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## Identification of bacteria contaminating pulp and a paper machine in a Canadian paper mill

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**Abstract** Over 100 bacteria from pulp and slime samples in a Canadian paper mill were identified by partial sequencing of their 16S rDNAs. Seventy-one percent of the isolates could be assigned to a bacterial genus with a high level of confidence. Another 12% exhibited at least 95% similarity within their 16S rDNA sequence with unidentified organisms that originate from warm or wet environments. *Pseudomonas*, *Bacillus*, and *Pseudoxanthomonas* isolates were represented at a relatively high proportion in both pulp and slime samples. This is the first time that *Pseudoxanthomonas* strains have been isolated from pulp and slime samples on a paper machine.

**Keywords** 16S rDNA · Biofilm · Pulp · Slimes · Paper machine · *Pseudoxanthomonas*

### Introduction

Paper machines offer an environment suitable for microbial growth because of their operating temperatures (30–50 °C), the white water pHs (5–8), and the presence of cellulose, starch, and other nutrients. Bacteria are constantly introduced in paper machines via fresh-water, fibers, filling agents, and recycled pulp. While free-living bacteria are not necessarily deleterious for machine operation, the formation of bacterial slimes needs to be controlled. Since the presence of slimes on machinery affects paper quality as well as the runability of machines [10], papermakers utilize biocides to control bacterial growth in their production systems. These biocides are periodically added to the white water and are not specific to slime-producing bacteria.

Over the last two decades, studies dedicated to the identification of bacteria in pulp samples and slimes present on the surfaces of paper mill machines have been conducted in several countries. Bacterial communities associated with paper mills were studied using techniques such as microscopic observation [13,14,16,17,18], physiological and biochemical characterization [7,11,13,14,17], fatty acids analysis [13,16,17,18], and sequencing of the 16S rDNA gene of bacterial isolates [13,14,16]. These studies demonstrated that the bacterial communities in paper machines depend on the paper machine environment [7,8,13,16], on the papermaking process and the additives used [7,8], on the type of paper being produced [7], and on the paper mill location [8,11]. Bacterial genera such as *Bacillus*, *Pseudomonas*, *Sphaerotilus* and *Alcaligenes* were isolated in most paper mills [3,7,8,11].

Evenleigh and Brewer [5] observed the variation of bacteria in slime accumulations in a Canadian paper mill over a 2-year period using morphological, physiological, and biochemical tests. Their study revealed that bacterial flora were mainly composed of pseudomonads, bacilli, and flavobacters. In spite of all the improvements of the paper fabrication processes since 1964, no other study on the characterization of bacterial contaminants on paper machines has been published in Canada.

This paper presents the first step in a study on bacterial communities associated with a Canadian paper mill. The taxonomic identity of over 100 bacterial isolates from slimes and pulp samples was determined by partial sequencing of their 16S rDNA. The results suggest that bacteria from the genus *Pseudoxanthomonas* represent important contaminants of the Canadian alkaline paper machine that was studied.

### Materials and methods

#### Bacterial isolates and culture media

Bacterial isolates analyzed in this study are listed in Table 1. The type strain *Pseudoxanthomonas broegbernensis* (ATCC BAA-10) was obtained from the American Type Culture Collection

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**Table 1** Bacterial isolates used in this study

Bacterial isolate	Isolation condition		
	Origin	Temperature (°C)	Medium
CA-05, CA-06, CA-08, CA-09, CA-11, CA-12, CA-13, CA-15, CA-16, CA-17, CA-20, CA-21, CA-22, CA-23, CA-25, CA-26, CA-27, CA-29, CA-30, CA-31, CA-32, CA-33, CA-34, CA-35, CA-36, CA-37, CA-38	Headbox samples	50	PCA
CA-40, CA-41	Headbox samples	50	R2A
S4-01, S4-09, S4-12, S4-15, S4-18, S4-20, S4-21, S4-22, S4-23, S4-24, S4-25, S4-26, S4-27, S4-28, S4-29, S4-32, S4-33, S4-34, S4-35, S4-37, S5-01, S5-02, S5-04, S5-09, S5-11, S5-13, S5-15, S5-16, S5-22, S5-24, S5-25, S5-26, S5-27, S5-28, S5-30, S5-31, S5-32, S5-33, S5-34, S5-35, S5-36, S5-39, S5-40, S5-41, S5-42, S5-44, S5-46, S7-02, S7-06, S7-07, S7-08, S7-09, S7-10, S7-11, S7-12, S7-13, S7-14, S7-15, S7-16, S7-17, S7-18, S7-19, S7-20, S7-21, S7-22, S7-23, S7-27, S7-28, S7-30, S7-31	Slime samples	37	PCA
S4-30, S4-31, S4-39, S4-40, S4-41, S5-38, S7-24, S5-47, S5-48, S7-32, S7-33, S7-34,	Slime samples	37	R2A

(Manassas, Va.). Bacteria were cultivated in plate count broth (PCB; 5 g tryptone, 2.5 g yeast extract, 1 g glucose per liter; pH 7.0) or in R2B (0.5 g yeast extract, 0.5 g proteose peptone N3, 0.5 g casamino acids, 0.5 g glucose, 0.3 g soluble starch, 0.3 g sodium pyruvate, 0.3 g potassium phosphate dibasic, 0.05 g magnesium sulfate per liter; pH 7.2) for 48-h at 37 °C or 50 °C. For plating, 15 g of agar per liter was added to the growth media.

#### Isolation of bacteria from pulp and paper mill slimes

Slimes and pulp samples were collected in sterile flasks from the wet-end steel surfaces and from the headbox of an alkaline printing paper machine, respectively. Bacteria were isolated from slime and pulp samples as follows. Serial dilutions of the samples were spread onto PCA and R2A plates and incubated at 37 °C (slime samples) or at 50 °C (pulp material) for 48 h. Pure cultures were obtained by a random process and serial passages of the colonies on their isolation media.

#### DNA extraction

Bacterial DNA was extracted from 48-h-old cultures in PCB. After centrifugation, the cell pellets were resuspended in one-half volume of Tris-EDTA pH 8.0. Sodium dodecyl sulfate was added to the cell suspension at a final concentration of 10% and the mixture was incubated at 50 °C for 30 min. Proteins and polysaccharides were extracted with an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1). The DNA was then recovered from the aqueous phase and dissolved in sterile distilled water according to the procedure of Merk et al. [12].

#### 16S rDNA sequencing

PCR amplification of the 16S rDNA gene fragments was carried out using BSF8/20 and BSR926/20 primers (Table 2) (BIO/CAN

Scientific, Mississauga, Canada). DNA amplification was carried out in a total volume of 50 µl containing template DNA (200 ng·µl<sup>-1</sup>), 1× PCR buffer (Amersham Biosciences, Baie d'Urfé, Quebec, Canada), 0.25 µM dNTPs, 0.2 pmol·µl<sup>-1</sup> primers and 5 U *Taq* DNA polymerase (Amersham Biosciences). PCR conditions were the following: a 5-min initial denaturation step at 94 °C, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C and 2 min elongation at 72 °C. The PCR products were immobilized on streptavidin-coated paramagnetic beads M-280 (DYNAL, Oslo, Norway) and single-stranded DNA templates for sequencing were prepared according to the manufacturer's instructions. The biotinylated strand was sequenced by the dideoxynucleotide chain-termination method using the ALFlexpress automatic DNA sequencer and the Autocycle Sequencing kit (Amersham Biosciences). DNA sequence homology searches were performed using the BLAST programs [1].

## Results

Partial 16S rDNA sequences (235–815 bp) were obtained for more than 100 bacterial isolates sampled from a Canadian paper mill. DNA sequence homology searches in databases allowed the association of 71% of our isolates with a specific bacterial genus (more than 95% sequence similarity [2]) (Table 3). Some 12% of the 16S rDNA sequences of other isolates also exhibited a level of similarity higher than 95% but with DNA sequences that belonged to unidentified microorganisms reported in the Genbank (Table 4). Almost 17% of our isolates could not be assigned to any microbial genus with a high level of confidence due to their low similarity scores. Identification efficiency was not correlated to the length of the sequences analyzed. In a previous study,

**Table 2** Oligonucleotide primers used for DNA sequencing

Primer	Nucleotide sequence	Corresponding region of the 16SrDNA <sup>a</sup>
BSF 8/20	5'-AGAGTTTGATCCTGGCTCAG-3' <sup>b</sup>	8–27
BSR 926/20	5'-CCGTCAATTTYTTTTRAGTTT-3' <sup>c</sup>	907–926

<sup>a</sup>Numbers refer to the corresponding nucleotide positions of the *Escherichia coli* 16S rRNA [4]

<sup>b</sup>Labeled at the 5' end with cyanine

<sup>c</sup>Labeled at the 5' end with biotin

**Table 3** Nearest GenBank neighbors to the bacterial isolates from this study

Isolate	Accession number (isolates)	Nearest GenBank neighbor <sup>a</sup>	Accession number (homologs)	Similarity (%)
S7-22; S7-15; S7-24 <sup>b</sup>	AF530265	<i>Acidovorax temperans</i>	AF078766	97.4–98.1
S7-09	AF530266	<i>Aeromonas salmonicida</i>	AB027006	96.5
S4-31	AF530267	<i>Allorhizobium undicola</i>	Y17047	95.3
S7-27	AY135478	<i>Azorhizophilus paspali</i>	AJ308318	96.7
S4-09	AF530268	<i>Azospirillum dobereinerea</i>	AJ238567	96.1
S5-22	AY124484	<i>Azospirillum</i> sp.	AF413109	96.8
S5-48	AY124483	<i>Bacillus flexus</i>	AB021185	99.2
S4-34	AF530269	<i>Bacillus methanolicus</i>	X64465	96.1
S4-12; S5-02 <sup>b</sup>	AF530271	<i>Bacillus</i> sp.	AF286485	95.0–97.9
S5-27	AF530270	<i>Bacillus</i> sp.	AJ276809	97.4
S7-33	AY135480	<i>Bacillus</i> sp.	AF417874	97.0
CA-09	AF530291	<i>Bacillus subtilis</i>	AB018487	97.5
S7-18	AF530273	<i>Blastobacter</i> sp.	U20772	95.8
S5-11; S5-01 <sup>b</sup>	AF530274	<i>Brevibacillus agri</i>	AB039334	98.8–99.7
S5-16	AF530275	<i>Brevibacillus</i> sp.	AF228763	97.5
S4-27	AF530276	<i>Enterobacter agglomerans</i>	AF157688	98.5
S7-16	AY124487	<i>Hydrogenophaga palleronii</i>	AF078769	96.3
S7-11	AF530277	<i>Hydrogenophaga</i> sp.	AF078768	95.5
CA-20; CA-32 <sup>b</sup>	AY135484	<i>Leptothrix</i> sp.	AF385534	95.4–96.6
S4-24	AF530278	<i>Microbacterium aurum</i>	Y17229	97.3
S4-26; S5-09; S4-21 <sup>b</sup>	AY124486	<i>Microbacterium barkeri</i>	X77446	95.1–97.2
S4-20; S7-14; S4-29 <sup>b</sup>	AF530280	<i>Microbacterium</i> sp.	AB027702	96.9–97.9
S4-23; S5-31; S5-47 <sup>b</sup>	AF530281	<i>Microbacterium testaceum</i>	AF474330	96.9–98.6
S4-18; S4-28; S5-32; S7-23; S7-30; S7-31 <sup>b</sup>	AF530272	<i>Pseudomonas alcaligenes</i>	AF094721	96.7–99.5
<b>S5-26</b>				
CA-11; CA-05; CA-08; S5-24; CA-33 <sup>b</sup>	AY124485; AF530290	<i>Pseudomonas</i> sp.	AJ278108, AF311980	98.1
		<i>Pseudomonas thermotolerans</i>		96.1–99.1
S5-25	AF530282	<i>Pseudoxanthomonas broeghermensis</i>	AJ012231	97.3
S7-07; CA-21; CA-06; CA-12; S4-01; S5-13; S5-15; S7-06; CA-22; CA-25; CA-26; CA-30; CA-31; CA-34; CA-36 <sup>b</sup>	AF530283	<i>Pseudoxanthomonas</i> sp.	AB039330	95.5–100
CA-23; CA-27; CA-29 <sup>b</sup>	AF530289	<i>Pseudoxanthomonas</i> sp.	AB039336	97.0
CA-15; CA-16; CA-17; CA-37; CA-41 <sup>b</sup>	AY135482	<i>Pseudoxanthomonas</i> sp.	AF427039	96.0–99.7
S7-10				
S4-30; S7-20 <sup>b</sup>	AF530281, AF530285	<i>Rhizobium</i> sp.	AF345551, AF345553	95.4
		<i>Rhizobium</i> sp.		98.0
S5-42	AY135479	<i>Sphingomonas subterranea</i>	AB025014	97.6
S5-34	AF530286	<i>Staphylococcus capitis</i>	AF193885	97.6
S4-25; S7-21 <sup>b</sup>	AF530287	<i>Xanthobacter flavus</i>	X94206	95.2–96.1
S7-19	AF530288	<i>Xanthobacter</i> sp.	AJ313028	96.1

<sup>a</sup>Only the organisms with at least 95% similarity between their 16S rDNA sequences were considered

<sup>b</sup>The same nearest GenBank neighbor was found for this group of isolates. Only the sequence of the first isolate of this group was submitted to GenBank

**Table 4** Bacterial isolates whose partial 16S rDNA sequence has 95% or higher similarity with that of an unidentified microbial strain

Isolate	Accession number	Nearest neighbor in GenBank		
		Provenance of the unknown organism	Accession number	Similarity (%)
CA-13	AY123960	Compost during thermophilic phase	AF213286	99.2
S4-22	AY123961	Medium containing dehydroabietic acid	AF125877	95.7
S7-02	AY123962	Metal contaminated soil	AF145849	96.9
S5-35; S5-44 <sup>a</sup>	AY123963	Biodeteriorated mural paintings	AJ315073	95.0–95.3
S5-28; S7-17 <sup>a</sup>	AY123967	Industrial waste gas biofilter	AJ318141	95.0–95.8
S7-28	AY124463	Travertine edepositional facies	AF445688	95.8
S5-38	AY123966	River Taffepilithon	AY038761	96.4
S7-13	AY123965	Aquifercontaminated with chlorinated solvent	AF050533	97.5
S7-32; S5-30; S5-04 <sup>a</sup>	AY124462	Activated sludge plant	X85208	95.2–98.2

<sup>a</sup>The same nearest GenBank neighbor was found for this group of isolates. Only the sequence of the first isolate of this group was submitted to GenBank

**Table 5** Distribution of bacteria within the pulp of the headbox and the slimes present on the wet-end steel surfaces

Genus	Proportion of isolates (%)	
	Headbox	Slime
<i>Acidovorax</i>	0	3.7
<i>Aeromonas</i>	0	1.2
<i>Allorhizobium</i>	0	1.2
<i>Azorhizophilus</i>	0	1.2
<i>Azospirillum</i>	0	2.4
<i>Bacillus</i>	3.4	7.3
<i>Blastobacter</i>	0	1.2
<i>Brevibacillus</i>	0	3.7
<i>Enterobacter</i>	0	1.2
<i>Hydrogenophaga</i>	0	2.4
<i>Leptothrix</i>	6.9	0
<i>Microbacterium</i>	0	13.4
<i>Pseudomonas</i>	13.8	9.8
<i>Pseudoxanthomonas</i>	62.1	7.3
<i>Rhizobium</i>	0	3.7
<i>Sphingomonas</i>	0	1.2
<i>Staphylococcus</i>	0	1.2
<i>Xanthobacter</i>	0	3.7
Unidentified isolates <sup>a</sup>	13.8	34.2

<sup>a</sup>The 16S rDNA sequences of these isolates did not match the corresponding sequences filed in the databases or were similar to those of microorganisms which are still lacking identification

Kataoka et al. [9] used 16S rDNA nucleotide sequences as short as 120 bp to identify *Streptomyces* species. In this study, about the same percentage of successful identification was obtained with sequences longer or shorter than 500 bp.

There is a differential distribution of the bacterial genera within the pulp in the headbox of the paper machine and the slimes present on the wet-end steel surfaces (Table 5). Bacteria belonging to four described genera (*Bacillus*, *Leptothrix*, *Pseudomonas* and *Pseudoxanthomonas*) were found in the headbox. The *Pseudoxanthomonas* strains represented 62% of all the headbox isolates. On the other hand, bacteria from 17 described genera were isolated from the slimes (Table 5), with *Microbacterium* as the most frequently isolated genus. The isolation medium also influenced bacterial distribution (Table 6). For example, *Bacillus* species are more frequently represented on R2A whereas *Pseudoxanthomonas* strains were more often isolated on PCA.

## Discussion

Studies on the identification of the microflora present on paper machines have been carried out in different countries [7,11,17]. In this study, we isolated a large number of bacteria from a Canadian paper mill over a 1-year period in order to obtain a representation of the bacterial communities inhabiting the pulp and the slimes present on a papermaking machine. Over 100 isolates were identified with a high level of confidence following the analysis of portions of their 16S rDNA sequences. There were some limitations to the isolation method

**Table 6** Distribution of bacteria found in pulp or in slimes on a paper machine according to the isolation medium

Genus	Proportion of isolates (%)	
	R2A	PCA
<i>Acidovorax</i>	7.1	2.1
<i>Aeromonas</i>	0	1.0
<i>Allorhizobium</i>	7.1	0
<i>Azorhizophilus</i>	0	1.0
<i>Azospirillum</i>	0	2.1
<i>Bacillus</i>	14.3	5.2
<i>Blastobacter</i>	0	1.0
<i>Brevibacillus</i>	0	3.1
<i>Enterobacter</i>	0	1.0
<i>Hydrogenophaga</i>	0	2.1
<i>Leptothrix</i>	0	2.1
<i>Microbacterium</i>	7.1	9.3
<i>Pseudomonas</i>	0	12.4
<i>Pseudoxanthomonas</i>	7.1	23.7
<i>Rhizobium</i>	7.1	2.1
<i>Sphingomonas</i>	0	1.0
<i>Staphylococcus</i>	0	1.0
<i>Xanthobacter</i>	0	3.1
Unidentified isolates <sup>a</sup>	50	26.8

<sup>a</sup>The 16S rDNA sequences of these isolates did not match the corresponding sequences filed in the databases or were similar to those of microorganisms which are still lacking identification

used. For example, a selection was generated by the culture media used in this study. The media selected for aerobic bacteria capable for growth on PCA or R2A media at the chosen temperatures (37 °C or 50 °C). Therefore, anaerobic and uncultivable bacteria were not taken into account in this study. It is also possible that some of the isolated species represented dormant cells that were inactive in the paper machine but developed well on the isolation media tested. In a previous study, Evenleigh and Brewer [5] characterized the microflora associated with slime accumulation in a Canadian paper mill. They established that the bacterial communities in these slimes were mainly composed of pseudomonads, flavobacters, and bacilli. While our study also indicates the presence of bacilli and pseudomonads in high proportions, particularly in the pulp samples, we did not detect any flavobacters.

Bacterial genera such as *Bacillus*, *Pseudomonas*, and *Microbacterium* are common inhabitants of papermaking environments in Finland, New Zealand, and the USA [3,7,8,11,15,16,18]. In this study, partial sequencing of the 16S rDNA of the isolates confirmed the occurrence of these three genera in a Canadian paper mill.

A high proportion of the isolates obtained in this study were associated with the genus *Pseudoxanthomonas* since their sequences exhibited a level of similarity higher than 95% with those of the *Pseudoxanthomonas* strains. This is the first report of the occurrence of bacteria from the genus *Pseudoxanthomonas* in pulp as well as in slimes on paper machines. The genus *Pseudoxanthomonas* is a single-species genus that was recently described by Finkmann et al. [6]. *Pseudoxanthomonas broegbernensis* is not known as a thermophilic

species. However, some sequences corresponding to thermophilic *Pseudoxanthomonas* strains isolated from hot springs were recently deposited in GenBank. These strains would belong to a novel *Pseudoxanthomonas* species that has to be further characterized and named.

It remains unknown whether the presence of *Pseudoxanthomonas* is specific to conditions prevalent in the Canadian paper mill studied here. However, it is possible that a fraction of the isolates identified as *Pseudomonas* and *Xanthomonas* in previous studies were misidentified [5,7,8,11,16]. Various physiological and biochemical tests have often been used to characterize microbial isolates from paper machines. Since *P. broegbernsensis* phenotypically resembles bacteria from both *Pseudomonas* and *Xanthomonas* genera, it is quite possible that some *Pseudoxanthomonas* strains could have been confused with members of either the *Pseudomonas* or the *Xanthomonas* genera.

In this study, *Pseudoxanthomonas* isolates were found both in the pulp and in the slime samples. It would appear that they are capable of resisting the relatively elevated temperatures (around 50 °C) present in the headbox and, at least under certain conditions, of colonizing the wet-end machinery steel surfaces. However, we observed that the *Pseudoxanthomonas* isolates did not exhibit diffuse growth in liquid media but rather formed bacterial flocks (data not shown) suggesting that *Pseudoxanthomonas* might play an important role in the formation of slimes on machinery. The role of this species in the formation of slimes is being studied.

A higher proportion of *Pseudoxanthomonas* isolates were recovered from the headbox than from the machine surfaces whereas *Bacillus*, *Microbacterium*, and *Pseudomonas* isolates were predominant in slime samples. This differential distribution of the bacterial genera could be explained by various factors including the ability of certain types of bacteria to attach and develop on metal surfaces and the temperatures used during the isolation process (50 °C for pulp isolates and 37 °C for slime isolates). These temperatures were the same as those of the headbox and the wet-end surfaces, respectively.

Sequencing of portions of the 16S rDNA allowed us to identify a large number of isolates in a limited period of time. Nonetheless, a relatively high proportion of the strains isolated in this study (about 29%) could not be identified because their 16S rDNA sequences did not match known sequences filed in databases or because their sequences were similar to those of still unidentified microorganisms. Interestingly, most of these unidentified microorganisms originated, as did our isolates, from warm or wet environments (activated sludge, rivers, hot springs, etc.) (Table 4). The fact that a significant proportion of the microorganisms isolated from paper industries could not be identified by the sequencing of their 16S rDNA reflects the need for further research on bacterial communities inhabiting those environments as well as those of other industrial processes.

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