

Full Paper

Oceanobacillus soja sp. nov. isolated from soy sauce production equipment in Japan

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A Gram-positive, spore-forming, motile rod-shaped bacterium, designated strain Y27^T, was isolated from the bottom of a mold fermenter used in the process of soy sauce production. Phylogenetic analysis of the 16S rRNA gene sequence from this strain placed it within the genus *Oceanobacillus*, and further sequence analysis revealed that this strain has a sequence similarity of 95.0–98.7% to other known species of *Oceanobacillus*. The DNA-DNA relatedness between strain Y27^T and related type strains of the genus *Oceanobacillus* is below 43%, indicating that it should be considered a separate species. Characterization of strain Y27^T revealed that the major cellular fatty acid is anteiso-C_{15:0}, the cell wall contains meso-diaminopimelic acid-type peptidoglycans, the major menaquinone is MK-7, and the major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The genomic DNA G+C content of the strain is 38.0 mol%. On the basis of these phylogenetic, physiological and chemotaxonomic data, we propose that this isolate represents a novel species of the genus *Oceanobacillus*, and propose the name *Oceanobacillus soja* sp. nov. The type strain is strain Y27^T (=JCM 15792^T=NRRL B-59181^T=NBRC 105379^T=NCIMB 14542^T).

Key Words—*Oceanobacillus soja* gen. nov., sp. nov.; soy sauce; spore

Introduction

Soy sauce is a traditional fermented seasoning. During the soy sauce production process, soybeans are soaked and steamed, mixed with roasted and crushed wheat, and then combined with seed mold. The mixture is then cultured for a few days using a koji production process, and subsequently mixed with brine and matured by fermentation. The koji production process involves the growth of mold strains that excrete neces-

sary amylases and proteases. These enzymes metabolize the starches and proteins of the soybean/wheat mixture into the glucose and amino acids that give soy sauce its flavor. Therefore, the koji production step is a critical control point of soy sauce production. As such, the temperature and air currents of the mold culture room are strictly controlled to promote proper mold growth.

Producers fear *Bacillus* contamination of koji, because *Bacillus* compete with the desirable mold, causing marked declines in amylase and protease production (Takazane et al., 1998). In addition, *Bacillus* spores may persist through the production process and wind up in the final soy sauce product (Takazane et al., 1998). The bottom of the koji fermenter is aerated by a blower that is attached under the fermenter for the pur-

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pose of permeating the fermenting mixture with oxygen and exhausting the generated heat. The fermenter bottom itself is porous and difficult to clean, contributing to concerns about contamination of koji with *Bacillus*, especially as spores. Thus, we examined the numbers and types of *Bacillus* spores that could be recovered from fermenter bottoms used in soy sauce production in four plants (in Saitama Prefecture). Among the isolates, we identified a previously unknown bacterial strain that appears to be a novel member of the genus *Oceanobacillus*.

This genus, which was created by Lu et al. (2001), consists of a group of halotolerant and alkaliphilic bacteria comprising six recognized species and one subspecies, as follows: *O. iheyensis* (Lu et al., 2001), *O. oncorhynchi* (Yumoto et al., 2005), *O. oncorhynchi* subsp. *incaldanensis* (Romano et al., 2006), *O. picturæ* (which was transferred from *Virgibacillus picturæ*; Heyrman et al., 2003; Lee et al., 2006), *O. chironomi* (Raats and Halpern, 2007), *O. profundus* (Kim et al., 2007) and *O. caeni* (Nam et al., 2008).

Here, we report the isolation of a novel bacterium from the bottom of a mold fermenter used in the soy sauce production process, and its characterization as a novel member of the genus *Oceanobacillus*.

Materials and Methods

Bacterial strains and isolation. The surface of the koji fermenter bottom was swabbed with cotton-tipped swabs (Fukifuki-check, Eiken Chemical, Tokyo, Japan). Swabbed samples were incubated for 10 min at 80°C, and spread on Brain Heart Infusion (BHI) (Oxoid, Hampshire, United Kingdom) agar. After aerobic incubation of the samples for 24 h at 30°C, we isolated strain Y27^T from the grown colonies. *Oceanobacillus oncorhynchi* JCM 12661^T was cultured in Horikoshi-I medium (Horikoshi, 1999) containing the following components: glucose 10.0 g; Polypepton (Nihon Pharmaceutical, Tokyo, Japan) 5.0 g; yeast extract 5.0 g; K₂HPO₄ 1.0 g; MgSO₄·7H₂O 0.2 g; agar 15.0 g; distilled water 900 ml. The medium was sterilized by autoclaving, and then aseptically supplemented with 100 ml of 10% Na₂CO₃ solution. The final pH of the medium was around 10.0. *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T was cultured in BHI agar. *Oceanobacillus iheyensis* JCM 11309^T was cultured in modified Horikoshi-I medium containing the following components: glucose 10.0 g; Polypepton (Nihon Phar-

maceutical) 5.0 g; yeast extract 5.0 g; NaCl 10.0 g; K₂HPO₄ 1.0 g; MgSO₄·7H₂O 0.2 g; agar 15.0 g; distilled water 900 ml. The medium was sterilized by autoclaving, and then aseptically supplemented with 100 ml of 10% NaHCO₃ solution. The final pH of the medium was around 9.0. All strains were cultured aerobically at 30°C.

Morphological, physiological and biochemical assessments. The cell morphology of strain Y27^T was examined using a BX50F4 microscope (Olympus, Tokyo, Japan). Colony morphology was observed after incubation on LB agar (Becton Dickinson, MD, USA) at 30°C. Gram-staining was performed using Favor G "Nissui" (Nissui Pharmaceutical, Tokyo, Japan). For examination of optimal growth temperature, the incubation temperature was varied in 5°C increments between 5°C and 45°C. Growth in the presence of NaCl was examined in BHI broth containing NaCl at 0, 5, 7.5, 10, 15, or 20% (all w/v). The pH range for growth was determined using BHI broth adjusted to various pH values with HCl or NaOH. Anaerobic growth was investigated using an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) on LB agar, with incubation at 30°C. Motility was observed on BHI agar. General physiological tests were performed using conventional methods (Barrow and Feltham, 1993). The hydrolysis of Tween 20, 40, 60 and 80 was tested by addition of 1% of Tween and 0.01 g of CaCl₂ to the following growth media: BHI agar for strain Y27^T and *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; Horikoshi-I medium for *Oceanobacillus oncorhynchi* JCM 12661^T; and modified Horikoshi-I medium for *Oceanobacillus iheyensis* JCM 11309^T.

Biolog test and API strips. Biolog tests were carried out according to the manufacturer's instructions (Biolog, CA, USA). The provided GN/GP inoculating fluid was used to suspend the bacteria to around OD₆₀₀ = 0.4, and the provided GP2 microplate was used to examine carbohydrate utilization. The plates were incubated at 30°C for 24 h and color changes were checked. API strips (API 20E, Rapid 20E and API 50CHB; bioMérieux, Lyon, France) were used, according to the manufacturer's instructions, to determine the physiological and biochemical characteristics of strain Y27^T. The strips were incubated for 24 h at 36°C for API 20E and Rapid 20E, and for 24 h at 30°C for API 50CHB.

16S rRNA gene sequencing, phylogenetic analysis and DNA-DNA hybridization. Genomic DNA was iso-

lated using an ISOPLANT kit (Nippongene, Tokyo, Japan), according to the manufacturer's instructions. The 16S rRNA gene was sequenced as described previously (Klijn et al., 1991; Mori et al., 1997). Multiple alignment was performed using CLUSTAL W (Thompson et al., 1994) followed by phylogenetic tree construction using Kimura's two-parameter model (Kimura, 1980). Clustering was performed using the neighbor-joining algorithm (Saitou and Nei, 1987) implemented in the MEGA program, version 4.0 (Tamura et al., 2007). The resultant tree topologies were evaluated by a bootstrap analysis based on 1,000 re-samplings. For DNA-DNA hybridization, genomic DNA was extracted from 400 ml of bacterial culture as described previously (Saito and Miura, 1963), and DNA-DNA hybridization was performed using the photobiotin-labeling method (Ezaki et al., 1989). The hybridization was carried out at 41°C for 3 h, and the results were measured fluorometrically (Cytofluor; Perseptive Biosystems, MA, USA).

Quinone, cell wall peptidoglycan, fatty acid, polar lipid and G+C content analysis. Respiratory quinones were extracted and purified according to a published protocol (Nishijima et al., 1997), and analyzed by HPLC (Waters 600 series; Waters, MA, USA). Cell wall peptidoglycans were prepared and analyzed by HPLC (Waters). For analysis of fatty acid methyl esters, strain Y27^T was incubated at 27°C for 2 days on Trypticase Soy plates (Becton Dickinson (BD), NJ, USA), and fatty acid methyl esters were extracted and prepared using the Sherlock Microbial Identification System Version 5.0 (MIDI, DE, USA), according to the provided protocol. Polar lipids were extracted from freeze-dried bacteria and analyzed as described previously (Minnikin, 1979; Tindall, 1990a,b). The G+C content of DNA was determined by HPLC as described previously (Mesbah et al., 1989).

Nucleotide sequence accession numbers. The nucleotide sequence determined in this study has been deposited with the DDBJ under accession number AB473561.

Results and Discussion

Phylogenetic analysis

The generated phylogenetic tree positioned strain Y27^T within genus *Oceanobacillus* (Fig. 1). Strain Y27^T showed highest sequence similarity to type strains *O. oncorhynchi* (98.7%) and *O. oncorhynchi* subsp. *incal-*

danensis (98.7%), followed by type strains *O. iheyensis* (97.2%), *O. chironomi* (95.7%), *O. picturae* (95.6%), *O. profundus* (95.6%), and *O. caeni* (95.0%). Lower levels of sequence similarity up to 94.3% were found for species from other genera. These data indicated that strain Y27^T is a member of the genus *Oceanobacillus*.

Strain Y27^T, *O. oncorhynchi* JCM 12661^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *O. iheyensis* JCM 11309^T formed a cluster with 97% bootstrap support, suggesting that we should compare the DNA relatedness between strain Y27^T and the other three strains. This analysis revealed that the levels of DNA-DNA relatedness between strain Y27^T and *O. oncorhynchi* JCM 12661^T (43%), *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (33%) and *O. iheyensis* JCM 11309^T (17%), were all much less than the 70% suggested by Wayne et al. (1987) as a threshold for species differentiation. These results indicate that strain Y27^T is a novel species that is distinct from the other, previously known *Oceanobacillus* species.

Morphological, physiological and biochemical analysis

The morphological, physiological, and biochemical characteristics of strain Y27^T are given in the species description below. Table 1 compares various characteristics between Y27^T and the closely related species, *O. oncorhynchi* JCM 12661^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *O. iheyensis* JCM 11309^T. Consistent with the other *Oceanobacillus* species, strain Y27^T was halotolerant and alkaliphilic. However, while the other species could grow in media containing up to 20–22% NaCl, strain Y27^T tolerated only up to 15% NaCl, showing less halotolerance than the others. However, strain Y27^T could grow at up to 45°C, showing good heat tolerance. Unlike *O. oncorhynchi* JCM 12661^T and *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, strain Y27^T could not reduce nitrate. Unlike *O. iheyensis* JCM 11309^T, strain Y27^T could not hydrolyze casein or gelatin. Finally, of the tested species, only strain Y27^T was unable to hydrolyze Tween 40.

Carbohydrate utilization analysis

A Biolog test was used to examine carbohydrate utilization by strain Y27^T and its related strains (Table 2). Only strain Y27^T could utilize D-melezitose, D-arabitol and D-sorbitol, though the latter two sugars were utilized only weakly. Lu et al. (2001) previously showed

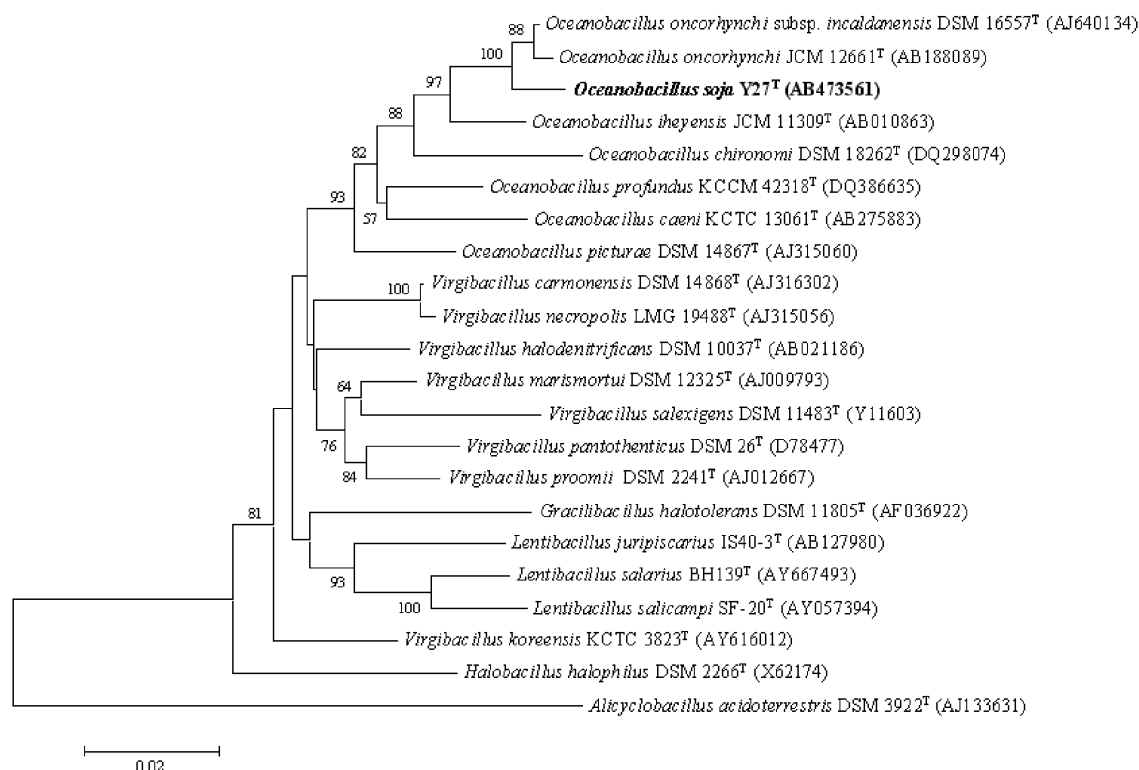


Fig. 1. Phylogenetic relationships among strain Y27^T, recognized members of the genus *Oceanobacillus* and other related species based on 16S rRNA gene sequences.

The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Alignment gaps and ambiguous bases were not taken into consideration so that 1,276 bases of the 16S rRNA gene nucleotide were compared. Numbers at nodes represent levels of bootstrap support (%) based on 1,000 resampled datasets. Bootstrap percentages more than 50% are shown. DDBJ accession numbers are given in parentheses. Bar, 1% sequence divergence.

Table 1. Differential phenotypic characteristics among strain Y27^T, *O. oncorhynchi* JCM 12661^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *O. iheyensis* JCM 11309^T.

Characteristic	1	2	3	4
Cell size (μm)	0.8–0.9 × 1.5–2.0	0.4–0.6 × 1.1–1.4	0.5–0.8 × 1.2–2.0	0.6–0.8 × 2.5–3.5
Colony color	Cream	White	Cream-beige	Creamy-white
Spore formation	+	+	–	+
Spore position	ST	ST	–	ST or T
NaCl range for growth (%)	0–15	0–22	5–20	0–21
Growth pH range	6–10	9.0–10.0	6.5–9.5	6.5–10.0
Optimum pH	8.5	9.0–10.0	9.0	7.0–9.5
Growth temperature range (°C)	15–45	15–40	10–40	15–42
Optimum growth temperature (°C)	30–35	30–36	37	30
Anaerobic growth	–	+	–	–
Oxidase	+	+	+	v
Nitrate reduction	–	+	+	–
Hydrolysis of:				
Casein	–	–	–	+
Gelatin	–	–	–	+
Tween 20	+	–	– ^a	+
Tween 40	–	+	+	+
Tween 60	+	–	+	+
DNA G + C content (mol%)	38.0	38.5	40.1	35.8

Strains: 1, Y27^T (*Oceanobacillus soja* sp. nov.); 2, *O. oncorhynchi* JCM 12661^T (data from Yumoto et al., 2005); 3, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (Romano et al., 2006); 4, *O. iheyensis* JCM 11309^T (Lu et al., 2001). +, Positive; –, negative; v, variable. ST, subterminal; T, terminal. ^aDetermined in this study.

Table 2. Differential carbohydrate utilization characteristics among strain Y27^T, *O. oncorhynchi* JCM 12661^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *O. iheyensis* JCM 11309^T.

	1	2	3	4
N-Acetyl-D-glucosamine	+	+	+	–
N-Acetyl-β-D-mannosamine	+	+	+	– ^a
D-Arabitol	w	–	–	– ^a
Arbutin	–	–	+	–
D-Cellobiose	+	–	+	–
D-Fructose	+	+	+	–
Gentiobiose	–	–	+	–
D-Gluconic acid	w	–	+	–
Maltose	w	–	+	+
Maltotriose	–	–	+	–
D-Mannitol	+	–	+	– ^a
D-Melezitose	+	–	–	–
β-Methyl-D-glucoside	–	–	+	–
D- Psicose	+	+	+	–
Salicin	–	w	+	–
D-Sorbitol	w	–	–	–
Sucrose	–	–	+	–
D-Trehalose	+	+	+	–
Glycerol	+	–	+	–
Turanose	–	–	–	+

Strains: 1, Y27^T (*Oceanobacillus soja* sp. nov.); 2, *O. oncorhynchi* JCM 12661^T; 3, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; 4, *O. iheyensis* JCM 11309^T (data from Lu et al., 2001). +, Positive; –, negative; w, weak. ^aDetermined in this study.

that of the strains tested in that case, only *Gracilibacillus dipsosauri* utilized D-melezitose, while numerous other *O. iheyensis*-related bacteria, including *Gracilibacillus halotolerans*, *Salibacillus salexigens*, *Halobacillus halophilus*, *Halobacillus trueperi* and *Halobacillus litoralis*, could not utilize this carbohydrate. This may suggest that the ability of strain Y27^T to utilize D-melezitose could be relatively rare among related bacteria.

Fermentation analysis

Acid production from various carbohydrates was tested using API 50CH (Table 3). Few sugars were found to be was fermented by *O. iheyensis* JCM 11309^T. Acid production from D-sorbitol was observed only by strain Y27^T. Conversely, acid production from dulcitol, amygdalin, D-melibiose and D-raffinose was observed from *O. oncorhynchi* JCM 12661^T and *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, but not strain Y27^T.

Chemotaxonomic analysis

Consistent with other *Oceanobacillus* species, strain Y27^T contained MK-7 as its major menaquinone. Its cell wall contained peptidoglycans of the meso-diaminopimelic acid type. The predominant cellular fatty

acid of strain Y27^T was anteiso-C_{15:0}; this is consistent with *O. oncorhynchi* JCM 12661^T (Yumoto et al., 2005), *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (Romano et al., 2006) and *O. iheyensis* JCM 11309^T (Lu et al., 2001). A comparison of fatty acid profiles is shown in Table 4. The major polar lipids of strain Y27^T were diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C content of strain Y27^T was 38.0 mol%.

Distribution

We examined spores isolated from koji fermenter bottoms in four soy sauce-producing plants, but obtained strain Y27^T from only one of them. The other three plants showed no evidence of isolates similar to strain Y27^T so that strain Y27^T might not be a normal inhabitant in soy sauce-producing plants. Notably, strain Y27^T can grow effectively under the same conditions used to grow mold during the koji production process. Thus, it will be desirable to eliminate such bacteria, in order to improve the efficiency of the mold cultures.

In conclusion

Based on the phenotypic, phylogenetic, and genomic evidence presented herein, we propose that

Table 3. Differential fermentation characteristics among strain Y27^T, *O. oncorhynchi* JCM 12661^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *O. iheyensis* JCM 11309^T.

Acid production from	1	2	3	4
D-Ribose	+	— ^a	+	— ^a
D-Xylose	—	—	+	— ^a
β-Methyl-D-xyloside	—	— ^a	+	— ^a
D-Galactose	—	+	—	—
D-Sorbose	—	+ ^a	—	— ^a
Dulcitol	—	+ ^a	+	— ^a
D-Mannitol	+	+ ^a	+	— ^a
D-Sorbitol	+	—	—	— ^a
N-Acetyl-glucosamine	+	+ ^a	+	— ^a
Amygdalin	—	+ ^a	+	— ^a
Arbutin	+	+ ^a	+	— ^a
Aesculin	+	+ ^a	+	— ^a
Salicin	+	+ ^a	+	— ^a
D-Cellobiose	+	+ ^a	+	— ^a
D-Melibiose	—	+	±	—
D-Sucrose	+	+	+	— ^a
D-Trehalose	+	+	+	—
D-Raffinose	—	+	±	— ^a
Gentiobiose	—	+ ^a	+	— ^a
D-Tagatose	+	+ ^a	+	— ^a

Strains: 1, Y27^T (*Oceanobacillus soja* sp. nov.); 2, *O. oncorhynchi* JCM 12661^T (data from Yumoto et al., 2005); 3, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; 4, *O. iheyensis* JCM 11309^T (Lu et al., 2001). +, Positive; —, negative; ±, could not determine positive or negative. ^aDetermined in this study.

Table 4. Fatty acid profiles for strain Y27^T, *O. oncorhynchi* JCM 12661^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *O. iheyensis* JCM 11309^T.

Fatty acid	1	2	3	4
Saturated fatty acids				
C _{14:0}	0.5	—	—	—
C _{15:0}	—	—	0.5	—
C _{16:0}	4.8	2.1	2.8	1.1
C _{17:0}	—	—	0.2	—
Unsaturated fatty acids				
C _{16:1}	—	—	0.5	—
Branched fatty acids				
iso-C _{14:0}	5.4	1.1	0.4	13.0
anteiso-C _{14:0}	—	—	21.0	—
iso-C _{15:0}	11.9	22.7	22.8	34.3
anteiso-C _{15:0}	45.8	49.3	46.5	38.7
iso-C _{16:0}	12.4	3.2	—	7.8
iso-C _{17:0}	4.3	4.3	2.0	1.1
anteiso-C _{17:0}	14.7	18.0	1.9	4.1
iso-C _{18:0}	—	—	1.1	—
anteiso-C _{18:0}	—	—	0.1	—
Summed feature 5	0.4	—	—	—

Strains: 1, Y27^T (*Oceanobacillus soja* sp. nov.); 2, *O. oncorhynchi* JCM 12661^T (data from Yumoto et al., 2005); 3, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (Romano et al., 2006); 4, *O. iheyensis* JCM 11309^T (Lu et al., 2001). Summed feature 5 : C_{18:2} ω6, 9c and/or anteiso-C_{18:0}.

strain Y27^T should be considered a novel species of the genus *Oceanobacillus*, and suggest the name *Oceanobacillus soja* sp. nov.

The type strain is strain Y27^T (=JCM 15792^T=NRRL B-59181^T=NBRC 105379^T=NCIMB 14542^T), which was isolated from the bottom of a mold fermenter used

in the soy sauce production process in Japan.

Description of *Oceanobacillus soja* sp. nov.

Oceanobacillus soja (so' ja. L. fem. adj. *soja* associated with the isolation origin).

Cells are Gram-positive, spore-forming, motile rods,

0.8–0.9 µm by 1.5–2.0 µm in size. Colonies formed on LB agar are cream, smooth, circular, and convex, with entire margins after 48 h incubation at 30°C. Endospores are positioned subterminally. Produces catalase and oxidase. Growth occurs at 15–45°C and pH 6.0–10.0, with optimum growth at 30–35°C and approximately pH 8.5. Growth occurs in the presence of 0–15% (w/v) NaCl. Negative for anaerobic growth on LB medium. Negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, tryptophan deaminase, indole production, the Voges-Proskauer reaction, gelatinase and nitrate reduction. Positive for oxidase and catalase. Negative for hydrolysis of casein and starch. Positive for hydrolysis of Tween 20 and Tween 60. Negative for hydrolysis of Tween 40 and Tween 80. Acid is produced from D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, sorbitol, N-acetyl-glucosamine, arbutin, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose and D-tagatose, but not from the other carbohydrates of the API 50CHB test suite. The following substrates are assimilated in the Biolog test: N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, D-arabitol, D-cellobiose, D-fructose, D-gluconic acid, α-D-glucose, maltose, D-mannitol, D-mannose, D-melezitose, D-psicose, D-sorbitol, D-trehalose and glycerol. The major menaquinone is MK-7. The cell wall contains peptidoglycan of the meso-diaminopimelic acid type. The predominant cellular fatty acids are anteiso-C_{15:0}. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C content of the type strain is 38.0 mol%. The type strain is strain Y27^T (=JCM 15792^T=NRRL B-59181^T=NBRC 105379^T=NCIMB 14542^T), which was isolated from the surface of soy sauce production equipment in Japan.

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