Description of *Rahnella perminowiae* sp. nov., *Rahnella bonaserana* sp. nov., *Rahnella rivi* sp. nov. and *Rahnella ecdela* sp. nov. from diverse environmental sources and the emended description of the genus *Rahnella*.

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1.4 Keyword

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ABSTRACT

Bacteria isolated from onion bulbs suffering from bacterial decay in the United States and Norway were previously shown to belong to the genus *Rahnella* based on partial housekeeping gene sequences and/or fatty acid analysis. However, many strains could not be assigned to any existing *Rahnella* species. Additionally, strains isolated from creek water and oak as well as a strain with bioremediation properties were assigned to *Rahnella* based on partial housekeeping gene sequences. The taxonomic status of these 21 strains was investigated using multilocus sequence analysis, whole genome analyses, phenotypic assays and fatty acid analysis. Phylogenetic and phylogenomic analyses separated the strains into five clusters, one of which corresponded to *Rahnella aceris*. The remaining four clusters could be differentiated both genotypically and phenotypically from each other and existing *Rahnella* species. Based on these results, we propose the description of four novel species: *Rahnella perminowiae* sp. nov. (type strain SL6^T = LMG 32257^T = DSM 112609^T), *Rahnella bonaserana* sp. nov. (type strain H11b^T = LMG 32256^T = DSM 112610^T), *Rahnella rivi* sp. nov. (type strain FC061912-K^T = LMG 32259^T = DSM 112611^T) and *Rahnella ecdela* sp. nov. (type strain FRB 231^T = LMG 32255^T = DSM 112612^T).

Rahnella is a genus of environmentally-linked species in the family *Yersiniaceae* [1]. For many years *Rahnella aquatilis* was the only validly described species in the genus *Rahnella* [2], although two genomospecies were proposed containing strains that could not be phenotypically differentiated from *R. aquatilis* [3]. *R. aquatilis* has long been acknowledged as a truly ubiquitous bacterium and has been isolated from a diverse range of sources, both environmental and clinical [4]. The genus *Rahnella* has expanded exponentially in recent years with the description of six novel species from a range of ecological niches and the elevation of the two genomospecies to validly described species [5–7]. These eight species contributed to the existing diversity of *Rahnella* with isolations of *Rahnella victoriana*, *Rahnella variigena* and *Rahnella inusitata* from bleeding cankers of oak; *R. victoriana*, *R. variigena* and *R. woolbedingensis* from asymptomatic alder and walnut; *Rahnella bruchi* from the gut of the *Agrilus biguttatus* beetle; *Rahnella aceris* and *Rahnella laticis* from sap of *Acer pictum* and *Rahnella contaminans* as a contaminant from MRSA agar plates [5–7]. In addition to their isolation from the

natural environment, *Rahnella* species have been linked to nitrogen-fixation [8], metal and radionuclide sequestration [9] and biological control [10]; and more recently as possible pathogens of oak [11], poplar [12] and onion [13].

A study by Asselin *et al*. [13] indicated the existence of several potential novel *Rahnella* species, isolated over a number of years from onion bulbs with signs of bacterial decay in the United States and Norway, and from creek water in the United States. Multilocus sequence analysis of a selection of onion isolates placed them in four separate clusters without reference strains in the *Rahnella* genus, suggesting they belong to four novel taxa [13]. A further potential novel *Rahnella* taxon was identified in this study following *gyrB* gene sequencing of a strain previously isolated from a *Quercus* species displaying symptoms of Acute Oak Decline (AOD) in the Netherlands. The above mentioned strains were examined using a polyphasic approach based on genotypic, phenotypic, genomic and fatty acid assays to clarify their taxonomic position. Based on the results, we propose four novel *Rahnella* species: *Rahnella perminowiae* sp. nov., *Rahnella bonaserana* sp. nov., *Rahnella rivi* sp. nov. and *Rahnella ecdela* sp. nov.

Isolation and Ecology

Bacterial strains were previously isolated from onion bulbs in the United States (New York State and Oregon) and Norway (Vestfold, Østfold, Oppland and Hedmark) as described in Asselin *et al.* 2019 [13], either directly from onion tissue or following soaking and crushing in buffer or sterile water. Strain FC061912-K^T was isolated from creek water following high-speed centrifugation and culturing. A *Rahnella* strain Y9602 able to sequester metals was isolated from a mixed-waste-contaminated subsurface in Tennessee, United States [14]. FRB 231^T was isolated from the bleeding lesion on a symptomatic oak in the Netherlands displaying symptoms of Acute Oak Decline (AOD). A swab was taken from the lesion, suspended in sterile Ringers solution and the resulting suspension plated onto Luria-Bertani (LB) agar. All strains can be routinely cultured on LB agar or in LB broth incubated at 28 °C, and stored in 40 % glycerol at -80 °C. See Suppl. Table S1 for a list of strains investigated in this study.

Genotypic characterisation

DNA for all PCR reactions was extracted using alkaline lysis [15] and stored at -20 °C. Multilocus sequence analysis (MLSA) was performed on strains which weren't included in the study by Asselin *et*

al. [13], by amplification and sequencing of the *gyrB*, *rpoB*, *infB* and *atpD* housekeeping genes as previously described [16]. The following modifications were used: annealing temperature of 46 °C for the *gyrB* PCR, alternative *rpoB* amplification and sequencing primers designed for *Rahnella* species [13] and an alternative *atpD* reverse sequencing primer atpD-08R 5' CCCAGAAGTGCGGACACTTC 3'. Almost complete 16S rRNA gene sequencing was performed on a selection of strains (AR20, L31-1-12, C60, SL6^T, H11b^T, FC061912-K^T and FRB 231^T) using the primers from Coenye *et al.* [17] and standard amplification cycles with an annealing temperature of 55 °C. Additional sequences for the closest phylogenetic relatives were downloaded from GenBank and added to the datasets which were aligned and trimmed in BioEdit v7.2.5 [18] to the following lengths: *gyrB* – 741 bp, *rpoB* – 636 bp, *infB* – 615 bp, *atpD* – 642 bp and 16S rRNA – 1346 bp. Following concatenation of the four housekeeping genes, Smart Model Selection (SMS) [19] was performed on both the MLSA and 16S rRNA gene datasets before maximum likelihood phylogenetic analysis using PhyML 3.0 [20]. Reliability of the generated clusters was assessed with 1000 bootstrap replicates.

In the maximum likelihood phylogenetic tree based on concatenated multilocus gene sequences (Fig. 1), the strains isolated from onion bulbs in the United States and Norway were separated into three clusters. The first cluster (*Rahnella* clade 1) comprised eight strains isolated from onion in the United States and Norway, strain Y9602 which can sequester heavy metals and the type strain of a recently described *Rahnella* species, *R. aceris* [6]. As the cluster was strongly supported with a bootstrap value of 100 % and there was little sequence variation amongst the strains, it was concluded that strains in this cluster belonged to *R. aceris*. The remaining isolates from onion bulbs did not cluster with any reference or type strains. *Rahnella* clade 2, another large, well-supported group of nine strains from onion in the United States and Norway, clustered on the border of the type species, *R. aquatilis*, but was clearly separated and constituted a potential novel species. The remaining strain isolated from onion, H11b^T (*Rahnella* clade 3) was situated on a separate branch between *R. victoriana* and *R. variigena*. Strains FRB 231^T, isolated from *Quercus* sp. (*Rahnella* clade 4), and FC061912-K^T (*Rahnella* clade 5), isolated from creek water adjacent to an onion field, also had separate positions within the phylogenetic tree with no close association to a type strain indicating these three strains belonged to further novel *Rahnella* species.

The 16S rRNA gene sequence pairwise similarity for the selected strains was calculated using EZBioCloud [21], and was 99.3 % to *R. aceris* and *R. aquatilis* for strains from *Rahnella* clade 2; 99.2 – 99.5 % similar to *R. variigena, R. bruchi*, *R. wooldbedingensis* and *R. victoriana* for H11b^T, FRB 231^T and FC061912-K^T (*Rahnella* clades 3, 4 and 5). As expected and as previously observed [5–7], due to the

recognised high degree of homogeneity in the 16S rRNA gene of genera in the *Enterobacterales*, the taxonomic position of the potential novel species was not clearly or reliably represented by the 16S rRNA gene phylogenetic tree (Suppl. Fig. S1). The existing *Rahnella* species did not form a monophyletic clade and are interspersed by *Rouxiella* species and *Ewingella americana*.

BOX and ERIC PCR (repetitive element-based PCR) were performed on all isolates to examine their genetic diversity using the primers BOX-A1R and ERIC-2 and -1R, respectively [22]. Included in the analyses were two representative strains from each existing *Rahnella* species. Amplicons were separated in 1.5 % agarose at 50 V for ~3 h. BOX PCR provided the best resolution for all strains tested and allowed the differentiation of the four potential novel species, not only from each other but also from existing *Rahnella* species (Suppl. Fig. S2). Although the fingerprint patterns for strains from *Rahnella* clade 2 had similar patterns, they were isolated from onion bulbs in different areas and countries and therefore cannot be clones.

Genome features

The whole genome sequences of nine strains isolated from symptomatic onion bulbs (*Rahnella* clade 1: AR20, F57b, L31-1-12, R92a; *Rahnella* clade 2: C60, L72c, L151-1A, SL6^T and *Rahnella* clade 3: H11b^T) and two single strains (*Rahnella* clades 4 and 5) from *Quercus* sp. (FRB 231^T) and creek water (FC06191-K^T) were sequenced by Microbes NG (Birmingham, UK) on the Illumina HiSeq platform, following DNA extraction by cell lysis and DNA purification with SPRI (Solid Phase Reversible Immobilization) beads. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [23]. *De novo* assembly was performed using SPAdes version 3.11.1 [24] and the resulting contigs were annotated in Prokka 1.11 [25]. Genome sizes from 5.40 to 5.75 Mbp and DNA G + C contents ranging from 51.4 to 53.2 mol % were observed for the sequenced strains. The genome sequences were submitted to Genbank under the BioProject number PRJNA706176); genome features and assembly accessions are listed in Suppl. Table S2. The 16S rRNA sequences derived by genome sequencing of the above strains were compared to those obtained with Sanger sequencing to ensure there was no contamination of the whole genome sequences.

To infer the phylogenomic position of the strains, pairwise comparisons between the genomes were conducted using Genome Blast Distance Phylogeny (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d_5 [26] with 100 distance replicates each. The resulting intergenomic distances were used to construct a balanced minimum evolution tree

including Subtree Pruning and Regrafting (SPR) post-processing using FASTME 2.1.6.1 [27]. Branch support was inferred from 100 pseudo-bootstrap replicates and the tree was rooted at the midpoint [28]. In the resulting phylogenomic tree (Fig. 2), all representative *Rahnella* strains from the present study formed a robust clade with existing *Rahnella* species with 100 % bootstrap support. The clustering of the strains agreed with that observed in the MLSA phylogenetic tree (Fig. 1), with five strains assigned to the recently validated species *R. aceris* and four strains isolated from onion forming a separate cluster representing a novel taxon. The remaining representative strains from various sources had unique positions within the *Rahnella* clade confirming their taxonomic status as three novel species.

Whole genome comparisons were performed between representative *Rahnella* strains from the present study and existing *Rahnella* species using average nucleotide identity with FastANI [29]. Strains from *Rahnella* clade 1 shared ANI values of 98.2 to 99.7 % with each other and 98.4 to 99.4 % with the type strain of *R. aceris* SAP-19^{T} (Suppl. Table S3). These values are above the suggested species limit of 95 % [30] confirming that the strains from onion and strain Y9602 belong to the same taxon, *R. aceris*. ANI values ranging from 99.1 – 99.3 % were observed amongst strains from *Rahnella* clade 2 confirming they belong to a single taxon. The strains from the three single-strain species (*Rahnella* clades 3 – 5) exhibited ANI values of less than 91.2 % to each other, and to strains from *Rahnella* clade 2. Furthermore, representative strains from *Rahnella* clades 2 – 5 were less than 94 % related in terms of ANI to all existing *Rahnella* species (Table 1). The conclusions drawn from ANI analysis were confirmed by *in silico* DNA-DNA hybridisation (*is*DDH) using the genome-to-genome distance calculator (GGDC) [26] and are also presented in Table 1.

Physiology and Chemotaxonomy

Cell size, morphology and motility were determined using light microscopy and the microscopy imaging software CellSens version 1.11 (Olympus Life Science, Tokyo, Japan). Flagella arrangement for all proposed species, and existing *Rahnella* species (except *R. contaminans* and *R. laticis*) was observed by transmission electron microscopy (FEI Tecnai 12 120kV BioTwin Spirit TEM) following negative staining. Briefly, grids were floated on mid-log phase bacterial suspensions for 2 mins, washed 3 times in distilled water, stained with 3 % uranyl acetate for 30 sec and washed again 3 times before wicking away excess liquid and air drying. Colony morphology was examined following growth on tryptone soya agar (TSA, Sigma) incubated at 28 °C for 48 h, while the growth temperature range was determined on TSA incubated at 4, 10, 25, 28, 30, 37 and 41 °C in triplicate. Ranges for pH were tested in triplicate in tryptone soya broth (TSB, Oxoid) with the pH adjusted to 4 – 10 (in increments of 1)

with sodium acetate/acetic acid and carbonate/bicarbonate buffers. Salt tolerance was tested in saline-free nutrient broth (3 g l^{-1} beef extract, 5 g l^{-1} peptone) with the salt concentration adjusted to 1 – 7 % (in increments of 1 % w/v) by supplemented NaCl. These were incubated overnight at 28 °C with shaking. Included in the temperature, pH and salt tolerance tests were the type strains for existing *Rahnella* species (except *R. aceris, R. contaminans and R. laticis*). Catalase and oxidase activity were determined by bubble production in 3 % v/v H_2O_2 and staining with Kovács reagent (1 % tetra-methyl-*p*-phenylenediamine dihydrochloride), respectively.

Cells from all strains are straight rods with an average size of 0.6 x 1.6 µm. They occur singly, or in pairs and are motile by peritrichous flagella (Fig. 3). Members of the order *Enterobacterales* are known for their motility by several peritrichous flagella, and a recent study confirmed that all examined *Rahnella* species possess the primary peritrichous flagella locus (*flag*-1), with most strains of *R. variigena* encoding an additional secondary predicted peritrichous locus (*flag*-3b) [31]. However, the recently described *R. aceris*, *R. contaminans* and *R. laticis* are indicated to be motile by a single, polar flagellum [6, 7] and it was suggested that as all *Rahnella* species have similar flagella gene profiles, they could all be motile by a polar flagellum [7]. The original description of the genus *Rahnella* describes *R. aquatilis* as motile by peritrichous flagella, although electron microscopy images were not published [2, 4]. To clarify the flagella arrangement of the existing *Rahnella* species, strains of these were also imaged by TEM as described above (with the exception of *R. contaminans* and *R. laticis*). All species examined clearly displayed multiple flagella on the surface of the cells, not at the poles, providing evidence that the majority of *Rahnella* species are motile by peritrichous flagella (Suppl. Fig. S3). Additionally, the genomes of all existing *Rahnella* species and the proposed four novel species were screened for the presence of *flag* loci. All species were found to possess the primary peritrichous flagella locus (*flag*-1), while the additional secondary *flag*-3b locus was encoded in the genome of strain H11b^T (*Rahnella* clade 3) along with the *flag*-1 locus (data not shown).

Following growth on TSA for 48 h, the resulting colonies are cream-coloured, round, slightly convex, smooth with entire margins and 2 – 3 mm in diameter. All strains tested grew at 4, 10, 25, 28, 30 and 37 °C but not at 41 °C. Growth for all *Rahnella* strains included in this study was observed in the pH range of 5 to 8, with weak growth at pH 9. Strains from the four proposed novel species and existing *Rahnella* species grew well in nutrient broth supplemented with up to 6 % NaCl, while weak growth was observed at 7 % NaCl. All strains from the proposed novel species are oxidase negative and catalase positive.

Phenotypic testing was performed on a selection of strains from *Rahnella aceris* (AR20, C1b, F57b, L31-1-12, L172-1A, R92a and Y9602), *Rahnella* clade 2 (A66, C60, L72c, L51-1-12, L151-1A, L173-1B and SL6^T) and the three strains from clades 3 – 5 (H11b^T, FRB 231^T and FC061912-K^T) using the commercial assays API 20E, API 50 CH/B (bioMérieux) and GEN III GN/GP microplates (Biolog). These were performed according to the manufacturer's instructions. GEN III plates were scored after 6 h and again after 24 h, while API 20 E and 50CH/B galleries were read after 24 h and 24 - 48 h, respectively. The type strain of *R. aquatilis*, LMG 2794^T, was included as a positive control in the API 20E and 50 CH/B tests. Due to a lack of phenotypic data for existing *Rahnella* species based on the GEN III microplate system, the type strains and reference strains for these were included in the GEN III assays (with the exception of the type strains of *R. aceris, R.contaminans* and *R. laticis*).

Strains from the four proposed novel species were clearly differentiated from each other, and from the existing species in the genus *Rahnella* based on phenotypes. Even the three proposed single-strain species have clearly distinguishable phenotypic profiles. The most useful phenotypic characteristics for species differentiation are listed in Table 2. The full phenotypic profiles for each proposed species are described in the below protologues. It is acknowledged that the phenotypic profiles for the single-strain species may change as further strains belonging to these taxa are isolated. Differing results for several phenotypic characteristics for existing *Rahnella* species were observed by Jeon *et al*. [7], such as citrate utilization, arginine dihydrolase, gelatinase and acetoin production. The phenotypic data generated in the present study and previous studies [5, 6] were obtained following the manufacturer's instructions for incubation temperature and time, whereas the data presented by Jeon *et al*. was generated under different incubation conditions. This could account for the differences observed between the studies.

Fatty acid methyl ester (FAME) analysis was performed on selected strains from *Rahnella* clade 1, now confirmed as belonging to *R. aceris* (AR20, L31-1-12, R92a and Y9602), clade 2 (C60, L72c, L151-1A and SL6^T) and the three strains from clades 3 - 5 (H11b^T, FRB 231^T and FC061912-K^T) by Fera Science Ltd. (York, UK). Strains were cultivated on TSA at 28 °C for 24 h and the protocol followed was based on the Sherlock Microbial Identification System Version 6.4 (MIDI Inc.). The results obtained were compared against the library RTSBA6 6.21. The fatty acid profiles obtained for all strains were similar in composition to those of existing *Rahnella* species [5, 6]. Complete fatty acid profiles for all *Rahnella* species are presented in Table 3.

In the past eight years, the genus *Rahnella* has evolved from a monotypic genus to a genus comprising species from a diverse range of hosts, sources and locations. The description of four novel *Rahnella* species contributes to an already extensive list of environmental niches and highlights a possible role for several species in bacterial decay of onion and AOD. Additionally, a large number of strains from onion bulb decay in the USA and Norway have been assigned to *R. aceris*, along with strain Y9602 that has the ability to sequester heavy metals, enhancing the description of this former single-strain species.

Based on the genotypic, genomic, phenotypic and chemotaxonomic data generated in this study, we conclude that the strains represent four novel species and propose the description of: *Rahnella perminowiae* sp. nov. (type strain SL6^T = LMG 32257^T = DSM 112609^T), *Rahnella bonaserana* sp. nov. (type strain = H11b^T = LMG 32256^T = DSM 112610^T), *Rahnella rivi* sp. nov. (type strain = FC061912-K^T = LMG 32259^T = DSM 112611^T) and *Rahnella ecdela* sp. nov. (type strain = FRB 231^T = LMG 32255^T = DSM 112612^T).

Description of *Rahnella perminowiae* sp. nov.

Rahnella perminowiae (per.mi.no'wi.ae. N.L. gen. fem. n. *perminowiae*, pertaining to Perminow, named after Juliana I.S. Perminow for her work on bacterial plant diseases, including diseases of onion, at the Norwegian Institute of Bioeconomy Research since 1993).

Gram-negative rods (0.6 – 0.8 x 1.5 – 1.8 μm) which occur singly or in pairs and are motile. Colonies are cream on tryptone soya agar, round, convex and smooth with entire margins, facultatively anaerobic, oxidase negative and catalase positive. Optimum growth is at 28 °C, although strains can grow within the range 4 – 37 °C. Strains grow well at pH 5 - 8 and in broth supplemented with NaCl up to 6 %. Positive β-galactosidase, arginine dihydrolase and citrate utilization. Negative for lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophan deaminase, indole production, acetoin production and gelatinase. Nitrate is reduced to nitrite. Acid is produced from: glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, *N*-acetylglucosamine, methyl-αD-glucopyranoside, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, gentiobiose, D-fucose and L-fucose (API 50CHB/E). Dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, α-D-glucose, D-mannose, D-fructose, D-

galactose, D-fucose, L-fucose, L-rhamnose, D-sorbitol, D-mannitol, glycerol, α-D-glucose-6-phosphate, β-D-fructose-6-phosphate, glycyl-L-proline, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, citric acid, L-malic acid, bromosuccinic acid, acetic acid and formic acid are utilised (Biolog GEN III). L-lactic acid and α-ketoglutaric acid are variable (type strain is weakly positive). Major fatty acids include $C_{16:0}$, $C_{18:1}$ ω7*c*, $C_{17:0}$ cyclo, summed feature 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and summed feature 3 ($C_{16:1}$ ω7*c* and /or iso-$C_{15:0}$ 2-OH).

The DNA G + C content of the type strain is 51.8 mol %.

The type strain SL6^T (= LMG 32257^T = DSM 112609^T) was isolated from onion in Hedmark, Norway.

Description of *Rahnella bonaserana* sp. nov.

Rahnella bonaserana (bo.na.se.ra'na. N.L. fem. adj. *bonaserana*, pertaining to Bonasera, named after Jean M. Bonasera for her work on bacterial plant diseases at Cornell University over 22 years developing culturing and identification techniques)

Gram-negative rods (0.5 – 0.6 x 1.3 – 1.6 μm) which occur singly or in pairs and are motile. Colonies are cream on tryptone soya agar, round, convex and smooth with entire margins, facultatively anaerobic, oxidase negative and catalase positive. Optimum growth is at 28 °C, although strains can grow within the range 4 – 37 °C. Strains grow well at pH 5 - 8 and in broth supplemented with NaCl up to 6 %. Positive for β-galactosidase, arginine dihydrolase and citrate utilization. Negative for lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophan deaminase, indole production, acetoin production and gelatinase. Nitrate is reduced to nitrite. Acid is produced from: glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, D-fucose and L-fucose (API 50CHB/E). Dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, glycerol, α-D-glucose-6-phosphate, β-D-fructose-6-phosphate, D-aspartic acid, D-serine, glycyl-L-proline, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric

acid, L-malic acid, acetic acid and formic acid are utilised (Biolog GEN III). Major fatty acids include $C_{16:0}$ and $C_{17:0}$ cyclo.

The DNA G + C content of the type strain is 51.9 mol %.

The type strain H11b^T (= LMG 32256^T = DSM 112610^T) was isolated from onion in New York State, USA.

Description of *Rahnella rivi* sp. nov.

Rahnella rivi (ri'vi. L. gen. n. *rivi*, of a river or creek, referring to the isolation source of the type strain)

Gram-negative rods (0.6 – 0.7 x 1.5 – 1.8 μm) which occur singly or in pairs and are motile. Colonies are cream on tryptone soya agar, round, convex and smooth with entire margins, facultatively anaerobic, oxidase negative and catalase positive. Optimum growth is at 28 °C, although strains can grow within the range 4 – 37 °C. Strains grow well at pH 5 - 8 and in broth supplemented with NaCl up to 6 %. Positive for β-galactosidase, arginine dihydrolase and acetoin production. Negative for lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S, urease, tryptophan deaminase, indole production and gelatinase. Nitrate is reduced to nitrite. Acid is produced from: glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, methyl-αD-glucopyranoside, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, gentiobiose and D-turanose (API 50CHB/E). Dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, inosine, D-mannitol, glycerol, α-D-glucose-6-phosphate, β-D-fructose-6-phosphate, D-aspartic acid, D-serine, glycyl-L-proline, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, D-saccharic acid, L-lactic acid, citric acid, L-malic acid, tween 40, acetoacetic acid, acetic acid and formic acid are utilised (Biolog GEN III). Major fatty acids include $C_{16:0}$, $C_{18:1}$ ω7*c*, $C_{17:0}$ cyclo, summed feature 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and summed feature 3 ($C_{16:1}$ ω7*c* and /or iso-$C_{15:0}$ 2-OH).

The DNA G + C content of the type strain is 53.2 mol %.

The type strain FC061912-K^T (= LMG 32259^T = DSM 112611^T) was isolated from river water in New York State, USA.

Description of *Rahnella ecdela* sp. nov.

Rahnella ecdela (ec.de'la. N.L. fem. adj. *ecdela* from Gr. adj. *ekdélos* meaning clear or manifest, referring to the clear separation from other species in this genus)

Gram-negative rods (0.5 – 0.6 x 1.3 – 1.5 μm) which occur singly or in pairs and are motile. Colonies are cream on tryptone soya agar, round, convex and smooth with entire margins, facultatively anaerobic, oxidase negative and catalase positive. Optimum growth is at 28 °C, although strains can grow within the range 4 – 37 °C. Strains grow well at pH 5 - 8 and in broth supplemented with NaCl up to 6 %. Positive for β-galactosidase. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S, urease, tryptophan deaminase, indole production, acetoin production and gelatinase. Nitrate is reduced to nitrite. Acid is produced from: glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, *N*-acetylglucosamine, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, gentiobiose, D-fucose, L-fucose and D-arabitol (API 50CHB/E). Dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-raffinose, α-D-lactose, D-melibiose, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-mannitol, D-arabitol, glycerol, α-D-glucose-6-phosphate, β-D-fructose-6-phosphate, D-aspartic acid, D-serine, glycyl-L-proline, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, L-lactic acid, citric acid, L-malic acid, tween 40, acetoacetic acid, acetic acid and formic acid are utilised (Biolog GEN III). Major fatty acids include $C_{16:0}$, $C_{17:0}$ cyclo and summed feature 2.

The DNA G + C content of the type strain is 51.9 mol %.

The type strain FRB 231^T (= LMG 32255^T = DSM 112612^T) was isolated from *Quercus* spp. exhibiting AOD symptoms in the Netherlands.

Emended description of the genus *Rahnella* (Izard et al. 1981 emend. Brady *et al.* 2014, Jeon *et al*. 2021)

Rahnella (Rah.nel'la. N.L. dim. ending -*ella*; N.L. fem n. *Rahnella* named after Otto Rahn, the German-American microbiologist who proposed the name *Enterobacteriaceae* in 1937)

The description is based on the data from Brenner *et al*. 1998 [3], Kämpfer, 2005 [4], Brady *et al*. 2014 [5], Lee *et al*. 2020 [6], Jeon *et al*. 2021 [7] and this study.

Gram-negative straight rods (0.5 – 1.0 x 1.0 – 3.0 μm), facultatively anaerobic, oxidase negative and catalase positive. Cells occur singly or in pairs and are motile by peritrichous flagella when grown at 25 °C, although some species possess a single flagellum. Colonies are white to cream on nutrient or tryptone soya agar, round, slightly convex and smooth with entire margins. Strains can grow at temperatures between 4 and 30 °C with optimum growth at 28 – 30 °C, growth at 37 °C varies depending on the species. Strains can grow within the pH range 5 – 8 and in the presence of 0 – 6 % (w/v) NaCl. Positive for β-galactosidase activity but negative for H_2S, urease and indole production. Lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activity are all negative. Arginine dihydrolase, citrate utilization, acetoin and gelatinase production are variable. Nitrate is reduced to nitrite. Acid is produced from: L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose and D-trehalose. The following carbon sources are utilized at 28 °C: D-maltose, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose.

Frequently isolated from fresh water and various environmental habitats including soils, the rhizosphere, woody tissues of oak, alder and walnut, tree sap and onion bulbs. Also found in the intestines of snails and insects such as beetles and moths. Can be isolated from foods or human clinical specimens, especially from immunocompromised patients. Major fatty acids include $C_{16:0}$ and $C_{17:0}$ cyclo. The presence of $C_{18:1}$ ω7*c*, summed feature 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and summed feature 3 ($C_{16:1}$ ω7*c* and /or iso-$C_{15:0}$ 2-OH) as major fatty acids is variable.

The G + C content ranges from 51.3 to 53.7 mol %.

The type species is *Rahnella aquatilis*.

AUTHOR STATEMENTS

1.6 Authors and contributors

CB was involved in the conceptualisation, data curation, formal analysis, investigation, methodology, validation, visualisation, writing, reviewing and editing of the work. JA, SB, MB, BC and SV were involved in the provision of resources and the conceptualisation, writing, reviewing and editing of the manuscript. DA and SD were responsible for funding acquisition and the conceptualisation, writing, reviewing and editing of the manuscript.

1.7 Conflicts of interest and disclaimers

The authors declare that there are no conflicts of interest.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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FIGURES AND TABLES

Table 1: Percentages of average nucleotide identity (fastANI – lower left, orange) and *in silico* DNA-DNA hybridization (*is*DDH – upper right, blue) between *Rahnella perminowiae* sp. nov., *Rahnella bonaserana* sp. nov., *Rahnella rivi* sp. nov., *Rahnella ecdela* sp. nov. and existing species of the genus *Rahnella* (type strain columns are shaded in grey). Percentages above cut-off value for species delimitation (>95 % for ANI and >70 % for *is*DDH) are shaded.

*is*DDH / fastANI	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	100	96.2	93.4	93.9	32.2	28.2	31.6	49.2	42.3	31.4	28.6	25.3	28.2	32.8	33.9	31.6
2	99.3	100	93.1	93.6	31.7	27.8	31.2	45.0	41.8	31.1	28.3	26.5	27.9	32.4	33.8	31.2
3	99.1	99.1	100	94.4	31.8	27.7	31.2	44.8	41.8	31.0	28.3	26.5	27.8	32.4	33.7	31.2
4	99.2	99.1	99.3	100	31.9	27.9	31.3	45.1	42.0	31.3	28.4	26.6	28.1	32.6	33.8	31.3
5	87.4	87.8	87.8	87.8	100	28.8	42.8	34.1	34.1	42.1	29.9	26.0	28.9	54.8	33.2	42.2
6	85.5	86.4	86.3	86.4	86.3	100	28.6	29.0	28.7	28.5	27.8	31.7	27.8	29.2	28.8	28.7
7	87.1	87.5	87.5	87.6	91.2	86.2	100	33.3	33.2	53.5	29.6	25.4	28.7	44.3	33.2	47.6
8	91.7	91.6	91.6	91.6	88.4	86.3	88.0	100	49.2	32.9	29.8	25.5	29.5	34.4	37.5	33.1
9	90.6	90.6	90.7	90.7	88.6	86.1	88.1	92.9	100	33.0	29.4	27.4	28.9	35.3	36.5	33.2
10	86.9	86.9	86.6	86.9	90.7	86.1	93.8	87.9	87.9	100	29.6	25.9	28.7	42.8	33.0	53.0
11	85.7	85.6	85.7	85.6	87.0	85.8	86.9	86.5	86.4	86.8	100	27.5	60.8	30.4	30.0	29.8
12	84.6	84.5	84.6	84.6	85.2	88.0	85.4	85.4	85.3	85.2	85.5	100	27.9	25.8	26.6	25.7

13	85.5	85.4	85.4	85.4	86.2	85.8	86.3	86.5	86.2	86.2	95.3	85.6	100	29.4	29.5	28.8
14	87.6	87.7	87.6	87.7	94.0	86.6	91.6	88.5	89.2	91.2	87.3	85.7	86.8	100	34.3	43.6
15	88.1	88.0	88.1	88.0	88.7	86.4	88.2	90.0	89.2	88.1	86.8	85.8	86.5	89.3	100	33.4
16	87.1	87.0	86.9	87.0	91.0	86.2	92.5	88.1	88.0	93.6	86.8	85.5	86.3	91.5	88.2	100

(1) *Rahnella perminowiae* SL6^{T} (GCA_019049755.1), (2) *Rahnella perminowiae* C60 (GCA_019049695.1), (3) *Rahnella perminowiae* L72c (GCA_019049715.1), (4) *Rahnella perminowiae* L151-1A (GCA_019049735.1), (5) *Rahnella bonaserana* H11b^{T} (GCA_019049675.1), (6) *Rahnella rivi* FC061912-K^{T} (GCA_019049655.1), (7) *Rahnella ecdela* FRB 231^{T} (GCA_019049625.1), (8) *Rahnella aquatilis* CIP 78.65^{T} (GCA_000241955), (9) *Rahnella aceris* SAP-19^{T} (GCA_011684115), (10) *Rahnella bruchi* DSM 27398^{T} (GCA_003614975), (11) *Rahnella contaminans* Lac-M11^{T} (GCA_011065485), (12) *Rahnella inusitata* DSM 30078^{T} (GCA_003602055), (13) *Rahnella laticis* SAP-17^{T} (GCF_015644585), (14) *Rahnella variigena* CIP 105588^{T} (GCA_003602185), (15) *Rahnella victoriana* DSM 27397^{T} (GCA_004330295), (16) *Rahnella woolbedingensis* DSM 27399^{T} (GCA_003602095).

^{T} = type strain.

582 **Table 2:** Phenotypic characteristics allowing differentiation of *Rahnella perminowiae* sp. nov., *Rahnella bonaserana* sp. nov., *Rahnella rivi* sp. nov. and
583 *Rahnella ecdela* sp. nov. from each other and existing *Rahnella* species
584
585 1 = *Rahnella perminowiae* sp. nov. (*n* = 7), 2 = *Rahnella bonaserana* sp. nov. (*n* = 1), 3 = *Rahnella rivi* sp. nov. (*n* = 1), 4 = *Rahnella ecdela* sp. nov. (*n* = 1), 5 =
586 *Rahnella aquatilis* (*n* = 1), 6 = *Rahnella aceris* (*n* = 7, type strain not included), 7 = *Rahnella bruchi* (*n* = 3), 8 = *Rahnella contaminans* (*n* = 1), 9 = *Rahnella*
587 *inusitata* (*n* = 3), 10 = *Rahnella laticis* (*n* = 3), 11 = *Rahnella variigena* (*n* = 5), 12 = *Rahnella victoriana* (*n* = 7), 13 = *Rahnella woolbedingensis* (*n* = 3). Data for
588 type strain of 6 taken from [6] and data for 5, 7, 9, 11 – 13 taken from [5] except for carbohydrate utilisation data which was obtained from the present
589 study. *n* = number of strains.
590

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Arginine dihydrolase	+	+	+	-	-	+[a]	-	-	-	-	-	-	-
Acetoin production	-	-	+	-	+[a]	-	-	-	+[a]	-	+	+[a]	-
Gelatinase production	-	-	-	-	-	-	+	-	+[b]	-	+[b]	w+	w+
Acid from:													
D-sorbitol	+	+	-	-	+	+	v[c]	+	v[c]	-	+	+	-
methyl-α-D-glucopyranoside	+	-	+	-	-	v	v[c]	-	v[c]	-	-	-	-
N-acetylglucosamine	(+)	-	-	+	+	+	+	+	+	v[c]	+	+	+
D-melezitose	-	-	-	-	-	+	-	-	-	-	-	-	-
gentiobiose	(+)	-	+	+	+	(+)	+	+	+	+	+	+	+

D-turanose	-	-	+	-	-	+	-	-	v[c]	-	-	-	-
D-fucose	+	+	-	+	+[a]	(+)[d]	-	-	-	-	(+)[d]	+	-
D-arabitol	-	-	-	+	-	-	+	+	+	-	-	-	-
Utilisation of:													
D-turanose	-	-	+	-	-	+	v	ND	+	ND	-	-	-
stachyose	-	-	+	-	-	v	+	ND	+	ND	v	-	-
N-acetyl-D-galactosamine	-	-	w+	+	w+	-	+	ND	-	ND	-	-	+
N-acetyl neuraminic acid	-	+	+	-	w+	-	+	ND	+	ND	-	+	+
3-methyl glucose	-	+	-	+	+	-	+	ND	-	ND	-	-	+
D-serine	-	+	+	+	+	-	+	ND	+	ND	+	+	+
D-sorbitol	+	+	-	-	+	+	-	ND	v	ND	+	+	-
D-arabitol	-	-	-	+	-	-	+	ND	+	ND	-	-	-
D-aspartic acid	-	+	+	+	+	+	+	ND	-	ND	+	v	+
D-serine	-	+	+	+	+	-	+	ND	-	ND	+	+	+
minocycline	-	+	-	-	-	-	-	ND	-	ND	+	-	-
quinic acid	+	+	-	+	+	+	+	ND	-	ND	+	+	+
tween 40	-	-	+	+	+	v	+	ND	+	ND	-	-	+
acetoacetic acid	-	-	+	+	+	-	+	ND	-	ND	-	+	+
sodium butyrate	-	+	-	+	-	-	-	ND	-	ND	+	-	-

+, 90 – 100 % strains +; (+), 70 – 89 % strains +; -, 91 – 100 % strains -; w+, weakly positive; v, variable; ND, not determined

[a] Differs from Jeon *et al*., 2021

[b] Late reaction for type strain

[c] Positive for type strain

[d] Negative for type strain

Table 3: Fatty acid composition (percentage of peak areas) of *Rahnella* species.

1 = *Rahnella perminowiae* sp. nov. (*n* = 4), 2 = *Rahnella bonaserana* sp. nov. (*n* = 1), 3 = *Rahnella rivi* sp. nov. (*n* = 1), 4 = *Rahnella ecdela* sp. nov. (*n* = 1), 5 = *Rahnella aquatilis* (*n* = 1), 6 = *Rahnella aceris* (*n* = 4, type strain not included), 7 = *Rahnella bruchi* (*n*=3), 8 = *Rahnella contaminans* (*n* = 1), 9 = *Rahnella inusitata* (*n*=3), 10 = *Rahnella laticis* (*n* = 2), 11 = *Rahnella variigena* (*n*=4), 12 = *Rahnella victoriana* (*n*=4), 13 = *Rahnella woolbedingensis* (*n*=3). Values are expressed as the average if more than one strain per species were investigated, with the standard deviation shown in parentheses. Data for 5, 7, 9, 11 – 13 taken from [5], data for 8, 10 taken from [7] . *n* = number of strains.

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8***	**9**	**10***	**11**	**12**	**13**
Saturated fatty acids													
$C_{12:0}$	4.1 (± 0.1)	3.9	3.9	4.5	4.2	4.1 (± 0.0)	3.5 (± 0.0)	3.2	3.6 (± 0.2)	3.4 (± 0.07)	3.6 (± 0.1)	3.6 (± 0.3)	3.2 (± 0.0)
$C_{14:0}$	5.5 (± 0.1)	5.7	5.8	6.1	6.4	5.5 (± 0.1)	6.0 (± 0.3)	7.0	6.2 (± 0.2)	6.6 (± 0.8)	6.6 (± 0.3)	6.6 (± 0.3)	6.4 (± 0.1)
$C_{16:0}$	33.2 (± 0.4)	34.6	33.0	38.5	33.1	33.6 (± 0.3)	34.1 (± 0.5)	31.1	34.4 (± 1.4)	30.9 (± 5.9)	34.4 (± 2.1)	34.8 (± 0.3)	34.2 (± 1.1)
Unsaturated fatty acids													
$C_{18:1}$ ω7*c*	9.3 (± 0.9)	4.6	12.1	7.3	8.4	9.0 (± 0.2)	2.6 (± 0.9)	8.1	9.6 (± 0.8)	10.1 (± 3.4)	5.4 (± 1.1)	6.9 (± 0.5)	8.2 (± 1.3)
Cyclopropane fatty acids													
$C_{17:0}$	27.7 (± 1.3)	29.7	19.8	28.7	28.7	23.7 (± 0.5)	30.7 (± 0.7)	28.0	24.2 (± 2.4)	24.2 (± 3.1)	30.3 (± 0.5)	28.1 (± 0.6)	28.4 (± 1.2)
$C_{19:0}$ ω8*c*	2.6 (± 0.2)	4.7	0.3	5.7	2.3	1.6 (± 0.1)	7.9 (± 0.7)	2.4	1.0 (± 0.4)	1.6 (± 0.1)	5.0 (± 0.5)	3.6 (± 0.6)	3.9 (± 0.5)
Summed features													

2: $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$	9.5 (± 0.2)	8.9	9.5	9.5	6.9	9.3 (± 0.1)	9.0 (± 0.1)	9.0	9.1 (± 0.4)	8.5 (± 1.9)	8.9 (± 0.2)	8.8 (± 0.3)	8.7 (± 0.1)
3: $C_{16:1}$ ω7*c* and/or $C_{16:1}$ ω6*c*	8.4 (± 1.3)	4.2	12.6	4.4	8.1	9.9 (± 0.8)	1.8 (± 0.2)	7.2	10.1 (± 1.8)	10.2 (± 5.2)	2.9 (± 0.7)	5.0 (± 0.6)	4.2 (± 1.4)

[*] Fatty acid analysis for these two species was performed by Jeon *et al*. [7] following growth on nutrient agar, whereas the remaining *Rahnella* species were cultured on TSA prior to analysis by Fera Science Ltd. (York, UK).

Figure 1: Maximum likelihood tree based on concatenated partial *gyrB*, *rpoB*, *atpD* and *infB* gene sequences of proposed novel *Rahnella* species, existing *Rahnella* species and the closest phylogenetic neighbours. Bootstrap values after 1000 replicates are expressed as percentages (values > 50 % shown). *Hafni aalvei* (ATCC 13337^T) is included as an outgroup. The scale bar indicates the fraction of substitutions per site. ^{T} = type strain

Figure 2: Phylogenomic tree of proposed novel *Rahnella* species, existing *Rahnella* species and the closest phylogenetic neighbours. GBDP pseudo-bootstrap support values > 60 % shown at the nodes (from 100 replicates), with an average branch support of 85.4 %. The branch lengths are scaled in terms of GBDP distance formula d_5. The tree is rooted at the midpoint. GenBank assembly and accession numbers are given in parentheses. ^{T} = type strain

Figure 3: Transmission electron microscopy of proposed novel *Rahnella* species displaying their flagella arrangement: (a) *Rahnella perminowiae* SL6^T, (b) *Rahnella bonaserana* H11b^T, (c) *Rahnella rivi* FC061912-K^T, (d) *Rahnella ecdela* FRB 231^T. Scale bar, 1 µm.