Ammoniphilus resinae sp. nov., an endospore-forming bacterium isolated from resin fragments

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A polyphasic approach was used to characterize an endospore-forming bacterium, designated strain CC-RT-ET, isolated from resin fragments. Strain CC-RT-ET was Gram-stain-variable and facultatively anaerobic, able to grow at 20–37 °C (optimal 30 °C) and pH 6.0–9.0 (optimal pH 7.0). Ellipsoidal and terminal endospores were observed. The 16S rRNA gene sequence analysis of strain CC-RT-ET showed highest sequence similarity to Ammoniphilus oxalivorans RAox-FS1 (97.8 %) and Ammoniphilus oxalatus RAox-1T (97.7 %). According to the DNA–DNA hybridization, the relatedness values of strain CC-RT-ET with Ammoniphilus oxalivorans RAox-FS1 and Ammoniphilus oxalatus RAox-1T were 21 % and 29 %, respectively. The DNA G+C content was 39.2 mol% and the predominant quinone system was menaquinone-7 (MK-7). The major fatty acids are 12-methyl tetradecanoic, cis-hexadec-9-enoic and hexadecanoic acids; the common major respiratory quinone is MK-7. The current study which aimed to determine the taxonomic position of strain CC-RT-ET based on polyphasic analyses was performed in comparison with reference strains of the genus Ammoniphilus, for which the name Ammoniphilus resinaesp. nov. is proposed. The type strain is CC-RT-ET (=BCRC 80314T=DSM 24739T).

The genus Ammoniphilus is a member of the family Paenibacillaceae in the phylum Firmicutes, and at the time of writing includes only two species with validly published names (LPSN, http://www.bacterio.net/ammoniphilus.html). Ammoniphilus oxalivorans and Ammoniphilus oxalatus were isolated from the rhizosphere of sorrel (Rumex acetosa) in Russia and described by Zaitsev et al. (1998). The typical characters of members of the genus Ammoniphilus are Gram-stain-variable, obligately oxalotrophic, mesophilic, haloalkalitolerant, oxidase- and catalase-positive, and motile by peritrichous flagella (Zaitsev et al., 1998). High concentrations of ammonium ions are required for growth (optimal at ≥0.07 M NH4+). The cell wall contains two electron-dense layers; the external layer consists of a chain of electron-dense granules morphologically resembling the cellulosomes of Clostridium thermocellum (Zaitsev et al., 1998). The major fatty acids are 12-methyl tetradecanoic, cis-hexadec-9-enoic and hexadecanoic acids; the common major respiratory quinone is MK-7. The current study which aimed to determine the taxonomic position of strain CC-RT-ET based on polyphasic analyses was performed in comparison with reference strains of the genus Ammoniphilus. The morphological, biochemical and phylogenetic characteristics of the novel bacterium were determined according to the minimal standards described by Logan et al. (2009).

A resin sample was collected from a tropical forest in Indonesia. The resin sample was stored in the dark in a sweatbox, surface-sterilized with 75 % alcohol, ground and used to isolate bacterial strains by the standard serial dilution-plating technique on nutrient agar (NA; Hi-Media). After

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC-RT-ET is HM193518.

Two supplementary figures are available with the online Supplementary Material.

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incubation for 3 days at 30°C, a presumably novel bacterium (designated strain CC-RT-E-T) was purified and used for further taxonomic analyses. Strain CC-RT-E-T was routinely grown aerobically on NA at 30°C for 2 days and stored at -80°C in nutrient broth (NB; Himedia) supplemented with 30% (v/v) glycerol for long-term preservation. For taxonomic purposes, Ammoniphilus oxalivorans DSM 11537T and Ammoniphilus oxalaticus DSM 11538T were used as reference strains for the comparisons of phenotypic properties. Due to the required utilization of ammonium ions, species of the genus Ammoniphilus were grown at 30°C for 2 days on ammonium-based media (OM-2) (Zaitsev et al., 1998); strain CC-RT-E-T was grown at 30°C for 2 days on NA for colony morphology, presence of flagella and morphology.

A commercial DNA extraction kit (MO BIO UltraClean) was used to extract the genomic DNA for 16S rRNA gene amplification. The PCR was performed with bacterial universal primers pA and pB (Edwards et al., 1989). DNA fragments encoding the 16S rRNA gene were assembled using Vector NTI 9.0 software (IBI) and deposited in the Genbank database using Sequin software tool. The almost-complete 16S rRNA gene sequence (1497 nt) of strain CC-RT-E-T was compared with all type strains using the Nucleotide Similarity Search program (EZTaxon, http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Phylogenetic analysis was performed with MEGA 6 software (Molecular Evolutionary Genetics Analysis, version 6.0; Tamura et al., 2013). Closely related 16S rRNA gene sequences were retrieved from the EzTaxon-e and GenBank databases and aligned by using the CLUSTAL X (1.83) program (Thompson et al., 1997). Phylogenetic trees were reconstructed by using 16S rRNA gene sequences with neighbours-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods and evaluated by bootstrap analyses (Felsenstein, 1985) after 1000 replications.

During 16S rRNA gene sequence analysis, the pairwise comparison indicated that strain CC-RT-E-T shared a similarity to Ammoniphilus oxalivorans RAOx-FS-T (97.8% 16S rRNA gene sequence similarity) and Ammoniphilus oxalaticus RAOx-1T (97.7%). Based on the genomic distinction by using the established molecular criteria for species-level relatedness (Wayne et al., 1987), DNA-DNA association was conducted between strain CC-RT-E-T, A. oxalivorans DSM 11537T (=RAOx-FS-T) and A. oxalaticus DSM 11538T (=RAOx-1T). Bacterial genomic DNA was isolated by using UltraClean Microbial Genomic DNA Isolation Kits (MO BIO) according to the manufacturer’s instructions. DNA samples from strains CC-RT-E-T, A. oxalivorans DSM 11537T and A. oxalaticus DSM 11538T were loaded onto positively charged membranes as described by Seldin & Dubnau (1985). Chromosomal DNA of both reference strains was used to construct hybridization probes by labelling with digoxigenin–11-dUTP (DIG). The experiment was carried out in triplicate for each sample.

The DNA-DNA relatedness values of strain CC-RT-E-T with A. oxalivorans DSM 11537T and A. oxalaticus DSM 11538T were 21±2 and 29±2%, respectively (the reciprocal value were 26±1% and 18±1%, respectively), which were well within the reported threshold described by Wayne et al. (1987), and indicated that strain CC-RT-E-T might be a putative novel species of the genus Ammoniphilus. Furthermore, the phylogenetic trees reconstructed by using 16S rRNA gene sequences with neighbour-joining, maximum-likelihood and maximum-parsimony methods also indicated that strain CC-RT-E-T forms a separate lineage distantly associated with with A. oxalivorans and A. oxalaticus in the family Paenibacillaceae (Fig. 1).

Colony morphology, presence of flagella and morphology of the cells of strain CC-RT-E-T were investigated using the colonies/cells after incubation for 48 h on NA at 30°C. The Gram staining reaction was performed as described by Murray et al. (1994). A sporangial appearance was observed after malachite-green staining of strain CC-RT-E-T, grown on NA (supplemented with 5 mg MnSO₄·H₂O⁻¹) for one week (Murray et al., 1994). Cell morphology was observed by transmission electron microscopy (JEM-1400; JEOL) after staining with 0.2% uranyl acetate, and also by light microscopy (model A3000; Zeiss). For scanning electron microscopy, culture broth was placed through 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, and then the samples were rinsed, dehydrated in a graded series of ethanol, critical-point-dried, coated with gold-palladium and observed under a field emission scanning electron microscope (JSM-7401F; JEOL). For the preparation of 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.

Catalse activity was determined by assessing bubble production by cells in 3% (v/v) H₂O₂, and oxidase activity was determined by using 1% (v/v) N,N,N,N-tetramethyl-1,4-phenylenediamine reagent (bioMérieux). Anaerobic test was performed using NB supplemented with 0.1% KNO₃, and incubated in an anaerobic chamber (COY). Hydrolysis of gelatin, skimmed milk, starch (1%), Tween 20, 40, 60 and 80 (1%) and DNase test (Hi-Media) were determined as previously described (Lai et al., 2015; Lin et al., 2013; Hameed et al., 2013). H₂S production was checked on both 3 g l⁻¹ Na₂S₂O₃ and 0.1 g l⁻¹ cysteine. Growth at different temperatures was investigated in NB at 4, 15, 20, 25, 30, 37, 40, 45 and 50°C with a 48 h incubation period. The requirement for NaCl was determined using NB containing 0–10% NaCl (in 1% increments). Cell growth at various pH (4.0–10.0, in intervals of 1.0 pH unit) was examined using the following buffer systems: 0.1M citric acid/0.1M trisodium citrate for pH 4.0–5.0; 0.2M Na₂HPO₄/0.2M NaH₂PO₄ for pH 6.0–8.0; and 0.1M NaHCO₃/0.1M Na₂ CO₃ for pH 9.0–10.0. Additionally, strain CC-RT-E-T was tested in ammonium-based medium (OM-2) with various concentrations (1, 3, 5, 7, 9, 11, 13 and 15 g l⁻¹) of ammonium ions that required for species of the genus Ammoniphilus (Zaitsev et al., 1998). Oxidation of carbohydrates was
tested by using API ZYM and API Staph strips (bioactivities of various enzymes and acid production were performed according to manufacturer
strates were tested with API 20NE strips (bioM
sis of aesculin and gelatin, and assimilation of different sub-
Acid production from
KOH) and VP 2 ((40 %
mannitol, and assimilation of different substrates were tested with API 20NE strips (bioMérieux). The activities of various enzymes and acid production were tested by using API ZYM and API Staph strips (bioMérieux). All experiments conducted using commercial kits were performed according to manufacturer’s instructions. Acid production from d-glucose was confirmed by using methyl red and Voges-Proskauer tests with glucose phosphate peptone medium consisting 0.5 % glucose, 0.5 % peptone and 0.5 % K2HPO4 (w/v) per 100 ml; a few drops of a solution of 0.04 % (w/v) methyl red in ethanol, VP 1 (40 % KOH) and VP 2 (α-naphthol) reagents were added at intervals as described in the individual experiments.

Colonies of strain CC-RT-E
were white, raised and circular after incubation for 2 days on NA. Transmission electron micrographs of thin sections did not exhibit electron-dense granules (Fig. 2). Strain CC-RT-E
is Gram-stain-negative in young cultures (<48 h) and turns to Gram-stain-positive at the late stage of growth. Growth occurs under anaerobic conditions in NB supplemented with 0.1 % KNO3 and at 20–37 °C (optimum 30 °C), at pH 6.0–9.0 (optimum pH 7.0), and in the presence of 0–2 % (w/v) NaCl (optimum 1 %). Ellipsoidal and terminal endospores were observed. Positive reactions for oxidase and catalase activities, but negative for DNase activity. Distinguished from Ammoniphilus oxalivorans and Ammoniphilus oxalaticus, strain CC-RT-E
can grow on rich media such as NA, tryptic soy agar (TSA; Bacto) and R2A agar (BD Difco), but not in ammonium-based medium (OM-2) with different concentrations of ammonium ions as described by Zaitsev et al. (1998). Additionally, strain CC-RT-E
showed several distinct physiological and biochemical characteristics; a comparison of the phenotypic properties between strain CC-RT-E
and the type strains of recognized species of the genus Ammoniphilus is given in Table 1. Detailed phenotypic characteristics of strain CC-RT-E
are given in the species description.

For the investigation of chemotaxonomic characteristics, strain CC-RT-E
and reference strains were harvested at a similar physiological age (48 h) on NA and OM-2 plates, respectively. Polar lipids were extracted and analysed by two-dimensional TLC (Minnikin et al., 1984), and total lipids profiles were stained with 10 % ethanolic molybdophosphoric acid. Aminolipids were detected with 0.2 % (w/v) solution of ninhydrin in butanol, phospholipids were detected with Dittmer and Lester’s Zinzadze reagent, and glycolipids were detected with α-naphthol spray reagent. Isoprenoid quinones were purified by the methods according to Minnikin et al. (1984) and analysed by HPLC as described by Collins (1985). The cell-wall diamino acid was

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain CC-RT-E
and related species. Bootstrap values (>50 %) based on 1000 replications are shown at the branching points. Open circles indicate that the corresponding nodes were recovered in the tree reconstructed based on the maximum-likelihood algorithm, while filled circles indicate that the corresponding nodes were also recovered based on maximum-likelihood and maximum-parsimony algorithms. Burkholderia sabiae Br3407
was used as an outgroup. Bar, evolutionary distance (Knuc) of 0.02.

The cell–wall peptidoglycan of strain CC-RT-E
was of the AB type (Table 1). The muropeptide units were glycyl-L-glutamyl-L-lysine (GGDL) and meso-diaminopimelyl-L-glutamic acid (CDMGA). The diamino acid, which was found in 90 % of the strains, was diamino-pimelic acid. The G+C content of the cell–wall peptidoglycan was 53.6 mol % (Table 1). A 16S rRNA gene sequence of strain CC-RT-E
was determined using reverse primer 16S Fw1 (Taniguchi et al., 1989) and a universal primer 16S Rev. The sequence showed 99.9 % identity with the type strain of Aneurinibacillus terranovensis (KM178533). Phylogenetic analysis (Fig. 1) showed a relatively close relationship between CC-RT-E
and other members of the genus Ammoniphilus. According to the same analysis, strain CC-RT-E
was placed in a separate lineage, although it showed 99.2 % 16S rRNA gene identity with the type strain of Aneurinibacillus oxalivorans (AB271756)
. (Fig. 1). The major menaquinone was Q-10, which was also detected in other members of the genus Ammoniphilus. A aerobic Gram-negative bacillus from anoxic soil is described in this paper and given the name Aneurinibacillus amnoniphilus sp. nov.
determined from whole-cell hydrolysates (6 N HCl, 100 °C, 18 h) subjected to TLC on cellulose plates using the solvent system of Rhuland et al. (1955). For analysis of DNA G+C content, DNA samples were prepared and degraded enzymatically into nucleosides as described by Mesbah et al. (1989). The nucleoside mixture obtained was then separated via HPLC [Hitachi L-2130 chromatograph equipped with a Hitachi L-2200 autosampler, Hitachi L-2455 Diode array detector, and a reverse-phase C18 column (Phenomenex Synergi 4 µ Fusion-RP80 250 × 4.60 mm)]. Polyamines were extracted as described by Scherer & Kneifel (1983) and analysed by HPLC. The dansyl derivatives were separated by using a Hitachi L-2130 chromatograph equipped with a Hitachi L-2200 autosampler, Hitachi L-2485 fluorescence detector (excitation at 360 nm and emission at 520 nm), and a reverse-phase C18 column (Phenomenex Synergi Fusion-RP80, 250×4.60 mm, 4 µm particle size). Fatty acid methyl esters were prepared, separated and identified according to the standard protocol (Paisley, 1996) of the Microbial Identification System (MIDI) (Sasser, 1990) using a gas chromatograph (Agilent 7890A) fitted with a flame ionization detector. Grown cultures were harvested from the plate and subjected to saponification, methylation and extraction (Miller, 1982). Identification and comparison were made by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0).

The DNA G+C content analysis of strain CC-RT-E T showed that the genomic G+C content was 39.2 mol%, which is close to the genera Ammoniphilus (42–46 mol%) (Zaitsev et al., 1998), Aneurinibacillus (43–47 mol%) (Shida et al., 1994; Goto et al., 2004), Brevibacillus (40–57 mol%) (Logan & De Vos, 2009), and Paenibacillus (35–59 mol%) (Shida et al., 1997; Montes et al., 2004; Takeda et al., 2005; Yao et al., 2014). The predominant quinone system was menaquinone-7 (MK-7, 91 %), which is consistent as for the other genera in the family Paenibacillaceae. A minor amount of MK-6 (9 %) was also detected. The polyamine pattern of strain CC-RT-E T showed predominance of sym-homospermidine, putrescine and 2-hydroxyputrescine as major polyamines. The polar lipid profile of strain CC-RT-E T consists of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol,

Fig. 2. Scanning electron micrograph (a) and transmission electron micrographs using thin section (b) and negative staining (c) of strain CC-RT-E T. Arrow indicates the formation of endospore. Bars, 100 nm (a), 500 nm (b) and 1 µm (c).

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phosphatidylmethylmethanolamine, an unknown phospholipid and an unknown aminolipid which is similar to the other two species of the genus *Ammoniphilus* except that phosphatidylmethylmethanolamine present in strain CC-RT-E³ was not present in other members of the genus *Ammoniphilus* (Fig. S1, available in the online Supplementary Material). Analysis of the cell-wall diamino acid of strain CC-RT-E³ revealed meso-diaminopimelic acid as the diagnostic diamino acid, which was also detected in reference strains *A. oxalivorans* DSM 11537ᵀ and *A. oxalaticus* DSM 11538ᵀ (Fig. S2). The fatty acid profile of strain CC-RT-E³ was similar to those for recognized species of the genus *Ammoniphilus*. The major fatty acids (>5%) were C₁₄:₀ (12.3%), iso-C₁₅:₀ (18.0%), anteiso-C₁₅:₀ (33.3%), and C₁₆:₁ω7t/C₁₆:₁ω6c (summed feature 3; 13.5%). Strain CC-RT-E³ contained qualitative and quantitative differences (C₁₄:₀, iso-C₁₅:₀, anteiso-C₁₅:₀, iso-C₁₆:₀, anteiso-C₁₇:₀, and anteiso-C₁₇:₁ω9c) in fatty acid methyl esters when compared with *A. oxalivorans* DSM 11537ᵀ and *A. oxalaticus* DSM 11538ᵀ. The fatty acid components which distinguished strain CC-RT-E³ from those of recognized species of the genus *Ammoniphilus* are listed in Table 2.

Based on the distinct polyphasic data provided, including morphological, biochemical and phylogenetic (based on 16S rRNA gene sequences) characteristics, DNA–DNA hybridization, and polyamine, polar lipid and cellular fatty acid compositions, strain CC-RT-E³ was in accordance with the description of the genus *Ammoniphilus* and is therefore proposed to represent a novel species of the genus *Ammoniphilus*.

**Description of Ammoniphilus resinae sp. nov.**

*Ammomiphilus resinae* (re.si’nae. L. gen. n. *resinae* of resin, referring to the aged resin from which the type strain was isolated).

Cells are Gram-stain-variable, rod-shaped (2.8–3.4 µm in length and 0.5–0.7 µm in diameter), oxidase- and catalase-positive, and lack flagella. Tiny colonies display bright appearance after being incubated for 48–72 h. Grows at 20–37 °C (optimum 30 °C) and pH 6.0–9.0 (optimum pH 7.0), and tolerates less than 2% (w/v) NaCl (optimum 1%). Growth does not occur in ammonium-based medium (OM-2) with different concentrations (1, 3, 5, 7, 9, 11, 13 and 15 g l⁻¹) of ammonium ions. Facultatively anaerobic in NB supplemented with 0.1 % KNO₃. Ellipsoidal endospores are observed at terminal position. Utilizes numerous compounds as carbon sources, including L-arabinose, β-hydroxybutyric acid, γ-hydroxybutyric acid and

**Table 1. Differential characteristics within the genus Ammoniphilus**

<table>
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<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
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<tr>
<td>Ammonium ions requirement</td>
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<td>Indole production</td>
<td>+</td>
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<td>−</td>
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<td>Nitrate reduction</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>Oxidation of carbohydrates (Biolog GP2)</td>
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<td>t-Arabinose</td>
<td>+</td>
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<td>β-hydroxybutyric acid</td>
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<td>γ-hydroxybutyric acid</td>
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<td>Succinamic acid</td>
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<td>Enzymatic reaction (API ZYM)</td>
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<td>Esterase lipase (C8)</td>
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<td>Leucine arylamidase</td>
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<td>β-galactosidase</td>
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<td>β-glucuronidase</td>
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<td>Assimilated reaction (API 20NE)</td>
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<tr>
<td>Maltose</td>
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<td>t-Mannitol</td>
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<td>Potassium gluconate</td>
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<td>Trisodium citrate</td>
<td>−</td>
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<td>DNA G+C content (mol%)</td>
<td>39.2</td>
<td>42*</td>
<td>45–46*</td>
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*Data from Zaitsev et al. (1998).*
succinic acid. Positive for nitrate and nitrite reduction, indole production and β-glucosidase, and assimilation of D-glucose, D-mannose, potassium gluconate and malic acid. Aesculin and starch are hydrolysed but casein, gelatin and Tweens 20, 40, 60 and 80 are not. Positive for Voges-Proskauer reaction; negative for DNase activity and production of H₂S. Alkaline phosphatase, esterase (C4), esterase lipase, leucine arylamidase, cystine arylamidase, α-chymotrypsin, and naphthol-AS-Bl-phosphohydrolase activities are present. Acid is produced from the following substrates: D-arabinose, L-arabinose, D-ribose, D-xylene, D-galactose, D-glucose, L-rhamnose, D-mannitol, D-sorbitol, maltose, lactose, trehalose, glycerogen, xylitol, L-fucose, and D-arabitol. The major fatty acids are C₁₄:0, iso-C₁₅:0, anteiso-C₁₅:0 and C₁₆:0<sub>ω₆c</sub>. The cell-wall diamino acids meso-diaminopimelic acid. The polar lipid profile includes diphosphatidyglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethyl ethanolamine, an unknown phospholipid and an unknown aminolipid. The major polyamines are sym-homospermidine and putrescine. The predominant quinone system is menaquinone 7 (MK-7).

The type strain is CC-RT-E<sup>T</sup> (=BCRC 80314<sup>T</sup>=DSM 24738<sup>T</sup>) isolated from aged resin samples. The DNA G+C content of the type strain is 39.2 mol%.

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**References**


