Short communication

An attempt at isolation of lactic acid bacteria from soil samples collected in Aokigahara Jukai Forest, Japan

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Aokigahara Jukai Forest in Japan is not only a dense forest but it is also a mystical zone at the foot of Mt. Fuji. To maintain the primitive condition of Aokigahara Jukai, people are not permitted to live there. The ecology of microorganisms in the rhizospheres of trees in Aokigahara Jukai Forest is unknown. In previous studies, diverse lactic acid bacteria (LAB) or spore-forming LAB (*Sporolactobacillus*) were isolated from the soil in the rhizospheres of vines and fruit trees¹⁻⁵⁾. These studies provided the motivation to survey the ecological distribution of LAB existing in the soil in Aokigahara Jukai Forest. In this study, we collected soil samples and attempted to isolate LAB from soil in the Aokigahara Jukai.

Twelve soil samples were collected in the rhizospheres of trees in Aokigahara Jukai Forest (LAT.35 ° 28'N/ LON.138 ° 38'E; Shoji, Fujikawaguchiko-Cho, Minamitsuru-Gun, Yamanashi Prefecture, Japan) on April 10, 2003 (Fig. 1). The enrichment procedure described by Chen *et al.*¹⁾ was used for the isolation of acid-producing bacteria. Each collected sample was incubated in 5 ml of MRS (de Man, Rogosa, and Sharpe) me-

dium under anaerobic conditions (BBL™ GasPak™, H₂+ CO₂) at 30° C for 2-3 d. After incubation, cultures were appropriately diluted and spread onto the surface of MRS agar plates and then incubated at 30° C for 2-3d. To distinguish acid-producing bacteria from other bacteria, 1% of CaC O₃ was added to the MRS agar plates. After incubation, colonies were randomly selected from MRS plates and purified by replating on MRS agar plates. Isolates were tested with the Gram stain, and only Gram-positive bacteria were chosen for further study.

Tests of spore formation were performed with methods described by Kozaki *et al.*⁶⁾ The DNA of isolates was extracted using the methods described by Kozaki *et al.* and then identified with 16S rDNA sequencing analysis. Four primers of 27 F (5'-AGAGTTTGATCCTGGCTCAG-3'), 520 R (5'-ACCGCGGCTGCTGGC-3'), 920 R (5'-GTCAATTCCTTTGAGTTT-3'), and 1492 R (5'-GGTTACCTTGTTACGACTT-3') were used in this study. Sequence homologies were examined by comparing the obtained sequences with those in the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>) using FASTA.

Only 6 isolates were obtained from these soil samples; all were rods in their cell morphology. Examined sequence homologies showed the following results: 2 *Bacillus coagulans*, 2 *Clostridium baratii*, 1 *Bacillus* sp., and 1 *Clostridium*

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Fig. 1 The inside of Aokigahara Jukai Forest.

perfringens. The sequences determined in this study have been deposited in the DDBJ database with accession numbers from AB 240204 to AB 240209 (Table 1). Further, based on their sequences, the evolutionary distance (the Knuc value) and similarity values of representative strains of each group were generated using the software CLUSTAL W (<http://www.ddbj.nig.ac.jp/search/clustalw-e.html>), and a phylogenetic tree was constructed by the neighbor-joining method with the program TreeView (version 1.66)⁷⁾ (Fig. 2).

The aim of this study was to isolate LAB from soil in Aokigahara Jukai Forest; however, no LAB were found, only *Bacillus* and *Clostridium* species were isolated. It is well known that rich nutrition, with carbohydrates, minerals, and nitrogen compounds or other substances, is

Fig. 2 Phylogenetic relationships of representative strains. The tree was constructed using the neighbor-joining method.

Table 1 Results of identification.

Isolated strain	Gram stain	Catalase reaction	Spore formation	Closest DDBJ match / % homology
T3 (AB240204)	+	+	+	<i>Bacillus coagulans</i> (AB271752) / 99.9
T5 (AB240205)	+	+	+	<i>Bacillus coagulans</i> (AJ563373) / 99.6
T6 (AB240206)	+	—	+	<i>Clostridium perfringens</i> (AB045286) / 99.6
T8 (AB240207)	+	—	+	<i>Clostridium baratii</i> (AY341242) / 99.9
T9 (AB240208)	+	+	+	<i>Bacillus</i> sp. (DQ448756) / 95.2
T10 (AB240209)	+	—	+	<i>Clostridium baratii</i> (AY341242) / 98.8

* +: positive; —: negative.

necessary for the growth of LAB. As previously described, it was reported that diverse LAB could be isolated in the rhizospheres of various fruit trees¹⁻³⁾. Unlike the situation with fruit trees, it is probable that less carbohydrate exists in Aokigahara Jukai Forest. A low-nutrient environment is therefore considered to be the main reason for the failure to isolate LAB. In addition, both *Bacillus* and *Clostridium* species have the ability to form spores under ill conditions but LABs do not. This could be another reason for the failure to isolate LAB.

In summary, no LAB were found in soil in Aokigahara Jukai Forest in this study. Although

several *Bacillus* and *Clostridium* species were identified, the nutritional conditions in the rhizospheres of trees in Aokigahara Jukai Forest were considered to be too poor to allow the growth of LAB. Although few, the results obtained in this study may provide useful information in clarifying the ecology of microorganisms in soil in Aokigahara Jukai Forest.

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青木ヶ原樹海の土壌からの乳酸菌の分離の試み

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我々は、過去にブドウ園、桑の木など様々な果樹から土壌を採取して、その中に存在する乳酸菌叢に関する調査を行った。今回の研究においては、青木ヶ原樹海の奥から土壌サンプル12点を採取して、乳酸菌の分離を行った。その結果、6株の桿菌が分離された。これらの分離菌株は16S rDNA塩基配列解析にて、*Bacillus coagulans*が2株、*Clostridium baratii*が2株、*Clostridium perfringens*が1株と*Bacillus* sp.が1株に同定された。今回、青木ヶ原樹海内の土壌から乳酸菌の発見は至らなかった。青木ヶ原樹海内の土壌は、栄養要求性の高い乳酸菌にとって、相応しくない環境だと推測した。今回の研究結果は乳酸菌の分離に有用な情報を提供したい。