Rouxiella badensis sp. nov. and Rouxiella silvae sp. nov. isolated from peat bog soil and emendation description of the genus Rouxiella

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Abstract

Four bacterial strains isolated from peat bog soil or swampy meadow in Baden-Württemberg (Germany) and found to have rrs sequences close to that of Rouxiella chamberiensis were compared to this species by using multi-locus sequence analysis and phenotypic tests. The four strains constituted two discrete groups (referred to as the Baden and the Silva groups) belonging to the genus Rouxiella. These groups differed in their ability to grow at 37°C, reduce nitrate into nitrite, and to produce acid from several carbohydrates. Two novel species are, therefore, proposed: Rouxiella badensis sp. nov. for the Baden group (type strain, 323=CIP 111153=DSM 100043) and Rouxiella silvae for the Silva group (type strain, 213=CIP 111154=DSM 103735). The definition of the genus Rouxiella has also been emended in order to take these two novel species into account.

In 2013, parenteral nutrition bags for newborns were found to be contaminated by a previously unknown bacterium that was named thereafter Rouxiella chamberiensis [1], type-species by monotypy of the novel genus Rouxiella.

Four surfactant-producing strains isolated from peat bog soil (strain 323) or swampy meadow (strains 213, 223, and 421) near Kaltenbronn in the northern Black Forest (Baden-Württemberg, Germany) were provisionally identified as Rouxiella sp. by rrs gene (encoding 16S rRNA) sequencing [2, 3]. These four isolates were studied using molecular methods and phenotypic tests. The result is the following description of a proposed two novel species of the genus Rouxiella.

For rrs sequencing, the exact method used was as published [1]. This extended the sequences determined by Kügler et al. [2] and Kügler [3] to 1357 bp.

Multi-locus sequence analysis (MLSA) was used, based on partial sequences of the housekeeping genes fusA (634 bp), pyrG (306 bp), rplB (333 bp), rpoB (949 bp) and sucA (501 bp). The rationale for this choice and the exact methods used have been described previously [1].

A whole-genome shotgun sequencing experiment and assembly of strains DSM 100043 (Baden group) and CIP 111154 (Silva group) was performed using the Next Generation Sequencing (NGS) technique (Illumina MiSeq). Average nucleotide identities (ANI) [4, 5] and Genome-to-Genome Distance (GGD) [6] were computed on whole genome sequences to measure the genetic and evolutionary relatedness among strains, and to help consolidate the existing taxonomic ranks of bacterial strains. The ANI calculations were performed using the in silico DNA–DNA hybridization method [4, 7] implemented in the JSPECIES software (http://imedea.uib-csic.es/jspecies/about.html; [8]) with default BLAST parameters. GGD calculations were performed using the Genome-to-Genome Distance Calculator, version 2, available at (http://ggdc.dsmz.de/).

For the rrs gene, sequences were compared to all bacterial sequences available from the GenBank database by using...
the BLAST program (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Related sequences were downloaded, compared and phylogenetic trees were generated with the MEGALIGN module of the Lasergene software (DNASTAR), using the neighbour-joining (NJ) algorithm [9]. Bootstrap analysis with 1000 replicates was performed to assess the reliability of tree branching. GenBank accession numbers of the sequences used in this study are listed in Fig. 1. The NJ tree derived from rrs sequences (1357 bp) (Fig. 1) showed the novel strains to constitute two discrete branches close to the type strain of Rouxiella chamberiensis. Table S1 (available in the online Supplementary Material) gives the nucleotide substitution ratios among strains. Rouxiella sp. strains 213T and 223 had identical rrs sequences. Rouxiella sp. strains DSM 100043T and 421 shared 99.9% rrs sequences. The pair comprising Rouxiella sp. strains 213T and 223 (provisionally referred to as the Silva group) and that comprising Rouxiella sp. DSM 100043T and 421 (provisionally referred to as the Baden group) shared 99.4 to 99.5% rrs sequences. R. chamberiensis shared 99.4 to 99.5% rrs sequences with the Baden group, and 99.0% with the Silva group. Species with more than 98% similarity with the Baden group, and 99.0% with the Silva group showed 94.83% similarity. The two strains in the Baden group (strains DSM 100043T and 421) were 99.93% similar in terms of concatenated sequences. Strains in the Baden group and the Silva group showed 94.83% similarity and 93.16 and 92.84% similarity to Rouxiella chamberiensis, respectively. The other species closest to the Baden group were Rahnella aquatilis (91.25% similarity) and Ewingella americana (90.25% similarity). Other genera were less than 87.96% (Serratia marcescens) related to the Baden group. The other species of genera closest to the Silva group were Ewingella americana (90.71% similarity) and Rahnella aquatilis (90.58% similarity). Other genera were less than 87.61% (Serratia marcescens) related to the Silva group.

The individual contribution of genes to MLSA was examined. Fig. S1 shows the five NJ trees and Tables S3 to S7, the five nucleotide substitution ratio matrices. In fusA-, pyrG- and sucA trees, the branch formed by the Baden and Silva groups formed with high bootstrap values (>75%) before joining with R. chamberiensis. In the rpoB tree, the bootstrap value of Baden and Silva branching was 74.8% before joining with R. chamberiensis. In the rplB tree, the Baden group branched with R. chamberiensis before joining with the Silva group. In all trees, R. chamberiensis, Baden and Silva formed a group distinct from other genera. The best separation of this Rouxiella group from other genera was shown in the pyrG tree where the lowest similarity within the Rouxiella group was 94.6% and the highest similarity between the Rouxiella group and other genera (Rahnella) was 87.2%.

Both strains in the Silva group (strains 213T and 223) had identical concatenated sequences. The two strains in the Baden group (strains DSM 100043T and 421) were 99.93% similar in terms of concatenated sequences. Strains in the Baden group and the Silva group showed 94.83% similarity and 93.16 and 92.84% similarity to Rouxiella chamberiensis, respectively. The other species closest to the Baden group were Rahnella aquatilis (91.25% similarity) and Ewingella americana (90.25% similarity). Other genera were less than 87.96% (Serratia marcescens) related to the Baden group. The other species of genera closest to the Silva group were Ewingella americana (90.71% similarity) and Rahnella aquatilis (90.58% similarity). Other genera were less than 87.61% (Serratia marcescens) related to the Silva group.

The individual contribution of genes to MLSA was examined. Fig. S1 shows the five NJ trees and Tables S3 to S7, the five nucleotide substitution ratio matrices. In fusA-, pyrG- and sucA trees, the branch formed by the Baden and Silva groups formed with high bootstrap values (>75%) before joining with R. chamberiensis. In the rpoB tree, the bootstrap value of Baden and Silva branching was 74.8% before joining with R. chamberiensis. In the rplB tree, the Baden group branched with R. chamberiensis before joining with the Silva group. In all trees, R. chamberiensis, Baden and Silva formed a group distinct from other genera. The best separation of this Rouxiella group from other genera was shown in the pyrG tree where the lowest similarity within the Rouxiella group was 94.6% and the highest similarity between the Rouxiella group and other genera (Rahnella) was 87.2%.

**Fig. 1.** Neighbour-joining unrooted tree based on rrs gene sequences. Bootstrap values >75% (based on 1000 replicates) are indicated by thick lines. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.
Gene concatenation with bootstrap for MLSA, although a traditional procedure in phylogenetic studies, has been shown to be misleading [10]. Bootstrap for individual genes (NJ), averaging distance matrices and bootstrap on the gene set were shown to be more accurate [10]. Since our MLSA included only five genes, resampling of genes would have been meaningless. An average distance matrix was obtained from the five distance matrices (\textit{fusA}, \textit{pyrG}, \textit{rplB}, \textit{rpoB} and \textit{sucA}) and an NJ tree was reconstructed (Fig. S2). The corresponding nucleotide substitution ratio matrix is given in Table S8. Similarity between the Silva group and the Baden group was 95.08% and similarities between R. chamberiensis and the Silva and Baden groups were 92.92 and 93.45%, respectively. The highest similarity between the \textit{Rouxiella} group and other genera was 90.3% (\textit{Rahnnella}).

The sequence comparisons detailed above support the inclusion of the Baden group and Silva group in the genus \textit{Rouxiella} and the recognition of two novel species, \textit{Rouxiella badensis} sp. nov. (Baden group) and \textit{Rouxiella silvae} sp. nov. (Silva group).

To confirm these novel species, ANI and GGD were calculated. The ANI values computed on the assembled whole-genome shotgun sequence of strains representing \textit{Rouxiella chamberiensis}, \textit{Rouxiella badensis} sp. nov. (Baden group), \textit{Rouxiella silvae} sp. nov. (Silva group), \textit{Ewingella americana}, \textit{Rahnnella aquatilis}, \textit{Serratia marcescens} and \textit{Yersinia enterocolitica} are given in Table S9. Among the three species of the genus \textit{Rouxiella}, ANI values were 79.59 to 80.70%, well below the species limit of 95.0% ANI [7]. Between the three species of the genus \textit{Rouxiella} and closely related genera (\textit{Ewingella}, \textit{Rahnnella}, \textit{Serratia} and \textit{Yersinia}), ANI values were 72.51 to 76.42%.

GGD calculations using the version 2 GGD calculator, formula 2 (as recommended by the site), and expressed as a percent in silico DNA–DNA hybridization (DDH), showed \textit{Rouxiella badensis} sp. nov. DSM 100043\textsuperscript{T} to be 24% related to both \textit{Rouxiella chamberiensis} CIP 110714\textsuperscript{T} and \textit{Rouxiella silvae} sp. nov. CIP 111154\textsuperscript{T}. This value is far below the accepted 70% for species limits for species delineation [11]. \textit{Rouxiella badensis} sp. nov. DSM 100043\textsuperscript{T} was 21% related (by in silico DDH) to \textit{Ewingella americana} ATCC 33852\textsuperscript{T}, \textit{Rahnnella aquatilis} CIP 78.65\textsuperscript{T} and \textit{Serratia marcescens} ATCC 13880\textsuperscript{T}. This Whole Genome Shotgun project has been deposited at the DDBJ/ENA/GenBank under the accession number MRWE00000000 for \textit{Rouxiella badensis} sp. nov. DSM 100043\textsuperscript{T} and under the accession number MRWD00000000 for \textit{Rouxiella silvae} sp. nov. CIP 111154\textsuperscript{T}. The version described in this paper is version MRWE01000000 for \textit{Rouxiella badensis} sp. nov. DSM 100043\textsuperscript{T} and version MRWD01000000 for \textit{Rouxiella silvae} sp. nov. CIP 111154\textsuperscript{T}.

Phenotypic tests were performed as published [1]. The phenotypic features of the novel strains are given in the species description.

A number of phenotypic traits allow the differentiation of the novel species of the genus \textit{Rouxiella} from each other...
and from *R. chamberiensis* (Table 1). Both strains of the species *Rouxiella badensis* sp. nov. grew at 37 °C and reduced nitrate to nitrite, whereas *R. chamberiensis* and *Rouxiella silvae* sp. nov. failed to grow at 37 °C and to reduce nitrate. Furthermore, acid was produced diversely from several carbohydrates. Acid was produced from seven carbohydrates by *Rouxiella badensis*, sp. nov. but not by *R. chamberiensis* (D-fucose, glycero, maltose, melezitrose, raffinose, sucrose, trehalose). Acid was produced from ten carbohydrates (N-acetyl-glucosamine, D-arabitol, L-lyxose, D-maltose, melezitose, raffinose, sucrose, trehalose, turanose and D-xylitol) by *Rouxiella silvae* sp. nov. and not *R. chamberiensis*. Acid was produced from N-acetyl-glucosamine, D-lyxose and D-xylitol by *R. silvae* and not by *Rouxiella badensis* sp. nov., and acid was produced from glycero and L-rhamnose by *Rouxiella badensis* sp. nov. and not by *Rouxiella silvae* sp. nov. Different reactions were observed for the production of acid from D-arabitol and trehalose by strains of the species *Rouxiella badensis* sp. nov. and from methyl-D-glucose by strains of the species *Rouxiella silvae* sp. nov., showing that the strains in these two novel species are different (and not isolates of a single strain).

This study shows clearly that the novel strains referred to as the Baden group and the Silva group belong to the genus *Rouxiella*. Furthermore, both strains of the Baden group had almost identical sequences in five gene portions, and both strains of the Silva group had identical sequences in these five gene portions. Both groups were clearly separated in MLSA and all individual gene studies. Furthermore, genome comparisons showed the two novel species to be distinct from each other and from *Rouxiella chamberiensis*. We have presented data that strongly supports the recognition of two novel species of the genus *Rouxiella*: *Rouxiella badensis* sp. nov. (Baden group) and *Rouxiella silvae* sp. nov. (Silva group). This is supported by physiological and biochemical features, such as growth at 37 °C, nitrate reduction and carbohydrate fermentation. However, as the description of the genus *Rouxiella* (Le Flèche-Mateos, Levast, Lomprez, Arnoux, Andonian, Perraud, Vincent, Ar Gouilh, Thiberge, Vandenbogaert, Diancourt, Caro, Burguière and Manuguerra, 2015) was based on a single species, this description now needs to be emended.

**EMENDED DESCRIPTION OF THE GENUS ROUXIELLA (LE FLÈCHE-MATÉOS, LEVAST, LOMPREZ, ARNOUX, ANDONIAN, PERRAUD, VINCENT, AR GOUILH, THIBERGE, VANDENBOGAERT, DIANCOURT, CARO, BURGUIÈRE AND MANUGUERRA, 2015)**

The following sentences should be substituted to the corresponding items in the genus description. Produces whitish or lemon-yellow colonies. Growth is facultatively anaerobic and occurs at 4–30 °C. Some species fail to grow at 37 °C (21 days). Some species fail to reduce nitrate to nitrite. Acid is produced after 48 h from L-arabinose, cellobiose, D-glucose, myo-inositol, lactose, D-mannitol, D-mannose, melibiose, salicin and D-xyllose; acid is not produced from D-adonitol, dulcitol and D-sorbitol.

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**Table 1. Differential characters among species and strains of species of the genus Rouxiella.** +, Positive after incubation for 2 days; −, negative after incubation for 2 days.

<table>
<thead>
<tr>
<th>Character</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Colony colour</td>
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<td>No pigment</td>
<td>No pigment</td>
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<td>No pigment</td>
</tr>
<tr>
<td>Growth at 37 °C</td>
<td>–</td>
<td>+</td>
<td>No pigment</td>
<td>No pigment</td>
<td>–</td>
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<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Acid produced from:</td>
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<td>–</td>
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<tr>
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<td>+</td>
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<td>D-Sucrose</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>D-Trehalose</td>
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<td>+</td>
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<tr>
<td>D-Turanose</td>
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<tr>
<td>D-Xylitol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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</tr>
</tbody>
</table>
DESCRIPTION OF **ROUXIELLA BADENSIS** SP. NOV.

*Rouxiella badensis* (ba.den’sis N.L. fem. adj. *badensis* from Baden in Germany).

Gram-stain-negative rods fitting the definition of the genus *Rouxiella*. Whitish colonies formed on solid media. Growth occurs at 4 to 37 °C. Nitrate reduced to nitrite. Acid is produced from D-fucose, glycerol, maltose, melezitose, raffinose, L-rhamnose, sucrose and turanose. No acid is produced from N-acetyl-glucosamine, D-lyxose, methyl-D-glucose and D-xylitol. Different reaction in acid production from D-arabitol and trehalose. It produces acid from D-arabitol, but not from trehalose. Produces surfactant.

The type strain is DSM 100043T (CIP 111153T, strain 323T) occurs in peat bogs or swampy soils. The DNA G+C content of the type-strain is 53 mol%.

DESCRIPTION OF **ROUXIELLA SILVAE** SP. NOV.

*Rouxiella silvae* (sil’vae L. gen. *silvae* of forest).

Gram-stain-negative rods fitting the definition of the genus *Rouxiella*. Whitish colonies formed on solid media. Growth occurs at 4 to 30 °C. No growth occurs at 37 °C. Nitrate is not reduced to nitrite. Acid is produced from N-acetyl-glucosamine, D-arabitol, D-fucose, D-lyxose, maltose, melezitose, raffinose, sucrose, trehalose, turanose and D-xylitol. No acid is produced from glycerol and L-rhamnose. Different reactions in terms of acid production from methyl-D-glucose. Produces a surfactant. It does not produce acid from methyl-D-glucose.

The type strain is strain CIP 111154T (DSM 103735T, strain 213T) occurs in swampy soil. The DNA G+C content of the type-strain is 51 mol%.

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Conflicts of interest

All authors declare that there is no conflicts of interest.

References


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