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Nanomechanical probing of the septum and surrounding substances on *Streptococcus mutans* cells and biofilms

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ABSTRACT

We report a unique bio-nanomechanical behavior at the septum (Z-ring) of *Streptococcus mutans*-containing biofilm through *in situ* measurements obtained by atomic force microscopy. A distinct serrated pattern on the releasing force–displacement curves can only be observed with the use of a sharp nanosized probe tip, and this was found at the septum of *S. mutans*. Further investigations suggested the serrated patterns could be due to the unfolding of some sub-surface divisome proteins. Seismometer measurements were conducted at the septum by placing an ultra-sensitive atomic force microscope probe on the surface. Unique periodic vibrations were observed at the septum under various biofilm conditions. This finding suggests the possibility of remodeling of the cell wall nanostructure at the septum of *S. mutans*.

1. Introduction

Streptococcus mutans is one of the cariogenic bacteria that colonize human teeth. The glucans synthesized by *S. mutans* attach bacterial communities to the tooth surface, forming biofilm (dental plaque), and the acidic environment in this eventually leads to the demineralization of enamel, the formation of cavities and the development of periodontitis [1–3]. In addition, wounds from dental surgery and poor personal oral hygiene habits may cause dissemination of oral bacteria into the bloodstream. With the aid of the collagen-binding protein Cnm [4], it is generally believed that *S. mutans* may adhere to heart tissues and invade human coronary artery endothelial cells, causing infective endocarditis [5], while other health risks, such as pneumonia and diabetes, may be exacerbated by the pathogens in biofilms [6,7].

A better understanding of the physiology of *S. mutans* is crucial for the control and elimination of biofilms. Prior studies investigated the relationship between the cariogenic nature and surface roughness of *S. mutans*, and also tied the surface-cell adhesion of the surface polysaccharides to the different mutations of *S. mutans* cells [8,9]. However, to date there have been very few studies that undertake nanoscale characterizations of specific locations of biofilms, despite the importance of this issue. It is therefore the aim of this paper to uncover the bio-nanomechanical behaviors of *S. mutans* cells to achieve a better understanding of cariogenic pathogens.

This work focuses on the septum (Z-ring) region of *S. mutans*. The septum is where cell dividing occurs, and this location naturally has the most active metabolism. There are several prokaryotic cytoskeleton proteins, such as FtsZ and FtsA, that are involved in the cell division process. FtsZ is the primary cytoskeletal protein in nearly all bacterial and archaeal species, and this assembles into a ring structure (Z-ring) that consists of FtsZ, FtsA, and other division proteins. Recently, a new component of divisome, called septum forming (SepF) protein, was identified in Gram-positive bacteria [10,11], and it is believed that SepF proteins are required for regular assembling of FtsZ protofilaments, and that the absence of these will lead to a malformed septum [10]. Several proteins work together in arranging the Z-ring protein complex and tethering it to the cell membrane, and the cell wall of the division site then invaginates and forms a septum.

Scanning probe techniques, such as atomic force microscopy (AFM), have a unique combination of advantages in the study of biomaterials. For instance, AFM needs little or no sample preparation, allows *in situ* and *in vivo* measurements, is ultra-sensitive in tip-sample interactions, and produces results on a nanoscale resolution, and these attributes are especially useful for the understanding of pathogens. Furthermore, recent advances in probe technology and instrumentation have enabled local mapping of nanomechanical properties, and such approaches have the potential to reveal the functionality of microbes.

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Based on the AFM platform, several derivative measurement techniques are of particular interest in the study of biofilms [12,13]. For instance, force spectroscopy, which measures ultra-small forces between the AFM tip and sample surface, has proven useful in the study of cells. Cross *et al.* characterized the stiffness of cancer cells by AFM nanoindentation, and used their biomechanical properties to distinguish live metastatic cancerous cells from normal ones, even when they showed a similar morphology [14]. The retracting force during tip-sample interaction is also useful in interpreting local adhesion, and it is therefore possible to analyze adhesion force or the bond-breaking (or unfolding) force of the surface proteins or molecules [12,15–19].

Another valuable technique adopted in our study is the "seismometer" mode. As the name suggests, in this an ultra-sensitive AFM probe is stationary and kept in contact with the sample surface to serve as the detector of "seismic waves". By using this technique, researchers were able to discover the distinct periodic nanomechanical motion of yeast cells, which was identified as a metabolic process [20,21].

2. Materials and methods

2.1. S. mutans culture

S. mutans (ATCC 25175) biofilm samples (Fig. 1(a)) were prepared and cultivated in Brain Heart Infusion liquid (BHI, Merck, Darmstadt, Germany) at 37 °C for 24 h and 48 h, respectively. The standard biofilm cultivation steps used for this are described elsewhere [22]. Several 5 mm × 5 mm sterilized glass substrates were placed into a 24-well micro-plate. 5 μL of *S. mutans* aqueous solution with a 5×10^8 colony-forming-unit per milliliter (CFU/mL) concentration was dispersed on the top surface of the glass substrate. 1000 μL BHI consisting of 0.15% sucrose was added into the well and cultured at 37 °C. The BHI liquid was changed every 24 h. Following these procedures, *S. mutans* biofilm samples were prepared with 24- and 48-h growth times. In addition, preparation of single (isolated) *S. mutans* specimens was completed by dispensing 5 μL of bacteria aqueous solution (5×10^8 CFU/mL) on the top surface of the glass substrate.

A fluorescent microscope (DMIRB, Leica, Germany) and the live/dead BacLight Bacterial Viability Kits (Molecular Probe Inc., Eugene, OR, USA) were used to confirm the vitality of *S. mutans*. For AFM measurements on living cells, the specimen was taken out of the liquid right before the measurements, and was exposed to the air (relative humidity 70%, room temperature ~25 °C). The experiments were performed at less than 30 min exposure in air, and the specimens were checked with the Viability Kit, whose nucleic acid stains (SYTO 9 and propidium isodide) reveal green for live and red for dead, to confirm the cells were still alive. The alcohol-treated single cell and biofilm specimens were prepared by soaking the cultivated substrates in 95% (v/v) alcohol for 1 h. Alcohol-treated specimens were also confirmed dead (red fluorescent) under FM.

The porous EPS structure of the biofilm helped to contain fluids and keep it moist after the specimens were exposed to air [23]. In our preliminary study ($n = 4$) that assessed the vitality of the *S. mutans* biofilm with fluorescent dye, more than 90% *S. mutans* were still alive after 30 min of exposure in the air, and about 70% *S. mutans* were still alive after 1 h of exposure.

2.2. In situ AFM measurements

The single *S. mutans* and 24 h- or 48 h-cultivated *S. mutans* biofilm specimens were taken out of BHI fluid and transferred to a commercial AFM system (BASO AFM, Force Precision Instrument, Co., Taiwan) for *in situ* nanomechanical measurements. The specimen surface was survey-scanned under AFM in tapping (AC) mode to identify the specific area of interest (Fig. 1(b)), and measurements of localized biomechanical properties were then carried out by AFM nanoindentation and seismometer scans. The AFM probes (ContAl, Budget Sensors, Bulgaria) used in this work were prescreened for quality, and had a typical tip radius of curvature (ROC) of less than 5 nm and spring constant in the range of 0.25–0.3 N/m.

All of the experiments were conducted in an acoustically isolated chamber to avoid disturbance from the environment. To make sure the fluctuations of the force–displacement curves in our

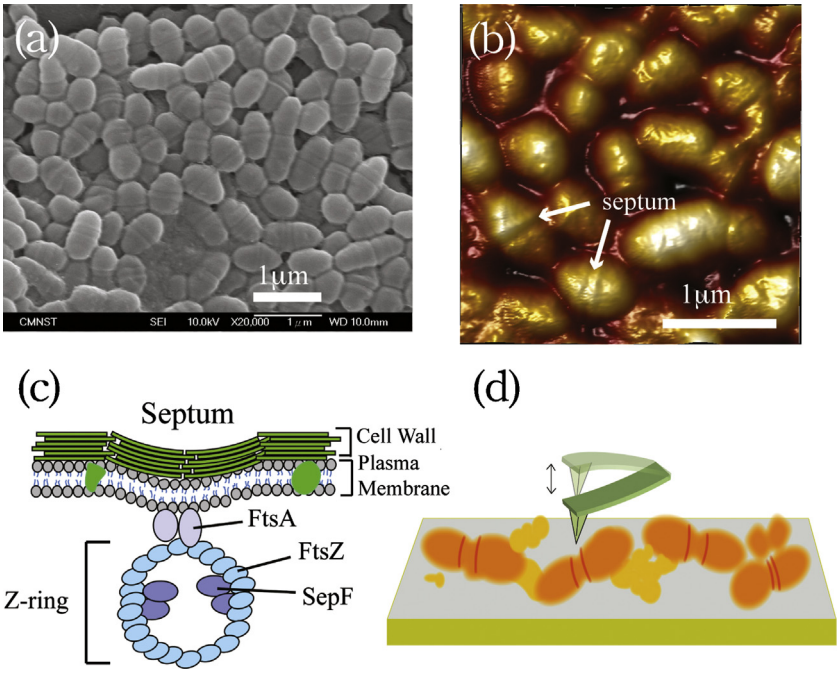

Fig. 1. (a) The SEM and (b) AFM images of *S. mutans* biofilm after 24-h culture. The septum formed between two cells for cell division. (c) The Z-ring structure inside *S. mutans*. FtsZ assembled into a closed ring structure and was tethered to plasma membrane by FtsA. (d) The experimental setup of AFM-based measurements.