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4 Flow Cytometry-Based Method for Isolating Live Bacteria with *Meta*-Cleavage Activity on

5 Dihydroxy Compounds of Biphenyl

6 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

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69 opening the benzene ring (Fig. 1). *meta*-Cleavage is a key enzymatic step in the metabolism

70 of aromatic compounds. The *meta*-cleavage enzyme, BphC, catalyzes the addition of two

71 atoms of oxygen to 2,3-dihydroxybiphenyl (2,3-DHBP) and produces a yellow *meta*-cleavage

72 product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) (18-20). Bacteria with a

73 *meta*-cleavage enzyme form yellow colonies when grown on biphenyl (21, 22), which is a

74 typical indicator of biphenyl degradation ability. Many PCB degraders have been isolated by

75 this conventional screening method (23).

76 There must be new and unique PCB-degrading bacterial resources in nature which can be

77 utilized as genetic resources for bioremediation. The aforementioned conventional screening

78 methods do not serve for isolation of bacteria that do not form colonies. In this study, we

79 constructed and optimized the experimental condition for a new FCM-assisted method for

80 direct isolation of bacteria with *meta*-cleavage activity. Furthermore, the efficacy of the

81 method was proven using a mixture of *Comamonas testosteroni* TK102 and indigenous

82 bacteria derived from an underground water sample.

83

84 **MATERIALS AND METHODS**

85 **Bacterial strains and culture conditions** A PCB degrader, *C. testosteroni* strain TK102,

86 and its *bphC* mutant were used in this study (24, 25). Strain TK102 is a Gram-negative

87 bacterium isolated from soil contaminated with PCBs (20). The expression of PCB

88 degradative genes of strain TK102 is constitutive (25). A *bphC* mutant of TK102 was

89 constructed by insertion of kanamycin resistance gene (24). Strain TK102 was maintained on

90 W-solid medium (9.8 g/l Na_2HPO_4, 1.7 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $MgSO_4 \cdot 7H_2O$,

91 10.75 mg/l MgO, 9.5 mg/l $FeSO_4 \cdot 7H_2O$, 2.0 mg/l $CaCO_3$, 1.44 mg/l $ZnSO_4 \cdot 7H_2O$, 1.22 mg/l

92 $MnCl_2 \cdot 4H_2O$, 0.28 mg/l $CoSO_4 \cdot 7H_2O$, 0.25 mg/l $CuSO_4 \cdot 5H_2O$, 0.06 mg/l H_3BO_3, 51.3 μl

93   concentrated HCl, and 15 g/l agar, pH 7.9) at 30°C and biphenyl (Nacalai Tesque, Inc., Japan)

94   as sole carbon source was given as vapor phase on the lid of petri dish. The *bphC* mutant of

95   strain TK102 was maintained on 1/3-diluted Luria-Bertani solid medium (1/3LB, 3.3 g/l

96   tryptone, 1.7 g/l yeast extract, 5.0 g/l NaCl, and 15 g/l agar) with 300 μg/ml of kanamycin at

97   30°C. R2A Agar (Becton, Dickinson and Company, Sparks, MD) was used for measuring

98   sorting efficiency.

99   *E. coli* MV1184 (26) was grown in 5 ml of LB medium (10.0 g/l tryptone, 5.0 g/l yeast

100   extract, 5.0 g/l NaCl) for 12 h at 37°C, and used for the experiments. *E. coli* transformants

101   were cultivated on LB medium supplemented with 100 μg/ml ampicillin at 37°C.

102   **Construction of plasmids**   Plasmid pKTF18, which contains the *bphABC* gene from

103   *Pseudomonas pseudoalcaligenes* KF707, was kindly supplied by Dr. Kensuke Furukawa,

104   Beppu University (27). A 5.7-kb *Xho*I/*Cla*I fragment from pKTF18 that contains the *bphAB*

105   region was introduced into pBluescriptII KS+ vector and designated as pSIbphAB. A 2.1-kb

106   *Sal*I/*Sac*I fragment from pKTF18 that contains the *bphC* region was introduced into

107   pBluescriptsII KS+ vector and designated as pSIbphC.

108   **Fluorescence spectrum measurement of HOPDA**   *E. coli* MV1184 (pSIbphC) was

109   cultivated in 100-ml medium for 6 h at 37°C. The cells were collected and washed twice with

110   phosphate buffer (50 mM, pH 7.0). The collected cells were suspended in the same buffer

111   (OD_{600}=3.0). One microliter of 100 mM 2,3-DHBP solution (9.3 mg of 2,3-DHBP dissolved

112   in 0.5 ml ethanol) was added to the 1-ml cell suspension (28, 29). The mixture was incubated

113   at room temperature for 10 min, and the supernatant was collected by centrifugation. The

114   supernatant was diluted 10-fold with phosphate buffer (50 mM, pH 7.0, 7.5, 8.0, or 8.5), and

115   its fluorescence was measured by a fluorescence spectrophotometer (F-4500, HITACHI High

116   Technologies Co., Tokyo, Japan) in three-dimensional measurement mode. Analytical

117 conditions were as follows: scan speed, 30,000 nm/min; scanning wavelength range, (Ex)

118 350–550 nm (Em) 450–600; sampling, every 2.0 nm; contour line, each 4.0 (relative

119 intensity); and voltage of photomultiplier tube, 700 V.

120 **Distribution of 2,3-dihydroxybiphenyl and its alkylated derivatives** The cellular

121 persistence of 2,3-DHBP and its alkylated derivatives was analyzed with the *bphC* mutant of

122 strain TK102. One milliliter of the *bphC* mutant preculture was inoculated into 100-ml

123 medium supplemented with 30 mg/l of biphenyl or one of its biphenyl-alkylated derivatives,

124 4-*n*-ethylbiphenyl, 4-*n*-butylbiphenyl (Wako Pure Chemical Industries Ltd., Osaka, Japan),

125 and 4-*n*-heptylbiphenyl (Avocado Research Chemicals Ltd., Lancaster, UK) and incubated at

126 30°C for 24 h. The supernatant and cell fraction were separated by centrifugation, and the cell

127 fraction was washed with phosphate-buffered saline (PBS, pH 7.4, 1.64 g/l Na_2HPO4 $7H_2O$,

128 0.28 g/l NaH_2PO_4 $2H_2O$, 8.0 g/l NaCl) twice. The cell fraction was disrupted by an ultrasonic

129 disruptor (VC-505, Sonics & Materials, Newtown, CT) for metabolite extraction. The

130 supernatant and the disrupted samples were acidified to pH 2 by the addition of hydrochloric

131 acid. An equal volume of ethyl acetate was added to extract 2,3-DHBP and its derivatives.

132 The extraction was done twice.

133 **GC-MS analysis** The collected 2,3-DHBP and its derivatives were analyzed by a gas

134 chromatograph (MODEL 5980 series 2, Agilent Technologies, Santa Clara, CA) equipped

135 with an Ultra-2 capillary column (50 m, 0.2 mm, 0.33 μm, Agilent Technologies) and a mass

136 selective detector (model 5971, Agilent Technologies). Analytical conditions were as follows:

137 sample volume, 1 μl; mode, splitless; column temperature, 60°C to 300°C (16°C/min) and

138 following 300°C for 12 min; injector temperature, 250°C; transfer line temperature, 280°C;

139 carrier gas, helium; and flow rate, 1 ml/min.

140 **Production and purification of 2,3-dihydroxy-4'-butylbiphenyl (2,3-DHBBP)** Since

141 2,3-DHBBP was not commercially available, we used *E. coli* cells harboring *bphAB* to

142 synthesize 2,3-DHBBP from 4-*n*-butylbiphenyl. *E. coli* MV1184 (pSIbphAB) was cultivated

143 at 37°C, 80 rpm for 7 h then 4-*n*-butylbiphenyl was added to a final concentration of 30 mg/l

144 and incubated for an additional 16 h. The cells were collected by centrifugation and

145 suspended with 20-ml water. The cells were disrupted by the ultrasonic disruptor, and the cell

146 suspension was acidified (pH 2.0) by the addition of 50-μl concentrated hydrochloric acid. A

147 half volume of ethyl acetate (10 ml) was added to extract 2,3-DHBBP. The extraction was

148 done twice. The ethyl acetate layer was concentrated with a rotary evaporator (REN-1 Series,

149 IWAKI, AGC Techno Glass Co., Ltd., Japan). The 2,3-DHBBP was further purified as

150 follows.

151 *Silica gel column chromatography* The dried sample was dissolved in 1.5-ml ethyl

152 acetate, 0.2 g of Wakogel C-100 (Wako Pure Chemical Industries Ltd.) was added to absorb

153 the extracted sample while gently drying under a stream of nitrogen. Dried sample powder

154 was transferred on a prepared column (1 g of Wakogel C-100 in a pasteur pipet, 7 mmϕ x 146

155 mm) pre-equilibrated with hexane. Hydrophobic substances were eluted by hexane:ethyl

156 acetate (2:1). The eluted samples were collected in 1-ml aliquots and analyzed with the TLC

157 plates (Silicagel 60 F254, layer thickness = 0.25 mm, Merck & Co., Inc., Whitehouse Station,

158 NJ) with hexane:ethyl acetate (2:1). Detection of 2,3-DHBBP was performed by

159 UV-visualization (λ = 254 nm, R_f = 0.54) and spraying a crude extract of *E. coli* containing

160 *bphC* from strain TK102 (24). The fractions that turned yellow based on the enzymatic

161 reaction of BphC were collected. Crude extract from *E. coli* without *bphC* did not show any

162 yellow color with fractions.

163 *C_{18} reversed-phase column chromatography* The collected fractions were dried gently

164 under a stream of nitrogen and resuspended in methanol. The resulting solution was applied to

165   an Inertsil WP300 C18 HPLC column (7.6 mmϕ x 250 mm, 5 μm, GL Science Inc., Tokyo,

166   Japan) connected to HPLC PX8020 system (TOSOH, Tokyo, Japan). The compounds were

167   eluted using the gradient method (flow rate, 3 ml/min) from 60% to 100% methanol (0 min to

168   10 min) and following 100% methanol (10 min to 20 min). One major peak (retention time,

169   10.0 min) detected with UV detector ($\lambda = 254$ nm) was collected. The peak was identified as

170   2,3-DHBBP with TLC analysis as described above. The collected sample was concentrated

171   under a stream of nitrogen and kept at -80°C. An aliquot of the sample was modified by

172   trimethyl silylating agent (BSTFA+TMCS, 99:1, Sigma-Aldrich, St. Louis, MO) and its mass

173   fragmentation pattern was checked with the previously purified 2,3-DHBBP by GC-MS

174   analysis.

175   **Microscopic observation of HOPDA-labelled cells**   The strain TK102 cells at

176   stationary phase (OD_{610}=1.6) grown in 1/3LB medium were collected by centrifugation,

177   washed with PBS twice, and resuspended in PBS (OD_{600}=0.2). Ten microliters of the cell

178   suspension was centrifuged, and the pellet was suspended with 4 μl of various concentrations

179   (50, 100 and 500 mg/l) of 2,3-DHBP and 2,3-DHBBP. Cells were observed with a fluorescent

180   microscope equipped with a 100-W Hg lamp (BX-50, Olympus, Tokyo, Japan) and Olympus

181   DP70 digital camera. Images of the labeled cells were acquired with an excitation of 460 to

182   490 nm with a long-pass emission filter at 510 nm.

183   **FCM analysis and sorting**   Cell number was estimated by using the Bacteria Counting

184   Kit for Flow Cytometry (Invitrogen, Carlsbad, CA) and FCM analysis (Epics Altra, Beckman

185   Coulter Inc., Fullerton, CA). The kit provides a convenient and accurate means for assessing a

186   bacterial population. By adding the microsphere standard at a given density and SYTO BC

187   nucleic acid stain to the diluted bacterial suspension, we could determine the density of

188   bacterial cells in the sample from the ratio of bacterial signals to microsphere signals in the

9

189 cytogram. The procedure for counting was based on the instruction supplied with the kit.

190 FCM analysis and cell sorting were carried out with an Epics Altra instrument equipped with

191 a sapphire laser providing 20 mW at 488 nm (Coherent Inc., Santa Clara, CA). The samples

192 were evaluated by forward scatter, side scatter, and fluorescence. The photodiode detector of

193 forward scatter was changed to a photomultiplier tube, which provided higher sensitivity than

194 a photodiode. Band-pass filters for green (525 nm), orange (575 nm), and red (610 nm) were

195 installed into fluorescent detectors and were used to detect each signal from particles. The

196 side scatter was used as a main parameter and discriminator. The samples were analyzed at a

197 data rate 100–2500 events/sec. Data were acquired using Expo32 software (Applied

198 Cytometry, Sheffield, UK). Particles in the objective gate region were sequentially sorted in

199 the complete abort option.

200   The cells for FCM analysis were prepared as follows. PBS was filtered through 0.2-μm

201 filter before use. The buffer was aerated through 0.2-μm air filter (50 mmϕ, cellulose acetate,

202 Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for at least 10 min when used as the reaction buffer

203 with the addition of 2,3-DHBBP. Strain TK102 was cultivated on W-solid medium with

204 biphenyl as vapor phase for 36 h at 30°C. TK102 cell suspensions in PBS (10^7, 10^8 cells/ml)

205 were prepared after counting the cell number by the Bacteria Counting Kit. The cell

206 suspensions were diluted 10-fold with aerated PBS and adjusted to 10^6 and 10^7 cells/ml and

207 allowed to stand at room temperature for 20 min. One or two microliters of 2,3-DHBBP

208 ethanol solution was added to 400 μl of cell suspension to prepare 1, 5, 10, and 50 mg/l of

209 2,3-DHBBP reaction mixture and incubated at 30°C for 5 min. The mixtures were diluted

210 with PBS to show the event rates at 100–250 events/sec.

211   In the case of analysis and cell sorting for HOPDA green fluorescence, the band-pass filter

212 (525 nm) set in the green fluorescence detector (FL1) was removed. Since the maximum

213 emission wavelength of HOPDA was shorter than 525 nm, the filter interfered with the

214 passage of HOPDA fluorescence. Green HOPDA fluorescence was picked up within the

215 wavelength range of 488–550 nm (550 nm is the wavelength of the dichroic long-pass filter

216 assembled in front of the band-pass filter). Sterile polypropylene FCM tubes containing

217 500-μl sterile PBS were used to receive the sorted cells and all of the sorted samples were

218 spread on an R2A agar plate to cultivate the cells at 28°C. After 2 days, the colonies of sorted

219 samples were isolated with R2A agar plates.

220 **Sorting test with artificial bacterial mixture**   We examined the efficacy of our method

221 using a bacterial mixture containing 10% of strain TK102 and 90% of indigenous bacteria

222 derived from an underground water sample. The underground water sample was collected

223 from a water pump in the experimental field at Research Institute for Bioresources, Okayama

224 University (Aug. 14, 2009). The sample was filtered through a 35-μm mesh (NYTAL, SEFAR,

225 Thal, Switzerland). The cell number was 1.1 x 10^4 cells/ml which was measured with the kit

226 described above. Bacterial cells were harvested on 0.2-μm polycarbonate membranes (47

227 mmφ, Toyo Roshi Kaisha, Ltd.) with vacuum. Trapped cells were suspended in PBS at the cell

228 number 10^7 cells/ml. A mixture containing 10^6 cells/ml of strain TK102 and 0.9 x 10^7 cells/ml

229 of underground water sample was subjected to FCM analyses and sorting. Diluted samples

230 after reaction gave the event rates at 1800–2500 events/sec because of solid particles in

231 underground water.

232 **Identification of bacteria by mass spectrometry**   The identity of the colonies was

233 evaluated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

234 (MALDI-TOF/MS, Ultraflex, Bruker Daltonics Inc., Billerica, MA). Mass-spectrum of whole

235 cellular proteins detected by MALDI-TOF/MS can be used for accurate classification and

236 identification of bacteria even at species level (30). Strain TK102 was used as a control for

237   spectra comparison.

238

239   **RESULTS**

240   **Characterization of HOPDA fluorescence**   The fluorescence of HOPDA was

241   characterized to set up the analytical conditions for FCM. An excitation-emission matrix of

242   HOPDA is shown in Fig. 2. The fluorescence increased with increased pH in the range pH

243   7.0–8.5, and the maximum excitation wavelength shifted from 463 to 471 nm. The maximum

244   emission wavelength was 514 nm and this value was not influenced by pH change. It was

245   estimated that blue light or a laser would be able to excite the HOPDA to emit green

246   fluorescence.

247   **Modification of the substrate for efficient labeling**   We then examined whether or not

248   the cells could be labeled with HOPDA using 2,3-DHBP as a substrate. A suspension of

249   TK102 incubated with 2,3-DHBP turned yellow, suggesting the accumulation of HOPDA.

250   However, the fluorescence was observed from the background rather than from the cells by

251   microscopic observation, indicating that the fluorescent intermediate metabolite was released

252   from the cells and diffused into the medium. To prevent the release of the intermediate

253   metabolite, alkylated derivatives of biphenyl were added to the TK102 *bphC* mutant and the

254   distribution of 2,3-dihydroxyalkylbiphenyl in the supernatant and cells was then measured by

255   GC-MS (Table 1). By introducing butyl- and heptyl- tails, the cellular accumulation of

256   2,3-dihydroxyalkylbiphenyl in the *bphC* mutant was significantly increased. At the same time,

257   the wild-type TK102 cells emitted bright green fluorescence. Since the transformation

258   efficiency of 4-*n*-heptylbiphenyl was low compared to that of 4-*n*-butylbiphenyl (data not

259   shown), 2,3-dihydroxy-4'-butylbiphenyl (2,3-DHBBP) was used for labeling in the following

260   experiments. Fluorescence characteristic of HOPDA-derivative from 2,3-DHBBP showed

12

261 almost the same pattern of that from HOPDA derived from 2,3-DHBP. To check dose

262 dependence, various amounts of 2,3-DHBP and 2,3-DHBBP were added (Fig. 3). The former,

263 again failed to label the cells. With 500 mg/l of 2,3-DHBBP, the cells were strongly labeled

264 by fluorescence, which persisted for 15 min (data not shown). The fluorescence decayed

265 gradually in 15 minutes after reaction suggesting further degradation of *meta*-cleavage

266 metabolite of 2,3-DHBBP.

267 **Optimization of flow cytometry condition** Fig. 4 shows a dot plot of non-labeled

268 TK102 cells obtained with flow cytometry. Gates A and B were defined as cells and labeled

269 cells, respectively. The A-gated cells (events) were equivalent to 93.2% of the total events,

270 which suggested that the residual 6.8% would be non-bacterial particles that remained after

271 the preparation, although the buffer was filtered in advance. The gate B was set in the

272 relatively high intensity region to avoid sorting non-fluorescent cells. Only 0.2% events were

273 detected in gate B. The A-gated events were sorted and the collected sample was spread on

274 R2A agar. The sorting efficiency of the sample was $46.7 \pm 2.5\%$ (Colony number / sorted

275 events x 100). This considerable loss of cells should be taken into consideration as described

276 below.

277 **Optimization of substrate concentration** Flow cytometric analyses by the addition of

278 1, 5, 10 and 50 mg/l of 2,3-DHBBP are shown in Fig. 5. The shift of the intermediate value of

279 the histogram peak to a higher fluorescent intensity according to the increase of substrate

280 concentration can be noted in this figure. The addition of 1 mg/l 2,3-DHBBP gave only 0.4%

281 events in gate B, which was not practical for sorting experiment although sorting efficiency

282 was the best among the tested conditions (Table 2). When 50 mg/l 2,3-DHBBP was used, the

283 sorted cells were not able to form colonies. When 5 and 10 mg/l 2,3-DHBBP were added (10^7

284 cells/ml condition), 6.2% and 5.8% of events were detected in gate B and sorting efficiencies

285   were 14.9% and 15.6%, respectively. Similar results were obtained in 10^6 cell/ml condition.

286   Since the sorted cells from the region A in Fig. 4 showed sorting efficiency of 46.7% without

287   the addition of 2,3-DHBBP, 31.9 (14.9/0.467) to 43.0% (20.1/0.467) of sorted cells

288   maintained their colony forming abilities after the reaction with 2,3-DHBBP at various

289   experimental concentrations. As 5 and 10 mg/ml 2,3-DHBBP gave similar results based on

290   percentages of gate B and sorting efficiency, lower concentration (5 mg/l) was used for the

291   next experiment.

292   **Isolation of fluorescence-labeled cells by cell sorting**   TK102 cells (10^6 cells/ml) were

293   mixed with bacteria derived from underground water (0.9 x 10^7 cells/ml). Five mg/l of

294   2,3-DHBBP was added to the cell suspension and measured by FCM. The region A in Fig.

295   6-A originated from bacterial cells which was observed in the control experiment (Fig. 4). The

296   dot plots in the region A were picked up and their green fluorescence was analyzed as shown

297   in Fig. 6-B. The plots in gated region B were sorted in complete abort mode. The experiment

298   was repeated 5 times of which, one was with sorted events 800, and the other four with sorted

299   events 700. As a result, 235 colonies were observed on R2A plates from a total of 3600 sorted

300   events. All bacterial colonies were isolated and subjected to TK102 identification by

301   MALDI-TOF/MS. One hundred ninety six colonies were identified as TK102 and the other 39

302   colonies were classified into 9 strains. TK102 strains, with colony forming abilities, constitute

303   5.44% (196 / 3600 x 100) of the dot plots. Since 17.5% of the cells maintained their colony

304   forming abilities after the reaction and sorting at 10^6 cells/ml (Table 2), 31.1% of total sorted

305   events were TK102.

306   **Calculation of actual enrichment coefficient**   Contamination of soil particle during

307   cell-sorting was measured as follows. After 10,000 events in the gated region B were sorted

308   from the underground water sample, the sorted sample was stained with 1x SYTO BC of

309   Bacteria Counting Kit. FCM analysis of the sorted sample showed that 77.2% of sorted event

310   equivalents was soil particles. Since 0.29% of the total plots was detected in gate B, 0.22% of

311   sorted events was contaminated soil particle (data not shown).

312   After the reaction with 2,3-DHBBP, 0.88% of the total plots appeared in gate B region (Fig.

313   6), suggesting that 25.0% (0.22/0.88) of sorted event equivalents were soil particles (75.0%

314   were cells). In addition, 5.44% of total sorted events was identified as strain TK102. Taking

315   this amount into consideration, 7.25% (5.44/0.750) of sorted bacterial cells formed colonies.

316   Since 17.5% of sorted events formed colony after reacting with 2,3-DHBBP in 10^6 cells/ml

317   (Table 2), 41.4% (7.25/0.175) of total sorting event equivalents were assumed strain TK102.

318   These numbers indicate a 4.1-fold enrichment of TK102 cells (from 10% to 41%).

319

320   **DISCUSSION**

321   HOPDA (Fig. 1), an intermediate metabolite of 2,3-DHBP formed by BphC, is known as a

322   "yellow ring-cleavage compound" (17, 21, 22) with an absorption maximum of 434 nm (21,

323   28, 29). It is used as a marker for screening cells that possess a *bphC* gene. The fluorescent

324   properties of HOPDA were measured by a fluorescence spectrophotometer and it revealed

325   that HOPDA was excited by blue light and emitted green fluorescence at intensities that

326   increased in higher pH buffers (pH 8.0 and 8.5, Fig. 2). This suggests that the electron

327   charge of HOPDA might become poor in lower pH conditions as a result of electron release

328   from the two-hydroxyl groups, as with other fluorescent probes (31). This paper presents the

329   concept of the use of fluorescent properties of intermediate metabolite of biphenyls for

330   labeling bacterial cells possessing BphC activity and that these could be specifically isolated

331   with sensitive and high-throughput FCM sorting.

332   The fluorescence of intermediate metabolite from 2,3-DHBP was observed more from the

333   background than from the cells (Fig.3). This may be due to the release of the metabolite by

334   the cells by an unknown mechanism. When we introduced alkyl tail to the substrate, labeling

335   the cells with 2,3-DHBBP was successful (Fig. 3). The accumulation of metabolite increased

336   with the longer tail, suggesting that the hydrophobic property of the introduced alkyl chain

337   played a role in the metabolite's persistence in the cell membrane (Table 1). To establish the

338   labeling method for FCM analysis and cell sorting, we had to optimize the substrate

339   concentration while considering the toxic effect of the intermediate metabolites (28, 33, 34).

340   Thus, the minimum substrate concentration of 5 mg/l was used for specific isolation of live

341   cells.

342   The cell suspension contained many non-bacterial particles and so it was essential to

343   clearly distinguish labeled cells from particles and non-labeled cells. Thus, gate A was set to

344   eliminate non-bacterial cells and gate B was set to detect labeled cells (Fig. 4). As setting

345   parameters influenced overall efficiency and specificity of detection and isolation, these were

346   carefully defined. Gate B was defined in the relatively high intensity region which

347   emphasized more on specificity than on efficiency. The gated events in gate A were sorted

348   without the addition of the substrate and the collected sample was spread on R2A plates. The

349   sorting efficiency was 46.7% and this value suggested that cells lost their colony forming

350   abilities when they were subjected to the entire process of sorting and plating (32).

351   In order to prove the applicability of our method, strain TK102 cells were sorted from an

352   artificial mixture with indigenous bacteria of underground water by FCM sorting. Because

353   such environmental water samples contained many particles including soil, contamination of

354   soil particle during sorting was evaluated. Since the total sorted events contained

355   non-bacterial particles, actual enrichment coefficient was 4.1 fold in this method. These

356   results confirmed the effectiveness of the method for recovering BphC-positive cells in a

357 mixture with other bacteria even if it contained non-bacterial particles.

358

359 In this study, we developed a new method for sorting bacterial cells with specific functions.

360 This method does not require staining of cells with fluorescent dyes but rather utilizes the

361 unique fluorescent intermediate metabolite during degradation of biphenyls. Although the

362 experimental parameters (cell and substrate concentrations, reaction time, and gate definition)

363 and settings of the sorting apparatus (FCM and cell sorter) need to be carefully defined, this

364 method serves as a basic technology for the isolation of live bacteria with specific functions

365 from environmental samples.

366 One of the difficulties of this method is the fast degradation of the fluorescent compound.

367 Accumulation of the fluorescent metabolite is dependent on the relative rate of its formation

368 and degradation. In this study, the fluorescent compound was rapidly degraded (in 10 min) in

369 the cells. The proportion of bacterial cells to soil-derived particles in the ground water sample

370 was 1 to 10. In such condition, the sorting experiment took longer than 10 min.

371 Combining this method with the use of a more advanced FCM apparatus (35),

372 gel-microdroplet technique (13, 14, 36) and million-well growth chip (37), the sorted bacterial

373 cells can be further purified and analyzed in a high-throughput manner. Although the concept

374 of this method depends on the fluorescence of the intermediate metabolite of biphenyl, a

375 similar approach using appropriately designed substrates can be applied for other chemicals

376 and such approach will extend the range of our knowledge on microorganisms with unique

377 functions but are yet to be isolated.

378

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512

513

Figure legends

515

516   Fig. 1. Upper pathway of the aerobic microbial degradation of biphenyl in *C. testosteroni*

517   TK102. Enzymes: BphA, biphenyl 2,3-dioxygenase; BphB, *cis*-biphenyl-2,3-dihydrodiol

518   2,3-dehydrogenase; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; and BphD,

519   2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase

520

521   Fig. 2. Three-dimensional measurement of HOPDA fluorescence. The synthesized HOPDA

522   preparation was diluted (10-fold) with phosphate buffer of different pH. The pH, maximum

523   excitation wavelength (Ex), maximum emission wavelength (Em), and maximum

524   fluorescence (Max) of each sample were as follows. A, pH 7.0, Ex 463 ± 6 nm, Em 515 ± 1

525   nm, Max 16 ± 2; B, pH7.5, Ex 465 ± 6 nm, Em 514 ± 2 nm, Max 19 ± 3; C, pH8.0, Ex $467 \pm$

526   4 nm, Em 514 ± 2 nm, Max 26 ± 1; D, pH 8.5, Ex 471 ± 3 nm, Em 513 ± 3 nm, Max 30 ± 2.

527

528   Fig. 3. Fluorescence microscopic observation of reaction of strain TK102 with 2,3-DHBP and

529   2,3-DHBBP of varied concentrations. All images were obtained 3 min after the reaction. Bars,

530   10 μm.

531

532   Fig. 4. Flow cytometric analysis of unreacted TK102 cells. A, side versus forward scatter of

533   TK102 cell suspension. Region A defined bacterial cells according to size throughout this

534   experiment. B, histogram analysis. Boxed events in region A were picked up and analyzed as

535   B.

536

537   Fig. 5. Flow cytometric analysis of TK102 (10^7 cells/ml) with addition of 2,3-DHBBP. A, C,

538 E and G (left column), Histogram analysis of TK102 added with 2,3-DHBBP at 1, 5, 10 and

539 50 mg/l concentrations, respectively. B, D, F and H (right column), Green fluorescence versus

540 forward scatter with the same concentrations as the left column. Control samples (TK102

541 without 2,3-DHBBP) were analyzed to determine the level of background fluorescence

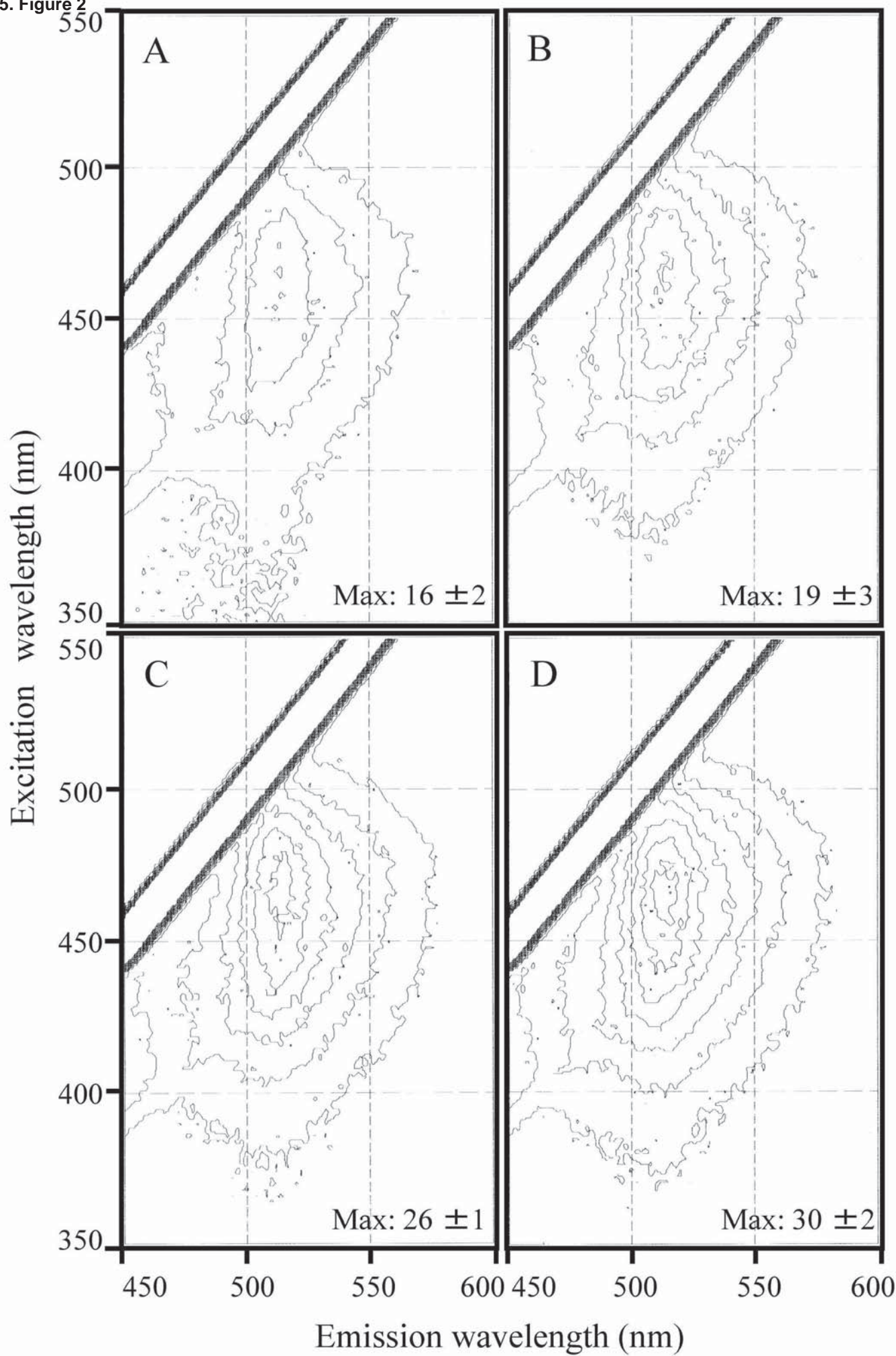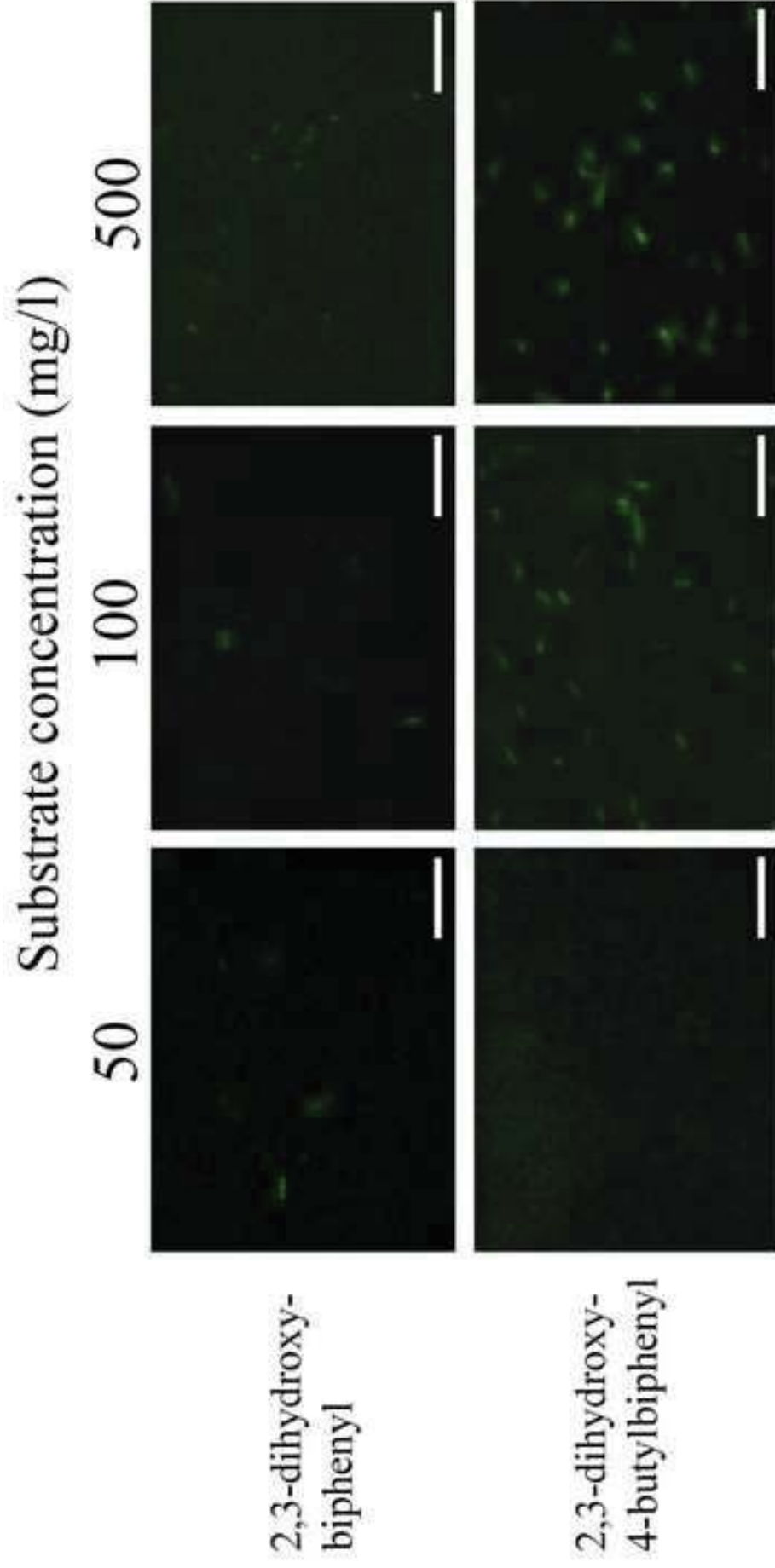542 occurring within gate B and the mean number of gated ratio was $0.2 \pm 0.1\%$ during analysis of

543 5000 total events.

544

545 Fig. 6. Flow cytometric analysis of TK102 suspended in the underground water added with

546 2,3-DHBBP. A, side versus forward scatter of TK102 cell suspension. B, green fluorescence

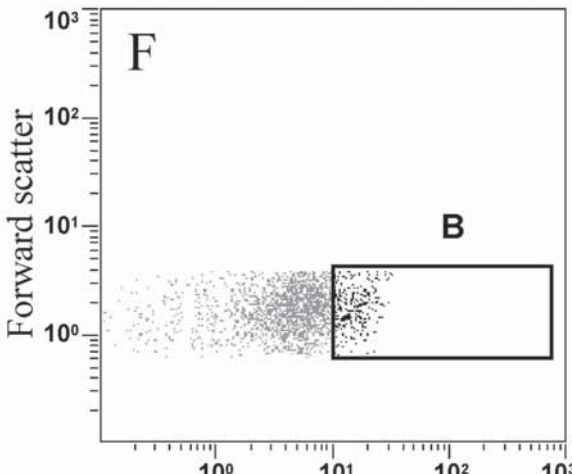547 versus forward scatter of TK102 cell suspension. Dot plot B contains 5000 events.

548

Figure 1

A — Max: 16 ± 2
B — Max: 19 ± 3
C — Max: 26 ± 1
D — Max: 30 ± 2

Excitation wavelength (nm)

Emission wavelength (nm)

Figure 3

Substrate concentration (mg/l)

500   100   50

2,3-dihydroxy-biphenyl
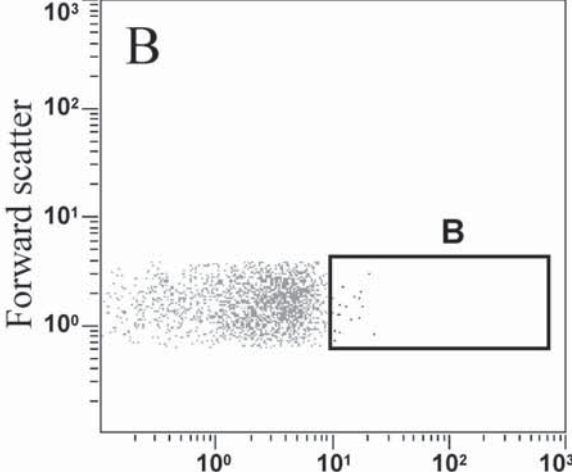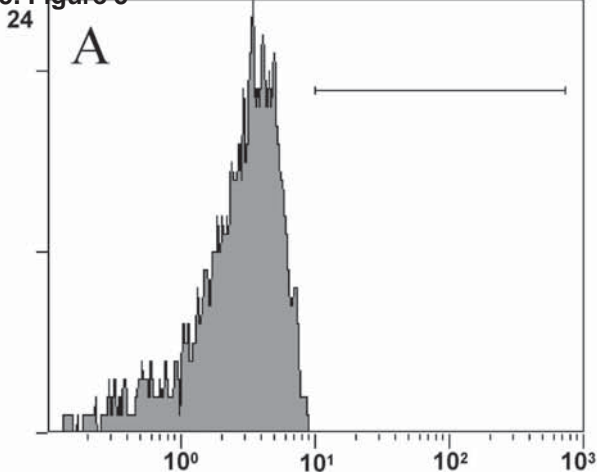
2,3-dihydroxy-4-butylbiphenyl
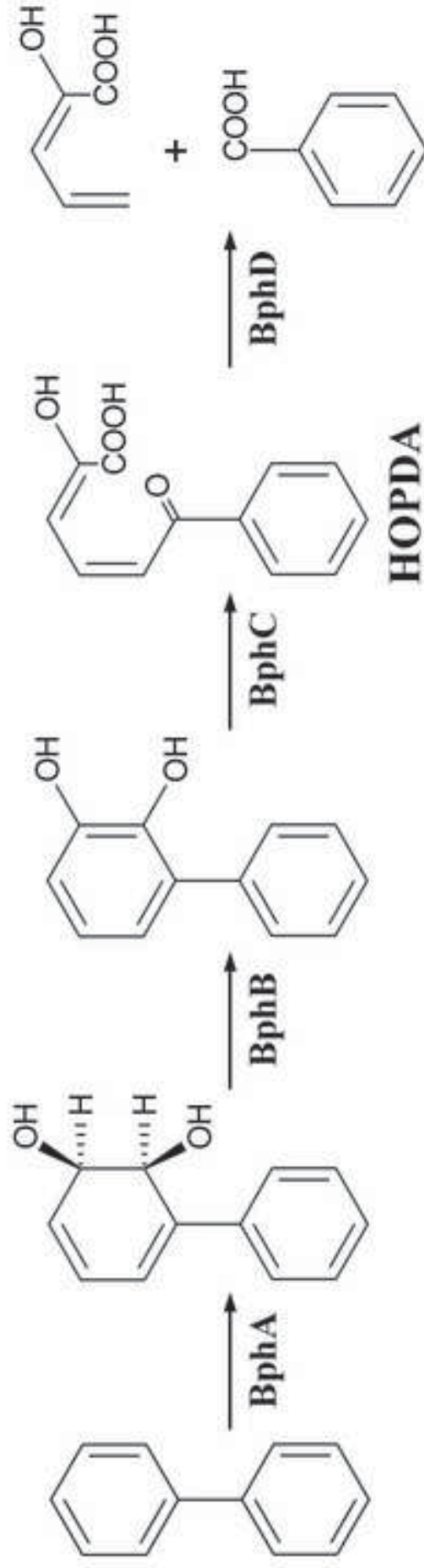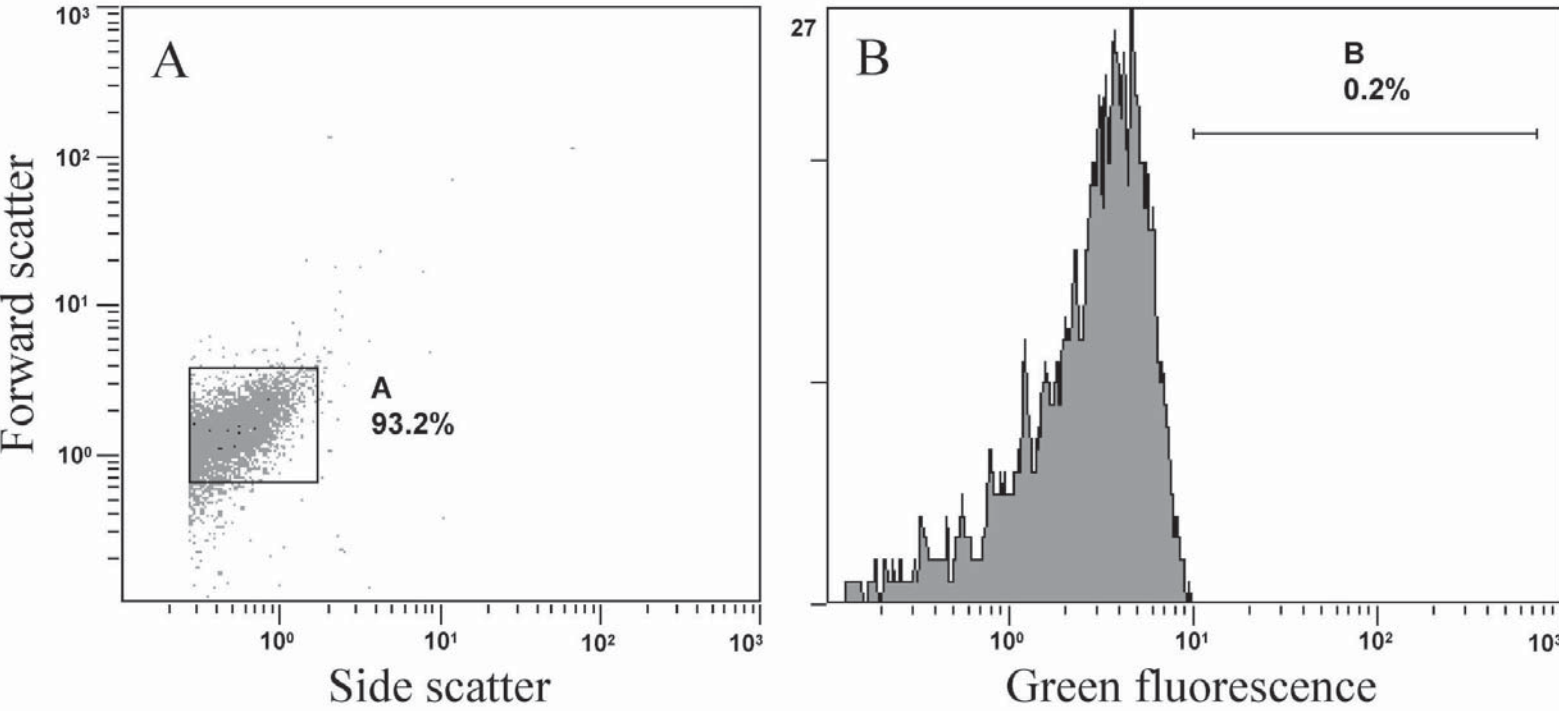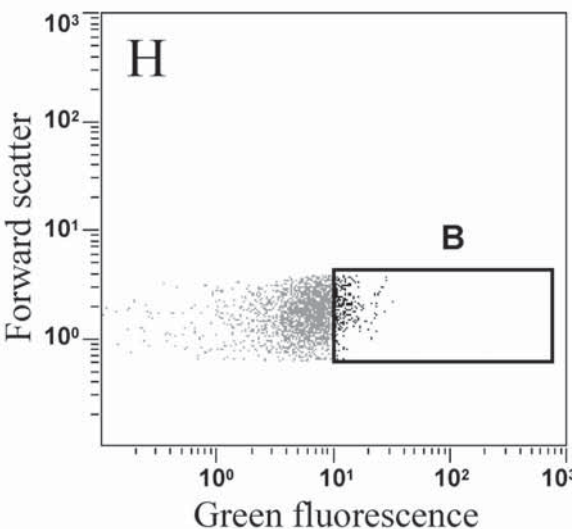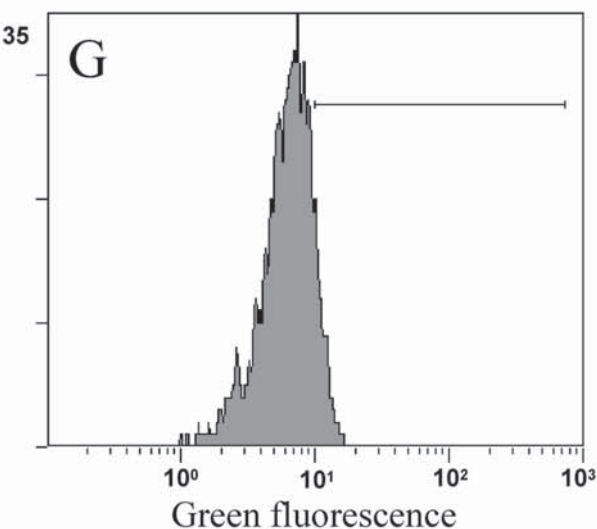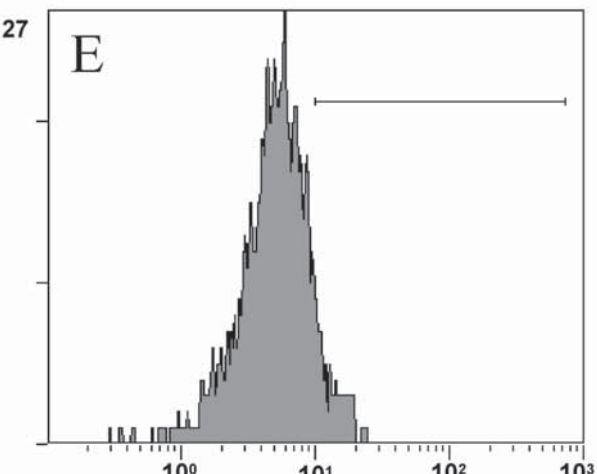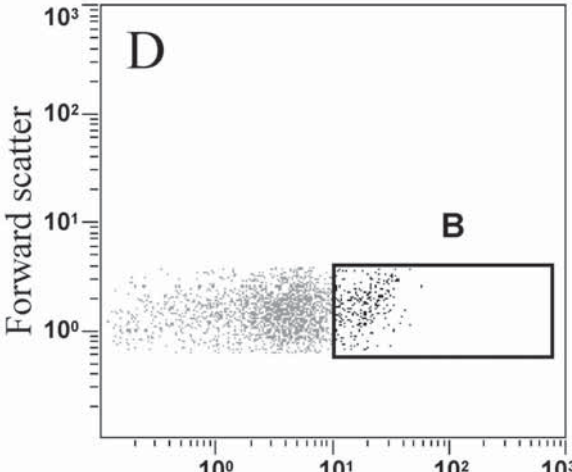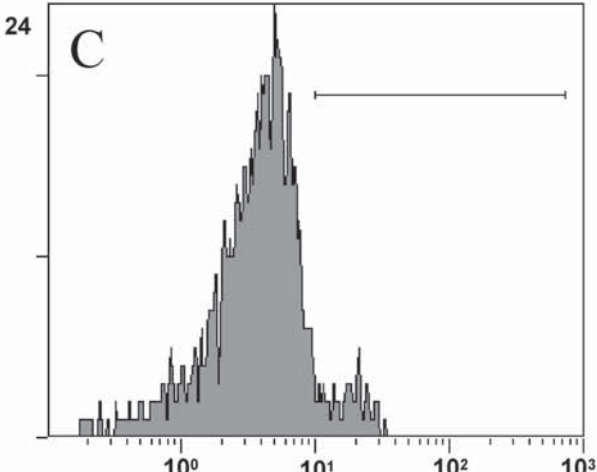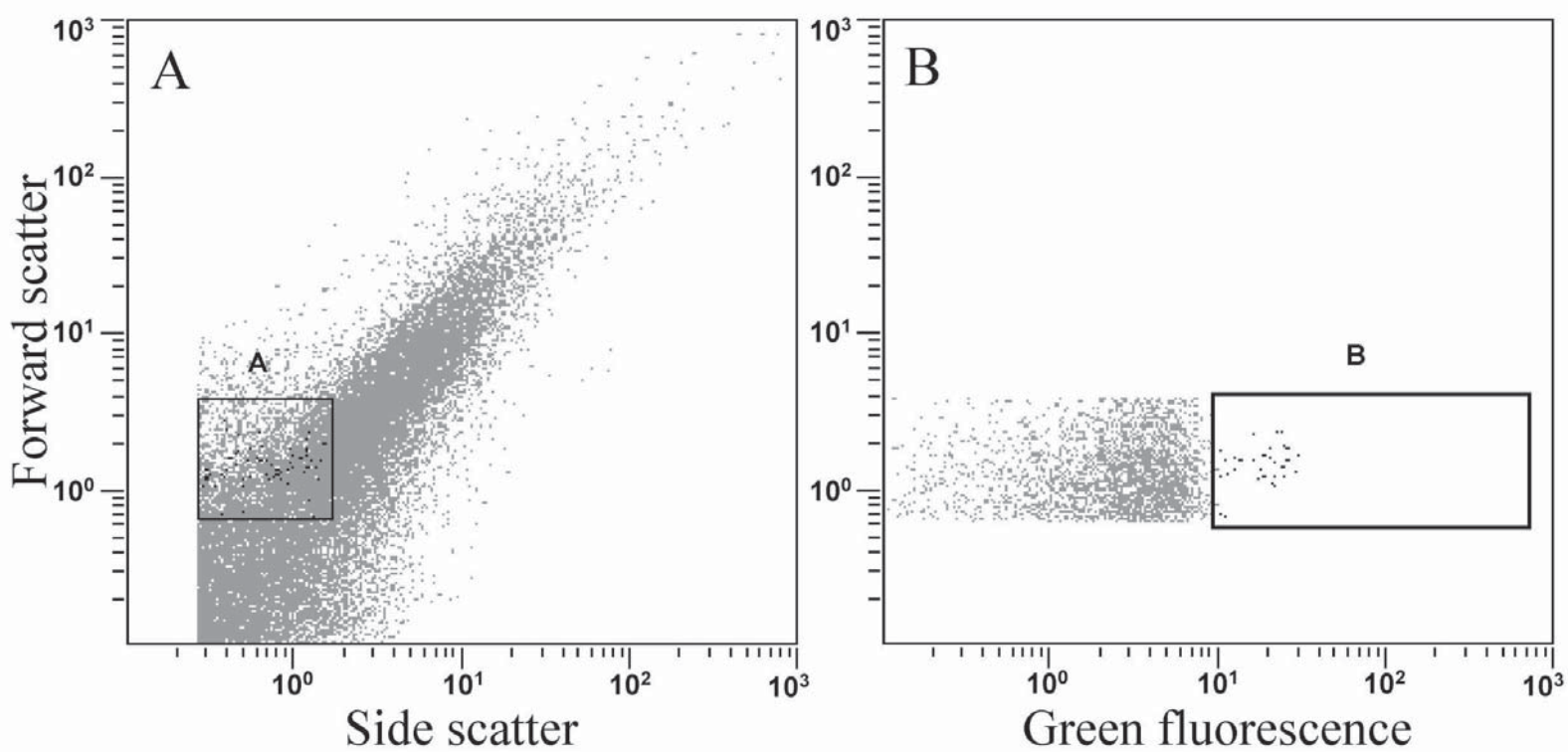
5. Figure 4

5. Figure 6

6. Table

TABLE 1. Distribution of 2,3-dihydroxybiphenyl and its derivatives extracted from the culture supernatant and the cells of *C. testosteroni* TK102[a].

Fractions	% 2,3-dihydroxybiphenyl and its derivatives			
	2,3-dihydroxy-biphenyl	2,3-dihydroxy-ethylbiphenyl	2,3-dihydroxy-butylbiphenyl	2,3-dihydroxy-heptylbiphenyl
Whole cells	0.2 ± 0.1	6.3 ± 0.4	46.1 ± 3.4	54.5 ± 2.2
Supernatant	99.8 ± 0.1	97.3 ± 0.4	53.9 ± 3.4	45.5 ± 2.2

[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 $\pm$ standard deviation, (n=3).

.

Table 2. Sorting efficiency of green fluorescent cells[a].

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

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.