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ORIGINAL ARTICLE

Genetic characterization of microbial communities living at the surface of building stones

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Abstract

Aims: The aim of the present study was to reveal the microbial genetic diversity of epilithic biofilms using a DNA-based procedure.
Methods and Results: A DNA extraction protocol was first selected to obtain PCR-amplifiable metagenomic DNA from a limestone biofilm. Extracted DNA was used to amplify either 16S rRNA genes or ITS regions from prokaryotic and eukaryotic genomes, respectively. Amplified DNAs were subsequently cloned, amplified by colony PCR and screened by restriction analysis [restriction analyses of amplified ribosomal DNA (ARDRA)] for DNA sequencing. Phylogenetic analysis using 16S rDNA sequences showed that predominating bacteria were *Alphaproteobacteria* belonging to the genera *Sphingomonas*, *Erythrobacter*, *Porphyrobacter*, *Rhodopila* and *Jannashia*; *Cyanobacteria* and *Actinobacteria* were also identified. Analysis of ITS rDNA sequences revealed the presence of algae of the *Chlorophyceae* family and fungi related either to *Rhinocladiella* or to a melanized ascomycete. Statistical analysis showed that the specific richness evidenced was representative of the original sampled biofilm.
Conclusions: The molecular methodology developed here constitutes a valuable tool to investigate the genetic diversity of microbial biofilms from building stone.
Significance and Impact of the Study: The easy-to-run molecular method described here has practical importance to establish microbiological diagnosis and to define strategies for protection and restoration of stone surfaces.

Introduction

Stone monuments and buildings are continuously exposed to weathering agents such as air, sunlight and rain. Although ineluctable, evolution of stone decay depends on its mineral composition and environmental conditions, mainly influenced by climate and human activities (Warscheid and Braams 2000). Moreover, besides physical and chemical factors, biological agents also play an important role in the deterioration of stone material. Knowledge of such processes is crucial to define strategies for restoration and conservation of historic monuments, and thus, is an economic issue (Wakefield and Jones 1998).

Micro-organisms classically found on building surfaces are autotrophic and heterotrophic bacteria, fungi, algae and lichens (Gorbushina 2007). This consortium of heterogeneous microbial species forms a biofilm whose internal cohesion and adhesion to underlying surface are ensured by extracellular polymeric substances. Main detrimental effects of microbial biofilms are: (i) discolouration of stone surface because of the presence of photosynthetic or protective pigments, (ii) physical alteration of the material structure by penetration of bacterial and fungal hyphae and by differential mechanical pressure imposed by shrinking and swelling cycles of the adhesive biofilms, and (iii) chemical modification of the mineral support by acidolytic and oxidoreductive corrosion processes generated by products of the microbial metabolism (Warscheid and Braams 2000).

The characterization of microbial communities colonizing stone surfaces is a prerequisite to monitor

deteriogenic micro-organisms and to set up suitable treatments to circumvent their undesirable effects. Because culture-dependent methods conduce inevitably to underestimate the microbial diversity (Ward *et al.* 1990), DNA-based typing methods have been widely used to study the microbial diversity of communities from various ecological niches. However, it should be noted that molecular techniques share some limitations that are mainly caused by inherent biases of the PCR technology, as primer specificity or formation of chimeric sequences (Nocker *et al.* 2007). As yet, only few reports mentioned their use in the genotyping of biofilms present at the surface of building stones (Gonzàlez and Saiz-Jiménez 2005). Moreover, all previous molecular studies were focused on specific components of stone biofilms – for example, bacteria (Zimmermann *et al.* 2005; McNamara *et al.* 2006; Imperi *et al.* 2007) or fungi (Möhlenhoff *et al.* 2001) – but to our knowledge, studies concerning the entire consortium remained very scarce. The objective of this study is to develop an easy-to-run molecular procedure suitable to assess the genetic diversity of the overall microbial community from building stone biofilms.

Materials and methods

Sampling

Sampled microbial biofilms appeared as rosy areas on the face-wall of 'Villa Isabel', a private house located in the centre of Biarritz city (France). Samples were taken by scraping off limestone material with sterile scalpels and microtubes, maintained at 4°C during the transport to the laboratory and stored at −80°C until use.

DNA extraction and purification

DNA extractions were carried out using two commercial kits: the Easy-DNA™ kit (Invitrogen, Carlsbad, CA, USA) and the UltraClean™ Soil DNA Isolation kit (MoBio Laboratories Inc, Solana Beach, CA, USA), following furnisher's instructions. The two conditions of cell lysis proposed by the furnisher of the later DNA extraction kit were essayed, consisting in submitting cells either to bead beating for 5 min at room temperature or to four cycles of heating (65°C, 5 min) and brief (5 s) bead beating (soft lysis). In any cases, DNA extractions started by resuspending 100 mg of sample material in lysis solution.

rDNA amplification

The 16S rDNA was amplified by PCR using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′), previously designed by Weisburg *et al.* (1991). For PCR amplification of the ITS region, primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used (White *et al.* 1990). PCRs were carried out in 50 μl final volume containing 20–50 ng of extracted DNA, 20 pmol of each primer, 100 μmol l^{-1} of each deoxyribonucleotide triphosphate, 5 μl of 10× buffer (500 mmol l^{-1} KCl, 15 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} Tris–HCl pH 8·3) and 2·5 U of Taq DNA polymerase (5 PRIME Inc., Gaithersburg, MD, USA). PCR was performed in a robocycler (Themocycler PTC-100; MJ Research, Waltham, MA) with the following thermocycling programme: 5 min denaturation at 94°C, followed by 35 cycles at 94°C for 1 min, 48°C for 1 min and 72°C for 1 min. 10 min at 72°C was used as a final extension step. Amplified DNA was separated by electrophoresis in TEA 1% w/v agarose gel, cut out and purified on column using the GFX™ PCR and DNA Gel Band Purification kit (GE Healthcare Life Sciences, Uppsala, Sweden). 16S rDNA PCR amplifications gave an amplimer of 1500 bp. ITS rDNA PCR amplifications yielded two amplimers of 650 and 750 bp, which were grouped before purification.

rDNA cloning

Four microlitres of purified PCR products was cloned with the pCR 2·1-TOPO vector (Invitrogen), following the manufacturer's protocol. The ligation mixture was transformed into One Shot® TOP10 Electrocomp™ *E. coli* cells (Invitrogen), and recombinant clones were selected on LB solid medium containing ampicillin (100 μg ml^{-1}) and X-Gal (0·1 mmol l^{-1}).

Inserts amplification and purification

White colonies randomly picked were screened for inserts by performing colony PCR with the vector specific primers M13 F (5′-GTAAAACGACGGCCAG-3′) and M13 R (5′-CAGGAAACAGCTATGAC-3′). PCRs were carried out in 25 μl final volume containing 20 pmol of each primer, 100 μmol l^{-1} of each deoxyribonucleotide triphosphate, 2.5 μl of 10× buffer, 1·25 U of Taq DNA polymerase (5 PRIME Inc.). Thermal cycling conditions were as follows: 10 min denaturation at 94°C, followed by 35 cycles at 94°C for 45 s, 56°C for 45 s and 72°C for 1 min. A final extension step of 10 min at 72°C was applied. The amplimer of all clones containing inserts of the correct size was purified using GFX™ PCR and Gel Band Purification kit (GE Healthcare Life Sciences), following the manufacturer's protocol.

Restriction analyses of amplified ribosomal DNA (ARDRA)

Purified amplimers were separately digested with restriction enzymes *Hinf*I and *Hae*III (10U, New England Biolabs) for 4 h at 37°C. The restricted DNA fragments were separated on a 1·5% w/v agarose gel containing ethidium bromide (0·5 μg ml^{-1}). After migration in TBE buffer at 25 V/h for 1 h 30 min, DNA fragments were visualized under UV illumination at 312 nm. The restriction profiles were captured as JPEG format with a CDD camera and compared manually for grouping clones into ribotypes.

Sequencing and phylogenetic analysis

rDNA inserts representative of each distinct ARDRA pattern were selected and sequenced with Big Dye Terminator ver. 1·1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Concretely, one DNA clone was sequenced when the number of clones grouped under the same ARDRA pattern ranged between 1 and 3, two when it ranged between 4 and 10, three when it ranged between 10 and 20, four when it ranged between 20 and 30 and five when it encompassed 30. Sequencing reactions were analysed with ABI 330X sequencer at the Genotyping and Sequencing facility of Bordeaux (France). The presence of possible chimeric DNA sequences was investigated by using the CHIMERA_CHECK program of the Ribosomal Database Project II (Cole *et al.* 2003). Clones containing chimeric sequences were excluded from further analysis. All DNA sequences were compared with sequences in the Genbank/EMBL/DDBJ database using BLASTN (Basic Local Alignment Search Tool, http://blast.ncbi.nih.gov/Blast.cgi). 16S or ITS rDNA clones were clustered into phylotypes at an overlap percentage identity cut-off of 97 using CLUSTALW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html?). Evolutionary distances were calculated by the Kimura-2-parameter algorithm, and the phylogenetic trees were constructed by the neighbour-joining method, using MEGA3 program, ver. 3·0 (Kumar *et al.* 2004). Confidence in tree topology was determined by bootstrap analysis using 100 resamplings of the sequence. Sequences reported in this study can be accessed under numbers FJ028664 to FJ028676 and FJ028701 to FJ028712.

Statistical analysis of phylotype richness from DNA clone libraries

To assess whether DNA clone libraries were large enough to be representative of phylotype richness from environmental samples, we used the web interface constructed by Kemp and Aller (2004) and located at http://www.aslo.org/lomethods/free/2004/0114a.html. Author instructions were followed for calculations using the abundance-based richness estimator S_{Chao1} and Good's C index of coverage (Kemp and Aller 2004).

Results

Whole community DNA extraction from epilithic biofilms

Comparison of DNA preparations obtained from the different procedures (Table 1) showed that DNA extraction with the UltraClean™ Soil DNA Isolation kit using a soft cell lysis procedure yielded pure and stable DNA of high molecular weight from stone microbial biofilms.

Phylogenetic analysis of 16S and ITS rDNA

Analysis of ARDRA patterns from cloned 16S rRNA genes (SLB clones) and ITS regions (ILB clones) allowed to group 67 SLB clones into eight ribotypes and 76 ILB clones into three ribotypes (Figs 1 and 2). In order to further characterize microbial communities from building stone biofilms under study, DNA clones with identical ARDRA patterns were randomly selected and sequenced. A total of 27 clones were partially sequenced, two of them contained chimeric sequences and were discarded from phylogenetic analysis. Pairwise alignments of DNA clone sequences using 97% similarity as the cut-off allowed to discriminate 11 phylotypes: SLB clones clustered into eight phylotypes and ILB clones into 3 (Figs 1 and 2).

Phylogenetic analysis of SLB clones (16S rRNA genes from limestone biofilm, Fig. 1) revealed that 40% of clones were distantly related to chloroplast rDNA sequences of an unknown alga (79% identity with chloroplastic rDNA sequence of *Monomorphina pyrum*). Other clones belonged to *Alphaproteobacteria* division (55%), *Cyanobacteria* (3%) and *Actinobacteria* (2%). The two most important groups of sequences that fell into the *Alphaproteobacteria* division were related to *Sphingomonas* sp. *KIN169* (20 clones, 93–95% identity) and to *Erythrobacter* sp. *R14* (11 clones, 96–100% identity), two species of the *Sphingomonadaceae*

Table 1 DNA extraction protocol comparison

DNA extraction protocol	Purity (A_{260}/A_{280})	DNA fragment length	Stability*
Easy-DNA™ kit	1·7–1·8	>23 kbp	No
UltraClean™ Soil DNA Isolation kit	1·7–1·8	~20 kbp	Yes
UltraClean™ Soil DNA Isolation kit with soft lysis	1·7–1·8	>23 kbp	Yes

*Estimated by electrophoresis analysis after a storage for 6 days at 4°C.

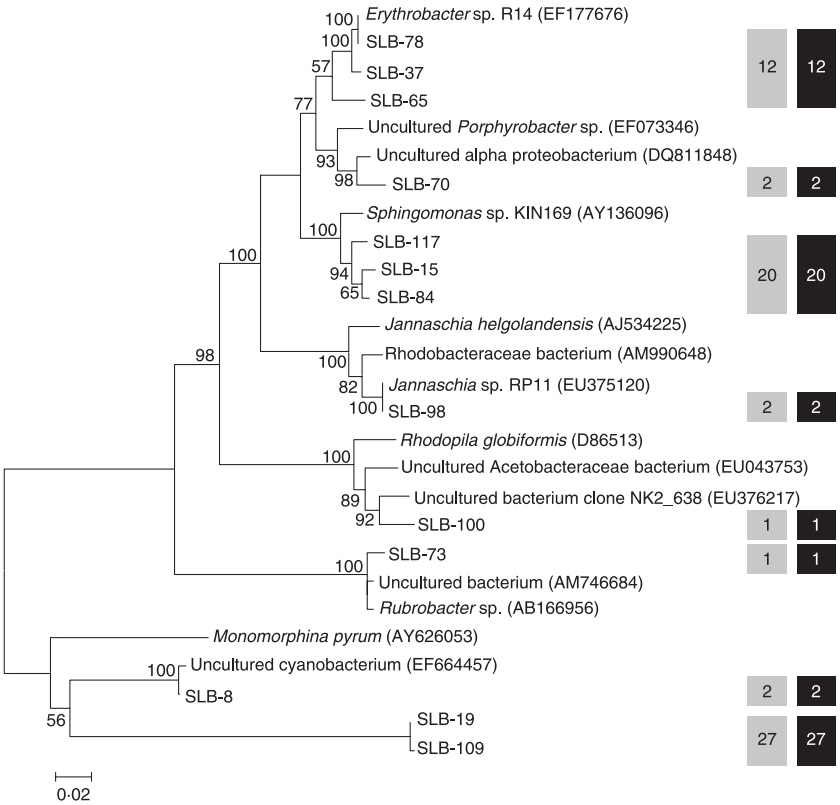

Figure 1 Phylogenetic tree of partial 16S rDNA gene sequences (613 bp) of SLB clones isolated from microbial community of a limestone biofilm. Percentages of 100 bootstrap resamplings that support branching points above 50% confidence are indicated. GenBank accession numbers are given in parentheses. The number of clones having the same ribotype is indicated within grey boxes. The number of clones grouped into the same phylotype is given within black boxes. Scale bar represents 0·02 nucleotide changes per position.

family. Among minor groups of sequences also belonging to *Alphaproteobacteria* division, one is similar (97%) to an uncultured bacterium belonging to the genus *Porphyrobacter*, a second to an uncultured bacterium of the *Rhodopila* genus (95%) and a third to *Jannaschia* sp. *RP11* (99% identity). The clone SLB-73, affiliated to *Actinobacteria* (High GC gram-positive bacteria), is closely related (98% identity) to an uncultured bacterium of the genus *Rubrobacter* already identified on limestone monuments (Ortega-Morales *et al.* 2004). The 16S rDNA sequences of two clones matched (98% identity) with that of an uncultured cyanobaterium.

DNA sequencing of eukaryotic rDNA sequences (ITS regions) isolated from limestone biofilm allowed to separate ILB clones into three phylotypes (Fig. 2). The most abundant comprised 37 clones that were affiliated to an uncultured chlorobiont of the *Chlorophyceae* family (94% identity). The two other phylotypes contained fungal ITS sequences related either to *Rhinocladiella* sp. (24 clones, 86–95% identity) or to a melanized limestone ascomycete (15 clones, 95% identity).

Reliability of phylotype richness

Phylotype richness (S_{Chao1} estimator) was compared against sampling effort (library size) using the free software tool developed by Kemp and Aller (2004). The obtained curves (Fig. 3) showed that in all cases, richness estimates reached a stable asymptotic value indicating that the number of clones analysed were sufficient to yield a reliable estimate of phylotype richness. Richness estimates (9·9 and 3 for SLB and ILB clone library, respectively) were identical or slightly greater than the actual number of phylotypes (9 and 3 for SLB and ILB clone library, respectively). Calculations of Good's C indexes (Fig. 3) confirmed that clone libraries were large enough to capture most of the diversity of microbial biofilms sampled. Coverage was 0·91 and 1 for SLB and ILB clone library, respectively.

Discussion

The goal of this study was to develop a molecular methodology, based on a single DNA extraction procedure and to assess the genetic diversity of all members (prokaryotes and eukaryotes) of microbial communities living at the surface of building stones. As previously reported for samples derived from soil or concrete (Yeates and Gillings 1998; Giannantonio *et al.* 2009), the bead beating DNA extraction procedure we used in this study produced high quality DNA suitable for PCR amplification and cloning of either prokaryotic 16S rRNA genes or eukaryotic ITS regions. It appeared efficient to isolated DNA from any

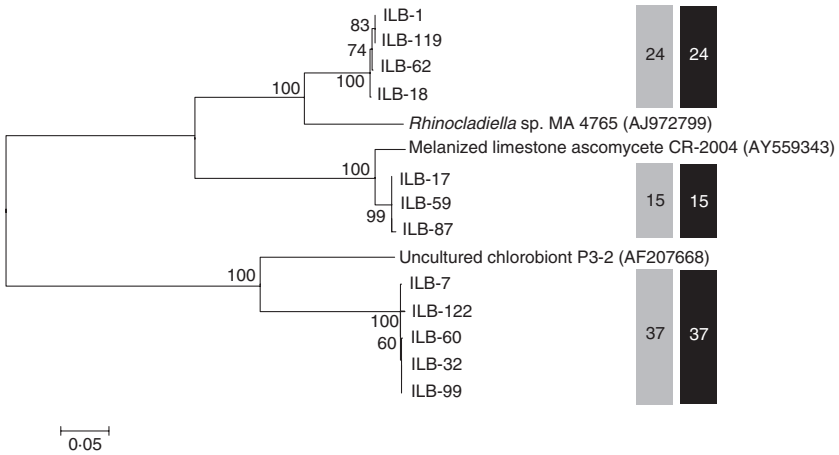

Figure 2 Phylogenetic tree of partial ITS region sequences (513 bp) of ILB clones isolated from microbial community of a limestone biofilm. Percentages of 100 bootstrap resamplings that support branching points above 50% confidence are indicated. GenBank accession numbers are given in parentheses. The number of clones having the same ribotype is indicated within grey boxes. The number of clones grouped into the same phylotype is given within black boxes. Scale bar represents 0·05 nucleotide changes per position.

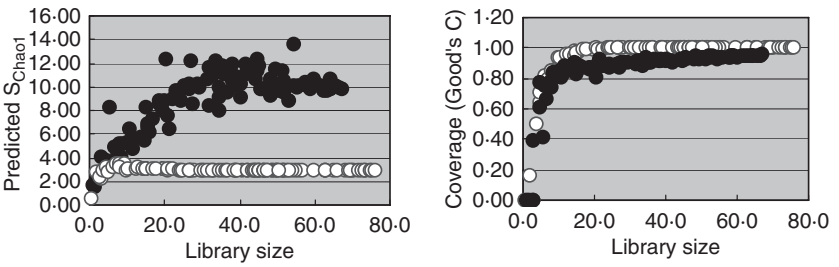

Figure 3 Estimated phylotype richness (left panel) and coverage (right panel) *vs* library size for the 16S rDNA clone library (SLB clones, filled symbols) and the ITS rDNA clone library (ILB clones, open symbols).

micro-organism present at the surface of building stone, including DNA from organisms that are hard to lyse, such as *Actinobacteria* (Kauffmann *et al.* 2004) or thick-walled *Cyanobacteria*. Although *Cyanobacteria* were poorly represented in the studied biofilm, this could not be because of a lack of the DNA extraction method because its use to characterize a sandstone biofilm containing 60% of *Cyanobacteria* was conclusive (data not shown).

Phylogenetic analysis of partial 16S rRNA gene sequences showed that *Alphaproteobacteria* are predominant in the studied limestone biofilm, as in Altamira cave biofilms within which they have been considered as the most metabolically active group (Portillo *et al.* 2009). Most of them are affiliated either to the halotolerant *Sphingomonas* sp. (Pinhassi and Berman 2003) or to the marine *Erythrobacter* sp. (Yurkov and Beatty 1998), indicating that most of bacteria present within the investigated biofilm are well adapted to the coastal environment where they originate from. Phototrophs such as *Cyanobacteria* and algae where also identified. They are supposed to be the first colonizers, and so, to support the growth of subsequent heterotrophs (bacteria and fungi). Analysis of ITS region of rRNA genes confirmed the presence of algae and allowed to identify two ascomycetous fungi previously described as typical inhabitants of stone surfaces (Chertov *et al.* 2004; Sert *et al.* 2007). On the whole, the combined 16S and ITS DNA analysis revealed a relatively low microbial diversity (11 phylotypes). However, this result is not surprising, as it could be assumed that stone surfaces are oligotrophic substrata. Comparable specific richnesses were reported following DNA analyses of microbial biofilms from frescoes or concrete surfaces (Imperi *et al.* 2007; Giannantonio *et al.* 2009). Statistical evaluation of phylotype richness of 16S and ITS rDNA clone libraries confirmed that microbial diversity evidenced in our study was representative of the original stone biofilm. As mentioned by Kemp and Aller (2004), such an evaluation is missing in the most of the published works dealing with the assessment of microbial diversity.

Concerning the methodology used in this study, our results showed that clones grouped under a same ARDRA pattern were closely related to each other, and thus, confirmed that ARDRA profiling is a good screening tool to identify clones of interest for sequencing (Nocker *et al.* 2007). This method of screening has often been used to investigate the microbial diversity living at the surface of stone material (Imperi *et al.* 2007; Giannantonio *et al.* 2009), but DGGE has frequently been used too (Möhlenhoff *et al.* 2001; Imperi *et al.* 2007); besides this, some studies have been performed by direct sequencing of rDNA cloned sequences (Zimmermann *et al.* 2005; McNamara *et al.* 2006). Such a practice may become more and more spread as costs for DNA sequencing are decreasing.

In conclusion, whether a screening step is used or not, the development of any molecular procedure suitable to assess the genetic diversity of microbial communities needs

to ensure that DNA isolation is efficient for the totality of members who make up the consortium and that the number of analysed clones is sufficient to yield a genetic diversity representative from the original sample. As it was the case for the molecular methodology described here, we adopted it for further studies. Its use in preliminary studies designed to follow microbial colonization of building stone revealed that it was convenient to identify micro-organisms even on poorly colonized stones (data not shown).

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