Ferrous-iron-dependent Uptake of L-Glutamate by a Mesophilic, Mixotrophic Iron-oxidizing Bacterium Strain OKM-9

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Strain OKM-9 is a mesophilic, mixotrophic iron-oxidizing bacterium that absolutely requires ferrous iron as its energy source and L-amino acids (including L-glutamate) as carbon sources for growth. The properties of the L-glutamate transport system were studied with OKM-9 resting cells. Plasma membranes, and actively reconstituted proteoliposomes. L-Glutamate uptake into resting cells was totally dependent on ferrous iron that was added to the reaction mixture. Potassium cyanide, an iron oxidase inhibitor, completely inhibited the activity at 1 mM. The optimum pH for Fe

Key words: iron-oxidizing bacterium; mixotroph; L-glutamate transport system; iron oxidase; L-glutamate transporter

The acidophilic, obligately autotrophic iron-oxidizing bacteria Acidithiobacillus ferrooxidans[1] [formerly Thiobacillus ferrooxidans[2]] and Leptospirillum ferrooxidans[3] can use ferrous iron and pyrite (FeS2) as energy sources, and carbon dioxide as a carbon source, for their growth. They are important in the inorganic iron and sulfur cycles on earth. Ferric iron produced by the oxidation of Fe

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Abbreviations: OGL, n-octyl-β-D-glucopyranoside; 2,4-DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide m-chlorophenylhydrazone
no acids are transported into these bacterial cells via proton- or sodium-dependent amino acid transport systems. The uptake of amino acids by the extremely acidophilic, thermophilic archaeon *Pyrococcus furiosus* has been reported. Its optimum pH and temperature for growth are 0.7 and 60°C. The uptake of glutamate by this archaeon is dependent on the ΔpH, which seems to be the main constituent of the proton motive force. In contrast, Gram-negative bacteria such as *Salmonella typhimurium* and *Rhodobacter sphaeroides* have L-amino acid-binding proteins in the periplasmic space and ATP-binding proteins in the plasma membrane involved in L-amino acid transport. Iron-oxidizing bacteria studied extensively to date, *Acidithiobacillus ferrooxidans* and *L. ferrooxidans*, are obligate chemolithotrophs and cannot use organic compounds as energy and carbon sources. Therefore, there have been few reports on the study of a transport system for organic compounds in iron-oxidizing bacteria other than glucose transport by the thermoacidophilic iron-oxidizing bacterium *Leviphilus* strain ALV, glutathione transport by *Acidithiobacillus ferrooxidans*, and L-glutamate transport by the moderately thermophilic iron-oxidizing bacterium strain TI-1.

In this study, we characterized the Fe²⁺-dependent L-glutamate uptake activity of the mesophilic, mixotrophic iron-oxidizing bacterium strain OKM-9, using resting cells, plasma membranes, and actively reconstituted proteoliposomes to obtain a biochemical base for bacterial mixotrophy. To our knowledge, this is the first report on L-glutamate transport system in a mesophilic, mixotrophic iron-oxidizing bacterium.

**Materials and Methods**

*Bacterium, medium, and cultivation conditions.* A mesophilic, mixotrophic iron-oxidizing bacterium, strain OKM-9, was used in this study. Strain OKM-9 was cultured at 30°C under aerobic conditions in a Fe²⁺-yeast extract (0.03%) medium (pH 2.5) containing FeSO₄·7H₂O (3%), (NH₄)₂SO₄ (0.3%), K₂HPO₄ (0.05%), MgSO₄·7H₂O (0.05%), KCl (0.01%), and Ca(NO₃)₂ (0.001%).

**L-Glutamate uptake.** The [U-¹⁴C] L-glutamate that was taken up into strain OKM-9 cells was measured by a method previously described. The composition of the reaction mixture was as follows: 2.0 ml of acidic water the pH of which was adjusted to 3.5 with sulfuric acid, washed resting cells (1 mg of protein), the plasma membrane (1 mg of protein), the solubilized fraction from plasma membranes (0.1 mg of protein) or reconstituted proteoliposomes (0.1 mg), 100 μmol of Fe²⁺, 40 nmol of L-glutamate, and 2.7 nmol of L-[U-¹⁴C] glutamate (0.5 μCi). The total volume was 2.25 ml. The reaction started with the addition of L-glutamate and Fe²⁺ to the reaction mixture, which had already been incubated at 30°C for 10 min. After a 0.2-ml sample was withdrawn, 50 μl of 20 mm HgCl₂ was added to the reaction mixture to stop the reaction. The reaction mixture was passed through a 0.2-μm cellulose ester membrane filter (Advantec, Toyo Roshi Kaisha, Ltd., Japan). The filter, which had caught the cells, was washed twice with 10 ml of distilled water and then put into 4 ml of a counting sol (Dojin, Kumamoto, Japan; Scintisol EX-H). After the filter had been completely solubilized in the counting sol, the radioactivity was measured with an Aloka LSC-5100 liquid scintillation system.

**Iron-oxidizing activity.** The iron-oxidizing activity was measured via the amount of oxygen uptake because of Fe²⁺ oxidation with a biological oxygen monitor (Yellow Spring Instrument Co., Inc., Ohio USA). The composition of the reaction mixture used to measure the iron-oxidizing activity was: resting cells (0.1 mg of protein), plasma membrane (0.1 mg of protein) or solubilized fraction from the plasma membrane (0.1 mg of protein), Fe²⁺ (100 μmol), and acidic water (pH 3.5) (2.5 ml). The total volume was 3.0 ml. The reaction mixture, without the ferrous iron, was incubated at 30°C for 10 min. The addition of ferrous iron to the reaction mixture started the reaction.

**Reconstitution of an L-glutamate transport system into proteoliposomes.** Cell-free extract was prepared from the resting cells of strain OKM-9 by sonication in 50 mm sodium phosphate buffer (pH 7.5) at 100 W for 10 min and then centrifugation at 12,000×g for 10 min to remove cell debris. The extract was centrifuged at 105,000×g for 1 h to obtain the plasma membrane. The membrane was solubilized in 1% n-octyl-β-D-glucopyranoside (OGL) for 12 h at 4°C in the presence of 0.4 m sodium sulfate and 25% (v/v) glycerol. The solution was centrifuged at 105,000×g for 1 h to obtain the solubilized supernatant solution. Lecithin (40 mg) and the supernatant (0.6 mg of protein) were mixed and dialyzed twice against 11 of acidic water (pH 3.5) for 6 h for the first dialysis and for 24 h for the second time dialysis for a proteoliposome preparation.

**Protein concentration.** Protein was measured by the method of Lowry et al. with crystalline bovine serum albumin as the standard.

**Results**

**Uptake of L-glutamate into resting cells of OKM-9**

The acidophilic, mesophilic, and mixotrophic iron-oxidizing bacterium OKM-9 can grow in a FeSO₄·7H₂O (3%) medium (pH 2.5) with 20 L-amino acids,
but not in a 20 l-amino acid medium without FeSO₄·7H₂O (pH 2.5). To obtain biochemical bases of a mixotrophic nature of strain OKM-9, we studied the properties of L-glutamate uptake with resting cells. The resting cells incorporated L-glutamate with an initial rate of 0.36 nmol·min⁻¹·mg protein⁻¹ in the presence of 44.4 mM FeSO₄·7H₂O and 19 µM L-glutamate (Fig. 1). However, uptake of L-glutamate was not observed in the reaction mixture without Fe²⁺ or with 1 mM of potassium cyanide, an inhibitor of iron oxidase in this strain. The amount of L-glutamate taken up into OKM-9 cells increased in proportion to the Fe²⁺ concentration in the reaction mixture. The amount of Fe²⁺ oxidized during L-glutamate uptake was measured under the same conditions (Fig. 2); 0.3 nmol of L-glutamate was transported when 810 nmol of Fe²⁺ was oxidized by the OKM-9 iron oxidase. These results suggest that the L-glutamate uptake by strain OKM-9 was dependent directly or indirectly on Fe²⁺ oxidation. The optimum pH for the L-glutamate uptake was 3.5–4.0 (data not shown). L-Glutamate uptake activity was not observed at pHs of 2.0 or below. The optimum pH for iron-oxidizing activity of this strain was 2.0. However, same 50% of the total iron-oxidizing activity observed at pH 2.0 was present between pH 3.0 and 4.5. The amount of L-glutamate taken up into OKM-9 cells increased with increase of L-glutamate concentration in the reaction mixture (Fig. 3). From the Eadie-Hofstee plot, the Kₘ and Vₘₐₓ for L-glutamate were 0.4 mM and 11.3 nmol·min⁻¹·mg protein⁻¹, respectively (Fig. 3, inset).

Compounds that affected L-glutamate uptake
L-Glutamate uptake was measured in the presence of various L- or D-amino acids. The activity was strongly inhibited by 5 mM L-aspartate, D-aspartate, D-glutamate, L-glutamine, L-asparagine, or L-cysteine (Table I). L-Glutamate and L-aspartate are
transported with the same transporter. Therefore, L-glutamate uptake activities were measured in the presence of different concentrations of L-aspartate. A Lineweaver-Burk plot showed a curve of typical competitive inhibition (data not shown) with an apparent $K_i$ for L-aspartate of 75.9 $\mu$M. L-Glutamate uptake was slightly increased by L-proline, glycine, or L-alanine. Inhibition of L-glutamate uptake by L-amino acids was stronger than inhibition by the corresponding D-amino acids.

Uncouplers, which disrupt the transmembrane pH gradient, such as 2,4-dinitrophenol (2,4-DNP) (1 mM) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (0.1 mM), did not inhibit L-glutamate uptake. The ionophores valinomycin (0.1 mM), monensin (0.1 mM), and gramicidin D (100 $\mu$g/ml) did not affect the uptake activity.

$L$-Glutamate uptake by plasma membranes from strain OKM-9

$L$-Glutamate uptake was observed when the OKM-9 cell-free extract was used as the enzyme source. Almost all of the $L$-glutamate uptake of the cell extract was present in the plasma membranes, but not in the cytosol fraction (Table 2). The plasma membranes had about 85% of the Fe$^{2+}$-dependent $L$-glutamate uptake activity and 40% of the iron-oxidizing activity of the resting cells. A cytosol fraction prepared from a cell extract did not affect the $L$-glutamate uptake of the plasma membranes. Experiments to check whether $L$-glutamate was actually taken up into the cells or merely bound to the $L$-glutamate-binding protein on the surface of the plasma membrane by specific affinity or to the membrane surface by hydrophilic interaction were done. First, to check the involvement of $L$-glutamate-binding protein, which binds $L$-glutamate with specific affinity, resting cells or the plasma membranes were treated with $L$-glutamate by the method described above. After the reaction was stopped, nonradioactive $L$-glutamate was added at the final concentration of 10 mM and after that the membrane filters loaded with cells or the plasma membranes were washed twice with 10 ml of 10 mM nonradioactive $L$-glutamate instead of the same volume of distilled water. The amounts of radioactivity were incorporated by resting cells or the plasma membranes washed with distilled water, suggesting that the radioactivity retained on the membrane filter after washing was not due to radioactive $L$-glutamate bound to $L$-glutamate-binding protein. Secondarily, to check the nonspecific binding of $L$-glutamate to resting cells and the membranes with hydrophilic interaction, resting cells or the plasma membranes were treated with $L$-glutamate as described above. After the reaction was stopped, the membrane filters with the samples were washed twice with 10 ml of 1 mM NaCl instead of the same volume of distilled water. Nearly the same amounts of radioactivity were incorporated by resting cells or the plasma membranes after the washing with distilled water and 1 mM NaCl, suggesting that nonspecific binding of $L$-glutamate to the cells or the plasma membranes was negligible. These results indicate that the radioactivity retained on the membrane filter after washing was due to the radioactive $L$-glutamate taken up into the cells and the membrane vesicles.

Reconstitution of $L$-glutamate transport system in proteoliposomes

$Fe^{2+}$-dependent $L$-glutamate transport activity similar to that observed in the resting cells (0.36 mmol·min$^{-1}$·mg$^{-1}$) was observed when the plasma membranes were used as the enzyme source, but the proteinous fraction obtained by solubilization of the plasma membranes with 1% OGL did not have $L$-glutamate uptake activity (Table 2). This finding suggests that the proper structure of the membrane vesicles in which $L$-glutamate is accumulated was decomposed by the detergent treatment. Therefore, we tried to prepare proteoliposomes containing iron oxidase and $L$-glutamate transporter from the

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Iron-oxidizing activity (µO$_2$·min$^{-1}$·mg$^{-1}$)</th>
<th>$L$-Glutamate uptake activity (nmol·min$^{-1}$·mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting cells</td>
<td>4.86</td>
<td>0.36</td>
</tr>
<tr>
<td>Cell extract</td>
<td>1.59</td>
<td>0.32</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>2.01</td>
<td>0.31</td>
</tr>
<tr>
<td>105,000×g supernatant</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fraction solubilized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with 1% OGL</td>
<td>2.33</td>
<td>0.07</td>
</tr>
<tr>
<td>Lecithin proteoliposomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with 44.4 mM Fe$^{2+}$</td>
<td>1.94</td>
<td>2.79</td>
</tr>
<tr>
<td>without Fe$^{2+}$</td>
<td>—</td>
<td>0.01</td>
</tr>
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</table>

Table 1. Effects of Amino Acids on $L$-Glutamate Uptake by a Mesophilic, Mixotrophic Iron-oxidizing Bacterium, Strain OKM-9

<table>
<thead>
<tr>
<th>Amino acid (5 mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>D-Glutamate</td>
<td>17</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>3</td>
</tr>
<tr>
<td>D-Aspartate</td>
<td>15</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>50</td>
</tr>
<tr>
<td>D-Glutamine</td>
<td>88</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>59</td>
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<tr>
<td>D-Asparagine</td>
<td>98</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>6</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>100</td>
</tr>
<tr>
<td>L-Proline</td>
<td>155</td>
</tr>
<tr>
<td>Glycine</td>
<td>125</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>110</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>104</td>
</tr>
</tbody>
</table>

Table 2. Iron-oxidizing and $L$-Glutamate Uptake Activities of Resting Cells, Cell Extract, the Plasma Membranes, and the Lecithin Proteoliposomes
OKM-9 plasma membranes. The solubilized fraction (0.6 mg) and lecithin (40 mg) were mixed and then dialyzed against acidic water. The proteoliposomes thus formed had both iron-oxidizing activity (1.94 µl/min⁻¹·mg⁻¹) and Fe²⁺-dependent L-glutamate uptake activity. The latter activity was 8-fold that of the resting cells (Table 2). In contrast, Fe²⁺-dependent L-glutamate uptake was not observed when the sample containing the solubilized fraction and Fe²⁺, but not lecithin, and the samples containing lecithin and Fe²⁺, but not the solubilized fraction, were used for the uptake experiment. The results suggest that iron oxidase and the L-glutamate transporter were reconstituted into a lecithin proteoliposome, into which L-glutamate was transported and accumulated, using the energy produced by Fe²⁺ oxidation.

**Discussion**

The characteristics of the L-glutamate transport system of the mesophilic, mixotrophic iron-oxidizing bacterium strain OKM-9 were first described in this study that used resting cells, plasma membranes, and actively reconstituted proteoliposomes. The most unusual points of the L-glutamate transport system observed in OKM-9 were that transport of L-glutamate into the cells was driven by Fe²⁺ oxidation and that uncouplers such as 2,4-DNP and CCCP and ionophores valinomycin, monensin, and gramicidin D did not inhibit the transport.

Involvement of the Fe²⁺ oxidation in L-glutamate transport is supported by the following results: (1) no transport occurred in the reaction mixture without Fe²⁺, (2) the level of transport activity depended on the concentration of Fe²⁺ added to the reaction mixture, and (3) the activity was completely inhibited by 1 mM potassium cyanide which completely inhibited the iron oxidase of this strain. Strain OKM-9 can grow mixotrophically with Fe²⁺ as the sole energy source and organic compounds as carbon sources, but cannot grow autotrophically or heterotrophically. This pattern is understandable because the strain cannot fix carbon dioxide. Why does strain OKM-9 not grow heterotrophically? Probably, the inability of the strain to use organic compounds as the sole source of energy is due to its lack of NADH oxidase activity. We have found that the plasma membranes of strain OKM-9, like those of moderately thermophilic, mixotrophic iron-oxidizing bacterium strain TI-1, do not have NADH oxidase activity. The Fe²⁺-dependent L-glutamate uptake observed in this study can explain the mixotrophic nature of this strain, because L-glutamate was not transported into the cells when Fe²⁺ was not present around the cells as an energy source.

The substrate specificity of the OKM-9 L-glutamate transporter was similar to that reported previously for other microorganisms. L-Glutamate transporters reported up to now can generally transport not only L-glutamate but also L-aspartate. The L-glutamate uptake of *P. oshimae* was strongly inhibited by L-aspartate and to a lesser extent by L-glutamine. The Fe²⁺-dependent L-glutamate transport system of strain OKM-9, which was inhibited by L-asparaginase, differed from that of *P. oshimae*.

L-Glutamate uptake of the acidophilic archaean *P. oshimae* is dependent on the proton gradient (ΔpH) between the plasma membranes, and thus is inhibited by 1 µM nigericin and gramicidin D. Aerobic microorganisms with ΔpH-dependent L-amino acid uptake obtain the ΔpH needed for L-amino acid transport by NADH oxidation via a terminal electron transport system. Presently, we do not have experimental evidence to explain why the uncouplers and the ionophores usually used in transport experiments did not inhibit L-glutamate uptake, because 10 µM CCCP did inhibit the growth of strain OKM-9. Perhaps these inhibitors did not reach the OKM-9 membrane under the conditions used, or these inhibitors did not work for the L-glutamate transporter of strain OKM-9; a pH gradient or membrane potential may not be needed when L-glutamate is transported by the Fe²⁺-dependent L-glutamate transport system of strain OKM-9. The iron oxidase of this strain is especially important. So we think that iron oxidase itself may be able to transport L-glutamate.

**Acknowledgments**

We thank Mrs. Gail Bower-Irons for valuable discussion.

**References**


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