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The distribution of plasmids determining citrate utilization in citrate-positive variants of *Escherichia coli* from humans, domestic animals, feral birds and environments

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SUMMARY

Sixty-seven isolates of citrate-positive variants of *Escherichia coli* were isolated from human, domestic animal, feral bird and environmental sources. With the exception of citrate utilization, all isolates were identified as typical *E. coli* by their biochemical reactions. The transmission of the ability to utilize citrate on Simmons' citrate agar was demonstrated in 53 (79·1%) out of the 67 citrate-positive *E. coli* variants obtained from various sources. Drug resistance determinants and citrate utilizing character were co-transmitted into *E. coli* K-12 by conjugation among citrate-positive *E. coli* isolates carrying R plasmids except for that isolated from horses. The other characters (haemolysin or colicin production, raffinose or sucrose fermentation) were not transmitted together with the citrate utilizing character. These facts suggested that the structural gene responsible for citrate utilizing ability in citrate-positive variants of *E. coli* was located on a conjugative plasmid.

INTRODUCTION

The isolation of three citrate-positive *Escherichia coli* variants from human sources has been reported by Washington & Timm (1976). More recently, Ishiguro, Oka & Sato (1978) reported that 27 citrate-positive variants of *E. coli* were isolated from domestic pigeons and domestic animals at high rates. With the exception of a strain from horses, these citrate-positive variants from non-human sources were resistant to three to five antibiotics and most variants carried conjugative R plasmids showing thermosensitive transfer. Furthermore, the structural gene for citrate utilizing ability, like those for the hydrogen sulphide-producing variants of *E. coli* (Ørskov & Ørskov, 1973), was located on a transmissible plasmid (Sato *et al.* 1978). The citrate utilizing ability in the variants was always found in association with the conjugative R plasmids. However, it remained to be solved whether the Cit plasmid is self-transmissible. Further study on the citrate-utilizing plasmid indicated that the plasmid was self-transmissible and also transferable to *Shigella* spp. and *Salmonella* spp. lacking citrate

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utilization. Moreover, the transferability of the plasmid was enhanced by the R plasmid coexisting in the same host cells (unpublished data). These facts suggested that there might be the potential spread of citrate utilizing ability within *E. coli* strains in nature with the prevalence of conjugative R plasmids. Little, however, is known about the distribution of citrate-positive *E. coli* in nature.

This paper deals with the isolation of citrate-positive *E. coli* variants from human, domestic animal, feral bird and environmental specimens, and with further confirmation as to whether the citrate utilizing ability is located on a transmissible plasmid.

MATERIALS AND METHODS

Isolation procedures of E. coli*-like colonies from human, domestic animal, feral bird and environmental sources*

Human. Stool samples were obtained from 486 humans in Obihiro. Each of the stool samples was plated directly onto a deoxycholate-hydrogen sulphide-lactose agar (DHL, Eiken) plate by a toothpick. The DHL agar plates were incubated at 37 °C for 24 h. At least 10–15 *E. coli*-like colonies grown on each DHL agar plate were used in this study.

Domestic animals. A total of 490 cattle rectal faeces were collected from 53 locations from 1975 to 1977 in Hokkaido. Twenty composite faecal samples were collected from five pig farms in Obihiro and 396 rectal faeces were obtained from 396 horses from 1976 to 1977 in Tokachi and Kushiro areas. The rectal faeces and composite faecal samples from domestic animals were incubated in 10 ml or about ten-fold amounts of selenite brilliant green (SBG, Nissui) broth at 43 °C overnight for *Salmonella* isolation. These broth cultures were left at 37 °C overnight and were subcultured onto DHL agar plates with or without the following antibiotics: chloramphenicol (Cm, 25 μg/ml), tetracycline (Tc, 25 μg/ml) or streptomycin (Sm, 12·5 μg/ml) to select drug-resistant *E. coli*. Three to six *E. coli*-like colonies on each DHL agar plate with or without the addition of antibiotics were obtained from cattle samples, and ten *E. coli*-like colonies grown on each DHL agar plate from pig or horse samples were tested in this study.

Feral pigeons, crows and other wild birds. In all feral pigeons, crows and other wild birds, the lower parts of the intestines of the carcasses were put into heart infusion broth (Eiken) and incubated overnight at 37 °C. These broth cultures were subcultured onto DHL agar plates with or without the addition of Cm, Tc or Sm. *E. coli*-like colonies on DHL agar plates were used in this study.

Environmental sources. Ten farm sewage samples were collected from five dairy farms in the Tokachi area and nine sewage samples were obtained from five pig farms in Obihiro. These sewage samples obtained from cattle and pig farms were serially diluted in sterile saline, and then 0·1 ml of appropriate dilutions in saline were plated onto DHL agar plates with or without the addition

of antibiotics such as Cm, Tc and Sm. A total of 234 and 527 isolates obtained from dairy farm sewage and pig farm sewage respectively, were examined for citrate utilization.

Isolation of citrate-positive E. coli *variants and differential tests of* E. coli

All the *E. coli*-like colonies grown on DHL agar plates from human, domestic animal, feral bird and environmental sources were streaked onto Simmons' citrate agar and incubated at 37 °C for 4 days. Colonies grown on Simmons' citrate agar were examined by an initial set of tests that are used routinely in this laboratory for identification of gram-negative bacilli: triple sugar iron agar, sulphide-indole-motility medium, Voges–Proskauer reaction, lysine–iron agar, malonate and KCN medium. With the exception of citrate utilization, the isolates identified as the *E. coli*-like form by the initial tests were differentiated in more detail by biochemical tests described by Edwards & Ewing (1972). No serological studies was performed.

Antibiotic susceptibility tests

Antibiotic susceptibility testing of citrate-positive *E. coli* variants was performed as described in a previous paper (Ishiguro *et al.* 1978), using 12 antibiotics at the following final concentrations (μg/ml): Cm, 25; Tc, 25; Sm, 12·5; kanamycin (Km), 25; aminobenzylpenicillin (Ap), 25; cephaloridine (Cer), 25; gentamicin (Gm), 12·5; colistin (CL), 12·5; furatrizine (Ft), 6·3; nalidixic acid (Nal), 25; rifampin (Rif), 25; sulfadimethoxine (Su), 800. Heart infusion agar (Eiken) was used for the tests except in the test with Su, in which Mueller Hinton agar (Eiken) was used.

Examination for haemolysin and colicin production

Fresh whole blood from sheep was collected with 3·8% sodium citrate as anticoagulant, and washed packed human O red cells were also used. The red cells were washed three times in saline and the packed cells were added in heart infusion agar to give 5% (v/v) concentrations. All the citrate-positive *E. coli* variants were tested for the production of haemolysin (Hly) on washed 5% sheep blood agar and washed 5% human O group blood agar. Colicin production (Col) was determined by overlaying a lawn of indicator strain over 48 h old chloroform-killed colonies on nutrient agar. *E. coli* Row was used as the indicator strain (Ørskov & Ørskov, 1973).

Detection of conjugative R plasmids

The detection of conjugative R plasmids was made according to the procedures described by Ishiguro *et al.* (1978). *E. coli* ML1410, a nalidixic acid-resistant, methionine-requiring F^- derivative of K-12, was used as a recipient. Three colonies of transconjugants on each selective medium were purified and examined for citrate utilization on Simmons' citrate agar containing methionine (50 μg/ml), as well as for drug resistance to the antibiotics applied. The transconjugants were also examined for production of haemolysin and colicin.

Transfer experiment of citrate utilizing character

All isolates identified as citrate-positive *E. coli* variants by biochemical tests were used for transfer experiments. Transfer experiments on their citrate utilizing ability were performed by mating, as well as detection of R plasmid (Sato *et al.* 1978). *E. coli* ML1410 which does not ferment raffinose and sucrose was used as a recipient. Colonies grown on Simmons' citrate agar plates were used as donor and cultivated in 2 ml of penassay (Difco) broth at 25 °C for 18 h. The *E. coli* ML1410 was cultured in a similar way. Equal volumes of donor and recipient cultures were mixed and incubated at 25 or 37 °C for 18 h. The selective media used were Simmons' citrate agar to which were added methionine (50 μg/ml) and nalidixic acid (50 μg/ml). A loopful of each mixed culture was subcultured onto a selective agar plate, and was incubated at 37 °C for 4 days. To determine transconjugant recipients and their characters, three colonies of transconjugants on the selective media were purified on DHL agar plates and examined for citrate utilizing ability on the same media and for resistance to antibiotics. The purified colonies were also tested for the production of haemolysis and colicin. With any donor isolates that fermented raffinose or sucrose promptly, fermentation of these carbohydrates was tested for in transconjugant strains in bromcresol purple semisolid medium (Eiken) containing 0·5 % of raffinose or sucrose. Also, utilization of raffinose or sucrose as a sole carbon source by transconjugants were made on M9 minimal salts medium (Shipley, Giles & Falkow, 1978) containing methionine (50 μg/ml) and 0·5 % of raffinose or sucrose.

For further study of the relation between R plasmids and citrate-utilization, representative isolates from various sources were investigated by performing transfer tests. Donor and recipient were precultured at 25 °C for 4 h with shaking. Then, 0·1 ml of donor and 1 ml of recipient cultures were mixed in 4·5 ml of fresh penassay broth, and incubated for 2 h at 25 or 37 °C with gentle shaking. In this experiment, a heart infusion agar was used for Tc, Mueller Hinton agar was used for Su, DHL agar was used for Cm, Sm, Km and Ap, and Simmons' citrate agar containing methionine (50 μg/ml) was used for citrate utilization. Transfer frequencies were determined after 2 h of mixed incubation as transconjugants per donor. Colonies grown on each selective medium were purified and examined for their characters.

RESULTS

Isolation of citrate-positive variants of E. coli

Table 1 shows the incidence of citrate-positive variants in *E. coli* isolated from human, domestic animal, feral bird and environmental sources. Of 5459 *E. coli*-like colonies obtained from 486 humans, 24 (0·44 %) were identified as citrate-positive *E. coli* variants by the initial tests used in this study. These 24 variants were isolated from stool samples of seven humans.

In cattle, 12 (0·55 %) out of a total 2170 *E. coli* colonies examined were found to be citrate-positive. No citrate-positive *E. coli* variant was detected from

Table 1. *Isolation of citrate-positive variants of* E. coli *from humans, domestic animals, feral birds and environmental samples*

Host	Locality (no. of locations)	Periods of isolation	Specimens examined	No. of specimens examined	No. of *E. coli*-like colonies tested	No. of isolates which utilized citrate (No. of specimens)	No. of isolates obtained on selective media containing antibiotics
Humans	Obihiro	1977	Stool samples	486	5459	24 (7) 0·44%	—*
Cattle	Hokkaido (53)	1975–7	Rectal faeces	490	2170	12 (6) 0·55%	0
	Tokachi (5)	1977	Farm sewage samples	10	234	0 . .	.
Pigs	Obihiro (5)	1977	Composite faecal samples	20	297	0 . .	.
	Obihiro (5)	1977	Farm sewage samples	9	527	22 (6) 4·17%	11
Horses	Tokachi and Kuchiro	1976–7	Rectal faeces	396	3816	5 (4) 0·13%	4
Feral pigeons	Hokkaido (15)	1976–7	Intestinal contents	168	395	2 (1) 0·51%	2
Crows	Horonobe (2)	1977	Intestinal contents	10	44	2 (2) 4·54%	2
Wild birds	Tokachi	1976–7	Intestinal contents	6	80	0 . .	.

* Not used for selective media.

20 pig composite faecal samples. Of 3816 *E. coli*-like colonies obtained from 396 horses, only 5 (0·13 %) were identified as citrate-positive *E. coli* variants by the initial biochemical tests. Four of these 5 isolates were obtained on selective media containing antibiotics. Four citrate-positive *E. coli* variants were isolated from 168 feral pigeons and 10 crows, whereas no citrate-positive variants were detected from six wild birds (Table 1). The four variants isolated from one feral pigeon and two crows were obtained on selective media containing Cm.

Though no citrate-positive *E. coli* variant was found among 234 colonies isolated from ten dairy farm sewage samples, 22 (4·17 %) out of 527 colonies obtained from pig farm sewage samples were found to be citrate-positive, and half of these were detected on selective media containing antibiotics. Excepting that the isolates from human stools were obtained without selection by antibiotics and that those from feral pigeons and crows were obtained on media containing antibiotics, no significant difference in the isolation rates of citrate-positive variants of *E. coli* was observed between selective and non-selective media.

Biochemical characteristics

All the citrate-positive strains isolated alkalized Simmons' citrate agar within 4 days at 37 °C. The citrate-positive colonies isolated on Simmons' citrate agar plates were always confirmed by streaking on Simmons' citrate agar slants. With the exception of citrate utilization on Simmons' citrate agar, 67 citrate-positive isolates closely resembled *E. coli*, according to the initial tests used. All citrate-positive isolates from various sources were identified as *E. coli* by biochemical reactions described by Edwards & Ewing (1972), as shown in Table 2.

Antibiotic susceptibility and detection of conjugative R plasmids

The antibiotic susceptibility of 67 citrate-positive *E. coli* variants from human, domestic animal, feral bird and environmental sources and the resistance patterns of their conjugative R plasmids are shown in Table 3. All the variants tested were susceptible to the following antibiotics: CL, Cer, Gm, Nal, Rif. Twenty-two variants isolated from pig sewage samples and four variants isolated from feral birds were resistant to two or more antibiotics such as Tc, Sm, Su, Cm, Km and Ft. Of 67 citrate-positive *E. coli* variants, 50 (74·6 %) were resistant to drugs tested, and 25 (50 %) of drug resistant isolates carried conjugative R plasmids. As shown in Table 4, it was of interest that 19 (86·4 %) of 22 resistant *E. coli* isolates obtained from pig sewage samples carried thermosensitive R plasmids, whereas only three (16·7 %) of 18 resistant isolates from humans had conjugative R plasmids.

Incidence of plasmids (R^+, Hly and Col) in citrate-positive E. coli *isolates*

Table 4 shows the incidence of plasmids (R^+, Hly and Col) among the 67 isolates of citrate-positive *E. coli* examined during this investigation. It should be noted that no citrate-positive isolates possessing the three characters, drug

Table 2. *Thirty-four biochemical reactions of 61 citrate-positive E. coli isolates from human, domestic animal, feral bird and environmental sources*

	Humans (24)*		Cattle (12)		Pigs (22)		Horses (5)		Feral pigeons (2)		Crows (2)	
Test or substrate	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Citrate (Simmons')	24	100	12	100	22	100	5	100	2	100	2	100
ONPG	24	100	12	100	22	100	5	100	2	100	2	100
H_2S (TSI)	0	0	0	0	0	0	0	0	0	0	0	0
Urease (Christensens)	0	0	0	0	0	0	0	0	0	0	0	0
Indole	24	100	12	100	22	100	5	100	2	100	2	100
Methyl red	24	100	12	100	22	100	5	100	2	100	2	100
Voges–Proskauer	0	0	0	0	0	0	0	0	0	0	0	0
KCN	0	0	0	0	0	0	0	0	0	0	0	0
Motility	15	62·5	10	83·3	22	100	5	100	0	0	2	100
Gelatin, 22 °C	0	0	0	0	0	0	0	0	0	0	0	0
Lysine decarboxylase	24	100	12	100	22	100	5	100	2	100	2	100
Arginine dihydrolase	0	0	0	0	0	0	0	0	0	0	0	0
Ornithine decarboxylase	4	16·7	10	83·3	20	90·9	1	20·0	2	100	2	100
Phenylalanine deaminase	0	0	0	0	0	0	0	0	0	0	0	0
Malonate	0	0	0	0	0	0	0	0	0	0	0	0
Glucose	24	100	12	100	22	100	5	100	2	100	2	100
Lactose	24	100	12	100	22	100	5	100	2	100	2	100
Sucrose	2	8·3	11	91·7	1	4·5	4	80·0	2	100	2	100
Mannitol	24	100	12	100	22	100	5	100	2	100	2	100
Dulcitol	9	37·5	12	100	22	100	5	100	2	100	2	100
Salicin	9	37·5	12	100	19	86·4	3	60·0	2	100	2	100
Adonitol	0	0	0	0	2	9·1	0	0	0	0	0	0
Inositol	0	0	4	33·3	0	0	0	0	0	0	0	0
Sorbitol	21	87·5	12	100	14	63·6	5	100	2	100	2	100
Arabinose	24	100	12	100	22	100	5	100	2	100	2	100
Raffinose	2	8·3	10	83·3	13	59·1	4	80·0	2	100	0	0
Rhamnose	24	100	12	100	22	100	4	80·0	2	100	2	100
Maltose	24	100	12	100	22	100	5	100	2	100	2	100
Xylose	24	100	12	100	22	100	5	100	2	100	2	100
Trehalose	24	100	12	100	22	100	5	100	2	100	2	100
Nitrate to Nitrite	24	100	12	100	22	100	5	100	2	100	2	100
Sodium acetate	24	100	12	100	22	100	5	100	2	100	2	100
Oxidase	0	0	0	0	0	0	0	0	0	0	0	0
Esculin	9	37·5	12	100	17	77·3	3	60·0	2	100	2	100

* No. of isolates tested.

Table 3. *Drug-resistance patterns and conjugative R plasmids in* 67 *citrate-positive* E. coli *isolates*

Host	No. of isolates tested	Drug-resistance: Resistance patterns	Drug-resistance: No. of isolates	Resistance patterns of conjugative R plasmids	No. of R+ isolates	No. of ts* R+ isolates
Humans	24	Tc, Km	2	Tc, Km	2	0
		Su	16	Su	1	0
		(S)†	6			
Cattle	12	Su	2	—‡	0	0
		(S)	10			
Pigs	22	Tc, Sm, Su, Cm, Km, Ft	1	Tc, Sm, Su, Cm, Km	1	1
		Tc, Sm, Su, Cm, Km	2	Tc, Sm, Su, Cm, Km	2	2
		Tc, Sm, Su, Cm, Ft	1	—		
		Tc, Sm, Su, Cm	3	Tc, Sm, Su, Cm	3	3
		Tc, Sm, Su, Km	2	Tc, Km	1	1
				Tc, Sm, Km	1	1
		Tc, Sm, Su	1	Tc	1	1
		Tc, Km	11	Tc, Km	10	10
		Tc, Su	1	—		
Horses	5	Tc, Sm, Su, Cm, Ap	1	Tc, Sm, Su, Cm, Ap	1	0
		Tc, Sm, Su	2	—		
		Sm	1	—		
		(S)	1			
Feral pigeons	2	Sm, Su, Cm	2	—		
Crows	2	Tc, Sm, Su, Cm, Km, Ft	2	Tc, Sm, Su, Cm, Km	2	1

Tc, Tetracycline; Sm, streptomycin; Su, sulphadimethoxine; Cm, chloramphenicol; Km, kanamycin; Ap, aminobenzylpenicillin; Ft, furatrizine.

* ts, R plasmids showing thermosensitive transfer.

† (S), Susceptibility to antibiotics used in the present study.

‡ Resistance not transferred.

Table 4. *The distribution of plasmids (R+, Col, Hly)* in citrate-positive* E. coli *isolates and the relationships between conjugative Cit character and other plasmids*

Source	No. of isolates	Properties of isolates			No. containing conjugative Cit characters (ts)†		No. containing transmissible plasmids with Cit character‡		
		R+	Hly	Col			R+	Hly	Col
Humans	24	3	0	2	24	100%	3	0	0
Cattle	12	0	1	3	6	50%	0	0	0
Pigs	22	19	13	1	19 (19)	86·3%	19	0	0
Horses	5	1	0	0	0	0%	0	0	0
Feral pigeons	2	0	0	0	2	100%	0	0	0
Crows	2	2	0	0	2 (1)	100%	2	0	0

* R+, Drug resistance plasmid; Col, Colicin production; Hly, Haemolysin production.

† ts, Cit character showing thermosensitive transfer.

‡ In each experiment, three transconjugants were picked, diluted in 0·05 ml saline, and grown on non-selective media. The purified colonies were examined for characters.

resistance (R+), haemolysin and colicin production, in the same cell were detected. In particular, it can be seen that 13 (59·1%) of 22 isolates from pig sewage samples were found to harbour the haemolysin production plasmid. However, these 13 isolates were obtained from one sewage sample on a pig farm.

Transfer of citrate-utilizing ability

All 67 citrate-positive *E. coli* isolates were tested for donor ability of the citrate-utilizing (Cit) character in mating with *E. coli* K-12 strain. The numbers of isolates containing conjugative Cit characters are listed in Table 4. In each experiment, three Cit+ recipient colonies grown on Simmons' citrate agar plates were purified on non-selective media. The purified colonies were examined for the fermentation of raffinose and sucrose, for resistance to antibiotics, and for haemolysin and colicin production. All 24 isolates from humans, and four isolates from feral pigeons and crows had transmitted Cit character to ML1410 Nal^r with selection for the Cit marker, while five isolates from horses had not done so. Table 4 shows that all citrate-positive strains isolated from pig sewage, and one of two strains from crows, transferring Cit character are temperature-sensitive. Tests for resistance to antibiotics showed that R plasmids of the isolates from humans, sewage samples and crows were co-transferred with Cit character. No citrate-positive isolate possessing the ability to ferment raffinose or sucrose was demonstrated. Also, none of the transconjugant receiving Cit character by conjugation produced haemolysin or any colicin to which strain Row was sensitive.

The relationship between R plasmids and transmissible Cit character

Table 5 summarizes the relationship between drug resistance patterns of R plasmids and Cit character transferred in transconjugants selected for either drug resistance or Cit character. It can be seen that the segregation of resistance patterns of R plasmids and Cit character occurred in four recipient strains selected

Table 5. *The relationship between drug-resistance patterns of R plasmids and simultaneous transfer of Cit character in citrate-positive* E. coli *variants carrying conjugative R plasmids*

Host	No. of isolates tested	Resistance patterns of conjugative R plasmids	No. of R+ isolates	Characters of transconjugants selected for R plasmid	No. of isolates (ts)*	Characters of transconjugants selected for Cit character	No. of isolates (ts)
Humans	3	Tc, Km	2	Tc, Km, Cit+	2	Tc, Km, Cit+	2
		Su	1	{Su, Cit+ (2/3); Su (1/3)} †	1	Su, Cit+	1
Pigs	19	Tc, Sm, Su, Cm, Km	3	Tc, Sm, Su, Cm, Km, Cit+	2 (2)	Tc, Sm, Su, Cm, Km, Cit+	2 (2)
				{Tc, Sm, Su, Cm, Km (2/3); Tc, Km, Cit+ (1/3)}	1 (1)	Tc, Km, Cit+	1 (1)
		Tc, Sm, Su, Cm	3	Tc, Sm, Su, Cm, Cit+	2 (2)	Tc, Sm, Su, Cm, Cit+	1 (1)
						Tc, Sm, Su, Cit+	1 (1)
				{Tc, Sm, Cm, Cit+ (1/3); Tc, Su, Cit+ (2/3)}	1 (1)	Tc, Su, Cit+	1 (1)
		Tc, Sm, Km	1	{Tc, Cit+ (1/3); Tc, Sm, Km (2/3)}	1 (1)	Tc, Cit+	1 (1)
		Tc, Km	11	Tc, Km, Cit+	11 (11)	Tc, Km, Cit+	11 (11)
		Tc	1	Tc, Cit+	1 (1)	Tc, Cit+	1 (1)
Horse	1	Tc, Sm, Su, Cm, Ap	1	Tc, Sm, Su, Cm, Ap	1	Not obtained	
Crows	2	Tc, Sm, Su, Cm, Km	2	Tc, Sm, Su, Cm, Km, Cit+	2 (1)	Tc, Sm, Su, Cm, Km, Cit+	2 (1)

* ts, No. of isolates carrying plasmids showing thermosensitive transfer.

† In each experiment, three transconjugants were picked, diluted in 0·05 ml saline, and grown on non-selective media. The purified colonies were examined for characters.

Table 6. *Transfer frequency of R plasmids and Cit characters of citrate-positive* E. coli *isolates*

Donor	Source	Recipient	Selector*	Cultivation temperature (°C)	Transfer frequency†	Character of transconjugants
Hul99 (Cit$^+$, Col)	Human	ML1410	Sim (Met + Nal)	37	$1{\cdot}7 \times 10^{-4}$	20/20 Cit$^+$
Ps60 (Cit$^+$)	Horse	ML1410	Sim (Met + Nal)	37	$< 10^{-8}$	—
3E30 (Su, Cit$^+$)	Cattle	ML1410	Su + Nal	37	$< 10^{-8}$	—
			Sim (Met + Nal)		$1{\cdot}5 \times 10^{-6}$	20/20 Cit$^+$
B229 (Tc, Km, Cit$^+$, Hly)	Pig farm sewage sample	ML1410	Tc + Nal	25	$1{\cdot}8 \times 10^{-3}$	11/20 Tc, Km, Cit$^+$ 9/20 Tc, Km
			Sim (Met + Nal)		$8{\cdot}5 \times 10^{-3}$	20/20 Tc, Km, Cit$^+$
			Tc + Nal	37	$4{\cdot}0 \times 10^{-7}$	4/10 Tc, Km, Cit$^+$ 6/10 Tc, Km
			Sim (Met + Nal)		$1{\cdot}8 \times 10^{-7}$	4/8 Tc, Km, Cit$^+$ 4/8 Cit$^+$
D79 (Sm, Su, Cm, Cit$^+$)	Feral pigeon	ML1410	Sm + Nal	25	$< 10^{-7}$	—
			Sim (Met + Nal)		$6{\cdot}7 \times 10^{-7}$	2/2 Cit$^+$
			Sm + Nal	37	$< 10^{-8}$	—
			Sim (Met + Nal)		$1{\cdot}9 \times 10^{-5}$	20/20 Cit$^+$
755 (Tc, Sm, Su, Cm, Cit$^+$)	Crow	ML1410	Tc + Nal	25	$< 10^{-8}$	—
			Sim (Met + Nal)		$< 10^{-8}$	—
			Tc + Nal	37	$1{\cdot}2 \times 10^{-6}$	19/20 Tc, Sm, Su, Cm, Km, Cit$^+$ 1/20 Tc, Sm, Su, Cm, Km
			Sim (Met + Nal)		$7{\cdot}6 \times 10^{-7}$	4/4 Cit$^+$

Tc, tetracycline; Sm, streptomycin; Su, sulphonamide; Cm, chloramphenicol; Km, kanamycin.

* Sim (Met + Nal): Simmons citrate agar containing methionine (50 μg/ml) and nalidixic acid (50 μg/ml) was used as selective media for citrate character in each transfer experiment.

† Determined from 2 h mating at 25 and 37 °C, measured as transconjugants per donor.

for drug resistance, while the drug resistance patterns of R plasmid and Cit character were transferred jointly in all cases selected for Cit character. When selection was made for transfer of drug resistance of R plasmids, the segregation of their characters occurred obviously.

In order to determine what relationship there is between the Cit characters and R plasmids, transfer experiments with the representative isolates from various sources were carried out both at 25 and 37 °C in more detail. As shown in Table 6, Cit character was transmitted with considerable frequency from *E. coli* Hu199 to the recipient *E. coli* ML1410. The other five representative donor isolates from various sources except horses transferred Cit character at frequencies ranging from $< 10^{-8}$ to $> 10^{-3}$ per donor cell. Though Cit character and drug resistance were transferred jointly in the isolates (B229 and 755) from pig farm sewage or crow samples (Table 5), different segregation patterns on their characters of transconjugants were observed in further experiments as shown in Table 6. Many transconjugants obtained on Simmons' citrate agar plates carried only citrate-utilizing ability without drug resistance markers.

This indicates that Cit character in these citrate-positive isolates are plasmid-mediated and not located on the R plasmids. In *E. coli* B229, the Cit character was more efficiently transferred to *E. coli* ML1410 at 25 °C than at 37 °C, indicating that this Cit character is as thermosensitive as the R plasmid.

DISCUSSION

This investigation confirms the existence of citrate-positive variants of *E. coli* in human, domestic animal, feral bird and environmental samples. In a previous report (Ishiguro *et al.* 1978), 13 (36·1%) citrate-positive isolates from domestic pigeons and 12 (36·3%) citrate-positive isolates from pigs were obtained from one location, suggesting that these isolates were highly related and probably derived from a single ancestral organism. Moreover, since the citrate-utilizing abilities have been reported to be transmitted with considerable frequency from the parent isolates to *E. coli* K-12 (Sato *et al.* 1978), the citrate-utilizing abilities observed in the previous study may have been spread by plasmids conferring the citrate-utilizing ability. According to the present extensive survey, the incidence of citrate-positive *E. coli* variants is very low. Our isolation rate of citrate-positive *E. coli* variants from human sources corresponded to that described by Edwards & Ewing (1972). It is of interest that citrate-positive *E. coli* variants were isolated from crows and pig sewage samples with high frequency. Moreover, in one of seven human stool samples, 13 out of 15 colonies examined were positive on Simmons' citrate agar. These facts indicate that citrate-positive *E. coli* isolates carrying the Cit plasmid may be rather more dominant than those without the plasmid and that there is potential spread of citrate-utilizing ability inside the body. Since citrate-positive *E. coli* variants were isolated from pig sewage samples with high frequency, and since these isolates carried conjugative and thermosensitive citrate utilization plasmids as well as R plasmids, the transfer

of the citrate-utilizing ability from citrate-positive *E. coli* cells to citrate-negative cells may be enhanced at temperatures below 37 °C outside the body, for instance, in sewage, where transmissible substrate-utilizing ability has been described by Smith & Parsell (1975). No attempt to explain this phenomenon has yet been made.

With regard to other biochemical properties, the behaviour of 67 citrate-positive isolates from various sources was typical of *E. coli*, except in the arginine hihydrolase test. The transmission of ability to ferment raffinose or sucrose among *E. coli* strains has been demonstrated (Ørskov & Ørskov, 1973; Smith & Parsell, 1975). The analysis of transfer experiments with raffinose- or sucrose-positive isolates in this study showed that the raffinose or sucrose character was not carried by the same transfer factor possessing Cit character. Also, attempts to transfer the ability to ferment raffinose or sucrose into *E. coli* K-12 were unsuccessful. Furthermore, the production of haemolysin or colicin was not transmitted together with the Cit character in the haemolysin- or colicin-positive isolates used in this study.

It was most interesting that many of the citrate-positive *E. coli* isolates except those isolated from cattle were resistant to antibiotics and were found to carry conjugative R plasmids (Tables 3 and 4). The present studies have shown the intimate relation between Cit character and R plasmid. The Cit character and drug resistance markers of R plasmids were co-transmitted by conjugation in many transconjugants tested. But, since transconjugants carrying only the citrate-utilizing determinant were found during transfer experiments except in some of the human isolates (Table 6), it can be concluded that the Cit character, like those for an H_2S-positive *E. coli* strain, is located on a transmissible plasmid (Cit plasmid) which differs from R plasmid, as described by Sato *et al.* (1978). However, further studies on the association between Cit plasmid and R plasmid are needed.

Though no analysis of enterotoxin (Ent) plasmid, K88 and K99 plasmids in the 67 citrate-positive *E. coli* isolates was performed, it is clear that Cit plasmids are physically independent and conjugative plasmids. Therefore, this Cit character can now be added to the list of biochemical characters determined by transferable plasmids. No transfer of Cit character in the variants isolated from horses was demonstrated. Citrate-negative cells were obtained, however, from one of five isolates tested by treating with acridine orange (unpublished data). From these results, the existence of a non-conjugative plasmid conferring citrate-utilizing ability is also apparent.

The citrate reaction on Simmons' citrate agar is a very important character for identification in the family *Enterobacteriaceae*. The evidence in this study suggests that one more biochemical character used as a classification of *Enterobacteriaceae* can be spread by plasmids. A study of the genetic properties of Cit plasmid isolated from various sources is now in progress.

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