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ORIGINAL PAPER

Uptake and degradation of EDTA by *Escherichia coli*

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Abstract It was found that *Escherichia coli* exhibited a growth by utilization of Fe(III)EDTA as a sole nitrogen source. No significant growth was detected when Fe(III)EDTA was replaced by EDTA complexes with other metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. When EDTA uptake was measured in the presence of various ions, it was remarkable only when Fe^{3+} was present. The cell extract of *E. coli* exhibited a significant degradation of EDTA only in the presence of Fe^{3+}. It is likely that the capability of *E. coli* for the growth by utilization of Fe(III)EDTA results from the Fe^{3+}-dependent uptake and degradation of EDTA.

Keywords EDTA uptake · EDTA degradation · Fe^{3+}-dependent

Introduction

Ethylenediaminetetraacetic acid (EDTA) is a synthetic chelating agent for most metal ions. Because of high stability and low toxicity, EDTA is applied in various industrial processes such as paper bleaching, metal processing, photographic industry, and pharmacy (Bohuslavek et al. 2001; Bucheli-Witschel and Egli 2001; Eroshin et al. 2002). Usage of a large quantity of EDTA is causing water pollution by discharge of the waste solution. For the purpose of biodegradation of EDTA before its release into the environment, several EDTA-degrading bacteria have been isolated (Fang et al. 2003; Lauff et al. 1990; Miyazaki et al. 1999; Nörtemann 1992; Payne et al. 1998; Witschel et al. 1997). The bacteria exhibited EDTA degradation differently depending on the species of its complexes with metal. For instance, while *Burkhol cepacia* degraded Fe(III)EDTA and Cu(II)EDTA, an *Agrobacterium* sp. (ATCC55002) degraded only Fe(III)EDTA. The bacterial strains, BNC1 and DSM9103, degraded preferentially the EDTA complex with Mg^{2+}, but not Fe(III)EDTA.

The enzymes, monooxygenases, involved in the degradation of EDTA have been purified in BNC1 and DSM9103 (Payne et al. 1998; Witschel et al. 1997). Both enzymes oxidize EDTA to ethylenediaminetriacetate (ED3A) and glyoxylate. ED3A are further degraded to ethylenediaminediacetate (EDDA) and glyoxylate. In each degradation step, EDTA monooxygenases require reduced flavin mononucleotide ($FMNH_2$) supplied by NADH-dependent FMN oxidoreductase.

Escherichia coli grows in a minimal medium (M9 medium) containing glucose and NH_4Cl as carbon and nitrogen sources, respectively (Sambrook et al. 1989). It was found that *E. coli* exhibited a significant growth when NH_4Cl of the medium was replaced by

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Fe(III)EDTA. To examine the utilization of Fe(III)EDTA as a nitrogen source, in this study, uptake and degradation of EDTA by *E. coli* were measured in the presence of various metal ions. The cell free extract of *E. coli* significantly degraded EDTA only in the presence of Fe^{3+}. The enzyme system of *E. coli* involved in the EDTA degradation might be useful for pretreatment of the waste solution.

Materials and method

Strains and culture

Escherichia coli BL21 [F^-omp Thsd S_B ($R_B^-M_B^-$) gal dcm] and ΔNikA (KP7600 but NikA::KmR), a transposon insertion disruptant obtained from National BioResource Project (NIG, Japan): *E. coli*, were used. The bacteria were grown aerobically at 37°C in Luria–Bertani broth (LB broth). When the growth was measured in a minimal medium (M9 medium), 50 µl of the cells grown at a late logarithmic stage in LB broth was directly transferred into 10 ml of medium (pH 7.5) of the following composition (g/l): Na_2HPO_4, 6.0; Na_2HPO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 0.1; glucose, 2.0 (Sambrook et al. 1989). When EDTA was used as a nitrogen source, NH_4Cl was replaced by EDTA, which was added as the complexes with various metal ions, respectively. For determination of the growth rate, the bacterium was aerobically grown at 35°C. Since Fe(III)EDTA complex has been suggested to undergo photolysis, the chemical was carefully kept in the dark (Kari and Giger 1996). Furthermore, all cultures in the presence of EDTA were conducted in the dark box.

EDTA uptake

The bacterium grown at a late logarithmic stage in 50 ml of LB broth was collected by centrifugation (1,500*g*, 5 min) and washed once with 40 mM HEPES buffer (pH 7.5) containing 200 mM KCl and 50 mM NaCl (Buffer A). The bacteria were suspended in 3 ml of Buffer A and used for the uptake experiment, the procedure of which was essentially the same as that described previously (Koyama et al. 1987). Briefly, 1.0 ml of Buffer A containing 10 mM glucose and 10 µM [^{14}C]EDTA (192 GBq/mol, MP Biomedicals, USA) in the absence and presence of 500 µM chloride salts of the various ions, respectively, was incubated at 30°C for 2 min. EDTA uptake was initiated by the addition of 50 µl (approx. 5×10^{14} CFU) of the bacterial suspension. After an appropriate time of the incubation with shaking, 5 ml of ice-cold Buffer A was added to stop the reaction. Immediately, the cells were quickly collected on a membrane filter (0.65 µm/pore size, Advantec, Japan) by filtration using an aspirator (Circulating aspirator WJ-20, Sibata, Japan), and assayed for radioactivity as described previously (Liquid scintillation counter LSC-5100, Aloka, Japan).

Assay of EDTA degradation

The bacterium grown at a late logarithmic stage in 200 ml of LB broth was collected by centrifugation and washed once with Buffer A containing 8% glycerol. The bacteria thus obtained were suspended in 10 ml of the same buffer and treated with 1.0 mg of lysozyme at 35°C for 30 min. The bacteria were frozen and kept at −20°C at least for 2 h. The frozen cells were defrozen at 35°C, and then centrifuged at 40,000*g* for 8 min. After the pellet was suspended in 4 ml of Buffer A, the suspension was treated with a bath-type sonicater (Sono cleaner 50a, Kaijo Denki, Japan) at 0°C for 5 min and centrifuged at 40,000*g* for 15 min at 4°C (Model 50B-7, Sakuma, Japan). The supernatant was used for the assay of EDTA degradation.

EDTA degradation was examined by glyoxylate production (Bohuslavek et al. 2001; Payne et al. 1998; Witschel et al. 1997). Reaction was initiated by adding 50 µl of the extract to 750 µl of the reaction mixture consisting of 50 mM Tris–HCl (pH 8), 3.0 mM NADH, 5 µM FMN, 10 mM EDTA, and 10 mM chloride salts of various metal ions, respectively. After 10 min at room temperature, the reaction was stopped by adding 400 µl of concentrated HCl. Glyoxylate produced by the reaction was determined by the fluorescence at 530 nm with excitation at 490 nm (Spectrofluorophotometer RF-1500, Shimadzu, Japan) (Zarembski and Hodgkinson 1965).

Protein determination

Protein concentration was determined by Protein assay rapid kit (Wako, Japan) using bovine serum albumin as standard.

Result and discussion

Growth

BL21 grew well in a minimal medium (M9) containing glucose and NH_4Cl as carbon and nitrogen sources, respectively (Fig. 1). Slight growth was observed even when NH_4Cl was omitted, which is likely to result from the contamination of LB medium transferred into the culture medium together with the inoculated bacteria. When NH_4Cl was replaced by Fe(III)EDTA, a significant growth was observed depending on its concentration. In the presence of 20 mM Fe(III)EDTA, the final growth level was almost equivalent to that in M9 medium, although the growth rate was lower. Some absorbance observed at zero time in the presence of Fe(III)EDTA was due to the brown color of the complex. In the presence of 10 mM EDTA complexes with metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, only slight growth was detected similarly to that in the absence of nitrogen sources (data not shown).

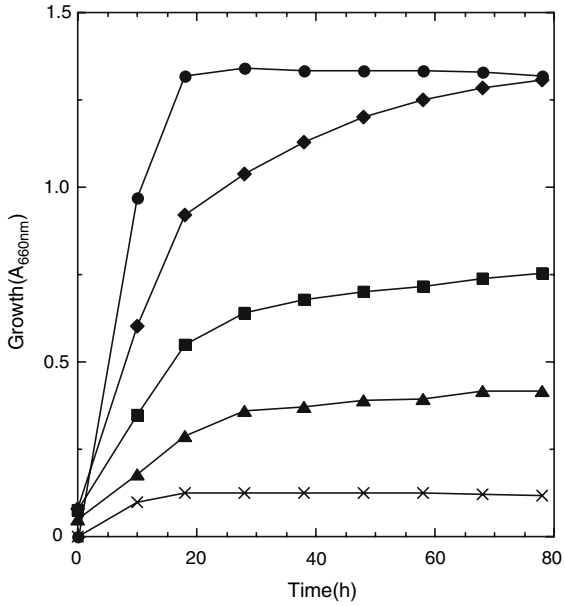

Fig. 1 Effect of Fe(III)EDTA on the growth of BL21. The bacterium was aerobically cultured in the various compositions of medium. Standard medium was M9 medium (●) which contains NH_4Cl as a sole nitrogen source. In other mediums, the following concentrations of Fe(III)EDTA were added instead of NH_4Cl: ×, 0; ▲, 5; ■, 10; ◆, 20 mM, respectively

EDTA uptake

For the utilization of EDTA as nitrogen sources, BL21 may take up the chemical into the cell. EDTA-degrading strain DSM9103 exhibited uptake of free EDTA and unstable EDTA complexes with metal ions such as Ba^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+} (Witschel et al. 1999; Sillen and Martell 1964). DSM9103 showed no uptake of stable EDTA complexes such as Fe(III)EDTA and Cu(II)EDTA. In contrast to DSM9103, BL21 exhibited a significant uptake of EDTA in the presence of Fe^{3+} (Fig. 2). The uptake increased linearly for 1 min with almost saturated within 2 min. For the comparison of initial uptake rate, EDTA uptake for 1 min was measured in the absence and presence of various metal ions (Table 1). BL21 exhibited some uptake of EDTA in the absence and presence of metal ions such as Ca^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}, respectively. While slight increase was observed by the addition of Co^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+}, respectively, EDTA uptake was most enhanced by Fe^{3+}. EDTA uptake rate in the presence of Fe^{3+} was approximately 10-fold that of DSM9103 in the absence and presence of Ba^{2+}, Ca^{2+}, and Mg^{2+} (Witschel et al. 1999).

Active transport is driven by the various energy sources such as ATP, a proton electrochemical potential ($\Delta\mu_H+$), a sum of transmembrane pH gradient (ΔpH, alkaline inside) and membrane potential ($\Delta\psi$, negative inside), and sodium electrochemical potential ($\Delta\mu_{Na}+$), a sum of transmembrane sodium gradient (ΔpNa, outside > inside) and $\Delta\psi$ (Ames and Lecar 1992; Dean et al. 1989; Harold 1972; Sugiyama et al. 1986; Ueno et al. 2000). EDTA uptake of DSM9103 was drastically inhibited by uncouplers, suggesting that it is driven by $\Delta\mu_H+$ (Witschel et al. 1997). In the EDTA-degrading strain, BNC1, ABC-type transport system, which is energized by ATP, appears to be involved in the uptake of free EDTA (Zhang et al 2007). To examine the driving force for EDTA transport of BL21, the influences of carbonylcyanide-m-chlorophenylhydrazone (CCCP), a potent uncoupler, and gramicidin, a sodium ionophore, on EDTA uptake in the presence of Fe^{3+} was determined. As shown in Table 1, the drugs had essentially no effect on the uptake, suggesting that it is neither driven by $\Delta\mu_H+$ nor $\Delta\mu_{Na}+$ (Sugiyama et al. 1986; Ueno et al. 2000). EDTA uptake of BL21 seems to be energized by ATP (Ames and Lecar 1992; Dean et al. 1989).

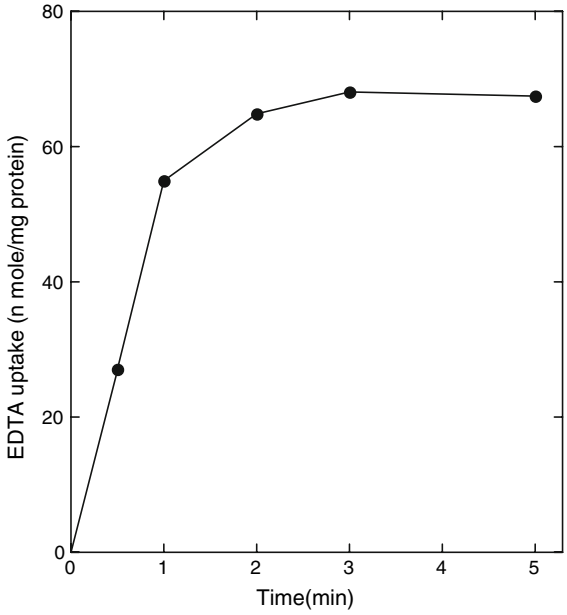

Fig. 2 Uptake of EDTA in the presence of Fe^{3+}. Reaction was carried out at 30°C. Control experiments were conducted by stopping the reaction immediately after the addition of the cells. Uptake was estimated by subtracting the control value

Echerichia coli possesses a nickel transport system, which is encoded by the operon of NikABCDE and expressed under anaerobic conditions (Navarro et al. 1993). The transport system is a kind of ABC-type transporters (Eitinger 2000). It has been revealed that NikA, a nickel-binding periplasmic protein, showed high affinity for $Fe(III)EDTA(H_2O)^-$ (Cherrier et al. 2005). EDTA uptake of BL21 in the presence of Ni^{2+} was approximately 10% of that in the presence of Fe^{3+}, which was decreased to 39 and 34% of the original level by the addition of 10 and 100 μM $NiCl_2$, respectively (Table 1). Since EDTA forms much more stable complex with Fe^{3+} than that with Ni^{2+}, an inhibitory effect of $NiCl_2$ on EDTA tranport is unlikely to result from the preferential formation of NiEDTA complex (Sillen and Martell 1964). EDTA uptake in the presence of Mn^{2+} and Zn^{2+}, respectively, was as low as that in the presence of Ni^{2+}. However, EDTA uptake in the presence of Fe^{3+} was neither inhibited by Mn^{2+} nor Zn^{2+}, suggesting that the inhibition is rather specific for Ni^{2+}. Thus, it might be expected that a NikA protein is involved in the EDTA uptake. To examine the possibility, EDTA uptake of a NikA-deficient mutant was measured (Table 1). Similarly to BL21, the

Table 1 EDTA Uptake in the presence of various ions and chemicals

Addition	EDTA uptake (nmol/min/mg protein)	
	BL21	NikA-mutant
None	6.0 ± 0.7	11.1 ± 2.3
Ca^{2+}	8.4 ± 2.5	11.7 ± 0.7
Cd^{2+}	7.6 ± 4.5	15.3 ± 2.5
Co^{2+}	10.4 ± 1.2	14.1 ± 5.3
Cu^{2+}	12.7 ± 4.4	22.8 ± 4.5
Mg^{2+}	10.6 ± 3.1	12.9 ± 1.3
Mn^{2+}	9.3 ± 2.1	15.8 ± 2.0
Ni^{2+}	6.1 ± 3.6	15.1 ± 1.7
Zn^{2+}	7.4 ± 1.8	13.8 ± 5.1
Fe^{3+}	58.3 ± 8.9	64.6 ± 3.5
+CCCP (30 μM)	46.7 ± 9.5	61.0 ± 12.5
+Gramicidin (0.1 μM)	51.6 ± 4.0	62.7 ± 11.7
+$NiCl_2$ (10 μM)	22.7 ± 2.6	19.8 ± 2.7
+$NiCl_2$ (100 μM)	20.0 ± 2.0	16.9 ± 7.0
+$MnCl_2$ (100 μM)	60.6 ± 4.5	–
+$ZnCl_2$ (100 μM)	55.5 ± 2.3	–

Reaction was conducted at 30°C for 1 min in the presence of 500 μM chloride salts of various metal ions, respectively. In several experiments in the presence of Fe^{3+}, the indicated concentrations of chemicals were added, respectively. Values were means ± SD, which were from at least three independent experiments

mutant exhibited EDTA uptake in the absence and presence of various ions, respectively. The highest uptake, unexpectedly, was observed in the presence of Fe^{3+}, which was equivalent to that of BL21. EDTA uptake of the mutant in the presence of Fe^{3+} was also inhibited by Ni^{2+}. The mutant was able to grow in the presence of Fe(III)EDTA as a sole nitrogen sources (data not shown). The facts indicate that NikA protein does not participate in the main uptake system of EDTA. The reason why $NiCl_2$ inhibited EDTA uptake in the presence of Fe^{3+} is unknown at present.

EDTA degradation

EDTA was degraded by the bacterial monooxygenases, which resulted in the production of glyoxylate (Payne et al. 1998; Witschel et al. 1997). To examine the EDTA degradation by BL21, it was determined whether glyoxylate is produced by the reaction of the cell extract with EDTA in the absence and presence of various metals (Fig. 3). In the absence of metal

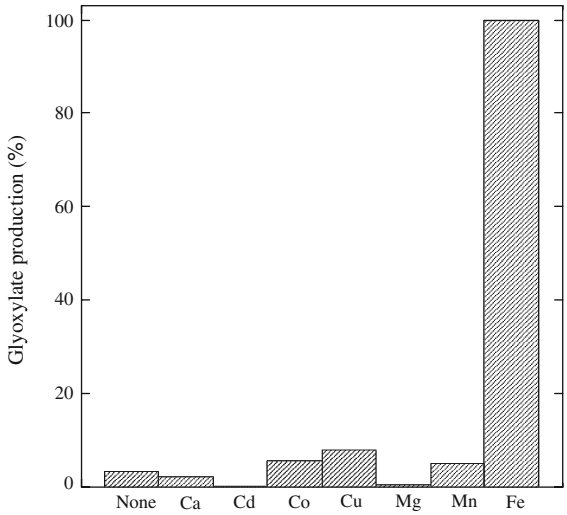

Fig. 3 EDTA degradation by the cell extract in the presence of various metal ions. EDTA degradation was examined by glyoxylate production, which was expressed as the ratio compared to that (0.085 ± 0.005 μmol/mg protein/min) in the presence of Fe^{3+}. The production was estimated by subtracting the control value obtained without the extract. Values were means from at least two experiments; standard deviations were <6%

ions, slight glyoxylate productions were detected. Upon the addition of Fe^{3+}, glyoxylate production was remarkably increased. The activity for glyoxylate production in the presence of Fe^{3+} was comparable to that of the cell extract of DSM9103 in the presence of Mg^{2+}, and approximately a half of that of BNC1 in the presence of Mg^{2+} (Payne et al. 1998; Witschel et al. 1997). In the presence of other metal ions, glyoxylate productions were <10% of that in the presence of Fe^{3+}, respectively. Thus, BL21 fairly depends on the presence of Fe^{3+} for the uptake and degradation of EDTA, which may contributes to the utilization of Fe(III)EDTA as a nitrogen source for the growth.

Since EDTA monooxygenase requires FMN that was reduced by NADH-dependent FMN oxidoreductase, both NADH and FMN are essential for the EDTA degradation (Payne et al. 1998; Witschel et al. 1997). When the extract of BL21 was reacted with Fe(III)EDTA in the absence of NADH, essentially no production of glyoxylate was detected. When the addition of FMN alone was omitted, however, approximately 70% of glyoxylate was produced as compared with that in the presence of FMN (data not shown). Since only small amount of FMN was necessary, it might be possible that the flavin contaminated in the cell extract contributed to the reaction. EDTA monooxygenase whose sequence of amino acid residues has been so far determined is only from a bacterial strain BNC1 (Bohuslavek et al. 2001). While the genome sequence of *E. coli* is available, we could find no open reading frame for the bacterial protein whose amino acid sequence shows higher than 20% identity with that of the BNC1 enzyme. Although EDTA degradation of *E. coli* might need NADH and FMN, the enzyme system remains to be clarified.

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