Canadian Journal of Microbiology

Aerobic polychlorinated biphenyl-degrading bacteria isolated from the Tohoku region of Japan are not regionally endemic

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2021-0056.R2
Manuscript Type:	Article
Date Submitted by the Author:	15-Nov-2021
Complete List of Authors:	Hara, Tomijiro; Kyoto University Takatsuka, Yumiko; Kyoto University
Keyword:	Polychlorinated biphenyls, biphenyl dioxygenase, <i>Comamonas testosteroni</i>
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

**Aerobic polychlorinated biphenyl-degrading bacteria isolated from the Tohoku region
of Japan are not regionally endemic**

Tomijiro Hara* and Yumiko Takatsuka

Environmental Microbiology Research Section, Laboratory for Complex Energy
Processes, Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto 611-
0011, Japan

***Corresponding author**

Tomijiro Hara, Ph.D.

Environmental Microbiology Research Section

Laboratory for Complex Energy Processes

Institute of Advanced Energy, Kyoto University

Gokasho, Uji, Kyoto 611-0011, Japan

Phone and Fax: +81-774-38-3551

E-mail: t-hara@iae.kyoto-u.ac.jp

Word count: 6,647

Abstract: 200

Main text excluding references: 5,066

Abstract

In the Tohoku region of Japan, 72% of the land comprises mountain forest zones; during winter, the severe climatic conditions include heavy snowfall. In an environment such as this, which is considered high in biodiversity, we assumed that aerobic bacteria would be diverse and would possess the ability to degrade polychlorinated biphenyls (PCBs). In this study, 78 environmental samples were collected from the Tohoku region and 56 aerobic PCB-degrading bacterial strains were isolated. They belonged to the genera *Achromobacter*, *Rhodococcus*, *Pseudomonas*, *Stenotrophomonas*, *Comamonas*, *Pigmentiphaga*, *Xenophilus*, *Acinetobacter* and *Pandoraea*. Previously reported aerobic PCB-degrading bacterial strains isolated in Japan belonged to the same genera, except that the genera *Acidovorax* and *Bacillus* were not identified in the present study. In particular, the isolated *Comamonas testosteroni* strains YAZ2 and YU14-111 had high PCB-degrading abilities. Analysis of the sequences of YAZ2 and YU14-111 strains showed that the gene structures of the *bph* operon encoding enzymes associated with PCB degradation were the same as that of the *Acidovorax* sp. KKS102 strain. Moreover, 2,3-

biphenyl dioxygenase activity was responsible for the degradation characteristics of all the isolated strains. Overall, this study suggests that aerobic PCB-degrading bacteria are not specifically endemic to the Tohoku region but distributed across Japan.

Keywords

Polychlorinated biphenyls, biphenyl dioxygenase, *Comamonas testosteroni*

Abbreviations

PCBs: polychlorinated biphenyls

Introduction

Polychlorinated biphenyls (PCBs) are well-known organochlorine environmental pollutants, the structure of which involves the replacement of 10 hydrogen atoms in the biphenyl core with chlorine in various combinations of positions and numbers, which has led to 209 congeners. PCBs are characterised by their toxicity, lack of degradability and persistence (Pieper and Seeger 2008). Therefore, abolishing the use of PCBs is an internationally important issue with relevance to the formation of a globally sustainable society. To 'clean up' PCBs from various environments, it will be necessary to utilise the

metabolic functions of microorganisms that have adapted to severe natural environments in remediation technologies. Two important metabolic reactions are known to be involved in the degradation of PCBs by microorganisms: aerobic oxidation and anaerobic dechlorination. Here, we focus only on aerobic oxidation. In 1970, Lunt and Evans (1970) first reported the isolation of gram-negative bacteria possessing the ability to mineralise biphenyl. Subsequently, Ahmed and Focht (1973) reported of two *Achromobacter* strains that could mineralise biphenyls and co-metabolise PCBs. These two findings are regarded the cornerstones of the field of research into oxidative PCB degradation by microorganisms. Besides *Achromobacter*, numerous aerobic gram-negative and gram-positive PCB-degrading bacteria have been isolated from other genera, including gram-negative bacteria from *Pseudomonas*, *Alcaligenes*, *Burkholderia*, *Acinetobacter*, *Comamonas*, *Sphingomonas* and *Ralstonia* and gram-positive bacteria from *Arthrobacter*, *Corynebacterium*, *Rhodococcus* and *Bacillus* (Shuai et al. 2016). The mechanism of PCB degradation used by these bacteria involves the biphenyl metabolic enzyme group, with initial degradation catalysed by biphenyl dioxygenase, which catalyses the incorporation of two oxygen atoms into one of the aromatic rings of PCBs and induces phenyl ring cleavage (Pieper and Seeger 2008).

The microbial communities of aerobic PCB-degrading bacteria have been characterised in previous research. Correa et al. (2010) reported that the β -Proteobacteria *Comamonas* sp. and *Burkholderia* sp. comprised the major bacterial groups in synthetic sediments exposed to PCBs. They reported that *Rhodococcus* sp. belonging to Actinobacteria was also present. In another study, Nogales et al. (1999) investigated soil samples collected from a PCB-contaminated site at a former Soviet missile base north of Wittenberg, Germany; they found that *Rhodopila globiformis*, species from *Burkholderia*, *Variovorax* and *Sphingomonas* and the *Acidobacterium* cluster were dominant.

Unlike research on microbial communities conducted after the year 2000, research published in the 1970s and 1980s did not typically report the locations where the aerobic PCB-degrading bacterial strains were isolated. Similarly, the relationship between these locations and the genetic structure of the PCB-metabolising enzymes was not discussed. This may have been because many aerobic PCB-degrading bacteria were isolated from soils, sediments or waters at sites polluted by either PCBs or other hazardous substances, and the researchers may not have wished to reveal the location of such sites. This also implies that PCBs were recognised as extremely harmful substances back then.

The present study shows that aerobic PCB-degrading bacteria can potentially

be isolated relatively easily from natural landscapes uncontaminated with PCBs. Sample collection in this study was restricted to the Tohoku region of Japan, mainly in Yamagata Prefecture, including municipalities adjacent to mountains and forests. Many bacterial strains capable of degrading PCBs were isolated from the collected samples. However, in environments similar to those studied here, ligninolytic fungi with oxidative degradation effects on PCBs may also exist. Although PCB mineralisation by ligninolytic fungi was first reported in 1985, the metabolism of PCBs and the individual involvement of enzymes is not yet fully understood (Cajthaml 2015). Therefore, we investigated only bacterial populations with co-metabolic processes involving biphenyl catabolic enzymes.

Furthermore, we analysed and compared the gene structures of the *bph* operon, which is a cluster of genes encoding PCB-degrading enzymes, between two *Comamonas testosteroni* strains with high PCB-degrading ability, which was identified in many isolates in the present study as well as in aerobic PCB-degrading bacterial strains previously isolated in Japan (Kimbara et al. 1989; Seto et al. 1995; Shimura et al. 1999). The results showed that aerobic PCB-degrading bacteria are not endemic to the Tohoku region but are in fact also found in other areas of Japan.

Materials and Methods

Chemicals

The PCBs Kanechlor KC-300 and KC-500 were purchased from GL Sciences Inc. (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO) and ethyl acetate at concentrations of 10 mg/ml and 5 mg/ml, respectively, to prepare stock solutions; they were further diluted with DMSO if necessary. Other general reagents were obtained from Fujifilm-Wako Pure Chemical Corp. (Osaka, Japan).

Analysis of polychlorinated biphenyls (PCBs) in soil

Soil samples (10 g each) were placed in a pre-washed flask to which 50 ml of potassium hydroxide–ethanol solution (10-g potassium: 50-ml ethanol: 50-ml distilled water) was added. A reflux condenser was then assembled and heated at 120°C for 1 h to extract PCBs from the soil. Subsequently, 50 ml of hexane was added to the extract and mixed before it was filtrated using glass wool. The remaining PCBs in the residue were further extracted initially with 20 ml of ethanol-hexane (1:1) and subsequently with 30 ml of hexane, and each filtrated extract was collected. All the filtrated extracts were then mixed, after which 50 ml of distilled water was added to the extract and the mixture was shaken. Subsequently, the hexane layer was recovered. The remaining water layer was extracted twice with 50 ml of hexane for each extraction. Approximately 190 ml of all

the collected hexane extract was dehydrated by adding anhydrous sodium sulphate, after which it was concentrated using a rotary evaporator. Finally, the extract was adjusted to 100 ml with hexane.

PCB 169 (3,3',4,4',5,5'-hexachlorobiphenyl) was added as a clean-up spike to 1 ml of pre-extracted sample and this entire amount was applied to a multilayer silica gel column. The column was heated at 85°C for 1 h and cooled to $\leq 40^\circ\text{C}$ afterwards. Next, an alumina column was connected. PCBs were eluted with 20 ml of hexane and 600 μl of toluene. Finally, a syringe spike solution (PCB 209; 2,2',3,3',4,4',5,5',6,6'-decachlorobiphenyl) was added.

PCBs were analysed using a gas chromatography/electron capture detector (GC/ECD) (model 7890A; Agilent Technologies, Santa Clara, CA, USA). GC separation was completed using a DB-5 capillary column (20 m $\times$ 0.18 mm $\times$ 0.18 μm). The carrier gas (H_2) was maintained at a constant flow rate of 3 ml/min, and a volume of 2 μl was used in the splitless injection mode. The column temperature initially was 100°C (held for 1.0 min) but was increased to 160°C in 40°C/min increments (held for 1.0 min), to 250°C in 15°C/min increments, and finally to 280°C in 20°C/min increments (held for 1.6 min).

Analytical data were analysed using OpenLAB CDS ChemStation Edition for

GC Systems, Revision C.01.07 SR3 [465] (Agilent Technologies). Equation 1 shows the quantitation calculation formula. 'Undetected' (uncontaminated) was defined as not more than the analytical quantification limit (0.5 mg/kg). These analyses were performed by an external company, namely Miura Co., Ltd. (Ehime, Japan).

$$\text{PCBs (mg/kg)} = \text{PCBs reference (}\mu\text{g/ml)} \times \frac{\sum \text{CB sample}(\%)}{\sum \text{CB reference}(\%)} \times \frac{\text{extraction(ml)}}{\text{soil(g)}} \times \frac{100}{\text{Clean – up spike recovery rate}(\%)} \quad (1)$$

Isolation of biphenyl-utilising bacterial strains as candidates for PCB degraders

Environmental samples were collected from various sites in the towns and cities of Yamagata, Fukushima, Miyagi, Iwate and Niigata prefectures in the Tohoku region. Each environmental sample was added to 2–10 ml of W-minimal medium, which had the same composition as that of a previously reported mineral salts medium (Kimbara et al. 1988), containing 0.1% biphenyl, after which it was incubated at 30°C with shaking at 120 rpm. Enrichment cultures were performed under three different conditions in which the pH of the W-minimal medium was adjusted to 4.5, 7.5 or 8.5, respectively. Samples in which culture growth was observed were used for inoculation with 10× dilution in the subsequent incubation. After several serial transfers, an aliquot of each sample was first

spread on a W-medium plate supplied with biphenyl as vapour and then incubated at 30°C. The growth of the resulting colonies was evaluated in both liquid- and solid-W-media. The bacteria were repeatedly cultured in liquid and solid media, and the isolates that grew on biphenyl in W-liquid medium were selected as biphenyl-utilising bacterial strains; these were stored at -80°C with 15%–18% glycerol. The ability of all strains to degrade PCB was analysed (described below).

16S rRNA gene analysis

A heat extract containing genomic DNA from each isolated strain was used as a template for polymerase chain reaction (PCR). Briefly, bacterial cells were suspended in TE buffer (10-mM Tris-HCl, 1-mM ethylenediaminetetraacetic acid; pH 8.0), heated at 98°C for 20 min and then centrifuged. The supernatant was then collected and used for PCR. For each strain, approximately 1,500 base pairs (bp) of the partial 16S rRNA gene were amplified using the primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'); the resulting fragment was purified and directly sequenced using the primers 8F, 1492R and/or 907R (5'-CCCGTCAATTCCTTTGAGTTT-3') via the sequencing service provided by Fasmac Co., Ltd. (Kanagawa, Japan). DNA sequences were analysed using the BLAST tool of

the National Center for Biotechnology Information to determine their phylogenetic affiliations. Phylogenetic trees were reconstructed with partial 16S rRNA gene sequences using Genetyx-Mac version 20 (Genetyx Corp., Tokyo, Japan) and the unweighted pair group method with arithmetic mean (UPGMA) method. The nucleotide sequences were deposited in the DDBJ/EMBL/GenBank database under the accession numbers LC529433–LC529488.

PCB degradation assay employing bacterial cells and gas chromatography–mass spectrometry (GC–MS) analysis

Each strain was grown in W-minimal medium containing 0.1% biphenyl to an optical density at 660 nm (O.D.₆₆₀) of >0.5 at 30°C. The cells were then harvested at 15°C and used immediately or stored at –80°C until use. For the PCB degradation assay, the cells were washed twice and resuspended in 20-mM sodium phosphate buffer (pH 7.5). The reaction was completed in a 4-ml borosilicate glass vial sealed with Teflon-covered packing (As One Corp., Osaka, Japan); the final concentration of cells was adjusted to an O.D.₆₆₀ of 10–60 in a reaction mixture with a total volume of 0.5 ml. Kanechlor KC-300 or a mixture of KC-300 and KC-500 (1:1) was added at final concentrations of 5–100 µg/ml. If necessary, Triton X-100 was added at a final concentration of 0.005%,

especially when the reaction was performed with a high PCB concentration (e.g., at 100 µg/ml). A control reaction mixture, in which the buffer was added instead of the cell suspension, was prepared for each reaction. Reaction mixtures were incubated in glass vials for 24 h at 30°C with rotation at 50 rpm produced by a HulaMixer (Thermo Fisher Scientific KK, Tokyo, Japan).

After the reaction was stopped by adding 20 µl of 1 N HCl, anthracene dissolved in toluene was added as an internal standard at a concentration of 1.6 µg/ml. The remaining PCBs were then extracted twice with 1 ml of ethyl acetate used for each extraction. Approximately 2 ml of ethyl acetate extract was dehydrated with sodium sulphate and then the PCBs were analysed using gas chromatography–mass spectrometry (GC–MS) (model 7890A/5975C; Agilent Technologies) with an HP-5ms column (30 m × 0.25 mm × 0.25 µm). Helium was used as the carrier gas at a flow rate of 1.5 cm/s. The column temperature for GC was as follows: 80°C for 2 min, followed by an increase to 130°C at the rate of 20°C/min, followed by a further increase to 300°C at 8°C/min. A quadrupole detector was used with the following temperatures for the ion source, quadrupole and transfer line: 230°C, 150°C and 300°C, respectively. Data acquisition was performed using MSD ChemStation software (Agilent Technologies). Compounds were identified and confirmed using the NIST 08 Mass Spectral Library. The percentage

degradation of each reaction mixture was calculated by dividing the concentration of PCBs from the reaction mixture by the concentration of PCBs in the control mixture (without bacterial cells), multiplying the result by 100, and then subtracting the resulting value from 100.

Sequencing of genes encoding biphenyl-metabolising enzymes from the PCB degraders YU14-111 and YAZ2

The oxidative PCB biodegradation pathway in bacteria is well known: biphenyl dioxygenase introduces two oxygen molecules at the 2nd and 3rd positions on the phenyl ring, which is followed by several enzymatic reactions, including ring cleavage, ultimately leading to the formation of chlorinated benzoate via hydrolysis (Bedard et al. 1986; Pieper and Seeger 2008). Based on the alignment of the amino acid sequences of the biphenyl-2,3-dioxygenase large subunit (BphA1) in PCB-degrading bacterial strains (GenBank accession numbers: AAB63425: *Burkholderia xenovorans* LB400; AAC44526: *Pandoraea pnomenusa* B-356; BAC01052: *C. testosteroni* TK102; AAA25743: *Pseudomonas pseudoalcaligenes* KF707; ACN62349: *Pseudomonas putida* B6-2; BAA04137: *Pseudomonas* sp. KKS102), three highly conserved regions of Lys⁹⁵-Val⁹⁶-Phe⁹⁷-Lue⁹⁸-Asn⁹⁹-Gln¹⁰⁰-Cys¹⁰¹, Gln²²⁷-Phe²²⁸-Cys²²⁹-Ser²³⁰-Asp²³¹-Met²³²-

Tyr²³³ and Glu³⁸⁴-Gln³⁸⁵-Asp³⁸⁶-Asp³⁸⁷-Gly³⁸⁸-Glu³⁸⁹-Asn³⁹⁰ (residue numbers are for BphA1 of the *B. xenovorans* LB400 strain) were selected. Subsequently, three degenerate primers were synthesised: BphA-95F (5'-AA(A/G)GT(G/C)TTCCTGAACCAGTG-3'), BphA-227F (5'-CA(A/G)TTCTGCAG(C/T)GACATGTA-3') and BphA-390R (5'-TT(C/T)TC(A/T/G)CC(A/G)TCGTCCTGCTC-3'). PCR was performed with the primer sets BphA-95F/BphA-390R (forward/reverse primers) and BphA-227F/BphA-390R using heat extract from YU14-111 or YAZ2 as a template; consequently, DNA fragments with sizes of approximately 900 bp and 500 bp, respectively, were obtained. Using sequencing analysis, these fragments were confirmed to contain the partial *bphA1* gene. Based on information on the partial *bphA1*, the DNA sequences of the upstream and downstream unknown regions were analysed using a combination of Southern hybridisation and inverse PCR (Triglia et al. 1988). Genomic DNA was prepared from YU14-111 and YAZ2 strains using a Bacterial DNA Preparation kit (Jena Bioscience, Jena, Germany) and digested using various restriction enzymes (Takara Bio Inc., Shiga, Japan). Southern hybridisation was performed with a PCR-amplified 900-bp DNA fragment containing part of *bphA1* as a probe using ECL direct nucleic acid labelling and detection systems (GE Healthcare, Boston, MA, USA). Subsequently, *Xho*I-, *Sac*II- and *Sma*I-digested fragments were selected as targets for inverse PCR. After determining the

sequences and confirming that the inverse PCR-amplified fragments encoded biphenyl-metabolising enzymes, further sequencing of upstream and downstream flanking regions was performed by repeating Southern hybridisation and inverse PCR. Sequencing analysis details of the *bph* operons from YU14-111 and YAZ2 strains obtained using the primers shown in Table S1 are available on request. Finally, 13,974-bp-long DNA sequences from both strains were determined; these included 15 open reading frames (ORFs) coding for biphenyl-metabolising enzymes and related proteins. The nucleotide sequences of YU14-111 and YAZ2 were deposited in the DDBJ/EMBL/GenBank database under the accession numbers AB706355 and LC528624, respectively.

Matrix-assisted laser desorption/ionisation–time of flight mass spectrometry analysis used to compare the ribosomal protein profiles of YU14-111 and YAZ2

The ribosomal protein profiles of YU14-111 and YAZ2 were analysed using matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI–TOF MS) and the resultant profiles were compared. MALDI–TOF MS analysis was conducted according to the method described by Lohmann et al. (2013) and the resource manufacturer's instructions (Shimadzu Corp., Kyoto, Japan). Each strain was cultured on biphenyl vapour-containing W-medium plates as described above; one colony was

collected using a toothpick and then spotted onto a steel target plate. Subsequently, 1 μ l of α -cyano-4-hydroxycinnamic acid matrix solution containing 3% trifluoroacetic acid, 33% acetonitrile and 33% ethanol (Shimadzu Corp.) was added to the spot, and this was allowed to dry at room temperature. Measurements were taken using a MALDI-TOF MS AXIMA Confidence with Microorganism Identification System (Shimadzu) with a laser frequency of 50 Hz, acceleration voltage of 20 kV and extraction delay time of 200 ns. Spectra were recorded in the linear positive mode within a mass range of 2–20 kDa. Plates were calibrated and validated using *Escherichia coli* as the control, and tests were conducted at the beginning and end of use of each plate. All mass fingerprints were analysed using SARAMIS Premium software (Shimadzu), which first compares the fingerprints to SuperSpectra.

Results

Isolation of aerobic PCB-degrading bacteria from samples obtained from the Tohoku region

The Tohoku region is situated in the northern part of the main island of Japan; it is separated into the Sea of Japan-side and the Pacific Ocean-side by the Ou Mountains, which pass through the centre of the Tohoku region from north to south and are dotted

with plains and basins (Fig. 1A). The region is divided into the Aomori, Akita, Iwate, Miyagi, Fukushima and Yamagata prefectures (and may include Niigata prefecture) (Fig. 1A), and it covers an area of 66,950 km² (79,530 km², including Niigata prefecture). The climate is relatively cool in summer but extremely cold with substantial snowfall during the long winter. Yamagata prefecture faces the Sea of Japan and contains many volcanic hot springs. The forest area of this prefecture is approximately 6,700 km² in size and accounts for approximately 72% of its total land area.

We collected 78 environmental samples from six towns and nine cities of the Tohoku region (Fig. 1B and Table 1). The samples were subjected to aerobic enrichment culture using biphenyl as the sole carbon source. Consequently, 56 biphenyl-utilising bacterial strains were isolated from 31 of 78 environmental samples (Table 1); thus, the isolation rate from the collected samples was 39.7%. The genera and proportions (in parentheses) of the isolated bacterial strains were as follows: *Achromobacter* (30%), *Rhodococcus* (23%), *Pseudomonas* (20%), *Stenotrophomonas* (16%), *Comamonas* (3%), *Pigmentiphaga* (2%), *Xenophilus* (2%), *Acinetobacter* (2%) and *Pandoraea* (2%) (Fig. 2A).

All isolates except one strain grew well in W-minimal medium supplied with biphenyl (Fig. S1). During culture under such conditions, all 56 isolates were found to

produce a yellow-pigmented substance (data not shown) that could be a characteristic product of biphenyl metabolism by bacteria, i.e. 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA), the previous metabolite of benzoic acid (Pieper and Seeger 2008). Biphenyl-utilising bacteria are known to biodegrade PCBs to chlorinated benzoic acid by co-metabolism through biphenyl catabolism (Pieper and Seeger 2008). Figure S1 shows that the isolated strains had the ability to degrade PCBs by co-metabolism. Indeed, when the 56 isolates were plotted based on PCB congeners that were easily or not easily degraded in a mixture of Kanechlor KC-300 and KC-500 (1:1), the data indicated that all isolates had a similar tendency for PCB degradability (Figures S2 and S3).

The PCB contamination analysis of 31 environmental samples showed that none of the samples were contaminated with PCBs (Table 2). Therefore, the 56 isolated biphenyl-utilising bacterial strains were obtained from a vast environment uncontaminated with PCBs, suggesting that such bacteria with the potential to degrade PCBs are widely distributed in the Tohoku region.

Phylogenetic analysis of the isolated aerobic PCB-degrading bacterial strains

A phylogenetic tree was constructed using 16S rRNA gene sequences from the 56 aerobic PCB-degrading bacterial strains as well as strains belonging to the β - and γ -

Proteobacteria subdivisions and Actinobacteria (Fig. 2B). This analysis was aimed at determining how closely related the isolated bacteria in the Tohoku region were to each other in terms of genus classification.

Among β -Proteobacteria, *Achromobacter* was the most isolated genus; this genus was dominant in β -Proteobacteria and most closely related to *Pigmentiphaga*. Among γ -Proteobacteria, the genera *Pseudomonas* and *Stenotrophomonas* were almost equally dominant; however, *Stenotrophomonas* was more closely related to β -Proteobacteria than *Pseudomonas* was to β -Proteobacteria. Among Actinomycetes, *Rhodococcus* strains were dominant. Overall, the aerobic PCB-degrading bacteria isolated from environments uncontaminated with PCBs in the Tohoku region belonged predominantly to the class β -Proteobacteria.

Williams et al. (1997) were the first to perform phylogenetic analyses of aerobic PCB-degrading bacteria. In particular, they examined and described how closely the identified strains were related to the existing aerobic PCB-degrading bacteria. Thus, our results were investigated in line with the work of Williams et al. (1997).

Phylogenetic analysis of the isolated strains and representative aerobic PCB-degrading bacterial strains previously isolated in Japan

We compared the aerobic PCB-degrading bacterial strains isolated from the Tohoku region with the bacterial strains previously isolated from other regions in Japan (Fig. 2C). The strains used for this comparison were *Acidovorax* sp. KKS102 (Kimbara et al. 1989; Ohtsubo et al. 2006), *P. furukawaii* KF707 (Furukawa and Miyazaki 1986; Kimura et al. 2018), *C. testosteroni* TK102 (Hiraoka and Kimbara 2002) and *Rhodococcus jostii* RHA1 (Seto et al. 1995).

Close relationships were found to exist between the following strains: *Xenophilus* sp. R7 was related to *Acidovorax* sp. KKS102; *Pseudomonas* sp. YAZ51 was related to *P. furukawaii* KF707; *C. testosteroni* YAZ2 was related to *C. testosteroni* TK102; and *Rhodococcus* sp. YAZ79 was related to *R. jostii* RHA1 (Fig. 2C). These close relationships showed that the bacterial strains isolated from uncontaminated environments in the Tohoku region were not endemic to the region but could be isolated from across Japan.

Characterisation of the isolates from the Tohoku region with strong PCB-degrading ability

The PCB-degrading ability of the isolated bacterial strains cultured with biphenyl as the sole carbon source was examined; specifically, two strains of *C.*

testosteroni YAZ2 and YU14-111 possessed high PCB-degrading ability (detailed data not shown). Figure 3C shows the GC–MS results for the degradation characteristics of Kanechlor KC-300, in which 50% of the congeners present were 3-chlorine-substituted PCBs. YAZ2 and YU14-111 strains degraded $83.1\% \pm 1.5\%$ and $82.6\% \pm 2.5\%$ of PCBs, respectively, in Kanechlor KC-300. Furthermore, the strains had similar degradation specificities for individual congeners. In addition, these two strains had similar morphological characteristics, as observed under a scanning electron microscope (SEM) (Fig. 3A), and similar protein mapping of intact cells according to MALDI–TOF MS analysis (Fig. 3B). In addition, the biochemical characteristics of these two strains, which were examined using an API kit for bacterial identification (bioMérieux, Lyon, France; data not shown), were highly similar.

The oxidative PCB degradation pathway in aerobic bacteria is well known and biphenyl-metabolising enzymes are known to play a crucial role in this pathway (Pieper and Seeger 2008). To confirm whether *C. testosteroni* strains YU14-111 and YAZ2 had the same ability to aerobically degrade PCB, we determined the DNA sequences (13,974-bp in length) corresponding to the *bph* operon region of both strains; the sequences included 15 ORFs coding biphenyl-metabolising enzymes and related proteins (Fig. 4A). Based on the sequences determined from the two strains, the entire structure of the *bph*

operon (i.e. the genetic organisation of the *bph* genes) and each of the *bph* genes encoding the biphenyl-metabolising enzymes were found to be identical. Thus, the two strains isolated from the uncontaminated environment in the Tohoku region were apparently the same.

Phylogenetic analysis of the amino acid sequences of BphA1, BphB, BphC and BphD in *C. testosteroni* strain YAZ2 deduced from *bph* operon gene data (which were identical in strain YU14-111) was performed using previously reported aerobic PCB-degrading bacterial strains (shown on the phylogenetic trees in Fig. 4B). BphA1 is a large subunit of biphenyl 2,3-dioxygenase; BphB is a cis-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase; BphC is a 2,3-dihydroxybiphenyl 1,2-dioxygenase; and BphD is HOPDA hydrolase; overall, these upstream enzymes lead to the degradation of biphenyls to benzoic acid (Pieper and Seeger 2008). According to this analysis, *C. testosteroni* strains YAZ2 and YU14-111 possessed enzyme groups that were identical to those of *Acidovorax* sp. KKS102. DNA sequencing data revealed that the structures of the *bph* operon from the strains YAZ2 and YU14-111 were closely related to that of strain KKS102. The pE promoter on the *bph* operon is controlled by catabolite regulation in *Acidovorax* sp. KKS102. The presence of HOPDA, a pathway intermediate, has been reported to induce activation of the pE promoter (Ohtsubo et al. 2006). These results

suggest that *C. testosteroni* strains YAZ2 and YU14-111 also degrade PCBs via the same control mechanism as that used by *Acidovorax* sp. KKS102.

Discussion

In previous studies of aerobic PCB-degrading bacterial strains, Furukawa and Miyazaki (1986) reported that the strain KF707 was ‘isolated from soil near a biphenyl manufacturing plant’. Later, Kimura et al. (2018) indicated that strain KF707 was isolated in Kitakyushu city. Furthermore, Seto et al. (1995) reported that strain RHA1 was ‘isolated from soil samples contaminated with insecticide’. However, Kimbara et al. (1988) reported that strain KKS102 was ‘isolated from soil samples obtained from industrial regions in Tokyo and Kawasaki city’, and Shimura et al. (1999) reported that strain JF8 was ‘isolated from compost obtained from a field in Okayama city’, whereas Fukuda et al. (2014) indicated that strain TK102 was ‘isolated from soil contaminated with PCBs in Hamamatsu city’. These prominent aerobic PCB-degrading bacterial strains all appear to have originated in Japan. Given these findings, we asked the following question: are aerobic PCB-degrading bacteria endemic to specific regions? *Pseudomonas paucimobilis* strain Q1, a bacterium that utilises biphenyl, can grow not only on biphenyl but also on xylene, toluene or both combined, as well as on salicylate and octane

(Furukawa et al. 1983). These aromatic hydrocarbons exist in various forms in nature, suggesting that such bacteria grow in many habitats and are not, therefore, endemic.

Because 72% of the land in the Tohoku region is covered by forests, we randomly collected environmental samples from a wide area including highlands or forest areas leading to mountain slopes (Fig. 1B). The results showed that 56 bacterial strains with the ability to degrade PCBs were isolated from 78 samples collected in 15 cities and towns that had not been contaminated with PCBs. Further analysis showed that the bacteria isolated from the Tohoku region belonged to the genera that were similar to those of the prominent aerobic PCB-degrading bacterial strains previously isolated in Japan (with the exception of *Acidovorax* and *Bacillus*, which were not identified in this study). Moreover, strains were isolated in 11 of 15 cities and towns; thus, the isolation rate across all sample collection sites was 73%. Of note, there was a close genetic relationship between the strains isolated in the present study from the Tohoku region and the representative strains originating from Japan. Therefore, aerobic PCB-degrading bacteria are extensively distributed across the Tohoku region; moreover, strains isolated from similar environments are not endemic to the region but are generally present across Japan.

Shuai et al. (2016) reported that *Stenotrophomonas*, *Pseudomonas* and *Rhodococcus* spp. were isolated from environmental samples collected in Heilongjiang

Province (northeastern region), Shanxi Province (northern region) and Shanghai Municipality (eastern region) in China (all areas where industry had quickly developed). Our results indicate that the aerobic PCB-degrading bacteria isolated in China were similar to those isolated in Japan. Shuai et al. (2010) also collected samples from an environment possibly contaminated with PCBs, i.e. four Shanghai Municipality landscapes, and isolated aerobic PCB-degrading bacteria from these areas; these authors found that the bacteria belonged to the genera *Rhodococcus*, *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter* and *Pandoraea*, which is in agreement with our results. Therefore, the studies by Shuai et al. also strongly support that aerobic PCB-degrading bacteria are not endemic to specific habitats, whether or not they are contaminated with PCBs.

In this study, we investigated the PCB degradation ability of 56 biphenyl-utilising bacterial strains isolated from the Tohoku region, mainly using Kanechlor KC-300 and KC-500 (Figs. S2 and S3), and selected *C. testosteroni* strains YAZ2 and YU14-111 for further analysis. Morphological observations from SEM images and homology testing using protein mapping of intact bacterial cells conducted via MALDI-TOF MS (many mapped peaks of which were derived from ribosomes) (Claydon et al. 1996) suggested that strains YAZ2 and YU14-111 were identical. Importantly, these strains

were isolated from samples collected from different environments, i.e. Yonezawa city and its suburbs, which are linearly approximately 6 km apart. Therefore, we further tested the strains in terms of their PCB degradation ability using Kanechlor KC-300; the percentage of Kanechlor KC-300 degradation was highly similar between the two strains (YAZ2 vs YU14-111: $83.1\% \pm 1.5\%$ vs $82.6\% \pm 2.5\%$) and both showed 2,3-dioxygenase activity in their degradation of PCB congeners (Fig. 3C).

From the sequencing analysis of the *bph* operon of YU14-111 and YAZ2, the genetic organisation and sequences of the *bph* genes were found to be identical between the two strains. We accessed the draft genome data of strain YAZ2 (data not shown), which revealed that it was composed of a 5.45-Mbp chromosome-like section and an 87-kbp circular plasmid, with the *bph* operon confirmed to be located on the chromosome. Although there are no equivalent data for strain YU14-111, if the *bph* operon is located on the chromosome in both strains, their mother strain is likely to have had the same origin. Overall, these analyses show that bacteria of the same species with the same gene structure and PCB degradation characteristics inhabit and are distributed across the natural landscape of municipalities in Japan (i.e. an area of 10 km²).

Because the structure of the YAZ2 strain *bph* operon was determined, we also conducted comparative phylogenetic analysis of *bph* operon gene data from prominent

aerobic PCB-degrading bacterial strains known to have originated in Japan. The structures of *bph* gene clusters in strains YU14-111 and YAZ2 were closely related to that of strain KKS102. According to the YAZ2 strain draft genome data, the *bph* operon of strain YAZ2 is located in the integrative and conjugative element (ICE), which contributes to horizontal gene transfer, similar to that in *Acidovorax* KKS102 (data not shown). The total gene length of the ICE in strain YAZ2 (ICE_{YAZ2}) was estimated at 50 kbp, whereas the total length of ICE_{KKS102} was approximately 62 kbp (Ohtsubo et al. 2012). Among these regions, a gene sequence of approximately 19 kbp that included the *bph* operon and its flanking regions was identical in the YAZ2 and KKS102 strains; however, the homology with ICE_{KKS102} in other regions was low. In ICE_{YAZ2}, the conjugation-related genes located in the right-wing downstream of the *bph* operon were 88%–90% homologous to those of *Pseudomonas* and *Stenotrophomonas*. In addition, the ~19-kbp sequence corresponding to the left-wing upstream of the *bph* operon includes a mosaic structure of genes that are 83%–91% homologous with gene sequences from a wide range of Proteobacteria including *Comamonas*, *Cupriavidus*, *Delftia* and *Citrobacter*. We speculate that, in the process of biological evolution, ICE_{YAZ2} has been repeatedly transferred horizontally among these bacterial species in the same manner as that in the KKS102 strain.

Overall, our results suggest that aerobic PCB-degrading bacteria are not regionally endemic. Specifically, we argue that such bacteria isolated from an environment uncontaminated with PCBs of the Tohoku region of Japan are not endemic species, at least in Japan and East Asia. However, as we did not perform investigations outside Japan, it remains unclear whether our findings are common to all natural environments. Because aerobic PCB-degrading bacteria do not appear to be regionally endemic, optimisation of adapted bacterial species, such as those described herein, may occur naturally in the environment. This should be clarified in future research to help develop new bioremediation techniques.

Acknowledgments

The authors would like to thank Dr Ian Laycock from Enago for the English language review. This work was supported by JSPS KAKENHI grant numbers JP26550066 and JP18K19881.

References

Ahmed, M. and Focht, D.D. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can J Microbiol **19**(1):47–52. doi:10.1139/m73-007.

- Bedard, D.L., Unterman, R., Bopp, L.H., Brennan, M.J., Haberl, M.L., and Johnson, C. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Appl Environ Microbiol* **51**(4):761–768. doi:10.1128/aem.51.4.761-768.1986.
- Cajthaml, T. 2015. Biodegradation of endocrine-disrupting compounds by ligninolytic fungi: mechanisms involved in the degradation. *Environ Microbiol* **17**(12):4822–4834. doi:10.1111/1462-2920.12460.
- Claydon, M.A., Davey, S.N., Edwards-Jones, V., and Gordon, D.B. 1996. The rapid identification of intact microorganisms using mass spectrometry. *Nat Biotechnol* **14**(11):1584–1586. doi:10.1038/nbt1196-1584.
- Correa, P.A., Lin, L., Just, C.L., Hu, D., Hornbuckle, K.C., Schnoor, J.L., and Van Aken, B. 2010. The effects of individual PCB congeners on the soil bacterial community structure and the abundance of biphenyl dioxygenase genes. *Environ Int* **36**(8):901–906. doi:10.1016/j.envint.2009.07.015.
- Fukuda, K., Hosoyama, A., Tsuchikane, K., Ohji, S., Yamazoe, A., Fujita, N., Shintani, M., and Kimbara, K. 2014. Complete genome sequence of polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 (NBRC 109938). *Genome Announc* **2**(5):e00865-14. doi:10.1128/genomeA.00865-14.

- Furukawa, K., and Miyazaki, T. 1986. Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J Bacteriol 166(2):392–398. doi:10.1128/jb.166.2.392-398.1986.
- Furukawa, K., Simon, J.R., and Chakrabarty, A.M. 1983. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J Bacteriol 154(3):1356–1362. doi:10.1128/jb.154.3.1356-1362.1983.
- Hiraoka, Y. and Kimbara, K. 2002. Rapid assessment of the physiological status of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 by flow cytometry. Appl Environ Microbiol 68(4):2031–2035. doi:10.1128/aem.68.4.2031-2035.2002.
- Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M., and Yano, K. 1988. Isolation and characterisation of a mixed culture that degrades polychlorinated biphenyls. Agric Biol Chem 52(11):2885–2891. doi:10.1080/00021369.1988.10869161.
- Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M., and Yano, K. 1989. Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading

- soil bacterium *Pseudomonas* sp. strain KKS102. *J Bacteriol* **171**(5):2740–2747. doi:10.1128/jb.171.5.2740-2747.1989.
- Kimura, N., Watanabe, T., Suenaga, H., Fujihara, H., Futagami, T., Goto, M., Hanada, S., and Hirose, J. 2018. *Pseudomonas furukawaii* sp. nov., a polychlorinated biphenyl-degrading bacterium isolated from biphenyl-contaminated soil in Japan. *Int J Syst Evol Microbiol* **68**(5):1429–1435. doi:10.1099/ijsem.0.002670.
- Lohmann, C., Sabou, M., Moussaoui, W., Prévost, G., Delarbre, J.-M., Candolfi, E., Gravet, A., and Letscher-Bru, V. 2013. Comparison between the Biflex III-Biotyper and the Axima-SARAMIS systems for yeast identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry. *J Clin Microbiol* **51**(4):1231–1236. doi:10.1128/jcm.03268-12.
- Lunt, D. and Evans, W.C. 1970. The microbial metabolism of biphenyl. *Biochem J* **118**(3):54–55. doi:10.1042/bj1180054pb.
- Nogales, B., Moore, E.R., Abraham, W.R., and Timmis, K.N. 1999. Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environ Microbiol* **1**(3):199–212. doi:10.1046/j.1462-2920.1999.00024.x.
- Ohtsubo, Y., Goto, H., Nagata, Y., Kudo, T., and Tsuda, M. 2006. Identification of a

- response regulator gene for catabolite control from a PCB-degrading β -proteobacteria, *Acidovorax* sp. KKS102. *Mol Microbiol* **60**(6):1563–1575. doi:10.1111/j.1365-2958.2006.05197.x.
- Ohtsubo, Y., Ishibashi, Y., Naganawa, H., Hirokawa, S., Atobe, S., Nagata, Y., and Tsuda, M. 2012. Conjugal transfer of polychlorinated biphenyl/biphenyl degradation genes in *Acidovorax* sp strain KKS102, which are located on an integrative and conjugative element. *J Bacteriol* **194**(16):4237–4248. doi:10.1128/JB.00352-12.
- Pieper, D.H. and Seeger, M. 2008. Bacterial metabolism of polychlorinated biphenyls. *J Mol Microbiol Biotechnol* **15**(2–3):121–138. doi:10.1159/000121325.
- Seto, M., Kimbara, K., Shimura, M., Hatta, T., Fukuda, M., and Yano, K. 1995. A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. *Appl Environ Microbiol* **61**(9):3353–3358. doi:10.1128/aem.61.9.3353-3358.1995.
- Shimura, M., Mukerjee-Dhar, G., Kimbara, K., Nagato, H., Kiyohara, H., and Hatta, T. 1999. Isolation and characterization of a thermophilic *Bacillus* sp. JF8 capable of degrading polychlorinated biphenyls and naphthalene. *FEMS Microbiol Lett* **178**(1):87–93. doi:10.1111/j.1574-6968.1999.tb13763.x.
- Shuai, J., Yu, X., Zhang, J., Xiong, A.-S., and Xiong, F. 2016. Regional analysis of

- potential polychlorinated biphenyl degrading bacterial strains from China. *Braz J Microbiol* **47**(3):536–541. doi:10.1016/j.bjm.2014.12.001.
- Shuai, J.-J., Tian, Y.-S., Yao, Q.-H., Peng, R.-H., Xiong, F., and Xiong, A.-S. 2010. Identification and analysis of polychlorinated biphenyls (PCBs)-biodegrading bacterial strains in Shanghai. *Curr Microbiol* **61**(5):477–483. doi:10.1007/s00284-010-9641-2.
- Triglia, T., Peterson, M.G., and Kemp, D.J. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucleic Acids Res* **16**(16):8186. doi:10.1093/nar/16.16.8186.
- Williams, W.A., Lobos, J.H., and Cheetham, W.E. 1997. A phylogenetic analysis of aerobic polychlorinated biphenyl-degrading bacteria. *Int J Syst Bacteriol* **47**(1):207–210. doi:10.1099/00207713-47-1-207.

Figure Legends

Fig. 1. Geographical locations of environmental samples collected from the Tohoku region of Japan

(A) Map of Japan including part of East Asia showing the prefectures of Aomori, Akita, Iwate, Miyagi, Yamagata and Fukushima (with Niigata) belonging to the Tohoku region.

(B) Sampling locations in Tohoku region. The numbers in parentheses indicate the number of isolated aerobic PCB-degrading bacterial strains relative to the number of samples collected. These maps were created using Google Earth Pro provided by Google Inc.

Fig. 2. Isolation and classification of PCB-degrading bacteria from the Tohoku region

(A) The overall status of PCB-degrading bacterial strains isolated from the Tohoku region.

(B) 16S rRNA gene phylogenetic tree of the isolated strains. Using the UPGMA method, the tree was generated using approximately 200 bp of partial 16S rRNA gene sequences corresponding to the V4–V5 regions. (C) Phylogenetic analysis of the strains isolated in this study and representative PCB-degrading strains previously isolated in Japan. Using the UPGMA method, the tree was generated using approximately 800 bp of partial 16S rRNA gene sequences corresponding to the V1–V5 regions. The sequences from representative PCB-degrading strains previously isolated in Japan were acquired from the GenBank database (accession numbers: LC151978 for *P. furukawaii* KF707; CP003872 for *Acidovorax* sp. KKS102; CP006704 for *C. testosteroni* TK102; and CP000431 for *R. jostii* RHA1).

Fig. 3. Comparison of the isolated *C. testosteroni* strains YAZ2 and YU14-111

(A) SEM images. (B) MALDI–TOF MS profiles of ribosomal proteins from each colony. (C) PCB-degrading profiles. The cells of each strain ($\text{O.D.}_{660} = 10$) grown in W-minimal medium supplied with biphenyl were subjected to a KC-300-degrading assay at 30°C for 24 h, and the remaining PCBs were analysed using GC–MS. I.S., internal standard.

Fig. 4. Comparative genetic analysis of *C. testosteroni* strains YU14-111 and YAZ2 (A)

Organisation of the *bph* gene cluster in *C. testosteroni* strains YU14-111 and YAZ2. The sequences of the *bph* gene cluster and neighbouring region in strains YU14-111 and YAZ2 were identical within the analysed 13,974-bp sequences. Grey arrows represent the *bph* gene cluster, whereas white arrows show ORFs sharing homology with known genes. The restriction enzyme sites used for inverse PCR are also shown. ORFs for the *bph* gene cluster were as follows: *bphS*: GntR family transcriptional regulator; *bphE*: 2-hydroxypenta-2,4-dienoate hydratase; *bphG*: acetaldehyde dehydrogenase; *bphF*: 4-hydroxy-2-oxovalerate aldolase; *bphX*: putative membrane protein; *bphA1*: biphenyl dioxygenase large subunit; *bphA2*: biphenyl dioxygenase small subunit; *bphA3*: biphenyl dioxygenase ferredoxin subunit; *bphB*: *cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase; *bphC*: dihydroxybiphenyl dioxygenase; *bphD*: 2-hydroxy-6-oxo-phenylhexa-2,4-dienoic

acid hydrolase; *bphW*: putative outer membrane protein; and *bphA4*: biphenyl dioxygenase ferredoxin reductase. (B) Phylogenetic trees of BphA1, BphB, BphC and BphD from *Comamonas testosteroni* YAZ2 and other well-known PCB-degrading bacterial strains previously isolated in Japan. The trees were constructed using the UPGMA method and the amino acid sequence of each biphenyl-metabolising enzyme. The GenBank accession numbers of the referred sequences for BphA1, BphB, BphC and BphD, respectively, in each strain were as follows: *Acidovorax* sp. KKS102: BAJ72245, BAJ72248, AFU46884 and AFU46885; *C. testosteroni* TK102: BAC01052, AIJ45794, BAC01056 and AIJ45792; *P. furukawaii* KF707: BAU73321, BAU73316, BAU73315 and BAU73310; *R. jostii* RHA1: ABG99107, ABG99101, BAA06872 and BAC92715.

Table 1. Distribution of PCB-degrading strains isolated from environmental samples in different prefectures

Table 2. Analysis of PCB contamination in the soil samples from which biphenyl-utilising strains were isolated

Table 1. Distribution of PCB-degrading strains isolated from environmental samples in different prefectures

Prefecture	Number of samples		PCB-degrading bacterial genera isolated and numbers						
	Total	PCB-degrading bacteria obtained	<i>Rhodococcus</i>	<i>Pseudomonas</i>	<i>Comamonas</i>	<i>Achromobacter</i>	<i>Stenotrophomonas</i>	Others	Total
Yamagata	64	23	10	11	2	10	7	2 ^a	42
Fukushima	7	5	2	–	–	5	2	1 ^b	10
Miyagi	1	1	–	–	–	–	–	1 ^c	1
Iwate	4	1	1	–	–	1	–	–	2
Niigata	2	1	–	–	–	1	–	–	1
Total	78	31	13	11	2	17	9	4	56

^a *Pigmentiphaga* and *Xenophilus*, ^b *Acinetobacter*, ^c *Pandreaea*.

Table 2. Analysis results on PCB contamination of soil in which biphenyl-utilising strains were isolated

No.	Collection site		Sample name	PCBs ^a [mg/kg]
	Prefecture	City/Town		
1	YAMAGATA	SAKATA	T1	≤ 0.5 (uncontaminated)
2		YONEZAWA	T7	≤ 0.5 (uncontaminated)
3			T12	≤ 0.5 (uncontaminated)
4			T14	≤ 0.5 (uncontaminated)
5			T15	≤ 0.5 (uncontaminated)
6			e	≤ 0.5 (uncontaminated)
7			R1	≤ 0.5 (uncontaminated)
8			R7	≤ 0.5 (uncontaminated)
9			R9	≤ 0.5 (uncontaminated)
10			R10	≤ 0.5 (uncontaminated)
11			R11	≤ 0.5 (uncontaminated)
12			R12	≤ 0.5 (uncontaminated)
13			H1	≤ 0.5 (uncontaminated)
14			H3	≤ 0.5 (uncontaminated)
15			H4	≤ 0.5 (uncontaminated)
16			H5	≤ 0.5 (uncontaminated)
17			H9	≤ 0.5 (uncontaminated)
18			H11	≤ 0.5 (uncontaminated)
19			H14	≤ 0.5 (uncontaminated)
20			H15	≤ 0.5 (uncontaminated)
21			H18	≤ 0.5 (uncontaminated)
22		KAWANISHI	Ho3	≤ 0.5 (uncontaminated)
23		IIDE	Ho4	≤ 0.5 (uncontaminated)
24	NIIGATA	SEKIKAWA	Ho8	≤ 0.5 (uncontaminated)
25	FUKUSHIMA	KORIYAMA	b	≤ 0.5 (uncontaminated)
26		INAWASHIRO	c	≤ 0.5 (uncontaminated)
27		MOTOMIYA	Ho10	≤ 0.5 (uncontaminated)
28		FUKUSHIMA	Ho11	≤ 0.5 (uncontaminated)
29			Ho12	≤ 0.5 (uncontaminated)

30	MIYAGI	TAIWA	Ho15	≤ 0.5 (uncontaminated)
31	IWATE	MIYAKO	Ho16	≤ 0.5 (uncontaminated)

a Uncontaminated with PCBs were defined as below 'the analytical quantification limit (0.5 mg/kg)', which was determined on the soil measurement method specified by the Ministry of the Environment of Japan.

Draft

Fig. 1. Geographical locations of environmental samples collected from the Tohoku region of Japan

(A) Map of Japan including part of East Asia showing the prefectures of Aomori, Akita, Iwate, Miyagi, Yamagata and Fukushima (with Niigata) belonging to the Tohoku region. (B) Sampling locations in Tohoku region. The numbers in parentheses indicate the number of isolated aerobic PCB-degrading bacterial strains relative to the number of samples collected. These maps were created using Google Earth Pro provided by Google Inc.

182x205mm (300 x 300 DPI)

Fig. 2. Isolation and classification of PCB-degrading bacteria from the Tohoku region
(A) The overall status of PCB-degrading bacterial strains isolated from the Tohoku region. (B) 16S rRNA gene phylogenetic tree of the isolated strains. Using the UPGMA method, the tree was generated using approximately 200 bp of partial 16S rRNA gene sequences corresponding to the V4–V5 regions. (C) Phylogenetic analysis of the strains isolated in this study and representative PCB-degrading strains previously isolated in Japan. Using the UPGMA method, the tree was generated using approximately 800 bp of partial 16S rRNA gene sequences corresponding to the V1–V5 regions. The sequences from representative PCB-degrading strains previously isolated in Japan were acquired from the GenBank database (accession numbers: LC151978 for *P. furukawii* KF707; CP003872 for *Acidovorax* sp. KKS102; CP006704 for *C. testosteroni* TK102; and CP000431 for *R. jostii* RHA1).

182x191mm (300 x 300 DPI)

Fig. 3. Comparison of the isolated *C. testosteroni* strains YAZ2 and YU14-111

(A) SEM images. (B) MALDI-TOF MS profiles of ribosomal proteins from each colony. (C) PCB-degrading profiles. The cells of each strain (O.D.₆₆₀ = 10) grown in W-minimal medium supplied with biphenyl were subjected to a KC-300-degrading assay at 30°C for 24 h, and the remaining PCBs were analysed using GC-MS. I.S., internal standard.

178x218mm (300 x 300 DPI)

Fig. 4. Comparative genetic analysis of *C. testosteroni* strains YU14-111 and YAZ2 (A) Organisation of the *bph* gene cluster in *C. testosteroni* strains YU14-111 and YAZ2. The sequences of the *bph* gene cluster and neighbouring region in strains YU14-111 and YAZ2 were identical within the analysed 13,974-bp sequences. Grey arrows represent the *bph* gene cluster, whereas white arrows show ORFs sharing homology with known genes. The restriction enzyme sites used for inverse PCR are also shown. ORFs for the *bph* gene cluster were as follows: *bphS*: GntR family transcriptional regulator; *bphE*: 2-hydroxypenta-2,4-dienoate hydratase; *bphG*: acetaldehyde dehydrogenase; *bphF*: 4-hydroxy-2-oxovalerate aldolase; *bphX*: putative membrane protein; *bphA1*: biphenyl dioxygenase large subunit; *bphA2*: biphenyl dioxygenase small subunit; *bphA3*: biphenyl dioxygenase ferredoxin subunit; *bphB*: *cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase; *bphC*: dihydroxybiphenyl dioxygenase; *bphD*: 2-hydroxy-6-oxo-phenylhexa-2,4-dienoic acid hydrolase; *bphW*: putative outer membrane protein; and *bphA4*: biphenyl dioxygenase ferredoxin reductase. (B) Phylogenetic trees of BphA1, BphB, BphC and BphD from *Comamomonas testosteroni* YAZ2 and other well-known PCB-degrading bacterial strains previously isolated in Japan. The trees were constructed using the UPGMA method and the amino acid sequence of each biphenyl-metabolising enzyme. The GenBank accession numbers of the referred sequences for BphA1, BphB, BphC and BphD, respectively, in each strain were as follows: *Acidovorax* sp. KKS102: BAJ72245, BAJ72248, AFU46884 and AFU46885; *C. testosteroni* TK102: BAC01052, AIJ45794, BAC01056 and AIJ45792; *P. furukawii* KF707: BAU73321, BAU73316, BAU73315 and BAU73310; *R. jostii* RHA1: ABG99107, ABG99101, BAA06872 and BAC92715.

119x118mm (300 x 300 DPI)