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# Degradation of Eu(III)–malic acid complexes by *Pseudomonas fluorescens*

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**Abstract**

We studied the biodegradation of Eu(III)–malic acid complexes by *Pseudomonas fluorescens*. The bacterium degraded 10 mM acid in the presence of 0.05, 0.1, and 0.2 mM Eu(III), and also in its absence. The rate of degradation increased with decreasing ratios of Eu(III) to malic acid. These results suggest that the toxicity of Eu(III) can be masked through its complexation with malic acid. Unidentified metabolites associated with Eu(III) were produced as malic acid was broken down. Analyses by electrospray ionization mass spectrometry (ESI-MS) showed that one of them was pyruvic acid. Our findings suggest that the metabolites can influence the environmental behavior of Eu(III) by forming complexes with it.
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*Keywords:* Bacteria; Degradation; Europium(III); Malic acid; Pyruvic acid

## 1. Introduction

Transuranic (TRU) elements, such as Am(III) and Cm(III), are highly toxic because they emit high-energy α particles and have long half-lives. Plans to dispose of TRU wastes in geological repositories have raised a number of concerns about polluting the environment through dissolution and subsequent mobilization of TRU [1,2]; thus, long-term assessments of safety are required. TRU wastes contain cellulosic materials, scintillation fluids, waste oils, decontamination reagents, and chemical reagents. Among them, organic acids, such as citric acid, EDTA, and NTA form stable complexes with trivalent radionuclides, enhancing their mobility by increasing their solubility [3]. The number of microorganisms in deep geological environments is almost equivalent to that found in surface soils [4]. This suggests that microbial metabolites, especially organic acids, may affect the long-term behavior of actinides [5]. To estimate such behavior, we need to elucidate the mechanisms of degradation of complexes of organic acids with radionuclides by microorganisms.

The U(IV)–citrate complex is recalcitrant to degradation by the soil bacterium *Pseudomonas fluorescens* [6]. Similarly, this bacterium could not break down a Eu(III)–citrate complex with a molar ratio of 1:1 [7]. However, scarcely anything is known about the degradability of complexes with trivalent f-elements by microorganisms.

We studied the biodegradation of Eu(III)–malic acid complexes by *P. fluorescens*. Malic acid has two carboxyl groups and one hydroxyl group. By comparison with the structure of citric acid, malic acid lacks one acetic acid moiety. Malic acid has two optical isomers, L- and D-malic acid. Unlike the latter that is not a natural isomer, L-malic acid occurs in the environment as one of the microbial metabolites formed in the TCA cycle; hence, we used it in our study.

Europium(III) is a good analogue for Am(III) and Cm(III). Thus, by using Eu(III)–malic acid complexes for the degradation experiments, we can simulate the degradability of

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complexes of Am(III)– and Cm(III)–malic acid. Knowledge of the environmental behavior of trivalent f-elements is very important because they can substitute for Ca(II) in a wide range of physiological processes in organisms [8,9].

## 2. Experimental

### 2.1. Culture

*P. fluorescens* (ATCC 55241) was isolated from the low-level radioactive waste disposal site at West Valley, N.Y. [6]. The mineral-salts solution contained the following components: NaCl, 5.6 mg $dm^{-3}$; $(NH_4)_2SO_4$, 88 mg $dm^{-3}$; KCl, 75 mg $dm^{-3}$; β-glycerophosphoric acid disodium salt, 22 mg $dm^{-3}$; 2-[4-(2-hydrixyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 4.76 mg $dm^{-3}$; and trace elements ($MgSO_4{\cdot}7H_2O$, 7.0 mg $dm^{-3}$; $CaCl_2{\cdot}2H_2O$, 2.8 mg $dm^{-3}$; $MnSO_4{\cdot}5H_2O$, 1.2 mg $dm^{-3}$; $CuSO_4{\cdot}5H_2O$, 0.15 mg $dm^{-3}$; $CoCl_2{\cdot}2H_2O$, 0.15 mg $dm^{-3}$; $FeSO_4{\cdot}7H_2O$, 1.5 mg $dm^{-3}$; $Na_2MoO_4{\cdot}2H_2O$, 0.090 mg $dm^{-3}$; and $ZnSO_4{\cdot}7H_2O$, 0.10 mg $dm^{-3}$). The pH of the mineral-salts solution was adjusted to $7.0 \pm 0.1$ with NaOH. It was sterilized by autoclaving at 120 °C for 20 min. Malic acid, glucose, and $Eu(III)Cl_3$ solutions were sterilized separately by passing them through a 0.20-μm pore size Millipore HA filter (ADVANTEC MFS Inc., DISMIC-25).

Three different mineral-salt media, the malic acid medium, the glucose medium, and the blank test medium, were used for the biodegradation experiments. Each medium was prepared as follows:

(i) The malic acid medium, at pH 6.5, contained 10 mM malic acid and 0, 0.05, 0.1, or 0.2 mM $Eu(III)Cl_3$. The molar ratios of malic acid to Eu(III) were 200:1, 100:1, and 50:1 in the media containing 0.05, 0.1, and 0.2 mM Eu(III), respectively.
(ii) The glucose medium contained 1 mM glucose and 0 or 0.05 mM $Eu(III)Cl_3$. The pH of the medium was adjusted to 6.0 by HCl.
(iii) The blank test medium whose pH was adjusted to 6.0, 6.7, and 6.9, contained 0.05 mM Eu(III) without any carbon source and bacteria. The concentrations of Eu(III) in solution at 0, 12, 24, 72, and 264 h, were measured by ICP-AES.

### 2.2. Biodegradation experiments

Small numbers of *P. fluorescens* cells were cultured in 8 $cm^3$ of the mineral-salts medium containing 1 mM malic acid or 1 mM glucose. Cell growth was estimated by measuring the media's optical density at 600 nm (OD). Cell suspensions of 0.5 $cm^3$ were withdrawn from both the malic acid and the glucose media at the late exponential growth phase (OD of 0.02) and added to 49.5 $cm^3$ of their respective media contained in 100 $cm^3$ Erlenmeyer flasks. The samples were incubated at 30 °C in the dark, while being shaken at 180 rpm. The blank test media were not inoculated with the bacteria. Aliquots were withdrawn from all the flasks every 24 h up to 264 h for measuring pH and the concentrations of organic acids and Eu(III). The concentration of malic acid was measured after filtering the culture media through a 0.20-μm pore size Millipore HA filter (ADVANTEC MFS Inc., DISMIC-25). The Eu(III) concentration was measured after acidifying 0.5 $cm^3$ of the filtered solution with 2 $cm^3$ of 0.2 M $HNO_3$.

### 2.3. Analytical methods

The concentration of malic acid was measured by introducing the filtered solutions into a high-pressure liquid chromatography system (Waters, Alliance 2695) equipped with an organic acid column (Waters, P/N 023694) with the mobile phase of 0.2 (v/v%) formic acid. The flow rate was 0.7 $cm^3$ $min^{-1}$. The column's temperature was 60 °C. The elutions were subjected to ESI-mass spectroscopy (Waters, ZQ 2000) operated in the negative ion mode for $m/z$ = 50–500 under the following conditions: capillary voltage, −3.07 V; cone voltage, −23 V; desolvation temperature, 150 °C; sheath gas, $N_2$; flow rate, 360 $dm^3$ $h^{-1}$. The concentration of malic acid was estimated from the ESI-mass spectra detected at $m/z$ = 133.1. The elutions also were introduced into photodiode array detector (Waters, Alliance 2996) to detect UV absorption by the organic acids coexisting with malic acids. The Eu(III) concentration was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Shimadzu, ICP-7000). To characterize organic acids produced by *P. fluorescens* during inoculation period, the elution time and ESI-mass spectra of standard acid solutions were measured by the same methods. The standards used were fumaric acid, maleic acid, succinic acid, oxaloacetic acid, citric acid, isocitric acid, glyceric acid, glyoxilic acid, and pyruvic acid.

## 3. Results and discussion

### 3.1. Blank test

Fig. 1 shows the changes with time of Eu(III) concentrations in the blank test medium. At pH 6.0, the concentration remained constant throughout the experiment. It decreased rapidly at pH 6.7 and 6.9 through precipitation or adsorption on the flask.

### 3.2. Cell growth in the glucose medium

Cell growth occurred in the glucose medium without Eu(III). In contrast, it was not observed in glucose medium containing 0.05 mM Eu(III) up to 200 h after inoculation (data not shown), suggesting that the cell did not grow and Eu(III) was toxic to *P. fluorescens*. The toxicity of Gd(III), used as an MRI contrast reagent, to *Escherichia coli* has been reported [10,11].

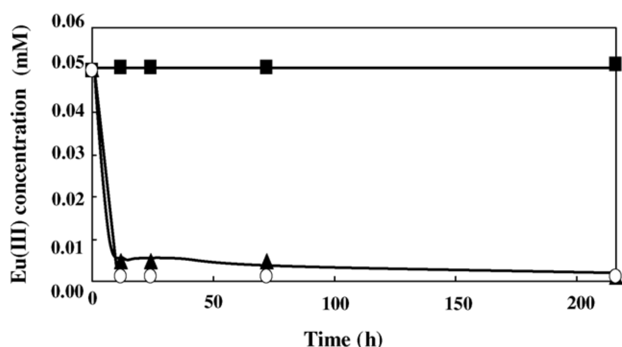


Fig. 1. Eu(III) concentration vs. contact time in the blank test medium at (■) pH 6.0; (▲) pH 6.7; and (◯) pH 6.9.

### 3.3. Cell growth in the malic acid medium

The concentrations of malic acid decreased with time after inoculating the cells into malic acid media without Eu(III) (Fig. 2a); it was not detected at 72 h after inoculation. Over the same time, the pH of the medium increased to about 6.85.

The amount of malic acid also declined with time in media containing 0.05, 0.1, or 0.2 mM Eu(III). The acidity (p$K$) of malic acid and malic acid mono-anion are 3.24 and 4.67, respectively [12]. The stability constant (log $K$) of the 1:1 molar ratio Eu(III)–malic acid complex is 4.85 and that of the 1:2 molar ratio complex is 8.11 [13]. Accordingly, more than 98% of Eu(III) formed complexes with malic acid in the medium. In the medium containing 0.05 mM Eu(III), the degradation of malic acid occurred as fast as that in the medium with no Eu(III). The rate of the degradation of malic acid decreased with increasing ratios of Eu(III) to malic acid. The pH also gradually rose with contact time; at 264 h after inoculation, it was between 6.7 and 6.9, indicating the metabolism of malic acid (Fig. 2b). The Eu(III) concentration in the medium did not change despite the complete degradation of malic acid in media with 0.05 and with 0.1 mM Eu(III) (Fig. 2c). The blank test suggests that, in the absence of malic acid, Eu(III) becomes insoluble and precipitates at pH 6.7–6.9.

Appanna et al. studied the association of *P. fluorescens* with Y(III) that has similar chemical properties to Eu(III) [14]. Biodegradation of citric acid proceeded in the presence of 1.0 mM Y(III) with 20.7 mM citric acid. On the other hand, its breakdown was inhibited in the presence of 5.0 mM Y(III) and 20.7 mM citric acid due to the adsorption of Y(III) on the cells. With higher concentrations of Y(III), the amounts of it accumulating on the cells increased, while cellular activity deteriorated due to its toxicity. These facts signify that higher concentrations of organic acids can more effectively mask the metal's toxicity thereby lowering the bioavailability of the toxic metal–organic acid complexes.

Fig. 3a–c show the time course of UV absorption intensity from 200–400 nm, detected by a photodiode array, showing the presence of organic acids eluted through the LC at 8.85, 9.90, and 12.03 min. Absorption intensity was normalized by the maximum intensity of each profile. The peak detected at 9.90 min was assigned to malic acid by comparison with a standard malic acid solution. The intensity of the peak for

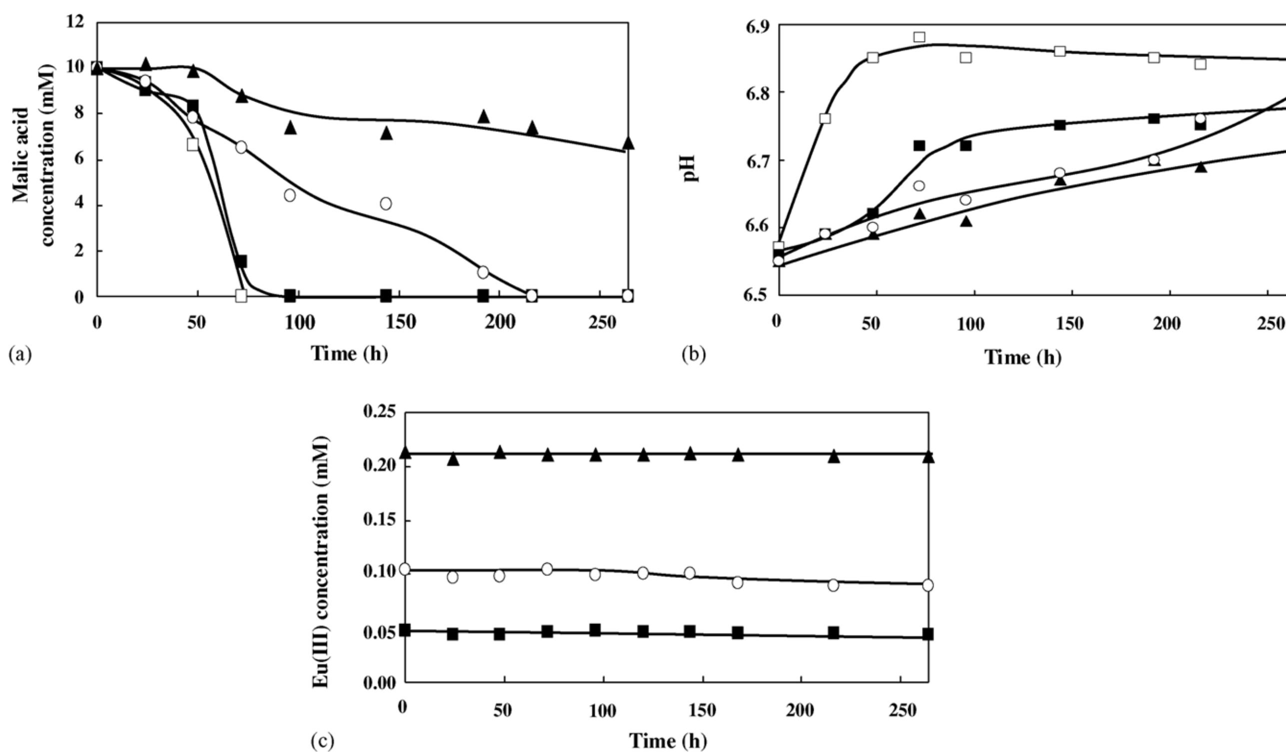


Fig. 2. Biodegradation of malic acid by *P. fluorescens* in the presence of (□) 0 mM; (■) 0.05 mM; (◯) 0.1 mM; and (▲) 0.2 mM of Eu(III)$Cl_3$. (a) Malic acid concentration vs. contact time; (b) pH vs. contact time; and (c) Eu(III) concentration vs. contact time.

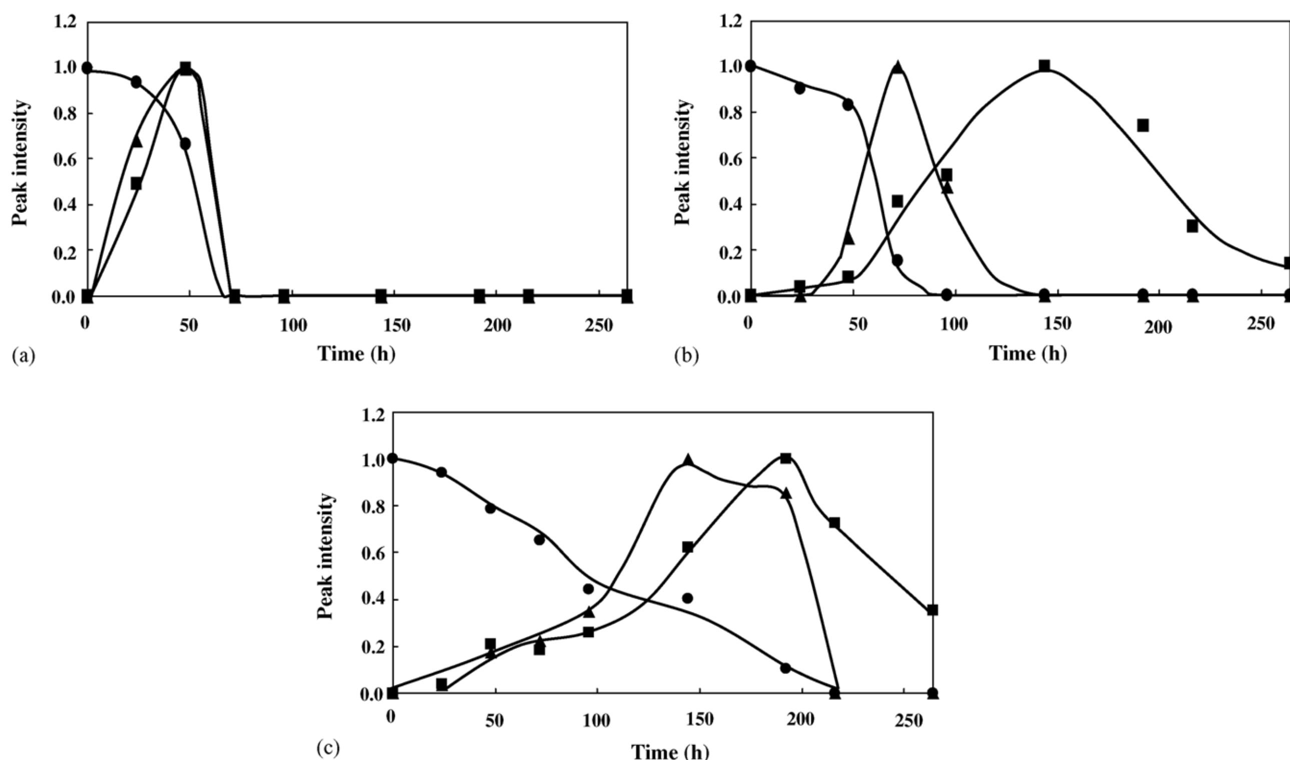


Fig. 3. Time course of the absorption intensity of the malic acid medium detected by a photodiode array at 200–400 nm after exposure to *P. fluorescens* for (●) malic acid and unidentified organic acids eluted at retention times of (■) 8.85 min and (▲) 12.03 by LC; (a) in the absence of Eu(III), and in the presence of Eu(III) with a malic acid to Eu(III) molar ratio of (b) 200:1 and (c) 100:1. The intensity is normalized by the highest intensity in each profile.

malic acid declined with time. In the medium lacking Eu(III), the peak intensities at 8.85 and 12.03 min rose with time after inoculation, reached a maximum at 48 h after inoculation, but then disappeared at 72 h (Fig. 3a). In the absence of Eu(III), these unidentified organic acids were degraded as fast as malic acid, indicating that their degradability is almost equivalent when they are not complexed with Eu(III). By contrast, the peak intensities at 8.85 and 12.03 min increased with time, with the former peak remaining after the disappearance of malic acid (Figs. 3b and c). The peak intensity at 8.85 min reached a maximum at approximately 144 h for 0.05 mM Eu(III), and 192 h for 0.1 mM Eu(III). The peak intensity at 12.03 min reached a maximum at approximately 72 h for the medium with 0.05 mM Eu(III) and at 144 h for 0.1 mM Eu(III).

Fig. 4a shows the $m/z$ of the unidentified organic acid detected at 8.85 min, analyzed by ESI-mass, where $m$ and $z$ are the molecular weight and the negative charge of sample fragments, respectively. Three strong peaks at 87, 91, and 105, and six weak peaks at 133, 175, 177, 193, 211, and 237 were found. Assuming that the ionic valence state of the unidentified organic acid was −1, the $m/z$ of 91 and 237 is the same mass as the monodeprotonated formic acid dimer and the HEPES (buffer) anion, while the $m/z$ of 87, the strongest peak, is the same mass as the pyruvic acid anion. Because the elution time of pyruvic acid was 8.85 min and its ESI-mass spectrum is similar to that of the organic acid eluted at 8.85 min (Fig. 4), the most probable designation of this unknown organic acid is pyruvic acid. The $m/z$ of the unidentified organic acid detected at 12.03 min was 115 and 161. Although we tried to relate these values to various organic acids in the TCA cycle, we could not assign them to any plausible organic acid. Identification of the organic acid is currently investigated.

Pyruvic acid is an intracellular metabolite of L-malic acid that reacts with the cellular enzyme malate dehydrogenase; the pyruvic acid so generated is [15] excreted into the medium. Appanna et al. studied the biodegradation of trivalent metal–citrate complexes by *P. fluorescens* [16,17]. They showed that Al(III)–citrate was metabolized intracellularly and that organic acids, such as oxalic acid and glyoxlic acid, then appeared in the medium. Their results suggest that the Al(III)–citrate complex entered the cell, releasing Al(III) after its breakdown into solution with these organic acids. With a Ga(III)–citrate, its β-hydroxyaspartate derivative was released into the solution, while Ga(III) predominated in the solution after the degradation of citric acid. In the case of Y(III)–citrate, however, Y(III) was thought to be precipitated in the bacterium's outer membrane by elaborating a novel protein there. In media containing Eu(III)–malic acid, a pyruvic-like acid was produced and released into the medium; Eu(III) did not accumulate on the outer membrane, even though Y(III) has similar chemical properties to Eu(III).

With an increasing ratio of Eu(III) to malic acid, degradation of the acid as well as the unidentified organic acid detected at 12.03 min and the pyruvic-like acid was retarded;

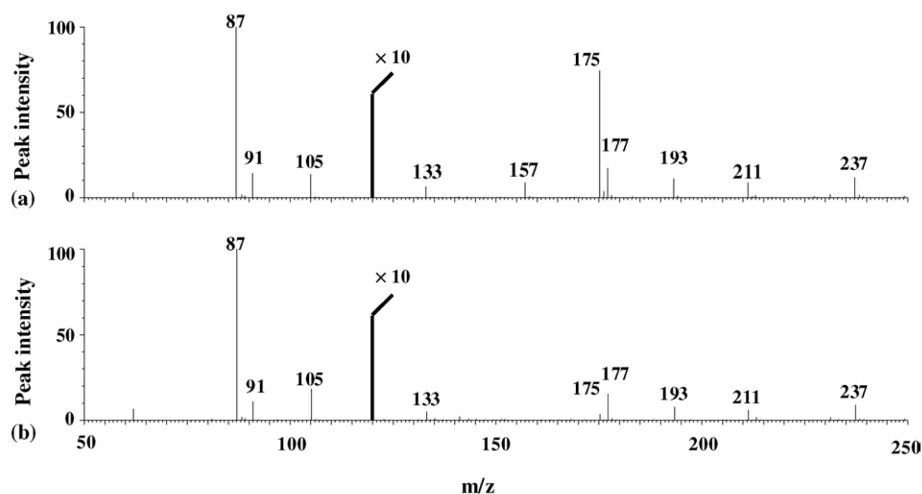


Fig. 4. ESI-mass spectra of (a) organic acid eluted at 8.85 min and (b) pyruvic acid. The intensity is normalized by the strongest intensity (the *m*/*z* of 87). The *m*/*z* of 91 and 237 have the same mass as the formic acid dimer and HEPES, respectively.

their degradation rates were malic acid > unidentified organic acid > pyruvic-like acid. The stability constant of the 1:1 Eu–pyruvic acid complex is 1.88, that of the 1:2 complex is 3.3, and that of the 1:3 complex is 3.8 [18]. Although the stability constants of the Eu(III)–malic acid complex are larger than those of pyruvic acid, its degradability is higher in the presence of Eu(III). Francis et al. studying metal–citric acid complexes found that biodegradability depends on the structure of the complex [6] (independent of the stability constant of the complexes). The Eu(III)–malic acid complex can readily be degraded, whereas the Eu(III)–pyruvic acid complex might be recalcitrant, independent of stability constants.

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