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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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ABSTRACT

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 20.1^T [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.

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

Abbreviations: BI, Bayesian inference; ML, maximum-likelihood; MLSA, multilocus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining; PP, posterior probabilities; SH, Shimodaira–Hasegawa.

☆ *Note*: The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this study are KF876184–KF876192. Those for the partial *atpD*, *gyrB* and *rpoB* gene sequences are KF876193–KF876201, KF876202–KF876210 and KF876211–KF876219, respectively.

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Table 1
Isolates used in this study.

Isolate	Host plant species (family)	Geographic origin	Latitude/longitude	Date of isolation	Reference
1.12A	*Epipactis palustris* (Orchidaceae)	Dune Dewulf, Gijvelde, France	51°3′48″ N, 2°28′9″ E	July 2012	This study
2.1A^T	*Epipactis palustris* (Orchidaceae)	Dune du Perroquet, Bray-Dunes, France	51°4′48″ N, 2°32′28″ E	July 2012	This study
2.6A	*Epipactis palustris* (Orchidaceae)	Dune du Perroquet, Bray-Dunes, France	51°4′48″ N, 2°32′28″ E	July 2012	This study
8.8A^T	*Epipactis palustris* (Orchidaceae)	Ter Yde, Oostduinkerke, Belgium	51°7′4″ N, 2°40′6″ E	July 2012	This study
SAP 86.2	*Narcissus papyraceus* (Amaryllidaceae)	Hinojos, Huelva, Spain	37°18′14″ N, 06°26′15″ E	January 2011	Álvarez-Pérez and Herrera (2013)
SAP 817.2	*Iris xiphium* (Iridaceae)	Hinojos, Huelva, Spain	37°18′14″ N, 06°26′15″ E	May 2011	Álvarez-Pérez and Herrera (2013)
CdVSA 20.1^T	*Protea roupelliae* (Proteaceae)	Mount Gilboa, South Africa	29°16′58″ S, 30°17′32″ E	January 2011	This study
CdVSA 21.1	*Protea roupelliae* (Proteaceae)	Mount Gilboa, South Africa	29°16′58″ S, 30°17′32″ E	January 2011	This study
CdVSA 50.1	*Protea subvestita* (Proteaceae)	Sani Pass, Southern Drakensberg, South Africa	29°36′40″ S, 29°21′40″ E	January 2011	This study

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, *Acinetobacer nectaris* and *A. boissieri* [4], and one of the family Enterobacteriaceae, *Rosenbergiella nectarea* [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 [2,3,46,47]. 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. nectarea* (LMG 26121^T, further referred to as 8N4^T), *Phaseolibacter flectens* (LMG 2186) and *Tatumella citrea* (LMG 22049^T). 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′-CTGCTGGGAC-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 200–10,000 bp DNA ladder (Smartladder, 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, 1x 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