Facultatively oligotrophic bacteria in Roman mural paintings

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ABSTRACT: Due to the limitation of nutrients during long periods, deteriorated monuments, and particularly their walls, represent an interesting ecosystem where oligotrophic bacteria can be isolated. Therefore, facultatively oligotrophic bacteria, present in the deteriorated mural paintings decorating the Tomb of Servilia, Roman Necropolis of Carmona, 1st and 2nd century AD, were studied. The most abundant genera were *Bacillus* and *Paenibacillus*, which were also the most abundant copiotrophs. Total cellular fatty acid methyl esters were analysed and a cluster analysis was performed. It was shown a close relationship between copiotroph and oligotroph isolates, as the copiotroph isolates resulted to be facultatively oligotrophs.

RESUMEN: Se han estudiado las comunidades microbianas presentes en las pinturas murales de la Tumba de Servilia, Necrópolis de Carmona, siglo I-II A.C. Se ha detectado la presencia de bacterias oligotrofas facultativas. Entre las bacterias copiotrofas o heterotrofas, los géneros más abundantes fueron *Bacillus* y *Paenibacillus*, que también resultaron ser los más abundantes entre las bacterias oligotrofas. Debido a la limitación de nutrientes los monumentos y, particularmente sus paredes, representan un interesante ecosistema que permite el aislamiento de bacterias oligotrofas.

1 INTRODUCTION

Organisms able to grow on rich media are referred to as heterotrophs or copiotrophs (Fry 1990) as they are especially suited for the exploitation of high nutrient flux habitats, and can be distinguished from the oligotrophs, able to develop at low concentrations of organic substances. Oligotrophic bacteria were investigated more frequently in marine than in soil samples, which is consistent with the fact that marine environment is an oligotrophic habitat. Williams (1985) also suggested that soil is a grossly oligotrophic environment. This could also be extended to monuments.

Culture media with high content of organic substances are generally used to isolate and grow environmental microorganisms. These culture media contain carbohydrates, proteins, etc. in concentrations far exceeding those the organism would ever encounter in nature, and to which was adapted for maximum efficiency of utilization. In the laboratory, rich nutrient media are utilized because they promote microbial growth, reduce incubation times and promote the production of industrially useful metabolites (Wainwright et al. 1991), but when dealing with the study of microbial communities they can mask the real microorganism distribution and isolate selectively airborne propagules instead of the active microorganisms colonizing the site of our interest. On the other hand, the abundant production of secondary metabolites, concomitant with rich nutrient media, seems to be a pathological behaviour resulting directly from a metabolic overflow and could not be extrapolated to nature (Saiz-Jimenez 1995). An oligotroph that excrete oxidizable organic substances as metabolic by-products would not be competitive in an environment of low organic flux. Therefore, oligotrophy is regarded as a specific set of properties that are especially suitable for exploitation of habitats extremely poor in organic nutrients (Poindexter 1981).

Monuments, frescoes and mural paintings are basically made of inorganic materials (stone, lime, pigments, etc.) and the organic matter content is low, arising from atmospheric deposition or water transport through rising damp (Saiz-Jimenez 1995). Ancient mural paintings subjected to the ravages of time have deteriorated for centuries and the original organic matter, if any, used in the preparation of the paintings, was metabolized by the microorganisms in periods of favourable environmental conditions.

In the European Commission project ENV4-CT98-0705: "Novel molecular tools for the analysis of unknown microbial communities of mural paintings and their implementation into the conservation/restoration practice", mural paintings from different sites were investigated (Heyrman et al. 1999, Möhlenhoff et al. 1999, Piñar et al. 1999, Saiz-Jimenez & Laiz 2000). One of the sites is the Roman Necropolis of Carmona, used

between the 1st and 2nd century AD, where most of the tombs, excavated in a calcarenite quarry, were decorated with paintings. The hypogean Necropolis was discovered in 1868 and the first excavations were carried out in 1874–1881. Between 1897 and 1931 more than 500 tombs were excavated, the last being discovered in 1985. During more than a century, exhumated tombs have deteriorated and not only the mural paintings but some of the tombs were heavily damaged due to environmental agents. Several reasons contributed to this destruction:

(i) the tomb entrances were not protected and the rain flooded the tombs,
(ii) annual plants and trees grew outside and inside, and the mechanical action of the roots was very important in promoting rock fissuration and cracking,
(iii) water infiltration through the rock fissures produced water tracks, and rock blocks fell down,
(iv) colonization by ferns, mosses, cyanobacteria and algae covered the walls (Ariño & Saiz-Jimenez 1996, 1997, Ariño et al. 1997).

The Tomb of Servilia (Fig. 1) was selected for a study on heterotrophic and oligotrophic microflora. This tomb is the most impressive and stand out from the rest of the tombs found in the Necropolis of Carmona. It shows hellenistic features and looks rather a luxurious house with a colonnaded courtyard onto which open rooms. The funeral chamber, with a large trapezoid entrance-hall covered by a pointed dome, is decorated with mural paintings (Fig. 2). In past decades a hole on top of the dome was closed turning the chamber into a greenhouse where phototrophic organisms grew luxuriously due to a high relative humidity and mild and constant temperature. The hole was re-opened but the dome walls present abundant remains from dead phototrophic organisms. Samples were taken in several sites in order to cope with the different ecological situations, including oligotrophic bacteria, as few studies, if any, were carried out on such group of bacteria on deteriorated monuments.

Figure 1. Tomb of Servilia.

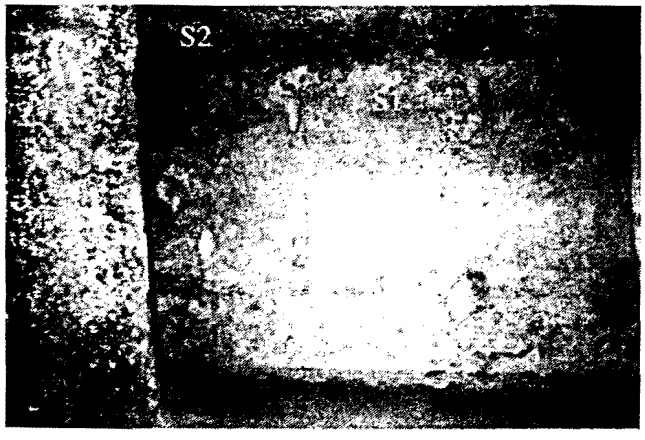

Figure 2. Mural painting where samples S1 and S2 were collected.

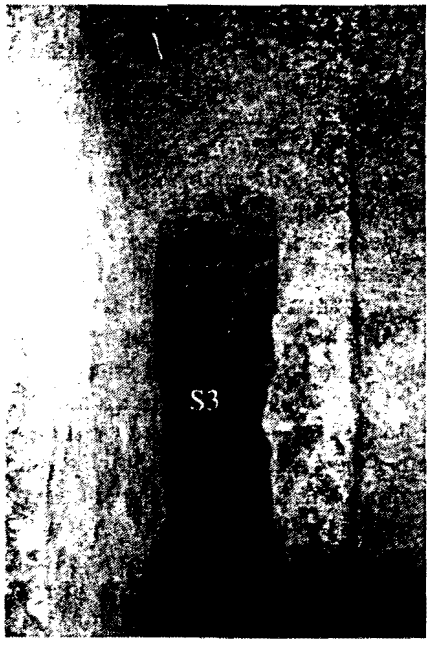

Figure 3. Small room where sample S3 was obtained.

2 MATERIALS AND METHODS

2.1 *Sampling*

Three samples were taken from the Tomb of Servilia, from the painted wall (S1) at 1.80 m height, and from the ceiling (S2) at some 2 m height (Fig. 2).

These samples had a powdery aspect due to wall deterioration. Sample S3 was taken from the wall of a small, inner room, with high humidity and colonized by phototrophic microorganisms, as sun light penetrates through the doors enlighting the wall (Figs 3 and 4). This sample was a biofilm and had a green colour due to the abundant biomass. For comparative purposes, samples from the mural paintings of the chapel from

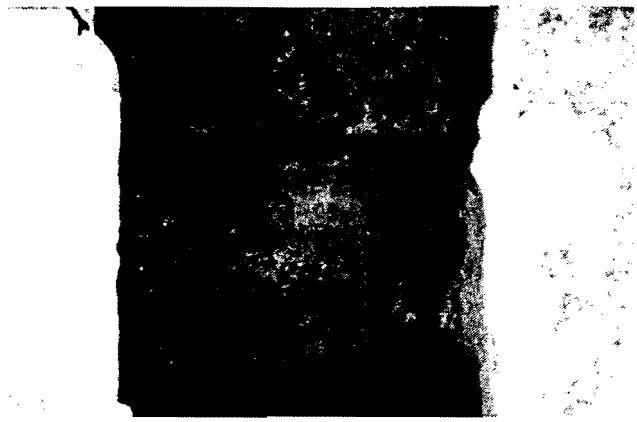

Figure 4. Detail of Figure 3 showing phototrophic microorganisms and sampling site

the Herberstein castle, Austria, were used (Piñar et al. 1999, Rölleke et al. 1996).

2.2 *Culturing*

Bacteria capable of growing and multiplying in diluted media were enumerated and isolated by plating directly on a 100-fold diluted TSA medium. Enumeration was also performed on standard TSA medium. The total organic content of the diluted medium was estimated to be around 100 mg C/litre. The same samples were plated onto both media. For actinomycetes, diluted starch–casein (SC) medium was used (Kuster & Williams 1964).

2.3 *Identification*

After enumeration, individual colonies were randomly isolated and purified by streak plating on diluted TSA until pure cultures were obtained. Isolates were characterized by morphological and physiological properties, the later using standard microbiological methods, API and Biolog. Identification were performed with APILab Plus and Biolog databases.

Total cellular fatty acid methyl esters (FAMEs) were analysed using the Microbial Identification System, Delaware, USA, MIDI, in accordance with protocols for cultures grown on solid medium and recommended instrument specifications. This permitted automatic identification of bacteria by comparison with the MIDI Sherlock Standard Aerobe and Actin1 databases. Cluster analysis were performed with 2D-plot and dendrogram programmes from MIDI.

3 RESULTS AND DISCUSSIONS

There is not a generally accepted limit for defining oligotrophy. Poindexter (1981) stated that oligotrophs are those bacteria that can be isolated on media containing 1–15 mg organic C/litre and can be subcultured on such media. They may also be able to grow on richer media on subsequent cultivation. Culture media containing 50 mg of yeast extract or 200–300 mg glucose or peptone per litre of water were used for isolation of oligotrophic bacteria (Kuznetsov et al. 1979).

Table 1. Copiotrophs and oligotrophs in Servilia Tomb.

Sample	TSA, CFU/g	TSA·10^{-2}, CFU/g
S1	614	1040
S2	150,012	40,223
S3	5180	1333

Table 2. Genera present in Servilia Tomb.

Genera	Sample S1	Sample S2	Sample S3
Arthrobacter	+	–	–
Bacillus	+++	++	+++
Cellulomonas	+	+	+
Micrococcus	–	–	+
Paenibacillus	–	+	+
Staphylococcus	–	+	–
Streptomyces	–	+	–

+++ >50%; ++ 25–50%; + <25% of isolates.

Nearly all natural environments contain only small amounts of nutrients. The soil is not a rich medium for bacteria but a rather poor medium as compared with one prepared in a laboratory. Hattori (1976) observed that many soil bacteria which were able to grow in 100-fold diluted nutrient broth were unable to grow in full strength broth and they were considered to be oligotrophic. It seems acceptable that amounts <10 mg C/litre define oligotrophy in marine isolates (Fry 1990) but probably the amount should be increased for terrestrial ecosystems. We used a 100-fold diluted medium containing about 100 mg C/litre. However, it must be recognized that nutrient amounts provided by most standard culture media bears little relationship with the nutrients contained in soils, waters or deteriorated monuments.

Table 1 shows enumeration of copiotrophs and oligotrophs in TSA media, in the samples from the Tomb of Servilia. Counts of oligotrophs decreased about four times with respect to copiotrophs in samples S2 and S3, but increased in sample S1.

Table 2 shows the genera identified from the different samples. In the walls of the Servilia tomb, among copiotrophs *Bacillus* species were the most abundant isolates followed by *Cellulomonas* and *Paenibacillus*. Similar data were reported by Heyrman et al. (1999). Interestingly, the oligotrophs were only represented by *Bacillus*, *Paenibacillus* and *Streptomyces* strains.

Table 3 indicates the percentages of the most abundant species with respect to total isolates, both for copiotrophs and oligotrophs. It is noteworthy that *Bacillus megaterium*, *Streptomyces* sp. and *Bacillus pumilus* which represented respectively 22% of copiotroph

Table 3. Percentage of most abundant species with respect to total isolates.

Isolates	% Isolates in TSA			% Isolates in TSA·10^{-2}		
	S1	S2	S3	S1	S2	S3
Bacillus megaterium	22.0	2.9	7.9	33.3		
Bacillus pumilus		5.7	23.7			50.0
Bacillus sp.	2.4	2.9	13.2		14.2	
Streptomyces sp.		11.3			28.6	
Paenibacillus polymyxa		5.7	2.6		14.3	
Bacillus subtilis	2.4		7.9			
Bacillus amyloliquefaciens	2.4		5.3			
Cellulomonas turbata	4.9	2.9				
Paenibacillus macerans		2.9	2.6			
Bacillus thuringiensis		8.5				
Bacillus licheniformis			5.3			
Bacillus brevis	4.9					
Bacillus sphaericus	4.9					
Paenibacillus alvei		2.9			14.3	
Bacillus cereus		2.9				
Staphylococcus warnei		2.9				
Staphylococcus xylosus		2.9				
Staphylococcus sp.		2.9				
Cellulomonas biazotea			2.6			
Micrococcus luteus			2.6			
Micrococcus lylae			2.6			
Paenibacillus sp.			2.6			
Arthrobacter viscosus	2.4					
Bacillus laterosporus	2.4					
Not identified	51.3	42.7	21.1	66.7	28.6	50.0

isolates in sample S1, 11% in S2, and about 24% in S3, also represented 33%, 29%, and 50% of the oligotroph isolates.

Cluster analysis based on FAME analysis shows the relationship between the isolates of *Bacillus megaterium, Bacillus pumilus* and *Paenibacillus polymyxa*, both copiotrophs and oligotrophs (Fig. 5). Most of the strains appeared grouped in three main clusters corresponding to each species. Only eight isolates were out of the clusters: five corresponded to *Bacillus megaterium* and three to *Bacillus pumilus*. One isolate of *Paenibacillus polymyxa* was included in the *Bacillus megaterium* cluster. The oligotrophic strains were grouped in the corresponding species cluster, indicating that no differences in FAME were observed between copiotrophs and oligotrophs. The copiotroph isolates were facultatively oligotrophs as, in fact, it was demonstrated by culturing them in 100-fold diluted TSA medium. On the other hand, all oligotroph isolates were able to grow on standard TSA medium indicating that they can be considered as facultatively oligotrophs.

Evidence for the existence of strictly oligotrophic soil bacteria is scanty and relatively few isolation procedures have been specifically designed to detect them (Williams 1985). Obligate oligotrophs are comparatively rare in nature and only can grow on low concentrations of the carbon while facultatively oligotrophs are common in nature and can grow at both very low and high concentrations of carbon.

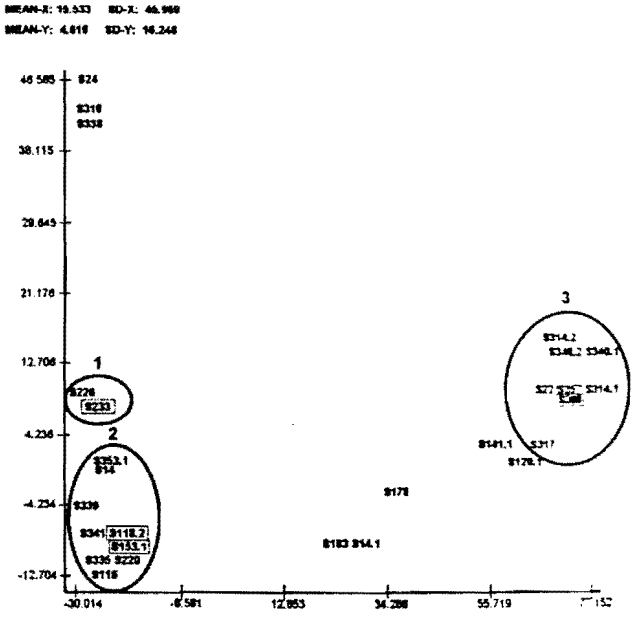

Figure 5. Cluster analysis of strains of *Paenibacillus polymyxa* (cluster 1), *Bacillus megaterium* (cluster 2) and *Bacillus pumilus* (cluster 3). Strains isolated in TSA·10^{-2} are inserted in a square.

Williams (1985) considered that the reaction of some soil bacteria in culture may be interpreted as evidence that they are facultatively oligotrophs, while Wainwright et al. (1991) stated that most microorganisms appear to

be facultatively oligotrophs and grow well in the presence of both low and high concentrations of nutrients. Also, many commonly used laboratory bacteria are facultative oligotrophs. Fry (1990) surveyed 40 pure cultures of laboratory bacteria and showed 95% to be facultatively oligotrophs, none were obligate oligotrophs. All the isolates from this study were also facultatively oligotrophs, which indicates that in deteriorated mural paintings there is an active microflora able to cope with periods of low nutrients.

Semenov (1991) reported that oligotrophs have been found among most taxonomic groups of Gram-positive and Gram-negative bacteria. Identification of oligotrophic bacteria was reported by Fry (1990) and mainly referred to water samples. Most of the isolates belong to the genera *Pseudomonas, Flavobacterium* or *Spirillum*. Few Gram-positive bacteria were identified, from which a *Bacillus pumilus* was isolated from a reservoir. For soil isolates only two unknown species of *Arthrobacter* and *Agromonas* were reported. The predominance of Gram-negative over Gram-positive isolates was already reported for karstic dripping waters (Laiz et al. 1999). In terrestrial environments it was found the opposite trend, and *Bacillus* strains predominated (Laiz et al. 2000). In the studied tomb *Bacillus* strains were also dominant (Tables 2 and 3). For comparison, the oligotrophs isolated from the deteriorated mural paintings of Herberstein chapel were included (Rölleke et al. 1996); *Bacillus* strains showed the same trend, although in this case *Arthrobacter, Flavobacterium* and *Cellulomonas* strains were also isolated (Table 4).

Lewis et al. (1988) suggested that bacteria on stone can be extremely versatile and could maintain their activity during nutrient perturbations, operating at low nutrient levels and utilizing what the environment has to offer. *Bacillus, Arthrobacter* and actinomycetes seems to be well adapted to thrive on deteriorated monuments during nutrient starvation conditions.

Bacillus were reported to be the most abundant isolates in many deteriorated monuments, frescoes, etc. (Lazar & Dumitru 1973, Incerti et al. 1997, Laiz et al. 2000a, b, Saiz-Jimenez & Laiz 2000). The abundance of bacilli was explained by Laiz et al. (2000a, b) as the result of an adaptation to halophilic conditions.

Arthrobacter may be classed as typical autochthonous microbes of the soil. They have often been isolated from soil using nutrient-poor media and although they are able to survive nutrient limitations, there is no convincing evidence that they are obligate oligotrophs (Williams 1985). Actinomycetes can also be readily isolated from terrestrial environments using low-nutrient media and particularly water-agar (Semenov 1991).

The presence of actinomycetes in hypogea environments was widely reported and they seem to be common inhabitants of underground churches, catacombs, tombs and karstic caves (Monte & Ferrari 1993, Groth & Saiz-Jimenez 1999). It appears that most cave microbial communities usually rely on allochthonous organic matter transported from the surface. This organic matter is composed of humic substances, refractory to microbial degradation, to which actinomycetes are well adapted. Even a culture medium based on humic acid

Table 4. Facultatively oligotrophic bacteria isolated from mural paintings.

Servilia Tomb			Herberstein Chapel		
Strain	Media	Identification*	Strain	Media**	Identification
S118.2	TSA·10^{-2}	*Bacillus megaterium*	1R7	TSA·10^{-2}	*Bacillus laterosporus*
S149	TSA·10^{-2}	NI	2R2	TSA·10^{-2}	*Flavobacterium resinovorum*
S153.1	TSA·10^{-2}	*Bacillus megaterium*	5R7	TSA·10^{-2}	*Bacillus* sp.
S170	SC·10^{-2}	NI	5R11	TSA·10^{-2}	*Bacillus subtilis*
S175.1	SC·10^{-2}	NI	5R15	TSA·10^{-2}	*Cellulomonas* sp.
S176.1	SC·10^{-2}	NI	6R4	TSA·10^{-2}	*Arthrobacter pascens*
S228	TSA· 10^{-2}	*Bacillus cereus/thuringiensis*	6R5	TSA·10^{-2}	*Bacillus* sp.
S231.2	TSA·10^{-2}	*Paenibacillus alvei*	6R19	TSA·10^{-2}	*Arthrobacter oxydans*
S233	TSA·10^{-2}	*Paenibacillus polymyxa*			
S250	SC·10^{-2}	*Streptomyces* sp.			
S251	SC·10^{-2}	NI			
S252.2	SC·10^{-2}	*Streptomyces* sp.			
S268.2	SC·10^{-2}	NI			
S357	TSA·10^{-2}	*Bacillus pumilus*			
S358	TSA·10^{-2}	NI			
S360	SC·10^{-2}	*Bacillus pumilus*			
S362	SC·10^{-2}	NI			

* NI: Not identified (S149, S170, S175.1, S176.1, S251, and S268.2 are actinomycetes).
** No isolates were obtained from medium SC·10^{-2}.

was developed for selective isolation of actinomycetes (Hayakawa & Nonomura 1987).

To conclude, the data reported indicated that facultatively oligotrophic bacteria are present in deteriorated mural paintings. Due to the limitation of nutrients during long periods, deteriorated monuments, and particularly their walls, represent an interesting ecosystem where probably obligate oligotrophic bacteria could be isolated, assuming appropriate isolation procedures, specifically designed to detect them, are used. Further research on this group of bacteria is needed.

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