Analysis of Biofilm Communities in Breweries

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Für meine Familie

Preface

This work was part of the joint research project “Development of innovative strategies for an efficient and environmentally sound abatement of biofilms in the food industry considering the filling of beer exemplarily” (title in German: “Entwicklung innovativer Strategien zur effizienten und umweltschonenden Bekämpfung von Biofilmen in der Lebensmittelindustrie am Beispiel der Bierabfüllung”). The project partners were the Lehrstuhl Aquatische Mikrobiologie, Universität Duisburg-Essen, Privatbrauerei A. Rolinck GmbH & Co, Steinfurt, the Bitburger Brauerei Th. Simon GmbH, Bitburg and the Department of Microbiology, University of Osnabrück.
The results are presented in nine Chapters. The Chapters 1 to 4 are manuscripts and represent the main part of this study. Additional data are reported in Chapters 5 to 9 to complete the picture of microbial communities of brewery biofilms obtained in this study.

Acknowledgments

First of all, I want to express my sincere thanks to many people, all of which contributed to this work in a scientific or private way.

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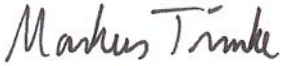

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Summary

The main objective of this study was the characterization of surface associated microbial communities in breweries. In addition, the beer-spoiling potential of isolated strains and biofilm samples was investigated. Some studies reported the identity of cultivatable organisms from industrial plants. However, there were no data available about the composition of biofilm communities from these habitats for cultivation-independent techniques. Consequently, the fatty acid methyl esters (FAMEs) analysis, the fluorescence *in situ* hybridization (FISH) and the construction and investigation of 16S rRNA gene clone libraries were applied to reveal the structure of these communities. All of these methods have different advantages and therefore, they complement each other to get a more reliable picture of the biofilm communities.

The cultivation method was included in this study because it enables a verification of results from other studies. Furthermore, the obtained strains are genuine brewery isolates and can be used for physiological tests. Isolates were obtained from seven different sample sites (Chapter 1 and 5). They were identified and affiliated to 25 different genera. Some of these strains were inoculated in beer but none of them was able to grow in it (Chapter 1 and 5). However, these strains can still be harmful for the industry, e.g. if they are able to form biofilms. This aspect was investigated by analyzing the potential of the isolates to produce acyl-homoserine lactones (AHLs) (Chapter 6). These quorum sensing mediating molecules are involved in the maturation process of biofilms. Indeed, some strains were found to secrete these autoinducer molecules, they mainly belonged to the genus *Pseudomonas*.

An abundant proportion among the isolates was constituted by members of the Enterobacteriaceae (Chapter 7). In the beginning of this study, there was a minor suspicion concerning their beer-spoiling potential. Indeed, all isolated Enterobacteriaceae were found to be able to multiply in non-alcoholic beer under access of oxygen but they represented no risk for filled beer.

The beer-spoiling potential of biofilm communities was investigated by inoculating them in beer (Chapter 3). These enrichments allowed the detection of minor proportions of beer-spoiling organisms. About 25% of the biofilms contained microorganisms which were able to multiply in beer with 4.8% of ethanol (v/v). The absence of anaerobic beer-spoiling bacteria in most of the biofilms was confirmed by using specific FISH probes for *Pectinatus* and *Megasphaera* cells (Chapter 9). However, *Pectinatus* cells constituted one of the most abundant groups in two biofilm communities. These samples clearly demonstrated that brewery biofilms can become hazardous for the quality of the product.

The acetic acid bacteria were supposed to be abundant brewery biofilm organisms. This was not confirmed by any method used (Chapter 8). Instead, FISH signals were found for many other taxa in considerable proportions, e.g. communities from the conveyors consisted of members of the Eukarya, Archaea, Alpha-, Beta-, Gammaproteobacteria, Cytophaga-

Flavobacteria, Planctomycetales, Actinobacteria and Firmicutes (Chapter 1). Such diverse communities were also evidenced for three other biofilms analyzed by FISH (Chapter 2 and 9).

Whereas the FISH technique allows the specific detection of single cells, the FAME analysis targets all organisms present, except the Archaea. The fatty acid profiles of 78 biofilms indicated significant differences between the communities, even between those which were exposed to similar conditions. In addition, repeated sampling of identical sites revealed a temporal variability of the microbial communities (Chapter 3). Characteristical fatty acids of beer-spoiling bacteria were almost absent. Typical fatty acids of Eukarya dominated nearly half of all biofilms. The high proportions of Eukarya in some biofilms was not confirmed, as these samples were also investigated by FISH. This divergence was found to be due to the higher biomass of eukaryotic cells compared to bacterial cells (Chapter 3). As some wild yeast strains were isolated and characterized, they are a potential source of these fatty acids. In contrast to the revealed bacterial diversity, most of the isolated yeasts were assigned to *Saccharomyces* or *Candida* spp. (Chapter 4). The *Saccharomyces* spp. showed a high beer-spoiling potential and many *Candida* species were able to form biofilms.

The construction of 16S rRNA gene clone libraries and the analysis of the clones with amplified ribosomal DNA restriction analysis (ARDRA) was performed with two biofilm communities (Chapter 2). Clones with identical ARDRA patterns were grouped and some representatives were identified by sequencing. These clone sequences were affiliated to 30 different genera, most of which were members of the Alpha- and Gammaproteobacteria and the Bacteroidetes. In addition, some clone sequences were assigned to uncultured organisms. Despite of the presence of 53 and 59 different ARDRA patterns in the two clone libraries, respectively, they had only four patterns in common. This result underlined the differences in the microbial composition of these communities.

In conclusion, breweries represent a habitat with high cleaning and disinfecting pressure, which might have selected for a limited number of more resistant or adopted species. Instead, the community structures of biofilms in industrial environments were found to be diverse and variable in their compositions.

Zusammenfassung

Das Hauptziel dieser Arbeit war die Charakterisierung von oberflächen-assoziierten mikrobiellen Gemeinschaften in Brauereien. Außerdem wurde das bierschädliche Potential von Isolaten und Biofilmen untersucht. Bisher wurden aus industriellen Habitaten lediglich kultivierbare Mikroorganismen beschrieben. Erst mit dieser Arbeit, die neben der Kultivierung auch kultivierungsunabhängige Methoden nutzt, wurde die gesamte mikrobielle Gemeinschaft auf industriellen Anlagen betrachtet. Jede der eingesetzten Methoden, die Kultivierung, die Analyse von Fettsäuremethylestern, die Fluoreszenz *in situ* Hybridisierung (FISH) sowie der Aufbau und die Analyse von 16S rRNA Gen Klonbibliotheken, besitzt individuelle Stärken, deren Kombination ein verlässlicheres Bild von den mikrobiellen Gemeinschaften ergibt.

Aus Proben von sieben verschiedenen Standorten wurden Stämme isoliert, die insgesamt 25 verschiedenen Gattungen zugeordnet wurden (Kapitel 1 und 5). Neben dieser Identifizierung sind solche authentischen Stämme aus den Brauereien eine Voraussetzung für physiologische Untersuchungen. So wurden einige von diesen Stämmen auf ihr bierschädliches Potential untersucht, keiner von diesen verursachte eine Trübung in Bier (Kapitel 1 und 5). Trotzdem können sie im industriellen Bereich Probleme bereiten, nämlich dann, wenn sie in der Lage sind, Biofilme zu bilden. Unter diesem Gesichtspunkt wurden die Isolate auf die Produktion von acylierten Homoserin-Laktonen (AHL) untersucht (Kapitel 6). Diese Moleküle vermitteln in der Zellkommunikation innerhalb der Bakterien und sind an der Ausbildung von reifen Biofilmen beteiligt. Einige Isolate waren in der Lage diese Autoinduktor-Moleküle abzugeben, es waren in erster Linie Vertreter der Gattung *Pseudomonas*.

Eine häufig isolierte Gruppe bildete die Familie der Enterobacteriaceae (Kapitel 7). Für diese bestand ein Anfangsverdacht eines möglichen bierschädlichen Potentials. In der Tat waren alle isolierten Enterobacteriaceae in der Lage in alkohlfreiem Bier unter Zugang von Luft eine Trübung zu verursachen. In verschlossenen Röhrchen konnten sie jedoch nicht anwachsen und stellen daher keine Gefahr für abgefülltes Produkt dar.

Um nun das bierschädliche Potential von kompletten Gemeinschaften zu untersuchen, wurden Biofilme in Bier angeimpft (Kapitel 3). Diese Anreicherungen ermöglichten den Nachweis von bierschädlichen Organismen, auch wenn sie in kleineren Anteilen in der Gemeinschaft vorkamen. Ungefähr ein Viertel der Biofilme war in der Lage eine Trübung in Bier mit 4.8% Ethanol (vol/vol) zu verursachen.

Die Abwesenheit von anaeroben bierschädlichen Bakterien wurde mit Hilfe spezifischer FISH Sonden gegen Zellen der Gattung *Pectinatus* sowie *Megasphaera* für mehrere Biofilme bestätigt (Kapitel 9). In zwei Biofilmen stellten Zellen der Gattung *Pectinatus* eine der am häufigsten mit der FISH Technik detektierten Gruppen dar. Diese beiden Proben belegen, dass die Biofilme zu einer Gefahr für die Qualität des Produktes werden können.

Die Hypothese, dass Essigsäurebakterien eine häufige und funktionell bedeutsame Gruppe in Biofilmen in den Brauereien seien, konnte durch keine in dieser Arbeit angewandte Methode gestützt werden (Kapitel 8). Mit der FISH Technik wurden jedoch zahlreiche andere Taxa nachgewiesen, dies waren in Biofilmen von dem Einlauf- und Auslauftransportband die

Eukarya, Archaea, Alpha-, Beta- und Gammaproteobakterien, Cytophaga/Flavobakterien, Planctomycetales, Actinobakterien und Firmicutes (Kapitel 1). Ähnliche Ergebnisse lieferten Untersuchungen an drei weiteren Standorten (Kapitel 2 und 9).
Während mit der FISH Methode einzelne Zellen spezifisch nachgewiesen werden, erfasst die Analyse der Fettsäuren alle vorhandenen Taxa, mit Ausnahme von den Archaea. Zwischen den verschiedenen untersuchten Biofilmen zeigten sich signifikante Unterschiede in den Fettsäureprofilen, selbst wenn es sich um benachbarte Standorte handelte (Kapitel 3). Durch eine wiederholte Beprobung identischer Standorte konnte zudem eine zeitliche Variabilität in der Zusammensetzung der Gemeinschaften nachgewiesen werden. Hinweise auf bierschädliche Bakterien waren kaum vorhanden, dagegen dominierten in etwa der Hälfte der Biofilme typische Fettsäuren für Eukarya. Eine Dominanz der Eukarya in den Gemeinschaften konnte durch die FISH Methode nicht bestätigt werden. Diese Abweichung ist auf den höheren Anteil von Biomasse einer eukaryotischen Zelle im Gegensatz zu einer bakteriellen Zelle zurückzuführen (Kapitel 3). Als eine mögliche Quelle der detektierten eukaryotischen Fettsäuren kommen Hefen in Betracht, die auch isoliert und charakterisiert wurden (Kapitel 4). Ein hohes bierschädliches Potential zeigten die Hefen der Gattung *Saccharomyces*. Biofilme konnten nur von Vertretern der Gattung *Candida* ausgebildet werden.
Für die Erstellung und Untersuchung von 16S rRNA Gen Klonbibliotheken wurden zwei Proben genutzt. Die Differenzierung der einzelnen Klone erfolgte durch eine Restriktionsanalyse. Klone mit identischen Mustern wurden gruppiert und repräsentative Vertreter durch eine Sequenzierung des Inserts identifiziert. Die klonierten Sequenzen wurden 30 verschiedenen Gattungen zugeordnet, von denen die meisten zu den Alpha- und Gammaproteobakterien sowie den Bacteroidetes gehörten. Ferner konnte durch diese Methode die Anwesenheit von bisher nicht kultivierbaren Bakterien nachgewiesen werden. Unter den analysierten Klonen war kein Muster eines bekannten bierschädlichen Organismus. Insgesamt wurden 53, bzw. 59 verschiedene Muster in den beiden Klonbibliotheken gefunden, von denen nur vier in beiden vorkamen. Auch dieses Ergebnis verdeutlicht die Diversität in der Zusammensetzung der untersuchten mikrobiellen Gemeinschaften.
Da Brauereien ein industrielles Habitat mit einem hohen Desinfektions- und Reinigungsdruck darstellen, lag die Annahme nahe, dass sich nur wenige robuste oder adaptierte Arten in dieser Umgebung dauerhaft ansiedeln können. Stattdessen wurden durch alle in dieser Arbeit eingesetzten Methoden ausschließlich diverse und in ihrer Zusammensetzung variierende mikrobielle Gemeinschaften nachgewiesen.

General Introduction

Microbial spoilage is a constant concern in the food processing industry. Even breweries have the risk of contamination of their product, although beer is very restrictive to bacteria due to the low pH value (3.8-4.7), the concentration of hop bitters (approximately 17-55 mg iso-alpha-acids/l) and the content of ethanol (0-8% w/v) (Jespersen and Jakobsen 1996). Therefore, the beer-spoiling organisms are limited to a few genera, which are listed in Table 1. The most important beer-spoiling organisms has been *Lactobacillus brevis*, which accounted for nearly the half of the analyzed contaminated samples (Back 2003). Other Lactobacillaceae like *L. lindneri* and *Pediococcus damnosus* were regularly identified and constituted higher proportions among the beer-spoiling organisms. In contrast to this, the main beer-spoiling organisms in one of the breweries considered in this study were the yeasts and *Pectinatus* cells (Methner 2003).

Table 1. Reclamations due to beer-spoiling organisms as reported by the Institute for Brewingtechnology I, Technical University Munich (Back 2003).

Species	1997	1998	1999	2000	2001	2002
Lactobacillus brevis	36.0	42.0	40.0	50.0	42.7	48.8
Lactobacillus lindneri	10.0	4.0	10.0	7.5	12.7	11.4
L. brevisimilis	5.0	1.0	1.0	1.9	0	2.4
L. plantarum	2.0	4.0	2.0	0.9	0.7	2.4
L. casei / *paracasei*	1.0	9.0	5.0	8.5	4.0	4.1
L. coryniformis	6.0	11.0	4.0	0.9	2.7	5.7
Pediococcus damnosus	4.0	14.0	12.0	14.2	21.3	12.2
Pectinatus sp.	31.0	3.0	6.0	4.7	10.0	6.5
Megasphaera sp.	6.0	2.0	4.0	3.8	4.0	1.6
Obesumbacterium proteus / *Enterobacter agglomerans*	2.0	1.0	1.0	4.7	0	0
Saccharomyces wild yeasts	0	6.0	11.0	4.7	2.0	3.3
Non-*Saccharomyces* wild yeasts	7.0	3.0	4.0	0	0	1.6

Growth of these organisms in beer has different impacts on the quality of the product, some examples are given in Table 2.

Table 2. Consequences of bacterial growth in beer.

Species	Effect on beer
Lactobacillus brevis	Turbidity, sediment
Lactobacillus lindneri	Turbidity, sediment
L. brevisimilis	Slight turbidity, slight sediment
L. frigidus	Strong turbidity, slime
L. casei	Slight turbidity, slight sediment, taste of diacetyl
Pediococcus damnosus	Sediment, in part slight turbidity, taste of diacetyl
Pectinatus cerevisiiphilus	Sediment, turbidity, unpleasant odour and taste
Megasphaera cerevisiae	Slight turbidity, in part slight sediment, nauseating odour and taste

Reference: Back *et al.* 1988.

Since all beer-spoiling bacteria are very sensitive in relation to heat, disinfectants and a dry environment, they are supposed to survive only in combination with more resistant microorganisms (Back 1994). There is a detailed model of the formation of biofilms in breweries available (Fig. 1) which explains the development of contaminations in breweries. One of its most important aspect is the outstanding meaning of acetic acid bacteria.

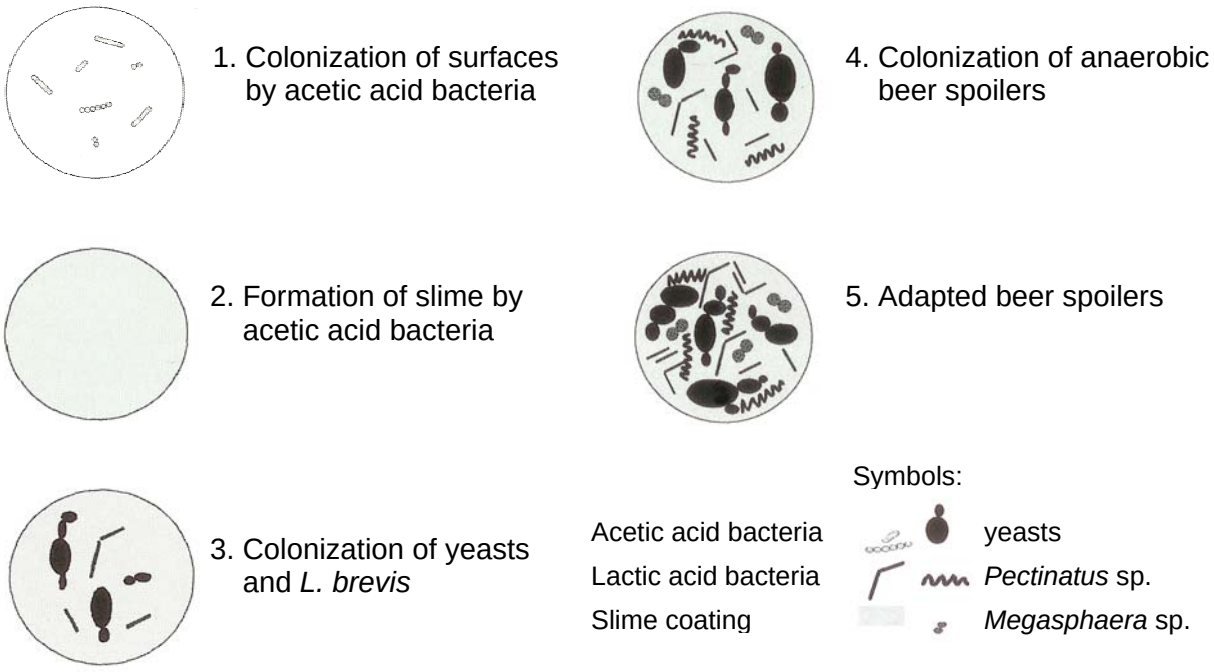

Figure 1. Development of contaminations in breweries according to Back (1994).

First of all, acetic acid bacteria are supposed to colonize the surfaces, in particular the species *Acetobacter pasteurianus* and *Gluconobacter frateurii*. In addition, ubiquitous slime producing microorganisms, e.g. *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Cryptococcus* sp., *Rhodotorula* sp. and others build up slime coatings in certain niches. The next postulated step is the settlement of yeasts and *Lactobacillus brevis*. The acidification of the environment by acetic acid bacteria brings an advantage to the acid-tolerant lactic acid bacteria. The acetic acid bacteria and yeasts provide anoxic conditions and a protective slime cover. The presence of lactate, which originated from the lactic acid bacteria, enables the colonization of strictly anaerobic beer spoilers like *Pectinatus* spp. and *Megasphaera* sp. Finally, remained beer and froth provide a constant nutrient supply. Therefore, the biofilms are considered as a reservoir of beer-spoiling organisms.

Out of the biofilm they can be carried off by persons, splashs or movements of the air to all parts of the plant and they might get into the bottles. This would be a so-called secondary contamination, which takes place during the filling procedure. An infection of beer during the production process is defined as a primary contamination (Back 1994). Breweries have to avoid any contamination of beer because of the decreasing product quality. The spoilage sometimes happens to a few bottles of a filling process. So it is hard to find the source and entry path of these microorganisms. There are two fundamental strategies to avoid microbial contamination. One is the thermal handling by pasteurization of filled beer. A disadvantage of this procedure is the high capital outlay and energy costs. Another aspect is the loss of quality, in particular in regard of the taste, because oxidation processes run faster under increased temperatures. The second strategy is the application of preserving agents, however, no such additives were added due to a voluntary self-commitment fixed in the German purity law. As a consequence, the complete plants and pipelines are prophylactical cleaned. This is connected to the need of more than 2000 tons of cleaning agents and disinfectants per year in the german beverage industry just in the filling area. Adding the costs of cold and heated water to these chemicals, about 4 millions € were spent for cleaning purposes each year, not included the human ressources costs. These cleaning actions target the complete microbial communities. However, next to the information concerning the beer-spoiling organisms, there is only little information available about the identity of all the other organisms present. In addition, the data base on cultivation studies and are therefore methodical biased. Consequently, the aim of this part of the joint research project was to reveal the compositions of microbial communities of brewery biofilms by cultivation independent techniques. Furthermore, the localization of some dangerous spots may lead to a more economic use of energy and chemicals in the brewing and food industry.

The communities analyzed originated from two breweries, a medium-scale company (designated: brewery A; it produces approximately 0.3 millions hl beer per year) and a

large-scale brewery (designated: brewery B; it produces approximately 4.3 millions hl beer per year). The bottling plant of brewery A is shown in Fig. 2. It points up the complexity of such a plant. There are a lot of corners and inner surfaces in the filling area, all of which can be niches for microorganisms. An investigation of many different samples from two filling plants was performed (Fig. 3).

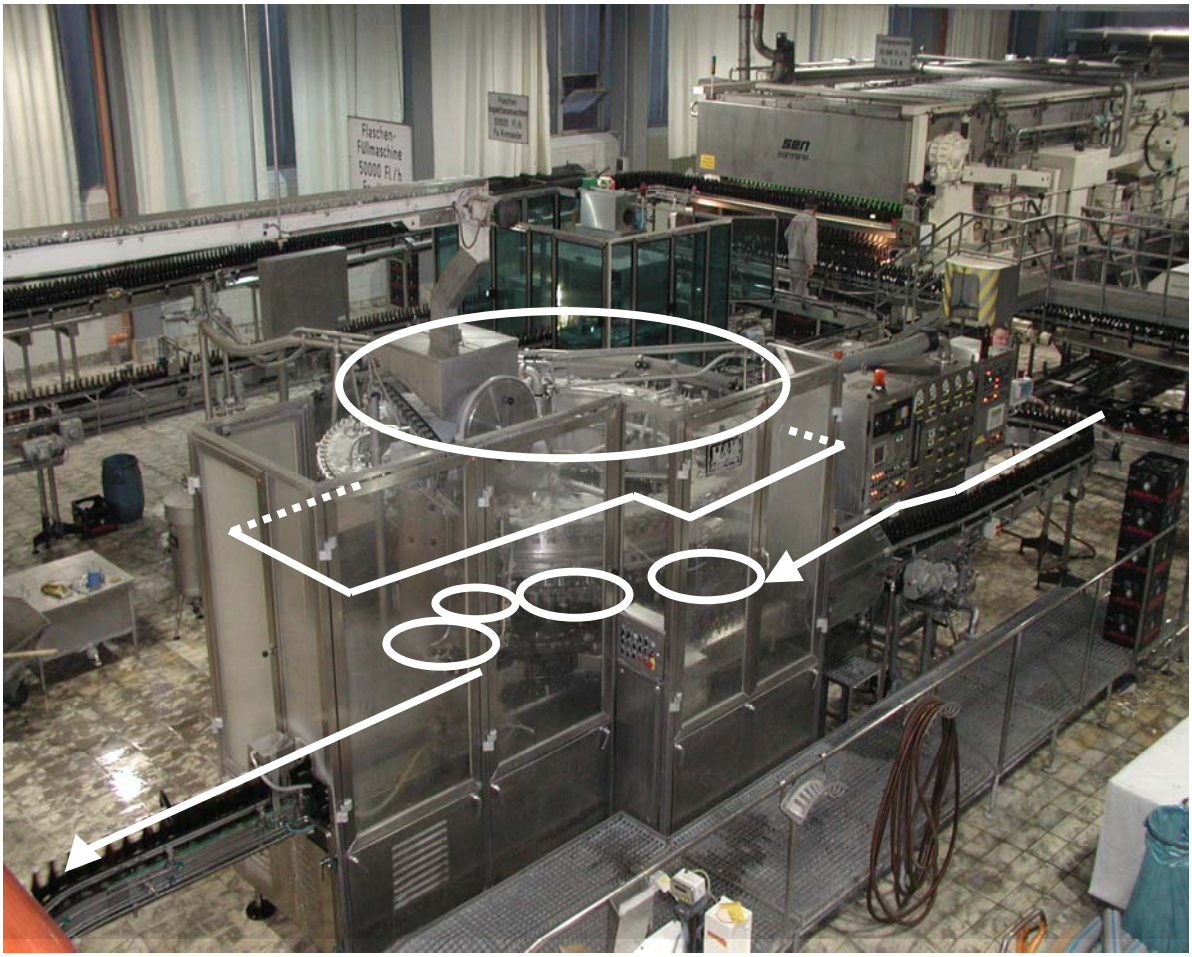

Figure 2. General view of the filling plant of brewery A. The white markings show important parts and the way of the bottles. They arrive on the right conveyor at the filling plant. The small circles represent star wheels which guide the bottles throughout the plant. The first one is the infeed star wheel, it directs the bottles into the filler, indicated by the big round circle. The bottle go around counter-clockwise and get filled. The discharge star wheel leads the bottles to the crowner (small circle) and the crowner star wheel guides the bottle to the discharge conveyor.

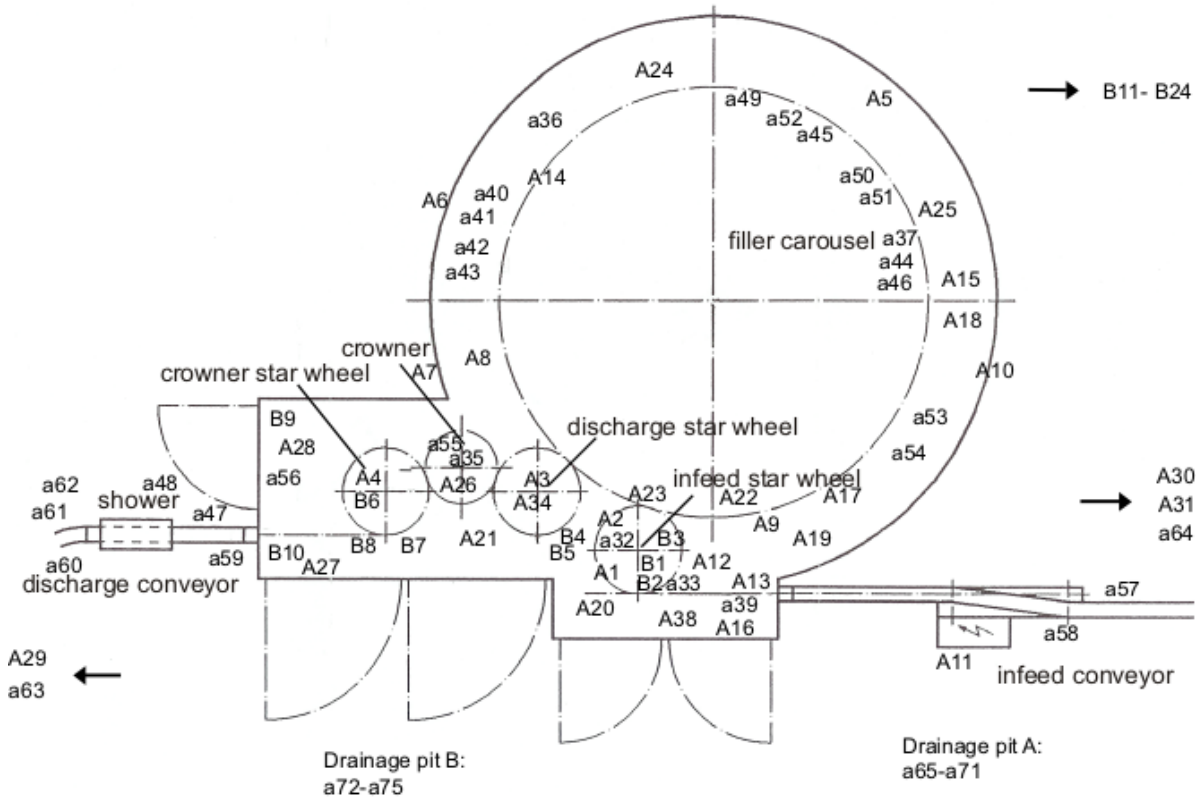

Figure 3. Schematical view a bottling plant with the approximate localization of the sample sites indicated by the sample abbreviations (for details Table 1, Chapter 3). Long distance related (> 10 m) sample locations are indicated by arrows.

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Chapter 1

Microbial Composition of Biofilms in a Brewery Investigated by Fatty Acid Analysis, Fluorescence *in Situ* Hybridization and Isolation Techniques

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Abstract

Biofilms associated with brewery plants can harbour spoiling microorganisms that potentially damage the final product. Most beer-spoiling microorganisms are thought to depend on numerous interactions with the accompanying microbiota. However, there is no information on the microbial community structure of biofilms from bottling plants. The conveyors that transport the bottles to and from the plant are known as potential sources of microbial contamination of beer. Consequently, the material buildup from two conveyors was analyzed using a cultivation/isolation approach, and the culture-independent techniques of whole cell fatty acid analysis and fluorescence *in situ* hybridization (FISH). Heterogeneous communities were present at both conveyors. Although characteristic fatty acids for Eukarya were present, FISH-signals for Eukarya were extremely low. The Proteobacteria, in particular the Gammaproteobacteria, were abundant at both sample sites. Bacterial isolates were obtained for every dominating group detected by FISH: the Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, the Xanthomonadaceae, the Actinobacteria, the Bacteroidetes and the Firmicutes.

Introduction

The presence of growth-supporting conditions such as humidity and nutrients in bottling plants make it practically impossible to keep their surfaces free of microbial coating. In beer-bottling plants, nutrients are supplied by the overfoaming amount of 1–2 ml of beer per bottle, which reduces the oxygen content in the bottle head space. Additionally, bottling plants of breweries are sprinkled with hot water at intervals of 1–2 h during the production process. The biofilms formed must be constantly disinfected and removed in order to prevent contamination of the product. Incubation of swab-samples in selective media is used for monitoring the hygienic condition of the plant. These samples are taken from different parts of the bottling plant and other relevant sites. If necessary, intensive cleaning procedures can be initiated. The conveyor from the bottling plant is in permanent contact with beer, which may result in an adaptation of microorganisms to beer (Back 1994a). The transport activity of the conveyors might even distribute microorganisms throughout the brewery. For all these reasons, conveyors have been recognised as one of the most critical spots for potential contamination and are therefore considered in the present study.

In general, beer is an unfavourable environment for microorganisms due to its low pH value (3.8–4.7), the concentration of hop bitters (ca. 17–55 mg iso-α-acids l^{-1}), the content of ethanol (0–8% w/w) and CO_2 (ca. 0.5% w/v) (Jespersen and Jakobsen 1996). Therefore, the main beer-spoiling bacteria belong to a limited number of taxa. These are members of

the Lactobacillaceae, in particular *Lactobacillus brevis*, *Lactobacillus lindneri*, *Pediococcus damnosus* and the Acidaminococcaceae *Pectinatus frisingensis*, *Pectinatus cerevisiiphilus* and *Megasphaera cerevisiae* (Back 1994a; Jespersen and Jakobsen 1996). They can cause turbidity and some produce metabolic end-products that result in off-flavours. These well known beer-spoiling bacteria could be opportunistic colonizers of biofilms, taking advantage of multiple interactions within complex communities. An initial step of biofilm formation is the adherence to surfaces. In vitro experiments demonstrated that primary colonizers enable secondary bacteria to become part of the biofilm. Pure cultures of *Listeria monocytogenes* formed no biofilms but, in mixed cultures with *Pseudomonas fragi*, attachment and microcolony formation by *L. monocytogenes* was enhanced (Sasahara and Zottola 1993). Furthermore, several *Bacillus* species, which cause problems in paper-producing machinery, were unable to colonize surfaces except in combination with *Deinococcus geothermalis* (Kolari *et al.* 2001). While *P. fragi* excretes extracellular polymeric substances, which entrap and embed microorganisms, *D. geothermalis* forms thin adhesion threads (Kolari *et al.* 2002). Many species, such as *Xanthomonas campestris*, *Klebsiella aerogenes* and *Pseudomonas aeroginosa*, produce exopolysaccharides (Sutherland 2001). Beer-spoiling bacteria are facultative or obligate anaerobes, and are acidophilic or at least acidotolerant. Therefore, they are supported by aerobes or facultative aerobes, which reduce oxygen, and produce anoxic microenvironments in biofilms (De Beer *et al.* 1994). Acetobacteraceae reduce the pH in the presence of oxygen while fermentative bacteria reduce the pH in the absence of oxygen. Cells in a biofilm are protected against chemicals, i.e. the organisms located in a biofilm are more tolerant of biocidal agents than planktonic organisms (LeChevallier 1991). In brewery biofilms, slime-producing Acetobacteraceae are considered as the primary colonizers (Back 1994a), providing favourable conditions for the aerotolerant anaerobic and acidophilic Lactobacillaceae and the anoxic environment indispensable for *Pectinatus* and *Megasphaera*. Much research has focussed on the rapid detection of beer-spoiling bacteria by fatty acid analysis, PCR or using antibodies (Helander and Haikara 1995; Mücher and Schönling 2000; Ziola *et al.* 2000). However, there is no information about the composition of brewery biofilms, particularly for culture-independent techniques and the impact of gushed beer on the different conveyor communities. For this reason an investigation of the rub-off material from conveyors to and from the bottling plant was performed.

Materials and Methods

Sampling. Biofilms, lubricants and rubbed off conveyor-material were collected. This material accumulates mainly at the rotating rollers of the conveyors. Samples were taken from the conveyor that carries clean bottles to the bottling plant (Conveyor I), and from the

rollers of the conveyor that transports filled bottles for packaging (Conveyor II). Samples were collected by means of sterilized spatula, transferred into sterile tubes and stored at 4°C until processing. Samples were taken on 20 August 2000, and on 11 January 2001 from the same locations.

Fatty acid analyses. The sample material contained a high proportion of lubricants. To prevent a huge background of abiotic compounds, these were removed by washing 200 mg sample material twice with 4 ml sterilised water and 4 ml decane on an end-over-end rotator. The water phase and the decane phase were separated by centrifugation. The organic phase and the interphase were rejected and the pelleted cells were stored at –20°C until use. In addition, three lubricants used at the conveyors were analyzed for their fatty acid profiles to identify the abiotic lipid background. Only one of them contained fatty acids (Optimol Olit CLS; Optimal Ölwerke Industrie, Munich, Germany). Saponification, methylation and extraction of fatty acid methyl esters (FAMEs) were performed according to Sasser (1990), and FAMEs were identified by GC-MS as described previously (Lipski and Altendorf 1997). Potential loss of biomass by the washing procedure was tested for the isolates *Pseudomonas* sp. DW115, *Bacillus* sp. DW137 and *Saccharomyces cerevisiae* DSM1334. For each strain, 100 mg cell material was analyzed in triplicate with and without the decane washing step. The total amount of fatty acids was used for Student's *t*-test calculation with a confidence level of 0.95 using SYSTAT software (SPSS Science SoftwareG, Erkrath, Germany). In addition, removal of the lubricant Optimol Olit CLS from artificial mixtures with each of the strains *Pseudomonas* DW115 and *Bacillus* DW137 (1:1 w/w) using the decane washing procedure was tested.

Whole cell hybridization of sample material. Sample materials were suspended in Ringer's solution (0.9% NaCl, 0.042% KCl, 0.024% $NaHCO_3$) before fixation. Sample Conveyor I from January and both samples from Conveyor II were washed as described for the fatty acid preparation, but the decane phase was removed without the interphase. Fixation and hybridization was carried out as described by Friedrich *et al.* (1999) using paraformaldehyde (PFA) or ethanol for fixation. Formamide concentrations in the hybridization buffers were used according to the references given below. Probe-positive counts were determined relative to 4′,6-diamidino-2-phenylindole (DAPI; 1 mg l^{-1})-stained cells. At least 400, and usually more than 600, cells per replicate and between two and normally three replicates were counted per probe. Bacteria were detected with the probe EUB338 (Amann *et al.* 1990), Eukarya and Archaea with probes EUK502 (Amann *et al.* 1995) and ARCH915 (Stahl and Amann 1991), respectively. The probe NON338 (Wallner *et al.* 1993) was used as a negative control. Proteobacteria were investigated with ALF968, a probe for Alphaproteobacteria (Neef 1997), Betaproteobacteria and Gammaproteobacteria with BET42a and GAM42a (Manz *et al.* 1992) and the *Xanthomonas* branch with XAN818

(Friedrich *et al.* 1999). Actinobacteria were detected with HGC69a (Roller *et al.* 1994), Firmicutes with LGC354A-C (Meier *et al.* 1999), a subset of Bacteroidetes with CF319a (Manz *et al.* 1996) and Planctomycetales with PLA46 (Neef *et al.* 1998). AG1427 (Neef 1997) was used for detection of cells of the genera *Acetobacter* and *Gluconobacter*, probe ENT183 for detection of Enterobacteriaceae cells (Friedrich *et al.* 2003) with probe Pae997 (Amann *et al.* 1996) accounting cells of the genus *Pseudomonas*. Because Pae997 gave only weak signals with *Pseudomonas pseudoalcaligenes* DSM50188T, two helper oligonucleotides (Fuchs *et al.* 2000) were designed. The helper oligonucleotides were used in equimolar amounts to Pae997 (Hpae1: 5′-GAA GGC ACC AAT CCA TC-3′, Hpae2: 5′ -TGT CAA GGC CWG GTA AGG-3′).

Isolation. Bacteria were isolated from rub-off material obtained in August 2000. The samples were suspended in 0.9% NaCl-solution, plated on Petri dishes and isolated until pure cultures were achieved. Media used were TSA (trypticase soy agar; Becton/Dickinson, Cockeysville, Md.), MRS (DeMan-Rogosa-Sharpe; Difco, Detroit, Mich.), DSM 105 medium (*Gluconobacter oxydans* medium; http//www.dsmz.de/media) and DSM 254 medium (*Acetobacter peroxydans* medium). MRS agar plates were incubated in an anaerobic jar at 25°C (GasPak, BD Diagnostic Systems, Heidelberg, Germany). The other media were used under aerobic conditions at 25°C. All isolates were examined for growth on TSA.

Identification. Pure cultures were grouped according to their catalase and oxidase activity (MacFaddin 1980), their Gram-reaction and their fatty acid profiles. For fatty acid analysis, cells were incubated for 3 days on TSA at 25°C and analyzed as described above, except for the decane procedure. Representative strains of each group were subjected to 16S rRNA gene sequencing. Genomic DNA was isolated with a DNeasy Tissue Kit according to the manufacturer′s instructions (Qiagen, Hilden, Germany). Nearly the complete 16S rRNA gene of the purified chromosomal DNA was amplified using the universal bacterial primers GM3F and GM4R (Muyzer *et al.* 1995). 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 *Taq* DNA polymerase (Gibco-Life Technologies, Karlsruhe, Germany) and a variable amount of template, adjusted to a final volume of 100 µl with autoclaved double distilled water. The PCR consisted of ten cycles with denaturation at 95°C for 1 min, annealing at 49°C for 1 min and elongation at 72°C for 2 min. In addition, 25 cycles were performed with an annealing temperature 44°C followed by a final extension at 72°C for 10 min. Sequencing of the PCR-product of some isolates was carried out by GAG BioScience (Bremen, Germany). The primers used were 518F, 787R (Buchholz-Cleven *et al.* 1997), 1114F (Lane 1991) or GM3F. Alternatively, the 16S rRNA gene was sequenced using approximately 350 ng 16S rRNA gene PCR-product, 0.8 µM primer 787R, 4 µl of ABI

PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Applied Biosystems, Foster City, Calif.) and water to a final volume of 10 µl. The PCR consists of 30 cycles, denaturation for 1 min at 96°C, annealing for 15 s at 50°C and elongation at 60°C for 4 min. The sequencing product was purified using the DyeEx Spin Kit (Qiagen). Electrophoresis was performed on an ABI377 (Applied Biosystems) sequencer in our faculty. The partial sequences obtained were compared to the National Centre for Biotechnology Information nucleotide sequence database using the BLAST algorithm (Altschul *et al.* 1997).

Growth of the isolates in a selective medium and in beer. The isolated strains were tested for growth in NBB medium (Döhler, Darmstadt, Germany). NBB contains a pH-indicator in order to detect acidification. A few colonies grown on TSA were suspended in 0.9% NaCl until a turbidity of 1.0 McFarland standard was achieved; 200 µl were used as inoculum for 5 ml NBB medium. The tubes were incubated at 25°C in an upright position without rotation. The test result was positive if the medium showed turbidity. In addition, acidification was noted. Furthermore, the strains were inoculated in 5 ml beer with 4.8% (v/v) ethanol and incubated in an anaerobic jar.

Accession numbers. The 16S rRNA gene sequences obtained in this study were submitted to the EMBL database and are available under the following accession numbers: AJ534843 and AJ534848 to AJ534870.

Results

Fatty acid analyses. The decane wash step has no effect on fatty acid recovery for the isolates *Pseudomonas* DW115 and *Bacillus* DW137. Only for the fatty acids of *Saccharomyces cerevisiae* DSM1334 could a significant decrease (P-value < 0.05) of the lipid yield be observed. One of the lubricants tested (Optimol Olit CLS) contained linoleic acid (Δ9-*cis*,Δ12-*cis*-octadecadienoic acid, 18:2 *cis*9,12), oleic acid (Δ9-*cis*-octadecenoic acid, 18:1 *cis*9), stearic acid (octadecanoic acid, 18:0), the short-chain octanoic, nonanoic and decanoic acids (8:0, 9:0, 10:0) and some non-fatty acid compounds. This lubricant was mixed with cells. After the decane washing procedure only small contamination with the lubricant lipids (<6%) was found in the fatty acid profiles of the test strains.

The August Conveyor I sample contained mainly short-chain and hydroxy-fatty acids, particularly dodecanoic acid (12:0) and 3-hydroxy-dodecanoic acid (12:0 3OH) (Table 1). Furthermore, 3-hydroxy-decanoic acid (10:0 3OH), 2-hydroxy-dodecanoic acid (12:0

2OH), 3-hydroxy-tetradecanoic acid (14:0 3OH) and 3-hydroxy-hexadecanoic acid (16:0 3OH) were abundant. Decanoic acid was also quite prevalent in August and tetradecanoic acid (14:0) at both sample times. In contrast, the Conveyor II sample consisted mainly of long-chain linoleic, oleic and vaccenic acids (Δ11-*cis*-octadecenoic acid, 18:1 *cis*11). Both profiles derived from this location were very similar, especially regarding of the predominance of the linoleic acid. Stearic acid was one of the most abundant fatty acids in August, but was detected in much lower amounts in January. Palmitoleic acid (Δ9-*cis*-hexadecenoic acid, 16:1 *cis*9) and palmitic acid (hexadecanoic acid, 16:0) were present at a constant proportion in all four samples. Vaccenic acid was detected at high concentrations, in particular in the Conveyor I sample in January. 13-Methyl 14:0 (15:0 iso) and 12-methyl 14:0 (15:0 anteiso) constituted a minor part of the community profiles and were absent from Conveyor I in January.

Table 1. Fatty acid composition of the rub-off samples. Percent values are related to the complete fatty acid profile of each sample.

	Conveyor I		Conveyor II	
Fatty acid	August 2000	January 2001	August 2000	January 2001
8:0		0.4		0.3
10:0	4.7	1.0	0.2	0.5
11:0 iso		0.2		0.6
11:0	2.0	0.7		
10:0 3OH	6.3	1.4	0.7	0.7
12:0	20.4	8.0	2.6	4.1
11:0 iso 2OH				0.4
13:0	0.9	0.5		
13:0 iso				0.2
12:0 2OH	3.0		0.3	
12:0 3OH	9.1	2.0	1.5	0.5
14:0 iso		0.5		1.2
14:1 *cis*7				0.3
14:0	5.5	3.7	1.1	2.6
15:0 iso		1.4	1.1	2.5
15:0 anteiso		1.1	0.8	4.3
15:0				1.0
14:0 2OH	1.0			
14:0 3OH	3.6	1.1		1.2
16:0 iso		0.4	0.3	1.1
16:1 *cis*		0.4		0.5
16:1 *cis*9	9.3	10.0	7.9	11.1
16:1 *trans*9	1.7	1.1	1.4	0.8

Table 1. continued

	Conveyor I		Conveyor II	
Fatty acid	August 2000	January 2001	August 2000	January 2001
16:1 *cis*11		0.7		1.4
16:0	8.2	11.8	18.2	11.4
17:0 iso				0.5
17:0 anteiso				0.3
17:1 *cis* 9				1.4
17:0 cyclo9-10	2.4	1.1	1.4	0.6
17:0				0.3
16:0 3OH	2.2	0.4		0.2
18:2 *cis*9,12	2.9	5.9	27.2	20.4
18:1 *cis*9	3.5	9.6	11.8	12.3
18:1 *cis*11	9.4	33.3	10.5	14.2
18:1 *trans*11				0.9
18:0	3.9	1.9	12.2	2.2
19:0 cyclo11-12		1.4	0.8	

Fluorescence *in situ* hybridization. Bacterial detection rates obtained with probe EUB338 varied between 65% in the January samples and 89.5% in the sample from Conveyor II in August (Fig. 1). Almost all EUB338-positive cells were also detected by the more specific probes. Regardless of the sampling location, the detection rate of all DAPI-stained cells with EUB338 was significantly lower in January compared to the samples from August. The Proteobacteria, detected with probes specific for alphaproteobacterial 16S rRNA, betaproteobacterial and gammaproteobacterial 23S rRNA and the Xanthomonadaceae, were the most dominant group in all four samples, ranging from 38.7% (Conveyor I, January) to 78.7% (Conveyor II, August) of all DAPI-stained cells.

The Alphaproteobacteria and Gammaproteobacteria were the most abundant groups in August, and in January in the sample derived from Conveyor I, although their detection rate, in particular for the Gammaproteobacteria was lower in January. The proportion of Betaproteobacteria was about 10%. Members of the Bacteroidetes (4.6%) and Firmicutes (7.4%) occurred quite frequently in August. The Bacteroidetes signals were somewhat higher in January but those of Firmicutes were lower. Actinobacteria and Planctomycetales signals represented around 2% in the sample taken in August, Actinobacteria were more common in January. Even Archaea were detected in Conveyor I at 2.4%. Relative to DAPI counts, Eukarya were of only minor importance in terms of abundance, representing maximally 0.9%.

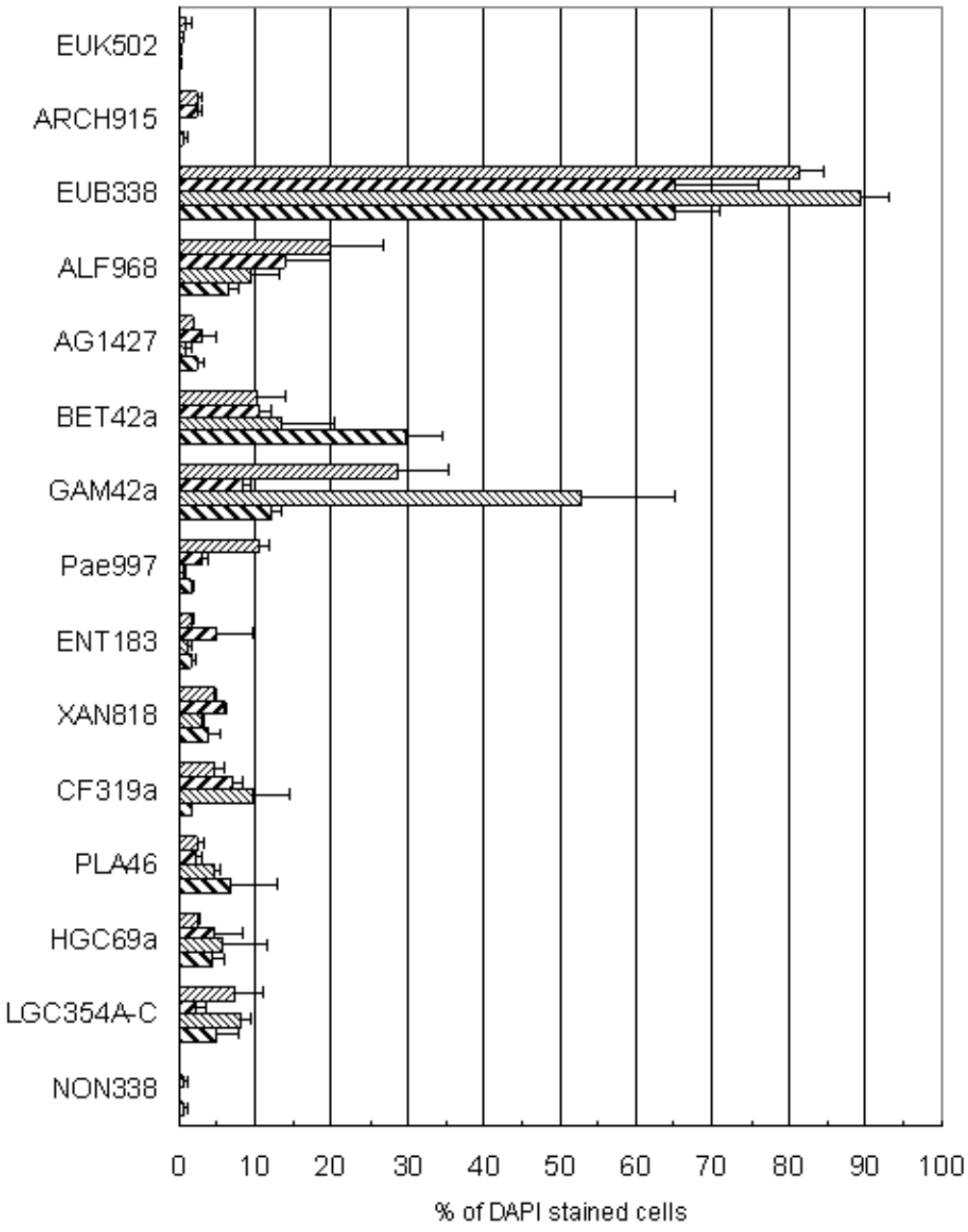

Figure 1. Percentages of probe specific counts, relative to direct enumeration with 4′,6-diamidino-2-phenylindole (DAPI). The probe names are given on the left. *Thin hatched lines sloping up left to right* Conveyor I, August 2000; *thick hatched lines sloping up left to right* Conveyor I, January 2001; *thin hatched lines sloping down left to right* Conveyor II, August 2000; *thick hatched lines sloping down left to right* Conveyor II, January 2001.

The Conveyor II population in August was clearly dominated by Gammaproteobacteria (53.0%), but their amount was lower in January (12.0%). On the other hand, the betaproteobacterial component of the population was about two times higher in January (13.4 vs 29.8%). In the Conveyor II sample the Alphaproteobacteria represented a smaller part of the population than in Conveyor I. The Bacteroidetes made up a large portion of the community in August (9.8%) but had almost disappeared in the sample taken in January (1.6%). Members of the Planctomycetales had a nearly constant abundance of approximately 5%. The proportion of Actinobacteria was in the same range and even more members of the Firmicutes were counted. Both fractions were slightly higher in the August sample compared to that from January. Even smaller was the proportion of the Xanthomonadaceae. The Archaea and Eukarya signals (0.5, 0.2%) were within the detection limit of the technique because the negative control probe also gave 0.6% signals in the Conveyor II sample from January.

Specific probes were used for the acetic acid bacteria, *Pseudomonas* cells and the Enterobacteriaceae. AG1427-specific signals were in the range 0.9 to 3.1% of all DAPI-stained cells. The Pseudomonadaceae FISH-signals revealed about one-third (36.9 and 33.3%) of the total gammaproteobacterial of Conveyor I. They were less abundant in Conveyor II. The proportion of Enterobacteriaceae was small in all samples.

Isolation and identification. In total, 117 strains were isolated from the different media. Sixty-four strains with different colony morphologies were subjected to catalase and oxidase test, Gram-stain and analysis of fatty acid composition after growth on TSA (data not shown). According to these results, isolates were grouped, and at least one representative of each cluster was used for partial sequencing of the 16S rRNA gene (Table 2). These representatives were members of different taxa with a focus on Gammaproteobacteria, particularly of the Enterobacteriaceae (isolates DW1, DW38, DW39, DW40, DW56, DW99, and DW143a1) and the Pseudomonadaceae (isolates DW90, DW101, DW115, and DW118). Three isolates belonging to the Alphaproteobacteria (DW78, DW79, DW114b), two isolates of the Betaproteobacteria (DW104, DW126), two members of the family Xanthomonadaceae (DW100, DW112a3), two strains of the genus *Acinetobacter* (DW112b, DW149) and two strains of the family Microbacteriaceae (DW8, DW129) were identified. Finally, one member of the Bacteroidetes (DW3) and the Firmicutes (DW137) were cultivated. No member of the Acetobacteraceae was isolated, even though two different well-suited media were used. Also, no typical beer-spoiling bacteria were cultivated on MRS medium under anaerobic conditions.

Growth in NBB and in beer. Nineteen isolates caused turbidity in NBB bouillon. Of these, 10 acidified the medium, particularly all strains of the family Enterobacteriaceae, two strains of the genus *Pseudomonas* (DW90, DW115) and one *Bacillus* strain (DW137). However, none of these isolates caused turbidity in beer.

Table 2. Similarities of the partial 16S rRNA gene sequences and growth of the isolates in NBB medium.

Strain	Similarity (%)[a]	Species[b]	Growth in NBB[c]
Conveyor I			
DW104	99% (1348/1359)	*Achromobacter xylosoxidans*	++
DW78	100% (693/693)	*Brevundimonas vesicularis*	-
DW99	99% (567/568)	*Citrobacter freundii*	++ acid
DW3	96% (1136/1175)	*Chryseobacterium scophthalmum*	+
DW143a1	98% (573/579)	*Enterobacter aerogenes*	++ acid
DW56	98% (524/531)	*Enterobacter asburiae*	++ acid
DW1	98% (506/514)	*Erwinia amylovora*	++ acid
DW129	98% (493/502)	*Microbacterium liquefaciens*	+
DW8	99% (550/553)	*Microbacterium testaceum*	+
DW101	98% (1254/1274)	*Pseudomonas alcaligenes*	-
DW79	99% (791/792)	*Rhizobium radiobacter*	-
DW100	99% (1373/1376)	*Stenotrophomonas maltophilia*	++
Conveyor II			
DW126	99% (1011/1014)	*Acidovorax temperans*	-
DW112b	98% (602/608)	*Acinetobacter haemolyticus*	++
DW149	99% (536/538)	*Acinetobacter johnsonii*	++
DW137	100% (626/626)	*Bacillus cereus*	++ acid
DW114b	100% (603/603)	*Brevundimonas bullata*	-
DW38	99% (514/516)	*Citrobacter freundii*	++ acid
DW39	99% (533/535)	*Enterobacter dissolvens*	++ acid
DW40	99% (600/603)	*Klebsiella oxytoca*	++ acid
DW118	100% (546/546)	*Pseudomonas fluorescens*	+
DW115	99% (553/557)	*Pseudomonas mosselii*	++ acid
DW90	100% (561/561)	*Pseudomonas putida*	++ acid
DW112a3	100% (541/541)	*Stenotrophomonas acidaminiphila*	++

[a] The number of identical/compared bases is given in parenthesis.

[b] Sequences were aligned according to entries in a public database using the BLASTN 2.2.5 program. The most similar species is given.

[c] + Turbidity in NBB within 3 days of incubation, + turbidity later than 3 days, – no growth, *acid* acidification.

Discussion

A polyphasic analysis of the rub-off material from the conveyors in a beer bottling plant was performed in order to reveal the structure of the microbial community associated with the surface of a technical plant. Furthermore, the effect of the overfoaming beer on the microbial community was investigated.
Material from Conveyor I in August showed a high portion of characteristic fatty acids of the genus *Pseudomonas*, which are 10:0 3OH, 12:0, 12:0 2OH and 12:0 3OH (Vancanneyt *et al.* 1996). This was supported by FISH-analyses, which revealed 10.6% of *Pseudomonas* cells detected by Pae997 and 28.7% of Gammaproteobacteria, which may contain further *Pseudomonas* species. These fatty acids constituted a much smaller proportion of the total in January. Consistently, the number of signals for members of the genus *Pseudomonas* detected with Pae997 decreased. In general, sample material from Conveyor I contained a higher percentage of fatty acids typical for *Pseudomonas* and Pae997-conferred FISH signals than Conveyor II. The high number of GAM42a-conferred signals was represented by 11 isolates, 4 of the genus *Pseudomonas* and 7 of the family Enterobacteriaceae, although the ENT183-signals made up only 1%. The presence of *Pseudomonas* species and Enterobacteriaceae is in accordance with several reports from breweries. These strains could occur in wort, but they do not multiply in bottled beer (Lawrence 1988; Back 1994b). Also, members of both families have been found on machinery in the paper industry (Väisänen *et al.* 1998), reflecting their presence on industrial plants. The isolation of Enterobacteriaceae strains from all samples coincided with the detection of 14:0 3OH in the biofilm samples, which is a characteristic fatty acid for Enterobacteria (Bryn and Rietschel 1978). This fatty acid was more abundant at Conveyor I than at Conveyor II, where 14:0 3OH was found only in January. However, there is no clear correlation between the proportion of 14:0 3OH and the amount of Enterobacteriaceae-specific FISH signals. This indicated that the majority of 14:0 3OH originated from other taxa. The isolated strains *Rhizobium radiobacter* (formerly: *Agrobacterium radiobacter*) DW79 and *Achromobacter* sp. DW104 also contained these fatty acids (data not shown). The alphaproteobacterial FISH signals in Conveyor I were nearly twice as high as those in Conveyor II. Accordingly, the long-chain fatty acids 18:1 *cis*11 and 19:0 cyclo11–12, which predominate within the genera *Rhizobium* and *Brevundimonas* (Lipski *et al.* 1992), were more common. Moreover, the samples from Conveyor I additionally contained 16:0 3OH, which is present in *Rhizobium* cells (Lipski *et al.* 1992), like in *Rhizobium radiobacter* DW79, isolated from this location.

At Conveyor II, the main long-chain fatty acids palmitic, linoleic, oleic and vaccenic acids appeared in the same range in both January and August. This indicated a more stable composition of the microbial community. Oleic acid occurs in Eukarya (Erwin and Bloch

1964) and Corynebacterineae (Kroppenstedt 1985), but also in the Gammaproteobacteria Pasteurellaceae (Schlater *et al.* 1989) and Moraxellaceae (Moss *et al.* 1988; Véron *et al.* 1993). Of these taxa, only the FISH signals for Gammaproteobacteria predominated in August in Conveyor II. Also, two strains of the Moraxellaceae belonging to the genus *Acinetobacter* were isolated from this location. It has to be mentioned that some bacteria contain 18:2 *cis*9,12 (Schlater *et al.* 1989), but generally, their presence is considered as a signature for Eukarya (Lechevalier and Lechevalier 1988; Stahl and Klug 1996) pointing to microeukaryotes. The low percentage of signals for probe EUK502 contradicts this supposition. Phase contrast microscopy also revealed that yeast cells were almost absent. The lubricant Optimol Olit CLS was another source of these lipids. However, extraction experiments with artificial bacteria/lubricant mixtures showed that the washing procedure with decane removes almost all of the lubricant-associated fatty acids.

We used whole cell fatty acid analysis to integrate information from all cell fatty acids. Moreover, this method was chosen to ensure comparability with the huge number of published fatty acid profiles from pure cultures obtained by this method. The widely used analysis of phospholipid fatty acids (PLFA) discriminates lipids from dead cells or detrital lipids but the method does not detect Glycolipids from living cells and LPS bound hydroxy-fatty acids. These markers were used for the detection of acetic acid bacteria, *Pectinatus* sp., *Pseudomonas* sp. and Enterobacteriaceae (Yamada *et al.* 1981; Helander and Haikara 1995; Vancanneyt *et al.* 1996; Bryn and Rietschel 1978).

The FISH analyses revealed an apparent shift in sample of Conveyor II concerning the Proteobacteria. Gammaproteobacteria represented the majority in August, while Betaproteobacteria predominated in January (Fig. 1). Characteristic differences between the fatty acid profiles of these two subgroups are scarce (Lipski *et al.* 1992); this finding could therefore not be corroborated by the lipid analyses.

Different microbial communities at the two conveyors may be related to variable conditions during the filling procedure, e.g. the influence of overfoamed beer. Both sample locations showed a higher detection rate for EUB338 in August, indicating a more active metabolic state of the cells in the summer. The variation in population composition from the same sample site could be due to changes in operating conditions. Specifically, bottling capacity is reduced in wintertime, consequently there is less overflow of beer and the cleaning is more intense. The bottling plant was completely cleaned after taking the samples in August 2000. Therefore, the biofilms from January 2001 were approximately 5 months old and were thus younger than those from August 2000.

Acetic acid bacteria are considered as important organisms in the initial phase of biofilm formation in breweries (Back 1994a). The FISH counts, in combination with the fatty acid analyses and the cultivation approach, showed that these bacteria are of only low quantitative importance in the biofilm communities analyzed. The proportion of probe signals for Acetobacteraceae in the August samples used for isolation were 1.8 and 0.9%. Even though two well-suited media were used, no strain of this group could be isolated. Moreover, 2-hydroxy-hexadecanoic acid, a fatty acid typical of Acetobacteraceae (Yamada *et al.* 1981), was not detected in any sample. As members of the Firmicutes, the families Lactobacillaceae, including *L. brevis*, *L. lindneri* and *Pediococcus damnosus* and the Acidaminococcaceae in particular *Pectinatus cerevisiiphilus*, *P. frisingensis* and *Megasphaera cerevisiae* are target organisms of the probes LGC354A-C (Meier *et al.* 1999). The signals obtained with these probes ranged from 2.1 to 8.1% of all DAPI-stained cells and LGC354A-C detects a large number of different taxa, most of which are not beer-spoiling taxa. If Lactobacillaceae are present, 18:1 *cis*11 and lactobacillic acid (19:0 cyclo11–12) should be present in the biofilm samples (Rizzo *et al.* 1987). In fact, small amounts of 19:0 cyclo 11–12 have been detected in two samples that contained 18:1 *cis*11, but this combination is also typical for most Alphaproteobacteria (Lipski *et al.* 1992). A biomarker of the genus *Pectinatus* - 3-hydroxy-tridecanoic acid (Helander and Haikara 1995) - was not found in the biofilm samples. The LGC354A-C-conferred signals were more likely related to members of the Bacillaceae. A *Bacillus cereus* strain was isolated, and its dominating fatty acids (15:0 iso and 15:0 anteiso; Lipski and Altendorf 1997) were present in the lipid profiles (Table 1), except in the August Conveyor I sample. Consequently, beer-spoiling bacteria represent a minority in these biofilms. This is also true for the communities from Conveyor II, which are exposed to beer. This was also confirmed by the cultivation approach, although ten strains grew in, and acidified, NBB, none actually caused turbidity in beer.

The aim of this work was the analysis of the microbial communities of biofilms in a technical plant. Heterogeneous communities were revealed by all three methods used, with an emphasis on Proteobacteria. The appearance of Archaea, restricted to Conveyor I, has even been shown. In addition, it was possible to identify at least one member of each dominating taxa discovered by FISH, in the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Xanthomonadaceae, Actinobacteria, Bacteroidetes and the Firmicutes. The microbial composition of the 5-month-old biofilms from January 2001 were similar to those of the much older biofilms from August 2000. This diversity indicates very little selective pressure on these environments by cleaning agents and disinfectants, or from the presence of beer.

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Chapter 2

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

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Abstract

Brewery biofilms can harbour beer-spoiling microorganisms, which for is tested routinely. Their presence is supposed to depend on accompanying microorganisms. While there is a substantial amount of information about isolates from bottling plants, cultivation-independent methods are rarely used for the microbial characterization of these surfaces. Therefore, biofilms from the inner part of the bottling plant (Center) and a biofilm from a screw closed to the star wheels (Screw) were examined. Two clone libraries were investigated by amplified 16S rDNA gene restriction analysis (ARDRA) with subsequent sequencing of representative clones. The majority of the clones from the Center sample were assigned to the Gamma- and Alphaproteobacteria and Bacteroidetes. The dominance of these groups in the sample was confirmed by fluorescence *in situ* hybridization (FISH). In addition, the fatty acid methyl esters (FAMEs) analysis of this biofilm indicated the presence of higher proportions of Eukaryotes. The majority of the clones from the Screw library were affiliated to the same main groups as mentioned for the Center sample. Nevertheless, only four ARDRA patterns were detected in both libraries. In addition, considerable numbers of clones were assigned to uncultivated organisms.

Introduction

Biofilms represent a potential source for product damage in many industries. Therefore, plant surfaces are subjected to intense hygienic control. The biofilms have to be disinfected and removed before they become a source of contamination. This is a requirement concerning all industrial plants which process biodegradable products in a humid environment. Problems can occur on paper-producing machineries by slime formation of *Bacillus* species (Blanco *et al.* 1996) and of course in the food processing industry (Zottola and Sasahara 1994; Wong 1997). It is impossible for breweries to keep bottling plants in a conventional filling area sterile. A constant presence of humidity guarantees basic requirements for life. Humidity is provided by cleaning procedures and over-foamed beer, which also supplies some nutrients. The brewery biofilms are supposed to be dominated by ubiquitous slime forming members of the Pseudomonadaceae and Enterobacteriaceae and in particular the Acetobacteraceae as well as accompanying organisms like *Acinetobacter calcoaceticus, Bacillus* sp., *Micrococcaceae,* and many other Gram-negative organisms (Back 2003). These organisms may form niches, which can be colonized by beer-spoiling bacteria, from where they can infect the product by the formation of aerosols. Important beer-spoiling bacteria are members of the Lactobacillaceae, e.g. *L. brevis* and *L. lindneri* and of the strictly anaerobic Acidominococcaceae, which are *Pectinatus cerevisiiphilus*, *P. frisingensis* and *Megasphaera cerevisiae.*

All of these organisms can be detected by a culture-independend approach based on the 16S rRNA gene. In addition, clone libraries enable to demonstrate the presence of unknown organisms, including those with unknown growth requirements. Therefore, this method was used to elucidate the microbial composition of brewery biofilms. They maybe constituted of a narrow spectrum of species due to selective pressure of intense cleaning procedures and application of disinfecting agents. The proportions of beer-spoiling and acetic acid bacteria were examined. Furthermore, the presence of isolates from this bottling plant in the clone libraries will demonstrate their distribution on the plant. In addition, strictly anaerobic bacteria would point to anaerobic microenvironments in the biofilms. These aspects were investigated for two biofilms. One sample was was located in the center of the bottling plant at the level of the open bottles (designated sample: Center). The other sample was taken from a screw nearby the stars (designated sample: Screw). The differentiation of clones was done by ARDRA followed by sequencing which enabled the identification at least at the genus level. The higher amount of biomass at the Center sample site allowed to analyze the biofilm by further methods. The fatty acid methyl ester (FAME) analysis and the fluorescence *in situ* hybridization (FISH) technique were used for getting a more reliable picture of the bacterial community.

Materials and Methods

Sampling. Biofilms were collected by means of sterilized spatulas, transferred into sterile tubes and stored at 4°C until processing. Samples were taken on January 11 (Screw) and on March 22, 2001 (Center). The Center sample site was in the middle of the bottling plant at the level of the open bottles. The biofilm was located around the threat of a screw with a diameter of approximately 5 cm. The biofilm was about 1 - 2 mm high and 6 mm in width, therefore it was considered as a mature biofilm. In contrast to this sample location, the Screw biofilm was only accessible by removing the screw. The biofilm developed at the bottom of the screw, which had a diameter of 1.6 cm. The biofilm was about 1-2 mm high. Because of its hidden position, the screw biofilm was not exposed directly to the cleaning procedures. In detail, the bottling plant was sprinkled regularly with water of 85°C for 3 min in intervals of 2 hours and at the end of a filling procedure it has been laid in foam with an alcalic cleaning agent.

Construction of the clone libraries. DNA extraction was carried out using a modified protocol described by Zhou *et al.* (1996). 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 sulphate (SDS) were added and incubated for 1 h. Hexadecylmethylammonium bromide (CTAB) and NaCl were added to final concentrations of 1% (w/v) and 1.5 M, respectively and incubated for 15 min at 65°C. Subsequently, a freeze and thawing procedure in liquid nitrogen and in a 65°C water bath was repeated three times. The material was centrifuged at 6000 × *g* at 4°C. The supernatant was transferred to a separate tube and one volume of chloroform-isoamyl alcohol (24:1, v/v) was added. The mixture was shaken and centrifuged at 6000 × *g* at 4°C. The aqueous phase was recovered and nucleic acids were precipitated by the addition of 0.7 vol isopropanol and 0.3 vol of 10 M ammonium acetate and pelleted by centrifugation at 16000 × *g* at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in 150 µl autoclaved double distilled water.

PCR amplification and cloning of the amplified 16S rRNA genes. The purified chromosomal DNA was used for amplification of nearly the complete 16S rRNA gene using the two universal primers GM3F (*Escherichia coli* 16S rRNA gene positions 8 to 23 [Brosius *et al*. 1978]) and GM4R *(E. coli* positions 1492 to 1507) (Muyzer *et al.* 1995). 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 Technologies, Karlsruhe, Germany) and a variable amount 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 using the PicoGreen® dsDNA quantification kit (Molecular Probes, Eugene, OR) and a Fluostar fluorescence reader (SLT Instruments, Crailsheim, Germany). The PCR products of three independent PCR reactions were pooled to avoid initial bias. The 16S rRNA gene products were ligated into the pCR®2.1-TOPO® vector. Plasmids were transformed into TOP10 One Shot® competent cells following the instructions of the manufacturer (Invitrogen, Groningen, NL). Recombinant transformants were selected by blue/white colony screening. Individual colonies were grown overnight in 5 ml Luria-Bertani (LB) medium. Two ml were used for plasmid preparation (QIAprep Spin Miniprepkit, Qiagen, Hilden, Germany), and 0.5 ml were mixed with 0.5 ml fresh LB medium containing 50 µl dimethylsulfoxide (DMSO) and stored at –80°C.

Screening for full-length inserts and ARDRA. The isolated plasmids were screened in a 0.8% agarose gel in 1 × TAE buffer (DNA Typing Grade®, Life Technologies, Gaitersburg, MD). Only plasmids with the expected insert of about 1.5 kb were further processed. The isolated plasmids were diluted 1:20 and used as templates (about 10-15 ng) in a PCR reaction which contained the components as described above but the annealing

temperature was reduced after ten cycles from 49°C to 44°C. The restriction mixtures contained 800 ng of the PCR product, 7.5 U *Alu*I (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 TBE-buffer (89 mM Tris, 89 mM boric acid, 2mM sodium EDTA × 2 H_2O) for 300 min at 70 V. A 50 bp DNA ladder (Life Technologies) was used as standard.

Selection of the restriction enzyme *Alu*I. The tetrameric restriction enzymes *Alu*I, *Bfa*I, *Bst*UI, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Rsa*I and *Taq*I were tested *in silico* using the TACG Restriction Enzyme analysis (Mangalam) tool from the Pasteur-Institut, Paris [http://bioweb.pasteur.fr/seqanal/interfaces/tacg.html]. Due to the expected presence of beer-spoiling bacteria in these biofilms, 28 16S rDNA sequences of the following species were digested: *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Lactobacillus brevis*, *L. casei*, *L. curvatus*, *L. fructivorans*, *L. lindneri*, *L. paracasei*, *L. plantarum*, *Lactococcus lactis*, *Megasphaera cerevisiae*, *Pediococcus damnosus*, *P. dextrinicus*, *P. inopinatus*, *P. pentosaceus*, *Weissella confusa*, *Zymomonas mobilis*.

The obtained fragments were analyzed using the Stanford Center for Tuberculosis Research Moelcular Fingerprint Analyzer (SCTR-MFA) [version 0.4.1 (beta), http://molepi.stanford.edu/free_software.html]. The best differentiation was achieved with *Alu*I, which resulted in 18 different clusters of the 28 sequences, followed by *Mbo*I with 13 clusters. The other enzymes tested were less suitable for the resolution of these brewery related bacteria. To compare the virtual patterns with real ones, the ARDRA was performed *in vitro* with the following strains: *Acetobacter pasteurianus* DSM 3509, *Gluconobacter oxydans* DSM 3503, *Kocuria kristinae* DSM 20321 (formerly: *Micrococcus kristinae*), *Lactobacillus brevis* DSM 20054^T, *L. brevis* DSM 6235, *L. brevis* Bi2, *L. brevisimilis* DSM 6265, *L. buchneri* La-1501, *L. casei* DSM 20011^T, *L. curvatus* DSM 20019, *L. coryniformis* subsp. *coryniformis* DSM 20001, *L. curvatus* subsp. *curvatus* DSM 20019, *L. lindneri* La-1805, *L. malefermentans* DSM 5705, *L. parabuchneri* DSM 5707, *L. paracasei* subsp. *paracasei* DSM 20006, *L. plantarum* DSM 20174, *Lactococcus lactis* Lc-0302, *Megasphaera cerevisiae* DSM 20461, *Pectinatus cerevisiiphilus* Pe-0104, *P. frisingensis* DSM 20465, *Pediococcus damnosus* DSM 20331, *P. inopinatus* VLB 8, *Selenomonas lacticifex* DSM 20757 and *Zymophilus raffinosivorans* DSM 20765.

The sequences of eleven species were restricted *in silico* and *in vitro*. Eight of the species gave the same restriction pattern both *in silico* and *in vivo*. One species differed by the absence of a fragment *in silico* which was due to an incomplete database sequence. Two patterns differed because of an additional cutting site *in silico*, the fragments of which were

not separated *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, the ARDRA procedure was performed with some isolates from this bottling plant. Strains from the conveyors have been published (Timke *et al*. 2005), except of strain DW119. The other isolates were obtained by impressing parts of the crowner die on different media. The sample locations of the strains are given in parenthesis: *Achromobacter* sp. DW104, *Brevundimonas* sp. DW78, *Citrobacter* sp. DW99, *Chryseobacterium* sp. DW3, *Enterobacter* sp. DW56, *Enterobacter* sp. DW143a1, *Microbacterium* sp. DW8, *Microbacterium* sp. DW129, *Stenotrophomonas* sp. DW100 (infeed conveyor); *Acidovorax* sp. DW126, *Acinetobacter* sp. DW149, *Bacillus* sp. DW137, *Brevundimonas* sp. DW114b, *Citrobacter* sp. DW38, *Enterobacter* sp. DW40, *Pseudomonas* sp. DW90, *Stenotrophomonas* sp. DW112a3, *Pseudomonas* sp. DW119 (discharge conveyor); *Arthrobacter* sp. DW28, *Deinococcus* sp. DW22, *Enhydrobacter* sp. DW32, *Escherichia* sp. DW33, *Micrococcus* sp. DW13, *Roseomonas* sp. DW26, *Staphylococcus* sp. DW16, *Staphylococcus* sp. DW21 (crowner die).

Analysis of the restriction patterns. The gels were stained with ethidium bromide (1 μg ml^{-1}). Images were acquired using a CCD camera and analysed using the software packages FragmentNT Analysis and ImageQuant (versions 1.2 and 5.2 respectively, Molecular Dynamics, Sunnyvale, CA). Clones with identical ARDRA patterns were grouped and they were referred to as ARDRA group, ARDRA cluster or ARDRA pattern, respectively.

Rarefaction analysis and diversity indices. The ARDRA patterns were also considered as operational taxonomic units (OTUs) and used for rarefaction analysis and calculation of diversity indices. Rarefaction calculations were done using the program aRarefactWin [version 1.3, S.M. Holland, http://www.uga.edu/~strata/software/]. The Shannon-Weaver index of general diversity (H) was calculated applying the formula $H = -\Sigma P_i \ln P_i$, where P_i is the proportion of the individuals of ARDRA pattern i relative to the total number of ARDRA patterns (Shannon and Weaver 1963). The Shannon evenness index (J) was calculated using the formula $J = H/\ln S$, where S is the total number of revealed ARDRA patterns. The Simpson`s index of dominance concentration (D) was calculated as follows: $D = \Sigma (P_i)^2$, where P_i is the proportion for each ARDRA pattern (Simpson 1949). Coverage of the clone libraries was calculated by the equation $C = [1 - (n/N)] \times 100$, where n is the number of unique clones and N is the total number of clones examined (Good 1953).

Sequencing of clones and phylogenetic affiliation. Several clones of every ARDRA cluster with more than two clones were sequenced, using approximately 350 ng of 16S

rRNA gene PCR-product, 0.8 µM primer 787R (Buchholz-Cleven *et al.* 1997), 4 µl of ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Applied Biosystems, Foster City, USA) and water to a final volume of 10 µl. The PCR consists of 30 cycles, denaturation for 1 min at 96°C, annealing for 15 s at 50°C and extension at 60°C for 4 min. The sequencing product was purified using the DyeEx^{TM} Spin Kit (Qiagen, Hilden, Germany). The electrophoresis was performed on a ABI377 (Applied Biosystems) sequencer. The obtained sequences were compared to the National Center for Biotechnology Information nucleotide sequence database using the BLAST algorithm (Altschul *et al.* 1997). 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 (Tables 1 and 4). In total, 15 16S rRNA gene inserts of the Center clones were completely sequenced, using the primers pUC/M13, 518F and 787R (Buchholz-Cleven *et al.* 1997). In addition, 63 partial sequences were obtained from the Center sample and 18 partial sequences from the Screw sample. All sequences were checked for chimera formation with the CHIMERA_CHECK software of the Ribosomal Database Project (Cole *et al.* 2003). Additionally, the phylogenetic affiliations of the 5`and 3` ends of putative chimeras were compared using the ARB software package (Ludwig *et al.* 2004). By this procedure seven potential chimeras (8.2% of all analysed sequences) were detected and rejected from further processing. Sequences of 25 clones were not submitted to the EMBL database because of an identity to already published ones or to other clone sequences from this study. The accession numbers of the 64 submitted sequences are AJ619025 to AJ619086, AJ715500 and AJ715501.

Fatty acid analyses. This method as well as the FISH technique were performed only with the Center sample due to the scarce biomass of the Screw biofilm. Sixty mg of the Center sample material were analyzed. To remove a background of abiotic lipids the material was washed twice with 3 ml sterilized Ringer`s solution (0.9% NaCl, 0.042% KCl, 0.024% $NaHCO_3$) and 3 ml of decane by rotation for 30 min. The organic-phase and the inter-phase were removed (Timke *et al.* 2005). The water-phase was centrifuged at 3400 × g for 20 min at 4°C and the pellet was stored at -20°C until preparation. Saponification, methylation and extraction of the fatty acid methyl esters were done according to Sasser (1990). Identification of the fatty acid methyl esters was performed by GC-MS as described previously (Lipski and Altendorf 1997).

Whole cell hybridization of sample material. Sample materials were suspended in Ringer`s solution before fixation. The fixation and hybridization was done as described by Friedrich *et al.* (1999) using paraformaldehyde (PFA) or ethanol for fixation. Formamide concentrations in the hybridization buffers were used according to the respective references. Probe-positive counts were determined relative to 4',6-diamidino-2-

phenylindole (DAPI; 1 mg l^{-1}) stained cells. At least 400 cells were counted in triplicates per probe. The probes used are given in Table 3, probe Pae997 was supplemented with two helper oligonucleotides (Timke *et al.* 2005).

Results

Sequencing and phylogenetic classification of Center sample clones. The number of analyzed clones was 216 for the Center library, which resulted in 53 different ARDRA patterns. Two or more representative clones of the ARDRA clusters consisting of at least two clones were sequenced. Furthermore, some clones of single member clusters were also sequenced. In total, 66 sequences of clones out of 24 ARDRA groups were analyzed for an evaluation of the cluster homogeneity. The sequences within seven groups were identical. In the remained 17 analyzed ARDRA clusters, the sequence similarities were above 97.8% for 13 of them. This emphasized the high discriminative power of the ARDRA. Thirty-six sequenced clones are shown in Table 1. They represented 29 different ARDRA groups with a total number of 192 clones.

The Gamma- and the Alphaproteobacteria made up 42.7% and 28.6% of these 192 taxonomical assigned clones (Table 1). The Gammaproteobacteria were the most abundant group and consisted of members of the Moraxellaceae and Xanthomonadaceae. In particular, the genera *Acinetobacter*, *Enhydrobacter* and *Luteimonas*, *Pseudoxanthomonas*, *Stenotrophomonas* and *Xanthomonas* were identified, respectively. The genera *Methylobacterium* and *Paracoccus* represented the most frequently detected clone groups of the Alphaproteobacteria. Further clones were assigned to sequences of the Alphaproteobacteria genera *Agrobacterium*, *Brevundimonas*, *Devosia*, *Erythrobacter*, *Porphyrobacter* and *Sphingomonas*.

Table 1. Phylogenetic affiliation of clones from the Center sample. Brackets indicate clones with identical ARDRA patterns.

Clone	Number of clones/cluster[a]	Phylogenetic relationship		
		Class or phylum[b]	Species, strain or clone	similarity (%)
MTAA10 }	22	α	*Methylobacterium extorquens* JCM 2802^T	100
MTAD2 }		α	*Methylobacterium extorquens* JCM 2802^T	99.4
MTAF47 }	10	α	*Paracoccus versutus* ATCC 25364^T	96.3
MTAD35[c] }		α	*Paracoccus yeeii* CDC G1212^T	100
MTAE39	3	α	*Agrobacterium tumefaciens* NCPPB 2437^T	99.8
MTAB20	2	α	*Devosia riboflavina* NBRC 13584^T	99.2
MTAE38	3	α	*Sphingomonas mali* NBRC 15500^T	99.6
MTAE11	3	α	*Porphyrobacter neustonensis* DSM 9434^T	99.7
MTAF30	2	α	*Erythrobacter flavus* SW-46^T	97.5
MTAD4	2	α	*Methylobacterium extorquens* JCM 2802^T	99.4
MTAG39[c]	2	α	*Paracoccus carotinifaciens* NBRC 16121^T	100
MTAB27[c]	3	α	*Brevundimonas vesicularis* LMG 2350^T	98.9
MTAG19	1	α	*Paracoccus aminophilus* JCM 7686^T	98.9
MTAD1[c]	1	α	*Ochrobactrum anthropi* LMG 3310^T	98.7
MTAD6	1	α	*Paracoccus versutus* ATCC 25364^T	99.0
MTAA39	2	β	*Xylophilus amelinus* DSM 7250^T	97.1
MTAG33[c]	1	β	*Acidovorax temperans* CCUG 11779^T	99.7
MTAG9	26	γ	*Acinetobacter johnsonii* DSM 6963^T	99.3
MTAC25	26	γ	*Luteimonas mephitis* B1953/27.1^T	95.8
MTAA44 }	14	γ	*Xanthomonas campestris* ATCC 33913^T	97.8
MTAB23 }		γ	*Xanthomonas vasicola* LMG 736^T	96.8
MTAD30 }	10	γ	*Pseudoxanthomonas broegbernensis B1616/1T*	97.4
MTAE42 }		γ	*Stenotrophomonas nitritireducens* L2^T	99.0
MTAD19	5	γ	*Enhydrobacter aerosaccus* LMG 21877^T	99.5
MTAC39	1	γ	*Xanthomonas codiaei* LMG 8678^T	95.7
MTAE19 }	10	B	*Chryseobacterium joostei* LMG 18212^T	95.7
MTAC14 }		B	*Chryseobacterium indoltheticum* LMG 4025^T	99.2
MTAC8 }	9	B	*Chryseobacterium defluvii* B2^T	96.4
MTAB26 }		B	*Chryseobacterium indoltheticum* LMG 4025^T	95.8
MTAG12	5	B	*Flexibacter sancti* ATCC 23092^T	89.6
MTAC21	4	B	*Sphingobacterium thalpophilum* DSM 11723^T	96.7
MTAB28	2	B	*Pedobacter heparinus* DSM 2366^T	99.7
MTAG14 }	11	Acid.	Bacterium Ellin6075	95.4
MTAF8 }		Acid.	Bacterium Ellin6099	91.2
MTAC17	9	TM7	Uncultured TM7 bacterium clone SM1G12	95.6
MTAA41	2	TM7	Uncultured TM7 bacterium clone SM1G12	95.0

[a] In addition, 25 ARDRA clusters were detected consisting of only one clone. These clones were not sequenced.

[b] The abbreviations represent the Alpha- (α), Beta- (β) and Gammaproteobacteria (γ), the Bacteroidetes (B), the Acidobacteria (Acid.) and the uncultured candidate division TM7 (TM7).

[c] Sequence has not been submitted, all other sequences were deposited at the EMBL database.

Also abundant taxa were the Flavobacteria/Sphingobacteria (15.6%) represented by Chryseobacterium species, which belong to the Flavobacteriaceae and by the genera *Flexibacter*, *Sphingobacterium* and *Pedobacter*, which are members of the Sphingobacteriales. Only 3 clones (1.6%) were assigned to the Betaproteobacteria *Xylophilus* and *Acidovorax*. The other clones were affiliated to the Acidobacteria (5.7%) and the uncultured candidate division TM7 (5.7%).

FAMEs of the Center sample. The fatty acid profile was dominated by C_{18} acids, particularly *cis*-vaccenic acid (Δ11-*cis*-octadecenoic acid, 18:1 *cis*11), linoleic acid (Δ9-*cis*,Δ12-*cis*-octadecadienoic acid, 18:2 *cis*9,12) and oleic acid (Δ9-*cis*-octadecenoic acid, 18:1 *cis*9) (Table 2). Furthermore the methyl branched 12-methyl 14:0 (15:0 anteiso) and 14-methyl 16:0 (17:0 anteiso) made up an important part of the fatty acid composition. Some iso branched acids were detected. Also abundant fatty acids were the palmitic acid (hexadecanoic acid, 16:0), palmitoleic acid (Δ9-*cis*-hexadecenoic acid, 16:1 *cis*9) and Δ9-*cis*-heptadecenoic acid (17:1 *cis*9). The lipopolysaccharides bound hydroxy-fatty acids were found in a small portion.

Table 2. Fatty acid composition of the Center sample.

Fatty acids	Percentages 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

Table 2. continued

Fatty acids	Percentages of the complete profile
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

FISH of the Center sample. The bacterial detection rate obtained with a combination of the probes EUB338 (Amann et al. 1990), EUB338II and EUB338III (Daims et al. 1999) (designated: EUB338-III) was 60.7% (Table 3). The probe signals for Eukarya and Archaea made up a little proportion (2.6% and 1.6%) so in total, about two-thirds of all DAPI stained cells were detected. The overall percentages for the probes PLA46, CF319a, ALF968, BET42a, GAM42a, HGC69a, LGC354A-C and XAN818 were 40.1% indicating that two-thirds of the cells detectable with the EUB338-III probes were targeted with the probes for higher taxa.

Table 3. Fluorescence *in situ* hybridization results of the Center sample. Values are percentages of DAPI-stained cells. Standard deviations (SD) are given in parenthesis.

Probe	Mean value[a] ± SD	Target	Reference
EUB338-III	60.7 (5.1)	Bacterial 16S rRNA	Amann *et al.* 1990, Daims *et al.* 1999
EUK502	2.6 (1.1)	Eucaryal 18S rRNA	Amann *et al.* 1995
ARCH915	1.6 (0.4)	Archaeal 16S rRNA	Stahl and Amann 1991
PLA46	2.0 (1.0)	Planctomycetal 16S rRNA	Neef *et al.* 1998
CF319a	9.6 (3.2)	Cytophaga-Flavobacterium cluster of the CFB phylum 16S rRNA	Manz *et al.* 1996
ALF968	11.7 (0.9)	α- proteobacterial 16S rRNA	Neef 1997
BET42a	5.0 (2.3)	β- proteobacterial 23S rRNA	Manz *et al.* 1992
GAM42a	4.8 (1.2)	γ- proteobacterial 23S rRNA	Manz *et al.* 1992
HGC69a	1.9 (1.2)	Actinobacterial 23S rRNA	Roller *et al.* 1994
HGC69a	3.8 (1.4)	Actinobacterial 23S rRNA (Ethanol fixed cells)	Roller *et al.* 1994
LGC354A-C	3.2 (3.1)	Firmicutes 16S rRNA	Meier *et al.* 1999
LGC354A-C	3.8 (1.2)	Firmicutes 16S rRNA (Ethanol fixed cells)	Meier *et al.* 1999
ACA652	0.9 (0.4)	*Acinetobacter* sp. 16S rRNA	Wagner *et al.* 1994
AG1427	0.1 (0.1)	*Acetobacter* sp. and *Gluconobacter* sp. 16S rRNA	Neef 1997
ENT183	0.0 (0.0)	Enterobacterial[b] 16S rRNA	Friedrich *et al.* 2003
Pae997	1.0 (0.8)	*Pseudomonas aeruginosa* and further *Pseudomonas* sp. 16S rRNA	Amann *et al.* 1996
XAN818	1.9 (0.8)	Xanthomonas branch 16S rRNA	Friedrich *et al.* 1999
NON338	0.0 (0.0)	Negative control	Wallner *et al.* 1993

[a] All values originated from PFA fixed samples except the mentioned HGC69a and LGC354A-C percentages.

[b] The target sequence has been found for the genera *Enterobacter*, *Citrobacter*, *Erwinia*, *Escherichia*, *Hafnia*, *Klebsiella*, *Pantoea*, *Salmonella*, *Serratia*, and *Shigella*.

The Alphaproteobacteria were the most abundant FISH group (11.7%), followed by the Bacteroidetes (9.6%). FISH percentages for the Gammaproteobacteria were 4.8%, of which an abundant proportion corresponded to the genus *Acinetobacter* detected by probe ACA652 (0.9% of all DAPI stained cells). The Xanthomonadaceae as a group of the Gammaproteobacteria accounted for 1.9% of the FISH signals. The Betaproteobacteria (5.0%), the Gram-positive Actinobacteria (3.8%) and Firmicutes (3.8%) constituted considerable proportions of the FISH signals. Whereas the fixation procedure had nearly no effect for the Firmicutes, the number of actinobacterial probe signals increased for ethanol fixed cells compared to PFA fixed ones. Signals for the Planctomyces group were 2%. The more specific probe for *Pseudomonas* cells detected a proportion of about 1%. No signals were found for the enterobacterial as well as for the acetic acid bacterial probe.

Sequencing and phylogenetic classification of Screw sample clones. Eighty clones of this library were analyzed by ARDRA, of which 59 different patterns were detected. Sixteen clones were sequenced, each of them represented an ARDRA cluster with one up to six members (Table 4). Therefore, they covered 37 clones of the 80 examined ones. This part of the clone library contained members of the same classes or phyla as the Center library, except for the Acidobacteria. The main groups were the Alpha- and Gammaproteobacteria with 29.7% and 18.9%, respectively. Sequences of Alphaproteobacteria were assigned to members of the genera *Devosia*, *Sphingopyxis* and *Blastochloris*. The most similar sequences for some clones were found to be members of the Gammaproteobacteria including the genera *Acinetobacter*, *Citrobacter*, *Enhydrobacter*, *Thermomonas* and *Xanthomonas*. The soil clone SM1G12 of the uncultured candidate division TM7 sequence was similar to the one of clone MTBII30, which belonged to a six member clone group (16.2%). Another abundant ARDRA pattern of this library comprising five clones (13.5%) was affiliated to an uncultured clone which originated from a soil sample. One pattern was assigned to *Achromobacter*, a member of the Betaproteobacteria (5.4%). Two ARDRA patterns were affiliated to members of the Flavobacteria/Sphingobacteria (10.8%), a *Flavobacterium* sp. and an uncultured Cytophagales clone. Another partial clone sequence was identical to a *Lactococcus lactis* sequence (2.7%) and next to this member of the Firmicutes a representative of the Actinobacteria was detected by a sequence assigned to *Microbacterium* sp.

Table 4. Phylogenetic affiliation of clones from the Screw sample.

Clone	Number of clones/cluster[a]	Phylogenetic relationship		
		Class or phylum[b]	Species, strain or clone	similarity (%)
MTBI8	5	α	*Devosia riboflavina* NBRC 13584^T	99.4
MTBI42	2	α	*Sphingopyxis witflariensis* W-50^T	98.5
MTBII35	2	α	*Blastochloris viridis* ATCC 19567^T	94.9
MTBII11	2	α	*Sphingpyxis macrogoltabidus* NBRC 15033^T	99.1
MTBI32	2	β	*Achromobacter xylosoxidans* DSM 30026^T	99.0
MTBI36	2	γ	*Thermomonas fusca* LMG 21737^T	99.8
MTBII46	2	γ	*Xanthomonas campestris* ATCC 33913^T	95.6
MTBI44	1	γ	*Citrobacter freundii* DSM 30039^T	99.7
MTBIII10	1	γ	*Acinetobacter johnsonii* DSM 6963^T	99.0
MTBII12[c]	1	γ	*Enhydrobacter aerosaccus* LMG 21877^T	99.7
MTBI24	2	B	*Flavobacterium ferrugineum* ATCC 13524^T	96.3
MTBII23	2	B	Uncultured Cytophagales clone 26	92.8
MTBII7[c]	1	LGC	*Lactococcus lactis* subsp. *lactis* NCDO 604^T	100
MTBIII7	1	HGC	*Microbacterium leavaniformans* DSM 20140^T	97.2
MTBII30	6	TM7	Uncultured TM7 bacterium clone SM1G12	93.9
MTBII31	5	Uncul.	uncultured bacterium #0319-6E22	93.2

[a] In addition, 43 ARDRA clusters were detected consisting of only one clone. These clones were not sequenced.

[b] The abbreviations represent the Alpha- (α), Beta- (β) and Gammaproteobacteria (γ), the Bacteroidetes (B), the Firmicutes (LGC), the Actinobacteria (HGC), the uncultured candidate division TM7 (TM7) and uncultured cloned sequences (Uncul.).

[c] Sequence has not been submitted, all other sequences were deposited at the EMBL database.

Comparison of both clone libraries. The ARDRA groups showed two different compositions of the clone libraries. The clone library from the Center sample contained more multiple member clusters and fewer unique patterns than the Screw library. The number of clones per cluster are given in Tables 1 and 4. A rarefaction analysis of these results obtained by ARDRA was done in order to estimate to what extent the diversity of the samples studied can be described by examining 216 and 80 clones, respectively (Fig. 1). The rarefaction curve of the Center 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 of 86.6% for the 216 examined clones. In contrast, the much steeper curve of the Screw clone library and the Coverage value of 40% confirmed the higher diversity in this clone library. Thus, further analysis of clones would have discovered additional diversity. The Shannon-Weaver diversity index (*H*) provides more information about community structure than species richness, it accounts for both abundance and evenness of the species present. The calculated *H*-indices were 3.27 (Center) and 3.91 (Screw). The value also depends on the number of considered OTUs. If every ARDRA pattern found would have been unique, the maximal possible values for the clone libraries would have

been 5.38 (Center) and 4.38 (Screw). These theoretical maximal values underlined the higher diversity in the Screw library, the *H*-value of which was closer to the maximal possible values. The Shannon evenness index (*J*) can vary between 0 and 1, where 1 means, that all species are present at the same abundance. The smaller *J*-value (0.82) for the Center sample indicates a higher dominance of a few species in comparison to the Screw library (0.96), where the *J*-value approached 1 which pointed to a higher evenness of the distribution of ARDRA types over the patterns found. The same aspect is expressed by a higher Simpson`s index of dominance concentration of 0.059 for the Center library and 0.026 for the Screw clones, repectively.

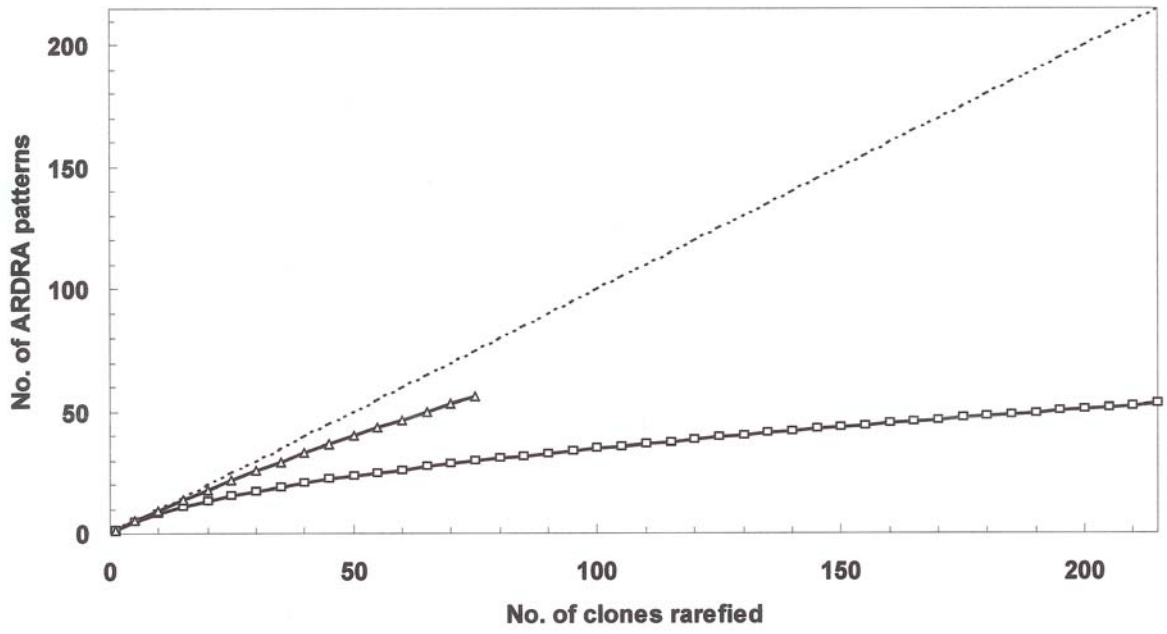

Figure 1. Rarefaction curves calculated for the different ARDRA patterns. The spotted line equals slope 1, which is the case if every new examined clone has a new pattern. The curve for the Center sample (open squares) flattens and indicates a nearly complete represented diversity in this library. The slope for the curve of the Screw library (open triangles) is much steeper and far from saturation indicating a higher diversity and a high number of not detected ARDRA patterns.

Next to the diversity found within each of the clone libraries, there were also great differences between these two clone libraries. Comparing the 53 and 59 different ARDRA patterns of the Center and Screw sample, respectively, only four patterns were identical. The large group of the clone library from the Center sample with clone MTAG9 identified as *Acinetobacter johnsonii* was present in the Screw library as a single pattern. A pattern assigned to *Enhydrobacter aerosaccus* has been detected five times in the Center library

and once in the Screw library (MTBII12). A pattern affiliated to *Devosia riboflavina* has been present three times in the Center and five times in the Screw sample, respectively. The ARDRA type of clone MTAB30 from the Center sample was found two times in the Screw library and MTBI24 has been assigned to a *Flavobacterium* species.

Comparison of ARDRA patterns from clone libraries with those of reference strains. The 108 different ARDRA patterns of both clone libraries were compared to *in silico* and *in vitro* generated patterns from beer-spoiling bacteria. One clone from the Screw library (MTBII7) matches with the pattern of *Lactococcus lactis* Lc-0302. This species is considered as a potential beer-spoiling organism (Back 1994). No other restriction pattern of the tested beer-spoiling bacteria was found in the total number of 296 analyzed clones. Their absence was confirmed by the obtained sequences (Tables 1 and 4).

Bacteria were isolated from this bottling plant (Timke *et al.* 2005; unpublished) and their ARDRA patterns were compared to the ones of the clones. In total, eight ARDRA patterns of the isolates were identical to clone patterns. Five ARDRA patterns of isolates were present in the Center clones library and also five in the Screw sample, respectively. The ARDRA patterns of the isolated strains *Acinetobacter* sp. DW149 and *Enhydobacter* sp. DW32 were detected in both libraries. Details of their identity and sequence similarities are given in Table 5.

Table 5. 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% (1484)	*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% (1378)	*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.
[b] The number of compared bases is given in parenthesis.

Discussion

While selective detection methods like cultivation, specific PCR or FISH could only detect the respective target organisms, 16S rRNA gene clone libraries are non-discriminative. Therefore, a clone library is a powerful tool for answering the question of biodiversity in biofilms, for which no data from beer bottling plants were available.

The Center and the Screw samples have been processed separately and were not combined because of the opportunity to find differences between the two clone libraries. Nearly every processing step in the construction of a 16S rRNA gene clone library leads to some bias (von Wintzingerode *et al*. 1997). Therefore, the composition of a clone library differs from the one of the microbial community *in vivo*. Despite all critical steps, the clone libraries of this study revealed a high diversity. This result implied that the methodology works effectively for various phylogenetic groups and the confirmed diversity has to be considered as the minimal one present.

The obtained clones were differentiated by ARDRA which discriminative power has been shown by Moyer *et al.* (1996). They demonstrated in a computer-simulated restriction digestion that at least three tetrameric restriction enzymes were needed to detect > 99% of 106 taxa spanning the domain bacteria, also including some species of the same genus. Recently, the ARDRA was deployed for the fast identification of filamentous actinomycetes to the genus level with four different restriction endonucleases (Cook and Meyers 2003). In this study, *Alu*I has been used. Consequently, the sequencing approach showed that only seven of the 24 ARDRA groups analyzed for homogeneity had identical sequences. Therefore, the ARDRA differentiation could overlook species diversity and the revealed diversity represented the minimal level of species richness present. At least, the number of examined clones limits the revealed diversity. Whereas the rarefaction analysis (Fig. 1) and the coverage values indicated additional diversity in the Screw clone library, only little would have been found in the Center clone library.

Differences of the diversity between the two clone libraries were evidenced by the calculated diversity indices. This can be due to several reasons. The Center site was accessible for the cleaning procedures and the complete plant was cleaned intensively in August 2000. In March 2001 the sample was taken, consequently, this community was somewhat older than half a year and has been exposed to cleaning stress like disinfectants and sprinkling hot water. In contrast, the bottom side of the screw was not accessible and could have been reached only by unscrewing it also for cleaning purposes, so it has been an older biofilm. This could explain the different diversity levels (Fig. 1) based on the assumption that further species colonized the biofilm with time. An alternate explanation is

that the composition of the communities have changed between the time the two samples were taken. Indeed, the composition of microbial communities of breweries varied. Temporal and spatial variations in the compositions of microbial communities were detected by analyzing the lipid profiles of brewery biofilms (Timke *et al*. submitted).

The diversity indices enable a direct comparison with other studies. Although the cross-study comparison is often hampered by the various methods used to cluster clone phylotypes. A clone library from a waste gas degrading biofilter revealed a higher diversity with *H* and *J* indices of 5.68 and 0.98 (Friedrich *et al.* 2002). The corresponding values for the Center and Screw clone libraries were 3.27 (*H*) and 0.82 (*J*) as well as 3.91 (*H*) and 0.96 (*J*), respectively. In contrast, clone libraries of arctic and antarctic samples revealed Shannon-Weaver indices (*H*) of 1.09 and 0.81 (Brinkmeyer *et al.* 2003). The values reflected a lower diversity in these extreme habitats. Another study, methodological very similar to this one, examined the bacterial population of an aquifer (Cho and Kim 2000). The restriction of 225 clones with *Hae*III resulted in 38 different restriction fragment patterns. The *H*-value was 2.59, so the diversity has been somewhat lower than for the Center brewery biofilm.

Next to these diversity aspects, the data will be discussed in context of the identity of the major groups. First of all it has to be mentioned that Eukarya and Archaea were also present. The fatty acid profile of the Center sample indicated a high proportion of microeukaryotes by the presence of linoleic (Lechevalier and Lechevalier 1988; Stahl and Klug 1996) and oleic acids (Erwin and Bloch 1964). However, the detection of single cells with the fluorescent-labeled probe EUK502 detected only 2.6% probe conferred signals of all DAPI stained cells. A part of the oleic acid may originate from *Acinetobacter* cells, in which it is the main fatty acid (Moss *et al.* 1988; Véron *et al.* 1993). Clones affiliated to *Acinetobacter johnsonii* represented one of the most dominating groups. Important is also the different amount of biomass of a single bacterial and eukaryotical cell. This leads to diverging proportions of bacteria and yeasts comparing values obtained for biomass (FAMEs analysis) or cell numbers (FISH analysis) (Timke *et al*. submitted). The archaeal proportion of the Center community was 1.6% revealed by FISH. The presence of both domains on this plant was also confirmed by PCR products (data not shown) obtained with extracted DNA from the Screw sample and primers for the archaeal (Arch21F and Arch958R) or eukaryotical (EukF and EukR) small-subunit rRNA genes (DeLong *et al.* 1992).

No ARDRA pattern of an important beer-spoiling species was detected. This minor abundance or even absence of beer-spoiling bacteria is in line with results from a study in which the infeed and the outlet conveyor communities of this brewery were analyzed

(Timke *et al*. 2005). In contrast, some ARDRA patterns of isolates obtained from this plant were part of the clone libraries (Table 5). These strains confirmed the presence of the corresponding clone groups on 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.

In accordance with the proportion of Alphaproteobacteria in the Center clone library (28.6%), probe ALF969 detected the most DAPI stained cells of all probes used (11.7%). Correspondingly, the fatty acid *cis*-vaccenic acid was the dominating compound in the fatty acid profile of the Center sample (Table 2). This lipid is the major component of membranes of Alphaproteobacteria (Lipski *et al.* 1992). Many bacteria cyclisize the *cis*-vaccenic acid, but no 19:0 cyclo11-12 has been found. Interestingly, *Methylobacterium* species lack this ability (Doronina *et al.* 1998). One of the most abundant clone groups was affiliated to *Methylobacterium extorquens*. Additionally, this species produces pink pigments (Guckert *et al.* 1991) which could be the reason for the pink to brown colour of the Center biofilm. The presence of *Paracoccus* sp. in the Center biofilm has been supported by the detection of 12:1 *cis*5 which is a component of *Paracoccus* cells as well as the 18:0 (Lipski *et al.* 1998), which can be also found in *Methylobacterium* cells (Guckert *et al.* 1991). 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 (Yamada *et al.* 1981) has not been detected in the lipid extract.

A comparison of the proportions of Gammaproteobacteria in the clone library (42.7%) 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 only three clones (1.6%) affiliated to this class detected in the Center library. This shows again that no reliable quantitative information can be generated by clone libraries. Consistently, clones affiliated to *Acinetobacter* sp. made up 13.6% of the identified clones and only 0.9% of ACA652 probe signals were detected. The target sequence of probe ACA652 is present in *Acinetobacter* sp. DW149 and in the clones MTAC10, MTAG9 and MTAF5. Several strains of the Pseudomonadaceae and the Enterobacteriaceae were isolated from the plant (Timke *et al.* 2005, unpublished) and they were supposed to be part of the community (Back 2003). However, no sequence of these families was detected in the Center clone library. Accordingly, only 1% signals for the probe Pae997 and 0% signal for probe ENT183 were obtained.

The Flavobacteria/Sphingobacteria constituted a proportion of 15.3% in the Center clone library. The probe CF319a revealed 9.6% probe conferred signals for this sample which confirmed the quantitative importance of these organisms in the biofilm. However, the main fatty acid of these groups, 15:0 iso (Yabuuchi *et al.* 1983; Lipski *et al.* 1992) was only present in a low quantity.

FISH signals for the Gram-positive phyla Actinobacteria and Firmicutes indicated 3.8% of all DAPI stains cells for each of these phyla (Table 3). Their presence has been supported by fatty acid analysis, the detected methyl-branched 15:0 anteiso and 17:0 anteiso are main fatty acids in Microbacteriaceae (Bendinger *et al.* 1992). In contrast to this, no representative of these taxa was detected in the Center clone library. However, a *Microbacterium* sp. has been isolated from the infeed conveyor and another *Microbacterium* sp. was found in the Screw library. In general, clone libraries seem to underestimate the proportion of Gram-positive bacteria found by a further method like cultivation (Borneman *et al.* 1996; Smit *et al.* 2001) or FISH (Friedrich *et al.* 2002). Gram-positives may be more resistant to cell lysis but the DNA extraction method has been developed for soil communities (Zhou *et al.* 1996) and the clones MTBII7 and MTBIII7 from the Screw biofilm demonstrated that the detection of Gram-positives has been possible.

Additionally, members of the Acidobacterium group and the uncultured candidate division TM7 were found in the Center clone library, which next relative 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.

The Screw clone library revealed also the Alpha- and Gammaproteobacteria and the Flavobacteria/Sphingobacteria as main groups, although a comparison of the ARDRA patterns indicated two very different communities. In addition, there was a higher proportion of sequences in the Screw library which were assigned only to uncultured bacteria. The next similar sequence of the TM7 affiliated clone was the same as the one for the Center library, indicating the presence of this unclassified bacteria on a technical plant.

One of the first steps in characterizing an ecosystem is to identify the organisms inhabiting it, this was the main aspect of this work. Stainless steel as support material, regularly treatment with disinfectants and hot water let to the development of a community which was significantly different to other communities analyzed by the cloning approach as reviewed in Hugenholtz *et al.* (1998), including freshwater communities. The next step is to assess functional aspects of the identified organisms. This is feasible if there is a good

linkage of phylogeny and the investigated functions, e.g. nitrifying bacteria are located in certain phylogenetic groups. The detection of functional groups is hardly possible if the function can be fulfilled by a variety of different phylogenetic taxa, e.g. denitrifying bacteria are distributed among 50 genera (Zumft 1992). 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 is xanthan, produced by *Xanthomonas campestris* (Sutherland 2001), which was the next relative type strain for clones from both sample sites (MTAA44 and MTBII46). EPS production has been also described for *Acinetobacter* sp. (Lewis *et al.* 1989; Pirog *et al.* 2002). So this genus may be important for the formation of brewery biofilms. The oxygen requiring metabolism for establishing anaerobic microenvironments can be performed by every taxon which has been affiliated to the clone library sequences, except the *Lactococcus* sp. Therefore, both functions could obviously be fulfilled in mature biofilm communities like the examined ones. Nevertheless, no clone sequence was assigned to strictly anaerobic bacteria. Their absence has been supported by the fatty acid profile of the Center sample. There was no indication for the presence of plasmalogenes which are present in many anaerobic bacteria (Goldfine 1982).

In conclusion, the number of species affiliated to brewery biofilms was expanded by this cloning study including also some uncultured taxa. The diversity found for the industrial habitat was in the same range as reported for environmental samples. Although these bottling plant has been intensively cleaned, this did not selected for only a few species. The diversity indicated the presence of a large gene pool, which would enable the biofilm communities to react on changing cleaning procedures and different disinfecting agents. This strengthens the demand for preventing mature biofilms, before they become a serious problem.

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Chapter 3

Fatty Acid Analysis and Spoilage Potential of Mature Biofilms from Two Breweries

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Abstract

The whole cell fatty acid profiles of 78 biofilms from bottling plants and adjacent areas of two breweries were analyzed. About half of the lipid profiles were dominated by oleic and linoleic acid, which refer to a high proportion of Eukarya. Typical fatty acids of Gram-negative and Gram-positive bacteria were found in many samples. In addition, more than half of all samples contained dimethylacetals indicating the presence of strictly anaerobic bacteria in these biofilms. Typical fatty acids for potentially beer-spoiling genera were detected in three biofilms. However, growth experiments in beer demonstrated the presence of beer-spoiling organisms in about 25% of the investigated biofilms. The majority of the biofilms contained no beer-spoiling organisms or just those ones which caused turbidity in non-alcoholic beer but not in beer with 4.8% v/v of ethanol. The lipid profiles were not grouped in brewery associated clusters by statistical analyses indicating no brewery specific microbial communities. In addition, even biofilms from similar or adjacent sample locations clustered in different groups signifying a high heterogeneity of microbial brewery biofilm communities.

Introduction

The extraction and analysis of fatty acids is well-established in microbial ecology for characterizing microbial communities (Vestal and White 1989; Haack *et al.* 1994). This method allows the processing of large, representative samples of biomass and it detects all taxa present, except the Archaea. Marker lipids of certain groups allow to draw conclusions about the community structure (Vestal and White 1989, Zelles 1999). In addition, rarely appearing fatty acids indicate the presence of specific taxa. Consequently, the microbial communities of soil samples (Bossio *et al.* 1998; Steinberger *et al.* 1999), freshwater (Smoot and Findlay 2001), estuarine (Guckert *et al.* 1985) and marine sediments (White *et al.* 1979) have been studied by lipid profiles. Next to these natural habitats, there are also data available for communities from technical environments like drinking water supply systems (Keinänen *et al.* 2004) and wastewater treatment systems (Werker and Hall 1998). Knowlegde about these communities is of great interest because it could help to maintain and control the systems and is therefore also of economic relevance. The removal and disinfection of biofilms from industrial plants requires a lot of chemicals, energy and working time. Biofilms can cause problems in the paper producing industry by slime formation (Blanco *et al.* 1996) and in the food industry if they harbour spoiling organisms (Zottola and Sasahara 1994). Data of the fatty acid composition of microbial communities from industrial plants is scarce. Therefore, the biofilm communities of bottling plants and adjacent areas of two breweries were investigated in this study.

Beer bottling plants are complex machines with a lot of niches in which biofilms can develop. They can harbour beer-spoiling organisms and protect them against dessication, heat and disinfectants. In particular, the acetic acid bacteria are supposed to be associated with beer-spoiling bacteria and enable them to survive in the brewery environment (Back 1994). The main spoiling organism for beer is *Lactobacillus brevis*, further important beer-spoiling members of the Lactobacillaceae are *L. lindneri*, *L. coryniformis* and *Pediococcus damnosus*. In addition, the strictly anaerobic genus *Pectinatus* and wild yeasts are also harmful for beer (Back 2003). The beer can get turbid and some microorganisms, in particular *Pectinatus* sp. produce off-flavour compounds (Back *et al.* 1988). These organisms can be identified by their lipid profiles. The main fatty acids for the Lactobacillaceae are *cis*-vaccenic acid and lactobacillic acid (Rizzo *et al.* 1987). The genus *Pectinatus* has 13:0 3OH and 18:0 cyclo9-10 as signature fatty acids (Helander and Haikara 1995) and yeast cells are dominated by the typical eukaryotic fatty acids oleic (Erwin and Bloch 1964) and linoleic acid (Lechevalier and Lechevalier 1988; Stahl and Klug 1996). Interestingly, the genus *Saccharomyces* lacks significant amounts (< 0.8%) of linoleic acid (Malfeito-Ferreira *et al.* 1997), so it is possible to discriminate between *Saccharomyces* yeasts and non-*Saccharomyces* yeasts. The *Saccharomyces* spp. are considered to be the most hazardous brewing contaminant yeasts (van der Aa Kühle and Jespersen 1998). Non-*Saccharomyces* beer-spoiling yeasts are e.g. *Pichia membranefaciens* and *Hansenula anomala* as well as members of the genera *Torulopsis*, *Schizosaccharomyces*, *Brettanomyces*, *Kloeckera* and *Candida* (Jespersen and Jakobsen 1996). In addition, the detection of dimethylacetals points to the presence of plasmalogene containing strictly anaerobic organisms (Goldfine 1982; Moore *et al.* 1994). Thus, the potential of biofilms in oxic environments as habitats for strictly anaerobic bacteria such as beer spoilers could be evaluated. The beer-spoiling organisms can be distributed e.g. by persons, splashs or movements of the air from the biofilms and reach the product in form of aerosols. The infection appears randomly and affects only very few bottles, so it is very difficult to locate the source of the microorganisms and eliminate them. If their proportion of the total community is low, their typical fatty acids can be below the detection limit of this method. To overcome this, some biomass was inoculated in beer and checked for turbidity.

Materials and Methods

Sampling. The bottling plants were partly disassembled for taking the samples in order to reach even hardly accessible sites. In addition, biofilms were taken from other machines and places in the filling area, e.g. the bottle washing machine or drainage pits and water samples were also taken from a drainage pit and from a groove in the basement. Biofilms were collected by means of sterilized spatula, transferred into sterile tubes and stored at 4°C until

processing. Samples from brewery A were taken on March 22, 2001 (designated A1 - A31) and on July 3 and 4, 2002 (designated a32 - a75) and from brewery B on October 25, 2001 (designated B1 - B24), respectively. In total, 99 samples were taken (Table 1). Samples from brewery A of both sampling periods originated mainly from the bottling plant including the filler carousel. The biofilms of brewery B were taken from the region of the star wheels and the crowner and the majority of the samples represented locations in the filling area.

Fatty acid analyses. At least 50 mg of biomass were used for lipid analysis of 78 biofilm samples. Some samples had a higher proportion of lubricants. Therefore, all samples were washed with decane as described previously (Timke *et al.* 2005) to remove a background caused by abiotic fatty acids and to standardize the procedure. The pellet was stored at -20°C until processing. Saponification, methylation and extraction of the fatty acid methyl esters (FAMEs) were done according to Sasser (1990). Identification of the fatty acid methyl esters was performed by GC-MS as described previously (Lipski and Altendorf 1997). Dimethylacetals (DMAs) were identified by the ion m/z 75 as the base peak and the ion $M\text{-}31^{+}$ (Miyagawa 1982).

Fatty acid nomenclature. The fatty acids are named by their trivial names. If there are no common ones, shorthand designations were used, as the number of carbon atoms in the chain is given first. After a colon the number of double bonds is specified. The configuration and location of functional groups are given from the carboxyl end of the molecules. The abbreviation cyclo determines a cyclopropyl group.

Growth tests in beer. Enrichments in beer were done for testing the microbial communities for the presence of viable beer-spoiling organisms including those with proportions too low for being detected by the lipid analysis. The test was performed with all samples of which sufficient biomass for the inoculation of two test tubes was available. In total, 93 samples were tested. Biomass of the amount of a tip of a little spatula was used (approximately 0.1 g). It was inoculated in 21 ml beer in tubes sealed with screw caps. The tubes were filled completely with non-alcoholic beer, or beer with 4.8% v/v of ethanol (designated: beer). The samples were inoculated in beer of the corresponding brewery, which has been heated at 70°C for 30 min before use. The tubes were incubated at 25°C without any rotation and examined regularly for growth up to 133 days for the brewery A samples from 2001, for 79 days for the ones from 2002 and for 55 days for the brewery B samples. All tubes were checked for turbidity in comparison to control tubes by the same investigator. Samples showing only a slight turbidity were evaluated to exclude false positive results due to a dissolving of the inoculum. In those cases, 100 µl of the sample in question were transferred into fresh beer and re-examined for growth.

Statistical analysis of the fatty acid profiles and correlation of fatty acids to the growth potential in beer. All calculations were done with the SYSTAT® software package (SPSS Science SoftwareG, Erkrath, Germany). The hierarchical cluster analysis was calculated with the average linkage and euclidean distance methods (Fig. 1). Data reduction was performed using the principal component analysis procedure of the software (Fig. 2).

All fatty acid profiles of biofilms from identical sites which were sampled in 2001 and 2002 and lipid profiles from sites with similar exposition were selected for a more in depth analysis. The designated names of these groups used in Fig. 1 and Fig. 2 and the sample numbers are given in parenthesis. One of the most intensive investigated sample sites were the drainage pits with nine samples (gully: a65, a67, a68, a69, a72, a74, a75, B23, B24) and the inner parts of the star wheels with six samples (star: A2, A3, A4, a34, a35 and B3). The engine compartments of the bottling plants and the conveyor belts were each represented by five samples (engine: A27, A28, a56, B9 and B10; conv.: A29, a60, a61, a63 and a64). Four comparable sampling sites originated from the control frames (frames: A6, A7, A8 and a36). Three fatty acid patterns were obtained from biofilms from the lower part down inside the filler (filler: A24, A25 and a52), from different places of a mine chamber (chamber: A14, A15 and a41) and from a screw or a pipe respectively, inside the filler just next to the open bottles (screw: A23, a45 and a46). Finally, the edge between a stainless steel plate and cast iron inside the filler carousel was sampled in 2001 and 2002 (edge: A22 and a44).

To detect any correlation between the presence or absence of fatty acids and growth of members of the biofilm communities in non-alcoholic beer and beer the Pearson correlation coefficient was calculated. The percentages of each fatty acid or DMA in 72 different samples were correlated to the growth in non-alcoholic beer or beer indicated by 0 for no growth and 1 for turbidity in the data matrix.

Proportions of fatty acids in defined mixtures of yeasts and bacteria. The contribution of eukaryotic and bacterial cells to a community fatty acid profile may differ from their proportions of cell numbers, e.g. a minor number of yeast cells could dominate a mixed community fatty acid profile. To investigate such an effect, defined mixtures of *Candida* sp. Ro5a and *Pseudomonas* sp. DW115 were analyzed. Both strains were isolated from bottling plant A. The *Candida* sp. Ro5a cells were oval shaped with a size of approx. 3 to 8 µm. The cells were grown overnight at 30°C in Trypticase Soy broth (Becton/Dickinson, Cockeysville, USA), harvested by centrifugation (3400 × *g*, 10 min, 4°C) and fixed in 3% paraformaldehyde solution overnight at 4°C. After washing with phosphate-buffered saline (PBS, 10 mM $Na_2HPO_4 \cdot 2H_2O$ adjusted to pH 7.4 with $NaH_2PO_4 \cdot H_2O$; final concentration of NaCl, 130 mM) the cells were resuspended in 50% PBS/ethanol. The cell concentrations were determined by a Thoma counting chamber. The cell suspensions were mixed to get

yeast:bacterium cell number rations of: 100:0; 75:25; 50:50; 25:75; 10:90; 5:95; 2:98; 1:99; 0.5:99.5 and 0:100. The analysis of the fatty acids was done in triplicates as described above. Oleic and linoleic acid were only present in the fatty acid profile of the yeast. Their proportion in a pure culture was 80.8% ± 0.1% this maximal possible value represented 100% for the values obtained for the mixtures. In this way their proportions in the fatty acid profiles were calculated and the percentages could be compared to the percentages of the cell number. The same was done for the fatty acids only present in *Pseudomonas* sp. DW115 cells and not in yeasts with a higher proportion of 3% in a pure culture (ECL 10.460 (Equivalent chain length value, Sasser 1990); 10:0 3OH; 12:0, 12:0 3OH; 16:1 *trans*9 and 17:0 cyclo9-10). In addition, the fatty acid content per cell and per mg wet weight of *Candida* sp. Ro5a and *Pseudomonas* sp. DW115 was calculated.

Results

Fatty acid analysis. The fatty acid profiles of 78 samples revealed 65 different FAMEs with carbon chain lengths ranging from eight to 20 and 18 different DMAs (data not shown), some occurred only once and some were determined only at the level of the ECL value. Twenty-four fatty acids have been listed in Table 2, which were selected because of their presence in many samples, their higher proportions or because of their importance as signature fatty acids. The highest variation of the relative concentration of an individual fatty acid was found for linoleic acid (18:2 *cis*9,12) which ranged from 0% to 55.8% of the total lipid content. In general, many of the samples analyzed contained the same fatty acids but their relative amounts were different. The only fatty acid detected in every lipid profile and one of the most abundant ones was palmitic acid (16:0). Many lipid profiles were dominated by linoleic and oleic acid (18:1 *cis*9), which made up more than 25% of the total fatty acid content of the biomass of 41 samples (52.6%) and even more than 50% in 27 samples (34.6%). There were no distinct areas of the bottling plants with a noticeable accumulation or absence of both fatty acids. In many samples *cis*-vaccenic acid (18:1 *cis*11) was also present in relatively high concentrations, in 17 biofilms their proportion was higher than 10%. Further fatty acids with higher proportions were the stearic (18:0), palmitoleic (16:1), lauric (12:0), myristic (14:0) acid and 15:0 anteiso. Hydroxy fatty acids were detected in 39 samples within a range of 0.1% up to 18.6% in a sample from the discharge conveyor. The 13:0 3OH was only detected in a sample from the inner part of a pipe from the conveyor belt support of brewery B. Interestingly, it was the only hydroxy fatty acid detected in a lipid profile of the brewery B samples. Only two lipid profiles contained small amounts (< 1%) of 16:0 2OH, which originated from a drawer sheet metal at the discharge and down inside the filler carousel from a cable. The lactobacillic acid (19:0 cyclo11-12) has been detected in two samples in minor proportions (< 2%), one originated from a

flushing band and the other one from the bottom part of the infeed star wheel. Nine lipid profiles revealed a small proportion of tuberculostearic acid (18:0 10methyl). They came from the star wheel and filler carousel region from plant A as well as from the periphery of the bottling plants. Other fatty acids like capric acid (10:0), 15:0 iso, 16:0 iso, 17:0 anteiso, 17:1 *cis*9 and 17:0 cyclo9-10 were somewhat frequently detected in lower amounts of the total fatty acid contents.

Dimethylacetales (DMAs) were detected in 40 out of the 78 fatty acid profiles, however, in nine of them they made up less than 2% of the total amount of FAMEs and DMAs. They were abundant in all drainage pit samples with a proportion of 19.7% to 47.4% in the lipid profile (Table 2). Most samples from the conveyors and the biofilms down inside the filler carousel showed significant proportions of DMAs. They were also detected at the outlet of the bottling plant A and at different places at the filler carousel.

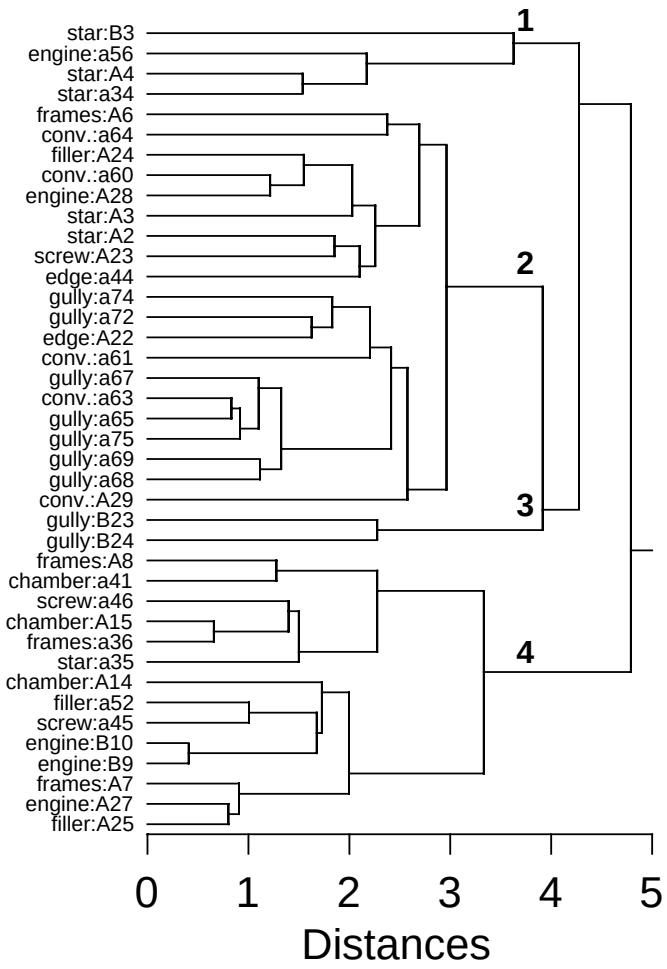

Figure 1. Dendrogram calculated with the percentages of fatty acids and DMAs in selected biofilm samples using the averge linkage and the euclidean distance.

Statistical analysis of the fatty acid profiles. A hierarchical cluster analysis performed with the 40 selected lipid profiles resulted in 4 major clusters (Fig. 1). The main difference for cluster 4 (Fig. 1) has been the presence of high proportions of oleic and linoleic acid. Cluster formation did not correlate with any parameter, e.g. brewery, origin of the biofilms, conditions of the sites, e.g. humidity or constant presence of beer.

The principal component analysis (PCA) was used as an additional statistical method in order to reveal similarities of the fatty acid profiles because of its inherent data reduction. The plot (Fig. 2) was done for the selected samples. However, the main aspect of this plot is the fact that the areas of the different groups were overlapping.

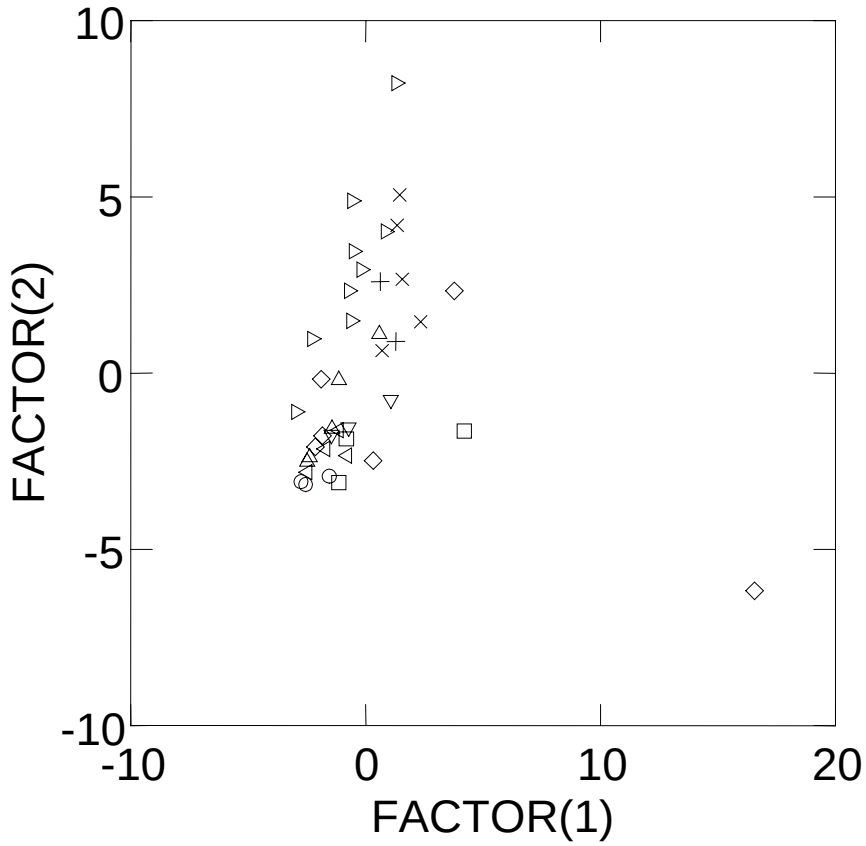

Figure 2. Principal component analysis of community FAME profiles of 40 different samples. They were grouped in nine clusters according to their sample locations as described in the Materials and Methods section. The symbols for the groups are the following: chamber (○), conveyor (×), edge (+), engine (△), filler (▽), frames (◁), gully (▷), screw (□), star (◇).

Growth tests in beer. The results of the inoculation of biofilms in beer are listed in Table1. Different kinds of turbidity were considered as a positive result. Some test tubes showed a turbidity within the complete tube, sometimes a sediment was formed, or fluffs or a slight turbidity which becomes visible only after turning the test tube upside down. Most of the samples from brewery A contained organisms which were able to grow in non-alcoholic beer, in total, 52.0% and 68.9% from the 2001 and 2002 samples (Fig. 3), respectively. In contrast, only 16.0% of the samples from March 2001 caused turbidity in beer. In summer 2002 the infective proportion increased up to 28.9% compared to the proportion of the March 2001 samples. The main proportion (70.8%) of the biofilms from brewery B contained no microorganisms able to multiply in beer. If there was growth in non-alcoholic beer (29.2%) it has been found also for beer (25.0%). This was the main difference between the two breweries. In general, nearly all samples positive in beer were also positive in non-alcoholic beer. Only one biofilm sample caused turbidity in beer but not in non-alcoholic beer (A18). The incubation time until turbidity became visible in beer was around 50 days for the brewery A samples from 2001. The brewery A samples from 2002 needed between three and 17 days for visible growth in non-alcoholic beer and somewhat longer in beer. Most of the subcultures became turbid after seven days and at least after 21 days. Brewery B samples had shorter initial growth phases in beer, in most cases seven or even less days, but maximal 20 ones.

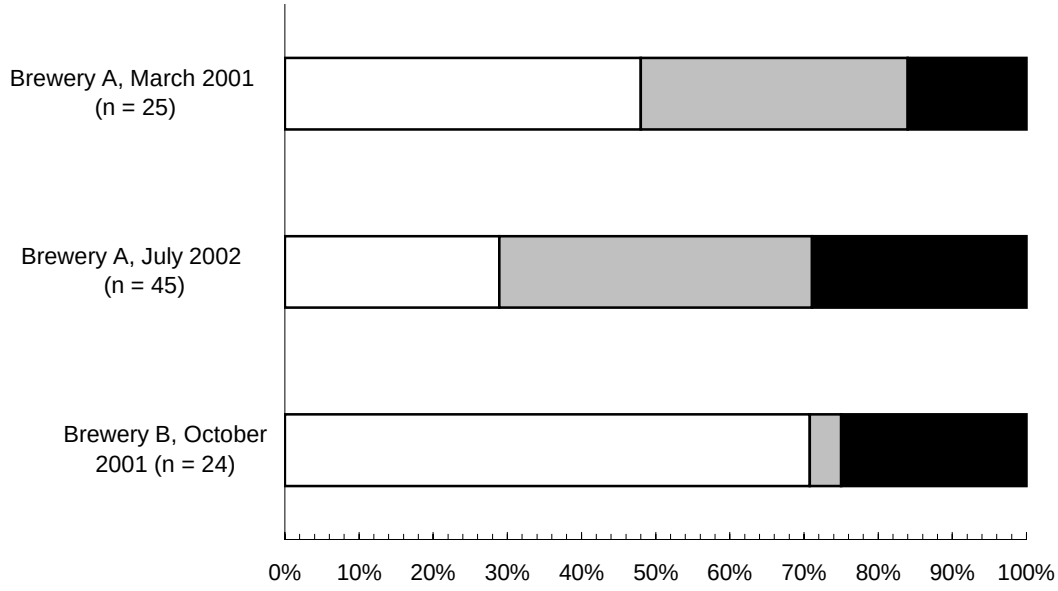

Figure 3. Spoiling potential of biofilms from the three sampling periods. The percent values for the samples which caused no turbidity are indicated by the open part of the bars, samples grown in non-alcoholic beer are given in gray and the ones who caused turbidity in non-alcoholic and also in beer are indicated by the black proportions of the bars.

The positive samples in non-alcoholic beer from brewery A originated from sites all over the plant. This may have been also an inherent consequence of the high number of these samples. Although there were only 11 samples from brewery A identified as being harmful for beer, the locations were nearly as distributed as mentioned for the samples grown in non-alcoholic beer. Just the absence of viable beer-spoiling organisms in biofilm communities in the area of the star wheels, the infeed and discharge area and from the drainage pits was noticeable. Only two critical spots were located on the bottling plant of brewery B. The samples B4 and B5 originated from a cam groove change part between the infeed and discharge star wheels. This plastic part was divided into two components and they were treated as independent samples because of potential microheterogeneities. The same was true for the two samples from the left and right side in the engine compartment (B9, B10), which both caused turbidity in beer. Other hot spots were further away from the bottling plant, like the pipe of the conveyor belt support (B15), a sample from the labeler (B16) and water from the basement (B20).

In total, 72 samples were tested for their growth in beer and analyzed by fatty acid profiling. The Pearson correlation coefficient was calculated for all fatty acids and DMAs detected. A value of 0 indicated that neither the percentage of a fatty acid or DMA nor a turbidity in beer could be predicted from each other by using a linear equitation. A Pearson correlation of 1 or –1 indicated that one variable can be predicted perfectly by a linear function of the other. The highest correlation values were 0.270 and –0.309 for 15:0 anteiso and stearic acid considering the non-alcoholic beer. For normal beer the maximal correlation values were 0.268 and -0.248 for 17:1 *cis*11 and 11:0 iso, respectively. These low values demonstrated, that no reliable prediction of the beer-spoiling potential using the lipid profile of a biofilm community can be made.

Correlation of the abundance of fatty acid proportions and cell numbers. The proportions of the total amounts of oleic and linoleic acid of *Candida* sp. Ro5a and the ones found exclusively in *Pseudomonas* sp. DW115 of defined mixtures are shown in Fig. 4. The concentration of cells was determined by direct counting. A linear correlation between the amounts of lipids of the two organisms according to their cell number was not found. Instead, the curve for the yeast fatty acids could be described by the exponentional function: $y = 100\% \cdot (1\text{-}e^{(-x/t)})$ with $t = 15.64 \pm 0.77$. Figure 4 shows a drastic impact of low numbers of yeast cells to the lipid profile, e.g. only 10% yeast cells contributed to 47.3% of the total fatty acid profile of a mixed culture. Therefore, a high prevalence of bacterial cells is not reflected in lipid profiles of mixed communities. This finding is underlined by the calculation of the fatty acid content of a single cell which resulted in 415 fg for *Candida* sp. Ro5a and 52 fg for *Pseudomonas* sp. DW115, respectively. The amount of extractable

fatty acids was somewhat higher for the Ro5a yeast cells with 15.59 ± 0.20 µg/mg wet weight than for *Pseudomonas* sp. DW115 with 13.62 ± 0.63 µg/mg wet weight.

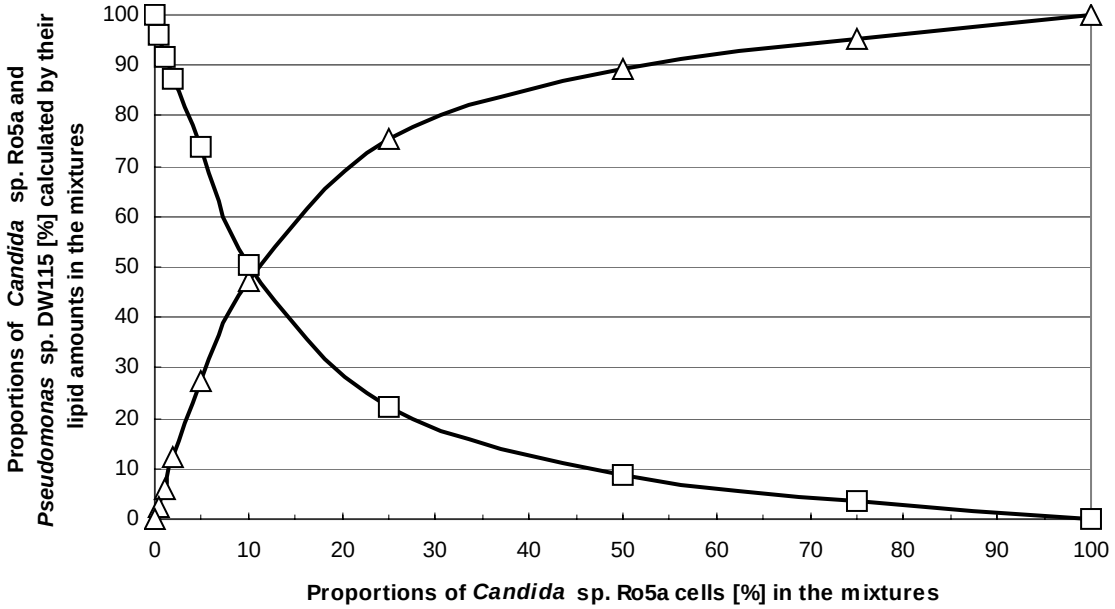

Figure 4. Proportions of yeast and bacteria fatty acids in defined mixtures. The proportions of oleic and linoleic acid in the defined mixtures in comparison to pure culture lipid profiles are indicated by open triangles. The proportions of bacterial fatty acids are given by the open squares.

Table 1. Origin of the samples and results of the enrichment cultures in beer.

Sample	Sample location	Turbidity in beer	
		Non-alcoholic	4.8% v/v ethanol
	star wheels and adjacent parts		
A1	Infeed star wheel, fastener	n. d.	n. d.
A2	Inside the infeed star wheel	-	-
a32	Inside the infeed star wheel	+	-
a33	Beneath the infeed star wheel	+	-
B1	Screw and thread from the infeed star wheel	-	-
B2	Infeed star wheel	-	-
B3	Infeed star wheel, thread	-	-
A3	Inside the discharge star wheel	-	-
a34	Inside the discharge star wheel	+	-
B4	Cam groove, change part between infeed star wheel and discharge star wheel	+	+
B5	Like B4, from the complement part of B4	+	+
A4	Inside the crowner star wheel	+	-
a35	Inside the crowner star wheel	+	-
B6	From the tunnel at the discharge star wheel	-	-
	filler carousel, upper part		
A5	Control frame no. 2	-	-
A6	Control frame no. 6	+	-
A7	Control frame no. 9, release up	+	-
A8	Control frame no. 9, setting screw for the discharge	+	+
A9	Control frame of the half filling	n. d.	n. d.
A10	Sleeve which fixes the control bents	n. d.	n. d.
a36	Suspension gear, material from the spring	-	-
a37	Filler carousel, upper part	-	-
	filler carousel, middle height		
A11	From a ring of the control unit of the bottling plant	-	-
B7	Beneath a sheet metal at the cone belt	-	-
A12	Infeed worm	-	-
a38	Infeed worm, left	-	-
a39	Infeed worm, right	-	-
A13	Beneath the infeed worm, under a removed sheet metal	-	-
A14	Mine chamber, sheet metal	-	-
a40	Mine chamber, top	+	-
a41	Mine chamber, right	+	-
a42	Mine chamber, right to a41	+	+
a43	Mine chamber, left	+	-
A15	Protective plate for the mine chamber	+	-
A16	Bottle stop	+	+
A17	Half filling	+	-

Table 1. continued

Sample	Sample location	Turbidity in beer	
		Non-alcoholic	4.8% v/v ethanol
A18	Flushing path 1	-	+
A19	Flushing path 2	n. d.	n. d.
A20	Vacuum path 1, sheet metal	+	+
A21	Vacuum path 2	n. d.	n. d.
A22	Inside the filler carousel, edge between cast iron and stainless steel table	-	-
a44	Exactly like A22 but from 2002	+	+
A23	Screw inside the filler carousel at the height of the bottles	+	-
a45	Screw inside the filler, like A23	+	-
a46	Pipe, inside the filler, at the height of the bottles	+	+
a47	Drawer at the discharge, sheet metal	+	-
a48	Drawer at the discharge, sheet metal, left to a47	+	+
	filler carousel, lower part		
A24	From a cable, down inside the filler carousel	-	-
A25	Like A24 but 90° in the filler carousel apart from it	+	-
a49	From a cable, down inside the filler carousel	+	+
a50	Similar to a49, from another cable	+	+
a51	Similar to a49, from another cable	+	+
a52	Down and inside the filler carousel, below the cables	+	-
a53	Beneath the filler, outside near the infeed region	-	-
a54	Beneath the filler, outside near the infeed region, left from a53 from a screw	+	-
	region of the crowner		
A26	Crowner, sealing cone	-	-
a55	Distributor of the crowner, lower part	-	-
B8	Metall powder at the crowner, behind a metal sheet	-	-
A27	Engine compartment, beneath the crowner	+	-
A28	Engine compartment, next to the conveyor	+	-
a56	Engine compartment	+	+
B9	Engine compartment, left	+	+
B10	Engine compartment, right	+	+
	conveyor belts		
a57	Infeed conveyor, in front of the infeed star wheel	-	-
a58	Rubbed off material from the infeed conveyor, behind the infeed star wheel	+	-
a59	Discharge, between the crowner star wheel and the conveyor	+	-
a60	About 10 cm beneath the discharge conveyor, near by the filler	+	+

Table 1. continued

Sample	Sample location	Turbidity in beer	
		Non-alcoholic	4.8% v/v ethanol
a61	Beneath the discharge conveyor	+	-
a62	Plastic panelling at the discharge conveyor	-	-
a63	Discharge conveyor, directly beneath the chain links of the conveyor	+	+
A29	Discharge conveyor, approx. 5 m behind the filling plant	+	-
a64	Beneath a conveyor for boxes	+	-
	surroundings of the filling plant		
B11	Bottle washer, in the guide rails	-	-
A30	Cable conduit, between the bottle washer and the filling plant	-	-
A31	Like A30 but directly from the cable	n. d.	n. d.
B12	Cullet collecting sheet under a phosphate shower	-	-
B13	Water from the phosphate shower	-	-
B14	Conveyor belt support at the filler discharge	-	-
B15	Inside the pipe of a conveyor belt support	+	+
B16	Labeler, down at the level of the brushes	+	-
B17	Parts of a broken tile	-	-
B18	Water beneath the broken tile (B17)	-	-
B19	Loose seam sealing next to a drainage pit	-	-
B20	Water from a joint in the basement	+	+
	drainage pits		
a65	Gully A, upper area	-	-
a66	Gully A, edge	+	-
a67	Gully A, inner part beneath the round cover	-	-
a68	Gully A, inner edge	+	+
A69	Gully A, inside the pipe	-	-
a70	Water from gully A (100 ml centrifuged)	-	-
a71	Crown cap from gully A with deposits inside	+	-
a72	Gully B, upper edge	+	+
a73	Gully B, inner edge	+	-
a74	Gully B, inside the pipe	+	-
a75	Gully B, inner part beneath the round cover	+	+
B21	Cover of a gully	-	-
B22	Like B21, but different part from the cover	-	-
B23	Gully, inside the pipe	-	-
B24	Edge of the gully, with cullet	-	-

n. d.: not determined.

+/-: turbidity or no turbidity of beer.

Table 2. Selected fatty acids of biofilm communities. The values represent percentages of the fatty acids of the total lipid profile. The number and the sum of percentages of the not listed fatty acids are summarized in separate rows. In addition, the number of different DMAs and their proportions of the lipid profile are given. The order of the samples is the same as in Table 1.

Fatty acid	A1	A2	B3	A3	a34	B4	B5	A4	a35	A5	A6	A7	A8	A9	A10	a36	a37	a38	A13	A14
10:0		0.5	2.1	0.7	0.6	1.2	1.9	0.8	1.0							0.2	1.0	0.6	4.6	
10:0 3OH		2.4		2.0	0.2				1.9								0.7			
12:0	0.5	10.7	55.2	8.3	20.7	24.9	55.0	32.4	8.0	1.4	0.6	0.5	0.7		3.1	0.1	18.4	2.2	55.2	
12:0 3OH		2.4		1.6					0.7			0.1					1.9			
14:0		3.8	15.9	3.8	5.0	9.9	15.9	9.8	3.7		0.4	0.8	1.1		2.7	0.7	4.2	0.9	12.9	0.7
13:0 3OH																				
15:0 iso		2.7		2.3	0.3				0.6		0.7	0.8	1.2			0.1				
15:0 anteiso		2.0		1.1	0.7				1.3	1.5	0.9	4.2	5.7	1.5		0.9	0.5			0.8
15:0		3.4		0.9	0.1				0.2				1.0				0.8			
14:0 3OH		3.2									0.8						1.1			
16:0 iso		2.8		9.4	0.4				0.6		0.7	0.6	1.9			0.1				
16:1 cis9	0.5	6.9		12.3	2.3	1.9		1.3	2.9		6.4	1.9	4.0	1.3	5.9	1.6	1.3	1.5		
16:1 trans9									0.4											
16:0	16.5	10.1	12.7	9.1	21.2	14.9	10.4	19.1	23.6	20.4	12.4	12.5	12.2	19.3	29.3	27.1	9.3	27.4	9.6	17.2
17 :0 anteiso				1.1	0.5				0.6		0.6	0.5				0.2				
17 :1 cis9		0.9		6.0	0.3				0.3		0.7		2.5							
17.0 cyclo9-10		0.6																		
16:0 2OH																				
18:2 cis9,12	41.6	7.8		9.0	13.1	8.1	1.9	9.7	18.4	43.8	21.8	55.8	12.8	37.7	14.3	25.8	6.3	30.3		37.8
18:1 cis9	33.9	10.6	6.5	14.1	27.6	31.6	9.7	22.0	28.9	25.3	24.1	17.9	47.4	32.6	19.8	36.7	4.7	29.5		34.4
18 :1 cis11	1.2	19.5		3.3	1.7			1.7	1.9	1.8	23.4	1.8	3.9	3.1	10.9	1.9	3.0			
18 :0	5.8	2.7		1.8	2.9	6.0		3.2	2.5	5.8	2.3	2.2	1.5	4.4	11.0	4.0	43.5	7.6	17.7	5.4
18 :0 10methyl		0.5		0.3							3.7									
19.0 cyclo11-12		1.5																		
No. of further fatty acids		8	2	15	4	2	2	1	5		1	1	2	1	3	3	4			1
% of further fatty acids		7.8	7.6	12.3	2.3	7.6	5.2	0.5	1.8		0.6	0.3	3.7	0.1	3.0	0.2	2.4			3.7
No. of DMAs				2					2				1				2			
% of DMAs				0.4					0.5				3.3				1.0			

Table 2. continued

Fatty acid	a41	a42	A15	A16	A17	A18	A19	A20	A21	A22	a44	A23	a45	a46	a47	a48	A24	A25	a49	a50
10:0								0.4	0.5	1.5	0.7		0.2		0.7	0.6	0.7			0.1
10:0 3OH								0.5	0.5	0.8		1.7	1.1		0.3	0.2		0.5		
12:0	0.4	0.2	0.6		0.3	0.8		1.1	3.7	7.9	3.7	0.6	0.2		18.9	17.3	1.5	0.3	0.6	0.4
12:0 3OH									0.4	2.3	0.6				1.1	1.2	1.1			
14:0	0.9	1.1	1.2		0.7	1.8	0.7	0.9	3.0	7.4	6.0	0.6	0.4	0.5	7.2	6.5	1.9	1.0	0.8	0.9
13:0 3OH																				
15:0 iso		0.5	0.2		0.1	2.3		0.9	1.7	1.8	2.3	1.8	0.9	0.8	2.6	1.7	3.1	1.6	1.6	2.0
15:0 anteiso	2.6	4.3	1.8		0.9	7.1	1.5	5.9	1.6	5.9	16.7	11.0	4.0	3.0	4.7	1.5	13.4	6.3	19.0	17.6
15:0	0.6	0.5			0.1	1.2		0.3	0.4	2.1	3.2	1.0	0.5	0.8	1.0	0.1	1.5	0.7	1.7	2.1
14:0 3OH									0.6	4.1	2.2	1.7	0.3		2.6	2.2		0.6		
16:0 iso		0.4	0.4		1.2	2.2		0.8	1.9		1.1	2.9	0.6	1.2	0.4	0.2	3.4	1.6	2.0	0.9
16:1 cis9	2.4	2.4	2.5	1.3	3.3	5.2	1.6		8.2	17.6	8.9	4.8	2.0	1.7	23.7	23.2	9.6	4.7	1.7	2.0
16:1 trans9																	0.5			
16:0	15.2	21.1	26.0	34.5	20.1	11.8	23.0	13.3	18.2	14.8	15.7	7.8	15.8	18.5	13.3	17.4	9.8	8.5	11.7	13.3
17 :0 anteiso		0.3			0.2	0.7		0.7	0.3		2.4	4.3	0.4	1.4			2.4	0.5	1.3	0.7
17 :1 cis9	3.8	1.0				1.7			0.8		1.0	4.8	1.5	2.7	0.5		1.8	0.7		2.3
17.0 cyclo9-10									0.8	2.1	3.0				2.9	1.1	0.8			
16:0 2OH															0.2					0.6
18:2 cis9,12	22.5	22.7	25.2	16.8	29.4	8.3	22.9	43.9	12.7	3.8	3.7	12.5	40.0	27.2	0.4	0.4	22.5	52.7	15.9	24.1
18:1 cis9	43.9	35.9	32.5	36.0	35.6	16.6	40.2	25.5	25.4	5.6	8.1	11.4	18.5	26.6	6.2	8.2	15.5	12.6	7.6	19.4
18 :1 cis11	3.7	1.6		2.2	1.5	25.0	2.4		8.4	15.3	13.2	23.1	5.5	4.3	7.1	10.1	5.0	4.1	1.3	2.8
18 :0	2.9	5.2	7.4	8.2	6.7	2.8	7.7	4.9	2.8	1.1	1.9	2.1	3.7	9.0	0.4	1.0	1.5	1.2	3.2	2.9
18 :0 10methyl						0.6			1.7					0.7						
19.0 cyclo11-12						0.8														
No. of further fatty acids	1	2	3	1	1	7			8	2	3	6	6	1	5	4	3	2	2	7
% of further fatty acids	1.0	0.8	2.4	1.0	0.1	9.9			5.9	2.9	3.4	7.8	4.2	1.8	2.2	1.2	3.3	1.7	2.0	3.9
No. of DMAs		2				2				1	2		1		3	4		1	4	6
% of DMAs		2.1				1.2				3.0	2.3		0.3		3.6	5.7		0.6	5.1	4.2

Table 2. continued

Fatty acid	a51	a52	a54	a55	A27	A28	a56	B9	B10	a57	a58	a59	a60	a61	a62	a63	A29	a64	B11
10:0			2.2	7.0	0.2	1.5	1.0			0.5		0.5	0.6	2.3	0.3	0.6	2.6	1.8	
10:0 3OH						1.0				0.9			1.1	1.1	0.2	1.1	5.7	2.3	
12:0	0.5	0.8	7.5	34.2	3.7	8.3	32.6	0.5	0.2	2.7	3.4	9.0	8.0	21.2	10.7	6.4	22.3	4.5	0.2
12:0 3OH						2.2	1.0					0.7	0.8	4.4	0.1	1.5	7.1	0.6	
14:0	1.0	1.4	4.9	15.4	3.4	3.4	10.7			0.7		4.1	3.1	3.7	3.0	2.2	4.2	1.4	0.5
13:0 3OH																			
15:0 iso	2.3	0.9	2.4		1.3	2.3	1.3			1.2		2.2	3.0	2.4	0.6	2.5	2.0	2.7	3.9
15:0 anteiso	28.3	7.5	19.9		3.8	9.6	3.7			0.8		2.3	12.3	4.0	7.1	5.8	4.2	3.3	12.5
15:0	3.4	0.7	2.0	0.6	0.4	1.2	0.8					0.3	2.0	0.3	0.4	1.2			0.8
14:0 3OH						1.3	1.0			1.2		1.0	2.3	2.4		2.1	4.1	0.7	
16:0 iso	1.2	1.1	3.9		0.5	1.5	0.9			0.9			1.2	0.4	1.0	0.8		0.6	15.1
16:1 cis9	2.5	3.9			3.3	10.3	8.5	1.6	1.2	5.4	3.9	30.5	14.5	22.5	2.9	9.9	6.3	10.0	4.8
16:1 trans9									1.2			1.0	0.5	0.6		0.6		0.7	
16:0	9.3	13.7	8.2	18.2	11.5	7.9	16.0	11.2	11.0	16.5	5.3	25.8	10.0	9.8	12.0	7.6	4.5	9.8	6.9
17 :0 anteiso	0.9		5.4		0.3	0.6			1.0				0.5		0.4	0.4			10.9
17 :1 cis9	2.5		3.1										0.6		0.5	0.7			5.1
17.0 cyclo9-10							0.9					2.6	1.7	1.1		1.2			
16:0 2OH																			
18:2 cis9,12	15.0	35.8	6.9	0.9	49.4	13.2	2.0	48.6	47.6	31.5			16.1	0.4	26.6	3.8		28.1	
18:1 cis9	13.8	25.1	12.6	2.8	15.2	13.8	11.7	29.4	26.4	18.1	11.7		6.9	8.1	24.8	7.7		10.9	2.8
18 :1 cis11	2.6	5.6			3.4	11.4	4.5	3.0	4.1	7.8	13.7	16.1	6.4	6.1	3.9	7.9	11.4	13.4	23.2
18 :0	2.3	2.1	6.6	18.4	2.2	1.9	3.0			4.1	2.1		1.1	0.3	2.7	2.3		2.2	
18 :0 10methyl																			0.6
19.0 cyclo11-12																			
No. of further fatty acids	7	2	6	3	2	5	1	2	2	2	1	1	11	7	7	10	2	8	8
% of further fatty acids	7.1	1.4	11.2	1.9	0.9	6.7	0.5	5.8	7.3	2.0	1.6	0.7	4.4	4.0	2.1	4.8	3.0	7.1	12.3
No. of DMAs	7		2	1		2				2	7	1	2	3		8	5		
% of DMAs	7.1		3.3	0.6		1.8				5.6	58.3	3.0	3.0	4.9		29.0	22.5		

Table 2. continued

Fatty acid	A30	A31	B12	B15	B16	B20	a65	a66	a67	a68	A69	a70	a71	a72	a73	a74	a75	B23	B24
10:0				1.7			1.1	0.9	3.3	0.9	0.6		1.2		0.5	0.3	0.8	3.2	
10:0 3OH							2.5	1.3	2.2	1.6	0.8		1.9	1.1	0.9	1.1	0.7		
12:0		8.9	0.8	22.5	1.8	1.6	8.5	6.3	8.7	8.0	4.3	1.5	8.6	6.2	6.9	6.1	6.3	12.0	16.1
12:0 3OH	0.6						2.8	2.1	6.0	1.4	1.9		3.1	1.9	1.7	1.0	0.7		
14:0		5.6	1.0	7.9	1.6	6.0	2.3	1.8	1.9	2.9	1.3	3.2	2.7	2.6	1.8	4.2	2.2	11.5	
13:0 3OH				2.6															
15:0 iso			1.4		3.3	1.4	2.0	1.8	1.2	1.4	1.2		1.6	0.8	0.6	1.4	1.3		
15:0 anteiso	0.9		1.2	6.6	21.4	6.1	4.0	4.3	1.9	1.4	1.2	1.4	3.5	2.4	0.6	0.9	7.1		
15:0		1.5	1.0	1.9	1.2	0.8		1.1	1.1			0.9	1.3			0.8	1.7	2.0	
14:0 3OH							0.9	1.5	2.1	1.2	1.1		2.2	1.5	1.8	5.5	0.7		
16:0 iso		1.5	0.8		7.6		0.9	0.8			0.3	0.9	0.9				1.3		
16:1 cis9	17.1	9.4	3.2	2.2	11.1	29.2	7.7	7.4	6.3	7.6	4.6	5.5	6.2	14.6	6.7	26.3	6.2		
16:1 trans9					1.2	1.4	1.0	1.0	1.3				0.9	4.3	1.0	1.0			
16:0		29.6	17.6	10.2	14.5	14.2	10.9	8.5	7.7	8.7	11.0	25.5	8.8	16.0	5.3	13.3	11.2	11.7	13.2
17 :0 anteiso				4.5	7.8														
17 :1 cis9			4.3	2.7				0.8					0.8						
17.0 cyclo9-10									0.6		1.0			1.8	0.7	3.4	1.0		
16:0 2OH																			
18:2 cis9,12	26.9	16.0	23.3		3.5		3.0	7.9	2.2	4.0	5.9	6.9	2.5	1.8	1.0	0.4	7.1		
18:1 cis9	42.7	12.6	31.7		12.3	3.0	7.6	7.8	10.9	8.4	11.5	22.6	6.2	8.6	4.7	2.7	10.5	11.6	19.7
18 :1 cis11	4.2	5.9	6.5	2.9	8.4	30.8	9.6	8.3	9.0	9.0	10.4	6.8	6.5	15.5	5.6	9.9	8.1		
18 :0	7.0	2.8				1.5	4.9	4.2	2.3	2.7	2.4	9.2	3.9	1.5	1.4	0.2	5.0	4.5	
18 :0 10methyl									0.5		0.9								
19.0 cyclo11-12																			
No. of further fatty acids	1	3	5	4	3	2	3	10	8	2	4	1	7	2	3	5	4		
% of further fatty acids	0.7	4.0	7.4	14.5	4.0	3.0	3.9	7.3	5.9	1.8	2.9	0.9	6.9	2.3	1.4	2.4	4.0		
No. of DMAs		1		5		1	7	11	9	6	8	5	8	4	9	5	7	5	3
% of DMAs		2.2		19.7		1.0	25.0	24.7	24.9	39.0	36.5	14.7	30.6	16.9	57.4	19.4	30.8	43.4	51.0

Discussion

The aim of the study was to investigate the structure of biofilm communities from different sites of two bottling plants to reveal similarities or differences between them. This was done by whole cell fatty acid analysis (MIDI-FAME). Whole cell fatty acid and phospholipid fatty acid (PLFA) analyses are used for investigating fatty acid profiles of environmental samples. The PLFA procedure discriminates between lipids from dead and living cells (White *et al.* 1979) and is therefore more suitable for characterizing the living organisms. However, the glycolipids, hydroxy fatty acids and the DMAs were not detected by the PLFA method. Especially the detection of hydroxy fatty acids was essential for this study because they are signature lipids for acetic acid bacteria, *Pectinatus* sp., *Pseudomonas* sp. and Enterobacteriaceae (Yamada *et al.* 1981; Helander and Haikara 1995; Vancanneyt *et al.* 1996; Bryn 1978). In addition, the DMAs are suitable to detect strictly anaerobes (Goldfine 1982; Moore *et al.* 1994) which indicate the presence of anaerobic microenvironments. These can be colonized by anaerobic beer-spoiling bacteria. The usage of the FAME analysis for differentiating complex artificial communities has been validated by Haack *et al.* (1994) and it has been succesfully used for the characterization of soil communities (Schutter and Dick 2000; Drenovsky *et al.* 2004). An advantage of the MIDI FAME procedure is the about 10-fold lower amount of required sample mass for getting a reliable community fingerprint than for analyzing PLFA (Drenovsky *et al.* 2004). This was essential for the analysis of small volume samples of this study.

Nearly half of the lipid profiles were dominated by oleic and linoleic acid indicating a high proportion of Eukarya. These samples were distributed on the bottling plant and in the adjacent regions in the filling area. No area on the bottling plant was exclusively dominated by yeasts. The presence of non-*Saccharomyces* yeasts in many biofilms was indicated by the high proportions of linoleic acid which is present in *Saccharomyces* cells only in traces (Malfeito-Ferreira *et al.* 1997), if at all. Yeasts unable to multiply in beer can cause problems too, if they can attach to surfaces and become pioneer organisms for the development of biofilms. The clinical important species *Candida albicans* is such a well-studied biofilm forming yeast (Kumamoto 2002; Douglas 2003). The identification of yeasts isolated from brewery bottling plants and their ability to form biofilms will be published elsewhere (Timke *et al.* submitted).

The lipid profiles reflect the proportions of groups according to their biomass. The exponential character of the correlation between cell number and fatty acid content in mixed cultures (Fig. 4) underlined the importance to differentiate between these two parameters. Therefore, the bacteria are quantitatively more important than reflected by the abundance of their fatty acids in yeasts and bacteria containing mixed samples.

The typical fatty acids for beer beer-spoiling bacteria were scarce in the lipid profiles. The 13:0 3OH has been found only once in the pipe of a conveyor belt support. The presence of *Pectinatus* sp. cells in this sample was confirmed by fluorescence *in situ* hybridization showing a proportion of 29.7% ± 3.2% of all 4',6-diamidino-2-phenylindole (DAPI; 1 mg l^{-1}) stained cells (data not shown). The main fatty acids of Lactobacillaceae are *cis*-vaccenic and lactobacillic acid (Rizzo *et al.* 1987) and the latter has been detected in only two samples. Whereas the *Pectinatus* harbouring biomass caused turbidity in beer, the ones with lactobacillic acid did not. This may be due to the presence of lactobacillic acid in many other bacteria, e.g. in Alphaproteobacteria, in which it is a major fatty acid (Lipski *et al.* 1992). The typical fatty acid of acetic acid bacteria (16:0 2OH, Yamada *et al.* 1981) has been detected in small amounts in only two lipid profiles. This finding does not support the proposed significance of acetic acid bacteria in brewery biofilms (Back 1994).

Lipid profiles of 24 samples had small proportions of 10:0 3OH and 12:0 3OH, which is a typical combination for *Pseudomonas* cells (Vancanneyt *et al.* 1996). The highest proportions of these fatty acids were detected in samples from extremely moistures sites (A29, a67). In line with this, is the finding that the regularly sprinkled region of the star wheels and the discharge region harbours most of the 10:0 3OH and 12:0 3OH containing biofilms from the brewery A samples taken in 2001. In 2002, this combination has been present in all drainage pit biofilms, in all biofilms at the outlet area and also in some biofilms located at the filler carousel. The distribution of 30 lipid profiles with 14:0 3OH was very similar to the ones discussed above. It is a typical fatty acid of the outer membrane of Enterobacteriaceae (Bryn *et al.* 1978). These organisms are reported to occur in brewery environments (Jesperson and Jakobsen 1996; Back 2003). Despite of the abundant use of water in the brewing industry, none of these hydroxy fatty acids were detected in brewery B samples. This was the main difference in the lipid profiles between the two breweries but it was not reflected in the statistical analyses (Fig. 1, Fig. 2), supposedly due to their low proportions. In addition to these marker lipids for Gram-negative bacteria, nine samples contained tuberculostearic acid, which is a signature fatty acid for Actinomycetes (Kroppenstedt 1992). These nine samples represented no distinct area.

The detection of DMAs indicated the presence of plasmalogenes in these samples. Strictly anaerobic bacteria, including both Gram-positive and Gram-negative species, have plasmalogenes (Goldfine 1982). For example, in pure cultures of *Pectinatus frisingensis* DSM 20465 and *P. cerevisiiphilus* Pe-0104 the DMAs made up a proportion of 12.5% and 16.4% and in *Megasphaera cerevisiae* DSM 20461 26.7% of the lipid profile (data not shown), respectively. These DMAs were also detected in some lipid profiles, however the appropriate 13:0 3OH of *Pectinatus* cells has been found only in sample B15. Consequently, this sample had significant proportions of the corresponding *Pectinatus* sp. DMAs.

Pectinatus sp. has been isolated for the first time from a brewery drainage system (Lee *et al.* 1978). Although no 13:0 3OH has been detected in the drainage pit samples, the high proportions of DMAs identified them as habitats for anaerobic bacteria.

FAME profiles of 40 yeasts including *Saccharomyces* spp. and *Candida* spp. isolated from both breweries revealed no DMAs (data not shown). But Protozoa contain plasmalogenes (Thompson and Nozawa 1972), e.g. in *Tetrahymena* sp. and *Paramecium* sp. about 59.5% and 80% of the phosphatidylcholine aliphatic residues are glyceryl ether linked (Lechevalier and Lechevalier 1988). To confirm the presence of Protozoa some longer chained fatty acids like arachidonic acid (Vestal and White 1989) should have been detected but it was absent in all lipid profiles. However, *Tetrahymena* lacks fatty acids with more than 18 carbon atoms (Lechevalier and Lechevalier 1988). Therefore, it is still possible that DMAs may originate from Protozoa the presence of which was confirmed by microscopy. The very humid places with high amounts of DMAs like the drainage pits and the discharge conveyor belt may be appropriate habitats for Protozoa. In conclusion, DMAs have been detected in 40 different lipid profiles. These biofilms could be a habitat for strictly anaerobic bacteria some of which may have a beer-spoiling potential. However, the Pearson correlation coefficients of the percentages of the total amounts of DMAs in a sample and the ability to cause turbidity in non-alcoholic beer or beer was –0.042 and 0.006, respectively. Accordingly, DMAs were no suitable indicators for the beer-spoiling potential of a biofilm community. They were absent in nearly half (48.0%) of all samples and thus excluded the presence of strictly anaerobic bacteria in these biofilms in significant proportions.

In general, the hygienic condition of a bottling plant has to comply with the requirements of the most vulnerable product bottled at this plant. For this reason, also non-alcoholic beer was used for the detection assay. Indeed, *Megasphaera* has been reported to be suppressed by an ethanol content above 2.8% (Haikara and Lounatmaa 1987) and *Pediococcus inopinatus* and *P. dextrinicus* are only capable to grow in beer with lower ethanol concentrations (Lawrence 1988). Some species grow very slowly in beer, e.g. *Lactobacillus brevisimilis* produces a slight turbidity or a little sediment very often only after weeks in bottled beer (Back *et al.* 1988). Therefore, the tubes were checked for at least seven weeks. A high number of biofilms inoculated in beer caused no turbidity (Fig. 3) despite of the high amounts of inoculated biomass. The proportions of biofilms with spoiling potential for non-alcoholic beer and beer increased slightly in July 2002 compared to the March 2001 samples from brewery A. In general, the problems with beer-spoiling organisms are reported to increase in summer times (Back 1994). Considering the three sampling periods, about one quarter of all biofilms examined caused turbitity of beer. In particular the star wheels and the discharge area where expected to harbour higher amounts of beer-spoiling organisms

due to the permanent contact to overfoamed beer. However, there was no spatial concentration of these biofilms.

There was no correlation between any fatty acid and the ability to cause turbidity in beer. This may be due to the low number of samples containing characteristic fatty acids of beer-spoiling bacteria. They have been absent or were present in not detectable proportions. In addition, the accompanying organisms, which constituted the majority of the lipid profile, varied significantly in space and time. Even higher amounts of fatty acids of eukaryotic origin e.g. from wild yeasts, did not indicate a higher risk for beer. This can be due to Protozoa and moulds which can not grow in beer but the microscopy of all samples with more than 25% of oleic and linoleic acid revealed only in nine out of these 41 samples higher amounts of hyphae from filamentous fungi. All the other samples were dominated by yeast cells.

Some samples have been suitable to compare the lipid profiles of spatially adjacent biofilms and from sites with similar exposition. The hierarchical cluster analysis and the PCA (Fig. 1, Fig. 2) revealed no distinct clusters of these sample sites. The same has been found for identical sites investigated at different times. This finding is in line with data obtained from conveyor communities of the same sample location. They showed different proportions of the main groups in August 2000 and in January 2001 (Timke *et al.* 2005). Considering the lipid profiles of 78 different samples, there was no typical brewery biofilm community, not even for distinct regions of the bottling plant. Instead, these communities seemed to be subjected to shifts of populations.

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Chapter 4

Characterization of Yeasts from Beer Bottling Plant Associated Biofilms

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Abstract

Wild yeasts were isolated from process surfaces of two breweries. In total, 41 strains were obtained and 34 (82.9%) of them were able to grow in beer. A first differentiation was achieved by cultivation on $CuSO_4$ or cristal violet containing selective media. The fatty acid profiling resulted in a separation of the yeasts in three groups and has therefore been less suitable. Restriction patterns of the region spanning the internal transcribed spacers (ITS1 and ITS2) and of the 5.8S rRNA gene made it possible to differentiate the yeasts in eleven groups. Most of these patterns could be assigned to a species by using the API ID 32 C kit. The wild yeasts identified consisted of *Saccharomyces cerevisiae* (41.5%) and *Candida* spp. (46.3%); 12.2% of the isolated yeasts had an assimilation profile with which no reliable species identification was possible. The most abundant species *S. cerevisiae* and *C. pelliculosa* were part of the microbial communities of both breweries. In addition, the biofilm forming potential has been tested. Twelve yeasts were able to attach to a surface and seven of them were identified as *C. pelliculosa*.

Introduction

Yeasts were found to constitute an important part of the microbial community in many mature brewery biofilms from bottling plants and adjacent areas (Timke *et al.* submitted). Therefore, it is important to have suitable and reliable methods for the detection of yeast contaminants in order to guarantee a high quality product and to trace and eliminate sources of contamination. The brewery associated yeasts are traditionally divided into non-*Saccharomyces* and *Saccharomyces* yeast (Jespersen and Jakobsen 1996). The *Saccharomyces* yeasts e.g. *S. bayanus*, *S. cerevisiae*, *S. pastorianus* or *S. paradoxus*. are considered as the most hazardous ones for beer (van der Aa Kühle and Jespersen 1998).

Four different methods were used to get information about the diversity of the isolated yeasts. A first differentiation was achieved by cultivation on selective media, containing crystal violet or $CuSO_4$. This technique is widely used in breweries. The lipid profiles of all yeasts were compared. In addition, these data can give valuable hints for the presence of these yeasts in brewery biofilm communities, of which lipid profiles had been analyzed previously (Timke *et al.* submitted). A restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer region (ITS) of the 5.8S rRNA gene was done. Finally, the carbon assimilation pattern of representative isolates were obtained by using the API ID 32 C kit. Both methods were used for identification of the isolates. The ability of the yeasts to spoil beer has been tested. In addition, even non beer-spoiling yeasts can be harmful for the breweries, e. g. if they are able to form biofilms. Such yeasts could become

biofilm pioneer organsims which enable secondary biofilm organisms, in particular the beer-spoiling bacteria, to colonize them. Such a relationship has been revealed for the bacterium *Deinococcus geothermalis* in paper machine environments and the problem causing *Bacillus* species (Kolari *et al.* 2001). *Candida albicans* is a biofilm forming yeasts (Kumamoto 2002; Douglas 2003), other species are *C. tropicalis* and *C. parapsilosis* if grown in a medium with 8% of glucose (Shin *et al.* 2002). Consequently, the ability of the isolated yeasts to form biofilms has been tested.

Materials and Methods

Origin and isolation of the strains. The yeast strains were isolated from two breweries. One brewery represented a medium-scale company with one filling plant (designated: brewery A). The strains with the abbreviations Ro and DW originated from this brewery. Biofilms were taken and inoculated in non-alcoholic beer and beer with 4.8% of ethanol (designated: beer). Hundred µl of the turbid beer were plated on MRS (DeMan-Rogosa-Sharpe, Difco Laboratories, Detroit, USA) medium and incubated in an anaerobic jar at 25°C (GasPak^{TM}, BD Diagnotic Systems, Heidelberg, Germany). Eleven yeast strains were obtained from five different biofilms (strains: Ro3a, Ro3b, Ro3-16, Ro3-20b, Ro5a, Ro5b, Ro5-2, Ro6, Ro9a, Ro9b, Ro32). Furthermore, the sealing cone of the crowner of this bottling plant was impressed on DSM 105 medium (*Gluconobacter oxydans* medium, [http://www.dsmz.de/media]) and resulted in the isolation of yeast strain DW14. Yeast strain 6-2St2a was isolated from a sterile stainless steel coupon, which was laid out overnight on the filler discharge star wheel. The coupon was plated on R2A medium (Reasoner and Geldreich 1985).

Further yeasts were obtained from a large-scale brewery (designated: brewery B). Eight yeasts originated from seven different biofilm samples; they were isolated as described above (strains: Bt6, Bt8, Bt11, Bt12, Bt15-7a, Bt15-7b, Bt16, Bt35). In addition, swab samples were incubated in NBB (detection agent for beer-spoiling bacteria; Döhler, Darmstadt, Germany) or non-alcoholic beer from different locations in this brewery. Turbid medium was plated on MRS and incubated as decribed above. This approach resulted in 19 strains from 12 different sample sites (NBB, strains: MS13, MS19b, MS32, E7H; non-alcoholic beer, strains: MS29, MS30, MS31a, MS31b, MS33, MS34, MS35a, MS35b, MS36a, MS36b, MS36c, MS37, MS38a, MS38b, MS38c). In total, 41 isolated yeasts were analyzed.

The brewing yeast strain of brewery B (MS39) was also included. As reference organisms *Pichia anomala* CECT 1114 (*Candida pelliculosa* var. *pelliculosa*) and *Hypomyces*

chrysospermus CECT 10573 were obtained from the Coleccion Española de Cultivos Tipo (University of Valencia, Spain). All yeasts were transferred to TS agar (Trypticase Soy Broth, Becton/Dickinson, Cockeysville, USA).

Cultivation on selective media. Wort agar (Becton/Dickinson) was prepared according to the manufacturer`s instructions. The hop-content of this medium suppressed the growth of many bacteria. The incubation time was three to five days at 30°C.

Crystal violet medium corresponded to wort medium supplemented with 200 mg crystal violet (Roth, Karlsruhe, Germany) per liter medium dissolved in 0.5 ml ethanol (Katô 1967). This medium was used for the detection of *Saccharomyces* wild yeasts. *Saccharomyces* culture yeast strains and non-*Saccharomyces* yeasts are not able to grow on this medium (Back 1994). The incubation time was two to three days at 28°C.

$CuSO_4$ medium according to Lin (1981) consisted of 4 g yeast extract (Becton/Dickinson), 2 g malt extract (Becton/Dickinson), 2 g meat peptone (Merck, Darmstadt, Germany), 10 g D(+)glucose (Merck), 1 g K_2HPO_4 (Merck), 0.5 g NH_4Cl (Merck), 0.5 g $CuSO_4$ (Riedel-de Haën, Seelze, Germany) per liter. The pH was adjusted to 6.2. Twenty g of agar (Invitrogen, Karlsruhe) were added before autoclaving. $CuSO_4$ medium was used for the detection of non-*Saccharomyces* yeasts because members of the genus *Saccharomyces* are not able to grow on this medium (Back 1994). The incubation time was four to five days at 28°C.

Growth test in beer. The yeast strains were inoculated in beer with 4.8% (v/v) of ethanol for testing the beer-spoiling potential. Test tubes were filled with 5 ml beer and cell material was transferred from solid media with an inoculation loop. The test tubes were incubated at 30°C without any rotation, but access of air was possible.

Fatty acid methyl ester analysis. The yeasts were incubated for three days on TS agar at 25°C. Approximately 40 mg of the biomass was analyzed. Saponification, methylation and extraction of the fatty acid methyl esters (FAMEs) were done according to Sasser (1990) and the identification of the FAMEs was performed by GC-MS as described previously (Lipski and Altendorf 1997). The linolenic acid co-eluted with oleic acid. Therefore, linolenic acid was detected only qualitatively based on the presence of the characteristic ions m/z 292, m/z 79 and m/z 136. The fatty acids are named by their trivial names. If there are no common ones, shorthand designations were used, as the number of carbon atoms in the chain is given first. After a colon, the number of double bonds is specified. The configuration and location of functional groups are given from the carboxyl end of the molecules.

API ID 32 C. Assimilation of carbon compounds was tested using the API ID 32 C kit (Bio Merieux SA, Marcy-L`Etoile, France) according to the manufacturer`s instructions. Identification of the isolates was done with the software Apilab Plus (version 3.3.3, Bio Merieux). The assimilation patterns were used in a hierarchical cluster analysis (Fig. 1). It was calculated with the average linkage and euclidean distance methods using the SYSTAT® software package (SPSS Science SoftwareG, Erkrath, Germany).

RFLP analysis of the ribosomal ITS region. DNA of the yeasts was isolated by incubation in a lysis buffer and subsequent boiling. The rRNA gene region was amplified by PCR with ITS1 and ITS4 primers and the products were analyzed by electrophoresis. The PCR products were digested with 5 U of *Hae*III (Promega, Mannheim, Germany) and separated in NuSieve® 3:1 agarose gels (BMA, Rockland, ME). Details are given in Jespersen *et al.* (2000). A 50 bp ladder marker (Life Technologies, Gaithersburg, MD) served as the size standard. Images were acquired and analyzed using the software package FragmentNT Analysis (version 1.2, Molecular Dynamics, Sunnyvale, CA).

Biofilm formation in microtiter plates. The test was performed in 96-well polystyrene microtiter plates (Greiner Bio-One, Frickenhausen, Germany). The design of this test has been adapted from Kolari *et al.* (2002). Each well was filled with 250 µl TS broth and inoculated with 2.5 µl of an overnight grown (30°C) culture. The yeasts were incubated for 1 day at 30°C without agitation, the A_{550} was measured with a microplate reader (340 ATTC, SLT). The wells were emptied, and rinsed with 0.9% NaCl (300 µl). Three-hundred µl of a crystal violet solution (4 g l^{-1} in 20% [v/v] methanol) were used to stain the remaining biofilm for 5 min. The nonabsorbed crystal violet was removed by washing with water three times. The remaining stain was dissolved from the biomass by addition of 330 µl ethanol (1 h at ambient temperature) and the A_{550} was determined. All measurements were done in four replicates.

Results and Discussion

The yeasts originated from many different places of two breweries, e.g. from the discharge star wheel (MS30) or the conveyors (MS32, MS35, MS36a, MS36b, MS38), from mature biofilms or swab samples. However, they all were associated to process surfaces, they have been part of the brewery associated microbioata and could be potential beer spoilers.

All yeasts, including the brewing culture yeast MS39, grew on wort medium. The majority of the yeasts were able to grow on $CuSO_4$ agar (79.5%) and seven (18.0%) on crystal violet agar (Table 1). This suggested a high proportion of non-*Saccharomyces* yeasts among the

isolates. No yeast was positive on both media which confirmed the discriminatory power of these media. Thirty-two isolates (82%) caused turbidity in beer (Table 1). All yeasts which grew on crystal violet medium have been potential *Saccharomyces* sp. They are considered as the most harmful ones for beer (van der Aa Kühle and Jespersen 1998) and in line with this, they all were able to grow in beer (Table 1). The ones grown on $CuSO_4$ agar were separated in beer spoilers (64.1%) and non beer spoilers.

Table 1. Growth of the yeasts on selective media and in beer. Summary of ITS-RFLP results, the presence of linoleic acid and the API identification. The ability to form a biofilm is given.

Strain	Growth tests on/in:			ITS-RFLP[b]	18:2 [%][c]	API ID 32 C identification[d]	Biofilm formation[e]
	$CuSO_4$[a]	Crystal violet[a]	Beer[a]				
MS32a	+	-	+	A		*S. cerevisiae* (99.9%)	-
MS31a	+	-	+	A		*S. cerevisiae* (99.9%)	-
Bt15-7a	+	-	+	A		*S. cerevisiae* (99.7%)	-
MS32b	+	-	+	A		*S. cerevisiae* (99.7%)	-
MS39	+	-	+	A		*S. cerevisiae* (99.7%)	-
Bt11	-	+	+	A		*S. cerevisiae* (99.7%)	-
Ro3-20b	+	-	+	A		*S. cerevisiae* (99.9%)	-
Ro3a	+	-	+	A		*S. cerevisiae* (99.9%)	-
Bt35	+	-	+	A		*S. cerevisiae* (99.9%)	-
Ro3b	+	-	+	A		n. d.[f]	-
Ro32	+	-	+	A		n. d.	-
Bt12	-	+	+	A		n. d.	-
Bt15-7b	+	-	+	A		n. d.	-
MS33	-	+	+	A		n. d.	-
MS34	-	+	+	A		n. d.	-
MS35b	-	+	+	A		n. d.	-
MS38a	-	+	+	A		n. d.	-
MS38b	+	-	+	A		n. d.	-
MS31b	+	-	+	A		No species identified	-
Ro5b	+	-	+	K	21.3	*C. valida* (99.9%)	-
MS36b	+		-	E		No species identified	-
MS36a	+	-	-	E		No species identified	-
MS19b	+	-	+	C		*C. glabrata* (99.1%)	-
MS30	-	+	+	C		*C. glabrata* (99.1%)	-
MS29	+	-	+	C		n. d.	-
Bt8	+	-	+	I	17.7	No species identified	+
Ro6	+	-	+	F	15.1	*C. colliculosa* (99.6%)	-
Bt16	+	-	+	J	36.3	No species identified	+
MS36c	+	-	+	B	33.4	*C. pelliculosa* (99.9%)	+
MS38c	+	-	-	B	29.9	*C. pelliculosa* (99.9%)	+
Ro5-2	+	-	+	B	38.3	*C. pelliculosa* (99.8%)	+

Table 1. continued

Strain	Growth tests on/in: $CuSO_4$[a]	Crystal violet[a]	Beer[a]	ITS-RFLP[b]	18:2 [%][c]	API ID 32 C identification[d]	Biofilm formation[e]
Ro5a	+	-	+	B	38.4	*C. pelliculosa* (99.9%)	+
MS35a	+	-	+	B	30.9	*C. pelliculosa* (99.9%)	+
CECT1114	+	-	+	B	37.2	*C. pellicolosa* (99.9%)	+
CECT10573	+	-	+	B	30.7	*C. pellicolosa* (99.9%)	+
Bt6	+	-	+	B	24.7	*C. pelliculosa* (99.9%)	-
Ro9b	+	-	-	B	28.6	*C. pelliculosa* (99.2%)	+
MS13	+	-	+	B	28.4	*C. pelliculosa* (99.7%)	+
Ro9a	+	-	-	B	28.4	n. d.	-
MS37a	+	-	-	B	34.1	n. d.	-
E7H	+	-	+	H	23.4	*C. guilliermondii* (98,8%)	+
6-2St2a	+	-	+	D	19.6	*C. sake* (99.7%)	+
Ro3-16	+	-	+	D	20.0	*C. sake* (99.4%)	n.d.
DW14	+	-	-	G	22.0	*C. parapsilosis* (99.9%)	+

[a] +/- indicates growth or no growth, respectively.

[b] For details of the restriction fragments see Table 3.

[c] The precentage of linolic acid of the whole lipid profile is given.

[d] The validity of the API result is given in parenthesis.

[e] +, indicates a A_{550} of at least 0.12; –, accounts for the inability of biofilm formation with the highest A_{550} of 0.09 ± 0.05.

[f] n. d.: not determined.

The fatty acid analysis revealed two main patterns (Table 2). One group was dominated by palmitoleic and oleic acid and the second one by linoleic and oleic acid, respectively. Interestingly, *Saccharomyces cerevisiae* contains no polyunsaturated fatty acids due to the absence of a Δ^{12} desaturase (Ratledge and Evans 1989). In accordance with this, all yeasts grown on crystal violet medium had no linoleic acid. However, the absence of linoleic acid is not an exclusive characertistic of *Saccharomyces* spp. It is also true for e.g. *Hanseniaspora* sp. or *Schizosaccharomyces* sp. (Loureiro and Querol 1999). Malfeito-Ferreira and colleagues (1997) emphasized the discriminatory power of the analysis of yeast long-chain fatty acids from yeasts at wine bottling plants. They identified three groups according to the composition of the fatty acids with 18 carbon atoms. Group I consisted of yeasts only with oleic acid, group II included species with oleic and linoleic acid and group III was based on the additional presence of linolenic acid. Moreira da Silva and coworkers (1994) also differentiated brewery associated yeasts by FAME analysis and found three main clusters. The first one corresponded to group I of Malfeito-Ferreira *et al.* (1997). However, members of group II were absent in the brewery related yeasts (Moreira da Silva *et al.* 1994, this study); this could have been *Zygosaccharomyces bailii* and *Brettanomyces*/*Dekkera* spp. (Malfeito-Ferreira *et al.* 1997) or *Torulaspora* spp. (Loreiro

and Querol 1999). Instead, Moreira da Silva *et al.* differentiated the linolenic acid containing yeasts by the proportion of oleic acid, which made up about 25% in one group (group IIIa) and approximately 47% in the other one (group IIIb). Accordingly, almost all isolated yeasts of this study belonged to group I and IIIb, only strain Ro6 was affiliated to group II. Consequently, the FAME profiles accounted for little discrimination of the brewery associated yeasts, which were assigned only to *Saccharomyces cerevisiae* and *Candida* spp.

Table 2. Fatty acid profiles of analyzed yeasts. The values are percentages of the total FAMEs. The fatty acids 12:0; 14:1 *cis*9; 14:0; 15:1 *cis*9 and 15:0 were omitted because of their minor proportions (< 1%).

strain	16:1 *cis*9	16:1 *trans*9	16:0	17:1 *cis*9	17:0	18:2 *cis*9,12	18:1 *cis*9	18:1 *trans*9	18:0
MS32a	53.8		14.6				28.8		1.5
MS31a	56.0	1.3	6.4	0.3			32.0	1.5	1.0
Bt15-7a	43.5	0.5	8.2				43.2	1.6	2.8
MS32b	48.0	0.4	7.2				37.1	3.5	3.4
MS39	57.1	0.7	7.8	0.4			31.1	1.4	1.2
Bt11	44.4	0.8	10.5				37.6	3.3	3.0
Ro3-20b	60.7	0.7	9.4				25.9	1.6	1.0
Ro3a	45.4	0.6	10.7				37.9	1.6	3.6
Bt35	42.7	0.5	6.9				45.5	1.9	2.4
Ro3b	39.0	0.5	8.0				46.1	1.5	4.5
Ro32	49.8	0.9	10.3				33.6	2.1	2.9
Bt12	50.6	0.6	11.0				33.4	1.7	2.5
Bt15-7b	44.0	0.5	8.4				42.7	1.5	2.7
MS33	54.5	1.0	7.5				32.3	2.3	1.3
MS34	70.1	1.5	5.5				19.6	1.8	0.6
MS35b	54.1	1.0	7.5				33.2	2.2	1.5
MS38a	63.3	1.4	6.9				24.7	2.3	0.8
MS38b	70.7	2.1	7.7				16.5	1.5	0.8
MS31b	47.7	1.0	8.6				36.0	2.9	2.8
Ro5b	13.9		12.9	0.5		21.3	49.8[a]		1.6
MS36b	66.7		21.1				8.6		1.6
MS36a	63.9	1.2	15.9				13.1	1.6	2.3
MS19b	49.7	0.6	3.7				36.3	5.4	4.1
MS30	54.4	1.1	6.1	0.2			33.5	2.8	1.0
MS29	63.8	1.6	4.3				28.6		0.5
Bt8	6.7		9.8	1.9	0.5	17.7	58.8[a]	3.4	1.2
Ro6	43.9		11.0			15.1	28.6		0.4
Bt16	4.3		13.5	0.5		36.3	45.0[a]		0.3
MS36c	2.0		12.0	3.2	0.8	33.4	46.9[a]		1.2

Table 2. continued

strain	16:1 *cis*9	16:1 *trans*9	16:0	17:1 *cis*9	17:0	18:2 *cis*9,12	18:1 *cis*9	18:1 *trans*9	18:0
MS38c	2.2		12.7	4.1	0.9	29.9	47.9[a]		1.8
Ro5-2	2.9		16.1	0.7	0.2	38.3	39.6[a]		1.8
Ro5a	3.7		14.8	1.0		38.4	40.8[a]		0.9
MS35a	2.7		12.8	5.2	0.9	30.9	45.3[a]		1.4
CECT1114	1.0		15.1	0.5		37.2	44.9[a]		0.9
CECT10573	2.1		19.1			30.7	47.1[a]		1.0
Bt6	2.4		18.0	3.3	0.8	24.7	46.8[a]		3.2
Ro9b	2.7		13.4	2.8	0.7	28.6	49.8[a]		1.4
MS13	2.7		15.4	2.4	0.7	28.4	47.5[a]		2.1
Ro9a	2.8		15.6	2.6	0.5	28.4	47.8[a]		1.8
MS37a	2.6		11.3	4.2	0.8	34.1	45.3[a]		1.2
E7H	6.1		9.4	2.4	0.9	23.4	56.8[a]		0.7
6-2St2a	3.5		7.8	10.9	2.2	19.5	54.4[a]		0.9
Ro3-16	5.3		10.0	7.3	1.3	20.0	52.2[a]	1.0	2.4
DW14	0.7		7.7	8.3	2.5	22.0	56.9[a]		1.7

[a] Linolenic acid was present, identified by characteristic ions.

The 41 isolated yeasts revealed eleven different ITS-RFLP patterns (Table 3). The most abundant one was assigned to *Saccharomyces cerevisiae*, as confirmed by API test (Table 4) and the pattern resembled that one of the reference strain *S. cerevisiae* CECT 1942^T given in Guillamón *et al.* (1998). The patterns were labeled from A to K (Table 3) according to their abundance. Pattern B was detected ten times and allocated to *Candida pelliculosa*. The other patterns were found one to three times. The size of the ITS PCR products made it possible to differentiate the isolates in at least seven clusters. However, it is recommended to use the restriction enzyme for additional information and especially for a confirmation of the concise four fragments pattern of *Saccharomyces cerevisiae*. The species *Saccharomyces bayanus* and *S. pastorianus* reveal a distinct pattern with only three fragments (Guillamón *et al.* 1998). *Saccharomyces paradoxus* CECT 1939^T and *S. paradoxus* CECT 11143 share the pattern with *S. cerevisiae*. The ITS-PCR product can be digested with *Hin*fI to achieve a distinction of these species (Esteve-Zarzoso *et al.* 1999). In this study, the ITS-RFLP was the method used with the highest resolution, next to the API ID 32 C kit.

Table 3. Size in bp of the ITS PCR products and the *Hae*III restriction fragments. Strains with similar patterns were grouped, average values and standard deviations are given.

Strains	PCR products	Restriction fragments				Pattern[a]
Ro3a, Ro3b, Ro3-20b, Ro32, Bt11, Bt12, Bt15-7a, Bt15-7b, Bt35, MS31a, MS32a, MS32b, MS33, MS34, MS35b, MS38a, MS38b	828 ± 29	308 ± 9	222 ± 23	170 ± 5	129 ± 4	A
Ro5a, Ro5-2, Ro9a, Ro9b, Bt6, MS13, MS35a, MS36c, MS37a, MS38c	594 ± 27	618 ± 14				B
MS19b, MS29, MS30,	881 ± 48	653 ± 6	221 ± 2			C
Ro3-16, 6-2St2a	511 ± 9	456 ± 0	80 ± 2			D
MS36a, MS36b	693 ± 13	514 ± 0	114 ± 0			E
Ro6	814	800				F
DW14	517	407	107			G
E7H	612	395	116	79		H
Bt8	486	381	87			I
Bt16	549	367	168			J
Ro5b	418	309	82			K
S. cerevisiae MS39	851	308	228	167	132	A
Pichia anomala CECT 1114, *Hypomyces chrysospermus* CECT 10573	652 ± 19	633 ± 0				B

[a] Similar patterns were labeled with A to K.

Information about the identity of the isolated yeasts was obtained by the API ID 32 C kit (Table 1). The 29 tested yeasts revealed 24 different assimilation patterns and only four were found twice. The cluster analysis (Fig. 1) revealed seven main clusters. Cluster 1 consisted of yeasts with the ITS-RFLP pattern A, the ones with pattern B comprised also one cluster. Three yeasts were single member clusters in Fig. 1 and in the ITS-RFLP analysis. The yeasts in API clusters 2 and 7 were multiple ITS-RFLP clusters. All isolates were capable of glucose assimilation, 78.6% and 71.4% were able to assimilate saccharose and maltose, repectively, which are the two major carbohydrates in brewing wort. The isolates were assigned to eight species, their frequency is given in parenthesis: *Saccharomyces cerevisiae* (9), *Candida pelliculosa* (8), *Candida glabrata* (2), *Candida sake* (2), *Candida colliculosa* (1), *Candida guilliermondii* (1), *Candida parapsilosis* (1) and *Candida valida* (1). Five strains could not be identified by the API database. This may be due to the focus of the database on *Candida* spp., which were represented by 38 species and only eleven other genera have been included.

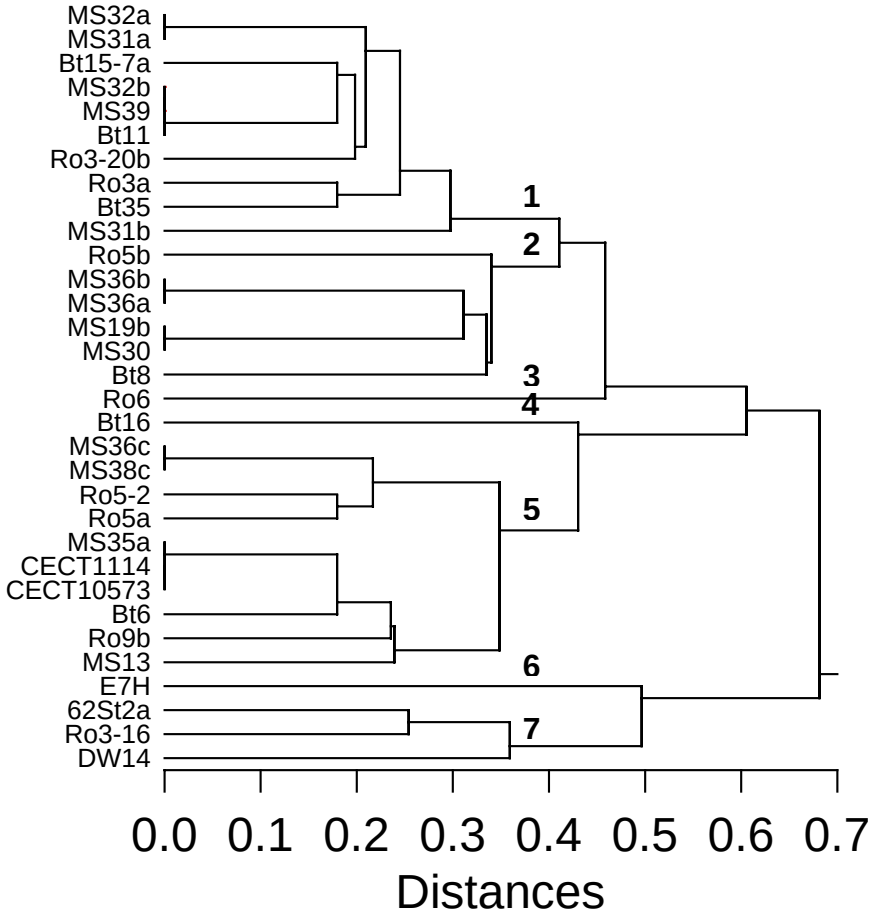

Figure 1. Dendrogram calculated with the API ID 32 C assimilation patterns of the yeasts using the averge linkage and the euclidean distance. The numbers indicated the main clusters.

The small number of genera is in line with data of van der Aa Kühle and colleagues (1998). They identified 124 wild yeast isolates from breweries and found mainly *Saccharomyces cerevisiae* (57.2%), *Pichia* spp. (28.3%) and *Candida* spp. (14.5%). The species *Pichia guillermondii* has been also isolated in this study, but it has been named as the corresponding anamorph *Candida guillermondii* (as enclosed in the API database). No other *Pichia* sp. has been isolated. In contrast, the *Candida* species *C. tropicalis*, *C. sake* and *C. parapsilosis* were listed in van der Aa Kühle *et al.* (1998) and they were also isolated in this study. Moreira da Silva *et al.* (1994) also identified wild brewery yeasts and among the *Candida* spp., there were *C. tropicalis*, *C. utilis* and the species *C. guilliermondii*, *C. pelliculosa*, *C. sake*, and *C. valida*, which were isolated in this study too. This signified these *Candida* spp., in addition to *Saccharomyces cerevisiae*, as typical members of brewery associated microbial communities. The beer-spoiling yeasts have been distributed on the

bottling plant and the adjacent regions in both breweries. This is in accordance with the distribution of biofilms dominated by oleic and linoleic acid (Timke *et al.* submitted). The situation has been found to be similar for wine bottling plants, where the yeast contaminants were present all over the bottling line (Malfeito-Ferreira *et al.* 1997).

Growth tests on selective media are easy to perform and there is no need for expensive equipment but the data indicated some unreliable results for $CuSO_4$ and crystal violet medium. From 18 strains identified as *Saccharomyces cerevisiae* by ITS RFLP patterns, only six were negative on $CuSO_4$ agar and twelve were positive. The differentiation in non-*Saccharomyces* and *Saccharomyces* yeasts could be improved by using further selective media for yeasts, e.g. lysine medium (Morris and Eddy 1957) and Lin`s wild yeast medium (Lin 1974).

Twelve of the isolated yeasts were able to form biofilms ($A_{550} \geq 0.12$). Seven of them belonged to the ITS-RFLP group with the pattern B, which has been assigned to *Candida pelliculosa.* Two yeasts could not be identified by the API profile. The others were affiliated to *C. guilliermondii*, *C. parapsilosis* and *C. sake*. In line with this, there are some studies reporting a biofilm forming potential of *Candida* spp, e.g. for *C. albicans* (Kumamoto 2002; Douglas 2003), *C. dubliniensis* (Ramage *et al.* 2001), *C. parapsilosis* and *C. tropicalis*, (Shin *et al.* 2002). There was no member of the *Saccharomyces* strains positive in the biofilm test system. There is a report, which described adhesion of *S. cerevisiae* to a number of plastic surfaces (Reynolds and Fink 2001), however Chandra *et al.* (2001) stated that *S. cerevisiae* failed to form extracellular material-encased biofilms similar to those formed by *C. albicans*. In contrast to the inability of biofilm formation, all *S. cerevisiae* strains were able to grow in beer. On the other hand, some biofilm positive *Candida* spp. could not multiply in beer. Interestingly, many lipid profiles of mature brewery biofilms had high proportions of linoleic acid (Timke *et al.* submitted), which indicated *Candida* spp. rather than *Saccharomyces* spp. to be one of the main yeasts in these microbial communities.

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Chapter 5

Isolation and Identification of Bacterial Isolates from Two Breweries

Abstract

Isolates were obtained from five different biofilms of two breweries. The strains were identified as members of the Alpha- and Gammaproteobacteria, Firmicutes, Deinococcaceae, Flavobacteriaceae and Micrococcaceae. In addition, in NBB medium grown swab samples and some turbid beer samples were used for isolation. All strains which originated from NBB or beer samples belonged to the Firmicutes. A beer-spoiling potential was found for some of the Firmicutes.

The aim of this study was to isolate and identify microorganisms of brewery biofilm samples and biofilm enrichments in beer. The latter ones particularly were used for isolation of beer-spoiling bacteria. The cultivation of beer-spoiling bacteria would have marked their isolation sources as hot spots for the hygienic control. Furthermore, unknown species with beer-spoiling potential can be isolated by this approach. Also non beer-spoiling bacteria were cultivated and identified. They can fulfill important functions like the primary colonization of surfaces (Kolari *et al.* 2001), the matrix production, acidification and anaerobification of the environment (Back 1994) for the beer-spoiling bacteria. In addition, pure cultures are indispensible for a reliable identification and description of species, including their physiological capabilities.

Parts from the bottling plant of brewery A were demounted and used for isolation. The crowner die was impressed on solid DSM 105 medium (*Gluconobacter oxydans* medium; http://www.dsmz.de/media), DSM 254 medium (*Acetobacter peroxydans* medium) and TSA (Trypticase Soy Agar, Becton/Dickinson, Cockeysville, USA) and incubated at 25°C. The following strains were obtained from DSM 105 medium: DW13, DW15, DW16, DW25, DW26, DW28; from DSM 254 medium: DW21, DW22, DW33 and isolate DW32 originated from TSA. All strains were transferred to TSA. Furthermore, shims from the discharge star wheel (Fig. 1) and the crowner star wheel were impressed on TSA medium, three (strains: MTTF9, MTTF13, MTTF14) and five strains were obtained (strains: MTTV4b, MTTV8, MTTV10S, MTTV12, MTTV12S), respectively.

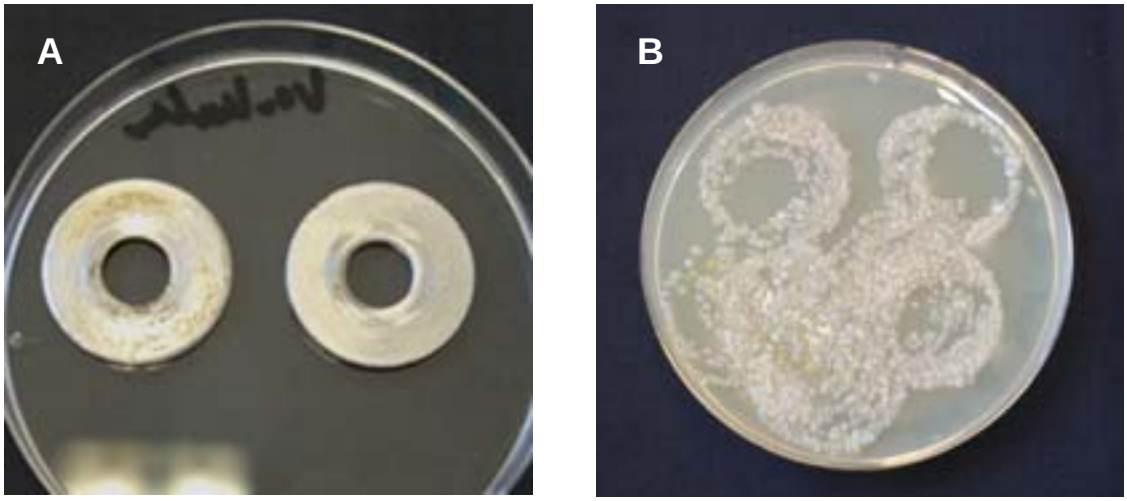

Figure 1. Picture A shows two demounted shims of the feeder discharge star wheel. One of them has a slight brown coating. Photograph B was taken from colonies grown on TSA from these impressed shims.

Two mature biofilms were suspended in 0.9% NaCl of which 100 µl were plated on solid media. This was done with a biofilm from a process water pipe of brewery B and with biomass from the inner part of the discharge star wheel of brewery A. The water pipe strains were DW6, isolated from TSA, and DW67 and DW68 which originated from R2A agar plates, respectively. R2A medium was developed for the cultivation of bacteria from potable water samples (Reasoner and Geldreich 1985). The biofilm from the discharge star wheel was plated on TSA, DSM 254 medium and DSM 92 medium (trypticase soy yeast extract medium). Four strains were obtained from TSA (strains: MQ1, MQ2, MQ5, MQ6.1), one strain from DSM 254 medium (strain MQ50) and DSM 92 medium (strain MQ64), respectively.

In addition, bacteria were isolated and identified from enrichments in beer. Hundred µl of beer inoculated with biofilms from the Rolinck brewery (samples: A4, A6, A8, A18, A20, A23, A26, A28, A29; for details: Table 1, Chapter 3) were plated on TSA and MRS (DeMan-Rogosa-Sharpe, Difco Laboratories, Detroit, USA). All plated samples were grown in non-alcoholic beer or beer with 4.8% (v/v) of ethanol, except the enrichment of sample A28. The MRS agar plates were incubated in an anaerobic jar at 25°C. Thirty-one bacterial strains were obtained, which were not further characterized. The same was done with samples from the Bitburger brewery (samples: B4, B5, B9, B10, B15, B20). Twenty-three bacterial strains were isolated. All obtained pure cultures were identified as yeasts or bacteria using a microscope. The yeasts have been discussed in Chapter 4.

Further isolates were obtained from turbid swab samples in NBB (selective agent for beer-spoiling bacteria; Döhler, Darmstadt, Germany) from the filling area of brewery A. They were grown on MRS medium in an anaerobic jar. Four strains were isolated and identified (strains: Thü4.2, Thü8.1, Thü8.2 and Thü11). The same cultivation conditions were provided for samples of beer taken from four contaminated bottles of beer which resulted in the isolation of the strains DWAlt2, Abf, Ro and WR. In addition, some NBB grown pure cultures from brewery A were kindly provided (strains: Ro2, Ro10, Ro11 and Ro13b) and strain GK from brewery B.

Most of the strains were tested for their beer-spoiling potential. Strains were inoculated in 21 ml tubes completely filled with beer and closed with screw caps. The cultures were incubated at 25°C.

The cultivation studies with biofilm samples revealed members of five different phyla. The majority of the isolates from the brewery biofilms belonged to the Proteobacteria. In particular, the isolates from the shims were all members of the Gammaproteobacteria next to one Alphaproteobacterium (strain MTTF13). Another Gram-negative phylum was represented by strain DW67 which has been a member of the Bacteroidetes/Chlorobi group. As Gram-positives, strains were affiliated to species of the Actinobacteria (strains DW13 and DW28) and the Firmicutes (DW16 and DW22). In addition, a member of the Deinococcus/Thermus phylum (strain DW22) was cultivated. The species affiliations of isolates from bottling plant parts impressed on solid media and the suspended biofilms are given in Table 1.

Table 1. Identification of strains isolated from brewery biofilms. The isolation sites were the crowner die (strains: DW13 – DW33), shims from the discharge star wheel (strains: MTTF9 - MTTF14), shims from the crowner star wheel (strains: MTTV4b – MTTV12S), the inner part of the discharge star wheel (strains: MQ1 – MQ64) and a process water pipe (strains: DW6 – DW68).

Strain	Similarity [a]	Species[b]	Accession number
DW13	99.7% (687)	*Micrococcus luteus* DSM 20030	AJ536198
DW16	100% (396)	*Staphylococcus warneri* ATCC 27836	Z26903
DW21	99.8% (569)	*Staphylococcus saprophyticus* NCTC 7292	Z26902
DW22	96.7% (674)	*Deinococcus grandis* DSM 3963	Y11329
DW26	99.2% (391)	*Roseomonas mucosa* ATCC BAA-692	AF538712
DW28	96.7% (786)	*Arthrobacter methylotrophus* DSM 14008	AF235090
DW32	99.7% (664)	*Moraxella osloensis* NCTC 10465	X74897
DW33	99.8% (618)	*Shigella flexneri* ATCC 29903	X96963

Table 1. continued

Strain	Similarity [a]	Species[b]	Accession number
MTTF9	99.2% (709)	*Pseudomonas pseudoalcaligenes* LMG 1225	Z76666
MTTF13	99.6% (470)	*Agrobacterium tumefaciens* NCPPB 2437	D14500
MTTF14	99.7% (660)	*Pseudomonas pseudoalcaligenes* LMG 1225	Z76666
MTTV4b	99.7% (642)	*Pseudomonas pseudoalcaligenes* LMG 1225	Z76666
MTTV8	99.4% (668)	*Acinetobacter johnsonii* DSM 6963	X81663
MTTV10S	99.2% (507)	*Escherichia fergusonii* ATCC 35469 / *Shigella flexneri* ATCC 29903	AF530475 / X96963
MTTV12	99.1% (690)	*Acinetobacter baumannii* ATCC 19606	Z93435
MTTV12S	98.9% (530)	*Citrobacter rodentium* CDC 1843-73	AF025363
MQ1	98.9% (652)	*Aeromonas punctata* ATCC 15468	X74674
MQ2	98.8% (420)	*Raoultella planticola* ATCC 33558[c]	AF129444
MQ3.1	93% (190/203)	*Pseudomonas flavescens* ATCC 51555	AJ308320
MQ5	98.3% (564)	*Raoultella planticola* ATCC 33558	AF129444
MQ6.1	99.8% (529)	*Enterobacter amnigenus* JCM1237 / *Buttiauxella izardii* DSM 9397	AB004749 / AJ233404
MQ41	97% (380/389)	*Acinetobacter calcoaceticus* DSM 30006	AJ633632
MQ49	98% (389/394)	*Pseudomonas plecoglossicida* FPC951	AB009457
MQ50	99.8% (621)	*Raoultella planticola* ATCC 33558	AF129444
MQ52	97% (447/459)	*Pseudomonas graminis* DSM 11363	Y11150
MQ64	99.3% (603)	*Acinetobacter johnsonii* DSM 6963	X81663
DW6	99.3% (606)	*Pseudomonas mandelii* CIP 105273	AF058286
DW67	96.8% (526)	*Flavobacterium hibernum* ATCC 51468	L39067
DW68	100% (613)	*Pantoea agglomerans* JCM 1236	AB004691

[a] The number of compared bases is given in parenthesis.

[b] The best fit for a type strain provided by the BLAST algorithm (Altschul *et al.* 1997) was aligned and similarity was calculated.

[c] formerly: *Klebsiella planticola* earlier synonym of *K. trevesanii* (Gavini *et al.* 1986, Drancourt *et al.* 2001).

All isolates listed in Table 2 have been assigned to the Firmicutes. They represented four families, the Bacillaceae (strains B3-27 and B5-3), Leuconostocaceae (strains B3-31, Thü4.2), Enterococcaceae (strain Thü11) and Lactobacillaceae. They were isolated from enrichments in NBB, which consisted to nearly 50% (v/v) of beer (Jespersen and Jakobsen 1996), from enrichments in beer or were provided brewery isolates.

Table 2. Identification of strains isolated from enrichments of brewery samples in NBB or beer. The isolates originated from biofilm enrichments in beer (strains: B3-27 – Bt15-2), from swabs in NBB (strains: Thü4.2 – Thü11), from contaminated bottles of beer (strains: DWAlt2 – WR) and some were provided brewery isolates (Ro2aan – GK).

Strain	Similarity[a]	Species[b]	Accession number
B3-27	99.8% (637)	*Bacillus weihenstephanensis* DSM 11821	AB021199
B3-31	96.9% (589)	*Leuconostoc pseudomesenteroides* NCDO 768	X95979
B5-3	99.4% (624)	*Bacillus horikoshii* DSM 8719	AB043865
Bt15-2	98.9% (459)	*Lactobacillus brevis* ATCC 14869	M58810
Thü4.2	98.5% (456)	*Leuconostoc gelidum* DSM 5578	AF175402
Thü8.1	100% (533)	*Lactobacillus ferintoshensis* R7-84	AF275311
Thü8.2	100% (547)	*Lactobacillus paracasei* subsp. *paracasei* JCM 8130	D79212
Thü11	100% (618)	*Enterococcus flavescens* CECT 4481	AJ420802
DWAlt2	99.7% (582)	*Lactobacillus brevis* ATCC 14869	M58810
Abf	98.4% (556)	*Lactobacillus brevis* ATCC 14869	M58810
Ro	96.2% (371)	*Lactobacillus coryniformis* DSM 20001	M58813
WR	96.3% (408)	*Lactobacillus coryniformis* DSM 20001	M58813
Ro2aan	99.3% (565)	*Lactobacillus paracasei* subsp. *paracasei* JCM 8130	D79212
Ro10an	98.9% (535)	*Lactobacillus paracasei* subsp. *paracasei* JCM 8130	D79212
Ro11an	98.3% (639)	*Lactobacillus perolens* L532	Y19167
Ro13ban	98.2% (612)	*Lactobacillus perolens* L532	Y19167
GK	99.7% (660)	*Lactobacillus collinoides* JCM 1123	AB005893

[a] The number of identical and compared bases is given in parenthesis.

[b] The best fit for a type strain provided by the BLAST algorithm (Altschul *et al.* 1997) was aligned and similarity was calculated.

In total, 15 strains of the ones listed in Table 1 have been tested for growth in beer (strains: DW13, DW16, DW21, DW22, DW28, DW32, DW33, MQ1, MQ2, MQ5, MQ6.1, MQ50, MQ64, DW6 and DW68). None of them was able to grow in beer. Also, all strains isolated from enrichments in NBB or beer were tested for growth in beer. Eight strains of the ones listed in Table 2 were able to grow in beer (strains: Bt15-2, Thü4.2, Thü11, DWAlt2, Ro, WR, Abf, GK). None of the 31 cultivated strains from brewery A biofilm samples inoculated in beer was able to cause turbidity in beer, three of which were identified by sequencing (strains: B3-27, B3-31 and B3-5). No beer-spoiling potential was found for 22 brewery B biofilm isolates, except for the *Lactobacillus brevis* affiliated strain Bt15-2.

Some biofilm samples were inoculated in beer prior to isolation. None out of 53 obtained bacterial strains from these enrichments isolated on TS or MRS medium was able to cause turbidity in beer. This indicated that beer-spoiling bacteria were scarce in the biofilms. The

cultivation conditions were chosen to be optimal for Lactobacillaceae including anerobic conditions, nevertheless, the MRS medium is not selective due to the high nutrient requirements of lactic acid bacteria. Therefore, many microorganisms have been isolated which were able to survive in beer for many weeks up to months. This signified a bacteriostatic effect of beer on many bacteria, rather than a bacteriocidal one. Interestingly, no beer-spoiling bacteria were isolated from the inner part of the discharge star wheel, although 96 strains were isolated on nine different media including aerobic and anaerobic incubation conditions. This sample location has been extremely exposed to beer, which may have provided optimal conditions for beer-spoiling bacteria. In addition, even the isolation of bacteria from samples which have been grown in beer resulted in a very low number of beer-spoiling bacteria. This is in accordance with the data presented in Chapter 1, 2, 3 and 9, which also indicated a low number of beer-spoiling bacteria in the analyzed microbial communities. The reason for the turbidity in beer of the enrichments, although no beer-spoiling bacteria were cultivated, may be the presence of yeasts. They were isolated from five out of six brewery B biofilm enrichments in beer. Nevertheless, the well-known beer-spoiling bacteria *Lactobacillus brevis* and *L. coryniformis* were detected. In addition, the strains Thü11 and Thü4 which were assigned to *Enterococcus flavescens* and *Leuconostoc gelidum* showed a beer-spoiling potential. *Leuconostoc* spp. have been considered as potential beer-spoiling bacteria according to Back (1994). *Enterococcus* spp. have been described as wort bacteria which can multiply mainly in mash and malt (Back 1994).

The majority of the isolates were members of the Proteobacteria, in particular Enterobacteriaceae and *Pseudomonas* spp. were often isolated. Both taxa have been reported to occur frequently in brewery biofilm communities (Back 2003). Interestingly, two strains were assigned to the genus *Acinetobacter*, of which *A. calcoaceticus* has been mentioned as a typical organism in brewery biofilms (Back 2003). Another member of this genus, *A. radioresistens*, has been isolated from slimes of paper-producing machineries (Väisänen *et al.* 1998). This is one of the few industrial habitats, which microbiota has been studied intensively. For instance, *Deinococcus geothermalis* has been described as a primary colonizer in paper machine biofilms (Kolari *et al.* 2001) and interestingly, an *Deinococcus* sp. strain DW22 has been isolated from the crowner die. Another *Deinococcus* strain was isolated from a steel coupon which was laid out overnight at the discharge star wheel of this bottling plant (Winkler 2003). The 16S rRNA gene sequence similarity of both *Deinococcus* isolates was 99.5%. These data suggest that *Acinetobacter* and *Deinococcus* maybe two abundant and functional important genera of brewery biofilms.

In general, the proportion of cultivatable microorganisms of a natural community is low. Some taxa are just represented by cloned 16S rRNA gene sequences, e.g. the candidated phlogenetic division OP11 (Harris *et al.* 2003). Furthermore, about 35% of members of the

Proteobacteria have not been cultivated (Hugenholtz *et al.* 1998). Despite of this methodological restrictions, the isolation studies revealed a high diversity for the brewery biofim communities. This is in line to data found for brewery biofilms (Back 2003) and also for the microbiota of printing paper machines (Väisänen *et al.* 1998) suggesting complex microbial communities on industrial plants.

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Chapter 6

Detection of Acyl-Homoserine-Lactones Secreting Isolates

Abstract

This study based on a cross-feeding assay for the detection of acyl-homoserine lactones (AHLs). In total, 106 isolates were investigated. The strains originated from two breweries, including seven different sample locations. Nine of the isolates were AHL positive. They all belonged to the Proteobacteria and most of them were members of the genus *Pseudomonas*. These strains derived from three different samples. Consequently, AHL mediated bacterial communication has been evidenced.

The main life-form of bacteria in the natural environment are sessile communities called biofilms (Costerton *et al*. 1995). The bacteria develop structures that are morphologically and physiologically different from their free-living counterparts (Davey and O'Toole 2000). A first step in the formation of biofilms is the attachment of cells to surfaces. Then, a differentiation process takes place which requires the induction of new metabolic pathways, e.g. the production of extracellular polymeric substances and export of enzymes. For *Pseudomonas aeruginosa* biofilms a cell-to-cell signal is involved in the development of biofilms (Davies *et al.* 1998). The population density is detected by the concentration of signal molecules (autoinducers) which are secreted by the cells consitutively. The autoinducers diffuse into the cells and by reaching a threshold concentration an induction of quorum sensing regulated genes takes place. This phenomenon has been called autoinduction and now it is known as quorum sensing (Fuqua *et al.* 1994b). A well-studied class of autoinducers are the acyl-homoserine lactones (AHLs). They are conserved molecules with a homoserine lactone moiety and different fatty acyl groups which vary in length, saturation levels and oxidation state. Another group of autoinducers are furanosylborate diester, which can be produced and recognized by Gram-negative and Gram-positive bacteria (Chen *et al.* 2002). A peptide mediated quorum sensing signalling system has been found for some Gram-positive bacteria (Kleerebezem *et al.* 1997) and some Gram-positive bacteria secrete γ-butyrolactones, which are structurally quite similar to AHLs (Takano *et al.* 2000). For *Candida albicans* farnesol has been described to mediate quorum sensing (Hornby *et al.* 2001). Bacteria which secrete such autoinducers are potential biofilm forming organisms. Therefore, brewery isolates were tested for AHLs secretion.

The secretion of AHLs was investigated by cross streaking experiments. The strains *Agrobacterium tumefaciens* NTL4 and KYC6 were generously provided by W. C. Fuqua (Department of Biology, Indiana University, Bloomington). The sensor strain *A. tumefaciens* NTL4 contains the plasmids pCF218 and pCF372. The strain NTL4 (Luo *et al.* 2001) is identical to the strain A136 (Ti plasmidless, multidrug resistant; Sciaky *et al.*

1978), but is offers a more stable maintenance of the plasmids (Fuqua, personal communication). The plasmid pCF218 contains multiple copies of *occR102 traR* (Fuqua and Winans 1994a) and the plasmid pCF372 which is a pUCD2 (Close *et al.* 1984) derivate containing a *traI-lacZ* gene fusion at positions –143 to +359 related to the *traI* transcription start site (Fuqua and Winans 1996). It provides extremely sensitive detection of AHLs (Zhu *et al.* 1998) and resistances against tetracycline and spectinomycine. *A. tumefaciens* KYC6 functions as a positive control. It is a *traM* null mutant bearing the *traR* overexpression plasmid pCF218. This strain is resistant against tetracycline and kanaymycine (Fuqua and Winans 1994a). It overproduces high levels of *Agrobacterium* autoinducers. The test was performed on AT medium (Tempe *et al.* 1977) with 0.5% of glucose (w/v) or on TSA medium (Trypticase Soy Agar, Becton/Dickinson, Cockeysville, USA). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) in dimethylformamide was plated on the solid medium at a final concentration of 40 μg ml^{-1} for detection of β-galactosidase activity. The strains were incubated at 30°C overnight.

In total, 106 strains have been tested. They originated from seven different sample sites. Some strains did not grow on AT medium and have been tested on TSA. Strains with the initial letters MT were only tested on TSA. The different sample locations, the medium on which the strains were tested on and the strains are given: biofilms, lubricants and rubbed off conveyor material of the infeed conveyor (AT: DW1, DW3, DW8, DW56, DW99, DW101, DW102, DW104, DW143a1; TSA: DW2, DW129), biofilms, lubricants and rubbed off conveyor material of the discharge conveyor (AT: DW38, DW39, DW40, DW41, DW91, DW92, DW115, DW118, DW126, DW137, DW149; TSA: DW114b, DW119, DW140), process water pipe (AT: DW6, DW66, DW68b, DW69, DW70, DW71, DW73, TSA DW68a), crowner die (AT: DW13, DW14, DW16, DW21, DW27, DW28, DW33; TSA: DW15), shims from the discharge star wheel (TSA: MTTF6, MTTF9, MTTF12, MTTF13, MTTF14, MTTF16, MTTF19), shims from the crowner star wheel (TSA: MTTV1, MTTV2, MTTV3, MTTV4b, MTTV6, MTTV7, MTTV8, MTTV10S, MTTV12, MTTV12S, MTTV13, MTTV14, MTTV15, MTTV16, MTTV19), inner part of the discharge star wheel (AT: MQ2, MQ3.1, MQ3.3, MQ5, MQ6.1, MQ6.2, MQ6.3, MQ7, MQ17, MQ18.1, MQ18.2, MQ19.2, MQ29, MQ41, MQ42, MQ47, MQ48, MQ49, MQ50, MQ51, MQ52, MQ53, MQ54, MQ55, MQ56, MQ58.1, MQ61, MQ62 ; TSA : MQ3.2.1, MQ3.2.2, MQ9, MQ10, MQ11, MQ12, MQ22.3, MQ23.1, MQ27, MQ28, MQ57, MQ59, MQ60.1, MQ60.2, MQ63.1, MQ64). All strains originated from brewery A, except the process water pipe isolates (brewery B). Positve test results were confirmed by repeating the cross streaking experiment. The 16S rRNA gene sequences of some strains were obtained as described in Chapter 1.

The majority of the tested isolates were negative in the test system, an example is shown in Fig. 1. The positive control strain KYC6 always caused a blue colour of the sensor strain NTL4. Some strains were also positive in the biosensor test system (Fig. 1).

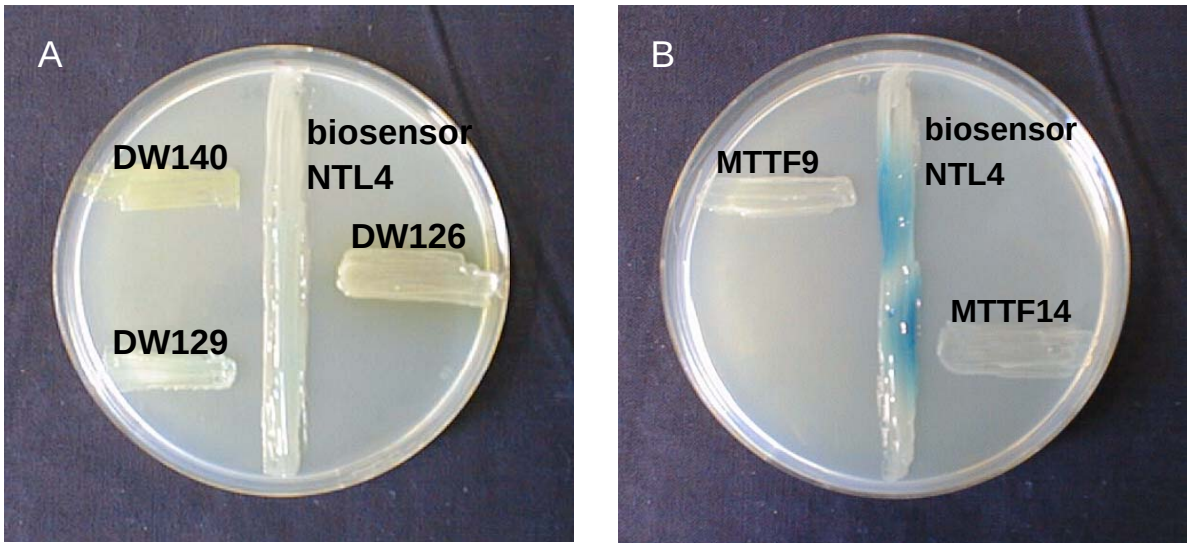

Figure 1. Cross streaking experiments for AHLs detection using the biosensor *A. tumefaciens* NTL4. (A) The biosensor was unaffected by the isolates strains DW140, DW129 and DW126. (B) An AHL positive result was obtained for the strains MTTF9 and MTTF14. A blue colour was visible and marked the edge of the AHLs diffusion front. Both pictures were taken after one day of incubation.

Strains from seven different sample locations were tested for the secretion of AHLs (Table 1). In total 106 isolates were investigated and nine (8.5%) of them induced the β-galactosidase activity of strain NTL4.

The 16S rRNA gene of the AHLs positive tested isolates was partly sequenced and affiliated to six *Pseudomonas* species (MTTV4b, MTTF9, MTTF14, MQ3.1, MQ49 and MQ52), an *Acinetobacter* (MQ41), an *Agrobacterium* (MTTF13) and a *Citrobacter* (MTTV12S) strain, details of the identification have been given in Table 1, Chapter 5.

Table 1. Origin of isolates and number of negative and positive tested strains in the AHLs biosensor cross-feeding assay.

Origin of the strains	Number of tested strains	Number of AHLs positive strains
• Biofilms, lubricants and rubbed off conveyor material of the infeed conveyor	11	0
• Biofilms, lubricants and rubbed off conveyor material of the discharge conveyor	14	0
• Process water pipe	7	0
• Crowner die	8	0
• Shims from the discharge star wheel	7	2
• Shims from the crowner star wheel	15	3
• Inner part of the discharge star wheel	44	4

The secretion of AHLs has been described for many Gammaproteobacteria, e.g. for *Pseudomonas* spp., *Erwinia* spp., *Seratia* spp., *Yersinia* spp., *Aeromonas* spp., *Vibrio* spp. and *Xenorhabdus nematophilus* (Whitehead *et al.* 2001). In addition, the Alphaproteobacteria *Agrobacterium tumefaciens*, *Rhizobium* spp. and *Rhodobacter sphaeroides* and the Betaproteobacterium *Chromobacterium violaceum* have been described to produce AHLs (Whitehead *et al.* 2001). However, it has to be mentioned that the AHLs secreted by the organisms listed are involved in the regulation of a broad range of biological functions (Whitehead *et al.* 2001). An impact of AHLs on the biofilm formation has been described for *Pseudomonas fluorescens* and *P. aeruginosa* (Allison *et al.* 1998; Davies *et al.* 1998). Other studies reported the requirement of an intact AHL system for the maturation of biofilms of *Aeromonas hydrophila* (Lynch *et al.* 2002), *Burkholderia cepacia* (Huber *et al.* 2001) and *Serratia liquefacies* (Labbate *et al.* 2004). Next to these species, the presence of AHLs has also been shown for naturally occurring aquatic biofilms (McLean *et al.* 1997) suggesting that AHLs play a role also in natural biofilms.

Therefore, all AHL synthesizing isolates could be potentially involved in the formation of biofilms, in particular the *Pseudomonas* spp. The autoinducer secreting isolates were often cultivated from the shims, which had a thin biofilm. This could be a hint for their meaning as surface colonizing organisms. In contrast, the plated crowner die showed no visible biofilm and here, all isolates were negative in the biosensor assay.

The AHL mediated communication of bacteria has been considered as a point of interference for circumventing the differentiation of biofilms. In this context, Dong *et al.* (2000) identified an autoinducer inactivation gene (*aii*A), coding for an AHL lactonase. AHL-inactivating enzymes have been also found in *Bacillus thuringiensis*, *B. cereus* and *B. mycoides* (Dong *et al.* 2002). Another source for AHL-degrading enzymes may be the species *Variovorax paradoxus* which has been reported to use AHL molecules as energy

and nitrogen sources (Leadbetter and Greenberg 2000). Such organisms or the purified AHLs degrading enzymes may be distributed on the plants for a prevention of biofilms. There has been also an effect described on *P. aeruginosa* biofilms by a halogenated furanone compound. This compound antagonizes AHLs by competition for the binding site on the receptor protein. This is produced by the Australian macroalgae *Delisea pulchra*. It did not effect the initial attachment but the architecture of the biofilm. In addition, the detachment of cells was enhanced, leading to a loss of biomass (Hentzer *et al.* 2002).

It has to be mentioned that AHLs are involved in the maturation of biofilms but they are not essential for their development. Other factors like nutrients and hydrodynamic conditions are also important for the determination of the biofilm structure (Heydorn *et al.* 2002; Purvedorj *et al.* 2002). Additionally, a suppression of the AHL mediated organisms may select for quorum sensing dependent organisms which use other autoinducers, e.g. yeasts. Nevertheless, this study confirmed the presence of AHL secreting strains in some biofilms.

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Chapter 7

Isolation of Enterobacteriaceae from a Beer Bottling Plant and Their Capability to Grow in Beer

Abstract

Twenty-eight strains belonging to the Enterobacteriaceae were isolated from brewery environments. They originated from 11 different sample sites. The strains were identified as members of the genera *Klebsiella* (12 strains), *Enterobacter* (10 strains), *Kluyvera* (3 strains), *Serratia* (2 strains) and *Citrobacter* (1 strain). Their beer-spoiling potential was tested in closed, completely filled tubes and there was no growth in beer or non-alcoholic beer. Therefore, Enterobacteriaceae represented no risk for bottled beer. However, eleven strains survived 14 weeks in beer, they belonged to the genera *Enterobacter* and *Klebsiella*. In addition, in non-alcoholic beer allowing the access of air, all Enterobacteriaceae caused turbidity. This was also found for some strains in beer with 4.8% (v/v) of ethanol, but the incubation time had to be at least ten weeks to detect an increase of the turbidity. A supplementation of beer with glucose had no growth promoting effects.

Introduction

Most of the Enterobacteriaceae are members of the risk group 2 (European classification), this means they can cause diseases and the risk of infection is low (anonymous 2000). Some Enterobacteriaceae even belong to risk group 3. Despite of the name of this family, many species are reported to occur in the natural environment and can be isolated from plant material, soil or water. Such free-living Enterobacteriaceae are e.g. members of the genera *Citrobacter*, *Enterobacter*, *Klebsiella*, *Hafnia*, *Proteus*, and *Serratia*. In particular the hop material may be a source of Enterobacteriaceae in breweries. Indeed, *Enterobacter cloacae* and *Klebsiella pneumoniae* can be present in brewery biofilms (Back 2003). Lawrence (1988) listed some species, which can be found in wort: *Citrobacter freundii*, *Enterobacter aerogenes*, *Ent. agglomerans*, *Klebsiella pneumoniae* and *Hafnia alvei*. Even four species are reported to survive the fermentation process, these are *Ent. agglomerans*, *Ent. cloacae*, *Hafnia alvei* and *H. protea* (Lawrence 1988). This means they cosediment with the culture yeast and may be passed to subsequent fermentations. The most hazardous species for beer are *Hafnia protea* and *Klebsiella oxytoca*, which can survive in beer (Lawrence 1988). However, in general, the Enterobacteriaceae are not considered as very significant because the drop in pH and the accumulation of ethanol during the brewing process quickly kills them (Lawrence 1988). So the Enterobacteriaceae are sometimes part of the bacterial communities in breweries, but they have not been supposed to cause serious damage. In this study, some Enterobacteriaceae were isolated from different sample sites, identified and tested in beer.

Materials and Methods

Isolation and identification. The strains were isolated from 11 different samples, they are listed in Table 1. The strains were cultivated on different media (TSA, MRS, DSM 105 medium, DSM 254 medium, or R2A; details are given in Chapter 1 and 5) and transferred to Trypticase soy agar (TSA). The sequencing of the 16S rRNA gene of the isolates was done as described in Chapter 1. A dendrogram was calculated with the obtained sequences applying the ARB software package (Ludwig *et al.* 2004). Assimilation of carbon compounds was tested using the API 20 E kit (Bio Merieux SA, Marcy-L`Etoile, France) according to the manufacturer`s instructions. Identification of the isolates was done with the software Apilab Plus (version 3.3.3, Bio Merieux).

Growth of the isolates in beer. A suspension in 0.9% NaCl of the strains according to McFarland standard 1 was prepared. Hundred µl of this suspension were used for inoculation of 21 ml beer, which has been heated to 70°C for 30 min before inoculation. In this study, beer with 4.8% (v/v) of ethanol was termed beer. The tubes were closed with screw caps and filled completely with beer (designated: anaerob). The strains were also inoculated in tubes filled half with beer with a possible exchange of air (designated: aerob). They were all incubated at 25°C without any shaking. The optical density was measured at 578 nm (A_{578}). Each of the strains was tested in duplicates. The blank value was beer without cells, which excluded false positive turbidity due to natural darken of beer.

Results

The isolation sources of the cultivated Enterobacteriaceae and the strain numbers are given in Table 1. All samples originated from brewery A, except of the process water pipe sample, which was taken in brewery B.

Table 1. Sample locations and isolated strains.

Sample locations	Isolated strains
• biofilms, lubricants and rubbed off conveyor material of the infeed conveyor	DW56, DW99, DW143a1
• shims from the discharge star wheel	MTTF6, MTTF19
• from the inner part of the discharge star wheel	MQ2, MQ5, MQ6.1, MQ50
• crowner die	DW33
• shims from the crowner star wheel	MTTV1, MTTV3, MTTV7a, MTTV7b, MTTV10S, MTTV12S, MTTV14, MTTV16, MTTV19
• steel coupon laid out overnight on the crowner star wheel	J8-5St2b
• biofilms, lubricants and rubbed off conveyor material of the discharge conveyor	DW38, DW39, DW40, DW41
• drawer at the discharge	IIRo15
• discharge conveyor	IIRo50
• drainage pit, inner edge of the lid	IIRo55
• process water pipe	DW68

Identification of the isolates. The lipid profiles of these strains were dominated by the fatty acids 16:0, 16:1 *cis*9, 18:1 *cis*11 and 17:0 cyclo9-10, the main fatty acids of Enterobacteriaceae (Brian and Gardner 1968) and at least 5% of 14:0 3OH, a typical fatty acid for Enterobacteriaceae (Bryn *et al.* 1978). Nearly all of the isolated strains were used for a partial sequencing of the 16S rRNA gene. The nearest relative type strains have been given (Table 2). The isolates were assigned to the genera *Enterobacter* (10 strains), *Shigella* or *Escherichia*, respectively (9 strains), *Klebsiella* (5 strains) and *Citrobacter* (2 strains).

In addition to the sequencing, the strains were identified by their assimilation profiles (Table 2). The same genus was found for 11 out of these 28 strains and 15 strains were affiliated to different genera as found for the sequencing. A dendrogram was calculated with the sequences of the isolates and some type strains (Fig. 1). *Enterobacter amnigenus* JCM 1237^T and *Ent. asburiae* JCM 6051^T clustered in one branch with the sequences of some isolates and with *Klebsiella oxytoca* JCM 1665^T, *Raoultella planticola* ATCC 33558^T (formerly: *Klebsiella planticola* an earlier synonym of *K. trevesanii*; Gavini *et al.* 1986, Drancourt *et al.* 2001), and *Citrobacter freundii* DSM 30039^T. Therefore, the partial sequence information is not sufficient for a reliable identification of Enterobacteriaceae. Consequently, the species affiliations in the following is based on the API 20 E identification, which resulted in 12 *Klebsiella*, 10 *Enterobacter*, 3 *Kluyvera*, two *Serratia*. and one *Citrobacter* strain (Table 2).

Table 2. Identification of the isolated Enterobacteriaceae by sequencing a part of the 16S rRNA gene and by physiological profiling using the API 20 E kit.

Isolate	Identification by sequencing		Identification by API 20 E	
	Strain[a]	Similarity[b]	Species	Validity[c]
DW56	*Ent. asburiae* JCM 6051	98.7% (531)	*Ent. cloacae*	59.4%
DW99	*C. freundii* DSM 30039	99.8% (568)	*K. oxytoca*	99.9%
DW143a1	*Ent. asburiae* JCM 6051	98.0% (579)	*Ent. sakazakii*	99.5%
MTTF6	*S. flexneri* ATCC 29903 / *E. coli* ATCC 11775	100% (565)	*Ent. cloacae*	38.4%
MTTF19	*S. flexneri* /*E. coli*[d]	100% (510)	*K. ornithinolytica*	91.0%
MQ2	*K. trevisanii* ATCC 33558	97.6% (413)	*K. pneum. pneumoniae*	97.6%
MQ5	*K. trevisanii* ATCC 33558	99.8% (544)	*K. pneum. pneumoniae*	97.6%
MQ6.1	*Ent. amnigenus* JCM 1237	98.6% (585)	*Ent. cloacae*	91.5%
MQ50	*K. trevisanii* ATCC 33558	99.8% (568)	*K. pneum. pneumoniae*	97.6%
DW33	*S. flexneri* /*E. coli*[d]	99.8% (618)	*Ent. cloacae*	95.1%
MTTV1	*S. flexneri* /*E. coli*[d]	100% (529)	*Kluyvera* spp.	93.9%
MTTV3	Not sequenced		*K. ornithinolytica*	92.1%
MTTV7a	Not sequenced		*S. liquefaciens*	67.4%
MTTV7b	*S. flexneri* /*E. coli*[d]	100% (586)	*Kluyvera* spp.	93.9%
MTTV10S	*S. flexneri* /*E. coli*[d]	99.4% (507)	*K. ornithinolytica*	92%
MTTV12S	*Ent. agglomerans* JCM 1236	97.7% (530)	*Ent. aerogenes*	52.8%
MTTV14	*S. flexneri* /*E. coli*[d]	100% (547)	*K. ornithinolytica*	91.0%
MTTV16	*S. flexneri* /*E. coli*[d]	100% (505)	*Kluyvera* spp.	83.6%
MTTV19	*S. flexneri* /*E. coli*[d]	100% (510)	*Ent. sakazakii*	99.5%
J8-5St2b	*Ent. dissolvens* LMG 2683	99.4% (471)	*S. odorifera*	95.2%
DW38	*C. freundii* DSM 30039	99.6% (516)	*C. brackii*	91.8%
DW39	*Ent. dissolvens* LMG 2683	99.6% (535)	*Ent. sakazakii*	99.2%
DW40	*K. oxytoca* JCM 1665	99.5% (603)	*K. oxytoca*	97.4%
DW41	*Ent. kobei* CIP 105566	98.0% (548)	*Ent. cloacae*	59.4%
IIRo15	*Ent. asburiae* JCM 6051	99.4% (543)	*K. ornithinolytica*	99.9%
IIRo50	*Ent. asburiae* JCM 6051	100% (401)	*K. oxytoca*	97.7%
IIRo55	*K. oxytoca* ATCC 13182	99.4% (510)	*K. ornithinolytica*	99.9%
DW68	*Ent. agglomerans* JCM 1236	100% (630)	*Ent. cloacae*	59.4%

[a] The best fit for a type strain provided by the BLAST algorithm (Altschul *et al.* 1997) was aligned and similarity was calculated.

[b] The number of compared bases is given in parenthesis.

[c] The likeliness of the identification determined by the software Apilab Plus is given.

[d] The *S. flexneri* and *E. coli* strains were the same as the ones affiliated to isolate MTTF6.

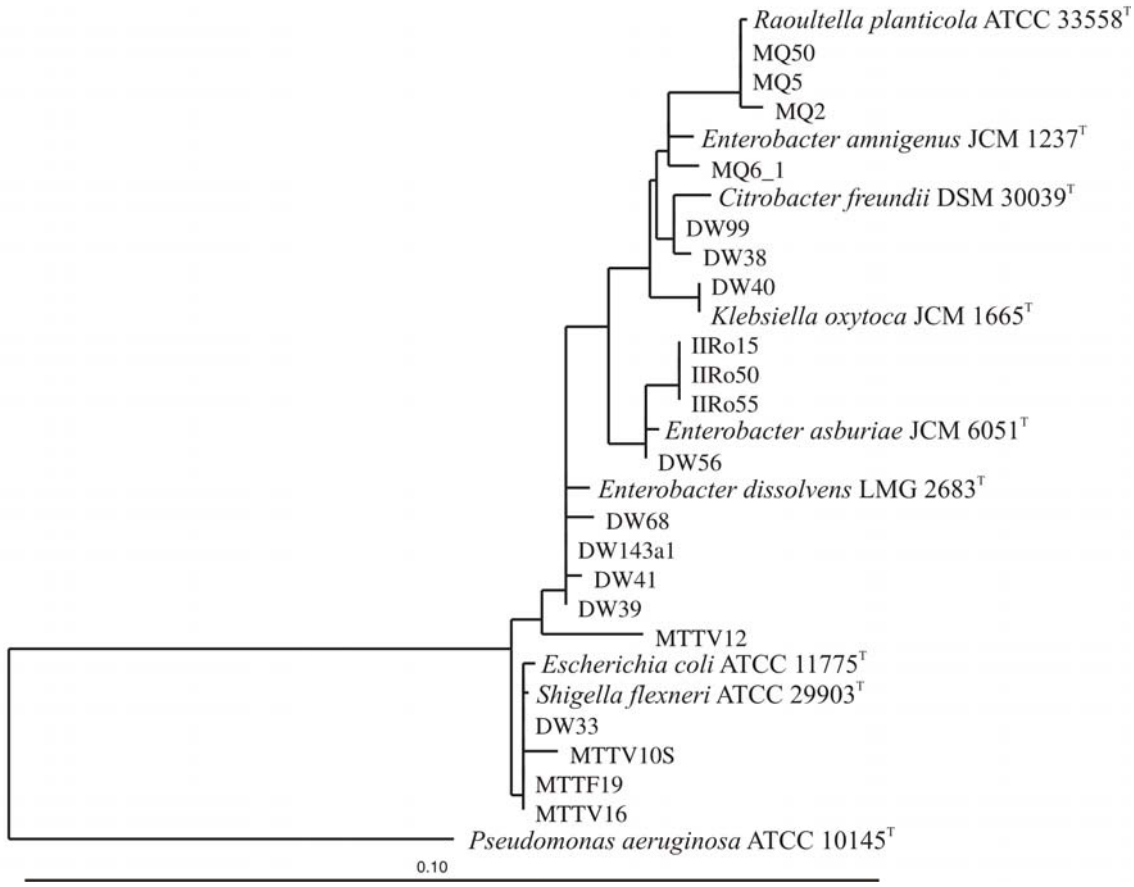

Figure 1. Phylogenetic tree showing the affiliations of partial 16S rRNA gene sequences to type strains. The tree was calculated by maximal likelihood analysis. A conservation filter of 50% for Gammaproteobacteria was used. The scale bar indicated 10% estimated sequence divergence. Five sequences of Deltaproteobacteria were used as outgroup.

Distribution of the isolates on the plant of brewery A. The strains affiliated to *Kluyvera* sp. were exclusively cultivated from the shims of the crowner star wheel. All other genera identified were distributed on the bottling plant, e.g. *Klebsiella* sp. and *Enterobacter* sp. were isolated from nearly every sample site.

Growth of the isolates in beer. The A_{578} for the isolates inoculated in non-alcoholic beer and beer at different incubation conditions are given in Fig. 2. All strains were able to grow in non-alcoholic beer, if access of oxygen was possible. After 22 days Each isolate reached a turbidity of at least 0.6 and some of them caused a A_{578} of even 1 after 22 days. In contrast to the aerobic incubated cultures, there was no growth detectable at anaerobic conditions in non-alcoholic beer, even though the A_{578} was measured for a period of 77 days. For beer

with access of oxygen, there was no increase of the turbidity detectable for the first nine weeks (Fig. 2). However, after 70 days the A_{578} increased for some of the strains (IIRo15, DW39, DW68, J8-5St2b, IIRo50, DW56, DW99, MTTV14, MQ5, IIRo55, DW38, DW143a1 and MTTV10S). The isolates were also tested in beer under anaerobic conditions. There was no increase of the A_{578} even after 77 days. In further experiments, non-alcoholic beer and beer was supplemented with 0.2% (w/v) of glucose. This supplement ensured the presence of a fermentable substrate. All strains were unable to grow in the glucose added non-alcoholic beers in a period of 45 days. The same was found for the ones in beer, which were investigated for 77 days. Only the strain IIRo15 (*Klebsiella ornithinolytica*) reached an A_{578} of about 0.1 in both replicates.

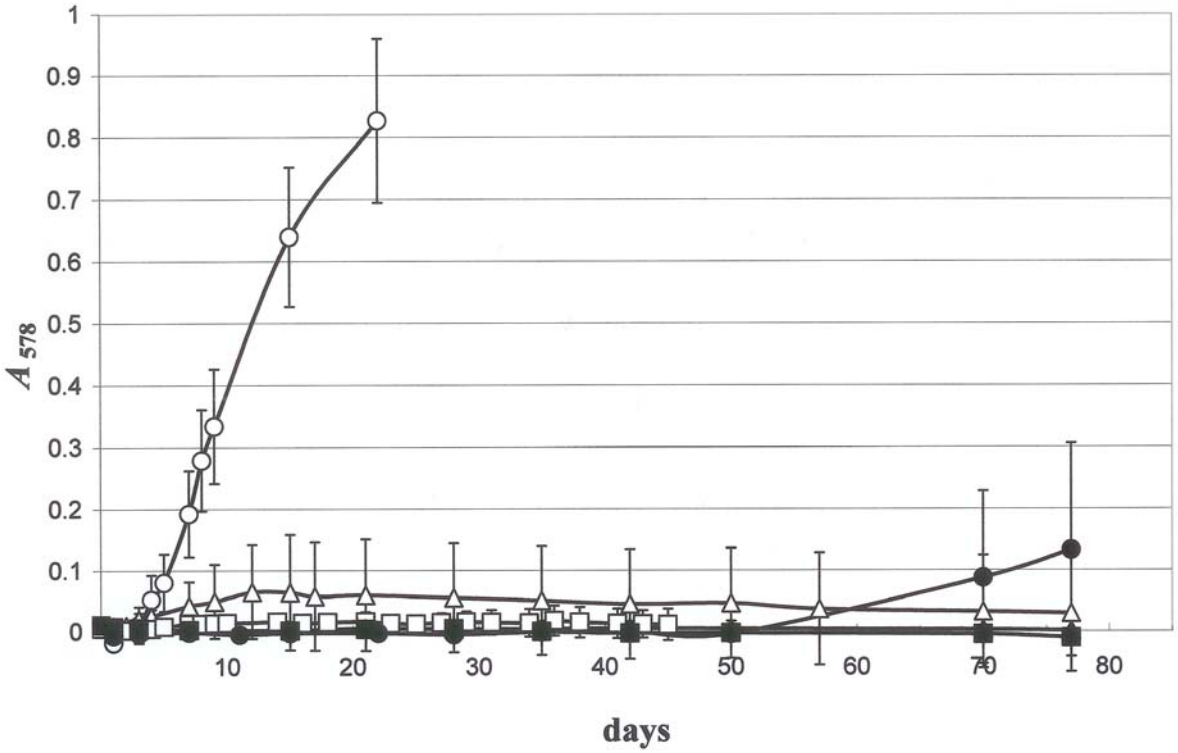

Figure 2. Growth tests of Enterobacteriaceae in beers at different conditions. Each data point is the average value of 28 strains measured in duplicates. The curves represented values obtained for: Non-alcoholic beer: aerob (open circles), anaerob (open triangles), anaerob with 0.2% of glucose (open squares); beer: aerob (closed circles), anaerob (closed triangles), anaerob with 0.2% of glucose (closed squares).

Verification of the re-isolated strains. The increase of the A_{578} after 60 days in beer was the reason for plating 100 µl of the beer on TSA medium after 14 weeks. Indeed, there were colonies grown from 11 different samples out of the 28 strains tested. All 11 strains belonged to the genera *Enterobacter* or *Klebsiella*. The identity of ten re-isolated strains was

confirmed by using the API 20 E kit. They had between none and maximal two diverging API test results comparing their profiles before and afterwards the incubation in beer, only one strain showed different results for four reactions.

Discussion

The isolation of Enterobacteriaceae from 11 different sample sites characterized them as constituent members of the microbial communities in breweries. However, their proportion was low, as revealed by FISH investigations (Chapter 1 and 2). The strains were assigned to five genera but the identification by sequencing and by API 20 E resulted in different genera for 13 out of 26 comparable results. One reason for the divergence of the results obtained by these methods was the difference between the partial 16S rRNA gene sequences and the next relative type strains. In most cases, they were not identical. Nevertheless, the type strains were given because of their reliable identity. This may not be true for every isolate which 16S rRNA gene sequence has been published, in particular for the closely related Enterobacteriaceae. In addition, the identification by sequencing was hampered by the low variability of the 16S rRNA gene sequences (Fig. 1). The maximal difference of the obtained 16S rRNA genes was found for MTTV16 and MQ50, which had only 14 diverging nucleotides out of 505 (2.8% difference). The comparison of 1434 nucleotides of the 16S rRNA genes of *Raoultella planticola* ATCC 33558^T and *E. coli* ATCC 11775^T revealed a difference of 5.2%.

The best match for nine sequences was *Shigella flexneri* ATCC 29903^T and *Escherichia coli* ATCC 11775^T (Table 1). This is in accordance to the close relationship of these genera. Brenner *et al.* reported already in 1973 the reassociation of the chromosomal DNA of both species to be close to 100%. However, whereas *E. coli* strains are usually able to ferment many sugars, *Shigella* spp. ferment only a few sugars (Germani and Sansonetti 2001). In addition, *Shigella flexneri* is able to form acetoin and to use citrate and *E. coli* is not. These examples justified the preference of the API identifications, which are based on 21 different reactions. An disadvantage of the API 20 E test may have been the database, which is limited to 70 Enterobacteriaceae and 32 other Gram-negative species. Indeed, six isolates had the highest sequence similarity to type strains which were not included in the database. The species *Enterobacter agglomerans, Ent. dissolvens, Ent. kobei* and *Raoultella planticola/Klebsiella trevesanii* were therefore not detectable by the API 20 E test. Nevertheless, the API 20 E demonstrated the absence of *S. flexneri* or *E. coli* strains in the breweries. All isolated strains belonged to genera with also free-living members. The presence of *Ent. cloacae* and *Klebsiella pneumoniae* in brewery biofilms was confirmed. Members of the genus *Hafnia* were not isolated.

All cultivated Enterobacteriaceae which have been tested in NBB, a selective agent for beer-spoiling bacteria, were able to proliferate in it (Table 2, Chapter 1). In addition, some were considered to cause turbidity in beer (data not shown), this spoiling potential was evaluated. The test conditions imitated a closed bottle of beer (anaerobic conditions) or remained and returned beer (aerobic conditions). Turbidity was detected for all strains in non-alcoholic beer with access of oxygen and for some strains in beer only after 60 days. The content of 4.8% (v/v) of ethanol is the most likely explanation for the inhibition of growth. Probably the ethanol evaporated during this long period of time and the situation became similar to the tests with non-alcoholic beer. However, the content of ethanol was not the only growth suppressing parameter. There was also no growth in non-alcoholic beer under anaerobic conditions. The pH-value was stable after five months, it was 4.41 for beer in the aerobic tubes and 4.38 in the closed tubes, respectively. An explanation may have been the absence of a fermentable substrate. However, the addition of 0.2% (w/v) of glucose as an additional energy source had no growth promoting effect under anaerobic conditions for non-alcoholic beer and for beer. Comprising the data (Table 2), the content of 4.8% (v/v) of ethanol suppressed growth and the access of oxygen had a major impact on the growth rate. If there was access of oxygen, respiration has been possible. The energy yield is much higher compared to the one of fermentation and to mulitply in beer may need a lot of energy. For instance, Sakamoto *et al.* (2002) described an overexpressed H^+-ATPase in *Lactobacillus brevis* cells exposed to hop compunds, for pumping of protons released from the hop compounds. In accordance with this, hop-resistant cells of *L. brevis* generated more ATP than hop-sensitive cells (Simpson and Fernandez 1994). The Enterobacteriaceae may have similar ATP depending resistance mechanisms.

Table 2. Summary of the growth tests in beer with different parameters.

	Varied parameter[a]			
	Exchange of air	4.8% (v/v) of ethanol	0.2% (w/v) of glucose	pH-value
Turbidity in non-alcoholic beer	+	-	-	4.54
No growth in non-alcoholic beer	-	-	-	4.52
No growth in non-alcoholic beer	-	-	+	n.d.
Slight turbidity in beer after 70 days	+	+	-	4.41
No growth in beer	-	+	-	4.38
No growth in beer	-	+	+	n.d.

[a] +/- indicates the presence or absence of the parameter.

Neither the strains with the highest A_{578} in non-alcoholic beer nor the ones with increased turbidity in beer after 70 days were found to be restricted to a single genus (data not shown). Only the strains which survived for 14 weeks in beer have been exclusively members of the genera *Klebsiella* and *Enterobacter*. *Klebsiella oxytoca* has been already described to

survive in beer (Lawrence 1988) for the other *Klebsiella* species and especially for the *Enterobacter* species it has not been reported so far. The eleven strains which survived in beer were also distributed on the plant as they were isolated from six different locations. Finally, the Enterobacteriaceae have been present in breweries but they were not able to spoil filled beer.

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Chapter 8

Occurrence and Quantitative Importance of Acetic Acid Bacteria in Brewery Biofilms

Abstract

The acetic acid bacteria are supposed to be numerous and functional important brewery biofilm organisms. Therefore, their presence was investigated by cultivation using five different brewery samples. Only one *Acetobacter* strain was isolated. This is in accordance with data obtained for lipid profiles of 78 biofilm samples. Only two of them had the fatty acid 16:0 2OH, which is characteristic for acetic acid bacteria. Direct microscopic detection of acetic acid bacteria by a fluorescently labelled rRNA directed probe identified them in only five out of 37 investigated biofilm samples. Concluding, they were indeed present in some biofilms but they were not obligate members of the microbial communities.

Introduction

The acetic acid bacteria such as *Acetobacter* and *Gluconobacter* species have been well-known beer-spoiling bacteria in the past. Nowadays, they do not represent a problem for filled beer any more. This is due to the drastically reduced oxygen content in filled beer by the improved brewing technology (Sakamoto and Konings 2003). However, the acetic acid bacteria are still considered as important brewery organisms because of their multiple physiological properties. They can provide a matrix as well as anaerobic and acidic microenvironments for beer-spoiling bacteria, like Lactobacillaceae (Back 1994). This predestinated them as accompanying organisms for beer-spoiling bacteria and even as indicator organisms. For these reasons, the occurrence of acetic acid bacteria has been examined by isolation techniques, detection of lipid markers and fluorescence *in situ* hybridization (FISH).

Materials and Methods

Isolation and identification. The media DSM 254 and DSM 105 (http://www.dsmz.de/media) were used for the isolation of acetic acid bacteria from samples of the brewery A, the infeed and discharge conveyor, the crowner die and the inner part of the discharge star wheel. Furthermore, a sample from the inner part of a pipe of the conveyor belt construction from brewery B was inoculated in beer. This enrichment was used for cultivation of beer-spoiling bacteria on the DSM 232 medium, which is recommended for the cultivation of *Pectinatus* spp. by DSMZ, Braunschweig, Germany. The DSM 232 agar plates were incubated at 25°C under anaerobic conditions. In addition, two enrichments of acetic acid bacteria from brewery B in liquid wort medium (10 ml)

supplemented with ethanol (150 µl) were provided. The strains E3E and E4E were isolated from these enrichments on DSM 105 medium. A part of the 16S rRNA gene of isolates was sequenced as described in Chapter 1. The obtained sequences were compared with public databases (DDBJ, EMBL, GenBank) using the BLASTN 2.2.9 program (Altschul *et al.* 1997).

Fatty acid analyses. The fatty acid analysis was performed as described in Chapter 1. Pure cultures were grown on DSM 254 medium (strain Bt31a) or on DSM 105 medium (strains E3E and E4E). The strains *Acetobacter pasteurianus* DSM 3509^T and *Gluconobacter oxydans* subsp. *oxydans* DSM 3503^T were purchased from the DSMZ and cultivated on the recommended media (DSMZ), respectively. The biofilm samples analyzed are given in Table 1 in Chapter 3. The nomenclature of the fatty acids is described in Chapter 3.

Whole cell hybridization of sample material. FISH was performed as described in Chapter 1, at least 400 DAPI stained cells were enumerated in duplicates for every sample. A combination of the probes EUB338 (Amann *et al*. 1990), EUB338II and EUB338III (Daims *et al*. 1999) were applied (designated: EUB338-III) for the detection of Bacteria. The FISH probe AG1427 was designed for cells of the genera *Acetobacter* and *Gluconobacter* (Neef 1997). It is specific for members of the Acetobacteraceae as verified by the "Probe Match" tool of the Ribosomal Database Project (Cole *et al*. 2003). Thirty-seven biofilm samples were investigated with the probes EUB338-III and probe AG1427. Some of them were also investigated with the negative control probe NON338 (Wallner *et al*. 1993). The samples are listed in Fig. 1 and details of them are given in Table 1 in Chapter 3. In addition, the samples B25 and B26 were taken from a cable of a labeler and from a centering tulip of a labeler of brewery B.

Results and Discussion

Only one of the isolates was a member of the Acetobacteraceae. The strain Bt31a originated from DSM 232 medium. Its partial sequence of the 16S rRNA gene (426 nucleotides) was identical to that one of *Acetobacter pasteurianus* subsp. *lovaniensis* LMG 1617^T. In addition, the strains E3E and E4E were affiliated to *Acetobacter cerevisiae* LMG 1625^T with 691 identical of 692 compared nucleotides and an identity of 642 nucleotides, respectively.

The fatty acid composition of reference strains and isolated strains of acetic acid bacteria are listed in Table 1. Their main fatty acid was 18:1 *cis*11. In addition, they contained 16:0 2OH in a proportion of 2.8% up to 7.6 %.

Table 1. Fatty acid profiles of acetic acid bacteria. The values are percentages of the fatty acids compared to the complete lipid profile. Only fatty acids with a proportion higher than 1% are listed.

	Acetobacter pasteurianus DSM 3509^T	*Gluconobacter oxydans* DSM 3503^T	Strain Bt31a	Strain E3E	Strain E4E
14:0	4.1		2.9		
14:0 2OH	7.9			2.0	
14:0 3OH				1.7	
16:0	16.0	12.2	10.6	12.1	
17:1 *cis*11				1.5	1.9
17:0				3.0	4.7
16:0 2OH	5.2	7.6	5.2	2.8	3.3
16:0 3OH	3.7		1.3	1.3	1.4
18:1 *cis*11	55.1	71.9	68.1	62.7	53.7
18:1 *trans*11				5.2	4.8
18:0		4.2	3.9	1.0	1.6
19:0 cyclo11-12	8.1	4.1	1.2	1.7	5.7
19:0 cyclo 2OH			3.6	2.7	4.6

The lipid profiles of 78 biofilm samples have been given in Chapter 3 (Table 2). Only sample a47 and sample a50 had detectable amounts of 16:0 2OH in proportions of 0.2% and 0.6%, respectively. The 18:1 *cis*11 was present in these samples in a proportion of 7.1% and 2.8%, respectively.

The FISH data obtained with probes EUB338-III and probe AG1427 for 26 biofilm samples of brewery A and 11 of brewery B samples are given in Fig. 1. The biofilm sample A8 had 62.9% signals for the probe EUB338 and probe AG1427 detected 37.8% of all DAPI stained cells, respectively. This was by far the highest detection rate of acetic acid bacteria for samples from brewery A. The sample location is shown in Fig. 2. Eight samples revealed probe AG1427 conferred signals in the range of 0.3% to 0.6%. These values are similar to the percentages obtained for the probe NON338, which were between 0% and 0.4%. The other biofilm samples showed no signals for the probe AG1427.

Four samples of brewery B had detectable amounts of acetic acid bacteria, out of 11 investigated ones. The highest proportions of probe AG1427 signals were found for the two engine compartment samples B9 (Fig. 2) and B10 with 12.0% and 28.0% of all DAPI stained cells, respectively. In accordance with their higher proportions of probe AG1427 signals, the lipid profiles of samples A8, B9 and B10 had proportions of 3.9%, 3.0% and 4.5% of 18:1 *cis*11. However, the 16:0 2OH fatty acid was not detected.

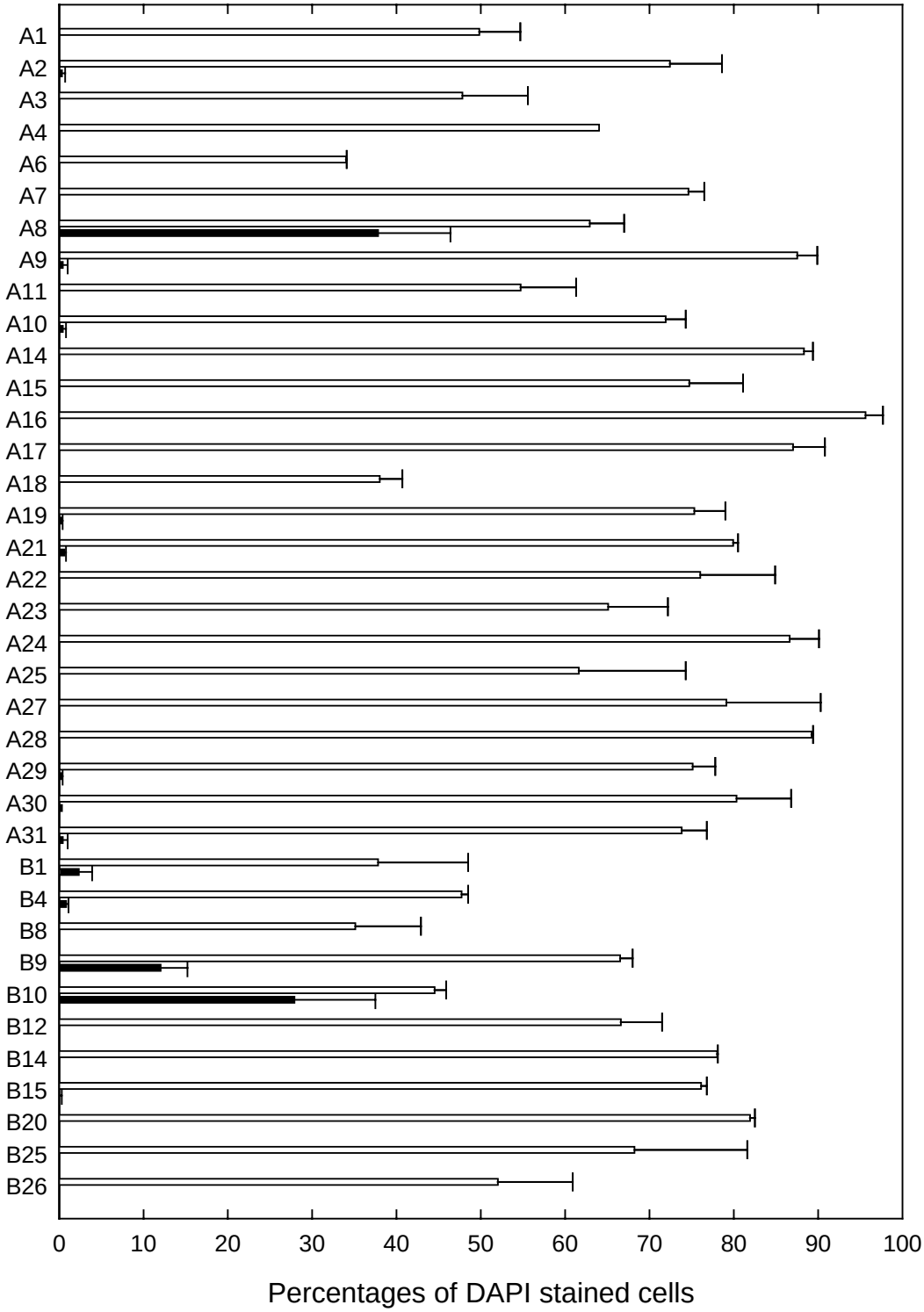

Figure 1. Percentages of FISH signals for Bacteria (white bars; probes EUB338-III) and the acetic acid bacteria (black bars; probe AG1427) of all DAPI stained cells. The samples are abbreviated as described in Chapter 3 (Table 1).

Two more samples of brewery B revealed minor proportions of probe AG1427 signals. Sample B1 originated from a screw of the infeed star wheel and sample B4 from a plastic part between the infeed and discharge star wheel, respectively. All biofilms harboring acetic acid bacteria (samples: A8, B1, B4, B9 and B10) were hardly accessible. This means that some parts of the bottling plants had to be demounted for taking the samples but this was also true for most of the samples analyzed. There was no conclusive explanation for the presence of acetic acid bacteria at these locations.

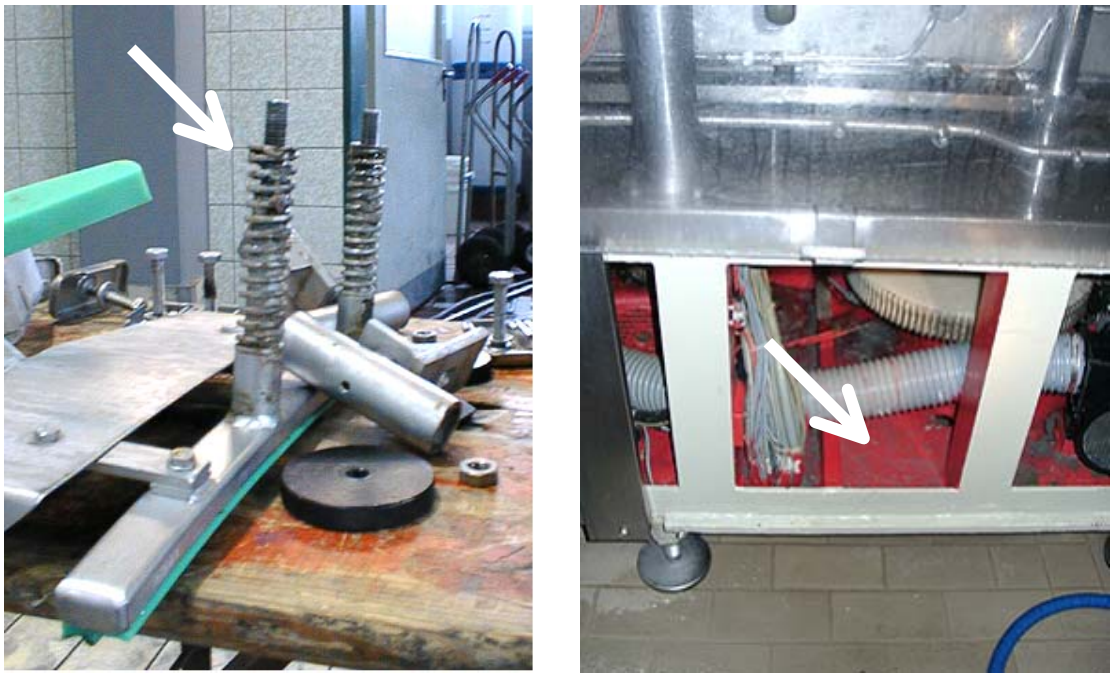

Figure 2. Two parts of the bottling plants with high proportions of acetic acid bacteria in the biofilms. The left picture was taken from the demounted setting screw for the discharge of a control frame (sample A8), the brown coatings (arrow) from the screw and from the coils of the spring were analyzed. The right picture shows the opened engine compartment of a bottling plant (sample B9). The acetic acid bacteria were found in the viscous grey compound from the bottom (arrow).

All of this biofilms investgated were visible ones (Fig. 2). In addition, the initial biofilm forming microorganisms were subject of another study, which identified no acetic acid bacteria by cultivation techniques and by FISH (Winkler 2003). Concluding, the acetic acid bacteria were no part of the microbial communities of the majority of the analyzed brewery biofilms.

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Chapter 9

Fluorescence *in Situ* Detection and Isolation of Anaerobic Beer-Spoiling Bacteria

Abstract

The presence of *Pectinatus* and *Megasphaera* cells in brewery biofilms was investigated by cultivation techniques and fluorescence *in situ* hybridization (FISH). Two areas of a bottling plant were found to harbour these beer-spoiling bacteria, the outlet region and the inner part of the filler of brewery A. They constituted small proportions (0% FISH signals) of the microbial communities. Some strains were isolated which partial 16S rRNA gene sequences differed slightly (99.4% similarity) from published *Pectinatus* sequences. *Pectinatus* cells were also present in the filling hall of brewery B, where they represented high proportions in two biofilm communities. However, *Pectinatus* cells were absent in most of the biofilms of both breweries analyzed by FISH. A new probe for the detection of *Megasphaera* cells was designed and evaluated. Several taxa were identified as accompanying organisms of strictly anaerobic beer-spoiling bacteria but none of them clearly dominated the communities. As a rare event, cells of *Pectinatus cerevisiiphilus* Pe-0104 and *Pectinatus frisingensis* DSM 20465 were able to form colonies in the presence of oxygen.

Introduction

The strictly anaerobic beer-spoiling bacteria *Pectinatus* spp. and *Megasphaera cerevisiae* were subject of this study. *Pectinatus* spp. are more important beer-spoiling organisms than *Megasphaera cerevisiae* (Table 1, General Introduction). Therefore this study was mainly focused on this genus, which consists of three species. *P. cerevisiiphilus* was described in 1978 (Lee *et al.* 1978). Very soon, *Pectinatus* sp. was reported to occur in Germany (Back *et al.* 1979), Sweden (Haukeli 1980), Norway (Kirchner *et al.* 1980), Finland (Haikara *et al.* 1981), Japan (Takahashi 1983) and France (Soberka *et al.* 1989). The species *P. frisingensis* and *P. portalensis* were described in 1990 and in 2004, respectively (Schleifer *et al.* 1990; Gonzalez *et al.* 2004). The simultaneous appearance of *Pectinatus* sp. in many countries can be explained by its anaerobic nature. The dissolved oxygen content of beer, as well as the volume of air in the headspace has declined considerably. Moreover, the isolation of *Pectinatus* sp. has been favored by the usage of less specific media and anaerobic incubation conditions for repressing aerobic bacteria (Seidel-Rüfer 1990). Considered as a strictly anaerobic organism, *Pectinatus* can not survive in the presence of oxygen. Nevertheless, *Pectinatus* cells are able to survive and to multiply in breweries maybe because of the presence of protecting organisms (Figure 1, General Introduction).

Beer-spoiling bacteria were expected to be present in brewery biofilms at least in some of them. For investigating the microbial compositions of biofilms, 78 lipid profiles were obtained (Chapter 3) and only one of them revealed the fatty acid 13:0 3OH. This fatty acid

is characteristical for *Pectinatus* spp. (Helander and Haikara 1995). Its rare detection is in accordance with data obtained from isolation studies (Chapter 5) and from 16S rRNA gene clone libraries (Chapter 2), which identified none of these taxa in brewery biofilm communities. In this study, the FISH technique and enrichment cultures were used for their detection. A probe for *Pectinatus* spp. was published (Yasuhara *et al.* 2001) but no probe was available for *Megasphaera* sp., therefore, it has been designed and evaluated in this study.

Materials and Methods

Fluorescence *in situ* hybridization. The target sequence of a probe specific for *Megasphaera* has been determined with the ARB software package using the probe design functions (Ludwig *et al.* 2004). The target sequence was also checked for specificies by using the PROBE_MATCH tool of the Ribosomal Database Projekt (Cole *et al.* 2003). The probe was named M1286 because of the target organism and the target sequence region (nucleotides 1286-1303) numbered according to the *E. coli* 16S rRNA gene (Brosius *et al.* 1978). The sequence of probe M1286 is: 5' CGAACTGGGACTCTGTTT 3'. Cy3 labeled probes were purchased from Thermo Hybaid (Interactiva Division, Ulm, Germany). The probe was hybridized with ethanol fixed cells of *Megasphaera cerevisiae* DSM 20461 at formamide concentrations in a range of 0% to 70% for assessing the optimal stringency conditions. The FISH procedure was performed as described in Chapter 1. Signal intensities of probe signals at different formamide concentrations were acquired using a CCD camera and the image analysis software package KS300 (Zeiss, Oberkochen, Germany). The mean probe conferred signal intensities per pixel of each cell were determined for each formamide concentration. An image analysis routine including shading correction, contrast enhancement, segmentation, filtering, and interactive particle removal was used (Friedrich *et al.* 2003). Background fluorescence was subtracted from the fluorescence intensity values obtained. The same procedure was done with *Megasphaera cerevisiae* DSM 20461 cells hybridized with probe EUB338, which represented the 100% signal intensity (Fig. 1). The next non-target organisms of probe M1286 have two mismatches to the probe sequence, one of these organisms is *Clostridium propionicum* DSM 1682^{T}. The probe P1447 was available for the detection of *Pectinatus* cells (Yasuhara *et al.* 2001). It was applied at 20% of formamide in the hybridization buffer.

Twenty-two biofilm samples of brewery A were analyzed with probe P1447 (samples: A0, A1, A2, A4, A6, A7, A9, A10, A11, A12, A13, A15, A16, A17, A18, A19, A21, A22, A23, A29, A30 and A31) and 14 biofilm samples from brewery B (B1, B4, B5, B9, B10, B11, B12, B14, B15, B16, B18, B20, B25, B26). In addition, six biofilm samples were

investigated with the probe M1286 (samples: A4, A13, A19, A21, A22, A23). Details of the sample locations are given in Chapter 3 (Table 1). In addition, the sample A0 originated from a plastic cladding of the infeed conveyor. The biofilms B25 and B26 were located on a cable of a labeler and on a centering tulip of a labeler.

Further FISH investigations were done using 16 probes within the biofilm samples B14 and B15 (Fig. 2). Details and references of the probes are given in Chapter 1. At least 400 DAPI (4',6-diamidino-2-phenylindole) stained cells were counted in triplicates per probe.

Isolation and identification. The isolation of *Pectinatus* sp. was performed with biofilm samples from brewery A taken in 2002. These biofilms were used for enrichments in beer and some of them showed a typical behavior for *Pectinatus* spp. in liquid medium. First, the beer got turbid in about one week then it became clear and a sediment developed. If the tube was turned around a slowly falling smear was formed. This notice was considered as a hint for the presence of *Pectinatus* cells in enrichments. The SMMP medium (selective medium for *Megasphaera* and *Pectinatus*, Lee 1994) was used for subcultures of the turbid enrichments. SMMP medium consists to 85% of beer supplemented with yeast extract, peptone and lactate. Additionally, it contained cycloheximide, crystal violet and sodium fusidate. The turbid enrichments in SMMP medium and the biofilms samples, which were originally used as inoculum for the enrichments, were investigated by FISH with probes P1447 and M1286 (Table 1). Additionally, 100 µl of the enrichments were plated on DSM 232 medium (http://www.dsmz.de/media) and incubated in an anaerobic jar (GasPak^{TM}, BD Diagnotic Systems, Heidelberg, Germany) at 30°C. DSM 232 medium corresponded to MRS medium (DeMan-Rogosa-Sharpe, Difco Laboratories, Detroit, USA), a medium for the cultivation of lactic acid bacteria, supplemented with 0.05% cysteine-hydrochloride. Grown colonies were picked and restreaked until pure cultures were obtained. The DNA of four isolates was extracted and the 16S rRNA gene was amplified and sequenced as described in Chapter 1. The phylogenetic analysis was performed with the ARB software package (Ludwig *et al.* 2004).

Results

Evaluation of FISH probe M1286. One of the most similar non-target sequence of the probe M1286 was the 16S rRNA gene of *Clostridium propionicum* DSM 1682^{T} with two central mismatches. The *Clostridium* cells gave almost no signals at a concentration of 10% formamide in the hybridization buffer and the fluorescence intensities of *Megasphaera* cells were still bright (Fig. 1). Hence, all subsequent hybridizations using M1286 were carried out applying a stringency of 10% formamide in the hybridization buffer.

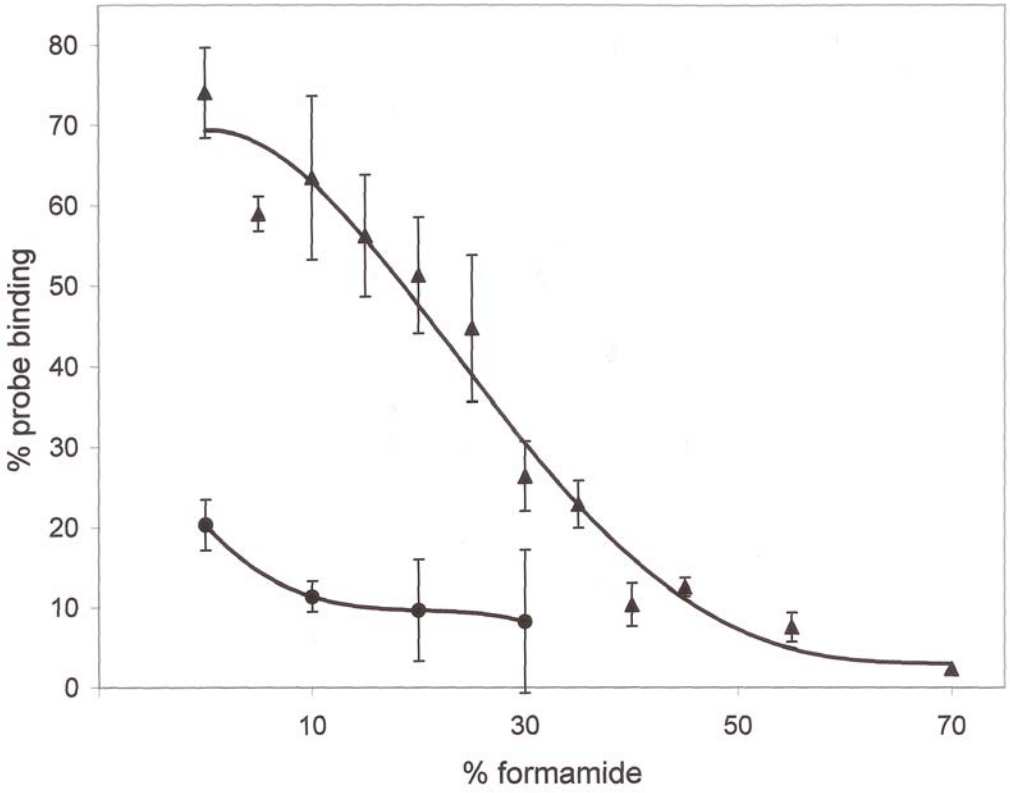

Figure 1. Quantification of binding of probe M1286 relative to that of EUB338 measured as fluorescence intensity. Different stringencies were obtained by varying the concentration of formamide in the hybridization buffer. Error bars indicate standard deviations. Symbols: triangles, *Megasphaera cerevisiae* DSM 20461; squares, *Clostridium propionicum* DSM 1682^{T}.

Signals of probe P1447 in brewery biofilm samples. The *Pectinatus* specific probe P1447 was used in 22 biofilm samples from brewery A and six biofilm communities were investigated with probe M1286. No target cells of these probes were detected. In addition, 14 biofilm samples from brewery B were analyzed with probe P1447. Only two biofilms contained *Pectinatus* cells. Their proportions were 15.7% and 29.7% of all DAPI stained cells in the paraformaldehyde fixed samples B14 and B15, respectively. Both sample locations were parts of the conveyor constructions (Fig. 2).

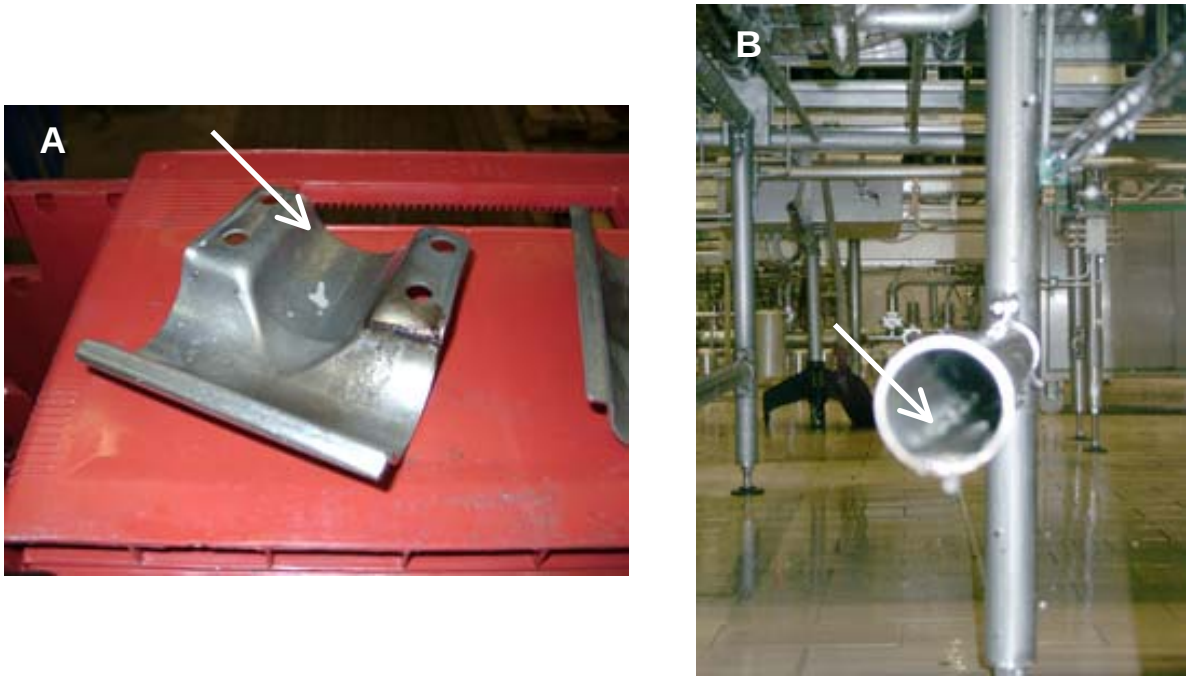

Figure 2. Picture A shows a conveyor belt support (sample B14). *Pectinatus* cells were detected in the solid brown coating (arrow). Picture B was taken from some pipes, on which the conveyors are located on (sample B15). There was a liquid, muddy material inside a pipe containing *Pectinatus* cells (arrow).

Enrichment cultures and isolation of *Pectinatus* sp. Fourty-five biofilms were inoculated in beer and 11 of them were supposed to contain *Pectinatus* cells because of the typical sediment. Hundred µl of these 11 samples were transferred to SMMP medium and six of them caused turbitity in it (Table 1). These biofilms have been mainly located at the inner part of the filler and at the outlet area of the bottling plant of brewery A. The enrichments were investigated by FISH with the probes P1447 and M1286 and five of them contained *Pectinatus* cells. *Megasphaera* cells were also present in five enrichments. Consequently, the original biofilm samples were analyzed by FISH for determining the proportions of *Pectinatus* and *Megasphaera* cells (Table 1). The FISH signals for the probes EUB338-III (Amann *et al.* 1990; Daims *et al.* 1999) were in a range between 68.5% and 96.7% of all DAPI stained cells, but no *Pectinatus* and almost no *Megasphaera* cells were detected.

The enrichment cultures in SMMP medium were also used for isolation studies. The four strains IIRo15, IIRo24.1, IIRo25 and IIRo28 were obtained from four different biofilm enrichments. The partial 16S rRNA gene sequences of these strains were identical for at least 613 compared nucleotides. The sequence of *Pectinatus* sp. IIRo28 was submitted to the EMBL database (accession number: AJ830008). The species with the most similar (99.4%) 16S rRNA gene sequence was *P. portalensis* (Fig. 3).

Table 1. FISH signals of probes P1447 and M1286 in original brewery biofilms and in enrichments of these biofilms in SMMP medium. The values are percentages of DAPI stained cells.

Sample locations	Probe signals (%) in original biofilm samples			Probe signals in enrichments[a]	
	EUB338-III	P1447	M1286	P1447	M1286
Drawer at the outlet, left side (sample: a47)	96.1	0	0	+	+
Drawer at the outlet, right side (sample: a48)	96.7	0	0	+	-
From a wire, down inside the filler (sample: a50)	68.5	0	0	+	+
From a wire, down inside the filler (sample: a51)	93.2	0	0.2	+	+
Down, inside the filler (sample: a52)	93.9	0	0.5	-	+
Beneath the conveyor at the outlet (sample: a60)	77.7	0	0	+	+

[a] + indicated the presence of probe P1447 or M1286 signals, – their absence, respectively. DAPI stained cells and probe signals were not enumerated.

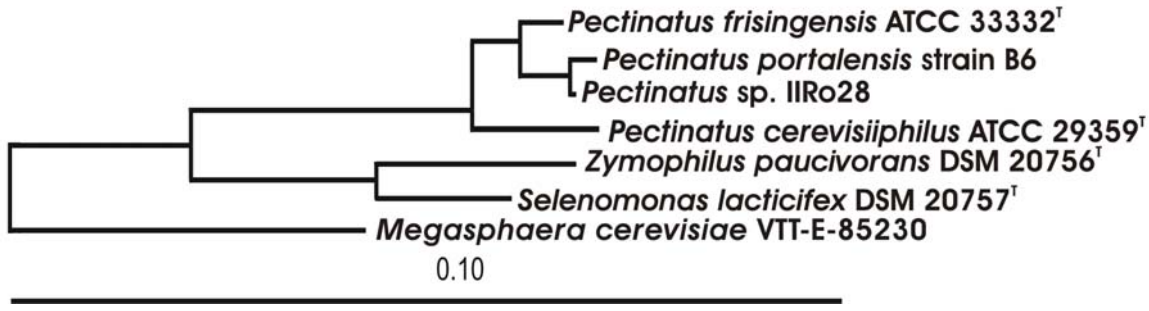

Figure 3. Phylogenetic tree showing the affiliations of 16S rRNA genes of the isolate *Pectinatus* sp. IIRo28 and described *Pectinatus* species. As members of the Acidaminococcaceae, sequences of *Megasphaera cerevisiae*, *Selenomonas lacticifex* and *Zymophilus paucivorans* were included. The dendrogram was calculated by maximum likelihood analysis with the 16S rRNA sequences of all organisms shown, except the one of strain *Pectinatus* sp. IIRo28. Sequences of five Deltaproteobacteria were used as outgroup. Finally, the partial sequence of strain IIRo28 was inserted. The scale bar indicates 10% estimated sequence divergence.

Accompanying organisms of *Pectinatus* cells. The biofilm samples B14 and B15 revealed high proportions of *Pectinatus* cells. Therefore, they were investigated with FISH probes to determine other abundant groups (Fig. 4).

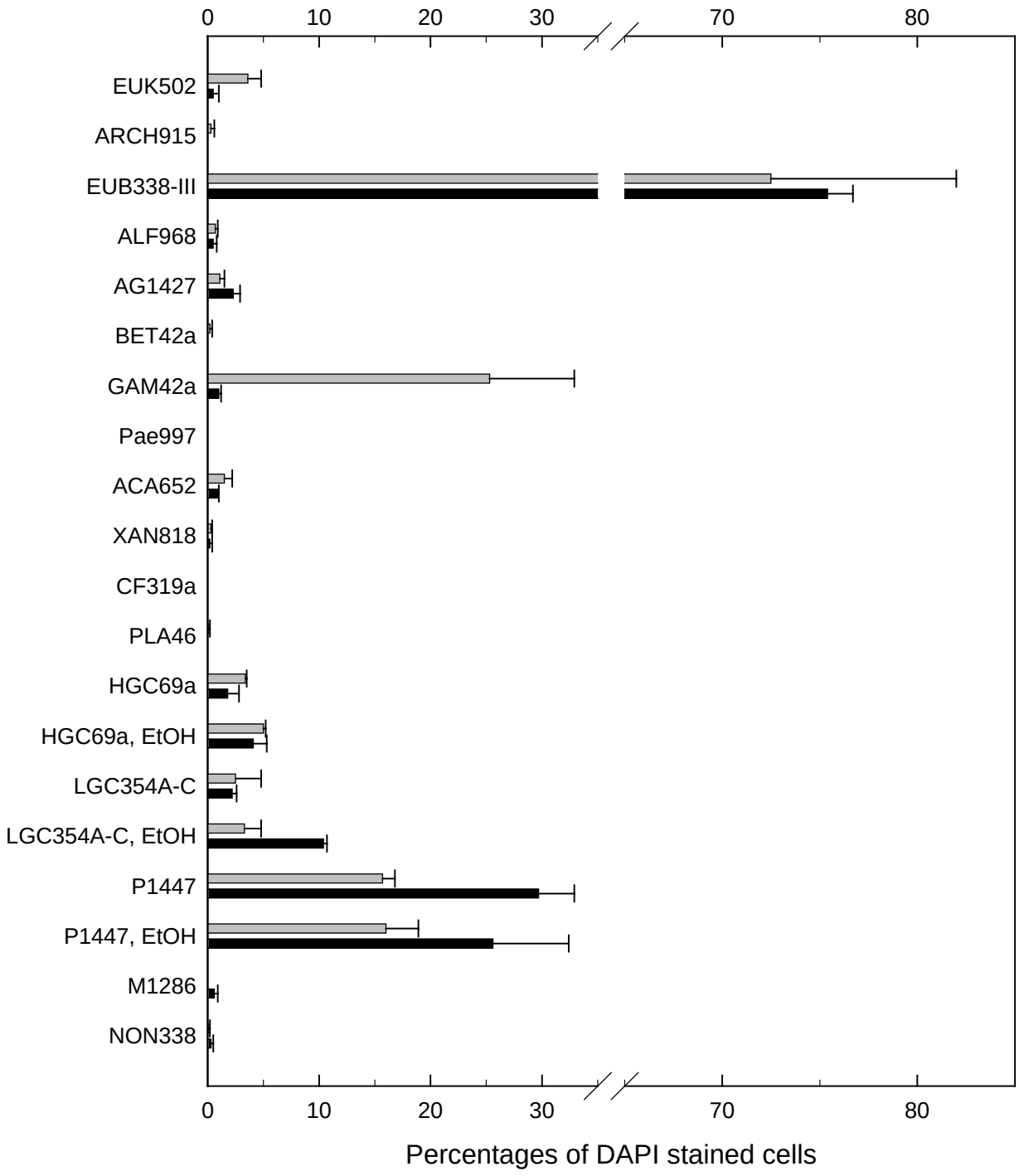

Figure 4. FISH investigations for determining accompanying organisms of *Pectinatus* cells in the samples B14 (gray bars) and sample B15 (black bars). Percentages of probe signals are shown. The target organisms of the probes have been given in Chapter 1. Most samples were fixed with paraformaldehyde, values for ethanol fixed samples are marked with EtOH.

Probes for the taxa Archaea, Alpha- and Betaproteobacteria, Xanthomonadaceae, Cytophaga/Flavobacteria, Planctomycetales and for *Megasphaera* detected less than 1% of all DAPI stained cells (Fig. 4). The Eukarya, and Actinobacteria were found in low proportions, as well as probe signals for the Firmicutes, except for the ethanol fixed sample B15, where they constituted about 10% of all DAPI stained cells. Next to *Pectinatus* cells, the Gammaproteobacteria were the most abundant taxa in sample B14. No cells of *Pseudomonas aeruginosa* and only small proportions of *Acinetobacter* cells were detected. The Gammaproteobacteria were present in a minor proportion in sample B15.

Oxygen tolerance of *Pectinatus*. *Pectinatus* cells were cultivated in an anaerobic jar on DSM 232 medium and harvested for FISH experiments. The remained biomass developed some new colonies after a few days exposed to the air (Fig. 5). This was found for *P. cerevisiiphilus* Pe-0104 and *P. frisingensis* DSM 20465. The cells did not only survived under atmospheric oxygen conditions, they even multiplied. The tower shaped colonies were analyzed by FISH and all DAPI stained cells were detected with probe P1447.

Figure 5. Three colonies of *P. frisingensis* DSM 20465 grown at atmospheric oxygen conditions. The colonies were about 2 mm in width and up to 4 mm high. A one Euro cent coin was placed in the background for scale.

Discussion

The FISH investigations revealed almost no *Pectinatus* and *Megasphaera* cells in biofilms from brewery A. This is in line with data obtained for biofilms of this brewery by cloning techniques, lipid profiling and cultivation studies (Chapter 2, 3 and 5). Nevertheless, they were present in brewery A as revealed by enrichment cultures. The outlet area and the inner part of the filler are in extensive contact to overfoamed beer but this did not select for higher proportions of *Pectinatus* or *Megasphaera* cells (Table 1).

High proportions of *Pectinatus* cells were identified in two biofilms from brewery B. Cultivation studies were performed with both samples, however, no *Pectinatus* strains were isolated. As members of the Firmicutes, the percentages of the probes LGC354A-C (Meier *et al*. 1999) should be as high as the probe P1447 conferred signals. However, a terminal mismatch is present in the 16S rRNA gene of *P. cerevisiiphilus* for probe P1447 and the sequences of all three described *Pectinatus* species have a central mismatch to probe LGC354B. Indeed, the *in situ* probe P1447 conferred signals for *P. frisingensis* DSM 20465 cells were brighter than for *P. cerevisiiphilus* Pe-0104 cells. The fluorescence signals of probes LGC354A-C for both strains were estimated to be of an weak intensity up to the detection limit of the cells. This pointed to the presence of *P. frisingensis* or *P. portalensis* in the biofilm samples B14 and B15 and it explains the diverging values obtained for the probes P1447 and LGC354A-C (Fig. 4).

Due to the low proportions of *Pectinatus* cells in many biofilms it would be important to find some more abundant indicator organisms. The strictly anaerobic beer-spoiling bacteria are supposed to need the presence of more robust microorganisms, like the acetic acid bacteria (Back 1994). Indeed, the probe for acetic acid bacteria detected a few signals in the *Pectinatus* spp. containing samples but the proportions of *Pectinatus* cells were more than ten times higher (Fig. 4). The Gammaproteobacteria were numerous in sample B14 (25.3% FISH signals), but they were nearly absent in sample B15 (1% FISH signals), which indicated that their presence was not necessary for *Pectinatus* cells. Alternatively, there may be no need of *Pectinatus* cells for accompanying organisms. This possibility was supported by the pure cultures of *Pectinatus* spp. which were grown on solid medium under atmospherical oxygen conditions (Fig. 5). It has to be mentioned that the colonies appeared seldom (up to 12 colonies per agar plate) and only from heavily inoculated cells. Nevertheless, *Pectinatus* was found to be micro-aerotolerant. The sulfate-reducing bacteria were also thought to be strict anaerobes, instead, various oxygen-reducing systems were found for *Desulfovibrio* species (Cypionka 2000). *Pectinatus* cells may have also properties that enable them to cope with oxygen and may explain their ability to reach and to colonize niches in the breweries, including bottled beer.

Interestingly, *Pectinatus* strains were exclusively isolated from breweries or beer except of the recently described species *P. portalensis*, which originated from a waste water treatment plant (Gonzalez *et al.* 2004). Therefore, the natural habitat is still unknown. In this study, *Pectinatus* cells were detected in extremely low proportions in brewery biofilms. However, under certain conditions they can multiply and become important members of the community, like in the conveyor construction samples.

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Contributions to meetings

Vereinigung für Allgemeine und Angewandte Mikrobiologie, Annual Meeting (March 24th – 27th, 2002) Polyphasic analysis of microbial communities in a brewery (oral presentation). Göttingen, Germany.

17th Forum for Applied Biotechnology (September 18th – 19th, 2003) Microbial composition of biofilms in a brewery investigated by a multiple approach including chemotaxonomic and genetic techniques (oral presentation). Gent, Belgium.

Vereinigung für Allgemeine und Angewandte Mikrobiologie, Annual Meeting (March 28th – 31st, 2004) Composition of two 16S rRNA gene clone libraries from brewery biofilms (oral presentation). Braunschweig, Germany.

1st European Conference on Biofilms – Prevention of Microbial Adhesion (March 31st - April 2nd, 2004) Aerobic biofilms as trojan horses for anaerobic product spoiling bacteria? (poster presentation). Osnabrück, Germany.

Further publication

Lipski, A., Timke, M., Altendorf, K. (2003) The microbial texture of bottling plant associated biofilms. European Brewery Convention, Monograph 32 Sanitary Engineering & HACCP, Fachverlag Hans Carl.

Affirmation

Hereby I declare to have prepared the present dissertation autonomously without illegitimate assistance. No other supplemental materials or references have been used than those, which are annotated.

Osnabrück, November 2004

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Curriculum Vitae

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