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Biofilm growth in human skeletal material from ancient Mesopotamia

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ABSTRACT

Investigators have long recognised the effects of microbial activity on archaeological bone. These investigators, however, have focused on single or groups of microbes rather than on complex microbial aggregates such as biofilms, a focus that has affected our understanding of archaeological bone biodeterioration. In this paper, we report on the investigation of a biofilm in archaeological human bone from the site of Tell Leilan, Syria (2900–1900 BCE). Scanning electron microscopy indicated that the biofilm is characterised by single cells and microcolonies of bacteria and fungi, as well as calcite crystals that were all embedded within extracellular polymeric substances. Using culture techniques and DNA sequencing, we isolated and identified several microbes from the biofilm including *Amycolatopsis* sp., *Penicillium chrysogenum*, *Aspergillus* sp., *Chaetomium* sp., and *Cladosporium* sp. Having characterised the Leilan biofilm, we are now closer to understanding the complex process of bone biodeterioration in archaeological bone collections.

1. Introduction

Researchers have long recognised the effects of microbial activity, also called biodeterioration, on archaeological bone (e.g., Bell, 1990; Garland, 1987; Grupe et al., 1993; Hackett, 1981; Jans et al., 2004; Marchiafava et al., 1974; Roux, 1887; Wedl, 1864). Microbial alteration to bone in the form of tunnels is typically assigned to the action of either fungi (Marchiafava et al., 1974; Wedl, 1864), bacteria (Baud and Lacotte, 1984; Hackett, 1981; Jackes et al., 2001), or cyanobacteria and algae (Ascenzi and Silvestrini, 1984; Davis, 1997). Although there have been cases in which the tunnels of several organisms have been recorded within the same specimen (e.g., Jans et al., 2004), most investigations, however, focus on single or small groups of microbes rather than on complex microbial aggregates such as biofilms—a tendency that may have a dramatic effect on our interpretations of the biodeterioration of archaeological bone.

In this paper, biofilm growth in the Tell Leilan skeletal material from ancient Syria is described. The ultrastructural characteristics of the biofilm were examined by scanning electron microscopy (SEM). The nature of the biofilm was explored using microbe isolation techniques and isolated organisms were identified based on morphology and on the similarity of their 16S rRNA gene sequence to known organisms. Although single and/or small groups of microorganisms have been characterized in archaeological bone, this is the first case of documented biofilm growth in archaeological human bone. Thus, this paper is a contribution to the understanding of the biodeterioration of bone through the characterization of the film and the identification of the microorganisms and their secretions within it.

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2. Biofilms

The term biofilm was coined by Costerton et al. (1978) and refers to surface-associated microbial aggregates that may contain millions of microbial cells including bacteria, protozoa, fungi, and algae. A biofilm, however, is distinguished from other microbial aggregates because embedded microorganisms are surrounded by a self-produced matrix of extracellular polymeric substances (EPS), including carbohydrates, proteins, and extracellular DNA (Allison, 1998; Blankenship and Mitchell, 2006; Davies, 2000; Flemming et al., 2000; Stoodley et al., 2002). EPS materials provide the structural support and protection for the embedded microbial cells (Stoodley et al., 2002).

Investigators have outlined the physiological events involved in biofilm formation (Blankenship and Mitchell, 2006; Busscher and van der Mei, 2000; Costerton et al., 1987; Davies, 2000; Kolenbrander et al., 2000; Stoodley et al., 2002). The initial phase of biofilm growth involves the attachment of an organism to a surface, facilitated by the secretion of EPS (Allison, 1998). A mature biofilm is characterised by a complex network of pores and channels through which nutrients are distributed (Davies, 2000). Maturation has been shown in some circumstances to take fewer than ten days to occur (Heydorn et al., 2000). Portions of the biofilm eventually slough off—a process referred to as detachment (Costerton et al., 1987; Stoodley et al., 2002).

Biofilms are highly structured communities and many members have specialised functions. Some may be parasites, and others scavengers or saprobes, in which case they actively digest the underlying materials (Costerton et al., 1987; Marsh and Bowden, 2000; Wimpenny, 2000). Organisms within a biofilm have been shown to work synergistically in the ultimate breakdown of a surface (Costerton et al., 1987; Wimpenny, 2000). For example, Bradshaw et al., 1994 have outlined how oral bacteria degrade hog mucin better when part of a biofilm. Throughout the growth of the biofilm, microorganisms will be imported and exported, and the community dynamic will change.

Being part of a biofilm confers important advantages to a microorganism by interfering with the establishment of antagonistic organisms (Busscher and van der Mei, 2000; Costerton et al., 1987; Marsh and Bowden, 2000) and by improving their ability to cope with a wider variety of environmental conditions (Allison, 1998; Allison et al., 2000; Gilbert et al., 1997; Wimpenny, 2000) such as extremes in temperature, in pH, and in RH, as well as from the harmful effects of chemicals (Davies, 2000). For example, although several oral bacteria are sensitive to low pH levels found in the mouth, they persist as a biofilm known as dental plaque (Kolenbrander, 2000; Kolenbrander et al., 2000, 2002; Marsh and Bradshaw, 1999). Ultimately, the advantages conferred to community members render biofilms more difficult to control (Donlan, 2000; Gilbert et al., 1997; Patel, 2005).

Biofilms have been recognised on a variety of surfaces including medical devices, water distribution pipes and fountains, and rocks in streams (Costerton et al., 1987; Flemming et al., 2000; Haupt et al., 2012; Revdiwala et al., 2012). Recently, Kaye et al. (2008) discussed possible biofilm growth in fossilized *Tyrannosaur* bone. In addition, biofilms have been observed on several surfaces (e.g., plaster, marble, and so forth) in a variety of contexts in which archaeological bone is present (Doggett, 2000; Saarela et al., 2004; Sanchez-Moral et al., 2003). Although biofilms have been recognised as dental plaque (Busscher and Evans, 1999; Kolenbrander et al., 2000, 2002; Marsh and Bradshaw, 1999) and in living patients suffering from bone and joint infections (Gristina and Costerton, 1984; Sedghizadeh et al., 2008, 2009; Toshiyuki, 2005), they have not been reported as having colonised archaeological bone.

3. Materials and methods

3.1. Skeletal material

The Tell Leilan skeletal material[2] consists of 59 adult and juvenile skeletons dating between 2900 and 1900 BCE. The skeletons were discovered in alkaline deposits while excavating building floors throughout the site. The bone was stored in paper bags and cardboard boxes in the US until 1992 when it was shipped to the University of Alberta (U of A).[3] Upon arrival at the U of A, the skeletal material was cleaned manually with bamboo sticks and brushes of varying firmness. Once skeletal elements were cleaned and inventoried, they were stored in 3 mil plastic bags. The skeletal material is presently stored in a centrally heated building that ranges in temperature and RH from 15 to 25 °C and 15–50% respectively depending on season.[4]

3.2. Scanning electron microscopy

A fragment was sampled from the left proximal radius of a juvenile between the ages of 4–6 years (L87 76G20 58 Phase 4 B2) and was prepared for SEM using ethanol and amyl acetate baths and CPD (see Tsuneda et al., 1991). This method was employed to ensure that constituents of the biofilm were distortion-free and that they did not suffer from cellular collapse (Kurtzman et al., 1974). The bone fragment was fixed in 3% glutaraldehyde for 8 h at 4 °C, was washed in distilled water, was postfixed in 2% tannic acid/guanidine hydrochloride solution for 3 h, was washed again in distilled water, and was postfixed in 2% osmium tetroxide (OsO_4). The fixed sample was dehydrated in an ethanol series (30%, 50%, 70%, 90%, 95%, 100%) followed by an amyl acetate series (50%, 100%).

The sample was critical-point dried in a Balzers CPD 030 Critical Point Dryer for 1.5–2 h, was coated with gold using a Nanotech SEMPrep 2 DC sputter coater, and was examined using a JEOL 6301F (field emission scanning electron microscope) fitted with a liquid nitrogen cooled lithium drifted silicon energy dispersive X-ray (EDX) detector with a Norvar window. Secondary electron (SE) micrographs were obtained using an Everhart-Thornley detector. In addition to the collection of SE micrographs, EDX was used to determine the nature of several crystalline inclusions within the biofilm.

3.3. Microbe isolation and identification

Samples from six skeletal elements were taken from burials located across the site and between excavation years and were assessed for their content of living microbes at the Department of Biological Sciences at the U of A. Each sample was rinsed in 95% ethanol, flamed briefly, wrapped in sterile foil, and crushed. Fragments and fine particulates were evenly distributed over three types of agar media: potato dextrose agar (PDA, Difco Bacto), oxytetracycline water media (16 g selected agar, 1 g oxytetracycline in 40 mL 70% ethanol, add 4 mL/L media for 0.01%), and oatmeal

[2] Tell Leilan is a 90-ha walled site in northeastern Syria. Excavations since 1979 have shown that the site was one of the three largest cities on the Habur Plains during the mid-third millennium BCE, occupied from the mid-sixth to early second millennium (Weiss, 1985; Weiss and Courty, 1993; Weiss et al., 1993). The site is situated on the left bank of the Wadi Jawah in a broad, undulating plain of flood deposits and sands underlain by Pleistocene gravel and plateau basalts (Besonen and Cremaschi, 2002). The area receives between 300 and 500 mm of rain per annum and experiences cool and wet winters and hot and dry summers (Besonen and Cremaschi, 2002; Cullen et al., 2000; Weiss, 1985; Weiss and Courty, 1993).

[3] Unfortunately there are no records describing the environment in which the skeletal material was stored or how the material was cared for prior to its arrival at the U of A.

[4] Records provided by Museum and Collection Services, U of A (P Mayne Correia, pers. comm.).