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Community Structure and Diversity of Biofilms from a Beer Bottling Plant as Revealed Using 16S rRNA Gene Clone Libraries†

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The microbial composition of biofilms from a beer bottling plant was analyzed by a cultivation independent analysis of the 16S rRNA genes. Clone libraries were differentiated by amplified 16S rRNA gene restriction analysis and representative clones from each group were sequenced. The diversity of the clone libraries was comparable with the diversity found for environmental samples. No evidences for the presence of strictly anaerobic taxa or important beer spoilers were found, indicating that biofilms developed for more than 6 months at the plant formed no appropriate habitat for those microorganisms. The genus *Methylobacterium* was one of the dominating groups of the clone libraries. The size of this population was assessed by fluorescence in situ hybridization and fatty acid analysis. In addition, considerable numbers of clones were assigned to uncultivated organisms.

Most industrial plants are inhabited by biofilms. Although these biofilms are usually without impact on the product, in a certain stage, these biofilms can be colonized by product-spoiling microorganisms. The interrelation and dependency of product-spoiling bacteria from other biofilm-forming microorganisms was shown for the paper industry (22) and also for the food industry (37). For breweries, the most important beer-spoiling organisms are members of the *Lactobacillaceae*, e.g., *Lactobacillus brevis* and *Lactobacillus lindneri*, and of the strictly anaerobic *Acidaminococcaceae*, particularly *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, and *Megasphaera cerevisiae*. All of these are supported by acidophilic and anaerobic conditions provided by the microbial biofilm community. According to these versatile interrelations between product spoilers and biofilm-forming microorganisms, the composition of the biofilm community is of interest. There were several attempts to characterize the biofilm-producing microorganisms by cultivation approaches (21, 45, 48). However, this approach provided only an incomplete picture of the community. To assess also those populations, which were not detectable by cultivation-based methods, we constructed two clone libraries of 16S rRNA genes from DNA extracted directly from the biofilms. This approach allows also the detection of strictly anaerobic taxa which would categorize the biofilm as a potential source of beer contaminants.

Biofilms were collected from the bottling plant of a brewery by means of sterilized spatulas, transferred into sterile tubes, stored at 4°C, and processed within 20 h. Samples were taken on January 11 (designated the Screw sample) and on March 22 (designated the Center sample), 2001. Both sample sites were near the route by which the open, filled bottles traveled to the capping element and therefore potential sources for product contaminations. The biofilm of the Center sample was located on a plastic seal. The Screw sample biofilm was taken from a part of a steel screw which was not accessible to daily cleaning procedures. Both biofilms were about 1 to 2 mm high. The daily cleaning procedures involved sprinkling with water of 85°C for 3 min in intervals of 2 h, and at the end of a filling procedure, the plant was foamed with an alkaline cleaner. The complete plant was cleaned intensively in August 2000. Therefore, both biofilms developed in about 7 months and at least the Center sample biofilm has been exposed to regular cleaning stress.

DNA was extracted by using a modified protocol described by Zhou et al. (54). The sample material was suspended in 12 ml DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate buffer [pH 8.0]) containing 0.085 mg ml^{-1} proteinase K and 3 mg ml^{-1} lysozyme. Samples were incubated at 37°C for 1 h on a horizontal shaker. Thereafter, 3 ml of 10% sodium dodecyl sulfate was added and incubated for 1 h. Hexadecylmethylammonium bromide and NaCl were added to final concentrations of 1% (wt/vol) and 1.5 M, respectively, and incubated for 15 min at 65°C. Subsequently, a freezing and thawing procedure in liquid nitrogen and in a 65°C water bath was repeated three times. The material was centrifuged at 6,000 × *g* at 4°C. The supernatant was transferred to a separate tube, and 1 volume of chloroform-isoamyl alcohol (24:1 [vol/vol]) was added. The mixture was shaken and centrifuged at 6,000 × *g* at 4°C. The aqueous phase was recovered, and nucleic acids were precipitated by the addition of 0.7 volumes of isopropanol and 0.3 volumes of 10 M ammonium acetate and pelleted by centrifugation at 16,000 × *g* at 4°C. The pellet was washed with 70% ethanol, dried, and resuspended in 150-μl autoclaved double-distilled water. The purified DNA was used for amplification of nearly the complete 16S rRNA gene by using the two universal primers GM3F (*Escherichia coli* 16S rRNA gene positions 8 to 23 [8]) and GM4R (*E. coli* positions 1492 to 1507) (33). PCR was carried out with 0.5 μM of each primer, 200 μM of each deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$, 1× PCR buffer, 2.5 U of *Taq* DNA polymerase (Gibco-Life Technolo-

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gies, Karlsruhe, Germany) and various amounts of template, adjusted to a final volume of 100 μl with autoclaved double-distilled water. The PCR consisted of denaturation at 95°C for 1 min, except that the initial denaturation was 5 min, annealing at 49°C for 1 min, and extension at 72°C for 2 min (30 cycles were done), followed by a final extension at 72°C for 10 min. The PCR products were purified (PCR purification kit; QIAGEN, Hilden, Germany) and quantified by using the PicoGreen double-stranded DNA quantification kit (Molecular Probes, Eugene, OR) and a Fluostar fluorescence reader (SLT Instruments, Crailsheim, Germany). The PCR products of three independent PCRs were pooled to avoid initial bias. The 16S rRNA gene products were ligated into the pCR2.1-TOPO vector. Plasmids were transformed into TOP10 One Shot-competent cells by following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Recombinant transformants were selected by blue/white colony screening. Individual colonies were grown overnight in 5 ml Luria-Bertani medium. Two milliliters was used for plasmid preparation (QIAprep Spin Miniprepkit; QIAGEN, Hilden, Germany).

Plasmid sizes were controlled in a 0.8% agarose gel in 1× Tris-acetate-EDTA buffer (DNA-typing grade; Life Technologies, Gaithersburg, MD). Only plasmids of approximately 5.4 kb carried the 16S rRNA gene insert of about 1.5 kb and were further processed. The plasmid preparations were diluted 1:20 and used as templates (about 10 to 15 ng) in a PCR which contained the components as described above, but the annealing temperature was reduced after 10 cycles from 49°C to 44°C. The PCR products were purified and quantified as described above.

For a restriction analysis, nine tetrameric restriction enzymes were tested in silico by using the TACG restriction enzyme analysis (Mangalam) tool from the Pasteur Institut, Paris (http://bioweb.pasteur.fr/seqanal/interfaces/tacg.html). Twenty-eight 16S rRNA gene sequences from the brewery-related genera *Acetobacter*, *Gluconobacter*, *Lactobacillus*, *Lactococcus*, *Megasphaera*, *Pediococcus*, *Weissella*, and *Zymomonas* were restricted in silico to obtain reference patterns. The fragment patterns were analyzed by using the Stanford Center for Tuberculosis Research Molecular Fingerprint Analyzer version 0.4.1 (beta). The best differentiation was achieved with AluI, which resulted in 18 different clusters of the 28 sequences, followed by MboI, with 13 clusters. The other enzymes tested were less suitable for the resolution of these bacteria. The predicted restriction patterns were checked by comparison with real patterns obtained by in vitro restriction of 25 strains from the genera *Acetobacter*, *Gluconobacter*, *Kocuria*, *Lactobacillus*, *Lactococcus*, *Megasphaera*, *Pectinatus*, *Pediococcus*, *Selenomonas*, and *Zymophilus*. The restriction mixtures contained 800 ng of the PCR-amplified 16S rRNA gene, 7.5 U AluI (New England Biolabs, Frankfurt am Main, Germany), 1× NEBuffer 2, and autoclaved double-distilled water was added to a final volume of 15 μl. They were incubated at 37°C for 3 h. The resulting fragments were separated in 3% NuSieve 3:1 agarose gels (BMA, Rockland, ME) in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM sodium EDTA · $2H_2O$) for 300 min at 70 V. A 50-bp DNA ladder (Life Technologies, Gaithersburg, MD) was used as a standard. The gels were stained with ethidium bromide (1 μg ml^{-1}). Images were acquired by using a charge-coupled-device camera and analyzed by using the software packages FragmentNT Analysis and ImageQuant (versions 1.2 and 5.2, respectively; Molecular Dynamics, Sunnyvale, CA). Eight of 11 species gave identical restriction patterns in silico and in vitro. To overcome these inconsistencies, the in vitro patterns were chosen for comparison to the ones of the clone libraries, if available. In addition, 16S rRNA gene restriction analysis (ARDRA) reference patterns were generated from 26 strains isolated previously from this bottling plant. The strains are members of the genera *Achromobacter*, *Acidovorax*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Brevundimonas*, *Citrobacter*, *Chryseobacterium*, *Deinococcus*, *Enhydrobacter*, *Enterobacter*, *Escherichia*, *Microbacterium*, *Micrococcus*, *Pseudomonas*, *Roseomonas*, *Staphylococcus*, and *Stenotrophomonas*. Most of these strains were described previously (47).

Clones with identical ARDRA patterns were grouped and were referred to as ARDRA groups. Several clones of every ARDRA group with more than two clones were sequenced by using approximately 350 ng of 16S rRNA gene PCR product, 0.8 μM primer 787R (9), 4 μl of Terminator Ready Reaction Mix (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit, version 2.0; Applied Biosystems, Foster City, Calif.), and water to a final volume of 10 μl. The PCR consists of 30 cycles of denaturation for 1 min at 96°C, annealing for 15 s at 50°C, and extension for 4 min at 60°C. For complete sequencing of the 16S rRNA gene inserts, the primers pUC/M13 and 518F were used in addition (9). The sequencing product was purified by using the DyeEx Spin Kit (QIAGEN, Hilden, Germany). The electrophoresis was performed on an ABI377 sequencer (Applied Biosystems). The obtained sequences were compared to those in the National Center for Biotechnology Information nucleotide sequence database by using the BLAST algorithm (1). Sequences of type strains were preferred for the taxonomic assignment rather than more similar sequences from clones or other strains because of the reliable identity of type strains. All sequences were checked for chimera formation with the CHIMERA_CHECK software of the Ribosomal Database Project (10). Additionally, the phylogenetic affiliations of the 5′ and 3′ ends of putative chimeras were compared by using the ARB software package (29). By this procedure, seven potential chimeras (8.2% of all analyzed sequences) were detected and rejected from further processing.

The number of analyzed clones was 216 for the Center sample library. These clones were differentiated into 24 different ARDRA groups and 29 single ARDRA patterns. At least two clones of each ARDRA group were sequenced. In total, 66 sequences were obtained from 24 different ARDRA groups and 5 single ARDRA patterns, representing a total of 192 clones. Twenty-four clones with unique ARDRA patterns were not subjected to sequence analyses. From the Screw sample library, 80 clones were analyzed by ARDRA, which resulted in 59 different patterns. Sixteen clones were sequenced; each of them represented an ARDRA cluster with one to six members. Therefore, they covered 37 clones of the 80 examined ones. From 24 ARDRA groups from both libraries analyzed for homogeneity, seven groups showed no sequence variations. The arithmetic average sequence similarity within all analyzed groups was 99.0%, with a median of 99.6%. This emphasized the high discriminative power of the ARDRA.

The sequence analyses of the Center sample library showed that gammaproteobacteria and alphaproteobacteria made up 42.7% and 28.7% of the 192 taxonomical assigned clones, respectively. The gammaproteobacteria were the most abundant group and consisted of members of the *Moraxellaceae* and *Xanthomonadaceae*. In particular, the genera *Acinetobacter*, *Enhydrobacter*, *Luteimonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, and *Xanthomonas* were identified (see the supplemental material). The genera *Methylobacterium* and *Paracoccus* represented the most frequently detected clone groups of the alphaproteobacteria. Further clones were assigned to sequences of the genera *Agrobacterium*, *Brevundimonas*, *Devosia*, *Erythrobacter*, *Porphyrobacter*, and *Sphingomonas*. Also abundant taxa were the flavobacteria/sphingobacteria (15.6%), represented by *Chryseobacterium* species and by the genera *Flexibacter*, *Sphingobacterium*, and *Pedobacter*. Only three clones (1.6%) were assigned to the betaproteobacteria genera *Xylophilus* and *Acidovorax*. The other clones were affiliated with the acidobacteria (5.7%) and the uncultured candidate division TM7 (5.7%).

The main groups of the Screw sample clone library were also the alphaproteobacteria and gammaproteobacteria with 29.7% and 18.9% of all clones, respectively. Alphaproteobacteria were represented by the genera *Devosia*, *Sphingopyxis*, and *Blastochloris*. Gammaproteobacteria were represented by the genera *Acinetobacter*, *Citrobacter*, *Enhydrobacter*, *Thermomonas*, and *Xanthomonas*. Two major groups of this library consisting of six and five clones, respectively, showed low sequence similarities only to the uncultured candidate division TM7 and to an uncultured clone which originated from a soil sample.

The comparison of the 112 ARDRA patterns from both samples (53 from the Center sample and 59 from the Screw sample) showed that four patterns were found in both of the libraries. This was the ARDRA group assigned to *Acinetobacter johnsonii*, which was one of the two dominating groups of the Center sample library, a group assigned to *Enhydrobacter aerosaccus*, a pattern affiliated with *Devosia riboflavina*, and an ARDRA group which was assigned to the species *Flavobacterium ferrugineum*.

With the exception of a single clone, the partial sequence and ARDRA pattern of which were in accord with those of the species *Lactococcus lactis*, no potential beer-spoiling species was detected. This negligible abundance or even absence of beer-spoiling bacteria is in line with results of a previous analysis of infeed and discharge conveyor biofilms from this brewery (47). Eight ARDRA patterns from the clone libraries were identical to those from strains isolated previously from this plant. The sequences of these clones were compared with the sequences of the isolates, and the similarities are presented in Table 1. These strains confirmed the presence of the corresponding clone groups in this bottling plant and indicated a distribution of these strains at different sites. However, the vast majority of the revealed ARDRA patterns of the clone libraries had no corresponding counterpart among the isolates.

The frequency of the sequences obtained from the clone libraries depends on the efficiencies of DNA extraction and of the PCR. Therefore, the quantitative composition of the community was only insufficiently reflected by the ARDRA group sizes. To evaluate the abundance of the dominating clone groups in situ, we analyzed the fatty acid composition of the Center sample biofilm and hybridized the samples with fluorescently labeled probes. These analyses were performed only with the Center sample due to the scarce biomass of the Screw sample biofilm. Sixty milligrams of the Center sample material was analyzed. Prior to the fatty acid preparation, abiotic lipids from lubricants were removed as described previously (47). Saponification, methylation, and extraction of the fatty acid methyl esters were done according to Sasser (38). Identification of the fatty acid methyl esters was performed by gas chromatography-mass spectrometry as described previously (26).

TABLE 1. Similarities of 16S rRNA gene sequences from clones and from cultivated strains showing identical ARDRA patterns

Clone[a]	Isolate	% Similarity[b]	Corresponding taxonomic assignment of clone and isolate
MTAG33	DW126	100 (587)	*Acidovorax*
MTAG9	DW149	99.4 (1,484)	*Acinetobacter*
MTBIII10	DW149	98.9 (718)	*Acinetobacter*
MTAB27	DW78	98.9 (627)	*Brevundimonas*
MTAD19	DW32	99.8 (666)	*Enhydrobacter*
MTBII12	DW32	100 (651)	*Enhydrobacter*
MTAE42	DW100	99.3 (1,378)	*Stenotrophomonas*
MTBI32	DW104	98.6 (589)	*Achromobacter*
MTBI44	DW99	99.6 (570)	*Citrobacter*
MTBIII7	DW129	97.2 (504)	*Microbacterium*

[a] The clones starting with MTA originated from the Center sample and the ones with MTB from the Screw sample, respectively. For detailed information, see also supplemental material.
[b] The numbers of compared bases are given in parentheses.

For fluorescence in situ hybridization (FISH) sample materials were suspended in Ringer's solution (0.9% NaCl, 0.042% KCl, 0.024% $NaHCO_3$) before fixation. The fixation and hybridization with Cy3-labeled probes was done as described by Friedrich et al. (15), by using paraformaldehyde or ethanol for fixation. Formamide concentrations in the hybridization buffers were used according to the respective references. Fluorescing cells were counted by means of an epifluorescence microscope (Zeiss Axioskop). Probe-positive counts were determined relative to 4′,6-diamidino-2-phenylindole (DAPI) (1 mg liter^{-1})-stained cells. At least 400 cells were counted in triplicates per probe. The probes used are given in Table 2; probe Pae997 was supplemented with two helper oligonucleotides (47). As a negative control, the sample was hybridized with probe NON338.

The bacterial detection rate obtained by fluorescence in situ hybridization with a combination of the probes EUB338 (3), EUB338II, and EUB338III (11) was 60.7% (Table 2). The probe signals for *Eukarya* and *Archaea* made up a little proportion (2.6% and 1.6%); therefore, about 65% of all DAPI-stained cells could be characterized by this approach.

In accordance with the abundance of alphaproteobacteria in the clone libraries (28.7% and 29.7%, respectively), probe ALF969 detected the most DAPI-stained cells of all probes used (11.7%). Correspondingly, the fatty acid *cis*-vaccenic acid (Δ11-*cis*-octadecenoic acid, 18:1 *cis*11) was the dominating compound in the fatty acid profile of the Center sample (Table 3). This lipid is the major component of membranes of alphaproteobacteria (27). One of the most abundant clone groups was affiliated with *Methylobacterium extorquens*. In contrast to

TABLE 2. Fluorescence in situ hybridization results of the Center sample

Probe	Mean percentage of DAPI-stained cells (SD)[a]	Target group, genus, and/or species	Reference(s)
EUB338[b]	60.7 (5.1)	Bacteria	3, 11
EUK502	2.6 (1.1)	Eukarya	4
ARCH915	1.6 (0.4)	Archaea	43
PLA46	2.0 (1.0)	*Planctomycetales*	34
CF319a	9.6 (3.2)	*Cytophaga-Flavobacterium* cluster of the CFB phylum	30
ALF968	11.7 (0.9)	Alphaproteobacteria	34
BET42a	5.0 (2.3)	Betaproteobacteria	31
GAM42a	4.8 (1.2)	Gammaproteobacteria	31
HGC69a	3.8 (1.4)[c]	*Actinobacteria*	36
LGC354A-C	3.8 (1.2)[c]	*Firmicutes*	32
ACA652	0.9 (0.4)	*Acinetobacter*	50
AG1427	0.1 (0.1)	*Acetobacter* sp. and *Gluconobacter* sp.	34
ENT183	0.0 (0.0)	Enterobacteria	17
Pae997	1.0 (0.8)	*Pseudomonas aeruginosa* and further *Pseudomonas* spp.	2
XAN818	1.9 (0.8)	Xanthomonadales	15
NON338	0.0 (0.0)	Negative control	51

[a] All values originated from paraformaldehyde-fixed samples except the HGC69a and LGC354A-C percentages.
[b] A mixture of EUB338, EUB338-II, and EUB338-III was used.
[c] Ethanol-fixed sample.

the majority of the alphaproteobacteria, the genus *Methylobacterium* does not transform 18:1 *cis*11 to 19:0 cyclo11-12 (14). This compound was also absent from the lipid profile of the biofilm, which indicates that this genus actually represents a large population in the biofilm community. The presence of *Paracoccus*-related sequences in the Center sample biofilm was also in accord with the detection of characteristic lipid components of this genus (10:0 3-OH, 12:1 *cis*5, 18:1 *cis*11, and 18:0 [12, 28]). In contrast to these taxa, the *Acetobacteraceae* were absent in both clone libraries and almost not detected by FISH (0.1%) in the Center sample. In addition, 2-hydroxy-hexadecanoic acid, a typical fatty acid for *Acetobacteraceae* (53), could not be detected in the lipid extract. Because of their acid production and oxygen consumption, the *Acetobacteraceae* are supposed to play a decisive role during the succession of biofilms in breweries. For the biofilms investigated in this study, we could not confirm their presence in the biofilm community.

A comparison of the proportions of gammaproteobacteria in the clone libraries (42.7% and 18.9%, respectively) and the FISH data, with 4.8% of GAM42a signals and 1.9% for the probe XAN818, indicated an overestimation of the gammaproteobacteria in the clone library. In contrast, 5.0% of FISH signals for betaproteobacteria corresponded to 1.6% of clones affiliated with this class in the Center sample library and 5.4% in the Screw sample library. This shows that no reliable quantitative information can be derived from the clone libraries generated in this study. Accordingly, clones affiliated with the genus *Acinetobacter* made up 13.6% of the identified clones in the Center sample library, but only 0.9% of the cells were labeled with probe ACA652. The target sequence of probe ACA652 is present in strain *Acinetobacter* sp. DW149 isolated from this plant and in all *Acinetobacter*-related clones from both libraries. Moreover, strain DW149 showed a bright signal when hybridized with probe ACA652.

Several strains of the *Pseudomonadaceae* and *Enterobacteriaceae* were isolated from the plant (47; our unpublished data), which was in accord with other reports about the biofilm composition in filling plants (5). However, no sequence of these families was detected in the Center sample clone library. Accordingly, only 1% signals for the probe Pae997 and no signal for probe ENT183 were obtained. These data suggest that cultivation-based analyses favored the detection of gammaproteobacteria. This phenomenon was already described for other technical plants (50).

The flavobacteria/sphingobacteria constituted a proportion of 15.3% in the Center sample clone library and 10.8% in the Screw sample library. The probe CF319a revealed 9.6% probe-conferred signals for the Center sample, which confirmed the quantitative importance of these organisms in the biofilm. Because two clones assigned to this group have more than two mismatches with probe CF319a, the portion of the flavobacteria/sphingobacteria is probably higher than indicated by this probe. However, the small amount of the main fatty acid of this group, 15:0 *iso* (27, 52) showed that the flavobacteria/sphingobacteria were not a dominating population of the biofilm community.

The gram-positive phyla *Actinobacteria* and *Firmicutes* were detected by FISH with percentages of 3.8% for each of these phyla (Table 2). Obviously, these populations were underrepresented in the clone libraries because of their higher resistance to cell lysis during the DNA extraction protocol. In this

TABLE 3. Fatty acid composition of the Center sample

Fatty acid	% of the complete profile
10:0	0.3
11:0 *iso*	1.0
10:0 3OH	1.7
12:1 *cis*5	2.4
12:0	0.5
11:0 *iso* 3OH	0.7
12:0 3OH	0.5
14:0 *iso*	0.4
14:0	0.6
14:1 *trans*2	1.2
15:0 *iso*	1.8
15:0 *anteiso*	10.6
15:0	1.0
14:0 3OH	1.6
16:0 *iso*	2.8
16:1 *cis*9	4.9
16:1 *trans* 9	0.4
16:0	7.6
17:1 *iso cis* 9	0.5
17:0 *iso*	0.6
17:0 *anteiso*	4.1
17:1 *cis*9	4.6
17:0 cyclo9-10	0.7
17:0	2.2
18:2 *cis*9,12	12.0
18:1 *cis*9	10.9
18:1 *cis*11	22.0
18:0	2.2
18:0 10 *methyl*	0.4

study, no additional mechanical lysis was used. Only one clone of each phylum could be found in the Screw sample library. These clones were assigned to the genera *Microbacterium* and *Lactococcus*, respectively. The detection of the major fatty acids of the genus *Microbacterium*, 15:0 *anteiso* and 17:0 *anteiso* (6), supported the presence of representatives of this group. The isolation of two *Microbacterium* strains from this plant in the course of a previous study (47) demonstrated the relevance of this taxon as part of the biofilm community. In contrast, the presence of the only potential beer-spoiling species, *Lactococcus lactis*, as suggested by one clone of the Screw sample library, could not be confirmed by isolation or fatty acid analysis. Lactobacillic acid (19:0 cyclo11-12), which is a major compound of most *Lactococcus* species (39), was not detected in the fatty acid profile of the Center sample (Table 3). In general, clone libraries seem to underestimate the proportion of gram-positive bacteria detected by other direct methods like cultivation (7, 42) or FISH (16).

Additionally, members of the *Acidobacterium* group and the uncultured candidate division TM7 were found in the clone libraries, for which the most similar sequences originated from a soil isolate and from a cloned sequence of hot spring samples, respectively. Because phenotypic information of these phyla is scarce or even absent, no prediction about the function or requirements of these organisms in the biofilm can be made.

In addition to the high bacterial diversity, representatives of the *Eukarya* and *Archaea* were also detected in the biofilms. *Eukarya* were detected by the presence of the polyunsaturated fatty acid linoleic acid (18:2 *cis*9,12), which is abundant in yeasts (23, 44) but rarely found in bacteria. The hybridization of single cells with the fluorescently labeled probe EUK502 detected 2.6% probe-conferred signals of all DAPI-stained cells. Because of the larger cell sizes of yeasts, a population of only 2.5% of yeasts cells can account for more than 10% of the fatty acid profile (47a). The archaeal proportion of the Center sample community was 1.6%, as revealed by FISH. The presence of *Eukarya* and *Archaea* in this plant was also confirmed by PCR (data not shown) obtained with extracted DNA from the Screw sample and primers for the archaeal (Arch21F and Arch958R) or eukaryotic (EukF and EukR) small-subunit rRNA genes (13). The FISH analyses showed that cell numbers of *Eukarya* and *Archaea*, in spite of their unambiguous presence in the biofilm, were below 3% of total cell numbers and bacteria formed the dominating biofilm community.

The ARDRA patterns were also used for calculation of diversity indices and for rarefaction analysis. A rarefaction analysis of the ARDRA patterns was done to estimate to what extent the diversity of the samples studied can be described by the number of clones examined (Fig. 1). Rarefaction calculations were done using the program aRarefactWin (version 1.3 [S. M. Holland]). The rarefaction curve of the Center sample clone library indicated that the majority of ARDRA profiles present in the sample was covered by the analysis. This was also confirmed by the coverage value (18) of 86.6% for the 216 examined clones. In contrast, the much steeper curve of the Screw sample clone library and the coverage value of 40% confirmed the higher diversity in this clone library. A Chao1 estimation (19) indicated a total pattern number of 113 for the Center sample and 203 patterns for the Screw sample. Thus, further analysis of clones would have discovered additional

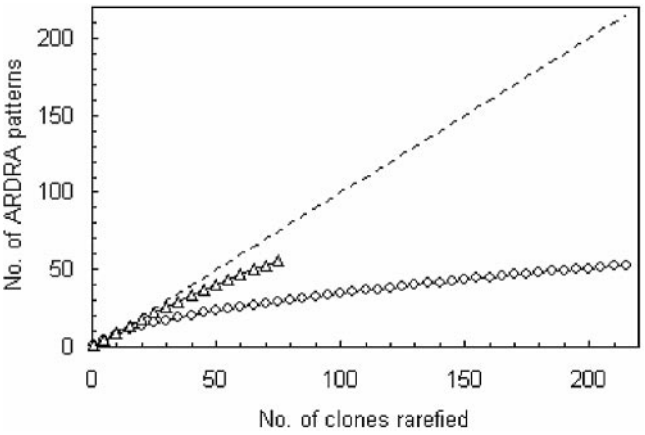

FIG. 1. Rarefaction curves calculated for the different ARDRA patterns. The dashed line equals a slope of 1, which is the case if every new examined clone has a new pattern. Different slopes of the curves for the Center sample (open circles) and for the Screw sample (open triangles) shows that the diversity of the Center sample was more completely covered than the diversity of the Screw sample.

diversity. The Shannon-Weaver diversity indices (40) were 3.27 for the Center sample and 3.91 for the Screw sample, respectively; the maximal possible values for the clone libraries would have been 5.38 (Center) and 4.38 (Screw). The Shannon evenness index was 0.82 for the Center sample library and 0.92 for the Screw sample library, which indicates a higher dominance of certain ARDRA patterns in comparison to the Screw sample library (0.96), where the Shannon evenness index approached 1. The same aspect is expressed by a higher Simpson's index of dominance concentration (41) of 0.059 for the Center sample library and 0.026 for the Screw sample clones, respectively.

Several processing steps in the construction of a 16S rRNA gene clone library may discriminate certain taxa (49). This results in differences of the composition of a clone library and the one of the microbial community in vivo. Despite all critical steps, the clone libraries of this study revealed a high diversity. This result implies that the methodology worked effectively for various phylogenetic groups and the confirmed diversity has to be considered to be the minimal one present.

This study shows that the microbial community of biofilms in technical plants was only insufficiently described by cultivation-based methods. This disadvantage affects also the cultivation independent PCR-based methods, because specific primers are usually designed based on sequence information from isolated strains. The cloning approach, in combination with complementing methods, shows that several taxa were not yet considered members of filling-plant-associated biofilms. One of the most interesting of these is the genus *Methylobacterium*, which can readily be overlooked by cultivation approaches. However, by use of appropriate medium, this taxon can easily be isolated for further physiological analysis. One of the most interesting putative properties of this group is the potential degradation of formaldehyde or formaldehyde-delivering sanitizers. This property may contribute to the remarkable resistance of biofilms against disinfectants (24).

This study shows also an unexpected high diversity for this habitat, which is similar to that reported for environmental

samples. Although the bottling plant is intensively cleaned, this process did not result in species depletion. The diversity indicated the presence of a large gene pool, which could enable the biofilm community to react on changing cleaning procedures and different disinfecting agents. This strengthens the demand for preventing mature biofilms before they become a serious problem.

The interesting functions of brewery biofilm organisms are those ones which enable the formation of biofilms and which promotes the colonization of the biofilm with beer-spoiling bacteria. In particular, these are the excretion of extracellular polymeric substances (EPS) and a high respiration rate. The secretion of EPS to produce a matrix is very widespread among bacteria. A number of microorganisms produce large amounts of EPS, a well-known example of which is xanthan, produced by *Xanthomonas campestris* (46), which was the next relative type strain for clones from both sample sites. EPS production has been also described for *Acinetobacter* sp. (25, 35). The oxygen-requiring metabolism for establishing anaerobic microenvironments can be performed by every taxon which has been affiliated with the clone library sequences, except the *Lactococcus lactis*. Therefore, both functions could obviously be fulfilled in mature biofilm communities like the examined one. Remarkably, no clone sequence was assigned to strictly anaerobic bacteria. This absence was supported by the fatty acid profile of the Center sample. In this analysis, no plasmalogenes which are present in many anaerobic bacteria (20) were detected.

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