Cerasibacillus quisquiliarum gen. nov., sp. nov., isolated from a semi-continuous decomposing system of kitchen refuse

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A moderately thermophilic and alkaliphilic bacillus, which had been reported and designated BLx (Haruta *et al*., 2002), was isolated from a semi-continuous decomposing system of kitchen refuse. Cells of strain BLx^T were strictly aerobic, rod-shaped, motile and spore forming. The optimum temperature and pH for growth were approximately 50 °C and pH 8–9. Strain BLx^T was able to grow at NaCl concentrations from 0·5 to 7·5 %, with optimum growth at 0·5 % NaCl. The predominant menaquinone was MK-7, and the major fatty acid was iso-$C_{15:0}$. Phylogenetic analysis showed that strain BLx^T was positioned in an independent lineage within the cluster that includes the genera *Virgibacillus* and *Lentibacillus* in *Bacillus* rRNA group 1. Strain BLx^T exhibited 16S rDNA similarity of 92·8–94·8 % to *Virgibacillus* species and 92·3 % to *Lentibacillus salicampi*. Phenotypic, chemotaxonomic and phylogenetic analyses supported the classification of strain BLx^T in a novel genus and species. *Cerasibacillus quisquiliarum* gen. nov., sp. nov. is proposed on the basis of phenotypic, chemotaxonomic and phylogenetic data. The type strain is BLx^T (DSM 15825^T = IAM15044^T = KCTC 3815^T).

Biological treatment is an efficient way to manipulate solid organic wastes that are produced in agriculture, industry and in residential areas. Composting by a microbial community is one solution for waste treatment. During the composting process, the temperature of the compost often reaches 80 °C because of the heat produced by the microbial community from the decomposition of organic matter (Finstein *et al.*, 1975). Studies of microbial communities during the composting process have been performed by several researchers using cultivation-based methods (Beffa *et al.*, 1996; Fujio & Kume, 1991; Pedro *et al.*, 2003; Nakasaki *et al.*, 1985; Ryckeboer *et al.*, 2003; Strom, 1985a, b). Various species of bacteria have been isolated from composting processes, and many of the isolates are thermophilic (or moderately thermophilic) and belong to the family *Bacillaceae*. They are thought to play important roles in the composting process.

We have been analysing the microbial community present during the decomposition of kitchen refuse in a laboratory-scale semi-continuous decomposing system (Haruta *et al.*, 2002). As the decomposition proceeded under conditions of high temperature (up to nearly 55 °C) and alkalinity (pH 8–9), a *Bacillus licheniformis*-like band was detected reproducibly under multiple operations using denaturing gradient gel electrophoresis (DGGE). The strain that was represented by the band was designated BLx. Fluorescence *in situ* hybridization (FISH) analysis reproducibly revealed that BLx was present as the dominant strain in the community. Furthermore, sequences with high similarity to that of strain BLx have been reported from other organic substance degradation processes: AB028110 (Kurisu *et al.*, 2002) and AB029411(Ishii *et al.*, 2000). Strain BLx is thought to play an important role in the degradation of organic materials because it appears to exist in decomposing systems under conditions of high temperature and alkalinity. In this paper, we report the isolation and characterisation of strain BLx from a kitchen refuse decomposing system. Based on our results, we propose *Cerasibacillus quisquiliarum* gen. nov., sp. nov., for strain BLx^T.

Strain BLx^T was isolated from a decomposed refuse sample from a decomposing system that was operated as previously described (Haruta *et al.*, 2002). Samples (10 g) were collected 12 h after addition of standard kitchen garbage and homogenized in 60 ml saline (0·85 % NaCl) with a POLYTRON homogenizer (Kinematica) at

Abbreviations: DAP, diaminopimelic acid; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain BLx^T is AB107894.

15 000 r.p.m. for 5 min. The homogenized sample was serially diluted with saline and spread on tryptic soy agar plates (TSA; tryptic soy broth, Difco, containing 1·5 %, w/v, agar). Plates were incubated at 37 or 50 °C. Colonies with different morphologies were isolated and purified at least three times. Isolates were then cultivated in tryptic soy broth (TSB; Difco). Cells were harvested by centrifugation, washed twice with saline and stored at −20 °C for DNA preparation. Isolates that grew on the plates were stored at 4 °C for further cultivation.

Strain BLx^T was screened by specific PCR from the isolates. Frozen cell pellets were suspended in sterilized distilled water, boiled for 5 min, and cooled immediately on ice. Extracted genomic DNA was recovered by centrifugation and used as a PCR template. Almost complete 16S rDNA sequences were amplified from these extracted genomic DNAs using the universal primers F (5′-AGAGTTTGA-TCCTGGCTCAG-3′; *Escherichia coli* positions 8–27) and R (5′-ACGGCTACCTTGTTACGACT-3′; *E. coli* positions 1512–1492) (Devereux *et al.*, 1995). Amplified products were applied to specific PCR, which was performed using the BLx^T-specific primer, BL3R (5′-ACCGRCCTATTCG-AACGGTAC-3′), whose sequence corresponds to a BLx^T-specific FISH probe, BL3 (Haruta *et al.*, 2002), and the primer F. The composition of 20 µl of PCR cocktail was as follows: PCR Gold buffer (Perkin-Elmer Biosystems), 1·5 mM $MgCl_2$, 0·2 mM dNTPs, 2·5 µM primer F, 2·5 µM BL3R, 0·5 U AmpliTaq Gold (Perkin-Elmer Biosystems) and 1 µl template (16S rDNA fragment). The conditions for specific PCR were as follows: 95 °C for 5 min, 30 cycles at 93 °C for 30 s and 72 °C for 30 s, 94 °C for 1 min, and finally 72 °C for 5 min. PCR products were confirmed by 2·0 % (w/v) agarose gel electrophoresis. Specific PCR products were identified from two isolates that were obtained from a sample whose temperature and pH were 46 °C and 8·6, respectively. 16S rDNA sequence analysis was performed with one of the isolates. The sequence of variable region 3 of the 16S rDNA of the isolate was identical to that of the DGGE bands of the *B. licheniformis*-like strain X68416. Based on this information, the isolate was identified as strain BLx^T.

Strain BLx^T was maintained in TSB or on TSA (pH 8·5, adjusted with NaOH). Cell wall and menaquinone composition and DNA were examined with cells cultured in these media. Marine agar (MA; Difco) was used for fatty acid methyl ester (FAME) analysis.

Colony morphology was observed after 4 days incubation on TSA at 37 °C. For sporulation, BLx^T was cultivated for 12 days at 37 °C on TSA containing the following trace elements: $MgSO_4$ (1 mM), $Ca(NO_3)_2$ (1 mM), $MnCl_2$ (10 µM) and $FeSO_4$ (1 µM). One loopful of colony material was swabbed onto a glass slide. Dried cells were observed with an Olympus BX60 phase contrast microscope. Gram reaction, oxidase production and catalase production were determined by conventional procedures. For anaerobic growth, BLx^T was cultivated on TSA in an AnaeroPack (Mitsubishi Gas Chemical) pouch bag with an oxygen absorber. The API 50 CHB system (bioMérieux) was employed, following the manufacturer's instructions, to identify the following biochemical properties: acid production from carbohydrates, hydrolysis of aesculin, gelatin, starch and urea, H_2S production and nitrate reduction. Casein hydrolysis was detected with TSA containing 5 % skimmed milk. BLx^T was cultivated at 50 °C in TSB containing various concentrations of NaCl and optical density at 660 nm was measured during incubation. BLx^T was cultivated in TSB at a range of temperatures (26·5–55 °C).

Genomic DNA was extracted from cells according to the procedure of Zhu *et al.* (1993). The isomer type of diaminopimelic acid (DAP) was determined by the method described by Schleifer (1985). Menaquinone was extracted from freeze-dried cells and analysed by the method of Collins & Jones (1982). For the analysis of fatty acid composition, one loopful of cell mass grown on MA for 7 days at 30 °C was obtained and FAMEs were prepared and identified following the Microbial Identification System (MIDI) instructions. The G+C content was determined by reverse-phase HPLC, as described by Tamaoka & Komagata (1984).

The almost complete 16S rDNA was amplified by PCR with the two universal primers, F and R, mentioned above. The PCR product was subjected to agarose gel electrophoresis and purified with a QIAEX gel extraction kit (QIAGEN). The purified 16S rDNA was sequenced directly using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM model 377 genetic analyser (Perkin-Elmer). The sequences obtained were aligned using the CLUSTAL X program (version 1.81; Thompson *et al.*, 1997). The multiple sequence alignment was then corrected manually when necessary. A phylogenetic tree was constructed from evolutionary distance data (Kimura, 1980) by applying the algorithm of the neighbour-joining method (Saitou & Nei, 1987) using MEGA version 2.1 (Kumar *et al.*, 2001). To evaluate the robustness of the inferred tree, the bootstrap resampling method of Felsenstein (1985) was used with 1000 replicates.

A number of strains belonging to the family *Bacillaceae* have been isolated from decomposition processes of solid organic waste (Fujio & Kume, 1991; Ryckeboer *et al.*, 2003; Strom, 1985b). Most of the strains are thermophilic and are thought to play important roles during the decomposition process. Molecular biological analyses have been used to observe the microbial community during these processes (Dees & Ghiorse, 2001; Ishii *et al.*, 2000; Peters *et al.*, 2000). Compared with conventional cultivation/isolation-based techniques, molecular biological techniques can indicate the existence of uncultured or viable but non-culturable (VBNC) microorganisms. However, these techniques are not specific for live cells, since DNA is extracted not only from living cells but also from dead cells in environmental samples. In addition, biases may have occurred because of differences in DNA-extraction efficiencies in different samples or during PCR (LaMontagne *et al.*, 2002; Watanabe *et al.*, 2001). These disadvantages should be considered when applying molecular biological techniques.

Strain BLx^T has been identified in the decomposition of kitchen refuse using the molecular biological technique, DGGE analysis (Haruta *et al.*, 2002). *B. licheniformis* was its closest relative, based on the sequence of the DGGE bands. Moreover, FISH analysis indicated that BLx^T was the dominant strain. Based on this information, the isolation of BLx^T on agar plates would have been expected, but was actually quite infrequent. The low rate of appearance on plates could be due to the reduced viability of BLx^T in the samples collected and/or the inability of BLx^T to compete effectively with other microorganisms for nutrients and/or inappropriate isolation conditions. The addition of an extract of decomposed matter was not effective in the isolation of BLx^T.

Strain BLx^T is a motile Gram-positive rod, 0·8 × 2·5–5·0 μm in size. A spherical terminal endospore was found in the cell (Fig. 1). The colonies were pigmented (light yellowish-brown), round and opaque after 4 days incubation on TSA plates at 37 °C. Further incubation at 37 °C or growth at 50 °C on TSA resulted in the appearance of amorphous translucent colonies.

Sequence analysis of the 16S rDNA was carried out with the PCR-amplified fragment (1484 bp). The sequence was aligned with 25 sequences of related species collected from the public databases. The alignment of 1331 bp of each species' sequence was used for phylogenetic analysis. Phylogenetic tree analysis based on the neighbour-joining algorithm showed that strain BLx^T belongs to *Bacillus* rRNA group 1 and is positioned within the cluster that includes the genera *Virgibacillus* (Arahal *et al.*, 1999, 2000; Heyndrickx *et al.*, 1999; Heyrman *et al.*, 2003; Garabito *et al.*, 1997; Proom & Knight, 1950; Wainø *et al.*, 1999) and *Lentibacillus* (Yoon *et al.*, 2002) (Fig. 2). The 16S rDNA sequence from strain BLx^T showed 92·8–94·8 % similarity to the genus *Virgibacillus*, 92·3 % similarity to the genus *Lentibacillus*, and 92·4–93·5, 92·8 and 92·5–93·1 % similarity, respectively, to the genera *Gracilibacillus* (Lawson *et al.*, 1996; Wainø *et al.*, 1999), *Paraliobacillus* (Ishikawa *et al.*, 2002) and *Halobacillus* (Spring *et al.*, 1996). The highest similarity of 16S rDNA to that of BLx^T was that of *Virgibacillus proomii* (94·8 %). However, *B. licheniformis* was originally identified as the closest relative. When the sequence of the DGGE band (variable region 3 of the 16S rDNA) of BLx^T was compared with that of *B. licheniformis* and *V. proomii*, the levels of similarity were 96·3 and 94·4 %, respectively. The bootstrap resampling value (66 %) supported the positioning of strain BLx^T in an independent lineage within the cluster that includes the genera *Virgibacillus* and *Lentibacillus*. The confidence level of the bootstrap analysis was 99 % between this cluster and that comprising the genera *Gracilibacillus*, *Paraliobacillus* and *Halobacillus*. From the results of the phylogenetic analysis, BLx^T was compared with members of the genera

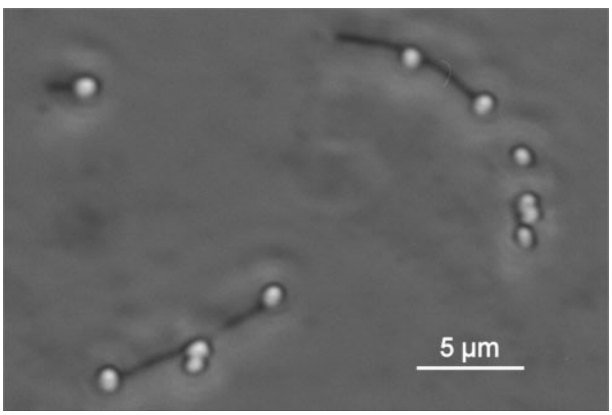

Fig. 1. Phase-contrast micrograph of cells of strain BLx^T after 12 days cultivation at 37 °C on TSA supplemented with trace metal salts.

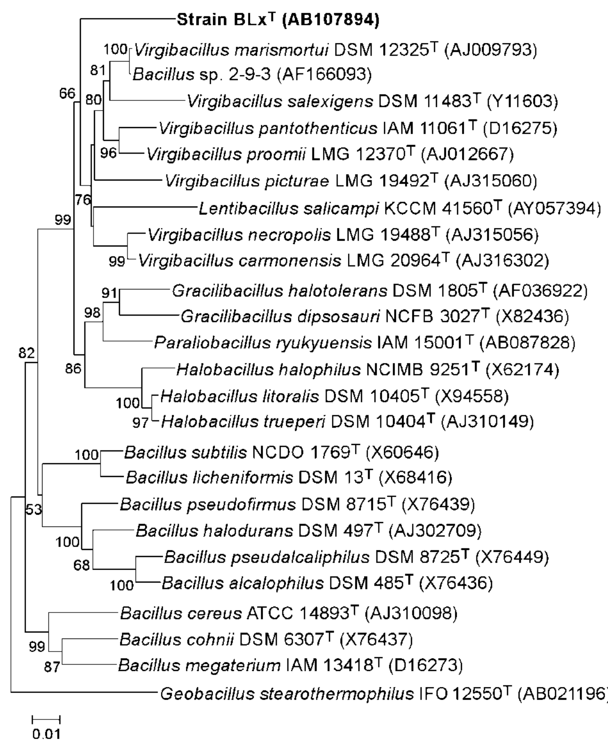

Fig. 2. Phylogenetic tree based on 16S rDNA sequences of strain BLx^T and related strains, constructed by neighbour-joining, with *Geobacillus stearothermophilus* as the outgroup. Numbers on the branches are bootstrap values (expressed as percentages) estimated by a bootstrap analysis performed with 1000 replicates. Bootstrap values less than 50 % not shown.

Table 1. Morphological, physiological and biochemical characteristics of strain BLx^T and related taxa

Strains: 1, BLx^T; 2, *V. pantothenticus* DSM 26^T (data from Heyndrickx *et al.*, 1999); 3, *V. proomii* DSM 13055^T (Heyndrickx *et al.*, 1999); 4, *V. salexigens* DSM 11483^T (data from Garabito *et al.*, 1997); 5, *V. marismortui* DSM 12325^T (data from Arahal *et al.*, 1999); 6, *V. carmonensis* LMG 26904^T (data from Heyrman *et al.*, 2003); 7, *V. necropolis* LMG 19488^T (Heyrman *et al.*, 2003); 8, *V. pictirae* LMG 19492^T (Heyrman *et al.*, 2003); 9, *L. salicampi* KCCM 41560^T (data from Yoon *et al.*, 2002). +, Positive, −, negative; w, weak; ND, not determined. Spore position: T, terminal; S, subterminal; C, central. Spore shape: E, ellipsoid; SP, spherical. None of the strains produce acid from lactose.

Characteristic	1	2	3	4	5	6	7	8	9
Morphology	Rods, singly, in pairs or in short chains	Rods, filaments, singly or in chains	Rods, filaments, singly or in chains	Rods, singly, in pairs or in short chains	Rods, singly, in pairs or in short chains	Rods, singly, in pairs or in short chains	Rods, singly, in pairs or in short chains	Rods, singly or in pairs	Rods
Colony pigmentation	Light yellowish-brown	Creamy-grey	Creamy-grey	None	Cream	Pink	None	None	Cream
Spore position	T	T(S)	T(S)	C(S)	T(S)	S	C(S, T)	T	T
Spore shape	SP	E, SP	E, SP	E	E	E, SP	E	E, SP	SP
Anaerobic growth	−	+	+	−	−	−	−	−	−
Optimum conditions for growth:									
Temperature (°C)	50	37	37	37	37	25–30	25–35	25–35	30
NaCl concn (%)	0·5	4	ND	10	10	5–10	5–10	5–10	4–8
Nitrate reduction	−	−	−	ND	+	+	+	+	ND
H_2S production	−	w	−	+	+	−	−	−	ND
Acid production from:									
Glycerol	−	+	−	+	+	−	w	w	−
D-Arabinose	−	+	−	−	−	−	−	−	−
D-Ribose	+	+	+	ND	ND	−	w	−	−
D-Xylose	+	−	−	−	−	−	−	−	−
D-Fructose	−	+	+	+	+	−	w	w	−
D-Galactose	−	+	+	−	−	−	−	w	−
D-Glucose	−	+	+	+	+	−	w	w	−
D-Mannose	−	+	+	+	ND	−	w	w	ND
Maltose	−	+	+	+	+	−	−	w	−
Sucrose	−	+	+	ND	−	−	−	−	ND
D-Trehalose	−	+	+	−	−	−	w	−	−
5-Ketogluconate	+	−	−	ND	ND	w	w	−	ND
Hydrolysis of:									
Gelatin	+	+	w	+	+	−	w	−	ND
Casein	−	+	+	+	+	+	+	+	+
Starch	−	+	+	−	−	−	−	−	−
Aesculin	−	+	+	+	+	w	−	−	−

Virgibacillus and *Lentibacillus* for the determination of its taxonomic position.

Strain BLx^T could grow in TSB at temperatures between 30 and 55 °C and at pH 7·5–10. Optimum growth was observed at 50 °C and pH 8–9. These conditions corresponded to those of the decomposed matter from which BLx^T was isolated. Members of the genera *Virgibacillus* and *Lentibacillus* are mesophilic, so the optimum growth temperature discriminates BLx^T from these genera. BLx^T preferred a low NaCl concentration for growth: the maximum growth rate was observed at 0·5 % NaCl (standard concentration of NaCl in TSB). No growth was observed at 10 % NaCl in TSB. Growth of *Virgibacillus* species is stimulated by the addition of NaCl, up to approximately 5 %. *Lentibacillus salicampi* grows optimally at 4–8 % NaCl. From this phenotypic viewpoint, BLx^T is quite different from these genera. Table 1 summarizes the morphological, physiological and biochemical characteristics of BLx^T compared to those of related taxa. BLx^T was capable of acid production from D-xylose, but could not hydrolyse casein. These biochemical properties also separate BLx^T from members of the genera *Virgibacillus* and *Lentibacillus*.

The chemotaxonomic properties of strain BLx^T were determined. The cell wall contained peptidoglycan of the *meso*-DAP type. The predominant quinone in the cell wall was unsaturated menaquinone with seven isoprene units (MK-7). The G+C content of the genomic DNA was 35·5 mol%. The chemotaxonomic properties of BLx^T were compared with those of the related genera. All strains have *meso*-DAP as the diamino acid in the cell wall and MK-7 as the predominant quinone. In general, these properties are not appropriate criteria for the discrimination of genera within *Bacillus* rRNA group 1. However, the fatty acid profile was remarkably different (Table 2). BLx^T contained iso-$C_{15:0}$ as the major component (67·2 %). Neither anteiso-$C_{15:0}$ nor anteiso-$C_{17:0}$ was detected. This composition of fatty acids discriminates BLx^T from *Virgibacillus* species and *Lentibacillus salicampi*, which contain anteiso-$C_{15:0}$ or both anteiso-$C_{15:0}$ and iso-$C_{15:0}$ as the major component(s), and anteiso-$C_{17:0}$ as one of the minor components (>10 %). These results of phylogenetic, phenotypic and chemotaxonomic analyses support the classification of BLx^T in a new genus, separated from the genera *Virgibacillus* and *Lentibacillus*. Therefore, we propose that BLx^T is designated the type strain of a novel species in a novel genus, *Cerasibacillus quisquiliarum* gen. nov., sp. nov.

Cerasibacillus quisquiliarum strain BLx^T was isolated from a semi-continuous decomposing system of kitchen refuse. The previous study indicated its abundance and its potential importance in garbage degradation. However, BLx^T was detected in very low numbers on nutrient agar plates and had only a limited ability to assimilate carbohydrates and hydrolyse biopolymers (Table 1; species description). Further research will be necessary to understand the reason for its low recovery on agar plates and what role(s) BLx^T plays during the decomposing process of kitchen refuse.

Table 2. Comparison of the cellular fatty acid profile of strain BLx^T with those of related taxa

Cells were cultivated on MA at 30 °C for 7 days. Values represent percentage of each fatty acid, as measured by the MIDI system. Strains: 1, Strain BLx^T; 2, *V. pantothenticus* DSM 26^T; 3, *V. proomii* DSM 13055^T; 4, *V. salexigens* DSM 11483^T; 5, *V. marismortui* DSM 12325^T; 6, *Lentibacillus salicampi* KCCM 41560^T. –, Not detected; tr, less than 1·0 %. Data from Yoon *et al.*, 2002 for *V. pantothenticus*, *V. proomii*, *V. salexigens* (transferred from *Salibacillus salexigens*), *V. marismortui* (transferred from *S. marismortui*) and *L. salicampi*.

Fatty acid	1	2	3	4	5	6
iso-14 : 0	2·4	1·4	5·2	3·0	3·5	12·0
iso-15 : 0	67·2	4·9	32·2	32·9	34·4	3·4
anteiso-15 : 0	–	51·7	33·1	41·7	31·4	38·6
16 : 1ω7*c* alcohol	2·6	–	–	1·5	1·0	tr
iso-16 : 0	2·9	6·0	7·1	4·7	7·6	30·1
16 : 0	10·9	1·0	tr	tr	1·1	1·4
iso-17 : 1ω10*c*	3·1	–	–	tr	–	–
iso-17 : 0	3·2	2·4	6·5	3·5	7·6	tr
anteiso-17 : 0	–	31·9	10·9	10·2	10·9	13·4
18 : 0	7·7	–	–	–	–	–

Description of *Cerasibacillus* gen. nov.

Cerasibacillus (Ce.ras.i.ba.cil′lus. L. neut. n. *cerasum* a cherry; L. masc. n. *bacillus* small rod, N.L. masc. n. *Cerasibacillus* a cherry *Bacillus*, as the appearance of its sporangium is cherry-like).

Cells are Gram-positive rods, 0·8 × 2·5–5·0 μm and motile and occur singly, in pairs or in short chains. Spherical endospores are produced terminally. Colonies are pigmented (light yellowish-brown), round and opaque at 37 °C on TSA. Strictly aerobic. Good growth occurs at low concentrations of NaCl. No growth within 6 days at 50 °C in TSB with 10 % NaCl. Grows at 30–55 °C (optimum 50 °C) and pH 7·5–10 (optimum pH 8–9). Catalase and oxidase positive. Nitrate not reduced. Voges–Proskauer test and indole production negative. G+C content 35·5 mol% (HPLC method). The cell wall contains peptidoglycan of the *meso*-DAP acid type. The major cellular fatty acid is iso-$C_{15:0}$ (cultured on MA at 30 °C for 7 days). The main menaquinone type is MK-7. Acid produced from D-xylose. Casein not hydrolysed. The type species of the genus is *Cerasibacillus quisquiliarum*.

Description of *Cerasibacillus quisquiliarum* sp. nov.

Cerasibacillus quisquiliarum (quis.qui.li.a′ rum. L. gen. pl. n. *quisquiliarum* of kitchen refuse).

Has the following properties in addition to those given in the genus description. Hydrolyses gelatin. No hydrolysis of starch, aesculin or urea. Acid produced from D-ribose, L-sorbose, D-tagatose and 5-ketogluconate. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, methyl β-D-xylose, galactose, glucose, fructose, mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannose, methyl α-D-glucose, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, glycogen, xylitol, gentiobiose, D-turanose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate.

The type strain, BLx^T (DSM 15825^T = IAM 15044^T = KCTC 3815^T), was isolated from a semi-continuous decomposing system of kitchen refuse.

Acknowledgements

This research was supported by the Research for the Future Program of the Japan Society for the Promotion of Sciences (JSPS) and the JSPS Research Fellowships for Young Scientists. We appreciate Mr L. Ding for helping us to operate the decomposing system. We thank Professor Dr H. G. Trüper for advice on nomenclatural etymology.

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