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Bacterial community diversity in paper mills processing recycled paper

Ulf Granhall · Allana Welsh · Ingela Noredal Throbäck · Karin Hjort · Mikael Hansson · Sara Hallin

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Abstract Paper mills processing recycled paper suffer from biofouling causing problems both in the mill and final product. The total bacterial community composition and identification of specific taxa in the process water and biofilms at the stock preparation and paper machine areas in a mill with recycled paper pulp was described by using a DNA-based approach. Process water in a similar mill was also analyzed to investigate if general trends can be found between mills and over time. Bacterial community profiles, analyzed by terminal-restriction fragment length polymorphism (T-RFLP), in process water showed that the dominant peaks in the profiles were similar between the two mills, although the overall composition was unique for each mill. When comparing process water and biofilm at different locations within one of the mills, we observed a separation according to location and sample type, with the biofilm from the paper machine being most different. 16S rRNA gene clone libraries were generated and 404 clones were screened by RFLP analysis. Grouping of RFLP patterns confirmed that the biofilm from the paper machine was most different. A total of 99 clones representing all RFLP patterns were analyzed, resulting in sequences recovered from nine bacterial phyla, including two candidate phyla. Bacteroidetes represented 45% and Actinobacteria 23% of all the clones. Sequences with similarity to organisms implicated in biofouling, like *Chryseobacterium* spp. and *Brevundimonas* spp., were recovered from all samples even though the mill had no process problems during sampling, suggesting that they are part of the natural paper mill community. Moreover, many sequences showed little homology to as yet uncultivated bacteria implying that paper mills are interesting for isolation of new organisms, as well as for bioprospecting.

Keywords Recycled pulp · Paper mill · Biofilm · Bacteria · Paper machine · Process water · T-RFLP · Clone library

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U. Granhall · A. Welsh · I. N. Throbäck · S. Hallin (✉)
Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, 750 07 Uppsala, Sweden
e-mail: sara.hallin@mikrob.slu.se

K. Hjort
Södertörn University, Huddinge, Sweden

M. Hansson
Swedish Institute of Agricultural and Environmental Engineering, Uppsala, Sweden

Introduction

Microorganisms constitute several problems in the modern papermaking industry. Biofouling causes machine breakage, reduced lifetime of the equipment, and shutdowns because of increased cleaning and maintenance of the machines. Other microbial-induced problems are corrosion as a result from the metabolism of specific microbial groups such as sulfur oxidizers and sulfate-reducing bacteria, and the production of explosive gases, colors, and bad odors in the mill and in the final product [3, 23]. Microorganisms arrive with the incoming water, the raw material, the solution or suspensions of additives, fillers, pigments, starches, and coatings [3] and they thrive due to the favorable conditions in the paper mill with suitable temperature (30–50°C) and the presence of abundant nutrients [44]. Paper mills with closed water circuits or mills using recycled pulp have more

microbial-induced problems [13]. With a closed process water circuit, the concentrations of organic compounds and electrolytes increase, and this leads to enhanced microbial growth. In comparison to wood pulp, pulp originating from recycled paper has much higher initial microbial numbers because various microbes often inhabit starches and coating products present in the recycled paper especially during long-term and outdoor exposures [13]. Adhesives present in the recycled pulp also constitute ideal places for attachment [3].

The majority of the microorganisms in paper mills live in biofilms, which are accumulations of microorganisms, extracellular polymers, organic and inorganic particles, and dissolved compounds [50]. Cells within the biofilms are more resistant to toxic substances such as antibiotics and detergents [48], and once established, the biofilm can remain long after the free-living organisms have died [10]. Within paper mills, the biofilms are often mixed with other materials to form a slime deposit [21, 46]. The most common solution to prevent biofilm formation and microbial growth in general has been to use biocides or enzymatic treatments [5, 26]. However there is a strong movement to limit the use of biocides worldwide due to their toxicity and the development of resistance by some microorganisms, which impacts human and environmental health [3]. More targeted approaches, for example, by enzymatic treatments, may allow the decreased use of biocides. However, our knowledge about the microorganisms biofouling paper mills is still so limited that enzymatic treatments, which may be more specific in their action towards particularly problematic organisms, are difficult to develop [45]. More targeted approaches at eliminating problematic organisms are further hampered because we lack the tools to identify and detect these organisms. Most information on microorganisms in paper mills is based on traditional bacterial isolation and characterization [23, 27, 31, 33, 34, 41, 44], which suffers from well-known bias associated with all cultivation-based approaches. Only a few studies using modern molecular methods are found that describe the diversity of bacteria potentially involved in biofouling in the actual papermaking process and they have focused on biofilms of paper machines using wood pulp [24] or wood pulp with a small fraction of recycled fibers [42] processed under thermophilic conditions. There are also a couple of studies on bacterial communities in the wastewater treatment process in paper mills [12, 16].

In our present work the total bacterial community composition and identification of specific taxa present in a paper mill processing recycled paper under mesophilic conditions is described by using a DNA-based approach. The main aim was to assess the bacterial community structure in the biofilms on the surfaces as well as in process water (surface water) samples in the stock preparation area and the paper machines downstream in the process in the mill. We hypothesized that the biofilms would harbor unique communities at each sampling location, whereas the communities in the process water would be similar throughout the mill because of the closed water circuit. As a reference, we also sampled process water in a similar mill at different time points to investigate if general trends can be found between mills and over time. The overall community structure at each sampling point was determined by using terminal-restriction fragment length polymorphism (T-RFLP). For in-depth comparative analysis of community membership and identification of planktonic and biofilm communities, 16S rRNA gene clone libraries were generated and analyzed by RFLP screening together with sequencing of specific clones.

Materials and methods

Sampling and DNA extraction

Samples were taken from paper mills A and C, both using recycled fibers and operated under mesophilic conditions. From mill A, we sampled the stock preparation area (S) refining pulp of recycled fibers and the paper machine (P) during a production period with normal operation and no particular process problems in November 2005. From both locations, three samples of biofilm (B) from the solid surfaces or process water sampled as surface water (SW), respectively, were collected. From mill C, surface water samples were collected from two different locations around the paper machine at two time points (December 2005 and April 2006). From both locations and time points, three replicates were sampled. The samples were mixed with EtOH 1:1 to preserve them and inhibit bacterial growth during delivery to the laboratory where the DNA extractions and subsequent analyses were performed.

DNA was extracted by using 1-ml samples from each sample with the DNA extraction kit FastPrep for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's instructions with one exception. The binding matrix (from the DNA extraction kit) with bound DNA was washed with 500 µl 5.5 M guanidine thiocyanate, the tube was mixed 4–6 times, and centrifuged for 30 s at 10,000*g* followed by discarding of the supernatant. This step was repeated five times.

Community structure analysis by T-RFLP of partial 16S rRNA genes

Partial 16S rRNA genes were amplified in duplicate PCR reactions from the 12 samples from mill A and the 12

samples from mill C as described in Hjort et al. [18]. The duplicate PCR samples from each DNA sample were pooled before further analysis. The PCR products were digested in parallel reactions with *Hae*III, *Hha*I, and *Msp*I (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. Fluorescently labeled TRFs were separated and detected by using an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA) with the GS-500 ROX size standard (Applied Biosystems) as described by Hjort et al. [18]. The TRF patterns were evaluated by using the Peak Scanner Software (Applied Biosystems) and TRFs smaller than 50 bp or contributing less than 1% of the total signal were excluded from the subsequent statistical analysis.

The bacterial community structure derived from T-RFLP fingerprints of the communities in mill A were analyzed by non-metric multidimensional scaling (NMS) using PC-ORD version 5.10 (MjM Software, Oregon, USA). The data matrix was arc-sinus square root transformed and the Bray–Curtis distance measure was used. The NMS was run using a random starting configuration, a maximum of 400 iterations, and an instability criterion of 0.00001 in standard deviations in stress over the last 200 iterations. A Monte Carlo test was performed on 300 runs with randomized data to test for the null hypothesis. For a two-dimensional solution with the lowest possible stress value, a final run using the best starting configuration from the first runs was performed.

Clone libraries, sequencing, and phylogenetic analysis

An in-depth analysis with identification of the community members was done for paper mill A. In total, four clone libraries were constructed by using pooled DNA samples from the three replicate samples from the biofilm and surface water, sampled both in the stock preparation and at the paper machine areas of the paper mill. Amplification of a 900-bp 16S rRNA gene fragment was performed in triplicate reactions for each DNA sample using the bacterial primers 27f [25] and 926r [49]. The PCR program consisted of an initial denaturation of the DNA at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C and a final elongation step at 72°C for 7 min. Prior to cloning, the PCR products from the same sampling point were pooled, separated by agarose gel (1%) electrophoresis, and purified with the MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA) to avoid insertions of incorrect amplicons. The PCR products were then cloned by using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Small amounts of cell material from randomly picked transformed clones were collected with toothpicks and resuspended in 25 µl pre-prepared PCR mixtures, and the inserts were amplified as described above. The inserts were digested overnight at 37°C using 3 U *Hae*III, *Hha*I, and *Msp*I, respectively, and then screened by restriction fragment length polymorphism (RFLP). The digested products were separated by electrophoresis in a 2% agarose gel at 100 V for 2.5 h.

The RFLP patterns were compared by using the Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) and grouped into RFLP pattern types. Library coverage (C) was estimated as $C = 1 - nN^{-1}$, where n is the number of different RFLP pattern types from a clone library that are encountered only once and N is the total number of clones analyzed. When at least 75% coverage was achieved for each of the clone libraries, plasmids in a selection of clones representing all RFLP pattern types were isolated by using the QIAprep Spin Miniprep Kit (Qiagen). The inserts were sequenced on one strand by Macrogen Inc. (Seoul, Korea) with an ABI3730 XL automatic DNA sequencer using the vector primer T3.

Amplified 16S rRNA gene fragments from clones representing each RFLP pattern type from each site were aligned with sequences retrieved from the public databases using Geneious [11] and the Ribosomal Database Project II data space and aligner [8, 9]. Derived sequences were checked for chimeric properties using Bellerophon [20] and Pintail [1]. The final dataset had 152 taxa and was trimmed to be 756-bp long and included 99 clone sequences. Maximum likelihood phylogenetic analyses for this dataset were completed by using the RAxML-HPC Blackbox (v 7.2.6) program [38, 39] on the computer cluster of the 'CyberInfrastructure for Phylogenetic RESearch' (CIPRES) [29]. TreeView X was used to display the treefiles [32]. Only bootstrap values of at least 70% demonstrate good support measures and thus were retained [17]. Presumptive classification of clones was assigned by using the RDP Classifier [47].

The partial 16S rRNA gene sequences were deposited in GenBank with accession numbers GU235876 to GU235980.

Results

T-RFLP analysis of 16S rRNA genes

Electropherograms resulting from the T-RFLP analysis of planktonic and biofilm communities in paper mill A contained about 170 terminal restriction fragments (TRFs) in each sample, with low relative abundance, indicating that there was a high bacterial diversity in all sample types. By contrast, profiles from the planktonic communities in the reference mill C were characterized by a few dominant peaks and many with low relative abundance, although the

total number of peaks was similar as for the other mill (Fig. S1). Nevertheless, the dominant peaks in mill C were also among the most dominant peaks retrieved in mill A, indicating potential similarities between the mills. The profiles from the reference mill C also showed that the bacterial community structure can be rather stable over time. Additionally, the community profiles were reproducible, as confirmed by similarities in the patterns among the replicate samples.

The T-RFLP bacterial community profiles analyzed by NMS to compare the planktonic and biofilm communities in paper mill A separated according to sampling location within the mill and sample type (Fig. 1). The biofilm samples from the paper machine (PB1-3) were most different from the other samples. The community structure in the biofilm and the surface water samples from the paper machine (PSW1-3) differed from each other as indicated by clear separation in the ordination analysis. By contrast, the bacterial communities in the biofilm (SB1-3) and the surface water (SSW1-3) in the stock preparation area showed similarities with each other and also resembled the process water sample from the paper machine. In total, 92.3% of the variance was represented in the two-dimensional space in the NMS analysis and the stress value was low.

RFLP pattern types in clone libraries

A total of 404 clones were screened by RFLP. The coverage for the four libraries based on RFLP pattern types was 91% for both the surface water and biofilm from the paper machine, and 82% and 90% for the surface water and biofilm, respectively, in the stock preparation area. In each library, about 23 different RFLP pattern types were found. Of the nine RFLP pattern types shared between at least two sampling sites, only three (A, E, and G) were detected at all the sites (Fig. 2), although the frequency of detection varied greatly. For example, RFLP pattern type E was discovered as a minor component in all but the stock preparation area biofilm where it was the dominant pattern type recovered. The RFLP pattern types of the paper machine biofilm area indicate that 80% of these clones were unique to this site as compared to the other sites that were more similar to each other with 22%, 33%, and 32% unique RFLP pattern types in the stock preparation area biofilm, surface waters, and the paper machine surface waters, respectively. The screen of RFLP pattern types supports the T-RFLP community profile analysis by revealing that the paper machine area biofilm harbored a large fraction of unique organisms not detected elsewhere in the paper mill and that the bacterial communities in the other samples were more similar.

16S rRNA gene sequencing

In total, 99 clones representing all RFLP pattern types from each site in the paper mill were sequenced and analyzed (Fig. 3). RFLP pattern types were adjusted based on sequence identity to verify those RFLP pattern types shared

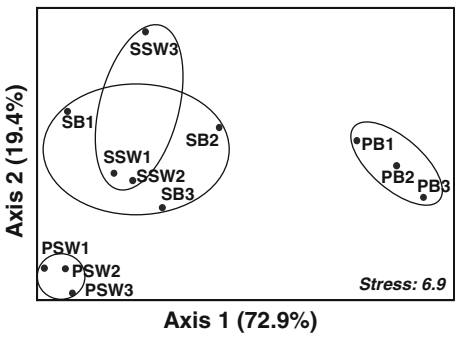


Fig. 1 Non-metric multidimensional scaling analysis of bacterial community profiles determined by T-RFLP analysis of partially PCR amplified 16S rRNA genes originated from four samples in the paper mill: the surface water (*SW*) and biofilm (*B*) in the paper machine (*P*) and stock preparation (*S*) areas, respectively. The *ellipses* indicate replicated samples from the four sampling sites ($n = 3$). In total 92.3% of the variance is represented in the two-dimensional space

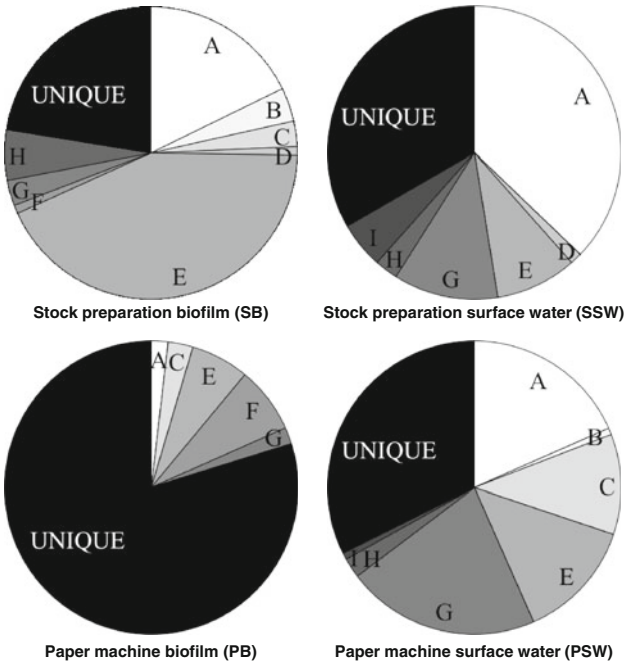


Fig. 2 Graphical representation of clones of the 16S rRNA gene grouped by restriction fragment length polymorphism (RFLP) patterns from clonal libraries generated at four sites in the paper mill: stock preparation biofilm (*SB*), stock preparation surface waters (*SSW*), paper machine biofilm (*PB*), and paper machine surface water (*PSW*) areas. *Letters* indicate those RFLP pattern types shared by more than one site and all other RFLP types unique to that site are gathered in the *black area*

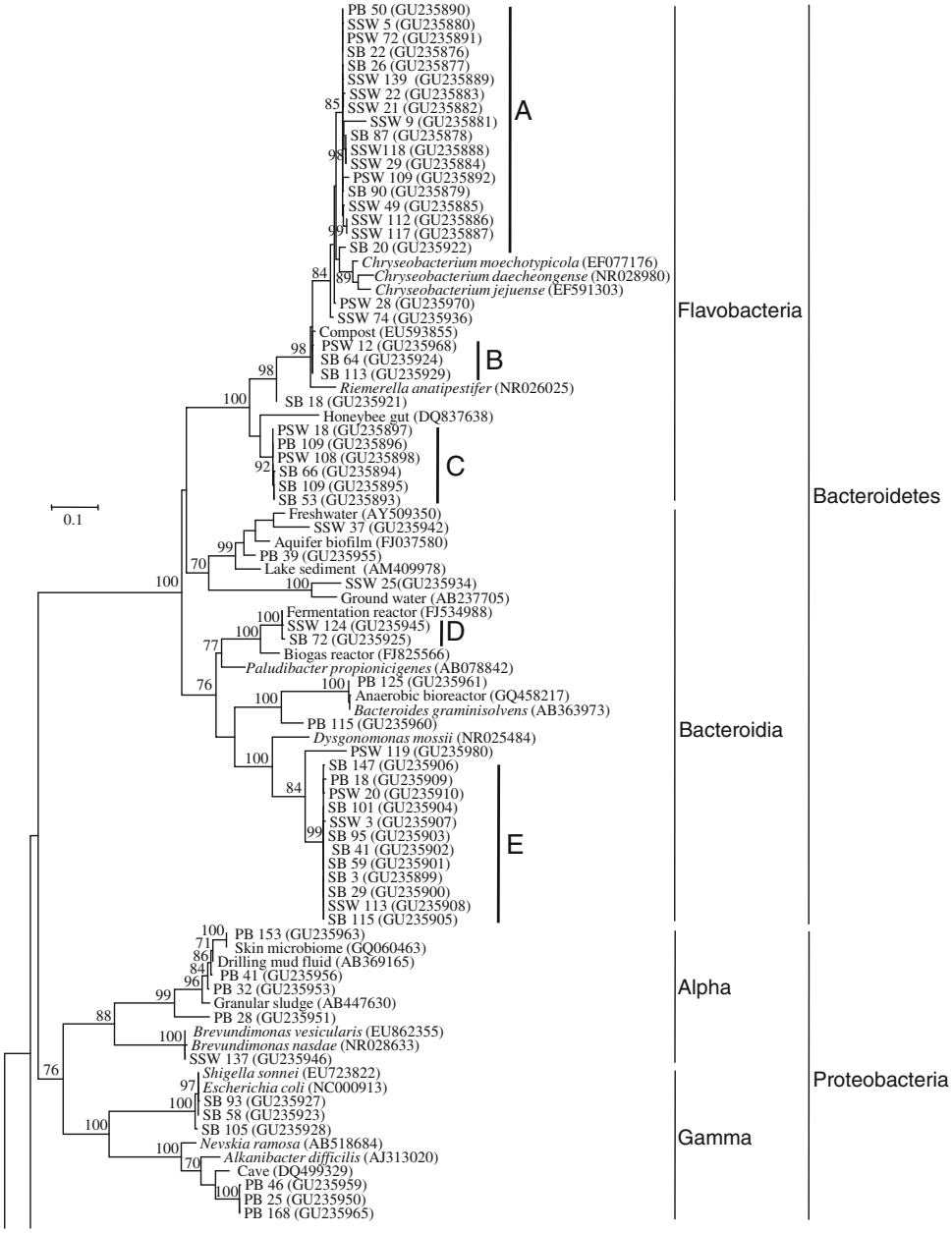
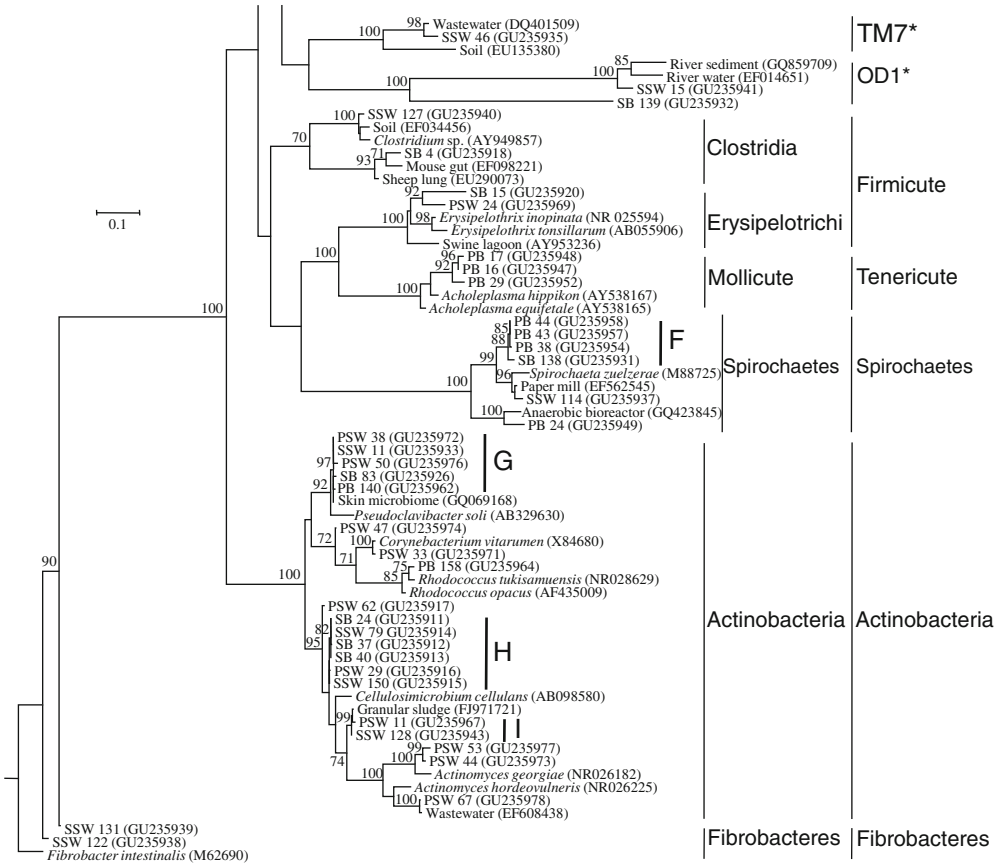


Fig. 3 Maximum likelihood tree created using 756 bp of the 16S rRNA gene from 99 clones representing RFLP groups amplified from DNA preparations from the stock preparation biofilm (*SB*), stock preparation surface water (*SSW*), paper machine biofilm (*PB*), and paper machine surface water (*PSW*) areas of the paper mill along with pure cultures and other clone sequences retrieved from the public database. *Numbers* reflect bootstrap support measures greater than 70% for each branch. *Letters* represent RFLP pattern type designations as in Fig. 2. On the *right side* is a presumptive classification by class and phyla for these sequences based on the guide tree created by using the RDP Classifier [47]. *Asterisks* indicate candidate phyla and are officially unclassified bacteria. *Fibrobacter intestinalis* was used as the outgroup

among sampling sites (Fig. 2). In most cases clones sequenced from one RFLP pattern type were more similar to each other than sequences from another pattern type, which suggests that the RFLP analysis reflected the taxonomic divergence among clones. Sequences from clones representing seven different phyla (Actinobacteria, Bacteroidetes, Fibrobacteres, Firmicute, Proteobacteria [α, γ], Spirochaetes, Tenericute) and two candidate phyla (OD1, TM7) were recovered (Fig. 3). Nodes differentiating class and candidate phyla were well supported by bootstrap values over 70%.

The most prominent group of clones was from the Bacteroidetes phylum constituting 45% of all the clones and five of the shared RFLP pattern types (A–E) retrieved

Fig. 3 continued



(Figs. 2, 3). The Bacteroidetes were most dominant in the biofilm in the stock preparation area and the largest RFLP type (E) among this phylum was similar to the Bacteroidia. By contrast, the process water at the same location was dominated by the Flavobacteria (RFLP type A) from the same phylum. Type A, most similar to *Chryseobacterium* spp., was found at all the sampling areas and represented 17% of all the clones. Actinobacteria represented 23% of the clones and three of the RFLP pattern types (G–I). Alpha- and Gammaproteobacteria sequences were only retrieved from biofilm samples, but at both locations. In samples from the stock preparation area biofilm, sequences were retrieved that were very similar to *Escherichia coli* and *Shigella sonni*. In the biofilm from the paper machine area, 30% of all the clones were similar to *Acholeplasma* sp. in the class Mollicutes and these were not found in any other sample.

Discussion

This study mainly compared planktonic bacterial communities in the process water with those in biofilms at different locations in a paper mill with a closed water circuit using pulp originating from recycled paper. The planktonic communities were further compared with those from a similar mill that was used as a reference in this study. Our data show that the bacterial community structure in surface water samples differ between mills processing pulp from recycled paper under mesophilic conditions, although the within-mill differences were small between different sampling locations and time points. Moreover, biofilm and surface water samples may be similar at the same location, as in the stock preparation area in mill A, but may also be different as in the paper machine area in the same mill. Similar results to ours have been found in freshwater biofilm and surface water samples exposed to differential flow rates where similarity in bacterial community structure was dependent on where in the process setup the surface water was sampled [35]. Lahtinen et al. [24] also showed that process water and biofilm communities from paper machines in wood pulp paper mills differed from each other [24]. Moreover, the bacterial community structure in biofilm and planktonic samples from a membrane bioreactor [19] and from a laboratory-scale wastewater treatment reactor have also been shown to be different [30]. The most different community in our paper mill was in the biofilm from the paper machine. Almost half of the unique clones from this site were similar to *Acholeplasma* spp., which are distinguished by their lack of a cell wall and a

particularly small and simple genome [43]. Other members of this class, i.e., some mycoplasmas, have the ability to form biofilms [28], and sequences similar to *Acholeplasma* have also been retrieved from a biofilm from a down-flow fluidized bed reactor [4]. The fact that distinct communities evolve in certain biofilms but not in others may be due to differences in adsorption material [40]. However, all bacteria in the biofilm are not necessarily responsible for the primary attachment and initial formation of the biofilm, since they may have colonized the biofilm later. In addition to surface properties, local conditions also play a role [10].

The clone libraries were dominated by sequences belonging to Bacteroidetes and Actinobacteria. By contrast, Lahtinen et al. [24] primarily retrieved sequences from Alphaproteobacteria in thermophilic paper machine biofilms in wood pulp paper mills, and the spectrum of ribotypes they detected differed significantly from what had been isolated from biofilms on paper machines in similar types of mills [23, 44]. Tiirola et al. [42] retrieved sequences from Alpha- and Betaproteobacteria in their study of bacterial succession in simulated paper mill biofilms in thermophilic conditions regardless of the pulp source. Interestingly, they retrieved sequences from Flavobacteria only from the mill using a small fraction of recycled fibers in the pulp. From recycled paper mills Suihko et al. [41] mainly found Gammaproteobacteria using culture-dependent techniques. With the limited number of studies available, it is not possible to conclude if the reported differences in community composition are due to the different types of pulp being processed, process management, environmental factors, or differences in methods used to describe bacterial diversity. However, it seems likely that unique communities of bacteria develop in the individual mills as was found in our study when comparing the planktonic communities between mills A and C. In agreement, unique bacterial communities have been found in wastewater treatment systems in different pulp mills, even though they may have had similar process management techniques and operations [16].

In our study, sequences similar to bacteria known to cause problems in paper mills and implicated in biofouling of paper mills or wastewater plants were detected at all sampling locations. For example, *Chryseobacterium* spp., predominant among the Bacteroidetes in our samples, are filamentous *Flavobacteria* involved in paper mill slime formation [31]. *Chryseobacterium*-like sequences were also recovered from biofilms analyzed by Tiirola et al. [42]. However, the *Chryseobacterium*-like clones from their study are only 87–93% similar to those from our study supporting the idea that each mill has unique, but related organisms. Among the Alphaproteobacteria in our samples clone SSW137 was identical to *Brevundimonas vesicularis*, which was isolated from slime deposits in a paper mill and shown to produce complex extracellular polymeric substances [45]. Sequences similar to odor-causing organisms were also recovered from all sites in this paper mill. Actinobacteria have been shown to generate foul odors by geosmin and 2-methylisoborneol (MIB) production [22, 37]. However, the detection of actinobacteria does not imply that they are the dominant organisms causing odors, and in fact they are often implicated in odor formation unjustifiably [51]. Similarly, *Desulfovibrio* species with the potential for causing odor problems were detected by using a sensitive nested-PCR technique in most biofilm and surface water samples in a paper mill with no obvious biofouling problems [27]. We also detected sequences that were related to known pathogenic bacteria, *E. coli*, in the biofilm in the stock preparation area. Fecal coliforms have been recovered from paper mill effluents previously [15]. Cultivation of such coliforms showed identical sequences to known pathogens like *E. coli*, but further characterization showed that they were non-pathogenic strains [14]. Thus, we have no sound argument to assume that the sequences we retrieved represent pathogenic organisms.

The paper mill was not experiencing any serious process problems at the time of sampling. Thus, the presence of these potentially problematic organisms suggests that they could be present in this paper mill without necessarily causing ill effects to the papermaking process or product. The natural presence of these organisms might help explain how slight changes in environmental conditions within a paper mill can lead to rapid development of biofouling problems and consequently to slime and odours [6, 36]. To detect and quantify changes in specific populations requires the development of highly specific, sensitive tools like fluorescent in situ hybridization (FISH) [10, 22, 30] or real-time PCR [12]. However, we still know so little about the microbial communities in paper mills using cultivation-independent methods that organisms instigating serious biofouling problems may as yet be undescribed.

Many sequences were recovered from our paper mill with little homology to as yet uncultivated bacteria. For example clones SB139 and SSW15 have closest homology to sequences in the candidate phylum OD1, whereas clone SSW46 was most similar to sequences in the candidate phylum TM7 [47]. Lahtinen et al. [24] also recovered sequences of organisms in the TM7 phylum, suggesting this as yet uncultured group may be common in several types of paper mills. Also present in our paper mill were the clones SSW131 and SSW122 with close sequence homology to *Fibrobacter intestinales*, which is the only genus in the whole phylum of Fibrobacteres [2]. The two species of cellulolytic *Fibrobacter* isolated thus far were from animals [2]. Our paper mill sequences may therefore represent some, as yet, uncultured *Fibrobacter* sp. from a quite different source of origin. The detection of

representatives from these less explored taxonomic groups together with the relatively high diversity of bacteria in our study underline that paper mills may be an excellent source for isolation of new uncultured and unique organisms as well as of interest for bioprospecting. In fact, paper mills have been used as a source for isolation of biotechnologically useful organisms like perchlorate-reducing bacteria [7], cellulose-degrading bacteria [33], and polysaccharide-producing bacteria [34]. Future research should aim at more sensitive detection and increased cultivation of novel and potentially useful organisms in industry.

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