Differential effects of distinct bacterial biofilms in a cave environment

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Abstract Current microbial surveys using molecular methods provide us with critical information on the major components of natural bacterial communities. However, limited investigation has been performed on the influence of bacterial metabolism on the environment. In this study, we analyzed the pH generated by distinct bacterial communities in a cave environment. Different bacterial biofilms developing on the walls of the cave were visually distinguished by their colorations (e.g., white, yellow, and grey) and mineral depositions, and previous studies have reported on their bacterial diversity and distribution. Using pH microelectrodes, we carried out *in situ* measurements and were able to detect differences among these bacterial biofilms. White biofilms and carbonate depositions resulted in alkaline pH values. Grey biofilms also increased the pH although these values remained lower than in white biofilms. A combination of grey-white biofilms resulted in alkaline pH values with highest values at the white edge of the colonies. Yellow biofilms generated a slightly acid pH. These results suggest that different bacterial communities can lead to distinct effects on their environment, for instance, precipitation or dissolution of carbonates in caves. These results add information about metabolic response to current knowledge from bacterial diversity surveys, providing information on the interaction between complex bacterial communities and the geological substrate.

Introduction

Microorganisms are able to develop in practically every habitat on Earth [10]. Their huge diversity and great metabolic capabilities allow these microorganisms to adapt to a variety of environmental conditions resulting in an almost unlimited ability to colonize new environments [11, 12]. In spite of these incredible capabilities, microbial activity

on natural substrates has been scarcely analyzed and the effects of bacterial growth in nature are barely understood [22, 23].

The environmental effect of microbial metabolism has been suggested for specific microbial groups such as sulfate-reducing bacteria [6, 13], sulfur-oxidizing bacteria [8, 20], iron-reducing bacteria [7, 18], and some other particular metabolic processes. Generally, complex microbial communities develop in natural environments. Recent methodological advances applying molecular techniques have permitted the survey of these communities and today, we have an acceptable knowledge of the major components of these communities. Nevertheless, in most cases, the consequences of the metabolism of these bacteria on the natural substrate (rock, soil, waters) remain to be proposed and understood.

An example of the importance of microbial transformations in the environment is related to the interest in preserving caves with unique prehistoric paintings and artistic representations. The consequences of microbial metabolism on these sites can be significant [3, 21]. These potential effects need to be predicted before major damage is caused to the environment and art work, so that actions can be taken to avoid the loss of a unique heritage for future generations.

In order to approach the effect of bacterial communities on an environment we propose the use of indicators of bacterial metabolism. The pH is a consequence of the generation of acidic or basic products during bacterial metabolism. Besides, the pH presents great interest in cave environments because calcium carbonate precipitation and dissolution are highly dependent on pH. These processes need to be monitored to avoid the

accumulation of carbonate deposits on artistic representations as a consequence of alkaline pHs or the dissolution of carbonate formations as a result of acidic pH values.

Several studies have described the interest, geological characteristics and evolution of environmental parameters in Altamira Cave [5, 9]. In the last years, complex microbial communities producing colored colonizations on Altamira Cave walls have been reported. White, yellow and grey biofilms have been analyzed and distinguished based on the bacterial communities forming these colonizations [14, 15]. In addition, a morphological study has been reported on the characteristics of these differently colored biofilms [5] suggesting that white and grey biofilms were associated with mineral deposits while yellow colonies did not present associated mineral formations.

Herein, we analyze the effect of distinctive bacterial biofilms developing in Altamira Cave. The pH was the selected indicator to evaluate the potential metabolic impact of these bacterial biofilms on cave walls.

Materials and Methods

Study site. This study was performed in Altamira Cave (Cantabria, Spain). This cave has been previously described from the geological [19] and microbiological [13, 14, 15], perspectives. Recently, the distribution and morphological characteristics of different bacterial biofilms developing on cave walls have been described [5]. In this cave white, yellow, and grey biofilms have been described [5, 14, 15] and the major bacterial components of these communities have been identified. Carbonate formations

in this cave have also been reported, and the formation of moonmilk deposits in this cave has been described in detail [3, 4].

Bacterial biofilms showing different colorations were sampled. The collection of samples included clay-rich substrate from cave walls to compare the estimated pH values in the bacterial biofilms, moonmilk depositions, and wall material.

***In situ* pH determinations.** Measurements of pH utilizing standard pH electrodes do not permit the determination of pH in microenvironments. In order to determine the pH in biofilms represented by bacterial colonizations on cave walls at early stages of development, microelectrodes able to determine the pH at these points were utilized. The pH in the different biofilms and moonmilk deposits was determined by using pH microelectrodes of 100 μm tip diameter (Unisense, Århus, Denmark) following the methods described in detail by Revsbech [17]. Bacterial biofilms were placed on a bed of filter paper on top of an agar block to avoid the breakage of the microelectrodes when touching the surface of the biofilms, clay-rich substrate, and moonmilk deposits.

Results and Discussion

The bacterial communities forming the differently colored biofilms detected in Altamira Cave have been previously described in detail [14, 15]. These studies have shown the existence of different compositions of bacteria in these communities. Thus, the different coloration of these biofilms is a result of their distinctive composition of bacterial phylotypes [14]. Besides, a morphological description of these biofilms forming

colonies on the cave walls have been reported [5]. However, the metabolic consequences of these distinctive communities remain to be investigated.

Knowing the composition of bacterial communities forming the reported biofilms represents a first approach to understanding the development of bacteria on cave walls. Nevertheless, a second and critical aspect is to evaluate the effect of those bacterial communities on the underlining substrate, and in the studied cave the potential risk for the conservation of the cave and its famous paleolithic paintings. One of the critical parameters in a carbonate cave, such as Altamira Cave, is the pH because calcium carbonate precipitation is highly affected by pH. Basic pH (8.0 and above) have been reported to allow the precipitation of calcium carbonate in caves and other environments [2]. Consequently, pH measurements represent a simple indication that can provide useful information to assess the effect of bacterial growth and metabolism on cave walls.

Figure 1 shows photographs and a schematic representation of the studied biofilms and moonmilk with the pH values measured at these sites. White biofilms showed basic pH values (Table 1) around one pH unit higher than estimates for the clay-rich substrate on cave walls. Grey biofilms also showed alkaline pH values (Table 1) although, in general, they presented slightly lower values than the white biofilms. Grey-white biofilms appeared to be a combination of grey and white biofilms. The white area of these grey-white biofilms corresponded to the external halo around the grey colored, central area of the biofilm. This white, external area showed higher pH values than the grey, central area (Figure 1). The white and grey areas presented pH values similar to those observed in separated white and grey colonies, respectively. Unlike white and

grey colonies, yellow biofilms showed slightly acidic pH values in the range from 6 to 7 (Table 1). The studied moonmilk deposits are soft fibers of calcium carbonate with pH values around 9 (Table 1). Alkaline pH values in these carbonate deposits were expected due to the requirements for carbonate precipitation under the cave conditions.

Moonmilk samples and white and grey colonies showed alkaline pH values above 8 and these three types of samples presented calcitic precipitates as previously shown [5]. Thus, white and grey biofilms due to their alkaline pH can induce the formation of speleothems that can cover the actual wall of the cave. This is a potential transformation resulting from bacterial metabolism of white and grey biofilms in the studied cave.

Yellow biofilms lack associated carbonate precipitates [5], which is a result of their acidic pH. Although yellow biofilms are not able to induce the precipitation of carbonate, their acidic pH can result in the dissolution of carbonate from walls and speleothems in the studied cave. Thus, different transformations can be predicted depending on the bacterial metabolism being carried out in distinct biofilms. While yellow biofilms can induce carbonate dissolution, white and grey biofilms can result in the precipitation of carbonate minerals.

A subject to be investigated is the possibility that different colored biofilms might preferentially develop on areas with slightly different organic nutrient supplies or environmental conditions. Stomeo et al. [21] have shown that different organic and inorganic compounds can limit growth of bacterial biofilms on cave substrate. As well, Portillo et al. [16] have reported that different nutrients affect the pH values reached in cultures of bacteria. If this would be the case, the bacteria community of yellow

biofilms might be processing different organic substrates than white and grey colonizations. Although this is a possibility to take into account, it is quite unlikely that relatively closed sites in a cave might receive a qualitatively different supply of organic nutrients and yellow, white and grey colonies in numerous cases develop in very close proximity [5]. Thus, it is expected that different bacterial communities composing the distinctively colored biofilms exert preferential metabolic processes with the available nutrients and the result is a different pH value in each of the types of samples under study. For example, the presence of a larger proportion of *Desulfovibrio*-related sulfate-reducing bacteria as metabolically active cells in yellow biofilms [14] than in biofilms with other colorations [14, 15] suggests a common presence of oxygen deprived microenvironments, and consequently, an increase of fermentation processes by the dominant metabolically active Gammaproteobacteria detected in the yellow biofilms [14]. Fermentation of organic matter by Gammaproteobacteria is known to induce acidification of the medium [10]. The consequences of bacterial metabolism represent the important aspect to be monitored during the assessments of conservation strategies and the evaluation of potential for the transformation of cave environments [1].

The assessment of the influence of bacteria on natural substrates, including those sites of singular cultural heritage interest, is a necessary approach to evaluate and predict potential risks and transformations as a result of bacterial development. Beyond the knowledge generated by understanding the bacterial composition of these communities forming biofilms on cave walls, indicators of the potential effects, or changes, on the natural substrate needs to be monitored to define and approach the risks involved on the development of distinctive bacterial biofilms. In this study, we have been able to distinguish the potential effects of differently colored bacterial biofilms under

development on cave walls and deduce their potential impact on calcium carbonate precipitation or dissolution. Thus, the consequences of bacterial metabolism in nature can be assessed, for instance through pH measurements, to complement results on bacterial diversity surveys and distribution in natural environments of distinctive and complex bacterial communities.

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Table 1. Results of pH measurements on white, yellow, grey, and grey-white biofilms and moonmilk deposits from the walls of Altamira Cave. The average values, standard deviation (SD), number of measurements (n) and the minimum (min) and maximum (max) measured values of pH are presented.

Biofilm/structure	Average	SD	n	Min	Max
White biofilm	8.72	0.23	15	8.44	9.10
Yellow biofilm	6.58	0.43	9	6.09	6.91
Grey biofilm	8.22	0.11	9	8.06	8.33
Grey-White biofilm (center)	8.12	0.05	3	8.06	8.16
Grey-White biofilm (white halo)	8.97	0.07	3	8.83	9.00
Moonmilk deposits	9.01	0.72	9	8.25	9.69
Clay-rich substrate	7.36	0.41	15	6.97	7.99

Figure legend

Figure 1. Schematic representation of the studied samples and their pH values measured during this study. Different types of bacterial biofilms and soft, carbonate formations (moonmilk) have been analyzed. Representative examples of the studied samples (bacterial biofilms and moonmilk) are presented. The schematic drawings are shown to help to understand the measurements and results. Scale bars indicate 3 mm.