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Isolating metal-tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood

Bioremediation of chromated copper arsenate-treated waste wood with one or more metal-tolerant bacteria is a potential method of naturally releasing metals from treated wood fibre. Sampling eight environments with elevated levels of copper, chromium, and arsenic resulted in the isolation of 28 bacteria with the capability of releasing one or more of the components from chromated copper arsenate-treated wood. The isolates represent 13 species of 8 different genera of soil-inhabiting bacteria. Three isolates, *Acinetobacter calcoaceticus* FN02, *Aureobacterium esteroaromaticum* VV03, and *Klebsiella oxytoca* CC08, were able to release 98% of the chromium, which is the most difficult component of chromated copper arsenate to remove from treated wood. *Bacillus licheniformis* CC01 released the highest percentage of copper, 93%, from treated wood. Eleven isolates, including *Bacillus licheniformis* CC01 and *Acinetobacter calcoaceticus* FN 02, released 44% to 48% of the arsenic.

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Introduction

The predominant inorganic compound used to preserve wood in the United States is chromated copper arsenate (CCA). More than 80% of all lumber and timber treated in the United States in 1993 was treated with CCA preservatives (Micklewright 1994). A notable increase in consumer demand for dimension lumber treated with CCA resulted from the popularity of its use in decks and outdoor structures in the past two decades. The average service life of 20 to 40 yrs for this product will eventually result in significant quantities of CCA-treated wood being removed from service and disposed in landfills. Cooper (1993) estimates that 19×10^6 m^3 of CCA-treated wood per year will be removed from service in the United States by the year 2020. This may be a modest estimate since a recent survey indicates that the average deck is removed after only 8 to 10 yrs of service (McQueen *et al.* 1998). Spent CCA-treated wood is a significant fibre source worthy of recycling consideration. Recycling the remediated fibre is one option for diverting this material from landfills.

Bioremediation of CCA-treated waste wood presents a unique recycling challenge. Indeed, several criteria set it apart from all other bioremediation methods. Bioremediation of spent CCA-treated wood involves actual treatment of the wood with one or more microorganisms that have the capability of releasing fixed metals from the wood. In contrast, most wood preservation remediation research to date has involved cleanup of contaminated sites, i.e. soil and groundwater, whether the source of contamination was from

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an accidental spill or accumulation of chemicals from years of normal operations of a wood preservation facility.

Nearly all bioremediation studies have used fungi (*Phanerochaete chrysosporium*) (Lamar & Dietrich 1990) or bacteria (*Rhodococcus phenolicus*) (Briglia *et al.* 1994) and *Flavobacterium* (Crawford & Mohn 1985) to mineralize phenolic compounds and have not involved heavy metals. Again, that research was directed at soil remediation. To date, little research has been done on devising methods for bioremediation of wood treated with inorganic arsenicals (Clausen 1996, 1997; Stephan *et al.* 1996; Cole & Clausen 1997; Clausen & Smith 1998). Stephan *et al.* (1996) have demonstrated that fungal isolates of *Antrodia vaillantii* and *Postia placenta* leach significant quantities of chromium and arsenic from CCA-treated wood by conversion of the metals into soluble compounds with little mass loss, while Clausen and colleagues (Clausen 1997; Cole & Clausen 1997; Clausen & Smith 1998) have demonstrated the ability of a metal-tolerant bacterium to liberate metals from CCA-treated wood with no mass loss. Illman & Highley (1996) reported that an isolate of *Meruliporia incrassata* has the ability to degrade CCA-treated southern pine with the same ease as untreated southern pine. Fungal degradation of spent CCA-treated wood is one option for releasing metals from the wood. As a result of composting, however, the wood fibre biomass is reduced by at least 40%, and methods for reclaiming released metals have yet to be developed. Treatment of spent CCA-treated wood with CCA-resistant *Bacillus licheniformis* CC01, however, leaves the wood fibre intact for recycling into composite products. The modulus of elasticity remains unaffected, and scanning electron microscopy reveals no damage to the wood cell wall by *B. licheniformis* CC01 (Clausen, unpublished data).

Intrinsic bioremediation has been studied quite extensively as a more economical, natural means of remediating chlorinated hydrocarbons. Intrinsic bioremediation examines the naturally occurring microbial flora of an area, sometimes including augmentation with composting matter, such as straw from mushroom plants or bark chips from sawmills (Laine & Jorgensen 1996, 1997). Intrinsic bacteria that are capable of biodegradation exist in soils, and those existing in soil on or near the site of contamination have adapted to the contaminant. Naturally occurring bacteria that are capable of mineralizing pentachlorophenol are recognized (Crawford & Mohn 1985; Briglia *et al.* 1994), but intrinsic bacteria that are capable of releasing heavy metals from CCA-treated wood have not been extensively studied.

It is difficult to imagine that a single bacterium could be capable of releasing efficiently all copper, chromium, and arsenic from treated wood. Yet single microbes are capable of degrading materials such as benzene, dioxin, DDT, PCB, styrene, xylene, tires, concrete, oil, gasoline, pentachlorophenol, and creosote. One isolate of *Bacillus licheniformis* CC01 can efficiently remove 93% of the copper and 45% of the arsenic from CCA sawdust in liquid culture (Clausen 1997). This isolate of *Bacillus* removes even more metal when CCA-treated chips are pretreated with oxalic acid (Clausen & Smith 1998). Under those conditions, Clausen & Smith (1998) have shown that 90% of the copper, 80% chromium, and 100% arsenic can be removed from CCA-treated chips. Still, a designer consortium of heterotrophic bacteria is desirable to remove efficiently 90% to 100% of the copper, arsenic, and chromium. One of the objectives of this survey was to identify bacteria suitable for inclusion in a designer consortium. Chromium has proved to be the most difficult metal to remove, not only microbially (Clausen 1997) but also by mechanical means such as steam explosion (Smith & Shiau 1997). Therefore, another objective of this survey was to identify bacteria that selectively remove chromium from CCA-treated wood.

Materials and methods

Sampling methods

Eight environments containing elevated levels of CCA were sampled for bacteria. First, CCA-treated stakes from four different test plots at the USDA Forest Service, Forest Products Laboratory's Valley View Experimental Exposure Site near Madison, WI (test plots started in 1949, 1972, 1976, and 1996) were sampled by swabbing the wood surface with a sterile cotton swab at the ground line. Test plot variables included Douglas-fir, spruce, and southern yellow pine 2 × 4 (standard 38 × 89 mm) lumber and incised, unincised, and plywood samples. Second, CCA-treated timbers in service since 1983 were sampled by swabbing the timber at the ground line with a sterile cotton swab. Third, a failed CCA-treated timber (after 13 yrs of service) was sampled. Fourth, a gravel storage yard and standing surface water outside a CCA treatment plant were sampled. This was an uncovered area where CCA-treated lumber is stored after initial drying. Finally, soil and wood samples were obtained from a closed treatment facility. The plant was in operation from 1950 to 1987, and areas surrounding the facility are known to be contaminated with CCA.

The soil-stake or soil-post interfaces were swabbed with a sterile cotton swab and then a single streak was aseptically

Table 1, Sample sites and summary of total bacterial isolates, CCA-tolerant bacteria, and CCA-resistant bacteria cultured from each site

Sample site	Number of samples	Number of tolerant isolates*	Number of resistant isolates†
1949 test plot	7	4	1
1972 test plot	4	7	1
1976 test plot	47	24	11
1996 test plot	7	7	4
13-yr-in-service pole	4	0	0
15-yr-old failed past	4	0	0
Treatment facility storage yard	8	20	7
Closed treatment facility	19	16	4

*Tolerance is defined as the ability of an organism to grow when subjected to CuO, CrO_3, and As_2O_5 in a nutrient medium. †Resistance is defined as the ability of an organism to withstand the toxic effects of CCA while releasing one or mare components of CCA (copper, chromium, and arsenic) from ground-treated wood.

made on a plate of nutrient agar (NA) containing 0.2% CCA Type C (CCA-NA) consisting of 23.75% CrO_3, 9.25% CuO, and 17.00% As_2O_5. Plates were further streaked in the laboratory with a sterile loop to isolate individual colonies and were incubated at 27°C. Isolated colonies were streaked on NA. Wood shavings from the failed timber were sprinkled on NA and incubated at 27°C for 7 d. Individual colonies were isolated by streaking onto fresh NA. Soils samples were hydrated with 5 ml of sterile distilled water, mixed thoroughly, streaked onto CCA-NA, and incubated at 27°C. Colonies were isolated by streaking onto NA.

Isolate identification

Purified isolates were identified by fatty acid methyl ester (FAME) speciation (Microcheck ID, Northfield, VT, USA), using Gram stain and API 20E confirmatory tests as necessary.

Isolate evaluation for metal tolerance and resistance

Individual isolates were evaluated for their ability to release metals from CCA-treated sawdust by exposure of 0.5 g of 20 mesh (0.841 mm sieve openings) CCA sawdust (6.4 kg m^{-3} retention) in 50 ml of nutrient broth (NB) to each isolated bacterium. The CCA sawdust was obtained by hammer milling a treated 2 × 4 (standard 38 × 89 mm) from a local retail lumber yard, and the retention levels of copper, chromium, and arsenic were determined by atomic absorption spectroscopy (AA). Cultures were incubated at 30°C for 7 d with mixing at 150 r.p.m. Controls consisted of 0.5 g of 20 mesh CCA sawdust in 50 ml NB without inoculum. Sawdust was collected by aspiration through Whatman no. 1 filter paper (Whatman, Hillsburo, OR) and was ovendried at 60°C. Dried sawdust samples were analyzed by AA for chromium, copper, and arsenic content and compared with control values according to AWPA A11-93 (American Wood Preservers' Association 1995).

Results and discussion

Sampling

Sampling environments that contain elevated concentrations of copper, chromium, and arsenic are a potential source of toxic-metal-tolerant bacteria. Such environments foster adaptation and selection for heavy metal resistance. Isolated bacteria can rapidly be screened for the ability to liberate chromium, copper, and arsenic from CCA-treated wood. Initial screening was conducted on CCA sawdust (20 mesh) to provide the maximum wood surface area to the bacterium.

Table 1 lists the sources of bacterial isolates, the sample number, and the number of resistant and tolerant bacterial isolates. Organisms cultured from various sites were notably different. Thirty-eight of 42 toxic-metal-tolerant bacteria from field test plots were isolated from unincised spruce 2 × 4 s (38 × 89 mm). Incising increases the surface area and allows greater preservative penetration, but it should not affect the endemic microbial population at the soil-stake interface nor how the microorganisms adapt to the metals in their immediate surroundings.

The one-year-old test plot represented an environment with initial leaching of surface residues into the immediate surroundings. Low numbers of resistant organisms reflect minimal time in service for selection of indigenous metal-tolerant organisms or adaptation to occur.

Forty-eight-year-old stakes have reached the end of their service life. There were few stakes left intact in this plot and any leached metals were likely to be present in low levels. Heterotrophic bacteria isolated from the failed and in-service timbers were uniformly nonresistant to heavy metals.

In the treatment facility storage yard, numerous tolerant

organisms were isolated from eight sampling sites. Metal-tolerant bacteria were also isolated from the standing surface water samples taken at the storage yard.

Isolates from most sampled areas of the closed treatment facility grew aggressively on CCA-NA. However, sampled areas with the highest levels of arsenic and chromium (up to 71 and 60 parts per thousand, respectively) yielded no isolates.

Metal tolerance

Table 1 demonstrates that metal-tolerant bacteria can be readily isolated from environments containing elevated levels of toxic metals. Some have adapted and some are endemic to their environment, while the environmental conditions may have selected for others. In general, heterotrophic bacterial adaptation to toxic metals is 2 to 4 orders of magnitude higher (millimolar) than levels of resistance displayed in fungi (micromolar) (Mergeay 1995). One unusual group of acidophilic chemolithoautotrophs, specifically *Thiobacillus ferrooxidans,* is tolerant of molar concentrations of toxic metals. However, they require fastidious growth conditions, which limit their consideration for bioremediation studies.

It is well recognized that plasmid-borne resistance to toxic metals is an important component of toxic metal tolerance (Mergeay 1995). It is also recognized that certain genera of heterotrophic bacteria, which are easily isolated from areas containing up to 1200 parts per million (p.p.m.) chromium, copper, and arsenic, produce a full complement of cellulolytic and pectinolytic enzymes. These enzymes may play a role in the release of metals fixed to wood, although studies have shown no damage to the wood cell wall of CCA-treated wood exposed to *Bacillus licheniformis* CC01, nor has cellulolytic or pectinolytic enzyme production been detected in this organism (Clausen, unpublished data).

Metal resistance

Table 2 identifies tolerant isolates from sampling sites and demonstrates their ability to remove copper, chromium, and arsenic from CCA-treated wood compared with an uninoculated wood control. Three isolates, *Acinetobacter calcoaceticus* FN02, *Aureobacterium estoroaromaticum* VV03, and *Klebsiella oxytoca* CC08, removed 94% or greater of the chromium. Only *Bacillus lichenformis* CC01 removed 93% of the copper. Eleven isolates, including *Acinetobacter calcoaceticus* FN02 and *Bacillus licheniformis* CC01, removed 44% to 48% of the arsenic.

Mergeay (1995) warns against using rich nutrient media when defining resistance in a bacterium. Toxic metals interact with medium components and carbon sources to give misleading results. Though CCA-NA contains copper, chromium, and arsenic in their least toxic states, the author believes this agar provided a selective medium designed to promote the growth of resistant bacterium by providing efficient counter-selection of undesired bacteria. A concentration of greater than 0.2% CCA in NA lowered the pH of the agar sufficiently to prevent gelling. Beyond the isolation

Table 2. Identification of CCA-resistant bacterial species and their relative removal rates of CCA metals from treated wood ground to 20 mesh (0.841 -mm sieve openings)

Bacterial species	Number of isolates	Metal removal (%) Cu	Cr	As
Acinetobacter calcoaceticus	2	25	0-97	15-48
Aureobacterium barkeri	1	50	68	37
Aureobacterium esteroaromaticum	1	—	97	—
Aureobacterium saperdae	1	25	—	48
Bacillus coagulans	2	39	—	—
Bacillus licheniformis	*2*	*93*	8	45
Bacillus sphaericus	1	—	—	44
Bacillus thuringiensis	1	—	—	44
Klebsiella oxytoca	2	25	0-94	3-48
Micrococcus kristinae	2		—	44-48
Pseudomonas fluorescens	1	50	—	48
Pseudomonas putida	1	25	—	—
Rhodococcus luteus	2	25	0-76	7-30
Stenotrophomonas maltophilia	5	50	—	0-33

stage, nutrient broth was used to provide a nutrient rich environment for the bacterium. Because CCA is fixed to the wood structure, atomic absorption spectroscopy was an accurate means of analyzing the remaining metals in the CCA sawdust after exposure to each bacterium, regardless of the affinity of the released metals to the medium components.

Conclusion

A survey of bacteria from eight environments with elevated levels of copper, chromium, and arsenic resulted in isolations of 28 bacteria with the ability to release one or more of these components from CCA-treated wood. The isolates, which represented eight genera of soil-inhabiting bacteria, included three that had the ability to release 94% or greater of the chromium from a sample of CCA-treated sawdust; *Aureobacterium esterouromaticum* VV03, *Klebsiella oxytoca* CC08, and *Acinetobacter calcoaceticus* FN02. *Bacillus licheniformis* CC01 released the highest amounts of copper (93%) and moderate amounts (45%) of arsenic. *Bacillus licheniformis* CC01, working in concert with an isolate that releases a large amount of chromium, has the potential to remove significant residual copper, chromium, and arsenic from CCA-treated wood.

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