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Article *in* International Journal of Systematic and Evolutionary Microbiology · February 2019

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Frateuria defendens sp. nov., bacterium isolated from the yellows grapevine's disease vector *Hyalesthes obsoletus*

Ofir Lidor,[1]† Diego Santos-Garcia,[1,2]† Netta Mozes-Daube,[1] Vered Naor,[3,4] Eyal Cohen,[5] Lilach Iasur-Kruh,[6] Ofir Bahar[7] and Einat Zchori-Fein[1,*]

Abstract

A *Dyella*-like bacterium was previously isolated from the planthopper *Hyalesthes obsoletus* (Hemiptera). Based on its 16S rRNA gene sequence, strain DHo^T was assigned to the family Rhodanobacteraceae with *Dyella* and *Frateuria* as its closest relatives. The closest 16S rRNA gene sequences were *Frateuria aurantia* DSM 6220^T (98.2 %), *Dyella thiooxydans* ATSB10^T (98 %), *Dyella terrae* JS14-6^T (97.8 %) and *Dyella marensis* CS5-B2^T (97.8 %). Strain DHo^T is a Gram-negative, aerobic, motile, yellow-pigmented, rod-shaped bacterium. Strain DHo^T cells grew well at 28–30 °C and at pH 6.5–7.5 on a nutrient agar plate. DNA–DNA hybridization showed that the relatedness between strain DHo^T and *D. jiangningensis* strain SBZ3-12^T, and *F. aurantia* DSM 6220^T was 42.7 and 42.6 %, respectively. Ubiquinone Q-8 was the predominant respiratory quinone, and the major fatty acids (>10 %) were iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$. *In silico* analysis based on phylogenetics and sequence identity at the nucleotide and protein levels suggests that *Frateuria* is the closest known relative of strain DHo^T. Based on the phenotypic, chemotaxonomic and phylogenetic data, strain DHo^T was designated as a novel species of the genus *Frateuria*, for which the name *Frateuria defendens* sp. nov. is proposed. The type strain is DHo^T (=NCCB 100648^T; =DLB^T=DSM 106169^T).

The genus *Frateuria* has been defined as a straight rod-shaped Gram-negative bacterium, motile by polar flagellum or non-motile, aerobic, oxidase-negative, and catalase-positive. It can produce acid from several carbon sources and hydrogen sulfide (H_2S) from thiosulfate, and has ubiquinone Q-8 [1, 2]. Currently, the genus *Frateuria* includes only two species with validly published names, *Frateuria aurantia* [1] and *Frateuria terrea* [3], the first being the type species. *F. aurantia* was originally isolated from the lily plant *Lilium auratum* and temporarily named *Acetobacter aurantius* [4]. *F. terrea* was originally isolated from forest soil [3].

A previously undescribed bacterium was isolated from the planthopper *Hyalesthes obsoletus* (Hemiptera: Cixiidae), an insect feeding on Abraham's balm (*Vitex agnus-castus*) bushes in the northern part of Israel. The isolate was temporarily designated as a *Dyella*-like bacterium (DLB) and its isolation procedure is detailed in Iasur-Kruh *et al.* [5]. Briefly, *H. obsoletus* individuals were surface-sterilized using antibiotics (100 and 30 µg ml^{-1} of ampicillin and tetracycline, respectively), ground in sterile saline solution (9 % NaCl), plated on CCT [6] agar plates with serial dilutions, and incubated at 28 °C in the dark for 48 h. Purified colonies were tentatively identified from partial 16S rRNA gene sequencing. Strain DHo^T genome (NCBI accession number: LFQR00000000) was previously sequenced by the Illumina MiSeq platform (2×100 bp) using a Nextera mate-pair library [7]. After a quality trimming step (<Q20), cleaned reads were assembled with CLC Genomics version 7 software. The assembly was composed of 333 contigs, with an average coverage of 2000×, an N50 of 42 Kb and an L50 of 23 contigs. No obvious contamination was detected in the assembly according to CheckM (completeness 98.5 %, contamination 0.8 %) [8]. The genome size is estimated at 4 191 200 bp, and the genome has a G+C content of 68.6 mol% [7].

Author affiliations: [1]Department of Entomology, Agricultural Research Organization, Newe Ya'ar Research Center, Ramat Yishay, Israel; [2]Department of Entomology, The Hebrew University of Jerusalem, Rehovot, Israel; [3]Shamir Research Institute, Katzrin, Israel; [4]Ohallo College, Katzrin, Israel; [5]Copia Agro & Food Ltd, Herzliya, Israel; [6]Department of Biotechnology Engineering, ORT Braude College of Engineering, Karmiel, Israel; [7]Department of Plant Pathology and Weed Research, Agricultural Research Organization, Rishon LeZion, Israel.
***Correspondence:** Einat Zchori-Fein, einat@agri.gov.il
Keywords: biological agent; DLB; phloem bacteria; plant pathogens.
Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; DLB, *Dyella*-like bacterium; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.
†These authors contributed equally to this work.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene of strain DHo^T is KX196280. The BioProject accession number is PRJNA286074. The genome assembly used in this work is LFQR00000000.
Eight supplementary figures are available with the online version of this article.

Table 1. Differential characteristics of strain DHo^T compared with closely related species

Taxa: 1, strain DHo^T; 2, *Frateuria aurantia* DSM 6220^T; 3, *Frateuria terrea* DSM 26515^T; 4, *Dyella thiooxydans* DSM 25733^T. All results are from the present work unless otherwise indicated. All strains are positive for activities of alkaline phosphatase, esterase, cysteinease arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM). All strains are negative for lipase, trypsin, chymotrypsin α-galactosidase, β-galactosidase, β-glucuronidase and α-fucosidase activities (API ZYM), as well as for indole production (API 20NE). All strains utilize D-glucose and do not assimilate L-arabinose, caproate, adipate, malate, tryptophane, arine di-hydrolase, urea, gluconate or phenyl-acetate. All strains produce acids from galactose or D-fucose and do not produce acids from glycerol, erythritol, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol α-D-mannopyranoside, lactose, inulin, melezitose, xylitol, turanose, D-tagatose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate (API 50 CH). +, Positive; –, negative; W, weakly positive; ?, not determined; NA, not available; *, data taken from other study. Additional data for taxon 2 was taken from Jung *et al.* [14], for taxon 3 from Zhang *et al.* [3] and for taxon 4 from Anandham *et al.* [38].

Characteristic	1	2	3	4
Oxidase	W	–*	+*	+*
Enzymatic activities (API ZYM):				
α-Mannosidase	–	–	+	+
α-Glucosidase	–	–	+	+
β-Glucosidase	+	–	–	+
N-Acetyl-β-glucosaminidase	+	–	+	+
Biochemical characteristics (API 20NE):				
Nitrate reduction	+	–	–	–
Aesculin hydrolysis	+	–	W	+
Gelatin hydrolysis	–	–	–	+
Assimilation of (API 20NE):				
D-Mannose	–	+	–	+
D-Mannitol	+	–	–	–
N-Acetylglucosamine	+	–	+	+
Maltose	+	–	+	+
Acid production (API 50 CH) from:				
Arbutin	+	–	–	–
D-Mannose	+	+	–	–
L-Arabinose	+	+	+	?
D-Ribose	+	+	W	–
D-Xylose	+	+	–	?
D-Fructose	W	W	–	–
D-Fucose	+	+	?	+
N-Acetylglucosamine	–	–	–	+
Inositol	–	+	–	–
Aesculin	+	–	+	+
Cellobiose	+	–	–	+
Melibiose	+	–	–	–
Gentobiose	?	–	–	–
Maltose	+	–	W	+
Trehalose	–	–	–	+
Sucrose	+	–	–	+
Amygdalin	+	–	–	–
Salicin	+	–	–	–
Starch	–	–	–	+
DNA G+C content (mol%)	68.6	63.5*	64.0*	66.0*

Strain DHo^T possesses endophytic properties, and retains a continuous *in planta* presence in its primary host plant *V. agnus-castus* [9]. The bacterium can penetrate and colonize plant species such as grapevine, carrot and others, but is not sustained longer than one month [9]. Because strain DHo^T inhibits the growth of spiroplasma *in vitro* [5] and reduces the symptoms of phytoplasma infection in grapevine plantlets *in vivo* [10], it is considered a potential control method against plant diseases. The description of strain DHo^T is a mandatory step before it can be further developed as a biocontrol agent.

Gram reaction was determined using a non-staining method [11], and a motility test was performed by allowing

Table 2. Fatty acid profile of strain DHo^T compared to closely related species

Taxa: 1, strain DHo^T; 2, *Frateuria aurantia* DSM 6220^T; 3, *Frateuria terrea* DSM 26515^T; 4, *Dyella thiooxydans* DSM 25733^T. All results are from the present work unless otherwise indicated.

Fatty acid	1	2	3	4
Saturated				
$C_{14:0}$	0.2	1.6	0.1	–
$C_{16:0}$	1.8	1.98	1.2	0.6
$C_{20:0}$	0.2	–	–	0.2
iso-$C_{11:0}$	3.8	0.2	3.2	3.5
iso-$C_{14:0}$	0.8	–	1.6	2.2
iso-$C_{15:0}$	20.4	55.1	10.4	12.2
iso-$C_{16:0}$	16	–	34.7	30.9
iso-$C_{17:0}$	11.1	3.8	6.6	3.8
iso-$C_{18:0}$	1	–	2.1	1.5
iso-$C_{19:0}$	0.4	–	–	0.4
iso-$C_{20:0}$	0.3	–	–	0.4
anteiso-$C_{11:0}$	0.1	–	–	–
anteiso-$C_{15:0}$	7.7	1.3	3.5	4.3
anteiso-$C_{17:0}$	6.7	–	1.6	0.6
$C_{17:0}$ cyclo	3.6	7.8	–	–
Hydroxy				
$C_{11:0}$ 2-OH	–	0.5	–	–
$C_{11:0}$ 3-OH	–	0.3	–	–
$C_{12:0}$ 2-OH	–	2.3	–	–
$C_{12:0}$ 3-OH	0.1	6.4	–	–
$C_{14:0}$ 2-OH	–	0.3	–	–
iso-$C_{11:0}$ 3-OH	4.6	0.6	4.0	4.8
iso-$C_{12:0}$ 3-OH	0.5	–	1.6	–
iso-$C_{13:0}$ 3-OH	2.9	2.3	1.8	2.2
iso-$C_{15:0}$ 3-OH	–	0.9	–	–
iso-$C_{16:0}$ 3-OH	–	–	–	0.3
iso-$C_{17:0}$ 3-OH	0.7	–	0.2	0.4
Unsaturated				
$C_{14:1}\omega5c$	–	0.6	–	–
iso-$C_{17:1}\omega9c$	9.4	1.4	20.1	20.1
anteiso-$C_{17:1}\omega9c$	0.7	–	–	–
Unknown (ECL 11.799)	3.1	1.1	1.7	2.2
Summed feature 3*	2.7	5.9	1.9	3.0

*Summed features are groups of two or three fatty acids that are not separated by GLC with the Sherlock Microbial Identification System. Summed feature 3 comprises iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$.

bacterial cells to grow on 0.7 % nutrient agar (NA) for 3 days. Catalase activity was determined by assessing bubble production in 3 % (v/v) hydrogen peroxide and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. Degradation of DNA (DNase activity) was tested using DNase agar (Scharlau) and flooding plates with 1 M HCl. The results of this as well as degradation tests for starch [12] and cellulose [13] were scored after 5 days. Bacterial growth on R2A agar, NA, tryptic soy agar (TSA) and MacConkey agar was evaluated at 28 °C for 3 days, after which colony morphology was recorded. Growth at different temperatures (ranging from 15 to 45 °C), pH values (4–10) and NaCl concentrations (0–4 % w/v) was evaluated after 5 days on R2A agar, as previously described [14].

Cells of strain DHo^T were Gram-negative, aerobic and motile. They were oxidase-positive, catalase-positive, and grew on NA and TSA, but not on MacConkey media. After 3 days of growth on R2A agar, the colonies were 1.0–1.5 mm in diameter, yellow-pigmented, smooth, circular, entire and transparent with clear edges. On R2A agar, the optimal growth temperature for the isolate was 28 °C; growth occurred at 15–42 °C, but not at 45 °C. Growth pH and NaCl ranges were 4–10 (optimal pH 7.0) and 0–2 %, respectively. Strain DHo^T hydrolysed starch but not DNA or cellulose.

Cell morphology was observed under a Nikon light microscope (×1000 magnification) and by scanning electron microscopy (SEM), the latter following Hover *et al.* [15]. Briefly, cells grown on a CCT agar plate at 28 °C for 48 h were fixed [16] and a 5 mm^2 zone of the fixed agar was excised and vapour-fixed with 8 % paraformaldehyde and 4 % glutaraldehyde dissolved in water for 1 h, followed by a second vapour fixation and additional immersion in 2 % osmium tetroxide for 1 h. The samples were submersed for 10 min in double-distilled water and then dehydrated in a series of increasing ethanol concentrations, from 7.5 to 100 %. Next, samples were critical-point dried using a K850 critical-point dryer (Quorum Technologies, UK). Coating was performed with 3 nm iridium using a Q150T coater (Quorum Technologies). Samples were imaged with a Merlin scanning electron microscope (Zeiss). Light and electron microscopy showed that strain DHo^T cells are rod-shaped, 0.2–0.3 µm wide and 1.0–1.3 µm long, and often presenting a single polar flagellum (Fig. S1, available in the online version of this article).

Enzyme activities and other physiological and biochemical characteristics were determined with API ZYM, API 20NE and API 50 CH galleries (bioMérieux). Tests involving commercial systems were performed according to the manufacturer's instructions; all strips (API 20NE and API 50 CH) were incubated at 30 °C and examined after 24 and 48 h, while the API ZYM test was performed at 37 °C for 4 h. The physiological characteristics of strain DHo^T along with the taxonomically related type strains *F. aurantia* DSM 6220^T, *F. terrea* DSM 26515^T and *D. thiooxydans* DSM 25733^T were determined in parallel (Table 1).

The cellular fatty acid profile was determined for strains DHo^T, *F. aurantia* DSM 6220^T, *F. terrea* DSM 26515^T and *D. thiooxydans* DSM 25733^T grown on R2A at 28 °C for 72 h. The cells were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analysed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package [17]. The value range was obtained using duplicate

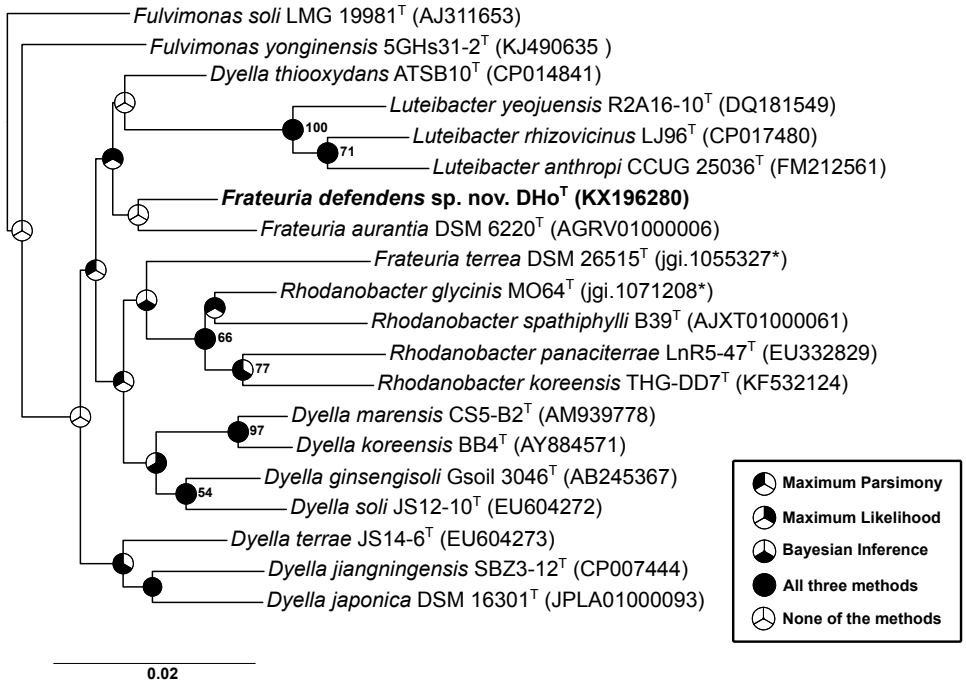

Fig. 1. Neighbour-joining tree based on partial 16S rRNA gene sequences (1424 bp) from several bacterial strains belonging to the family Rhodanobacteraceae. Strain DHo^T is highlighted in bold. Bootstrap support values >50 are displayed at each node. Nodes recovered by at least two other treeing methods are denoted by filled circles. *Xanthomonas vesicatoria* ATCC 35937^{T}16S rRNA sequence (CP018725.1) was used as outgroup (not shown). Clades recovered by other treeing methods are denoted at each node (Fig. S3-S5). * EZBioCloud accession numbers.

experiments. Strain DHo^T's fatty acid profile (Table 2) was mainly composed of the branched fatty acids of iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$ and iso-$C_{17:1}\omega9c$, which are also present in the genus *Frateuria* and *Dyella* [3, 18, 19]. *F. terrea* DSM 26515^T showed a fatty acid profile closer to *D. thiooxydans* DSM 25733^T than to *F. aurantia* DSM 6220^T, with fatty acids iso-$C_{16:0}$ and iso-$C_{17:1}\omega9c$ as the most abundant ones. In contrast, fatty acid iso-$C_{15:0}$ was, by far, the most abundant in *F. aurantia* DSM 6220^T. Interestingly, strain DHo^T presented a fatty acid profile that resembled both *F. aurantia* DSM 6220^T and *D. thiooxydans* DSM 25733^T with iso-$C_{15:0}$ and iso-$C_{16:0}$ as the two most abundant fatty acids. Finally, the most characteristic fatty acids of strain DHo^T were iso-$C_{17:0}$, anteiso-$C_{15:0}$, and anteiso-$C_{17:0}$, which were less abundant, or absent, in the other type strains tested.

Respiratory lipoquinones were extracted from 100 mg of freeze-dried cell material using a two-stage method [20, 21]; separation into different classes (menaquinones, ubiquinones, etc.) was performed using a thin-layer chromatography (TLC) technique followed by additional HPLC analysis. The predominant respiratory quinone system of strain DHo^T was Q-8, which is consistent with the quinone system of species of both *Dyella* and *Frateuria* [18, 22].

Polar lipid analysis was performed as a continuation of the respiratory lipoquinone extraction, the latter followed by separation by two-dimensional TLC; total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid for defined functional groups, as done previously [23]. The polar lipid profile of strain DHo^T was composed of lipids, phospholipids, glycolipids, and aminolipids (Fig. S2).

EzBioCloud's Identify service (database version 2018.05) was used to identify and retrieve the 16S rRNA gene sequences and proteomes of the type strains most closely related to strain DHo^T [24]. The highest identity values with strain DHo^T were found for *F. aurantia* DSM 6220^T (98.2 %), *Dyella thiooxydans* ATSB10^T (98 %), *D. terrae* JS14-6^T and *D. marensis* CS5-B2^T (97.8 %), *D. jiangningensis* SBZ3-12^T (97.6 %) and *Fulvimonas yonginensis* 5GHs31-2^T (97.5 %). 16S rRNA gene sequences were aligned with MAFFT version 7.215 (L-INS-i algorithm) [25] and pruned with Gblocks (no gaps allowed) [26]. A neighbour-joining (NJ) tree, and associated bootstrap values, were calculated with the FastME 2.0 server [27]. A maximum-parsimony (MP) tree was inferred using MPBoot version 1.1.0 (5000 ultrafast bootstrap) [28]. IQ-TREE version 1.6.2 [29] was used to compute the maximum-likelihood (ML) tree (5000 ultrafast bootstraps and 5000 SH-like approximate likelihood ratio test) under the winning model (TN+F+I+G4). A Bayesian tree was inferred using BEAST version 2.5.0 [30], by running four independent chains until convergence (TN+F+I+G4 model) and using the obtained ML tree as the

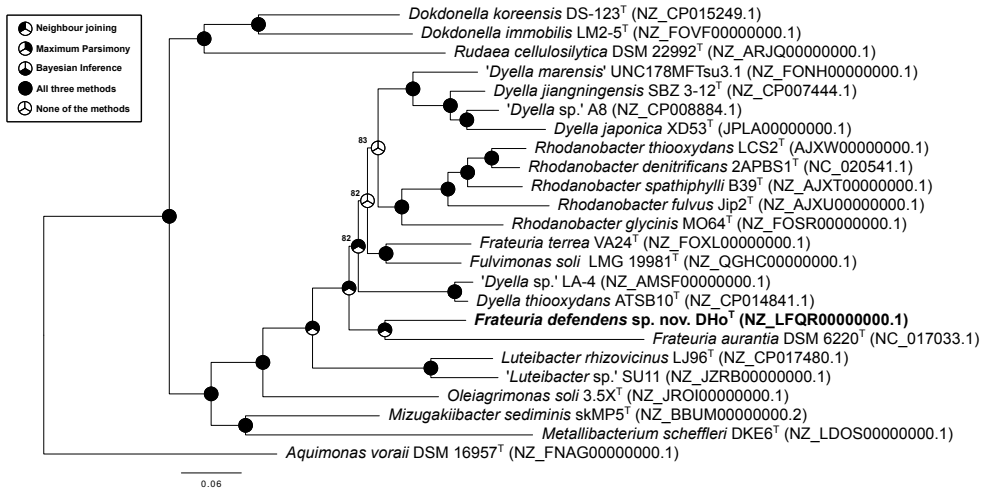

Fig. 2. Maximum-likelihood phylogenomic tree based on 258 conserved proteins (115350 amino acids) among several bacterial strains belonging to the family Rhodanobacteraceae. Strain DHo^T is highlighted in bold. Bootstrap support values (ultrafast bootstrap) are displayed at each node. Only support values <95 are displayed. Nodes recovered by at least two other treeing methods are denoted by filled circles. *Xanthomonas vesicatoria* ATCC 35937^T (NZ_CP018725.1) was used as outgroup (not shown). Clades recovered by other treeing methods are denoted at each node (Fig. S6-S8).

starting tree, until the chains converged. The phylogenetic position of strain DHo^T in the four treeing methods was as a part of a clade containing *F. aurantia* DSM 6220^T and *Dyella thiooxydans* ATSB10^T. *Luteibacter* type strains were also included in the *Frateuria* clade in three of the methods used (Figs 1, S3–S5). None of the methods included *F. terrea* DSM 26515^T in the same clade of *F. aurantia* DSM 6220^T and strain DHo^T. These results suggest that *F. aurantia* DSM 6220^T and *D. thiooxydans* ATSB10^T are the closest known relatives of strain DHo^T, but their phylogenetic relationships are not fully resolved.

To increase the confidence of strain DHo^T's phylogenetic classification, PhyloPhlan was used to select a subset of 258 proteins (from 400 conserved bacterial proteins) present in all of the downloaded proteomes [31]. For each protein, MAFFT (L-INS-i algorithm) [25] and Gblocks (no gaps allowed) [26] were used to generate a pruned multiple-sequence alignment before their concatenation. NJ, MP, ML (LG+F+R5) and Bayesian (LG+I+G4) trees were inferred as explained above, using the concatenated protein alignment as a single partition. According to this analysis, the phylogenetic position of strain DHo^T corroborated previous findings [5]. The isolate was placed in a basal position within the *Dyella–Frateuria–Rhodanobacter* clade, with the fast-evolving *F. aurantia* DSM 6220^T as the closest known relative in most of the treeing methods used (Figs 2, S6–S8).

In addition, average nucleotide identity (ANI) and average amino acid identity (AAI) [32] were calculated for some of the aforementioned genomes/proteomes using the Enveomics package [33]. The highest ANI value similarities to strain DHo^T were with *Fulvimonas soli* DSM 14263^T, *Dyella marensis* UNC178MFTsu3.1 and *F. terrea* DSM 26515^T (85.2, 82.5 and 81.8 %, respectively). However, when ANI values fall below 80 %, it is recommended that AAI values be used instead [32]. Strain DHo^T showed the highest AAI value similarities to *Fulvimonas soli* DSM 14263^T and *F. terrea* DSM 26515^T (77.6 and 74.5 %, respectively). The calculated ANI/AAI values suggested that the DHo^T strain is a new species because no related species showed ANI value similarities close to 95 % [34]. In addition, ANI/AAI values support the inclusion of strain DHo^T in the *Dyella–Frateuria–Rhodanobacter* clade.

DNA–DNA hybridization analysis was performed by first disrupting cells with a Constant Systems TS 0.75 KW (IUL Instruments) and purifying the DNA in the crude lysate by chromatography on hydroxyapatite, as described by Cashion *et al.* [35]. DNA–DNA hybridization was then carried out as described by De Ley *et al.* [36], with the modifications in Huss *et al.* [37], using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatic 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The DNA–DNA relatedness between strain DHo^T and *D. jiangningensis* SBZ3-12^T and *F. aurantia* DSM 6220^T was 42.7 and 42.6 %, respectively. The low DNA–DNA relatedness values (<70 %) confirmed the ANI/AAI results, and place the DHo^T strain as a novel species [34].

In summary, the characterisation of strain DHo^T was consistent with descriptions of the genus *Frateuria* with regard to morphological, biochemical and chemotaxonomic properties (Table 1), as well as fatty acid profile (Table 2). Phylogenetic analyses further supported the assignment of strain DHo^T to that genus (Figs 1 and 2). DNA–DNA hybridization relatedness and ANI/AAI confirmed strain DHo^T as a new species. It is thus suggested that strain DHo^T be assigned to the genus *Frateuria* as a representative of a novel

species, for which the name *Frateuria defendens* sp. nov. is proposed.

DESCRIPTION OF *FRATEURIA DEFENDENS* SP. NOV.

Frateuria defendens sp. nov. (de.fen′dens. L. part. adj. *defendens* protecting, as it protects plants from phloem-inhabiting pathogens).

Frateuria defendens strain DHo^T was isolated from the planthopper *Hyalesthes obsoletus* feeding on a *Vitex agnus-castus* (Abraham's balm) bush in northern Israel (32.988 N, 35.707 E) during the fall of 2011. The cells are Gram-negative, aerobic, motile and rod-shaped (0.2–0.3 µm wide and 1.0–1.3 µm long). Growth occurs between 15 and 42 °C, with tolerance to pH 4–10 and a maximum 2 % (w/v) NaCl. The strain can reduce nitrate and hydrolyse aesculin and cellulose, but not DNA, or gelatine. The bacterium can utilize the following substrates for its growth: L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, amygdalin, arbutin, salicin, maltose, melibiose, D-fucose, raffinose and sucrose. The following substrates are not utilized by the bacterium: glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, caprate, maleate, phenyl acetate, citrate, malate and adipate. Q-8 is the dominant respiratory quinone, and its major fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, and $C_{17:1}\omega 9c$. The G+C content of its genomic DNA is 68.6 mol%. Its primary polar lipids are lipids, phospholipids, glycolipids and aminolipids.

The type strain is DHo^T (=NCCB 100648^T;=DLB^T=DSM 106169^T), isolated from *Hyalesthes obsoletus* collected near a vineyard in the north of Israel. Strain DHo^{T}16S rRNA gene and genome assembly have been deposited in the GenBank under the accession numbers KX196280 and LFQR00000000, respectively.

Funding information
This work was supported in part by the Chief Scientist of the Israeli Ministry of Agriculture and Rural Development, and Copia Agro and Food Ltd. D.S-G. is a recipient of the Golda Meir Post-Doctoral Fellowship (HUJI).

Acknowledgements
The authors gratefully acknowledge Professors A. Oren and M. E. Trujillo for commenting on earlier drafts of the manuscript and advice on naming issues. The analysis of respiratory quinones, polar lipids, fatty acids, DNA–DNA hybridizations and the physiological and biochemical characteristics of API galleries (bioMérieux) was carried out by the identification service, Leibniz-Institute DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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