JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 6736–6742 Vol. 178, No. 23
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Overproduction of the *rbo* Gene Product from *Desulfovibrio* Species Suppresses All Deleterious Effects of Lack of Superoxide Dismutase in *Escherichia coli*

MARIA JULIA PIANZZOLA,[1] MATILDE SOUBES,[1] AND DANIÈLE TOUATI[2]*

Facultad de Quimica, Catedra de Microbiologia, Montevideo, Uruguay,[1] and Institut Jacques Monod, Centre National de la Recherche Scientifique-Université Paris 7, 75251 Paris Cedex 05, France[2]

Received 29 July 1996/Accepted 23 September 1996

In an attempt to isolate the superoxide dismutase (SOD) gene from the anaerobic sulfate-reducing bacterium *Desulfoarculus baarsii*, a DNA fragment was isolated which functionally complemented an *Escherichia coli* mutant (*sodA sodB*) deficient in cytoplasmic SODs. This region carries two open reading frames with sequences which are very similar to that of the *rbo-rub* operon from *Desulfovibrio vulgaris*. Independent expression of the *rbo* and *rub* genes from ptac showed that expression of *rbo* was responsible for the observed phenotype. *rbo* overexpression suppressed all deleterious effects of SOD deficiency in *E. coli*, including inactivation by superoxide of enzymes containing 4Fe-4S clusters and DNA damage produced via the superoxide-enhanced Fenton reaction. Thus, *rbo* restored to the *sodA sodB* mutant the ability to grow on minimal medium without the addition of branched amino acids, and growth on gluconate and succinate carbon sources was no longer impaired. The spontaneous mutation rate, which is elevated in SOD-deficient mutants, returned to the wild-type level in the presence of Rbo, which also restored aerobic viability of *sodA sodB recA* mutants. Rbo from *Desulfovibrio vulgaris*, but not *Desulfovibrio gigas* desulforedoxin, which corresponds to the NH_2-terminal domain of Rbo, complemented *sod* mutants. The physiological role of Rbo in sulfate-reducing bacteria is unknown. In *E. coli*, Rbo may permit the bacterium to avoid superoxide stress by maintaining functional (reduced) superoxide sensitive 4Fe-4S clusters. It would thereby restore enzyme activities and prevent the release of iron that occurs after cluster degradation and presumably leads to DNA damage.

All aerobic organisms produce toxic oxygen derivatives, such as superoxide, hydrogen peroxide, or hydroxyl radicals. They survive thanks to the coordinate expression of mechanisms including protective enzymes and repair functions which allow them to avoid or cope with the oxidative stress (44). The discovery in strictly anaerobic bacteria of protective enzymes including superoxide dismutase (SOD) raised the question of their role in anaerobic organisms and has been the subject of debate ever since (19, 22, 23).

Active oxygen species are a permanent threat to aerobic organisms. Bacteria have developed several ways to escape the deleterious effects of reactive oxygen species. Deficiency of one component of a protection system is often compensated for by expression of another component. Thus, in *Escherichia coli*, a deficiency of cytoplasmic SODs, which play a major role in defense by catalyzing dismutation of the superoxide radical, does not have drastic deleterious effects in rich medium (8). However, SOD is essential if homologous recombination (which repairs DNA strand breaks) is defective (26, 45).

In anaerobes, exposure to oxygen is transitory and protective mechanisms against oxygen toxicity appear to be less well developed. However, the few protection mechanisms present might be essential. The *sod* gene of the obligate anaerobe *Porphyromonas gingivalis* has been cloned, and a mutant was obtained by disruption (34). The mutant, unlike the wild type, showed a rapid loss of viability upon exposure to air, supporting the idea that SOD plays an essential role in aerotolerance of obligate anaerobes. However, the origin of superoxide generated by organisms which cannot use oxygen is obscure.

Sulfate-reducing bacteria have been classified as obligate anaerobes. However, they are found in a variety of environments, which suggests that they express a wide range of aerotolerance (36). Some are able to survive exposure to air for long periods (1, 11, 20, 31, 47), and there is evidence that dissimilatory sulfate reduction can occur in the presence of oxygen (7). A number of recent studies with *Desulfovibrio gigas* strongly suggests that O_2 may play a physiological role in these so-called strict anaerobes and could be a true electron acceptor (12). An aerobic respiratory chain is beginning to be elucidated (9, 10, 41). Oxygen exerts toxic effects at low concentrations, maybe in part by competition with sulfate as an electron acceptor, but also presumably by the generation of toxic oxygen derivatives. Actually, sulfate-reducing bacteria produce numerous electron carriers that can generate superoxide in the presence of oxygen (21). SOD and catalase have been detected in a few sulfate-reducing strains (23) and isolated from and characterized in *Desulfovibrio desulfuricans* (21). Abdollahi and Wimpenny further showed that the SOD activity which is low in anaerobiosis rises more than 10-fold in the presence of oxygen (1).

Sulfate-reducing bacteria play an important ecological role in recycling organic matter. *Desulfoarculus baarsii* (formerly *Desulfovibrio baarsii*) (48) which was originally isolated from a sediment is of particular interest, because unlike most sulfate-reducing bacteria that produce acetate, it can, in the presence of sulfate, completely oxidize organic matter to CO_2 and water (25).

To determine whether SOD is involved in aerotolerance of sulfate-reducing bacteria, we attempted to isolate the *sod* gene of *D. baarsii* by functional complementation of a SOD-defi-

* Corresponding author. Mailing address: Institut Jacques Monod, CNRS-Université Paris 7, 2 place Jussieu, 75251 Paris Cedex 05, France. Phone: 1 44 27 47 19. Fax: 1 44 27 76 67. Electronic mail address: touatida@ccr.jussieu.fr.

TABLE 1. Bacterial strains and plasmids

Bacterium or plasmid(s)	Genotype or relevant characteristic(s)	Source or reference
Desulfoarculus (Desulfovibrio) baarsii DSM 2075	DSM collection[a]	
E. coli strains		
CSR 603	*recA uvrA6 phr-1 thr-1 leuB6 proA2 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44*	39
DH5α	Δ(*lacZYA-argF*)*U169* ϕ*80* d*lacZ*Δ*M15 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1*	Gibco BRL
TG1	Δ(*lac-pro*) *supE thi-1 hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacIq lacZ*Δ*M15*	37
GC4468	Δ(*lacZYA-argF*)*U169 rps*L	45
QC 1891	Same as TG1 but ϕ(*sodA′-lacZ*)*49* ϕ(*sodB-kan*)Δ2	This work
QC 1799	F$^-$ Δ(*argF-lac*)*U169 rpsL* Δ*sodA3* ϕ(*sodB-kan*)Δ2	45
QC 2375	Same as QC 1799 but Δ*recA306 srl*::Tn*10*	45
Plasmids		
pUC18, pUC19	Cloning vectors, Ap^r	Laboratory stock
pJF119EH	Expression vector carrying ptac promoter, Ap^r	16
pJK15	pUC8 derivative carrying the *rbo* gene from *D. vulgaris*	5
pJK29	pUC8 derivative carrying the *rbo-rub* operon from *D. vulgaris*	5
pED6	pUC derivative carrying the *dsr* gene from *D. gigas*	6
pMJ3	pUC18 derivative carrying the *rbo-rub* region from *D. baarsii*	This work
pMJ12	pUC19 with a 0.7-kb *Sac*I-*Sma*I fragment from pMJ3	This work
pMJ13	pUC19 with a 1.7-kb *Sma*I-*Sac*I fragment from pMJ3	This work
pMJ24	pJF119EH derivative with the *Eco*RI-*Bam*HI fragment from pMJ12 carrying the *rbo-rub* genes under ptac promoter control	This work
pMJ25	pMJ24 with the 74-bp *Eag*I-*Eag*I fragment deleted	This work
pMJ26	pMJ24 with the 286-bp *Eco*RI-*Nco*I fragment deleted	This work
pMJ28	pMJ24 with the 412-bp *Nco*I-*Bam*HI fragment deleted	
pMJ30	pJF119EH derivative carrying a 234-bp *Eco*RI-*Hin*dII fragment generated by PCR from pDE6 and carrying the *dsr* gene from *D. gigas*	This work

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

cient mutant of *E. coli* (35). This, unexpectedly, led to isolation of a chromosomal region similar to the *rbo-rub* region of *Desulfovibrio vulgaris*, encoding a putative rubredoxin oxidoreductase (*rbo*) and rubredoxin (*rub*) (5, 46).

In this manuscript we describe the isolation and characterization of the *rbo-rub* genes of *D. baarsii*. We show that expression of *rbo* fully complements SOD deficiency in *E. coli*. Possible underlying mechanisms are discussed.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this work are described in Table 1 and Fig. 1. To construct pMJ30, a fragment of 230 bp containing the *dsr* gene was amplified from pED6 (6) by PCR, using the oligonucleotides 5′ CCGGAATTCCCCGCGGGCGTTTGCCC 3′ and 5′ CCCA AGCTTACAAGGGGGAAGAT 3′, with a 5′ *Eco*RI and *Hin*dIII restriction site, respectively. The amplified fragment was digested with *Eco*RI and *Hin*dIII and inserted into the corresponding sites in pJF119EH (16). The construct was verified by digestion with restriction enzymes and sequencing.

Media and growth conditions. *E. coli* was grown aerobically at 37°C in a rotary shaking bath at 200 rpm in LB (32) or minimal medium, as indicated. Minimal medium was M9 (32) with 0.4% glucose, succinate, or gluconate as indicated. Minimal medium was supplemented with 0.5 mM amino acids when required. Ampicillin was added to liquid medium at 500 and 50 μg/ml in plates as needed and isopropyl-β-D-thiogalactopyranoside (IPTG) at 2 mM as indicated. *E. coli* anaerobic cultures were grown in a Forma Scientific anaerobic chamber in LB medium containing 1% glucose. All media and materials were equilibrated in the anaerobic chambers 3 days before use. *D. baarsii* was grown anaerobically as described by Balch et al. (2), in reducing medium 208 recommended by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brauschweig, Germany.

Biochemical reagents. Enzymes were purchased from Biolabs or Boehringer Mannheim. Reagent-grade chemicals were from Sigma. Goldstar polymerase and PCR reagents from Eurogentech were used for DNA amplification. Bicinchoninic acid reagent (Pierce Chemical Company) was used to measure protein concentration, with bovine serum albumin as a standard. The Sequenase kit version 2.0 (U.S. Biochemical Corp.) was used for sequencing; [α-^{35}S]dATP was purchased from ICN.

Preparation of a *D. baarsii* library. High-molecular-weight chromosomal DNA from *D. baarsii* was prepared from a anaerobic culture in stationary phase obtained by 5 days of growth at 37°C. DNA was extracted by the method of Marmur (30). DNA was partially digested with *Sau3*AI, and fragments were fractionated as previously described (30 μg of DNA in 400 μl was loaded onto a 5 to 20% sucrose gradient and spun at 15°C for 14 h at 27,500 rpm in a SW41 Beckman rotor) (43). Fragments of 2 to 4 kb were collected and inserted into the *Bam*HI site of pUC18, previously digested, electroeluted, and treated with alkaline phosphatase. Over 6,000 transformants selected in strain QC 1891 were pooled in LB containing 17% glycerol and frozen at −80°C in aliquots.

General methods. Plasmid DNA was prepared by the alkaline extraction procedure of Birnboim and Doly (4) or by using Quiagen kits; standard procedures were used for agarose gel electrophoresis (38). SOD activity in polyacrylamide nondenaturing gels was assayed by staining by the method of Beauchamp and Fridovich (3). Plasmid proteins were labeled in maxicells as previously described (39, 43), except that a ^{35}S labeling mixture (ICN) was used in place of [^{35}S]methionine, and the cell lysate was spun after the freezing-thawing step to eliminate cell debris.

Mutation frequency measurements. The forward mutation test Rif^s to Rif^r was used. Rif^r mutants were scored by plating a culture grown overnight on LB plates containing 100 μg of rifampin per ml as previously described (45).

Nucleotide sequencing. Double-stranded DNA was sequenced by using a series of oligonucleotide probes and plasmids pMJ12, pMJ13, and pMJ27, by the Sanger dideoxy chain termination method (40). The sequence of the 0.7-kb *Sma*I fragment containing *rbo-rub* structural genes was confirmed by the Eurogentech sequencing service (Eurogentech S.A., Seraing, Belgium). The BLAST algorithm was used to search for similar sequences in data bases (Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, Calif.).

Nucleotide sequence accession number. A 916-bp nucleotide sequence including the *rbo* and *rub* genes from *D. baarsii* and starting 183 bp upstream of the start codon of *rbo* appears in the EMBL Nucleotide Sequence Database under the accession number X99543. This sequence has not been previously published.

RESULTS

Isolation of a putative *sod*$^+$ clone of *D. baarsii*. All phenotypes of *E. coli* SOD-deficient strains are complemented by expression of a functional SOD from any organism (35, 44a).

TABLE 2. Aerobic survival of a *sodA sodB recA* mutant transformed with various plasmids[a]

Plasmid	Survival[b] with the following concn of IPTG:	
	0	2 mM
None (control)	6×10^{-5} ($\pm 3 \times 10^{-5}$)	ND[c]
pMJ3 (*rbo rub*)	0.16 ($\pm$ 0.08)[d]	0.15 ($\pm$ 0.08)[d]
pJF119EH	2×10^{-5} ($\pm 3 \times 10^{-5}$)	3×10^{-5} ($\pm 3 \times 10^{-5}$
pMJ24 (*rbo rub*)	6×10^{-5} ($\pm 4 \times 10^{-5}$)	0.78 ($\pm$ 0.20)
pMJ25 (*rbo*)	1×10^{-4} ($\pm 5 \times 10^{-5}$)	0.82 ($\pm$ 0.20)
pMJ26 (*rub*)	4×10^{-5} ($\pm 4 \times 10^{-5}$)	7×10^{-5} ($\pm 4 \times 10^{-5}$)
pJK15 (*rbo*)	0.92 ($\pm$ 0.15)	ND
pJK29 (*rbo rub*)	0.84 ($\pm$ 0.16)	ND
pMJ30 (*dsr*)	6×10^{-5} ($\pm 3 \times 10^{-5}$)	7×10^{-5} ($\pm 4 \times 10^{-5}$)
pMJ28 (*rbo*)[e]	5×10^{-5} ($\pm 4 \times 10^{-5}$)	6×10^{-5} ($\pm 4 \times 10^{-5}$)

[a] Anaerobic cultures of QC 2375 transformed with various plasmids were plated under anaerobic and aerobic conditions, and CFUs were counted.
[b] Survival was calculated as the ratio of the number of colonies under aerobic conditions to those under anaerobic conditions. Colonies were counted after overnight incubation: there was no change in CFU after 48 h. Values are the means of at least three experiments, and standard errors are in parentheses.
[c] ND, not determined
[d] Small colonies after 24 h.
[e] Truncated *rbo* gene.

To clone a *sod* gene from *D. baarsii*, a library was constructed in an *E. coli sodA sodB* mutant deficient in both cytoplasmic SODs. *sodA sodB* strains are supersensitive to paraquat (methyl viologen), a compound which generates superoxide by redox cycling (8). Putative *sod*$^+$ transformants were therefore selected on LB containing 100 μM paraquat, a concentration lethal to pUC transformants.

DNA was extracted from several putative *sod*$^+$ plasmids and used to transform a *sodA sodB recA* mutant under anaerobic conditions. This mutant is unable to grow under aerobic conditions (26, 45). The high steady-state level of superoxide in SOD-deficient mutants leads to DNA damage via a Fenton reaction. Under normal aerobic growth conditions, the homologous recombination system efficiently repairs DNA strand breaks produced by the oxidative damage, allowing the *sodA sodB* mutant to survive in the presence of oxygen, but the double deficiency of SODs and of DNA strand break repair (*recA* or *recB* mutant) is lethal in aerobiosis. Of the plasmids conferring paraquat resistance, one plasmid, pMJ3, restored aerobic growth to the *sodA sodB recA* mutant (Table 2). Sensitivity to UV of the pMJ3 transformant was maintained, indicating the persistence of the defect in recombinational repair. pMJ3 was therefore presumed to carry a *sod*$^+$ gene.

Sequence of the complementing region and homology with the *rbo-rub* operon from *D. vulgaris*. The DNA region in pMJ3 responsible for complementation was mapped by deletion analysis, as summarized in Fig. 1. The 0.7-kb *Sac*I-*Sma*I fragment carried by pMJ12, which apparently contained part of the putative *sod* gene, was sequenced. Two major open reading frames of 381 and 162 bp were found. They were dissimilar to any *sod* gene sequences in data bases but were very similar to the *rbo-rub* operon of *D. vulgaris* (data not shown). Deduced

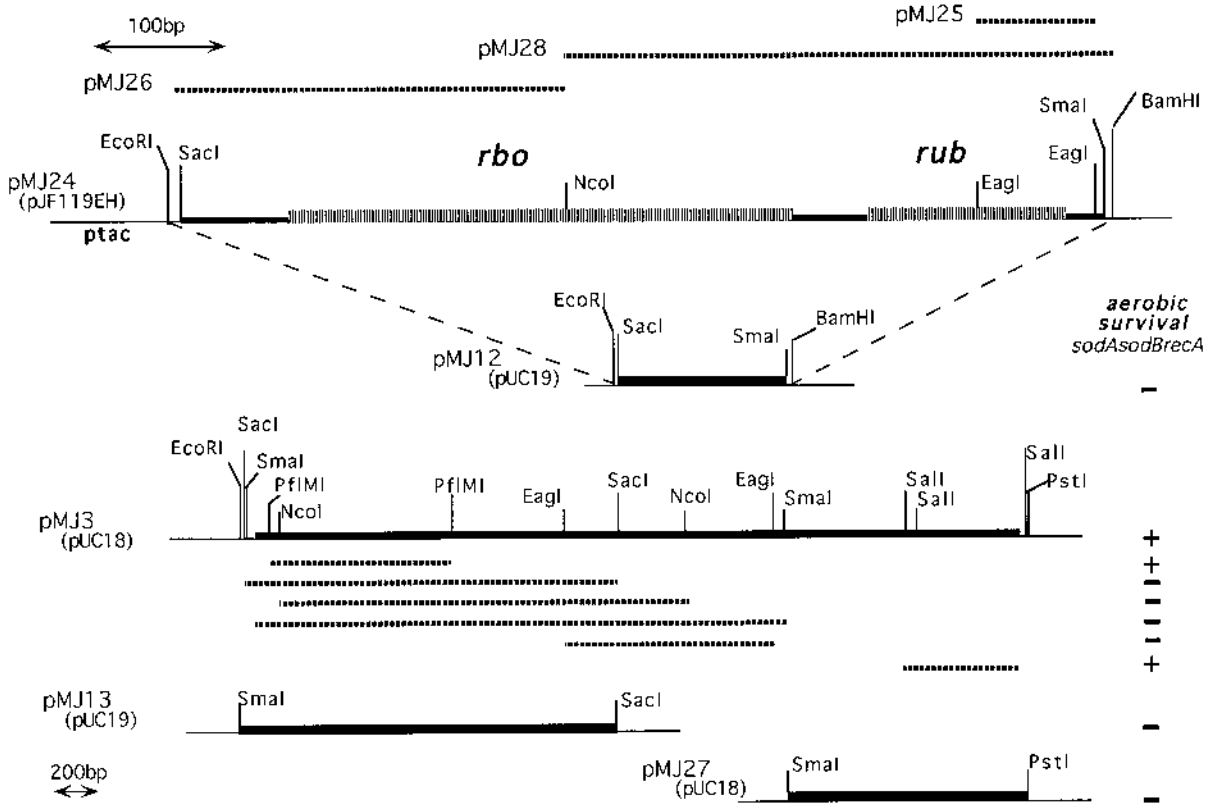

FIG. 1. Plasmids used in the study. DNA inserts (thick black lines) from *D. baarsii* are shown, and the names of vectors carrying the inserts are shown in parentheses after the plasmid names. Restriction map of pMJ3 is shown. Broken lines below represent internal deletions. The ability (+) or inability (−) of a plasmid to restore aerobic survival of a *sodA sodB recA* strain is shown to the right. Plasmids pMJ12, pMJ13, and pMJ27 have been used for sequencing. At the top are pJF119EH derivatives carrying inserts under the ptac control. Striped bars on pMJ24 show the *rbo* and *rub* genes. The broken lines above indicate the regions of pMJ24 that were deleted to create pMJ25, pMJ26, and pMJ28.

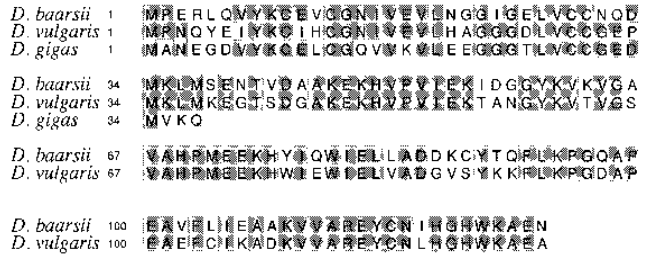

FIG. 2. Amino acid sequence comparison of Rbo proteins from *D. baarsii* and *D. vulgaris* (5) and Dx protein from *D. gigas* (6). The regions of sequence identity are shaded.

amino acid sequences encoded by the two open reading frames showed 70 and 77% identity with the Rbo (Fig. 2) and Rub (data not shown) amino acid sequences of *D. vulgaris* (5), respectively. Putative Shine-Dalgarno sequences are located just upstream of the initiation codons. A large stem-loop structure which is a probable transcription terminator is found downstream of the stop codon of the putative *rub* gene. The noncoding space between the two genes is larger than that between the *rbo* and *rub* genes of *D. vulgaris* (50 bp versus 16 bp) but does not contain an identifiable termination signal, suggesting that as in *D. vulgaris*, the two genes belong to the same operon. Although Rbo and Rub are produced in *E. coli* from their original promoter (Fig. 3, lane 1), no plausible promoter which resembles an *E. coli* consensus sequence was identified within 183 bp of the start codon of *rbo*. Surprisingly, a good sequence promoter was found 11 bp upstream from the *rbo* start codon and overlapping the Shine-Dalgarno sequence. As in *D. vulgaris*, the deduced NH_2-terminal sequence is similar to that of the *dsr* gene product from *D. gigas* (Fig. 2) and an ATG start codon is found in phase just at the end of the *dsr*-like sequence.

Production of the *rbo* gene product from pMJ3 is responsible for complementation. To determine whether the *rbo* or *rub* gene product was responsible for the complementation, constructs were made in which the *rbo* and *rub* genes were separately expressed under the control of the IPTG-inducible ptac promoter. The *rbo-rub* region was introduced into pJF119EH under ptac control (pMJ24), and either *rbo* or *rub* gene was partially deleted to give pMJ26 and pMJ25, respectively (Fig. 1 and Table 1). Production of proteins was verified in maxicells (Fig. 3). A 14-kDa polypeptide product corresponding to Rbo

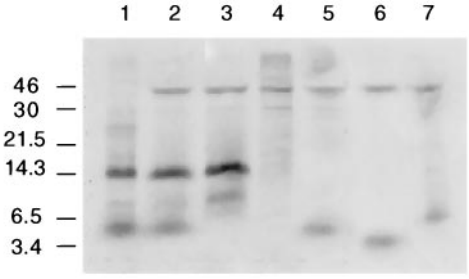

FIG. 3. Autoradiography of a 15% tricine–sodium dodecyl sulfate-polyacrylamide gel of polypeptides synthesized in the maxicell system in strain CSR603 transformed with plasmid pMJ3 (lane 1), pMJ24 (lane 2), pMJ25 (lane 3), pJF119EH (lane 4), pMJ26 (lane 5), pMJ30 (lane 6), or pMJ28 (lane 7). Strains were grown in the presence of 2 mM IPTG and 500 μg of ampicillin per ml. The positions (in kilodaltons) of Rainbow molecular mass markers (Amersham) are indicated to the left.

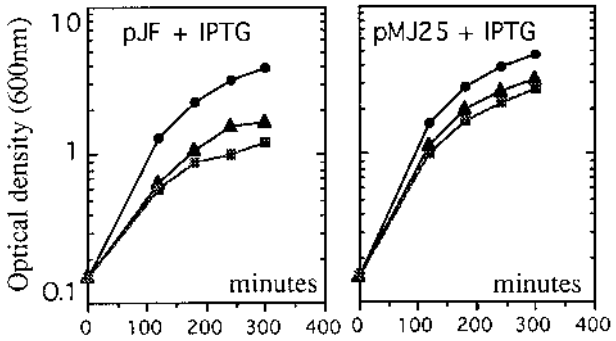

FIG. 4. Effect of Rbo on the paraquat sensitivity of *sodA sodB* strains. *sodA sodB* strains carrying the plasmid vector pJF119EH (pJF) and plasmid pMJ25 carrying the *rbo* gene were grown in the presence of paraquat. Paraquat was added to exponential cultures grown with 2 mM IPTG at 0 (circle), 50 (triangle), and 100 (square) μM.

was found in pMJ3, pMJ24, and pMJ25, and a 6-kDa product (rubredoxin) was found in pMJ3, pMJ24, and pMJ26.

Only conditions which led to Rbo production restored normal growth to the *sodA sodB recA* mutant in aerobiosis (Table 2). Similarly, resistance to paraquat was enhanced when Rbo was produced from pMJ25 (Fig. 4) or pMJ24 (data not shown) grown with IPTG but not when Rub was produced from pMJ26 (data not shown).

Overproduction of Rbo suppresses the increase of spontaneous mutagenesis in the *E. coli* SOD-deficient strain. Enhancement of Fenton reaction in strains lacking SOD leads to mutagenic DNA lesions, resulting in an increased oxygen-dependent spontaneous mutagenesis (13). Spontaneous mutagenesis, measured as the frequency of Rif^s to Rif^r mutations, returned to near-wild-type levels when Rbo was produced in SOD-defective strains (Fig. 5).

Overproduced Rbo restores growth of the *sodA sodB* mutant in minimal medium with glucose and enhances its growth on succinate and on gluconate. Excess superoxide can cause direct damage. The best superoxide targets identified in vivo are [4Fe-4S] clusters which are found in dehydratase-lyase enzymes (15). These superoxide-sensitive enzymes, including de-

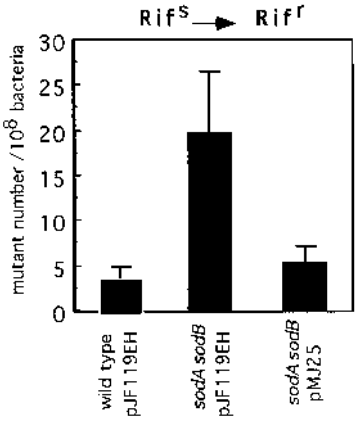

FIG. 5. Effect of Rbo on superoxide-enhanced mutagenesis. The wild-type strain was GC4468, and the *sodA sodB* mutant was QC 1799. The strain carrying pMJ25 was grown with 2 mM IPTG. Values are the means from five experiments. Bars represent standard errors. Differences are significant (*t* test) for the *sodA sodB* mutant carrying pJF119EH versus the *sodA sodB* mutant carrying pMJ25 ($P < 0.001$) but not for the wild type carrying pJF119EH versus the *sodA sodB* mutant carrying pJF119EH ($P = 0.15$)

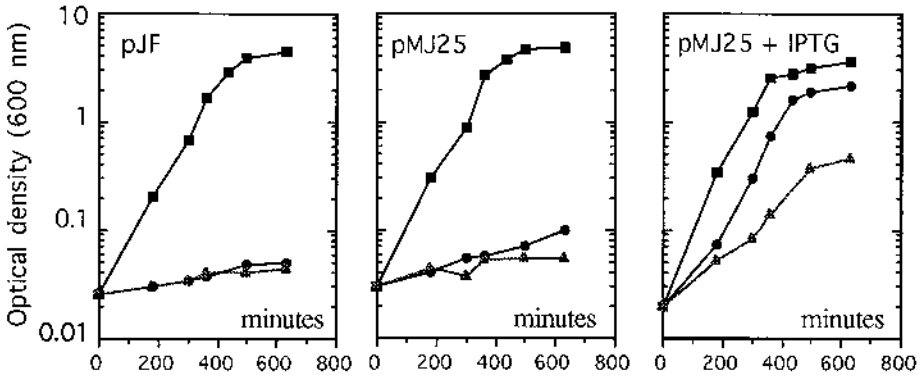

FIG. 6. Effect of Rbo on growth of the *sodA sodB* strain in minimal medium. *sodA sodB* strains carrying the plasmid vector pJF119EH (pJF) and plasmid pMJ25 were grown overnight in M9 medium plus glucose and all amino acids, thiamine, and biotin (enriched M9) and diluted 1/200 in preheated M9 glucose (triangles), enriched M9 without branched amino acids (circles), and enriched M9 (squares). IPTG at 2 mM was added. Growth of the strain harboring pJF119EH was not modified by the addition of IPTG (not shown).

hydroxyacid dehydratase, 6-phosphogluconate dehydratase, aconitase, and fumarases are totally or partially inactivated in SOD-lacking strains, leading to specific SOD^- phenotypes (14, 17, 18, 27, 28). Thus, inactivation of dehydroxyacid dehydratase, which is involved in the biosynthetic pathway of branched amino acids, results in the inability of *sodA sodB* mutants to grow in minimal medium unless these amino acids are provided (8). Expression of *rbo* restored growth in the absence of branched amino acids and to a lesser extent in the absence of all amino acids (Fig. 6). Similarly, impairment of growth of the *sodA sodB* strain on succinate and on gluconate due to aconitase and 6-phosphogluconate inactivation, respectively, was nearly suppressed by *rbo* expression (Fig. 7A and B, respectively). Thus, *rbo* expression suppressed direct and indirect (via Fenton reaction) damage produced by superoxide equally.

The *rbo* gene product from *D. vulgaris*, but not the *dsr* gene product from *D. gigas* complements an *E. coli* SOD-deficient mutant. The similarity between the deduced protein sequences of Rbo from *D. baarsii* and *D. vulgaris* led us to suppose that Rbo from *D. vulgaris* would also complement SOD deficiency. Anaerobic transformation of a *sodA sodB recA* mutant with the plasmids pJK29 and pJK15, in which *rbo* from *D. vulgaris* is expressed from its own promoter completely restored aerobic growth (Table 2).

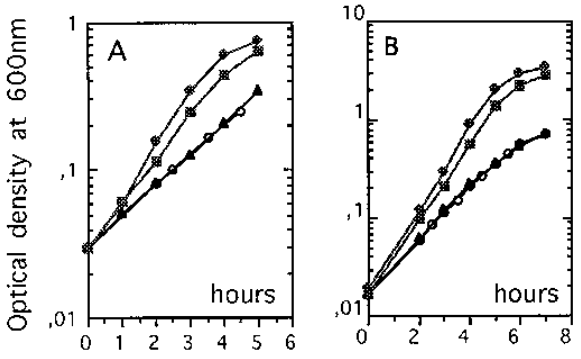

FIG. 7. Effect of Rbo on growth of the *sodA sodB* strain on succinate and gluconate as carbon sources. Cultures grown overnight in M9 medium plus glucose containing all amino acids, thiamine, and biotin were diluted 1/200 in the same medium but with succinate (A) or gluconate (B) instead of glucose. IPTG at 2 mM was added. The *sodA sodB* mutant was QC 1799, and the wild-type strain was GC4468. Symbols: ▲, *sodA sodB* mutant carrying pJF119EH; ○, *sodA sodB* mutant carrying pMJ25; ■, *sodA sodB* mutant carrying pMJ25 in the presence of IPTG; ◆, wild-type strain carrying pJF119EH.

The Rbo protein has NH_2-terminal 37-amino-acid domain similar to desulforedoxin (*dsr*), a small (4-kDa) redox carrier from *D. gigas*, with unknown physiological function (6) (Fig. 2). To determine whether the NH_2-terminal domain was sufficient to confer to Rbo the ability to complement SOD deficiency, plasmids were constructed in which the *dsr* gene (pMJ30) and a truncated *rbo* gene (pMJ28) are expressed from the ptac promoter (Fig. 1 and 3). Transformation of the *sodA sodB recA* strain with pMJ30 and pMJ28 did not restore aerobic growth upon IPTG induction (Table 2). Thus, the desulforedoxin-like domain alone cannot complement a SOD deficiency.

DISCUSSION

Defects in SOD lead to an increase in the superoxide steady-state level (24). This has been shown in various organisms to impair growth severely. Superoxide, however, is not extremely reactive per se, and few superoxide targets have been identified in vivo. Indeed superoxide toxicity is mostly due to an indirect effect. Excess superoxide increases the intracellular pool of reduced iron which together with hydrogen peroxide produces, via a Fenton reaction, highly reactive hydroxyl radicals. Various pieces of evidence, including an increase in DNA damage in SOD-deficient mutants which could not be due to direct action of superoxide (13, 45), suggest that the superoxide-enhanced Fenton reaction occurs in vivo. However, its process is unclear. In vitro superoxide can reduce ferric iron, but cells contain numerous and abundant reductants, and it is unlikely that there is any intracellular ferric iron available to be reduced by superoxide (26, 29). Thus, excess superoxide presumably promotes hydroxyl radical production in vivo by some more-specific pathways. Liochev and Fridovich (29) suggested that degradation of [4Fe-4S] clusters by superoxide oxidation and the subsequent iron release (15, 37) could increase the supply of intracellular free iron, allowing the Fenton reaction to proceed.

We show that overproduction of the *rbo* gene product of *Desulfovibrio* species efficiently suppresses the deleterious effects because of a lack of SOD in *E. coli.*

rbo was first characterized in *D. vulgaris* (strain Hildenborough) as part of an operon, *rbo-rub*, of which the downstream gene specifies rubredoxin (5). The name rubredoxin-oxidoreductase (Rbo) was tentatively given because of the

coordinated expression of the two genes, the likely redox function of Rbo, and the absence of a known redox partner of rubredoxin (46). The Rbo protein is 14 kDa, and its NH_2-terminal amino acid sequence is very similar to the sequence of desulforedoxin (Dx), a small (4-kDa) redox carrier protein from *D. gigas*. The nucleotide sequences of the two genes led Brumlick et al. to propose that *rbo* may have arisen by gene fusion (6). During isolation of iron-sulfur proteins from *D. desulfuricans*, new non-heme iron proteins very similar to Rbo were purified and named desulfoferrodoxins. They contain two active centers with mononuclear iron in different coordination environments and oxidation states (33, 42). The ferric site is similar to that in desulforedoxin, with a distorted tetrahedral sulfur coordination of iron, and the ferrous site is octahedrally coordinated with predominantly nitrogen- and/or oxygen-containing ligands. These proteins cannot accept electrons from reduced pyridine nucleotides. *D. vulgaris* possesses two desulfoferrodoxins, a *D. desulfuricans* desulfoferrodoxin type and the *rbo* gene product (33). The redox partners of those proteins have not been identified.

The sequence of the *rbo-rub* operon in *D. baarsii* and the results of its expression in maxicells suggest an organization similar to that in *D. vulgaris*, although these two species have distantly related metabolic pathways. As in *D. vulgaris*, the NH_2-terminal sequence of the *rbo* gene product is similar to that of desulforedoxin. However, this domain alone is not enough to complement SOD deficiency.

By which mechanism(s) does Rbo permit SOD-deficient strains to escape damage due to superoxide? Rbo may reduce the steady-state level of superoxide to a harmless level, either by reacting with it or by interfering with its production. In preliminary assays, only by loading activity gels with very large amounts of proteins could we (barely) detect a faint inhibition of the reduction of nitro blue tetrazolium (NBT) by superoxide (unpublished data). It is unclear whether such a low superoxide scavenging activity could account for the rescue of SOD^- strains. There is currently no data available indicating the threshold at which SOD activity suppresses the SOD^- phenotype in *E. coli*. Rbo may counteract the deleterious effects of the high level of superoxide in SOD^- mutants. Rbo complemented both the defects due to direct superoxide damage and those due to a superoxide-enhanced Fenton reaction. Therefore, it is not possible that overproduction of Rbo protected *sodA sodB* strains just by scavenging iron. Rbo may reduce the superoxide-oxidized [4Fe-4S] clusters. This would restore activity to the superoxide-sensitive enzymes and by avoiding the iron release that follows [4Fe-4S] cluster degradation, suppress the Fenton reaction.

Whether the Rbo properties reported in this study are relevant to the physiological function of Rbo in sulfate-reducing bacteria and their aerotolerance is not known. SOD activity has been reported in *D. vulgaris* (20), but neither the gene nor the protein have been characterized. In *D. desulfuricans*, which possesses proteins similar to Rbo, a SOD was clearly characterized (21). Thus, it is unlikely that Rbo is a SOD analog in these bacteria. It might, however, be involved in oxygen tolerance (15a).

Further studies will be necessary to determine whether other organisms have Rbo-like activities. Whatever the mechanism by which Rbo permits the bacterium to overcome the lack of SOD, it appears to be a new way to fight oxidative stress and an interesting tool that may well shed light on the mechanisms of superoxide toxicity.

ACKNOWLEDGMENTS

We thank G. Voordouw for providing plasmids and for communicating unpublished results.

This work was supported in part by the cooperative program France-Uruguay ECOS (Evaluation-Orientation de la cooperation Scientifique) U93B01 and in part by grant 6791 from the Association pour la Recherche sur le Cancer. M. J. Pianzzola had a 3-year fellowship from PEDECIBA (project URU 84/002) and was partly supported by CSIC.

REFERENCES

1. **Abdollahi, H., and J. W. T. Wimpenny.** 1990. Effects of oxygen on the growth of *Desulfovibrio desulfuricans*. J. Gen. Microbiol. **136:**1025–1030.
2. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **40:**260–296.
3. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44:**276–287.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Brumlik, M. J., and G. Voordouw.** 1989. Analysis of the transcriptional unit encoding the genes for rubredoxin (*rub*) and a putative rubredoxin oxidoreductase (*rbo*) in *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. **171:** 4996–5004.
6. **Brumlik, M. J., G. Leroy, M. Bruschi, and G. Voordouw.** 1990. The nucleotide sequence of the *Desulfovibrio gigas* desulforedoxin gene indicates that the *Desulfovibrio vulgaris rbo* gene originated from a gene fusion event. J. Bacteriol. **172:**7289–7292.
7. **Canfield, D. E., and D. J. Des Marais.** 1991. Aerobic sulfate reduction in microbial mats. Science **251:**1471–1473.
8. **Carlioz, A., and D. Touati.** 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J. **5:**623–630.
9. **Chen, L., M. Y. Liu, J. Legall, P. Fareleira, H. Santos, and A. V. Xavier.** 1993. Purification and characterization of an NADH-rubredoxin oxidoreductase involved in the utilization of oxygen by *Desulfovibrio gigas*. Eur. J. Biochem. **216:**443–448.
10. **Chen, L., M. Y. Liu, J. LeGall, P. Fareleira, H. Santos, and A. V. Xavier.** 1993. Rubredoxin oxidase, a new flavo-hemo-protein, is the site of oxygen reduction to water by the "strict anaerobe" *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. **193:**100–105.
11. **Cypionka, H., F. Widdel, and N. Pfennig.** 1985. Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. FEMS Microbiol. Ecol. **31:**39–45.
12. **Dilling, W., and H. Cypionka.** 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiol. Lett. **71:**123–128.
13. **Farr, S. B., R. D'Ari, and D. Touati.** 1986. Oxygen-dependent mutagenesis in *E. coli* lacking superoxide dismutase. Proc. Natl. Acad. Sci. USA **83:**8268–8272.
14. **Flint, D. H., M. H. Emptage, and J. R. Guest.** 1992. Fumarase A from Escherichia coli: purification and characterization as an iron-sulfur cluster containing enzyme. Biochemistry **31:**10331–10337.
15. **Flint, D. H., J. F. Tuminello, and M. H. Emptage.** 1993. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J. Biol. Chem. **268:** 22369–22376.

15a. **Fu, R., and G. Voordouw.** Personal communication.

16. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tac*P expression vector. Gene **48:**119–131.
17. **Gardner, P. R., and I. Fridovich.** 1991. Superoxide sensitivity of the *Escherichia coli* 6-phosphogluconate dehydratase. J. Biol. Chem. **266:**1478–1483.
18. **Gardner, P. R., and I. Fridovich.** 1991. Superoxide sensitivity of the *Escherichia coli* aconitase. J. Biol. Chem. **266:**19328–19333.
19. **Gregory, E. M., W. E. C. Moore, and L. V. Holdeman.** 1978. Superoxide dismutase in anaerobes: survey. Appl. Environ. Microbiol. **35:**988–991.
20. **Hardy, J. A., and W. A. Hamilton.** 1981. The oxygen tolerance of sulfate-reducing bacteria isolated from North sea waters. Curr. Microbiol. **6:**259–262.
21. **Hatchikian, E. C., and Y. A. Henry.** 1977. An iron containing SOD from the strict anaerobe *Desulfovibrio desulfuricans*. Biochimie **59:**153–161.
22. **Hatchikian, E. C., J. Le Gall, and G. R. Bell.** 1977. Significance of superoxide dismutase and catalase activities in the strict anaerobes, sulfate-reducing bacteria, p. 169–172. *In* A. M. Michelson, J. M. McCord, and I. Fridovich (ed.), Superoxide and superoxide dismutases. Academic Press, London.
23. **Hewitt, J., and J. G. Morris.** 1975. Superoxide dismutase in some obligately anaerobic bacteria. FEBS Lett. **50:**315–318.
24. **Imlay, J. A., and I. Fridovich.** 1991. Assay of metabolic superoxide production in *Escherichia coli*. J. Biol. Chem. **266:**6957–6965.
25. **Jansen, K., R. K. Thauer, F. Widdel, and G. Fuchs.** 1984. Carbon assimilation pathways in sulfate-reducing bacteria. Formate, carbon dioxide, carbon

monooxide, and acetate assimilation by *Desulfovibrio baarsii*. Arch. Microbiol. **138:**257–262.
26. **Keyer, K., A. Strohmeier Gort, and J. A. Imlay.** 1995. Superoxide and the production of oxidative DNA damage. J. Bacteriol. **177:**6782–6790.
27. **Kuo, C. F., T. Mashino, and I. Fridovich.** 1987. α, β Dihydroxyisovalerate dehydratase: a superoxide sensitive enzyme. J. Biol. Chem. **262:**4724–4727.
28. **Liochev, S. I., and I. Fridovich.** 1992. Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *soxRS* regulon. Proc. Natl. Acad. Sci. USA **89:**5892–5896.
29. **Liochev, S. I., and I. Fridovich.** 1994. The role of O2°- in the production of HO°: in vitro and in vivo. Free Radical Biol. Med. **16:**29–33.
30. **Marmur, J.** 1961. A procedure for the isolation of desoxyribonucleic acid from microorganism. J. Mol. Biol. **3:**208–218.
31. **Marschall, C., P. Frenzel, and H. Cypionka.** 1993. Influence of oxygen on sulfate reduction and growth of sulfate reducing bacteria. Arch. Microbiol. **159:**168–173.
32. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Moura, I., P. Tavares, J. J. G. Moura, N. Ravi, H. Huynh, M.-Y. Liu, and J. LeGall.** 1990. Purification and characterization of desulfoferrodoxin. J. Biol. Chem. **265:**21596–21602.
34. **Nakayama, K.** 1994. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. J. Bacteriol. **176:** 1939–1943.
35. **Natvig, D. O., K. Imlay, D. Touati, and A. Hallewell.** 1987. Human copper-zinc superoxide dismutase complements superoxide dismutase-deficient *E. coli* mutants. J. Biol. Chem. **262:**14697–14701.
36. **Odom, J. M., and J. Rivers Singleton.** 1993. The sulfate-reducing bacteria: contemporary perspectives. *In* T. D. Brook (ed.), Contemporary science. Springer-Verlag, New York.
37. **Rouault, T. A., and R. D. Klausner.** 1996. Iron-sulfur clusters as biosensors of oxidants and iron. Trends Biochem. Sci. **21:**174–177.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137:**692–693.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Santos, H., P. Fareleira, A. V. Xavier, L. Chen, M. Y. Liu, and J. LeGall.** 1993. Aerobic metabolism of carbon reserves by the "obligate anaerobe" *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. **195:**551–557.
42. **Tavares, P., N. Ravi, J. J. G. Moura, J. LeGall, Y.-H. Huang, B. R. Crouse, M. K. Johnson, B. H. Huynh, and I. Moura.** 1994. Spectroscopic properties of desulfoferrodoxin from Desulfovibrio desulfuricans (ATCC 27774). J. Biol. Chem. **269:**10504–10510.
43. **Touati, D.** 1983. Cloning and mapping of the manganese superoxide dismutase gene (*sodA*) of *E. coli* K-12. J. Bacteriol. **155:**1078–1087.
44. **Touati, D.** Superoxide dismutases in bacteria and pathogen protists. *In* J. Scandalios (ed.), Oxidative stress and the molecular biology of antioxidant defense. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, in press.
44a. **Touati, D.** Unpublished data.
45. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ *fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:** 2305–2314.
46. **Voordouw, G.** 1993. Molecular biology of sulfate reducing bacteria, p. 88–130. *In* J. M. Odom and J. Rivers Singleton (ed.), The sulfate-reducing bacteria: contemporary perspectives. Springer-Verlag, New York.
47. **Wall, J. D., B. J. Rapp-Giles, M. F. Brown, and J. A. White.** 1990. Response of *Desulfovibrio desulfuricans* colonies to oxygen stress. Can. J. Microbiol. **36:**400–408.
48. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3353–3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria. Ecophysiology, isolation, identification, application. Springer-Verlag, New York.