Ochrobactrum cytisi sp. nov., isolated from nodules of *Cytisus scoparius* in Spain

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Two strains named ESC1^T 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^T, *O. anthropi* DSM 6882^T and *Ochrobactrum tritici* DSM 13340^T. The predominant fatty acids were C_{18:1}ω7c and C_{18:1} 2-OH. Strains ESC1^T and ESC5 were strictly aerobic and were able to reduce nitrate and to hydrolyse aesculin. They produced β-galactosidase and β-glucosidase and did not produce urease after 48 h incubation. The G+C content of strain ESC1^T was 56.4 mol%. Both strains ESC1^T and ESC5 contained *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^T (=LMG 22713^T=CECT 7172^T).

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* and *Cicer*,

respectively (Trujillo *et al.*, 2005). In this work, we isolated two strains named ESC1^T 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^T 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

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Abbreviations: ITS, intergenic spacer; TP-RAPD, two-primers randomly amplified polymorphic DNA.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA, *nifH* and *nodD* gene sequences of strain ESC1^T are AY776289–AY776291, respectively.

TP-RAPD profiles, *nifH*- and *nodD*-based neighbour-joining trees, photos of nodules induced by strain ESC1^T and *R. etli* CFN42^T, fatty acid profiles and differentiating characters for all species of *Ochrobactrum* are available as supplementary material in IJSEM Online.

Fig. 1. Comparative sequence analysis of 16S rRNA genes of strain ESC1^T 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.

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^T and ESC5 and showed 100, 99.9 and 99.7 % similarity, respectively, to sequences from *O. anthropi* LMG 3331^T, *O. lupini* LUP21^T and *O. tritici* LMG 18957^T. Phylogenetic analysis of 16S rRNA gene sequences of the strains from this study showed clearly that they form a separate group within the genus *Ochrobactrum* together with *O. anthropi*, *O. lupini* and *O. tritici*, 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 *Ochrobactrum* 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^T and ESC5 and in *O. lupini* LUP21^T. 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 *O. anthropi*, *O. lupini* and *O. tritici* (Fig. 2). A pairwise analysis of the ITS sequences showed 91.8, 92.7 and 86.2 % identity between strain ESC1^T and *O. lupini* LUP21^T, *O. anthropi* LMG 3331^T and *O. tritici* LMG 18957^T, respectively. These values were lower than that found between *O. anthropi* LMG 3331^T and *O. tritici* LMG 18957^T (94.0 %) and suggested that the strains isolated in this work belong to a separate species within the genus *Ochrobactrum*.

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'-AAG-GAGGTGATCCANCCRCA-3') and also the primer pair 879F (5'-GCCTGGGAGTACGGCCGCA-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 *O. anthropi*, *O. lupini* and *O. tritici* (Trujillo *et al.*, 2005). Therefore, we analysed the TP-RAPD patterns of strains ESC1^T and ESC5 in comparison with those of the type strains from these three species (Supplementary Fig. S1). Strains ESC1^T 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

Fig. 2. Comparative sequence analysis of the 16S–23S rRNA ITS regions of strain ESC1^T 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 ESC1^T 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 hydroxyapatite by the procedure of Cashion *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 at 68 °C in 2 × SSC plus 10 % (v/v) DMSO. The resulting values between strain ESC1^T and strains *O. lupini* LUP21^T, *O. anthropi* DSM 6882^T and *O. tritici* DSM 13340^T were 70.4 % (individual values 69.8 and 71.0 %), 65.8 % (68.9 and 62.6 %) and 54.5 % (55.3 and 53.7 %), respectively (means of duplicates). The values obtained between *O. lupini* LUP21^T and *O. anthropi* DSM 6882^T and *O. tritici* DSM 13340^T were 69 and 63 %, respectively (means of duplicates). These values were nearly identical to those obtained in a previous work for *O. lupini* LUP21^T and *O. anthropi* LMG 3331^T using a different method for DNA–DNA hybridization (Trujillo *et al.*, 2005).

Fatty acids were extracted and analysed at the LMG as described previously (Trujillo *et al.*, 2005). The fatty acid pattern of strain ESC1^T (Supplementary Table S1) confirmed that it is a member of the genus *Ochrobactrum*, because it contains a major amount of C_{18:1}ω7c and moderate amounts (1–10 %) of C_{16:0}, C_{17:0}, C_{18:0}, C_{19:0} cyclo ω8c and summed feature 3 (C_{16:1}ω7c and/or C_{15:0} iso 2-OH). Like other *Ochrobactrum* species, strain ESC1^T contains C_{18:1} 2-OH, but the amounts detected in strains ESC1^T (20.4 %) and ESC5 (14.2 %) were higher than in their closest relatives *O. anthropi* and *O. lupini*. The novel strains also differ from *O. anthropi* in the amount of C_{19:0} cyclo ω8c. Several fatty acids present in small amounts in some closely related species were not detected in the strains from this study (see Supplementary Table S1).

Cells were stained according to the classical Gram procedure as 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 U), 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 LUP21^T and LUP23, *O. tritici* LMG 18957^T and *O. anthropi* LMG 3331^T were used as references in phenotypic characterization studies.

Cells of strains ESC1^T and ESC5 were Gram-negative, rod-shaped, non-sporulating, motile by means of a polar flagellum and commonly observed as single cells. Strains ESC1^T 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, α-glucosidase and lipase

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

Taxa: 1, strain ESC1^T; 2, strain ESC5; 3, *O. lupini* (unless indicated, data from Trujillo *et al.*, 2005); 4, *O. anthropi* (unless indicated, data from Holmes *et al.*, 1988); 5, *O. tritici* (unless indicated, data from Leubhn *et al.*, 2000). +, Positive; –, negative; V, variable; W, weak; ND, no data; S, sensitive; R, resistant; I, intermediate.

Character	1	2	3	4	5
Nitrate reduction	+	+	–	+	+
Aesculin hydrolysis	+	+	+	–	–
Urease (48 h)	–	–	+	+	+
API ZYM tests					
β-Galactosidase	+	–	V*	–	–†
α-Glucosidase	+	+	W*	+	–†
Lipase C14	+	+	W*	+	–†
Assimilation of:					
Gluconate	–	–	+	+	+
Citrate (24 h)	W	+	+	–	–
D-Mannose	+	+	+	+	–
L-Arabinose	+	+	+	+	–
D-Arabinose	+	+	–	+	ND
D-Turanose	+	+	–	+	+
L-Lyxose	+	+	–	+	ND
Antibiotic resistance					
Chloramphenicol	R	R	S–I	R	I
Polymyxin B (300 IU)	R	R	R	S	S

*Data from this study for *O. lupini* LUP21^T and LUP23.

†Data from this study for *O. tritici* LMG 18957^T.

‡Data from this study for *O. anthropi* LMG 3331^T; 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 ESC1^T 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 Rhijn *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 ESC1^T and ESC5 as described previously (Velázquez *et al.*, 2005). *Rhizobium etli* CFN42^T 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* CFN42^T (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* CFN42^T (data not shown).

In summary, on the basis of 16S rRNA gene and 16S–23S rRNA ITS sequences, strains ESC1^T 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 C_{18:1} ω7c, C_{18:1} 2-OH, C_{19:0} cyclo ω8c and C_{16:0}. The following fatty acids are detected in small amounts: summed features 2 and 3, C_{18:0}, C_{17:0}, C_{18:0} 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, lysine 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-rhamnose, arbutin, maltose, sucrose, turanose, L-xylose, tagatose, D-fucose, L-fucose, arabinol, 2-ketogluconate and 5-ketogluconate. Assimilation of D-xylose, L-xylose, galactose, D-fructose, lactose, melibiose, cellobiose, trehalose, dulcitol, glycerol, inositol, methyl α-D-glucoside and gluconate is weak. Caprate, adipate, methyl β-D-xyloside, L-sorbose, sorbitol, methyl α-D-mannoside, amygdalin, salicin, inulin, melezitose, D-raffinose, starch, glycogen, 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, cystine aminopeptidase, trypsin, chymotrypsin, phosphoamidase, 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, ESC1^T (= LMG 22713^T = CECT 7172^T), and strain ESC5 (= LMG 23703) were isolated from nodules of *Cytisus scoparius*.

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