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Enzymatic Reduction of Iron Oxide by Fungi

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The occurrence of the iron-reducing phenomenon among some common fungi was studied. Results indicated that (i) the reduction of ferric iron to the ferrous state by fungi seems to be restricted to nitrate reductase-inducible strains such as *Actinomucor repens*, *Alternaria tenuis*, *Fusarium oxysporum*, and *F. solani* and (ii) the amount of dissolved ferrous iron may be reduced progressively by increasing the amount of nitrate added to the medium. Compared with a complex medium (Sabouraud medium), less iron became reduced if NO_3^- was the only nitrogen source (Czapek Dox medium). These data strongly support the view that ferric iron is acting as an hydrogen acceptor in respiration, competing with nitrate for electrons that are mediated by the enzyme nitrate reductase. The significance of this property from an ecological viewpoint is discussed.

Fungi capable of reducing ferric compounds to the soluble ferrous state have not yet been reported. However, this property has been observed among a great variety of common bacteria, especially among members of the *Enterobacteriaceae*, *Bacillaceae*, *Pseudomonadaceae* (7; J. C. G. Ottow, Z. Pflanzenernaehr. Bodenk., *in press*), as well as among anaerobic, nitrogen-fixing clostridia (J. C. G. Ottow, Zentrabl. Bakteriol. Parasitenk. Abt. II, *in press*). These bacteria are widespread inhabitants of natural environments and are abundantly distributed in water (8) and soil (J. C. G. Ottow, Zentrabl. Bakteriol. Parasitenk. Abt. II, *in press*). Representatives of the same group of bacteria are probably involved in the reduction of manganese dioxide in soil, since a manganese dioxide-reducing strain of *Bacillus* species was recently isolated from manganese nodules (9). Evidence of iron reduction with any particular fungi has thus far never been reported, although two molds, *Verticillium* species and *Cladosporium herbarum*, are suspected of being capable of reducing iron (6). These organisms were isolated from a decaying mixture of soil and organic matter. Although considerable amounts of ferrous iron were detected, it was not evident whether these fungi were responsible for the reduction process. A great deal of gas was observed, which could have been of bacterial origin. This investigation was undertaken to determine the iron-reducing ability of some widespread fungi, some available from culture collections and some obtained by isolation from an iron oxide-enriched industrial waste product showing abundant fungal growth.

MATERIALS AND METHODS

Organisms. The fungi tested in this study are listed in Table 1. All were obtained from the culture collection at the Institut für Landwirtschaftliche Mikrobiologie, Giessen, Germany, except for the organisms *Aureobasidium* species, *Cephalosporium* species, *Chaetomium murinum*, *Fusarium solani*, and an unidentified species of *Spicaria*. These molds were isolated from an iron oxide-enriched, industrial waste product (*p*H approximately 7.5 to 8.0), rich in organic matter, by spreading mycelia-containing fragments on 2% malt extract agar. Fungi growing from these samples were subcultured for pure culture, stored, and identified by genus. The identification of *F. solani* was verified by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The test organisms were grown on a 2% malt extract agar (30 C), and a small agar block from an area of dense growth was selected as inoculum.

Media. The qualitative determination of iron-reducing ability was performed in 100-ml, conical flasks containing 2% glucose-asparagine broth (7), to which 0.1% finely powdered, reagent-grade hematite (α-Fe_2O_3) was added. Nitrate reduction was demonstrated in the same medium fortified with 0.1% KNO_3. Tests on nitrite were made after 3 and 5 days by the Griess-Ilosvay method with reagents I and II.

Quantitative determination of iron-reducing capacity (milligrams of Fe^{2+}/liter) was made in the same 2% glucose-asparagine broth as well as in Czapek Dox and Sabouraud media. All media received 0.1% finely powdered hematite and were dispensed while stirring the suspension in 25-ml volumes into 100-ml, conical flasks. The media were sterilized by autoclaving for 15 min at 121 C. Because of the many current modifications existing in the nutritional compositions of both Czapek Dox and Sabouraud media, the media used in this study are described be-

low. Czapek Dox solution contained K_2HPO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $NaNO_3$, 3.0 g; glucose, 30.0 g; tap water, 1,000 ml; final *p*H, 6.8. Sabouraud solution contained peptone, 10.0 g; maltose, 30.0 g; yeast extract (Difco), 0.5 g; tap water, 1,000 ml; final *p*H, 7.0.

Methods. The qualitative and quantitative determinations of iron-reducing capacity were performed as described previously (7). Cultures were incubated for 2 weeks (30 C) and the amount of dissolved ferrous iron was expressed in milligrams of Fe^{2+} per liter by using α,α'-dipyridyl as a specific reagent. The determinations were made on an Eppendorf photometer set at 546 nm.

RESULTS

Qualitative determination of iron-reducing ability. Among the organisms tested for their ability to reduce ferric oxide (Table 1), only *Alternaria tenuis*, *Actinomucor repens*, *F. oxysporum*, and *F. solani* were capable of reducing ferric iron to the ferrous state when grown in a 2% glucose-asparagine broth. All organisms showed fair to good growth in this medium. From Table 1 it is also evident that only those fungi endowed with the enzyme nitrate reductase, demonstrated as nitrite formation, are potentially capable of reducing ferric iron oxide.

TABLE 1. *Occurrence of iron-reducing ability among some common fungi in relation to their capacity to reduce nitrate into nitrite*

Organisms	Fe^{2+} formation	NO_2^- formation
Aureobasidium species	−	−
Actinomucor repens	+	+
Aspergillus ustus	−	−
Alternaria tenuis	+	+
Cephalosporium species	−	−
Chaetomium indicum	−	−
C. murinum	−	−
Cladosporium species	−	−
Fusarium oxysporum	+	+
F. solani	+	+
Penicillium species	−	−
Spicaria species	−	−
Trichoderma lignorum	−	−
Control[a]	−	−

[a] Uninoculated flasks.

Quantitative determination of iron-reducing capacity. Three organisms listed in Table 2 were selected and their iron-reducing capacity was evaluated in three different media. Growth of these organisms was abundant in all media used. In the shallow culture layer, a dense mycelium developed, reaching to the bottom of the flasks and thus enabling direct contact between iron oxide and the hyphae. To reveal the influence of nitrate on the iron-reducing capacity, increasing amounts of KNO_3 were added to the 2% glucose-asparagine medium. After each quantitative determination, the *p*H value of the culture was measured by use of a glass electrode (Table 2).

From Table 2, one may conclude that (i) the iron-reducing capacity differed greatly according to the organism and medium used; (ii) the amount of reduced iron was highest in the media free from nitrate; (iii) increasing the amount of nitrate in the glucose-asparagine medium decreased the extent to which iron is reduced. When the amount of dissolved iron was evaluated, neither nitrate nor nitrite could be demonstrated in the culture media, suggesting a complete reduction of nitrate. Indirectly the utilization of nitrate is evident through a raised final culture reaction, which is much more alkali in the samples that received KNO_3. Thus, in both Czapek Dox and glucose-asparagine medium (the latter fortified with 0.3% KNO_3), the final *p*H was shifted to alkalinity, whereas the amount of reduced iron

TABLE 2. *Iron-reducing capacity (milligrams of Fe^{2+}/liter) of three common fungi in relation to the media and amount of nitrate used*[a]

Organism	Media									
	Czapek Dox		Sabouraud		2% Glucose-asparagine					
					Without KNO_3		With 0.1% KNO_3		With 0.3% KNO_3	
	Fe^{2+}	*p*H	Fe^{2+}	*p*H	Fe^{2+}	*pH*	Fe^{2+}	*p*H	Fe^{2+}	*p*H
Actinomucor repens	2.3	8.9	7.2	8.2	6.2	7.8	4.7	8.3	1.8	8.9
Fusarium oxysporum	2.3	7.1	4.0	5.1	4.6	6.1	2.5	7.8	1.5	7.5
F. solani	6.0	7.9	17.3	6.5	9.7	7.4	6.5	7.9	4.0	8.2
Control[b]	0	6.8	0	7.0	0	7.0	0	7.0	0	7.0

[a] Values are an average of five determinations.
[b] Uninoculated media.

reached its minimum. Apparently nitrate and iron oxide are competitive substrates, and this is independent of whether NO_3^- was the only nitrogen source or was applied in combination with asparagine. These results indicate that iron reduction is not only restricted to those fungi capable of reducing nitrate, but, in addition, may even be mediated by the same enzyme, nitrate reductase.

DISCUSSION

The reduction of ferric iron is essentially an endergonic, energy-requiring process ($\Delta G > 0$): $Fe^{3+} + e \rightarrow Fe^{2+}$. If ferric iron could serve as an electron acceptor in respiration, when the demand for oxygen exceeds the supply, its reduction would be of great advantage from an ecological viewpoint. Solubilization as a result of acidifying the medium is doubtful, because in most cultures no relationship could be detected between the degree of acidification and the rate of iron reduction. More likely, iron reduction by fungi should be regarded as a physiologically linked, enzymatic process. This view is supported by the following observations: (i) the iron-reducing property is restricted to those fungi that are nitrate reductase-inducible, and (ii) the amount of reduced iron may be suppressed by the addition of nitrate to the medium. In soil, the sparing effect of nitrate on ferrous iron formation, when applied to it, has been noticed before (10) and could be confirmed by results obtained with iron-reducing bacteria in pure culture (8). In all probability, ferric iron is serving as an electron acceptor in a way similar to dissimilation of nitrate by denitrifying bacteria. However, nitrate reduction in fungi is believed to be strictly assimilatory in nature (2) and mediated by flavine enzymes rather than by cytochromes (5). But, even among bacteria, the differentiation between an "assimilatory" and a "dissimilatory" nitrate reductase seems to be doubtful, since these two enzymes in *Escherichia coli* could not even be distinguished serologically (4). Apparently one enzymatic pathway exists, functioning either in an assimilative or in a dissimilative manner according to the environmental conditions and nutritional needs. Actually such nitrate reductase-equipped microorganisms are well adapted to endure and survive in conditions of reduced oxygen tensions by replacing oxygen through nitrate or alternative electron acceptors such as ferric iron, manganese dioxide, or others. Even the reduction of ceric ions to the cerous state by *F. oxysporum* could be explained in this way (1). The ability of nitrate reductase to reduce substrates other than NO_3^- is well established, since both perchlorate (ClO_4^-) and chlorate (ClO_3^-) may act as suitable acceptors for this enzyme (3). The suggested mechanisms of enzymatic iron reduction by nitrate reductase-containing microorganisms may be summarized by the following reactions:

biological oxidation

$$\text{energy source} \xrightarrow{\text{dehydrogenases}} e^- + H^+ + \text{adenosine triphosphate} + \text{end products}$$

iron as hydrogen acceptor

$$Fe_2O_3 + H_2O \rightleftharpoons 2FeO{\cdot}OH$$

$$2FeO{\cdot}OH + 6H^+ + 2e^- \xrightarrow[\text{nitrate reductase}]{} 2Fe^{2+} + 4H_2O$$

Direct proof, however, must await enzymatic experiments.

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