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TAXONOMIC DESCRIPTION

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MICROBIOLOGY SOCIETY

Sphingomonas aliaeris sp. nov., a new species isolated from pork steak packed under modified atmosphere

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Abstract

Species belonging to the genus *Sphingomonas* have been isolated from environments such as soil, water and plant tissues. Many strains are known for their capability of degrading aromatic molecules and producing extracellular polymers. A Gram-stain-negative, strictly aerobic, motile, red-pigmented, oxidase-negative, catalase-positive, rod-shaped strain, designated DH-S5^T, has been isolated from pork steak packed under CO_2-enriched modified atmosphere. Cell diameters were 1.5×0.9 µm. Growth optima were at 30 °C and at pH 6.0. Phylogenetic analyses based on both complete 16S rRNA gene sequence and whole-genome sequence data revealed that strain DH-S5^T belongs to the genus *Sphingomonas*, being closely related to *Sphingomonas alpina* DSM 22537^T (97.4% gene sequence similarity), followed by *Sphingomonas qilianensis* X1^T (97.4%) and *Sphingomonas hylomeconis* GZJT-2^T (97.3%). The DNA G+C content was 64.4 mol%. The digital DNA–DNA hybridization value between the isolate strain and *S. alpina* DSM 22537^T was 21.0% with an average nucleotide identity value of 77.03%. Strain DH-S5^T contained Q-10 as the ubiquinone and major fatty acids were $C_{18:1}$ cis 11 (39.3%) and $C_{16:1}$ cis 9 (12.5%), as well as $C_{16:0}$ (12.1%) and $C_{14:0}$ 2-OH (11.4%). As for polar lipids, phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, dimethylphosphatidylethanolamine and sphingoglycolipid could be detected, alongside traces of monomethylphosphatidylethanolamine. Based on its phenotypic, chemotaxonomic and phylogenetic characteristics, strain DH-S5^T (=DSM 110829^T=LMG 31606^T) is classified as a representative of the genus *Sphingomonas*, for which the name *Sphingomonas aliaeris* sp. nov. is proposed.

INTRODUCTION

Modified atmosphere packaging of food is used to both reduce microbial growth and extend the shelf life of food. Some bacterial taxa associated with food spoilage, such as members of the family *Pseudomonadaceae* and *Bacillaceae*, are inhibited by high CO_2 levels in packaging, whereas others, like members of the *Lactobacillaceae* or *Brochothrix thermosphacta*, can still grow under these conditions [1, 2]. In order to assess the impact of modified atmospheres with elevated CO_2 concentrations on the food-associated microbial community, the selective effect of this treatment must be analysed to exclude the enrichment of microbial populations with elevated risk potential. In the course of the characterization of microbial isolates from food packed under elevated CO_2 concentrations, one strain was preliminarily allocated to the genus *Sphingomonas*. However, until now, species of this

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Keywords: *Alphaproteobacteria*; food spoilage ; modified atmosphere packaging; novel species; *Sphingomonadaceae*; *Sphingomonas*.

Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; DGTS, 1,2-diacylglyceryl-3-O-4′-(*N*,*N*,*N*-trimethyl)-homoserine; DMPE, dimethylphosphatidylethanolamine; DPG, diphosphatidylglycerol; GBDP, genome BLAST distance phylogeny; MHD, monohexosyldiacylglycerol; MMPE, monomethylphosphatidylethanolamine; NB, nutrient broth; ONT, Oxford Nanopore Technologies; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SGL, sphingoglycolipid; TSA, tryptic soy agar; TYGS, Type (Strain) Genome Server.

GenBank accessions for the 16S rRNA gene sequences are MT821129 (*Sphingomonas aliaeris* DH-S5^T) and MT821524 (*Sphingomonas alpina* DSM 22537^T). Genome sequences have been deposited at DDBJ/ENA/GenBank under the accessions CP061035–CP061037 (*Sphingomonas aliaeris* DH-S5^T) and CP061038 (*Sphingomonas alpina* DSM 22537^T). The versions described in this paper are versions CP061035.1, CP061036.1, CP061037.1 and CP061038.1.

One supplementary figure is available with the online version of this article.

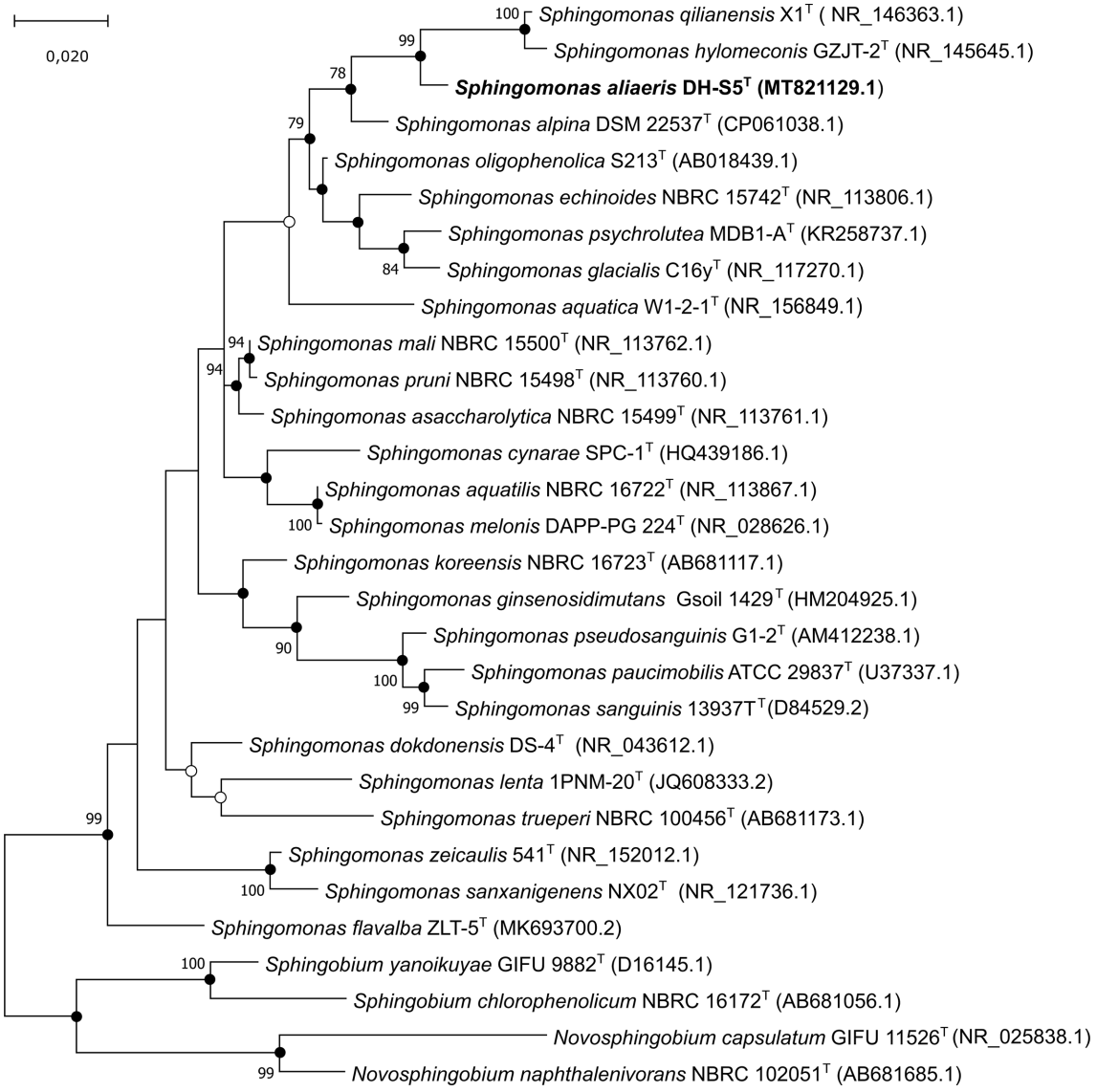

Fig. 1. The evolutionary history was inferred by using the maximum-likelihood method and Tamura's three-parameter model [21]. Bootstrap values >70% are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter=0.7019)). All positions with less than 90% site coverage were eliminated. There were a total of 1395 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [19]. Filled circles indicate that the corresponding branches were recovered with maximum-likelihood, maximum-parsimony and neighbour-joining method. Non-filled circles indicate that the corresponding branch was recovered in at least one of the two named methods (next to maximum-likelihood). Rooted outgroups were *Novosphingobium capsulatum* GIFU 11526T and *Novosphingobium naphthalenivorans* NBRC 102051T. Bar represents 0.02 substitutions per site.

genus were not associated with food matrices nor environments with high CO_2 levels.

The genus *Sphingomonas*, which was defined by Yabuuchi *et al.* in 1990 and lastly emended by Feng *et al.* in 2017, belongs to the family of *Sphingomonadaceae*, being part of the *Alphaproteobacteria* [1, 2]. About 30 years after its initial description, the genus currently consists of 135 species with validly published names, which have been isolated from various surroundings, such as plant tissues, water, contaminated and uncontaminated soils, and subsurface sediments [3]. The aerobic and Gram-stain-negative sphingomonads are characterized by the presence of ubiquinone Q-10 and the name-providing sphingoglycolipids [1]. 2-Hydroxy fatty acids, especially $C_{14:0}$ 2-OH, are abundant in all species [4]. *Sphingomonas* species are generally known in the field of bioremediation for their ability to degrade aromatic compounds and other refractory pollutants [3]. *Sphingomonas paucimobilis*, the type species of the genus, was described to degrade malachite green, a triphenylmethane dye released in textile industry processes [5]. Furthermore, other compounds highly toxic to humans

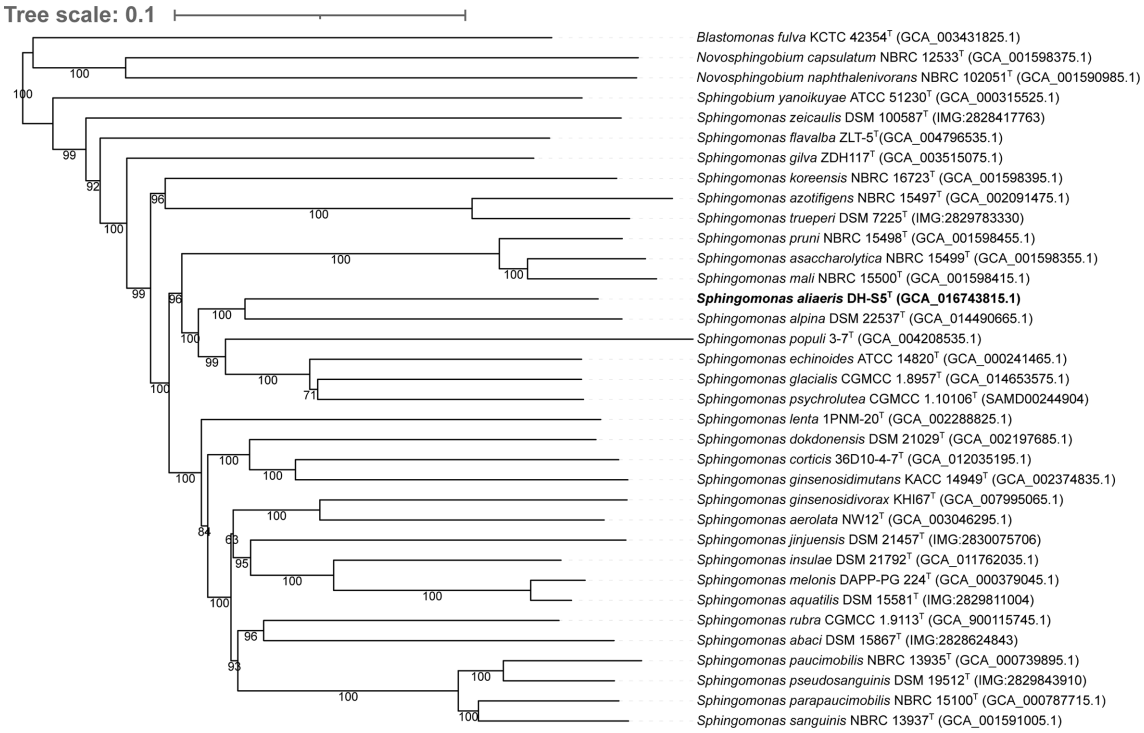

Fig. 2. Tree inferred with FastME 2.1.6.1 from whole-proteome-based GBDP distances. The branch lengths are scaled via GBDP distance formula d5. Branch values are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 90.4%. The tree was rooted at midpoint. Genome sequences obtained from the JGI/IMG database are provided by their IMG object id (indicated by the prefix 'IMG'), whereas sequences retrieved from GenBank are specified either by their assembly accessions (indicated by the prefix 'GCA') or biosample accession (indicated by the prefix 'SAM').

are reported to be degraded by different *Sphingomonas* strains [3, 6]. Furthermore, this ability is distributed among species of the closely related genera *Sphingobium* [7], *Novosphingobium* [8] and *Sphingopyxis* [9], which were separated from the genus *Sphingomonas* in 2001 due to the results of phylogenetic and chemotaxonomic analyses [10].

Another outstanding characteristic of this genus is the ability to produce so-called sphingans, bacterial exopolysaccharides. Among these polymers is gellan gum, which is used in the food industry as a stabilizing or thickening agent [11]. It is known as GelRite and, due to its higher melting point, it is used as an alternative to agar in microbial media for cultivating thermophilic micro-organisms [12]. Recently, other novel sphingans have been isolated and described [13] and are of great interest for industrial processes, such as for use as coatings or edible membranes for food packaging [14]. Additionally, only rare cases of infections caused by *Sphingomonas* strains, mainly *S. paucimobilis* [15], have been reported, making it less complicated to use them for biotechnological applications.

ISOLATION AND ECOLOGY

Strain DH-S5^T was obtained from pork steak samples packed under modified atmosphere. The packed sample was stored for 40 days at 4 °C. The modified atmosphere at time of packaging consisted of 75% O_2 and 20% CO_2, indicating that DH-S5^T is a psychrotolerant organism which endures concentrations of CO_2 500 times higher than in Earth's atmosphere. Ten grams of the sample was cut and homogenized with 90 ml Ringer's solution (Merck). The suspension (100 µl) was plated on plate count agar (VWR International), from which the isolate was recovered. Reference strain *Sphingomonas alpina* DSM 22537^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). For further experiments the strains were cultivated on Reasoner's 2A (R2A) agar (Merck) and nutrient broth (NB; 5 g meat extract, 5 g peptone and 2.5 g NaCl *ad* 1000 ml H_2O), as reference strain *S. alpina* DSM 22537^T did not grow on tryptic soy agar (TSA; Merck). The strains were subsequently stored at −80 °C using Cryobank (Mast Group).

SINGLE-GENE AND GENOME-SCALE PHYLOGENIES

Extraction of genomic DNA for 16S rRNA gene sequencing was performed with the DNeasy Blood and Tissue Kit (Qiagen) according to the instructions of the manufacturer. The 16S rRNA gene was amplified using the universal bacterial primers GM3 8F (5′-AGAGTTTGATCMTGGC-3′) and GM4 1507R (5′-TACCTTGTTACGACTT-3′) [16]. The PCR products were purified using the QIAquick PCR Purification

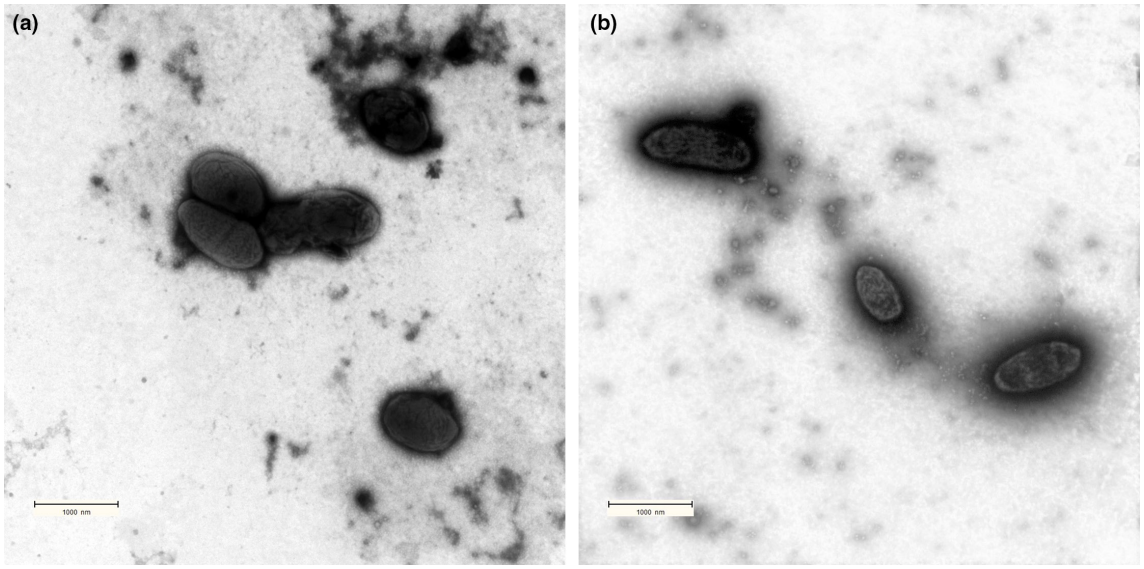

Fig. 3. Transmission electron microscope images of (a) strain DH-S5^T and (b) *Sphingomonas alpina* DSM 22537^T grown on R2A agar at 30 and 25 °C, respectively.

Kit (Qiagen) according to the manufacturer's instructions. The DNA was quantified with an Epoch microplate spectrophotometer (BioTek) and Sanger sequenced by Seqlab (Göttingen, Germany). The sequencing primers used were GM1F (5′-CCAGCAGCCGCGGTAAT-3′) and GM8R (5′-GGACTACCAGGGTATCTAAT-3′). Sequences were manually checked and edited with Chromas software (version 2.6.6, Technelysium).

The nearly complete 16S rRNA gene sequence of 1404 bp was compared with type strains of validly published species in GenBank using BLAST version 2.11.0 (National Center for Biotechnological Information [17]). Highest similarity of 97.4% was shown to *S. alpina* S8-3^T (CP061038.1) and 97.4% to *Sphingomonas qilianensis* X1^T (NR_146363.1), followed by 97.2% to *Sphingomonas hylomeconis* GZJT-2^T (NR_145645.1), 96.5% to *Sphingomonas psychrolutea* MDB1-A^T (NR_137233.1) and 96.4% to *Sphingomonas glacialis* C16y^T (NR_117270.1). Meier-Kolthoff *et al.* [18] proposed 98.8% similarity in the 16S rRNA gene sequence as a threshold for distinction between two species, which means that isolate DH-S5^T is not a member of these species.

The phylogenetic tree of 16S rRNA gene sequences of strain DH-S5^T and type strains of the genera *Sphingomonas*, *Sphingobium* and *Novosphingobium* was reconstructed using MEGA X (version 10.1.8 [19]). The sequences were aligned using MUSCLE multiple sequence alignment for DNA [20], which is implemented in MEGA X software. A maximum-likelihood phylogenetic tree was reconstructed using the Tamura three-parameter model [21]. Non-uniformity of evolutionary rates among sites were modelled by using a discrete gamma distribution (+G) with five rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Model parameters were estimated using the 'Find best DNA Models' option and selected according to the lowest Bayesian information criterion scores. Maximum-parsimony and neighbour-joining trees were reconstructed and information about tree topologies was integrated in the maximum-likelihood tree. The tree is given in Fig. 1. All trees were supported by a bootstrap test including 1000 replications.

The complete genomic DNA of DH-S5^T and *S. alpina* DSM 22537^T was purified with the MasterPure Gram Positive DNA Purification Kit (Epicentre) according to the manufacturer's instructions. The DNA was quantified and the sequencing libraries prepared, following the Oxford Nanopore Technologies (ONT) protocol for 'Native barcoding genomic DNA', using the SQK-LSK109 and EXP-NBD104 kits. The samples were sequenced on the ONT GridION X5 system. Basecalling and demultiplexing were performed with ONT MinKNOW (version 3.6.5) and Guppy (version 3.2.10).

Genome assemblies were produced from the sequencing data via the flye assembly tool (version: 2.7b; parameters: '--genome-size 5 m --asm-coverage 50' [22]). The resulting genome sequences were further processed by an initial polishing via the Racon tool (version 1.4.10; parameters: '-m 8 x −6 g −8 w 500' [23]) based on mapping results of ONT reads to the corresponding assemblies using minimap2 (version: 2.17; parameter: '-x map-ont' [24]). This procedure was applied once. Afterwards, Medaka was used for final polishing (version 1.0.3; parameter: '-m r941_min_high_g303'; https://github.com/nanoporetech/medaka). Chimaera check was performed with DECIPHER (version 2.17.1).

To additionally elucidate the evolutionary relatedness of strain DH-S5^T with its closest relatives at the whole-genome level, thus promising better-resolved trees in comparison to phylogenies only based on one to only a few marker genes, a

Table 1. Differentiating characteristics of strain DH-S5^T and the reference strain, as well as strains *Sphingomonas qilianensis* X1^T and *Sphingomonas hylomeconis* GZJT-2^T

Strains: 1, DH-S5^T; 2, *Sphingomonas alpina* DSM 22537^T; 3, *Sphingomonas qilianensis* X1^T; 4, *Sphingomonas hylomeconis* GZJT-2^T. w, Weakly positive; ND, no data available.

Characteristic	1	2	3†	4‡
Pigmentation	Red	Yellow	Light yellow	Yellow
Colony diameter (mm)	<1	1.5–2.0	1.0–1.5	1.0–2.0
Cell diameter (μm)	1.5×0.9	1.9×0.8	1.2–2.0×0.4	1.4–2.2×0.6–0.7
Optimum growth temperature (°C)	30	25	15–30 (range)	25
Growth on TSA	+	−	+	+
Growth on Columbia blood agar	−	+	ND	ND
Growth with 20% CO_2	+	−	ND	ND
Assimilation of:				
L-Arabinose	−	+	+	−
D-Mannitol	+	−	−	−
N-Acetyl-glucosamine	−	+*	−	+
Enzyme activity:				
α-Chymotrypsin	+	−*	−	−
β-Glucuronidase	+	−*	−	w
β-Galactosidase	−	+	+	−
Naphthol-AS-BI-phosphohydrolase	−	+	+	+
β-Glucosidase	−	+	+	+
β-Glucosidase (aesculin hydrolysis)	−	+	+	+

*Data differs from Margesin *et al.* [34].
†Data taken from Piao *et al.* [41].
‡Data taken from Akbar *et al.* [40].

genome-scale phylogenomic analysis was conducted via the Type (Strain) Genome Server (TYGS; https://tygs.dsmz.de) [25], as previously done for a large-scale genome-based taxonomic study of the *Alphaproteobacteria* [26]. Briefly, TYGS was used to conduct two independent GBDP (genome BLAST distance phylogeny) analyses, using either the whole-genome nucleotide sequences or amino acid sequences of the entire proteomes, including strain DH-S5^T and a set of 36 closely related type strains, which were automatically determined by TYGS. The resulting GBDP distances were used to infer balanced minimum-evolution trees via FastME 2.1.6.1 [27]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [28], and visualized with iTOL [29]. The whole-proteome sequence-based GBDP tree (Fig. 2) is considerably better supported than the whole-genome sequence-based GBDP tree (Fig. S1, available in the online version of this article) and reveals a maximally supported phylogenetic placement with the evolutionary distances to the phylogenetic neighbours supporting the status of a distinct species.

GENOME FEATURES

Meier-Kolthoff *et al.* [18] recommended a 16S rRNA gene sequence similarity of 98.8% or above to be the minimal standard for comparing genome sequences, which was not the case for the strains used in this study, as the similarity was lower. 16S rRNA gene Sanger sequences were compared with the ones obtained from the genome sequences using BLAST. G+C content and ANI (average nucleotide identity) values were determined using the EzBioCloud online ANI calculator [30]. Digital DNA–DNA hybridization (dDDH) of strains DH-S5^T and *S. alpina* DSM 22537^T was performed using the Genome-to-Genome Distance Calculator (version 2.1), as described by Meier-Kolthoff *et al.* [31].

The genome sizes of DH-S5^T (CP061035-CP061037) and *S. alpina* DSM 22537^T (CP061038) were 4.26 and 5.20 Mbp, respectively. The genome sequence of DH-S5^T included two plasmids, pSphA1 and pSphA2 (CP061036 and CP061037), consisting of 203 and 142 Kbp, respectively. Plasmid and genome sequences were compared with sequences from type

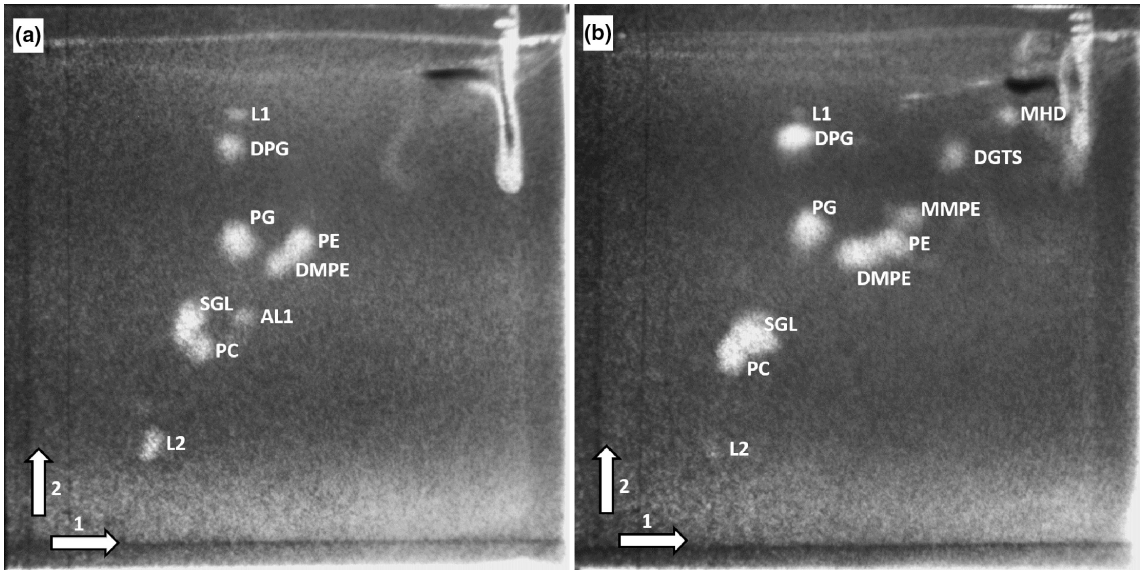

Fig. 4. Two-dimensional thin-layer chromatography showing polar lipid patterns of (a) strain DH-S5^T and (b) *Sphingomonas alpina* DSM 22537^T. Lipids are stained with primuline dye as described by White *et al.* [49]. DGTS, 1,2-diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine; DMPE, dimethylphosphatidylethanolamine; DPG, diphosphatidylglycerol; L1, unidentified lipid 1; L2, unidentified lipid 2; MHD, monohexosyldiacylglycerol; MMPE, monomethylphosphatidyl-ethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SGL, sphingoglycolipid. Dimensions are indicated by arrows.

strains of species with validly published names in GenBank using BLAST, as described above. Protein sequences were determined using the SEED Viewer and RAST (Rapid Annotation using Subsystem Technology, version 2.0 [32]). A plasmid with a high similarity of 98.5% to plasmid pSphA1 has been described for *Sphingobium cloacae* JCM 10874^T (AP017656.1). The highest similarity of 86.0% to plasmid pSphA2 was found to be an unnamed plasmid described for *Sphingomonas insulae* KCTC 12872^T (CP048421.1). Large plasmids have been found in various members of the family *Sphingomonadaceae* by several authors and are often associated with the capability of degrading aromatic molecules [33]. In the case of the plasmids found in strain DH-S5^T mainly genes related to membrane transport (cation transporters and type IV secretion system) and resistance to toxic compounds, such as copper, cobalt, zinc, cadmium and chromium, were identified. The 16S rRNA gene Sanger sequence of strain DH-S5^T included two base deletions in comparison to the ONT-based sequence. For strain *S. alpina* DSM 22537^T one deletion in the Sanger sequence could be detected. G+C content of the genome including plasmids was 64.4 mol% for DH-S5^T and 64.0% for *S. alpina* DSM 22537^T. Margesin *et al.* [34] described a highly similar content of 64.1% for *S. alpina* S8-3^T, Takeuchi *et al.* [10] described levels of 62–68% for the whole genus. The dDDH value between strain DH-S5^T and *S. alpina* DSM 22537^T was 21.0% and the ANI value was 77.03%. The proposed species boundaries for dDDH and ANI values are 70% and 95–96%, respectively [35, 36]. Therefore, based on the (phylo-)genomic data, DH-S5^T represents a novel species of the genus *Sphingomonas*.

PHYSIOLOGY AND CHEMOTAXONOMY

Physiological and chemotaxonomic characteristics of strain DH-S5^T and *S. alpina* DSM 22537^T were determined in addition to the phylogenetic and phylogenomic analyses. Colony morphology was assessed after incubation on R2A agar for 3 days at 30 °C. Additionally, growth on Columbia blood agar (Oxoid) and TSA was tested. Growth parameters were evaluated after incubation in NB by measuring the optical density at 600 nm after 24 h (for temperature optima) or 72 h, respectively. Tolerance range and optima for growth temperatures (2, 3, 5, 10, 15, 20, 25, 30, 33, 35 and 37 °C), NaCl concentrations (0, 0.25, 2, 4, 6, 8 and 10 %) and different pH values (4–10, in intervals of 1 pH unit) have been analysed. Acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0–8.0) and glycine buffer (pH 9.0 and 10.0) were used to adjust the pH in NB media, as previously described [37]. Anaerobic growth was tested on R2A agar in an anaerobic jar by using the Anaerocult system (Merck). Growth under modified atmosphere packaging was assessed by placing inoculated R2A agar petri dishes in a polypropylene tray (ES-Plastic), which was sealed with polyethylene foil (Südpack) by a Multivac T200 traysealer (Multivac). The most common gas composition for meat products [38], consisting of 80% O_2 and 20% CO_2, was used. The trays were incubated at 10 °C for 4 weeks after which growth was evaluated. Gas atmospheres were analysed with an Oxybaby M+ O_2/CO_2 gas analyser (WITT-Gasetechnik). Biofilm formation was analysed as previously described by Kolari *et al.* [39]. Cell morphology and dimensions, as well as motility and the presence of endospores were examined using a Zeiss Axio Observer microscope AxioScope.A1 (Carl Zeiss),

Table 2. Fatty acid patterns, detected quinones and polar lipid profiles of type strain DH-S5^T and reference strain, as well as *Sphingomonas qilianensis* X1^T and *Sphingomonas hylomeconis* GZJT-2^T

Strains: 1, DH-S5^T; 2, *Sphingomonas alpina* DSM 22537^T; 3, *Sphingomonas qilianensis* X1^T; 4, *Sphingomonas hylomeconis* GZJT-2^T; DGTS, 1,2-diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine; DMPE, dimethylphosphatidylethanolamine; DPG, diphosphatidylglycerol; MHD, monohexosyldiacylglycerol; MMPE, monomethylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SGL, sphingoglycolipid; ND, Not detected.

	1	2	3*	4†
Fatty acids				
$C_{14:0}$	1.5	1.8	ND	1.2
$C_{15:0}$	3.3	ND	ND	ND
$C_{14:0}$ 2-OH	11.4	15.6	11.1	7.5
$C_{16:1}$ cis 9	12.5	5.0	19.1	17.7‡
$C_{16:1}$ cis 11	2.0	0.8	1.2	2.7
$C_{16:0}$	12.1	12.5	15.6	10.1
ECL 16.150	1.6	ND	ND	ND
$C_{17:1}$ cis 7	2.0	1.3	ND	ND
$C_{17:1}$ cis 11	10.3	0.3	1.7	2.5
$C_{17:0}$	1.2	ND	ND	ND
$C_{18:1}$ cis 11	39.3	55.5	50.1	50.2§
$C_{18:0}$	ND	ND	ND	1.0
$C_{18:1}$ cis 11, 11 methyl	2.9	4.7	1.2	3.2
ECL 18.123	ND	2.3	ND	ND
Quinones	Ubiquinone Q-10	Ubiquinone Q-10	Ubiquinone Q-10	Ubiquinone Q-10
Polar lipids	DPG, DMPE, MMPE, PC, PE, PG, SGL	DGTS, DMPE, DPG, MHD, MMPE, PC, PE, PG, SGL	PE, PG, SGL	DPG, MMPE, PC PE, PG, SGL

*Data taken from Piao *et al.* [41].
†Data taken from Akbar *et al.* [40].
‡Not separated from $C_{16:1}$ cis 10 and given as summed feature 3.
§Not separated from $C_{18:1}$ cis 12 and given as summed feature 8.

including Zen2012 software. A minimum of at least 100 cells were measured. A single colony was taken from an agar plate, diluted with Ringer's solution and stained with uranyl acetate (2% in distilled H_2O, pH 4–4.5) for electron microscopy. A Zeiss 109T transmission electron microscope (Carl Zeiss) was used and images were taken with a 1K digital camera, including the ImageSP software.

After incubation for 3 days, strain DH-S5^T formed red, circular, convex and shiny colonies with a diameter of <1.0 mm on R2A agar. The two compared strains showed a clear difference in colony colour, as *S. alpina* DSM 22537^T grew in yellow, circular, convex and shiny colonies with a diameter of 1.5–2 mm, which was also observed by Margesin *et al.* [34]. Other closely related strains like *Sphingomonas qilianensis* X1^T and *Sphingomonas hylomeconis* GZJT-2^T have also been described to be light-yellow or yellow in colour, with circular, convex and smooth colonies [40, 41], consequently differing from DH-S5^T. Both strains had the same growth temperature range at 3–33 °C, with an optimum temperature of 30 °C for DH-S5^T and 25 °C for *S. alpina* DSM 22537^T, respectively. The species *S. qilianensis* is described to grow at elevated temperatures of 15–30 °C [41]; for *S. hylomeconis* a temperature range of 4–30 °C is mentioned [40]. Strain DH-S5^T and *S. alpina* DSM 22537^T grew at the same NaCl and pH values, ranging from 0 to 0.25% NaCl (optimum at 0.25%) and pH 6 to 7 (optimum at pH 6). Strain DH-S5^T was able to grow on TSA, but not on Columbia blood agar. On the other hand, *S. alpina* DSM 22537^T grew on Columbia blood agar, but not on TSA. No growth of the strains occurred under anaerobic conditions. DH-S5^T showed growth under 20% CO_2-containing modified atmosphere, in contrast to *S. alpina* DSM 22537^T, indicating its capability to colonize and spoil food packaged under CO_2-enriched atmosphere. No biofilm formation was observed for either strain. Cells of DH-S5^T were 1.5×0.9 µm in diameter and those of *S. alpina* DSM 22537^T were 1.9×0.8 µm. Cells of both strains showed motility and endospores were

not detected. Electron microscopy footage of both strains is displayed in Fig. 3. A summary of the differentiating characteristics, including for *S. qilianensis* X1^T and *S. hylomeconis* GZJT-2^T, is shown in Table 1.

The Gram reaction was tested by Gram-staining as previously described [42]. Catalase activity was analysed by observing the production of gas after adding a drop of 3% H_2O_2 (v/v) on colonies on R2A agar after incubation for 3 days. Cytochrome oxidase activity was determined with Bactident Oxidase test strips (Merck). Further biochemical and physiological characteristics, including enzyme activity, were tested with the API ZYM and API 20 NE systems (bioMérieux) at 30 °C.

Gram-staining was negative for both strains and catalase activity was positive. The two strains differed in cytochrome oxidase activity, as only *S. alpina* DSM 22537^T showed a positive reaction. Both strains tested positive on alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, trypsin and acid phosphatase. No enzymatic activity was detected for cystine arylamidase, α-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, nitrate reduction to nitrite and N_2, indole formation, glucose fermentation, arginine dihydrolase, urease and protease hydrolysis (gelatin). Additionally, DH-S5^T differed from *S. alpina* DSM 22537^T by a positive reaction for the enzymes α-chymotrypsin and β-glucuronidase and negative reactions for β-galactosidase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and β-glucosidase activities (aesculin hydrolysis). Both strains tested positive for the assimilation of D-glucose, D-mannose and maltose. Neither DH-S5^T nor *S. alpina* DSM 22537^T were able to metabolize the following carbohydrates: potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetate. Regarding the assimilation of carbohydrates, strain DH-S5^T could be differentiated from *S. alpina* DSM 22537^T by a positive result for D-mannitol and negative results for L-arabinose and *N*-acetyl-glucosamine.

Fatty acid methyl ester samples were prepared as described by Sasser [43] and analysed via gas chromatography (model 6980, Agilent) with a flame ionization detector, as previously described [44]. Verification of fatty acid identity was carried out using gas chromatography (model 7890A, Agilent Technologies) with mass spectrometry (model 5975C, Agilent Technologies). The chromatographic conditions were previously described by Lipski and Altendorf [45]. Fatty acids were extracted from cell material of colonies grown on R2A agar for 72 h at 30 °C. Extraction and analysis of isoprenoid quinones was performed as described by Minnikin *et al.* [46], as modified by Wiertz *et al.* [47]. Polar lipids were extracted and visualized using thin-layer chromatography (TLC), based on the Bligh and Dyer method [48] and modified by Minnikin *et al.* [46]. Primulin dye was used for visualization of all polar lipids [49]. Aminolipids were stained by ninhydrin reagent (Sigma-Aldrich). The same plates were sprayed with molybdenum blue reagent (Sigma-Aldrich) for visualization of phospholipids. Sphingoglycolipids were detected by spraying α-naphthol reagent (4 g in 250 ml methanol, mixed with 30 ml sulfuric acid) on TLC plates and heating them up at 137 °C for 10 min until staining appeared. Sugar-containing lipids produced red-brown spots. For verification, primulin-stained lipid spots were scratched off from the TLC plates, extracted and analysed by quadrupole time-of-flight mass spectrometry, as described by Hölzl *et al.* [50]. Polar lipid patterns of strain DH-S5^T and *S. alpina* DSM 22537^T are shown in Fig. 4.

The major fatty acids (>5%) of strain DH-S5^T were $C_{18:1}$ cis 11 (39.3%), $C_{16:1}$ cis 9 (12.5%) and $C_{16:0}$ (12.1%), as well as the hydroxylated fatty acids $C_{14:0}$ 2-OH (11.4%) and $C_{17:1}$ cis 11 (10.3%), as expected for the genus *Sphingomonas* [4]. The fatty acid profile of *S. alpina* DSM 22537^T differed from strain DH-S5^T in significantly lower amounts of $C_{17:1}$ cis 11 (0.3%) and higher amounts of $C_{18:1}$ cis 11 (55.5%). The closely related strains *S. qilianensis* X1^T and *S. hylomeconis* GZJT-2^T showed similarly low amounts of $C_{17:1}$ cis 11 (1.7 und 2.5%, respectively) and high amounts of $C_{18:1}$ cis 11 (50.1 and 50.2%, respectively) [40, 41]. IUPAC-IUB nomenclature of fatty acids is used [51]. Both strains contained ubiquinone Q-10 as their major respiratory quinone, which is in agreement with the findings of Margesin *et al.* [34]. A polar lipid pattern consisting of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), monomethylphosphatidylethanolamine (MMPE), dimethylphosphatidylethanolamine (DMPE) and sphingoglycolipid (SGL) was detected for strain DH-S5^T. In addition to these lipids, monohexosyldiacylglycerol (MHD) and 1,2-diacylglyceryl-3-O-4′-(*N*,*N*,*N*-trimethyl)-homoserine (DGTS) were detected in polar lipid extracts of *S. alpina* DSM 22537^T. In their emended description of the genus *Sphingomonas*, Busse *et al.* [52] mentioned the occurrence of PE, PG, DPG, SGL, PC, MMPE and DMPE [52]. Table 2 gives an overview of the fatty acid patterns, detected quinones and polar lipid profiles of the isolate and reference strain *S. alpina* DSM 22537^T, as well as the closely related strains *S. qilianensis* X1^T and *S. hylomeconis* GZJT-2^T.

DESCRIPTION OF *SPHINGOMONAS ALIAERIS* SP. NOV.

Sphingomonas aliaeris (a.li.a'e.ris. L. masc. adj. *alius* different; L. masc. n. *aer* air, atmosphere; N.L. gen. n. *aliaeris* of a different atmosphere).

Cells are Gram-stain-negative, oxidase-negative, catalase-positive, motile and rod-shaped. Cells are 1.5 (±0.4)×0.9 (±0.1) μm in diameter. Growth is strictly aerobic on R2A agar and TSA at 3–33 °C at pH 6.0; optimum growth at 30 °C. Tolerates 0.25% NaCl and 20% CO_2. No growth occurs on Columbia blood agar. Colonies are round, convex, red-pigmented, shiny and mostly less than 1 mm in diameter. Does not hydrolyse aesculin or gelatin. Positive for alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin and β-glucuronidase. Negative for cystine arylamidase, α-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, nitrate reduction to nitrite

and N_2, indole formation, glucose fermentation, arginine dihydrolase, urease, β-galactosidase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. D-Glucose, D-mannose, maltose and D-mannitol are used as carbon sources. Does not assimilate potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetate, L-arabinose and *N*-acetyl-glucosamine. Main fatty acids are $C_{18:1}$ cis 11, $C_{16:1}$ cis 9, $C_{16:0}$ and $C_{14:0}$ 2-OH. The ubiquinone is Q-10. The polar lipid profile consists of PC, PG, DPG, PE, DMPE, SGL and MMPE.

The type strain, DH-S5^T (=DSM 110829^T=LMG 31606^T), was isolated from refrigerated pork steak packed under modified atmosphere, containing 20% CO_2. The DNA G+C content of the type strain is 64.4 mol% based on the whole genome sequence.

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

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