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Orbus sasakiae sp. nov., a bacterium isolated from the gut of the butterfly *Sasakia charonda*, and emended description of the genus *Orbus*

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A novel Gram-stain-negative, facultatively anaerobic, non-motile and coccus-shaped bacterium, strain C7^T, was isolated from the gut of the butterfly *Sasakia charonda*. Strain C7^T grew optimally at 20–25 °C, at pH 7–8 and with 1 % (w/v) NaCl. The strain was negative for oxidase activity but positive for catalase activity. The 16S rRNA gene sequences of strain C7^T and *Orbus hercynius* CN3^T shared 96.8 % similarity. The major fatty acids identified were $C_{14:0}$, $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 2 (comprising $C_{14:0}$ 3-OH/iso-$C_{16:1}$). The major respiratory quinone was ubiquinone-8 (Q-8). The polar lipids of strain C7^T were phosphatidylethanolamine, phosphatidylglycerol, an unidentified phospholipid and two unidentified aminophospholipids. The G+C content of the genomic DNA extracted from strain C7^T was 32.1 mol%. Taken together, the phenotypic, genotypic and phylogenetic analyses indicate that strain C7^T represents a novel species of the genus *Orbus*, for which the name *Orbus sasakiae* sp. nov. is proposed. The type strain is C7^T (=KACC 16544^T=JCM 18050^T). An emended description of the genus *Orbus* is provided.

Orbus, a genus in the class *Gammaproteobacteria*, was first introduced by Volkmann *et al.* (2010). At the time of writing, *Orbus hercynius* is the only recognized species within this genus and was originally isolated from the faeces of wild boars in Germany. The major fatty acids of the genus *Orbus* are monounsaturated, even-numbered, straight-chain C_{18} ($C_{18:1}\omega7c$) and saturated, even-numbered, straight-chain C_{16} ($C_{16:0}$) components (Volkmann *et al.*, 2010). Here, we isolated an *Orbus*-like strain, designated C7^T, from butterfly gut. Based on taxonomic characterization and phylogenetic analysis, we propose that strain C7^T represents a novel species belonging to the genus *Orbus*.

Strain C7^T was isolated from the gut of the butterfly *Sasakia charonda* in South Korea. Cells were isolated by the standard dilution-plating method and were incubated under aerobic conditions at 20 °C on trypticase soy agar (TSA; Difco). To obtain pure cultures, a single colony was repeatedly transferred. Gram reactions were performed using a Gram staining kit (bioMérieux) according to the manufacturer's instructions. Cell morphology and Gram staining of strain C7^T were observed using a light microscope (ECLIPSE 50*i*; Nikon). Motility was examined by the method of Tittsler & Sandholzer (1936). Growth under anaerobic conditions was monitored after 7 days incubation in an anaerobic chamber ($N_2/H_2/CO_2$, 90 : 5 : 5) at 37 °C on TSA medium. The growth of strain C7^T on CIN agar medium (CIN; BBL) was tested. Based on the experiments described above, strain C7^T was determined to be coccoid (0.5–1.0 µm in diameter), Gram-stain-negative, facultatively anaerobic and non-motile. Growth of strain C7^T was observed on CIN agar. All experiments were performed in triplicate.

Growth at different temperatures (4, 15, 20, 25, 30, 37, 45 and 65 °C), pH values (pH 4.0–11.0, at intervals of 1.0 pH unit) and NaCl concentrations (0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 %, w/v) was tested in trypticase soy broth (TSB; Difco). pH was adjusted by adding the following buffers: 10 mM MES ($C_6H_{13}NO_4S$) (for pH 4, 5 and 6), 10 mM TAPS ($C_7H_{14}NO_6S$) (for pH 7, 8 and 9) or 10 mM Na_2HPO_4 (for pH 10 and 11). The strains were cultivated in TSB at 20 °C for all experiments, unless stated otherwise. After incubation for 24 h, 48 h or 7 days, the turbidity of the cultures was measured as the optical density at 600 nm (OD_{600}) using a spectrophotometer (SYNERGY MX; BioTek). Strain C7^T grew with 0–5 % (w/v) NaCl, at

Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C7^T is JN561614.

A supplementary figure is available with the online version of this paper.

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4–37 °C and at pH 6.0–10.0, while optimal growth was observed in the presence of 1 % (w/v) NaCl, and at 20–25 °C and pH 7.0–8.0.

For phylogenetic analysis, the 16S rRNA gene of strain C7^T was amplified by colony PCR using PCR pre-mix (iNtRon Biotechnology) and two universal bacterial-specific primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Baker *et al.*, 2003). The PCR product was purified (QIAquick PCR Purification kit) and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. The primers for the sequencing were as follows: 8F, 338F (5′-ACTCCTACGGGAGGCAGCAG-3′), 518R (5′-ATTACCGCGGCTGCTGG-3′) and 1492R (Weisburg *et al.*, 1991). Reaction mixtures were analysed using an automated DNA analyser system (PRISM 3730XL DNA analyser; Applied Biosystems). Fragments of the 16S rRNA gene sequence were assembled with SeqMan (DNASTAR), resulting in an almost full-length 16S rRNA gene sequence. A comparison of different 16S rRNA gene sequences was performed using the EzTaxon server (Chun *et al.*, 2007). As a result, strain C7^T displayed the highest level of 16S rRNA gene sequence similarity to *O. hercynius* CN3^T (96.8 %), while 16S rRNA gene sequence similarity with the type strains of other species was less than 90.4 %. The 16S rRNA gene was aligned with the most closely related species using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994). The MEGA5 software program (Tamura *et al.*, 2011) was used to determine the phylogenetic relationship between strain C7^T and its most closely related type strains. A phylogenetic tree based on the aligned 16S rRNA gene sequences was constructed using the maximum-parsimony (Kluge & Farris, 1969), maximum-likelihood (Felsenstein, 1981) and neighbour-joining methods (Saitou & Nei, 1987), with 1000 randomly selected bootstrap replicates. Phylogenetic analysis showed that strain C7^T clustered with species of the genus *Orbus* (Fig. 1). *O. hercynius* DSM 22228^T was obtained from the German Culture Collection (DSMZ) and used as a reference strain for comparison.

Biochemical analysis of strain C7^T and *O. hercynius* DSM 22228^T cultivated at 20 °C for 48 h in TSB or on TSA medium was performed. Catalase and oxidase activities were determined by observing bubble production in 3 % (v/v) hydrogen peroxide solution and reactivity with 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. The ability to assimilate a sole carbon source was determined using GN2 MicroPlates with GN/GP inoculating fluid (Biolog), according to the manufacturer's instructions. Acid production from carbohydrates was determined using API 50CH test strips with API 50CHB/E medium (bioMérieux), according to the manufacturer's instructions. Enzyme activities were determined using API20NE and API ZYM test strips (bioMérieux). As shown in Table 1, strain C7^T and *O. hercynius* DSM 22228^T were biochemically distinct.

For chemotaxonomic analyses, strain C7^T and *O. hercynius* DSM 22228^T were cultivated on TSA or in TSB medium at

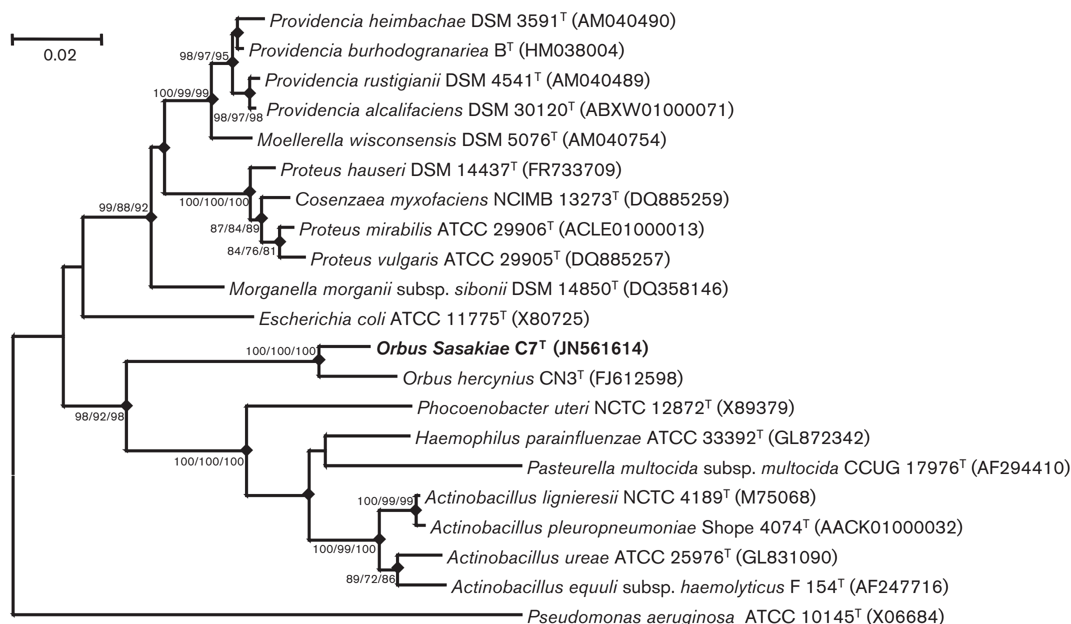

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic position of strain C7^T. Filled diamonds represent identical branches that are present in phylogenetic trees generated using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. Numbers at nodes represent bootstrap values (>70 %) based on 1000 replications. *Pseudomonas aeruginosa* ATCC 10145^T served as an outgroup. Bar, 0.02 accumulated changes per nucleotide.

Table 1. Differential biochemical characteristics between strain C7^T and *Orbus hercynius* DSM 22228^T

All data were obtained from the current study. Both strains assimilated *N*-acetyl-D-glucosamine, D-fructose, gentiobiose, α-D-glucose, D-mannose, sucrose, formic acid and D-glucose 6-phosphate (Biolog GN2). Both strains produced acid from D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, arbutin, aesculin, salicin, sucrose, raffinose, gentiobiose, D-tagatose, gluconate, 2-keto-gluconate and 5-keto-gluconate (API 50CHB). Both strains were positive for alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM). Both strains were positive for the reduction of nitrates to nitrites, D-glucose fermentation and aesculin ferric citrate hydrolysis (API 20NE). +, Positive; −, negative.

Characteristic	C7^T	*O. hercynius* DSM 22228^T
Oxidase activity	−	+
Assimilation of:		
Dextrin	+	−
Tween 40	+	−
Tween 80	+	−
N-Acetyl-D-galactosamine	+	−
Cellobiose	+	−
L-Fucose	+	−
D-Galactose	+	−
α-Lactose	+	−
Lactulose	+	−
Melibiose	+	−
β-Methyl D-glucoside	+	−
D-Psicose	+	−
Raffinose	+	−
Trehalose	−	+
Pyruvic acid methyl ester	+	−
Citric acid	+	−
α-Ketobutyric acid	+	−
Bromosuccinic acid	+	−
L-Threonine	+	−
Uridine	+	−
α-D-Glucose 1-phosphate	+	−
Enzyme activity		
Urease	−	+
β-Galactosidase (PNPG hydrolysis)	+	−
Esterase	+	−
N-Acetyl-β-glucosaminidase	+	−
Acid production from:		
D-Galactose	+	−
L-Sorbose	+	−
Dulcitol	−	+
Inositol	+	−
D-Mannitol	−	+
Amygdalin	+	−
Cellobiose	+	−
Lactose	+	−
Melibiose	+	−
Trehalose	−	+

20 °C for 48 h. Physiological ages of strain C7^T and *O. hercynius* DSM 22228^T were standardized as exponential phase. The cellular fatty acids of strain C7^T and *O. hercynius* DSM 22228^T were extracted as described by the Sherlock Microbial Identification System (MIDI, 1999), analysed by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sherlock software 4.0) (Sasser, 1990) based on the TSBA 40 database. The isoprenoid quinone was extracted from freeze-dried cells using chloroform/methanol (2 : 1, v/v) (Collins & Jones, 1981a) and was identified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and reversed-phase HPLC (Collins & Jones, 1981b) using a Thermo ODS HYPERSIL (250 × 4.6 mm) column. Polar lipids of strain C7^T and *O. hercynius* DSM 22228^T were extracted according to the procedures described by Xin *et al.* (2000), separated by two-dimensional TLC on silica gel plates (Merck) and detected by spraying the plate with appropriate detection reagents (Tindall, 1990): 10 % ethanolic molybdatophosphoric acid reagent for total lipids, ninhydrin reagent for amino-containing lipids, Zinzadze reagent for phospholipids and α-naphthol reagent for glycolipids. To identify the phospholipids of strain C7^T and *O. hercynius* DSM 22228^T, four standard phospholipid compounds (Sigma) were used: phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphoethanolamine (PE) and diphosphatidylglycerol (DPG). Standard compounds and phospholipids of strain C7^T were separated by one-dimensional TLC on a silica gel plate (Merck) using chloroform/acetic acid/methanol/water (50 : 6 : 6 : 1, v/v) as the solvent, and Zinzadze reagent was sprayed to detect each spot. The major fatty acids (>10 % of the total fatty acids) of strain C7^T in the exponential phase were $C_{18:1}\omega7c$, $C_{16:0}$, summed feature 2 (comprising $C_{14:0}$ 3-OH/iso-$C_{16:1}$) and $C_{14:0}$. The cellular fatty acid profile of strain C7^T was qualitatively similar to that of *O. hercynius* DSM 22228^T (Table 2). The polar lipids of strain C7^T were PE, PG, an unidentified phospholipid (PL), and two unidentified aminophospholipids (APL1 and 2). The polar lipids of *O. hercynius* DSM 22228^T were PE, PG and two unidentified aminophospholipids (APL1 and 2) (Fig. S1 in IJSEM Online). Strain C7^T contained Q-8 as the major quinone, as with *O. hercynius*, and MK-8(H_4) as a minor quinone.

Extraction of genomic DNA from strain C7^T was performed according to the method described by Rochelle *et al.* (1992). The DNA G + C content was determined by the fluorimetric method using SYBR Gold I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). The genomic DNA of *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Ruminococcus obeum* ATCC 29174^T was used as references for calibration. The G + C content of the genomic DNA extracted from strain C7^T was 32.1 mol%.

Based on phenotypic, phylogenetic and genotypic characteristics, it is clear that strain C7^T represents a novel species of the genus *Orbus*, for which the name *Orbus sasakiae* sp. nov. is proposed.

Table 2. Fatty acid compositions (%) of strain C7^T and *Orbus hercynius* DSM 22228^T

All data were obtained from the current study. Values are percentages of the total fatty acids. Fatty acids that represented <0.5 % in all species were omitted. tr, Trace amount (<0.5 %); −, not detected.

Fatty acid	C7^T	*O. hercynius* DSM 22228^T
Saturated		
$C_{10:0}$	tr	tr
$C_{12:0}$	0.6	0.6
$C_{14:0}$	11.2	11.7
$C_{16:0}$	28.0	31.2
$C_{18:0}$	tr	tr
Unsaturated		
$C_{14:1}\omega5c$	−	tr
$C_{16:1}\omega5c$	tr	tr
$C_{18:1}\omega5c$	−	tr
$C_{18:1}\omega7c$	29.4	28.0
Branched		
$C_{12:0}$ 3-OH	tr	tr
iso-$C_{17:0}$ 3-OH	−	tr
$C_{19:0}$ 10 methyl	−	tr
$C_{19:0}$ cyclo $\omega8c$	−	tr
Summed features*		
2	19.9	13.7
3	9.3	12.7
5	−	tr
Unknown		
ECL 14.502	tr	−

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I; summed feature 3 consisted of $C_{16:1}\omega7c$/iso-C_{15} 2-OH; summed feature 5 consisted of $C_{18:2}\omega6, 9c$/anteiso-$C_{18:0}$.

Emended description of the genus *Orbus*

The description of the genus *Orbus* is as described previously by Volkmann *et al.* (2010) with the following change: oxidase activity is species-dependent; the major polar lipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and two unidentified aminophospholipids.

Description of *Orbus sasakiae* sp. nov.

Orbus sasakiae [sa.sa'ki.ae. N.L. gen. n. *sasakiae* of *Sasakia*, isolated from the midgut of a butterfly (*Sasakia charonda* in the order *Lepidoptera*)].

Strain C7^T cells are non-motile, Gram-stain-negative, facultatively anaerobic and coccoid (0.5–1.0 µm in diameter). After cultivation at 20 °C for 3 days on TSA medium, the colonies are opaque, ivory, smooth, circular, raised and 1.0–2.0 mm in diameter. Grows at 4–37 °C, in the presence of 0–5 % (w/v) NaCl and at pH 6.0–10.0. The optimal growth conditions are 20–25 °C, 1 % NaCl and pH 7.0–8.0. Oxidase-negative and catalase-positive. Assimilates dextrin, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-lactose, lactulose, D-mannose, melibiose, methyl *β*-D-glucoside, D-psicose, raffinose, sucrose, pyruvic acid methyl ester, citric acid, formic acid, α-ketobutyric acid, bromosuccinic acid, L-threonine, uridine, α-D-glucose 1-phosphate and D-glucose 6-phosphate, but not α-cyclodextrin, glycogen, adonitol, L-arabinose, D-arabinose, i-erythritol, *myo*-inositol, maltose, D-mannitol, L-rhamnose, D-sorbitol, trehalose, turanose, xylitol, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, *β*-hydroxybutyric acid, *γ*-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, DL-carnitine, *γ*-aminobutyric acid, urocanic acid, inosine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol or DL-α-glycerol phosphate (Biolog GN2). Produces acid from D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, inositol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, melibiose, sucrose, raffinose, gentiobiose, D-tagatose, gluconate, 2-keto-gluconate and 5-keto-gluconate, but not from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, methyl *β*-D-xyloside, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, maltose, trehalose, inulin, melezitose, starch, glycogen, xylitol, turanose, D-lyxose, D-fucose, L-fucose, D-arabitol or L-arabitol (API 50CHB/E test strips). The following API ZYM test enzymes are scored as positive: alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-*β*-glucosaminidase. Esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, α-mannosidase and α-fucosidase are negative. Positive for the reduction of nitrates to nitrites, D-glucose fermentation, aesculin ferric citrate hydrolysis and *β*-galactosidase, but negative for the reduction of nitrates to nitrogen, indole production, L-arginine dihydrolase, urease and protease on API 20NE test strips. The major fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, summed feature 2 (consisting of $C_{14:0}$ 3-OH/iso-$C_{16:1}$) and $C_{14:0}$. The following polar lipids are detected: PE, PG, an unidentified phospholipid and two unidentified aminophospholipids. The major ubiquinone is Q-8.

The type strain is C7^T (=KACC 16544^T=JCM 18050^T), which was isolated from the gut of the butterfly *Sasakia*

charonda in South Korea. The DNA G + C content of the type strain is 32.1 mol%.

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