Environmental Microbiology (2007) **9**(12), 3035–3045 doi:10.1111/j.1462-2920.2007.01415.x

Fungal rDNA signatures in coronary atherosclerotic plaques

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Summary

Bacterial DNA has been found in coronary plaques and it has therefore been concluded that bacteria may play a role as trigger factors in the chronic inflammatory process underlying coronary atherosclerosis. However, the microbial spectrum is complex and it is not known whether microorganisms other than bacteria are involved in coronary disease. Fungal 18S rDNA signatures were systematically investigated in atherosclerotic tissue obtained through catheter-based atherectomy of 38 patients and controls (unaffected coronary arteries) using clone libraries, denaturating gradient gel analysis (DGGE), *in situ* hybridization and fluorescence *in situ* hybridization (FISH). Fungal DNA was found in 35 of 38 (92.11%) coronary heart disease patients by either polymerase chain reaction (PCR) with universal primers or *in situ* hybridization analysis ($n = 5$), but not in any control sample. In a clone library with more than 350 sequenced clones from pooled patient DNA, an overall richness of 19 different fungal phylotypes could be observed. Fungal profiles of coronary heart disease patients obtained by DGGE analysis showed a median richness of fungal species of 5 (range from 2 to 9) with a high interindividual variability (mean similarity 18.83%). For the first time, the presence of fungal components in atherosclerotic plaques has been demonstrated. Coronary atheromatous plaques harbour diverse and variable fungal communities suggesting a polymicrobial contribution to the chronic inflammatory aetiology.

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Introduction

Coronary heart disease (CHD) is a major health problem in the western industrialized world. However, the pathogenesis of the multifactorial disease remains unclear. As the classical coronary risk factors, such as arterial hypertension, diabetes mellitus or smoking, failed to explain the aetiology and clinical course of CHD adequately, inflammatory and infectious mechanisms are discussed. The general importance of an elevated serum C-reactive protein as a marker for disease progression (Ridker *et al*., 2002) also raised an interest in the role of infection as a pathogenic factor.

In fact, atherosclerotic plaques show diverse bacterial signatures with a high interindividual variability of microbial communities pointing to individual mechanisms of infection exposition and control (Ott *et al*., 2006). As most of the bacteria detected in coronary lesions are natural and indigenous members of human microbial communities (such as the flora of the skin, the oral cavity or the human intestine), an origin of bacterial material found on human barrier organs was suggested in CHD patients (Ott *et al*., 2006).

The first interventional trials with macrolide antibiotics that were conducted to specifically eradicate plaque infection by *Chlamydia* sp. after acute coronary syndrome consistently suggested a risk reduction for secondary events such as cardiovascular complications and restenosis (Etminan *et al*., 2004). However, large, randomized, controlled studies (WIZARD, PROVEIT, ROXIS, ACES) that have been recently completed have failed to show a benefit for specific antibiotics, such as roxithromycin or azithromycin, in the treatment of CHD (Gupta *et al*., 1997; Gurfinkel *et al*., 1999; O'Connor *et al*., 2003; Grayston *et al*., 2005; Ridker *et al*., 2005), which could point to a more complex nature of microbial plaque colonization.

Fungi form rich and diverse microbial communities in normal human microbial communities, such as the intestine or oral cavity (Bernhardt, 1996; 1998; Bernhardt and

Knoke, 1997). Fungal infections have been recognized as an important problem in intensive care and in cases of fever of unknown origin in hospitalized patients (Pizzo, 1999; Hotchkiss and Karl, 2003). However, understanding of the clinical importance of fungal infections is hampered by a lack of culture techniques and by – in comparison with bacterial pathogens – a much smaller knowledge about strains and taxa.

Based on cultivation studies, the total number of fungal microorganisms is estimated to be 10^0–10^2 colony-forming units (cfu) ml^{-1} in the oral cavity and increases up to 10^6 cfu ml^{-1} in the faeces (Simon and Gorbach, 1984; Bernhardt, 1996). *Candida* sp. are considered as part of the normal intestinal and oral microbiota and can cause infections under specific conditions (Diebel *et al*., 1999; Barcenilla *et al*., 2000; Krause *et al*., 2001; Mavromanolakis *et al*., 2001). As fungi live as opportunistic microbiota on body surfaces, it is reasonable to assume that fungi may cross human barriers as described for bacteria.

Due to the fastidious nature of bacterial and fungal physiology, only a small proportion of complex microbial ecosystems is amenable to classical cultivation (Hugenholtz *et al*., 1998). Molecular techniques based on ribosomal RNA marker gene signatures allow the description of diverse microbial communities including the uncultivable part (Pace, 1997). A rich and diverse bacterial community has previously been detected in atherosclerotic lesions from patients with CHD (Ott *et al*., 2006). In this study, we intended to detect fungal signatures in coronary plaques of the same cohort of patients and describe the richness and diversity of fungal communities in coronary lesions using molecular techniques.

Results

Detection of fungal DNA by broad-range 18S rDNA polymerase chain reaction

Total amounts of 60–610 ng (mean 135.6 ng) of genomic DNA were extracted from coronary atherectomy specimens. Fungal DNA was found in 35 of 38 (92.11%) CHD patient samples by either polymerase chain reaction (PCR) with universal primers or *in situ* hybridization analysis ($n = 5$). No fungal DNA was found in the control material.

Community structure analysis

Clone libraries are currently the most accurate 18S rDNA-based molecular method to represent the microbial composition of a complex habitat (Eckburg *et al*., 2005). We analysed a clone library from coronary atherosclerotic lesions based on pooled DNA from 10 subjects with the highest fungal diversity in the community fingerprint analysis. From a total of 384 sequenced clones 320 (83.3%) could be assigned to further analysis. Chimeric sequences and incomplete inserts were discarded.

The majority of sequences ($n = 250$) were unique; the remaining inserts ($n = 70$) were abundant. At a phylotype level of 0.03, which is a common cut-off for the classification of operational taxonomic units (OTUs), 19 OTUs were detected.

The rarefaction curve of the OTUs observed and the number of sequences sampled depicts that the slope of curves decreases as definition of OTUs relaxes towards 10% sequence difference, and reaches a plateau at a cut-off level of $\leq 3\%$ (Fig. 1), at which the value number of observed OTUs was 19. The rarefaction curves of the Chao1 estimator of the richness of OTUs with the function of sequences sampled indicate that at difference levels 1%, 3% and 10% the estimated OTUs revealed 94, 21 and 7 respectively (Fig. 2). For the 3% level the estimated number of OTUs ($n = 21$) is very close to the observed number of OTUs ($n = 19$).

The collector curves of Chao1 and ACE (abundance-based coverage estimator) analyse the completeness of sampling at different distance levels (Fig. 3). Chao1 and ACE estimators predict a minimum of 21 species [95% confidence interval (CI) between 19 and 29] at a 3% difference class. After sampling of 131 sequences no change at the species levels was observed; at this point the estimated richness was 20 OTUs for Chao1 and ACE, which is consistent with the total estimated richness. These results show that the Chao1 and ACE estimators are relatively independent of the sample size. The data also indicate that the total number of clones analysed is sufficient to demonstrate the total coverage of fungi in this clone library as defined for the 3% level.

There are six abundant OTUs with more than 10 sequences and 13 OTUs with 10 or fewer individuals. The Simpson index of diversity for the library was calculated to 0.18, and the Shannon–Weaver index of diversity was calculated to 2.13 (95% CI between 2.0 and 2.3). Evenness of the library was determined by using the species richness and Shannon–Weaver index of diversity as 0.72 corresponding to a low variation in the fungal library. Simpson diversity index is a measure of diversity. In ecology, it is often used to quantify the biodiversity of a habitat. It takes into account the number of species present, as well as the abundance of each species. With this index, 0 represents infinite diversity and 1, no diversity. Simpson index of 0.18 indicates high diversity. The Shannon–Weaver index was used to calculate the evenness. Evenness is a measure of the relative abundance of the different species making up the richness of an area. A maximum value of 1 indicates even distribution

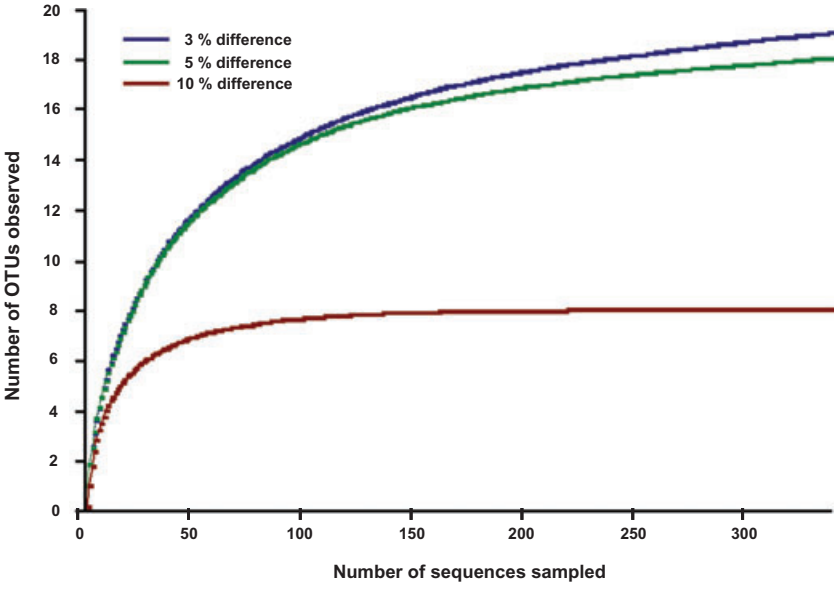

Fig. 1. Rarefaction curves of the OTUs observed and the number of sequences sampled. For phylotype analysis of OTUs the DOTUR software application was used. After alignment of sequences, Jukes-Cantor corrected distance matrices were generated and used as input files for DOTUR to calculate OTUs at various distance levels by using the furthest clustering algorithm. The rarefaction curve of the OTUs observed and the number of sequences sampled depicts that the slope of curves decreases as definition of OTUs relaxes towards 10% sequence difference, and reaches a plateau at a cut-off level of $\leq$ 3%, at which the value number of observed OTUs was 19. The rarefaction curves (number of OTUs observed on the *y*-axis against the number of sequences samples on the *x*-axis) for different cut-off levels (3%, 5% and 10%) are indicated.

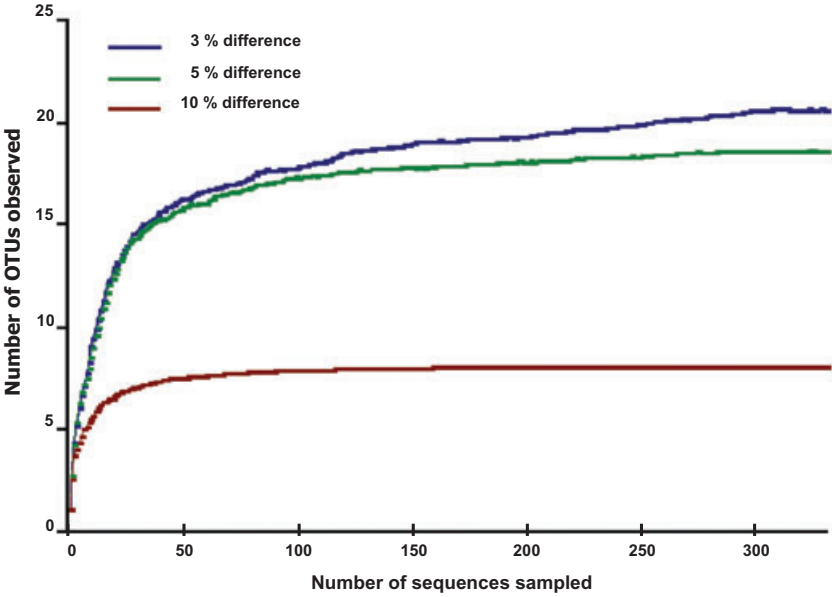

Fig. 2. Rarefaction curves of the Chao1 estimator. The rarefaction curves of the richness of OTUs with the function of sequences sampled indicate that at difference levels 1%, 3% and 10% the estimated OTUs revealed 94, 21 and 7 respectively. For the 3% level the estimated number of OTUs ($n = 21$) is very close to the observed number of OTUs ($n = 19$). The rarefaction curves of the Chao1 estimator of the richness of OTUs (Chao1 estimator on the *y*-axis against the number of sequences samples on the *x*-axis) for different cut-off levels (3%, 5% and 10%) are indicated.

of phylotypes, and low values indicate dominance in fungal library.

The taxonomic evaluation of the clone library by BLAST analysis showed a diversity of 23 OTUs, which is similar to the observed formal diversity at the 3% phylotype level as indicated above ($n = 19$). The species names and frequencies are listed in Table 1. The majority of OTUs is represented by *Yarrowia* sp. (57.2%), followed by *Candida* sp. (15.6%), *Cryptococcus* sp. (4.1%), *Gibberella pulicaris* (4.4%) and *Rhodosporidium* sp. (4%). A total of 16 sequences (5%) could not be assigned to known fungal representatives either by BLAST search or by using the ARB software package.

Formal evaluation of fungal richness and diversity

Community fingerprint analysis [denaturing gel gradient electrophoresis (DGGE)] using broad-range 18S rDNA primers was performed for each single specimen to formally investigate fungal diversity. Richness (number of bands) and a diversity score were calculated from the individual banding profiles as shown previously (Ott *et al.*, 2004; 2006). A separate statistical analysis was made for the demographic (age, gender) and clinical subgroups (restenosis, acute coronary syndrome, C-reactive protein elevation, cardiovascular risk factors). The median fungal richness of all individuals was 5 (range from 2 to 9); the

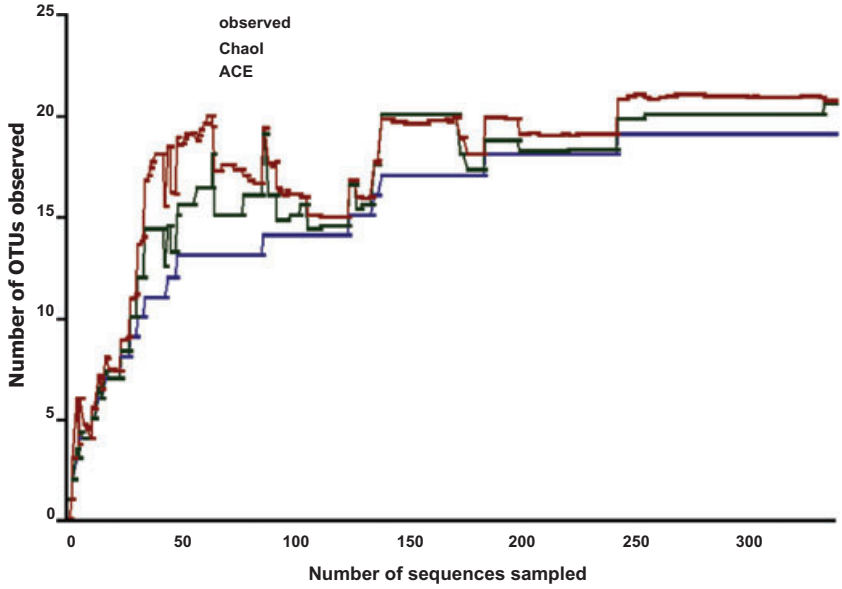

Fig. 3. Collector curves of Chao1 and ACE (abundance-based coverage estimator). The collector curves analyse the completeness of sampling at different distance levels. Chao1 and ACE estimators predict a minimum of 21 species (95% CI between 19 and 29) at a 3% difference class. After sampling of 131 sequences no instantaneous change at the species levels was observed; at this point the estimated richness was 20 OTUs for Chao1 and ACE, which is consistent with the total estimated richness. These results show that the Chao1 and ACE estimators are relatively independent of the sample size. The collector curves (number of OTUs observed on the *y*-axis against the number of sequences samples on the *x*-axis) for Chao1 and ACE estimators as well as the observed number of OTUs are indicated.

Table 1. Operational taxonomic units as assessed by BLAST analysis (indexed by frequency).

No.	Species[a]	Frequency *n* (%)
1	*Yarrowia* sp.	98 (30.6)
2	*Yarrowia lipolytica*	85 (26.6)
3	*Candida parapsilosis*	29 (9.1)
4	*Gibberella pulicaris*	14 (4.4)
5	*Candida buinensis*	11 (3.4)
6	*Rhodosporidium diobovatum*	11 (3.4)
7	Uncultured ascomycete	10 (3.1)
8	*Rhodotorula mucilaginosa*	10 (3.1)
9	*Ceratobasidium* sp.	9 (2.8)
10	*Cryptococcus albidus* var. *kuetzingii*	8 (2.5)
11	*Candida austromarina*	7 (2.2)
12	*Cryptococcus vishniacii*	5 (1.6)
13	Uncultured fungus	5 (1.6)
14	*Penicillium chrysogenum*	4 (1.3)
15	*Glyphium elatum*	3 (0.9)
16	*Candida* sp.	2 (0.6)
17	Uncultured *Pezizomycotina*	2 (0.6)
18	*Microxyphium citri*	2 (0.6)
19	*Candida albicans*	1 (0.3)
20	*Rhodosporidium fluviale*	1 (0.3)
21	*Rhodosporidium lusitaniae*	1 (0.3)
22	Uncultured basidiomycete	1 (0.3)
23	*Fusarium culmorum*	1 (0.3)

a. Operational taxonomic units were identified by NCBI BLAST analysis using search results of at least 97% similarity (NCBI BLAST, http://www.ncbi.nlm.nih.gov/).

median diversity was 0.58 (0.19–0.89). The statistical data for the richness for the different demographic and clinical subgroups showed no statistical significant differences (data not shown). The similarity of fungal profiles as determined by Pearson correlation revealed a high overall interindividual variability between the subjects of 18.83%. Figure 4 shows the DGGE profiles along with the similarity matrix demonstrating the variability of molecular patterns of fungal colonization.

Morphological identification of fungi by in situ *hybridization and fluorescence* in situ *hybridization*

In situ hybridization and fluorescence *in situ* hybridization (FISH) were performed to localize whether fungal signatures detected by PCR were visible by microscopic view. For morphological detection of fungal ribosomal genes within atherosclerotic plaques, *in situ* hybridization and FISH using a mix of broad-range 18S rRNA-targeted oligonucleotide probes were performed. Figure 5 demonstrates fungi in the cross-sections of atherosclerotic plaques.

Control individuals and technical controls

As a technical control blood samples, catheter material (catheter tips, ballons, wires, introducers), swabs and syringes were collected from five patients during heart catheter examination as shown previously (Ott *et al*., 2006). Direct universal PCRs as described above gave negative results. Although no positive bands could be obtained, the PCR products were examined using clone libraries as shown for the patient samples.

Discussion

Aetiology and pathogenesis of CHD are poorly understood, as the classical coronary risk factors do not fully explain the variable clinical nature of the disease. Our findings emphasize the infection hypothesis in the pathophysiology of coronary artherosclerosis. Recently, we

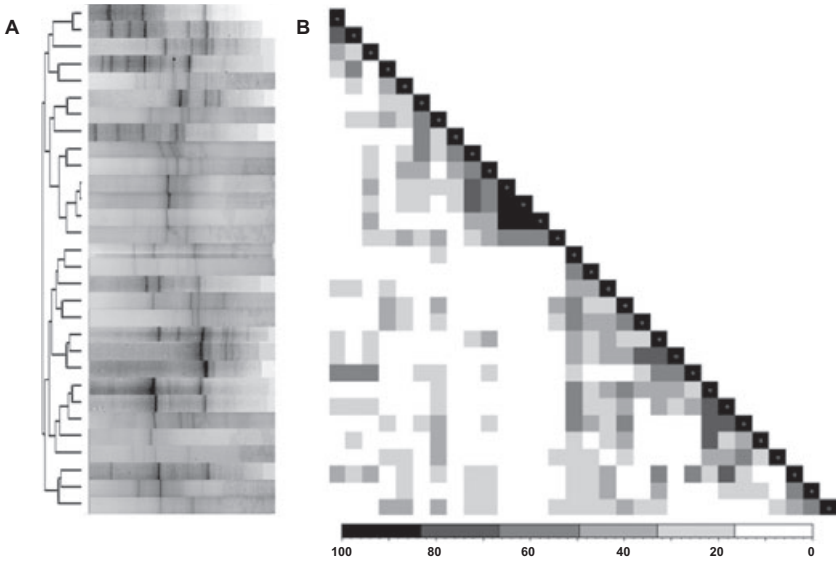

Fig. 4. Denaturating gradient gel analysis (DGGE) profiles of fungal consortia and similarity matrix.
A. The normalized DGGE profiles of the PCR-positive patients ($n = 30$). A cluster analysis revealed no significant correlation between the individual patterns and demographic or clinical factors.
B. The low interindividual similarity among the profiles. The degree of similarity is coded by intensity of grey tones (0% = white to 100% = black).

could demonstrate a rich and diverse bacterial colonization in atherosclerotic plaque specimens in the same cohort of CHD patients with a high interpatient variability of bacterial consortia (Ott *et al*., 2006). The lack of regular patterns in the bacterial colonization of atherosclerotic lesions and the homology of taxa with human microbial communities, e.g. the intestine or the oral cavity, suggest a general impairment and leakage of barriers facilitating bacterial invasion.

Fungi are omnipresent in the environment and represent an enormous natural diversity. Fungi also show an ubiquitous distribution on human surfaces, e.g. the intestine, from which they can be frequently isolated (Simon and Gorbach, 1984). Many of the fungal species found in the clone library, such as *Yarrowia* sp., *Candida* sp., *Penicillium* sp., *Rhodutorula* sp. and *Cryptococcus* sp., were previously isolated from the gastrointestinal tract or other human microbial communities (Haupt *et al*., 1983; Galan-Sanchez *et al*., 1999; Khatib *et al*., 2001). Contact with fungi or fungal components (e.g. mycotoxins) can also be mediated by dairy products, such as yoghurt or cheese (Cappa and Cocconcelli, 2001). The overall presence of fungi in our natural environment, the permanent fungal colonization of human microbial communities and the contact with fungi through food components make it likely that fungi cross human barriers leading to temporary fungaemia. Most of the contacts might be transient without causing manifest infection. For bacteria, translocation from the gastrointestinal tract, especially the oral cavity, is common. The incidence of bacteraemia following dental procedures, such as tooth extraction, endodontic treatment, periodontal surgery and root scaling, is well documented (Li *et al*., 2000). Transient bacteraemia is also common after surgical procedures, endoscopy, manipulation of infected tissue or local infections (Rijnders *et al*., 2001; Chowers *et al*., 2003).

Several explanations are conceivable for the presence of fungal DNA in coronary atherosclerotic lesions: Lehtiniemi and colleagues (2005) recently suggested that atheromas might act as mechanical sieves collecting microbiota from the circulation thereby assigning microorganisms a role as 'innocent bystanders'. While the concept of a destroyed coronary surface acting like a sieve is intriguing, the resulting bacterial and fungal colonization of the lesions could be still a (secondary) driver for a local inflammation process. Local macrophage and systemic monocyte activation supports this hypothesis (Dorffel *et al*., 1999; Hansson, 2005). Monocytes and macrophages themselves could also act as vehicles to transport material from bacterial cells by phagocytosis from the circulation into the plaque. Alternatively, primary infection of plaque lesion through microbiota could be a non-specific process that could accelerate disease progression. Association of high levels of serum C-reactive protein with a negative prognosis supports this hypothesis (Ridker *et al*., 2002). The high interindividual variability of bacterial profiles of fungal 18S rDNA fragments could point to additional individual mechanisms of infection control through host genetic and immunological factors that have to be considered in the pathophysiology of CHD.

In our study, no significant associations of demographic and clinical factors with the degree of fungal richness and diversity were detected. This could be due to a lack of statistical power through the limited numbers of patients in the subgroup analysis. On the other hand, the lack of association between fungal diversity with classical risk factors could be an argument for infection as independent risk factor in the aetiology and pathophysiology of CHD.

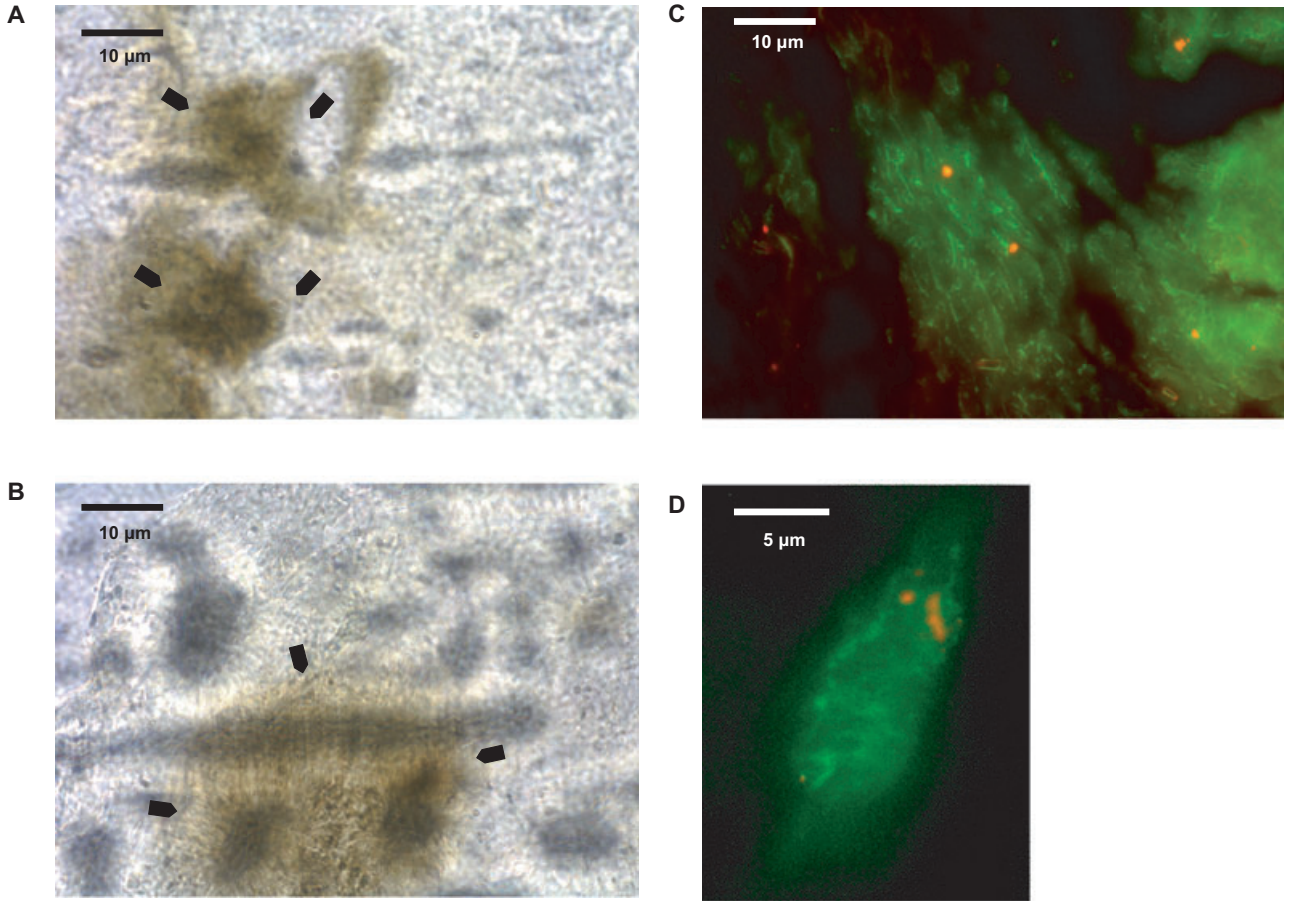

Fig. 5. Detection of 18S rRNA in tissue sections from coronary artery plaques using a mix of broad-range 18S rRNA-targeted Biotin-labelled and fluorescence-labelled oligonucleotide probes (FUN-1429 and MY-1574). The picture shows a representative experiment from five patients who were examined. The atherectomy specimens for *in situ* hybridization analysis and FISH were obtained from patients with stable angina undergoing elective catheterization. The arrows depict the signals (brown colour) from fungi (A and B). The FISH analysis in (C) and (D) shows that single cells or cell structures of fungi are clearly located within the plaque tissue. The detection level of this method is estimated to 100 fungal cells per 0.1 g of tissue.

Molecular techniques based on ribosomal genes allow a detailed description of microbial communities independent of the uncertainties of classical cultivation. However, several limitations have to be considered (Fredricks *et al*., 2005). The specificity of broad-range primers is limited, and PCR-based methods in general are susceptible to amplification bias (Wintzingerode *et al*., 1997). Although our statistical analysis showed that the number of clones is sufficient to cover the full richness of the clone library, the results should not be interpreted to indicate absolute fungal representation. Furthermore, due to inherited limitations of the experimental approach (species-specific number of rDNA copies, small amount of detectable 18S rDNA), no quantitative representation of fungal signatures/phylotypes can be achieved.

In conclusion, coronary atherosclerosis in a cohort of atherectomy patients is associated with rich and diverse fungal signatures detected in atherosclerotic plaques by different molecular techniques. The high general fungal richness, the interindividual variability of fungal signatures and the lack of association with demographic and clinical markers suggest that polymicrobial infection may be an independent risk factor in the pathophysiology of CHD. However, the aetiological and functional role of fungal microbiota in the pathophysiology of CHD remains to be defined.

Experimental procedures

Patients

All 38 patients underwent directional coronary atherectomy (DCA) under sterile conditions from non-ostial *de novo* ($n = 31$) or restenotic ($n = 7$) lesions in native coronary arteries at the Cardiac Catheterization Laboratories of the Cardiology Department, University Hospital Schleswig-Holstein, Campus Kiel, Germany. Six of the 38 patients who underwent successful DCA had acute coronary syndrome; the other 32 patients had stable angina pectoris. Acute coronary

syndrome was defined as new-onset or worsening angina that required hospitalization and was associated with alterations of the electrocardiogram, with or without elevation of cardiac markers, including creatinine kinase and troponin measurements. Stable angina (SAP) was defined as no change in frequency, duration or intensity of symptoms within 6 weeks before the intervention. A pre-defined subgroup analysis comprised patients who had an elevated C-reactive protein defined as serum levels exceeding 8 mg dl^{-1}. The baseline data of the patients included into the study are summarized elsewhere (Ott *et al*., 2006). The categories 'acute coronary syndrome', 'restenosis' and 'elevated C-reactive protein' describe partially overlapping subgroups. The study was approved by the ethics committee of the Medical Faculty of the Christian-Albrechts University Kiel, Germany. All patients considered for the study gave written informed consent prior to catheter examination.

Material for control experiments

Blood samples, catheter material (catheter tips, ballons, wires, introducers), swabs and syringes were collected from five patients during heart catheter examination and subjected to the same molecular detection techniques as technical controls. Biological material from 26 control individuals was analysed: tissue material from 15 post-mortem patients (kidney, liver, myocardium, coronary arteries, including eight splenetic tissue fragments) was obtained from the Institute of Pathology, University Hospital Schleswig-Holstein, Campus Kiel, Germany. The 15 post-mortem patients suffered from malignant diseases. These were selected because no current infectious disease was reported, and no antibiotic medication was administered within the last 3 months before death. Permission for post-mortem examination and research use of tissues was obtained from the next of kin. Native coronary arteries from explanted hearts were obtained from 11 heart-beating tissue donors [men : women 7:4, median age 52 years (34–64), left anterior descending artery (LAD) $n = 5$, right coronary artery (RCA) $n = 6$] from the Kiel transplantation programme. The explanted hearts were discarded from implantation due to morphological or technical reasons. None of these patients had an active infectious disease (C-reactive protein levels not elevated), recent or current treatment with antibiotics, or atherosclerosis of any coronary artery on macroscopic examination. The main inclusion criteria for the organ donors were that: (i) no external trauma had occurred, (ii) no infection had been detected/no antibiotics were administrated and (iii) age was between 30 and 70 years. The cause of death of the heart-beating donors according to the pre-transplantation documents is listed as follows: apoplectic stroke $n = 5$, subarachnoid haemorrhage $n = 2$, severe intracerebral haemorrhage $n = 3$, and cerebral hypoxaemic state after arythmia due to idiopathic dilatative cardiomyopathy $n = 1$. None of these patients had clinical or macroscopic signs of CHD.

Experimental design

As the PCR-based techniques used will require the full use of the entire specimen obtained by arterectomy, a hierarchical study design was employed. Thirty-three samples were extracted for analysis by DGGE to obtain a formal overview about fungal diversity signatures. The extracted material from the 10 patients showing the largest diversity on DGGE was then subjected to the generation of a library that allowed sequence analysis of the 18S rDNA gene in 384 clones, which resulted in a high-quality overview of the fungal strains involved and confirmed the results of the DGGE analysis. Last, the remaining five intact samples were subjected to *in situ* hybridization that detects live fungi within the tissue.

Treatment of atherectomy particles, control material and DNA extraction

DNA from blood, control material and tissue specimens was extracted following a protocol adapted for the characteristics of microorganisms (Ott *et al*., 2004). The solid catheter materials (swabs, introducers, wires, catheter tips, ballons) were washed in demineralized water for 30 min to dissolve cell and tissue fragments. All control material was investigated by the same experimental procedures that were used for the samples.

Selection of primers

In this study, an established nested PCR approach was used to avoid amplification of human DNA and to increase recovery of fungal rDNA. The full-length 18S rDNA was amplified during an initial PCR step using the conserved primers NS0 and EF3. The inner PCR primers NS1 and FR1 produce a fungal-specific amplicon of ~1650 bp for the use in DGGE analysis and for the generation of clone libraries (Vainio and Hantula, 2000). For DGGE analysis, a GC-rich sequence (GC clamp) was attached to 5′ end of the primer FR1 to prevent complete melting of double-strand PCR products during separation (Vainio and Hantula, 2000). The nested PCR primers BF2 and TR1-RP produce a fragment of 540 bp and were used for cloning and direct sequencing of DGGE bands.

Denaturing gradient gel electrophoresis

All 33 patients included in this study were investigated. DGGE analysis was performed with a denaturing gradient of 18–38% as previously described (Gomes *et al*., 2003). The reaction mixture of the initial PCR contained a 0.2 μM concentration of each primer (Eurogentec, Seraing, Belgium), a 0.2 μM concentration of each deoxynucleoside triphosphate (Qiagen, Hilden, Germany), 0.04% Dimethyl Sulfoxide (Sigma-Aldrich, Deisenhofen, Germany), 10× PCR buffer (Qiagen, Hilden, Germany), 15 mM $MgCl_2$, 1 U DNA Polymerase (Qiagen, Hilden, Germany) and 100 ng of sample DNA. Polymerase chain reaction was started with a denaturation step of 95°C for 3 min, followed by 30 cycles of 94°C for 15 s, 53°C for 30 s and 72°C for 3 min; the nested PCR was performed with 25 cycles of 94°C for 30 s, 48°C for 45 s and 72°C for 45 s. DNA concentrations were adjusted to 200 ng per lane. DGGE was performed in 1× Tris-acetate-EDTA buffer at 58°C at a constant voltage of 180 V for 18 h. After silver staining (Heuer *et al*., 2001), DGGE gels were air-dried and scanned for image analysis.

Bands from the silver-stained DGGE gels were excised with sterile scalpels, transferred into 1.5 ml reaction tubes containing 500 µl of destaining solution (6 mM $K_3Fe[CN]_6$), and incubated at 37°C in a heating block (Thermomixer, Eppendorf, Hamburg, Germany) for 10 min. The destaining solution was discarded and the gel slices were washed three times with distilled water (adapted protocol by Jin *et al.*, 2003). The isolation of DNA fragments from destained gel slices was performed as described by Schwieger and Tebbe (1998). The recovered DNA was re-amplified with Primers BF2 and TR1 (Table 2). The PCR products were cloned into competent *Escherichia coli* cells using the pCR™2.1 TOPO TA Cloning™ Kit for sequencing (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. A PCR using M13 primers with 5 µl of an overnight culture was performed to check the size of the inserts prior to sequencing. Direct sequencing of the inserts was performed with an ABI PRISM™ 3700 DNA Analyser (Applied Biosystems, Foster City, USA). The PCR product (8 µl) was digested with 0.3 U SAP (shrimp alkaline phosphatase) and 1.5 U ExoI (both Amersham Biosciences, Freiburg, Germany) for 15 min. The reaction was stopped by heating for 15 min at 72°C. The sequencing reaction was performed using 1 µl of ABI PRISM™ BigDye™ (Applied Biosystems, Foster City, USA), a 0.3 µM concentration of each sequencing primer (Eurogentec, Seraing, Belgium) and 2 µl of digested PCR product. The reaction conditions were 96°C for 10 s followed by 25 cycles of 95°C for 1 min and 60°C for 175 s.

Clone libraries

The nested PCR was performed as described above. After purification (MiniElute™ PCR Purification Kit, Qiagen, Hilden, Germany), the PCR products were pooled and cloned into chemically competent *E. coli* cells as described above.

In situ *hybridization*

In situ hybridization followed established protocols (Forghani *et al.*, 1985; Kerstens *et al.*, 1994). Briefly, atherectomy specimens were shock frozen in Tissue-Tek™ freezing medium, cut into 6 µm sections using a cryomicrotome and placed on super frost glass slides (Menzel-Glaser, Braunschweig, Germany). The 18S rRNA broad-range oligonucleotide probes FUN-1429 (3′-GTG ATG TAC TCG CTG GCC-5′, position 1429–1447, *Candida albicans* reference system) and MY-1574 (3′-TCC TCG TTG AAG AGC-5′, position 1574–1589, *C. albicans* reference system) (Baschien, 2003) are 3′-labelled with Biotin (Thermo Electron, Ulm, Germany). After air-drying, tissue sections were treated with 3% hydrogen peroxide for 10 min at 37°C to inactivate endogenous peroxidase. After a brief rinse in 0.01 M phosphate-buffered saline (PBS; pH 7.3), 15–20 ml of hybridization solution containing 60% deionized formamide (Sigma, Aldrich, Germany), 10% Dextran sulfate (Sigma, Aldrich, Germany), 2× SSC (standard saline citrate, pH 7.0) and 0.5 mg of each probe was applied to each tissue section. The slides were placed on a moistened filter paper in a plastic dish, and the dish was covered and floated in water bath at 80°C for 10 min to denature the target DNA, followed by overnight incubation at

Table 2. Polymerase chain reaction (PCR) and sequencing primers used in this study.

Primer	Application	Dir.	Position	L	T	C	Sequence (5′–3′)	Gene	Reference
NS0	Pre-PCR	f	1–18[a]	1650 bp	53°C	30	TACCTGGTTGATCCTGCC	18S	Messner and Prillinger (1995); Smit *et al.* (1999)
EF3		r	1653–1673[a]				TCCTCTAAATGACCAAGTTTG		
NS1	DGGE/cloning	f	19–38[a]	1550 bp	48°C	25	GTAGTCATATGCTTGTCTC	18S	White *et al.* (1990); Vainio and Hantula (2000)
FR1-GC[c]		r	1572–1589[a]				CCCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACG GGCCGAICCATTCAATCGGTAIT		
FR1	Cloning/sequencing	r	1572–1589[a]	–	48°C	–	AICCATTCAATCGGTAIT	18S	Vainio and Hantula (2000)
BF2	Cloning/sequencing	f	316–334[a]	520 bp	51°C	25	ACTTTCGATGGTAGGATAG	18S	Bock *et al.* (1994); Makimura *et al.* (1994)
TR1		r	838–856[a]				TACGGCGGTCCTAGAAAC		
M13(21)	Vector	f	389–404[b]	–	55°C	30	TGTAAACGACGGCCAGT	Vector	Guttman and Charlesworth (1998)
M13(24)		r	205–221[b]				AACAGCTATGACCATG		
GADPH_F2	Human DNA	f	–	270 bp	55°C	30	ACCCACTCCTCCACCTTT	GADPH	Mah *et al.* (2004)
GADPH_R2		r					CTGTTGCTGTAGCCAAATTCGT		

a. Position referring to the *Saccharomyces cerevisiae* reference sequence.
b. M13 priming sites on pCR™2.1 plasmid.
c. A GC-rich sequence (GC clamp) was attached to 5′ end to prevent complete melting of double-strand PCR products during separation (Dir., direction; L, length of fragment; T, annealing temperature; C, number of PCR cycles).

37°C. Slides were washed once for 5 min in 2× SSC (0.3 M Sodium Chloride and 0.03 M Sodium Citrate) buffer, once in 2× SSC containing 50% formamide and once again in 2× SSC. The slides were then immersed in PBS containing 0.1% Triton X-100 and the rinsed briefly in PBS. To detect the probe hybridized to fungal DNA, extra-avidin peroxidase conjugate (Sigma, Aldrich, Germany) was added to the tissue and incubated for 30 min at 37°C. The slides were than washed once with 2× SSC buffer for 5 min, rinsed briefly in PBS containing 0.1% Triton X-100 and rinsed in PBS for 3 min. The slides were kept in DAB (3-3-diaminobenzidine) substrate solution (Vector laboratories, CA, USA), a brown colour developed with 4–5 min. The enzymatic reaction was stopped by placing the slides in distilled water. The slides were air-dried and mounted in mounting media (Aquatex, Merck, Germany).

Fluorescence in situ *hybridization*

Preparation of tissue sections is performed as described above. After air-drying the specimens for 30 min at room temperature, slides were overlaid with 50 µl of hybridization buffer: NaCl 0.9 M, Tris-HCl 0.02 mM (pH 8.0), sodium dodecyl sulfate (SDS) 0.01%, formamide 30%, probe 5 ng μl^{-1}. A mixture of two broad-spectrum, fungi-specific 18S rRNA Cy3-labelled probes was used (Baschien, 2003): FUN1492 5′-GTGATGTACTCGCTGGCC-3′, MY1574 5′-TCCTCGTTGAAGAGC-3′. Hybridization was carried out at 46°C for 90 min in a humid chamber, and stringent washing was done at 48°C for 30 min in a buffer containing NaCl 0.112 M, Tris-HCl 0.02 (pH 8.0), SDS 0.01%. Sections were overlaid with Mounting medium (Clarion Mounting Medium, Sigma-Aldrich, Deisenhofen, Germany) and covered with a cover strip. Fluorescence was detected by an Axioimager Apotome-equipped microscope (Zeiss, Germany) with appropriate filter systems and pictures were captured by a digital camera system (Axiocam, Zeiss, Germany).

Data and sequence analysis

The sequences were analysed and aligned using Sequencher software package (Gene Codes, Ann Arbor, USA). For phylotype analysis of OTUs the computer-based program DOTUR (Schloss and Handelsman, 2005) was used (http://www.plantpath.wisc.edu/fac/joh/DOTUR.html). DNA sequences were aligned with CLUSTAL X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) with the output format option INPUT. Jukes-Cantor corrected distance matrices were calculated from the aligned sequences with the aid of the DNADIST program, which is part of the PHYLIP software package (http://evolution.genetics-washington.edu/phylip.html). Distance matrix files were used as input files for DOTUR to calculate OTUs at various distance levels by using the furthest clustering algorithm at different distance levels as shown elsewhere (Eckburg *et al*., 2005; Schloss and Handelsman, 2005). Rarefaction curves for the different precision levels using Chao1 estimator were calculated to estimate the richness of the clone library in relation to the sequences sampled (Eckburg *et al*., 2005; Schloss and Handelsman, 2005). We constructed collector curves of Chao1 and ACE to evaluate the completeness of sampling at different distance levels (Eckburg *et al*., 2005; Schloss and Handelsman, 2005).

The Simpson index of diversity for the clone library was calculated according to the following formula:

$$D = \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{N(N - 1)}$$

where n_i is the number of individuals of species counted, and N is the total number of all individuals counted.

The number of OTUs observed is also called species richness. Low values of the Simpson index indicate high diversity. The Shannon–Weaver index of diversity was determined using the classic formula:

$$H = \sum_{i=1}^{s} p_i \ln(n_i)$$

where p_i is the abundance of each species (abundance of the species/total abundances).

The evenness of the library represents the degree of genetic variation in a clone library. The evenness was calculated using species richness and Shannon–Weaver index of diversity:

$$E = \frac{H}{\ln(s)}$$

The values for the evenness are between 0 and 1; high E-values thereby indicate a low degree of variation in populations between the species. Taxonomic analysis of the OTUs was performed by NCBI BLAST search using results of at least 97% similarity (NCBI BLAST, http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were examined for chimera using the Chimera Check tool of the Ribosomal Data Projects (RDP) of the Center for Microbial Ecology, Michigan State University, MI, USA (RDP II, http://rdp.cme.msu.edu/). For the taxonomic classification of sequences that could not be assigned to known fungal species in the BLAST analysis, comparative sequence analysis using the ARB software package (http://www.arb-home.de) was employed.

Most of the numerical data generated by community fingerprint analysis did not follow a normal distribution. An explorative analysis was carried out to investigate relationships between diversity (assessed as number of bands and as diversity score according to Ott *et al*., 2004; 2006) and demographic (age, gender) or clinical characteristics (indication for examination: restenosis versus myocardial syndrome, elevated versus normal levels of C-reactive protein, cardiovascular risk factors). As the number of bands was asymmetrically distributed in some of the subgroups, data are represented by median values with quartiles. Statistical tests of differences in location parameters were performed by using two-sided Wilcoxon rank sum tests for independent samples at a 5% level of significance. The statistical analysis was conducted using SPSS (10.0, Chicago, IL, USA). Similarity between fingerprint profiles was determined using Pearson correlation as described elsewhere (Ott *et al*., 2004).

Acknowledgements

This work was supported by grants from the German National Genome Research Network (NGFN), the DFG (SFB415) and

the EU (EU QLG2-CT-2001-02161). We gratefully acknowledge Meike Barche and Ulrike Panknin for their assistance with the generation of clone libraries and sequencing. We also thank Kornelia Smalla from the Institute for Plant Virology, Microbiology, and Biosafety, Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany, for their technical support with DGGE profiling. Nils Haake (Department of Cardiovascular Surgery, UKSH Campus Kiel, Germany) has provided the control material from heart-beating donors.

References

Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C., and Flint, H.J. (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* **66:** 1654–1661.

Baschien, C. (2003) Development and evaluation of rRNA targeted *in situ* probes and phylogenetic relationships of freshwater fungi. In *School of Process Sciences and Engineering*. Berlin, Germany: Technical University of Berlin, pp. 30–32.

Bernhardt, H. (1996) [*Candida* in the ecological system of the orointestinal tract]. *Mycoses* **39:** 44–47.

Bernhardt, H. (1998) [Fungi in the intestine – normal flora or pathogens?]. *Z Arztl Fortbild Qualitatssich* **92:** 154–156.

Bernhardt, H., and Knoke, M. (1997) Mycological aspects of gastrointestinal microflora. *Scand J Gastroenterol Suppl* **222:** 102–106.

Bock, M., Maiwald, M., Kappe, R., Nickel, P., and Naher, H. (1994) Polymerase chain reaction-based detection of dermatophyte DNA with a fungus-specific primer system. *Mycoses* **37:** 79–84.

Cappa, F., and Cocconcelli, P.S. (2001) Identification of fungi from dairy products by means of 18S rRNA analysis. *Int J Food Microbiol* **69:** 157–160.

Chowers, M.Y., Gottesman, B., Paul, M., Weinberger, M., Pitlik, S., and Leibovici, L. (2003) Persistent bacteremia in the absence of defined intravascular foci: clinical significance and risk factors. *Eur J Clin Microbiol Infect Dis* **22:** 592–596.

Diebel, L.N., Liberati, D.M., Diglio, C.A., Dulchavsky, S.A., and Brown, W.J. (1999) Synergistic effects of *Candida* and *Escherichia coli* on gut barrier function. *J Trauma* **47:** 1045–1050; discussion 1050–1041.

Dorffel, Y., Latsch, C., Stuhlmuller, B., Schreiber, S., Scholze, S., Burmester, G.R., and Scholze, J. (1999) Preactivated peripheral blood monocytes in patients with essential hypertension. *Hypertension* **34:** 113–117.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., *et al.* (2005) Diversity of the human intestinal microbial flora. *Science* **308:** 1635–1638.

Etminan, M., Carleton, B., Delaney, J.A., and Padwal, R. (2004) Macrolide therapy for *Chlamydia pneumoniae* in the secondary prevention of coronary artery disease: a meta-analysis of randomized controlled trials. *Pharmacotherapy* **24:** 338–343.

Forghani, B., Dupuis, K.W., and Schmidt, N.J. (1985) Rapid detection of herpes simplex virus DNA in human brain tissue by *in situ* hybridization. *J Clin Microbiol* **22:** 656–658.

Fredricks, D.N., Fiedler, T.L., and Marrazzo, J.M. (2005) Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med* **353:** 1899–1911.

Galan-Sanchez, F., Garcia-Martos, P., Rodriguez-Ramos, C., Marin-Casanova, P., and Mira-Gutierrez, J. (1999) Microbiological characteristics and susceptibility patterns of strains of *Rhodotorula* isolated from clinical samples. *Mycopathologia* **145:** 109–112.

Gomes, N.C., Fagbola, O., Costa, R., Rumjanek, N.G., Buchner, A., Mendona-Hagler, L., and Smalla, K. (2003) Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. *Appl Environ Microbiol* **69:** 3758–3766.

Grayston, J.T., Kronmal, R.A., Jackson, L.A., Parisi, A.F., Muhlestein, J.B., Cohen, J.D., *et al.* (2005) Azithromycin for the secondary prevention of coronary events. *N Engl J Med* **352:** 1637–1645.

Gupta, S., Leatham, E.W., Carrington, D., Mendall, M.A., Kaski, J.C., and Camm, A.J. (1997) Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. *Circulation* **96:** 404–407.

Gurfinkel, E., Bozovich, G., Beck, E., Testa, E., Livellara, B., and Mautner, B. (1999) Treatment with the antibiotic roxithromycin in patients with acute non-Q-wave coronary syndromes. The final report of the ROXIS Study. *Eur Heart J* **20:** 121–127.

Guttman, D.S., and Charlesworth, D. (1998) An X-linked gene with a degenerate Y-linked homologue in a dioecious plant. *Nature* **393:** 263–266.

Hansson, G.K. (2005) Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352:** 1685–1695.

Heuer, H., Wieland, G., Schönfeld, J., Schönwälder, A., Gomes, N.C.M., and Smalla, K. (2001) *Bacterial Profiling Using DGGE or TGGE Analysis*. Wymondham, UK: Horizin Scjientific Press.

Haupt, H.M., Merz, W.G., Beschorner, W.E., Vaughan, W.P., and Saral, R. (1983) Colonization and infection with *Trichosporon* species in the immunosuppressed host. *J Infect Dis* **147:** 199–203.

Hotchkiss, R.S., and Karl, I.E. (2003) The pathophysiology and treatment of sepsis. *N Engl J Med* **348:** 138–150.

Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180:** 4765–4774.

Jin, J., Gao, Z., Guo, L., Yang, J., and Yu, Y. (2003) Altered expression of zinc finger proteins, ADAMs, and integrin-related proteins following treatment of cultured human cells with a low concentration of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine. *Environ Mol Mutagen* **41:** 344–352.

Kerstens, H.M., Poddighe, P.J., and Hanselaar, A.G. (1994) Double-target *in situ* hybridization in brightfield microscopy. *J Histochem Cytochem* **42:** 1071–1077.

Khatib, R., Riederer, K.M., Ramanathan, J., and Baran, J., Jr (2001) Faecal fungal flora in healthy volunteers and inpatients. *Mycoses* **44:** 151–156.

Krause, R., Schwab, E., Bachhiesl, D., Daxbock, F., Wenisch, C., Krejs, G.J., and Reisinger, E.C. (2001) Role of *Candida* in antibiotic-associated diarrhea. *J Infect Dis* **184:** 1065–1069.

Lehtiniemi, J., Karhunen, P.J., Goebeler, S., Nikkari, S., and Nikkari, S.T. (2005) Identification of different bacterial DNAs in human coronary arteries. *Eur J Clin Invest* **35:** 13–16.

Li, X., Kolltveit, K.M., Tronstad, L., and Olsen, I. (2000) Systemic diseases caused by oral infection. *Clin Microbiol Rev* **13:** 547–558.

Mah, N., Thelin, A., Lu, T., Nikolaus, S., Kuhbacher, T., Gurbuz, Y., *et al.* (2004) A comparison of oligonucleotide and cDNA-based microarray systems. *Physiol Genomics* **16:** 361–370.

Makimura, K., Murayama, S.Y., and Yamaguchi, H. (1994) Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Med Microbiol* **40:** 358–364.

Mavromanolakis, E., Maraki, S., Cranidis, A., Tselentis, Y., Kontoyiannis, D.P., and Samonis, G. (2001) The impact of norfloxacin, ciprofloxacin and ofloxacin on human gut colonization by *Candida albicans*. *Scand J Infect Dis* **33:** 477–478.

Messner, R., and Prillinger, H. (1995) *Saccharomyces* species assignment by long range ribotyping. *Antonie Van Leeuwenhoek* **67:** 363–370.

O'Connor, C.M., Dunne, M.W., Pfeffer, M.A., Muhlestein, J.B., Yao, L., Gupta, S., *et al.* (2003) Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. *JAMA* **290:** 1459–1466.

Ott, S.J., Musfeldt, M., Wenderoth, D.F., Hampe, J., Brant, O., Folsch, U.R., *et al.* (2004) Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* **53:** 685–693.

Ott, S.J., El Mokhtari, N.E., Musfeldt, M., Hellmig, S., Freitag, S., Rehman, A., *et al.* (2006) Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. *Circulation* **113:** 929–937.

Pace, N. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276:** 734–740.

Pizzo, P.A. (1999) Fever in immunocompromised patients. *N Engl J Med* **341:** 893–900.

Ridker, P.M., Cannon, C.P., Morrow, D., Rifai, N., Rose, L.M., McCabe, C.H., *et al.* (2005) C-reactive protein levels and outcomes after statin therapy. *N Engl J Med* **352:** 20–28.

Ridker, P.M., Rifai, N., Rose, L., Buring, J.E., and Cook, N.R. (2002) Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* **347:** 1557–1565.

Rijnders, B.J., Wilmer, A., Van Eldere, J., and Van Wijngaerden, E. (2001) Frequency of transient streptococcal bacteremia following urgent orotracheal intubation in critically ill patients. *Intensive Care Med* **27:** 434–437.

Schloss, P.D., and Handelsman, J. (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71:** 1501–1506.

Schwieger, F., and Tebbe, C.C. (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16s rRNA gene-based microbial community analysis. *Appl Environ Microbiol* **64:** 4870–4876.

Simon, G.L., and Gorbach, S.L. (1984) Intestinal flora in health and disease. *Gastroenterology* **86:** 174–193.

Smit, E., Leeflang, P., Glandorf, B., Dirk van Elsas, J., and Wernars, K. (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl Environ Microbiol* **65:** 2614–2621.

Vainio, E.J., and Hantula, J. (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol Res* **104:** 927–936.

White, T.J., Bruns, T.D., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylognetics. In *PCR Protocols: A Guide to Methods and Applications*. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds). New York, USA: Academic Press, Harcourt Brace Jovanovich, pp. 315–322.

Wintzingerode, V., Göbel, U.B., and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21:** 213–229.

