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Influence of metal complexation on the metabolism of citrate by *Klebsiella oxytoca*

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The uptake of ^{14}C-labeled cadmium–, copper– and zinc–citrate into cells of *Klebsiella oxytoca* was followed. The study was made in order to examine if the earlier reported disability of the bacterium to degrade these complexes was due to an inhibition in transport through the cell membrane. Citrate complexed to cadmium, copper or zinc was taken up at a similar rate to the free citric acid. However, the metal–citrate complexes were not metabolized as shown by the marked accumulation of ^{14}C in the cells as compared with the ^{14}C content in the cells incubated with free citric acid. This was confirmed by the results from trichloroacetic acid-precipitation showing that no ^{14}C was incorporated into macromolecules when the citrate was complexed to the different metals. It is suggested that the inhibited degradation was due to effects on the interaction between enzyme (aconitase) and substrate in the conversion of citrate to iso-citrate. The role of complex configuration on the mineralization of metal–citrate is discussed and also tested in mineralization studies of other metal–citrate complexes (aluminum–, calcium–, cobalt–, manganese– and nickel–citrate).

Keywords: biodegradation, *Klebsiella oxytoca*, metal–citrate complexes

Introduction

Microbial processes play an important role in regulating the transfer and bioavailability of metals in the environment. Metal complexing organic acids are normal constituents in soil and natural waters, and the metal–organic complexes formed control the solubility and bioavailability of many metals. There is a scarcity of knowledge concerning the effects of metal complexation on the bioavailability and degradation of organic compounds. Moreover, it is often difficult to interpret the data from the few studies done because of uncertain chemistry or toxicity effects. However, there are reports which state that metals may have an effect on the biodegradability of organic compounds (Björndal *et al.* 1972, Firestone & Tiedje 1975, Juste *et al.* 1975, Griffith & Schnitzer 1976, Andreux 1981, Madsen & Alexander 1985, Boudot *et al.* 1986, Brunner & Blaser 1989).

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Madsen & Alexander (1985) reported that chemical speciation influenced the mineralization of organic compounds by naturally occurring microbial communities and by individual bacterial populations. The media used were chemically defined with compositions favoring particular chemical species of oxalate, citrate, nitrilotriacetate (NTA) and EDTA. The data suggested that discrimination among different forms of citric acid and oxalic acid occurs in nature and that the magnesium complex of both chemicals may be acted on more slowly than other complexes. The only species of NTA that was degraded in this case was Ca–NTA, while none of the EDTA complexes was degraded. In contrast, we recently reported that cadmium–, copper– and zinc–citrate were not decomposed by a *Klebsiella* sp. while magnesium–citrate and free citric acid were easily degraded (Brynhildsen & Rosswall 1989). We suggested that an explanation of the results could be that cadmium–, copper– and zinc–citrate were not recognized by any membrane transport system in the *Klebsiella* sp. leading to an inhibited cellular uptake. In this paper we have tested the hypothesis and try to explain why the metal–organic complexes could not be degraded by

the bacteria. The results from degradation studies of other metal–citrate complexes are also discussed.

Materials and methods

Organism

A citrate-degrading *Klebsiella oxytoca* strain TV1 (= CCUG 26509) was used in the study. The isolation of the bacteria was described previously (Brynhildsen *et al.* 1988).

Inocula

The *K. oxytoca* was grown in citrate enrichment media (Brynhildsen *et al.* 1988) and harvested at late exponential phase centrifugation (15 700 × *g* for 10 min at 4 °C). The cells were washed twice in 0.1 M KCl and resuspended in KCl. The cell density of the bacterial suspension was determined in terms of the optical density (A_{550}) correlated to viable count.

Experimental media

The media were specially devised to avoid alteration of the metal–organic complex under study, through reactions between the complex and medium constituents. Water of Milli-Q quality was used in all solutions and the concentrations of essential inorganic ions were kept at a minimum. The medium, which was designed by Madsen & Alexander (1985), was also described in our preceding article (Brynhildsen & Rosswall 1989). The different metals (aluminum, calcium, cadmium, cobalt, copper, magnesium, manganese, nickel and zinc) to be complexed with citrate were added as chloride salts in quantities high enough that a minimum of 95% of the total citrate would be bound to the metal as a metal–citrate complex (at pH 6.0) calculated from stability constants given by Sillén & Martell (1964, 1971), Martell & Smith (1977) and Perrin (1979). The ionic strength of all media was adjusted to 0.1 M by the addition of KCl. The pH was adjusted to between 6.6 and 6.8 by 0.1 M KOH, since it was known to drop considerably as a result of autoclaving. The reasons for the various pHs used is that the extent of the drop was different in the different media dependent on metal addition. After sterilization (autoclaving at 120 °C for 20 min), the pH of the media was 6.0. All glassware and polypropylene containers were cleaned by being soaked in HNO_3:H_2O (1:3) overnight and then rinsed thoroughly. The Erlenmeyer flasks used in the experiments were also filled with 0.1 M KCl and heated for 40 min at 120 °C in order to remove acid from the plastic pores.

Citrate uptake experiments

The uptake of citrate was studied in cadmium-, copper- and zinc-containing media and in media with no metal added by measuring the uptake of [1,5-^{14}C]citric acid into the cells. Citrate was added to a final carbon concentration of 1 μg C l^{-1}, compared with the previous study (Brynhildsen & Rosswall 1989) where the carbon concentration was 10 μg l^{-1}. The uptake experiments were run in acid-washed 125 ml Erlenmeyer flasks (polypropylene) containing 50 ml portions of the media and the bacteria at a density of 3×10^7 cells ml^{-1}. ^{14}C-labeled citric acid was added to yield a final radioactivity of 3500 d.p.m. ml^{-1}. The experiments were conducted at 22 °C. Bacterial samples were collected after 0.5, 1, 1.5, 2, 3, 3.5, 4, 5, 6, 7, 10, 20 and 30 min by filtration through membrane filters (Nuclepore polycarbonate filters; pore size, 0.2 μm; diameter, 25 mm). The filters were washed three times with 1 ml 0.1 M KCl and placed in 20 ml scintillation vials containing 15 ml of scintillation cocktail (HP Ready Solve; Beckman Instruments, Fullerton, CA). The vials were shaken, placed in the dark for 10 min, and then assayed for radioactivity with a liquid scintillation counter (Beckman LS 100). The duration of counting for each sample was programmed such that the 2σ error was less than 5% in each determination.

Trichloroacetic acid (TCA) precipitation

Incorporation of ^{14}C into macromolecules was detected by assessing the amount of radioactivity precipitable in cold 5% TCA. Samples (1 ml) were taken out at 30 min, and 1 h, 2 h and 3 h, and mixed with cold TCA. After 15 min the samples were filtered through membrane filters (Nuclepore polycarbonate filters; pore size, 0.2 μm; diameter, 25 mm). The filters were washed with 5 ml of TCA, placed in scintillation vials containing 15 ml scintillation cocktail and assayed for radioactivity as above.

Citrate degradation experiments

The degradation of aluminium–, calcium–, cobalt–, manganese– and nickel–citrate complexes was followed by measuring the degradation of [^{14}C]citric acid (10 μg of C l^{-1}). Citric acid was added under sterile conditions to yield a final activity of 5000 d.p.m. ml^{-1}. Tests of mineralization were performed in acid-washed 125 ml Erlenmeyer flasks (polypropylene) containing 60 ml portions of the media and the bacteria at a density of 10^6 cells ml^{-1}. Flasks with identical metal concentrations (Al, 6×10^{-6} M; Ca, 5×10^{-3} M; Co, 3×10^{-4} M; Mn, 5×10^{-3} M; Ni, 3×10^{-4} M), but with glucose instead of citric acid, were also incubated in order to check the toxicity of the different metals. The glucose solution contained ^{14}C-labeled glucose to yield a final radioactivity of 2000 d.p.m. ml^{-1}. Rates of citrate and glucose decomposition were determined in three parallel flasks incubated at room temperature on a rotary shaker (120 r.p.m.). Samples were taken from flasks after 1, 3, 5, 24, 48 and 72 h for assay of radioactivity as described by Brynhildsen & Rosswall (1989).

Special chemicals

All inorganic salts were analytical reagent grade. [1,5-^{14}C]Citric acid (111 mCi mmol^{-1}) was obtained from

Amersham International and D-[U-^{14}C]glucose (3.7 mCi mmol^{-1}) from E.I. du Pont de Nemours & Co., Boston, MA.

Results

Citrate complexed to cadmium, copper or zinc was taken up at a similar rate as the free citric acid (Figure 1). After 1.5 min about 0.4×10^{-12} mol of the different citrate complexes were assimilated per 3×10^7 cells. After 10 min the ^{14}C-accumulation of the free citric acid leveled of at 0.7×10^{-12} mol citrate per 3×10^7 cells and no further accumulation could be seen even after 40 min. A marked accumulation of ^{14}C in the cells was observed when cadmium, copper or zinc was bound to the organic anion. After 40 min, 1.9×10^{-12} mol copper–citrate, 1.7×10^{-12} mol zinc citrate and 1.65×10^{-12} mol cadmium citrate were taken up per 3×10^7 cells compared with 0.7×10^{-12} mol for the free citric acid. Accordingly it seems that the metal–citrate complexes are not metabolized in the cells, whereas the free citric acid is easily metabolized leading to loss of ^{14}C in the form of CO_2.

Data from TCA precipitation showed that ^{14}C from the free citric acid was incorporated into macromolecules, whereas no incorporation was detected when the organic acid was complexed to cadmium, copper or zinc (Figure 2).

Uncomplexed citric acid and calcium citrate were mineralized at almost the same rates by *K. oxytoca* (Figure 3). After 72 h, 13% of the uncomplexed citrate and 15% of the calcium citrate remained in the media (and/or in the cells). When aluminum, cobalt, manganese or nickel were bound to the organic anion there was a marked effect on the citrate degradation. At the end of incubation 66%

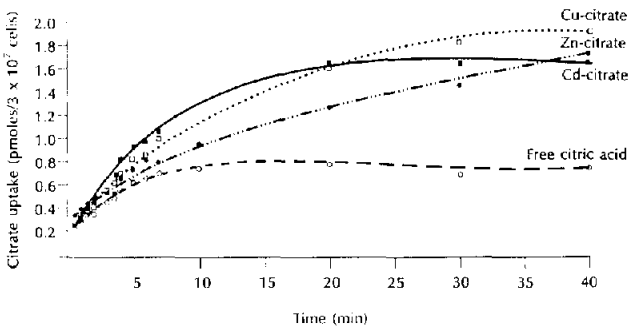

Figure 1. Intracellular uptake of citrate species by *K. oxytoca* (initial cell density 3×10^7 cells ml^{-1}): ○, free citric acid; ■, cadmium–citrate; □, copper–citrate; ●, zinc–citrate. Total carbon concentration 1 μg C l^{-1}. SD was generally less than 5% except for the copper system when it was 7–14%.

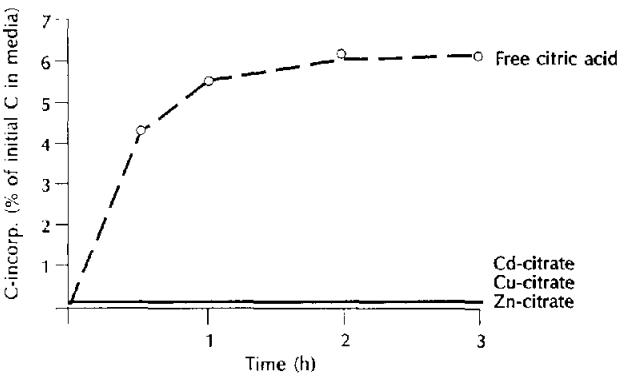

Figure 2. Carbon incorporation in macromolecules by *K. oxytoca* (initial cell density 3×10^7 cell ml^{-1}) in media containing, free citric acid (– – –); cadmium–citrate, copper–citrate or zinc–citrate (——). Total carbon concentration 1 μg C l^{-1}.

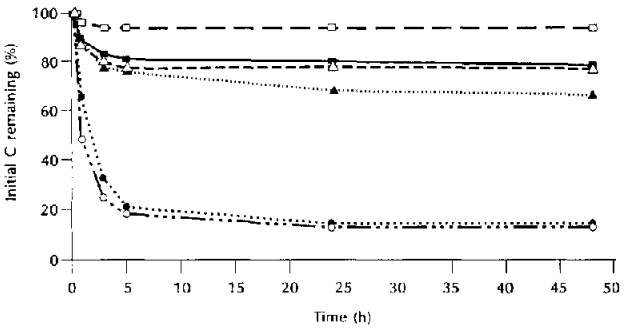

Figure 3. Mineralization of various metal–citrate complexes (10 μg C l^{-1}) by *K. oxytoca*: ○, free citric acid; ●, calcium–citrate; □, nickel–citrate; ■, cobalt–citrate; △, aluminum–citrate; ▲, manganese–citrate. SD generally less than 6%.

(manganese–citrate), 77% (aluminum–citrate), 78% (cobalt–citrate), and 94% (nickel–citrate) of the initially added carbon remained in the media. It is very difficult to prepare a medium containing exactly the predicted amount (95%) of the citric acid bound to the different metals. Thus, it is possible that the relatively small amounts of citrate mineralized were due to degradation of uncomplexed citric acid.

In contrast to citrate, glucose was decomposed in all of the different metal-containing media. However, a marked retardation in decomposition was seen in the media containing nickel and cobalt. After 48 h, 69% of the initially added carbon was detected in the Ni^{2+} containing medium and 66% in the Co^{2+} containing medium, whereas 52% remained in the 'metal-free' medium. In the media containing Ca^{2+}, Mn^{2+} or Al^{3+}, the amounts of carbon left in the media were 54, 31 and 22%, respectively, at the end of incubation. The higher

amount of decomposition in the media containing Mn^{2+} or Al^{3+} most probably reflects a lower carbon incorporation in the cells due to stress effects of the metals.

Discussion

One of the most important physiological processes when discussing bioavailability is the mechanism of uptake. At neutral pH, citrate exists mainly as the trianion and co-transport with at least three positive charges seems necessary to compensate for the continuous inflow of negative charges (Dimroth & Thomer 1986). The ability of some bacterial species to grow on citrate has been demonstrated to be associated with cation-dependent transport systems. Citrate utilization by *Klebsiella aerogenes* was reported to be sodium-dependent (O'Brian *et al.* 1969, Johnson *et al.* 1975). It is thought that this sodium requirement is due to the sodium requirement of the oxalacetate decarboxylase, the second enzyme in the pathway for anaerobic metabolism of citrate. Citrate transport in membrane vesicles of *Bacillus subtilis* is strictly dependent on the presence of divalent cations of metals such as cobalt, magnesium, manganese and nickel (Willecke *et al.* 1973, Bergsma & Konings 1983).

Some of our findings are contradictory to those results, since aluminum, cadmium, cobalt, copper, nickel and zinc inhibited the degradation of citrate (Figure 3; and Brynhildsen *et al.* 1989). However, the former reports only discuss the uptake of citrate and not its metabolism, since the fate of the metal–citrate complexes after being taken up of the cells was not studied. Firestone & Tiedje (1975) showed that a NTA degrading *Pseudomonas* sp. was not able to degrade nickel–NTA, while other metal chelates of NTA were easily degraded. Even though a specific NTA-degrading protein as a part of the transport system was not demonstrated, the authors suggested that the specificity of such a protein would dictate which NTA forms to be transported, i.e. metabolized.

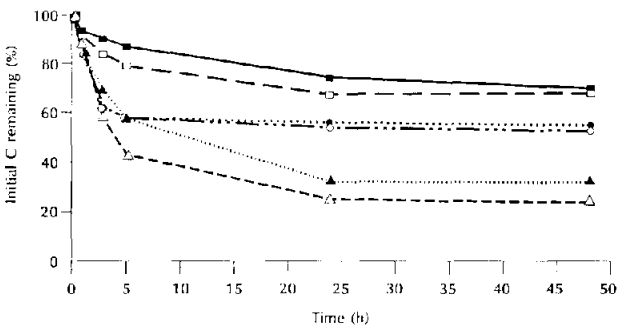

Figure 4. Mineralization of glucose (10 μg C l^{-1}) by *K. oxytoca* in media containing: ○, no metal; ●, calcium; □, nickel: ■, cobalt; △, aluminum; ▲, manganese. SD generally less than 4%.

In our preceding article we discussed the possible reasons for the fact that cadmium–, copper– and zinc–citrate could not be degraded by *K. oxytoca* (Brynhildsen & Rosswall 1989). Our hypothesis was that the different metal–citrate complexes were not recognized by any membrane transport system in the *Klebsiella* strain.

The results from this study do not support the hypothesis, since cadmium–, copper– and zinc–citrate were taken up at a similar rate as the free citric acid (Figure 1). However, the metal citrate complexes were not metabolized as shown by the marked accumulation of ^{14}C in the cells as compared to the ^{14}C content in the cells incubated with free citric acid.

The accumulation of ^{14}C was due to non-metabolized citrate and not to a higher incorporation of ^{14}C into macromolecules as confirmed by the results from the TCA precipitation (Figure 2). Carbon from the free citric acid was incorporated into macromolecules, whereas no incorporation was detected with the metal–citrate complexes. Accordingly, the effect of the metals, rendering the organic anion undegradable to the *K. oxytoca*, seems to be on the metabolism rather than the uptake.

Because of its ubiquitous distribution, citrate can easily be degraded by a large variety of bacterial species via the tricarboxylic acid cycle or the citrate fermentation pathway (Antranikian & Giffhorn 1987). The tricarboxylic acid cycle is the expected pathway for citrate degradation under the aerobic conditions of this investigation. Aconitase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] is a member of the group of enzymes involved in this cycle. The enzyme catalyzes the stereospecific interconversion of citrate and isocitrate via the dehydrated intermediate *cis*-aconitate (Kennedy *et al.* 1987). Citrate complexes differ in configuration depending on the metal involved (Glusker 1980). This could be the reason for cadmium–, copper– and zinc–citrate not being metabolized by *K. oxytoca*, as the altered configuration may lead to a stereochemical hindrance of the contact between enzyme and substrate.

The mechanism behind the interconversion of citrate catalyzed by aconitase has been extensively studied (Rose & O'Connel 1967, Glusker 1971, Villafranca & Mildvan 1971, 1972, Glusker 1980). The enzyme is activated by Fe(II), which is bound to

the aconitase and serves as a bridging atom that binds the citrate molecule. It is also established that other divalent metals (manganese, cobalt, nickel, copper, zinc, cadmium, calcium and magnesium) do not have the same activating role (Villafranca & Mildvan 1971), but rather exhibit an inhibiting effect (e.g. manganese; Villafranca & Mildvan 1972). The reason for this could either be differences in molecular structure between different metal citrate species (related to the metal) or possibly to competition for the binding positions between Fe(II) (on the aconitase) and the other metals (on the citrate complex or in solution).

Since magnesium–citrate was degraded at nearly the same rate as free citric acid by *K. oxytoca* (Brynhildsen & Rosswall 1989), while the metabolism of cadmium–, copper– and zinc–citrate was inhibited, we divided different metal–citrate complexes into isomorphous series of crystals. Our hypothesis was that metal–citrate complexes with structures identical to magnesium–citrate would be degraded, while complexes with the same structures as cadmium–, copper– or zinc–citrate would not be degraded. There are at least three different types of molecular structures that have been identified (X-ray diffractometry) for divalent metal citrates (H_3A = citric acid): (i) dimeric species, $[MA_2\ H_2O]_2^{2-}$ or $[MA_2\ 2H_2O]_2^{2-}$, coordination number 6; (ii) monomeric species, $[MA_2]^{4-}$, coordination number 6; and (iii) monomeric species, [MHA], coordination number 7.

Structure type III is preferred for large ions (ionic radius > 0.9–1 Å) and type I for small ions (ionic radius < 0.7 Å). Structure type and stability constants for the 1:1 complex $M(II):A^{3-}$ are summarized in Table 1.

Since manganese– and magnesium–citrate have identical structures except for the presence and size of the metal ion (Glusker 1980), we expected manganese–citrate to be recognized by aconitase leading to degradation of the complex. The results did not support our hypothesis, since only 34% of the manganese–citrate was mineralized. Thus, there is no obvious correlation between observed degradation (Figure 3; and data in Brynhildsen & Rosswall 1989) and the structure type (Table 1). However, the order of degradation appears to be the reverse of the order of the stability constants of the complex. Thus, all of copper, nickel, cobalt and zinc, as well as to some extent cadmium and manganese may inhibit the interconversion for the simple reason that the corresponding metal–citrate complex is stronger than the Fe(II) complex that has to be formed on the aconitase. The other complexes (with calcium and magnesium, as well as hydrogen) are weaker than the Fe(II) complex and will therefore decompose to form the aconitase–Fe(II)–citrate moiety. Possibly, there is some specific inhibiting effect of manganese for other reasons. The Mn^{2+} ions have been shown to compete with Fe^{2+} for binding at the active site of the enzyme, thereby serving as a competitive inhibitor of aconitase (Villafranca & Mildvan 1972). In contrast to many metals, Mn^{2+} ions bind to exactly the same site of the enzyme as the enzyme activator Fe^{2+}, but the manganese complex of aconitase does not provide the appropriate conformation for catalysis (Villafranca & Mildvan 1971). It is possible that the manganese–citrate complexes in our mineralization studies was degraded initially (Figure 3) but that the liberated Mn^{2+} ions inactivate aconitase by binding to the active site of the enzyme leading to a hindrance of further degradation. For the cobalt–

Table 1. Stability constants and structure type for metal complexes with citric acid

Element	Ionic radius[a] (Å)	log β_1[b]	Structure[c]	Reference
Ca	1.00	3.55	III	Sheldrick (1974)
Cd	0.95	3.75	III[d]	
Zn	0.75	4.98	II	Swanson & Isley (1983)
Cu	0.73	5.90	Ib	Sheldrick (1974)
Mg	0.72	3.40	Ia	Johnson (1965)
Ni	0.70	5.40	Ib	Baker *et al.* (1983)
Mn	0.67	3.67	Ia	Carrel & Glusker (1973)
Co	0.65	5.00	Ia[d]	
Fe	0.61	4.40	Ia	Strouse *et al.* (1977)

[a]For CN 6 (from Jesson & Muetterties 1969).
[b]Defined by $M^{n+} + A^{3-} = MA^{n-3}$; H_3A = citric acid; data from Perrin (1979).
[c]III, monomeric, [MHA], 7-coordination; II, monomeric, $[MA_2]^{4-}$, 6-coordination; Ia, dimeric, $[MA\ H_2O]_2^{2-}$, 6-coordination; Ib, dimeric, $[MA\ 2H_2O]_2^{2-}$, 6-coordination.
[d]Assumed structure type, based on ionic radius and expected coordination properties.

and nickel–systems, the concentrations are high enough that a toxic effect cannot be excluded in the present study.

In conclusion, this study has shown that the inhibiting effects of metal complexation on citrate degradation by *K. oxytoca* was not due to a hindrance in cellular uptake but to a inhibited metabolism. We suggest that the reason for this could be inhibition of the aconitase catalyzed interconversion of citrate.

Further, complex strength, as indicated by the stability constants for the metal–citrate complex in relation to the corresponding iron–aconitase complex, rather than the detailed geometry of the metal–citrate complex, may be the parameter that regulates the extent of aconitase activity and metal–citrate degradation. Since aconitase is distributed among almost all respiring organisms, the results from the citrate mineralization experiments by *K. oxytoca* may be expected to apply for other organisms as well. Naturally, this hypothesis has to be confirmed by studies using mixed cultures of microorganisms and those kind of experiments are in progress.

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