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Flow Cytometry-Based Method for Isolating Live Bacteria with Meta-Cleavage Activity on Dihydroxy Compounds of Biphenyl

Running Title: Flow cytometry-based method for isolating bacteria

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ABSTRACT

A new method for isolating targeted live bacterial cells was established with the use of cell sorting by flow cytometry (FCM) based on the fluorescence of the intermediate metabolite of biphenyl degradation. During biphenyl degradation, a PCB degrader, Comamonas testosteroni TK102, produces a meta-cleavage intermediate metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), which emits green fluorescence. HOPDA was produced from 2,3-dihydroxy biphenyl as a substrate, but it was not appropriate for labeling cells because it was released from the cells into the medium. When we used 4-n-butylbiphenyl and 4-n-heptylbiphenyl, we found that the cells produced and accumulated 2,3-dihydroxy intermediate metabolites. By the addition of synthesized 2,3-dihydroxy-4'-butylbiphenyl (2,3-DHBBP), we were able to label the cells with strong green fluorescence, suggesting the persistence of fluorescent intermediate metabolite in the cells by the introduction of the alkyl tail. 2,3-DHBBP was then used to label strain TK102 and the cells were sorted with FCM. The sorting efficiency of FCM was defined as the percentage of colony numbers per sorting events. Strain TK102 cells were successfully enriched by 4.1-fold from the mixture with environmental indigenous bacteria with a sorting efficiency of 7.3%. The method we present here serves as a basic technique for the specific and direct isolation of live bacterial cells which contain dioxygenases active on dihydroxylated aromatic compounds.
INTRODUCTION

The establishment of a method for isolating targeted bacteria from the environment is a fundamental goal in environmental microbiology. Conventional screening methods, such as enrichment culture and plating on selective media, have been used in most cases where nonpredominant cells might be eliminated in the competition and many kinds of bacteria are unable to form colonies. Recently, researchers have been trying to isolate bacterial cells directly from environmental samples using flow cytometry (FCM), which enables high-throughput detection and sorting of fluorescence-labeled bacteria (1-4). The staining targets of fluorescent dyes are DNA, RNA, and other cellular components. Sorted bacteria are subjected to 16S rDNA analysis to elucidate the microbial community structure of environmental samples (5-9). Fluorescence in situ hybridization (FISH) coupled with FCM sorting has also been applied to specifically isolate the cells that contain unique sequences from complex microbial communities (10, 11). A gel microdroplet technique and a water-in-oil emulsion technique, both of which are based on the combination of a single-cell encapsulation procedure with FCM sorting, have enabled the isolation and screening of cells that express certain cloned genes and do not grow on nutrient medium (12-14). However these FCM-assisted methods have not yet led to isolate live specific bacteria which have unique metabolic ability. In this study, we constructed a new method which uses sorting technology of FCM based on fluorescent metabolite accumulating in bacterial cells.

Polychlorinated biphenyls (PCBs), which are serious environmental pollutants, are recalcitrant to biodegradation (15, 16). Many Gram-positive and Gram-negative PCB-degraders, such as *Pseudomonas*, *Burkholderia*, *Comamonas*, and *Rhodococcus* species were isolated and the microbial degradation pathways of these species have been extensively studied (17). Every aerobic PCB degrader found thus far has a meta-cleavage enzyme for
opening the benzene ring (Fig. 1). *meta*-Cleavage is a key enzymatic step in the metabolism of aromatic compounds. The *meta*-cleavage enzyme, BphC, catalyzes the addition of two atoms of oxygen to 2,3-dihydroxybiphenyl (2,3-DHBP) and produces a yellow *meta*-cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) (18-20). Bacteria with a *meta*-cleavage enzyme form yellow colonies when grown on biphenyl (21, 22), which is a typical indicator of biphenyl degradation ability. Many PCB degraders have been isolated by this conventional screening method (23).

There must be new and unique PCB-degrading bacterial resources in nature which can be utilized as genetic resources for bioremediation. The aforementioned conventional screening methods do not serve for isolation of bacteria that do not form colonies. In this study, we constructed and optimized the experimental condition for a new FCM-assisted method for direct isolation of bacteria with *meta*-cleavage activity. Furthermore, the efficacy of the method was proven using a mixture of *Comamonas testosteroni* TK102 and indigenous bacteria derived from an underground water sample.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**  A PCB degrader, *C. testosteroni* strain TK102, and its *bphC* mutant were used in this study (24, 25). Strain TK102 is a Gram-negative bacterium isolated from soil contaminated with PCBs (20). The expression of PCB degradative genes of strain TK102 is constitutive (25). A *bphC* mutant of TK102 was constructed by insertion of kanamycin resistance gene (24). Strain TK102 was maintained on W-solid medium (9.8 g/l Na₂HPO₄, 1.7 g/l KH₂PO₄, 1.0 g/l (NH₄)₂SO₄, 0.1 g/l MgSO₄·7H₂O, 10.75 mg/l MgO, 9.5 mg/l FeSO₄·7H₂O, 2.0 mg/l CaCO₃, 1.44 mg/l ZnSO₄·7H₂O, 1.22 mg/l MnCl₂·4H₂O, 0.28 mg/l CoSO₄·7H₂O, 0.25 mg/l CuSO₄·5H₂O, 0.06 mg/l H₃BO₃, 51.3 μl
concentrated HCl, and 15 g/l agar, pH 7.9) at 30°C and biphenyl (Nacalai Tesque, Inc., Japan) as sole carbon source was given as vapor phase on the lid of petri dish. The $bphC$ mutant of strain TK102 was maintained on 1/3-diluted Luria-Bertani solid medium (1/3LB, 3.3 g/l tryptone, 1.7 g/l yeast extract, 5.0 g/l NaCl, and 15 g/l agar) with 300 µg/ml of kanamycin at 30°C. R2A Agar (Becton, Dickinson and Company, Sparks, MD) was used for measuring sorting efficiency.

$E. coli$ MV1184 (26) was grown in 5 ml of LB medium (10.0 g/l tryptone, 5.0 g/l yeast extract, 5.0 g/l NaCl) for 12 h at 37°C, and used for the experiments. $E. coli$ transformants were cultivated on LB medium supplemented with 100 µg/ml ampicillin at 37°C.

**Construction of plasmids** Plasmid pKTF18, which contains the $bphABC$ gene from *Pseudomonas pseudoalcaligenes* KF707, was kindly supplied by Dr. Kensuke Furukawa, Beppu University (27). A 5.7-kb $XhoI/ClaI$ fragment from pKTF18 that contains the $bphAB$ region was introduced into pBluescriptII KS+ vector and designated as pSIbphAB. A 2.1-kb $SalI/SacI$ fragment from pKTF18 that contains the $bphC$ region was introduced into pBluescriptsII KS+ vector and designated as pSIbphC.

**Fluorescence spectrum measurement of HOPDA** $E. coli$ MV1184 (pSIbphC) was cultivated in 100-ml medium for 6 h at 37°C. The cells were collected and washed twice with phosphate buffer (50 mM, pH 7.0). The collected cells were suspended in the same buffer (OD$_{600}$=3.0). One microliter of 100 mM 2,3-DHBP solution (9.3 mg of 2,3-DHBP dissolved in 0.5 ml ethanol) was added to the 1-ml cell suspension (28, 29). The mixture was incubated at room temperature for 10 min, and the supernatant was collected by centrifugation. The supernatant was diluted 10-fold with phosphate buffer (50 mM, pH 7.0, 7.5, 8.0, or 8.5), and its fluorescence was measured by a fluorescence spectrophotometer (F-4500, HITACHI High Technologies Co., Tokyo, Japan) in three-dimensional measurement mode. Analytical
conditions were as follows: scan speed, 30,000 nm/min; scanning wavelength range, (Ex) 350–550 nm (Em) 450–600; sampling, every 2.0 nm; contour line, each 4.0 (relative intensity); and voltage of photomultiplier tube, 700 V.

**Distribution of 2,3-dihydroxybiphenyl and its alkylated derivatives** The cellular persistence of 2,3-DHBP and its alkylated derivatives was analyzed with the bphC mutant of strain TK102. One milliliter of the bphC mutant preculture was inoculated into 100-ml medium supplemented with 30 mg/l of biphenyl or one of its biphenyl-alkylated derivatives, 4-\textit{n}-ethylbiphenyl, 4-\textit{n}-butylbiphenyl (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 4-\textit{n}-heptylbiphenyl (Avocado Research Chemicals Ltd., Lancaster, UK) and incubated at 30°C for 24 h. The supernatant and cell fraction were separated by centrifugation, and the cell fraction was washed with phosphate-buffered saline (PBS, pH 7.4, 1.64 g/l Na$_2$HPO$_4$ 7H$_2$O, 0.28 g/l NaH$_2$PO$_4$ 2H$_2$O, 8.0 g/l NaCl) twice. The cell fraction was disrupted by an ultrasonic disruptor (VC-505, Sonics & Materials, Newtown, CT) for metabolite extraction. The supernatant and the disrupted samples were acidified to pH 2 by the addition of hydrochloric acid. An equal volume of ethyl acetate was added to extract 2,3-DHBP and its derivatives. The extraction was done twice.

**GC-MS analysis** The collected 2,3-DHBP and its derivatives were analyzed by a gas chromatograph (MODEL 5980 series 2, Agilent Technologies, Santa Clara, CA) equipped with an Ultra-2 capillary column (50 m, 0.2 mm, 0.33 μm, Agilent Technologies) and a mass selective detector (model 5971, Agilent Technologies). Analytical conditions were as follows: sample volume, 1 μl; mode, splitless; column temperature, 60°C to 300°C (16°C/min) and following 300°C for 12 min; injector temperature, 250°C; transfer line temperature, 280°C; carrier gas, helium; and flow rate, 1 ml/min.

**Production and purification of 2,3-dihydroxy-4'-butylbiphenyl (2,3-DHBBP)** Since
2,3-DHBBP was not commercially available, we used *E. coli* cells harboring *bphAB* to synthesize 2,3-DHBBP from 4-*n*-butylbiphenyl. *E. coli* MV1184 (pSIbphAB) was cultivated at 37°C, 80 rpm for 7 h then 4-*n*-butylbiphenyl was added to a final concentration of 30 mg/l and incubated for an additional 16 h. The cells were collected by centrifugation and suspended with 20-ml water. The cells were disrupted by the ultrasonic disruptor, and the cell suspension was acidified (pH 2.0) by the addition of 50-μl concentrated hydrochloric acid. A half volume of ethyl acetate (10 ml) was added to extract 2,3-DHBBP. The extraction was done twice. The ethyl acetate layer was concentrated with a rotary evaporator (REN-1 Series, IWAKI, AGC Techno Glass Co., Ltd., Japan). The 2,3-DHBBP was further purified as follows.

**Silica gel column chromatography** The dried sample was dissolved in 1.5-ml ethyl acetate, 0.2 g of Wakogel C-100 (Wako Pure Chemical Industries Ltd.) was added to absorb the extracted sample while gently drying under a stream of nitrogen. Dried sample powder was transferred on a prepared column (1 g of Wakogel C-100 in a pasteur pipet, 7 mmφ x 146 mm) pre-equilibrated with hexane. Hydrophobic substances were eluted by hexane:ethyl acetate (2:1). The eluted samples were collected in 1-ml aliquots and analyzed with the TLC plates (Silicagel 60 F254, layer thickness = 0.25 mm, Merck & Co., Inc., Whitehouse Station, NJ) with hexane:ethyl acetate (2:1). Detection of 2,3-DHBBP was performed by UV-visualization (λ = 254 nm, Rf = 0.54) and spraying a crude extract of *E. coli* containing *bphC* from strain TK102 (24). The fractions that turned yellow based on the enzymatic reaction of BphC were collected. Crude extract from *E. coli* without *bphC* did not show any yellow color with fractions.

**C18 reversed-phase column chromatography** The collected fractions were dried gently under a stream of nitrogen and resuspended in methanol. The resulting solution was applied to
an Inertsil WP300 C18 HPLC column (7.6 mmø x 250 mm, 5 μm, GL Science Inc., Tokyo, Japan) connected to HPLC PX8020 system (TOSOH, Tokyo, Japan). The compounds were eluted using the gradient method (flow rate, 3 ml/min) from 60% to 100% methanol (0 min to 10 min) and following 100% methanol (10 min to 20 min). One major peak (retention time, 10.0 min) detected with UV detector (λ = 254 nm) was collected. The peak was identified as 2,3-DHBBP with TLC analysis as described above. The collected sample was concentrated under a stream of nitrogen and kept at -80°C. An aliquot of the sample was modified by trimethyl silylating agent (BSTFA+TMCS, 99:1, Sigma-Aldrich, St. Louis, MO) and its mass fragmentation pattern was checked with the previously purified 2,3-DHBBP by GC-MS analysis.

Microscopic observation of HOPDA-labelled cells The strain TK102 cells at stationary phase (OD_{610}=1.6) grown in 1/3LB medium were collected by centrifugation, washed with PBS twice, and resuspended in PBS (OD_{600}=0.2). Ten microliters of the cell suspension was centrifuged, and the pellet was suspended with 4 μl of various concentrations (50, 100 and 500 mg/l) of 2,3-DHPB and 2,3-DHBBP. Cells were observed with a fluorescent microscope equipped with a 100-W Hg lamp (BX-50, Olympus, Tokyo, Japan) and Olympus DP70 digital camera. Images of the labeled cells were acquired with an excitation of 460 to 490 nm with a long-pass emission filter at 510 nm.

FCM analysis and sorting Cell number was estimated by using the Bacteria Counting Kit for Flow Cytometry (Invitrogen, Carlsbad, CA) and FCM analysis (Epics Altra, Beckman Coulter Inc., Fullerton, CA). The kit provides a convenient and accurate means for assessing a bacterial population. By adding the microsphere standard at a given density and SYTO BC nucleic acid stain to the diluted bacterial suspension, we could determine the density of bacterial cells in the sample from the ratio of bacterial signals to microsphere signals in the
cytogram. The procedure for counting was based on the instruction supplied with the kit.

FCM analysis and cell sorting were carried out with an Epics Altra instrument equipped with a sapphire laser providing 20 mW at 488 nm (Coherent Inc., Santa Clara, CA). The samples were evaluated by forward scatter, side scatter, and fluorescence. The photodiode detector of forward scatter was changed to a photomultiplier tube, which provided higher sensitivity than a photodiode. Band-pass filters for green (525 nm), orange (575 nm), and red (610 nm) were installed into fluorescent detectors and were used to detect each signal from particles. The side scatter was used as a main parameter and discriminator. The samples were analyzed at a data rate 100–2500 events/sec. Data were acquired using Expo32 software (Applied Cytometry, Sheffield, UK). Particles in the objective gate region were sequentially sorted in the complete abort option.

The cells for FCM analysis were prepared as follows. PBS was filtered through 0.2-μm filter before use. The buffer was aerated through 0.2-μm air filter (50 mmφ, cellulose acetate, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for at least 10 min when used as the reaction buffer with the addition of 2,3-DHBBP. Strain TK102 was cultivated on W-solid medium with biphenyl as vapor phase for 36 h at 30°C. TK102 cell suspensions in PBS (10^7, 10^8 cells/ml) were prepared after counting the cell number by the Bacteria Counting Kit. The cell suspensions were diluted 10-fold with aerated PBS and adjusted to 10^6 and 10^7 cells/ml and allowed to stand at room temperature for 20 min. One or two microliters of 2,3-DHBBP ethanol solution was added to 400 μl of cell suspension to prepare 1, 5, 10, and 50 mg/l of 2,3-DHBBP reaction mixture and incubated at 30°C for 5 min. The mixtures were diluted with PBS to show the event rates at 100–250 events/sec.

In the case of analysis and cell sorting for HOPDA green fluorescence, the band-pass filter (525 nm) set in the green fluorescence detector (FL1) was removed. Since the maximum
emission wavelength of HOPDA was shorter than 525 nm, the filter interfered with the passage of HOPDA fluorescence. Green HOPDA fluorescence was picked up within the wavelength range of 488–550 nm (550 nm is the wavelength of the dichroic long-pass filter assembled in front of the band-pass filter). Sterile polypropylene FCM tubes containing 500-μl sterile PBS were used to receive the sorted cells and all of the sorted samples were spread on an R2A agar plate to cultivate the cells at 28°C. After 2 days, the colonies of sorted samples were isolated with R2A agar plates.

**Sorting test with artificial bacterial mixture** We examined the efficacy of our method using a bacterial mixture containing 10% of strain TK102 and 90% of indigenous bacteria derived from an underground water sample. The underground water sample was collected from a water pump in the experimental field at Research Institute for Bioresources, Okayama University (Aug. 14, 2009). The sample was filtered through a 35-μm mesh (NYTAL, SEFAR, Thal, Switzerland). The cell number was 1.1 x 10^4 cells/ml which was measured with the kit described above. Bacterial cells were harvested on 0.2-μm polycarbonate membranes (47 mmΦ, Toyo Roshi Kaisha, Ltd.) with vacuum. Trapped cells were suspended in PBS at the cell number 10^7 cells/ml. A mixture containing 10^6 cells/ml of strain TK102 and 0.9 x 10^7 cells/ml of underground water sample was subjected to FCM analyses and sorting. Diluted samples after reaction gave the event rates at 1800–2500 events/sec because of solid particles in underground water.

**Identification of bacteria by mass spectrometry** The identity of the colonies was evaluated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS, Ultraflex, Bruker Daltonics Inc., Billerica, MA). Mass-spectrum of whole cellular proteins detected by MALDI-TOF/MS can be used for accurate classification and identification of bacteria even at species level (30). Strain TK102 was used as a control for
RESULTS

Characterization of HOPDA fluorescence  The fluorescence of HOPDA was characterized to set up the analytical conditions for FCM. An excitation-emission matrix of HOPDA is shown in Fig. 2. The fluorescence increased with increased pH in the range pH 7.0–8.5, and the maximum excitation wavelength shifted from 463 to 471 nm. The maximum emission wavelength was 514 nm and this value was not influenced by pH change. It was estimated that blue light or a laser would be able to excite the HOPDA to emit green fluorescence.

Modification of the substrate for efficient labeling  We then examined whether or not the cells could be labeled with HOPDA using 2,3-DHBP as a substrate. A suspension of TK102 incubated with 2,3-DHBP turned yellow, suggesting the accumulation of HOPDA. However, the fluorescence was observed from the background rather than from the cells by microscopic observation, indicating that the fluorescent intermediate metabolite was released from the cells and diffused into the medium. To prevent the release of the intermediate metabolite, alkylated derivatives of biphenyl were added to the TK102 *bphC* mutant and the distribution of 2,3-dihydroxyalkylbiphenyl in the supernatant and cells was then measured by GC-MS (Table 1). By introducing butyl- and heptyl- tails, the cellular accumulation of 2,3-dihydroxyalkylbiphenyl in the *bphC* mutant was significantly increased. At the same time, the wild-type TK102 cells emitted bright green fluorescence. Since the transformation efficiency of 4-*n*-heptylbiphenyl was low compared to that of 4-*n*-butylbiphenyl (data not shown), 2,3-dihydroxy-4’-butylbiphenyl (2,3-DHBBP) was used for labeling in the following experiments. Fluorescence characteristic of HOPDA-derivative from 2,3-DHBBP showed
almost the same pattern of that from HOPDA derived from 2,3-DHBP. To check dose
dependence, various amounts of 2,3-DHBP and 2,3-DHBBP were added (Fig. 3). The former,
again failed to label the cells. With 500 mg/l of 2,3-DHBBP, the cells were strongly labeled
by fluorescence, which persisted for 15 min (data not shown). The fluorescence decayed
gradually in 15 minutes after reaction suggesting further degradation of meta-cleavage
metabolite of 2,3-DHBBP.

Optimization of flow cytometry condition  Fig. 4 shows a dot plot of non-labeled
TK102 cells obtained with flow cytometry. Gates A and B were defined as cells and labeled
cells, respectively. The A-gated cells (events) were equivalent to 93.2% of the total events,
which suggested that the residual 6.8% would be non-bacterial particles that remained after
the preparation, although the buffer was filtered in advance. The gate B was set in the
relatively high intensity region to avoid sorting non-fluorescent cells. Only 0.2% events were
detected in gate B. The A-gated events were sorted and the collected sample was spread on
R2A agar. The sorting efficiency of the sample was 46.7 ± 2.5% (Colony number / sorted
events x 100). This considerable loss of cells should be taken into consideration as described
below.

Optimization of substrate concentration  Flow cytometric analyses by the addition of
1, 5, 10 and 50 mg/l of 2,3-DHBBP are shown in Fig. 5. The shift of the intermediate value of
the histogram peak to a higher fluorescent intensity according to the increase of substrate
concentration can be noted in this figure. The addition of 1 mg/l 2,3-DHBBP gave only 0.4%
events in gate B, which was not practical for sorting experiment although sorting efficiency
was the best among the tested conditions (Table 2). When 50 mg/l 2,3-DHBBP was used, the
sorted cells were not able to form colonies. When 5 and 10 mg/l 2,3-DHBBP were added (10^7
cells/ml condition), 6.2% and 5.8% of events were detected in gate B and sorting efficiencies
were 14.9% and 15.6%, respectively. Similar results were obtained in 10^6 cell/ml condition. Since the sorted cells from the region A in Fig. 4 showed sorting efficiency of 46.7% without the addition of 2,3-DHBBP, 31.9 (14.9/0.467) to 43.0% (20.1/0.467) of sorted cells maintained their colony forming abilities after the reaction with 2,3-DHBBP at various experimental concentrations. As 5 and 10 mg/ml 2,3-DHBBP gave similar results based on percentages of gate B and sorting efficiency, lower concentration (5 mg/l) was used for the next experiment.

**Isolation of fluorescence-labeled cells by cell sorting**

TK102 cells (10^6 cells/ml) were mixed with bacteria derived from underground water (0.9 x 10^7 cells/ml). Five mg/l of 2,3-DHBBP was added to the cell suspension and measured by FCM. The region A in Fig. 6-A originated from bacterial cells which was observed in the control experiment (Fig. 4). The dot plots in the region A were picked up and their green fluorescence was analyzed as shown in Fig. 6-B. The plots in gated region B were sorted in complete abort mode. The experiment was repeated 5 times of which, one was with sorted events 800, and the other four with sorted events 700. As a result, 235 colonies were observed on R2A plates from a total of 3600 sorted events. All bacterial colonies were isolated and subjected to TK102 identification by MALDI-TOF/MS. One hundred ninety six colonies were identified as TK102 and the other 39 colonies were classified into 9 strains. TK102 strains, with colony forming abilities, constitute 5.44% (196 / 3600 x 100) of the dot plots. Since 17.5% of the cells maintained their colony forming abilities after the reaction and sorting at 10^6 cells/ml (Table 2), 31.1% of total sorted events were TK102.

**Calculation of actual enrichment coefficient**

Contamination of soil particle during cell-sorting was measured as follows. After 10,000 events in the gated region B were sorted from the underground water sample, the sorted sample was stained with 1x SYTO BC of
Bacteria Counting Kit. FCM analysis of the sorted sample showed that 77.2% of sorted event equivalents was soil particles. Since 0.29% of the total plots was detected in gate B, 0.22% of sorted events was contaminated soil particle (data not shown).

After the reaction with 2,3-DHBBP, 0.88% of the total plots appeared in gate B region (Fig. 6), suggesting that 25.0% (0.22/0.88) of sorted event equivalents were soil particles (75.0% were cells). In addition, 5.44% of total sorted events was identified as strain TK102. Taking this amount into consideration, 7.25% (5.44/0.750) of sorted bacterial cells formed colonies. Since 17.5% of sorted events formed colony after reacting with 2,3-DHBBP in 10^6 cells/ml (Table 2), 41.4% (7.25/0.175) of total sorting event equivalents were assumed strain TK102. These numbers indicate a 4.1-fold enrichment of TK102 cells (from 10% to 41%).

DISCUSSION

HOPDA (Fig. 1), an intermediate metabolite of 2,3-DHBP formed by BphC, is known as a “yellow ring-cleavage compound” (17, 21, 22) with an absorption maximum of 434 nm (21, 28, 29). It is used as a marker for screening cells that possess a bphC gene. The fluorescent properties of HOPDA were measured by a fluorescence spectrophotometer and it revealed that HOPDA was excited by blue light and emitted green fluorescence at intensities that increased in higher pH buffers (pH 8.0 and 8.5, Fig. 2). This suggests that the electron charge of HOPDA might become poor in lower pH conditions as a result of electron release from the two-hydroxyl groups, as with other fluorescent probes (31). This paper presents the concept of the use of fluorescent properties of intermediate metabolite of biphenyls for labeling bacterial cells possessing BphC activity and that these could be specifically isolated with sensitive and high-throughput FCM sorting.

The fluorescence of intermediate metabolite from 2,3-DHBP was observed more from the
background than from the cells (Fig. 3). This may be due to the release of the metabolite by
the cells by an unknown mechanism. When we introduced alkyl tail to the substrate, labeling
the cells with 2,3-DHBBP was successful (Fig. 3). The accumulation of metabolite increased
with the longer tail, suggesting that the hydrophobic property of the introduced alkyl chain
played a role in the metabolite’s persistence in the cell membrane (Table 1). To establish the
labeling method for FCM analysis and cell sorting, we had to optimize the substrate
concentration while considering the toxic effect of the intermediate metabolites (28, 33, 34).
Thus, the minimum substrate concentration of 5 mg/l was used for specific isolation of live
cells.

The cell suspension contained many non-bacterial particles and so it was essential to
clearly distinguish labeled cells from particles and non-labeled cells. Thus, gate A was set to
eliminate non-bacterial cells and gate B was set to detect labeled cells (Fig. 4). As setting
parameters influenced overall efficiency and specificity of detection and isolation, these were
carefully defined. Gate B was defined in the relatively high intensity region which
emphasized more on specificity than on efficiency. The gated events in gate A were sorted
without the addition of the substrate and the collected sample was spread on R2A plates. The
sorting efficiency was 46.7% and this value suggested that cells lost their colony forming
abilities when they were subjected to the entire process of sorting and plating (32).

In order to prove the applicability of our method, strain TK102 cells were sorted from an
artificial mixture with indigenous bacteria of underground water by FCM sorting. Because
such environmental water samples contained many particles including soil, contamination of
soil particle during sorting was evaluated. Since the total sorted events contained
non-bacterial particles, actual enrichment coefficient was 4.1 fold in this method. These
results confirmed the effectiveness of the method for recovering BphC-positive cells in a
mixture with other bacteria even if it contained non-bacterial particles.

In this study, we developed a new method for sorting bacterial cells with specific functions. This method does not require staining of cells with fluorescent dyes but rather utilizes the unique fluorescent intermediate metabolite during degradation of biphenyls. Although the experimental parameters (cell and substrate concentrations, reaction time, and gate definition) and settings of the sorting apparatus (FCM and cell sorter) need to be carefully defined, this method serves as a basic technology for the isolation of live bacteria with specific functions from environmental samples.

One of the difficulties of this method is the fast degradation of the fluorescent compound. Accumulation of the fluorescent metabolite is dependent on the relative rate of its formation and degradation. In this study, the fluorescent compound was rapidly degraded (in 10 min) in the cells. The proportion of bacterial cells to soil-derived particles in the groundwater sample was 1 to 10. In such condition, the sorting experiment took longer than 10 min.

Combining this method with the use of a more advanced FCM apparatus (35), gel-microdroplet technique (13, 14, 36) and million-well growth chip (37), the sorted bacterial cells can be further purified and analyzed in a high-throughput manner. Although the concept of this method depends on the fluorescence of the intermediate metabolite of biphenyl, a similar approach using appropriately designed substrates can be applied for other chemicals and such approach will extend the range of our knowledge on microorganisms with unique functions but are yet to be isolated.

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REFERENCES


19. Furukawa, K. and Arimura, N.: Purification and properties of 2,3-dihydroxybiphenyl dioxygenase from polychlorinated biphenyl-degrading


Vieira, J. and Messing, J.: Production of single-stranded plasmid DNA, *Methods in*


Figure legends

Fig. 1. Upper pathway of the aerobic microbial degradation of biphenyl in *C. testosteroni* TK102. Enzymes: BphA, biphenyl 2,3-dioxygenase; BphB, *cis*-biphenyl-2,3-dihydrodiol 2,3-dehydrogenase; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; and BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase

Fig. 2. Three-dimensional measurement of HOPDA fluorescence. The synthesized HOPDA preparation was diluted (10-fold) with phosphate buffer of different pH. The pH, maximum excitation wavelength (Ex), maximum emission wavelength (Em), and maximum fluorescence (Max) of each sample were as follows. A, pH 7.0, Ex 463 ± 6 nm, Em 515 ± 1 nm, Max 16 ± 2; B, pH 7.5, Ex 465 ± 6 nm, Em 514 ± 2 nm, Max 19 ± 3; C, pH 8.0, Ex 467 ± 4 nm, Em 514 ± 2 nm, Max 26 ± 1; D, pH 8.5, Ex 471 ± 3 nm, Em 513 ± 3 nm, Max 30 ± 2.

Fig. 3. Fluorescence microscopic observation of reaction of strain TK102 with 2,3-DHBP and 2,3-DHBBP of varied concentrations. All images were obtained 3 min after the reaction. Bars, 10 μm.

Fig. 4. Flow cytometric analysis of unreacted TK102 cells. A, side versus forward scatter of TK102 cell suspension. Region A defined bacterial cells according to size throughout this experiment. B, histogram analysis. Boxed events in region A were picked up and analyzed as B.

Fig. 5. Flow cytometric analysis of TK102 (10^7 cells/ml) with addition of 2,3-DHBBP. A, C,
E and G (left column), Histogram analysis of TK102 added with 2,3-DHBBP at 1, 5, 10 and 50 mg/l concentrations, respectively. B, D, F and H (right column), Green fluorescence versus forward scatter with the same concentrations as the left column. Control samples (TK102 without 2,3-DHBBP) were analyzed to determine the level of background fluorescence occurring within gate B and the mean number of gated ratio was 0.2 ± 0.1% during analysis of 5000 total events.

Fig. 6. Flow cytometric analysis of TK102 suspended in the underground water added with 2,3-DHBBP. A, side versus forward scatter of TK102 cell suspension. B, green fluorescence versus forward scatter of TK102 cell suspension. Dot plot B contains 5000 events.
TABLE 1. Distribution of 2,3-dihydroxybiphenyl and its derivatives extracted from the culture supernatant and the cells of *C. testosteroni* TK102a.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>2,3-dihydroxy-biphenyl</th>
<th>2,3-dihydroxy-ethylbiphenyl</th>
<th>2,3-dihydroxy-butylbiphenyl</th>
<th>2,3-dihydroxy-heptylbiphenyl</th>
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</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>0.2 ± 0.1</td>
<td>6.3 ± 0.4</td>
<td>46.1 ± 3.4</td>
<td>54.5 ± 2.2</td>
</tr>
<tr>
<td>Supernatant</td>
<td>99.8 ± 0.1</td>
<td>97.3 ± 0.4</td>
<td>53.9 ± 3.4</td>
<td>45.5 ± 2.2</td>
</tr>
</tbody>
</table>

*a* The *bphC* mutant was cultivated in 1/3LB medium supplemented with 30 mg/l of a biphenyl or alkyl biphenyls. The resting-cell reaction products, 2,3-dihydroxybiphenyl and its derivatives, were extracted from the supernatant and the cells. The extracts were analyzed by GC-MS. Data are presented as average value ± standard deviation, (n=3).
Table 2. Sorting efficiency of green fluorescent cells.

<table>
<thead>
<tr>
<th>2,3-DHBBP (mg/l)</th>
<th>10^7 cells/ml</th>
<th>Gate B (%)</th>
<th>Sorting efficiency (%)</th>
<th>10^6 cells/ml</th>
<th>Gate B (%)</th>
<th>Sorting efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4 ± 0.1</td>
<td>32.4 ± 7.2</td>
<td>0.5 ± 0.1</td>
<td>100</td>
<td>0.5 ± 0.1</td>
<td>20.0 ± 7.0</td>
</tr>
<tr>
<td>5</td>
<td>6.2 ± 2.6</td>
<td>14.9 ± 10.4</td>
<td>8.7 ± 3.4</td>
<td>17.5 ± 1.7</td>
<td>8.7 ± 3.4</td>
<td>17.5 ± 1.7</td>
</tr>
<tr>
<td>10</td>
<td>5.8 ± 0.2</td>
<td>15.6 ± 0.7</td>
<td>7.2 ± 0.6</td>
<td>20.1 ± 7.2</td>
<td>7.2 ± 0.6</td>
<td>20.1 ± 7.2</td>
</tr>
<tr>
<td>50</td>
<td>9.8 ± 2.5</td>
<td>0</td>
<td>1.0 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>1.0 ± 0.5</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as average value ± standard deviation, (n=3). Collected cells were spread on R2A agar and incubated for 2 days.

a Two concentrations of strain TK102 suspension were used.

b Gate B (%) means percentage of the detected events in gate B where the events in gate A was regarded as 100%.

c Sorting efficiency (%) means percentage of colony number / sorted events (1000) in gate B.