Description of *Herbiconiux flava* sp. nov. and emended description of the genus *Herbiconiux*

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A non-motile and non-endospore-forming rod, strain NBRC 16403^T, was isolated from the phyllosphere of a sedge (*Carex* sp.). 16S rRNA gene sequence analysis indicated that strain NBRC 16403^T was closely related to *Herbiconiux solani* DSM 19813^T (98.6% 16S rRNA gene sequence similarity), *Herbiconiux ginsengi* wged11^T (97.8%) and *Herbiconiux moechotypicola* RB-62^T (97.8%). The peptidoglycan (B2 γ type) contained D- and L-2,4-diaminobutyric acids, D-alanine, glycine and *threo*-3-hydroxyglutamic acid, which replaced glutamic acid almost completely. The predominant menaquinones were MK-10 and MK-11. The polar lipid pattern comprised diphosphatidylglycerol, phosphatidylglycerol, three glycolipids and minor amounts of other polar lipids. The major fatty acids were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}; no cyclohexyl-C_{17:0} was detected. The DNA G + C content was 71.0 mol%. The results of phylogenetic and DNA–DNA relatedness studies, along with phenotypic differences between strain NBRC 16403^T and recognized members of the genus *Herbiconiux*, indicated that the isolate should be assigned to a novel species of the genus *Herbiconiux*, for which the name *Herbiconiux flava* sp. nov. is proposed. The type strain is NBRC 16403^T (=VKM Ac-2058^T).

The genus *Herbiconiux* was proposed by Behrendt *et al.* (2011) as a member of the family *Microbacteriaceae*. The peptidoglycan of members of this genus is of the B2 γ type with D- and L-2,4-diaminobutyric acid as the diagnostic diamino acids and glycine, alanine and *threo*-3-hydroxyglutamic acid. The predominant menaquinone is MK-11, with MK-10 detectable as a minor component. The major fatty acids are cyclohexyl-C_{17:0} and anteiso-C_{15:0}. The genus *Herbiconiux* currently comprises three species: *Herbiconiux solani* (Behrendt *et al.*, 2011), isolated from the phyllosphere of potato plants, *Herbiconiux ginsengi*, reclassified from *Leifsonia ginsengi* Qiu *et al.* 2007 by Behrendt *et al.* (2011), and *Herbiconiux moechotypicola* (Kim *et al.*, 2012), isolated from the gut of hairy long-horned toad beetles. During the course of quality control of the collections of the NITE Biological Resource Center (NBRC), it was revealed that strain NBRC 16403^T, which

was labelled as *Curtobacterium* sp., was phylogenetically related to members of the genus *Herbiconiux*.

Strain NBRC 16403^T (originally designated DL 374^T) was isolated from the phyllosphere of *Carex* sp. collected in the Central Chernozem Nature Park (Belgorod Region, Russia) on *Corynebacterium* agar (DSMZ medium 53: containing 1.0% casein peptone tryptic digest, 0.5% yeast extract, 0.5% glucose, 0.5% NaCl and 1.5% agar; pH 7.2) after incubation at room temperature (18–24 °C) in daylight for 1 month, as described by Dorofeeva *et al.* (2002). The strain was Gram-stain-positive and the plate from which it was isolated bore Gram-negative colonies in the majority. NBRC medium 802 [1.0% polypeptone (Wako), 0.2% yeast extract (Difco), 0.1% MgSO₄·7H₂O; pH 7.0; 1.5% agar if required] was used as the basal medium for this study. Biomass for chemotaxonomic and molecular systematic studies (except for polar lipid analysis) was obtained by incubating the isolate in shake flasks (100 r.p.m.) for 48 h at 28 °C.

Colony appearance was examined after incubation at 28 °C for 3 days. Cell morphology was investigated by examining cells from cultures over a whole growth cycle (up to 5 days) under a light microscope (BX-51; Olympus) and a scanning electron microscope (JSM-6060; JEOL). Growth

Abbreviations: DAB, 2,4-diaminobutyric acid; Hyg, *threo*-3-hydroxyglutamic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NBRC 16403^T is AB583921.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

under anaerobic conditions was determined by incubation in an anaerobic chamber with an O₂-absorbing and CO₂-generating agent (Anaero-Pack; Mitubishi Gas Chemical). Cell motility and growth at different pH and with different NaCl concentrations were determined using liquid basal medium, as outlined by Hamada *et al.* (2009). Growth at 5–25 °C (at intervals of 5 °C), 28, 37 and 45 °C was determined on solid basal medium after 3 days and growth at 5 and 10 °C was also evaluated after 10 days. Gram-staining was performed using a Faver G Nissui kit (Nissui). Other physiological and biochemical tests were performed using the API ZYM, API Coryne, API 20 E and API 50 CH systems (bioMérieux), according to the manufacturer's instructions. API 50 CH B/E medium was used to prepare cell suspensions for the API 50 CH test. *H. ginsengi* NBRC 104580^T and *H. solani* NBRC 106740^T were used as reference strains. The results of physiological and biochemical analysis are summarized in the species description and Table 1. A scanning electron micrograph of cells of strain NBRC 16403^T and all of the data from the API tests are given in Fig. S1 and Table S1, respectively (available in IJSEM Online).

PCR amplification and sequencing of the 16S rRNA gene of strain NBRC 16403^T was performed as described previously (Hamada *et al.*, 2009). Phylogenetic neighbours were identified and pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The 16S rRNA gene sequence determined in this study was aligned with

reference sequences of the genus *Herbiconiux* and some related taxa using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms in MEGA 4.1 (Tamura *et al.*, 2007). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain NBRC 16403^T formed a cluster with members of the genus *Herbiconiux* and was most closely related to *H. solani* DSM 19813^T (Fig. 1). 16S rRNA gene sequence similarity between the isolate and *H. solani* DSM 19813^T, *H. ginsengi* wged11^T and *H. moechotypicola* RB-62^T was 98.6, 97.8 and 97.8 %, respectively.

Cell-wall samples were prepared from approximately 1 g wet cells by mechanical disruption with an ultrasonic oscillator and glass beads. Cell walls were separated from unbroken cells by differential centrifugation in distilled water and further purified in boiling 4 % SDS (100 °C, 30 min), followed by several washings with distilled water. The molar ratio of amino acids in cell-wall hydrolysates (4 M HCl, 16 h) were determined using the method described by Hamada *et al.* (2009). Amino-acid isomers in cell-wall hydrolysates were examined using the method described by Nozawa *et al.* (2007) using liquid chromatography MS (model LCMS-2020; Shimadzu). Preparation and analysis of cellular fatty acid methyl esters were performed using the protocol of the MIDI Sherlock Microbial Identification System (Sasser, 1990). Fatty acids

Table 1. Differential phenotypic characteristics of strain NBRC 16403^T and the type strains of species of the genus *Herbiconiux*

Strains: 1, *Herbiconiux flava* sp. nov. NBRC 16403^T; 2, *H. solani* NBRC 106740^T; 3, *H. ginsengi* NBRC 104580^T; 4, *H. moechotypicola* RB-62^T (data from Kim *et al.*, 2012). Data are from this study unless indicated. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4
Predominant menaquinones	MK-10, MK-11	MK-11 ^{a*}	MK-11 ^b	MK-11
Temperature range (°C)	5–37	10–28	5–28	15–30
pH range	5.0–9.0	5.0–8.0	5.0–9.0	5.0–8.5
Enzyme activities				
Acid phosphatase	–	+	–	ND
Alkaline phosphatase	–	+	–	ND
α-Galactosidase	+	–	w	ND
β-Galactosidase	+	–	+	+
β-Glucosidase	–	+	+	ND
Acetoin production	w	–	+	ND
Acid production from:				
Amygdalin	w	+	–	–
Arbutin	–	+	w	–
Glycerol	+	+	–	+
Lactose	–	–	+	–
L-Rhamnose	–	+	–	+
D-Ribose	–	+	+	+
DNA G + C content (mol%)	71.0	71.1	69.1	70.3

*Data from: a, Behrendt *et al.* (2011); b, Qiu *et al.* (2007).

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences of strain NBRC 16403^T and its taxonomic neighbours. Bootstrap values ($\geq 70\%$) based on 1000 replicates are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Cellulomonas flavigena* NCIMB 8073^T was used as the outgroup. Bar, 0.01 K_{nuc} substitutions per nucleotide position.

were identified using Sherlock Microbial Identification software (ACTINO database, version 4.0). Cyclohexyl fatty acids were identified using GC-MS (model 5973; Agilent). For polar lipid analysis, the isolate was grown in PYGP broth (containing 0.5 % peptone, 0.3 % yeast extract, 0.5 % glucose, 0.02 % K_2HPO_4 ; pH 7.2) at 24 °C on a rotary shaker (120 r.p.m.) and harvested at the exponential growth stage (after 28 h). The analysis was performed as described by Minnikin *et al.* (1977), with the following slightly modified solvents: chloroform/methanol/water (65:25:4, by vol.) for the first direction and chloroform/acetic acid/methanol/water (80:15:12:5, by vol.) for the second direction. Isoprenoid quinones, cell-wall sugars and DNA G+C content were analysed according to the methods outlined by Hamada *et al.* (2009).

The obtained peptidoglycan sample of strain NBRC 16403^T contained Ala, Gly, *threo*-3-hydroxyglutamic acid (Hyg) and 2,4-diaminobutyric acid (DAB) in a molar ratio of 0.7:1.0:0.4:1.3. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, Gly, D-Hyg and D- and L-DAB. Taking into account the above results and the fact that all known B-type peptidoglycans contain an L-isomeric amino acid in position 3 of the peptide subunit (DSMZ, 2001), it was concluded that the cell-wall peptidoglycan of strain NBRC 16403^T is of the B2 γ type (Schleifer & Kandler, 1972; Sasaki *et al.*, 1998) in which glutamic acid is almost completely replaced by Hyg. Similarly, peptidoglycan samples from *H. ginsengi* NBRC 104580^T and *H. solani* NBRC 106740^T contained Ala, Gly, Hyg and DAB in molar ratios of 0.6:1.0:0.4:1.1 and 0.8:1.0:0.5:1.1, respectively, and both strains also possessed D-Ala, Gly, D-Hyg and D- and L-DAB in their peptidoglycan. Therefore, it was concluded that strain

NBRC 16403^T had the same peptidoglycan composition as the two reference strains. The major cellular fatty acids of strain NBRC 16403^T were anteiso- $C_{15:0}$ (64.6 %), iso- $C_{16:0}$ (16.3 %) and anteiso- $C_{17:0}$ (16.1 %) (Table S2). The menaquinone system contained MK-10 and MK-11 (molar ratio 3:2). The principal polar lipids were diphosphatidylglycerol, phosphatidylglycerol and one glycolipid, along with minor or trace amounts of two additional glycolipids and three unidentified polar lipids (Fig. S2). The DNA G+C contents of strain NBRC 16403^T, *H. ginsengi* NBRC 104580^T and *H. solani* NBRC 106740^T were 71.0, 69.1 and 71.1 mol%, respectively.

The microplate-hybridization method developed by Ezaki *et al.* (1988, 1989) was used to determine DNA–DNA relatedness. DNA–DNA relatedness between strain NBRC 16403^T and *H. solani* NBRC 106740^T and *H. ginsengi* NBRC 104580^T was 4–9 and 6–14 %, respectively, which were well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the assignment of bacterial strains to different genomic species.

The results of phylogenetic analysis based on 16S rRNA gene sequences suggested that strain NBRC 16403^T belonged to the genus *Herbiconiux*. Also, the chemotaxonomic characteristics of the strain generally corresponded to those of the genus *Herbiconiux* except for differences in the major fatty acids and menaquinones. It has been reported that *H. ginsengi*, *H. solani* and *H. moechotypicola* contain cyclohexyl- $C_{17:0}$ as their major fatty acid (Qiu *et al.*, 2007; Behrendt *et al.*, 2011; Kim *et al.*, 2012). In this study, an unidentified fatty acid (identified as summed feature 7 in the MIDI Sherlock system) was detected in *H. ginsengi* NBRC 104580^T and *H. solani* NBRC 106740^T,

which was identified as cyclohexyl- $C_{17:0}$ by the mass spectrum (m/z 282 [M^+], cyclohexyl- $C_{17:0}$ methyl ester) and the retention time. However, this fatty acid was not detected in strain NBRC 16403^T. Similarly, some species of the genus *Curtobacterium* contain cyclohexyl- $C_{17:0}$ but others do not (Suzuki & Komagata, 1983; Aizawa *et al.*, 2007). Thus, the presence of cyclohexyl- $C_{17:0}$ may be considered as variable within the genus *Herbiconiux* as well. In addition, strain NBRC 16403^T contained two major menaquinones, MK-10 and MK-11, whereas *H. ginsengi*, *H. solani* and *H. moechotypicola* have MK-11 as the predominant menaquinone and MK-10 as a minor component (Qiu *et al.*, 2007; Behrendt *et al.*, 2011; Kim *et al.*, 2012). Hence, the description of the genus *Herbiconiux* should be emended to reflect these variations in the fatty acid and menaquinone compositions.

DNA–DNA relatedness between strain NBRC 16403^T and the two reference strains was low and the isolate also differed from the reference strains in some phenotypic characteristics (Table 1). Therefore, strain NBRC 16403^T represents a novel species of the genus *Herbiconiux*, for which the name *Herbiconiux flava* sp. nov. is proposed.

Emended description of the genus *Herbiconiux* Behrendt *et al.* 2011

The description is as given by Behrendt *et al.* (2011) with the following modifications. The predominant menaquinone is MK-11; major amounts of MK-10 may also be present. Cellular fatty acids mainly comprise iso- and anteiso-branched fatty acids, with anteiso- $C_{15:0}$ as a major component. Cyclohexyl- $C_{17:0}$ may be present. The DNA G + C content is 69–71 mol%.

Description of *Herbiconiux flava* sp. nov.

Herbiconiux flava (fla'va. L. fem. adj. *flava* golden yellow, referring to the colour of the colonies).

Cells are Gram-stain-positive, non-motile and non-endospore-forming rods ($0.5\text{--}0.7 \times 1.0\text{--}2.0$ μm). Cells fragment via septa formation; bud-like cells also often occur. Cells in older cultures tend to be shorter and coccoid cells may be present; however, no distinct rod–coccus life cycle was observed under the test conditions. Colonies are circular, smooth and pale yellow. Catalase-positive and oxidase-negative. No anaerobic growth. Grows at 5–37 °C (optimum 28 °C) and pH 5.0–9.0 (optimum pH 7.0). Grows with 0–3 % (w/v) NaCl (optimum growth in the absence of NaCl), but not with 5 % NaCl. Acid is produced from cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inositol, maltose, D-mannose, D-mannitol, salicin, sucrose and D-xylose. Esterase (C4), esterase lipase (C8), leucine arylamidase, α - and β -galactosidases and α -glucosidase are present, but acid and alkaline phosphatases, lipase (C14), trypsin, chymotrypsin, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -fucosidase, α -mannosidase, pyrrolidonyl arylamidase, arginine dihydrolase, lysine and ornithine

decarboxylases and tryptophan deaminase are absent. H_2S and indole are not produced. Aesculin is hydrolysed, but gelatin and urea are not hydrolysed. Nitrate is not reduced. The peptidoglycan is of the B2 γ type, with D- and L-DAB as the diagnostic diamino acids, and contains *threo*-3-hydroxyglutamic acid. The predominant menaquinones are MK-10 and MK-11. The cell-wall sugars are galactose, mannose and rhamnose. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and one glycolipid. The major cellular fatty acids are anteiso- $C_{15:0}$, iso- $C_{16:0}$ and anteiso- $C_{17:0}$. The DNA G + C content of the type strain is 71.0 mol%.

The type strain, NBRC 16403^T (=VKM Ac-2058^T), was isolated from the phyllosphere of a sedge from Belgorod region, Russia.

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