Molecular characterization of the stomach microbiota in patients with gastric cancer and controls

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Abbreviations: T-RFLP, terminal restriction fragment length polymorphism; TRF, terminal restriction fragment; NOC, N-nitroso compounds; RFLP, restriction fragment length polymorphism

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ABSTRACT

Persistent infection of the gastric mucosa by *Helicobacter pylori*, can initiate an inflammatory cascade that progresses into atrophic gastritis, a condition associated with reduced capacity for secretion of gastric acid and an increased risk in developing gastric cancer. The role of *H. pylori* as an initiator of inflammation is evident but the mechanism for development into gastric cancer has not yet been proven. A reduced capacity for gastric acid secretion allows survival and proliferation of other microbes that normally are killed by the acidic environment. It has been postulated that some of these species may be involved in the development of gastric cancer, however their identities are poorly defined. In this study, the gastric microbiota from ten patients with gastric cancer was characterized and compared with five dyspeptic controls using the molecular profiling approach, terminal-restriction fragment length polymorphism (T-RFLP), in combination with 16S rRNA gene cloning and sequencing. T-RFLP analysis revealed a complex bacterial community in the cancer patients that was not significantly different from the controls. Sequencing of 140 clones revealed 102 phylotypes, with representatives from five bacterial phyla (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria). The data revealed a relatively low abundance of *H. pylori* and showed that the gastric cancer microbiota was instead dominated by different species of the genera *Streptococcus*, *Lactobacillus*, *Veillonella* and *Prevotella*. The respective role of these species in development of gastric cancer remains to be determined.

INTRODUCTION

Gastric cancer is a global health problem, with the highest rates in developing countries. Research during the past few decades has shown that *Helicobacter pylori* is the major causative agent in gastric cancer, although the etiological mechanism is not yet fully understood. The majority of the individuals that are infected by *H. pylori* develop mild pangastritis, where the gastric physiology is maintained and not associated with disease (Smith *et al.*, 2006). However, *H. pylori* infection can also lead to severe diseases. In antral predominant gastritis the production of gastric acid is increased (hyperchlorhydria), which is associated with a high risk to develop duodenal ulcer disease, but protective against gastric cancer development (Hansson *et al.*, 1996). By contrast, corpus predominant gastritis leads to lower production of gastric acid (hypochlorhydria) and may lead to atrophic gastritis, a condition with increased risk in developing gastric cancer (Uemura *et al.*, 2001). The hypochlorhydric condition creates an environment suitable for other microorganisms that can enter and colonize the stomach. Some of these bacteria are nitrogen-reducing bacteria that are able to produce carcinogenic N-nitroso compounds (NOC) through conversion of nitrates or nitrites from the saliva (Ziebarth *et al.*, 1997). Secretion of Vitamin C to gastric juice by healthy mucosa inhibits the bacterial synthesis of nitrites to NOC. However, in hypochlorohydric gastric juice, vitamin C levels are reduced (Schorah *et al.*, 1991). Instead, the nitrite levels are increased and available for the bacteria that are present in the hypochlorhydric stomach (Mowat *et al.*, 2000). This raises the question whether other bacteria than *H. pylori* contribute to development of cancer. In fact, *H. pylori* colonizes the atrophic stomach poorly, and in intestinal metaplasia hardly at all, suggesting that *H. pylori* creates an environment (atrophy and hypochlorhydria) suitable for gastric carcinogenesis rather than causing the cancer directly (Blaser & Atherton, 2004).

It is known that the microbiota in individuals with gastric cancer or precancerous conditions is altered from the normal gastric microbiota. However, information on which species are present during these altered physiological states is scarce. The gastric microbiota has, however, been studied in hypochlorhydric conditions in subjects using acid reducing drugs, but these studies have relied on cultivation approaches (Adamsson *et al.*, 1999; Mowat *et al.*, 2000). A large fraction of the microbes residing in the gut have not yet been cultivated (Bik *et al.*, 2006; Eckburg *et al.*, 2005) therefore suggesting a skewed representation from culture-based studies. Although the development of molecular tools has bypassed the necessity for cultivation, very few studies have investigated the microbial composition of the stomach using culture independent approaches (Bik *et al.*, 2006; Kato *et al.*, 2006; Monstein *et al.*, 2000; Andersson *et al*., 2008).

In the present study, terminal restriction fragment length polymorphism (T-RFLP) was used in combination with cloning and sequencing of 16S rRNA genes to assess the composition of the gastric microbiota of subjects with gastric cancer. The aim was to describe the composition of the gastric microbiota in patients with gastric cancer, compared to subjects with normal gastric mucosal morphology.

MATERIAL AND METHODS

Patient cohort

The clinical material in this study derived from a hospital-based study described previously (Enroth *et al.*, 2000). Stomach biopsies from ten patients with non-cardia gastric cancer (five with intestinal type and five with diffuse type) and five dyspeptic control patients with normal gastric mucosal morphology, were included in this study. Biopsies derived from non-

cancerous gastric mucosa of antrum and corpus were pooled together. All subjects were included after providing informed consent, and the study was approved by the ethics committee of the Medical Faculty, Uppsala University DNR UU96/95 (Uppsala, Sweden). None of the individuals included in this study had taken antibiotics five years prior to sampling. Eight out of ten cancer patients were positive for *H. pylori*, by one or more of four tests; culturing, immunohistochemistry, ELISA and immunoblotting (Enroth *et al.*, 2000). However, only three of the patients (patients designated: 2, 7 and 10) were positive for *H. pylori* by culturing alone. None of the controls were positive for *H. pylori* in any of the above-mentioned tests. Information about sociodemographic and clinical characteristics can be found in Table 1.

DNA isolation and PCR conditions

Biopsies from antrum and corpus were homogenized together and stored in a freezing buffer containing glycerol at -70°C. 100 µl of the homogenized suspension were transferred to a microcentrifuge tube containing 0.25 ml of 0.1 mm zirconium beads with 180 µl of ATL lysis buffer solution (Qiagen, Hilden, Germany). In addition, 20 µl of proteinase K were added to the tube. The bacterial cells in the samples were lysed by bead beating two times 45 s at a setting of 5.0 using a FastPrep Instrument (MP Biomedicals, Solon, OH). The DNA from the samples was then recovered using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions for tissue samples, starting at step 3 in the protocol.

16S rRNA genes were amplified from the isolated DNA in three technical replicates per sample using broad range bacterial primers Bact 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (Edwards *et al.*, 1989), 5′ end-labelled with 6-carboxyfluorescein (6-FAM), and 926r (5'-CCGTCAATTCCTTTRAGTTT-3') (Muyzer *et al.*, 1993). Amplification was carried out in

25 µl reaction mixtures under conditions that have been described previously (Dicksved *et al.*, 2007), with the exception that the number of PCR cycles was increased to 35 instead of 30. PCR amplified DNA product amounts and sizes were confirmed by agarose gel electrophoresis using GeneRuler 100bp DNA ladder Plus (Fermentas Life Sciences, Burlington, Canada) as a size marker.

Terminal-restriction fragment length polymorphism (T-RFLP)

PCR products were digested with the *Hae*III restriction enzyme (GE Healthcare, Uppsala, Sweden) and the digested fragments were separated on an ABI 3700 capillary sequencer, as previously described (Hjort *et al.*, 2007). The sizes of the fluorescently labeled fragments were determined by comparison with the internal GS ROX-500 size standard (Applied Biosystems (ABI), Foster city, CA, USA). T-RFLP electropherograms were imaged using GeneScan software (ABI). Relative peak areas of each terminal restriction fragment (TRF) were determined by dividing the area of the peak of interest by the total area of peaks within the following threshold values; lower threshold at 50 bp and upper threshold at 500 bp. A threshold for relative abundance was applied at 0.5% and only TRFs with higher relative abundances were included in the remaining analyses.

Cloning and sequencing

Cloning and sequencing of 16S rRNA genes that were PCR amplified from DNA extracted from the biopsy samples was performed to confirm the identities of bacterial species corresponding to the dominant TRFs. DNA samples from six cancer patients (No; 4, 5, 6, 7, 8 and, 9) were selected for amplification with the general primers Bact-8F and 926r. Three replicate PCR products from each individual were pooled and gel purified using the Qiagen gel extraction kit (Qiagen). The pooled PCR products were cloned into the TOPO TA pCR

4.0 vector, followed by transformation into *Escherichia coli* TOP 10 competent cells (Invitrogen, Carlsbad, CA). A total of 64 inserts from each library were amplified using vector primers M13f and M13r (Invitrogen) with the same thermal cycling program as for T-RFLP analysis. The PCR products were digested with *Hae*III and *Hha*I (GE Healthcare) and digested fragments were separated in a 2.5% agarose gel. Band patterns, i.e restriction fragment length polymorphism types (RFLP types), were analyzed using Gelcompare software (Applied Maths, Gent, Belgium). Plasmids from all clones with unique RFLP types and several redundant RFLP types were purified and selected prior to sequence analysis, in total 140 clones. Obtained sequences were examined using MacVector 8.1.1 (Accelrys Software Inc, San Diego, CA), to identify their corresponding TRF sizes, and for removal of redundant sequences. The remaining sequences were aligned against GenBank database entries using standard nucleotide BLAST at NCBI (URL: www.ncbi.nlm.nih.gov). Hits defined as unknown or uncultured bacteria were subsequently aligned against sequenced bacterial genomes (genomic BLAST at NCBI), as well as examined with the Ribosomal Database Project II Sequence Match, in an attempt to classify them. Sequences with 99-100% similarity were given the same name as the species hit whereas sequences with 97-99% identity were assigned the genus name followed by "sp". Sequences were aligned online using MAFFT aligner version 6 with the Q-INS-i strategy (Katoh & Toh, 2008). A phylogenetic tree was constructed using PHYML v2.4.5 (Guindon & Gascuel, 2003), and displayed using Figtree v1.0 (http://tree.bio.ed.ac.uk/software/figtree/), where large clusters were collapsed.

Statistical analysis

T-RFLP data from each individual was entered into a data matrix that consisted of the terminal restriction fragments as variables and individuals as objects. A consensus T-RFLP profile was constructed for each of the technical duplicates. For significance testing of TRF

distribution between the cancer patients and the controls, multi response permutation procedures (MRPP) were used with Bray Curtis metrics as distances measure (PC-ORD, MjM Software, Oregon). Cluster analysis was based on Bray Curtis distances and a dendrogram was created using the software PAST (URL: http://folk.uio.no/ohammer/past/). Diversity, defined as evenness and richness of the bacterial community members detected as TRFs by T-RFLP analysis, was calculated using Simpson's index of diversity (D) (Begon *et al.*, 2006). Differences in TRF abundances between groups were tested using Mann Whitney's test and P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The inhospitable acidic milieu in the stomach provides an effective barrier, killing many of the microbes that enter the gastrointestinal tract. However, a change of the physiological conditions of the stomach, as occurs during acid reducing drug therapy, corpus atrophy or gastric cancer, provides an opportunity for foreign microbes to enter and colonize the stomach. The evidence chain that targets *H. pylori* as a causative agent in gastric cancer development is long, but proof of the progression into cancer is lacking. Eradication of *H. pylori* was recently shown to decrease the incidence of gastric cancer (Fukase et al., 2008), however, the prophylactic effect of such eradication remains controversial (Malfertheiner *et al.*, 2006). Duodenal ulcer disease, with overproduction of gastric acid, has been shown to protect against development of gastric cancer (Hansson *et al.*, 1996). By contrast, several studies have suggested that an altered gastric physiology, as in corpus predominant gastritis, promotes bacterial overgrowth of the stomach and survival of potential genotoxic species with an unknown impact on the human health (Calmels *et al.*, 1987; Heavey & Rowland, 2004). However, current knowledge of the identities of these microbial immigrants has been biased

by the inability to cultivate a large fraction of the gut microbiota. Therefore, we used molecular approaches in this study to describe the gastric microbiota of patients suffering from gastric cancer.

Analysis of clone libraries

Clone libraries were generated from six cancer patients, and a total of 384 clones were screened for their individual RFLP types. Each RFLP type was expected to originate from similar target sequences. Therefore, to reduce the number of clones to sequence, all unique and redundant RFLP types were selected for sequencing. Out of the 140 clones sequenced, 102 phylotypes were found. These clustered into five bacterial divisions: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria (Fig. 1). Members of the Firmicutes were most highly represented, with the majority corresponding to *Streptococcus*, *Lactobacillus* and different *Clostridiales,* such as *Veillonella*. The Bacteroidetes phylum was mainly represented by different species of *Prevotella*. All four subdivisions of Proteobacteria (alpha, beta, gamma and delta/epsilon subdivisions) were found in the clone libraries with *Neisseria* and *Haemophilus* as the most dominant genera. *H. pylori* was only found in one of the libraries (No. 7), which was in concordance with previous culture based results from this material (Table 1). In addition, Actinobacteria and Fusobacteria were found, but clones corresponding to these phyla were not abundant.

Correlation of clones to TRFs

The terminal restriction fragment (TRF) sizes of the sequenced clones were calculated *in silico* for their predicted lengths (base pairs) upon cleavage with the *Hae*III enzyme. Positive correlations between sequenced clones and TRFs were judged based on the presence of both a TRF and a clone with the same TRF size (base pairs) in the same samples. Most of the TRFs

could be correlated to the sequence data (Fig. 2). For many of the TRFs, only one genus matched with the particular fragment size, but a few TRFs had several genera that matched, e.g. TRFs 64, 205, 271 and 331. The most dominant TRFs, found in the majority of the individuals, were related to *Streptococcus* sp. (TRFs 307, 309 and 317), *Prevotella* (TRF 264), *Veillonella* (TRF 212), and different Proteobacteria (TRF 205) (Fig. 2). For some TRFs, mainly rare ones with abundances lower than 5 %, no matching sequences could be found in the libraries. In addition, some of the species found in the clone libraries had the restriction site outside the T-RFLP length-threshold values. This was the case for some of the Proteobacteria, such as *Klebsiella pneumoniae*. By correlating TRFs to the sequence data, we found that the abundance of TRFs corresponding to Firmicutes represented 60% of the total population, followed by Bacteroidetes and Actinobacteria with relative abundances of 11% and 7% respectively (Fig. 3). TRFs that correlated to Proteobacteria and Fusobacteria were present in lower abundances, 6% and 3% respectively (Fig. 3). Approximately 12 % of the T-RFLP abundance data could not be correlated to any sequence data (Fig. 3). This problem was mainly encountered for TRFs with low relative abundances, and many of the corresponding clones would probably have been found if more clones were sequenced. On the other hand, clones identified as *Prevotella multiformis* could be correlated to two different TRFs (177 and 264) possibly due to variations in the 16S rRNA gene sequences within this species.

Analysis of the T-RFLP profiles

The approach that we used in this study did not enable us to determine whether there were any differences in total abundances of bacteria between the cancer patients and the controls, but we could determine differences in relative abundances of some phyla. Analysis of the T-RFLP data revealed a total of 49 distinct TRFs and the average number of TRFs found in an

individual was 14 (range; 7-20). None of the TRFs were present in all individuals and none were common for all cancer patients (Fig. 2). The diversity of the microbiota was estimated based on the T-RFLP data using Simpson's index of diversity. There were no significant differences in diversity indices between the cancer patients and the control group. When the samples were clustered according to their TRF patterns, four out of five healthy controls clustered together (Fig. 3), suggesting that these bacterial communities are more similar to each other than to the cancer patients. In addition, three of the cancer patients differed significantly (P = 0.001) in their TRF distribution compared with the others (No 5, 6 and 10; Fig. 2). Mainly, the TRFs that corresponded to streptococci (274, 307, 309 and 317) were absent or very low in these three cancer patients. There was, however, no significant difference in the TRF distribution between all cancer patients and the healthy controls (P = 0.12) tested with MRPP.

Abundance of *Helicobacter pylori*

The abundance of *H. pylori* in all samples included in this study was very low. The TRF (271) that corresponds to *H. pylori* was not among the most abundant ones and is not unique for *H. pylori*. The low abundance of *H. pylori* is in agreement with what has previously been reported in hypochlorhydric stomachs (Houben & Stockbrügger, 1995). In a previous study, based on molecular approaches, *H. pylori* was detected in subjects that were considered negative based on traditional culturing or serological methods (Bik *et al.*, 2006). In our study, clone libraries were created from six cancer patients and we only detected *H. pylori* in the one patient that was positive by culture, suggesting that *H. pylori* was absent in the other five individuals. However only a limited number of clones were screened from each library and it is possible that we would have found *H. pylori* clones in these libraries by increasing the number of sequenced clones. Nevertheless, our results demonstrate a high microbial diversity

in the stomach and is in accordance with a recent cloning survey of the stomach microbiota in hospitalized individuals (Bik *et al.*, 2006), although the previous study did not include gastric cancer patients. The main difference in bacterial composition between this and the study of Bik, (2006) was an overall lower prevalence of *H. pylori* and higher prevalence of clones or TRFs matching with lactobacilli in this study.

Presence of *Streptococcus bovis*, a bacterium associated with colorectal cancer

It has previously been shown that bacteria more frequently colonize gastric tumors compared to the surrounding mucosal tissues and the most frequent colonizers of tumors are gram-positive cocci such as streptococci (Sjöstedt *et al.*, 1988). Streptococci are commonly isolated from the stomach and they have a wide repertoire of clinical features that can affect the host. For example, among the clones in this study, we found some that corresponded to *Streptococcus bovis*, a species that has been associated with colorectal cancer (Biarc *et al.*, 2004; Ellmerich *et al.*, 2000). It has been demonstrated that *S. bovis* or its antigens were able to promote cancer in rats, and that it exerted its pathological activity in the colonic mucosa only when pre-neoplastic lesions were established (Biarc *et al.*, 2004). As for CagA positive strains of *H. pylori*, *S. bovis* or its wall extracted antigens are able to induce an over-expression of inflammatory mediators known to be associated with carcinogenesis, such as IL 8, prostaglandin E_2, (PGE_2), and cyclooxygenase (COX-2) (Jüttner *et al.*, 2003). Further analysis of this group of bacteria is warranted to elucidate their potential involvement in gastric cancer progression.

Acid reducing therapy

The control group in this study was hospital-based, and the individuals were relatively old, with a possible deficiency in acid production that may have influenced the results. Although it

would have been interesting to compare pH values in the gastric juice of individuals in the control group and the cancer patients, this data was not available for this sample material. In hypochlorhydric stomachs more bacteria may be in an actively growing state because of the more favorable pH. This hypothesis is supported by previous investigations on persons that have taken acid reducing drugs where a higher microbial diversity than normal was detected, and a correlation was found to species normally found in the oro-pharyngeal tract (Adamsson *et al.*, 1999; Mowat *et al.*, 2000). The microbial species composition, found in the stomach during acid reducing drug therapy, shares many similarities with that found in the stomach of gastric cancer patients. This emphasizes the need for further research to characterize the structure and the activity of the microbiota in gastric cancer patients as well as in individuals undergoing acid reducing drug therapy.

N-nitroso compounds and gastric cancer

Another factor that is thought to be a risk factor in developing gastric cancer is formation of N-nitroso compounds. Correa (1992) postulated that bacteria present in the hypochlorhydric stomach have the ability to reduce dietary nitrate to nitrite, and subsequently produce carcinogenic N-nitroso compounds using secondary amines from food (Correa, 1992). It has also been reported that some of the species commonly found in the oral cavity have the ability to promote these reactions (Calmels *et al.*, 1987; Ziebarth *et al.*, 1997). In this study several of the clones identified are normally found in the oral cavity, and may be potent promoters of this reaction, although this requires further investigation to confirm.

Conclusion

In the current study, the gastric microbiota of patients with gastric cancer were characterized for the first time using molecular approaches and compared with dyspeptic controls having a

normal healthy mucosa. Representatives of several genera were detected with a predominance of Firmicutes, mainly of oral origin. However, no significant differences in microbial compositions were found between cancer patients and controls. The prevalence of *H. pylori* was very low and this bacterium was only found in samples where it had previously been isolated with culture-based techniques. Although we only examined a limited number of subjects, this study contributes the first DNA-based description of the bacterial community composition in the stomachs of individuals with gastric cancer and shows a presence of a complex gastric microbiota with potential as a maintainer or bystander in the progression of gastric cancer.

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Table 1. Clinical characteristics for gastric cancer patients and dyspeptic controls

Sample ID	Birth year	Gender	Group	Culture	Serology			H.p total
				H. pylori	***ELISA***	***imm***	***p116***	
1	88	M	Cancer	0	0	0	0	0
2	52	M	Cancer	1	1	1	1	1
3	58	M	Cancer	0	1	1	1	1
4	66	F	Cancer	0	1	0	0	1
5	67	M	Cancer	0	1	1	1	1
6	77	M	Cancer	0	1	1	1	1
7	73	M	Cancer	1	1	1	1	1
8	77	M	Cancer	0	1	1	1	1
9	77	F	Cancer	0	0	0	0	0
10	72	M	Cancer	1	ND	1	1	1
C11	68	M	Control	0	0	0	0	0
C12	75	M	Control	0	0	0	0	0
C13	75	M	Control	0	0	0	0	0
C14	73	F	Control	0	0	0	0	0
C15	75	F	Control	0	0	0	0	0

Figure 1

A phylogenetic tree, showing different phylotypes found in the stomach of six gastric cancer patients based on 16S rRNA gene sequences.

Figure 2

Relative abundances of terminal restriction fragments (TRFs) corresponding to dominant bacterial populations in the stomach of gastric cancer patients and controls with a normal gastric mucosa. The explanation of the relative abundance intervals is shown in a progressive grey scale with higher abundances correlating with darker shades. The abundance profile for each sample represents a column and the abundance for each TRF as a row. To reduce space in the figure, TRFs that were only present in one of the samples and not correlated to sequence data were removed. The samples were sorted according to their clustering with Bray Curtis index as distance matrix and UPGMA as dendrogram. Branches in black represent gastric cancer patients and in grey represent controls. *Samples that were selected for clone library construction and sequencing of the 16S rRNA genes (see Figure 1). Sequences from the 16S rRNA gene clone library having matching TRF sizes when digested with *Hae*III *in silico* and their closest blast hits are shown to the left of their corresponding TRF.

Figure 3

Pie chart that shows the relative abundances of the different bacterial phyla found in six gastric cancer patients. The proportions of the different phyla are based on T-RFLP data, i.e abundance of TRFs that were correlated to sequence data. A grey scale represents different bacterial phyla according to the legend. TRFs with no matches in the clone libraries were classified as uncorrelated (white area).

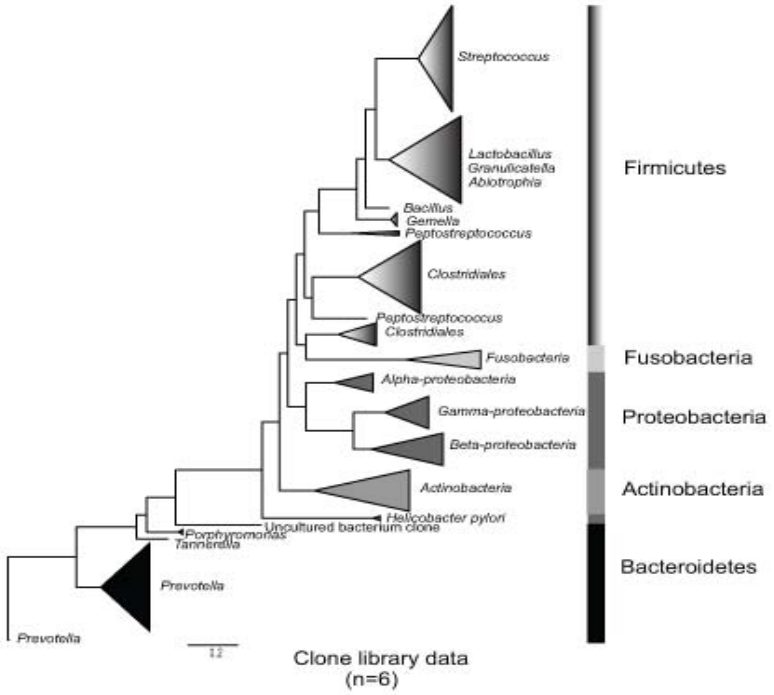
Streptococcus
Lactobacillus
Granulicatella
Abiotrophia
Bacillus
Gemella
Peptostreptococcus
Clostridiales
Peptostreptococcus
Clostridiales
Fusobacteria
Alpha-proteobacteria
Gamma-proteobacteria
Beta-proteobacteria
Actinobacteria
Helicobacter pylori
Uncultured bacterium clone
Porphyromonas
Tannerella
Prevotella
Prevotella
1.2
Clone library data
(n=6)
Firmicutes
Fusobacteria
Proteobacteria
Actinobacteria
Bacteroidetes

Figure 1

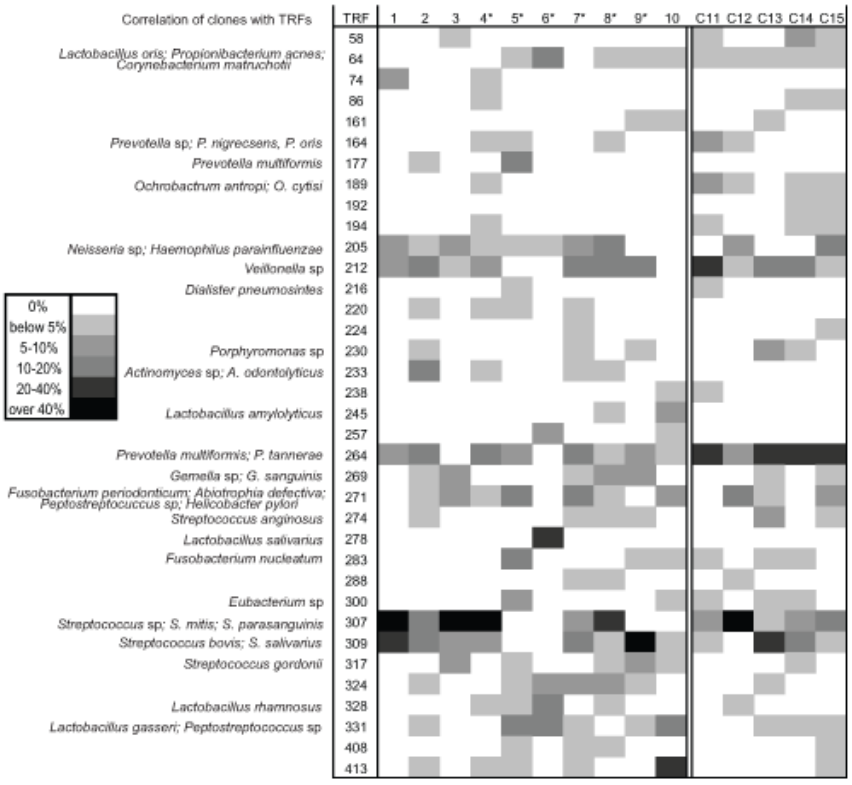
Correlation of clones with TRFs
TRF
1
2
3
4*
5*
6*
7*
8*
9*
10
C11
C12
C13
C14
C15
Lactobacillus oris; Propionibacterium acnes; Corynebacterium matnuchotii
Prevotella sp; P. nigrecsens, P. oris
Prevotella multiformis
Ochrobactrum antropi; O. cytisi
Neisseria sp; Haemophilus parainfluenzae
Veillonella sp
Dialister pneumosintes
Porphyromonas sp
Actinomyces sp; A. odontolyticus
Lactobacillus amylolyticus
Prevotella multiformis; P. tannerae
Gemella sp; G. sanguinis
Fusobacterium periodonticum; Abiotrophia defectiva; Peptostreptococcus sp; Helicobacter pylori
Streptococcus anginosus
Lactobacillus salivarius
Fusobacterium nucleatum
Eubacterium sp
Streptococcus sp; S. mitis; S. parasanguinis
Streptococcus bovis; S. salivarius
Streptococcus gordonii
Lactobacillus rhamnosus
Lactobacillus gasseri; Peptostreptococcus sp
0%
below 5%
5-10%
10-20%
20-40%
over 40%
58
64
74
86
161
164
177
189
192
194
205
212
216
220
224
230
233
238
245
257
264
269
271
274
278
283
288
300
307
309
317
324
328
331
408
413

Figure 2

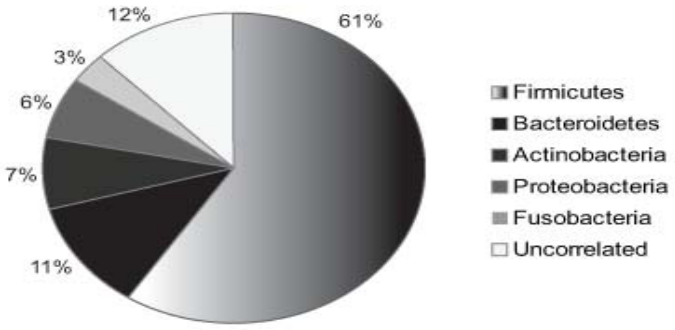
12%
61%
3%
6%
7%
11%
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Fusobacteria
Uncorrelated

Figure 3