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Heterotrophic microorganisms in air and biofilm samples from Roman catacombs, with special emphasis on actinobacteria and fungi

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Abstract

The aim of the study was to characterize the heterotrophic microbiota in surface samples and in the air of the Roman catacombs of St. Callistus and St. Domitilla. The microbiotas inhabiting different environments and substrates, including plaster, marble and tufa in illuminated and dark sites, were studied. Microbial groups examined were aerobic microorganisms, anaerobic and facultatively anaerobic bacteria, proteolytic microorganisms, actinobacteria, yeasts and moulds. Filamentous actinobacteria, *Streptomyces* spp., were dominant in the biofilm samples. 16S rDNA sequence similarity analysis indicated that many of the isolates were novel species. Gram-negative bacteria were a minority among the isolated bacteria: few slime forming bacteria or types most closely related to them, like *Pseudomonas* spp., *Stenotrophomonas nitritireducens*, *Sinorhizobium morelense* and *Bosea thiooxidans*, were isolated. Fungi, mainly white *Lecanicillium psalliotae*, *Torrubiella* spp., *Beauveria alba* and *L. aranearum*, were isolated from both air and biofilm samples.

Keywords: Catacomb; Mould; Actinobacterium; Air; Biofilm; Heterotroph

1. Introduction

Biodeterioration problems in historic buildings, churches and hypogea are well recognized and during the past few decades the role of the microbes in biodeterioration processes has been acknowledged. Biodeterioration cannot usually be related to single microbial groups; rather, they are a result of complex microbial interactions. A multitude of factors determine the course of the process. Local differences in these factors, e.g., exposure to light and humidity and the supply of nutrients for microbes, have a great impact on biodeterioration and its outcome. Further, the microbiota contaminating a historical site shows varying levels of harmful metabolic activities at different times. After colonization, microbes can initiate damaging biodeterioration processes or they can continue living at a low level of metabolic activity in a biofilm. Typically, although microbes can colonize permanently various materials in historical sites, their damaging activity is not constant, but rather periodical. The impact of this varying combination of physical, chemical and biological effects leads to a situation where, at different locations in a historical site, the extent of damage caused by biodeterioration ranges from inconspicuous to obvious aesthetic and physical and/or chemical damage (Albertano, 1995; Ciferri, 1999; Dornieden et al., 2000; Warscheid and Braams, 2000; Raschle, 2001).

Roman hypogea are typically habitats where in-site variations in environmental factors such as illumination and microclimate influence the development and composition of the microbiota involved in biodeterioration. Microbial cells and spores are transported to the hypogean sites by airflow, rain, animals and visitors. Their fate, e.g., whether they die, remain alive but dormant, or actively grow and contribute to the biodeterioration, is determined by environmental factors such as humidity, temperature, light and the nature of the substrate they are inhabiting, its chemical and biological components, including the resident microbiota (Albertano, 1995). The substrates in Roman hypogea vary: some were built above the ground and covered by earth, whereas others were dug into the tufa rock of volcanic origin (Albertano, 1995). These rock deposits are tuffs and the so-called pozzuolana (scoriar in an ash matrix) of alkaline and potash

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composition (Sanchez-Moral et al., 2003). In addition, other materials, usually mixtures of rock fragments in a carbonated matrix that form a mortar and a thin layer of slaked lime or plaster on which paintings were made, form potential habitats for microbes in Roman hypogea (Sanchez-Moral et al., 2003).

The role of photosynthetic microbes such as cyanobacteria and microalgae in the hypogean biodeterioration processes is well recognized. Initial colonization by photosynthetic microbes occurs when the site is exposed to prolonged periods of illumination and visitors, which provide a source of heat and CO_2 (Albertano et al., 2003). Cyanobacteria are photosynthetic microbes that can use CO_2 as a carbon source for growth. When these pioneering microbes have established themselves, other microbes, including heterotrophic bacteria, colonize the site and sustain themselves by exploiting the metabolites and biomass produced by the primary colonizers. Heterotrophic microbes act in synergy with the primary colonizers thus enhancing the deterioration process (Albertano, 1995; Albertano and Urzi, 1999). The photosynthetic primary colonizers of hypogean environments include cyanobacterial genera *Eucapsis*, *Leptolyngbya*, *Scytonema* and *Fischerella*, chlorophytes and diatoms, several of the species seeming to be unique to Roman catacombs (Albertano and Urzi, 1999). Actinobacteria (previously actinomycetes, especially *Streptomyces*) and filamentous fungi (*Sporotrichum*, *Aspergillus*, *Cladosporium*, *Penicillium*, etc.) are commonly detected together with photosynthetic microbes in the Roman catacombs (Albertano and Urzi, 1999). Besides actinobacteria, other heterotrophic bacteria that are co-habitants of such hypogean environments are not well recognized. Of the heterotrophic microbes, *Streptomyces* has been found in most Roman hypogean samples as major colonizers with *Bacillus*, *Micrococcus* and fungi being noted as part of the accompanying microbiota, especially in samples obtained near to the entrance of the catacombs (Albertano and Urzi, 1999).

The aim of the present study was to characterize in more detail the heterotrophic microbiota (aerobic and anaerobic bacteria, yeasts and moulds) of Roman catacombs. The microbiota inhabiting different environments, including stone, frescoes and air at illuminated and dark sites, were studied in addition to the impact of the visitors on the airborne microbiota.

2. Material and methods

2.1. Sampling and detection

Sampling was performed in catacombs of St. Callistus and St. Domitilla in Rome (Italy), in March 2001. Both airborne and biofilm-associated heterotrophic microorganisms were studied. Biofilms were collected from different surfaces (plaster, marble, and tufa) in six locations consisting of rooms and corridors. Samples were taken on consecutive days: on the first sampling day, the catacombs were open to the visitors (visitors day) and on the following day they were closed (no visitors day). More detailed information about the sampling locations is presented in Table 1. In some cases the 'visitors' were other research groups (Albertano et al., 2003).

Microbial groups examined were aerobic microorganisms, anaerobic and facultatively anaerobic bacteria, proteolytic microorganisms, actinobacteria, yeasts and moulds. The growth media and incubation conditions are presented in Table 2. Viable heterotrophic microorganisms in the catacomb air were sampled using an MAS-100 Microbial Air Sampler (single-stage sieve impactor, Merck, Switzerland) for standard 100 mm Petri dishes to collect a 100-l air sample. The numbers of colony forming units (CFU) in samples were calculated referring to the positive hole conversion table for the MAS-100 as per manufacturer's instructions. Total numbers of microbes growing on various agar media were enumerated. Since plate count agar (PCA) and Ca-caseinate agar plates did not contain fungal antibiotics, fungi as well as bacteria were also able to grow in these media. Citrate buffered calcium caseinate agar (Martley et al., 1970) was included as culture medium for examination of proteolytic bacteria. Total and proteolytic (clear zone around the colony or precipitation of casein) counts were calculated from the agar.

Table 1
Description of sampling places studied in Christian catacombs of Domitilla (CD) and St. Callistus (CSC)

		Sampling sites and materials	
CD	12	First acrosolium on the left after the entrance, accessible to visitors, artificial light	(a) Plaster
	13	Cubiculum of 'Apostoli Piccoli',	(a) Marble
		accessible to visitors, artificial light	(b) Marble
			(c) Tufa
	17	Cubiculum of 'Buon Pastore', no access for visitors, no light	Marble (fresco)
CSC	13	Cubiculum of 'Oceano', no direct access for visitors, artificial light	Marble (fresco)
	17	Acrosolium along the corridor, accessible to visitors, artificial light	Marble, roof
	18	Cubiculum of 'Le Pecorelle', no access for visitors, no light	Marble

Table 2
Microbial groups determined during sampling in Rome; used culture media and incubation conditions

Microbial group	Medium	Incubation conditions
Aerobic microorganisms	Plate count agar (PCA, Difco)	25°C, 7 days
Proteolytic microorganims (Total count and proteolytic microorganisms)	Citrate buffered Ca-caseinate agar (Martley et al., 1970)	25°C, 7 days
Anaerobic and facultatively anaerobic microorganisms	Reinforced clostridial medium (RCMA, Difco)	25°C, 10 days, anaerobic (Anaerocult®, Merck, Germany)
Yeast and moulds	Potato dextrose agar (PDA, Difco) + 0.01% chloramphenicol + 0.01% chlortetracycline + 0.02% Tween Triton × − 100	25°C, 7 days
Actinobacteria	Actinomycete isolation agar (AIA, Difco) + 0.05% cyclohexamide	25°C, 21 days

Sterile cotton swabs moistened with sterile distilled water were used for non-destructive contact sampling of the surfaces. The biofilm mass that was removed by swabbing a 5 cm^2 area of biofilm with a swab was directly spread over the agar surfaces for qualitative microbiota analysis. After both biofilm and air sampling reinforced clostridial medium agar (RCMA) plates used for isolation of anaerobic microorganisms were immediately sealed in plastic bags in which an anaerobic atmosphere was generated with the Anaerocult® system (Merck, Germany). Representative isolates of different colonial morphologies were selected from agar plates for further identification.

2.2. Identification of fungi

A stereoscopic light microscope with a magnification of 400× was used for preliminary identification of the moulds to generic level (including *Penicillium*, *Aspergillus*, *Cladosporium*, *Acremonium*, *Fusarium*) according to typical colonial and conidial morphology. At this stage, the dominant white isolates could not be identified and were therefore sent with selected isolates of other moulds and yeasts to Centraalbureau voor Schimmelcultures (CBS), the Netherlands, for final identification. The mould isolates ($N = 20$) were identified on the basis of morphological characters (Domsch et al., 1980; de Hoog et al., 2000; Samson et al., 2000). Yeast isolates ($N = 8$) were identified phenotypically according to Barnett et al. (2000) on the basis of morphology, fermentation, and growth on carbon and nitrogen compounds.

2.3. Identification of bacteria

Preliminary identification of bacterial isolates from RCMA, citrate buffered Ca-caseinate agar, actinomycete isolation agar (AIA) and PCA was made on the basis of colony and cell morphology, Gram-staining, catalase, and API identification strips (bioMeriéux, France) ID 32 GN or API 20NE for Gram-negative rod-shape bacteria and APIStaph for Gram-positive, catalase-positive cocci.

Prior to further analysis isolates grown on RCMA ($N = 79$) were screened for genotypic similarity using RAPD fingerprinting as previously described (Alander et al., 2001) using random primers OPA-2 (5′-TGCCGAGCTG-3′) and OPA-3 (5′-AGTCAGCCAC-3′). For RAPD analysis the template DNA was prepared by mechanical lysis of the cells in a Ribolyser (Hybaid Ltd., UK). One isolate representing each different RAPD-genotype was further identified using ribotyping or partial 16S rDNA sequencing.

Representatives of dominant actinobacterial isolates growing on AIA plates ($N = 27$), as well as some dominant heterotrophic bacterial isolates growing on PCA or Ca-caseinate plates ($N = 15$), were selected for ribotyping and partial 16S rDNA sequencing.

Ribotyping was performed using the standard method of the automated ribotyping device RiboPrinter® System (DuPont Qualicon™, USA) according to the manufacturer's instructions, as described by Bruce (1996). The restriction enzyme used for actinobacteria was *Pvu*II and for other bacteria *Eco*RI. From the ribogroups generated and isolates partially sequenced, new identification patterns to the RiboPrint® database of VTT were created. The similarity in identification must be > 0.85, preferably around 0.90. Owing to the low number of reference patterns for actinobacteria in the existing identification database of the manufacturer (Release Qualicon 2002A) identification of these isolates was not possible. Therefore, selected isolates were further analysed using partial 16S rDNA sequencing.

The first set of isolates ($N = 17$) were sequenced by the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), following Suihko and Stackebrandt (2003). The sequences were compared with those of the type strains as well as with 16S rDNA reference sequences obtained from the Ribosomal Database Project (Maidak et al., 1999).

The second set of isolates ($N = 46$) was sequenced at VTT Biotechnology. Total genomic DNA from actinobacter isolates grown in trypticase soya broth was extracted with the FastDNA spin kit for soil (Q-BIO gene, USA) according to the manufacturer's instructions using a Fast Prep™ cell homogenizer (Labnet, USA). Total genomic DNA from other heterotrophic isolates was performed from 2 to 3 day old colonies on a non-selective agar (TSA, PCA) by lysing the cells mechanically with the cell homogenizer.

Nearly complete small-subunit rRNA genes (1500 bp) were PCR-amplified using bacterium specific primers BSF8/20 and BSR1541 (Wilmotte et al., 1993). PCR was performed in a total volume of 50 μl containing 1 μl appropriately diluted template DNA (50–100 ng), 0.2 μM of both primers, 0.2 mM dNTP, 3 units Dynazyme II polymerase (Finnzymes Ltd., Finland) in a reaction buffer with 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 1.5 mM $MgCl_2$. The PCR consisted of a thermal cycling program with the following steps: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 45 s, primer annealing at 56°C for 45 s and elongation at 72°C for 2 min and a final 7-min incubation at 70°C (Mastercycler Gradient, Eppendorf, Germany). Prior to sequencing, amplification products were purified using Qiaquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. Sequencing reactions of PCR products were performed with the ABI PRISM BigDye terminator Cycle sequencing kit v. 3.0 (Applied Biosystems, USA) according to the manufacturer's instructions using primer BSF8/20 (and BSR 534/18) (Wilmotte et al., 1993). Sequences were analysed with ABI PRISM 3000100 automated capillary DNA cycle sequencer (Applied Biosystems, USA) and corrected manually (Chromas v. 2.13). Sequences were aligned with DNAMAN. Similarity searches of partial 16S rDNA sequences were performed using BLAST (NCBI) analysis (Altschul et al., 1997).

3. Results

3.1. Airborne microbes

3.1.1. Quantitative analysis

The numbers of airborne microbes detected in the Christian catacombs of Domitilla (samples CD12, CD13 and CD17) and St. Callistus (samples CSC13, CSC17 and CSC18) are presented in Table 3. Total numbers of viable aerobic microbes in air were slightly higher in Domitilla (530–1450 CFU m^{-3}) than in St. Callistus (180–1440 CFU m^{-3}). The highest numbers of airborne aerobic microbes in the individual samples were detected in the sampling site CD13 after visitors and in CSC17 on 'no visitors day', whereas the lowest numbers were detected in the sampling site CSC13 after visitors and in the sampling site CSC18 on 'no visitors day'. Airborne microbes growing on Ca-caseinate agar were generally more common in St. Callistus (270–2930) compared to Domitilla (430–1330). Less than 5% of the total numbers of colonies were proteolytic. Proteolytic (caseinate hydrolysing) bacteria occurred in the air samples of St. Callistus on 'no visitors day'. In St. Callistus, the numbers of airborne fungi were lower than in Domitilla, 40–250 CFU m^{-3} vs. 450–2650 CFU m^{-3}. The highest numbers of airborne fungi were detected in Domitilla, at the sampling site CD12 (near the entrance) on 'visitors day'. The numbers of anaerobically growing bacteria were low both in Domitilla (20–90 CFU m^{-3}) and in St. Callistus ($<$ 1–80 CFU m^{-3}). Since the analysis of the airborne microbes was performed only once, the quantitative analysis reflect the situation of the air at that time alone.

3.1.2. Qualitative analysis

The results of the qualitative microbiological analysis of air samples for fungi are shown in Table 4 and for other heterotrophic bacteria in Tables 5a and b. The most dominant airborne fungi were identified as *Torrubiella* spp., *Beauveria alba*, *Acremonium bacillisporum*, *Lecanicillium psalliotae* and *L. aranearum*, all white filamentous fungi (Table 4). In Domitilla the white fungus comprised over 90% of the total viable fungal microbiota, whereas in St. Callistus the incidence of white fungus was lower (ca. 50%). Other filamentous fungi isolated included *Aspergillus ustus*, *Penicillium chrysogenum* and *P. glabrum*. Yeasts were mainly detected in the air samples from St. Callistus and were identified as *Cryptococcus albidus*, *C. curvatus*, *Rhodotorula minuta*, *R. glutinis*, *Sporobolomyces salmonicolor* and *Debaryomyces hansenii* var. *hansenii*.

With 11 species, the actinobacterial microbiota of St. Callistus was slightly more diverse than that from Domitilla with its eight species (Table 5). The partial 16S rDNA sequence analysis indicated that many of the actinobacterial isolates were novel species (similarity $<$ 98%). For example, the closest similarity was 96.8% to *Amycolatopsis coloradensis*, 96.4% to *Amycolatopsis azurea* and 97.6% to *Nocardia asteroides*. The only actinobacterium identified by ribotyping was *Micrococcus luteus. Promicromonospora* and *Mycobacterium* could not be typed with *Pvu*II when the standard method was used.

Other bacteria detected were mostly Gram-positive chemoorganotrophs belonging to the genera *Staphylococcus*, *Microbacterium*, *Bacillus* and *Paenibacillus* (Table 5). Gram-negative bacteria detected belonged to the genera *Pseudomonas*, *Stenotrophomonas*, *Sinorhizobium*, *Pectobacterium* and *Acinetobacter*. The occurrence of proteolytic microbes, i.e., *Micrococcus* spp., *Pseudomonas* spp. or *Microbacterium* spp., in the samples was sporadic. Anaerobically growing bacteria were mainly Gram-positive cocci, members of the genus *Staphylococcus*, which are chemoorganotrophic facultative anaerobes. Other facultatively anaerobic bacteria isolated included *Paenibacillus*.

A greater diversity in the microbiota was seen on 'visitors day' than on 'no visitors day'. Several fungi and bacteria, e.g., *Pseudomonas* spp., were isolated in 'visitors day' samples that were absent from 'no visitors day' samples (Tables 4 and 5). However, at one sample site at St. Callistus (CSC17, Table 4) both the 'visitors day' and the 'no visitors day' mycobiota were rather variable. On 'visitors day' the incidence of *Penicillium* and *Aspergillus* species in the airborne samples was higher than on the day without visitors. The airborne microbiota at sampling sites CD17 and CSC18, where there was no illumination and no

Table 3
Numbers of airborne microbes grown on different media

Sampling place		CFU m^{-3} on visitors/no visitors days				
		PCA (aerobic microorganisms)	Ca-caseinate agar (total count and proteolytic microorganisms)	PDA (yeasts and moulds)	RCMA (anaerobic and facultatively anaerobic microorganisms)	AIA (actinobacteria)
Domitilla	CD12	1030/1090	1330/430	2650/450	30/10	50/50
	CD13	1450/[a]	970/[a]	1960/[a]	30/40	110/60
	CD17[b,c]	530/530	970/760	630/610	90/20	170/30
St. Callistus	CSC13[d]	180/440	270/1130	80/40	< 1/70	60/480
	CSC17	530/1440	1580/2930	250/150	30/80	420/730
	CSC18[b,c]	670/290	1020/590	50/40	50/ < 1	270/310

See Table 2 for description of growth media.
[a]Overgrown with fungi (results discarded).
[b]Sampling site was dark (other sites were artificially illuminated).
[c]No access to visitors.
[d]No direct access to the public.

Table 4
Yeasts and moulds present in the air and biofilm samples of Domitilla and St. Callistus

Identified fungi	Domitilla						St. Callistus					
	CD12		CD13		CD17		CSC13		CSC17		CSC18	
	V	NV	V	NV	V	NV	V	NV	V	NV	V	NV
Arthroderma curreyi	−	−	−	−	−	−	−	−	−	+	−	−
Acremonium bacillisporum	−	+[a]	+	+[a]	+	+[a]	−	−	−	−	−	+
Acremonium sp.	−	−	−	−	−	−	−	−	+	−	−	−
Aspergillus ustus	−	−	−	−	−	−	−	−	−	−	−	+
Aspergillus sp. #1	−	+	+	−	+	+	−	−	−	−	−	−
Aspergillus sp. #2	−	−	−	−	−	−	−	−	+	−	+	−
Beauveria alba	+	+[a]	+	+[a]	+	+[a]	−	−	−	−	−	−
Cladosporium sp.	−	−	−	−	−	−	+	+	+	−	−	+
C. cladosporioides	−	−	−	−	−	−	−	−	−	+	−	−
Cryptococcus albidus (y)	−	−	−	−	−	−	−	−	−	+	−	−
C. curvatus (y)	−	−	−	−	−	−	−	−	−	+	−	−
Debaryomyces hansenii var. *hansenii* (y)	−	−	+	+	−	−	−	−	−	−	−	−
Fusarium sp.	−	−	−	−	+	−	−	−	−	−	−	−
Lecanicillium aranearum	−	−	−	−	−	−	+	−	+	+[a]	−	−
L. psalliotae	+	+[a]	+	−	−	−	−	−	−	−	−	−
Penicillium brevicompactum	−	−	−	−	−	−	+	+	+	−	−	−
P. chrysogenum	+	−	+	−	−	−	−	−	−	−	−	−
P. glabrum	−	−	−	−	+	+	−	−	−	−	−	−
P. minioluteum	−	−	−	−	−	−	−	−	+	−	−	−
P. pinophilum	−	−	−	−	−	−	+	+	−	+	−	−
Penicillium sp. #1	+	+	−	−	−	−	−	−	−	−	−	−
Penicillium sp. #2	−	−	−	−	+	+	−	−	−	−	−	−
Phialophora cinerescens	+	−	+	−	−	−	−	−	−	−	−	−
Rhodotorula glutinis (y)	−	−	−	−	−	−	−	−	+	−	+	−
R. minuta (y)	−	−	−	−	−	−	−	−	−	+	−	−
Sporobolomyces salmonicolor (y)	−	−	−	−	−	−	−	−	−	−	+	−
Torrubiella sp.	+	+[a]	+	+[a]	+	+[a]	+	+[a]	+	−	−	−

V = visitors, NV = no visitors, + = present, − = not present, *y* = yeast.
[a]Detected also in biofilm.

visitors were allowed to enter, was qualitatively as diverse as at the other sampling sites. At these two sites, some genera of fungi and bacteria were detected that were not in the other air samples from the same catacomb, and vice versa.

3.2. Biofilm samples

The summary of the microbes isolated and their identification is presented in Tables 4 and 5. The results are

Table 5
Occurrence of heterotrophic bacteria in the air and biofilm samples

Source	Microbial group	Species	Identification		Incidence of microbes		
			Partial 16S rDNA sequence (%)	Ribotyping similarity	Air		Biofilm
					Visitors	No visitors	
(a) *Christian Catacombs of Domitilla*							
CD12	Actinobacteria	"*Streptomyces vulgaris*"[a]	100	unID	x	x	x
		Closest *S. exfoliatus/venezuelae/zaomyceticus*	100	unID	x	x	x
		Closest *Nocardia asteroides*[b]	na	na	x	x	x
	Other bacteria	*Bacillus thuringiensis*	100	0.91	x	x	x
		closest *Microbacterium resistens*	97.7	na	x	x	x
		Staphylococcus epidermidis	na	0.96	x	x	
		Closest *Stenotrophomonas maltophilia*	100	0.84	x	x	x
		Closest *Pectobacterium cypripedii*	96.4	na	x	x	x
		Closest *Pseudomonas gingeri/putida*[c]	na	na	x		x
CD13	Actinobacteria	Closest *Amycolatopsis coloradensis*[b]	na	na	x	x	x
		Closest *Nocardia asteroides*	97.6-98.9	unID	x	x	x
		Closest *Promicromonospora aerolata*[b]	na	na	x	x	x
		Streptomyces vulgaris[a]	100	unID	x	x	x
	Other bacteria	Closest *Bacillus thuringiensis*[b]	na	na			x
		Closest *Enterobacter intermedius/Kluyvera cochleae*	100	unID	x	x	x
		Closest *Buttiauxella izardii*	99.0	unID	x		x
		Closest *Microbacterium liquefaciens/maritypicum*	100	unID	x		x
		Closest *Paenibacillus polymyxa/burgondia*	99.5/99.3	0.73 *P. polymyxa*	x		x
		Closest *Pseudomonas alcaligenes*	100	0.84 *P. putida*	x		x
		Closest *P. fuscovaginae/putida/asplenii*	99.1	0.78 *P.putida*	x	x	x
		Closest *P. gingeri/putida/aspleniil/fuscovaginae*	99.2	unID	x		x
		Closest *P. putida/jessenii*	99.1/ 98.9	unID	x		x
		Sinorhizobium morelense	100	unID	x	x	x
		Stenotrophomonas nitritireducens	99.6–99.8	0.97	x	x	x
		Staphylococcus epidermidis	na	0.96	x	x	x
		S. warneri	na	0.96	x	x	x
		S. hominis	na	0.96	x	x	x
CD17	Actinobacteria	Closest *Crossiella cryophila*	99.8	unID			x
		Closest *Dermacoccus nishinomiyaensis*	99.8	unID	x	x	x
		Micrococcus luteus	100	0.98	x	x	x
		Closest *Nocardia asteroides*[b]	na	na	x	x	
		Closest *Promicromonospora aerolata*[b]	na	na	x	x	x
		Closest *Streptomyces exfoliatus/venezuelae/ zaomyceticus*[b]	na	na	x	x	x
	Other bacteria	Closest *Bacillus mycoides*	100	0.93 *B. weihenstephanensis*			x
		Closest *Bosea thiooxidans*	99.0	unID			x
		Closest *Bacillus simplex/macroides*	99.4	0.88 *B. simplex*			x
		Closest *Pseudomonas oleovorans*	99.1	0.94 *P. oryzihabitans*	x	x	x
		Staphylococcus epidermidis	100	0.94	x	x	x
(b) *Christian Catacombs of St. Callistus*							
CSC13	Actinobacteria	Closest *Janibacter limosus*	98.5	unID	x	x	x
		Micrococcus luteus	na	0.98	x	x	x
		Closest *Nocardioides albus/fulvus/flavus*	98.6/98.4	unID	x	x	x
		Closest *Promicromonospora aerolata*	99.1	no disc.	x	x	x
		Streptomyces griseus	100	unID	x	x	x
	Other bacteria	*Staphylococcus capitis*	na	0.92		x	
		S. hominis	na	0.96		x	
		S. warneri	na	0.92		x	
CSC17	Actinobacteria	Closest *Amycolatopsis azurea*	96.4	unID	x	x	x
		Closest *A. coloradensis*	96.8	unID	x	x	x
		Closest *Nocardia ignorata*	98.8	unID	x	x	x

Table 5 (*continued*)

Source	Microbial group	Species	Identification		Incidence of microbes		
			Partial 16S rDNA sequence (%)	Ribotyping similarity	Air		Biofilm
					Visitors	No visitors	
		Micrococcus luteus	na	0.97	x	x	x
		Closest *Mycobacterium fortuitum* subsp. *acetamidolyticum*	97.9	no disc.			x
		Closest *Promicromonospora aerolata*	99.1	no disc.	x	x	x
		Streptomyces virginiae/lavendulae	99.7–99.5	unID	x	x	x
		S. griseomycini	100	unID	x	x	x
	Other bacteria	Closest *Dietzia maris*	96.8	unID			x
		Closest *Oxalobacter* sp.	97.3	unID	x	x	x
		Staphylococcus capitis	na	0.92	x	x	
		S. hominis	na	0.87	x	x	
		S. warneri	na	0.92	x	x	
		Stenotrophomonas nitritireducens	99.8	0.81	x	x	x
CSC18	Actinobacteria	Closest *Amycolatopsis coloradensis*	na	[d]	x	x	x
		Closest *Micrococcus luteus*	na	0.93	x	x	x
		Streptomyces griseomycini	100	[d]	x	x	x
	Other bacteria	*Acinetobacter radioresistens*	99.3	0.88		x	x
		Bacillus pumilus	100	0.89	x	x	
		Closest *Pectobacterium cypripedii*	96.4	unID	x	x	x
		Staphylococcus hominis	na	0.92	x	x	
		S. warneri	na	0.92	x	x	

unID = un identified, na = not analysed, no disc. = no discrimination.
[a]Not a validly described species.
[b]Same morphology with a sequenced isolate.
[c]API results.
[d]Same riboprint with a sequenced isolate.

qualitative, but give an indication of the occurrence of different types of microorganism. Fungi, mainly white *Torrubiella* sp., *B. alba*, *L. psalliotae* and *A. bacillisporum*, were more dominant in samples taken from Domitilla (CD) than in samples from St. Callistus (CSC) (Table 4). Actinobacteria, mainly streptomycetes, were the most common bacteria in the biofilm samples (Table 5). The occurrence of proteolytic microbes was sporadic. Isolated proteolytic bacteria belonged to the genera *Micrococcus*, *Pseudomonas* and *Microbacterium*. Some Gram-negative slime forming bacteria, belonging to the genera *Pseudomonas*, *Enterobacter*, *Pectobacterium*, *Stenotrophomonas*, *Sinorhizobium* or *Bosea* were also isolated. Facultatively anaerobic bacteria were isolated from biofilm samples taken from CD13 and CD17. Bacteria isolated from CD13 belonged to the genera *Paenibacillus*, *Enterobacter* and *Staphylococcus*, and those isolated from CD17 to the genus *Bacillus*. Qualitatively, a more diverse biofilm microbiota was identified in Domitilla than in St. Callistus.

3.3. Qualitative comparison of airborne and biofilm microbiotas

Within the same sampling site the airborne and biofilm microbiotas showed both similarities and differences (Tables 4 and 5). At both Domitilla and St. Callistus, the airborne microbiota, especially the fungal and the actinobacterial components, was more diverse than in the biofilm. At Domitilla, *Stenotrophomonas* spp., *Crossiella cryophila* and *Bosea* spp. were isolated from biofilm but not from air samples; at St. Callistus, *Mycobacterium* spp. and *Dietzia* spp. were in the biofilm but not the air. From one site (CD13), three biofilm samples were obtained from two different materials, i.e., two samples from marble and one from tufa. Just as some similarities and some differences were observed between airborne and biofilm samples taken at the same site, so similarities and differences in the microbiota of different materials at the same site were evident (data not shown).

4. Discussion

As the previous section shows, the quantitative analysis of the airborne microbiota in the catacombs indicated that the air was often heavily contaminated, with the total numbers of airborne microbes isolated under less selective conditions, i.e., aerobically on non-selective agar, varying from ca. 300 to 2650 CFU m^{-3} air. The ambient,

humid air of the catacombs is ideally suited to microbial growth on surfaces, the temperature being fairly stable at 16–19°C and the relative humidity being 90–99% (Albertano and Urzi, 1999; Sanchez-Moral et al., 2003) further promote it.

The investigation clearly showed the importance of the media employed in surveys for isolation of airborne microorganisms. Although the total numbers of viable airborne aerobic microorganisms (detected on PCA) and airborne fungi (on PDA) were generally higher in Domitilla than in St. Callistus, the reverse was the case for bacteria on AIA. Numbers of anaerobically growing bacteria (on RCMA) were low in both catacombs, whilst numbers on Ca-caseinate agar were more numerous in St. Callistus than in Domitilla. The fact that in eight of the 12 air samples collected, the highest microbial numbers were detected on Ca-caseinate agar, not on PCA, may have been due to the Ca^{2+} concentration (20 mM) in the medium enhancing recovery and growth of bacteria adapted to demineralization of calcium and growth in calcium-rich environments. The caseinate in the medium would also enable the growth of various actinobacteria. However, strongly halophilic bacteria are not culturable on this media. Halo-adapted bacterial communities have been reported to occupy niches in deteriorated monuments and to be involved in biogeochemical processes (Saiz-Jimenez and Laiz, 2000).

At two sampling sites, CD17 and CSC18 (one in each catacomb), without illumination and visitors not permitted, and an area with artificial light and not directly accessible to visitors (CSC13), airborne numbers were generally lower than at other sampling sites within the same catacomb. The recent cleaning of the frescoes of CSC13 could explain the counts there being even lower than at CSC18.

No obvious trends to increasing or diminishing numbers in the airborne microbiota related to visitors were detected. Possibly only in the case of fungi was there evidence that the presence of visitors might lead to increases in microbial numbers. The number of airborne microorganisms recorded by air sampling is sensitive to a multitude of confusing factors, including airflow, ventilation and movements of people (Griffiths and DeCosemo, 1994). Since the present investigation involved visits of other sampling groups at different sites, our air samples on 'no visitors day' could have been affected by these other groups, a further factor introducing uncertainty. For further information, see Albertano et al. (2003); Urzi et al. (2003).

Although the choice of air sampling equipment, in our case an MAS-100 impactor, is an additional factor affecting counts, Nesa et al. (2001) reported that in a hospital air results obtained with the MAS-100 correlated well with those with two other impactor samplers, the Air Test Omega and the Bioimpactor 100-08. The calculated D_{ae50} value for MAS-100 is 1.72 μm (Li and Lin, 1999) and it has been reported that air samplers with a $D_{ae50} < 2$ μm should theoretically be able to precipitate practically any airborne microbiological contaminant-carrying particle (Meier and Zingre, 2000). Lighthart (1997) reported that airborne bacteria in the outdoors are associated with particles, such as amorphous plant, fungal and soil debris, that vary in size from < 0.65 to > 7.0 μm. As catacombs provide underground habitats where the microclimatic conditions are a combination of outdoor and indoor environments, it is possible that some of the larger airborne particles (and the microorganisms attached to them) may have escaped the air sampling.

As can be seen from Tables 5a and b, the airborne microbiota in both catacombs was qualitatively diverse. The most dominant airborne fungi in catacombs were *Torrubiella* sp., *L. psalliotae*, *L. aranearum*, *B. alba* and *A. bacillisporum*, all of which are white filamentous fungi. The absence of *Cladosporium* from the Domitilla samples can partially be due to the seasonal variation. European studies have reported that in the winter time the occurrence of *Cladosporium* is at its lowest level (Medrela-Kuder, 2003). Yeasts, with the exception of *Debaryomyces hansenii* var. *hansenii*, were detected only in the air samples of St. Callistus. The most frequently occurring actinobacteria were streptomycetes, *Nocardia* spp. and *Micrococcus* spp. Partial 16S rDNA sequence analysis revealed that many isolates are of novel species. Other bacteria detected in the air samples were mostly Gram-positive chemoorganotrophs such as *Staphylococcus* spp., *Microbacterium* spp. and *Bacillus* spp. Gram-negative bacteria were detected more commonly in St. Callistus than in Domitilla and they belonged to the genera *Pseudomonas*, *Stenotrophomonas*, *Pectobacterium* and *Acinetobacter*. The occurrence of proteolytic microbes, *Pseudomonas* spp. and *Microbacterium* spp., in the samples was sporadic. Most anaerobically growing bacteria were Gram-positive cocci, mainly facultative anaerobic *Staphylococcus* spp. No obligate anaerobes could be identified in the air samples of the present study. Lighthart (1997) reported that majority (73–90%) of the genera cultivated from air samples of outdoor air are Gram-positive. The high moisture is likely to enhance the survival of airborne microbes. In addition, increased pigment production in microbes adapted to survive solar or other radiation has been reported (Lighthart, 1997), and indeed most of the airborne yeasts and bacteria isolated in the present study were pigmented.

Although no clear trend of the presence of visitors increasing the numbers of airborne microbes was detected, the microbiota seemed to be more diverse on 'visitors day'. In addition, on 'visitors day' the incidence of *Penicillium* and *Aspergillus* species in the airborne samples was higher than on the day without visitors. However, at the sampling sites with no illumination and with no visitors the airborne microbiota was as diverse as elsewhere. Since the aerobiological investigation was not repeated, the present results give information only about the temporary incidence of airborne microbes, so no seasonal or other variations can be seen from the results. To obtain more comprehensive information on the microbiological air quality in the catacombs,

sampling would require to be repeated several times and during different seasons. The concentration of airborne bacteria has been shown to vary greatly in the short term, diurnally and annually (Lighthart, 1997). In urban outdoor air, the number of airborne microbes has been reported to vary greatly, from < 1 to > 7000 CFU m^{-3} (Lighthart, 1997). In one study, culturable airborne fungi in lecture hall air have been reported to vary in number seasonally from 170 to 1200 CFU m^{-3} (Medrela-Kuder, 2003).

Biofilms are complex microbial ecosystems with active and efficient interactions between microorganisms (Costerton et al., 1999). In mineral building materials, fungi have been recognized as being one group of pioneering and highly aggressive microbes causing biodeterioration (Gorbushina and Petersen, 2000; Warscheid and Braams, 2000), but less is known about the possible role of bacteria as pioneering microbes (Ciferri, 1999). In the present study, actinobacteria, mainly *Streptomyces* spp., were the most commonly detected bacteria in the biofilm samples of the catacombs. Fungi, mainly white filamentous fungi, were more abundant in the biofilm samples from Domitilla than in the samples from St. Callistus. As in the air samples, the occurrence of proteolytic microbes in the biofilm samples was sporadic. A more diverse biofilm microbiota could be identified in the biofilm samples from Domitilla than in those from St. Callistus.

As mentioned previously, at any individual sampling site the microbiota in air showed both similarities to and differences from that of biofilm, as did the microbiota of biofilms on different materials at the same sampling site. Interestingly, although the mycobiota and actinobacterial microbiota of the air were more diverse than in the corresponding biofilm, within a catacomb several bacterial taxa, e.g., *Crossiella* sp., *Bosea* sp., *Mycobacterium* sp. and *Dietzia* sp., were detected in biofilm but not air samples.

While interpreting the qualitative results of the microbial analysis it has to be kept in mind that the description of the microbiota is always dependent on the culture media and conditions used and on the interactions between microbes present in the sample. Thus results of the microbiota analysis by culture-dependent methods can always give only an estimate of the complex microbiota present. There has been speculation that with culture-dependent methods the numbers of spore-forming bacteria and fungi might be overestimated and only species resistant to desiccation can be retrieved (Laiz et al., 2003). However, in the present study we were able to isolate relatively many Gram-negative isolates that have been reported to be sensitive to desiccation and form dormant stages (Laiz et al., 2003).

With culture-independent methods, an even more versatile picture of the microbial populations thriving on deteriorated monuments could have been achieved. However, there are still limitations in the molecular-based methods, e.g., the methods are only as reliable as the DNA extraction method (critical step with complex and inhibitor-rich environmental samples), and all PCR-based methods inherently produce a bias in the result due to preferential amplification of some target DNAs. Furthermore, when molecular detection methods are applied no information on the viability and physiological status of microbes is obtained (Gorbushina et al., 2003).

Some especially interesting microorganisms were identified from the catacomb samples. One of these was a bacterial isolate closest related to *Bosea thiooxidans* isolated from a dark biofilm growing on fresco painted on marble (CD17, Cubiculum of 'Buon Pastore', dark site where visitors had no access). Bacteria in the genus *Bosea* are chemolithoheterotrophic, thiosulfate-oxidizing bacteria (Das et al., 1996), which obtaining energy from the oxidation of inorganic compounds and fix CO_2 from the atmosphere, resulting in release of corrosive acids, e.g., sulphuric acid. According to Warscheid and Braams (2000), the colonization of marble by sulphur-oxidizing bacteria in Western Europe had been previously reported by Italian groups. Warm, wet conditions similar to those in the underground hypogea are known to support the growth of chemolithotrophic bacteria (Warscheid and Braams, 2000).

Some of the Gram-negative isolates were most closely related to *Sinorhizobium morelense*. These soil bacteria are opportunistic pathogens and have been reported to be highly resistant to multiple antibiotics (Wang et al., 2002). A group of yellow-pigmented Gram-negative isolates were most closely related to *Stenotrophomonas nitritireducens*. Finkmann et al. (2000) have reported *S. nitritireducens* strains isolated from ammonia-supplied biofilters to reduce nitrite and produce nitrous oxide (N_2O). Certain actinobacteria isolated both from catacomb air and biofilm were most closely related to *Janibacter limosus*. Strains of this genus have been isolated from sludge and environmental samples and described (Martin et al., 1997; Imamura et al., 2000).

In conclusion, air and biofilm samples of Roman catacombs have a complex microbiota with several possible novel species. The present study is the first one studying the heterotrophic microbiota of catacombs in more detail. The presence of diverse fungal and actinobacterial microbiota in the air of the catacombs warrants further studies, since these microbial groups contain several representatives that can be harmful to the human health. It is unlikely that these microbes form a risk to occasional visitors, instead occupational hazards to the health of the people working in the catacombs should be considered. Furthermore, many of the isolated microbes are known to produce extracellular compounds (enzymes, acids) that can cause biodeterioration of frescoes (discolouration and mineral precipitation). Future characterization of the isolates and microbial interactions should be performed to obtain more information about the production of biodegradative metabolites and bioactive compounds. Identification and understanding of the complex ecosystem is essential when planning microbial growth-limiting actions.

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