Molecular Analysis of Phenol-Degrading Microbial Strains

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In an attempt to estimate the occurrence of phenol hydroxylase-related gene sequences we performed a dot blot hybridization assay with DNA from phenol utilizing *Trichosporon cutaneum* R57 strain NBIMCC 2414 and microbial isolates from different wastewaters. The used oligonucletides were homologous to the 5′-end of TORPHD locus (NCBI)-coding phenol hydroxylase in *Trichosporon cutaneum* ATCC 46490 and to the 5′-end of TORCCMLE locus (NCBI)-coding *cis,cis*-muconate-lactonizing enzyme in *Trichosporon cutaneum* ATCC 58094. Two microbial strains, *Escherichia coli* JM 109 and *Lactobacillus acidophilus* ATCC 4356, incapable to degrade phenol were used as negative controls.

We established the presence of hybridization with both used oligonucleotide probes in *T. cutaneum* R57 and *T. cutaneum* ATCC 46490 yeast strains. The experiments implemented with microbial isolates obtained from three industrialized areas in Bulgaria showed that 7 of them may carry sequences hybridizing with a phenol hydroxylase oligonucleotide probe. A subsequent hybridization test for the *cis,cis*-muconate-lactonizing enzyme showed that only 3 of them displayed a positive signal. *Lactobacillus acidophilus* ATCC 4356 and *Escherichia coli* JM 109 strains' DNA used as negative controls in the experiments did not reveal any sequence similarity to the both applied oligonucleotides.

The partial nucleotide sequences of 16S rDNAs of the isolated strains C1 and K1 obtained as PCR products were determined and sequenced. A comparison of these nucleotide sequences with similar sequences in NCBI Data Bank indicated that both C1 and K1 strains are closely related to the genera *Acinetobacter* and *Burkholderia*.

Key words: Trichosporon cutaneum, Dot Hybridization, 16S rDNA

Introduction

Phenol and its various derivatives, as well as many other aromatic compounds, are known as hazardous pollutants. Because of their industrial significance, the strains expressing phenol-degrading activity have been studied for many years. The advent of molecular techniques has improved our understanding of the microbial cultures responsible for degradation of xenobiotics in the environment and their role in controlling bioremediation processes. The utilization of molecular methods could be useful not only to identify species, but to discover new genes involved in the catabolism of aromatics for the purpose to innovate and improve the technological processes of biodegradation (Watanabe *et al.*, 1998; Cafaro *et al.*, 2005; Lau *et al.*, 2007).

The first key enzyme of aromatics degradation is phenol hydroxylase (EC 1.14.13.7) which catalyzes the conversion of phenols to their *o*-diol derivatives. It has been reported that further degradation of the phenol hydroxylase reaction product catechol may occur via the *ortho*- or *meta*-fission pathway in microorganisms. The both modes of catechol degradation might be found in bacteria (Sauret-Ignazi *et al.*, 1996; Polymenakou and Stephanou, 2005; O'Sullivan *et al.*, 2007). The mechanism of phenol utilization most common for yeast and micelial fungi goes through the *ortho*-fission pathway (Spånning and Neujahr, 1991; Yan *et al.*, 2005).

Phenol hydroxylase shares overall mechanistic features with flavin-containing monooxygenases acting on phenolic acids which have mainly been studied with prokaryotes (Cafaro *et al.*, 2004). Phenol hydroxylase has been isolated, purified and characterized from the basidiomycetous yeast *Trichosporon cutaneum* (Enroth *et al.*, 1998). Using hybridization methods the gene coding phenol hydroxylase (*phyA*) was detected in one strain of *T. cutaneum* and one strain of *Candida elinovii.* DNA from *T. pullulans, T. penicillatum* and *C.*

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tropicalis did not show any sequence similarity to the *phyA* sequence (Kalin *et al*., 1992).

Cycloisomerases catalyze the conversion of *cis,cis*-muconate to (+)-muconolactone in the *ortho*-mechanism of phenol degradation. According to Mazur *et al*. (1994), several notable differences occur between bacterial and eukaryotic muconate-lactonizing enzymes (MLEs), suggesting that these enzymes might be unrelated. The authors of the cited investigation presume that MLEs of *T. cutaneum* ATCC 58094 and bacterial MLEs do not share a common genetic origin. Another possible interpretation is that the similar enzyme mechanism of action might be due to a common evolutionary origin (Mazur *et al*., 1994).

Synthetic oligonucleotides are used as probes for the detection and isolation of specific genes and as primers for DNA sequencing (Itakura *et al*., 1984). Methods for biotin-labeling of DNA have been developed and widely accepted because of their safety and sensitivity to detection (Langer *et al*., 1981; Murasugi and Wallace, 1984). Oligonucleotide probes may originate from ribosomal RNA gene sequences (Blasco *et al*., 2003), randomly cloned chromosomal fragments or known genes (Daly *et al*., 2003). They are usually more specific than total chromosomal DNA probes. A synthetic biotin-labeled oligonucleotide can be used directly as a hybridization probe or to prepare longer probes by enzymatic reactions. As in the study of biotin-labeled RNA molecules, these DNA probes have been used in locating genes and specific sequences of nucleotides (Brigati *et al*., 1983; Puvion-Dutilleul and Puvion, 2005).

In the present work we have investigated the occurrence of phenol hydroxylase and/or *cis,cis*-muconate cyclase-related gene sequences in both taxonomically determinated and unknown phenol utilizing microbial strains. For this purpose we used two gene-specific biotin-labeled oligonucleotides in dot blot hybridization experiments. The partial nucleotide sequences of 16S rDNAs of two isolated strains obtained as PCR products were determined and sequenced. The phylogenetic analysis of both new isolated strains indicated their relatedness to the genera *Acinetobacter* and *Burkholderia*, respectively.

Materials and Methods

Microorganisms and cultivation conditions

The *Trichosporon cutaneum* R57 strain is registered in Bulgarian National Bank of Industrial Microorganisms and Cell Cultures (N2414/94). The culture was grown on YPD broth (Fluka, Seelze, Germany). On the same medium *Trichosporon cutaneum* ATCC 46490 strain was cultivated. Sediments and water samples were obtained from three industrialized areas in Bulgaria: wastewater treatment plant (C1), oil refinery (B4, B6, B7, B8, B11) and wastewater of charcoal-producing factory (K1). The microbial isolates were grown on Luria broth (Fluka, Seelze, Germany) and basic salt medium (Futamata *et al.*, 2001), where phenol was the sole source of carbon and energy. The cultivation was carried out on a New Brunswick rotary shaker at 28–30 °C and 200 rev/min. The strains *Escherichia coli* JM 109 and *Lactobacillus acidophilus* ATCC 4356 were maintained at 37 °C, on Luria broth and de Man-Rogosa-Sharpe medium (Oxoid Limited, Basingstoke, UK), correspondingly.

Genomic DNA extraction procedure

The cells of *T. cutaneum* R57 strain were disrupted by the three cycle's freeze-thaw procedure including liquid nitrogen treating and heating to 90 °C. The following extraction of yeast and bacterial genomic DNA was performed by using standard methods (Maniatis *et al*., 1982). Final DNA purification was conducted with a GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). The quantity and quality of the extracted DNA were checked by measuring the UV absorption spectrum (Futamata *et al*., 2001).

Biotin labeling

The single stranded oligonucleotides used in this work are as given in Table I. All used oligonucleotides and primers were obtained from Applied Biosystems (Foster City, CA, USA). OligoD1 was homologous to the 5′-end of TORPHD locus (NCBI)-coding phenol hydroxylase in *T. cutaneum* ATCC 46490 (Kalin *et al*., 1992). OligoZA1 was designed to be homologous to positions 18 to 63 of the 5′-end of the TORCCMLE locus (NCBI)-coding *cis,cis*-muconate-lactonizing enzyme in *T. cutaneum* ATCC 58094 (Mazur *et al*., 1994).

For biotin labeling of single stranded oligonucletides the Biotin ULS® Labeling Kit (Fermentas UAB, Vilnius, Lithuania) was used. The reaction mixture consisted of 10 μl fOligoD1 or fOligoZA1 (0.1 μg/μl), 5 μl Biotin ULS® reagent (0.5 U/μl)

Table I. Oligonucleotides for dot blot hybridization and primers for PCR amplification of 16S rDNA.

	Sequence (5′ to 3′)	Designed for
Oligonucleotides[a]		
fOligoD1	AAGTACTCCGAGTCCTACTGCGACGTCCTCATCGTCGG	Kalin *et al*., 1992
OligoD1	CCGACGATGAGGACGTCGCAGTAGGACTCGGAGTACTT	
fOligoZA1	AGCTATGATATTTTGATGGGCACGTTCCGCTCGCCCTACCTCTAC	this work
rOligoZA1	GTAGAGGTAGGGCGAGCGGAACGTGCCCATCAAAATATCATAGCT	
Primers		
fD1	AGAGTTTGATCCTGGCTCAG	Weisburg *et al*., 1991
rD1	AAGGAGGTGATCCAGCC	Weisburg *et al*., 1991

[a] f, forward; r, reverse.

and 5 μl labeling solution. The next step of the procedure included incubation for 30 min at 85 °C followed by centrifugation at 12,000 rev/min for 30 s. The reaction was stopped with 5 μl stop solution. The mixture was then incubated for 10 min at room temperature and precipitated with 4.5 μl 4 M LiCl and 71 μl 100% ethanol (Fluka). The probes were centrifuged at 12,000 rev/min for 15 min after overnight incubation at −20 °C. The sediments were washed with 70 μl 70% ethanol, and centrifuged again at 12,000 rev/min for 15 min, and dried at room temperature and dissolved in 10 μl sterile water.

Dot blot hybridization

Positively charged nylon membranes (Boehringer, Mannheim, Germany) for dot blot analysis were prepared by spotting 1 μl (300 ng) genomic DNA, following the recommendation of the manufacturer. They were treated for 5 min on filter papers wetted with denaturing solution (0.5 M NaOH, 1.5 M NaCl), for 30 s in 0.5 M tris(hydroxymethyl)methylamine-hydrochloride (tris-HCl) buffer, pH 7.5, and for 5 min in neutralizing solution consisting of 0.5 M tris-HCl buffer, pH 7.5, and 1.5 M NaCl. The genome DNA was fixed by UV treatment (λ = 254 nm) for 3 min. A pre-hybridization was performed by incubation for 1.5 h at 42 °C in hybridization solution: 5× sodium chloride/sodium citrate buffer (SSC), pH 7.0, 0.1% sodium lauryl sulfate (SLS), 0.02% sodium dodecyl sulfate (SDS), 0.5% blocking reagent (Boehringer) in a hybridization chamber (Techne, Cambridge, UK). The biotin-labeled oligonucleotides (1 μl/ml) were added to the hybridization solution used in the process of hybridization. The temperature conditions in the hybridization chamber were as follows: 15 min at 66 °C, 15 min at 60 °C, 1 h at 55 °C, 1 h at 50 °C, 1 h at 45 °C, 18 h at 42 °C and 1 h at 35 °C (Petrova *et al*., 2003). The membranes were washed in 6× solution consisting of sodium chloride/sodium citrate buffer, pH 7.0, and 0.1% sodium dodecyl sulfate as follows: twice at 25 °C for 15 min with constant agitation and once at 42 °C for 15 min. The detection was done according to the instructions of the DNA Labeling and Detection kit (Boehringer).

PCR conditions and sequencing

The primers used for PCR amplification of conserved regions within rDNA genes are listed in Table I. The amplification was performed on a thermocycler Eppendorf Mastercycler personal (Eppendorf AG, Hamburg, Germany) by using PuReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences). The PCR reactions were carried out in a 25 μl volume containing 10 pmol of each primer and 50 ng genomic DNA. PCR procedure in the present study was carried out using an initial 5 min step at 95 °C followed by a 30 s denaturizing step at 95 °C, a 30 s annealing step at 55 °C, and then a 2 min extension step at 72 °C. Steps 2 to 4 were repeated 35 additional times, with a final 5 min extension step at 72 °C before storage of the PCR products at 4 °C. The obtained PCR products were purified by GFX™ PCR DNA and Gel Band Purification Kits (Amersham Biosciences). The sequencing of amplified fragments was performed on an ABI Prism 310 Genetic Analyzer by using BigDye® Terminator Kit version 3.1. The row data from the genetic analyzer were edited by sequence scanner version 1.0 software (Applied Biosystems).

Results and Discussion

The microbial isolates were maintained on both solid and liquid basic salt medium containing phe-

nol as a sole carbon source. After repeated subcultivation pure cultures able to utilize phenol were isolated. The preliminary morphological analyses showed that the investigated microorganisms were Gram-negative bacteria. The designation of isolated strains was kept the same as the water and sediment samples described in Materials and Methods. The isolated total DNAs from these cultures as well as DNAs from *T. cutaneum* R57 and *T. cutaneum* ATCC 46490 were used in the following experiments.

The results from performed dot blot hybridization analyses are presented on Fig. 1. Both *T. cutaneum* strains as well as all of the isolated microbial cultures showed positive signals.

In the next step of our analyses we carried out another set of dot blot hybridizations using an oligonucleotide probe for the detection of sequences coding the *cis,cis*-muconate-lactonizing enzyme. The fOligoZA1 was designed on the basis of published data in the NCBI TORCCMLE locus sequence coding *cis,cis*-muconate-lactonizing enzyme in *Trichosporon cutaneum* ATCC 58094 (Mazur *et al*., 1994). The purpose of these experiments was to find out if there are any sequence similarities between isolated total DNA probes in this study and the TORCCMLE locus sequence. The obtained results are presented on Fig. 2.

Together with the both strains of *T. cutaneum* only C1, B8 and B11 of the investigated microbial isolates displayed a positive hybridizing signal with fOligoZA1. These results would rather support the opportunity of a common evolutionary origin

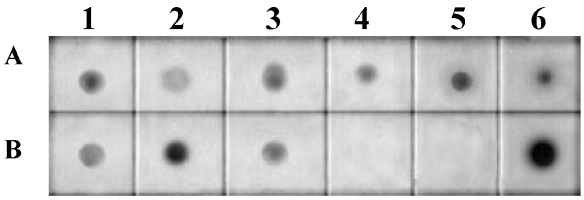

Fig. 1. Dot blot analyses of total DNA hybridized with fOligoD1. A (1–6): *Trichosporon cutaneum* R57 strain; *Trichosporon cutaneum* ATCC 464901; C1 isolated from wastewater treatment plant; K1 isolated from wastewater of charcoal producing factory; 4 and 6 activated sludge isolates from biological purification step of oil refinery. B (1–6): 7 and 11 wastewater isolates from biological purification step of oil refinery; 8 activated sludge isolate from biological purification step of oil refinery; negative controls: *Lactobacillus acidophilus* ATCC 4356; *Escherichia coli* JM 109; positive control: synthetic oligonucleotide "rOligoD1".

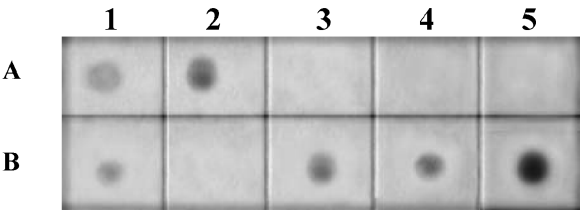

Fig. 2. Dot blot analyses of total DNA hybridized with fOligoZA1. A (1–5): *Trichosporon cutaneum* R57 strain; *Trichosporon cutaneum* ATCC 46490; K1 isolated from wastewater of charcoal producing factory; 4 and 6 activated sludge isolates from biological purification step of oil refinery. B (1–5): C1 isolated from wastewater treatment plant; 7 and 11 wastewater isolates from biological purification step of oil refinery; 8 activated sludge isolate from biological purification step of oil refinery; positive control: synthetic oligonucleotide "rOligoZA1".

of prokaryotic and eucaryotic MLEs-coding sequences (Mazur *et al*., 1994). It could confirm as well that the diversity of genes involved in biodegradation processes is very broad.

If we combine the data received from both hybridization sets of experiments with the obtained ability of these microbes to grow and develop in the medium containing phenol as a sole carbon source we could presume that the mechanism of phenol biodegradation in these three microbial isolates was the *ortho*-cleavage type. It was previously proved that both *T. cutaneum* ATCC 46409 strain and *T. cutaneum* R57 strain are able to utilize phenol *via* the *ortho*-pathway (Spånning and Neujahr, 1991; Alexievaa *et al*., 2004). The other four microbial cultures (K1, B4, B6 and B7) did not hybridize with fOligoZA1, *i.e.* they did not contain any similarity with oligonucleotide probes for MLE of *T. cutaneum* ATCC 58094. Hypothetically, in these isolates the pathway of phenol degradation might go through the *meta*-cleavage mechanism.

Lactobacillus acidophilus ATCC 4356 and *Escherichia coli* JM 109 strains' DNA used as negative controls in the experiments were not able to utilize phenol. Accordingly they did not reveal any sequence similarity to the both applied oligonucleotides.

The primer pair used (Table I) in the PCR procedure with the purpose to obtain prducts from 16S rDNA genes of the strains C1 and K1 was previously described (Weisburg *et al*., 1991). The partial sequences of the PCR products were determined. The sizes of the both nucleotide sequences obtained were 139 and 670 bases correspondingly

to C1 and K1. The comparison of our data with NCBI Gene Bank Data Base reference sequences was conducted by Blast program. The phylogenetic analyses of 16S rDNA sequences were performed by using CLUSTAL W version 1.7 (Thompson *et al.*, 1994). *Acinetobacter radioresistans* appeared to be closely related to strain C1 while the most close to strain K1 was *Burkholderia vietnamiensis*. The nucleotide sequences obtained in this investigation have been deposited in the NCBI nucleotide sequence databases under accession numbers EU118562 and EU118563. The results obtained in the present study correlate with previous works reporting about members of the *Acinetobacter* and *Burkholderia* genera as effective biodegraders of aromatic compounds. According to Geng *et al*. (2006), *Acinetobacter* species decompose phenolic compounds through the β-ketoadipate pathway, which involves an *ortho*-cleavage mechanism. It is known that among the strains belonging to *Burkholderia* there are some able to utilize phenol by two metabolic pathways – *ortho* and/or *meta* (Rogers *et al*., 2000). O'Sullivan *et al*. (2007) report a study defining a strain of *Burkholderia vietnamiensis* able to degrade a number of toxic aromatic compounds through the *meta*-cleavage pathway of degradation.

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Alexievaa Z., Gerginova M., Zlateva P., and Peneva N. (2004), Comparison of growth kinetics and phenol metabolizing enzymes of *Trichosporon cutaneum* R57 and mutants with modified degradation abilities. Enz. Microb. Technol. **34**, 242–247.

Blasco L., Ferrer S., and Pardo I. (2003), Development of specific fluorescent oligonucleotide probes for *in situ* identification of wine lactic acid bacteria. FEMS Microbiol. Lett. **225**, 115–123.

Brigati L., Myerson D., Leary J., Spalholz B., Travis Z., Fong Y., Hsiung D., and Ward C. (1983), Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labeled hybridization probes. Virology **126**, 32–50.

Cafaro V., Izzo V., Scognamiglio R., Notomista E., Capasso P., Casbarra A., Pucci P., and Donato A. (2004), Phenol hydroxylase and toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1: Interplay between two enzymes. Appl. Environ. Microbiol. **70**, 2211–2219.

Cafaro V., Notomista E., Capasso P., and Donato A. (2005), Regiospecificity of two multicomponent monooxygenases from *Pseudomonas stutzeri* OX1: Molecular basis for catabolic adaptation of this microorganism to methylated aromatic compounds. Appl. Environ. Microbiol. **71**, 4736–4743.

Daly K., Soraya P., and Shirazi-Beechey S. (2003), Design and evaluation of group-specific oligonucleotide probes for quantitative analysis of intestinal ecosystems: Their application to assessment of equine colonic microflora. FEMS Microbiol. Ecol. **44**, 243–252.

Enroth C., Neujahr H., Schneider G., and Lindqvist Y. (1998), The crystal structure of phenol hydroxylase in complex with FAD and phenol provides evidence for a concerted conformational change in the enzyme and its cofactor during catalysis. Structure **6**, 605–617.

Futamata H., Harayama S., and Watanabe K. (2001), Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. Appl. Environ. Microbiol. **67**, 4671–4677.

Geng A., Soh A., Lim C., and Loke L. (2006), Isolation and characterization of phenol-degrading bacterium from an industrial activated sludge. Appl. Microbiol. Biotechnol. **71**, 728–735.

Itakura K., Rossi J., and Wallace B. (1984), Synthesis and use of synthetic oligonucleotides. Annu. Rev. Biochem. **53**, 323–356.

Jiang Y., Wen J., Bai J., Jia X., and Hu Z. (2007), Biodegradation of phenol at high initial concentration by *Alcaligenes faecalis*. J. Haz. Mater. **147**, 672–676.

Kalin M., Neujahr H., Weissmahr R., Sejlitz T., Johl R., Fiechter A., and Reiser J. (1992), Phenol hydroxylase from *Trichosporon cutaneum*: gene cloning, sequence analysis, and functional expression in *Escherichia coli*. J. Bacteriol. **174**, 7112–7120.

Langer R., Waldrop A., and Ward C. (1981), Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. Proc. Natl. Acad. Sci. USA **80**, 4045–4049.

Lau S. C. K. and Liu W.-T. (2007), Recent advances in molecular techniques for the detection of phylogenetic markers and functional genes in microbial communities. FEMS Microbiol. Lett. **275**, 183–190.

Maniatis T., Fritsch F., and Sambrook J. (1982), Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Mazur P., Pieken A., Budihas R., Williams E., Wong S., and Kozarich J. (1994), *Cis,cis*-muconate lactonizing enzyme from *Trichosporon cutaneum*: evidence for a novel class of cycloisomerases in eucaryotes. Biochemistry **33**, 1961–1970.

Murasugi A. and Wallace B. (1984), Biotin-labeled oligonucleotides: enzymatic synthesis and use as hybridisation probes. DNA **3**, 269–277.

O'Sullivan A., Weightman J., Jones H., Marchbank M., Tiedje M., and Mahenthiralingam E. (2007), Identifying the genetic basis of ecologically and biotechnologically useful functions of the bacterium *Burkholderia vietnamiensis*. Environ. Microbiol. **9**, 1017–1034.

Petrova P., Miteva V., Ruiz A., and del Solar G. (2003), Sequencing and analysis of the *Streptococcus thermophilus* plasmid pt38. Plasmid **50**, 176–189.

Polymenakou N. and Stephanou G. (2005), Effect of temperature and additional carbon sources on phenol degradation by an indigenous soil Pseudomonad. Biodegradation **16**, 403–413.

Puvion-Dutilleul F. and Puvion E. (2005), Ultrastructural localization of defined sequences of viral RNA and DNA by *in situ* hybridization of biotinylated DNA probes on sections of herpes simplex virus type 1 infected cells. J. Electron Microsc. Tech. **18**, 336–353.

Rogers J., DuTeau N., and Reardon K. (2000), Use of 16S-rRNA to investigate microbial population dynamics during biodegradation of toluene and phenol by a binary culture. Biotechnol. Bioeng. **70**, 436–445.

Sauret-Ignazi G., Gagnon J., Béguin C., Barrelle M., Markowicz Y., Pelmont J., and Toussaint A. (1996), Characterisation of a chromosomally encoded catechol 1,2-dioxygenase (E.C. 1.13.11.1) from *Alcaligenes eutrophus* CH34. Arch. Microbiol. **166**, 42–50.

Spånning Å. and Neujahr H. (1991), Enzyme patterns during substrate shifts between phenol, acetate and glucose in continuous culture of *Trichosporon cutaneum*. Appl. Microbiol. Biotechnol. **35**, 525–529.

Thompson D., Higgins G., and Gibson J. (1994), CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673–4680.

Watanabe K., Teramoto M., Futamata H., and Harayama S. (1998), Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl. Environ. Microbiol. **64**, 4396–4402.

Weisburg W., Barns S., Pelletier D., and Lane D. (1991), 16S Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**, 697–703.

Yan J., Jianping W., Hongmei L., Suliang Y., and Zongding H. (2005), The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*. Biochem. Eng. J. **24**, 243–247.