Note

Lactobacillus thermotolerans sp. nov., a novel thermotolerant species isolated from chicken faeces

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Five strains of thermotolerant lactic acid bacteria (G 12, G 22, G 35^T, G 43 and G 44) isolated from chicken faeces were characterized taxonomically. The strains were facultatively anaerobic, Gram-positive, catalase-negative, non-motile, non-spore-forming rods. They were heterofermentative lactobacilli that produced DL-lactic acid. Growth of the strains occurred at 45 °C but not at 15 °C. The optimum temperature for growth was 42 °C, as determined from the specific growth rate. The highest permissive temperatures for growth were 50 °C for strain G 35^T and 48 °C for the other four strains. DNA G+C content of the strains was between 49 and 51 mol%. Complex fatty acid patterns of the strains showed the presence of $C_{14:0}$, $C_{16:0}$, sometimes $C_{18:0}$, $C_{18:1}$ and $C_{19:0\ cyclo}$ in the cell walls. Phylogenetic analysis of the 16S rRNA gene sequences of the five strains placed them in the *Lactobacillus casei/Pediococcus* group, with *Lactobacillus fermentum* as their closest relative (about 95 % sequence similarity). DNA–DNA hybridization data indicated that the thermotolerant strains were not *L. fermentum*. Taken together, the findings of this study show that the five strains isolated from chicken faeces represent a novel species within the genus *Lactobacillus*, for which the name *Lactobacillus thermotolerans* is proposed (G 35^T = DSM 14792^T = JCM 11425^T).

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, non-spore-forming bacteria (Kandler & Weiss, 1986), and are widely involved in the production of fermented foods. Recently, a great deal of interest has been focused on some members of the LAB with regard to their use as probiotics (Tannock, 1999). LAB belonging to the genus *Lactobacillus* have been isolated from a variety of habitats, including plant and dairy products, meat products, sewage and manure, and humans and animals (Kandler & Weiss, 1986). Some species of *Lactobacillus* isolated from chicken faeces and intestine have been reported previously. Fujisawa *et al.* (1984) isolated a novel *Lactobacillus* species, *Lactobacillus aviarius*, from the intestine of chickens, which consists of two subspecies, *L. aviarius* subsp. *aviarius* and *L. aviarius* subsp. *araffinosus*. Fujisawa *et al.* (1992) also isolated other novel *Lactobacillus* spp. from chicken faeces, namely *Lactobacillus gallinarum* and *Lactobacillus johnsonii*. When investigating acid and bile tolerance among intestinal strains of *Lactobacillus*, Jin *et al.* (1998) isolated 12 *Lactobacillus* strains (six strains of *Lactobacillus brevis*, three of *Lactobacillus*

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Abbreviations: LAB, lactic acid bacteria; TLAB, thermotolerant lactic acid bacteria.

The GenBank accession numbers for the 16S rDNA sequences of strains G 12, G 22, G 35^T, G 43 and G 44 are AF308146, AF308147, AF317702, AF317703 and AF317704, respectively.

A fuller phylogenetic tree showing the placement of *Lactobacillus thermotolerans* within the genus *Lactobacillus* (Fig. I) and a growth curve for strain G 35^T (Fig. II) are available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).

fermentum, two of *Lactobacillus acidophilus* and one of *Lactobacillus crispatus*) from chicken intestine. Gusils *et al.* (1999) isolated *L. fermentum*, *L. fermentum* subsp. *cellobiosus* and *Lactobacillus animalis*, when studying lectin-like protein fractions in *Lactobacillus* strains isolated from the gastrointestinal tracts of chickens. Although several *Lactobacillus* spp. have been isolated from chicken faeces, most of the strains isolated so far have been mesophiles. The isolation of *Lactobacillus* spp. from chicken faeces under high temperatures has not been reported. Some LAB used in the dairy industry, for example, *Lactobacillus delbrueckii*, *Lactobacillus helveticus* and *Streptococcus thermophilus*, are already known as thermotolerant starter species from their temperature range for growth (Delcour *et al.*, 2000). However, we still have little knowledge about the biodiversity of thermotolerant lactic acid bacteria (TLAB) in nature, because very few studies have been done that focus on LAB from the standpoint of their thermotolerance.

In the course of studies to isolate thermotolerant microorganisms for use in the fermentation industry, we have isolated a large number of LAB from various types of natural samples at relatively high temperatures, i.e. 40–50 °C. 16S rDNA sequence analysis of these LAB raised the possibility that some of the TLAB isolated from chicken faeces comprised a novel species within the genus *Lactobacillus*. In the present study, five *Lactobacillus* strains isolated from chicken faeces that were known to be TLAB were characterized. On the basis of physiological, biochemical and genetic data, it is shown that the five strains represent a novel species within the genus *Lactobacillus*, for which the name *Lactobacillus thermotolerans* is proposed.

Strains G 12, G 22, G 35^T, G 43 and G 44, isolated as TLAB, were used in this work. For the isolation of TLAB, fresh faecal samples from chickens were collected from the chicken coop of Kasetsart University, Bangkok, Thailand. These faecal samples were inoculated into glucose/peptone/yeast extract (GPY) broth and incubated anaerobically at 40, 45 and 50 °C for 24 h using mixed gases ($N_2/H_2/CO_2$, 8 : 1 : 1). GPY broth contained (g l^{-1}): glucose, 10; peptone, 5; yeast extract, 10; sodium acetate trihydrate, 2; NaCl, 0·01; Tween 80, 0·5; $MgSO_4.7H_2O$, 0·2; $MnSO_4.4H_2O$, 0·01; $FeSO_4.7H_2O$, 0·035. NaOH was used to adjust the broth to pH 6·8. After appropriate dilutions had been prepared, culture broths were spread onto GPY/BCP agar plates which were prepared by the addition of 5 g $CaCO_3$ l^{-1}, 0·06 g bromocresol purple l^{-1}, 0·05 g cycloheximide l^{-1} and 20 g agar l^{-1} to GPY broth. The plates were incubated anaerobically at 40, 45 and 50 °C in anaerobic jars having a $H_2 + CO_2$ environment generated with a BBL GasPak (Becton Dickinson Microbiology Systems). Colonies that were yellow in colour and formed clear zones were selected as TLAB. In the experiments detailed in this study, cultures were routinely grown anaerobically in MRS broth (Difco) using mixed gases at 42 °C (for strains G 12, G 22, G 35^T, G 43 and G 44) and 37 °C (for reference strains). For the preparation of DNA for DNA–DNA hybridization studies and PCR, cells grown in MRS broth were harvested in the late-exponential phase and DNA was extracted essentially as described by Marmur (1961). The 16S rRNA genes of the five novel strains (corresponding to positions 27–1522 of the *Escherichia coli* 16S rRNA gene) were amplified by PCR. The six oligonucleotide primers used in the PCR amplification have been described previously (Mori *et al.*, 1997). The purified PCR products were sequenced directly using a dRhodamine dye terminator cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (model 377; Applied Biosystems). The resulting sequences were subjected to similarity searches against sequences within the public databases, to determine a possible phylogenetic classification for the novel strains. To determine the closest known relatives of the novel strains, based on 16S rDNA sequences, primary searches were performed in GenBank using the FASTA program (Devereux *et al.*, 1984). The database sequences representing the best matches to the 16S rDNA sequences of the novel strains were retrieved, and all of the sequences were aligned using the CLUSTAL W software (Thompson *et al.*, 1994). Sequences in the alignment were corrected manually; approximately 1500 nt, covering the whole range of 16S rDNA sequences, were used in the phylogenetic analysis. A distance matrix was calculated using DNADIST, contained within the PHYLIP package (Felsenstein, 1993), and the Kimura two-correction parameter, and a phylogenetic tree was constructed using NEIGHBOR, contained within PHYLIP, using the neighbour-joining method (Saitou & Nei, 1987). The reliability of the individual branches of the tree was assessed by the bootstrap method (1000 replications) using SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (all within the PHYLIP package). The novel strains were further distinguished from their nearest relatives on the basis of physiological and biochemical comparisons. Cells were grown anaerobically on MRS agar or in MRS broth at 42 °C (strains G 12, G 22, G 35^T, G 43 and G 44) or 37 °C (reference strains) for 24 h. Catalase and oxidase activity, gas production from glucose, ammonia production from arginine and isomeric type of lactic acid were determined for each strain after they had been cultured in MRS broth. Configuration of the lactic acid was determined by HPLC using an Aminex HPX-87H 300 × 7·8 mm column (Bio-Rad) by the method described by Otsuka *et al.* (1994). The production of organic acids by each strain was determined by ion-exclusion HPLC using the culture supernatants as samples by the method described by Hoshi *et al.* (1994). Carbohydrate utilization and acid production by the strains were determined using the API 50 CHL system (bioMérieux) with CHL medium, as recommended by the manufacturer. API strips were incubated at 42 °C (novel strains) and 37 °C (reference strains) for up to 48 h. Tests for growth of the strains at 15 and 45 °C were performed in MRS broth; results were recorded after 48 h. To evaluate the thermotolerance of the strains isolated from chicken faeces, optimum temperatures for growth of the novel strains were determined in comparison with reference strains. Each strain was cultured in half-strength MRS broth at temperatures ranging between 15 and 50 °C in waterbaths with standard

thermometers. Growth of the strains was determined by measuring the OD_{660} values of the culture broths with a 20D spectrophotometer (Milton Roy). To determine the optimum temperature for growth of each of the strains, the specific growth rate was calculated from each growth curve for each strain. Detection of diaminopimelic acid in the cell walls of the strains was done with 50–100 mg washed cells that had been suspended in 100 µl of potassium phosphate buffer (pH 6·8) containing 0·38 g KH_2PO_4 l^{-1}, 0·39 g K_2HPO_4 l^{-1} and 7·46 g KCl l^{-1}. The cell suspension was treated with 1 ml of 6 M HCl at 100 °C for 18 h. The hydrolysate was applied to a cellulose TLC plate (no. 1.05552; Merck), and developed using methanol/water/6 M HCl/pyridine (80 : 26 : 3 : 10, by vol.) (Komagata & Suzuki, 1987). The resulting spots were visualized with ninhydrin. For the analysis of the cellular fatty acid composition of the strains, whole-cell fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl. Methyl esters were extracted with n-hexane (Komagata & Suzuki, 1987) and analysed by using a GC-MS (Hitachi M7200A GC/3DQMS) equipped with a DB-5ms capillary column coated with 5 % phenylmethylpolysiloxane to a thickness of 250 nm (Hanada *et al.*, 2002). DNA base content of the strains was determined by the method of Tamaoka & Komagata (1984) using HPLC following enzymatic digestion of genomic DNA to deoxyribonucleosides. DNA–DNA hybridization was performed essentially according to the membrane method described by Johnson (1973). Tritium-labelled DNA for DNA–DNA hybridization studies was prepared by using the nick-translation system (Amersham Pharmacia Biotech).

Strains G 12, G 22, G 35^T, G 43 and G 44, which were isolated from chicken faeces, were Gram-positive, non-motile, non-spore-forming rods. When grown in MRS broth at 42 °C, cells appeared as short rods of 1 × 2–3 µm in size, which occurred singly, in pairs or occasionally in short chains. Good growth of the strains was observed when they were grown under anaerobic conditions in liquid and on solid media. After incubation on MRS agar for 2 days, colonies were white, circular, convex, smooth, opaque and approximately 1–1·5 mm in diameter. The 16S rDNA sequences of the novel strains were subjected to similarity searches against sequences within GenBank, to infer a possible phylogenetic classification for the strains. The results of these searches revealed that the five novel strains were members of the genus *Lactobacillus*. This classification was confirmed by a 16S-rDNA-based phylogenetic analysis (Fig. 1) and by nucleotide sequence similarity values. The phylogenetic tree revealed that the novel strains were included in the *Lactobacillus casei/Pediococcus* group of the genus *Lactobacillus* (Collins *et al.*, 1991). The closest relatives of strain G 35^T were found to be *Lactobacillus mucosae* DSM 13345^T (Roos *et al.*, 2000) and *L. fermentum* ATCC 14931^T, with moderate similarity values of 95·1 and 95·0 %, respectively (Fig. 1). The branching pattern of the strain cluster with *L. fermentum* ATCC 14931^T was supported by a high bootstrap value (95 %) (Fig. 1). A fuller phylogenetic tree showing the placement of the five novel strains within the genus *Lactobacillus* is available as supplementary data in IJSEM Online (Fig. I; http://ijs.sgmjournals.org).

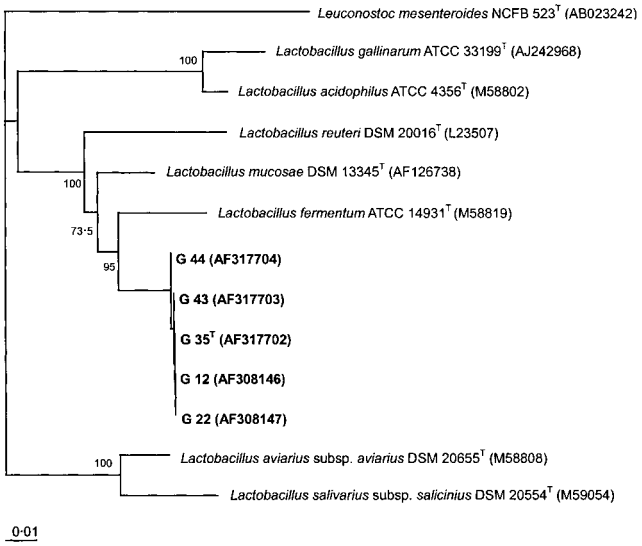

Fig. 1. Neighbour-joining tree, based on 16S rDNA sequences, showing the phylogenetic positions of strains G 12, G 22, G 35^T, G 43 and G 44 and some *Lactobacillus* spp. Bar, 0·01 substitutions per nucleotide position. Bootstrap values greater than 50 % (expressed as a percentage of 1000 replicates) are shown at the branch points.

The novel strains were further distinguished from their closest relatives, *L. mucosae* DSM 13345^T [obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)] and *L. fermentum* JCM 1173^T [obtained from the Japan Collection of Microorganisms (JCM)], on the basis of physiological and biochemical comparisons. None of the five novel strains exhibited oxidase or catalase activities. No diaminopimelic acid was detected in their cell walls. The five strains produced DL-lactic acid and gas from glucose. The amounts of lactate and other organic acids produced from glucose are shown in Table 1. Analyses revealed that the novel strains apparently produced about 100 mM of lactate and 67–95 mM of acetate as the main products when 111 mM glucose (20 g l^{-1}) in MRS broth was completely consumed, indicating that these organisms were obligately heterofermentative, as were the reference strains *L. fermentum* JCM 1173^T and *L. mucosae* DSM 13345^T. Results of carbohydrate fermentation tests determined using the API 50 CHL system are shown in Table 1. Only those carbohydrates that gave different fermentation patterns for the novel strains and the reference strains are listed. Clear differences were found between the novel strains and the reference strains in the fermentation of L-arabinose and galactose. All the novel strains fermented L-arabinose, whereas it was not fermented by the reference strains. However, the reference strains fermented galactose,

Table 1. Physiological and biochemical characteristics of the novel strains, G 12, G 22, G 35^T, G 43 and G 44, and selected reference organisms

Strains: 1, G 12; 2, G 22; 3, G 35^T; 4, G 43; 5, G 44; 6, *L. fermentum* JCM 1173^T; 7, *L. mucosae* DSM 13345^T. Organic acid production was measured after anaerobic culture of the strains in MRS broth containing 111 mM glucose at 42 °C (for the novel strains) and 37 °C (for *L. fermentum* and *L. mucosae*) for 24 h, after which time the glucose in the MRS broth was completely consumed. All the novel strains produced DL-lactate and gas from glucose. Acid production (determined using the API 50 CHL system) from glucose, ribose and aesculin, and ammonia production from arginine were observed in all the novel strains. +, Positive reaction; −, negative reaction.

Characteristic	1	2	3	4	5	6	7
Organic acid productivity (mM):							
Lactate	98·0	102·9	103·5	104·3	103·3	111·5	109·3
Succinate	0·6	0·4	0·4	0·4	0·4	0·7	10·7
Acetate	95·8	67·8	67·6	68·7	67·2	70·1	87·5
Propionate	1·5	1·3	1·4	1·3	1·6	1·9	2·1
Acid production from:							
L-Arabinose	+	+	+	+	+	−	−
D-Xylose	+	+	+	+	+	−	+
Galactose	−	−	−	−	−	+	+
D-Fructose	+	+	+	+	+	+	−
Lactose	−	−	−	−	−	+	−
Melibiose	+	+	+	−	−	+	+
Raffinose	+	+	+	+	−	+	+
Gluconate	+	+	+	+	−	+	+

which was not utilized by any of the novel strains. D-Xylose, which was fermented by all the novel strains, was not fermented by *L. fermentum* JCM 1173^T, the species most closely related to the novel strains. Also, fermentation of lactose occurred only in *L. fermentum* JCM 1173^T. Among the novel strains, strain G 44 showed a rather distinct fermentation pattern as demonstrated by its lack of raffinose and gluconate fermentation. The optimum temperature for growth of all the novel strains, determined from the specific growth rate, was 42 °C, whereas it was 40 °C for *L. fermentum* JCM 1173^T and *L. mucosae* DSM 13345^T. Also, *L. delbrueckii* subsp. *lactis* JCM 1248^T and *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T, known as thermophilic LAB, appeared to have their optimum growth at 40 °C, while *L. helveticus* JCM 1120^T showed its optimum growth at 37 °C. All the novel strains grew at 45 °C but not at 15 °C. The temperature range for growth of the novel strains was between 20 and 48 °C, except for strain G 35^T, which had a slightly higher permissive temperature (up to 50 °C) with a specific growth rate of 0·20 h^{-1} (Fig. II, supplementary data; http://ijs.sgmjournals.org). The temperature ranges for growth of the reference strains were 20–50 °C for *L. fermentum* JCM 1173^T and *L. mucosae* DSM 13345^T, 25–48 °C for *L. delbrueckii* subsp. *lactis* JCM 1248^T and *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T, and 25–46 °C for *L. helveticus* JCM 1120^T.

As shown in Table 2, strains G 12, G 22, G 35^T and G 43 contained an unsaturated straight chain acid, $C_{18:1}$, as their major component, and strain G 44 contained both $C_{18:1}$ and $C_{16:0}$ as major components. Straight chain acids of $C_{14:0}$ and $C_{18:0}$ (occasionally), and a cyclopropane acid, $C_{19:0\ cyclo}$, were also detected. Therefore, the profiles of the novel strains were clearly different from those of *L. fermentum* JCM 1173^T and *L. mucosae* DSM 13345^T, in which $C_{16:0}$ was found to be the major component. The DNA G+C contents of strains G 12, G 22, G 35^T, G 43 and G 44 were 50·6, 50·8, 50·5, 49·4 and 50·4 mol%, respectively. These G+C values are within the range reported for members of the genus *Lactobacillus*, i.e. 32–53 % (Kandler & Weiss, 1986). The DNA G+C contents of *L. fermentum* JCM 1173^T and *L. mucosae* DSM 13345^T, the species most closely related to the novel strains, were 53·5 and 48·4 mol%, respectively. DNA–DNA reassociation tests were performed between the five novel strains, *L. fermentum* JCM 1173^T and *L. mucosae* DSM 13345^T. Strain G 35^T and *L. fermentum* JCM 1173^T exhibited reciprocal values of DNA–DNA relatedness of 18·4 and 17·6 %. Reciprocal values between strain G 35^T and *L. mucosae* DSM 13345^T were 21·4 and 14·3 %. The other novel strains also showed low reciprocal values with the reference strains, suggesting that the novel strains isolated from chicken faeces represented a separate genomic species. The reciprocal values between the five novel strains were about 70 %, except for strain G 44. This strain showed levels of DNA homology of between 53 and 68 %. These values are not above the 70 % level seen as being indicative of single species status (Wayne *et al.*, 1987), but they may be seen as being indicative of closely related species or subspecies. According to the phylogenetic analysis of the 16S rRNA gene sequences of the novel strains and related *Lactobacillus* spp. (Fig. 1), strain G 44 formed a different branch to the other novel strains. Moreover, strain G 44 showed a rather distinct fermentation pattern as demonstrated by its lack of raffinose and gluconate fermentation (Table 1), and contained $C_{18:1}$ and $C_{16:0}$ fatty acids as major components, whereas the

Table 2. Cellular fatty acid profiles of the novel strains, G 12, G 22, G 35^T, G 43 and G 44, and selected reference organisms

Strains: 1, G 12; 2, G 22; 3, G 35^T; 4, G 43; 5, G 44; 6, *L. fermentum* JCM 1173^T; 7, *L. mucosae* DSM 13345^T. Strains were cultured anaerobically in MRS broth at 42 °C (for the novel strains) or 37 °C (for *L. fermentum* and *L. mucosae*) overnight. Each value shown is expressed as a percentage of the total fatty acids. ND, Not detected.

Fatty acid	1	2	3	4	5	6	7
Saturated fatty acid:							
$C_{14:0}$	0·5	1·2	0·9	1·5	2·4	0·9	4·4
$C_{16:0}$	5·2	8·5	6·6	7·2	41·3	68·1	69·0
$C_{18:0}$	ND	ND	ND	3·6	2·6	6·9	1·3
Unsaturated fatty acid:							
$C_{18:1}$	80·9	81·6	84·3	76·0	49·6	10·3	21·2
Branched-chain fatty acid:							
$C_{19:0\ cyclo}$	8·9	5·4	3·3	11·7	4·1	13·8	0·7
Summed features*:							
1	4·5	3·3	4·9	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	1·6
3	ND	ND	ND	ND	ND	ND	1·8

*Summed features represent groups of fatty acids that could not be separated by GC-MS. Summed feature 1 contained an unknown fatty acid at 15·00 min. Summed feature 2 contained an unknown fatty acid at 12·73 min. Summed feature 3 contained an fatty acid at 12·78 min.

other novel strains contained only an unsaturated straight chain fatty acid, $C_{18:1}$, as their major component (Table 2).

From the results presented here, it can be seen that the novel strains isolated from chicken faeces can be distinguished from all validly described *Lactobacillus* spp. on the basis of their biochemical, physiological and chemotaxonomic characteristics, their 16S rDNA sequences and the results of DNA–DNA hybridization studies. Consequently, we conclude that the TLAB strains isolated from chicken faeces represent a novel species within the genus *Lactobacillus*, for which we propose the name *Lactobacillus thermotolerans*.

Description of *Lactobacillus thermotolerans* sp. nov.

Lactobacillus thermotolerans (therm.o.tol.er′ans. Gr. n. *thermos* heat; L. pres. part. *tolerans* tolerating; N.L. part. adj. *thermotolerans* heat-tolerating).

Cells are Gram-positive, non-motile, non-spore-forming, catalase-negative rods of 1 × 2–3 μm in size, which occur singly, in pairs or as short chains. After anaerobic growth at 42 °C for 2 days, colonies on MRS agar are 1–1·5 mm in diameter, white, circular, convex, smooth and opaque. Obligately heterofermentative and produces D- and L-lactic acid. Grows up to 50 °C, but not at 15 °C; optimum temperature for growth is 42 °C. Aesculin is hydrolysed. Arginine is cleaved. Hydrogen sulfide is not produced. Nitrate is not reduced. Gelatin is not liquefied. No dextran is produced from sucrose. Acid is produced from glucose, ribose, L-arabinose, D-xylose and D-fructose. The majority of strains also ferment melibiose and D-raffinose. Glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β-xyloside, dulcitol, inositol, methyl α-D-mannoside, arbutin, lactose, inulin, melezitose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate are not fermented. *meso*-Diaminopimelic acid is not present in the cell wall. DNA G+C content of the type strain is 50·5 mol%. Major cellular fatty acid is a straight chain unsaturated acid, $C_{18:1}$. Isolated from the faeces of chickens in Thailand. The type strain is G 35^T (=DSM 14792^T =JCM 11425^T).

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