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Application of Molecular Systematics to Study of Bacterial Cultures Consuming Volatile Organic Compounds

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Abstract—A range of species of four mixed bacterial cultures was studied by molecular systematics methods with the use of 16S rRNA genes. The cultures had been developed for application in minireactors, to degrade volatile organic compounds (VOCs): ethyl benzene, *m*-xylene, styrene, and *o*-xylene. A sample of 30 plasmid rDNA clones was obtained for each of the mixed cultures. The clones were analyzed by RFLP according to two restriction sites. Major variants of the 16S-rDNA sequences, corresponding to the most abundant species, were determined for each association. Sequencing of four clones of predominant 16S-rDNAs showed that the culture consuming ethyl benzene was dominated by *Pseudomonas fluorescens*; *o*-xylene, by *Achromobacter xylosoxydans*; styrene, by *Pseudomonas veronii*; and *m*-xylene, by *Delftia acidovorans*. Minor components of all four cultures were generally similar. They included species of the genera *Sphingobacter*, *Rhizobium*, *Mesorhizobium*, *Pedobacter*, and *Paenibacillus*. Sampling sequencing of genes for 16S rRNA cloned from total genomic DNA allowed quantitative determination of the composition of actual bacterial associations consuming VOCs in minireactors.

At present, owing to the rapid development of industrial biotechnology, microbiological methods are in increasing use for purification of industrial waste air containing volatile organic compounds (VOCs) [1–4].

Use of microbiological filters is based on microbial degradation of organic substrates (xenobiotics) to carbon dioxide and water. Unlike physicochemical purification methods (catalytic or common incineration), 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 simple to operate 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 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.

The goal of the present work is to investigate the composition of microbial associations developing during VOC consumption 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 proximate methods for inspection of industrial devices and natural ecosystems.

MATERIALS AND METHODS

Source lyophilized cultures were taken from the microbial collection of the Laboratory of Industrial Enzymology, Bach Institute of Biochemistry, RAS, and from accessions formerly deposited in the All-Russia Collection of Industrial Microorganisms.

Cultures degrading particular xenobiotics were placed into flow pilot minireactors for air purification from VOCs [1–4]. The overall VOC load on each minireactor was 300–350 mg/m^3. The aerial and aqueous phases of the minireactors were nonsterile.

The specificity of the source cultures with respect to the corresponding substrates was checked. The effi-

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ciency of VOC degradation was determined by GLC, as described in [1].

Microbial associations were fairly stable as biocatalysts. They provided a high degree of VOC degradation. Samples of microflora from the biocatalyst surfaces were inoculated onto 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 conditions in the biocatalyst, 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.

The composition of the mineral medium for cultivating microbial degraders was as follows (g/l): KH_2PO_4 (3.4), K_2HPO_4 (4.3), $(NH_4)_2SO_4$ (2.0), $MgSO_4$ (0.2), $CaCl_2$ (0.04), $FeSO_4$ (0.03), $MnCl_2$ (0.001), Na_2MoO_4 (0.0003), $CuSO_4$ (0.0005), H_3BO_3 (0.0035), $ZnSO_4$ (0.001), and distilled water; pH 7.3–7.4. If necessary, pH was adjusted with 1 N NaOH or HCl [1].

Escherichia coli strains were grown on conventional LB or M9 media [5].

Total genomic DNA was isolated from cultures grown on the minimal mineral medium with the presence of corresponding VOCs.

The following strains were used for transformation and cloning of plasmid constructs: BMH 7118 (*thi*, *sup*E Δ(*lac-pro*AB) [*mut*S::Tn10] [F', *pro*AB, *lac*I^{q}Z ΔM15]), NM522 (*sup*E *thi-l* Δ(*lac-pro*AB) Δ(*mcr*B-*hsd*SM) ($r_k - m_k$) [F', *pro*AB, *lac*I^{q}Z ΔM15]).

To isolate total genomic DNA, degrader cultures were grown on petri dishes with agar medium in vapors of corresponding VOCs at 28°C for 48 h. The biomass was harvested by washing with 1.5 ml of sterile minimal mineral medium and placed in a plastic test tube for lysis and DNA isolation.

For isolating DNA, use was made of a Bacterial Genomic DNA Mini-prep Kit (50), manufactured by V-Gene Biotechnology Ltd. (China). The isolation was performed according to the manufacturer's recommendations.

The following primers, described in [5], 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. Polymerase chain reaction (PCR) was carried out in 30 μl of a mixture containing 100 ng DNA, 200 μmol deoxyribonucleotide phosphates (Sibenzyme, Russia), 1.5 mM $MgCl_2$ (Sigma, USA), 200 pmol of each primer, 10 μl 10X Taq buffer (Promega, USA) and 2.5 U Taq polymerase (Bionem, Russia).

The reaction was carried out in a Tertsik MS-2 thermocycler (DNK-Tekhnologiya, Russia) according to the following program: 94°C, 1 min and 25 cycles: 94°C, 10 s; 60°C, 6 s; 72°C, 40 s. The reaction products were stored at 4°C.

Samples of 1–5 pmol of each amplicon were taken for cloning.

The cloning into the pGEM T vector was carried out according to the conventional protocol with pGem®-T Vector System I kits (Promega, USA).

The resulting clones were discriminated by a color plate test on a medium with isopropyl-β-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as an inducer. The primary clones supposed to harbor an insert were screened by PCR with the matter of an *E. coli* recombinant colony as a template.

Plasmid DNA constructs were isolated with a Rapid Plasmid DNA Daily Mini-prep Kit (50) (V-Gene Biotechnology Ltd.) according to the manufacturer's recommendations. The final plasmid concentration for each clone was 20 ng/μl.

To obtain secondary amplicons of cloned 16S rRNA gene sequences, formerly obtained plasmid constructs based on pGem®-T Vector System I (Promega, USA) were used as templates.

Polymerase chain reaction was carried out in 20 μl of a common mixture containing 30 ng of plasmid DNA, 200 μmol deoxyribonucleotide triphosphates (Sibenzyme, Russia), 1.5 mM $MgCl_2$ (Sigma, USA), 200 pmol of each primer, 10 μl 10X Taq buffer (Promega, USA), and 2.5 U Taq polymerase (Bionem, Russia).

Before running the restriction fragment length polymorphism (RFLP) test, amplicons were purified using a PCR Clean-Up Kit (50) (V-Gene Biotechnology, China) according to the manufacturer's recommendations.

For RFLP, we chose two restriction endonucleases, produced by MBI Fermentas (Lithuania): *Msp*I (recognition sequence 3'-G G C^C-5') and *Rsa*I (recognition sequence 3'-C A^T G-5'). The volume of the digestion mixture was 30 μl. The reaction was carried out in Yellow Tango™ buffer (Fermentas, Lithuania).

Restriction products were analyzed by electrophoresis in 1.5% agarose gel and 6% polyacrylamide gel. The results were recorded with a ViTran image recording system (Biokom, Russia).

Cloned amplicons were sequenced on an AbiPrism 3100 automated sequencer (Applied Biosystems, USA) using a standard primer pair:

pUC/M13 for(3'-CGCCAGGGTTTTCCCAGTCACGAC-5');
pUC/M13 rev(3'-AGCGGATAACAATTTCACACAGGA-5').

The results of sequencing were recorded in digital form and processed with the DNA-star (unit SeqMan) and N-BLAST program packages.

The 16S rRNA gene sequences were deposited in digital form with the use of the SEQIN program package.

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.

RESULTS AND DISCUSSION

Nonsterile industrial associations degrading various xenobiotics under both aerobic and anaerobic conditions consist of 2–10 strains in different proportions [7–14]. As a rule, an association is dominated by one or two strains [8, 11], 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 [9, 10] 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 [12, 13]. 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 [11]. 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 [14].

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 main stages of cloning, RFLP analysis, and sequencing of 16S rDNA are schematically shown in Fig. 1.

In cloning and sorting for individual restriction patterns (schizotypes), we used both plasmid DNAs harboring 16S rDNA inserts and DNA fragments obtained by PCR of pGem T-vector-based constructs with standard *pUC-M13* primers.

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 schizotypes 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 a closely 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,

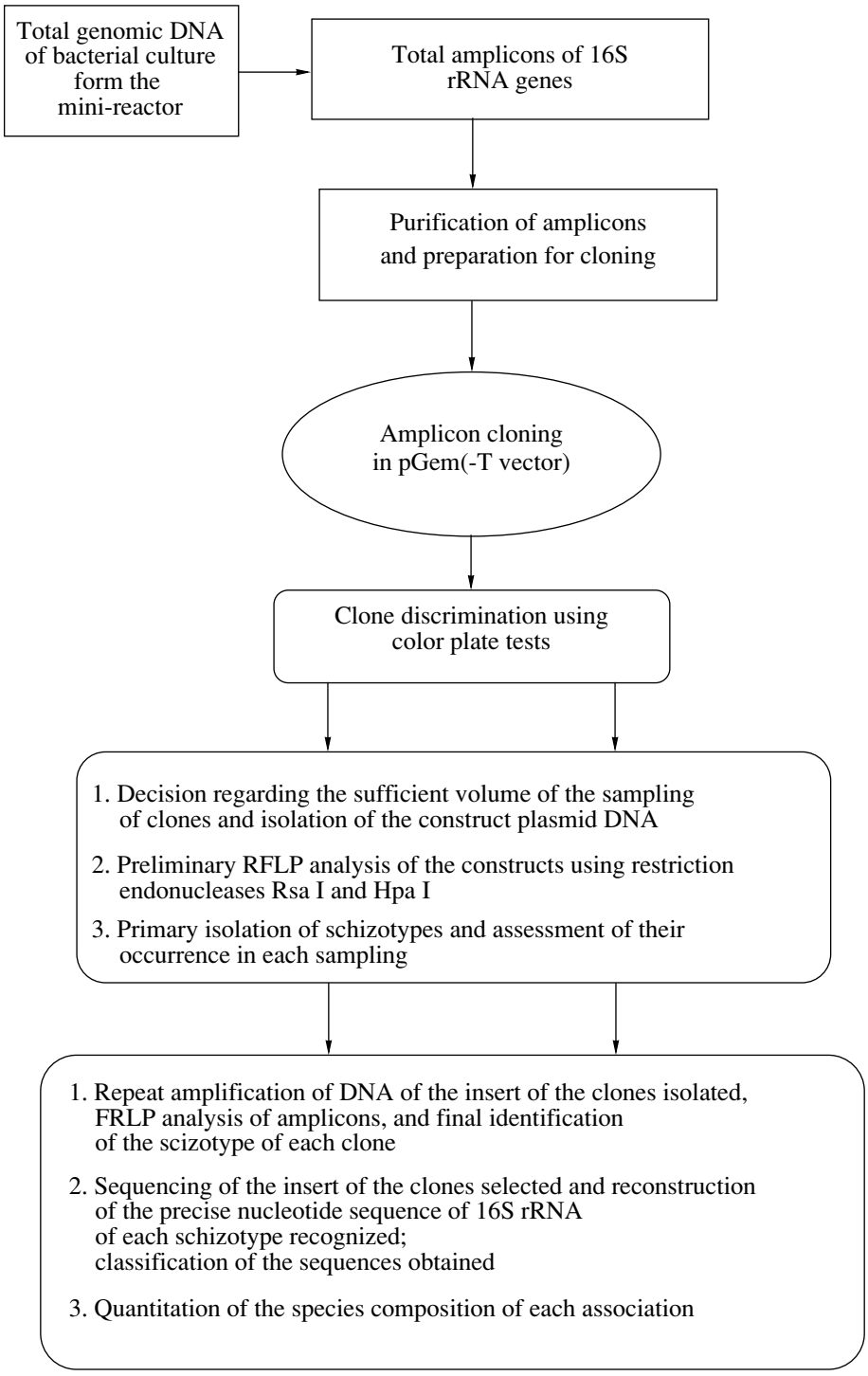

Fig. 1. Design of the experiment.

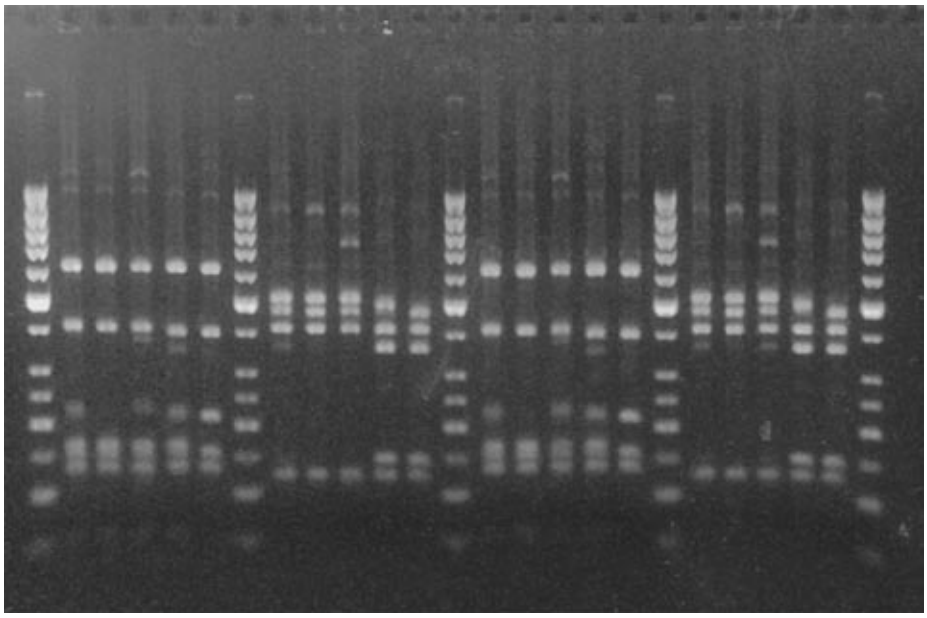

Fig. 2. Restriction analysis of the total products of amplification of 16S rDNA with *Msp*I and *Rsa*I:
(*1*, *11*) *m*-Xyl (*Hpa*II); (*2*, *12*) EtBz (*Hpa*II); (*3*, *13*) Tol (*Hpa*II); (*4*, *14*) *o*-Xyl (*Hpa*II); (*5*, *15*) Sty (*Hpa*II); (*6*, *16*) *m*-Xyl (*Rsa*I); (*7*, *17*) EtBz (*Rsa*I); (*3*, *13*) Tol (*Hpa*II); (*4*, *14*) *o*-Xyl (*Hpa*II); (*5, 15*) Sty (*Hpa*II); (*6*, *16*) *m*-Xyl (*Rsa*I); (*7*, *17*) EtBz (*Rsa*I); (*8*, *18*) Tol (*Rsa*I); (*9*, *19*) *o*-Xyl (*Rsa*I); (*10*, *20*) Sty (*Rsa*I); L, Gene ruler (100 bp DNA ladder).

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 (Fig. 4 and Table 2) showed that the newly found

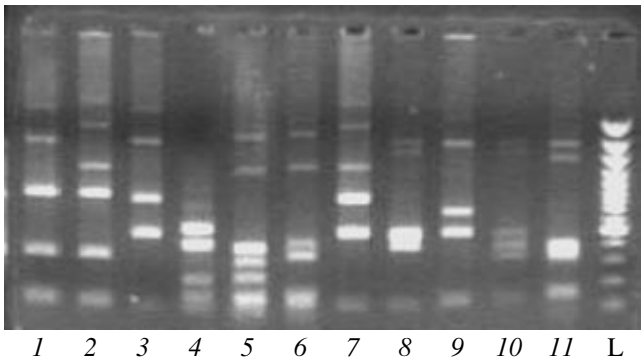

Fig. 3. Electrophoretic patterns of major schizotypes present in the associations according to the restriction endonuclease *Rsa*I.
(*1–11*) Schizotypes; L, Gene ruler (100 bp DNA ladder).

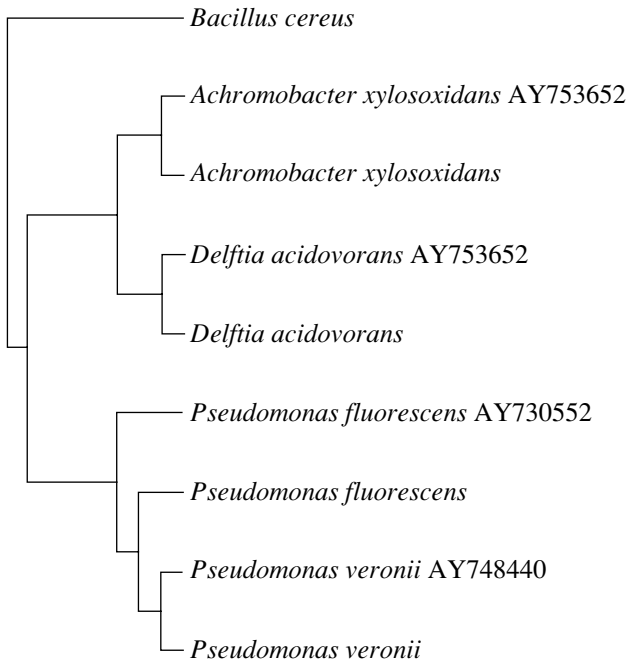

Fig. 4. Phylogenetic tree reflecting the systematic positions of the degrader strains recognized.

Table 1. Sequencing of 16S rRNA genes and accession numbers of sequences stored in NCBI

Consumed substrate	Strain	No.
m-Xylene	*Delftia acidovorans*	AY753653
o-Xylene	*Achromobacter xylosoxidans*	AY753652
Styrene	*Pseudomonas veronii*	AY748440
Ethyl benzene	*Pseudomonas fluorescens*	AY730552

sequences are indeed close to the reference species and genera. This similarity cannot be attributed to insufficient data on particular eubacterial taxa (Table 3).

In this work, we studied four cultures of similar origin, which had long been adapted to consumption of one of the following VOCs: *o*-xylene, styrene, *m*-xylene, or ethyl benzene. It should be noted that the biochemical and enzymological features of formerly reported strains suggest that one pool of enzymes coded by the same or similar gene set is applicable to render the majority of Gram negative bacteria able to consume all the VOCs listed. Our study demonstrated the presence of different major components (predominant strains) in each of the mixed cultures. The association consuming ethyl benzene contained four schizotypes (1, 2, 8, and 10), of which the predominant schizotype 10 (*Pseudomonas fluorescens*) comprised 27 of 30 clones examined (90%). The three remaining strains comprised no more than 10% of the association.

Similar distribution of the abundance of species was observed in other mixed cultures as well, although the predominant strains were different: for *o*-xylene, *Achromobacter xylosoxydans* (80% of the population); for styrene, *Pseudomonas veronii* (83%); and for *m*-xylene, *Delftia acidovorans* (83%).

Different microorganisms dominated in different cultures. They were specific for a particular degrading culture but did not occur in other associations even as accessory components. In contrast, minor components of all mixed cultures studied were generally the same. The most typical of them were species of the genus *Sphingobacterium* and, to a lesser extent, nitrogen-fixing bacteria of the genera *Rhizobium* and *Mesorhizobium*, *Pedobacter*, and a psychrophilic gram-positive strain of the genus *Paenibacillus*.

Thus, the range of species and abundance of minor components of bacterial associations were stable, in contrast to predominant strains, specific to VOC types. This suggests that dominant and minor components complement each other in the course of adaptation to the environment in minireactors. This favors the stability and viability of the association but hampers its separation by conventional microbiological methods.

Table 2. Statistical processing of aligned sequences coding for 16S rRNA

	Matches, %											
		1	2	3	4	5	6	7	8	9		
Difference, %	1		97.8	65.9	81.8	84.0	80.2	79.0	77.5	79.3	1	*Achromobacterxylosoxydans* AY753652
	2	0.6		65.9	83.8	84.6	81.3	81.5	79.5	80.7	2	*Achromobacter xylosoxydans*
	3	28.0	27.5		64.4	67.1	74.1	70.3	68.1	71.5	3	*Bacillus cereus*
	4	14.0	13.4	29.3		97.5	79.0	79.1	77.9	78.5	4	*Delftia acidovorans* AY753653
	5	13.2	12.8	28.3	0.9		80.7	79.3	78.3	80.1	5	*Delftia acidovorans*
	6	17.4	17.1	22.8	18.5			94.8	97.9	98.9	6	*Pseudomonas fluorescens*
	7	16.8	16.4	22.0	17.8	17.2	0.5		97.8	98.9	7	*Pseudomonas fluorescens* AY730552
	8	19.4	18.9	24.5	19.4	19.6	1.9	1.5		98.2	8	*Pseudomonas veronii* AY748440
	9	18.3	17.8	23.5	18.8	18.5	1.0	0.6	1.1		9	*Pseudomonas veronii*
		1	2	3	4	5	6	7	8	9		

Table 3. Frequencies of occurrence of schizotypes of rDNA clones in associations adapted to various VOCs

Maximum similarity to the NCBI accession, %	Fragments		Occurrence of a schizotype in a sample depending on the consumed substrates, clone				Schizotype no.
	according to *Rsa*I	according to *Msp*I	ethyl benzene	*o*-xylene	styrene	*m*-xylene	
AB020205	900	500	1	1	1	–	1
Sphingobacterium sp.,	300	400					
97%	200	200					
	60	180					
		90					
		90					
AF433165	800	540	1	–	1	1	2
Paenibacillus sp.,	300	380					
95.4%	200	260					
	160	100					
		90					
		90					
U50168	900	550	–	1	1	1	3
Rhizobiom sp. CJ5, 90.4%	300	400					
	160	200					
	100	180					
		100					
AJ491845	600	800	–	24	–	–	4
Achromobacter xylosoxydans,	500	300					
95%	200	200					
	160	160					
AY206686	400	700	–	1	1	1	5
Mesorirhizobium ciceri,	350	490					
97.7%	300	180					
	200	90					
	160						
AF181575	580	600	–	–	–	25	6
Delftia acidovorans, 97%	450	400					
	240	200					
	160	190					
		80					
AB020205	700	500	–	1	–	1	7
Sphingobacterium	560	390					
multivorum, 96.2%	160	200					
		180					
		100					
		90					
AY599662	560	500	1	1	1	–	8
Pedobacter sp.	530	400					
TB2-14II, 96%	200	200					
	100	180					
		100					
		80					

Table 3. (Contd.)

Maximum similarity to the NCBI accession, %	Fragments		Occurrence of a schizotype in a sample depending on the consumed substrates, clone				Schizotype no.
	according to *Rsa*I	according to *Msp*I	ethyl benzene	*o*-xylene	styrene	*m*-xylene	
AB020205	680	500	–	1	–	1	9
Sphingobacterium sp., 98.6%	560	390					
	180	200					
		180					
		100					
		90					
D84013	600	800	27	–	–	–	10
Pseudomonas fluorescens, 99.2%	560	300					
	200	200					
	160	160					
AB021411	560	800	–	–	25	–	11
Pseudomonas veronii, 99.6%	470	300					
	230	200					
	160	160					

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