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Article *in* International Journal of Systematic and Evolutionary Microbiology · June 2005

DOI: 10.1099/ijs.0.63399-0 · Source: PubMed

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Lysobacter concretionis sp. nov., isolated from anaerobic granules in an upflow anaerobic sludge blanket reactor

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The taxonomic positions of *Lysobacter* species with validly published names and a novel strain Ko07^T, which was newly isolated from an upflow anaerobic sludge blanket reactor treating wastewater from a brewery, were (re)estimated on the basis of results obtained by using a polyphasic taxonomy approach. Phylogenetic inference based on 16S rRNA gene sequences showed that strain Ko07^T and all *Lysobacter* species with validly published names clustered together in a phylogenetic branch within the class '*Gammaproteobacteria*'. The sequence similarity of strain Ko07^T to the type strains of established *Lysobacter* species was in the range 94·9–96·7 %. Ubiquinone Q-8 and branched fatty acids, $C_{11:0}$ iso, $C_{15:0}$ iso, $C_{16:0}$ iso, iso $C_{17:1}\omega 9c$ and $C_{11:0}$ iso 3OH, predominantly appeared in strain Ko07^T as well as in all type strains of the recognized *Lysobacter* species. The DNA–DNA hybridization values of strain Ko07^T with those of recognized *Lysobacter* species were estimated to be 2–20 %. Despite sharing common taxonomic features in important phenotypic characteristics, such as gliding movement, long-rod shape and proteolytic activity, strain Ko07^T could be distinguished from the *Lysobacter* species with validly published names by its low DNA–DNA hybridization value, a comparatively low DNA G+C content (63·8 mol%), substrate utilization and some physiochemical characteristics. On the basis of the results obtained in this study, it is proposed that strain Ko07^T should be classified as representing a novel member of the genus *Lysobacter*, for which the name *Lysobacter concretionis* sp. nov. is proposed. The type strain is Ko07^T (=KCTC 12205^T=DSM 16239^T).

The genus *Lysobacter* was established by Christensen & Cook (1978) for non-fruiting-body, gliding bacteria with high G+C content. The *Lysobacter* species with validly published names are *Lysobacter antibioticus*, *Lysobacter brunescens*, *Lysobacter enzymogenes* and *Lysobacter gummosus*. Although the description of an antibiotic-producing species, '*Lysobacter lactamgenus*', has been published (Ono *et al.*, 1984; Kimura *et al.*, 1996), its taxonomic position has not yet been confirmed and its name has not been validated; thus, no novel species of the genus *Lysobacter* has been proposed since 1978. Fatty acid profiles for two type strains, *L. enzymogenes* ATCC 29487^T and *L. antibioticus* ATCC 29479^T, are available from the MIDI database, but identifying bacterial strains as belonging to the genus *Lysobacter* by using the MIDI system only is insufficient. Furthermore, as the *Lysobacter* species were classified originally on the basis of only a few phenotypic characteristics (Christensen & Cook, 1978), it was unclear whether they could still retain their taxonomic positions if phylogenetic and/or chemotaxonomic features were considered.

Anaerobic granules are bacterial aggregates that result from the flocculation of sludge in an upflow anaerobic sludge blanket (UASB) reactor (de Zeeuw & Lettinga, 1980). They are thought to be composed of micro-organisms, inorganic nuclei and extracellular polymers (Fukuzaki *et al.*, 1991; Shen *et al.*, 1993). Great attention has been paid to the internal structure and catalytic activities of the granules (MacLeod *et al.*, 1990; Schmidt & Ahring, 1996). In our laboratory, the relationship between the structure and resistance to toxic chemicals in anaerobic granules from a

Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain Ko07^T, *Lysobacter gummosus* ATCC 29489^T and *Lysobacter brunescens* ATCC 29482^T are AB161359, AB161361 and AB161360, respectively.

16S rRNA gene sequence identity data for strain Ko07^T and closely related bacteria are available as supplementary material in IJSEM Online.

brewery wastewater-treatment UASB reactor was studied (Bae & Lee, 1999; Bae *et al.*, 2000). In a series of studies, we attempted to isolate micro-organisms from the anaerobic granules in order to investigate the community structure based on a culture system. Interestingly, the granules contained aerobic bacteria, even though they had been kept under anaerobic conditions for 2 years. Strain Ko07^T was one of the dominant bacterial isolates to grow under aerobic conditions. In this study, a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences, DNA–DNA relatedness, and chemotaxonomic and phenotypic properties, was used to determine the precise taxonomic position of strain Ko07^T. The results obtained indicate that strain Ko07^T should be classified as representing a novel species of the genus *Lysobacter*, and that it can be clearly distinguished from *Lysobacter* species with validly published names. In this report, we propose Ko07^T as the type strain of a novel species, for which the name *Lysobacter concretionis* sp. nov. is proposed. We also present the interspecies relationships of *Lysobacter* species newly inferred by the polyphasic approach used.

For the isolation of aerobic bacteria, brownish-black granules (around 2 mm in diameter) from a brewery wastewater-treatment UASB reactor, which had been operated anaerobically for 2 years, were homogenized by using an Ace Homogenizer (Nihonseiki Kaisha). The suspension was spread on R2A agar plates (Difco), after being serially diluted with 50 mM phosphate buffer (pH 7·0). The plates were incubated at 30 °C for 2 weeks. Single colonies were purified by transfer onto fresh plates, followed by a second incubation under the same conditions. The purified colonies were tentatively identified by partial 16S rRNA gene sequencing. Ko07^T was one of the dominant isolates to grow on the plates under aerobic conditions. The isolate was deposited in the Korean Collection for Type Cultures as KCTC 12205^T (=DSM 16239^T). *Lysobacter* species with validly published names were obtained from BCCM^{TM}/LMG (Belgian Co-ordinated Collections of Microorganisms/Laboratorium voor Microbiologie).

Morphological characteristics and motility were observed by using differential interference contrast microscopy. Gram staining and tests for catalase and oxidase were performed according to protocols outlined by Smibert & Krieg (1981). Some physiological properties and substrate utilizations were determined by using API 20 NE, API ID 32 GN and API 50 CHB kits (bioMérieux). Degradation of chitin, starch and Tween 80 was also investigated, by using the protocols outlined by Atlas (1993). Cells grown on R2A agar plates at 30 °C for 2 days were used for the experiments mentioned above. To determine whether strain Ko07^T had nitrite reductase genes (*nirK* and *nirS*), PCR was performed by using two different primer systems developed by Braker *et al.* (1998), *nir*K1F/*nir*K5R for *nirK* and *nir*S1F/*nir*S6R for *nirS*, under various thermal profiles.

Cells grown on R2A agar plates for 2 days were used for the analysis of cellular fatty acid composition. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed by using a gas chromatograph (6890; Hewlett Packard), equipped with the Microbial Identification software package (Sasser, 1990). Ubiquinones were extracted from cells grown in liquid medium with the same composition as R2A but without the agar, according to the protocol described by Komagata & Suzuki (1987). The ubiquinones were analysed by HPLC using a C_{18} reversed-phase column at 254 nm, and a mobile phase comprising acetonitrile and iso-propanol (65 : 35, v/v) at 1 ml min^{-1}.

Genomic DNA was isolated by using the protocol described by Schmidt *et al.* (1991). RNA was removed by treatment with a mixture of RNase A and T1 (each at 20 U ml^{-1}), at 30 °C for 1 h. The DNA extract was used for the genomic and the phylogenetic studies. The genomic DNA G+C content was determined as described by Mesbah *et al.* (1989). The DNA–DNA hybridization value was estimated fluorometrically by using photobiotin-labelled DNA probes and a microdilution well, according to the method of Ezaki *et al.* (1989).

The 16S rRNA gene was amplified by using the bacterial universal primer set 9F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1512R [5′-ACG G(A/T/C)T ACC TTG TTA CGA CTT-3′]. The PCR product was purified with a GFX PCR DNA and a Gel Band Purification kit (Amersham Biosciences). It was sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an automatic DNA sequencer (model 310; Applied Biosystems). For full sequencing, the following primers were also used: 519F (5′-CAG CAG CCG CGG TAA TAC-3′), 907F [5′-AAA CTC AAA (G/T)GA ATT GAC GG-3′], 536R (5′-GTA TTA CCG CGG CTG CTG-3′) and 1100R (5′-GGG TTG CGC TCG TTG-3′). The partial sequences were aligned and combined by using the BioEdit program (Hall, 1999). A BLAST search was performed to obtain the 16S rRNA gene sequences from related taxa in GenBank. These collected sequences were aligned and edited with the CLUSTAL_X program (Thompson *et al.*, 1997) and the BioEdit program (Hall, 1999), respectively. The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983), and the phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA 2 program (Kumar *et al.*, 2001). A bootstrap method was used to obtain the confidence levels for the neighbour-joining analysis, with a 1000 bootstrap data set (Felsenstein, 1985).

Strain Ko07^T is aerobic, Gram-negative and rod-shaped (0·7–1·0 × 1·0–13·5 μm). The size of the cells changed with time. Cells grown on R2A agar plates for 5 days appeared as curved rods longer than 5 μm, whereas cells incubated for 30 days were shorter, as shown in Fig. 1. Flagella movement was not observed by microscopy. The colonies grown on R2A agar plates for 2 days were yellowish in colour, smooth, circular, convex and non-glossy, and 2–4 mm in

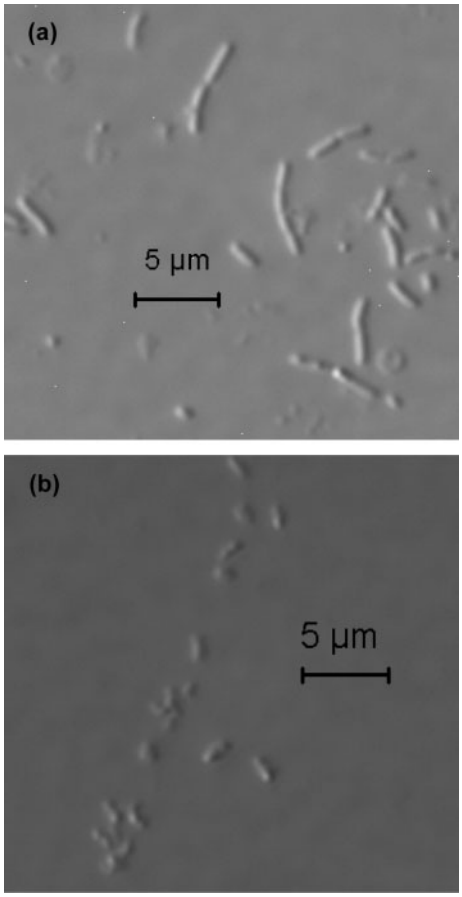

Fig. 1. Morphological features of strain Ko07^T grown on an R2A agar plate for 5 days (a) and 30 days (b), observed by differential interference contrast microscopy.

diameter. The colour changed to a weak brown on longer incubation ($\geqslant$3 weeks). Spreading and swarming colonies, indicating gliding movement, were observed on an R2A agar plate that was incubated for 1 month, and on YA [0·5 % (w/v) yeast extract plus 1·5 % Bacto agar] and CCA (0·2 % tryptone plus 1·5 % Bacto agar) plates that had been incubated for 2 weeks. Tests for catalase and oxidase were positive. Gelatin was liquefied within 1 day, indicating proteolytic activity; this was also observed for all the type strains of species of the genus *Lysobacter*. The capability to degrade polysaccharide on chitin and starch that is present in some *Lysobacter* species (Christensen & Cook, 1978) was not observed in strain Ko07^T. Lipase activity on Tween 80 was also not detected, whereas most *Lysobacter* species have this activity (Christensen & Cook, 1978). Differences in phenotypic characteristics including substrate utilization between Ko07^T and type strains of some *Lysobacter* species are summarized in Table 1; the results suggest that Ko07^T is different from the other type strains in phenotypic aspects. Nitrate was not reduced to nitrite. The nitrite reductase genes *nirK* and *nirS* were not amplified by the primer systems *nir*K1F/*nir*K5R and *nir*S1F/*nir*S6R, respectively. Ko07^T grew well at 25–30 °C and pH 6·8–7·5, but not at 4 or 45 °C.

Q-8 was the major quinone in strain Ko07^T and in all the type strains of the recognized *Lysobacter* species. The predominant cellular fatty acids observed in Ko07^T and in the type strains of all the recognized *Lysobacter* species were $C_{16:0}$, $C_{11:0}$ iso, $C_{11:0}$ iso 3OH, $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$ iso, $C_{17:0}$ iso, iso $C_{17:1}\omega9c$ and summed feature 4 ($C_{15:0}$ iso 2OH/$C_{16:1}\omega7c$), although their relative amounts were slightly different (Table 1). The fatty acid profiles obtained matched well those of *L. antibioticus* ATCC 29479^T and *L. enzymogenes* ATCC 29487^T from MIDI, and also those of strains N4-7 and C3, which have been identified as belonging to the genus *Lysobacter* (Sullivan *et al.*, 2003). The results indicate that strain Ko07^T has chemotaxonomic features in common with the recognized *Lysobacter* species. The genomic DNA G+C content of strain Ko07^T was 63·8 mol%, which is lower than those of the type strains of recognized *Lysobacter* species, 65·7–69·2 mol% (Table 2), indicating that strain Ko07^T should be considered as representing a novel species of the genus *Lysobacter*.

For the phylogenetic analysis, an almost complete 16S rRNA gene sequence of strain Ko07^T (1481 nt) was obtained. 16S rRNA gene sequences of *L. gummosus* ATCC 29489^T (1482 nt) and *L. brunescens* ATCC 29482^T (1467 nt) were also determined in this study; those of *L. enzymogenes* DSM 2043^T and *L. antibioticus* DSM 2044^T were obtained from GenBank. As shown in the phylogenetic tree (Fig. 2), strain Ko07^T is closely clustered with the type strains of *Lysobacter* species with validly published names and unnamed *Lysobacter* species, in an independent phylogenetic branch within the class '*Gammaproteobacteria*' that is clearly distinct from the neighbouring genera, *Xanthomonas* and *Thermomonas*. The sequence similarity of strain Ko07^T to those organisms in the cluster was 94·9–96·7 % (see Supplementary Table in IJSEM Online). The high bootstrap value (98 %) provides strong support for the inclusion of strain Ko07^T in the genus *Lysobacter*. This was also supported by the aforementioned taxonomic characteristics and the typical phenotypic properties described originally by Christensen & Cook (1978), e.g. gliding movement, long-rod shape, proteolytic activity and the high G+C content of genomic DNA. *L. brunescens* ATCC 29482^T was also placed in the *Lysobacter* cluster, but in a branch with a low bootstrap value, 42 % (Fig. 2). This organism has a sequence similarity of 94·1–96·1 % with some *Thermomonas* species and *Xanthomonas melonis*, of which some values are higher than those for type strains of the recognized *Lysobacter* species (95·2–95·6 %) (see Supplementary Table in IJSEM Online). In spite of the weak phylogenetic relationship with other *Lysobacter* species, strain Ko07^T still shares the common features of members of the genus *Lysobacter*, with respect to the presented phenotypic and chemotaxonomic aspects (Table 1).

In order to determine whether strain Ko07^T represents a novel species of the genus *Lysobacter*, DNA–DNA hybridization values between Ko07^T and the recognized *Lysobacter* species were estimated. The DNA–DNA hybridization

Table 1. Comparison of characteristics of strain Ko07^T and *Lysobacter* species with validly published names

Taxa: 1, Ko07^T; 2, *L. enzymogenes* DSM 2043^T; 3, *L. gummosus* ATCC 29489^T; 4, *L. antibioticus* DSM 2044^T; 5, *L. brunescens* ATCC 29482^T. All strains could liquefy gelatin. +, Positive; −, negative.

Characteristic	1	2	3	4	5
Cell size (μm)	0·7 × 1·0–13·5	0·5 × 38·0	0·4 × 2·0	0·4 × 6·5	0·3 × 11·0
$NO_3^- \rightarrow NO_2^-$ / $NO_2^- \rightarrow N_2$	−/−	−/−	−/−	−/− (+/−)*	−/−
Aesculin hydrolysis	−	+	+	+	+
Indole production	−	−	−	−	+
Glucose acidification	−	+	−	+	−
Arginine dihydrolase	−	−	−	−	+
Urease	−	−	−	−	+
β-Galactosidase	−	+	+	+	−
Growth on:					
N-Acetylglucosamine	−	+	+	−	−
Maltose	−	+	+	+	−
Acetate	+	−	+	−	+
Glycogen	+	+	+	+	−
L-Serine	−	+	−	−	−
D-Glucose	−	+	+	−	−
Salicin	−	+	−	−	−
D-Melibiose	−	+	+	−	−
Arabinose	−	−	+	−	−
Valerate	+	−	+	+	−
Citrate	−	+	+	−	−
Histidine	−	−	+	−	−
3-Hydroxybutyrate	+	+	+	+	−
L-Proline	+	+	+	+	−
Mannose	−	+	−	+	−
Malate	−	+	−	−	−
Major ubiquinone	Q-8	Q-8	Q-8	Q-8	Q-8
Cellular fatty acids (%):†					
$C_{10:0}$	−	−	−	0·88	1·29
$C_{14:0}$	0·82	1·15	−	1·67	0·67
$C_{16:0}$	**2·43**	**5·05**	**7·79**	**10·45**	**1·48**
$C_{16:1}\omega7c$ alcohol	−	−	1·06	3·00	0·75
$C_{16:1}\omega11c$	−	−	−	5·54	−
$C_{11:0}$ iso	**6·42**	**4·25**	**3·95**	**3·47**	**7·03**
$C_{11:0}$ iso 3OH	**5·55**	**6·01**	**4·44**	**5·17**	**5·61**
$C_{14:0}$ iso	2·56	−	−	2·25	6·67
$C_{15:0}$ iso	**36·06**	**42·98**	**39·29**	**19·87**	**23·79**
$C_{15:0}$ anteiso	**1·15**	**1·93**	**5·05**	**5·43**	**1·73**
$C_{16:0}$ iso	**19·92**	**3·00**	**4·66**	**12·08**	**21·88**
$C_{16:0}$ iso H	−	−	−	−	2·74
$C_{17:0}$ iso	**2·93**	**4·35**	**12·96**	**1·86**	**0·68**
Iso $C_{17:1}\omega9c$	**13·9**	**8·84**	**12·60**	**4·53**	**11·48**
$C_{17:0}$ cyclo	2·49	10·55	−	8·06	−
Summed feature 4‡	**0·91**	**8·27**	**4·88**	**11·16**	**9·00**
Summed feature 7‡	−	1·58	−	1·33	−

*Results in parentheses were taken from Christensen & Cook (1978).

†Bold type indicates predominant fatty acids.

‡Summed feature 4 contained $C_{15:0}$ iso 2OH and/or $C_{16:1}\omega7c$; summed feature 7 contained $C_{18:1}\omega7c/\omega9t/\omega12t$ and/or $C_{18:1}\omega7c/\omega9c/\omega12t$, which could not be separated by GLC with the Microbial Identification System.

Table 2. Genetic properties of strain Ko07^T and recognized *Lysobacter* species

Taxa: 1, Ko07^T; 2, *L. enzymogenes* DSM 2043^T; 3, *L. gummosus* ATCC 29489^T; 4, *L. antibioticus* DSM 2044^T; 5, *L. brunescens* ATCC 29482^T.

Strain	DNA G+C content (mol%)	DNA–DNA hybridization (%)				
		1	2	3	4	5
1. Ko07^T	63·8	100				
2. *L. enzymogenes* DSM 2043^T	69·0*	20±2	100			
3. *L. gummosus* ATCC 29489^T	65·7*	2±2	25±2	100		
4. *L. antibioticus* DSM 2044^T	69·2*	14±3	23±2	14±2	100	
5. *L. brunescens* ATCC 29482^T	67·7±0·1*	20±4	33±3	24±1	12±2	100

*Results were taken from Christensen & Cook (1978).

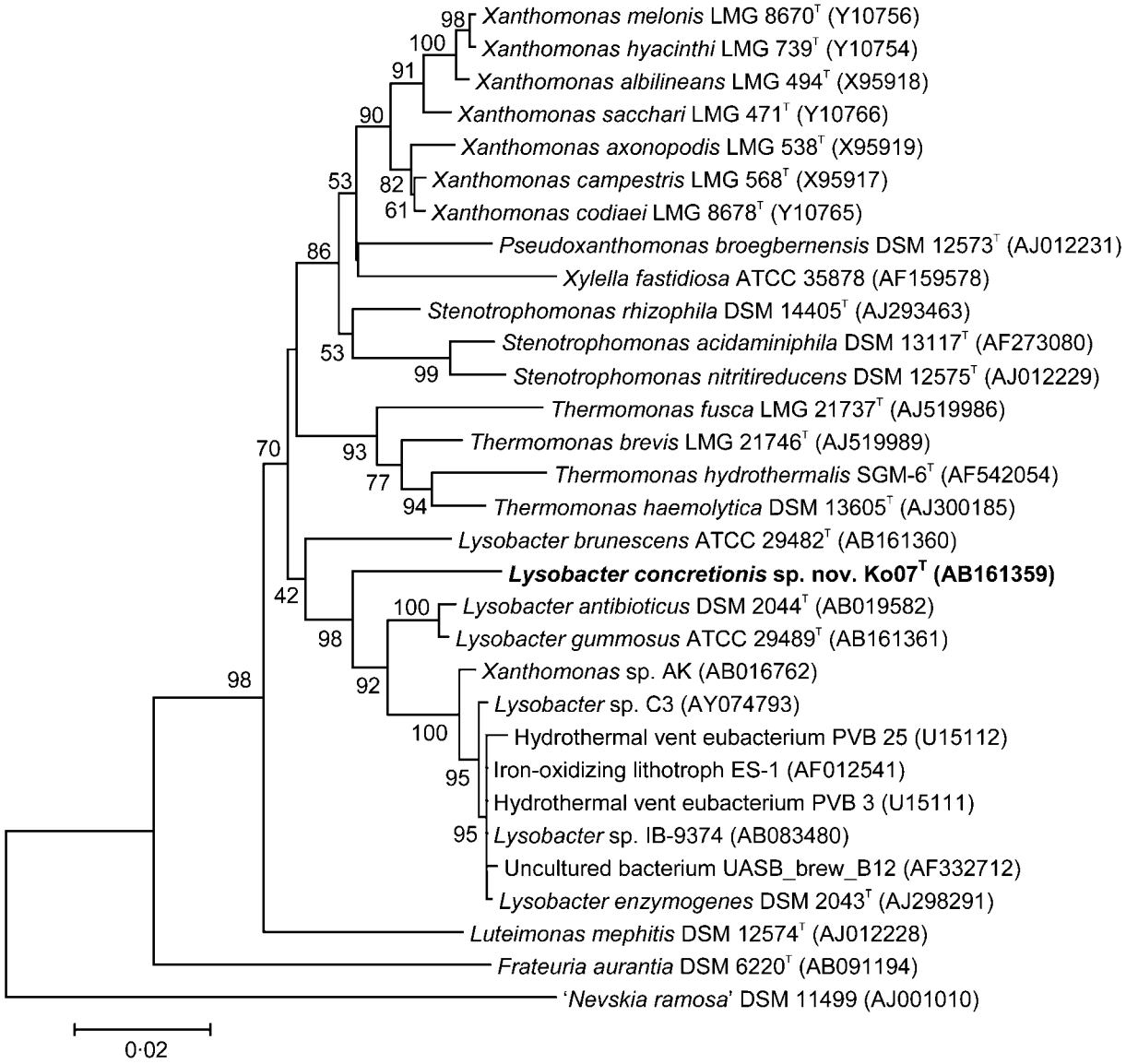

Fig. 2. Phylogenetic relationships of strain Ko07^T with recognized *Lysobacter* species and other related species of the class '*Gammaproteobacteria*'. The tree was constructed by using the neighbour-joining method based on 16S rRNA gene sequences. For the alignment, 1343 nucleotides were used from each sequence. Bar, 0·02 substitution per nucleotide position. Bootstrap values (expressed as a percentage of 1000 replications) are shown at the branch points.

values obtained were 2–20 % (Table 2), which are low enough to confirm that Ko07^T should be classified as representing a novel *Lysobacter* species. Its lower DNA G+C content (63·8 mol%) and the differences in phenotypic characteristics (Table 1) also suggest that Ko07^T should be considered as a novel species. Therefore, on the basis of the results obtained in this study, we propose that strain Ko07^T should be classified as a novel species within the genus *Lysobacter*, for which the name *Lysobacter concretionis* sp. nov. is proposed.

Description of *Lysobacter concretionis* sp. nov.

Lysobacter concretionis (con.cret.i.on′is. L. gen. n. *concretionis* of a compacting, condensing, congealing).

Gram-negative, aerobic rod or filamentous shape, of various sizes (0·7–1·0 × 1·0–13·5 μm), after growth on R2A agar plates at 25 °C for 10 days. Does not move by means of flagella, but by gliding on the surface of the agar. Colonies grown on an R2A agar plate for 2 days are yellow and circular, but become a weak brownish-yellow-coloured swarming form after 1 month. Optimum growth temperature and pH are 25–30 °C and 6·8–7·5, respectively. The DNA G+C content is 63·8 mol%, as determined by HPLC. Q-8 is the predominant quinone. The major cellular fatty acids are $C_{16:0}$ (2·4 %), $C_{11:0}$ iso (6·42 %), $C_{15:0}$ iso (36·1 %), $C_{16:0}$ iso (19·9 %), iso $C_{17:1}\omega 9c$ (13·9 %) and $C_{11:0}$ iso 3OH (5·6 %). Does not reduce nitrate. Can liquefy gelatin, but cannot degrade chitin, Tween 80 or starch. Other phenotypic characteristics, such as substrate utilization and enzyme production, are summarized in Table 1.

The type strain is Ko07^T (=KCTC 12205^T=DSM 16239^T), isolated from a UASB reactor treating brewery wastewater.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Science & Technology (Grant MG02-0101-001-2-2-0), Republic of Korea.

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