Species-Specific Oligonucleotides for Enumeration of *Pseudomonas putida* F1, *Burkholderia* sp. Strain JS150, and *Bacillus subtilis* ATCC 7003 in Biodegradation Experiments

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Species-specific sequences were identified within the V4 variable region of 16S rRNA of two bacterial species capable of aromatic hydrocarbon metabolism, *Pseudomonas putida* F1 and *Burkholderia* sp. strain JS150, and a third, *Bacillus subtilis* ATCC 7003, that can function as a secondary degrader. Fluorescent in situ hybridization (FISH) with species-specific oligonucleotides was used for direct counting of these species throughout a phenol biodegradation experiment in batch culture. Traditional differential plate counting methods could not be used due to the similar metabolism and interactions of the primary degraders and difficulties in selecting secondary degraders in mixed culture. In contrast, the FISH method provided reliable quantitative results without interference from those factors.

Direct methods for the detection of individual species in a mixed population are needed for studying population dynamics in the biodegradation of pollutant mixtures. These methods overcome problems encountered with indirect counting methods with organisms that are unculturable on selective media (20, 29); are difficult to select in the presence of related species (13, 17, 28); or produce toxins, inducers, or inhibitors (22, 24). Indirect methods, such as differential plating and most-probable-number (MPN) dilution, involve coculturing species on a counting medium separate from the original sample. Finding the factor that differentiates one organism from another can be problematic for closely related species that metabolize a similar range of substrates and have similar antibiotic resistance. In such cases, other differences have been exploited. For example, to distinguish *Pseudomonas putida* and *Pseudomonas resinovorans*, closely related species with similar morphology and metabolic pathways, Dikshitulu et al. (13) developed a differential plating method based on different growth rates by using sodium citrate as the sole carbon source. However, this method cannot be extended to more than two microorganisms, cannot be used when the population sizes of the two species are significantly different, and gives erroneous results if an inhibitory substance is secreted by one of the species. Cord-Ruwisch et al. (10) used direct microscopic enumeration of two morphologically distinct organisms to demonstrate interactions in mixed culture. This method cannot be used for organisms with similar morphology. Kampfer (20) compared classical methodologies with rRNA analysis of filamentous bacteria from activated sludge. He concluded that "Results of these investigations have outlined a high degree of genetic diversity hidden by identical morphology."

Another problem in differentiating organisms in mixtures for enumeration is metabolic interactions among species. Lewis et al. (22) showed that culture filtrates, mixed populations, and

microbial exudates altered plate counts of organisms and rates of transformation of pollutants. Moller et al. (24) demonstrated induction of a degradative gene in *P. putida* by products of an *Acinetobacter* species during growth on toluene and related aromatic compounds. Secondary degraders are organisms that do not grow on the primary substrate to be degraded, but rather utilize secondary metabolites produced by organisms able to grow on the primary substrate (27). They pose a unique problem because they are usually present in smaller numbers and do not have readily exploitable metabolic pathways for selection in the presence of primary degraders. Jimenez et al. (19) characterized a four-member aerobic bacterial consortium capable of mineralizing alkylbenzene sulfonate. Three members of the consortium could grow on alkylbenzene sulfonate as primary degraders, but all four members were necessary for mineralization.

Molecular biology offers new methods for direct quantitative determination of species mixtures in samples (3). Ribosomal DNA sequences have been used in a variety of techniques, including directed PCR, fluorescent in situ hybridization (FISH), in situ PCR, slot blotting, MPN PCR, flow cytometry, Southern hybridizations, and colony hybridizations for identification of microbes at several taxonomic levels (2, 6, 7, 11, 12, 16, 17, 20, 21, 24, 28–30). These sequences are highly conserved, well-characterized gene families with high copy numbers and distinct variable regions. rRNA genes contain conserved regions of sequences that are the same within a kingdom and variable regions that contain differences between taxonomic groups down to the species level (16). A databank of ribosomal DNA sequences has been created to aid in designing probes for single species or groups of related species (23), and a collection of oligonucleotide probe sequences has also been developed for organized distribution of published probes and conditions for their use (1). Because these species-specific sequences can be used in more than one quantitative molecular technique, once developed, they can be used for a variety of lab and environmental samples (2, 16, 28, 30).

P. putida F1 and *Burkholderia* sp. strain JS150 are ideal organisms for the study of population dynamics in the biodeg-

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TABLE 1. 16S rRNA primers and oligonucleotides used in this study

Standardized name ^a	Target group	Probe sequence (5' to 3')	Old probe name (reference)
S-K-Mon-0515-a-S-18	Kingdom Monera	TGC CAG CAG CCG CGG TAA	515FPL (25)
S-D-Bact-1390-a-A-20	Domain <i>Bacteria</i>	AGG CCC GGG AAC GTA TTC AC	13B (25)
S-D-Bact-0806-a-A-18	Domain <i>Bacteria</i>	ACT ACC AGG GTA TCT AAT	806R (25)
S-S-P.put-0597-a-S-20	Species <i>P. putida</i>	ACT GCA TCC AAA ACT GGC AA	Pp16S
S-S-B.spJ-0597-a-S-20	Species <i>Burkholderia</i> strain JS150	ACT GCA TTG GTG ACT GCA TC	JS16S
S-G-Bacil-0597-a-S-22	Genus <i>Bacillus</i>	GGG TCA TTG GAA ACT GGG GAA C	Bs16S
S-S-P.put-0597-a-A-20	Species <i>P. putida</i>	TTG CCA GTT TTG GAT GCA GT	Pp16S-R
S-S-B.spJ-0597-a-A-20	Species <i>Burkholderia</i> strain JS150	GAT GCA GTC ACC AAT GCA GT	JS16S-R
S-G-Bacil-0597-a-A-22	Genus <i>Bacillus</i>	GTT CCC CAG TTT CCA ATG ACC C	Bs16S-R

^a Based on nomenclature for the Oligonucleotide Probe Database (1).

radiation of pollutant mixtures. Both grow on aromatic hydrocarbons, such as toluene and phenol, which are common groundwater contaminants. In addition, their catabolic pathways are well characterized (15, 18). Because they can utilize many of the same compounds as carbon and energy sources, their interactions as primary degraders are of interest. However, because of their similar metabolic pathways, these two organisms are difficult to distinguish by using traditional selective counting media. *Bacillus subtilis* is a common soil bacterium that can function as a secondary degrader of aromatic hydrocarbons. Little is known of its interactions with the other species, as might be expected for organisms in microbial consortia.

We report here the development of a FISH method based on 16S rRNA species-specific sequences for direct counting of these organisms in a mixed culture over time to study population dynamics during the biodegradation of phenol. Our development and use of this technique was motivated by the failure of traditional methods in our system.

MATERIALS AND METHODS

Bacterial species and growth media. *P. putida* F1, obtained from David Gibson (15), was grown with shaking at 30°C on mineral salts-based medium (MSB) with toluene vapors. MSB contains 7.5 mM ammonium sulfate, 40 mM phosphate buffer (pH 7.25), and a modified Hutner's mineral base (9). *Burkholderia* sp. strain JS150, obtained from Jim Spain (18), was grown with shaking at 30°C on MSB with 0.5 mM phenol. *B. subtilis* ATCC 6633 was obtained from the American Type Culture Collection, grown on trypticase soy agar (TSA) (Becton Dickinson) plates or in trypticase soy broth with shaking at 37°C. *P. putida* F1 and *Burkholderia* strain JS150 both grow on phenol and toluene, while *Burkholderia* strain JS150 grows on chlorobenzene (15, 18). *B. subtilis* does not grow on phenol, toluene, or chlorobenzene, but does grow on phenyl ethyl alcohol plates (8). Experiments in our laboratory demonstrated that this strain can grow on metabolites released by both *Burkholderia* strain JS150 and *P. putida* F1 (data not shown).

Design of oligonucleotides. Sequences of 16S rDNA from several *P. putida* strains, *Burkholderia* species, and *Bacillus* species were retrieved from GenBank (4). Comparison of these sequences showed several regions suitable for species-specific forward primers for PCR amplification reactions. The V4 variable region was chosen between bases 515 and 806 in *Escherichia coli* (25). Available sequences from the following closely related organisms were examined to confirm the uniqueness of these sequences (accession no. in parentheses): *P. putida* F1 (L37365), *P. putida* Env2.9 (Z36533), *P. putida* PaW1 mt-2 (L28676), *P. putida* JCM6156 (D37924), *Burkholderia* strain KB740 (X77679), *Burkholderia* strain H1 (U16144), *B. subtilis* W168 PY79 (K00637), *Bacillus megaterium* IAM 13418 (D16273), *Bacillus aerotrophus* NCIMB 12899 (X60607), *B. subtilis* 168 (D64126), and *B. subtilis* (X00007).

DNA was isolated from 1 ml of late-log-phase culture of *P. putida* F1 (24 h old) and *Burkholderia* strain JS150 (18 h old) by using the sodium dodecyl sulfate method of Black and DuTeau (5). DNA was isolated from 24-h-old colonies of *B. subtilis* ATCC 6633 by using their hexadecyl trimethyl ammonium bromide procedure (5). An aliquot of DNA from each species and *E. coli* B DNA (Sigma) was diluted to 1 ng/μl for use in PCR with universal bacterial primers flanking the V4 variable region of 16S rRNA (Table 1). Products of the following lengths were obtained: 875 bp for *E. coli*, 876 bp for *P. putida* F1, 870 bp for *Burkholderia* strain JS150, and 873 bp for *B. subtilis* ATCC 6633 (see Fig. 1). Directed PCR was performed in 25-μl reactions (50 mM KCl, 10 mM Tris [pH 9.0], 1.5 mM

MgCl₂, 200 μM dNTPs, 25 pM each primer, 1 ng of template DNA, 0.01% gelatin [wt/vol], and 0.1% Triton X-100) and 1 U of *Taq* polymerase (Promega) on an MJ Research PTC-100 thermal cycler (5). Thermal cycling conditions were 1.5 min at 92°C, 30 s at 56°C, and 1 min at 72°C for 35 cycles with a final extension of 7 min at 72°C. Primers were commercially synthesized (Operon Technologies). Products of directed PCR were separated for 1 h at 60 V on a 1.2% agarose gel (50 ml of minigel) containing 25 μg of ethidium bromide with Tris-borate-EDTA buffer.

PCR products from *P. putida* F1, *Burkholderia* strain JS150, and *B. subtilis* ATCC 6633 were purified with the PCR Wizard kit (Promega). Following the manufacturer's instructions for cycle dideoxy termination and automated sequencing with Applied Biosystems, Inc. Model 377, PCR products were sequenced in both directions by using universal primers as sequencing primers (CSU MacroMolecular Resources).

FISH. To maximize signal for detection by in situ hybridization with species-specific oligonucleotides, one needs to use an oligonucleotide that hybridizes to the abundant 16S rRNA in the fixed bacterial cells (12). We used the complementary (antisense) sequence of the species-specific PCR forward primer (Table 1). Primers were synthesized commercially and 5' labeled with fluorescein isothiocyanate (FITC) (Operon Technologies).

Slides were cleaned with 95% ethanol and allowed to air dry. An aliquot of 30 μl of cell suspension was dropped into the center of each glass slide. Slides were run through a graded series of 50, 70, and 95% ethanol and then fixed in a solution of 10% formaldehyde–90% ethanol (6). To promote cell adhesion, 30 μl of a bind silane solution (5 μl of g-methacryloxy-propyl-trimethoxysilane [Sigma] in 100 μl of a solution of 95% ethanol with 0.5% glacial acetic acid) was dropped onto the cells (14). For hybridization, 50 μl of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.1% sodium dodecyl sulfate 8 ng of FITC-labeled oligonucleotide/μl [21]) was dropped onto the slide. A cover slip was sealed on each slide with rubber cement. Slides were placed in an MJ Research PTC-16S thermal cycler with a petri dish of distilled, deionized water to maintain humidity for 16 h at 45°C. Coverslips were removed and slides were washed in 50 ml of hybridization solution for 15 min at 45°C. Slides were examined on a Leitz Aristoplan epifluorescence microscope with 15 μl of 1:1 glycerol-PBS with 0.25% NaI, using a Bioquant Image Analysis system (Geo-Centers, Inc.).

FISH for enumeration of samples from batch cultures. Duplicate flasks were inoculated with *P. putida* F1, *Burkholderia* strain JS150, and *B. subtilis* ATCC 7003 from overnight cultures of each organism grown with shaking at 30°C on MSB with 0.5 mM phenol. The initial cell concentrations were approximately 10⁵ cells of each species/ml. These mixed culture flasks were grown with shaking for 35 h at 30°C on MSB with an initial phenol concentration of 0.7 mM. Samples were taken at 6, 13, 20, 24, 26, 28.5, and 35 h. Duplicate slides were prepared from each sample. Twenty fields were randomly chosen on each slide for counting. Phenol levels were determined with an HP 5890 gas chromatograph equipped with an HP model 5971 mass selective detector. Samples were extracted (0.75 ml of aqueous sample to 0.75 ml of chloroform containing 25 mg of *p*-xylene/liter as an internal standard) and injected onto a 50-m HP-5 (cross-linked 5% phenyl methyl silicone) capillary column (Hewlett Packard) with an inner diameter of 0.2 mm and a film thickness of 0.33 μm which was equipped with an HP model 7673 autosampler. The carrier gas was high-purity helium at 1.9 ml/min and 45 psi. The temperatures of the inlet and detector were 200 and 280°C, respectively. The oven temperature was held at an initial temperature of 35°C for 1.5 min, raised at a rate of 50°C/min to 57°C where it was held for 3 min, then increased at 10°C/min to 100°C and finally 15°C/min to 160°C.

Nucleotide sequence accession number. The *Burkholderia* strain JS150 16S rDNA sequence obtained in this study has been assigned GenBank accession no. AF092889.

RESULTS

Plate counts. The initial experimental plan called for the use of differential plate counts as a validation of the molecular mi-

FIG. 1. Agarose gel of 16S rDNA fragments amplified with species-specific primers. Size markers are a 1 Kb plus ladder (Gibco-BRL). All PCR reactions used the universal reverse primer 13B (Table 1). Forward primers (Table 1) are indicated across the top of the figure. Template DNA used in the reaction is given above each lane. The negative control was 1× PCR mix with no added template DNA. P. p. F1, *P. putida* F1; Bu. Sp. JS150, *Burkholderia* sp. strain JS150; Ba. s. 6633, *B. subtilis* ATCC 6633.

crobal methods under investigation, since *P. putida* F1, *Burkholderia* strain JS150, and *B. subtilis* are readily cultured on a variety of media. However, developing a differential plate counting method for a mixture of these organisms proved to be difficult. No medium or growth condition that enabled growth of *B. subtilis* but not the other strains was found. Furthermore, devising a differential plate counting method for *P. putida* F1 and *Burkholderia* strain JS150 was also problematic, since they have similar metabolic capabilities. Both organisms were primary degraders of phenol and toluene and exhibited similar morphologies on plates using either of these substrates as the sole source of carbon and energy. Plating of *Burkholderia* strain JS150 on chlorobenzene was originally viewed as an option, since *P. putida* F1 is unable to utilize chlorobenzene. However growth of *Burkholderia* strain JS150 on plates with chlorobenzene vapor was extremely slow, and the accumulation of degradation products colored the plates brown, making enumeration of colonies difficult. A plate counting method based on differential growth (13) using one-half-strength TSA (1/2 TSA) plates was chosen. On this medium, *P. putida* F1 produced countable colonies in approximately 24 h and *Burkholderia* strain JS150 produced countable colonies in approximately 48 h. Use of this plate count method was limited in that it was unable to detect the species in smaller concentrations when the biomass ratio was greater than five. If one species was present at a much lower concentration than another, the plate dilutions required for enumeration of the more abundant species would fail to pick up the species in lower concentration.

This plate count method eventually proved untenable because interactions between the two species were observed when they were grown together on the same plate of 1/2 TSA medium. Specifically, *P. putida* F1 inhibited the growth of *Burkholderia* strain JS150 in inhibition tests on 1/2 TSA plates. This was demonstrated by streaking one species in a line down the middle of a 1/2 TSA plate and incubating the plate for 24 h at 30°C. After 24 h, the second species was streaked in several lines perpendicular to that of the first species, with the streak

beginning as close as possible to the line with the first species. The plate was then allowed to grow for another 24 h. After the second growth period, plates were checked for inhibition zones. When *P. putida* F1 was streaked first, it clearly inhibited the growth of the *Burkholderia* sp. strain JS150 as evidenced by a zone of at least 1 cm around the *P. putida* F1 in which the other species was unable to grow. Conversely, when the *Burkholderia* strain JS150 was streaked first, no zone of clearing (and thus no inhibition of growth) was observed for *P. putida* F1. This inhibition was also noted when known mixtures of the two species were plated and counted using this method; counts of *Burkholderia* strain JS150 were always lower than the correct value.

Design of species-specific oligonucleotides. The sequences from PCR products amplified from *P. putida* F1 and *B. subtilis* ATCC 6633 were compared with GenBank sequences to verify the identities of the organisms used. The *Burkholderia* sp. strain JS150 16S rDNA sequence has not been reported previously. Comparison of sequences showed several regions suitable for species-specific forward primers for PCR amplification reactions. One region between bases 550 and 650 in *E. coli* was chosen. Primers were designed (Table 1) and synthesized commercially (Operon Technologies). Oligo 4.0 (National Biosciences, Inc.) was used to verify the suitability of these primers as forward primers with the universal reverse primers 806R or 13B (25).

A one-step PCR program with an annealing temperature of 60°C was used to amplify fragments with species-specific primers. The annealing temperature was raised by 2°C from 48°C until specific amplification occurred. Species-specific primers were used in PCR with DNA from all three species to demonstrate discrimination (Fig. 1). Detectable PCR amplification occurred only with the DNA and the forward primer from a given species. Products of 761, 755, and 759 bp were obtained for *P. putida* F1, *Burkholderia* strain JS150, and *B. subtilis* ATCC 6633, respectively.

FIG. 2. FISH of 16S rRNA with 5' FITC-labeled species-specific oligonucleotides of a mixture of *P. putida* F1, *Burkholderia* strain JS150, and *B. subtilis* ATCC 7003. (A) Hybridization with *P. putida*-specific oligo PP16S-R. (B) Field shown in panel A but stained with PI to show all three species. (C) Hybridization with *Burkholderia* strain JS150-specific oligo JS16S-R. (D) Field shown in panel C but stained with PI to show all three species. (E) Hybridization with *B. subtilis*-specific oligo BS16S-R. (F) Field shown in panel E but stained with PI to show all three species. Arrows indicate the locations of reference bacteria to orient the field. Images were produced with Adobe Photoshop 2.5 for Windows and printed on a Sony Digital Color Printer UP-D7000.

FIG. 3. Biodegradation of phenol by a 1:1:1 inoculum of *P. putida* F1, *Burkholderia* strain JS150, and *B. subtilis* ATCC 7003.

FISH. Slides were prepared with single species and mixtures of species to confirm discrimination of species by the antisense oligonucleotides. Hybridization with labeled species-specific oligonucleotides and counter-staining with propidium iodide (PI) demonstrated discrimination between species with these probes (Fig. 2). The efficiency of staining single species was calculated by counting six fields per slide for a total of at least 300 cells per slide for slides of single species hybridized with their corresponding oligonucleotide. Fields were counted again after PI counter-staining. Sensitivities were $89.3\% \pm 9.1\%$ for *Burkholderia* sp. strain JS150 with S-S-B.spJ-0597-a-A-20 (JS16S-rev), $93.0\% \pm 8.9\%$ for *P. putida* F1 with S-S-P.put-0597-a-A-20 (Pp16S-rev), and $88.3\% \pm 11.0\%$ for *B. subtilis* ATCC 7003 with S-G-Bacil-0597-a-A-22 (Bs16S-rev).

Biodegradation. The biodegradation of phenol and growth of the three species in one flask, as measured by the FISH method described above, is shown in Fig. 3. Growth of all three species occurred, and phenol was consumed. *Burkholderia* strain JS150 had a shorter lag time and a faster growth rate and grew to higher cell densities than either of the other two species. The secondary degrader, *B. subtilis* ATCC 7003, also grew, though to a relatively small extent. Phenol was completely mineralized as evidenced by carbon balances in the duplicate flasks of $97\% \pm 11\%$ and $94\% \pm 10\%$.

DISCUSSION

The results in Fig. 3 demonstrate the ability of the FISH method to produce reliable, quantitative cell density data for a mixture of three bacterial species. It is important to note that these results were produced in the presence of interspecies interactions that were problematic for growth-based, indirect counting methods. Furthermore, the FISH method yielded results when there were 10-fold differences in population size between *Burkholderia* strain JS150 and the other two species. Finally, the data obtained with this method have a high degree of precision. The larger errors of the two stationary-phase measurements of *Burkholderia* sp. strain JS150 were caused by clumping of those cells, a phenomenon that plagues most cell-counting methods.

Biodegradation in natural environments is carried out by mixed microbial populations. The application of laboratory-derived processes for remediation of contaminated sites with microbes is dependent upon knowledge of the response of

microbes of interest to environmental influences, such as the addition of nutrients, the interactions between native organisms, and interactions with added microbes when bioaugmentation is used. If understood, these relationships could be important for the design of bioreactors and in situ bioremediation processes (26). As we begin to explore population dynamics of organisms in mixed cultures, we need to apply techniques that are quantitative, have a high degree of specificity, and do not require subculturing of organisms.

The use of traditional methods for single organisms raised several problems in our mixed culture study. Specific issues to be considered in distinguishing these organisms were relatedness of species (17, 28), relative numbers or biomass ratio (7), interactions between species (22), and the presence of secondary degraders (19, 24, 27). Finding substrate-specific plates to distinguish species with similar metabolic pathways was difficult. The chlorobenzene plates were impractical for distinguishing *P. putida* F1 and *Burkholderia* sp. strain JS150 since slow growth allowed the accumulation of an intermediate that discolored the plates. Selection by differential growth rate was limited, in part, by the small range of concentration difference detectable, problems that began at only a fivefold concentration difference.

Although a differential plate count method was devised for *P. putida* F1 and *Burkholderia* strain JS150, it could not be used because the inhibition of *Burkholderia* strain JS150 skewed the counts. This sort of inhibition by colonies growing on agar medium plates for counting has rarely been described in biodegradation studies, and provides a good example of the complexity of the interactions between microbial populations.

Secondary degraders in microbial consortia are of increasing interest (7, 19), but their low numbers and the difficulty in selectively culturing them from the mixture are problems. No selective carbon source for *B. subtilis* ATCC 7003 was known. Phenyl ethyl acetate agar was the recommended selective medium (8), but *P. putida* F1 grew on this medium also. The search for a differential medium for *B. subtilis* ATCC 7003 would have been time consuming. Another basis for choosing direct counting methods is their ability to track an unlimited number of species in the same sample. In contrast, all culture-based (indirect) counting methods become nearly impossible to apply as the number of different organisms in a population increases.

The use of 16S rRNA sequences allows us to detect each species based on differences in DNA sequence. This specificity allows us to exploit the uniqueness of the species without requiring the biochemical expression of phenotypic traits. How does species specificity work in environmental samples with many other species? We know by the genetic definition of relatedness that closely related species have similar sequences; less-related organisms differ more. The purpose of designing species-specific oligonucleotides is to find a variable region within the 16S rRNA gene that is different even in closely related species. Other organisms in a field trial, like fungi and bacteria from other orders, families, and even genera, will differ more and will not pose a problem. The problem will be the presence of closely related species within the same genus. Controls can be designed to check for the presence of related species. Sequences of 16S rRNA can be compared, adjusting oligonucleotide sequences to maximize differences (17, 28). The oligonucleotide sequences described here have also been used for discrimination of these species in MPN PCR, colony hybridizations, and slot blotting (data not shown), techniques used in direct counting of organisms in soil, ground water, biofilms, and continuous culture (2, 3, 7, 11, 20, 24, 28–30). Development of genetic markers for multiple molecular bi-

ological techniques creates opportunities for researchers in microbial ecology to directly detect and enumerate organisms in mixed cultures.

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