Two new *Rahnella* genomospecies that cannot be phenotypically differentiated from *Rahnella aquatilis*

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Fifty-one *Rahnella aquatilis* and *R. aquatilis*-like strains from water, snails and human sources were characterized by routine biochemical tests, carbon source utilization tests, DNA relatedness (hydroxyapatite method) and 16S rRNA sequencing. The results of the genetic methods indicated that the strains comprised three closely related species within the genus *Rahnella*. It was not possible to differentiate *R. aquatilis* from the two newly recognized species. The new species were therefore given the vernacular names *Rahnella* genomospecies 2 and *Rahnella* genomospecies 3.

Keywords: *Rahnella aquatilis*, *Rahnella aquatilis*-like strains, *Rahnella* genomospecies, taxonomy, DNA relatedness

INTRODUCTION

During a numerical taxonomic study of the genus *Enterobacter*, Gavini *et al.* (13) used the name Group H₂ for phenotypically similar, phenylalanine deaminase-positive, lysine- and ornithine-negative, arginine dihydrolase-negative strains. Subsequent DNA relatedness studies on Group H₂ led Izard *et al.* (25) to propose the new genus and species name *Rahnella aquatilis* for these organisms. All the strains reported by Gavini *et al.* (13) were isolated from water in France. Farmer *et al.* (10) isolated *R. aquatilis* from water in the United States and documented the first human isolate from a burn infection (10). Subsequent reports indicated that *R. aquatilis* is widely distributed in nature (2, 19, 20, 23, 24, 36), present in foods (8, 17), and can occasionally be isolated from a variety of

human sources, especially from immunocompromised patients (1, 5, 6, 12, 14, 16, 18, 22, 27–29, 33, 34).

Between 1984 and 1988, Müller *et al.* (30–32) cultured the intestinal contents of snails, slugs and earthworms for *Enterobacteriaceae*. Among the 2446 isolates obtained were a number of new species of *Buttiauxella* and *Kluyvera*, as well as other infrequently seen species, including *Ewingella americana* and *Serratia fonticola* (30–32), and nine strains that resembled *R. aquatilis*. In this report, we present the phenotypic and genotypic data used to characterize these *Rahnella*-like strains, as well as human and water isolates. Three DNA hybridization groups (genomospecies) were evident, one of which corresponded to *R. aquatilis*. The two new genomospecies could not be phenotypically differentiated from *R. aquatilis* with certainty and are therefore described, but not formally named.

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Abbreviations: pNP, *para*-nitrophenyl; pNA, *para*-nitroanilide; CDC, Centers for Disease Control and Prevention; JLU, Justus-Liebig-Universität; SM, Staatliches Medizinaluntersuchungsamt.

The GenBank accession numbers for the sequences reported in this paper are U88434–U88439, U90757 and U90758.

METHODS

Bacterial strains. Fifty-one *R. aquatilis* and *R. aquatilis*-like strains were studied (Table 1). Cultures were cultivated at 30 °C and stored on nutrient agar slants.

Table 1. List of *R. aquatilis* and *R. aquatilis*-like strains

The following abbreviations are used in this table: CCUG, Culture Collection, University of Göteborg, Sweden; CDC, Centers for Disease Control and Prevention, USA; CUETM, Collection de l'Unité d'Ecotoxicologie Microbienne, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; MCCM, Mannheim, Germany; SHD, State Health Department, USA; SM, Staatliches Medizinaluntersuchungsamt, Germany.

Strain designation	Source	Country	Donor
Genomospecies 1			
CCUG 14185 ^T (MCCM 01700; CUETM 77-115)	Water	France	W. Mannheim
CCUG 21234 (MCCM 01659)	Not given	Not given	Not given
CDC 5686-72	Not given	USA	Virginia SHD
CDC 2042-78	Water	USA	Oregon SHD
CDC 1327-79 ^T (CUETM 77-115; ATCC 33071)	Water	France	H. Leclerc
CDC 2987-79 (CUETM 78-71)	Water	France	H. Leclerc
CDC 2989-79 (CUETM 77-143)	Water	France	H. Leclerc
CDC 1129-80 (CUETM 77-142)	Water	France	F. Gavini
CDC 2789-79A	Not given	Not given	Not given
CDC 1131-80 (CUETM 78-68)	Water	France	F. Gavini
CDC 1132-80 (CUETM 78-80)	Water	France	F. Gavini
CDC 1133-80 (CUETM 78-85)	Water	France	F. Gavini
CDC 1134-80 (CUETM 78-99)	Water	France	F. Gavini
CDC 1135-80 (CUETM 78-101)	Water	France	F. Gavini
CDC 1136-80 (CUETM 77-133)	Water	France	F. Gavini
CDC 742-81	Human stool	USA	California SHD
CDC 4301-95	Human blood	Switzerland	G. Funke
CDC 4591-86	Human sputum	USA	North Carolina SHD
DSM 4594 ^T (CUETM 77-115)	Water	France	DSM
DSM 30075	Not given	Not given	DSM
Genomospecies 2			
CDC 301-78	Water	USA	Georgia SHD
CDC 301-78A	Water	USA	Georgia SHD
CDC 658-79	Human burn	USA	North Carolina SHD
CDC 1130-80 (CUETM 77-146)	Water	France	F. Gavini
CCUG 21213 (MCCM 01948)	Not given	Not given	W. Mannheim
SM Bonn 1	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 3	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 5	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 6	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 7	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 9	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 10	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 11	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 12	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 13	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 14	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 15	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 16	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bonn 17	Human blood*	Germany	R. Schulze-Röbbbecke
SM Bremen 24	Human	Germany	E. von Pritzbuier
SM Hamburg 224-36	Human	Germany	J. Bockemühl
SM Italy 2383	Snail	Italy	H. E. Müller
SM SA 1224	Snail	Germany	H. E. Müller
SM SA 1245	Snail	Germany	H. E. Müller
SM S7/1-576	Snail	Germany	H. E. Müller
SM S7/1-682	Snail	Germany	H. E. Müller
SM S7/1-852	Snail	Germany	H. E. Müller
SM S7/1-862	Snail	Germany	H. E. Müller
SM S7/2-1213	Snail	Germany	H. E. Müller
SM S7/2-1215	Snail	Germany	H. E. Müller
Genomospecies 3			
DSM 30078	Not given	Not given	DSM

* Stored donated blood.

DNA hybridization. The methods used to prepare, isolate and purify labelled and unlabelled DNA, the method used for DNA reassociation, and the method used to separate single-stranded and double-stranded DNA on hydroxyapatite have been described previously (3). DNA relatedness was determined at optimal (60 °C) and, on selective strains, at stringent (75 °C) reassociation criteria (Table 2). Percentage divergence was calculated to the nearest 0.5 %.

16S rRNA gene sequencing. DNA was purified as previously reported (3). Purified genomic DNA was diluted to 1 µg ml⁻¹ in sterile water. Ten microlitres of the diluted DNA was used in a 100 µl PCR containing 200 µM dNTPs, 1 mM MgCl₂, 1 × PCR buffer II (Perkin-Elmer), 0.1 µM fD1 primer, 0.1 µM rD1 primer, and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer). The primers fD1 and rD1 were originally described by Weisburg *et al.* (41) as suitable for amplifying the 16S gene of many eubacteria. We omitted the linker sequences from the primers as previously described (7). The parameters for the amplification were: 94 °C for 5 min; 35 cycles at 94 °C for 15 s, 50 °C for 15 s and 72 °C for 1.5 min; followed by a 72 °C extension for 5 min before cooling to 4 °C.

The results of the PCR reaction were checked by running 10 µl from each reaction on a 1 % agarose gel. The 16S DNA was then purified and concentrated by using a High Pure PCR Purification kit (Boehringer Mannheim). The concentration of the 16S PCR product was determined by using a Fluorescence Concentration Analyzer (IDEXX Laboratories). Sixty nanograms of PCR product was used for each sequencing reaction. The sequencing reaction consisted of 16S DNA, 8 µl ABI PRISM from the dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and 3.2 pmol primer and was made up to 20 µl with sterile water. The primer set used for sequencing was derived from those designed by Stackebrandt & Charfreitag (37). The manufacturer's instructions for the cycle sequencing kit were followed for the thermal cycler conditions. The extension products from each sequencing reaction were purified through a Centriscap column (Princeton Separations) and dried in a vacuum centrifuge for 20 min.

The sequencing reactions were resolved on a 5 % acrylamide/8M urea gel electrophoresed on an ABI 373S automated sequencer (Perkin-Elmer). The sequence data were edited and compiled using the Genetics Computer Group (GCG) Sequence Analysis Package (University of Wisconsin, USA). *Rahnella* 16S rRNA sequences were aligned with 16S sequences from GenBank using the GCG program PILEUP and the multiple sequence file was edited by hand. The edited alignment was used in PHYLIP (version 3.5, J. Felsenstein, University of Washington, Seattle, USA) to derive a phylogenetic dendrogram using the nucleotide substitution model of Jukes & Cantor (26) and the neighbour-joining method of Saitou & Nei (35). The plotfile from PHYLIP 3.5 was reformatted for the HP Laserjet 4 printer with PRINTGL software (Ravitz Software).

The GenBank accession numbers for the 16S rRNA sequences are U88434–U88439, U90757 and U90758. GenBank sequences for five strains deposited as *R. aquatilis* (GenBank accession nos X79936–X79940 corresponding to strain nos 334, 2-87, 339, ATCC 33989 and 3-88, respectively) were used in the dendrogram.

Biochemical characterization. Routine biochemical tests were carried out at the Staatliches Medizinaluntersuchungsamt (SM), Braunschweig, Germany, at the Centers for Disease Control and Prevention (CDC), Georgia, USA, and at the Justus-Liebig-Universität (JLU), Giessen, Germany. At the SM, tests were done as described previously; reactions were incubated at 30 and 36 °C, and results were recorded at 48 h and 7 d (30). At CDC, tests were done as described previously; reactions were incubated at 35 °C, unless noted (see Table 3), and results were recorded at 48 h and 7 d (8, 21). At the JLU, reactions were incubated at 35 °C and results were recorded at 48 h as described previously (20, 39). The results obtained at all institutions are shown in Table 3. Carbon source utilization tests and qualitative enzyme tests were done at the JLU using microtitre plates as described previously (30, 39).

RESULTS

DNA relatedness

DNA relatedness among *R. aquatilis* and *R. aquatilis*-like strains was determined using radiolabelled DNA from the type strain of *R. aquatilis* (CDC 1327-79 = CUETM 77-115 = ATCC 33071) and from two *R. aquatilis*-like strains. Three DNA relatedness groups or genomospecies were evident. The *R. aquatilis* genomospecies contained the labelled type strain (a water isolate from France), two additional cultures of the type strain from different culture collections, nine of the remaining 10 original water isolates from France, a water isolate from the United States, three human isolates and four strains of unknown origin (Table 2). Mean relatedness among these strains was 88 % in 60 °C reactions, with a range of 72–100 %. Mean divergence within related sequences was 3.5 %, with a range of 0.0–7.0 %, and mean relatedness in 75 °C reactions was 81 %, with a range of 60–100 %. The *R. aquatilis* type strain was 70 % related, with 8 % divergence, to 30 strains subsequently designated genomospecies 2 (see below) in 60 °C reactions, and 50 % related to them in 75 °C reactions. The type strain was 64 % related with 9 % divergence at 60 °C and 48 % related at 75 °C to strain DSM 30078, which was subsequently designated genomospecies 3 (see below).

Labelled DNA from strain SM S7/1-576, a snail isolate, was highly related to a group of 29 strains that included the seven other snail isolates, one of the original French water isolates, two water isolates from the United States, 14 strains from contaminated stored human blood in Germany, one human isolate from the United States and four isolates of unknown origin. These strains were designated *Rahnella* genomospecies 2. Mean relatedness of strain SM S7/1-576 to other genomospecies 2 strains was 93 % (range of 73–100 %) with mean divergence of 1.5 % (range 0.5–5.5 %) in 60 °C reactions, and mean relatedness was 91 % (range of 68–100 %) in 75 °C reactions. Strain SM S7/1-576 DNA showed a mean relatedness in 60 °C reactions of

Table 2. DNA relatedness among *Rahnella* strains

%D represents divergence, calculated to the nearest 0.5%.

Source of unlabelled DNA	Relatedness (%) to labelled DNA from:							
	CDC 1327-79 ^T			SM S7/1-576			DSM 30078	
	60 °C	%D	75 °C	60 °C	%D	75 °C	60 °C	%D
Genomospecies 1								
CDC 1327-79 ^T	100	0.0	100	55	8.0	46	53	11.5
DSM 4594 ^T	100	0.0	100	50	7.5	43		
CCUG 14185 ^T	100	0.0	100	60	7.5	56		
CDC 2987-79	100	0.5		61	9.5	39		
CDC 1135-80	100	0.5	100	61	9.5	64		
CDC 2789-79A	99	1.0	100	42	9.0	57		
CDC 5686-72	93	2.5	92	58	8.0	46		
DSM 30075	91	4.0	82	60	7.5	60		
CDC 1136-80	88	5.0	73	60	8.5	46	57	11.0
CDC 1129-80	91	4.5	81	60	7.5	52		
CDC 1132-80	87	5.0	73	68	7.0	55		
CDC 1133-80	86	4.5	75	61	9.0	51	54	10.5
CDC 1134-80	85	7.0	63	54	8.5	51	55	11.0
CDC 2989-79	83	1.0	97	47	8.5	44		
CDC 742-81	82	0.0	87	44	9.0	34		
CDC 4591-86	81	5.0	74	45	8.5	44	45	12.0
CDC 4301-95	79	5.0	73	52	8.5	43	46	11.5
CDC 2042-78	77	4.0	72	59	8.5	44	56	10.5
CCUG 21234	74	6.5	62	63	7.0	49		
CDC 1131-80	72	6.0	60	56	6.5	56		
SM S7/1-576	71	7.5	51	100	0.0	100	61	11.0
Genomospecies 2								
SM S7/2-1215	70	8.5	51	100	0.5	95		
SM Bonn 15	76	9.0	50	100	0.5	100		
SM Bonn 16	75	9.0	50	99	0.5	99		
SM Bonn 17	76	9.0	60	99	1.0	98		
SM Bremen 24	81	6.0	59	98	2.0	86		
SM SA 1245	66	7.0	45	97	1.5	89		
SM SA 1224	66	7.0	48	97	1.5	90		
CDC 658-79	70	6.5	52	97	1.5	97		
SM S7/2-1213	69	7.5	49	96	0.5	96		
SM S7/1-852	65	7.5	48	96	1.0	87		
CCUG 21213	64	7.5	47	96	1.0	91		
SM Bonn 14	69	7.5	48	95	0.5	96		
SM Bonn 1	72	8.0	51	95	1.0	97		
SM Bonn 3	68	8.0	51	94	1.0	94		
SM S7/1-682	69	7.5	50	94	1.0	93		
SM Bonn 12	68	8.0	51	94	1.0	96		
SM Bonn 7	70	7.5	45	93	1.0	95		
SM Bonn 6	65	8.0	48	93	1.0	92		
SM Bonn 9	68	8.0	49	93	1.0	94		
SM Bonn 5	66	8.0	45	92	1.0	92		
SM Bonn 11	71	7.5	50	92	1.5	94		
SM Bonn 13	72	8.0	51	91	0.5	95		
SM S7/1-862	73	8.0	49	91	1.0	91		
SM Bonn 10	68	8.0	46	90	1.0	91		
SM Hamburg 224-36/89	66	8.0	45	89	1.5	83		
CDC 301-78	67	6.0	56	83	2.0	78		
CDC 301-78A	75	8.5	46	82	1.5	90		
SM Italy 2383	68	8.0	47	74	5.0	68	55	11.0
CDC 1130-80	76	9.5	49	70	5.5	74	49	11.0
Genomospecies 3								
DSM 30078	64	9.0	48	53	9.0	39	100	0.0

56%, with 8% divergence, to DNAs from the 20 strains in the *R. aquatilis* genomospecies. Mean relatedness to these strains in 75 °C reactions was 49%. Relatedness of strain SM S7/1-576 to strain DSM 30078 was 48% with 11% divergence at 60 °C.

Labelled DNA from strain DSM 30078 was 53% related to the type strain of *R. aquatilis*, with related sequences exhibiting 11% divergence. Its mean relatedness to six other *R. aquatilis* (*sensu stricto*) strains was 52% with 11% divergence. Relatedness of DSM 30078 to the reference strain of *Rahnella* genomospecies 2 was 61% with 11% divergence. DSM 30078 showed 52% relatedness, with 11% divergence, to two other strains from genomospecies 2. Since DSM 30078 was not related at the species level to either *R. aquatilis* or to *Rahnella* genomospecies 2, it was designated *Rahnella* genomospecies 3.

16S rRNA sequencing

Eight representative strains from the three *Rahnella* hybridization groups were used for 16S rRNA gene amplification and sequencing: CDC 2987-79, CDC 1327-79, DSM 30078, SM Bonn 7, SM S7/1-576, SM Italy 2383, CDC 658-79 and CCUG 21234. The 16S sequences were aligned and edited. The hypervariable regions V1 and V5 were omitted (15), resulting in a sequence that corresponded to *Escherichia coli* base positions 147–1490 (4). The dendrogram derived from the distance matrix is shown in Fig. 1.

The eight *Rahnella* strains fell into three distinct groups (Fig. 1). The *R. aquatilis* type strain group contained five strains: CDC 2987-77 and CDC 1327-79 and three *R. aquatilis* sequences previously deposited in GenBank, which were 99.70–100.00% similar to each other. The second group contained a total of seven strains: SM Bonn 7, SM S7/1-576, SM Italy 2383, CDC 658-79, CCUG 21234, and two sequences retrieved from GenBank that had been deposited as *R. aquatilis*, which were 99.40–99.92% similar to each other, and 97.77–98.37% similar to the *R. aquatilis* type strain group. The third group contained only strain DSM 30078, which was 98.29–98.58% similar to the other *Rahnella* genomospecies.

Routine biochemical characterization

The biochemical test results for strains assigned to *R. aquatilis* (GS 1) and to GS 2 and GS 3 were obtained independently in three laboratories. The data obtained at CDC (9) are shown in Table 3. Agreement of results obtained in the three laboratories was generally good, and results obtained for GS 1 strains were in close agreement with previously published biochemical profiles of *R. aquatilis* (10, 13). For GS 1, different results were obtained for ornithine decarboxylase (0% at CDC, 50% at SM) and methyl α -D-glucoside (0% at CDC, 25% at SM, and 75% at JLU). For GS 2, methyl α -D-glucoside was 0% at CDC and JLU, and 70% at SM. A number of discrepancies was seen in

reactions for the single strain (DSM 30078) in GS 3. This strain was methyl red-positive, citrate-negative, phenylalanine deaminase-negative, did not produce gas from D-glucose and was acetate-negative in tests at CDC, whereas the opposite results were obtained at SM (phenylalanine deaminase was delayed positive). In tests for acid production from carbohydrates, DSM 30078 was cellobiose-negative at CDC (positive at SM and JLU), raffinose-positive at CDC and JLU (negative at SM), and L-rhamnose-negative at CDC (positive at SM and JLU). GS 1 and GS 2 could not be differentiated on the basis of this battery of biochemical tests. GS 3 could be differentiated from GS 1 and GS 2 on the basis of its positive D-arabitol reaction and its negative dulcitol reaction.

Carbon source utilization and hydrolysis tests

Strains were tested for their ability to utilize a battery of carbohydrates and to hydrolyse a battery of substrates (Table 3). No test or test battery served to differentiate GS 1 and GS 2 strains. A combination of utilization of L-histidine, L-ornithine and L-proline, and hydrolysis of L-proline-pNA appears to be helpful. GS 3 is separable from GS 1 and GS 2 strains on the basis of its negative reactions in tests for the utilization of DL-lactate and L-glutamate- γ -3-carboxy-pNA.

DISCUSSION

Since its recognition from water isolates in 1976 (13) and its description in 1979 (25), *R. aquatilis* has been isolated from a relatively large number of environmental and human sources (1, 2, 5, 6, 8, 10, 12, 14, 16–20, 22–24, 27–29, 33, 34, 36). Our isolates from snails enlarge its habitat still further. Our DNA relatedness studies were designed to confirm the identity of these and other unusual isolates from molluscs. To our surprise, strains with the biochemical profile of *R. aquatilis* were in three separate DNA relatedness groups or genomospecies.

GS 1 consisted of 20 strains, including three separate cultures of the *R. aquatilis* type strain. It therefore corresponded to *R. aquatilis*. Seventeen of these strains conformed fully to the molecular definition of a bacterial species as a group of strains whose DNAs share at least 70% relatedness at optimal criteria for DNA reassociation (60 °C reactions in this study), and whose related sequences show 5% or less divergence (40). The three remaining strains in GS 1, CDC 1134-80, CCUG 21234 and CDC 1131-80, were 72–85% related to the type strain of *R. aquatilis*, with divergence within related sequences of 6.0–7.0%. Their relatedness to the type strain of *R. aquatilis* at stringent reassociation criteria (75 °C reactions in this study) was 60–63%. In our laboratory, we consider 60% or greater relatedness at stringent reassociation criteria to also represent species level relatedness. We tend to place strains that fulfil at least two of the three criteria for species level relatedness within the species. Re-

Table 3. Reactions of *R. aquatilis* and *R. aquatilis*-like strains in routine biochemical tests

All strains gave positive reactions within 48 h in tests for fermentation of L-arabinose, D-galactose, D-glucose, lactose, D-mannitol, ONPG, salicin and trehalose, and in the test for nitrate reduction. All strains gave negative reactions after 7 d in tests for fermentation of adonitol, erythritol and iso-inositol, and in tests for DNase, hydrogen sulfide, indole production, oxidase, tyrosine deaminase, urease and yellow pigment. All strains gave positive reactions in tests for utilization of *N*-acetyl-D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-galactose gluconate, D-glucose, D-maltose, D-mannose, α -D-melibiose, L-rhamnose, D-ribose, salicin, D-trehalose, D-xylose, D-mannitol, D-sorbitol, citrate, fumarate, L-malate, pyruvate and L-serine, and positive reactions in tests for hydrolysis of pNP- β -D-xylopyranoside, *bis*-pNP-phosphate, pNP-phenylphosphonate, pNP-phosphoryl choline and L-alanine-pNA. All strains gave negative reactions in tests for utilization of adonitol, iso-inositol, putrescine, propionate, adipate, azelate, glutarate, DL-3-hydroxybutyrate, mesaconate, suberate, β -alanine, L-leucine, L-tryptophan, 3-hydroxybenzoate and 4-hydroxybenzoate. +, Positive reaction; –, negative reaction; (+), delayed (3–7 d) positive reaction; numbers indicate the percentage of strains giving a positive reaction within 48 h; numbers in parentheses indicate the percentage of strains giving a positive reaction within 7 d. pNP, *para*-nitrophenyl; pNA, *para*-nitroanilide.

Biochemical reaction	GS 1	GS 2	GS 3
Methyl red	89	52	+
Voges-Proskauer	100	72	+
Citrate utilization	83 (94)	48 (79)	–
Phenylalanine deaminase	94	72	+
Arginine dihydrolase	0	0 (3)	–
Lysine decarboxylase	0	0	–
Ornithine decarboxylase	0 (11)	0	–
Motility	33 (44)	24 (31)	–
Gelatin liquefaction (22 °C)	0 (6)	0 (34)	–
KCN, growth	0 (11)	3	–
Malonate	89	52 (79)	+
Gas from D-glucose	83	76	–
Acid from:			
D-Arabitol	0	3	+
Cellobiose	100	90 (93)	+
Dulcitol	89 (94)	76 (83)	–
Maltose	100	90 (100)	+
D-Mannose	100	100	+
Melibiose	94 (100)	100	+
Methyl α -D-glucoside	0	0	–
Raffinose	94	97	+
L-Rhamnose	100	100	–
D-Sorbitol	94	97	+
Sucrose	100	97	+
D-Xylose	100	100	(+)
Glycerol	0 (56)	7 (34)	–
Aesculin hydrolysis	100	97	+
Mucate	28 (44)	0	–
Tartrate	33	86	+
Acetate	11	3	–
Lipase (corn oil)	0	0	–
Lipase (Tween 80)	75	63	–
Utilization of:			
<i>N</i> -Acetyl-D-galactosamine	0	4	–
D-Fructose	75	65	+
D-Sucrose	100	96	+
Acetate	0	27	–
<i>cis</i> -Aconitate	25	15	–
<i>trans</i> -Aconitate	0	4	–

Table 3 (cont.)

Biochemical reaction	GS 1	GS 2	GS 3
4-Aminobutyrate	0	4	—
DL-Lactate	100	96	—
2-Oxoglutarate	25	4	—
L-Alanine	100	73	+
L-Aspartate	100	92	+
L-Histidine	50	8	+
L-Ornithine	50	0	—
L-Phenylalanine	0	4	—
L-Proline	50	4	—
Phenylacetate	0	4	—
Hydrolysis of:			
pNP- β -D-galactopyranoside	0	8	—
pNP- α -D-glucopyranoside	25	4	—
pNP- β -D-glucopyranoside	100	12	+
2-Deoxythymidine-5'-pNP-phosphate	0	12	—
L-Glutamate- γ -3-carboxy-pNA	100	85	—
L-Proline-pNA	75	4	+

Fig. 1. Phylogenetic dendrogram based on a neighbour-joining comparison of a 1350 nucleotide 16S rRNA gene sequence. The scale bar represents a 2% difference. The accession numbers for the sequences retrieved from GenBank are: X79936, *R. aquatilis* 334; X79937, *R. aquatilis* 2-87; X79938, *R. aquatilis* 339; X79939, *R. aquatilis* ATCC 33989; X79940, *R. aquatilis* 3-88; M59155, *Hafnia alvei*; M59292, *Yersinia enterocolitica*; Z75317, *Yersinia pestis*; X75273, *Yersinia frederiksenii*; X82252, *Xenorhabdus bovienii*. The accession numbers for the strains sequenced in this study are: U88436, CDC 2987-79; U90757, CDC 1327-79; U90758, DSM 30078; U88438, SM Bonn 7; U88434, SM 57/1-576; U88439, SM Italy 2383; U88437, CDC 658-79; U88435, CCUG 21234. The sequence of CCUG 21234 (genomospecies 1) clusters with the strains in genomospecies 2.

ciprocal DNA relatedness tests using type or reference strains for GS 1 and GS 2 and a stringent, 75 °C incubation temperature serve to separate all strains placed in these genomospecies. It is possible that strains CCUG 21234 and CDC 1131-80 represent a

fourth *Rahnella* genomospecies. However, for the reasons stated above, and for convenience pending study of further strains, these strains were provisionally included in GS 1.

Thirty strains were placed in GS 2. All but two of these were 83% or more related with 2% or less divergence within related sequences. The two other strains were at the border of species level relatedness (73–74% relatedness at 60 °C, 5.0–5.5% divergence, and 68–74% relatedness at 75 °C) and were included in GS 2. All 14 Bonn strains were isolated from contaminated stored blood. It is possible that they represented multiple isolates of a single strain. If this were true, their inclusion does not significantly skew the data since almost all other GS 2 strains show comparable levels of relatedness to the labelled GS 2 reference strain, as do the Bonn strains.

Mean relatedness of the 20 GS 1 strains to labelled DNA from a GS 2 strain in 60 °C reactions was 56% with 8% divergence. In reciprocal reactions at 60 °C, GS 2 strains were 64–81% (70% mean) related to labelled DNA from GS 1; however, the mean divergence was 8%. Relatedness of each of these strains to labelled DNA from a GS 2 strain was significantly higher, and divergence was significantly lower. In 75 °C reactions, GS 1 strains were 81% interrelated compared to 48% relatedness to GS 2 strains. Reciprocally, GS 2 strains were 91% interrelated at 75 °C, compared to 49% relatedness to GS 1 strains. We therefore felt justified in designating the GS 1 and GS 2 strains as separate species. One strain, DSM 30078, was not related to either GS 1 or GS 2 at the species level. It was therefore designated GS 3.

One of the two strains (CCUG 21234) provisionally

placed in GS 1 was sequenced. It grouped in GS 2, showing 98.06–98.14% similarity to GS 1 strain sequences and 99.18–99.85% similarity to GS 2 strain sequences. It is possible that its grouping at the species level on the basis of sequence is misleading, as has been seen on several occasions with other genera (11, 38). Perhaps the grouping of CCUG 21234 with GS 2 strains indicates a back-mutation or recombinational event in the 16S rRNA gene. The more likely of these two possibilities is the recombinational event. In a 26 base region beginning at position 454 [*E. coli* numbering system (4)] the GS 1 strains differ from the GS 2 strains by nine bases. Strain CCUG 21234 is 100% homologous to all of the GS 2 strains in this variable region.

The level of relatedness, in excess of 40%, between the three genomospecies was compatible with placing them in the single genus *Rahnella*. This conclusion was strongly confirmed by their high level of 16S rRNA similarity and supported by their extremely similar biochemical profiles. 16S rRNA sequence studies confirmed the existence of three phylogenetic groups within the test strains and similar strains whose sequences had previously been deposited in GenBank. Further, the close relatedness between the sequences of the three 16S rRNA groups confirmed that they all were most closely related to one another and should remain in the single genus *Rahnella*. GS 1 corresponds to *R. aquatilis*, GS 2 should be referred to as *Rahnella* genomospecies 2 and GS 3 should be referred to as *Rahnella* genomospecies 3.

It should be noted that the five strain sequences previously deposited in the GenBank were all called *R. aquatilis*. The type strain was not included, and similarity values among the strains were 97.84–100.00%. Two of these strains, 2-87 and 3-88, group with our GS 2 strains (99.40–99.55% similarity). This observation again points out the fact that, while sequence differences of more than 3% are sufficient to exclude a strain from a species, it is prudent to do DNA hybridization on strains exhibiting 97% or greater sequence similarity before concluding that they belong to the same species (11, 38).

It was not possible to identify GS 1 and GS 2 solely on the basis of their biochemical characteristics. It appeared that GS 3 could be identified biochemically but, since only one GS 3 strain is known, this observation is tentative. We hesitate to formally name genomospecies that cannot be differentiated biochemically. We therefore refer to them as *Rahnella* genomospecies 2 and *Rahnella* genomospecies 3.

Isolates from a variety of human sources have been identified as *R. aquatilis*. On the basis of our DNA relatedness data, we know that some of these strains were actually *Rahnella* genomospecies 2. Molecular identification of all clinical isolates would be required to attempt a determination of the relative clinical incidence and significance of *R. aquatilis* and *Rahnella*

genomospecies 2. We do not know the source of the *Rahnella* genomospecies 3 strain.

REFERENCES

1. Alaallaa, S. R., Qadri, S. M. H., Al-Furayh, O. & Al-Qatary, K. (1992). Urinary tract infection due to *Rahnella aquatilis* in a renal transplant patient. *J Clin Microbiol* **30**, 2948–2950.
2. Berge, O., Heulin, T., Achouak, W., Richard, C., Bally, R. & Balandreau, J. (1991). *Rahnella aquatilis*, a nitrogen-fixing enteric bacterium associated with the rhizosphere of wheat and maize. *Can J Microbiol* **37**, 195–203.
3. Brenner, D. J., McWhorter, A. C., Leete-Knutson, J. K. & Steigerwalt, A. G. (1982). *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J Clin Microbiol* **15**, 1133–1140.
4. Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Biochem* **75**, 4801–4805.
5. Caraccio, V., Rocchetti, A. & Garavelli, P. L. (1994). *Rahnella aquatilis* bacteremia in a patient with chronic renal failure. *G Mal Infect Parassit* **46**, 330–331.
6. Christiaens, E., Hansen, W. & Moinet, J. (1987). Isolement des expectorations d'un patient atteint de leucémie lymphoïde chronique et de broncho-emphysème d'une *Enterobacteriaceae* nouvellement décrite: *Rahnella aquatilis*. *Med Mal Infect* **17**, 732–734.
7. Daly, J. S., Worthington, M. G., Brenner, D. J., Moss, C. W., Hollis, D. G., Weyant, R. S., Steigerwalt, A. G., Weaver, R. E., Daneshvar, M. I. & O'Connor, S. P. (1993). *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J Clin Microbiol* **31**, 872–881.
8. Davis, J. A. & Eyles, M. J. (1992). Discolouration of cottage cheese caused by *Rahnella aquatilis* in the presence of glucono- δ -lactone. *Aust J Dairy Technol* **47**, 62–63.
9. Farmer, J. J., III, Asbury, M. A., Hickman, F. W., Brenner, D. J. & the *Enterobacteriaceae* Study Group (1980). *Enterobacter sakazakii*: a new species of '*Enterobacteriaceae*' isolated from clinical specimens. *Int J Syst Bacteriol* **30**, 569–584.
10. Farmer, J. J., III, Davis, B. R., Hickman-Brenner, F. W. & 12 other authors (1985). Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J Clin Microbiol* **21**, 46–76.
11. Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.
12. Funke, G. & Rosner, H. (1995). *Rahnella aquatilis* bacteremia in an HIV-infected intravenous drug abuser. *Diagn Microbiol Infect Dis* **22**, 293–296.
13. Gavini, F., Ferragut, C., Lefebvre, B. & Leclerc, H. (1976). Étude taxonomique d'entérobactéries appartenant ou apparentées au genre *Enterobacter*. *Ann Microbiol (Paris)* **127B**, 317–335.
14. Geiges, O., Staehlin, B. & Baumann, B. (1990). The microbiological evaluation of prepared salad vegetables and sprouts. *Mitt Geb Lebensmittelunters Hyg* **81**, 684–721.
15. Gray, M. W., Sankoff, D. & Cedergren, R. J. (1984). On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in a small subunit ribosomal RNA. *Nucleic Acids Res* **12**, 5837–5852.

16. Goubau, P., Van Aelst, F., Verhaegen, J. & Boogaerts, M. (1988). Septicaemia caused by *Rahnella aquatilis* in an immunocompromised host. *Eur J Clin Microbiol Infect Dis* 7, 697–699.
17. Hamze, M., Mergaert, J., van Vuuren, H. J., Gavini, F., Beji, A., Izard, D. & Kersters, K. (1991). *Rahnella aquatilis*, a potential contaminant in lager beer breweries. *Int J Food Microbiol* 13, 63–68.
18. Harrell, L. J., Cameron, M. L. & O'Hara, C. M. (1989). *Rahnella aquatilis*, an unusual Gram-negative rod isolated from the bronchial washing of a patient with acquired immunodeficiency syndrome. *J Clin Microbiol* 27, 1671–1672.
19. Heron, S. J. E., Wilkinson, J. F. & Duffus, C. M. (1993). Enterobacteria associated with grass and silages. *J Appl Bacteriol* 75, 13–17.
20. Heulin, T., Berge, O., Mavingui, P., Gouzou, L., Hebbar, K. P. & Balandreau, J. (1994). *Bacillus polymyxa* and *Rahnella aquatilis*, the dominant N₂-fixing bacteria associated with wheat rhizosphere in French soils. *Eur J Soil Biol* 30, 35–42.
21. Hickman, F. W. & Farmer, J. J., III (1978). *Salmonella typhi*: identification, antibiograms, serology, and bacteriophage typing. *Am J Med Technol* 44, 1149–1159.
22. Hoppe, J. E., Herter, M., Aleksic, S., Klingebiel, T. & Niethammer, D. (1993). Catheter-related *Rahnella aquatilis* bacteremia in a pediatric bone marrow transplant recipient. *J Clin Microbiol* 31, 1911–1912.
23. Horie, S., Yanagida, Y., Saheki, K., Hiraishi, A. & Cho, C. (1985). Occurrence of *Rahnella aquatilis* psychrotrophic coliforms in mountain soils. *J Food Hyg Soc Jpn* 26, 573–578.
24. Iimura, K. & Hosono, A. (1996). Biochemical characteristics of *Enterobacter agglomerans* and related strains found in buckwheat seeds. *Int J Food Microbiol* 30, 243–253.
25. Izard, D., Gavini, F., Trinel, P. A. & Leclerc, H. (1979). *Rahnella aquatilis*, nouveau membre de la famille des Enterobacteriaceae. *Ann Microbiol (Paris)* 130, 163–177.
26. Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
27. Lebossi, E., Paraskaki, I., Grigoraki, K., Cosmidis, H. & Deliyianni, V. (1990). *Rahnella aquatilis*: saprophytic or opportunistic pathogenic bacterium. *Acta Microbiol Hell* 35, 569–574.
28. Maraki, S., Samonis, G., Marnelakis, E. & Tselentis, Y. (1994). Surgical wound infection caused by *Rahnella aquatilis*. *J Clin Microbiol* 32, 2706–2708.
29. Matsukura, H., Katayama, K., Kitano, N., Kobayashi, K., Kanegane, C., Higuchi, A. & Kyotani, S. (1996). Infective endocarditis caused by an unusual gram-negative rod, *Rahnella aquatilis*. *Pediatr Cardiol* 17, 108–111.
30. Müller, H. E., Brenner, D. J., Fanning, G. R., Grimont, P. A. D. & Kämpfer, P. (1996). Emended description of *Buttiauxella agrestis* with recognition of six new species of *Buttiauxella* and two new species of *Kluyvera*: *Buttiauxella ferragutiae* sp. nov., *Buttiauxella gaviniae* sp. nov., *Buttiauxella brennerae* sp. nov., *Buttiauxella izardii* sp. nov., *Buttiauxella noackiae* sp. nov., *Buttiauxella warmboldiae* sp. nov., *Kluyvera cochleae* sp. nov., and *Kluyvera georgiana* sp. nov. *Int J Syst Bacteriol* 46, 50–63.
31. Müller, H. E., Fanning, G. R. & Brenner, D. J. (1995). Isolation of *Ewingella americana* from mollusks. *Curr Microbiol* 31, 287–290.
32. Müller, H. E., Fanning, G. R. & Brenner, D. J. (1995). Isolation of *Serratia fonticola* from mollusks. *Syst Appl Microbiol* 18, 279–284.
33. Oh, H. M. & Tay, L. (1995). Bacteremia caused by *Rahnella aquatilis*: report of two cases and review. *Scand J Infect Dis* 27, 79–80.
34. Reina, J. & Lopez, A. (1996). Clinical and microbiological characteristics of *Rahnella aquatilis* strains isolated from children. *J Infect* 33, 135–137.
35. Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–425.
36. Selenska-Pobell, S., Evguenieva-Hackenberg, E. & Schwickerath, O. (1995). Random and repetitive primer amplified polymorphic DNA analysis of five soil and two clinical isolates of *Rahnella aquatilis*. *Syst Appl Microbiol* 18, 425–438.
37. Stackebrandt, E. & Charfrenit, O. (1990). Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii*-specific oligonucleotide probe. *J Gen Microbiol* 136, 37–43.
38. Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44, 846–849.
39. Véron, M. (1975). Nutrition et taxonomie des enterobactéries. I. Méthode d'étude des auxanogrammes. *Ann Microbiol (Paris)* 126A, 267–274.
40. Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37, 463–464.
41. Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173, 697–703.