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Occurrence of Two Resin Acid-Degrading Bacteria and a Gene Encoding Resin Acid Biodegradation in Pulp and Paper Mill Effluent Biotreatment Systems Assayed by PCR

Running title: Resin Acid-Degrading Bacteria in Biotreatment Systems

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ABSTRACT

We examined the distribution of two dehydroabietic acid-degrading bacteria, Pseudomonas abietaniphila BKME-9 and Zoogloea resiniphila DhA-35, in biotreatment systems for pulp and paper mill effluents (PPMEs) using PCR assays. These two bacteria were first isolated from two PPME biotreatment systems and can degrade both dehydroabietic acid (DhA) and other abietane resin acids. We also examined the distribution of a catabolic gene, ditA1, encoding the α subunit of an aromatic ring-hydroxylating dioxygenase involved in DhA degradation by BKME-9. PCR primers specific for the 16S rDNA of BKME-9 and of DhA-35 and specific for ditA1 were used. Among three laboratory- and 17 full-scale PPME biotreatment systems, 10 contained BKME-9, three contained DhA-35, and 11 contained ditA1, indicating the wider distribution of BKME-9 than of DhA-35. Both BKME-9 and ditA1 were detected in the biotreatment system from which BKME-9 was originally isolated in 1994, suggesting the persistence of BKME-9 in that biotreatment system. The detection limit of the PCR assay was one cell per PCR reaction, which corresponds to one BKME-9 cell per $6x10^7$ total sludge bacteria. A competitive PCR assay indicated that ditA1 ranged from 51 to 250 copies/mg of dry biomass. BKME-9 appears to contribute to the biodegradation of resin acids in some PPME biotreatment systems. Using degenerate PCR primers and touchdown PCR, we obtained from our DhA-degrading strain collection six DNA sequences putatively homologous to that of ditA1. Cluster analysis of these DNA sequences suggests that ditA1 encodes a representative of a novel class of dioxygenase enzymes.

Introduction

Resin acids are toxic, tricyclic, diterpenoid compounds present in wood that occur in pulp and paper mill effluents (PPMEs) [13, 20, 42]. Although resin acids account for only a small portion of the total organic matter in PPMEs, they contribute to the major toxicity of PPMEs [13, 20, 42]. Therefore, efficient removal of resin acids from PPMEs is essential for biotreatment of PPMEs. However, biotreatment systems occasionally fail to remove resin acid toxicity when upsets occur due to causes such as extreme temperature, organic loading, pH, sulfite concentrations, or resin acid concentrations. These failures result in toxicity breakthroughs [23, 34, 37].

Our understanding of biodegradation of resin acids in biotreatment systems is severely hindered by our limited understanding of the microbial ecology of resin acid-degrading populations in these systems. Numerous resin acid-degrading bacteria from a variety of sources including forest soil [28], Arctic soil (unpublished data), PPME treatment systems [7], and laboratory bioreactors [26, 28, 45, 46], and compost [46] have been isolated and characterized. However, little information is known about their ecology, including distribution, abundance, and population dynamics. Our knowledge of resin acid-degrading microorganisms and resin acid biodegradation is mostly based on laboratory studies of pure cultures, but, microorganisms almost certainly behave differently in pure cultures than in mixed communities where complex interactions occur. In addition, most microorganisms (85% to 99.999%) in natural microbial communities are thought to be nonculturable [2, 6, 15, 39, 43], and cultivation in the laboratory possibly alters the structure of microbial communities. Therefore, conclusions drawn from pure culture studies may not be valid for microorganisms in situ. Investigation of the distribution and abundance of indigenous resin acid-degrading bacteria, and their catabolic genes in various PPME biotreatment systems, could reveal factors affecting these populations and help us understand their ecology and relative importance to the overall removal of resin acids in these systems. Measuring the population of a specific group of resin acid-degrading organisms in a PPME biotreatment system could also allow estimation of the capacity of resin acid degradation by that group and the importance of that group for resin acid removal. The information obtained from such ecological studies will help us understand system failure and contribute to the optimization of existing PPME biotreatment systems as well as development of new biotreatment systems.

Resin acid-degrading populations in biotreatment systems are likely small, because resin acids are a small fraction of total organic matter in PPMEs, and most resin acid degraders appear to have narrow substrate ranges [26, 28, 46]. Therefore, a very sensitive method is required to study resin acid-degrading populations. Hybridization of oligonucleotide probes to nucleic acids directly extracted from samples has been used to study species within complex communities [2, 15, 30, 39], but this hybridization method was found not sensitive enough to detect resin acid-degrading populations in PPME biotreatment systems [30]. PCR assays are more sensitive and can be made quantitative by using either most probable number PCR or competitive PCR. Most-probable-number PCR (MPN-PCR) is based on most-probable-number analysis of microorganisms, in which PCR is used to screen for the presence of a specific target sequence in samples diluted to extinction [9, 10]. In competitive PCR, a competitive internal standard (CIS), with known copy number and identical primer-binding sequences as on the target, is co-amplified together with the target of interest [11, 17, 21, 22]. Competitive PCR has the advantage that, once the CIS has been constructed, many samples can be analyzed relatively more efficiently and with a higher precision than that would be achieved by MPN-PCR, which requires a greater number of reactions and is less precise.

Genes involved in DhA biodegradation by Pseudomonas abietaniphila BKME-9, isolated four years ago from a PPME biotreatment system [7], were recently identified and sequenced [25]. One of the genes, ditA1, encodes the α subunit of a diterpenoid dioxygenase. The ditA1 gene was found to be required for growth of BKME-9 on DhA. The sequences of ditA1 and the 16S rRNA genes of BKME-9 and of DhA-35 [28] enabled us to design PCR primers to analyze DhA-degrading populations in various samples. The former sequence also enabled us to obtain sequences of putative ditA1 homologues in other resin acid-degrading bacteria.

The objectives of this study were (i) to develop very sensitive PCR assays for resin acid-degrading bacteria in PPME biotreatment systems, (ii) to investigate the distribution and abundance of those bacteria, and (iii) to isolate and compare ditA1 homologues from other resin acid-degrading bacteria in order to design guild-specific primers. For the first time we report the distribution of resin acid-degrading bacteria indigenous to PPME biotreatment systems using a PCR assay more sensitive than any previously reported. The partial sequences of putative ditA1 homologues from six out of 15 other DhA-degrading bacteria were also determined, and ditA1 homologues appear to encode a distinct class of aromatic ring-hydroxylating dioxygenases.

Materials and Methods

Cultures and sludge samples

Pseudomonas abietaniphila BKME-9 was kindly provided by P. Bicho, PAPRICAN, Canada. Other resin acid degraders were from our own collections. Other Pseudomonas spp. were a gift from E. Moore, GBF-National Research Center for Biotechnology, Germany. Burkholderia (formerly Pseudomonas) cepacia LB400 was given by L. Eltis, University of Laval. Zoogloea ramigera ATCC 19544^T was purchased from ATCC. All the pure cultures used in this study were grown in tryptic soy broth (TSB) overnight for DNA extraction. The sludge (mixed liquor) samples from laboratory-scale activated sludge bioreactors treating bleached kraft mill effluent (BKME) were kindly provided by S. Duff and E. Hall, Pulp and Paper Centre, University of British Columbia, Canada. All sludge (also mixed liquor) samples from full-scale biotreatment systems were kindly collected and shipped to us on ice by the pulp and paper mills and municipal treatment plants listed in Table 2. The biomass was harvested from the mixed liquor samples (1-2 ml for samples with high biomass concentrations, or 50-130 ml for samples with low biomass concentrations) by centrifugation at 16,000 x g for 5 min at 4^oC and was frozen immediately at -70^oC until use for DNA extraction.

DNA extraction

DNA from pure cultures was extracted by using the CTAB method [5]. DNA from activated sludge samples was extracted by using a mini-bead beating plus ammonium acetate precipitation method [47]. In order to avoid potential cross contamination, aerosol barrier pipet tips were used for all liquid handling, and DNA extractions were carried out in a sample preparation room which is separate from the PCR product analysis room.

Primer design and construction of the competitive internal standard (CIS)

PCR primer pairs used in this study (Table 1) were synthesized at the Nucleic Acid/Protein Service (NAPS), University of British Columbia. The 16S rDNA sequences of BKME-9 and DhA-35, and ditA1 gene sequence of strain BKME-9 were first analyzed using the BLAST program [1]. Related sequences were retrieved and aligned with GeneWorks (Intelligenetics, Inc., CA). PCR primers were designed from the regions where specificity would best be achieved. Finally, the primer sequences were analyzed with the computer program Amplify [12]. To design degenerate PCR primers for amplification of ditA1 homologues from other resin acid degraders, the ditA1 sequence was aligned with other aromatic ring-hydroxylating dioxygenase gene sequences retrieved from GenBank. The forward primers (ditA1-Y263f and ditA1-R263f) were designed to target the [2Fe-2S]-binding site of these dioxygenases, and the reverse primer (ditA1-1196r) was designed after analyzing the alignment of ditA1 and sequences of two PCR products amplified from two other resin acids degraders (IpA-51 and DhA-51) using the ditA1-719f/ditA1-1212r primers at low annealing temperature (50^oC).

To construct a competitive internal standard (CIS) for quantitative PCR amplification of the ditA1 gene, one longer primer (the deletion-1212r) was also synthesized. The construction of the CIS for ditA1 is shown schematically in Fig. 1A. PCR amplification of ditA1 with primers ditA1-719f and deletion-1212r produced the CIS for ditA1.

Qualitative PCR amplification

The 16S rDNAs and the ditA1 fragment were amplified separately with their specific primer pairs in a thermal cycler (PowerBlock II, ERICOMP, San Diego). PCR amplification was conducted in a total volume of 50 µL containing 0.5 pmol of each primer per µL, 200 µM (each) dNTP, 1.75 mM $MgCl_2$, 1x Taq buffer, 670 µg/ml bovine serum albumin (BSA), and 1.25 U of Taq DNA polymerase (Sigma, St Louis). The DNA templates were first subjected to an initial denaturation step for 2 min at 95°C with simplified hot start. The subsequent cycles consisted of a 0.5-min denaturation step at 94°C, a 0.5-min annealing step at 66°C (60°C for Zre-57f/Zre-423r), and a 1-min extension step at 72°C. A final 5-min extension at 72°C was included after 40 cycles of amplification. Negative controls containing all components except DNA templates and positive controls using BKME-9 or DhA-35 genomic DNA as template were included in parallel. The PCR products were electrophoresed on standard 1.0% agarose gels, which were then stained with 0.5 µg/ml ethidium bromide. In order to prevent contamination, the preparation of individual PCR reactions was carried out in a PCR chamber equipped with a UV lamp, located in a separate room. The PCR chamber was irradiated with UV for 10 min before and after each use. The PCR products were analyzed in a separate room to minimize chances of cross contamination.

Determination of the detection limit of the PCR method

To determine the overall detection limit, a culture of BKME-9 grown to early stationary phase was enumerated by direct counting under a phase contrast microscope. This culture was then diluted (1:10) in a series of tubes, and the dilutions with 10^0 to 10^6 cells/ml were mixed into sludge samples obtained from laboratory bioreactor C, which did not have PCR-detectable BKME-9 or ditA1. The DNA was extracted from these samples as described above and subjected to PCR amplification using the PCR conditions described above. The detection limit was calculated from the sample with the lowest number of added BKME-9 cells that gave the expected PCR products.

Quantitative PCR

To quantify the ditA1 gene copy number in a sample, 1.0 µL of DNA sample and 1.0 µL of diluted CIS with known copy number were co-amplified in a series of PCR tubes. The PCR conditions were similar to those specified above with the exception of dNTP concentrations. Instead of 200 µM of each dNTP, 50 µM each of dATP, dTTP, dGTP, and 10 µM of dCTP were used. For each 50-µL quantitative PCR reaction, 2.0 µCi of [α–^{32}P]dCTP (3,000 Ci/mmol) (NEN Life Science Products, Boston, MA) was included to label the PCR products, which were then separated on a 5% polyacrylamide gel and quantified with a PhosphorImager SI (Molecular Dynamics, Inc., CA). The copy number of ditA1 was calculated according to the following equation: Number of ditA1 gene/ml = $C \times R_l \times R_v$ (1), where C = copy number of the CIS yielding the same amount of PCR product as the target per PCR reaction, R_l = the ratio of the CIS sequence length (bp) over the target sequence length (bp), R_v = the ratio of the DNA volume (μL) resulting from DNA extraction per sample over the DNA volume (μL) used per PCR reaction. The copy number of ditA1 gene/mg of biomass was calculated from the volatile suspended solid (VSS) concentration of each sample and the quantitative data obtained from the equation above.

The amplification efficiencies of ditA1 and its CIS were determined by using the competitive PCR assay described above. A varying number of CIS and 1.5 x 10^3 ditA1 fragments were co-amplified, and the PCR products were quantified. The ratios of input ditA1 and CIS were plotted against the ratios of the PCR products of ditA1 and its CIS. Also, BKME-9 cells were seeded into a sludge sample, which did not have PCR-detectable ditA1 or BKME-9, to the density of 10^4 BKME-9 cells/ml. Then, the seeded BKME-9 cells were quantified.

Restriction of the PCR products

The identities of the PCR products were verified by restriction analysis. The PCR products amplified from the sludge samples with the ditA1 primers were digested with restriction enzymes AccI and AlwNI according to the conditions specified by the manufacturer (Boehringer Mannhem, GmbH, Germany). The resultant restriction fragments were resolved on 1.0% agarose gels and visualized after staining with ethidium bromide.

PCR amplification, sequencing, and phylogenetic analysis of ditA1 homologues from resin acid degraders

Genomic DNA extracted from pure cultures of all resin acid degraders was subjected to PCR amplification using either ditA1-Y263f or ditA1-R263f and ditA1-1196r. The PCR conditions were similar to those used in the qualitative PCR reactions described above except for a longer extension step (1.5 min) and the touch-down PCR (TD-PCR) cycling, during which the annealing temperature decreased 2^oC at every other cycle from 55^oC to 31^oC, followed by 20 cycles with an annealing temperature at 50^oC. After PCR amplification and confirmation of the product size by electrophoresis, the PCR products were cloned into pCR2.1 vectors using a TOPO TA Cloning Kit (Invitrogen, CA). After screening by restriction analysis, the chosen clones were sequenced with an Applied Biosystem 373A DNA sequencer as specified by the manufacturer (Perkin-Elmer, Applied Biosystem, CT) for Taq cyclic sequencing with fluorescent-dye-labeled dideoxynucleotides. ClustalX [38] and TreeCon [41] were used to align the sequences and generate the phylogenetic tree.

Nucleotide sequence accession number

Not yet available.

Determination of resin acid concentrations and VSS

The resin acids were analyzed by gas chromatography as previously described [26] with minor modification in the extraction step. Four ml of sludge sample (mixed liquor) was added to a 9-ml PYREX tube containing two ml of ethyl acetate and one gram of glass beads with a diameter of two mm. The tube was shaken vigorously by hand for one min to break the sludge flocs. The identity of resin acids was confirmed by mass spectroscopy using previously described methods [27]. The VSS contents of all the samples were determined according to standard procedures [3].

Results and Discussion

The specificity of the PCR assays

The 16S rDNA primer pair for BKME-9 (Pab-613f/Pab-832r) was designed not to complement any known DNA sequences in the GenBank or Ribosomal Database Project (RDP). Analysis of the most closely related 16S rDNA sequences using the computer program Amplify showed that the Pab-613f/Pab-832r primer pair was specific to P. abietaniphila BKME-9. In qualitative PCR assays, PCR product of the expected size was amplified from strain BKME-9, but not from five phylogenetically related Pseudomonas species, including P. agarici LMG 2112^T, P. amygdali LMG 2123^T, P. putida ATCC 12633, P. syringae LMG 1247t1^T, and P. stutzeri ATCC 17588. This indicates the high specificity of this primer pair and PCR conditions as predicted by the sequence analysis. Using the same experimental approach, the Zre-57f/Zre-423r primer pair was also shown to be specific to Zoogloea resiniphila DhA-35.

The 5’ region of the ditA1 gene sequence (about 400 bp) had some similarity (<60%) to a few other genes encoding the α subunit of aromatic ring-hydroxylating dioxygenases found in the GenBank. Therefore, the primer pair (ditA1-719f/ditA1-1212r) was designed to amplify the 3’ region of ditA1. This appeared logical as the C-terminal region of the α subunit of the dioxygenase has been shown to determine substrate specificity [19, 31]. The specificity of the ditA1 primer pair was tested against metabolically related bacteria, which include 13 resin acid-degrading isolates [Sphingomonas sp. DhA-33, Zoogloea resiniphila DhA-35, Ralstonia sp. BKME-6, Burkholderia sp. DhA-53, Burkholderia sp. DhA-54, Burkholderia sp. IpA-51, Pseudomonas vancouverensis DhA-51, Pseudomonas multiresinivorans IpA-1, Pseudomonas sp. IpA-2, Mycobacterium sp. IpA-13, Mycobacterium sp. DhA-55, β-Proteobacteria DhA-71 and DhA-73 [7, 26, 45, 46]] and Burkholderia cepacia LB400, a biphenyl-degrading bacterium [16]. Under the PCR conditions used in this study, the only PCR product was from BKME-9. Thus, the ditA1-719f/ditA1-1212r primer pair appears to be specific for the ditA1 gene of BKME-9. The failure to amplify the expected PCR products with both the ditA1 and 16S-rDNA primers from some sludge samples (see below), which presumably contain very diverse microbial communities, also suggests that the primers used in this study are specific to BKME-9 and DhA-35. Due to the high specificity of the PCR assays used in this study, we could not detect resin acid-degrading populations as a whole. A guild-specific PCR assay would allow for the detection of most resin acid degraders, but the ditA1 assay was too specific for this purpose.

The PCR detection limit

The PCR detection limit depends on the combined efficiencies of cell lysis, DNA recovery, and PCR amplification. The PCR assay developed in this study is very sensitive. When BKME-9 cells were mixed with sludge samples containing no PCR-detectable BKME-9, and the total genomic DNA was subsequently extracted, the detection limit of the PCR assay was approximately one ditA1 copy per PCR reaction. This detection limit is lower than that reported by Tsai et al. [40], who could detect four or more E. coli cells per PCR reaction when PCR and hybridization were used together. This detection limit is also lower than those reported for soil and sediment samples [14, 32, 36, 44]. The great sensitivity of this method can be attributed to the thorough cell lysis by mini-bead beating [29] and omission of extensive purification steps, which lead to DNA loss [21, 48]. Apparently, the absence of humic substances, which are abundant in soil and sediment samples, and the high density of biomass in activated sludge samples made both DNA extraction and subsequent PCR amplification more efficient.

The PCR assay described in this study is also very sensitive in terms of ratio of target over non-target sequences. It has been estimated that each E. coli cell on the average, contains $9x10^{-9}$ μg DNA [18]. Assuming that this holds true for average bacteria, the 53.7 μg of total DNA recovered from one ml of sludge of laboratory bioreactor C would represent $6x10^9$ bacteria, which is a typical bacterial density for such a bioreactor. Our PCR amplification method detected one copy of ditA1 (one BKME-9 cell) out of approximately $6x10^7$ non-target bacteria in the sludge per PCR reaction. This sensitivity is two orders of magnitude greater than that reported by Andersen and Omiecinski [4]. These results indicate that the DNA extraction and PCR assay developed in this study are well suited for the investigation of microorganisms and genes found in low abundance in complex microbial communities such as PPME biotreatment systems.

Distribution of BKME-9, DhA-35, and the ditA1 gene

Pseudomonas abietaniphila BKME-9 and the ditA1 gene are widespread, but not ubiquitous, in PPME biotreatment systems in British Columbia. BKME-9 and ditA1 were each detected in nine of 17 full-scale biotreatment systems (Table 2). These results suggest that BKME-9 may contribute to resin acid removal in those biotreatment systems. BKME-9 was originally isolated from the biotreatment system of Weyerhaeuser Canada in 1994 [7], and interestingly, BKME-9 and ditA1 were relatively abundant in that biotreatment system, when assayed in this study. Thus, BKME-9 appears to be a stable member of the microbial community in that biotreatment system. Further purification of the template DNA with Chroma Spin Columns (Clonetech Laboratories, Inc., CA) prior to PCR amplification did not cause previously negative samples to yield the PCR products. This suggests that the negative results were due to below detectable levels of ditA1 and BKME-9, rather than inhibition of PCR amplification.

Pseudomonas abietaniphila BKME-9 and ditA1 were detected in a laboratory-scale activated sludge system treating BKME at 35°C, while only ditA1 was detected in a parallel laboratory-scale bioreactor treating BKME at 45°C (Table 2). This is consistent with the observation that BKME-9 does not grow above 37°C in pure culture (unpublished data). This suggests the presence of a gene homologous to ditA1 in an organism that grows at 45°C. Neither BKME-9 nor ditA1 was detected in a third bioreactor operated at 55°C or in the two municipal biotreatment systems. Surprisingly, the municipal sludge samples did contain resin acids, including DhA, which was confirmed by GC-mass spectroscopy. Leachate from trees and timber are possible sources of these resin acids.

In a few PPME biotreatment systems, the 16S rDNA of BKME-9 or ditA1 was detected in the absence of the other gene (Table 2). However, in most samples in which only the 16S rDNA of BKME-9 or the ditA1 was found, the detection was close to the lower detection limit of the assay. Near the lower detection limit, experimental variability may be one reason for these results. Further, if more than one copy of the 16S rRNA gene is present on the BKME-9 chromosome and if ditA1 is present as a single copy [preliminary studies suggested that ditA1 is a single-copy gene likely present on the BKME-9 chromosome or a mega plasmid (data not shown)], only the former may have been detectable where the BKME-9 population was small. On the other hand, where only ditA1 was detected, it seems likely that species other than Pseudomonas abietaniphila exist, having genes homologous to ditA1, which were detected by the PCR assay. Using degenerate primers and touch-down PCR, we identified six putative ditA1 homologues from other resin acid degraders (see below).

Strain DhA-35 was not distributed as widely as strain BKME-9, as DhA-35 was detected in only three of the 17 biotreatment systems (Table 2). The two municipal sludge samples were also shown to have barely detectable DhA-35. It is possible that resin acids present in the sewage select resin acid degrading populations. It is not known what are the main parameters affecting the distributions of resin acid degraders. However, levels of resin acids, as growth substrates, should be an important factor affecting the population sizes and dynamics of resin acid-degrading bacteria in PPME biotreatment systems.

Restriction analysis of the PCR products

Restriction analysis with AccI and AlwNI of the PCR products amplified with the ditA1 primers from all but two positive sludge samples produced the expected band patterns (data not shown). In addition to the expected restriction fragments, the PCR products from the Howe Sound Pulp and Crestbrook Pulp and Paper Industries samples yielded small amounts of two additional restriction fragments with AccI and partially digested PCR products with AlwNI. These results indicate the presence of ditA1 in all positive samples and the presence of possible ditA1 homologues in two of the samples. We suspect that there are ditA1-homologous genes present in a group of species, which may share the ability to degrade DhA in those PPME biotreatment systems. This hypothesis is supported by our finding ditA1 homologues from other resin acid-degrading bacteria using a less stringent PCR assay (see below).

Construction of CIS and evaluation of amplification efficiency of ditA1 and CIS

The CIS was produced by the looped template method (Fig. 1A). The PCR products from ditA1 and its CIS could be separated by electrophoresis (Fig. 1B and 2A). The looped template method used in this study to produce the CIS is simpler and more cost-efficient than Porcher's method which needs four PCR primers and two rounds of PCR amplification [33] and the looped-oligo method which cannot generate a separable CIS for long amplicons [35].

To quantify a DNA target by competitive PCR, the competitor and the target have to be amplified at the same efficiency. To test if ditA1 and its CIS are amplified similarly, a calibration curve was generated by amplifying a range of CIS copies in the presence of a constant ditA1 copy number. We determined that ditA1 and its CIS were amplified at the same efficiency (Fig. 2). This is consistent with other reports in which similar approaches were used [11, 17, 21, 22]. To ensure that ditA1 and its CIS are also amplified at the same efficiency in the presence of non-target DNA and potential impurities that can be co-extracted with DNA from sludge samples, 10^4 cells of BKME-9 were added to 1.0 ml of sludge sample from laboratory bioreactor C and quantified using the quantitative PCR assay. The added BKME-9 cells were detected at 10^4 cells/ml level, indicating that ditA1 and its CIS were amplified without detectable bias in our PCR assays. This demonstrates that the primers, CIS, and PCR conditions used in this study are suitable for quantitative PCR assays of specific resin acid-degrading bacteria in sludge samples.

The abundance of ditA1 gene

In most samples assayed, the abundance of ditA1 and BKME-9 were very low and near the detection limit (Table 2). In the samples where ditA1 was relatively abundant, it was quantified (Fig. 3 and Table 3). The R^2 values of the plots for other assays ranged from 0.9861 to 0.9986. It was found that the laboratory bioreactor A had the highest copy number of ditA1, and the two full-scale PPME biotreatment systems of Crestbrook Forest Industries and Weyerhaeuser Canada had lower copy numbers of ditA1 (Table 3). Considering that DhA only accounts for a very small portion of the BOD in the PPMEs and that most resin acid degraders appear to have a narrow substrate range [26, 45, 46], DhA-degrading populations are probably small fractions of the microbial communities in PPME biotreatment systems, but these populations are critical for effective PPME treatment.

The concentration of resin acids in the influents to the PPME biotreatment systems varies dramatically, from undetectable levels to 1,000 mg/L [24], and DhA is only one of several resin acids. If one BKME-9 cell degrades 217 fg of DhA per hour (this rate was estimated from the data reported by Bicho [8]), and the BKME-9 abundance is 10^2 to 10^3 cells/ml, the DhA degradation capacity would be 22-220 ng h^{-1} $liter^{-1}$ of mixed liquor. If the DhA concentrations in PPME biotreatment systems are in the range of 1.0 to 10 μM (our analysis of two untreated PPME samples determined DhA concentrations of 1.8 and 4.2 μM), and the DhA is completely degraded, the BKME-9 population we detected could only degrade a small portion (0.1% to 1%) of the DhA in PPME biotreatment systems. However, we do not know the loading and removal rates of DhA in those PPME biotreatment systems, so the above is only a rough estimate of the relative importance of strain BKME-9 to the overall DhA removal in those systems. These results suggest that although organisms with ditA1 are present and contribute to the biodegradation of resin acids in some PPME biotreatment systems, microorganisms containing ditA1 detected in this study are quantitatively minor members of the resin acid-degrading populations in those PPME biotreatment systems. Therefore, a guild-specific PCR assay that can detect all or most resin acid-degrading populations is desirable.

Putative Homologues of ditA1

With the use of degenerate primers and touchdown PCR, we amplified and sequenced six putative ditA1 homologues from a total of 15 other resin acid-degrading bacteria. Cluster analysis of the partial translated protein sequences (310 aa) from these ditA1 homologues and the α subunit of some other aromatic ring-hydroxylating dioxygenases suggests that DitA1 and its homologues form a distinct group of dioxygenases (Fig. 4A). There appear to be three groups of ditA1 homologues based on the translated protein sequences. At the ditA1-719f annealing site, there are few matches between ditA1-719f primer and these ditA1 homologues (Fig. 4B). This further shows the high specificity of the ditA1-719f/ditA1-1212r primers for BKME-9. These results supports the hypothesis that there are more ditA1 homologues in biotreatment systems that can not be detected with the ditA1-719f/ditA1-1212r primers.

Resin acids have existed on the Earth as long as have trees. Even if homologues of the ditA1 gene are from a common ancestor, they had much time to diverge. However, because they use the same substrate, there should be some conservation among ditA1 homologues. A guild-specific PCR assay would be a more powerful tool for ecological studies, allowing one to investigate a functional group of microorganisms (e.g., resin acid degraders), rather than individual species. However, further investigation of the distribution and conservation of DhA degradation genes is required to design such guild-specific PCR primers and to interpret results from their use. We are currently isolating and sequencing more ditA1 gene homologues from other resin acid-degrading isolates in order to find conserved regions from which to design guild-specific primers for further ecological studies of resin acid-degrading bacteria. Information from such guild-specific PCR assays of PPME biotreatment systems will provide us more useful information on resin acid-degrading populations.

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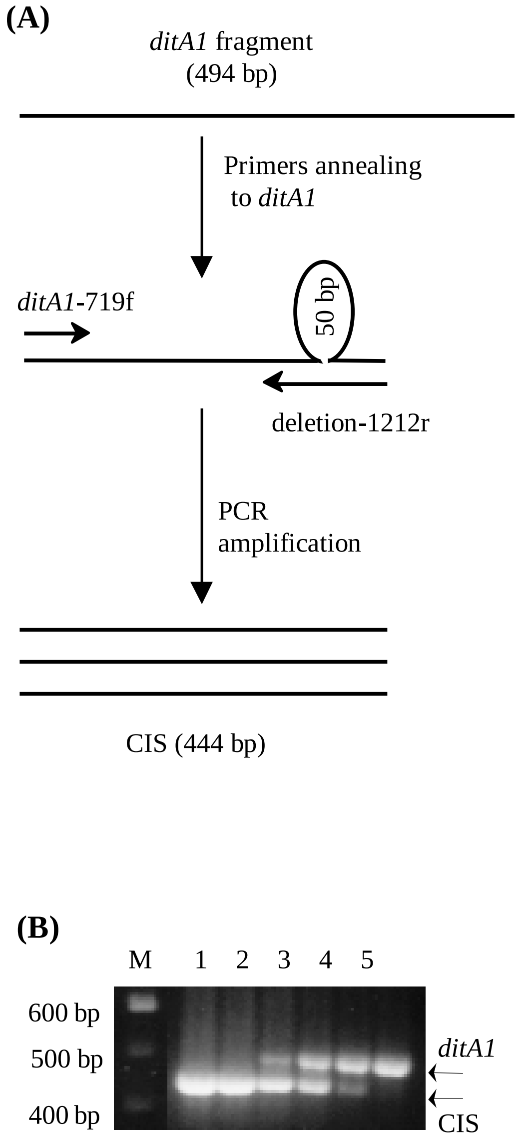


FIG. 1. Schematic representation of the construction of the competitive internal standard (CIS) for ditA1 (A) and separation of the PCR products from this CIS and ditA1 after co-amplification (B). PCR amplification of the ditA1 fragment with primers ditA1-719f and deletion-1212r resulted in a DNA fragment, which is identical to the ditA1 template except a 50-bp deletion. This deletion enables the separation of the CIS (444 bp) and the target (494 bp) sequences. Lane M: molecular weight marker; Lane 1 to 6, decreasing number of CIS with same amount of ditA1.

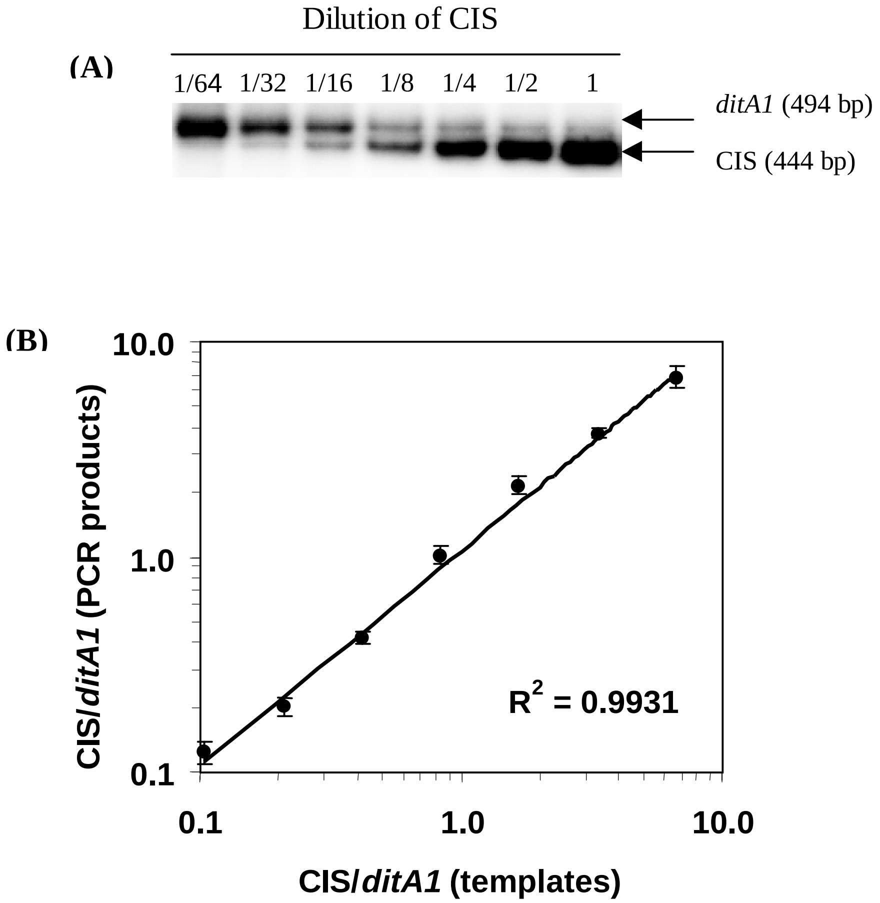


FIG.2. Validation of the quantitative PCR assays. (A) A range of CIS copies (2 x10^4 x dilution factor) were co-amplified with a constant number (1.5 x 10^3) of ditA1 using the conditions described in Quantitative PCR. The PhosphorImager image is shown. Calculation of ditA1 and CIS signals was performed in each lane. (B) Calibration curve of competitive PCR of ditA1 and CIS. The ratios of CIS-to-ditA1 signal intensities were plotted against the ratios of CIS-to-ditA1 template input. Error bars indicate standard deviation (n=2).

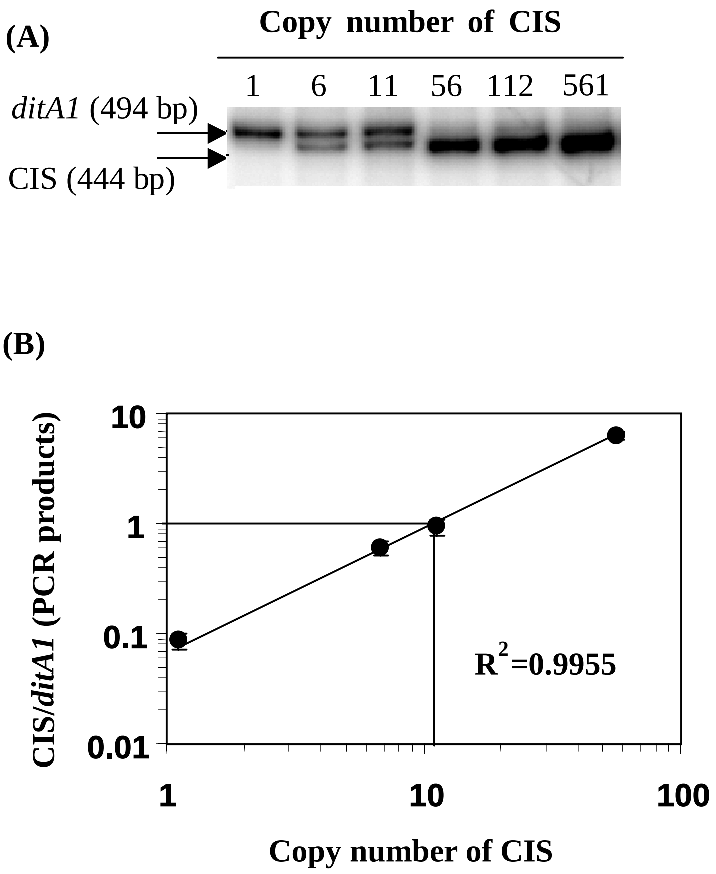
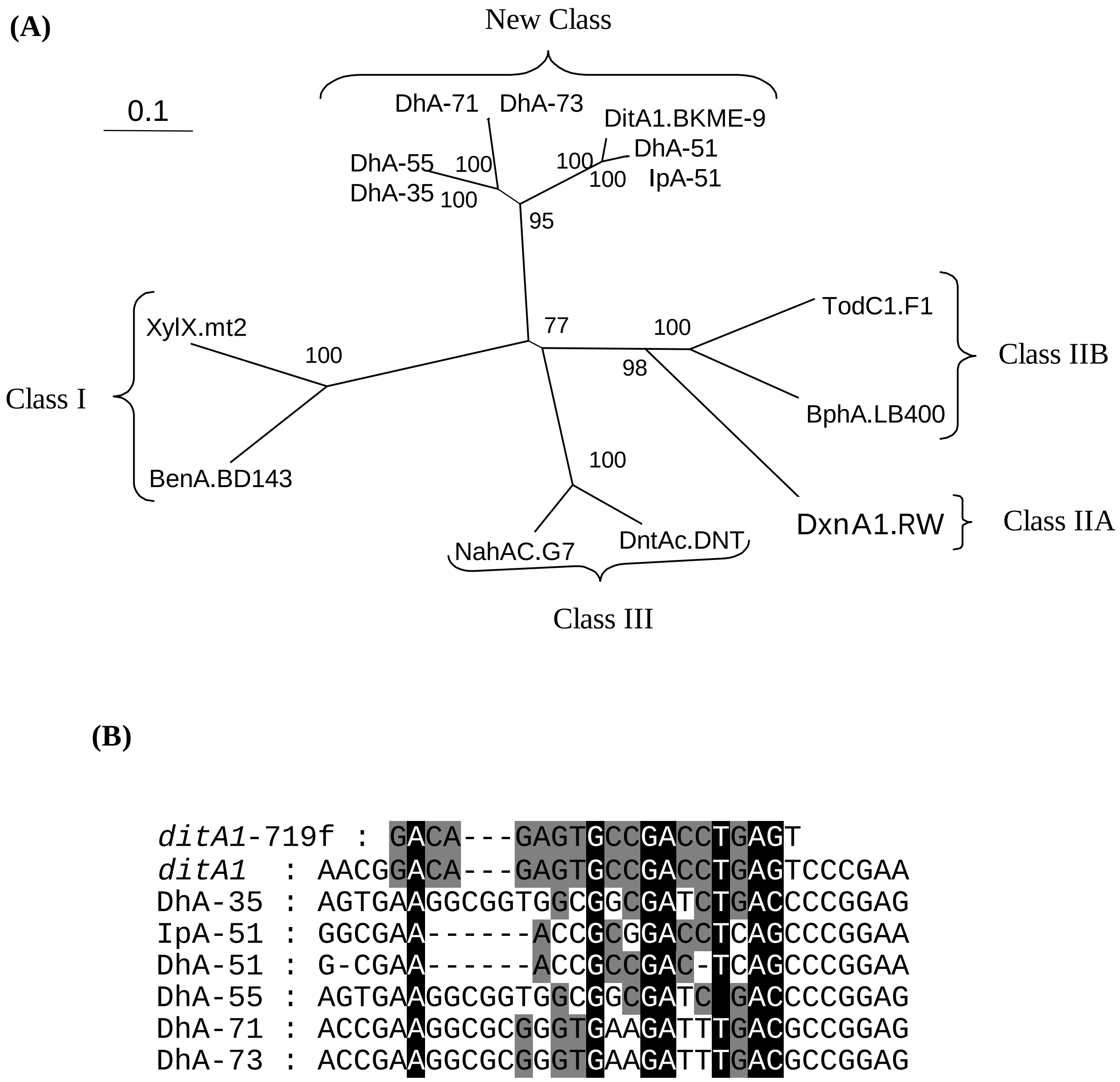


FIG. 3. Quantitation of the ditA1 gene in the bioreactor A. A pre-determined amount of the DNA was co-amplified in competitive PCR reactions with increasing number of added competitive internal standard (CIS) of ditA1. After separation by electrophoresis and analysis by phosphorImager (A), the CIS and the target product bands were quantified and plotted (B). When the ratio of PCR products from CIS and ditA1 equals to one, the CIS copies are equal to the ditA1 copies in the sample. The copy number of ditA1 in the sample was calculated according to equation [1]. Error bars indicate standard deviation (n=2).



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ditA1-719f : GACA---GAGTGCCGACCTGAGT
ditA1      : AACGGACA---GAGTGCCGACCTGAGTCCCGAA
DhA-35 : AGTGAAGGCGGTGGCGGCGATCTGACCCCGGAG
IpA-51 : GGCGAA------ACCGCGGACCTCAGCCCGGAA
DhA-51 : G-CGAA------ACCGCCGAC-TCAGCCCGGAA
DhA-55 : AGTGAAGGCGGTGGCGGCGATC GACCCCGGAG
DhA-71 : ACCGAAGGCGCGGGTGAAGATTTGACGCCGGAG
DhA-73 : ACCGAAGGCGCGGGTGAAGATTTGACGCCGGAG
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Fig. 4. Phylogenetic tree (unrooted) of ditA1 and its homologues (A) and alignment of the corresponding sequences at the ditA1-519f binding site (B). The sequences of ditA1 homologues (310 aa, corresponding to aa 88 to 398) and sequences of the α subunit of other dioxygenases were aligned using ClustalX. Evolutionary distances were calculated according to the model of Jukes and Cantor as implemented in TreeCon. A neighbor joining phylogenetic tree was inferred by using TreeCon. Confidence levels were determined by bootstrap analysis. The scale bar represents 0.1 estimated change per nucleotide. Numbers on branches represent percent confidence of 100 replicate analyses. The putative ditA1 homologues were named after the representative resin acid-degrading bacterial strains. The sequence abbreviation, enzyme substrate, species and GenBank references are as follows (accession number in parentheses): BenA.BD143 = benzoate,

Acinetobactor calcoaceticus BD143 (M76990); XylX.mt2 = toluate, Pseudomonas putida mt2 (M64747); NahAC.G7 = naphthalene, Pseudomonas putida G7 (M83949); DntAc.DNT = 2,4-dinitrotoluene, Burkholderia sp. DNT (U62430); DxnA1.RW1 = dioxin, Sphingomonas sp. RW1 (AJ223219/223220); BphA1.LB400 = biphenyl, Burkholderia cepacia LB400 (M86348); TodC1.F1 = toluene, Pseudomonas putida F1 (J04996); DhA-35 = DhA, Zoogloea resiniphila DhA-35 (to be included); DhA-55 = DhA, Mycobacterium sp. DhA-55 (to be included); DhA-71 = DhA, β Proteobacterium DhA-71 (to be included); DhA-73 = DhA, β Proteobacterium DhA-73 (to be included); DhA-51 = DhA, Pseudomonas vancouverensis DhA-51 (to be included); IpA-51 = IpA, Burkholderia sp. IpA-51 (to be included); DitA1.BKME-9 = DhA, Pseudomonas abietaniphila BKME-9 (to be included).

TABLE 1. PCR primers used in this study.

Primer	Length	Primer sequence	Targets
Pab-613f	20 nt	5’-GCAAGCTAGAGTAGGGCAGA-3’	BKME-9
Pab-832r	20 nt	5’-TTAGCTGCGCCAATAAGAGT-3’	BKME-9
ditA1-719f	20 nt	5’-GACAGAGTGCCGACCTGAGT-3’	ditA1
ditA1-1212r	20 nt	5’-CGGCCAAGTGTCAGAGTCAT-3’	ditA1
ditA1-R263f	16 nt	5’-TYAAYGT**R**TGYCC**R**CA-3’	ditA1
ditA1-Y263f	16 nt	5’-TYAAYGT**Y**TGYCC**Y**CA-3’	ditA1
ditA1-1196r	17 nt	5’-TCRTCYTGYTCNACCAT-3’	ditA1
deletion-1212r	37 nt	5’-CGGCCAAGTGTCAGAGTCAT atcttctcgcgcagctc*-3’	ditA1
Zre-57f	18 nt	5’-AACGGCAGCACGGGCTTC-3’	DhA-35
Zre-423r	20 nt	5’-CCCAGGGTATTAACCCAAGA-3’	DhA-35

* Nucleotides in upper case are the sequence of ditA1-1212r, and nucleotides in lower case indicate the sequence corresponding to a sequence 50-nt downstream from ditA1-1212r.

TABLE 2. The distribution of the ditA1 gene, BKME-9, and DhA-35 determined by a qualitative PCR assay

Source of samples	Pulping process	Biotreatment systems	DhA (µM)	VSS[1] (mg/L)	ditA1 gene	16S rDNA of BKME-9	16S rDNA of DhA-35
Crown Packing Ltd.	P[2]	AS[3]	1.21	4780	(+)	(+)	-
Squamish Pulp	K[4]	AS (UNOX)	0.79	4060	-	-	-
Eurocan Pulp & Paper	K, M[5]	AL[6]	8.96	110	(+)	-	-
Howe Sound Pulp & Paper	K, M	AS (UNOX)	2.66	2620	(+)	(+)	-
Western Pulp at Port Alice	K	AS	0.63	9800	(+)	-	-
Harmac Pacific Inc.	K	AS	0.00	2650	-	-	-
Powell River Div.	K, M	O_2 AS	1.02	1723	(+)	(+)	-
Alberni Specialties Div.	K, M	AB[7]	0.96	4520	-	-	(+)
Crestbrook Forest Industries	K	AB	2.54	1495	+	+	-
Quesnel River Pulp Co.	K	AB	1.73	1075	-	-	+
Celgar Pulp Co.	K	AL	0.00	1620	(+)	(+)	-
Prince George Pulp & Paper	K	AL	0.00	720	-	-	-
Avenor Inc.	K	AS	1.47	1416	(+)	(+)	-
Fibreco Pulp Co.	M	AS	7.72	2350	-	-	+
Northwood Pulp & Timber Ltd.	K	AL	1.38	44	-	(+)	-
Cariboo Pulp & Paper Co.	K	AL	0.00	73	-	(+)	-
Weyerhaeuser Canada	K	AL	0.00	86	+	+	-
Laboratory bioreactor A (35°C)	K	AS	0.96	4000	+	+	-
Laboratory bioreactor B (45°C)	K	AS	0.74	2766	+	-	-
Laboratory bioreactor C (55°C)	K	AS + UF[8]	283.50	24230	-	-	-
UBC pilot plant (municipal)		AS	2.08	2924	-	-	(+)
James Plant (municipal)		AS	6.49	2875	-	-	(+)

[1]Volatile suspended solids (dry biomass); [2]paper mill; [3]activated sludge; [4]kraft pulping; [5]mechanical pulping; [6]aeration lagoon; [7]aeration basin; [8]ultrafiltration. Symbols: -, not detectable; +, detectable; (+), detectable but near detection limit.

TABLE 3. The abundance of the ditA1 gene determined by quantitative PCR.

Source of sample	Copies of ditA1/mg VSS[a]	Copies of ditA1/ml	Relative abundance[b] of ditA1
Crestbrook Forest Industries	94	1.4×10^2	$1/1.6 \times 10^7$
Weyerhaeuser Canada	198	1.7×10^1	$1/3.5 \times 10^6$
Laboratory bioreactor A (35°C)	250	1.0×10^3	$1/6.5 \times 10^6$
Laboratory bioreactor B (45°C)	51	1.4×10^2	$1/4.5 \times 10^7$

[a] Volatile suspended solids.

[b] Estimated from DNA yield of each sample and assuming the average DNA content of 9×10^{-9} µg/cell.