *dorC2* (Fig. 5, lanes 4 and 6). The above data show that the 46 kDa cytochrome is the *dorC* gene product. It was also observed that, unlike wild-type cells, neither *dorC1* nor *dorC2* was able to

grow anaerobically in the dark with DMSO as electron acceptor (data not shown) and this provides further evidence that DorC is essential for DMSO respiration.

Expression of DMSO reductase in *dorA*, *dorC*, *dorD* and *dorB* mutants

A *dorA* mutant was constructed as described in Methods. This mutant exhibited no DMSO reductase activity with reduced methylviologen and no DMSO reductase polypeptide could be detected using an anti-DMSO reductase antibody in cell-free extracts of the mutant (data not shown). The sequence and mutational analysis of the *dor* genes predicts that DorC and DorA are electron transport proteins. This raised the question of the role of DorD and DorB, as well as the question of whether the *dor* genes form an operon. To address these questions, the effects of mutations in three *dor* genes on the expression of DMSO reductase were analysed. Mutant *dorC1* showed a loss of reduced methylviologen-DMSO oxidoreductase activity (Table 2). A Western blot of cell extracts from *dorC1* showed that the DorA polypeptide was not produced in this mutant (Fig. 6a, lane 2). Mutant *dorC2* showed a decrease in reduced methylviologen-DMSO reductase activity to a level equal to 45 % of wild-type activity (Table 2). A Western blot of cell-free extracts from *dorC2* showed that the DorA polypeptide could be detected (Fig. 6a, lane 1). This suggested that a polar mutation in *dorC* abolished *dorA* expression while this continued in cells containing a non-polar mutation in *dorC*.

Two *dorD* mutants were constructed as described in the Methods. In *dorD1* the Gm cassette is transcribed in a direction opposite to that of the *dor* operon while in mutant *dorD2* transcription of the Gm cassette proceeds in the same direction as *dorD*. Mutants *dorD1* and *dorD2* showed a complete loss of both reduced methylviologen-DMSO oxidoreductase activity (Table 2) and polypeptide production, as indicated by Western blotting (data not shown). Chromosomal *dorA::lacZ* transcriptional fusions were constructed in the *dorD* mutants to determine whether *dorA* was transcribed. As expected

Table 2. DMSO reductase activity and expression of *dorA* and *moaA* transcriptional fusions in wild-type *R. capsulatus* 37b4 and *dor* mutants

All cells were grown phototrophically in the presence of DMSO as described in the Methods. One unit of DMSO reductase activity is defined as 1 µmol methylviologen oxidized min⁻¹ (mg protein)⁻¹. ND, Not detected.

Sample	DMSO reductase activity (units)	ϕ[<i>dorA::lacZ</i>] activity (Miller units)	ϕ[<i>moaA::lacZ</i>] activity (Miller units)
37b4	28.0	29.3 (± 3.9)	69.8 (± 1.3)
<i>dorC1</i>	0	ND	ND
<i>dorC2</i>	12.6	ND	ND
<i>dorD1</i>	0	0	ND
<i>dorD2</i>	0	13.7 (± 0.5)	ND
<i>dorB1</i>	5.0	ND	0
<i>dorB2</i>	11.5	ND	22.3 (± 1.7)

Fig. 6. (a) Western blot of cell extracts of *dorC* mutants probed with anti-DorA antibody. Each lane contains 20 µg protein from cells grown in the presence of DMSO. Lanes: 1, *dorC2*; 2, *dorC1*; 3, 37b4. (b) Western blot of cell extracts of *dorB* mutants probed with anti-DorA antibody. Each lane contains 20 µg protein from cells grown in the presence of DMSO. Lanes: 1, 37b4; 2, *dorB1*; 3, *dorB2*.

$\phi[dorA::lacZ]$ activity in *dorD1* was zero but in *dorD2* the activity was slightly under 50% of the activity in wild-type cells (Table 2). Thus, the failure to detect

DMSO reductase polypeptide in *dorD2* was not due to the abolition of the transcription of *dorA*.

Two *dorB* mutants were constructed as described in Methods. Table 2 shows that polar and non-polar mutations in *dorB* reduced, but did not abolish, the activity of DMSO reductase. DMSO reductase activity in mutants *dorB1* and *dorB2* was 18 and 41% of that in wild-type cells, respectively. Western blots of crude extracts of both mutants showed that two polypeptides reacted with anti-DMSO reductase (Fig. 6b, lanes 2 and 3). The smaller polypeptide had a molecular mass of 85 kDa, identical to that of purified DMSO reductase. The larger polypeptide was not observed in cell-free extracts from wild-type cells and had a mass about 5 kDa greater than the smaller polypeptide. The organization of the *dor* gene cluster (Fig. 1) leads to the possibility that a polar mutation in *dorB* might abolish the expression of the Moco biosynthetic genes. Hence, the expression of a *moaA::lacZ* transcriptional fusion was measured in mutants *dorB1* and *dorB2*. The results showed that the $\phi[moaA::lacZ]$ activity was 0 and 22.3 Miller units in *dorB1* and *dorB2*, respectively, compared to 70 Miller units in wild-type cells.

Regulation of the expression of the *dorCDA* operon by Fnr and the presence of an electron acceptor

Some of the physiological factors regulating the expression of DMSO reductase in *R. capsulatus* have already been identified but the details of the regulation of transcription have not been established. To address this question chromosomal *dorA::lacZYA* transcriptional fusions were constructed in wild-type *R. capsulatus* and a number of mutants and $\phi[dorA::lacZ]$ activity was measured in cells grown aerobically and anaerobically as described in Methods section. Expression of the *dorCDA* operon was not detectable in cells grown at high oxygen tension but a low level of expression could be observed when the cells were grown at low oxygen tension (data not shown). The presence of DMSO in anaerobic cultures led to a greater than 40-fold increase in $\phi[dorA::lacZ]$ activity while addition of TMAO led to only about a 7-fold increase (Table 3).

Table 3. $\phi[dorA::lacZ]$ expression in *R. capsulatus* strains grown under phototrophic (anaerobic conditions) in the presence and absence of DMSO and TMAO

Sample	β -Galactosidase activity (Miller units)
37b4 (wild-type)	ND
37b4 $\phi[dorA::lacZ]$	1.6 $\pm$ 0.3
37b4 $\phi[dorA::lacZ]$ + DMSO	71.8 $\pm$ 2.2
37b4 $\phi[dorA::lacZ]$ + TMAO	11.4 $\pm$ 1.6
RGK295 (Δfnr) $\phi[dorA::lacZ]$	8.3 $\pm$ 0.5
RGK295 (Δfnr) $\phi[dorA::lacZ]$ + DMSO	3.5 $\pm$ 0.8
RGK295 (Δfnr) $\phi[dorA::lacZ]$ + TMAO	2.6 $\pm$ 0.9
37b4 $\Delta dorR$ $\phi[dorA::lacZ]$	ND
37b4 $\Delta dorR$ $\phi[dorA::lacZ]$ + DMSO	ND

ND, Not detected.

It has been reported that Fnr is essential for the synthesis of DMSO reductase in *R. capsulatus* (Zeilstra-Ryalls *et al.*, 1997), although no study of transcriptional regulation was made. Table 3 shows that in an FNR mutant of *R. capsulatus* a basal level of $\phi[dorA::lacZ]$ activity was detected in microaerobic cells. However, the addition of DMSO or TMAO failed to induce expression of the *dorCDA* operon, in contrast to the situation in wild-type cells. In view of the observations made in *R. sphaeroides* (Mouncey & Kaplan, 1998) and *E. coli* (Jourlin *et al.*, 1996a, b), it seems likely that the DorS/DorR two-component system is responsible for the induction of *dorCDA* operon expression by DMSO and TMAO. This was confirmed by constructing a *dorR* mutant and in this case no $\phi[dorA::lacZ]$ activity could be detected under any growth conditions (Table 3).

DISCUSSION

Although many facultative aerobic bacteria are able to use DMSO and TMAO as electron acceptors in anaerobic respiration, it is only in *E. coli* that the operons encoding the components of these respiratory pathways have been investigated in detail (Mejean *et al.*, 1994). The *tor* operon encoding the TMAO respiratory proteins was shown to contain two genes, *torC* and *torD*, in addition to the TMAO reductase structural gene, *torA* (Mejean *et al.*, 1994). The results described in this paper have shown that genes homologous to *torC* and *torD* are linked to *dorA* of *R. capsulatus*. A second oxidoreductase in *E. coli* uses DMSO as the preferred substrate and can be purified as a menaquinol-DMSO oxidoreductase (Weiner *et al.*, 1988). This DMSO reductase is encoded by the *dmsABC* operon (Weiner *et al.*, 1992). The sequence analysis of *dorCDA* from *R. capsulatus* confirms that it should be compared with *torCAD* and not *dmsABC* from *E. coli*. The experiments with the *dorC* and *dorD* mutants in which the Gm cassette was inserted so that there is a polar effect on transcription resulted in no DMSO reductase polypeptide being produced. This observation is consistent with the view that there is no promoter for *dorA* expression within *dorC* or *dorD* and that *dorCDA* forms an operon. The presence of a likely transcription terminator immediately after the translation stop codon of *dorA* suggests that these three cistrons are co-transcribed. However, the presence of an additional ORF (*dorB*) and a Moco biosynthetic gene cluster which is essential for DMSO reductase activity (Solomon *et al.*, 1999) suggests that transcription of the *dor* gene cluster may be complex. One possibility is that *dorCDA* transcripts plus larger transcripts might be generated by read-through beyond the putative transcription terminator. This possibility is currently under investigation.

The electron transfer components connecting the ubiquinol pool to DMSO reductase have been identified as cytochromes using spectroscopic methods (McEwan *et al.*, 1989). DorC has five C-X-X-C-H motifs, indicating that this protein represents the *c*-type cytochromes which can be detected in reduced-minus-DMSO oxidized difference spectra in *R. capsulatus* (McEwan *et al.*,

1989). A similar protein, TorC, is present in *E. coli* (Mejean *et al.*, 1994). The broad α absorption band of the cytochrome in reduced-minus-DMSO oxidized spectra of whole cells of *R. capsulatus* is consistent with the oxidation of a cytochrome with several haem centres with absorption maxima ranging from 550 to almost 560 nm (McEwan *et al.*, 1989). Indeed, since DorD is highly unlikely to be a haemoprotein, it is probable that DorC is responsible for all of the absorption changes observed in the difference spectra and that no *b*-type cytochrome, in addition to *c*-type cytochrome(s), is involved in this pathway, in contrast to the suggestion of McEwan *et al.* (1989).

In *E. coli* the presence of *torC* has also been correlated with the production of a haem-staining polypeptide of apparent molecular mass 46 kDa and this is almost certainly TorC (Ujjiye *et al.*, 1996). In *R. capsulatus* the situation is more complicated because of the presence of a DMSO-induced 44 kDa cytochrome (Hanlon *et al.*, 1992) which is almost certainly a cytochrome *c* peroxidase (CCP). However, CCP is mainly located in the periplasm of *R. capsulatus*, in contrast to DorC that is a membrane-bound protein (A. L. Shaw & A. G. McEwan, unpublished observations). The data shown in Fig. 5 are consistent with the view that the membrane-bound 44 kDa cytochrome induced by DMSO is the *dorC* gene product. A similar conclusion has been made by Mouncey *et al.* (1997) in *R. sphaeroides*.

It seems probable that DorC is bound to the periplasmic face of the cytoplasmic membrane via its hydrophobic N-terminal sequence. Although we identified a potential N-terminal signal polypeptide in DorC, it is possible that it is not cleaved; this is the case in another membrane-bound protein which faces the periplasm, cytochrome *c_v* of *R. capsulatus* (Myllykallio *et al.*, 1997). Sequence analysis showed that DorC, like TorC, is a member of the NirT class of cytochromes. However, these two proteins differ from the other members of the NirT class in that they possess an additional C-terminal domain containing a haem *c* binding motif. It seems likely that these proteins possess a tetrahaem domain that receives electrons from ubiquinol, as suggested for other members of this class. Electrons would then be transferred to *c*-type cytochrome at the C terminus and it is this component which may donate electrons to DMSO reductase. In other systems, such as Nap, a distinct periplasmic cytochrome is involved in electron transfer from the NirT class cytochrome to the terminal reductase (Berks *et al.*, 1995a).

Sequence alignment of DorD with TorD of *E. coli* and *Haemophilus influenzae* highlights their similarities but offers little clue to their function. DorD is the homologue of DorB from *R. sphaeroides* (Ujjiye *et al.*, 1996; Mouncey *et al.*, 1997) and, in view of the relationship between these proteins and those in enteric bacteria, it is suggested that DorD is a more appropriate name. Although DorD exhibits hydrophobic regions near the N and C termini, we have observed that an N-terminal histidine-tagged DorD is water-soluble, suggesting a cytoplasmic location for this protein (A. L. Shaw & A.

G. McEwan, unpublished observations). Until recently, the function of the TorD/DorD class of proteins has eluded definition. However, it has now been shown that *E. coli* TorD acts as a cytoplasmic chaperone-like protein and interacts with unfolded TorA (Pommier *et al.*, 1998). Our results are consistent with a similar role for DorD in the biogenesis of DMSO reductase in *R. capsulatus*. The comparison of mutants *dorC2* and *dorD2* showed that DorD was required to allow production of active DMSO reductase. Since *dorA* was transcribed in the *dorD2* mutant, it seems likely that DorA is not stabilized in the absence of DorD and that it is rapidly degraded. However, pulse-chase experiments would be required to verify this view. In earlier work it has been shown that a *torD* mutation results in lowered amounts of TMAO reductase (Mejean *et al.*, 1994) but the effect was not as dramatic as that of a *dorD* mutation on the level of DMSO reductase in *R. capsulatus*. One explanation for this could be the presence of a second TorD-like protein, ORF Ycac, which is located close to the *dms* operon in *E. coli* (Blattner *et al.*, 1997). Perhaps ORF Ycac has a role in the biogenesis of DMSO reductase and TMAO reductase in *E. coli*.

The phenotype of the *dorB* mutant suggests that it is also involved in the biogenesis of DMSO reductase. Both polar and non-polar mutations in *dorB* appeared to cause a decrease in the level of *moaA* expression and as a result the phenotype of the *dorB* mutant resembled that of a *moaA* mutant (Solomon *et al.*, 1999). In these mutants two forms of DMSO reductase polypeptide were observed. We have shown that the small form of DMSO reductase is probably periplasmic mature DMSO reductase while the larger polypeptide is probably cytoplasmic apo-DMSO reductase (Solomon *et al.*, 1999). A polar mutation in *moaA* caused a 97% decrease in DMSO reductase activity but did not affect the activity of other molybdoenzymes (Solomon *et al.*, 1999). In contrast, a polar mutation in *dorB* caused an 82% decrease in DMSO reductase activity while a non-polar mutation in *dorB* caused a 42% decrease. This distinguishes the phenotype of the *dorB* mutant from the *moaA* mutant. Although our experiments do not identify the function of DorB, it is suggested that it has a role in Moco insertion into DorA. If this were the case then DorB may have a role in Moco insertion that is similar to that proposed for NarJ in the assembly of nitrate reductase (Blasco *et al.*, 1998).

A major difference between the *dor* and *tor* systems is their regulation by oxygen. In *E. coli* there is no evidence that Fnr controls the expression of the *tor* operon and the reason why the *tor* operon is not expressed under aerobic conditions is not clear. The periplasmic TorT protein could have a role (Jourlin *et al.*, 1996a, b) and it is interesting that the homologue of *torT* is absent from the *dor* gene cluster in *R. sphaeroides* and *R. capsulatus*. The results in this paper confirm that expression of DMSO reductase in *R. capsulatus* is dependent on Fnr and establish that it affects the transcription of *dorCDA*. However, it seems unlikely Fnr acts directly to induce

expression of this operon; no consensus binding-site for Fnr was identified in the *dorCDA* regulatory sequence, even though recognizable Fnr consensus sequences are readily identifiable in *R. capsulatus* (Zeilstra-Ryalls *et al.*, 1997). In *R. sphaeroides* it appears that Fnr exerts an effect on *dor* operon expression by positively regulating the expression of the sensor *dorS* (Mouncey & Kaplan, 1998). This also seems to be the case in *R. capsulatus* (A. L. Shaw & A. G. McEwan, unpublished observations) but further analysis is required to determine whether the organization of the *dor* regulatory region 5' of *dorR* in *R. capsulatus* is the same as that described by Mouncey & Kaplan (1998) in *R. sphaeroides*.

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