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 BLxT 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 BLxT 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-C15:0. Phylogenetic analysis showed that strain BLx was positioned in an independent lineage within the cluster that includes the genera Virgibacillus and Lentibacillus in Bacillus rRNA group 1. Strain BLxT 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 BLxT 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 BLxT (DSM 15825T = IAM15044T = KCTC 3815T).

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 BLxT.

Strain BLxT 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
15000 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 BLxT 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'-AGAGTTTGATCCTGGCTCAG-3')
and R (5'-ACGGCTATCTTGTGCTACG-3'); E. coli positions
1512–1492) (Devereux
et al., 1995). Amplified products
were applied to specific PCR, which was performed using
the BLxT-specific primer, BL3R (5'-ACGGRCTATTTGC-AACGGTAC-3'),
whose sequence corresponds to a BLxT-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 MgCl2, 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 identi-
cal to that of the DGGE bands of the B. licheniformis-like
strain X68416. Based on this information, the isolate was
identified as strain BLxT.

Strain BLxT was maintained in TSB or on TSA (pH 8.5,
adjusted with NaOH). Cell wall and menaquinone com-
position 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, BLxT was cultivated for
12 days at 37 °C on TSA containing the following trace
elements: MgSO4 (1 mM), Ca(NO3)2 (1 mM), MnCl2
(10 μM) and FeSO4 (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, BLxT 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 produc-
tion from carbohydrates, hydrolysis of aesculin, gelatin,
starch and urea, H2S production and nitrate reduction.
Casein hydrolysis was detected with TSA containing 5 %
skimmed milk. BLxT was cultivated at 50 °C in TSA
containing various concentrations of NaCl and optical
density at 660 nm was measured during incubation. BLxT
was cultivated in TSA 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 Identifi-
cation System (MIDI) instructions. The G+C content
was determined by reverse-phase HPLC, as described by

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 electropho-
resis 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 con-
structed 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 & Ghirose, 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 BLxT 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 BLxT was the dominant strain. Based on this information, the isolation of BLxT 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 BLxT in the samples collected and/or the inability of BLxT 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 BLxT.

Strain BLxT 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 BLxT 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; Heyman 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 BLxT 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), Paralibacillus (Ishikawa et al., 2002) and Halobacillus (Spring et al., 1996). The highest similarity of 16S rDNA to that of BLxT 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 BLxT 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 BLxT 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, Paralibacillus and Halobacillus. From the results of the phylogenetic analysis, BLxT was compared with members of the genera Virgibacillus, Paralibacillus, Halobacillus, and the newly proposed genus Cerasibacillus (Lawson et al., 1996; Wainø et al., 1999), Paralibacillus (Ishikawa et al., 2002), Halobacillus (Spring et al., 1996) and Cerasibacillus quisquiliarum gen. nov., sp. nov. (Garabito et al., 1997; Proom & Knight, 1950; Wainø & Altenburger, 1999).

Fig. 1. Phase-contrast micrograph of cells of strain BLxT 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 BLxT 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 BLxT and related taxa

Strains: 1, BLxT; 2, *V. pantothenticus* DSM 26T (data from Heyndrickx *et al.*, 1999); 3, *V. proomii* DSM 13055T (Heyndrickx *et al.*, 1999); 4, *V. salexigens* DSM 11483T (data from Garabito *et al.*, 1997); 5, *V. marismortui* DSM 12325T (data from Arahal *et al.*, 1999); 6, *V. carmoneensis* LMG 26904T (data from Heyrman *et al.*, 2003); 7, *V. necropolis* LMG 19488T (Heyrman *et al.*, 2003); 8, *V. pictirae* LMG 19492T (Heyrman *et al.*, 2003); 9, *L. salicampi* KCCM 41560T (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.

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<td>Rods, filaments, singly or in chains</td>
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<td>w</td>
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<td>Aesculin</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
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Virgibacillus and Lentibacillus for the determination of its taxonomic position.

Strain BLx\textsuperscript{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\textsuperscript{T} was isolated. Members of the genera Virgibacillus and Lentibacillus are mesophilic, so the optimum growth temperature discriminates BLx\textsuperscript{T} from these genera. BLx\textsuperscript{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\textsuperscript{T} is quite different from these genera. Table 1 summarizes the morphological, physiological and biochemical characteristics of BLx\textsuperscript{T} compared to those of related taxa. BLx\textsuperscript{T} was capable of acid production from D-xylose, but could not hydrolyse casein. These biochemical properties also separate BLx\textsuperscript{T} from members of the genera Virgibacillus and Lentibacillus.

The chemotaxonomic properties of strain BLx\textsuperscript{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\textsuperscript{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 RNA group 1. However, the fatty acid profile was remarkably different (Table 2). BLx\textsuperscript{T} contained iso-C\textsubscript{15:0} as the major component (67.2 %). Neither anteiso-C\textsubscript{15:0} nor anteiso-C\textsubscript{17:0} was detected. This composition of fatty acids discriminates BLx\textsuperscript{T} from Virgibacillus species and Lentibacillus salicampi, which contain anteiso-C\textsubscript{15:0} or both anteiso-C\textsubscript{15:0} and iso-C\textsubscript{15:0} as the major component(s), and anteiso-C\textsubscript{17:0} as one of the minor components (> 10 %). These results of phylogenetic, phenotypic and chemotaxonomic analyses support the classification of BLx\textsuperscript{T} in a new genus, separated from the genera Virgibacillus and Lentibacillus. Therefore, we propose that BLx\textsuperscript{T} is designated the type strain of a novel species in a novel genus, Cerasibacillus quisquiliarum gen. nov., sp. nov.

Cerasibacillus quisquiliarum strain BLx\textsuperscript{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\textsuperscript{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\textsuperscript{T} plays during the decomposing process of kitchen refuse.

### Table 2. Comparison of the cellular fatty acid profile of strain BLx\textsuperscript{T} with those of related taxa

<table>
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<th>Fatty acid</th>
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### Description of Cerasibacillus gen. nov.

Cerasibacillus (C.e.ri.s.i.ba.ci.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\textsubscript{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-sorbos, 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, glycosyl, xylitol, gentiobiose, D-turanose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate.

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

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References


