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Rubrerythrin from *Clostridium perfringens*: Cloning of the Gene, Purification of the Protein, and Characterization of Its Superoxide Dismutase Function

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The food-borne pathogen *Clostridium perfringens*, which is an obligate anaerobe, showed growth under conditions of oxidative stress. In protein extracts we looked for superoxide dismutase (SOD) activities which might scavenge highly toxic superoxide radicals evolving under such stress conditions. Using the classical assay to detect SOD activity on gels after electrophoresis of *C. perfringens* proteins, we obtained a pattern of three major bands indicating SOD activity. The protein representing the brightest band was purified by three chromatographic steps. On the basis of 20 amino acids determined from the N terminus of the protein, we designed a degenerate oligonucleotide probe to isolate the corresponding gene. We finally sequenced an open reading frame of 195 amino acids (molecular mass, 21,159 Da) with a strong homology to the *Desulfovibrio vulgaris* rubrerythrin; therefore, we assumed to have cloned a rubrerythrin gene from *C. perfringens*, and we named it *rbr*. The C-terminal region of the newly detected rubrerythrin from *C. perfringens* contains a characteristic non-heme, non-sulfur iron-binding site -Cys-X-X-Cys-$(X)_{12}$-Cys-X-X-Cys- similar to that found in rubrerythrin from *D. vulgaris*. In addition, three -Glu-X-X-His- sequences could represent diiron binding domains. We observed SOD activity in extracts of *Escherichia coli* strains containing the recombinant *rbr* gene from *C. perfringens*. A biological function of rubrerythrin as SOD was confirmed with the functional complementation by the *rbr* gene of an *E. coli* mutant strain lacking SOD activity. We therefore suppose that rubrerythrin plays a role as a scavenger of oxygen radicals.

Rubrerythrin is a non-heme, non-sulfur iron protein that was first isolated from the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) (13). Rubrerythrin was first described as a homodimer containing four iron atoms which are arranged into two rubredoxin-like FeS_4 centers and one hemerythrin-like binuclear iron cluster (13). Later on, six iron atoms per rubrerythrin dimer were reported to be distributed in two mononuclear centers and two binuclear clusters (21). The molecular mass of each subunit was estimated to be approximately 21,500 Da (12). The rubrerythrin gene (*rbr*) of *D. vulgaris* has been cloned and sequenced (23). The analysis of the C-terminal portion of the amino acid sequence confirmed spectroscopic studies (7) which predicted structural similarities to rubredoxin iron centers, and on this basis, four cysteines probably representing iron ligands could be readily identified (23). Recently, it has been suggested that the oxidized form of the diiron cluster bears a close structural (but not necessarily functional) resemblance to that of the (μ-oxo)(μ-carboxylato) diiron cluster in oxidized ribonucleotide reductase (7). Moreover, the visible absorption, electron paramagnetic resonance, and Mössbauer spectra of the FeS_4 site in rubrerythrin are quite similar to those of rubredoxin in both oxidized and reduced forms of the proteins. The midpoint reduction potential of the FeS_4 in the rubrerythrin (approximately +230 mV) is at least 200 mV more positive than that of rubredoxin (21). This high reduction potential suggests that rubrerythrin is not directly involved in the electron transport chain of *D. vulgaris*, which has a highly reducing growth requirement. The physiological function of rubrerythrin is not known. Despite the apparent spectroscopic resemblance to hemerythrin, rubrerythrin does not bind O_2 reversibly (10). However, interactions of rubrerythrin with O_2 or its immediate reduction products in vivo cannot be ruled out a priori (10). Tests of rubrerythrin for catalase, superoxide dismutase (SOD), nitrate reductase, phosphatase, and pyrophosphatase activities were reported to be negative (12, 21, 23).

In this paper, we report the isolation and characterization of a rubrerythrin from a species other than *D. vulgaris*, namely, *Clostridium perfringens*, and we define a role for its function. Physiological and structural implications of these results are examined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The culture medium A used for *C. perfringens* consisted of brain heart infusion broth (Oxoid) containing 0.05% (wt/vol) cysteine hydrochloride, 0.1% (vol/vol) $MnSO_4 \cdot H_2O$, 0.01% (vol/vol) $FeSO_4 \cdot H_2O$, and 0.1% (vol/vol) resazurine, pH 7.2. Anaerobic growth on solid medium containing 1.6% (wt/vol) agar (Difco) was performed in an anaerobic gas chamber (Coy Laboratories, Ann Arbor, Mich.) under an atmosphere of nitrogen containing 6% hydrogen at 37°C. Anaerobic growth in liquid medium was routinely done in 500-ml serum flasks containing 400 ml of medium by shaking at 37°C. Large-scale cell preparations of *C. perfringens* were carried out in 5,000-ml Erlenmeyer flasks containing 4,000 ml of medium A. The sensitivity of the *Clostridium* strains to oxidative stress was assessed by comparing the cell growths obtained under partially aerobic and anaerobic conditions. The partially aerobic environment was produced by adding both 40 ml of oxygen per liter and 10^{-4} M paraquat (methylviologen) to the serum flasks when the cultures reached an optical density of 0.2 to 0.23 at 600 nm. *Escherichia coli* was routinely grown at 37°C in Luria-Bertani medium (25) or minimal medium (5) for complementation of *E. coli sod* mutations by the cloned *rbr* gene from *C. perfringens*. If required, the following supplements were added to the media: thiamine, 50 μg/ml; ampicillin, 50 μg/ml; kanamycin, 15 μg/ml; tetracycline, 10 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 20 μg/ml; isopropylthio-β-D-galactopyranoside (IPTG), 240 μg/ml.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
C. perfringens NCIMB8875	Cs^r, Nm^r, Km^r	NCIMB[a]
E. coli XL1-Blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*, Tet^r)	4
E. coli QC774	F^- Δ*lac4169 rpsL* ϕ(*sodA-lacZ*)*49* ϕ(*sodB-kan*)*1*-Δ_2 Cm^r Km^r	5
Plasmids		
pUC18	Ap^r, LacZ′, cloning vector	31
pRub1	Ap^r, 1.0-kb *Hin*dIII-*Hin*dIII fragment from strain NCIMB8875 in pUC18	This study
pRub2	Ap^r, 0.95-kb *Nde*II-*Nde*II fragment from strain NCIMB8875 in pUC18	This study
pRr	Ap^r, 1.0-kb *Hin*dIII-*Hin*dIII fragment from pRub1 in pRub2	This study
pLME1	Ap^r, 2.7-kb *Eco*RI-*Pst*I fragment from pME2040 in pMS119EH	16

[a] National Collection of Marine and Industrial Bacteria, Aberdeen, Scotland.

Preparation of protein extracts. *C. perfringens* cells which were cultured at 37°C under oxidative stress (see above) in medium A were harvested in the postexponential growth phase (A_{600} between 0.8 and 1.0) by centrifugation (10,000 × *g* for 20 min). The following procedures were carried out at 4°C. The cells were washed twice with a saline solution (0.85 M NaCl) and resuspended in 4 ml/g of cells (wet weight) of buffer A composed of 50 mM potassium phosphate containing 21.75% (vol/vol) glycerol, 30 mM KCl, 0.2 mM EDTA, 0.5 mM $MgCl_2 \cdot 6\ H_2O$, 0.2 mM phenylmethylsulfonyl fluoride, and 2 mM 1,4-dithio-DL-threitol at pH 7.6. The cells were disrupted by six passages through a French pressure cell (SLM Amico; Lightnight Instruments, Lausanne, Switzerland) at approximately 120 MPa. After centrifugation at 20,000 × *g* for 30 min, the clarified supernatant was further treated with 0.1 M $MnCl_2$ to precipitate nucleic acids (3). Then, the centrifugation step was repeated and the resulting supernatant was extensively dialyzed against buffer A to get a so-called crude extract.

Purification of rubrerythrin. All purification procedures were performed at 4°C.

(i) Step 1: ion-exchange chromatography. The crude extract was loaded onto a column (12 by 2.6 cm) of DEAE Sepharose Fast Flow (Pharmacia LKB, Uppsala, Sweden) equilibrated with buffer A. Proteins were eluted in four steps with buffer A at potassium phosphate concentrations of 50 mM, 90 mM, 150 mM, and 1 M, respectively, and at a flow rate of 1 ml/min (corresponding to 12 cm/h). In each step, the column was eluted with 4 bed volumes of the corresponding buffer. The fractions containing rubrerythrin (displaying SOD activity) which eluted at 90 mM phosphate buffer were pooled and concentrated by membrane filtration. The concentrated sample was dialyzed overnight against 1 mM potassium phosphate buffer (pH 7.2) containing 30 mM KCl, 0.2 mM EDTA, 2 mM 1,4-dithio-DL-threitol, and 0.2 mM phenylmethylsulfonyl fluoride (buffer B).

(ii) Step 2: hydroxylapatite chromatography. The resulting sample was applied to a Bio-Gel HT hydroxylapatite column (10 by 1.6 cm) (Bio-Rad Laboratories, Hercules, Calif.) which was equilibrated by a three-step procedure: 2 bed volumes, each, of 10 and 1 mM Tris-HCl buffer (pH 7.2), and then 2 bed volumes of buffer B. The sample was eluted in five steps with buffer B containing 1, 10, 50, 100, and 300 mM potassium phosphate, respectively, at a flow rate of 1 ml/min (corresponding to 30 cm/h). In each step, the column was eluted with 3 bed volumes of the corresponding phosphate buffer. The fractions containing rubrerythrin were pooled and concentrated by membrane filtration.

(iii) Step 3: immobilized metal affinity chromatography. After a buffer exchange by dialysis against buffer C (0.02 M Na_2PO_4, 1 M NaCl, pH 6.8), the sample from step 2 was subjected to immobilized metal-affinity chromatography and applied onto a HiTrap affinity chelating column (5 ml) (Pharmacia LKB). The column was first washed with 3 bed volumes of distilled water and then loaded with a half bed volume of 0.1 M metal salt solution (0.1 M $CuSO_4$). After this stage, the column was washed again with 3 bed volumes of distilled water and finally equilibrated according to the manufacturer's instructions (Pharmacia LKB). Elution of the sample was achieved by running a gradient of NH_4Cl through the column (linear gradient from 0 to 100% NH_4Cl). Thus, the elution system was a linear gradient of 10 bed volumes from 100% of buffer C to 100% of elution buffer (0.02 M Na_2PO_4, 1 M NH_4Cl, pH 7.8). The proteins were eluted at a flow rate of 0.5 ml/min, with fractions collected at 4-min intervals. The pooled fractions containing rubrerythrin were stored at −20°C until analytical measurements were carried out.

SOD assays. SOD activity was measured by two methods. The first method (in vitro assay) was based on the ability of the enzyme to inhibit the autoxidation of pyrogallol (15). One unit of SOD is defined as the amount of extract that caused 50% inhibition of the rate of the autoxidation of pyrogallol (15). The SOD activity was determined by quantifying the inhibition by the sample of pyrogallol autoxidation, which was monitored kinetically at 420 nm. In the second method, SOD activity was determined qualitatively by visualization of the activity on gels (1) after polyacrylamide gel electrophoresis (PAGE) of protein extracts under nondenaturing conditions in the discontinuous Tris-glycine system originally described by Davis (8). The assay utilized photochemical events to generate $O_2^{\cdot -}$ and used nitroblue tetrazolium to detect this radical. SOD signaled its location by causing achromatic zones on otherwise uniformly blue gels.

Determination of hydrogen peroxide. In order to verify the SOD activity by analysis of enzymatic end products, the production of hydrogen peroxide by the purified enzyme was determined by the method of Saikumar et al. (24). The method is based on the oxidation of NADH. In the presence of SOD, the 2 mol of $O_2^{\cdot -}$ produced from the H_2O_2-horseradish peroxidase-scopoletin system is converted to 1 newly generated mole of H_2O_2. Consequently, 2 mol of NADH is oxidized, and H_2O_2 is completely regenerated in this system. The decrease in absorbance at 340 nm (oxidation of NADH) was recorded in a spectrophotometer (UVIKON 922; Kontron).

Protein determination. Protein concentrations were routinely determined by the method of Lowry et al. (14), using bovine serum albumin as a standard.

Molecular weight determination. The subunit molecular weight was estimated by the method of Weber and Osborn (30). As the molecular weight marker, a sodium dodecyl sulfate (SDS)-PAGE standard (Bio-Rad Laboratories) was used.

Amino acid analysis. Following native PAGE or SDS-PAGE, the rubrerythrin was blotted onto a polyvinylidene difluoride Immobilon membrane (Millipore, Bedford, Mass.). The membrane and the gels were pretreated according to the protocol of the manufacturer (Millipore). The transfer was carried out overnight at room temperature by 40 mA with a tank blotting apparatus (Bio-Rad Laboratories), and the protein was sequenced in an Applied Biosystems 476A Protein Sequencer (Foster City, Calif.). Sequencing cycles were run according to standard protocols provided by the manufacturer.

DNA techniques. Total cellular DNA was prepared from *C. perfringens* cells as previously described (2). Small-scale plasmid DNA isolation from *E. coli* was carried out according to the boiling method (25). Plasmid DNA for nucleotide sequencing was purified by using QIAGEN-tip 100 according to the manufacturer's protocol (QIAGEN Inc., Studio City, Calif.). Routine DNA manipulations were performed as described by Sambrook et al. (25). DNA restriction enzyme fragments were isolated from agarose gels with the QIAEX II gel extraction kit (QIAGEN Inc.) as recommended by the manufacturer. *E. coli* XL1-Blue cells were transformed by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories) (9), and *E. coli* QC774 cells were transformed by the $CaCl_2$ treatment (25). Transfer of DNA from 0.8% agarose gels to a Nylon Plus membrane (QIAGEN) was performed according to the method of Southern (25). Synthetic oligonucleotides (approximately 5 pmol) were end labelled by phosphorylation with T4 polynucleotide kinase and 100 μCi of [γ-^{32}P]ATP (5×10^3 Ci/mmol) (25). The conditions for prehybridizations and hybridizations with oligonucleotide probes were carried out at 45°C in the absence of formamide, as previously described (17).

Cloning the *C. perfringens* rubrerythrin gene. Fragments of *Hin*dIII-digested chromosomal DNA of strain NCIMB8875 between 0.8 and 2 kb in size were purified from agarose gels by a QIAGEX gel extraction procedure (QIAGEN Inc.) and ligated to pUC18 which had been linearized with *Hin*dIII. The reaction products were used to transform electrocompetent *E. coli* XL1-Blue cells. Transformants were picked to an array, and a colony blot was probed with the radioactive labelled oligonucleotide Lm59 (5′-GCNGARAAYCTIATGAAR-3′) to identify clones bearing the upper part of the *C. perfringens rbr* gene. An overlapping *Nde*II-*Nde*II fragment hybridizing with oligonucleotide Lm60 (5′-TAAATCCACGGTTTCCAC-3′) was cloned into *Bam*HI-restricted pUC18 to analyze the lower and downstream part of the *rbr* gene.

Nucleotide sequence determination. Nucleotide sequencing of both strands from cloned DNA was performed by the dideoxy-chain termination method (26), using a Sequenase, version 2.0, kit (U.S. Biochemicals, Cleveland, Ohio) and α-^{32}S-dATP as a label. Oligonucleotide primers were the universal forward (−40) and reverse sequencing primers and some synthetic oligonucleotides (Microsynth GmbH, Balgach, Switzerland).

Computer analysis. Nucleotide sequences were analyzed on the VAX with the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison), whereas multiple alignments of amino acid sequences were carried out with the CLUSTAL program, and similarities were calculated with the GAP program as previously described (17).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in GenBank under accession number X92844.

TABLE 2. Purification of rubrerythrin from *C. perfringens* and its SOD activity

Step	Vol (ml)	Total units	Total protein (mg)	Sp act (Units[a]/mg)	Purification factor	Yield (%)
Crude extract	70	1,967	903	2.2	1.0	100
Crude extract after treatment with $MgCl_2$ and dialysis	65	2,080	748	2.8	1.3	105
DEAE-cellulose	138	1,314	97	13.5	6.1	67
Hydroxylapatite	39	1,095	26	42	19.1	56
IMAC[b]	4	120	1.0	120	54.5	7

[a] Fridovich units: One unit of superoxide dismutase is defined as the amount of extract that caused 50% inhibition of the rate of the autoxidation of pyrogallol (15).
[b] Immobilized metal-affinity chromatography.

RESULTS

Isolation and purification of a protein demonstrating SOD activity. *C. perfringens* NCIMB8875, which by definition is an obligately anaerobic bacterium lacking a respiration chain, shows good growth under conditions of partial aeration and in the presence of 0.1 mM of the redox cycling agent paraquat (methylviologen) (data not shown). One probable reason is the presence of SOD activity which might scavenge highly toxic superoxide radicals generated by both paraquat and oxygen in vivo. To verify this, we looked for SOD activity in cell extracts. After a nondenaturing PAGE and SOD activity staining procedure (1), we observed a major distinct achromatic band which is hardly visible (see Fig. 6, lane 1). This qualitative SOD activity was confirmed with a reasonable level of SOD activity measured by monitoring the inhibition of the autoxidation of pyrogallol (15) in crude extracts (approximately 2.2 U/mg of protein). We purified the protein corresponding to the major SOD-positive band. The purification procedures for this SOD activity are summarized in Table 2. The protein was purified 55-fold from the initial crude extract to a specific SOD activity of 120 U/mg of protein. The purified protein from *C. perfringens* was judged to be homogeneous according to the following criteria: electrophoresis on a polyacrylamide gel under native conditions (pH 8.8) indicated the presence of a single SOD-positive band (Fig. 1, lane 1), and a single band at the same migration distance was exclusively visible after protein staining with Coomassie brilliant blue R-250 (Fig. 1, lane 2).

The purified protein has an N terminus homologous to that of rubrerythrin. By SDS-PAGE in the presence of β-mercaptoethanol, the purified enzyme displayed only a single protein band, with an apparent molecular mass of approximately 23,000 Da. The N-terminal sequences of both proteins derived from SDS-PAGE and from native PAGE were determined. The first 20 residues were determined (MKSLKGTKTAEN LMKSFAGE) and were identical from both sources. This sequence was subjected to the FASTA sequence comparison program. Surprisingly, none of the amino acids of the N terminus corresponded to conserved amino acids of classical SODs (20), but we observed a high homology with the N-terminal amino acid sequence of rubrerythrin from *D. vulgaris* (Hildenborough) which is demonstrated in the alignment of both N termini (see Fig. 4).

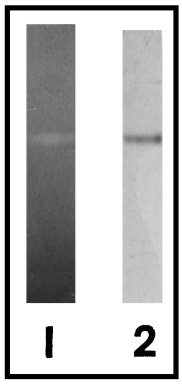

FIG. 1. Polyacrylamide gel electrophoresis of *C. perfringens* rubrerythrin. The gels were stained for SOD activity (lane 1) and with Coomassie brilliant blue R-250 (lane 2). Five micrograms of purified rubrerythrin was loaded in each lane.

Cloning of the rubrerythrin encoding gene. Successful cloning of the gene which corresponds to the rubrerythrin-like N terminus relied upon use of a synthetic 18-mer oligonucleotide (Lm59) as a gene-specific probe. The oligonucleotide was deduced from residues AENLMK and had the sequence (5′→3′) GCNGARAAYCTIATGAAR containing 5 degeneracies. A Southern blot of *C. perfringens* chromosomal DNA cut with *Hin*dIII and probed with ^{32}P-labelled Lm59 generated a signal of 1 kb in size (data not shown). The corresponding *Hin*dIII fragment was cloned into pUC18, identified by colony hybridization with the same probe after screening approximately 1,000 *E. coli* transformants, and the recombinant plasmid was named pRub1 (Table 1). An overlapping *Nde*II-*Nde*II fragment (approximately 1 kb in size) cloned into pUC18 (pRub2) was identified by hybridizing with the primer Lm60 (reversed to nucleotides 942 to 960 as indicated in Fig. 3), which was selected after DNA sequencing of the pRub1 insertion. A Southern blot of *C. perfringens* DNA cut with various restriction enzymes and probed with labelled Lm60 verified the origin of the cloned *Hin*dIII fragment and the *Nde*II fragment (Fig. 2, lanes 7 and lanes 6, respectively). We composed a coherent insertion from pRub1 and pRub2 by subcloning the *Hin*dIII fragment of pRub1 into pRub2 which has been digested with *Hin*dIII. This new clone was named pRr (Table 1), and after sequencing, it turned out that it contained a DNA sequence which was highly similar to that of the rubrerythrin gene from *D. vulgaris* (see below; see Fig. 4). Therefore, we assumed to have cloned a rubrerythrin gene (*rbr*) from *C. perfringens.*

DNA sequence of the *rbr* gene. The nucleotide sequence of the 1.6-kb insertion of pRr containing the *rbr* gene is shown in Fig. 3 together with the deduced amino acid sequence. A notable feature of the nucleotide sequence is a ribosome binding site (AGGAGG) 15 to 9 nucleotides upstream from the translational start site of the protein. Its deduced amino acid sequence has a strong homology (52% identity, 82% similarity) with the *D. vulgaris* rubrerythrin (Fig. 4). The homology was further confirmed by the sequence of the C-terminal region of *C. perfringens* which contains the characteristic non-heme, non-sulfur iron-binding site -Cys-X-X-Cys-$(X)_{12}$-Cys-X-X-Cys- of the *Desulfovibrio* rubrerythrin at the identical positions (23). In addition, two -Glu-X-X-His- sequences (proposed as diiron-binding sites) are present in the N-terminal region in both bacteria (positions 53 to 56 and 133 to 136 in Fig. 4). An additional -Glu-X-X-His- motif is resident at positions 96 to 99

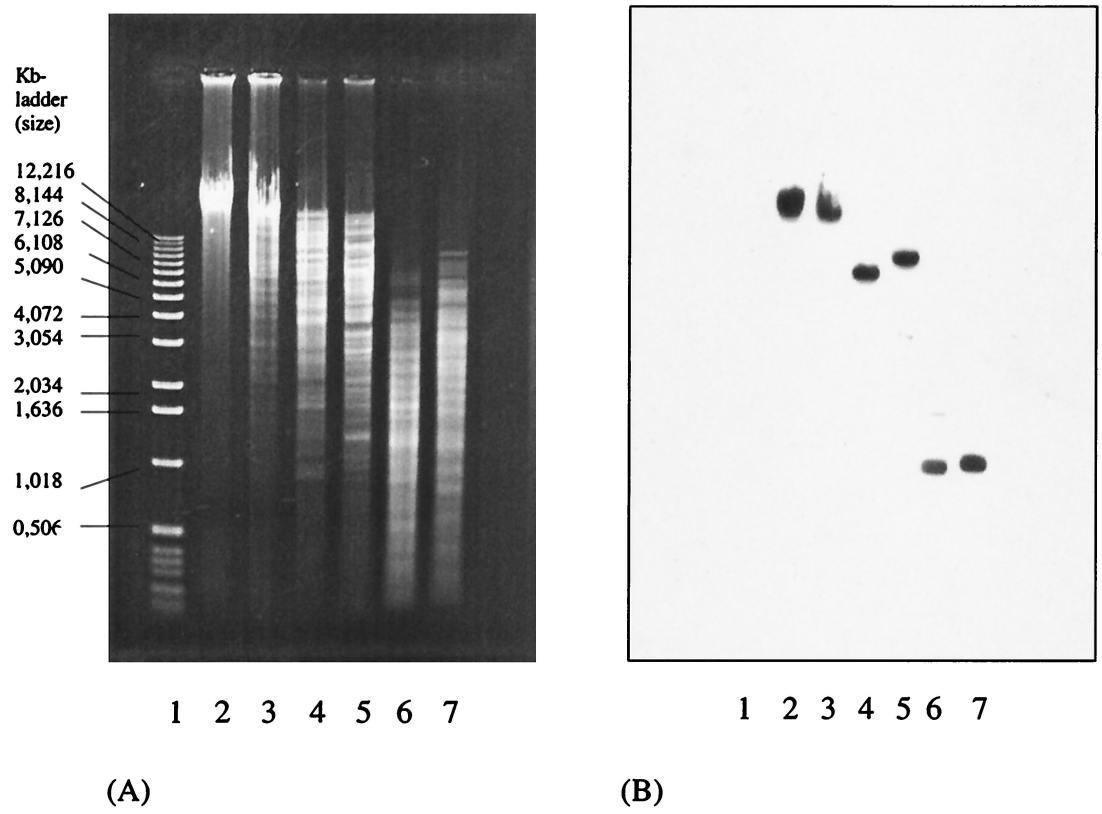

FIG. 2. Southern hybridization analysis of *C. perfringens* chromosomal DNA digested with various restriction enzymes. (A) Ethidium bromide-stained 0.8% agarose gel. (B) Autoradiography of membrane-bound DNA that hybridized with γ-^{32}P-labelled oligonucleotide Lm60. Lanes: 1, 1-kb DNA molecular size ladder; 2, undigested *C. perfringens* DNA; 3, DNA digested with *Pst*I; 4, DNA digested with *Eco*RI; 5, DNA digested with *Hin*dII; 6, DNA digested with *Nde*II; 7, DNA digested with *Hin*dIII.

in the *C. perfringens* rubrerythrin (Fig. 4). The subunit molecular mass of rubrerythrin calculated from the amino acid sequence is 22,159 Da and confirms the result obtained with SDS-PAGE. The calculated isolelectric point of the *C. perfringens* rubrerythrin is 5.30.

Expression of the cloned *rbr* gene in *E. coli*. To test whether the putative *rbr* gene plays a role as functional SOD, it was expressed in *E. coli* QC774. The *sodA* and *sodB* genes of this strain are insertionally inactivated (Table 1), and it fails to grow under aerobic conditions on minimal medium (5). Transformants harboring the recombinant plasmid pRr with the complete *rbr* gene regained the ability to grow aerobically on minimal medium containing 0.01 μM paraquat. However, the growth rate was lower than the growth rate of QC774 cells containing the *sod* gene of *Methanobacterium thermoautotrophicum* in pLME1 (Fig. 5) (16). Negative controls carrying the vector plasmid did not grow under these conditions within 2 to 3 days (Fig. 5). Moreover, protein extracts were prepared from selected transformants which had been grown on Luria-Bertani medium in the presence of kanamycin (15 μg/ml), ampicillin (50 μg/ml), and paraquat (0.01 μM). PAGE of such extracts under native conditions and subsequent SOD activity staining clearly demonstrated in the case of recombinant pRr a SOD-positive band (Fig. 6, arrows in lanes 2 and 5) with similar migration behavior as the homologous rubrerythrin from *C. perfringens* NCIMB8875 (Fig. 6, arrowhead in lane 1). The additional bands observed in Fig. 6, lanes 2 and 3, are originally from Mn-SOD, Fe-SOD, and MnFe-hybrid-SOD in *E. coli* XL1-Blue. As controls, extracts from the SOD-negative background (*E. coli* QC774) didn't generate a band (Fig. 6, lane 4) whereas *E. coli* QC774/pLME1 expressed its recombinant SOD (Fig. 6, lane 6). In order to stress a SOD-like activity and to rule out the possibility of a metal reducing effect of the purified rubrerythrin, we performed the H_2O_2 determination method of Saikumar et al. (24). This method allowed us to measure the production of rubrerythrin-dependent H_2O_2, the product evolved also in classical SOD reactions, as shown in Fig. 7. In the applied assay system, the purified rubrerythrin (10 μg) was able to oxidize 36 nmol of NADH within 4 min, whereas bovine CuZn-SOD (10 μg) oxidized 69 nmol of NADH in the same time (Fig. 7). The oxidized amount of NADH corresponding to the produced H_2O_2 was in the same range in reaction mixtures containing 10 μg of crude extract from *E. coli* QC774/pLME1 (Table 1) containing a recombinant SOD from *M. thermoautotrophicum*. These findings therefore demonstrated the rubrerythrin-dependent production of H_2O_2 by an established method and confirmed the SOD-like functional characteristics of rubrerythrin.

DISCUSSION

Although *C. perfringens* does not contain hemoproteins such as cytochrome *c* or catalase and is classified as a strict anaerobe because of its inability to respire oxygen and grow under aerobic conditions, we observed the growth of *C. perfringens* in aerobic conditions as well as in the presence of paraquat. Consequently, *C. perfringens* should possess mechanisms to protect itself against free oxygen radicals. We presumed that *C. perfringens* contained SOD activity to scavenge superoxide anion radicals. Hewitt and Morris (11) have already proposed the

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                HindIII
   1  AAGCTTTTATTCTAAGGGCATCTTTATCACTATTATCAAGCATATATCTTTAACTTTATC
  61  TAAAGTGTTTGGATGTATTTTAACCTTCACAATAAAAACCTCCTTATAAATTTTAGTGAA
 121  TTTAATATATAATTAACTTTATTTATTAAATTATATACGCATATTAAAAATAGAGCTATA
 181  GTATGTAATTTTAAGGGAATAAAAAAGCCGAAATAGTGTTATTATTTTTTTATATATTTG
 241  AATGATATATATGAATTTAGAAATAATATGAATATTTATATGATAAAATAGTTTTATTAA
 301  CAAAATGTTCAAAAAGTACTTGAAATTTTCCCCAAAGTGATATTATAAATTATATAAGGA
 361  ATAACAATTATTAATTAATATGGCTTAAAAATTATTTATTTGGAAAAATAATTTTAATTG
                      *****          rbr
 421  TGTTTTTTCAAATAAGTGATTGTAGGAGGTTTTTTATTATGAAAAGTTTAAAAGGTACTA
                                     M  K  S  L  K  G  T  K
 481  AAACTGCTGAAAATCTAATGAAATCATTCGCTGGTGAATGCCAAGCAAGAACAAGATATA
        T  A  E  N  L  M  K  S  F  A  G  E  C  Q  A  R  T  R  Y  T
 541  CTTATTTTTCAAGTACAGCTAGAAAAGAAGGTTATGTTCAAATATCAAATATATTTTTAG
        Y  F  S  S  T  A  R  K  E  G  Y  V  Q  I  S  N  I  F  L  E
                                                        NdeII
 601  AAACTGCTGAAAATGAAAAAGAACACGCTAAGAGATTTTATAAATTCTTAAAAGACGATC
        T  A  E  N  E  K  E  H  A  K  R  F  Y  K  F  L  K  D  D  L

 661  TTCAAGGAGAAGCTGTAGAAATAAATGCTGCTTACCCAGTAGAATTACCTACAGATACAT
        Q  G  E  A  V  E  I  N  A  A  Y  P  V  E  L  P  T  D  T  L

 721  TAACTAACTTAAAATTTGCTGCTGAGGGAGAACATGATGAATTAAGTAATCTTTATCCAT
        T  N  L  K  F  A  A  E  G  E  H  D  E  L  S  N  L  Y  P  S

 781  CATTTGCTGATGTAGCTGACGAAGAAGGATTCCCAGAAGTAGCTGCTGCTTTCAGAATGA
        F  A  D  V  A  D  E  E  G  F  P  E  V  A  A  A  F  R  M  I

 841  TAGCTAAAGCAGAAACTGCTCACTATAATAGATTTATGAAATTAGCTAAAAATATGGAAG
        A  K  A  E  T  A  H  Y  N  R  F  M  K  L  A  K  N  M  E  E

 901  AAGGAAAAGTATTTAAAAAAGACGAAGTAGTACTTTGGAAATGTGGAAACTGTGGATTTA
        G  K  V  F  K  K  D  E  V  V  L  W  K  C  G  N  C  G  F  I
                                                        HindIII
 961  TTTGGGAAGGTGCAGAAGCTCCATTAAAATGCCCTGCATGCTTACATCCACAAGCTTTCT
        W  E  G  A  E  A  P  L  K  C  P  A  C  L  H  P  Q  A  F  F

1021  TTGAAGTTTTCAAAGAAACTTATTAAAAAATAGATTTATTCATGCTTAACTCGTATAATT
        E  V  F  K  E  T  Y  *
1081  TATCAATTGAATGTAAATATAATAAACTAAAACTTATTTCAGAGAGAAGAAATTCTCTCT
1141  GATTTTTTTATAAATAAAGAGCGTATATCAAAATAACTTTTGTATACGCTCTATAAATTT
1201  GCAATTTCAATGTAGTGGATAGCTAAACACTACCATTTGCTCTCATCATTTATTGGTCTC
1261  CAAAATTGAATTTCATCAATTAAAAGTTCTCTATCAAAATTATAATCTTGAAGTTTTGTA
1321  TAAGCTGACTCAAACTCATCCTCTCCCCATACTCTCTGCAAAATATGGGTTAGCTAAGGA
1381  TACATGAAGTTGCCATGTATCTGGGTTAGAAGTCAATTTAAATCCTTCTTTTGATAAATT
1441  CTCATAAAGTTCTCTAGAAAGTTTCTTTATTACTCCACTTTCTTCATCTACCTTTAAATT
1501  TACAGACCTATAAGGTTCTCCAAATTTAATTAGTCCATTTAAATGAACTTCAAATTTTTT
1561  TATATGATTTTAAAGTGTTTTTTACTACTTCATTTAATTTATCCATATCAGGATC
                                                        NdeII
```

FIG. 3. Nucleotide sequence of *rbr* gene and its corresponding amino acids of *C. perfringens* rubrerythrin. The sequence covers the insertion in pRr (Table 1). The underlined amino acids indicate the region from which oligonucleotide Lm59 was deduced. The line over nucleotides 942 to 960 indicates the sequence of primer Lm60. A potential ribosome binding site is marked by asterisks.

presence of SOD in *C. perfringens*, and indeed, we discovered SOD activity bands using the activity staining method of Beauchamp and Fridovich (1) in cell extracts (Fig. 1). This was the incentive to attempt a purification of the responsible enzyme (Fig. 1). The purified enzyme possesses a high SOD activity (Table 2), and its apparent molecular mass of 23,000 Da suggested that this enzyme is a classical Mn-SOD or Fe-SOD (20). In fact, we were able to amplify a partial *sod* gene encoding the Mn/Fe-SOD from *C. perfringens* NCIMB8875 by

```
                10        20        30        40        50        60
CpRr   MKSLKGTKTAENLMKSFAGECQARTRYTYFSSTARKEGYVQISNIFLETAENEKEHAKRF
DvRr   MKSLKGSRTEKNILTAFAGESQARNRYNYFGGQAKKDGFVQISDIFAETADQEREHAKRL
       ******..*  *.. .**** *** ** **.. *.*.*.****.** ***..*.*****.
                70        80        90       100       110       120
CpRr   YKFLKDDLQGEAVEINAAYPVELPTDTLTNLKFAAEGEHDELSNLYPSFADVADEEGFPE
DvRr   FKFL----EGGDLEIVAAFPAGIIADTHANLIASAAGEHHEYTEMYPSFARIAREEGYEE
       .***    .*  .** **.*  . .** .**  .* ***.* ...***** .* ***. *
              130       140       150       160       170       180
CpRr   VAAAFRMIAKAETAHYNRFMKLAKNMEEGKVFKKDEVVLWKCGNCGFIWEGAEAPLKCPA
DvRr   IARVFASIAVAEEFHEKRFLDFARNIKEGRVFLREQATKWRCRNCGYVHEGTGAPELCPA
       .*  *  ** **  * .**. .*.*. **.** ...   *.* ***.. **. **  ***
              190
CpRr   CLHPQAFFEVFKETYX
DvRr   CAHPKAHFELLGINWX
       * **.* **..
```

FIG. 4. Alignment of *C.* rubrerythrin (*Cp*Rr) and *D. vulgaris* rubrerythrin (*Dv*Rr) amino acid sequences. Identical amino acid residues are indicated by asterisks, and similar amino acid residues are indicated by dots. The EXXH and CXXC clusters are written in boldface capital letters.

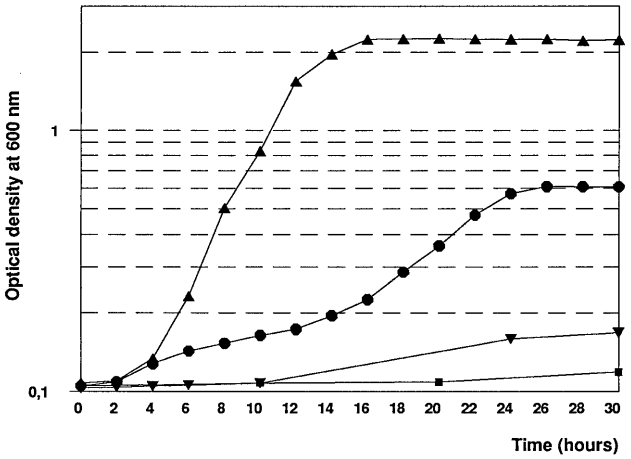

FIG. 5. Growth behavior of *E. coli* QC774 on minimal medium in the presence of paraquat (0.01 μM) and kanamycin (15 μg/ml). For growth of QC774 containing recombinant plasmids, ampicillin (50 μg/ml) was included in the minimal medium. Preculture (1%) was inoculated into minimal medium, and the cells were incubated at 37°C. Symbols: ■, *E. coli* QC774; ▼, *E. coli* QC774/pUC18; ●, *E. coli* QC774/pRr; ▲, *E. coli* QC774/pLME1.

PCR technique, and a partial *sod* gene sequence was also recently described (22), but the N terminus analysis of our purified protein contradicted what we expected before. The first 23 amino acid residues from its N-terminal region have no similarity with SOD proteins but show a strong homology with the rubrerythrin N terminus from *D. vulgaris* (Fig. 4). This finding was later confirmed by the nucleotide sequence (Fig. 3) and its deduced amino acid sequence (Fig. 4).

Rubrerythrin was so far found exclusively in the anaerobic sulfate-reducing bacterium *D. vulgaris*. It is the first example in which, after extensive spectroscopic analyses, the presence of both a rubredoxin type and a hemerythrin type of polypeptide chain have been discovered in the protein (13). The primary structure determined here shows that the fusion of the name rubredoxin and hemerythrin to create the name rubrerythrin is justified. Rubrerythrin from *C. perfringens* is composed of 195

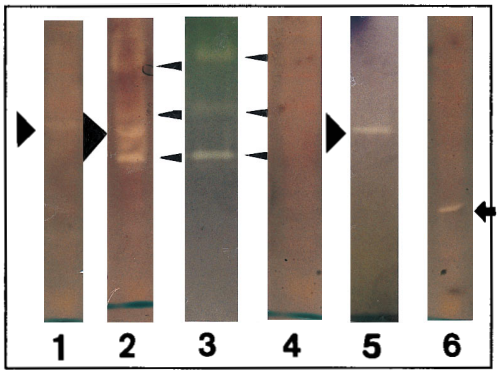

FIG. 6. Expression of recombinant rubrerythrin from *C. perfringens* NCIMB8875 in *E. coli*. Approximately 50 to 100 μg of protein extracts was run in each lane on a nondenaturing 10% polyacrylamide gel and stained for SOD activity. Lanes: 1, *C. perfringens* NCIMB8875; 2, XL1-Blue/pRr; 3, XL1-Blue; 4, QC774; 5, QC774/pRr; 6, QC774/pLME1. Large arrowheads indicate the positions of the authentic rubrerythrin (lane 1) and the recombinant rubrerythrin (lane 2 and lane 5). The small arrowheads to the right of lanes 2 and 3 indicate the positions of Mn-SOD, MnFe-hybrid SOD, and Fe-SOD, respectively (from top to bottom). The arrow to the right of lane 6 points to the position of the recombinant SOD from *M. thermoautotrophicum*.

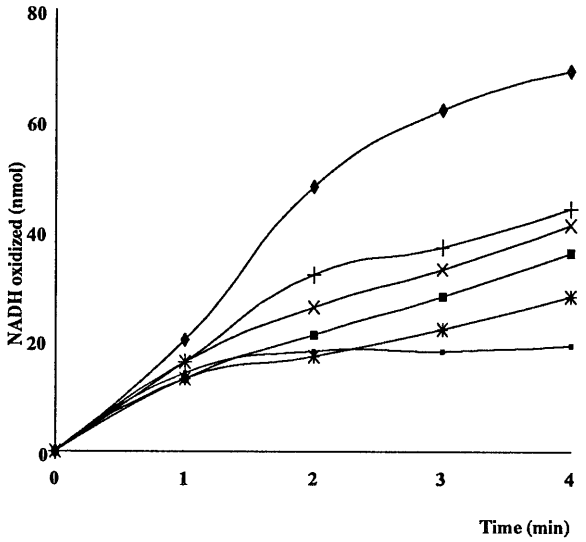

FIG. 7. Oxidation of NADH by horseradish peroxidase in the presence of recombinant rubrerythrin from QC774/pRr. The reaction mixture (1 ml) consisted of 50 mM phosphate buffer (pH 7.2), 2 μM scopoletin, 10 μg of horseradish peroxidase, 0.1 mM NADH, and various amounts of rubrerythrin. NADH oxidation was monitored at 340 nm after adding 1 μM H_2O_2. Symbols: ◆, 10 μg of Cu/Zn-SOD; ●, 0 μg of rubrerythrin as a blank; *, 5 μg of purified rubrerythrin from QC774/pRr; ■, 10 μg of purified rubrerythrin from QC774/pRr; ×, 10 μg of partially purified rubrerythrin from QC774/pRr; +, 10 μg of crude extract from QC774/pLME1 containing Fe-SOD.

amino acids, and it is noticeable that this amino acid sequence is 4 residues longer than that of *D. vulgaris* which was published previously (28). The primary structure of rubrerythrin from *C. perfringens* contains one short loop between positions 63 and 67 (Leu, Lys, Asp, Asp) which does not occur in the primary structure of *D. vulgaris* rubrerythrin. The rubrerythrin amino acid compositions of both bacteria are very similar, except for more Arg residues (+8) in the *D. vulgaris* rubrerythrin (Fig. 4). This finding partially explains the higher pI (7.1) of the *D. vulgaris* rubrerythrin compared with the pI (5.3) of rubrerythrin from *C. perfringens*. The 50-residue C-terminal portions of both rubrerythrins show some sequence homologies to rubredoxins (data not shown), especially the presence of two -Cys-X-X-Cys- pairs. In fact, the rubrerythrin from *C. perfringens* contains 5 cysteine residues, 4 of which are located in the C-terminal part of the protein at positions 162, 165, 178, and 181, respectively (Fig. 4). The spacing of the Cys residues in rubrerythrin -Cys-X-X-Cys-$(X)_{12}$-Cys-X-X-Cys- is exactly identical to that in rubrerythrin from *D. vulgaris* (Fig. 4) and different from that in rubredoxins where there are 28 or 29 residues between the two clusters (23). In contrast to the C-terminal part, which shows moderate but distinct sequence similarities with rubredoxins, it is difficult to demonstrate sequence similarities for the N-terminal part of rubrerythrin with any other known protein. Gupta et al. (10) proposed a similarity between the -Glu-X-X-His- in the N-terminal region and the diiron clusters of methane monoxoygenase and ribonucleotide reductase (R2 subunit). After sequence comparisons, they suggested that each subunit of the rubrerythrin homodimer accommodates one FeS_4 site and one diiron cluster, and they proposed for the rubrerythrin a (μ-oxo)diiron(III) cluster, a structure similar to that found in ribonucleotide reductase (R2 subunit). The N-terminal portion contains two -Glu-X-X-His- sequences. Moreover, in contrast to the *Desulfovibrio* protein, the rubrerythrin from *C. perfringens* contains an additional -Glu-X-X-His- cluster in the region 95 to 98. In this region, both rubrerythrins have the same primary structure, with the notable exception that the Glu residue in *C. perfringens* rubrerythrin is replaced by an Ala residue in *D. vulgaris* rubrerythrin.

Figure 4 demonstrates that there is no evidence for the presence of a leader sequence upstream of the encoding gene. A biological function of rubrerythrin has not been defined yet. Although different biological functions have been proposed, no one of these appears reasonable to define the function of the protein in vivo. Some studies propose pyrophosphatase activities (6, 21) and iron storage functions (10) for the rubrerythrin. With the existing data, it is difficult to speculate on the evolutionary origin of rubrerythrin. Van Beeumen et al. (28) suggested that rubrerythrin was an ancestral type of protein that existed long before the oxygen-respiring eukaryotes developed on earth. However, other studies (10, 13) suggested that it is impossible for rubrerythrin to have a function as an electron carrier like the rubredoxins or to be an oxygen transport protein like hemerythrin, because the redox potential for the rubredoxin-like center in rubrerythrin is too high (estimated at about +230 mV), also over 200 mV more positive than those of authentic rubredoxins. Interactions of rubrerythrin with O_2 or its immediate reduction products cannot be ruled out a priori (10). We suggest that rubrerythrin plays a role in the detoxification of oxygen radicals in *C. perfringens* growing in the presence of oxygen. The efficiency is less than that of a classical SOD but enough for the protection of the cells against oxygen during the transfer of the bacteria from one anaerobic niche to another. The SOD activity has been measured by two different methods, and both methods gave comparable results (Fig. 1 and Table 2). Although rubrerythrin shows SOD activity, it is so far impossible to define whether this activity is a primary or secondary function. Moreover, it cannot be excluded that rubrerythrin does not have a catalytic function and that its interaction with $O_2^{\cdot -}$ causes its own denaturation. It is known that some proteins are able to catalyze a dismutation, although at low reaction rates (19, 27, 29). It is therefore correct to propose a scavenger function of oxygen radicals for rubrerythrin. The capacity of the transformed QC774/pRr growing on minimal medium (Fig. 5) and producing H_2O_2 confirms the rubrerythrin function as a scavenger of oxygen radicals in vivo. On the other hand, we cannot conclude that the efficiency of rubrerythrin as a scavenger of oxygen radicals in vivo is equivalent to that of SODs, because the tolerance of paraquat is lower for the QC774/pRr recombinant. This difference may result from the fact that classical Mn-SOD is induced to a high level under conditions of oxidative stress, whereas rubrerythrin is probably not. Obviously, the physiological function of this protein requires further scrutiny to confirm this finding. Even if the appearance of SOD activity is probably not of physiological significance, it has led to the discovery of this rather unusual protein in a gram-positive bacterium.

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ADDENDUM IN PROOF

After acceptance of this paper, the X-ray crystallography of the *D. vulgaris* rubrerythrin became known (F. deMare, D. M. Kurtz, Jr., and P. Nordlund, Nature Struct. Biol. **3:**539–

546, 1996). In addition, this rubrerythrin was proposed to have a ferroxidase activity in vitro (F. Bonomi, D. M. Kurtz, Jr., and X. Cui, J. Biol. Inorg. Chem. **1:**67–77, 1996). The most significant development, however, was the detection of a putative rubrerythrin gene in the genome of *Methanococcus jannaschii* which is positioned in the neighborhood of several putative electron transfer proteins like rubredoxin, alkyl hydroperoxide reductase, desulfoferrodoxin, heterodisulfide reductase, and some others (C. J. Bult et al., Science **273:**1058–1073, 1996). A preliminary comparison of the GC content and the codon usage in our laboratory revealed that the rubrerythrin genes of all three species have values specific for the respective species. This implies that the rubrerythrins have evolved by longitudinal gene transfer and may be "ur-proteins" of anaerobic procaryotes already present before the evolutionary division of the Archaea from the Bacteria. The conservation of the ferredoxin-like FeS_4 and the ferritin-like diiron domains in almost identical positions of all three rubrerythrins is remarkable.

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