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mRNA Differential Display in a Microbial Enrichment Culture: Simultaneous Identification of Three Cyclohexanone Monooxygenases from Three Species

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mRNA differential display has been used to identify cyclohexanone oxidation genes in a mixed microbial community derived from a wastewater bioreactor. Thirteen DNA fragments randomly amplified from the total RNA of an enrichment subculture exposed to cyclohexanone corresponded to genes predicted to be involved in the degradation of cyclohexanone. Nine of these DNA fragments are part of genes encoding three distinct Baeyer-Villiger cyclohexanone monooxygenases from three different bacterial species present in the enrichment culture. In *Arthrobacter* sp. strain BP2 and *Rhodococcus* sp. strain Phi2, the monooxygenase is part of a gene cluster that includes all the genes required for the degradation of cyclohexanone, while in *Rhodococcus* sp. strain Phi1 the genes surrounding the monooxygenase are not predicted to be involved in this degradation pathway but rather seem to belong to a biosynthetic pathway. Furthermore, in the case of *Arthrobacter* strain BP2, three other genes flanking the monooxygenase were identified by differential display, demonstrating that the repeated sampling of bacterial operons shown earlier for a pure culture (D. M. Walters, R. Russ, H. Knackmuss, and P. E. Rouvière, Gene 273:305–315, 2001) is also possible for microbial communities. The activity of the three cyclohexanone monooxygenases was confirmed and characterized following their expression in *Escherichia coli*.

It is now well recognized that the diversity of microbial species and their metabolic capabilities constitute a tremendous source of biocatalysts (6, 10, 39). Only a small fraction of microorganisms in most environments can be readily isolated (1, 58); therefore, gene discovery techniques which overcome the need for strain isolation provide access to the diversity of microbial chemistry. Direct cloning approaches can be very successful (21, 27, 28, 48), but they require a genetic selection or an easy screen as well as the efficient expression of the cloned DNA in an appropriate host (15). Other approaches, based on PCR amplification from environmental DNA, target only highly conserved gene families (50). While these techniques are powerful, they often are not applicable. Differential display (DD) is an alternate technique that can be used for the discovery of bacterial genes, requiring neither a genetic selection or screen nor the presence of highly conserved genes. This technique of DD involves the reproducible amplification of DNA fragments from the mRNA population at arbitrary sites by reverse transcription (RT) followed by PCR (RT-PCR) (36, 37, 57). DD is used to compare the mRNA pools from cells grown under different physiological conditions. Genes expressed at the same level in all cultures will be amplified equally from all cultures, while genes expressed only under a specific condition will give rise to RT-PCR bands only under that condition. DD is a gene discovery technique that can be applied to identify differentially expressed genes. It does not rely on prior knowledge of the genes targeted or on a genomic sequence but only on the fact that the activity that these genes encode is inducible.

DD has been applied extensively to eukaryotic systems and takes advantage of the poly(A) tails of eukaryotic mRNA by using poly(dT) primers to synthesize cDNAs by RT (36, 37, 57). This approach of DD cannot be applied to prokaryotes, which lack stable poly(A) tails. A second variation of DD uses arbitrary oligonucleotide primers to initiate RT of the message at random sites (57) and thus can be applied to archaeal and bacterial species. Application of prokaryotic DD has been limited to fewer than 25 studies, half of them published in the last 2 years (2–4, 9, 16, 25, 26, 44, 46, 47, 52, 56). We have recently shown that a high-throughput approach to DD, using a large set of arbitrary oligonucleotides to initiate RT-PCR, resulted in the repeated identification of an operon responsible for the degradation of 2,4-dinitrophenol (56). We called this high-throughput approach to DD high-density sampling differential display.

Our objective for the present study was to apply high-density sampling DD to identify multiple genes or operons carrying out the dominant physiology of a microbial community. The culture used for this work originated from a wastewater bioreactor and was enriched for growth on cyclohexanone. We show here that DD is a robust technique for gene discovery in prokaryotes and is well suited for isolating genes encoding metabolic enzymes from complex microbial communities.

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MATERIALS AND METHODS

Enrichment for cyclohexanone-degrading bacteria. An enrichment culture degrading cyclohexanone was obtained by the successive transfers of wastewater

bioreactor sludge in mineral salt medium [50 mM $KHPO_4$ (pH 7.0), 10 mM $(NH_4)SO_4$, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MnCl_2$, 1 μM $FeCl_3$, 1 μM $ZnCl_3$, 1.72 μM $CuSO_4$, 2.53 μM $CoCl_2$, 2.42 μM Na_2MoO_2, and 0.0001% $FeSO_4$] with 0.1% cyclohexanone added as a carbon source.

Community analysis. A terminal-restriction fragment length polymorphism (T-RFLP) analysis was performed to determine the complexity of the community (33, 40). For T-RFLP, DNA was extracted from 1 ml of enrichment culture that was resuspended in 200 μl of buffer P1 from the RNeasy RNA purification kit (Qiagen, Valencia, Calif.). Buffer P2 from the same kit and 0.3 ml of zirconia beads (Biospec Products, Bartlesville, Okla.) were added to the resuspended cells in bead beating tubes. The cells were then disrupted at 2,400 beats per min for 2 min in a bead beater (Biospec Products). DNA was purified by standard phenol extraction and ethanol precipitation protocols (49). 16S ribosomal DNA (rDNA) genes were amplified in a standard PCR by using *Taq* (Qiagen), a rhodamine-labeled primer (5′-ACGGGCGGTGTGTAC-3) and a second non-labeled primer (5′-GAGTTTGATCCTGGCTCAG-3′). The PCR conditions included a single 5-min cycle at 94°C, 20 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and one final elongation cycle at 72°C for 7 min. Following amplification, four separate PCRs were purified by using the QIAquick PCR purification kit (Qiagen) and eluted in 80 μl of H_2O each. Thirty-eight microliters of this product was used in a 50-μl digestion reaction volume with either *Alu*I, *Mse*I, or *Nla*III restriction enzymes used as indicated by the manufacturer (New England Biolabs, Beverly, Mass.). Restriction fragment lengths were determined on an ABI 3700 sequencer in the GeneScan mode. The data were collected by using GeneScan Analysis 3.1 software (Applied Biosystems, Foster City, Calif.). Finally, the results were analyzed internally by using PatScan. The fluorescence threshold was placed at 100 relative fluorescence units. Fragments with sizes smaller than 50 bp were not included in the analysis. Predicted lengths of T-RFLP fragments for identified species were matched with chromatograms within 2 bp (29).

Individual strains were isolated from the community by spreading the enrichment culture on R2A Agar (Difco, Sparks, Md.) at 30°C. Strains were streaked to purity on the same medium and were identified by 16S rDNA sequence analysis. 16S rDNA was amplified from chromosomal DNA by using several primers corresponding to conserved regions of the 16S rDNA gene (32). The following temperature program was used: 95°C for 5 min; 25 cycles of 95°C for 1 min; 55°C for 1 min; 72°C for 1 min, followed by 72°C for 8 min, and then a 4°C hold.

Induction of cyclohexanone oxidation genes. One milliliter of the culture was suspended in 25 ml of minimal medium (described above) with 0.1% yeast extract, Casamino Acids, and peptone (YECAAP) and incubated overnight at 30°C with agitation. During this incubation residual cyclohexanone was consumed. The next day 10 ml of the overnight culture was resuspended in a total volume of 50 ml of minimal medium with 0.1% YECAAP to an optical density at 600 nm of 0.29. After equilibration at 30°C for 30 min, the culture was split into two separate flasks. Cyclohexanone (0.1%) was added to one of these 25-ml cultures, and both cultures were incubated for an additional 3 h. After that time, the cultures were chilled on ice, harvested by centrifugation in a rotor cooled to −4°C, washed with 2 volumes of ice-cold minimal salts medium, and diluted to an optical density of 1 at 600 nm. Six milliliters of culture were placed in a water-jacketed respirometry cell equipped with an oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) at 30°C. After establishing the baseline respiration for each cell suspension, cyclohexanone was added to a final concentration of 0.1% and the rate of O_2 consumption was further monitored. To confirm the viability of the control culture, 2 mM potassium acetate was added after the cyclohexanone.

Isolation of total cellular RNA. RNA isolation was performed with the same cultures that were used for the respirometry experiment. After the 3-h induction period with cyclohexanone that was described above, 2 ml each of the control and induced samples was harvested by centrifugation at 17,000 × *g* in a rotor cooled to −4°C and resuspended in 900 μl of buffer RLT (Qiagen). A 300-μl volume of zirconia beads (Biospec Products) was added, and cells were disrupted by use of a bead beater (Biospec Products) at 2,400 beats per min for 3 min. Each of these samples was split into six aliquots for nucleic acid isolation by the RNeasy RNA purification kit (Qiagen), and each was eluted with 100 μl of RNase-free distilled water supplied with the kit. DNA was degraded in the samples by using 10 mM $MgCl_2$–60 mM KCl–2 U of RNase-free DNase I (Ambion, Austin, Tex.) at 37°C for 4 h. Following testing for total DNA degradation by PCR with one of the arbitrary oligonucleotides used for RT-PCR, RNA was purified by use of the RNeasy minikit in the same manner as described above. The RNA was eluted from the column in 100 μl of RNase-free H_2O.

Generation of randomly amplified polymorphic DNAs from arbitrarily reverse-transcribed total RNA. A set of 240 primers with the sequence CGGAG CAGATCGAWXYZ, where WXYZ represents all but four of the 244 combinations of the three bases A, G, and C, were used in 480 separate RT-PCRs with RNA from either control or induced cells (56). These 480 reactions were performed in five 96-well PCR plates in which each primer was distributed in two adjacent wells. The four primer variants that were predicted to form the strongest primer dimers were omitted from the experiment.

The SuperScript one-step RT-PCR system (Life Technologies Gibco BRL, Rockville, Md.) reaction mixture was used with 2 to 5 ng of total RNA per individual 25-μl reaction volume. For each 96-well PCR plate, two 2.5-ml reaction mixtures sufficient for 48 reactions were prepared according to the manufacturer's instructions. Each contained buffer, nucleotides, RNA and DNA polymerase, and one of the two RNA samples (0.1 to 0.2 μg of total RNA). Each mixture was dispensed with a multichannel pipette in the odd or even wells of the 96-well PCR plates containing the prealiquoted oligonucleotide primers.

The following temperature program was used: 4°C (2 min), 5-min ramp to 37°C (1 h), followed by 95°C incubation (3 min), 1 cycle with 94°C (1 min), 40°C (5 min), and 72°C (5 min), 40 cycles with 94°C (1 min), 60°C (1 min), and 72°C (1 min), followed by an incubation at 70°C (5 min) and 4°C. Products of these PCR amplifications were separated by electrophoresis at 1 V/cm in polyacrylamide gels (Amersham Pharmacia Biotech, Piscataway, N.J.). Products resulting from the control mRNA (no cyclohexanone induction) and from the mRNA from induced cells were analyzed side by side and visualized by silver staining by use of an automated gel stainer (Amersham Pharmacia Biotech).

Reamplification of differentially expressed DNA fragments. A 25-μl volume of DNA elution buffer (10 mg of NaCN/ml, 20 mM Tris-HCl [pH 8.0], 50 mM KCl, and 0.05% NP-40) was incubated with each excised gel band containing a differentially amplified DNA fragment at 95°C for 20 min. Reamplification of this DNA fragment was achieved in a PCR by using 5 μl of the elution mixture in a 25-μl reaction volume with the primer used in the RT-PCR. The temperature program for reamplification was as follows: 94°C (5 min), 20 cycles of 94°C (1 min), 55°C (1 min), and 72°C (1 min), followed by 72°C (7 min). The reamplification products were directly cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, Calif.) and were sequenced by using an ABI 377 DNA sequencer with ABI BigDye terminator sequencing chemistry (Applied Biosystems). To compensate for the possible reamplification of background DNA excised with the RT-PCR bands, eight clones were sequenced for each band reamplified. The nucleotide sequences of the cloned fragments were compared against the nonredundant GenBank database by using the BlastX program (National Center for Biotechnology Information).

Sequencing of cyclohexanone oxidation pathway genes. *Rhodococcus* sp. strain Phi2 and *Arthrobacter* sp. strain BP2 cosmid libraries were constructed with the pWEB cosmid cloning kit (Epicentre Technologies, Madison, Wis.). The *Rhodococcus* strain Phi1 cosmid library was constructed with the SuperCos 1 cosmid vector kit (Stratagene, La Jolla, Calif.). Cosmids were screened by PCR with primers designed against the differentially amplified fragments with homology to known cyclohexanone degradation genes (Table 1). Recombinant *Escherichia coli* strains carrying the cosmid clones were used as the template in these PCRs with 1 μl of cell culture added to 24 μl of PCR mixture. Cosmids from recombinant *E. coli* from any of the three libraries screened, yielding a product of the size corresponding to a monooxygenase gene, were partially digested with Sau3A1. Fragments with sizes between 10 and 15 kb from these partial digests were subcloned into the cloning vector pSU19 (41). These subcloned plasmids

TABLE 1. 16S rDNA typing of strains isolated from the cyclohexanone degrading enrichment[a]

Isolate (strain)[b]	Closest relative	Accession no.	% Identity
a (BP2)	*Arthrobacter keyseri* strain 12B	AF256196	99
b (Phi1)	*Rhodococcus pyridinovorans*	AF173005	99
c (Phi2)	*Rhodococcus ruber*	RR16SR6	99
d	*Caulobacter intermedius*	AB023784	96
e	*Brevundimonas diminuta*	BD16S1635	97
f	*Stenotrophomonas* sp. strain DB1	AF309081	98
g	*Rhizobium* sp. strain X59	AF345555	95
h	*Bacillus* sp.	AB017592	91

[a] Strains were isolated on R2A medium. *Arthrobacter* strain BP2, *Rhodococcus* strain Phi1, and *Rhodococcus* strain Phi2 can grow on cyclohexanone as a sole carbon and energy source.

[b] The predicted T-RFLP fragment for each strain is indicated in Fig. 1.

were isolated by using Qiagen Turbo96 Miniprep kits and rescreened by PCR as described above. Plasmids carrying the correct sequence were disrupted by in vitro transposition using the GPS-1 genome priming system kit (New England Biolabs, Inc.). Plasmids carrying randomly inserted transposons were sequenced from each end of the transposon to obtain the sequence of kilobase-long DNA fragments. Sequence assembly was performed with the Sequencher program (Gene Codes Corp., Ann Arbor, Mich.).

Sequence analysis. Sequences obtained from the cosmids were compared to the nonredundant GenBank Database at the National Center for Biotechnology Information by using the BlastX program. Multiple sequence alignments were generated by use of the ClustalW program. Phylogenetic trees were calculated with the neighbor-joining tree method.

Biochemical characterization of monooxygenases. The cyclohexane monooxygenase genes from *Arthrobacter* strain BP1, *Rhodococcus* strain Phi1, and *Rhodococcus* strain Phi2 were cloned into the expression vector pTrcHis-topo (Invitrogen) such that the expressed proteins contained an N-terminal histidine tag. To overexpress each of these proteins, a 1-liter *E. coli* culture was grown in Luria-Bertani broth with riboflavin (1 μg/ml) at 30°C until the absorbance at 600 nm reached 0.5. At this point, the temperature was shifted to 16°C and the cultures were allowed to equilibrate for 0.5 h, and then IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mM to induce expression. Culture growth was allowed to proceed at 16°C overnight (~14 h), and cells were harvested and resuspended in 5 ml of buffer A (300 mM NaCl, 5% glycerol, 20 mM Tris-HCl [pH 8.0]) containing 10 mM EDTA and 10 μg of lysozyme/ml. Following a 30-min incubation on ice, cells were disrupted by sonication and the particulate fraction was removed by centrifugation. The supernatant was mixed for 1 h at 4°C with 500 μl of a metal chelation agarose (Ni-nitrilotriacetic acid Superflow; Qiagen). The resin was washed batchwise with a series of 10-ml volumes of buffer A containing 0.15, 0.3, 0.45, 0.6, 1.2, and 60 mM imidazole in order to remove proteins binding nonspecifically. The bound proteins were then eluted with 300 mM imidazole buffer, the eluted proteins were concentrated by ultrafiltration with a Centricon device (cutoff, 10,000 Da; Amicon, Danvers, Mass.), and the buffer was replaced by buffer A such that the final concentration of protein was 1 mg/ml. The monooxygenase activity of each overexpressed enzyme was assayed spectrophotometrically by monitoring the decrease of absorbance at 340 nm which corresponds to the co-oxidation of NADPH. Assays were performed in quartz cuvettes that contained the following in a 400 μl volume: 31.7 mM morpholineethanesulfonic acid (MES)-HEPES-sodium acetate buffer (pH 7.5), 15 mM NADPH, 1.25 mM of each substrate, and 25 ng of enzyme solution/ml.

Nucleotide sequence accession numbers. The sequences of the three gene clusters have been deposited in GenBank under the accession numbers AY123972, AY123973, and AY123974.

RESULTS

Enrichment for a cyclohexanone-degrading microbial population. To test the applicability of DD to mixed microbial communities as a method to identify genes involved in cyclohexanone degradation, we established an enrichment culture growing on 0.1% cyclohexanone. The overall complexity of this microbial population was assessed by T-RFLP analysis. T-RFLP identifies "ribotypes," i.e., groups of species generating DNA fragments of the same length when analyzed with a specific fluorescently labeled primer and restriction enzyme (33). T-RFLP analysis cannot identify species or even genera precisely, since distantly related genera can yield the same T-RFLP fragment of identical length. For our purpose this technique provides an estimation of the complexity of the population and sets a measure of its minimum complexity. The electrophoretic patterns of T-RFLP fragments obtained with three different restriction enzymes are presented in Fig. 1

At the sensitivity and resolution of the ABI3700 sequencing machine, more than 50 peaks, each with individual fluorescence greater than 0.1% of the total fluorescence, were seen for each digest. Each peak corresponds to a different ribotype. These data indicate that at least 50 species were present in the enrichment in an abundance >0.1%. The combined fluorescences of the rare ribotypes (fluorescence between 0.1 and 1% of the total fluorescence) contribute to a significant fraction of the population, accounting for between 6 and 18% of the total fluorescence according to the restriction enzyme used. On the other hand, 7 or 8 ribotypes (according to the restriction enzyme used) account for approximately 70% of the fluorescence. As judged by the complexity of the T-RFLP patterns, such a population is qualitatively as complex as those reported for activated sludges from municipal wastewater treatment plants (29, 38, 40) but not as complex as those in natural environments such as soils (40), aquifers (38,), or open sea waters (43). However, the population was more complex than specialized natural populations such as arid soil populations (20) or those surrounding rice roots (17).

Strain isolation and analysis were carried out in addition to the T-RFLP analysis of this enrichment. Serial dilutions of the enrichment were spread at 30°C on R2A medium, a low-nutrient medium used for environmental isolates. After 72 h, eight strains with different colony morphologies or colors were isolated. These strains were typed by sequencing of their 16S rDNA genes (Table 1). The positions of the T-RFLP fragments predicted from their sequence are shown in Fig. 1. Three of the eight strains isolated, *Arthrobacter* strain BP2, *Rhodococcus* strain Phi1, and *Rhodococcus* strain Phi2, could use cyclohexanone as a sole source of carbon and energy.

The predicted T-RFLP fragments for *Arthrobacter* strain BP2 correspond to a predominant peak in samples digested with all three restriction enzymes. Assuming that all species yield a T-RFLP fluorescence signal representative of their abundance (despite many caveats relative to cell disruption, PCR amplification bias, and number of RNA operons [29, 33]), the calculated *Alu*I T-RFLP fragment of *Arthrobacter* strain BP2 (129 bp matching peaks at 131 bp) indicates that that species probably does not account for more than 15% of the enrichment culture. Similarly, the two *Rhodococcus* species cannot be distinguished by the three restriction enzymes used in our analysis. The calculated *Mse*I T-RFLP fragment (120 bp) indicates that these two *Rhodococcus* species combined account for no more than 2% of the population.

Induction of cyclohexanone oxidation genes. To test for induction, the enrichment culture was grown overnight in minimal medium supplemented with 0.1% YECAAP but lacking cyclohexanone, in order to allow the cells to return to an uninduced state. Subsequently the culture was diluted fivefold in fresh minimal medium, with YECAAP being then split into two separate cultures, one of which received 0.1% cyclohexanone. After 3 h, oxygen consumption in each culture was tested. As shown in Fig. 2, the culture previously exposed to cyclohexanone increased its rate of O_2 consumption upon addition of cyclohexanone (Fig. 2, top panel), indicating that the genes responsible for cyclohexanone oxidation were induced. The control culture, not previously exposed to cyclohexanone, showed no increase in O_2 consumption upon addition of cyclohexanone (Fig. 2, bottom panel). A decrease in O_2 saturation was observed when acetate was added to the control culture, confirming that the cells were metabolically active.

DD analysis of the enrichment culture. Following the demonstration that the degradation of cyclohexanone was inducible in at least some members of the enrichment community, we performed the DD analysis of the genes induced by cyclo-

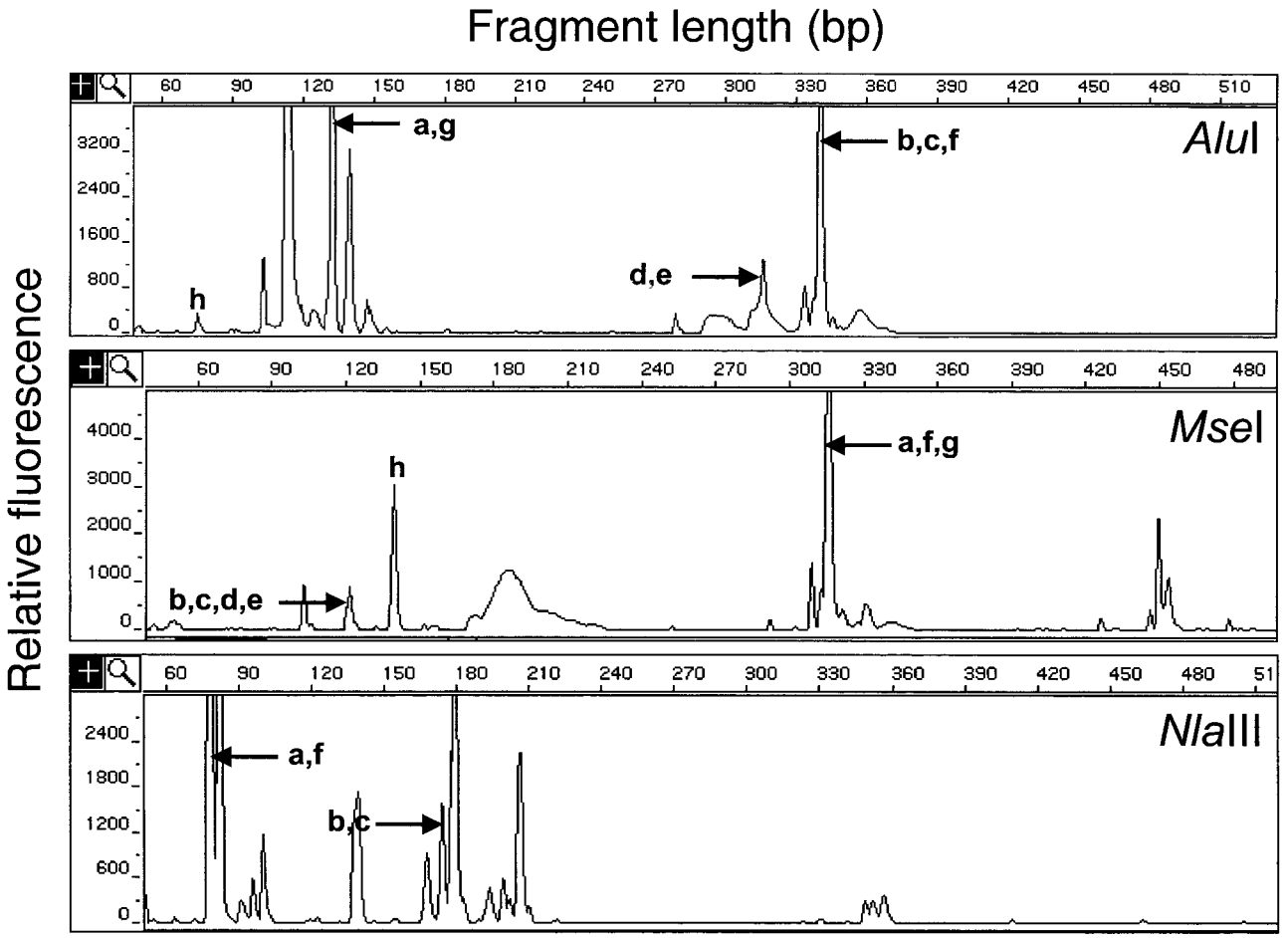

FIG. 1. Complexity of the cyclohexanone degradation enrichment. T-RFLP analysis was performed with DNA extracted from the enrichment culture. Three restriction enzymes, *Alu*I, *Mse*I, and *Nla*III, were used. Letters indicate the T-RFLP fragment predicted for each of the eight bacterial strains isolated from the enrichment: a, *Arthrobacter* strain BP2; b, strain *Rhodococcus* strain Phi1; c, strain *Rhodococcus* strain Phi2. Other isolates (d to h) are listed in Table 1. Fragments smaller than 50 bp were not recorded. Isolates d, e, g, and h have a predicted T-RFLP fragment 41 bp long with *Nla*III.

hexanone. Randomly amplified DNA fragments were generated from total RNA of the cyclohexanone-induced and control enrichment cultures in 240 parallel RT-PCR experiments. Each pair of RT-PCRs was directed with a different primer (56). From approximately 10,000 RT-PCR DNA fragments visualized on polyacrylamide gels, we chose to analyze 59 bands that were amplified from RNA of the cyclohexanone-induced culture but not from RNA of the control culture. After excision from the gel, these differentially amplified fragments (100 to 400 bp) were reamplified by PCR and cloned. The sequences from eight isolates of each cloning experiment were obtained. Twelve fragments among the 59 analyzed were recognized as putative homologues of genes necessary for biochemical conversion of cyclohexanone to adipic acid as described for *Acinetobacter* sp. strain SE19 (Fig. 3). Nine of the 12 fragments encoded protein sequences similar to that of the ChnB cyclohexanone monooxygenase from *Acinetobacter*. One of them overlapped two open reading frames (ORFs) (Fig. 4). The upstream region encoded a homologue of the C terminus of a cyclohexanone monooxygenase, while the downstream region encoded the N terminus of an aldehyde dehydrogenase similar to the 6-oxohexanoate dehydrogenase from *Acinetobacter* (12, 30). Of the other four fragments, two fragments were homologues of the hydrolases similar to the ε-caprolactone hydrolase, and two were homologues of a hydroxy-acid dehydrogenase like the 6-hydroxycaproate dehydrogenase. Several of these fragments had sequence overlaps as depicted in Fig. 4. Homologues identified for the remaining 46 differentially amplified fragments not predicted to be involved in cyclohexanone oxidation included metabolic genes as well as genes coding for core physiological functions such as ribosomal proteins or DNA polymerase. With the exception of stable RNA genes that were differentially amplified in eight RT-PCR DNA fragments, no gene outside those proposed to be involved in cyclohexanone oxidation was sampled more than once. These genes were not studied further.

Identification and sequencing of genes encoding cyclohexanone oxidation enzymes. Following the DD experiment, we tried to determine whether the differentially expressed gene fragments that potentially belong to cyclohexanone oxidation genes originated from one of the isolated strains. Primers based on the sequences of the differentially amplified mono-

TABLE 2. Sequence similarity of ORFs involved in cyclohexanone degradation[a]

ORF	Closest homologues involved in cyclohexanone degradation (organism)	Accession no. or reference	Identity (%)	Similarity (%)	E value[b]
Arthrobacter strain BP2 ChnD	6-Hydroxycaproate dehydrogenase (*Arthrobacter oxydans*)	13	90	93	e-121
Arthrobacter strain BP2 ChnC	Caprolactone hydrolase (*Arthrobacter oxydans*)	13	85	90	e-153
Arthrobacter strain BP2 ChnC	Cyclohexanone monooxygenase (*Xanthobacter flavus*)	CAD10801	60	74	0.0
	ChnB Cyclohexanone monooxygenase (*Acinetobacter* sp. strain SE19)	AAG10026	57	72	e-180
Arthrobacter strain BP2 ChnE	Succinic semialdehyde dehydrogenase (*Deinococcus radiodurans*)	BAA21377	51	67	e-131
	ChnE 6-oxohexanoic dehydrogenase (*Acinetobacter*)	AAG10022	33	52	4e-66
Arthrobacter strain BP2 ChnR	TetR/AcrR family transcriptional regulator (*Bacillus subtilis*)	BAA19366	33	55	e-20
Rhodococcus strain Phi2 ChnR	ChnR2 transcriptional regulator (*Brevibacterium* sp. strain HCU)	AAK73166	31	44	8e-54
Rhodococcus strain Phi2 ChnD	ChnD 6-hydroxyhexanoate dehydrogenase (*Brevibacterium* sp. strain HCU)	AAK73165	48	71	2e-99
Rhodococcus strain Phi2 ChnC	ChnC 6-hexanolactone hydrolase (*Acinetobacter* sp. strain NCIMB9871)	BAB61745	59	75	2e-96
Rhodococcus strain Phi2 ChnB	Cyclohexanone monooxygenase (*Xanthobacter flavus*)	CAD10801	59	72	0.0
Rhodococcus strain Phi2 ChnE	Succinic semialdehyde dehydrogenase (*Deinococcus radiodurans*)	BAA21377	53	68	e-137
	ChnE 6-oxohexanoic dehydrogenase (*Acinetobacter*)	AAG10022	32	50	e-62
Rhodococcus strain Phi1 ChnB	Cyclohexanone monooxygenase (*Xanthobacter flavus*)	CAD10801	58	71	0.0

[a] The predicted functions of surrounding genes not involved in cyclohexanone degradation pathways are shown in Fig. 4.
[b] Probability that sequence similarity is due to chance.

oxygenase gene fragments were used to screen by PCR for the presence of the various gene fragments in the eight strains isolated. DNA extracted from the whole enrichment culture was used as a positive control template. These fragments could be specifically amplified from either the *Arthrobacter* sp. strain BP2, *Rhodococcus* sp. strain Phi1, or *Rhodococcus* sp. strain Phi2 but not from the other isolated strains (data not shown). Products of these amplifications were sequenced to confirm their identity.

The DNA regions flanking each monooxygenase were cloned and sequenced (Fig. 4). Contigs assembled from *Arthrobacter* strain BP2 and *Rhodococcus* strain Phi2 carried the four genes required for oxidation of cyclohexanone to adipic acid as determined for *Acinetobacter* sp. strain SE19 (12) (Fig. 3, Table 2). The organization of the gene clusters in *Arthrobacter* strain BP2 is identical to that of *Rhodococcus* strain Phi2 with respect to the sequence and position of the metabolic genes. However, the organization differs from that of the cyclic ketone degradation gene clusters in *Acinetobacter* strain SE19 (12), *Brevibacterium* strain HCU (8), *Arthrobacter* (13), and *Rhodococcus* strain SC1 (34). Both *Arthrobacter* strain BP2 and *Rhodococcus* strain Phi2 gene clusters also lack a short-chain Zn-independent alcohol dehydrogenase homologue of the cyclohexanol dehydrogenases found in *Acinetobacter* strain SE19 (12), *Arthrobacter* (13), and *Brevibacterium* strain HCU (8).

Once the sequences of the three gene clusters were determined, we compared them to those of all the differentially expressed bands identified in the DD experiment. As represented in Fig. 3, each of the 12 fragments predicted to be involved in cyclohexanone oxidation were included in one of the three gene clusters sequenced. Four of the five cyclohexanone oxidation genes from *Arthrobacter* strain BP2 were represented in the differentially expressed fragments, but only the cyclohexanone monooxygenase was sampled from each of the *Rhodococcus* species. A 13th differentially amplified fragment with homology to the gene of a TetR family transcriptional regulator was found later to be also located on the *Arthrobacter* gene cluster (Fig. 4).

The regions surrounding the cyclohexanone degradation genes in both *Arthrobacter* strain BP2 and *Rhodococcus* strain Phi2 include genes characteristic of the degradation pathways of aromatic compounds. ORF7 in *Arthrobacter* strain BP2 as well as the partial ORF1 in *Rhodococcus* strain Phi2 encode carboxy-muconolactone decarboxylases. The fragment of a carboxy-muconolactone decarboxylase gene was similarly found upstream of one of the two cyclohexanone gene clusters in *Brevibacterium* strain HCU (8). ORF8 in *Rhodococcus* strain Phi2 encodes a protocatechuate dioxygenase homologue.

The genes upstream of the *Rhodococcus* strain Phi1 cyclohexanone monooxygenase gene have not been sequenced and may also be involved in the degradation cyclohexanone. However, the downstream genes code for conserved hypothetical proteins, a phytohemagglutinin synthase, and a polyketide and/or fatty acid synthase, suggesting that the monooxygenase of *Rhodococcus* strain Phi1 could be part of a biosynthetic pathway. This is the case for the *Emericella nidulans* Baeyer-Villiger monooxygenase StcW gene that is part of the sterigmatocystin biosynthetic gene cluster (7).

Relationships of the newly identified cyclohexanone monooxygenases to other Baeyer-Villiger flavin monooxygenases. Sequence comparison using the BLAST programs against the nonredundant GenBank database showed that these newly

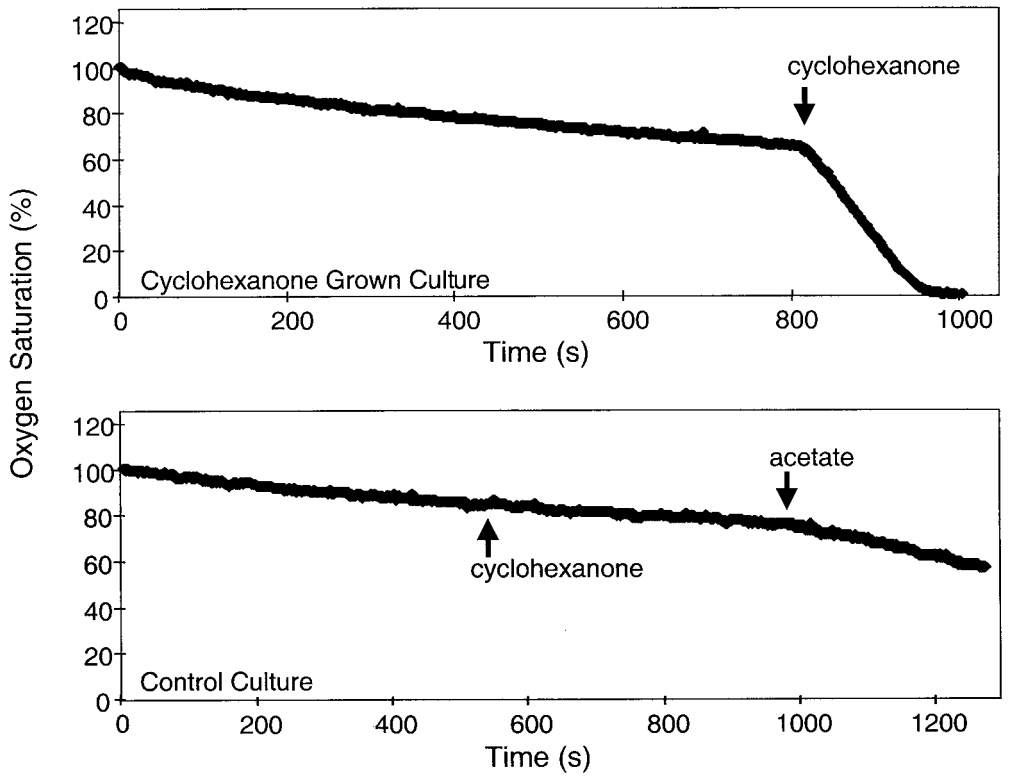

FIG. 2. Inducibility of cyclohexanone degradation in the enrichment culture. The oxygen consumption of cultures grown on acetate or cyclohexanone was measured before and after addition of acetate or cyclohexanone (indicated by arrows). Cyclohexanone-exposed enrichment culture (top panel) but not control culture (bottom panel) can oxidize cyclohexanone, indicating the inducibility of this pathway in at least some species of the enrichment.

identified cyclohexanone monooxygenases, along with the previously identified *Brevibacterium* sp. strain HCU and *Acinetobacter* sp. strain SE19 cyclohexanone monooxygenases, are part of the large family of flavin-dependent monooxygenases (23, 24). The monooxygenases (ChnB) from the two *Rhodococcus* species are relatively similar and share 90% amino acid identity and 89% nucleotide identity. The *Arthrobacter* enzyme is more distantly related, with 84% amino acid identity to both the *Rhodococcus* strain Phi1 and *Rhodococcus* strain Phi2 enzymes. All three enzymes cluster together in the family of BV monooxygenases. The other three genes involved in the degradation of cyclic ketones (*chnC*, *chnD*, and *chnE*) and their corresponding proteins show the same sequence divergence between the two species, between 78 and 84% of nucleotide identity and between 80 and 87% of amino acid identity.

Biochemical characterization of the cyclohexanone monooxygenases. To confirm that the genes identified by DD were those of the targeted pathway, cultures of *E. coli* cells carrying the cosmids encoding the cyclohexanone oxidation operons from *Arthrobacter* strain BP2 or *Rhodococcus* strain Phi2 were grown in the presence of mineral medium containing glucose and 0.1% cyclohexanone as described previously (8). Complete oxidation of cyclohexanone was not observed, but traces of adipic acid (~5% conversion of the substrate added) were detected by gas chromatography-mass spectrometry. No adipic acid was seen in the control culture lacking the cosmids (data

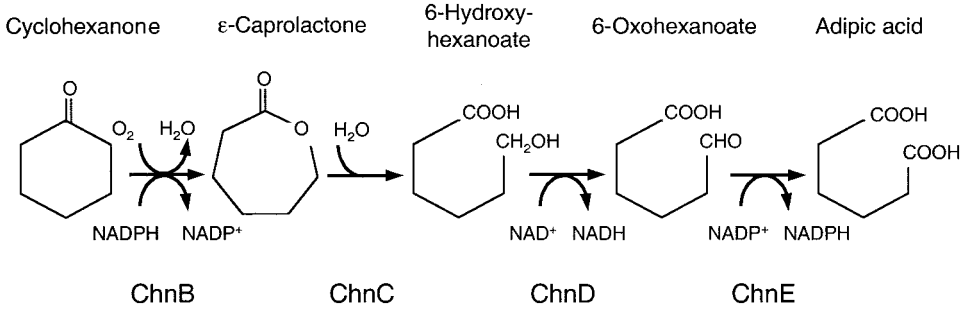

FIG. 3. Oxidation pathway of cyclohexanone into adipic acid. The nomenclature of the cyclohexanone genes is derived from that of *Acinetobacter* (12, 30).

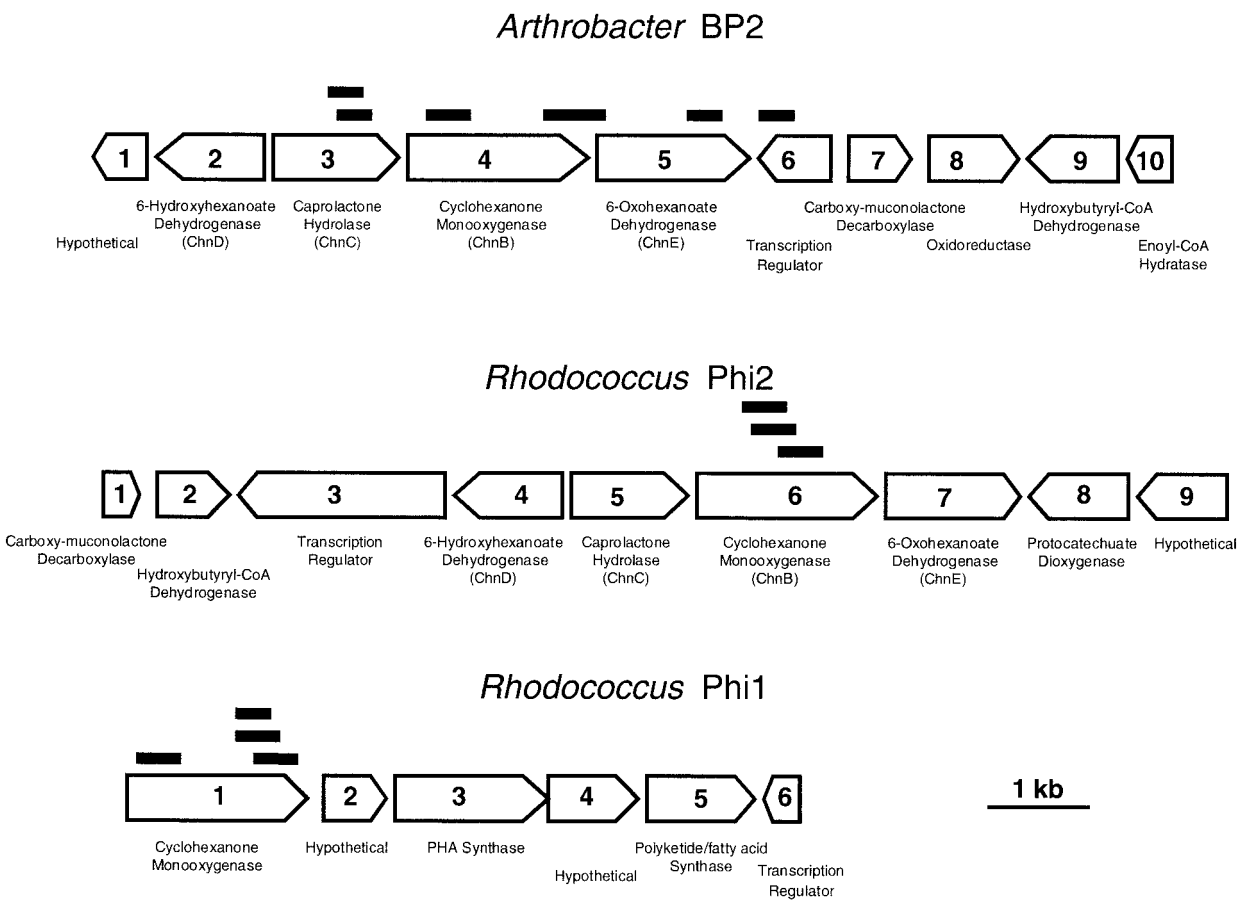

FIG. 4. Organization of the gene clusters identified though DD in three bacteria. Black bars correspond to RT-PCR bands specifically amplified from the RNA of a cyclohexanone-induced culture. Names of genes follow the nomenclature chosen for *Acinetobacter* genes (12, 30).

not shown). The low levels of conversion most likely result from inefficient expression of high G+C gram-positive genes and operons in *E. coli* (8, 35).

Previous work with the cyclohexanone degradation genes of *Brevibacterium* had shown that the flavin cyclohexanone monooxygenases can be easily expressed in *E. coli* in an active form, unlike the other genes of the pathway (8, 9). We therefore cloned and expressed in *E. coli* the three putative cyclohexanone monooxygenase genes to produce His_6 tag fusion proteins. The purified proteins all oxidized cyclohexanone as well as a large variety of cyclic and linear ketones. The specific activities (Table 3) are in the range of those previously reported for the monooxygenases of *Acinetobacter*, *Brevibacterium*, *Rhodococcus*, *Nocardia*, and *Pseudomonas* (9, 11, 18, 31, 34, 53–55). While following the overall similar patterns of activity for most substrates, the enzymes exhibited different specific activity signatures for some of the substrates. For example, the *Rhodococcus* strain Phi2 enzyme readily oxidized the linear 2-tridecanone while no activity was detected with the *Arthrobacter* strain BP2 enzyme (Table 3). We anticipate that these three enzymes will find useful applications in biocatalysis. Investigations are under way to characterize further their substrate specificity as well as the enantiomeric specificity of their products.

TABLE 3. Substrate specificity of the cyclohexanone monooxygenases identified[a]

Substrate	Activity (μmol of substrate/min/mg of protein)		
	Arthrobacter strain BP2	*Rhodococcus* strain Phi1	*Rhodococcus* strain Phi2
Cyclobutanone	0.13	0.10	0.15
Cyclopentanone	1.49	1.37	2.45
2-Methylcyclopentanone	3.51	3.39	6.45
Cyclohexanone	3.57	3.68	3.75
2-Methylcyclohexanone	4.21	4.77	5.95
Cyclohex-2-ene-1-one	2.74	2.69	3.09
1,2-Cyclohexanedione	0.24	0.08	ND
1,3-Cyclohexanedione	0.40	ND	0.14
1,4-Cyclohexanedione	3.99	3.30	6.15
Cycloheptanone	3.85	3.62	6.23
Cyclooctanone	0.65	0.63	0.14
Cyclodecanone	0.17	0.08	0.21
2-Tridecanone	ND	0.16	1.69
Dihexyl ketone	ND	0.16	ND
2-Phenylcyclohexanone	ND	1.05	0.73
Dimethyl-2-piperidone	4.15	3.54	6.51
Phenylboronic acid	0.19	ND	0.11
β-Ionone	1.49	2.71	0.54
Norcamphor	2.84	1.50	2.82
Dimethyl sulfoxide	0.42	0.52	0.54

[a] Assays were performed as described in Materials and Methods. Substrates, as provided by the manufacturers, were added at 1.25 mM in the presence of 15 mM NADPH and 10 μg of enzyme at room temperature. The co-oxidation of NADPH was monitored at 340 nm. ND, no activity detected.

DISCUSSION

In the last 3 years, an increasing number of articles have reported the use of DD for the identification of regulated bacterial genes in isolated strains. The goal of this work is to assess the ability of this technique to identify specific metabolic genes in a microbial community. Previous experiments by Fleming et al. have shown that a toluene degradation gene identified by DD in a pure culture could also be detected in a reconstituted microcosm made by adding cells of a characterized species to a soil sample to a final abundance of approximately 80% of heterotrophic bacteria present (22). We set out to test the applicability of DD to the microbial community of an enrichment culture. As a test case, we chose to look for genes involved in the degradation of cyclohexanone for three reasons: first, this degradation pathway is inducible in several organisms (9, 19, 45); second, these genes have been well characterized (8, 9, 11–13, 30, 31, 34) and can be recognized by sequence similarity to gauge the success of the DD experiment; and third, the genes uncovered have potential utility for chiral biocatalysis (5, 14, 42, 51).

In this work we used high-density sampling DD (56). This approach addresses the limitations of older DD protocols, namely, the generation of false positives. It is often observed that RT-PCR bands amplified differentially from the RNA of cells grown under inducing physiological conditions do not actually reflect a difference in gene expression. These false positives are thought to arise from variability in the RT-PCR amplification process, and as such, they should arise randomly from the mRNA population. Thus, genes with unchanged levels of expression are unlikely to be sampled multiple times. In contrast, the repeated sampling of the same genes or operons is an indication that these genes are truly differentially expressed. In the DD analysis of a microbial population in which the complexity of the mRNA pool is greater than for a pure culture, the multiple sampling of a gene not actually differentially expressed is even more unlikely.

We set out to identify genes involved in the degradation of cyclohexanone in an enrichment culture and sampled six genes involved in cyclohexanone oxidation in 13 independent RT-PCRs. These genes are part of three gene clusters belonging to three different species. Two of the gene clusters encode all the genes required for the conversion of cyclohexanone into adipic acid. The organization schemes of these two clusters are very similar, with the exception of the presence of a transcriptional regulator and the surrounding genes in the *Rhodococcus* strain Phi2 cluster (Fig. 4).

The first cluster was sampled at the highest density with six DNA fragments identifying four genes. This gene cluster is found in *Arthrobacter* strain BP2, which appears to be a predominant species in the enrichment, although not accounting for more than 15% of the population. This corresponds to a sevenfold increase in the quantitative complexity of the mRNA pool (from all cells) relative to that of the *Arthrobacter* cyclohexanone operon. The qualitative complexity (number of different mRNA species) is much greater since 85% of the total RNA of the enrichment comes from at least 50 bacterial strains. The two other cyclohexanone monooxygenase genes present in two different *Rhodococcus* species (strains Phi1 and Phi2) were sampled in four and three RT-PCRs, respectively, each driven by a different primer. T-RFLP analysis showed that *Rhodococcus* strains Phi2 and Phi1 accounted together for less than 2% of the population. Thus, the corresponding mRNA was sampled in an RNA pool with at least a 50-fold increase in quantitative complexity. The difference in abundance between *Rhodococcus* strain Phi2 and *Arthrobacter* strain BP2 may explain the difference in the density of the sampling of their cyclohexanone degradation operons.

This work further supports the use of DD for the discovery of inducible metabolic prokaryotic genes. In one DD experiment, we identified three new Baeyer-Villiger flavin monooxygenase genes. In this work, a known metabolic pathway was used as a proof of concept case for the identification of genes in complex microbial communities. Because the multiple sampling of metabolic genes or operons is a strong lead for the identification of genes expressed under specific physiological conditions, this same approach can be applied to the discovery of other uncharacterized metabolic pathways in complex microbial populations. We believe that high-density sampling DD can be applied to other microbial populations for the discovery of enzymes that cannot be screened or selected for or for which there is insufficient sequence information for PCR amplification. The experiments described in this report provide a foundation for building on the application of DD methodology to environmental samples.

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