Listeria rocourtiae sp. nov.

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A *Listeria*-like strain isolated in Austria from pre-cut lettuce fitted the description of the genus *Listeria* although it could not be assigned to any of the known species. Comparison of the *rrs* gene (encoding 16S rRNA) sequence and gene content by DNA-array indicated affiliation to the genus *Listeria*. Phylogenetic distance from known species of the genus *Listeria* indicated that it represents a novel species. Since it can be differentiated from all other known species of the genus *Listeria* by using phenotypic tests, the name *Listeria rocourtiae* sp. nov. is proposed for the novel species. The type strain is CIP 109804^T (=DSM 22097^T =Allerberger 700284/02^T). The type strain is avirulent as assessed by cell culture assays and inoculation of mice.

Molecular methods have deeply modified our vision of the taxonomic structure of the genus *Listeria* (Rocourt *et al.*, 1982; Seeliger & Jones, 1986). At the time of writing, the genus *Listeria* is composed of six species: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi* and *L. ivanovii*, the latter comprising *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Rocourt & Buchrieser, 2007). Although species of the genus *Listeria* are environmental bacteria, two species (*L. monocytogenes* and *L. ivanovii*) are pathogenic for animals and humans. *L. monocytogenes* is the most commonly isolated member responsible for listeriosis in humans and animals.

The purposes of this work were to (*i*) characterize an isolate resembling members of the genus *Listeria* but not identified as belonging to any known species, (*ii*) position the isolate in the bacterial phylogenetic tree, and (*iii*) describe the isolate as representing a novel species for which the name *Listeria rocourtiae* sp. nov. is proposed.

Strain CIP 109804^T was recovered from a single sample of pre-cut lettuce at the Federal Food Safety Microbiology Laboratory in Salzburg (Austria) in 2002. This sample was detected positive for the presence of *Listeria monocytogenes* by the enzyme-linked fluorescence assay Vidas *Listeria monocytogenes* II (bioMérieux). The strain was isolated on RAPID' *L.mono* (Bio-Rad), an agar medium based on the chromogenic detection of phosphatidylinositol-specific phospholipase C (PIPLC) and xylose fermentation (Leclercq, 2004). Multiple colonies with identical phenotypic characteristics according to the RAPID' *L.mono* manufacturer's instructions for positive confirmation were obtained and incorrectly identified as *L. monocytogenes*. No other known species of the genus *Listeria* was detected. One isolate was then sent to the National Reference Laboratory (NRL) of Austria for *Listeria monocytogenes* for confirmation of its identification. This isolate was not confirmed as *L. monocytogenes* by NRL but as a potential new species and

Abbreviations: PIPLC, phosphatidylinositol-specific phospholipase C.

The GenBank/EMBL/DDBJ accession number for the *rrs* gene sequence of strain CIP 109804^T is FJ557241.

A dendrogram of hierarchical clustering reconstructed with the J-Express program based on the presence and the absence of genes, and a table detailing the origins and descriptions of reference strains used in the comparative genomic DNA array hybridization and analysis are available with the online version of this paper.

was sent to the WHO Collaborating Centre for Foodborne Listeriosis for its characterization.

In order to characterize the isolate, the following phenotypic and genotypic methods were undertaken. Colonies were observed on ALOA (AES laboratoire), a medium based on the simultaneous detection of PIPLC and β-glucosidase activities. Gram staining, respiratory characteristics, catalase and oxidase production and the presence of a capsule by the use of a wet Indian ink film were determined. Motility was tested by stab-inoculating mannitol-mobility semi-solid agar (Bio-Rad) and semi-solid nutrient agar containing 0.4 % agar in a U-shaped tube. Haemolysis was determined on Columbia agar containing 5 % defibrinated horse blood (Oxoid). Furthermore, the CAMP test (Christie *et al.*, 1944) was performed on Columbia agar containing 5 % defibrinated sheep blood (Oxoid) using *Staphylococcus aureus* CIP 5710 and *Rhodococcus equi* NCTC 1621. Growth at different temperatures was determined in trypto-casein-soy broth and agar (Difco) after incubation at 4 °C for 10 days and at 22, 30, 37 and 42 °C for 7 days. Acid production from carbohydrates was determined by using the API50CH system (bioMérieux) as recommended by the manufacturer. Reactions were recorded after 2, 5, 10 and 15 days of incubation at 30 °C. Biochemical tests were completed with API-*Listeria* strips (bioMérieux) as recommended by the manufacturer (Bille *et al.*, 1992). Serotyping of strain CIP 109804^T was performed as described by Seeliger & Höhne (1979).

The *rrs* gene (encoding 16S rRNA) was amplified by PCR using the universal primers A, 5′-AGAGTTTGATCATG-GCTCAG-3′ (position 8–27, *Escherichia coli* numbering system), and H, 5′-AAGGAGGTGATCCAACCGCA-3′ (1541–1522) (Böttger, 1989), in a GeneAmp thermal cycler (Perkin-Elmer) with the following cycle parameters: 4 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 57 °C and 2 min at 72 °C; final extension step at 72 °C for 5 min. Each PCR product was purified by filtration on P100 Gel Fine (Bio-Rad) and then sequenced by using primers A, H and eight other sequencing primers (*E. coli* numbering system): B, 5′-CTCCTACGGGAGGCAGCAGT-3′ (339–358); C, 5′-ACTGCTGCCTCCCGTAGGAG-3′ (358–339); D, 5′-CGTGCCAGCAGCCGCGGTAAT-3′ (514–534); E, 5′-TTACCGCGGCTGCTGGCACGT-3′(533–514); F, 5′-GATTAGATACCCTGGTAG-3′ (786–803); G, 5′-GCATG-TGGTTTAATTCGA-3′ (947–964); I, 5′-TCGAATTAAAC-CACATGC-3′ (964–947) and J, 5′-AGGGTTGCGCTC-GTTGCGG-3′ (1115–1097) with the ABI PRISM BigDye terminator cycle sequencing kit and the ABI PRISM 3700 DNA sequencer according to the manufacturer's instructions (Applied Biosystems). The almost-complete *rrs* gene sequence for CIP 109804^T (1539 nt; GenBank accession FJ557241) was obtained by using the Script Assembler Tool Kit developed by Eric Deveaud and Betina Setterblad, Groupe Logiciels et Banques de Données, Institut Pasteur (unpublished program).

Phylogenetic analysis based on the *rrs* gene sequence of strain CIP 109804^T and reference type strains belonging to the genus *Listeria* retrieved from GenBank was performed. Sequences were aligned by using CLUSTAL X (Thompson *et al.*, 1997) and Jukes–Cantor evolutionary distances were calculated (Jukes & Cantor, 1969). A neighbour-joining (Saitou & Nei, 1987) phylogenetic tree was reconstructed by using the Taxotron software (P. Grimont, Paris, France).

A specific PCR analysis, allowing separation of the four major serotypes (1/2a, 1/2b, 1/2c and 4b) of *L. monocytogenes* into four distinct PCR groups was performed as described by Doumith *et al.* (2004a).

A *Listeria* biodiversity DNA array (Doumith *et al.*, 2004b; Hong *et al.*, 2007; Volokhov *et al.*, 2007) was used to detect the presence of known *L. monocytogenes* genes, including those involved in virulence. The binary data obtained from 710 probes were analysed by hierarchical clustering using the program J-Express (Dysvik & Jonassen, 2001).

Virulence tests commonly used for species of the genus *Listeria* were undertaken as described by Roche *et al.* (2001). These included the plaque-forming assay on confluent human adenocarcinoma HT-29 cell monolayers (ECACC no. 85061109) (Fogh & Trempe, 1975; Roche *et al.*, 2001) and an *in vivo* test after subcutaneous inoculation into the left footpad of conventional Swiss mice (Charles River, L'Arbresle, France) (Audurier *et al.*, 1981). HT-29 cell monolayers were infected with 2 to 7 log *Listeria* per well for 2h at 37 °C. Conventional Swiss mice were inoculated with 4 log c.f.u. *Listeria* in 50 µl for subcutaneous injection. The *L. monocytogenes* strain EGDe (BUG 1600) was used as a positive control, the *L. innocua* strain BUG 499 as a negative one.

Finally, susceptibility to a wide range of antibacterial agents was determined with the disk diffusion method on Mueller–Hinton agar plates (Bio-Rad) and minimal inhibitory concentrations were determined for the antibiotics that are clinically relevant for listeriosis. Susceptibility to β-lactams and trimethoprim was also tested by the E-test procedure (AB Biodisk), according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (Anonymous, 2006). The natural susceptibility to antibiotics of strain CIP 109804^T was deduced by using the database on natural susceptibility of *Listeria* species (Troxler *et al.*, 2000). Moreover, we used the interpretative criteria and recommendations from the CA-SFM (freely available at http://www.sfm.asso.fr) and the European Committee on Antibiotic Susceptibility Testing (EUCAST, freely available at http://www.escmid.org/research_projects/eu_cast/) for the interpretation of the *in vitro* susceptibility of *L. monocytogenes* to antibiotics.

The results of these different tests showed that the food isolate (CIP 109804^T) was a Gram-stain-positive, non-spore-forming, non-capsulated, rod-shaped bacterium that was non-motile at 37 °C. At 4, 22 and 30 °C, the isolate swarmed through semisolid medium in a U-shaped tube and showed a typical 'umbrella' motility in tubes of mannitol-mobility semi-solid agar. Colonies on trypto-casein-soy agar plates

had a diameter of 0.5 to 1.0 mm after 48h of growth at 30 °C. They were round, translucent with dew-drop appearance and low convex with a finely textured surface and entire margin. Colonies appeared blue-green when viewed under a magnifying glass with oblique transmitted light, and bluish grey by normal illumination. The strain showed an optimal growth temperature of 30 °C. This strain did not grow at 42 °C and was able to grow at 4 °C (only in trypto-casein-soy broth and in mannitol-mobility semi-solid agar) within 48h. It was facultatively anaerobic, catalase-positive, nitrate reductase-positive, mannitol-positive and oxidase-negative. It was non-haemolytic and gave negative results for the CAMP test. Thus, this isolate (CIP 109804^T) could be readily distinguished from the other species of the genus *Listeria* according to the results of the biochemical tests shown in Table 1. The API-*Listeria* strips showed negative results for arylamidase, D-arabitol, glucose-1-phosphate and D-tagatose, and positive results for aesculin hydrolysis, α-mannosidase and acidification of methyl α-D-glucoside, D-xylose, rhamnose and ribose. This API-*Listeria* profile (7630) did not fit any known profile in the profile database provided by the manufacturer. Based on results of the API 50CH system, acid was produced from glycerol, erythritol, ribose, D-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, aesculin, cellobiose, maltose, lactose, melibiose, soluble starch, trehalose, D-raffinose, glycogen and β-gentiobiose. Acid was not produced from D-arabinose, L-arabinose, L-xylose, methyl β-xyloside, methyl α-D-mannoside, sucrose, inulin, melezitose, xylitol, L-fucose, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. On the RAPID' *L.mono* medium, colonies were white surrounded by a yellow halo, indicating that this strain was PIPLC negative and xylose-positive. This isolate did not grow on the chromogenic medium ALOA (Leclercq, 2004). The multiplex PCR (Doumith *et al.*, 2004a) was negative. This strain was not agglutinated by sera I to XV targeting known somatic antigens, and did not react with sera directed against flagellar antigens A, AB, C, D and E. Thus, this strain had no specific serotype or PCR group described for the genus *Listeria* to date.

Table 1. Characteristics that distinguish strain CIP 109804^T from closely related species of the genus *Listeria*

Species: 1, *L. rocourtiae* sp. nov.; 2, *L. monocytogenes*; 3, *L. innocua*; 4, *L. seeligeri*; 5, *L. ivanovii* subsp. *londoniensis*; 6, *L. ivanovii* subsp. *ivanovii*; 7, *L. welshimeri*; 8. *L. grayi*. All species are aesculin hydrolysis positive. +, Positive reaction; (+), weak or delayed positive reaction; −, negative reaction; V, variable reaction. Data from this study and Seeliger & Jones (1986).

Characteristic	1	2	3	4	5	6	7	8
β-haemolysis	−	+	−	+	+	+	−	−
CAMP test								
S. aureus	−	+	−	(+)	−	−	−	−
R. equi	−	−	−	−	+	+	−	−
Arylamidase	−	−	+	+	V	V	V	+
PIPLC	−	+	−	−	+	+	−	−
α-Mannosidase	+	+	+	−	−	−	+	V
Fermentation of:								
Mannitol	+	−	−	−	−	−	−	+
D-Arabitol	−	+	+	+	+	+	+	+
D-Xylose	+	−	−	+	+	+	+	−
Rhamnose	+	+	V	−	−	−	V	−
Methyl α-D-glucoside	+	+	+	+	+	+	+	V
Ribose	+	−	−	−	−	+	−	+
Glucose-1-phosphate	−	−	−	−	V	V	−	−
D-Tagatose	−	−	−	−	−	−	+	−

The *rrs* gene sequence determined consisted of a continuous stretch of 1539 nt. A comparison of the *rrs* gene sequence of strain CIP 109804^T with that of other members of the genus *Listeria* is shown in Table 2 (1467 nt compared). Per cent *rrs* gene sequence similarities between *L. rocourtiae* sp. nov. and other species were 95.84 (*L. monocytogenes*, *L. welshimeri*), 95.64 (*L. innocua*, *L. grayi*), 95.57 (*L. ivanovii*), 94.96 (*L. seeligeri*), 91.89 (*Brochothrix thermosphacta*) and 91.68 (*Brochothrix campestris*). Jukes–Cantor distance values ranged from 0.042 to 0.046 between strain CIP 109804^T and all other type strains of the genus *Listeria*. These values are comparable with those between *L. grayi* and other species of the genus *Listeria* (0.037 to 0.043). The tree in Fig. 1 shows the phylogenetic relationships of strain CIP 109804^T and other members of the genus *Listeria* based on the *rrs* gene sequence (Stackebrandt & Goebel, 1994). This tree clearly shows that the newly characterized strain CIP 109804^T represents a distinct species and belongs to the genus *Listeria* as long as this genus includes *L. grayi*.

The gene content of strain CIP 109804^T was surveyed by using a *Listeria* biodiversity DNA array. When compared with 27 strains representative of the different serogroups of *L. monocytogenes* and 13 isolates representing all other species of the genus *Listeria* (Supplementary Table S1, available in IJSEM online), strain CIP 109804^T clustered within the genus *Listeria* and was closely related to *L. ivanovii* subsp. *ivanovii* (Supplementary Fig. S1). The clustering derived from the biodiversity DNA array should not be taken as a phylogenetic tree since genes on the array represented only *L. monocytogenes* and *L. innocua*. It shows that *L. rocourtiae* sp. nov. is not excluded at the periphery of the tree and shares some genes with *L. ivanovii*. The number of reacting probes per species were as follows: *L. monocytogenes* (263–513, mean 403), *L. innocua* (219–311, mean 262), *L. welshimeri* (162–206, mean 184), *L. seeligeri* (31–119, mean 75), *L. ivanovii* (87–235, mean 146), *L. grayi* (322) and *L. rocourtiae* sp. nov. (189). Typical *Listeria* genes, such as those known to encode 11 LPxTG anchor-containing surface proteins, were detected in strain CIP 109804^T, further supporting that this strain should be classified in the genus *Listeria*. Furthermore, many transcriptional regulators and teichoic acid biosynthesis genes present in other *Listeria* genomes were identified.

Table 2. Jukes–Cantor distance for a 1467 nt region of the *rrs* gene of strain CIP 109804^T and some representatives of the genera *Listeria* and *Brochothrix*

1, *Listeria monocytogenes* NCTC 10357^T (accession no. X56153.1); 2, *Listeria innocua* NCTC 11288^T (X56152.1); 3, *Listeria welshimeri* NCTC 11857^T (X56149.1); 4, *Listeria seeligeri* NCTC 11856^T (X56148.1); 5, *Listeria ivanovii* subsp. *ivanovii* NCTC 11846^T (X56151.1); 6, *Listeria grayi* CIP 68.18^T (X56150.1); 7, *Brochothrix thermosphacta* NCDO 1676^T (X56155.1); 8, *Brochothrix campestris* ATCC 43754^T (X56156.1).

Strain	1	2	3	4	5	6	7	8
Listeria rocourtiae CIP 109804^T	0.042	0.045	0.043	0.042	0.046	0.045	0.088	0.089
Listeria monocytogenes NCTC 10357^T		0.005	0.010	0.008	0.015	0.043	0.083	0.080
Listeria innocua NCTC 11288^T			0.008	0.008	0.013	0.043	0.084	0.080
Listeria welshimeri NCTC 11857^T				0.006	0.010	0.037	0.086	0.081
Listeria seeligeri NCTC 11856^T					0.008	0.037	0.078	0.074
Listeria ivanovii subsp. *ivanovii* NCTC 11846^T						0.037	0.083	0.079
Listeria grayi CIP 68.18^T							0.085	0.082

Strain CIP 109804^T did not form plaques in HT-29 cell culture and was unable to colonize the spleens of Swiss mice 3 days after subcutaneous inoculation. Strain CIP 109804^T should be classified within the avirulent group of species of the genus *Listeria*, exhibiting no virulence according to the Roche *et al.* (2001) classification.

Strain CIP 109804^T was naturally sensitive or showed intermediate sensitivity to carbapenems, rifampicin, macrolides, lincosamides, linezolid, glycopeptides, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, fusidic acid and chloramphenicol. However, strain CIP 109804^T was resistant to cephalosporins (>32 mg l^{-1}), sulfamethoxazole (0.023 mg l^{-1}) and nalidixic acid (>256 mg l^{-1}), similar to other species of the genus *Listeria* (Troxler *et al.*, 2000), and resistant to tetracyclines (1.5 mg l^{-1}), trimethoprim (0.032 mg l^{-1}), fosfomycin (96 mg l^{-1}), kanamycin (>4 mg l^{-1}) and streptomycin (8 mg l^{-1}). *In vitro* resistance to β-lactams was unexpected, with minimal inhibitory concentrations to penicillin, ampicillin and amoxycillin >256 mg l^{-1}.

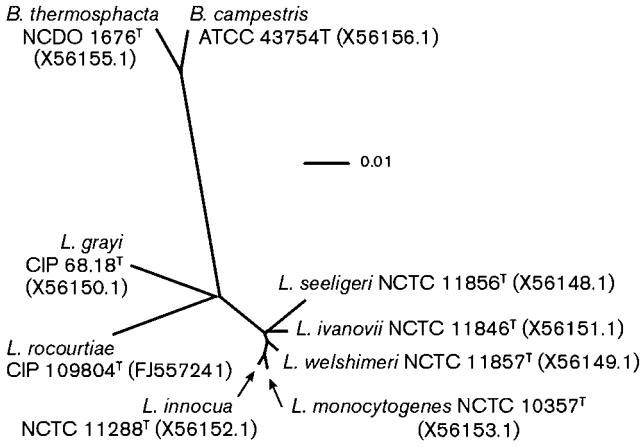

Fig. 1. Unrooted tree showing the phylogenetic relationship of strain CIP 109804^T and species of the genera *Listeria* and *Brochothrix* (the closest genus), based on a distance matrix of *rrs* gene sequences (1467 nt). The tree is based on an analysis of a continuous stretch of approximately 1467 nt. Bar, 1 substitution per 100 nt.

Thus, on the basis of the molecular findings described above as well as the phenotypic distinctiveness of strain CIP 109804^T, we propose that this strain should be classified as a member of a novel species, *Listeria rocourtiae* sp. nov.

Description of *Listeria rocourtiae* sp. nov.

Listeria rocourtiae (ro.cour′ti.ae. N.L. fem. gen. n. *rocourtiae* named in honour of Jocelyne Rocourt, French bacteriologist, whose work had a major impact on the taxonomy of the genus *Listeria*).

Cells are regular, short rods, 0.4–0.5 μm in diameter and 1–2 μm in length with rounded ends. Gram-stain-positive. Not acid-fast. Capsule not formed. Does not form spores. Motile when cultured at 4 to 30 °C. Aerobic and facultatively anaerobic. Colonies appear bluish grey by normal illumination and a characteristic blue-green sheen is produced by obliquely transmitted light. After 48 h of incubation on trypto-casein-soy agar at 30 °C, colonies are 0.5–1.0 mm in diameter, round, translucent and low convex with a finely textured surface and entire margin. Acid is produced from glycerol, erythritol, ribose, D-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, soluble starch, trehalose, D-raffinose, glycogen and β-gentiobiose. Acid is not produced from D-arabinose, L-arabinose, L-xylose, methyl β-xyloside, methyl α-D-mannoside, sucrose, inulin, melezitose, xylitol, L-fucose, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The type strain is non-haemolytic, CAMP-test negative and avirulent.

The type strain is strain CIP 109804^T (=DSM 22097^T =Allerberger 700284/02^T) and was isolated from pre-cut

lettuce in Salzburg, Austria, in 2002. The lettuce was grown and processed in Siezenheim, a village near Salzburg.

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