Anaerobic Elemental Sulfur Reduction by Fungus Fusarium oxysporum

Tsuyoshi Abe, Takayuki Hosino, Akira Nakamura, and Naoki Takaya

Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

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Reduction of inorganic sulfur compounds by the fungus Fusarium oxysporum was examined. When transferred from a normoxic to an anoxic environment, F. oxysporum reduced elemental sulfur to hydrogen sulfide (H2S). This reaction accompanied fungal growth and oxidation of the carbon source (ethanol) to acetate. Over 2-fold more of H2S than of acetate was produced, which is the theoretical correlation for the oxidation of ethanol to acetate. NADH-dependent sulfur reductase (SR) activity was detected in cell-free extracts of the ethanol to acetate. NADH-dependent sulfur reductase reduces NO3⁻ to NO2⁻ in fungal denitrification. This reaction accompanies ATP synthesis through oxidative phosphorylation in the mitochondrial membrane, indicating that the reaction is significant to NO3⁻ respiration in fungi as it is in bacteria. Zhou et al. discovered and characterized the mechanism of ammonia fermentation, in which NO3⁻ is reduced to ammonium by successive reactions of cytosolic NO3⁻ and nitrite reductases that couple to substrate-level phosphorylation. Other fungal species also denitrify and/or anaerobically produce ammonium. These findings indicate that fungi utilize NO3⁻ as a terminal electron acceptor when O2 is restricted. Despite these studies of the NO3⁻ and O2-dependent dissimilation mechanisms, those dependent on other inorganic compounds remain elucidative in fungi.

Some bacteria and archaea are able to dissipate inorganic sulfur compounds in an anaerobic environment. Obligatory anaerobic sulfur-reducing bacteria acquire energy by a sulfate respiration mechanism that uses sulfate as a terminal electron acceptor. This sulfate respiration mechanism reduces sulfate to H2S with eight electrons equivalents. Elemental sulfur (S8) is ubiquitous in the environment, and bacteria utilize it in various biological reactions. It has been reported that sulfur-reducing bacteria and archaea such as Desulfurobacterium acetoxidans, Desulfovibrio baculatus, and Staphylothermus marinus produce ATP by oxidizing organic compounds that couple S8 reduction to sulfide. S8 is also used as an energy source by some photo- and chemolithotrophic archaea and bacteria. By contrast, little is known about how eukaryotes respond to and utilize inorganic sulfur compounds (especially S8) under anaerobic conditions, although fungi as well as bacteria assimilate sulfite and sulfate to synthesize amino acids, proteins, and other compounds containing sulfur.

In this study we found that F. oxysporum anaerobically reduced S8 and lives in an anaerobic environment. Our results indicate that the reaction is of significance for the fungus in dissipilating S8 and adapting to O2 scarcity.

Key words: elemental sulfur; sulfur reductase; Fusarium oxysporum

Oxygen (O2) respiration is a characteristic feature of mitochondria, due to which eukaryotes inhabit aerobic environments. The process produces ATP for cell growth, and thus is physiologically significant as a dissimilation mechanism. Recent reports have shown that some lower eukaryotes express other dissimilation mechanisms and survive under anoxic conditions. Dissimilatory nitrate (NO3⁻) reduction, for example, is performed by fungi and a ciliate. Dissimilatory NO3⁻ reduction by the Ascomycota fungus, Fusarium oxysporum, is the most characterized of these mechanisms in eukaryotes. This fungus dissimilates NO3⁻ through denitrification and ammonia fermentation, which are induced under O2-limited and more anoxic conditions respectively. Denitrification is a process through which NO3⁻ or nitrite is reduced to gaseous nitrogen or nitrous oxide. It was discovered as a bacterial mechanism and, is thought to be important to the global nitrogen cycle. Kobayashi et al. have found that mitochondrial NO3⁻ reductase reduces NO3⁻ to NO2⁻ in fungal denitrification.

To whom correspondence should be addressed. Tel./Fax: +81-298-53-4937; E-mail: ntakaya@sakura.cc.tsukuba.ac.jp

Abbreviations: COX, cytochrome c oxidase; SR, sulfur reductase
Materials and Methods

Strains, cultures, and media. F. oxysporum JCM11502 originated in the Japanese Collection of Microorganisms. The fungus was aerobically incubated at 30 °C for 72 h with shaking at 120 rpm on a rotary shaker in 300 ml of GP medium (3% glycerol, 0.2% polypeptone, 10 mM KH₂PO₄ (pH 7.2), 2 mM MgSO₄, and 0.1% trace elements) in 500 ml flasks. The mycelia were harvested by centrifugation and washed three times with 0.9% NaCl, and portions of the mycelia (corresponding to the amount of cells obtained from 20 ml precultures) were used to inoculate 100 ml of MMEA medium (300 mM ethanol, 10 mM NH₄Cl, 10 mM KH₂PO₄, 2 mM MgSO₄, and 0.1% trace elements) containing 20 mM sulfur compounds or 0.64 g l⁻¹ S₈ in 500 ml Erlenmeyer flasks sealed with butyl rubber stoppers. Anaerobic or initially aerobic conditions were maintained with or without purging headspace air in the flasks with N₂ gas for 10 min. Aerobic conditions were maintained by sealing the flasks with cotton plugs without purging. The effect of the carbon source was examined in test tubes containing 3.6 ml of MMEA medium, in which the ethanol was replaced with 100 mM of each carbon source. The test tubes were incubated at 30 °C with shaking at 120 rpm under anaerobic conditions.

Analytical methods. To determine total sulfide in the cultures, culture broth was processed for the methylene-blue reaction and centrifuged at 17,400 × g for 5 min to remove cell debris, and the absorbance at 665 nm was measured. The amount of H₂S in the gas phase was calculated by assuming that 2.014 liter of H₂S is solved into 1 liter of water at 30 °C, 1 atm, and that the gas dissolves relative to its partial pressure (Henry’s Law). The dry cell weight was determined after drying the washed cells at 94 °C for 24 h. Acetate was determined by high-performance liquid chromatography (HP-1100 system, Hewlett Packard, Palo Alto, CA, USA) equipped with an SCR-101H column (Shimadzu, Kyoto, Japan). Perchloric acid (0.09% in water) was the eluent, and absorbance at 210 nm was monitored. The levels of CO₂ were determined by gas chromatography as described previously.

Preparation of cell free extract of F. oxysporum. F. oxysporum was rotary-shaken in Erlenmeyer flasks containing 2.51 of MMEA medium with 0.64 g l⁻¹ S₈ under the anaerobic or aerobic conditions. After 48 h, the cells were harvested, suspended in buffer (20 mM potassium phosphate, pH 7.2, 10% glycerol, and 0.6 M sucrose), disrupted using aluminum oxide powder, and centrifuged at 2,000 × g for 30 min to obtain cell-free extracts. A crude mitochondria fraction was obtained by centrifuging the cell-free extracts at 10,000 × g for 60 min.

H₂S production and O₂ consumption by intact cells. F. oxysporum was rotary-shaken in Erlenmeyer flasks containing 100 ml of MMEA medium with 0.64 g l⁻¹ S₈ under anaerobic or aerobic conditions as above, and collected by centrifugation. The cells (70 mg) were transferred to 100 ml of MMEA medium in the 500 ml flask. After incubation for 6 h, total sulfide in the culture was determined as above. To measure the O₂ consumption rate, cells (0.7 mg) were incubated in 5 ml of MMEA medium in an air-tight vessel at 30 °C for 5 min. O₂ was monitored with a Clark-type O₂ electrode YSI 5300 (YSI, Yellow springs, OH, USA).

Enzyme assay. We measured sulfur reductase activity in assay mixtures (1 ml) containing 10 mg S₈, 20 mM Tris–HCl (pH 7.2), 10 mM glucose, 0.5 units of glucose oxidase, 250 units of catalase, and 1 mM NADH. Polysulfide (1 mM) and colloidal sulfur (1.1 mM) were replaced with S₈ to assay their reduction. Headspace gas in the tube was replaced by purging with N₂ gas for 2 min. After incubation at 30 °C for 10 min, the reaction was started by adding NADH. Levels of produced H₂S were determined by the methylene blue method. Cytochrome c oxidase activity was measured as described previously by monitoring the change in absorbance at 549 nm due to the oxidation of ferrocyanochrome c₅₅₉, prepared as described previously.

Preparation of polysulfide and colloidal sulfur. Polysulfide was prepared from crystalline sodium sulfide and sulfur powder in O₂-free water as described previously. Colloidal sulfur was prepared by acidifying sodium thiosulfate with concentrated H₂SO₄, as described previously.

Results

Anaerobic sulfur reduction by F. oxysporum. We cultured F. oxysporum JCM11502 in minimum medium containing various inorganic sulfurs under anaerobic conditions, and found that it evolved H₂S in the presence of S₈ (Fig. 1A). The culturing medium lacked NO₃⁻, which previous studies indicate is required for cell growth under anaerobic conditions, but we found that the cell mass increased to 65 mg flask⁻¹ after culturing 2 d, indicating that reduction of S₈ to H₂S supported fungal growth under anaerobic conditions. By contrast, none of the other tested inorganic sulfurs (sulfate, sulfite, thiosulfate, and tetrathionate) contributed to H₂S production. Cell growth supported by these compounds was less significant (below 9 mg flask⁻¹) (Fig. 1A). These results indicate that anaerobic production of H₂S and cell growth is specific to S₈. Time-dependent production of H₂S was investigated. The results showed that F. oxysporum produced H₂S immediately after the start of culture, and that its amount reached 0.27 mmol flask⁻¹ after 30 h (Fig. 1B). We found that F. oxysporum concomitantly produced 0.14
and 0.04 mmol flask$^{-1}$ of acetate and CO$_2$ respectively (Fig. 1B), indicating that the carbon source (ethanol) was oxidized to acetate and CO$_2$ while the fungus reduced S$_8$. Because of its high reactivity against cellular compounds, the amount of H$_2$S should be underestimated, and hence, these results indicate that over twice as much H$_2$S was produced as acetate.

**Effect of O$_2$ on H$_2$S production**

When cultured with S$_8$ under aerobic conditions, *F. oxysporum* accumulated little H$_2$S (data not shown). Under aerobic conditions, no significant difference was observed in cell growth (390 ± 30 mg flask$^{-1}$) in the presence and absence of S$_8$. When starting the culture under the initially aerobic conditions, where the head space of the flasks was air, the fungus initiated H$_2$S production after a lag of about 10 h (Fig. 2A), and this was not observed under the anaerobic conditions (Fig. 1B). During the lag period, O$_2$ was consumed and CO$_2$ and acetate were produced, indicating that the fungus respired with O$_2$. The fungus produced H$_2$S after the O$_2$ in the culture flasks was consumed to a level below 0.04 mmol flask$^{-1}$ (Fig. 2A). Figure 2B shows the activity of S$_8$- and O$_2$-reduction by aerobically and anaerobically grown cells. These results show that the aerobically grown cells produced little activity for S$_8$ reduction, in contrast to the anaerobically grown cells. They imply that O$_2$ repressed expression of the mechanism for S$_8$ reduction. Both O$_2$ consumption by the cells (Fig. 2B) and cytochrome c oxidase (COX) activity in the mitochondrial fractions (Fig. 2C) were decreased under anaerobic conditions, whereas S$_8$-reduction was up-regulated. The anaerobically grown cells showed higher activity in O$_2$ consumption than in COX activity in the mitochondria. This is probably due to the reduction in O$_2$ by H$_2$S, which we detected in considerable amounts in the cells but less in the mitochondria preparation.

**Effect of carbon source on H$_2$S production**

We examined anaerobic S$_8$ reduction by *F. oxyspo-
Results showed that the fungus produced H$_2$S by using ethanol (Figs. 1 and 2), glucose, butanol, malate, succinate, fumarate, pyruvate, propionate, and glycolate as sole sources of carbon under anaerobic conditions (Table 1), amounts of H$_2$S produced and of biomass produced did not greatly differ, among the carbon sources. By contrast, the fungus neither grew nor produced H$_2$S when anaerobically cultured using acetate, lactate, glycerol, glutamate, formate, or citrate as carbon sources, indicating that the carbon source affected the S$_8$-to-H$_2$S reaction.

**Cell-free activity for sulfur reduction**

Cell-free activity for S$_8$ reduction (S$_8$ reductase, SR) was reconstituted using NADH as an electron donor. We detected considerable SR activity in cell-free extract prepared from the S$_8$-reducing cells, while that prepared from the aerobically-grown cells showed less activity (Fig. 2C). These results indicate that the fungus induced SR activity under anaerobic conditions and repressed it under aerobic conditions. This is in consistent with the intact cell assay (Fig. 2B).

Since, solubility of S$_8$ is extremely low (0.16 μm) under physiological conditions, it has been assumed that the true substrate of the known SR might be more soluble form of sulfur.22-20 Hence we examined the reducing activity of more soluble polysulfide (S–S$_n$=–S$^-$) and colloidal sulfur (HS$_x$S$_2$O$_y$–) to H$_2$S using fungal cell-free extract. The results showed that the NADH-dependent reducing activity for these substrates was 1.6- and 2.8-nmol min$^{-1}$mg$^{-1}$ respectively (Fig. 3), and that it was comparable to S$_8$ reducing activity (2.2 nmol min$^{-1}$mg$^{-1}$), indicating that *F. oxysporum* SR utilized polysulfide or colloidal sulfur as well as S$_8$.

**Discussion**

This study found that the fungus *F. oxysporum* reduces S$_8$ to H$_2$S under anaerobic conditions. The addition of S$_8$ to the medium was necessary for the growth of the fungus under these conditions, indicating that reduction of S$_8$ to H$_2$S contributed to cell growth. S$_8$-dependent growth is also of characteristic of S$_8$ reducing bacteria and archaea.8,13 The growth of the fungus per S$_8$ reduction was calculated from the data in Fig. 1A to be 0.3 g mmol$^{-1}$ H$_2$S, comparable to the value of *D. acetoxidans* dissimilating S$_8$ (0.2 g mmol$^{-1}$).8 indicating that S$_8$ reduction to H$_2$S was sufficient for the fungus to acquire energy for growth. These results imply that the reaction is physiologically important as a dissimilatory mechanism of the fungus. Although researchers have studied bacterial and archaeal S$_8$ dissimilation mechanisms, little is known about the dissimilatory use of S$_8$ by eukaryotes. This study is the first to provide evidence of eukaryotic S$_8$ dissimilation.

At present, mechanism by which the fungus produces ATP remains to be identified, but the fact that H$_2$S production accompanied formation of acetate suggests a possible pathway of ATP production. Previously, we and others reported that *F. oxysporum* reduced NO$_3^-$ to ammonia by the ammonia fermentation mechanism under anaerobic conditions.3 This mechanism oxidizes ethanol to acetate the amount of which is stoichiometrical relative to the reduced NO$_3^-$, and which generates ATP by substrate-level phosphorylation.3 This study found that the fungal S$_8$-reduction mechanism produced twice as much H$_2$S as acetate. Since oxidation of an ethanol to acetate produces four electron equivalents while reduction of S$_8$ to H$_2$S requires two electron equivalents, the results are consistent with the theoretical correlation of the oxidation of ethanol to acetate that concomitantly proceeds with the reduction of S$_8$ to H$_2$S, calculated value from the published data for the anaerobic cell mass increase was 0.7 g l$^{-1}$ for ammonia fermenting culture,3 which resembles to our results on S$_8$-dependent cell mass increase (0.65 g l$^{-1}$).

This suggests that the S$_8$ reducing mechanism shares an ATP-generating mechanism with ammonia fermentation system.

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**Table 1.** Substrate Utilized as Electron Donors and Carbon Source by *F. oxysporum*

Cells (2.7 ± 0.3 mg) were cultured in minimum medium with 100 mM of each carbon source and S$_8$ for 48 h under anaerobic conditions. Data are mean values of three measurements.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>H$_2$S (nmol tube$^{-1}$)</th>
<th>Cells produced (mg tube$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>65.5 ± 12</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50.1 ± 6.5</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Butanol</td>
<td>94.4 ± 10</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Malate</td>
<td>65.8 ± 8.4</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Succinate</td>
<td>55.8 ± 6.3</td>
<td>4.4 ± 1.9</td>
</tr>
<tr>
<td>Fumarate</td>
<td>68.8 ± 9.5</td>
<td>9.4 ± 2.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>72.7 ± 19</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Propionate</td>
<td>73.3 ± 1.6</td>
<td>3.1 ± 2.4</td>
</tr>
<tr>
<td>Glycolate</td>
<td>69.3 ± 10</td>
<td>1.8 ± 0.8</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Reduction of Polysulfide and Colloidal Sulfur.

NADH-dependent reduction of polysulfide and colloidal sulfur by the cell-free extract assayed as described in “Materials and Methods.” Symbols represent S$_8$ (circles), colloidal sulfur (squares), and polysulfide (triangles). Values are the means of three experiments.
This study found the first fungal SR and showed that it is soluble and that it uses NADH as a reductant. We used ethanol as a carbon source and cultured the fungus under anaerobic conditions where no electron acceptor other than $S_8$ was supplied. This implicates that the fungus must remove excess redox equivalents generated by the oxidation of ethanol for growth. It is highly possible that the fungus uses SR for $S_8$ to serve as an electron sink. To date, similar soluble NADH-dependent SRs have been found in *Thiobacillus ferrooxidans* and *Pyrococcus furiosus*. These organisms oxidize ferrous iron and organic acids respectively to generate ATP, and the resulting redox equivalent (NADH) was re-oxidized by organic acids. The fungus uses SR for $S_8$ oxidation for growth. It is highly possible that the fungus uses SR for $S_8$ to serve as an electron sink. To date, similar soluble NADH-dependent SRs have been found in *Thiobacillus ferrooxidans* and *Pyrococcus furiosus*. These organisms oxidize ferrous iron and organic acids respectively to generate ATP, and the resulting redox equivalent (NADH) was re-oxidized by organic acids.

Since $S_8$ is much less soluble and membrane-permeable, it is thought that it is not directly reduced by bacteria, but is converted to a more soluble compound that serves as the actual substrate for reduction. In the case of *Wolinella succinogenes*, polysulfide was found to be the soluble intermediate for sulfur reduction, and its polysulfide reductase was identified. Polysulfide is generated chemically from $S_8$ and trace amounts of sulfide or polysulfide by the following reaction scheme: $S_8 + S^{2-} \rightarrow S_2 + m^2$. The typical SR assays in this study contained considerable amounts of sulfide (about 0.24 $\mu$m) derived from cell-free extracts (data not shown), strongly suggesting that $S_8$ reacts with it and dissolves as polysulfide upon the assay. Our finding that fungal cell extracts exhibited reducing activity for $S_8$, despite of its low solubility, as high as that for polysulfide (Fig. 3) supports this notion. Future studies should determine whether polysulfide or $S_8$ is the actual substrate for the fungal SR.

This study found that the fungus repressed H$_2$S production under an atmosphere of O$_2$. By contrast, O$_2$ respiration activity is down-regulated during the production of H$_2$S (Fig. 2). These observations imply that the fungal $S_8$-reduction mechanism is an adaptation mechanism to anaerobiosis that replaces O$_2$ respiration. It has been reported that the fungus induced anaerobic dissimilation mechanisms by which the fungus respires with NO$_3^-$ and NH$_4^+$ in addition, the fungus induces cytoplasmic substrate level phosphorylation mechanisms (fermentation) by utilizing NO$_3^-$ (ammonium fermentation). The midpoint potential for $S_8$ is considerably lower (79/HS$^-$, $E_0 = -0.28$ V) than that for NO$_3^-$ (NO$_3^-$/NO$_2^-$, $E_0 = 0.43$ V), which serves as an electron acceptor for the fungus. The finding that the fungus reduces $S_8$ indicates that it adapts to more reductive environments than has been imagined. That a single species of *F. oxysporum* expresses such versatile dissimilation mechanisms is notable. This variety of metabolic mechanisms might contribute to the ability of the fungus to thrive under various environmental conditions with respect to electron acceptors.

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**References**


