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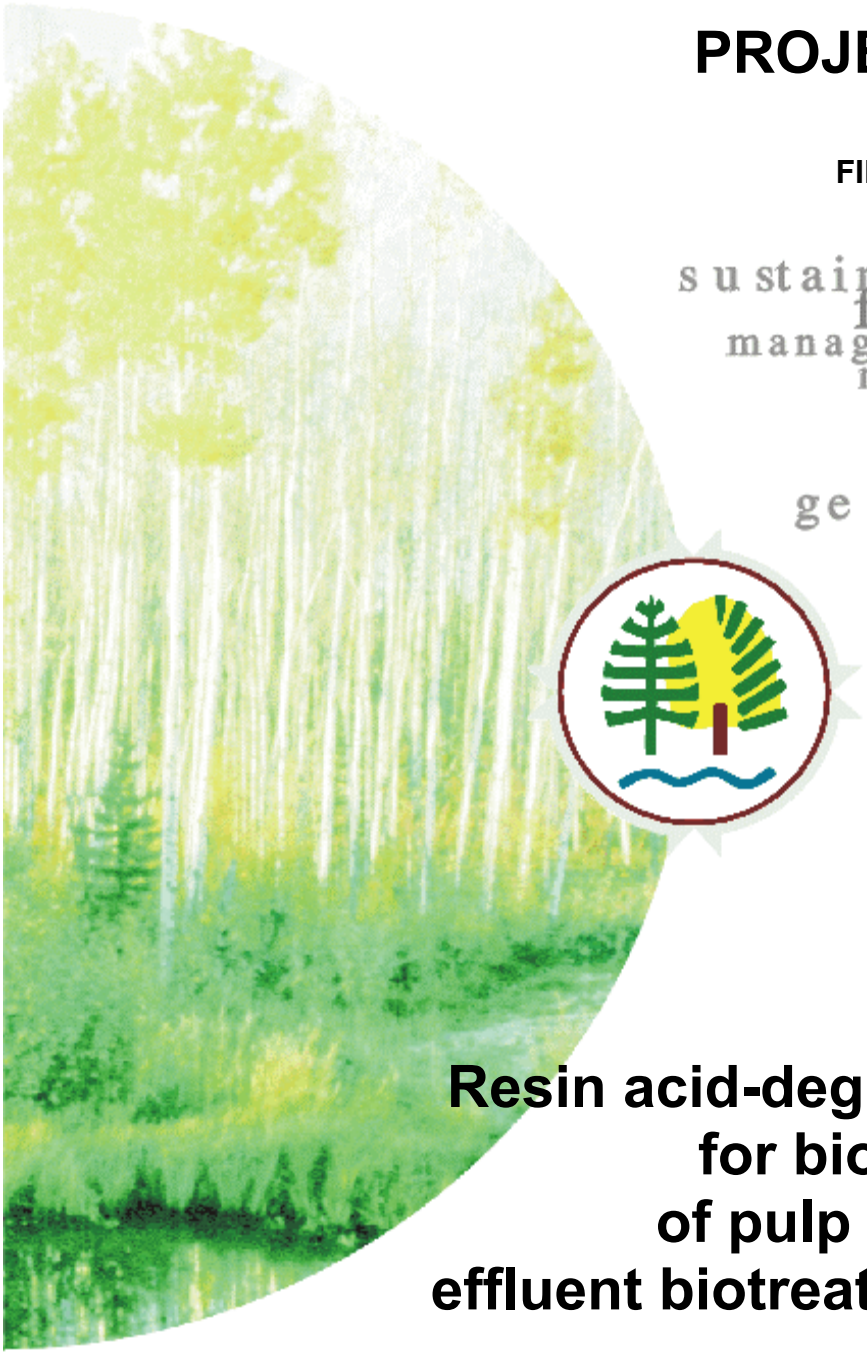

Resin acid-degrading inocula for bioaugmentation of pulp and paper mill effluent biotreatment systems

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Resin acid-degrading inocula for bioaugmentation of pulp and paper mill effluent biotreatment systems

SFM Network Project:
Biotechnology for detoxication of pulp and paper mill effluents

by

William W. Mohn

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May 2001

EXECUTIVE SUMMARY

Removal of resin acids is a critical function of biotreatment systems for pulp and paper mill effluents. Perturbations of these systems, such as black liquor spills in a pulp mill, can result in inhibition of resin acid biodegradation activity and breakthrough of resin acids in system discharges. Bioaugmentation with resin acid-degrading bacteria may be a practical response to inhibited biotreatment systems or means to prevent inhibition.

Resin acid-degrading bacteria were isolated, characterized and tested for their abilities to function as inocula for biotreatment systems. The range of environmental conditions that can be tolerated by these organisms was investigated. Strains were identified that tolerated extreme temperatures. Putatively nitrogen-fixing organisms were also isolated. Isolates were also characterized with respect to their phylogeny and their catabolic capabilities, including the range of resin acids on which they can grow. In all, over 18 unique isolates were characterized in detail.

Various stresses were tested for their effects on microbial communities of activated sludge and aerated lagoon biotreatment systems. Extreme pH had a particularly strong inhibitory effect on resin acid-degrading populations in such systems. In some cases, this effect was shown to be due to killing of resin acid degraders. Selected isolates were used to inoculate microbial communities from biotreatment systems. Three isolates, *Pseudomonas abietaniphila* BKME-9, *Sphingomonas* sp. DhA-33 and *Zoogloea resiniphila* DhA-35, restored resin acid removal activity to inhibited communities. Assays targeting unique DNA sequences of these organisms demonstrated that the organisms grew and persisted in the biotreatment system communities. In some cases, the inocula were shown to cause changes in the other populations in the communities. In most cases, the inocula did not adversely affect general biodegradation activity of the communities, measured as removal of total organic carbon. Bioaugmentation appears to be feasible as a means to restore resin acid removal activity in biotreatment systems inhibited by stresses to the microbial community. Larger scale testing is required to confirm the efficacy of this strategy and to determine its practicality.

ACKNOWEDGEMENTS

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The work described was conducted by Zhongtang Yu, a postdoctoral fellow; Vincent Martin, Annette Muttray and Daryl Smith, graduate students; and Paul Sahota, an undergraduate student.

INTRODUCTION

Resin acids are the major cause of acute toxicity in pulp mill effluents (Environmental Protection Service Canada 1987, Priha and Talka 1986, Walden & Howard 1981). Most resin acids are biodegradable under favorable conditions (Martin et al. 1999), and biological treatment systems are generally effective in removing resin acids from pulp mill effluents. However, experience has shown that treatment systems commonly experience temporary failure to remove resin acids and to meet effluent toxicity standards (Liss and Allen 1992, Richardson and Bloom 1982, Taylor et al. 1988, M. Hirvi, Weyerhaeuser Canada, pers. comm.; L. Olsen, Canadian Forest Products Ltd., pers. comm.). These episodes can result in environmental damage, penalties to the mill operators and damage to mill public relations. The most common causes of these system failures are periods of system shut down, seasonal temperature changes and chemical shocks to the system caused by mill operations (e.g., black liquor spills). These episodes apparently are due to inhibition of the resin acid-degrading populations in biotreatment systems. These populations are likely small, because resin acids are only a small fraction of the organic loading of those systems. Such small populations are relatively less stable than larger populations and may be more susceptible to perturbations. This may explain why some systems have been found to have poor resin acid removal efficiently while simultaneously having normal biological oxygen demand (biodegradable organic matter) removal efficiencies (Liss and Allen 1992).

Inocula have the potential of reducing or eliminating periods of toxicity removal failure by establishing microbial populations capable of toxicity removal. Inocula for treatment systems are commercially available; however, these products do not specifically target toxic compound removal, were not developed on the basis rigorous scientific research and have not been conclusively demonstrated to be effective. Developing effective inocula is not a trivial matter, as the organisms used must tolerate the physicochemical conditions in the treatment system and must successfully compete or co-exist with indigenous populations. This project identified inocula capable of establishing resin acid-degrading populations in treatment systems. Strains we previously isolated (Mohn et al. 1999) and newly isolated ones were evaluated for their potential use as inocula. The organisms were characterized with respect to useful properties. In laboratory experiments, under conditions where they were expected to be of practical use, the organisms were added to treatment system communities following various stresses, which inhibited resin acid removal. Molecular probing methods, which we have developed, were used to conclusively determine the fate of the inoculum strains after they were added to treatment system microbial communities. The ability of the inocula to stimulate detoxication of effluent was simultaneously determined.

A fundamental understanding of resin acid degraders has made the above biotechnology development possible and has numerous other benefits. Understanding resin acid biodegradation

helps to determine the fate of resin acids in treatment systems, and so, to evaluate the efficacy and sustainability of such systems. Such detailed understanding of treatment processes is valuable for comprehensive planning processes, such as life cycle analysis. Understanding resin acid degraders and their diversity helps to determine the limits of biological treatment of resin acids, and so, facilitates design of new systems to meet overall environmental objectives (e.g., high temperature systems allowing recycling of process waters). Finally, identifying resin acid degraders allows the development of molecular probes necessary to study their populations in treatment systems. The resulting ecological understanding allows a more rational (as opposed to empirical) approach to optimization of existing treatment systems and development of new ones.

SUMMARY OF DATA ANALYSIS

Characterization of resin acid degraders

Phylogenetic and physiological diversity of resin acid degraders

A large collection of resin acid-degrading bacteria was characterized. Details of this work are in several publications (Mohn 1995, Mohn et al. 1999, Wilson et al. 1996, Yu and Mohn 1999, Yu et al. 2000). The organisms were found to be phylogenetically diverse, including gram-positive bacteria and members of three gram-negative proteobacterial sub-classes (Fig. 1). The organisms were also physiologically diverse, being adapted to a wide range of environments. Cold tolerant (psychrotolerant) organisms, growing well on resin acids at 4˚C, were obtained from Arctic soil. Heat-loving (thermophilic) organisms, growing on resin acids at up to 60˚C, were obtained from a laboratory bioreactor treating bleached kraft mill effluent at 55˚C. Most of the isolates were found to degrade only abietane resin acids. However, some isolates were specific for pimaranes, and some could degrade both. One of the isolates, *Zoogloea resiniphila* DhA-35, has a floc-forming growth habit typical of many *Zoogloea* spp.

Microaerophilc, nitrogen-fixing resin acid degraders

Bacteria were enriched and isolated on resin acids in medium with very low oxygen concentrations and with no added nitrogen source (other than N_2). These bacteria were obtained from both forest soil and a pulp and paper mill aerated lagoon effluent treatment system, which are both low-nitrogen environments. Enrichment cultures were in tubes in soft agar medium. An oxygen gradient formed in the tubes because of biological oxygen demand in the medium and limited diffusion of O_2 through the agar medium. Growth occurred in bands below the agar surface where the O_2 concentration was below the ambient atmospheric concentration. Growth occurred on medium with dehydroabietic acid (DhA) as the sole organic substrate, but no growth occurred with isopimaric acid. Colonies were isolated on petri plates with solid medium incubated in an N_2 atmosphere with low added O_2 concentrations. Purified cultures were incubated in serum bottles on liquid medium under the same controlled atmosphere. Growth and DhA removal occurred in bottles with 0.1 to 1.0%, but not with higher, O_2 concentrations in the headspace. Thus, the organisms are obligately microaerophilic. Attempts failed to confirm nitrogenase activity in the cultures using the acetylene reduction assay. We hypothesize that

growth and N fixation by these cultures is too slow to support nitrogenase activity above our detection limit. We are doing further experiments to test this hypothesis. The alternative, which appears unlikely, is that the organisms somehow obtain sufficient fixed N for growth from trace quantities of impurities in the culture system.

Selection of inocula for bioaugmentation of treatment systems

The physiological diversity of resin acid degraders permits the selection of inocula with traits useful for particular applications. Extreme temperatures occur during winter and summer in aerated lagoon systems in which temperature control is very difficult. Bioaugmentation with psychrotolerant or thermophilic resin acid degraders could help these systems quickly adapt to extreme temperatures. Nitrogen fixing, microaerophilic strains could help to achieve efficient resin acid removal in systems without the addition of nitrogen as a nutrient. Currently nitrogen is commonly added, despite the expense of doing so. If a system has poor capacity to remove particular resin acids, such as pimaranes, inocula capable of degrading those compounds may be useful. Floc-forming organisms, such as strain DhA-35, may be particularly useful as inocula. The flocs may help the inoculum to be retained in the system, by improving its settleability, and may serve as a physical barrier, protecting the inoculum and other organisms from physicochemical stresses, such as transient pH extremes.

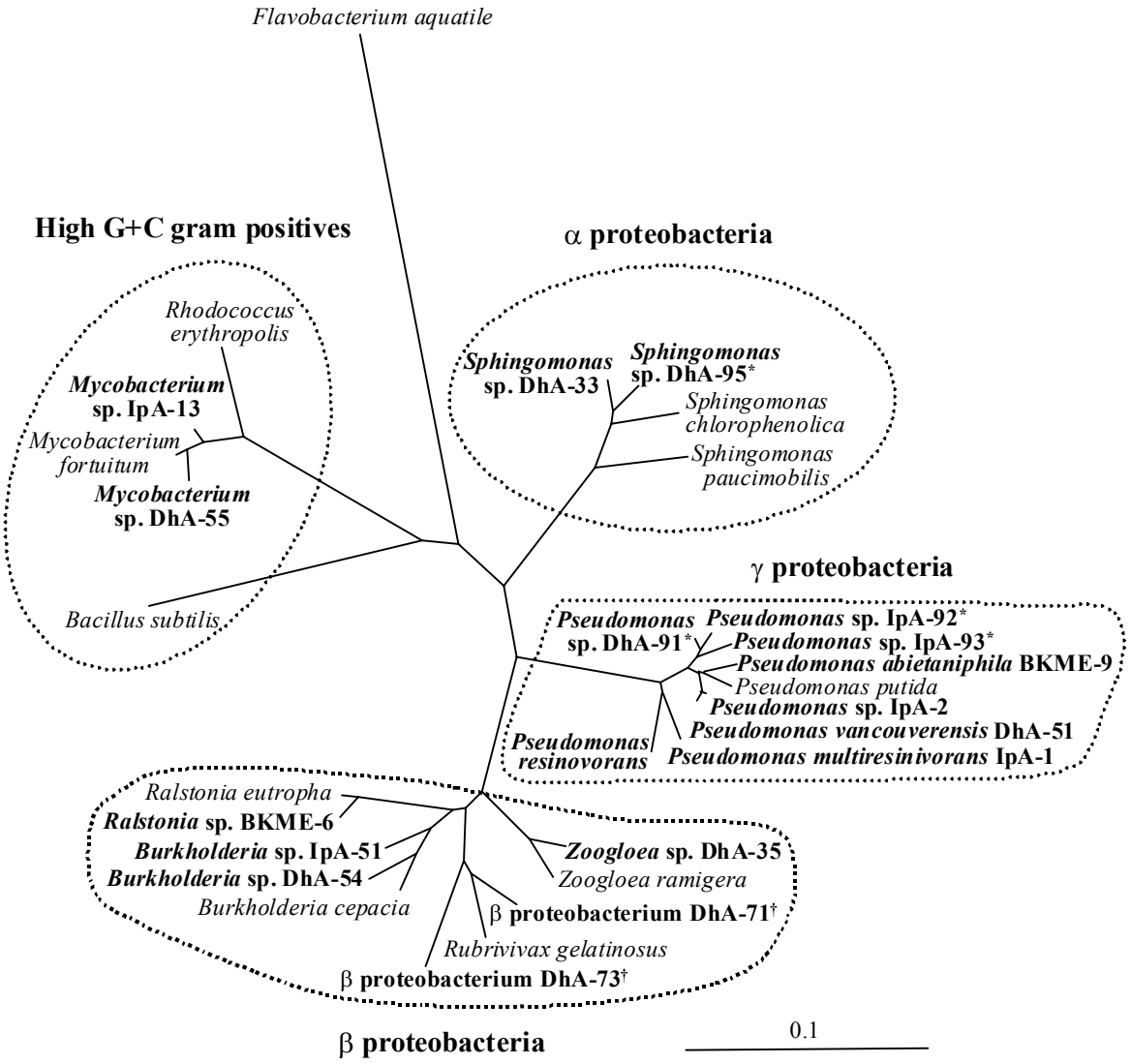

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences indicating that resin acid-degrading bacteria are found among distantly related phylogenetic groups. Resin acid-degrading strains are shown in bold. Distance scale corresponds to 0.1 mutation per nucleotide position. *Indicates psychrotolerant strains. † Indicates thermophilic strains.

Bioaugmentation of treatment system communities

Bioaugmentation of an activated sludge community with DhA-33

One of our resin acid-degrading isolates, *Sphingomonas* sp. DhA-33, was used to bioaugment an activated sludge community from a pulp and paper mill effluent treatment system (Muttray and Mohn 2000). The sludge and DhA-33 were incubated in a batch cultures in bleached kraft mill effluent (BKME) with added dehydroabietic acid (DhA). The addition of DhA-33 increased the rate of DhA removal by the co-culture (Fig. 2). The population size and metabolic activity of DhA-33 were monitored by assaying that organism's ribosomal RNA genes (rDNA) and its ribosomal RNA (rRNA). These nucleic acid-based assays permitted specific monitoring of DhA-33 while it grew within a very complex microbial community of the activated sludge. DhA-33 grew in the co-culture during the first 24 hours, coinciding with the period when DhA removal occurred. This suggests that DhA-33 was directly responsible for the stimulation of DhA removal in the co-culture. A larger DhA-33 population resulted when it was cultured alone on the same medium than in the co-culture, suggesting that it was affected by competition for nutrients in the co-culture. However, the growth rate of DhA-33 was equal in the pure and co-cultures, and so not detectably affected by competition. The rRNA:rDNA ratio of DhA-33 peaked during DhA degradation, indicating that this was the period of maximum metabolic activity of DhA-33. This further supports the role of DhA-33 in DhA biodegradation and indicates that it did not substantially metabolize other components of the BKME, after the DhA was depleted. After the period of growth on DhA, DhA-33 persisted in an inactive state in the sludge community for an additional 48 hours. This experiment demonstrates that DhA-33 can survive and metabolize a resin acid when it is added to a complex activated sludge community.

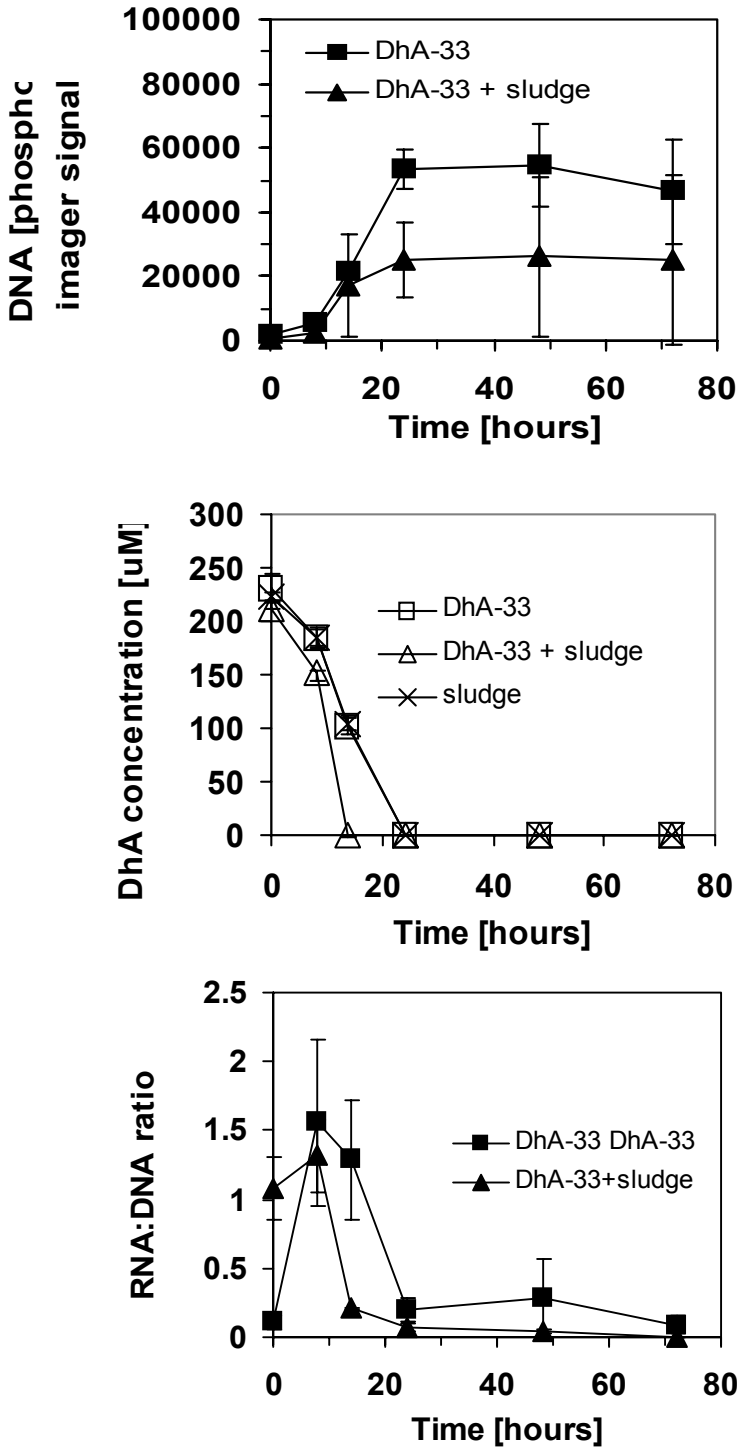

Fig. 2. Growth of activated sludge and strain DhA-33 on BKME plus DhA. Top panel shows relative DhA-33 population measured as rDNA using a competitive PCR assay. Middle panel shows DhA removal. Bottom panel shows relative metabolic activity of DhA-33 measured as rRNA:rDNA ratio using competitive RT-PCR and PCR assays.

Bioaugmentation of an activated sludge community with BKME-9 or DhA-35

A series of experiments were performed in laboratory sequencing batch reactors to determine the effects of various stresses on activated sludge and the capacity of inocula to restore resin acid removal efficiency to the stressed microbial communities (Yu and Mohn 2001). The reactors treated BKME with added DhA. A shock with high pH (pH 9.5), similar to that occurring during a black liquor spill, inhibited the resin acid removal rate of activated sludge by approximately 30%. Inoculation of the sludge community with *Pseudomonas abietaniphila* BKME-9 or *Zoogloea resiniphila* DhA-35 enhanced the resin acid removal activity (Fig. 3).

To simulate conditions during a mill shutdown, activated sludge was starved by incubation at 7˚C for three weeks. Starvation reduced the resin acid removal rate of the sludge by 50%. Bioaugmentation with BKME-9 or DhA-35 restored resin acid removal activity to the starved sludge community. Activated sludge was also shocked by overloading with DhA, as might occur if a mill shifted from a wood furnish low in resin acids to one high in resin acids. Again, bioaugmentation with BKME-9 or DhA-35 enhanced resin acid removal activity to the starved sludge community (Fig. 4). However, at this DhA concentration, BKME-9 was significantly more effective than DhA-35. Strains BKME-9 and DhA-35 did not substantially degrade the total organic carbon in BKME, most of which is not resin acids, nor did they stimulate total organic carbon by activated sludge (Fig. 5). This is not surprising since the two strains do not degrade the major components of BKME, methanol, lignin, cellulose, hemicellulose, lipids, etc. Further, the inocula were small relative to the total microbial population in the sludge. Thus, these inocula were specific for resin acid biodegradation.

Both BKME-9 and DhA-33 persisted in activated sludge during long-term incubations of a sequencing batch reactors with 48-hour cycles (Fig. 6). Neither strain was detectable in the sludge prior to inoculation. During the first two weeks, both populations declined gradually; then, the populations remained stable. Clearly these organisms are both able to compete and function in the sludge community. DhA-35 forms flocs, which would confer upon it good settleability, a characteristic essential for retention in this system. The persistence of BKME-9 suggests that it was incorporated in flocs formed by other organisms in the sludge. When BKME-9 alone was incubated in a sequencing batch reactor, it was washed out (not shown). Both strains were clearly able to compete successfully within the sludge community for required nutrients.

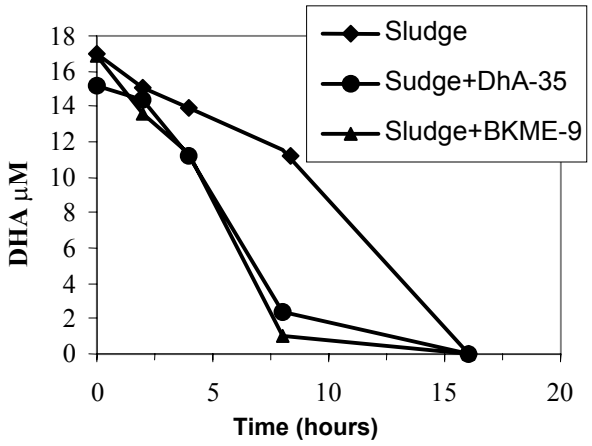

Fig. 3. Removal of DhA by high-pH-shocked (pH 9.5 for 30 min.) activated sludge bioaugmented with strains BKME-9 or DhA-35 (10^6 cells/ml).

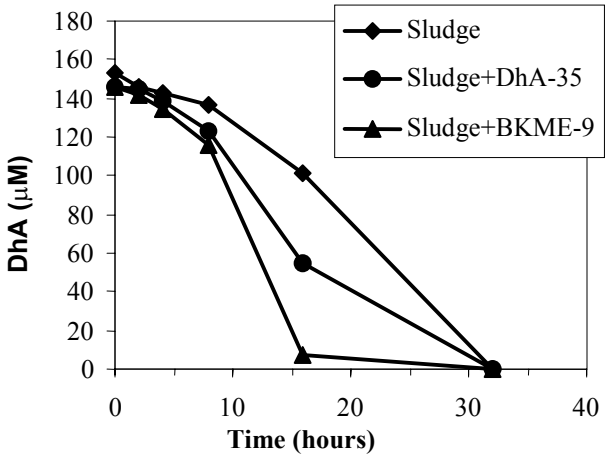

Fig. 4. Removal of DhA by activated sludge overloaded with DhA (60 mg/L added) and bioaugmented with strains BKME-9 or DhA-35 (10^6 cells/ml).

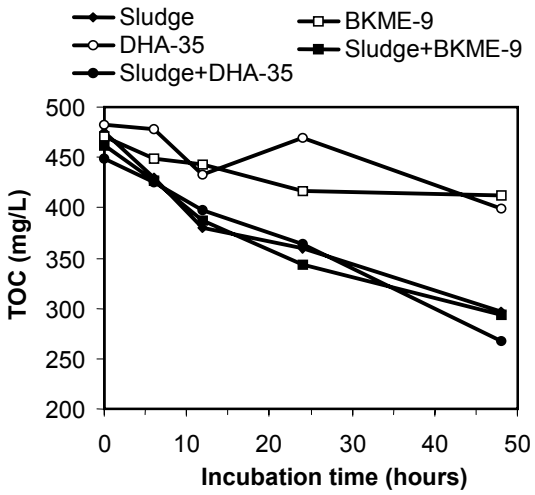

Fig. 5. Total organic carbon removal from BKME medium in sequencing batch reactors during one 48-hour cycle.

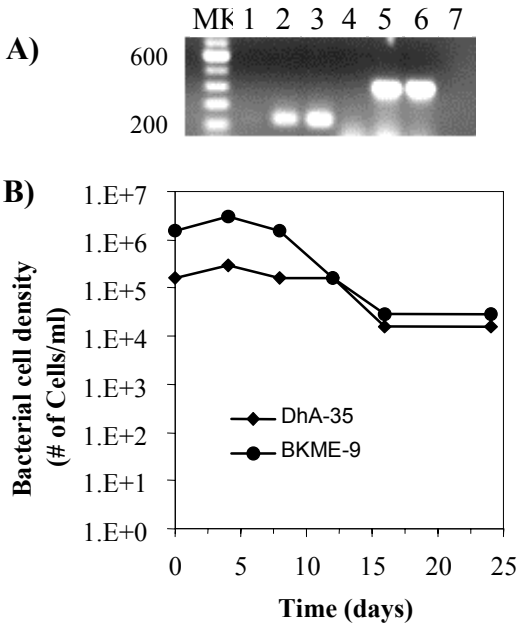

Fig. 6. Population changes of BKME-9 and DhA-35 in bioaugmented sequencing batch reactors. Strain-specific MPN-PCR assays were used to determine the populations in samples taken at the end of each cycle. (**A**) Qualitative PCR assays of BKME-9 and DhA-35 in SBRs. MK: molecular marker; lane 1: sludge only SBR; lane 2: BKME-9 only SBR; lane 3:SBR of sludge plus BKME-9; lane 4: sludge only SBR; lane 5: DhA-35 alone SBR; lane 6: SBR of sludge plus DhA-35; lane 7: negative control. (**B**) Population changes of BKME-9 and DhA-35 in SBRs of sludge plus BKME-9 or sludge plus DhA-35.

Bioaugmentation of an aerated lagoon community with BKME-9

A series of experiments were done examining the effects of pH shocks and bioaugmentation with *Pseudomonas abietaniphila* BKME-9 on an aerated lagoon microbial community (Muttray and Mohn submitted). Aerated lagoons are more commonly used than activated sludge systems to treat pulp and paper mill effluents. The biomass in lagoons is less dense than in sludge, and the biomass in lagoons does not typically exist in flocs, as it does in sludge. Thus, due to a lack of physical protection, lagoon communities may be more susceptible to environmental stresses than sludge communities.

Biomass from an aerated lagoon, with and without added BKME-9, was incubated in a batch culture on BKME plus DhA. As expected, the lagoon community degraded DhA (Fig. 7). Bioaugmentation with BKME-9 stimulated DhA removal, approximately doubling the removal rate. Analysis of BKME-9 rDNA indicated that the inoculum, which had previously been acclimated to BKME, grew immediately without a lag and then persisted at a density close to the maximum. Thus BKME-9 competed successfully in the community. The metabolic activity of BKME-9, indicated by the rRNA:rDNA ratio, was initially at its highest level and may have decreased in response to the shock of transfer and competition for nutrients. Metabolic activity of BKME-9 remained essentially steady during DhA removal; then, it decreased to a very low level after depletion of DhA. It appears that BKME-9 persisted in a state of very low activity during the final days of incubation. This experiment demonstrates that BKME-9 can persist and be metabolically active in a lagoon community, and so, has potential for bioaugmentation of lagoon systems.

Biomass from an aerated lagoon plus BKME-9 were maintained in a continuous bioreactor on glucose plus DhA (Fig 8). The system reached an apparent steady state after 20 hours. Visible precipitation of DhA in the medium reservoir appeared to retard the metabolic activity of BKME-9 at 50 hours. At this time, there was a slight decrease in the BKME-9 rRNA, which caused a large change in the rRNA:rDNA ratio. A high-pH shock, as might result from a black liquor spill in a pulp mill, immediately inhibited DhA removal, reduced the BKME-9 rDNA and reduced the rRNA:rDNA ratio of BKME-9. This indicates that the pH shock killed a large part of the BKME-9 population and inhibited the metabolic activity of the surviving portion of the population. Recovery of DhA removal activity occurred over the following 16 hours, accompanied by increases of BKME-9 rRNA and rDNA to previous levels. Thus, recovery of DhA removal activity was associated with re-growth of the BKME-9 population.

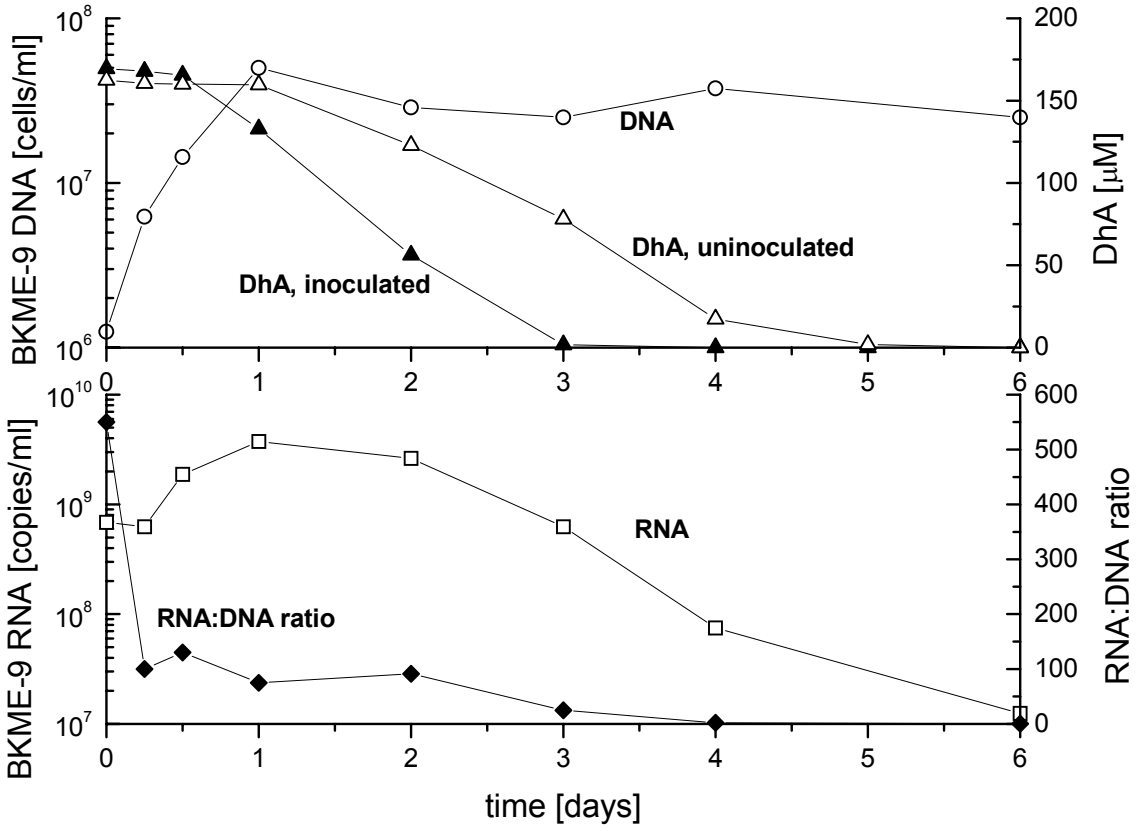

Fig. 7. A batch culture of aerated lagoon biomass (approx. 10^5 cells/mL) incubated at 22˚C on BKME plus DhA with and without bioaugmentation with BKME-9 (approx. 10^5 cells/mL). The rRNA and rDNA of BKME-9 were assayed by species-specific competitive RT-PCR and PCR assays, respectively.

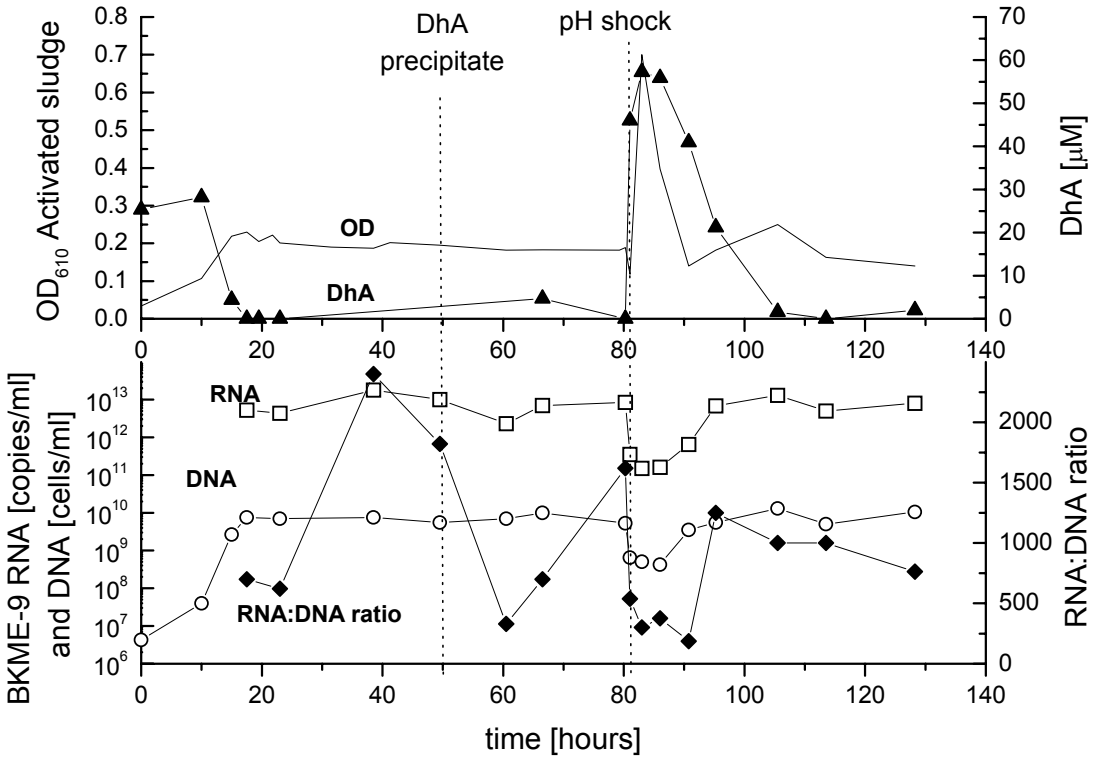

Fig. 8. A continuous bioreactor with aerated lagoon biomass plus BKME-9. The total volume was 240 mL, and a synthetic medium with 0.1 g/L glucose plus 60 mg/L DhA was added at a flow rate of 1.4 L/d. The pH was 6.5, except during a 30-min pH 12.5 shock at 80 h. Total microbial density was determined as optical density (OD). BKME-9 rRNA and rDNA were measured as in Fig. 7.

Bioaugmentation of an aerated lagoon community with DhA-35

Another series of experiments were done examining the effects of pH shocks and bioaugmentation with *Zoogloea resiniphila* DhA-35 on an aerated lagoon microbial community (Yu and Mohn 2001). In agreement with the hypothesis that resin acid degraders in aerated lagoon communities are more susceptible to environmental stresses than those in activated sludge communities, we found that low- and high-pH stresses dramatically inhibited resin acid removal by a lagoon community (Fig. 9). Either stress completely inhibited DhA removal by the community. After 4 days post-stress incubation (pH 6.5), DhA removal activity recovered in the high-pH-stress treatment, but after 6 days, it did not recover in the low-pH-stress treatment. Bioaugmentation with DhA-35, after low- and high-pH stresses, completely restored DhA removal activity to a level as high or higher than in the non-stressed control. The biomass in bioaugmented treatments formed visible flocs during incubation. Strain DhA-35 normally forms such flocs, which are typical of other *Zoogloea* spp.

The above pH stresses dramatically decreased the populations of culturable DhA degraders in the lagoon community (Fig. 10). This indicates that the inhibition of DhA removal activity by pH stresses was probably due to killing of the responsible organisms. The greater population reduction due to the low-pH stress is consistent with the lack of recovery of DhA removal activity in that treatment. The DhA-35 population in the bioaugmented treatments was initially approximately 7 to 8 x 10^6 cells/ml (Fig. 11). This population increased in both treatments during the period of DhA removal and then declined. Consistent with this, the metabolic activity of DhA-35, based on its cellular rRNA:rDNA ratio, was highest immediately after depletion of DhA. The DhA-35 cells remaining after 6 days incubation, appeared to have low metabolic activity, suggesting that they could use little substrate in the BKME after depletion of the DhA. These general trends are consistent with the previously discussed fate of DhA-35 after addition to the activated sludge community (Fig. 6).

The pH stresses to the aerated lagoon community had little effect on total organic carbon removal (Fig. 12) or total microbial populations in (Fig. 13). These results are consistent with the previously discussed affect of high pH on activated sludge. The concomitant removal of organic carbon and microbial population increases indicates that organic matter in the BKME supported growth. After 3 days, counted cells decreased; while, culturable cells did not decrease (Fig. 12). We interpret this inconsistency to be due to floc formation which made cell counts difficult and caused under-estimation at high cell densities. The fact that pH stresses had little effect on total organic carbon removal and total microbial populations suggests that the resin acid-degrading population in the lagoon community is particularly susceptible to pH stress. Bioaugmentation with DhA-35 did not stimulate removal of organic carbon, in accordance with the small size of the inoculum, relative to the total microbial population, and the fact that DhA-35 is unable to degrade the major components of BKME other than resin acids. Surprisingly, addition of DhA-35 appeared to inhibit removal of organic carbon after 3 days of incubation. We interpret this inhibition to be due to effects on community composition (see below).

In order to assess the effects of pH stresses and bioaugmentation on the structure of the aerated lagoon community, we analyzed ribosomal intergenic spacer length polymorphism fingerprints. The different bands on the electrophoretic gel represent different bacterial populations, and the intensity of each band is a function of population size. As expected, every reactor initially had a similar fingerprint (Fig. 14A), and the time-zero samples clustered together on the basis of similarity (Fig. 14B). This is not surprising because, while the low- and high-pH treatments may have killed many bacteria, the rDNA of those bacteria probably remained detectable for some time until the cells decomposed. The percent similarity among the time-zero samples ranged from 84 to 63%. One intense band of approximately 1.4 kb was common to all the treatments, suggesting the presence of one or more predominant populations that yield this band.

In every bioreactor, temporal changes in community composition were apparent (Fig. 14). The control day 3 fingerprint appears anomalous; otherwise, trends are consistent within each treatment, evidenced by the fact that day 3 and 6 samples of each treatment cluster. The relatively subtle changes in the control from days 0 to 6 may be due to adaptation of the microbial community to the experimental conditions, particularly the temperature (30^{o}C), which was lower than in the source lagoon (42^{o}C), as well as to depletion of substrates (TOC). It was expected that the low- and the high-pH stresses would selected acid-tolerant and alkaline-tolerant populations, respectively. Accordingly, the pH stresses, without bioaugmentation, had clear affects on the fingerprints, consistent with increases in some populations and decreases in others. Some of the changes were common to low- and high-pH stresses; while others were unique to one or the other. The most intense bands generally persisted in all non-bioaugmented reactors.

Bioaugmentation had a dramatic impact on community fingerprints, evidenced by the very distinct clustering of the fingerprints from samples from the bioaugmented reactors (Fig. 14). A single band of approximately 1.3 kb, attributable to DhA-35, is clearly evident in samples from bioaugmented treatments. In agreement with the PCR anaysis (Fig. 11), the RIS-LP analysis shows extensive growth of the inoculum. Several other intense bands entirely disappear from or appear in the fingerprints of the inoculated treatments, suggesting that the presence of DhA-35 led to large decreases or increases of certain populations in the community. Notably, the combination of low-pH stress and bioaugmentation led to the loss of the 1.4 kb band, which remained predominant in all other treatments. It is interesting to note, that this apparently substantial change in the community composition occurred in the one treatment with low TOC removal activity (Fig. 12). Aside from the 1.4 kb band, most major changes were common to both low- and high-pH-stressed, bioaugmented treatments. Floc formation associated with DhA-35 may have caused strong selective pressure for or against certain populations.

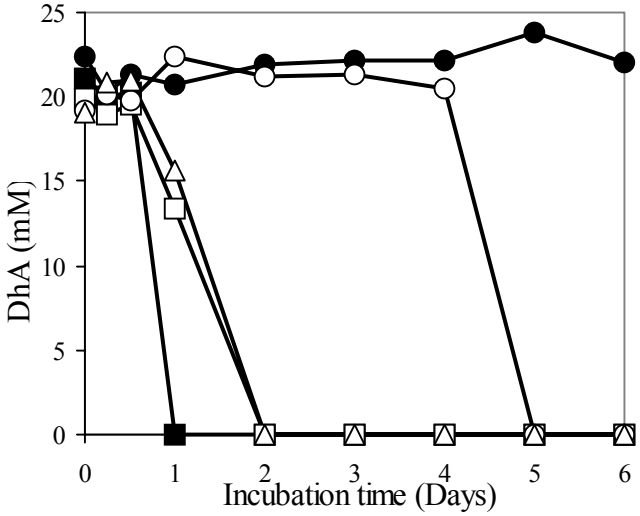

Fig. 9. DhA removal by aerated lagoon biomass stressed with high pH (pH 10) for 1 hour (open squares and open circles) or low pH (pH 3) for 1 hour (closed squares and closed circles). Squares indicate treatments bioaugmented with strain DhA-35. Triangles indicate control with no pH stress.

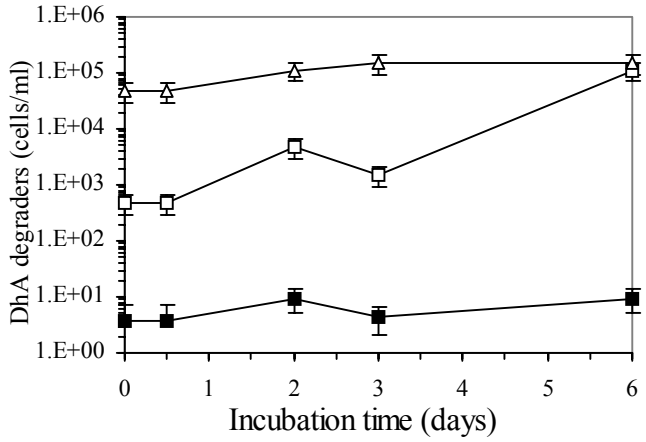

Fig. 10. Culturable DhA degraders in an aerated lagoon community. Triangles, control; open squares, high-pH stress; closed squares, low-pH stress. Populations were estimated by a most-probable number assay.

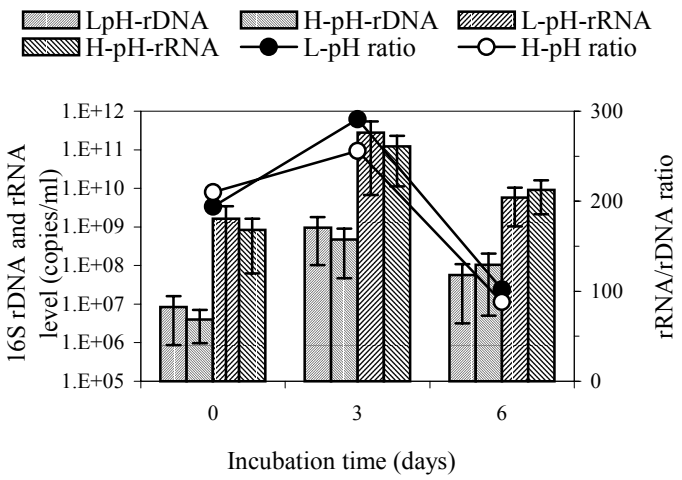

Fig. 11. Changes in the population (rDNA) and metabolic activity (rRNA:rDNA ratio) of strain DhA-35 in an aerated lagoon community. The rRNA and rDNA were assayed by species-specific competitive PCR and RT-PCR assays.

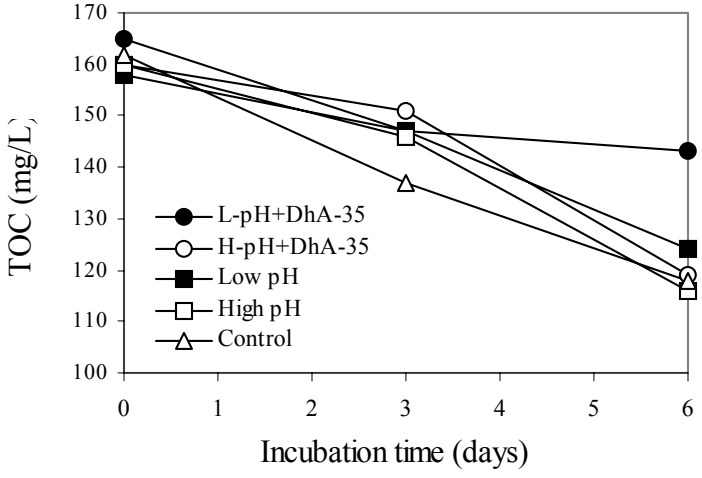

Fig. 12. Removal of total organic carbon from BKME by an aerated lagoon community.

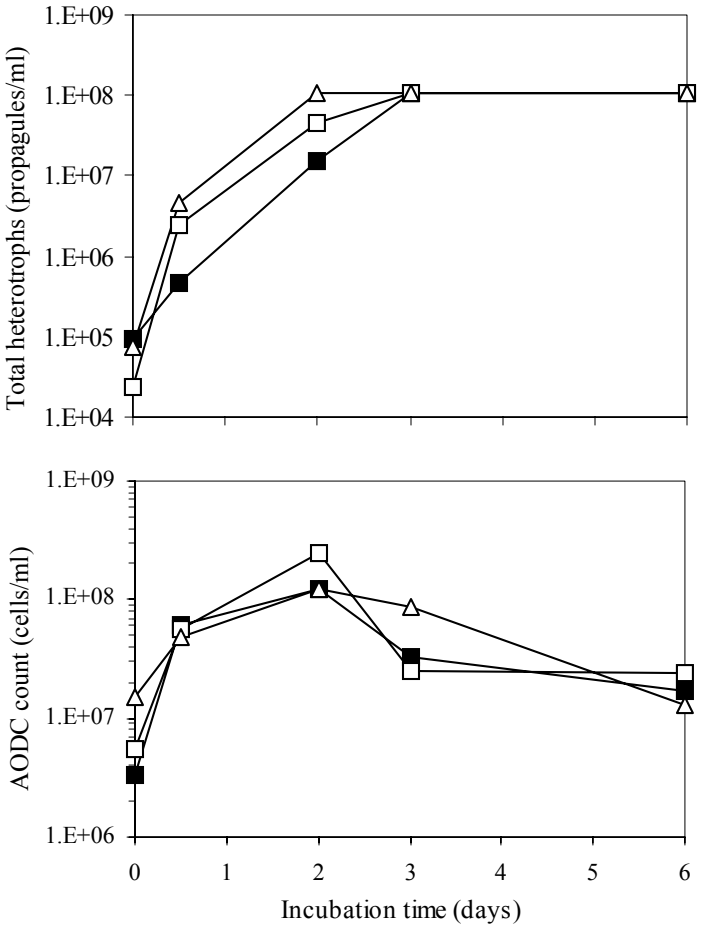

Fig. 13. Total microbial populations in non-bioaugmented treatments of an aerated lagoon community. Upper panel, culturable heterotrophs estimated by most-probable number assay (values on days 3 and 6 may have exceeded upper limit of assay). Lower panel, acridine orange direct counts of cells. Triangles, non-stressed control; solid squares, low-pH treatment; open squares, high-pH treatment.

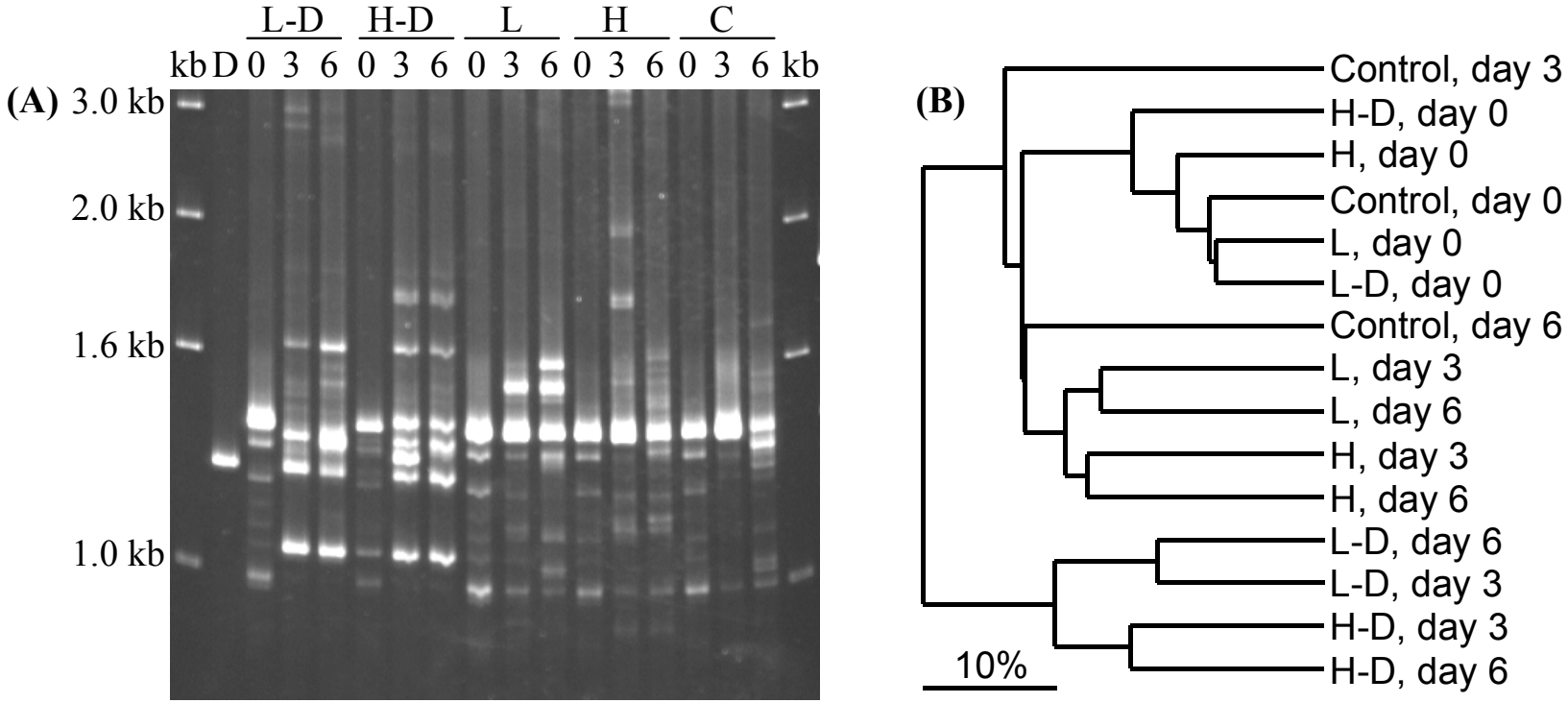

Fig. 14. Ribosomal intergenic spacer length polymorphisms fingerprints of bacterial communities (A) and cluster analysis based on percent similarity of fingerprints (B). Lane designations: kb, 1 kb ladder; D, DhA-35; L-D, low pH treatment augmented with DhA-35; H-D, high pH treatment augmented with DhA-35; L, low pH treatment; H, high pH treatment; C, control. The numbers 0, 3, and 6 indicate the sampling time (days). Bar indicates 10% dissimilarity.

CONCLUSIONS AND MANAGEMENT APPLICATIONS

1. Resin acid-degrading bacteria are adapted to a wide range of environmental conditions, including extreme temperatures, scarce oxygen and scarce nitrogen. Many of these conditions occur in biotreatment systems, consistently or occasionally. Resin acid-degrading strains with appropriate characteristics have potential for bioaugmentation of treatment systems in order to improve the efficiency of resin acid removal.

2. Resin acid-degrading populations are relatively small components of microbial communities in treatment systems and appear to be particularly susceptible to inhibition by environmental stresses. This inhibition can cause toxicity breakthrough in the systems. In the case of pH stresses, inhibition involves death of a large fraction of the resin acid degraders.

3. Molecular probing methods were developed to monitor the population size and metabolic activity of individual resin acid degraders. These methods can be used to specifically monitor a particular strain introduced into a very complex microbial community.

4. Molecular fingerprinting methods were developed to monitor changes in the composition of bacterial communities in treatment systems. These methods can detect the impact on the microbial community of environmental stresses and introduced strains. These methods are not yet practical for routine use in mills, but it should be possible to achieve this goal.

5. Resin acid-degrading bacterial strains consistently survived when added to microbial communities from activated sludge or aerated lagoon systems. These strains were metabolically active and increased the resin acid removal activity of the communities. Inocula of 10^5 cells/mL were effective, and lower cell densities should be tested. The efficacy of inocula should be confirmed on larger experimental scales, and the cost of bioaugmentation of full-scale treatment systems should be determined.

6. Resin acid-degrading strains may have useful applications in response to treatment system inhibition caused by transient environmental stresses such as black liquor spills.

7. Resin acid-degrading strains may have useful applications in preventing failure of toxicity removal in treatment systems. When conditions known to be potentially inhibitory occur, such as high summer or low winter operating conditions, system shut-downs for mill maintenance or shifts in system pH, appropriate strains can be added to help the microbial community adapt to the new conditions before toxicity breakthrough occurs.

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