

Gulosibacter molinativorax gen. nov., sp. nov., a molinate-degrading bacterium, and classification of '*Brevibacterium helvolum*' DSM 20419 as *Pseudoclavibacter helvolus* gen. nov., sp. nov.

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A Gram-positive, molinate-degrading bacterium, strain ON4^T (=DSM 13485^T =LMG 21909^T), was isolated from a mixed bacterial culture able to mineralize the herbicide molinate. The strain was strictly aerobic, oxidase- and catalase-positive and non-acid-fast, with a growth temperature of 10–41 °C. It contained the major menaquinone MK-9 and a cell-wall peptidoglycan based on D-ornithine. 16S rDNA sequence analysis revealed that the strain formed a distinct line of descent in the family *Microbacteriaceae*, showing the highest 16S rDNA similarity (~95%) to members of the genus *Curtobacterium* and '*Brevibacterium helvolum*' DSM 20419 (=ATCC 13715). The latter was reported to have the cell-wall peptidoglycan type B2_γ and the major menaquinone MK-9, which are typical of *Clavibacter*, but it is clearly separated from this genus at the phylogenetic level. Based on low values of 16S rDNA sequence similarity to previously described genera and their distinctive phenotypic characteristics, it is proposed that strains ON4^T and '*B. helvolum*' DSM 20419 be classified as two novel genera and species, with the respective names *Gulosibacter molinativorax* gen. nov., sp. nov. and *Pseudoclavibacter helvolus* gen. nov., sp. nov.

INTRODUCTION

The family *Microbacteriaceae* (Park *et al.*, 1993; Stackebrandt *et al.*, 1997) embraces a large group of actinomycetes with cell-wall peptidoglycan of the B-type (Schleifer & Kandler, 1972). The family currently contains 16 genera, distinguished at the phenotypic level by a number of

chemotaxonomic characteristics, including peptidoglycan diamino acids and respiratory menaquinone composition (Collins & Bradbury, 1992; Evtushenko & Takeuchi, 2003; Sheridan *et al.*, 2003).

'*Brevibacterium helvolum*' DSM 20419 (=ATCC 13715), an organism isolated from butter, has peptidoglycan B2_γ, [L-DAB]-D-Glu-D-DAB (DAB is 2,4-diaminobutyric acid), unsaturated menaquinone with nine isoprene units (MK-9) (Schleifer & Kandler, 1972; Sasaki *et al.*, 1998) and the polyamines 2,3-diaminopropane and cadaverine (Altenburger *et al.*, 1997). Based on comparative analysis of 16S rDNA, Rainey *et al.* (1994) showed that '*B. helvolum*' DSM 20419 formed a separate subline of descent within the radiation of actinomycetes belonging to the family *Microbacteriaceae*. According to Rainey *et al.* (1994), this strain could represent the nucleus of a novel genus.

Abbreviation: DAB, 2,4-diaminobutyric acid.

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Strain ON4^T was isolated from a microbial mixed culture enriched in molinate, which also included four strains of the genera *Pseudomonas*, *Stenotrophomonas* and *Achromobacter* (Barreiros *et al.*, 2003). The strain used molinate as the only carbon, nitrogen and energy source and grew at herbicide concentrations up to 2 mM, although it did not perform its complete degradation (Barreiros *et al.*, 2003). It exhibited the highest 16S rDNA similarity to the misclassified strain '*B. helvolum*' DSM 20419 and could not be affiliated to any previously described genus. In this paper, we describe the morphological, physiological, chemotaxonomic and phylogenetic characteristics of these organisms and propose to accommodate them into two novel genera.

METHODS

Determination of morphological, growth and biochemical characteristics. Wet mounts and staining methods followed by optical microscopic examination were used to assess cell morphology, Gram and Ziehl-Neelsen reactions, production of spores, accumulation of poly-hydroxybutyrate granules and motility, according to procedures described previously (Doetsch, 1981; Smibert & Krieg, 1981). Cell morphology of isolate ON4^T was further studied by electron microscopy after negative staining with 2% (w/v) sodium phosphotungstate (pH 7.0) using a Hitachi H-600 transmission electron microscope at 75 kV.

Growth temperature, pH and salinity ranges were examined by measuring turbidity (at 610 nm) of cultures in 250 ml screw-capped Erlenmeyer flasks containing 50 ml LB medium (Carlton & Brown, 1981) incubated in an orbital water-bath shaker. The pH range for growth was examined in medium buffered with 12 mM MES (Sigma) at pH 5.0–6.0 or with 15 mM CAPS (Sigma) at pH 9.5–11.0. Anaerobic growth of strains and oxidase (tetramethyl-*p*-phenylenediamine), catalase, nitrate reductase and Tweenase reactions were examined at 30 °C as described by Smibert & Krieg (1981) using 3-day cultures grown on LB agar (LA). Other enzymic activities were tested using the API ZYM system, following the instructions of the manufacturer. The nutritional pattern was characterized using the API 50CH system and a defined medium B (Manaiá *et al.*, 2003). The ability of strain ON4^T to use API 50CH carbon sources was also tested in mineral medium supplemented with different concentrations of yeast extract (0.01–0.2%, w/v). Additionally, the aerobic dissimilation of 95 carbon sources was tested using the Biolog GP microplate system, according to the manufacturer's indications.

Determination of chemotaxonomic characteristics. The polar lipids of strains ON4^T and DSM 20419 were characterized using cultures grown in LB medium, at 30 °C, at the late exponential phase of growth. Lipid extractions were performed as described previously (Tindall, 1990). Individual polar lipids were separated by two-dimensional TLC as described by Minnikin *et al.* (1977). For the analysis of methylated fatty acids (FAMES) of strains ON4^T and DSM 20419, cells were grown for 3 days on LA medium at 30 °C. FAME extraction and analysis were performed as described by Moreira *et al.* (2000). For the analysis of respiratory quinones, strains ON4^T and DSM 20419 were cultured on LA medium, harvested, freeze-dried and extracted according to Tindall (1989), and the extracts were analysed as described by Moreira *et al.* (2000). The peptidoglycan of strain ON4^T was analysed as described previously (Schleifer & Kandler, 1972), using TLC on cellulose sheets instead of paper chromatography. L- and D-ornithine could be distinguished by their small but significant mobility in the solvent system used for the characterization of diamino acids. For the determination of DNA base composition of strains ON4^T and DSM

20419, genomic DNA was isolated as described by Cashion *et al.* (1977) and the DNA G + C content was analysed by HPLC (Mesbah *et al.*, 1989).

16S rRNA sequence determination and phylogenetic analysis.

The sequence of the 16S rRNA gene of isolate ON4^T was determined after PCR amplification from total DNA extracts using primers 27f and 1492r (Lane, 1991) as described previously (Nogales *et al.*, 2001). The nucleotide sequence of purified PCR products was determined by using the BigDye Terminator cycle sequencing kit and ABI377 and 310 sequencers (Applied Biosystems), according to the manufacturer's instructions. The nucleotide sequence was aligned with reference sequences using the ARB package (<http://www.arb-home.de>). Phylogenetic trees were constructed using the ARB package and the distance methods of neighbour-joining and Fitch [using the correction of the Jukes & Cantor (1969) for calculation of evolutionary distances] as well as parsimony methods. Bootstrap analysis (1000 replicates) was done using the PHYLIP package (Felsenstein, 1989). Ambiguous nucleotide positions were excluded from the calculations.

RESULTS AND DISCUSSION

Morphological, biochemical and growth characteristics

On LA medium, strain ON4^T formed white, opaque colonies, 1 mm in diameter, while colonies of strain DSM 20419 were yellow and 2 mm in diameter. Both strains formed irregularly rod-shaped cells (see Supplementary Figure in IJSEM Online), and strain ON4^T had a tendency to form short filaments. Strains ON4^T and DSM 20419 stained as Gram-positive, were non-acid-fast and non-spore-forming, had no visible deposits of poly- β -hydroxybutyrate and were non-motile and catalase-positive. Both organisms were observed to be strictly aerobic and positive for oxidase, although a very weak reaction was observed for strain DSM 20419. Isolate ON4^T was able to reduce nitrate under both aerobic and anaerobic conditions, although growth did not occur in the absence of oxygen (Table 1).

The growth temperature optima were 28–30 °C for strain DSM 20419 and 35–37 °C for strain ON4^T, although the latter also grew well at 28–30 °C. Metabolic activities of strain ON4^T studied in the presence of vitamins or yeast extract were restricted to only 15 of 110 compounds tested and, of the few carbon sources utilized, most contained nitrogen. A slow reaction was observed with the only sugar, α -D-glucose. Strain DSM 20419 was much more versatile metabolically and utilized a variety of organic compounds, including sugars, alcohols, organic acids, amino acids and nucleotides. Details of the physiological and biochemical characteristics of the strains are given in Table 1 and in the species descriptions.

Chemotaxonomic characteristics

Cell-wall analysis revealed that strain ON4^T had a B-type peptidoglycan based upon D-ornithine. Strains ON4^T and DSM 20419 had the major isoprenoid quinone MK-9 (about 95%), with MK-8 as a minor component. The polar

Table 1. Phenotypic characteristics of strains ON4^T and DSM 20419^T

Both strains produced alkaline phosphatase, esterase (C₄), esterase lipase (C₈), lipase (C₁₄), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Neither strain produced trypsin, chymotrypsin, α -galactosidase, β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase or amylase. Both strains were positive or showed weak reactions for the utilization of pyruvic acid, methyl pyruvate, L-glutamic acid, 2,3-butanediol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate and uridine 5'-monophosphate. Neither strain was able to use the following carbon sources: adonitol, dulcitol, arbutin, starch, methyl β -D-glucoside, salicin, α - and β -cyclodextrin, glycogen, inulin, mannan, *N*-acetyl-D-mannosamine, amygdalin, D- and L-arabinose, D- and L-arabitol, erythritol, D- and L-fucose, D-galactose, D-galacturonic acid, maltotriose, D-lyxose, D-melezitose, D-melibiose, L-xylose, methyl α - and β -D-galactoside, methyl β -xyloside, 3-methyl-D-glucose, methyl α -D-glucoside, methyl α -D-mannoside, palatinose, L-rhamnose, D-ribose, L-sorbose, sedoheptulosan, stachyose, D-tagatose, xylitol, acetic acid, α - and β -hydroxybutyric acid, α -ketoglutaric acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, D- and L-malic acid, monomethyl succinate, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, alaninamide, D-alanine, L-alanyl glycine, L-asparagine, glycyl L-glutamic acid, L-pyroglytamic acid, L-serine, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and DL- α -glycerol phosphate. w, Weak.

Characteristic	Strain ON4 ^T	Strain DSM 20419 ^T
Colony colour	White	Yellow
Range of growth:		
Temperature (°C)	10–41	10–35
pH	5.5–10.5	6–10
NaCl (% w/v)	0–7	0–6
Reduction of nitrate	+ (nitrite)	–
Presence of:		
β -Galactosidase	–	+
α -Glucosidase	–	+
Utilization of:		
Tween 40, <i>N</i> -acetyl D-glucosamine, cellobiose, D-fructose, gentiobiose, lactulose, D-mannose, D-psicose, sucrose, D-trehalose, turanose, D-xylose, glycerol, D-mannitol, D-sorbitol, D-gluconic acid, γ -hydroxybutyric acid, L-lactic acid	–	+
<p><i>p</i>-Hydroxyphenylacetic acid</p>	+	–
α -D-Glucose	w	+
Dextrin	–	w
L-Alanine, aesculin	–	+
Putrescine	+	w

lipid patterns of both strains were composed of diphosphatidylglycerol, phosphatidylglycerol and one unknown minor glycolipid. The predominant cellular fatty acids detected in strains ON4^T and DSM 20419 were anteiso-C_{15:0} (46.9 and 45%), iso-C_{16:0} (33.2 and 23.3%) and anteiso-C_{17:0} (9.7 and 19.4%) (see Supplementary Table in IJSEM Online). The DNA G+C contents were 64.5 mol% for strain ON4^T and 67.0 mol% for DSM 20419. The above characteristics are typical of the family *Microbacteriaceae* (Collins & Bradbury, 1992; Evtushenko & Takeuchi, 2003).

16S rDNA sequence analysis

Nearly the complete nucleotide sequence of the 16S rRNA gene (1430 nt) of isolate ON4^T was determined. Phylogenetic analysis based on 16S rDNA sequences supported the affiliation of the strain to the family *Microbacteriaceae*. The strain formed a distinct lineage adjacent to '*B. helvolum*' DSM 20419 (Fig. 1) when a different set of strains and different algorithms were used to generate the phylogenetic trees, although the stability of the branch was not confirmed by a high bootstrap resampling value (not shown). Pairwise sequence similarities calculated for 16S rDNA

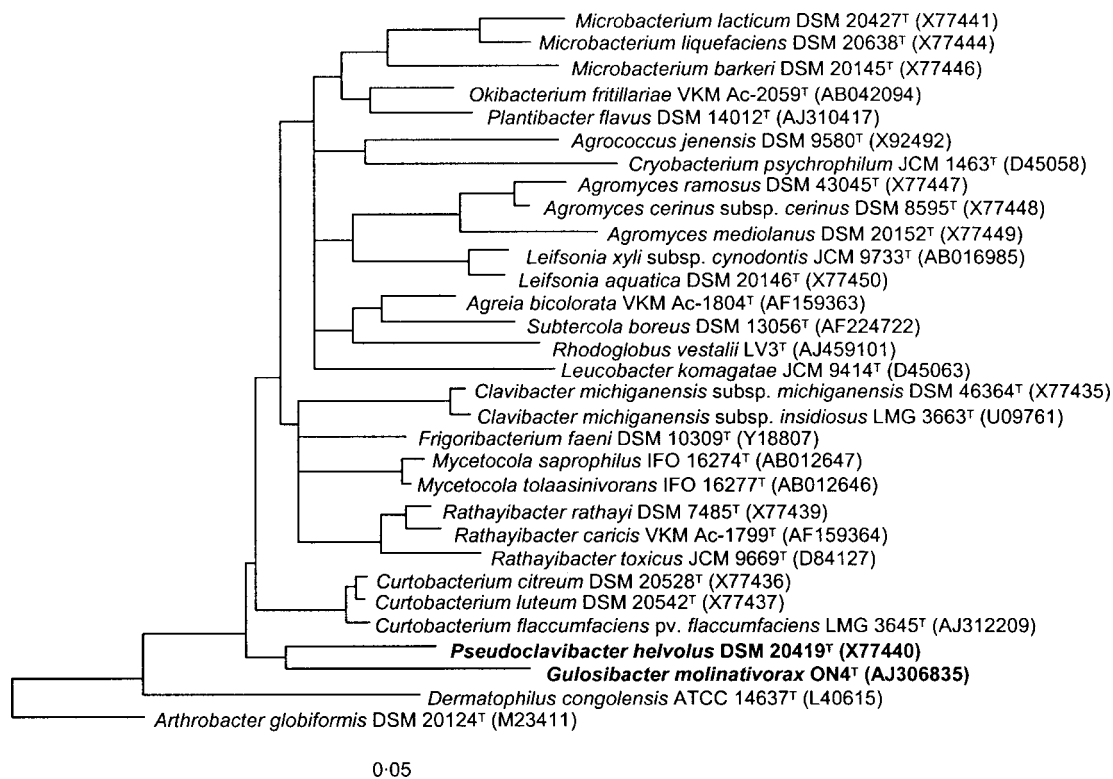


Fig. 1. Phylogenetic relationships of the 16S rDNA sequences of strains ON4^T and '*B. helvolum*' DSM 20419 with related genera within the family *Microbacteriaceae*. Evolutionary distance matrices were calculated using the correction of Jukes & Cantor (1969). The dendrogram was constructed using the neighbour-joining algorithm (Saitou & Nei, 1987), using a 50% conservation filter (total positions compared, 1331). Multifurcations were introduced using the ARB package for those branches that did not show a stable position when different algorithms were used for the analysis, which corresponded to branches with low bootstrap values (1000 replicates were done). The 16S rDNA sequence of *Arthrobacter globiformis* DSM 20124^T was used as the outgroup.

sequences in the region between positions 52 and 1459 (*Escherichia coli* numbering) revealed that ON4^T exhibited the highest similarity to '*B. helvolum*' and representatives of *Curtobacterium flaccumfaciens* (~95%). The 16S rDNA sequence of '*B. helvolum*' was most similar to the sequence of *Curtobacterium luteum* (95.4%).

Taxonomic conclusions

The similarities of the 16S rDNA sequences of strains ON4^T and '*B. helvolum*' DSM 20419 and each of these strains and other members of the *Microbacteriaceae* are within the range of 16S rRNA gene sequence similarities observed between those of the genera of the family. The organisms are also distinguished from most genera of the family by a combination of their menaquinone and peptidoglycan composition, which are salient chemotaxonomic markers of the genera belonging to the family *Microbacteriaceae* (Sasaki *et al.*, 1998; Evtushenko & Takeuchi, 2003). Although strain ON4^T is similar in these characteristics to *Curtobacterium*, it can be differentiated from this genus by positive oxidase reaction, a higher optimal temperature for growth, a lower G + C content and

the specific nutritional pattern (Table 2). Strain DSM 20419 has the peptidoglycan and the major menaquinone typical of the genus *Clavibacter* (Sasaki *et al.*, 1998). However, a distinctive polyamine pattern (Altenburger *et al.*, 1997) along with some physiological properties clearly separate it from *Clavibacter* species. Thus, based on both molecular genetic and phenotypic data, it is proposed to classify the studied organisms in two novel genera and species, *Gulosibacter molinativorax* gen. nov., sp. nov. (type strain ON4^T = DSM 13405^T = LMG 21909^T) and *Pseudoclavibacter helvolus* gen. nov., sp. nov. (type strain DSM 20419^T = ATCC 13715^T).

Description of *Gulosibacter* gen. nov.

Gulosibacter (Gu.lo'si.bac.ter. L. adj. *gulosus* fond of titbits, dainty feeder, N.L. masc. n. *bacter* rod; N.L. masc. n. *Gulosibacter* rod fond of titbits).

Irregular rod-shaped cells; may tend to form short filaments and to branch. Gram-positive. Non-spore-forming. Aerobic. Catalase-positive. Chemo-organotrophic. The diamino acid of peptidoglycan is D-ornithine. The major respiratory quinone is menaquinone MK-9. Polar lipids

Table 2. Differentiating characteristics of strains ON4^T and DSM 20419^T and related genera of the family *Microbacteriaceae*

Taxa: 1, strain ON4^T; 2, strain DSM 20419^T; 3, *Curtobacterium*; 4, *Clavibacter*. Data were obtained from Collins & Bradbury (1992), Altenburger *et al.* (1997), Sasaki *et al.* (1998), Behrendt *et al.* (2002) and this study. Characteristics are scored as: +, positive; −, negative; NA, no data available; W, weak; d, variable characteristic among the genus. Abbreviations: DAP, 2,3-diaminopropane; CAD, cadaverine; SPD, spermidine; SPM, spermine.

Characteristic	1	2	3	4
Optimal growth temperature (°C)	35–37	28–30	25–30	21–26
Oxidase test	+	W	−	−
Utilization of:				
m-Inositol	−	W	+	d
α-D-Lactose	−	+	+	d
D-Maltose	−	+	+	d
D-Melibiose	−	−	+	−
D-Melezitose	−	−	+	−
D-Raffinose	−	+	−	−
D-Ribose	−	−	+	d
Tween 80	−	+	d	−
Diamino acid of peptidoglycan	D-Orn	DL-DAB	D-Orn	DL-DAB
Major polyamines	NA	DAP, CAD	SPD, SPM	SPD, SPM
DNA G + C content (mol%)	65	67	68–75	65–78
Common habitat/site of isolation	Herbicide-contaminated soil and water	Butter	Plant material	Plant material

are diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The predominant fatty acids are 12-methyl-tetradecanoic acid (anteiso-C_{15:0}), 14-methyl-pentadecanoic acid (iso-C_{16:0}) and 14-methyl-hexadecanoic acid (anteiso-C_{17:0}). The DNA G + C content is about 65 mol%. The type species is *Gulosibacter molinativorax*.

Description of *Gulosibacter molinativorax* sp. nov.

Gulosibacter molinativorax [mo.li.na'ti.vo.rax. N.L. n. *molinas* molinate (a herbicide); L. adj. *vorax* devouring, ravenous, voracious; N.L. masc. adj. *molinativorax* molinate-degrading].

Irregular rod-shaped cells, 0.8–1.0 µm long and 0.5–0.6 µm wide, with tendency to form short filaments and branching. Non-motile. Colonies grown on LA medium are white and about 1 mm in diameter after 48–72 h of growth. Gram-positive. Strictly aerobic. Oxidase test is positive. Mesophilic: growth occurs at 10–41 °C, with optimum growth at 35–37 °C. The pH growth range is 5.5–10.5. Maximal growth rate is observed in the presence of 1% (w/v) NaCl; 8% NaCl inhibits growth. Nitrate is reduced to nitrite. A few organic compounds, including putrescine, methyl pyruvate, *p*-hydroxyphenylacetic acid, adenosine, inosine, thymidine and uridine, are utilized. Growth occurs in mineral medium supplemented with the thiocarbamate herbicide molinate. The DNA G + C content is 64.5 mol%.

The type strain, ON4^T (= DSM 13485^T = LMG 21909^T), was

isolated from a mixture of contaminated soil and water collected from a site of effluent discharge of a molinate-producing chemical plant in southern Portugal.

Description of *Pseudoclavibacter* gen. nov.

Pseudoclavibacter (Pseu.do.cla'vi.bac.ter. Gr. adj. *pseudēs* false; N.L. masc. n. *Clavibacter* a bacterial generic name; N.L. masc. n. *Pseudoclavibacter* false *Clavibacter*).

Forms rod-shaped cells. Non-spore-forming. Gram-positive. Aerobic, catalase-positive. Chemo-organotrophic; various organic compounds are used as carbon and energy sources, including sugars, alcohols, organic acids and nitrogenated bases. The peptidoglycan is B2γ [L-DAB]–D-Glu–D-DAB (Schleifer & Kandler, 1972; Sasaki *et al.*, 1998). Menaquinone MK-9 is the major respiratory quinone. Polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The predominant fatty acids are 12-methyl-tetradecanoic acid (anteiso-C_{15:0}), 14-methyl-pentadecanoic acid (iso-C_{16:0}) and 14-methyl-hexadecanoic acid (anteiso-C_{17:0}). Major polyamines are 1,3-diaminopropane and cadaverine (Altenburger *et al.*, 1997). The G + C content of DNA is about 67 mol%. The type species is *Pseudoclavibacter helvolus*.

Description of *Pseudoclavibacter helvolus* sp. nov.

Pseudoclavibacter helvolus (hel.vo'lus. L. adj. *helvolus* pale yellow, yellowish).

Irregular rod-shaped cells, 0.9–1.1 µm long and 0.4–0.5 µm wide. Non-motile. Colonies grown on LA medium are yellow and about 2–3 mm in diameter after 48–72 h of growth. Gram-positive. Strictly aerobic. Oxidase reaction is weakly positive. Mesophilic; growth occurs at 10–35 °C, with optimum growth at 28–30 °C. The pH growth range is 6–10. Maximal growth rate is observed in the presence of 1% (w/v) NaCl; 8% NaCl inhibits growth. Aesculin, L-alanine, α-D-glucose, D-fructose, α-D-lactose, D-maltose, sucrose, D-raffinose, N-acetyl-D-glucosamine, glycerol, D-mannitol, pyruvic acid, methyl pyruvate, Tween 80, adenosine, 2'-deoxyadenosine, inosine, thymidine and uridine are utilized. Lipases are produced. The DNA G+C content is 67 mol%.

The type strain is DSM 20419^T (= ATCC 13715^T), isolated from butter.

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