JOURNAL OF CLINICAL MICROBIOLOGY, May 1981, p. 919-933
0095-1137/81/050919-15$02.00/0

Vol. 13, No. 5

Kluyvera, a New (Redefined) Genus in the Family *Enterobacteriaceae*: Identification of *Kluyvera ascorbata* sp. nov. and *Kluyvera cryocrescens* sp. nov. in Clinical Specimens

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Received 12 January 1981/Accepted 23 January 1981

Kluyvera is proposed as a new genus for the group of organisms formerly known as Enteric Group 8 (synonym = API group 1). Strains of *Kluyvera* share the properties of most members of the family *Enterobacteriaceae*: they are gram-negative rods, motile with peritrichous flagella, catalase positive, and oxidase negative; they grow on MacConkey agar, ferment D-glucose with the production of acid and gas, and are susceptible to many antibiotics. Strains are usually indole positive, methyl red positive, Voges-Proskauer negative, citrate positive, H_2S (triple sugar iron) negative, urea negative, phenylalanine deaminase negative, lysine decarboxylase positive, arginine dihydrolase negative, and ornithine decarboxylase positive. *Kluyvera* strains ferment many of the sugars and polyhydroxyl alcohols used in identification. By deoxyribonucleic acid-deoxyribonucleic acid hybridization, strains of *Kluyvera* were divided into three groups. *Kluyvera ascorbata* is proposed as the type species for the genus. Most strains of *K. ascorbata* have been isolated from clinical specimens. *K. cryocrescens* is proposed as the second species. It was occasionally isolated from clinical specimens, but it was isolated more commonly from the environment. *Kluyvera* species group 3 was heterogeneous, but was distinct from the two named species by deoxyribonucleic acid hybridization. This group was rare, so no species names will be proposed at this time. *K. ascorbata* can be differentiated from *K. cryocrescens* by its positive ascorbate test, inability to grow at 5°C in a refrigerator, and smaller zones of inhibition around carbenicillin and cephalothin disks. The test normally used for identification does not clearly differentiate these two species. *Kluyvera* species are probably infrequent opportunistic pathogens. The most common source is sputum, where they are probably not clinically significant. Five strains have been from blood cultures. More information is needed about the incidence and clinical significance of the genus *Kluyvera*.

Since 1965 we have received over 100 strains of a gram-negative, oxidase-negative, fermentative bacterium which does not fit any of the defined species in the family *Enterobacteriaceae*. Most of these strains were from clinical specimens, and originally they were reported simply as "unidentified." However, the biochemical reactions of the new group were tabulated in 1977, and it was given the vernacular name "Enteric Group 8" (7, 21). A synonym of Enteric Group 8 is "API group 1," used by Analytab Products, Plainview, N.Y. Since 1977 we have reported cultures as Enteric Group 8 and requested that other biochemically similar strains be sent for further study. In about 1978, one of us (B.H.) noticed that the biochemical reactions of Enteric Group 8 were almost identical to a group of organisms that had been discovered in Japan (2), given the name *Kluyvera* (4, 5), and later moved to the genus *Escherichia* (3).

In 1956 to 1957 Asai and co-workers (4, 5) in Japan proposed the genus *Kluyvera* for a group of polarly flagellated bacteria which produced large amounts of α-ketoglutaric acid (2) during the fermentation of glucose. Five strains were originally studied (2): one from soil and four from sewage. Two species names were proposed (4), *Kluyvera citrophila* and *Kluyvera noncitrophila*, which were based on the difference between the two species in utilizing citric acid as

the sole source of carbon and energy. Asai and co-workers state (based on their interpretation of the Kluyver and van Niel paper [17]) that the genus was named (4, 5) to honor A. J. Kluyver who, with C. B. van Niel, in 1936 postulated (17) that there may be a group of polarly flagellated organisms in the tribe *Pseudomonadeae* which have a mixed-acid type of fermentation similar to the genus *Escherichia* (which was called *Bacterium* in the paper). If such a group were to be discovered, it could be a separate genus, which would differentiate it from the genus *Aeromonas*, which has a butylene glycol-fermentative pathway rather than a mixed-acid pathway (17). Asai et al. (4) thought that they had discovered this postulated group of polarly flagellated organisms in 1956 to 1957 and named the group *Kluyvera* in honor of A. J. Kluyver for his prediction and many contributions to microbial metabolism and physiology. The genus *Kluyvera* was classified in the tribe *Pseudomonadeae*, which at that time included nonfermentative genera, but also included the fermenters of the genus *Aeromonas*. Today, the family *Pseudomonadaceae* is restricted to bacteria which do not ferment glucose.

In 1962, Asai et al. (3) confirmed the observations (unpublished data) of J. M. Shewan and Rudolph Hugh (3) that all five of their *Kluyvera* strains actually had peritrichous rather than polar flagella, so they proposed that the two species *K. citrophila* and *K. noncitrophila* be transferred to the genus *Escherichia* in the family *Enterobacteriaceae*. Thus, the genus *Kluyvera* was abolished in 1962 by its original proposers, although reports in the literature still refer to *Kluyvera* (6, 16, 19, 20, 22, 25, 26). *Kluyvera* lost all standing in nomenclature when the genus name and the two species names did not appear on the "Approved Lists of Bacterial Names" compiled and edited by Skerman et al. (23). Since 1 January 1980, none of the *Kluyvera* names has had standing in nomenclature.

The purpose of this study was to better define the group of organisms that we have been reporting as Enteric Group 8 and to compare our strains with strains of *Kluyvera*. On the basis of deoxyribonucleic acid (DNA)-DNA hybridization, biochemical reactions, and antibiotic susceptibility, we propose *Kluyvera* as a new genus in the family *Enterobacteriaceae* with two species, *K. ascorbata* and *K. cryocrescens*. The remainder of this paper discusses the identification of these organisms which occur in clinical specimens.

MATERIALS AND METHODS

General. Unless exceptions are given, the following statements hold throughout this paper: all experiments were done in the Enteric Section, Centers for Disease Control; the temperature of incubation was 36 ± 1°C; water refers to glass-distilled water; commercial media were used whenever possible (the terms "from individual ingredients" or "was made with" appear if a commercial medium was not used); media were sterilized in an autoclave at 121°C for 15 min; optical density was measured in a Bausch & Lomb Spectronic 20 spectrophotometer at 650 nm in disposable glass tubes (13 by 100 mm); filter sterilization was through a 0.22-μm nitrocellulose filter; refrigeration was at a temperature of 5 ± 1°C; all results are based on cultures picked twice, each time from a single isolated colony; names of enzymes are put in quotation marks unless actual enzyme assays were done; and the term "antibiotic" refers to true antibiotics and to synthetic antimicrobial agents. In most instances organic chemicals were from Sigma Chemical Co., St. Louis, Mo., and inorganic chemicals were Baker Analyzed Reagent grade and were from J. T. Baker Chemical Co., Phillipsburg, N.J.

Nomenclature. The names used are from the "Approved Lists of Bacterial Names" (23). In addition, we use *Citrobacter amalonaticus* as a synonym of *Levinea amalonatica*. The genus *Kluyvera* is proposed in this paper as a new (redefined) genus in the family *Enterobacteriaceae*. The genus *Kluyvera* has two new named species, *K. ascorbata* and *K. cryocrescens*, and a third unnamed group, *Kluyvera* species group 3 (see Results).

Strains. As of 1 November 1980 we had identified 144 strains as *Kluyvera* species. For this study 100 strains were examined (in 1978 to 1980) in more detail: 78 strains of *K. ascorbata*, 17 strains of *K. cryocrescens*, and 5 strains of *Kluyvera* species group 3. Their sources are given in Table 1. Several strains, including the five original isolates of Asai and co-workers, are important for historical or nomenclatural reasons, so more information about them is given in Table 2.

Stock cultures. A single colony was picked from a 24-h-old Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plate, which had been streaked to give good isolated colonies. This step was done at least twice. The streak plates were then kept for 14 to 28 days at room temperature before they were discarded. Stock cultures were stored on Trypticase soy agar (BBL) or blood agar base (Difco Laboratories, Detroit, Mich.) in sealed tubes (in the dark at room temperature) and have remained viable for 1 to 10 years without transfer.

DNA-DNA hybridization. The genetic relatedness of strains was determined by DNA-DNA hybridization on hydroxyapatite with ^{32}P-labeled DNA. Reassociation was often done at both 60 and 75°C, but, unless otherwise indicated, all data refer to reassociations done at 60°C. The methods used have been described by Brenner et al. (8, 24), and the original papers should be consulted for technical details too lengthy to repeat here. Closely or highly related strains (that is, belonging to the same species) usually have a relatedness (based on relative binding of the DNAs) of 70% or more (occasionally down to 60%) when reassociation is done at 60°C (and relatedness of 50% or more when reassociation is done at 75°C). Species

TABLE 1. *Sources of 100 Kluyvera strains*

Source	*K. ascorbata* (78 strains)	*K. cryocrescens* (17 strains)	*Kluyvera* species group 3 (5 strains)
Human clinical specimens:			
Sputum	28	2	2
Urine	11	2	
Stool	8		
Throat	5	1	2
Blood	1	1	
Other, or not given	8	1	1
Water	1		
Sewage	1	3	
Soil		1	
Milk	1		
Hospital sink		1	
Cow	1		
Unknown	13	5	

TABLE 2. *Important strains of Kluyvera*

Species	Previously designated	CDC number	ATCC number	Source
Kluyvera ascorbata	Enteric Group 8	0648-74	33433	Human, sputum, North Carolina
Kluyvera cryocrescens	Enteric Group 8	2065-78	33435	Kitchen food, Persian Gulf
Kluyvera ascorbata	*K. citrophila*	0408-78[a]	14236	Sewage (Asai's designation = 6, Beta)
Kluyvera cryocrescens	*K. citrophila*	0409-78[a]	14237	Soil (Asai's designation = 84C, Alpha)
Kluyvera cryocrescens	*K. citrophila*	0410-78[a]	14238	Sewage (Asai's designation = 11, Gamma)
Kluyvera cryocrescens	*K. noncitrophila*	0411-78[a]	14239	Sewage (Asai's designation = 4)
Kluyvera cryocrescens	*K. noncitrophila*	0412-78[a]	14240	Sewage (Asai's designation = 10)
Kluyvera ascorbata	Enteric Group 8	2221-78	33434	Human ?, ?, New York

[a]These cultures were studied twice at CDC. Numbers were assigned in 1961 also: 0408-78 = 2567-61, 0409-78 = 2568-61, 0410-78 = 2569-61, 0411-78 = 2570-61, 0412-78 = 2571-61.

are considered to belong in the same genus if they are more closely related to each other than to species in other genera and if they are similar in their phenotypic properties.

G+C content of DNA. The moles percent of guanine plus cytosine (G+C) was determined by the buoyant density method (cesium chloride centrifugation) in the laboratory of Manley Mandel, M.D. Anderson Hospital, Houston, Tex., from unsheared DNA prepared by A. G. Steigerwalt. Values were determined twice in the presence of reference DNAs from *Bacillus subtilis* bacteriophage 2C (density, 1.742 g/cm^3). Because of the variables in the technique, the results of G+C content are sometimes rounded to two significant figures.

Biochemical tests used for identification. Biochemical tests were basically done according to the methods of Edwards and Ewing (10); modifications and more complete documentations have been given in considerable detail in more recent publications (12–14). The biochemical reactions of the 100 *Kluyvera* strains were done twice, first by the standard tube method (10) as they arrived at the Enteric Section during the period 1969 to 1980. They were then all retested in 1979 to 1980 in glass tubes (100 by 13 mm) by using the 72-prong multi-inoculator shown in Fig. 1. This later testing was done with the same media and was essentially the same as the standard testing in the Enteric Section; however, the advantage was that all strains could be compared at the same point in time. Two racks, or a total of 144 tubes, were used for each test so that all 100 strains could be studied at one time.

The following tests were done by C. Richard at the Institut Pasteur. "Beta-xylosidase" was tested by the method of Brisou et al. (9) which depends on the formation of yellow *p*-nitrophenol from colorless *p*-nitrophenyl-β-D-xylopyranoside. "Beta-glucuronidase" was tested in a similar manner by the method of Kilian and Bülow (15), but with *p*-nitrophenyl-β-D-glucuronide as the colorless substrate. "Tetrathionate reductase" was tested by the method of LeMinor and Pichinoty (18) and is based on acid production in a peptone medium from tetrathionate, which is reduced to thiosulfate with the production of H^+.

"Ascorbate" test. The ascorbate test is a new test which was devised to differentiate the two named *Kluyvera* species. Ascorbate medium 7795 (a number is given to all media used in the Enteric Section) contains 10 g of peptone, 3 g of meat extract, 5 g of NaCl, 10 g of L-ascorbic acid-sodium salt, and 0.004 g of bromothylmol blue (Difco furnished the first three

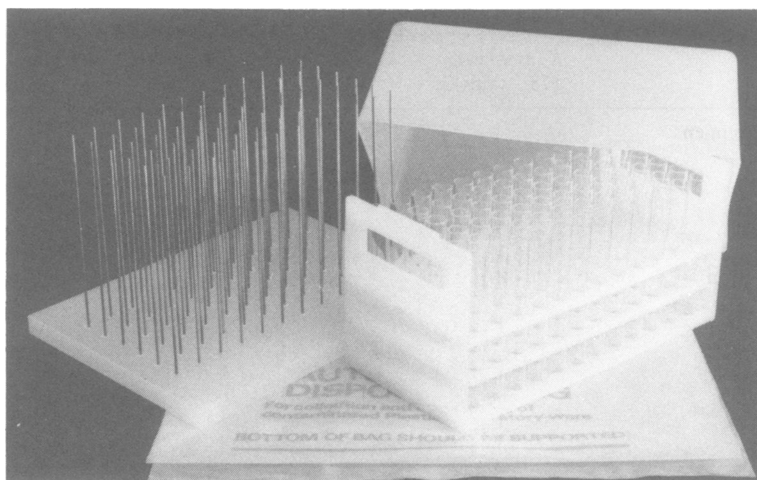

FIG. 1. *A 72-prong multi-inoculator (fabricated in the Centers for Disease Control machine shop), polypropylene 72-hole test tube rack (Scientific Products, McGraw Park, Ill.), polypropylene instrument tray (Bel-Art Products, Pequannock, N.J.) used as a top to prevent airborne contamination of tubes, and polypropylene autoclave bag (York Scientific Inc, Ogdensburg, N.Y.) used to cover racks during incubation to prevent contamination.*

ingredients as "Enteric Fermentation Base" no. 836-01, which can be purchased by anyone, even though it does not appear in the Difco catalog). All ingredients are dissolved in 900 ml of water, and 0.1 N NaOH is added until pH 7.5 is reached (dark blue in color). Water is added to make the final volume 1,000 ml. The medium is then dispensed into 16- by 125-mm tubes (10 ml each), and a Durham tube is added. A plastic cap is added, and the medium is autoclaved. As soon as the tubes are removed from the autoclave, 4 ml of sterile mineral oil is aseptically added to each tube. The medium is stored immediately in the refrigerator and discarded if the pH drops to below 6.8 (green in color), or 0.1 N NaOH can be added to change the color back to light blue (pH 7.4). The ascorbate test is inoculated with about 10^4 to 10^5 organisms (for example, 0.001 ml of a 24-h-old heart infusion broth culture) under the mineral oil. A positive reaction is the production of acid (pH to below 6.0; color change from blue to yellow) and usually gas (for those strains which produce gas during fermentation). Strains which are negative usually lower the pH to around 6.5 to 6.8 (green to green-yellow) but do not produce gas. A control tube of uninoculated ascorbate medium must be included in the incubator since the pH of uninoculated medium drops. The drop in pH of the uninoculated medium is much faster at 36°C than in the refrigerator.

Glucose fermentation at 5°C. Peptone water with Andrade indicator is satisfactory for this test, or the ascorbate medium referred to above can be used (except 10 g of D-glucose is substituted for the 10 g of L-ascorbic acid-sodium salt, the final pH is adjusted to 7.6 before autoclaving, and the mineral oil overlay is omitted). The tube is inoculated with about 10^4 to 10^5 organisms and "incubated" in a refrigerator maintained at 5 ± 1°C. The tubes are observed for 3 weeks for the production of acid. A positive test is growth and glucose fermentation to a pH of <6.0 within 21 days.

Susceptibility to antibiotics—disk method. The standardized single-disk method (Kirby-Bauer) was used (13), and the zones of complete inhibition around the antibiotic disks (BBL) were measured. Table 3 gives the antibiotic disks used, potencies, and abbreviations. Conversion of the zone sizes into "susceptible" (or "sensitive"), "intermediate," and "resistant" was based on the zone size interpretative chart supplied with the disks. In addition, the zone of "any inhibition" was measured around the carbenicillin disk (100 μg) and cephalothin disk (30 μg). This was defined to be twice the distance from the center of the disk to the area of complete growth with no inhibition. Colonies in the zone were ignored in measuring this zone of any inhibition which is different from the usual measurement which considers these resistant colonies. This zone of any inhibition was found useful in differentiating the two *Kluyvera* species.

Susceptibility to antibiotics—minimum inhibitory concentrations in Mueller-Hinton broth. The Sensititre-Gram Negative Plate (made by Seward Laboratory, London, England; purchased from GIBCO Diagnostics, Lawrence, Mass.) was used according to the manufacturer's instructions. Strains were picked from 24-h Trypticase soy agar plates and inoculated (about 10^6 cells) into 0.5 ml of brain heart infusion broth. They were grown for 4 to 6 h (to about 10^9 cells per ml), and a 0.001-ml loopful was transferred to 10 ml of Mueller-Hinton broth (GIBCO) to give about 10^5 bacteria per ml. Then 0.05 ml of this diluted suspension was added with an eight-channel dispensor (GIBCO) to each of 96 wells of a disposable plastic plate containing the dehydrated antibiotics (13). After overnight incubation the wells were observed for turbidity.

Requirements for vitamins or amino acids. To

TABLE 3. *Disk-diffusion, antibiotic susceptibility of Kluyvera*

Antibiotic	*K. ascorbata* (73 strains)			*K. cryocrescens* (16 strains)		
	Mean	Standard deviation	% susceptible	Mean	Standard deviation	% susceptible
Colistin (10)[a]	12.2[b]	0.9	99	12.5[a]	0.6	100
Nalidixic acid (30)	19.5	3.1	63	22.1	2.5	94
Sulfadiazine (250)	18.9	3.5	78	22.4	3.9	94
Gentamicin (10)	19.1	1.5	100	20.2	2.6	100
Streptomycin (10)	15.0	1.9	65	16.1	2.5	69
Kanamycin (30)	19.2	1.4	95	20.3	2.5	94
Tetracycline (30)	19.9	2.5	85	22.0	1.9	100
Chloramphenicol (30)	24.2	3.2	97	25.5	5.7	94
Penicillin G (10 U)	6.3	1.2	0	6.9	2.3	0
Ampicillin (10)	9.3	3.1	7	11.4	3.7	25
Carbenicillin (100)	12.0	3.8	4	16.9	5.2	13
Cephalothin (30)	12.9	2.8	6	15.8	3.1	31

[a]Figures in parentheses are potencies of the disks in micrograms (or units if the number is followed by a U).
[b]The mean value of the zones of complete inhibition

avoid any contamination by organic matter, only new glassware was used. Growth on D-glucose as the sole source of carbon and energy was determined in Centers for Disease Control medium 7727H, which contained 3 g of Na_2HPO_4, 2 g of KH_2PO_4, 0.5 g of NH_4Cl, 0.5 g of $(NH_4)_2SO_4$, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of ethylenediaminetetracetic acid-disodium salt, 0.004 g of bromothymol blue, and 1 g of D-glucose, all in 1,000 ml of water. The final pH was 6.8. This medium was filter sterilized, and 4 ml was dispensed into sterile disposable borosilicate glass tubes (100 by 13 mm). Each tube was inoculated with 0.001 ml (72-prong inoculator) of a 24-h heart infusion broth culture. Serial transfers (0.001 ml into 4 ml of fresh medium) were made at 1- to 3-day intervals for seven transfers (estimated to be about 70 cell divisions since the original inoculum) to eliminate any nutrients carried over from the original heart infusion broth inoculum. If a culture grew and fermented glucose for all seven transfers, we considered it able to grow on glucose as the sole source of carbon and energy. A vitamin requirement was established if the culture would not grow for seven transfers in medium 7727H, but would grow through seven transfers (0.001 ml into 4 ml of fresh medium each time) of medium 7727H supplemented with the following vitamins: 0.813 mg of thiamine, pyridoxine, pyridoxamine dihydrochloride, pyridoxal hydrochloride, pantothenic acid (hemi-calcium salt), riboflavin, nicotinamide, niacin, *p*-aminobenzoic acid, folic acid, lipoic acid, and glutathione per liter; and 0.0813 mg each of biotin, cyanocobalamine (vitamin B_{12}), and menadione per liter.

Lactose fermentation, the "coliform" concept, and the "fecal coliform" concept. These tests were done according to instructions in the 14th edition of *Standard Methods for the Examination of Water and Wastewater* (1), except that pure cultures were used. Each tube was inoculated with 10^3 to 10^4 organisms grown in brain heart infusion broth (except brilliant green bile broth and EC medium, which were inoculated with 0.001 ml of a 48-h-old culture in lauryl tryptose broth as required in *Standard Methods*). The insert tubes were observed for gas information; the tubes were gently shaken, and the presence or absence of gas bubbles was noted.

RESULTS

Nomenclature. On 1 January, 1980, the following names lost standing in nomenclature: the genus *Kluyvera* Asai, Okumura, and Tsunoda 1957; the species *Escherichia* ("*Kluyvera*") *citrophila* (Asai, Okumura, and Tsunoda 1957) Asai, Iizuka, and Komagata 1962; and the species *Escherichia* ("*Kluyvera*") *noncitrophila* (Asai, Okumura, and Tsunoda 1957) Asai, Iizuka, and Komagata 1962. The species definitions eventually proposed in our study did not correlate with the ability to utilize citric acid as postulated by Asai and co-workers (4, 5) so we thought it unwise to "revive" the names "*citrophila*" and "*noncitrophila*" (see reference 23, p. 230). To avoid confusion we felt it best to propose the genus name *Kluyvera*, but without either species name used previously. Enteric Group 8 and API group 1 are synonyms of *Kluyvera*. When we made a preliminary proposal on *Kluyvera* (G. R. Fanning, J. J. Farmer III, J. N. Parker, G. P. Huntley-Carter, and D. J. Brenner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, I30, p. 100), we were sure that the two species names would appear on the "Approved Lists of Bacterial Names" (23) since they were validly published and were represented by type strains. They were not, however, included on the Approved Lists. In this article, we propose *Kluyvera* gen. nov. as a new genus in the family *Enterobacteriaceae* with two species, *K. ascorbata* sp. nov. (pronunciation = ah skohr bah' tah; holotype strain =

American Type Culture Collection [ATCC] 33433) and *K. cryocrescens* sp. nov. (pronunciation = cry oh kress' enz; holotype strain = ATCC 33435). *Kluyvera* is a Latinized form of Kluyver, after the late A. J. Kluyver, who many consider to be the father of microbial physiology. The genus name is treated as a modern (neo) Latin feminine noun. The species name "*ascorbata*" is derived from the modern chemical term "ascorbate" designating the salt of ascorbic acid. "Ascorbate" is derived from the modern Latin word "ascorbic," which is derived from "a" (Greek, for "negative") and "scorbutus" (Latin, for "scurvy"). The intended meaning is "the ascorbate *Kluyvera*," or less literally, "the *Kluyvera* which has a positive ascorbate test." The species name "*cryocrescens*" is derived from "kryos" (Greek noun, for "cold") and from "crescens" (Latin feminine participle, for "growing"). The intended meaning is "the *Kluyvera* which grows in the cold," because *K. cryocrescens* can grow and ferment glucose at 5°C. Both species names are treated as modern Latin feminine adjectives which agree in gender with the feminine generic name *Kluyvera*. The type species for the genus *Kluyvera* is *K. ascorbata*. Several cultures of *Kluyvera* did not fit *K. ascorbata* or *K. cryocrescens*. We propose the vernacular name *Kluyvera* species group 3 for them. No scientific name(s) will be proposed until more strains become available.

DNA-DNA hybridization. Table 4 and Fig. 2 show that *Kluyvera* is made up of three hybridization groups. Strain 408-78 was more than 80% related to 26 strains and was 69 and 77% related to 2 other strains. This group of 29 strains which was highly related to strain 408-78 was initially called "*Kluyvera* hybridization group 1" and was eventually named *K. ascorbata*. Strain 409-78 was related by 75% or more to nine other strains. This group of 10 strains was initially called "*Kluyvera* hybridization group 2" and evantually was named *K. cryocrescens*. (The name Enteric Group 12 has been used to report these cultures.) Five other strains were 60 to 68% related to both *K. ascorbata* and *K. cryocrescens*. These probably represent one or more additional species of *Kluyvera*. The vernacular name *Kluyvera* species group 3 will be used until more strains become available for study. (The names Enteric Group 36 and Enteric Group 37 have been used to report these cultures.) Figure 2 shows that all three *Kluyvera* groups were much more closely related to each other than to other genera of *Enterobacteriaceae*. This closer genetic relatedness and striking phenotype similarity argue that *Kluyvera* is a distinct genus in the family *Enterobacteriaceae*. The closest relatives of *Kluyvera* species were species of the genera *Klebsiella*, *Enterobacter*, and *Citrobacter*.

G+C content of DNA. The moles percent of G+C were as follows: *K. ascorbata* ATCC 33433 = 56.1 (buoyant densities, 1.715 and 1.715 g/

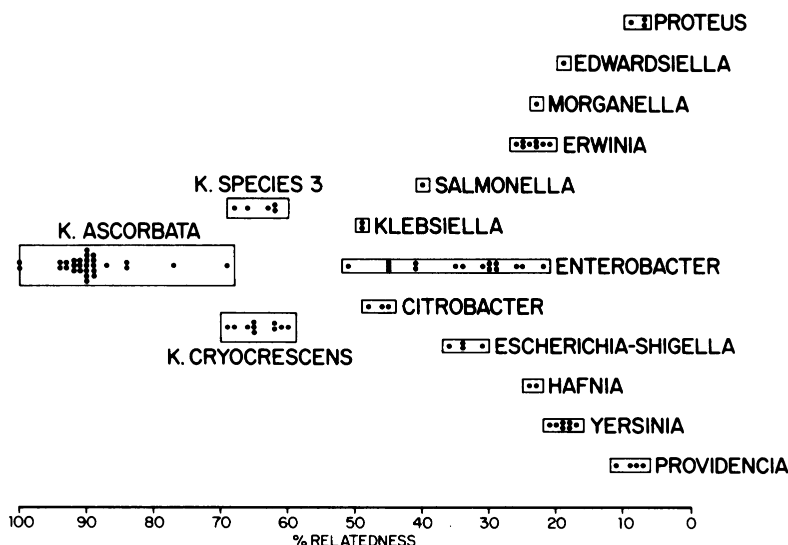

FIG. 2. *Relatedness by DNA-DNA hybridization of Kluyvera to other genera in the family Enterobacteriaceae. The other strains were compared to strain ATCC 14236 of K. ascorbata labeled with* ^{32}P. *Percent relatedness is based on the percent DNA reassociation at 60°C. The spatial location on the undesignated vertical axis is arbitrary.*

TABLE 4. *Identification of 44 Kluyvera strains by DNA-DNA hybridization*

Test strain		% Relatedness of test strain to reference strain:					
		K. ascorbata 0408-78			*K. cryocrescens* 0409-78		
		60°C	Divergence	75°C	60°C	Divergence	75°C
K. ascorbata	0408-78	100[a]	0[a]	100[a]	65	11	45
	1322-75	100	1	95	69	12	44
	2589-70	94	2	86	72	12	38
	0972-78	94	1	88	65	12	36
	1402-74	93	2	86	71	12	47
	2221-78	93	1	85	66	12	35
	1058-74	92	2	86	68	13	41
	0412-75	92	1	84	66	11	46
	2439-76	92	1	86	66	11	45
	0842-74	91	1	88	68	11	45
	3214-75	91	1	88	67	11	40
	2070-78	91	1	89	63	11	43
	0573-74	90	1	83	66	11	44
	0648-74	90	2	95	67	12	42
	4417-74	90	1	89	63	12	40
	0204-75	90	1	90	64	11	45
	0055-76	90	1	95	69	11	46
	2892-76	90	1	89	70	10	50
	3198-77	90	1	85	64	12	44
	3858-69	89	1	86	65	12	45
	4450-74	89	1	85	65	11	43
	0720-76	89	0	85	60	12	44
	1432-76	89	1	84	63	11	44
	1153-77	89	1	91	67	12	45
	0762-75	87	1	88	63	11	46
	1220-73	84	1	82	63	11	42
	2064-78	84	5	80	66	10	49
	2066-78	77	7	62	66	11	44
	0556-74	69	6	65	55	11	38
K. cryocrescens	0409-78	62	12	41	100	0	100
	3317-75	60	10	44	96	0	103
	2065-78	69	11	46	94	1	93
	0412-78	65	11	43	88	0	87
	0411-78	61	11	39	86	1	88
	1396-73	68	11	47	80	8	69
	0410-78	65	12	44	80	8	64
	0546-78	65	11	43	80	9	64
	2063-78	66	11	44	78	7	61
	1734-74	62	11	42	75	8	63
K. species group 3	2774-70	68	9	53	64	11	43
	2065-76	66	11	49	67	12	43
	2891-76	63	10	45	61	11	40
	4246-74	62	11	45	60	11	49
	3108-76	62	10	43	61	11	42

[a]The first value is the % relatedness of the reference strain to the test strain when the reassociation is done at 60°C. The second value is the percent divergence (synonym = ΔTm) which is a measurement of the relatedness of the DNA nucleotide sequences of the pair of strains. The larger the value of the divergence, the less related the strains are (for each percent of unpaired bases, divergence will increase by about 1°C). The third value is the % relatedness of the reference strain to the test strain when the reassociation is done at 75°C.

cm^3), *K. ascorbata* 1058-74 = 56.6 (buoyant densities, 1.715 and 1.716 g/cm^3), *K. cryocrescens* ATCC 14237 = 55.1 (buoyant densities, 1.714 and 1.714 g/cm^3) and *K. cryocrescens* 0546-78 = 55.1 (buoyant densities, 1.714 and 1.714 g/cm^3).

TABLE 5. *Biochemical reactions for 100 strains of Kluyvera*

Test	Cumulative % positive at:			Reactions for type strains:	
	24h	48h	7d	*K. ascorbata* ATCC 33433	*K. cryocrescens* ATCC 33435
Indole production	83	89	ND	+[a]	+
Methyl red	100	100	ND	+	+
Voges-Proskauer	0	0	ND	–	–
Citrate (Simmons') utilization	93	97	97	+	+
H_2S production (TSI[b] or PIA[b])	0	0	0	–	–
Urea-Christensen's	0	0	0	–	–
Phenylalanine "deaminase"	0	ND	ND	–	–
Lysine "decarboxylase" – Moeller's	87	87	87	+	–
Arginine "dihydrolase" – Moeller's	0	0	2	–	–
Ornithine "decarboxylase" – Moeller's	100	100	100	+	+
Motility	94	97	97	+	+
Gelatin liquefaction at 22°C	0	0	0	–	–
KCN, growth in	93	95	96	+	+
Malonate utilization	3	94	94	$+^2$	$+^2$
D-Glucose-acid production	100	100	100	+	+
D-Glucose-gas production	81	83	83	+	+
Acid production from:					
Lactose	96	98	99	+	+
Sucrose	99	99	99	+	+
D-Mannitol	99	99	99	+	+
Dulcitol	27	29	29	–	–
Salicin	100	100	100	+	+
Adonitol	0	0	0	–	–
i-(myo) Inositol	0	0	0	–	–
D-Sorbitol	41	41	41	–	–
L-Arabinose	99	100	100	+	+
Raffinose	98	98	98	+	+
L-Rhamnose	100	100	100	+	+
Maltose	100	100	100	+	+
D-Xylose	97	98	98	+	+
Trehalose	100	100	100	+	+
Cellobiose	92	100	100	+	+
α-Methyl-D-glucoside	97	98	98	+	+
Erythritol	0	0	0	–	–
Melibiose	97	98	98	+	+
D-Arabitol	0	0	0	–	–
Glycerol	13	15	95	$+^7$	$+^7$
D-Mannose	100	100	100	+	+
D-Galactose	100	100	100	+	+
Mucate – acid production	95	96	99	+	+
Tartrate, Jordan's	0	0	0	–	–
Esculin hydrolysis	100	100	100	+	+
Acetate utilization	19	69	100	$+^3$	$+^3$
Citrate – Christensen's	94	95	98	+	+
Lipase (corn oil)	0	0	0	–	–
"Deoxyribonuclease" – 25°C or 36°C	0	0	0	–	–
Oxidase – Kovacs'	0	ND	ND	–	–
$NO_3^- \rightarrow NO_2^-$	100	ND	ND	+	+
ONPG test	100	100	100	+	+
Pectate hydrolysis	0	0	0	–	–
Pigment production	0	0[c]	0[c]	–	–
Tyrosine clearing	0	0	0	–	–

[a]Symbols: ND = Not Done, + = Positive at 24h, – = Negative at end of the incubation period, $+^2$ = positive at day 2 (the superscript gives the day the reaction became positive).
[b]Abbreviations: TSI = Triple Sugar Iron Agar, PIA = Peptone Iron Agar, ONPG = O-Nitrophenyl-β-D-galactoside.
[c]4% of the strains produced red-blue crystals in and around the colonies with prolonged incubation.

Biochemical reactions. Table 5 gives the biochemical reactions of *Kluyvera* in the tests often used in American clinical laboratories for identification. The strains were very uniform in their reactions and were distinct from any other described species of *Enterobacteriaceae*, which was the original reason we reported them as unidentified and later defined Enteric Group 8. The biochemical reactions for the type strains of *K. ascorbata* (ATCC 33433) and *K. cryocrescens* (ATCC 33435) are also given in Table 5.

Description of *Kluyvera*. A brief description of the genus *Kluyvera* would include: gram-negative rods, motile with peritrichous flagella (however, arrangement of flagella may be interpreted as polar; see reference 3), oxidase negative, catalase positive, nitrate reduced to nitrite, D-glucose and other carbohydrates fermented with the production of acid and usually gas, large amounts of α-ketoglutaric acid produced during fermentation (2), G+C content of DNA of 55 to 57 mol% (buoyant density), and isolated from soil, water, sewage, food and human clinical specimens. A further description of the genus and its two species is given in the tables, figures, and text. The type species is *K. ascorbata*.

Differentiation of *K. ascorbata* and *K. cryocrescens*. None of the 50 tests routinely used for identifying members of the family *Enterobacteriaceae* (12–14) correlated exactly with the two distinct groups obvious after DNA-DNA hybridization. Other tests were therefore tried. Two of these, the ascorbate test and glucose fermentation at 5°C, correlated almost exactly with the hybridization groups (Table 6). Other biochemical tests which correlated somewhat with the hybridization data are also listed. Strains which were indole negative or lysine negative or which did not produce gas were more likely to be *K. cryocrescens* than *K. ascorbata*. A total of 45% of the *K. ascorbata* strains fermented dulcitol, but none of *K. cryocrescens* strains did.

Differentiation of *Kluyvera* from other, similar members of the family *Enterobacteriaceae*. Table 7 gives the tests (results from tests done at the Institut Pasteur) which should prove useful in differentiating *Kluyvera* species from other indole-positive, Voges-Proskauer-negative species in the family. *Kluyvera* is usually positive in Moeller's lysine "decarboxylase" and is raffinose positive. Few other indole-positive groups share these properties. The test for β-xylosidase, which is often used in France, may also prove useful in identifying *Kluyvera* species.

Biogroups. Although the biochemical reactions of *Kluyvera* were fairly uniform, there was some variation among the strains (Table 5). This variation allows the definition of biogroups, which are defined and summarized in Tables 8 and 9 (gas$^-$ refers to gas production from D-glucose). The biogroups should be helpful in identifying newly isolated strains and can be used as markers to study the ecology and epidemiology of *Kluyvera*.

Production of blue and red crystals. As previously mentioned, streak plates on Trypticase soy agar were held at room temperature for several weeks before they were discarded. It was noted that occasional colonies of four strains turned blue-purple with time; typical colonies are shown in Fig. 3. With magnification (×10 to ×60 with a dissecting microscope), a mixture of tiny red and blue crystals was noted in and around the colonies which appeared blue. We propose the name "kluyveramycin" for this mixture of blue and red crystals in the hope that it may have a use in microbiology or medicine. Strain 2221-78 of *K. ascorbata* was a strong producer of kluyveramycin and it was deposited in the ATCC as no. 33434.

Antimicrobial susceptibility. *Kluyvera* was generally susceptible to antibiotics. Table 3 gives the mean zones of inhibition and their standard deviations for both species. Early in the study it was apparent that *K. cryocrescens* often had large zones of inhibition (often with colonies in the zones) around the disks for car-

TABLE 6. *Differentiation of K. ascorbata and K. cryocrescens with biochemical tests*

Test	*K. ascorbata*[a]		*K. cryocrescens*[a]	
Ascorbate test	+[b]	97%[b]	–	0%
D-Glucose-acid (5°C, 21 d)	–	3%	+	100%
Lysine "decarboxylase" – Moeller's	+	100%	V	50%
D-Glucose-gas	⊕	86%	V	50%
Dulcitol-acid	V	45%	–	0%

[a] Based on the 29 strains of *K. ascorbata* and 10 strains of *K. cryocrescens* definitively identified by DNA-DNA hybridization.
[b] The percent positive at 48h, 36°C unless otherwise stated, + = 90-100% positive, ⊕ = 75-89% positive, V = 26-74% positive, ⊖ = 11-25% positive, – = 0-10% positive.

TABLE 7. *Differentiation of indole⁺, Voges-Proskauer⁻ species of Enterobacteriaceae isolated in clinical microbiology laboratories*

Test	Reaction for:					
	Escherichia coli	*Citrobacter diversus*	*Citrobacter "Levinea" amalonaticus*	*Kluyvera*	*Enterobacter agglomerans* biogroup G3	*Enterobacter agglomerans* biogroup 5
Indole production	+[a]	+	+	⊕	+	+
Citrate-Simmons'	–	+	+	+	+	+
Lysine "decarboxylase" – Moeller's	⊕	–	–	⊕	–	–
Arginine "dihydrolase" – Moeller's	–	–	⊖	–	–	–
Ornithine "decarboxylase" – Moeller's	V	+	+	+	–	–
KCN-growth in	–	–	+	+	⊕	V
Malonate	–	+	–	+	⊕	V
Yellow pigment	–	–	–	–	⊕	⊕
"β-Xylosidase"	–	–	–	+	V	V
"β-Glucuronidase"	⊕	–	–	–	–	–
"Tetrathionate reductase"	–	–	+	–	⊖	V
D-Glucose-gas production	⊕	+	+	⊕	+	–
Acid production from:						
Lactose	+	–	–	+	V	–
Adonitol	–	+	–	–	⊖	–
L-Sorbose	⊖	–	+	–	–	–
D-Sorbitol	+	+	+	V	V	V
Dulcitol	V	V	–	V	V	V
Sucrose	V	V	–	+	–	–
Salicin	V	–	–	+	V	V
Raffinose	⊖	–	–	+	–	–
Melibiose	⊕	–	–	+	⊕	V

[a] + = 90-100% positive, ⊕ = 75-89% positive, V = 26-74% positive, – = 11-25% positive, ⊖ = 0-10% positive. Data are based on reactions which occur within 48 hours (Institute Pasteur's data).

TABLE 8. *Biogroups of Kluyvera ascorbata*

Biogroup	Number of strains	Fermentation of: Dulcitol	Fermentation of: D-Sorbitol	Other phenotypic characteristics which differ from the wild type
1	37	–[a]	–	
2	18	+	+	
3	5	–	+	
4	4	+	+	Indole⁻[b]
5	2	–	–	Gas⁻
6	2	–	–	Lysine⁻
7	2	+	+	Gas⁻, KCN⁻
8	1	–	–	Mucate⁻
9	1	–	–	α-methyl-D-glucoside⁻
10	1	–	–	Citrate⁻, motility⁻
11	1	–	–	Raffinose⁻, melibiose⁻
12	1	–	–	Indole⁻, lysine⁻, motility⁻
13	1	+	+	Gas⁻, malonate⁻
14	1	+	+	Indole⁻, gas⁻, lactose⁻
15	1	–	+	Indole⁻, gas⁻

[a] Symbols: + = positive within 2 days, – = negative at 2 days.
[b] Negative reactions are for the end of the incubation period.

TABLE 9. *Biogroups of Kluyvera cryocrescens*

Biogroup	Number of strains	Reactions For: Lysine	D-Sorbitol	Gas	Other phenotypic characteristics which differ from the wild type
1	3	+[a]	+	+	
2	2	−	−	−	
3	2	−	−	+	
4	1	+	+	+	Citrate⁻, KCN⁻[b]
5	1	+	+	+	Motility⁻, KCN⁻
6	1	−	−	−	Indole⁻, D-Xylose⁻
7	1	−	−	−	Citrate⁻, ONPG⁻
8	1	+	+	−	
9	1	+	−	−	Indole⁻, D-Mannitol⁻, α-methyl-D-glucoside⁻
10	1	−	+	−	
11	1	−	+	+	
12	1	−	−	+	Malonate⁻
13	1	−	−	+	Indole⁻, D-Xylose⁻

[a] Symbols: + = positive within 2 days, − = negative at 2 days
[b] Negative reactions are for the end of the incubation period

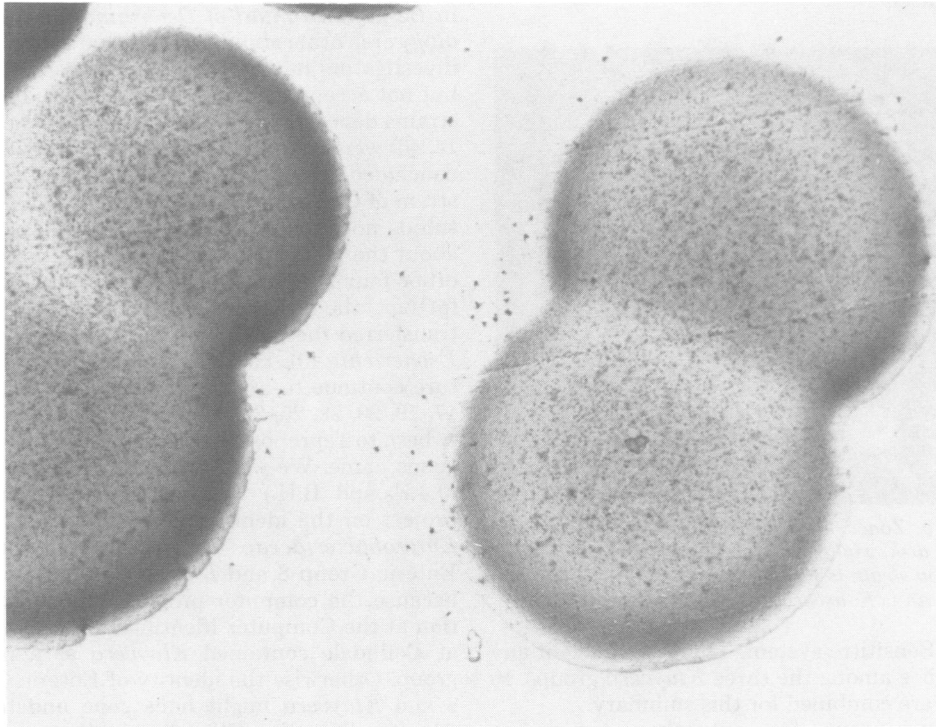

FIG. 3. *Kluyveramycin crystals in and around the colonies of K. ascorbata ATCC 33434.*

benicillin and cephalothin. Before the ascorbate test and glucose fermentation at 5°C were developed, these large zones on the antibiogram plate were the only way to differentiate the two species. Figure 5 shows the zones of complete inhibition (see Materials and Methods for the definition of this term) against carbenicillin and cephalothin. Two distinct groups are formed when these zones are plotted (Fig. 4), and only 2 of the 39 strains gave results which did not agree with their species assignment based on DNA-DNA hybridization. The difference in these zone sizes (Fig. 4) provides an additional way to differentiate *K. ascorbata* and *K. cryocrescens*. Table 10 gives the minimum inhibitory concentrations (broth dilution) of *Kluyvera*

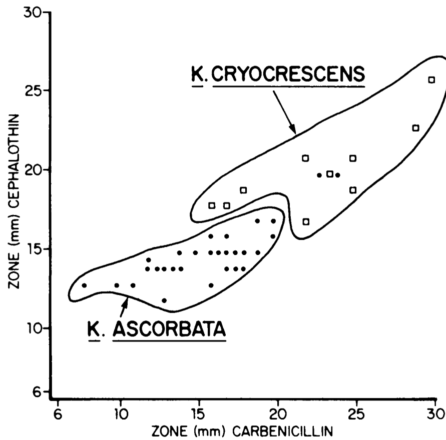

FIG. 4. *Differentiation of K. ascorbata and K. cryocrescens based on the zones of complete inhibition around carbenicillin (100-μg disk) and cephalothin (30-μg disk).*

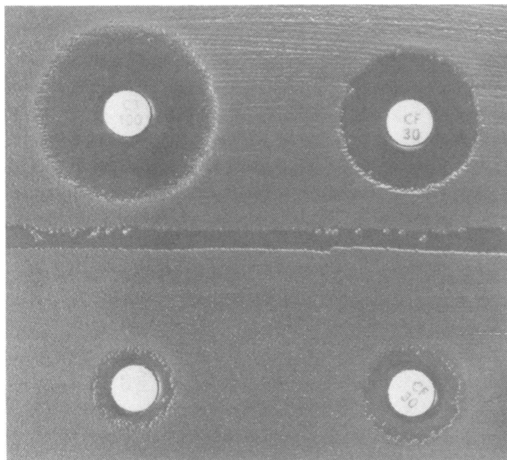

FIG. 5. *Zones of inhibition around cephalothin (30-μg, disk, right) and carbenicillin (100-μg disk, left). Top strain is K. cryocrescens ATCC 33435; bottom strain is K. ascorbata 1402-74.*

in the Sensititre system. There was little if any difference among the three *Kluyvera* groups, so they were combined for this summary.

Requirement for vitamins or amino acids. Twenty-seven of the 29 strains of *K. ascorbata* grew through seven transfers on glucose as the sole source of carbon, as did 5 of 10 strains of *K. cryocrescens* and 5 of 5 strains of *Kluyvera* species group 3. All of the strains grew through seven transfers when trace amounts of 15 vitamins were added to the glucose-basal salts medium. Thus, it appears that none of the *Kluyvera* strains required an amino acid, but that two strains of *K. ascorbata* and five strains of *K. cryocrescens* required one or more vitamins.

Coliform concept. In the test for lactose fermentation used for identification (enteric fermentation base with Andrade indicator and 1% filter-sterilized lactose), 90% of the *Kluyvera* strains fermented lactose with the production of acid and gas. At 36°C, 89% of the strains also produced gas from lactose in the liquid media used in coliform analysis. In the confirmatory test, 87% produced gas in brilliant green bile lactose broth. At 45°C in EC medium, however, none of the strains formed gas. Thus, *Kluyvera* can be considered as "coliform," but not a "fecal coliform."

DISCUSSION

The genus *Kluyvera* has had a turbulent history since it was first proposed by Asai and his co-workers in 1956. Few workers were familiar with the genus, because it had not been included in *Bergey's Manual of Determinative Bacteriology* or in other standard reference books. Some investigators have tested the *Kluyvera* strains, but not accepted them as new species. The five strains deposited in the ATCC (ATCC 14236 to 14240) were examined by Ewing (10, p. 278) who concluded, "One of these (84C) proved to be a strain of *C. freundii* (indole produced, hydrogen sulfide not produced)." No comment was made about the identity or taxonomic position of the other four strains (10). To complicate matters further, the original proposers of the genus transferred the two named species to the genus *Escherichia* (3). However, reports in the literature continue to use the name *Kluyvera* (6, 16, 17, 19, 20, 22, 25, 26), which is why we thought it best to repropose it rather than coin a new genus name. We were fortunate that two of us (J.J.F. and B.H.) were doing a collaborative project on the identification of new groups of *Enterobacteriaceae*. The relationship between Enteric Group 8 and *Kluyvera* was discovered because the computer programs for identification at the Computer Identification Laboratory at Colindale contained *Kluyvera* as a named group. Otherwise the identity of Enteric Group 8 and *Kluyvera* might have gone undetected. Almost all of the *Kluyvera* strains were sent because the original laboratory observed that their isolate did not fit any of the existing species in the family *Enterobacteriaceae* (7, 21). Several of the strains were sent as "*E. coli* citrate positive??"; others were referred as "*Enterobacter*?" or "*Citrobacter*?" Our first impression after studying the biochemical reactions of the new group was that it was a *Citrobacter-Enterobacter* "intermediate" (7). All these vernacular (common) names can now be dropped in favor

TABLE 10. *Minimum inhibitory concentrations for 9 antibiotics*[a]

Antibiotic	Cumulative percent of strains inhibited at antibiotic concentration (μg/ml) of:											
	.25	.5	1	2	4	8	16	32	64	128	256	512
Ampicillin		4			12	31	50	81[b]				
Carbenicillin						4	12	15	31	42	77	81
Cephalothin					4	12	35	58	81	96		
Amikacin		12	69	92	100							
Gentamicin	50	96	100									
Kanamycin			23	81	96	100						
Tobramycin	35	92	96	100								
Chloramphenicol			4	19	62	92	96	96	96			
Tetracycline		4	15	50	81	88	88	96				

[a]Based on 10 strains each of *K. ascorbata* and *K. cryocrescens* and 6 of *Kluyvera* species group 3.
[b]If the last value is not 100, the test system did not include any higher concentrations of antibiotic.

of the scientific name *Kluyvera*.

Our data indicate that *Kluyvera* is a new genus in the family *Enterobacteriaceae*. Biochemically, *Kluyvera* is distinct from all other named genera, and it is also distinct by DNA-DNA hybridization (Fig. 2). Our concept of a genus is a group of distinct species which are more closely related to each other by DNA hybridization than they are to other species or genera. A new genus should also be phenotypically distinct from other genera, and all species in the genus should be similar enough so that a genus definition can be logically formed. Ideally, the species should be phenotypically distinct from each other, so that species definitions can be easily formed. *Kluyvera* is a good genus based on the first criterion, but the two named species are phenotypically very similar.

The two species of *Kluyvera* were initially very hard to differentiate. None of the 47 biochemical tests routinely done in the Enteric Section at the Centers for Disease Control clearly differentiated the two groups which were formed by DNA hybridization. For over a year the only tests that showed a good correlation were the zones of complete inhibition around the antibiotic disks carbenicillin and cephalothin. *K. cryocrescens* had large zones. often with resistant colonies, but *K. ascorbata* had much smaller zones. Finally, two characteristics, the ascorbate test and glucose fermentation at 5°C, were found which correlated almost exactly with the DNA hybridization data. Clinical laboratories should be able to recognize a strain of *Kluyvera* because of its differences from the other named groups in *Enterobacteriaceae*. The ascorbate test is recommended for those who wish to differentiate the two *Kluyvera* species. Others may wish to report "*Kluyvera* species," which would include all three groups. We encourage laboratories to identify *Kluyvera* to the species level so that more can be learned about the ecology, epidemiology, and role in human diseases of *K. ascorbata*, *K. cryocrescens*, and *Kluyvera* species group 3. We have now reported 11 strains as *Kluyvera* species group 3 because they were either different from both named species by DNA hybridization or different in their phenotypic reactions. These strains probably represent additional species of *Kluyvera*, but more study is required before a scientific name(s) can be assigned.

Concerning the clinical significance of *Kluyvera*, Schwach (21) reported three isolates of Enteric Group 8, all from upper respiratory tract specimens, which we subsequently received and identified (phenotypically) as *K. ascorbata*. Since the strains were in mixed cultures, and not detected in subsequent culture, she concluded that they were probably not clinically significant. Braunstein and co-workers (7) recently reported on two cultures that we had identified as Enteric Group 8 (both are *K. ascorbata*). One of these was from the sputum of a 6-year-old boy with pulmonary tuberculosis. This isolate was not considered clinically significant. A second isolate was from gall bladder drainage fluid of a 63-year-old woman with acute pancreatitis. On the basis of chart review, this isolate was considered clinically significant. We are aware of no other published reports assessing the clinical significance of Enteric Group 8 or *Kluyvera*. Of our 144 *Kluyvera* strains, none have been from spinal fluid, but 5 strains have been from blood: 3 strains (2 from France) of *K. ascorbata*, 1 strain of *K. cryocrescens* (from a 3-month-old child, at autopsy), and 1 strain of *Kluyvera* species group 3. No other information was included to assist in the evaluation of the five isolates. These five blood isolates and the report

of Braunstein and co-workers (7) suggest that *Kluyvera* is more than a benign saprophyte. Most new species of *Enterobacteriaceae* have at least attained the status of "infrequent opportunistic pathogen." On the basis of present knowledge this status also seems appropriate for *Kluyvera*. The respiratory tract was the most common source for *Kluyvera*, but there is no strong evidence that it is clinically significant. (One isolate of *K. ascorbata* was, however, from a lung at autopsy.) The respiratory tract (particularly sputum) is notoriously difficult to evaluate for clinical significance except in carefully designed prospective studies which include patients' antibody responses. The urinary tract was the next most common source, but there was no mention of more than 100,000 organisms per ml of urine or the presence of leukocytes or erythrocytes. Feces was a common site of isolation, and the documented presence of *Kluyvera* in food (16; Table 1) is an obvious source of these isolates. Since fecal cultures are usually taken because the patient has diarrhea, the role of *Kluyvera* as a possible cause should be investigated prospectively. Further study is needed to fully understand the ecology and epidemiology of *Kluyvera* and to define its role in disease. We hope that this report will stimulate others to identify strains of *Kluyvera* and determine their source and significance.

ACKNOWLEDGMENTS

We thank Manley Mandel for doing the G+C determinations, A. G. Steigerwalt for preparing the DNA for these determinations, Martin Busse for *Kluyvera* cultures isolated from food (16) and water (26), T. Schwach for more information on her three case reports (21), Karla Tomfohrde for furnishing *Kluyvera* cultures referred to Analytab Products for identification, John Holt and Warren Armstrong for suggesting possible names for the *Kluyvera* species, Kathleen Ann Kelley, Emory University, for helping with the idiosyncrasies of Greek and Latin grammar and for writing the section on the origins of the species names "*ascorbata*" and "*cryocrescens*," and Robert Gherna for furnishing the ATCC strains.

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