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The Effect of Media Composition on EDTA Degradation by *Agrobacterium* sp.†

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ABSTRACT

EDTA degradation by an *Agrobacterium* sp. has been examined by quantifying ^{14}C-labeled CO_2 produced from iron-[2-^{14}C] EDTA and by measured loss of nonlabeled EDTA by HPLC. Fe-EDTA degradation resulted in a rise in pH, nitrate concentration, and ammonia concentration. Addition of glycerol resulted in suppression of Fe-EDTA degradation and in a decrease in pH and NH_4^+ concentration in the media. Addition of peptone or yeast extract did not affect degradation. Some of the components (e.g., biotin) of the media are not necessary for growth and biodegradation. Although cobalt-EDTA cannot be degraded, ferrous iron can be added to displace the cobalt.

Index Entries: EDTA; bacteria; degradation; *Agrobacterium*; cobalt.

INTRODUCTION

Groundwater contamination by organic compounds has been recognized as a serious problem at DOE sites. At some sites, such as the Trench 7 area at Oak Ridge National Laboratory (ORNL), organics in groundwater chelate radionuclides, increasing the mobility of the radioactive materials.

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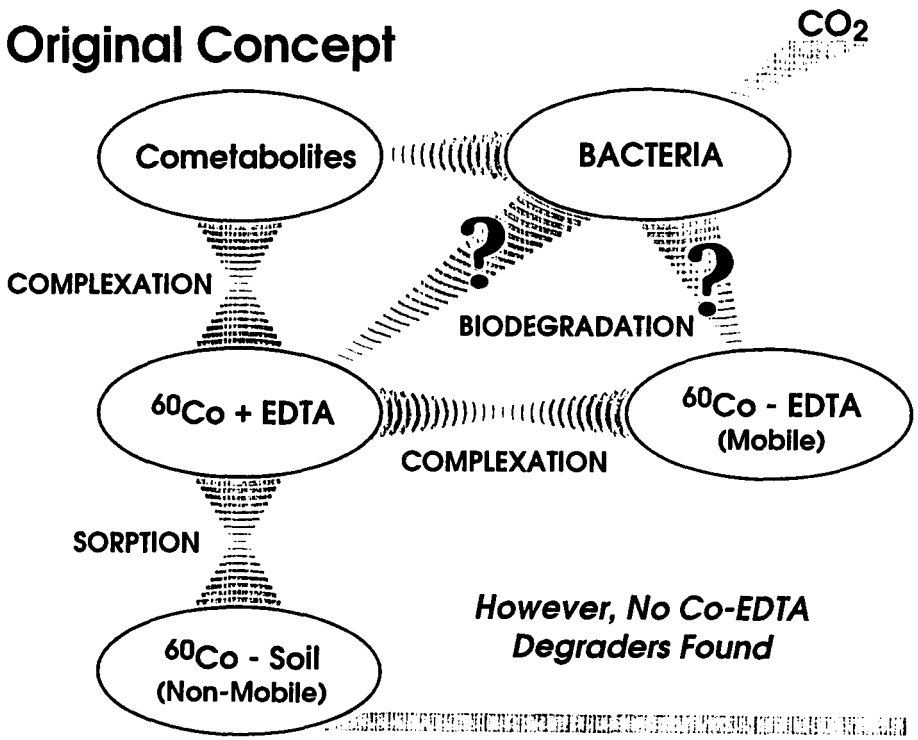

Fig. 1. Original concept for immobilization of cobalt in groundwater by degradation of EDTA.

It has been well-documented that ethylenediaminetetraacetate (EDTA) mobilizes ^{60}Co (*1–3*). EDTA is also found associated with other radionuclides (such as uranium and plutonium) disposed of in the same areas and thus is probably increasing the mobility of these radionuclides.

Both the persistence of EDTA in the subsurface environment and as demonstrated in laboratory studies (*4,5*), indicate degradation of EDTA is very slow despite its relatively simple structure. Biodegradation of EDTA has been enhanced to a degree in soil and water systems by the addition of cometabolites (specifically glycine, glucose, and peptone) but the extent and rate of degradation is limited (*2*).

The objective of the present research was to develop new approaches to stimulate microbial degradation of EDTA, particularly EDTA chelated to cobalt. Biodegradation of the organic compound (EDTA) keeping the ^{60}Co in solution would permit the ^{60}Co to associate with the soil and reduce its mobility. Our initial approach to EDTA removal was to attempt to isolate strains capable of EDTA degradation. Later we worked with a strain known to degrade EDTA and some related strains. These approaches were based on direct degradation of free EDTA, which would shift the equilibrium and causing a release of the chelated cobalt, or on direct degradation of Co(III)-EDTA (Fig. 1).

MATERIALS AND METHOD

Cell Culture and Media Preparation

A known Na-Fe(II)-EDTA degrading strain (2) *Agrobacterium* sp (ATCC #55002) was obtained from Genencor (Rochester, NY) and its nutritional requirements and application to degradation of Co-EDTA was assessed. An *Agrobacterium radiobacter* strain and a strain of *Agrobacterium tumifaciens* were obtained from E. Brown at Iowa State University. Neither of these isolates had been previously tested for EDTA degradation ability. Mixed aerobic cultures, designated MC1 and SWSA6-1, were obtained from Na-EDTA enrichments of sewage sludge and water from Solid Waste Storage Area 6 at ORNL, respectively.

Starter cultures of the Genencor *Agrobacterium* isolate were initiated by inoculating either 25 mL of Difco T-soy broth or Fe-EDTA in mineral salts media with 0.1 mL from glycerol stocks and incubating for ~24 h at room temperature. Cells were either harvested by centrifugation, washed twice, and suspended in NATE medium (7) without a carbon source or were inoculated directly into the medium without washing. The other *Agrobacterium* iolates were grown and maintained on Difco (Surrey, UK) nutrient broth and on nutrient agar slants. The MC1 and SWSA6-1 mixed cultures were maintained on NATE media containing 1 g/L yeast extract and 20 m*M* EDTA. The pH was 7.2 unless otherwise stated.

The *Agrobacterium* strain from Genencor was examined using the Biolog Bacterial Identification system. This system identifies bacteria based on their ability to oxidize 95 different carbon substrates.

Preparation of K-Co(III)-EDTA

Experiments were run with ^{14}C labeled and unlabeled K-Co(III)-EDTA prepared by the method of Logaken (*8*). Briefly, 8 g of cobalt (II) chloride hexahydrate, 20 g of potassium acetate, 10 g of Na-EDTA, and ~250 μCi of radiolabeled Na-EDTA (Amersham, Arlington Heights, IL) were combined in 60 mL of deionized water and heated to near boiling. After gradual addition of 30 mL of 3% hydrogen peroxide the solution was cooled to room temperature. A total of 400 mL of 95% ethanol was then added and the flask was cooled overnight in an ice water bath. The liquid was decanted and deionized water was added to the precipitate until it was redissolved. The solution was passed through a filter (pore size 0.22 μm) and reprecipitated by the addition of 95% ethanol followed by cooling in an ice water bath. The decanting, dissolution, and reprecipitation were repeated once more and after the final precipitation the liquid was decanted and the remaining salt was dried for 24 h at a temperature of 70.6°C. This yielded a total of 1 gm of K-Co(III)-EDTA with a specific activity of ~ ~2.36 μCi/mol^{-1}. Unlabeled K-Co(III)-EDTA was prepared in the same manner except that no ^{14}C EDTA was added.

Measurement of EDTA Degradation

In all experiments using radiolabeled Na-EDTA, Fe-EDTA, or K-Co-EDTA, mineralization was assessed by liquid scintillation counting of the $^{14}CO_2$ generated during the experiment. After 4–14 d of incubation the cultures were harvested and $^{14}CO_2$ was quantified. NaOH was injected into 250 mL culture bottles to trap the CO_2 in solution and the bottles were opened. A test tube containing NaOH (2 mL) or another CO_2 trapping solution was inserted and the bottle was resealed. The solution was then acidified, driving the CO_2 into the headspace and into the trapping solution. After equilibration overnight the bottles were opened and ^{14}C in the trapping solution was quantified. Tests with this procedure have indicated that it was >98% effective in trapping the $^{14}CO_2$.

In experiments without radiolabeled EDTA, the disappearance of EDTA was followed using HPLC techniques described by Lauff et al. (*6*) except that isocratic rather than gradient elution was used, 50 μL injections were made into a 50 μL sample loop and standards were made in the salts media (*6*) rather than in unbuffered distilled water. Standard curves were prepared and uninoculated sterile controls were run to confirm that degradation had occurred.

Assessing Degradation of Na-Fe(III)-EDTA by Direct Aerobic Mechanisms

To confirm our ability to detect Na-Fe(III)-EDTA degradation by the *Agrobacterium* obtained from Genencor using HPLC techniques, experiments were run in the mineral salts media and under growth conditions similar to those described by Lauff et al. (*6*). An experiment was run at a pH of 7.4 with 35 and 100 m*M* EDTA at room temperature. Duplicate bottles and uninoculated controls were used for each treatment and degradation was assessed by HPLC measurements of the loss of EDTA from solution. On occasion EDTA degradation was assessed by following changes in nitrate in the media. Nitrate is liberated on degradation of the EDTA.

Experiments were run using the MC1 and SWSA6-1 mixed cultures and the two *Agrobacterium* isolates from Iowa State to determine if the ability to degrade Fe(III)-EDTA was present in related species or in bacteria from a site contaminated with a small amount of EDTA. In these experiments, degradation was followed using radiolabel techniques. Experiments with these cultures were run with ~0.25 μCi Na-EDTA (specific activity of 108 mCi/mmol^{-1}) yielding a concentration of radiolabeled EDTA of ~20 n*M*. Na-Fe(III)-EDTA at a final concentration of 20 m*M* and 1 g/L yeast extract were also added.

An experiment with radiolabeled K-Co(III)-EDTA was designed to test the degradative ability of the bacteria at very low EDTA concentrations. The bacteria were grown in NATE media with ~1 μCi K-Co(III)-EDTA (0.4*M*) and either 0 or 0.1 g ferrous iron. Additional carbon was provided

by adding 2 mL of T-soy broth 4 d after inoculation. Prior to this addition, no obvious increase in turbidity was observed in the treatments with or without iron, and minimal oxygen consumption, as measured by GC techniques, was observed in any of the bottles. The radioactivity remaining in the water phase and in $^{14}CO_2$ produced by EDTA degradation were quantified 3 d after addition of the T-soy broth.

Determination of the Effect of Iron Addition on Cobalt Speciation

Replacement of ^{60}Co in the Co-EDTA complex by ferrous and ferric iron additions, so that degradation of the EDTA as Fe-EDTA could occur, was assessed. It was expected that ferrous iron would displace the Co to a greater degree than ferric iron and would subsequently result in greater EDTA degradation. Twenty milliliters of 1 m*M* Co(III)-EDTA was added to 40 mL centrifuge tubes with and without 5 g of Oak Ridge Reservation soil. The soil was a shaly clay Bt horizons of Typic Hapludult and provides a natural matrix containing competing ions and natural levels of inorganic nutrients and organic carbon. After addition of 0.5 or 5.0 mL of 10 m*M* ferrous or ferric iron solutions to the tubes, the pH was adjusted to 7.5–8.0 with 0.1*M* NaOH. After 2 d of equilibration, solids were removed by centrifugation and radioactivity remaining in solution was measured by gamma spectroscopy. Replacement of Co with iron results in loss of cobalt from solution because only complexed Co-EDTA will remain in solution. The loss of ^{60}Co from solution was quantified by gamma counting.

Statistical Analysis

Results were tabulated using Lotus 1-2-3 and analyzed using the analysis of variance procedure (ANOVA) of SAS software run on an IBM compatible computer. The 95% confidence level was used for all tests of significance.

RESULTS

Bacterial Identification

Based on the capability to oxidize the 95 substrates on the GN plate the Biolog system identified the Genencor *Agrobacterium* strain as *Agrobacterium radiobacter.* The isolate was capable of oxidizing a wide variety of substrates but lacked the ability to oxidize a few of the substrates expected of the "typical" *Agrobacterium radiobacter* isolate (Table 1) used to calibrate the data base. Thus, the identification is based on comparisons to the data base and should be considered tentative.

Table 1
Results of Uses of Biolg GN Plates on the Genencor *Agrobacterium* sp[a]

α-cyclodextrin		τ-hydroxybutyric acid	
dextrin	Yes	*p*-hydroxyphenlyacetic acid	
glycogen	Yes	itaconic acid	
tween 40		α-ketobutyric acid	Yes
tween 80		α-ketoglutaric acid	Yes
N-acetyl-D-glactosamine		α-ketovaleric acid	
N-acetyl-D-glucosamine	Yes	D, L-lactic acid	Yes
adonitol A.r.		malonic acid	
L-arabinose	Yes	propionic acid	Yes
D-arabitol	Yes	quinic acid	Yes
cellobiose	Yes	D-saccharic acid	
i-erythritol		sebacic acid	
D-fructose	Yes	succinic acid	Yes
L-frucose	Yes	bromosuccinic acid	Yes
D-galactose	Yes	succinamic acid	A.r.
gentiobiose	Yes	glucuronamide	
α-D-glucose	Yes	alaniamide	
m-inositol	Yes	D-alanine	Yes
α-lactose	Yes	L-alanyl-glycine	Yes
lactulose	Yes	L-aspargnine	Yes
maltose	Yes	L-aspartic acid	Yes
D-maltose	Yes	L-glutamic acid	Yes
D-mannitol	Yes	glycyl-L-aspartic acid	Yes
D-mannose	Yes	glycyl-L-glutamic acid	Yes
D-melibiose	Yes	L-histidine	Yes
β-methylglucoside	A.r.	hydroxy L-proline	Yes
psicose	Yes	L-leucine	Yes
D-raffinose	Yes	L-ornithine	Yes
L-rhamnose	A.r.	L-phenylalanine	
D-sorbitol	Yes	L-proline	Yes
sucrose	Yes	L-pyroglutamic acid	Yes
D-trehalose	Yes	D-serine	
turanose	Yes	L-serine	Yes
xylitol	Yes	L-threonine	Yes
methylpyruvate	Yes	D,L-camitine	
momo-methylsuccinate	Yes	τ-aminobutyric acid	Yes
acetic acid	Yes	urocanic acid	A.r.
cis-aconitic acid	Yes	inosine	
citric acid		uridine	Yes
formic acid	Yes	thymidine	
D-galactonic acid lactone	Yes	phenylethylamine	
D-galacturonic acid		putrescine	
D-gluconic acid	Yes	2-aminoethanol	
D-glucosaminic acid		2,3, butanediol	
D-glucuronic acid		glycerol	Yes
α-hydroxybutyric acid	Yes	D,L-α-glycerolphosphate	
β-hydroxybutyric acid	Yes	glucose-1-phosphate	A.r.
		glucose-6-phosphate	

[a]The isolate can oxidize those substrates marked as "yes". The data base indicates that *Argobacterium radiobacter* can also oxidize those substrates marked "A.r."

Fe-EDTA Degradation

Mineralization of Iron EDTA could be demonstrated in the radiolabel experiments using the Genencor *Agrobacterium* isolate. However, in many cases the percent converted to CO_2 was low. For example, in an experiment with 10 m*M* Fe-EDTA approx 17% of the EDTA was mineralized. Although the $^{14}CO_2$ production with the isolate was considerably greater than the production in the sterile control (0.06% mineralized) many of our subsequent experiments were carried out using the HPLC techniques since the extent of degradation could be better documented.

In an experiment using the Genencor *Agrobacterium* isolate monitored by the HPLC techniques, the concentration of Fe-EDTA fell from 35 m*M* to less than 15 m*M*. In this 68 h experiment the initial pH was 6.45 and the final pH was greater than 7.5. Standard deviations for the analysis of the EDTA were less than 1 m*M*. During this experiment nitrate concentrations in the media rose to greater than 320 μg/L.

There was no detectable degradation of Co-EDTA by the Genencor *Agrobacterium* isolate, by other *Agrobacterium* strains, or by the mixed isolates (data not shown). We found no other isolate or consortia that was capable of degrading either Fe-EDTA or cobalt-EDTA. The capability of the Genencor strain to degrade Na-Fe-EDTA is not indicative of the degradative capabilities of other strains of *Agrobacterium* or bacteria isolated from surrogate sources. The *Agrobacterium* cultures from Iowa State and two additional cultures produced minimal amounts of $^{14}CO_2$ (maximum of 6.5%) when grown on 20 m*M* Fe-EDTA and yeast extract spiked with ^{14}C Na-EDTA. Addition of iron chloride in the range of 50–400 $\mu g/L^{-1}$ did not increase the degradation of Na-EDTA by the *Agrobacterium radiobacter* from Iowa State in nutrient broth with radiolabeled Na-EDTA at 20 n*M*. This strain showed little ability to degrade the EDTA with a maximum of 3.5% degradation observed in this experiment.

Effect of Biotin and Addition of Yeast Extract and Peptone on Fe-EDTA Degradation

Removal of biotin from the growth media had no effect on degradation of 35 m*M* Fe-EDTA by the Genencor *Agrobacterium*. Over the course of the experiment approximately the same amount of the EDTA was left undegraded in treatments with biotin (19 m*M*) and without biotin (18.5 m*M*).

In a similar experiment, examining the degradation of 5 m*M* Fe-EDTA with addition of yeast extract and peptone, there again was no effect on the EDTA degradation. Because of evaporation during this long experiment EDTA concentration in the sterile controls had risen to over 9 m*M*. However, there was significant degradation to less than 3 m*M* with and without the added yeast extract and peptone.

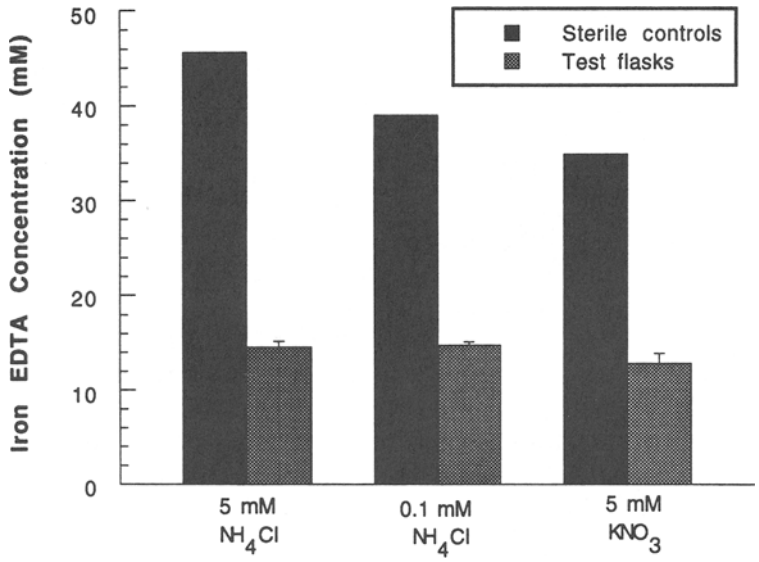

Fig. 2. The effect of nitrogen form (NH_4 and NO_3) and concentration 0.1 m*M* and 5 m*M* on degradation of 35 m*M* Fe-EDTA by Genencor *Agrobacterium* sp.

Effect of Nitrogen Form and Concentration of Fe-EDTA Degradation

There was no difference among treatments containing different concentrations and forms of nitrogen in the amount of Fe-EDTA degradation observed in an experiment with 35 m*M* Fe-EDTA (Fig. 2). A drop in concentration of ammonium chloride from 5 m*M* in the base media to 0.1 mM had no effect on EDTA degradation. Similarly, a change in the form of nitrogen from NH_4Cl to KNO_3 had no effect.

Effect of Glycerol

Glycerol and pH did have significant effects on EDTA degradation by the Genencor isolate. Addition of glycerol to the growth media resulted in a reduction of EDTA degradation by *Agrobacterium*, as shown by HPLC data (Fig. 3). Also, initiation of the EDTA degradation experiments at a pH of 7 also inhibited EDTA degradation by this organism (Fig. 3). During EDTA degradation in the absence of glycerol ammonia concentration in the growth media increases, presumably as a result of liberation of nitrogen from degradation of the EDTA. With glycerol in the media the ammonia completely disappears from the media (Fig. 4A). The same phenomena was noted at pH 7. Interestingly, there were small amounts of nitrate produced in all incubations (Fig. 4B) but there were significantly higher concentrations produced when glycerol was added to the media.

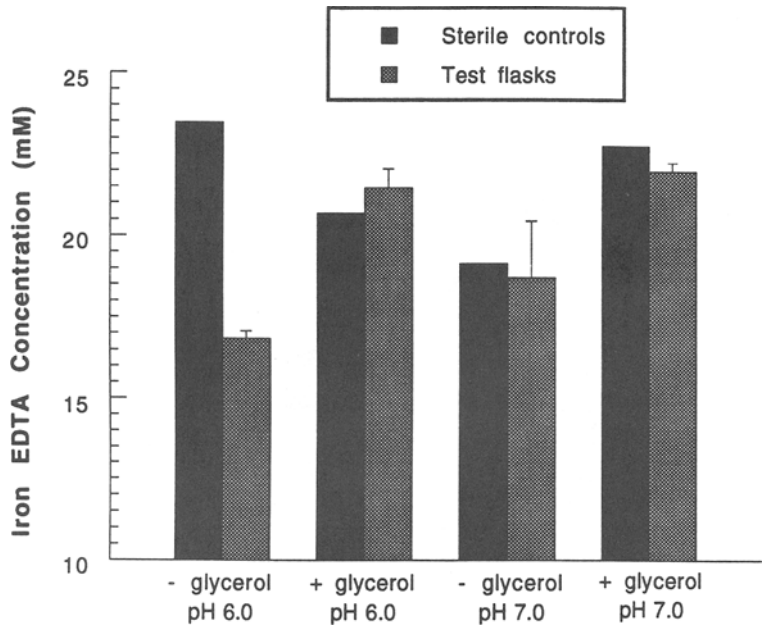

Fig. 3. The effect of glycerol and pH on Fe-EDTA degradation by Genencor *Agrobacterium* sp.

Ability of Ferric and Ferrous Iron to Displace Cobalt Complexed to EDTA

Results of iron addition experiments showed that addition of ferrous iron resulted in precipitation of the cobalt and its loss from solution (Table 2). Addition of the ferric iron resulted in removal of less cobalt from the solution. The process appears very slow and depends on both pH and EDTA concentration (S. Y. Lee, unpublished data). These experiments confirm that iron does replace cobalt and thus organisms capable of degrading Na-FeEDTA could be useful in biodegradation of Co-EDTA.

DISCUSSION

Effect of Media Formulation and Strain of *Agrobacterium* on EDTA Degradation

Chelator degradation has been an environmental concern from a number of aspects (*9,10*). Although other chelators such as NTA and DTPA have proven to be degradable (e.g., *11,12*), only a few species of bacteria have been reported to degrade EDTA (*5,6*). The specific requirements for growth of these few organisms have not been extensively examined. Initial isolation of bacteria are often made in media containing

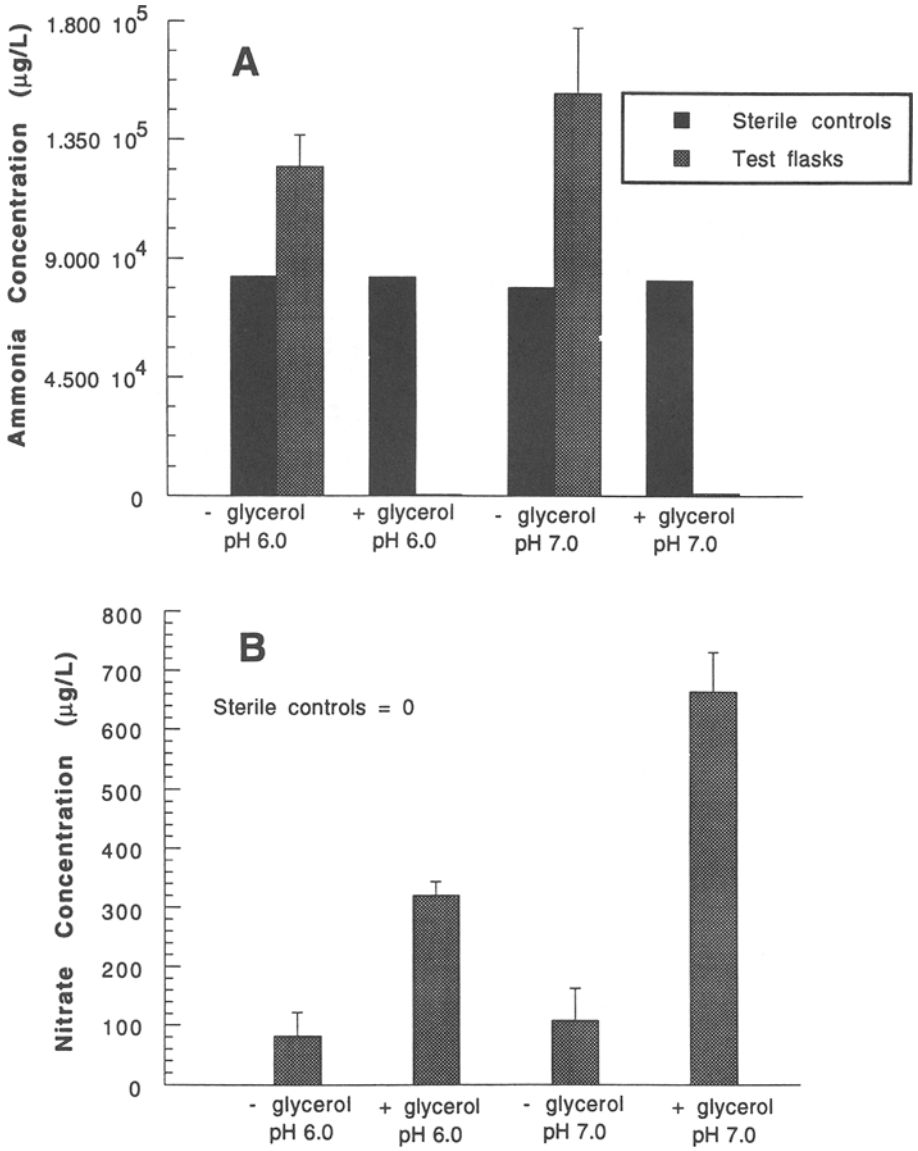

Fig. 4. Nitrate (A) and ammonia (B) production by *Agrobacterium* sp. during growth on Fe-EDTA with and without added glycerol.

vitamins and other factors (e.g., yeast extract) that may not be needed for growth of the organisms. They are in the media to increase chances for isolating a degrader that has some nutritional requirements beyond that provided for by the primary carbon source. From our research it appears that many of the components of the media used to isolate the *Agrobacterium* were not necessary for growth and that many substrates may be capable of supporting growth of this isolate (Table 1).

Table 2
Cobalt Removal from Solution After Addition of $10^{-2}M$ Fe(II) and Fe(III) Solutions With and Without Oak Ridge Reservation Soil

Treatments	Soil	Cobalt removed, %
0.5 mL Fe(II)	NO	42
0.5 mL Fe(II)	YES	70
5.0 mL Fe(II)	NO	45
5.0 mL Fe(II)	YES	77
0.5 mL Fe(III)	NO	20
0.5 mL Fe(III)	YES	34

The ability to degrade EDTA appears to be rare. Neither other *Agrobacterium* strains nor isolates or consortia from a contaminated area were capable of degrading EDTA. Other investigators have been unable to isolate EDTA degrading pure cultures (*5,12*).

Biotin was not required for EDTA degradation by the Genencor isolate and nitrogen may also not be required. A 50-fold dilution of the nitrogen did not result in a change in the amount of EDTA remaining after incubation. Although we did not run a test without any nitrogen, it is likely that nitrogen can be completely supplied by degradation of the EDTA.

Addition of peptone and yeast extract as supplemental carbon and trace organic sources also did not effect EDTA degradation. Yeast extract can increase growth or activity of bacteria by supplying small quantities of vitamins or other organics that can promote growth. Peptone could have been used as a supplemental carbon or nitrogen source. It is evident that the *Agrobacterium* neither needs nor benefits from addition of these compounds during EDTA degradation.

Effect of Glycerol and pH on EDTA Degradation

Preservation of bacterial cells in glycerol during freezing is a common technique for long term storage of isolates. We have noted that upon incubation of frozen cultures of the Genencor *Agrobacterium* it was often very difficult to demonstrate EDTA degradation despite copious growth of the bacteria. This prompted an examination of the effect of glycerol on EDTA degradation.

Addition of glycerol had a pronounced effect on EDTA degradation, whereas addition of other carbon sources did not effect EDTA degradation. The effect of the glycerol may be linked to the change in pH that occurred during growth in the presence of the glycerol. It is evident from our experiments that elevated pH can inhibit EDTA degradation. The change in pH could be owing to production of organic acids from the glycerol.

Potential Remediation Approach

The addition of the iron to the cobalt-EDTA had the desired effect of forming iron-EDTA from the cobalt-EDTA. Presumably, the iron destabilized the cobalt association with the EDTA and replaced the cobalt resulting in precipitation of the cobalt. These experiments confirm that iron does replace cobalt and thus organisms capable of degrading Na-FeEDTA could be useful in biodegradation of Co-EDTA. Problems to examine include the ability of the isolate to degrade Fe-EDTA in the presence of large excesses of iron and the potential for percipitation of the EDTA owing to the iron addition.

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