Catenulispora acidiphila gen. nov., sp. nov., a novel, mycelium-forming actinomycete, and proposal of *Catenulisporaceae* fam. nov.

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A novel, Gram-positive bacterial strain was isolated from forest soil. Among species with validly published names, the 16S rRNA gene sequence is related most closely (approx. 93 % similarity) to that of *Sporichthya polymorpha* DSM 43042^T. However, differently from this species, it forms both vegetative and aerial mycelia. The aerial hyphae are straight to slightly flexuous, starting to septate to form chains of more than 20 cylindrical spores with a rugose surface. The strain is acidophilic, with a pH range for robust growth between 4.3 and 6.8 and an optimum around 6.0. The peptidoglycan type is A3_γ LL-Dpm–Gly. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and two unknown phospholipids. Predominant menaquinones are MK-9(H₆) and -9(H₄), and iso- and anteiso-branched C_{16:0} and C_{17:0} are the main cellular fatty acids. The DNA G + C content is 71.9 mol%. The distinct phylogenetic position and the unusual combination of chemotaxonomic characteristics justify the proposal of *Catenulispora* gen. nov., with the type species *Catenulispora acidiphila* sp. nov. (type strain, ID139908^T = DSM 44928^T = NRRL B-24433^T). *Catenulisporaceae* fam. nov. is also proposed.

The order *Actinomycetales* within the class *Actinobacteria* represents a large and diverse group of common soil inhabitants (McCarthy & Williams, 1992) and antibiotic producers (Demain, 1998). Recent efforts in strain isolation (Sait *et al.*, 2002; Joseph *et al.*, 2003) and evidence from soil DNA (Monciardini *et al.*, 2002) indicate the existence of novel taxa in the order *Actinomycetales* that may represent promising sources of novel metabolites (Bull *et al.*, 2000; Donadio *et al.*, 2002).

Isolation and cultivation of strain ID139908^T

A soil sample collected from a wooded area in Gerezano, Italy, was dried at 30 °C under vacuum for 7 days, resuspended in 18.2 mM citric acid, 164 mM Na₂HPO₄ buffer

(pH 7), and aliquots from serial dilutions were deposited onto GTV plates [GTV contains 500 ml soil extract l⁻¹ (prepared by autoclaving 100 g fresh soil resuspended in 500 ml H₂O, followed by filtering through sterile gauze), 10 g gellan gum ml⁻¹, 3 mM CaCl₂ (resulting pH, 5) and was supplemented after autoclaving with 0.1 % (v/v) CMM vitamin solution (25 µg thiamin hydrochloride ml⁻¹, 250 µg calcium pantothenate ml⁻¹, 250 µg nicotinic acid ml⁻¹, 500 µg biotin ml⁻¹, 1.25 mg riboflavin ml⁻¹, 6 µg vitamin B₁₂ ml⁻¹, 25 µg *p*-aminobenzoic acid ml⁻¹, 500 µg folic acid ml⁻¹ and 500 µg pyridoxal hydrochloride ml⁻¹) and 50 µg cycloheximide ml⁻¹] and incubated at 28 °C. Seven strains, which appeared after an 8 week incubation, were related closely on the basis of their 16S rRNA gene sequences. One of these isolates was chosen for further studies and maintained on ISP2 agar (Shirling & Gottlieb, 1966) adjusted to pH 5.5–6.0 with HCl, and was designated strain ID139908^T.

Morphology

Morphology of aerial mass was examined directly on HSA5 agar plates [HSA5 consists of 0.5 g humic acid l⁻¹,

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Abbreviation: FESEM, field emission scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ID139908^T is AJ865857.

Supplementary figures showing a FESEM image, polar lipid composition and phylogenetic position of strain ID139908^T are available in IJSEM Online.

Fig. 1. FESEM of strain ID139908^T grown on HSA5 agar for 3 weeks at 28 °C. Cylindrical arthrospores (0.4–1.0 µm length by approx. 0.5 µm diameter) with a rugose surface are visible. Collapsing of the central part of spore walls results in a wavy appearance of spore chains. Bar, 1 µm.

1 mg FeSO₄·7H₂O l⁻¹, 1 mg MnCl₂·4H₂O l⁻¹, 1 mg ZnSO₄·7H₂O l⁻¹, 1 mg NiSO₄·6H₂O l⁻¹, 10 mM *N*-cyclohexyl-2-aminoethanesulfonic acid, 20 g agar l⁻¹, adjusted to pH 5.5 and supplemented after autoclaving with 0.1 % (v/v) CMM vitamin solution] after a 3–4 week incubation at 28 °C, under a light microscope equipped with a ×40 long working distance objective (model ULWD-CDPlan; Olympus) and fitted with a 3CCD camera (Sony). Strain ID139908^T forms a branched, non-fragmenting, vegetative mycelium and straight to slightly flexuous aerial hyphae. When drops of an H₂O suspension of aerial mass from 3- or 4-week-old cultures were incubated at 28 °C and checked under light microscopy at 30 min intervals for 2 h, motile elements were not observed.

Field emission scanning electron microscopy (FESEM) was performed as described elsewhere (Hammerschmidt *et al.*, 2005) by fixing samples of 3-week-old HSA5 agar cultures of strain ID139908^T with glutaraldehyde (2 %) and formaldehyde (5 %). FESEM revealed long filaments of aerial

hyphae that showed marked septation, resulting in chains of more than 20 cylindrical arthrospores (see Supplementary Fig. S1, available in IJSEM Online). Spores show a rugose surface and are 0.4–1.0 µm long, with a mean diameter of 0.5 µm (Fig. 1).

Cultural and physiological characteristics

The media used were those recommended by Shirling & Gottlieb (1966), but acidified to pH 5.5–6.0 with HCl. Inocula for cultural and physiological analyses were obtained by growing strain ID139908^T in ATSB medium (17 g casitone l⁻¹, 3 g soytone l⁻¹, 2.5 g glucose l⁻¹, 10 mM MES, adjusted to pH 5.8 with HCl) for 1 week at 28 °C on a rotary shaker at 200 r.p.m. Mycelium was washed with sterile H₂O and then diluted to a suitable inoculum. Aliquots of the suspension were streaked in a cross-hatched manner onto the media used. Unless stated otherwise, all evaluations were made after 3 weeks incubation at 28 °C. The pH range for growth was determined by using ISP2 as basal medium, adjusted to the desired values with HCl or NaOH. Temperature range for growth and NaCl and lysozyme tolerance were determined by using acidified (pH 5.5–6.0) ISP2 medium.

Table 1 shows the appearance of strain ID139908^T on various agar media. It grew well on most of the tested media, with production of brownish pigments and whitish aerial mass, turning to yellow/green with age. However, the brownish pigments were not observed in tyrosine-supplemented Suter medium (Suter, 1978), suggesting that they are not melanin-related. Strain ID139908^T grew well on media at initial pH between 4.3 and 6.8, with an optimum around pH 6.0, but scant growth was visible up to pH 7.5. This strain must thus be considered an acidophile. Optimal growth occurred between 22 and 28 °C and measurable growth also occurred at 11 and 32 °C.

Strain ID139908^T could grow in the presence of up to 3 % (w/v) NaCl, albeit with a progressive reduction of pigmentation starting at 1 % NaCl, and was resistant to at least 100 µg lysozyme ml⁻¹. Among carbon sources, as recorded

Table 1. Culture characteristics of strain ID139908^T after 3 weeks growth at 28 °C

All media were acidified to pH 5.5–6.0.

Medium	Growth	Reverse*	Aerial mass	Diffusible pigment
ISP2 (yeast extract–malt extract agar)	Abundant, convolute	Dark brown/nutmeg (15E8)	Absent	Abundant, brown
ISP3 (oatmeal agar)	Good, thin	Cream to light brown (12C6)	Moderate, whitish	Good, brownish
ISP4 (inorganic salts–starch agar)	Good	Cream to light orange (11F6)	Absent	Good, brownish
ISP5 (glycerol–asparagine agar)	Good	Brown/sienna (5G12)	Scant, whitish	Abundant, light brown
ISP6 (peptone–yeast extract–iron agar)	Absent	–	–	–
ISP7 (tyrosine agar)	Abundant	Brown/mahogany (6L12)	Moderate, whitish	Abundant, brown

*Colour codes in parentheses are according to Maerz & Paul (1950).

on acidified ISP9 medium (Shirling & Gottlieb, 1966), glucose, arabinose, xylose, mannitol, fructose and glycerol supported good growth, whereas no visible colonies were formed with sucrose, inositol, rhamnose or cellulose; scant growth was observed with raffinose. Aerobiosis-related properties were analysed on acidified ISP2 agar medium (plates were stored at 4 °C and evaporated before use) under an aerobic, microaerophilic (GasPac system with a Gas Generating kit for *Campylobacter*; Oxoid BR056A) or anaerobic atmosphere. Strain ID139908^T grew better under aerobic conditions, but reduced, non-pigmented growth was also obtained in microaerophilic and anaerobic atmospheres. The strain was positive for catalase (evaluated by mixing 0.8 ml of a 1-week-old ATSB culture with 0.5 ml freshly prepared 3 % hydrogen peroxide), starch hydrolysis and gelatin liquefaction (Gottlieb, 1961), casein degradation [evaluated on acidified 1.5 % (w/v) skimmed-milk agar] and H₂S production (detected 2 days after inserting sterile lead acetate filter-paper strips into the necks of culture tubes containing acidified ISP6 medium). Strain ID139908^T was negative for nitrate reduction [evaluated after 3 and 7 days in ATSB medium supplemented with 2 g KNO₃ l⁻¹, using Bacto-Nitrite test strips (Difco) to reveal the presence of nitrites]. When 15 µl of an antibiotic solution (10 µg ml⁻¹) was spotted onto previously inoculated, acidified ISP2 plates, after 1 week at 28 °C, strain ID139908^T was found to be resistant to nalidixic acid, oxacillin, apramycin, daunomycin, GE2270, nisin, rifampicin and kanamycin, but sensitive to novobiocin, thiostrepton, A40926 and ramoplanin.

Chemotaxonomic characteristics

Freeze-dried biomass was obtained from a 1-week culture in ATSB medium. The peptidoglycan of strain ID139908^T, analysed as described by Schleifer & Kandler (1972) and modified according to Willems *et al.* (1997), contained LL-diaminopimelic acid (Dpm), glycine, glutamic acid and alanine. From the two-dimensional TLC pattern (data not shown) of peptides in the partial peptidoglycan hydrolysate (4 M HCl, 100 °C, 45 min), the peptidoglycan was assigned to type A3γ LL-Dpm-Gly (A41.1 according to <http://www.dsmz.de/species/murein.htm>). Menaquinones [determined as described by Groth *et al.* (1997)] were MK-9(H₆), -9(H₄) and -9(H₈) (ratio of peak areas, 4.5:2.8:1.0, respectively). Whole-cell sugars [determined according to Stanek & Roberts (1974)] contained large amounts of arabinose, together with xylose, ribose, rhamnose and glucose. The polar lipid pattern [determined according to Groth *et al.* (1997)] consists of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and two unknown phospholipids (see Supplementary Fig. S2, available in IJSEM Online). The cellular fatty acid profile of strain ID139908^T, analysed as described by Miller (1982), is composed of i-C_{16:0} (47.05 %), ai-C_{17:0} (12.73 %), i-C_{17:0} (5.73 %), C_{16:0} (5.61 %), i-C_{17:1ω9c} (4.69 %), i-C_{15:0} (4.26 %), i-C_{16:1} (3.41 %), C_{16:1ω7c} (3.16 %), ai-C_{17:1ω9c} (2.84 %), ai-C_{15:0} (2.25 %),

C_{18:1ω9c} (1.43 %), C_{17:0} cyclo (1.17 %) and C_{17:1ω8c} (1.06 %) (fatty acids representing <1.00 % of the total are not reported). The DNA G+C content of strain ID139908^T, determined according to Mesbah *et al.* (1989), is 71.5 mol%.

Although strain ID139908^T and members of the family *Streptomycetaceae* share the presence of LL-diaminopimelic acid in the peptidoglycan and type 2c fatty acids (*sensu* Kroppenstedt, 1985), their phospholipid and whole-cell sugar patterns, as well as the menaquinone composition, clearly differentiate them (Korn-Wendisch & Kutzner, 1992; Zhang *et al.*, 1997; Kim *et al.*, 2003).

Phylogenetic analysis

The almost-complete 16S rRNA gene sequence for strain ID139908^T [1441 nt, corresponding to 93.3 % of the *Escherichia coli* sequence (Brosius *et al.*, 1978)] was determined and compared with all GenBank entries as described previously (Monciardini *et al.*, 2003). The partial 16S rRNA gene sequence of strain ID139908^T is 98.6–99.5 % identical to those of the actinomycete strains Ellin 5093, 5062, 5034, 5119 and 5116 (Joseph *et al.*, 2003), recently isolated from a pasture soil in Ellinbank, Australia, and >99 % identical to those of 'bacterium 12202' (GenBank accession no. AY639903) and 'Actinobacterium Aac30' (GenBank accession no. AB180773). No description of these strains has, to our knowledge, appeared in the literature. Among species with validly published names, the highest binary similarity values are with members of the suborder *Frankineae*, namely *Sporichthya polymorpha* DSM 43042^T (93 %) and *Geodermatophilus obscurus* DSM 43161 (92.5 %). Representatives of several actinomycete lineages have 16S rRNA gene sequence similarities to ID139908^T of between 91 and 92 %. Although strain ID139908^T clearly belongs to the order *Actinomycetales*, it does not seem to be affiliated to any of the described lineages within the order. Of the two described bacterial strains with the highest 16S rRNA gene sequence relatedness to strain ID139908^T, *S. polymorpha* DSM 43042^T also contains LL-diaminopimelic acid in its peptidoglycan, but the menaquinones differ, as strain ID139908^T has MK-9(H₆) and -9(H₄) as major components, in contrast to DSM 43042^T, which has MK-9(H₆) and -9(H₈) (Rainey *et al.*, 1993). The fatty acid patterns also differentiate the two strains, as *S. polymorpha* is a 3c type, in contrast to ID139908^T, which is of the 2c type (Kroppenstedt, 1985). Morphologically, the two strains are also completely different, as *S. polymorpha* exhibits a unique life cycle with the absence of a vegetative mycelium and the aerial mycelium dividing into rod-shaped to coccoid spores that are motile (Lechevalier & Lechevalier, 1989). *G. obscurus* DSM 43161 is even more different from ID139908^T, as the two strains do not share any chemotaxonomic markers (Goodfellow, 1989) and *G. obscurus* does not show the growth-cycle characteristics of filamentous actinomycetes (Luedemann & Fonseca, 1989). Phenotypic characteristics of other members of the suborder *Frankineae*

can be found elsewhere (Tamura *et al.*, 1998; Maszenan *et al.*, 2005).

When the sequence of strain ID139908^T was aligned with those deposited for the Ellinbank isolates and with those obtained from similar strains isolated in our laboratory, we could identify the signature nucleotides of the order *Actinomycetales* with the exception of position 449, where a C is found instead of the A proposed for the order (Stackebrandt *et al.*, 1997). However, we could not find the signature nucleotide pattern of any of the other described suborders (Stackebrandt *et al.*, 1997). Of the six signatures described for the suborder *Frankineae*, three are not found in ID139908^T and related strains, namely 141:222 (A–U instead of G–C), 371:390 (A–U instead of G–C) and 1003:1037 (G–C instead of G–G).

The 16S rRNA gene sequence of strain ID139908^T was aligned with those of the type species of the major actinomycete lineages and analysed as described previously (Monciardini *et al.*, 2003). The resulting phylogenetic tree is shown in Fig. 2. As no obvious relatives of the novel strain could be identified, we included in the analysis representatives of different families of the various suborders, with a

particular focus on filamentous actinomycetes. Strain ID139908^T, together with the Ellinbank isolates (Joseph *et al.*, 2003), forms a coherent clade within the *Actinomycetales* lineage, clearly distinguished from other described strains. Although the closest relatives of the novel lineage appear to be members of the suborder *Frankineae*, bootstrap values are too low to allow definitive phylogenetic placement within this suborder, as also suggested by the low pairwise identity of 16S rRNA gene sequences and by the differences in the pattern of signature nucleotides. A phylogenetic tree obtained from the alignment of the 16S rRNA gene sequences of representatives of the described families of the suborder *Frankineae* (Tamura *et al.*, 1998; Maszenan *et al.*, 2005) with those of strain ID139908^T and related strains further confirms that the two lineages are independent (see Supplementary Fig. S3, available in IJSEM Online). Additional phylogenetic analyses, performed with different sequences and treeing methods, confirm the high divergence of ID139908^T and related sequences from representatives of described families of the *Actinomycetales* (not shown). Altogether, phylogenetic data indicate that strain ID139908^T is sufficiently divergent from known bacterial species as to be described as being representative of a novel genus. In

Fig. 2. Neighbour-joining tree based on 1324 aligned positions within the 16S rRNA gene. GenBank accession numbers for the sequences are in parentheses. The tree is rooted by using the 16S rRNA gene sequence of *Bifidobacterium bifidum* NBRC 14252^T (GenBank accession no. S83624). Numbers at nodes are bootstrap values based on 100 resamplings; only values >65 are shown. Bar, 1 inferred nucleotide substitution per 100 nt.

addition, these data support the hypothesis that strain ID139908^T, along with the closely related Ellinbank strains, represents a novel family within the order *Actinomycetales*. Thus, it is proposed that *Catenulispora* gen. nov. should be established in order to harbour strain ID139908^T. Following the guidelines for the affiliation to higher hierarchical taxa in the class *Actinobacteria* proposed by Stackebrandt *et al.* (1997), which base the clustering solely on phylogenetic relationships, we propose the description of *Catenulisporaceae* fam. nov. to accommodate the proposed genus *Catenulispora*.

Description of *Catenulisporaceae* fam. nov.

(Ca.te.nu.li.spo.ra'ce.ae. N.L. fem. n. *Catenulispora* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Catenulisporaceae* the *Catenulispora* family).

The family contains the type genus *Catenulispora*. The pattern of 16S rRNA gene signatures consists of nt 127:234 (G–C), 129:232 (U–A), 449 (C), 580:761 (U–A), 586:755 (U–A), 591:648 (C–G), 824:876 (A–U), 825:875 (A–U), 834:852 (G–U), 838:848 (U–G), 952:1229 (U–A), 999:1041 (U–U) and 1000:1040 (U–U). A phylogenetic analysis is presented in this study.

Description of *Catenulispora* gen. nov.

Catenulispora (Ca.te.nu.li.spo'ra. L. fem. n. *catenula* small chain; Gr. fem. n. *spora* seed; N.L. fem. n. *Catenulispora* a thin chain of spores).

Gram-positive, acidophilic, non-acid-fast, aerobic organisms forming branching hyphae. Non-fragmentary vegetative mycelium and aerial hyphae starting to septate in chains of cylindrical arthrospores are produced. Motile elements are not produced. Peptidoglycan contains LL-diaminopimelic acid, glycine, glutamic acid and alanine. Glucose, xylose, ribose, rhamnose and arabinose (the latter in large amounts) are detected as whole-cell sugars. i-C_{16:0} and ai-C_{17:0} are present as major cellular fatty acids. Menaquinones MK-9(H₆) and MK-9(H₄) are predominant, but MK-9(H₈) is also detected. Polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and two unknown phospholipids. The G + C content of the DNA is 71 mol%. The type species is *Catenulispora acidiphila*.

Description of *Catenulispora acidiphila* sp. nov.

Catenulispora acidiphila (a.ci.di'phi.la. N.L. neut. n. *acidum* acid; Gr. adj. *philos* loving; N.L. fem. adj. *acidiphila* acid-loving).

Chemotaxonomic and general characteristics are the same as given above for the genus. Acidophilic: grows well in a pH range from 4.3 to 6.8, optimal around 6.0. Mesophilic: best growth occurs at 22–28 °C, but significant growth can also be observed at 11 and 37 °C; no growth occurs at 4 or 40 °C. Aerial hyphae are relatively short, straight to flexuous and

produce chains of more than 20 cylindrical arthrospores. Spores have a length in the range 0.4–1.0 µm and a mean diameter of around 0.5 µm. Spore surface is rugose. The organism grows well on various media, better on acidic yeast extract–malt extract agar and acidic tyrosine agar. In general, the soluble pigments produced by the type strain, as well as the vegetative mycelium, are brownish, whilst the aerial mass is whitish, turning to yellow/green with ageing. Brown mahogany soluble pigment is produced on tyrosine agar; no pigmentation is observed on tyrosine-supplemented Suter synthetic medium. H₂S is produced. Nitrates are not reduced. Starch and casein are hydrolysed. Gelatin is liquefied. Catalase-positive. Up to 3 % (w/v) NaCl is tolerated, as well as 100 µg lysozyme ml^{−1}. Glucose, arabinose, xylose, mannitol, fructose and glycerol are utilized, whereas sucrose, inositol, rhamnose and cellulose are not. Resistant to nalidixic acid, oxacillin, apramycin, daunomycin, GE2270, nisin, rifampicin and kanamycin at 10 µg ml^{−1}, but sensitive to the same concentration of novobiocin, thiostrepton, A40926 and ramoplanin. The G + C content of the DNA is 71.5 mol%.

The type strain, ID139908^T (=DSM 44928^T=NRRL B-24433^T), was isolated from temperate forest soil.

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