JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7617–7624 Vol. 179, No. 24
0021-9193/97/$04.00+0

Characterization of Genes Encoding Dimethyl Sulfoxide Reductase of *Rhodobacter sphaeroides* 2.4.1^T: an Essential Metabolic Gene Function Encoded on Chromosome II

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Received 4 August 1997/Accepted 7 October 1997

***Rhodobacter sphaeroides* 2.4.1^T is a purple nonsulfur facultative phototrophic bacterium which exhibits remarkable metabolic diversity as well as genomic complexity. Under anoxic conditions, in the absence of light and the presence of dimethyl sulfoxide (DMSO) or trimethylamine *N*-oxide (TMAO), *R. sphaeroides* 2.4.1^T utilizes DMSO or TMAO as the terminal electron acceptor for anaerobic respiration, which is mediated by the molybdoenzyme DMSO reductase. Sequencing of a 13-kb region of chromosome II revealed the presence of 10 putative open reading frames, of which 5 possess homology to genes encoding the TMAO reductase (the *tor* system) of *Escherichia coli*. The *dorS* and *dorR* genes encode a sensor-regulator pair of the two-component sensory transduction protein family, homologous to the *torS* and *torR* gene products. The *dorC* gene was shown to encode a 44-kDa DMSO-inducible *c*-type cytochrome. The *dorB* gene encodes a membrane protein of unknown function homologous to the *torD* gene product. The *dorA* gene encodes DMSO reductase, containing the molybdopterin active site. Mutations were constructed in each of these *dor* genes, and the resulting mutants were shown to be impaired for DMSO-dependent anaerobic growth in the dark. The mutant strains exhibited negligible levels of DMSO reductase activity compared to the wild-type strain under similar growth conditions. Further, no DorA protein was detected in DorS and DorR mutant strains with anti-DorA antisera, suggesting that the products of these genes are required for the positive regulation of *dor* expression in response to DMSO. This characterization of the *dor* gene cluster is the first evidence that genes of chromosome CII encode metabolic functions which are essential under particular growth conditions.**

Rhodobacter sphaeroides is a gram-negative purple nonsulfur bacterium belonging to the alpha-3 subdivision of the *Proteobacteria* (49). This organism is capable of a wide range of metabolic lifestyles, exhibiting growth chemoautotrophically, photoautotrophically, photoheterotrophically, chemoheterotrophically, and diazotrophically (reviewed in reference 24). The ability of purple nonsulfur bacteria, as well as other bacterial species, to utilize dimethyl sulfoxide (DMSO) and trimethylamine *N*-oxide (TMAO) as terminal electron acceptors in the absence of oxygen for chemoheterotrophic growth has been well documented (12, 23, 52). Both DMSO and TMAO are commonly occurring compounds in nature and play roles in sulfur and nitrogen cycling (1).

Under anoxic conditions in the presence of DMSO or TMAO and in the absence of light, *R. sphaeroides* can grow via a periplasmic DMSO reductase (DMSOR), which serves as the terminal electron acceptor in a cascade in which electrons are transferred from quinol to DMSO or TMAO (24). This process does not require the cytochrome bc_1 complex, and electrons are transferred from quinol to DMSOR via a membrane-bound *b*-type cytochrome and a periplasmic *c*-type cytochrome (25). DMSOR from both *R. sphaeroides* and the closely related bacterium *R. capsulatus* have been crystallized and shown to contain the molybdopterin cofactor (Moco) as the sole prosthetic group (for a review, see reference 21).

Studies on the genetics of DMSO reduction in both *R. sphaeroides* and *R. capsulatus* have been limited mainly to several reports which describe the sequence of the gene encoding DMSOR (2, 22, 37, 51). The *dmsA* or *dorA* gene product has extensive homology to other molybdoenzymes such as biotin sulfoxide reductase and TMAO reductase (50). Recently, the sequences of two genes upstream of the *dmsA* gene of *R. sphaeroides* f. sp. *denitrificans* were reported (45). The *dmsC* and *dmsB* gene products were shown to be homologous to the *torC* and *torD* gene products, which encode a *c*-type cytochrome and a membrane protein, respectively, and it was suggested that the *dmsCBA* genes form a transcriptional unit, similar to the *torCDA* genes in *E. coli* (27).

Studies from our laboratory revealed that *R. sphaeroides* 2.4.1^T possesses two different circular chromosomes, of ~3.0 Mbp (CI) and ~0.9 Mbp (CII) (42, 43). A number of genes have been shown to be duplicated between CI and CII (5). In contrast, we have previously shown by Tn*5* mutagenesis that some pathways, e.g., *p*-aminobenzoic acid, uracil, histidine, and thymine biosynthesis, are partitioned rather than duplicated between CI and CII (4). These results suggested that CII is an essential genomic element, having unique as well as shared and duplicated functions.

Recently, we used a low-redundancy sequencing strategy for analysis of the genetic content of CII (5). We analyzed ~300 kb of unique DNA which identified approximately 200 putative open reading frames (ORFs) representing a wide variety of functions, e.g., amino acid biosynthesis, nutrient transporters, redox-active systems, and a number of regulatory functions, indicating that CII does not contain genes specialized for any particular metabolic function, physiologic state, or growth condition.

In an attempt to understand the functional role of genes of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or characteristics	Reference or source
R. sphaeroides		
2.4.1^T	Wild-type	46
NM15	*dorS*::ΩSt/Sp	This study
NM16	*dorR*::ΩSt/Sp	This study
NM17	*dorC*::ΩSt/Sp	This study
NM18	*dorB*::ΩSt/Sp	This study
NM19	*dorA*::ΩSt/Sp	This study
E. coli		
DH5α*phe*	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_{K^-} m_{K^+}) *supE44* λ$^-$ *thi-1 gyrA relA1 phe*::Tn*10d*Cm	11
HB101	F$^-$ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 glaK2 lacYI* Δ(*mcrC-mrr*) *rpsL20* (St^r) *xyl-5 mtl-1 recA13*	3
Cosmids		
pUI8508	pLA2917 derivative + ca. 15 kb of *R. sphaeroides* 2.4.1^T DNA containing *orf1 orf2 orf3 dorS dorR dorC dorB dorA moeA moaA*	10
pUI8519	pLA2917 derivative + ca. 25 kb of *R. sphaeroides* 2.4.1^T DNA containing *orf1 orf2 orf3 dorS dorR dorC dorB dorA moeA moaA*	10
Plasmids		
pUI1087	Cloning vector	54
pBS II	Cloning vector, Amp^r, with T3 and T7 promoters	Stratagene
pHP45Ω	Source of the ΩSt/Sp cassette	31
pSUP202	Mob$^+$ Amp^r Cm^r Tc^r	40
pRK2013	Conjugative helper plasmid	13
pRK415	pRK404 derivative with modified polylinker; Tc^r	20
pNMT2	pUI1087 containing 2,054-bp *Eco*RI fragment (*dorC dorB*)	This study
pNMT4	pUI1087 containing 4,680-bp *Eco*RI fragment (*orf2 orf3 dorS dorR dorC*)	This study
pNMT5	pUI1087 containing 2,814-bp *Eco*RI fragment (*dorA moeA moaA*)	This study
pNMT6	pBluescript II containing 3,191-bp *Bam*HI fragment (*dorS dorR dorC dorB*)	This study
pNMT15	pUI1087 containing 1,769-bp *Eco*RI-*Pst*I fragment (*orf2 orf3 dorS*)	This study
pNMT16	pUI1087 containing 2,911-bp *Eco*RI-*Pst*I fragment (*dorS dorR dorC*)	This study
pNMT20	pNMT16 containing ΩSt/Sp cassette inserted into *Bam*HI site in *dorS*	This study
pNMT23	pSUP202 containing 4.8-kb *Eco*RI-*Pst*I fragment from pNMT20	This study
pNMT24	pNMT2 containing ΩSt/Sp cassette inserted into *Stu*I site in *dorC*	This study
pNMT25	pSUP202 containing 4.0-kb *Eco*RI fragment from pNMT24	This study
pNMT26	pBS II containing 3,479-bp *Pst*I fragment (*dorB dorA moeA*)	This study
pNMT27	pNMT26 containing ΩSt/Sp cassette inserted into *Stu*I site in *dorA*	This study
pNMT28	pSUP202 containing 5.5-kb *Pst*I fragment from pNMT27	This study
pNMT37	pNMT6 containing ΩSt/Sp cassette inserted into *Nru*I site in *dorR*	This study
pNMT40	pSUP202 containing 3.0-kb *Xho*I fragment from pNMT37	This study
pNMT41	pUI1087 containing 1.5-kb *Eco*RI-*Stu*I fragment from pNMT2 (*dorC dorB*)	This study
pNMT42	pNMT41 containing ΩSt/Sp cassette inserted into *Bam*HI site in *dorB*	This study
pNMT43	pSUP202 containing 3.5-kb *Eco*RI-*Pst*I fragment from pNMT42	This study
pNMT59	pBS containing 4,316-bp *Sal*I fragment (*dorC dorB dorA moeA*)	This study
pNMT61	pRK415 containing 3,479-bp *Pst*I fragment (*dorB dorA moeA*)	This study
pNMT66	pRK415 containing 4.4-kb *Kpn*I-*Hin*dIII fragment from pNMT59	This study

CII, we present here the first detailed genetic analysis of a large DNA sequence from CII. We describe the sequencing and characterization of a 13-kb region of CII which contains both structural and regulatory genes for DMSOR from *R. sphaeroides* 2.4.1^T. We show that mutations in these genes, herein designated *dor*, impair anaerobic-dark DMSO growth and block DMSOR activity. We also present evidence indicating that the expression of DMSOR is induced by both anaerobiosis and the presence of DMSO. These results are the first examples of an essential function for the products of genes encoded on CII for any particular growth condition.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium, and *R. sphaeroides* strains were grown at 30°C in Sistrom's minimal medium A containing succinate as the carbon source (6, 35). Where appropriate, DMSO was added at a final concentration of 60 mM and TMAO was added at a final concentration of 30 mM. The cells were grown anaerobically in sealed glass tubes, which were first sparged with nitrogen gas, and were incubated in the dark for chemoheterotrophic growth or in front of a 10-W-m^{-2} light source for photoheterotrophic growth. Aerobic cultures were grown on a rotary shaker in glass flasks. Antibiotics were used as follows to maintain selection for plasmids or to select for recombinant strains: ampicillin, 100 μg ml^{-1} (*E. coli*); kanamycin, 25 μg ml^{-1} (*R. sphaeroides*) and 50 μg ml^{-1} (*E. coli*); spectinomycin, 25 μg ml^{-1} (*R. sphaeroides* and *E. coli*); streptomycin, 25 μg ml^{-1} (*R. sphaeroides* and *E. coli*); and tetracycline, 1 μg ml^{-1} (*R. sphaeroides*) and 10 μg ml^{-1} (*E. coli*).

Materials and reagents. All reagents and materials used were of analytical grade and, except where noted, were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Construction of mutants. Standard recombinant DNA techniques were used for construction of mutants throughout (35). Enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.), Promega Corp. (Madison, Wis.), and Boehringer Mannheim Biochemicals, Bethesda Research Laboratories Life Technologies Inc. (Gaithersburg, Md.).

To construct a *dorS* insertion mutation, the 2.9-kb *Pst*I-*Eco*RI fragment containing the 3′ half of *dorS* and the whole of *dorR* was cloned into pUI1087, resulting in plasmid pNMT16. The ΩSt/Sp cassette from *Bam*HI-digested pHP45Ω was inserted into the *Bam*HI site of pNMT16, resulting in plasmid

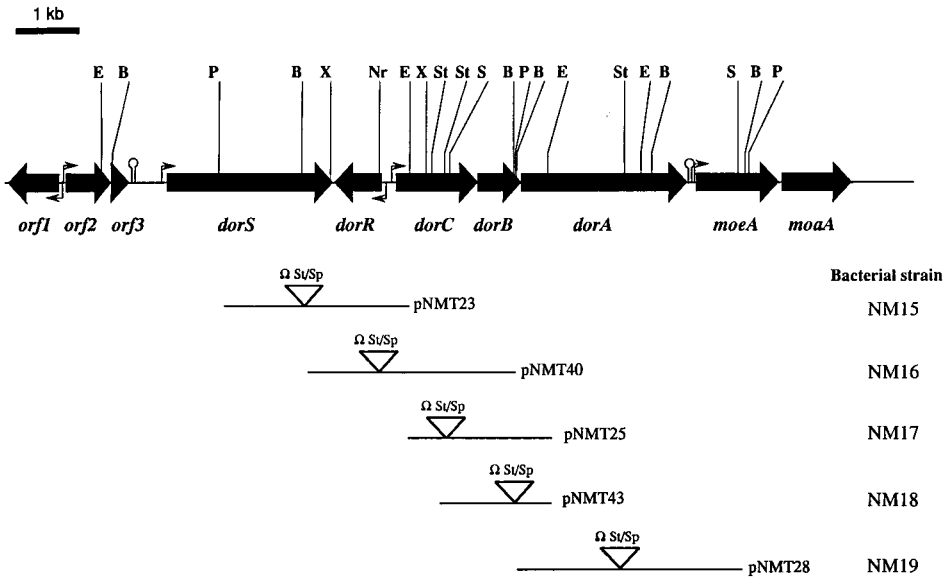

FIG. 1. Physical map of the *dor* region of *R. sphaeroides* 2.4.1^T. The large arrows show the genes and the directions of transcription. The positions of insertion and/or deletion mutations in the *dor* genes constructed by insertion of the ΩSt/Sp resistance cassette are shown below the map. The plasmids and resulting strains constructed are listed. Putative promoters are shown by the small arrows, and terminators are shown by the stem-loop structures. Endonuclease sites: B, *Bam*HI; E, *Eco*RI; N, *Not*I; Nr, *Nru*I; P, *Pst*I; S, *Sal*I; St, *Stu*I; X, *Xho*I.

pNMT20. The 4.8-kb *Eco*RI-*Pst*I fragment from pNMT20 was cloned into pSUP202 to give pNMT23. To construct a *dorR* insertion mutation, the 3.2-kb *Bam*HI fragment containing the complete *dorR* and *dorC* genes was cloned into pBluescript II to give plasmid pNMT6. *Nru*I-digested pNMT6 was ligated to the *Sma*I-digested ΩSt/Sp cassette, resulting in plasmid pNMT37. A 3.0-kb *Xho*I fragment from pNMT37, containing the *dorR*::Ω insertion, was ligated to *Sal*I-digested pSUP202 to form plasmid pNMT40. The *dorC* insertion-deletion mutation was constructed by cloning the 2.1-kb *Eco*RI fragment, which contains the 3′ half of *dorC* and the 5′ half of *dorB* into pUI1087, generating plasmid pNMT2. The *Sma*I-digested ΩSt/Sp cassette was ligated to *Stu*I-digested pNMT2, resulting in plasmid pNMT24. *Eco*RI-digested pNMT24 was cloned into *Eco*RI-digested pSUP202 to give plasmid pNMT25. The *dorB* insertion-deletion mutation was constructed by ligating the 1.5-kb *Eco*RI-*Stu*I fragment from pNMT2 to *Eco*RI-*Sma*I-digested pUI1087, resulting in plasmid pNMT41. The *Bam*HI-digested ΩSt/Sp cassette was ligated to *Bam*HI-digested pNMT41 to give plasmid pNMT42. The 3.5-kb *Pst*I-*Eco*RI fragment from pNMT42 was cloned into *Pst*I-*Eco*RI-digested pSUP202, resulting in plasmid pNMT43. The *dorA* insertion mutation was constructed by cloning the 3.5-kb *Pst*I fragment containing the complete *dorA* gene into pBluescript II, generating plasmid pNMT26. The *Sma*I-digested ΩSt/Sp cassette was ligated into *Stu*I-digested pNMT26, resulting in plasmid pNMT27. *Pst*I-digested pNMT27 was cloned into *Pst*I-digested pSUP202 to give plasmid pNMT28.

All of the *dor*::Ω insertion or insertion-deletion mutation pSUP202-derivative plasmids were conjugated into *R. sphaeroides* 2.4.1^T by triparental matings with pRK2013, as described previously (7). The plasmid-borne mutated genes were integrated into the *R. sphaeroides* genome by homologous recombination. Putative double-crossover candidates were screened for antibiotic sensitivity and anaerobic growth in the dark with DMSO. The integration sites of the cassettes in the *R. sphaeroides* genome were confirmed by nonradioactive Southern hybridizations of restriction digests of genomic DNA probed with appropriate biotinylated probes, as described previously (35).

Construction of clones for complementation. Clones for complementation of DorC, DorB, and DorA mutants were constructed as follows. The 3.5-kb *Pst*I fragment from pNMT26 was cloned into *Pst*I-digested pRK415, resulting in plasmid pNMT61, which places the *dorA* gene in the orientation where it is under control of the vector *lac* promoter. To construct a clone containing both *dorB* and *dorA*, the 4,316-bp *Sal*I fragment containing the *dorB* and *dorA* genes was cloned into *Sal*I-digested pBluescript II to give plasmid pNMT59. The 4.4-kb *Kpn*I-*Hin*dIII fragment from pNMT59 was cloned into pRK415, resulting in plasmid pNMT66, in which the *dorB* and *dorA* genes are in the correct orientation, under control of the *lac* promoter.

DNA sequencing. Automated DNA sequencing was performed with an ABI 373A automatic DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center, Houston, Tex. Oligonucleotides used for priming the sequencing reactions were purchased from Bethesda Research Laboratories Life Technologies. The sequences were analyzed with the Genetics Computer Group (GCG) programs and the BLAST server at the National Center for Biotechnology Information (8).

Enzyme assays. Cells for DMSOR activity were grown to mid-log phase under appropriate conditions, washed twice with degassed 0.1 M Tris-HCl (pH 8.0) buffer, and resuspended in 50 mM Tris-HCl (pH 8.0) buffer. The cells were broken by passage through a French press (Aminco, Urbana, Ill.), and the resulting extracts were stored at 4°C under N_2 until required. DMSOR activity was assayed by measuring the DMSO-dependent oxidation of partially reduced methyl viologen, as previously described (17, 26).

Immunoblotting. Crude cell extracts, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels, were transferred to nitrocellulose membranes by wet electrotransfer in 50 mM Tris–380 mM glycine–0.1% SDS–20% methanol buffer. DMSOR (DorA) polypeptide was detected on the protein blots by the alkaline phosphatase color detection system (Promega Corp., Madison, Wis.) with polyclonal rabbit antiserum against purified *R. capsulatus* DorA protein (1:2,500 dilution) and secondary goat anti-rabbit alkaline phosphatase-linked immunoglobulin (1:25,000 dilution).

Heme staining. Heme staining of polypeptides electrophoresed on 15% polyacrylamide gels was performed with 3,3′,5,5′-tetramethylbenzidine by the previously described method of Thomas et al. (44).

Nucleotide sequence accession number. The nucleotide sequence of the *dorSRCBA* genes has been deposited in the nucleotide sequence databases under accession no. AF016236.

RESULTS

Sequence of *dor* gene cluster. As part of the low-redundancy sequencing strategy for CII, overlapping cosmids which contained genes possessing extensive homology to the previously sequenced *dmsCBA* genes from *R. sphaeroides* f. sp. *denitrificans* were identified (45). These cosmids mapped to the *Dra*I R fragment from CII of *R. sphaeroides* 2.4.1^T (42). Subclones were constructed from cosmids pUI8508 and pUI8519, which contain approximately 25 kb of unique insert DNA. Sequencing from both ends of each subclone resulted in approximately 85% coverage of a 13-kb region of DNA containing genes homologous to the *dmsCBA* genes. The remaining gaps were filled by further subcloning and primer walking strategies. This 13-kb DNA sequence encodes 10 putative ORFs, of which only 7 will be discussed in this paper (Fig. 1; Table 2). Since the DMSOR from *R. sphaeroides* 2.4.1^T is more closely related to the Tor TMAO reductase of *E. coli* than to the *E. coli* Dms

TABLE 2. Characteristics of genes in the *dor* region of *R. sphaeroides* 2.4.1^T

Gene	Start position[a]	Stop position[a]	Size of product[b]	Similar protein in database	Identity observed or BLAST probabilities[c]	Shine-Dalgarno sequence[d]
dorS	2534	4978	815	LemA sensor kinase, *P. syringae*	$9.7e^{-50}$	GGAAGGAGTTTGGTCG**ATG**
dorR	5697	5002	232	ArcA anaerobic response regulator, *E. coli*	$7.1e^{-31}$	CAGGAGACCTCGCGCGTC**ATG**
dorC	5930	7142	404	DmsC pentaheme cytochrome, *R. sphaeroides* f. sp. *denitrificans*	98%	GGGGAAGGACCGGCGCGCCCGCCGC ATTCCTGCGG**ATG**
dorB	7141	7818	226	DmsB membrane protein, *R. sphaeroides* f. sp. *denitrificans*	97%	GGAAGGAGCTGCGGA**ATG**
dorA	7818	10283	822	DmsA DMSO reductase, *R. sphaeroides* f. sp. *denitrificans*	98%	GGAAGAAAGGAAAGCCAG**ATG**
moeA	10421	11652	411	MoeA molybdopterin biosynthesis protein, *E. coli*	$1.7e^{-38}$	GAGGAGACAAGCCG**GTG**
moaA	11712	12739	343	MoaA molybdopterin biosynthesis protein, *E. coli*	$7.0e^{-20}$	AAAGGATGTCGCC**ATG**

[a] Numbers refer to the 13-kb sequence as shown in Fig. 1.
[b] Product sizes represent the number of amino acids.
[c] The probability computed by the BLASTX program that sequences found during the search matched by chance.
[d] Underlined nucleotides represent Shine-Dalgarno sequences upstream of the start codon shown in bold type.

DMSOR, we have used the *dor* designation for genes involved in DMSO reduction in *R. sphaeroides* 2.4.1^T, as proposed by Shaw et al. (37).

(i) *dorS* and *dorR*. The *dorS* and *dorR* gene products are homologous to the LemA sensor kinase of *Pseudomonas syringae* (40% identity, 62% similarity) and to the *E. coli* ArcA response regulator (42% identity, 63% similarity), respectively, of the two-component sensory transduction family (41). Interestingly, the *dorS* gene product belongs to the subfamily of sensor kinase proteins which possess three phosphorylation sites: a classical transmitter domain, a receiver domain, and an additional carboxy-terminal transmitter domain (16). In *E. coli*, the *tor* operon, encoding TMAO reductase, is preceded by the *torS* and *torR* genes, encoding a TMAO-dependent sensory-regulatory system (18). A third gene, *torT*, which is also required in *E. coli* for TMAO-dependent regulation, is lacking in this region of the *R. sphaeroides* genome (19). The homologies and locations of the *dorS* and *dorR* genes suggest that their gene products may be involved in TMAO and DMSO sensing and regulation, analogous to the TorSTR system.

(ii) *dorC*, *dorB*, and *dorA*. The *dorCBA* genes show >95% identity to the previously sequenced *dmsCBA* genes from *R. sphaeroides* f. sp. *denitrificans* (45). Preceding the *dorC* gene is a putative promoter region. The *dorC* gene encodes a membrane-bound pentaheme *c*-type cytochrome homologous to TorC of *E. coli* and NapC of *R. sphaeroides* (27, 33). The *dorB* gene is predicted to encode a membrane protein homologous to TorD from *E. coli*, for which no specific function has been assigned (27). The *dorA* gene encodes the DMSOR, which belongs to the family of molybdoenzymes which possess only molybdopterin as their sole prosthetic group; these include TorA, TMAO reductase, BisC (biotin sulfoxide reductase of *E. coli*), and NapA (periplasmic nitrate reductase of *R. sphaeroides*) (27, 29). Since the *dorCBA* genes are so closely related to the *dmsCBA* genes, the homology profiles of the *dorCBA* genes will not be presented here, and the reader is referred to the *dmsCBA* gene profiles published elsewhere (45, 51). The start and stop codons between *dorC* and *dorB* and between *dorB* and *dorA* overlap, suggesting that the *dorCBA* genes are transcribed in a single transcriptional unit. Downstream of the *dorA* gene is a stem-loop structure, similar to those associated with *rho*-independent transcriptional termination in bacteria, suggesting that the *dorCBA* genes form a complete transcriptional unit (30).

(iii) *moeA* and *moaA*. Downstream of the *dor* genes are two ORFs whose products have significant homology to two proteins involved in the molybdopterin biosynthetic pathway in a number of different organisms. The *moeA* gene product possesses 45% identity and 59% similarity to MoeA from *E. coli*, which is involved in converting factor activity in the formation of molybdopterin (32). Downstream of *moeA* is *moaA*, whose product is 43% identical and 63% similar to MoaA from *E. coli*, which is involved in the early steps of molybdopterin biosynthesis (34). Partial sequencing reveals that downstream of *moaA*, there are additional ORFs, which have no homology to any protein in the databases.

Characterization of the Dor mutants. To determine the requirement of the products of the *dor* genes for DMSO reduction in *R. sphaeroides* 2.4.1^T, insertion or insertion-deletion mutations in the *dorS*, *dorR*, *dorC*, *dorB* and *dorA* genes were constructed by introduction of the ΩSt/Sp cassette. As predicted, strains with mutations in any of the *dor* genes did not

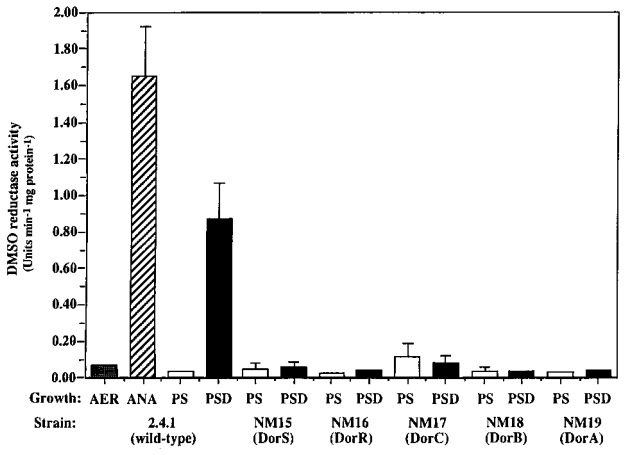

FIG. 2. DMSOR activities of *R. sphaeroides* wild-type and Dor mutant strains. Growth conditions are as follows: AER (▨), aerobic plus DMSO; ANA (▨), anaerobic-dark plus DMSO; PS (□), photosynthetic; PSD (■), photosynthetic plus DMSO. Units represent micromoles of methyl viologen oxidized. Results are the mean of triplicate assays from at least three separate growth experiments.

TABLE 3. Complementation of *dor* polar mutants

Strain/plasmid	Anaerobic growth with DMSO in[a]:		DMSOR activity[b]
	Light	Dark	
2.4.1 (wild type)	+	+	0.826
2.4.1/pNMT66 (*dorBA*)	+	+	1.564
2.4.1/pNMT61 (*dorA*)	+	+	1.299
NM17 (*dorC*::Ω)	+	−	0.080
NM17/pNMT66 (*dorBA*)	+	−	0.143
NM17/pNMT61 (*dorA*)	+	−	0.245
NM18 (*dorB*::Ω)	+	−	0.039
NM18/pNMT66 (*dorBA*)	+	+	0.296
NM18/pNMT61 (*dorA*)	+	−	0.298
NM19 (*dorA*::Ω)	+	−	0.044
NM19/pNMT66 (*dorBA*)	+	+	0.110
NM19/pNMT61 (*dorA*)	+	+	0.225

[a] Growth was measured after 8 days of incubation at 30°C on solid Sistrom's medium in the presence of 60 mM DMSO in anaerobic jars placed in the dark or in front of a light source. +, presence of growth; −, absence of growth.

[b] DMSOR activities were measured with cell extracts from liquid cultures grown photosynthetically in the presence of 60 mM DMSO. Values are micromoles of methyl viologen oxidized per minute per milligram of protein. Results are the means of activities from triplicate assays.

exhibit anaerobic growth in the dark with 60 mM DMSO or 30 mM TMAO as the terminal electron acceptor.

DMSOR activity was measured by monitoring the DMSO-dependent oxidation of partially reduced methyl viologen (17, 26). After aerobic growth, even in the presence of DMSO, the wild-type strain 2.4.1^T exhibited a very low level of DMSOR activity (Fig. 2). After anaerobic growth in the dark with DMSO, a 24-fold increase in activity compared with aerobic levels was observed. DMSOR activity after photosynthetic (anaerobic-light) growth was similar to that after aerobic growth. Intriguingly, after photosynthetic growth in the presence of DMSO, DMSOR activity was approximately 55% of the activity under dark growth conditions. This result was surprising but reproducible, suggesting that some factor, possibly regulatory, is limiting for DMSOR activity under photosynthetic conditions. This is under investigation.

Since the Dor mutants are unable to grow anaerobically in the dark with DMSO, DMSOR activity was measured after photosynthetic growth in both the presence and absence of DMSO. The Dor mutants exhibited similar low levels of DMSOR activity after growth in either the presence or absence of DMSO, in contrast to the wild-type strain, in which a large amount of DMSOR activity was observed in the presence of DMSO (Fig. 2). Similar results were obtained after growth in the presence of absence of TMAO. The residual low levels of activity are likely to be nonspecific since the Dor mutants are unable to grow with DMSO or TMAO as the terminal electron acceptor and since no differences in the activity in the presence and absence of either electron acceptor for the mutant strains are apparent. This demonstrates that the products of the *dor* genes are responsible for DMSO and TMAO reduction in *R. sphaeroides*, with the *dorC*, *dorB*, and *dorA* genes encoding structural components of DMSO reductase and the *dorS* and *dorR* genes encoding necessary regulatory functions. However, it is to be noted that the *dorC* and *dorB* insertion mutations are polar on *dorBA* and *dorA* expression, respectively (see below).

Polar effects of *dorC* and *dorB* mutations. It has previously been noted that the ΩSt/Sp cassette can cause polar effects on downstream genes due to bidirectional transcriptional terminators present within the cassette (31). To determine whether the cassette is polar for downstream gene expression in the *dorC* and *dorB* mutations, immunoblotting of these mutants was performed with antisera raised against the DorA protein from *R. capsulatus* (15). Indeed, the DorC and DorB mutant strains, NM17 and NM18, respectively, do not exhibit cross-reacting protein with the DorA antisera after photosynthetic growth in the presence of DMSO, suggesting that the inserted cassette does prohibit downstream transcription (data not shown). This is in contrast to the wild-type strain, which shows an 82-kDa cross-reacting band under similar growth conditions.

To circumvent the polar effects of the inserted cassette, plasmids which place the *dorBA* and *dorA* genes under control of the *lac* promoter of the vector were introduced into strains NM17, NM18, NM19 and the wild-type strain 2.4.1^T. The resulting strains were tested for DMSO-dependent anaerobic-dark growth and DMSOR activity. The DorC mutant NM17 was unable to grow, even with plasmids containing *dorBA* and *dorA*, indicating an absolute requirement for DorC in the DMSO reduction pathway (Table 3). The DorB mutant NM18 was able to grow only when a plasmid containing both the *dorB* and *dorA* genes was introduced, demonstrating that the *dorB* gene product is essential for DMSO reduction. As predicted, DMSO-dependent anaerobic growth in the dark was restored to the DorA mutant NM19 when plasmids containing either the *dorBA* or *dorA* gene were introduced. Interestingly, the DMSOR activities of the mutant strains containing the plasmids were approximately 20 to 25% of the activities measured for the wild-type strain, although these activities were significantly higher than those for the mutant strains which lack the plasmids. This observation is explained by the results of immunoblotting of these strains, which showed that the expression of DorA from the plasmids is much lower than the expression of DorA in the wild-type strain (data not shown), reflecting the lower promoter activity of the vector *lac* promoter relative to that of the wild-type *dor* promoter. This is in accordance with previous observations which suggested that the DorA protein represents more than 10% of the total cell protein during anaerobic-dark growth with DMSO (36). Thus, although the level of DorA protein expressed from the plasmids is sufficient to restore growth to the appropriate mutants, the amount of DorA is lower than the wild-type strain, accounting for the lower DMSOR activities.

***dorC* encodes a 44-kDa DMSO-inducible *c*-type cytochrome.** It was previously shown that a 44-kDa *c*-type cytochrome is induced by *R. sphaeroides* under anaerobic growth conditions in the presence of DMSO (47). It was suggested that in the closely related bacterium *R. capsulatus*, this cytochrome rep-

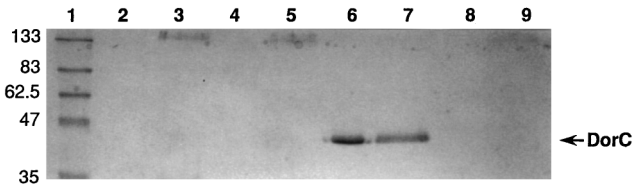

FIG. 3. Heme-stained protein gel of SDS-PAGE-treated polypeptides from *R. sphaeroides* wild-type and DorC mutant strains. Lanes: 1, molecular weight markers (in thousands) (Bio-Rad); 2, 2.4.1^T −DMSO soluble fraction; 3, 2.4.1^T −DMSO insoluble fraction; 4, NM17 −DMSO soluble fraction; 5, NM17 −DMSO insoluble fraction; 6, 2.4.1^T +DMSO soluble fraction; 7, 2.4.1^T +DMSO insoluble fraction; 8, NM17 +DMSO soluble fraction; 9, NM17 +DMSO insoluble fraction.

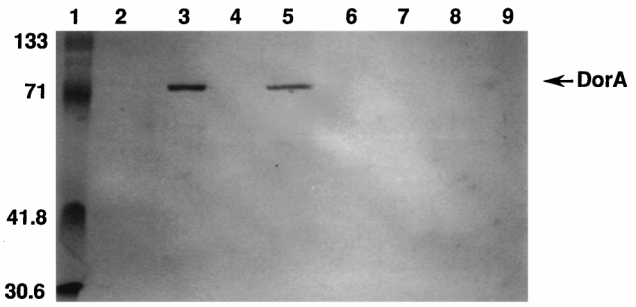

FIG. 4. Synthesis of DorA by wild-type and DorS and DorR mutant strains of *R. sphaeroides*. Whole-cell proteins were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. The presence of DorA was detected with polyclonal antisera to DorA and visualized by the AP detection system (Promega). Lanes: 1, molecular weight markers (in thousands) (New England BioLabs); 2, 2.4.1^T aerobic plus DMSO; 3, 2.4.1^T anaerobic-dark plus DMSO; 4, 2.4.1^T photosynthetic; 5, 2.4.1^T photosynthetic plus DMSO; 6, NM15 photosynthetic; 7, NM15 photosynthetic plus DMSO; 8, NM16 photosynthetic; 9, NM16 photosynthetic plus DMSO.

resents a cytochrome *c* peroxidase (14). To determine whether this cytochrome is the product of the *dorC* gene, polypeptides from photosynthetic cultures, grown in the presence or absence of DMSO, were heme stained after being subjected to SDS-polyacrylamide gel electrophoresis. The wild-type strain 2.4.1^T synthesized an approximately 44-kDa *c*-type cytochrome only in the presence of DMSO (Fig. 3, lanes 6 and 7). Most of this cytochrome was present in the soluble fraction, although some remained in the insoluble fraction after cell fractionation. In contrast, the DorC mutant strain NM17 lacked this cytochrome (lanes 8 and 9), indicating that the cytochrome is likely to be the product of the *dorC* gene.

Regulation of DorA expression. Since the *dorS* and *dorR* gene products show homology to the TorS and TorR proteins of *E. coli*, we investigated the regulation of DorA protein expression in DorS and DorR mutant backgrounds. Immunoblot analysis of crude protein extracts from cultures grown aerobically and anaerobically, in both the presence and absence of DMSO, showed that the wild-type strain 2.4.1^T expressed the DorA protein only in the absence of oxygen and the presence of DMSO (Fig. 4, lanes 3 and 5). Wild-type cells grown either aerobically in the presence of DMSO or photosynthetically in the absence of DMSO did not express DorA polypeptide (lanes 2 and 4). These results suggest a requirement for both the presence of DMSO and the absence of oxygen for DorA expression. In contrast to the wild-type strain, the DorS mutant strain NM15 and the DorR mutant strain NM16 did not produce DorA after photosynthetic growth, even in the presence of DMSO (lanes 6 through 9). This suggests that the *dorS* and *dorR* gene products are essential for induction of DorA protein expression in the presence of DMSO and the absence of oxygen.

DISCUSSION

In this report, we describe the DNA sequencing and characterization of a 13-kb DNA sequence from CII of *R. sphaeroides* 2.4.1^T. This is the first detailed analysis of a large contiguous sequence from CII and represents the first reported case of genes from CII being essential for a particular growth condition. Sequence analysis revealed a total of 10 putative ORFs present in this region, representing approximately 90% of the coding capacity. Nine of these ORFs possessed homology to genes present in the databases. These ORFs reveal a cluster of genes whose products are related functionally. The *dor* genes encode components of the DMSO reduction pathway in *R. sphaeroides* 2.4.1^T. Downstream of the *dor* cluster are two genes from the molybdopterin biosynthetic pathway, *moeA* and *moaA*. Although DMSOR contains a molybdopterin cofactor, the products of the *moeA* and *moaA* genes are unlikely to be solely responsible for the biosynthesis of the DMSOR molybdopterin cofactor, since their function in other organisms appears to be limited to the early steps of molybdopterin biosynthesis (32). Indeed, recent findings have suggested an essential role for the *mobA* gene product in DMSOR function in *R. sphaeroides* (28a). Interestingly, the *moaE* gene, whose product is also involved in molybdopterin biosynthesis, has been partially sequenced from CI of *R. sphaeroides* 2.4.1^T (9). In *E. coli*, the *moaA* and *moaE* genes are present in a single operon (34). It is not known whether a second copy of *moaA* is linked to *moaE* on CI of *R. sphaeroides* or whether genes for molybdopterin biosynthesis are partitioned between the two chromosomes.

The *dor* gene cluster of *R. sphaeroides* 2.4.1^T contains genes with homology to the TMAO reductase gene cluster of *E. coli* (27). The *dorCBA* genes are almost identical to the previously sequenced *dmsCBA* genes of *R. sphaeroides* f. sp. *denitrificans*, and the *dorA* gene product has extensive homology to the *dorA* gene product of *R. capsulatus* (22, 37, 45, 51). Mutations in the *dorCBA* genes resulted in the inability to use DMSO or TMAO as the terminal electron acceptor in anaerobic respiration and in greatly diminished in vitro DMSOR activity. The residual DMSOR activities observed are probably nonspecific, because these low levels are unaffected by growth in either the presence or absence of DMSO and because the Dor mutants are unable to grow with DMSO or TMAO. These results suggest that the *dorCBA* genes encode the sole DMSOR in *R. sphaeroides* 2.4.1^T. This is different from the situation in *E. coli*, where DMSOR and TMAO reductase are encoded by separate enzymes (48). The *dmsCBA* genes of *E. coli* encode a constitutive, cytoplasmic DMSOR which has both molybdopterin and [Fe-S] clusters as prosthetic groups (48). Further, this enzyme requires no cytochromes and derives its electrons directly from menaquinone (48). In contrast, the *torCDA* genes encode an inducible, periplasmic TMAO reductase which requires both *b*- and *c*-type cytochromes for electron transfer to the molybdopterin cofactor (27). Further, the TorA TMAO reductase contains only molybdopterin in the active site.

Adjacent to the *dorCBA* genes of *R. sphaeroides* 2.4.1^T are the *dorS* and *dorR* genes, which encode a sensor-regulator pair of the two-component sensory-transduction protein family (41). We have shown here that the products of these two genes are required for induction of the *dor* genes in response to DMSO and/or anaerobiosis. In a separate study, we demonstrated that the FnrL protein of *R. sphaeroides* 2.4.1^T is responsible for the anaerobic induction of DMSOR (53). This would suggest that the DorS and DorR proteins control DMSO induction of the *dor* operon. In the *E. coli* Tor system, a homologous sensor-regulator pair, TorS and TorR, is required for the TMAO-dependent (but not DMSO-dependent) induction of *torCDA* expression (18, 38, 39). In addition, a periplasmic protein, TorT, is involved, although its exact role is unclear (19). No homolog of TorT was found in or near the *dor* gene cluster of *R. sphaeroides* 2.4.1^T, suggesting that regulation may be different in the Dor system. In addition to DorR, we have identified an additional DNA-binding regulatory protein, encoded by *orf2* upstream of *dorS* (Fig. 1), which appears to be involved in *dor* gene expression (28). The presence of multiple regulatory proteins suggests that the expression of the *dor* genes is complex and requires multiple signals. Further exper-

iments are under way to investigate *dor* expression and to characterize the role of the *orf2* gene product.

This characterization of the *dor* gene cluster and the roles of its products in DMSO reduction is the first example of a case where such a detailed genetic and biochemical characterization has directly shown that CII of *R. sphaeroides* 2.4.1^T encodes an essential gene function, revealing its critical role in cell metabolism under anaerobic, dark growth conditions. We define essentiality here as the requirement for such gene products in a particular growth state, not just for housekeeping functions. Since DMSO and TMAO are abundant compounds in the physiological niches for *R. sphaeroides*, it is likely that DMSO reduction and TMAO reduction are important metabolic processes when oxygen becomes limited for this organism. Further, given that *dor* expression is regulated by not only the DorR and DorS proteins, encoded by genes on CII, but also by FnrL, encoded by the *fnrL* gene on CI, it would appear that essentiality of this metabolic system is governed by the presence of genes on both chromosomes of *R. sphaeroides* 2.4.1^T (53). Since auxotrophic mutants have been identified by transposon mutagenesis of CII, it is expected that further essential functions will be identified upon further genetic analysis of genes from CII and that this study will not represent the sole case.

ACKNOWLEDGMENTS

We thank A. G. McEwan for generously providing antiserum to the *R. capsulatus* DorA protein. We also thank J. Zeilstra-Ryalls, T. Palmer, and A. G. McEwan for helpful suggestions and technical assistance.

This work was supported by a grant from the Clayton Foundation for Research (to M.C.) and by U.S. Public Health Service grants GM15590 and GM55481 (to S.K.).

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