Two strains named ESC1<sup>T</sup> and ESC5 were isolated from nodules of *Cytisus scoparius* growing in a Spanish soil. Phylogenetic analysis of the 16S rRNA gene showed that these strains belong to the genus *Ochrobactrum*, their closest relatives being *Ochrobactrum anthropi* and *Ochrobactrum lupini*, with 100 and 99.9% similarity to the respective type strains. Despite this high similarity, the results of DNA–DNA hybridization, phenotypic tests and fatty acid analyses showed that these strains represent a novel species of genus *Ochrobactrum*. The DNA–DNA hybridization values were respectively 70, 66 and 55% with respect to *O. lupini* LUP21<sup>T</sup>, *O. anthropi* DSM 6882<sup>T</sup> and *Ochrobactrum tritici* DSM 13340<sup>T</sup>. The predominant fatty acids were C<sub>18:1</sub>ω7c and C<sub>18:1</sub>ω9c containing nodD and *nifH* genes on megaplasmids that were related phylogenetically to those of rhizobial strains nodulating *Phaseolus*, *Leucaena*, *Trifolium* and *Lupinus*. From the results of this work, we propose that the strains isolated in this study be included in a novel species named *Ochrobactrum cytisi* sp. nov. The type strain is ESC1<sup>T</sup> (=LMG 22713<sup>T</sup> = CECT 7172<sup>T</sup>).

The genus *Ochrobactrum* currently contains six species, including human pathogens such as *Ochrobactrum anthropi* (Holmes et al., 1988), rhizospheric bacteria such as *Ochrobactrum tritici* (Lebuhn et al., 2000) and legume endosymbionts such as *Ochrobactrum lupini* (Trujillo et al., 2006). The strains of the latter species carry symbiotic genes *nodD* and *nifH* phylogenetically related to those of different rhizobial strains nodulating *Phaseolus*, *Leucaena*, *Trifolium* and *Lupinus*. From the results of this work, we propose that the strains isolated in this study be included in a novel species named *Ochrobactrum cytisi* sp. nov. The type strain is ESC1<sup>T</sup> (=LMG 22713<sup>T</sup> = CECT 7172<sup>T</sup>).

respectively (Trujillo et al., 2005). In this work, we isolated two strains named ESC1<sup>T</sup> and ESC5 from *Cytisus scoparius* nodules in a contaminated soil from southern Spain (Sevilla) near to the Guadiamar river, which has received spills from the Aznalcollar mines containing several heavy metals. The data obtained in this study show that these strains belong to a novel species of *Ochrobactrum*.

Strains ESC1<sup>T</sup> and ESC5 were isolated from root nodules of *Cytisus scoparius* according to Vincent (1970) using yeast mannitol agar (YMA); Bergersen, 1961). The cultures used in further phenotypic and molecular studies were purified from a single colony after 2 days incubation at 28°C on YMA. The colonies were white, mucoid, translucent and convex on this medium.

Sequencing of the nearly complete 16S rRNA gene was performed as described previously (Rivas et al., 2002b). The 16S–23S rRNA intergenic spacer (ITS) region was amplified and sequenced as described by Lebuhn et al. (2006). Sequence comparison and alignment were performed by using the BLASTN program (Altschul et al., 1990) and
CLUSTAL W software (Thompson et al., 1997), respectively. Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar et al., 2001) was used for all analyses.

The 16S rRNA gene (rrn) sequences obtained (1476 nucleotides) were identical in strains ESC1<sup>T</sup> and ESC5 and showed 100, 99.9 and 99.7% similarity, respectively, to sequences from <i>O. anthropi</i> LMG 3331<sup>T</sup>, <i>O. lupini</i> LUP21<sup>T</sup> and <i>O. tritici</i> LMG 18957<sup>T</sup>. Phylogenetic analysis of 16S rRNA gene sequences of the strains from this study showed clearly that they form a separate group within the genus <i>Ochrobactrum</i> together with <i>O. anthropi</i>, <i>O. lupini</i> and <i>O. tritici</i>, being most closely related to the first two of these species (Fig. 1).

Sequence comparison of 16S–23S rRNA ITS regions provides a fast way of assessing relatedness between species of the genus <i>Ochrobactrum</i> because good correlations have been found among ITS1 and rrn sequences and DNA–DNA hybridization values (Lebuhn et al., 2006). Therefore this region was sequenced in strains ESC1<sup>T</sup> and ESC5 and in <i>O. lupini</i> LUP21<sup>T</sup>. In agreement with phylogenetic analyses based on the rrn sequences, phylogenetic analysis of the ITS sequences showed that the strains from this study are closely related to <i>O. anthropi</i>, <i>O. lupini</i> and <i>O. tritici</i> (Fig. 2). A pairwise analysis of the ITS sequences showed 91.8, 92.7 and 86.2% identity between strain ESC1<sup>T</sup> and <i>O. lupini</i> LUP21<sup>T</sup>, <i>O. anthropi</i> LMG 3331<sup>T</sup> and <i>O. tritici</i> LMG 18957<sup>T</sup>, respectively. These values were lower than that found between <i>O. anthropi</i> LMG 3331<sup>T</sup> and <i>O. tritici</i> LMG 18957<sup>T</sup> (94.0%) and suggested that the strains isolated in this work belong to a separate species within the genus <i>Ochrobactrum</i>.

Two-primers randomly amplified polymorphic DNA (TP-RAPD) patterns were analysed according to the method described by Rivas et al. (2002a) using the primer pair 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1522R (5′-AAGGAGGTGATCCANCCRCA-3′) and also the primer pair 879F (5′-GCCTGGGGAGTACGGCCGCA-3′) and 1522R (5′-AAGGAGGTGATCCANCCRCA-3′), which correspond to Escherichia coli positions 8–27, 879–898 and 1509–1522, respectively. TP-RAPD patterns of strains from the same species are identical (Rivas et al., 2001, 2004) and allowed differentiation among <i>O. anthropi</i>, <i>O. lupini</i> and <i>O. tritici</i> (Trujillo et al., 2005). Therefore, we analysed the TP-RAPD patterns of strains ESC1<sup>T</sup> and ESC5 in comparison with those of the type strains from these three species (Supplementary Fig. S1). Strains ESC1<sup>T</sup> and ESC5 showed identical TP-RAPD patterns (Supplementary Fig. S1, lanes 1 and 2 and lanes 6 and 7), suggesting that they belong to the same species. The TP-RAPD patterns obtained with the two

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**Fig. 1.** Comparative sequence analysis of 16S rRNA genes of strain ESC1<sup>T</sup> and representative related species. The tree was constructed by the neighbour-joining method. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions.

**Fig. 2.** Comparative sequence analysis of the 16S–23S rRNA ITS regions of strain ESC1<sup>T</sup> and representative related species. The tree was constructed by the neighbour-joining method. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 5 substitutions per 100 nucleotide positions.
primer pairs used in this study differ from those of O. tritici (lanes 3 and 8), O. anthropi (lanes 4 and 9) and O. lupini (lanes 5 and 10). These results are congruent with the ITS sequence data and suggest that the strains from this study belong to a different species.

The G+C content of strain ESC1T was 56.4 mol%, as determined by HPLC (Rivas et al., 2003). DNA–DNA hybridization analyses were performed at the DSMZ. DNA was isolated by chromatography on hydroxypatite by the procedure of Cashon et al. (1977), which was carried out as described by De Ley et al. (1970) with the modifications described by Huß et al. (1983) and Escara & Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992) and DNA–DNA relatedness was tested as described previously (Rivas et al., 2005). Cells were stained according to the classical Gram procedure (Doetsch, 1981) and motility was checked by phase-contrast microscopy. Catalase and oxidase activities were described by Doetsch (1981) and motility was checked by phase-contrast microscopy. Catalase and oxidase activities were tested as described previously (Rivas et al., 2003). Physiological studies were done using API 20NE and API 20E systems following the manufacturer’s instructions (bioMérieux). API 50CH strips were inoculated with suspensions of the strains in a basal medium containing YNB (yeast nitrogen base; Difco) adjusted to pH 7. For API ZYM strips, suspensions of cells growing for 24 h on TSA plates were used for inoculation as recommended by the manufacturer. Susceptibility to various antibiotics was examined as described previously (Valverde et al., 2005) using discs (Becton Dickinson) containing (per disc) penicillin (10 μg), ampicillin (2 μg), oxytetracycline (30 μg), neomycin (5 μg), cloxacillin (1 μg), erythromycin (2 μg), cefuroxime (30 μg), ciprofloxacin (5 μg), polymyxin B (300 IU) and gentamicin (10 μg) and antibiotic agar 11 (Oxoid) as the basal medium. O. lupini strains LUP21T and LUP23, O. tritici LMG 18957T and O. anthropi LMG 3331T were used as references in phenotypic characterization studies.

Cells of strains ESC1T and ESC5 were Gram-negative, rod-shaped, non-sporulating, motile by means of a polar flagellum and commonly observed as single cells. Strains ESC1T and ESC5 differ in the production of β-galactosidase in API ZYM (Table 1 and Supplementary Table S2). They differ from O. lupini in nitrate reduction, urease production after 48 h incubation and gluconate, D-arabinose, D-turanose and L-lyxose assimilation and from O. anthropi in aesculin hydrolysis, urease production after 48 h incubation, production of β-galactosidase in API ZYM, citrate (24 h) and gluconate assimilation and resistance to polymyxin B. The two strains differ from O. tritici in aesculin hydrolysis, urease production after 24 h incubation, production of β-galactosidase, a-glucosidase and lipase.

Table 1. Differentiating physiological characters between the novel strains and the closest phylogenetically related species of genus Ochrobactrum

<table>
<thead>
<tr>
<th>Character</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tr>
<td>Nitrate reduction</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin hydrolysis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Urease (48 h)</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>API ZYM tests</td>
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<td></td>
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<tr>
<td>β-Galactosidase</td>
<td>+</td>
<td>–</td>
<td>V*</td>
<td>–</td>
<td>–†</td>
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<tr>
<td>a-Glucosidase</td>
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<td>+</td>
<td>W*</td>
<td>+</td>
<td>+‡</td>
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<td>+</td>
<td>W*</td>
<td>+</td>
<td>+‡</td>
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<td>Assimilation of:</td>
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<td></td>
</tr>
<tr>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Citrate (24 h)</td>
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<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>ND</td>
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<td>Antibiotic resistance</td>
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<tr>
<td>Chloramphenicol</td>
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<td>S†</td>
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<tr>
<td>Polymyxin B (300 IU)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*Data from this study for O. lupini LUP21T and LUP23.
†Data from this study for O. tritici LMG 18957T.
‡Data from this study for O. anthropi LMG 3331T; reported as negative by Holmes et al. (1988).
C14 in API ZYM and citrate, D-mannose and L-arabinose assimilation and in resistance to polymyxin B.

Partial sequences of nodD (296 nt) and nifH (320 nt) genes were amplified by PCR and sequenced as described previously (Rivas et al., 2002b). The nodD and nifH sequences of strains ESC1T and ESC5 were identical (data not shown). A comparison of the nifH gene sequence of the strains from this study against those held in databases showed that it is closely related (98.7 % similarity) to the nifH gene of Ensifer sp. GR-06 and GR-X8, two strains isolated from Phaseolus vulgaris in Spain near to Sevilla (Herrera-Cervera et al., 1999) (Supplementary Fig. S2). The nodD gene sequence is closely related (97.2 % similarity) to the nodD genes of strains of Rhizobium rhizogenes (Supplementary Fig. S3), a species recently found to be able to nodulate Phaseolus (Velázquez et al., 2005), and Ensifer sp. Br816, a strain with a broad host range able to nodulate Phaseolus vulgaris, Leucaena and Trifolium (van Rijn et al., 1996). These results are in agreement with those obtained in previous studies showing the lateral transfer of symbiotic genes from rhizobia to several non-rhizobia from the Alphaproteobacteria in the rhizosphere (Rivas et al., 2002b; Sy et al., 2001; Trujillo et al., 2005; van Berkum & Eardly, 2002). Concretely, the results of nodD and nifH gene sequencing suggest that the strains from this study have acquired these genes from rhizobia nodulating hosts from the cross-inoculation group of Phaseolus. Therefore, Phaseolus vulgaris was used to confirm nodulation by strains ESC1T and ESC5 as described previously (Velázquez et al., 2005). Rhizobium etli CFN42T was used as a positive control. As a negative control, P. vulgaris plants were watered with nitrogen-free Rigaud and Puppo solution. Both strains generated nodules on P. vulgaris after 6 weeks inoculation. They formed white nodules (Supplementary Fig. S4a) with a morphology similar to that of nodules induced by R. etli CFN42T (Supplementary Fig. S4b), although they were white and smaller in size than those elicited by R. etli. Plants inoculated with these strains developed a significantly smaller number of nodules than those inoculated with R. etli CFN42T (data not shown).

In summary, on the basis of 16S rRNA gene and 16S–23S rRNA ITS sequences, strains ESC1T and ESC5 belong to the genus Ochrobactrum, being closely related to O. anthropi, O. lupini and O. tritici. Nevertheless, DNA–DNA hybridization values and chemotaxonomic and phenotypic data indicate that they represent a taxon that merits species status within the genus Ochrobactrum, for which the name Ochrobactrum cytisi sp. nov. is proposed.

Description of Ochrobactrum cytisi sp. nov.

Ochrobactrum cytisi (cy.ti’.si. N.L. masc. n. Cytisus botanical genus name of the legume Cytisus scoparius N.L. gen. n. cytisi of Cytisus, referring to the isolation source of the first strains, nodules of C. scoparius).

Cells are motile, non-spore-forming. Gram-negative rods. Good growth occurs on YMA and nutrient agar at 25–30 °C. Colonies on these media are white to beige, mucoid with entire edges and 2–3 mm in diameter within 24 h. Oxidase- and catalase-positive. The fatty acid profile is composed mainly of C18:0, C19:0 cyclo o8c and C16:0. The following fatty acids are detected in small amounts: summed features 2 and 3, C18:0, C17:0, C18:0 2-OH and an unknown fatty acid at ECL 11.799. The following tests were done by using API 20E and API 20NE systems. Nitrate is reduced to nitrite. Voges–Proskauer reaction, indole production and aesculin hydrolysis are positive. Production of β-galactosidase is variable. Production of urease (after 48 h incubation), arginine dihydrolase, lysis decarboxylase, ornithine decarboxylase and gelatinase is negative. Carbon sources utilized include D-glucose, L-arabinose, D-mannose, mannitol, N-acetylglucosamine, maltose, citrate, erythritol, D-arabinose, ribose, adonitol, dulcitol, L-threomannose, arbutin, maltose, sucrose, turanose, L-lyxose, tagatose, D-fucose, L-fucose, arabitol, 2-ketogluconate and 5-ketogluconate. Assimilation of D-xylose, L-xylose, galactose, D-fructose, lactose, melibiose, cellubiose, trehalose, dulcitol, glycerol, inositol, methyl D-glucoside and glucoside is weak. Caprate, adipate, methyl β-D-xyloside, L-sorbite, sorbitol, methyl β-D-mannoside, amygdalin, salicin, inulin, melezitose, D-rafinose, starch, glycopycin, xylitol, β-gentiobiose and L-arabitol are not assimilated. The following enzymes are detected by using API ZYM strips: alkaline and acid phosphatases, esterase C4, lipase C8, lipase C14, leucine aminopeptidase, valine aminopeptidase, cysteine aminopeptidase, trypsin, chymotrypsin, phosphomimidase, β-galactosidase and α-glucosidase. Production of N-acetyl-β-glucosaminidase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase is negative. Resistant to ampicillin, penicillin, cefuroxime, cloxacillin, oxytetracycline, polymyxin B, erythromycin, neomycin and chloramphenicol. Sensitive to ciprofloxacin and weakly sensitive to gentamicin. The G + C content of the type strain is 56.4 mol%.

The type strain, ESC1T (= LMG 22713 = CECT 7172), and strain ESC5 (= LMG 23703) were isolated from nodules of Cytisus scoparius.

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References


