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Characterization of the complex bacterial communities colonizing biliary stents reveals a host-dependent diversity

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This study provides a comprehensive survey of the spatial and temporal bacterial composition of biliary stent biofilms. The bacterial diversity, distribution and dynamics of 59 biliary and 4 pancreatic stent communities from 40 patients being treated at two different hospitals, which implant stents either simultaneously or consecutively, were characterized by single-strand conformation polymorphism (SSCP) analysis. Fifty-one phylotypes belonging to 5 bacterial phyla and 24 bacterial families were detected across 63 stents. This is a much broader diversity than previously detected through culture-dependent methods, particularly in regard to the diversity of obligate anaerobes. Stent bacterial diversity was patient-dependent and more similar when stents were implanted simultaneously rather than consecutively. Stent bacterial community composition differed between hospitals specifically because of the difference in abundance of *Bifidobacteria*. Co-colonization of *Veillonella* sp., *Streptococcus anginosus* and organisms closely related to *Fusobacterium nucleatum* revealed a potentially important attachment and survival strategy that has yet to be reported in biliary stents. This work reveals a more complete survey of the identities of bacterial species that form biofilms in biliary stents, their co-colonization patterns and the natural variation in species composition between different patients, hospitals and locations along the stent. Consideration of the community composition from individual patients will allow tailoring of prophylactic antibiotic treatments and thus will make the management of stent biofilms more effective.

Introduction

Biliary stents are used to treat jaundice and cholangitis resulting from bile duct obstructions largely caused by malignancies of the pancreas and the hepatobiliary system. However, the positioning of biliary stents results in the disruption of the sphincter of Oddi and microbial contamination through an ascending infection by duodenal biliary reflux (Sung *et al.*, 1992). The major limitation of stent therapy is stent occlusion by a microbial biofilm (Donelli *et al.*, 2007; Nocker *et al.*, 2007), in which the stent lifetime decreases with repeated replacement, being possibly because of earlier

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microbial contamination of a usually sterile biliary system (Matsuda *et al.*, 1991). There has been some effort to identify the microbial species responsible for stent colonization using culture-dependent approaches (Donelli *et al.*, 2007). From work to date, it is thought that these biofilms are predominantly composed of facultative anaerobic Gram-positive *Enterococcus* sp. of the class 'Bacilli', facultative anaerobic Gram-negative *Escherichia sp.* and *Klebsiella* sp. of the class γ-Proteobacteria (Speer *et al.*, 1988; Dowidar *et al.*, 1991; Molinari *et al.*, 1996; Di Rosa *et al.*, 1999; Zhang *et al.*, 2002), and also *Clostridium perfringens* (Dowidar *et al.*, 1991; Leung *et al.*, 2000). However, cultivation methods are labour-intensive and only limited numbers of stents can be intensively analyzed at any given time. In addition, inconsistencies in species compositions between recent studies (Donelli *et al.*, 2007) are most likely because of differences in the cultivation media and the techniques used rather than the true differences in diversity among the different stents.

Moreover, as only a limited fraction of known bacterial species can be cultivated with the currently available knowledge, surveys of bacterial diversity and distribution should rely on culture-independent molecular approaches. Molecular fingerprinting methods, predominantly targeting the 16S rDNA as a phylogenetic marker, have been developed in the recent decade to allow for a rapid, simultaneous and reproducible analysis of multiple samples, providing information on the microbial diversity in complex habitats (Smalla et al., 2007). Single-strand conformation polymorphism (SSCP) is a powerful fingerprinting technique, which takes advantage of the fact that single-stranded DNA (ssDNA) under non-denaturant conditions acquires a secondary conformation of intramolecular loops and foldings because of complementarity of the bases on the same strand. The application was originally conceived to compare amplifications from single templates to quickly identify single-nucleotide polymorphisms in the spanned sequence fragment (Orita et al., 1989) and is still one of the most effective and widely used methods to identify single-nucleotide polymorphisms (Larsen et al., 2001). SSCP was optimized for applications in microbial ecology through the use of a phosphorylated primer, which allows the selective digestion of the phosphorylated strand by lambda exonuclease (Schwieger and Tebbe, 1998). In this study, SSCP analysis of 16S rRNA gene fragments was used to obtain detailed insights into biofilm community structures across 63 different biliary and pancreatic stents belonging to 40 patients at two different hospitals.

By surveying either end of each of the stents, we were able to elucidate (i) whether stent bacterial community composition differs between different hosting hospitals; (ii) whether the composition differs between patients; (iii) whether the composition is more similar between stents implanted simultaneously rather than consecutively; (iv) whether the composition differs between the distal and proximal liver extremes of the stents; and (v) whether there are patterns in stent colonization. Such information will lead to a greater understanding into the mechanisms that determine stent biofilm composition and dynamics, in which more appropriate prophylactic antibiotic treatments can be tailored to prevent biofilm occlusion.

Materials and methods

Biliary and pancreatic stents
Sixty-three stents comprising 59 biliary and 4 pancreatic stents from 40 patients being treated at two different hospitals in Lower Saxony, Germany were obtained. The Surgery Clinic of Braunschweig (herein abbreviated as 'BS') provided 27 stents from 18 patients, among whom 5 patients provided multiple stents, and the Medical Clinic of Salzgit-ter-Lebenstedt (herein abbreviated as 'SZ') provided 36 stents from 22 patients, among whom 10 patients provided multiple stents. The internal biofilm of both the distal and proximal liver ends of each stent were analyzed independently. In addition, for six stents the exterior biofilm was also surveyed. In total, 132 samples were analyzed.

Both the SZ and BS hospitals exchanged stents when occluded, dislocated or histology samples were required. At SZ, several stents (up to 3) were inserted simultaneously into the biliary and/or pancreatic ducts. By contrast, at BS a clogged biliary stent was consecutively replaced with a new stent, resulting in several stents being collected over time. All stents were positioned and removed by endoscopy. Each removed stent was immediately stored at $-20\,^{\circ}C$ until further DNA extraction.

Treatment of removed stents and DNA extraction
Stents were thawed at $4\,^{\circ}C$, swabbed with 70% ethanol and sectioned in longitude under sterile conditions. The inner biofilm was collected by scraping a 3 cm length from both the distal and proximal ends of each stent. The exterior biofilm was removed from the proximal liver end by scraping the outside of the stent. DNA was extracted using the FastDNA Spin Kit (MP Biomedicals, Irvine, CA, USA) for Soil. The amount of double-stranded DNA was determined by staining with PicoGreen fluorescent dye (Invitrogen, Carlsbad, CA, USA).

SSCP analysis
The bacterial diversity was analyzed using SSCP as described earlier (Schwieger and Tebbe, 1998). In brief, a 10 ng template DNA was amplified using Com-1 (forward) (5′-CAGCAGCCGCGGTAATAC-3′) and Com2-Ph (reverse) (5′-CCGTCAATTCCTTTGA GTTT-3′) primers, producing an approximately 410 bp fragment of the eubacterial 16S rDNA enclosing the variable regions IV and V. Double-stranded PCR products were purified with QIAquick (Qiagen, Dusseldorf, Germany). ssDNA was produced by lambda exonuclease digestion, purified as described earlier (Witzig et al., 2006) and quantified by staining with OliGreen fluorescent dye (Invitrogen). For fingerprinting analysis, 100 ng of ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 8 µl. A mixture of ssDNA from four isolates identified as Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis and Agrobacterium radiobacter served as markers. After incubation for 3 min at $95\,^{\circ}C$, the ssDNA samples were stored on ice, loaded onto an MDE gel ($0.6\times$ MDE gel solution; Cambrex BioScience, Charles City, IA, USA), electrophoretically separated at $20\,^{\circ}C$ at 400 V for 16 h on a Macrophor sequencing apparatus (GE Healthcare, Munich, Germany) and silver-stained (Schwieger and Tebbe, 1998). Bands were excised and DNA

recovered as described earlier (Witzig et al., 2006). DNA was reamplified using the Com primers with an annealing temperature of 58 °C, and sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Sequencing trace files were checked for quality and assembled using Sequencher (v.4.0.5, Gene Codes Corporation, Ann Arbor, MI, USA). In a few cases, multiple bands showing $>98.9\%$ sequence identity (up to 4 bases difference) were observed only concurrently, indicating the multiplicity to be either because of multiple conformations of the same ssDNA molecule (Schwieger and Tebbe, 1998; Schmalenberger et al., 2001) or because of highly similar 16S rDNA variants of the same organism. For this reason, sequences showing $>98.9\%$ sequence identity were grouped as 'phylotypes' using MEGA4. Phylogenetic placement of the sequences was performed using the Ribosomal Database Project (RDP-II) Classifier program (Wang et al., 2007) at a confidence threshold of 80%.

Clone libraries
16S rDNA clone libraries were prepared using primers F27 (forward) (5′-AGAGTTTGATCMTGGC TCAG-3′) and R1492 (reverse) 5′-TACGGYTACCTTG TTA-3′) (Lane, 1991). PCR products were purified by using the QIAquick PCR purification kit (Qiagen) and cloned in pGEM-T easy vector system (Promega, Madison, WI, USA). Plasmid inserts were amplified by PCR with vector-specific M13 forward and reverse primers (Sambrook et al., 1989) on randomly selected transformant colonies, which were suspended in sterile water and incubated at 95 °C for 10 min. Amplicons were sequenced as described earlier (Lane, 1991) and the 16S rDNA clone libraries screened for putative chimaera using Mallard (Ashelford et al., 2006) according to the Jukes–Cantor model. Chao-1 richness estimators (Chao, 1987) and rarefaction analyses were used to estimate phylotype richness (Schloss and Handelsman, 2005).

The most closely related 16S rDNA sequences were identified in the RDP-II database using the Seqmatch program against sequences obtained from validly described type strains and isolates. Alignments were performed using ClustalW implemented in MEGA4 (Tamura et al., 2007). Phylogenetic analyses were performed with MEGA4 using the neighbour-joining method with Jukes–Cantor correction and pair wise deletion of gaps/missing data. A total of 100 bootstrap replications were performed to test for branch robustness. Nucleotide sequences reported in this study are available under GenBank accession numbers EU704129-EU704250 and EU915501-EU915504.

Statistical analyses
Non-parametric multivariate statistical analysis was performed using PRIMER (v.6.1.6, PRIMER-E, Plymouth Marine Laboratory, UK) (Clarke, 1993; Clarke and Warwick, 2001). All the following routines were computed on binary (presence/absence) data. A sample-similarity matrix was generated using the Bray–Curtis coefficient by comparing the presence/absence of each of the 51 phylotypes with regard to every pair wise combination of all the 132 samples. Visual comparisons between stent bacterial communities of predefined groups such as hospital, patient and stent-end were explored by ordination using non-metric multidimensional scaling (nMDS) (50 random restarts and 999 iterations). A stress value below 0.2 corresponds to a useful ordination indicating a valuable two-dimensional representation (Clarke and Warwick, 2001). Data are presented in such a way that samples with a similar community composition are closer in spatial proximity on the plot, whereas samples that are more spatially separated on the plot have less species in common. The relative distance between samples on the plot is thus not arbitrary (Clarke, 1993). However, as the nMDS plot is arbitrary oriented, the axes are usually not presented (Clarke, 1993). Analysis of similarity (ANOSIM) was used to test for statistically significant differences in bacterial communities between predefined groups using 999 permutations. The predefined groups consisted of samples representing different hospitals, patients or stent-ends. Groups of stents are considered significantly different if the P-value falls <0.05. The accompanying R statistic measures the degree of separation between groups and ranges from -1 to 1, in which the higher its value (closer to 1) the more distinct the groups (Clarke and Warwick, 2001). A global R statistic and P-value first report whether there are any overall differences in the bacterial communities between groups. If there are, then the R statistic and P-value are reported for each pair wise comparison between each pair of groups. If differences were found between groups, similarity percentage analysis (SIMPER) was used to compute which SSCP phylotypes were responsible for such differences. Lastly, multivariate dispersion analysis was used to calculate the closeness of grouping among samples originating from the same patient. A low dispersion index indicates low within-group heterogeneity (Clarke and Warwick, 2001).

Nonrandom associations between pairs of phylotypes were assessed using the PAIRS program (Ulrich, 2008). Only relationships between phylotypes that were present in at least 10% of samples were evaluated. Random matrices for generating standardized scores (C-score) (Stone and Roberts, 1990) and significance levels (P-values) were obtained using the fixed row (phylotypes)—fixed column (stent samples) constraints algorithm and 100 random matrices were computed. Significant species under-dispersion or over-dispersion results in Z-transformed scores (observed C-score—expected C-score/s.d.) above 1.96 or below -1.96 (at

the 5% error level) (Ulrich and Zalewski, 2006). False detection error rates were corrected for by a sequential Bonferroni correction (BY criterion) (Benjamini and Yekutieli, 2001).

Results

Stent bacterial diversity

Single-strand conformation polymorphism analysis of 16S rDNA gene fragments of 59 biliary and 4 pancreatic stent bacterial communities detected 51 bacterial phylotypes belonging to 5 bacterial phyla and 24 families (see Figure 1 and Table 1 as well as Supplementary Figure 1). *Firmicutes* and *Proteobacteria* were represented by 27 and 13 of the 51 phylotypes, respectively, whereas the remaining 11 phylotypes belonged to the phyla *Actinobacteria*, *Fusobacteria* and *Bacteroidetes* (Figure 1).

The most frequent occurring phylotypes across all 132 samples were those related to *Enterococcus* sp. (phylotype 34, observed in 66% of samples, see Figure 1), *Veillonella* sp. (phylotype 35, 62% of samples), *Fusobacterium sp.* (phylotype 40, 52%) *Bifidobacterium sp.* (phylotype 23, 50%) and *Streptococcus anginosus* (phylotype 31, 48%) which is in contrast to culture-dependent studies, in which only *Enterococcus* sp. *and Streptococcus* sp. are abundant (Speer *et al.*, 1988; Dowidar *et al.*, 1991; Molinari *et al.*, 1996; Di Rosa *et al.*, 1999; Zhang *et al.*, 2003; Cole *et al.*, 2007).

To reveal any fine-scale diversity, a representative stent, in which four highly abundant phylotypes had been detected, was analyzed through clone library sequencing. The 60 almost complete 16S rDNA sequences analyzed (33 sequences derived from the proximal end and 27 sequences from the distal end) covered the majority of the expected diversity of the stent, as indicated by rarefaction analysis (data not shown) and the Chao-1 richness indicator, which showed that the community consisted of 13.5 (12.2, 24.5; 95% confidence intervals) OTUs out of which 12 had been sampled (see Figure 2). Both SSCP and clone library analysis indicated bacteria related to *S. anginosus* to be predominant at the distal liver end, those related to *F. magna* to be present in high abundance at the proximal liver end and bacteria related to *Veillonella* sp., *Enterococcus* sp. or *Enterobacteriaceae* to be abundant at both extremes of the stent (see Figure 2). Sequences of the 16S rDNA fragments identified by SSCP fingerprinting to be predominant were identical to those obtained from the clone libraries in the majority of the respective clone sequences. However, closely related 16S rDNA sequences of the major clusters shown in Figure 2 differed by up to 6, 10, 11 and 8 bases, respectively, significantly exceeding possible PCR errors, indicating the stent to be colonized not by single clones but by closely related, genotypically distinct *Veillonella*, *Steptococcus*, *Finegoldia* and *Enterococcus* strains.

Enterobacteriaceae sequences indicated, as expected, the presence of members of different genera, which could not be separated by SSCP analysis.

### Co-aggregation of species

Analysis of phylotype distribution across stent communities indicates that they were not completely randomly distributed, but that there were statistically significant co-occurrences of certain pairs of phylotypes, whereas other phylotypes seemingly avoided each other (Table 2). Of the 66 pairs of phylotypes analyzed, five pairs showed a significant co-occurrence ($P < 0.05$), indicating a positive interaction between *Fusobacterium* sp., *Veillonella* sp. and *S. anginosus* in stent communities. Three pairs of phylotypes showed significant avoidance (see Table 2).

Comparing bacterial diversity between hospitals and stent ends

Members of five bacterial families (*Enterobacteriaceae*, *Streptococcaceae*, *Enterococcaceae*, *Veillonellaceae* and *Fusobacteriaceae*) were observed in ≥50% of stent communities, independent of the location inside the stent or the hospital (Table 1). However, there was a striking difference between the stent communities of both hospitals, with a higher abundance of organisms related to *Bifidobacterium animalis* (the only *Bifidobacteriaceae* detected) in SZ stents being observed in >80% of stents, whereas such a band was observed only in approximately 20% of stent communities from BS. Also *Lactobacillaceae* were more abundant in stent communities of SZ (Table 1). This difference could be attributed to the frequent presence of organisms related to *L. sobrius* and *L. frumenti* (phylotypes 41 and 46, see Figure 1) in stents from SZ.

To evaluate the differences between the global bacterial compositions of stents between the two hospitals, the SSCP fingerprints across all stents were compared using ordination (Figure 3). Even though the nMDS plot showed no distinct clustering, there was a divergence of BS samples (open symbols) to the left and SZ samples (filled symbols) to the right of the plot, and ANOSIM confirmed a statistically significant difference of the global bacterial composition of stents between hospitals ($P < 0.001$). However, the Global R statistic, which measures the overall divergence between BS and SZ samples, has a value of $R = 0.166$ and is considered to be low, indicating that although bacterial community structures are statistically different, they are still barely separable. SIMPER confirmed that the most likely reason for this divergence was the higher abundance of *Bifidobacterium animalis* at SZ (contributing 10% to the overall dissimilarity between the two hospitals).

There was no statistically significant difference between the global stent community structures
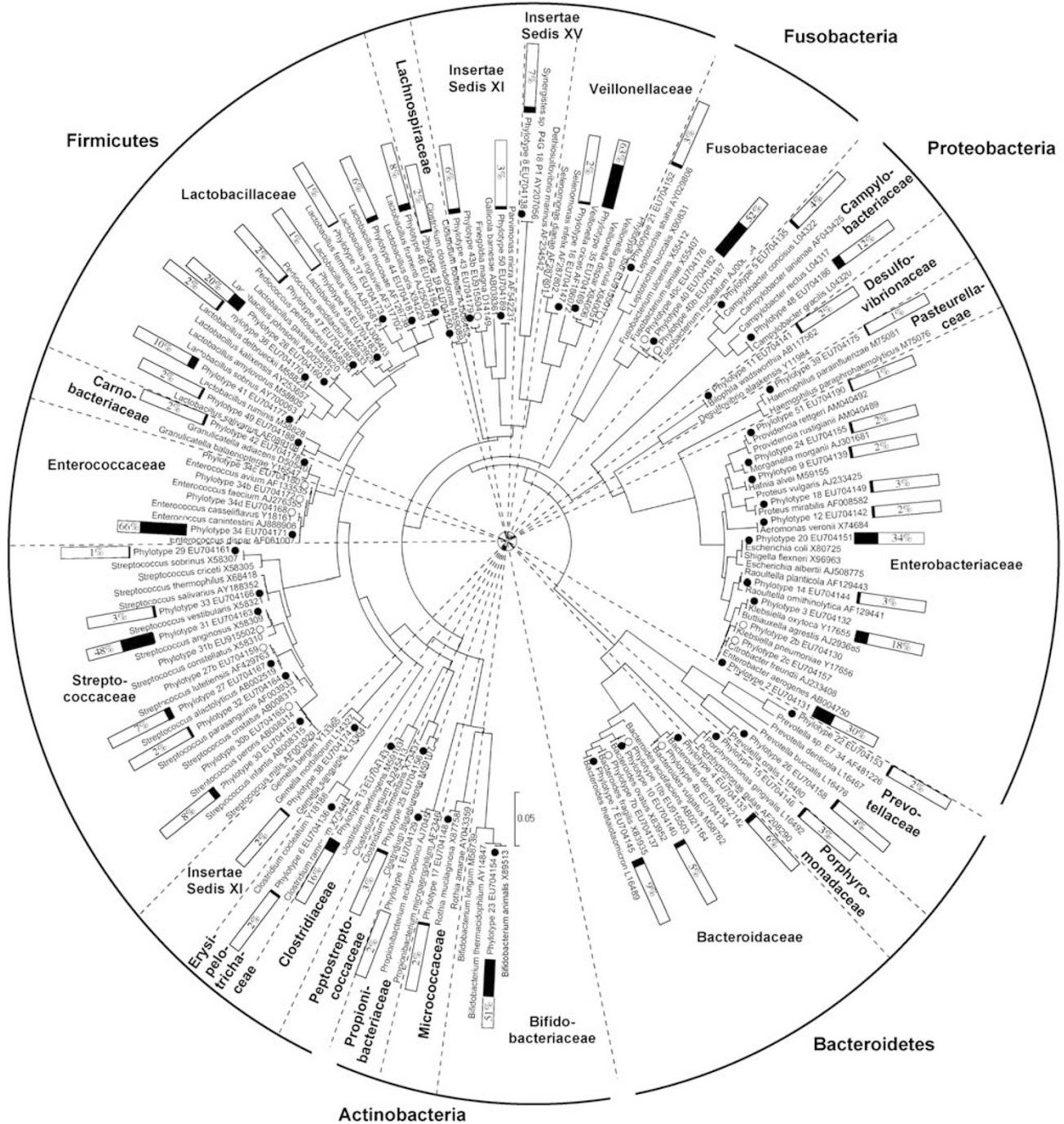
Figure 1 Taxonomical distribution of the 16S rRNA gene sequences retrieved by single-strand conformation polymorphism profiling of stent microbial communities (indicated by circles). For further analyses, sequences showing > 98.9% sequence identity were grouped as 'phylotypes' and representative phylotypes are indicated by filled circles. The abundance of a phylotype is given as % of total samples ($n = 132$) indicated as a bar behind the respective phylotype. The evolutionary history was inferred using the Neighbour-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons. Phylogenetic analyses were conducted in MEGA4 using partial 16S rDNA sequences (position 537–906 in the *Escherichia coli* numbering system). Sequences of closely related bacterial type strains and isolates were included in the analysis. The different bacterial families identified are separated by dashed lines. GenBank accession numbers are given after the strain name or the phylotype.

of the distal and proximal liver ends at either the BS ($P = 0.640$, $R = -0.012$) or the SZ ($P = 0.803$, $R = -0.014$) hospitals (see Figure 3).

However, an overview of the prevalence of different families in proximal and distal stent ends indicated a higher frequency of *Lactobacilli* being

Table 1 Families of the domain bacteria identified by single-strand conformation polymorphism (SSCP) fingerprinting as abundant in biliary stent microbial communities from Surgery Clinic of Braunschweig (BS) and Medical Clinic of Salzgitter (SZ)

Bacterial family	Occurrence in biliary stents (% of samples analyzed)[a]								
	SZ			BS			Total		
	T[b] n = 32	D[c] n = 32	P[d] n = 32	T n = 27	D n = 27	P n = 27	T n = 59	D n = 59	P n = 59
Streptococcaceae	59.4	40.6	56.3	74.1	63.0	70.4	66.1	50.8	62.7
Enterobacteriaceae	78.1	68.8	71.9	66.7	63.0	66.7	72.9	66.1	69.5
Veillonellaceae	78.1	62.5	65.6	66.7	63.0	59.3	72.9	62.7	62.7
Enterococcaceae	87.5	62.5	87.5	55.6	55.6	51.9	72.9	59.3	71.2
Fusobacteriaceae	53.1	50.0	53.1	55.6	48.1	51.9	54.2	49.2	52.5
Lactobacillaceae	50.0	46.9	25.0	33.3	33.3	7.4	42.4	40.7	16.9
Bacteroidaceae	15.6	15.6	15.6	25.9	22.2	25.9	20.3	18.6	20.3
Bifidobacteriaceae	93.8	81.3	84.4	22.2	22.2	7.4	61.0	54.2	49.2
Clostridiaceae	12.5	12.5	12.5	22.2	14.8	22.2	16.9	13.6	16.9
Campylobacteraceae	28.1	15.6	25.0	11.1	11.1	3.7	20.3	13.6	15.3

[a]A sample was scored positive if at least one band indicative for the presence of members of the respective bacterial family was observed by SSCP fingerprinting in the evaluated compartment.
[b]T, occurrence in either stent communities of samples distal or proximal to the liver.
[c]D, only stent communities of samples distal to the liver were evaluated
[d]P, only stent communities of samples proximal to the liver were evaluated.

observed in distal stent communities across both hospitals.

There were also no statistically significant differences either between the interior and exterior surfaces of biofilms of biliary stents (P-values were always <0.05) or between the communities of the pancreatic and the biliary distal and proximal liver end stent communities at SZ hospital (P = 0.645, R = −0.049 and P = 0.855, R = −0.101), respectively. This can also be visually observed in Figures 3 and 4. In particular, the pancreatic stent communities of two patients ordinate with the biliary stent communities of those same patients.

Simultaneously and consecutively implanted stents
A comparison of bacterial community structures in stents implanted consecutively (originating from patients A–E from BS) and simultaneously (originating from patients F–O from SZ) showed that communities of different stents from the same patient generally show similar composition (Figure 4). By contrast, there were statistically significant differences between the global communities originating from individual patients. A global R statistic of 0.709 at a significance level of P<0.001 indicates that the groups can be regarded as very well separated. Pair wise comparisons between patient stent communities (n = 105) proved that only three pairs of patients; patients A and D, patients I and L, and patients M and O shared similar bacterial communities (Figure 4). All other pair wise comparisons (n = 102) were significantly different (P<0.05), indicating that each patient had their own distinct bacterial community.

Dispersion analysis revealed that bacterial communities established on the stents of individual SZ patients have considerably lower variability than those of individual BS patients (see Figure 4). This indicates that communities of stents that are implanted consecutively show a larger within-group heterogeneity compared with the more tightly grouped communities of stents that are implanted simultaneously.

Discussion

This work identified 51 distinct bacterial phylotypes belonging to 5 bacterial phyla and 24 families from stent microbial communities revealing a greater diversity of bacteria compared with earlier studies (Speer et al., 1988; Dowidar et al., 1991; Molinari et al., 1996; Di Rosa et al., 1999; Leung et al., 2000; Zhang et al., 2003). These six former studies collectively identified members of 23 bacterial genera (but only 16 families) from 138 stents using cultivation approaches and usually indicated Gram-negative facultative anaerobic γ-Proteobacteria (predominantly Enterobacteriaceae comprising >55% of isolates) and Gram-positive facultative anaerobic 'Bacilli' (comprising >30% of isolates) as predominant. An exception to this is the study of Leung et al. (2000), who identified C. perfringens as a dominant stent colonizer of patients that had received antibiotic prophylaxis against Gram-negative bacterial infections.

In contrast to these culture-dependent studies, a high abundance of strict anaerobic bacteria, particularly Fusobacterium sp. and Veillonella sp., was observed here, indicating the bias of relying solely on isolation methods. In accordance with culture-dependent studies, a high abundance of Enterococci and Enterobacteriaceae was evident in
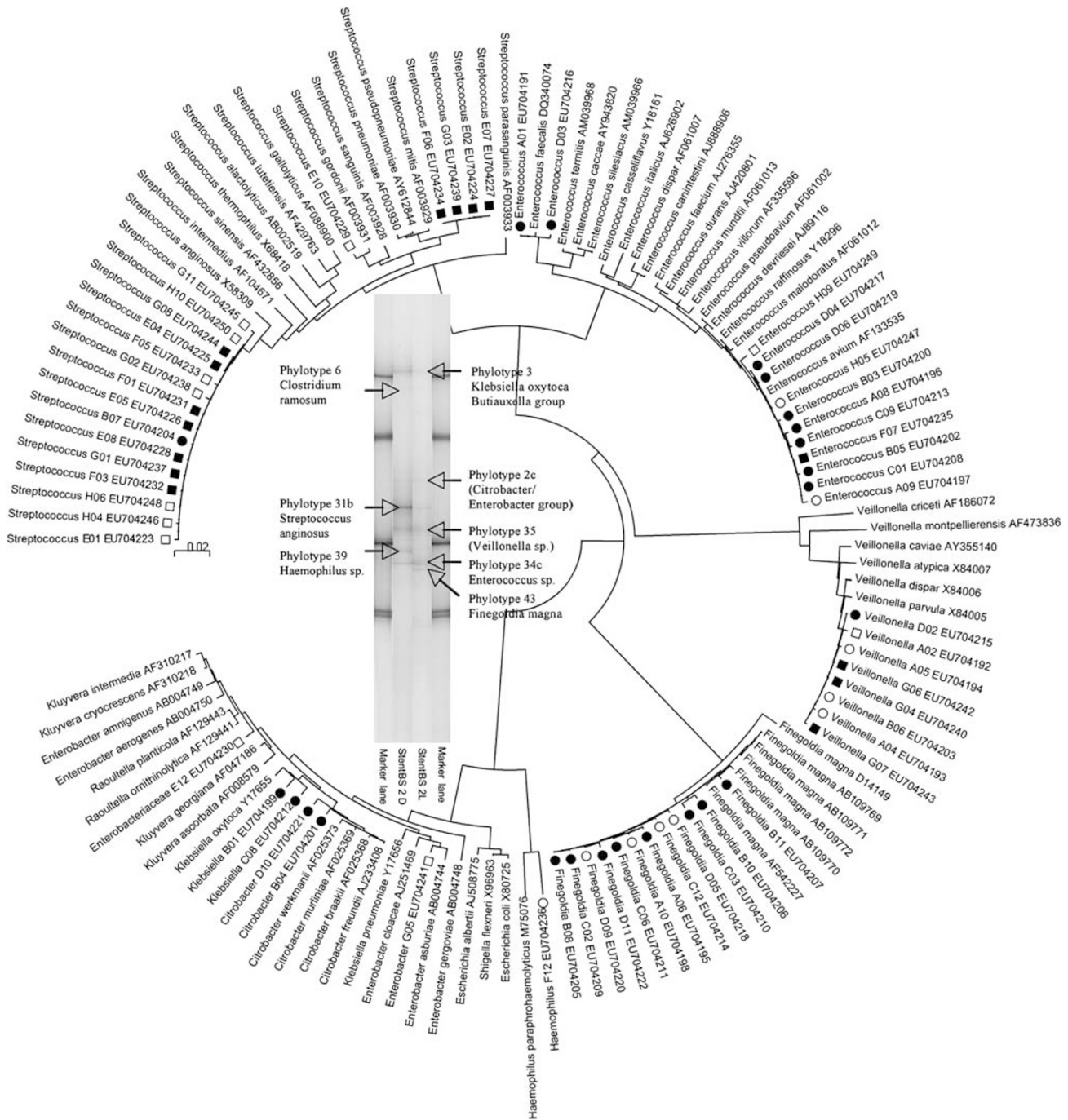
Figure 2 Taxonomical distribution of the 16S rRNA gene sequences retrieved from a clone library of an exemplary biliary stent. Sequences retrieved from the stent end distal to the liver are indicated by squares and those from the stent end proximal to the liver are indicated by circles. Sequences identical in the central fragment corresponding to position 537–906 in the *E. coli* numbering system to a single-strand conformation polymorphism (SSCP)-fragment are indicated by filled symbols. The evolutionary history was inferred using the Neighbour-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons. Phylogenetic analyses were conducted in MEGA4 using nearly complete 16S rDNA sequences. Sequences of closely related bacterial type strains and isolates were included in the analysis. GenBank accession numbers are given after the strain name or the phylotype. The PCR-SSCP analysis of 16S rRNA gene segments generated from the stent microbial community from the Surgery Clinic of Braunschweig (BS) is shown as an insert. The marker lane contains a mixture of single-stranded DNA from *K. oxytoca*, *S. epidermidis*, *E. faecalis* and *A. radiobacter*. D, biliary stent community—stent end distal to the liver; L, biliary stent community—stent end proximal to the liver.

Table 2 Co-occurrence of phylotypes in stent microbial communities

Phylotype 1–Phylotype 2	Abundance (n = 132)			Z-score[b]	P-value
	Phylotype 1	Phylotype 2	Co-occurrence[a]		
Fusobacterium sp. – *Streptococcus anginosus*	68	64	49	−3.74	0.0001
Veillonella sp.—*Fusobacterium* sp.	83	68	57	−3.50	0.0004
Enterococcus sp.—*Bifidobacterium* sp.	87	67	55	−2.69	0.0069
Lactobacillus sp. (28)[c]—*Lactobacillus* sp. (41)	27	13	8	−2.71	0.0066
Veillonella sp.—*Streptococcus anginosus*	83	64	51	−2.47	0.0133
Streptococcus anginosus—*Lactobacillus* sp. (41)	64	13	1	2.86	0.0041
Escherichia/Shigella group—*Klebsiella oxytoca/ Buttiauxella* group	45	24	3	2.18	0.0286
Fusobacterium sp.—*Lactobacillus* sp. (41)	68	13	2	2.22	0.0258

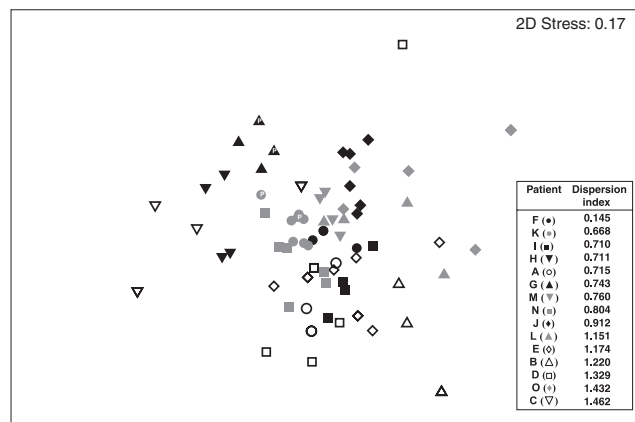[a]The number of samples in which presence of both phylotypes were observed is given.
[b]A *Z*-score below −1.96 indicates significant co-occurrence of the respective two phylotypes, whereas a *Z*-score above 1.96 indicated significant avoidance.
[c]Numbers in parentheses indicate phylotype number.
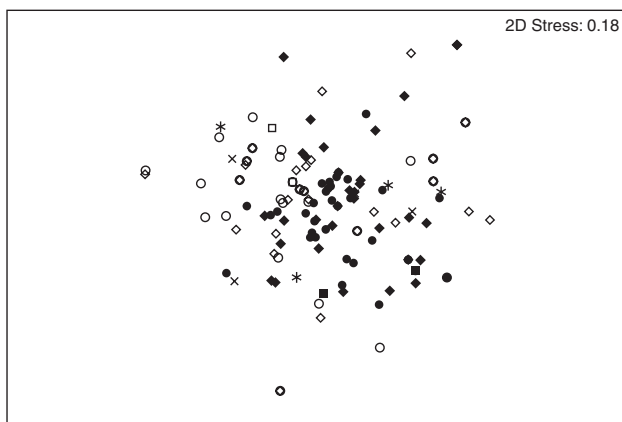
Figure 3 Differences in structure between stent microbial communities of stents from BS (open symbols) and SZ (filled symbols). Communities having developed at the distal end of biliary stents are indicated by diamonds, and those at the proximal end of biliary stents by circles. Pancreatic stent samples originated exclusively from SZ, communities having developed at the distal end are indicated by crosses, and those at the proximal end by asterisks. Communities having developed at the exterior of the stent proximal to the liver end are indicated by squares. The two-dimensional (2D) non-metric multidimensional scaling plot is based on the Bray–Curtis similarity coefficient computed using the presence/absence of data from single-strand conformation polymorphism analysis. The stress value of 0.18 corresponds to a useful 2D interpretation. The spatial closeness of points on the plot indicates their relative closeness in terms of their bacterial communities.

Figure 4 Differences in stent bacterial community structure between 15 patients with multiple stents implanted either simultaneously (SZ hospital, open symbols) or consecutively (BS hospital, filled symbols). The letter P indicates a pancreatic stent. The two-dimensional non-metric multidimensional scaling plot is based on the Bray–Curtis similarity coefficient computed using the presence/absence of data from single-strand conformation polymorphism analysis. Dispersion indices of communities from patients A–O were calculated using PRIMER6 (multivariate dispersion analysis routine). The stress value of 0.17 corresponds to a useful two-dimensional interpretation. The spatial closeness of points on the plot indicates their relative closeness in terms of their bacterial communities.

stent communities. Culture-dependent studies relatively frequently isolated *Pseudomonas* sp. (Dowidar *et al.*, 1991; Molinari *et al.*, 1996); however, members of this family are not considered as intestinal bacteria and there was no sign of their prevalence in any of the stents analyzed in this work. By contrast, *Bacteroides* sp. are among the most predominant genera of intestinal microbiota (Hold *et al.*, 2002) and have been found to be dominant among bacteria on the ileal mucosal surface (Wang *et al.*, 2005) and in accordance with the hypothesis of invasion of microorganisms through an ascending infection by duodenal biliary

reflux (Sung *et al.*, 1992) were observed in a high number (approximately 20%) of stent communities. This also explains the high prevalence in stent communities of acid-tolerant *Streptococcus* spp. and *Lactobacillus* spp., which were reported to be highly abundant in the ileum (Hayashi *et al.*, 2005; Wang *et al.*, 2005) and probably are major colonizers of the duodenum (Wilson, 2005).

The immense prevalence of *Veillonella* sp. in biliary stent biofilms is at first astonishing. However, there was a strong correlation between the presence of organisms related to *S. anginosus*, *Veillonella* sp. and *Fusobacterium* sp. in stent communities. In dental plaque biofilms, *Streptococci* have been described as the primary colonizers

(Nyvad and Kilian, 1990; Diaz et al., 2006), a process mediated by adhesins (Demuth et al., 1996; Jado et al., 2001). Adhesins to fibronectin and vitronectin known to coat biliary stent surfaces introduced into the human host (Yu et al., 1996) may also play a role in primary colonization of biliary stents. The co-aggregation properties of Veillonella sp. isolates from the oral cavity with multiple streptococcal strains have recently been reported (Hughes et al., 1988), and in particular, isolates related to V. parvula and V. dispar showed strong co-aggregation properties (Palmer et al., 2006). It can be suggested that Veillonella sp. may also rely on its promiscuous co-aggregation properties to integrate into biliary and pancreatic stent biofilms. In addition, Fusobacterium sp. have been reported to co-aggregate with early biofilm colonizers such as Streptococcus sp. (Bolstad et al., 1996) having a special role as bridging organisms (Kolenbrander et al., 2002) allowing other bacteria to attach to biofilms. Whether F. nucleatum plays a similar role in biliary stent biofilms remains to be elucidated.

Although the global differences between stent communities from different hospitals were only minor, the slight divergence between the BS and SZ samples was because of the higher prevalence of Bifidobacteria in biofilms from SZ stents. Bifidobacteria are autochthonous bacteria in the gastrointestinal tract (Klijn et al., 2005), but so far they have not been characterized as abundant in biliary stents. Culture-independent studies on the small intestine found them to be in low abundance (Hayashi et al., 2005; Wang et al., 2005), whereas they are more abundant in the colon and have been reported to comprise 3.3% in faeces (Vaughan et al., 2002). However, the sequences obtained were related to B. animalis, which has been extensively used in functional dairy products (Masco et al., 2005), rather than to B. longum, B. bifidum, B. adolescentis and B. angulatum, which are assumed to be the predominant species in the human gastrointestinal tract (Gueimonde et al., 2004; Mangin et al., 2006).

Stent bacterial community composition seems to be highly dependent on the individual patient. Although sharing some species in common, most patients still had their own distinct bacterial fingerprint and only some patients shared a similar bacterial community. However, patients who had several stents implanted simultaneously (in SZ) had less bacterial heterogeneity across multiple stents than patients who had several stents implanted consecutively (BS). This increased similarity in bacterial compositions between stent biofilms from patients with simultaneously introduced stents seems reasonable because those stents are assumed to be concurrently inoculated by an ascending infection of the duodenal microbiota (Sung, 1995; Liu et al., 1996; Donelli et al., 2007). As the duodenal bacterial composition and thus the possibly contaminating bacterial community is supposed to be subject to major changes over time because of rapid peristalsis and the short transit time of the lumenal contents (Wilson, 2005), this may explain the higher heterogeneity in communities of stents that have been implanted consecutively.

The gastrointestinal bacterial composition is strongly host-dependent and pronounced interindividual differences have been observed earlier (Zoetendal et al., 2002; Eckburg et al., 2005; Hayashi et al., 2005). Differences in host physiology, host genetic background or environmental factors have all been implicated to account for such host-dependency. For example, microbial communities of monozygotic twins have a higher similarity to each other than their marital partners, indicating a strong influence of the host genotype (Zoetendal et al., 2001). Similarly, a large individual variability, which could not be explained by geographic origin, age or gender, has been reported (Lay et al., 2005). In addition, microbial community composition in the intestine depends to a large extent on the supply of dietary carbohydrates that resist digestion in the upper tract, and it is conceivable that diets have an impact on bacterial community composition (Gibson et al., 1995; Kleessen et al., 2001; Duncan et al., 2003). Thus, it is not surprising that most individuals have a distinct bacterial community that will colonize their implanted stents. However, this may provide drawbacks for using prophylactic antibiotic treatments to prevent bacterial attachment and growth on such stents.

In conclusion, this work provides a comprehensive survey of the important bacterial species prevalent in biliary and pancreatic stents. It is now fundamental to consider those prevalent anaerobic species, their co-aggregation properties and possible interactions with other bacteria that will enhance their attachment, growth and survival strategies. With such knowledge, more appropriate treatments can be tailored to prevent biofilm occlusion of stents.

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