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# Prokaryotic Suppression Subtractive Hybridization PCR cDNA Subtraction, a Targeted Method To Identify Differentially Expressed Genes▽†

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**Molecular biology tools can be used to monitor and optimize biological treatment systems, but the application of nucleic acid-based tools has been hindered by the lack of available sequences for environmentally relevant biodegradation genes. The objective of our work was to extend an existing molecular method for eukaryotes to prokaryotes, allowing us to rapidly identify differentially expressed genes for subsequent sequencing. Suppression subtractive hybridization (SSH) PCR cDNA subtraction is a technique that can be used to identify genes that are expressed under specific conditions (e.g., growth on a given pollutant). While excellent methods for eukaryotic SSH PCR cDNA subtraction are available, to our knowledge, no methods previously existed for prokaryotes. This work describes our methodology for prokaryotic SSH PCR cDNA subtraction, which we validated using a model system: *Pseudomonas putida* mt-2 degrading toluene. cDNA from *P. putida* mt-2 grown on toluene (model pollutant) or acetate (control substrate) was subjected to our prokaryotic SSH PCR cDNA subtraction protocol to generate subtraction clone libraries. Over 90% of the sequenced clones contained gene fragments encoding toluene-related enzymes, and 20 distinct toluene-related genes from three key operons were sequenced. Based on these results, prokaryotic SSH PCR cDNA subtraction shows promise as a targeted method for gene identification.**

Molecular tools such as quantitative PCR (8, 15, 16, 21, 22), fluorescence in situ hybridization (2, 31, 37–39), and microarrays (19, 20, 24) are increasingly employed to interrogate biological treatment processes (23, 32, 35). When functional genes (i.e., those that encode proteins) are assessed with these tools, the results provide direct evidence of a microbial community's capabilities and activities. Few, if any, relevant functional gene sequences are known for many systems, and this lack of sequences is a fundamental barrier to the effective application of molecular tools in environmental systems (1).

Several methods for identifying prokaryotic functional genes are available. Transposon mutagenesis has been used, but it requires an appropriate phenotypic screen. Methods based on differential gene expression, including microarrays, differential display, RNA arbitrarily primed PCR, and representational difference analysis (RDA), also have been used (3, 5, 9, 10, 30, 33, 36). Although microarrays are powerful tools for identifying differentially expressed genes, the design of new microarrays requires significant investment. Also, differential display, RNA arbitrarily primed PCR, and RDA often have high false-positive rates and might miss some differentially expressed genes due to PCR biases (6, 10, 27, 30, 33, 36). Furthermore, RDA uses multiple high-stringency hybridizations, which could result in the loss of low-abundance, up-regulated genes (3, 5).

Suppression subtractive hybridization (SSH) PCR cDNA subtraction is an alternative gene identification method that has gained favor for eukaryotic applications but has not yet been applied to prokaryotes (8a, 14a). This method can identify rare and abundant genes, potentially yielding a more diverse gene pool. The existing eukaryotic SSH PCR cDNA subtraction methods cannot be applied directly to prokaryotes, so we have adapted those methods to develop a prokaryotic SSH PCR cDNA subtraction protocol. A bacterium with a fully sequenced genome, *Pseudomonas putida* mt-2, degrading toluene was selected as a model system for developing and validating this methodology (13, 28). While our particular interest is to identify pollutant biodegradation genes in prokaryotes with unsequenced genomes, other potential applications for this technique exist (e.g., identifying genes involved in pathogenesis or antibiotic resistance).

## MATERIALS AND METHODS

**Method synopsis.** Here we briefly outline the steps in SSH PCR cDNA subtraction. The microorganism of interest is cultured under two conditions: for example, with a control substrate, such as acetate (i.e., driver culture), and a pollutant, such as toluene (i.e., tester culture). RNA is extracted and used to generate double-stranded cDNA. The cDNA is digested with a restriction enzyme to produce fragments of suitable size and to prepare the cDNA for adaptor ligation. Tester cDNA is split into two pools, and each pool is ligated to adaptor 1 or adaptor 2 (Fig. 1, step I). The tester cDNA pools are heat denatured and separately hybridized to an excess of heat-denatured driver cDNA (Fig. 1, step II). These two pools are mixed together with additional heat-denatured driver cDNA for a second hybridization, and the overhang ends are filled in with DNA polymerase (Fig. 1, step III). cDNA fragments in the tester 1 pool that were single stranded after the first hybridization can now hybridize to their complements in the tester 2 pool. This results in double-stranded cDNA fragments of differentially expressed genes that have adaptor 1 on one end and adaptor 2 on the other end.

Suppression PCR selectively amplifies up-regulated genes, which have different adaptors on each end (type a in Fig. 1, step III), by using primers that are complementary to the adaptors (Fig. 1, step IV). Fragments with no adaptors or those with adaptors on one end only (types b, e, f, and g in Fig. 1, step III) cannot be amplified because they do not have primer binding sites on both ends.

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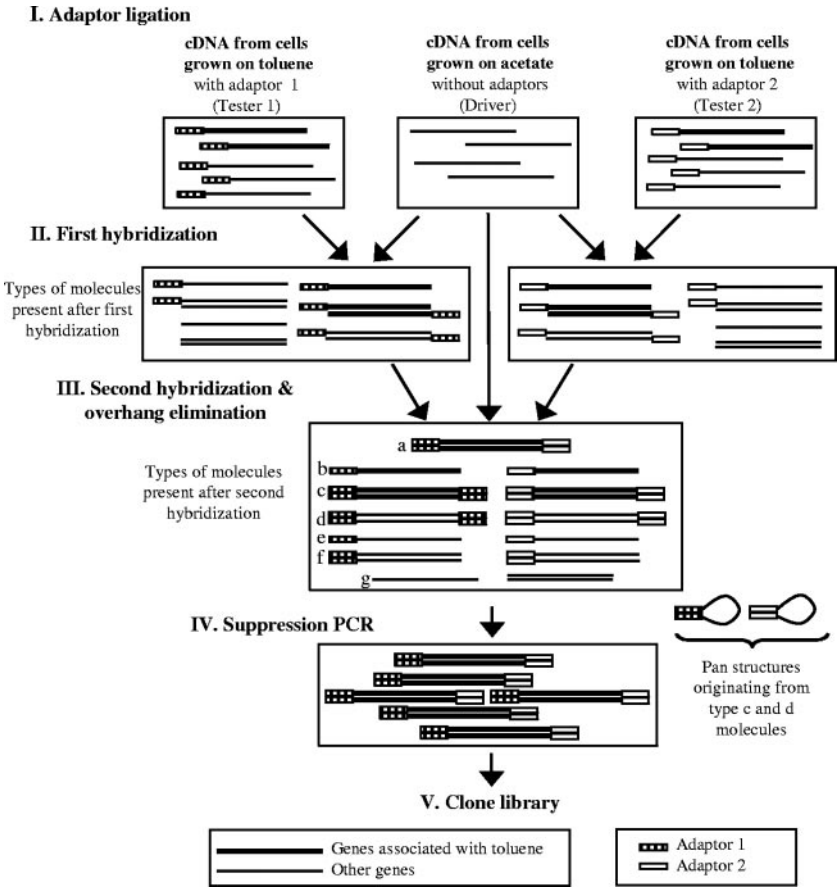


FIG. 1. Schematic of SSH PCR cDNA subtraction for the model system. (Adapted from reference 8a with permission of the publisher.)

Amplification of molecules with the same adaptor on both ends (types c and d in Fig. 1, step III) is suppressed because of intramolecular hybridization between the adaptor on the 5′ end of the molecule and its complement on the 3′ end of the molecule. During the first hybridization, abundant genes form more hybrids with the same adaptor on both ends than do rare genes because hybridization rates are governed by second-order kinetics. Suppressing the amplification of these molecules reduces the amount of abundant genes, thereby increasing the diversity of the subtracted gene pool. The suppression PCR amplicon is used to generate a clone library (Fig. 1, step V), and clone inserts are sequenced.

Reagents for eukaryotic SSH PCR cDNA subtraction are available commercially (PCR-Select cDNA subtraction kit; Clontech, Mountain View, CA). Whenever possible, reagents from this kit were used according to the manufacturer's instructions. SSH PCR cDNA subtractions were run with two sets of independently cultured cells (one set for development and one set for the replicate subtraction).

**Bacterial strain and culturing conditions.** *Pseudomonas putida* mt-2 (ATCC 33015) was grown at 30°C in 250-ml glass bottles containing 50 ml of M9 medium (25), supplemented with 50 μl of stock salt solution (4), and sealed with Teflon-lined Mininert caps (Alltech, Deerfield, IL). To ensure the TOL plasmid was present, cultures were initially grown in M9 medium with 675 mg/liter of *m*-toluate and then transferred to medium with 50 mg/liter of toluene or 600 mg/liter acetate. Toluene headspace samples were analyzed with an isothermal program at 60°C in a Hewlett-Packard 5890 gas chromatograph equipped with a Restek RTX-624 capillary column and a flame ionization detector.

**mRNA isolation.** Total RNA was isolated from ~$1 \times 10^{10}$ log-phase *P. putida* cells (measured by plate counts) grown on toluene or acetate by use of a RiboPure-Bacteria kit (Ambion, Austin, TX). Total RNA was DNase treated with DNA-free (Ambion, Austin, TX), and the absence of contaminating DNA was verified by PCR (see primer sequences for *rplU21* in Table 1). mRNA was isolated from 180 μg total RNA for each substrate by use of a MICROBExpress bacterial mRNA enrichment kit (Ambion, Austin, TX). Multiple preparations for each substrate were pooled before ethanol precipitation. Only 10 μl of glycogen (5 mg/ml) was added to each pooled sample. Residual rRNA was removed by repurifying 20 μg of the ethanol-precipitated RNA with a MICROBExpress kit. No glycogen was added during the ethanol precipitation of the second round of mRNA purification to ensure that the glycogen concentration remained below the inhibitory level (2 mg/ml) (12). RNA quantity was measured by absorbance at 260 nm, and quality was assessed on a 6.7% formaldehyde gel.

**cDNA synthesis.** We designed a prokaryotic cDNA subtraction (PCS) primer (5′-TTTTGTACAAGCTN$_8$-3′), which contains an RsaI site (underlined) close to a random-octamer (N$_8$) mRNA-binding region. For first-strand cDNA synthesis, 2 μg of mRNA was mixed with 2 μl of PCS primer (10 μM) and nuclease-free water to a final volume of 11 μl. The mixture was denatured at 70°C for 10 min and placed on ice. Four microliters of 5× first-strand buffer, 2 μl of 0.1 M dithiothreitol, 1 μl deoxynucleoside triphosphate (dNTP) mix (10 mM each dNTP; New England Biolabs, Beverly, MA), and 2 μl of SuperScript III reverse transcriptase (200 U/μl) were added to each reaction mixture, which was incubated for 10 min at 25°C followed by 1.5 h at 42°C. Then, an additional 2 μl of SuperScript III reverse transcriptase was added, and the incubation continued for 1.5 h at 42°C.

Second-strand synthesis was performed according to the instructions in the PCR-Select cDNA subtraction kit. Afterward, the reaction mixtures were heated to 70°C for 10 min to inactivate the polymerases and placed on ice. One microliter of DNase-free RNase (500 μg/ml) (Roche, Germany) was added to each tube, and the tubes were incubated at 37°C for 30 min and then placed on ice. This step enabled accurate cDNA quantification but may be omitted. Three to four cDNA synthesis reaction mixtures were pooled and purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA). cDNA yields were quantified by absorbance at 260 nm.

**cDNA digestion and adaptor ligation.** Tester and driver cDNA were digested with RsaI and purified using a MinElute reaction cleanup kit (Qiagen, Valencia, CA). Digested cDNA was electrophoresed on a 1% agarose gel and stained with SYBR gold (Molecular Probes, Eugene, OR).

TABLE 1. Primer sequences

| Gene description (designation) | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| Benzyl alcohol dehydrogenase (*xylB*) | 5′-TGG TTT GTC GCG ATC AGC AT-3′ | 5′-ACC GAG CGC CCC ATA AAG TT-3′ |
| Benzaldehyde dehydrogenase (*xylC*) | 5′-TCC AGG GCG CAT GAA TCT TT-3′ | 5′-AAT TCC GGA GTT CGC AAC CA-3′ |
| 4-Hydroxy-2-oxovalerate aldolase (*xylK*) | 5′-CGA AGT GAC CCA CGG TGA TG-3′ | 5′-GTG TCC ATG CCC AGT TCA CG-3′ |
| 2-Hydroxymuconic semialdehyde dehydrogenase (*xylG*) | 5′-CGG TGG TCA CCG AGG AAA TC-3′ | 5′-CCA GCT GTT GAC CCA GAC GA-3′ |
| Catechol 2,3-oxygenase (*xylE*) | 5′-GTC GAG TTG CTG GGC CTG AT-3′ | 5′-CTC CAG TTG CCG GAG AGC AT-3′ |
| Toluate 1,2-dioxygenase subunit (*xylX*) | 5′-GAC CAG TTC GGC TCG CAG TT-3′ | 5′-CCC CTC GAT CCA GTG TTT GG-3′ |
| Regulatory protein (*xylR*) | 5′-TAT GCG CTC AAG GGG ATG GT-3′ | 5′-ATC AGG CCC AGC TCA GTT CG-3′ |
| Regulatory protein (*xylS*) | 5′-AAT GCT GGG CAG CAA TGT CA-3′ | 5′-GAG CGA GCG TGG ACT CAT CA-3′ |
| Benzoate dioxygenase large subunit (*benA*) | 5′-AGG CGG GTG ACG AGA TCA AG-3′ | 5′-GGG GTA CAG GCA CAG GTT GC-3′ |
| Benzoate dioxygenase reductase subunit (*benC*) | 5′-ACG GCG TGA CCA ACG ACT TT-3′ | 5′-GGT ACT GGC TGT CCG GGT TG-3′ |
| Acetylornithine aminotransferase (*argD*) | 5′-TCG CGA GCT GAT CGA CTT TG-3′ | 5′-GGA GAC GTG CCA GAG GGT GT-3′ |
| Ribosomal protein L21 (*rplU21*) | 5′-GGT CGC CAA CGG TGA AGA AG-3′ | 5′-TGC CGG TGA TTT TGA TCT CG-3′ |
| RNA polymerase sigma factor (*rpoD*) | 5′-CGA ATA TGA CCG CGT CAC CA-3′ | 5′-GAT CGG ATA CCG CAC CGA AG-3′ |

Adaptors 1 and 2 from the PCR-Select cDNA subtraction kit were ligated to tester cDNA. To assess ligation efficiency, a PCR amplification test was performed according to the protocol in the PCR-Select cDNA subtraction kit by use of two housekeeping genes not differentially expressed in the presence of toluene (9, 26): genes for RNA polymerase sigma factor (*rpoD*) and ribosomal protein L21 (*rplU21*) (Table 1). (The adaptors and corresponding secondary PCR primers provided in the PCR-Select cDNA subtraction kit used for this study are labeled 1 and 2R by Clontech [Mountain View, CA]. For simplicity, we refer to adaptor 2R and nested-PCR primer 2R as adaptor 2 and nested-PCR primer 2, respectively.)

**First and second hybridizations and suppression PCR.** The first and second hybridizations were performed according to the PCR-Select cDNA subtraction kit protocol, with the exception that 4 μl of freshly denatured driver cDNA was added during the second hybridization. Primary and secondary nested suppression PCRs were run with Advantage cDNA polymerase mix (Clontech, Mountain View, CA) according to the PCR-Select cDNA subtraction kit protocol, except where noted. The primary PCR primer (PCR primer 1, which is complementary to both adaptors) and secondary nested-PCR primers (nested-PCR primer 1 and nested-PCR primer 2, which are complementary to adaptors 1 and 2, respectively) were provided by the PCR-Select cDNA subtraction kit.

To test the effect of the nested-PCR primer 2 concentration on suppression efficiency, we conducted suppression PCR experiments using 50 to 400 nM nested-PCR primer 2 and 400 nM nested-PCR primer 1. Purified plasmid DNA from cDNA subtraction clones with an insert containing adaptor 1 at both ends (1,1-clone), adaptor 2 at both ends (2,2-clone), or adaptor 1 at one end and adaptor 2 at the other end (1,2-clone) was used as the template. Identities of the clone inserts are shown in Table 2. Twenty-seven cycles of suppression PCR were conducted according to the secondary PCR protocol using 10 ng of plasmid DNA from one or all three clones as a template. PCR products were electrophoresed, and product size and mass were determined with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA).

To optimize the nested-PCR primer 2 concentration for sufficient suppression efficiency, secondary PCRs were run on hybridized cDNA with a nested-PCR primer 2 concentration of 50 to 400 nM. An amplicon from each suppression PCR was cloned, and clones were screened using a PCR adaptor screen as described below. For subsequent subtractions, suppression PCR was conducted with 50 nM nested-PCR primer 2.

**Clone screening and fragment sequencing.** An amplicon from the secondary nested PCR was cloned using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). Twenty to forty clones were selected and screened using the following PCR adaptor screen. For each clone, three PCRs were run using the following thermocycler program: 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, followed by 72°C for 10 min. The reaction mixtures contained nested-PCR primer 1 only, nested-PCR primer 2 only, or both primers. A master mix was prepared for each primer combination by mixing 18.9 μl of water, 2.5 μl of 10× Thermopol reaction buffer (New England Biolabs, Beverly, MA), 0.5 μl dNTP mix (10 mM of each dNTP; New England Biolabs, Beverly, MA), 2 μl total of primers (10 μM), and 0.1 μl *Taq* DNA polymerase (5 U/μl) (New England Biolabs, Beverly, MA) per reaction mixture. Cells were used directly as the PCR template. The amplicon was visualized on a 2% agarose gel stained with ethidium bromide. Clones yielding a band when only nested-PCR primer 1 was present were identified as 1,1-clones. Clones yielding a band when only nested-PCR primer 2 was present were identified as 2,2-clones. Clones that produced a band only when both primers were present were identified as 1,2-clones.

Those identified as 1,2-clones were screened further using a HaeIII digest. Five microliters of each 1,2-clone amplicon was incubated with 0.5 μl of HaeIII

TABLE 2. Clones used for suppression PCR optimization

| Clone name | Actual length (bp)[a] | Adaptors | Gene identity[b] | Gene name | % Identity[c] |
|---|---|---|---|---|---|
| 31 | 634 | 1,1 | 16S rRNA gene | 16S rRNA | 99 (633/634) |
| 32 | 456 | 1,2 | pWW0:72946-73401 | *xylC* | 98 (449/456) |
| 33 | 731 | 2,2 | pWW0:74007-74737 | *xylW* | 99 (730/731) |

[a] Lengths do not include adaptors.
[b] The pWW0 accession number is AJ344068 (the numbers refer to the nucleotide positions for that accession number), and the *Pseudomonas putida* KT2440 accession number is AE015451.
[c] The ratio in parentheses represents the number of matching bases over the total number of bases used by the blastn algorithm.

(New England Biolabs, Beverly, MA) at 37°C for 1 h, followed by enzyme inactivation at 65°C for 20 min. The amplicon was electrophoresed on a 2% agarose gel. Gel images were captured using a Gel Logic 100 imaging system, and Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY) was used to determine approximate molecular weights for each band. Clones showing a unique restriction pattern were retained for sequencing.

Plasmids were isolated from selected clones by use of a FastPlasmid mini kit (Eppendorf, Westbury, NY). Clone inserts were sequenced at the University of Texas at Austin DNA sequencing facility by use of M13 forward and reverse primers. The sequences were compared to publicly available sequences by use of the blastn algorithm (www.ncbi.nlm.nih.gov/BLAST).

**Reverse transcription, quantitative real-time PCR (RT-qPCR).** To synthesize cDNA, 4 μg of DNase-treated total RNA (isolated as described above) was mixed with 2 μl of random hexamer primer (10 μM) and nuclease-free water (Ambion, Austin, TX) to a final volume of 10 μl. The mixture was denatured at 70°C for 5 min and placed on ice. Four microliters of 5× first-strand buffer, 2 μl of dNTP mix (10 mM of each dNTP; New England Biolabs, Beverly, MA), 2 μl of water, and 2 μl of avian myeloblastosis virus reverse transcriptase (Roche, Germany) were mixed and incubated at 42°C for 1.5 h. Negative controls, used to verify the absence of contaminating genomic DNA, were prepared by omitting the reverse transcriptase. qPCR reactions were run on a 7900HT real-time PCR system (Applied Biosystems, Foster City, CA) using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 0.3 μM of each primer (Table 1). The following thermocycler program was used: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. Fivefold serial dilutions of cDNA from toluene-grown cells were used to construct a standard curve. Transcript quantities of each target gene were normalized to the transcript quantities of housekeeping gene *rplU21* for the same culturing conditions. Then, up-regulation was calculated for toluene-grown cultures compared to acetate-grown cultures.

## RESULTS AND DISCUSSION

While some steps of SSH PCR cDNA subtraction required significant methodology development for application to prokaryotes, other steps needed only verification that methods previously developed for eukaryotes remained valid for prokaryotes. Adaptations of the eukaryotic protocol as well as results of the prokaryotic SSH PCR cDNA subtractions conducted for our model system are presented.

**cDNA synthesis and adaptor ligation.** We designed a PCS primer with an RsaI site at the 5′ end (to enable adaptor ligation) and a random-octamer mRNA-binding region at the 3′ end. Because only the random-octamer region is likely to bind to mRNA while the RsaI site remains single stranded, binding of the PCS primer to mRNA is not as energetically favorable as is binding of a standard random primer or the eukaryotic cDNA subtraction primer, which has 30 thymine residues that can bind to the poly(A) tail of eukaryotic mRNA. cDNA yields with the PCS primer were significantly lower than those with a random hexamer primer or with the eukaryotic cDNA subtraction primer (data not shown). To improve cDNA yield, SuperScript III was used in a 3-h incubation for first-strand synthesis and a second aliquot of SuperScript III was added after the first 1.5 h. By use of this protocol, two to four cDNA synthesis reactions produced sufficient cDNA (1 to 2 μg) (data not shown). Additionally, this cDNA had an adaptor ligation efficiency of nearly 100% (data not shown).

**Hybridizations, suppression PCR, and cloning.** A set of clones for our model system was generated initially according to the eukaryotic suppression PCR protocol, and we observed that 75% of 22 clone inserts were 2,2-clones, 0% were 1,1-clones, and 25% were the desired 1,2-clones. The cause of the high percentage of 2,2-clones remains unknown; the predominance of 2,2-clones was reproducible in the model system but did not occur when we performed a control cDNA subtraction with eukaryotic mRNA as prescribed by the PCR-Select cDNA subtraction kit (data not shown). Because clones with the same adaptor on both ends (e.g., 2,2-clones) represent nontarget gene fragments, we modified suppression PCR to reduce the amplification of 2,2-fragments.

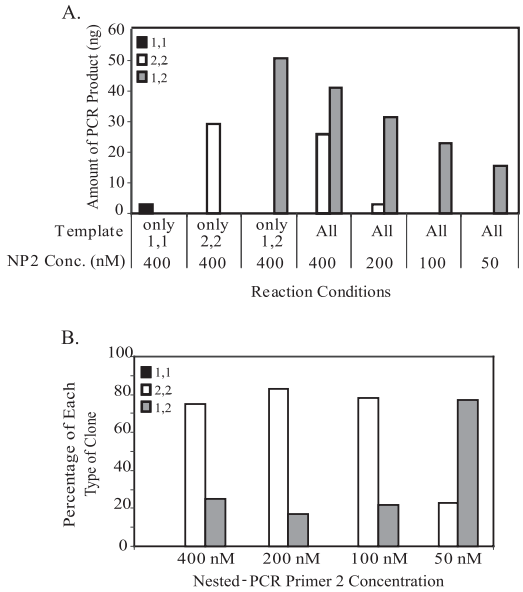


FIG. 2. Effect of nested-PCR primer 2 concentration (NP2 Conc.) on the abundance of 1,1-, 2,2-, and 1,2-clones. (A) Amount of each clone type when suppression PCR was conducted using purified plasmids as the template. Each reaction mixture contained a plasmid with an insert with adaptor 1 on both ends (1,1-clone), a plasmid with an insert with adaptor 2 on both ends (2,2-clone), a plasmid with an insert with one of each adaptor (1,2-clone), or all three plasmids (All). (B) Percentage of each clone type when suppression PCR was conducted using hybridized cDNA as the template. Prokaryotic SSH PCR cDNA subtraction clone libraries were generated with the specified nested-PCR primer 2 concentration, and the percentages of 1,1-, 2,2-, and 1,2-clones were determined by the PCR adaptor screen. A minimum of 20 clones from each library was screened.

During suppression PCR, primer annealing competes with intramolecular pan structure formation (Fig. 1, step IV). Lower primer concentrations favor pan formation, and temperature dictates the stability of binding. The nested-PCR primer 1 binding region within adaptor 1 has a melting temperature that is 4 to 5°C greater than that of the nested-PCR primer 2 binding region within adaptor 2. Therefore 1,1-pan structures are more stable than 2,2-pan structures once formed, which supports the observed bias toward amplification of 2,2-fragments.

We conducted suppression PCR experiments with a purified 1,1-clone, 2,2-clone, and 1,2-clone; when all three templates were present with a nested-PCR primer 2 concentration of 400 nM, 2,2-clones and 1,2-clones were amplified exponentially, while 1,1-clones were not (Fig. 2A). Furthermore, we determined that decreasing the nested-PCR primer 2 concentration to 50 nM resulted in significant quantities of 1,2-amplicon while keeping the 2,2-amplicon below the detection limit (0.5 ng) (Fig. 2A).

To select an appropriate nested-PCR primer 2 concentra-

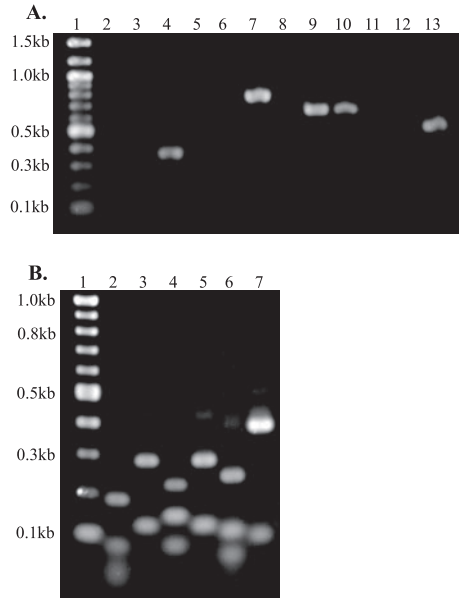


FIG. 3. Representative electrophoresis gels for clone screening. (A) Electrophoresis gel of four clones screened to identify 1,2-clones by use of the PCR adaptor screen. Lanes: 1, DNA marker; 2 to 4, clone 11; 5 to 7, clone 14; 8 to 10, unsequenced 2,2-clone; 11 to 13, clone 21. Reaction mixtures from lanes 2, 5, 8, and 11 contained nested-PCR primer 1 only. Reaction mixtures from lanes 3, 6, 9, and 12 contained nested-PCR primer 2 only. Reaction mixtures from lanes 4, 7, 10, and 13 contained nested-PCR primers 1 and 2. Lanes 8 to 10 show a 2,2-clone, and the other three clones shown are 1,2-clones. See Table S1 in the supplemental material for clone identities. (B) Electrophoresis gel for six clones screened using the HaeIII digest screen. Lanes: 1, DNA marker; 2, clone 16; 3, clone 19; 4, clone 1; 5, clone 30; 6, clone 21; 7, clone 2.

tion for prokaryotic SSH PCR cDNA subtraction, new suppression PCRs were run on hybridized cDNA (Fig. 1, step III) with 400 nM, 200 nM, 100 nM, or 50 nM nested-PCR primer 2, and subtracted clone libraries were generated. Selected clones were screened using the PCR adaptor screen; a representative gel is shown in Fig. 3A. The percentages of 1,2-clones were 25%, 17%, 22%, and 77% for 400 nM, 200 nM, 100 nM, and 50 nM nested-PCR primer 2, respectively (Fig. 2B). Therefore, 50 nM nested-PCR primer 2 was selected as the optimal concentration.

Reducing the nested-PCR primer 2 concentration from 400 nM to 50 nM significantly increased the percentage of 1,2-clones, but it resulted in a mild increase in the number of chimeric clones (from 5% to 14%). In an attempt to reduce chimera formation, a shorter denaturation time (10 s instead of 30 s) was used in suppression PCR because heating DNA can lead to depurination (14, 34) and thereby induce chimera formation (29). However, this modification did not reduce chimera formation.

**Screening clones for sequencing.** The PCR adaptor screen can be used to discard 1,1- and 2,2-clones. The amplicon from 1,2-clones should be digested and electrophoresed to select unique clone inserts for sequencing. The amplicon from several 1,2-clones was digested with HaeIII and analyzed by gel electrophoresis. Figure 3B shows a representative gel. Clones 19 and 30 appeared to be identical by this screen, which was confirmed by sequencing; the other clones appeared to be distinct from one another by this screen, which was confirmed by sequencing. All 28 1,2-clones from the subtraction library generated using 50 nM nested-PCR primer 2 were sequenced, and the validity of the PCR adaptor screen and the restriction digest screen was verified (data not shown).

For future applications, it will be necessary to verify that sequences obtained via prokaryotic SSH PCR cDNA subtraction represent differentially expressed genes. Screening clones for differential expression can be conducted prior to sequencing (e.g., by generating Northern blot probes from cloned fragments) or after sequencing (e.g., by RT-qPCR). Because prokaryotic SSH PCR cDNA subtraction produces gene fragments, a downstream tool, such as gene walking, is required to obtain complete gene sequences.

**Analysis of gene fragments isolated using prokaryotic SSH PCR cDNA subtraction.** Seventy-one clones were sequenced during the development of the prokaryotic SSH PCR cDNA subtraction protocol. Ninety-two percent of these clones contained fragments of genes known to be involved in toluene degradation (data not shown). The majority of these clones contained fragments of genes from the TOL plasmid upper or *meta* operons, which contain the key metabolic genes involved in toluene degradation (7, 11). These upper and *meta* operon genes have been shown to be highly up-regulated in the presence of toluene (9) and therefore are the key genes that prokaryotic SSH PCR cDNA subtraction was expected to identify in the model system.

Following the development phase, we examined the reproducibility of the prokaryotic SSH PCR cDNA subtraction protocol by performing a replicate subtraction on an independent cell culture for the model system. Eighty-eight percent of 24 unique clones sequenced from this replicate subtraction contained sequences of genes previously shown to be related to toluene degradation (see Table S1 in the supplemental material) (9). Twenty of these clone fragments were from genes in the upper and *meta* operons. Figure 4 is an alignment between clone fragments from this replicate subtraction and the upper and *meta* operons. With only 24 clones, we identified fragments of 9 of the 20 genes found in the upper and *meta* operons. Because each operon is transcribed as a single mRNA molecule, some clones contain fragments of two genes within an operon as well as the intergenic sequence.

In addition to the plasmid-borne genes of the upper and *meta* operons, fragments of four chromosomal genes were obtained (see Table S1 in the supplemental material). Clone 21 contained a benzoate transporter gene (*benK*), which is part of the *benABCDKEZF* operon that is involved in benzoate metabolism (18). Benzoate is a by-product of toluene degradation; although there is functional redundancy between many of the *ben* genes and upper operon genes, *ben* genes have been shown to be up-regulated in the presence of toluene (9). Overall, the replicate subtraction successfully identified fragments representing 10 distinct toluene-related genes within three key operons (upper, *meta*, and *ben*). Clone 22 contained an acetylornithine aminotransferase gene (*argD*), which is involved in arginine biosynthesis. Other genes involved in arginine biosynthesis (i.e., *argA*, *argF*, and *argJ*) have previously been shown to be up-regulated in the presence of toluene (9). Two clones (23 and 24) contained rRNA sequences. A small percentage of

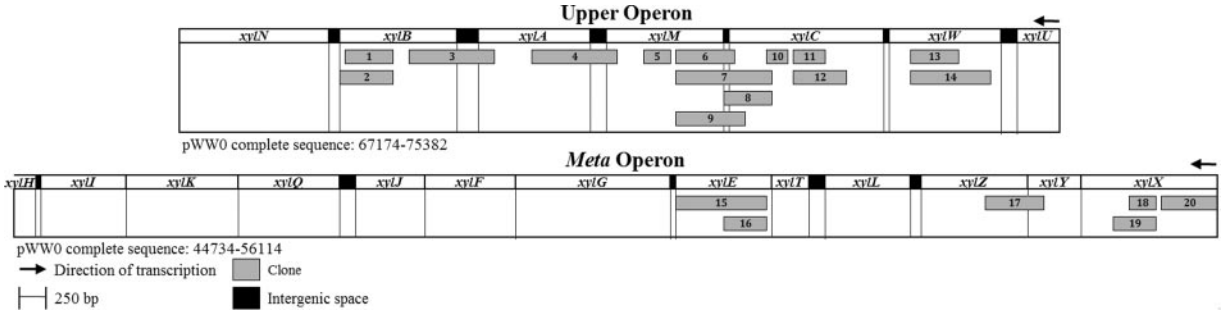


FIG. 4. Comparison of clones obtained from the replicate prokaryotic SSH PCR cDNA subtraction to the corresponding segments of the upper and *meta* operon genes of the TOL plasmid. Clone fragments have arbitrarily been designated fragments 1 to 20. See Table S1 in the supplemental material for the sequence information. Values 67174–75382 and 44734–56114 are nucleotide positions.

rRNA clones were expected because rRNA is still present in the purified mRNA.

Overall, 95 clones were sequenced from the compiled subtractions (developmental plus replicate subtractions). In addition to the genes shown in Fig. 4 from the replicate subtraction, *xylR*, *xylN*, *xylI*, *xylK*, *xylQ*, *xylF*, *xylT*, *xylL*, *benA*, and *benD* were isolated from the developmental subtraction (genes *xylK* and *xylQ* were found in chimeric clones). Additional clone sequencing should yield sequences for the remaining genes found in the upper, *meta*, and *benABCDKEZF* operons. Alternatively, these sequences could be obtained via gene walking, which is likely to yield sequences of functionally related genes in prokaryotes where operons are common.

**RT-qPCR.** To assess the up-regulation of *xyl* and *ben* genes under the culturing conditions used for prokaryotic SSH PCR cDNA subtraction, RT-qPCR was conducted for selected genes (Fig. 5). *xylB* and *xylC* were highly up-regulated as a result of toluene exposure, and almost all of the upper operon genes were identified from the compiled prokaryotic SSH PCR cDNA subtractions. *xylK*, *xylG*, *xylE*, and *xylX* were not quite as highly up-regulated, and many *meta* operon genes were identified by prokaryotic SSH PCR cDNA subtraction. The regulatory genes *xylR* and *xylS* were up-regulated 16-fold and 70-fold, respectively, which is consistent with these genes being identified less frequently than were upper and *meta* operon genes (only 1 of 95 clones contained a fragment of the *xylR* gene). The selected chromosomal genes *benA* and *benC* were up-regulated to levels similar to or greater than levels for the upper operon genes. Consistent with RT-qPCR data, *benA*, *benD*, and *benK* were isolated by prokaryotic SSH PCR cDNA subtraction. Prokaryotic SSH PCR cDNA subtraction identified *argD*, which has not previously been shown to be up-regulated in response to toluene, and RT-qPCR showed that *argD* was fourfold up-regulated when cells were grown on toluene compared to acetate.

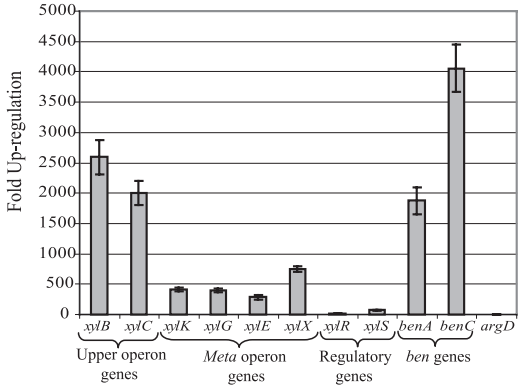


FIG. 5. Up-regulation of selected genes in toluene-grown cultures relative to acetate-grown cultures. Transcript quantities of each target gene were normalized to the housekeeping gene *rplU21* for each culturing condition prior to calculating up-regulation. All qPCRs were conducted in triplicate, and error bars represent 1 standard deviation. Similar results were obtained when transcript quantities were normalized to housekeeping gene *rpoD* (data not shown).

Prokaryotic SSH PCR cDNA subtraction was highly successful at isolating genes involved in toluene degradation; however, a few highly up-regulated genes were not among the sequenced clones. For example, although *xylG* and *xylE* were up-regulated to nearly the same level (Fig. 5), *xylE* was isolated by prokaryotic SSH PCR cDNA subtraction while *xylG* was not. Although the up-regulation may be a predictor of which genes might be isolated by prokaryotic SSH PCR cDNA subtraction, the absolute quantity of gene transcripts (i.e., not relative to levels present in acetate-grown cells) also is important. When a small number of clones is selected for sequencing, the probability that fragments of a given gene will be sequenced depends on the prevalence of an amplicon from that gene in the suppression PCR pool. Some degree of equalization between high- and low-abundance genes occurs during SSH PCR cDNA subtraction due to the second-order kinetics of hybridization; however, the frequency of a given gene in the suppression PCR pool depends on its up-regulation, the absolute quantity of transcripts for that gene in the mRNA pool, and the size and number of cDNA fragments for that gene. For the eukaryotic protocol, a target gene must be more than 0.01% of the total cDNA by mass and more than fivefold up-regulated to be isolated (17). For some genes, the restriction enzyme used may not produce a sufficient number of gene fragments of the appropriate size (approximately 100 to 900 bp) for isolation via SSH PCR cDNA subtraction. A combination of these factors results in some genes being isolated repeatedly while others are not among the selected clones.

We have demonstrated that the methodology developed for prokaryotic SSH PCR cDNA subtraction can be used to identify pollutant degradation genes from prokaryotes. Overall, 91% of the 95 clones sequenced from the compiled prokaryotic

SSH PCR cDNA subtractions contained fragments of genes previously shown to be related to toluene degradation, and we successfully identified fragments of 20 genes known to be relevant to toluene degradation. Just 6% of the clones from the compiled subtractions contained only rRNA fragments (false positives). Domínguez-Cuevas et al. (9) showed that 180 genes are up-regulated in *P. putida* mt-2 to various degrees in response to toluene. The pool of genes isolated via prokaryotic SSH PCR cDNA subtraction did not show this kind of diversity. Rather, the pool was dominated by genes encoding enzymes involved in toluene degradation, which would be most useful for interrogating biological treatment systems. Prokaryotic SSH PCR cDNA subtraction makes it possible to obtain the sequences of key genes within a few weeks. Because prokaryotic SSH PCR cDNA subtraction requires no a priori knowledge of the genetics of a given bacterium, this technique can be used on any isolated bacterium for which appropriate culturing conditions have been identified. Future work will focus on applying this new methodology to environmentally relevant prokaryotes for which little or no gene sequence information is available. It might also be possible to use this tool to identify genes from mixed cultures, but future work is required to investigate its utility for this purpose.

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## REFERENCES

1. **Alleman, B., et al.** 2005. SERDP and ESTCP expert panel workshop on research and development needs for the environmental remediation application of molecular biological tools. Final report. http://docs.serdp-estcp.org/viewfile.cfm?Doc=MBT%20Workshop%20Report%2Epdf.
2. **Amann, R., B. M. Fuchs, and S. Behrens.** 2001. The identification of microorganisms by fluorescence in situ hybridisation. Curr. Opin. Biotechnol. **12:**231–236.
3. **Bathe, S., and P. R. Norris.** 2007. Ferrous iron- and sulfur-induced genes in *Sulfolobus metallicus*. Appl. Environ. Microbiol. **73:**2491–2497.
4. **Bauchop, T., and S. R. Elsden.** 1960. The growth of micro-organisms in relation to their energy supply. J. Gen. Microbiol. **23:**457–469.
5. **Becker, P., W. Hufnagle, G. Peters, and M. Herrmann.** 2001. Detection of differential gene expression in biofilm-forming versus planktonic populations of *Staphylococcus aureus* using micro-representational-difference analysis. Appl. Environ. Microbiol. **67:**2958–2965.
6. **Bowler, L. D., M. Hubank, and B. G. Spratt.** 1999. Representational difference analysis of cDNA for the detection of differential gene expression in bacteria: development using a model of iron-regulated gene expression in *Neisseria meningitidis*. Microbiology **145:**3529–3537.
7. **Burlage, R. S., S. W. Hooper, and G. S. Sayler.** 1989. The TOL (pWW0) catabolic plasmid. Appl. Environ. Microbiol. **55:**1323–1328.
8. **Cole, A. C., M. J. Semmens, and T. M. LaPara.** 2004. Stratification of activity and bacterial community structure in biofilms grown on membranes transferring oxygen. Appl. Environ. Microbiol. **70:**1982–1989.

8a. **Diatchenko, L., Y.-F. C. Lau, A. P. Campbell, A. Chenchik, F. Mogadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov, and P. D. Siebert.** 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA **93:**6025–6030.

9. **Domínguez-Cuevas, P., J. E. González-Pastor, S. Marqués, J. L. Ramos, and V. de Lorenzo.** 2006. Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. J. Biol. Chem. **281:**11981–11991.
10. **Fleming, J. T., W. H. Yao, and G. S. Sayler.** 1998. Optimization of differential display of prokaryotic mRNA: application to pure culture and soil microcosms. Appl. Environ. Microbiol. **64:**3698–3706.
11. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
12. **Gerard, G.** 1994. Inhibition of SuperScript II reverse transcriptase by common laboratory chemicals. Focus **16:**102–103.
13. **Greated, A., L. Lambertsen, P. A. Williams, and C. M. Thomas.** 2002. Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. Environ. Microbiol. **4:**856–871.
14. **Greer, S., and S. Zamenhof.** 1962. Studies on depurination of DNA by heat. J. Mol. Biol. **4:**123–141.

14a. **Gurskaya, N. G., L. Diatchenko, A. Chenchik, P. D. Siebert, G. L. Khaspekov, K. A. Lukyanov, L. L. Vagner, O. D. Ermolaeva, S. A. Lukyanov, and E. D. Sverdlov.** 1996. Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: cloning of Jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate. Anal. Biochem. **240:**90–97.

15. **Hall, S. J., P. Hugenholtz, N. Siyambalapitiya, J. Keller, and L. L. Blackall.** 2002. The development and use of real-time PCR for the quantification of nitrifiers in activated sludge. Water Sci. Technol. **46:**267–272.
16. **Harms, G., A. C. Layton, H. M. Dionisi, I. R. Gregory, V. M. Garrett, S. A. Hawkins, K. G. Robinson, and G. S. Sayler.** 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ. Sci. Technol. **37:**343–351.
17. **Ji, W., M. B. Wright, L. Cai, A. Flament, and K. Lindpaintner.** 2002. Efficacy of SSH PCR in isolating differentially expressed genes. BMC Genomics **3:**12.
18. **Jiménez, J. I., B. Miñambres, J. L. García, and E. Díaz.** 2002. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**824–841.
19. **Kelly, J. J., S. Siripong, J. McCormack, L. R. Janus, H. Urakawa, S. El Fantroussi, P. A. Noble, L. Sappelsa, B. E. Rittmann, and D. A. Stahl.** 2005. DNA microarray detection of nitrifying bacterial 16S rRNA in wastewater treatment plant samples. Water Res. **39:**3229–3238.
20. **Koizumi, Y., J. J. Kelly, T. Nakagawa, H. Urakawa, S. El-Fantroussi, S. Al-Muzaini, M. Fukui, Y. Urushigawa, and D. A. Stahl.** 2002. Parallel characterization of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-denaturing gradient gel electrophoresis, RNA-DNA membrane hybridization, and DNA microarray technology. Appl. Environ. Microbiol. **68:**3215–3225.
21. **Layton, A. C., H. Dionisi, H. W. Kuo, K. G. Robinson, V. M. Garrett, A. Meyers, and G. S. Sayler.** 2005. Emergence of competitive dominant ammonia-oxidizing bacterial populations in a full-scale industrial wastewater treatment plant. Appl. Environ. Microbiol. **71:**1105–1108.
22. **Lee, P. K. H., D. R. Johnson, V. F. Holmes, J. Z. He, and L. Alvarez-Cohen.** 2006. Reductive dehalogenase gene expression as a biomarker for physiological activity of *Dehalococcoides* spp. Appl. Environ. Microbiol. **72:**6161–6168.
23. **Lovley, D. R.** 2003. Cleaning up with genomics: applying molecular biology to bioremediation. Nat. Rev. Microbiol. **1:**35–44.
24. **Loy, A., C. Schulz, S. Lücker, A. Schöpfer-Wendels, K. Stoecker, C. Baranyi, A. Lehner, and M. Wagner.** 2005. 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "*Rhodocyclales*". Appl. Environ. Microbiol. **71:**1373–1386.
25. **Maniatis, T., J. Sambrook, and E. F. Fritsch.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. **Marlowe, E. M., J. M. Wang, I. L. Pepper, and R. M. Maier.** 2002. Application of a reverse transcription-PCR assay to monitor regulation of the catabolic *nahAc* gene during phenanthrene degradation. Biodegradation **13:**251–260.
27. **Nagel, A. C., J. T. Fleming, G. S. Sayler, and K. L. Beattie.** 2001. Screening for ribosomal-based false positives following prokaryotic mRNA differential display. BioTechniques **30:**988–996.
28. **Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. C. Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Düsterhöft, B. Tümmler, and C. M. Fraser.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**799–808.
29. **Pääbo, S., D. M. Irwin, and A. C. Wilson.** 1990. DNA damage promotes jumping between templates during enzymatic amplification. J. Biol. Chem. **265:**4718–4721.
30. **Paulino, L. C., M. P. de Mello, and L. M. M. Ottoboni.** 2002. Differential gene expression in response to copper in *Acidithiobacillus ferrooxidans* analyzed by RNA arbitrarily primed polymerase chain reaction. Electrophoresis **23:**520–527.
31. **Pernthaler, A., and R. Amann.** 2004. Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. Appl. Environ. Microbiol. **70:**5426–5433.

32. **Rittmann, B. E.** 2002. The role of molecular methods in evaluating biological treatment processes. Water Environ. Res. **74:**421–427.
33. **Rivera-Marrero, C. A., M. A. Burroughs, R. A. Masse, F. O. Vannberg, D. L. Leimbach, J. Roman, and J. J. Murtagh.** 1998. Identification of genes differentially expressed in *Mycobacterium tuberculosis* by differential display PCR. Microb. Pathog. **25:**307–316.
34. **Roger, M., and R. D. Hotchkiss.** 1961. Selective heat inactivation of pneumococcal transforming deoxyribonucleate. Proc. Natl. Acad. Sci. USA **47:** 653–669.
35. **Sharkey, F. H., I. M. Banat, and R. Marchant.** 2004. Detection and quantification of gene expression in environmental bacteriology. Appl. Environ. Microbiol. **70:**3795–3806.
36. **Shepard, B. D., and M. S. Gilmore.** 1999. Identification of aerobically and anaerobically induced genes in *Enterococcus faecalis* by random arbitrarily primed PCR. Appl. Environ. Microbiol. **65:**1470–1476.
37. **Silyn-Roberts, G., and G. Lewis.** 2001. In situ analysis of *Nitrosomonas* spp. in wastewater treatment wetland biofilms. Water Res. **35:**2731–2739.
38. **Stoffels, M., R. Amann, W. Ludwig, D. Hekmat, and K. H. Schleifer.** 1998. Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. Appl. Environ. Microbiol. **64:**930–939.
39. **Zhang, H., B. E. Logan, J. M. Regan, L. A. Achenbach, and M. A. Bruns.** 2005. Molecular assessment of inoculated and indigenous bacteria in biofilms from a pilot-scale perchlorate-reducing bioreactor. Microb. Ecol. **49:** 388–398.