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Associations of Eu(III) with Gram-Negative Bacteria, *Alcaligenes faecalis, Shewanella putrefaciens*, and *Paracoccus denitrificans*

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We studied the association of Eu(III) with Gram-negative bacteria, *Alcaligenes faecalis*, *Shewanella putrefaciens*, and *Paracoccus denitrificans* by a batch method and time-resolved laser-induced fluorescence spectroscopy (TRLFS). The kinetics study showed that the Eu(III) adsorption on the bacteria rapidly proceeded. The Eu(III) adsorption on *A. faecalis* and *P. denitrificans* at pHs 3, 4, and 5, and that on *S. putrefaciens* at pHs 4 and 5 reached a maximum within 5 minutes after contact. For *P. denitrificans*, the percent adsorption of Eu(III) decreased after the maximum percent adsorption was attained, which suggests the existence of exudates with an affinity with Eu(III). TRLFS showed that the coordination of Eu(III) on these bacteria is multidentate through an inner-spherical process. The ligand field of Eu(III) on *P. denitrificans* was as strong as the ones observed for halophilic microorganisms, while that of *A. faecalis* and *S. putrefaciens* was the typical one observed for non-halophilic microorganisms. The coordination environment of Eu(III) on the bacteria differed from each other, though they are categorized as Gram-negative bacteria with the similar cell wall components.

1. Introduction

Migration behavior of radioactive nuclides in the environment is greatly affected by bacteria.[1, 2] Gram-negative bacteria are ubiquitously found in the environment. Francis et al. showed that a Gram-negative bacterium *Pseudomonas fluorescens* selectively affects the environmental behavior of bidentate citric-acid metal complexes through their degradation while it shows no association with tridentate or binuclear complexes because they are recalcitrant to its degradation.[3] In this study, we used three Gram-negative bacterial species, *Alcaligenes faecalis*, *Shewanella putrefaciens*, and *Paracoccus denitrificans* to study the association of Gram-negative bacteria with Eu(III). *A. faecalis* widely prevails in soil, waters, and even in digestive organs of mammals. This bacterial species is famous for the ability to oxidate $As(III)O_2^-$ to $As(V)O_4^{3-}$, wherein the protein involved in the reaction has been shown to contain Mo.[4, 5] *S. putrefaciens* is outstanding for its ability to grow on a wide variety of compounds as a terminal electron acceptor, such as O_2, NO_3^-, NO_2^-, Mn(IV)-oxides, Fe(III)-oxides, and U(VI).[6, 7] *P. denitrificans* grows both aerobically and anaerobically, and it anaerobically reduces a fixed nitrogen oxide.[8, 9] However, reduction of metal oxides, such as Mn(IV)-oxides and Fe(III)-oxides is not reported.

Associations of trivalent f-elements (lanthanides and actinides) with bacteria, especially coordination characteristics of these elements on them have not clearly been understood. Main functional groups on the cell surface of bacteria are carboxyl and phosphate groups.[10–12] Despite the relatively small variety in the kinds of functional groups, a wide variety of coordination environments are observed for Eu(III) on Gram-positive, Gram-negative, and halophilic bacteria,[13] which suggests the steric effects derived from the polymeric structure on the adsorption characteristics as well as the contribution from minor functional groups to its adsorption on the cell surface. It is important to precisely assess the association of f-elements with bacteria in that these elements may be leaked out of radioactive contaminants. In this study, we used Eu(III) because of its informative fluorescence properties. Europium(III) is a good analogue of Am(III) and Cm(III), and a study on the association of Eu(III) with bacteria is of great help in assessing that of Am(III) and Cm(III), whose long half-lives and high-energy α-particle emission can pose high risks to human health.

Time-resolved laser-induced fluorescence spectroscopy is a powerful tool to characterize the coordination environment of Eu(III) with regard to the number of water molecules in the inner-sphere of Eu(III) and the strength of its ligand field both in the inner- and outer-sphere.[14] Previously, we used this technique and successfully characterized the association of Eu(III) with biopolymers (cellulose, chitin, and chitosan) and a unicellular alga *Chlorella vulgaris*.[15, 16]

In the present study, we examined the adsorption behavior of Eu(III) on Gram-negative bacteria *A. faecalis*, *S. putrefaciens*, and *P. denitrificans* by a batch method. Using time-resolved laser-induced fluorescence spectroscopy (TRLFS), we examined the coordination environment of Eu(III) adsorbed on them.

2. Experimental

2.1. Preparation of the microorganisms. One cubic centimeter of cell suspensions at the exponential growth phase from *Alcaligenes faecalis*, *Shewanella putrefaciens*, and *Paracoccus denitrificans* were transferred to 250 cm^3 of culture media. These bacteria were grown aerobically at 30 °C in a nutrient medium at pH 6.8 containing the following components: polypepton, 5 g dm^{-3}; beef extract, 3 g dm^{-3}; and NaCl, 5 g dm^{-3}. The cultures were incubated on a rotary shaker at 100 rpm.

2.2. Measurement of adsorption behavior of Eu(III) on the bacteria. We used radioactive ^{152}Eu(III) in this study. The adsorption kinetics and distribution ratios (log K_d) of Eu(III) was studied by a batch method at pHs 3, 4, and 5. At these pHs, the hydrolysis of Eu(III) can be neglected. Bacterial cells were harvested at the late exponential phase by centrifugation for 10 minutes at 10000 *g*. The cell pellets were washed four

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times with a 0.5% NaCl solution and resuspended in it. For the adsorption kinetics study, 0.75 cm^3 of cell suspension was added to a 15-cm^3 Eu(III) solution containing 0.5% NaCl and 5.25 cm^3 of the suspension was withdrawn at 5, 10, and 15 minutes after contact. Subsequently, the cells were filtered through a 0.20-μm membrane filter (ADVANTEC MFS, Inc., DISMIC-25). To measure the log K_d, 0.25 cm^3 of bacterial suspension was added to 5-cm^3 solutions of Eu(III). After 10 minutes of contact, the cells were filtered through a 0.20-μm membrane filter (ADVANTEC MFS, Inc., DISMIC-25). The pH of the contact solution was maintained at 3.00 ± 0.05, 4.00 ± 0.05, or 5.00 ± 0.05, using NaOH or $HClO_4$ during the contact. The concentration of Eu(III) in the contact solution was approximately $1 \cdot 10^{-6}$ mol dm^{-3}. Europium(III) in the supernatant was analyzed by γ-spectroscopy (Packard Co., COBRAII). The distribution ratio K_d (cm^3 g^{-1}) was calculated according to the eq A:

$$K_d = (C_0 - C)\, V / C\, W, \qquad \text{(A)}$$

where C_0 is the initial concentration of Eu(III) in the aqueous phase; C, the equilibrium concentration of the metal ion in the aqueous phase; V, the volume of the aqueous phase (cm^3); and, W, the dry-weight of the bacterial cells (g). The dry-weight of the bacterial cells were 0.004 to 0.009 g.

2.3. TRLFS. The coordination environment of Eu(III) adsorbed on bacteria was studied by TRLFS. Samples were prepared by adding bacterial cells to a solution of $1 \cdot 10^{-3}$ mol dm^{-3} Eu(III) at pHs approximately 4–6 containing 0.5% NaCl. The cells were kept standing for approximately 2 hours after contact. Aliquots of cell suspension were centrifuged to settle the cells, which then were exposed to light of a wavelength of 394 nm from a XeCl excimer laser pumped dye laser (Lambda Physik, COMPex 201) pumping PBBO (Lambda Physik) in a 1,4-dioxane solution in a dye laser head (Lambda Physik, SCANmate 2).[17]

To measure the luminescence lifetime of Eu(III), the emission light was collected into a monochromator (Oriel, 77257) using an optical fiber and detected by a photomultiplier tube (Hamamatsu, R3896). The signal was fed into a digitizing oscilloscope (Hewlett Packard, 54519A) connected to a personal computer through a GP-IB interface. The luminescence decay curves were fitted by a single component exponential function. The number of water molecules, N_{H_2O}, of Eu(III) adsorbed on bacterial cells was estimated according to the eq B:

$$N_{H_2O} = 1.05 \cdot 10^{-3} (1 / \tau_{obs}) - 0.44, \qquad \text{(B)}$$

where τ_{obs} (s) is the luminescence lifetime.[18]

To obtain the emission spectra of Eu(III), the emission light was focused on a polychromator (ISA Jogin-Yvon, HR-320) using an optical fiber and detected by a gated multichannel diode array (Princeton Instruments, Inc., DIDA-512). The spectrometer was connected to Spectrometric Multichannel Analyzer system (Tokyo Instruments, Inc., SMA) installed on the personal computer. The relative peak intensity ratio ($R_{E/M}$) is defined by the ratio (C)

$$R_{E/M} = I\,(^5D_0 \rightarrow {}^7F_2) / I\,(^5D_0 \rightarrow {}^7F_1), \qquad \text{(C)}$$

where I ($^5D_0 \rightarrow {}^7F_2$) and I ($^5D_0 \rightarrow {}^7F_1$) are calculated from the peak areas at 614 nm ($^5D_0 \rightarrow {}^7F_2$) and 592 nm ($^5D_0 \rightarrow {}^7F_1$), respectively.

3. Results

3.1. Kinetics of Eu(III) adsorption on the bacteria. Figures 1a-c show the adsorption kinetics of Eu(III) at pHs 3, 4, and 5 for *Alcaligenes faecalis*, *Shewanella putrefaciens*, and *Paracoccus denitrificans*. Non-specific adsorption of Eu(III) on the membrane filter and the wall of the contact vessels was not observed at these pHs.

Figure 1a shows the adsorption kinetics of Eu(III) for *A. faecalis*. At all pHs, the equilibrium was attained within 5 minutes. The percent adsorption increased with an increase in pH. Similarly, the adsorption of Eu(III) by *S. putrefaciens* increased with a rise in pH (Figure 1b). The Eu(III) adsorption reached equilibrium within 5 minutes at pHs 4 and 5, while kinetics at pH 3 slowed and Eu(III) adsorption did not reach equilibrium up to 15 minutes. For the adsorption of Eu(III) on *P. denitrificans*, the equilibrium at pH 3 was attained within 5 minutes, and afterward the percent slightly decreased (Figure 1c). At pHs 4 and 5, the adsorption of Eu(III) reached a maximum within 5 minutes. The percent adsorption at 15 minutes was higher at higher pHs.

3.2. Distribution ratio of Eu(III) for the bacteria. Figures 2a-c show the logarithmic distribution coefficient (log K_d) of Eu(III) for the bacterial cells at 10 minutes after contact at pHs 3, 4, and 5. The values are the average of 3 replicates. Figure 2a shows log K_d of Eu(III) for *A. faecalis*. *A. faecalis* exhibited a higher log K_d for Eu(III) at higher pHs. log K_d at pHs 3, 4, and 5 was approximately 2.4, 3.6, and 4.6, respectively. *S. putrefaciens* also showed an increasing tendency in log K_d with a rise in pH (Figure 2b). log K_d at pHs 3, 4, and 5 was 2.3, 3.8, and 5.1, respectively. Figure 2c shows that log K_d for *P. denitrificans* was higher than those for *A. faecalis* and *S. putrefaciens*. log K_d at pHs 3, 4, and 5 was approximately 3.6, 4.6, and 5.8, respectively.

3.3. Coordination environment of Eu(III) on the bacteria. Figure 3 shows the Coordination-Environment diagram (CE diagram), on which ΔN_{H_2O} and $R_{E/M}$ for Eu(III) adsorbed on the

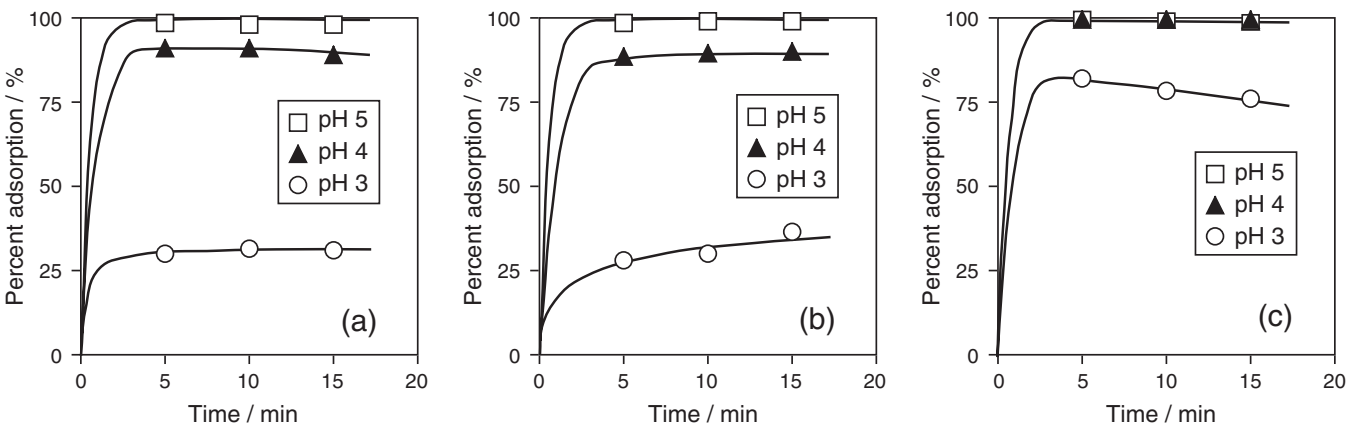

Figure 1. Time dependence in the percent adsorption of Eu(III) on (a) *Alcaligenes faecalis*, (b) *Shewanella putrefaciens*, and (c) *Paracoccus denitrificans*.

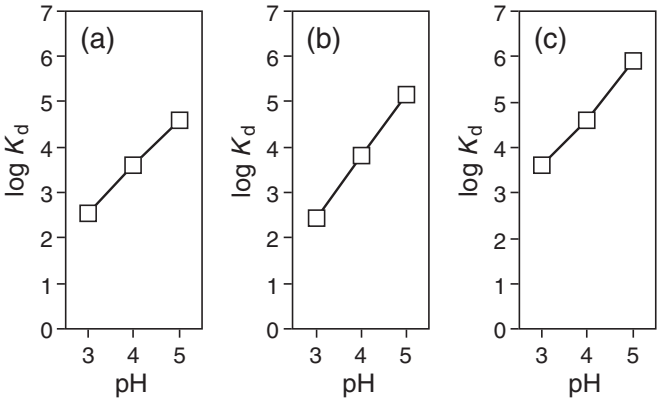

Figure 2. Logarithmic distribution coefficient (log K_d) of Eu(III) determined for (a) *Alcaligenes faecalis*, (b) *Shewanella putrefaciens*, and (c) *Paracoccus denitrificans*.

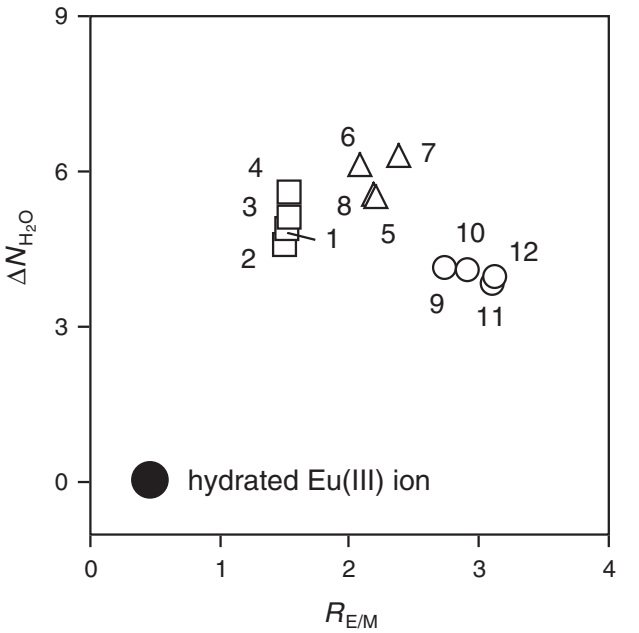

Figure 3. CE diagram obtained for the bacterial species listed above. The large closed circle represents the result obtained for hydrated Eu(III) ion with no interaction other than water molecules. pHs are as follows: (1) 4.62, (2) 5.29, (3) 5.62, and (4) 5.73 for *Alcaligenes faecalis*, (5) 4.46, (6) 4.86, (7) 5.34, and (8) 5.67 for *Shewanella putrefaciens*, (9) 4.26, (10) 4.68, (11) 5.26, and (12) 5.68 for *Paracoccus denitrificans*.

three bacterial species were plotted. On the CE diagram, ΔN_{H_2O} indicates the number of water molecules in the inner-sphere of Eu(III), calculated according to the equation $\Delta N_{H_2O} = 9 - N_{H_2O}$. In this equation, we assumed that the number of water molecules in the inner-sphere of hydrated Eu(III) ion is 9 based on previous research.[19] ΔN_{H_2O} represents the number of coordination sites occupied in the inner-sphere of Eu(III) by ligands other than water molecules. *A. faecalis* showed a ΔN_{H_2O} of 4.60 at pH 5.3 to 5.61 at pH 5.7. $R_{E/M}$ was between 1.50 and 1.54. No clear correlation among pH, ΔN_{H_2O} and $R_{E/M}$ was observed. The ΔN_{H_2O} in a 0.5% NaCl solution was almost 0 and the $R_{E/M}$ in the solution was not affected by the presence of NaCl.

S. putrefaciens showed a large ΔN_{H_2O}. The largest ΔN_{H_2O} was 6.33 at pH 5.3, and the smallest one was 5.51 at pH 4.5. The $R_{E/M}$ for Eu(III) on *S. putrefaciens* ranged from 2.07 to 2.37. No clear pH dependence was observed. *P. denitrificans* showed a small ΔN_{H_2O}, which ranged from 3.99 to 4.15. On the other hand, $R_{E/M}$ observed for *P. denitrificans* was larger than those on the other two bacterial species: the maximum was 3.13 at pH 5.7 and the smallest was 2.73 at pH 4.3.

4. Discussion

Takahashi et al. showed that the hydrolyzed Eu(III) species exhibits an extremely large τ_{obs}^{-1}.[20] In this study, τ_{obs}^{-1} smaller than those corresponding to $N_{H_2O} = 9$ was found, showing that the Eu(III) species on the cell surface was not precipitated. The Eu(III) concentration used in the adsorption experiments was lower than that used in the TRLFS experiments. These facts signify that the accumulation of Eu(III) in the bacterial cells is not due to its precipitation on the cell surface.

Accumulation of metals in bacteria usually involves two steps. The first step is the metal adsorption on the bacterial cell surface without involving biological activity. The second step, which generally proceeds very slowly, is their absorption into the cytoplasm of the bacterial cells.[21, 22] Our observations suggest that the association observed between Eu(III) and *A. faecalis* can be attributed to the first step. Previously, we reported a rapid adsorption of trivalent f-elements on microorganisms.[13, 23] Similarly, the Eu(III) adsorption on *S. putrefaciens* at pHs 4 and 5 and that on *P. denitrificans* at pHs 3–5 rapidly reached a maximum but the slow increase in the adsorption as the second step was not observed. The adsorption of Eu(III) on *P. dinitrificans* at pH 3 showed a maximum at 5 minutes after contact and then the percent adsorption decreased. It is suggested that *P. dinitrificans* exudes some substance with an affinity with Eu(III) at the pH. *Chlorella vulgaris* was shown to excrete some exudates which desorb Eu(III) and Cm(III) from its cell surface.[16]

The adsorption of cations on bacterial cells through the exchange of H^+ on the functional groups is commonly observed.[24, 25] If the adsorption of Eu(III) on cell surfaces is through the exchange with 3 H^+ in functional groups on the cell surface, the slope of the log K_d versus pH plots is predicted to be 3 in the pH range where the hydrolysis of Eu(III) does not occur. However, all bacterial species showed a smaller slope in the log K_d versus pH plots than the predicted value: *A. faecalis*, *S. putrefaciens*, and *P. denitrificans* showed a slope of approximately 1.1, 1.4, and 1.1, respectively. This cannot be explained by the mechanisms mentioned above. The presence of exudates cannot clearly explain this, either. The adsorption of Eu(III) on biopolymers, cellulose, chitin, and chitosan produced a slope of the log K_d versus pH plots of approximately 1 at acidic pHs, wherein the coordination of ether oxygen was suggested.[26] In the adsorption of Eu(III) on these three bacterial species, involvement of functional groups such as the ether oxygen might be present. *P. fluorescens* showed a negative slope in the log K_d versus pH plots between pH 3–5.[13] The main cell wall components of Gram-negative bacteria are lipopolysaccharide and peptidoglycan, where the possible adsorption sites for Eu(III) are carboxyl groups and phosphate groups.[27] These findings indicate that the structure of coordination sites on *P. fluorescens* and that on the three other bacterial species used in this study is not identical to each other, though all four species are categorized as Gram-negative bacteria based on the characteristics of the cell wall structure.

TRLFS shows high selectivity and sensitivity for trivalent f-elements. This technique is applicable to the adsorbed species on the surface of a solid phase in the presence of a water phase. Ligands in the inner-sphere and the ones in the outer-sphere of an atom produce its coordination environment, with the characteristic ligand field surrounding the central atom. TRLFS can predict the coordination environment of Eu(III) including both the inner- and outer-sphere, owing to its luminescence properties.[14] The fluorescence lifetime of excited Eu(III) is related to the number of water molecules in the inner-sphere (N_{H_2O}), which is calculated by the eq B.[28] The relative intensity of $^5D_0 \rightarrow {}^7F_2$ (electric dipole) and $^5D_0 \rightarrow {}^7F_1$ (magnetic dipole) emissions ($R_{E/M}$) is related to the strength of ligand field of Eu(III), based on which we can characterize the

coordination environment for both the inner- and outer-sphere.[29] Unknown coordination environments of Eu(III) can be characterized based on the location of a $R_{E/M}$-ΔN_{H_2O} (= 9 – N_{H_2O}) plot of the Eu(III) on the CE diagram.[14] We previously showed that the ligand field of Eu(III) ($R_{E/M}$) on halophilic microorganisms, such as *Halomonas* sp. and *Halobacterium salinarum* is stronger than that on the non-halophilic microorganisms, such as *C. vulgaris* and *B. subtilis*.[13] In this study, $R_{E/M}$ increased in the increasing order of *A. faecalis*, *S. putrifaciens*, and *P. denitrificans*. Note that $R_{E/M}$ for *P. denitrificans* was larger than that for non-halophilic microorganisms and comparable to that observed for Eu(III) adsorbed on halophilic microorganisms.[13] This suggests that the structure of the coordination site for Eu(III) on *P. denitrificans* is similar to that of halophilic Gram-negative bacteria, such as *Halomonas* sp., implying its adaptability to saline conditions.

Coordination sites of trivalent f-elements on a Gram-negative bacterium, *Pseudomonas aeruginosa* are reported to be carboxyl groups and phosphate groups.[30] One molecule of these groups can provide at most bidentate coordination with Eu(III). However, ΔN_{H_2O} on the bacteria in this study was larger than 3. Especially, *S. puterfaciens* showed ΔN_{H_2O} of as large as 6. These facts signify that the adsorption of Eu(III) on the bacteria is through inner-spherical coordination, wherein more than one functional groups are involved. Reportedly, ΔN_{H_2O} on a Gram-negative bacterium *P. fluorescens* was larger than that on a Gram-positive bacterium *B. subtilis*.[13] ΔN_{H_2O} obtained for *A. faecalis* and *S. putrefaciens* is almost equal to that for *P. fluorescens*, while that for *P. denitrificans* is comparable to that of *B. subtilis*. These findings demonstrate that the coordination environment of Eu(III) differs even in Gram-negative bacteria, though only a few kinds of functional groups are involved in the coordination. Further investigation would be required to achieve the overall understanding of the association mechanisms between f-elements and bacteria.

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