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Analysis of the Transcriptional Unit Encoding the Genes for Rubredoxin (*rub*) and a Putative Rubredoxin Oxidoreductase (*rbo*) in *Desulfovibrio vulgaris* Hildenborough

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The nucleotide sequence of a 2.0-kilobase-pair *Eco*RI restriction fragment upstream from the gene (*rub*, 162 base pairs) encoding rubredoxin from *Desulfovibrio vulgaris* Hildenborough indicates that it is part of a larger transcriptional unit, containing an additional 378-base-pair open reading frame which terminates 16 nucleotides from the translational start of the *rub* gene and could encode a polypeptide of 14 kilodaltons (kDa). Northern (RNA) blotting of RNA isolated from both *D. vulgaris* Hildenborough and *Escherichia coli* TG2 transformed with plasmid pJK29, which contains both genes on a 1.1-kilobase-pair *Sal*I insert, confirms that the genes for this 14-kDa polypeptide and rubredoxin are present on a single transcript of 680 nucleotides. Strong evidence that the 14-kDa polypeptide is also a redox protein is provided by the fact that its NH_2 terminus is homologous to desulforedoxin, which has been isolated from *D. gigas* as a small dimeric redox protein (36 amino acids per monomer), coordinating two iron atoms. Since rubredoxin is a potential redox partner for the 14-kDa protein, it has been tentatively named rubredoxin oxidoreductase, produced by the *rbo* gene. Southern blotting indicates that the *rbo-rub* operon is present in several species and strains of sulfate-reducing bacteria.

Rubredoxins are small, electron-carrying proteins (molecular mass, 6 kilodaltons [kDa]), which are found in the cytoplasm of several anaerobic bacteria, e.g., *Desulfovibrio gigas* (8), *D. vulgaris* Hildenborough (7), *D. desulfuricans* (15), *Thermodesulfobacterium commune* (24), *Clostridium pasteurianum* (36), *Peptostreptococcus elsdenii* (4), and *Peptococcus aerogenes* (3). The amino acid sequences of these rubredoxins have been determined (3, 4, 7, 8, 15, 36), and the three-dimensional structures of rubredoxins from *C. pasteurianum*, *D. vulgaris*, *D. gigas*, and *D. desulfuricans*, which has a very low molecular mass (only 5.2 kDa) have been solved (2, 13, 14, 32, 41–43). The redox center of rubredoxin consists of a single iron atom (redox potential, −50 to 0 mV), coordinated to four cysteinyl sulfurs. This redox potential is relatively high, since dissimilatory sulfate reduction by *Desulfovibrio* species requires electrons at −400 to −200 mV (17, 18, 23, 27), and it is not clear, therefore, which electron transfer reaction is catalyzed by rubredoxin. Several attempts have been made to define the redox partners of rubredoxin by biochemical experiments. By using the rubredoxin-mediated reduction of eucaryotic cytochrome *c* by NADH as an assay, an NADH-rubredoxin oxidoreductase was (partially) purified from *D. gigas* (16) and *C. acetobutylicum* (25). The *Clostridium* enzyme was found to have flavin adenine dinucleotide as the prosthetic group and to consist of a single subunit with a molecular mass of 41 kDa (25). The *D. gigas* enzyme shows specificity for rubredoxin from *D. gigas* (K_m = 6.2 × 10^{-6} M) relative to rubredoxins from *D. vulgaris* (K_m = 5.3 × 10^{-5} M) and *C. pasteurianum* (K_m = 1.0 × 10^{-4} M), which show 71 and 63% sequence identity, respectively, with *D. gigas* rubredoxin. The subunit molecular mass and nature of the cofactor have not been reported for the *D. gigas* enzyme, and it has not been isolated from *D. vulgaris*.

A different approach to the identification of possible redox partners for rubredoxin, which makes use of the fact that the gene encoding rubredoxin (*rub*) from *D. vulgaris* Hildenborough was recently cloned from a λ library of the *D. vulgaris* chromosome and its nucleotide sequence was determined (37), is taken here. No typical promoter consensus sequence (29) was found in the region immediately upstream from the *rub* gene, which instead appears to contain the 3′ end of another reading frame, indicating that the *rub* gene may be part of a larger transcriptional unit. Since at least one gene encoding a redox protein interacting with rubredoxin could be present on this larger transcript, the region upstream from the *rub* gene is characterized in detail in the present paper.

MATERIALS AND METHODS

Strains, vectors, and media. The bacterial strains, plasmids, and cloning vectors used in this work are described in Table 1. For the isolation of RNA, *D. vulgaris* Hildenborough was grown anaerobically in a medium made up from the following solutions, described by Pfennig et al. (26): solution 1 (1 liter, kept under 90% N_2–10% CO_2), solution 2 (1 ml), solution 3 (1 ml), solution 4 (30 ml), solution 5 (3 ml), 15% (wt/vol) of sodium lactate (10 ml), solution 8 (1 ml), solution 9 (0.1 ml), and Wolin vitamins (10 ml) (44). This medium allows a high growth rate of *D. vulgaris*, and RNA can successfully be isolated from cells grown for 16 h at 30°C at a 5% (vol/vol) inoculum. Chromosomal DNA, isolated from 15 different species and strains of sulfate-reducing bacteria (see Table 2) after growth on Postgate medium C (27), was a gift from Helen Kent, AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton, England. DNA from *D. vulgaris* Miyazaki was a gift from T. Yagi, Department of Chemistry, Shizuoka University, Shizuoka, Japan, and DNA from *D. salexigens* NCIMB 8365 was donated by D. W. S. Westlake, Department of Microbiology, University of Alberta, Edmonton, Canada. *Escherichia coli* TG2 was grown in TY medium (22) containing 10 g of tryptone, 5

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TABLE 1. Bacterial strains and DNA vectors used in this study

Strain or vector	Genotype, phenotype, comments, reference
Strains	
D. vulgaris subsp. *vulgaris* Hildenborough[a]	NCIMB 8303; isolated from clay soil near Hildenborough, U.K. (27)
E. coli TG2[b]	Δ(*lac-pro*) *supE thi hsdM hsdR recA* F′ (*traD36 proAB*$^+$ *lacZ*ΔM15I^q); from T. J. Gibson.
Vectors	
pUC8, pUC9	Amp^r (35)
pJK15	This study (Fig. 1)
pJK29	This study (Fig. 1)
pRbo1	This study (Fig. 1)
pRub1	This study (Fig. 1)

[a] Abbreviated as *D. vulgaris* Hildenborough. A bacteriophage λ gene library has been constructed for this organism (37).

[b] Constructed from *E. coli* JM101 [Δ(*lac-pro*) *supE thi* F′ (*traD36proAB*$^+$ *lacZ*ΔM15 I^q] by T. J. Gibson and M. D. Biggin, Laboratory of Molecular Biology, Medical Research Council Centre, Cambridge, U.K.

g of yeast extract, and 5 g of NaCl per liter of water at pH 7.4.

Biochemical reagents. All enzymes were obtained from Pharmacia, Inc., with the exception of calf alkaline phosphatase, which was from Boehringer Mannheim Biochemicals. The radioisotopes [α-^{35}S]dATP (400 Ci/mmol; 10 mCi/ml), [α-^{32}P]dATP (3,000 Ci/mmol; 10 mCi/ml), and [γ-^{32}P]ATP (3,000 Ci/mmol; 10 mCi/ml) were purchased from Amersham Corp. and were used for dideoxynucleotide sequencing, nick translation, and 3′ and 5′ end labeling, respectively. Ficoll 400 was purchased from Pharmacia, Inc. Polyvinylpyrrolidone (molecular weight 40,000), bovine serum albumin (fraction V), molecular biology grade sodium dodecyl sulfate (SDS), bakers' yeast tRNA, salmon sperm DNA (sodium salt), and dextran sulfate (molecular weight 500,000) were purchased from Sigma Chemical Co. Low- and high-gelling-temperature (LGT and HGT) agarose were obtained from Bethesda Research Laboratories, Inc. Nitrocellulose and Hybond-N hybridization transfer membranes were obtained from Schleicher & Schuell, Inc., and Amersham, respectively. All other reagent grade chemicals were purchased from either Sigma or Fisher Scientific Co.

DNA cloning. Several recombinant bacteriophages carrying the *rub* gene were isolated from a λ library, as described previously (37). These clones cover 35 kilobase pairs (kb) of the *D. vulgaris* Hildenborough chromosome, and an *Eco*RI restriction map of a part of this region is shown in Fig. 1. A 2.0-kb *Eco*RI fragment and a 1.1-kb *Sal*I fragment (Fig. 1) were cloned into the *Eco*RI and *Sal*I sites of pUC8 (35), generating plasmids pJK15 and pJK29, respectively, which were purified as described elsewhere (40).

Shotgun nucleotide sequencing. The procedure followed for shotgun nucleotide sequencing was essentially that outlined by Bankier and Barrell (5). Plasmid pJK15 was sonicated, and the resulting fragments were end repaired and size fractionated by gel electrophoresis on 1% (wt/vol) LGT agarose. The 400- to 1,000-base-pair (bp) fraction was excised and isolated from the gel and ligated to the replicative form of M13mp8 (21), digested with *Sma*I and calf alkaline phosphatase (5). After being transfected into competent, $CaCl_2$-treated *E. coli* TG2 cells, the ligation mixtures were spread onto TY plates with top agar, containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (5). Single-stranded DNA was isolated from white recombinant phage plaques by using a 1.5-ml minipreparation procedure (5). The purified DNAs (2 μl) were spotted on a nitrocellulose filter, which was subsequently baked at 80°C for 1 h under vacuum. The 2.0-kb *Eco*RI insert from pJK15 was isolated by LGT agarose gel electrophoresis, radiolabeled by nick translation

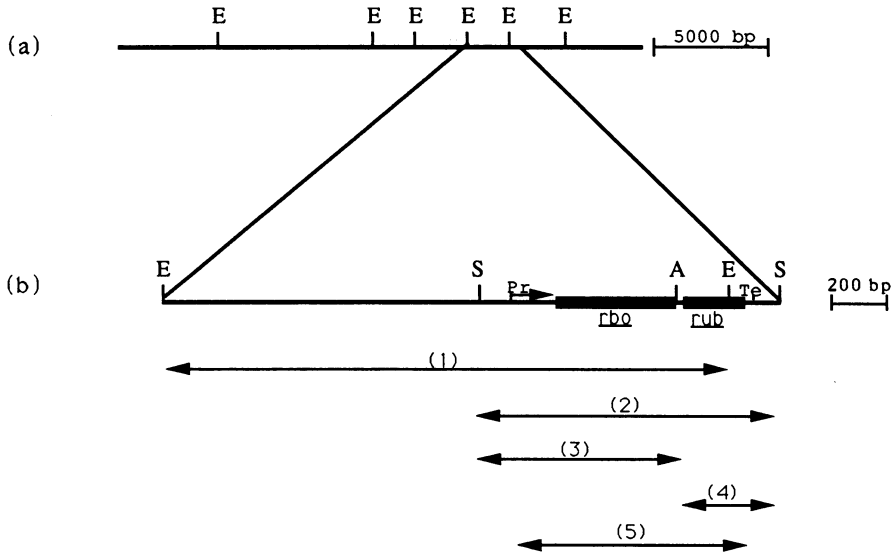

FIG. 1. Survey of DNA containing the *rub* gene of *D. vulgaris* Hildenborough. (a) Location of *Eco*RI (E) restriction sites as determined earlier (37). (b) Map of a 2,234-bp *Eco*RI-*Sal*I region of DNA for which the nucleic acid sequence was determined. The location of restriction sites for *Eco*RI, *Sal*I (S), and *Ava*I (A), the coding regions for the genes encoding rubredoxin (*rub*) and the putative rubredoxin oxidoreductase (*rbo*), and the location of promoter (Pr) and transcription terminator (Te) sequences, are indicated. The following plasmids (inserts) were constructed: 1, pJK15 (2.0 kb EE); 2, pJK29 (1.1 kb SS); 3, pRbo1 (746 bp AS) and 4, pRub1 (347 bp AS). The position of the nucleic acid sequence shown in Fig. 3 is indicated in region 5.

(20), and denatured by boiling. The filter was prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2])–10× Denhardt solution (20)–0.5% (wt/vol) SDS for 15 min at 68°C. The 2.0-kb DNA probe was then added to the prehybridization solution, and hybridization was continued for 16 h at 68°C. After hybridization, the filter was washed in 6× SSC–0.5% (wt/vol) SDS at 68°C for 1 h. The filter was then dried and wrapped in Saran wrap, and positive clones were identified by autoradiography. These were then sequenced by the dideoxy-chain termination procedure developed by Sanger et al. (31), as detailed by Bankier and Barrell (5). The sequencing data were processed and analyzed by using the programs of Staden and McLachlan (33, 34).

Construction of gene-specific probes. The completed nucleotide sequence indicated the presence of a unique *Ava*I site in the 19-bp region separating the *rbo* and *rub* genes (Fig. 1; see also Fig. 3). Plasmid pJK29 was digested with *Ava*I, and the digested DNA was end repaired with the Klenow fragment of DNA polymerase I to generate blunt ends (20). Following inactivation of the polymerase at 68°C, pJK29 was further digested with *Sal*I, resulting in the excision of both the *rbo* and *rub* genes on 746- and 347-bp fragments of DNA, respectively; these were isolated by electrophoresis on 1.5% (wt/vol) LGT agarose. The purified fragments were ligated into pUC8, previously digested with *Sal*I and *Sma*I, to give plasmids pRbo1 and pRub1, respectively. The insert of pRbo1 was gel isolated following digestion with *Hin*dIII and *Eco*RI as a 767-bp *rbo* gene-specific probe, whereas the presence of an *Eco*RI site near the 3′ end of the *rub* gene (Fig. 1; see also Fig. 3) allowed the isolation of a 162-bp *rub* gene-specific probe by digestion of pRub1 with *Eco*RI and gel electrophoresis.

RNA isolation and Northern blotting. RNA was isolated from cultures of *D. vulgaris* Hildenborough and *E. coli* TG2 by the hot-phenol extraction method (11) and stored as the ethanol precipitate at −20°C. Samples of RNA were collected by centrifugation, washed with 70% (vol/vol) ethanol, dried under vacuum, and redissolved in 20 μl of loading buffer, prepared as described elsewhere (10). For Northern (RNA) blotting, RNA samples were electrophoresed on gels containing 1.0% (wt/vol) HGT agarose in 1× MOPS buffer (10× MOPS buffer is 0.2 M 3-*N*-morpholinopropanesulfonic acid, 10 mM EDTA, and 50 mM sodium acetate [pH 7.0]) containing 2% (vol/vol) formaldehyde and then blotted onto a Hybond-N hybridization transfer membrane. Following transfer, the membrane was exposed to shortwave UV light to cross-link the RNA to the filter. Blots were prehybridized for 4 h at 42°C in a solution containing 0.75 M NaCl, 0.075 M sodium citrate (pH 7.0), 50% (vol/vol) formamide, 1% (wt/vol) SDS, 0.1% (wt/vol) Ficoll 400, 0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) bovine serum albumin, and 0.16 mg of boiled bakers' yeast tRNA per ml. Following prehybridization, the blots were hybridized at 42°C for 16 h in the solution described above, also containing 10% (wt/vol) dextran sulfate as a hybridization enhancer, with a nick-translated probe derived from either plasmid pRbo1 or pRub1. Following hybridization, the blots were washed twice for 5 min at room temperature with 100 ml of 0.3 M NaCl–0.06 M Tris hydrochloride (pH 8.0)–0.002 M EDTA, then twice for 15 min at 60°C with 100 ml of the same solution with 0.5% (wt/vol) of SDS added, and, finally, twice for 15 min at room temperature with 0.003 M Tris base, after which the filters were dried and autoradiographed.

S1 nuclease mapping. A probe covering the region upstream from the *rbo* gene was prepared by digestion of pJK29 with *Eco*RI and calf alkaline phosphatase and gel isolation of the 913-bp *Eco*RI fragment, containing the relevant *Sal*I-*Eco*RI fragment. The probe was 5′ end labeled by adding T4 polynucleotide kinase (1 μl; 10 U/μl) and [γ-^{32}P]ATP (2.5 μl; 10 μCi/μl) in the presence of 50 mM Tris hydrochloride (pH 7.6)–10 mM $MgCl_2$–3 mM dithiothreitol–0.1 mM EDTA in a total volume of 10 μl at room temperature for 1 h. The labeled probe was phenol extracted, ethanol precipitated, and dissolved in 20 μl of 80% (vol/vol) formamide–20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.5)–400 mM NaCl (hybridization buffer). RNA (100 μg) was dissolved in 40 μl of hybridization buffer and incubated with the probe at 44°C for 12 h. Following the addition of 240 μl of S1 nuclease buffer (300 mM NaCl, 18 mM sodium acetate [pH 4.6], 10 mM $ZnSO_4$) and 2 μl of S1 nuclease (384,000 U/μl), the hybridization solution was incubated at room temperature for 30 min. The reaction was terminated by the addition of 10 μl of denatured bakers' yeast tRNA (1 μg/μl), and then phenol extraction and ethanol precipitation of the DNA-RNA hybrids for 1 h at −70°C were carried out. The vacuum-dried samples were dissolved in 10 μl of formamide dye mixture, prepared as described by Bankier and Barrell (5), and heated at 80°C for 5 min before being loaded on either a 4 or 6% (wt/vol) denaturing acrylamide gel (5), which was run at 39 W for 2 to 3 h. A partial *Hin*fI digest of plasmid pUC8, labeled by filling the ends with Klenow polymerase in the presence of dGTP, dCTP, dTTP, and [α-^{32}P]dATP, was used as a molecular size marker (1,613, 913, 517, 396, 140, 75, and 65 nucleotides).

Southern blotting. Samples of genomic DNA (3 to 10 μg) from 17 different species and strains of sulfate-reducing bacteria were digested with *Eco*RI, electrophoresed on a 0.7% (wt/vol) HGT agarose gel as described elsewhere (40), and blotted on Hybond-N hybridization transfer membrane. The DNA was bound to the membrane by UV cross-linking as described above, and the blots were prehybridized for 1 h at 42°C with a solution (pH 7.4) containing 50% (vol/vol) formamide, 1 M NaCl, 0.1% (wt/vol) sodium PP_i, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) bovine serum albumin, 0.2% (wt/vol) Ficoll 400, 10% (wt/vol) dextran sulfate, and 0.175 mg of denatured salmon sperm DNA per ml. A nick-translated pRub1 probe (20) was then added, and hybridization was continued for 16 h. The Southern blots were washed similarly to the Northern blots, dried, and autoradiographed. The pRub1 probe was removed by washing with 0.5 M NaOH–1.5 M NaCl for 30 min and neutralizing for 30 min with 1 M Tris hydrochloride (pH 8.0)–1.5 M NaCl. Removal of the probe was confirmed by autoradiography, after which the blots were rehybridized with the nick-translated pRbo1 probe.

RESULTS

Nucleotide sequence upstream from *rub*. The nucleotide sequence of the 2.0-kb *Eco*RI fragment, containing the region upstream from the *rub* gene, was determined by the random cloning and dideoxy sequencing procedure of Bankier and Barrell (5), and these data were combined with the sequence of the *rub* gene (37) to obtain a sequence of 2,234 nucleotides (nt) extending from an *Eco*RI to a *Sal*I site (Fig. 1b). An identification of possible coding regions with the aid of the codon probability method of Staden and McLachlan (34) in this 2.2-kb sequence is shown in Fig. 2. Apart from the *rub* gene, this analysis indicates the presence of two other plausible genes in frames a and b. The amino acid sequences derived from these possible genes were compared

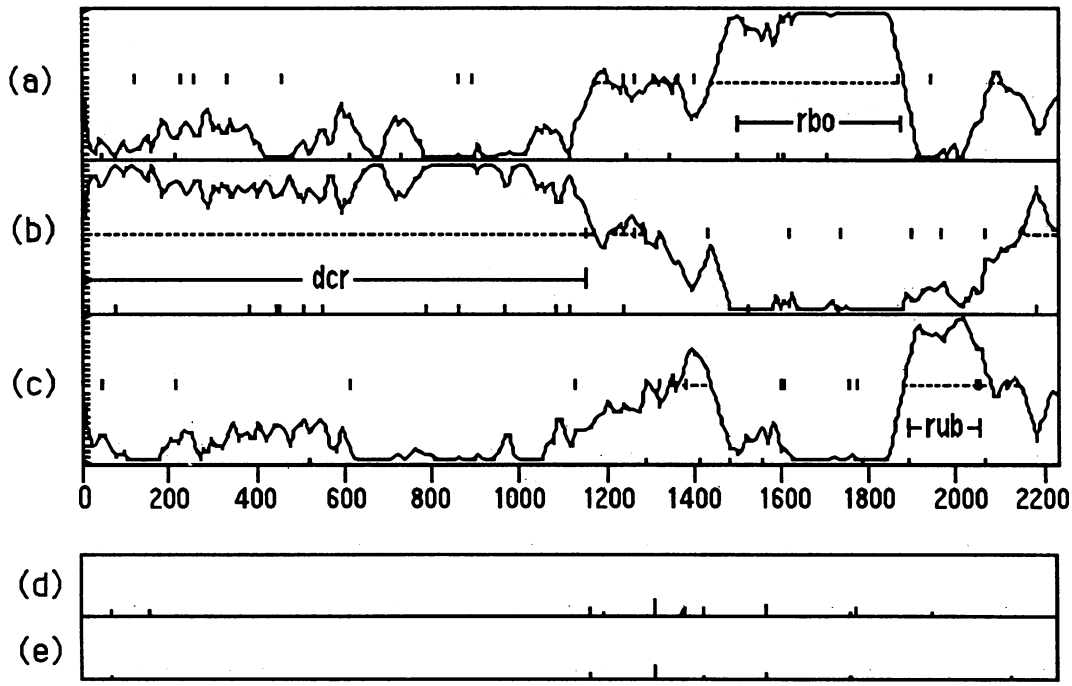

FIG. 2. Statistical analysis of the 2,234-bp region outlined in Fig. 1b. (a to c) The probability that a stretch of sequence (25 bases) is coding is calculated and plotted for each of the three reading frames a, b, and c (36). A codon usage table, which includes all codons of structural genes from *D. vulgaris* Hildenborough, was used as the standard in the calculation. Methionine (ATG) start codons are indicated on the base line, and stop codons are indicated at the half level of each frame. The coding regions for a putative rubredoxin oxidoreductase (*rbo*), a putative *D. vulgaris* chemoreceptor (*dcr*), and rubredoxin (*rub*) are indicated by the calculation as shown in frames a to c, respectively. (d and e) Search for *E. coli* promoters. Sequences with homology to the consensus −35 (TTGACA) and −10 (TATAAT) *E. coli* promoter sequence are indicated in frames d and e, respectively. The scale is in base pairs.

with sequences in the Bionet data base, which indicated unique and extensive homologies with, respectively, desulforedoxin of *D. gigas* (9) and the aspartate and serine chemoreceptors of *Salmonella typhimurium* (30) and *E. coli* (6), respectively. The amino acid sequence that can be derived from the sequenced portion of the *dcr* gene (nt 1 to 1158) lacks approximately 140 NH_2-terminal amino acids, and further nucleic acid sequencing is required for the completion of the sequence of the putative *Desulfovibrio* chemoreceptor protein. The homology of the *dcr* gene product with these two known chemotactically active proteins will therefore not be further discussed here, allowing a focus on the gene immediately preceding the *rub* gene. In view of the homology with desulforedoxin, as discussed below, the upstream gene appears to also encode a redox protein and is tentatively referred to as rubredoxin oxidoreductase, the product of the *rbo* gene.

The nucleotide sequence of the *rub* gene and its upstream region (nt 1301 to 2140) is shown in Fig. 3. Each nucleotide was determined four times on average, at least once on each strand. The gene encoding rubredoxin (nt 1896 to 2057) and the putative *rbo* gene predicted by the codon probability calculations in Fig. 2a (nt 1499 to 1879) have been translated into amino acid sequences. Each gene is preceded by a plausible ribosome-binding site. A search for *E. coli* promoters (Fig. 2d and e) indicates the presence of a promoter consensus sequence (−35, −10) upstream from these two genes, and a potential promoter has been indicated in Fig. 3. A hairpin-loop-forming structure of nine G · C base pairs, which may serve as a transcription terminator, is present immediately downstream from the *rub* gene. Thus, an analysis of the nucleic acid sequence indicates that the two genes may form an operon. Direct experimental evidence for the presence of both genes on a single transcript is presented below.

Northern blotting and S1 nuclease mapping. Northern blots of RNA isolated from both *D. vulgaris* and *E. coli* TG2(pJK29) indicate that a single transcript of approximately 680 nucleotides hybridizes with both the pRbo1 and the pRub1 probes (Fig. 4). The 1.1-kb insert of pJK29 is in the correct orientation for transcription of the *rbo* and *rub* genes from the *E. coli lac* promoter present on the pUC vector. However, a transcript originating from the *lac* promoter and containing both the *rbo* and *rub* genes would be approximately 1,100 nt long, and this size is not consistent with the data in Fig. 4C. The observation of similarly sized transcripts in *D. vulgaris* and *E. coli* indicates that the *D. vulgaris* promoter controlling the transcription of these two genes functions in *E. coli* and may thus resemble the *E. coli* consensus. The observed size of the transcript is sufficiently large to accommodate both the *rbo* and the *rub* genes. Assuming that the transcript terminates at position 2110, on the 3′ side of the hairpin indicated in Fig. 3, the transcriptional start site is placed in the vicinity of position 1430, which is 70 nt upstream from the translational start of the *rbo* gene. The transcriptional start site was next defined more precisely by S1 nuclease mapping. Use of the 913-bp probe gives rise to a major protected fragment of 600 ± 50 nt (average of three experiments), while minor protected fragments of variable, smaller sizes are also observed (Fig. 5). The 600-nt fragment ends at nt 2041 (Fig. 3), indicating a transcriptional start site at nt 1441 ± 50. The presence of a promoter sequence with reasonable homology to the *E. coli* consensus at nt 1355 to 1380 (Fig. 3) suggests a potential start

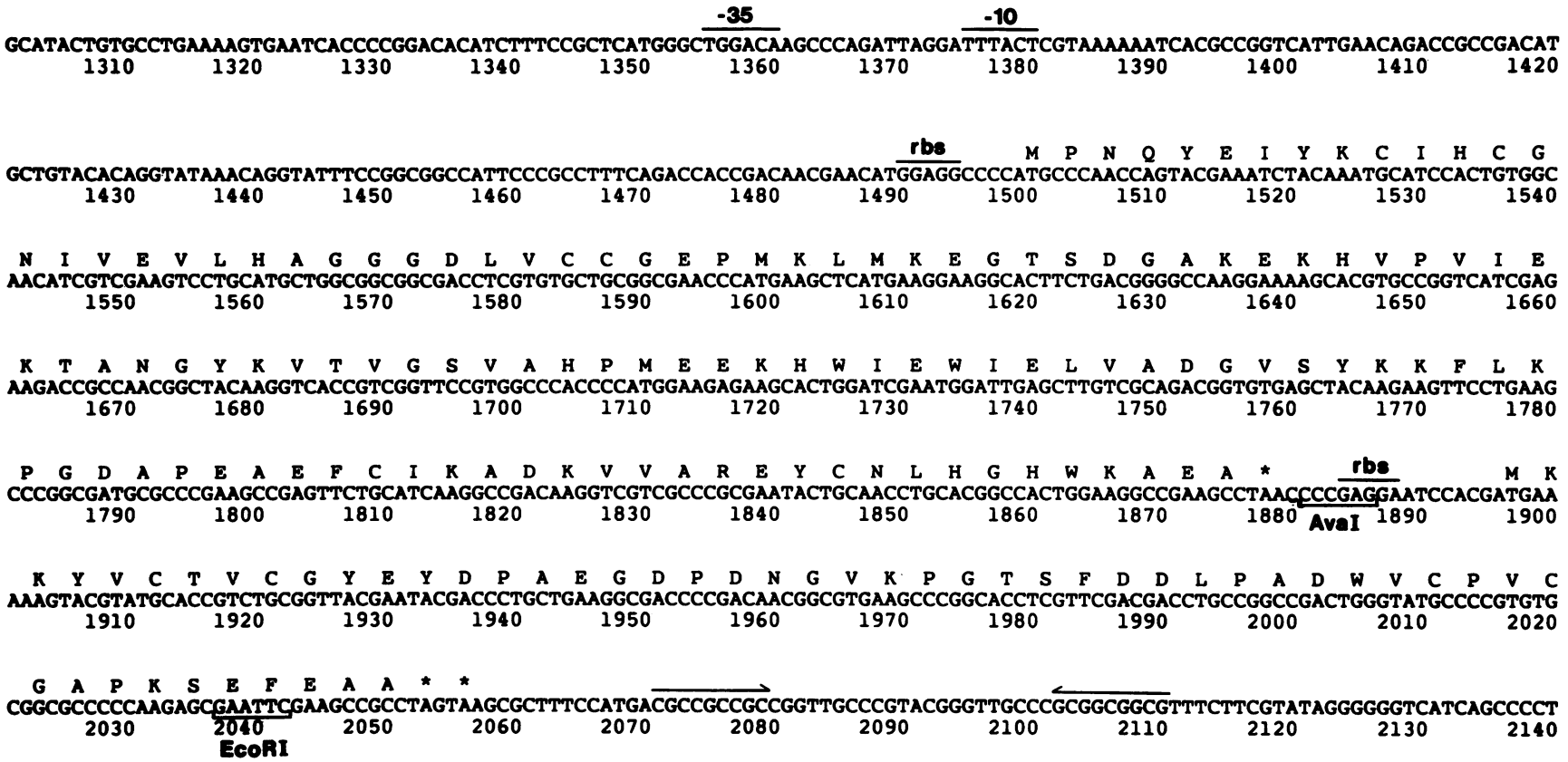

FIG. 3. Nucleotide sequence of the *rbo-rub* operon. The numbering of the sequence is the same as in Fig. 1 and 2. The coding regions of the *rbo* (nt 1499 to 1879) and *rub* (nt 1896 to 2057) genes have been translated with the single-letter amino acid code. Translation stop codons are designated by asterisks. The ribosome-binding sites (rbs) and a plausible promoter (−35, −10) are indicated. A G+C-rich stem-and-loop structure (→←) which could serve as a transcription termination signal is shown.

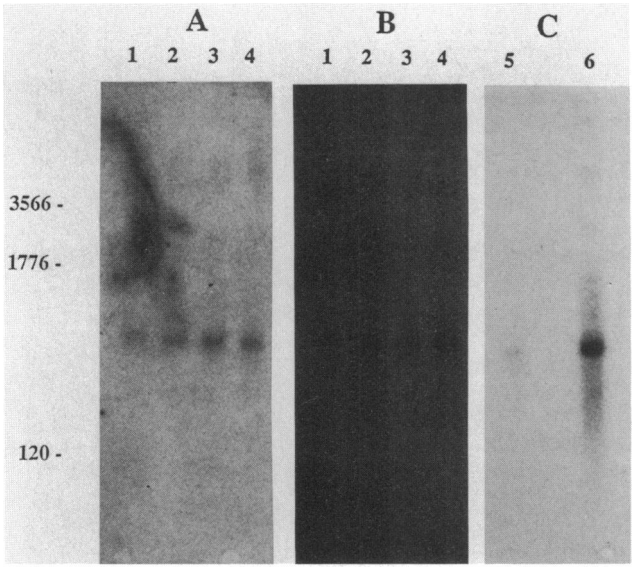

FIG. 4. Northern blotting of total RNA isolated from *D. vulgaris* Hildenborough and *E. coli* TG2(pJK29). (A) Increasing amounts of *D. vulgaris* RNA (25, 50, 75, and 100 μg) were loaded in lanes 1 to 4, respectively. The blot was hybridized to the pRbo1 probe and autoradiographed for 5 days at −70°C. The size markers, 25S rRNA (3,566 bases), 16S rRNA (1,776 bases), and 5S rRNA (120 bases), were visualized by fluorography of the blot following transfer of the ethidium bromide-stained gel. (B) The same blot as in panel A was incubated for 2 min in boiling distilled water to dissociate the pRbo1 probe. Following autoradiography to confirm the dissociation of this probe, the blot was rehybridized to the pRub1 probe. Autoradiography was carried out for 7 days at −70°C. (C) *D. vulgaris* RNA (25 μg) and RNA isolated from *E. coli* TG2 transformed with plasmid pJK29 (100 μg) were loaded in lanes 5 and 6, respectively. Following electrophoresis, the blot was incubated with the pRbo1 probe. Autoradiography was performed for 14 days at −70°C. Use of the pRub1 probe gave identical results (not shown).

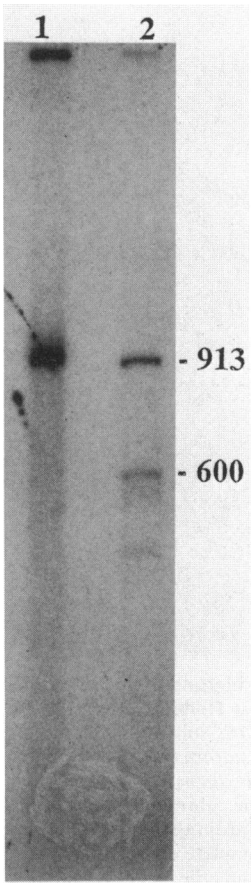

FIG. 5. S1 nuclease mapping of the transcriptional start site of the *rbo-rub* operon. Lanes: 1, 913-nt probe control; 2, *D. vulgaris* RNA (100 μg) hybridized with the 913-nt probe and treated with S1 nuclease; a major protected fragment of 600 nt is indicated. The size of this fragment was derived from the positions of pUC8 × *Hin*fI markers (not shown). Autoradiography was performed for 4 weeks at −70°C.

site in the vicinity of nt 1390, which is within the range predicted by the S1 nuclease mapping results. The smaller (<600-nt) fragments do not map to the same position and are presumed to be artifacts arising from preferential S1 nuclease cleavage (12).

Southern blotting. Southern blots of chromosomal DNA digested with *Eco*RI from 17 different species and strains of sulfate-reducing bacteria, including *D. vulgaris* Hildenborough, were hybridized with the inserts from plasmids pRub1 and pRbo1 as probes. The results obtained are shown in Fig. 6A and B, respectively, as well as in Table 2. These results are discussed in more detail below.

DISCUSSION

Homology of the *rbo* gene product with desulforedoxin. Desulforedoxin is a small redox protein, which has been isolated from *D. gigas* but has so far not been found in other sulfate-reducing bacteria. Its polypeptide chain is only 36 amino acids long and has been determined by protein sequencing (9). As discussed by Le Gall et al. (18), the protein is isolated as a dimer (molecular mass, 7.9 kDa; 3.9 kDa per subunit) with two bound Fe atoms as redox centres and has rubredoxinlike spectroscopic properties. The product of the *rbo* gene (378 nt) is much larger than desulforedoxin (126 amino acids; molecular mass, 14 kDa). A comparison of the two amino acid sequences shows that the homology resides entirely at the NH_2 terminus (Fig. 7). Thus, of the first 36 amino acids of the *rbo* gene product, 19 are identical to desulforedoxin, whereas conservative amino acid changes are frequently found in the nonidentical positions. The four cysteine residues (C-9, C-12, C-28, and C-29), which have been proposed to coordinate to the iron (18), are among the conserved amino acid residues. Another interesting region of homology comprises the three glycine residues (G-22, G-23, G-24), which must represent an important flexible (e.g., loop) region of the molecule. The high degree of homology indicates that at its NH_2 terminus, the *rbo* gene product must have a 4-kDa desulforedoxin domain, which has been fused to a larger polypeptide of 10 kDa. This situation is very reminiscent to that in [Fe] hydrogenase of *D. vulgaris* Hildenborough, which is known to have three iron-sulfur clusters as prosthetic groups coordinated by cysteine residues of the large (46-kDa) subunit. The NH_2 terminus of this large subunit is homologous to bacterial 8Fe-8S ferredoxin (38), indicating that two electron-transferring 4Fe-4S clusters are likely to be present in this part of the molecule in a structure resembling that of ferredoxin (1), while a third unique hydrogen-binding cluster could be coordinated by some of the 10 cysteine residues found in the 40-kDa COOH-terminal part of the molecule. It was proposed, therefore, that the hydrogenase gene originated from a

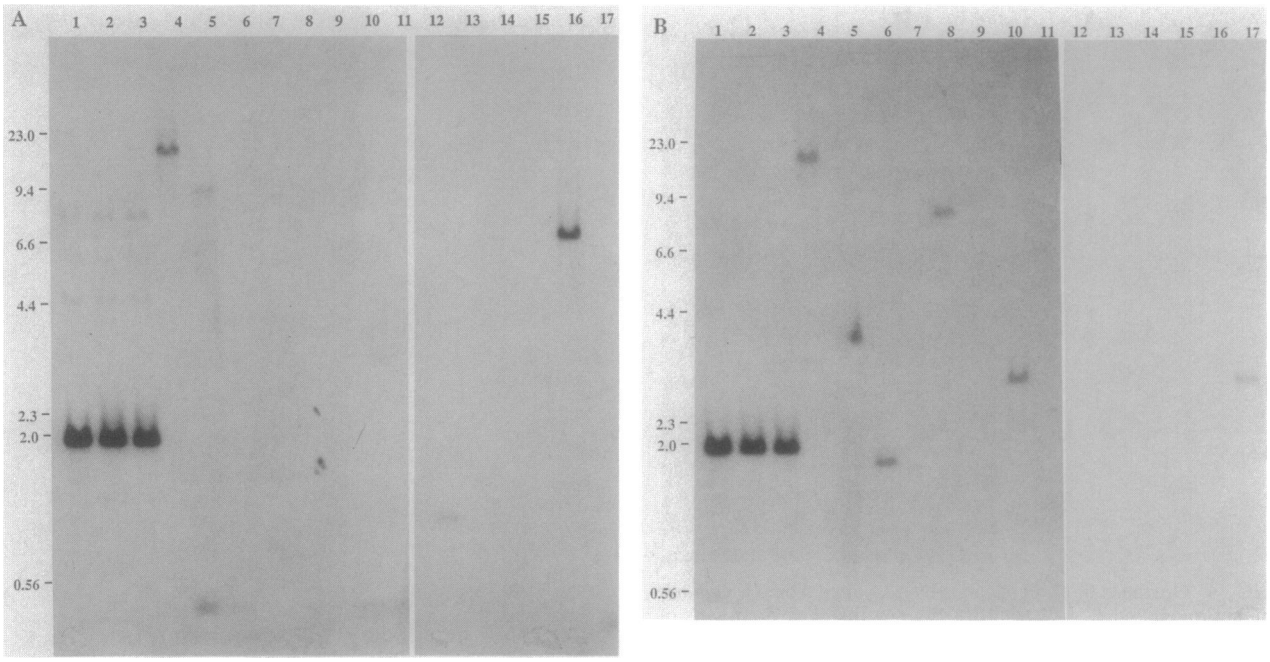

FIG. 6. Southern blotting of *Eco*RI-digested chromosomal DNA (5 to 10 μg) from 17 species and strains of sulfate-reducing bacteria. Lanes: 1, *D. vulgaris* Hildenborough; 2, *D. vulgaris* Wandle; 3, *D. vulgaris* Brockhurst Hill; 4, *D. vulgaris* Groningen; 5, *D. vulgaris* Miyazaki; 6, *D. vulgaris* subsp. *oxamicus* Monticello; 7, *D. desulfuricans* Norway; 8, *D. desulfuricans* Teddington R; 9, *D. desulfuricans* El Agheila Z; 10, *D. desulfuricans* Berre Sol; 11, *D. desulfuricans* Canet 41; 12, *D. gigas*; 13, *D. salexigens* British Guiana; 14, *D. salexigens* California; 15, *D. salexigens*; 16, *D. africanus* Walvis Bay; 17, *D. africanus* Bhengazi. (A) The blots were hybridized with the pRub1 probe (containing part of the rubredoxin gene from *D. vulgaris* Hildenborough) and subjected to autoradiography for 8 days at −70°C. The probe was then dissociated from the blots by washing in 0.5 M NaOH–1.5 M NaCl and neutralized by washing with 0.5 M Tris hydrochloride (pH 7.4)–1.5 M NaCl. Complete dissociation of the pRub1 probe was confirmed by autoradiography. (B) The blots were then hybridized with the pRbo1 probe (containing the gene of a putative rubredoxin oxidoreductase from *D. vulgaris* Hildenborough) and subjected to autoradiography for 2.5 days at −70°C. The positions of molecular size markers (bacteriophage λ DNA digested with *Hin*dIII) are indicated in kilobases.

TABLE 2. Hybridization of *Eco*RI-digested chromosomal DNA from 17 different sulfate-reducing bacteria with an *rub*-specific and an *rbo*-specific probe from *D. vulgaris* Hildenborough

Species	Strain	NCIMB no.	Size of hybridizing fragment (kb) for:	
			rub	*rbo*
D. vulgaris subsp. *vulgaris*	Hildenborough	8303	2.0	2.0
	Wandle	8306	2.0	2.0
	Brockhurst Hill	8306	2.0	2.0
	Groningen	11779	15.4	15.4
	Miyazaki		0.5	3.9
D. vulgaris subsp. *oxamicus*	Monticello 2	9442	0.5	1.8
D. desulfuricans subsp. *desulfuricans*	Norway 4	8310		
	Teddington R[a]	8312		7.8
	El Agheila Z	8318		
	Berre Sol	8388	0.5	2.8
	Canet 41	8393	0.5	
D. gigas		9332	1.2	
D. salexigens	British Guiana	8403		
	California 43:63	8364		
		8365		
D. africanus	Walvis Bay	8397	7.1	
	Bhengazi	8401	0.4	3.0

[a] Reclassified as *D. vulgaris* (28).

fusion of genes encoding an electron-transferring ferredoxin and a larger hydrogen-binding polypeptide (38). In the present case, the desulforedoxin domain coincides precisely with the 36 NH_2-terminal amino acids.

Another example of a redox protein that could have arisen by gene fusion is rubrerythrin, which was recently isolated (19) and shown to contain both rubredoxinlike and hemerythrinlike redox centres. The putative *rbo* gene product is distinct from rubrerythrin in its molecular mass (14 versus 22 kDa) and amino acid composition. Also, preliminary protein sequence data on rubrerythrin (D. M. Kurtz, Jr., and J. Le Gall, personal communication) indicate that this protein does not resemble the *rbo* gene product.

Two possible modes of coordination of the two iron atoms by the eight cysteines of a desulforedoxin dimer have been considered by Le Gall et al. (18): the four cysteines of a single subunit could coordinate to the same iron, in which case dimer formation is achieved by noncovalent interactions of the two subunits, or cysteines from both subunits (e.g., C-9, C-12, and C-28 from one and C-29 from the other) could contribute to the coordination of the two irons, causing a partially covalent connection of the two subunits via the two Fe redox centres. In view of its homology with desulforedoxin, the functional form of the *rbo* gene product is also proposed to be a dimer. Only two additional cysteine residues (C-103 and C-115) are present in the remainder of the sequence of the *rbo* gene product (Fig. 7), which is insufficient for the coordination of a third Fe-containing redox centre (e.g., the coordination of a single Fe, a single

```
                    5                   10                  15
pro-asn-gln-tyr-glu-ile-tyr-lys-cys-ile-his-cys-gly-asn-ile-
ala-asn-glu-gly-asp-val-tyr-lys-cys-glu-leu-cys-gly-gln-val-

                   20                  25                  30
val-glu-val-leu-his-ala-gly-gly-gly-asp-leu-val-cys-cys-gly-
val-lys-val-leu-glu-glu-gly-gly-gly-thr-leu-val-cys-cys-gly-

                   35                  40                  45
glu-pro-met-lys-leu-met-lys-glu-gly-thr-ser-asp-gly-ala-lys-
glu-asp-met-val-lys-gln

                   50                  55                  60
glu-lys-his-val-pro-val-ile-glu-lys-thr-ala-asn-gly-tyr-lys-

                   65                  70                  75
val-thr-val-gly-ser-val-ala-his-pro-met-glu-glu-lys-his-trp

                   80                  85                  90
ile-glu-trp-ile-glu-leu-val-ala-asp-gly-val-ser-tyr-lys-lys-

                   95                 100                 105
phe-leu-lys-pro-gly-asp-ala-pro-glu-ala-glu-phe-cys-ile-lys-

                  110                 115                 120
ala-asp-lys-val-val-ala-arg-glu-tyr-cys-asn-leu-his-gly-his-

                  125
trp-lys-ala-glu-ala
```

FIG. 7. Comparison of the amino acid sequence of the *rbo* gene product (Fig. 3) with that of desulforedoxin (9). Regions of sequence identity are highlighted by boxes.

2Fe-2S cluster, or a single 4Fe-4S cluster requires four cysteines). However, a third redox centre could be present in the 14-kDa *rbo* gene product, if one assumes that the two subunits can donate both cysteine ligands to the same coordination site. The *rbo* gene product appears quite different from the NADH-rubredoxin oxidoreductase purified from *C. acetobutylicum* which was discussed in the Introduction. The subunit molecular mass is different (41 kDa for the *Clostridium* enzyme), and the search of the Bionet data base did not reveal homologies of the *rbo* gene product with flavin-binding proteins.

In analogy with the [Fe] hydrogenase gene, one could suggest that the *rbo* gene has arisen by fusion of the gene for desulforedoxin with that encoding a 10-kDa redox protein containing a single redox centre. The observation that this gene forms a single operon with the gene encoding rubredoxin could mean that the two proteins are redox partners, but definitive proof for this proposal must await the purification and characterization of this novel redox protein.

Distribution of the *rbo* gene in sulfate-reducing bacteria. The *rub* and *rbo* genes are present on a similarly sized *Eco*RI fragment (Fig. 6) and may thus form an operon as in Fig. 3 in *D. vulgaris* Wandle, Brockhurst Hill, and Groningen. This is no surprise for the first two strains, which are practically identical to the Hildenborough strain (39). However, the Groningen strain is quite distinct from Hildenborough and was shown to lack a gene for [Fe] hydrogenase and to have only a low degree of homology in its gene for cytochrome c_3 (39). Four species of sulfate-reducing bacteria have homologous *rub* and *rbo* genes on different restriction fragments (Table 2; Fig. 6). This could indicate a separation of the two genes in these species or the presence of an additional *Eco*RI site in their *rbo-rub* operon. Finally, a homologous *rub* gene in the absence of a detectable, homologous *rbo* gene is found in three, whereas the converse is found in a single one of the species of sulfate-reducing bacteria examined. *D. gigas*, which has a rubredoxin with 71% sequence identity (37 identical residues in a total of 52) with the protein from *D. vulgaris* Hildenborough (36), the pRub1 probe appears sufficiently homologous for the detection of the *rub* gene on a 1.2-kb *Eco*RI fragment (Fig. 6A, lane 12; Table 2). However, the 53% identity of *D. gigas* desulforedoxin with the N terminus of the *D. vulgaris rbo* gene product (Fig. 7; 19 identical residues in a total of 36) appears insufficient for the detection of the desulforedoxin gene in *D. gigas* (Fig. 6B, lane 12). The data in Fig. 6 should therefore not be interpreted in terms of the presence or absence of a gene encoding a desulforedoxinlike polypeptide in species that fail to hybridize with the pRbo1 probe. They indicate that the *rbo* gene is present in nine other species of sulfate-reducing bacteria and is spatially linked to the *rub* gene in at least four species. The *rbo* gene product could thus function as a redox protein in some but not all species of sulfate-reducing bacteria.

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