Polynucleotide Sequence Divergence in the Genus *Citrobacter*

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SUMMARY

Deoxyribonucleic acid reassociation was used to assess divergence in species of *Citrobacter* and *Citrobacter*-like strains. Typical strains of *Citrobacter freundii* were highly related. Antigenically atypical strains and most biochemically atypical strains showed a lower, but species level, of relatedness to typical *C. freundii*. Four additional relatedness groups were detected. One of these corresponded to *C. diversus*, including *Levinea malonatica*. A second contained *L. amalonatica*. A third group contained two indole⁺ strains which exhibited only 40 to 50 % relatedness to other groups of *Citrobacter*. The fourth group contained H₂S⁻, lysine⁺ organisms that are more closely related to *Serratia* than to *Citrobacter*.

INTRODUCTION

In a study on polynucleotide sequence relationships in the tribe Salmonelleae, our findings indicated that bacteria included in the genus *Citrobacter* have substantially diverged from other members of the tribe (Crosa, Brenner, Ewing & Falkow, 1973).

The genus *Citrobacter* is composed of strains that are, in general, biochemically similar. Until the past few years all strains, including slow lactose-fermenting Bethesda strains and various biotypes, were designated as *C. freundii*. Recently, several laboratories have described additional species of *Citrobacter* or proposed new genera for groups of bacteria previously considered as *C. freundii*.

Macierewicz (1966) proposed the genus *Padlewskia* for hydrogen sulphide negative, adonitol positive strains. Fredriksen (1970) described a similar set of strains and proposed the name *C. koseri*. Young, Kenton, Hobbs & Moody (1971) proposed a new genus, *Levinea*, with two species, *L. malonatica* and *L. amalonatica*. These strains also ferment adonitol and are hydrogen sulphide negative and indole positive. The two species differ in their ability to utilize malonate. Ewing and Davis (Davis & Ewing, 1966, 1971; Ewing & Davis, 1972) described a large number of strains that ferment adonitol, and are hydrogen sulphide and potassium cyanide negative and indole positive, naming them *Citrobacter diversus* (Davis & Ewing, 1971; Ewing & Davis, 1972). These authors think that *Padlewskia* and *C. koseri* are similar to *C. diversus*, and that *Levinea* species are KCN positive variants of *C. diversus*.

Another group of atypical 'Citrobacter-like' strains was reported by Leclerc & Buttiaux (1965). These bacteria were hydrogen sulphide negative and decarboxylated lysine. Data from Edwards & Ewing (1972), Ewing & Davis (1972) and Young *et al.* (1971) indicate that neither *Citrobacter* nor *Levinea* can decarboxylate lysine.

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In this paper we discuss results obtained from interspecific DNA reassociation tests on representatives of typical and atypical *C. freundii*, *C. diversus*, and *Levinea* species.

METHODS

Bacteria and media. The strains used (unless designated below) were obtained from W. H. Ewing and G. Hermann and had been characterized biochemically and antigenically at the Center for Disease Control, Atlanta, Georgia, U.S.A. *Enterobacter agglomerans*, *Erwinia dissolvens* and *E. amylovora* strains were from M. P. Starr (International Collection of Phytopathogenic Bacteria), and *Pectobacterium carotovorum* from the American Type Culture Collection. *Citrobacter* strains 1C, 184-6, H310, 517-60, P11C, Zurich and 3796 were obtained from S. Schaeffer, *Enterobacter cloacae* 165 from V. J. Brenner, strains of *Levinea* species including type strains 25408 (*L. malonatica*) and 25406 (*L. amalonatica*) from V. M. Young, and *Proteus mirabilis* 1 from S. Falkow. *Salmonella typhimurium* LT2 and *Shigella flexneri* 24570 have been described previously (Brenner *et al.* 1969*a*). Bacteria were maintained on meat extract agar slants and cultivated on brain heart infusion broth (Difco) at 37 °C. The medium employed for labelling the cells was as described by Crosa *et al.* (1973).

Preparation of DNA and DNA reassociation. Both unlabelled and ³²P-labelled DNA were prepared, purified and sheared as described previously (Crosa *et al.* 1973). DNA reassociation reactions were carried out to a *Cot* (concentration × time, parameters of DNA reassociation, Britten & Kohne, 1966) of about 100 for the unlabelled DNA in mixtures containing 0.1 µg of sheared, denatured ³²P-labelled DNA/ml from the reference strain and a 1500-fold excess of sheared, denatured, unlabelled bacterial DNA, in 0.28 M-phosphate buffer (PB: 0.14 M-Na₂HPO₄ + 0.14 M-NaH₂PO₄, pH 6.8). Incubations were carried out at either 60 or 75 °C. Estimation of DNA reassociated in the above conditions was made by the use of hydroxyapatite (HA) for the separation of single-stranded DNA from reassociated DNA in 0.14 M-PB + sodium dodecyl sulphate, by means of a batch procedure (Brenner, Fanning, Rake & Johnson, 1969*b*). The thermal stability of reassociated DNA was assessed by a stepwise elution at increasing temperatures, as described by Crosa *et al.* (1973).

In control experiments the reassociation of labelled DNA was 2.5 % or less.

Spectrophotometric determination of genome size. The molecular complexity of bacterial DNA was determined as previously described (Crosa *et al.* 1973).

RESULTS

DNA relatedness among citrobacteria was first assessed using labelled reference DNA from a typical *C. freundii* strain, 460-61 (Table 1). Typical strains of *C. freundii* are hydrogen sulphide positive, indole negative, potassium cyanide positive, and malonate negative. These reactions will henceforth be referred to by a + or - sign (e.g. + - + - indicates H₂S positive, indole negative, KCN positive, malonate negative). DNA reassociation reactions were carried out at 60 °C and, in many cases, at 75 °C. The stability of a given DNA duplex is assessed by determining its *T_{m(c)}*, i.e. that temperature at which 50 % of reassociated DNA is eluted from HA.

We assumed that each 1 % of unpaired bases in reassociated DNA causes a 1° decrease in *T_{m(c)}* (Laird, McConaughy & McCarthy, 1969). Therefore the $\Delta T_{m(c)}$ (*T_{m(c)}* homoduplex - *T_{m(c)}* heteroduplex) can be used as an index of base sequence divergence in related DNA.

In 60 °C reactions, four typical strains of *C. freundii* and seven typical or malonate positive

Table 1. *Relative relatedness of enterobacteria to the typical C. freundii strain 460-61*

Source of unlabelled DNA†	60 °C		75 °C	
	Binding (%)	$\Delta T_{m(e)}$ *	Binding (%)	$\Delta T_{m(e)}$ *
<i>C. freundii</i> 460-61 + - + -	100	—	100	—
<i>C. freundii</i> 3062-62 + - + -	96	0.7	93	1.9
<i>C. freundii</i> 4675-65 + - + -	96	0.9	—	—
<i>C. freundii</i> 5245-60 + - + -	92	0.5	—	—
Bethesda 1A + - + +	90	3.6	—	—
Bethesda 2A + - + +	95	0.4	85	0.7
Bethesda 3A + - + -	97	0.9	—	—
Bethesda 4A + - + +	94	0.9	—	—
Bethesda 7 + - + -	94	0.8	—	—
Bethesda 8A + - + +	95	3.3	83	4.0
Bethesda 9A + - + -	97	0.7	—	—
<i>C. freundii</i> 1C‡	83	5.8	—	—
<i>C. freundii</i> H310‡	76	9.4	52	8.5
<i>C. freundii</i> P11C‡	82	6.5	—	—
<i>C. freundii</i> 184-6‡	82	7.0	—	—
<i>C. freundii</i> 3796‡	81	6.5	—	—
<i>C. freundii</i> Zurich‡	83	6.4	—	—
<i>C. freundii</i> 5118-60 - - + -	97	0.8	—	—
<i>C. freundii</i> 2987-57 - - + -	85	4.8	75	4.8
<i>C. freundii</i> 6440-59 - - + +	93	0.9	—	—
<i>C. freundii</i> 876-58 - - + +	80	7.7	—	—
Bethesda 6 + - + +	86	6.5	64	5.2
Bethesda 29A + - + +	87	6.5	66	6.6
<i>C. diversus</i> 1066-70 - + - +	70	11.4	37	9.4
<i>C. diversus</i> 1381-70 - + - +	65	11.8	—	—
<i>C. diversus</i> 2292-70 - + - +	68	9.7	43	—
<i>C. diversus</i> 3613-63 - + - +	68	11.9	—	—
<i>L. malonatica</i> 25408 - + + +	64	12.0	—	—
<i>L. malonatica</i> 25409 - + + +	68	11.8	—	—
<i>L. malonatica</i> 25410 - + + +	63	11.6	—	—
<i>L. amalonatica</i> 25406 - + + -	66	12.3	32	9.0
<i>L. amalonatica</i> 25407 - + + -	62	12.8	—	—
<i>S. typhimurium</i> LT2	60	13.5	26	7.8
<i>S. marinus</i> 1112	60	13.7	—	—
<i>S. chameleon</i> 1110	61	13.7	—	—
Arizona 1	58	12.5	—	—
Arizona 62	55	14.0	—	—
Arizona 111	61	12.8	—	—
Arizona 118	56	12.6	—	—
<i>E. coli</i> K12	59	14.3	25	8.1
<i>E. coli</i> B	59	—	—	—
<i>S. flexneri</i> 24570	57	—	23	—
<i>E. aerogenes</i> 1627-66	47	14.3	17	—
<i>E. cloacae</i> 165	44	16.0	—	—
<i>E. cloacae</i> 1347-71	55	14.0	22	—
<i>E. dissolvens</i> ED114	44	14.6	18	—
<i>K. pneumoniae</i> 2	52	13.9	19	—
<i>E. agglomerans</i> 2553	30	17.8	—	—
<i>E. hafniae</i>	20	16.6	—	—
<i>S. marcescens</i> SM6-W2	34	15.5	—	—
<i>Edwardsiella tarda</i> 1126-64	25	17.5	—	—
<i>Erwinia amylovora</i> EA169	28	18.4	—	—
<i>Pectobacterium carotovorum</i> 495	30	17.2	—	—
<i>Proteus mirabilis</i> 1	11	20.1	—	—

* $T_{m(e)}$, thermal elution midpoint; that temperature at which 50 % of the DNA bound to HA at the 60 or 75 °C incubation temperature is eluted. $\Delta T_{m(e)}$ is the decrease in $T_{m(e)}$ between heterologous DNA reactions and the homologous *C. freundii* reaction.

† The plus and minus symbols represent positive or negative reactions for H₂S, indole, KCN and malonate, respectively (see text).

‡ These strains have atypical antigens.

Table 2. *Relative relatedness of Citrobacter strains to the atypical C. freundii strain 876-58*

Source of unlabelled DNA†	Binding at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C
<i>C. freundii</i> 876-58 -- + +	100	—
<i>C. freundii</i> 6440-59 -- + +	70	7.0
<i>C. freundii</i> 2987-57 -- + --	68	8.2
<i>C. freundii</i> 5118-60 -- + --	75	7.1
Bethesda 6 + + + +	77	6.2
Bethesda 29A + + + +	74	6.2
<i>C. freundii</i> 5245-60	72	7.1
<i>C. freundii</i> 460-61 + + + -	69	7.0
<i>C. freundii</i> 3062-62 + + + --	77	7.2
<i>C. freundii</i> 4675-65 + + + --	73	7.1
Bethesda 3A + + + -	71	6.6
'Citrobacter-like' 4552-71 -- + + lys ⁺	34	18.5
'Citrobacter-like' 4553-71 -- + + lys ⁺	31	17.6
'Citrobacter-like' 4554-71 -- + + lys ⁺	31	16.6
'Citrobacter-like' 4556-71 -- + + lys ⁺	34	17.8
'Citrobacter-like' 4557-71 -- + + lys ⁺	34	18.0
<i>C. diversus</i> 1066-70 - + - +	59	10.4
<i>C. diversus</i> 1381-70 - + - +	60	10.1
<i>C. diversus</i> 2292-70 - + - +	60	10.1
<i>C. diversus</i> 3613-63 - + - +	61	9.8
<i>L. malonatica</i> 25408 - + + +	58	9.9
<i>L. malonatica</i> 25409 - + + +	59	9.9
<i>L. malonatica</i> 25410 - + + +	55	9.8
<i>L. amaltonatica</i> 25405 - + + -	57	11.3
<i>L. amaltonatica</i> 25407 - + + -	56	11.4

* See Table 1 for a definition of $\Delta T_{m(e)}$.

† See Table 1 for plus and minus code.

Bethesda strains exhibited an average of 95 % relatedness to *C. freundii* 460-61. The related sequences exhibited only 0.5 to 3.6 % unpaired bases. At 75 °C, relatedness and thermal stability of related sequences remained high. Two other Bethesda strains (+ + + +) exhibited somewhat lower relatedness and duplex stability in reactions with *C. freundii* 460-61.

Citrobacter freundii which are atypical in that they possess *Salmonella* or *Escherichia coli* antigens showed some 80 % relatedness to *C. freundii* 460-61. In this case 6 to 9 % divergence is present in related sequences. Biochemically atypical *C. freundii* appeared to show two patterns of relatedness to 460-61. Two strains show some 95 % relatedness and less than 1 % divergence; five other strains exhibit 80 to 87 % relatedness with 5 to 8 % divergence. A 10 to 20 % reduction in relatedness occurs in reactions carried out at 75 °C. There is no observable correlation between the pattern of relatedness and the biochemical reactions in -- + - and -- + + strains.

Citrobacter diversus, *Levinea malonatica* and *L. amaltonatica* strains are 60 to 70 % related to *C. freundii* 460-61. Extensive (10 to 13 %) divergence is present in these heteroduplexes. Approximately half of these related sequences are sufficiently related to remain stable in 75 °C incubations.

C. freundii 460-61 is 45 to 60 % related to *Salmonella*, *Arizona*, *Shigella*, *E. coli*, *Klebsiella*, *Enterobacter cloacae*, *E. aerogenes* and *Erwinia dissolvens*. Divergence in these reactions is 12 to 16 %. Relatedness falls by 2.5- to 3-fold when these reactions are carried out at 75 °C. Relatedness between *C. freundii* 460-61 and representatives of *Enterobacter agglomerans*, *E. hafniae*, *Serratia marcescens*, *Erwinia amylovora*, *Pectobacterium carotovorum* and

Table 3. *Relative relatedness of Citrobacter strains to H₂S⁺, indole⁺, KCN⁺, malonate⁻ strains of C. freundii*

Source of unlabelled DNA†	<i>C. freundii</i> 3158-63		<i>C. freundii</i> 80-58		<i>C. freundii</i> 1636-61	
	Binding at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C	Binding at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C	Binding at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C
<i>C. freundii</i> 3158-63 + + + -	100	—	71	2.2	43	—
<i>C. freundii</i> 80-58 + + + -	87	2.7	100	—	48	13.6
<i>C. freundii</i> 1636-61 + + + -	55	12.8	46	—	100	—
<i>C. freundii</i> 1637-61 + + + -	56	11.9	41	—	98	0.0
<i>C. freundii</i> 3104-61 + + + -	78	8.0	64	7.2	76	3.0
<i>C. freundii</i> 2970-59 + + + -	73	7.8	54	8.8	48	—
<i>C. freundii</i> 2990-58 + + + -	89	0.0	73	2.5	48	—
<i>C. freundii</i> 892-61 + + + -	—	—	70	5.1	—	—
Bethesda 6 - - + +	86	3.1	77	1.7	48	—
Bethesda 29A - - + +	85	3.2	76	1.3	51	—
<i>C. freundii</i> 876-58 - - + +	82	8.2	69	6.0	51	—
<i>C. freundii</i> 6440-59 - - + +	76	7.5	62	—	52	—
<i>C. freundii</i> 2987-57 - - + -	67	8.9	58	9.1	46	—
<i>C. freundii</i> 5118-60 - - + -	84	8.1	69	8.1	50	—
<i>C. diversus</i> 1381-70 - - + +	62	11.6	50	11.7	53	—
<i>C. diversus</i> 3613-63 - - + +	—	—	50	—	—	—
<i>L. malonatica</i> 25409 - - + +	—	—	51	12.0	—	—
<i>L. amalonatica</i> 25406 - - + +	—	—	49	12.8	—	—
<i>L. amalonatica</i> 25407 - - + +	—	—	52	—	—	—
<i>C. freundii</i> 460-61 - - + -	78	7.3	65	7.4	49	—
<i>C. freundii</i> 3062-62 - - + -	80	7.8	67	7.0	51	—
<i>C. freundii</i> 4675-65 - - + -	77	9.0	66	7.3	44	—
<i>C. freundii</i> 5245-60 - - + -	69	7.2	65	—	52	—
Bethesda 1A - - + +	70	7.6	60	7.5	47	13.7
Bethesda 4A - - + +	68	7.5	61	6.9	50	—
<i>C. freundii</i> Zurich‡	83	6.0	70	5.6	50	—
<i>C. freundii</i> P11-C‡	—	—	81	0.9	—	—

* See Table 1 for a definition of $\Delta T_{m(e)}$.

† See Table 1 for plus and minus code.

‡ These strains have atypical antigens.

Edwardsiella tarda are 20 to 35 %, with 15 to 18 % divergence in related sequences. Minimal relatedness is present between *C. freundii* 460-61 and *Proteus mirabilis*.

Relatedness among atypical *C. freundii* was tested with reference DNA from the - - + + strain *C. freundii* 876-58 (Table 2). Both atypical and typical *C. freundii* exhibited some 70 to 75 % relatedness with *C. freundii* 876-58 in 60 °C reassociation reactions. Related sequences exhibited 6 to 8 % divergence. Based on their reactions with *C. freundii* 460-61 (Table 1), it was expected that the two - - + + strains, 876-58 and 6440-59, should be about 70 % related. Reciprocal reassociation reactions between strains 460-61 and 876-58 show similar thermal stability, but different levels of relatedness; 69 % of strain 876-58 DNA is related to 80 % of strain 460-61 DNA. Therefore, the genome of strain 876-58 is some 16 % larger than that of strain 460-61.

A group of H₂S⁻, lysine positive strains described as perhaps 'Citrobacter-like' by Leclerc & Buttiaux (1965) exhibited only 31 to 34 % relatedness (16.5 to 18.5 % divergence) to *C. freundii* 876-58.

Heterogeneity in atypical *C. freundii* was also assessed using reference DNAs from three + + + - strains (1636-61, 3158-63 and 80-58). These data, shown in Table 3, indicate extensive divergence among + + + - strains, as well as substantial differences in genome

Table 4. *Relatedness of Citrobacter strains to Levinea species*

Source of unlabelled DNA†	<i>L. malonatica</i> 25408				<i>L. amalonatica</i> 25406			
	Binding		Binding		Binding		Binding	
	at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C	at 75 °C (%)	$\Delta T_{m(e)}$ * at 75 °C	at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C	at 75 °C (%)	$\Delta T_{m(e)}$ * at 75 °C
<i>L. amalonatica</i> 25405 - + + +	56	11.9	35	8.0	100	0.1	100	0.1
<i>L. amalonatica</i> 25406 - + + +	56	11.9	34	8.7	100	—	100	—
<i>L. amalonatica</i> 25407 - + + +	66	11.3	42	6.7	94	0.1	93	0.0
<i>L. malonatica</i> 25408 - + + +	100	—	100	—	63	1.3	37	7.3
<i>L. malonatica</i> 25409 - + + -	88	0.9	86	0.7	58	12.1	33	7.8
<i>L. malonatica</i> 25410 - + + -	86	0.3	88	0.0	57	11.0	36	6.8
<i>C. diversus</i> 1066-70 - + - +	84	0.6	84	—	58	12.7	32	—
<i>C. diversus</i> 1381-70 - + - +	85	0.5	86	—	58	12.9	32	—
<i>C. diversus</i> 2292-70 - + - +	83	0.3	84	—	57	13.0	33	—
<i>C. diversus</i> 3613-63 - + - +	84	0.3	86	—	58	14.2	32	—
Bethesda 1A - + - +	51	12.2	26	8.5	54	14.0	25	8.6
Bethesda 3A - + - -	54	12.0	28	8.9	53	13.7	26	9.1
Bethesda 8A - + - +	58	13.8	28	9.5	56	14.2	24	10.0
<i>C. freundii</i> 517-60 ????	55	12.9	31	8.9	90	0.8	90	0.6
<i>C. freundii</i> 3796‡ - + - +	58	13.4	28	9.4	53	13.4	30	8.8
<i>C. freundii</i> 5118-60 - - + -	52	12.7	29	—	52	14.4	26	—
<i>C. freundii</i> 876-58 - - + +	52	12.9	31	—	51	13.2	26	—
<i>C. freundii</i> 460-61 - + - -	55	12.6	30	—	55	14.2	28	—
<i>C. freundii</i> 4675-65 - + - -	54	13.2	33	—	55	13.5	27	—
<i>C. freundii</i> 5245-60 - + - -	52	12.5	31	—	53	13.0	26	—

* See Table 1 for a definition of $\Delta T_{m(e)}$.

† See Table 1 for plus and minus code.

‡ These strains have atypical antigens.

size. Strain 1636-61 is highly related to two other - + + - strains (1637-61 and 3104-61), but exhibits only 54 to 55 % relatedness with other citrobacteria. Strains 3158-63 and 80-58 form highly stable duplexes. Since 87 % of strain 3158-63 is related to 71 % of strain 80-58 DNA, the 80-58 genome is about 23 % larger than that of 3158-63. This genome size difference apparently explains the consistently higher relatedness of strain 3158-63 to all citrobacteria tested, whereas the $\Delta T_{m(e)}$ values of heteroduplexes are similar whether the reference DNA is from strain 3158-63 or strain 80-58.

We next tested relatedness of *L. malonatica* and *L. amalonatica* to various groups of citrobacteria. Brenner, Fanning, Steigerwalt & Young (1972) reported that these two *Levinea* species were 55 to 65 % related, with 11 to 12 % divergence in related sequences. *Citrobacter diversus* strains are - + - + and *L. malonatica* strains are - + + +. The positive KCN reactions presented for *L. malonatica* are from the data of Young *et al.* (1971). These were reported as delayed reactions. The data of Ewing & Davis (1972) indicate that *C. diversus* (as well as strains of *L. malonatica* from Young's Laboratory) are KCN negative after 48 h. These bacteria, therefore, may well be identical in this respect and we thank Dr Ewing for bringing this information to our attention. *Citrobacter diversus* and *L. malonatica* are extremely closely related (Table 4). *Levinea amalonatica* is closely related to the atypical *C. freundii* strain 517-60. DNA from 517-60 was prepared some years ago. Lyophiles of this strain were not viable when the present study was initiated, therefore this culture was not available for biochemical characterization. Both *Levinea* species exhibit approximately 55 % relatedness to other groups of biochemically atypical citrobacteria and to typical *C. freundii*. The amount of divergence in these reactions is 12 to 14 %. In 75 °C reactions, relatedness falls approximately twofold with a concomitant increase in heteroduplex thermal stability.

Table 5. *Relative relatedness of enterobacteria to lysine positive 'Citrobacter-like' strain 4556-71*

Source of unlabelled DNA†	Binding at 60 °C (%)	$\Delta T_{m(e)}$ * at 60 °C
'Citrobacter-like' 4556-71 - - + + lys ⁺	100	—
'Citrobacter-like' 4552-71 - - + + lys ⁺	96	0.8
'Citrobacter-like' 4553-71 - - + + lys ⁺	94	1.3
'Citrobacter-like' 4554-71 - - + + lys ⁺	93	1.2
'Citrobacter-like' 4557-71 - - + + lys ⁺	94	1.5
<i>C. freundii</i> 5245-60 + - + -	28	17.3
<i>C. freundii</i> 460-61 + - + -	25	16.6
<i>C. freundii</i> 3062-62 + - + -	28	17.7
<i>C. freundii</i> 4675-65 + - + -	30	17.7
Bethesda 3A + - + -	28	15.1
<i>C. freundii</i> 876-58 - - + +	27	18.1
<i>C. freundii</i> 6440-59 - - + +	28	17.6
<i>C. freundii</i> 2987-57 - - + -	25	18.3
<i>C. freundii</i> 5118-60 - - + -	28	17.5
Bethesda 6 + - + +	27	16.5
Bethesda 29A + - + +	26	15.1
<i>C. diversus</i> 1066-70 - + - +	30	17.6
<i>C. diversus</i> 1381-70 - + - +	28	16.7
<i>C. diversus</i> 2292-70 - + - +	28	17.1
<i>C. diversus</i> 3613-63 - + - +	28	17.0
<i>L. malonatica</i> 25408 - + + +	26	14.5
<i>L. malonatica</i> 25409 - + + +	28	14.6
<i>L. malonatica</i> 25410 - + + +	27	15.1
<i>L. amalonatica</i> 25405 - + + -	27	16.9
<i>L. amalonatica</i> 25407 - + + -	28	14.9
<i>E. coli</i> K12	25	14.7
<i>S. typhimurium</i> LT2	22	15.0
<i>K. pneumoniae</i> 2	31	—
<i>E. aerogenes</i> 1627-66	28	—
<i>E. cloacae</i> 1347-71	28	—
<i>E. agglomerans</i> EH103	25	—
<i>E. hafniae</i>	27	—
<i>Edwardsiella tarda</i> 1795-62	23	—
<i>P. carotovorum</i> 495	24	—
<i>E. amylovora</i> EA178	22	—
<i>S. marcescens</i> SM6-W2	47	—

* See Table 1 for a definition of $\Delta T_{m(e)}$.

† See Table 1 for plus and minus code.

Table 5 shows data obtained with reference DNA from a lysine⁺ 'Citrobacter-like' strain. The five lysine⁺ strains are extremely closely related. These strains are only distantly related to any group of Citrobacter strains. The only enterobacterium to which they exhibit greater than 30 % relatedness is *S. marcescens*.

Reciprocal DNA reassociation reactions at 60 °C are shown in Table 6. These data clearly demonstrate non-reciprocal levels of reassociation in almost all cases. The non-reciprocity reflects differences in the genome size of these organisms. For instance, the larger values obtained using labelled DNA from *C. freundii* 460-61 indicate that this organism has a smaller genome than *C. freundii* 876-58, *Levinea* species or *Salmonella typhimurium*.

Relative genome sizes calculated from reciprocal reassociation data are shown in Table 7. Also shown in Table 7 are relative genome sizes obtained spectrophotometrically.

Table 6. *Reciprocal DNA reactions*

Source of labelled DNA	Source of unlabelled DNA	Relative reassociation at 60 °C
<i>C. freundii</i> 460-61	<i>C. freundii</i> 876-58	80
<i>C. freundii</i> 876-58	<i>C. freundii</i> 460-61	69
<i>C. freundii</i> 460-61	<i>L. malonatica</i> 25408	64
<i>L. malonatica</i> 25408	<i>C. freundii</i> 460-61	55
<i>C. freundii</i> 460-61	<i>L. amalonatica</i> 25406	66
<i>L. amalonatica</i> 26406	<i>C. freundii</i> 460-61	55
<i>C. freundii</i> 460-61	<i>S. typhimurium</i> LT2	60
<i>S. typhimurium</i> LT2*	<i>C. freundii</i> 460-61	50
<i>C. freundii</i> 3158-63	<i>C. freundii</i> 1636-61	55
<i>C. freundii</i> 1636-61	<i>C. freundii</i> 3158-63	43
<i>C. freundii</i> 3158-63	<i>C. freundii</i> 80-58	87
<i>C. freundii</i> 80-58	<i>C. freundii</i> 3158-63	71
<i>C. freundii</i> 1636-61	<i>C. freundii</i> 80-58	48
<i>C. freundii</i> 80-58	<i>C. freundii</i> 1636-61	46

* Data from Crosa, Brenner, Ewing & Falkow (1973).

Each reaction is normalized to that of the respective homologous reassociation.

Table 7. *Determination of genome size relative to that of C. freundii 460-61*

Organism	Relative genome size from spectro-photometric determination	Relative genome size from reciprocal hybridization reactions
<i>C. freundii</i> 460-61	100	100
<i>C. freundii</i> 5118-60	112	—
<i>C. freundii</i> 4675-65	126	—
<i>C. freundii</i> 876-58	114	116
Bethesda 8A	106	—
Bethesda 29A	113	—
<i>C. diversus</i> 1066-70	123	—
<i>L. malonatica</i> 25408	121	118
<i>L. amalonatica</i> 25406	—	120
<i>S. typhimurium</i> LT2	116	120
<i>E. coli</i> K12	108	—

The estimation of the genome size spectrophotometrically was accomplished by a modification of the method of Gillis, De Ley & De Cleene (1970). Sheared DNA at an absorbance at 260 nm of 2.000 ± 0.010 in $2 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M-NaCl} + 0.015 \text{ M-sodium citrate}$) was denatured by heating in a boiling water bath for 5 min. The DNA was then transferred to 10 mm cuvettes in a 70 °C chamber of a Gilford 2000 Spectrophotometer set at a ΔE of 0.25 in the vicinity of 2. The absorbancy of each sample and the temperature were recorded automatically at 40 s intervals on a Minneapolis Honeywell recorder for 60 min. The decrease in absorbancy during 30 min, beginning 10 min after initiation of the renaturation process, was employed in the calculation of the molecular complexity of the DNA as described by Crosa *et al.* (1973). Each reaction was repeated five or more times.

Data obtained by these two methods are similar, and indicate that the extremes of genome size in citrobacteria vary by at least 25 %.

Citrobacteria form several relatedness groups. Typical *C. freundii* exhibit 40 to 60 % relatedness to most other enterobacteria. These data are perhaps best demonstrated by plotting the distribution of relatedness of groups of organisms against a reference strain. Figure 1 shows relatedness at 60 °C of enterobacteria to *C. freundii* 460-61. The mode for typical strains of *C. freundii*, including most strains of Bethesda, is 91 to 95 %. Biochemically

Fig. 1. Distribution of relatedness of strains to *Citrobacter freundii* 460-61. Values shown were obtained from 60 °C reassociation reactions in which *C. freundii* 460-61 was the reference strain.

and antigenically atypical *C. freundii* strains are distributed around a mode of 81 to 85 %. There is some overlap between the atypical and typical strains. Relatedness of both *C. diversus* and Levinea species is distributed between 61 and 70 %. Relatedness of *C. freundii* to Salmonelleae is no higher than relatedness of *C. freundii* to Escherichieae.

DISCUSSION

Polynucleotide sequence relatedness studies at both optimal (60 °C) and stringent (75 °C) DNA reassociation criteria were carried out to determine the extent of divergence in bacteria biochemically and/or immunologically related to *C. freundii*. Although *Citrobacter*, *Levinea* and Bethesda (with the exception of the H_2S^- , lysine⁺ 'Citrobacter-like' strains) all exhibit about 50 % relatedness to Salmonelleae (Crosa *et al.* 1973), these bacteria are not a homogeneous group. The diversity exhibited among members of the genus *Citrobacter* is not surprising in view of their antigenic variability (Davis & Ewing, 1966, 1971; Ewing & Davis, 1972), and the differences in fatty acid composition (Machtiger & O'Leary, 1973).

In agreement with the conclusions reached by several investigators (see Edwards & Ewing, 1972), we find no reason to distinguish the Bethesda group from *C. freundii* solely on the basis of slow lactose fermentation.

Biochemically typical (+ - + -) *C. freundii* (and Bethesda) belong to one relatedness group. Two H_2S^- strains, 5118-60 (- - + -) and 6440-59 (- - + +) are included in this group. The level of relatedness among these organisms is approximately 95 %, with an average of less than 1.5 % divergence within the related sequences. This relatedness group may be broadened to include most of the antigenically and biochemically atypical citrobacteria. *Citrobacter freundii* 460-61 exhibits an average of 80 % relatedness to these strains.

The biochemically atypical strains exhibit considerable heterogeneity. *Citrobacter freundii* 876-58, a - - + + strain, shows similar relatedness to typical + - + - strains, to atypical

— — + +, — — + —, — + + + (Table 2), and to + + + — strains (Table 3). In all cases, the level of relatedness is 70 to 80 %, with 6 to 8 % divergence. Further evidence for heterogeneity among biochemically atypical strains is found among + + + — strains (Table 3). These bacteria can be divided into three groups based on reactions with + + + — reference strains 3158-63 and 80-58. Strains 3158-63, 80-58, 2990-58, Bethesda 6 and Bethesda 29A are most highly related, based both on percentage relatedness and on heteroduplex stability. Relatedness of + + + — strains 3104-61 and 3970-59 to + + + — reference strains 3158-63 and 80-58 is no higher than that of other atypical or typical strains. In general, relatedness is higher with strain 3158-63 than with strain 80-58. This is presumably due to the larger genome size of 80-58.

The + + + — strains 1636-61 and 1637-61 form a separate relatedness group. These strains are essentially identical and exhibit only some 50 % relatedness to all other strains of *C. freundii*. Strain 3104-61 appears to be a link between these two strains and other *C. freundii* (Table 3).

Levinea malonatica (— + + +) and *C. diversus* (— + — +) comprise one relatedness group. These organisms are 50 to 60 % related to other citrobacteria. *Levinea amalonatica* strains (— + + —) are significantly different from *L. malonatica*, although utilization of malonate seems to be the only biochemical characteristic in which they differ (Young *et al.* 1971).

H₂S⁺, lysine⁺ 'Citrobacter-like' strains form a very closely related group. These bacteria are not closely related to any member of the genus *Citrobacter*. Of the bacteria tested they are most highly related to *S. marcescens*, although the level of relatedness is certainly less than that present among strains of *S. marcescens*.

As shown in Tables 5 and 6, genome sizes in the genus *Citrobacter* can vary by at least as much as 25 %. Therefore it is possible that the heterogeneity observed among strains of *C. freundii* is the result of genome size differences. For example, suppose that *C. freundii* strains A and B contain identical sequences of 2×10^9 daltons, and that the genome in strain A is 2×10^9 daltons compared with 3×10^9 daltons in strain B. Labelled DNA from strain A will exhibit 100 % relatedness to DNA from strain B; however, labelled DNA from strain B will exhibit only 67 % relatedness to DNA from strain A. In this case the thermal stability of A/B and B/A reactions will be identical and identical to the thermal stability of A/A and B/B reactions. In the *Citrobacter* reactions reported here, a lower level of relatedness is accompanied by a decrease in thermal stability. We therefore feel certain that the heterogeneity observed among atypical strains of *C. freundii* (except where specifically correlated with genome size differences) is not simply the result of differences in genome size.

Differences in H₂S, indole, KCN and malonate reactions were used by Ewing (Davis & Ewing, 1966, 1971; Ewing & Davis, 1972; Edwards & Ewing, 1972) as the basis for differentiating between *C. diversus* and *C. freundii* and for the formation of biogroups in *C. freundii*. In some hybridization groups there is a good correlation between DNA relatedness and the biochemical reactions for H₂S, indole, KCN and malonate. In atypical *C. freundii*, differences in these biochemical reactions cannot account for the large differences in relatedness, nor can they account for the relatedness differences present between *C. diversus* and *C. freundii*. Other than malonate utilization there is at present no biochemical basis for distinguishing *L. malonatica* from *L. amalonatica*. Both Ewing (Davis & Ewing, 1966, 1971; Ewing & Davis, 1972; Edwards & Ewing, 1972) and Bascomb, Lapage, Curtis & Willcox (1973) have examined a great many additional biochemical characters in *Citrobacter*. They found some 6 to 8 additional biochemical differences

between *C. freundii* and *C. diversus* (and *Levinea* species), but these differences are not present in all strains of one group and absent in all strains of the other group. Furthermore, the inclusion of these differences cannot account for more than a small portion of the relatedness differences.

Bascomb *et al.* (1973) were successful in identifying 93 % of 61 *C. freundii* biotype 1 strains and 95 % of 20 *C. freundii* biotype 2 strains using a numerical taxonomy program. They arbitrarily placed all *C. freundii* strains in biotype 1 and all *Levinea* (including *C. diversus*), *Padlewskia* and *C. koseri* strains in biotype 2. The amount of heterogeneity present between strains of both biotypes is emphasized by the presence of 20 different biopatterns in the 20 biotype 2 strains and 43 biopatterns in the 61 biotype 1 strains examined by Bascomb *et al.* (1973).

Another approach that is potentially useful in determining the extent of divergence among citrobacteria is that of gene transfer and recombination (de Graff & Stouthamer, 1971; 1972; de Graaff, Barendsen & Stouthamer, 1973; de Graaff, Kreuning & van de Putte, 1973; de Graaff, Kreuning & Stouthamer, 1974). The rationale for these studies is that the efficiency of gene transfer and recombination is highest between closely related strains and falls off rapidly as relatedness between strains decreases.

With respect to speciation in the genus *Citrobacter*, we feel the following scheme is appropriate (for the + - + - code, see p. 272): (i) *C. freundii* is comprised of typical + - + - strains, as well as atypical strains of biotype - - + -, - - + +, and + + + - (unless otherwise specified). (ii) The + + + - strains 1636-61 and 1637-61 require further study both biochemically and by hybridization, before a decision is made as to whether they should be placed in a species separate from *C. freundii*. (iii) The - + - + strains (*C. diversus* and *L. malonatica*) belong to one species separate from *C. freundii*. *Citrobacter diversus* is the prior designation; therefore this species should include *L. malonatica* strains. (iv) The - + + - strains belong to a species distinct from *C. freundii* and *C. diversus*. *Levinea amaltonatica* is a properly published name with priority. It seems, however, that this species should also be included in *Citrobacter*.

H₂S⁻, lysine⁺ strains are not members of the genus *Citrobacter*. They show relatedness indicative of the genus level to *S. marcescens*. Further study is necessary before these organisms are speciated.

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