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Isolation and Characterization of Thallium-tolerant Bacteria from Heavy Metal-polluted River Sediment and Non-polluted Soils

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Thallium (Tl) is a heavy metal found in trace amounts in the earth's crust and has been studied to a much lesser degree than other heavy metals. Since the discovery of high-temperature superconducting components in the Tl-Ca-Ba-Cu-O system, Tl has attracted greater attention as a potential major pollution source of the future. In this study, the response of soil culturable bacterial communities to the addition of Tl was examined with fresh samples of non-polluted garden and arable soils. A preserved air-dried sample from heavy metal-polluted river sediment was also used as a reference. Soil suspension experiments were performed to homogenize the soils to evenly distribute both the microbial populations and Tl and reduce spatial variability. From soil suspensions loaded with Tl at 0.24 to 0.98 mM, bacteria tolerant to 0.49 mM Tl (100 mg Tl l^{-1}) were isolated and characterized phylogenetically. The background level of culturable Tl-tolerant bacterial populations was 0.0003–0.013% for the non-polluted soils. The Tl-tolerant fraction increased rapidly in response to Tl loading and dominated the bacterial community, which might be attributed mainly to an immediate effect due to the death of Tl-sensitive microorganisms and leaking of nutrients from cell lysis of sensitive species supporting the growth of primarily Tl-tolerant heterotrophs. Such Tl-tolerant culturable bacteria were isolated and classified into 9 groups of *Alphaproteobacteria*, those from the arable soil were identified as *Pseudomonas* spp., and those from the contaminated river sediment were identified as *Bacillus niacini*.

Key words: Thallium, heavy metals, *Bacillus niacini*, *Pseudomonas* spp., *Alphaproteobacteria*

Thallium (atomic number, 81; relative atomic mass, 204.37; chemical symbol, Tl) is a metal found in trace amounts in the earth's crust and has been studied to a much lesser degree than other heavy metals such as lead, cadmium, and mercury[36]. In general, Tl concentrations in surface soils range from 0.1 to 2 mg kg^{-1}, with most reported concentrations being less than 1 mg per kg[1,26,28,37,42]. However, the content of Tl in soils seems to depend largely on the geological origin of the parent material[33]. The concentrations of Tl in the clayey soils developed on Sinemurian limestone in France are as high as 55 mg per kg[42]. Other examples of soils contaminated with Tl include soils near cement plants in Germany with concentrations as high as 15 mg per kg[37], soils near old mines in Germany with concentrations of up to 73 mg per kg[37], and soils originating from a mining area in China with Tl concentrations of up to 61 mg per kg[49]. In Poland, an average Tl concentration of 43 mg kg^{-1} was detected in a 100-year-old calamine waste heap soil[46]. Thallium-contaminated soils have also been found in Japan, including river sediments (4.8–79.9 mg kg^{-1}) and soils (0.26–2.4 mg kg^{-1}) near the Hosokura mine and smelter[2]. From these data, pollution from Tl is thought to be restricted to local places such as in the vicinities of non-ferrous metal mines, smelters, and factories using Tl. However, since the discovery of high-temperature superconducting components in the Tl-Ca-Ba-Cu-O system, thallium has

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Abbreviations. Metals are referred to by their recognized atomic symbols (e.g. Tl=Thallium; Pb=lead; Zn=zinc)

attracted greater attention as a potential major pollution source of the future[1].

With respect to degree of toxicity, Tl ranks alongside Pb, Hg, and Cd. The maximum admissible daily dose of Tl for humans is 15.4 μg kg^{-1} dry wt[41,42]. Because thallium's ionic radius (1.40 Å) is similar to that of potassium (1.33 Å), the toxicity results from Tl^+ mimicking K^+ ions in many metabolic processes. Thallium may also bind with sulfhydryl groups of proteins to inactivate many enzymatic reactions. Thus, it is known to have caused many accidental, occupational, and therapeutic poisonings since its discovery[17,39]. Tremel *et al.*[42] studied the uptake of Tl and its accumulation in several species of crop plants growing on soils with high Tl levels of geochemical origin (from 0.3 to 40 mg kg^{-1} dry wt soil). They found that rape seeds (*Brassica napus*) contained the largest amounts of Tl, up to 33 mg kg^{-1} dry wt (shoots, up to 20 mg kg^{-1} dry wt). Maize (*Zea mays*), on the other hand, contained up to 0.343 mg Tl kg^{-1} dry wt in shoots, but nearly no Tl in the grain. Related studies on the uptake of Tl by plants in Tl-polluted soils in China[47,48] and Poland[46] have also been reported.

Concerning the effects of Tl on microorganisms, considerable variation in the sensitivity of bacteria to Tl has been described, with 0.6 mM Tl acetate reported sufficient to prevent the growth of *Pseudomonas aeruginosa* while 39 mM Tl did not prevent the growth of *Staphylococcus aureus* and *Streptococcus faecalis*[13]. Thallium's inhibition of the photosynthesis of *Chlamydomonas reinhardii* principally resulted from inhibition of NADP reduction or dark reactions, in contrast to the inhibition by Cd and methyl-Hg of the Hill and modified Mehler reactions[24]. Norris *et al.*[21] studied the toxicity and accumulation of Tl in *Saccharomyces cerevisiae*, *Bacillus megaterium*, and *Escherichia coli*. They found that Tl was rapidly bound, presumably to cell surfaces, by *S. cerevisiae* and *E. coli*, and was progressively accumulated by energy-dependent transport systems with both organisms. Further, apparent K_m and K_i values for competitive inhibition of Tl uptake by potassium indicated *S. cerevisiae* to have a higher affinity for Tl than for potassium, while *E. coli* had a transport system with a higher affinity for potassium than for Tl. Afterwards, Damper *et al.*[4] reported that the accumulation of $^{204}Tl^+$ by *E. coli* occurred primarily via either of two K^+ transport systems called Kdp and Trk.

To the best of our knowledge, very little is known about the effects of Tl on soil microbial communities. In this study, therefore, we examined the response of soil cultivable communities to the addition of Tl. We performed soil suspension experiments to homogenize soils to evenly distribute both the microbial populations and Tl and reduce spatial variability. Our objectives were to characterize the Tl-tolerant isolates, identified by partial 16S rRNA gene sequencing, with regard to their profiles of metal tolerance.

Materials and Methods

Samples

A heavy metal-contaminated sediment sample and two uncontaminated surface soil samples were used in this study. The sediment sample had been taken from the Namari River (at site R3: 38°48'7"N, 140°54'36"E) near the Hosokura mine and smelter, Miyagi Prefecture, in September 1994 and contained 79.9 mg Tl kg^{-1} dry wt and the following heavy metals (mg kg^{-1} dry wt)[2]: Cu, 1,730; Zn, 104,000; As, 499; Ag, 125; Cd, 257; Sb, 367; Te, 46.4; Pb, 152,000; Bi, 183. This sample had been air-dried and stored at room temperature before this study. The surface soils were sampled from a garden area on the campus of Ibaraki University College of Agriculture (36°2'7"N, 140°12'45"E) and arable land at the Agricultural Experimental Station (36°1'51"N, 140°12'45"E), Ibaraki University College of Agriculture. In the arable field, yacon plants (*Polymnia sonchifolia*) had been cultivated before soil sampling. No heavy metal pollution was recorded for these sampling sites but the soil samples were found to contain background levels of Tl (garden soil, 0.5 mg Tl kg^{-1} dry wt; arable soil, 0.57 mg Tl kg^{-1} dry wt) by flame atomic absorbance spectrometry (see below). The unpolluted surface soils were stored at 4°C in the dark without air-drying, and subjected to microbiological analysis within 2 weeks after sampling.

Effect of thallium loading on soil bacterial populations and isolation of bacteria

One gram (fresh weight) of the unpolluted soils or 1 g (dry weight) of the polluted river sediment was suspended in 50 ml of Tl-added distilled water (in a 500-ml volume shaking flask) and incubated with shaking (300 rpm) at 30°C for 4 weeks. Thallium nitrate was added at 0.49 mM (100 mg Tl l^{-1}) for the sediment sample, 0.24 and 0.98 mM (50 and 200 mg Tl l^{-1}, respectively) for the garden soil, and 0.38 mM (77 mg Tl l^{-1}) or 0.98 mM for the arable soil. Control soil suspensions without Tl addition were also incubated in the same way. To examine the counter ion effect, Tl sulfate was also used in an experiment.

Changes in bacterial populations were assessed by plate counting on 100-fold diluted nutrient broth (DNB) agar[23] and DNB agar supplemented with 0.049 mM (10 mg Tl l^{-1}) or 0.49 mM Tl nitrate. The nutrient broth (NB, pH 7.0) was

composed of 1% (w/v) meat extract (Kyokuto Seiyaku, Tokyo, Japan), 1% (w/v) Bacto™ Peptone (Becton, Dickinson and Company, MD, USA) and 0.5% (w/v) NaCl. Culturable bacteria were enumerated after incubation at 30°C for 4 weeks and then isolated from the plates. The isolation procedure was essentially the same as previously described[22] and bacteria were maintained at room temperature in semisolid DNB stab cultures. Pure cultures of isolates were examined for their cell morphology and Gram staining as described by Ohta and Hattori[23].

Phylogenetic characterization

Genomic DNA was extracted from bacterial cells grown on DNB agar plates by the method of Wang and Wang[44] and used as a template for the amplification of the 16S rRNA gene via PCR with the primers 10F, 5'-AGTTTGATCCTGGCTCAG-3' (*Escherichia coli* positions 8–27), and 1541R, 5'-AAGGAGGTGATCCAGCCG-3' (*E. coli* positions 1542–1525)[45]. The PCR conditions, the electrophoresis of amplified DNA, and the purification of PCR products were essentially as described previously[22]. Nucleotide sequences were determined using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit and read on an Applied Biosystems 3100 DNA sequencer[35]. The primer 1541 R was used in sequencing reactions to obtain partial DNA sequences. All sequences determined in this way were compared to similar DNA sequences retrieved from the DDBJ/EMBL/GenBank databases by using the BLAST program[25]. For the phylogenetic analysis of the sequence data sets, the CLUSTAL W program[40] was utilized and a phylogenetic tree was constructed by the neighbor-joining method[34].

Growth responses to heavy metals

To determine the growth response to Tl, bacteria were grown in batch cultures (5 ml of medium in 20-ml test tubes) of a 10-fold diluted nutrient broth (10^{-1} NB) medium containing different concentrations of Tl nitrate (0 to1.96 mM) at 30°C on a shaker at 300 rpm. The growth was assessed by measuring the optical density at 660 nm (OD_{660}) of the culture. Growth responses to the other heavy metals were also determined by culturing on a 10^{-1} NB agar medium supplemented with different concentrations of the following analytical-grade reagents: $CuSO_4 \cdot 5H_2O$, $Cd(NO)_2 \cdot 4H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 5H_2O$. Metal stock solutions were sterilized by membrane filtration and added to the medium aseptically. Each metal in the stock solution was determined by flame atomic absorbance spectrometry as described by Asami *et al.*[2]. Growth was recorded after a week of incubation at 30°C. The lowest concentration of metal that completely prevented growth was termed the minimal inhibitory concentration (MIC). For all the growth experiments, liquid cultures on 10^{-1} NB at mid-log phase were used as the inoculum: the ratio of the inoculum volume to culture volume was 1:20.

Nucleotide sequence accession numbers

The 16S rDNA sequence data for isolates in this study have been deposited under DDBJ accession numbers AB231940 to AB232044 and AB269670 to AB269686.

Results

Effects of thallium loading on bacterial populations of uncontaminated soils

The garden soil was used to determine the background levels of culturable bacteria able to grow in the presence of 0.049 and 0.49 mM Tl (10 and 100 mg Tl l^{-1}, respectively) which were about 10 times lower than and similar to, respectively, compared with the reported inhibitory concentration for *P. aeruginosa* (0.6 mM)[13] and wild-type cells of *E. coli* (0.5 mM)[21]. The percentage of bacteria tolerant to 0.049 and 0.49 mM Tl among all the culturable bacteria was 1.3 and 0.0003% (mean value of a duplicate determination), respectively.

To determine the effect of Tl loading on soil bacterial populations, the garden soil suspension was incubated in the presence of 0.24 and 0.98 mM Tl (50 and 200 mg Tl l^{-1}, respectively). The population level of total culturable heterotrophic bacteria in the Tl-added soil suspensions was roughly constant (10^8 CFU per g soil) during the 28-day incubation period (Figs. 1B and 1C). In the 0.24 mM Tl-loaded soil suspension (Fig. 1B), the number of bacteria tolerant to 0.049 mM Tl increased from 10^6 CFU per g soil on day 0 to about 10^8 on day 7 and this level was maintained for the next 3 weeks. In the case of bacteria tolerant to a higher concentration of Tl, 0.49 mM, the number increased sharply from 10^4 CFU per g soil on day 0 to about 10^7 on day 7 and remained in the order of 10^7 with further incubation. In the soil suspension loaded with 0.98 mM Tl (Fig. 1C), the number of bacteria tolerant to 0.049 and 0.49 mM Tl increased rapidly to about 10^8 on day 7 and these population levels were maintained for the rest of the incubation period.

On day 28, the proportion of bacteria tolerant to 0.049 mM Tl among the total culturable population was 50% and 100% for the 0.24 and 0.98 mM Tl-loaded soil suspensions, respectively. Bacteria tolerant to 0.49 mM Tl accounted for

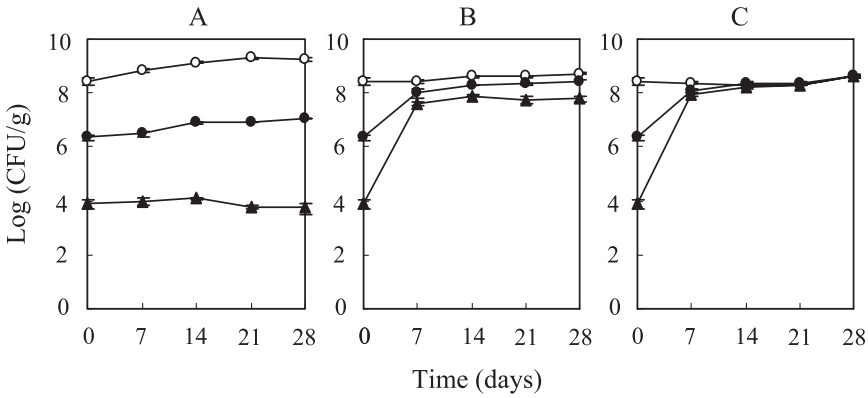

Fig. 1. Numbers of culturable bacteria in the non-polluted garden soil suspensions that received 0 mM (A), 0.24 mM (B), and 0.98 mM (C) thallium. Symbols: ○, total number of CFU on DNB; ●, CFU of bacteria tolerant to 0.049 mM thallium; ▲, CFU of bacteria tolerant to 0.49 mM thallium. Values are the means±SD of 4 replicate plates.

12% and 93% for the 0.24 and 0.98 mM Tl-loaded soil suspensions, respectively. In a control experiment without Tl, no increase in the number of bacteria tolerant to 0.49 mM Tl was detected during the 28 days of incubation while the number of Tl (0.049 mM)-tolerant bacteria showed a 5-fold increase (Fig. 1A). This small increase seemed to be related to an 8-fold increase in the total number of CFU on DNB.

To examine the reproducibility and a counter ion-effect, two more suspensions of the same garden soil were prepared, with one loaded with Tl nitrate (0.98 mM) and the other with Tl sulfate (0.98 mM). When culturable bacteria were enumerated after 15 days of incubation, bacteria tolerant to 0.049 and 0.49 mM Tl (Tl nitrate) accounted for 75% and 73%, respectively, in the Tl nitrate-loaded suspension and 97% and 100%, respectively, in the Tl sulfate-loaded suspension.

The same experiment was also carried out with the normal arable soil. The background number of bacteria tolerant to 0.049 and 0.49 mM Tl was 10^6 CFU per g soil (2.5% of total culturable bacteria) and 10^4 CFU per g soil (0.013%), respectively. When 0.98 mM Tl nitrate and Tl sulphate were added to the soil suspension and incubated for 15 days, the proportion of 0.049 mM Tl-tolerant bacteria among all culturable bacteria increased to 86% (Tl nitrate-loaded suspension) and 52% (Tl sulphate-loaded suspension). The Tl (0.49 mM)-tolerant bacteria accounted for 79% (Tl nitrate-loaded suspension) and 65% (Tl sulphate-loaded suspension) of the population on day 15. These results clearly showed that the normal soils harbored Tl-tolerant bacteria in the order of 10^4 CFU per g (for 0.49 mM Tl-tolerant bacteria) and the population of Tl-tolerant fractions increased rapidly in response to Tl loading.

Isolation and phylogenetic characterization of thallium-tolerant bacteria

After the enumeration of bacteria on day 28, bacteria were isolated from plates of DNB supplemented with 0.49 mM Tl and isolates were characterized by an analysis of their 16S rRNA gene sequences (Table 1). Ten Tl-tolerant isolates were obtained from the control garden soil suspension without Tl-loading and contained Gram-positive (6 isolates) and Gram-negative (4 isolates) bacteria: *Firmicutes*, 3 isolates; *Actinobacteria*, 3 isolates; *Alphaproteobacteria*, 4 isolates. All of the 59 Tl-tolerant isolates from the Tl-loaded suspensions of the garden soil were Gram-negative, rod-shaped bacteria and fell into several groups in the *Alphaproteobacteria* (Fig. 2). In contrast, all of the 23 isolates from the arable soil were affiliated with *Pseudomonas* in the *Gammaproteobacteria*. The garden soil was used for the two separate runs of soil suspension experiments and Tl-tolerant bacteria were obtained from each experiment. Although they were affiliated with *Alphaproteobacteria*, they showed a trans-family level of phylogenetic diversity (Fig. 2).

With respect to the isolation frequency of specific bacteria, two groups (A and B) were reproducibly isolated from the two separate soil suspension experiments: representative strains of Group A included SK50-12 (from run 1 with 0.24 mM Tl), SK200a-9 (from run 1 with 0.98 mM Tl), and SK200b-8 (from run 2 with 0.98 mM Tl); representative strains of Group B included SK50-23 (from run 1 with 0.24 mM Tl), and SK200b-17 (from run 2 with 0.98 mM Tl). Five strains (representative strain, SK200a-15) closely related to *Bradyrhizobium* strain Cp5-3, were found only in

Table 1. Phylum level affiliations of thallium-tolerant bacteria[a]

Sample, loading amount of Tl[b]	Number (and percentage) of isolates belonging to				Total No. (%) of isolates
	Alphaproteobacteria	*Gammaproteobacteria*	*Firmicutes*	*Actinobacteria*	
Garden soil					
Run 1					
No added Tl	4 (40)	0 (0)	3 (30)	3 (30)	10 (100)
0.24 mM Tl	25 (100)	0 (0)	0 (0)	0 (0)	25 (100)
0.98 mM Tl	19 (100)	0 (0)	0 (0)	0 (0)	19 (100)
Run 2					
0.98 mM Tl	15 (100)	0 (0)	0 (0)	0 (0)	15 (100)
Arable soil					
0.38 mM Tl	0 (0)	23 (100)	0 (0)	0 (0)	23 (100)
River sediment					
No added Tl	0 (0)	0 (0)	7 (100)	0 (0)	7 (100)
0.49 mM Tl	0 (0)	0 (0)	23 (100)	0 (0)	23 (100)
Total	63	23	33	3	122

[a] Thallium-tolerant bacteria able to grow in the presence of 0.49 mM Tl were isolated from soil suspensions loaded with thallium and incubated for 28 days.
[b] Thallium nitrate was used.

the isolates from run 1 with 0.98 mM Tl while 6 strains (representative strain, SK200b-10) closely related to *Bosea thiooxidans* strain DSM 9653^T, were the isolates from run 2 with 0.98 mM Tl.

Soil suspension experiments were also conducted with the river sediment sample polluted with heavy metals to isolate Tl-tolerant bacteria. The number of bacteria tolerant to Tl at 0.49 mM was below 10^2 CFU per g (<0.002% of total cultivable bacteria) on day 0. When a soil suspension was incubated, Tl-tolerant bacteria increased to 10^4 CFU per g on day 7 in parallel with an increase in total culturable bacteria (to 10^8 CFU per g). When the suspension was loaded with 0.49 mM Tl, the Tl-tolerant bacterial fraction increased to 10^7 CFU per g by day 7 and remained in the order of 10^7 CFU on days 14 to 28. The proportion of Tl-tolerant bacteria increased to 96–100% on days 7 to 14 and then declined to 13% by day 28. From the Tl-loaded suspension, 23 Tl-tolerant bacteria were isolated and all of their 16S rRNA gene sequences were identical to those of *Bacillus niacini* in *Firmicutes* (nucleotide sequence positions 813–1399 of *E. coli*) (Table 1, Fig. 2).

Comparison of thallium tolerance of isolates

Six representative Tl-tolerant strains from the 3 samples were grown in media containing different amounts of Tl and their maximum growth rates were determined. The following strains were tested (source): strain SK50-23 (run 1 of garden soil with 0.24 mM Tl), SK200a-9 (run 1 of garden soil with 0.98 mM Tl), SK200b-10 (run 2 of garden soil with 0.98 mM Tl), NH-10 and NH-16 (arable soil with 0.38 mM Tl), and HK1 (river sediment with 0.49 mM Tl). The relative growth rates of the tested strains declined successively with increasing Tl concentrations over 0.49 mM and the percent inhibition at 1.96 mM Tl ranged from about 50 to 80% (Fig. 3). The Tl concentration leading to 50% growth inhibition was estimated to be 1.2–1.3 mM for strains SK200b-10 and NH10, 1.5 mM for strain SK50-23, and 1.8–1.9 mM for strains SK200a-9, NH16, and HK1.

Profiles of the tolerance of isolates to heavy metals

The river sediment sample was highly contaminated with not only Tl but also Pb (152,000 mg kg^{-1}), Zn (104,000 mg kg^{-1}), and Cu (1,730 mg kg^{-1})[2]. Therefore, the representative strains were tested for their multi-heavy-metal cotolerance. Table 2 summarizes the MICs of Cd, Co, Cu, Ni, Pb, Tl, and Zn against strains HK1, SK200a-9, SK50-23, NH10, and NH16. Strain HK1, closely related to *B. niacini*, from the contaminated river sediment exhibited a higher MIC value for Zn than for Tl. The order of toxicity of the metals to the bacterium was found to be Cd>Pb>Co=Cu>Tl>Ni>Zn. Zinc was also less toxic toward strains SK200a-9 and SK50-23 (*Alphaproteobacteria*) from the nonpolluted garden soil. In contrast, strains NH10 and NH16, affiliated with *Pseudomonas*, showed a narrow range of metal tolerance and specific tolerance to Tl.

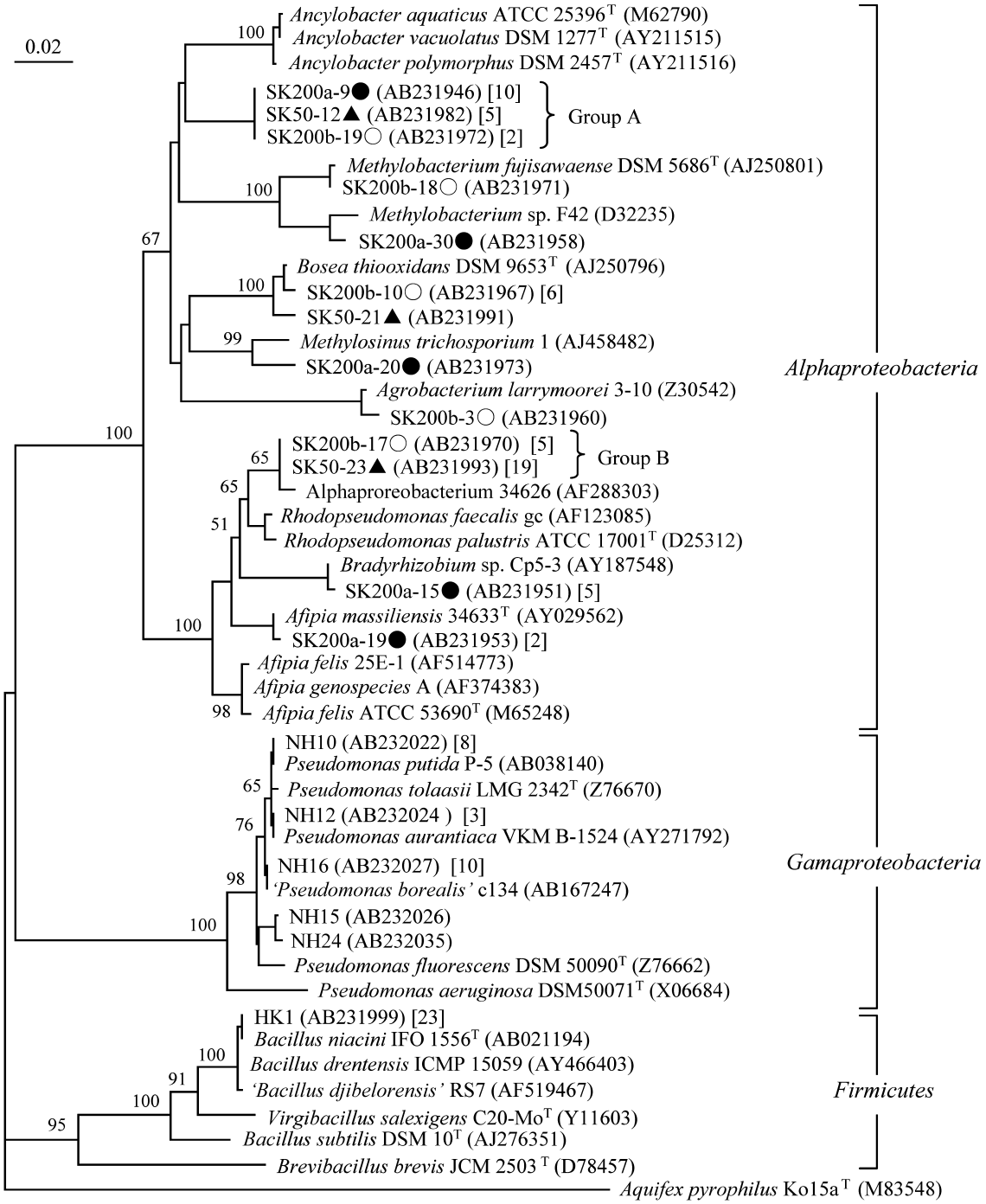

Fig. 2. Phylogenetic position of the thallium-tolerant bacteria based on the 16S rRNA gene sequences. The tree was constructed by the neighbor-joining method. Strains from garden soil, arable soil, and river sediment are designated by the letters SK, NH, and HK, respectively. Accession numbers are indicated in parentheses. The numbers in the brackets show the numbers of strains possessing complete or almost-complete sequences of representative strains. Bootstrap values greater than 50% (percentages of 1000 replications) are shown at nodes. The sequence from *Aquifex pyrophilus* Ko15a^T was used as an outgroup. Symbols: ●, strain from the first run of soil suspension loaded with 0.98 mM Tl; ○, strain from the second run suspension loaded with 0.98 mM Tl; ▲, strain from the soil suspension loaded with 0.24 mM Tl. Bar, 0.02 substitutions per nucleotide position.

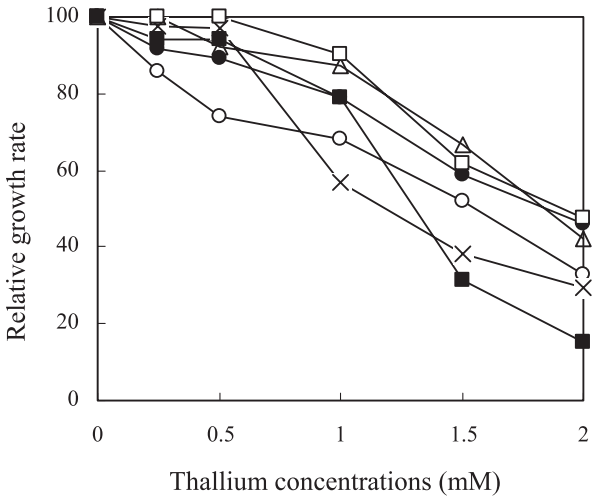

Fig. 3. Effect of thallium on the growth of thallium-tolerant bacteria. Symbols: ●, strain SK200a-9; ○, strain SK50-23; ×, strain SK200b-10; △, strain HK1; □, strain NH16; ■, strain NH10.

Discussion

The major findings reported in this paper are that the background level of culturable bacterial populations tolerant to about 0.5 mM Tl was 0.0003–0.013% in the non-polluted fresh soils, the tolerant fraction increased rapidly in response to Tl loading, and the Tl-tolerant culturable populations were identified as several groups in the *Alphaproteobacteria*, *Pseudomonas*, and *Bacillus*.

An increase in the tolerant fraction vis-a-vis a heavy metal has been reported in a number of studies on the effect of heavy metal addition in soil microcosms[5,6,16,19,29,30,32] and heavy metal polluted environments[11,14,18,27,31]. Díaz-Raviña *et al.*[6] for example, examined the development of metal tolerance in soil bacterial communities exposed to different heavy metals (Cu, Cd, Zn, Ni, and Pb) under laboratory conditions by thymidine incorporation and plate count techniques. They determined the numbers of CFU on plates containing metals in the range from 3×10^{-3} to 1×10^{-7} M to calculate the logarithms of the concentrations that resulted in 50% inhibition (IC_{50}) (i.e., a 50% decrease in the number of CFU). The IC_{50} values showed that Cd (mean IC_{50}, −5.29) was the most toxic metal for bacteria extracted from unpolluted agricultural soils, followed by Zn (IC_{50}, −4.36). Cu and Ni had similar values (IC_{50}, −3.83), while Pb was the least toxic metal (IC_{50}, −3.05). The addition of Cd at 16 mmol kg^{-1} and Zn at 32 mmol kg^{-1} enabled the largest increase in the metal tolerance to be detected: from IC_{50}= −5.29 to −4.10 for Cd and from IC_{50}=−4.36 to >−3.30 for Zn. In the case of the least toxic metal, Pb, the metal's addition at 32 mmol kg^{-1} effected the least increase in tolerance to Pb (from IC_{50}=−3.05 to −2.90). In our data, the addition of Tl to the garden soil suspension at a final concentration of 0.24 mM resulted in an increase in the 0.049 mM Tl-tolerant fraction from 1.3% to 50% and that in the 0.49 mM Tl-tolerant fraction from 0.0003% to 12%. When the IC_{50} value for the Tl-loaded bacterial community was estimated from our two point data (i.e., at 0.000049 and 0.00049 M Tl), the value appeared to approximate −4.3 [=log (0.000049)] for the Tl-loaded suspension. Further, assuming that the slope of the dose-response curve for the Tl-loaded soil suspension was identical to that for the control suspension without Tl loading, the IC_{50} value was estimated as −5.5 [=log (0.000003)] from the shifted curve. This calculation suggests that the toxicity of Tl was as high as that of Cd for culturable populations in the uncontaminated soil: IC_{50}=−5.5 for Tl from our calculation versus IC_{50}=−5.29 for Cd from the data of Díaz-Raviña *et al.*[6].

The effect of Tl on the bacterial community was seen within 7 days after the metal addition for the uncontaminated soils. Such an immediate response of the metal-resistant fraction and a large decrease in microbial community diversity have often been reported in studies on, for exam-

Table 2. Minimum inhibitory concentrations (MICs) of seven heavy metals against thallium-tolerant strains

Strain	Source	Class; group[a] or the closest relative (% identity)[b]	MIC (mM)						
			Tl	Cd	Co	Cu	Ni	Pb	Zn
SK200a-9	garden soil	*Alphaproteobacteria*; Group A	4.4	0.4	1.7	0.8	1.7	1.4	3.1
SK50-23	garden soil	*Alphaproteobacteria*; Group B	3.4	0.9	1.7	1.6	3.4	1.0	4.6
NH10	arable soil	*Pseudomonas putida* P-5 (100%)	2.9	0.4	0.8	0.8	0.9	1.4	0.8
NH16	arable soil	*'Pseudomonas borealis'* c134 (100%)	2.9	0.4	0.8	0.8	0.9	1.0	0.8
HK1	river sediment	*Bacillus niacini* IFO15566 (100%)	3.9	0.4	3.4	3.1	5.1	1.4	>12

[a] Groups A and B as indicated in the phylogenetic tree (Fig. 2).
[b] Percent identity of nucleotide sequence positions 813–1399 of *E. coli*.

ple, Cr[16], Cu[38], and Hg[30] contamination.

The Tl-tolerant bacteria isolated in this study were identified as several groups in the *Alphaproteobacteria*, *Pseudomonas*, and *Bacillus* (Fig. 2 and Table 1). Among these taxonomic groups, *Pseudomonas* and *Bacillus* are often found to dominate some metal-contaminated soils[7,16,27,31,32]. Ellis *et al.*[7] reported that isolates belonging to *Firmicutes* (mainly *Bacillus* spp.) had a higher relative abundance in the most contaminated samples (Cu, 13–14 mg kg^{-1}; Pb, 13–19 mg kg^{-1}; Zn, 9.6–14 mg kg^{-1}), whereas the *Gammaproteobacteria* (mainly *Pseudomonas* spp. and a *Xanthomonas* sp.) increased in relative abundance in the least-contaminated samples (Cu, 0.5–0.6 mg kg^{-1}; Pb, 0.9–1.2 mg kg^{-1}; Zn, 0.7–0.9 mg kg^{-1}). Because an air-dried river sediment sample was used in this study, spore-forming bacteria could be selectively retained and the Tl-tolerant fraction sensitive to dryness was probably eliminated from the sample. This may explain the exclusive isolation of *Bacillus* from the heavy metal-polluted river sediment. Our Tl-tolerant *Bacillus* isolates exhibited 16S rDNA sequence (sequence positions 813–1399 of *E. coli*) identities to *B. niacini* (16S r DNA sequence accession number, AB021194) of 100%. *B. niacini* is frequently found in arable soils[20] but its metal tolerance is not fully described. To our knowledge, there is only one report that mentions the bacterium in relation to metal-contaminated soils. That study involved experiments on soil microcosms contaminated with heavy metals (Pb and Cr) and xylene and showed that one of ten prominent bands from denatured gradient gel electrophoresis (DGGE) profiles of the microcosms matched the sequence of *B. niacini* (AY167809) (sequence identity, 96%)[19]. This may explain our finding that our *B. niacini*-related isolates showed a high tolerance to not only Tl but also other metals (Table 2).

Twenty-three Tl-tolerant bacterial strains isolated from the arable soil were all affiliated with *Pseudomonas* and might be assigned to one of 4 species (Fig. 2): *Pseudomonas ptuida* (8 strains), *Pseudomonas aurantiaca* (3 strains), *'Pseudomonas borealis'* (10 strains), and *Pseudomonas fluorescens* (2 strains). *Pseudomonas putida* and *P. fluorescens* have been often found as metal-tolerant species in metal-polluted soils[7,12,27].

Recently, Nakatsu *et al.*[19] reported that the community-wide effects of heavy metal addition differed between two carbon sources (glucose and xylene). For glucose (a substrate that probably could be used by a wide range of microbes in the soil), either Pb or Cr produced large changes and replacement with new phylotypes. In contrast, many phylotypes selected by xylene (a substrate that requires a specialized catabolic pathway) treatment were retained when either metal was added. In that study, *P. fluorescens* was found in the retained phylotypes that responded to xylene alone and xylene plus Pb or Cr. In our *Pseudomonas* isolates, 8 and 10 strains showed 16S rDNA sequence (sequence positions 813–1399 of *E. coli*) identities to *P. putida* strain P-5 (AB038140) of 100% and to *'P. borealis'* strain c134 (AB167247) of 100%, respectively. Interestingly, since these strains of *P. putida* P-5 and *'P. borealis'* c134 were isolated from soil polluted with trichloroethylene[9,10], it may be postulated that such *Pseudomonas* groups responded to the contamination in the arable soil and were retained after the Tl treatment. However, contamination from trichloroethylene and its related compounds is not likely in the arable land of this study. Therefore, further information on the properties of bacterial isolates and also on chemical pollutants in the soil is needed to elucidate the predominance of the *Pseudomonas* spp. in the arable soil.

The Tl-tolerant isolates from the garden soil were affiliated with the *Alphaproteobacteria* and divided into 9 clusters on the phylogenetic tree (Fig. 2), indicating higher diversity compared with the Tl-tolerant isolates (*Bacillus*) from the river sediment and those (*Pseudomonas*) from the arable soils. The isolates of Groups A and B were reproducibly isolated from repeated soil suspension experiments: Group A, 17 strains with the representatives SK200a-9, SK50-12, and SK200b-8; Group B, 24 strains with the representatives SK200b-17 and SK50-23. The closest relative of strain SK200a-9 (Group A) is *Ancylobacter aquaticus* ATCC 25396^T (M62790) with a 16S rDNA sequence identity of 93%. Similar to the above mentioned *Pseudomonas* spp., *A. aquaticus* has been isolated from river samples contaminated with chlorinated aliphatic compounds[8,43]. Based on the 16S rDNA sequence identity, the strains of Group A can not be identified as *Ancylobacter* and should be studied further.

The closest relative of the Group B strains is alphaproteobacterium strain 34626 isolated from hospital water supplies by direct plating and amoebal co-culture procedures[15], and the 16S rDNA sequence identity of which is 99.3%. Strain 34626 was characterized as being fastidious and not able to be cultivated on standard microbiological media, and its possible role in hospital-acquired human infections is not known[15]. The second relative of Group B is *Rhodopseudomonas palustris* ATCC 17001^T (D25312) with a 16S rDNA sequence identity of 97.8%. Further taxonomic characterization of Group B strains is also now in progress in our laboratory.

With respect to the mechanism of Tl tolerance, Norris *et al.*[21] isolated and characterized a mutant of *E. coli* with ten-fold decreased sensitivity to Tl. The mutant effected binding of Tl in amounts equivalent to the wild-type strain, but showed no subsequent uptake and accumulation of the metal from buffer. Therefore, the lack of Tl uptake by the Tl-tolerant mutant appears to be a basis for tolerance. Although the accumulation of Tl by *E. coli* occurs primarily via either of two K^+ transport systems called Kdp and Trk[4], the Tl-tolerant mutant was able to accumulate K to normal intracellular concentrations during growth and thus apparently its K uptake systems were unimpaired. Norris *et al.*[21] discussed a possibility that the ability to transport Tl was lost with suspension of the mutant cells in buffer due to the loss of a vital component of the transport system. In the discussion, this system was not characterized but assumed to be related to the observation that osmotically shocked *E. coli* and membrane vesicle ghosts from *E. coli* cells lost the ability to accumulate K by active transport[3]. In addition to the relative affinities for K and Tl uptake, the difference in the sensitivity to Tl in bacteria might be related to different sites of Tl action. Further studies on the physiology of our Tl-tolerant isolates are now in progress by measuring Tl accumulation during growth in media containing different Tl: K ratios.

The increase in Tl-tolerant fractions of the soil community after the adding of metal can be attributed mainly to an immediate effect due to the death of sensitive species as described by Díaz-Ravińa *et al.*[6]. Presumably, leaking nutrients released by cell lysis from Tl-sensitive microorganisms is the basis for the growth of primarily Tl-tolerant heterotrophs. In addition, the total number of CFU increased about 8-fold when the uncontaminated garden soil suspension was incubated without the addition of Tl (Fig. 1A). This suggests that the leaking nutrients are also derived from water-soluble organic matter present in the soil. The utilization of these leaking nutrients by microorganisms might be dependent on the ability of the surviving bacteria to compete and adapt. It can be concluded that such bacteria surviving in response to Tl loading were isolated in our study. They were identified as 9 clusters in the *Alphaproteobacteria*, including two novel clusters (Groups A and B), in the garden soil, *Pseudomonas* spp. in the arable soil, and *B. niacini* in the heavy metal-polluted river sediment. The competitive abilities and adaptation of these specific bacteria should be explored further.

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