WORKING PAPER
2000-4

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Bioaugmentation with resin acid-degrading bacteria enhances resin acid removal in sequencing batch reactors treating pulp mill effluents

RUNNING TITLE: BIOAUGMENTATION WITH RESIN ACID DEGRADERS

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DATE: JANUARY 26, 2000.

ABSTRACT

Resin acids are the major toxicants in pulp and paper mill effluents (PPMEs), and they form pitch interfering with papermaking. Efficient and reliable resin acid removal is critically important to prevent toxicity discharge and ensure runability of paper machines. Two resin acid-degrading bacteria, *Pseudomonas abietaniphila* BKME-9 and *Zoogloea resiniphila* DhA-35, were tested in laboratory sequencing batch reactors (SBRs) for their ability to enhance resin acid removal by biomass from a full-scale biotreatment system treating PPMEs. Both bacteria enhanced resin acid removal but not removal of total organic carbon (TOC) by either pH-shocked or starved activated sludge. These two bacteria also increased resin acid removal when the sludge was given high concentration (200 μM) of resin acid. A most-probable-number polymerase chain reaction (MPN-PCR) assay showed that these two bacteria were initially not detectable (detection limit: 10^2 bacterial cells/ml) in the sludge community and were persistent after inoculation. Both bacteria did not substantially change the indigenous microbial community composition, as assayed by ribosomal intergenic spacer analysis (RISA). Our results suggest that it is feasible and potentially useful to enhance resin acid removal by bioaugmentation using special resin acid-degrading bacteria such as BKME-9 and DhA-35.

Key words -- resin acids, dehydroabietic acid, activated sludge, bioaugmentation, ribosomal intergenic spacer, PCR, RISA, pulp and paper effluents.

INTRODUCTION

Resin acids are toxic, tricyclic, diterpenoid compounds naturally occurring in trees, especially in softwood trees in which resin acids can account for a few percent of the tree biomass. Resin acids are biodegradable, and a number of resin acid-degrading bacteria have been isolated (reviewed by Martin *et al*., 1999). Resin acids are released into pulp and paper mill effluents (PPMEs) during pulping processes. Although resin acids account for only a small portion of the total organic matter in PPMEs, they are the major cause of the toxicity of PPMEs to aquatic life (Environmental Protection Services Canada, 1987; Leach and Thakore, 1973; Priha and Talka, 1986; Walden and Howard, 1981). Thus, there is great concern over discharges of PPMEs containing resin acids. Efficient removal of resin acids from PPMEs is required during biotreatment of PPMEs. However, biotreatment systems occasionally fail to remove resin acids when upsets occur, such as low temperature, high pH, or high resin acid concentrations, resulting in toxicity breakthroughs (Liss and Allen, 1992; Richardson and Bloom, 1982; Taylor *et al*., 1988).

The above-mentioned system failures apparently stem from inhibition of the resin acid-degrading populations in the biotreatment systems. Resin acid-degrading populations in biotreatment systems are likely small because resin acids only account for a small fraction of the total organic loading of those systems. Such small bacterial populations are generally less stable than larger bacterial populations in biotreatment systems. This may explain why some biotreatment systems had resin acid breakthroughs, even when their overall BOD removal was not significantly affected (Liss and Allen, 1992).

The most straightforward strategy to remediate such system failures is bioaugmentation using resin acid-degrading bacteria. Bioaugmentation has been reported to enhance removal of 3-chlorobenzoate, 4-methyl benzoate, toluene, phenol, and chlorinated solvents (Ahring *et al*., 1992; McClure *et al*., 1991; Nüßlein *et al*., 1992; Selvaratnam *et al*., 1997), but it has not been reported to enhance resin acid removal from PPMEs. The bacteria suitable for bioaugmentation have to meet at least three criteria. First, they must be catabolically active to degrade resin acids in the complex microbial communities of biotreatment systems in the presence of other potentially inhibitory organic and inorganic pollutants commonly found in PPMEs. Second, they must be competitive, and hence persistent, after being introduced into biotreatment systems. Third, they should be compatible with indigenous microbial communities so that they will not

adversely affect the indigenous microbial communities. Therefore, candidate bacteria for bioaugmentation should be carefully selected among resin acid degraders and tested.

Numerous resin acid-degrading bacteria from a variety of sources including forest soil, Arctic soil, PPME treatment systems, laboratory bioreactors, and compost have been isolated and characterized in our laboratory (Mohn, 1995; Mohn *et al*., 1999; Wilson *et al*., 1996; Yu and Mohn, 1999a; Yu *et al*., 2000). Although these bacterial isolates provided us a rich source for selecting candidates for bioaugmentation, we have little information about their ability to degrade resin acids in biotreatment systems, their competitiveness (persistence), or their effects on the indigenous communities, which are far more complex than pure cultures. *Pseudomonas abietaniphila* BKME-9 and *Zoogloea resiniphila* DhA-35 are two bacteria isolated from PPME biotreatment systems (Bicho *et al*., 1994; Mohn, 1995). These two bacteria degrade abietane resin acids such as dehydroabietic acid (DhA) and abietic acid (AbA) in pure culture in mineral medium. The 16S rRNA gene sequences of DhA-35 (GenBank accession number: AJ011506) and BKME-9 (GenBank accession number: AJ011504) (Mohn, *et al*., 1999) enabled us to design specific polymerase chain reaction (PCR) assays, which allow monitoring the populations of BKME-9 and DhA-35 in complex microbial communities.

Biotreatment systems host complex microbial communities. Such complex communities cannot be comprehensively characterized with available methods. Bacterial ribosomes are the machinery synthesizing proteins in bacterial cells and contain three species of ribosomal RNAs: 5S, 16S, and 23S rRNAs. The 16S and the 23S rRNA genes (rDNAs) are in one transcriptional unit, but are separated by a ribosomal intergenic spacer (RIS), which varies in length and DNA sequence among different bacterial species, and even among different strains of a species (reviewed by Gürtler and Stanisich, 1996). The 16S and the 23S rDNAs flanking the RIS allow the design of universal primers for PCR, which can exponentially amplify DNA fragments containing the RIS from all or most of the bacteria in microbial communities. Ribosomal intergenic spacer analysis (RISA) of a community generates a fingerprint that indicates the structure of that community. Changes in microbial community structures can be detected by RISA (Yu and Mohn, 2000).

The objectives of this study were (i) to test the effects of bioaugmentation of stressed activated sludge samples with *Pseudomonas abietaniphila* BKME-9 and *Zoogloea resiniphila* DhA-35 on DhA removal, (ii) to investigate the competitiveness and persistence of these two

bacteria in complex microbial communities common to PPME biotreatment systems, and (iii) to assess the effects of bioaugmentation on indigenous microbial populations using RISA. Our results showed that these two bacteria meet the three criteria of successful bioaugmentation and that it may be feasible and potentially useful to enhance resin acid removal by bioaugmentation using resin acid-degrading bacteria. This is the first study reporting bioaugmentation of PPME biotreatment using resin acid-degrading bacteria. This is also the first time to use RISA to assess the effects of inocula on indigenous populations in sludge microbial communities.

MATERIALS AND METHODS

Medium, pure cultures, and activated sludge samples

The medium used in this study was untreated bleached kraft mill effluent (BKME) supplemented with 0.02% NH_4NO_3. *Pseudomonas abietaniphila* BKME-9 and *Zoogloea resiniphila* DhA-35 were from our own stocks. They were grown on 200 μM DhA in the above BKME medium for two days to prepare the inocula. The cell densities of these two cultures were estimated microscopically. Samples of fresh activated sludge (mixed liquor) were taken from the UNOX system of Western Pulp at Squamish, British Columbia, Canada. This pulp mill also provided the untreated BKME.

Sequencing batch reactors

The sequencing batch reactors (SBRs) were 500-ml flasks containing 200 ml of BKME medium with different inocula (see below). DhA was added to a final concentration of 20 or 200 μM. The SBRs were incubated at 30^{o}C on a shaker. Each cycle was 48 hours. At the end of each cycle, samples were taken while mixing with a stir bar. The biomass was then retained by settling for 30 min, and the supernatant (180 ml) was removed by aspiration and replaced with fresh BKME medium containing the same amount of DhA.

pH shock

The pH of a fresh sludge sample was adjusted to 9.5 using 10 N NaOH. This pH-shocked sludge was incubated for 30 min at 30^{o}C before its pH was adjusted to 7 using 10 N HCl. Then, 20 ml aliquots of this sludge sample were placed in each of three SBRs containing 180 ml of BKME medium: one containing about 10^6 cells/ml of BKME-9, one containing about 10^6

cells/ml of DhA-35, and one containing neither bacterium. DhA was added to a final concentration of 20 μM. The SBRs were incubated as described above, and samples for analysis were taken during the incubation.

Starvation

To test how bioaugmentation helps DhA removal by sludge biomass starved at low temperature, fresh mixed liquor was incubated at 7^{o}C for three weeks. We chose 7^{o}C in this study because pulp mill shutdowns likely occur in winter, and during such shutdowns the temperature of the biotreatment system drops to 7^{o}C or lower, depending on the location of the pulp mills and the duration of shutdowns (Hirvi, 1999). Twenty ml of the starved sludge biomass was inoculated into each of three SBRs containing 180 ml of BKME and 20 mM DhA: one containing about 10^6 cells/ml of BKME-9, one containing about 10^6 cells/ml of DhA-35, and one containing neither bacterium. These SBRs were incubated and sampled as described above.

DhA overloading

Twenty ml aliquots of a fresh sludge sample were placed in each of three SBRs containing 180 ml of BKME medium: one containing about 10^6 cells/ml of BKME-9, one containing about 10^6 cells/ml of DhA-35, and one containing neither bacterium. DhA was added to a final concentration of 200 μM. The incubation and sampling were the same as described in the pH shock experiment.

DNA extraction, PCR, and electrophoresis

The DNA was extracted using the method of Yu and Mohn (1999b). For the analysis of most-probable-number PCR (MPN-PCR), serial 10-fold dilutions of the DNA were made. The species-specific primers for BKME-9 (*Pab*-613f/*Pab*-832r) and DhA-35 (*Zre*-57f/*Zre*-423r) were described previously (Yu *et al*., 1999a). For the MPN-PCR assays, the above DNA dilutions were used as the DNA templates and amplified for 35 cycles according to the conditions reported previously (Yu *et al*., 1999a). The MPN-PCR products were resolved on 1.0% agarose gels, which were stained with ethidium bromide and photographed. The population sizes of BKME-9 and DhA-35 were calculated from the MPN-PCR results using the computer program Quality (http://ubik.microbiol.washington.edu/cbu/quality/jquality.html). The detection limit of the PCR

assay is one cell per PCR reaction, equivalent to 10^2 bacterial cells/ml of mixed liquor, or one targeted bacterial cell out of 10^6 total bacterial cells of sludge community (Yu, *et al*. 1999a).

For the RISA, 10-fold diluted DNA extracts were used as the templates. The universal primers used in the RISA were S926f (5'-CTYAAAKGAATTGACGG-3') and L189r (5'-TACTGAGATGYTTMARTTC-3'). The PCR amplification of RIS was conducted in a total volume of 50 μL containing 0.5 pmol of each primer per μL, 200 μM (each) dNTP, 1.5 mM $MgCl_2$, 1x PCR 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^{o}C with a simplified hot start. The subsequent cycles consisted of a 0.5-min denaturation step at 94^{o}C, a 0.5-min annealing step at 47^{o}C, and a 2-min extension step at 72^{o}C. A final 5-min extension at 72^{o}C was included after 35 cycles of PCR amplification. The resultant PCR products of RIS were resolved on native polyacrylamide (3.5%; 38:1) gels, which were stained with GelStar according to the manufacturer's recommendation (MFC BioProducts, Rockland, ME).

Determination of resin acid concentrations and total organic carbon

The concentrations of resin acids were determined using gas chromatography as reported previously (Mohn, 1995). The total organic carbon (TOC) contents were determined according to standard procedures (AWAH, 1995).

RESULTS AND DISCUSSION

pH shock

The DhA removal by the pH-shocked activated sludge in the laboratory SBRs is shown in Fig. 1. Un-shocked sludge removed DhA faster than the pH-shocked sludge, suggesting that exposure to pH 9.5, a pH value encountered in cases such as black liquor spills in pulp mills, did adversely affect the indigenous DhA-degrading populations. However, the reduction in DhA removal was not dramatic. This may be explained by the fact that a large portion of the biomass in activated sludge exists as granules, which provide buffering capacity and protect the cells inside the granules.

Bioaugmentation with either BKME-9 or DhA-35 greatly enhanced the DhA removal by the pH-shocked sludge sample (Fig. 1). Thus, both BKME-9 and DhA-35 were catabolically

active in the PPME environment. The bioaugmented samples also removed DhA faster than the un-shocked sample. After a longer incubation, the pH-shocked sludge also removed all the DhA, indicating the presence and survival of the indigenous DhA-degrading populations. The absence of detectable BKME-9 and DhA-35 in the non-bioaugmented sludge (see Fig. 5) indicates that the indigenous DhA-degrading populations did not include BKME-9 or DhA-35. This is consistent with the fact that we did not detect these strains in a sample of sludge taken from the same biotreatment system in a previous study (Yu *et al*., 1999a). Given the limited hydraulic retention time in any biotreatment system, only efficient removal of resin acids within the hydraulic retention time can prevent resin acid toxicity breakthroughs. These results indicate that bioaugmentation using either BKME-9 or DhA-35 can help pH-shocked biotreatment system recover and minimize resin acid breakthroughs. These two bacteria were also shown to meet the first criterion for successful bioaugmentation: active in degrading resin acids in sludge microbial communities.

Starvation

Due to various reasons, pulp and paper mills shut down from time to time. During mill shutdowns, the biotreatment systems receive no influent so the biomass starves. Activated sludge samples starved for three weeks at 7^{o}C showed significantly lower DhA removal compared to the fresh sludge sample (Fig. 2). Comparing the DhA removal by the pH-shocked sludge and the starved sludge (Fig. 1 and 2), starvation had a more severe effect on DhA-degrading populations than pH shock. As reported previously (Martin *et al*. 1999; Mohn *et al*., 1999), resin acid degraders usually can use only a limited number of substrates. Therefore, during the starvation, the resin acid-degrading populations would likely become much smaller after resin acids are depleted.

The bioaugmentation with BKME-9 or DhA-35 increased the rate of DhA removal by the starved sludge to a rate slightly greater than that of the fresh sludge (Fig. 2). This experiment demonstrated that bioaugmentation with resin acid-degrading bacteria is a potentially useful strategy to enhance resin acid removal by starved sludge biomass. Some mills use methanol to feed the biomass in their biotreatment systems during mill shutdowns. Because most resin acid-degrading bacteria can not use methanol, this methanol-feeding strategy does not likely maintain the resin acid-degrading populations in the biotreatment systems. The expense of purchasing

resin acids make it impractical to include resin acids as a co-substrate for the starved sludge biomass. Therefore, bioaugmentation with resin acid-degrading bacteria like BKME-9 and DhA-35 may be the most useful and practical strategy to prevent resin acid toxicity breakthroughs during the re-startup of mill shutdowns. Again, BKME-9 and DhA-35 were proved to meet the first criterion for successful bioaugmentation.

DhA overloading

When the sludge was challenged with 200 μM (60 ppm) of DhA, bioaugmentation with BKME-9 or DhA-35 enhanced DhA removal (Fig. 3). This is consistent with the results of the pH-shock (Fig. 1) and the starvation experiments (Fig. 2). However, in this experiment, DhA-35 showed less enhancement than BKME-9. This is not consistent with the pH shock and the starvation experiments. One possible explanation is that the cell density of DhA-35 in the inoculum was overestimated, because *Zoogloea resiniphila* DhA-35 is a flock-forming organism and is difficult to enumerate microscopically. The sludge sample without bioaugmentation removed all the DhA after 30 hours, which is several times longer than the hydraulic retention time of typical activated sludge systems. When softwood trees are pulped, higher loading of resin acids is expected in the biotreatment systems. Under such circumstance, the small indigenous resin acid-degrading populations may be overwhelmed, and resin acid breakthroughs may occur. Therefore, bioaugmentation with resin acid degraders such as BKME-9 and DHA-35 may prevent potential resin acid breakthroughs in this situation. Both BKME-9 and DhA-35 meet the first criterion for successful bioaugmentation when resin acid overloading occurs.

TOC removal

Bioaugmentation with either BKME-9 or DhA-35 failed to enhance TOC removal by un-shocked sludge sample (Fig. 4). Consistent with this observation, pure cultures of BKME-9 or DhA-35 did not remove much TOC in PPME (Fig. 4). This is not surprising because, as stated above, resin acids account for only a small portion of the total organic matter in PPMEs entering biotreatment systems, and most resin acid degraders so far characterized appear to be nutritional specialists that can use only a few organic substrates other than resin acids. The majority of the organic substances in PPMEs are methanol, lignin, cellulose, semicellulose, wood sugars, lipids, etc. that BKME-9 and DhA-35 can not degrade. In addition, compared to the microbial

populations in the sludge community, the populations of the inoculated BKME-9 and DhA-35 in the SBRs were very small. It is expected that such small populations may not contribute much to the overall TOC removal.

It would be preferable if the inocula enhance the removal of resin acids as well as the above mentioned substances. White rot fungi such as *Trametes versicolor* were shown to remove a variety of the organic substances present in PPMEs (Cai *et al*., 1998), but not resin acids. In fact, while some fungi hydroxylate resin acids, no fungus has been shown to degrade resin acids (Martin *et al*., 1999). A mixed culture of white rot fungus and resin acid-degrading bacteria might make an inoculum that can remove both resin acids and TOC.

Persistence of Pseudomonas abietaniphila BKME-9 and Zoogloea resiniphila DhA-35

After being introduced, both BKME-9 and DhA-35 were persistent in the sludge microbial community (Fig. 5). Neither strain was detectable in sludge before the inoculation (Fig. 5A). These results indicate that both bacteria were competitive in the sludge microbial community and were retained well during settling of the biomass. During the first two weeks, both the BKME-9 and DhA-35 populations decreased slightly. This decrease may be attributed to their adaptation to the new experimental conditions. In cases where biotreatment systems are stressed, resin acid removal can be restored within two weeks. Even after the two weeks, the populations of BKME-9 and DhA-35 were still more than 10^4 cells/ml, a population relatively large for small amount of resin acids present in most PPMEs.

It is worth pointing out that it is difficult to enumerate a bacterial population in a complex microbial community like activated sludge using conventional methods. In this study, we used species-specific MPN-PCR assays, which are culture-independent, specific, and sensitive (Degrange *et al*. 1998; Leung *et al*. 1997), to monitor the population dynamics of BKME-9 and DhA-35 in bioaugmented SBRs. Because the target is the 16S rDNA, the numbers obtained from the MPN-PCR assays are proportional to the population sizes but are not the actual cell numbers. However, because one bacterium generally has one to several copies of 16S rDNA, our MPN-PCR data should be within one order of magnitude of the actual population sizes.

It is interesting that although BKME-9 is not a flock-forming bacterium, it was well retained by the settling procedure used for the SBRs. In an SBR containing BKME-9 alone, the population of BKME-9 decreased rapidly during the first two cycles, and after that, much fewer

BKME-9 cells remained (data not shown). Possible explanations of this phenomenon include (i) activated sludge harbors numerous flock-forming bacteria (e.g. *Zoogloea* spp.), which can incorporate other non-flocculating bacteria including BKME-9 into sludge flocks, so improving the settleability of BKME-9, or (ii) some members of the sludge community may have provided beneficial effects to BKME-9 so that BKME-9 grew better. These results showed that even non-flocculating bacteria can be used as inocula for bioaugmentation of activated sludge systems.

It is also interesting that neither bacterium was indigenous to the sludge sample used to inoculate the SBRs, but after introduction, both BKME-9 and DhA-35 could compete well with the indigenous bacteria in the sludge community and persist. This indicates that the inoculum for bioaugmentation may not necessarily have to be from the microbial community to be bioaugmented. Therefore, both BKME-9 and DhA-35 meet the second criterion for successful bioaugmentation: competitive and persistent in sludge microbial communities.

Community structure

The bioaugmentation did not greatly affect the RISA banding patterns of the sludge community (Fig. 6), indicating that the microbial community structures were not substantially affected by the bioaugmentation with either BKME-9 or DhA-35. Bacterial RIS differ in length and sequence among different bacterial species, even among different strains of a species. In this study, the numerous PCR-amplified RIS products were resolved only on the basis of size, and as such, the resolution power of this assay is limited. It is certain that any change in RISA banding patterns would indicate a change in microbial community structure, but a change in microbial community structure may not be detectable by such RISA because of the complexity of the microbial community. Such a minor change in community structure could substantially affect resin acid removal capacity but would not likely affect TOC removal.

The RISA is subject to certain bias occurring during DNA extraction and PCR amplification. Thus, each RIS band does not necessarily proportionally represent a population within the community. However, these biases do not invalidate comparison of samples analyzed by an identical method. Relative differences in major populations, spatial or temporal, will be detectable. The great similarity in RISA patterns and in the relative intensity of RIS bands of the samples from this study demonstrates that the variability between samples is low for this assay.

Comparing the samples taken at different time points, it can be seen that all the microbial

communities changed slightly over time. Such change may be attributed to the adaptation of the communities to the SBRs during the three weeks of operation. Nevertheless, as indicated by the RISA, addition of BKME-9 or DhA-35 did not change the presence of the major populations indigenous to the sludge community, nor did the inocula substantially change the relative abundance of these populations. This is consistent with the fact that bioaugmentation did not affect TOC removal (Fig. 4). Both BKME-9 and DhA-35 proved themselves to meet the third criterion for successful bioaugmentation: compatible with microbial communities indigenous to activated sludge.

CONCLUSION

The study showed that both BKME-9 and DhA-35 meet the three criteria for successful bioaugmentation of PPME biotreatment systems to enhance DhA removal. They are catabolically active in pH-shocked activated sludge, starved sludge and sludge overloaded with DhA; they are competitive and persistent in the activated sludge community; and they did not appear to adversely affect the indigenous microbial community. As pulp and paper mills are moving towards recycling their effluents, they require more efficient and reliable removal of resin acids from their process water to avoid accumulation of resin acids, which interfere with paper making. Therefore, bioaugmentation with resin acid-degrading bacteria may be useful to pulp and paper mills in the near future.

ACKNOWLEDGEMENT

We thank Jeanne Taylor at the Western Pulp for providing us the samples of activated sludge and untreated BKME. We also thank Paul Sahota for his technical assistance.

This research was funded by the Sustainable Forest Management Network.

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Figure Legend

Fig. 1. Removal of DhA by pH-shocked (pH 9.5 for 30 min) activated sludge samples bioaugmented with BKME-9 or DhA-35 (10^6 cells/ml). Symbols: closed diamonds, pH-shocked sludge without bioaugmentation; open diamonds, un-shocked sludge without bioaugmentation; closed circles, pH-shocked sludge augmented with DhA-35; closed triangles, pH-shocked sludge augmented with BKME-9.

Fig. 2. Removal of DhA by starved activated sludge (for three weeks at 7^oC) bioaugmented with BKME-9 or DhA-35 (10^6 cells/ml). Symbols: closed diamonds, starved sludge without bioaugmentation; open diamonds, un-starved sludge without augmentation; closed circles, starved sludge augmented with DhA-35; closed triangles, starved sludge augmented with BKME-9.

Fig. 3. Removal of DhA by activated sludge overloaded with DhA (200 μM) and bioaugmented with BKME-9 or DhA-35 (10^6 cells/ml). Symbols: closed diamond, sludge without bioaugmentation; closed circle, sludge augmented with DhA-35; closed triangle, sludge augmented with BKME-9.

Fig. 4. TOC removal by un-shocked sludge and pure cultures of BKME-9 and DhA-35. Symbols: open circles, DhA-35 alone; open triangles, BKME-9 alone; closed diamonds, sludge alone; closed circles, sludge bioaugmented with DhA-35; closed triangles, sludge bioaugmented with BKME-9.

Fig. 5. Population changes of BKME-9 and DhA-35 in bioaugmented SBRs. (A) A gel image showing the specific PCR amplification of the 16S rDNA of DhA-35 and BKME-9. Lane M: 100 bp ladder; lane 1: DNA of sludge alone; lane 2: BKME-9 DNA; lane 3: DNA of sludge six days after bioaugmentation with BKME-9; lane 4: DNA of sludge alone; lane 5: DhA-35 DNA; lane 6: DNA of sludge six days after bioaugmentation with DhA-35; lane 7: negative control without DNA template. (B) Population changes of BKME-9 and DhA-35 in the bioaugmented SBRs. Symbols: closed circles, DhA-35; closed triangles, BKME-9.

Fig. 6. Microbial community structures in SBRs inoculated with fresh activated sludge samples, as revealed by RISA assays. The ribosomal intergenic spacers (RIS) were PCR amplified from the total community DNA, resolved on polyacrylamide gels, and stained with GelStar. Lane M: 100 bp ladder; lane 1, 4, 7, 10: sludge alone; lane 2, 5, 8, 11: sludge bioaugmented with BKME-9; lane 3, 6, 9, 12: sludge bioaugmented with DhA-35.

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