Pseudomonas coleopterorum sp. nov., a cellulase-producing bacterium isolated from the bark beetle Hylesinus fraxini

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We isolated a strain coded Esc2AmT during a study focused on the microbial diversity of adult specimens of the bark beetle Hylesinus fraxini. Its 16S rRNA gene sequence had 99.4 % similarity with respect to its closest relative, Pseudomonas rhizosphaerae IH5T. The analysis of partial sequences of the housekeeping genes rpoB, rpoD and gyrB confirmed that strain Esc2AmT formed a cluster with P. rhizosphaerae IH5T clearly separated from the remaining species of the genus Pseudomonas. Strain Esc2AmT had polar flagella and could grow at temperatures from 4–38°C. The respiratory quinone was Q9 and the main fatty acids were C16 : 0, C18 : 1ω7c and/or C18 : 1ω6c in summed feature 8 and C16 : 1ω7c and/or C16 : 1ω6c in summed feature 3. DNA–DNA hybridization results showed 51 % relatedness with respect to P. rhizosphaerae IH5T. Oxidase, catalase and urease-positive, the arginine dihydrolase system was present but nitrate reduction and β-galactosidase production were negative. Aesculin hydrolysis was positive. Based on the results from the genotypic, phenotypic and chemotaxonomic analyses, we propose the classification of strain Esc2AmT as representing a novel species of the genus Pseudomonas, for which we propose the name Pseudomonas coleopterorum sp. nov. The type strain is Esc2AmT (=LMG 28558T = CECT 8695T).

Bacteria classified within the genus Pseudomonas are naturally spread in nature thanks to their capability of utilizing a wide range of organic and inorganic compounds and adaptation to diverse environmental conditions. Essentially, any habitat with a temperature range of 4–42°C, a pH between 4 and 8 and containing simple or complex organic compounds is a potential habitat for members of the genus Pseudomonas (Moore et al., 2006). Strains classified within the genus Pseudomonas have been isolated from several sources, including insects, from which the species Pseudomonas entomophila was isolated (Opota et al., 2011; Mulet et al., 2012). Recent studies revealed the predominance of this genus in the gut microbiomes of some plant-related insects (Bansal et al., 2014) and they have also been found in haematophagous insects (Alvarez et al., 2012; Maleki-Ravasan et al., 2014), cockroaches (Zhang et al., 2013) and bark beetles (Morales-Jiménez et al., 2013). Bacteria associated with the gut of bark beetles of the genus Dendroctonus are able to fix nitrogen and to produce cellulases that are involved in insect development and fitness (Morales-Jiménez et al., 2012). Cellulolytic members of the genus Pseudomonas have been found in the gut microbiota of these insects in China (Hu et al., 2014). During a screening of cellulolytic bacteria from bark beetles, we isolated a strain named Esc2AmT from a Hylesinus fraxini beetle, which had been collected from its gallery caved in a Fraxinus excelsior tree growing in the locality of Jilove u Prahy (Czech Republic). Based on its genotypic, chemotaxonomic and phenotypic characteristics, strain Esc2AmT should be classified as representing a novel species of the genus Pseudomonas for which we propose the name Pseudomonas coleopterorum sp. nov.
The bacterium was isolated from an adult specimen of the bark beetle *Hylesinus fraxini*, for which individuals were rinsed in distilled water, surface disinfected with 70 % ethanol for 5 min and again rinsed twice in sterile distilled water. The samples were then crushed and the contents were serially diluted in sterile distilled water and spread onto nutrient agar plates (Difco). Cells grew as round and convex yellow colonies on nutrient agar. Gram staining was performed according to the protocol of Doetsch (1981) and motility was checked by phase-contrast microscopy after growing the cells in nutrient agar medium at 22 °C for 48 h. The flagellation type was determined after 24 h of incubation in nutrient broth at 24 °C by electron microscopy as previously described by García-Fraile et al. (2015). Strain Esc2AmT was a Gram-stain-negative, non-sporulated rod motile by means of polar flagella (Fig. S1 available in the online Supplementary Material).

The 16S rRNA gene was amplified and sequenced as described by Rivas et al. (2007). The amplification and partial sequencing of gyrB, rpoB and rpoD housekeeping genes was performed using the primers specified in Table S1. PCR amplification was carried out in a final volume of 25 μl containing 12.5 μl Plain PP Master Mix (Top-Bio), 1 μl each primer at a concentration of 10 μM, 1 μl template DNA and 9.5 μl PCR H2O. PCR amplification was performed with the following conditions: an initial denaturation step at 94 °C for 90 s, 40 cycles of denaturation at 94 °C for 10 s, annealing at 50 °C for 20 s and extension at 72 °C for 50 s, and a final extension step at 72 °C for 5 min. The sequences were compared with those from GenBank using BLASTN (Altschul et al., 1990) and in the case of the 16S rRNA gene with EzTaxon-e (Kim et al., 2012). Sequence alignments were done using CLUSTAL W (Thompson et al., 1994; Larkin et al., 2007). The distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining (NJ; Saitou & Nei, 1987) and maximum-likelihood (ML; Rogers & Swofford, 1998) analyses using *Mega* software (Tamura et al., 2011).

The comparison of the 16S rRNA gene sequence of strain Esc2AmT with those held in the EzTaxon-e database allowed us to classify it as representing a member of the genus *Pseudomonas* with *Pseudomonas rhizophaerea* IH5T as the most closely related species, with 99.4 % similarity. The remaining species of the genus *Pseudomonas* presented less than 99 % similarity. The NJ (Fig. 1) and ML (Fig. S2) phylogenetic analyses showed that strain Esc2AmT grouped in a branch formed by the species *P. rhizophaerea* IH5T, *Pseudomonas abietanaphila* DSM 17554T, *Pseudomonas graminis* DSM 11363T and *Pseudomonas lutea* OK2T, where it formed a cluster with *P. rhizophaerea* IH5T (Fig. 1).

These results were confirmed by the analysis of the housekeeping *rpoD*, *rpoB* and *gyrB* genes that presented, respectively, 95 %, 97.0 % and 92.1 % similarity between strain Esc2AmT and *P. rhizophaerea* IH5T. These values are similar to those commonly found among different species of the genus *Pseudomonas* (Ramírez-Bahena et al., 2014), suggesting that Esc2AmT represents a novel species of the genus *Pseudomonas*. The remaining species showed less than 85 %, 94 % and 86 % similarity with respect to strain Esc2AmT for the *rpoD*, *rpoB* and *gyrB* genes, respectively. The NJ phylogenetic analysis of concatenated *rpoD*, *rpoB* and *gyrB* genes confirmed that Esc2AmT and *P. rhizophaerea* IH5T formed an independent cluster within the genus *Pseudomonas* (Fig. 2). The same tree topology was found after ML phylogenetic analysis (not shown).

DNA–DNA hybridization was, therefore, performed between strain Esc2AmT and *P. rhizophaerea* IH5T as described by Ezaki et al. (1989), following the recommendations of Willems et al. (2001). The mean percentage DNA–DNA similarity between the two strains was 51 % (+3 %). Taking into account a threshold value of 70 % DNA–DNA similarity for definition of a bacterial species (Wayne et al., 1987), strain Esc2AmT represents a distinct species of the genus *Pseudomonas*.

The G+C content of DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G+C content of strain Esc2AmT was 62.5 mol%. This value is similar to those obtained for *P. rhizophaerea* IH5T (Peix et al., 2003) and it is within the range of 58–69 mol% DNA G+C found in the species of the genus *Pseudomonas* (Palleroni, 2005).

To perform cellular fatty acids analysis, strain Esc2AmT and the closely related species grouping together in the 16S rRNA gene phylogeny (*P. rhizophaerea* IH5T, *P. abietanaphila* DSM 17554T, *P. graminis* DSM 11363T and *P. lutea* OK2T) were grown on TSA plates (Becton Dickinson, BBL) for 24 h at 28 °C. The extraction of fatty acids was carried out as described by Sasser (1990) and analysed by using the Microbial Identification System (MIDI) Sherlock 6.1 and the library RTSBA6. The major fatty acids of strain Esc2AmT were C18 : 1ω7c and/or C18 : 1ω9c in summed feature 8 (26.9 %), C16 : 0 (25.9 %) and C16 : 1ω7c and/or C16 : 1ω6c in summed feature 3 (26.6 %). This fatty acid profile is characteristic of members of the genus *Pseudomonas* and is similar to those of closely related species according to the 16S rRNA gene sequence analysis (Table 1). For analysis of respiratory quinones, cells were cultivated overnight in nutrient broth at 24 °C with shaking at 200 r.p.m. Then, biomass was harvested by centrifugation and freeze-dried. Quinone extraction and analysis was performed at the Identification Service of the DSMZ (Braunschweig, Germany). Our isolate Esc2AmT contained Q9 as the respiratory quinone (100 %). Strain Esc2AmT was also confirmed to produce fluorescent pigment in nutrient agar plates, when observed under a UV lamp.

Phenotypic characterization was performed for strain Esc2AmT and the type strains of the closely related species included in cellular fatty acids analysis by using API 20NE (bioMérieux) and the Biolog system (GN2 plates).
Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences of strain Esc2AmT and closely related species of the genus *Pseudomonas*. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branching points. Bar, one substitution per 100 nucleotides.
**Fig. 2.** Neighbour-joining phylogenetic tree based on concatenated partial rpoD, rpoB and gyrB gene sequences of strain Esc2AmT and closely related species of the genus *Pseudomonas*. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branching points. Bar, two substitutions per 100 nucleotides.
Table 1. Cellular fatty acid composition of strain Esc2AmT and representatives of the most closely related species and the type species of the genus Pseudomonas, P. aeruginosa

<table>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>C10:0 3-OH</td>
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<td>5.0</td>
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<td>2.6</td>
<td>1.7</td>
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<td>2.9</td>
<td>2.4</td>
<td>3.5</td>
<td>4.5</td>
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<td>0.1</td>
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*Summed feature 3: C16:1ω7c, C16:1ω6c.
†Summed feature 8: C18:1ω7c/C18:1ω6c.

according to the manufacturers’ instructions. Growth temperature range was determined on nutrient agar from 4°C to 42°C. Salt tolerance was checked on the same medium containing 0–10% (w/v) NaCl. The same medium with a final pH between 4 and 10 was used to analyse the pH range for growth. Phenotypic features of strain Esc2AmT are detailed in the species description and the main differences with respect to the closely related species of the genus Pseudomonas are listed in Table 2.

Although cellulase activity has no taxonomic relevance in the genus Pseudomonas, considering that cellulolytic activity is important for insect development and fitness (Morales-Jiménez et al., 2012), we analysed the cellulase activity in strain Esc2AmT. This activity was detected in minimal extracts stored at 28°C. Phenotypic features of strain Esc2AmT are detailed in the species description and the main differences with respect to the closely related species and the type species of the genus Pseudomonas, P. aeruginosa are listed in Table 2.

Quantitative determination of cellulase activity in cell extracts was performed using the 2,2′-bicinchoninic acid (BCA)-reducing sugar assay (Waffenschmidt & Jaenicke, 1987; Mateos et al., 1992). Cellulolytic activity of cell extracts on CM-cellulose was measured at 4, 22, 28, 37, 45 and 55°C using PCA buffer (pH 5), at pH 3, 5 and 8 using PCA buffer and at pH 10 using carbonate/bicarbonate buffer (Table S2). Considering all the results of this study, phylogenetic and phenotypic data support the classification of strain Esc2AmT as representing a novel species within the genus Pseudomonas, for which the name Pseudomonas coleopterorum sp. nov. is proposed.

Description of Pseudomonas coleopterorum sp. nov

Pseudomonas coleopterorum (co.le.op.te.ro’rum. N.L. gen. pl. n. coleopterorum of Coleoptera, referring to the order of insects from which the organism was first isolated). Gram-stain-negative, strictly aerobic, non-spore-forming, rod-shaped cells of 1.6–2.3 μm in length and 0.8–0.9 μm.
in diameter, motile by means of polar flagella. Colonies are yellow, circular, convex and fluorescent and 1.5–2.0 mm in diameter after 48 h of growth at 24°C in nutrient agar. Temperature for growth ranges between 4 and 30°C, growth pH ranges between 4 and 10. Optimal growth occurs at 20–24°C and pH 6–8. Able to grow with up to 5% NaCl in nutrient agar. The respiratory ubiquinone is Q9. C18 : 0 η6c and/or C18 : 1 η6c in summed feature 8, C16 : 0 and C16 : 1 η6c and/or C17 : 1 η6c in summed feature 3 are the main fatty acids. Oxidase- and catalase-positive. Arginine dihydrolase system, aesculin hydrolysis and urease are positive. Gelatinase is negative. Indole and β-galactosidase production as well as nitrate reduction are negative. Assimilation of glucose, caprate, malate, citrate, Tween 40, Tween 80, L-arabinose, D-arabitol, D-fructose, D-galactose, α-D-glucose, D-mannitol, D-mannose, sucrose, methyl-pyruvate, cis-aconitate, citrate, D-galactonate lactone, D-galacturonate, D-glucuronate, β-hydroxybutyrate, itaconate, α-ketoglutarate, D,L-lactate, propionate, quinate, D- saccharate, succinate, bromosuccinate, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, D-aspartate, L-glutamate, glycol L-glutamate, L-proline, L-pyroglutamate, L-serine, D,L-carnitine, γ-aminobutyrate, inosine, uridine, D,L-α-glycerol phosphate and glycerol is positive. Negative results are obtained for α-cyclodextrin, dextrin, glucogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, cellulbiose, i-erythritol, L-fucose, gentiobiose, myo-inositol, α-lactose, lactulose, maltose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, trehalose, turanose, xylitol, D-glucuronate, D-glucosamine, γ-hydroxybutyrate, p-hydroxyphenylacetate, α-ketobutyrate, γ-ketovalerate, malonate, sebacate, succinamate, glucuronamide, L-alaninamide, L-histidine, D-serine, L-aspartate, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-threonine, urocanate, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glucose 1-phosphate, glucose 6-phosphate, adipate and phenylacetate. Assimilation of monomethyl succinate, acetate, formate and α-hydroxybutyrate is weakly positive.

The type strain, Esc2AmT (=LMG 28558T=CECT 8695T), was isolated from bark beetles sampled in the Czech Republic. The DNA G+C content of the type strain is 62.5 mol%.

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References


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