Taxonomic Description

Acinetobacter pollinis sp. nov., *Acinetobacter baretiae* sp. nov., and *Acinetobacter rathckeae* sp. nov., isolated from floral nectar and honeybees

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The GenBank/ENA/DDBJ accession numbers for the partial nucleotide sequences of the 16S rRNA gene are indicated in Table S1 and the accession numbers for partial *rpoB* gene sequences are indicated in Table S2. The whole genome shotgun projects have been deposited at DDBJ/ENA/GenBank under the accession numbers VTDL00000000-VTDT00000000.

ABSTRACT

A detailed evaluation of eight bacterial isolates from floral nectar and animal visitors to flowers shows evidence that they represent three novel species in the genus *Acinetobacter*. Phylogenomic analysis shows the closest relatives of these new isolates are *A. apis*, *A. boissieri*, and *A. nectaris*, previously described species associated with floral nectar and bees, but high genome-wide sequence divergence defines these isolates as novel species. Pairwise comparisons of the average nucleotide identity (ANI) of the new isolates compared to known species is extremely low (<83%), thus confirming that these samples are representative of three novel *Acinetobacter* species, for which the names *Acinetobacter pollinis* sp. nov., *Acinetobacter baretiae* sp. nov., and *Acinetobacter rathckeae* sp. nov. are proposed. The respective type strains are SCC477^T (= TSD-214^T = LMG 31655^T), B10A^T (= TSD-213^T = LMG 31702^T), and EC24^T (= TSD-215^T = LMG 31703^T).

The genus *Acinetobacter* (*Gammaproteobacteria*) is a physiologically and metabolically diverse group of bacteria currently including 65 validly published and correct names, plus several other tentative designations and effectively but not validly published species names (https://lpsn.dsmz.de/genus/acinetobacter, last accessed on March 5, 2021). *Acinetobacter* species are ubiquitous in natural and human-associated environments, and a substantial proportion of them are associated with animal and plant hosts [e.g. 1–6].

Detailed analysis of a collection of 14 *Acinetobacter* strains obtained from the floral nectar of Mediterranean wild plants in southern Spain led to the description of two new species, namely *A. nectaris* and *A. boissieri* [7]. Soon thereafter, Kim et al. [8] isolated from the intestinal tract of a honey bee a bacterial strain which clustered with the *A. nectaris-A. boissieri* clade in both 16S rRNA and *rpoB* gene trees, but was nevertheless identified and described as a new species with the name *Acinetobacter apis*. However, the diversity of acinetobacters associated with flowering plants and their natural visitors remains mostly unknown, even when the genus *Acinetobacter* seems to rank among the main bacterial inhabitants of the floral nectar of angiosperms [9–15], and the mouth and digestive tract of flower-visiting hummingbirds [15] and bumblebees [16]. In this study, we explored the phylogenomic affiliation and physiology of a collection of eight bacterial isolates representing three new *Acinetobacter* species associated with floral nectar and animal visitors to flowers.

Isolation and Ecology

The eight isolates investigated in this study are listed in Table 1. Six of these isolates were obtained from nectar samples of three plant species (namely *Diplacus (Mimulus) aurantiacus*, *Epilobium canum*, and *Scrophularia californica*, two isolates from each species) collected at different locations in California, USA. The other two isolates were retrieved from the mouth and gut of honey bees (*Apis mellifera*) sampled on the Stanford University campus (Stanford, CA, USA). Nectar samples were diluted in 500 µL of saline solution (0.85% w/v NaCl, Merck) and a 25-µL aliquot of each was streaked on tryptone soy agar (TSA, Oxoid) [9]. Immediately after capture, honey bees were kept individually in sterile containers and anaesthetized by placing them inside a polystyrene box with ice for 10-15 min, after which they were allowed to feed on sugar water (20% w/v sucrose, Sigma-Aldrich) and then dissected to extract their gut. Honey bee guts were immediately ground inside a microtube containing 1 mL of saline solution using a disposable pellet pestle. Aliquots of the remaining sugar water and homogenized gut samples (2 µL and 10 µL, respectively) were streaked on TSA. All cultures were incubated at 25 °C for 7 days, and a colony of each phenotypically distinct microbial type was picked and separately subcultured on TSA to obtain axenic cultures. All isolates were stored at -20 °C in lysogeny broth (LB, Difco) containing 25% glycerol (Sigma-Aldrich).

16S ribosomal RNA and *rpoB* gene sequencing

Genomic DNA was extracted from the eight isolates using the Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol for bacterial samples. 16S rRNA gene

amplification was performed using the New England Biolabs (Ipswich, MA, USA) standard Taq polymerase kit and primers 27F and 1494R [17], with the following thermocycling conditions: initial denaturation at 95 °C for 3 minutes, 35 amplification cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR cleanup was performed using the QIAquick PCR purification kit (Qiagen) and the amplicons were sent to Cornell University Institute of Biotechnology Resource Center Genomics Facility for Sanger sequencing. Forward and reverse sequences were trimmed for quality, joined using GeneStudio, and trimmed to the same start and stop site and were >1149 bp in length (GenBank accessions MN701871 – MN701875, MN701877, MN701878 and MN709041). Sequence comparisons using BLAST showed that the studied isolates had the highest sequence identity with members of genus *Acinetobacter* (Table S1).

PCR amplification and sequence analysis of two variable regions (zones 1 and 2, 861 bp in total) of the *rpoB* gene, which encodes the β subunit of RNA polymerase, was performed following the procedures described in Álvarez-Pérez et al. [7]. Sequence comparisons using BLAST identified *A. nectaris* and *A. boissieri* as the closest relatives of all studied isolates (with four isolates most closely matching each species), but sequence similarity was ≥93.6% in all cases (Table S2).

Genome Features and Phylogenomic Analysis

Nextera skim libraries were prepared using genomic DNA and run on a 2×250 paired end Rapid Run HiSEQ 2500 platform at the Cornell University Institute of Biotechnology Resource Center Genomics Facility. Genomic sequencing and annotation resulted in high quality bacterial genomes that were submitted to GenBank (VTDL00000000- VTDT00000000) (Table 2). Genomes were assembled using Discovar de novo (version 52488) and authenticated through comparison of the 16S rRNA gene sequence isolated by BLAST from genomic assembles to the Sanger-sequenced 16S rRNA gene from the extracted DNA of each culture. These 16S rRNA gene sequences were 99.5-99.8% similar, confirming that the genomic assemblies represented the isolated cultures. The N50 for the assembled contigs were between 66 and 302, as calculated by the program stats.sh provided by the Joint Genomics Institute (JGI, Walnut Creek, CA). The average coverage depth of the assembled *Acinetobacter* genomes was 200× or greater as estimated by BBMap [18]. Genome completeness was estimated by checkM [19]. All assemblies were at least 98% complete, and the majority of genomes were more than 99% complete (Table 2). Using OrthoANIu [20], genome size of the isolates was found to vary between 2.59 and 2.75 Mbp and GC content between 36.6 and 39.3% (Table 2).

Genetic similarity between the isolates investigated in this study and previously documented *Acinetobacter* species was evaluated using phylogenomic analysis. A phylogenomic tree was constructed by concatenating the DNA of single-copy protein-coding genes shared by the bacterial isolates and those *Acinetobacter* species identified as being reference or representative sequences in PATRIC (Table S3). PhyloPhLan3 identified 261 shared proteins from RAST annotations [21]; shared protein sequences concatenated and aligned by PhyloPHLan were used to construct a phylogenomic tree using IQ-TREE [22]. Modelfinder selected the general amino-acid exchange rate matrix with empirical base frequencies and two rate categories (LG+F+R2) [23] and a consensus tree was constructed using 1000 bootstrap replicates. The phylogenomic analysis of the *Acinetobacter* isolates resulted in three novel clades (Fig. 1). Four isolates (*Acinetobacter* sp. SCC474, SCC477^T, FNA3, and FNA11) clustered near *A. nectaris* (clade I). Additionally, isolates EC115 and EC24^T formed a clade (II) closely related to *A. boissieri* ANC4422^T, as did *Acinetobacter* sp. B10A^T and B5B (clade III).

To evaluate if the isolates in this study represent novel species, overall genome similarity was evaluated using pairwise digital DNA-DNA hybridization (DDH) as implemented in Jspecies (version 3.7.3), and pairwise average nucleotide identity (ANI) generated using orthoANIu [20]. The ANI generated by orthoANIu, which was generated using the USEARCH program [24], was compared to ANI based on BLAST+ and MUMmer as well as correlation indexes of tetra-nucleotide signatures as implemented in JSpecies [25]. Pairwise comparisons within the proposed new clades resulted in greater than 90% DDH and greater than 98% ANI [26,27]. All isolates evaluated in this study had a 28% or fewer DDH and an 85% or lower ANI when compared to closely related previously described *Acinetobacter* species (Table 3). Pairwise comparisons found three groups of isolates that represented distinct species, and for which we propose the following names: (I) *Acinetobacter baretiae* sp. nov., including isolates B10A^T and B5B; (II) *Acinetobacter rathckeae* sp. nov., including isolates EC24^T and EC115; and (III) *Acinetobacter pollinis* sp. nov., including isolates FNA11, FNA3, SCC474, and SCC477^T (Table 4). Type strains were chosen from representative isolates with the most complete and least contaminated genomes, which were all at least 99.3% complete (based on checkM, Table 2). Notably, despite the high ANI and DDH values obtained for isolates FNA3 and FNA11, SCC474 and SCC477^T, and EC24^T and EC115, which suggest a potential clonal origin, the members of each of these pairs of isolates came from nectar samples obtained from different plants and displayed some phenotypic differences (see below), so we finally retained all isolates listed in Table 1 in our analyses.

Physiology

Metabolic and physiological features were assessed using the set of tests previously described for the genus *Acinetobacter* [28] (Table 4). Assimilation tests were performed in liquid basal mineral medium [29] supplemented with 0.1% (w/v) of nutrient source. Temperature growth tests were carried out in tryptone soy broth (TSB, Oxoid). Salt tolerance was determined by culturing isolates on LB agar (Difco) containing 0, 1, 3, 5, 7, and 10% NaCl (w/v). The ability to grow in the presence of sucrose was determined by culturing the studied isolates in glass tubes containing 5 ml of LB broth (Difco) supplemented with 0, 10, 20, 30, 40 or 50% sucrose (w/v, Sigma–Aldrich). Except for the temperature growth tests, the incubation temperature was 25 °C. All tests were carried out in duplicate on different days and results were observed after three, six and ten days of incubation. Gram-staining and tests for oxidase, catalase, growth in anaerobiosis and microaerobiosis, haemolysis, gelatinase and acid production from sugars were performed as detailed in Álvarez-Pérez et al. [7].

All tested isolates were catalase positive, oxidase negative, non-hemolytic, and grew well under microaerobic as well as aerobic conditions, but not in anaerobiosis. Growth was observed on TSB at 25 °C and 30 °C, but some isolates were able to grow at 12 °C and/or 37 °C. Isolates of *A. pollinis* and *A. rathckeae* tolerated sucrose concentrations up to 40%, and *A. rathckeae* EC115 and some strains of *A. nectaris* could even grow at 50% sucrose. None of the isolates of *A. baretiae* showed growth in media containing 0% or ≥30% sucrose, and isolate B10A^{T} only tolerated 10% sucrose. In addition, all isolates could grow in LB agar containing 0% and 1% NaCl. All *A. pollinis* isolates except SCC474 and the type strain of *A. nectaris* also tolerated 3% NaCl, but none of the tested isolates grew in media containing ≥5% NaCl.

Like their close phylogenetic relatives *A. nectaris* and *A. boissieri*, all isolates of the new species here described except *A. pollinis* SCC474 assimilated fructose. Furthermore, *A. nectaris* and four of the five isolates of *A. pollinis* assimilated sucrose, whereas *A. boissieri*, *A. pollinis* isolate SCC474, and all the isolates of *A. baretiae* and *A. rathckeae* could not grow on this carbon source. With the exception of *A. baretiae* and *A. rathckeae*, which only seem to grow on fructose, the isolates described here tested positive in other assimilation assays. The results for these and the other phenotypic analyses are summarized in Table 4.

Finally, we noticed some discrepancies between the results obtained in this study for assimilation of some sugars and those previously obtained for the type strains of *A. nectaris* and *A. boissieri* using Biolog's phenotype microarray (PM) technology, which tests for oxidation of carbon sources [7]. In

particular, the type strain of *A. nectaris* cannot oxidize but seems to assimilate D-glucose, a discrepancy which may be due to the stringent criteria used in the interpretation of PM profiles (e.g., reactions were considered positive only if the net area under the curve exceeded 5000 units, and no cutoff values for "weak positive" reactions were set [7]). On the other hand, the type strain of *A. boissieri* can oxidize but does not seem to assimilate sucrose. It should be taken into account that Biolog's PM technology detects metabolic activity through production of NADH that engenders a redox potential and flow of electrons to reduce a tetrazolium dye [30], rather than actual substrate consumption. Therefore, this PM technology does not necessarily yield the same results as assimilation assays.

Description of *Acinetobacter pollinis* sp. nov.

Acinetobacter pollinis [pol.li'nis. L. gen. n. *pollinis* of pollen, because isolates are often obtained from pollen as well as nectar [31]].

The description is based on the characteristics of four isolates from the floral nectar of *Diplacus* (*Mimulus*) *aurantiacus* and *Scrophularia californica* plants collected in California, USA (isolates FNA3, FNA11, SCC474, SCC477^T). Cells are Gram-negative, aerobic, oxidase-negative, catalase-positive, non-motile coccobacilli, generally occurring in pairs. All isolates tested can grow at decreased oxygen concentrations, but not under anaerobic conditions. Growth occurs at 12, 25, 30, and 37 °C, but not at 4 and 41 °C. Colonies on TSA medium are round and smooth, raised or slightly umbonate, cream, slightly opaque, with entire margins and 1–6 mm in diameter after 4 days of incubation at 25 °C. Growth is observed on Columbia agar supplemented with sheep blood, but all isolates are non-hemolytic on this medium. All isolates are negative for gelatin hydrolysis and utilization of Simmons' citrate, and positive for D-fructose, D-glucose and sucrose acidification. The only carbon source assimilated by all isolates tested is 4-aminobutyrate. Variable results were observed for L-aspartate, D-fructose, L-glutamate and sucrose (negative for SCC474, positive for the rest), and D-gluconate and D-glucose (negative for SCC474 and SCC477^T, positive for the rest). No growth occurs on acetate, trans-aconitate, adipate, β-alanine, L-arabinose, L-arginine, azelate, benzoate, 2,3-butanediol, citraconate, ethanol, gentisate, glutarate, histamine, L-histidine, 4-hydroxybenzoate, DL-lactate, L-leucine, levulinate, D-malate, malonate, L-ornithine, phenylacetate, L-phenylalanine, putrescine, D-ribose, L-tartrate, tricarballylate, trigonelline, and tryptamine. Sucrose is tolerated at concentrations ranging from 0% to 40% (w/v). Growth occurs in media containing 0% and 1% NaCl (w/v) and all tested isolates except SCC474 also grow in LB agar supplemented with 3% NaCl.

The type strain SCC477^T (= TSD-214^T = LMG 31655^T) was isolated from the floral nectar of a *Scrophularia californica* (California figwort) plant collected at Stebbins Cold Canyon Reserve (CA, USA). The genome size of the isolates tested ranged from 2.67 to 2.76 Mb (2.75 Mb for the type strain) with GC content of 36.6-36.7 mol%.

The genome assemblies, 16S rRNA and *rpoB* gene sequences corresponding to the four tested isolates have been deposited in the GenBank/EMBL/ DDBJ data bases under the following accession numbers: GCA_015627175.1, MN701878 and MN389214 for SCC477^T; GCA_015627215.1, MN701875 and MN315325 for FNA3; GCA_015627205.1, MN701874 and MN315322 for FNA11; and GCA_015627235.1, MN701877 and MN389213 for SCC474.

Description of *Acinetobacter rathckeae* sp. nov.

Acinetobacter rathckeae [rath.cke'ae. N.L. gen. fem. n. *rathckeae* of Rathcke, named after the American evolutionary ecologist Beverly Rathcke (1945–2011), in recognition of her contribution to pollination ecology].

The description is based on the characteristics of two isolates (EC24^T and EC115) which were isolated from the floral nectar of *Epilobium canum* plants in California, USA. Cells are Gram-negative, aerobic, oxidase-negative, catalase-positive, non-motile coccobacilli. The isolates tested can grow at decreased oxygen concentrations, but not under anaerobic conditions. Growth occurs at 25 and 30 °C, but not at 4, 37 and 41 °C. Only one of the two isolates (EC115) can grow at 12 °C. Colonies on TSA medium are round and smooth, flat, white-cream, with entire margins and <1–2 mm in diameter after 4 days of incubation at 25 °C. Growth is observed on Columbia agar supplemented with sheep blood, but the isolates are non-hemolytic on this medium. Both isolates are negative for gelatin hydrolysis and utilization of Simmons' citrate, and positive for D-fructose, D-glucose and sucrose acidification. The only carbon source assimilated by the two isolates tested is D-fructose. No growth occurs on acetate, trans-aconitate, adipate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, L-aspartate, azelate, benzoate, 2,3-butanediol, citraconate, ethanol, gentisate, D-gluconate, D-glucose, L-glutamate, glutarate, histamine, L-histidine, 4-hydroxybenzoate, DL-lactate, L-leucine, levulinate, D-malate, malonate, L-ornithine, phenylacetate, L-phenylalanine, putrescine, D-ribose, sucrose, L-tartrate, tricarballylate, trigonelline, and tryptamine. Sucrose is tolerated at concentrations ranging from 0% to 40% (w/v), and isolate EC115 also grows at 50% sucrose. Growth occurs in media containing 0% and 1% NaCl (w/v), but not ≥3% NaCl.

The type strain EC24^T (= TSD-215^T = LMG 31703^T) was isolated from the floral nectar of an *E. canum* (California fuchsia) plant collected at UC Davis campus (Davis, CA, USA). The genome size is 2.75 Mb for EC24^T and 2.62 Mb for EC115, with GC content of 39.3 and 39.2 mol%, respectively.

The genome assemblies, 16S rRNA and *rpoB* gene sequences corresponding to these isolates have been deposited in the GenBank/EMBL/ DDBJ data bases under the following accession numbers: GCA_015627125.1, MN701873 and MN389216 for EC24^T; and GCA_015627165.1, MN701872 and MN389215 for EC115.

Description of *Acinetobacter baretiae* sp. nov.

Acinetobacter baretiae [ba.re'ti.ae N.L. gen. fem. n. *baretiae* of Baret, named after the French botanist Jeanne Baret (1740–1807), in recognition of her contribution to botanical expeditions and her pioneering role as a female explorer and scientist].

The description is based on the characteristics of two isolates (B5B and B10A^T) obtained from the mouth and midgut of honey bees, *Apis mellifera*, collected in the Merck Green, located between the Gilbert Building and the Herrin Laboratories on the Stanford University campus in Stanford, CA, USA. Cells are Gram-negative, aerobic, oxidase-negative, catalase-positive, non-motile coccobacilli. The isolates tested can grow at decreased oxygen concentrations, but not under anaerobic conditions. Growth occurs at 12, 25 and 30 °C, but not at 4, 37 and 41 °C. Colonies on TSA medium are round, smooth, flat or slightly umbonate, white, slightly opaque, with entire margins and <1–2 mm in diameter after 4 days of incubation at 25 °C. Growth is observed on Columbia agar supplemented with sheep blood, but the isolates are non-hemolytic on this medium. Both isolates are negative for gelatin hydrolysis and utilization of Simmons' citrate, and positive for D-fructose, D-glucose and sucrose acidification. The only carbon source assimilated by the two isolates tested is D-fructose. No growth occurs on acetate, trans-aconitate, adipate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, L-aspartate, azelate, benzoate, 2,3-butanediol, citraconate, ethanol, gentisate, D-gluconate, D-glucose, L-glutamate, glutarate, histamine, L-histidine, 4-hydroxybenzoate, DL-lactate, L-leucine, levulinate, D-malate, malonate, L-ornithine, phenylacetate, L-phenylalanine, putrescine, D-ribose, sucrose, L-tartrate, tricarballylate, trigonelline, and tryptamine. Sucrose is tolerated at 10% (w/v, both isolates) and 20% (only isolate B5B), and tested isolates failed to grow in LB broth containing no sucrose. Growth occurs in media containing 0% and 1% NaCl (w/v), but not ≥3% NaCl.

The type strain B10A^T (= TSD-213^T = LMG 31702^T) was isolated from the gut of an *A. mellifera* (honey bee) individual sampled at Stanford campus (Stanford, CA, USA). The genome size is 2.70 Mb for B10A^T and 2.59 Mb for B5B, with GC content of 37.5 mol% for both isolates.

The genome assemblies, 16S rRNA and *rpoB* gene sequences corresponding to these isolates have been deposited in the GenBank/EMBL/ DDBJ data bases under the following accession numbers: GCA_015627105.1, MN709041 and MN315286 for B10AT; and GCA_015627115.1, MN701871 and MN315310 for B5B.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Ethical approval

Not required for this study.

Consent for publication

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ABBREVIATIONS

ANI: average nucleotide identity

DDH: DNA-DNA hybridization

LB: lysogeny broth

TSA: tryptone soy agar

TSB: tryptone soy broth

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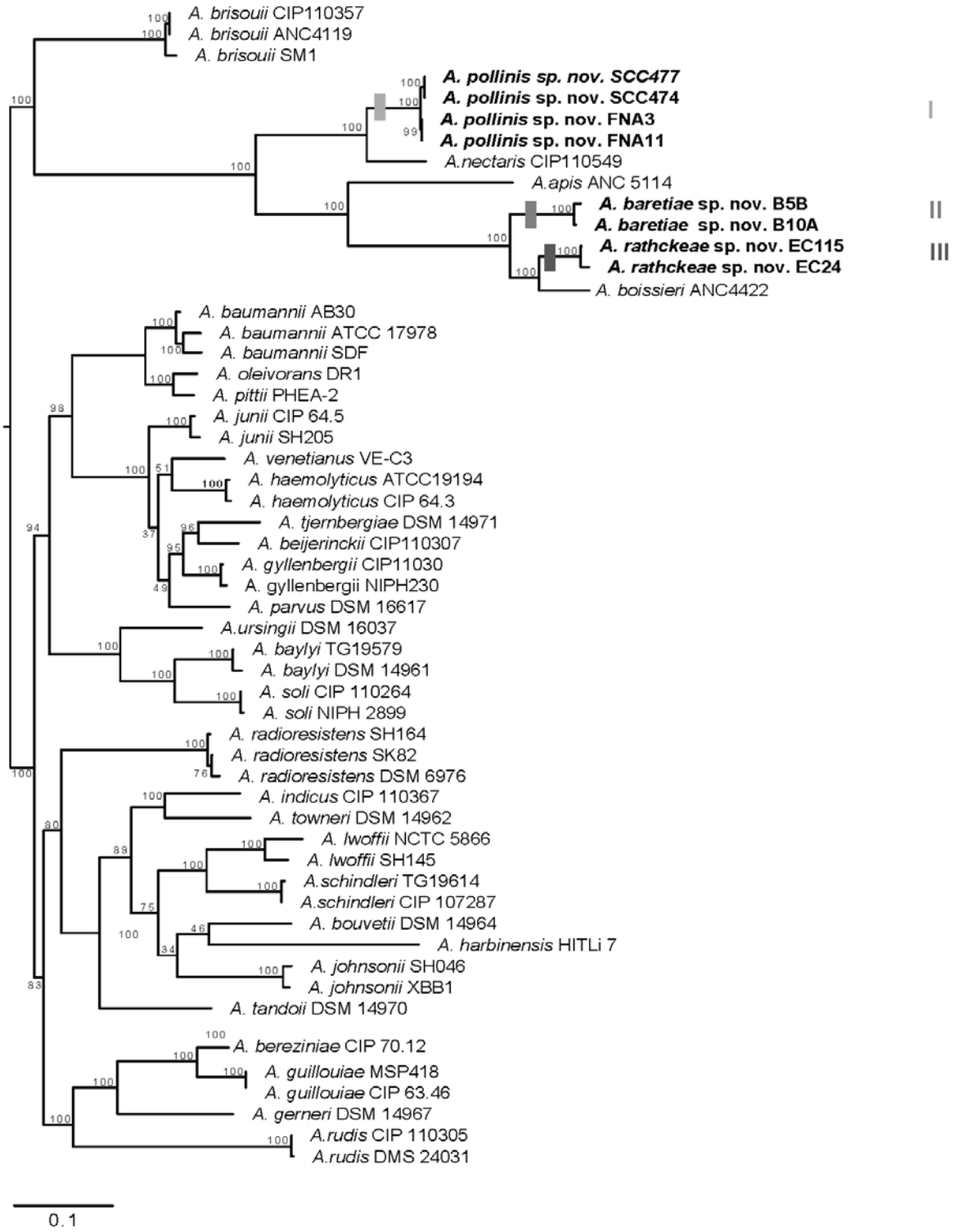
FIGURES AND TABLES

Figure 1: Maximum-likelihood phylogenomic tree constructed from shared concatenated RAST proteins identified by PhyloPHLan3 and constructed using IQTREE. The proposed new species are delineated by grey markings within the tree and numbered clades. The tree was constructed using the general time reversible model and bootstrap values based on 1000 replicates are listed at nodes.


A. brisouii CIP110357
A. brisouii ANC4119
A. brisouii SM1
A. pollinis sp. nov. SCC477
A. pollinis sp. nov. SCC474
A. pollinis sp. nov. FNA3
A. pollinis sp. nov. FNA11
A.nectaris CIP110549
A.apis ANC 5114
A. baretiae sp. nov. B5B
A. baretiae sp. nov. B10A
A. rathckeae sp. nov. EC115
A. rathckeae sp. nov. EC24
A. boissieri ANC4422
I
II
III
A. baumannii AB30
A. baumannii ATCC 17978
A. baumannii SDF
A. oleivorans DR1
A. pittii PHEA-2
A. junii CIP 64.5
A. junii SH205
A. venetianus VE-C3
A. haemolyticus ATCC19194
A. haemolyticus CIP 64.3
A. tjernbergiae DSM 14971
A. beijerinckii CIP110307
A. gyllenbergii CIP11030
A. gyllenbergii NIPH230
A. parvus DSM 16617
A.ursingii DSM 16037
A. baylyi TG19579
A. baylyi DSM 14961
A. soli CIP 110264
A. soli NIPH 2899
A. radioresistens SH164
A. radioresistens SK82
A. radioresistens DSM 6976
A. indicus CIP 110367
A. towneri DSM 14962
A. lwoffii NCTC 5866
A. lwoffii SH145
A.schindleri TG19614
A.schindleri CIP 107287
A. bouvetii DSM 14964
A. harbinensis HITLi 7
A. johnsonii SH046
A. johnsonii XBB1
A. tandoii DSM 14970
A. bereziniae CIP 70.12
A. guillouiae MSP418
A. guillouiae CIP 63.46
A. gerneri DSM 14967
A.rudis CIP 110305
A.rudis DMS 24031
0.1

417

418

419 **Table 1**: Overview of the *Acinetobacter* isolates analyzed in this study.

Species	Isolate	Source	Sampling location	Year of isolation	Isolate donor*
Acinetobacter pollinis sp. nov.	FNA3	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford (California, USA)	2017	KT, TF
	FNA11	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford (California, USA)	2017	KT, TF
	SCC474	Floral nectar of *Scrophularia californica* (Scrophulariaceae)	Stebbins Cold Canyon Reserve (California, USA)	2016	RV, GH
	SCC477^T	Floral nectar of *Scrophularia californica* (Scrophulariaceae)	Stebbins Cold Canyon Reserve (California, USA)	2016	RV, GH
Acinetobacter rathckeae sp. nov.	EC115	Floral nectar of *Epilobium canum* (Onagraceae)	UC Davis campus (California, USA)	2015	RV, MM
	EC24^T	Floral nectar of *Epilobium canum* (Onagraceae)	UC Davis campus (California, USA)	2015	RV, MM
Acinetobacter baretiae sp. nov.	B10A^T	Gut of *Apis mellifera*	Stanford campus (California, USA)	2018	TF, SAP
	B5B	Mouthparts of *Apis mellifera*	Stanford campus (California, USA)	2018	TF, SAP

420

* Isolate donors: GH, Griffin Hall (UC Davis, USA); KT, Kaoru Tsuji (Center for Ecological Research-Kyoto University, Japan); MM, Megan Morris (Stanford University, USA); RV, Rachel Vannette (UC Davis, USA); SAP, Sergio Álvarez-Pérez (KU Leuven, Belgium); TF, Tadashi Fukami (Stanford University, USA).

Table 2: Genome characteristics of the *Acinetobacter* isolates investigated in this study. Genome size, GC content, and contig number was assessed by ORthoANIu, N50 was estimated by stats.sh in JGI, and coverage was as generated by BBMap and completeness was estimated by checkM. 16S rRNA gene similarity between genomic and Sanger sequencing efforts was generated using BLAST.

Isolate	Size (Mb)	GC content (%)	Contigs (#)	N50 (Kb)	Ave coverage	Stdev coverage	Complete (%)	16S similarity
A. baretiae sp. nov. B10A^T	2.70	37.5	64	104	799	412	99.9	99.6
A. baretiae sp. nov. B5B	2.59	37.5	68	96	830	497	99.5	99.6
A. rathckeae sp. nov. EC115	2.62	39.2	25	208	461	1397	99.3	99.8
A. rathckeae sp. nov. EC24^T	2.75	39.3	17	302	407	1586	99.9	99.8
A. pollinis sp. nov. FNA11	2.67	36.6	75	117	1223	573	99.9	99.6
A. pollinis sp. nov. FNA3	2.67	36.6	84	97	865	9520	99.3	99.5
A. pollinis sp. nov. SCC474	2.75	36.7	127	71	216	417	98.1	99.9
A. pollinis sp. nov. SCC477^T	2.75	36.7	90	66	274	599	99.3	99.7

428

429 **Table 3**: Digital DNA-DNA hybridization (DDH) (Jspecies) and pairwise average nucleotide identity
430 (ANI) (orthoANIu) for the isolates used in this study and closely related *Acinetobacter* species. DDH
431 values greater than 70% and ANI values greater than 96% denote the pair are the same species and
432 are highlighted in light grey (DDH) and dark grey (ANI); species groupings are labeled.

		I					II		III				
		A. pollinis sp. nov. FNA3	*A. pollinis* sp. nov. FNA11	*A. pollinis* sp. nov. SCC474	*A. pollinis* sp. nov. SCC477^T	*A. rathckeae* sp. nov. EC24	*A. rathckeae* sp. nov. EC115	*A. baretiae* sp. nov. B5B	*A. baretiae* sp. nov. B10A	*A.apis* ANC 5514	*A. boissieri* ANC 4422	*A. nectaris* CIP 110549	*A. brisouii* ANC 4119 ^{T}
I	*A. pollinis* sp. nov. FNA3		100.0	97.0	97.0	20.1	19.7	19.9	20.1	20.0	19.1	19.3	24.6
	A. pollinis sp. nov. FNA11	100.0		97.0	97.1	20.1	19.7	20.0	20.1	19.1	19.3	24.5	20.0
	A. pollinis sp. nov. SCC474	99.7	99.7		99.9	19.6	19.8	20.0	20.0	19.1	19.4	24.7	20.1
	A. pollinis sp. nov. SCC477^T	99.6	99.7	100.0		19.6	19.8	19.9	19.9	19.0	19.4	24.6	20.1
II	*A. rathckeae* sp. nov. EC24^T	75.3	75.0	74.8	74.6		98.1	23.6	23.5	19.6	28.4	20.4	19.9
	A. rathckeae sp. nov. EC115	74.7	82.1	74.7	74.6	99.7		24.1	23.5	19.6	28.4	20.0	20.0
III	*A.baretiae* sp. nov. B5B	74.6	74.6	74.4	74.4	81.2	81.8		94.4	19.5	23.9	19.7	19.9
	A.baretiae sp. nov. B10A^T	74.7	74.6	74.4	74.8	81.2	81.3	99.3		19.5	23.9	19.7	20.0
	A.apis ANC 5514	74.8	74.7	74.4	74.3	74.1	74.5	73.1	73.5				
	A. boissieri ANC 4422	84.7	84.9	74.8	74.4	74.6	74.5	73.3	73.2				
	A. nectaris CIP 110549	75.2	75.5	82.0	82.1	82.4	82.2	73.4	73.7				
	A. brisouii ANC 4119^T	73.1	73.0	73.2	73.3	73.3	73.2	75.9	75.8				

DDH

ANI

433

434

435 **Table 4:** Metabolic and physiological characteristics of the *Acinetobacter* isolates included in this study in comparison with their closest phylogenetic
436 relatives.*

	***A. pollinis* sp. nov.**				***A. rathckeae* sp. nov.**		***A. baretiae* sp. nov.**		***A. nectaris*†**		***A. boissieri*†**		***A. apis***
Characteristic	**FNA3**	**FNA11**	**SCC474**	**SCC477^T**	**EC24^T**	**EC115**	**B10A^T**	**B5B**	**SAP 763.2^T**	**Other isolates‡**	**SAP 284.1^T**	**Other isolates‡**	**HYN18^T**
Growth on TSB at:													
4°C	–	–	–	–	–	–	–	–	–	–	w	+	–
12°C	+	+	+	+	–	+	+	+	+	ND	w	ND	ND
25°C	+	+	+	+	+	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	–	–	–	–	–	–	–	–	–
41°C	–	–	–	–	–	–	–	–	–	–	–	–	–
Acid from sucrose	+	+	+	+	+	+	+	+	+	+	+	+	ND
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from fructose	+	+	+	+	+	+	+	+	+	ND	+	ND	ND
Citrate (Simmons)	–	–	–	–	–	–	–	–	–	ND	–	ND	+

Growth on LB agar plus NaCl at:													
0% (w/v)	+	+	+	+	+	+	+	+	+	ND	+	ND	+
1% (w/v)	+	+	+	+	+	+	+	+	+	ND	+	ND	+
3% (w/v)	+	+	–	+	–	–	–	–	+	ND	–	ND	–
5% (w/v)	–	–	–	–	–	–	–	–	–	ND	–	ND	–
7.5% (w/v)	–	–	–	–	–	–	–	–	–	ND	–	ND	–
10% (w/v)	–	–	–	–	–	–	–	–	–	ND	–	ND	–
Growth on LB broth plus sucrose at:													
0% (w/v)	+	+	+	+	+	+	–	–	+	+	+	+	ND
10% (w/v)	+	+	+	+	+	+	+	+	+	+	+	+	ND
20% (w/v)	+	+	+	+	+	+	–	+	+	+	+	+	ND
30% (w/v)	+	+	+	+	+	+	–	–	+	+	w	w	ND
40% (w/v)	+	+	+	+	+	+	–	–	+	+	w	w	ND
50% (w/v)	–	–	–	–	–	+	–	–	w	+	w	w	ND
Assimilation of:													
Acetate	–	–	–	–	–	–	–	–	– (–)	(–)	– (–)	(–)	–
4-Aminobutyrate	+	+	–	+	–	–	–	–	+	ND	–	ND	–
L-Arabinose	–	–	–	–	–	–	–	–	– (–)	(–)	– (–)	(–)	–
L-Aspartate	+	+	–	+	–	–	–	–	+ (+)	(+)	– (–)	(–)	–

2,3-Butanediol	–	–	–	–	–	–	–	–	–	ND	–	ND	–
Ethanol	–	–	–	–	–	–	–	–	–	ND	–	ND	–
D-Gluconate	+	+	–	–	–	–	–	–	+ (+)	(v)	– (–)	(–)	+
D-Glucose	+	+	–	–	–	–	–	–	+ (–)	(v)	– (–)	(–)	+
L-Glutamate	+	+	–	+	–	–	–	–	+ (+)	(+)	– (–)	(v)	+
DL-Lactate	–	–	–	–	–	–	–	–	–	ND	–	ND	–
Phenylacetate	–	–	–	–	–	–	–	–	–	ND	–	ND	–
D-Ribose	–	–	–	–	–	–	–	–	– (–)	–	– (–)	–	–
Trigonelline	–	–	–	–	–	–	–	–	–	ND	–	ND	–
Sucrose	+	+	–	+	–	–	–	–	+ (+)	(+)	– (+)	(+)	ND
D-Fructose	+	+	–	+	+	+	+	+	+ (+)	(+)	+ (+)	(+)	ND

*All tested isolates were positive for catalase production and growth in microaerobiosis, and negative for oxidase and gelatinase production, haemolysis and growth in anaerobiosis. Furthermore, all isolates tested negative for assimilation of the following nutrient sources: *trans*-aconitate, adipate, β-alanine, L-arginine, azelate, benzoate, citraconate, gentisate, glutarate, histamine, L-histidine, 4-hydroxybenzoate, L-leucine, levulinate, D-malate, malonate, L-ornithine, L-phenylalanine, putrescine, L-tartrate, tricarballylate, and tryptamine. +, positive reaction; –, negative reaction; w, weak growth; v, variable results; ND, not determined.

†Results shown in parentheses refer to oxidation of carbon sources, as determined in a previous study using Phenotype MicroArray (PM) technology (see Ref. [7]). Note the discrepancies observed between assimilation and oxidation of D- glucose and sucrose for the type strains of *A. nectaris* and *A. boissieri*, respectively.

‡Results obtained by Álvarez-Pérez *et al.* [7] for other conspecific isolates.