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Molecular characterizations of microbial communities fouling painted and unpainted concrete structures

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ABSTRACT

This study reports the use of culture-independent and culture-dependent approaches to identify naturally occurring communities of *Bacteria* and *Fungi* fouling the surfaces of concrete structures with and without an acrylic paint coating in Georgia, USA. Genomic DNA was extracted from four different sites and PCR amplification of bacterial ribosomal RNA (16S rRNA) genes and the internal transcribed spacer (ITS) region of fungal rRNA genes was conducted. Bacterial and fungal community composition was determined by restriction analysis of amplified DNA of eight clone libraries and sequencing. Five bacterial phyla were observed, and representatives of the phylum *Cyanobacteria* and the classes *Betaproteobacteria* and *Gammaproteobacteria* dominated the bacterial clone libraries. The ITS region of rRNA gene sequences revealed the dominant phylotypes in the fungal clone libraries to be most closely related to *Alternaria*, *Cladosporium*, *Epicoccum* and *Udeniomyces*. The majority of these fungal genera could be cultured from the sites and successfully used to foul concrete in laboratory-based experiments. While the fungal sequences were most closely related to cultured isolates, the vast majority of bacterial sequences in the libraries were related to uncultured environmental clones. Results show phylogenetically distinct microbial populations occurring at the four sites.

1. Introduction

Microorganisms that foul concrete and stone surfaces can have deleterious effects on structural integrity and aesthetic appeal (Dubosc et al., 2001). Phylogenetically diverse microorganisms have been reported to colonize these surfaces, including *Cyanobacteria* that appear as black crusts on historic buildings in Latin America (Gaylarde et al., 2007), and *Chlorophyceae* and *Cyanobacteria* that coat the exterior surfaces of buildings in the south of France (Dubosc et al., 2001). Examples of surface fouling fungi include *Cladosporium* spp. that colonize the interior mortars of buildings (Shirakawa et al., 2003), as well as *Phoma leveillei* and *Trichoderma citrinoviride* isolated from natural stone surfaces (Bartosch et al., 2003). Communities of bacteria (e.g. *Pseudomonas*, *Bacillus*, and *Xanthomonas* spp.) and fungi (e.g. *Cladosporium*, *Alternaria*, and *Fusarium* spp.) have also been found to co-inhabit limestone surfaces (Mitchell and Gu, 2000). The diversity of microorganisms that inhabit these particular surfaces may be dependent upon environmental factors (e.g. temperature and humidity) as well as the nature of the surface itself (e.g. the presence of paint) (Gaylarde and Gaylarde, 2005).

Biologically mediated deterioration of concrete structures can occur by different mechanisms, such as through the alteration of surface pH (Sand, 1997). Sulfur-oxidizing bacteria have been shown to deteriorate concrete sewer pipes through the generation of sulfuric acid (Parker, 1945; Milde et al., 1983; Sand and Bock, 1991; Nica et al., 2000). Similarly, concrete surfaces can be corroded by biogenic nitrous and nitric acid produced by nitrifying bacteria (Sand, 1997). A *Fusarium* species has also been shown to expedite the deterioration of concrete through the surface penetration of hyphae and possible organic acid production (Gu et al., 1998). *Fungi* such as *Penicillium frequentans* and *Cladosporium cladosporioides* have, however, been found to damage stone surfaces by releasing acids and other metabolites, respectively (De La Torre et al., 1993). While the microorganisms fouling many of these concrete and stone surfaces have been identified based on morphological attributes and their mechanisms of deterioration characterized, studies in this particular field have relied primarily on culture-based techniques for microbial characterizations. Although this approach is valuable in obtaining isolates, it can only identify a small fraction of the microbial community, as most microorganisms are resistant to cultivation (Staley and Konopka, 1985). Molecular methods, such as 16S ribosomal RNA (16S rRNA) gene analysis, have been used to identify microbial communities from a variety of environments (Horton and Bruns, 2001; Mills et al., 2003; Martinez et al., 2006; Michaelsen et al., 2006). To date, few studies have conducted

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molecular-based characterizations of the microbial communities on concrete and stone surfaces (Crispim et al., 2006). Recently, the molecular diversity of endolithic microbial communities inhabiting the pore space in rocks has been described (McNamara et al., 2006; Norris and Castenholz, 2006; Walker and Pace, 2007).

This study reports the use of both culture-independent and culture-dependent techniques to identify *Bacteria* and *Fungi* fouling concrete structures in the state of Georgia, USA. To identify microbial communities, total genomic DNA was extracted from four geographically distinct sites and subjected to PCR by amplifying 16S rRNA genes and the internal transcribed spacer (ITS) region (i.e. ITS1, 5.8S and ITS2) of fungal rRNA genes, an increasingly used sequence region for fungal molecular taxonomy studies (Torzilli et al., 2006; Midgley et al., 2007). Restriction fragment length polymorphism (RFLP) and phylogenetic analyses were performed on the amplified rRNA genes to determine the microbial community composition at each site. Culturable isolates determined to be among the most frequently detected fungal genera by molecular-based analysis were tested for their ability to foul concrete under controlled laboratory conditions. Our results indicate that distinct microbial communities are present at sites exhibiting visibly similar fouling appearances. We also confirmed that the culturable fungal isolates obtained are capable of causing biofouling when tested in laboratory-based experiments.

2. Materials and methods

2.1. Site description and sampling

Four outdoor concrete sites located throughout the state of Georgia, USA (Atlanta, 33°48′ N, 84°22′ W; Gainesville, 34°15′ N, 83°57′ W; LaGrange, 33°03′ N, 84°57′ W; Savannah, 32°13′ N, 81°37′ W) and a control site north of Atlanta (33°52′ N, 84°27′ W) that lacked any visible fouling were chosen for study. A coating of acrylic paint was present at two of the sites, LaGrange and Savannah. All four of the study sites had visibly fouled surface characteristics, black crusts on the concrete appearing as either vertical striations or covering the entire surface. To collect samples for DNA extraction, 5–10 strips (5 cm × 1 cm) of heterotrophic plate count agar (Difco, USA) and potato dextrose agar (EMD, USA) were aseptically pressed against the concrete surfaces. Concrete was also chipped directly off the surfaces for environmental scanning electron microscopy (ESEM) and photo-pigment extractions. Samples were stored at 4 °C until DNA extraction (1–3 days). Additional agar strips of the same media type were used to culture isolates, which were collected from concrete surfaces as described above. These agar strips, however, were placed directly onto solid media, incubated at 25 °C for 7 days and re-streaked multiple times to ensure purity. Purity was verified microscopically and by molecular analysis of the ITS region. The pH of the study sites were determined using a mixture of 1% phenolphthalein, 80% ethanol, and 19% water (v/v) was sprayed onto the surface of the sites and the underlying concrete (McPolin et al., 2007).

2.2. DNA extraction and PCR amplification

DNA was extracted and purified from agar strip samples taken from the concrete surfaces using an UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.) per manufacturer's instructions. Bacterial 16S rRNA genes were amplified as previously described (Martinez et al., 2006). The ITS region of fungal rRNA genes were amplified using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990). Three separate DNA extractions per sampling site were performed and samples were pooled. PCR reactions were performed using GoTaq® Green Master Mix (Promega), 2.5 μM of each primer and 50 ng extracted DNA. Amplification of 16S rRNA genes and ITS region of rRNA genes were performed as previously described (Mills et al., 2003; Michaelsen et al., 2006). Amplified PCR products were separated on 1.0% agarose gels and purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.3. Clone library construction and RFLP grouping

Clone library construction and RFLP analysis was performed as previously described (Mills et al., 2005; Martinez et al., 2006). Briefly, purified amplicons were cloned into the TOPO cloning vector pCR2.1 and transformed into TOP10 electrocompetent cells according to the manufacturer's instructions (Invitrogen, USA). Two clone libraries (16S and ITS) per site were constructed. Inserts were subsequently PCR amplified from lysed colonies with vector-specific primers M13F (5′-GTA AAA CGA CGG CCA G-3′) and M13R (5′-CAG GAA ACA CGT ATG AC-3′), which were used in order to prevent amplification of the host *Escherichia coli* 16S rRNA gene. PCR products from bacterial and fungal rRNA gene libraries were double digested with HhaI/MspI and HaeIII/RsaI (Promega, USA), respectively. Clones were grouped according to RFLP banding patterns, and representative clones were sequenced from RFLP groups containing three or more members. Clones containing 16S rRNA genes were sequenced using primers as previously described (Mills et al., 2003). Clones containing the ITS region of rRNA genes were sequenced using primers M13F and M13R. Sequencing was performed at the Georgia Tech core DNA facility using a BigDye Terminator v3.1 Cycle sequencing kit on an automated capillary sequencer (model 3100 Gene Analyzer, Applied Biosystems).

2.4. Phylogenetic and rarefaction analysis

Phylogenetic and rarefaction analysis was performed as previously described (Martinez et al., 2006). Sequences obtained for individual clones were aligned and assembled using the program BioEdit v7.0.9.0 (Hall, 1999). Sequences were checked for chimeras using Chimera Check from the Ribosomal Database Project II (Maidak et al., 1999). Sequences from this study, as well as reference sequences obtained from the GenBank database provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), were aligned and neighbor-joining trees constructed in MEGA 4 (Tamura et al., 2007) using the Jukes–Cantor model (Jukes and Cantor, 1969). An average of 1500 nt for 16S sequences, and 600 nt for ITS sequences were used in all analyses. Bootstrap data represent a percentage of 1000 samplings. Rarefaction analysis was performed using equations as previously described (Heck et al., 1975). Sequences have been deposited in the GenBank database under accession numbers EU409843–EU409865 and EU409876–EU409893.

2.5. Biofouling laboratory experiments

Representative fungal isolates obtained from the four sample sites via agar strips were cultured using potato dextrose agar (EMD, USA) and identified by analysis of the ITS region of rRNA genes as described above. Three isolates, most closely related to *Cladosporium cladosporioides*, *Epicoccum nigrum*, and an *Alternaria* sp., were selected for biofouling assays. Concrete tiles (made with commercially available Type I/II cement and a water/cement ratio of 0.50 by mass) with approximately 36 cm^2 exposed surface area were cast. Tiles were carbonated in a chamber (Nuaire US Autoflow NU-4850) at 20% CO_2, 40 °C, and 55% relative humidity for 30 days to reduce their surface pH. Tiles were sterilized by autoclaving prior to inoculation. Incubation chambers capable of spraying liquid media onto concrete tile surfaces and recycling runoff were constructed and sterilized by flushing with 10% bleach solution (Kurth, 2008). Individual fungal strains were resuspended in 0.85% saline and 100 μl was used to inoculate the surface of tiles. Incubations were run for 1 week at 25 °C, with 300 ml of growth media (i.e., either 20% potato dextrose broth (Difco, USA) or sterile water) sprayed onto tile surfaces and recycled over 6-h time intervals. Relative humidity of incubation chambers was 100% during spray cycles, and 95% relative humidity during non-spray cycles. Following incubation, tiles were stored at 25 °C until imaged by ESEM within 14 days.

2.6. Chlorophyll and carotenoid detection

To determine if fouled concrete samples contained photosynthetic pigments, which would indicate the presence of photoautotrophs, chlorophyll and carotenoid extraction procedures were conducted essentially as described in Parsons et al., 1984 with minor modifications. Triplicate powdered concrete samples (5 g) obtained from each site were ground and suspended in 15 ml sterile saline and filtered onto 0.22-μm membrane filters (Millipore). Filters were suspended in 90% acetone and incubated at 4 °C in the dark for 12 h. The resulting solution was centrifuged for 5 min at 5000 rpm and photometrically quantified as previously described (Parsons et al., 1984). Controls were conducted that included using known chlorophyll *a* standards (Turner Designs, USA) and extractions of clean (non-fouled) concrete samples and clean concrete samples mixed with known concentrations of pigment standard to validate the procedure and to ensure that the concrete suspension did not significantly quench or inhibit chlorophyll measurements.

2.7. Environmental scanning electron microscopy

Surface samples from each site as well as concrete tiles inoculated with fungal isolates were imaged on a FEI Quanta 200 environmental scanning electron microscope (ESEM) at the Tennessee Technological University (Cookeville, TN). Samples were examined in an 85% relative humidity atmosphere at stage temperature 5 °C and 733.3 Pa. Samples from concrete biofouling experiments were gold-sputtered and viewed in high vacuum mode to maintain adherence of the abundant growth for high resolution imaging.

3. Results

3.1. Study site characteristics

Visual observations during sampling at each of the four sites indicated that fouling appeared apparently exclusively on the

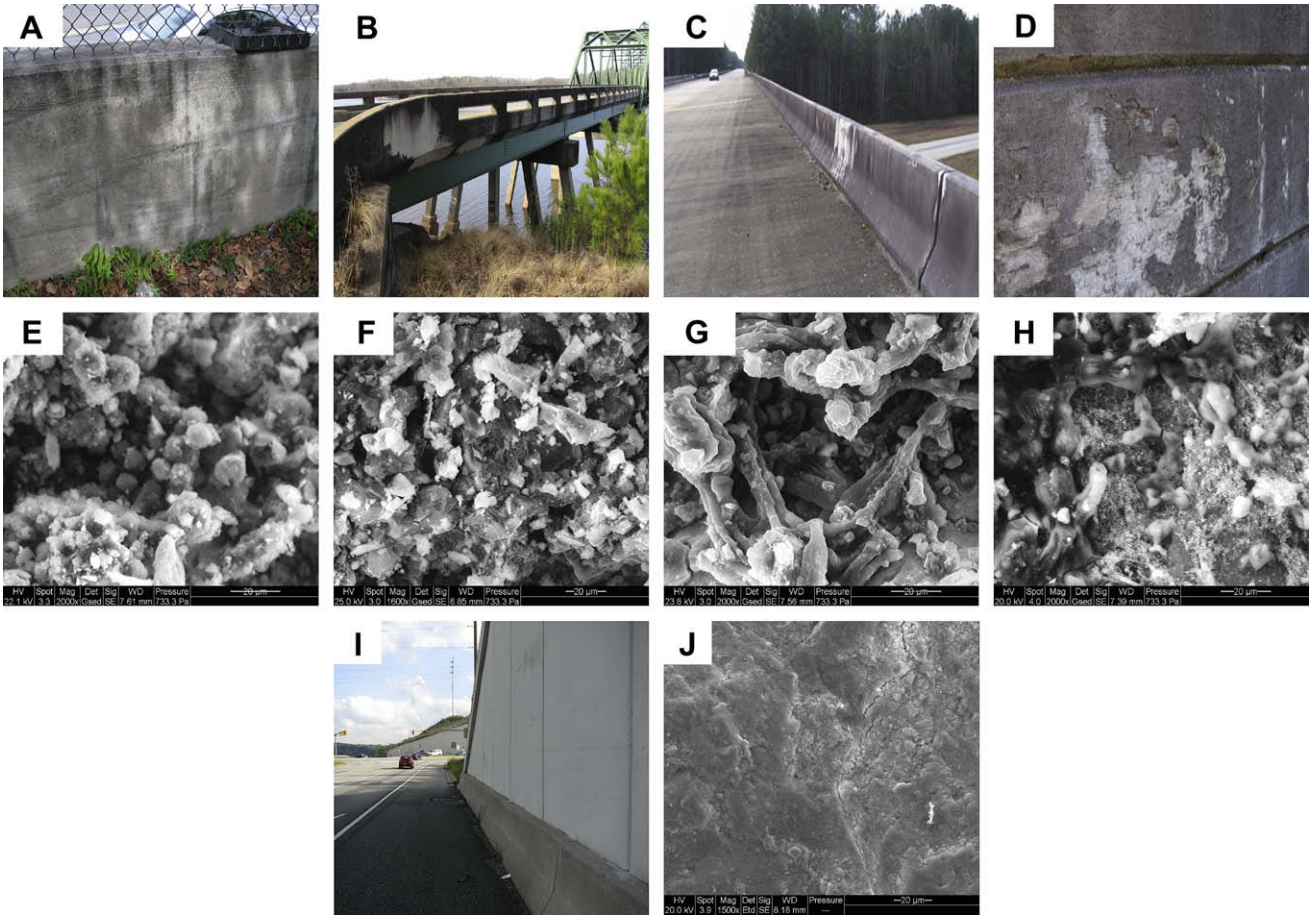

Fig. 1. Examples of fouled concrete surfaces (A–D). (A) Atlanta site with black biofilm on unpainted surface. (B) Gainesville site with black crust on unpainted surface. Note removal of the crust by power washing as seen in the lighter areas. (C) LaGrange site with black crust on painted concrete surface. Lighter areas denote spots that have been cleaned. (D) Savannah site with black crust on painted surface. Note areas of peeling show underlying concrete with no visible biofilms. Examples of ESEM images from samples collected at: (E) Atlanta; (F) Gainesville; (G) LaGrange; (H) Savannah. Example of a non-fouled surface (I) and corresponding ESEM (J).

surface (Fig. 1A–D), and that the underlying concrete was devoid of any apparent discoloration (i.e., Fig. 1D). At the LaGrange and Savannah sites fouling occurred mainly on surface coatings composed of paint, which could easily be peeled from the structure (Fig. 1C,D). The amount of fouling (measured as the percentage of surface coverage over a 2 m^2 sample site area) typically appeared greatest at those sites with surface coatings. The pH of all the concrete surfaces was less than 9.0, while underlying concrete surfaces were more alkaline (>9.0), as expected.

3.2. Phylogenetic diversity of bacterial communities

The composition of the bacterial communities was determined by 16S rRNA gene phylogenetic analysis. PCR amplifiable DNA was recovered from all four of the sampled sites. DNA was not recovered from samples collected from a control site (i.e., no visible fouling; Fig. 1I). A total of 180 clones representing the four sites were grouped into 40 RFLP patterns (data not shown). Rarefaction analysis was applied to determine if a sufficient number of clones were screened to estimate diversity within each of the clone libraries sampled (Fig. 2A). For those bacterial clones obtained from the Atlanta site, the curve indicated saturation (Fig. 2A). Thus, a sufficient number of clones were sampled representative of the diversity of the bacteria in this library. Numerically dominant RFLP groups, ranging from 8% to 23% of all clones were obtained for each of the four libraries (Table 1). In contrast, rarefaction curves generated for rRNA gene clones obtained from Gainesville, LaGrange and Savannah did not indicate saturation (Fig. 2A). Although additional sampling of clones would be needed to reveal the full extent of the diversity, numerically dominant RFLP groups were obtained (Table 1). Specifically, one dominant group of clones from each of the libraries (Gainesville, LaGrange and Savannah, represented by RFLP groups 1, 18, and 30, respectively) comprised 12%, 17% and 8% of all clones, respectively (data not shown).

Analysis of 156 *Bacteria* clones from RFLP groups with three or more members revealed a greater diversity relative to the fungal clone library (Table 1) and included predominately uncultured bacterial lineages (Fig. 3). A considerable majority of the clone sequences (102 of 156) were representative of the phylum *Proteobacteria*, (Table 1). Of these, 75% were related to the *Gammaproteobacteria*, including the most numerically dominant phylotype represented by the clone 16A1 (23% of the total clone library; Table 1). This phylotype, present at the Atlanta, Gainesville and Savannah sites, was most closely related (99% similar) to a non-cultured organism (uncultured clone ctg_CGOF104; Penn et al., 2006) obtained from deep-sea coral on a Gulf of Alaska seamount (Fig. 3). The second most dominant phylotype (17% of all clones; Table 1), belonging to the *Cyanobacteria*, was most closely related (93% similar) to a non-cultured organism obtained from a spacecraft assembly clean room (uncultured clone JSC2-A6; Moissl et al., 2007) (Fig. 3). This phylotype, represented by clone 16L3, was only found at the LaGrange site (Table 1). The third most dominant phylotype (8% of all clones; Table 1), found

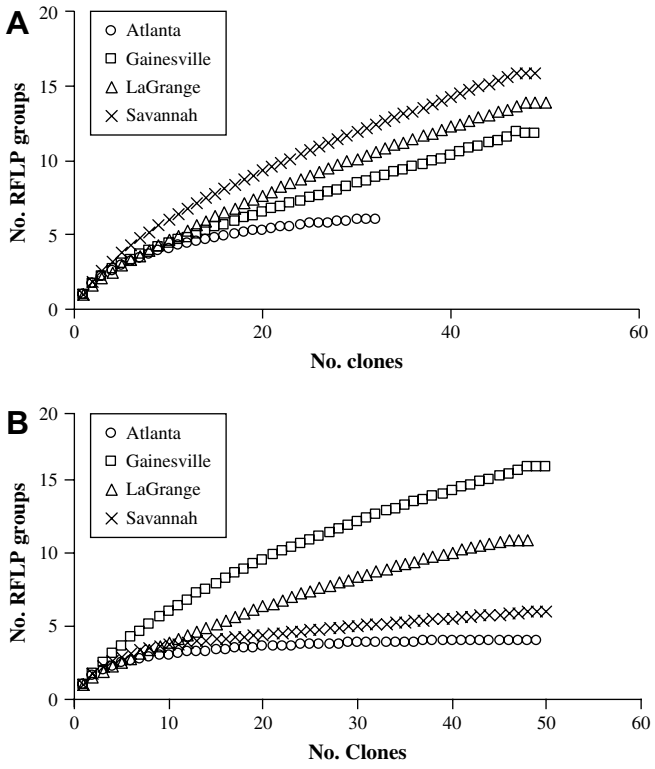

Fig. 2. Rarefaction curves determined for the different RFLP patterns of (A) bacterial 16S rRNA gene clones and (B) ITS region of fungal rRNA gene clones obtained from each study site.

in Savannah, was most closely related (99% similar) to *Pantoea ananatis* BD 561 (Fig. 3), a gammaproteobacterium found to be the causal agent of brown stalk rot in maize grown in South Africa (Goszczynska et al., 2007).

Comparisons of bacterial community diversity between concrete sites without (Fig. 4A,B) and with (Fig. 4C,D) acrylic paint coating were also conducted. Although there were differences in the diversity patterns between unpainted and painted sites, there were also different patterns amongst all four sites (Fig. 4A–D). For example, clone 16A1 (Table 1), the most frequently detected phylotype, was recovered from painted and unpainted sites (Fig 4A,B,D), while the second most frequently detected phylotype, 16L3, was only detected at one of the two sites with paint coating (Fig. 4C). However, additional bacterial lineages were also found to occur on one or both surfaces, and/or at one of the four sites (Table 1 and Fig. 4A–D).

3.3. Phylogenetic diversity of fungal communities

The composition of fungal communities was determined by examining a total of 197 clones containing the ITS region of rRNA genes, which grouped into 34 RFLP patterns. Rarefaction analysis was applied to determine if a sufficient number of clones were screened to estimate diversity within each of the fungal clone libraries sampled (Fig. 2B). For those clones obtained from the Atlanta and Savannah sites, the curves indicated saturation (Fig. 2B). Thus, a sufficient number of clones were sampled representative of the diversity of the fungi in each respective library. Numerically dominant RFLP groups, ranging from 10% to 18% of all clones were obtained for each of the four libraries (Table 1). In contrast, curves generated for clones obtained from Gainesville and LaGrange did not indicate saturation (Fig. 2B). Additional sampling of clones would be needed to reveal the full extent of the diversity. However, as observed with the bacterial libraries, numerically dominant RFLP groups were obtained (Table 1). Specifically, one dominant group of clones from each of the libraries (Gainesville, represented by RFLP group 8 and LaGrange, represented by RFLP group 21) comprised 10% and 17% of all clones, respectively (data not shown).

Table 1
Summary of representative sequences from 16S and ITS rRNA gene clone libraries

	Representative sequence	No. related clones	Site acquired	Nearest relative	Phylogenetic group	Similarity (%)
16S	16A1	41	A, G, S	ASC clone ctg_CGOF104 [DQ395781]	*γ-Proteobacteria*	99
	16A12	4	A	ID clone BF0002D02 [AM697512]	*β-Proteobacteria*	92
	16A18	7	A	RS bacterium m5 [DQ453814]	*γ–Proteobacteria*	96
	16G1	14	G	OCS clone OCS7 [AF001645]	*β-Proteobacteria*	93
	16G10	6	A,G, S	GW clone 005C-B03 [AY661994]	*γ-Proteobacteria*	93
	16G48	5	G, L, S	*Janthinobacterium lividum* GA01 [DQ473538]	*β-Proteobacteria*	93
	16L2	4	A,L	ARV clone AYRV1-102 [DQ990927]	*Cyanobacteria*	98
	16L3	30	L	SAC clone JSC2-A6 [DQ532167]	*Cyanobacteria*	93
	16L4	3	L	SAC clone KSC6-17 [DQ532358]	*Acidobacteria*	95
	16L13		L	ID clone BF0001B010 [AM696984]	*β-Proteobacteria*	97
	16L33	2	L	clone ODP1230B22.23 [AB177177]	*Cyanobacteria*	98
	16S2	11	S	*Pedobacter steynii* WB2.3-45T [AM491372]	*Bacteroidetes*	99
	16S3	7	A, S	*Pseudomonas lutea* OK2 [AY364537]	*γ-Proteobacteria*	98
	16S5	15	S	*Pantoea ananatis BD* 561 [DQ133546]	*γ-Proteobacteria*	99
	16S32	2	S	*Rhodococcus erythropolis* EPWF [AY822047]	*Actinobacteria*	99
	16S46	2	S	SAC clone JSC9-A3 [DQ532271]	*Cyanobacteria*	87
ITS	IA1	27	A,G	*Alternaria* sp. CID62 [EF589849]	*Pleosporales*	100
	IA3	17	A	*Hypocrea lixii* [AJ608956]	*Hypocreales*	99
	IA5	5	A	*Aspergillus niger* 16888 [AY373852]	*Eurotiales*	99
	IG2	6	G	*Trichosporon pullulans* CBS 2541 [AF444418]	*Cystofilobasidiales*	100
	IG3	3	G	soil fungus clone 164-21 [DQ420945]	*Pleosporales*	100
	IG6	3	G	*Mucor racemosus* UWFP 1053 [AY213661]	*Mucorales*	99
	IG22		G	*Udeniomyces megalosporus* CBS 7236 [AF444408]	*Cystofilobasidiales*	100
	IG24		G	*Davidiella tassiana* claO63 [EF589965]	*Capnodiales*	99
	IL2	33	L	*Udeniomyces pseudopyricola* [AY841862]	*Cystofilobasidiales*	98
	IL40	20	L, S	*Epicoccum* sp. G7A [EF432273]	*Pleosporales*	99
	IS1	13	S	*Cladosporium cladosporioides* [DQ810182]	*Capnodiales*	100
	IS8	35	G, S	*Cladosporium* sp. B5B [EF432298]	*Capnodiales*	100

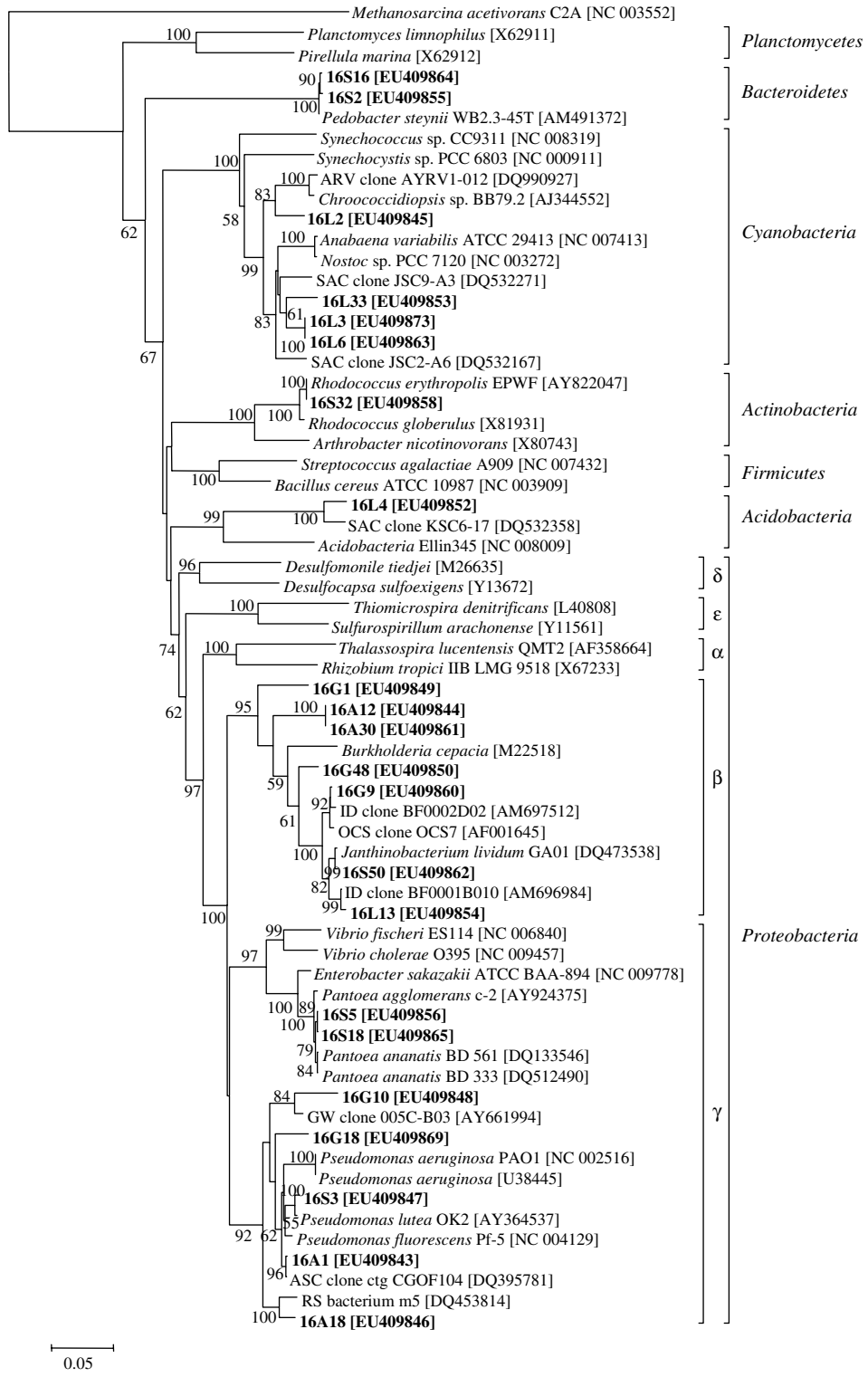

Fig. 3. Phylogenetic analysis of bacterial 16S rRNA gene clone sequences, as determined by distance Jukes–Cantor analysis, from concrete surfaces in Georgia, USA (in boldface), to selected cultured isolates and uncultured clones. Sequences derived from 16S rRNA gene templates are denoted by 16, and study site is denoted by: A, Atlanta; G, Gainesville; L, LaGrange; S, Savannah. Designations of environmental clone sequences are: ARV, Atacama Desert rock varnish; ASC, Alaskan seamount coral; GW, groundwater; ID, indoor dust; OCS, Oregon continental shelf; RS, red soil; SAC, spacecraft assembly cleanroom. GenBank accession numbers are in brackets. Bootstrap values represent 1000 replicates and only values greater than 50% are reported. The scale bar represents 0.05 substitutions per nucleotide position.

Analysis of the 168 fungal clones from RFLP groups with three or more members revealed the greatest similarity to previously cultured organisms (Fig. 5). A large number of clones (51 of 168) were representative of the order *Capnodiales* (Table 1). Of these, 90% were related to the genus *Cladosporium*, including the most numerically dominant phylotype, represented by clone IS8 (18% of total clones; Table 1). This phylotype, present in Gainesville and Savannah, was most closely related (100% identity) to *Cladosporium* sp. B5B (Fig. 5). The second most dominant phylotype, represented by clone IL2 (17% of total clones; Table 1), only found at the LaGrange

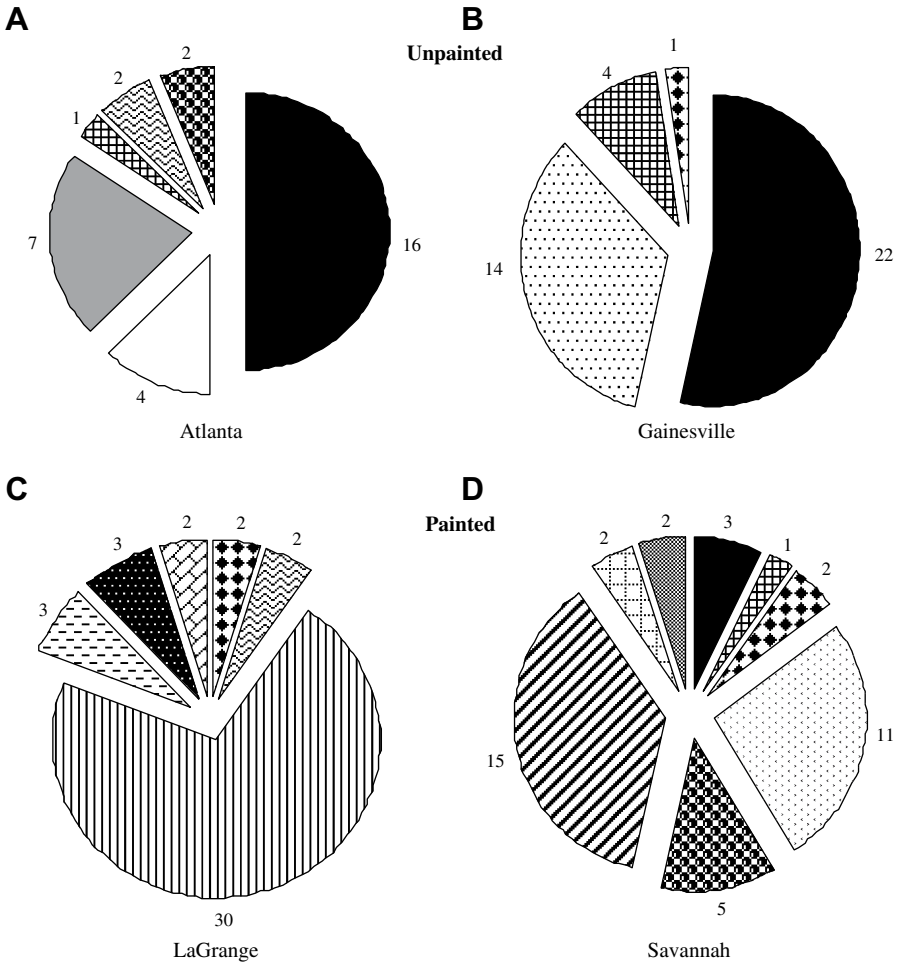

Fig. 4. Phylotypes detected in 16S rRNA gene clone libraries derived from (A) Atlanta, (B) Gainesville, (C) LaGrange and (D) Savannah sites. Numbers of clones associated with each phylotype from which a representative clone had been sequenced are shown. Phylotypes include ASC clone ctg_CGOF14 (■), ID clone BF0002D02 (□), RS bacterium m5 (▩), OCS clone OCS7 (▨), GW clone 005C-B03 (▦), *Janthinobacterium lividum* GA01 (▩), ARV clone AYRV1-102 (▨), SAC clone JSC2-A6 (▥), SAC clone KSC6-17 (▨), ID clone BF0001B010 (▩), clone ODP1230B22.23 (▨), *Pedobacter steynii* WB2.3-45T (▨), *Pseudomonas lutea* OK2 (▩), *Pantoea ananatis* BD 561 (▨), *Rhodococcus erythropolis* EPWF (▨), and SAC clone JSC9-A3 (▨).

site, was most closely related (98% similar) to *Udeniomyces pseudopyricola* (Fig. 5). The order *Pleosporales* was also highly represented (47 of 168; Table 1). This lineage included the third most dominant phylotype (14% of total clones; Table 1), which was found at Atlanta and Gainesville and was most closely related (100% identity) to *Alternaria* sp. CID62 (Fig. 5), as well as the phylotype most closely related (99% similarity) to *Epicoccum* sp. G7A, which was found at LaGrange and Savannah (10% of total clones; Table 1 and Fig. 5).

As observed with bacterial community diversity, fungal diversity between concrete sites without (Fig. 6A,B) and with (Fig. 6C,D) acrylic paint coating indicated that fungal phylotypes were not distributed solely according to surfaces coatings. Similar results were obtained for fungal biodiversity patterns, i.e., different phylotypes were detected at each of the four sites with some phylotypes occurring on both painted and unpainted surfaces. For example, clone type IS8 (Table 1) was recovered from painted and unpainted sites (Fig. 6B,D), while the second most frequently detected phylotype, IL2, was only detected at one of the two sites with paint coating (Fig. 6C). In contrast, IA1 was detected on unpainted surfaces only (Fig. 6A,B).

3.4. Chlorophyll and carotenoid detection

The detection of chlorophyll and carotenoids would indicate the presence of active oxygenic photoautotrophs on the fouled concrete surfaces. However, repeated attempts to detect chlorophyll *a*, *b*, *c*, and carotenoids did not reveal significant concentrations of any of these biomarkers at any of the study sites (data not shown).

3.5. Biofouling laboratory-based assays and ESEM

To complement our culture-independent characterizations of fouling microbial communities, we conducted a culture-based approach to obtain isolates for use in laboratory-based assays. Only fungal isolates were recovered from these culturing experiments. Cultured fungal isolates identified as *Alternaria* sp., *E. nigrum*, and *C. cladosporioides* were selected for testing. These fungal strains were representative of dominant genera detected by our culture-independent analyses (Table 1). To determine if any of these fungal isolates could colonize concrete, biofouling experiments were conducted. All three cultured isolates were able to colonize concrete tiles within 7 days when supplied with a carbon source (Fig. 7A–C). The *C. cladosporioides* isolate was able to rapidly colonize nearly the entire concrete surface (Fig. 7A), while the *Alternaria* sp. isolate showed only partial colonization (Fig. 7B). Colonization patterns of the *E. nigrum* isolate (Fig. 7C) revealed a black, crust-like growth, which is the most similar to fouling patterns observed in the field (Fig. 1A–D). Colonization was only observed when a carbon source was provided, as incubations using sterile water did not

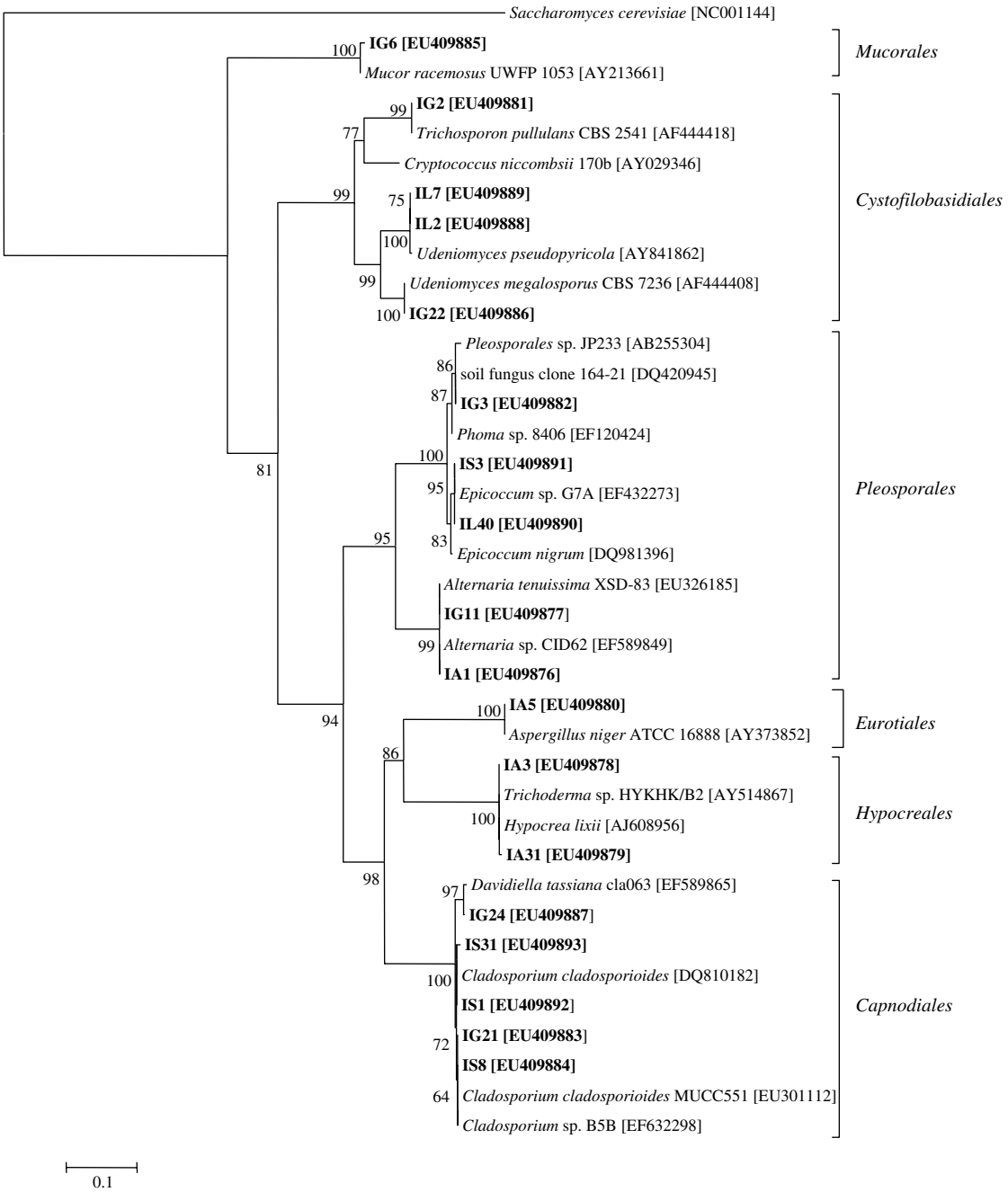

Fig. 5. Phylogenetic analysis of ITS region of fungal rRNA gene clone sequences, as determined by distance Jukes–Cantor analysis, from concrete surfaces in Georgia, USA (in boldface), to selected cultured isolates and uncultured clones. Sequences derived from the ITS region of rRNA gene templates are denoted by I, and study site is denoted by A, Atlanta; G, Gainesville; L, LaGrange; S, Savannah. GenBank accession numbers are in brackets. Bootstrap values represent 1000 replicates and only values greater than 50% are reported. The scale bar represents 0.1 substitutions per nucleotide position.

produce any noticeable growth (Fig. 7D). ESEM images of tiles revealed abundant hyphae and spores present on the fouled surfaces (Fig. 7E–G). These fungal structures were similar to what was observed in ESEM images of fouled sample sites (Fig. 1E–H). No putative fungal structures were present on the surfaces of non-fouled concrete tiles (Fig. 7H) or the sample site exhibiting no biofouling (Fig. 1J).

4. Discussion

The growth of microorganisms on concrete infrastructure detracts from the appearance of bridges, walls, pavements, canals and mortared joints. The discoloration of these structures by microbial biofilms may lead to public misconceptions regarding the performance and maintenance of the affected structures. Microbial activity can also significantly decrease the performance of concrete structures by the production of biogenic acids, which can result in deterioration of concrete and can accelerate corrosion of metals (Sand, 1997; Gu et al., 1998; Warscheid and Braams, 2000). Growth of filamentous microorganisms such as algae on concrete-lined canals, drainage ditches and spillways may slow the flow of water and increase the concrete permeability. In some instances, such as on the wearing surface of rigid pavements and bridge decks, microbial growth could cause slippery surfaces reducing skid

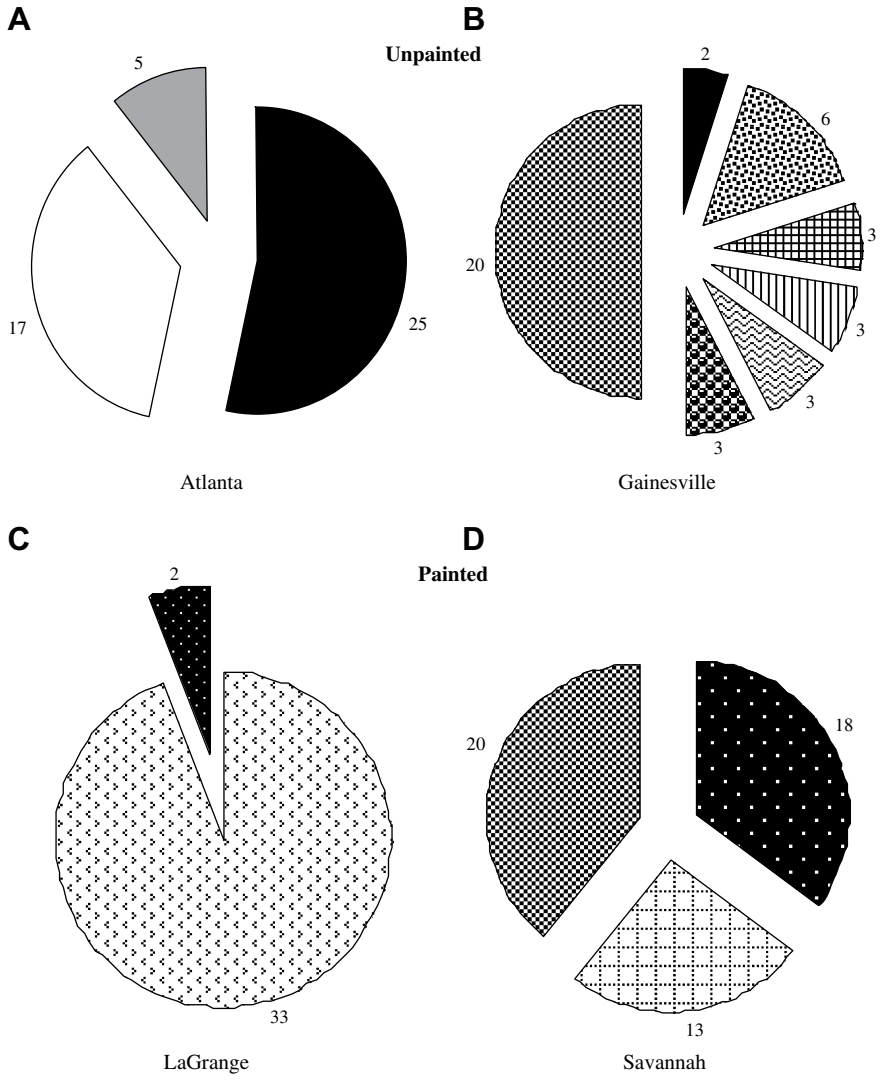

Fig. 6. Phylotypes detected in ITS rRNA gene clone libraries derived from (A) Atlanta, (B) Gainesville, (C) LaGrange and (D) Savannah sites. Numbers of clones associated with each phylotype from which a representative clone had been sequenced are shown. Phylotypes include *Alternaria* sp. CID62 (■), *Hypocrea lixii* (□), *Aspergillus niger* 16888 (▣), *Trichosporon pullulans* CBS 2541 (⚄), soil fungus clone 164-21 (▦), *Mucor racemosus* UWFP 1053 (▥), *Udeniomyces megalosporus* CBS 7236 (▨), *Davidiella tassiana* cla063 (▩), *Udeniomyces pseudopyricola* (⬚), *Epicoccum* sp. G7A (▪), *Cladosporium cladosporioides* (⬚), and *Cladosporium* sp. B5B (▨).

resistance. The presence of microbes within the small cracks and pores of stones and rocks can cause damage via swelling due to the cellular uptake of water (Gaylarde and Morton, 1997). Similarly, fissures can be created by the penetration of fungal hyphae into concrete (Gu et al., 1998).

This study is the first to report the composition of microbial assemblages fouling concrete infrastructures in the state of Georgia. To date, the majority of studies that have characterized microbial communities on exterior concrete and similar surfaces (e.g. indoor mortars, stone) have relied on culturing and morphological analyses (Mitchell and Gu, 2000; Dubosc et al., 2001; Shirakawa et al., 2003; Gaylarde and Gaylarde, 2005; Gaylarde et al., 2007). The present study employed a combination of culture-independent and culture-dependent methods to identify the types of microorganisms affecting concrete in different geographical locales throughout the state. The biodiversity patterns of bacterial and fungal communities could not be entirely explained by the presence or absence of a paint coating. Many of the clone types, including a number of frequently detected phylotypes, were present on painted and unpainted surfaces. We did observe a lower fungal diversity on coated surfaces though there were differences in fungal diversity between sites as well. In contrast, bacterial community diversity was somewhat higher on painted relative to unpainted surfaces. However, there were distinctly different bacterial communities present at each of the four sites. Thus, while paint may promote the colonization and/or growth of certain microorganisms, it appears that additional factors such as site location are also contributing to differences in observed microbial biodiversity patterns. Although there was a phylogenetically distinct bacterial community present at each site, the majority of the bacterial sequences present on the concrete surfaces belonged to just two bacterial phyla (i.e. *Cyanobacteria* and *Proteobacteria*), revealing low microbial diversity. This is not an unexpected finding, as one would consider concrete surfaces to be an inhospitable, extreme environment, thereby limiting the types of bacterial populations able to tolerate such conditions. Of those bacterial sequences that were most closely related (e.g., >98% similar) to previously cultured chemoorganotrophic bacteria, we postulate that they may have originated from soils or been associated with plants. For example, approximately 10% of the total bacterial sequences obtained were phylogenetically related to *P. ananatis*, a plant pathogen that has been recently implicated in

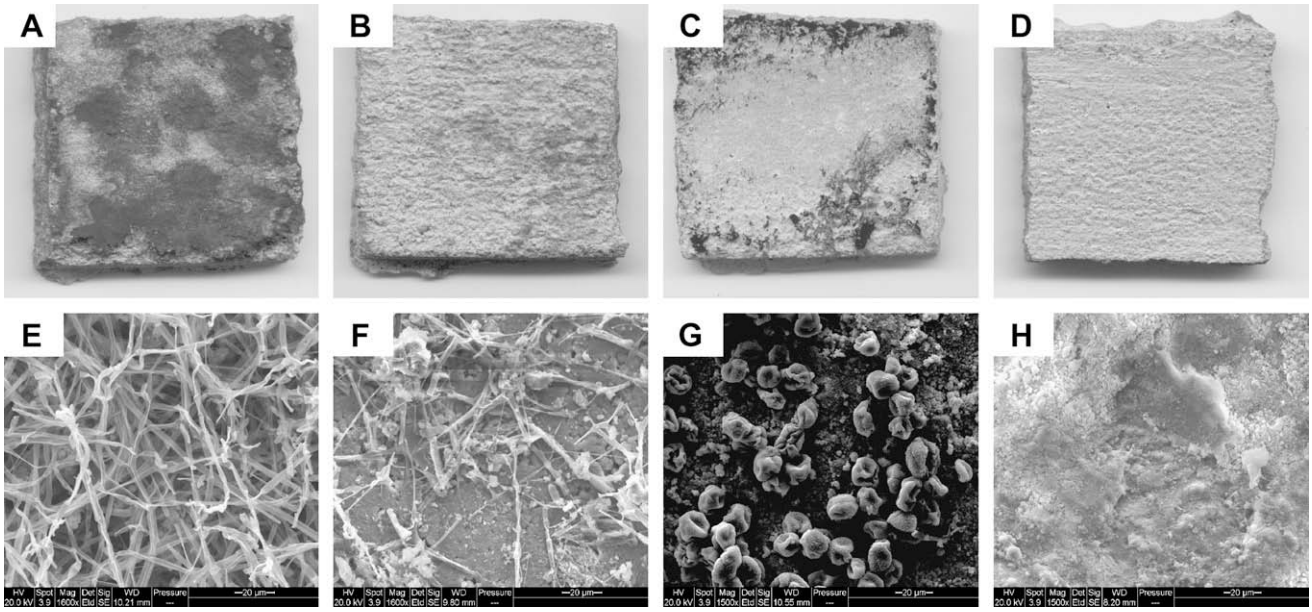

Fig. 7. Examples of laboratory incubated concrete tiles fouled by fungal isolates cultured from the study sites (A–D) and corresponding ESEM images (E–H) with: (A, E) *Cladosporium cladosporioides*; (B, F) *Alternaria* sp.; (C, G) *Epicoccum nigrum*; (D, H) inoculated tile incubated with sterile water that exhibited no fouling.

outbreaks of center rot disease of onion, and theorized to have been transferred to Georgia via seeds produced in South Africa (Walcott et al., 2002).

The vast majority of the bacterial 16S rRNA gene sequences that were obtained, however, were only related to environmental clone sequences. Many of these environmental clone sequences were isolated from extreme environments, such as deep-sea corals on Gulf of Alaska seamounts (Penn et al., 2006) and spacecraft assembly cleanrooms (Moissl et al., 2007). The most common sequences belonged to cyanobacterial, gammaproteobacterial and betaproteobacterial lineages, which accounted for the largest fractions of each of the communities. In addition, distinct patterns of microbial communities between sites were observed. For example, cyanobacterial sequences dominated the LaGrange site while gammaproteobacterial sequences were present only at the Atlanta, Gainesville and Savannah sites. Oxygenic phototrophs such as *Cyanobacteria* typically serve as primary producers supporting heterotrophic (chemoorganotrophic) microbial populations (e.g., *Proteobacteria*). The absence of *Cyanobacteria* from the other study sites is intriguing and may suggest that other autotrophic metabolic capabilities could be contributing to primary productivity in these microbial communities (Warscheid and Braams, 2000; De La Torre et al., 2003), or that phototrophic colonizers have been succeeded. It is also possible that environmental pollutants associated with automobile and industrial activities serve as a source of carbon and energy for these microbial communities (Warscheid et al., 1991; Gu et al., 1998). Alternatively, chemoorganotrophic bacteria and fungi with or without the presence of photoautotrophs may collectively act as primary microbial colonizers, conditioning the surface for subsequent microbial succession (Warscheid and Braams, 2000).

In contrast to the bacterial community composition, fungal sequences were most closely related to cultured isolates at an average similarity of 99%. As observed with the bacterial communities, distinct fungal communities occurred at each site. Many of the fungal genera detected in this study have been reported to cause concrete and stone deterioration, including *Alternaria*, *Cladosporium*, *Epicoccum, Mucor* and *Aspergillus* (Gaylarde and Morton, 1997; Mitchell and Gu, 2000; Shirakawa et al., 2002). Targeting of the ITS region of fungal rRNA genes for phylogenetic analysis was valuable in revealing the fungal diversity present on the concrete surfaces. Methodology based on the sequencing of this region coupled with the characterization of RFLP banding patterns has recently been used by others for the species-level identification of fungi in different ecosystems (Gardes and Bruns, 1993; Bougoure et al., 2005; Torzilli et al., 2006; Midgley et al., 2007; Slippers et al., 2007). It is important to note that the ITS PCR primers used in this study are considered universal, as the ITS1 and ITS4 primers are not specific to fungal nuclear regions, and have been reported to co-amplify plant host rRNA genes in the case of fungal symbionts present in the *Monotropoideae* (Gardes and Bruns, 1993; Horton and Bruns, 2001).

Efforts to culture fungal isolates directly from the concrete surfaces were successful. However, bacterial isolates were not obtained, suggesting that more intensive culturing efforts might need to be undertaken to isolate bacteria from these surfaces (Connon and Giovannoni, 2002; Kaeberlein et al., 2002; Zengler et al., 2002; Bollmann et al., 2007). The inability to culture bacteria from the concrete surfaces at our sites is likely due to the fact that the majority of bacterial sequences we detected were most closely related to as yet uncultured clones, while nearly all of the fungal sequences detected were most closely related to previously cultured isolates. We show that fungal isolates obtained from field samples colonized concrete tiles in laboratory-based assays when supplied with moisture and a carbon source. Differences were observed in fungal colonizing abilities, as a *C. cladosporioides* strain exhibited more robust and rapid growth compared to an *Alternaria* sp. isolate that was tested. The fouling observed on concrete tiles inoculated with the *E. nigrum* strain cultured from the concrete closely resembled the black stain frequently observed at our study sites. *Epicoccum* is a ubiquitous dematiaceous mold commonly isolated from air, soil and decaying plant matter, and can frequently colonize building materials (Mitchell and Gu, 2000; Shirakawa et al., 2002; Basilico et al., 2007). The staining and discoloration of the concrete surfaces are likely caused by the excretion of melanins and melanin-related pigments that provide cellular protection against UV irradiation, desiccation and temperature changes

(Warscheid and Braams, 2000). As these pigments are metabolic by-products produced by some dematiaceous fungi as well as pseudomonads (Warscheid and Braams, 2000), it is possible that both fungal and bacterial populations contribute to the discoloration process at our study sites.

In conclusion, this study provides the first insight into the microbial community diversity occupying the surfaces of outdoor concrete structures in the state of Georgia. Molecular analysis of extracted bacterial rRNA genes revealed a predominance of *Proteobacteria* at all sample sites except for one, which was dominated by *Cyanobacteria*. Analysis of the ITS region of fungal rRNA genes revealed genera previously shown to foul concrete and stone surfaces (e.g. *Cladosporium, Alternaria, Epicoccum*). The potential role in concrete biofouling of several of these genera was demonstrated in laboratory-based experiments. As more knowledge of microbial communities inhabiting concrete surfaces is attained in the future, microbial community colonization processes, mechanisms of deterioration, and the role of concrete surface characteristics in biofouling may be elucidated.

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