Brenneria goodwinii sp. nov., associated with acute oak decline in the UK

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A group of nine Gram-negative staining, facultatively anaerobic bacterial strains isolated from native oak trees displaying symptoms of acute oak decline (AOD) in the UK were investigated using a polyphasic approach. 16S rRNA gene sequencing and phylogenetic analysis revealed that these isolates form a distinct lineage within the genus Brenneria, family Enterobacteriaceae, and are most closely related to Brenneria rubrifaciens (97.6% sequence similarity to the type strain). Multilocus sequence analysis based on four housekeeping genes (gyrB, rpoB, infB and atpD) confirmed their position within the genus Brenneria, while DNA–DNA hybridization indicated that the isolates belong to a single taxon. The isolates can be differentiated phenotypically from their closest phylogenetic neighbours. The phylogenetic and phenotypic data demonstrate that these isolates from oak with symptoms of AOD represent a novel species in the genus Brenneria, for which the name Brenneria goodwinii sp. nov. (type strain FRB 141T = R-43656T = BCC 845T = LMG 26270T = NCPPB 4484T) is proposed.

An episode of acute oak decline (AOD) has recently been identified in the UK by Denman & Webber (2009) and the condition has a rapid effect on tree health. Mortalities are reported to occur within 3 to 5 years of the onset of symptom development (Denman et al., 2010). Affected trees are identified by stem bleeding or oozing of a dark sticky fluid from small (5–10 cm) vertical cracks formed between bark plates on tree trunks. Tissues underlying the stem bleed (i.e. periderm, phloem, cambium and, in some cases, part of the sapwood) are stained and/or necrotic (particularly the phloem tissue). Frequently, but not always, larval galleries of the bark-boring buprestid Agrilus biguttatus are found in close proximity to or traverse the necrotic patches. Mature and even veteran oak trees native to the UK, e.g. Quercus robur (pedunculate oak) and Q. petraea (sessile oak), appear to be most affected in the Midlands (particularly in East Anglia), but there are an increasing number of reports from southern and south-eastern regions of England. Recent reports of a similar condition in Spain (Biosca et al., 2003; Pozo-Carrión et al., 2008) and Belgium (Vansteenkiste et al., 2004) have been documented.

During 2008–2010, numerous cream-coloured, Gram-negative bacterial strains were isolated from necrotic lesions, fluid exudates and occasionally from larval galleries in symptomatic oak at a number of sites in the UK. The majority of these isolates were identified as belonging to a novel genus and species, Gibbsiella quercinecans (Brady et al., 2010), while a second group of the isolates was identified as representing a novel subspecies of Lonsdalea quercina (formerly Brenneria quercina) (Brady et al., 2012). A third group of the isolates was tentatively identified as belonging to a novel species of the genus Brenneria based on partial 16S rRNA gene and gyrB sequencing. In the present study, the taxonomic position of these Brenneria isolates is investigated further using a polyphasic approach based on multilocus sequence analysis (MLSA), DNA–DNA hybridization, phenotypic assays and fatty acid analyses. Further studies to elucidate whether these taxa play a role in the current episode of AOD are under way.

Abbreviations: AOD, acute oak decline; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 26270T, LMG 26272 and LMG 26271 are JN544202–JN544204, respectively. The accession numbers for the housekeeping gene sequences determined in this study are JN544205–JN544213 (atpD), JN544214–JN544222 (gyrB), JN544223–JN544231 (infB) and JN544232–JN544240 (rpoB), as detailed in Table S1.

Two supplementary tables and a supplementary figure are available with the online version of this paper.
The oak isolates and reference strains investigated in this study are listed in Table S1, available in IJSEM Online. Genomic DNA for sequencing was extracted using an alkali extraction method (Niemann et al., 1997) and stored at -20 ºC. Almost-complete (1346 bp) 16S rRNA gene sequences were determined for three oak isolates (LMG 26270T, LMG 26271 and LMG 26272) using the primers and conditions described by Coenye et al. (1999). Sequences for the closest phylogenetic neighbours were downloaded from GenBank and aligned with the oak sequences using the CLUSTAL W application in BioEdit version 7.0.9.0 (Hall, 1999) and the overlaps were trimmed.

MODELTEST 3.7 (Posada & Crandall, 1998) was applied to the dataset to determine the best-fit evolutionary model. Maximum-likelihood and neighbour-joining trees were reconstructed using PhyML (Guindon & Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the models and parameters set by MODELTEST (Tamura–Nei model including proportion of invariable sites and gamma distribution). Bootstrap analysis with 1000 replications was performed on the trees to assess the reliability of the clusters generated. As the topology of the two trees was similar, only the maximum-likelihood tree is shown. The three oak isolates demonstrated >99.5 % pairwise 16S rRNA gene sequence similarity to each other and >97.0 % to the type strains of Brenneria rubrifaciens (97.6 %) and Lonsdalea quercina (97.1 %). They formed a single cluster with 100 % bootstrap support on a separate branch in the 16S rRNA gene phylogenetic tree (Fig. S1), and demonstrated a close phylogenetic relationship to B. rubrifaciens and B. salicis (the type species of Brenneria), although there was no significant bootstrap support for this clade. The oak isolates were far removed from members of Lonsdalea, despite sequence similarities above 97 %. The remaining two Brenneria species, Brenneria nigrifluens and B. alni, cluster with Samsonia erythrinae, separate from the type species of Brenneria. Like many genera in the Enterobacteriaceae, Brenneria is polyphyletic when analysis is based on 16S rRNA gene sequencing. It is possible that a degree of homoplasy exists in the hypervariable regions of this gene, as a result of tolerance to mutation, or horizontal gene transfer could have taken place in these regions without affecting gene function, but disrupting the phylogenetic signal (Naum et al., 2008). However, as the investigated oak isolates are closely associated with the type species of the genus Brenneria, it is probable that they constitute a single novel species in this genus.

MLSA based on partial gene sequencing of gyrB, rpoB, infB and atpD was recently used to evaluate the phylogenetic position of species belonging to the genus Brenneria within the Enterobacteriaceae (Brady et al., 2012). The MLSA scheme was proven to be very useful for this purpose, and therefore the same four housekeeping genes were sequenced for nine Brenneria isolates from oak. Amplification and sequencing of the above genes were carried out as described previously (Brady et al., 2008). Additional sequences for the closest phylogenetic neighbours were downloaded from GenBank, and are listed in Table S1. Sequence analysis and tree reconstruction (applying the general time reversible model including proportion of invariable sites and gamma distribution) were performed as for 16S rRNA gene sequencing. MLSA revealed a high degree of sequence similarity between the oak isolates for all four housekeeping genes, with <0.9 % gyrB, <0.7 % infB, <0.4 % rpoB and <0.5 % atpD sequence variation. The oak isolates form a well-supported cluster within the Brenneria clade, far removed from Lonsdalea, in the phylogenetic tree based on the concatenated sequences of the four housekeeping genes (Fig. 1). This confirms the identity of the isolates as members of the genus Brenneria and also indicates that they possibly represent a single novel species in this genus.

High-quality DNA for DNA–DNA hybridizations was extracted from four oak isolates (LMG 26270T, LMG 26271, LMG 26272 and R-43657) and the type strains B. salicis LMG 2698T and B. rubrifaciens LMG 2709T, using a modification (Cleenwerck et al., 2002) of the method of Wilson (1987). DNA–DNA hybridizations were performed using the microtitre plate method (Ezaki et al., 1989) with minor modifications (Cleenwerck et al., 2002). The hybridization temperature was 43 ºC. Reciprocal reactions (A × B and B × A) were performed for each possible DNA pair and the variation observed was within the limits of this method (Goris et al., 1998). Values presented in Table 1 are based on a minimum of four replicates. When hybridized against each other, the four oak isolates exhibited high levels of DNA–DNA relatedness, ranging from 90 to 100 %. This confirms that these isolates belong to a single species. By contrast, low levels of DNA–DNA relatedness (28–34 %) were observed following hybridization of DNA from LMG 26270T and LMG 26271 with DNA from B. salicis LMG 2698T and B. rubrifaciens LMG 2709T, confirming that the isolates belong to a novel species. The DNA G + C content of the oak isolates LMG 26270T, LMG 26271, LMG 26272 and R-43657, measured using HPLC (Mesbah et al., 1989), was 52.5, 52.6, 52.7 and 52.3 mol%, respectively. This is within the DNA G + C content of 50.1–56.1 mol% generally observed for recognized Brenneria species (Hauben & Swings, 2005; Brady et al., 2012).

Biochemical and physiological tests were performed on all nine oak isolates listed in Table S1 using API 20E and API 50CHB/E test kits (bioMérieux). The results were compared with those for reference strains of each recognized Brenneria species and L. quercina, generated under identical conditions (Brady et al., 2012). Additionally, Biolog GN2 MicroPlate tests were carried out on the same nine oak isolates to determine carbon-source utilization. The tests were performed according to the manufacturers’ instructions and were incubated for 24 h (API 20E, Biolog GN2) or 48 h (API 50CHB/E). Results are listed in Table 2 and in the species description. The oak isolates can be distinguished from recognized Brenneria species by various features such as their ability to produce acid from inositol (differentiation from B. salicis, B. alni and B. rubrifaciens), amygdalin (differentiation from B. salicis, B. nigrifluens and...
B. rubrifaciens), D-galactose (differentiation from B. salicis, B. nigrifluens and B. rubrifaciens) and raffinose (differentiation from B. alni and B. rubrifaciens). Additionally, these oak isolates differ from L. quercina by their inability to utilize citrate, their ability to produce acid from L-arabinose and several additional characteristics listed in Table 2.

Whole-cell fatty acid composition was determined for four oak isolates (LMG 26270T, LMG 26271, LMG 26272 and R-43657) using an Agilent Technologies 6890N gas chromatograph. Cultivation of the isolates, and extraction and analysis of the fatty acid methyl esters were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI). Cells were harvested from cultures grown on trypticase soy agar (BBL 11768) for 24 h at 28°C. The peaks of the profiles were identified using the TSBA50 identification library version 5.0. Profiles obtained for the oak isolates were compared with profiles of phylogenetically related strains, generated under the same conditions (Brady et al., 2012), and were found to be similar to those of recognized species.
Table 1. DNA–DNA relatedness amongst the type strains of B. salicis and B. rubrifaciens and strains of Brenneria goodwinii sp. nov.

Values are expressed as percentages (± difference between reciprocal values/2). ND, Not determined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>B. goodwinii sp. nov.</td>
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<tr>
<td>1. LMG 26270T</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. LMG 26271</td>
<td>93 ± 0.5</td>
<td>100</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>3. R-43657</td>
<td>95 ± 2.0</td>
<td>94 ± 4.5</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. LMG 26272</td>
<td>92 ± 2.5</td>
<td>101 ± 2.5</td>
<td>90 ± 2.5</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. B. salicis LMG 2698T</td>
<td>29 ± 1.5</td>
<td>34 ± 2.0</td>
<td>ND</td>
<td>ND</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>6. B. rubrifaciens LMG 2709T</td>
<td>28 ± 1.5</td>
<td>34 ± 1.0</td>
<td>ND</td>
<td>ND</td>
<td>47 ± 4.0</td>
<td>100</td>
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</table>

Table 2. Phenotypic characteristics that distinguish Brenneria goodwinii sp. nov. from recognized Brenneria and Lonsdalea species

Species: 1. B. goodwinii sp. nov. (9 strains); 2. B. salicis (3); 3, B. alni (5); 4, B. nigriflueus (5); 5, B. rubrifaciens (5); 6, L. quercina (17). All data were generated under the same conditions using API tests (bioMérieux); data for reference strains were taken from Brady et al. (2012). +, 90–100% strains positive in 1–2 days; –, negative; d, 11–89% strains positive in 1–4 days. All reference Brenneria strains were positive for acid production from D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetylgalactosamine and sucrose. All reference Brenneria strains were negative for acid production from erythritol, L-xylene, D-adonitol, methyl D-xylpyranoside, L-sorbose, dulcitol, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D- and L-fucose and L-arabitol.

- Characteristics

- Acid production from:
  - L-Arabinose
  - Amygdalin
  - D-Galactose
  - Gentiose
  - Inositol
  - Melibiose
  - Potassium gluconate
  - Raffinose
  - D-Sorbitol
  - Turanose
  - D-Xylose

- Gram-negative, short rods (0.8 × 1–1.3 μm), facultatively anaerobic, oxidase-negative and catalase-positive. Cells occur singly and are motile by means of peritrichous flagella (determined by TEM). Colonies are pale cream on nutrient agar, round, convex and smooth with entire margins. Strains can grow at temperatures between 10 and 40 °C. Positive for β-galactosidase and acetoin production.

**Description of Brenneria goodwinii sp. nov.**

*Brenneria goodwinii* (good.wi.ni.i. N.L. masc. gen. n. *goodwinii* of Goodwin, named in honour of Peter John Goodwin, for his major contribution to promoting the health and prosperity of oak in Britain).

Hypersensitivity reaction tests were conducted in duplicate on eight wild tobacco seedlings (*Nicotiana sylvestris*) following the method described by Lelliot & Stead (1987). Bacterial suspensions (10⁹ c.f.u. ml⁻¹) were injected into four intercellular spaces per leaf with a fine needle and syringe. The seedlings were incubated at 26 °C and assessed after 48 h, and again after 72 h. Of the eight isolates tested, only two elicited a hypersensitivity response (data not shown). However, pathogenicity tests are currently under way to determine whether these isolates contribute to lesion formation in the phloem tissue of oak with symptoms of AOD in the UK.
but negative for activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase, production of indole and H₂S and utilization of citrate. Nitrate is not reduced to nitrite, but is weakly reduced to N₂ gas. Acid is produced from glycerol, L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, N-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, lactose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose and potassium 5-ketoglucanone (API 50CHB/E). Reactions for D-xylose (type strain positive), methyl α-D-glucopyranoside (type strain negative), cellobiose (type strain negative) and potassium gluconate (type strain negative) are variable. The following carbon sources are utilized at 28 °C: N-acetyl-D-glucosamine, L-arabinose, D-fructose, D-galactose, gentiobiose, α-D-glucose, inositol, lactose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, D-sorbitol, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, formic acid, D-gluconic acid, succinic acid, bromosuccinic acid, L-asparagine, L-aspartic acid, L-serine, glycerol, DL-glycerol phosphate and α-D-glucose 6-phosphate (Biog N24). The G+C content of the type strain is 52.5 mol%.

The type strain is FRB 141 T (= = BCC 845 T =LMG 26270 T =NCPPB 4484 T), isolated from Quercus robur in Outwood, Loughborough, Leicestershire, UK. Strains of this species have been isolated from English and sessile oak exhibiting symptoms of AOD.

Acknowledgements

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References


oak trees: molecular characterization and development of PCR

Surico, G., Mugnai, L., Pastorelli, R., Giovannetti, L. & Stead, D. E.
(1996). Erwinia alni, a new species causing bark cankers of alder


Predispositions and symptoms of Agrilus borer attack in declining oak

Current Protocols in Molecular Biology, pp. 2.4.1–2.4.5. Edited by
F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman,