Differentiation of the *Oxytocom* Group from *Klebsiella* by Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization

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By deoxyribonucleic acid-deoxyribonucleic acid molecular hybridization, evidence was obtained that the bacteria of the *Oxytocom* group represent a distinct deoxyribonucleic acid homology group and are not indole-forming and gelatin-liquefying variants of *Klebsiella aerogenes* and *Klebsiella pneumoniae* (sensu lato), respectively, and not a subgroup within *Klebsiella*, as had been suggested previously. The establishment of a new, but presently unnamed, genus of *Enterobacteriaceae*, with one species, is proposed, represented by strain 497-2 of R. Hugh (ATCC 13182). Furthermore, none of a series of bacterial strains classified as *Enterobacter*, *Hafnia*, or *Erwinia* species was found to be related to *Klebsiella* or to the *Oxytocom* strains. The deoxyribonucleic acid reassociation values do not allow species differentiation within the true, indole-negative klebsiellae.

Attempts to give an unambiguous definition of *Klebsiella* by means of phenotypic criteria have led to contradictory viewpoints. Recent suggestions have been reviewed by Bascomb et al. (1) and, in part, by Stenzel et al. (26). Traditional enterobacteriological methods have failed to produce an acceptable classification of what has been named the *Klebsiella-Enterobacter-Serratia* division (10), perhaps because such tests cover only a small and nonrepresentative part of genome information (cf. 7). Further progress in this field, however, can be expected from investigations of taxonomic relatedness on the molecular level.

By means of deoxyribonucleic acid (DNA) reassociation, Brenner et al. (3) showed that the klebsiellae, on the one hand, and typical strains of *Enterobacter aerogenes*, on the other hand, represent clearly different DNA homology groups at the same rank as conventional genera. Separation of species within *Klebsiella* was, however, impossible, but the systematic position of the so-called *Oxytocom* strains was not considered. *Bacillus oxytocom pernicius* (13) has so far been classified as: (i) a distinct taxonomic unit within the family *Enterobacteriaceae* (21), (ii) a *Klebsiella* species (20, 26), and (iii) a subspecies of *Klebsiella pneumoniae* (11) or *Klebsiella aerogenes* (1). The present investigation was undertaken to examine the relationship between *Oxytocom* strains and members of the *Klebsiella* group by means of DNA-DNA hybridization

MATERIALS AND METHODS

Microorganisms. The bacterial strains used are listed in Table 1. They were kept in a freeze-dried state under vacuum and for short periods (weeks) on tryptic soy agar (Difco Laboratories) at 4 C.

Media and cultivation. Fernbach flasks containing 200 ml of medium were inoculated with 1% (vol/vol) of late exponential cultures and incubated aerobically (G-25 gyratory shaker, New Brunswick Scientific Co, 200 rpm) at 30 C. Unlabeled DNA was prepared from bacteria grown in a complex medium containing 2.0 g of proteose peptone no. 3 (Difco), 0.5% (wt/vol) NaCl, and tris(hydroxymethyl)amino-methane q.s. (pH 7.4), sterilized for 15 min at 121 C, and 0.2% (wt/vol) D(+)-glucose, sterilized by filtration. Labeled DNA was recovered from bacteria grown in ammonium glucose medium [0.5 g of NaCl, 0.02 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of $(\text{NH}_4)_2\text{HPO}_4$, and 0.1% (wt/vol) K_2HPO_4 , sterilized by heat; 0.05 M D(+)-glucose and 0.01 M NaHCO_3 , sterilized by filtration] which had been supplemented with 1 μCi of [*methyl*- ^3H]thymidine per ml before inoculation (Amersham Buchler Ltd., specific activity ca. 20 Ci/mmol). The cultures were harvested in the late exponential phase at $10,000 \times g$ (4 C), washed twice with 1 to $1.5 \times \text{SSC}$ (0.15 M NaCl plus 0.15 M sodium citrate) and stored at -25 C.

Preparation, purification, and estimation of bacterial DNA base composition. DNA was extracted from washed bacterial sediments according to a modification (18) of the method described by Marmur

TABLE 1. Identity and source of bacterial strains used^a

Organism	Strain	Source
<i>Klebsiella oxytoca</i>	497-2	ATCC 13182
<i>K. oxytoca</i>	533-1	ATCC 13183
<i>K. oxytoca</i>	25871/68	HIM 382-3
<i>K. oxytoca</i>	E 172/68	Stenzel
<i>K. pneumoniae</i>	CDC 298/53	NCTC 9633
<i>K. pneumoniae</i>	E 121/68	Stenzel
<i>K. pneumoniae</i>	V 1942/69	Stenzel
<i>K. pneumoniae</i>	35584/69	HIM 341-5
<i>K. aerogenes</i>	E 672	Lapage
<i>K. aerogenes</i>	E 674	Lapage
<i>K. edwardsii</i>		
subsp. <i>edwardsii</i>	F1	NCTC 5054
<i>K. edwardsii</i>		
subsp. <i>atlantae</i>	CDC 4610/53	NCTC 9496
<i>Klebsiella</i> sp. (unnamed)	E 45/67	Lapage
<i>Klebsiella</i> sp. (unnamed)	E 46/67	Lapage
<i>K. ozaenae</i>	McLurry	NCTC 9657
<i>K. ozaenae</i>	AMC 35-E6	NCTC 5051
<i>K. rhinoscleromatis</i>	R 70	NCTC 5046
<i>K. rhinoscleromatis</i>	5 Sumatra	NCTC 1936
<i>K. rhinoscleromatis</i>	Muggia	ATCC 9436
<i>Enterobacter aerogenes</i>	819/56	NCTC 10006
<i>E. cloacae</i>	279/56	NCTC 10005
<i>E. carotovorus</i> ^b	904	CCM 1008
<i>E. agglomerans</i> ^c	CDC 1461/67	NCTC 9381
<i>Hafnia alvei</i>	Cadaveris	NCTC 6578
<i>Erwinia amylovora</i>	S59/5	CCM 114
<i>Escherichia coli</i>	U 5/41	NCTC 9001
<i>E. coli</i>	K-12, W-1485	ATCC 12435
<i>E. coli</i>	Luria B	Martin

^a ATCC, American Type Culture Collection; CCM, Czechoslovak Collection of Microorganisms; HIM, Culture Collection of Institute of Hygiene, Faculty of Medicine, Philipps University, Marburg/Lahn, West Germany; Lapage, S.P. Lapage, London; NCTC, National Collection of Type Cultures, London; Martin, H. H. Martin, Darmstadt; Stenzel, W. Stenzel, Moers.

^b cf. Martinec and Kocur (24).

^c cf. Ewing and Fife (12).

(23). DNA was treated further with 0.01% (wt/vol) acid-washed Norite (Serva Feinbiochemica GmbH, Co., Heidelberg, Germany) to remove traces of polysaccharide and protein (5). Ultraviolet spectra of the final DNA preparation dissolved in $0.1 \times$ SSC were recorded (i) to detect residual protein contamination, (ii) to determine DNA concentration, and (iii) to estimate base composition by the method of Ulitzur (27). Specific activities of different radioactive DNA preparations were between 3,000 and 6,000 counts per min per μ g of DNA.

DNA-DNA hybridization. DNA-DNA hybridization was carried out by using the membrane filter method of Denhardt (9) with the following modifications. Cellulose membrane filters (pore width, 0.45μ m; diameter, 25 mm; no. SM 11 306, Sartorius, Göttingen, Germany) were loaded with 5 μ g of nonradioactive heat-denatured DNA, dried for 4 h at 80 C, and incubated with a 10-fold excess (near saturation of homologous DNAs) of sonically denatured radioactive DNA, in 1.2 ml of buffer solution containing $5.2 \times$ SSC and 48% (wt/vol) formamide (24), for 48 h at 46 C. Thus, reassociation took place under restrictive conditions corresponding to about 8 to 10 C below the melting temperature of *Klebsiella* DNA. After incubation, filter disks were washed thoroughly with $2 \times$ SSC, dried, and counted in a toluene-based scintillation fluid in a Nuclear-Chicago liquid scintillation counter. Counting efficiency was approximately 38%. On the basis of specific radioactivities, binding values for the homologous reactions were about 2% of the input radioactive DNA.

RESULTS

Klebsiella oxytoca strain 497-2 of Hugh (ATCC 13182), *Klebsiella pneumoniae* (sensu strictiori, cf. Cowan et al. [6]) strain CDC 298/53 (NCTC 9633), and *Klebsiella aerogenes* strain E 672 of Lapage (1) were selected as donors of radioactively labeled DNA. Binding values between these DNAs and those of the organisms listed in Table 1, i.e., representatives of a variety of taxa considered to belong to the *Klebsiella* or *Enterobacter*, were determined. In addition, three representative *Escherichia* strains were included. As indicated by ultraviolet absorbance ratios (245/270 nm; approximately 0.9 for all DNAs used), the DNA base composition of all samples appeared to be in the same range, in agreement with data published so far (cf. 8, 17). Hybridization conditions were, therefore, applied uniformly to all tests. Binding values are summarized in Table 2.

Reassociation of radioactive Oxytocum DNA. Labeled DNA of strain 497-2 was bound by the DNAs of two other *Oxytocum* strains to a high extent and by the DNA of a third *Oxytocum* strain to an intermediate extent. In contrast, reactions with DNAs of the indole-negative klebsiellae and the other organisms tested yielded moderate to low binding values.

Reassociation of radioactive DNAs of *K. pneumoniae* and *K. aerogenes*. Labeled DNAs of strains CDC 298/53 and E 672 exhibited comparable affinities, yielding intermediate to high reassociation values with DNAs of indole-

TABLE 2. Reaction of [methyl-³H]-labeled DNA fragments from representative *K. oxytoca*, *K. pneumoniae*, and *K. aerogenes* strains with DNA from various *Enterobacteriaceae*

Source of immobilized, unlabeled DNA	Labeled DNA, relative binding ^a (%)		
	<i>K. oxytoca</i> 497-2	<i>K. pneumoniae</i> CDC 298/53	<i>K. aerogenes</i> E 672
<i>K. oxytoca</i> :			
497-2	100	22	15
533-1	90	19	NT ^b
25871/68	34	18	NT
E 172/68	70	13	NT
<i>K. pneumoniae</i> :			
CDC 298/53	22	100	86
E 121/68	13	72	52
V 1942/69	7	47	NT
35884/69	8	45	NT
<i>K. aerogenes</i> :			
E 672	12	66	100
E 674	8	48	72
<i>K. edwardsii</i> :			
F 1	5	56	77
CDC 4610/53	11	58	73
<i>Klebsiella</i> sp.:			
E 45/67	12	61	85
E 46/67	8	58	NT
<i>K. ozaenae</i> :			
McLurry	15	48	69
AMC 35-E6	10	83	71
<i>K. rhinoscleromatis</i> :			
R 70	10	74	89
5 Sumatra	10	67	78
Muggia	23	55	NT
<i>E. aerogenes</i> 819/56	12	17	22
<i>E. cloacae</i> 279/56	11	11	16
<i>E. carotovorus</i> 904	3	5	NT
<i>E. agglomerans</i> CDC 1461/67	5	7	NT
<i>H. alvei</i> Cadaveris	2	4	NT
<i>E. amylovora</i> S59/5	4	5	NT
<i>E. coli</i>			
U 5/41	7	NT	NT
K-12, W-1485	6	10	11
Luria B	6	8	NT

^a Specific radioactivities of different DNA preparations were between 3,000 and 6,000 counts per min per μ g of DNA. Mean values of at least three experiments are listed.

^b NT, Not tested.

negative klebsiellae, moderate binding values with *Oxytocom*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Escherichia* DNAs, and low binding values with *Enterobacter carotovorus*, *Enterobacter agglomerans*, *Hafnia alvei*, and *Erwinia amylovora* DNAs.

DISCUSSION

Since the results given in Table 2 represent reassociation values under rather restrictive conditions, they cannot be compared directly with those obtained by more permissive procedures. The reactions observed under the conditions described have to be considered, however, as highly indicative of the degree of homology between the participants of the reaction. Heat stability of the heteroduplexes as an exact criterion of the degree of homology was not examined.

The results presented might be invalidated by differences in length of chromosomal DNA molecules, which cannot be ruled out according to data reported (cf. 8), but which were considered of minor importance for the present study. However, the majority of both recently isolated and old collection cultures of *Enterobacteriaceae* may contain one or more species of plasmid DNA (4). This was confirmed for the organisms that were investigated by detection of multiple drug resistance (Table 3) and/or by demonstration of circular extrachromosomal DNA by ethidium bromide-cesium chloride density centrifugation (unpublished data). In general, the amount of plasmid DNA is considered to be relatively small compared to the amount of chromosomal DNA (15, 25). However, different kinetics of DNA synthesis during early growth of bacterial cultures may yield labeled extrachromosomal DNA of relatively high specific activity. Therefore, interference of reassociation of non-chromosomal DNA cannot be ruled out for the results reported here.

Although an entirely different set of bacterial strains was investigated, the results presented exhibit the same tendency as those of Brenner et al. (3), which show a clear distinction between *Klebsiella* and *Enterobacter* species, including *E. aerogenes*, but no significant differences between species within the indole- and gelatin-negative klebsiellae. In particular, the binding values observed do not indicate whether *K. aerogenes*, *K. pneumoniae* (sensu strictiori), *K. edwardsii*, and the "unnamed" subgroup described by Bascomb et al. (1) are different species or not. This holds true also for the recognized species *K. ozaenae* and *K. rhinoscleromatis*.

TABLE 3. Drug resistance and circular DNA

Organism and strain	Drug resistance pattern ^a	Circular DNA present ^b
<i>K. oxytoca</i>		
497-2	A/S	—
533-1	A	NT
25871/68	A/Ca/Te/Chl/Sm/S	(+)
E 172	A/Ca/S	NT
<i>K. pneumoniae</i>		
CDC 298/53	A	(+?)
E 121/68		NT
V 1942/69	A/Ca/Ce/Sm/K/S/Tri/Ni/Na	+
35884/69	A/Ca/Ni	NT
<i>K. aerogenes</i>		
E 672	A/Ca	+
E 674	A/Ca	NT
<i>K. edwardsii</i>		
F 1		NT
CDC 4610/53	A	NT
<i>Klebsiella</i> sp.		
E 45/67	A/Ca/Sm/S	NT
E 46/67	A/Ca/Sm/S	NT
<i>K. ozaenae</i>		
McLurry		NT
AMC 35-E6		NT
<i>K. rhinoscleromatis</i>		
R 70		NT
5 Sumatra		NT
Muggia	A	NT
<i>E. aerogenes</i>		
819/56	A/Ce	++
<i>E. cloacae</i>		
279/56	A/Ce/Sm/S	NT
<i>E. carotovorus</i>		
904	S	NT
<i>E. agglomerans</i>		
CDC 1461		NT
<i>H. alvei</i>		
Cadaveris		NT
<i>E. amylovora</i>		
S59/5	A/Ce/Chl/Sm/K/Co/Tri/Na	NT
<i>E. coli</i>		
U 5/41		(+)
K-12, W-1485		—
Luria B		(+)

^a Multidisc (Oxoid) diffusion test on Mueller-Hinton agar; A, ampicillin (10 µg); Ce, Cephalothin (15 µg); Te, tetracycline (10 µg); Chl, chloramphenicol (10 µg); S, sulfafurazole (100 µg); Tri, trimethoprim (1.25 µg); Ca, carbenicillin (100 µg); Sm, streptomycin (2 µg); K, kanamycin (5 µg); Co, colistin (50 µg); Ni, nitrofurantoin (50 µg); Na, nalidixic acid (30 µg).

^b Isolated by isopycnic centrifugation in cesium chloride-ethidium bromide by the method of Goebel and Schrempf (16). NT, Not tested.

Furthermore, the data of Table 2 suggest that the variety of phenotypically related organisms that have been classified in *Enterobacter* may contain several other DNA homology groups of *Enterobacteriaceae* (2, 14). As for *Erwinia amylovora*, Jediss (19) suggested that this taxon does not belong to *Enterobacteriaceae*.

In spite of the small number of *Oxytocum* strains (two American strains and two isolated in Germany) investigated, the results presented clearly indicate that the *Oxytocum* group represents a distinct DNA homology group that should be classified separately from *Klebsiella*. This finding confirms Lautrop's view (21) that the *Oxytocum* strains form a distinct generic group of organisms. The phylogenetic relatedness of the *Oxytocum* group to *Klebsiella* may be approximately similar to that of *Enterobacter aerogenes*, and the relatedness to *Escherichia* may be even closer than to *Klebsiella*. Consequently, on the basis of Lautrop's proposal and the data presented, a new genus with one species should be established that will conform widely to the phenotype of *Klebsiella oxytoca* as described by Kaluzewski (20) and by Stenzel et al. (26). Strain 497-2 of R. Hugh (ATCC 13182) is a typical representative of this taxon. However, the questions of the type strain and name of the new taxon have to await further investigations.

The proposal of another new genus of *Enterobacteriaceae* might contribute to further confusion for the bacteriologist, since *Oxytocum* organisms look like true klebsiellae in nearly every respect except indole reaction. However, the evaluation of clinical, epidemiological, and ecological properties of the *Oxytocum* group will depend on their proper distinction from *Klebsiella*. For the purposes of food and water control, the new taxon may be useful, since production of both gas and indole at 45 C will be restricted to one genus of *Enterobacteriaceae*, *Escherichia*, whereas klebsiellae retain only the ability to form gas in Eijkman's test, and the new taxon retains only the ability to produce indole (cf. 26).

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REPRINT REQUESTS

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