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Correlation between Phylogeny and Zn-Resistance in *Methylobacterium* Species Isolated from Non-Polluted Soil Environments

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SUMMARY

Zn-resistant *Methylobaterium* strains were isolated from several non-polluted soil samples collected in all over Japan. Zn-resistant *Methylobaterium* strains were predominantly detected in all soil samples and they were also characterized as a UV-tolerant bacteria. The MIC test revealed that the isolates have high zinc resistance in comparison with that of reference *Methylobacterium* strains obtained from culture collections. The 16S rDNA-based phylogenetic analysis showed that all strains were divided into two clusters designated as cluster A and cluster B in the present study. All isolates were distributed only in the cluster A. The phylogenetic clustering also well coincided with the differences in the pattern of carbon source utilization.

INTRODUCTION

The genus *Methylobacterium* (1) is a group of strictly aerobic, facultatively methylotrophic, gram-negative, rod-shaped and pink-pigmented bacteria. The member of this genus had been often isolated as a heavy metal resistant or a toxin resistant bacteria. *M. radiotolerans* was originally isolated as a radiation-resistant *Pseudomonas* species from rice samples irradiated by γ-ray, and the radio-resistance of this species was 10 to 40 times higher than that of other *Pseudomonas* species, such as *Pseudomonas fluorescens*. (2, 3).

Zn has been used to galvanize iron and is one of the most indispensable heavy metals for daily life. It is known that Zn affects soil microorganisms and their metabolisms (4). In this study, we determined physiological characteristics and phylogenetic positions of Zn-resistant *Methylobacterium* isolated from non-polluted Japanese soil samples.

MATERIALS AND METHODS

Bacterial strains and cultivation *Methylobacterium*-like bacteria were isolated from the non-polluted soil samples. Sampling sites were shown in Fig. 1. Diluted soils were plated onto 10-fold diluted nutrient agar medium which was supplemented with cycloheximide (50 mg liter^{-1}) to inhibit fungal growth, and 10 mM of Zn. To estimate the number of *Methylobacterium*-like bacteria by the colony forming units (CFU), all pink pigment-producing colonies were calculated as *Methylobacterium*-like bacteria.

Zn resistance and UV tolerance The minimum inhibitory concentration (MIC) of Zn on bacterial growth was measured by the growth on nutrient agar media containing 1 mM to 25 mM of zinc after 1 week of incubation. The UV tolerance was estimated by the fatal UV dose. Cells were exposed to UV radiation (UV-C, 100-280nm) by placing the plates at 60 cm under a UV lamp (Toshiba, Japan). Irradiation was for 0 to 5 minutes at an interval of one minute and the fatal UV dose was determined by survival after incubation.

Carbon source utilization and enzyme profile Colonies were suspended in Stanier's broth and the suspension was inoculated into the liquid media containing various carbon source. Utilization of each carbon compound was determined by measuring turbidity after incubation. Enzyme profiles were determined using the API ZYM systems (BioMerieux, Montalieu-Vercieu. France) according to the manufacturers' instruction.

Amplification and sequencing of partial 16S rDNA All DNAs were extracted using the benzylchloride method (5) and purified by a MicroSpin S-400 HR column (Pharmacia Biotech, USA). 16S rDNAs were amplified by PCRwith universal primers. Automated fluorescent sequencing was performed on PCR products with a automatic sequencer.

Phylogenetic analysis Maximum likelihood analysis (ML) was carried out using the program package MOLPHY (version 2.3b3) (6). For maximum likelihood analysis, the initial tree was constructed by the maximum parsimony method using the program PAUP (version 3.1.1) (7). The applied ratio of transition and transversion was 2:1. A heuristic search was used with a random stepwise addition sequence of 30 replicates, tree-bisection-reconnection branch swapping, and the MULPARS option. The 10 most parsimonious trees were obtained, and they were subjected to the Kishino-Hasegawa test (8) for selection of the best tree having the highest log-likelihood value among them. The maximum likelihood tree was reconstructed using the NucML program of the MOLPHY package based on the HKY model (9).

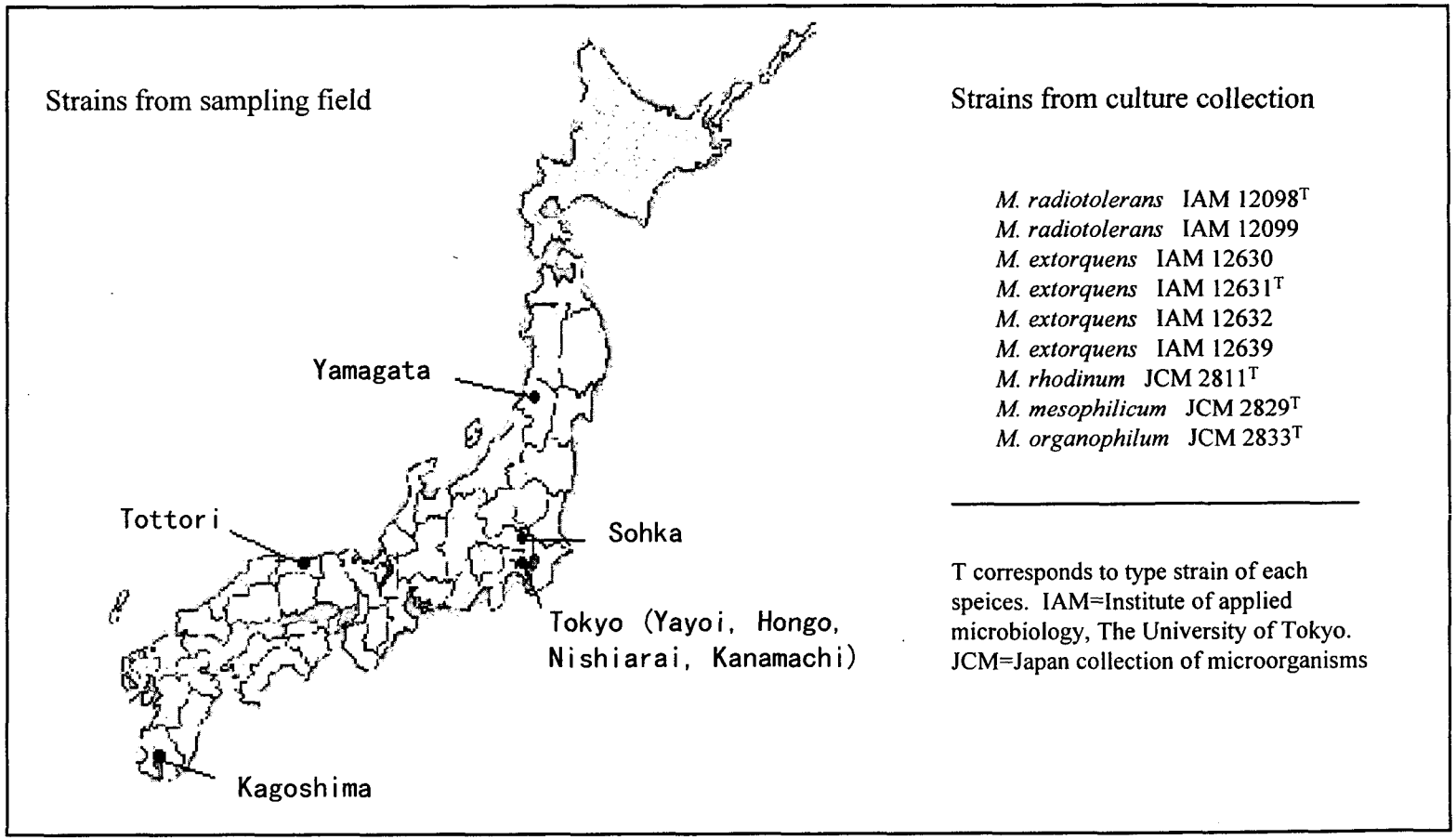

Fig. 1. Sampling location of isolates and strains obtained from culture collections.

RESULTS AND DISCUSSION

The isolation of Zn-resistant *Methylobacterium* species Zn-resistant pink-pigmented bacteria had the same colony morphology and seemed to be a *Methylobacterium* species. The numbers of CFU were $2.1x10^3$, $3.4x10^3$, $8.7x10^2$, $1.4x10^2$, $5.6x10^2$, $1.0x10^3$, $3.0x10^3$ and $8.4x10^2$ per gram dry soil collected in Yamagata, Tottori, Kagoshima, Sohka, Yayoi, Hongo, Nishiarai and Kanamachi, respectively. Pink-pigmented bacteria was predominantly detected in all soil samples used in this study. Blast search using the partial 16S rDNA sequences of the representative pink-pigmented bacteria revealed that they belonged to the genus *Methylobacterium*.

The Zn resistance of *Methylobacterium* MIC values of representative strains among all isolates and reference strains were measured. The MIC value of *Escherichia coli* was lower than 1 mM, and most of the soil bacteria are considered to have this level of MIC (10). Bacteria resistant to 10 mM Zn are categorized as Zn-resistant bacteria (11). Judging from the MIC values, almost isolates from non-polluted soils are considered as strong Zn-resistant bacteria having a high level of MIC. These facts suggest that the Zn-resistant *Methylobacterium* is widely distributed in non-polluted fields. However, the MIC values of CT4, NI2, NI4, and NI7 were relatively lower than the other isolates. Reference *Methylobacterium* strains obtained from the culture collections could be considered as Zn-resistant bacteria, although their overall MIC values are lower than those of all isolated strains in this study.

The UV tolerance of *Methylobacterium* All the *Methylobacterium* species showed strong UV tolerance of the degree of 9.6 to 48 mW $(cm^2)^{-1}$. The UV tolerance of *Methylobacterium* species was at least 12-fold higher than that of *Escherichia coli*. Therefore, the widely distributed *Methylobacterium* species could be considered as the UV-tolerant bacteria. The UV tolerance of the *Methylobacterium* species seems to be related to pink pigment production. Pink pigment probably protects against cell membrane damage due to UV light. All tested strains showed strong UV tolerance and this fact demonstrates that UV tolerance is an inherent characteristic of *Methylobacterium*.

The carbon source utilization and enzyme activities of *Methylobacterium* Utilization of carbon compounds by *Methylobacterium* strains was determined. The carbon source utilization of isolates was differentiated by two patterns corresponding to the *M. radiotolerans* group and the *M. extorquens* group, respectively. However, examined enzyme activities of all *Methylobacterium* strains were quite similar. Different activities were shown in the only 5 kinds of enzymes such as, leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase among all strains. All strains showed negative reaction for alkaline phosphatase, esterase (C4), lipase (C14), valine

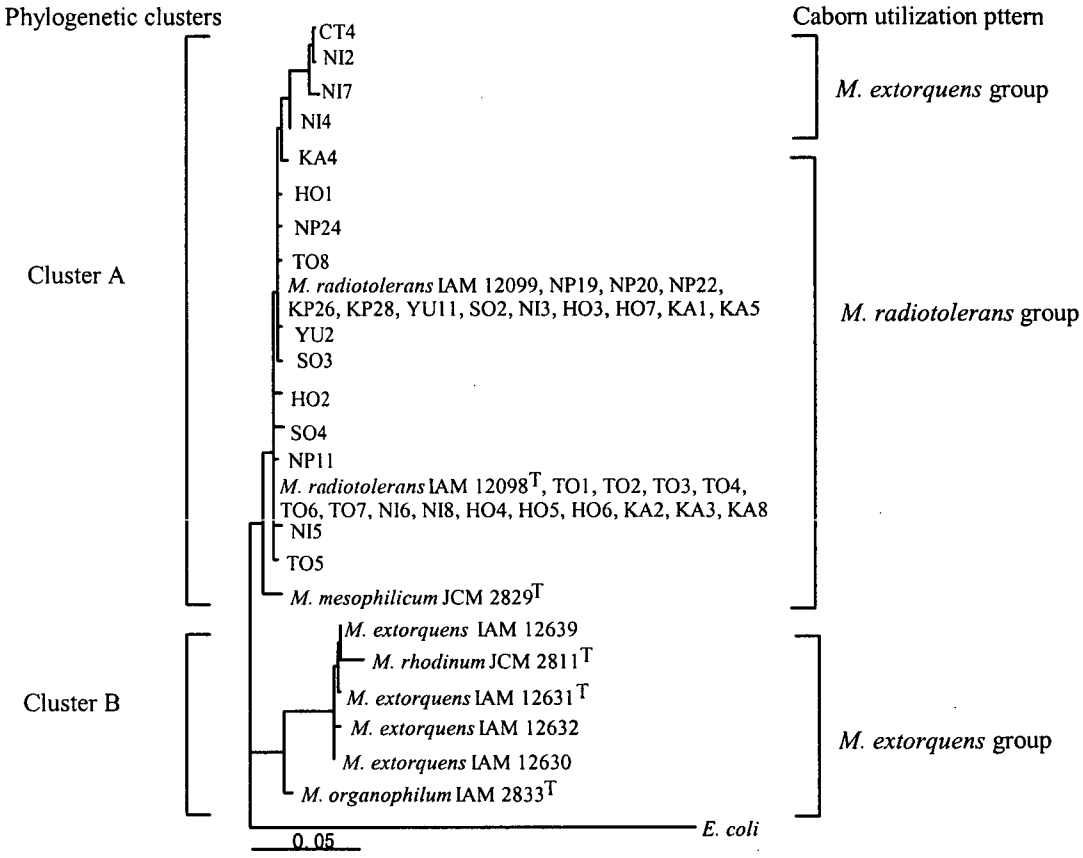

Fig. 2. Phylogenetic relationships of *Methylobacterium* species inferred from the maximum likelihood method Scale bar means substitutions per site.

arylamidase, cystine arylamidase, chymotrypsin, α -galactosidase, β-galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase. All strains also showed positive reaction for ester lipase (C8).

Phylogenetic analysis and the relationship with physiological properties The phylogenetic tree constructed by the ML method is shown in Fig. 2. All *Methylobacterium* strains were divided into two clusters. This topology supports that phylogenetic positions of the Zn-resistant *Methylobacterium* strains well coincide with the carbon source utilization patterns. All strains isolated in this study were placed in the cluster A. Strong zinc-resistant strains were distributed in this cluster and they belonged to the *M. radiotolerans* group. CT4, NI2, NI4, and NI7 belonging to the *M. extorquens* group, which showed moderate Zn-resistance, were also placed in the cluster A. Cluster B consists of moderate Zn-resistant strains corresponding to the *M. extorquens* group. We can not find the evolutionary relationship between strong- and moderate-zinc resistant strains in the cluster A. However, all of strong Zn-resistant *Methylobacterium* isolated in this study are placed in the cluster A and this fact indicates that Zn resistance of *Methylobacterium* seems to be correlated with the phylogenetic position.

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