Lactobacillus ingluviei sp. nov., isolated from the intestinal tract of pigeons

Margo Baele,¹ Marc Vancanneyt,² Luc A. Devriese,¹ Karen Lefebvre,² Jean Swings² and Freddy Haesebrouck¹

Correspondence

Margo Baele

Margo.Baele@rug.ac.be

¹Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

²BCCM/LMG Bacteria Collection, Laboratory of Microbiology, Faculty of Sciences, Ghent University, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

Lactic acid bacteria were isolated from the crop and intestines of pigeons. One group of strains, showing similar genomic patterns after screening with tRNA intergenic spacer PCR, could not be identified to the species level. Sequencing of the 16S rRNA gene of one representative strain revealed about 96% similarity to sequences from *Lactobacillus fermentum* and *Lactobacillus mucosae*. Determination of the DNA base composition, DNA–DNA hybridization experiments, SDS-PAGE of whole-cell proteins and biochemical testing confirmed that the seven strains studied constitute a single novel *Lactobacillus* species, for which the name *Lactobacillus ingluviei* sp. nov. is proposed. The type strain is strain KR3^T (=LMG 20380^T =CCUG 45722^T).

INTRODUCTION

In a previous study on the enterococcal, streptococcal and lactobacillar crop flora of pigeons, *Lactobacillus agilis* was the most frequently isolated species and was present in eight of 10 pigeon crops examined (Baele *et al.*, 2001a). Five of the 10 pigeons were also colonized by an unknown bacterium, which showed a tRNA intergenic spacer PCR (tDNA-PCR) fingerprint different from those of all lactic acid bacteria available in the database. tDNA-PCR makes use of universal bacterial primers directed against the edges of the tRNA genes, amplifying the spacers between the genes. After determination of the amplicon lengths by means of capillary electrophoresis, a peak pattern is obtained that is characteristic for a given species (Baele *et al.*, 2000, 2001b, 2002). The taxonomic position of this group of strains was investigated in a polyphasic study.

METHODS

Strains. Seven analogous strains were isolated from crop-sac samples of five euthanized pigeons (A–E, Table 1) belonging to different pigeon-fanciers; this was performed both anaerobically on Rogosa SL agar (Becton-Dickinson) and on Slanetz & Bartley agar (Oxoid) in a CO₂-enriched atmosphere after 48 h incubation. From two of these pigeons, strains with identical tDNA fingerprints were also

isolated from intestinal samples. In the present study, these isolates were investigated further (Table 1). The strains were designated as KR3^T (=LMG 20380^T), KR10 (=LMG 20381), KR16 (=LMG 20382), KR36 (=LMG 20383), KR47 (=LMG 20384), KR21 (=LMG 20978) and KR45 (=LMG 20979), and all were deposited in the BCCM/LMG Bacteria Collection, Ghent, Belgium.

tDNA-PCR. Cultures were grown overnight on MRS (de Man–Rogosa–Sharpe) agar (Lab M) under anaerobic conditions at 37 °C. For DNA preparation, a small loopful of cells was suspended in 20 µl lysis buffer (0.25% SDS, 0.05 M NaOH) and heated at 95 °C for 5 min. After brief centrifugation at 16 000 g, lysed cells were resuspended in 180 µl distilled water and centrifuged again for 5 min at 16 000 g to remove the cell debris. Supernatants were used as the DNA in the PCR or were frozen at –20 °C until further use.

The PCR was carried out using outwardly directed tRNA-gene consensus primers T5A (5′-AGTCCGGTGCTCTAACCACTGAG-3′) and T3B (5′-AGGTCGCGGGTTCGAATCC-3′) as described earlier (Baele *et al.*, 2000). Cycle reactions were carried out as described previously.

The samples were subjected to capillary electrophoresis using an ABI-Prism 310 Genetic Analyzer (Perkin-Elmer Cetus). Electrophoregrams were normalized using internal size standards with GENESCAN analysis software, version 2.1. Electrophoregrams were compared with the database by using a software program developed at our laboratory (Baele *et al.*, 2000, 2001b). After calculation of a distance matrix, clustering was done with NEIGHBOR software (PHYLIP; <http://evolution.genetics.washington.edu/phylib.html>), using the UPGMA algorithm.

16S rDNA sequencing. DNA extraction was performed as described for tDNA-PCR analysis. 16S rDNA was amplified using the commercially available Qiagen Taq Mastermix, to which primers αβ-NOT (5′-TCAAACCTAGGACCGAGTC-3′) and ω_{MB} (5′-TACC-TTGTACTTCACCCCA-3′) were added at a concentration of 0.2 µM. The PCR products were sequenced using the BigDye

Abbreviation: tDNA-PCR, tRNA intergenic spacer PCR.

The GenBank accession number for the 16S rRNA sequence of strain LMG 20380^T is AF333975.

A tDNA-PCR-based dendrogram including a wider sample of reference taxa is available as supplementary material in IJSEM Online (<http://ij.s.sgmjournals.org/>).

Table 1. Strains used in this study

Strain	Pigeon	Origin	Isolation medium
KR10 (= LMG 20381)	A	Crop	Slanetz & Bartley
KR21 (= LMG 20978)	B	Crop	Slanetz & Bartley
KR47 (= LMG 20384)	B	Intestines	Rogosa SL
KR36 (= LMG 20383)	C	Crop	Rogosa SL
KR45 (= LMG 20979)	C	Intestines	Rogosa SL
KR3 ^T (= LMG 20380 ^T)	D	Crop	Slanetz & Bartley
KR16 (= LMG 20382)	E	Crop	Slanetz & Bartley

Terminator sequencing kit (Applied Biosystems) and primers pD, Gamma*, 3 and O* (Coenye *et al.*, 1999), as described before (Baele *et al.*, 2001), and determined on an automatic DNA sequencer (ABI Prism 310 Genetic Analyzer). The electrophoregrams were exported and converted to GeneBase (Applied Maths) using Abiconv (Applied Maths). The sequences were compared with the NCBI GenBank by using the BLAST search tool. Phylogenetic analysis was performed using the Bionumerics software (Applied Maths) after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from GenBank. Multiple alignment was calculated using an open gap penalty of 100% and a unit gap penalty of 0%. A tree was constructed using the neighbour-joining method.

DNA base composition. Strains LMG 20380^T and LMG 20383 were grown on MRS broth and incubated for 24 h at 37 °C under anaerobic conditions. High-molecular-mass native DNA was extracted from 0.75–1.25 g wet weight of cells by using the protocol described by Pitcher *et al.* (1989), with the following modifications: the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) containing RNase (200 µg ml⁻¹; Sigma), mutanolysin (100 U ml⁻¹; Sigma) and lysozyme (25 mg ml⁻¹; Serva) for 1 h at 37 °C. Before the addition of GES reagent, proteinase K (200 µg ml⁻¹; Merck) was added to the mixture for 15 min.

For determination of the DNA base composition, DNA was enzymically degraded into nucleosides and then separated by HPLC as described previously (Vancanneyt *et al.*, 2001).

DNA–DNA hybridization experiments. High-molecular-mass native DNA was prepared as described above for determination of the DNA base composition. DNA–DNA hybridizations were performed as described by Vancanneyt *et al.* (2001) by using a micro-plate method and fluorescence measurements for calculation of the binding values. Hybridizations were performed at 41 °C.

PAGE analysis of whole-cell protein. Cells were cultivated as indicated for determination of DNA base composition. Whole-cell protein extracts were prepared and PAGE was then performed as described by Pot *et al.* (1994). Registration of the protein patterns, normalization of the densitometric traces, pattern storage and grouping of the strains using Pearson's product-moment correlation coefficient (*r*) and UPGMA analysis were performed as described by Pot *et al.* (1994) by using the software GelCompar (Applied Maths).

Biochemical activity and growth characteristics. All seven strains were tested biochemically as described previously. Growth was tested on MRS agar at 25, 30, 37 and 42 °C under anaerobic conditions. The influence of the gas atmosphere was determined: growth yield under aerobic and anaerobic (H₂+CO₂, GasPak Plus; BBL) conditions and in a 5% CO₂-enriched atmosphere were

Fig. 1. Dendrogram obtained from tDNA-PCR fingerprints, after similarity calculation with in-house software and clustering with UPGMA using the program NEIGHBOR. The bar represents a distance of 10%. A dendrogram including a wider sample of reference taxa is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

compared. Carbohydrate acidification tests were carried out with API 50 CH galleries according to the instructions of the manufacturer (bioMérieux).

RESULTS AND DISCUSSION

Genotypic studies

Screening of the isolates by tDNA-PCR revealed an atypical pattern different from all profiles of lactic acid bacteria available in the database. Capillary electrophoresis of the amplicons revealed mean peak positions of 162.5, 176.5, 185.5 and 255.2 bp. Numerical analysis clearly confirmed the aberrant position of the strains (Fig. 1).

To determine their phylogenetic position, one representative strain, LMG 20380^T, was selected for 16S rDNA sequence analysis. Highest similarities, of 96 and 95.4%, were obtained to the sequences of *Lactobacillus fermentum* and *Lactobacillus mucosae*, respectively (Fig. 2). These values clearly indicate that strain LMG 20380^T belongs

Fig. 2. Phylogenetic tree showing the relationships among *Lactobacillus ingluviei* sp. nov., related species of the *Lactobacillus reuteri* group and species representing different lineages within the genus *Lactobacillus*. The tree was constructed by neighbour-joining analysis of a distance matrix from a multiple-sequence alignment. *Lactococcus lactis* was used as the outgroup, and bootstrap values are indicated at the branch-points (100 trees resampled). Accession numbers are given.

to the genus *Lactobacillus*, but the similarities are too low for possible relatedness at the species level with one of the currently described *Lactobacillus* species (Stackebrandt & Goebel, 1994).

Two strains, LMG 20380^T and LMG 20383, were subsequently subjected to DNA–DNA hybridization analysis. A relatedness value of 77% confirmed that the two strains constitute a single genomic species. Determination of the DNA G+C content of the two strains revealed respective values of 49.4 and 49.2 mol%.

Phenotypic studies

SDS-PAGE analysis of whole-cell proteins yielded highly similar patterns in the seven strains, confirming that they represent a single species. The profiles were different from all patterns of lactic acid bacteria in the database (data not shown), confirming their separate species status. The patterns of three of the strains and of *L. fermentum* and *L. mucosae*, their phylogenetically closest neighbours, are shown in Fig. 3.

The growth characteristics of all seven strains were compared. Six strains showed smooth colonies on MRS agar, but strain LMG 20383 showed dry, crumbly colonies. On Columbia blood agar, the strains appear streptococcus-like and without further testing, might be confused with the latter taxon. Other growth characteristics and differentiating phenotypic features between the novel taxon and its phylogenetic neighbours, *L. fermentum* and *L. mucosae*, are described below and are summarized in Table 2.

The results of the present study allowed us to assign the strains to a novel species, for which we propose the name *Lactobacillus ingluviei* sp. nov.

Description of *Lactobacillus ingluviei* sp. nov.

Lactobacillus ingluviei (in.glu'vi.ei. L. n. *ingluvies* crop sac; L. gen. n. *ingluviei* of a crop sac).

Cells are Gram-positive, non-motile, very short, plump rods, rapidly decolorizing in the Gram-stain procedure. Cells mostly occur singly or in pairs, and some appear to be slightly longer than others. They are non-sporulating and catalase-negative. Colonies are white and smooth or crumbly and dry. Growth is enhanced under anaerobic conditions and also slightly in the presence of 5% CO₂, compared with aerobic growth. Better growth is obtained at 42 °C than at 37 °C. No growth occurs at 25 °C and growth is poor at 30 °C. The strains grow as non-haemolytic streptococcus-like colonies on Columbia blood agar with

Fig. 3. Protein profiles of three representative strains of *L. ingluviei* sp. nov., *L. mucosae* and *L. fermentum* and the corresponding dendrogram derived from UPGMA cluster analysis of correlation coefficients *r* (expressed as percentages). The molecular mass markers (lane M) are (from left to right): lysozyme, 14 500; trypsin inhibitor, 20 100; trypsinogen, 24 000; carbonic anhydrase, 29 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000; egg albumin, 45 000; BSA, 66 000; and β -galactosidase, 116 000.

Table 2. Characteristics that differentiate *L. ingluviei* sp. nov. from closely related species

Data for reference taxa were obtained from Hammes & Vogel (1995) and Roos *et al.* (2000). Characters are scored as follows: +, positive; D, strain-dependent; D+, usually positive; –, negative.

Production of acid from:	<i>L. ingluviei</i>	<i>L. fermentum</i>	<i>L. mucosae</i>
D-Fructose	+	+	–
Galactose	–	+	D
Melibiose	–	+	D+

diameters of up to 0.5 mm. Acid is produced from L-arabinose, D-fructose, methyl β -xyloside, ribose, sucrose and D-xylose. No acid is produced from adonitol, amygdalin, D-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D- and L-fucose, galactose, β -gentiobiose, 2-ketogluconate, 5-ketogluconate, *N*-acetylglucosamine, glycerol, glycogen, methyl α -D-glycoside, inositol, inulin, lactose, D-lyxose, mannitol, methyl α -D-mannoside, melizitol, melibiose, rhamnose, salicin, sorbitol, L-sorbose, starch, D-tagatose, trehalose, D-turanose, xylitol or L-xylose. Acidification of aesculin, gluconate (weak reaction), D-glucose, maltose, D-mannose and D-raffinose is variable. The DNA G + C content is 49 mol% and the characteristic tDNA-PCR fingerprint is composed of fragments with lengths of 162.5, 176.5, 185.5 and 255.2 bp, as determined by fluorescent capillary electrophoresis. The habitat is pigeon crop and intestines. The type strain is strain KR3^T (=LMG 20380^T =CCUG 45722^T).

ACKNOWLEDGEMENTS

This work was supported by the Research Fund of the University of Ghent, Belgium (Codennr. BOF98/GOA/014). The authors are grateful to Arlette Vandekerckhove for skilled technical assistance.

REFERENCES

- Baele, M., Baele, P., Vanechoutte, M., Storms, V., Butaye, P., Devriese, L. A., Verschraegen, G., Gillis, M. & Haesebrouck, F. (2000). Application of tRNA intergenic spacer PCR for the identification of *Enterococcus* species. *J Clin Microbiol* **38**, 4201–4207.
- Baele, M., Devriese, L. A. & Haesebrouck, F. (2001a). *Lactobacillus agilis* is an important component of the pigeon crop flora. *J Appl Microbiol* **91**, 488–491.
- Baele, M., Storms, V., Haesebrouck, F., Devriese, L. A., Gillis, M., Verschraegen, G., De Baere, T. & Vanechoutte, M. (2001b). Application and evaluation of the interlaboratory reproducibility of tRNA intergenic length polymorphism analysis (tDNA-PCR) for identification of *Streptococcus* species. *J Clin Microbiol* **39**, 1436–1442.
- Baele, M., Vanechoutte, M., Verhelst, R., Vancanneyt, M., Devriese, L. A. & Haesebrouck, F. (2002). Identification of *Lactobacillus* species using tDNA-PCR. *J Microbiol Methods* **50**, 263–271.
- Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J. R. W., Kersters, K. & Vandamme, P. (1999). Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *Int J Syst Bacteriol* **49**, 405–413.
- Hammes, W. P. & Vogel, R. F. (1995). The genus *Lactobacillus*. In *The Genera of Lactic Acid Bacteria*, pp. 19–54. Edited by J. B. Wood-Brian & W. H. Holzappel. London: Blackie.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Roos, S., Karner, F., Axelsson, L. & Jonsson, H. (2000). *Lactobacillus mucosae* sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine. *Int J Syst Evol Microbiol* **50**, 251–258.
- Stackebrandt, E. & Goebel, 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.
- Vancanneyt, M., Snauwaert, C., Cleenwerck, I. & 8 other authors (2001). *Enterococcus villorum* sp. nov., an enteroadherent bacterium associated with diarrhoea in piglets. *Int J Syst Evol Microbiol* **51**, 393–400.