Thermoleophilum album and Thermoleophilum minutum are culturable representatives of group 2 of the Rubrobacteridae (Actinobacteria)

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Analysis of the morphological and genotypic properties of three obligately thermophilic strains of Thermoleophilum album and Thermoleophilum minutum, originally described as green non-sulfur bacteria, indicates that these taxa belong to the Rubrobacter subdivision of the Actinobacteria. EM of the cell wall clearly showed morphology typical of Gram-positive bacteria. A comparison of 16S rRNA gene sequences, including signature nucleotide pairs and secondary structural features considered diagnostic for the subclass Rubrobacteridae, revealed that the three strains of Thermoleophilum were highly similar and should be considered as members of group 2 of this subdivision, represented up to now only by uncultured organisms.

Almost 20 years ago, Zarilla & Perry (1984, 1986a) described aerobic, obligately thermophilic bacteria restricted to growth on a narrow range of n-alkanes. The cell wall was characterized as Gram-negative. Based on the unique substrate specificity and the absence of phenotypic similarity to any of the previously described genera of obligate thermophiles, the genus Thermoleophilum was proposed. Six known strains of Thermoleophilum have been isolated from several thermal and non-thermal areas across the USA (Phillips & Perry, 1976; Merkel et al., 1978; Zarilla & Perry, 1984). The bacteria can grow only on C12–19 n-alkanes, cyclohexane, cycloheptane, C12–18 alcohols and C13–19 ketones. No other carbon sources have been shown to support growth (Zarilla & Perry, 1984). These bacteria are rods, 0·4 μm in diameter and 0·7–1·5 μm long. Colonies are small and translucent or white. Diaminopimelic acid has been found in the cell wall of all strains and lysine or ornithine is also found in some strains. The DNA G+C composition is 68–70 mol%. Optimal pH for growth is between 6·5 and 7·5. Growth occurs at temperatures of 45–70 °C, with optimum growth at 60 °C. All strains of Thermoleophilum were initially classified within the single species Thermoleophilum album but later, a second species, Thermoleophilum minutum, was described (Zarilla & Perry, 1984, 1986a) [T. minutum was validly described before the valid publication of the names Thermoleophilum and T. album (Zarilla & Perry, 1986a, b)]. T. minutum is represented by strain YS-4T (=ATCC 35265T), which was isolated from a hot spring in Yellowstone National Park, USA (63 °C at source), and strain PTA-1 (=ATCC 35268), which was formerly described as Thermomicrobium fosteri (Phillips & Perry, 1976) and was isolated from soil in North Carolina, USA (ambient temperature at source). The type strain of T. album, HS-5T (=ATCC 35263T), was isolated from a hot spring in Arizona, USA (61 °C at source). Three other strains of T. album, YS-3 (=ATCC 35264), NM (=ATCC 35266) and RR-D (=ATCC 35267), were respectively isolated from hot springs at Yellowstone and Fayedook (>60 °C at source) and from soil in North Carolina (ambient temperature at source). All of these strains were deposited in the ATCC and they were described briefly by Perry (1984) as ‘other organisms’ within the genus Thermomicrobium (green non-sulfur bacteria).

During long-term study of different hydrocarbonoclastic micro-organisms that are highly specialized for alkane degradation, Thermoleophilum strains were ordered and examined. The present study was performed on three of the four strains available at the ATCC, namely T. album strains HS-5T and NM and T. minutum PTA-1. T. album RR-D (=ATCC 35267) was unrecoverable; this was also observed during sequential cultivation passages with ‘Candidatus Microthrix parvicella’ (Rheims et al., 1996). The two remaining strains, including the type strain of T. minutum, are not available in public collections of micro-organisms and are apparently lost.
For ultrastructural analysis, mid-exponentially grown cells were fixed and embedded by conventional methods. Ultrathin sections were examined by TEM and revealed to be bacillus-like rods with a dense cytoplasm, which often contained holes derived from inclusions that were withdrawn from the section during the cutting process (Fig. 1a). The diameter of the cells was 140–217 nm. The focus of interest was the ultrastructural organization of the cell wall. At high magnification, the cell wall generally displayed a series of layers typical of Gram-positive bacteria. The cytoplasmic membrane had a mean thickness of 5.4 ± 0.8 nm (range 4.4–6.7 nm; Fig. 1b, labelled ‘cm’). It formed a layer internal to the homogeneous murein matrix (Fig. 1b, ‘cw’), limited on both sides by electron-dense structures. The murein matrix thickness ranged from 12.5 to 15.5 nm, with a mean of 14.4 ± 0.8 nm.

Fig. 1. Ultrastructure of mid-exponential phase cells of *T. album* HS-5<sup>T</sup>. (a) The rod-like cells often contain electron-translucent holes, which are inclusions torn out during the sectioning process; bar, 200 nm. (b) Cell wall ultrastructure. The massive murein layer (cw) covers the electron-translucent cytoplasmic membrane (cm), which is in contact with the cytoplasm (cp); bar, 50 nm. (c) Mean density plot of the cell-wall area boxed in (b), with murein and cytoplasmic membrane regions indicated by bars.

Comparative analysis of the almost complete 16S rDNA sequences of *Thermoleophilum* strains (1460 bp) using FASTA and BLAST (Altschul et al., 1997) to search the databases and the Ribosomal Database Project revealed that these strains clustered together with uncultured organisms belonging to group 2 of the subclass *Rubrobacteridae*. Using the approach of Anzai et al. (2000), by eliminating from analysis the hypervariable regions at positions 70–100, 181–219, 447–487, 1004–1036, 1133–1141 and 1446–1456 (in the *Escherichia coli* numbering system), the placement of these organisms within group 2 was evident (Fig. 2). Moreover, comparison with environmental *Rubrobacter*-like 16S rDNA clone sequences, for which a common fragment of about 330 nt (corresponding to *E. coli* positions 93–436) is available, indicated that all *Thermoleophilum* strains formed a deep branch together with riboclone YNPFP59 (uncultured thermal soil bacterium) and exhibited a moderate degree of relatedness within group 2 of the *Rubrobacteridae* (Fig. 3). Using least-squares, Jukes–Cantor and maximum-likelihood methods, identical branching patterns were obtained; differences were found only in the length of the branches.

However, placement of the *Thermoleophilum* strains within this group of the *Rubrobacteridae* is not definite. Accordingly to Rheims et al. (1996), comparison of the 1329–1373 nt region revealed that this characteristic fragment of the *Thermoleophilum* sequence was related more closely to that of the group 3 type sequence G9 (96–98% sequence similarity in this region) than to the group 2 type sequence TM36 (91–93%). The relatedness between *Rubrobacter radiotolerans* (group 1) and *Thermoleophilum* sequences in this region was 85–87%. Signature nucleotide pairs for the subclass *Rubrobacteridae* had been tentatively proposed on the basis of positions that were uniquely present in the type species, *R. radiotolerans* (Stackebrandt et al., 1997). Environmental clones belonging...
Fig. 2. Inferred rooted neighbour-joining tree based on 16S rDNA sequences showing the relationship of Thermoleophilum strains to the Actinobacteria, generated using the approach of Anzai et al. (2000). Methanococcus jannaschii and Chloroflexus aggregans sequences were respectively used for rooting and outgrouping. Bootstrap values (calculated from 100 trees) obtained for Thermoleophilum strains and related Rubrobacter group 2 representatives are given. Bar, 0.1 fixed-point changes per nucleotide position.

Fig. 3. Neighbour-joining tree based on 16S rDNA sequences representing the phylogenetic placement of Thermoleophilum strains among known groups of rubrobacteria. The tree was constructed from analyses of 329 nt corresponding to E. coli positions 93–436. Methanococcus jannaschii and Acidothermus cellulolyticus sequences were respectively used for rooting and outgrouping. Bootstrap values lower than 50 (calculated from 100 trees) are not shown. Bar, 0.05 fixed-point changes per nucleotide position.
Table 1. Nucleotide signatures for the subclass *Rubrobacteridae* present in 16S rDNA clones known to be phylogenetically related to *R. radiotolerans*

Source of sequences: 1, *R. radiotolerans* (type sequence group 1); 2, peat bog clone TM36 (type sequence group 2); 3, soil 0649 clone 1G9 (type sequence group 3); 4, *T. album* ATCC 35263T. Numbering of signature nucleotides for *E. coli* as defined by Holmes *et al.* (2000). +, Signature similar to that of *R. radiotolerans*.

<table>
<thead>
<tr>
<th>Positions</th>
<th>Source of sequences</th>
<th>Signature nucleotide pairs for:</th>
</tr>
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<tbody>
<tr>
<td>941–1342</td>
<td>1, <em>R. radiotolerans</em></td>
<td>1234 G–C + + +</td>
</tr>
<tr>
<td>568–779</td>
<td>2, TM36</td>
<td>1234 G–C + + +</td>
</tr>
<tr>
<td>865–1071</td>
<td>3, 1G9</td>
<td>1234 G–C + + +</td>
</tr>
<tr>
<td>1115–1326</td>
<td>4, <em>T. album</em> ATCC 35263T</td>
<td>1234 G–C + + +</td>
</tr>
</tbody>
</table>

Table 1.

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References


To both groups 2 and 3 showed three exceptions (Holmes *et al.*, 2000). However, these signature nucleotide pairs, considered to be diagnostic for group 1, have limited value in the consideration of the group of *Rubrobacteridae* to which the genus *Thermoleophilum* should be assigned. Four and three mismatches were found in relation to group 1 and groups 2/3, respectively (Table 1).

To sum up, *T. album* HS-5T, *T. album* NM and *T. minutum* PTA-1 were found to be phylogenetically affiliated with the actinomycete line of descent, specifically with group 2 of the *Rubrobacteridae*. Thus, they are the first known culturable representatives of this intriguing group of ‘yet-to-be-cultured’ soil actinobacteria.