Deoxyribonucleic Acid Relatedness Between *Enterobacter cloacae* and *Enterobacter amnigenus* sp. nov.

D. IZARD, F. GAVINI, P. A. TRINEL, AND H. LECLERC

Institut Pasteur de Lille, Unité INSERM 146, Université de Lille II, Domaine du C.E.R.T.I.A., 59651 Villeneuve d'Ascq Cedex, France

A deoxyribonucleic acid (DNA)-DNA hybridization study was carried out to determine the taxonomic position of a new group of enterobacters (group H₃, Gavini et al. [Ann. Microbiol. (Inst. Pasteur) 127B:317-335, 1976]) previously studied by numerical taxonomy. The reassociation binding ratios with the group H₃ strain ATCC 33072 ranged from 61 to 97%. With respect to DNA hybridization, this group, which represents a new species in the genus *Enterobacter*, *Enterobacter amnigenus*, is closely related (49 to 66%) to *Enterobacter cloacae*. A difference in genome size between *E. amnigenus* ATCC 33072 and *E. cloacae* Collection de l'Unité d'Écotoxicologie Microbienne (CUETM) 77-120 was observed after reciprocal binding: the genome of ATCC 33072 was 24% larger. *E. amnigenus* was defined from phenotypic and genetic data. The type strain of *E. amnigenus* is CUETM 77-118 (ATCC 33072).

In a previous numerical taxonomy study of the genus *Enterobacter*, Gavini et al. (11) showed clearly the existence of three new groups (H₁, H₂, and H₃) phenotypically related to *Enterobacter cloacae*. The purpose of this study is to define the taxonomic position of group H₃ by deoxyribonucleic acid (DNA)-DNA hybridization.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were previously listed in detail (9, 16). All of the Collection de l'Unité d'Écotoxicologie Microbienne (CUETM) strains have been studied by Gavini et al. (10-13) with a numerical taxonomy procedure.

Methods. DNA extraction was performed by Marmur's method (20) as slightly modified by Ferragut and Leclerc (8). The purity and quality of each DNA preparation was checked by determination of the absorbancy ratio at 260 nm/280 nm (1.8 to 1.9) and 260 nm/230 nm (2 to 2.3) as described by Marmur (20) and by determination of melting point (T_m) curves, which include hyperchromicity values from 30 to 40% (19).

The strains chosen for DNA labeling were the centrostrain (the strain most phenotypically similar to the others in a taxon) of group H₃, CUETM 77-118 (Gavini et al. [11]), and the centrostrain of group G (*Enterobacter cloacae*; Gavini et al. [11]), CUETM 77-120. For the preparation of labeled DNA, 1 mCi of [³H]thymidine (specific activity, 10 to 25 Ci/mmol) was added to 500 ml of a synthetic medium containing 4 g of glucose, 4 g of Casamino Acids, and 250 µg of deoxyadenosine per liter to inhibit the activity of thymidylate synthetase. The cells were harvested in the late-log phase. The specific activity was 80,000 cpm/µg for ³H-labeled DNA. The unlabeled DNA of high molecular weight was denatured and fixed on nitrocellulose filters by the technique described by De Ley and Tijtgaat (6). Fragmentation of unlabeled and

labeled DNA was carried out in a French pressure cell at a pressure of 20,000 lb/in². DNA reassociation was done by competition reaction under the conditions employed by De Ley and Tijtgaat and described by Legault-Demare et al. (18). The optimal temperature of renaturation ($T_{OR,D}$) was calculated from the equation of De Ley and Tijtgaat: $T_{OR,D} = 0.51 \text{ mol\% of guanine plus cytosine} + 28.0$. The guanine plus cytosine content of labeled DNAs equaled 54.3 mol% for strain CUETM 77-118 (15) and 55.7 mol% for strain CUETM 77-120 (15). Consequently, the optimal temperatures of incubation were 55.7 and 56.4°C, respectively. The time of incubation was 16 h. Radioactivity counting was done by scintillation counting, using 10 ml of Bray's solution (1) in vials.

The rate of reassociation was calculated by the formula: counts per minute without competitor DNA – counts per minute with heterologous DNA/counts per minute without competitor DNA – counts per minute with homologous DNA × 100.

RESULTS

The phenotype data of Gavini et al. (11) on the group H₃ strains are summarized in Table 1.

The reassociation data obtained with labeled DNA of group H₃ strain CUETM 77-118 are presented in Table 2. The DNA relatedness of strain CUETM 77-118 to 18 other isolates of the same group ranged from 97 to 61%. The reference strain of group H₃ shared 49 to 66% of its genome with *E. cloacae* and 52 to 65% with the strains of group E (*E. cloacae* acetoin-negative strains; Gavini et al. [13]). These ranges of relative binding ratios with *E. cloacae* (acetoin-positive or -negative strains) showed a genetic relationship between group H₃ and the genus *Enterobacter*. Strain 77-118 exhibited 46 to 54% relatedness with the genus *Levinea* and 46 to

TABLE 1. *Phenotype characterization of group H₃ strains^a*

Characteristic	Group H ₃ (N = 28) ^b		Strain CUETM 77.118 (ATCC 33072) reaction
	Reaction ^c	+ (+) Strains (%)	
Growth at temp (°C):			
4	+ ₄	96	+ ₄
41	—	0	—
44.5	—	0	—
Motility at temp (°C):			
30	+	100	+
37	+ or —	89.3	+
KCN	+ or —	53	—
Lysine decarboxylase	—	0	—
Arginine dihydrolase	+	96	+
Ornithine decarboxylase	+	100	+
Gelatin liquefaction (20°C)	—	0	—
Urease	+ or —	50	+ ₄
Indole	—	0	—
Methyl red test (30°C)	—	0	—
Methyl red test (37°C)	—	0	—
Voges-Proskauer reaction (30°C)	+	100	+
Voges-Proskauer reaction (37°C)	+ or —	89.3	+
Citrate (Simmons) (30°C)	+	100	+
Citrate (Simmons) (37°C)	+	100	+
Malonate	+	100	+
D-Tartrate ^d	—	0	—
L-Tartrate ^d	—	0	—
m-Tartrate ^d	—	0	—
Mucate ^d	+	100	+
Jordan tartrate	—	0	—
Phenylalanine deaminase	—	0	—
Nitrate to nitrite	+	100	+
H ₂ S production	—	0	—
Tetrathionate reductase	—	0	—
Tween esterase	—	0	—
Deoxyribonuclease	—	0	—
Chitinolysis	—	0	—
β-Galactosidase	+	100	+
β-Xylosidase	+	100	+
Fermentation of:			
L-Arabinose	+	100	+
D-Xylose	+	96	+
D-Ribose	+	100	+
Maltose	+	100	+
D-Glucose (gas)	+	100	+
D-Lactose	(+) or +	21 (78.6)	+ ₅
D-Galactose	+	100	+
L-Rhamnose	+	96	+
D-Mannose	+	100	+
D-Fructose	+	100	+
α-Methyl D-glucoside	(+) or +	35	+ ₄
D-Trehalose	+	100	+
Raffinose	+	96	+
L-Sorbose	—	0	—
Melibiose	+	100	+
D-Melezitose	—	0	—
D-Sucrose	+ or —	64	+
D-Mannitol	+	100	+
D-Dulcitol	—	3	—
meso-Inositol	—	0	—

TABLE 1.—Continued

Characteristic	Group H ₃ (N = 28) ^b		Strain CUETM 77.118 (ATCC 33072) reaction
	Reaction ^c	+ (+) Strains (%)	
D-Cellobiose (acid)	+	100	+
D-Cellobiose (gas)	+	100	+
D-Glycerol (gas)	—	0	—
D-Sorbitol	— or +	46	—
Adonitol	—	0	—
meso-Erythritol	—	0	—
Salicin	+ or —	89	+
Inulin	—	0	—
Glycogen	— or +	28	—
Esculin hydrolysis	+	100	+

^a Gavini et al. (11) and unpublished data.^b N, Number of strains tested.^c Numbers after symbols indicate number of days needed for reaction to take place. +, Positive reaction for at least 90% of the strains within 24 to 48 h; (+), slow positive reaction (i.e., 2 to 30 days); —, negative reaction for at least 90% of the strains.^d Kauffmann's method.

51% relatedness with group L of Gavini et al. (12), which is phenotypically related to the genus *Klebsiella*.

The DNA relatedness values between the reference strain of group H₃ and the cultures belonging to the different species of *Enterobacteriaceae* and to the new groups of Gavini et al. (groups D and F related to the genus *Citrobacter* [7, 9, 13]; groups H₁ and H₂ related to the genus *Enterobacter* [11, 15–17]; and groups K and L related to the genus *Klebsiella* [12]) are also listed in Table 2. The results are given in descending order for each species or group. For all of these taxa (except *E. cloacae*, the genus *Levinea*, and group L) the relatedness results were in the range of 8 to 46%.

The reassociation data obtained with labeled DNA of *E. cloacae* CUETM 77-120 are given in Table 3. *E. cloacae* CUETM 77-120 was selected as the donor of radioactively labeled DNA, for it is the centrostrain of group G (*E. cloacae*; Gavini et al. [11]). As previously shown by Steigerwalt et al. (22), strains of *E. cloacae* show only 62 to 97% interrelatedness. Our study confirmed the existence of a distinct group of acetoin-negative strains of *E. cloacae* (group E of Gavini et al. [13]).

We next tested the relatedness of *E. cloacae* and group H₃. Reciprocal binding values between strains CUETM 77-118 and CUETM 77-120 were used to approximate their relative genome size. These results showed that the values obtained in the two series of reactions were not identical (60 and 46%). The genome of *E. cloacae* CUETM 77-120 was 24% larger than that of CUETM 77-118. Such differences in genome size in the family *Enterobacteriaceae* have been

mentioned previously by different authors (Brenner et al. [2, 3]; Gillis et al. [14]; Crosa et al. [4, 5]; and Steigerwalt et al. [22]).

DISCUSSION

The DNA-DNA hybridization results obtained in this study correlate well with the results obtained for the same strains by numerical taxonomy (11). Both types of data confirm that *E. cloacae* and group H₃, although closely related, are distinct both phenotypically and genetically (Fig. 1). The characteristics used in differentiating *E. cloacae* from group H₃ are listed in Table 4. We therefore treat group H₃ as a new species, *Enterobacter amnigenus* (L. adj. *amnigenus*: coming from water), because all of the strains of this taxon have been isolated from well waters and springs.

E. amnigenus sp. nov. The following description of *E. amnigenus* is based on the data obtained by Gavini et al. (11) and Izard et al. (15) from studies of 28 strains.

Gram-negative, peritrichous, nonsporeforming, straight rods (0.5 by 30 µm) resembling those of the other members of *Enterobacteriaceae*. Never encapsulated. Growth on nutrient agar occurs at 30 and 37°C but not at 41°C.

The following biochemical reactions are positive: acetoin production, citrate (Simmons), mucate and malonate utilization, reduction of nitrates to nitrites, ornithine decarboxylase and arginine dihydrolase production, production of β-galactosidase and β-xylosidase, and fermentation of the following carbohydrates within 24 to 48 h (for 80 to 100% of the strains): glucose (with gas production), arabinose, xylose, ribose, mal-

TABLE 2. Relative reassociation of labeled DNA from strain CUETM 77-118 (centrostrain of *H*₃, 28 strains) with DNA from members of the family Enterobacteriaceae^a

Source of unlabeled DNA	Relative binding at <i>T</i> _{OR,D} (55.7°C) (%)	Source of unlabeled DNA	Relative binding at <i>T</i> _{OR,D} (55.7°C) (%)
Group H ₃		ATCC 6750	36
CUETM 77-118	100	ATCC 8090	32
CUETM 78-65	97	" <i>Salmonella houtenae</i> " CRR 498	40
CUETM 78-78	96	<i>Salmonella arizonae</i> CDC 62	31
CUETM 78-83	96	<i>Salmonella typhimurium</i> LT 2	28
CUETM 78-97	94	<i>Shigella sonnei</i> WRAIR Virulent	37
CUETM 78-73	89	<i>Shigella flexneri</i> ATCC 12661	36
CUETM 77-127	88	<i>Shigella dysenteriae</i> WRAIR A1	30
CUETM 78-96	86	<i>Escherichia coli</i> ATCC 10536	35
CUETM 78-86	74		
CUETM 78-77	71	Group F	
CUETM 78-70	70	CUETM 78-135	35
CUETM 78-75	70	CUETM 78-3	30
CUETM 78-89	70		
CUETM 78-98	67	<i>Enterobacter aerogenes</i>	
CUETM 78-79	64	ATCC 13048	34
CUETM 78-100	64	CUETM 78-160	33
CUETM 77-132	64		
CUETM 78-81	62	Group E	
CUETM 78-74	61	CUETM 77-160	65
<i>E. cloacae</i>		CUETM 78-37	62
CUETM		CUETM 78-2	59
77-125	66	CUETM 78-1	59
77-116	62	CUETM 78-42	58
77-41	61	CUETM 77-158	54
77-120	60	CUETM 77-150	52
77-135	60		
ATCC 13047	58	<i>Levinea amalonatica</i>	
CUETM		R 17996/73	54
77-126	57	CUETM 78-327	54
77-138	54	CDC 25406	52
77-121	53		
77-119	49	<i>Levinea malonatica</i>	
<i>Klebsiella pneumoniae</i>		CDC 25408	50
CUETM		LM 2-70	46
77-169	42	R 16	44
77-170	41		
77-180	39	<i>Citrobacter diversus</i> CDC 3613-63	46
77-174	39		
LM 47-70	37	Group L	
CUETM 77-181	35	CUETM 77-177	51
		CUETM 77-146	46
Group H ₁			
CUETM 77-130	42	Group D	
CUETM 77-147	36	CUETM 78-325	46
CUETM 77-145	32	CUETM 78-324	40
CUETM 77-148	30	CUETM 78-13	35
CUETM 77-139	29	CUETM 78-30	30
CUETM 77-123	26		
CUETM 77-140	26	<i>Klebsiella oxytoca</i>	
CUETM 77-124	25	LM 25-70	44
CUETM 78-87	25	CUETM 78-323	44
<i>Citrobacter freundii</i>			
CUETM 77-166	41	<i>Klebsiella ozaenae</i> CDC 276-71	34
CDC 2987-57	39	<i>Serratia rubidaea</i> CDC 2199-72	32
		<i>Serratia marcescens</i> CDC 868-57	31
		<i>Serratia marcescens</i> ATCC 13880	25

TABLE 2.—Continued

Source of unlabeled DNA	Relative binding at $T_{OR,D}$ (55.7°C) (%)	Source of unlabeled DNA	Relative binding at $T_{OR,D}$ (55.7°C) (%)
<i>Serratia liquefaciens</i> LM 3-70	29	CUETM 77-108	20
<i>Serratia fonticola</i> ATCC 29848	29	CUETM 77-115	11
<i>Serratia fonticola</i> ATCC 29844	27	CUETM 77-146	7
<i>Serratia plymuthica</i> B 36-17	27		
<i>Enterobacter agglomerans</i>			
R 6	27	<i>Edwardsiella tarda</i> CUETM 77-185	21
ATCC 27155	16	<i>Proteus mirabilis</i> ATCC 9240	19
		<i>Hafnia alvei</i> CUETM 78-268	15
" <i>Rahnella aquatilis</i> " (group H ₂)		<i>Yersinia enterocolitica</i> CUETM 77-75	10
CUETM 78-68	27	<i>Yersinia pseudotuberculosis</i> LM 48-1	8

^a Names in quotation marks are not on the Approved Lists of Bacterial Names (21). ATCC, American Type Culture Collection, Rockville, Md.; B, P. Brisou, University of Poitiers, Poitiers, France; CDC, Centers for Disease Control, Atlanta, Ga.; CRR, Center for Reference and Research on *Salmonella* (L. Le Minor, Institut Pasteur de Paris, Paris, France); CUETM, Collection de l'Unité d'Ecotoxicologie Microbienne, 59651 Villeneuve d'Ascq, France; LM, L. Le Minor, Institut Pasteur de Paris, Paris, France; R, C. Richard, Institut Pasteur de Paris, Paris, France; WRAIR, Walter Reed Army Institute of Research, Washington, D.C.; $T_{OR,D}$, temperature of optimal renaturation.

TABLE 3. Relative reassociation of labeled DNA from *E. cloacae* CUETM 77-120 with DNA from groups E and H₃

Source of unlabeled DNA	Relative binding at $T_{OR,D}$ ^a (56.4°C) (%)	Source of unlabeled DNA	Relative binding at $T_{OR,D}$ ^a (56.4°C) (%)
<i>E. cloacae</i>		Group H ₃	
CUETM		CUETM 78-75	56
77-120	100	CUETM 78-70	55
77-121	97	CUETM 78-100	54
77-126	91	CUETM 77-132	53
77-116	86	CUETM 78-74	53
77-41	86	CUETM 78-98	52
77-134	74	CUETM 78-89	52
77-135	73	CUETM 77-137	52
77-138	70	CUETM 78-77	50
ATCC 13047	69	CUETM 78-79	48
CDC 1347-71	66	CUETM 78-73	48
CUETM 77-119	62	CUETM 78-96	47
		CUETM 78-81	46
Group E		CUETM 77-118	46
CUETM 78-2	75	CUETM 78-78	44
CUETM 78-1	73	CUETM 78-83	44
CUETM 77-160	70	CUETM 78-65	42
CUETM 77-158	68	CUETM 78-86	42
CUETM 77-137	68	CUETM 78-97	40
CUETM 77-150	64		
CUETM 78-42	63		

^a $T_{OR,D}$, Temperature of optimal renaturation.

tose, galactose, rhamnose, mannose, fructose, raffinose, melibiose, cellobiose, mannitol, salicin, and esculin. The following reactions are negative: indole production, methyl red test, tartrate utilization (Jordan), H₂S production, phenylal-

anine deaminase production, lysine decarboxylase, gelatinase, Tween esterase, tetrathionate reductase, deoxyribonuclease, and fermentation of the following carbohydrates within 30 days (for 80 to 100% of the strains): lactose, sorbose,

RBR with *E. cloacae* CUETM 77_120

FIG. 1. Genetic relationships between *E. cloacae* and group *H*₃. RBR, Relative binding ratio; ●, *E. cloacae* acetoin-positive group *G*; ○, *E. cloacae* acetoin-negative group *E*; △, group *H*₃; *, labeled DNA.

TABLE 4. Characteristics useful in differentiating *E. cloacae* from *E. amnigenus* (group *H*₃)

Characteristics	<i>E. cloacae</i>		<i>E. amnigenus</i> (N = 28) ^a	
	Reaction ^b	+ (+) Strains (%)	Reaction ^b	+ Strains (%)
Growth at temp (°C):				
4	d	9.5	+	100
41	+	100	—	0
Gelatin liquefaction at 20°C	(+)	(98.4)	—	0
Fermentation				
Sorbitol	+	96.8	— or +	46
Esculin	— or +	31.7	+	100
Salicin	(+) or +	39.7 (57.1)	+ or —	89
Carbon source utilization				
Aconitate	+	92.1	—	0
Phenyl acetate	+ or —	81	—	0
Asparagine	+ or —	73	—	0
L-Serine	+	90.9	— or +	32.1

^a N, Number of strains tested.

^b Symbols: +, positive reaction for at least 90% of the strains within 24 to 48 h; (+), slow positive reaction (i.e., between 2 and 30 days for fermentation reactions); + or —, — or +, the first sign corresponds to the more frequent result; —, negative reaction for at least 90% of the strains; d, different biochemical types [+ , (+), —].

melezitose, dulcitol, inositol, glycerol, adonitol, erythritol, inulin, dextrin, starch, pectin, and chitin.

The following are utilized (by 80 to 100% of the strains) as sole carbon sources: D-fructose, D-galactose, D-glucose, D-mannose, D-maltose, D-trehalose, D-cellobiose, D-ribose, D-xylose, L-arabinose, L-rhamnose, starch, salicin, gluconate, D-mannitol, DL-glycerate, citrate, fumarate, DL-lactate, L-malate, pyruvate, L- α -alanine, D- α -alanine, and L-glutamate. The following are not utilized (by 80 to 100% of the strains within 5 days) as sole carbon sources: D-fucose, inulin, ethanol, methanol, *n*-propanol, *iso*-propanol, geraniol, 2,3-butylene glycol, ethylene glycol, propylene glycol, erythritol, adonitol, D-dulcitol, *meso*-inositol, propionate, D-tartrate, L-tartrate, *meso*-tartrate, levulinate, citraconate, itaconate, mesaconate, glycolate, aconitate, D-malate, 2-ke-toglutarate, oxalate, acetate, DL-3-hydroxybutyrate, maleate, pimelate, suberate, azelate, glutarate, sebacate, salicylate, butyrate, *iso*-butyrate, *n*-valerate, *iso*-valerate, *n*-caproate, heptanoate, adipate, caprate, pelargonate, caprylate, phenol, phthalate, *iso*-phthalate, *tere*-phthalate, benzylformate, D- and L-mandelate, *p*-hydroxybenzoate, phenyl acetate, benzoate, *m*-hydroxybenzoate, D-quininate, glycine, β -alanine, L-leucine, L-isoleucine, L-valine, L-lysine, L-citrulline, L-methionine, DL-2-aminobutyrate, DL-3-aminobutyrate, DL-4-aminobutyrate, L-aspartate, L-arginine, L-ornithine, L-threonine, L-cysteine, 5-aminovalerate, L-phenylalanine, L-tyrosine, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, L-tryptophan, histamine, putrescine, spermine, tryptamine, amylamine, ethanolamine, butylamine, benzylamine, diphenylamine, urea, urate, sarcosine, betaine, creatine, hippurate, pantothenate, nicotinate, trigonelline, asparagine, acetamide, potassium nitrate, naphthalene, dodecane, and hexadecane.

Mean guanine plus cytosine content of DNA: 54.2 mol% (5 strains analyzed [15]).

Genetic relatedness (DNA-DNA hybridization) with the most related species or genus: *E. cloacae* (49–66%; 17 strains analyzed); *Levinea* (46–54%; 7 strains analyzed).

Found in water (drinking water and surface water) and unpolluted soils.

Type strain: CUETM 77-118 (ATCC 33072).

ACKNOWLEDGMENTS

We thank J. De Ley and M. Véron for their constructive comments and continued interest in our work. We also thank E. Dewailly and A. Bernigaud for their technical assistance.

REPRINT REQUESTS

Address reprint requests to: Daniel Izard, Unité INSERM 146, Domaine du C.E.R.T.I.A., B.P. 39, 59651 Villeneuve d'Ascq Cedex, France.

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