Description of *Microbacterium foliorum* sp. nov. and *Microbacterium phyllosphaerae* sp. nov., isolated from the phyllosphere of grasses and the surface litter after mulching the sward, and reclassification of *Aureobacterium resistens* (Funke et al. 1998) as *Microbacterium resistens* comb. nov.

Undine Behrendt,1 Andreas Ulrich2 and Peter Schumann3

The taxonomic position of a group of coryneform bacteria isolated from the phyllosphere of grasses and the surface litter after sward mulching was investigated. On the basis of restriction analyses of 16S rDNA, the isolates were divided into two genotypes. According to the 16S rDNA sequence analysis, representatives of both genotypes were related at a level of 99.2% similarity and clustered within the genus *Microbacterium*. Chemotaxonomic features (major menaquinones MK-12, MK-11 and MK-10; predominating iso- and anteiso-branched cellular fatty acids; G+C content 64–67 mol%; peptidoglycan-type B2/β with glycolyl residues) corresponded to this genus as well. DNA–DNA hybridization studies showed a reassociation value of less than 70% between representative strains of both subgroups, suggesting that two different species are represented. Although the extensive morphological and physiological analyses did not reveal any differentiating feature for the genotypes, differences in the presence of the cell-wall sugar mannose enabled the subgroups to be distinguished from one another. DNA–DNA hybridization with type strains of closely related *Microbacterium* spp. indicated that the isolates represent two individual species, which can also be differentiated from previously described species of *Microbacterium* on the basis of biochemical features. As a result of phenotypic and phylogenetic analyses, the species *Microbacterium foliorum* sp. nov., type strain P 333/02T (＝ DSM 12966T ＝ LMG 19580T), and *Microbacterium phyllosphaerae* sp. nov., type strain P 369/06T (＝ DSM 13468T ＝ LMG 19581T), are proposed. Furthermore, the reclassification of *Aureobacterium resistens* (Funke et al. 1998) as *Microbacterium resistens* (Funke et al. 1998) comb. nov. is proposed.

**Keywords:** *Microbacterium foliorum* sp. nov., *Microbacterium phyllosphaerae* sp. nov., plant-associated, phenotypic and phylogenetic analysis, *Microbacterium resistens* comb. nov.

INTRODUCTION

Strains of the genus *Microbacterium* are widespread and can be isolated from different sources (Collins & Bradbury, 1992). The above-ground parts of plants have also been reported as a habitat of such bacteria possessing the group B type of peptidoglycan. Thompson et al. (1993) and Legard et al. (1994)
isolated strains of the species *Microbacterium lacticum*, *Microbacterium liquefaciens* and *Microbacterium saperdae* from the phyllospheres of sugar beet (*Beta vulgaris*) and spring wheat (*Triticum aestivum*). McInroy & Kloepper (1995) detected strains of *Microbacterium* spp. as well as *Aureobacterium* spp. living endophytically in sweet corn (*Zea mays*) and cotton (*Gossypium hirsutum*). However, the species of the genus *Aureobacterium* were accommodated in the genus *Microbacterium* as a consequence of a thorough taxonomic reinvestigation of these organisms (Takeuchi & Hatano, 1998a). Coincidental with this reclassification of *Aureobacterium* species, a new species of this genus, *Aureobacterium resistent*is*, was described (Funke et al., 1998) and therefore could not be included in the study of Takeuchi & Hatano (1998a). Although *A. resistentis* displays all the taxonomic characteristics of the redefined genus *Microbacterium*, the reclassification of *A. resistens* as *Microbacterium resistens* comb. nov. is still pending.

In the course of studying the composition of phyllosphere microbial communities of grasses and the microbes in decaying surface litter after sward mulch, the microbial communities of grasses and the *saperdae* (Funke et al., 1998). The hydrolysis of Tween 60, Tween 80, and starch was assayed according to Sands (1990). Growth on TTC and CNS medium was tested according to Vidaver & Davis (1994). The capacity for anaerobic growth was tested using Anercult A (Merck). API 20NE (bioMérieux) and the API 50CH gallery using the API CHE suspension medium (bioMérieux) were applied to determine additional physiological and biochemical characteristics.

Cluster analysis of physiological features was performed by using the unweighted pair group arithmetic average-linkage algorithm method, which was based on Pearson correlation or squared Euclidean distances (Sneath & Sokal, 1973).

**Determination of chemotaxonomic characteristics.** Methods for the determination of peptidoglycan structure, menaquinone patterns and DNA base composition have been described previously (Groth et al., 1999). The peptidoglycan structure was elucidated by two-dimensional ascending TLC of amino acids and peptides in cell-wall hydrolysates on cellulose plates. Menaquinones were analysed by reversed-phase HPLC. G+C contents were determined by HPLC of nucleosides. The determination of the glycolate content was performed according to the colorimetric method of Uchida et al. (1999). Fatty acid methyl esters were analysed by GC as described by Stead et al. (1992). Sugars in cell-wall hydrolysates were analysed by TLC as described by Komagata & Suzuki (1987).

**16S rDNA sequence determination and phylogenetic analysis.** Restriction analyses of amplified 16S rDNA were performed as described previously (Behrendt et al., 1999). For 16S rDNA sequence determination, total DNA of the representative strains P 333/02 and P 369/06 was obtained. Cells grown overnight in 2 ml media were washed with TE buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA) and frozen in liquid nitrogen. The frozen cells were crushed using a glass pestle, and the lysate was resuspended in 200 µl TE buffer. Further purification was carried out using the QiaAmp Blood Kit (Qiagen) according to the manufacturer's instructions. The 16S rDNA was amplified with *Pfu* DNA polymerase (Promega) and cloned into the vector PCR4Blunt-TOPO using the Zero Blunt TOPO cloning kit for sequencing (Invitrogen). A cycle sequencing protocol was applied for sequencing both complementary strands with a Li-Cor Sequencer (model 4200; MWG Biotech). The similarity values were based on pairwise comparisons of sequences. For phylogenetic analyses, the DNA sequences were aligned using the clustal W algorithm (program version 1.74; Thompson et al., 1994) and the trees were constructed using the neighbour-joining and maximum-likelihood algorithms (phylip computer program package, version 3.57; Felsenstein, 1993). The neighbour-joining algorithm (neighbor; Saitou & Nei, 1987) is based on a matrix of pairwise distances corrected for multiple base substitutions by the method of Kimura (1980) (dnadist with a transition/transversion ratio of 2:0). The maximum-likelihood method (dnaml; Felsenstein, 1981) was applied with three jumbles of the dataset and without global rearrangement. The 16S rDNA sequence of *Clavibacter michiganensis* was used as the outgroup in both calculations. The neighbour-joining tree was generated using the neighbour-joining and maximum-likelihood algorithms. The neighbour-joining tree was generated using the neighbour-joining and maximum-likelihood algorithms. The neighbour-joining tree was generated using the neighbour-joining and maximum-likelihood algorithms.

**METHODS**

**Bacterial strains and cultivation.** The bacterial strains examined in this study were isolated from grasses and surface litter, as described previously (Behrendt et al., 1997). The histories and corresponding numbers of the isolates and type strains of *Microbacterium* spp. and *A. resistens* used for comparative studies are listed in Table 1. General laboratory methods were performed as described previously (Behrendt et al., 1997). The *SIFIN* or in nutrient broth II (*SIFIN*) at 25 °C unless otherwise stated. Stacks of all cultures were maintained at −79 °C, using the Microbank storage system (Pro-Lab Diagnostics).

**Morphological, physiological and biochemical characterization.** Cell morphology was determined by light microscopy after 24 and 72 h cell growth. The motility of cells was tested by using the hanging-drop method and staining of flagella, according to the method described by Rudolph & Marvids (1990). The Gram reaction was tested by using the classical staining procedure, as described by Süssmuth et al. (1987). The rapid KOH string test (Ryu, 1938), growth on MacConkey agar (Merck), and the test for L-alanine aminopeptidase (Bactident test strips; Merck) were also applied. Most tests for characterizing the biochemical profiles of studied strains were performed as described previously (Behrendt et al., 1999). The production of acetoin (the Voges–Proskauer reaction) was determined according to Süssmuth et al. (1987). The hydrolysis of Tween 60, Tween 80, and starch was assayed according to Sands (1990). Growth on TTC and CNS medium was tested according to Vidaver & Davis (1994). The capacity for anaerobic growth was tested using Anercult A (Merck). API 20NE (bioMérieux) and the API 50CH gallery using the API CHE suspension medium (bioMérieux) were applied to determine additional physiological and biochemical characteristics.

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Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Isolate no./species</th>
<th>Collection number(s)</th>
<th>Source</th>
<th>Isolation date</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 206/02</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>20/04/93</td>
</tr>
<tr>
<td>P 206/08</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>22/06/93</td>
</tr>
<tr>
<td>P 315/01</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>15/06/93</td>
</tr>
<tr>
<td>P 333/02</td>
<td>DSM 12966&lt;sup&gt;T&lt;/sup&gt;, LMG 19580&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Phylophores of grasses</td>
<td>29/06/93</td>
</tr>
<tr>
<td>P 334/05</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>29/06/93</td>
</tr>
<tr>
<td>P 369/06</td>
<td>DSM 13468&lt;sup&gt;T&lt;/sup&gt;, LMG 19581&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Phylophores of grasses</td>
<td>13/07/93</td>
</tr>
<tr>
<td>P 375/05</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>–</td>
</tr>
<tr>
<td>P 403/11</td>
<td>–</td>
<td>Decaying grasses in the litter layer</td>
<td>20/07/93</td>
</tr>
<tr>
<td>P 416/01</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>27/07/93</td>
</tr>
<tr>
<td>P 421/05</td>
<td>–</td>
<td>Decaying grasses in the litter layer</td>
<td>07/08/93</td>
</tr>
<tr>
<td>P 423/09</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>10/08/93</td>
</tr>
<tr>
<td>P 434/29, P 437/09</td>
<td>–</td>
<td>Decaying grasses in the litter layer</td>
<td>10/08/93</td>
</tr>
<tr>
<td>P 438/12, P 439/06</td>
<td>–</td>
<td>Decaying grasses in the litter layer</td>
<td>17/08/93</td>
</tr>
<tr>
<td>P 444/21</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>24/08/93</td>
</tr>
<tr>
<td>P 447/09, P 448/05</td>
<td>–</td>
<td>Decaying grasses in the litter layer</td>
<td>24/08/93</td>
</tr>
<tr>
<td>P 449/03, P 449/26, P 449/29, P 450/03</td>
<td>–</td>
<td>Phylophores of grasses</td>
<td>05/10/93</td>
</tr>
<tr>
<td>P 469/32</td>
<td>–</td>
<td>Sewage</td>
<td>–</td>
</tr>
</tbody>
</table>

* Isolate nos relate to the collection at the Institute of Primary Production and Microbial Ecology, Centre for Agricultural Landscape and Land Use Research Müncheberg (ZALF), Paulinenaue, Germany.

RESULTS

Restriction analysis of 16S rDNA

To analyse the phylogenetic heterogeneity of the isolates (Table 1), 16S rDNA genes were amplified with primers described by Weisburg et al. (1991), resulting in a single band of approximately 1500 bp. Digestion of these PCR products (using the endonucleases TaqI, HinfI, AluI, MspI, ScrFI and Sau3A) led to identical restriction patterns for all isolates. However, one different band per restriction pattern of the 16S rDNA sequences resulted from the endonucleases CfoI and HaeIII. The isolates were nearly equally divided into two genotypes, as follows: genotype I: P 315/01, P 333/02<sup>T</sup>, P 375/05, P 403/11, P 437/09, P 444/21, P 447/09, P 448/05, P 449/03, P 449/26, P 449/29, P 469/32; genotype II: P 206/02, P 286/08, P 334/05, P369/06<sup>T</sup>, P 416/01, P 421/05, P 423/09, P 434/29, P 438/12, P 439/06, P 450/03. One strain of each genotype, P 333/02<sup>T</sup> (I) and P 369/06<sup>T</sup> (II), was chosen as a representative strain for certain investigations.

Morphological, physiological and biochemical characteristics

All of the isolates studied were Gram-positive, strictly aerobic, non-spore-forming, irregularly rod-shaped organisms. Some cells were arranged at angles, forming V-shapes, but primary branching was not observed. In older cultures, rods were shorter, but a marked rod–coccus growth cycle did not occur. Some cells were motile by means of a single polar or lateral flagellum.

The optimum growth temperature was 25 °C. At 37 °C, growth was strain-dependent, whereas none of the isolates was capable of growing at 42 °C. On solid media, colonies were circular, slightly convex with entire margins, shiny and moist. The pigment of colonies was translucent yellow and became lemon-yellow in older cultures.

Determination of the Gram reaction by means of classical staining procedures gave uncertain results for most of the strains, as they are decolorized easily and react like Gram-negative bacteria. However, the rapid
Table 2. Physiological tests showing differing results among the 23 isolates tested

<table>
<thead>
<tr>
<th>Test</th>
<th>P 333/02T (I) (DSM 12966T)</th>
<th>P 369/06T (II) (DSM 13468T)</th>
<th>No. of strains (%)</th>
<th>Strains showing the less common response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 37 °C</td>
<td>w+</td>
<td>w+</td>
<td>15/8</td>
<td>P 444/21; P 449/29; P 286/08; P 334/05; P 434/29; P 438/12; P 439/06; P 450/03</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>–</td>
<td>–</td>
<td>1/22</td>
<td>P 206/02</td>
</tr>
<tr>
<td>Tween 60</td>
<td>+</td>
<td>+</td>
<td>21/2</td>
<td>P 437/09; P 206/02</td>
</tr>
<tr>
<td>Tween 80</td>
<td>+</td>
<td>+</td>
<td>7/16</td>
<td>P 286/08; P 334/05; P 369/06T; P 434/29; P 438/12; P 439/06; P 450/03</td>
</tr>
<tr>
<td>Starch</td>
<td>w+</td>
<td>+</td>
<td>22/1</td>
<td>P 206/06</td>
</tr>
<tr>
<td>Oxidative acid production from glucose (API 20NE)</td>
<td>+</td>
<td>+</td>
<td>22/1</td>
<td>P 438/12</td>
</tr>
<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>–</td>
<td>3/20</td>
<td>P 333/02T; P 444/21; P 447/09</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>–</td>
<td>+</td>
<td>3/20</td>
<td>P 447/09; P 369/06T; P 421/05</td>
</tr>
<tr>
<td>Acid production from (API 50CH):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Arabinose</td>
<td>–</td>
<td>–</td>
<td>13/10</td>
<td>P 333/02T; P 375/05; P 403/11; P 417/09; P 447/09; P 449/26; P 286/08; P 369/06T; P 416/01; P 450/03</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>+</td>
<td>22/1</td>
<td>P 206/02</td>
</tr>
<tr>
<td>Methyl D-xylose-glucoside</td>
<td>–</td>
<td>–</td>
<td>19/4</td>
<td>P 333/02T; P 449/29; P 369/06T; P 434/29</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>+</td>
<td>+</td>
<td>15/8</td>
<td>P 444/21; P 449/26; P 286/08; P 416/01; P 421/05; P 438/12; P 439/06; P 450/03</td>
</tr>
<tr>
<td>β-Gentiobiose</td>
<td>–</td>
<td>+</td>
<td>21/2</td>
<td>P 333/02T; P 449/26</td>
</tr>
<tr>
<td>Glycogen</td>
<td>–</td>
<td>–</td>
<td>5/18</td>
<td>P 444/21; P 449/26; P 416/01; P 421/05; P 438/12</td>
</tr>
<tr>
<td>Inulin</td>
<td>–</td>
<td>–</td>
<td>5/18</td>
<td>P 444/21; P 449/26; P 416/01; P 421/05; P 438/12</td>
</tr>
<tr>
<td>Inositol</td>
<td>–</td>
<td>–</td>
<td>2/21</td>
<td>P 423/09; P 434/29</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>–</td>
<td>7/16</td>
<td>P 444/21; P 449/29; P 206/02; P 416/01; P 421/05; P 438/12</td>
</tr>
<tr>
<td>Methyl D-xylose-mannoside</td>
<td>+</td>
<td>+</td>
<td>11/12</td>
<td>P 333/02T; P 375/05; P 403/11; P 444/21; P 449/26; P 449/29; P 206/06; P 369/06T; P 416/01; P 421/05; P 438/12</td>
</tr>
<tr>
<td>Melibiose</td>
<td>–</td>
<td>+</td>
<td>13/10</td>
<td>P 315/01; P 333/02T; P 403/11; P 447/09; P 448/05; P 449/32; P 206/02; P 286/08; P 434/29</td>
</tr>
<tr>
<td>Melezitose</td>
<td>+</td>
<td>+</td>
<td>21/2</td>
<td>P 449/29; P 434/29</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>21/2</td>
<td>P 447/09; P 206/06</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
<td>16/7</td>
<td>P 375/05; P 403/11; P 444/21; P 447/09; P 449/03; P 449/26; P 449/29</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>+</td>
<td>18/5</td>
<td>P 3705/0; P 403/11; P 447/09; P 423/09; P 450/03</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>–</td>
<td>–</td>
<td>2/21</td>
<td>P 449/26; P 438/12</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>6/17</td>
<td>P 444/21; P 449/26; P 286/08; P 416/01; P 438/12; P 439/06</td>
</tr>
<tr>
<td>Xylitol</td>
<td>–</td>
<td>–</td>
<td>1/22</td>
<td>P 206/02</td>
</tr>
<tr>
<td>α-Xylose</td>
<td>+</td>
<td>+</td>
<td>22/1</td>
<td>P 206/02</td>
</tr>
<tr>
<td>Methyl D-xylose</td>
<td>+</td>
<td>+</td>
<td>5/18</td>
<td>P 375/05; P 444/21; P 423/05; P 438/12; P 439/06</td>
</tr>
</tbody>
</table>

* Weak hydrolysis of starch, with the exception of strain P 416/01.
† Strains belonging to genotype I are indicated by the use of normal lettering; strains belonging to genotype II are indicated by bold lettering.

KOH string test resulted in a Gram-positive reaction, which was supported by the absence of L-alanine aminopeptidase. No growth was observed on MacConkey agar.

All strains showed positive results for catalase, β-galactosidase, DNase, aesculin and gelatin hydrolysis, as well as for growth in the presence of 2% NaCl. They assimilated arabinose, gluconate, glucose, malate, maltose, mannitol, mannose, N-acetylglucosamine and produced acid from amygdalin, arbutin, cellobiose, d-fructose, galactose, glycerol, maltose, man- nitol, D-mannose, salicin, sucrose and trehalose. The following characteristics were negative for all strains: assimilation of adipate and caprate; acid production from adonitol, D-arabinol, L-arabinol, dulcitol, D-fucose, erythritol, galactone, 2-ketogluconate, 5-ketogluconate, L-sorbose, D-tagatose and L-xylitol; the oxidase reaction; reduction of nitrate to nitrite; indole production; H₂S production from sodium thiosulphate; urease; arginine dihydroxase and the Voges–Proskauer reaction. Oxidative and fermentative production of acid from glucose, according to the method of Hugh & Leifson (1953) did not occur, but oxidative acid production was positive for all of the strains tested with API 50CH and for nearly all tested with API 20NE.

Physiological test results demonstrating the differences between the strains studied are given in Table 2. These differences did not correspond to genotype affiliations. Cluster analysis of physiological characteristics showed no clustering according to the genotypes (data not shown). The results of additional physiological tests for representative strains P 333/02T (I) and P 369/06T (II) also failed to reveal any discriminatory features. Growth on CNS and TTC media was positive, whereas hydrolysis of cellulose and production of levan from sucrose were negative for both strains. Thus, it was not possible to differentiate effectively between all strains of genotypes I and II on the basis of the morphological or physiological features investigated.

Chemotaxonomic characteristics

Strains P 333/02T and P 369/06T were analysed with respect to their menaquinone composition, cellular fatty acid profile, cell-wall sugars and G+C content.
The menaquinones were fully unsaturated and ranged from MK-9 to MK-13, for P 333/02T, and from MK-10 to MK-13, for P 369/06T, respectively. MK-12 and MK-11 were the predominant menaquinones for both strains, constituting more than 70% of the peak area ratio; MK-10 followed at a value of approximately 10%. Strain P 333/02T contained 56-2% 12-methyl tetradecanoic acid, 17-2% 14-methyl hexadecanoic acid, 13-5% 14-methyl pentadecanoic acid, 7-5% hexadecanoic acid, 3-9% 13-methyl tetradecanoic acid, 1-2% 15-methyl hexadecanoic acid, and 0-5% 12-methyl tridecanoic acid. Strain P 369/06T contained 46-1% 12-methyl tetradecanoic acid, 16-3% 14-methyl pentadecanoic acid, 15-4% 13-methyl tetradecanoic acid, 14-7% 14-methyl hexadecanoic acid, 3-7% 15-methyl hexadecanoic acid, 3-2% hexadecanoic acid and 0-7% 12-methyl tridecanoic acid. The G + C contents of P 333/02T and P 369/06T were 67 and 64 mol%, respectively.

The cell-wall peptidoglycan type for P 333/02T and P 369/06T was found to be B2β (Schleifer & Kandler, 1972) (l-homoserine)-d-Glu→Gly→d-Orn with glycolyl residues. The cell-wall sugars of strain P 369/06T were galactose and rhamnose; strain P 333/02T additionally contained mannosyl residues.

Phylogenetic analysis

The 16S rDNA sequencing of P 333/02T and P 369/06T gave a similarity level of 99-2%, which represents differences at only 12 of the 1480 bp determined (10 substitutions and 2 additional bases in P 333/02T). All of the divergent nucleotides of the two sequences were found to be variable among Microbacterium spp. Moreover, the different nucleotides of P 333/02T and P 369/06T corresponded to those of at least two other Microbacterium species. Seven of these differences were located in a variable region that was significantly distinct within the genus Microbacterium (positions 74–97; Escherichia coli numbering system; Brosius et al., 1978).

To characterize the relationships of the isolates at the species level, the DNA–DNA similarity of strains P 333/02T and P 369/06T, as well as that of P 439/06 and P 449/03, representing two pairs of both subgroups, were examined. The results of DNA–DNA reassociation revealed similarities of 41 and 42-7%, suggesting that the strains represent different species. DNA–DNA hybridization between P 333/02T and the second strain of genotype II (P 439/06) revealed a similarity of 33-6%, supporting the above findings. However, the reassociation between P 369/06T and P 439/06, both of which are members of genotype II, showed 86-3% similarity, demonstrating that they belong to the same species.

Comparison of 16S rDNA sequences with validly described Microbacterium spp. showed that both strains (P 333/02T and P 369/06T) evidently belong to the genus Microbacterium (Fig. 1). The strains were clustered together using the neighbour-joining method and the maximum-likelihood method, which was supported by high bootstrap values. The best conformations were found with the rRNA sequences of Microbacterium maritipicum, Microbacterium oxydans, M. liquefaciens, Microbacterium keratolyticum, M. saperdae and Microbacterium luteolum, which were clustered together by both methods. Species displaying similarity values higher than 97% relative to P 333/02T (genotype I) are listed in Table 3. To clarify the taxonomic position at the species level,
DNA–DNA similarity was examined. All DNA–DNA reassociation values between strain P 333/02\(^T\) and the closely related *Microbacterium* spp. were lower than 70\% (Table 3), indicating that the isolate represents a separate species (Wayne et al., 1987).

Hybridization studies performed for the closest phylogenetic neighbours of P 369/06\(^T\) (genotype II) showed similar DNA–DNA reassociation values (Table 3), indicating that genotype II represents a new species as well.

**DISCUSSION**

Restriction analysis of 16S rDNA revealed two different genotypes among the isolates. Comparison of 16S rDNA sequences between representative strains of both subgroups and all validly described species of *Microbacterium* showed a clear affiliation to this genus (Fig. 1). The isolates clustered unambiguously within the monophyletic branch of *Microbacterium*, showing a high level of similarity to the type species *M. lacticum* (DSM 20427\(^T\)). The results of chemotaxonomic examinations focusing on selected strains supported these findings. Thus, the tested isolates displayed the major menaquinones MK-12, MK-11 and MK-10, as well as predominating iso- and anteiso-branched fatty acids, as described for the genus *Microbacterium* (Takeuchi & Hatano, 1998a). The G+C content (64 and 67 mol\%) is also within the range typical for *Microbacterium* (Takeuchi & Hatano, 1998a). The cell-wall peptidoglycan is of the B2\(\text{a}\) type based on D-ornithine (Schleifer & Kandler, 1972). These findings are in accordance with the original description of the genus *Aureobacterium* (Collins et al., 1983), accommodated recently in a redefined genus *Microbacterium* (Takeuchi & Hatano, 1998a). Thus, all of the chemotaxonomic features of the isolates correspond to the general phenotypic characteristics of the genus *Microbacterium*.

To clarify the relationship of both genotypes at the species level, DNA–DNA hybridization studies were performed. The results showed reassociation values far below 70\%, the threshold value proposed by Wayne et al. (1987) as indicating species status. From these data, it was concluded that both genotypes differentiated by 16S rDNA restriction analysis represent individual species. Comparison of the 16S rDNA sequences of strains representing the genotypes revealed a high level of similarity. Both strains formed a separate branch in the phylogenetic tree, with high bootstrap support (Fig. 1). However, because they demonstrate greater differences in their 16S rDNA sequences than are shown, for example, by the more closely related species *M. oxydans*, *M. liquefaciens* and *M. maritypicum*, 16S rDNA analysis also supported the differentiation of both genotypes at the species level. In contrast to the phylogeny, no physiological feature was found to differentiate all strains of the subgroups. The physiological characteristics of the isolates were similar, although strain-specific differences for several features are demonstrated (Table 2). Clustering on the basis of these divergent features showed no correspondence to the assignment of genotypes (data not shown). Similar results were found for species of the genus *Sulfitobacter* by Pukall et al. (1999). *Sulfitobacter mediterraneus* showed the same physiological properties as *Sulfito-
Microbacterium spp. nov.

Microbacterium foliorum (fo.li.o.’rum. L. pl. gen. neut. n. foliorum of the leaves).

Cells are Gram-positive, strictly aerobic, non-spore-forming, irregularly shaped rods, which sometimes form V-shapes and are motile by means of a single polar or lateral flagellum. In older cultures, rods are shorter, but a marked rod–coccus cycle does not occur. Colonies are yellow, shiny, slightly convex and round with entire margins. Oxidase, urease, arginine dihydrolase and Voges–Proskauer reactions are negative. Hydrogen sulphide and indole are not produced. Nitrate is not reduced to nitrite. Positive for catalase and β-galactosidase. Gelatin, DNA and aesculin are hydrolysed. Utilization of starch is weak. None of the strains hydrolyses casein and Tween 80, whereas hydrolysis of Tween 60 is strain-dependent. Arabinose, gluconate, glucose, malate, maltose, mannotol, mannose and N-acetylglucosamine are assimilated, but caprate and d-xylene are not used. Citrate and phenyl-acetate are utilized only by certain strains. Acid production is positive from amygdalin, 1-arabinose, arbutin, cellobiose, D-fructose, galactose, glycerol, maltose, mannotol, D-mannose, salicin, sucrose, trehalose and d-xylene, but negative from adonitol, D-arabitol, L-arabitol, dulcitol, D-fucose, erythritol, gluconate, 2-ketogluconate, 5-ketogluconate, inositol, L-sorbose, D-tagatose, xylitol and L-xylene. Acid production is variable between strains of D-arabinose, methyl α-D-glucoside, L-fucose, β-gentiobiose, glyce

gen, inulin, lactose, methyl α-D-mannoside, melibiose, melezitose, raffinose, rhamnose, ribose, sorbitol, starch and methyl β-D-xylodiside. Negative for the fermentative and oxidative production of acid from glucose, according to the method of Hugh & Leifson (1953), but positive for oxidative acid production when tested using API 20NE and API 50CH. Growth occurs in the presence of 2% NaCl. The optimum temperature for growth is approximately 25 °C. At 37 °C, growth is strain-dependent, but growth does not occur at 42 °C. The major menaquinones are MK-12, MK-11 and MK-10. The predominant cellular fatty acids are 12-methyl tetradecanoic acid, 14-methyl hexadecanoic acid and 14-methyl pentadecanoic acid. Contains peptidoglycan of the B2β-type peptidoglycan and an unknown cell-wall sugar composition, differed from both genotypes by the motility of the cells, H₂S production, and the assimilation of N-acetylglucosamine and malate.

Since both groups of grass-associated isolates can be distinguished from all validly described Microbacterium and Aureobacterium species on the basis of phenotypic and phylogenetic characteristics, and from each other by analysis of the cell-wall sugars, 16S rDNA restriction analysis using the enzymes CfoI and HaeIII, and DNA–DNA hybridization, we conclude that both groups deserve a separate species status. Consequently, the names Microbacterium foliorum sp. nov. and Microbacterium phyllosphaerae sp. nov. are proposed.

Furthermore, the reclassification of A. resistens (Funke et al. 1998) as Microbacterium resistens (Funke et al., 1998) comb. nov. is proposed. A. resistens (Funke et al. 1998) displayed the general characteristics of the redefined genus Microbacterium, and should be reclassified as a result of the taxonomic unification (Takeuchi & Hatano, 1998a).

Description of Microbacterium phyllosphaerae sp. nov.

Microbacterium phyllosphaerae (phy.l.o.sphaë.æae. Gr. n. phyllon leaf; Gr. fem. n. sphaira ball, sphere; M.L. gen. fem. n. phyllosphaerae of the phyllosphere).

The morphological and physiological properties are as
Table 4. Differential characteristics of *Microbacterium* spp. and *A. resistens*

Data from this study and Funke *et al.* (1998), Matsuyama *et al.* (1999), Schumann *et al.* (1999), Takeuchi & Yokota (1994), Takeuchi & Hatano (1998a, b), Yokota *et al.* (1993a, b). Abbreviations: +, positive reaction; +w, weakly positive; −, negative; +/−, different results in cited references; d, reaction differs among strains; ND, not determined; y, yellow;yw, yellow white; yb, yellow beige; ly, light yellow; o, orange; GEL, gelatine; starch; H2S, H2S production; VP, Vöges-Proskauer test; ADH, arginine dihydrolase; ARA, arabinose; NAG, N-acetylglucosamine; MLT, malate; CIT, citrate; PAC, phenyl acetate; GLC, glucose; Rha, rhamnose; Gal, galactose; Man, mannose; 6dTal, 6-deoxytalose; Fuc, fucose; Xyl, xylose.

<table>
<thead>
<tr>
<th>Species</th>
<th>Colour of colony</th>
<th>Motility</th>
<th>Growth at 37°C</th>
<th>Hydrolysis of GEL</th>
<th>H2S</th>
<th>VP</th>
<th>ADH</th>
<th>Assimilation of:</th>
<th>Acid from GLC</th>
<th>Major Menhamnone (MK)</th>
<th>Cell wall sugar(s)</th>
<th>Type of Peptidoglycan</th>
<th>Cell wall diamino acid</th>
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<td>d</td>
<td>+</td>
<td>+w</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>B20</td>
<td>Orn</td>
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<td>d</td>
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<td>+w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+w</td>
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<td>+</td>
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<td>Orn</td>
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<td>Orn</td>
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<td>+w</td>
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<td>+w</td>
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<td>+</td>
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<td>+w</td>
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<td>+</td>
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<td>d</td>
<td>ND</td>
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<td>+w</td>
<td>+</td>
<td>+</td>
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<td>Orn</td>
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<td>+</td>
<td>+w</td>
<td>+</td>
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<td>+</td>
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<td>Orn</td>
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<td>+w</td>
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<td>+</td>
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<td>+</td>
<td>+w</td>
<td>+</td>
<td>+</td>
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<td>Orn</td>
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<td>−</td>
<td>+</td>
<td>+w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>Gal, Glc</td>
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<tr>
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<td>+</td>
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<td>+w</td>
<td>+</td>
<td>+</td>
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<td>+w</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>6dTal, Man, Gal</td>
<td>B10</td>
<td>Lys</td>
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<tr>
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<tr>
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<td>B10</td>
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<td>12, 11, ND</td>
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<td>Orn</td>
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</table>

* The majority of strains showed a weak reaction with the exceptions of one strain with strong hydrolysis and one without hydrolysis.
† Determined by API 20NE; one tested strain was not able to produce acid.
‡ Traces.
§ Depending on strain; Gal, Glc, Rha, Man.
is positive. The type strain, isolated from human arginine dihydrolase and Voges–Proskauer reactions

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Balows, H. G. Tru

enaue) for their excellent technical assistance. Furthermore, we wish to thank Professor Dr H. G. Tru


Complete nucleotide sequence of a 16S ribosomal RNA gene from

Escherichia coli

Complete nucleotide sequence of a 16S ribosomal RNA gene

from

Escherichia coli


