Yersinia aleksiciae sp. nov.

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Yersinia kristensenii consists of phenotypically heterogeneous strains. This is reflected by the existence of strains with various multilocus enzyme electrophoresis and 16S rRNA gene sequence types. Strains originally phenotyped as members of *Y. kristensenii* were studied using 16S rRNA gene sequencing, DNA–DNA hybridization, determination of the DNA base composition and various phenotypic tests. The results were compared to those of *Yersinia* type strains. Based on levels of DNA–DNA relatedness, a specific 16S rRNA gene sequence type and the presence of lysine decarboxylase activity, a novel species, *Yersinia aleksiciae* sp. nov., is proposed. The type strain is Y159^T (= WA758^T = DSM 14987^T = LMG 22254^T).

Yersinia kristensenii was described to accommodate strains that are trehalose-positive, ornithine decarboxylase-positive and rhamnose-negative. These are mostly adapted to the soil ecosystem. The G+C content of the DNA has been determined to be 48.5 ± 0.5 mol% and two hybridization clusters have been found (Bercovier *et al.*, 1980). Of 115 *Y. kristensenii* strains investigated, 7% belonged to serogroup O:16 (Bercovier *et al.*, 1980). Aleksic & Bockemühl (1984) discovered an O:16 antigen specific for *Y. kristensenii*. In successive studies it was noted that *Y. kristensenii* strains with various multilocus enzyme electrophoresis (MLEE) clusters exist (Goullet & Picard, 1988; Caugant *et al.*, 1989; Dolina & Peduzzi, 1993). Neubauer *et al.* (2000a) were able to demonstrate the existence of at least two distinct 16S rRNA gene sequence types for *Yersinia enterocolitica* and *Y. kristensenii*. The possibility that *Y. kristensenii* might therefore comprise at least two distinct species was suggested (Sulakvelidze, 2000; Neubauer *et al.*, 2000a).

The aim of this present study was to investigate whether isolates originally phenotyped as members of *Y. kristensenii*

displaying different 16S rRNA gene sequence types may be representative of a novel species.

Five *Yersinia* isolates originally phenotyped as *Y. kristensenii* serotype O:16 were supplied by Dr G. Wauters (Université Catholique de Louvain, Brussels, Belgium; strain names prefixed by WA). Strains Y159^T (= WA758^T; GenBank/EMBL accession no. AJ627597) and Y390 (= WA948; AJ627599) were isolated from human faeces in Finland (by M. Skurnik in 1981). Strain Y221 (= WA120; GenBank/EMBL accession no. AJ627594) was isolated from pork products in Toronto, Canada (by D. A. Schiemann in 1979). Strain Y389 (= WA31A/89; GenBank/EMBL accession no. AJ627600) was isolated from rats and moles in Japan (by H. Fukushima in 1989). Strain Y388 (= WA8/90; GenBank/EMBL accession no. AJ627601) was isolated from dairy products in Australia (by D. Hughes in 1990). Strains Y156 (O:12,25) (GenBank/EMBL accession no. AJ627595) and Y216 (O:nt) phenotyped as *Y. kristensenii* were of unknown origin. Additionally, a panel of *Yersinia* strains phenotyped as *Y. kristensenii* was investigated. These strains were isolated from reindeer faeces in Finland during the years 2000–2002: Y479 (Lappi, Finland), Y489 and Y490 (Tromsö, Finland) and Y473 (Näkkälä, Finland); and strain Y475 was isolated from soil (by N. Kemper, Kiel, Germany). The serogroup of these strains is unknown. Two *Y. kristensenii* strains (Y157 O:11,23 and Y160 O:28; GenBank/EMBL accession no. AJ627598) were of the 16S rRNA gene sequence type A [5'-AAGGCARTCGTGTTAATAGCACGRTTGATT-3'; representing positions 451–480 of the type strain ATCC 33638^T (= Y110^T) (Neubauer *et al.*, 2000a)]. The following type strains of *Yersinia* species or subspecies were also investigated: *Yersinia enterocolitica* subsp. *paleartica* DSM 13030^T, *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610^T, *Yersinia ruckeri* ATCC 29473^T, *Yersinia pseudotuberculosis* ATCC 29833^T, *Yersinia frederiksenii* ATCC 33641^T, *Yersinia bercovieri* ATCC 43970^T, *Yersinia rohdei* ATCC 43380^T,

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Abbreviations: LDC, lysine decarboxylase; MLEE, multilocus enzyme electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Y221, Y156, Y159^T, Y160, Y390, Y389 and Y388 are AJ627594, AJ627595 and AJ627597–AJ627601, respectively.

Phenotypic characteristics and DNA–DNA hybridization results for various *Yersinia* isolates are available as supplementary tables in IJSEM Online.

Yersinia mollaretii ATCC 43969^T, *Yersinia aldovae* ATCC 35236^T (=Y112^T) and *Yersinia intermedia* ATCC 29909^T (=Y118^T). The last of these is lysine decarboxylase (LDC)-negative. For comparative studies, *Y. aldovae* strain Y186 and *Y. intermedia* strains Y20 and Y154 (both the latter strains are LDC-positive) were used. Strains were maintained on standard nutrient agar I (Oxoid) at 4 °C. Subcultivation was on blood agar overnight at 28 °C. Broth culture was carried out overnight in LB medium (Difco) at 28 °C and 150 r.p.m. in controlled environment shakers (New Brunswick). Cells were harvested by centrifugation (1200 g) and stored in 70 % 2-propanol at 4 °C until used for DNA–DNA hybridization experiments.

Identification of *Yersinia* isolates was performed as described by Neubauer *et al.* (2000b) except that incubation was for 72 h. An LDC cut-off value of an absorbance of 0.1 was used. API 20E (bioMérieux) tests were performed as described by the manufacturer. PCR assays for genes encoding pathogenicity factors (*yadA*, V-antigen) were performed as described by Neubauer *et al.* (2000d).

Spectroscopic DNA–DNA hybridization experiments were performed at 62 °C with 10 % DMSO. DNA was isolated by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was performed by spectroscopy in 10 % DMSO at 62 °C using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter (De Ley *et al.*, 1970; Huß *et al.*, 1983; Escara & Hutton, 1980). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). A deviation of 5 % in DNA–DNA relatedness values can be tolerated when comparing individual experiments (see Supplementary Tables B and C in IJSEM Online). The DNA G+C content was determined in three independent measurements. Cells were disrupted using a French pressure cell and the DNA was purified as described by Mesbah *et al.* (1989) and Tamaoka & Komagata (1984). An HPLC system (Shimadzu Corp.) and NUCLEOSIL 100-5C18 in combination with a pre-column NUCLEOSIL 100-5C18 (both from Macherey and Nagel) were used. Both analyses were carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Determination of the 16S rRNA gene sequence type of *Yersinia* isolates was performed as described by Neubauer *et al.* (2000a) using the sequencing primer pair SP1 (5'-GAATATTGCACAATGGGCGCA-3') and SP3 (5'-AACAAACCGCCTGCGTGCGC-3'). The amplicons produced from two independent cultures were sequenced in both directions. Partial sequencing of the 16S rRNA gene of *Yersinia* strains was performed using primers 16-1 (5'-AGAGTTTGATCCTGGCTCA-3'), 16-2 (5'-CACCGGCTAACTCCGTGCCA-3'), 16-3 (5'-GAGACAGGTGCTGCATGGCT-3'), PT2 (5'-GGCAACAAAGGATAA-3') (Neubauer *et al.*, 2000e), 16-4 (5'-TACCTTGTTACGACTTCTC-3') and 16-5 (5'-TTCCGATTAACGCTTGCAAC-3'). PCR products were sequenced in both directions.

Interpretation of the results was aided by use of Lasergene software (DNASTAR) based on the CLUSTAL method.

Cells of strains isolated from reindeer, including Y159^T, Y216, Y221, Y388, Y389 and Y390, were small, Gram-negative, motile, coccoid rods. All isolates were catalase- and urease-positive and oxidase-negative. They were trehalose-positive, ornithine decarboxylase-positive and rhamnose-negative. The principal API 20E profile obtained was 1114703 (data not shown). Based on these findings, their classification within *Y. kristensenii* was justified (Bercovier *et al.*, 1980). However, these strains differed from other *Y. kristensenii* strains identified by a semi-automated system for identification of species within the genus *Yersinia* (Neubauer *et al.*, 2000b) in that all were LDC-positive. This feature is usually not used for the differentiation of *Yersinia* species (Supplementary Table A). In a recent investigation we found that LDC activity was only present in strains Y159^T, Y216 and Y221 of *Y. kristensenii* and in 42 % of the isolates of *Y. intermedia*, including Y154 and Y20, but not in the type strain (Neubauer *et al.*, 2000b). These LDC-positive *Y. intermedia* strains, in contrast to the LDC-positive strains Y159^T, Y216, Y221, Y388, Y389, Y390 and the LDC-positive reindeer strains, were aesculin-, salicin-, citrate- and sucrose-positive. A highly fermentative phenotype clearly distinguishes both groups of isolates. In accordance with these findings, LDC-positive *Y. intermedia* strains showed only 37.5–48.7 % DNA–DNA relatedness to LDC-positive *Y. kristensenii* isolates (Supplementary Table B). Furthermore, these strains showed only a low level of DNA–DNA relatedness to the *Y. kristensenii* type strain. Pathogenicity in *Yersinia* is linked to the presence of the 64 kDa *Yersinia* virulence plasmid encoding a type III secretion system and various effector proteins (Cornelis, 2002). Neither the plasmid-borne gene of the *Yersinia* adhesin nor the V-antigen, which plays a role as a regulator in the secretion of effector proteins, generated PCR products in the *Y. kristensenii*-like LDC-positive strains investigated here. Thus, the plasmid-borne markers for *Yersinia* pathogenicity are missing. Nonetheless, these strains should be grouped into risk class 2 until apathogenicity has been confirmed.

A further apparent difference was that strains Y159^T, Y216, Y221, Y388, Y389, Y390 and all reindeer strains belonged to 16S rRNA gene sequence type B, representing positions 451–480 of the *Escherichia coli* 16S rRNA gene sequence (IUB nomenclature, 5'-AAGGGTTCAGTGTTAATAGCACTGAGCATT-3') (Neubauer *et al.*, 2000a). For the description of the 16S rRNA gene sequence type, only 30 nt have been taken into consideration (Neubauer *et al.*, 2000a, c). In *Y. enterocolitica*, specific 16S rRNA gene sequence types have been used to define the subspecies *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *paleartica*. The American phenotype biovar 1B strains are represented by a unique 16S rRNA gene sequence type which is clearly distinct from that of the European phenotype of biovars 1A, 2, 3, 4 and 5 (Ibrahim *et al.*, 1993; Neubauer *et al.*, 2000c). It therefore seemed clear that the

16S rRNA gene sequence type could be used for the definition of a novel species. Three strains of 16S rRNA gene sequence type A (*Y. kristensenii*: Y110^T, Y157, Y160) and two strains of 16S rRNA gene sequence type B (Y159^T, Y221) were subjected to DNA–DNA hybridization analysis (Supplementary Table C). *Y. kristensenii* strains Y157 and Y160 clustered into the group represented by the type strain of *Y. kristensenii* (Y110^T) with an intragroup relatedness of 86.6–94.7 %. This result demonstrated that both isolates had been correctly phenotyped. These strains are members of the genomic species *Y. kristensenii* (Johnson, 1984; Wayne *et al.*, 1987; Stackebrandt & Liesack, 1993). A further group was formed by isolates Y159^T and Y221, with an intragroup similarity of 89.0 % (Supplementary Table C). Strains Y388 and Y216 have levels of relatedness of 84.3 and 83.9 % to Y159^T, respectively (Supplementary Table B). Their intergroup relatedness ranged from 28.6 to 47 %, demonstrating that neither strain belongs to *Y. kristensenii* (Supplementary Table C). Strains Y159^T and Y221 had levels of relatedness of 22.5–61.7 % to those *Yersinia* type strains that are most closely related to *Y. mollaharii* (Supplementary Table D). A high similarity value between the 16S rRNA gene sequence type B was demonstrated for the 16S rRNA gene sequence type found in *Y. aldovae* (Neubauer *et al.*, 2000a). However, DNA–DNA hybridization studies of strains Y159^T and Y221 and two *Y. aldovae* strains (Y112^T, Y186) revealed an intergroup relatedness of only 22.5–46.9 % (Supplementary Table C). Consequently, strains Y159^T and Y221 comprise a genomic species (Johnson, 1984; Wayne *et al.*, 1987; Stackebrandt & Liesack, 1993; Stackebrandt *et al.*, 2002) characterized by their LDC-positive phenotype and a specific 16S rRNA gene sequence type. Hereafter, we refer to these strains as strains of *Yersinia aleksiciae* sp. nov. We can conclude that the 16S rRNA gene sequence type A is a species-specific marker for *Y. kristensenii* and 16S rRNA gene sequence type B for *Y. aleksiciae*.

A 16S rRNA gene sequence similarity of 97 % corresponds to a 70 % level of DNA–DNA relatedness. This value is considered to be the standard for species definition (Stackebrandt & Liesack, 1993; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). In comparing the 16S rRNA gene sequences of the type strains of the species of the genus *Yersinia*, levels of DNA–DNA relatedness never fall below 97.4 %. To determine whether LDC-positive *Y. aleksiciae* strains of the 16S rRNA gene sequence type might fulfil this criterion, nearly complete 16S rRNA gene sequences (1419 nt) of strains Y110^T, Y156, Y157, Y160, Y159^T, Y216, Y388, Y389, Y390 and Y221 were determined. LDC-positive strains showed a 16S rRNA gene sequence similarity of 99.9 %. Similarity to *Y. kristensenii* strains Y110^T, Y156, Y157 and Y160 and to the type strains of other *Yersinia* species, however, was 98.3–98.8 %, which would not allow the definition of a novel species (data not shown). However, in cases where 16S rRNA gene sequence similarity is not discriminatory, DNA–DNA hybridization can be used to delineate a novel species (Stackebrandt *et al.*, 2002).

The DNA G+C content has thus to be determined (Stackebrandt *et al.*, 2002). The mean G+C content of *Y. kristensenii* type strain Y110^T was 50.0 mol% (two readings). This was 1.5 mol% above the previously published value (Bercovier *et al.*, 1980). Strains Y156, Y157 and Y160 of this species had values of 50.1, 49.2 and 50.5 mol%, respectively. The G+C content of *Y. aleksiciae* Y159^T was 51.1 and 45.2 mol% (two readings), giving a mean value of 48.1 mol%. Other strains of *Y. aleksiciae*, Y216, Y221 and Y388, had G+C values of 45.7, 50.9 and 46.1 mol%, respectively. These estimates are within the accepted limits for the genus *Yersinia* of 46–50 mol% (Bercovier & Mollaret, 1984). The differences of up to 5 mol% observed within individual series of measurements were the result of the technique and equipment used.

The taxonomic position of strain Y156 remains unclear because this isolate is a member of 16S rRNA gene sequence type B but is LDC-negative, thus displaying the classical metabolic activity of *Y. kristensenii*. Strain Y156 was clearly separated from the 16S rRNA gene sequence similarity cluster formed by strains Y110^T, Y157 and Y160 within the species *Y. kristensenii*, and with a DNA–DNA relatedness level of 70.8 % to the type strain was at the limit for inclusion in this species. However, strain Y156 had a high 16S rRNA gene sequence similarity (99.2 %) to the type strain of *Y. kristensenii*. Its 16S rRNA gene sequence also showed higher similarity to that of the type strains of *Y. aldovae* and *Y. mollaharii*. Genomic relatedness of strains identified as *Y. kristensenii* to *Y. mollaharii* has been previously demonstrated in MLEE studies (Gouillet & Picard, 1988; Caugant *et al.*, 1989; Dolina & Peduzzi, 1993). Data from published studies cannot be used to clarify whether these strains might be members of the genomic species *Y. aleksiciae* sp. nov. The role of the O-antigen O:16 as a phenetic marker could not be elucidated here. However, there is strong evidence that it is specific to *Y. aleksiciae*.

The ad hoc committee for the re-evaluation of the species definition in bacteriology of the ICSP encouraged ‘the use of innovative methods for prokaryotic systematics, e.g. 16S rDNA sequencing’ (Stackebrandt *et al.*, 2002). Using 16S rRNA gene sequence type analysis in combination with DNA–DNA hybridization on a collection of *Yersinia* strains, we were able to define the novel species *Y. aleksiciae* within the genus *Yersinia*. Nonetheless, except for the LDC reaction, phenotypic tests are unable to differentiate the type strains of *Y. kristensenii* and *Y. aleksiciae*. However, our DNA–DNA relatedness studies clearly support the differentiation of the two species into *Y. kristensenii sensu stricto* and *Y. aleksiciae*. Single phenetic and genomic markers have previously been used in systematics to characterize a species (Yassin *et al.*, 2002).

The importance of this novel species for ecologists, evolution geneticists and clinical microbiologists must be emphasized. Members of the novel species seem to be well adapted to warm-blooded animals, including humans. Strains have been isolated from the faeces of humans,

rats, moles, reindeer and pigs, and from dairy products. Therefore, it can be concluded that *Y. aleksiciae* is not host specific. *Y. aleksiciae* is widely distributed and has been isolated in Europe, America, Australia and Asia. It remains to be proven whether it is a member of the normal flora in the digestive tract of various mammals. Members of *Y. aleksiciae* have been isolated regularly within the past few years from reindeer in Finland and Norway using 16S rRNA gene sequence type analysis and LDC reactivity as markers (data not shown).

Description of *Yersinia aleksiciae* sp. nov.

Yersinia aleksiciae (al.ek'sic.i.ae. N.L. gen. n. *aleksiciae* in honour of Professor Stojanca Aleksic, Hamburg, Germany, to whom we owe much of our knowledge on the epidemiology and microbiology of *Yersinia*).

Gram-negative, motile, coccoid rods. Cells are catalase- and urease-positive, oxidase-negative and reduce nitrate. Trehalose-, catalase- and urease-positive, oxidase-negative, ornithine decarboxylase-positive, rhamnose-negative but LDC-positive. Aesculin-, salicin-, citrate- and sucrose-negative. Strains belong to the 16S rRNA gene sequence type 5'-AAGGGTTCAGTGTTAATAGCACTGAGCATT-3', representing positions 451–480 of the *E. coli* 16S rRNA gene sequence (IUB nomenclature). The DNA G + C content of the type strain is 48.1 mol%.

The type strain is strain Y159^T (=WA758^T=DSM 14987^T=LMG 22254^T), belonging to serogroup O:16.

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