New aerobic ammonium-dependent obligately oxalotrophic bacteria: description of *Ammoniphilus oxalaticus* gen. nov., sp. nov. and *Ammoniphilus oxalivorans* gen. nov., sp. nov.

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The genus *Ammoniphilus* is proposed for aerobic endospore-forming Gram-variable rod-shaped bacteria, which are ammonium-dependent, obligately oxalotrophic and haloalkalitolerant, oxidase- and catalase-positive, mesophilic and motile by peritrichous flagella. Cell wall contained two electron-dense layers. The external layer consists of a chain of electron-dense granules morphologically resembling the cellulosomes of *Clostridium thermocellum*. Two species are described, *Ammoniphilus oxalaticus* gen. nov., sp. nov. and *Ammoniphilus oxalivorans* gen. nov., sp. nov. The type strains of these species are strains RAOx-1 (= DSM 11538) and RAOx-FS (= DSM 11537), respectively. *Ammoniphilus* strains were isolated from the rhizosphere of sorrel (*Rumex acetosa*) and from decaying wood. The strains require a high concentration of ammonium ions and use oxalate as the sole organic source of carbon and energy for growth; no growth factors were required. Growth occurred at pH 6.8–9.5. The optimum temperature and pH for growth were 28–30 °C and 8.0–8.5. All strains grew in a saturated solution of ammonium oxalate, and tolerated 3% NaCl. Whole-cell hydrolysates contain meso-diaminopimelic acid and glucose. The menaquinone of the strains was MK 7, and the major cellular fatty acids were 12-methyl tetradecanoic, cis-hexadec-9-enoic and hexadecanoic acids. The G+C content of the DNA was 45–46 mol% for *A. oxalaticus* and 42 mol% for *A. oxalivorans*. The almost complete 16S rDNA sequence of three strains of the two species of *Ammoniphilus* shows that the genus falls into the radiation of the *Clostridium*–*Bacillus* subphylum of Gram-positive bacteria. The closest phylogenetic neighbour of *Ammoniphilus* is *Oxalophagus oxalicus*. The DNA–DNA hybridization value between strains RAOx-1 and RAOx-FS was 39.7%.

Keywords: *Ammoniphilus oxalaticus* gen. nov., sp. nov., *Ammoniphilus oxalivorans* gen. nov., sp. nov., oxalotrophic bacteria

INTRODUCTION

Oxalic acid and its salts are widespread in nature, being produced by many species of plants, algae and

fungi (37). Wood-rotting basidiomycetes (both white-rot and brown-rot) are known to excrete oxalic acid and it has been demonstrated that oxalic acid has multiple roles in lignocellulose degradation and depolymerization of cellulose, affecting the activity of an extracellular H₂O₂-dependent lignin peroxidase and a manganese-dependent peroxidase and many other functions important in fungal ecology and patho-

The EMBL accession numbers for the 16S rDNA sequences from strains RAOx-1^T, RAOx-FS^T and RAOx-FS are Y14578, Y14580 and Y14579, respectively.

genicity to plants [for a review, see (19)]. Oxalic acid also complexes divalent ions that are important for the activity of many degradative enzymes. Oxalates are important in the cycling of both cations and phosphates in soil, effectively increasing the availability of the latter (24, 32). Thus, oxalic acid has an important ecological significance.

Since oxalic acid has an oxidation state that is ultimate for an organic compound, energy release during its full oxidation to CO_2 and H_2O is low, and the molar growth yield on oxalate is also low (26). Therefore microbes that mineralize and/or convert oxalic acid to biomass may be important modulators in the biological carbon cycle. Their biochemistry is incompletely known and the taxonomic position of most strains described is unclear (30, 31, 33, 36, 40, 42). Among aerobic bacteria, '*Pseudomonas oxalaticus*' strain Ox1 (41), *Methylobacterium extorquens* strain AM1, previously classified as *Pseudomonas* sp. (5, 15, 45), and among the anaerobic bacteria *Oxalobacter formigenes* strain OxB (1, 3, 38) and *Oxalophagus oxalicus* strain AltOx1, previously classified as *Clostridium oxalicum* (10, 12), have been described most completely from the physiological and biochemical points of view.

We isolated from the rhizosphere of sorrel (*Rumex acetosa*) and from decaying wood, bacteria that utilize oxalate as the sole carbon and energy source. One strain, RAOx-1, isolated from the Belarus region was preliminarily referred to as *Bacillus oxalophilus* (53, 54). The isolation, characterization and taxonomic position of eight additional obligately oxalotrophic, ammonium-dependent strains RAOx-FF, RAOx-FS, RAOx-RF, RAOx-RM, RAOx-RS from the rhizosphere of sorrel, DWOx-RM from decaying pine wood in Finland, and RAOx-PF and RAOx-PM from the rhizosphere of sorrel in Russia, are described in this paper. We propose a new genus, *Ammoniphilus* gen. nov., and the species *Ammoniphilus oxalaticus* sp. nov. and *Ammoniphilus oxalivorans* sp. nov. for these strains.

METHODS

Medium. Medium OM-2, used for the enrichment and cultivation of oxalate-utilizing strains, contained 10–20 g $(\text{NH}_4)_2\text{C}_2\text{O}_4$ (ammonium oxalate), 10 g NaHCO_3 , 10 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.7 g NaCl, 0.57 g KCl, 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (sodium thiosulfate) in 1 l of deionized water, pH 6.8–7.0. The first three salts must be dissolved in the indicated sequence. After autoclaving for 15 min at 121 °C the pH of the medium shifted to 8.5–9.0. Solid medium contained 1.5–2.0% (w/v) Bacto Agar (Difco).

Ability for growth of oxalotrophic strains on rich medium was tested on nutrient agar no. 1, malt extract peptone agar no. 90, potato dextrose agar no. 129 and trypticase soy agar no. 535, and under anaerobic conditions in oxalate medium no. 495 as described in the *DSMZ Catalogue of Strains* (18).

Enrichment and isolation. Strain DWOx-RM was isolated

on OM-2 medium from pine wood decaying in the forest and strains RAOx-RF, RAOx-RM and RAOx-RS were isolated from the rhizosphere of sorrel (*Rumex acetosa*) growing along a forest footpath, Rovaniemi (66°30' N, 25°45' E), Finland, strains RAOx-FF and RAOx-FS in a public garden, Helsinki (60°10' N, 24°57' E), Finland, and strains RAOx-PF and RAOx-PM in a kitchen garden, Serpukhov (54°54' N, 37°30' E), Moscow region, Russia. Strain RAOx-1 was isolated from the rhizosphere of sorrel growing in a kitchen garden, Slutsk (53°02' N, 27°33' E), Belarus, as described by Zaitsev *et al.* (53). Five grams of soil with roots or 10 g decaying wood were incubated in a 500 ml flask with 100 ml OM-2 medium stationary at 28 °C. After 20 d, 10 ml enrichment culture from the flasks where ammonia release had occurred and 90 ml OM-2 medium were transferred into a conical flask (500 ml) and incubated on a gyratory shaker at 180 r.p.m., diluted 1:10 (v/v) after 5 d with OM-2 medium and incubated until visibly turbid. The culture was then plated on the OM-2 agar and incubated at 28 °C and the colonies streaked pure on OM-2 agar medium.

Cultivation and measurement of growth. Pure cultures of oxalate-utilizing bacteria were cultivated in 3 l conical flasks with 500 ml OM-2 medium on a gyratory shaker (180 r.p.m.) at 28–30 °C. Growth was estimated by absorbance at 540 nm ($A_{540} = 1$ corresponded to 272 mg dry cells l^{-1}).

Morphology. Gram staining was performed by the modified Hucker method (43). The morphology of negatively stained cells was determined by electron microscopy as described previously (44). For scanning electron microscopy, 50–200 μl of culture was placed onto a Millipore filter (0.45 μm) fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. Then the samples were rinsed three times with buffer, dehydrated in a graded series of ethanol and critical-point-dried. The filters were coated with gold palladium and observed with SEM Zeiss DSM 962 microscope. For thin sections, the cells were prefixed with 2% (v/v) glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature and washed three times in the same buffer. Thin sections were prepared and examined as described previously (52).

Utilization of carbon sources. The utilization of 53 different organic compounds was tested in OM-2 medium where ammonium oxalate was replaced by 6 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$. Stock solutions were filter sterilized, and the carbon sources were added to final concentrations of 1 and 10 g l^{-1} . The pH was adjusted to 7.0–7.5 with sterile 1 M NaOH or 1 M HCl after the carbon source was added. Media containing 1 or 10 g sodium oxalate l^{-1} were used as positive controls; the same medium without carbon source was used as a negative control.

Utilization of nitrogen sources. The ability of the isolates to utilize $(\text{NH}_4)_2\text{SO}_4$, KNO_3 , KNO_2 or urea as the sole source of nitrogen was tested on OM-2 agar in which ammonium oxalate was replaced by sodium oxalate (10 g l^{-1}). Nitrogen sources were added to the medium to the final concentrations of 0.01–0.1 M with steps of 0.01 M.

NaCl tolerance. NaCl tolerance of the oxalate-utilizing strains was tested on solid OM-2 medium containing 10 g ammonium oxalate l^{-1} and 0–7% (w/v) NaCl.

Antibiotic susceptibilities. Antibiotic sensitivity was tested on the OM-2 agar. Stock solutions of antibiotics (Sigma)

ampicillin, chloramphenicol, chlortetracycline, penicillin G, polymyxin B, rifampicin and streptomycin were filter-sterilized and added to autoclaved, cooled (55 °C) medium.

Biochemical characteristics. Nitrate reduction was tested by monitoring for nitrite accumulation and for gas in tubes containing 10 ml OM-2 medium with 0.1 or 1 g KNO₃ l⁻¹ and with sodium oxalate (10 g l⁻¹) and no ammonium oxalate. Hydrolysis of gelatin and starch were tested in OM-2 medium supplemented with 12% (w/v) gelatin or 0.2% (w/v) starch, respectively. Urea hydrolysis was tested by monitoring for ammonium accumulation with Nessler's Reagent after 10 h incubation of cells (40–50 mg) suspended in 1 ml OM-2 medium with 1 or 5 g urea l⁻¹ and with sodium oxalate (10 g l⁻¹) and no ammonium oxalate. Indole production was tested with Ehrlich's reagent in OM-2 medium supplemented with 1 g L-tryptophan l⁻¹. H₂S production was tested in tubes with OM-2 agar supplemented with 0.25 g ferric ammonium citrate l⁻¹ and in OM-2 liquid medium in which thiosulfate was replaced by 0.5 g cysteine l⁻¹ with lead acetate strip of filter paper; oxidase and catalase were analysed as described by Smibert & Krieg (49).

Chemotaxonomic characteristics. Cells for chemotaxonomic analyses were grown in OM-2 medium to the middle of the exponential phase on a rotary shaker, then centrifuged and freeze-dried. Diaminopimelic acid, cell wall sugars, menaquinones, whole-cell fatty acids and DNA base composition were analysed as described previously (55).

DNA isolation and characterization. Isolation of DNA and DNA–DNA hybridization (13, 20), and determination of renaturation rates were as described previously (28, 29).

16S rDNA sequence determination and analysis. Genomic DNA was extracted from the strains investigated in this study and used for the PCR-mediated amplification of the 16S rDNA (46). The purified PCR products were cloned, the 16S rDNA inserts reamplified and sequenced as described previously (47) using a model 373A automatic DNA sequencer (Applied Biosystems).

To determine the closest relatives of strains of RAOx-1, RAOx-FS and RAOx-FF, their phylogenetic positions were initially determined using the database ARB (50). A fine resolution of relatedness between RAOx strains and their closest relatives was performed using the ae2 editor (39). Phylogenetic dendrograms were reconstructed using the treeing algorithm of De Soete (14) and Saitou & Nei (48), contained in the PHYLIP package (22). Bootstrap values were determined using the PHYLIP package (22). The accession numbers of the 16S rDNAs of reference strains were: *Alicyclobacillus acidoterrestris* (X60743), *Aneurinibacillus aneurinolyticus* (X60645), *Bacillus alcalophilus* (X76436), *Bacillus sphaericus* (D16280), *Bacillus stearothermophilus* (X57309), *Bacillus subtilis* (Z82044), *Brevibacillus brevis* (X60612), *Listeria monocytogenes* (X98530), *Oxalophagus oxalicus* (Y14581), *Paenibacillus polymyxa* (D16276) *Sporolactobacillus inulinus* (D16283), *Staphylococcus aureus* (X68417) and *Tetragenococcus halophilus* (D88668). The sequences for *Enterococcus faecalis* and *Oxalobacter formigenes* were obtained from the Ribosomal Database Project (22).

Ribotype analysis. Ribotype analysis based on the simultaneous separation and transfer of *Eco*RI DNA restriction fragments followed by hybridization with a chemiluminescently labelled rDNA probe from *Escherichia coli* was performed using an automated Riboprinter (Qualicon).

RESULTS

Isolation of oxalate-utilizers

Oxalate-utilizing bacterial strains were readily isolated from the rhizosphere of sorrel or decaying wood by enrichment culture in OM-2 medium. Nine of the 58 isolated strains were investigated in detail and are described below: RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM.

Colony morphology

Visible colonies appeared on OM-2 agar after 2 d incubation. After 4–8 d, the colonies of strains RAOx-1, RAOx-PM, RAOx-FF and RAOx-RF were light brown, convex and circular with entire margin and smooth surface, ≥ 2 mm in diameter (Fig. 1a). Old colonies (14 d) were flat, light brown in the centre and transparent at the edges, up to 4 mm in diameter, usually with salt crystals (Fig. 1b). Colonies of strains RAOx-RM and DWOx-RM were light brown in the centre and bright-beige at the edges, those of RAOx-

Fig. 1. Colonies of strain RAOx-1 on OM-2 agar with 20 g ammonium oxalate l⁻¹. (a) 4-d-old colonies and (b) 14-d-old colony with salt crystals. The emergence of salt crystals was typical for old colonies of DWOx-RM and all RAOx strains. Bar, 1 mm (both parts).

Fig. 2. (a) Scanning electron micrograph of cells of strain RAOx-1. Bar, 2 μ m. (b) Electron micrograph of a negatively stained cell. Bar, 0.5 μ m.

PF were bright beige, mucoid, 2–5 mm in diameter, and those of RAOx-FS and RAOx-RS were white, 0.5–2 mm in diameter.

Cell morphology and ultrastructure

Cells of strains RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM were straight or slightly curved motile rods occurring mostly as single cells (Figs 2 and 3), sometimes in pairs, in short or long chains. The cells of the strain RAOx-1 were 1.0–3.8 μ m $\times$ 0.6–1.1 μ m and RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM were 1.0–8.0 μ m $\times$ 0.6–1.1 μ m in size, and those of RAOx-FS (Fig. 4) and RAOx-RS 1.1–1.4 μ m $\times$ 0.7–1.1 μ m. All strains formed endospores (Figs 4a, 5 and 6) and lacked capsules. Peritrichous flagella were revealed by electron microscopy (Fig. 2b). Cell wall of the strains contained two electron-dense layers, an internal layer (IL) and an external layer (EL), which is atypical for Gram-

Fig. 3. Thin section of vegetative cells of the strain RAOx-1. The cell wall consisted of two electron-dense layers: external layer (EL) and internal layer (IL). EDG, electron-dense granules surrounding the external cell surface. Bar, 0.5 μ m (all parts).

positive bacteria (Figs 3b, c and 4b). The external layer consisted of electron-dense granules (EDG) (Figs 3a, b and 4b) interconnected by thin fibrous material surrounding the cell surface like a skin (Fig. 3c). The electron-dense granules had a hemiellipsoid form (Fig. 4b) with a height of 40–90 nm (as measured from the plane of the outer cell wall) and a width of 100–250 nm (measured at the base of the cell surface). The endospores were oval (Fig. 6b) and located centrally (Figs 5a and 6a) or subterminally in non-swollen or slightly swollen sporangia (Fig. 5b). The endospores were stable for 10 min at 80 °C but not at 85 °C. All strains stained Gram-negative in young cultures but became Gram-positive in the mid-exponential growth stage and variable at the late stage of growth.

Chemotaxonomic characteristics

Whole-cell hydrolysates of strains RAOx-1 and RAOx-FS contained *meso*-diaminopimelic acid and glucose. Menaquinone MK 7 was the sole respiratory quinone in each of the nine strains. The whole-cell fatty acid compositions of the strains are shown in

Fig. 4. Thin section of vegetative and old cells of the strain RAOx-FS with spores at different stages of maturation. EL and IL, external and internal electron-dense layers; EDG, electron-dense granule. Bar, 0.5 μ m (both parts).

Table 1. The main fatty acids were straight-chain saturated hexadecanoic acid (22–29%), unsaturated *cis*-hexadec-9-enoic acid (28–36% for strains RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM, DWOx-RM and 13% for the strain RAOx-FS and RAOx-RS) and branched chain 12-methyl tetradecanoic acids (8–15% for strains RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM, DWOx-RM and 36–37% for strains RAOx-FS and RAOx-RS). Total amount of branched-chain fatty acids ranged from 26–36% (strains RAOx-

Fig. 5. Sporulation of the strain RAOx-1. Thin section of cells shows ovoid spores located centrally (a) or terminally within slightly swollen sporangia (b). Bar, 0.5 μ m (both parts).

1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM, DWOx-RM) to 51–52% (strains RAOx-FS and RAOx-RS). Fig. 7 shows the Euclidian distances of the strains in the whole-cell fatty acid based dendrogram. Strains RAOx-FS and RAOx-RS clustered separate from the remaining strains. The G+C content of the DNA of strains RAOx-FS and RAOx-RS was 42 mol% and for the other seven strains it was 45–46 mol% (Table 2).

Physiological and biochemical characteristics

No growth was observed for any of the nine strains under anaerobic conditions (80% N_2 + 10% CO_2 + 10% H_2) in 14 d on OM-2 medium or anaerobic

Fig. 6. (a) Thin section of cell of the strain RAOx-FS shows ovoid spores located centrally, and (b) mature spore of the strain RAOx-1. Bar, 0.5 μ m (both parts).

Fig. 7. Euclidian distance dendrogram based on the whole-cell fatty acid compositions of the nine oxalate-utilizing strains.

oxalate medium N 495. All strains thus were obligately aerobic chemoorganotrophs. All strains were catalase- and oxidase-positive, produced H_2S from cysteine but not from thiosulfate and did not reduce nitrate or form indole. They grew in liquid culture without cell aggregation forming no pigment in liquid or solid medium. No vitamins were required. All strains were sensitive to low concentrations of antibiotics: 5 μ g ampicillin ml^{-1} , 3 μ g chloramphenicol ml^{-1} and 80 units polymyxin B ml^{-1} (RAOx-1), and 3 μ g ampicillin ml^{-1} , 1 μ g chloramphenicol ml^{-1} and 20 units polymyxin B ml^{-1} (RAOx-FS). Both strains were sus-

Table 1. Composition of whole-cell fatty acids of nine oxalate-utilizing strains

Strains: 1, RAOx-FS; 2, RAOx-RS; 3, RAOx-1; 4, RAOx-PF; 5, RAOx-PM; 6, RAOx-FF; 7, RAOx-RF; 8, RAOx-RM; 9, DWOx-RM.

Systematic name	Shorthand designation	Fatty acid composition (% w/w) of strains:								
		1	2	3	4	5	6	7	8	9
12-Methyl tridecanoic acid	14:0-iso	3	3	4	5	5	7	7	8	7
Tetradecanoic acid	14:0	2	3	3	5	4	7	8	7	9
13-Methyl tetradecanoic acid	15:0-iso	4	4	4	1	2	3	2	3	3
12-Methyl tetradecanoic acid	15:0-anteiso	37	36	15	8	9	12	8	15	14
cis-Pentadec-9-enoic acid	15:1 ω 6c	—	< 1	2	2	2	2	2	2	2
Pentadecanoic acid	15:0	2	2	2	3	3	3	4	3	3
14-Methyl pentadecenoic acid	16:1-iso H	1	< 1	3	3	2	2	2	3	2
14-Methyl pentadecanoic acid	16:0-iso	5	4	6	7	6	5	5	5	4
cis-Hexadec-9-enoic acid	16:1 ω 7c	13	13	29	36	34	30	31	28	29
cis-Hexadec-11-enoic acid	16:1 ω 5c	—	—	< 1	1	< 1	< 1	< 1	< 1	1
Hexadecanoic acid	16:0	26	28	28	26	29	25	29	22	22
14-Methyl hexadec-7-enoic acid	anteiso-17:1 ω 9c	1	1	1	< 1	< 1	< 1	< 1	< 1	< 1
14-Methyl hexadecanoic acid	17:0-anteiso	4	3	2	1	1	1	< 1	1	< 1
Other		< 2	< 2	< 1	< 2	< 2	< 2	< 2	< 3	< 3

Table 2. Physiological and biochemical characteristics of the oxalate-utilizing strains

Strains: 1, RAOx-FS; 2, RAOx-RS; 3, RAOx-1; 4, RAOx-PF; 5, RAOx-PM; 6, RAOx-FF; 7, RAOx-RF; 8, RAOx-RM; 9, DWOx-RM. All tests except for the temperature range test were performed at 28 °C. R, Rapid; S, slow; +, positive; -, negative; $\pm$, poor growth. Numbers for growth on ammonium oxalate indicate yield of cells (mg dry wt l⁻¹) after 70 h, 115 h or 8 d incubation on a gyratory shaker (155 r.p.m.) on 20, 60 or 100 g ammonium oxalate l⁻¹, respectively.

Characteristic	Reaction of strain:								
	1	2	3	4	5	6	7	8	9
Growth rate	S	S	R	R	R	R	R	R	R
Urease	-	-	-	+	+	+	-	+	+
Hydrolysis of:									
Gelatin	+	+	-	+	+	+	+	-	+
Starch	+	+	+	+	+	+	-	+	+
Growth at:									
5 °C/30 d	-	-	-	-	-	-	-	-	-
10 °C/30 d	-	-	-	+	-	+	+	+	+
14 °C/20 d	-	-	+	+	+	+	+	+	+
20 °C/5 d	+	+	+	+	+	+	+	+	+
38 °C/2 d	+	+	+	+	+	+	+	+	+
40 °C/10 d	-	-	+	+	-	$\pm$	-	$\pm$	$\pm$
41 °C/10 d	-	-	-	-	-	-	-	-	-
Growth with NaCl (%):									
3	+	+	+	+	+	+	+	+	+
4	+	+	+	-	-	+	+	+	+
5	+	+	-	-	-	-	-	-	-
6	-	+	-	-	-	-	-	-	-
Growth with (NH ₄) ₂ SO ₄ (M)									
0.01	-	-	-	-	-	-	-	-	-
0.015	$\pm$	$\pm$	-	$\pm$	$\pm$	-	$\pm$	-	-
0.02	$\pm$	$\pm$	-	$\pm$	$\pm$	-	$\pm$	$\pm$	$\pm$
0.025	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
0.03	$\pm$	$\pm$	$\pm$	+	+	$\pm$	$\pm$	$\pm$	$\pm$
0.035	+	+	+	+	+	+	+	+	+
Growth on ammonium oxalate (g l ⁻¹):									
2	-	-	-	-	-	-	-	-	-
3	-	-	-	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	-
4	-	-	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
5	+	+	+	+	+	+	+	+	+
20 for 70 h	350	260	240	310	190	340	340	500	420
60 for 115 h	300	250	210	420	240	410	330	540	340
100 for 8 d	420	320	440	70	350	400	390	410	80
Glyoxylate (2 g l ⁻¹)	-	-	-	+	+	-	-	-	$\pm$
Growth on mixture (g l ⁻¹) of:									
Formate (4) and glyoxylate (2)	-	-	+	+	+	+	+	$\pm$	+
Methanol (0.2-2) and glyoxylate (2)	-	-	$\pm$	+	+	$\pm$	+	$\pm$	+
G + C content (mol %)*	42	42	46	46	45	46	46	46	45

(46.7)

*The G + C content of strain RAOx-1, determined by thermal denaturation (53), is given in parentheses.

ceptible to 3 μ g chlortetracycline ml⁻¹, 1 μ g streptomycin ml⁻¹, 0.5 μ g rifampicin ml⁻¹ and 0.1 μ g penicillin ml⁻¹. Other biochemical and physiological properties of the strains are listed in Table 2. Strains

RAOx-FS and RAOx-RS differed from the remaining strains by slower growth and their inability to grow at low temperatures (14 °C), higher tolerance of NaCl, requirement of a higher concentration of ammonium

Fig. 8. Ribotype patterns of the nine oxalate-utilizing strains and reference strains *Bacillus cereus* DSM 31^T and *Paenibacillus polymyxa* DSM 36^T. Restriction was by *Eco*RI.

oxalate, and inability to grow on mixtures of glyoxylate with formate or methanol.

Phylogenetic analyses

The almost complete 16S rDNA of strains RAOx-1, RAOx-FS and RAOx-FF were sequenced [97% of the *E. coli* 16S rDNA sequence (6)] and the phylogenetic position of the strains was determined. It was found that the 16S rDNA sequences of strains RAOx-1, RAOx-FS and RAOx-FF were highly similar, with greater than 98.9% similarity. The binary value for strains RAOx-1 and RAOx-FF was 99.3%, while these two strains were slightly less highly related to strain RAOx-FS (98.7 and 98.9%, respectively). As measured by the renaturation method, the DNA-DNA similarity between strains RAOx-1 and RAOx-FS was 39.7%, indicating that these two strains are separate species.

The results of ribotyping of the nine RAOx strains, using *Eco*RI for restriction cleavage showed that the strains had only one identical band and displayed four ribotypes: (i) for RAOx-FS and RAOx-RS; (ii) for RAOx-1; (iii) for RAOx-PF and RAOx-PM; and (iv) for RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM (Fig. 8).

16S rDNA sequence alignment showed that the closest relative of strains RAOx-1, RAOx-FF and RAOx-FS was *Oxalophagus oxalicus*, a strict anaerobe. The 16S rDNA similarity between the RAOx strains and *O. oxalicus* DSM 5503^T was 96–96.5%. The relationships between these organisms and closest members of the genus *Bacillus* and their relatives was below 92% and for another oxalotroph *Oxalobacter formigenes* ATCC 35274^T it was below 79%. The position of the RAOx strains among these organisms was stable irrespective

Fig. 9. Phylogenetic dendrogram based on the result of 16S rDNA sequence comparison. The neighbour-joining tree was derived from transversion distances. Bootstrap values (expressed as percentages of 500 replications) are shown at the branch points; values above 95% were considered significant. Bar, 5 substitutions per 100 nucleotides.

of the algorithm used. A neighbour-joining tree is depicted in Fig. 9. The relatedness between the RAOx strains and *O. oxalicus* was recovered in 100% of the trees generated, as demonstrated by the bootstrap values for this group. The 16S rDNA sequences of these organisms shared the lack of 3 bp in the helical region between positions 455–462 and 470–477 (6).

Substrate utilization

Oxalate was used as the sole organic source of carbon and energy. No growth was detected in media with one-carbon compounds formate or methanol or on polycarbon compounds acetate, adipate, adonitol, β -alanine, L-arabinose, L-asparagine, L-aspartate, D-cellobiose, citrate, dulcitol, ethanol, fructose, fumarate, D-galactose, gluconate, glutamate, D-glucose, glycerate, glycerol, β -glycerophosphate, meso-inositol,

α -ketoglutarate, L-lactate, lactose, L-leucine, D-lyxose, malate, maleate, malonate, maltose, D-mannitol, D-mannose, DL-methionine, DL-norleucine, β -phenylalanine, L-proline, pyruvate, raffinose, L-rhamnose, D-ribose, sarcosine, L-serine, D-sorbitol, succinate, sucrose, DL-threonine, L-tryptophane, L-tyrosine, L-valine, D-xylose. The strains did not grow on any rich medium tested: nutrient agar no. 1, malt extract peptone agar no. 90, potato dextrose agar no. 129 and trypticase soy agar no. 535; and they did not grow in OM-2 medium if ammonium oxalate was replaced by 6 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$ plus yeast extract, malt extract, peptone or tryptone. Strains RAOx-PF, RAOx-PM and DWOx-RM grew slowly on OM-2 medium if ammonium oxalate was replaced by 2 g glyoxylate l^{-1} plus 6 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$. In addition, weak growth was observed for strains RAOx-1, RAOx-FF, RAOx-RF, RAOx-RM in OM-2 medium if ammonium oxalate was replaced by a mixture of formate (4 g l^{-1}) and glyoxylate (2 g l^{-1}) plus 6 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$; very weak growth occurred on a mixture of methanol (0.2 g l^{-1}) and glyoxylate (2 g l^{-1}) plus 6 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$ (Table 2). None of the strains grew on mixtures of formate (4 g l^{-1}) and glycerol (1 g l^{-1}), formate (4 g l^{-1}) and glycerate (1 g l^{-1}), formate (4 g l^{-1}) and acetate (2 g l^{-1}), formate (4 g l^{-1}) and citrate (2 g l^{-1}), formate (4 g l^{-1}) and succinate (1 g l^{-1}), formate (4 g l^{-1}) and pyruvate (2 g l^{-1}) or methanol (0.2–2 g l^{-1}) and glycerate (1 g l^{-1}) plus 6 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$. These findings indicate that the strains were obligately oxalotrophic.

Nitrogen and sulfur sources

Ammonium but not nitrate, nitrite or urea was used as the nitrogen source. All nine strains had an absolute requirement for NH_4^+ . They did not grow on media with less than 0.03 M NH_4^+ ; poor growth was observed with 0.03–0.06 M NH_4^+ ; and good growth occurred only with ≥ 0.07 M NH_4^+ (Table 2).

The strains used thiosulfate, sulfate, sulfite, sulfide, DMSO, methionine, cysteine, glutathione and thio-uridine as sources of sulfur.

Growth parameters

All nine strains grew in batch culture with a high concentration of oxalate (Table 2). The growth rate and the growth yield of the strain RAOx-1 in OM-2 medium containing 7–14 g l^{-1} oxalate were 0.35 h^{-1} and 0.9–1.7 g dry cells (mol substrate carbon used) $^{-1}$, respectively. All strains were able to grow in saturated solution of ammonium oxalate with a total concentration of salts in the medium of about 8.5% (w/v).

DISCUSSION

Ammonium oxalate enrichment cultures from the rhizosphere of sorrel and from decaying pine wood yielded aerobic, ammonium-dependent, oxalate-utilizing bacterial strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF,

RAOx-RM and DWOx-RM which differed markedly from the oxalate-utilizers reported earlier. (i) Our isolates were Gram-variable, endospore-forming and peritrichously flagellated bacteria whereas the majority of aerobic oxalate-grown bacteria were described as Gram-negative monotrichously flagellated organisms (2, 7, 9, 30, 31, 33, 35, 40, 45). (ii) Electron microscopic examination revealed cell wall atypical for Gram-positive bacteria consisting of two electron-dense layers and electron-dense granules attached to the external cell surface. (iii) All strains had an absolute requirement for ≥ 0.07 M NH_4^+ , not replaceable by Na^+ or K^+ . (iv) The nine strains grew at high concentrations of oxalate, with an optimum of 20 g l^{-1} . Good growth occurred even at the saturating concentration of ammonium oxalate, 60 g l^{-1} (~ 0.4 M), at 30 °C. Most oxalate-utilizing micro-organisms described previously grew at lower concentrations of substrate. *Pseudomonas* sp. OD1 grew on potassium oxalate up to 20 g l^{-1} (30), and '*P. oxalaticus*' OX1 grew on potassium oxalate up to 180 mM (~ 30 g l^{-1}), with an optimum of 15 mM (~ 2.5 g l^{-1}) (16). Concentrations of oxalate greater than 108 mM inhibited growth of *Pseudomonas* sp. KOx, and 270 mM inhibited growth of *Thiobacillus novellus* (7, 9). (v) Biochemical study of the carbon-assimilation pathway of the strain RAOx-1 showed the presence of NAD-independent, dye-linked (phenazine methosulfate/dichlorophenol indophenol-linked) formate dehydrogenase. NAD-dependent formate dehydrogenase (EC 1.2.1.2) was not found (53). Only dye-linked formate dehydrogenases were also found in the strict anaerobic, oxalotrophic bacterium *Oxalobacter formigenes* OxB (11). Oxalotrophic bacteria '*P. oxalaticus*' Ox1 (17), *Alcaligenes eutrophus* H16 (23), *Alcaligenes* sp. LOx and *Thiobacillus novellus* (8, 9) were reported to possess one NAD- and one dye-linked or particulate formate dehydrogenase. Other oxalotrophic bacteria, *Methylobacterium extorquens* (*Pseudomonas* sp.) strain AM1 (5) and *Pseudomonas* sp. strain RJ₁ (40), were reported to have NAD-linked formate dehydrogenase.

An important feature and distinctive characteristic of strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM is their inability to grow on formate as the growth substrate. For the strain RAOx-1 formate served only as an energy source but not as the carbon source, since high activity of dye-linked formate dehydrogenase was detected while the other enzymes of formate metabolism were absent (53), resembling in this respect the oxalotrophic bacteria '*P. oxalaticus*' strains Ox4 and Ox6 (35), *Pseudomonas* sp. strains OD1 (30), RJ₁ (40) and *Alcaligenes* sp. LOx (8).

Autotrophic oxalate assimilation after its oxidation to CO_2 via the ribulosebiphosphate cycle in the strain RAOx-1 is unlikely because D-ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) was absent (53). Moreover, the organism was unable to grow autotrophically under 75% H_2 + 15% CO_2 + 10% O_2 atmosphere.

Further enzymological analyses described elsewhere (53) showed that strain RAOx-1 lacked a number of tricarboxylic acid cycle enzymes, in particular α -ketoglutarate dehydrogenase. This is typical for the obligate oligotrophs (51). Ammonium was assimilated via alanine (EC 1.4.1.1), glutamate (EC 1.4.1.4) and glycine (EC 1.4.1.10) dehydrogenases. The strain RAOx-1 had an oxalate-CoA transferase (EC 2.8.3.2). This reaction of primary substrate activation is widespread among oxalotrophic bacteria (5). Activity of PMS-dependent oxalyl-CoA decarboxylase was also detected. We suggest that these enzymes provided for oxalate oxidation to CO_2 as follows:

The alternative pathway of oxalate oxidation via glyoxylate bypass did not function due to lack of isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2); the activity of malate dehydrogenase (EC 1.1.1.39) was significantly lower than the activity of formate dehydrogenase. Assimilation of oxalate proceeded by a variant of the serine pathway, since serine-glyoxylate aminotransferase (EC 2.1.6.45) and hydroxypyruvate reductase (EC 1.1.1.29) were found (53).

The oxalotrophic strains described in this paper were readily isolated from four regions distant to each other: Belarus, central Russia, southern and northern Finland. Probably obligate oxalotrophs occur widely in oxalate containing natural habitats such as roots of oxalate-accumulating plants and decaying wood and may be involved in the degradation of lignocellulose by scavenging the oxalate produced by wood-rotting fungi (19). The electron-dense granules surrounding the external cell surface of the obligate oxalotrophs might represent organelles (i) that promote adherence of the bacterium to the roots of oxalate-accumulating plants or to the hyphae of oxalate-producing wood-rotting basidiomycetes and/or (ii) that contain enzymes for the oxidation of oxalate (oxalyl-CoA decarboxylase, data not presented). We tentatively propose the term 'oxalosome' for these granules in analogy to the cellulosome described for *Clostridium thermocellum* (4, 21), similar in form and size.

The presence in the strain RAOx-1 of the dye-linked formate dehydrogenase only arouses the question how the strain RAOx-1 obtains NAD(P)H to supply the reactions of the serine pathway, resembling in this respect the strict anaerobe *Oxalobacter formigenes* OxB in which also only dye-linked formate dehydrogenase was found (11). It is possible that in strains RAOx and strain DWOx-RM the NAD(P) is reduced through the process of reverse electron transport. In this connection, the strict dependence of these strains on a high concentration of ammonium may indicate a role of ammonium ion gradient for providing for the synthesis of ATP or reducing equivalents.

The morphology and the physiological characteristics

of the oxalotrophic strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM place them in the group of endospore-forming Gram-positive rods and cocci in group 18 in *Bergey's Manual of Systematic Bacteriology* (27).

Strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM are aerobic, catalase- and oxidase-positive, endospore-forming Gram-variable rods, motile by peritrichous flagella, produce menaquinone MK-7 and have 42–46 mol% G + C in their DNA. These characteristics are consistent with membership of the genus *Bacillus* but distinguish the strains from the other genera of group 18.

Analyses of whole-cell fatty acids of the strains revealed a high level of *cis*-hexadec-9-enoic acid (Table 1), which is atypical for the genus *Bacillus* (34).

Phenotypically, strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM differed from all aerobic spore-forming taxa in utilizing oxalate as a sole organic source of carbon and energy and in their inability to utilize any of the other organic compounds utilized by members of the genus *Bacillus*. Yeast extract or Casamino acids were not required and did not enhance growth.

The other significant characteristic of strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM was their strict ammonium-dependence, which distinguishes these strains from all other spore-forming taxa.

The highly unusual metabolism of strains RAOx-FS, RAOx-RS, RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM, together with their cell wall morphology, chemotaxonomic and physiological characteristics, and phylogenetic position indicates that these micro-organisms merit a new genus. Out of the 58 oxalate-utilizers isolated in this study, two groups were distinguishable on the basis of their growth rate, tolerance to NaCl, ability to grow at different temperature, at low concentrations of ammonium oxalate and on the mixtures of formate + glyoxylate or methanol + glyoxylate, whole-cell fatty acid composition, DNA base ratios and DNA-DNA homology data. Phylogenetically closest (Fig. 9) was the strictly anaerobic, catalase-negative, spore-forming bacterium *Oxalophagus oxalicus* with a low G + C content (35.4–37.2 mol%) of the DNA (10). It used oxalate as a sole energy source excreting formate as a dead-end product and required acetate for cell carbon synthesis (10, 12). On the basis of the data described above, we propose the genus *Ammoniphilus* gen. nov. The new genus comprises two species, *Ammoniphilus oxalaticus* gen. nov., sp. nov. represented by strains RAOx-1, RAOx-PF, RAOx-PM, RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM, and *Ammoniphilus oxalivorans* gen. nov., sp. nov., represented by strains

RAOx-FS and RAOx-RS. The proposed type strains of the two species show less than 40% DNA–DNA hybridization which supports their genomic distinction.

Ribotyping after *Eco*RI restriction showed one identical band in all strains of both species, which is characteristic of the genus. Strains of *A. oxalivorans* share one riboprint pattern while the strains of *A. oxalaticus* displayed different ribogroups, one by strain RAOx-1, another by strains RAOx-PF and RAOx-PM, and a third by strains RAOx-FF, RAOx-RF, RAOx-RM and DWOx-RM. The ribogroups reflect the geographic origins of the strains. A recent study of DNA fingerprints of *Mycobacterium africanum* also suggested that the number of bands and the variability of pattern reflected the geographic origins of the strains (25).

Description of *Ammoniphilus* gen. nov.

Ammoniphilus (am.mo.ni.phi'lus. M.L. neut. n. *ammonium* ammonia (NH₃); Gr. adj. *philos* loving, friendly to; M.L. masc. n. *ammoniphilus* ammonia lover).

Cells are straight or slightly curved rods. Motile by means of peritrichous flagella. Endospores are formed. Gram reaction is variable. Cell wall consists of two electron-dense layers and electron-dense granules. Obligate aerobe. Catalase and oxidase-positive. The menaquinone is MK 7. Requires high concentrations of ammonium ions for growth. Growth is optimal at ≥ 0.07 M NH₄⁺. Chemoorganotroph. Oxalate is used as the sole organic source for carbon and energy. Isolated from *Rumex acetosa* roots or decaying wood. Temperature optimum is 28–30 °C, pH optimum is 8.0–8.5. Cell hydrolysates contain meso-diaminopimelic acid and glucose. According to the almost complete sequence of 16S rDNA of three of its members, this genus belongs to the *Clostridium*–*Bacillus* subphylum of Gram-positive bacteria. The type species is *Ammoniphilus oxalaticus*.

Description of *Ammoniphilus oxalaticus* sp. nov.

Ammoniphilus oxalaticus (o.xa.la'ti.cus. M.L. adj. *oxalaticus* pertaining to oxalate).

Straight short or long slightly curved rods 1.0–8.0 $\mu\text{m} \times 0.6$ –1.1 μm in size, occurring singly, in pairs, in short or long chains. Endospores are oval, centrally or subterminally formed, in non-swollen, slightly swollen or swollen sporangia, moderately heat-resistant to 80 °C. On agar medium (4–8 d) colonies are light brown or bright beige, convex, circular form with entire margin and smooth surface or mucoid, up to 5 mm in diameter. Some strains grow poorly at 0.03 M NH₄⁺ and no growth occurs at ≤ 0.02 M NH₄⁺. Obligate oxalotroph. Growth occurs up to 100 g ammonium oxalate l⁻¹. Good growth on 5–40 g ammonium oxalate l⁻¹, poor growth at the concentration of 3–4 g l⁻¹ and no growth ≤ 2 g l⁻¹. Some strains can use glyoxylate as the sole organic source for carbon

and energy. Weak growth occurs on mixtures of formate and glyoxylate or methanol and glyoxylate. No growth occurs with other organic acids, sugars or alcohols. Mesophilic, the optimum temperature for growth is 28–30 °C. Most strains grow at 10–40 °C, pH 6.8–9.5, and are tolerant to 4% NaCl. Some strains slowly hydrolyse starch, gelatin and urea. All strains form H₂S from cysteine but not from thiosulfate. Nitrates, nitrites or urea are not utilized as sole nitrogen sources. No reduction of nitrate to nitrite or N₂; no requirement for vitamins; no production of indole. The major cellular fatty acids are 12-methyl tetradecanoic (8–15%), *cis*-hexadec-9-enoic (28–36%) and hexadecanoic acids (22–29%). The G + C content of the DNA is 45–46 mol%. The type strain RAOx-1 was isolated from the rhizosphere of sorrel (*Rumex acetosa*), and is deposited in the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, as strain DSM 11538, and in the University of Helsinki HAMBI Collection as strain HAMBI 2283.

Description of the type strain

Straight short or long slightly curved rods 1.0–3.8 $\mu\text{m} \times 0.6$ –1.1 μm in size which occur singly or in pairs with peritrichous flagella. Cells in young cultures are Gram-negative, in the mid exponential growth are Gram-positive, and in old cultures the Gram reaction is variable. Poor growth occurs at 0.05 M NH₄⁺ and no growth occurs at ≤ 0.04 M. Glyoxylate does not support growth as the sole organic source for carbon and energy. Good growth on ≥ 5 g ammonium oxalate l⁻¹, poor growth on 4 g l⁻¹, and no growth on ≤ 3 g l⁻¹. Minimum doubling time is 2 h. Growth occurs at 14 °C but not at 10 °C. Oxalate is oxidized by oxalyl-CoA decarboxylase and formate dehydrogenase (dye-linked). Assimilation of oxalate proceeds by the serine pathway. The Krebs cycle is incomplete at the level of α -ketoglutarate dehydrogenase. The glyoxylate bypass does not function. Ammonium is assimilated by reductive amination of keto acids. Tolerant to 4% of NaCl. Urease is not produced. Gelatin is not liquefied. The G + C content of the DNA is 46 mol%. The other properties of this strain are the same as the properties described above for the species.

Description of *Ammoniphilus oxalivorans* sp. nov.

Ammoniphilus oxalivorans (o.xa.li.vo'rans. M.L. neut. n. *oxalatum* oxalate; L. part. pres. *vorans* eating; M.L. adj. *oxalivorans* oxalate-eating).

Straight short or long slightly curved rods (1.1–1.4 $\mu\text{m} \times 0.7$ –1.1 μm in size which occur singly, in pairs, in short or long chains. Endospores are oval, centrally or subterminally formed, in non-swollen or slightly swollen sporangia. On agar medium (4–8 d) colonies are white, convex, circular form with entire margin and smooth surface, up to 2 mm in diameter. Poor growth occurs at 0.03–0.6 M NH₄⁺ while no growth occurs at ≤ 0.02 M NH₄⁺. Obligate oxalotroph.

Growth occurs up to 100 g ammonium oxalate l⁻¹, with good growth in 5–60 g ammonium oxalate l⁻¹. No growth occurs in ≤ 4 g ammonium oxalate l⁻¹, with glyoxylate or other organic acids, sugars or alcohols, or on mixtures of formate and glyoxylate or methanol and glyoxylate. Mesophilic, growth occurs at 20–38 °C, with an optimum of 28–30 °C. The pH range for growth is 6.8–9.5. Tolerant to 5% NaCl. Starch and gelatin are hydrolysed. H₂S formed from cysteine but not from thiosulfate. Nitrates, nitrites or urea are not utilized as the sole nitrogen sources. No reduction of nitrate to nitrite or N₂; no requirement for vitamins; no production of urease and indole. The major cellular fatty acids are 12-methyl tetradecanoic (37%), *cis*-hexadec-9-enoic (13%) and hexadecanoic acids (26–28%). The G+C content of the DNA is 42 mol%. The type strain RAOx-FS was isolated from the rhizosphere of sorrel (*Rumex acetosa*) and is deposited in the DSMZ as strain DSM 11537 and in the University of Helsinki HAMBI Collection as strain HAMBI 2284. The properties of this strain are the same as the properties described above for the species.

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