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Degradation of Estrogens by *Rhodococcus zopfii* and *Rhodococcus equi* Isolates from Activated Sludge in Wastewater Treatment Plants

Takeshi Yoshimoto,[1]* Fumiko Nagai,[1] Junji Fujimoto,[1] Koichi Watanabe,[1] Harumi Mizukoshi,[1] Takashi Makino,[1] Kazumasa Kimura,[1] Hideyuki Saino,[2] Haruji Sawada,[1] and Hiroshi Omura[1]

Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo,[1] and National Institute for Land and Infrastructure Management, Tsukuba, Ibaraki,[2] Japan

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We have isolated four strains of *Rhodococcus* which specifically degrade estrogens by using enrichment culture of activated sludge from wastewater treatment plants. Strain Y 50158, identified as *Rhodococcus zopfii*, completely and rapidly degraded 100 mg of 17β-estradiol, estrone, estriol, and ethinyl estradiol/liter, as demonstrated by thin-layer chromatography and gas chromatography-mass spectrometry analyses. Strains Y 50155, Y 50156, and Y 50157, identified as *Rhodococcus equi*, showed degradation activities comparable with that of Y 50158. Using the random amplified polymorphism DNA fingerprinting test, these three strains were confirmed to have been derived from different sources. *R. zopfii* Y 50158, which showed the highest activity among these four strains, revealed that the strain selectively degraded 17β-estradiol during jar fermentation, even when glucose was used as a readily utilizable carbon source in the culture medium. Measurement of estrogenic activities with human breast cancer-derived MVLN cells showed that these four strains each degraded 100 mg of 17β-estradiol/liter to 1/100 of the specific activity level after 24 h. It is thus suggested that these strains degrade 17β-estradiol into substances without estrogenic activity.

Natural estrogens, including 17β-estradiol (E2), estrone (E1), and estriol (E3), are excreted in the urine of humans and cattle, most of which flows into wastewater treatment plants. As some natural estrogens are discharged into environments without processing, it is estimated that estrogen discharge is increasing in urban areas (14, 15, 17). Feminization of a male organism has been noted in rivers and lakes into which sewage is discharged from wastewater treatment plants, and loss of ecological balance is causing concern (7, 20, 21, 29). Since the use of low-dosage oral contraceptive pills was approved by the Central Pharmaceutical Affairs Council in Japan in 1999, environmental loading of ethinyl estradiol (EE2), a synthetic estrogen, is expected to increase (27; T. Yoshimoto, S. Murakami, K. Hamasato, H. Omura, Y. Goda, A. Kobayashi, and S. Fujimoto, Abstr. 4th Annu. Meet. Jpn. Soc. Endocr. Disrupt. Res., p. 155, 2001).

Between 1998 and 2000, the Ministry of Land, Infrastructure and Transport conducted a 3-year nationwide survey of 25 endocrine disruptors at 47 wastewater treatment plants in 13 districts of Japan (Tokyo, Sapporo, Sendai, Ibaraki, Saitama, Kawasaki, Yokohama, Nagoya, Shiga, Kyoto, Osaka, Kobe, and Fukuoka). E2, E1, and EE2 concentrations were measured in wastewater and treated wastewater flowing into wastewater treatment plants. EE2 was not detected in any sample in the treatment plants, but E1 and E2 were detected in all plants. The measurement values of E2 and E1 in wastewater influent and treated wastewater were 0.0091 to 0.094 μg/liter (125 samples), <0.0002 (not detected) to 0.066 μg/liter (146 samples), 0.015 to 0.077 μg/liter (23 samples), and <0.0005 (not detected) to 0.063 μg/liter (24 samples), respectively. The concentrations of E2 were analyzed by an enzyme-linked immunosorbent assay kit (in 1998 to 2000), and the concentrations of E1 were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (in 2000 only). Using the median of the concentration of E2 and E1 of each year, it was found that 33% (1998), 23% (1999), and 21% (2000) of E2 and 14% (2000) of E1 in wastewater influent was discharged into the environment (22). Together with the Ministry of Land, Infrastructure and Transport (National Institute for Land and Infrastructure Management), we have investigated microorganisms that degrade the four principal estrogens (E2, E1, E3, and EE2) selectively to develop an efficient method of degrading estrogens by activated sludge processing at wastewater treatment plants.

Microorganisms such as *Rhodococcus erythropolis* and *Mycobacterium fortuitum*, sensu stricto, isolated from garden compost and soil, have been found to degrade the cholesterol skeleton, which has a molecular structure resembling that of estrogens (6, 34). *Novosphingobium tardaugens* sp. nov has been isolated from activated sludge as an E2-degrading microorganism (8), and *Fusarium proliferatum* has been isolated from cowshed samples as an EE2-degrading microorganism (27). Shi et al. (27) have reported the existence of EE2-degradative products, but these substances were not yet identified. It is not clear that these degrade the pathways of estrogens. In addition, *Streptomyces* sp. strains have been reported as microorganisms that perform the microbial transformation of E2 into E1 (2, 18, 35).

Other groups have reported the estrogenic activities of principal estrogens by using human breast cancer cells (MCF-7 ERE-luciferase; MVLN cells) (4, 5, 23, 24, 28, 37). This report has shown that if the specific activity of E2 is defined as 1.0, the specific activity of E1 is 0.14, that of E3 is 0.026, and that of EE2 is 0.58 (9, 10; T. Yoshimoto, M. Nakao, K. Hamasato, H.

* Corresponding author. Mailing address: Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan. Phone: 81-42-577-8960. Fax: 81-42-577-3020. E-mail: takeshi-yoshimoto@yakult.co.jp.

Omura, K. Kannan, J. P. Giesy, A. Horiuchi, and S. Hashimoto, Abstr. 9th Symp. Environ. Chem., p. 458-459, 2000). The effects of E1, E3, and EE2 as well as E2 on wildlife, when environmental loading increases, are causing concern. Microorganisms that degrade various estrogens, i.e., E2, E1, E3, and EE2, have been studied both more efficiently and more rapidly than bacterial strains previously described, with the aim of allowing simultaneous micro-organic degradation at wastewater treatment plants (T. Yoshimoto, F. Nagai, H. Omura, J. Fujimoto, K. Watanabe, T. Makino, and H. Saino, Abstr. 5th Annu. Meet. Jpn. Soc. Endocr. Disrupt. Res., p. 432, 2002). Using enrichment culture of activated sludge from wastewater treatment plants, which mainly receive domestic effluent, we have found four strains of microorganisms that are able to degrade these four estrogens simultaneously, very efficiently and rapidly. We describe these estrogen-degrading strains.

MATERIALS AND METHODS

Chemicals. Sigma E-4876, E-4244, E-5521, and E-4877 (St. Louis, Mo.) were used as estrogens E2, E1, E3, and EE2, respectively. For isolation of bacterial strains from enrichment culture solutions, Difco 277010, 218263, and 271120 (Sparks, Md.) were used as ISP culture medium, R_2A culture medium, and YM culture medium, respectively. The ISP culture medium contained 4 g of yeast extract, 10 g of malt extract, and 4 g of dextrose per liter (pH was adjusted to 7.2); R_2A culture medium contained 0.5 g of yeast extract, 0.5 g of Proteose peptone, 0.5 g of Casamino Acids, 0.5 g of dextrose, 0.5 g of soluble starch, 0.3 g of sodium pyruvate, 0.3 g of dipotassium phosphate, and 0.05 g of magnesium sulfate per liter (pH was adjusted to 7.2); and YM culture medium contained 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 10 g of dextrose per liter (pH was adjusted to 6.2). The solid phase extraction was performed by using Sep-Pak Plus cartridges (no. 036810; Waters, Milford, Mass.) and GF/B filter paper (no. 1821-047; Whatman, London, England). Silica gel 60 F_{254} plates (no. 1-13727; Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). The surrogate substance, estradiol-2,4,16,16-d_4, under the operating conditions of gas chromatography-mass spectrometry (GC-MS) was DLM-2487 from Cambridge Isotope Laboratories (Andover, Mass.). *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) (no. 38832; Pierce, Rockford, Ill.) was used as the silylating reagent. The glucose concentration in the jar fermentor culture was measured by using the glucose test kit (no. 273-13901; Wako, Tokyo, Japan). Sigma P-5655 and S-5761 and HyClone (Logan, Utah) SH30070 and SH30068 were used for the measurement of estrogenic activities with Dulbecco's modified Eagle's medium with Ham's F-12, sodium bicarbonate, fetal bovine serum (FBS), and charcoal-dextran-treated FBS, respectively. Luciferase was measured with the LucLite kit (no. 6016911; Packard, Meriden, Conn.).

Enrichment culture of estrogen-degrading strains. Enrichment culture of an activated sludge in a wastewater treatment plant was used to obtain estrogen-degrading strains. We selected four sites, Fushimi Wastewater Treatment Plant (plant A; Kyoto, Japan), Morigasaki Treatment Center (plant B; Ohta-ku, Tokyo, Japan), Tamagawa-joryu Treatment Plant (plant C; Akishima, Tokyo, Japan), and Todoroki Environment Center (plant D; Kawasaki, Kanagawa, Japan), and sampled activated sludge from their bioreactors. Using these sludge samples as seeds, enrichment culture was performed with modified Dominic and Graham's (MDG) culture medium (3, 13, 31, 32, 33). The MDG medium contained 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.5 g of NaCl, 0.15 g of $MgSO_4 \cdot 7H_2O$, and 1.0 ml of trace elements per liter and 0.005% (wt/vol) yeast extract. The trace elements contained 2.0 g of $NaHCO_3 \cdot 10H_2O$, 0.3 g of $MnSO_4 \cdot 4H_2O$, 0.2 g of $ZnSO_4 \cdot 7H_2O$, 0.02 g of $(NH_4)Mo_7O_{24} \cdot 4H_2O$, 0.1 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $CoCl_2 \cdot 6H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$, and 0.5 g of $FeSO_4 \cdot 7H_2O$ per liter. The pH of MDG medium was adjusted to 7.0. Enrichment culture was performed in a cylindrical column (500 mm in length, 80 mm in diameter) to which 2 liters of MDG medium and 20 ml of activated sludge were added and 60 to 100 ml of MDG medium containing E2 (0.1% wt/vol)/h was fed continuously. Air was pumped into the column at a flow rate of 50 to 100 ml/min, and culture was continued under aerobic conditions for about 10 weeks.

Isolation of strains. After enrichment culture, an appropriate amount (1 ml) of culture solution was taken from each column (included each sludge from plants A to D), and serial 10-fold dilutions were prepared with physiological saline. Then, 100-μl samples of the 10^1 to 10^9 dilutions were plated on agar medium prepared with ISP, R_2A, or YM culture medium. The plates were subsequently incubated at 28°C for 5 days (aerobically), and the colonies formed were isolated to slants.

Determination of estrogen-degrading activity. The strains obtained were inoculated into 10 ml of MDG medium containing 100 or 0 mg of E2/liter. E2 was prepared to a concentration of 25 g/liter in methanol, and 40 μl was added to 10 ml of MDG medium. As a control, only 40 μl of methanol was added. Those for which turbidity (absorbance at 620 nm) increased in shaking culture were selected, and strains increased by the control were omitted. The selected strains were then inoculated into test tubes, each containing 10 ml of MDG medium, together with 1 mg of each of the four estrogens (E2, E1, E3, and EE2) at a final concentration of 100 mg/l (added 40 μl of 25-g/liter concentrations of four estrogens in methanol) and cultured at 25°C for 24 h by shaking. After cultivation, the cell mass was filtered off through GF/B filter paper and extracted with methanol (ultrasonication for 15 min twice). These methanol extracts were combined with about 10 ml of the filtrate, and the mixture was subjected to solid phase extraction with Sep-Pak Plus cartridges and then eluted with methanol. The eluate was concentrated by nitrogen gas evaporation and analyzed by TLC and GC-MS. The selection criterion for strains was whether they could degrade 100-mg/liter concentrations of each of the four estrogens to 20 mg/liter or less within 24 h.

Estrogen-degrading activity. Degrading activities of the obtained strains for E2, E1, E3, or EE2 were measured precisely by shaking culture in test tubes. The estrogens used in the test were dissolved in methanol and prepared to concentrations of 25 g/liter and sterilized by using 0.22-μm-pore-size membrane filters. Test tubes were charged with 10 ml of MDG medium and one of the four substances (40 μl of a 25-g/liter concentration) at a final concentration of 100 mg/liter and shaken. Shaking was stopped at 0, 1 to 6 (at hourly intervals), 8, 10, or 24 h after the start. The number of bacterial cells increased to 10^6 to 10^8 CFU by preculture and was inoculated into the MDG medium. As a control, test tubes which did not contain estrogen (only 40 μl of methanol) were prepared and inoculated with each strain (only estrogens in methanol). Shaking culture was started at 25°C. At the respective time points, shaking was stopped and E2, E1, E3, and EE2 were extracted from the culture solutions. The entire culture solution was filtered to separate the cell mass from the filtrate, and the cell mass was extracted with methanol (ultrasonication for 15 min twice). The extracts were combined with about 10 ml of the filtrate, and the mixture was subjected to solid phase extraction. The eluate was analyzed by TLC and GC-MS after concentration by nitrogen gas evaporation. Each control was taken into consideration in each determination.

TLC analysis. TLC analysis was performed by using silica gel 60 F_{254}. The developing solvent was a mixture of benzene and ethanol (9:1). The concentrated sample of 10 μl was spotted and developed. At that time, the development time was about 16 min/100 mm, and R_f values were as follows: E2, 0.66; E1, 0.84; E3, 0.40; EE2, 0.76.

GC-MS analysis. GC-MS analysis was carried out on a GC (no. 6890; Agilent, Wilmington, Del.) equipped with an HP-5 column (30 m by 0.32-mm inner diameter, 0.25-μm film thickness; Agilent) coupled to the JMS-700 mass spectrometer (JEOL, Tokyo, Japan). The mass spectrometer was operated in the electron impact mode at 70 eV, and in the ion monitoring at 416 (TMS-E2), 342 (TMS-E1), 504 (TMS-E3), 440 (TMS-EE2), and 420 (TMS-E2-d_4) *m*/*z*. The mass resolution, detector voltage, and sampling rate were 1,000, 1.0 kV, and 50 ms, respectively. Helium was used as a carrier gas at a flow rate of 1.5 ml/min. The oven temperature was maintained at 150°C for 1 min and then increased to 250°C at a rate of 50°C/min (maintained for 1 min), followed by an increase to 300°C at a rate of 5°C/min (maintained for 1 min). Samples (1 μl) were injected into the GC, which was operating in the splitless mode with an injector port temperature of 280°C. The concentrated sample of 10 to 100 μl was taken, and estradiol-d_4 was added as a surrogate substance. After evaporation in a nitrogen stream, the residue was heated at 100°C for 1 h with 100 μl of BSTFA for trimethylsilylation. The product was analyzed by GC-MS in the selected ion monitoring mode.

Jar fermentation of estrogen-degrading strains. For one strain (Y 50158) that showed strong activity, E2 and glucose, as a carbon sources, were added to the culture solution in a 10-liter jar fermentor culture system at the start of culture. Elimination of the substances was measured daily. In a 10-liter jar fermentor, 7 liters of MDG medium was added, followed by 70 g of glucose and 3.5 g of E2, to make up concentrations of 10 g/liter and 500 mg/liter, respectively. Bacterial cells proliferated to 10^6 CFU by preculture (100 ml twice) were inoculated into the jar fermentor. The cells were cultured with a 0.5 air vol/medium vol/min (3.5 liters/7 liters/1 min) ventilation ratio at 200 rpm and 28°C, and 150 ml of the culture solution was collected as samples for analysis in time sequence from the start of the culture. The glucose concentration was determined by the Wako

glucose test kit, and the E2 concentration was determined by the following method. The culture solution was boiled for 15 min immediately after sampling to stop the enzyme reaction from E2 to E1. The boiled culture solution was filtered to separate the cell mass from the filtrate. The cell mass was extracted with methanol (ultrasonication for 15 min twice), and the extract, together with about 150 ml of the filtrate, was subjected to solid phase extraction. The methanol eluate was concentrated by nitrogen gas evaporation. The concentrated samples were analyzed for E2 and E1 by GC-MS. The increase in cell mass was determined by measuring the turbidity of the culture solution (absorbance at 620 nm) with the absorption spectrophotometric analyzer (Tecan Spectra Classic; Wako).

Identification of strains by 16S rDNA sequences. The strains that showed activity were identified by determining their 16S ribosomal DNA (rDNA) sequences. DNA was extracted from the cultured cell of the respective strains by the benzyl chloride method (38) as follows. The strains were cultured in YM medium at 30°C for 3 days, and cells were collected and shaken with DNA extracting buffer (250 μl; 100 mmol of Tris-HCl/liter, 40 mmol of EDTA/liter [pH 9.0]), benzyl chloride (200 μl), and 10% sodium dodecyl sulfate (50 μl) at 50°C for 30 min. After adding 3 M sodium acetate (150 ml), the mixture was centrifuged and the supernatant was precipitated with isopropanol. The precipitate was washed with 70% ethanol, then dried, and dissolved in Tris-EDTA buffer (100 μl; 10 mmol of Tris-HCl/liter, 1 mmol of EDTA/liter [pH 8.0]). As PCR primers, 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 15R (5′-AAG GAGGTGATCCARCCGCA-3′) were used. The whole volume was made up to 50 μl, and PCR was conducted in a reaction solution containing 10 mmol of Tris-HCl (pH 8.3)/liter, 50 mmol of KCl/liter, 1.5 mmol of $MgCl_2$/liter, 200 μl of deoxynucleoside triphosphate mixture, 1 U of *Taq* DNA polymerase (Takara, Shiga, Japan), 50 ng of template DNA, and 0.4 μmol of PCR primers 8F and 15R/liter by using a DNA thermal cycler PTC200 (MJ Research, Waltham, Mass.) at 94°C for 20 s, 55°C for 15 s, and 72°C for 180 s for 25 cycles. Then the PCR product was purified by using Microcon-PCR (Millipore, Billerica, Mass.). The 16S rDNA sequence was analyzed by the dye terminator method. The ABI PRISM dye terminator kit (Perkin-Elmer, Wellesley, Mass.) and the ABI PRISM 373A sequencer (Applied Biosystems, Foster City, Calif.) were used. Sequence data were aligned with the Auto Assembler (Perkin-Elmer), and phylogenetic analyses were performed by using data which we have analyzed and data from the DNA Data Bank of Japan. The phylogenetic trees were constructed by the neighbor-joining method (26). Bootstrap resampling analysis of 1,000 replicates was performed to estimate the confidence of tree topologies.

Estrogenic activity of strains by MVLN human breast cancer cells. The amount of the decrease in estrogenic activity during the degradation of estrogens by the microorganism was measured by using human breast cancer cells, modified MCF-7 cells (MVLN cells). The measurement was performed by using a previously described method (10, 11, 16). MVLN cells were precultured for 4 to 5 days and distributed to each well of a 96-well microplate, adjusting the cell number to 15,000 cells/well. The culture solution was commercial Dulbecco's modified Eagle's medium (15.6 g) to which sodium bicarbonate (1.2 g) and distilled water were added to make 1 liter, and the pH was adjusted to 7.4. As it has been reported that phenol red shows estrogen activity (16), a cell culture medium that contained no phenol red was used. To this culture solution, FBS was added to a 10% concentration and used for cell culture. After a 24-h cultivation at 28°C, the culture solution in each well was replaced with a culture solution containing 10% commercial charcoal-dextran-treated FBS from which serum estrogens were eliminated. To the wells containing the culture solution, the reference standard product of E2 and the concentrated degradation culture solution (culture solutions obtained after 0, 3, 5, and 24 h of E2 degradation) were added. After a 3-day cultivation, the amount of luciferase increased by cell proliferation was determined by the Lumicount microplate luminometer with a LucLite kit. The cell growth rate was calculated from the difference in luminescence intensity from the control, and the estrogen activity was expressed as a fraction of the reference standard (E2).

RESULTS

Isolation and characterization of estrogen-degrading bacteria. From the enrichment culture solution of plant A sludge, 2, 7, and 5 colonies were obtained with ISP, R_2A, and YM medium dishes, respectively. From plant B, 6, 5, and 5 colonies were obtained with ISP, R_2A, and YM medium, respectively. From plant C, 7, 5, and 4 colonies were obtained with ISP, R_2A, and YM medium, respectively. From plant D, 3, 3, and 4 colonies were obtained with ISP, R_2A, and YM medium, respectively. In total, 56 strains were obtained. Fourteen, 16, 16, and 10 strains were obtained from the activated sludge of plants A, B, C, and D, respectively.

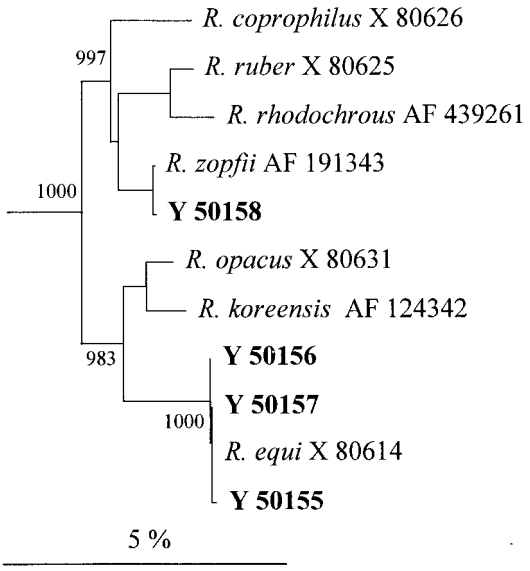

FIG. 1. Phylogenetic tree showing the relationship of 16S rDNA sequences within Y 50155, Y 50156, Y 50157, and Y 50158 and closely related *Rhodococcus* sp. The tree was constructed by neighbor-joining analysis based on 16S rDNA sequences. Bootstrap confidence values obtained with 1,000 resamplings are given at the branch points (only values of >950 are shown). The bar indicates a genetic distance of 0.05.

When the strains obtained were cultured in MDG medium containing E2, turbidity increased in 25 of 56 strains, demonstrating proliferation. There were no strains for which turbidity rises in the control. The 9 strains with marked increases in turbidity were selected, and the degrading activities on E2, E1, E3, and EE2 after 24 h of culture were measured by GC-MS and TLC. Four strains, Y 50155, Y 50156, Y 50157, and Y 50158, degraded all four substances. Strain Y 50155 was obtained from the sludge of plant A, strain Y 50156 was obtained from plant B, and strains Y 50157 and Y 50158 were obtained from plant C.

The 16S rDNA sequences of Y 50155, Y 50156, Y 50157, and Y 50158 strains were determined to identify the bacterial species. The 16S rDNA sequence (about 1,400 bases) of Y 50158 was identical to that of *Rhodococcus zopfii* (DDBJ accession no. AF191343) at 99.9% similarity, and the strain was identified as *R. zopfii*. As the 16S rDNA sequences of Y 50155, Y 50156, and Y 50157 were identical to that of *Rhodococcus equi* (accession no. X80614) at 99.9% similarity, these three strains were identified as *R. equi*. Figure 1 shows the phylogenetic tree constructed by the neighbor-joining method for these four strains and closely related *Rhodococcus* sp. Table 1 shows the 16S rDNA (about 1,400 bp) similarity.

Random amplified polymorphism DNA fingerprinting (RAPD) (1) was performed for the three strains, Y 50155, Y 50156, and Y 50157, identified as *R. equi*. For discrimination of strains, electrophoretic patterns of the PCR products were compared by using primers 1254 (CCGCAGCCAA), 1281 (A ACGCGCAAC), and 1280 (GAGGACAAAG), with the ex-

TABLE 1. Similarity of 16S rDNA (about 1,400 bp) of Y 50155, Y 50156, Y 50157, and Y 50158 and closely related *Rhodococcus* spp.[a]

Strain no.	Strain	% Similarity to strain no.:										
		1	2	3	4	5	6	7	8	9	10	11
1	*R. ruber* X 80625											
2	*R. rhodochrous* AF 439261	98.8										
3	*R. zopfii* AF 191343	98.0	97.8									
4	**Y 50158**	97.8	97.7	**99.9**								
5	*R. coprophilus* X 80626	97.2	96.4	98.0	97.9							
6	*R. koreensis* AF 124342	95.7	95.3	97.2	97.2	96.5						
7	*R. opacus* X 80631	95.9	95.6	96.8	96.8	96.5	98.8					
8	*R. equi* X 80614	95.8	95.4	96.4	96.4	95.8	97.3	97.7				
9	**Y 50155**	95.8	95.4	96.3	96.3	95.7	97.3	97.6	**99.9**			
10	**Y 50156**	95.8	95.4	96.5	96.5	95.8	97.4	97.8	**100**	**99.9**		
11	**Y 50157**	95.8	95.4	96.4	96.4	95.8	97.3	97.7	**100**	**99.9**	**100**	
12	*B. subtilis* YIT 6112	81.3	81.2	81.2	81.1	80.3	81.2	80.7	80.3	80.2	80.3	80.3

[a] Data specific to strains Y 50158, Y 50155, Y 50156, and Y 50157 are shown in boldface type.

tracted DNA as the template. The three strains showed different band patterns, as shown in Fig. 2, and were identified as different strains.

Estrogen-degrading activity of the strains. Strains Y 50155, Y 50156, Y 50157, and Y 50158 showed stronger estrogen-degrading activities than those previously seen. In the control experiment, each estrogen was not detected (<0.1 μg/liter) in all samples from the test tube of MDG medium to which only methanol was added, without each estrogen. In the MDG medium which does not inoculate the strain, the examination of the recovery of each estrogen at concentrations of 100 mg/liter showed a value of 92 to 106%.

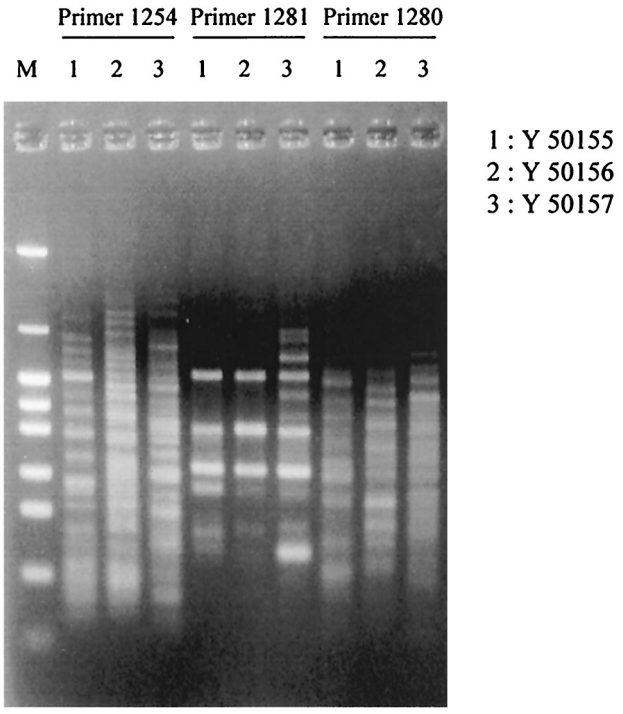

FIG. 2. Random amplified polymorphism DNA band patterns of Y 50155, Y 50156, and Y 50157, identified as *R. equi*. Primers 1254 (CCGCAGCCAA), 1281 (AACGCGCAAC), and 1280 (GAGGACA AAG) were used as random primers. Lane M, DNA markers (pHY Marker; Takara).

Three strains, Y 50155, Y 50156, and Y 50157, degraded E2 (100 mg/liter, 10 ml) by 80% or more in 5 h and by 99% in 24 h. All of these strains degraded E1 at the same concentration by 80% or more in 8 h and by 99% in 24 h. All of these strains degraded E1 at the same concentration by 80% or more in 8 h and by 99% in 24 h. Strains Y 50156 and Y 50157 degraded E3 at the same concentration by 80% or more in 5 h and by 95% or more in 24 h, whereas strain Y 50155 showed degrading activity of 72% in 24 h. All three strains degraded EE2 at the same concentration by 70% or more in 8 h and by 80% in 24 h, and strain Y 50156 showed degrading activity of 96% in 24 h (data not shown).

Figure 3 shows the degrading activities of strain Y 50158 for E2, E1, E3, and EE2. Strain Y 50158 degraded E2 (100 mg/liter, 10 ml) by 81% in 2 h, E1 at the same concentration by 91% in 3 h, E3 by 96% in 4 h, and EE2 by 70% in 6 h. This strain degraded all four substances completely in 24 h. The viable cell count was 3.8×10^8 CFU after a 24-h degradation of E2 (2.8×10^8 CFU at 0 h), 2.1×10^8 CFU after a 24-h degradation of E1 (1.0×10^8 CFU at 0 h), 2.5×10^8 CFU after a 24-h degradation of E3 (1.6×10^8 CFU at 0 h), and 3.0×10^8 CFU after a 24-h degradation of EE2 (2.0×10^8 CFU at 0 h), showing that strain Y 50158 decomposed high concentrations of the respective substances in a short period of time and increased in viable cell count by assimilating these estrogens.

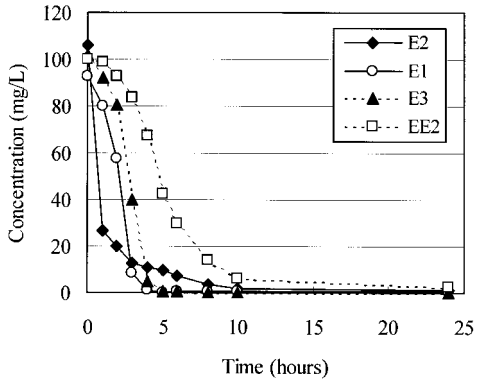

FIG. 3. Degradation activities of *R. zopfii* Y 50158 for E2, E1, E3, and EE2 (in test tube culture).

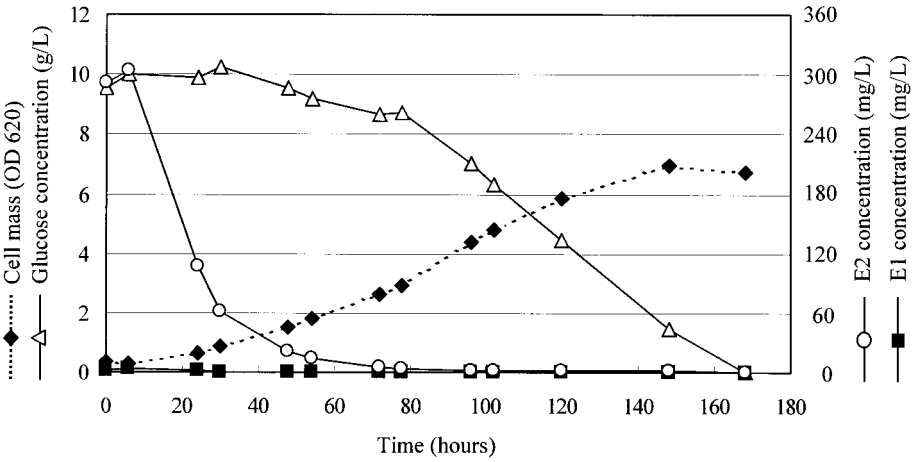

FIG. 4. Changes in cell mass and glucose, E2, and E1 concentrations during jar fermentation of *R. zopfii* Y 50158. OD 620, optical density at 620 nm.

Estrogen-degrading activity of *R. zopfii* Y 50158 in the presence of glucose. Strain Y 50158, which had shown particularly strong degrading activity, was inoculated into a culture solution containing E2 and glucose as a carbon source, and the elimination of estrogens was examined in a time series. Figure 4 shows changes in the amount of cell mass, glucose concentration, and concentrations of E2 and E1, analyzed by GC-MS. As strain Y 50158 degraded E2 selectively, even in the presence of glucose in the culture solution, it was found that the strain consumed E2 preferentially and completely, without catabolite repression. As the E1 concentration did not increase during E2 degradation, it was confirmed that the decrease in E2 concentration was not brought about by microbial transformation of E2 into E1.

Degrading estrogenic activities of the strains. Table 2 shows the results of measurements of estrogenic activities in E2 degradation culture solutions (at 0, 3, 5, and 24 h) with strains Y 50155, Y 50156, Y 50157, and Y 50158. The four strains were shown to decrease estrogenic activities in the culture solution to 1/5 to 1/10 in 3 h and to 1/100 in 24 h, expressed as a fraction of the initial activity.

DISCUSSION

In this study, *R. zopfii* Y 50158 and *R. equi* Y 50155, Y 50156, and Y 50157, microorganisms capable of degrading estrogens E2, E1, E3, and EE2, were isolated from the activated sludge of wastewater treatment plants. According to GC-MS analysis, the four strains began degrading E2, E1, E3, and EE2 (100 mg/liter, 10 ml) immediately after starting culture. E2 and E1 were degraded completely in 24 h, and E3 and EE2 were degraded by about 80% of their concentrations in 24 h. *R. zopfii* Y 50158 showed particularly strong degrading activities, with all four substances completely degraded in 24 h. This strain was also shown to degrade E2 selectively without catabolite repression, even in the presence of glucose as a readily utilizable carbon source. Although, it usually degraded the substance of a low molecule. First, strain Y 50158 consumed E2 preferentially, whereas E2 and glucose were mixed. It turns out that the start of glucose consumption was suppressed by this. As one reason, strain Y 50158 may own the enzyme system which degrades both glucose and E2, and the latter may be committing it preferentially. However, research of this mechanism is a future research subject. Moreover, the degradation rate of Y 50158 was not affected by the administration of a complex medium such as yeast extract or polypeptone in the jar fermentor culture. Thus, although bioreactors at a wastewater treatment plant were estimated to operate under eutrophic conditions, it was suggested that this bacterial strain could possibly degrade estrogens selectively in such an environment.

TABLE 2. Estrogen-degrading activities of the studied strains

Time (h)	Estrogenic activity of strain[a]:			
	Y 50155	Y 50156	Y 50157	Y 50158
0	1	1	1	1
3	0.10	0.25	0.22	0.19
5	0.03	0.15	0.15	0.14
24	0.03	0.09	0.08	0.02

[a] The estrogenic activities at 0 h are indicated as 1.

Measurement of estrogenic activities by MVLN cells showed that the four strains decreased estrogenic activities to 1/100 of the specific activity level after 24 h of degradation of 100 mg of E2/liter. Thus, it was suggested that these four strains degraded E2 to a substance that had no estrogenic activity. In the present study, the entire volume of the culture solution (10 ml), after shaking culture in a test tube, was concentrated, and the cultured cell mass of each strain was extracted with methanol by ultrasonication for measurement of the concentrations of substances and estrogenic activities. Thus, it was thought that the concentration of each substance was decreased by degradation rather than by biosorption to cell mass. At present, decomposition pathways and degrading products are being analyzed in detail. According to GC-MS analysis, there were no estrogen-degradative products. Although we think that estrogens are decomposed completely, we are conducting the experiment clarified now.

We confirmed that these strains degraded the estrogens in far higher concentrations (100 mg/liter) than those concentrations actually measured with the wastewater influent and treated wastewater of the wastewater treatment plants. Therefore, it is considered that that the low concentration of the level measured by the survey is decomposed immediately. In the future, we hope that concentrations and activities of estrogens released into the environment can be greatly reduced by culturing these strains in bulk, introducing them into the bioreactors of wastewater treatment plants, and slightly prolonging the retention of activated sludge in wastewater processing, which is now typically about 3 to 8 h (12).

Fujii et al. have isolated a new bacterial species that degrades E2, but a long time was needed for degradation (8). By contrast, *R. zopfii* Y 50158 and *R. equi* Y 50155, Y 50156, and Y 50157 isolated in the present study are very effective for practical applications, with their strong and rapid estrogen-degrading activities on E2, E1, E3, and EE2, dependence on E2 as a carbon source, and reduction of estrogenic activities. In addition, *Rhodococcus* sp. strains found in the present study have been reported to degrade cholesterol that possesses a steroidal skeleton and to degrade aromatic compounds and aniline as well as environmental pollutants such as 2,4-dinitrophenol, polychlorinated biphenyl, and 2,4,6-trichlorophenol, which are difficult to degrade (19, 25, 30, 34, 36). We consider that some species of the genus *Rhodococcus* may possess an enzyme system that degrades the steroidal skeleton. As microorganisms belonging to the genus *Rhodococcus* are probably capable of eliminating various environmental pollutants, we want to further study the possibility of degrading each pollutant with the obtained strains to reduce the environmental loading of toxic compounds.

We have started a study in an operating wastewater treatment plant by adhering these four strains, independently or in combination, to carriers. We hope to develop a wastewater treatment plant that will efficiently reduce the amount of environmental loading of estrogens.

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