INHIBITION OF BACTERIAL GROWTH BY CITRATE

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According to Bergey's Manual of Determinative Bacteriology, 7th edition, the genus *Arthrobacter* is divided into two groups, one of which utilizes citrate as a sole organic nutrient and the other does not utilize citrate. Among authentic strains of this genus, the growth of *Arthrobacter pascens* IFO 12139 and *Arthrobacter simplex* IFO 12069 was inhibited by citrate. It was found that *A. pascens* IFO 12139 required bivalent cation(s) for growth and failed to grow when the medium contained excess of citrate against bivalent cations. On the other hand, *A. simplex* IFO 12069 could not utilize citrate as a sole source of carbon, and citrate inhibited glucose utilization of the organism. Among many strains belonging to the genera *Corynebacterium*, *Brevibacterium*, and *Bacillus*, growth was inhibited by citrate.

Utilization of citrate is one of important keys in the genus *Arthrobacter* and in the family Enterobacteriaceae. Some investigators stated that many of the so-called "citrate-negative" Enterobacteria in Koser's medium metabolized citrate if the medium was supplied with an energy source and/or growth factors (1–3). The genus *Arthrobacter* is divided into two groups; one of them utilizes citrate as a sole source of organic nutrient and the other does not. When six authentic strains belonging to the former group of the genus were reexamined for their ability to utilize citrate, it was found that the growth of *A. pascens* IFO 12139 and *A. simplex* IFO 12069 was inhibited by citrate. The present paper describes the details of this growth inhibition by citrate.

MATERIALS AND METHODS

Organisms. All the strains used in these experiments have been maintained in this Institute. Six authentic strains of the genus *Arthrobacter* used in this work were received from American Type Culture Collection (ATCC), Rockville, Md., U.S.A. The ATCC strain number of *Arthrobacter* is shown in Table 2.

Media. Two kinds of media for the test of citrate utilization were

Table 1. Constituents of the assay medium for CO₂ production from glucose in *A. pascens* IFO 12139.

Tris-HCl buffer (pH 7.2)	100 μ moles
Glucose	10 μ moles
Glucose [U- ¹⁴ C] (22 mCi/mmole)	0.1 μ Ci
MgCl ₂	0 or 5 μ moles
Sodium citrate	0 or 1.0 %
Cell suspension	0.5 ml
Total volume	3.0 ml

prepared by Koser's and Christensen's methods. The constituents of the two media were as follow: Koser's medium: Citric acid 2 g, NaCl 5 g, MgSO₄·7H₂O 0.2 g, NH₄H₂PO₄ 1 g, distilled water 1,000 ml, pH 6.8. Christensen's medium: Sodium citrate 3 g, glucose 0.2 g, yeast extract 0.5 g, ferric ammonium citrate 0.4 g, KH₂PO₄ 1 g, NaCl 5 g, agar 20 g, distilled water 1,000 ml, Phenol Red (0.2% soln.) 6 ml, pH 6.8–6.9.

Influence of citrate on bacterial growth was examined with a basal medium and nutrient both. The basal medium contained (NH₄)₂SO₄ 2 g, KH₂PO₄ 0.25 g, K₂HPO₄ 0.25 g, MgSO₄·7H₂O 0.25 g, FeSO₄·7H₂O 0.01 g, MnSO₄·6H₂O 0.01 g, biotin 1 μ g, calcium pantothenate 200 μ g, folic acid 1 μ g, inositol 1,000 μ g, niacin 200 μ g, *p*-aminobenzoic acid 100 μ g, pyridoxine-HCl 200 μ g, riboflavin 100 μ g, thiamine-HCl 200 μ g, choline 500 μ g, distilled water 1,000 ml, pH 7.2. Composition of the nutrient broth was beef extract 3 g, peptone 5 g, yeast extract 3 g, distilled water 1,000 ml, pH 7.2. The supplements, such as citrate, glucose, pyruvate, and malate, were autoclaved separately and added to the above media.

Determination of bacterial growth. Young bacterial cells in 5 ml of nutrient broth were collected by centrifugation, washed with sterilized water, and resuspended in 5 ml of sterilized water. An aliquot of the bacterial suspension was inoculated into tubes containing 5 ml of the basal medium supplemented with the test material. These tubes were incubated on a reciprocal shaker at 28° for 7 days. After incubation, the optical density was measured at 600 m μ in 12-mm tube by a Shimadzu Spectronic 20 (Shimadzu Seisakusho Ltd.).

Another examination of *A. simplex* IFO 12069 was made in a series of nutrient broth media by the same method of incubation. Growth was determined by measuring the optical density at 600 m μ in 18-mm Pyrex tube every 2 hr with the use of a Coleman Spectrophotometer (Coleman Instruments Corporation).

Preparation of intact cells. Young bacterial cells in 5 ml of nutrient broth culture were inoculated into 500 ml of the basal medium supplemented with 0.3% glucose and 0.3% yeast extract, and was incubated on a reciprocal shaker at 28° for 20 hr. Cells were harvested by centrifugation, washed

Table 2. Citrate utilization by the genus *Arthrobacter*.

Species	Strain No. IFO (ATCC)	Koser's medium a)	Christensen's medium b)
<i>Arthrobacter globiformis</i>	12137 (8010)	+	+
<i>Arthrobacter pascens</i>	12139 (13346)	—	+
<i>Arthrobacter simplex</i>	12069 (6946)	—	—
<i>Arthrobacter oxydans</i>	12138 (14358)	+	+
<i>Arthrobacter aurescens</i>	12136 (13344)	+	+
<i>Arthrobacter ureafaciens</i>	12140 (7562)	+	+

a) Citrate utilization was judged by whether the organisms could grow in the medium.

b) Citrate utilization was judged by color change of the pH indicator.

twice with distilled water, and resuspended in 25 ml of 0.1 M Tris-HCl buffer (pH 7.0). The intact cell suspension was used in the following experiment.

Radioisotope experiment. An assay medium (Table 1) was poured into Warburg flasks sealed with a rubber cap and was incubated at 28°. After 30-min incubation, 0.5 ml of 1 N H₂SO₄ was injected into the medium with a syringe through the rubber cap and 0.3 ml of hyamine-ethanol solution was then injected with a syringe into the center well of each Warburg flask. The Warburg flasks were further incubated at 28° for 20 min. Radioactivity of 0.2 ml of each hyamine-ethanol solution absorbed CO₂ was counted in a vial containing 10 ml of scintillation fluid (DPO 150 mg, POPOP 6,000 mg, toluene 1,000 ml), by a Packard Tri-Carb Liquid Scintillation Counter.

Assay method for glucose in media. Content of glucose was determined by Glucostat (Worthington Biochemical Corporation).

RESULTS AND DISCUSSION

The ability of six authentic strains of *Arthrobacter* to utilize citrate in Koser's and Christensen's media is shown in Table 2. Among the strain tested, *A. pascens* IFO 12139 could not grow in Koser's medium and *A. simplex* IFO 12069 did not utilize citrate in both media. However, in a medium without vitamins such as Koser's medium, a bacterial strain may not grow if the strain has a nutritional requirement. For this reason, the basal medium containing vitamins was used for further experiments stated below.

Citrate assimilation by *A. pascens* IFO 12139 and *A. simplex* IFO 12069 was tested in the basal medium supplemented with either glucose, pyruvate, or malate, and combined with various concentrations of sodium citrate. As shown in Fig. 1, *A. pascens* IFO 12139 could utilize citrate as a sole carbon source when the concentration of citrate was 0.1% or less, but the organism

Fig. 1. Growth inhibition by citrate.

Growth in the basal medium supplemented with glucose, pyruvate, or malate, and with various concentrations of sodium citrate was determined by measuring the optical density at 600 $m\mu$ after 7 days' incubation

could not grow when sodium citrate concentration was 1%, even if the medium was supplemented with glucose, pyruvate, or malate. On the other hand, *A. simplex* IFO 12069 could not utilize citrate as a sole source of carbon and, when one of available carbon sources was added in the basal medium, the growth decreased in proportion to the concentration of sodium citrate for all kinds of supplemented carbon sources.

The above phenomena were observed not only for sodium salt but also for potassium and ammonium salts of citric acid.

Growth inhibition of A. pascens IFO 12139 by citrate

It is noteworthy that the assimilation of citrate in *A. pascens* IFO 12139 is influenced by concentration of the compound in the medium. Citric acid has a chelating effect against metals. It was anticipated that the phenomenon was caused by chelating of citrate with metals in the medium. *A. pascens* IFO 12139 was incubated in a series of media containing various concentrations of sodium citrate and of bivalent cations (Table 3). The results are shown in Fig. 2. As *A. pascens* IFO 12139 could not grow in the medium without bivalent cations (O), it was evident that the organism required one or more of these cations for growth. When the bivalent cations were added to the medium at the ordinary concentration (S), growth of the organism was inhibited by 0.5 to 1% of sodium citrate but, when the concentration of

Table 3. Constituents of the medium for testing the effect of bivalent cations on growth inhibition of *A. pascens* IFO 12139 by citrate.

Glucose	1 g
(NH ₄) ₂ SO ₄	2 g
KH ₂ PO ₄	0.25 g
K ₂ HPO ₄	0.25 g
Vitamins (biotin 1, calcium pantothenate 200, folic acid 1, inositol 1,000, niacin 200, <i>p</i> -aminobenzoic acid 100, pyridoxine-HCl 200, riboflavin 100, thiamine-HCl 200, choline 500 μ g)	
Distilled water	1,000 ml
Sodium citrate	0, 1, 5 or 10 g

Cations	(0)	(S)	(5S)	(10S)
MgSO ₄ ·7H ₂ O	0	0.25	1.25	2.5 g
FeSO ₄ ·7H ₂ O	0	0.01	0.05	0.1 g
MnSO ₄ ·6H ₂ O	0	0.01	0.05	0.1 g

(pH 7.2)

Fig. 2. Effect of bivalent cations on growth inhibition of *A. pascens* IFO 12139 by citrate.

Growth in the medium shown in Table 3 was determined by measuring optical density at 600 $m\mu$ after 7 days' incubation.

the cations was 5- or 10-fold of the ordinary concentration (5S or 10S), the organism could grow even in the medium containing 1% of sodium citrate.

In addition, influence of citrate on glucose metabolism in intact cells of *A. pascens* IFO 12139 was examined. As shown in Fig. 3, citrate did not have any influence on CO₂ production from glucose even in the assay medium containing 1% of sodium citrate.

Fig. 3. Influence of citrate on CO₂ production from glucose by *A. pascens* IFO 12139.

CO₂ production from glucose by intact cells of *A. pascens* IFO 12139 was carried out in the assay medium (Table 1).

- a) +Assay medium with 5 μ moles MgCl₂.
 -Assay medium without MgCl₂.
 b) +Assay medium with 1% sodium citrate.
 -Assay medium without citrate.

Fig. 4. Inhibition of glucose utilization of *A. simplex* IFO 12069 by citrate.

- N: Nutrient broth.
 NG: Nutrient broth+0.1% glucose.
 NC: Nutrient broth+1% sodium citrate.
 NGC: Nutrient broth+0.1% glucose+1% sodium citrate.

Table 4. Utilization of carbon sources by *A. simplex* IFO 12069 and growth inhibition by citrate in the medium containing available carbon sources.

Carbon source	Utilization	Growth in the medium containing 1% citrate
	a)	b)
Glucose	+	—
Galactose	+	—
Mannose	—	
Fructose	—	
Xylose	—	
Ribose	—	
Gluconate	—	
Glycerol	—	
Pyruvate	+	+
Malate	+	+
Fumarate	+	+
Glutamate	+	+

a) + utilization, — no utilization.

b) + growth, — no growth.

Table 5. Citrate utilization of bacteria.

Species	Strain No.	Koser's medium		Christensen's medium
		0.2%	0.07% a)	
<i>Corynebacterium flaccumfaciens</i>	IFO 12156	—	+	+
" <i>michiganense</i>	" 12158	—	+	+
" <i>aquaticum</i>	" 12154	—	+	+
" <i>rathayi</i>	" 12162	—	—	—
" <i>tritici</i>	" 12164	—	+	+
<i>Brevibacterium divaricatum</i>	" 12132	—	+	+
" <i>vitarumen</i>	" 12143	—	—	—
" <i>incertum</i>	" 12145	—	—	—
" <i>fuscum</i>	" 12127	—	+	+
<i>Bacillus magerans</i>	" 3490	—	+	+
" <i>pumilus</i>	" 12111	—	+	+
" <i>firmus</i>	" 3330	—	—	—
" <i>brevis</i>	" 12334	—	—	—

a) Citric acid concentration was reduced to 0.07%.

From these results, it seems that growth inhibition of *A. pascens* IFO 12139 by citrate is due to deficiency of bivalent cations from their chelation with citrate.

Growth inhibition of A. simplex IFO 12069 by citrate

When 1% of sodium citrate was added, *A. simplex* IFO 12069 failed to grow in the basal medium containing glucose as carbon source, but did grow in the basal medium containing pyruvate or malate (Fig. 1). It was expected that glucose utilization of the organism was inhibited by citrate. Growth was tested in four kinds of nutrient broth supplemented with glucose and citrate (Fig. 4). When the culture medium contained 1% of sodium citrate, growth of the organism was repressed and glucose content in the medium (NGC) did not diminish even after 7 days' incubation (Fig. 4). It is evident that glucose utilization of *A. simplex* IFO 12069 is inhibited by citrate.

A. simplex IFO 12069 utilized glucose, galactose, pyruvate, malate, and glutamate, but did not utilize mannose, fructose, xylose, ribose, gluconate, or glycerol as a sole source of carbon (Table 4). An examination was made to see whether the organism could grow in the basal medium containing 1% of sodium citrate and one of these available carbon sources. Growth of *A. simplex* IFO 12069 was inhibited by citrate when the source of energy was glucose or galactose, but was not arrested when pyruvate, malate, glutamate, or fumarate was added (Table 4). It is evident that galactose utilization of the organism is also inhibited by citrate. This result will be published in more detail elsewhere.

Growth inhibition of gram-positive bacteria by citrate

It is an interesting fact that citrate, a member of the TCA cycle, inhibits growth of bacteria. Nobody has discussed the influence of this phenomenon on bacterial taxonomy. It is necessary to investigate the distribution of such inhibition reaction of citrate in bacteria and the examination was carried out on 23 strains of *Corynebacterium*, 33 strains of *Brevibacterium*, and 16 strains of *Bacillus* by the use of the basal medium containing 0.1% of glucose and 1% of sodium citrate. The growth of 13 strains shown in Table 5 was inhibited by citrate.

Citrate utilization of the above 13 strains was tested in Christensen's medium and Koser's medium supplemented with vitamins and amino acids. All the 13 strains, including the 8 strains which utilized citrate in Christensen's medium, could not grow in Koser's medium. When the citric acid concentration in Koser's medium was changed from 0.2% to 0.07%, these 8 strains, which utilized citrate in Christensen's medium, grew in that medium.

When utilization of citrate as a sole source of carbon is examined, bacteria which were judged as positive for citrate utilization in Christensen's medium have to be retested in the newly modified Koser's medium in which

citric acid concentration was reduced to 0.07%, and vitamins and amino acids were supplemented.

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