

Agreia bicolorata* gen. nov., sp. nov., to accommodate actinobacteria isolated from narrow reed grass infected by the nematode *Heteroanguina graminophila

Lyudmila I. Evtushenko,¹ Lubov V. Dorofeeva,¹ Tatyana G. Dobrovolskaya,² Galina M. Streshinskaya,³ Sergey A. Subbotin⁴ and James M. Tiedje⁵

Author for correspondence: Lyudmila I. Evtushenko. Tel: +7 095 9257448. Fax: +7 095 9563370. e-mail: evtushenko@ibpm.serpukhov.su

¹ All-Russian Collection of Microorganisms (VKM), G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

^{2,3} Department of Soil Science² and School of Biology³, Lomonosov Moscow State University, Moscow 119899, Russia

⁴ Institute of Parasitology, Russian Academy of Sciences, Leninskii prospect 33, Moscow 117071, Russia

⁵ Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-1325, USA

***Agreia bicolorata* gen. nov., sp. nov. (type strain VKM Ac-1804^T = UCM Ac-620^T) is proposed to accommodate aerobic, oxidase- and catalase-positive, weakly motile, coryneform actinobacteria isolated from leaf galls induced by the plant-parasitic nematode *Heteroanguina graminophila* in narrow reed grass, *Calamagrostis neglecta*. Bacteria assigned to *Agreia bicolorata* gen. nov., sp. nov. form a distinct lineage within the phylogenetic branch of the family *Microbacteriaceae* and possess the following chemotaxonomic characteristics: B-type peptidoglycan containing 2,4-diaminobutyric acid, ornithine, alanine, glycine, glutamate and hydroxyglutamate; cell wall sugars rhamnose, fucose and mannose; MK-10 as major menaquinone; phosphatidylglycerol and diphosphatidylglycerol as principal phospholipids; and 12-methyl-tetradecanoic acid (anteiso-15:0), 14-methyl-pentadecanoic acid (iso-16:0) and 14-methyl-hexadecanoic acid (anteiso-17:0) as predominant fatty acids. The DNA G+C content of *Agreia bicolorata* is about 67.0 mol%.**

Keywords: *Actinomycetales*, *Agreia bicolorata* gen. nov., sp. nov., plant-parasitic nematode, *Heteroanguina graminophila*

INTRODUCTION

A number of coryneform bacteria have been reported to be involved in an intimate relationship with plant-parasitic nematodes of the genus *Anguina* (Gupta & Swarup, 1972; Gummins *et al.*, 1975; Price *et al.*, 1979; Bird, 1981; Riley, 1987; Riley *et al.*, 1988; Riley & McKay, 1990; Riley & Ophel, 1992; McKay & Ophel, 1993). Bacteria attached to the surface of the infective nematode are carried into the growing points of host plants and can multiply there causing certain plant diseases (Gummins *et al.*, 1975; Vidaver, 1982; Bradbury, 1986; Collins & Bradbury, 1991). They may also inhabit many galls induced by nematodes in seeds, leaves, stems and roots (Bird, 1981; Evtushenko *et al.*, 1994). These bacterial associates of nematodes may facilitate development of nematode larvae within the

galls and release of nematodes from the galls, destroying the gall envelope by fermentative activities (Bird, 1981, 1985). The unambiguously identified coryneform bacteria of such *Anguina*-bacterial communities have been assigned to different species of the genus *Rathayibacter* (Zgurskaya *et al.*, 1993; Sasaki *et al.*, 1998). *Rathayibacter toxicus* (formerly *Clavibacter toxicus*, also previously referred to as *Corynebacterium* sp., *Corynebacterium rathayi*, annual ryegrass toxicity bacteria or 'ARGT' bacteria) may produce neurotoxins of glycolipid character in the infected plants which are harmful for grazing animals (Vogel *et al.*, 1982; Jago *et al.*, 1983; Riley, 1987; McKay & Ophel, 1993). The toxin responsible for annual ryegrass toxicity was shown to be produced when bacteria were infected with a bacteriophage (Ophel *et al.*, 1993). Other microorganisms may also be involved in *Anguina*-bacteria disease complexes in the absence of the rathayi-bacterial type associates (Wen & Viglierchio, 1992).

Very little is known about micro-organisms associated with gall-forming nematodes of other genera of the

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank accession number for the 16S rRNA sequence of strain VKM Ac-1804^T is AF159363.

subfamily Anguininae (Krall, 1991; Subbotin & Ivanova, 1991). Our electron microscopic study of galls induced by nematodes of the genera *Heteroanguina*, *Mesoanguina* and *Subanguina* in different grasses showed a significant accumulation of irregular Gram-positive rods in the cavities, intracellular space and destroyed cells of the galls. Some coryneform bacteria isolated from these galls have been identified as members of the genera *Curtobacterium*, *Microbacterium* and *Rhodococcus* (Evtushenko *et al.*, 1994), which are known to be widely spread in the phyllosphere of different plants as saprophytes or plant pathogens (Vidaver, 1982; Collins & Bradbury, 1991). The bacteria found in root galls of annual meadow grass infected by the nematode *Subanguina radicola* have been described as *Leifsonia poae* gen. nov., sp. nov. (Evtushenko *et al.*, 2000). Results of a taxonomic study of coryneform bacteria isolated from leaf galls induced by the nematode *Heteroanguina graminophila* in narrow reed grass, *Calamagrostis neglecta*, are presented. Based on the data obtained, a new genus and species, *Agreia bicolorata* gen. nov., sp. nov., is proposed.

METHODS

Isolation and cultivation of bacteria. Narrow reed grass (*Calamagrostis neglecta*) samples infected by the gall-forming nematode *Heteroanguina graminophila* were collected in Moscow Region in June 1991 and stored dry in sterile Petri dishes at 8 °C for 2 years. The galls were cut from the leaves with a sterile scalpel, treated with a detergent and washed with sterile water. They were then cut into pieces, added to 2 ml 0.85% NaCl (w/v) solution and ground with a pestle. One drop of this suspension was plated onto nutrient agar (2 g peptone, 1 g glucose, 1 g yeast extract, 1 g casein peptone, 10 ml glycerol, 5 g chalk, 100 ml wort, 15 g agar, 900 ml distilled water, pH 7.2–7.4) and incubated for 2 weeks at room temperature (17–24 °C). Bacteria from representative colonies were selected and regrown on the same medium or corynebacterial (CB) agar (pH 7.2–7.4), which contained (l⁻¹): 5 g peptone, 5 g glucose, 3 g yeast extract, 10 g casein peptone, 5 g NaCl and 15 g agar. Bacteria were stored at 8 °C or freeze-dried. For the chemotaxonomic study, shake cultures were grown in liquid CB medium for 18 h.

Morphology, physiology and cell chemistry. Morphology and life cycle were studied in cultures grown on CB agar by phase-contrast and electron microscopy as described previously (Evtushenko *et al.*, 1994). Motility was studied by the hanging drop method. Physiological features were examined as described by Zgurskaya *et al.* (1993) and Evtushenko *et al.* (2000). For extraction of cell walls, crude cells were disrupted by sonication, separated from unbroken cells by fractional centrifugation, and purified using trypsin and 2% SDS as reported previously (Evtushenko *et al.*, 2000). The presence of 2,4-diaminobutyric acid (DAB) and ornithine was detected by the TLC method (Bousfield *et al.*, 1985). Quantitative determination of amino acids was performed with an LC 600 E amino acid analyser (Biotronic) after acid hydrolysis (6 M HCl, 105 °C, 6 h) as described by Schleifer & Kandler (1972). Sugars of whole cells and cell walls, the presence of mycolic acids, and the composition of

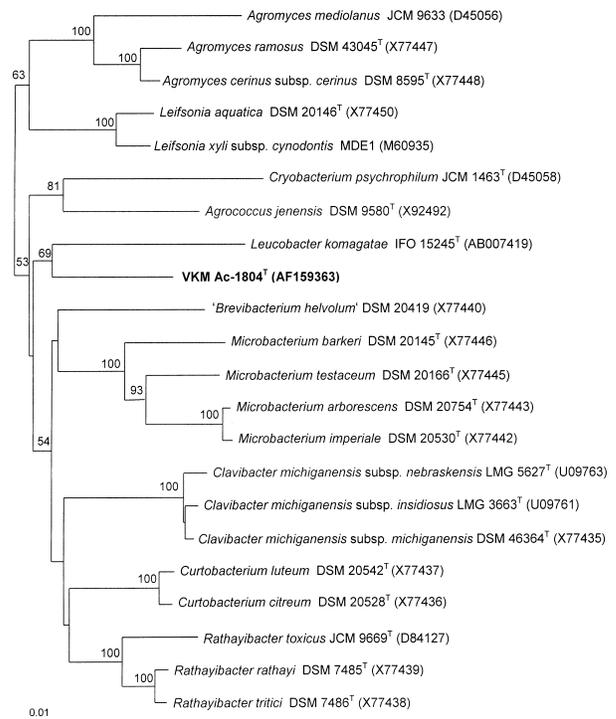


Fig. 1. Phylogenetic tree showing the position of strain VKM Ac-1804^T based on 16S rDNA analysis. The sequence of *Brevibacterium linens* DSM 20425^T (X77451) served as the outgroup (not presented). Accession numbers of nucleotide sequences are given in parentheses. Numbers within the dendrogram indicate the percentages of occurrence of the branching order in 1000 bootstrapped trees. Bar, 1 nt substitution per 100 nt.

menaquinones, fatty acids and phospholipids were studied as reported previously (Evtushenko *et al.*, 1989, 2000).

DNA base composition, DNA–DNA reassociation and 16S rDNA sequence analyses. The methods used for extraction and purification of DNA and determination of the G + C content by thermal denaturation have been described previously (Evtushenko *et al.*, 1989). DNA–DNA hybridization was performed using the membrane filter method (Evtushenko *et al.*, 2000). ³H-labelled DNAs from reference strains were obtained by *in vitro* nick-translation. The 16S rRNA was amplified using the PCR method and prokaryotic 16S rDNA universal primers fD1 and rP1 (Weisburg *et al.*, 1991) as described previously (Zhou *et al.*, 1997). PCR products were detected by agarose gel electrophoresis, visualized by UV fluorescence after ethidium bromide staining, and then purified and concentrated using a Wizard PCR Preps DNA purification system (Promega). The 16S rDNA sequences were analysed directly using purified PCR products as the sequencing template. Sequencing reactions were performed with automated fluorescent *Taq* cycle sequencing using the ABI Catalyst 800 and a model ABI 373A automatic DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. Nucleotide substitution rates were calculated as described by Kimura & Ohta (1972) and the phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with

CLUSTAL W software (Thompson *et al.*, 1994). Tree topologies were evaluated by bootstrap analysis of the sequence data with the same software. GenBank, DDBJ and EMBL accession numbers for reference 16S rDNA sequences of the strains used in this analysis are given in Fig. 1. *Brevibacterium linens* DSM 20425^T (X77451) was used as the outgroup.

RESULTS AND DISCUSSION

Isolation

Numerous coryneform bacteria that formed colonies of different tints of yellow and orange developed from the ground gall suspensions plated on the isolation media. About 20–25% semi-liquid bacterial colonies with brown-orange pigment were observed after 2 weeks incubation. Two representatives of the latter group, strains VKM Ac-1804^T and VKM Ac-1805, were characterized in detail.

Morphology

The colonies of both strains were round, glistening, opaque, butyrous, semi-fluid or fluid, at first deep-yellow or yellow-orange and later becoming red-orange or brown-orange on CB agar. Abundant polysaccharide capsules were usually formed on some agar media and liquid culture produced viscous slime. The young cells (18–20 h) were non-spore-forming, weakly motile, irregular rods (0.4–0.5 × 1.5–2.5 µm); primary branching was rarely observed. In older cultures, shorter irregular rods predominated as single cells; pairs or short chains with diphtheroid arrangements were also observed. However, a marked rod-coccus growth cycle did not occur. Electron microscopy revealed that the cell wall composition was typical of that of Gram-positive bacteria.

Cell chemistry

Cell walls contained DAB, ornithine, alanine, glycine and glutamate plus hydroxyglutamate in a molar ratio close to 1:1:2:2:2, as well as muramic acid and glucosamine. Whole-cell sugars of all strains were similar and included predominantly glucose and rhamnose; fucose, mannose and ribose were minor components. Quantitative cell wall sugar analysis of strain VKM Ac-1804^T revealed rhamnose, fucose and mannose in a molar ratio of 7:1:0.5. Mycolic acids were not present. The major menaquinone was MK-10 and minor amounts of MK-9 were found in both strains; traces of MK-8 and MK-11 were also detected in VKM Ac-1804^T. Phosphatidylglycerol and diphosphatidylglycerol were the principal phospholipids. The major fatty acids were anteiso- and iso-branched (31.3 and 30.2% anteiso-15:0, 26.6 and 27.1% iso-16:0, 39.4 and 41.3% anteiso-17:0 in VKM Ac-1804^T and VKM Ac-1805, respectively). Other fatty acids (iso-15:0, 15:0, 16:0, iso-18:1, 18:1 and iso-18:0) were present in small amounts (0.1–0.8%).

16S rDNA gene sequence analysis

An almost complete 16S rRNA sequence (1468 nt) was determined in strain VKM Ac-1804^T. Phylogenetic analysis indicated that this strain formed a distinct lineage within the phylogenetic cluster of the actinomycete genera that comprise the family *Microbacteriaceae*. It was linked to the type strain of *Leucobacter komagatae*, IFO 15245^T, with a 69% bootstrap replication value, showing 16S rDNA gene sequence similarity of 94.1% (Fig. 1).

DNA base composition and DNA–DNA hybridization

The DNA G+C contents of strains VKM Ac-1804^T and VKM Ac-1805 were 67.0 and 67.2 mol%, respectively. The DNA–DNA similarity between these strains was 87.4%, clearly indicating that both strains belong to a common genomospecies.

Physiology

Strains VKM Ac-1804^T and VKM Ac-1805 were almost identical in more than 100 physiological characteristics studied, including sensitivity to different antibiotics. The strains were aerobic, catalase- and oxidase-positive, mesophilic, with a growth optimum at 24–26 °C. Voges–Proskauer test was positive and methyl red test was negative. H₂S was weakly produced. All strains used L-arabinose, D-glucose, D-galactose, cellobiose, fructose, inositol (weak), inulin, lactose, maltose, mannose, mannitol, melibiose, raffinose, L-rhamnose, ribose, salicin, sorbitol, trehalose, turanose and D-xylose as a carbon source for growth in salt medium supplemented with 0.1% (w/v) yeast extract and 0.1% (w/v) casitone; none of the strains utilized adonitol, dextran, lyxose, *meso*-erythritol or sorbose as a carbon source. An alkaline reaction was observed with citrate, fumarate, gluconate (weak), α-ketoglutarate and malate in both strains, but no reaction occurred with oxalate or tartrate. Utilization of succinate, acetate and propionate was negative only in strain VKM Ac-1804^T. A few amino acids (DL-leucine, DL-methionine and DL-proline) were weakly utilized as a source of nitrogen. Tween 40, Tween 80 and aesculin were hydrolysed, but starch, hypoxanthine and xanthine were not decomposed. Growth occurred in the presence of neomycin and streptomycin (both at 10 µg ml⁻¹), but was inhibited by 6% NaCl and the following antibiotics (at 10 µg ml⁻¹): ampicillin, doxycycline, gentamicin, penicillin, rifampicin and tetracycline.

Taxonomic affiliation of strains

At the time of writing this report, the family *Microbacteriaceae* proposed by Park *et al.* (1993) contained ten genera which are characterized by B-type peptidoglycan (Table 1). Seven of them, *Agromyces* (Gledhill & Casida, 1969), *Clavibacter* (Davis *et al.*, 1984), *Rathayibacter* (Zgurskaya *et al.*, 1993), *Agrococcus* (Groth *et al.*, 1996), *Leucobacter* (Takeuchi *et al.*,

Table 1. Salient characteristics that differentiate *Agreia* gen. nov. from other genera of the family *Microbacteriaceae*

Data from Gledhill & Casida (1969), Komagata & Suzuki (1986), Davis *et al.* (1984), Zgurskaya *et al.* (1993), Groth *et al.* (1996), Takeuchi *et al.* (1996), Suzuki *et al.* (1997), Takeuchi & Hatano (1998), Kämpfer *et al.* (2000) and Evtushenko *et al.* (2000). +, Positive; -, negative.

Genus	Colony colour*	Motility	Diamino acid†	Major MK‡	Fatty acid type§	G + C (mol%)	Growth at 28 °C
<i>Agreia</i> gen. nov.	Y, O, RO, BO	+	DAB, Orn	MK-10	S, A, I	67.0	+
<i>Agrococcus</i>	O, Y	-	DAB	MK-11,12	S, A, I	74	+
<i>Agromyces</i>	Y, W	-	DAB	MK-12	S, A, I	71-76	+
<i>Clavibacter</i>	Y, O, P	-	DAB	MK-9	S, A, I	67-78	+
<i>Cryobacterium</i>	P	-	DAB	MK-10	S, A, I, (12H)	65	-
<i>Curtobacterium</i>	Y, O	+/-	Orn	MK-9	S, A, I, (H)	68-75	+
<i>Frigoribacterium</i>	Y	+	Lys	MK-9	S, A, I	71.7	-
<i>Leifsonia</i>	Y	+	DAB	MK-11	S, A, I	66-73	+
<i>Leucobacter</i>	W	-	DAB	MK-11	S, A, I	66.2	+
<i>Microbacterium</i>	Y, O, W	+/-	Orn/Lys	MK-11, -12, -13, -14	S, A, I	65-72	+
<i>Rathayibacter</i>	Y	-	DAB	MK-10	S, A, I	63-72	+

* BO, Brown-orange; O, orange; P, pink; RO, red-orange; Y, yellow; W, white.

† DAB, 2,4-Diaminobutyric acid; Orn, ornithine; Lys, lysine.

‡ Designation of Collins & Jones (1981).

§ S, Straight-chain saturated; A, anteiso-methyl-branched; I, iso-methyl-branched; H, cyclohexyl fatty acid; 12H, 12-methyl-tetradecenoic acid; components given in parentheses are sometimes present.

1996), *Cryobacterium* (Suzuki *et al.*, 1997) and *Leifsonia* (Evtushenko *et al.*, 2000) possess the DAB-based peptidoglycans, whereas ornithine is the characteristic diamino acid of the genus *Curtobacterium* (Komagata & Suzuki, 1986) and most *Microbacterium* spp. (Takeuchi & Hatano, 1998). The morphology, amino acid composition, acyl type of peptidoglycan, menaquinone profile, phospholipid pattern, fatty acid type and growth temperature were proposed as salient characteristics to differentiate genera of the family at the phenetic level. In addition, D- or L-isomers of DAB (Sasaki *et al.*, 1998), the polyamine pattern (Altenburger *et al.*, 1997) and the presence of cell wall teichoic acids (Shashkov *et al.*, 1993, 1995; Gnizozub *et al.*, 1994) were indicative of some DAB-containing genera or species.

Isolates VKM Ac-1804^T and VKM Ac-1805 were notably distinguished among all the above genera of the family *Microbacteriaceae* at the phenetic level. The most striking features differentiating these strains from other genera of the family were the orange to red-orange or brown-orange pigments of the colonies and the unusual composition of amino acids in their cell walls (see above). No bacteria having a combination of DAB and ornithine as diamino acids in their peptidoglycans have been described so far and the presence of both ornithine and glutamate plus hydroxyglutamate has been reported only in peptidoglycans of *Microbacterium* spp. (Takeuchi & Hatano, 1998). Our strains also differed from most genera of the family *Microbacteriaceae* in their major menaquinone, which is an essential differentiating characteristic of genera con-

taining DAB in their cell walls (Zgurskaya *et al.*, 1993; Groth *et al.*, 1996; Takeuchi *et al.*, 1996; Suzuki *et al.*, 1997; Sasaki *et al.*, 1998). The above differences are in line with the significant phylogenetic separation of strain VKM Ac-1804^T within the *Microbacteriaceae* cluster (Fig. 1). Thus, on the basis of phenetic and phylogenetic distinctions of the gall bacteria from validly described genera of the family *Microbacteriaceae*, it is proposed that these organisms should be assigned to a novel genus, *Agreia* gen. nov. The high level of DNA-DNA similarity between VKM Ac-1804^T and VKM Ac-1805, along with the identity of these strains in their phenotypic characteristics, including fatty acid profiles and the numerous growth and physiological properties, indicate that both strains are members of one species, *Agreia bicolorata* gen. nov., sp. nov.

While this report was being reviewed, the description of another novel genus belonging to the family *Microbacteriaceae*, *Subtercola* gen. nov., was published (Männistö *et al.*, 2000). Strains of this genus were isolated from boreal groundwater and differed markedly from our strains in some essential chemotaxonomic characteristics, e.g. the cell wall peptidoglycan was of the B-type and contained only DAB as diamino acid and lacked ornithine, the major menaquinones were MK-9 and MK-10 in almost equal proportions, and the fatty acid composition and temperature growth optimum differed. The phenotypic differences between our strains and *Subtercola* spp. are supported at the phylogenetic level. The type strain of *Agreia bicolorata* gen. nov., sp. nov. showed

96.8–97.1% 16S rDNA sequence similarity with members of *Subtercola* spp., which is approximately in the same range as similarities reported for other closely related genera of the family *Microbacteriaceae*, e.g. *Subtercola* and *Clavibacter* (95.1–97.1%), or *Clavibacter*, *Frigoribacterium* and *Rathayibacter* (96.1–97.1%) (Kämpfer *et al.*, 2000; Männistö *et al.*, 2000).

Ecology

There is no unambiguous evidence to show whether bacteria assigned to *Agreia bicolorata* gen. nov., sp. nov. are nematode-associates like *Rathayibacter* spp., i.e. carried by nematodes into plants and developing inside plant galls, or secondary settlers of the galls (their surface sections), descending from the microbial communities of the phyllosphere. For the first scenario, arguments may be as follows: (i) *Agreia bicolorata* gen. nov., sp. nov. comprised a significant proportion of the population grown from the ground gall suspension; (ii) the bacteria were not found in plants that lacked nematode galls; (iii) the bacteria formed abundant slime which is thought to be a characteristic feature of nematode-associated bacteria (Bird, 1985); and (iv) their morphology was similar to that of bacteria observed within the galls by electron microscopy (Evtushenko *et al.*, 1994). Nevertheless, there is a probability that the particular bacterial associates of the nematodes are unculturable under conditions used for isolation of gall bacteria and that *Agreia bicolorata* gen. nov., sp. nov. is a representative of the secondary bacterial infection of galls. Further study should elucidate the ecological niche of these bacteria and their relationship with nematodes and other micro-organisms of the nematode–bacteria multi-component complexes.

Description of *Agreia* gen. nov.

Agreia (Ag.re'i.a. N.L. gen. fem. n. *Agreia* of Agre, named to honour Nina S. Agre, a Russian microbiologist).

Colonies are yellow or yellow-orange in young cultures and can be red-orange or brown-orange with age; round, glistening, opaque, butyrous, sometimes fluid. Cells are Gram-positive, non-spore-forming, weakly motile, irregularly shaped rods. Primary branching is rarely observed. Usually mesophilic. Obligately aerobic. Catalase- and oxidase-positive. Cell wall peptidoglycan is of the B-type and contains DAB and ornithine as diamino acids; hydroxyglutamate may be present instead of some of the glutamate. Mycolic acids are lacking. The major menaquinone is MK-10, with minor amounts of MK-9. The principal phospholipids are phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids are anteiso-15:0, anteiso-17:0 and iso-16:0. Forms a distinct lineage within the phylogenetic cluster of actinomycetes of the family *Microbacteriaceae*. The type species is *Agreia bicolorata*.

Description of *Agreia bicolorata* gen. nov., sp. nov.

Agreia bicolorata (bi.co.lo.ra.ta. M.L. n. *bis* two; M.L. adj. *coloratum* coloured; M.L. fem. adj. *bicolorata* two-coloured).

General morphological, chemotaxonomic and growth characteristics are as given above in the genus description. Colonies are yellow-orange to orange in young cultures and red-orange to brown-orange with age, semi-fluid or fluid. Abundant capsule is usually formed. Cells are irregular rods (0.4–0.5 × 1.2–2.5 µm) that are weakly motile. In older cultures, shorter irregular rods predominate as single cells; pairs or short chains with diphtheroid arrangements may be present. A marked rod–coccus growth cycle does not occur. Mesophilic. Thermal optimum for growth is 24–26 °C; growth occurs at 37 °C. Cell wall peptidoglycan is of the B-type and contains DAB, ornithine, alanine, glycine and glutamate plus hydroxyglutamate (1:1:2:2:2 molar ratio). Cell wall sugars are rhamnose, mannose and fucose. DNA G+C content is about 67.0 mol%. A wide range of carbohydrate and organic acids are used for growth; utilization of succinate, acetate and propionate varied in the strains. Only a few amino acids are utilized as a source of nitrogen. H₂S is weakly produced. Voges–Proskauer test is positive and methyl red test is negative. No growth with 6% NaCl. Strains are susceptible to 10 µg ml⁻¹ ampicillin, doxycycline, gentamicin, penicillin, rifampicin and tetracycline, but tolerate the same concentration of neomycin and streptomycin. Isolated from leaf galls on *Calamagrostis neglecta*, induced by the plant-parasitic nematode *Heteroanguina graminophila* (Moscow region, Russia). Type strain is VKM Ac-1804^T (= UCM Ac-620^T), deposited in the All-Russian collection of Microorganisms (VKM) and Ukrainian Collection of Microorganisms (UCM).

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