

## NOTE

***Sphingomonas melonis* sp. nov., a novel pathogen that causes brown spots on yellow Spanish melon fruits**

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**A polyphasic taxonomic study was performed on the phytopathogenic bacterial strains DAPP-PG 224<sup>T</sup> and DAPP-PG 228, which cause brown spot on yellow Spanish melon (*Cucumis melo* var. *inodorus*) fruits. Based on the presence of glucuronosyl ceramide (SGL-1) in cellular lipids, the results of fatty acid analysis and 16S rDNA sequence comparison, the strains had been identified as belonging to the genus *Sphingomonas* and as phylogenetically related to *Sphingomonas mali*, *Sphingomonas pruni* and *Sphingomonas asaccharolytica*. The levels of 16S rDNA sequence similarity of these three species to strain DAPP-PG 224<sup>T</sup> were respectively 98.0, 98.0 and 97.4%. DNA–DNA hybridization experiments between strains pathogenic on melon fruit and *S. mali*, *S. pruni* and *S. asaccharolytica* revealed ≤ 16% relatedness. Based on these results, the two isolates studied are regarded as independent from the type strains of the three species mentioned above. *Sphingomonas* strains from melon fruits are recognized as forming a genetically and phenotypically discrete species and to be differentiated by phenotypic characteristics from all 29 named species of the genus. Thus, the name *Sphingomonas melonis* sp. nov. is proposed for the isolates from diseased melon fruits. The type strain is DAPP-PG 224<sup>T</sup> (= LMG 19484<sup>T</sup> = DSM 14444<sup>T</sup>). The G+C content of DNA of the type strain is 65.0 mol%.**

**Keywords:** brown spot on melon fruit, *Cucumis melo* var. *inodorus*, polyphasic taxonomy, *Sphingomonas melonis* sp. nov.

Brown spots caused by *Sphingomonas* sp. have been reported on yellow Spanish melons (*Cucumis melo* var. *inodorus* Naud.) cultivated in greenhouses in Almeria, Spain (Buonauro *et al.*, 2001). When yellow Spanish melon fruits and leaves were inoculated with the bacterium, only fruits showed disease symptoms. 16S rDNA sequence analysis performed on one bacterial isolate revealed that it was phylogenetically closely related to *Sphingomonas mali*, *Sphingomonas pruni*

and *Sphingomonas asaccharolytica* (Buonauro *et al.*, 2001).

In this paper, we have determined the taxonomic position of two strains associated with brown spot disease of yellow Spanish melons by a polyphasic taxonomic study. All the species transferred to three new genera proposed by the splitting of the genus *Sphingomonas* (Takeuchi *et al.*, 2001) are recognized as junior subjective synonyms of the corresponding *Sphingomonas* species (Yabuuchi & Kosako, 2003). Thus, we show here that our two strains are members of a novel species in the genus *Sphingomonas*, for which the name *Sphingomonas melonis* sp. nov. is proposed.

Phenotypic and genotypic characterization was carried out on two isolates from melon fruits, DAPP-PG 224<sup>T</sup> (= LMG 19484<sup>T</sup> = DSM 14444<sup>T</sup>) and DAPP-PG 228

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The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of strain DAPP-PG 224<sup>T</sup> is AB055563.

**Table 1.** Physiological and biological characteristics of *Sphingomonas melonis* sp. nov. and the type strains of four other *Sphingomonas* species

Strains are identified as: 1, *S. melonis* sp. nov. DAPP-PG 224<sup>T</sup>; 2, *S. melonis* sp. nov. DAPP-PG 228; 3, *S. pruni* EY 4228<sup>T</sup>; 4, *S. mali* EY 4341<sup>T</sup>; 5, *S. asaccharolytica* EY 4229<sup>T</sup>; 6, *S. paucimobilis* EY 2395<sup>T</sup> (type species of the genus). Characteristics are scored as: +, positive reaction within 3 days; (+), positive reaction after more than 4 days; –, negative reaction, NG, no growth. In basic microbiological tests, all six strains were Gram-negative, rod-shaped, positive for hydrolysis of aesculin and negative for alcapton production, lysine and ornithine decarboxylases, arginine dihydrolase base Moeller and urease in Christensen's medium. In the API 20NE system, all strains were positive for aesculin hydrolysis, *p*-nitrophenyl  $\beta$ -D-galactopyranoside and assimilation of glucose, L-arabinose, *N*-acetyl D-glucosamine and maltose and negative for reduction of nitrate, indole from tryptophan, glucose fermentation, arginine dihydrolase, urease, gelatinase and assimilation of D-mannitol and *n*-caprate. In oxidative acid production tests, all six strains produced acid oxidatively from L-arabinose, cellobiose, fructose, galactose, lactose, maltose, mannose, trehalose and xylose and none of the strains produced acid oxidatively from adonitol or D-ribose. In Biotype 100 assimilation tests, all six strains assimilated  $\alpha$ -D(+)-glucose, D(+)-trehalose, D(+)-mannose, sucrose, D(+)-cellobiose, aesculin, fumarate and D(+)-galactose and none of the strains assimilated L(+)-sorbitol, D(–)-ribose, D(+)-arabitol, L(–)-arabitol, dulcitol, D-tagatose, *myo*-inositol, D-sorbitol, adonitol, hydroxyquinoline  $\beta$ -glucuronide, D-lyxose, *i*-erythritol, 3-*o*-methyl DS-glucopyranose, L(+)-tartrate, D(–)-tartrate, 2-keto-D-gluconate, 5-keto-D-gluconate, L-tryptophan, (–)-quinic acid, *m*-hydroxybenzoate, benzoate, 3-phenylpropionate, *m*-coumarate, trigonelline, histamine, L-histidine, DL- $\alpha$ -amino-*n*-valerate, ethanolamine, tryptamine, D-glucosamine, malonate or 2-oxoglutarate.

Characteristic	1	2	3	4	5	6
<b>Basic microbiological tests</b>						
Motility*	–	–	+	+	+	+
Colony colour†	Y	Y	Y	LY	Y	Y
Oxidase, Kovacs'	+	+	+	+	+	–
Growth in the presence of 3% NaCl	+	+	+	+	+	–
Hydrolysis of:						
Gelatin	–	–	–	–	–	+
Starch	–	–	+	–	+	+
Tween 80	–	–	+	+	+	+
Citrate, Simmons'	–	–	–	–	–	+
DNase	NG	NG	NG	+	NG	–
Phenylalanine deaminase	–	–	–	–	–	+
Acylamidase	–	–	NG	–	NG	–
Malonate	(+)	(+)	(+)	+	(+)	–
<b>API 20NE system tests</b>						
Assimilation of:						
D-Mannose	+	+	–	+	+	+
DL-Malate	+	+	–	–	–	+
Phenylacetate	+	+	–	–	–	–
Gluconate	–	–	–	+	–	+
Adipate	–	–	–	–	–	+
Sodium citrate	–	–	–	–	–	+
Oxidase	+	+	+	–	+	–
<b>Oxidative acid production from:</b>						
Glucose	+	+	(+)	–	+	+
Rhamnose	+	+	–	+	+	–
Melezitose	(+)	+	–	–	–	+
Sucrose	+	+	–	–	–	+
D-Arabinose	–	–	–	+	+	+
Ethanol	–	–	–	+	+	+
Melibiose	–	(+)	–	–	–	(+)
Dulcitol	–	–	–	+	+	–
Glycerol	+	–	–	–	–	–
Inositol	–	–	–	+	–	–
Mannitol	–	–	–	–	+	–
Raffinose	–	–	–	–	–	(+)
Sorbitol	–	–	(+)	–	–	–
Salicin	NG	–	–	–	–	(+)
Inulin	NG	–	–	–	–	–

**Table 1** (cont.)

Characteristic	1	2	3	4	5	6
<b>Assimilation tests (Biotype 100)</b>						
Maltotriose, L-glutaminate	+	+	+	–	+	+
Maltose, D(+)-xylose	+	+	+	+	–	+
L(–)-Malate	+	+	–	+	+	+
α-D(+)-Melibiose	–	–	+	+	+	+
β-D(+)-Fructose, L(+)-arabinose, <i>N</i> -acetyl D-glucosamine, DL-lactate, succinate	+	+	–	–	+	+
L-Aspartate	–	–	+	–	+	+
Phenylacetate, L-proline	+	+	–	–	+	–
α-L-Rhamnose	–	–	+	+	–	–
D-Galacturonate	+	+	–	–	–	–
Paratinose, D(+)-raffinose, α-L(–)-fucose, D(+)-melezitose, maltitol, D(+)-turanose, 1- <i>o</i> -methyl α-D-glucopyranoside, <i>cis</i> -aconitate, citrate, glutarate, DL-β-hydroxybutyrate, L-tyrosine, L-alanine, L-serine, gentisate	–	–	–	–	+	+
1- <i>o</i> -Methyl β-D-glucopyranoside, <i>trans</i> -aconitate, α-lactose, lactulose, 1- <i>o</i> -methyl β-galactopyranoside, 1- <i>o</i> -methyl α-galactopyranoside, protocatechuate, <i>p</i> -hydroxybenzoate, betaine	–	–	–	–	–	+
β-Gentiobiose, D-saccharate, D-mannitol, xylitol, glycerol, mucate, <i>meso</i> -tartrate, D(+)-malate, tricarballylate, D-glucuronate, putrescine, D-gluconate, DL-α-amino- <i>n</i> -butyrate, caprate, caprylate, DL-glycerate, itaconate, D-alanine, propionate	–	–	–	–	+	–

\* Spreading growth on semi-solid agar (Gard) plate.

† Y, Deep yellow; LY, lemon yellow.

(= LMG 19485 = DSM 14445), in comparison with *Sphingomonas paucimobilis* EY 2395<sup>T</sup>, *S. mali* EY 4341<sup>T</sup>, *S. pruni* EY 4228<sup>T</sup> and *S. asaccharolytica* EY 4229<sup>T</sup>.

The morphology of the isolates was observed by Gram staining and under the transmission electron microscope (Philips EM 400) after negative staining of the cells with an aqueous solution of 1% sodium phosphotungstate and 0.4% sucrose (pH 6.5). Phenotypic tests listed in Table 1, referred to as 'basic microbiological tests', were performed as reported previously (Yabuuchi *et al.*, 1990). The API 20NE system (bioMérieux) was used according to the manufacturer's instructions. Oxidative acid production was tested by using Bacto-OF basal medium (Difco) supplemented with a total of 26 carbohydrates, sugar alcohols (1%) and ethanol (3%). Biotype 100 strips (bioMérieux) were used to estimate the nutritional profiles of bacteria following the manufacturer's instructions.

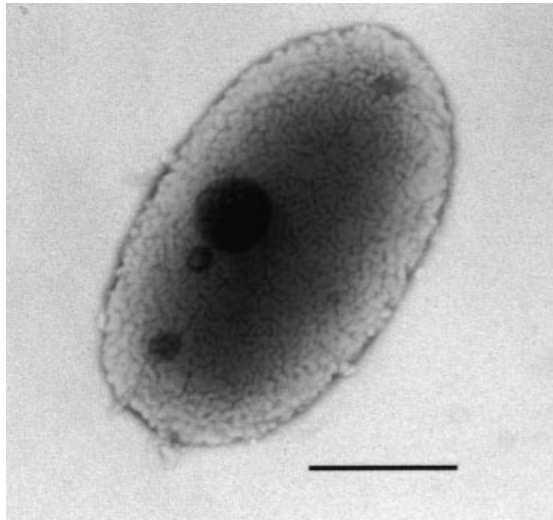
Antimicrobial susceptibility of the organisms was tested using 36 kinds of Sensi-discs and ready-to-use Mueller–Hinton II medium (Becton-Dickinson), as described previously (Yabuuchi *et al.*, 1999).

For the analyses of cellular lipids and fatty acids, cells grown on agar media were harvested and cellular lipids

were extracted twice with chloroform/methanol, first 2:1, then 1:3 (v/v). Extracted lipids were hydrolysed with 0.5 M NaOH, as described previously (Yabuuchi *et al.*, 1990, 1999). The total extractable lipids and their alkaline hydrolysates were analysed by TLC with an acidic solvent system composed of chloroform/methanol/acetic acid/water (100:20:12:5, by vol.). Purified glucuronosyl ceramide (SGL-1) of *S. paucimobilis* EY 2395<sup>T</sup> was used as the standard. Cellular fatty acid analysis was performed as described previously (Yabuuchi *et al.*, 1990, 1999).

Ubiquinones were purified from lipid extracts on silica gel TLC plates (Kiesel gel 60 F254, 20 × 20 cm; Merck), developed with benzene and separated by reverse-phase TLC (HPTLC RP-18 F254; Merck), developed with acetone/acetonitrile (80:20, v/v) and visualized under UV light (254 nm) (Collins & Jones, 1981). The ubiquinone type was confirmed by comparing *R<sub>f</sub>* values with standard ubiquinones (Collins & Jones, 1981; Komagata & Suzuki, 1987), including Q-7, Q-9 and Q-10 (Sigma) and Q-8 from *Ralstonia pickettii* EY 3254<sup>T</sup> (Yabuuchi *et al.*, 1995).

Genomic DNA was prepared according to the procedure of Wilson (1987). The G+C content of DNA was determined by the HPLC method described by

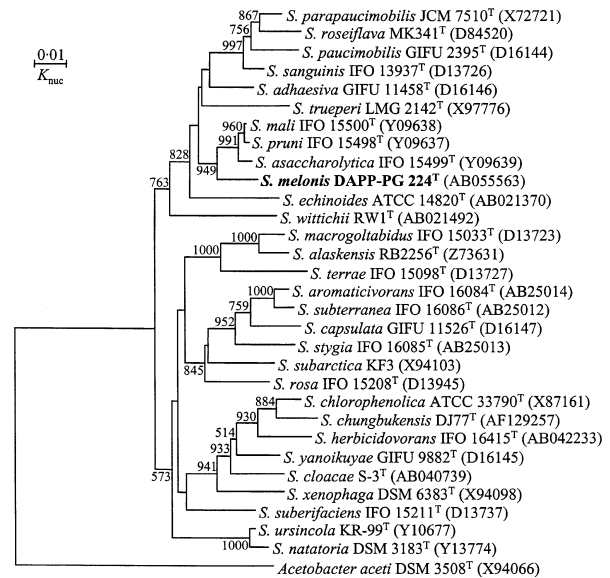


**Fig. 1.** Electron micrograph of a negatively stained cell of *Sphingomonas melonis* sp. nov. strain DAPP-PG 224<sup>T</sup>. Cells were stained with a solution of 1% sodium phosphotungstate and 0.4% sucrose (pH 6.5) and observed under a Philips EM 400 transmission electron microscope. Plait-like structures can be observed on the cell surface. Bar, 0.5 µm.

Tamaoka & Komagata (1984). DNA–DNA hybridization was performed at 50 °C according to the method described by Ezaki *et al.* (1989).

The 16S rDNA sequence of strain DAPP-PG 224<sup>T</sup> has been deposited in the DDBJ under accession no. AB055563. As reference sequences, the sequences of the type strains of 29 validly named *Sphingomonas* species and *Acetobacter aceti* were obtained from the NCBI (National Center of Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>). CLUSTAL W (Thompson *et al.*, 1994) was used to align multiple sequences and to calculate nucleotide substitution rates ( $K_{nuc}$  values; Kimura, 1980). The neighbour-joining method (Saitou & Nei, 1987) was used to reconstruct a phylogenetic tree from the distance matrices by NJPLOT (written by Manolo Gouy, Laboratoire de Biométrie, Université Claude Bernard – Lyon 1, Villeurbanne, France). To draw our phylogenetic tree, *A. aceti* DSM 3508<sup>T</sup> (X94066) was used as the outgroup. Alignment positions that included gaps and/or unidentified bases were not taken into consideration for calculations. To evaluate the topology of our phylogenetic tree, the sequence data were sampled 1000 times for bootstrap analysis (Felsenstein, 1985).

On nutrient agar, colonies of DAPP-PG 224<sup>T</sup> and DAPP-PG 228 were 0.4–0.5 mm in diameter, circular, entire, domed, deep-yellow-pigmented with a smooth surface and entire margins after 2 days of incubation at 27 °C. Cells of the two strains were Gram-negative, non-spore-forming, rod-shaped (0.68–0.85 × 1.2–1.9 µm), non-motile without flagella. However, plait-like structures were observed on the cell surface by electron microscopy (Fig. 1).



**Fig. 2.** Phylogenetic position of *Sphingomonas melonis* sp. nov. strain DAPP-PG 224<sup>T</sup> among the type strains of 29 validly named *Sphingomonas* species. Sequence accession numbers are given in parentheses.

Results for strains DAPP-PG 224<sup>T</sup> and DAPP-PG 228 in basic microbiological and API 20NE system tests are given in Table 1. Both strains produced acid oxidatively from 13 of 26 carbohydrates, sugar alcohols and ethanol in OF basal medium. Results that differed for the two strains were that strain DAPP-PG 224<sup>T</sup> produced acid from glycerol while strain DAPP-PG 228 produced acid from melibiose (Table 1).

The Biotype 100 system revealed that both strains assimilated 21 compounds as sole carbon sources. In contrast to the other four *Sphingomonas* species compared, they assimilated D-galacturonate and not α-D(+)-melibiose (Table 1).

Both strains DAPP-PG 224<sup>T</sup> and DAPP-PG 228 were resistant to amoxicillin, penicillin, ampicillin, piperacillin, cefoperazone, moxalactam, cefaclor, ceftazidime, flomoxef, ceftazolin, cefmetazole, cefotaxime, aztreonam, carumonam, panipenem, polymyxin B and trimethoprim. Strain DAPP-PG 228 was also resistant to roxithromycin, norfloxacin and sparfloxacin.

TLC analysis developed with an acidic solvent system revealed the presence of glucuronosyl ceramide (SGL-1) in extractable lipids of both melon strains as well as in extracts from the other four *Sphingomonas* type strains examined.

Octadecenoic acid (C18:1) was the major component of total fatty acids and 2-hydroxytetradecanoic acid (2-OH C14:0) was the major 2-hydroxy acid in all strains tested except for *S. asaccharolytica* EY 4229<sup>T</sup>. In the type strain of *S. asaccharolytica*, C<sub>17</sub>-cyclopropanoic acid (C17:0 Δ) and 2-hydroxypentadecanoic

**Table 2.** Phenotypic characteristics of *Sphingomonas melonis* sp. nov. and 11 phylogenetically related *Sphingomonas* species

Species are identified as: 1, *S. melonis* sp. nov.; 2, *S. paucimobilis* (data from Yabuuchi *et al.*, 1990); 3, *S. adhaesiva* (Yabuuchi *et al.*, 1990); 4, *S. asaccharolytica* (Takeuchi *et al.*, 1995); 5, *S. echinoides* (Denner *et al.*, 1999); 6, *S. mali* (Takeuchi *et al.*, 1995); 7, *S. parapaucimobilis* (Yabuuchi *et al.*, 1990); 8, *S. pruni* (Takeuchi *et al.*, 1995); 9, *S. roseiflava* (Yun *et al.*, 2000); 10, *S. sanguinis* (Takeuchi *et al.*, 1993); 11, *S. suberifaciens* (van Bruggen *et al.*, 1990); 12, *S. trueperi* (Kämpfer *et al.*, 1997). Assimilation data were obtained from the API 20 NE, API 50 CH or Biotype 100 systems. +, Positive or most strains positive when  $n > 1$ ; –, negative or most strains negative when  $n > 1$ ; ND, not determined.

Substrate or test	1	2	3	4	5	6	7	8	9	10	11	12
Number of strains examined	2	7	1	1	1	3	4	1	5	1	12	1
Yellow or deep-yellow colonies	+	+	+	–	–	–	+	–	–	+	–	–
Phenylalanine deaminase	–	+	–	–	–*	–	–	–	–*	+	ND	–*
Nitrite from nitrate	–	–	–	–	+	–	+	–	+	–*	+	–
Hydrolysis of gelatin	–	–	–	–	–	–	–	–	+	+	ND	+
Acid produced from:												
Glycerol	–	–	+	–	+	–	–	–	–*	+	–	–*
Rhamnose	+	–	+	–	–	+	+	–	–*	+	–	–
Salicin	–	+	+	–	–	–	–	–	–*	–*	+	–
Assimilation of:												
Gluconate	–	–	–	–	–	+	+	+	+	–	ND	–
Adipate	–	–	–	–	–*	+	–	–	+	–*	ND	–
Malate	+	–	+	–	+	–	–	+	+	–	ND	+
Citrate	–	+	–	–	–	–	+	–	+	–*	ND	–
Fructose	+	+	+	–	–	+	+	+	+	+	ND	+
Galactose	+	+	–	–	–*	+	+	+	+	+	ND	+
Melezitose	–	+	–	–	–*	–	+	–	–*	+	ND	+

\* Data taken from Yabuuchi & Kosako (2003).

acid (2-OH C15:0) were respectively the major component of the total fatty acids and the major 2-hydroxy acid. No 3-hydroxy acid was detected in any strains tested.

The major ubiquinone of both isolates from melon fruit was Q-10, with Q-8 and Q-9 as minor components. The ubiquinone composition was consistent with that of *S. paucimobilis*.

The 16S rDNA sequence of the type strain, DAPP-PG 224<sup>T</sup> (1439 nt determined), revealed high similarity to *S. mali* (98%), *S. pruni* (98%), *S. asaccharolytica* (97.4%), *Sphingomonas adhaesiva* (96.7%), *Sphingomonas sanguinis* (95.8%), *Sphingomonas parapaucimobilis* (95.6%), *Sphingomonas trueperi* (95.4%), *Sphingomonas echinoides* (95.4%), *Sphingomonas roseiflava* (95.2%), *Sphingomonas suberifaciens* (94.5%) and *S. paucimobilis* (94.1%). Fig. 2 shows the position of strain DAPP-PG 224<sup>T</sup> within the radiation of species of the genus *Sphingomonas* on the basis of a neighbour-joining analysis of 16S rDNA sequences. The G + C content of the DNA was respectively 65.0 and 64.9 mol% for strains DAPP-PG 224<sup>T</sup> and DAPP-PG 228. The level of DNA–DNA reassociation between strains DAPP-PG 224<sup>T</sup> and DAPP-PG 228 was 97%, while it was respectively 14, 11 and 11% between DAPP-PG 224<sup>T</sup> and the type strains of *S. mali*, *S. pruni* and *S. asaccharolytica*.

Differential phenotypic characteristics for the strains from melon fruit and 11 *Sphingomonas* species with the high values (94.1–98%) of 16S rDNA sequence similarity are listed in Table 2.

Among the members of the genus *Sphingomonas*, as described in the next volume of the new edition of *Bergey's Manual of Systematic Bacteriology* by Yabuuchi & Kosako (2003), five species have been reported to be associated with plants. *Sphingomonas rosa*, *S. pruni*, *S. asaccharolytica* and *S. mali* have been isolated from the roots of different plants (Takeuchi *et al.*, 1995), while *S. roseiflava* has been isolated from the ears of some Gramineae (Yun *et al.*, 2000). Whilst these five species are not known as phytopathogenic, *S. suberifaciens* (originally placed erroneously in the rejected genus ‘*Rhizomonas*’ as ‘*Rhizomonas suberifaciens*’) causes corky root disease of lettuce (van Bruggen *et al.*, 1990). A phytopathogenic *Sphingomonas* species has been reported to cause brown spot on yellow Spanish melon (*Cucumis melo* var. *inodorus*) fruit (Buonauro *et al.*, 2001). Since it has been reported that a large population of *Sphingomonas* species grows on the surface of many plants (Kim *et al.*, 1998), it could be hypothesized that this *Sphingomonas* species grows as an epiphyte on melon fruit and that this capacity increases the likelihood of fruit infection.

The 16S rDNA sequence of the melon fruit isolates has 97% or more similarity to those of *S. mali*, *S. pruni* and *S. asaccharolytica*. The DNA–DNA hybridization values ( $\leq 16\%$ ) obtained in the present study between the two isolates from melon fruit and three phylogenetically related *Sphingomonas* species indicate clearly that the two isolates represent a novel species in the genus. Thus, we propose the name *Sphingomonas melonis* sp. nov. for these isolates.

The plait-like structures on the *S. melonis* cell surface were similar to those described by Hisano *et al.* (1996) for *Sphingomonas* strain A1. They also reported that these structures became larger (mouth-like) when the bacterium was grown in the presence of alginate. Hashimoto *et al.* (1999) suggested that *Sphingomonas* strain A1 incorporated alginate directly through mouth-like pit structures using a novel ATP-binding cassette transporter (ABC transporter).

#### Description of *Sphingomonas melonis* sp. nov.

*Sphingomonas melonis* [me.lo'nis. L. gen. n. *melonis* of melon (*Cucumis melo* var. *inodorus*, Spanish melon), referring to the fruit of the plant for which the organism is pathogenic].

Colonies are pinpoint after 2 days incubation and are never larger than 0.7–0.8 mm in diameter on subsequent days. They are circular and domed with a smooth surface and entire margins and are deep-yellow-pigmented. Cells are Gram-negative, rod-shaped (0.68–0.85  $\mu\text{m}$  wide, 1.2–1.9  $\mu\text{m}$  long), non-spore-forming and non-motile and present plait-like structures on the cell surface. Oxidase-positive. Metabolism is respiratory, never fermentative or photosynthetic. Hydrolyses aesculin and alkalizes malonate broth, but does not alkalize Simmons' citrate medium. Does not hydrolyse gelatin, starch or Tween 80. Grows in the presence of 3% NaCl and is positive for *p*-nitrophenyl  $\beta$ -D-galactopyranoside. Assimilates glucose, L-arabinose, *N*-acetyl D-glucosamine, maltose, D-mannose, DL-malate and phenyl acetate. Other properties are given in Table 1. The major component of alkali-stable cellular lipids is SGL-1. The major fatty acid of cellular lipid is C18:1; the only hydroxy acid is 2-OH C14:0. No 3-OH acid are present. The major isoprenoid quinone is ubiquinone Q-10. Susceptible to aminoglycosides, imipenem, tetracyclins and new quinolones, while resistant to penicillins and cephalosporins. Isolated from the brown spots of yellow Spanish melon fruits (*Cucumis melo* var. *inodorus*) in Almeria (Spain) as the causative agent of the disease. The type strain is DAPP-PG 224<sup>T</sup> (= LMG 19484<sup>T</sup> = DSM 14444<sup>T</sup>). The G+C content of DNA is 65.0 mol %.

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