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TAXONOMIC DESCRIPTION
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Pseudochrobactrum algeriensis sp. nov., isolated from lymph nodes of Algerian cattle

Maite Loperena-Barber[1], Mammar Khames[2], Sébastien O. Leclercq[3], Michel S. Zygmunt[3], Esteban D. Babot[4], Amaia Zúñiga-Ripa[1], Ana Gutiérrez[4], Mustapha Oumouna[2], Ignacio Moriyón[1], Axel Cloeckaert[3]† and Raquel Conde-Álvarez[1,*],†

Abstract

Three Gram-negative, rod-shaped, oxidase-positive, non-spore-forming, non-motile strains (C130915_07^T, C150915_16 and C150915_17) were isolated from lymph nodes of Algerian cows. On the basis of 16S rRNA gene and whole genome similarities, the isolates were almost identical and clearly grouped in the genus *Pseudochrobactrum*. This allocation was confirmed by the analysis of fatty acids ($C_{19:cyclo}$, $C_{18:1}$, $C_{18:0}$, $C_{16:1}$ and $C_{16:0}$) and of polar lipids (major components: phosphatidylethanolamine, ornithine-lipids, phosphatidylglycerol, cardiolipin and phosphatidylcholine, plus moderate amounts of phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine and other aminolipids). Genomic, physiological and biochemical data differentiated these isolates from previously described *Pseudochrobactrum* species in DNA relatedness, carbon assimilation pattern and growth temperature range. Thus, these organisms represent a novel species of the genus *Pseudochrobactrum*, for which the name *Pseudochrobactrum algeriensis* sp. nov. is proposed (type strain C130915_07^T=CECT30232^T=LMG 32378^T).

The genus *Pseudochrobactrum* was proposed by Kämpfer *et al.* [1], and at present it comprises four species: *P. saccharolyticum*, *P. asaccharolyticum*, *P. kiredjianiae* and *P. lubricantis* [1–3]. This genus is clearly differentiated from the genera *Ochrobactrum* and *Brucella* based on phenotypic features and phylogenetic position [1, 4, 5].

ISOLATION AND GROWTH CHARACTERISTICS

Here we report the characterization of three strains (C130915_07^T, C150915_16 and C150915_17) obtained during a bacteriological survey for *Brucella* in farms and slaughterhouses of the Algeria central region (Medea and El-Azizia) [6]. During this survey, 30 retropharyngeal and/or mammary lymph nodes of *Brucella* seropositive animals were collected. After necropsy, lymph nodes were degreased and surface-sterilized by brief immersion on ethanol and gentle burning, placed in sterile plastic bags, sealed and transported to the laboratory in iceboxes. There, they were homogenized in sterile saline and seeded on CITA *Brucella* selective agar [7]. Unexpectedly, three isolates (C130915_07^T, C150915_16 and C150915_17), obtained as pure culture from mammary lymph nodes (*circa* 10–30 colonies/organ) of three different cows without pathological signs, were negative in Bruce-ladder PCR for *Brucella* species and did not agglutinate with anti-smooth brucellae serum. Upon subculture on TSA (tryptone soy agar) at 37 °C for 24–48 h, the three isolates showed beige-coloured colonies of *circa* 2 mm in diameter. Growth at 37 °C was also observed on nutrient agar, CPSE (chromogenic medium for identification of urinary pathogens) and MacConkey agar (non-lactose fermenting), but not on de Man–Rogosa–Sharpe (for *Lactobacillus*) or *Salmonella–Shigella* agars (all from

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Keywords: cattle; *Proteobacteria*; *Pseudochrobactrum*; *Pseudochrobactrum algeriensis sp. nov.*.
Abbreviations: CPSE, chromogenic medium for identification of urinary pathogens; dDDH, digital DNA–DNA hybridization; gANI, genomic average nucleotide identity; NAG, *N*-acetyl glucosamine; PDME, phosphatidyldimethylethanolamine; PMME, phosphatidylmonomethylethanolamine; TSA, tryptone soy agar.
16S rRNA gene sequences: MZ227818–MZ227820. Genome assembly: CP075348–CP075362.
†These authors contributed equally to this work
Two supplementary tables and one supplementary figure are available with the online version of this article.
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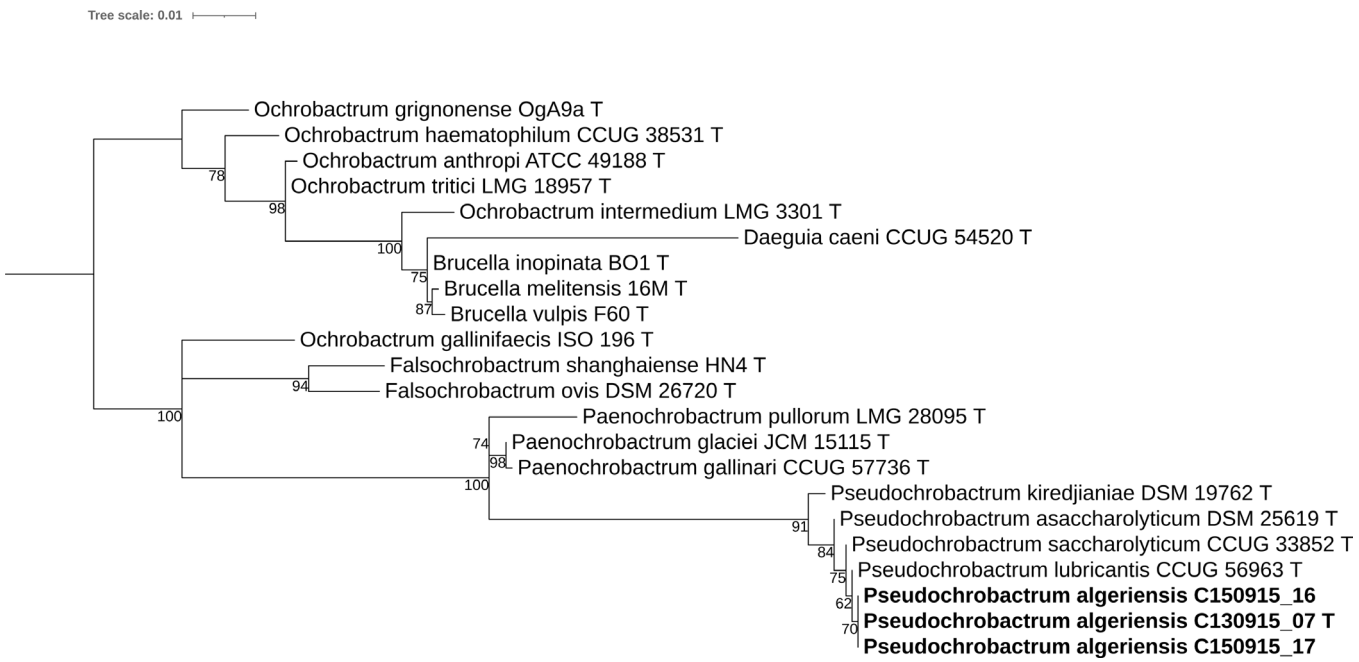

Fig. 1. 16S rRNA gene minimum-likelihood phylogeny of a selected subset of *Brucellaceae* type strains. Bootstrap support over 1000 replications are indicated at each branching point. The tree is rooted according to a previously published *Brucellaceae* 16S rRNA topology conducted with several Rhizobiales outgroups [4].

bioMérieux). On TSA, the three strains were able to grow at 18–38 °C, but not at 10 or 40 °C. Cells of colonies grown for 3 days at 37 °C on TSA were 0.5–0.7×0.8–1.5 µm coccobacilli that stained Gram-negative using conventional protocols [8, 9].

16S rRNA PHYLOGENY

The phylogenetic position of the three isolates was determined based on comparative analysis of their 16S rRNA genes with *Brucellaceae* members. The 16S rRNA gene was amplified for each strain using universal primer sets 27F/1492R and 518F/800R. Direct sequencing of purified products was conducted by Macrogen with sequencing primers (27F, 1492R, 518F and 800R) and an ABI 3730xl automated sequencer (Applied Biosystems). Sequences were deposited in the NCBI GenBank database under the accession numbers MZ227818 (C130915_07^T), MZ227819 (C150915_16) and MZ227820 (C150915_17). The 16S rRNA gene sequences were aligned with MAFFT version 7.31 [10] to the 16S rRNA sequences from the genomes of 19 *Brucellaceae* type strains. These genomes were downloaded from GenBank as genome assemblies or raw sequence reads, depending on the availability (Supplementary Table S1, available in the online version of this article). Downloaded raw sequence reads were assembled using SPAdes version 3.13.1 [11] with default parameters, and scaffolds shorter than 200 bp or with a read coverage lower than 20 ([10] for *Paenochrobactrum pullorum*) were discarded. Pairwise similarities (gaps excluded) were calculated from the alignment, using the software package UGENE version 37.1 [12]. The 16S rRNA gene sequences of the three strains were 100% identical, and exhibited more than 99% similarity to the 16S rRNA genes of all the other *Pseudochrobactrum* type strains. The most similar sequences were those of *P. lubricantis* CCUG 56963^T and *P. saccharolyritum* CCUG 33852^T, with one and two differences out of the 1406 aligned nucleotides, respectively.

A maximum-likelihood (ML) phylogenetic tree was reconstructed from the multiple alignment using RaXML version 8.2.11 [13] with the GTRCAT model of evolution and 1000 bootstrap replications. The resulting topology was displayed using the iTOL web interface version 5.7 [14]. Consistent with the pairwise similarity analysis, C130915_07^T, C150915_16 and C150915_17 branched together, inside a well-supported clade (91% bootstrap support) composed of all previously described *Pseudochrobactrum* type strains (Fig. 1). This clade was distant from type strains of other genera of the family, indicating that strains C130915_07^T, C150915_16 and C150915_17 belong to the genus *Pseudochrobactrum*.

OVERALL GENOME FEATURES

The complete genomes of strains C130915_07^T, C150915_16 and C150915_17 were sequenced using the PacBio RSII sequencing system (Pacific Biosciences) and assembled by GenoScreen (Lille, France). The complete genome sequences were annotated

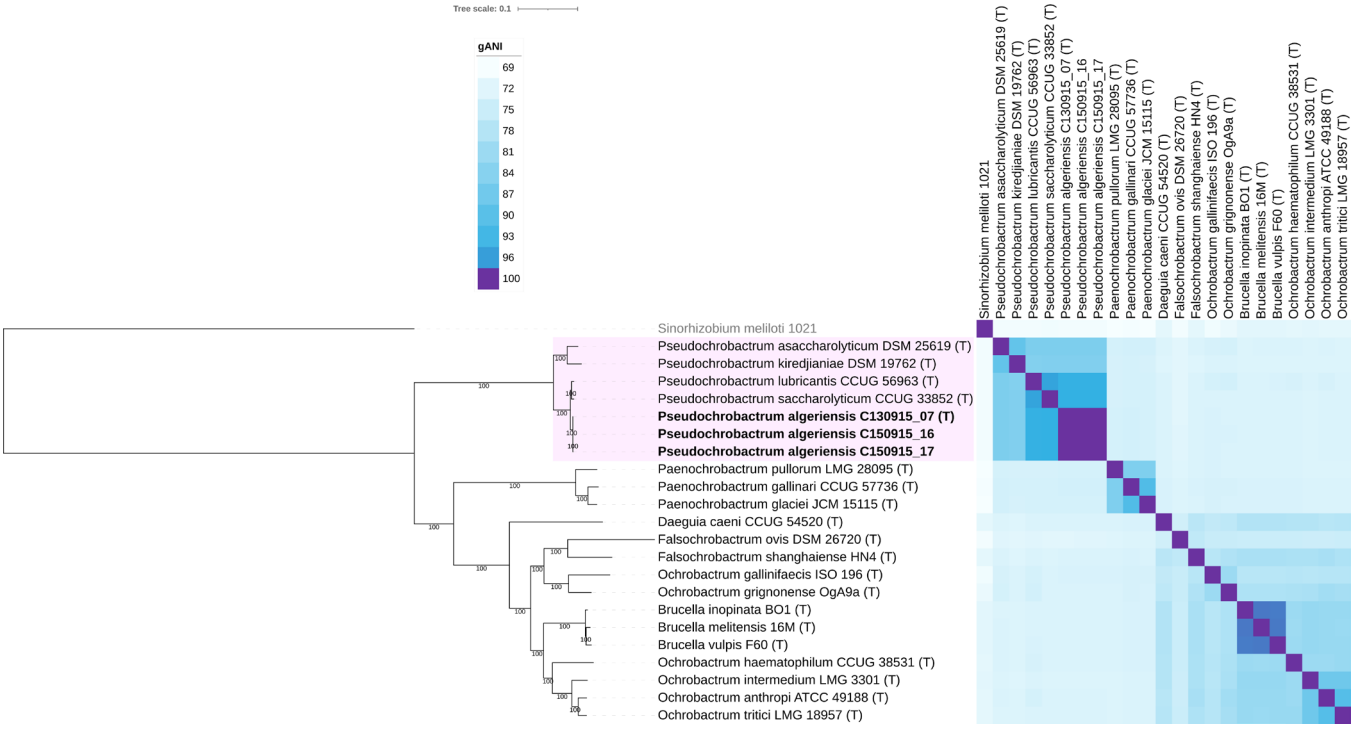

Fig. 2. Whole-genome maximum-likelihood phylogeny of a selected subset of *Brucellaceae* type strain genomes, based on the alignment of 854 core proteins. Bootstrap support over 100 replications are indicated at each branching point. The *Pseudochrobactrum* clade is boxed in pink. Pairwise gANI values are given as a heatmap, with values below the species threshold of 96.5 coloured as a white-to-blue gradient, and values above the threshold coloured as a blue-to-purple gradient.

using Prokka 1.12 [15] with default parameters. The genome sequences were deposited in the NCBI GenBank database under the accession numbers CP075358–CP075362 (C130915_07^T), CP075353–CP075357 (C150915_16) and CP075348–CP075352 (C150915_17).

The genomes of the three strains are extremely similar and consist of a chromosome of ~3.46 Mbp with ~51.6mol% G+C content carrying 3104–3127 protein-coding genes, 15 rRNA genes and 58 tRNA genes. All three strains also carry four plasmids. The first plasmid is 432 kbp long with ~51.4mol% G+C content and carries 391–392 protein-coding genes and two tRNA genes (Phe_{gaa} and Thr_{ggt}) unique to this replicon (Supplementary Table S2). Two other plasmids are also almost identical in all strains and are 33.6 kbp and 21.7 kbp long, respectively, while the third plasmid is 43.3 kbp long in strain C150915_16 and 76 kbp long in strains C130915_07^T and C150915_17.

The genome sequences of the three strains were combined with the 19 genome sequences of *Brucellaceae* type strains used in the 16S rRNA gene analysis to produce a phylogenomic tree as described in [4], with the genome sequence of *S. meliloti* as an outgroup and 100 bootstrap replications (Fig. 2). The ML tree reconstructed from the global alignment of 854 orthologous proteins provided a completely resolved phylogeny consistent with other whole-genome based phylogenies of the family *Brucellaceae* [4, 5]. In this tree, the three isolates C130915_07^T, C150915_16 and C150915_17 clustered together as a specific branch within the *Pseudochrobactrum* clade, similar to the position observed with the 16S rRNA gene. They appeared closely related to *P. lubricantis* CCUG 56963^T and *P. saccharolyritum* CCUG 33852^T, which form a sister clade.

The genomic average nucleotide identity (gANI) between strains C130915_07^T, C150915_16 and C150915_17 as well as with the other *Brucellaceae* genomes used in this study was calculated using ANIcalculator version 1 [16] with rRNA genes excluded. All pairwise comparisons of the three isolates exhibited a gANI value of 100 (Fig. 2), far above the 96.5 threshold recommended by [16] as a species limit. On the other hand, they showed gANI values of 96.03–96.05 when compared to *P. saccharolyticum* CCUG 33852^T and of 95.85–95.88 when compared to *P. lubricantis* CCUG 56963^T. To confirm these results using other indicators, the digital DNA–DNA hybridization (dDDH) values were calculated, using the server-based Genome-to-Genome Distance Calculator version 2.1 [17]. The average nucleotide identity (ANI) values based on the BLAST+ algorithm (ANIb) and the MUMmer alignment tool (ANIm) were also calculated for the three isolates and the other *Pseudochrobactum* type strains using the JSpeciesWS website [18]. All dDDH results were below the generally recommended species threshold limit of 70%, with values ranging between 66.6 and 66.7% (C.I. 63.7–69.5%) when compared to *P. lubricantis* CCUG 56963^T, and equal to 67.4% (C.I. 64.4–70.2%)

Table 1. Differentiating physiological reactions of strains C130915_07^T, C150915_16 and C150915_17, in comparison with the other *Pseudochrobactrum* type strains

Strains: 1, C130915_07^T, C150915_16 and C150915_17 (same results for the three strains); 2, *P. Asaccharolyticum*; 3, *P. saccharolyticum*; 4, *P. Kiredjianiae;* 5, *P. Lubricantis*. +, Positive; –, negative; (+) and (–), weak reaction.

Assimilation of	1	2*	3*	4*	5*
Gluconate	–	–	–	–	–
L-Arabinose	–	–	+	–	–
D-Glucose	+	–	+	+	+
D-Mannose	+	–	+	–	+
L-Malate	+	–	+	+	+
Citrate	+	–	–	–	–
Maltose	–	–	–	–	–
N-Acetyl-D-glucosamine	+	+	–	(+)	+
D-Mannitol	–	–	–	–	–
Phenylacetate	–	–	–	–	–
L-Lactate	–	+	+	+	–
D-Sorbitol	–	–	–	–	–
Adonitol	–	–	–	–	–
L-Histidine	(–)	–	+	–	+

*Data from [1–3].

when compared to *P. saccharolyticum* CCUG 33852^T. On the contrary, ANIb and ANIm values were all slightly higher than the recommended species threshold of 95%, ranging from 95.59 to 95.63% when compared to *P. lubricantis* CCUG 56963^T and from 95.91 to 95.93 % when compared to *P. saccharolyticum* CCUG 33852^T.

In summary, the genomic analyses indicated that strains C130915_07^T, C150915_16 and C150915_17 belong to the genus *Pseudochrobactrum*, and that they can be considered as a novel species closely related to *P. saccharolyticum* and *P. lubricantis* at genomic level.

PHYSIOLOGY AND CHEMOTAXONOMY

The differential phenotypic features of strains C130915_07^T, C150915_16 and C150915_17, and the other *Pseudochrobactrum* type strains are given in Table 1. Biochemical tests were carried out using API 20NE and Vitek 2 systems as described by the manufacturer (both from bioMérieux). The three strains were positive for urease, cytochrome oxidase, citrate utilization and assimilation of D-glucose, D-mannose, L-malate and *N*-acetyl-D-glucosamine (NAG). They produced negative reactions in the following tests: denitrification, indole production, D-glucose fermentation, arginine dihydrolase, aesculin hydrolysis (β-glucosidase), gelatin hydrolysis (protease), β-galactosidase and assimilation of capric acid, gluconate, adipic acid, phenylacetic acid, L-arabinose, maltose and D-mannitol.

Strains C130915_07^T, C150915_16 and C150915_17 could be distinguished from other *Pseudochrobactrum* species by their carbon assimilation pattern (Table 1). They similarly differed from all previously described *Pseudochrobactrum* species [1–3] in the assimilation of citrate. In addition, they differed from *P. asaccharolyticum* in the ability to assimilate D-glucose, D-mannose, L-lactate and L-malate; from *P. saccharolyticum* in the ability to assimilate L-arabinose, L-lactate, L-histidine and NAG; from *P. kiredjianiae* in the ability to assimilate L-lactate, NAG and D-mannose; and from *P. lubricantis* in the ability to assimilate L-histidine.

Chemotaxonomic characterization of strains C130915_07^T, C150915_16 and C150915_17 included the analysis of fatty acids and polar lipids. For fatty acid analysis, dried bacteria were saponified with 15% NaOH in 50% (v/v) methanol, acidified with HCl, the product extracted with hexane–methyl *terc*-butyl ether (1:1), and the extracts evaporated to dryness and dissolved in chloroform. GC-MS analyses were performed in a Shimadzu GC–MS QP 2010 Ultra system, using a fused-silica DB-5HT capillary column (30m×0.25 mm internal diameter, 0.1 µm film thickness) from J and W Scientific. The oven was heated from 120 °C (1.0 min) to 300 °C (15 min) at 5 °C min^{-1}. The injection was performed at 300 °C, and the transfer line was kept at 300 °C. Compounds

Table 2. Mayor fatty acid composition (relative percentage) of strain C130915_07^T and other *Pseudochrobactrum* type strains

Strains: 1, C130915_07^T; 2, *P. asaccharolyticum*; 3, *P. saccharolyticum*; 4, *P. kiredjianiae*; 5, *P. lubricantis*.

Fatty acid	1	2*	3*	4*	5*
Saturated acids:					
$C_{12:0}$	–	–	0.7	–	–
$C_{14:0}$	–	–	–	–	–
$C_{16:0}$	7.6	5.5	1.9	2.8	4.5
$C_{17:0}$	–	–	–	–	–
$C_{18:0}$	31.3	11.0	7.4	7.7	7.8
Unsaturated acids:					
$C_{13:1}$ at 12–13	–	–	–	–	–
$C_{16:1}$	0.3	–	–	–	–
$C_{17:1}\omega6c$	–	–	–	–	–
$C_{18:1}\omega7c$	59.4	74.9	74.6	33.4	29.1
$C_{18:3}\omega6c$ [6, 9, 12]	–	–	0.5	–	–
11-Methyl-$C_{18:1}\omega7t$	–	–	–	1.0	1.8
$C_{20:1}\omega7c$	–	–	–	0.8	–
$C_{20:2}\omega6,9c$	–	–	–	0.9	1.4
Hydroxy acids:					
$C_{18:1}$ 2-OH	–	–	–	–	–
$C_{18:0}$ 3-OH	–	–	–	–	–
Summed feature 3†	–	0.8	–	–	–
Cyclopropane acids:					
$C_{17:0}$ cyclo	–	–	–	0.5	–
$C_{19:0}$ cyclo$\omega8c$	1.4	7.3	14.3	53.0	53.9
Unknown 13.957‡	–	–	–	–	–
Unknown 14.959‡	–	0.6	0.9	–	1.2

*Data from original authors [1–3].
†Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total.
‡Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore could not be identified; they are referred to by their equivalent chain length.

were identified by mass fragmentography and by comparing their mass spectra with those of the Wiley and NIST libraries. The fatty acid profile (Table 2) of strain C130915_07^T was composed of $C_{19:cyclo}$ (1.4%), $C_{18:1}$(59.4%), $C_{18:0}$(31.3%), $C_{16:1}$(0.3%) and $C_{16:0}$(7.6%). This fatty acid profile and the fatty acid profiles of C130915_16 and C130915_17 showed a high similarity to the profiles of members of *Pseudochrobactrum*.

For the polar lipid analyses of C130915_07^T, C150915_16 and C150915_17 isolates, the strains were cultivated on TSA at 37 °C for 2 days, free-lipids were extracted [19] and analysed on silica gel 60 high-performance thin-layer chromatography plates (Merck). Bidimensional chromatography was performed with chloroform–methanol–water (14:6:1) in the first dimension and chloroform–methanol–acetic acid (13:5:2) for the second dimension. Plates were developed with copper (II) sulphate for the identification of total lipids (Supplemental Fig. S1). The polar lipid composition of strains C130915_07^T, C150915_16 and C150915_17 was similar in its major components to those of recognized *Pseudochrobactrum* species [1–3], and included phosphatidylethanolamine, ornithine-lipids, phosphatidylglycerol, cardiolipin, phosphatidylcholine and several unidentified lipids, including large amounts of aminolipids. Among the latter there were two ninhydrin-positive spots compatible with phosphatidylmonomethylethanolamine (PMME) and

phosphatidyldimethylethanolamine (PDME). This indicates that the phospholipid methyltransferase (PmtA) pathway is active. Although the PmtA pathway was proposed to be a trait distinguishing the *Pseudochrobactrum* and *Ochrobactrum* clades from *Brucella* [1], it has been recently shown that some *Brucella* species have an active PmtA pathway and thus produce PMME and PDME [20], in contrast to *B. abortus* and *B. melitensis* where PMME and PDME are not or barely detectable [21, 22].

ECOLOGY AND PRACTICAL IMPLICATIONS

It is important to stress that this new species grows on selective media designed to isolate an important pathogen; however, some simple tests might allow a prompt differentiation. These include lack of agglutination with anti-smooth *Brucella* sera, ability to grow on MacConkey and negative Bruce-ladder PCR [23]. The first characteristic is consistent with the absence in C130915_07^T, C150915_16 and C150915_17 of homologues of the genes necessary for perosamine synthesis, the only sugar in the O-polysaccharide of the smooth lipopolysaccharide of classical brucellae (Loperena *et al*., manuscript in preparation). Therefore, the brucellosis seropositivity observed cannot be due to these isolates but rather to the presence of the animals in a *Brucella* infected herd.

Other *Pseudochrobactrum* species appear to be environmental inhabitants [1–3], and this may also be the origin of the newly proposed species. The presence in local ganglia (which were surface-sterilized) could be the result of a transient bacteraemia but, its repeated isolation (three animals sampled in two different days) in the inimical environment within lymph nodes is remarkable, particularly when considering that it is phylogenetically related to the opportunistic pathogens of the genus *Ochrobactrum* [24] and the facultative intracellular parasites of the genus *Brucella* [25]. A search for putative genes coding for the enzymes involved in the synthesis of the lipid A component of the lipopolysaccharide suggests that C130915_07^T, C150915_16 and C150915_17 share with *Brucella* and other closely related genera the genes of the lipid A classical pathway (i.e., Raetz pathway), as well as others putatively involved in lipid A remodelling (Loperena et al., manuscript in preparation). Thus, it is plausible that, like *Brucella* and *Ochobactrum*, these isolates bear lipid A with a reduced pathogen-associated molecular pattern, a trait that could delay immunity activation and hamper a prompt clearance of the bacteria in lymph nodes [25]. The large amounts of cationic aminolipids present in their envelope should account for the resistance to the cationic peptides in CITA medium. Resistance to this cationic lipopeptide parallels resistance to bactericidal peptides of innate immune system [25] and could also favour a more or less transitory colonization. Research is in progress to test these hypotheses, an investigation that could also improve our understanding of the evolutionary adaptations of *Brucellaceae* members.

DESCRIPTION OF *PSEUDOCHROBACTRUM ALGERIENSIS* SP. NOV.

Pseudochrobactrum algeriensis (al.ge.ri.en´sis. N.L. masc. adj. algeriensis of or belonging to Algeria, referring to the isolation place).

Aerobic, Gram-stain-negative, oxidase- and catalase-positive, non-motile and rod-shaped. Non-pigmented, beige colonies with regular edges with a diameter of about 2 mm. Good growth occurs at 18–38 °C within 24–48 h on TSA, nutrient agar, MacConkey and CPSE. In API 20NE tests, urease, cytochrome oxidase, citrate utilization and assimilation of D-glucose, D-mannose, L-malate, and *N*-acetyl-D-glucosamine are positive. Denitrification, indole production, D-glucose fermentation, arginine dihydrolase, aesculin hydrolysis (β-glucosidase), gelatin hydrolysis (protease), β-galactosidase and assimilation of gluconate, capric acid, adipic acid, phenylacetic acid, L-arabinose, maltose and D-mannitol are negative. The major fatty acids are $C_{18:0}$ and $C_{18:1.}$ The polar lipid profile consists of the major compounds phosphatidylethanolamine, ornithine-lipids, phosphatidylglycerol, cardiolipin and phosphatidylcholine, plus moderate amounts of PMME, PDME and other aminolipids. The type strain is C130915_07^T (CECT 30232^T=LMG 32378^T) isolated from lymph nodes of an Algerian cow.

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Author contributions
R.C.-A., M.S.Z., A.C. and I.M. conceived and coordinated the study. M.K., M.L.-B., A.Z.-R., E.D.B., A.G. and M.O. performed and/or supervised experiments. SOL performed the genomic analyses. R.C.-A., M.L.-B., S.O.L., M.S.Z., A.C. and I.M. wrote the manuscript. All authors read and agreed to the published version of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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