ABSTRACT

JI, MIKYOUNG LEE. Functional evaluation of the mechanisms of reactive oxygen detoxification in the hyperthermophilic archaeon *Pyrococcus furiosus* using complementation studies. (Under the direction of Amy Grunden)

*Pyrococcus furiosus* is an obligatory anaerobic hyperthermophilic archaeon (optimal growth temperature is 100°C) which can be isolated from deep and shallow marine hydrothermal vent systems. Initially, due to the obligatory anaerobic nature of *P. furiosus*, it was thought that this organism did not possess the ability to detoxify reactive oxygen species. Recent studies, however, have shown that *P. furiosus* produces the enzyme, superoxide reductase (SOR) which catalyzes the reduction of the reactive oxygen specie, superoxide, to hydrogen peroxide. This is in contrast to aerobic organisms which use superoxide dismutase (SOD) to convert superoxide to hydrogen peroxide and O₂. Surprisingly, *P. furiosus* SOR, unlike many *P. furiosus* enzymes, was shown to function at low temperature (<25°C). A model for superoxide reduction by SOR has been developed by Jenney et al. (1999) where the electrons used by SOR to reduce superoxide are supplied by the Fe-S protein, rubredoxin (Rd) and Rd is reduced by the oxidoreductase, NAD(P)H-rubredoxin oxidoreductase (NROR). Previous studies have shown that SORs from *Desulfovibrio vulgaris* and *Desulfovibrio gigas* can complement defects in *Escherichia coli* superoxide dismutases (sodA, sodB). In addition, *E. coli* has a gene, *norW* that is homologous to *P. furiosus* NROR (42% similarity) and also has the gene, *norV* which codes for a protein containing a rubredoxin domain that is 49% similar to *P. furiosus* Rd. The *norV* and *norW* genes exist together as part of an operon. In this
study, *P. furiosus* SOR, Rd, NROR genes which are thought to be involved in SOR detoxification of $O_2^-$ were cloned and expressed in *E. coli* strain JM105. Using complementation studies, *P. furiosus* SOR was shown to detoxify $O_2^-$ generated in the *E. coli sodA, sodB* strain (NC906) and restore growth under formerly nonpermissive conditions. *E. coli* strains with deletion of the putative flavorubredoxin (*norV*) and NROR (*norW*) genes have been constructed and verified by PCR. *P. furiosus* SOR has shown a low level of complementation in the *E. coli sodA, sodB*, and *norV* mutant suggesting that the *E. coli* flavorubredoxin (Fl-Rd) may be supplying the electrons to recombinant *P. furiosus* SOR in vivo.
Functional Evaluation of the Mechanisms of Reactive Oxygen Detoxification in the Hyperthermophilic Archaean *Pyrococcus furiosus* Using Complementation Studies

by

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INTRODUCTION

Reactive Oxygen Species Detoxification in Aerobes

Oxygen is a required element for most living organisms. Without it, much of the life on Earth today would not exist. Oxygen is used in aerobic organisms as a final electron acceptor in the respiratory electron transport chain, which ultimately provides energy for the cell via oxidative phosphorylation. Oxygen is also required as a substrate in many biosynthetic reactions but does pose a risk of generating reactive oxygen species (Hassan & Schrum, 1994). These reactive oxygen species (ROS) include superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (HO’), which are all partially reduced intermediates of oxygen. In the normal operation of the electron transport chain, O₂ is reduced directly to H₂O by the cytochrome oxidase system. However, some intermediates along the chain can transfer a single electron to oxygen to produce an O₂⁻ radical and a further single electron transfer generates H₂O₂ and HO’ (O₂ → O₂⁻ → H₂O₂ → HO’ + H₂O → H₂O). These oxygen species are reactive to various kinds of cellular structures and can for instance cause direct oxidation and inactivation of enzymes, receptors, and ion pumps, induce mutations by damaging DNA molecules, and destroy cellular membranes through the peroxidation of membrane lipids (Punchard & Kelly, 1996). Due to the potentially destructive nature of the reduced oxygen intermediates, aerobic cells must have efficient protective mechanisms to detoxify the reactive oxygen species. Several enzymes are present that repair cells damaged by reactive oxygen species and that modify the reactive oxygen species to less reactive forms (Fridovich, 1998). One of the most important oxygen detoxifying enzymes is superoxide dismutase (SOD), which converts
two superoxide anions to hydrogen peroxide and water molecules by dismutation. The hydrogen peroxide is then immediately converted into water molecules by an enzyme called catalase as shown in Figure 1.

There are three known SODs in *Escherichia coli*: MnSOD, FeSOD and CuZnSOD. All the SODs contain specific metal centers in their active sites to mediate electron transfer between the reactants (Fridovich, 1995). The iron-containing enzyme (FeSOD) and manganese-containing enzyme (MnSOD) are located in the cytoplasm and the copper-zinc-containing enzyme (CuZnSOD) is in the periplasmic space in *E. coli*. MnSOD is a basic dimeric protein having 205 amino acid residues per subunit and a molecular weight (M.W.) of 22.9 kDa, while FeSOD is an acidic dimeric protein having 192 amino acid residues per subunit and a M.W. of 21.1 kDa (Beyer et al., 1989). These enzymes have significant levels of identity in their amino acid sequences and have been shown to assume very similar structural folds, suggesting that they have evolved from a common ancestral protein (Fee, 1991; Stallings et al., 1984; Hassan, 1989; Carlioz et al., 1988). The copper-zinc superoxide dismutase (CuZnSOD) in *E. coli* is a homodimer having 173 amino acid residues and a M.W. of 15.7 kDa per subunit (Imlay & Imlay, 1996), It contains one atom of both Cu and Zn per subunit, where Cu is known to function in enzyme catalysis and Zn serves a structural role to stabilize the enzyme (Hassan, 1989). The enzyme is unrelated to Fe-MnSOD in either sequence homology or secondary structure and it plays a protective role against superoxide generated outside of the cell or in the periplasm itself as opposed to the cytoplasmic localization of the Fe or MnSODs (Battistoni et al., 2000).
It had been thought that no life could survive without oxygen until studies conducted by Louis Pasteur in 1861 showed that yeast could thrive under anaerobic conditions in fermenting cultures. Pasteur determined that oxygen inhibits the process of fermentation, and he went on to differentiate microorganisms into ‘aerobic’, ‘facultative’ and ‘anaerobic’ based on whether the organisms absolutely required oxygen for growth, could grow with or without oxygen, or could not survive in the presence of oxygen, respectively. In the 1880’s, work was initiated in the development of techniques for growing anaerobes in pure culture, and in 1950, Robert Hungate had devised techniques that could be routinely used for the isolation of pure cultures of anaerobes.

It had been thought that strict anaerobes are by necessity anaerobic because they do not have any mechanisms to protect their cells from oxygen toxicity. This assumption that obligate anaerobes lacked oxygen detoxification capabilities arose from the observations that these organisms typically contain little or no cellular SOD activity (McCord et al., 1971). Therefore, sensitivity of anaerobes to oxygen suggested the inability to detoxify superoxide molecules due to a lack of the required enzymes. However, most of the anaerobic organisms, which often lack these classical ROS defense enzymes, show various degrees of tolerance to oxygen when they are occasionally exposed to oxygen in their environments.

**Hydrothermal Vent Organisms and the Discovery of a New Enzyme for Detoxification of Superoxide**

With the discovery of deep-sea hydrothermal vents, a new, unusual habitat for life was found at the bottom of the ocean, where the environment in the hydrothermal vent
system is strictly anoxic and very hot (>350 °C). Interestingly, observations indicated that microbes from hydrothermal vents are able to survive exposure to oxygen under conditions of cold temperatures (~4 °C) (Marteinsson et al., 1997), suggesting that these organisms possess some form of oxygen detoxification mechanism.

Researchers once thought that no living organisms could survive around hydrothermal vents located on the ocean floor since the environment was judged to be too extreme for life, as they contained toxic chemicals, high temperature, enormous pressure, extreme acidity, and total darkness. However, in the late 1970’s, scientists discovered extensive communities of organisms such as six foot, red-tipped tube worms, large white clams, yellow mussels and pale crabs that congregate around hydrothermal vents in the deep sea. These vent chimneys are formed by cold seawater penetrating through cracks in the ocean floors and interacting with heated and chemically altered rocks of volcanic crust. This modified hot liquid is forced back to the ocean floor for tens to hundreds of meters to become plume spreads. By mixing with cold, oxygenated water, deposits of metal precipitation in the plume spread, creating chimney like structures called hydrothermal vents (Prieur et al., 2001). Vent fluids contain gases, especially hydrogen sulfide, and microbes utilizing hydrogen sulfide as their energy source. Microbes from the vents serve as the primary food source for clams, mussels and other invertebrates, thereby forming the base of food chain in this ecosystem (Van Dover et al., 2002).

The microbes found in deep-sea hydrothermal vents are predominantly anaerobic hyperthermophilic species that can grow optimally above 80° C (Stetter, 1996). *Pyrococcus furiosus*, one of the microbes found in the vent, is classified as a strict anaerobic hyperthermophilic heterotroph, which grows at 100°C optimally and ferments
carbohydrates and peptides producing organic acids, CO$_2$, H$_2$, and H$_2$S as by-products (Fiala & Stetter, 1986). *P. furiosus* is classified as a member of the domain Archaea, which was identified as the third domain of life based on the 16S r RNA sequence-derived phylogenetic trees developed by Carl Woese (Woese *et al*., 1990). Phylogenetic trees constructed using 16S rRNA homology have indicated that thermophiles are in the most deeply rooted and shortest lineages, and are therefore regarded as the most primitive and slowest evolving organisms (Stetter, 1996).

For anaerobic hyperthermophilic microbes that live in deep-sea vents, exposure to cold, oxygen-containing water in their environment was thought to cause cell death. However, studies later showed that anaerobic thermophilic microbes including *Pyrococcus furiosus* could survive during long periods of time after exposure to cold, oxygenated seawater (Huber *et al*., 1990). However, the mechanisms used by the thermophilic anaerobes to survive the oxygen exposure had not been identified at the time.

In aerobic organisms, the primary source of the superoxide comes from the one-electron reduction of dioxygen by the components of electron transport chains. Superoxide is primarily produced as a result of oxygen reacting with flavin, a common cofactor found in membrane-bound electron carriers in the electron transport chain. The normal activity of flavins is to transfer electrons from univalent redox cofactors, such as metal centers. When oxygen molecules diffuse into active sites of flavoproteins, reduced flavins transfer a single electron to oxygen producing superoxide, flavosemiquinone, and even hydrogen peroxide. This superoxide easily attacks the [4Fe-4S] clusters within cellular proteins. The iron-sulfur clusters oxidized by superoxide are unstable. They
release iron into the cytoplasm forming inactive \([3\text{Fe}-4\text{S}]^+\) centers in the enzymes. The free iron is maintained in reduced forms, and it further reduces the hydrogen peroxide creating hydroxyl radicals, which bind nonspecifically to every type of biomolecule, such as DNA, RNA, protein, polysaccharide, and lipids (Imlay, 2002). Furthermore, in anaerobes, which often feature enzymes containing flavoproteins in their central metabolic pathways, superoxide generated by flavoproteins and oxygen can inactivate key metabolic enzymes that contain iron-sulfur clusters (Pan & Imlay, 2001).

In archaea, including *P. furiosus*, a family of ferredoxin-dependent enzymes that oxidize glycerol aldehyde-3-phosphate, formaldehyde, and glyceraldehyde contain tungsten and pterin cofactors as well as iron-sulfur clusters. These are irreversibly damaged by oxygen in vitro (Imlay, 2001). Thus, superoxide is produced by reactive interaction between oxygen molecules and flavoproteins and ultimately causes the inactivation of central catabolic pathways in strict anaerobes.

For most anaerobes that live in an environment transiently exposed to air, detoxification of reactive oxygen species is necessary for their survival. They show various degrees of tolerance to oxygen, and SOD and/or catalase enzymes are generally not present in their genomes. Therefore, there has been much interest in determining what mechanism other than SOD is being used by these organisms to detoxify oxygen.

The discovery of the detoxification of reactive oxygen species in anaerobes began with the isolation of two iron-sulfur containing proteins named desulfoferrodoxin (Dfx) from *Desulfovibrio desulfuricans* and neelaredoxin (Nlr) from *Desulfovibrio gigas* in the early 1990’s (Moura *et al.*, 1990; Chen *et al.*, 1994). Dfx has two mononuclear iron centers: center I and center II. Center I is located in the N-terminal region of Dfx and has
ferric iron coordinated by four cysteines. Center II has ferrous iron bound in an octahedral geometry to four histidines in the equatorial plane, and one cysteine and one glutamate in axial positions. Center II of Dfx is homologous to Nlr in *D. gigas* while center I of Dfx is similar to desulfurodoxin (Dx) in *D. gigas* (Abreu *et al.*, 2002). It is known that the center II of Dfx and Nlr are the sites where superoxide is bound. The enzyme’s function was first studied in 1996 in the laboratory of Danièle Touati. Dfx was cloned from *Desulfovibrio baarsii* and it showed complementation of SOD activity in *E. coli* SOD deficient mutants. Soon after, Liochev and Fridovich (1997) proposed that Dfx catalyzes the reduction of superoxide rather than dismutation at the expense of cellular reductant such as NAD(P)H. Later, this was confirmed as an oxidoreductase by Lombard *et al.* (2000a).

In 1999, Jenny *et al.* identified SOD activity in *P. furiosus*. The putative SOD was purified and was determined to have a M.W. of 14.3 kDa. The gene encoding this protein was identified using N-terminal sequencing and was found to contain 124 amino acids. It shows high homology to Dfx and Nlr of the sulfate reducing bacteria. High SOD activity was measured by a standard SOD assay at 25°C. However, there is a fundamental difference in terms of enzyme property. In the assay, an increasing amount of bovine SOD inhibited cytochrome c reduction, which means that bovine SOD reduces the superoxide, and a similar pattern was observed in the case of putative SOD in *P. furiosus*. However, when an excess amount of bovine SOD is added to the reaction mix, no further effect had been observed, while an excess amount of *P. furiosus* “SOD” caused oxidation of the reduced cytochrome c (Figure 2). Therefore, it was realized that *P. furiosus* “SOD”
is an oxidoreductase, not a dismutase and has accordingly been named superoxide oxidoreductase (SOR).

In the process of identifying and biochemically characterizing *P. furiosus* SOR, the small Fe-containing redox protein (~5.3kDa) rubredoxin (Rd) was identified as a physiological electron carrier for SOR reduction (Jenney *et al.*, 1999). The Rd gene is located next to SOR in the *P. furiosus* genome, and is reduced by an NAD(P)H dependent oxidoreductase (NROR) using NAD(P) H as its electron donor (Auchere, 2002). NROR is a monomer with a M.W. of ~45 kDa. and contains one flavin adenine dinucleotide (FAD) per mole. Its physiological role is known to be reduction of rubredoxin in *P. furiosus* (Ma & Adams, 1999). A model for the detoxification of reactive oxygen species by SOR has been proposed and is illustrated in Figure 3. In this model, the direct electron donor to SOR is Rd, and the Rd is in turn reduced through a second electron donor, most likely NAD(P)H oxidoreductase (NROR). The further conversion of hydrogen peroxide to water molecules is thought to occur via peroxidase as genes encoding peroxidases have been identified in the *P. furiosus* genome (Jenney *et al.*, 1999).

Most of the *P. furiosus* enzymes are not very active below 80°C, but SOR, NROR, and Rd are still active at low temperature (< 25°C) (Ma & Adams, 1999, Grunden *et al.* 2003), which would obviously be advantageous for the ROS detoxification in *P. furiosus* since *P. furiosus* lives in an environment in which it would periodically encounter oxygen-containing cold seawater (Huber *et al.*, 1990).

SOD and SOR can be distinguished from one another in that SOD generates one-half mole of oxygen and one-half mole of hydrogen peroxide per oxygen molecule while SOR produces only one mole of hydrogen peroxide (Fig.1, Fig.4). The generation of
oxygen by SOD would be disadvantageous to anaerobes because this oxygen could potentially produce yet more superoxide as a result of cellular metabolism, and it is known that some of the enzymes in the fermentation pathways of anaerobes have reaction centers and iron-sulfur clusters that are especially sensitive to damage by superoxide. The hydrogen peroxide generated by SOR is a strong oxidant that is easily reduced within anaerobic cells where cellular reducing agents are abundant (Auchere & Rusnak, 2002).

Recent studies have confirmed that the enzymatic reactions involving SOR, Rd and NROR, which serve as the basis of the proposed model for oxygen detoxification in *P. furiosus*, can successfully reduce superoxide to hydrogen peroxide *in vitro* (Grunden *et al.*, 2003). However, feasibility of the SOR pathway *in vivo* is still needed to unambiguously prove that this is the mechanism responsible for oxygen detoxification in *P. furiosus*. To date, *in vivo* studies in *P. furiosus* have been hampered because of an absence of a usable genetic system in *P. furiosus*. As of now, tools for targeted gene disruptions, which would be required for *in vivo* investigation of oxygen detoxification in *P. furiosus*, are not available, and there are currently no stable selectable markers that can be used for introducing vectors into *P. furiosus*. As a result in the study presented here, *E. coli*, which is very amenable to genetic manipulation and has functional homologs to SOR, Rd, and NROR encoded in its genome (see below), will be used for *in vivo* feasibility of the oxygen detoxification pathway of *P. furiosus*.

**Homologs of Rubredoxin and NAD(P)H:Rubredoxin Oxidoreductase Present in *E. coli***
*E. coli* contains the nor*VW* genes (previously designated, *ygaK* and *ygaD*) which encode a flavorubredoxin (Fl-Rd, *norV*) and NADH:(flavo) rubredoxin reductase (NROR, *norW*), respectively which are involved in nitric oxide reduction under anaerobic conditions. NorV and NorW are 49% and 42% similar to the sequence of *P. furiosus* Rd and NROR, respectively (In this study, Fig.18; Grunden et al., 2003). In *E. coli*, *norV* and *norW* have overlapping coding regions suggesting that they likely comprise a single transcriptional unit. YgaA, which has been identified as a transcriptional regulator of the *norVW* operon, is located upstream of the *norVW* operon and is transcribed in the opposite direction (Fig. 5) (Gardner et al., 2002; Hutchings et al., 2002). YgaA is ~ 42% similar to the sequence of the nitric oxide reductase regulator (*NorR*) protein of *Ralstonia eutropha*, which activates expression of nitric oxide reductase in response to NO and reactive nitrogen intermediates (Pohlmann et al, 2000). *E. coli* flavorubredoxin (*norV*) belongs to a family of flavin proteins, called A type flavoproteins. Anaerobic archaea including *Methanococcus* and *Pyrococcus* and facultative/anaerobic eubacteria including *Desulfovibrio, Dehalococcus, Treponoma, Clostridium, Salmonella, Klebsiella*, and *Synechocystis*, all have this type of protein (Wasserfallen et al., 1998; Frazão et al., 2000). These flavoproteins contain bound flavin in the form of FMN or FAD cofactors, and these cofactors are required to catalyze one or two electron(s) transfer reactions. *E. coli* flavorubredoxin contains two fused protein domains, one, which has a diiron center and a tightly bound FMN, and a second rubredoxin-like domain which has a mononuclear iron. It is suggested in *E. coli* that the NADH-dependent flavorubredoxin reductase (NorW) is responsible for supplying two electrons to the diferric center via the
rubredoxin domain and the proximal FMN in flavorubredoxin and that these electrons are ultimately used for nitric oxide reduction (Gardner et al., 2002).

Based on the sequence and functional similarities that exist between *E. coli* NorV and NorW and *P. furiosus* Rd and NROR, respectively, knockout mutants of these genes in *E. coli* have been constructed for use in complementation experiments with the *P. furiosus* homologs. These complementation experiments ultimately will be able to test whether these enzymes participate in SOR-mediated oxygen detoxification *in vivo*.
\[ O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad \text{(SOD)} \]

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(Catalase)} \]

Figure 1. Common oxygen detoxification enzymes present in aerobes and the reactions they catalyze.
Figure. 2. A standard SOD assay of *P. furiosus* superoxide reductase (SOR).

For this assay, superoxide is produced by Xanthine/ Xanthine oxidase, and 

O$_2^-$ is reduced by cytochrome c. O$_2^-$ is removed by adding SOD.

In graph A, increasing amounts of bovine SOD inhibit the cytochrome c reduction 

indicating that SOD has removed the O$_2^-$. Adding excess bovine SOD has no 

further effect. In graph B, the *P. furiosus* SOD-like enzyme was assayed. Addition of 

excess SOR caused the direct oxidation of cytochrome c indicating that it functions as 

an oxidoreductase, not a dismutase.

Graph modified from Jenney *et al*, (1999)
Figure 3. A model for the detoxification of oxygen in the hyperthermophilic, anaerobe *P. furiosus*

Figure from Jenney *et al.*, (1999)
$O_2^- + 2H^+ + e^- \rightarrow H_2O_2$ (SOR)

Figure 4. Oxygen detoxification enzyme in most of anaerobes.
Figure 5. The *E. coli* FL-Rd (*norV*) and NROR (*norW*) operon contains homologs of *P. furiosus* genes that are likely involved in SOR expression. The Rd-domain of *norV* has 49% similarity to *P. furiosus* Rd (PF Rd), *NorW* has 42% similarity to *P. furiosus* NROR. YgaA is a the regulator of the operon.
MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Construction of recombinant *P. furiosus* SOR, Rd, NROR expression plasmids. *P. furiosus* SOR, Rd, and NROR genes were PCR amplified using boiled genomic DNA as the template. The primers used for PCR amplification of the *P. furiosus* genes are indicated in Table 2 and the specific restriction enzyme sites engineered into the amplified genes that were used for recombinant cloning are designated in bold print. The PCR amplification was performed using Accupol DNA polymerase (Gene Choice, Gaithersburg, MD) and an Icycler (BioRad, Hercules, CA) programmed with the following parameters: one cycle of denaturation at 95°C for 4 min., 30 cycles of 30 sec. denaturation at 95°C, 30 sec. annealing at 55°C, and extension at 72°C for 2 min. The amplified genes (SOR gene, 375 bp; Rd gene, 165 bp; and NROR gene, 1080 bp) were purified using the Qiaquick PCR purification kit (Qiagen, Valencis, CA). For construction of the recombinant plasmids, the restriction enzymes, *Nco*I and *Sac*I, were used for digestion of the *P. furiosus* SOR gene, BamHI and *Sal*I for the *P. furiosus* Rd gene, and *Sac*I and BamHI for the *P. furiosus* NROR gene. The corresponding restriction enzymes were used to digest the vector pTrc99A in its multiple cloning site. The expression plasmids were produced by ligating the digested *P. furiosus* genes to the appropriate digested vector DNA and the resulting plasmids were named pMJ1 (*P.*
furiosus SOR in pTrc99A), pMJ2 (P. furiosus Rd in pTrc99A), and pMJ3 (P. furiosus NROR in pTrc99A). These plasmids were transformed into the E. coli strain XL1-Blue using the CaCl$_2$ method. To confirm the presence of the P. furiosus SOR, Rd and NROR genes in the recombinant expression vectors, restriction enzyme analysis and PCR verification using the appropriate primers (see Table 2) were done to ensure the presence of the insert DNA.

Construction of compatible recombinant P. furiosus Rd and SOR expression plasmids and P. furiosus SOR and Rd co-expressing plasmids. There were a few attempts to clone both P. furiosus Rd and SOR genes into the same expression vector to achieve co-expression. Because the genes are next to each other (10 nucleotides apart), there was an attempt to clone the two genes as a single sequence into vector pTrc99A. The Rd-SOR DNA sequence was amplified by PCR and purified (Qiagen, Hercules, Ca). The resulting Rd-SOR PCR product was engineered to contain SalI and BamHI restriction sites, and the PCR product was digested in overnight restriction reactions for cloning into similarly digested pTrc99A vector DNA. The digested DNA was ligated together using T4 DNA ligase and the resulting plasmid construct was transformed into the E. coli XL1- Blue by the conventional CaCl$_2$ method. Transformants were picked and DNA was isolated from the transformants using the Qiagen Miniprep system to determine if the P. furiosus insert DNA was present in the constructs. None of the screened transformants appeared to contain the expected insert DNA based on visualization of DNA-agarose gels.
As an alternate method, the cloning vector, pPCR-Script was employed to clone these two genes as a single sequence. Because the restriction enzyme sites of the Rd-SOR PCR product are located at the ends of the DNA fragments, there is a possibility that digestion at these restriction sites is inefficient. To alleviate this potential problem, the Rd-SOR PCR product was first cloned into pPCR-Script that had been digested with the blunt-end cutter EcoRV. The resulting ligation mix was transformed into XL1-Blue and plated onto LB+Amp+Tet + X-gal media. Only white colonies, which indicated the presence of the DNA inserted into the multiple cloning site of pPCR-Script, were picked, and plasmid isolations were performed using the Quiagen Miniprep kit. One of the transformants was shown to contain the insert by restriction site and PCR analysis. The plasmid, which contained the *P. furiosus* Rd- SOR insert in pPCR-Script was digested with *Sal*I/*Bam*HI and visualized on a 1% agarose gel. The DNA fragment that contains the Rd-SOR genes with *Sal*I and *Bam*HI sites was cut and gel purified. The Rd-SOR DNA fragment with appropriate restriction sites was ligated with pTrc99A cut with corresponding restriction sites. The ligation mix was transformed into XL1-Blue and plated into LB+Amp+ 0.3% glucose agar. The transformants were picked and plasmid DNA isolated. However, none of the transformants was found to contain the Rd-SOR insert.

A second attempt to clone the two genes into a single vector was prepared by digesting the already cloned expression plasmids, pMJ1(*P. furiosus* SOR in pTrc99A) with the restriction enzymes NcoI and *Sac*I and pMJ2 (*P. furiosus* Rd in pTrc99A) with the restriction enzymes *Bam*HI and *Sal*I. The digested *P. furiosus* SOR and Rd DNA fragments were separated in a 1% agarose gel and purified using the Qiagen Gel
Purification kit. For the vectors, pMJ2 (*P. furiosus* Rd in pTrc99) and pMJ1 (*P. furiosus* SOR in pTrc99A) were digested with *Nco*I/SacI and *Bam*HI/SalI, respectively. The digested vectors were gel purified. The SOR gene with the *Nco*I/SacI sites was ligated into pMJ2 digested with *Nco*I and SacI, and the *P. furiosus* Rd DNA fragment with *Bam*HI/SalI sites was ligated into pMJ1 restricted with *Bam*HI and SalI. The ligation mixes were transformed into XL1- Blue Super competent cells (StrataGene, Kirkland, WA). Transformants were successfully isolated that contained the expression plasmid where the *P. furiosus* Rd gene had been ligated into the existing SOR expressing plasmid, pMJ1. Plasmids were isolated from these transformants and the presence of the appropriate insert DNA was verified by PCR and restriction site analysis. This construct was transformed into JM105 to co-express the proteins following the procedure described in the expression section. However, the expression studies later conducted with this Rd-SOR expression clone did not indicate successful co-expression of both the *P. furiosus* Rd and SOR genes in the *E. coli* expression strain JM105.

Since none of the previous attempts to produce a plasmid that could support co-expression of *P. furiosus* Rd and SOR were successful, a strategy to clone *P. furiosus* Rd in a plasmid that is compatible with the existing SOR expression plasmid was used instead. In this case, a DNA fragment containing both the Trc promoter and Rd from plasmid pMJ2 would be cloned into the compatible low copy number plasmid pACYC184. To do this, plasmid pMJ2 was digested with *Sph*I to release a DNA fragment that contained the Rd gene and Trc promoter. Plasmid, pACYC184 was digested with the blunt end cutter *Sca*I. Since *Sph*I, which was used to isolate the Rd-containing DNA fragment, produces sticky ends, the DNA required treatment to yield
compatible blunt ends for ligation. Attempts to produce blunt-ended DNA involved treatment with Mung Bean Nuclease (New England BioLab) or T4 DNA Polymerase (New England BioLab) following the supplier’s recommendations. No clones containing the \textit{P. furiosus} Rd DNA were isolated even with some modification of the protocols for producing blunt end DNA. A second attempt to produce a compatible Rd-expressing plasmid was also done, and in this case pACYC177 is to be used as the vector. No clones were obtained yet.

**Expression of recombinant \textit{P. furiosus} SOR, Rd, NROR proteins in the \textit{E. coli} strain JM105.** Plasmids containing the \textit{P. furiosus} SOR, Rd, and NROR genes (pMJ1, pMJ2, and pMJ3) were transformed into the \textit{E. coli} strain JM105 for recombinant protein expression. Expression of the recombinant genes is under the control of the Trc promoter, a hybrid \textit{lac} and \textit{trp} promoter, which can be induced with isopropyl-\textit{\beta}-D-thiogalactopyranoside (IPTG), a non-degradable analog of allolactose. 30mL cultures of JM105/pTrc99A, JM105/pMJ1, JM105/pMJ2, and JM105/pMJ3 were grown in LB media at 37°C with shaking. Once the cultures reached an O.D.\textsubscript{600} of \sim 1.0, IPTG was added to a final concentration of 1mM to induce expression of the recombinant \textit{P. furiosus} genes. The cultures were incubated 3 more hours before harvesting. To determine if recombinant proteins were successfully produced in whole cell samples, 1 mL aliquots of the cultures were collected just prior to IPTG-induction and 1, 2, and 3 h. post induction. The OD\textsubscript{600} was measured for every time-point sample. The samples were centrifuged and the resulting pellets were then resuspended with differing amounts of 2X protein denaturing dye (0.125M tris-Cl, 4% SDS, 20% v/v glycerol, 0.2M DTT, 0.02%
bromophenol blue, pH 6.8) according to the O.D. by normalizing cell density at the time of the IPTG induction. The samples were loaded in 12.5% polyacrylamide SDS gels, and the protein expression was monitored using visual detection of Coomassie Blue-stained gels.

Pellets from the 30mL cultures were resuspended in lysozyme buffer (0.03mg/mL lysozyme in 50mM Tris, pH 8.0) and sonicated on ice for bursts of 30 to 60 sec. three times. The cell free extracts were centrifuged at 15,300 rpm in Beckman F2402H rotor for 20 min. Since the recombinant *P. furiosus* proteins should be heat stable, while the majority of *E. coli* proteins are heat labile, aliquots of the cell free extracts were subjected to heat-treatment which involved incubation of the samples in an 80°C water bath for 20 min. and centrifugation of the sample at 15,300 rpm in Beckman F2402H rotor for 20 min. to remove denatured *E. coli* protein. Protein concentrations of the samples were determined based on the Bradford method (Bradford, 1976). 2X protein loading dye was added and the samples were boiled at 100°C for 5 min. Samples (3~5 μg each) were loaded onto 12.5% polyacrylamide SDS gels. Protein detection was done by staining the gel with Coomassie-Blue.

**E. coli norV and nor W mutant strain construction.** For disrupting *norV* (Flavorubredoxin) and *norW* (NAD(P)H dependant rubredoxin oxidoreductase) which are homologous to *P. furiosus* Rd and NROR genes in *E. coli*, the PCR-based one step inactivation of chromosomal gene method by Datsenko & Wanner (2000) was used. The basic explanation for the strain construction is described in Figure 6. Plasmid, pKD3, which contains a chlororamphenicol resistance gene cassette, is used as the template for
generation of PCR fragments encoding the chloramphenicol resistance marker and homologous DNA regions for the target genes (\textit{norV} and \textit{norW}). The primers consist of 60 bases, which include 40 that are homologous to the flanking region of the target genes and 20 bases that are complementary to the chloramphenicol resistance cassette (see Table 2). The PCR products were gel purified (Qiagen, Valencia, CA). pKD46 is a helper plasmid which carries the phage \(\lambda\) Red recombinase that is L-arabinose inducible. pKD46 is cured from cells when the incubation temperature is raised to 37\(^\circ\)C. The \textit{sodA}, \textit{sodB} deficient strain NC906 was used in the initial attempts to construct the \textit{norV}, \textit{sodA}, \textit{sodB} deficient strain. However, this approach did not yield the appropriate mutant construct and as a result the \textit{E. coli} strain LMG194 was used instead. The use of LMG194 is recommended when expression vectors containing the arabinose promoter (pBAD) such as pKD46 are used. The LMG194/pKD46 transformants were plated in LB + ampicillin + 0.3\% glucose agar and incubated overnight at 30\(^\circ\)C. Transformants carrying pKD46 were grown in 10 mL SOB medium containing 0.2\% of L-arabinose and ampicillin at 30\(^\circ\)C until the O.D.600 reached 0.6. The cultures were spun down at 9,500 rpm in Beckman C1015 rotor for 10 min. and washed three times with ice-cold 10\% glycerol in order to make the cells electrocompetent. The cells (LMG194/pKD46 and NC906/pKD46) were suspended in 100\(\mu\)L of 10\% glycerol and 50 \(\mu\)L aliquots were placed into two separate microfuge tubes. The \textit{norV} specific PCR products (concentration \(\sim\)1 \(\mu\)g) were added to the 50\(\mu\)L of the suspended cells and no DNA was added to the other 50 \(\mu\)L of cells as a negative control. Electroporation was done by using a Gene Pulser (Bio Rad, Hercules, CA) set at 2.5V, 200\(\Omega\), and 25\(\mu\)F according to the manufacturer’s instruction. Shocked cells were added to 1 mL of pre-warmed SOC
medium and incubated for 1-hour at 37°C. 10% of the electroporated cells were employed in mutant strain construction. LMG194 was transformed with plasmid pKD46 using the CaCl₂ method. The cells were plated into LB+ chloramphenicol agar and incubated at 37°C overnight. The remainder of the cells was incubated overnight at room temperature before plating the next day. For HMJI (LMG194, norV) strain, P1 transduction was done to transfer the disrupted norV gene into NC906 (Miller, 1992).

PCR fragments, which have the norW homologous region and the chloramphenicol resistance gene, were used for disrupting the norW gene region in E. coli strain NC906. The procedures used were the same as described above for construction of the norV gene disruption in LMG194. BW545 (wild type) and MJ100 (norV, sodA, sodB deficient strain), and MJ200 (norW, sodA, sodB deficient strain) genomic DNAs were purified using the GNOME DNA Kit (Bio 101, Carlsbad, CA) and the isolated DNA was sheared with BstEII. To verify the mutants of norV and norW in the strains MJ100 and MJ200, PCR analysis was conducted using the respective genomic DNAs as the templates and the primers that are specific to outer region of the genes as well as the primers specific to the disrupted genes (see Table 2 for primers).

**In vivo P. furiosus SOR complementation studies in an E. coli sodA sodB deficient strain grown in M63 minimal media.** The ability of the P. furiosus SOR gene to complement an E. coli strain deficient in sodA and sodB was examined by growth activity in M63 minimal medium without amino acid supplement, where restoration of growth indicates complementation of SOD. The expression plasmid, pMJ1 (P. furiosus SOR in pTrc99A) was transformed into the E.coli sodA, sodB (NC906) mutant strain. The freshly
transformed cells (MJ002) were grown overnight aerobically at 37 °C in M63 minimal medium, containing glucose as its carbon source, 0.2% casamino acids (a source of amino acids), and 100 µg/mL ampicillin (M63 glucose M.M. w. A.A + Amp). The overnight cultures were used to inoculate 30 mL of M63 minimal medium that contained glycerol as the carbon source and the appropriate antibiotics, but lacked amino acid supplementation (M63 glycerol M.M. w/o. A.A.+ Amp). For each culture, the appropriate volume of inoculum was added to give a starting OD600 of 0.06. As controls, cultures of NC905, NC 906, and MJ001 (NC906/pTrc99A) were prepared as above. Prior to incubating the 30 mL cultures, IPTG was added to a final concentration of 0.1 mM and the cultures were incubated aerobically at 37°C with shaking (225 rpm). For the complementation experiments, MJ002 (P. furiosus SOR in the E. coli sodA, sodB mutant) cultures were prepared with and without IPTG supplementation in order to demonstrate the effect of P. furiosus SOR expression on culture growth. During the growth experiments, the OD600 of the cultures was measured at 3, 5, 7, 9, and 11 h. after inoculation.

**In vivo P. furiosus SOR complementation studies using the E. coli sodA, sodB, norV deficient strain.** The ability of the P. furiosus SOR gene to complement an E. coli strain deficient in sodA, sodB and the norV gene was examined by growth activity in M63 minimal medium without amino acid supplementation as indicated in the section above. The plasmids pTrc99A and pMJ1 (pTrc99A-SOR) were transformed into a sodA, sodB, and norV deficient E. coli strain. The transformed strains (MJ101 and MJ102, respectively) were cultured overnight at 37°C in M63 minimal media that contained
glucose, casamino acids, ampicillin and chloramphenicol (30 µg/mL). These overnight cultures were then inoculated into 30 mL of M63 minimal media that contained glycerol, ampicillin and chloramphenicol. For each culture, sufficient inocula were used to provide an initial culture OD$_{600}$ of ~0.06. Growth of the cultures was monitored as indicated above. Cultures of NC905, NC906, MJ001, MJ002, and MJ101 were prepared and grown with the appropriate antibiotics to serve as experimental controls.
Table 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>LMG194</td>
<td>F' Δlac X 74 galE thi rpsL ΔphoA Δara174Leu:: Tn10</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>XL1-BLUE</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA lac [F' proAB lacI'ZΔM15Tn10(Tet')]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>JM105</td>
<td>F' traD36 proA⁺ proB⁺ lacIq delta(lacZ)M15 delta(pro-lac) hsdR4 sbcB15 rpsL thi endA1 lambda-</td>
<td>Lab collection</td>
</tr>
<tr>
<td>BW545</td>
<td>Δ(lacU)169 rpsL</td>
<td>Lab collection</td>
</tr>
<tr>
<td>HMJ1</td>
<td>LMG194norV::Cm⁰</td>
<td>In this study</td>
</tr>
<tr>
<td>NC905</td>
<td>F' lambda- ilvG⁻ rfb⁻50 rph⁻1</td>
<td>H. Hassan</td>
</tr>
<tr>
<td>NC906</td>
<td>NC 905sodA, sodB</td>
<td>H. Hassan</td>
</tr>
<tr>
<td>MJ001</td>
<td>NC906/ pTrc99A</td>
<td>In this study</td>
</tr>
<tr>
<td>MJ002</td>
<td>NC906/ pMJ1</td>
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</tr>
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<td>NC906norV::Cm⁰</td>
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<td>MJ101</td>
<td>MJ100 /pTrc99A</td>
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<td>MJ102</td>
<td>MJ100/pMJ1</td>
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<tr>
<td>MJ200</td>
<td>NC906norW::Cm⁰</td>
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<th>Plasmids</th>
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<td>pTrc99A</td>
<td>Trc promoter, Amp⁰ ColEl ori</td>
<td>Amersham Pharmacia Biotech</td>
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<td>pMJ1</td>
<td>pTrc99A P. furiosus SOR</td>
<td>In this study</td>
</tr>
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<td>pMJ2</td>
<td>pTrc99A P. furiosus Rd</td>
<td>In this study</td>
</tr>
<tr>
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<td>pTrc99A P. furiosus NROR</td>
<td>In this study</td>
</tr>
<tr>
<td>pKD3</td>
<td>Amp⁰, Cm⁰</td>
<td>B. Wanner</td>
</tr>
<tr>
<td>pKD46</td>
<td>Amp⁰, Temperature sensitive, γ Rec</td>
<td>B. Wanner</td>
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<td>pACYC184</td>
<td>Cloning vector, p15A ori, Cm⁰, Tet⁰</td>
<td>New England BioLab</td>
</tr>
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<td>pACYC177</td>
<td>Cloning vector, p15A ori, Amp⁰, Kan⁰</td>
<td>New England BioLab</td>
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<td>pPCR-Script</td>
<td>Lac promoter, Amp⁰, pUC ori, blue/white screen system</td>
<td>StrataGen</td>
</tr>
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<td>Gene name</td>
<td>Primer sequences</td>
<td>Features of the primer</td>
</tr>
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<td><strong>P. furiosus sor</strong></td>
<td>Forward 5’-GGTGGTG GCCATGGTTAGTGAAAACCATAAG-3’</td>
<td>NcoI site presence</td>
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<td>Reverse 5’-GATGAAGAA GAGCTC ACTGACTTCTAT-3’</td>
<td>SacI site presence</td>
</tr>
<tr>
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<td>Forward 5’-GGATCC GGAAGGAGGTATCATGGCAAGGTG-3’</td>
<td>BamHI site presence</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TAATCAT GTGAC CCACCTCAATCT-3’</td>
<td>SalI site presence</td>
</tr>
<tr>
<td><strong>P. furiosus nror</strong></td>
<td>Forward 5’-GAGCTC CAAGGAGATGAAATGAGGTAGTT-3’</td>
<td>SacI site presence</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’- GTTGGGACTTCACATGGATCCTAGTGGAGA-3’</td>
<td>BamHI site presence</td>
</tr>
<tr>
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<td>Forward 5’-CCAGCACATCAACGGAAAAACTC-3’</td>
<td>outer region of the gene (-210 to -188)</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-ATTCCTGGCTTTTCAACCACAGGGGC-3’</td>
<td>outer region of the gene (+158 to +181)</td>
</tr>
<tr>
<td><strong>E. coli norW (outer region)</strong></td>
<td>Forward 5’-CCAGGACGCTCTGAAGTTATGC-3’</td>
<td>outer region of the gene (-303 to -283)</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GTATTTAGCGGCGGGTATATC-3’</td>
<td>outer region of the gene (+283 to +305)</td>
</tr>
<tr>
<td><strong>PCR product (norV-Cm specific)</strong></td>
<td>Forward 5’- TAAATTGAGGTGTATGTCTTATTTGTGTTGA AAAAAATAACA TGTAGGCTGAGCTGTTCG-3’</td>
<td>*Homologous region of the gene *chloramphenicol gene cassette region</td>
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<tr>
<td></td>
<td>Reverse 5’- CTTTGGCCGAGGAGCATTCCGGGCAGAGGA AGTTATCCGGCATATGAATAATCCTCCTTACG-3’</td>
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<tr>
<td><strong>PCR product (norW-Cm specific)</strong></td>
<td>Forward 5’- GCCCCGCAA CTTGGAATATCGCAAAC- CAGAGGCCATGTAAGGCTGAGCTGTTCG-3’</td>
<td>*Homologous region of the gene *chloramphenicol gene cassette region</td>
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<tr>
<td></td>
<td>Reverse 5’- CGACATT TTACGACAGTAGCCCACTCACTAC- TCGGCAATGCTATGTAATATCCTCCTTACG-3’</td>
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Figure 6. Gene replacement by PCR product mediated transformation.

A. Design of drug resistant cassette that has flanking region of the resistance.

B. Design of target gene that has flanking region of the gene.

C. Primer design that has the flanking regions of the drug resistance cassette (about 20 nt.) and target gene (about 40 nt)

D. Linear DNA fragment that has drug resistance and flanking portions of the target gene generated by PCR amplification.

E. Gene disruption by homologous recombination between the linear DNA fragment and the target gene.

Figure modified from <www. Biology.utah.edu/protocols/Lin.Transform.html>
RESULTS

Recombinant expression of \textit{P. furiosus} SOR, Rd, and NROR in \textit{E. coli}. In order to study the \textit{P. furiosus} oxygen detoxification mechanism, each of the \textit{P. furiosus} SOR, Rd, and NROR genes was PCR amplified and cloned into the expression vector, pTrc99A. To confirm the presence of the correct insert, the plasmids were digested with the appropriate restriction enzymes and DNA gels were used to determine if the expected fragment sizes resulted. In some cases, PCR analysis was additionally performed to verify the presence of the insert gene(s). For the \textit{P. furiosus} SOR and Rd plasmids, expected sizes of DNA fragments (375 bp and 165 bp respectively) were observed for both the restriction site and PCR analysis (Figures 7 and 8). For the \textit{P. furiosus} NROR plasmid, the expected sizes DNA fragment (~1100bp) was obtained by restriction site analysis (Figure 9). The \textit{P. furiosus} SOR, Rd, and NROR plasmid constructs were transformed into the \textit{E. coli} strain JM105 for protein expression. For the expression, a final concentration of 1 mM IPTG was added to the cultures to induce expression of the recombinant \textit{P. furiosus} proteins. Harvested cells were broken and the cell free extracts were loaded onto 12.5%-polyacrylamide-SDS gels for visual confirmation of successful recombinant protein expression. A distinct band corresponding to the expected size for the \textit{P. furiosus} SOR protein (14.5 kDa) was observed in the cell free extracts of the \textit{E. coli} samples that contained the SOR expression plasmid and were induced with IPTG (Figure 10). The cell free extract samples were then subjected to heat treatment in an effort to demonstrate the heat stability of the recombinant \textit{P. furiosus} SOR protein. SDS-PAGE analysis of the heat-treated SOR expression samples resulted in the detection of one particularly noticeable
band that migrated at the expected size for SOR, thus confirming that the recombinant \textit{P. furiosus} SOR was thermostable, unlike the majority of the \textit{E. coli} proteins that were denatured during the heat treatment.

For Rd protein expression, the expected size of the protein, which is \(\sim 6\) kDa, was observed in both the untreated and heat-treated samples. A distinct band in the heat treated samples was found in the Rd lane at the bottom of the gel (Figure 10) and similar bands were observed in the untreated cell-free extract of the Rd expression samples (Figure 11).

For the \textit{P. furiosus} NROR protein expression experiments, it was often difficult to identify an expressed protein band corresponding to the expected size (\(\sim 45\) kDa) for NROR. From several trials, putative expression of NROR was observed in the whole cell pellet samples on one occasion (Figure 12). The difficulty in over-expression of \textit{P. furiosus} NROR was thought to be due to the prevalence of rare codons (AGG and AGU coding for arginine, AUA coding for isoleucine, and CUA coding for leucine) in the \textit{P. furiosus} NROR gene sequence. Of the 361 amino acids in the NROR sequence, 7 are encoded by rare Arg codons, 17 by rare Ile codons, and 17 by rare Leu codons, which can result in poor over-expression or in the production of truncated proteins in \textit{E. coli} cells. To overcome this problem, a rare codon plasmid, pRIL, which contains genes for the tRNAs for the rare Arg, Ile, and Leu codons, AGG, AGA, AUA, and CUA, respectively, was transformed into the \textit{E. coli} expression strain JM105 which also harbors pMJ3 (\textit{P. furiosus} NROR in pTrc99A). NROR protein over-expression was still not detected by visualization in a 12.5%-polyacrylamide-SDS gel even with coexpression of the rare tRNAs. Expression experiments will be continued in order to identify conditions under which \textit{P. furiosus} NROR is over-expressed in \textit{E. coli}. Additionally, expression of \textit{P. furiosus} NROR will be
confirmed by both visual identification as well as by enzyme activity as described by Ma & Adams. (1999).

**E. coli norV (Fl-Rd⁻) and norW (NROR⁻) mutant strain constructs.** The “One-step inactivation of chromosomal genes in E. coli K-12 using PCR products” method by Datsenko and Wanner (1999) was used in this study to inactivate specific target genes, and it is based on PCR-mediated gene replacement. For construction of the norV (Fl-Rd⁻) specific gene disruption, two colonies from overnight-incubated cells were isolated on the LB+ Cm selection plate, which were putative norV specific mutants (norV in LMG194 strain). These two isolates along with the wild type strain, BW545, were subjected to PCR analysis for verification of the targeted gene disruption. For the PCR analysis, sheared genomic DNA from each isolate and the control strain were used as the DNA templates and primers that are specific to either the outer region of the genes or located within the disrupted genes were used (See Table 2). The different primer combinations would yield different sized PCR products. The expected sizes of the NorV disrupted DNA are 1.1 kb, 1.4 kb, 1.3 kb, and 1.7 kb when the following primer combinations are used: 5’ norV:Cm specific and 3’ norV:Cm specific primers, 5’ outer region and 3’ norV:Cm specific primers, 5’ norV:Cm specific and 3’ outer region primers, and 5’ outer region and 3’ outer region primers, respectively. The expected DNA size when using the wild type DNA as the template DNA and the 5’ outer region and 3’ outer region primers is about 2.0 kb. PCR analysis of one of the two isolates did result in the production of PCR fragments that matched the expected sizes. The verified strain was designated HMJI (norV deficient strain in LMG194). The Cm-disrupted norV gene from strain HMJI was transduced into the sodA,
sodB mutant strain NC906 using P1 phage in order to produce a strain for use in the SOR complementation studies. The successful introduction of the disrupted norV gene into NC906 by P1 transduction (Miller, 1992) was verified using PCR analysis (Figure 13), and the resulting strain was named MJ100. For construction of an E. coli strain with a norW gene disruption, four colonies were isolated from the overnight-incubated cells. In this case the norW - Cm\(^{\prime}\) PCR fragment could be directly integrated into E. coli strain NC906. The four isolates were subjected to PCR analysis to confirm the correct location of the chromosomal insertion using the appropriate primers (Table 2). One of the isolates gave the expected PCR fragment sizes and was given the strain name MJ200 (Figure 14).

**P. furiosus SOR is able to complement the growth defect in a sodA, sodB deficient E. coli strain.** Under aerobic conditions, bacterial mutants defective in sodA and sodB are not able to grow in minimal media lacking amino acid supplementation because the protective enzymes against superoxide toxicity are impaired. Excessive amounts of superoxide can cause irreparable damage to the cell. A primary target of superoxide-mediated damage is [4Fe-4S] cluster-containing enzymes. One particular enzyme, which is known to incur superoxide damage, is dihydroxyacid dehydratase, a key enzyme involved in branched amino acid biosynthesis (Kuo et al., 1987). Thus, amino acid biosynthesis is expected to be impaired in cells lacking SOD activity, and as a result, there will be poor growth of a sodA, sodB mutant E. coli strain in minimal media lacking amino acid supplementation. In this study, it was observed that recombinant expression of the *P. furiosus* SOR restored the growth of the sodA, sodB mutant E. coli strain in the M63 minimal medium lacking amino acid supplementation (Figure 15). As indicated in Figure 15, MJ002 (E. coli sodA, sodB
mutant strain expressing *P. furiosus* SOR grew up to approximately 62% of the O.D. level as compared to the wild type strain, NC905, while NC906, the *sodA*, *sodB* mutant strain showed poor growth in the M63 minimal media in the absence of amino acid supplementation. It was also shown that the amount of SOR expression in the cell is critical to restoring growth since the MJ002 strain only grew well in the minimal media without amino acid supplementation when IPTG was added to induce over-expression of *P. furiosus* SOR. Growth was also monitored for NC906 containing the empty expression vector pTrc99A to show that the restored growth capabilities for strain MJ002 is due only to the presence of the SOR-expressing vector. The growth experiments were repeated five times and the data shown in Figure 15 are the average of all five trials. Bars indicate the standard deviation of the data sets.

*P. furiosus* SOR is only able to support a low level of growth restoration in a *sodA*, *sodB*, and *norV* defective *E. coli* strain. It is proposed that rubredoxin is the primary electron donor to SOR in *P. furiosus* reactive oxygen species detoxification based on SOR activity data (Jenney *et al*., 1999; Figure 3). The Rd and SOR genes in the *P. furiosus* genome, as well as in other anaerobes that contain SOR, are adjacent to each other suggesting a relationship between the two proteins (Adams *et al*., 2002). In the present work, the functional relationship between SOR and Rd is explored using the growth-based complementation studies discussed above.

In this case, the *P. furiosus* SOR gene was transformed into the *sodA*, *sodB*, and *norV* deficient *E. coli* strain (MJ100), and transformants (MJ102) were tested for their growth levels in M63 minimal medium lacking amino acid supplementation. For this
particular complementation study, poor growth in the media would imply a functional relationship between SOR and Rd (assuming that the Rd domain in norV is indeed responsible for transferring electrons to SOR in E. coli), whereas good growth would indicate that SOR is receiving the necessary electrons from another source. The results of this experiment showed that there is only a low level of growth restoration in the sodA, sodB, and norV deficient strain when P. furiosus SOR is overexpressed in the strain (Figure 16). MJ102 showed about 15% of the growth compared to the wild type strain. It is thought that major electron donor to the P. furiosus SOR is not functioning properly due to the disruption of flavorubredoxin gene (norV), but the SOR still receives the electrons inefficiently from an unknown factor in the cell. The MJ002 strain showed 70% restored growth compared to the wild type O.D. level, which is consistent with the previous P. furiosus SOR complementation study in E. coli sodA, sodB mutant. The MJ100 strain which is a triple mutant (sodA, sodB, norV) showed the poorest growth overall. The data presented in Figure 16 are the average of five separate growth experiments. Bars indicate the standard deviation of the data. The requirement of Rd for SOR activity will be further evaluated once the Rd and SOR coexpression construct has been successfully developed.

Co-expression of recombinant P. furiosus SOR and Rd in E. coli. This study presented the preliminary evidence that P. furiosus SOR and Rd are both required for full complementation of E. coli SOD activity. However, in order to further investigate the functional relationship between the P. furiosus SOR and Rd, P. furiosus SOR and Rd need to be co-expressed in the E. coli strain deficient in sodA, sodB, and norV.
The first attempt to clone both the *P. furiosus* Rd and SOR genes as a single PCR fragment into a vector, pTrc99A, was not successfully achieved. A number of clones were screened for the presence of plasmids containing the Rd-SOR genes from among 44 that were originally isolated; however, no plasmids containing the desired insert DNA were identified. In order to minimize the possibility of vector ligation without insert, the ligation mix was digested with *Xba*I. An *Xba*I restriction site is located in the multiple cloning site region in plasmid pTrc99A between the *Sal*I and *Bam*HI restriction sites which were used to clone in the Rd-SOR DNA, and therefore, only empty religated vector would still contain the *Xba*I site. The *Xba*I-cut ligation mix was then transformed into XL1-Blue cells. Nine transformants were recovered and used for plasmid isolation, but again none of them contained plasmids with insert DNA. Since it was possible that the uncontrolled coexpression of Rd and SOR was preventing isolation of a stable Rd-SOR containing plasmid, the *Xba*I-cut ligation mix was also transformed into JM105 and plated on LB-glucose-ampicillin plates in an effort to prevent unintended expression of Rd and SOR. Again none of the resulting transformants contained plasmids with the Rd-SOR insert.

Since it was possible that the failure to isolate a plasmid containing the Rd-SOR insert DNA was due to inefficient digestion at either the *Sal*I or *Bam*HI restriction sites in the Rd-SOR PCR product because these sites are situated very near the ends of the DNA fragment, an alternate method for cloning was used in which the Rd-SOR PCR fragment was directly cloned into the cloning vector pPCR-Script that had been digested with the blunt-end cutter EcoRV. Furthermore, use of pPCR-Script as the cloning vector has the additional advantage of blue/white screening to indicate whether transformed colonies of *E. coli* strain XL1-Blue contain plasmids with the insert DNA. Four of the white colonies
that resulted from the transformation were used for plasmid isolation, and one of the isolates did appear to contain insert DNA of the appropriate size based on restriction and PCR analysis. The Rd-SOR DNA was subsequently cut from this vector using BamHI and SalI and cloned into the appropriately restricted expression vector pTrc99A. Using this cloning strategy, no plasmids were successfully isolated that contained the Rd-SOR insert DNA.

At this point a different cloning approach was used which involved cloning SOR or Rd into the already existing Rd (pMJ2) and SOR (pMJ1) expression vectors, respectively. Using this approach, the *P. furiosus* Rd DNA was successfully inserted into the SOR expressing plasmid pMJ1. Three separate SOR-Rd expression clones were transformed into JM105 for coexpression studies. None of the three SOR-Rd expression clones appeared to support expression of both the SOR and Rd, as only one distinct overexpressed band migrating at ~14 kDa was observed (Figure 17). Thus, it seems that only SOR is overexpressed using the SOR-Rd expression plasmids. The inability to overexpress *P. furiosus* Rd on the same transcript as SOR may be due to mRNA instability or inefficient binding at the ribosomal binding site located upstream of the Rd gene.

This failure to achieve coexpression of *P. furiosus* Rd and SOR prompted use of a different cloning and expression strategy in which SOR and Rd are expressed from separate but compatible expression plasmids in the same *E. coli* cell. In this case there was an attempt to clone *P. furiosus* Rd along with the Trc promoter into the low-copy plasmid pACYC184. However, technical difficulties involved in preparing blunt end pTrc-Rd DNA for ligating into the *Sca*I digested pACYC184 vector have to date prevented the successful cloning of pTrc-Rd into this vector. Several attempts were done to clone the
pTrc-Rd DNA into another compatible low-copy plasmid pACYC177, which has the benefit of having a stable kanamycin selection marker rather than the somewhat light sensitive tetracycline marker from pACYC184. No clone was obtained yet in this method.
Figure 7. Restriction site analysis for the cloned *P. furiosus* SOR and Rd. L: DNA size standards, Lane 1: pTrc99A, (undigested), Lane 2: pMJ2 (pTrc99A-Rd, undigested), Lane 3: pMJ1 (pTrc99A-SOR, undigested), Lane 4: pTrc99A (digested by *Nco*I/*Sac*I) Lane 5: pMJ1 (digested with *Nco*I/*Sac*I) Lane 6: SOR (PCR product) Lane 7: pMJ2 (digested with *Sal*I/BamHI) Lane 8: Rd (PCR product)
Figure. 8. PCR analysis of *P. furiosus* SOR and Rd. L: DNA size standards Lane 1: *P. furiosus* SOR PCR product using genomic DNA as the template, Lane 2: cloned *P. furiosus* SOR #1, Lane 3: cloned *P. furiosus* SOR #2, Lane 4 *P. furiosus* Rd PCR product using genomic DNA as the template, Lane 5: cloned *P. furiosus* Rd #1, Lane 6: cloned *P. furiosus* Rd #2.
Figure 9. Restriction site analysis of the cloned *P. furiosus* NROR. L: DNA size standards Lane 1: pTcr99A (undigested), Lane 2: pMJ3 (pTrc99A-NROR, undigested), Lane 3: pTrc99A (digested with SacI/BamHI) Lane4: pMJ3 (digested with SacI/BamHI) Lane 5: *P. furiosus* NROR PCR product
Figure 10. *P. furiosus* SOR expression in *E. coli* strain JM105.

Expected M.W. is 14.5k Da. L: M.W. standards,  C: control, pTrc99A only,  
Rd: pTrc99A-Rd, SOR: pTrc99A-SOR  
H.T.: cell free extract, heat treated for 20 min at 80°C (3 µg per lane)  
N.H.T.: cell free extract, not heat treated (5 µg per lane)
Figure 11. *P. furiosus* Rd expression in *E. coli* strain JM10. Expected M.W. is ~6kDa

L: M.W. standards  C: control, pTrc99A only  Rd: pTrc99A-Rd

N.H.T.: Cell free extract, not heat treated (5µg per lane)
Figure 12. *P. furiosus* NROR expression in *E. coli* strain JM105. Expected M.W. is about 45 kDa. L: M.W. standards, C: control, pTrc99A only, NROR: pTrc99A-NROR t stands for the time (hours) after IPTG induction.
Figure 13. PCR verification of *E. coli* norV mutant (MJ100) using different combinations of gene-Cm specific or outer gene specific primers.

L stands for the DNA size markers.
Figure 14. PCR verification of *E. coli* norW mutant (MJ200) using different combinations of gene-Cm specific or outer gene specific primers.

L stands for the DNA size markers.
Figure 15. Complementation study of the ability of *P. furiosus* SOR to restore growth of an *E. coli* *sodA, sodB* experimental mutant in M63 glycerol minimal medium without amino acid supplementation. Inocula of the cultures were prepared from overnight cultures grown in M63 glucose minimal media with amino acid supplementation. The experimental cultures were inoculated with enough inoculum to give a starting O.D. of 0.06. NC905: wild type *E. coli* strain, NC906: *sodA, sodB* mutant *E. coli* strain, MJ002: pTrc99A-SOR in *sodA, sodB* mutant, MJ001:pTrc99A in *sodA, sodB* mutant. Where indicated, a total concentration of 0.1 mM of IPTG was added at the time of the culture inoculation. The experiments were repeated five times. Bars indicate the standard deviation of the data set.
Figure 16. Complementation study of the ability of P. furiosus SOR to restore growth of an E. coli sodA, sodB, norV mutant in M63 glycerol minimal medium without amino acid supplementation. The experimental cultures were inoculated with enough inoculum to give a starting O.D. of 0.06. NC905: wild type E. coli strain, NC906: sodA, sodB E. coli mutant, MJ002: pTrc99A-SOR in sodA, sodB mutant, MJ001: pTrc99A in sodA, sodB mutant, MJ102: pTrc99A-SOR in sodA, sodB, norV mutant, MJ101: pTrc99A in sodA, sodB, norV mutant, MJ100: sodA, sodB, norV mutant. Experiments were repeated five separate times. Bars indicate the standard deviation of the data set.
Figure 17. *P. furiosus* SOR-Rd co-expression in *E. coli* strain JM105. Expected M.W. is 14.5 kDa (SOR) and 6 kDa (Rd). L: M.W. standards, 1: pTrc99A, 2: pTcr99A-Rd, 3: pTrc99A-SOR, 4: clone #2, 5: clone #3, 6: clone# 8. All the clones showed the SOR band only, but not the Rd band.
DISCUSSION

Initial in vivo complementation studies in E. coli confirm aspects of the proposed P. furiosus oxygen detoxification pathway

It is known that anaerobic microorganisms can periodically be exposed to oxygen in their environments, and therefore, having some protective mechanism to detoxify reactive oxygen species would be highly advantageous for their survival. It was recently proposed by Jenney et al. (1999) that the hyperthermophilic anaerobe, Pyrococcus furiosus, has an oxygen detoxification capability. In P. furiosus, the reactive oxygen specie superoxide is reduced to hydrogen peroxide by the enzyme superoxide reductase (SOR). The electrons required for this reduction are thought to be provided by rubredoxin (Rd), which in turn receives it electrons from NAD(P)H:rubredoxin oxidoreductase (NROR). There has been accumulating evidence that this pathway is valid when investigated through in vitro experimentation. However, this proposed detoxification mechanism has not been functionally studied in in vivo systems. Therefore, to more fully explore the P. furiosus oxygen detoxification mechanism in vivo, functional complementation studies were performed as part of this study. Due to the fact that P. furiosus is not very amenable to genetic manipulation, E. coli mutant strains, which are deficient in the homologs of P. furiosus genes involved in its oxygen detoxification mechanism, were employed in the complementation study.

The complementation studies in here showed that high growth levels could be restored to the E. coli sodA, sodB mutant when it was cultured aerobically in M63 minimal
medium without amino acid supplementation when *P. furiosus* SOR was expressed in *E. coli*. This result indicates that the *P. furiosus* SOR detoxified the superoxide generated in the *E. coli* cell that would otherwise have accumulated and caused cellular damage in the *sodA*, *sodB* mutant, preventing growth of the cells in the amino acid deficient medium. For further confirmation of *P. furiosus* SOR activity in the *E. coli sodA*, *sodB* mutant, SOR enzyme activity assays should be conducted as well.

The ability of *P. furiosus* SOR to complement for *E. coli* SOD is consistent with other complementation studies that used recombinant SORs from other diverse anaerobes such as from *Desulfovibrio vulgaris*, *Desulfovibrio gigas*, *Desulfoarcululus baarsii*, and *Treponema pallidum* (Lumppio et al., 2001; Silva et al., 2001; Pianzzola et al., 1996; Lombard et al., 2000b). In these studies, the SOR analogs were shown to suppress the deleterious effects of superoxide when they were expressed in *E.coli* SOD mutant strains (Adams et al., 2002). From these complementation studies it is evident that the recombinant SORs are efficiently receiving electrons from a donor in the *E. coli* cell.

It is important to note that the *P. furiosus* SOR complementation experiments reported in this study were conducted at 37 °C, the normal growth temperature for *E. coli*, but a temperature well below that which is optimal for the activity of most enzymes from hyperthermophiles (> 80 °C). Therefore, the fact that the recombinant *P. furiosus* SOR could successfully restore growth of the *E. coli sodA*, *sodB* mutant strain in amino acid deficient minimal medium, indicates that the SOR is still enzymatically active at a mesophilic temperature. This finding is consistent with the previously reported observation that *P. furiosus* SOR is enzymatically active in *in vitro* assays conducted at 23 °C, a temperature far below the optimal growth temperature of 100 °C seen for *P. furiosus*.  

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(Jenney et al., 1999). Furthermore, these findings of the ability of *P. furiosus* SOR to catalyze superoxide reduction at low temperature lend support to the idea that *P. furiosus*, in its normal environment, would only be exposed to oxygen when it is expelled from the hydrothermal vent system into cold, oxygenated seawater, and as such, any oxygen detoxification mechanism must be active at low temperature to adequately provide protection to the cell.

The second complementation study in which the *E. coli* *sodA* *sodB*, and *norV* mutant strain was tested for the ability of *P. furiosus* SOR to restore wild type growth levels in amino acid deficient minimal medium, resulted in only a low level of growth restoration, indicating that in this strain *P. furiosus* SOR has a lower activity compared to its activity in the *E. coli* strain that is only lacking active SOD but not NorV. Since NorV is the only protein in *E. coli* known to contain a rubredoxin-like domain (49% similarity between *P. furiosus* rubredoxin and the rubredoxin-like domain of NorV) and that rubredoxin appears to be the direct electron donor for *P. furiosus* SOR based on *in vitro* assays, the limited growth restoration of the *NorV* mutant strain expressing *P. furiosus* SOR is most likely the result of inefficient electron transfer to SOR in the *NorV* deficient *E. coli* strain. Thus, it is assumed that NorV in *E. coli* is serving as the primary electron donor for *P. furiosus SOR* in the complementation of the *E. coli* SOD.

There are many anaerobes besides *P. furiosus* that have rubredoxin, but its exact physiological function is not well established although it is presumed to be an electron carrier (Das et al., 2001). The genes encoding Rd and SOR are adjacent to each other in several species of anaerobes further suggesting a direct relationship between the two proteins since it is known that genes that are transcribed on a single mRNA are typically
functionally linked (Adams et al., 2002). Furthermore, studies have shown that in a few of the anaerobic species, NROR serves as the physiological electron donor to rubredoxin in the oxygen detoxification process (Guedon & Petitdemange, 2001). In *P. furiosus*, NROR has been shown to function in the reduction of rubredoxin using NADPH as the source of electrons (Ma et al., 1999; Jenney et al., 1999). Given that the reduction of superoxide by SOR has been shown to proceed in *in vitro* assays only when rubredoxin and NAD(P)H-reduced NROR are also supplied, a mechanism for superoxide reduction has been proposed for *P. furiosus* by Jenney et al. (1999) in which Rd functions as the election carrier for the SOR, and the rubredoxin receives the electrons from NROR that has been reduced by NAD(P)H. Further, it has been suggested that the oxygen detoxification system present in *P. furiosus* may be representative of systems that have recently been described in other anaerobes (Lumppio et al., 2001; Silva, 2001 et al.; Pianzzola et al., 1996; Lombard et al., 2000b, Abreu et al., 2000).

**The oxygen detoxification system in *P. furiosus* may be representative of a general oxygen detoxification pathway for anaerobes and microaerophiles**

About 60 microbes are classified as either anaerobic, microaerophilic, or facultative organisms among the 148 microorganisms that have had their genomes completely sequenced. Thirteen of them have SOR sequences that have significant similarity to SOR from *P. furiosus* and include anaerobic archaea (*Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, *Methanosarcina acetivorans*, *Methanosarcina mazei Goel*, *Pyrococcus abyssi*, and *Pyrococcus horikoshii*), anaerobic bacteria (*Clostridium acetobutylicum*, *Thermotoga maritima*) and
microaerophilic bacteria (Magnetococcus spp., Thermoanaerobacter tencongensis and Treponema pallidum) (Table 3). Seven of the SOR possessing microorganisms also have Rd and NROR in their genomes (Archaeoglobus fulgidus, Methanobacterium thermoautotrophicum, Methanococcus jannaschii, Pyrococcus abyssi, Pyrococcus furiosus, Thermotoga maritima, and Magnetococcus spp.). The taxonomic distributions of the organisms that appear to have SOR genes are not only limited to thermophilic archaea but also include mesophilic bacteria as well, indicating that the use of SOR for oxygen detoxification is widespread among anaerobic microorganisms. Also given that a number of the anaerobic microorganisms that have SOR, also appear to have Rd, and NROR-like enzymes, it is suggested that the SOR reduction pathway involving Rd and NROR can be described as a generalized detoxification system of reactive oxygen species in anaerobes.

**Future studies suggested by this work**

There has been a recent study confirming the SOR/ Rd/ NROR superoxide reduction system of *P. furiosus* using an *in vitro* assay system (Grunden *et al.*, 2003). This present study has provided preliminary evidence that rubredoxin is the physiological electron donor to SOR *in vivo* through the SOR complementation studies using the *E. coli sodA, sodB* and *E. coli sodA, sodB, norV* mutant strains. However, to further confirm this assessment, it will be necessary to co-express the *P. furiosus* Rd and SOR in the *E. coli sodA, sodB*, and *norV* mutant strain and show that full growth restoration occurs. The construction of a *P. furiosus* Rd-SOR co-expression system to use in the indicated complementation study is currently ongoing.
*E. coli* has the gene, *norW*, which shows 42% similarity to *P. furiosus* NROR encoding gene. It is located adjacent to the flavorubredoxin gene (*norV*), and NorW is thought to transfer electrons to the rubredoxin-like domain of the NorV using NADPH as the electron source (Gardner *et al.*, 2002). In order to confirm the function of *P. furiosus* NROR in oxygen detoxification *in vivo*, a complementation study of *P. furiosus* NROR and SOR expressed in a *sodA*, *sodB*, and *norW* *E. coli* mutant strain is needed. The construction of a recombinant *P. furiosus* NROR expression vector for use in *E. coli* is ongoing, while the *sodA*, *sodB*, and *norW* mutant *E. coli* strain has been constructed already as part of this study and has been confirmed. The co-expression of the *P. furiosus* NROR and SOR should also be investigated in a future study.

The functional relationship between the *P. furiosus* rubedoxin and NROR could also be examined in an *E. coli norV*, *norW* double mutant. It will also be desirable to co-express *P. furiosus* SOR, Rd, and NROR in an *E. coli* strain mutant in SOD, NorV, and NorW. In addition, attempts could be made to directly probe the *in vivo* activity of SOR, Rd, and NROR in *P. furiosus* by using a recently described method for the generation of gene knockouts in *P. furiosus* (Sato *et al.*, 2003). Disruption of SOR, Rd, and NROR, singly and in combination could be used to study the resulting effects on superoxide reduction by both enzymatic assay and oxygen tolerance profiles.
Table 3. The distribution of SOR, Rd, and NROR in microorganisms that have been sequenced.

<table>
<thead>
<tr>
<th>Name of species</th>
<th>SOR presence</th>
<th>Rubredoxin presence</th>
<th>NROR presence</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Archaeoglobus fulgidus</em></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Anaerobic archaea</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Anaerobic archaea</td>
</tr>
<tr>
<td>Methanosarcina acetivorans</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Anaerobic archaea</td>
</tr>
<tr>
<td>Methanosarcina mazei Goel</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Anaerobic archaea</td>
</tr>
<tr>
<td><em>Pyrococcus furiosus</em></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Anaerobic archaea</td>
</tr>
<tr>
<td><em>Pyrococcus horikoshii</em></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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</tr>
<tr>
<td><em>Pyrococcus abyssi</em></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Anaerobic archaea</td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
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</tr>
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</tr>
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<td>Treponema pallidum</td>
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<td>No</td>
<td>Microaerophilic bacteria</td>
</tr>
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</table>

*Bold type indicates organisms that have genes encoding SOR, Rd, and NROR*
Figure 18. Alignment of *P. furiosus* Rd with the *E. coli* Rd-domain of NorV. Asterisks denote the identical residues between the sequences (35% identity). Dots indicate similar residues from the alignment (14% similarity). A total of 49% similarity is observed between the sequences.
REFERENCES


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