New Taxa: Proteobacteria

Rosenbergiella australoborealis sp. nov., Rosenbergiella collisarenosi sp. nov. and Rosenbergiella epipactidis sp. nov., three novel bacterial species isolated from floral nectar

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A B S T R A C T

The taxonomic status of nine strains of the family Enterobacteriaceae isolated from floral nectar of wild Belgian, French, South African and Spanish insect-pollinated plants was investigated following a polyphasic approach. Confirmation that these strains belonged to the genus Rosenbergiella was obtained from comparative analysis of partial sequences of the 16S rRNA gene and other core housekeeping genes (atpD [ATP synthase β-chain], gyrB [DNA gyrase subunit B] and rpoB [RNA polymerase β-subunit]). DNA–DNA reassociation data, determination of the DNA G+C content and phenotypic profiling. Two strains belonged to the recently described species Rosenbergiella nectarea, while the other seven strains represented three novel species within the genus Rosenbergiella. The names Rosenbergiella australoborealis sp. nov. (with strain CdVSA 20.1T [LMG 27954 T = CECT 8500 T] as the type strain), Rosenbergiella collisarenosi sp. nov. (with strain 8.8A T [LMG 27955 T = CECT 8501 T] as the type strain) and Rosenbergiella epipactidis sp. nov. (with strain 2.1A T [LMG 27956 T = CECT 8502 T] as the type strain) are proposed. Additionally, the description of the genus Rosenbergiella is updated on the basis of new phenotypic and molecular data.

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Introduction

Floral nectar is regarded as the key component in the mutualism between animal-pollinated plants and their pollinators, which use this sugar-rich solution as a reward for their pollination services [14,37]. Floral nectar is assumed to be initially sterile [15], but floral visitors can actively vector different microorganisms, most often yeasts and bacteria [1,8,41]. Some of these yeasts and bacteria, are particularly well adapted to flourish in this rather unique harsh environment of high sugar concentrations and antimicrobial compounds [4,41].

Although it has already been known for decades that microbes are common inhabitants of floral nectars [48], the ecological importance of nectar-associated microorganisms for the plant–pollinator mutualisms is only now beginning to be understood. Floral microbes can have a negative impact on plant–pollinator mutualisms by decreasing floral attractiveness through a reduction of nectar nutritional value [24,38] and interfering with pollen germination and damaging pollen tubes [27]. However, it has also been suggested that floral microorganisms enhance pollination by producing volatiles or fermentation by-products that attract pollinators [57,59] and by raising flower temperature [40]. In this regard, Herrera et al. [42] recently demonstrated that nectar yeasts...
increased pollinator visitation, and may have important consequences for the fecundity of plants. In particular, Vannette et al. [68] further showed that nectar bacteria, rather than yeasts, reduced pollination success, seed set and nectar consumption by pollinators, thereby weakening the plant–pollinator mutualism.

Current studies have highlighted that nectar-associated microbial communities are generally species-poor (but see [29]), but may nevertheless represent a reservoir of unexplored microbial biodiversity [15,16,22,39,46,55,58]. However, research efforts to find new species associated with floral nectar have been significantly more intensive for yeasts (e.g. [23,30,62]) than for bacteria and to date only two studies have described novel bacterial species in nectar [4,35]. The newly described bacterial species all belonged to the Gammaproteobacteria, and include two new species of the family Moraxellaceae, Acinetobacter nectaris and A. boissieri [4], and one of the family Enterobacteriaceae, Rosenbergiella nectaris [35].

The Enterobacteriaceae are a large family that live in a wide variety of habitats, including plants, food and environmental sources and clinical samples [11,12,43,50]. With regard to flowers, Junker et al. [49] demonstrated that bacteria of this family dominate the epiphytic bacterial communities in petals. Additionally, several members of this family have also been found inhabiting the floral nectar of some cultivated plant species from Northern Israel [1,29] and diverse wild plant species from Belgium, Spain and South Africa [2,3,4,6,47]. Strikingly, some Enterobacteriaceae isolates were found that could not be identified conclusively to the genus or species level based on 16S ribosomal RNA (rRNA) gene sequence analysis. In this study, we investigated the taxonomic status of nine of these Enterobacteriaceae isolates obtained from floral nectar from phylogenetically diverse wild plant species across different study sites on two continents. Europe (Belgium, France and Spain) and Africa (South Africa). Additionally, we provide an updated description of the genus Rosenbergiella on the basis of new physiological and molecular data.

Materials and methods

Bacterial strains and DNA extraction

Nine strains isolated from floral nectar according to the procedure described by Álvarez-Pérez et al. [2] were used in this study (Table 1). Strains were isolated in 2011 and 2012 from five insect-pollinated plant species, including Epipactis palustris (Orchidaceae; Belgium and France), Iris xiphium (Iridaceae; Spain), Narcissus papyraceus (Amaryllidaceae; Spain), Protea roupelliae and P. subvestita (Proteaceae; South Africa) (Table 1). In addition, reference strains of the most closely related species to our strains were included in the study for comparative phenotypic analysis, including R. nectaris (LMG 26121, further referred to as BN41), Phascolobacter flecens (LMG 2186) and Tatumella citrea (LMG 22049). Cultures were preserved at −80 °C in trypticase soy broth (Oxoid, Basingstoke, UK), containing 32.5% glycerol. Genomic DNA was extracted from five-day old cultures grown on trypticase soy agar (TSA; Oxoid) using the phenol–chloroform extraction method described previously [53].

RAPD fingerprinting

In order to assess whether our isolates represented different strains, each of the nine isolates was subjected to Random Amplified Polymorphic DNA (RAPD) analysis. Amplification was performed using a Bio-Rad T100 thermal cycler in a total volume of 20 µl containing 0.5 µM of primer OPB-10 (‘5’-CTGCTTGGGC-3’) (Operon, Huntsville, USA), 0.15 mM of each dNTP, 1.0 unit Titanium Taq DNA polymerase, 1× Titanium Taq PCR buffer (Clontech Laboratories, Palo Alto, CA, USA), and 5 ng genomic DNA (as measured by a Nanodrop spectrophotometer). Samples were subjected to the following PCR conditions: denaturation at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 30 °C and 2 min at 72 °C, with a final extension at 72 °C for 10 min. Obtained PCR products were separated by loading 6.5 µl of the reaction volume on 1.5% agarose gels followed by gel electrophoresis in 1× Tris/acetate EDTA (TAE) buffer at 100 V for three hours. Gels were stained with ethidium bromide and visualized with UV light. A 200–100,000 bp DNA ladder (Smartadder, Eurogentec, Seraing, Belgium) was used as size marker for comparison. The BioChemi System (UVP, Upland, CA, USA) was used to acquire image data. The analysis was performed twice with identical fingerprints, and indicated that our isolates represented different strains (Fig. S1).

PCR amplification and sequencing of selected loci

For each of the nine nectar strains, the almost complete 16S rRNA gene was PCR amplified using the primers 27F and 1492R [52]. PCR amplification was performed in a reaction volume of 20 µl, containing 312.5 µM of each dNTP, 1.0 µM of each primer, 1.25 units TaKaRa Ex Taq Polymerase, 1× Ex Taq Buffer (Clontech Laboratories, Palo Alto, CA, USA) and 5 ng genomic DNA. Before amplification, DNA samples were denatured at 94 °C for 2 min, followed by 35 cycles of 45 s at 94 °C, 45 s at 59 °C and 45 s at 72 °C, with a final extension at 72 °C for 10 min. Following agarose gel electrophoresis, target amplicons were cut from the gel and purified.

Table 1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host plant species (family)</th>
<th>Geographic origin</th>
<th>Latitude/longitude</th>
<th>Date of isolation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.12A</td>
<td>Epipactis palustris (Orchidaceae)</td>
<td>Dune Dewulf, Belgium</td>
<td>51°3’48” N, 2°28’9” E</td>
<td>July 2012</td>
<td>This study</td>
</tr>
<tr>
<td>2.1A</td>
<td>Epipactis palustris (Orchidaceae)</td>
<td>Dune du Perroquet, France</td>
<td>51°4’48” N, 2°32’8” E</td>
<td>July 2012</td>
<td>This study</td>
</tr>
<tr>
<td>2.6A</td>
<td>Epipactis palustris (Orchidaceae)</td>
<td>Dune du Perroquet, Bray-Dunes, France</td>
<td>51°4’48” N, 2°32’8” E</td>
<td>July 2012</td>
<td>This study</td>
</tr>
<tr>
<td>8.8A</td>
<td>Epipactis palustris (Orchidaceae)</td>
<td>Ter Yde, Oostduinkerke, Belgium</td>
<td>51°7’4” N, 2°40’6” E</td>
<td>July 2012</td>
<td>This study</td>
</tr>
<tr>
<td>SAP 86.2</td>
<td>Narcissus papyraceus (Amaryllidaceae)</td>
<td>Hinojos, Huelva, Spain</td>
<td>37°18’14” N, 06°26’15” E</td>
<td>January 2011</td>
<td>Álvarez-Pérez and Herrera (2013)</td>
</tr>
<tr>
<td>SAP 87.2</td>
<td>Iris xiphium (Iridiaceae)</td>
<td>Hinojos, Huelva, Spain</td>
<td>37°18’14” N, 06°26’15” E</td>
<td>May 2011</td>
<td>Álvarez-Pérez and Herrera (2013)</td>
</tr>
<tr>
<td>CdVSA 20.1</td>
<td>Protea roupelliae (Proteaceae)</td>
<td>Mount Gilboa, South Africa</td>
<td>29°16’58” S, 30°17’32” E</td>
<td>January 2011</td>
<td>This study</td>
</tr>
<tr>
<td>CdVSA 21.1</td>
<td>Protea roupelliae (Proteaceae)</td>
<td>Mount Gilboa, South Africa</td>
<td>29°16’58” S, 30°17’32” E</td>
<td>January 2011</td>
<td>This study</td>
</tr>
<tr>
<td>CdVSA 50.1</td>
<td>Protea subvestita (Proteaceae)</td>
<td>Sani Pass, Southern Drakensberg, South Africa</td>
<td>29°36’40” S, 29°21’40” E</td>
<td>January 2011</td>
<td>This study</td>
</tr>
</tbody>
</table>
using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Purified PCR products were cloned into *Escherichia coli* plasmids using the pCR2.1 vector and the Topo-ITA cloning kit (Invitrogen™ Life Technologies™, Carlsbad, CA, USA) and sequenced with the primers M13F and M13R. To improve the accuracy of the sequencing, inserts of at least three individual clones were sequenced. Subsequently, obtained sequences were individually trimmed for quality, using a minimum Phred score of 20. For each target fragment, accurate consensus sequences were obtained for each isolate by aligning the acquired sequences with MEGA v.5 [67] and manual sequence editing of ambiguous base calls based on the obtained electropherograms, resulting in a sequence length of 1501 bp. Partial fragments of the housekeeping genes atpD (ATP synthase β-chain), gyrB (DNA gyrase subunit B) and rpoB (RNA polymerase β-subunit) were amplified using the primer pairs atpD 01-F/atpD 02-R [10], gyrB 01-F gyrB 02-R [21] and rpoB ML01-F (5'-AGTTATGGACGACAAACC-3')/rpoB ML02-R (5'-TTGCAATTTGACACCATTCA-3'). In all cases, PCR amplification was performed in a reaction volume of 20 μL, consisting of 15.0 mM of each dNTP, 0.5 μM of each primer, 1 unit Titanium Taq DNA polymerase, 1X Titanium Taq PCR buffer (Clontech Laboratories), and 5 ng genomic DNA. PCR conditions were as described above, with the exception of the annealing time and temperature, which were 1 min at 55 °C for atpD and gyrB, and 45 s at 63 °C for rpoB. Cloning, sequencing (using M13F) and subsequent assembly of consensus sequences were performed as described above and resulted in sequences of 856, 914 and 507 bp for atpD, gyrB and rpoB, respectively.

Taxonomy of nectar isolates and phylogenetic reconstruction

In order to determine the taxonomic affiliation of our nectar strains, the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/), last accessed 15 November 2013; [51]) was used to search for neighbours among validly named bacterial species on the basis of 16S rRNA gene sequences. Closest relatives of the studied nectar strains were also identified by a BLAST search of 16S rRNA sequences in GenBank [5]. Nucleotide sequences of the nectar isolates and type strains of closely related species for the four studied loci were included in multiple alignment generations by MUSCLE (http://www.ebi.ac.uk/Tools/maa/muscle/,[26]). The resulting alignments were trimmed with BioEdit v.7.0.9.0 [33] to ensure that all sequences had the same start and end point.

Phylogenetic trees for each individual gene and a concatenation of the three protein-encoding genes (atpD, gyrB and rpoB, in this order) were constructed using four different methods in order to provide solid evidence of the classification: neighbour-joining (NJ), maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML). NJ and MP trees were obtained using MEGA v.5. In NJ analyses, the evolutionary distances were computed by the Tamura-Nei method [66], and the rate of variation among sites was modelled by a gamma distribution, with shape parameters set at the values estimated by PhyML in ML analyses (see below). MP trees were obtained using the close-neighbor-interchange (CNI) algorithm with search level 3, in which the initial trees were obtained with the random addition of sequences (ten replications). In both the NJ and MP methods, 1000 bootstrap replications were used to infer consensus trees.

BI analyses were performed with MrBayes v.3.1.2 [61]. The simplest models of sequence evolution among those available in MrBayes that best fitted the sequence data were determined using the jModeltest v.0.1.1 package [56], and resulted in selection of the following: HKY+G+I for the 16S rRNA gene, HKY+G for gyrB, SYM+G+I for atpD, and GTR+G+I for rpoB. A partitioned model was used to obtain the atpD + gyrB + rpoB concatenated tree. Four Metropolis-coupled Markov chains (five for the 16S rRNA dataset) were run twice, until average standard deviation of split frequencies fell below 0.01 (1.4 x 10⁶ to 1.5 x 10² generations). The chains were sampled each 100 generations and chain temperature was set to 0.2. Fifty percent majority rule consensus trees were calculated using the sumt command and discarding the first 25% of the trees to yield the final Bayesian estimates of phylogeny. Posterior probabilities (PP) from the 50% majority rule consensus trees were used as estimates of robustness.

ML analyses were carried out using the online version of PhyML v.3.0 (http://www.atgc-montpellier.fr/phyml/,[32]), under the HKY+G+I (16S rRNA), HKY+G (gyrB) or the GTR+G+I (atpD, rpoB and concatenated dataset) models of molecular evolution, with four substitution rate categories, starting trees generated by BIONJ, and SPR tree search algorithms. Model parameters were estimated from the dataset and support for the inferred topologies was tested using 1000 bootstrap replications.

The confidence of alternative tree topologies based on single gene and concatenated datasets was evaluated by the Shimodaira–Hasegawa (SH) test [64]. This analysis was performed with TREE-PUZZLE v.5.2 [63], and tree topologies with p-values < 0.05 were considered to be incongruent with the dataset under analysis.

**DNA–DNA hybridisation and DNA G+C content analysis**

In order to perform DNA–DNA hybridisations between the strains 1.12A, 2.1A, 8.8A, GvSA20.1, and R. nec-tarea 8N4² as well as to determine the DNA G+C content of the novel type strains, high-quality DNA was prepared by the method of Wilson [70], with some minor adjustments [19]. DNA–DNA hybridisations were performed at 41 °C using the microplate method [28] with some modifications [19,31]. Reciprocal reactions were performed for every pair. For determination of the DNA G+C content the DNA was enzymatically degraded into nucleosides and the DNA base composition was determined by HPLC [54]. Three independent analyses were conducted for each DNA sample, and mean DNA G+C content values were calculated.

**Physiological and biochemical characterisation**

For physiological and biochemical characterisation, strains were grown aerobically on TSA at 25 °C for 24 h unless otherwise indicated. Tests for motility, hydrogen sulphide production and indole production were performed in SIM (Sulphide Indole Motility) medium (Becton, Dickinson and Company, Le Pont de Clai, France) at 25 °C. Growth at 4, 22, 25, 30, 37 and 42 °C, haemolysis of sheep erythrocytes on Columbia blood agar (bioMérieux, Marcy l’Étoile, France) and sucrose tolerance (0, 10, 20, 30, 40, 50, 60% sucrose (w/v, Sigma–Aldrich) in Luria–Bertani (LB) broth (Difco)) was determined as described previously [4]. Catalase activity was determined by observing bubble production with a 3% (v/v) hydrogen peroxide solution [17]. Oxidase activity was tested with oxidase test strips (Oxoid) and DNase plates (Oxoid) were used for investigating DNase activity. Atoxin (3-hydroxy-2-butanone) production was determined with the Voges–Proskauer reaction [7]. Production of acid from glucose, fructose and sucrose in Hugh–Leifson medium was performed as described previously [44]. Fermentation of lactose was examined using MacConkey agar (TecLaim, Madrid, Spain) with confirmation in Phenol Red Lactose Broth [7]. Further, the ability to hydrolyse gelatin was determined by the gelatin plate assay described by Atlas [7] and growth on Brain Heart Infusion agar (Panreac, Castellar del Vallés, Barcelona) was evaluated as well. Finally, Phenotype MicroArray (PM) tests (Biolog, Hayward, CA, USA) were performed [9] using PM Plate 1, containing 95 different single carbon sources in addition to a negative control. Plates were incubated during four days at 25 °C in the OmniLog automated
Results and discussion

BLAST analysis of the 16S rRNA gene sequences against GenBank revealed that all nectar strains studied belonged to the Enterobacteriaceae. Additionally, using the EzTaxon server, R. nectarae 8N4T was found to be the most closely related species to our nectar strains, followed by Phaseolobacter flectens ATCC12775T (Table S1). The 16S rRNA gene sequence identity level between all our strains varied between 99.27% and 100%. Whereas bacterial species delineation is generally based on a 16S rRNA gene similarity cut-off level of 97% [65], different Enterobacteriaceae species may share over 99% 16S rRNA gene sequence similarity [12], while 98% is generally considered as a reasonable cut-off value to delineate different genera in this family [25,45]. On the other hand, Halpern et al. [35] defined a specific signature region in the 16S rRNA gene for the genus Rosenbergiella. While this signature perfectly matched sequences for the strains 1.12A, 2.1A1, 2.6A and 8.8AT, four mismatches were found when compared with sequences of the other five nectar strains (SAP 86.2, SAP 817.2, CdVSA 20.1T, CdVSA 21.1 and CdVSA 50.1). It has to be noted, however, that the proposed signature region was found based on a single Rosenbergiella species only. To further assess the phylogenetic position of our strains, partial atpD, gyrB and rpoB gene sequences were analysed. For these individual markers, it has been shown that species from different Enterobacteriaceae genera generally share less than 94% gene similarity [25,60]. Nevertheless, specific limits for species or genus differentiation of the Enterobacteriaceae based on these genes are not defined so far [13]. Highest levels of sequence similarity were again found with R. nectarae 8N4T, ranging from 94.86 to 97.30%, from 82.12 to 98.47% and from 84.84 to 96.21% for atpD (740 bp), gyrB (850 bp) and rpoB (343 bp), respectively (Table S2).

Phylogenetic analysis of the 16S rRNA gene sequences showed that the Belgian (8.8AT) and French (1.12A, 2.1A1 and 2.6A) nectar strains clustered with R. nectarae 8N4T, but that grouping was only well-supported in the NJ tree (bootstrap support for the clade was <75% in the ML and MP trees, and the posterior probability was 50% in the BI tree. Fig. 1). The Spanish (SAP 86.2 and SAP 817.2) and South African (CdVSA 20.1T, CdVSA 21.1 and CdVSA 50.1) nectar strains formed another poorly supported clade in the 16S rRNA trees obtained by all phylogenetic methods (<75% bootstrap support or posterior probability). These strains formed separate clusters, but were closely together to R. nectarae 8N4T and the other nectar strains. The combined analysis of atpD, gyrB and rpoB sequences provided a better resolution and allowed classifying the studied strains in four clades (Fig. 2). The first clade, which was highly supported by all the phylogenetic methods, included strains 1.12A and 2.6A, as well as R. nectarae 8N4T. Strain 2.1A1 was close to but clustered apart from the former strains in all concatenated trees, whereas the Spanish and South African strains showed limited sequence variability (as inferred from the length of tree branches) and formed a third robust cluster. Strain 8.8AT was basal to the rest of nectar strains in the concatenated NJ and MP trees with >99% bootstrap support, but clustered with the Spanish and South African strains in the trees obtained by the ML and BI methods (75.5% bootstrap support and 88% posterior probability, respectively; data not shown). These four clusters were also shown in the independent analyses of the three protein-encoding genes, although their support values and phylogenetic relations were variable (Fig. S2a–S2c). The results of the SH test performed on individual gene sequences and on the concatenated dataset showed that most NJ trees were incongruent with each other, but were not significantly different from the NJ tree based on the alignment of concatenated sequences (Table S3). Furthermore, most datasets supported the trees obtained by the ML and BI methods for the concatenation of individual loci, and the gyrB and rpoB datasets also supported the concatenated MP tree (Table S3). Therefore, although the studied genes may not exhibit the same evolutionary history, in general terms the trees based on the alignment of concatenated sequences did not contradict the information brought by each individual locus. DNA–DNA hybridisation confirmed the MLSA analysis. Strains 2.1A, 8.8AT and CdVSA 20.1T showed between 23 ± 15% and 53 ± 9% DNA–DNA relatedness among each other and with 8N4T (Table 2). Strain 1.12A showed 77 ± 7% DNA–DNA relatedness with the type strain of R. nectarae (Table 2), which is above the generally accepted limit for species delineation (i.e. 70%; [69]).

All strains tested were facultative anaerobic, catalase-positive, oxidase-negative and DNase-negative, and could grow at 22, 25 and 30 °C, but not at 4 °C (Table S4). Most strains also grew at 37 °C, with the exception of strains 8.8AT and 2.1AT (Tables 3 and S4). All nectar strains were able to grow at sucrose concentrations up to 50% (w/v), while no growth was observed at 60% sucrose. In contrast to Halpern et al. [35], who observed growth for R. nectarae 8N4T up to 60% sucrose concentration, in our assay 8N4T was only found to grow up to 50% sucrose. All strains were non-haemolytic on Columbia blood agar and were motile in SIM medium. None of them produced indole or hydrogen sulphide, nor were able to hydrolyse gelatin. All strains produced acid from sucrose, glucose and fructose. When streaked on MacConkey agar, all strains were positive for lactose fermentation, which was also confirmed using Phenol Red Lactose Broth. Further, all strains were able to produce acetoin (positive Voges–Proskauer reaction) (Table S4). Clear differences were observed among our nectar strains and between these and the reference strains of R. nectarae, P. flectens and T. citrea when phenotyped by the Biolog system (Tables 3 and S4).

In contrast to the other nectar strains, strains 1.12A, 2.6A, R. nectarae 8N4T and T. citrea LMG 22049T were the only strains able to oxidise 2-deoxyadenosine. Oxidation of phenylethylamine could only be performed by the strains 1.12A and 2.6A. T. citrea LMG 22049T, and strains 1.12A and 2.6A were the only strains that could oxidise citric acid. Together with R. nectarae 8N4T and T. citrea LMG 22049T, strain 8.8AT was the only nectar isolate that could oxidise D-glucose-6-phosphate. Further, together with the Spanish and South African strains (CdVSA 20.1T, CdVSA 21.1, CdVSA 50.1, SAP 86.2, and SAP 817.2), these four isolates could oxidise β-methyl-D-glucoside. The Spanish and South African strains differed from the other nectar strains in the inability to oxidise glycol-L-aspartic acid (Gly-Asp), glycol-L-glutamic acid (Gly-Glu), d,L-α-glycerol phosphate, uridine and d-ribose. Some key biochemical features (namely D-glucose-6-phosphate, β-methyl-D-glucoside, glycol-L-aspartic acid, glycol-L-glutamic acid, uridine and d-ribose) were also assessed using more conventional tests using the basal mineral medium of Cruze et al. [20], supplemented with 0.1% (w/v) of the tested carbon source (incubated for 10 days at 25 °C), and confirmed the Biolog fingerprinting results.

Table 2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% DNA–DNA relatedness</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1.12A</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<td></td>
<td>33 ± 7</td>
</tr>
<tr>
<td></td>
<td>28 ± 2</td>
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<td></td>
<td>77 ± 7</td>
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Table 3
Distinctive\(^1\) phenotypic characteristics for the three novel Rosenbergiella species in comparison with their most related species.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(R.) epipactidis (2.1A)</th>
<th>(R.) collisarenosi (8.8A)</th>
<th>(R.) australoborealis (CdVSA 20.1)(^1)</th>
<th>(R.) nectarea (8N4)</th>
<th>(P.) flectens LMG 2186</th>
<th>(T.) citrea LMG 22049(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth (72h) on TSA at:</td>
<td></td>
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<tr>
<td>4°C</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>37°C</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>42°C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>Growth on MacConkey agar (48h)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Oxidation of(^6):</td>
<td></td>
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</tr>
<tr>
<td>Ala-Gly</td>
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\(^{+}\): positive reaction; \(^{-}\): negative reaction; \(^{w}\): weak growth.

\(^{1}\) A comprehensive overview of the results obtained for the different phenotypic tests performed in this study is given in Table S4.

\(^{6}\) Oxidation of carbon sources was determined by Phenotype MicroArray (PM) technology (Biolog) using PM Plate 1. Plates were incubated in the OmniLog\(^{5}\) incubator-reader for 4 days at 25\(^\circ\)C and were read every 15 minutes. OmniLog\(^{5}\) PM software was used to calculate the area under the curve. Reactions were considered positive when the net area under the curve exceeded the value for the blank three times. All isolates were positive for oxidation of the following substrates not shown in the table: \(l\)-asparagine, \(l\)-glutamic acid, \(\alpha\)-D-glucose, \(d\)-fructose, \(d\)-galactose, glycerol, inosine and sucrose. All isolates were negative for oxidation of the following substrates not shown in the table: 1,2-propanediol, 2-aminoethanol, glucuronamide, phenylglycine, tyramine, \(d\)-aspartic acid, \(d\)-serine, \(d\)-threonine, \(l\)-threonine, adonitol, \(\alpha\)-methyl-\(d\)-galactoside, \(\alpha\)-methyl-\(d\)-galactose, \(d\)-mehlsieux, \(d\)-sorbitol, dulcitol, \(l\)-fucose, N-acetyl-\(d\)-mannosamine, acetic acid, acetoacetic acid, \(\alpha\)-hydroxybutyric acid, \(\alpha\)-hydroxyglutaric acid-\(\gamma\)-lactone, \(\alpha\)-ketoisocaproic acid, \(\alpha\)-ketoisocaproic acid, \(\alpha\)-hydroxybutyric acid, \(\alpha\)-methyl-D-glucosamine, formic acid, glycolic acid, glyoxylic acid, m-hydroxyphenyl acetic acid, m-tartaric acid, p-hydroxyphenyl acetic acid, propionic acid, tricarballylic acid, Tween 20, Tween 40 and Tween 80.
Taken together, the results of these genotypic and phenotypic analyses support the classification of 1.12A and 2.6A within the species *R. australoborealis*. Additionally, our results support the distinction of three additional *Rosenbergiella* species isolated from floral nectar. For these species, we propose the names *Rosenbergiella australoborealis* sp. nov., *Rosenbergiella epipactidis* sp. nov. and *Rosenbergiella collisarenosi* sp. nov., represented by the type strains CdVSA 20.1T, 2.1A² and 8.8A², respectively. The DNA G+C content of the novel type strains CdVSA 20.1T, 2.1A² and 8.8A² was 45.3, 47.2 and 47.6 mol\%, respectively, which is similar to the DNA G+C content of *R. australoborealis* 8N4T (46.8 mol\%) and slightly higher than that of the related species *P. flectens* (44.3 mol\%) [34,35]. The DNA G+C content of strains 1.12A and 2.6A was 47 mol\%, which is very close to the G+C content of the type strain of *R. australoborealis*, supporting again that both strains belong to the same species.

The results of the present study also provide new insights into the taxonomy and phylogenetic affiliation of nectar-dwelling prokaryotes, which complement the recent description of bacteria...
Fig. 2. Neighbour-joining (NJ) consensus tree, based on phylogenetic analysis of concatenated atpD + gyrB + rpoB sequences, displaying the relationships of the nectar strains of Rosenbergiella australoborealis sp. nov., Rosenbergiella collisarenosi sp. nov., Rosenbergiella epipactidis sp. nov. and Rosenbergiella nectarea characterised in this study with respect to R. nectarea BNA4\(^T\) and other representatives belonging to the family Enterobacteriaceae. Evolutionary distances were computed using the Tamura-Nei method and are in the units of number of base substitutions per site. There were a total of 1404 positions in the final dataset. Node support values (bootstrap percentages, based on 1000 simulations) \(\geq\) 80% are shown next to the branches. Maximum parsimony (MP) and maximum likelihood (ML) bootstrap node support values \(\geq\) 90% and Bayesian inference (BI) posterior probabilities \(\geq\) 90% are marked by asterisks, filled squares and long crosses, respectively. Pseudomonas aeruginosa LMG 1242\(^T\) was used as an outgroup to root the tree. GenBank accession numbers are provided in Figure S2.
in the Gammaproteobacteria of similar ecology [4,35]. Rosen-
bergiella is a recently described new genus of Enterobacteriaceae
that was previously isolated from floral nectar of two cultivated
plant species in Israel, Amygdalus communis (Almond) and Cit-
rus paradisi (Grapefruit). In this study, we report the description
of novel Rosenbergiella species isolated from wild phylogeneti-
cally diverse plant species with a diverse array of pollinators
including bees, beetles, birds, butterflies, flies, and wasps [6,18,36].
As microbes are vectored from flower to flower by floral visi-
tors [1.8,41] and the observed vectors are common in nature,
it may be assumed that the bacteria they are transporting are also
widespread among different plant hosts. The diverse vector and
host sources together with its wide geographical distribution in
both hemispheres suggests that Rosenbergiella is a well-established
 genus, which has adapted to the conditions of nectar, being able to
withstand high sugar concentrations (up to 50%, w/v) and to oxy-
dise the major sugars of nectar (i.e. sucrose, glucose and fructose).
The ecological role of Rosenbergiella species in the multi-kingdom
interactions taking place within and around floral nectar remains
unclear to date, but clearly deserves further studies.

Update of Rosenbergiella Halpern et al. 2013

The genus Rosenbergiella (Ro.sen.ber.gi.e'll.a. N.L. fem. dim. n.,
named after Prof. E. Rosenberg, an Israeli microbiologist) has been
described previously by Halpern et al. [35] based on three strains
and a single species (R. nectarea), with 8N4 as the type species
[35]. Our study allows updating the description of the genus. Cells
are Gram-negative, yellow/orange (pale) beige pigmented, rods
that are facultatively anaerobic and motile. Strains grow well at
22–30 °C, some also at 37 °C, but not at 4 °C (but see Halpern et al.
[35]) and 42 °C. Catalase-positive, oxidase-, DNase- and gelatinase-
negative. Strains grow on Brain Heart Infusion agar, MacConkey
agar and Columbia blood agar but are not haemolytic on the latter
medium. Sucrose is tolerated up to a concentration of 50% (w/v).
Indole and H2S are not produced. Positive for Voges–Proskauer reac-
tion. Produce acid from sucrose, glucose and fructose and oxidise
glycolyl-β-proline (Gly-Pro), l-asparagine, l-aspartic acid, l-glutamic
acid, l-proline, adenosine, α-d-glucose, d-fructose, d-galactose,
glycerol, inosine, l-arabinose, N-acetyld-glucosamine, sucrose and d-
glucosic acid. Lactose is fermented. At the genetic level, all Rosen-
bergiella strains (12 investigated, including nine from this study
and the three strains used by Halpern et al. [35]) have over 99% (≥99.4% on a total of ~1350 bp) sequence similarity for the 16S
rRNA gene. Additionally, all strains share >80% sequence similarity for the rpoB (343 bp investigated), gyrB (850 bp) and atpD (740 bp)
genes, respectively. G+C content for the different species varies
between 45.3 and 47.6 mol%. Major cellular fatty acids, as deter-
dined for the type species R. nectarea 8N4, are C16:0, C17:0, CYCLO
C18:1 ω7c and C16:1 ω7c [iso-C15:0 20H] [35].

Description of R. australoborealis sp. nov.

R. australoborealis [aus.tra.lo.bo.re.a'lis L. gen., referring to the
fact that strains of this species were discovered in floral nectar
from different plants from the southern (australis) and the northern
(borealis) hemispheres.

Cells are Gram-negative rods that are facultatively anaero-
bic and motile. After 24 h aerobic incubation at 25 °C on TSA
medium, colonies are pale beige pigmented, convex and smooth
with entire margins. Catalase-positive, oxidase-, DNase-
and gelatinase-negative. Colonies grow on Brain Heart Infusion agar,
MacConkey agar and Columbia blood agar but all strains are not
haemolytic on the latter medium. R. australoborealis strains pro-
duce acid from sucrose, glucose and fructose, and ferment lactose.
Indole and H2S are not produced. Positive for Voges–Proskauer
reaction. Colonies grow well at 22–37 °C, but not at 4 °C and 42 °C.
Sucrose is tolerated at concentrations ranging from 10% to 50%
(w/v). Oxidation of glycy1-L-proline, l-asparagine, l-aspartic acid,
l-glutamic acid, l-proline, adenosine, α-d-glucose, β-methyl-d-
glucoside, d-fructose, d-galactose, glyceral, inosine, l-arabinose,
N-acetyld-glucosamine, sucrose and d-glucuronic acid as Biolog PM
1 substrates, while negative for the other tested sources. Oxidation
of l-glutamine and d-mannose is strain-dependent.

The description of the species is based on the characteristics
of five strains which were isolated from floral nectar of different
plant species from Spain and South Africa. The type strain is CdVSA 20.1T
(=LMG 27954T = CECT 8500T), isolated from floral nectar of Protea
roupeilliae (Proteaceae) in South Africa (Mount Gilboa). The DNA G+C
content of the type strain is 45.3 mol%.

Description of R. collisarenosi sp. nov.

R. collisarenosi [co.li.sar.e.no'si L. gen. collisarenosi, named after
the environment of the isolation source, found in nectar of an Epi-
pactus palustris plant living in a dune habitat].

Cells are Gram-negative rods that are facultative anaero-
bic and motile. After 24 h aerobic incubation at 25 °C on TSA
medium, colonies are pale beige pigmented, convex and smooth
with entire margins. Catalase-positive, oxidase-, DNase- and
gelatinase-negative. Colonies grow on Brain Heart Infusion agar,
MacConkey agar and Columbia blood agar without haemolysis
activity. Acid is produced from sucrose, glucose, and fructose.
Lactose is fermented, indole and H2S are not produced. Posi-
tive for Voges–Proskauer reaction. Strain 8.8AT grows well at
22–30 °C, but not at 4 °C, 37 °C and 42 °C. Sucrose is toler-
ated at concentrations ranging from 10% to 50% (w/v). The
studied strain oxidises glycy1-l-aspartic acid, glycy1-l-glutamic
acid, glycy1-l-proline, l-asparagine, l-aspartic acid, l-glutamic
acid, l-glutamine, l-proline, adenosine, α-d-glucose, β-methyl-
d-glucoside, D, D-α-glycerol phosphate, d-fructose, d-galactose,
d-glucoheptose, d-mannose, d-ribose, glycerol, inosine,
l-arabinose, N-acetyld-glucosamine, sucrose, uridine and d-
glucuronic acid as determined with the Biolog PM 1 assay.

The type strain is 8.8AT (=LMG 27953T = CECT 8501T), isolated
from floral nectar of Epipactus palustris (Orchidaceae) collected
in Belgium (Ter Yde, Oostduinkerke). The DNA G+C content of
the type strain is 47.6 mol%.

Description of R. epipactidis sp. nov.

R. epipactidis [epi.pac.ti.dis. L. gen. f. s. Epipactus -idis, referring to
the genus name of the host (Epipactis); the first strain of this
bacterial species found in nectar of this host plant].

Cells are Gram-negative rods that are facultative anaerobic,
motile and catalase positive. After 24 h aerobic incubation at 25 °C
on TSA, colonies are beige-pigmented, convex and smooth with
entire margins. Growth is observed on Brain Heart Infusion agar,
MacConkey agar and Columbia blood agar without haemolysis
activity. Strain 2.1AT grows well at 22–30 °C, but not at 4 °C, 37 °C
and 42 °C. Acid is produced from sucrose, glucose and fructose.
Lactose is fermented, indole and H2S are not produced. Positive
for Voges–Proskauer reaction. Sucrose is tolerated at concentrati-
ons ranging from 10% to 50% (w/v). Liquefaction of galactose,
oxidase and DNase activity is absent. The tested strain oxidises
the following Biolog PM 1 substrates: glycy1-l-aspartic acid, glycy1-
l-glutamic acid, glycy1-l-proline, l-asparagine, l-aspartic acid,
l-glutamic acid, l-proline, adenosine, α-d-glucose, D,L-α-glycerol
phosphate, d-fructose, d-galactose, d-mannose, d-ribose, glycerol,
inosine, l-arabinose, N-acetyld-glucosamine, sucrose, uridine and
d-glucuronic acid.

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http://dx.doi.org/10.1016/j.syapm.2014.03.002
The type strain is 2.1A (＝LMG 27956 ＝ CECT 8502) and was isolated from floral nectar of Epipactis palustris (Orchidaceae) growing in Dune du Porquerot, France. The G+C content of the type strain is 47.2 mol%.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2014.03.002.

References


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Among-population variation in microbial community structure in the floral nectar of the bee-pollinated forest herb *Pulmonaria officinalis* L. *PLoS ONE* 8, e56917, http://dx.doi.org/10.1371/journal.pone.0056917.


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