Gluconobacter nephelii sp. nov., an acetic acid bacterium in the class *Alphaproteobacteria*

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Three strains, RBY-1^T, PHD-1 and PHD-2, were isolated from fruits in Thailand. The strains were Gram-negative, aerobic rods with polar flagella, produced acetic acid from ethanol and did not oxidize acetate or lactate. In phylogenetic trees based on 16S rRNA gene sequences and 16S–23S rRNA gene internal transcribed spacer (ITS) sequences, the strains formed a cluster separate from the type strains of recognized species of the genus *Gluconobacter*. The calculated 16S rRNA gene sequence and 16S–23S rRNA gene ITS sequence similarities were respectively 97.7–99.7 % and 77.3–98.1 %. DNA G + C contents ranged from 57.2 to 57.6 mol%. The strains showed high DNA–DNA relatedness of 100 % to one another, but low DNA–DNA relatedness of 11–34 % to the tested type strains of recognized *Gluconobacter* species. Q-10 was the major quinone. On the basis of the genotypic and phenotypic data obtained, the three strains clearly represent a novel species, for which the name *Gluconobacter nephelii* sp. nov. is proposed. The type strain is RBY-1^T (=BCC 36733^T=NBRC 106061^T=PCU 318^T), whose DNA G + C content is 57.2 mol%.

In the genus *Gluconobacter*, 12 species have been described at the time of writing: *Gluconobacter oxydans*, *Gluconobacter cerinus*, *Gluconobacter frateurii*, *Gluconobacter asaii*, *Gluconobacter albidus*, *Gluconobacter thailandicus*, *Gluconobacter kondonii*, *Gluconobacter roseus*, *Gluconobacter japonicus*, *Gluconobacter sphaericus*, *Gluconobacter kanchanaburiensis* and *Gluconobacter wancherniae* (De Ley, 1961; Skerman *et al.*, 1980; Gosselé *et al.*, 1983; Yamada & Akita, 1984a, b; Mason & Claus, 1989; Yukphan *et al.*, 2004a, 2005, 2010; Tanasupawat *et al.*, 2004, 2005; Malimas *et al.*, 2007,

2008a, b, c, 2009a, b). However, *G. asaii* was shown to be a later heterotypic synonym of *G. cerinus* (Katsura *et al.*, 2002; Tanaka *et al.*, 1999; Yamada *et al.*, 1999). This paper describes *Gluconobacter nephelii* sp. nov. for three strains isolated in Thailand.

The three strains, RBY-1^T, PHD-1 and PHD-2, were isolated from rambutan and litchi by an enrichment-culture approach using the glucose/ethanol/acetic acid medium described previously (Yamada *et al.*, 1976, 1999, 2000; Yukphan *et al.*, 2004c). *G. oxydans* NBRC 14819^T, *G. cerinus* NBRC 3267^T, *G. frateurii* NBRC 3264^T, *G. albidus* NBRC 3250^T, *G. thailandicus* BCC 14116^T, *G. kondonii* NBRC 3266^T, *G. roseus* NBRC 3990^T, *G. sphaericus* NBRC 12467^T, *G. japonicus* NBRC 3271^T, *G. kanchanaburiensis* BCC 15889^T, *G. wancherniae* BCC 15775^T and *Acetobacter aceti* NBRC 14818^T were used as reference strains.

Phylogenetic analyses based on 16S rRNA gene sequences and 16S–23S rRNA gene internal transcribed spacer (ITS) sequences were determined for strains RBY-1^T, PHD-1 and

Abbreviations: ITS, internal transcribed spacer; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S–23S rRNA gene ITS sequences of strains RBY-1^T, PHD-1 and PHD-2 are respectively AB540145, AB540146 and AB540147, and those for their 16S rRNA gene sequences are respectively AB540148, AB540149 and AB540150.

A supplementary table and five supplementary figures are available with the online version of this paper.

PHD-2 as described previously (Malimas *et al.*, 2007; Yukphan *et al.*, 2004a, b, c). Multiple sequence alignments were performed with the program CLUSTAL_X (version 1.81) (Thompson *et al.*, 1997). Sequence gaps and ambiguous bases were excluded. Distance matrices were calculated by the two-parameter method of Kimura (1980). The neighbour-joining (Saitou & Nei, 1987), maximum-parsimony and maximum-likelihood (Felsenstein, 1981, 1983) methods were used for constructing phylogenetic trees. The confidence values of individual branches in the phylogenetic trees were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 replications with the program MEGA (version 4.0; Tamura *et al.*, 2007). Pairwise sequence similarities were calculated for strain RBY-1^T based on 16S rRNA gene and 16S–23S rRNA gene ITS sequences. In particular, the program PHYLIP (version 3.6; Felsenstein, 1995) was used to construct a phylogenetic tree using the maximum-likelihood method.

In a phylogenetic tree based on 16S rRNA gene sequences (1402 bases), constructed by the neighbour-joining method, strains RBY-1^T, PHD-1 and PHD-2 constituted an independent cluster, connected to a cluster comprising the type strains of *G. cerinus*, *G. frateurii*, *G. thailandicus* and *G. japonicus* with a bootstrap value of 94 % and forming a cluster comprising the species with lower DNA G+C contents (Fig. 1a). The neighbour-joining tree (Fig. 1a) was slightly different from the maximum-parsimony tree (see Supplementary Fig. S1, available in IJSEM Online)

and had the same topology as the maximum-likelihood tree (Supplementary Fig. S2); however, both trees showed clearly that strains RBY-1^T, PHD-1 and PHD-2 belonged to the genus *Gluconobacter* and, more precisely, to the subcluster containing *G. japonicus*, *G. frateurii*, *G. thailandicus* and *G. cerinus* NBRC 3267^T. In a phylogenetic tree based on 16S–23S rRNA gene ITS sequences (715 bases), the three strains constituted a cluster along with the type strain of *G. japonicus* with a bootstrap value of 99 %, and also formed a cluster comprising the species with lower DNA G+C contents (Fig. 1b). In addition, the phylogenetic trees constructed by the neighbour-joining method showed the same topology as those constructed by the maximum-parsimony and maximum-likelihood methods (Supplementary Figs S3 and S4).

The calculated pairwise 16S rRNA gene sequence similarities of strain RBY-1^T for 1402 bases were 99.7, 99.5, 99.4, 99.1, 98.0, 97.9, 97.7, 97.9, 97.9, 98.0 and 98.9 % respectively to *G. japonicus* NBRC 3271^T, *G. frateurii* NBRC 3264^T, *G. thailandicus* BCC 14116^T, *G. cerinus* NBRC 3267^T, *G. albidus* NBRC 3250^T, *G. kondonii* NBRC 3266^T, *G. kanchanaburiensis* BCC 15889^T, *G. roseus* NBRC 3990^T, *G. oxydans* NBRC 14819^T, *G. sphaericus* NBRC 12467^T and *G. wancherniae* BCC 15775^T. The three strains had 16S rRNA gene sequence similarities of 100 % to one another. In contrast, the calculated pairwise 16S–23S rRNA gene ITS sequence similarities of strain RBY-1^T for 715 bases were 98.1, 94.4, 94.0, 92.3, 81.0, 79.2, 77.3, 81.5,

Fig. 1. Phylogenetic relationships of *Gluconobacter nephelii* RBY-1^T, PHD-1 and PHD-2. The phylogenetic trees, based on 16S rRNA gene sequences (a) and on 16S–23S rRNA gene ITS sequences (b), were constructed by the neighbour-joining method. The type strain of *Acetobacter aceti* was used as an outgroup. Numbers at nodes indicate bootstrap percentages derived from 1000 replications. Bars, 0.005 K_{nuc} (a); 0.05 K_{nuc} (b).

80.9, 80.9 and 80.4% respectively to *G. japonicus* NBRC 3271^T, *G. frateurii* NBRC 3264^T, *G. thailandicus* BCC 14116^T, *G. cerinus* NBRC 3267^T, *G. albidus* NBRC 3250^T, *G. kondonii* NBRC 3266^T, *G. kanchanaburiensis* BCC 15889^T, *G. roseus* NBRC 3990^T, *G. oxydans* NBRC 14819^T, *G. sphaericus* NBRC 12467^T and *G. wancherniae* BCC 15775^T. The data obtained above support the hypothesis that strain RBY-1^T is separated phylogenetically from the type strains of recognized *Gluconobacter* species in 16S rRNA gene and 16S–23S rRNA ITS gene sequences.

Extraction of bacterial chromosomal DNAs was performed by modifications of the methods of Marmur (1961), Saito & Miura (1963) and Ezaki *et al.* (1983). DNA base compositions were determined by the method of Tamaoka & Komagata (1984). DNA–DNA hybridizations were carried out by the photobiotin-labelling method using microplate wells, as described by Ezaki *et al.* (1989). Levels of DNA–DNA hybridization were determined colorimetrically (Verlander, 1992). Colour intensity was measured as A_{450} on a VersaMax microplate reader (Molecular Devices).

When single-stranded and labelled DNA from strain RBY-1^T was hybridized with DNAs from test strains in 2 × SSC containing 50% formamide at 49.0 °C for 15 h, strain RBY-1^T demonstrated levels of DNA–DNA relatedness of 100, 100, 100, 34, 34, 29, 27, 23, 18, 15, 12, 11, 11 and 6% respectively to strains RBY-1^T, PHD-1, PHD-2, *G. japonicus* NBRC 3271^T, *G. frateurii* NBRC 3264^T, *G. thailandicus* BCC 14116^T, *G. cerinus* NBRC 3267^T, *G. albidus* NBRC 3250^T, *G. kondonii* NBRC 3266^T, *G. kanchanaburiensis* BCC 15889^T, *G. roseus* NBRC 3990^T, *G. oxydans* NBRC 14819^T, *G. sphaericus* NBRC 12467^T and *A. aceti* NBRC 14818^T (Supplementary Table S1). The data obtained above indicate that the three strains are separated genetically at the species level from the type strains of recognized *Gluconobacter* species and constitute a single species.

A computerized restriction analysis was performed for restriction endonucleases that differentiate strains RBY-1^T, PHD-1 and PHD-2 from the tested type strains of recognized *Gluconobacter* species based on approximately 715 bp of the 16S–23S rRNA gene ITS sequences with the program NEBcutter (version 2.0; New England BioLabs) (Yukphan *et al.*, 2004a, b; Malimas *et al.*, 2006). The combination of three restriction endonucleases, *Bst*NI, *Mbo*II and *Mbo*I, distinguished strain RBY-1^T particularly from *G. japonicus* NBRC 3271^T, producing restriction fragments respectively comprising 499, 111, 98 and 7 bp, 360 and 355 bp and 306, 302, 85, 14 and 8 bp.

The 16S–23S rRNA gene ITS PCR products of strain RBY-1^T, *G. oxydans* NBRC 14819^T, *G. cerinus* NBRC 3267^T, *G. frateurii* NBRC 3264^T, *G. albidus* NBRC 3250^T, *G. thailandicus* BCC 14116^T, *G. kondonii* NBRC 3266^T, *G. roseus* NBRC 3990^T, *G. sphaericus* NBRC 12467^T, *G. japonicus* NBRC 3271^T and *G. kanchanaburiensis* BCC 15889^T were prepared and examined for restriction analysis using the restriction endonucleases *Bst*NI, *Mbo*II and *Mbo*I (Fermentas). As shown in Supplementary Fig. S5, the three

strains were distinguished from the type strains of *G. cerinus*, *G. frateurii*, *G. albidus*, *G. thailandicus*, *G. kondonii*, *G. roseus*, *G. sphaericus* and *G. kanchanaburiensis* by *Bst*NI digestion as well as from the type strain of *G. oxydans* by *Mbo*II and *Mbo*I digestions. Additionally, the three strains were distinguished from the type strain of *G. japonicus* by *Mbo*I digestion. The data obtained indicate that the three strains are discriminated from the type strains of the recognized *Gluconobacter* species by 16S–23S rRNA gene ITS restriction analysis using *Bst*NI, *Mbo*II and *Mbo*I.

Strains RBY-1^T, PHD-1 and PHD-2 were examined for morphological, physiological, biochemical and chemotaxonomic characteristics as described previously (Asai *et al.*, 1964; Yamada *et al.*, 1969, 1976, 1999; Gossele *et al.*, 1983; Mason & Claus, 1989; Katsura *et al.*, 2002; Yukphan *et al.*, 2004c; Malimas *et al.*, 2007). Flagella were observed using cells cultivated on mannitol agar slants at 30 °C for 24 h (Yamada *et al.*, 1976) by staining (Forbes, 1981) and transmission electron microscopy (TEM). For observation of cell morphology by TEM, cells were coated with copper grids and stained by submerging the grids in 2% (w/v) uranyl acetate for 1 min. The grids were rinsed with distilled water (Dijkstra & De Jager, 1998) and examined with a transmission electron microscope (JSM-1230, JEOL Ltd). The phenotypic and chemotaxonomic characteristics of strains RBY-1^T, PHD-1 and PHD-2 are given in the species description.

Strains RBY-1^T, PHD-1 and PHD-2 are Gram-negative, aerobic rods with a polar flagellum (Fig. 2). They produce acetic acid from ethanol, as clear zones appeared on GEY/CaCO₃ agar plates, but they do not oxidize acetate or lactate. They have Q-10 as the major ubiquinone. The DNA G + C contents of strains RBY-1^T, PHD-1 and PHD-2 were 57.2, 57.6 and 57.4 mol%, respectively. Strains RBY-1^T, PHD-1 and PHD-2 did not produce acid from melibiose or L-sorbose, which differentiated them from the phylogenetically most closely related species, *G. japonicus* NBRC 3271^T, and type strains of some other *Gluconobacter* species

Fig. 2. Transmission electron micrograph of *Gluconobacter nephelii* RBY-1^T. Bacterial cells were cultivated on a mannitol agar slant at 30 °C for 24 h. Bar, 1 µm.

Table 1. Differential characteristics of *G. nephelii* RBY-1^T, PHD-1 and PHD-2

Strains: 1, *G. nephelii* RBY-1^T; 2, *G. nephelii* PHD-1; 3, *G. nephelii* PHD-2; 4, *G. japonicus* NBRC 3271^T; 5, *G. frateurii* NBRC 3264^T; 6, *G. thailandicus* BCC 14116^T; 7, *G. cerinus* NBRC 3267^T; 8, *G. oxydans* NBRC 14819^T; 9, *G. albidus* NBRC 3250^T; 10, *G. kondonii* NBRC 3266^T; 11, *G. roseus* NBRC 3990^T; 12, *G. sphaericus* NBRC 12467^T; 13, *G. kanchanaburiensis* BCC 15889^T; 14, *G. wancherniae* BCC 15775^T. +, Positive; w, weakly positive; –, negative; ND, not determined; Ga, *G. albidus*; Gc, *G. cerinus*; Gf, *G. frateurii*; Gka, *G. kanchanaburiensis*; Gko, *G. kondonii*; Gn, *G. nephelii*; Go, *G. oxydans*; Gt, *G. thailandicus*.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Water-soluble brown pigment	–	–	–	–	–	–	–	–	–	–	–	+	+	+
2,5-diketo-D-gluconate formation	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Growth at 37 °C	–	–	–	–	–	w	–	+	–	–	–	–	–	–
Acid production from:														
<i>meso</i> -Erythritol	+	+	+	+	w	+	+	+	w	+	–	–	w	–
Glycerol	w	w	w	+	w	–	+	w	+	+	w	w	w	w
Maltose	w	w	w	–	w	–	+	w	w	w	–	–	w	–
D-Mannitol	w	w	w	+	–	w	+	w	w	+	w	w	+	+
Melibiose	–	–	–	+	w	–	+	+	–	+	–	+	w	w
L-Sorbose	–	–	–	+	–	–	+	w	w	w	–	–	w	w
Growth on:														
D-Arabitol	+	+	+	w	+	+	+	–	–	w	w	w	+	+
L-Arabitol	+	+	+	w	+	w	–	–	–	–	–	–	–	–
<i>meso</i> -Erythritol	w	w	w	+	–	w	+	w	–	+	+	–	w	w
<i>meso</i> -Ribitol	w	w	w	+	+	w	w	–	–	–	–	–	–	–
Restriction pattern with:														
<i>Bst</i> NI	Go	Go	Go	Go	Gf	Gf	Gc	Go	Ga	Gc	Gc	Gc	Gka	ND
<i>Mbo</i> II	Gf	Gf	Gf	Gf	Gf	Gf	Gc	Go	Ga	Gko	Go	Go	Go	ND
<i>Mbo</i> I	Gn	Gn	Gn	Gt	Gf	Gt	Gc	Go	Ga	Gc	Gt	Gc	Gka	ND

(Table 1). In addition, acid production from *meso*-erythritol, glycerol (weakly positive), maltose (weakly positive) and D-mannitol (weakly positive), growth at 37 °C and growth on D- and L-arabitol, *meso*-erythritol (weakly positive) and *meso*-ribitol (weakly positive) supported the differentiation of the strains from the type strains of recognized *Gluconobacter* species (Table 1).

As described above, strains RBY-1^T, PHD-1 and PHD-2 are distinguished genetically, phylogenetically, morphologically, physiologically, biochemically and chemotaxonomically from the type strains of recognized *Gluconobacter* species (Table 1). A novel species, namely *Gluconobacter nephelii* sp. nov., is therefore proposed for the three strains.

Description of *Gluconobacter nephelii* sp. nov.

Gluconobacter nephelii (ne.phe'li.i. N.L. gen. n. *nephelii* of *Nephelium*; N.L. neut. n. *Nephelium* the generic name of rambutan, *Nephelium lappaceum*, a tropical fruit, from which the type strain was isolated).

Cells are Gram-negative rods, measuring 0.8–1.0 × 1.0–2.0 µm. Motile with a polar flagellum. Grows at pH 3.0 and at 35 °C. Optimum growth occurs at pH 5.5 and 25 °C. Does not oxidize acetate or lactate. Grows on mannitol agar, but not on glutamate agar. Dihydroxyacetone is produced

from glycerol, and 2- and 5-keto-D-gluconate, but not 2, 5-diketo-D-gluconate or a water-soluble brown pigment, are produced from D-glucose. Acid is produced from D- and L-arabinose, *meso*-erythritol, ethanol, D-fructose, D-galactose, D-glucose, glycerol (weakly positive), maltose (weakly positive), D-mannitol (weakly positive), D-mannose, L-rhamnose (weakly positive), raffinose, D-sorbitol, sucrose and D-xylose. Acid is not produced from dulcitol, lactose, melibiose or L-sorbose. Grows on D- and L-arabinose, *meso*-erythritol (weakly positive), D-fructose, glucose, glycerol, maltose, D-mannitol, raffinose, L-rhamnose, D-sorbitol, L-sorbose and sucrose. Concerning growth on pentitols, D- and L-arabitol are positive and *meso*-ribitol is weakly positive. Does not grow on dulcitol, ethanol, D-galactose, lactose, D-mannose, melibiose or D-xylose. Major ubiquinone is Q-10. DNA G + C content is 57.2–57.6 mol%.

The type strain is RBY-1^T (=BCC 36733^T=NBRC 106061^T=PCU 318^T), isolated from rambutan. Its DNA G + C content is 57.2 mol%.

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