J Ind Microbiol Biotechnol (2009) 36:929–937
DOI 10.1007/s10295-009-0571-6

ORIGINAL PAPER

Early succession of bacterial biofilms in paper machines

Marja Tiirola · Tomi Lahtinen · Matti Vuento · Christian Oker-Blom

Received: 10 March 2009 / Accepted: 1 April 2009 / Published online: 24 April 2009

Abstract Formation of biofilms causes severe problems in paper machines, and hence financial costs. It would be preferable to prevent attachment of the primary-colonizing bacteria than to control the growth of secondary communities, which are sheltered by exopolysaccharide slime layers. We have therefore investigated the early succession of paper-machine biofilms by incubating stainless-steel test coupons in the process water-flow lines in two paper machines operating in slightly alkaline conditions in temperatures (45 and 49°C) supporting thermophilic microbes. Microbial succession was profiled using length heterogeneity analysis of PCR-amplified 16S rRNA genes (LH-PCR) and linking the sequence data of the created 16S rRNA gene libraries to the dominant LH-PCR peaks. Although the bacterial fingerprints obtained from the attached surface communities varied slightly in different samples, the biomarker signals of the dominating primary-colonizing bacterial groups remained high over time in each paper machine. Most of the 16S rRNA gene copies in the early biofilms were assigned to the genera *Rhodobacter*, *Tepidimonas*, and *Cloacibacterium*. The dominance of these sequence types decreased in the developing biofilms. Finally, as phylogenetically identical primary-colonizers were detected in the two different paper mills, the machines evidently had similar environmental conditions for bacterial growth and potentially a common source of contamination.

M. Tiirola (✉) · T. Lahtinen · M. Vuento · C. Oker-Blom
Department of Biological and Environmental Science,
University of Jyväskylä, P.O. Box 35,
40014 Jyväskylä, Finland
e-mail: mtiirola@jyu.fi

Keywords Paper machines · Biofilms · Succession · Bacterial diversity · 16S rRNA gene · LH-PCR · *Rhodobacter* · *Tepidimonas* · *Cloacibacterium*

Introduction

Formation of microbial biofilms on machine surfaces is an economically important problem in the operation of paper mills, due to an increased number of web breaks and more frequent down time for cleaning and maintenance of the machinery. Previous electron-microscope studies have shown that paper-mill slime biofilms morphologically resemble wastewater biofilms with complicated biological structures [26]. Culture techniques have identified many different Gram-positive and Gram-negative bacterial genera and species within paper-machine biofilms and slimes, including *Flavobacterium*, *Bacillus*, *Enterobacteriaceae*, *Klebsiella, Methylobacterium, Burkholderia cepacia, Deinococcus, Pseudomonas*, and *Pseudoxanthomonas* [6, 19, 27, 28]. Comparison of the sugar composition of paper-mill and pure-culture slimes has suggested that the betaproteobacterium *Burkholderia cepacia* might be responsible for much of the slime formation in paper mills [15]. Recent molecular studies have shown that the diversity of both free-living and immobilized bacteria in paper-mill machinery varies over time and among different processes [13]. Sequencing of the 16S rRNA gene library also showed that mature biofilms can be colonized by nitrogen-fixing rhizobial organisms [13], which might be responsible for preventing nitrogen limitation for microbial growth in the carbon-rich paper-machine process waters.

The biofilm formation starts with rapid establishment of a monomolecular layer upon all surfaces and by attachment of primary bacteria to this layer for feeding [3]. Since it is

probably easier to prevent these early stages of biofilms than to limit the growth of secondary communities, more information about the primary-colonizing bacterial groups, their surface attachment mechanisms, and biocide susceptibility are required. Because paper-machine slimes are often pink, colored bacteria capable of primary attachment have been of special interest. Kolari and coworkers [11] isolated several pink-pigmented *Deinococcus-Thermus* phylum bacteria and red-pigmented *Meiothermus* species from slimes of paper-mill machines operating under acidic conditions, and red-pigmented alphaproteobacterial *Rhodobacter*-related strains from neutral or alkaline machines. By using quantitative PCR, it was later shown that *Meiothermus* spp. were common contaminants in colored-process deposits, and members of this genus were ubiquitous in many paper-mill process samples and end products [7]. However, all previous studies of paper-machine biofilms have been based on characterization of bacterial communities from deposits and visible biofilms (slimes), and the early succession of biofilms has not been studied by molecular methods. Direct culture-independent analysis of the early biofilm succession could help to design specific antimicrobial treatments against the primary-colonizers and thus reduce the use of biocides.

We investigated the succession of paper-machine biofilms by incubating stainless-steel test coupons in two paper-machine water-flow lines. Sample incubations were performed for various time periods and the follow-up lasted 9 and 6 months for the separate machines. The microbial succession was characterized by length heterogeneity analysis of PCR-amplified 16S rRNA genes (LH-PCR) [23] and sequencing of the 16S rRNA gene clone libraries. DNA-based analyses showed that, although the bacterial fingerprints obtained from surface-attached communities varied over time, the fingerprints of key bacterial groups could be identified. The DNA-based approach showed that the same primary-colonizing biofilm bacteria were detected in different paper mills, implying that the machines studied shared closely similar environmental conditions and potentially a common source of contamination.

Materials and methods

Sampling

The study was done with paper machines B (PM B) and C (PM C) during a period of 9 and 6 months, involving 48 and 22 biofilm sampling timepoints and 27 and 11 process water sampling timepoints, respectively. PM B produced lightweight coated (LWC) catalogue paper from thermomechanical spruce pulp, sulphate pulp, and recycled fibers, operating at pH 7.3 ± 0.5 (average ± SD), 49 ± 2°C. PM C produced coated fine paper from refined pine and birch sulphate pulp, operating at pH 7.9 ± 0.4, 45 ± 1°C. Both machines used starch and several other additives for coating the paper. The machines were located in different factories, which were beside different water bodies. Biofilm formation was simulated in Robbin's devices [16], consisting of an oval stainless-steel chamber with detachable test coupons (25 × 80 mm). The devices were connected to the water-flow line of the clean filtration tanks. Plastic tubes were connected to the top and lower part of the chamber for process water circulation. The void volume of the chamber was 3 l, and the speed of water feed varied from 10 to 50 l min^{-1}. The flow speed in the Robbin's device was adjusted experimentally so that it allowed formation of a thin (<1 mm) biofilm within 1 week. The duration of the incubation varied from 1 to 15 days in random order, and seven to nine incubations were performed for each incubation time class, for PM B and C, respectively. The bacterial DNA was extracted from two to four replicate test coupons, which were processed separately, but the output data from each sampling were combined for the statistical analysis. Process water samples were prefiltered through a 2.0-mm-pore-size cellulose filter to remove fibers and deposits. Bacteria were then concentrated from 90 ml of water by centrifugation (4,500×*g*, 30 min). Samples were analyzed immediately or stored at −20°C.

DNA extraction

One milliliter of biofilm sample suspension or concentrated process water was centrifuged at 12,000×*g* for 10 min, and the pellet was used for the analysis. The pellet was re-suspended 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 the samples, and the tubes were shaken at speed 4.0 for 30 s with FastPrep Instrument (Qbiogene, 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 and 0.6 vol alcohol) and a 70% ethanol wash, and finally dissolved into 100 µl of TE-buffer (pH 8.0).

LH-PCR analysis

Specific amplification of bacterial 16S rRNA gene sequences was performed with ird700 phosphoramidite-labelled primer 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and an unlabelled primer PRUN518r (5′-ATTACCGCGG

CTGCTGG-3′). In the PCR reactions, 2 µl of purified DNA (or sterile water in the blank controls) was used as a template in 50 µl PCR mixture containing 0.2 mM of 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 an initial denaturing step at 95°C for 5 min and 30 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, NE, USA) in 6% Long Ranger denaturing polyacrylamide gel (FMC Bioproducts, Rockland, ME, USA). Data were analyzed using Quantity One software (Bio-Rad Laboratories, CA, USA). Relative fluorescence of different-sized peaks in each sample was calculated and drawn to average LH-PCR profiles for each month. Statistical analysis of the biofilm profiles was performed using one-way ANOVA and Tukey's *t* test using software SPSS 14.0 (SPSS, Chicago, IL). Phylogenetic assignments of distinct LH-PCR peaks were determined by comparing the observed lengths with the simulated lengths and identities from the 16S rRNA gene clone libraries.

Cloning and sequencing

The PCR products of 1- and 7-day-old biofilm samples from PM B and of 3-day-old biofilm sample from PM C were selected for cloning. The 16S rDNA PCR products were run in a 1% agarose gel and purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences, Freiburg, Germany). The products were cloned into the pDrive TA-vector (Qiagen, CA, USA) and transformed to the *E. coli* JM-109 strain for mass culture and plasmid purification with NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany) of different clones. Sequencing was performed from 60 PM B clones and 42 PM C clones by simultaneous bidirectional cycle sequencing with a SequiTherm Excel II DNA Sequencing Kit (Epicentre Technologies, Madison, WI, USA), sequencing primer pairs T7 and SP6 with IRD800 and IRD700 dyes, respectively, and an automated LI-COR 4200 sequencer. Sequences were edited using Contig Express software (Invitrogen) and compared with the EMBL database using the BLAST algorithm [2]. Most closely related type strains were also searched from RDPII database (release 10, 6 March 2009, http://rdp.cme.msu.edu/). Selected sequences and reference sequences were aligned and neighbor-joining trees were calculated using the program ClustalX [24]. Sequences PM-b1 to PM-b33 were obtained from 1-day biofilm and PM-b34 to PM-b60 from 7-day biofilm from paper machine B. All PM-c sequences were obtained from 3-day biofilm from paper machine C.

Nucleotide sequence accession numbers

Sequences of the clones were deposited in the EMBL database under the accession numbers AM988673 to AM988774.

Results

The long-term biofilm succession was followed in paper machines operating under slightly alkaline conditions at temperatures close to 50°C. The biofilms of the test coupons were less than 1 mm thick at all the sampling times, and their appearance was light gray and not slimy. During the pre-experimental testing of the Robbin's device, it was determined that the bacterial fingerprints of the replicate biofilm coupons were very similar. The average standard deviation of the four major peaks in replicate samples was ±8.2% of the relative percentage of fluorescence intensity, and the standard deviation of the peak size determination between replicate samples ranged from 0.17 to 0.30 bp (average SD 0.22 bp). Comparison of the average LH-PCR patterns from the biofilm and process water community profiles (Fig. 1) showed distinct bacterial profiles, although some common peak sizes were observed, such as peak P3, 529 bp, which was one of the dominant peaks in the biofilms but also in the process waters of both machines (abundance on average 26 and 17%, respectively, in PM B and PM C biofilms). The microbial profiles differed slightly between biofilm sampling times, but some peaks (e.g., peaks P1 and P3) were consistently abundant in all biofilm samples and in both paper mills. The LH-PCR patterns could be compared to the sequence lengths simulated from the international databases [25]. Based on this simulation of the EMBL database, the specific peak sizes were 501 bp for *Deinococcus geothermalis* and 483 bp for *Meiothermus* sp. and *Thermus* sp., which have been considered as models for primary-colonizing bacteria [7, 11]. These specific peak sizes or sizes close to them were seldom observed in the LH-PCR profiles in biofilms or process waters in paper machines B or C, but the peak 483 bp matching *Thermus/Meiothermus* was detected in another machine running under acidic conditions (pH 4.7) and high temperatures 69°C (unpublished observation).

Peaks P1–P4 indicate LH-PCR size classes (Fig. 1) that were most abundant and were present in all of the biofilm monthly summary profiles, and therefore this biomarker was selected for more detailed study. When the PM B biofilm samples were grouped by the length of the incubation, the proportion of peaks P1–P3 averaged 72% of all the fragments in the early (1–2 days) biofilms, but decreased to 44% in the older biofilms (10–15 days) (Fig. 2). The relative abundance of peak P3 decreased significantly within

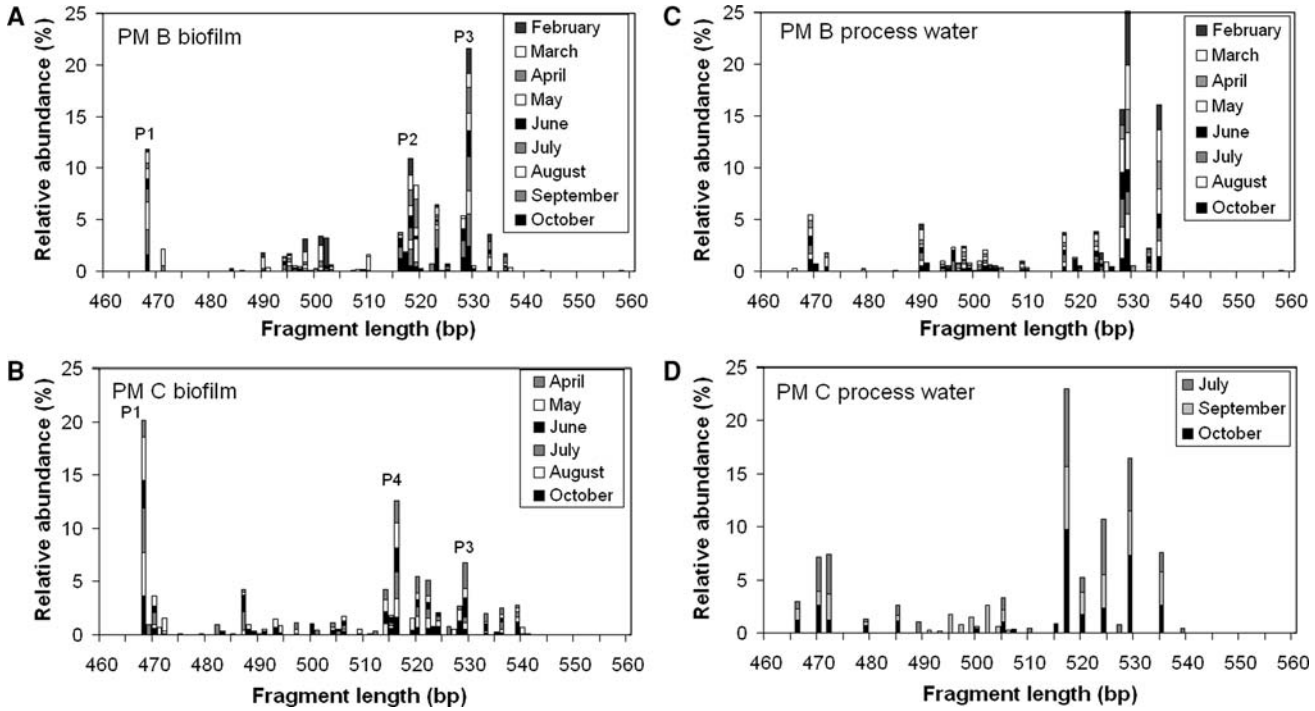


Fig. 1 Relative abundance of LH-PCR peaks from the paper machine (PM) B and C biofilms (**a**, **b**) and process water samples (**c**, **d**), respectively. The *bars* are divided into monthly distributions of relative peak abundance. Peaks *P1–P4* indicate size classes that were selected for more detailed study (Fig. 2)

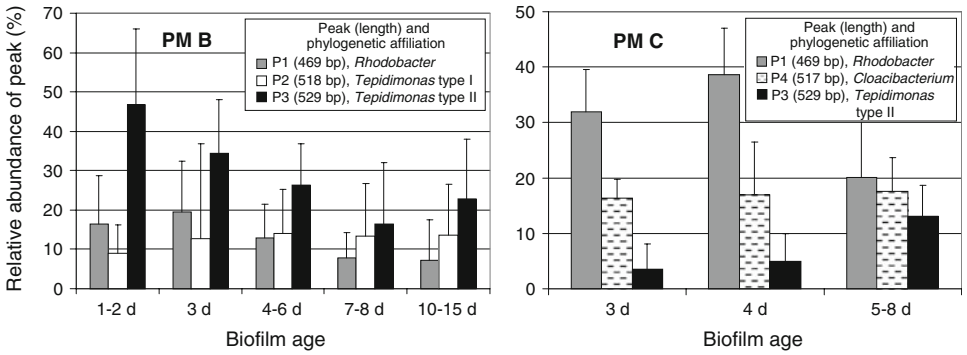


Fig. 2 Relative abundance of LH-PCR peaks P1–P4 (average ± SD) from PM B (*left*) and PM C (*right*) biofilm samples grouped by the biofilm age. $N = 9$ and 7 (on average) in each biofilm age class in PM B and PM C, respectively. *Tepidimonas* type I refers to the *Tepidimonas taiwanensis/Tepidimonas ignava*-clustering sequences and *Tepidimonas* type II refers to the sequences clustering with *Tepidimonas 'arfidensis'*

7 days ($P < 0.05$). For peak P1, the decreasing trend was not statistically significant. The peak P1 size could be assigned to the alphaproteobacterial genus *Rhodobacter* by comparing the peak lengths and sequenced insert lengths and identities in the 16S rRNA gene clone libraries. The closest *Rhodobacter* strain was *Rhodobacter 'gluconicum'* M37P (Fig. 3a) (EMBL accession number DQ363135, sharing 95.5–96.4% sequence identity with our clones), which has been isolated from hot 37°C geyser water. Peak P2 was assigned to *Tepidimonas ignava* or *Tepidimonas taiwanensis* (>99% sequence identity) and P3 to *Tepidimonas 'arfidensis'* SMC-6271 (>99% sequence identity), all belonging to the betaproteobacterial *Burkholderiales* group (Fig. 3b). Interestingly, all the *Tepidimonas ignava/taiwanensis* type (type I) partial 16S rRNA gene sequences were 11 bp shorter than the *Tepidimonas 'arfidensis'*-type sequences (type II). This was due to an extra hairpin loop in the rRNA sequences of the latter type. However, exclusion of this region did not change the topology of the tree in the phylogenetic analysis.

In addition to the sequence types mentioned, the 16S rRNA gene clone library from 1 day PM B biofilm included

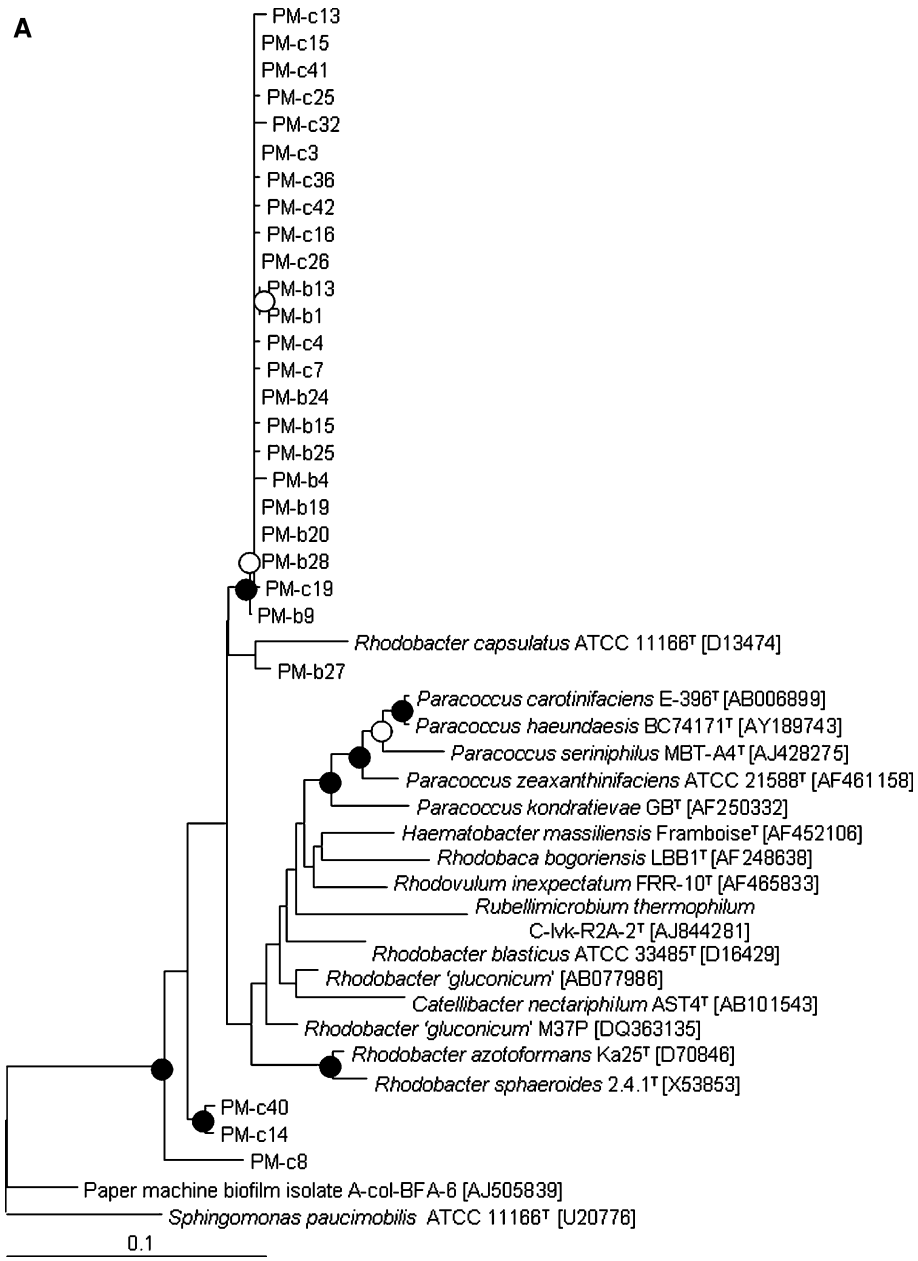
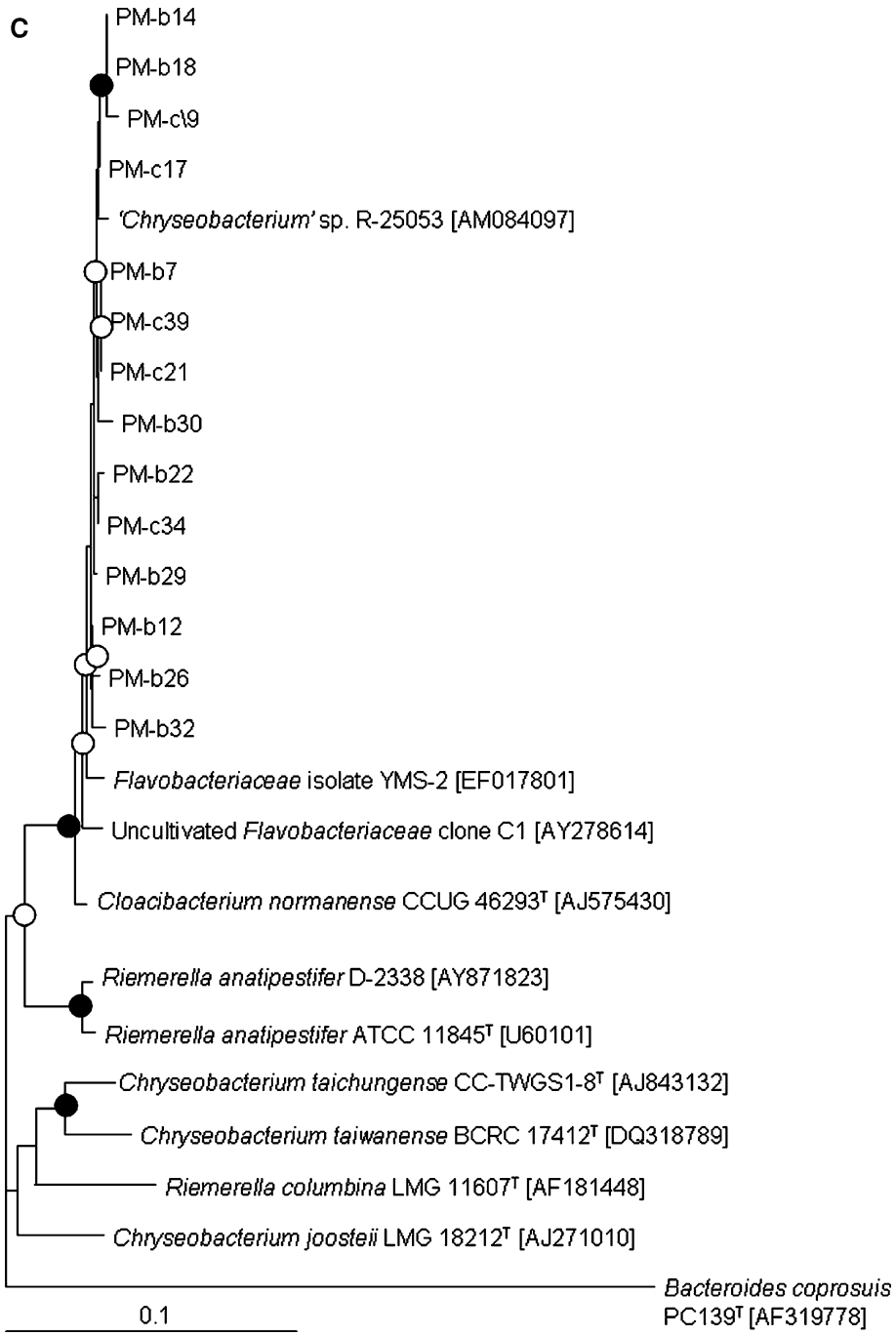


Fig. 3 Neighbor-joining trees showing the relationships of the biofilm 16S rRNA gene sequences matching peaks P1 (**a**), P2 and P3 (**b**), and P4 (**c**). The *scale bar* indicates 0.1 or 0.01 nucleotide changes per nucleotide; *dark* and *light circles* indicate ≥ 70% and ≥ 50% bootstrapping values, respectively

single sequences with closest matches to the alphaproteobacterial *Rhizobium, Azospirillum,* and *Pheospirillum* genera as well as to the actinobacterial species *Microbacterium barkeri*. The clone library from the 7-day biofilm also included several different *Clostridium*-type clones as well as *Petrobacter*, *Desulfocaldus, Pseudomonas thermotolerans, Fulvimonas, Deferribacter*, and *Fervidobacterium*-resembling clones, and clones closest to the unidentified strain RSI-24 isolated from rhizospere soil. However, the LH-PCR lengths of these clones did not match peaks P1–P3. The distribution of the LH-PCR sizes of cloned sequences did not totally cover all the lengths that were expected to appear, which might have been due to the moderately small size of the libraries.

The study of PM C covered biofilms of moderate age (3–8 days). The relative abundance of the three most common fragment sizes (P1, P3, and P4) was 50–60% in all samples. The proportion of P1 was also higher in the younger (3–4 days) biofilms than in older (5–8 days) biofilms ($P < 0.05$). In contrast, the proportion of P3 increased significantly between 3- and 4-day biofilms and 5- to 8-day biofilms. Again, all peak P1 matching clones were assigned to *Rhodobacter* sp., and the peak P3 matching single clone was assigned to *Tepidimonas 'arfidensis.'* The number of *Tepidimonas*-type clones was low in the PM C clone library but fitted well to the low proportion of the LH-PCR peak P3 in the specific PM C sample that was cloned. All the clones matching peak P4 were

Fig. 3 continued



assigned to the family *Flavobacteriaceae* with closest match to type strain of *Cloacibacterium normanense* (with ~99% identity with the type strain) (Fig. 3c). As there were no other closely related (>95% similarity) type strain sequences of other genera and the phylogenetic affiliation in the neighbor-joining tree was further confirmed with high (99.7%) bootstrapping confidence, all the flavobacterial clones were affiliated to *Cloacibacterium*. Closely identical *Cloacibacterium* sequences were also abundant in the PM B clone library, but only in the 1-day-old sample. Other sequence types with several [3–5] clones in the PM C library were closest to *Bacteriovorax* sp., *Bdellovibrio bacteriovorus*, *Pseudomonas thermotolerans*, and to an unidentified strain RSI-24, the sequence type that was also detected in PM B. Single clone sequences were closest to the *Azorhizobium, Schlegelella*, and *Terrimonas* 16S rRNA gene sequences.

Phylogenetic trees showed a narrow diversity in the obtained *Rhodobacter*-like sequences (Fig. 3a): nearly all sequences were identical or closely identical with only small 1- to 4-bp microheterogeneity, which might have been derived from PCR biases or from heterogeneity in 16S

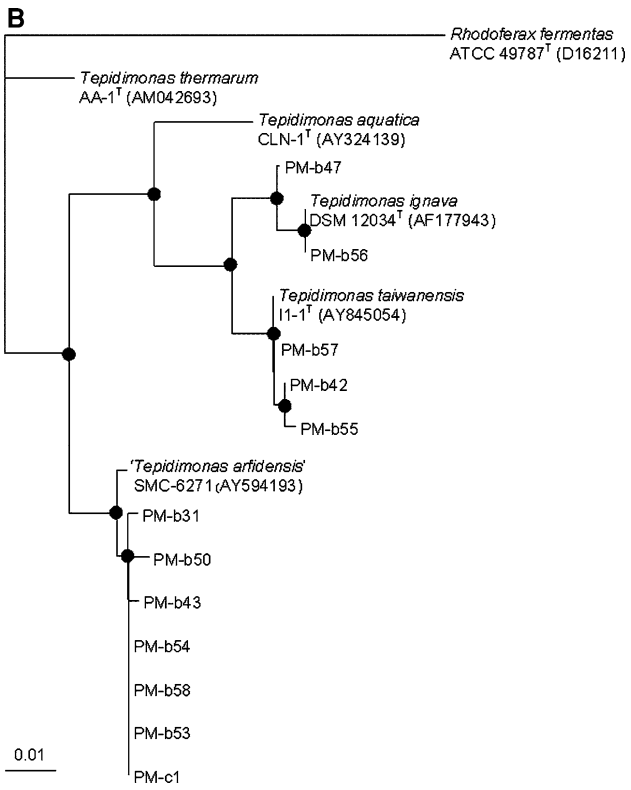


Fig. 3 continued

rRNA operons within organisms. The same 16S rRNA gene sequences were obtained from both the paper machines, which were located in different factories over 150 km apart. The *Tepidimonas* sequences were derived from nearly identical type II sequences present in both factories and from more diverse type I sequences, which were only obtained from older (7 day) PM B biofilms (Fig. 3b). The *Cloacibacterium* clones matching peak P4 also formed a phylogenetically coherent group of sequences. As with *Rhodobacter* and *Tepidimonas*-type sequences, identical *Cloacibacterium* sequences were obtained from both the paper mills.

Discussion

The aim of this study was to investigate the first steps of biofilm formation in paper machines and to reveal the bacterias' ability to colonize fresh stainless-steel surfaces. The most notable primary-colonizers in the slightly alkaline high temperature paper-machine conditions belonged to two major Gram-negative phyla: *Proteobacteria* (classes *Alphaproteobacteria* and *Betaproteobacteria*) and *Bacteroidetes*. A longer-term follow-up approach was selected to give more reliable and generalized data from each paper machine under real and slightly differing factory circumstances. The different incubation and biofilm formation times allowed some conclusions to be drawn about the succession and trends of bacterial communities specific to paper machines. Using DNA methods, the bias of culture techniques was avoided and the number of sampling times could be increased. Although PCR-based methods have their own problems with sample preparation and amplification, replicate samples showed acceptable precision for the quantity and base-pair size determination between replicate sample preparations. Combining data from LH-PCR profiles and clone libraries offered benefits from both of these methods. Fragment-length analysis is a cost-efficient, repeatable, and numerical profiling method [17], suitable for large sample sets of tens or hundreds of samples. Investigating fragment profiles may show the change in the community structure and reveal which samples are worth investigating using other methods. As sequencing using either the traditional cloning or modern pyrosequencing [20] approaches is costly, microbial profiling by fragment-length analysis offers a powerful and economical tool for microbial community studies.

This study focused on the abundance of the three dominant LH-PCR biomarkers (fragment sizes) detected in the paper machines. To identify the organisms behind these fragments, biofilm samples with high proportions of these LH-PCR sizes were selected for cloning and sequencing. The major LH-PCR fragment sizes in the early biofilms were assigned to the *Rhodobacter* type, *Tepidimonas* sequence types (type I and II), and the *Cloacibacterium* type. The LH-PCR analysis suggested that while *Tepidimonas* was the most common genus in the early PM B biofilms, *Rhodobacter* was the dominant primary-colonizer in PM C. The dominance of the biomarkers of these genera decreased in developing biofilms. The trend in the biomarker proportions suggested that the biofilm development process was not random, but that competition and stepwise succession were taking place in the multisubstrate environment. Besides the major LH-PCR sizes, several other sizes were detected in the LH-PCR profiles, but many of these were only detected during some samplings or some months. Also many other sequences were also obtained from the clone libraries but did not carry the sizes of the major LH-PCR peaks, and thus their relevance was not so clear.

The genus *Tepidimonas* includes four validly described, non-pigmented species, all isolated from hot springs or water tanks, but not previously from paper factories. The members of this genus are reported to grow aerobically in neutral or slightly alkaline conditions with their optimum temperature around 50–55°C, and from this perspective it is a unique genus in the *Betaproteobacteria*. The members of this genus are able to oxidize thiosulfate to sulphate in the presence of a metabolizable carbon source, and to assimilate organic acids and amino acids, but not carbohydrates or polyols [1, 4, 9, 18]. *Tepidimonas 'arfidensis'* SMC-6271

has the same features, but is not a validated species although it is fully described [10].

Rhodobacter is an alphaproteobacterial genus of mesophilic, anaerobic, phototrophic, purple nonsulfur bacteria including eight valid species, of which very many were earlier classified as *Rhodopseudomonas. Rhodobacter* species grow photoorganotrophically on a variety of organic compounds, including short-chain fatty acids, sugars, amino acids, and aromatic compounds, and members of this genus have frequently been isolated from sulfide-containing effluents [8], paper-mill slimes [11], and wastewater-treatment biofilms [21] and were also present in the biofilm of galvanized iron pipe after 1 day of model operation of the drinking-water distribution system [14]. The Robbin's device used for our biofilm study was made of stainless steel and was thus well-shaded from daylight, as are the paper machines. The high dominance of the *Rhodobacter* sequences in the early biofilms shows that light is not a precursor for the growth of these organisms, but they must be at least facultatively chemoorganotrophic. *Rhodobacter* '*gluconicum*' sequences (EMBL accession numbers DQ363135 and AB077986) were obtained from organisms transferring glucose to gluconic acid efficiently (information from the EMBL sequence annotation page). The new genus *Rubellimicrobium* [5] was described to accommodate red-pigmented, strictly aerobic, thermophilic biofilm bacteria from fine-paper machines and pulp driers within the deep line of descent in the family *Rhodobacteraceae.* However, all but one (PM-c8) of our biofilm clones were more closely related to *Rhodobacter* species than to *Rubellimicrobium thermophilum.* The sequence of *Rhodobacter* sp. A-col-BFA-6 isolated from a slightly alkaline high-temperature paper-machine environment [11] did not group with the clones of this study.

Flavobacteriaceae sequences of the biofilm libraries were all associated with *Cloacibacterium normanense*, a recently defined species of the *Bergeyella-Chryseobacterium-Riemella* branch of the family *Flavobacteriaceae. Cloacibacterium normanense* has been characterized as a facultatively anaerobic, rod-shaped bacterium and has been isolated from several paper mills from pulps containing 100% recycled fibers [22].

Many environmental factors, including temperature, oxygen concentration, pH, and availability of substrates, affect the final succession of microbial communities and thus can make biofilm structures highly variable. In the case of paper-mill biofilms, pH might be a principal parameter, since some machines operate under acidic and others under neutral or alkaline conditions. The pH conditions and running temperatures in both the machines we studied were slightly alkaline and supported growth of thermophilic bacteria (pH 7–8, 45–50°C). Of the known adhering paper-machine bacteria, *Deinococcus geothermalis*, e.g., has actually been isolated from acidic environments [12]. This might explain why the clone libraries as well as the LH-PCR profiles of newly formed biofilms did not have signs of this bacterium. On the other hand, several *Rhodobacter* and *Rubellimicrobium* isolates were obtained from slightly alkaline processes in previous studies [5, 11].

From the factory point of view, an important question is the origin of microbial contamination. The phylogenetic trees showed persistence of identical primary-colonizing bacteria in different paper mills, implying that the paper machines must share quite similar environmental conditions for bacteria and, potentially, a common source for contamination. The factories used different wood sources and different processes for making pulp. Considering the common feed material of the factories, both PM B and PM C used starch in the paper-coating process. The degradation products of starch provide simple sugars, which can either select for some of the species, or carry the specific primary-colonizing bacteria into the paper-mill process line.

This study was focused on the identification of the primary-colonizing bacteria able to attach to the fresh stainless-steel surfaces in slightly alkaline, high temperature, paper-machine conditions. This is the first step towards controlling the primary-colonizers by antimicrobials or anti-adhesion signals, and needs to be followed by culturing of the corresponding strains and characterization of their surface-attachment mechanisms and signalling systems. Although evidently important for the early biofilm succession on virgin stainless-steel surfaces, it would be important to know how the identified primary-colonizers survive in the later biofilm succession stages and in the clean-up procedures, and whether they subsidize colonization of biofilms after incomplete pipeline cleansing. Since established biofilms are notoriously difficult to remove, it is of fundamental interest to identify which bacteria remain after clean-up procedures—and whether these bacteria are the same as the primary-colonizers on virgin surfaces.

Acknowledgments We are grateful to Päivi Korhonen and Mirja Salkinoja-Salonen for valuable comments during the data collection period and afterwards and for Roger Jones for revising the style of the paper. The project was funded by the Technology Development Center of Finland (TEKES), Kemira Pulp&Paper and the Academy of Finland.

References

1. Albuquerque L, Tiago I, Verissimo A, da Costa MS (2006) *Tepidimonas thermarum* sp. nov., a new slightly thermophilic betaproteobacterium isolated from the Elisenquelle in Aachen and emended description of the genus *Tepidimonas*. Syst Appl Microbiol 29:450–456. doi:10.1016/j.syapm.2005.12.004
2. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. doi:10.1093/nar/25.17.3389

3. Blanco MA, Negro C, Gaspar I, Tijero J (1996) Slime problems in the paper and board industry. Appl Biochem Biotechnol 46:203–208
4. Chen TL, Chou YJ, Chen WM, Arun B, Young CC (2006) *Tepidimonas taiwanensis* sp. nov., a novel alkaline-protease-producing bacterium isolated from a hot spring. Extremophiles 10:35–40. doi:10.1007/s00792-005-0469-9
5. Denner EB, Kolari M, Hoornstra D, Tsitko I, Kampfer P, Busse HJ, Salkinoja-Salonen M (2006) *Rubellimicrobium thermophilum* gen. nov., sp. nov., a red-pigmented, moderately thermophilic bacterium isolated from coloured slime deposits in paper machines. Int J Syst Evol Microbiol 56:1355–1362. doi:10.1099/ijs.0.63751-0
6. Desjardins E, Beaulieu C (2003) Identification of bacteria contaminating pulp and a paper machine in a Canadian paper mill. J Ind Microbiol Biotechnol 30:141–145
7. Ekman J, Kosonen M, Jokela S, Kolari M, Korhonen P, Salkinoja-Salonen M (2007) Detection and quantitation of colored deposit-forming *Meiothermus* spp. in paper industry processes and end products. J Ind Microbiol Biotechnol 34:203–211. doi:10.1007/s10295-006-0187-z
8. Ferrera I, Massana R, Casamayor EO, Balague V, Sanchez O, Pedros-Alio C, Mas J (2004) High-diversity biofilm for the oxidation of sulfide-containing effluents. Appl Microbiol Biotechnol 64:726–734. doi:10.1007/s00253-004-1581-y
9. Freitas M, Rainey FA, Nobre MF, Silvestre AJ, da Costa MS (2003) *Tepidimonas aquatica* sp. nov., a new slightly thermophilic beta-proteobacterium isolated from a hot water tank. Syst Appl Microbiol 26:376–381. doi:10.1078/072320203322497400
10. Ko KS, Lee NY, Oh WS, Lee JH, Ki HK, Peck KR, Song JH (2005) *Tepidimonas arfidensis* sp. nov., a novel Gram-negative and thermophilic bacterium isolated from the bone marrow of a patient with leukemia in Korea. Microbiol Immunol 49:785–788
11. Kolari M, Nuutinen J, Rainey FA, Salkinoja-Salonen MS (2003) Colored moderately thermophilic bacteria in paper-machine biofilms. J Ind Microbiol Biotechnol 30:225–238
12. Kolari M, Schmidt U, Kuismanen E, Salkinoja-Salonen MS (2002) Firm but slippery attachment of *Deinococcus geothermalis*. J Bacteriol 184:2473–2480. doi:10.1128/JB.184.9.2473-2480.2002
13. Lahtinen T, Kosonen M, Tiirola M, Vuento M, Oker-Blom C (2006) Diversity of bacteria contaminating paper machines. J Ind Microbiol Biotechnol 33:734–740. doi:10.1007/s10295-006-0105-4
14. Lee DH, Lee JH, Kim SJ (2005) Diversity and dynamics of bacterial species in a biofilm at the end of the Seoul water distribution system. World J Microb Biotechnol 21:155–162. doi:10.1007/s11274-004-2890-0
15. Lindberg LE, Holmbom BR, Väisänen OM, Weber AM, Salkinoja-Salonen MS (2001) Degradation of paper mill water components in laboratory tests with pure cultures of bacteria. Biodegradation 12:141–148. doi:10.1023/A:1013175421662
16. McCoy WF, Bryers JD, Robbins J, Costerton JW (1981) Observations in fouling biofilm fonnation. Can J Microbiol 27:910–917
17. Mills DK, Fitzgerald K, Litchfield CD, Gillevet PM (2003) A comparison of DNA profiling techniques for monitoring nutrient impact on microbial community composition during bioremediation of petroleum-contaminated soils. J Microbiol Methods 54:57–74. doi:10.1016/S0167-7012(03)00007-1
18. Moreira C, Rainey FA, Nobre MF, da Silva MT, da Costa MS (2000) *Tepidimonas ignava* gen. nov., sp. nov., a new chemolitho-heterotrophic and slightly thermophilic member of the beta-Proteobacteria. Int J Syst Evol Microbiol 50:735–742
19. Oppong D, King V, Zhou X, Bowen J (2000) Cultural and biochemical diversity of pink-pigmented bacteria isolated from paper mill slimes. J Ind Microbiol Biotechnol 25:74–80. doi:10.1038/sj.jim.7000036
20. Ronaghi M, Karamohamed S, Pettersson B, Uhlén M, Nyrén P (1996) Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem 242:84–89. doi:10.1006/abio.1996.0432
21. Satoh H, Yamakawa T, Kindaichi T, Ito T, Okabe S (2006) Community structures and activities of nitrifying and denitrifying bacteria in industrial wastewater-treating biofilms. Biotechnol Bioeng 94:762–772. doi:10.1002/bit.20894
22. Suihko ML, Skyttä E (2009) Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres. J Ind Microbiol Biotechnol 36:53–64
23. Suzuki M, Rappe MS, Giovannoni SJ (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Appl Environ Microbiol 64:4522–4529
24. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882. doi:10.1093/nar/25.24.4876
25. Tiirola MA, Suvilampi JE, Kulomaa MS, Rintala JA (2003) Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR). Water Res 37:2259–2268. doi:10.1016/S0043-1354(02)00631-0
26. Väisänen OM, Nurmiaho-Lassila EL, Marmo SA, Salkinoja-Salonen MS (1994) Structure and composition of biological slimes on paper and board machines. Appl Environ Microbiol 60:641–653
27. Väisänen OM, Weber A, Bennasar A, Rainey FA, Busse HJ, Salkinoja-Salonen MS (1998) Microbial communities of printing paper machines. J Appl Microbiol 84:1069–1084. doi:10.1046/j.1365-2672.1998.00447.x
28. Verhoef R, Schols HA, Blanco A, Siika-aho M, Ratto M, Buchert J, Lenon G, Voragen AG (2005) Sugar composition and FT-IR analysis of exopolysaccharides produced by microbial isolates from paper mill slime deposits. Biotechnol Bioeng 91:91–105. doi:10.1002/bit.20494