Lactobacillus ceti sp. nov., isolated from beaked whales (*Ziphius cavirostris*)

A. I. Vela,[1] A. Fernandez,[2] A. Espinosa de los Monteros,[2] J. Goyache,[1] P. Herraez,[2] B. Tames,[1] F. Cruz,[1] L. Domínguez[1] and J. F. Fernández-Garayzábal[1]

Correspondence
J. F. Fernández-Garayzábal
garayzab@vet.ucm.es

[1]Laboratorio de Vigilancia Sanitaria Veterinaria (VISAVET), Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

[2]Departamento de Anatomía Patológica, Instituto Universitario de Sanidad Animal, Facultad de Veterinaria, Universidad de Las Palmas de Gran Canaria, 35016 Gran Canaria, Spain

Biochemical and molecular genetic studies were performed on three isolates of an unknown Gram-positive, catalase-negative and rod-shaped organism isolated from the lungs and liver of two beaked whales. The organisms were tentatively identified as *Lactobacillus* spp. based on cellular morphology and biochemical tests. 16S rRNA gene sequencing studies confirmed the provisional identification of the novel isolates as members of the genus *Lactobacillus*, but the isolates did not correspond to any recognized species of this genus. The novel strains shared the same phenotypic characteristics and exhibited 100 % 16S rRNA gene sequence similarity. The nearest phylogenetic relatives of the novel isolates were *Lactobacillus satsumensis* DSM 16230^T (94.2 % 16S rRNA gene sequence similarity), *Lactobacillus salivarius* JCM 1047 (94.0 %), *Lactobacillus nagelii* ATCC 700692^T (94.0 %) and *Lactobacillus saerimneri* DSM 16049^T (93.8 %). The novel isolates could be distinguished from these species and other related species of the genus *Lactobacillus* by physiological and biochemical tests. On the basis of these phenotypic, physiological and phylogenetic findings, it is proposed that the new isolates from whales be classified as a novel species of the genus *Lactobacillus*, *Lactobacillus ceti* sp. nov. The type strain is 142-2^T (=CECT 7185^T=CCUG 53626^T).

The genus *Lactobacillus* represents a large group within the Gram-positive bacteria and is presently classified within the order '*Lactobacillales*' according to the latest release of the Taxonomic Outline of the Prokaryotes (Garrity *et al.*, 2004). The genus *Lactobacillus* has undergone considerable expansion in the past decade and, at the time of writing, over 114 species and 16 subspecies (Euzéby, 1997; Felis & Dellaglio, 2007) are recognized and novel taxa are continuously arising within this group. Lactobacilli have been isolated from a wide range of environments and can be found on plants or material of plant origin, in manure and man-made habitats such as sewage, and in fermenting or spoiled food. Lactobacilli have also been associated with the intestinal tract and mucous membranes of man and many animals (Wibowo *et al.*, 1985; Hammes *et al.*, 1992; Aguirre & Collins, 1993; Roos *et al.*, 2000). During an investigation into the microbiota of stranded beaked whales (*Ziphius cavirostris*), three unidentified Gram-positive, rod-shaped organisms were recovered from the liver (strain 159-2) and lungs (strains 142-2^T and 160-1) of two animals. Neither of these two animals showed clinical signs of disease and no lesions were apparent after post-mortem examination. Strains were isolated on Columbia blood agar plates (bioMérieux) after incubation at 37 °C for 24 h under aerobic and anaerobic conditions. On the basis of the phenotypic and phylogenetic results, a novel species of the genus *Lactobacillus* is proposed.

Gram determination was performed by Gram staining (Merck). The isolates were tested for a number of key characteristics by using standard procedures (Barrow & Feltham, 1993). Growth in brain heart infusion broth was assessed at 4 and 15 °C for up to 14 days, at 22 °C for up to 7 days and at 30, 37 and 42 °C for 48 h. Growth in the presence of 3, 4.5 and 6.5 % NaCl (w/v) and under anaerobic (with 4–10 % CO_2) and microaerobic (with 5–15 % O_2 and 5–12 % CO_2) conditions using GasPak Plus and CampyPak Plus systems (BBL), respectively, was assessed at 37 °C for 48 h. Growth on MRS agar (bioMérieux) was tested at 37 °C for 48 h. The strains were biochemically characterized using the API Coryne, API 50 CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The API 50 CH strips were read after 7 days incubation at 37 °C. Using the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 142-2^T is AM292799.

miniaturized biochemical kits, the novel isolates displayed identical phenotypic profiles. The lactate isomer was determined enzymically using the DL-lactate test kit (Boehringer Mannheim). A detailed description of the morphological, physiological and biochemical characteristics of the isolates is given in the species description and in Table 1.

Analysis of the peptidoglycan structure of one isolate (strain 142-2^T) was performed as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose was used instead of paper chromatography. Analysis of the cell-wall composition of strain 142-2^T revealed the presence of lysine and serine, indicating the presence of the A3α L-Lys–D-Ser peptidoglycan type (DSMZ, 2001).

To establish the phylogenetic affinities of the novel isolates, the 16S rRNA gene sequences were determined as described previously (Vela *et al.*, 2005) and were subjected to a comparative analysis. The almost complete sequences (>1400 nucleotides) of the three novel isolates were determined and pairwise analysis revealed that the isolates were phylogenetically identical (100 % gene sequence similarity). Sequence searches of GenBank using the FASTA program (Pearson, 1994) revealed that the novel isolates had <95 % gene sequence similarity with any recognized species. The novel bacteria were phylogenetically closely related to *Lactobacillus satsumensis* DSM 16230^T (94.2 % 16S rRNA gene sequence similarity), *Lactobacillus salivarius* JCM 1047 (94.0 %), *Lactobacillus nagelii* ATCC 700692^T (94 %) and *Lactobacillus saerimneri* DSM 16049^T (93.8 %). These sequences, and those of other known related strains, were retrieved from GenBank and aligned with the newly determined sequences using the DNAtools program (Rasmussen, 1995). Phylogenetic trees were constructed according to three different methods, a neighbour-joining algorithm (Saitou & Nei, 1987), performed with the DNAtools and TREEVIEW (Page, 1996) programs, maximum-likelihood analysis conducted using PHYML software (Guindon & Gascuel, 2003) and the maximum-parsimony method carried out using the MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 software package (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated by the Kimura two-parameter method (Kimura, 1980) and close-neighbour-interchange (search level=2, random additions=100) was applied in maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). Phylogenetic trees obtained by using the neighbour-joining (Fig. 1) and the other two methods revealed a clear affiliation of the novel strains (as exemplified by strain 142-2^T) to the genus *Lactobacillus*, as they were positioned as a separate branch close to *L. saerimneri* DSM 16049^T, *Lactobacillus aviarius* ATCC 43234^T, *L. salivarius* JCM 1047and *Lactobacillus acidipiscis* JCM 10692^T. Although bootstrap resampling analysis did not reveal the affiliation between strain 142-2^T and the aforementioned species to be statistically significant (50 % value), the branching position of strain 142-2^T within this clade was relatively stable according to the three tree-making algorithms used in this study (Fig. 1). These data together with 16S rRNA gene sequence divergence values of >4 % between the novel isolates and other species of the genus *Lactobacillus* suggest they represent a separate species

Table 1. Characteristics that differentiate *Lactobacillus ceti* sp. nov. from other members of the *L. salivarius* subgroup of lactobacilli

Taxa: 1, *L. ceti* sp. nov.; 2, *L. saerimneri*; 3, *L. nagelii*; 4, *L. mali*; 5, *L. salivarius*; 6, *L. aviarius*; 7, *L. acidipiscis*; 8, *L. ruminis*; 9, *L. agilis*; 10, *L. equi*; 11, *L. animalis*; 12, *L. murinus*; 13, *L. vini*; 14, *L. satsumensis*; 15, *L. apodemis*. Data for *Lactobacillus* species were obtained from Kandler & Weiss (1986); Hammes *et al.* (1992); Edwards *et al.* (2000); Tanasupawat *et al.* (2000); Morotomi *et al.* (2002); Pedersen & Roos (2004); Endo & Okada (2005); Osawa *et al.* (2006) and Rodas *et al.* (2006). +, ⩾90 % strains positive; −, ⩾90 % strains negative; d, 11–89 % strains positive; NA, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Acid from:															
L-Arabinose	−	−	−	+	−	NA	d	−	−	−	d	+	+	−	−
Ribose	+	−	−	NA	−	NA	+	−	+	d	−	+	NA	−	d
Galactose	−	−	+	+	+	d	+	+	+	+	+	+	NA	d	+
Salicin	−	−	+	+	d	+	d	+	+	d	+	d	+	+	d
Cellobiose	−	−	+	d	−	+	−	+	+	−	+	+	+	−	d
Melibiose	−	−	−	−	+	d	−	+	+	+	+	+	−	−	+
Sucrose	−	+	−	+	+	+	d	+	+	−	+	+	+	+	+
Trehalose	−	+	+	+	+	+	d	−	+	−	−	d	+	+	+
Raffinose	−	−	−	−	+	+	−	+	+	−	−	+	−	−	+
Hydrolysis of aesculin	+	−	+	NA	d	NA	−	+	+	+	+	+	+	NA	+
Growth at 45 °C	−	+	+	NA	+	NA	−	d	+	+	+	+	+	+	+
Lactate isomer	L	DL	DL	L	L	DL	L	L	L	DL	L	L	DL	L	L
Peptidoglycan type	Lys–L-Ser	*m*-DAP	NA	*m*-DAP	Lys–D-Asp	Lys–D-Asp	Lys–D-Asp	*m*-DAP	*m*-DAP	NA	Lys–D-Asp	Lys–D-Asp	Lys–D-Asp	*m*-DAP	Lys–D-Asp

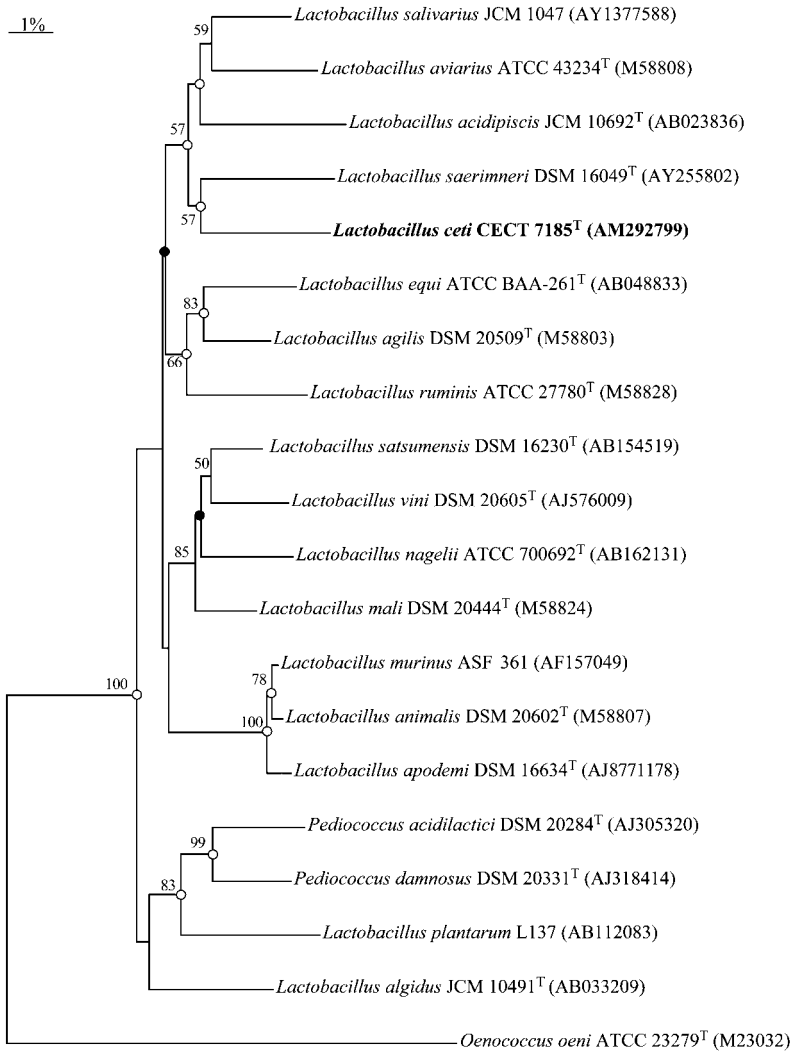

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of *Lactobacillus ceti* sp. nov. with other species of the genus *Lactobacillus*. *Oenococcus oeni* ATCC 23279^T was used as an outgroup. Bootstrap values (expressed as a percentage of 1000 replications) >50 % are given at the branching points. Filled circles indicate that the corresponding nodes (groupings) were also obtained in maximum-likelihood trees. Open circles indicate that the corresponding nodes (groupings) were also obtained in maximum-likelihood and maximum-parsimony trees. Bar, 1 % sequence divergence.

(Stackebrandt & Goebel, 1994). In order to differentiate the three novel isolates, pulsed-field gel electrophoresis (PFGE) with the restriction enzyme *Sma*I (MBI Fermentas) was performed as described previously (Vela *et al.*, 2003). Strains 159-2 and 142-2^T isolated from different whales generated distinguishable PFGE fingerprint profiles that revealed their genotypic differences at the strain level. On the other hand, strains 160-1 and 159-2, isolated from different organs of the same animal, exhibited undistinguishable PFGE profiles (data not shown).

Overall, the results of the present study show that the novel isolates from whales constitute a distinct branch and do not display a close relationship with any recognized organism (Fig. 1). Moreover, the novel isolates could be distinguished from their closely phylogenetic relatives on the basis of phenotypic characteristics (Table 1). Therefore, based on both phylogenetic and phenotypic criteria, it is evident that the new isolates merit classification as a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus ceti* sp. nov. is proposed. Strains 142-2^T, 159-2 and 160-1 showed identical phenotypic and physiological properties; these are given in the species description. Characteristics that are useful in differentiating *Lactobacillus ceti* sp. nov. from other members of the *L. salivarius* group of lactobacilli are summarized in Table 1.

Description of *Lactobacillus ceti* sp. nov.

Lactobacillus ceti (ce.ti. L. gen. n. *ceti* of a whale).

Cells are Gram-positive, 0.5 μm wide and 2.7 μm long, catalase-negative, non-spore-forming and non-motile rods. Cells are found singly, in pairs and in short chains. Facultatively anaerobic. Colonies are non-haemolytic, circular, smooth, entire and approximately 1 mm diameter on Columbia blood agar after 2 days incubation at 37 °C. Growth does not occur on MRS agar after 48 h. Grows at 22, 30 and 37 °C after 2 days incubation and at 15 °C after 5 days. Growth is not detected at 42 or 4 °C. Growth occurs in broth containing 3 % NaCl (w/v), but not with 6.5 % NaCl. L-Lactate is exclusively produced as the end product from hexoses and pentoses. Aesculin and urea are not hydrolysed.

Gelatin is hydrolysed after 2 days incubation. Nitrate is not reduced. Acidification is detected from 5-ketogluconate and ribose after 48 h. Acid is not produced from D-glucose, D- or L-xylose, D-mannitol, maltose, D-lactose, sucrose, *N*-acetyl-β-glucosamine, glycerol, erythritol, L-arabinose, D-adonitol, methyl β-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, melibiose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol or 2-ketogluconate. Activity is detected for acid phosphatase, naphthol-AS-BI-phosphohydrolase, alkaline phosphatase, esterase C4 (weak), ester lipase C8 (weak) and leucine arylamidase (weak). Pyrazinamidase, lipase C14, valine arylamidase pyrrolidonyl arylamidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, α-chymotrypsin, trypsin and cystine arylamidase are not produced. The peptidoglycan type is A3α L-Lys–D-Ser.

The type strain, 142-2^{T} (=CECT 7185^{T}=CCUG 53626^{T}), was isolated from the lungs of a beaked whale.

Acknowledgements

A. I. V. has a fellowship from the Ramon y Cajal Program (Spanish Ministry of Science and Technology/U.C.M.). The authors thank A. Casamayor for technical assistance, Juncal Fenández-Garayzábal for her assistance with the English reviewing of the manuscript, and the Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany, for the analysis of peptidoglycan.

References

Aguirre, M. & Collins, M. D. (1993). Lactic acid bacteria and human clinical infection. *J Appl Bacteriol* **75**, 95–107.

Barrow, G. I. & Feltham, R. K. A. (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd edn. Cambridge: Cambridge University Press.

DSMZ (2001). *Catalogue of Strains*, 7th edn, p. 617. Braunschweig: DSMZ. http://www.dsmz.de/species/murein.htm

Edwards, C. G., Collins, M. D., Lawson, P. A. & Rodriguez, A. V. (2000). *Lactobacillus nagelii* sp. nov., an organism isolated from a partially fermented wine. *Int J Syst Evol Microbiol* **50**, 699–702.

Endo, A. & Okada, S. (2005). *Lactobacillus satsumensis* sp. nov., isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials. *Int J Syst Evol Microbiol* **55**, 83–85.

Euzéby, J. P. (1997). List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *Int J Syst Bacteriol* **47**, 590–592 (Last full update May 03, 2007).

Felis, G. E. & Dellaglio, F. (2007). Taxonomy of Lactobacilli and Bifidobacteria. *Curr Issues Intest Microbiol* **8**, 44–61.

Garrity, G. M., Bell, J. A. & Lilburn, T. G. (2004). Taxonomic outline of the prokaryotes. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, release 5.0. New York: Springer. DOI:10.1007/bergeysoutline200405

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**, 696–704.

Hammes, W. P., Weiss, N. & Holzapfel, W. (1992). The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes*, 2nd edn, pp. 1535–1594. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Kandler, O. & Weiss, N. (1986). Genus *Lactobacillus* Beijerinck 1901, 212^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1209–1234. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Morotomi, M., Yuki, N., Kado, Y., Kushiro, A., Shimazaki, T., Watanabe, K. & Yuyama, T. (2002). *Lactobacillus equi* sp. nov., a predominant intestinal *Lactobacillus* species of the horse isolated from faeces of healthy horses. *Int J Syst Evol Microbiol* **52**, 211–214.

Osawa, R., Fujisawa, T. & Pukall, R. (2006). *Lactobacillus apodemi* sp. nov., a tannase-producing species isolated from wild mouse faeces. *Int J Syst Evol Microbiol* **56**, 1693–1696.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Pearson, W. R. (1994). Using the FASTA program to search protein and DNA sequence databases. *Methods Mol Biol* **24**, 307–331.

Pedersen, C. & Roos, S. (2004). *Lactobacillus saerimneri* sp. nov., isolated from pig faeces. *Int J Syst Evol Microbiol* **54**, 1365–1368.

Rasmussen, S. W. (1995). *DNA Tools, a Software Package for DNA Sequence Analysis*. Copenhagen: Carlsberg Laboratory.

Rodas, A. M., Chenoll, E., Macián, M. C., Ferrer, S., Pardo, I. & Aznar, R. (2006). *Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses. *Int J Syst Evol Microbiol* **56**, 513–517.

Roos, S., Karner, F., Axelsson, L. & Jonsson, H. (2000). *Lactobacillus muscosae* sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine. *Int J Syst Evol Microbiol* **50**, 251–258.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schleifer, K. H. (1985). Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Tanasupawat, S., Shida, O., Okada, S. & Komagata, K. (2000). *Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., isolated from fermented fish in Thailand. *Int J Syst Evol Microbiol* **50**, 1479–1485.

Vela, A. I., Goyache, J., Tarradas, C., Luque, I., Mateos, A., Moreno, M. A., Borge, C., Perea, J. A., Dominguez, L. & other authors (2003). Analysis of genetic diversity of *Streptococcus suis* clinical isolates from pigs in Spain by pulsed-field gel electrophoresis. *J Clin Microbiol* **41**, 2498–2502.

Vela, A. I., Collins, M. D., Lawson, P. A., García, N., Domínguez, L. & Fernández-Garayzábal, J. F. (2005). *Uruburuella suis* gen. nov., sp. nov., isolated from clinical specimens of pigs. *Int J Syst Evol Microbiol* **55**, 643–647.

Wibowo, D., Eschenbruch, R., Davis, C. R., Fleet, G. H. & Lee, T. H. (1985). Occurrence and growth of lactic acid bacteria in wine: a review. *Am J Enol Vitic* **36**, 302–313.