RESEARCH LETTER

Description of *Oxalicibacterium horti* sp. nov. and *Oxalicibacterium faecigallinarum* sp. nov., new aerobic, yellow-pigmented, oxalotrophic bacteria

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Abstract

Three strains of aerobic, Gram-negative, rod-shaped, non-spore-forming, yellow-pigmented bacteria (OD1^T, YOx^T and NS13), which were isolated in previous studies by enrichment in a mineral medium with potassium oxalate as the sole carbon source, were characterized. On the basis of 16S rRNA gene sequence similarity, strains OD1^T, YOx^T and NS13 belong to the *Betaproteobacteria*, most closely related to *Oxalicibacterium flavum* TA17^T (97.2–99.7% sequence similarity). The major whole-cell fatty acids were $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{17:0}$ cyclo. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strains OD1^T and YOx^T from *O. flavum* TA17^T and from each other. Therefore, it is concluded that the strains OD1^T and YOx^T represent novel species within the genus *Oxalicibacterium*, for which the names *Oxalicibacterium horti* sp. nov. (type strain OD1^T = DSM 21640^T = NBRC 13594^T) and *Oxalicibacterium faecigallinarum* sp. nov. (type strain YOx^T = DSM 21641^T = CCM 2767^T) are proposed.

Introduction

The genus *Oxalicibacterium*, with the type species *Oxalicibacterium flavum*, was established by Tamer *et al.* (2002) in the order *Burkholderiales*, class *Betaproteobacteria*, to accommodate an aerobic oxalic acid-utilizing yellow-pigmented bacterium isolated from a litter of oxalate-producing plants (*Rumex* sp. and *Mesembryanthemum* sp.). *Oxalicibacterium flavum* was characterized by a relatively high growth rate and yield in a basal mineral medium with oxalate as the sole carbon source, together with a limited substrate spectrum. Later, the genus has been assigned to the family *Oxalobacteraceae* according to Garrity *et al.* (2005).

In the present article, we describe the physiological, chemotaxonomic and phylogenetic characteristics of three aerobic, yellow-pigmented, oxalate-utilizing strains sharing the highest 16S rRNA gene sequence similarity to *O. flavum* TA17^T.

Materials and methods

Bacterial strains

Strain NS13 was isolated from a forest soil sample (Sahin *et al.*, 2002), strain OD1^T was from a garden soil sample (Jayasuriya, 1955) and strain YOx^T was from a chicken dung sample (Chandra & Shethna, 1975). All of the strains were isolated by enrichment in a mineral medium with potassium oxalate as the sole carbon source.

Phenotypic characterization

Physiological and biochemical tests were performed at 28 °C. Conventional biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). API 20NE and API ZYM strips (BioMérieux) and Biolog GN plates were used according to the manufacturer's instruction, except that nitrate reduction and indole production

from tryptophan were read after 2 days, while other reactions of the API 20NE strips were observed for 7 days. Utilization and assimilation of carbohydrates was determined on Biolog GN plates and incubated for 7 days before reading.

Tolerance to metals was tested by growing the isolates on nutrient lactate agar that contained 5 mM of one of the following compounds: $Cd(NO_3)_2$, $NiCl_2 \cdot 6H_2O$ and ZnCl. Antimicrobial susceptibility testing was performed by the disk-diffusion method using antibiotic-impregnated disks (Oxoid). The following antibiotics were tested: chloramphenicol (30 mg), colistin sulfate (10 mg), erythromycin (15 mg), penicillin G (10 IU) and streptomycin (10 mg). An inhibition zone of 12 mm or more in diameter was scored as sensitive to that antibiotic.

Lipophilic pigments were extracted with acetone–methanol (3 : 1 v/v); spectra were determined in the same solvent using a UV/visible spectrophotometer (Shimadzu 1601).

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequences were analyzed as described by Sahin *et al*. (2008). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980) without taking into account the alignment gaps and unidentified base positions. Phylogenetic trees were constructed from the distance data using the neighbor-joining method of Saitou & Nei (1987). The robustness for individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

Chemotaxonomic characterization

For analysis of fatty acids, cells were grown on tryptic soy broth agar (Difco) at 28 1C for 4 days. Cells were saponified, methylated to create fatty acid methyl esters and extracted as described previously (Kämpfer & Kroppenstedt, 1996). Peaks were automatically integrated, and fatty acid names and percentages were determined using the MICROBIAL IDENTIFICATION standard software package MIDI (Sasser, 1990).

Ribotyping, DNA base compositions and DNA--DNA hybridizations

Ribotyping of strains was performed with the automated RiboPrinter Microbial Characterization System (Qualicon, DuPont, Wilmington, DE). Riboprint analyses, using EcoRI, followed described methods (Bruce, 1996).

For determination of the G1C content, DNA was degraded to nucleosides using P1 nuclease and bovine intestinal mucosa alkaline phosphatase, as described by Mesbah *et al*. (1989). The nucleosides were separated by reversed-phase HPLC (Shimadzu Apparatus) according to the method described by Tamaoka & Komagata (1984), and the G1C content was calculated from the ratio of deoxyguanosine to thymidine.

The degree of DNA–DNA relatedness between the three isolates and previously described *O. flavum* type strain ($TA17^T$) was determined by measuring the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (DT_m) as described by González & Sáiz-Jiménez (2005).

Results and discussion

Phenotypic characteristics

The strains had a very limited substrate spectrum regarding the substrates in the API 20NE and Biolog GN plates. All strains utilized oxalate, formate, glycollate, lactate, pyruvate, succinate and malate. Other carboxylic acids, alcohols and all amino acids, except alanine, were not utilized. Differential phenotypic characteristics between the type strains of the three species of the genus *Oxalicibacterium* are given in Table 1. All strains produced a yellow, water-insoluble intracellular pigment. The absorption spectrum of the

Table 1. Differential characters of *Oxalicibacterium* strains

Reaction	1	2	3	4
Biolog GN				
cis-Aconitic acid				w
α-Hydroxybutyric acid		1	1	1
α-Ketobutyric acid				w
α-Ketoglutaric acid	w	w	1	1
Succinic acid			1	w
L-Alanine			1	1
L-Glutamic acid	1	w		1
API ZYM				
Alkaline phosphatase	1	1		
Valine arylamidase	1	1		
Acid phosphatase		1		
Naphthol phosphohydrolase	w	1		w
Resistance to				
Chloramphenicol (30 mg)	1	1	1	
Erythromycin (15 mg)				
Streptomycin (10 mg)				
Penicillin (10 mg)	1	1	1	
Colistin (10 mg)				
Tolerance to heavy metals (5 mM)				
$Cd(NO_3)_2$	1	1	1	
ZnCl	1	1	1	1
$NiCl_2 \cdot 6H_2O$	1			
DNA G1C content (mol%)	63.5	63.4	59.7	55.6

Strains: 1, *Oxalicibacterium flavum* $TA17^T$; 2, *O. flavum* NS13; 3, $OD1^T$; 4, YOx^T. Biolog GN and API 20NE reactions after 7-day incubation at 28 1C. All strains were positive for: (API 20NE) malate, (API ZYM) esterase (C4), esterase lipase (C8), leucine arylamidase and (Biolog GN plates), formic acid, DL-lactic acid, bromosuccinic acid, alanine amide and D-alanine, but negative for the other substrates or reactions of the panels not mentioned. , negative; 1, positive; w, weakly positive.

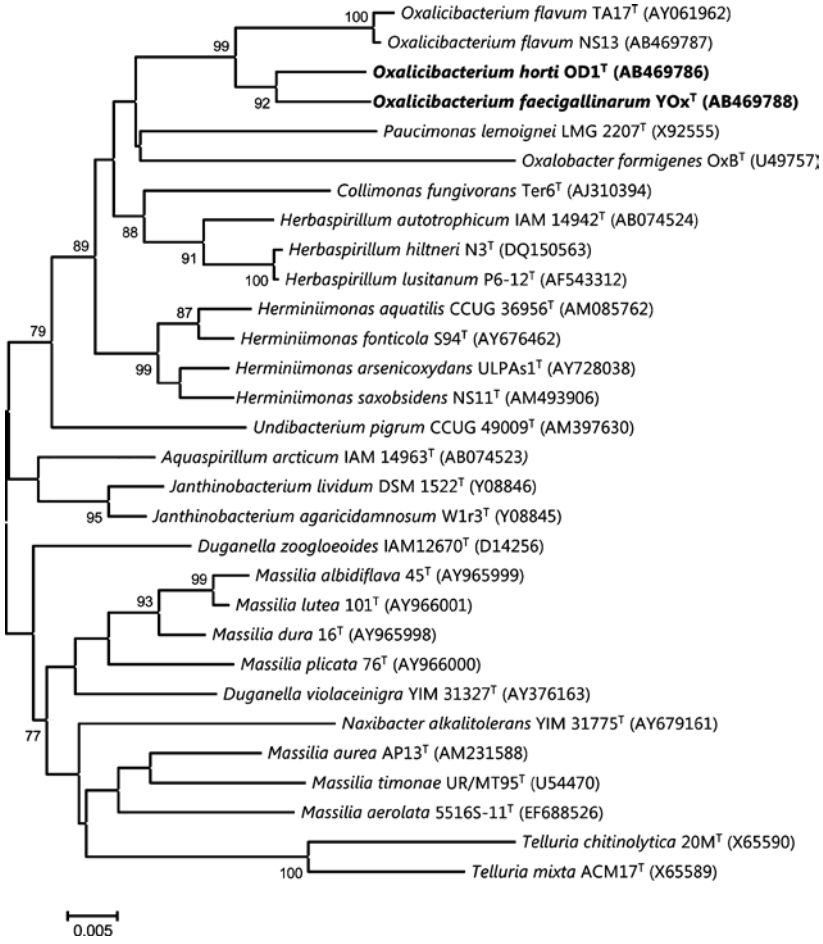

Fig. 1. Phylogenetic tree, based on neighbor-joining method (Saitou & Nei, 1987), derived from an alignment comprising 16S rRNA gene partial region sequences (1367 bp). The data set was resampled 1000 times using the bootstrap option, and the percentage values are given at the nodes. The scale bar indicates the number of substitutions per nucleotide position. Bold type indicates two new species.

pigment is almost the same for all strains, which showed major peaks between 430–435 and 450–455 nm, with a shoulder at 480 nm. During the cultivation on tryptic soy agar or nutrient lactate agar plates, a variation in the colony appearance was observed, especially for strains OD1^T and YOx^T. The variants are (1) circular, raised, more or less translucent, with an entire margin and/or (2) a wrinkled surface with an undulate margin. All variants had similar protein and FT-IR patterns (data not shown). Strains OD1^T, YOx^T and NS13 were able to grow in the presence of 25 g L^{1} potassium oxalate. The optimal growth in mineral medium with potassium oxalate as the sole source of carbon and energy occurred as 8 g L^{1}.

Phylogenetic and chemotaxonomic characteristics

Phylogenetic analyses using the 16S rRNA gene sequences indicated that strains OD1^T, YOx^T and NS13 belong to the family *Oxalobacteraceae* of the b-subclass of the *Proteobacteria*; *O. flavum* TA17^T showed 97.17%, 97.44% and 99.73% sequence similarity in the 16S rRNA gene with strains OD1^T, YOx^T and NS13, respectively. 16S rRNA gene sequence similarities were **O** 96.2% with all established type species of all the genera of the family *Oxalobacteraceae* (Fig. 1).

The fatty acid compositions of the genus *Oxalicibacterium* were not included in the species descriptions earlier. The major fatty acids were $C_{16:0}$ (14–24%), summed feature 3 ($C_{16:1}$o7c/$C_{15:0}$ iso 2OH; 11.9–37.3%) and moderate amounts of $C_{18:1}$o7c (4.2–17%). In addition, $C_{10:0}$ 3OH was the only hydroxylated fatty acid detected (Table 2). Furthermore, $C_{18:1}$o7c (17%) and summed feature 3 (37.3%) seems to be present in strain YOx^T in significantly higher amounts than all other *Oxalicibacterium* type species examined. The fatty acids $C_{18:1}$o6c (11%) and $C_{13:0}$ (0.5%) were detected only in strains YOx^T and OD1^T, respectively, in contrast to others. In conclusion, *Oxalicibacterium* type strains can be distinguished from each other based on their fatty acid profiles.

DNA--DNA hybridizations and ribotyping

The fragment patterns of strain *O. flavum* TA17^T and of the strains OD1^T, YOx^T and NS13 varied, resulting in a unique pattern for each of the strains (Fig. 2). The lack of a high

similarity of strains OD1^T and YOx^T to the reference type strain *O. flavum* TA17^T excludes the possibility of their affiliation to the *O. flavum* genomospecies. As displayed in the dendrogram, the patterns of the type strains of *O. flavum* TA17^T and the strain NS13 showed high similarities, and most of the differences observed were within the target recognition sites of the restriction enzymes used in the analysis.

The DNA–DNA relatedness studies among strains OD1^T, YOx^T and *O. flavum* TA17^T, sharing a 16S rRNA gene sequence similarity of 97.4–97.2%, showed that the DT_m values were 4 6 lC, which confirms that strains OD1^T and YOx^T do not belong to the genospecies *O. flavum*. The DT_m value of *O. flavum* TA17^T and strain NS13, sharing a 16S rRNA gene sequence similarity value of 99.73%, is 4.3 lC. The reassociation values of strains TA17^T-OD1^T, TA17^T-YOx^T, YOx^T-NS13 and YOx^T-OD1^T were 9.7, 6, 14 and 8 lC, respectively, which are well above the 5 lC cut-off point recommended for the delineation of species (Wayne *et al*., 1987; Rosselló-Mora & Amann, 2001).

Table 2. Whole-cell fatty acid composition of *Oxalicibacterium* strains

Fatty acids	1	2	3	4
$C_{13:0}$	–	–	0.5	–
$C_{14:0}$	0.9	1.2	5.4	4.2
$C_{16:0}$	24.1	22.6	14.0	20.1
$C_{17:0}$	3.7	5.0	3.8	1.3
$C_{18:0}$	TR	–	–	0.7
$C_{9:0}$ 3OH	TR	–	–	–
$C_{10:0}$ 3OH	8.4	8.4	6.6	4.6
$C_{15:1}$o6c	1.9	1.3	1.8	–
$C_{16:1}$o5c	0.9	–	0.8	TR
$C_{18:1}$o9c	–	–	1.7	17.0
$C_{18:1}$o7c	4.9	4.2	11.9	11.1
$C_{18:1}$o6c	–	–	–	–
$C_{18:1}$o5c	1.6	1.6	2.7	2.2
$C_{17:0}$ cyclo	19.7	19.6	17.1	1.1
$C_{19:0}$ cyclo o8c	14.7	24.4	14.9	37.3
Summed feature 3	18.4	11.9	18.8	

Strains: 1, *Oxalicibacterium flavum* TA17^T; 2, *O. flavum* NS13; 3, OD1^T; 4, YOx^T. Cells were grown on tryptic soy broth (TSB) agar at 28 lC for 3 days. Data are percentages of total fatty acids. TR, fatty acid content o 0.5%. Summed feature 3 comprises 16:1o7c, 15 iso 2OH or any combination of these fatty acids.

On the basis of the results described above, it can be concluded that the strains OD1^T and YOx^T represent novel species within the genus *Oxalicibacterium*, for which the names *Oxalicibacterium horti* sp. nov. and *Oxalicibacterium faecigallinarum* sp. nov. are proposed, respectively.

Description of *Oxalicibacterium horti* sp. nov.

Oxalicibacterium horti (hor'ti. L. gen. n. *horti*, of a garden, isolated from a garden soil).

Gram-negative, small rods 0.5 x 1.5 mm, motile by polar flagella. No spores found. Colonies on nutrient agar (Oxoid CM3) with 0.2% lactate are yellow pigmented. Forms smooth, raised, opaque with entire edges; diameter is up to 1.5–2 mm after 3 days of incubation at 28 lC. Growth occurs weakly at 37 lC, but not at 42 lC. Optimum growth occurs at 25 lC and pH 7.0. Grows in media containing 3% NaCl. Oxidase and catalase positive. Nitrate is not reduced to nitrite. Poly-b-hydroxybutyrate is accumulated. Negative for indole production, arginine dihydrolase, urease, esculin, casein and gelatine hydrolysis and b-galactosidase. Oxalate, DL-lactate, glycollate, DL-malate and succinate are utilized as the sole carbon and energy sources, but not other carbohydrates or carboxylic acids, acetate, citrate, malonate, methanol or ethanol. Substrate utilization, enzyme production and other physiological characteristics are given in Table 2.

The main fatty acids are summed feature 3 ($C_{16:1}$o7c/$C_{15:0}$ iso 2OH; 18.8%), $C_{16:0}$ (14%), $C_{17:0}$ cyclo (17.1%), $C_{18:1}$o7c (11.9%) and $C_{19:0}$ cyclo o8c (14.9%). Does not contain $C_{18:0}$ and $C_{18:1}$o6c fatty acids. The G1C content of DNA is 59.7 mol% (as determined by HPLC).

The type strain, OD1^T (= DSM 21640^T = NBRC 13594^T), was isolated from garden soil after enrichment with oxalate in a mineral medium.

Description of *Oxalicibacterium faecigallinarum* sp. nov.

Oxalicibacterium faecigallinarum (L. n. *faex faecis*, the dregs, feces; L. gen. pl. n. *gallinarum*, of hens; N.L. gen. pl. n. *faecigallinarum*, isolated from feces of hens).

Gram-negative, small rods 0.75 x 1.5 mm, motile by polar flagella. No spores found. Colonies on nutrient agar (Oxoid

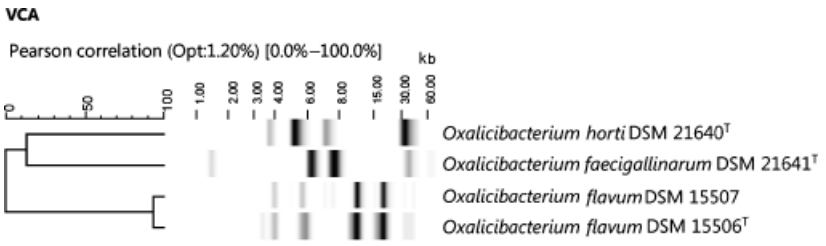

Fig. 2. Diversity of normalized ribotype patterns found in type strains of *Oxalicibacterium*. Cluster analysis was performed using the unweighted pair group method with the arithmetic averages method based on the Pearson correlation coefficient. VCA indicates a standard EcoRI batch.

CM3) with 0.2% lactate are yellow pigmented. Forms smooth, glistening, raised, translucent with entire edges; the diameter is up to 1–1.5 mm after 3 days of incubation at 28 lC. Growth occurs at 37 lC, but not at 42 lC. Optimum growth occurs at a pH between 7.0 and 7.5. Grows in media containing 3% NaCl. Oxidase and catalase positive. Very limited substrate spectrum. Oxalate, DL-lactate and DL-malate are utilized as the sole carbon and energy sources, but not other carbohydrates or carboxylic acids, acetate, citrate, malonate, methanol or ethanol. No acid produced from glucose, galactose, arabinose, sucrose and lactose. Nitrate is not reduced to nitrite. Indole and H_2S are not produced. Substrate utilization, enzyme production and other physiological characteristics are given in Table 2. The main fatty acids are summed feature 3 ($C_{16:1}$O7c/$C_{15:0}$ iso 2OH; 37.3%), $C_{16:0}$ (20.1%), $C_{18:1}$O7c (17%) and $C_{18:1}$O6c (11.1%). Does not contain $C_{15:1}$O6c, $C_{16:1}$O5c and $C_{18:1}$O5c fatty acids. The G1C content of DNA is 55.6 mol% (as determined by HPLC).

The type strain, YOx^T (= DSM 21641^T = CCM 2767^T), was isolated from chicken dung after enrichment with oxalate in a mineral medium.

Acknowledgement

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Statement

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains OD1^T, NS13 and YOx^T are AB469786, AB469787 and AB469788, respectively.

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