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Cascade Regulation of Dimethyl Sulfoxide Reductase (*dor*) Gene Expression in the Facultative Phototroph *Rhodobacter sphaeroides* 2.4.1^T

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Under anaerobic-dark growth conditions, in the presence of the alternative electron acceptor dimethyl sulfoxide (DMSO) or trimethylamine *N*-oxide (TMAO), *Rhodobacter sphaeroides* 2.4.1^T respires anaerobically using the molybdoenzyme DMSO reductase (DMSOR). Genes encoding DMSOR and associated proteins are encoded by genes of the *dor* locus. Previously, we demonstrated that the expression of DMSOR is regulated by both the oxygen status of the cell via the FnrL protein and by the presence of DMSO or TMAO, presumably through the DorS-DorR two-component sensor-regulator system. Here we further investigate expression of the *dor* genes through the use of transcriptional *lacZ* fusions to the *dorS*, *dorR*, and *dorC* promoters. The expression of *dorC*::*lacZ* was strongly induced by the absence of oxygen and presence of DMSO. In accordance with our previous findings of DMSOR activity, *dorC*::*lacZ* expression was reduced by up to one-third when cells were grown photosynthetically in the presence of DMSO with medium or high light, compared to the expression observed after anaerobic-dark growth. The induction of *dorC*::*lacZ* expression in the presence of DMSO was dependent on the DorS and DorR proteins. Expression of the *dorS* and *dorR* genes was also induced in the absence of oxygen. In an FnrL mutant, *dorS*::*lacZ* expression was not induced when oxygen tensions in the media were lowered, in contrast to what occurred in the wild-type strain. The expression of *dorS*::*lacZ* and *dorR*::*lacZ* was dependent on the DorS and DorR proteins themselves, suggesting the importance of autoregulation. These results demonstrate a cascade regulation of *dor* gene expression, where the expression of the regulatory proteins DorS and DorR governs the downstream regulation of the *dorCBA* operon encoding the structural proteins of DMSOR.

Rhodobacter sphaeroides 2.4.1^T is a facultative phototrophic bacterium which is capable of a wide range of metabolic lifestyles including aerobic, anaerobic, photosynthetic, and diazotrophic growth modes. Under anoxygenic growth conditions, in the absence of light, *R. sphaeroides* 2.4.1^T respires anaerobically using dimethyl sulfoxide (DMSO) or trimethylamine *N*-oxide (TMAO) as the terminal electron acceptor (for a review see reference 14). Reduction of both compounds is achieved by a single enzyme, DMSO reductase (DMSOR), which is a monomeric periplasmic protein containing a molybdopterin cofactor and whose structure has recently been determined (12, 20).

As part of our low-redundancy sequencing strategy for chromosome II of *R. sphaeroides* 2.4.1^T, we sequenced a 13-kb region containing genes homologous to the previously sequenced *dmsCBA* genes of *R. sphaeroides* f. sp. *denitrificans* and to the *tor* genes of *Escherichia coli*, which encode components of TMAO reductase (2, 9, 15, 16, 22, 25, 28). The *dorC*, *dorB*, and *dorA* genes form a single operon, and, respectively, encode a soluble *c*-type cytochrome, a membrane protein of unknown function, and DMSOR (16). Upstream of the *dorCBA* operon are two adjacent genes, *dorS* and *dorR*, that are transcribed divergently inward toward each other and which, respectively, encode putative sensor kinase and response regulator proteins of the two-component signal transduction family of proteins (16, 23). Strains with mutations in any of the *dorS*, *dorR*, or *dorCBA* genes are unable to grow anaerobically in the dark when DMSO or TMAO is supplied as the terminal electron acceptor and show negligible amounts of DMSOR-specific activity (16). These results indicate that the *dor* genes encode the sole system responsible for the reduction of both DMSO and TMAO in this bacterium.

We showed that the expression of the DorA protein was regulated by both the oxygen status of the cell and by the presence of DMSO or TMAO in the growth medium (16). We also demonstrated that the FnrL protein has a positive role in the regulation of DorA expression, suggesting that the expression of the *dorCBA* operon is responsive to redox control (31). Further, DorS and DorR mutants failed to accumulate DorA under any growth condition, demonstrating a positive role for these proteins in *dorCBA* expression (16). In a separate study, it was shown that the DmsR protein of *R. sphaeroides* f. sp. *denitrificans* induced the *dmsCBA* operon in response to DMSO, by binding to specific sites in the *dmsC* promoter (26). The DmsR protein of *R. sphaeroides* f. sp. *denitrificans* and the DorR protein of *R. sphaeroides* 2.4.1^T are almost identical at the amino acid level, and thus it seems likely that the DorR protein plays a similar role in *R. sphaeroides* 2.4.1^T. However, the authors did not report a corresponding DorS homolog and the cognate sensor protein for DmsR has not been identified.

We wished to further investigate how the *dor* genes of *R. sphaeroides* 2.4.1^T are regulated by both oxygen and DMSO and at what level these two signals interact. Here we examine transcriptional regulation of the *dorS*, *dorR*, and *dorC* promoters and present data which demonstrate the requirement for DMSO and anaerobiosis for the regulation of these promoters.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype and/or characteristics	Reference or source
R. sphaeroides		
2.4.1^T	Wild type	27
NM15	*dorS*::ΩSt^r/Sp^r	16
NM16	*dorR*::ΩSt^r/Sp^r	16
JZ1678	Δ*fnrL*::ΩKm^r	32
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	6
HB101	F$^-$ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 glaK2 lacYI* Δ(*mcrC-mrr*) *rpsL20* (St^r) *xyl-5 mtl-1 recA13*	1
Plasmids		
pUI1087	Cloning vector	33
pBS II	Cloning vector, Amp^r, with T3 and T7 promoters	Stratagene
pRK2013	Conjugative helper plasmid	7
pML5	Promoterless *lacZ* transcriptional fusion vector; Tc^r	13
pNMT68	pBS II containing 659-bp *dorC* URS PCR product	This study
pNMT69	pBS II containing 662-bp *dorS* URS PCR product	This study
pNMT77	pML5 containing *dorS*::*lacZ*	This study
pNMT78	pML5 containing *dorC*::*lacZ*	This study
pNMT92	pBS II containing 525-bp *dorR* URS PCR product	This study
pNMT94	pML5 containing *dorR*::*lacZ*	This study

We also show that *dorC* expression is governed either directly or indirectly by light intensity. We further demonstrate that this system is under autoregulation and propose a cascade model for the regulation of DMSOR expression in *R. sphaeroides* 2.4.1^T.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *E. 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 (3, 19). Where appropriate, DMSO was added at a final concentration of 60 mM and TMAO was added at a final concentration of 30 mM. 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, except in the experiments involving different light intensities. Aerobic cultures were grown by continuous sparging with a mixture of 30% O_2–69% N_2–1% CO_2. For oxygen shift assays, cultures were grown aerobically for five to six culture doublings and then shifted to 2% O_2–97% N_2–1% CO_2. One culture sample was removed prior to the oxygen shift, and further samples were assayed at appropriate intervals after the shift.

The media were supplemented with antibiotics, where appropriate, to maintain selection for plasmids or to select for recombinant strains. The final concentrations were as follows: ampicillin, 100 μg ml^{-1} (*E. coli*); kanamycin, 25 μg ml^{-1} (*R. sphaeroides*); spectinomycin, 25 μg ml^{-1} (*R. sphaeroides*); streptomycin, 25 μg ml^{-1} (*R. sphaeroides*); 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 *lacZ* reporter fusion plasmids. Standard recombinant DNA techniques were used throughout (19). Enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.), Stratagene (La Jolla, Calif.), Promega Corp. (Madison, Wis.), and Boehringer Mannheim Biochemicals, Bethesda Research Laboratories Life Technologies Inc. (Gaithersburg, Md.).

The upstream regulatory sequences (URS) of the *dorS*, *dorR*, and *dorC* genes were amplified by the PCR using Vent DNA polymerase (New England Biolabs) and oligonucleotides purchased from Bethesda Research Laboratories Life Technologies. The reaction conditions for each of the PCR amplifications were identical, consisting of 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. PCR amplification of the *dorS* promoter region was performed using the primers DORSP1 (5′-CGCCTAGG ACCTCGCGGATCGG-3′) and DORSP2 (5′-GCGTTCGAACCCGCGCCTC GGCG-3′), generating a 662-bp product. The PCR product was purified by using the Wizard PCR purification kit (Promega Corp.), and the ends were filled in by using *Pfu* polymerase (Stratagene). The blunt-ended PCR product was cloned into *Sma*I-digested pBS II, resulting in plasmid pNMT69. The *dorR* promoter region was amplified by using the primers DORRPBAM (5′-CGGCGCGGAT CCCGCATCGAGTGGC-3′) and DORRPHIND (5′-CGCGGCAAGCTTGCG CAGATACATCG-3′) to generate a 525-bp product. The PCR product was cloned into pBS II, as above, to give plasmid pNMT92. The *dorC* promoter region was amplified by using the primers DORCP1 (5′-GCGCGACGTCGCG CGCTTCGCTGACTTCG-3′) and DORCP2 (5′-GCGCGACGTCCCGCATC GAGTGG-3′) to generate a 659-bp product. The PCR product was cloned into pBS II, as above, to give plasmid pNMT68. The sequence and orientation of the cloned products were confirmed before proceeding with additional cloning steps. All of the cloned products were subcloned into the promoterless *lacZ* vector pML5, using *Bam*HI-*Hin*dIII double digestions. This resulted in the following plasmids: *dorS*::*lacZ*, pNMT77; *dorR*::*lacZ*, pNMT94; and *dorC*::*lacZ*, pNMT78 (Fig. 1). Each of the *lacZ* fusion plasmids was conjugated into *R. sphaeroides* 2.4.1^T by triparental matings with pRK2013, as described previously (4).

DNA sequencing. Automated DNA sequencing was performed using 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. Oligonucleotides used for priming the sequencing reactions were purchased from Bethesda Research Laboratories Life Technologies. Sequences were analyzed by using the Genetics Computer Group programs and the BLAST server at the National Center for Biotechnology Information (5).

Cell extract preparation and assays of β-galactosidase activity. Preparation of crude cell extracts and determination of β-galactosidase activities were performed as described previously (18, 24). Cell extract protein concentrations were determined by using the Pierce BCA Protein Assay Reagent (Pierce, Rockford, Ill.) with bovine serum albumin as a reference standard.

RESULTS

Regulation of *dorC*::*lacZ* expression. Previous experiments revealed that the DorA protein was produced only in the absence of oxygen and in the presence of DMSO or TMAO (16). Since the *dorA* gene is in an operon downstream of the *dorB* and *dorC* genes, with a putative promoter region upstream of *dorC*, we constructed a *dorC*::*lacZ* fusion in order to measure expression from the *dorC* URS. After introduction of this fusion plasmid (pNMT78) (Fig. 1) into wild-type or various mutant strains of *R. sphaeroides* 2.4.1^T, we measured β-galactosidase activities after growth under a number of different conditions.

dorC::*lacZ* expression was maximal after anaerobic-dark growth in the presence of DMSO (Fig. 2). In contrast, very little or no β-galactosidase activity was evident after aerobic growth or photosynthetic growth in the absence of DMSO. Intriguingly, the expression of *dorC*::*lacZ* was approximately ninefold lower after anaerobic growth in the dark with TMAO than after growth with DMSO under similar conditions. Most

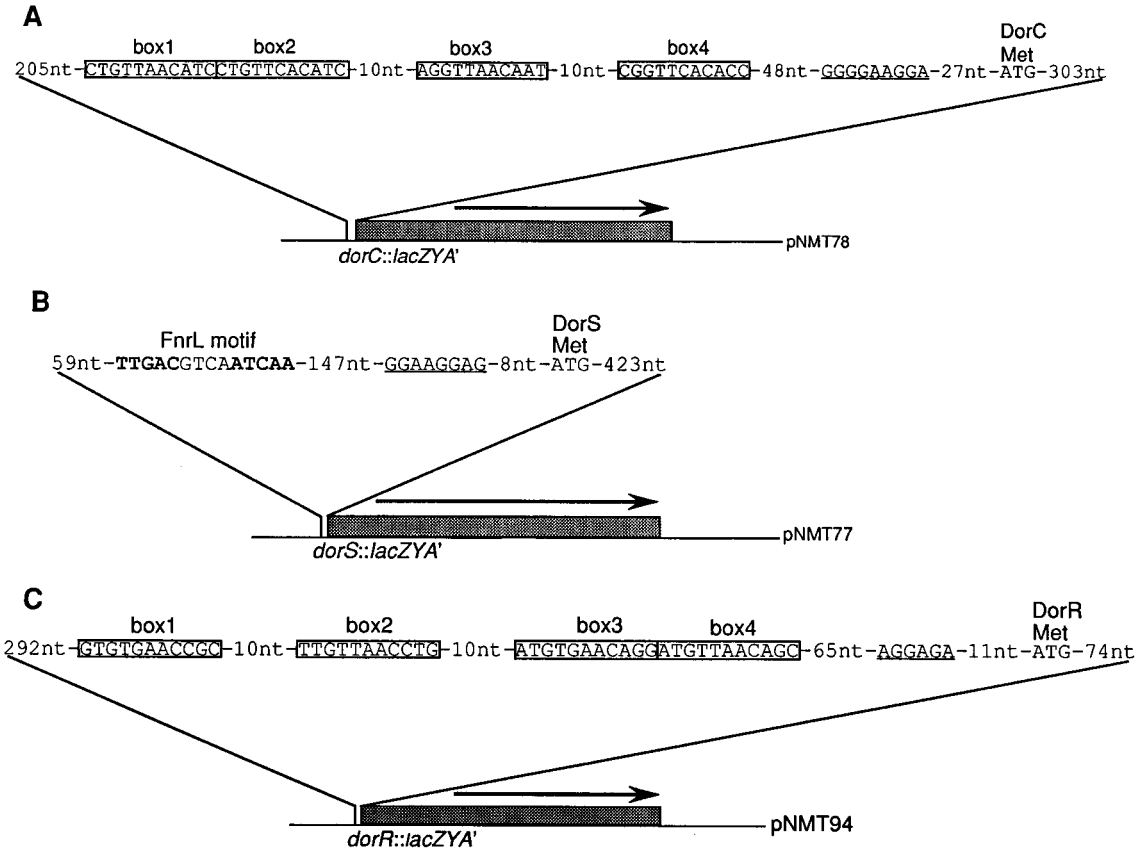

FIG. 1. Physical map of plasmids containing transcriptional *lacZ* fusions to the *dorC* (A), *dorS* (B), and *dorR* (C) URS of *R. sphaeroides* 2.4.1^T. Putative DorR binding sites in the *dorC* and *dorR* URS are boxed (boxes 1 to 4). The putative FnrL binding motif in the *dorS* URS is shown in bold type. Putative Shine-Dalgarno sequences are underlined. The arrows indicate the direction of transcription. See the text for further details.

interesting is the lower activity observed during photosynthetic growth in the presence of DMSO when compared to the activity after growth under anaerobic-dark conditions (Fig. 2). We previously observed that the specific activity of DMSOR after photosynthetic growth in the presence of DMSO was approximately 50% of that for anaerobic-dark conditions (16). Therefore, it appears that there is a good correlation between *dorC* transcription and DMSOR activity. We grew wild-type cells containing the various *dor*::*lacZ* fusions photosynthetically in the presence of DMSO at different light intensities and assayed β-galactosidase activities of extracts from these cultures in order to determine if there was an effect of light intensity on *dor* gene expression.

For the *dorC*::*lacZ* fusion, decreased β-galactosidase activities were observed after cells were grown under low (3 W m^{-2}), medium (10 W m^{-2}), or high (100 W m^{-2}) light intensity compared to the activity after anaerobic-dark growth (Table 2). Growth under high light intensity resulted in the maximal decrease in activity, resulting in a level approximately threefold lower than that observed under anaerobic-dark conditions (Table 2). In contrast, expression of *dorS*::*lacZ* did not vary significantly with differing light intensity, although a small decrease in β-galactosidase activity was observed when cells were grown under low or medium light intensity (Table 2). Only when cells were grown under high light, did *dorR*::*lacZ* expression decrease, to a level approximately 50% of that observed in the dark or under low or medium light intensity (Table 2).

It is clear that maximum expression of *dorC*::*lacZ* is observed after growth in the dark, in the absence of oxygen and in the presence of DMSO. We previously observed that the DorA protein was absent in DorR and DorS mutants, and it is believed that DorS and DorR form a two-component sensor-regulator system (16). To determine whether the DorSR system acts at the *dorC* URS, we examined the expression of *dorC*::*lacZ* in DorR and DorS mutant backgrounds after photosynthetic growth in the presence of DMSO, since the mutants are unable to grow anaerobically in the dark with DMSO or TMAO. The β-galactosidase activities in both mutants were very low when compared to activities in the wild-type background (Fig. 2). Similar low levels of activity were observed after photosynthetic growth in the absence of DMSO (data not shown). These results demonstrate a positive role for the DorSR system in the control of the *dorCBA* operon.

***dorS*::*lacZ* and *dorR*::*lacZ* expression.** To further establish the roles of the DorS and DorR proteins in the regulation of *dor* expression, we were interested to learn how the genes encoding these proteins are themselves regulated. We constructed *dorS*::*lacZ* and *dorR*::*lacZ* fusions and measured the resulting β-galactosidase activities from these fusions in wild-type and mutant backgrounds after growth under different conditions. For both fusions, only very low levels of activity were observed after aerobic growth (Fig. 3 and 4). An approximately 20-fold induction of *dorS*::*lacZ* expression was observed after anaerobic-dark growth in the presence of DMSO (Fig. 3). The activity after anaerobic-dark growth with TMAO was approximately 45% of that when cells were grown with DMSO, in contrast to the approximate 90% decrease in

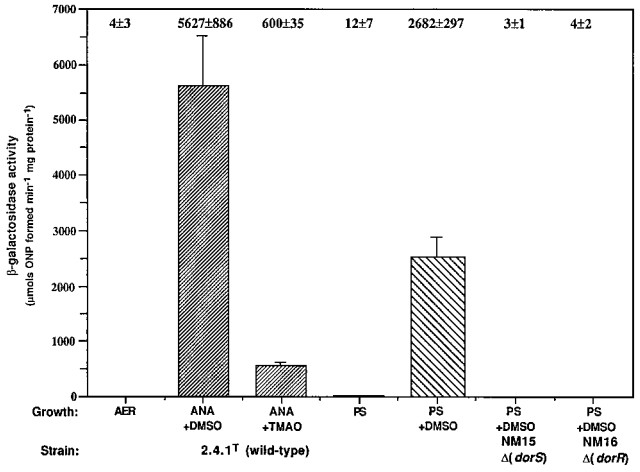

FIG. 2. β-Galactosidase activities from cell extracts of *R. sphaeroides* strains containing the *dorC*::*lacZ* transcriptional fusion plasmid pNMT78. Growth conditions are as follows: AER (▤), aerobically with 60 mM DMSO; ANA+DMSO (▨), anaerobically in the dark with 60 mM DMSO; ANA+TMAO (▨), anaerobically in the dark with 30 mM TMAO; PS (▩), photosynthetically; PS+DMSO (▧), photosynthetically with 60 mM DMSO. Photosynthetic cultures were grown with a light intensity of 10 W m^{-2}. Results are the mean values from triplicate assays of at least three independent cultures and are corrected for activity from the vector alone under the same conditions (pML5, <35 μmol of *o*-nitrophenol formed min^{-1} mg of $protein^{-1}$). Vertical bars represent the standard deviation from the mean.

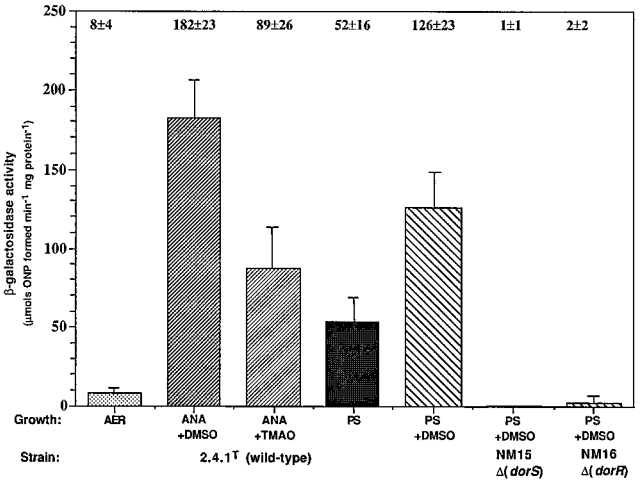

FIG. 3. β-Galactosidase activities from cell extracts of *R. sphaeroides* strains containing the *dorS*::*lacZ* transcriptional fusion plasmid pNMT77. Growth conditions are as follows: AER (▤), aerobically with 60 mM DMSO; ANA+DMSO (▨), anaerobically in the dark with 60 mM DMSO; ANA+TMAO (▨), anaerobically in the dark with 30 mM TMAO; PS (▩), photosynthetically; PS+DMSO (▧), photosynthetically with 60 mM DMSO. Photosynthetic cultures were grown with a light intensity of 10 W m^{-2}. Results are the mean values from triplicate assays of at least three independent cultures and are corrected for activity from the vector alone under the same conditions (pML5, <35 μmol of *o*-nitrophenol formed min^{-1} mg of $protein^{-1}$). Vertical bars represent the standard deviation from the mean.

dorC::*lacZ* expression observed between TMAO- and DMSO-grown cultures. In further contrast to *dorC*::*lacZ* expression, *dorS*::*lacZ* expression decreased only slightly after photosynthetic growth in the presence of DMSO, compared to the expression observed after anaerobic-dark growth. The expression of *dorS*::*lacZ* also appeared to be under DMSO-dependent control, as an approximately twofold increase in activity was observed after photosynthetic growth in the presence of DMSO compared to that in the absence of DMSO.

The expression of *dorR*::*lacZ* was much lower relative to that of the *dorS*::*lacZ* and *dorC*::*lacZ* fusions, but since the values presented are the results of three independent growths, each performed in triplicate, we believe that these represent the true values. Measurement of β-galactosidase activities from the *dorR*::*lacZ* fusion revealed that the expression of *dorR*, like that of *dorS*, is also induced by anaerobiosis, although only an approximately fivefold induction is observed in this case (Fig. 4). Similar to the reduction in *dorS*::*lacZ* expression, anaerobic-dark growth with TMAO resulted in an approximately 40% reduction in *dorR*::*lacZ* expression when compared to the levels observed when cells were grown with DMSO. When cells were grown photosynthetically, *dorR*::*lacZ* expression was stimulated twofold by the presence of DMSO. No significant differences were observed in the levels of *dorR*::*lacZ* expression between anaerobic-dark- and photosynthetically grown cultures in the presence of DMSO, in contrast to levels of both

TABLE 2. Effects of light intensity on *dorC*, *dorS*, and *dorR* expression

Growth condition[a]	β-Galactosidase activity[b]		
	dorC::*lacZ*	*dorS*::*lacZ*	*dorR*::*lacZ*
Anaerobic-dark	5,627 ± 886	182 ± 23	31 ± 12
Photoheterotrophic			
3 W m^{-2}	4,446 ± 289	165 ± 12	33 ± 5
10 W m^{-2}	2,682 ± 297	125 ± 22	38 ± 5
100 W m^{-2}	1,946 ± 375	175 ± 17	14 ± 3

[a] Cultures were grown to mid-log phase in the presence of 60 mM DMSO under the conditions indicated and assayed for β-galactosidase activity.

[b] Units of activity are micromoles of *o*-nitrophenol (ONP) formed $minute^{-1}$ milligram of $protein^{-1}$. Values represent the mean values ± the standard deviation of triplicate assays of at least three independent cultures and are corrected for activity from the vector alone under the same conditions (pML5, <35 μmol of ONP formed min^{-1} mg of $protein^{-1}$).

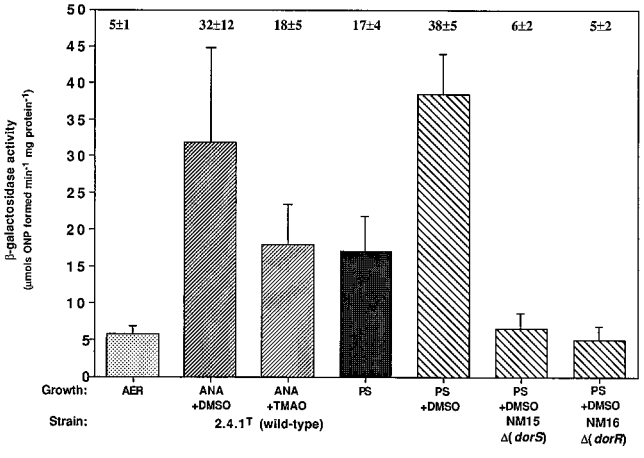

FIG. 4. β-Galactosidase activities from cell extracts of *R. sphaeroides* strains containing the *dorR*::*lacZ* transcriptional fusion plasmid pNMT94. Growth conditions are as follows: AER (▤), aerobically with 60 mM DMSO; ANA+DMSO (▨), anaerobically in the dark with 60 mM DMSO; ANA+TMAO (▨), anaerobically in the dark with 30 mM TMAO; PS (▩), photosynthetically; PS+DMSO (▧), photosynthetically with 60 mM DMSO. Photosynthetic cultures were grown with a light intensity of 10 W m^{-2}. Results are the mean values from triplicate assays of at least three independent cultures and are corrected for activity from the vector alone under the same conditions (pML5, <35 μmol of *o*-nitrophenol formed min^{-1} mg of $protein^{-1}$). Vertical bars represent the standard deviation from the mean.

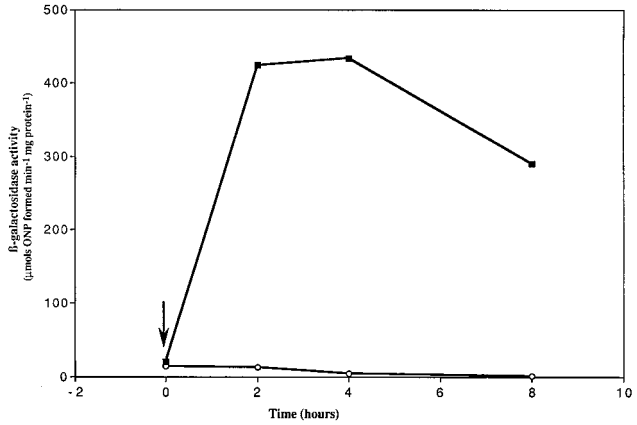

FIG. 5. Kinetics of induction of *dorS*::*lacZ* transcriptional fusion in the wild-type strain 2.4.1^T (■) and FnrL mutant strain JZ1678 (○) following a shift from 30 to 2% oxygen, indicated by the vertical arrow. Cultures were sampled at the times indicated, and extracts from 15-ml samples were assayed for β-galactosidase activity. The values represent the means of triplicate assays from two independent growth experiments. The standard error in each case did not exceed 20% of the mean value.

dorC::*lacZ* and *dorS*::*lacZ* expression (Fig. 4). When the light intensity was increased to 100 W m^{-2}, *dorR*::*lacZ* expression was reduced by approximately 50% relative to that seen under low or medium light intensity (Table 2). This demonstrates that *dorR* expression is also under light-responsive regulation, but not as stringently as *dorC* expression.

Since the DorSR system positively regulated *dorC*::*lacZ* expression, we wondered if the expression of the *dorS* and *dorR* genes themselves was under autoregulation by the DorS and DorR proteins. Both the *dorS*::*lacZ* and *dorR*::*lacZ* fusions were introduced into the respective DorS and DorR mutant strains, NM15 and NM16, and the β-galactosidase activities were measured after photosynthetic growth in the presence of DMSO. The activities from both fusions were much lower than those for the wild-type strain under similar growth conditions and resembled the levels observed under aerobic conditions for each fusion, indicating a positive role for the DorS and DorR proteins in the autoregulation of *dorS* and *dorR* gene expression (Fig. 3 and 4). Further, it should also be noted that we assayed *dorS*::*lacZ* and *dorR*::*lacZ* expression in the mutant strains after photosynthetic growth in the absence of DMSO and found the levels of expression to be very similar irrespective of whether DMSO was present or not (data not shown).

FnrL positively regulates *dorS*::*lacZ*. It was previously demonstrated than an FnrL mutant was unable to synthesize the DorC *c*-type cytochrome or the DorA protein, even when grown in low oxygen in the presence of DMSO (31). This suggested that at least one component of the *dor* cluster was under FnrL-mediated regulation. Upon inspection of the *dor* sequences, it was observed that within the *dorS* URS, there is a putative FnrL binding site, TTGAC-N_4-ATCAA, differing from the consensus Fnr motif by only one nucleotide change (Fig. 1) (32). To determine whether the expression of *dorS* is regulated by FnrL, the *dorS*::*lacZ* fusion plasmid pNMT77 was introduced into the FnrL mutant strain JZ1678 and an oxygen shift experiment was performed where high-oxygen (30%) cultures were shifted to low-oxygen (2%) conditions in the presence of DMSO.

Again, an extremely low level of β-galactosidase activity was observed for both the wild-type and FnrL mutant strains under high-oxygen (30%) conditions (Fig. 5). After the cultures were shifted to 2% oxygen, a rapid increase in *dorS*::*lacZ* expression was observed in the wild-type strain which attained a plateau level 2 to 4 h postshift. After this time, *dorS*::*lacZ* expression decreased toward the steady-state level previously observed for anaerobic-dark cultures (Fig. 3). In contrast, no such increase was observed for *dorS*::*lacZ* expression in the FnrL mutant strain. In fact, *dorS*::*lacZ* expression actually decreased after the oxygen shift.

DISCUSSION

Our previous results indicated that the expression of the *dorCBA* operon, encoding the structural components of DMSO reductase, is dually regulated by oxygen and DMSO (16). In order to further investigate the regulation of the *dor* genes in *R. sphaeroides* 2.4.1^T, we constructed transcriptional *lacZ* fusions to the upstream regulatory sequences of the *dorS*, *dorR*, and *dorC* genes for use as reporters of promoter activity.

As predicted, expression of *dorC*::*lacZ* was induced by both the presence of DMSO and the absence of oxygen. After aerobic growth or photosynthetic growth in the absence of DMSO, negligible levels of β-galactosidase activity are observed. Further, it would appear that TMAO is less efficient as an inducer of *dorC*::*lacZ* expression than DMSO. We previously demonstrated that the *dorCBA*-encoded DMSOR is also responsible for TMAO reductase activity and that Dor mutants are unable to utilize TMAO as the terminal electron acceptor (16). The weaker induction by TMAO may be related to the possibility that the DorSR system is responsible for *dorCBA* induction in response to the presence of either DMSO or TMAO (17). A twofold decrease in *dorS*::*lacZ* and *dorR*::*lacZ* expression was observed after growth with TMAO compared with expression after growth with DMSO. Further, in DorS and DorR mutants *dorC*::*lacZ* was expressed at a similar low level when the cells were grown photosynthetically with either DMSO or TMAO (data not shown). These data suggest that the DorSR system is responsible for both DMSO- and TMAO-dependent sensing and regulation.

We also showed that DorS and DorR mutants are unable to synthesize the DorA protein (16). The expression of *dorC*::*lacZ* in the DorS and DorR mutant backgrounds was negligible after cells were grown photosynthetically in the presence of DMSO or TMAO. Since the DorS and DorR proteins are required for the induction of *dorS*::*lacZ*, *dorR*::*lacZ*, and *dorC*::*lacZ* expression in response to DMSO (or TMAO), we propose that the DorS and DorR proteins form a DMSO (and TMAO)-responsive regulatory system, homologous to the TorS and TorR proteins of *E. coli* (9, 22). However, since *dorS*::*lacZ* and *dorR*::*lacZ* expression in the absence of either DMSO or TMAO is still dependent on the DorSR system, we believe that this system is responsive to an additional signal. The fact that expression of *dorC*::*lacZ* is not induced in the absence of DMSO, in contrast to that of *dorS*::*lacZ* or *dorR*::*lacZ*, even when DorS and DorR are present, is explained by our recent finding that an additional regulatory system, which we term DorXY, is positively required for *dorC*::*lacZ* expression but not for *dorS*::*lacZ* or *dorR*::*lacZ* expression (17). We are currently investigating whether this system is an additional DMSO-dependent regulatory system.

Presumably, DorR is able to activate transcription by binding to conserved motifs in the *dorC* URS (Dor box, consensus A/CG/TGTTA/CACANC), which were previously identified as DmsR binding sites in *R. sphaeroides* f. sp. *denitrificans* (Fig. 1) (26). These motifs are very similar to the TorR binding sites identified in the *torCDA* URS in *E. coli* (21). This suggests that DorR is able to activate transcription in a manner similar to that by DmsR and TorR. In this respect, it is of importance to note the absence of a homolog of the TorT protein in *R.*

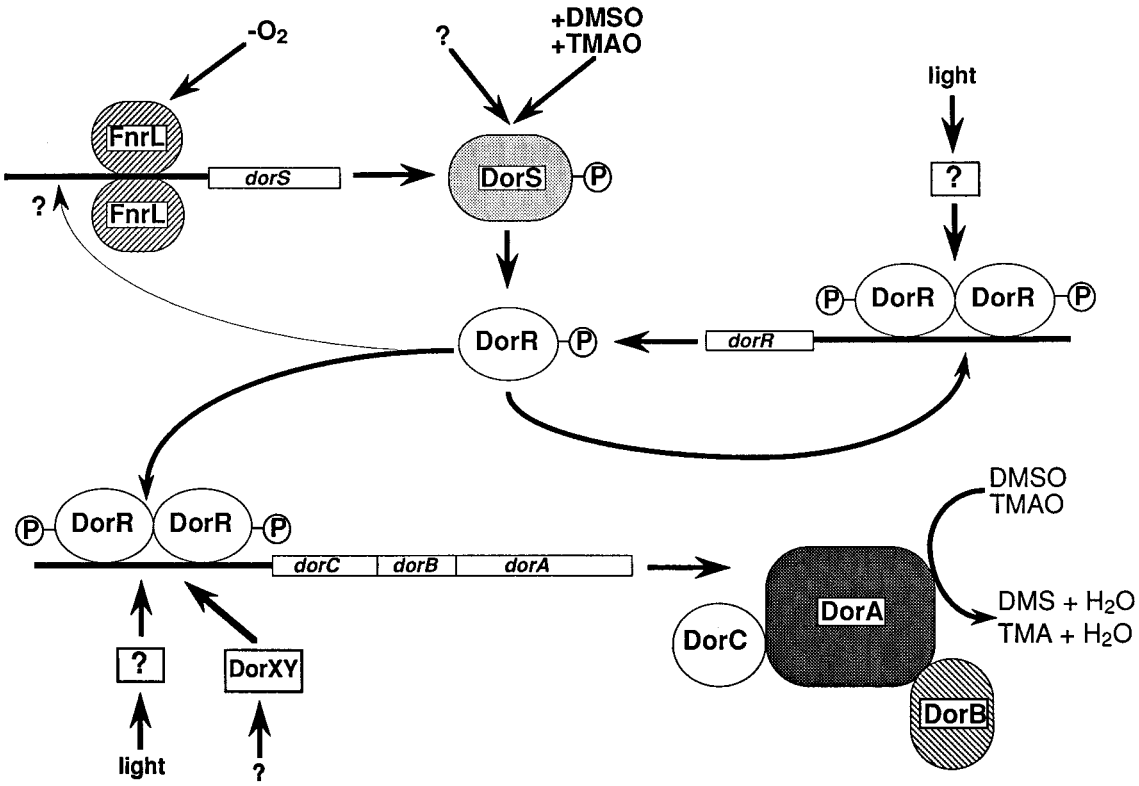

FIG. 6. Model for the regulation of DMSO reductase (*dor*) gene expression in *R. sphaeroides* 2.4.1^T. See the text for further details.

sphaeroides 2.4.1^T. In *E. coli* the TorT protein is required for *torCDA* expression, acting upstream of the TorS sensor protein (9, 10). The authors conclude that TorT is probably not a TMAO-binding protein, and its exact role remains unclear. Presumably, the absence of a TorT homolog in *R. sphaeroides* 2.4.1^T reflects a functional difference in the signal transduction pathway between the Tor and Dor systems.

The reduction in *dorC*::*lacZ* expression when cells were grown photosynthetically with DMSO compared to anaerobic-dark growth was in accordance with the 50% decrease in DMSOR specific activity that we previously observed (16). Further, we found a correlation between the decrease in expression of *dorC*::*lacZ* and the increase in light intensity (Table 2). The observation that *dorCBA* expression is possibly light responsive is exciting since mechanisms for light-responsive gene regulation in *R. sphaeroides* 2.4.1^T are poorly characterized. It has previously been demonstrated that several genes required for photosynthetic growth, namely *crtA*, *crtI*, *puc*, and *bchF*, are under light-dependent transcriptional regulation (30). In addition, increases in light intensity have been shown to affect the accumulation of bacteriochlorophyll in the cell, levels of *puc* (which encodes the B800-850 components of the light-harvesting complex) mRNA, and carotenoid accumulation (11, 29). It has been demonstrated that the PpsR protein functions in the light-responsive regulation of B800-850 abundance (8). Recently, it was shown that the activity of PpsR depends on the AppA protein, which may serve as a redox-dependent modulator of PpsR activity (8). Since *dorS*::*lacZ* and *dorR*::*lacZ* expression are much less affected than *dorC*::*lacZ* expression, we believe that the major target for light-dependent regulation is at the *dorC* URS. We are currently investigating whether the recently identified DorXY regulatory system is affected by light intensity or redox and whether *dorC*::*lacZ* expression is dependent on any of the previously identified redox-dependent regulatory systems of *R. sphaeroides* 2.4.1^T (17).

In addition to examining the regulation of *dorCBA* expression, we were interested in how the genes encoding the DorS and DorR regulatory proteins themselves were regulated. *dorS*::*lacZ* expression was shown to be dependent on the absence of oxygen and the presence of DMSO. By performing an oxygen shift experiment using an FnrL mutant, we demonstrated the positive role for this protein in the induction of *dorS*::*lacZ* expression in response to lowering the oxygen concentration (Fig. 5). The presence of an Fnr binding motif in the *dorS* URS suggests that this FnrL-dependent regulation occurs directly at the *dorS* promoter. *dorS*::*lacZ* expression was also shown to be increased by the presence of DMSO after photosynthetic growth (Fig. 3). As for *dorC*::*lacZ* expression, we believe that this induction is due to the activities of the DorS and DorR proteins, since in the DorS and DorR mutant strains *dorS*::*lacZ* expression was extremely low. At present it is unclear as to how the DorS and DorR proteins affect *dorS* expression, since there are no putative DorR binding motifs present in the *dorS* URS. Experiments are currently under way to examine this further.

The expression of *dorR*::*lacZ* was also shown to be dependent on the absence of oxygen and the presence of DMSO. Further, *dorR*::*lacZ* expression was also dependent on the presence of the DorSR system. Since there are putative DorR binding motifs present in the *dorR* URS, it is believed that the positive regulation by DMSO occurs via DorR binding and activation through these sites. Since *dorR*::*lacZ* expression returned to aerobic levels in the DorS mutant, even when cells were grown photosynthetically in the presence of DMSO, we believe that the anaerobic induction of *dorR*::*lacZ* expression

observed is dependent on the induction and activity of the DorS protein. It was therefore surprising to us to find that the DorS- and DorR-mediated effects on *dorS* and *dorR* expression were manifested even in the absence of DMSO. This suggests that the DorSR system may be responsive to an additional signal other than DMSO (or TMAO). It was previously demonstrated that the DmsR protein of *R. sphaeroides* f. sp. *denitrificans* was able to bind to and retard DNA on a gel of the *dmsCBA* URS in the absence of DMSO (26). This would suggest that DMSO is not the sole signal for the DorSR system.

Taking these results together, we would like to propose the following model for the regulation of DMSO reductase (*dor*) gene expression in *R. sphaeroides* 2.4.1^T. Under oxygen-limited conditions the FnrL protein is able to induce the transcription of the *dorS* gene, encoding the DorS sensor-kinase protein (Fig. 6). DorS is able to phosphorylate its cognate regulator, DorR, in response to the presence of DMSO (or TMAO) and to an additional uncharacterized signal. Phosphorylated DorR is then able to activate transcription from both the *dorR* and *dorCBA* promoters. This leads to an increase in the synthesis of the DorR protein itself and production of functional DMSOR. DorR also appears to affect transcription of the *dorS* gene, by an as yet uncharacterized mechanism. An additional regulatory system, encoded by the *dorX* and *dorY* genes, is also required for *dorCBA* expression. The *dorCBA* and *dorR* promoters are also subject to light-responsive control. Therefore, it appears that a regulatory cascade is involved, whereby the regulation of the *dorS* and *dorR* genes, encoding regulatory proteins, controls the downstream expression of the *dorCBA* operon, encoding the structural components of the DMSOR enzyme. Further, we have demonstrated that this regulation is complex, requiring multiple signals and multiple regulatory proteins.

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