**Rambilacter ginsenosidimutans** sp. nov., with Ginsenoside-Converting Activity

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A novel β-proteobacterium, designated BXN5-27T, was isolated from soil of a ginseng field of Baekdu Mountain in China, and was characterized using a polyphasic approach. The strain was Gram-staining-negative, aerobic, motile, non-spore-forming, and rod shaped. Strain BXN5-27T exhibited β-glucosidase activity that was responsible for its ability to transform ginsenoside Rb1 (one of the dominant active components of ginseng) to compound Rd. Phylogenetic analysis based on 16S rRNA gene sequences showed that this strain belonged to the family Comamonadaceae; it was most closely related to *Rambilacter henchiresiensis* TMB834T and *Rambilacter tataouinensis* TTB310T (96.4% and 96.3% similarity, respectively). The G+C content of the genomic DNA was 68.1%. The major menaquinone was Q-8. The major fatty acids were C16:0 summed feature 4 (comprising C16:1ω7c and/or iso-C15:0 2OH), and C17:0 cyclo. Genome and chemotaxonomic data supported the affiliation of strain BXN5-27T to the genus *Rambilacter*. However, physiological and biochemical tests differentiated it phenotypically from the other established species of *Rambilacter*. Therefore, the isolate represents a novel species, for which the name *Rambilacter ginsenosidimutans* sp. nov. is proposed, with the type strain being BXN5-27T (= DSM 23480T = LMG 24525T = KCTC 22276T).

**Keywords:** Rambilacter ginsenosidimutans, polyphasic taxonomy, 16S rRNA gene

A meteorite fell near Tataouine, Tunisia in 1931. Later, after the permission for a revisit in 1994, Thierry Heulin’s group [7] in France, while determining whether soil bacteria were responsible for the alteration and colonization of the meteorite from a meteorite fragment, isolated “nanometric” size bacterial strains collected from a fragment of weathered meteorite embedded in sandy soil in Tataouine, Tunisia. Afterwards, the genus *Rambilacter* was originally described by Heulin et al. [7]. Until now, only *R. tataouinensis* and *R. henchiresiensis* existed in the genus *Rambilacter*, and they are Gram-staining-negative, chemo-organotrophic, and cyst-producing soil bacterial strains.

During the course of a study to screen ginsenoside-converting aerobic bacterial strains in the soil of a ginseng field of Baekdu Mountain in China, several novel bacterial strains showing ginsenosides-converting activity were screened. Among them, a strain designated BXN5-27T, which could convert ginsenoside Rb1, to Rd became the subject of a taxonomic investigation.

Strain BXN5-27T was isolated from soil of a ginseng field of Baekdu Mountain in China. This soil sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and spread on modified xylan-nutrient agar (per liter: 0.02 g tryptone, 0.02 g yeast extract, 0.02 g malt extract, 0.02 g beef extract, 0.02 g casamino acid, 0.02 g soytryone, 1.0 g xylan, 0.1 g sodium pyruvate, 0.3 g K2HPO4, 0.05 g MgSO4, 0.05 g CaCl2, and 15 g agar, pH 7.0) plates after serial dilution with 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30°C for 1 month. One isolate, BXN5-27T, was cultured routinely on R2A agar (Difco) or 1/10 TSA (trypticase soy agar; Difco) at 25°C and preserved in a glycerol solution (20% [w/v]) at −70°C.

Extraction of genomic DNA was performed with a commercial genomic DNA extraction kit (Solgent), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Im et al. [8]. Full 16S rRNA gene sequences were compiled using SEQMAN software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained using the commercial genomic DNA extraction kit (Solgent), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Im et al. [8]. Full 16S rRNA gene sequences were compiled using SEQMAN software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained using the commercial genomic DNA extraction kit (Solgent), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Im et al. [8]. Full 16S rRNA gene sequences were compiled using SEQMAN software (DNASTAR).
from GenBank. Multiple alignments were performed with ClUSTAL_X [17] and gaps were edited with BIOEDIT [6]. Evolutionary distances were calculated using Kimura’s two-parameter model [9]. Phylogenetic trees were constructed by the neighbor-joining [13] and maximum-parsimony [5] methods using the MEGA3 [10], with bootstrap analysis based on 1,000 replications [4].

The nearly complete 16S rRNA gene sequence (1,465 bp) of strain BXN5-27T was determined and subjected to comparative analysis. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain BXN5-27T belonged to the family Comamonadaceae and was closely related to the genus Ramlibacter (Fig. 1). The closest phylogenetic neighbors of strain BXN5-27T were R. tataouinensis TTB310T and R. henchirenisis TMB834T (96.4% and 96.3% sequence similarity) using the EzTaxon server [http://www.eztaxon.org/][3]. The relationship that strain BXN5-27T and other members of the genus Ramlibacter had was also evident in the neighbor-joining tree, which was based on 1,350 nt (Fig. 1). These 16S rRNA gene sequence similarities of less than 97% with other strains suggests that strain BXN5-27T represents a novel species in the genus Ramlibacter, according to Stackebrandt and Goebel [15].

The Gram reaction was performed by the non-staining method as described by Buck [2]. The morphology of cells grown for 3 days at 30°C on 1/10 TSA was observed under a Nikon light microscope at ×1,000. Motility was determined with an optical microscope using the hanging drop technique. For the observation of flagella, cells grown on

![Fig. 1. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of Ramlibacter ginsenosidimutans BXN5-27T with other related species. This tree was made using the neighbor-joining method (Saitou and Nei [13]) with a Kimura [9] two-parameter distance matrix and pairwise deletion. Filled circles at nodes indicate generic branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1,000 replications) greater than 65% are shown at the branch points. Bar, 0.02 substitutions per 1 nucleotide position.]
1/10 TSA for 1 and 3 days were negatively stained with 1% (w/v) phosphotungstic acid, and after air-drying, grids were examined with a transmission electron microscope (model CM-20; Philips). Tests for the degradation of DNA [DNase agar (Scharlau) with DNase activity detected by flooding plates with 1 M HCl], starch [1], xylan, and hydroxyethyl-cellulose [16] were performed; evaluations were carried out after 7 days. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂, and oxidase activity was determined using 1% (w/v) tetramethyl p-phenylenediamine. Carbon source utilization and enzyme activities were tested by using the API 20 NE, ID 32GN, and API ZYM test kits (bioMérieux). Growth at different temperatures (4, 15, 20, 25, 30, 37, and 42°C) and various pH values (pH 5.0–10.0 at intervals of 0.5 pH units) was assessed after 10 days of incubation in 1/10 TSA and 1/2 R2A broth. For the pH experiments, three different buffers were used (final concentration, 50 mM): acetate buffer was used for pH 5.0–5.5; phosphate buffer was used for pH 6.0–8.0; and Tris buffer was used for pH 8.5–10.0. Salt tolerance was tested on 1/10 TSA supplemented with 1–10% (w/v) NaCl and incubated for 5 days at 30°C. Growth on nutrient agar and MRS agar was also evaluated at 30°C. The ginsenosides transformation method was described in our previous study [18].

Cells of strain BXN5-27ᵀ were aerobic, Gram-staining-negative, motile, non-spore-forming, rods 0.3–0.4 µm in diameter and 1.0–1.3 µm in length after culture on 1/10 TSA for 1 days (Fig. 2A), or curved rods 0.3–0.4 µm in diameter and 1.4–2.0 µm in length after culture on 1/10 TSA for 3 days (Fig. 2B), with peritrichous flagella. Cyst-forming was not observed from dried cells for 1 month, in contrast to previous reported strains of genus Ramlibacter. Owing to this reason, the genus emendation is required in this manuscript. Colonies grown on 1/10 TSA plates for 3 days were smooth, circular, and yellowish. The isolate grew on nutrient agar but not on MRS agar. β-Glucosidase activity was detected in strain BXN5-27ᵀ; these activities are responsible for the bioconversion of ginsenoside Rb₁ to Rd (Fig. 3). Other physiological characteristics of strain BXN5-27ᵀ are summarized in the species description, and the comparison of selective characteristics of strain BXN5-27ᵀ with those of closely related type strains is shown in Table 1.

![Fig. 2. TEM images of negatively stained cell of strain BXN5-27ᵀ. (A) Bar, 0.2 µm; (B) Bar, 0.5 µm.](image)

![Fig. 3. TLC analyses of time-course transformation of ginsenoside Rb₁ by strain BXN5-27ᵀ.](image)

### Table 1. Differentiating characteristics of strain BXN5-27ᵀ and related type strains of genus Ramlibacter.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods or curved rods</td>
<td>Rods or cysts</td>
<td>Rods or cysts</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reduction of nitrate to nitrite</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Assimilation of glucose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Assimilation of maltose</td>
<td>w</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Assimilation of malate</td>
<td>w</td>
<td>-</td>
<td>wᵇ</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>G+C content (mol %)</td>
<td>68.1</td>
<td>69.6ᵃ</td>
<td>66.6ᵇ</td>
</tr>
</tbody>
</table>

Strains: 1, Ramlibacter ginsenosidimutans BXN5-27ᵀ; 2, Ramlibacter tataouinensis TTB310ᵀ (KACC 11924ᵀ was used); 3, Ramlibacter henchirensis TMB834ᵀ (KACC 11925ᵀ was used).

All strains were positive for catalase and oxidase, and negative for reduction of nitrate to nitrogen, indole production, arginine dihydrolase, urease, and protease, and all strains were negative for assimilation of arabinose, mannose, mannitol, N-acetylglucosamine, gluconate, caprate, adipate, citrate, and phenyl-acetate. Discrepancy of protease activity of Ramlibacter henchirensis TMB834ᵀ was found from Heulin et al. [7]. All data for taxa were from this study. +, Positive; -, negative; w, weakly positive.

ᵃOnly these data were taken from Heulin et al. [7].
ᵇDiscrepancy was found from Heulin et al. [7].
described by Moore [12] and was enzymatically degraded into nucleosides; the G+C content was determined as described by Mesbah et al. [11] using a reverse-phase HPLC. Cellular fatty acids profiles were determined for strains grown on 1/10 TSA (Difco) for 3 days. Cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were identified by the Microbial Identification software package capillary GLC Hewlett Packard 6890 by the Sherlock system MIDI 6.0 and the Sherlock Aerobic Bacterial Database (TSBA60) [14].

The genomic DNA G+C content of strain BXN5-27T was 68.1 mol%. As shown in Table 2, the major fatty acids of strain BXN5-27T were C₁₀₀ (44.9%), C₁₆₋₁ ω7c and/or iso-C₁₅₀₂-2-OH (23.1%), and C₁₇₋₀ cyclo (15.0%), which is consistent with fatty acid data for R. tataouinensis TTB310T (KACC 11924T was used) and R. henchirensis TMB834T (KACC 11925T was used); however, qualitative and quantitative differences in the fatty acid compositions distinguished strain BXN5-27T from R. tataouinensis and R. henchiresis (Table 2).

In conclusion, phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain BXN5-27T was most closely related to genus Ramlibacter (Fig. 1) and genomic and chemotaxonomic data (i.e., C₁₆₋₀, C₁₆₋₁ ω7c and/or iso-C₁₅₀₂-2-OH, and C₁₇₋₀ cyclo as major fatty acids) supported the classification of strain BXN5-27T in the genus Ramlibacter. However, the biochemical tests (Table 1) and morphological difference enabled strain BXN5-27T to be differentiated from the other established species of Ramlibacter. Therefore, strain BXN5-27T should be assigned to the genus Ramlibacter as the type strain of a novel species, for which the name R. ginsenosidimutans sp. nov. is proposed.

**Emended Description of the Genus Ramlibacter**

This emended description is based on that given by Heulin et al. [7] but with the following amendment. Cells are non-motile or motile by means of polar flagella. Cells are pleomorphic or non-pleomorphic, negative or positive for nitrate reduction to nitrite.

**Description of Ramlibacter ginsenosidimutans sp. nov.**


Cells are Gram-staining-negative, aerobic, motile, and rod shaped after 1 day on 1/10 TSA and become curved rods after 3 days. Colonies grown on 1/10 TSA for 3 days are smooth, circular, convex, transparent, and yellowish. On 1/10 TSA, grows at 25–37°C, but not at 18 or 42°C. Growth occurs at pH 6.0–8.5 and in less than 1% NaCl, and optimally at pH 7.0 without NaCl. DNA, starch, xylan, and cellulose are not hydrolyzed. Substrate utilization, enzyme production, and other physiological characteristics are indicated in Table 1. In addition to the carbon sources listed in Table 1, it utilizes 4-hydroxybenzoate, 3-hydroxybenzoate, and ω-melibiose as sole carbon sources. Does not utilize arabinose, acetate, 1-alanine, N-acetylglucosamine, citrate, caprate, fucose, gluconate, glycoll, i-histidine, 3-hydroxybutyrate, itaconate, i-inositol, 2-ketogluconate, ni-lactate, mannotol, mannose, maltose, malate, phenyl-valerate, propionate, t-proline, β-ribose, rhamnose, β-sorbitol, β-sucrose, salicin, suberate, or l-serine as sole carbon sources. According to the API ZYM gallery, produces esterase (C4), β-glucosidase, and β-galactosidase, but not alkaline phosphatase, acid phosphatase, N-acetyl-β-glucosaminidase, esterase lipase (C8), α-fucosidase, α-galactosidase, α-glucosidase, β-glucuronidase, leucine arylamidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, and trypsin and valine arylamidase.

The C₁₆₋₀, summed feature 4 (comprising C₁₆₋₁ ω7c and/or iso-C₁₅₀₂-2-OH), and C₁₇₋₀ cyclo are the predominant cellular fatty acids. The G+C content of genomic DNA is 68.1 mol%.

| Table 2. Cellular fatty acid profiles of strain BXN5-27T and type related strains of genus Ramlibacter. |
|-----------------|---|---|---|
| **Fatty acid** | **1** | **2** | **3** |
| Saturated fatty acids | | | |
| C₁₂₋₀ | - | 8.6 | - |
| C₁₄₋₀ | 3.6 | - | 0.8 |
| C₁₆₋₀ | **44.9** | **25.5** | **38.1** |
| C₁₈₋₀ | 3.5 | 5.3 | - |
| Hydroxy fatty acids | | | |
| C₁₀₀ OH | - | 2.4 | - |
| C₁₂₀ OH | 2.2 | - | - |
| Cyclo fatty acid | | | |
| C₁₇₋₀ cyclo | **15.0** | **5.6** | **19.9** |


All data for taxa were from this study. Cells were cultured on 1/10 TSA agar for 3 days. Some fatty acids that account for less than 0.5% of the total fatty acids are not shown. Therefore, the percentages do not add up to 100%. Major fatty acids are shown in bold type.

Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed features consist of the following: 4, iso-C₁₅₀₂-2-OH and/or C₁₆₋₁ ω7c; 7, C₁₆₋₁ ω7c and/or C₁₈₋₁ ω7c; 10, C₁₆₋₁ ω7c and/or C₁₈₋₁ ω7c; 12, C₁₆₋₁ iso-C₁₅₀₂-2-OH; and C₁₇₋₀ cyclo as major fatty acids. The G+C content of genomic DNA is 68.1 mol%.
The type strain is BXN5-27T (= DSM 23480T = LMG 24525T = KCTC 22276T), isolated from soil of a ginseng field of Baekdu Mountain in China.

Acknowledgments

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Abbreviations


References