Characterization of bacteria isolated from a bleached kraft pulp mill wastewater treatment system

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Water samples from the wastewater treatment system of a bleach kraft mill and from the river that supplies the mill were plated on six different media and culturable isolates were screened for substrate utilization patterns, taxonomic characters, plasmid content, and resistance to ampicillin, streptomycin, kanamycin, tetracycline, naldixic acid, mercury, nickel, copper, cobalt, cadmium, and zinc. A cluster analysis of the substrate utilization profiles and taxonomic characters revealed that *Pseudomonas*, *Acinetobacter*, and *Acidovorax* spp. were common among the culturable isolates from the river, while *Ancylobacter aquaticus*, *Klebsiella* spp., and an unknown group of pleomorphic Gram-negative methylotrophs were common among the culturable isolates from the mill treatment system. Of isolates from the settling pond and aerated lagoon, 78 and 64% carried plasmids, while only 56% of isolates from the river carried plasmids. Plasmids were significantly associated with resistance to cadmium but not with any other resistance characters. Large numbers of plasmid-carrying *A. aquaticus* strains and pleomorphic methylotrophs accounted for high plasmid incidence levels in the mill treatment system, and the ability to dechlorinate simple aliphatic substrates was found in these two groups as well as in one *Pseudomonas* strain.

Key words: pulp and paper, wastewater treatment, chlorinated organics, culturable heterotrophs, methylotrophs, plasmids.

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Des échantillons d'eau prélevés d'un système de traitement des eaux usées d'une usine de blanchiment de pâte à papier ainsi que de la rivière qui alimente l'usine ont été étalés sur six milieux de culture différents. Des isolats cultivables ont été sélectionnés en vue d'établir leurs profils d'utilisation des substrats, leurs caractéristiques taxonomiques, leurs teneurs en plasmides, et leur résistance à l'ampicilline, la streptomycine, la kanamycine, la tétracycline, l'acide naldixique, le mercure, le nickel, le cuivre, le cobalt, le cadmium, et le zinc. Des analyses de groupe des profils d'utilisation des substrats et des caractères taxonomiques ont révélé que des espèces de *Pseudomonas*, *Acinetobacter* et *Acidovorax* se trouvaient parmi les isolats cultivables provenant de la rivière, tandis que l'*Ancylobacter aquaticus*, des espèces de *Klebsiella*, ainsi qu'un groupe inconnu de méthylotrophes pléiomorphes Gram négatifs, étaient parmi les isolats cultivables provenant du système de traitement d'eau de l'usine. Parmi les isolats prélevés de sédiments d'étangs et de lagunes oxygénées, 78 et 64% se sont avérés porteurs de plasmides, comparativement à 56% seulement des isolats provenant de la rivière. Les plasmides se sont révélés significativement associés à la résistance au cadmium, mais non à toute autre forme de résistance. Dans le système de traitement des eaux de l'usine, les souches d'*A. aquaticus* ont été pour un grand nombre porteuses de plasmides, et les méthylotrophes pléiomorphes ont eu les niveaux d'incidence plasmidique les plus élevés; d'autre part, l'aptitude à déchlorurer les substrats aliphatiques simples était présente chez les deux groupes, ainsi que chez une souche de *Pseudomonas*.

Mots clés : pâte et papier, traitement d'eaux usées, substances organiques chlorurées, hétérotrophes cultivables, méthylotrophes, plasmides.

[Traduit par la rédaction]

Introduction

The release of chlorinated organic compounds to the environment is of concern because many of these compounds are persistent, bioaccumulable, and toxic. The pulp and paper industry is a major source of chlorinated organics worldwide, since pulp bleaching usually involves the oxida-

tion of wood pulp with elemental chlorine or chlorine dioxide. It is estimated the industry collectively discharges 250 000 t of organically bound chlorine annually to fresh and marine waters (Kringstad and Lindstrom 1984). Wastewater from the production of bleached kraft pulp contains sugars, polysaccharides, organic acids, resin acids, and lignin transformation products and a variety of chlorinated derivatives, 200 of which have been identified (see Table 1 and Sunito *et al.* 1988; McKague and Kringstad 1989). Measurements of the organically bound chlorine (measured as total

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TABLE 1. Characteristics of mill effluent

Prior to treatment	
Biological oxygen demand ^a	204 mg/L
pH ^a	6.6
Temperature ^a	32°C
Organically bound chlorine ^a	
During softwood pulp production	38.5 mg/L
During hardwood pulp production	13.9 mg/L
Chlorinated organics ^b	
Total acids	5.0 (mg/L max)
Total phenolics	1.0 (mg/L max)
Total aldehydes, ketones, lactones	5.0 mg/L
High molecular weight lignin derivatives	40.0 (mg/L max)
After treatment ^c	
Biological oxygen demand	34.3 mg/L
pH	7.2
Total phosphorus	2.0 mg/L
Total Kjeldahl nitrogen	1.9 mg/L
Ammonia-ammonium	0.9 mg/L
Chlorinated organics	
Adsorbable organic chloride	25.4 mg/L
Chlorodehydroabietic acid	80 µg/L
Dichlorodehydroabietic acid	100 µg/L
2,4-Dichlorophenol	3.8 µg/L
2,4,5-Trichlorophenol	7.5 µg/L
1,2,3-Trichlorobenzene	0.8 µg/L
Metals	
Aluminum	1.2 mg/L
Copper	4.2 µg/L
Zinc	107.8 µg/L

^aTwelve-month averages; S.N. Liss, unpublished data.

^bCalculated from data in Kringstad and Lindstrom (1984) and the estimate that the mill produces 1000 t of pulp per 100 000 m³ of water discharged.

^cAverage concentrations detected in final effluent in the period January 1 to June 30, 1990; from Ontario Ministry of the Environment 1991.

adsorbable organic halogen (AOX) (Odendahl *et al.* 1989)) in kraft mill effluents show that a sizeable fraction of the carbon compounds in these effluents is chlorinated (Table 1).

Aerated stabilization basins or lagoons are traditionally employed to reduce the levels of suspended solids and biological oxygen demand (BOD) of the effluent prior to discharge to receiving waters. They consist of a vigorously aerated water column and an anoxic sludge layer and they perform well with respect to suspended solids and BOD reduction. However, many pulp and paper mills are relying on the same technology to reduce the toxicity of the effluent to fish and the overall levels of chlorinated organics. The performance of the secondary treatment lagoons in this regard is quite inconsistent (Ontario Ministry of the Environment 1991; Tomar and Allen 1991). Studies have established that a substantial fraction (10–50%) of the organically bound chlorine is removed in these treatment systems (Aprahamian and Stevens 1990; Bryant *et al.* 1987; Gergov *et al.* 1988; Lindstrom and Mohamed 1988; Tomar 1991; Tomar and Allen 1991) but the mechanisms of removal are unclear. Biosorption plays an important role, and mass balance studies suggest that mineralization of chlorinated organics does take place (Bryant *et al.* 1988; Chernysh *et al.* 1993; Tomar 1991). However, very little is known about the bacterial community operating in these systems. Improvements in the reduction of toxicity and the removal of chlorinated organics from bleach kraft pulp and paper mill

effluents require a better understanding of the bacterial population active in these traditional "black box" systems. Liss and Allen (1992) surveyed three kraft bleach mill lagoons in Northern Ontario and showed that aerobic and facultatively anaerobic heterotrophs dominated the culturable community of these systems. However, to date no report has looked at the taxonomic makeup or substrate utilization profiles of bacteria found in these communities, nor identified any bacterial species that may be involved in the degradation of chlorinated compounds.

In this study we identified the bacteria that make up the bulk of the culturable heterotrophic community and characterized these bacteria with respect to plasmid incidence, resistances to heavy metals and antibiotics, and substrate utilization profiles. We sampled the clarifier, aerated lagoon, and settling pond of a bleach kraft mill treatment system and contrasted the culturable heterotrophic community with that of the community from the influent river water to pinpoint bacterial species and community characteristics selected for in the mill wastewaters. We found that the dominant heterotrophic bacteria in the wastewaters are capable of dechlorinating simple aliphatic compounds and that plasmids are found in the majority of strains isolated from this system.

Materials and methods

Sampling location

The Canadian Pacific Forest Products kraft mill at Dryden is located on the banks of the Wabigoon River in Dryden, Ontario. Flowing through Canadian Shield country, the upstream river is typical for the area, being oligotrophic with a pH of 6.5–7.5. Water from the river is used in the log-debarking, pulping, pulp-chlorination, and paper-making processes in the mill. The mill bleaches approximately 1000 air-dry t of pulp/day with elemental chlorine and 5% chlorine dioxide. Wastewater (100 000 m³/day) from all processes is diverted to a primary clarifier for the settling of solids before being piped to a 23.5 ha aerated stabilization basin (ASB) where the reduction of BOD, suspended solids, and acute toxicity is designed to take place (retention time 7 days). Aeration is driven by 24 floating platform jet aerators. Temperatures reach a high of 25°C in July and a low of 13°C in January (Liss and Allen 1992). Separated from the ASB by large log booms is a final settling pond where no aeration takes place and accumulated biomass is allowed to settle (retention time 2 days). Odourless gas production is visibly vigorous in this pond: the fertilization of the effluent with ammonium nitrate and redox measurements taken in this pond (Liss and Allen 1992) are consistent with the hypothesis that high rates of denitrification are responsible for this gas production. Further details on the mill treatment system are given in Tomar and Allen (1991).

Media

Six media were used for the isolation of bacteria from the samples; five of these were 1.7% agar supplemented with various solutions: (i) A, mineral medium A lacking additional carbon sources (Wyndham 1986); (ii) R, river water (sampled May 28, 1991); (iii) CPS, casitone-peptone-starch (see Fulthorpe and Wyndham 1991); (iv) CE50, medium A made with 50% clarifier effluent (CE) in place of distilled water; (v) CE90, medium A made with 90% CE. The sixth medium was SPACE, standard plate-count agar supplemented with 30% by volume of effluent from the mill clarifier taken at the time of the sample.

Isolates originating on SPACE were purified on SPACE, those on A and R were purified on 0.1% tryptone – yeast extract agar, and those on CPS, CE50, and CE90 were purified on CPS. After purification all isolates were frozen in 20% glycerol at –20°C and

tested for growth on SPACE, CPS, TSA (trypticase soy agar), and one-tenth strength TSA and maintained as patches on all four media.

Sample collection and treatment

On May 28, 1991, samples from the upstream portion of the Wabigoon River, the ASB, and the settling pond of the treatment system were taken using sterile plastic vials. Samples were placed in a cooler and transported the same day to the laboratory in Toronto where they were stored at 4°C overnight. Samples were diluted and spread plated on six different media (A, R, CE50, CE90, CPS, and SPACE) and incubated at room temperature for 3 weeks. At that time, colony-forming units (cfu) were counted and a total of 163 colonies were picked from the plates that were judged to be representative of all sampling sites and colony types on each medium.

Throughout the winter of 1990–1991, samples of ASB fluid and clarifier fluid were shipped from the Dryden mill to Toronto for enumeration studies. Samples collected on March 21, 1991, were plated on CE50 medium and incubated at room temperature for 3 weeks. Colonies were picked from these plates, purified, and maintained. These 33 isolates were screened for plasmid content and other characteristics along with the 163 colonies described above and were included in all tests.

Finally, of the clarifier and ASB samples shipped throughout the year, a subset of isolates derived from colonies enumerated on SPACE agar was screened for plasmids using alkaline lysis (Gilbride and Liss 1991). These isolates were all rescreened using the in-well lysis method described below and were further characterized. Information derived from these isolates was included in the cluster analysis of the isolates and in tests of association between plasmids and resistance characters, but it was not used to compare levels of plasmid incidence between environments.

Plasmid screening

One hundred and ninety-five isolates were screened for plasmids using the method of Wheatcroft *et al.* (1990) designed to screen megaplasmids in *Rhizobium* species. Isolates were inoculated into 5-mL CPS broth cultures and incubated with shaking at 25°C to an absorbance at 620 nm of 0.1 or more. Most strains achieved this turbidity overnight or after 2 days; isolates that failed to grow on CPS broth within 2 days were grown in one-fifth strength nutrient broth. A volume equivalent to 1 mL of 0.2 absorbance units was used in the plasmid screen. Briefly, cells were washed in a dilute solution of sodium sarcosyl, treated with lysozyme, and loaded into the wells of a 0.8% agarose gel. The gel contained a band of sodium dodecyl sulfate (SDS) 1 cm behind the loading wells and cells lysed as this SDS moved forward with the electric current. If the cells lysed prior to being loaded, the lysate was aspirated with a pipette tip to reduce the viscosity and then loaded. Isolates that did not lyse at all were rerun using the same lysis agent supplemented with 2 mg/mL mutanolysin. Gels were run using pBRC60, an 85-kb plasmid, as a molecular weight marker. Plasmids were classified as small if they migrated in front of the band of chromosomal fragments.

Antibiotic and metal resistance profiles

Resistances of bacterial isolates to antibiotics and metal ions were determined by testing for growth on standard plate-count agar (SPA) supplemented with the test chemicals. Solutions of antibiotics and metal ions were filter sterilized (0.22 µm) before addition to sterile molten medium prior to pouring the plates. Antibiotics tested included ampicillin, kanamycin, streptomycin, tetracycline, and nalidixic acid. These were added to media to give a final concentration of 50 µg/mL, with the exception of ampicillin, which was added at a level of 60 µg/mL. Heavy-metal agar plates were prepared at three different concentrations as follows: cadmium sulfate (2×10^{-4} M, 1×10^{-4} M, 1×10^{-3} M), cobaltous sulfate (1×10^{-3} M, 5×10^{-3} M, 1×10^{-2} M),

FIG. 1. Log of the colony-forming units per millilitre as determined on each of six media for each of three sites sampled. Abbreviations are given in text. Error bars are standard deviations.

cupric sulfate (1×10^{-3} M, 5×10^{-3} M, 1×10^{-2} M), mercuric chloride (5×10^{-5} M, 1×10^{-4} M, 5×10^{-4} M), nickel chloride (1×10^{-3} M, 5×10^{-3} M, 1×10^{-2} M), and zinc sulfate (5×10^{-3} M, 1×10^{-2} M, 5×10^{-2} M). Plates were inoculated by streaking an inoculum from an overnight culture over a 2-cm line and incubated at room temperature (25°C) for 5 days. SPA plates without the antibiotics or metals were inoculated with the test strains as controls.

Isolate characterization and cluster analysis

All strains were tested for their Gram stains, oxidase and catalase reactions, and pigment formation on SPACE medium. In addition they were given scores of 0 or 1 for their insensitivity to lysozyme, slow growth (>2 days in CPS or nutrient broth (NB)), ability to grow on TSA agar, and ability to grow on SPA medium in the absence of oxygen. Wet mounts of strains were examined under phase-contrast microscopy prior to Biolog fingerprinting as an aid to their identification.

Substrate utilization profiles were obtained for all viable isolates using GN microplates (Biolog, Hayward, Calif.). Isolates were streaked onto TSA and incubated at 30°C for 18 h before fingerprinting. Slow-growing strains were left up to a maximum of 72 h prior to fingerprinting. Results of GN microplates were recorded after 24 h, or after a maximum of 72 h if they were slow to develop. Binary scores for the results of the antibiotic and metal resistance tests, for the Biolog results, and for the remaining characterization tests performed were entered into a BASIC program that calculated Jaccard similarity coefficients between each of the strains (program available on request from primary author). The similarity matrix generated was transformed into a distance matrix and used as input for the average linkage cluster procedure of SAS (Statistical Analysis System, release 6.03, SAS Institute Inc., Cary, N.C.).

Degradation of aromatic substrates and simple chlorinated organics

Biolog MT microplates were used to determine the capability of representative isolates to utilize lignin derivatives and a limited number of chlorinated organics as sources of carbon. Ten millimolar aqueous stock solutions of phenol, vanillin, benzoate, guaiacol, coumaric acid, ferulic acid, 2-chloroethanol, 2-chloroacetate, and 3-chlorobenzoate were made and filter sterilized through a 0.22-µm filter. Isolates were grown on nutrient agar for 24 h prior to testing. Fresh growth was rolled onto a sterile cotton swab and resuspended in sterile distilled water to a density that corresponded to an absorbance at 620 nm of 0.180. The cell suspension (120 µL) was pipetted into the wells of an MT plate and supplemented with 30 µL of the test carbon source solution,

giving a final test concentration of 2 mM. Each strain was tested on acetate and glucose as a positive control, and on water as a negative control.

Strains giving positive reactions in 2-chloroethanol wells were tested for choride release from this substrate in broth culture consisting of medium A supplemented with 5 mM 2-chloroethanol, 0.1% yeast extract, biotin (0.5 µg/L), and thiamine (0.25 µg/L). Strains were inoculated into 5 mL chloroethanol broth in test tubes and incubated at room temperature for 7 days. Free chloride ions were detected using an Orion chloride electrode. Chloride concentrations in inoculated cultures were compared with uninoculated controls. Growth of the strains was detected by reading the absorbance at 620 nm.

Statistical analysis

Characterization tests, plasmid screen results, and resistance profiles were entered into a data-base file. A dBase IV program was written to test associations between plasmid incidence and other characteristics using the G-test of independence (Sokal and Rohlf 1981).

Differences in the proportions of plasmid-bearing bacteria between sites were tested by comparing the difference in the proportion to the normal distribution using the test statistic

$$(p_2 - p_1) / \sqrt{[(\pi(1 - \pi)(1/n_1 - 1/n_2))]}$$

where n_1 and n_2 are the numbers of isolates at sites 1 and 2, p_1 and p_2 are the corresponding proportions of isolates with plasmids, and π is the proportion of isolates with plasmids in both samples together (Dixon and Massey 1969).

Results

Isolation of bacteria

Culturable bacteria were three orders of magnitude more abundant in the ASB and the settling pond systems than in the river (Fig. 1). Within each location, there were no significant differences between the cfu counts derived from each of the six media that were used. Bacteria isolated from the river environment were taken from agar plates of a 100-µL sample of a 10^{-2} or 10^{-3} dilution of the original samples; those from the mill treatment system were taken from a 100-µL sample of a 10^{-5} or 10^{-6} dilution of the originals. River isolates were thus originally present at concentrations equal to a minimum of 1×10^3 to 1×10^4 cells/mL; those from the treatment system were present at a minimum of 1×10^5 to 1×10^6 cells/mL. Therefore, each isolate represented at least 1–10% of the culturable population.

Cluster analysis

The Biolog GN microplate data base contains the fingerprints of over 500 species of bacteria, but the majority of the bacterial strains from the environments sampled did not match known fingerprints. As an aid to grouping the isolates obtained from the four study sites, results of the Biolog fingerprints and other taxonomic tests were used to calculate a matrix of phenotypic similarity coefficients. An average linkage cluster analysis of these coefficients yielded a dendrogram depicting the degree of phenotypic relatedness

TABLE 2. Distribution of isolates among phenetic groups

	Number of isolates found in ^a :			
	River	Clarifier	ASB ^b	Pond
<i>P. stutzeri</i>	3 (1)	2 (2)	3 (3)	1 (1)
<i>Acinetobacter</i>	3 (1)	0	0	1 (1)
<i>Pseudomonas</i>	3 (1)	4 (3)	1 (0)	0
<i>Comamonas</i>	0	2 (2)	1 (1)	0
<i>Klebsiella</i>	0	4 (4)	7 (5)	0
<i>Ancylobacter</i>	0	0	17 (16)	12 (11)
Cluster C	0	0	9 (4)	7 (7)
Intermediate	7 (4)	4 (3)	17 (10)	8 (6)
Various	8 (6)	1 (1)	12 (10)	11 (7)
Unreactive	12 (5)	1 (1)	10 (4)	8 (4)
Nonviable	8 (7)	0	3 (3)	3 (3)
Total	44 (25)	18 (16)	80 (56)	51 (40)

^aValues in parentheses are numbers of isolates containing plasmids.

^bAerated stabilization basin.

between the isolates (Fig. 2). All isolates sharing a distance of 0.5 or less were assigned to phenetic clusters that correspond roughly to bacterial genera. Phenetic clusters were formed by isolates identified as *Acinetobacter* spp., *Acidovorax* spp., *Ancylobacter aquaticus*, *Klebsiella* spp., *Comamonas testosteroni*, *Pseudomonas stutzeri*, and a group that included fluorescent pseudomonads such as *Pseudomonas fluorescens* and *Pseudomonas putida* as well as some strains that did not match fingerprints in the Biolog data base. These clusters and others are marked on Fig. 2 with solid lines.

A large proportion of the isolates lay outside these logical phenetic clusters. These include the 25 strains clustering to the left (top) of the dendrogram, marked as various, which are only loosely related to each other. Thirty-two strains grouping to the right (bottom) had a distance of 0.9 from all other isolates, and are marked as unreactive. They were distant from the majority of the isolates because all had fewer than three positive reactions in the GN microplates. They differed substantially from each other with respect to other traits, forming 19 phenetic clusters with similarities of 0.5 or more. We assume these strains were of various types that simply failed to react in GN microplates. A number of isolates fall between the well-defined clusters and these are classified as intermediate species throughout.

The proportion of isolates from each cluster found in each of the sites sampled is shown in Table 2. A large number of isolates from each environment fall into the intermediate, various, unreactive, or nonviable classes. The bacterial flora of the Wabigoon River was dominated by strains that were either nonviable or unreactive in Biolog GN plates. Of the viable strains that react in GN microplates, the majority did not match known fingerprints and fell into the various group. The dominant identifiable culturable isolates from the river were found in the *P. stutzeri*, *Acinetobacter*, other *Pseudomonas*, and *Acidovorax* clusters.

FIG. 2. Dendrogram showing the phenotypic relatedness of isolates from the Wabigoon River (no symbol), the mill clarifier (○), the aerated stabilization basin (■), and the settling pond (▲). Solid brackets indicate clusters of strains of distance 0.5 or less. Dotted line brackets indicate groups defined on the basis of their distance from the bulk of the strains for ease of reference. Abbreviations are as follows: Var, various strains; Com, *Comomonas testosteroni*; C, unidentified group of Gram-negative curved rods; Pstut, *Pseudomonas stutzeri* strains; Acin, *Acinetobacter* spp.; Ps, fluorescent pseudomonads and closely related strains; Acid, *Acidovorax* spp.; Kleb, *Klebsiella* spp.; Anc, *Ancylobacter aquaticus* strains; Unr, group of strains that were unreactive in GN microplates. Cluster analysis was performed on a matrix of distances (1 – Jaccard similarity coefficients) between isolates (see text).

TABLE 3. Substrates utilized by cluster C strains

Carbon source	Number of strains positive in group (total = 7)	
	Yellow pigmented	Orange pigmented
Tween 40	1	2
Tween 80	0	2
Mannose	1	0
Methyl pyruvate	7	6
Methyl succinate	7	7
Acetate	6	7
cis-Aconitate	5	5
Citrate	5	6
Formate	7	7
D-Galactonate lactone	5	5
D-Galactonate	5	0
D-Gluconate	7	6
α -Hydroxybutyrate	6	2
β -Hydroxybutyrate	7	7
γ -Hydroxybutyrate	3	3
Hydroxyphenylacetate	1	0
Itaconate	1	6
α -Ketobutyrate	1	2
α -Ketoglutarate	7	3
α -Ketovalerate	1	0
Lactate	7	6
Malonate	6	6
Propionate	6	6
Quinate	0	1
Sebacate	4	0
Succinate	6	6
Bromosuccinate	5	7
Succinamate	7	7
Glucuronamide	1	0
Alaninamide	4	0
D-Alanine	3	0
L-Alanine	5	0
L-Alanyl-glycine	1	0
L-Asparagine	1	2
L-Aspartate	4	2
L-Glutamate	7	1
L-Leucine	3	0
L-Pyrogutamate	6	5
L-Serine	0	2
L-Threonine	1	2
Thymidine	1	0
Methanol	7	0

Strains specific to mill wastewater

Strains that were isolated from the mill wastewater treatment system are marked on Fig. 2 (Wabigoon River, no symbol; mill clarifier, ○; aerated stabilization basin, ■; and settling pond, ▲). The bacteria of the treatment system overlapped with river microflora to some extent, in that strains were isolated in the *P. stutzeri* group and the other *Pseudomonas* group and many strains were intermediate to known clusters, of various types, or unreactive. However, it is clear from this figure and Table 2 that three clusters of strains were found only in the mill treatment system and not in the river environment.

The largest of these clusters is a group of *A. aquaticus* strains. Their Biolog identification was confirmed by an examination of their distinct cell morphology (forming C- to S-shaped cells on CPS or TSA medium), their pigmen-

tion (white to pale yellow-white on MS agar), their oxidase and catalase reactions (both positive), and their ability to use methanol as a sole carbon source. Similarities between *Ancylobacter* strains were low because of the high variation in their resistance characters. Fifty percent of the strains exhibited resistance to one or two of the following: nalidixic acid, ampicillin, streptomycin, kanamycin, copper, nickel, or cobalt at the concentrations tested. The other 50% of the strains did not show any resistance to the compounds tested. The majority of the *Ancylobacter* strains (69%) were isolated on media that contained some clarifier effluent (SPACE, CE50, or CE90). *Ancylobacter* strains were difficult to lyse in the plasmid screen procedures; the majority of them required mutanolysin for adequate lysis. Electrophoresis of the crude lysate of all but one of the *Ancylobacter* strains revealed the presence of 1–3 large plasmids (>85 kb).

The second cluster that was unique to the mill treatment system was that of *Klebsiella*. Isolates in this cluster were readily identified by their Biolog GN fingerprints, with some strains closely matching the substrate utilization patterns of *Klebsiella terrigena* and others those of *Klebsiella pneumoniae* ssp. *ozaenae*, while the remainder exhibited patterns intermediate to these two species. The generic taxonomic status of isolates in this cluster was confirmed by their colony morphology (white, mucoid) and by their oxidase reaction (negative). All but 2 of the 14 of these isolates contained at least one plasmid, and 7 strains harboured multiple plasmids ranging from 3 to 9 per strain. Each strain had a unique plasmid size distribution. Eleven of the 14 *Klebsiella* strains isolated were resistant to ampicillin, 4 to mercury, 6 to various combinations of zinc, nickel, cadmium, and copper, 3 to streptomycin, and 3 to nalidixic acid. Each strain was unique with respect to its complement of resistance characters, and individual resistance characters were not obviously associated with plasmids of particular sizes.

The third cluster unique to the mill treatment system is labelled as C in Fig. 2. All of the isolates in this group were isolated from either the ASB or the settling pond and they were comprised of oxidase- and catalase-positive, pleomorphic, irregularly thickened rods or L- or C-shaped cells. Cluster C isolates fell into two smaller groups that differed in their pigmentation. Strains R7 through CN8 in Fig. 2 were deep yellow, while strains SP15 through CN10 were pale orange. However, the substrate utilization profiles obtained with GN microplates were virtually identical for the two types (Table 3), and did not closely match those of any known species in the Biolog data base. The profiles were similar to those of *Methylobacterium* species, differing from *Methylobacterium* profiles by a minimum of 5 and a maximum of 10 reactions. All of the isolates could utilize formate as a carbon source and tests in broth culture showed that the yellow-pigmented strains could also utilize methanol. Their substrate utilization profile differed substantially from that of *A. aquaticus* and from those of other known aerobic, C-shaped heterotrophs such as *Spirosoma*, *Runella*, or *Flexibacter*. All of these known strains utilize sugars in preference to organic acids; in general, the cluster C strains did not utilize carbohydrates. On CPS or nutrient agar the cells resembled those of *Xanthobacter* species; however, they did not exhibit the coccoid forms, branching cells, or chains or rods exhibited by *Xanthobacter* species in various media,

TABLE 4. Isolates capable of utilizing lignin derivatives or chlorinated substrates

Substrate	Strain	Genus or group	Plasmids	Origin
Phenol	RC41.5	<i>Pseudomonas</i>	3	Clarifier
Vanillin				
Coumarate	CN19	Intermediate	1	SP
Ferulate				
Benzoate	RC41.5	<i>Pseudomonas</i>	3	Clarifier
	SP11	<i>Klebsiella</i>	9	ASB
	RC1.1	<i>Klebsiella</i>	3	Clarifier
	RC1.9	<i>Klebsiella</i>	1	Clarifier
	RC31.3	<i>Klebsiella</i>	1	Clarifier
	RD38.1	<i>Klebsiella</i>	4	ASB
	RC41.5	<i>Pseudomonas</i>	3	Clarifier
	RC42.5	<i>Comomonas</i>	2	Clarifier
	RC43.3	<i>Comomonas</i>	1	Clarifier
	R7	Cluster C	0	ASB
	R11	Cluster C	2	SP
	SP3	Cluster C	1	SP
	SP9	Cluster C	0	ASB
	CE1.4	Cluster C	0	ASB
	CN8	Cluster C	0	ASB
	CN11	Cluster C	2	SP
Chloroethanol	RC41.5	<i>Pseudomonas</i>	3	Clarifier
	A7	<i>A. aquaticus</i>	4	SP
	CE2.2	<i>A. aquaticus</i>	2	ASB
	CN13	<i>A. aquaticus</i>	1	SP
	SP22	<i>A. aquaticus</i>	2	ASB
Chloroacetate	CP13	Cluster C	1	ASB
	CF20	Cluster C	4	SP

NOTE: ASB, aerated stabilization basin; SP, settling pond.

nor did their colonies produce slime. A comparison of their characteristics to those of known methylotrophs published by Green and Bousfield (1982) shows that they differ from all strains described by these authors in their pigmentation, but are otherwise morphologically and phenotypically closest to the pink-pigmented methylotrophs represented in their analysis by cluster 10. Cluster C isolates and cluster 10 strains are both oxidase-positive pleomorphic rods, gelatinase negative, and sensitive to kanamycin and streptomycin, and they share common reactions to 27 sole carbon source tests, but differ in their reactions to 7 of these tests. Unlike cluster 10, cluster C isolates are able to use L-aspartate, L-glutamate, propionate, citrate, L-alanine, and L-leucine and are unable to use glycerol.

The yellow-pigmented cluster C strains exhibited some tolerance to heavy metals; four of the seven strains isolated were resistant to copper, zinc, or nickel. One strain exhibited resistance to streptomycin. None of the orange-pigmented strains exhibited metal tolerance, and only one expressed resistance to nalidixic acid. None of these characters were associated with plasmids in the strains. Most of the cluster C strains carried one or two large plasmids but plasmid hosts were unevenly distributed between the ASB and the settling pond environments. Of the seven yellow-pigmented strains analyzed, the four strains isolated from the ASB did not carry plasmids, while the three taken from the mill settling pond carried one or two large plasmids. Of the seven orange-pigmented strains analyzed, the three taken from the ASB carried fewer plasmids (two carried one large plasmid each, one carried two large plasmids) than did

FIG. 3. Percentage of isolates at each site containing plasmids.

those from the settling pond (one harboured one large plasmid, two harboured two large plasmids, and one carried three large plasmids and one small plasmid).

Degradation of aromatic substrates and simple chlorinated organics

The majority of strains, representative of each of the major clusters, were assayed in Biolog MT substrate utilization plates for the use of aromatic substrates and the use of 2-chloroethanol, 2-chloroacetate, and 3-chlorobenzoate. The results of these tests are shown in Table 4. Benzoate utilization was found in several of the clusters, namely *Comamonas* strains, *Klebsiella* species, and cluster C organisms. The use of a greater range of aromatic substrates was present only in *Pseudomonas* strain RC 41.5, in addition to its ability to use 2-chloroethanol as a carbon source. These characteristics were not shared by the other members of the *Pseudomonas* cluster from the treatment system. The ability to use chloroethanol was also detected in 4 of the 11 *A. aquaticus* strains tested (isolates CN13, A7, CE2.2, and SP22). Two members of the cluster C group (CP13, CF20) possessed the ability to utilize chloroacetate, but not chloroethanol, as a carbon source. The ability of all strains to liberate chloride ion from chloroethanol or chloroacetate was tested in broth culture; all exhibited growth and released free chloride ions in liquid media. All strains capable of utilizing 2-chloroethanol or 2-chloroacetate possessed at least one plasmid (Table 4).

Comparison of overall plasmid incidence rates between sampling sites

A total of 193 strains were successfully screened for plasmid content and a comparison of plasmid incidence rates between the environments sampled was carried out using only those strains isolated on March 21 or May 28 (i.e., those sampled randomly). There was a significantly higher number of plasmid-carrying isolates from the pond than from the river (78.4% versus 56.2%, $p < 0.05$). ASB isolates had a higher plasmid incidence (64.1%) than those from the river, but this difference was not statistically significant. Multiple plasmids per cell tended to be more common in the mill treatment system (Fig. 3). Large plasmids were the rule in all environments; small plasmids were observed but they were relatively rare (Table 5).

TABLE 5. Plasmid and resistance frequencies in sites sampled

Character	River	ASB	Pond
Resistance to:			
Antibiotics			
Ampicillin	52.1	29.6 ^a	15.7 ^b
Tetracycline	4.2	1.6	3.9
Streptomycin	16.7	23.4	21.6
Kanamycin	14.6	6.3	11.8
Nalidixic acid	22.9	18.8	31.4
Any 5 above	2.1	0.0	0.0
Any 4 above	2.1	1.6	3.9
Any 3 above	10.4	9.4	5.9
Any 2 above	16.7	12.5	15.7
Any 1 above	27.1	20.3	19.6
Metals			
Mercury	10.4	4.7	2.0
Nickel	8.3	6.3	13.7
Copper	4.2	12.5	3.9
Cadmium	0.0	1.6	2.0
Cobalt	0.0	1.6	7.8
Zinc	0.0	4.7	3.9
Any 3 metals	0.0	3.1	0.0
Any 2 metals	4.2	6.3	5.9
Any 1 metal	14.6	9.4	21.6
Carrying plasmids	56.2	64.1	78.4 ^a
Large (> 25 kb)	52.1	60.9	74.5 ^a
Small (< 25 kb)	10.4	10.9	13.7

NOTE: ASB, aerated stabilization basin.

^aSignificantly different from river isolates, $p < 0.05$.^bSignificantly different from river isolates, $p < 0.001$.

Ancyllobacter aquaticus strains accounted for 27% of the plasmid-bearing strains in the settling pond and 31% of those in the aerated stabilization basin, so it is clear they have a major impact on the overall plasmid incidence levels. Cluster C strains accounted for 17.5% of the plasmid-bearing strains in the settling pond.

When grouped overall, strains isolated from nutrient-deficient media (mineral medium A agar and river water agar) had the lowest plasmid incidence levels, whereas those isolated on rich media (CPS, SPACE) or on media containing a high clarifier effluent content (CE50, CE90) yielded isolates with high plasmid incidence levels (Fig. 4). Overall, CPS yielded strains with the highest proportions of both large and small plasmids. The proportion of plasmids in pigmented strains (66.1%) was not significantly different from that in nonpigmented strains.

Overall antibiotic and metal resistance profiles

Table 5 illustrates the frequency of isolates exhibiting resistance to each of five antibiotics and six heavy metals. Resistance to ampicillin was extremely common in isolates from the river, present in half of the isolates assayed, but significantly less common in both the ASB and pond isolates. Resistance to nalidixic acid, streptomycin, and kanamycin was also relatively common, while resistance to tetracycline was quite rare. There were no significant differences between the sites with respect to frequency of resistance to these four antibiotics. Multiple resistance tended to be more prevalent in the river isolates.

Resistances to the heavy metals at various concentrations are shown in Table 6. All isolates were resistant to 1 mM

FIG. 4. Percentage of isolates on each of six media that carried large, small, or any plasmids. Data from all of the environments sampled are pooled.

nickel agar, and 10 mM nickel agar precipitated salts, so only the results for 5 mM nickel are presented. Resistance to mercury and cadmium at the lower concentrations tested was prevalent in the river-water isolates. The isolates from the clarifier were enriched for resistance to mercury, cadmium, and zinc. The inclusion of clarifier effluent in isolation media appeared to select for some metal-resistant strains. While only 65.5% of all strains were isolated on media that contained clarifier effluent (CE50, CE90, or SPACE), 93.7% of nickel-resistant strains originated on these media, as did 91.7% of the copper-resistant strains and 83.3% of the zinc-resistant strains. Resistance to the highest concentrations of cobalt, cadmium, and zinc tested was only evident in ASB and pond isolates.

Associations between characters

Associations between resistance characters and the presence of plasmids was tested for each of the five antibiotics and six heavy metals, and for multiple combinations of these. For this analysis only resistances to the highest concentration of metals tested were scored as positive. There was a significant negative association between kanamycin resistance and the presence of plasmids ($p < 0.01$). There was a weak positive association between resistance to nalidixic acid and the presence of plasmids ($p < 0.10$). There was a weak association between the possession of strong resistance to two or more metals and the presence of small plasmids ($p < 0.10$). There was a strong positive association between cadmium resistance and the presence of small plasmids ($p < 0.005$).

Discussion

We have described, for the first time, the dominant culturable bacteria from a bleach kraft mill treatment system. Forty-one percent of the strains isolated from the aerated stabilization basin over the course of a year were either *Klebsiella* species, *A. aquaticus* strains, or members

TABLE 6. Levels of resistance to heavy metals

Metal	Concentration	Percentage of isolates resistant in:			
		river	clarifier	ASB	pond
Hg	5×10^{-5} M	29.2	50.0	23.4	19.6
Hg	1×10^{-4} M	22.9	44.4	21.9	17.6
Hg	5×10^{-4} M	10.4	5.6	7.8	2.0
Ni	5×10^{-3} M	8.3	5.5	6.3	13.7
Cu	1×10^{-3} M	4.2	5.6	15.6	11.7
Cu	5×10^{-3} M	4.2	0.0	12.5	9.8
Cu	1×10^{-2} M	4.2	0.0	6.2	3.9
Co	1×10^{-3} M	8.3	5.6	3.1	7.8
Co	5×10^{-3} M	0.0	0.0	1.6	7.8
Co	1×10^{-2} M	0.0	0.0	1.6	7.8
Cd	2×10^{-4} M	20.8	56.6	9.4	2.0
Cd	1×10^{-4} M	10.4	38.9	7.8	2.0
Cd	1×10^{-3} M	0.0	16.7	1.6	2.0
Zn	5×10^{-3} M	6.3	11.1	4.7	3.9
Zn	1×10^{-2} M	0.0	11.1	4.7	3.9
Zn	5×10^{-2} M	0.0	11.1	4.7	3.9

NOTE: ASB, aerated stabilization basin.

of an unknown group of pleomorphic methylotrophs referred to here as the cluster C group. Thirty-seven percent of strains isolated from the mill settling pond were either *A. aquaticus* or cluster C organisms. Clearly these three groups are important to the transformation of pulp and paper mill wastes. In addition, we have demonstrated that members of two of these major groups have the ability to dechlorinate simple chlorinated aliphatic molecules, and this constitutes the first report of the isolation of bacteria with dechlorination ability from a pulp mill treatment system.

This is only the second report of dechlorination ability in *A. aquaticus* strains. Strains isolated from the Rhine River and capable of degrading 1,2-dichloroethane were recently described by van den Wijngaard *et al.* 1992. *Ancylobacter aquaticus* has been characterized as an oligotrophic methylotroph (Raj 1989) because it can fix carbon dioxide under low nutrient conditions in reduced environments; however, this study and other references to its occurrence reveal that the organism has a strong affinity for carbon-rich wastewaters. Staley and Konopka (1984) report that *A. aquaticus* strains have been isolated from pulp mill lagoons, although no original studies are cited by them. Raj (1989) reviews the habitats in which *Ancylobacter* has been found and these include wood-lake ponds, aerated lagoons of a Weyerhaeuser pulp mill, the surface of granular activated carbon in a water treatment facility, and an activated sludge reactor used to treat spent cutting fluids, which include chlorinated paraffins. Strand *et al.* (1984) isolated *A. aquaticus* strains capable of degrading glucoisosccharinic acid, a normally recalcitrant substrate, from the influent and effluents of an aerobic wastewater treatment pond at a Pacific Northwest kraft paper mill. The abundance of *A. aquaticus* in pulp mills is attributable to the fact that they utilize galactose, glucose, arabinose, acetate, formate, and methanol, and all of these are found in the wastewaters of bleached kraft pulp mills (McKague *et al.* 1989). However, it is clear from this study that this species is also capable of exploiting simple chlorinated substrates that are present in the mill effluents.

The pleomorphic rods referred to here as the cluster C group are extremely important in that they utilize chloroacetate, are abundant in mill treatment systems, and to some extent utilize aromatic substrates. They share with *A. aquaticus* strains the ability to use methanol or other single carbon substrates such as formate. Methanol is produced in abundance in the kraft pulping process (Rydholm 1965), so it is not surprising that methylotrophic organisms should achieve high populations. The work here has focussed on the presence of facultative methylotrophs; however, obligate methylotrophs are also abundant (R.R. Fulthorpe, unpublished observation) and may possess broad substrate range methane monooxygenases. Many organisms possessing methane monooxygenases are now known to dechlorinate chloro-aliphatics (Oldenhuis *et al.* 1989; Fogel *et al.* 1986; Strand and Shippert 1986; Wilson and Wilson 1985).

Klebsiella species are common in the first portion of the treatment system. *Klebsiella* species are found in paper-making machinery, forming slimy biofilms that afford them some protection from antibacterial agents used (M. Salkinoja-Salonen, personal communication). The substrate utilization profile of *Klebsiella* species is extremely broad, but its apparent preference for the clarifier environment over the latter stages of the treatment process suggests that it is selected for starch utilization in the paper-making process and uses only labile substrates in the wastewater. However, *Klebsiella* strains can metabolize unusual aromatic substrates, such as benzonitrile (Nawaz *et al.* 1992) and possess some unusual hydroxybenzoate hydroxylases (Suarez *et al.* 1991), so their role in transforming aromatics in the mill effluents merits further study.

The broadest utilization of aromatic compounds was exhibited by a *Pseudomonas* strain (RC41.5), which could also utilize 2-chloroethanol as a carbon source. Unfortunately, while *Pseudomonas* strains were well represented in the clarifier, only one strain was isolated from the ASB and none from the final settling pond. This might have important implications for "bioaugmentation" studies such as that described by Christiansen *et al.* (1992) that seek to improve overall BOD removal in mill effluents by the addition of *Pseudomonas* species that may not persist in this environment.

This work shows that plasmid hosts are selected for in the mill treatment system, especially towards the latter stages. Plasmid incidence levels in culturable bacteria from diverse aquatic environments are remarkably similar in spite of differences in plasmid screening techniques: 34% in a dystrophic lake (Schutt 1989); 31% in antarctic waters (Kobori *et al.* 1984); 4–55% in the marine air–water interface (Hermansson *et al.* 1987); 13–47% in clean and sewage-contaminated ocean waters (Baya *et al.* 1986); 21–66% in marine *Vibrio* species (Hada and Sizemore 1981); 33% in marine subsurface sediments (Frederickson *et al.* 1988); 15% in river sediments (Day *et al.* 1988); and 24–32% in a quite different environment, cores of Kentucky soil (Wickham and Atlas 1988). Only one reported plasmid incidence level is comparable to that observed in the settling pond in this study (78%): plasmids were found in 79% of the bacteria isolated from the effluent of a wastewater treatment plant that handled 65% pharmaceutical and industrial waste (Baya *et al.* 1986). Several studies have tested the hypothesis that

bacterial plasmid incidence levels are higher in environments contaminated by hydrocarbons and other contaminants than in clean sites. Two such studies support this hypothesis (Hada and Sizemore 1981; Baya *et al.* 1986), while others present supportive "tendencies" in their data that are not statistically significant (Ogunseitan *et al.* 1987; Hermansson *et al.* 1987). Four studies show that overall plasmid incidence levels do not increase with degree of contamination (Leahy *et al.* 1990; Day *et al.* 1988; Wickham and Atlas 1988; Burton *et al.* 1982). Our data show that plasmid-encoded characteristics are important in the mill treatment system bacterial flora, more so than in the bacterial community of the influent river water; however, we have no strict control environment against which to compare the unique conditions of a pulp mill wastewater treatment system. What is clear is that the high plasmid incidence levels in the kraft mill treatment system are attributable to the dominance of the wastewaters by *A. aquaticus* and cluster C strains. The majority of the strains isolated that were capable of dechlorinating simple aliphatic molecules were found in these two groups, and all dechlorinating strains carried plasmids. *Ancylobacter aquaticus* strains isolated from the Rhine River were recently reported to utilize 1,2-dichloroethane and 2-chloroethanol and to carry the haloalkane dehalogenase gene (*dhlA*) previously characterized in *Xanthobacter autotrophicus* GJ10 and GJ11 (van den Wijngaard *et al.* 1992; Janssen *et al.* 1989). This gene is present on a large plasmid in *X. autotrophicus* (Tardif *et al.* 1991). Gerstenberg *et al.* (1982) report two large plasmids in *A. aquaticus*, but at this time these plasmids must be classified as cryptic, since no functional genes have been identified. Raj (1989) has speculated that hydrogen oxidation genes might be carried on them because a plasmid of exactly the same size is found in the lithoautotroph *Alcaligenes latus*, but this has not been confirmed. Our study shows that 1–4 large plasmids were readily visualized in plasmid screens of all pulp mill *A. aquaticus* strains. The four chloroethanol-degrading *A. aquaticus* strains isolated in this study each carried quite different complements of plasmids. Both of the orange-pigmented pleomorphic rods that utilized chloroacetate also carried plasmids. Current work is examining the possibility that the dechlorination genes are plasmid borne.

Apart from a significant association between cadmium resistance and low molecular weight plasmids, neither heavy metal resistances nor antibiotic resistances appear to be plasmid borne in this system, indicating that other characters, such as degradative genes, might explain their importance in the system. These results are in agreement with the findings of Kobori *et al.* (1984), Hermansson *et al.* (1987), Belliveau *et al.* (1991), and Wickham and Atlas (1988), all of whom noted no statistically significant correspondence between plasmid frequencies and resistances to common antibiotics and heavy metals in various environments.

In our study, the isolation media that yielded isolates with the highest plasmid incidence levels were rich media, in contrast to the findings of Kobori *et al.* (1984) who noted that nutrient-deficient media yielded isolates from the Antarctic with the highest plasmid incidence levels. In this study, Wabigoon River water agar (medium R) was a poor medium for recovering plasmid-bearing strains from the Wabigoon River, as was the nutrient-deficient medium A. The best

results were obtained with the nonselective medium CPS (casitone-peptone-starch). Others have noted that this medium yields the highest cfu counts from aquatic environments (Burton *et al.* 1982). In this work it did not produce higher cfu counts than any other medium used, but it may have been the least stressful medium of the six. Bacteria growing on CPS might have retained plasmids that were lost on medium A agar or river water agar because of the energy costs involved. The inclusion of clarifier effluent in medium A agar improves the cfu counts and the recovery of plasmid-bearing bacteria, but it does not make it any more selective for plasmid-bearing bacteria than the very general CPS agar.

The primary clarifier selects for heavy metal resistance: bacterial populations isolated from the clarifier were enriched for mercury, cadmium, and zinc resistances; the inclusion of clarifier effluent in isolation media selected for bacteria resistant to nickel, copper, and zinc. These observations suggest that the heavy metals are present in apparently high concentrations in the clarifier effluent. However, the concentrations of heavy metals are not high in effluents of either treated or untreated kraft pulp mills (Ontario Ministry of the Environment 1991). It may be that after the acidic bleaching process, metals are solubilized from soil and debris but are precipitated quickly in a clarifier.

In conclusion, this study has demonstrated that the culturable heterotrophic bacteria from a wastewater treatment system of a bleach kraft pulp and paper mill are dominated by populations of *A. aquaticus*, a group of unidentified pleomorphic methylotrophic rods, *Klebsiella* species, and *Pseudomonas* species. The ability to dechlorinate aliphatic compounds was detected in *A. aquaticus* strains, in orange-pigmented pleomorphic rods, and in one *Pseudomonas* strain. The ability to utilize aromatic lignin derivatives other than benzoate was found only in a *Pseudomonas* strain, while the ability to utilize benzoate was widespread in *Klebsiella* strains, in *Comamonas* strains, and the yellow-pigmented methylotrophic pleomorphic rods. The majority of isolates harboured large plasmids, with the incidence of plasmids in isolates increasing to 78% in the latter stages of the treatment process. This high plasmid incidence rate is largely due to the presence of *A. aquaticus* strains and the unidentified pleomorphic rods. The role of these species in the degradation and transformation of chlorinated organics in the treatment system merits further study.

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