Sulfate reducers have developed a multifaceted adaptative strategy to survive against oxidative stresses. Along with this oxidative stress response, we recently characterized an elegant reversible disulfide bond-dependent protective mechanism in the pyruvate:ferredoxin oxidoreductase (PFOR) of various Desulfovibrio species. Here, we searched for thiol redox systems involved in this mechanism. Using thiol fluorescent labeling, we show that glutathione is not the major thiol/disulfide balance controlling compound in four different Desulfovibrio species and that no other plentiful low molecular weight thiol can be detected. Enzymatic analyses of two thioredoxins (Trxs) and three thioredoxin reductases allow us to propose the existence of two independent Trx systems in Desulfovibrio vulgaris Hildenborough (DvH). The TR1/Trx1 system corresponds to the typical bacterial Trx system. We measured a TR1 apparent Km value for Trx1 of 8.9 μM. Moreover, our results showed that activity of TR1 was NADPH-dependent. The second system named TR3/Trx3 corresponds to an unconventional Trx system as TR3 used preferentially NADH (Km for NADPH, 743 μM; Km for NADH, 5.6 μM), and Trx3 was unable to reduce insulin. The Km value of TR3 for Trx3 was 1.12 μM. In vitro experiments demonstrated that the TR1/Trx1 system was the only one able to reactivate the oxygen-protected form of Desulfovibrio africanus PFOR. Moreover, ex vivo pulldown assays using the mutant Trx1C335S as bait allowed us to capture PFOR from the DvH extract. Altogether, these data demonstrate that PFOR is a new target for Trx1, which is probably involved in the protective switch mechanism of the enzyme.

Oxidative stress is a universal phenomenon experienced by both aerobic and anaerobic organisms from all three domains of life (1–3). To combat this problem, anaerobes have evolved multifaceted strategies to manage the deleterious effects of oxygen (O2) exposure. In this regard, these organisms demonstrate varying degrees of tolerance to O2, ranging from the extremely sensitive methanogens, which typically are inhibited by only a few ppm of O2 (4), to the much more aerotolerant Bacteroides (5) or sulfate-reducing Desulfovibrio species (6, 7). The extreme aerotolerance of these anaerobes can be related to their way of life. Abundance and metabolic activity of sulfate reducers in oxic zones of numerous biotopes (reviewed in Ref. 8) are frequently evaluated as higher than the ones found in neighboring anoxic zones. From the last decade, studies have uncovered original and complex adaptative strategies by which sulfate reducers seek to minimize the damage induced by oxidative conditions (8). One example is superoxide reductase, which is specific to anaerobes and scavenges superoxide ions by reduction. Although this enzyme is broadly distributed in sulfate-reducing bacteria (9–12), more species-specific mechanisms are also found as the disulfide bond-mediated protection of the pyruvate:ferredoxin oxidoreductase (PFOR)3 (13). In Desulfovibrio africanus, this enzyme, which contains three [4Fe-4S]2+/+/ clusters, shows an abnormally high oxygen stability (14, 15). Recently, we have pointed out the existence of the disulfide bond-dependent mechanism of protection against oxidative stress in PFOR of various Desulfovibrio species (13). We have shown that the formation of an intramolecular disulfide bond in the C-terminal domain of PFOR and the presence of a Met or His residue in close vicinity to the proximal [4Fe-4S] cluster are required for an oxygen-stable form of the enzyme. In vivo analyses demonstrated that PFOR was efficiently protected when cells were exposed to air or H2O2. The oxygen-stable form of the enzyme is less active but can be rapidly reactivated to its full activity level by the cleavage of the disulfide bond when cells return to more reductive conditions. Such a specific protection mechanism could be related to the crucial function of this enzyme in the carbon and energy metabolisms of Desulfovibrio (16–18).

Efficiency and reversibility of the protective mechanism of Desulfovibrio PFORs should be related to the thiol/disulfide redox balance of the cytoplasm. It is generally admitted that this redox balance is controlled by the presence of small proteins

3 The abbreviations used are: PFOR, pyruvate:ferrodoxin oxidoreductase; Trx, thioredoxin; TR, thioredoxin reductase; DvH, D. vulgaris Hildenborough; DTE, dithioerythritol; mBBr, monobromobimane; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); LMW, low molecular weight; FTR, ferredoxin-thioredoxin reductase; NEM, N-ethylmaleimide; Ni-NTA, nickel-nitritriacetic acid.
with redox-active cysteine residues, including the thioredoxins (Trxs) and the glutaredoxins as well as low molecular weight (LMW) thiols like the monocysteine tripeptide glutathione (GSH). Trxs and glutaredoxins are small thiol/disulfide oxidoreductases containing the two-cysteine disulfide motif CXXC. Trxs are characterized by the WCGPC motif, although most glutaredoxins contain the CPYC motif (19, 20). Trxs are widespread in all phylogenetic branches, although GSH is absent in many bacteria, including some anaerobic bacteria (21–23). Very few data are available on the anaerobe thiol systems, but it appears that the reduction of intracellular disulfides is mostly mediated by NADPH, which operates together with flavin adenine dinucleotide (FAD)-dependent thioredoxin reductase (TR) to convert oxidized Trx to the free thiol form (24–26). A Trx2 showing 47% amino acid sequence identity with Escherichia coli Trx2 has been characterized in Desulfovibrio desulfuricans and was proposed to be required for cell survival under either oxidative stress or metal ion hemostasis (26). The role of TR/Trx system is likely to be essential for aerotolerant anaerobes because it is required to minimize the occurrence of thiol oxidation by scavenging reactive oxygen species and to mitigate its consequence by repairing the inadvertent disulfides.

Our study aims at specifying the molecules involved in maintaining the thiol/disulfide redox balance of Desulfovibrio and in particular the system involved in the PFOR disulfide switch protective mechanism. Here, we present evidence showing that the TR/Trx systems are the major thiol/disulfide redox system in Desulfovibrio vulgaris Hildenborough (DvH) and probably in most Desulfovibrio species. We also demonstrated that PFOR is a new target for Trx1, which is probably involved in the protective switch mechanism of the enzyme. This result indicates that the TR/Trx system plays an important role in the adaptation strategy used by Desulfovibrio to cope with oxidative conditions.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**D. vulgaris Hildenborough (DvH (27)) was grown anaerobically at 33 °C in a basic medium C (27). E. coli strain DH5α was used for plasmid propagation, although E. coli TG1 or E. coli BL21-Star (DE3) was used for heterologous expression of the TR/Trx system genes. E. coli strains were cultured in either Luria-Bertani (LB), 2YT, or M9-glucose medium (28) in the presence of either ampicillin (100 µg/ml) or kanamycin (50 µg/ml) when required.

**Thiol Labeling and HPLC Fluorimetry Analysis**—Thiol labeling was performed as described previously (29). Briefly, Desulfovibrio and E. coli TG1 cells were cultured until the late log phase (A600 nm ~0.8 and ~5, respectively) and were then harvested by centrifugation. The pellet was washed twice with 50 mM NaCl, 50 mM Tris-HCl buffer (pH 8.0) and was resuspended in 5 mM sodium borate (pH 9.1) (buffer A) containing 1 mM deoxyribonuclease I (Fermentas) to a concentration of 200 µg wet weight of cells/ml. Cell disruption was achieved with a French press at 1000 p.s.i. All buffers were extensively bubbled with nitrogen to minimize thiol oxidation. Thiol groups were labeled by incubation of the protein extracts with 2 mM monobromobimane (mBBr) at 60 °C for 15 min in the dark. Thiol labeling was performed on both extracts treated with 10 mM N-ethylmaleimide (NEM) for 10 min at 60 °C in the dark to covalently cap thiol groups and untreated extracts. Samples were then precipitated with trichloroacetic acid (20%), and after centrifugation for 20 min at 13,000 × g at room temperature, supernatants were collected and diluted 50 times with 10 mM HCl before HPLC analysis. The thiol standards (1 mM final concentration) were treated in buffer A with 2 mM mBBr for 15 min at 60 °C in the dark. Standards were diluted to a concentration of 10 µM with HCl, 10 mM before HPLC analysis.

**Chromatographic analysis** was performed using a Waters Alliance® system equipped with a Waters 2690 XE separation module and a Waters 474 scanning fluorescence detector controlled by the Waters Millenium® chromatography manager software. Separation was achieved at room temperature on an Altima® HP C18 column (250 × 4.6 mm, 5 µm) with an isocratic flow rate of 0.8 ml/min⁻¹. Solvent A was 0.25% aqueous acetic acid (pH 3), and solvent B was methanol. The elution program with linear gradients was the following: 0 min, 5% B; 60 min, 95% B; 61 min 5% B; 70-min re-injection, 20 µl. Thiol derivatives were measured by excitation and emission wavelengths of 390 and 480 nm, respectively.

**RNA Preparation and Reverse Transcription**—RNAs were prepared from a DvH culture (40 ml) reaching the exponential phase (A600 nm ~0.4). The cells were harvested and resuspended in 200 µl of 10 mM Tris-HCl (pH 8.0). Total RNAs were isolated using the High Pure RNA kit from Roche Applied Science according to the manufacturer’s instructions with an extra DNase I digestion step to reduce the amount of contaminating DNA. RNA quality was checked by electrophoresis on agarose gel, and the absence of DNA contamination was confirmed by PCR. RNA was quantified spectrophotometrically at 260 nm (NanoDrop 1000 ThermoScience). For cDNA synthesis, 10 µg of total RNA and 3 µg of random primers (Invitrogen) were mixed, heated to 70 °C for 3 min, and placed in ice. The cDNA synthesis mixture (50 µl) was incubated at 42 °C for 15 s, and 200 µl of GoTaq DNA polymerase (Promega). The reaction was cycled for 30 cycles at 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 1 kb amplified in a Tgradient thermocycler (Biometra). As controls, PCRs were run under the same conditions with DvH genomic DNA (12.5 ng, positive control) and purified RNA (500 ng, negative control). The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

**Cloning of DvH Trx Systems Genes**—DVU_1838 (tr1), DVU_1839 (trx1), DVU_0377 (tr3), DVU_0378 (trx3), and DVU_1457 (tri) coding sequences were obtained by PCR using the Expand High Fidelity PCR system from Roche.
Pyruvate:Ferredoxin Oxidoreductase Is a Target of Thioredoxin

Applied Science and DvH genomic DNA as template. Forward and reverse oligonucleotide primers sequences are listed in supplemental Table S2. Cloning procedure was designed to introduce one N- or C-terminal His tag. The resulting amplimers were purified using the MinElute kit from Qiagen and digested with the appropriate restriction enzymes purchased from New England Biolabs (supplemental Table S2). All coding sequences were cloned into pJF119EH (30 except for the tr3 sequence that was cloned into pET28b+ (Invitrogen). The resulting constructs were transformed into E. coli DH5α and plated on LB agar containing ampicillin (100 μg/ml) when pJF119EH was used and kanamycin (50 μg/ml) for pET28b+. Clones were screened by PCR (GoTaq DNA polymerase, Promega), and restriction analysis and positive ones were further verified by DNA sequencing (Eurofins MWG Operon). This procedure led to the construction of p119trxl, p119trx3, p119TR1, p119TRi, and pET28TR3.

Protein Production—Confirmed constructs were transformed into E. coli TG1 cells except for the construct containing the tr3 gene, which was transformed into E. coli BL21- Star (DE3). For Trx production, after overnight inoculum culture on LB medium at 37 °C, 10 ml of this culture was added to 1 liter of 2YT medium. The cells were grown at 37 °C until the absorbance at 600 nm (A600 nm) reached 0.6. DvH Trxs production was then induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside final concentration. The cells were further grown for 4 h and harvested by centrifugation (20 min, 10,800 × g, 4 °C), and the pellet was stored at −80 °C. For TR production, after overnight inoculum culture on LB medium at 37 °C, 10 ml of this culture was added to 1 liter of M9-glucose medium. The cells were grown at 37 °C until the A600 nm reached 0.8. DvH TRs production was then induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside final concentration. The cells were grown for 12 h after induction and harvested by centrifugation (20 min, 10,800 × g, 4 °C), and the pellet was stored at −80 °C.

Protein Purification—The frozen cells pellets were thawed on ice and resuspended in 500 mM NaCl, 20 mM potassium phosphate (pH 8.0) (buffer B) containing 1 μM deoxyribonuclease I (Fermentas) and Complete (Roche Applied Science) protease inhibitor mixtures according to the manufacturer’s instructions. The cells were passed once through a French press cell at 1000 p.s.i., and cell debris was removed by centrifugation at 11,950 × g for 20 min at 4 °C. The supernatant was subsequently centrifuged at 208,000 × g for 75 min, and the supernatants that contained the soluble DvH His-tagged proteins were collected. In the case of the Trx purification, the proteins fraction was heated at 75 °C for 20 min and then centrifuged (13,000 × g, 15 min). Trx1 and Trx3 were found in the soluble fraction, which confirmed their thermostability. The various DvH His-tagged proteins were further purified by affinity chromatography on a Ni-NTA-agarose (Qiagen) column. All further purification steps were carried out at 4 °C with an automatic BioLogic LP 5 chromatography system (Bio-Rad). The fractions were loaded onto the column (3.2 × 1.5 cm) equilibrated with buffer B containing 20 mM imidazole. The column was washed with 30 ml of this buffer, and the His-tagged proteins were eluted with 20–500 mM imidazole gradient in buffer B. Peak fractions (1 ml) were collected, and the purity of the proteins was checked by using Coomassie Blue-stained SDS-PAGE. Fractions showing high levels of purity were pooled and concentrated on Amicon-ultra 15, 30K, and 3K (Millipore) for TRs and Trxs, respectively. The concentrated Trxs and TRs were further dialyzed against 50 mM NaCl, 50 mM Tris-HCl buffer (pH 8) and the same buffer containing 2 mM EDTA and 10% glycerol, respectively. The purity of the fractions was confirmed by SDS-PAGE on a 12.5% polyacrylamide gel (supplemental Fig. S1A). The protein concentration was measured using the BCA protein assay kit (Pierce). UV-visible spectra of the recombinant TRs were recorded on a Shimadzu UV-1601 spectrophotometer (supplemental Fig. S1B). TRs concentrations were calculated using the ε451 nm value of 11,300 M⁻¹ cm⁻¹.

Steady State Kinetic Analysis of TRs Using DTNB-linked Assay and Insulin Reduction Assay—Km and kcat measurements of TRs for the Trxs as substrates were carried out by using the DTNB reduction assay at saturating concentrations of the specific nicotinamide nucleotide (31). Briefly, the assay mixtures contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM DTNB, 2 mM EDTA, either 1 mM NADPH or 100 μM NADH and either 0.08–3.2 μM Trx3 or 1–25 μM Trx1. The reaction was started by adding either 20 μM TR3 or 100 μM TR1 in a final volume of 0.5 ml. A blank assay contained all the components except TR. The increase in absorbance at 412 nm due to production of 3-carboxy-4-nitrobenzenethiol (NBT) was followed for 5 min at 25 °C (Shimatzu UV-1601 spectrophotometer). Initial velocities were calculated by using a molar extinction coefficient of 2 × 13,600 M⁻¹ cm⁻¹, because the reduction of DTNB by 1 mol of Trx-(SH)₂, yields 2 mol of NBT. Apparent Vmax and Km values were obtained from three independent measures of the initial velocity by using a computer-aided direct fit to the Michaelis-Menten equation (Origin 6.1 software).

In the case of TR1, the Km value for its substrate Trx1 was also obtained using the insulin reduction assay (32). This assay contained 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM bovine insulin (Sigma), 2.5 mM EDTA, 1 mM NADPH, and 0.8–10 μM Trx1 (final volume 0.5 ml). The reaction was initiated by adding 100 nM of TR1. The increase in absorbance at 650 nm was monitored at 25 °C.

Cofactor specificity was determined using the DTNB reduction assay with either NADPH or NADH as a source of reducing equivalents. The concentrations of NADPH and NADH varied from 0.07 to 3 mM and 0.25 to 15 μM, respectively, with a constant Trx3 concentration of 1.4 μM. The reaction was started by adding either 100 nM of TR3 for measurements in the presence of NADPH or 5 nM in the other case.

PFOR Activation Assay—PFOR activity was determined as described previously (14). For the activation assay, samples were handled in an anaerobic chamber. D. africanus PFOR was preincubated for 30 min in a total volume of 100 μl containing 0.4 μM of the purified enzyme and a reducing agent of the disulfide bond. When DTE or Trx alone was used as reducing agent, PFOR was preincubated in 50 mM Tris-HCl (pH 8.5) buffer, whereas 100 mM potassium phosphate (pH 7.5) buffer was used when the TR/Trx systems were used as reducing source.
Site-directed Mutagenesis of Trx1 and Purification of the Protein Mutant Trx1C33S—Mutations were introduced into the trx1 gene by an overlapping PCR procedure (33). The primers pairs -200pTac/C33Srew and C33Sfw/pF119rev (supplemental Table S2) were used to generate the two DNA fragments having overlapping ends. Plasmid p119trx1 was used as the template in the PCRs. The resulting PCR products were combined and amplified by PCR using the primer pair -200pTac/pF119rev, leading to an amplicon of ~1050 bp. After digestion, the resulting EcoRI-BamHI fragment was introduced into the polylinker cloning site of plasmid pF119EH giving plasmid p119trx1C33S. Construction was then verified by sequencing. Production and purification of the His-tagged Trx1C33S were performed as described above.

Ex Vivo Pulldown Experiment Using Trx1C33S—Soluble extract of DvH was prepared as follows: after reaching late log phase, cells were harvested by centrifugation (10,800 × g, 20 min, 4 °C), and the pellet was washed three times with 50 mM NaCl, 50 mM potassium phosphate buffer (pH 7.5). Cells were then passed through a French press cell at 1000 p.s.i and centrifuged for 20 min at 11,900 × g and 4 °C. The soluble extract was obtained by an additional centrifugation at 208,000 × g during 45 min at 4 °C. Trx1C33S (1 mg) was incubated with 9 mg of the soluble extract (2.2 ml) for 1 h at 33 °C under gentle agitation. The mixture was further loaded onto a Ni-NTA-agarose (Qiagen) column. The column was washed with 500 mM NaCl, 50 mM potassium phosphate buffer (pH 7.5) (buffer C), and the washing steps were repeated until the absorbance of the washing solution at 280 nm was zero. An additional washing step was performed with 3 ml of buffer C containing 50 mM imidazole. Elution was achieved with 3 ml of buffer C containing 200 mM imidazole. The eluted proteins were separated by SDS-PAGE and silver-stained. Bands of interest were excised and then subjected to tryptic digestion and LC-ion trap MS/MS analysis (supplemental “Experimental Procedures”). To check that the interaction between Trx1 and the captured proteins was specific and based on the proposed disulfide-dependent mechanism, wild type Trx1 was employed for control pulldown experiments.

RESULTS

Searching for Glutathione in Desulfovibrio Strains—The presence of GSH in Desulfovibrio species is a controversial subject. Fahey et al. (21) reported the absence of GSH in DvH. However, more recently, a low concentration of glutathione was detected in Desulfovibrio gigas (34). We thus decided to search for the presence of GSH in extracts from four different strains of Desulfovibrio by using thiol derivatization and HPLC fluorimetry. E. coli was used as positive control for GSH detection. Fig. 1B shows that an important fluorescent peak was detected in the E. coli extract, corresponding to the retention time of GSH (Fig. 1A). Moreover, this peak disappeared when the E. coli extract was pretreated with NEM (Fig. 1C), confirming that it corresponded to GSH. On the contrary, DvH extracts exhibited only small fluorescent peaks, but none of these peaks had the same retention time as standard GSH (Fig. 1, D and E). Absence of the fluorescence peak corresponding to GSH was also observed in D. gigas, D. africanus, and D. desulfuricans strain 27774 extracts (supplemental Fig. S2). In the case of D. desulfuricans, nitrate can be substituted to sulfate as an alternative electron acceptor to prevent hydrogen sulfide (H2S) production. Even in the absence of H2S, no GSH peak could be detected in D. desulfuricans cells (supplemental Fig. S2). A 10 times lower dilution of the derivatized extracts did not show either GSH peak on the chromatograms for any Desulfovibrio strains (data not shown). Thus, no GSH could be detected in any of these four Desulfovibrio strains, suggesting that GSH does not control the thiol/disulfide redox balance in the cytoplasm of these bacteria.

Identification of Thioredoxin System Genes in the D. vulgaris Hildenborough Genome by in Silico Analysis—In silico analyses of the DvH genome (35) revealed the presence of two Trx homologues (DVU_1839 and DVU_0378) and three TR homologues (DVU_1838, DVU_0377, and DVU_1457). Amino acid sequences alignment of the two Trxs (DVU_1839 and DVU_0378) pointed out 47 and 16% sequence identity with DVU_1838, DVU_0377, and DVU_1457, respectively. B. E. coli cell extract was derivatized with mBBr. C. E. coli cell extract treated with NEM prior to reaction with mBBr. The arrow shows the position of the glutathione peak lost because of NEM treatment. D. DvH cell extract was derivatized with mBBr. E. DvH cell extract treated with NEM prior to reaction with mBBr.

FIGURE 1. HPLCs of low molecular weight thiols derivatized with mBBr. A, defined mixture of thiol standard: Cys, cysteine; GSH, glutathione; Na2S, disodium sulfide. Peaks at 15.2, 19, and 34.5 corresponded to Cys, GSH, and Na2S derivates, respectively. B. E. coli cell extract was derivatized with mBBr. C. E. coli cell extract treated with NEM prior to reaction with mBBr. The arrow shows the position of the glutathione peak lost because of NEM treatment. D. DvH cell extract was derivatized with mBBr. E. DvH cell extract treated with NEM prior to reaction with mBBr.
In addition, the two residues, Asp-26 and Lys-57, of *E. coli* Trx1 that have been suggested to deprotonate Cys-32 and to contribute to proton uptake accompanying the reduction of Trx (36) are conserved only in DVU_1839 (supplemental Fig. S3A). Concerning the TR homologues (DVU_1838, DVU_0377, and DVU_1457), their sequence identities with TrxB from *E. coli* were 34, 33, and 25%, respectively. Amino acid sequence alignment of these three putative TRs with TrxB also revealed some distinctive features of DVU_0377. For example, DVU_0377 exhibits the GXGXG motif sequence (common in NADH-dependent enzymes) (37) instead of the GXGXXG motif (common in NADPH-dependent enzymes) that is present in TrxB, DVU_1838, and DVU_1457 sequences (supplemental Fig. S3B). Furthermore, the FF residues following the active site motif that play a major role in recognition of Trx1 (38) are conserved in DVU_1838 and DVU_1457 TRs but not in DVU_0377 TR. From this *in silico* analysis, we can conclude that DvH genome encodes both classical (DVU_1838–1839 and DVU_1457) and atypical (DVU_0378-0377) TR/Trx systems.

**Genetic Organization of DvH Thioredoxin System Genes**—Genome analysis shows that DVU_0377 and DVU_0378 are integrated in a large gene cluster including 10 genes (DVU_0372–0381). To check for an operon organization, experiments were conducted to amplify intergenic regions from DVU_0371 to DVU_0372 on the cDNA templates. Fig. 2A shows that no amplification product was obtained for the intergenic regions between DVU_0371 and DVU_0372 or DVU_0372 and DVU_0381 and DVU_0382. The intergenic region between the other genes (from DVU_0372 to DVU_0381) was amplified, and it led to the respective amplifications with the same sizes as the ones obtained from the positive controls using genomic DNA as template. For all these PCRs, the absence of the amplification products when using RNA as template indicated that the cDNA was not contaminated by genomic DNA (Fig. 2A). DVU_0377 and DVU_0378 are thus included in a polycistronic unit, including eight other genes (supplemental Table S3). An interesting member of this transcriptional unit is DVU_0374. DVU_0374 encodes a protein showing 62% sequence identity with an oxidoreductase protein, MreG, from *D. desulfuricans* G20 (DdeG20). In DdeG20, it has been proposed that MreG reduces both U(VI) and Cr(VI) in the presence of *E. coli* Trx1 and TrxB (39). The same analysis performed on DVU_1838 and DVU_1839 showed that these genes are integrated into a tricistronic unit with an additional gene (DVU_1837) encoding a protein annotated as a putative competence protein (Fig. 2B and supplemental Table S3). In the case of DVU_1457 gene, Fig. 2C shows that no amplification was detected between DVU_1456 and DVU_1457, although a weak band was observed with the primers designed to amplify the intergenic region between DVU_1457 and DVU_1458 (Fig. 2C). However, the weak amplification might correspond to a leak of transcription by RNA polymerase because no strong intrinsic transcriptional terminator was found downstream of

**FIGURE 2.** RT-PCR analysis of the DVU_0377 (A), DVU_1838 (B), and DVU_1457 (C) gene clusters. RNAs were extracted, and after cDNA synthesis with reverse transcriptase, cDNAs were further amplified in PCR by addition of primer pairs (supplemental Table S1) to reveal transcriptional links between successive genes. The PCR products were analyzed by electrophoresis on agarose gel. −, PCRs performed on RNAs; +, PCRs performed on genomic DNA; c, PCRs performed on cDNAs. M, DNA size marker.
the DVU_1458 gene (data not shown). We can thus conclude that DVU_1458 is a monocistronic gene.

Taking into account all our results described above, DvH TR/Trx systems were renamed hereafter as follows: DVU_1839 and DVU_1838 as Trx1 and TR1, respectively, and DVU_0378 and DVU_0377 as Trx3 and TR3, respectively, to avoid confusion with *E. coli* Trx2 whose homologue gene is absent in the *Dv* genome. Finally, DVU_1458 was named TRi (i for isolated), because the encoding gene is not closely associated with a gene encoding a Trx.

**Catalytic Properties of DvH Thioredoxin System Proteins**—Trx-mediated reduction of DTNB was performed to examine whether the purified His-tagged recombinant proteins Trx1, Trx3, TR1, TR3, and TRi function as a thioredoxin system. The increase of absorbance at 412 nm when Trx1 and TR1 were used together in the test showed that the two proteins were able to reduce the artificial substrate (DTNB) (Fig. 3). The same observation was made in the presence of Trx3 and TR3 together (Fig. 3). When either the couple Trx1 and TR3 or Trx3 and TR1 was added to the assay, no significant increase of the absorbance was measured (Fig. 3). Finally, no DTNB reduction was observed when the reductase TRi was used with either Trx1 or Trx3, indicating that these two Trxs cannot form an efficient thioredoxin system with TRi as reductase (Fig. 3). It should be noted that TRi exhibited similar spectroscopic characteristics (absorption at 375 and 451 nm) as TR1 and TR3 demonstrated that all three TRs had incorporated the FAD cofactor (supplemental Fig. S1B). The protein/FAD ratio values were 0.85, 0.77, and 0.76 for TR1, TR3, and TRi, respectively. Absence of TRi activity is thus not due to an immature form of the recombinant protein. Altogether, these data show that TR1/Trx1 and TR3/Trx3 form two distinct general disulfide reductase systems capable of reducing artificial substrate (DTNB) in the presence of NADPH with no efficient cross-reactivity between them.

As described above, the TR3 sequence includes the GXGXGG motif common to many NADH-depending enzymes, whereas TR1 includes the GAGXXA motif suggesting its NADPH dependence (supplemental Fig. S3B). To experimentally determine their cofactor specificity, we performed the DTNB reduction assay under standard conditions, with either NADPH or NADH as a source of reducing equivalents. For the TR3/Trx3 system, the apparent \( K_m \) value for NADPH and the catalytic efficiency (apparent \( k_{cat}/K_m \)) were determined to be 5.6 ± 0.4 \( \mu \)M and 1.27 \( 10^6 \) \( \text{M}^{-1} \text{s}^{-1} \), respectively, which were about 130 times lower and about 250 times higher than the obtained values when NADPH was used, respectively (Table 1). In contrast, the TR1/Trx1 system showed a DTNB reducing activity only with NADPH as reductant, suggesting that TR1 is only able to use NADPH as cofactor (Table 1). Interestingly, TR3 alone was able to catalyze the reduction of DTNB using either NADH or NADPH as an electron donor, which is unusual for a low molecular weight reductase (~35 kDa) (data not shown).

The kinetic parameters for the interaction of the two DvH thioredoxin systems were determined (Table 2). The apparent \( K_m \) and \( k_{cat} \) values are consistent with results from other bacterial systems (26, 40). As thioredoxins are known as disulfide reductases that reduce insulin (40, 41), we determined the \( K_m \) value by using the insulin reduction assay. The apparent \( K_m \) value for Trx1 was determined to be 6.3 ± 0.6 \( \mu \)M, which was in the same range as the value determined from the DTNB-linked assay (apparent \( K_m \) value = 8.9 ± 1.7). Very surprisingly, Trx3 was found unable to reduce insulin in either the TR3- or DTT-mediated reduction assay (data not shown).

**DvH Thioredoxin1 System Activates *D. africanus* Pyruvate-Ferredoxin Oxidoreductase**—*D. africanus* PFOR activity measurements showed that the presence of DTE in the cuvette increased the initial rate of pyruvate oxidation (Fig. 4A). The kinetics obtained in presence of this sulfhydryl compound showed an induction phase related to an activation process of PFOR as pointed out previously (13, 14). On the contrary, no induction phase was observed when the enzyme was previously activated (data not shown).

**TABLE 1**

| Assay | \( K_m \) \( \mu \)M | \( k_{cat} \) \( s^{-1} \) | \( k_{cat}/K_m \) \( \text{M}^{-1} \text{s}^{-1} \) |
|-------|----------------|-----------|------------------|
| TR1/Trx1 | Not detectable | ND | ND |
| TR1/Trx1 | NADH 743 ± 92 | 3.66 ± 0.18 | 0.49 \( 10^5 \) |
| TR3/Trx3 | NADH 5.6 ± 0.4 | 7.10 ± 0.25 | 1.27 \( 10^6 \) |

* The activity was measured using the DTNB reduction assay as described under “Experimental Procedures.”

* ND means not determined.

* The assay was performed in the presence of 1 mM NADH.

**TABLE 2**

| Thioredoxin system | \( K_m \) \( \mu \)M | \( k_{cat} \) \( s^{-1} \) | \( k_{cat}/K_m \) \( \text{M}^{-1} \text{s}^{-1} \) |
|-------------------|----------------|-----------|------------------|
| TR1/Trx1 | 8.9 ± 1.7 | 0.98 ± 0.12 | 1.1 \( 10^5 \) |
| TR3/Trx3 | 1.12 ± 0.14 | 5.17 ± 0.30 | 4.6 \( 10^6 \) |

* The values corresponded to the DTNB reduction assay.

**FIGURE 3. Cross-reactivity of the DvH TR/Trx systems.** Kinetics of the DTNB reduction was obtained as described under “Experimental Procedures” in the presence of 1 mM NADPH. Proteins concentrations were as follows: 12 \( \mu \)M Trx1/100 nM TR1; 1.4 \( \mu \)M Trx3/100 nM TR3; 45 \( \mu \)M Trx3/100 nM TR1; and 50 \( \mu \)M Trx1/100 nM TR3. In the case of TRi (100 nM), 50 \( \mu \)M Trx1 or 45 \( \mu \)M Trx3 was used.

**Pyruvate:Ferredoxin Oxidoreductase Is a Target of Thioredoxin**

| Cofactor specificity | \( k_{cat}/K_m \) |
|---------------------|----------------|
| NADPH  \( <10^6 \) | ND |
| NADH 743 ± 92 | 0.49 \( 10^5 \) |
| NADH 5.6 ± 0.4 | 1.27 \( 10^6 \) |

* The activity was measured using the DTNB reduction assay as described under “Experimental Procedures.”

* ND means not determined.

* The assay was performed in the presence of 1 mM NADH.
Pyruvate:Ferredoxin Oxidoreductase Is a Target of Thioredoxin

A

B

C

FIGURE 4. Kinetics of the methyl viologen reduction by D. africanus pyruvate-ferredoxin oxidoreductase. The cuvette contains 0.4 nm purified PFOR. A, DTE-dependent activation of D. africanus PFOR. PFOR activity was measured as follows: (a) in the absence of DTE; (b) in the presence of 16 mM DTE; and (c) with the preincubation of PFOR (0.4 μM) with 100 mM DTE for 30 min before injection (1 μL) in the cuvette. B, activation assays of D. africanus PFOR by DvH DTE-reduced Trxs. 0.4 μM PFOR was preincubated with the following: (a) 5 mM DTE; (b) 60 μM DTE-reduced Trx1; (c) 60 μM DTE-reduced Trx3; and (d) 140 μM DTE-reduced Trx3. C, activation assays of D. africanus PFOR by DvH Trx systems. 0.4 μM PFOR was preincubated with the following: (a) 0.1 mM NADPH; (b) 0.1 mM NADH; (c) 0.1 mM NADPH, 200 nm TR1, and 60 μM Trx1; (d) 0.1 mM NADH, 200 nm TR3, and 60 μM Trx3; (e) 0.1 mM NADPH and 200 nm TR1; and (f) 0.1 mM NADH, and 200 mM TR3. All preincubations were carried out in an anaerobic chamber, in a total volume of 100 μL, during 30 min at 30 °C. 1 μL of the solution was then injected in a 1-ml cuvette that does not contain DTE.

FIGURE 5. Trx1 single-cysteine mutant pulldown experiment. SDS-PAGE profile of the high molecular weight captured proteins using Trx1C33S. The DvH proteins captured by His-tagged Trx1C33S were purified onto a Ni-NTA-agarose column, and the obtained protein samples were separated on 12% SDS-PAGE. Lane 1, molecular weight standard proteins (Bio-Rad); lanes 2 and 3, sample obtained in Trx1C33S pulldown experiment; lanes 4 and 5, control pulldown experiment using wild type Trx1. For lanes 2 and 4, DTT was omitted in the loading buffer.

The two most intense protein bands in this part of the gel were captured by His-tagged Trx1C33S. The profile of the high molecular weight captured proteins using Trx1C33S. The DvH proteins captured by His-tagged Trx1C33S were purified onto a Ni-NTA-agarose column, and the obtained protein samples were separated on 12% SDS-PAGE. Lane 1, molecular weight standard proteins (Bio-Rad); lanes 2 and 3, sample obtained in Trx1C33S pulldown experiment; lanes 4 and 5, control pulldown experiment using wild type Trx1. For lanes 2 and 4, DTT was omitted in the loading buffer.

anaerobically preincubated with DTE, and the maximum activity (91.9 units/mg) was obtained (Fig. 4A). It has been proposed that PFOR is able to switch, through a disulfide bond cleavage process, from a low activity oxygen-protected form to a high activity oxygen-sensitive form (13). To test whether DvH Trx systems were able to cleave the disulfide bond of D. africanus PFOR and thus activate the enzyme, the TR1/Trx1 and TR3/Trx3 systems were used as reducing systems in various preincubation mixtures with D. africanus PFOR. The kinetics obtained are shown in Fig. 4, B and C. D. africant PFOR preincubated with Trx1 reduced by DTE exhibited a specific activity of 83.7 units/mg. Moreover, the corresponding kinetics displayed no induction phase (Fig. 4B). On the other hand, preincubation with DTE-reduced Trx3 did not activate the enzyme (Fig. 4B). In the same way, PFOR preincubated with Trx1 reduced by its reductase (TR1) exhibited a specific activity of 72.1 units/mg without any induction phase, whereas preincubation with TR3 and Trx3 did not induce activation of the enzyme (Fig. 4C). Fig. 4C shows also that TRs alone were unable to activate PFOR. These results demonstrate that only the DvH TR1/Trx1 system is able to cleave the disulfide bond of D. africant PFOR and thus to activate the enzyme.

PFOR Is a Potential Trx1 Target Protein in D. vulgaris—To confirm the interaction between PFOR and Trx1, we developed a strategy based on the dithiol exchange mechanism catalyzed by Trx. In that way, the cysteine mutant Trx1 is thus able to covalently catch target proteins as a mixed disulfide intermediate. This method is well established for Trx (42) and has been applied successfully to a whole range of organisms (43-45). By using the His-tagged mutant Trx1C33S as bait, we captured ex vivo potential target proteins from DvH-soluble extract. Elution of mixed disulfide intermediates from the Ni-NTA resin was carried out with imidazole after extensive washing with NaCl-containing buffer to eliminate un specific bind proteins and thus increase the specificity of the eluted fraction. As control, the same pulldown experiment was performed with wild type Trx1, confirming that no proteins were bound unspecifically (Fig. 5, lanes 4 and 5). It should be noticed that a better resolution was obtained for the high molecular weight part of the gel (from 100 to 250 kDa) when the loading buffer contained DTT (Fig. 5, lanes 2 and 3). The two most intense protein bands in this part of the gel were cut off and analyzed by LC-MS/MS for identification (Fig. 5). DvH PFOR (locus tag DVU_3025) was identified in these two bands (Table 3). The apparent molecular weight of band B (Fig. 5) is in agreement with the calculated molecular weight of the
DvH PFOR subunit (~132 kDa). Identification of PFOR in band A is more intriguing and can be related with the oligomerization state of the protein. Indeed, it has been shown that DvH PFOR is a homo-octomeric complex of 1 MDa (46). One can imagine that this high level of oligomerization can induce electrophoresis artifacts of migration during electrophoresis. Identification of PFOR as mixed disulfide intermediate with Trx1C33S clearly indicates that this enzyme is a Trx1 target protein and can be correlated with the activation of D. africanus PFOR by the TR1/Trx1 system in vitro.

DISCUSSION

Low molecular weight thiols play critical roles in cell physiology. In most cells, the tripeptide glutathione (GSH) is the major LMW thiol. In this study, we demonstrated that all Desulfovibrio strains investigated, i.e. DvH, D. africanus, D. gigas, and D. desulfuricans ATCC 27774, do not contain detectable amounts of GSH using mBBr fluorescent labeling. This result is consistent with reports showing the absence of detectable GSH in DvH and others anaerobes (21, 25). Surprisingly, low levels of GSH and glutathione reductase activity were detected in D. gigas cell-free extract (34). However, analysis of Desulfovibrio genomes so far sequenced (www.ncbi.nlm.nih.gov) reveals the absence of γ-glutamyl-L-cysteine synthase- and GSH synthase-encoding genes, required for GSH synthesis. Consequently, GSH does not appear as the major thiol/disulfide balance controlling compound in Desulfovibrio species. DvH genome also encodes a protein annotated as putative glutaredoxin (DVU_0883). However, the corresponding protein sequence does not contain the CPYC motif found in most glutaredoxins (20). Analysis of the DVU_0883 genetic context indicates that DVU_0883 is rather the subunit of a putative ferredoxin-thioredoxin reductase (FTR). Indeed, DVU_0883 would be included in a dicstrionic unit, including the gene DVU_0884. This latter encodes a protein that displays sequence similarities with the catalytic subunit of a FTR. In all Desulfovibrio genomes sequenced so far, the gene encoding the putative glutaredoxin homologue is always associated with a gene encoding the catalytic subunit of FTR. Moreover, in Desulfotalea psychrophila, another sulfate reducer, a protein (locus Tag DP_2155) annotated as a putative FTR is encoding by a gene that corresponds to the fusion of the two genes, DVU_0883 and DVU_0884. The probable existence of this peculiar bacterial FTR in Desulfotalea has been previously reported (47). Altogether, these data strongly suggest that DVU_0883 is not a glutaredoxin, which is in accordance with the absence of the glutathione pathway in DvH.

Many microorganisms synthesize alternative LMW thiols. For example, mycothiol is the major LMW thiol in mycobacteria (48), and in Pyrococcus furiosus, CoASH may, at least partially, fulfill this role (49). More recently, a newly abundant thiol, named bacillithiol, has been described in Bacillus species and related bacteria (50). Our data show that in Desulfovibrio extracts, all fluorescent peaks are of low intensity and insensitive to NEM, suggesting that they correspond to background non-thiolic peaks (Fig. 1 and supplemental Fig. S2). The same result was obtained when separation of fluorescent peaks was achieved on a Nucleodur NH2-RP column with less hydrophobic properties than the Alltima™ HP C18 column (data not shown). The absence of a unique major LMW thiol could be related to a continuous large production of the reducing agent H2S during the sulfate reduction process. However, no significant peak can also be detected in D. desulfuricans cells when cultivated on nitrate as an alternative electron acceptor (supplemental Fig. S2). We can thus conclude that no abundant LMW thiol can be detected in the tested Desulfovibrio species.

The lack of the LMW thiol pathway in these Desulfovibrio strains suggests that the TR/Trx system might be the major and even the sole intracellular thiol/disulfide redox balance controlling system in these anaerobes. In this regard, analysis of the DvH genome revealed the presence of three putative TRs and two Trx homologues. We identified a homologue of E. coli Trx1. The available information on the abundance of Trx1 proteins indicates that it is an essential and ubiquitously distributed protein (23, 51–53). We show that the DvH trxl gene (DVU_1839) is expressed as part of a three-gene operon where the gene downstream trx1 encodes a TR (TR1) very similar to E. coli TrxB. Moreover, a high turnover number for TR1/Trx1 reaction (1.1 × 104 m−2 s−1) clearly indicates a close linked action in vivo. We thus propose that, in DvH, TR1 and Trx1 are the two components of the bacterial conventional Trx system. The corresponding operon also encodes a putative outer membrane lipoprotein (DVU_1837) as predicted by the LipoP 1.0 server. It should be noticed that comparison of all Desulfovibrio known genes indicates the same genetic organization in all Desulfovibrio strains for this operon. At the moment, no functional relation can easily be proposed with the TR1/Trx1 system, but the polycistronic mRNA structure implicates that their action has to be tightly coordinated in vivo. Interestingly, in the anaerobe Bacteroides fragilis, the trxB gene is the second gene of a bicistronic transcriptional unit that includes a gene encoding the lipoprotein chaperone, LolA (25).

The second DvH Trx homologue (DVU_0378, Trx3) contains the unconventional two-cysteine redox motif LCPhC. No reduction of insulin was observed by Trx3, and TR1 was unable to reduce it. However, this atypical Trx is reduced efficiently by another TR (DVU_0378 or TR3) showing 32% identities with TR1. Trx3 together with this TR formed a new and unconventional Trx system in DvH. The peculiarity of this system is confirmed by the catalytic properties of TR3. Indeed, unlike most of the TRs that use NADPH as cofactor, the apparent Keq value of TR3 for NADH is 130 times lower than the one for NADPH. Moreover, TR3 showed the capability to reduce DTNB directly. This indicates that TR3 resembles catalytic properties close to the ones of some archaeal type of TRs (54, 55). The only other bacterial TR showing this property has been isolated from Thermotoga maritima (56). In this study, we show that the

---

**TABLE 3**

Identification of the protein bands A and B by LC-MS/MS

| Band | Sequest score | Sequence coverage | No. of identified peptides | Accession no. | Genome annotation | Calculated mass |
|------|--------------|------------------|---------------------------|--------------|-------------------|----------------|
| A    | 280.2        | 17.49            | 38                        | DVU_3025 PFOR | 131.38            | DvH            |
| B    | 14015        | 8.56             | 17                        | DVU_3025 PFOR | 131.38            | DvH            |
genes encoding the Trx3 system are integrated in a polycistrionic unit with eight other genes. In *D. desulfuricans* G20, a similar operonic structure, namely the *mre* operon (metal reduction), was characterized (39). However, the *DvH* operon includes two additional genes, *DVUl_0375* and *DVUl_0372* encoding a Glu/Leu/Phe/Val dehydrogenase family protein and a putative membrane protein, respectively. It was suggested that the *DdetG20* Trx system encoding the *mre* operon together with the oxidoreductase MreG provided cells with a cytoplasmic mechanism of U(VI) and Cr(VI) reduction (39). We hypothesize that in *DvH* this operon plays a similar role in metal resistance. Interestingly, the homologous operon in *Desulfovibrio salesiens* contains only the first six genes. The absence of the oxidoreductase (MreG orthologue) gene in this sulfate-reducing bacterium suggests another role for the TR3/Trx3 system in *Desulfovibrio*.

A third putative TR (TRi) was predicted from the genome of *DvH*. It is not able to catalyze the reduction of *DvH* Trx1, although residues implicated in *E. coli* TrxB/Trx1 interfaces (38) are well conserved in the TRi sequence (supplemental Fig. S3). The absence of reduction of Trx3 by TRi is less unexpected taking into account the singularity of TR3 and Trx3 sequences. Thus, for the moment, no target for this putative TR can be proposed, especially as the corresponding encoding gene is monocistrionic. However, multiple genome-wide transcriptomic analyses designated to identify novel genes that respond to oxidative stress in *DvH* showed that the *DVUl_1457* gene was systematically up-regulated (57–60). This finding strongly suggests that TRi might be a part of the *DvH* survival strategy under transient oxygen exposure. In addition, TR1- and Trx1-encoding genes (*DVUl_1838–1839*) were also responsive to oxidative stress, suggesting that the conventional *DvH* Trx system might also be involved in oxidative protection (57, 58, 60). In contrast, the Trx3-encoding gene (*DVUl_0378*) was down-regulated after 30 min of exposure to air (57). A role of Trx systems in the oxidative stress response has already been shown for several bacterial species (reviewed in Ref. 61). For example, the gene encoding for *E. coli* Trx2 is under control of the oxidative stress transcription factor OxyR suggesting a specific role in response to oxidative stress (62, 63). It was proposed that *D. desulfuricans* G20 Trx2 might have the same role in this obligate anaerobe (26). However, analysis of the *Desulfovibrio* genome so far sequenced (www.ncbi.nlm.nih.gov) reveals the absence of Trx2 in *D. vulgaris* strains and four additional *Desulfovibrio* species. In *B. fragilis*, TrxB is essential for survival in an adverse aerobic environment (25). Moreover, aerobic conditions induced the expression of all *trx* genes in this highly aerotolerant anaerobe (23). *Clostridium acetobutilicum* also has an oxygen-responsive Trx-encoding gene (64), and the *Pyrococcus furiosus* genome encodes three homologues of Trx, one of them being up-regulated within 30 min of peroxide addition (65). Thus, the role of Trx systems appears as highly crucial for the growth and survival of obligate anaerobes.

The oxidative stress response of anaerobic bacteria is an elaborate response mounted to protect the organisms from lethal stress. Recent studies with anaerobes have resulted in the identification of new original mechanisms such as the induction of an aerobic class ribonucleotide reductase in *B. fragilis* under oxidative conditions (66). This enzyme may help to maintain dNTPs pools during the extended period of oxidative stress and allow the bacterium to recover at a faster rate than obligate anaerobes that do not contain an aerobically functioning enzyme. In the same way, we showed that an elegant disulfide bond-dependent reversible mechanism is able to protect *Desulfovibrio* PFOR against oxidative stress (13). The reversible formation of an intramolecular disulfide bond leads to an inactive but oxygen-stable form of PFOR during the stress period. Afterward, the enzyme is able to switch to its fully active form by the cleavage of the disulfide bond with no need of de novo synthesis, thus allowing quick growth recovery. Characterization of the *DvH* thiold redox systems allowed us to investigate their role in the disulfide bond-dependent reversible mechanism. First, we showed that the TR1/Trx1 system is the only reductive system able to activate *in vitro* *D. africanus* PFOR and switch it to its highly active form. Second, we brought evidence that the single-cysteine mutant Trx1-C*33S* was capable of forming a stable mixed disulfide bond with *DvH* PFOR, showing that this enzyme is a target of Trx1. In addition, the docking simulation between Trx1 and *D. africanus* PFOR is in agreement with an interaction site of Trx1 in the vicinity of the disulfide bond involved in the protective mechanism of the PFOR (supplemental Fig. S4). We thus conclude that the TR1/Trx1 system is the reductive system linked to the protective mechanism of PFOR induced in most *Desulfovibrio* cells following oxygen exposure. The role of the TR1/Trx1 system detected here may be highly crucial for rapidly resuming growth when cells re-enter anaerobic conditions.

It is likely that Trx systems are involved in multiple pathways as more than 80 *E. coli* proteins co-purifying with *E. coli* Trx1 have been identified implicating at least 26 distinct cellular processes (67). In the future, trapping Trxs targets in *DvH* should allow us to discover other important roles of *Desulfovibrio* Trx systems.

Acknowledgments—We acknowledge Y. Arfi for participation in the production and purification of Trx system components, M. C. Durand for technical assistance on RT-PCR experiments, and Sabrina Lignon and Régine Lebrun (IMM Proteomic Facilities) for the proteomics analyses.

**REFERENCES**

1. Imlay, J. A. (2008) *Mol. Microbiol.* **68**, 801–804
2. Imlay, J. A. (2008) *Annu. Rev. Biochem.* **77**, 755–776
3. Imlay, J. A. (2003) *Annu. Rev. Microbiol.* **57**, 395–418
4. Brukhman, A. L., and Nerusov, A. I. (2007) *Prakt. Biohim. Mikrobiol.* **43**, 635–652
5. Sund, C. I., Rocha, E. R., Tzianabos, A. O., Tzianabos, A. O., Wells, W. G., Gee, J. M., Reott, M. A., O’Rourke, D. P., and Smith, C. J. (2008) *Mol. Microbiol.* **67**, 129–142
6. Cypionka, H. (2000) *Annu. Rev. Microbiol.* **54**, 827–848
7. Baughn, A. D., and Malamy, M. H. (2004) *Nature* **427**, 441–444
8. Dolla, A., Fournier, M., and Dermoun, Z. (2006) *J. Biotechnol.* **126**, 87–100
9. Fournier, M., Zhang, Y., Wildschut, J. D., Dolla, A., Voordouw, J. K., Schrijemer, D. C., and Voordouw, G. (2003) *J. Bacteriol.* **185**, 71–79
10. Huang, V. W., Emerson, J. P., and Kurtz, D. M., Jr. (2007) *J. Biotechnol.* **126**, 87–100
11. Nivière, V., Asso, M., Weill, C. O., Lombard, M., Guigliarelli, B., Favadon, V., and Houéé-Levin, C. (2004) *Biochemistry* **43**, 808–818
