Bacterial consortia utilizing volatile organic compounds: mapping genomic determinants of essential biodegradation pathways

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ABSTRACT. Genotyping of a consortium of microorganisms used in biofiltration application is often required. A standard RT-PCR approach substantially improves qualitative resolution of the genotyping and makes it a really an express method. However, development of RT-PCR method for each type of consortium requires preliminary characterization of its polymorphism extent and identification of dominant and ubiquitous species in order to adjust RT-PCR primers to their genomic sequence tags e.g. 16S genes. The goal of the present study was mapping of genomic determinants of essential biodegradation pathways in microbial consortia developed under real experimental condition in laboratory trickling gas-phase biofilters and adapted to degrading a number of abundant VOC's: ethyl benzene, m-xylene, styrene and o-xylene. Herewith we report 16S-rRNA-based geno-systematic study of four related mixed bacterial cultures isolated from laboratory trickling biofilters. 16S-rDNA random cloning and RFLP-assay was applied to the total genomic DNA of the cultures. 16S-rDNA schizo-types belonging to the major species were identified with two restrictases. Sequencing of four dominant 16S-rDNA plasmid clones elucidated that *Pseudomonas fluorescens (**AY730552**)* was the principal specie in the ethylbenzene-utilizing culture. *Achromobacter xylosoxydans (**AY753652**)*, *Pseudomonas veronii(**AY748440**)* and *Delftia acidovorans (**AY753653**)* prevailed in o-xylene, styrene and m-xylene utilizing cultures respectively. Minor components in any of four mixed cultures were essentially the same and belonged to genera *Mesorhizobium*, *Pedobacter* and *Paenibacillus*. Therefore, random sequencing of 16S-rRNA cloned from total genomic DNA was demonstrated to be a suitable method for qualitative assay of VOC-utilizing mixed culture polymorphism under real experimental conditions. Species identification was accomplished by cloning and sequencing of key genes encoding major aromatic VOC biodegradation pathway enzymes such as cathechol-1,2-dioxygenase, cathechol-2,3-dioxygenase and xylene-monooxygenase. Certain alleles of these genes occurred in any population but their sequences were culture-specific. Results of cathechol-1,2- and cathechol-2,3-dioxygenase enzymatic activity assessment in crude extracts of the mixed cultures were in a good agreement with respective genotyping data.

1 INTRODUCTION

At present, due to the rapid development of industrial biotechnology, microbiological methods are increasingly used for purification of industrial waste air containing volatile

organic compounds (VOCs). Use of microbiological filters is based on microbial degradation of organic substrates (xenobiotics) to carbon dioxide and water. In contrast to chemical purification methods (catalytic or high-temperature burning), microbial filters do not produce chlorine, nitrogen oxides, or sulfur oxides. Unlike adsorption or filtration through selective membranes, the use of microbial filters does not result in accumulation of waste membranes or sorbents polluted with stable toxicants. Microbial filters do not pollute the environment. They are easily operated and efficient. For these reasons, they are applicable for degradation of the bulk of industrial and domestic organic pollutants. Application of microbial purification of waste air in industry requires modern methods of inspection of the state of the devices. Also, processes occurring in microbial filters in the course of operation and the state of the microorganisms should be well understood. Microbiological identification and classification of cultures, however useful they are, do not meet modern precision and speed requirements. The development of molecular methods of identification of microorganisms and their use in taxonomy and ecology allows appropriate use of molecular systematics and molecular biology in analyzing natural and industrial microbial cultures. The main advantage of methods of classification of bacterial cultures and associations, based on molecular genetics, is that they allow identification of microorganisms (down to species level) in various associations without cultivation. These methods provide statistically reliable data on the qualitative and quantitative composition of the associations.

Genotyping of bacterial species in consortia is prerequisite for applying molecular genetic methods to challenge their metabolic potential. The ability of Gram-negative bacteria to consume volatile organic compounds (VOCs) is determined by enzyme complexes responsible for scission of the aromatic ring via one of two pathways. Catechol dioxygenases are considered to be key enzymes of both pathways. In the meta pathway, catechol-2,3-dioxygenase (EC 1.13.11.2, C23O) cleaves the ring between carbon atoms 2 and 3. In the ortho pathway, catechol-2,3-dioxygenase (EC 1.13.11.1, C12O) cleaves it between carbon atoms 1 and 2 (Fig. 1). Both enzyme types are highly conserved regardless of their origin (An et al., 2001; Ocuta *et al.*, 1998). Usually catechol dioxygenase genes are located within operons encoding enzyme arrays from each pathway. Therefore, they may serve as relevant markers for retracing whole pathways of VOC consumption by polymerase chain reaction (PCR). Sets of primers for catechol dioxygenase genes of both types, proposed in (An et al., 2001; Ocuta *et al.*, 1998) have been repetitively tried previously. It was found that, in microorganisms of the orders *Pseudomonodales*, *Burkholderiales*, and *Ralstoniales*, genes encoding the meta- and para- cleavage of aromatic rings are mostly allocated to plasmids, including promiscuous plasmids *pWWO* and *pTOL*, occurring in natural populations. In contrast, genes for ortho pathway enzymes tend to be located on the bacterial chromosome, including mobile genetic elements. The lower pathways are virtually identical in either degrading microorganisms (Amann *et al.*, 1995).

The goal of the present work was to investigate the composition of microbial associations developing during aromatic VOC consumption in pilot mini-reactors and the role of each component of the association in the process. This would optimize VOC biodegradation in actual industrial devices and allow development of convenient methods for inspection of industrial devices and natural ecosystems.

2 MATERIALS AND METHODS

Source lyophilized cultures were taken from the microbial collection of the Laboratory of Enzyme Engineering, Bach Institute of Biochemistry RAS, and from accessions formerly deposited in the All-Russia Collection of Industrial Microorganisms. The specificity of the source cultures with respect to the corresponding substrates was checked Cultures degrading particular xenobiotics were placed into continuously operating flow laboratory scale minireactors for air purification as described earlier (Khomenkov *et al.*, 2005). Average VOC inlet concentration for each minireactor was kept at 300–350 mg/m^3. The efficiency of VOC degradation was determined by gas chromatography. Samples of microflora from the biocatalyst surfaces were inoculated on to Petri dishes with solidified minimal mineral medium and incubated in vapor of VOCs (ethyl benzene, *m* -xylene, styrene, or *o* -xylene) at 28□ C for 48 h. In spite of the selective pressure imposed on the biocatalyst during the run, it contained a notable amount of symbionts and oligocarbophilic organisms consuming metabolites of the main degrader species or inhabiting its debris. To remove the accompanying strains not related to the degraders and to stabilize the adaptation of the cultures to the consumption of a certain substance, colonies obtained by inoculation of biocatalyst samples were repeatedly passaged onto solidified minimal medium and cultivated as above. If necessary, pH was adjusted with 1 N NaOH or HCl. *E. coli* strains were grown on conventional LB or M9 media (Maniatis *et al.,* 1989). Total genomic DNA was isolated from the cultures as described previously (Khomenkov *et al.*, 2005). The following primers, described in (Anzai *et al.*, 2000), were used for amplification of 16S rRNA gene fragments and preparation of total amplicons from the genome of each culture: 8F (5'-AGAGTTTGATCCTGGCTCAG-3'); 1492R (5'-ACGGCTACCTTGTTACGACTT-3'). The expected size of amplified DNA was 1465 bp. PCR was carried out under standard conditions described elsewhere (Anzai *et al.*, 2000). The mixed PCR products were cloned in pGem-T Vector (Promega, USA).

To obtain pure DNA fragments of the cloned 16S rRNA genes, PCR was carried out using each plasmid clone as templates. Restriction fragment length polymorphism (RFLP) test was performed with the purified amplicons with two restriction endonucleases: *Msp*I and *Rsa*I. Restriction profiles were analyzed by electrophoresis in 1.5% agarose gel or 6% polyacrylamide gel. Sequencing plasmid DNA was performed on an AbiPrism 3100 automated sequencer. The 16S rRNA gene sequences were deposited in NCBI Gene Bank. Statistical evaluation, alignment of sequences and construction of phylogenetic trees were performed by the Clustal V method using the MegAlign unit of the DNA-star program package.

Genotyping the cultures with respect to challenge their metabolic potential was carried out by a similar routine using following primers, reported previously (An et al., 2001; Ocuta *et al.*, 1998), specific to catechol-1,2-dioxygenase and catechol-2,3-dioxygenase gene sequences:

C23O for 5'-ATGGATDTDATGGGDTTCAAGGT-3'
C23O rev 5'-ACDGTCADGAADCGDTCGTTGAG-3'
C12O for 5'-GCGHACVATGAAGGNCCRYTGTA-3'
C12O rev 5'-TCRCGSGTNGCAWANGCAAAGTC-3'

The expected sizes of amplified DNA fragments for the C12O and C23O genes were 462 and 721 bp, respectively. PCR cloning routine of this was formerly described in detail (Khomenkov *et al.*, 2005).

3 RESULTS AND DISCUSSION

3.1. Genotyping of bacterial species in VOC utilizing consortia

Nonsterile industrial associations degrading various xenobiotics under both aerobic and anaerobic conditions consist of 2–10 strains in different proportions (Stoffels *et al.*, 1998). As a rule, an association is dominated by one or two strains, which do the main work on consumption or conversion of a corresponding xenobiotic. Other members of the microbial association convert the products formed during the primary degradation of the xenobiotic or accompany the major strains as symbionts or consumers. The stability

of such associations is supported by the ability of symbionts to produce growth factors (Khomenkov *et al.*, 2005). The distribution of microbial associations depending on the location on the surface of the biocatalyst or concentrations of xenobiotics to convert as well as the vertical and horizontal layering over the biocatalyst were described in (Stoffels *et al.*, 1998). It is worth noting that even minute changes in the properties of the VOCs to be degraded, including use of their mixtures, can alter the strain proportions in a mature microbial association. The number of microbial strains forming a mixed population is significant for determining the size of a 16S rRNA clone sample in order to evaluate the proportions of the strains by RFLP. Statistically reliable analysis requires that a sequence be present in the sample more than once; therefore, the sample size meets the criterion of triple occurrence of any sequence to be analyzed. An additional task was to estimate the potential of degrading associations involving up to ten major microbial species; that is, 10% of dots of the total clone sample should belong to each of the major species equally represented in the association. As each species should be represented by no less than three independent rDNA clones, the size of an rDNA clone sample required for RFLP analysis should be no less than 30. In analyses of associations consisting of less than ten species, the proportion of rDNA of each type in the clone sample increases, and the results become statistically more reliable. In contrast, increased heterogeneity of an association and the presence of strains constituting less than 10% of the association reduces the faithfulness of the results. The differences between the patterns of the clones obtained by RFLP with plasmid DNAs harboring the 16S rDNA insert are determined by specific distributions of restriction sites in the sequences of 16S rRNA genes. This allows identification of the fragments according to restriction fragment patterns. However, plasmid-based RFLP is hampered by the fact that the pattern includes hybrid fragments, corresponding to vector–insert borders. Thus, the lengths of two fragments in the pattern may vary depending on the orientation of the cloned insert, and two different schizothymes may correspond to each 16S rRNA sequence. With large clone samples, this difficulty can be dealt with by mathematical processing of the results. Analysis of RFLP sequences of 16S rDNA obtained by secondary amplification of plasmid clones on DNA templates is much more informative and precise. However, being less laborious, analysis of plasmid RFLP is convenient for tentative evaluation and choice of the size of a plasmid clone sample. At the first stage, clones harboring inserts identical or similar in sequence were recognized by RFLP analysis of isolated plasmid DNA of these clones. The similarity or difference between the restriction patterns of the clones provided grounds for their grouping into schizotypes. Eleven schizotypes were recognized in four samples. The predominant schizotypes in each of the sample were fairly similar. Later, it was found that they belonged to one taxon. The RFLP results are shown in Figs. 2 and 3. The RFLP schizotyping of rDNA clones via plasmid DNA and PCR was followed by complete sequencing of type clones, each of which belonged to one of the major schizotypes in a sample. The resulting rDNA sequences of four clones were compared with sequences retrieved from the NCBI gene database (Table 1). For each of the schizotypes, we found aclosely similar (>96–98 matches) sequence. This allowed identification of the strains to genus or, sometimes, even to species. We found that schizotype 1 corresponded to a sequence of the species *Sphingobacterium multivorum* (97% matches); 2, to the genus *Paenibacillus* (95%); 3, to the genus *Rhizobium* (90%); 4, to *Alcaligenes xylosoxydans* (95%); 5, to *Mesorhizobium ciceri*, (98%); 6, to *Comamonas acidovorans* (97%); 7, to *Sphingobacterium multivorum* (96%); 8, to the genus *Pedobacter* (96%); 9, to *Sphingobacterium multivorum* (99%); 10, to *Pseudomonas fluorescens* (99%), and 11, to *Pseudomonas veronii* (99.6%).

To verify the correct assignment of particular clones to particular species or genera, we performed clade analysis of phylogenetic relations between the conjectured bacterial hosts of the cloned rDNAs and of their relations to rDNA sequences formerly entered into NCBI. We chose four found sequences and seven known ones most similar to those newly found. The rooted tree constructed by the algorithm implemented in the Clustal V package using *Bacillus cereus* ZK (NCBI accession no. CP000001) as an external group showed that the newly found sequences are indeed close to the reference species and genera. This similarity can not be attributed to insufficient data on particular eubacterial taxa.

Table 1. Frequencies of occurrence of schizotypes of rDNA clones in associations adapted to various VOCs

DIVERSITY,%	IDENTITY, %										
		1	2	3	4	5	6	7	8	9	
	1		97,8	65,9	81,8	84,0	80,2	79,0	77,5	79,3	1
	2	0,6		65,9	83,8	84,6	81,3	81,5	79,5	80,7	2
	3	28,0	27,5		64,4	67,1	74,1	70,3	68,1	71,5	3
	4	14,0	13,4	29,3		97,5	79,0	79,1	77,9	78,5	4
	5	13,2	12,8	28,3	0,9		80,7	79,3	78,3	80,1	5
	6	17,4	17,1	22,8	18,5			94,8	97,9	98,9	6
	7	16,8	16,4	22,0	17,8	17,2	0,5		97,8	98,9	7
	8	19,4	18,9	24,5	19,4	19,6	1,9	1,5		98,2	8
	9	18,3	17,8	23,5	18,8	18,5	1,0	0,6	1,1		9
		1	2	3	4	5	6	7	8	9	

Achromobacter xylosoxydans AY753652	
Achromobacter xylosoxydans	
Bacillus cereus	
Delftia acidovorans AY753653	
Delftia acidovorans	
Pseudomonas fluorescens	
Pseudomonas fluorescens AY730552	
Pseudomonas veronii AY748440	
Pseudomonas veronii	

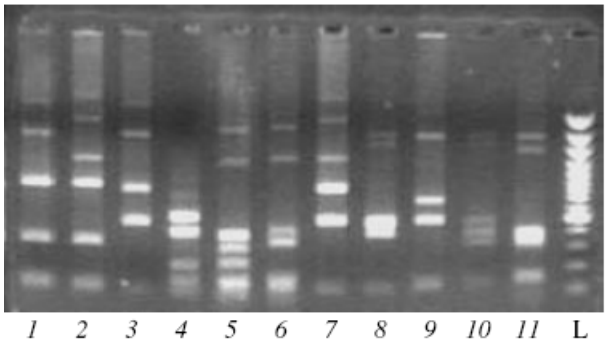

Figure 1. Electrophoretic patterns of major schizotypes present in the associations according to the restriction endonuclease *Rsa*I. (*1–11*) Schizotypes; L, Gene ruler (100 bp DNA ladder).

3.2 Challenging VOCs utilizing pathways by molecular genetic approaches

Associations adapted to one of the VOCs (toluene, styrene, ethyl benzene, *o*-xylene, *m*-xylene, or naphthalene) were developed on the basis of the industrial culture formerly studied. As a result of long-term adaptation to consumption of a certain xenobiotic, associations stably grew and degraded up to 95% of the target VOCs (at concentrations at the inlet of the minireactor varying within 300−350 mg/m^3). We isolated and studied the total genomic DNAs of six associations derived from the same original culture. The presence of genes for catechol dioxygenases was tested by PCR cloning with standard primer pairs for corresponding genes. Genes for C23O (catechol-2,3-dioxygenase) were found in cultures consuming toluene, naphthalene, and *m*-xylene. Genes for C12O (catechol-1,2-dioxygenase) were found in cultures consuming styrene, ethyl benzene, *o*-xylene, and *m*-xylene (Figs. 2a and 2b). For PCR, we chose optimum parameters and reaction conditions to obtain the most reproducible results. This is important for manipulations with putatively heterogeneous PCR products corresponding to several copies of homologous genes in the template DNA. The resulting PCR products were purified and cloned into pGem-T plasmid vector. Both strands of the amplicons were automatically sequenced. To verify the assignment of certain sequences to genes under study, C12O and C23O sequences were compared with similar sequences retrieved from the NCBI gene base. The comparison showed that all cloned PCR products were copies of C12O and C23O genes. We performed multiple alignment between the cloned sequences and those reported previously, to reveal polymorphic positions. Based on the alignment, we characterized phylogenetic differences between all available sequences. The rooted phylogenetic tree shown in Figs. 3a and 3b was constructed by the algorithm implemented in the Custal V package with most typical C12O representatives as a reference group: *CatA* gene from the mt-2 transposon of *P. putida* (NCBI AF363241) and Cat A from plasmids pND6 (NCBI NC005244) and pND6-1 (NCBI AY208917) of *P. putida* ND. The reference group for C23O included *XylE* genes located in plasmids *pWWO* (NCBI AJ344068), *pWW53*, and *pTOL* (NCBI V01161). The trees show that the newly found C12O and C23O genes are highly similar to the supposedly related genes. This similarity indicates that they are highly structurally and functionally conserved.

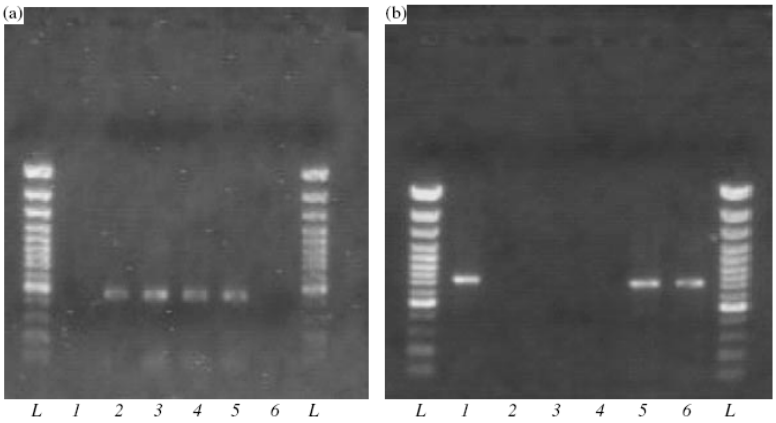

Fig. 2. Electrophoretic patterns of PCR products of the genes for (a) catechol-1,2-dioxygenase (462 bp) and (b) catechol-2,3-dioxygenase (721 bp).

Total genomic DNAs of associations consuming particular substrates were used as templates: *1*, toluene; *2*, styrene; *3*, ethyl benzene; *4*, *o*-xylene; *5*, *m*-xylene; *6*, naphthalene; *L* = 100 bp, DNA molecular weight reference.

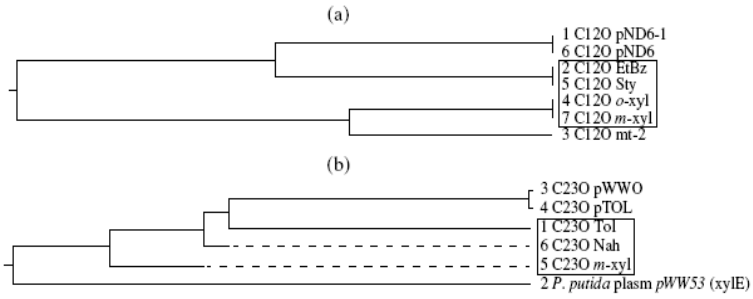

Fig. 3. Phylogenetic tree illustrating the systematic position of the newly found sequences with respect to the reference groups; (a) C12O, (b) C23O.

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