Yersinia nurmii sp. nov.

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This study was set up to identify three Gram-negative, rod-shaped strains originating from broiler meat packaged under a modified atmosphere. A polyphasic taxonomic approach, including multilocus sequence analysis (MLSA) of five genes (16S rRNA, *glnA*, *gyrB*, *recA* and *HSP60*), DNA–DNA reassociation between the closest phylogenetic neighbours and determination of relevant phenotypic properties, was applied. Phylogenetic analysis of the 16S rRNA gene sequences grouped these strains together and within the genus *Yersinia*. MLSA of the 16S rRNA gene and four housekeeping genes showed that the strains formed a monophyletic group separate from other *Yersinia* species in all phylogenetic trees constructed. The strains had a phenotypic profile different from those of other representatives of the genus *Yersinia*, but most similar to that of *Yersinia ruckeri*. Typical virulence markers for pathogenic *Yersinia* were not detected. Based on phylogenetic, phenotypic and DNA–DNA reassociation data, a novel species, *Yersinia nurmii* sp. nov., is proposed for the isolated strains. The type strain is APN3a-c^T (=DSM 22296^T =LMG 25213^T).

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and, at the time of writing, includes the species *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. ruckeri*, *Y. aldovae*, *Y. aleksiciae*, *Y. massiliensis* and *Y. similis*. Members of the genus *Yersinia* are Gram-negative, catalase-positive and oxidase-negative, facultatively anaerobic rods (Bottone *et al.*, 2005).

Strains APN5b-c, ABF6a-c and APN3a-c^T were isolated from three packages of broiler meat cuts, packaged under a modified atmosphere. The strains were recovered from VRBG (violet red bile agar with glucose) medium used for the enumeration of enterobacteria in the products. An attempt was made to identify these strains using a 16S and 23S rRNA gene *Hind*III and *Eco*RI RFLP (ribotype) library for members of the family *Enterobacteriaceae*, but the patterns did not cluster together with those of any known species.

To study the taxonomic position of these strains, the 16S rRNA gene and four housekeeping genes were sequenced and analysed. The partial housekeeping genes *glnA*, *gyrB*, *recA* and *HSP60* – which have been used previously in a multilocus sequence analysis (MLSA) study of *Yersinia* and

have sequence data available for many type strains of *Yersinia* species – were selected.

In this study, the 16S rRNA gene and housekeeping genes of strains APN5b-c, ABF6a-c and APN3a-c^T were sequenced in addition to those genes of strains *Y. aldovae* CIP 103162^T, *Y. aleksiciae* DSM 14987^T, *Y. enterocolitica* subsp. *palearctica* DSM 13030^T, *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610^T, *Y. intermedia* DSM 18517^T, *Y. massiliensis* CCUG 53443^T, *Y. pseudotuberculosis* CCUG 5855^T, *Y. rohdei* DSM 18270^T, *Y. ruckeri* ATCC 29473^T and *Y. similis* DSM 18211^T, which did not have public sequence data available (GenBank accession numbers FJ717338–FJ717391).

All strains used in this study were cultured aerobically for 24 h on blood agar at 30 °C. DNA from the strains was extracted by the guanidium thiocyanate method described by Pitcher *et al.* (1989) with the addition of 0.6 mg proteinase K ml^{−1} to the cell suspension buffer. The 16S and 23S gene RFLP (ribotyping) procedures were done according to Niskanen *et al.* (2009). Ribopatterns were scanned and analysed by using the BioNumerics 5.10 software (Applied Maths). Dendrograms were constructed by using UPGMA and the Dice coefficient correlation. *Yersinia* strains used for ribotyping are presented in Supplementary Fig. S1 (available in IJSEM Online).

The *glnA*, *gyrB*, *recA* and *HSP60* genes were amplified using primers described by Kotetishvili *et al.* (2005). The 16S rRNA gene was amplified with primers F19–38 (5′-CTGGCT-CAGGAYGAACGCTG-3′) and R1541–1522 (5′-AAGG-

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *glnA*, *gyrB*, *HSP60* and *recA* gene sequences determined in this study are FJ717338–FJ717391.

Five supplementary figures and a supplementary table are available with the online version of this paper.

AGGTGATCCAGCCGCA-3'). The PCR cycle for amplification of the *HSP60*, *glnA* and *recA* genes were described by Kotetishvili *et al.* (2005). For *gyrB*, the annealing temperature was increased to 62 °C. The PCR cycles for the 16S rRNA gene comprised denaturation at 98 °C for 2 min, followed by 30 amplification cycles (93 °C for 1 min, 54 °C for 2 min and 72 °C for 2.5 min) and final elongation at 72 °C for 8 min. Each 50 µl PCR mixture consisted of 200 mM each dNTP, 1 IU DyNAZyme II DNA polymerase (Thermo Fisher Scientific Inc.) and 0.5 µM each primer. PCR products were purified by using the MultiScreen PCR 96 system (Millipore). Bidirectional sequencing of the PCR products was done using an Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3130XL Genetic Analyzer. The consensus sequences of strains APN5b-c, ABF6a-c and APN3a-c^T were aligned with 16S rRNA and housekeeping gene sequences of type and reference strains of other *Yersinia* species retrieved from GenBank by using BioNumerics 5.10 software (Applied Maths). Only those *Yersinia* strains that had complete sequence data available for all five genes were used in the comparisons. Maximum-parsimony and neighbour-joining trees with the Jukes-Cantor correction and bootstrap analysis (1000 replicates) were constructed using BioNumerics 5.10 software (Applied Maths). *Serratia proteamaculans* was used as outgroup in the trees. The phylogenetic trees were constructed using the 16S rRNA gene (1425 bp) and housekeeping genes *glnA* (494 bp), *gyrB* (471 bp), *recA* (483 bp) and *HSP60* (486 bp). Concatenated trees were constructed by combining *glnA*, *gyrB*, *recA* and *HSP60* gene sequences. The GenBank accession numbers of the *glnA*, *gyrB*, *HSP60* and *recA* sequences used are presented in Supplementary Table S1 (available in IJSEM Online). The 16S rRNA gene sequences of strains APN5b-c, ABF6a-c and APN3a-c^T were also compared with those of type species of genera of the family Enterobacteriaceae.

DNA-DNA reassociation was performed at the DSMZ, Braunschweig, Germany. Briefly, DNA was isolated by using a French pressure cell (Thermo Scientific) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The DNA G+C content of strains APN5b-c, ABF6a-c and APN3a-c^T was determined as described by Xu *et al.* (2000). The melting-point curves were determined with a LightCycler (Roche Diagnostics) instrument using SYBR Green I dye (Roche Diagnostics). 1 × SSC was used to obtain sharp melting curves. *Y. aldovae* CIP 103162^T was used as the reference organism and *Y. mollaretii* DSM 18520^T was used as the control.

The possible pathogenicity of strains APN5b-c, ABF6a-c and APN3a-c^T was studied by determining the presence of

virulence genes *virF*, *inv*, *ail* and *yadA* (Fredriksson-Ahomaa *et al.*, 1999), which are used for the identification of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*. This was of interest because the strains were isolated from food. The *virF*, *inv* and *ail* genes were detected by PCR as described by Niskanen *et al.* (2009). Each primer for *virF*, *inv* or *ail* was used in a separate PCR. The *yadA* gene was detected by PCR as described previously (Fredriksson-Ahomaa *et al.*, 1999). *yadA*-, *inv*- and *virF*-positive *Y. pseudotuberculosis* and *yadA*-, *ail*- and *virF*-positive *Y. enterocolitica* were used as positive controls. Also, virulence-associated pyrazinamidase activity was tested according to Kandolo & Wauters (1985) at 28 °C for 48 h, and Congo red dye uptake and calcium-dependent growth at 37 °C were tested according to Riley & Toma (1989).

Morphological properties were determined by Gram staining and transmission electron microscopy. Oxidase activity was tested with oxidase strips and catalase activity with 3 % hydrogen peroxide. Biochemical characterization of strains APN5b-c, ABF6a-c, APN3a-c^T, *Y. similis* DSM 18211^T, *Y. mollaretii* DSM 18520^T, *Y. bercovieri* DSM 18528^T, *Y. kristensenii* CIP 80.30^T, *Y. rohdei* DSM 18270^T, *Y. ruckeri* ATCC 29473^T, *Y. aleksiciae* DSM 14987^T, *Y. intermedia* DSM 18517^T, *Y. aldovae* CIP 103162^T, *Y. enterocolitica* subsp. *paleartica* DSM 13030^T, *Y. frederiksenii* DSM 18490^T, *Y. enterocolitica* subsp. *enterocolitica* DSM 4780^T, *Y. massiliensis* CCUG 53443^T and *Y. pseudotuberculosis* DSM 8992^T was done using the API 20 E and API 50 CH strips according to instructions of the manufacturer (bioMérieux). The incubation temperature used was 28 °C and results were read after 24, 48 and 72 h. Each biochemical test was done at least twice. Motility testing was performed at 28 and 37 °C with Motility Test and Maintenance and Motility Test media (Atlas & Snyder, 2006).

Strains APN5b-c, ABF6a-c and APN3a-c^T had identical *glnA* (492 bp), *gyrB* (483 bp), *recA* (499 bp), *HSP60* (510 bp) and 16S rRNA (1452 bp) sequences. Strains shared 97.8–98.7 % 16 rRNA gene sequence similarity with other *Yersinia* species (Table 1). It has been stated that, within the genus *Yersinia*, the 16S rRNA gene sequence similarity of the type strains should be >97.4 %. Neighbour-joining trees of members of the genus *Yersinia*, based on the 16S rRNA and concatenated *glnA*, *gyrB*, *recA* and *HSP60* gene sequences, are presented in Figs 1 and 2, respectively. Trees constructed by the maximum-parsimony method are shown in Supplementary Figs S2 and S3 (available in IJSEM Online). Strains APN5b-c, ABF6a-c and APN3a-c^T formed a monophyletic group in all phylogenetic trees constructed. The group was separate from other *Yersinia* species in all individual housekeeping gene trees; in the tree derived from the concatenated *gyrB*, *recA* and *HSP60* gene sequences, *Y. ruckeri* is the closest phylogenetic neighbour of *Y. nurmii*, with a 100 % bootstrap value. In the trees formed individually from *glnA*, *recA* and *HSP60* gene sequences, *Y. ruckeri* is positioned as the closest phylogenetic neighbour, whereas

Table 1. 16S rRNA gene sequence similarities of strain APN3a-c^T with representatives of the genus *Yersinia*

16S rRNA gene sequence similarity was measured over 1432 bp.

Species	Similarity (%)
<i>Y. frederiksenii</i> CCUG 11293 ^T	98.6
<i>Y. massiliensis</i> CCUG 53443 ^T	98.3
<i>Y. aleksiciae</i> DSM 14987 ^T	97.8
<i>Y. bercovieri</i> ATCC 43970 ^T	98.1
<i>Y. mollaretii</i> ATCC 43969 ^T	98.7
<i>Y. enterocolitica</i> subsp. <i>paleartica</i> DSM 13030 ^T	98.0
<i>Y. enterocolitica</i> subsp. <i>enterocolitica</i> ATCC 9610 ^T	98.1
<i>Y. kristensenii</i> CCUG 33638 ^T	98.3
<i>Y. rohdei</i> ATCC 43380 ^T	98.5
<i>Y. intermedia</i> DSM 18517 ^T	98.3
<i>Y. aldovae</i> CCUG 18770 ^T	98.4
<i>Y. pseudotuberculosis</i> CCUG 5855 ^T	97.8
<i>Y. similis</i> Y228 ^T	97.9
<i>Y. ruckeri</i> ATCC 29473 ^T	98.0
<i>Y. pestis</i> KIM	97.9

in the *gyrB*-derived tree, *Y. nurmii* was fully separated from the other *Yersinia* species with a bootstrap value of 100 % (not shown). In the numerical analysis of both *Hind*III and *Eco*RI ribopatterns, strains APN5b-c, ABF6a-c and APN3a-c^T clustered together, differing from ribopatterns of type strains of recognized *Yersinia* species (Supplementary Fig. S1). In phylogenetic analysis of 16S rRNA gene sequences of type species of genera of the family *Enterobacteriaceae*,

strains APN5b-c, ABF6a-c and APN3a-c^T grouped with *Yersinia* species (Supplementary Fig. S4, available in IJSEM Online).

DNA–DNA reassociation was carried out, pairing the closest phylogenetic neighbour (according to *Hsp60*, *glnA* and *recA* gene analysis) *Y. ruckeri* ATCC 29473^T and two phylogenetically distant species, *Y. rohdei* DSM 18270^T and *Y. frederiksenii* DSM 18490^T, with strains APN3a-c^T and ABF6a-c, which were chosen to represent the novel species. The values varied from 17.5 to 33.4 %, indicating that strains APN3a-c^T and ABF6a-c do not belong to the any of the species studied, including the closest phylogenetic neighbour, *Y. ruckeri*. The DNA–DNA similarity level between strains APN3a-c^T and ABF6a-c was 81.7 %. This shows clearly that these strains represent the same species. The DNA G + C content of strains APN5b-c, ABF6a-c and APN3a-c^T was 48.7 ± 0.2 mol%.

Strains APN5b-c, ABF6a-c and APN3a-c^T were negative for virulence marker-encoding genes *virF*, *inv*, *ail* and *yadA*, did not have Congo red dye uptake or calcium-dependent growth, and were pyrazinamidase-positive.

Strains APN5b-c, ABF6a-c and APN3a-c^T were Gram-negative rods. The length of the cells varied between 0.8 and 1.9 µm after growth on blood agar for 24 h at 30 °C (Supplementary Fig. S5, available in IJSEM Online). The cells had multiple flagella. Colonies were convex and circular and the diameter of the colonies on blood agar after 24 h at 28 °C was 1.0–1.5 mm. The strains grew on blood, tryptic soy, MacConkey and nutrient agar at 25–37 °C. Cells were motile at 28 °C and under microscopical

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of *Yersinia nurmii* sp. nov. and type and reference strains of the genus *Yersinia*. Bootstrap values (%) for 1000 replicates are given at branch points. *S. proteamaculans* was used as outgroup. GenBank accession numbers are shown in parentheses. Bar, 2 % difference in nucleotide sequence.

Fig. 2. Neighbour-joining tree based on concatenated *glnA*, *gyrB*, *HSP60* and *recA* gene sequences of *Yersinia nurmii* sp. nov. and type and reference strains of the genus *Yersinia*. Bootstrap values (%) for 1000 replicates are given at branch points. *S. proteamaculans* was used as outgroup. Bar, 5% difference in nucleotide sequence.

observation. The strains were oxidase-negative and catalase-positive. The biochemical characteristics of the strains are presented in Table 2 and are compared with those of other *Yersinia* species. Strains ABF6a-c, APN5b-c and APN3a-c^T did not hydrolyse urea and did not ferment L-arabinose. The API 20E profile obtained for ABF6a-c, APN5b-c and APN3a-c^T was 3 3 0 7 1 2 1 (99.7% identity with *Cedecea davisae*). The phenotypic profile of strains APN5b-c, ABF6a-c and APN3a-c^T was most similar to that of *Y. ruckeri*.

Based on the results of the polyphasic taxonomic study described above, strains ABF6a-c, APN5b-c and APN3a-c^T represent a novel species of the genus *Yersinia*, for which the name *Yersinia nurmii* sp. nov. is proposed.

Description of *Yersinia nurmii* sp. nov.

Yersinia nurmii (nur'mi.i. N.L. gen. masc. n. *nurmii* of Nurmi, in honour of the late Professor Esko Nurmi, a distinguished researcher in the field of food hygiene, who, with his colleagues, introduced, among other things, the concept of competitive exclusion).

Cells are Gram-negative, motile rods with multiple flagella. Their length varies between 0.8 and 1.9 µm after 24 h incubation on blood agar at 28 °C. Oxidase-negative, catalase-positive and negative for urea hydrolysis. Positive for utilization of glycerol and ONPG, ornithine decarboxylase, Voges-Proskauer reaction and lipase. Hydrolyses gelatin. Ferments trehalose and sucrose. Ferments lactose and cellobiose weakly. Negative for utilization of D-xylose, adonitol, sorbose, aesculin, salicin and raffinose, fermentation of inositol, sorbitol, melibiose, rhamnose and L-arabinose, lysine decarboxylase and indole production. Grows on blood, tryptic soy, MacConkey and nutrient agar at 25–37 °C and produces 1.0–1.5 mm colonies on blood agar at 28 °C in 24 h. Colonies are circular and convex with elevated centres. The DNA G + C content is 48.7 mol%. 16S rRNA gene sequence similarity with the phylogenetically most closely related species, *Y. ruckeri*, is 98.0%.

The type strain is APN3a-c^T (=DSM 22296^T =LMG 25213^T), isolated from broiler meat packaged under a modified atmosphere. Reference strains are ABF6a-c and APN5b-c.

Table 2. Biochemical characteristics of *Yersinia nurmii* sp. nov. compared with those of type strains of other *Yersinia* species

Type strains: 1, *Y. nurmii* sp. nov.; 2, *Y. ruckeri*; 3, *Y. mollaretii*; 4, *Y. aldovae*; 5, *Y. bercovieri*; 6, *Y. intermedia*; 7, *Y. rohdei*; 8, *Y. enterocolitica* subsp. *enterocolitica*; 9, *Y. enterocolitica* subsp. *polarctica*; 10, *Y. aleksiciae*; 11, *Y. frederiksenii*; 12, *Y. kristensenii*; 13, *Y. pestis* (Sprague *et al.*, 2008); 14, *Y. pseudotuberculosis*; 15, *Y. similis*; 16, *Y. massiliensis*. All data are from this study unless otherwise indicated. +, Positive; –, negative; (+), weakly positive after 48 h incubation. All strains were positive for utilization of trehalose and glucose and negative for utilization of adonitol.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Utilization of:																
D-Xylose	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Sorbose	–	–	+	–	–	+	–	+	+	–	+	+	–	–	–	+
Aesculin	–	–	(+)	–	–	+	–	–	–	–	+	–	+	+	+	+
Salicin	–	–	(+)	–	(+)	+	(+)	–	–	(+)	+	+	–	+	(+)	+
Cellobiose	(+)	–	+	–	+	+	+	+	+	+	+	+	–	–	–	+
Lactose	(+)	–	(+)	–	–	–	(+)	–	–	–	–	+	–	–	–	–
Raffinose	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–
Glycerol	+	+	+	+	(+)	+	+	+	+	+	+	+	–	(+)	+	+
Citrate	+	–	+	+	–	+	+	–	–	–	+	–	–	–	+	+
Inositol	–	–	(+)	+	–	+	–	(+)	+	(+)	+	(+)	–	–	–	+
Sorbitol	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	+
Melibiose	–	–	–	–	–	+	+	–	–	–	–	–	–	+	–	–
Rhamnose	–	–	–	+	–	+	–	–	–	–	+	–	–	(+)	+	–
ONPG	+	+	+	–	+	+	+	+	+	+	+	+	–	+	+	+
Sucrose	+	–	+	–	+	+	+	+	+	–	+	–	–	–	–	(+)
L-Arabinose	–	–	+	+	+	+	+	+	+	+	+	+	+	–	+	+
Urea hydrolysis	–	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Lysine decarboxylase	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+
Voges-Proskauer reaction	+	–	–	+	–	+	–	–	–	–	+	–	–	–	–	–
Indole production	–	–	–	–	–	+	–	+	–	–	+	+	–	–	–	+

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