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Diversity of bacteria contaminating paper machines

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Abstract Formation of microbial biofilms and slimes is a general and serious problem in the operation of paper machines. Studies of microbial populations in paper machine-derived biofilms have been conducted using standard microbiological procedures; however, the bacterial genera present in this type of samples as well as their diversity are quite poorly known. Here, the bacterial diversity of 38 process water and 22 biofilm samples from four different Finnish paper machines were analyzed by length heterogeneity analysis of PCR-amplified 16S ribosomal DNA (LH-PCR). In addition, sequencing of the amplified 16S rRNA gene from 69 clones was conducted for characterization of the bacterial genera present in biofilm and slime samples. The LH-PCR profiles of both the free-living (process waters) and immobilized (biofilms) bacteria were diverse at all stages of the papermaking process. Out of the 69 sequenced clones, 44 belonged to alpha-*Proteobacteria*, most of which were close to the nitrogen-fixing root nodule genera *Sinorhizobium*, *Rhizobium* and *Azorhizobium*. Other clones were assigned to beta- and gamma-*Proteobacteria* and the phylum *Bacteroidetes*. In addition, eight of the clones were assigned to a yet uncultivated phylum, TM7. Finally, epifluorescence microscopy revealed that Gram-negative bacteria were predominant in both the biofilm (65%) and process water (54%) samples and a small coccoid cell morphology was most common in all samples. Together, our results show that the analysis of microbial samples from paper machines using modern molecular biology techniques adds valuable information and should, therefore, be useful as a more specific and sensitive microbiological method for the paper industry. This information could further be applied, e.g., in the development of more specific and environmental friendly antimicrobial agents for paper mills.

Keywords Biofilm · Slime · Pulp · Paper machine · 16S rRNA · LH-PCR · Rhizobia

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Introduction

Growth of microorganisms in paper machines and board mills may cause severe economical losses as a consequence of reduced production rates. This is due to the increased number of web breaks and more frequent down time for the cleaning and maintenance of the machinery. Microbial contaminations also cause higher production costs due to larger consumption of additives and biocides, reduction in the lifetime of the equipment as well as deterioration in the quality of the final product. The reuse of white water and recycling of the pulp are the main reasons for bacterial and fungal contamination [4]. Particularly problematic are those microbial contaminants that form biofilms and slimes. Therefore, it is necessary to obtain more detailed information regarding the bacterial groups forming these biofilms as well as the mechanisms by which they attach and/or detach the surfaces in paper machines.

Earlier studies on bacterial communities associated with paper mills have shown that several aerobic and anaerobic genera, such as *Bacillus*, *Sphaerotilus*, *Klebsiella*, *Achromobacter* and *Pseudomonas* and combinations thereof form typical bacterial microbiota in paper industry [4]. However, since most studies are based on physiological and biochemical characterization of the bacterial isolates, many of the strains may have been misidentified. By supporting the strain identification with phylogenetical methods, Vaisanen et al. [17] discovered that the most common bacteria present at the wet end of the paper machine belonged to the *Bacillus*,

Burkholderia, Ralstonia, *Deinococcus*, *Aureobacterium* and *Brevibacterium* genera. More recently, a study from a Canadian paper mill showed that the most typical bacterial isolates originating from the pulp and slime samples belonged to the *Bacillus*, *Pseudomonas* and *Pseudoxanthomonas* genera, although several other genera also were identified among the slime isolates [5]. A relatively high proportion (29%) of the bacterial isolates, however, could not be assigned to any genus with high level of confidence using their 16S rDNA sequences [5]. This clearly reflects the need for further research on the bacterial communities inhabiting the environments within the paper industry.

The disadvantages of traditional cultivation studies are generally known, as only a minor portion of microorganisms can be grown using current cultivation media and conditions. Therefore, modern techniques in molecular biology offer new possibilities for the profiling and characterization of microbial populations and dynamics and, thus, complete the results obtained from traditional studies. One of the recently introduced molecular methods is the length heterogeneity analysis of polymerase chain reaction amplified (LH-PCR) 16S rDNA genes [13]. The 16S rDNA region, naturally variable in length, is amplified by PCR using fluorescently labeled universal primers and the heterologous product analyzed using denaturing polyacrylamide gel electrophoresis in an automated sequencer. To date, the LH-PCR method has been applied in screening microbial diversities in soil [12], picoplankton samples [13], within fecal anaerobic populations [3] as well as in processes involved in the formation of biofilms [15, 16].

The objective of this study was to apply LH-PCR to characterize the diversity/similarity of bacterial communities in Finnish paper machines operating at temperatures between 40 and 54 °C. The bacterial communities in process waters as well as in biofilms attached to stainless steel surfaces were therefore analyzed from different spots and at different time points of the manufacturing process. In addition, sequencing of the amplified 16S rRNA gene from several clones was performed for characterization of the bacterial genera present in this type of biofilms.

Materials and methods

Collection of samples

Four Finnish paper machines (PM-A, PM-B, PM-C and PM-D) were sampled once a week during a period of 3–4 weeks in the summer of 2002. The fiber source, the final products, the conditions and the amount of samples taken regarding these machines are presented in Table 1. The process water samples were collected from the broke tower, head box, clear white water tower, white water tower, wire pit, save-all tray and the biofilm samples from the cloudy filtrate tank, wire pit, white water tower and the shower water tower, depending on the circumstances in the factory. After collection, samples were kept at +4 °C during transportation to the laboratory and thereafter stored at −20 °C.

Gram staining

The ViaGram™ Red$^+$ Bacterial Gram Stain and Viability kit (Molecular Probes, Inc., USA) were used for the primary characterization of the 21 biofilm samples and seven process water samples from paper machines PM-A, PM-B and PM-C. For examination by microscopy, the process water and biofilm samples were sonicated 2–6 times on ice for 20 s using a 13 mm standard horn and 18 W sonication capacity (Branson 450, Danbury, USA). After staining, samples were filtered using an Isopore 0.2 μm GTBP filter (Millipore, Ireland). Ten different areas were analyzed from each sample with an epifluorescence microscope (Zeiss, Germany) using 1,000× magnification and 20 cells were classified from each field of view.

DNA extraction

One milliliter of process water or biofilm sample (diluted 1:20 in water if necessary) was centrifuged for 10 min at 12,000×*g* and the pellet was used for analysis. The pellet was resuspended in 0.4 ml of extraction buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.2 mg ml^{-1} proteinase K, 1% sodium dodecyl sulfate) and incubated at 37 °C for 1 h. Cell lysis was ensured by bead-milling: 0.6 g of glass beads (diameter 0.1 mm) and 0.4 ml of phenol–chloroform–isoamyl alcohol (25:24:1) were added to each sample and the tubes shaken for 30 s at a speed of 4.0 with a FastPrep® Instrument (Qbiogene, Inc., CA, USA). The tubes were then centrifuged for 10 min at 12,000×*g*. The upper phase was re-extracted with chloroform–isoamyl alcohol (24:1), purified with isopropanol precipitation in the presence of 0.2 M NaCl, washed with 70% ethanol and finally dissolved in 100 μl of TE buffer (pH 8.0).

LH-PCR analysis

The primers for PCR were synthesized in T-A-G-Copenhagen ApS (Copenhagen, Denmark). Specific amplification of eubacterial sequences was performed with an ird700 phosphoramidite-labeled forward primer 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) [18] and a reverse primer PRUN518r (5′-ATTACCGCGGCTGCTGG-3′) [10]. In the PCR reactions, 2 μl of purified DNA solution was used as a template in 50 μl PCR mixture containing 0.2 mM dNTPs, 0.3 μM of each primer, 1× DynaZyme reaction buffer and 2 U DynaZyme F501-KL polymerase (FinnZymes, Espoo, Finland). The PCR procedure included

Table 1 Characteristics of the paper machines studied

Paper machine	Fiber source	Product	T (°C)	pH	Process water[a]	Biofilm[a]
A	Pine and birch pulp	Coated fine paper	43–47	6.8–7.5	20/4	1/1
B	Softwood and birch pulp	Self-fastening and packing paper	40–45	7.7–8.3	9/3	18/4
C	Spruce TMP, softwood and birch pulp	Machine finished coated paper	47–52	7.3–7.6	3/1	3/1
D	Spruce TMP, softwood and birch pulp	Supercalantered paper	52–54	3.8–5.0	6/2	0/0

TMP thermomechanical pulp
[a]Number of samples analyzed using LH-PCR/the number of sampling sites from which the samples were collected

an initial denaturing step at 95 °C for 5 min and 25 cycles of amplification (94 °C for 30 s, 55 °C for 1 min and 72 °C for 3 min). Gel electrophoresis was performed with an automated LI-COR 4200 sequencer (LI-COR Biosciences, Nebraska, USA) overnight in 6% Long Ranger denaturing polyacrylamide gel (FMC Bioproducts, Rockland, ME, USA). Size standards of 470, 527 and 553 bp were prepared as described by Tiirola et al. [16]. Data were analyzed using Quantity One software (Bio-Rad Laboratories, CA, USA).

Cloning and sequencing

The PCR product of the biofilm sample from the cloudy water tank of the paper machine A was selected for cloning. The 16S rDNA PCR product was run in a 1% agarose gel and purified using QIAguick® Gel Extraction kit (Qiagen, CA, USA). The products were cloned into the pDrive TA-vector (Qiagen, Netherlands) and transformed to JM-109 *Escherichia coli* strain for mass culture and plasmid purification with NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany) of different clones. Sequencing was performed by simultaneous bidirectional cycle sequencing with a SequiTherm Excel II DNA Sequencing Kit (Epicentre Technologies, Madison, USA), sequencing primer pairs T7 and SP6 with IRD800 and IRD700 dyes, respectively, and an automated LI-COR 4200 sequencer. Sequences were compared to databases with the BLAST program [1]. The clones that had less than 5 bp difference and gave the same matches and identity percentages in the BLAST search were considered as identical clones. The sequences were aligned and the neighbor-joining tree calculated using the ClustalX program [14].

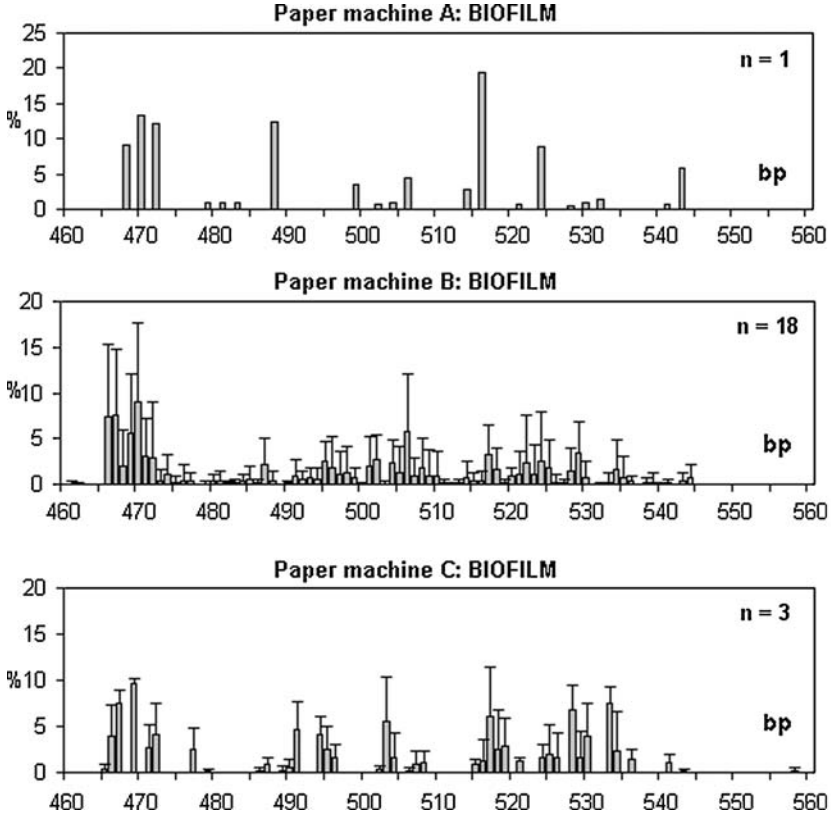

Fig. 1 Mean values and standard deviations of the pooled data from the LH-PCR analyses of 22 biofilm (BF) samples collected from four paper machines, PM-A, PM-B, PM-C and PM-D. The number of samples analyzed from each machine is shown in Table 1. The *X-axis* shows fragment lengths in base pairs (bp) and the *Y-axis* the proportion (%) of the single peak intensity of the total intensity of all peaks. PM-A: BF represents the LH-PCR profile of a sequenced sample from the corresponding paper machine

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the EMBL database under accession numbers AJ812332 to AJ812363.

Results

Microscopic examination

In the biofilm samples, the proportion of Gram-negative bacteria varied between 52 and 77% (65 ± 7%) and Gram-positive cells 23 and 48% (35 ± 7%). The analysis was conducted from samples obtained from paper machines PM-A, PM-B and PM-C that operated at slightly alkaline conditions. In the process water, the proportion of Gram-negative bacteria (54 ± 4%) was closer to that of the Gram-positive bacteria. Most cells in the biofilm samples were small coccoids (< 1 μm) or small rods (1–2 μm). Diplococcoids, larger coccoids (> 1 μm) and longer rods (length 3–5 μm) were also seen in some biofilm samples. The predominant morphology in the process water samples was also represented by small coccoid bacteria, but larger coccoids, long rods and filaments were seen more often.

LH-PCR results

The LH-PCR patterns, summarized in Figs. 1 and 2, showed that biofilms and process waters from paper machines had a high microbial diversity, which means that there must be several bacterial genera living in these environments. In the pooled data, the number of different bands increased, as the number of analyzed samples was higher. The average number of different LH-PCR peaks was 22.2 in the 22 biofilm samples and 17.1 in the 38 process water samples. Therefore, it seems that the microbial diversity was slightly lower in the process water than in the biofilms (significance: $P < 0.0003$). The lowest number of different LH-PCR

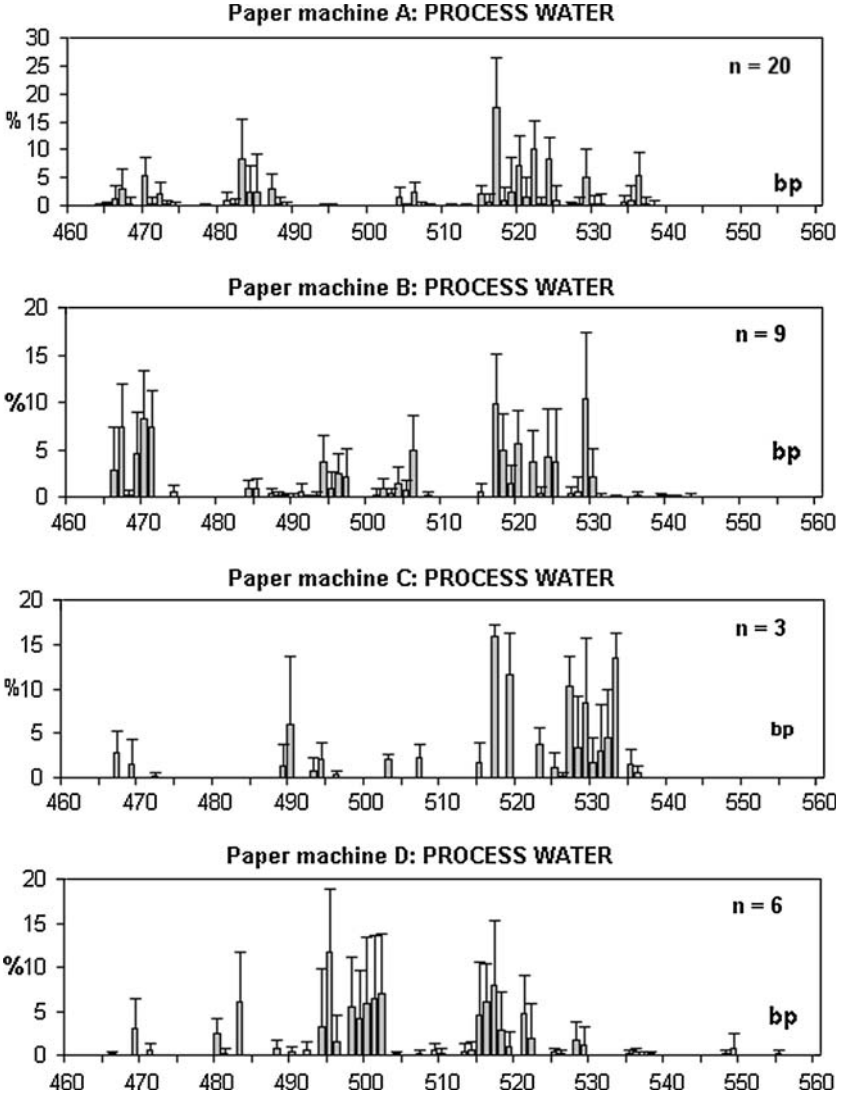

Fig. 2 Mean values and standard deviations of the pooled data from the LH-PCR analyses of 38 process water samples collected from four paper machines, PM-A, PM-B, PM-C and PM-D. The number of samples analyzed from each machine is shown in Table 1. The *X-axis* shows fragment lengths in base pairs (bp) and the *Y-axis* the proportion (%) of the single peak intensity of the intensity of all peaks

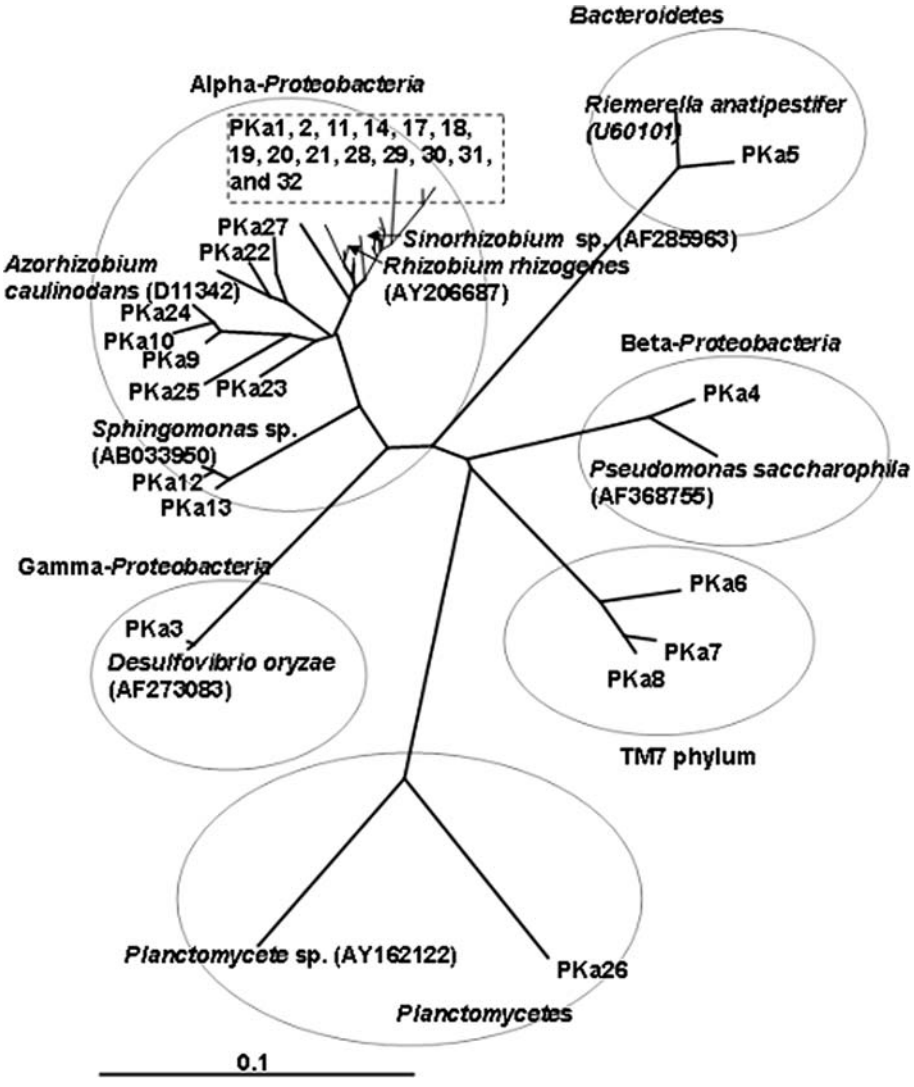

Fig. 3 A schematic representation of the phylogenetic tree based on the PKa 1–32 clones obtained from paper machine PM-A (biofilm) and 11 reference sequences. The total amount of analyzed clones was 69. The tree was calculated using neighbor-joining algorithm from partial 16S rDNA sequences (500 bp in length)

size classes (still as high as 15) was detected in the process water of paper machine PM-D, having an extreme environment for bacterial growth (pH 4.0–4.5, temperature 52–54 °C). The high standard deviation values of the LH-PCR peaks show that there was a high variation in the abundance and prevalence of different bacterial groups in the different paper machine samples during the study period. One of the most predominant LH-PCR peak was 517 bp in the process water samples (Fig. 2) (in average 19% of the total intensity), but short peaks (466–472 bp) dominated in the biofilm samples (Fig. 1). In comparison to the biofilm samples, the diversity of the short-length peaks was remarkably lower in the process water samples (16%, $P < 2.46 \times 10^{-9}$).

16S rDNA sequencing

The sequencing experiment of the biofilm sample from paper machine PM-A revealed that the predominant bacterial groups belonged at least to alpha-, beta- and gamma-*Proteobacteria* and to phylum *Bacteroidetes* (Fig. 3). A very high diversity of different clones was detected in alpha-*Proteobacteria*; the 41 sequenced alpha-proteobacterial clones were obtained from 24 different organisms. Many of the alpha-proteobacterial clones were closest to rhizobia *Rhizobium*, *Sinorhizobium* and *Azorhizobium*, and three of the clones were close to the genus *Sphingomonas*. However, most of the 69 sequences of the clone library were unique, showing that there must be a very high diversity in the bacterial community in such samples. The only clones, which were encountered several times in the clone library, were PKa16 (15 identical clones) closest to *Sinorhizobium* sp. C4 (identity 97%), PKa5 (13 clones) closest to *Riemerella anatipestifer* ATCC 11845^{T} (identity 94%) and clones PKa6 (6 clones), PKa18 (4 clones), PKa19 (3 clones) and PKa27 (2 clones). Over 60% of the clones (43 sequences) were obtained from short-length PCR fragments (467–472 bp), consisting of the very heterogeneous group of alpha-proteobacterial sequences of rhizobia and sphingomonads as well as one *Planctomycete* sequence.

The sequence length 488 bp was obtained from a yet uncultivated phylum TM7. The other clones had sequences with lengths of 517 bp (PKa5), 519 bp (PKa4), 532 bp (PKa3) and 543 bp (Pka1 and Pka2). These clone sizes did not cover all the major LH-PCR peaks, but short-length clones (the group of alpha-*Proteobacteria*) were over presented. No Gram-positive sequences were obtained, although microscopic examination of the sample showed that 27% of the bacterial cells in the sample should belong to the group of Gram-positive bacteria. The closest identity to cultivated organisms was below 97% in 38 clones and below 95% in 27 clones, showing that many of the clones belonged to the species or genera of uncultivated and unsequenced organisms.

Discussion

In this study, the bacterial diversity in process water and slime samples from four Finnish paper machines (A, B, C and D) was analyzed by LH-PCR. This method could offer a tool for rapid characterization of dominating groups of bacterial populations in several types of process industries. In total, 60 samples were analyzed and by combining the data, a variation in the bacterial diversity in each paper machine can be shown. The lowest number of different LH-PCR size classes was detected in the process water of paper machine PM-D operating under extreme acidic and high temperature conditions. It is known that acidic processes do not favor proliferation of bacteria, but fungal species can grow in these environments [8]. The average number of different LH-PCR peaks was slightly smaller in the process water samples, but in all four machines, the prevalence of different peaks and the intensity variation were very high in both the biofilm and process water samples.

The LH-PCR analysis of the biofilm sample of the cloudy water tank (paper machine A) showed 39% dominance of short-length LH-PCR peaks (467–472 bp), which was also common in other biofilm samples. Sequencing of the clone library of the sample revealed that these peak sizes belonged to alpha-proteobacterial organisms, which is well fitting to our earlier observation that in shaded industrial processes short-length LH-PCR peaks amplified by this primer pair most probably originate from alpha-proteobacterial organisms [16]. Most of the clones assigned to the short-length LH-PCR peaks belonged to the genera *Rhizobium*, *Sinorhizobium* and *Azorhizobium*. Bacteria belonging to these genera (referred to as rhizobia) grow in the soil as free-living organisms, but can also live as nitrogen-fixing symbionts inside root nodule cells of legume plants. Pulp and paper mill waters are typically rich in carbohydrates, but poor in fixed nitrogen, which is a consequence of the high C/N ratio typical of wood. The high prevalence of rhizobia may indicate that organisms able to fix atmospheric nitrogen represent an essential part of paper mill biofilms, although their presence in process waters and biofilms most likely originated from the root part of the raw material. At least *Klebsiella* species play a significant role as primary clarifiers and N_2-fixing organisms in pulp and paper effluent systems [6]. For the nitrogen-fixing organisms, on the other hand, inner parts of the biofilm could offer favorable microaerobic conditions needed for the function of the sensitive nitrogenase enzyme.

Interestingly, 14% of the cloned biofilm sequences of paper machine PM-A belonged to the TM7 phylum [7], which so far has not been cultivated. As the 488 bp LH-PCR band of these sequences was not predominant in other biofilms except for that of paper machine PM-A (Fig. 1), this group may not be a very common contaminant in papermaking processes. However, as the proportion of the sequence was high in both the clone library and the LH-PCR analysis, paper machine PM-A, with a pH of 7.5 and a temperature of 45 °C and which produces coated fine paper, could be a good source for cultivation trials of this phylum. The third interesting LH-PCR peak length was 517 bp, which belonged to *R. anatipestifer*—resembling clones in the sequenced biofilm sample. Our previous in silico analysis of the database has revealed [15] that this peak size indicates bacteria, which belong to the *Cytophaga*/*Flexibacter*/*Bacteroides* group.

The bacterial sequences obtained in this study represented species spectrum quite different from those typically isolated from biofilms. In previous studies many red to yellow colored and slimy biofilm bacteria belonging to the genera of *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Burkholderia*, *Deinococcus*, *Flectobacillus*, *Methylobacterium*, *Micrococcus* or *Roseomonas* have been identified [9, 11, 17]. In this study, however, the morphology of the analyzed biofilm sample was different in that the color of the biofilm was gray and the surface layer did not have a slimy appearance. Also, the ribosomal 16S DNA sequences were not analyzed from isolated bacterial cultures, but were amplified directly from the community DNA. However, it should be noticed that even this method cannot guarantee that all bacterial species are equally presented and identified from the entire population of the biofilm sample, but it prevents undeniable selection, which arises when bacteria are cultured and isolated on plates. It has been estimated that only 0.001–0.3% of bacterial species can be cultured using currently available media and methods [2]. Thus, direct amplification of 16S rRNA from a bacterial population in a biofilm can reveal species that are not possible to culture, as was the case with the sequences of the TM7 cluster. Other factors that affect the species composition include the state of the biofilm succession and possible contaminations with bacterial species, which are not actual, but live in a planktonic phase.

By microscopic examination, the proportion of Gram-negative bacteria was higher than that of the Gram-positive bacteria in both the biofilm and process water samples. This obviously shows that neutral or slightly alkaline conditions and temperatures between 40

and 50°C were not extreme enough to select for Gram-positives, which are usually thought to be more resistant when exposed to thermophilic conditions. Ultrasonication was necessary to facilitate fluorescent microscopy of slimy and aggregated samples. However, sonication may not have dispersed the rods and filaments from biofilms as efficiently as small coccoids, but it may, on the other hand, have detached diplococcoids and filamentous cells. Therefore, the microscopic findings can only be considered as rough estimates.

To our knowledge, this research is one of the first cultivation-independent studies of the paper machine process waters and biofilms, showing high diversity of the bacterial communities contaminating the paper-making process. Therefore, a comparison to earlier studies reveals that different results were obtained using modern molecular biology techniques. As shown in this study, many of the bacteria colonizing the machines may belong to bacterial species, genera and even phyla not yet cultivated or identified. The study indicates that nitrogen fixers may have some role in the formation and maintenance of biofilms in paper mill processes. However, it is obvious that all bacteria living in the biofilms are not responsible for primary attachment to the surface and the initial formation of the biofilm, but may have colonized the biofilm later. Identified primary biofilm formers have been found in the genera of *Deinococcus*, *Thermomonas*, *Meiothermus* and in a novel genus of "*Rhodobacter* and *Roseomonas*-like" alpha-*Proteobacteria* [9]. For the future, it would also be of importance to study the initial steps of biofilm formation by culture-independent methods. For this kind of work, LH-PCR and sequencing of selected samples may offer a valuable combination.

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