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NOTE

Distribution and isolation of strains belonging to the order *Solirubrobacterales*

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The number of prokaryotes on earth is estimated to be in the order of 10^{30} cells,[1] and it is well known that most microorganisms in the environment are as-yet-uncultured.[2] Many approaches have been tried to isolate unknown bacterial strains. Davis *et al.*[3] reported that medium choice and incubation time are significant for isolation of rare soil bacteria. *Patulibacter minatonensis*, belonging to the order *Solirubrobacterales*, was isolated using medium supplemented with superoxide dismutase and proposed as a novel genus in 2006.[4] At that time, the order *Solirubrobacterales*[5] consisted of only three families, three genera and three species; *Patulibacter minatonensis*, *Conexibacter woesei*[6] and *Solirubrobacter pauli*,[7] and presently there are still 11 species including the one species that is isolated in this study. We believe that as-yet-uncultured bacteria may exist within this taxon, and it is possible that additional strains could be used as an untapped resource. As the genera *Patulibacter*, *Conexibacter* and *Solirubrobacter* grow slowly, we tried to investigate their distribution and isolate them by cultivation over a long period of time in order to isolate strains belonging to the order *Solirubrobacterales*.

The strategy of detection and isolation of strains in the order *Solirubrobacterales* is shown in Figure 1. We designed specific primers for detecting *Solirubrobacterales* strains using the sequences of nine most closely related strains. The strains were as follows: *Patulibacter minatonensis* KV-614^T, *Conexibacter woesei* DSM 14684^T,[6] *Solirubrobacter pauli* B33D1^T,[7] *Rubrobacter radiotolerans* DSM 5868^T,[8] *R. taiwanensis* LS-286,[9] *R. xylanophilus* PRD-1^T,[10] *Symbiobacterium toebii* SC-1^T,[11] *Thermoleophilum album* ATCC 35266^{TM} and *T. minutam* ATCC 35268^T.[12,13] These gene sequences were aligned to obtain specific sequences and then visually compared to identify regions showing a high degree of conservation within the target order. The primer length was adjusted to give an appropriate T_m range to minimize T_m differences within primer pairs. Specific primer sets for strains related to the order *Solirubrobacterales* were designed as follows: 423PF: 5′-TCAGTTGGGACGAAGCTTC-3′ and 1012PR: 5′-AGGGAAGACGTGTTTCCAC-3′. PCR was performed initially with universal primers, and then nested PCR was performed for detection of target DNA in soil samples. PCR with universal primers (11F:5′-AGTTTGATCATGGCTCAG-3′, 1100R:5′-GGGTTGCGCTCGTTG-3′ or 1115R:5′-AGGGTTGCGCTCGTTG-3′) was performed as follows: initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min and an additional extension step at 72 °C for 2 min. Reaction mixtures (50 μl) containing 0.4 μl of Taq polymerase (TaKaRa, Shiga, Japan), 5.0 μl of 10 × Taq buffer, 2.0 μl of dNTP mixtures (2.5 μM), 29.6 μl of dH_2O, 4.0 μl of each primer (5 μM) and 5.0 μl of DNA were prepared. As a second step, PCR with specific primers (423PF-1012PR) was performed as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1.5 min and an additional extension step at 72 °C for 2 min. Reaction mixtures (25 μl) containing 0.2 μl of Taq polymerase (TaKaRa), 2.5 μl of 10 × Taq buffer, 1.0 μl of dNTP mixtures (2.5 μM), 14.3 μl of dH_2O, 2.0 μl of each primer (20 μM) and 3.0 μl of the first PCR products were prepared. The specific primers were tested using three genera belonging to the order *Solirubrobacterales* and specific bands of approximately 590 bp were detected in *P. minatonensis* KV-614^T and *C. woesei* NBRC 100937^T, but not in *S. pauli* JCM 13025^T. This result indicates that it was possible to detect strains related to them with some exceptions. Forty-four soil samples were collected from various environments in seven prefectures (Ibaraki, Saitama, Tokyo, Kanagawa, Chiba, Yamanashi and Okinawa) in Japan. Bacterial DNA was directly extracted from soil samples using a PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions and tested in the PCR as it was presumed that the primer set designed would be effective for investigating the distribution of *Solirubrobacterales* strains. Specific bands were detected in 31 (70%) of 44 soil samples by PCR (Figure 1-① and Figure 2, and Table 1). This result suggests that, although these are thought to be rare bacterial strains, they are widely distributed in soil. To confirm that these DNA fragments originated from target bacterial genomes, amplified DNA from soil sample no. 2, 3 and 5 was cloned using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and extracted using PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. DNA fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using a 3130 Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA). Sequences of seven clones from sample no. 2,

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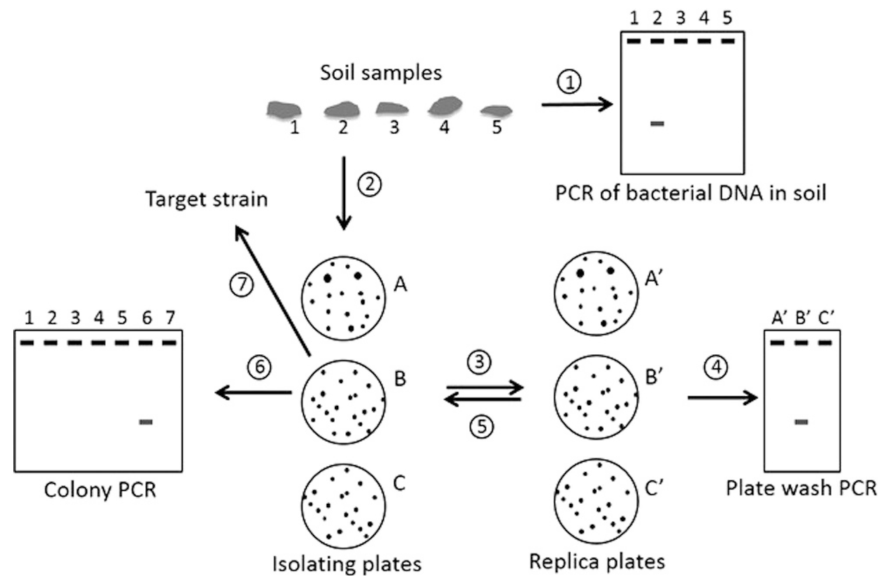

Figure 1 Strategy for distribution and isolation of order *Solirubrobacterales* strains. ① Detection of target DNA fragments by specific primers, ② isolation of bacteria from selected soil sample, ③ preparation of replica plates, ④ detection from all colonies on replica plate (Plate wash PCR), ⑤ selection of isolating plate, ⑥ detection of target colony (Colony PCR), ⑦ isolation of target strain (order *Solirubrobacterales* strain).

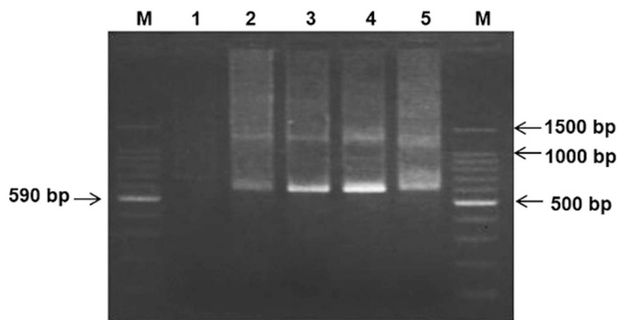

Figure 2 Detection of target DNA fragment from bacterial DNA of soil samples. Lane M: DNA marker. Lane 1: soil sample no. 1 from hill, Tokyo. Lane 2: soil sample no. 2 from field, Saitama prefecture. Lane 3: soil sample no. 3 from rice field, Saitama prefecture. Lane 4: soil sample no. 4 from forest, Saitama prefecture. Lane 5: soil sample no. 5 from sandy beach, Chiba prefecture

seven clones from no. 3 and six clones from no. 5 were analyzed and compared with two databases, DDBJ (http://blast.ddbj.nig.ac.jp/blastn?lang=ja) to find the related species and EzTaxon (http://www.ezbiocloud.net/eztaxon/identify) to identify the most closely validly recognized species (Table 2). The sequences have been deposited in DDBJ under accession numbers LC015776-LC015795. All of the clones were found to have highest similarities with the genera *Solirubrobacter* or *Conexibacter* strains in the order *Solirubrobacterales* (93.6 to 99.4%). Clones that were closest to *C. woesei* DSM 14684^T were detected in all soil samples. The amplified genes were confirmed to have originated from microorganisms related to the order *Solirubrobacterales* strains. Therefore, we next attempted to isolate bacterial strains from the soil sample collected from the field in Saitama Prefecture (sample no. 2 in Table 1 and Figure 2).

Isolation was carried out by a combination of Plate wash PCR using the previously described Stevenson procedure[14] with modifications and colony PCR. One gram of soil sample was serially diluted (10^{-1}–10^{-4}) and 100 µl of diluted samples were spread on

Table 1 Detection of target DNA from soil using specific primers

Source of soil sample	*Number of samples*	*Number of positive samples*	*Ratio (%)*
Agricultural land	11	7	64
Waterside	9	9	100
Under the trees	7	4	57
Others	17	11	65
Total	44	31	70

Agricultural land; rice field, field and fruit farm.
Waterside; sandy beach and around the lake.
Under the trees; under the tree exclude grove soil.
Others; track, rangeland, sidewalk, mountain path, botanical garden, grove, cemetery, flowerbed, sand in park, thicket, shrine and park.

1/5 strength nutrient agar (1/5 NA; Difco, Detroit, MI, USA) plates containing benlate (20 µg ml^{-1}; DuPont, Wilmington, DE, USA). Isolating plates were incubated at 27 °C for 20 days (Figure 1-②) and replica plates were made for each (Figure 1-③). Colonies grown on the replica plates for 9 days were collected into 500 µl sterile dH_2O using a cell scraper with modified Stevenson's method[14] (Figure 1-④). The cells were then washed twice with TE buffer (10 mM Tris-HCl, EDTA/2Na) with centrifugation at 7000 r.p.m. for 5 min, and suspended in 500 µl TE buffer. DNA extraction was performed by ultrasonic fragmentation according to the method of Yu *et al.*[15] Target DNA fragments were detected from only one (10^{-4} diluted) of the replica plates. Then, colony PCR was performed for all 91 colonies on the original isolating plate (Figure 1-⑤ and ⑥). Target DNA fragments were detected in seven colonies and the specific fragment sequences of these colonies were then analyzed. Five of these colonies were found to be closest to *P. minatonensis* KV-614^T with similarities of 98.5 to 100%. Two of the seven colonies, KV-962 and KV-963, were closest to *C. woesei* DSM 14684^T (98.3% similarity). Taxonomic studies of the two strains were carried out as the 16S rRNA gene sequences of KV-962 (accession no. AB597950) and KV-963 (accession no. AB597951) showed low similarity with those of *C. woesei* DSM

Table 2 The closely matched species based on DNA sequences cloned from amplified fragments

Soil no.	*Clone no.*	*Length of sequence*	*Accession no.*	*Closest match in EzTaxon database*	*Closest match in DDBJ database*
	1	506	LC015776	*Solirubrobacter phytolaccae* GTGR-8^T (99.4)	*Solirubrobacter* sp. L64, FJ459990 (99.8)
	2	534	LC015777	*Solirubrobacter ginsenosidimutans* BXN5–15^T (98.9)	*Solirubrobacter* sp. L64, FJ459990 (99.8)
	3	536	LC015778	*Solirubrobacter ginsenosidimutans* BXN5–15^T (99.3)	*Solirubrobacter* sp. L64, FJ459990 (99.3)
2	4	492	LC015779	*Solirubrobacter phytolaccae* GTGR-8^T (96.5)	*Solirubrobacter* sp. L64, FJ459990 (97.9)
	5	507	LC015780	*Solirubrobacter ginsenosidimutans* BXN5–15^T (99.2)	*Solirubrobacter* sp. L64, FJ459990 (99.8)
	6	536	LC015781	*Conexibacter woesei* DSM 14684^T (96.6)	*Conexibacter* sp. BS10, JF806520 (98.0)
	7	557	LC015782	*Conexibacter woesei* DSM 14684^T (97.1)	Bacterium Ellin504, AY960767 (97.5)
	8	542	LC015783	*Conexibacter woesei* DSM 14684^T (94.8)	*Conexibacter woesei* DSM 14684^T, CP001854 (95.3)
	9	528	LC015784	*Conexibacter woesei* DSM 14684^T (95.3)	*Conexibacter woesei* DSM 14684^T, CP001854 (95.7)
	10	531	LC015785	*Conexibacter woesei* DSM 14684^T (95.3)	*Conexibacter woesei* DSM 14684^T, CP001854 (95.7)
3	11	536	LC015786	*Conexibacter woesei* DSM 14684^T (95.0)	*Conexibacter woesei* DSM 14684^T, CP001854 (95.4)
	12	534	LC015787	*Conexibacter woesei* DSM 14684^T (95.3)	*Conexibacter woesei* DSM 14684^T, CP001854 (95.6)
	13	531	LC015788	*Conexibacter woesei* DSM 14684^T (95.3)	*Conexibacter woesei* DSM 14684^T, CP001854 (95.8)
	14	560	LC015789	*Conexibacter woesei* DSM 14684^T (93.6)	Bacterium Ellin325, AF498707 (96.3)
	15	557	LC015790	*Solirubrobacter ginsenosidimutans* BXN5–15^T (98.2)	*Solirubrobacter* sp. L64, FJ459990 (99.8)
	16	490	LC015791	*Solirubrobacter ginsenosidimutans* BXN5–15^T (97.8)	*Solirubrobacter* sp. L64, FJ459990 (98.7)
5	17	555	LC015792	*Conexibacter woesei* DSM 14684^T (94.8)	Bacterium Ellin504, AY960767 (97.3)
	18	526	LC015793	*Conexibacter woesei* DSM 14684^T (94.7)	Bacterium Ellin504, AY960767 (97.2)
	19	554	LC015794	*Conexibacter woesei* DSM 14684^T (94.9)	bacterium Ellin504, AY960767 (97.3)
	20	513	LC015795	*Solirubrobacter phytolaccae* GTGR-8^T (97.0)	*Conexibacter arvalis* KV-962^T, AB597950 (97.4)

The values within parentheses () represents similarity percentage of closest strain.
Soil sample number is same as the number mentioned in Figure 2.

14684^T, and they were proposed as a new species of *Conexibacter* and *C. arvalis*.[16] KV-962 and KV-963 were isolated from the soil samples from which clones no. 6 and no. 7 originated. The similarity values between these two isolates and two clones were 97.1–98.0%. The similarity values are not so high. This means that more related strains exist in this sample.

The order *Solirubrobacterales* is presently composed of only three genera containing 11 species. DNA fragments of strains belonging to the order *Solirubrobacterales* were detected from all prefectures examined (Data are not shown). This result indicates that they are cosmopolitan. According to isolation conditions, it is possible to isolate them more frequently. Especially, samples from waterside showed high rate of target DNA fragments (Table 1). Waterside may be suitable to inhabit as these strains have motility. We succeeded to isolate them using 1/5 NA by a combination of Plate wash PCR. These results show that bacterial strains belonging to the order *Solirubrobacterales* are widely found living in the soil.

P. minatonensis KV-614^T was isolated using a medium supplemented with SOD to remove oxygen species generated from nutrient agar.[17] Therefore, we used 1/5 NA for suppression of superoxide. *P. ginsengiterrae* P4–5^T was isolated using 1/10 NA,[18] and R2A agar was used for isolation of *S. soli* and *S. phytilica*.[19,20] These data show that nutritionally poor media are effective for isolation of *Solirubrobacterales* strains. Our clone library analyses in Table 2 indicated that the gene sequences are related to strains Ellin 325 or Ellin 504, which were originally isolated using nutritionally poor medium after cultivation for 3–6 months.[21,22] Although these isolates were isolated using poor media, it is unclear whether they are sensitive to reactive oxygen.

We expect that we will be able to obtain many as-yet-uncultured bacteria as untapped resources from the environment using new ideas for isolating and cultivating.

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