THE SEROLOGY OF *CITROBACTER KOSERI*, *LEVINEA MALONATICA*, AND *LEVINEA AMALONATICA*

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FREDERIKSEN (1970) described a collection of 30 strains belonging to the genus *Citrobacter*, but differing in several respects from *C. freundii*. Adonitol was fermented, malonate was utilised, indole was produced, and there was no growth in Moeller's potassium cyanide medium (KCN). Hydrogen sulphide (H_2S) production in ferric chloride gelatin was weak. Frederiksen considered that these strains should be regarded as a new species, and proposed the name *C. koseri*. Booth and McDonald (1971) examined 40 biochemically similar strains and proposed that they be regarded as a new species of *Citrobacter*.

Young *et al.* (1971) studied 108 strains and proposed the establishment of a new genus, *Levinea*, having two species, *L. malonatica* and *L. amalonatica*. The biochemical reactions described for *L. malonatica* were similar to those of *C. koseri*, but H_2S production was not detected in triple sugar iron agar (TSI agar). The reactions of *L. amalonatica* differed in that adonitol was not fermented, malonate was not utilised, and growth was always seen in KCN. Limited serological studies showed considerable antigenic sharing within the proposed species *L. malonatica*.

Gross, Rowe and Easton (1973) studied four cases of neonatal meningitis in a premature-baby unit; *C. koseri* was the causative organism in all, but serological studies showed that two distinct serotypes were involved.

We now report a biochemical and serological study of representative strains from all these authors, and a previously undescribed strain. An "O"-antigen scheme is proposed and its use is illustrated by the classification of 47 H_2S -negative strains of *Citrobacter* from clinical sources. A strain described by Ewing and Davis (1972) was also examined; this gave the biochemical reactions of *C. koseri*, but Ewing and Davis considered that the name *C. diversus* (Werkman and Gillen, 1932) was an earlier synonym and should have priority.

MATERIALS AND METHODS

Organisms. Twelve strains were subjected to detailed biochemical and serological examination. Strain no. NCTC10786 was in the National Collection of Type Cultures as a representative of *C. koseri* (Frederiksen, 1970); nos. NCTC 10768, 10769, 10770, and 10771 represented a proposed new *Citrobacter* sp. (Booth and McDonald, 1971); nos. NCTC 10805 and 10806 represented *L. amalonatica*, and NCTC 10810 and 10811 represented *L. malonatica* (Young *et al.*, 1971). Strains no. E1597/71 and E1599/71 were representatives of the two serotypes of *C. koseri* described by Gross *et al.* (1973) and E1558/72 was received at the Salmonella and Shigella Reference Laboratory, having been isolated from urine.

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TABLE I
 Biochemical reactions of 12 representative strains of *Citrobacter*

Test	Results of tests on strain no.											
	NCTC 10805	NCTC 10806	NCTC 10810	NCTC 10811	NCTC 10768	NCTC 10769	NCTC 10770	NCTC 10771	NCTC 10786	E1597/ 71	E1599/ 71	E1558/ 72
Motility (Craigie tube)	- ¹⁴	+	+	+	+	+	+	+	+	+	+	+
Methyl-red test: 37°C	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²	+ ²
20°C	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵	+ ⁵
Voges-Proskauer reaction: 37°C	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²
20°C	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵	- ⁵
Utilisation of: citrate (Simmons)	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
malonate	- ²	- ²	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
Growth in potassium cyanide	+ ¹	+ ¹	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²
Production of: Indole (Kovac's)	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
H ₂ S (TSI agar)	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷	- ⁷
β-galactosidase	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
Oxidation of gluconate	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²	- ²
Hydrolysis of urea (Christensen's)	+ ³	+ ⁷	+ ¹	+ ⁴	+ ⁷	+ ³	+ ¹	+ ³	+ ²	+ ³	+ ⁴	+ ¹
Deamination of phenylalanine	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹	- ¹
Liquefaction of gelatin	+ ¹⁴	+ ¹⁴	- ²⁸	- ²⁸	- ²⁸	- ²⁸	- ²⁸	- ²⁸	- ²⁸	- ²⁸	- ²⁸	- ²⁸
Decarboxylation of: arginine	+ ³	+ ²	+ ²	+ ²	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ³
lysine	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴	- ⁴
ornithine	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
Fermentation of: glucose; acid	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
glucose; gas	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
lactose	- ¹⁴	- ¹⁴	+ ⁷	+ ⁵	+ ⁷	+ ⁷	+ ⁷	+ ⁷	+ ⁷	+ ⁴	+ ⁷	- ¹⁴
mannitol	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
sucrose	- ¹⁴	- ¹⁴	- ¹⁴	+ ¹	+ ¹	- ¹⁴	- ¹⁴	+ ¹	+ ¹	+ ¹	- ¹⁴	+ ¹
salicin	+ ²	+ ⁵	+ ⁵	+ ⁵	+ ³	+ ²	+ ³	+ ³	+ ³	+ ³	+ ³	+ ⁷
dulcitol	- ¹⁴	- ¹⁴	- ¹⁴	+ ¹	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	+ ¹	+ ¹	+ ¹	- ¹⁴
inositol	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴
adonitol	- ¹⁴	- ¹⁴	+ ²	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
raffinose	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴
sorbitol	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
arabinose	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
rhamnose	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
xylose	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
trehalose	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
inulin	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴	- ¹⁴
glycerol	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
cellobiose	+ ²	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹
sorbose	+ ¹	+ ¹	+ ¹⁰	+ ⁵	+ ⁷	- ¹⁴	+ ⁷	+ ⁷	+ ³	+ ⁴	+ ⁷	+ ⁷
maltose	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹	+ ¹

+ = Test positive; - = test negative.
 The superscript numerals indicate the days of incubation.

Forty-seven strains isolated from clinical specimens were selected. Their biochemical reactions resembled those of *Citrobacter*, but H₂S production was not detected in TSI agar. They were re-examined biochemically and tested by the proposed "O"-antigen scheme. Strain no. NCTC10849, *C. diversus* of Ewing and Davis (1972), was similarly examined.

Biochemical tests were performed as described by Cowan and Steel (1965).

Preparation of antisera. O-antigen suspensions were prepared from the 12 strains by harvesting the growth from two nutrient agar slopes into 0.9% saline and heating to 100°C for 2½ hours. After centrifugation the heated organisms were resuspended in 15 ml of saline and commercial formalin was added to a final concentration of 0.3%. Rabbits were immunised by intravenous injections of 0.5 ml, 1.0 ml, 2.0 ml, 2.0 ml, and 2.0 ml of suspension at 5-day intervals; 40 ml of blood was taken 5 and 10 days after the final injection, and the rabbits were exsanguinated 5 days later. The products of all three bleedings were pooled.

O-antigen suspensions for agglutination tests were prepared by heating overnight broth-cultures at 100°C for 30 min. Commercial formalin was added to a final concentration of 0.3%.

Agglutination tests were performed in perspex agglutination trays, and were incubated at 50°C for 16 hours.

The antisera were tested against the O-antigen suspensions of all 12 strains, and their homologous and heterologous titres determined.

The 12 antisera were also tested against suspensions representing all the O-groups of *Salmonella* (1-65), all the O-groups of *E. coli* (1-157), and all serotypes of *Shigella*. O-antigen suspensions of the 12 strains were tested against antisera to all those antigens.

Absorption studies. Where cross-reactions were found among the 12 strains, mirror absorptions were performed to determine the relationships between the strains.

Examination of 47 wild strains and strain no. NCTC10849. O-antigen suspensions of all the strains were prepared and the agglutination titres of the 12 antisera against them were determined. Where necessary they were further tested with specific absorbed antisera.

RESULTS

Examination of 12 representative strains

Biochemistry. All the 12 strains were Gram-negative rods. They were oxidase negative, catalase positive, fermentative in the Hugh and Liefson test, and nitrate reducing. Their biochemical reactions are summarised in table I.

Ten strains formed a homogeneous group with biochemical reactions that conformed to Frederiksen's description of *C. koseri*, but strains no. NCTC 10805 and 10806 (*L. amalonatica*) differed in that they failed to ferment adonitol, and grew in KCN.

Antigenic relationships among the 12 representative strains. The serological cross-reactions within the group are summarised in table II. Absorption studies showed that the strains of *L. amalonatica* (nos. NCTC 10805 and 10806) had identical O antigens that were not related to the O antigens of the other strains studied. Strains no. NCTC 10810 and 10811 (*L. malonatica*) and NCTC10786 (*C. koseri*) had identical O antigens, and no. NCTC10786 was selected as a representative of this type. Its relationship with strains no. NCTC10768, E1597/71 and E1558/72 was studied by absorption techniques, and the results are shown in table III. Strain no. NCTC10771 showed numerous cross-reactions, and was considered to be degraded. The O antigens of nos. NCTC10769, NCTC10770, and E1599/71 were unrelated either to each other

TABLE II
Serological reactions of 12 representative strains of Citrobacter

Serum prepared against strain no.	Agglutination titre against suspension of indicated organism											
	NCTC 10805	NCTC 10806	NCTC 10810	NCTC 10811	NCTC 10786	NCTC 10768	E1558	E1597	NCTC 10769	NCTC 10770	NCTC 10771	E1599
NCTC10805	12800	12800	—	—	—	—	—	—	—	—	100	—
NCTC10806	25600	25600	—	—	—	—	—	—	—	—	800	—
NCTC10810	—	—	12800	6400	6400	400	6400	3200	—	—	12800	—
NCTC10811	—	—	6400	6400	6400	1600	6400	1600	—	—	6400	—
NCTC10786	—	—	25600	25600	12800	800	25600	6400	—	—	12800	—
NCTC10768	—	—	3200	1600	1600	25600	400	200	—	—	12800	—
E1558/72	—	—	12800	3200	3200	800	25600	6400	—	—	3200	—
E1597/71	—	—	400	200	200	—	—	800	—	—	3200	—
NCTC10769	—	—	—	—	—	—	—	—	25600	—	100	—
NCTC10770	—	—	—	—	—	—	—	—	—	12800	1600	—
NCTC10771	—	—	—	—	—	—	—	—	—	—	12800	—
E1599/71	—	—	—	—	—	—	—	—	—	—	1600	1600

— = > 100.

TABLE III
Serological relationship between strains no. NCTC10786, E1597/71, NCTC10768, and E1558/72

Serum		Agglutination titre against indicated serum of organism no.			
prepared against strain no.	absorbed with				
		NCTC10786	E1597/71	NCTC10768	E1558/72
NCTC10786	nil	6400	12800	800	25600
	E1597/71	400	—	—	—
	NCTC10768	800	3200	—	25600
	E1558/72	200	—	—	—
E1597/71	nil	100	1600	—	1600
	NCTC10786	—	400	—	—
	NCTC10768	—	200	—	—
	E1558/72	—	400	—	—
NCTC10768	nil	3200	800	25600	3200
	NCTC10786	—	—	3200	—
	E1597/71	—	—	3200	—
	E1558/72	—	—	3200	—
E1558/72	nil	3200	1600	1600	25600
	NCTC10786	—	—	—	12800
	E1597/71	100	—	—	3200
	NCTC10768	1600	1600	—	25600

— = < 100.

or to those of the interrelated group consisting of strains no. NCTC10786, NCTC10768, E1597/71, and E1558/72.

Antigenic relationships with other genera. Strains no. NCTC 10805 and 10806 (*L. amalonatica*) had significant reciprocal cross-reactions with *Escherichia coli* O137, and absorption studies showed that their O antigens were identical with each other and with the O antigen of *E. coli* O137; nos. NCTC 10805 and 10806 were also agglutinated by salmonella O65 antiserum, but reciprocal reaction was not observed.

The remaining 10 strains had no reciprocal cross-reactions with *E. coli*, *Shigella*, or *Salmonella* strains, but minor one-way cross-reactions occurred as follows: antiserum to strain no. E1599/71 agglutinated *E. coli* O29, that to

TABLE IV
Proposed O-antigen classification scheme

Specific O antigen	Type strain no.	Reference to type strain
O1	NCTC10786	<i>C. koseri</i> (Frederiksen, 1970)
O2	E1597/71	<i>C. koseri</i> (Gross, Rowe and Easton, 1973)
O3	NCTC10768	New <i>Citrobacter</i> sp. (Booth and McDonald, 1971)
O4	E1558/72	<i>C. koseri</i> (unpublished)
O5	NCTC10769	New <i>Citrobacter</i> sp. (Booth and McDonald, 1971)
O6	NCTC10770	New <i>Citrobacter</i> sp. (Booth and McDonald, 1971)
O7	E1599/71	<i>C. koseri</i> (Gross <i>et al.</i> , 1973)

Note: NCTC10849, *C. diversus* (Ewing and Davis, 1972) and NCTC 10810 and 10811, *L. malonatica* (Young *et al.*, 1971) also possess specific antigen O1.

strain no. E1597/71 agglutinated *E. coli* O79, that to no. NCTC10770 agglutinated *E. coli* O20 and O32, and that to no. NCTC10769 agglutinated *Sh. boydii* 7; E1597/71 was agglutinated by salmonella O1 antiserum.

Proposed O-antigen classification scheme

The scheme consists of seven specific antigenic types, and a type strain is proposed for each (table IV).

Application of the scheme to strains of Citrobacter. Forty-seven strains isolated from clinical specimens, and strain no. NCTC10849, the neotype strain of *C. diversus* (Ewing and Davis, 1971) were examined biochemically; 28 strains, including no. NCTC10849, were identified as *C. koseri*. They gave the biochemical reactions of the family Enterobacteriaceae and utilised Simmons' citrate, fermented adonitol, produced indole, and failed to grow in KCN—all tests considered important in the differentiation of *C. koseri* from other species. The remaining 20 strains failed to ferment adonitol and 19 of them grew in KCN.

Of the 28 strains identified biochemically as *C. koseri*, 22 were identified serologically according to the proposed scheme, three were rough, and three

were unidentifiable. Of the 22 strains identified serologically, seven possessed antigen O7, six O2, five O1, two O5, one O4, and one O6. *C. diversus*, no. NCTC10849, was found to possess antigen O1.

The 20 strains that failed to ferment adonitol could not be identified with the 12 antisera.

DISCUSSION

On the basis of the serological results an O-antigen scheme is proposed (table IV). The related strains no. NCTC10786, E1597/71, NCTC10768, and E1558/72 share common O-antigen components but can be distinguished by the use of absorbed antisera. Their specific O-antigen components are shown as O1, O2, O3, and O4 respectively. Those of the unrelated strains no. NCTC 10769, NCTC10770, and E1599/71 are proposed as O5, O6, and O7. Strain no. NCTC10771 is degraded, and is omitted from the scheme.

The strains described as *L. amalonatica* (Young *et al.*, 1971) do not belong to the species *C. koseri*. They grow in KCN and fail to ferment adonitol; they are not related serologically to the other strains studied, and show marked antigenic similarities with other genera. They are not included in the proposed antigenic scheme.

This scheme could be developed to form a more extensive serotyping scheme. Its application to 28 strains of *C. koseri*, mostly from clinical sources, illustrates its possible value in studies of the epidemiology of that organism.

SUMMARY

Various authors have described a group of organisms resembling *Citrobacter freundii*, but differing in several significant respects. It has been suggested that these organisms should be regarded as a new species, and the names *Citrobacter koseri*, *Levinea* sp. and *Citrobacter diversus* have been proposed for them. Representative strains described as *C. koseri*, *L. malonatica*, and *C. diversus* were shown to form a biochemically homogeneous group, and an antigenic-classification scheme is proposed for them. Strains of *L. amalonatica* differed biochemically and serologically from the other strains studied and are excluded from the antigenic scheme.

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