Original Article

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Isolation of antibiotic-producing *Pseudomonas* species with low-temperature cultivation of temperate soil

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Summary **We performed low-temperature cultivation of soil samples in Tokyo, Japan, and isolated 30 bacterial strains that formed colonies at 4°C. All the culture supernatants of these bacteria exhibited antibacterial activity against *Escherichia coli*. The 16S rDNA sequences of 29 strains showed similarity to that of the *Pseudomonas* genus, whereas the 16S rDNA sequence of one strain showed similarity to that of the *Janthinobacterium* genus. We classified the 29 strains into 10 groups according to the 16S rDNA sequence similarities, and performed two phylogenetic analyses using the 16S rDNA and *rpoD* gene sequences. Four groups formed a unique branch within *Pseudomonas* species in both phylogenetic analyses. Four other groups were closely related to the *Pseudomonas* species, but the most closely related species differed between the two phylogenic tree analyses. These results suggest that low-temperature cultivation of temperate soil is effective for isolating new bacterial sources for producing antibiotics.**

Keywords: Low-temperature cultivation, *Pseudomonas* species, phylogenetic analyses, soil bacteria

1. Introduction

Many antibiotics have been identified from bacterial culture supernatants. Unfortunately, however, the discovery rates of novel antibiotics from bacterial sources have been steadily decreasing over the years (*1*). Thus, new sources are needed for the development of novel antibiotics.

An estimated 99% of all bacterial species on earth are uncultured (*2*). These uncultured bacteria are expected to produce secondary metabolites distinct from those of known bacteria and are promising sources of new antibiotics (*3*). Thus, many methods have been investigated for culturing these bacteria or for isolating new genetic sources without culturing bacteria. A recent study identified a novel antibiotic from uncultured bacteria that is effective against drug-resistant bacteria (*4*).

Low temperature cultivation is a useful method of culturing bacteria from cold areas, because bacteria in cold areas can grow at low temperature. Such psychrophilic microorganisms have been targeted as new biologic resources for antibiotics, protein expression systems, or environmental pollution control (*5,6*). In contrast, bacteria in temperate regions are normally cultivated at 20-30°C, because many bacteria in these regions have optimal temperature growth at this range. The temperature of temperate soil falls to around 0°C in the winter with a year-round mean temperature below 20°C. In the present study, we performed low temperature cultivation of temperate soil bacteria to search for new bacterial sources for novel antibiotics.

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2. Materials and Methods

2.1. *Isolation of environmental strains*

Soil samples were collected from the ground at the University of Tokyo (35˚ 71' 20.56" N 139˚ 76' 2775" E) in February 2016 and March 2016. Approximately 5 g of soil was suspended in 50 mL of sterilized saline. One hundred microliters of the suspended solution was spread on a Luria-Bertani (LB) plate and incubated at 4°C for 7 to 10 days.

2.2. *Bacteria or fungi*

Escherichia coli JM109 was cultured in LB medium at 37°C, *Staphylococcus aureus* RN4220 was cultured in tryptic soy broth medium at 37°C, and *Candida albicans* ATCC10231 was cultured in YPD medium at 37°C.

2.3. *Assay of antimicrobial activities of the supernatants of the isolates*

The isolates were cultured for 1 week at 4°C on a rotary shaker at 150 rpm. The cultures were centrifuged at 20,400g for 5 min and the supernatants were filtered with 0.44-μm filters and stored at 4°C until the assay was performed. Overnight cultures of the microbes (*Escherichia coli* JM109, *Staphylococcus aureus* RN4220, and *Candida albicans* ATCC10231) were diluted 100-fold with Muller-Hinton broth. A 100-μL aliquot of the supernatant was serially diluted, mixed with 100 μL of the microbial solution in 96-well round bottom plates, and then incubated at 37°C overnight. The microbial growth in each well was observed by visual inspection. The maximum dilution that inhibited microbial growth was defined as the growth inhibitory activity.

2.4. *Extraction of genomic DNA*

Genomic DNA (gDNA) of the bacteria was extracted as described previously (*7*) with minor modification. Bacterial cells were collected from 2 mL liquid culture by centrifugation at 20,400g for 5 min. The bacteria were lysed in 200 μL extraction buffer (0.05 M Tris-HCl [pH 8.0], 0.05 M NaCl, 0.05 M EDTA, 0.5% sodium dodecyl sulfate), 25 mg acid-washed glass beads, and 200 μL PCI (phenol: chloroform: isoamyl alcohol = 25:24:1, v/v) by vortex for 5 min. Four hundred microliters of TE buffer (0.01 M Tris-HCl, 1 mM EDTA, pH8.0) and 200 μL of PCI was added to the sample, and the sample was centrifuged at 20,400g for 5 min. Forty microliters of 3 M sodium acetate (pH 7.0) and 400 μL of 2-propanol were added to the centrifuged supernatant (400 μL). After incubating at room temperature for 5 min, the samples were centrifuged at 20,400g for 10 min. The precipitate was washed with 70% ethanol and solved in 50 μL TE. The gDNA solutions were preserved at 4°C.

2.5. *16s rRNA gene phylogeny reconstruction*

The *16s rRNA* genes of isolates were amplified by polymerase chain reaction (PCR) from the extracted gDNA with four universal primers: E9F, E939R, E334F, and E1541R (*8*). After analysis of the products by electrophoresis on 1% agarose gels, the amplified PCR products were purified by phenol-chloroform extraction and ethanol precipitation. The purified PCR products were sequenced with an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. The sequences were compared with those of the type strains of bacterial species recorded in the EzTaxon database (*9*). The sequences of the isolates were aligned with the sequences of similar strains of the genus *Pseudomonas* using the ClustalW software (*10*). Based on the *16s rRNA* gene sequences, phylogenetic trees were reconstructed using the maximum-likelihood (*11*) and neighbor-joining (*12*) methods with the MEGA7 package (*13*). The *16s rRNA* gene sequences of the bacterial isolates in this study were registered in Genbank (LC230063-LC230092).

2.6. *rpoD gene phylogeny reconstruction*

The *rpoD* genes of isolates were amplified by PCR from gDNA extracted with two primers, PsEG30F and PsEG790R (*14*). The amplified *rpoD* gene was sequenced as described above. The obtained *rpoD* sequences were compared with those from the GenBank database in BLAST (*15*). The phylogenetic trees were constructed using the *rpoD* sequences that showed high similarity in the BLAST search, as described above. The *rpoD* sequences of the bacterial isolates in this study were registered in Genbank (LC230053-LC230062).

2.7. *Growth assay at different temperatures*

The isolates were cultured overnight at 17°C in LB medium. A 150-μL aliquot of the overnight cultures was inoculated into 5 mL of fresh LB broth. The cultures were incubated at four different temperatures (4, 17, 30, 37°C) on a rotary shaker at 150 rpm. The optical densities at 600 nm (OD_{600}) were measured at four time-points (0, 3, 6, and 24 h). The experiment was repeated three times. The OD_{600} values at each time-point were averaged and plotted with the standard errors of the mean.

3. Results

Five grams of soil samples were obtained in Tokyo, Japan, which is a temperate region. The samples were suspended in sterilized saline, spread on Luria-Bertani agar plates, and cultured at 4°C for 7 to 10 days. Thirty microbial strains that formed colonies were isolated.

To investigate whether the 30 isolated strains are potential sources of antibiotics, we examined the antimicrobial activities of the culture supernatants of these strains. All supernatants of the isolated strains inhibited the growth of *E. coli* (Table 1). A supernatant of one strain (P-11) weakly inhibited the growth of S. *aureus*, but the supernatants of the others did not inhibit *S. aureus* growth (Table 1). None of the supernatants inhibited the growth of *C. albicans* (Table 1). These

Table 1. Antimicrobial activities of the culture supernatants of the 30 isolated strains

Strain[1]	Growth inhibitory activity[2]		
	E. coli	*S. aureus*	*C. albicans*
P-1	2	ND	ND
P-2	2	ND	ND
P-3	2	ND	ND
P-4	2	ND	ND
P-5	2	ND	ND
P-6	2	ND	ND
P-7	4	ND	ND
P-8	4	ND	ND
P-9	4	ND	ND
P-10	4	ND	ND
P-11	16	2	ND
P-12	4	ND	ND
P-13	8	ND	ND
P-14	2	ND	ND
P-15	2	ND	ND
P-16	2	ND	ND
P-17	2	ND	ND
P-18	2	ND	ND
P-19	2	ND	ND
P-20	2	ND	ND
P-21	2	ND	ND
P-22	16	ND	ND
P-23	16	ND	ND
P-24	32	ND	ND
P-25	2	ND	ND
P-26	2	ND	ND
P-27	4	ND	ND
P-28	4	ND	ND
P-29	2	ND	ND
P-30	2	ND	ND

[1] "Strain" indicates the ID of the isolated strains. [2] "growth inhibitory activity" indicates the growth inhibitory activities of the supernatants of the isolates against microbes (*E. coli*, *S. aureus*, and *C. albicans*). The growth inhibitory activity was defined as the maximum dilution of the supernatants that inhibited the growth of the pathogens. ND means that the two-fold diluted culture supernatants did not inhibit microbial growth.

results suggest that all of the isolated strains secrete antimicrobial substances against *E. coli*.

To investigate the novelty of the isolated microorganisms, we analyzed the taxonomies of the isolates. We performed phylogenetic analyses with the *16s rRNA* gene sequences, which are widely used to classify bacteria (*16,17*). The full length of each of the *16s rRNA* genes was amplified with PCR and sequenced. Microbial species that have *16s rRNA* gene sequences highly similar to those of the isolated strains were searched using the EzTaxon-e database (*9*) (Table 2). One strain (P-20) had high similarity to the *Janthinobacterium* genus, and the other 29 strains had high similarity to the *Pseudomonas* genus. According to the closest match species, we classified the 29 strains into 10 groups as follows: *Pseudomonas helmanticensis* (*18*), *Pseudomonas moorei* (*19*), *Pseudomonas koreensis* (*20*), *Pseudomonas mohnii* (*19*), *Pseudomonas vancouverensis* (*21*), *Pseudomonas baetica* (*22*), *Pseudomonas frederiksbergensis* (*23*), *Pseudomonas congelans* (*24*), *Pseudomonas mandelii* (*25*), or *Pseudomonas mediterranea* (*26*) (Table 2). We selected 10 strains (P-1, P-7, P-12, P-13, P-14, P-16, P-22, P-23, P-25, and P-26) as representative strains of each group for further phylogenetic analysis (Table 2).

A phylogenic tree based on the *16S rRNA* sequences was constructed between the *Pseudomonas*-type strains and the 10 representative strains using the maximum likelihood method (*11*) (Figure 1). P-22, P-23, P-25, and P-26 formed a cluster that is clearly distinct from the *Pseudomonas* genus type strains. P-13 branched off from *P. mohnii* (*19*) and *P. umsongensis* (*20*). P-7 and P-14 branched off from a cluster including *P. moorei* (*19*), *P. vancouverensis* (*21*), and *P. jessenii* (*25*). P-1 and P-16, respectively, branched off from a cluster including *P. granadensis* (*27*) and *P. helmanticensis* (*18*). P-12 branched off from *P. batumici* (*28*). A phylogenic tree constructed by the neighbor-joining method (*12*) also confirmed the distinct cluster of P-22, P-23, P-25, and P-26 and the branches of P-13, P-14, P-12, and P-1, but not those of P-7 and P-16 (Figure 2).

The *16s rRNA* sequences are widely used in the phylogenetic analyses of bacteria including *Pseudomonads* (*16,17,29,30*), but phylogenic analysis based on the *16s rRNA* sequences does not have sufficient resolution to distinguish different species of the *Pseudomonas* genus. Thus, other housekeeping genes, including *gyrB*, *rpoB*, and *rpoD* are used for phylogenetic analyses of *Pseudomonas* species (*14,31-37*). Because analysis using the *rpoD* gene has the highest resolution (*14*), we used the *rpoD* gene for additional phylogenetic analyses. The *rpoD* gene was amplified by PCR and sequenced. Microbial species with similar *rpoD* sequences to those of the 10 representative strains were searched in the BLAST database (*15*). Eight representative strains other than P-7 and P-13 showed similarities lower than 96% to the *rpoD* sequences of known species (Table 3). A phylogenic tree based on the *rpoD* sequences was constructed between *Pseudomonas*-type strains and the 10 representative strains using the maximum likelihood method (Figure 3). P-7 branched off from *P. umsongensis* and P-13 branched off from *P. jessenii*. P-14 formed a distinct branch with P-7, P-13, *P. umsongensis*, and *P. jessenii*. P-12 and P-16, respectively, branched off from *P. koreensis* and *P. helmanticensis*. P-22, P-23, P-25, and P-26 formed a distinct cluster. P-1 formed a distinct branch from *P. proteolytica*. The phylogenetic tree branches, except those of P-1, P-14, and P-16, were also observed in the phylogenic tree constructed using the neighbor-joining method (Figure 4). The phylogenetic tree branches of P-1, P-14, and P-16 were slightly different between the maximum likelihood method and the neighbor-joining method, but both methods indicated that P-1 was related to *P. aeruginosa* and *P. proteolytica*, P-14 was related to *P. umsongensis*, *P. putida*, and *P. vancouverensis*, and P-16 was related to P. *marginalis* and *P. helmanticensis*.

Phylogenetic trees based on the *16S rRNA*

Table 2. Closest matched species estimated from *16S rRNA* gene sequences

Strain[1]	Closest Match[2]	Accession Number[3]	Similarity (%)[4]	Completeness (%)[5]
P-1*	*P. helmanticensis*	HG940537	99.4	100
P-2	*P. helmanticensis*	HG940537	99.4	100
P-3	*P. helmanticensis*	HG940538	99.6	100
P-4	*P. helmanticensis*	HG940539	99.4	100
P-5	*P. helmanticensis*	HG940539	99.6	100
P-6	*P. helmanticensis*	HG940539	99.6	100
P-15	*P. helmanticensis*	HG940537	99.7	100
P-7*	*P. moorei*	AM293566	99.7	100
P-8	*P. moorei*	AM293565	99.9	100
P-9	*P. mohnii*	AM293567	100	100
P-10	*P. mohnii*	AM293567	99.7	100
P-12*	*P. koreensis*	AF468452	99.6	99.7
P-30	*P. koreensis*	AF468452	99.7	99.7
P-13*	*P. mohnii*	AM293567	99.6	100
P-11	*P. vancouverensis*	AJ011507	99.9	100
P-14*	*P. vancouverensis*	AJ011507	99.7	100
P-16*	*P. baetica*	FM201274	99.4	99.1
P-21	*P. baetica*	FM201274	99.6	99.1
P-20*	*J. swalbardensis*	DQ355146	99.7	96.8
P-22*	*P. frederiksbergensis*	AJ249382	98.8	100
P-27	*P. frederiksbergensis*	AJ249383	98.6	100
P-28	*P. frederiksbergensis*	AJ249383	98.6	100
P-29	*P. frederiksbergensis*	AJ249383	98.7	100
P-23*	*P. congelans*	AJ492828	98.6	100
P-24	*P. congelans*	AJ492828	98.5	100
P-17	*P. mandelii*	AF058286	98.7	100
P-18	*P. mandelii*	AF058286	98.7	100
P-19	*P. mandelii*	AF058286	98.7	100
P-25*	*P. mandelii*	AF058286	98.7	100
P-26*	*P. mediterranea*	AUPB01000004	98.9	100

[1] "Strain" indicates the ID of the isolated strains. [2] "Closest match" indicates the species with which the *16s rRNA* gene sequences of the isolated strain were most similar. [3] "Accession Number" indicates the accession number of the sequence of the most similar species in Genbank. [4] "Similarity" indicates the similarity of the sequences of the isolated strain and the closest matched species. [5] "Completeness" indicates the ratio of the length of the query sequence to the full-length sequence. 11 groups are segmented by horizontal lines, and asterisks indicate the representative strain in each group.

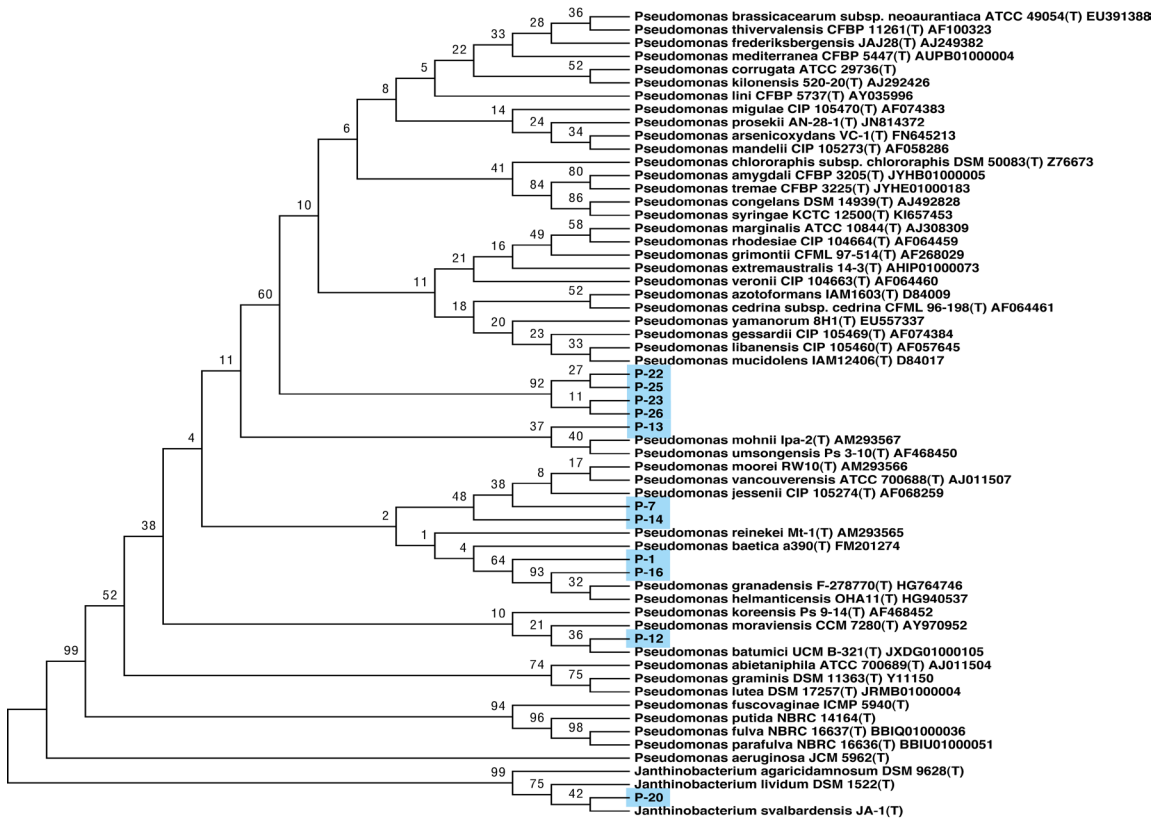

Figure 1. 16S rRNA-based phylogenetic tree by the maximum likelihood method. The evolutionary history was inferred with the maximum likelihood method. The bootstrap values (1,000 replicates) are shown above the branches. Only the topology is shown.

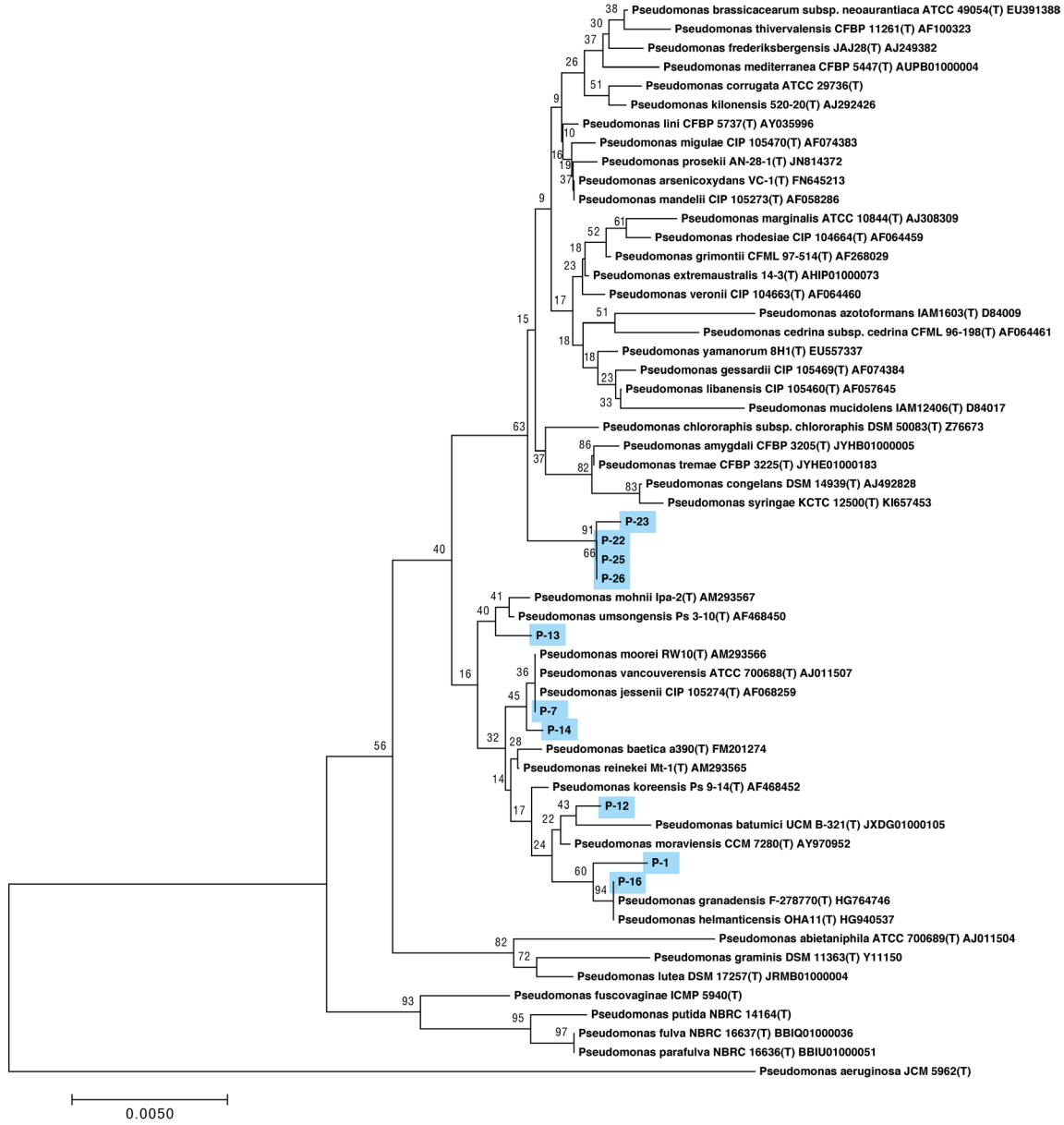

Figure 2. 16S rRNA-based phylogenetic tree by the neighbor-joining method. The evolutionary history was inferred with the neighbor-joining method. The bootstrap values (1,000 replicates) are shown above the branches. The evolutionary distances are in the units of the number of base substitutions per site.

Table 3. Closest matched species estimated from the *rpoD* gene sequences

Strain[1]	Closest Match[2]	Accession Number[3]	Identity (%)[4]	Query Cover (%)[5]
P-1	*P. marginalis*	AB039544.1	95	100
P-7	*P. putida*	D86031.1	98	99
P-12	*P. fluorescens*	CP000094.2	96	99
P-13	*P. jessenii*	FN678364.1	97	99
P-14	*P. reinekei*	FN678362.1	94	99
P-16	*P. marginalis*	AB039544.1	95	100
P-22	*P. mandelii*	CP005960.1	93	100
P-23	*P. mandelii*	CP005960.1	93	99
P-25	*P. mandelii*	CP005960.1	93	100
P-26	*P. mandelii*	CP005960.1	93	100

[1] "Strain" indicates the ID of the isolated strains. [2] "Closest Match" indicates bacterial species with the highest similarity to the *rpoD* gene sequences of the isolated strains. [3] "Accession Number" indicates the accession numbers of the *rpoD* gene sequences of the closest matched species in Genbank. [4] "Identity" indicates the similarity of the *rpoD* gene sequences of the isolated strains and closest matched species. [5] "Query Cover" indicates the ratio of the sequences of the isolated strains aligned to the those of the closest matched species.

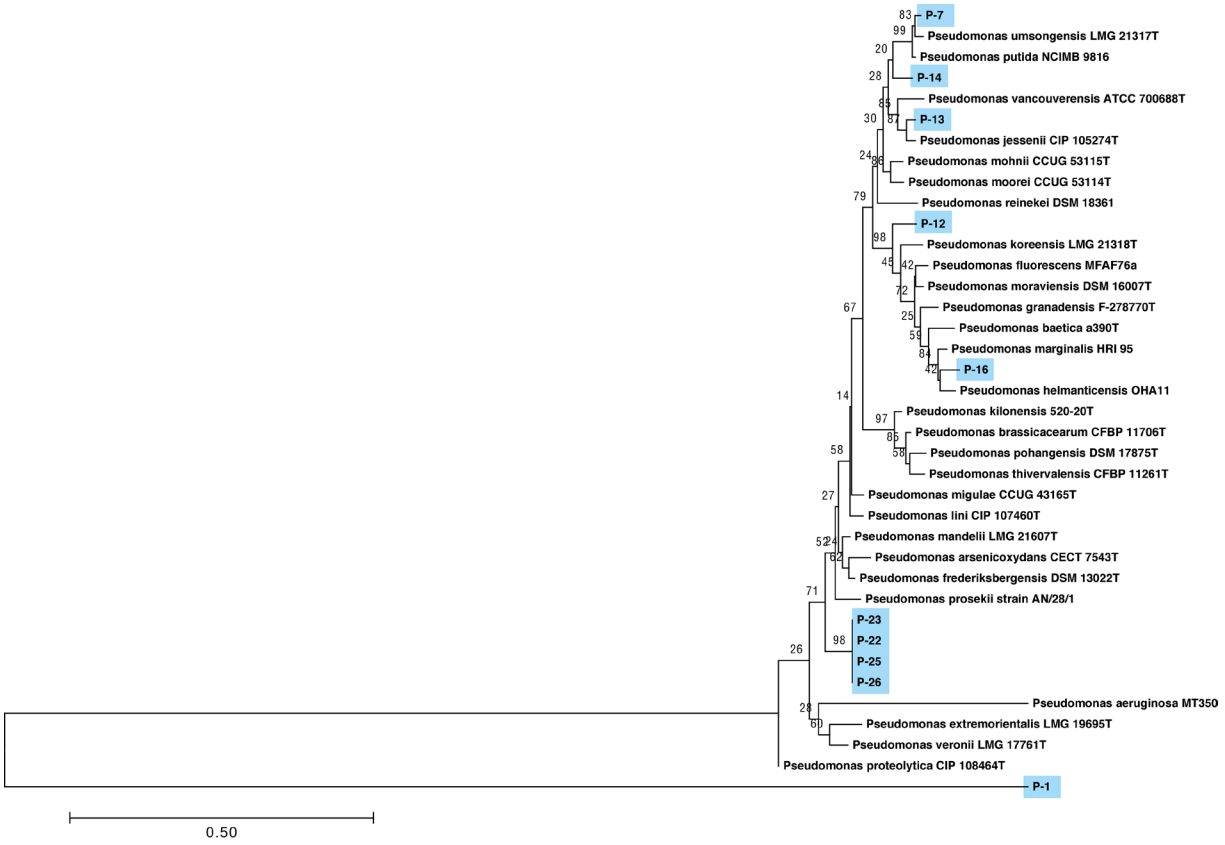

Figure 3. The *rpoD* gene-based phylogenetic tree by the maximum likelihood method. The evolutionary history was inferred with the maximum likelihood method. The bootstrap values (1,000 replicates) are shown above the branches. The branch lengths are measured in the number of substitutions per site.

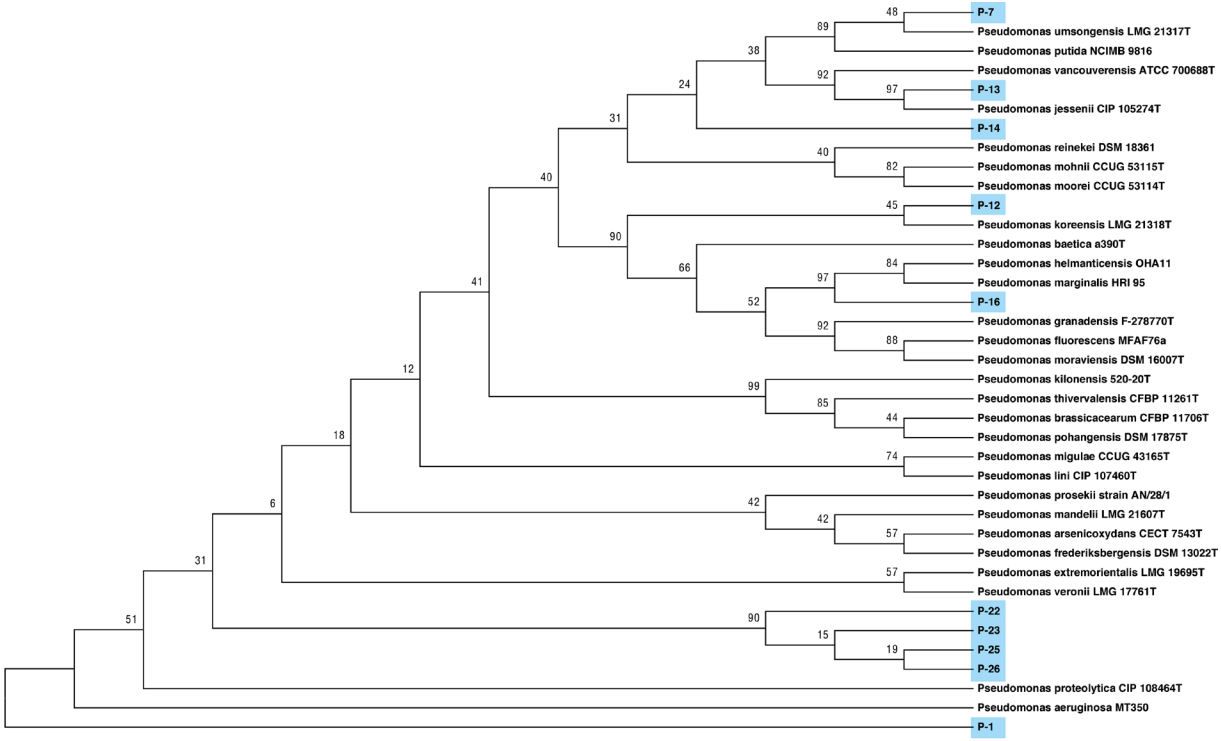

Figure 4. The *rpoD* gene-based phylogenetic tree by the neighbor-joining method. The evolutionary history was inferred with the neighbor-joining method. The bootstrap values (1,000 replicates) are shown above the branches.

Table 4. Comparison of the closest matched species between *16s rRNA*- or *rpoD*-based phylogenies

Strain[1]	Closest Match[2]	
	16s rRNA	*rpoD*
P-1	*P. helmanticensis, P. granadensis,* P-16	*P. aeruginosa, P. proteolytica*
P-7	*P. moorei, P. vancouverensis, P. jessenii*	*P. umsongensis*
P-12	*P. batumici*	*P. koreensis*
P-13	*P.mohnii, P.umsongensis*	*P. jessenii*
P-14	*P. vancouverensis, P. moorei, P.jessenii,* P-7	*P. vancouverensis, P. umsongensis, P. putida*
P-16	*P. helmanticensis, P. granadensis*	*P. helmanticensis, P. marginalis*
P-22	P-23, P-25, P-26	P-23, P-25, P-26

[1] "Strain" indicates the ID of the isolated strains. [2] "Closest match" indicates the closest match species estimated from the sequences of conserved genes.

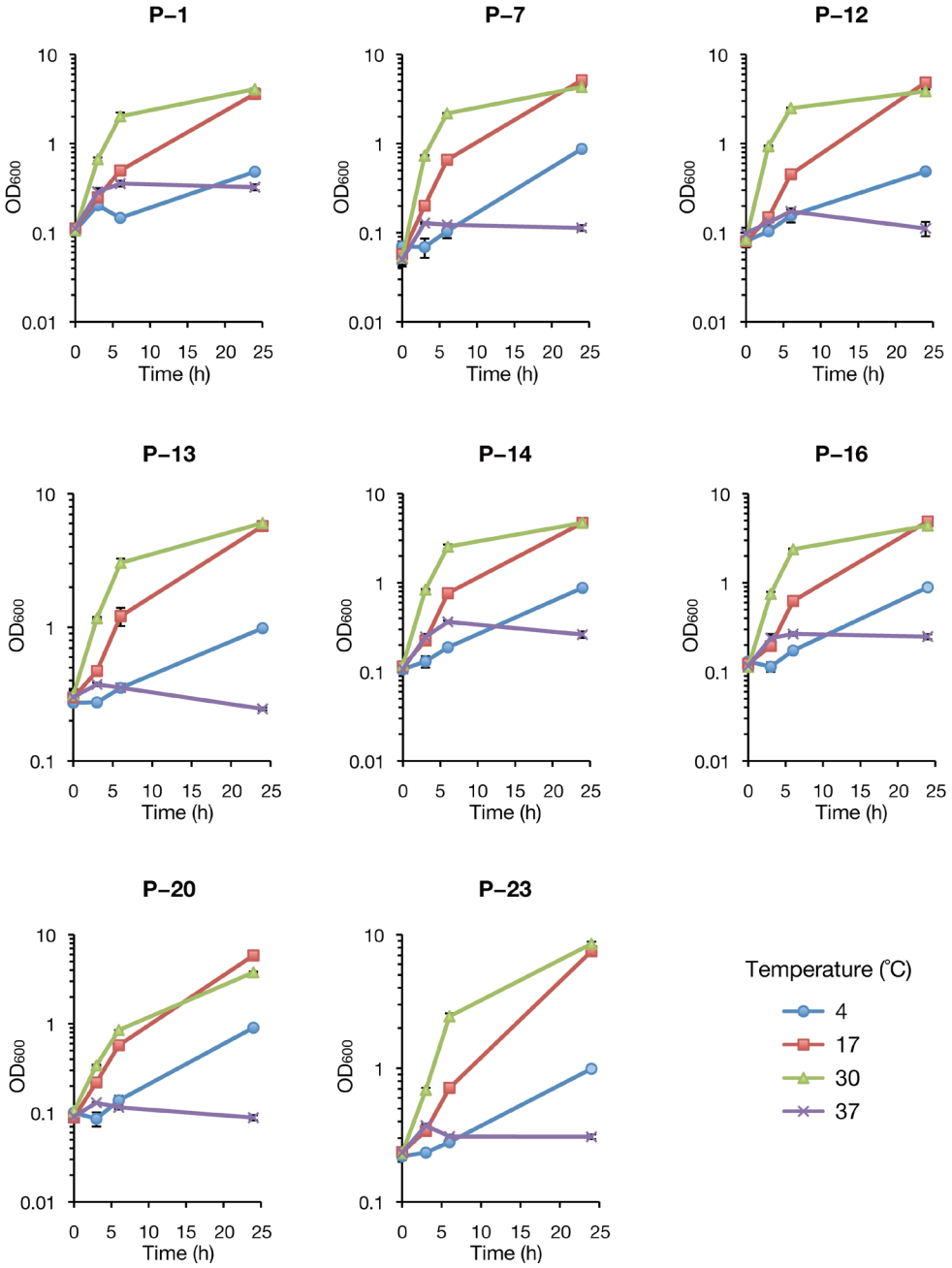

Figure 5. Growth curves of the isolated bacterial strains at different temperatures. The growth curves of the isolated strains were examined at several temperatures (4, 17, 30, or 37°C). The vertical axis represents the optical densities (OD_{600}) and the horizontal axis represents incubation time. The experiment was independently performed three times. Mean values with standard errors of the mean are shown.

sequences and *rpoD* gene sequences estimated the closest matched species of each representative strain (Table 4). Both analyses consistently estimated that P-22, P-23, P-25, and P-26 were related to each other, and formed a cluster distant from *Pseudomonas*-type strains. Also, both analyses estimated that P-14 was related to *P. vancouverensis*, and P-16 was related to *P. helmanticensis* (Table 4). The closest match species of P-1, P-7, P-12, and P-13, however, were not consistent between the analyses using *16S rRNA* sequences and the *rpoD* sequences.

Psychrophilic bacteria are defined as bacteria that grow optimally at or below 15°C, and psychrotolerant bacteria are defined as bacteria that can grow below 15°C, but have a higher optimal temperature (*5*). To determine whether the isolated strains were psychrophilic or psychrotolerant bacteria, growth curves of seven representative strains (P-1, P-7, P-12, P-13, P-14, P-16, and P-23), which were related to the *Pseudomonas* genus, and P-20, which was related to the *Janthinobacterium* genus, were measured at several temperatures (4, 17, 30, or 37°C; Figure 5). The optical densities of the culture medium of all strains increased over time at 4, 17, and 30°C. At 37°C, the optical densities increased from 0 to 3 h, but did not increase from 6 to 24 h. The rank order of optical density at 6 h was 30°C > 17°C > 37°C > 4°C. These results suggest that the optimal growth temperature of the isolates was between 17°C and 37°C and these bacteria are psychrotolerant.

4. Discussion

In the present study, we isolated bacteria from temperate soil by culturing at a low temperature and performed phylogenetic analyses of 10 representative isolates using the two housekeeping genes: *16S rRNA* and *rpoD*. Both analyses estimated that four representative isolates (P-22, P-23, P-25, P-26) formed a distinct cluster from *Pseudomonas*-type strains. The two phylogenetic analyses differed with respect to the related species for four other representative isolates (P-1, P-7, P-12, P-13). These results suggest that the bacterial strains isolated in this study are novel *Pseudomonas* species. All of the isolated bacteria exhibited antimicrobial activity against *E. coli*. Thus, we propose that low temperature incubation of temperate soil is an effective method of isolating novel bacterial sources of antibiotics.

In this study, most isolated strains belonged to the genus *Pseudomonas*. The genus *Pseudomonas* comprises 218 species of Gram-negative gamma proteobacteria. Members of the genus *Pseudomonas* are isolated from many environments including land, freshwater, and seawater. Its high stress-resistance capability is one reason for its wide range of habitats. Optimal temperatures of the genus *Pseudomonas* are around 25-30°C, but many of them can grow at low temperature, around 5°C (*38*). These characteristics are consistent with those of the *Pseudomonas* strains isolated in this study, which grew at 4°C and had a growth-optimal temperature of around 30°C.

The supernatants of the isolated *Pseudomonas* strains showed antibiotic activities against *E. coli*. The supernatants, except that of P-11, did not inhibit the growth of *S. aureus*, and none of them inhibited the growth of *C. albicans*. Several antimicrobial drugs have been discovered from *Pseudomonads*; two beta-lactams including sulfazecin and isosulfazecin (*39*) effective against both *S. aureus* and *E. coli*, and many anti-fungal chemicals (*40,41*). Because the antimicrobial activities of the isolates in this study are more effective against *E. coli* than *S. aureus* and *C. albicans*, the antimicrobial compounds are expected to differ from the known antibiotics obtained from other *Pseudomonas* species.

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