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Five-Gene Cluster in *Clostridium thermoaceticum* Consisting of Two Divergent Operons Encoding Rubredoxin Oxidoreductase-Rubredoxin and Rubrerythrin–Type A Flavoprotein–High-Molecular-Weight Rubredoxin

AMARESH DAS,[1] ERIC D. COULTER,[2] DONALD M. KURTZ, JR.,[2] AND LARS G. LJUNGDAHL[1]*

Center for Biological Resource Recovery and Department of Biochemistry and Molecular Biology,[1] and Department of Chemistry and Center for Metalloenzyme Studies,[2] University of Georgia, Athens, Georgia 30602

Received 18 August 2000/Accepted 6 December 2000

A five-gene cluster encoding four nonheme iron proteins and a flavoprotein from the thermophilic anaerobic bacterium *Clostridium thermoaceticum* (*Moorella thermoacetica*) was cloned and sequenced. Based on analysis of deduced amino acid sequences, the genes were identified as *rub* (rubredoxin), *rbo* (rubredoxin oxidoreductase), *rbr* (rubrerythrin), *fprA* (type A flavoprotein), and a gene referred to as *hrb* (high-molecular-weight rubredoxin). Northern blot analysis demonstrated that the five-gene cluster is organized as two subclusters, consisting of two divergently transcribed operons, *rbr-fprA-hrb* and *rbo-rub*. The *rbr*, *fprA*, and *rub* genes were expressed in *Escherichia coli*, and their encoded recombinant proteins were purified. The molecular masses, UV-visible absorption spectra, and cofactor contents of the recombinant rubrerythrin, rubredoxin, and type A flavoprotein were similar to those of respective homologs from other microorganisms. Antibodies raised against *Desulfovibrio vulgaris* Rbr reacted with both native and recombinant Rbr from *C. thermoaceticum*, indicating that this protein was expressed in the native organism. Since Rbr and Rbo have been recently implicated in oxidative stress protection in several anaerobic bacteria and archaea, we suggest a similar function of these proteins in oxygen tolerance of *C. thermoaceticum*.

Several studies indicate that anaerobic and microaerophilic bacteria can tolerate varying degrees of O_2 exposure. Superoxide dismutase (SOD) and catalase, which are known to relieve oxidative stress in aerobes, are often absent in anaerobic bacteria. Recently, nonheme iron proteins such as rubrerythrin (Rbr) and rubredoxin oxidoreductase (Rbo) (also known as desulfoferrodoxin) have been implicated in oxidative stress protection in anaerobes (1, 23, 27, 33, 42). So far, Rbr and Rbo or their genes have been found only in anaerobic or microaerophilic bacteria and archaea. The active sites of these proteins include a rubredoxin-type [$Fe(SCys)_4$] center in both Rbo and Rbr (3, 5, 11, 12, 22, 23, 35), a mononuclear [$Fe(NHis)_4SCys$] center in Rbo (6), and a nonsulfur, oxo-bridged di-iron center in Rbr (8, 17, 19, 23, 34). The latter two sites in their reduced forms react rapidly with superoxide in the case of Rbo (27) and with hydrogen peroxide in the case of Rbr (7, 8).

Clostridium thermoaceticum is a thermophilic gram-positive, obligately anaerobic bacterium that produces acetate from virtually any carbon source, including sugars, aromatic compounds, and C_1 compounds (25, 36, 44). It is not known how this bacterium responds to oxygen toxicity during growth. Determinations of catalase and SOD activities in *C. thermoaceticum* have been inconclusive. In this study we show that *C. thermoaceticum* contains genes encoding Rbo and Rbr and that these two genes are present in a cluster with three additional genes encoding a rubredoxin (Rub), a high-molecular-weight rubredoxin (Hrb) and a type A flavoprotein (FprA). All these genes have been expressed in *Escherichia coli*, and recombinant Rbr, Rub, and FprA were purified and partially characterized. Except for *rub*, none of these genes have previously been reported to be present in any acetogenic bacterium.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, A214 Life Sciences Building, The University of Georgia, Athens, GA 30602-7229. Phone: (706) 542-7640. Fax: (706) 542-2222. E-mail: larsljd@arches.uga.edu.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *C. thermoaceticum* was grown at 58°C in the presence of glucose (1%) under 100% CO_2, as previously described (26). All *E. coli* strains were routinely grown at 37°C in Luria-Bertani medium. All plasmids used in this study (Table 1) carry ampicillin resistance genes and were maintained in *E. coli* hosts in the presence of 100 μg of ampicillin per ml.

DNA and RNA sources and purification. Chromosomal DNA of *C. thermoaceticum* was isolated and purified as described previously (31). Plasmid DNA was isolated and purified using a QIAprep Spin Miniprep Kit from Qiagen Inc., Studio City, Calif. Lambda DNA was purified using the Wizard Lambda Preps DNA purification system from Promega, Madison, Wis. Total RNA was isolated from *C. thermoaceticum* harvested at exponential growth phase and purified using a RNeasy mini kit from Qiagen. Routine DNA manipulations were performed as described by Sambrook et al. (39).

DNA fragments to be cloned into plasmids were purified from 1% agarose gels after briefly staining with ethidium bromide using the QIAquick gel extraction kit from Qiagen. Plasmids used in cloning reactions were digested with the desired restriction enzymes and similarly gel purified. Purified, linearized plasmids were dephosphorylated by treatment with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, Id.) prior to ligation with target DNA fragments. The DNA ligation reactions were carried out using T4 DNA ligase from New England Biolabs (Beverly, Mass.) using the conditions outlined by the manufacturer. Synthesis of oligonucleotides used in PCR and in DNA sequencing experiments was carried out at the Molecular Genetics and Instrumentation Facility of the University of Georgia.

Cloning and sequencing strategy. Initially, we targeted to sequence the Rub gene. Two highly conserved regions of the amino acid sequences of Rub proteins

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Genotype or description	Source
Bacterial strains		
C. thermoaceticum (*Moorella thermoacetica*) ATCC 39073	Wild type	
E. coli		
XL1-Blue MRA	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 gyrA96 relA1 lac*	Stratagene
XL1-Blue MRA(P2)	XL1-Blue MRA (P2 lysogen)	
BL21(DE3)	*E. coli* B; F^- *ompT* r_B^- m_B^- (λDE3)	Novagen
INVαF′	F′ *endA*1 rec*A*1 *hsdR17* (r_K^- m_K^+) *supE44 thi*-1 *gyrA96 relA1* ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 λ^-	
Plasmids		
pCRII	Ap^r Km^r; vector for cloning PCR product	Invitrogen
pBluescript SK(+)	Ap^r, ColE1 origin, cloning vector	Stratagene
pET-21b(+)	Ap^r, T7 promoter expression vector	Novagen
pCRRub	pCRII derivative containing 96-bp PCR amplified Rub probe	This study
pRb5	pBluescript derivative containing *rbr, rbo, rrb*, and a part of *fprA* in a 3.0-kb *Pst*I fragment	This study
pRb48	pBluescript derivative containing *fprA, rbr, rbo, rub*, and a part of *hrb* in a 4.8-kb *Kpn*I fragment	This study
pRbo/Rub	pET-21(b+) derivative containing *rbo* and *rub*	This study
pFprA	pET-21(b+) derivative containing *fprA*	This study
pHrb	pET-21(b+) derivative containing *hrb*	This study

from different origins were used to design primers for PCR. The forward primer 5′-GTITG(TC)GG(TC)TA(TC)AT(TC)TA(TC)(AG)A(TC)C-3′ was designed from a conserved amino acid sequence, VCGYIYN/D, at the N-terminal ends of Rub proteins and the reverse primer 5′-G(AG)CAIACCCA(AG)TC(AG)T C(GC)G-3′ was designed from a conserved amino acid sequence, PCVWDDP, at the C-terminal end (29). The PCR was carried out with these primers using *C. thermoaceticum* genomic DNA as a template for 25 cycles under the following conditions for each cycle: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 1 min. A 96-bp PCR product was amplified. It was sequenced after cloning into the pCR 2.1 vector (Invitrogen, Carlsbad, Calif.). The deduced amino acid sequence of the PCR product was found to be highly homologous with those of Rub proteins from different sources (not shown). The PCR product was labeled with digoxigenin (DIG)–11-dUTP (Roche Molecular Biochemicals) and used as a probe to screen a genomic library of *C. thermoaceticum* constructed in λFIX II by Stratagene (La Jolla, Calif.) according to a method described previously (9). A Rub-positive clone designated λRd 2 was purified from the library, and its DNA was analyzed by Southern hybridization using the same 96-bp DIG-labeled PCR product as a probe. A 3.0-kb *Pst*I fragment and a 4.8-kb *Kpn*I fragment from λRd 2 were found to hybridize to the Rub probe. These two fragments were purified and cloned into pBluescript (Stratagene), and constructs designated pRb 5 (3.0-kb *Pst*I insert) and pRb 48 (4.8-kb *Kpn*I insert) were obtained (Table 1). The two constructs (pRb 5 and pRb 48) have the 3.0-kb *Pst*I fragment (see above) in common. The nucleotide sequences reported in this study were derived from these two clones, except for some sequences (described below) which were obtained directly from λRd 2.

Hybridization techniques. The genomic library of *C. thermoaceticum* in λFIX II was screened with the Rub probe (see above) by plaque hybridization experiments using the Genius system from Roche Molecular Biochemicals; Southern and Northern hybridization experiments were also carried out using the same Genius system as previously described (9). Individual genes were amplified by PCR with *C. thermoaceticum* genomic DNA as a template in the presence of DIG–11-dUTP and used as probes in Northern hybridization experiments.

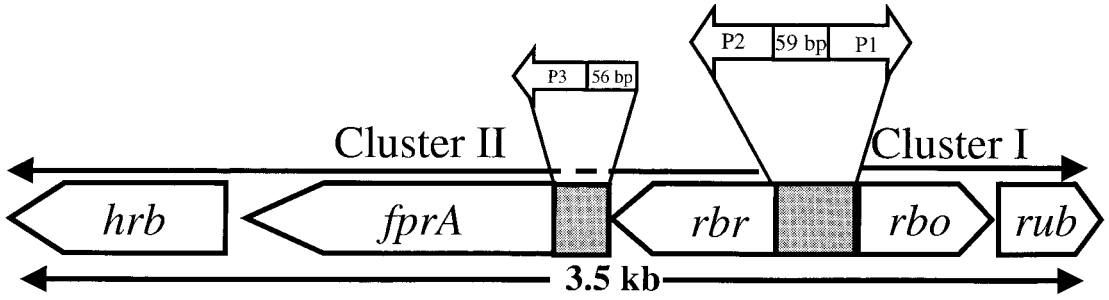
Heterologous expression of *rbr, fprA, rbo, rub*, and *hrb* in *E. coli*. The plasmid constructs used for the expression of the genes are listed in Table 1. Except for *rbr*, the genes were cloned into pET-21b (Novagen, Inc., Madison, Wis.) and expressed in *E. coli*. The *rbr* gene was expressed directly from its clone pRb5, which is a derivative of pBluescript. For cloning into pET-21b, the genes were amplified by PCR from *C. thermoaceticum* genomic DNA using specific primers. The primers were designed to have unique restriction sites at their 5′ ends, *Nde*I for forward primers and *Eco*RI for reverse primers. The PCR products were purified using a QIAquick PCR purification kit from Qiagen, digested with *Nde*I and *Eco*RI, and ligated into the corresponding restriction sites of pET-21b. *E. coli* strain BL21(DE3) was used for expression of *rbo, rub, fprA*, and *hrb*, and *E. coli* DH5α was used for expression of *rbr*. *E. coli* strains carrying recombinant plasmids were grown in 1- or 2-liter volumes of either Luria-Bertani medium or MZ9 salt medium (pET manual, Novagen). The MZ9 salt medium was supplemented with 20 mM ferrous sulfate for the expression of recombinant Rbr, Rbo, and Rub. After expression, recombinant Rbo (CthRbo) and Hrb (CthHrb) formed inclusion bodies, while recombinant Rbr (CthRbr), Rub (CthRub), and FprA (CthFprA) remained soluble upon cell lysis. The purification steps for the recombinant proteins are described below.

Purification of recombinant CthRbr, CthRub, and CthFprA. CthRbr was expressed without any inducer, while CthFprA and CthRub were expressed following induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The IPTG was added after the optical density at 600 nm of the cultures reached 0.6. Optimum expression of recombinant proteins occurred after 4 h of IPTG induction. After being harvested by centrifugation, cells were washed, resuspended in buffer A (20 mM Tris-HCl, pH 7.5) (1 g of cell paste in 3 ml of buffer), and sonicated. Crude cell lysates were centrifuged (100,000 × *g* for 1 h), and the supernatants were collected for purification of recombinant proteins.

For purification of CthRbr, supernatants were subjected to heat treatment at 65°C for 30 min. Most *E. coli* proteins precipitated in this step were removed by centrifugation at 25,000 × *g*. Crystalline ammonium sulfate was added to the clear light-red supernatant to 60% of saturation at room temperature. Proteins precipitated at this step were discarded, and ammonium sulfate was added to the supernatant to obtain 80% of saturation. Proteins, including CthRbr, precipitated at this step were collected by centrifugation and dissolved in 2 ml of buffer A. The CthRbr was purified from this suspension by repeated gel filtration on a TSK gel G 3000 SW column (Tosahaas) using the fast protein liquid chromatography system of Amersham-Pharmacia, Piscataway, N.J. The elution buffer was buffer A plus 0.1 M NaCl. Colored fractions from each gel filtration step were analyzed for the presence of Rbr by UV-visible spectroscopy. The characteristic spectra of oxidized Rbr proteins include peaks at 280, 370, and 492 nms. Fractions of Rbr collected after the second gel filtration were found to be pure based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recombinant CthRub and CthFprA were purified from the supernatants using anion-exchange chromatography on DEAE–Sepharose CL 6B (Pharmacia) followed by size exclusion chromatography on a TSK gel G 3000 SW column (Tosahaas). In the first step, supernatants containing soluble recombinant proteins were passed through a DEAE–Sepharose CL 6B column (300 by 25 cm) preequilibrated with buffer A. Proteins bound to the column were eluted by a salt gradient of 0 to 1 M NaCl in buffer A. Light-colored fractions (red for CthRub and yellow for CthFprA) eluted from the columns were analyzed for the presence of recombinant proteins by recording their UV-visible absorption spectra. Characteristic spectra for oxidized CthRub include peaks at 280, 492, and 380 nm, and those for CthFprA include peaks at 280, 350, and 450 nm. Fractions having these spectral properties were pooled, concentrated by Amicon ultrafiltrations, and applied to a TSK 3000 gel filtration column preequilibrated with buffer A plus 0.1 M NaCl. Fractions containing CthRub and CthFprA collected from the latter column were found to be more than 90% pure based on SDS-PAGE.

Spectral analysis and analytical methods. UV-visible absorption spectra of the recombinant proteins were obtained on a Shimadzu model UV 2100PC spectrophotometer. The electron paramagnetic resonance (EPR) spectra of recombinant proteins were obtained on a Bruker ESP-300E spectrometer as described previously (17). The molecular masses of the recombinant proteins were determined by gel filtration on Superose 12 column using the fast protein liquid



Size (nucleotides)	687	1197	573	375	159
Residues	229	399	191	125	53

FIG. 1. Organization of the five ORFs identified as *rbr, fprA, hrb, rbo*, and *rub* and of the putative promoters P1, P2, and P3, and relationship between the genes and their products.

chromatography system of Amersham-Pharmacia. The molecular mass standards used were chymotrypsin (25 kDa), egg albumin (45 kDa), and bovine serum albumin (68 kDa). Protein concentrations were determined by the Lowry method, as described previously (9). *Desulfovibrio vulgaris* Rbr (17) and Rub (4) were used as protein standards for quantitation of CthRbr and CthRub, respectively. The concentrations of these protein standards were determined using their well-established extinction coefficients at 492 nm, i.e., 5,400 M^{-1} cm^{-1} for *D. vulgaris* Rbr monomer (17) and 8,700 M^{-1} cm^{-1} for *D. vulgaris* rubredoxin (4). SDS-PAGE was carried out by the method of Laemmli (20) using 12% acrylamide in resolving gels and 4% acrylamide in stacking gels. Western blotting experiments were carried out according to Bio-Rad (Hercules, Calif.). Antibodies against *D. vulgaris* Rbr (17) were raised in rabbits at the Animal Care and Use Facility of the University of Georgia.

Identification of the flavin cofactor of recombinant CthFprA was carried out as follow. Recombinant CthFprA (25 mg per ml) was heated at 100°C for 20 min and then centrifuged at 14,000 × *g* to remove precipitated proteins. Four microliters of the supernatant was subjected to thin-layer chromatography on silica gel-coated glass plates along with flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Sigma, St. Louis, Mo.) as standards as described by Fetzner et al. (14). The molar content of FMN was determined spectrophotometrically using ε_{450} = 12,200 M^{-1} cm^{-1} (2).

For reconstitution, recombinant CthFprA (25 mg/ml) in a 100-μl reaction volume was treated with a 10-molar excess of FMN (Sigma) for 30 min at room temperature. The sample was diluted to 2 ml with 50 mM MOPS (morpholinepropanesulfonic acid) (pH 7.0) and concentrated to ~100 μl using an Ultrafree-15 protein concentrator from Fisher (Pittsburgh, Pa.). This dilution and reconcentration were repeated until the flowthrough contained no detectable FMN. The FMN content of reconstituted CthFprA was determined as described above.

Metal analysis of the recombinant proteins was carried out by inductively coupled plasma atomic emission at the Chemical Analysis Laboratory of the University of Georgia.

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been has been deposited at the GenBank, EMBL, and DDJB libraries with accession number AF202316.

RESULTS

Cloning, sequencing, and identification of the genes. Details of the cloning and sequencing strategy are described in Materials and Methods. The *rub* gene was sequenced from pRb5 carrying a 3.0-kb *Pst*I fragment isolated from a *rub*-positive lambda clone, λRd2. The latter clone was isolated from a *C. thermoaceticum* genomic library in λFIX II (9) after screening with a *rub* probe. Extended sequencing from both ends of *rub*, including the sequences of the 3.0-kb *Pst*I insert in pRb5 and the 4.8-kb *Kpn*I insert in pRb48, revealed the presence of four additional open reading frames (ORFs) at the 5′ end of the *rub* gene. Analysis of the deduced amino acid sequences of the five ORFs by FASTA identified them as homologs of rubredoxin (Rub), rubredoxin oxidoreductase (Rbo), rubrerythrin (Rbr), type A flavoprotein (FprA), and a protein with a C-terminal rubredoxin sequence motif which we referred to as high-molecular-weight rubredoxin (Hrb). The organization of the genes and the relationships between the genes and their products are summarized in Fig. 1. Except for Hrb, the predicted molar masses of the deduced proteins are in close agreement with those of the homologous proteins from other sources. The *C. thermoaceticum* FprA was identified by its sequence homology (between 28 and 48%) to FprA proteins from other microorganisms. The *C. thermoaceticum* Rub, Rbo, and Rbr were readily identified by their sequence identities (between 29 and 70%) to their homologs in other microorganisms and by their characteristic iron binding sequence motifs. Those motifs are $CX_2CX_{29}CX_2C$ for the single iron site in Rub, $EX_{30-34}EX_2HX_nEX_{30-34}EX_2H$ and $CX_2CX_{12}CX_2C$ for the diiron and rubredoxin sites, respectively, in Rbr, and $CX_2CX_{15}CC$ and $HX_{15}HX_9HX_{40}CX_2H$ for the $[Fe(SCys)_4]$ and $[Fe(NHis)_4SCys]$ centers, respectively, in Rbo (not shown). The deduced amino acid sequences of CthRub (70% identity), CthRbr (66% identity), and CthRbo (63% identity) are more homologous with the amino acid sequences of the corresponding proteins from *D. vulgaris* than with those from other sources.

C. thermoaceticum contains two rubredoxins, designated Rd I and Rd II (46). Rd I had a molar mass of 7.4 kDa, and Rd II had a molar mass of 6 kDa. Analysis of the amino acid compositions of the two rubredoxins shows the presence of six cysteine residues in Rd I and four cysteine residues in Rd II. The presence of four cysteine residues and the predicted molar mass of the protein encoded by *C. thermoaceticum rub* (5,745 Da [see Table 2]) are both in close agreement with the corresponding values for Rd II. Therefore, the protein encoded by *rub* is presumed to be Rd II. However, the deduced amino acid sequence of the 96-bp PCR product used as a Rub probe in this study does not match the corresponding amino acid sequence deduced from either *rub* or *hrb* (Fig. 2), indicating that

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Rub-probe    1  VCGYIYDPAK GDPEGGIAPG TAFEDLPDDW VC    32
CthRub       8  VCDYVYDPAE GDPDNGIAPG TPFADLPEDW VC    40
CthHrb     185  ICNYVYDPVQ GDPEHGIAPG TPFADLPEDW TC   217
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FIG. 2. Alignment of the deduced amino acid sequence of the 96-bp PCR-amplified *rub* probe with the corresponding sequences deduced from *rub* (CthRub) and *hrb* (CthHrb).

the *rub* probe belongs to another *rub* gene, which may encode Rd I.

No protein that is homologous to full-length CthHrb was found in the database. The carboxyl-terminal sequence of Hrb is homologous to those of rubredoxins (Fig. 3). The amino-terminal sequence of Hrb is homologous (28 to 44% sequence identities) to several proteins, including nitrilotriacetate monooxygenase component B from *Chelatobacter heinzii* (45), actinorhodin polyketide dimerase-related proteins from *Streptomyces coelicolor* (13) and *Thermotoga maritima* (accession no. C72410), a probable monooxygenase designated as b1007 from *E. coli* (accession no. E64842), a probable FMN:NADH oxidoreductase from *Streptomyces violaceoruber* (accession no. T46545), and a 63.5-kDa FprA (accession no. S75748) COOH-terminal sequence of *Synechocystis* sp. strain PCC 6803. The proteins listed above are also homologous among themselves (30 to 38% identical residues). The component B of nitrilotriacetate monooxygenase from *C. heinzii* was shown to have NADH:FMN oxidoreductase activity (45).

All of the newly identified genes of *C. thermoaceticum* were found to have an AUG start codon except the gene encoding Hrb, which has a UUG start codon. The five genes were organized in two divergently oriented subclusters separated by an AT-rich region, presumably containing regulatory sequences (Fig. 1). Subcluster I consists of *rbo-rub* (5′→3′), and subcluster II consists of *rbr-fprA-hrb* (5′→3′).

Northern blot analysis and regulatory sequences. Northern blot hybridization experiments on total RNA isolated from *C. thermoaceticum* confirmed the two predicted polycistronic operons within the five-gene cluster. The DIG-labeled DNA probes of *rbr, fprA*, and *hrb* were each found to hybridize to a 2.8-kb transcript (Fig. 4). Therefore, these three genes must be co-transcribed from a promoter located upstream of *rbr*. Similarly, the DIG-labeled DNA probes for *rbo* and *rub* each hybridized to a 1.5-kb transcript (Fig. 4), indicating an *rbo-rub* operon with a promoter apparently located upstream of *rbo*. We ruled out the possibility of any secondary promoter upstream of *rub* due to a very short intergenic region (20 bp) between the two genes. The 1.5-kb transcript hybridizing to *rbo* and *rub* is much larger than the total size of *rbo* and *rub* plus their intergenic region, 550 bp. No ORF or transcription terminator was apparent within the 300-bp sequence downstream of *rub*. In order to verify the cotranscription of *rbo* and *rub*, Northern hybridization experiments were carried out on total RNA isolated from *E. coli* BL21(DE3) harboring pRbo/Rub. The DIG-labeled *rbo* probe hybridized to a 0.6-kb transcript (Fig. 4), a size which is in close agreement with the expected size of the *rbo-rub* operon (550 bp). A similar size (~0.6 kb) of transcript was also found to hybridize DIG-labeled *rub* probe when a replicate RNA blot from this clone was used (not shown). The cotranscription of *rbo* and *rub* was further supported by their coexpression from pRbo/Rub in *E. coli* (Fig. 5).

Putative promoter sequences. Consistent with the Northern blot results, putative promoter sequences, P1 and P2, occur upstream of *rbo* and *rbr*, respectively, as diagramed in Fig. 1. The nucleotide sequences of P1 (5′-ATGACG-N_{15}—TAATA AT-N_{12}-AGGAG-3′) and P2 (5′-TTGACT-N_{17}—TACAAT–N_{21}-AGGAG-3′) are homologous to that of the *E. coli* consensus σ^{70} promoter (cTTGACa-N_{15-21}-TATAaT-N_x-AGG AG) (18), as we have shown previously for promoters of several *C. thermoaceticum* genes (30). A third putative promoter, P3 (5′-TTGATA-N_{21}—TATAAT–N_{32}-GGAGG-3′), was also found within the 79-bp *rbr-fprA* intergenic region, but no promoter-like sequence was found in the 133-bp *fprA-hrb* intergenic region. The presence of P3 upstream of *fprA* suggests that *fprA* might be subjected to secondary regulation of its own.

Expression of the *C. thermoaceticum* genes in *E. coli* and properties of the recombinant proteins. *C. thermoaceticum* Rbo and Rub were expressed in *E. coli* from the same plasmid, pRbo/Rub, while *C. thermoaceticum* Rbr, FprA, and Hrb were expressed from pRb5, pFprA, and pHrb, respectively (Table 1). The recombinant Rbo and Hrb formed inclusion bodies and were not further purified. Figure 5 shows SDS-PAGE of crude preparations of recombinant Rbo and Rub and of the purified recombinant Rbr, FprA, and Rub, all expressed in *E. coli*. Table 2 summarizes the properties of the recombinant proteins. The molar masses of the recombinant Rub and FprA were estimated by gel filtration as 6 and 90 kDa, respectively, indicating that, under nondenaturing conditions, Rub is present as a monomer and FprA is present as a dimer. Purification of Rbr by gel filtration yielded two major fractions with molar masses of about 66 and 44 kDa, respectively. On SDS-polyacrylamide gels, the two fractions ran as a single band both with an approximate molar mass of 22 kDa, which is in close agreement with the predicted molar mass of 21,348 Da calculated from the deduced amino acid sequence. The two fractions gave identical UV-visible absorption spectra (Fig. 6), indicating that they are the same protein. These results suggest that in solutions recombinant CthRbr is present as a mixture of dimers and trimers in a molar ratio of ~2:1.

Metal composition of recombinant *C. thermoaceticum* Rbr and Rub. The metal analyses of recombinant Rbr and Rub indicate the presence of iron and zinc in significant amounts in both proteins (Table 2). A mixture of iron and zinc forms is typically obtained when rubredoxins are overexpressed in *E. coli* (4). The iron-plus-zinc contents indicate that the single metal site in CthRub is occupied predominantly (~70%) by iron, with the remainder occupied by Zn^{2+}. The UV-visible

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LDTKALHTLT YGLYIITAKK GDRFNGQVAN TVFQITSDPP TIAVSINKQN 50

LTHEFIQAGQ GFVISVLARE VPLSLIGQFG FKSGREMDKF AGINYKLSEG 100

GLPYLADHTL AYLEASLNQT VDAGTHSIFI GTVTDAAVLL QGEPMTYAYY 150

HQVKRGTTPK TAPTFTVGRE KDKTALASPK YQCTICNYVY DPVQGDPEHG 200
                                  * *
IAPGTPFADL PEDWTCPICG AGKDAFEQI                        229
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FIG. 3. Deduced amino acid sequence of high-molecular-weight rubredoxin (Hrb). The rubredoxin domain of the protein (in boldface) and the cysteine ligands for the rubredoxin iron center (designated by asterisks) are marked as shown.

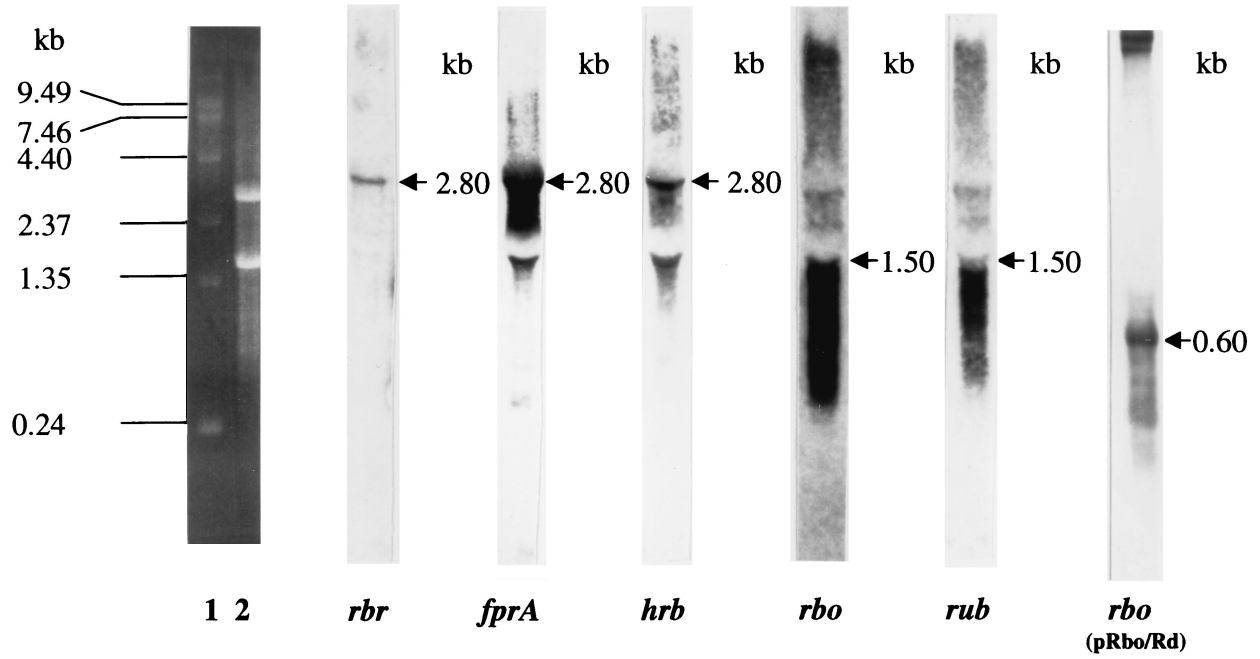

FIG. 4. Northern blots of total RNAs isolated from *C. thermoaceticum* and *E. coli* BL21(DE3)(pRbo/Rub) (far right lane) after hybridization with DIG-labeled *rub, rbo, rbr, fprA*, and *hrb*, as indicated below each lane. Lanes 1 and 2, positions of RNA markers and ribosomal RNA, respectively, in the ethidium bromide-stained gel.

absorption spectra of the recombinant CthRub and CthRbr are shown in Fig. 6, and the absorption maxima are listed in Table 2. The absorption features are typical of the respective homologous proteins from other species (17, 22, 27, 29, 42) and reflect their Fe^{3+} contents, i.e., any Zn^{2+}-occupied sites would have no optical absorption (4). The 492-nm absorption feature in the spectrum of Rbr is due almost entirely to the oxidized rubredoxin-type iron site (17). Comparing the ε_{492} of 10,800 M^{-1} cm^{-1} for the *D. vulgaris* Rbr homodimer to the ε_{492} of 3,200 M^{-1} cm^{-1} determined for CthRbr (using *D. vulgaris* Rbr as the protein standard), it was estimated that approximately 30% (i.e., 3,200/10,800) of the CthRbr rubredoxin sites contain iron. Since the metal analyses indicated that zinc was the only other heavy metal present in significant amounts (Table 2), it is assumed that the remaining 70% of CthRbr rubredoxin sites are occupied by Zn^{2+}. This interpretation is consistent with the higher-than-expected A_{280}/A_{492} absorbance ratio for CthRbr (~10) compared to that for *D. vulgaris* Rbr (~5.5), in which all rubredoxin-type sites are occupied by iron (17). The Rbr di-iron(III) site absorbs most intensely between 300 and 400 nm, but this feature is obscured by overlapping and more intense absorption from the rubredoxin-type site (17). Therefore, optical absorption cannot be used to quantitate iron occupancy of the Rbr di-iron sites. However, the estimated 70% Zn^{2+} oc-

TABLE 2. Properties of purified recombinant Rbr, Rub, and FprA from *C. thermoaceticum*

Parameter	Value for:		
	Rbr	FprA	Rub
Molecular mass (Da)			
Deduced	21,329	44,296	5,745
Native	44,000, 66,000[a]	90,000	6,000
Denatured	22,000	44,000	6,000
Content/mol			
Fe	3–4/dimer	~0.2/monomer	0.7/monomer
Zn	1–2/dimer	~0.1/monomer	0.2/monomer
FMN		0.5/monomer (reconstituted)	
UV-visible absorption maxima (nm)	280, 372, 492	280, 350, 456	280, 378, 492

[a] Two oligomers of Rbr were detected (see text).

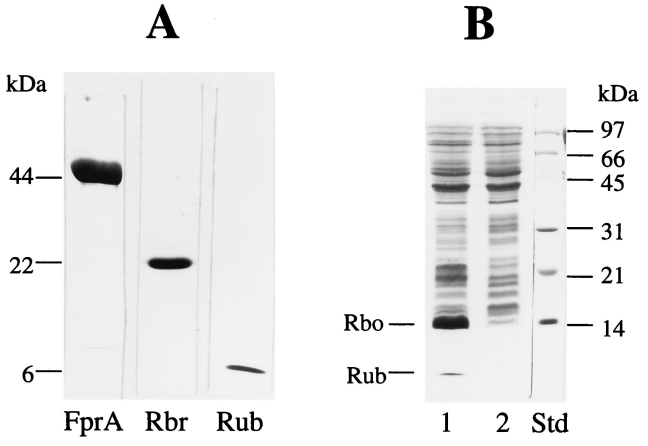

FIG. 5. (A) SDS-PAGE of the purified recombinant FprA (10 μg), Rbr (10 μg), and Rub (10 μg) expressed in *E. coli*. (B) Lane 1, SDS-PAGE of the extracts (40 μg) of *E. coli* harboring pRbo/Rub grown in MZ9 salt medium after induction with IPTG; lane 2, same as in lane 1 but without IPTG induction. The protein standards (std) and relative positions of the recombinant proteins are shown.

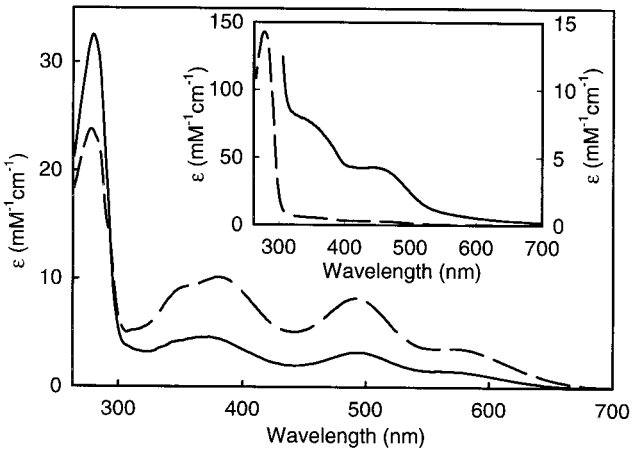

FIG. 6. UV-visible absorption spectra of as-isolated recombinant CthRub (57 μM monomer) (dashed line) and CthRbr (108 μM monomer) (solid line). Inset, UV-visible absorption spectra of as-isolated CthFprA (22 μM monomer [dashed line] and 206 μM monomer [solid line]). All spectra were recorded at room temperature in 50 mM MOPS, pH 7.0.

cupancy of the rubredoxin-type sites, together with the metal analysis of purified CthRbr, namely, 0.5 to 1 Zn atom per monomer and 1.5 to 2 iron atoms per monomer (Table 2), implies that most of the di-iron sites must be occupied by iron and not zinc. The recombinant CthRbr shows an oxidized rubredoxin-type EPR signal and a relatively weak, mixed-valent di-iron EPR signal (not shown), both of which closely resemble the corresponding signals in *D. vulgaris* Rbr and provide additional evidence for the metal site occupancies (17).

Cofactor content of recombinant *C. thermoaceticum* FprA. Thin-layer chromatography identified FMN and not FAD as a cofactor in the recombinant CthFprA. The FMN/FprA monomer molar ratio was quantitated as 0.2 in as-purified recombinant CthFprA and as 0.54 in the FMN-treated CthFprA (Table 2). No detectable FAD was bound to FAD-treated CthFprA. The A_{280}/A_{450} absorbance ratio of the as-purified CthFprA (Fig. 6, inset) is consistent with the substoichiometric FMN. Substoichiometric but significant levels of iron and zinc were also detected in the recombinant FprA (Table 2).

Antigenic relationship between Rbr proteins from *C. thermoaceticum* and *D. vulgaris*. Figure 7 shows that antibodies against *D. vulgaris* Rbr (17) reacted with both the recombinant CthRbr (dimer) and a protein present in crude cell extracts of *C. thermoaceticum* of the size expected for CthRbr. Both the dimer and trimer of recombinant CthRbr reacted similarly with the antibodies against *D. vulgaris* Rbr (not shown).

DISCUSSION

We have characterized a unique five-gene cluster consisting of two divergently transcribed operons, namely, *rbo-rub* and *rbr-fprA-hrb*. Anti-*D. vulgaris* Rbr antibodies reacted strongly with CthRbr, and this cross-reaction confirmed that the *C. thermoaceticum rbr* gene is expressed in the native organism. In other air-sensitive bacteria and archaea, Rub, Rbr, Rbo, and FprA have all been implicated in oxidative stress protection, and their genes often occur in tandem pairs (15, 28). However, these four genes have not previously been found to occur within the same cluster. The results described in this paper provide the first evidence for the presence of Rbr, Rbo, and FprA in any acetogenic bacterium. The *C. thermoaceticum* Hrb appears to be a unique protein with at least two domains. The sequence homologies of the C-terminal end to rubredoxins suggests a redox-linked function for Hrb. Since we were unable to express a recombinant Hrb in soluble form, we could not further characterize its properties.

The spectroscopic and physical properties of recombinant *C. thermoaceticum* Rub and Rbr expressed in *E. coli* were found to be very similar to those of the corresponding proteins from other sources (17, 22, 23, 32, 43). The metal analysis and spectroscopic properties indicate that the purified recombinant CthRbr contains significant amounts of both zinc and iron, with the majority of the zinc being in the rubredoxin-type site. Zinc can be incorporated into both the rubredoxin-type and di-iron sites of *D. vulgaris* Rbr (8, 41). However, Rbr and a closely related protein, nigerythrin, as isolated from *D. vulgaris*, each contain predominantly iron in both types of sites (22, 34).

The recombinant *C. thermoaceticum* FprA, when overexpressed in *E. coli*, contained substoichiometric amounts of FMN, iron, and zinc. In *Methanobacterium thermoautotrophicum* the function of FprA (designated FpaA) has been proposed to be an intermediate electron carrier between H_2 and CO_2 during methanogenesis (32). The *M. thermoautotrophicum fpaA* gene occurs in a cluster with two additional genes, organized in the order *fpaA-orfX-rdxA* (32). The deduced protein encoded by *orfX* has a 60-residue region that contains the di-iron site sequence motif found in Rbr, and the deduced protein encoded by *rdxA* contains a rubredoxin-type $CX_2CX_nCX_2C$ sequence motif. Thus, the *M. thermoautotrophicum fpaA-orfX-rdxA* gene cluster bears some resemblance to the *C. thermoaceticum rbr-fprA-hrb* operon. The FprA proteins have been previously reported for only two other bacterial species, *E. coli* and *Desulfovibrio gigas* (43). The 479-residue

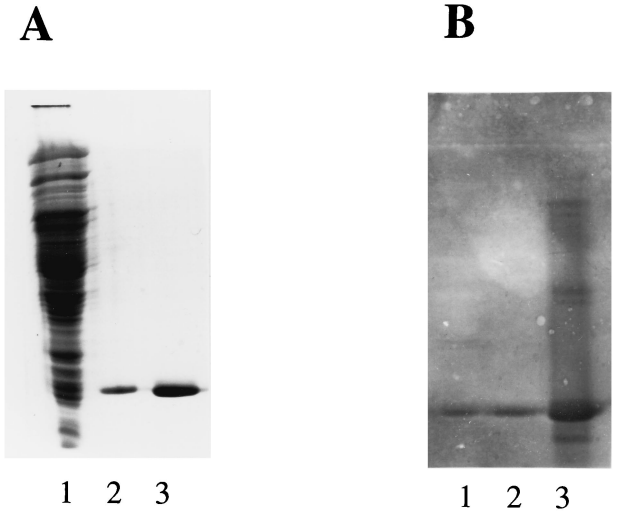

FIG. 7. Western blots showing interactions between recombinant CthRbr and antibodies against *D. vulgaris* Rbr. (A) SDS-PAGE of extracts of *C. thermoacetcicum* (lane 1) (50 μg) and of recombinant CthRbr (lane 2) (0 μg) and recombinant *D. vulgaris* Rbr (lane 3) (8 μg). (B) Western blot of a replica of the SDS gel shown in panel A after reaction with antibodies against *D. vulgaris* Rbr.

FprA from *E. coli* is sequentially homologous to archaeal FprA proteins but contains in addition a rubredoxin domain at the C-terminal end. In addition to flavin, the *D. gigas* FprA was reported to contain a di-iron site and to function as a rubredoxin:oxygen oxidoreductase (15, 16). The di-iron site ligands identified in the *D. gigas* FprA homolog are conserved in *C. thermoaceticum* FprA. The presence of a putative promoter structure, P3 upstream of *C. thermoaceticum fprA* (Fig. 2) suggests that its expression could be regulated independently from that of of *rbr*. Independent regulation of flavoproteins and a reverse relationship between the expression of flavoproteins and iron proteins (namely ferredoxins) were previously reported for methanogens (32) and acetogens (37). In any case, our results suggest that the expressions of Rbr, Rbo, and FprA are coregulated, and their homologies to known proteins suggest a cooperative role in oxidative stress protection.

The widespread occurrence of Rub in anaerobes is already well established, and while it is presumed to function as an intermediary electron carrier in various enzymatic reactions (21, 40, 47), its exact role(s) in anaerobes has never been established. In acetogens Rub has been proposed as an electron acceptor in the carbon monoxide dehydrogenase reaction (38) and as a terminal electron acceptor to the membrane electron transport chain (10, 24). Rub has been proposed to be a redox partner to *D. gigas* FprA, mostly on the basis that its genes are cotranscribed (15). However, the demonstration that *rbo* and *rub* genes are cotranscribed in *D. vulgaris* (4) and now in *C. thermoaceticum* (this study) suggests a functional relationship between Rbo and Rub.

Both Rbo and Rbr have been reported to restore aerobic growth to *sod* mutant strains of *E. coli* (1, 23, 27, 33). Furthermore, deletion of *rbo* led to increased dioxygen and superoxide sensitivities of *D. vulgaris* (28, 42). Evidence for superoxide reductase and NADH peroxidase activities for Rbo and Rbr, respectively, in vitro has been presented (7, 8, 27). Recently it was shown that *C. thermoaceticum*, which lacks any detectable catalase or SOD activities, could grow in the presence of trace amounts of O_2 in liquid media without any reducing agent (A. Karnholz, K. Küsel, and M. Drake, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. I-91, p. 401, 2000). This oxygen tolerance of *C. thermoaceticum* could result from expression of a novel five-gene cluster consisting of two divergently transcribed but coregulated operons, one containing Rbo and the other containing Rbr. With the addition of acetogenic bacteria to the list, it is becoming increasingly evident that, whatever their functions may be, Rbr, Rbo, and FprA are widespread in air-sensitive microorganisms.

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