Mar. Biotechnol. 4, 127–131, 2002
DOI: 10.1007/s10126-001-0073-7

MARINE BIOTECHNOLOGY

In Situ Microspatial Imaging Using Two-Photon and Confocal Laser Scanning Microscopy of Bacteria and Extracellular Polymeric Secretions (EPS) Within Marine Stromatolites

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Abstract: The combination of a hydrophilic embedding resin, Nanoplast, with fluorescent probes, and subsequent imaging using two-photon and confocal laser scanning microscopy (2P-LSM and CLSM) has allowed in imaging of the in situ microspatial arrangements of microbial cells and their extracellular polymeric secretion (EPS) within marine stromatolites. Optical sectioning by 2P-LSM and CLSM allowed imaging of endolithic cyanobacteria cells, *Solentia* sp., seen within carbonate sand grains. 2P-LSM allowed very clear imaging with a high resolution of bacteria using DAPI, which normally require UV excitation and reduced photo-bleaching of fluorescent probes.

Key words: confocal laser scanning microscopy, *Schizothrix* sp., *Solentia* sp., extracellular polymeric secretion, marine stromatolite, two-photon laser scanning microscopy.

INTRODUCTION

Many bacterial activities occur in microenvironments in which the microspatial arrangements of cells and their extracellular polymeric secretion (EPS) are especially important in regulating their activities (Azam, 1998; Azam et al., 1993). Observing the microspatial arrangements of bacteria and their EPS in natural sediment systems has been particularly difficult owing to the difficulty of preserving the delicate matrix of highly hydrated EPS due to chemical alteration that occurs during sample preparation and less penetration of light through sand grains in conventional light microscopy (Costerton et al., 1995; Decho, 1993; Decho and Lopez, 1993; Lavoie et al., 1995; Paterson, 1995). In this study we used modern marine stromatolites found in the Bahamas as an aquatic sediment model. Marine stromatolites are layered structures formed by complex interactions of heterotrophic bacteria, cyanobacteria, carbonate sand grains, and the environment (Reid and Browne, 1991). Previously we have reported a novel embedding method for confocal imaging to observe the microspatial relationship among microbial cells, their EPS, and carbonate sand grains in situ within the marine stromatolite using Nanoplast, a hydrophilic embedding resin (Decho and Kawaguchi, 1999).

Two-photon laser scanning microscopy (2P-LSM) has

Received April 26, 2001; accepted August 8, 2001.

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Figure 1. Confocal and two-photon laser scanning microscopes images of marine stromatolites, Bahamas. (A) Overlay image of autofluorescence carbonate sand grains (blue) trapped by propidium iodide labeled *Schizothrix* sp. filaments (red) and FITC–concanavalin A-labeled EPS (green) secreted by *Schizothrix* sp. by single photon confocal microscopy. (Scale: 50 μm.) (B) Overlay image of DAPI-labeled endolithic cyanobacterium, *Solentia* sp. (blue), within carbonate sand grains by 2P-LSM and autofluorescence carbonate sand grains (red) taken by CLSM. EPS (green) secreted by *Schizothrix* sp. can be seen around carbonate sand grains. (Scale: 50 μm.) (C) 2P-LSM image of DAPI-labeled *Schizothrix* sp. filaments (blue) extending from carbonate sand grains and DAPI-labeled heterotrophic bacteria (blue small dots) surrounding *Schizothrix* sp. filaments. Endolithic cyanobacterium, *Solentia* sp., was seen within carbonate sand grains (blue). (Scale: 50 μm.) (D) Cell division of DAPI-labeled endolithic cyanobacterium, *Solentia* sp. (blue), was captured by 2P-LSM. (Scale: 2 μm.) (E) Three-dimensional confocal image of propidium iodide labeled *Schizothrix* sp. filaments (red), heterotrophic bacteria (red dots), and $CaCO_3$ precipitates (blue). 3D projection from 20 sections. Increment in *z* between two consecutive optical sections: 0.5 μm. (Scale: 50 μm.)

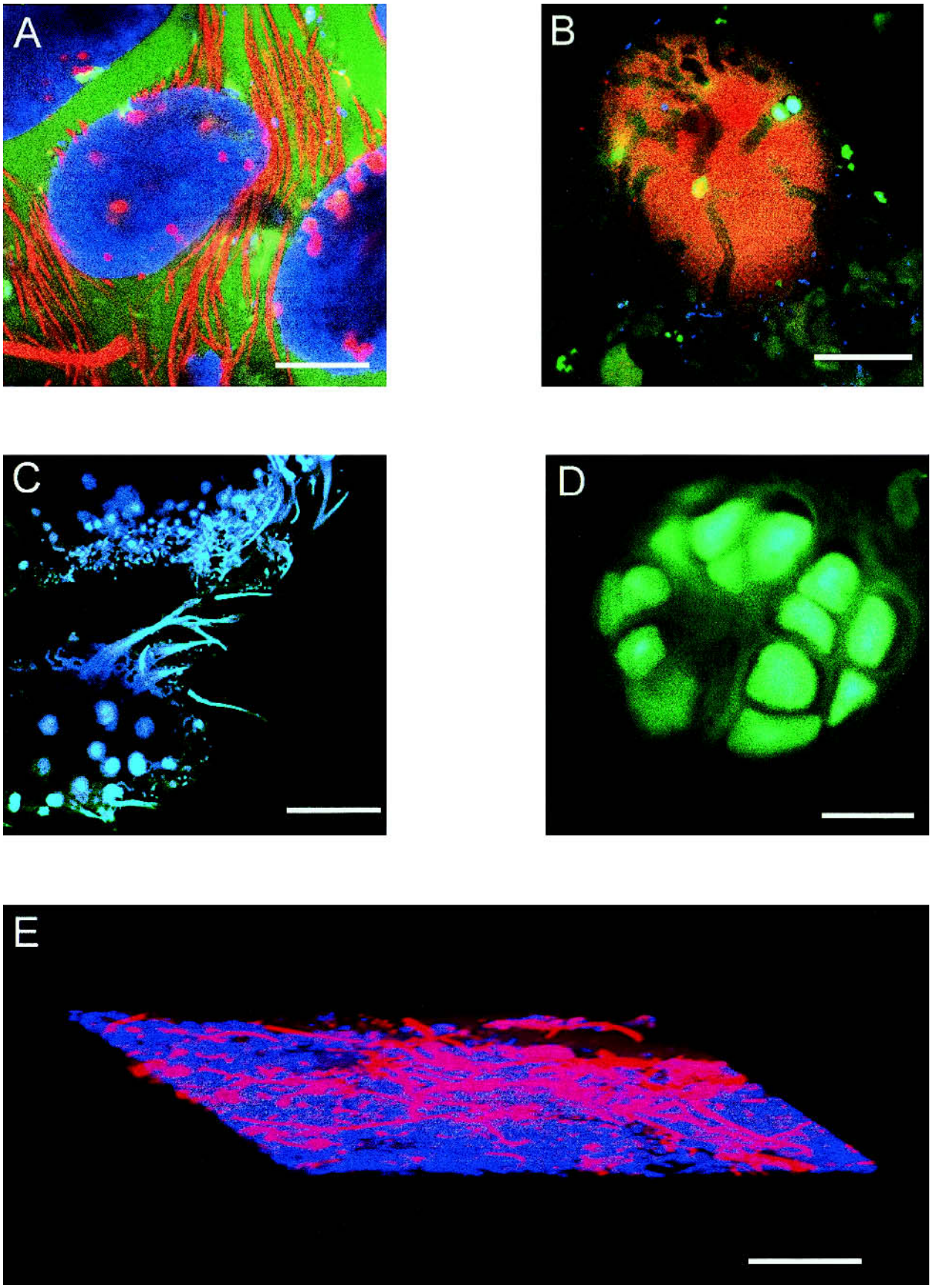
received much attention over conventional confocal microscopy in 3D imaging of biological structures in recent years (Denk et al., 1990; Diaspro and Robello, 2000; Konig, 2000). Two-photon excitation provides advantages over conventional confocal microscopy by reducing photoxicity and photobleaching of fluorescent probes and by increasing the resolution (Diaspro and Robello, 2000; Konig, 2000). Two-photon excitation also allows imaging of UV fluorophores such as 4′, 6-diamidino-2-phenylindole:2 HCl(DAPI), which normally require UV excitation (Diaspro and Robello, 2000). We applied 2P-LSM combined with CLSM to visualize bacteria and extracellular polymeric secretions (EPS) within marine stromatolites. Here we report the microspatial arrangement of bacteria, carbonate sand grains, and EPS within the marine stromatolite in situ using 2P-LSM and CLSM.

Materials and Methods

All stromatolite samples were collected from an intertidal marine environment at Highborne Cay in the Exuma Chain of islands in the Bahamas (Reid and Browne, 1991) and are under current investigation through the Research Initiative on Bahamian Stromatolites (RIBS) project.

Sample preparation was carried out according to the method of Decho and Kawaguchi (1999). Freshly collected stromatolite samples were sectioned using sterile razor blades. Immediately after collection, the sections of stromatolite were preserved in 3% formaldehyde in seawater. A double-labeling experiment was performed on the sections to determine the spatial relationship of microbial cells and their EPS, using DAPI or propidium iodide (Molecular Probes, Eugene, Or., U.S.A.) for cellular DNA and FITC concanavalin A (Molecular Probes) for EPS (Decho and Kawaguchi, 1999). Sections were trimmed (3 × 8 × 5 mm) using a sterile razor blade (2.5 × 4 cm), then placed in BEEM embedding molds (5 × 10 × 5 mm). Resin and catalyst were thoroughly mixed on site and then added to molds containing the stromatolites samples. The molds were placed in a temperature-controlled heat block at 25°C for 48–60 hours to allow slow penetration and complete mixing of the Nanoplast resin (Ted Pella, Redding, Calif., U.S.A.) with the hydrated sample. After penetration of the medium, the temperature was raised to 40°C for 48 hours to dry and then 60°C for 48 hours to harden the medium into blocks. The resulting blocks were thick sectioned (approximately 2 mm), mounted on glass microscope slides using Epon 812, and then observed using confocal or two-photon laser scanning microscopy (CLSM and 2P-LSM, respectively).

All CLSM and 2P-LSM were conducted using a MRC 1024MP system (BioRad Laboratories, Hercules, Calif., U.S.A.) equipped with a Nikon Eclipse TE 300 compound microscope (Nikon, Tokyo, Japan). Sample slides were viewed using either Nikon Plan Apo 60×/1.40 oil or Nikon Plan Apo 100×/1.40 oil objective lenses. A single photon (Krypton-Argon) laser 488-nm line was used for imaging FITC–concanavalin A-labeled EPS (emission = 522 nm). Autofluorescence was used for imaging carbonate sand grains (i.e., ooids), and $CaCO_3$ precipitates (emission = open). The *Z* optical sections were collected at 0.5-μm intervals to reconstruct a 3D projection image using Laser Sharp program (Bio-Rad Laboratory). A Titanium-Sapphire laser (Coherent, Santa Clara, Calif., U.S.A.) was used for the imaging of DAPI-labeled microbial cells. The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Ti-sapphire source of the two-photon system is an infrared wavelength laser that produces photon pulses at femtosecond intervals (Denk et al., 1990) and was set at 780 nm.

Results and Discussion

2P-LSM and CLSM images are able to provide a high level of spatial resolution. EPS and the cyanobacterial community (*Schizothrix* sp.) were very nicely imaged in the interstitial spaces between sand gains using a combination of the FITC–concanavalin A probe and propidium iodide (Figure 1A). *Schizothrix* sp. is a dominant species in marine stromatolite, which produces EPS (Reid et al., 2000). Optical sectioning made the image of endolithic cyanobacteria cells, *Solentia* sp., easily seen in the tunnels within carbonate sand grains (Figure 1B). *Solentia* is an endolith, which bores into carbonate sand grains (Reid et al., 2000). The use of a multiphoton laser enhanced our capability to image microbial systems, by providing the ability to image fluorophores (e.g., DAPI), which usually require UV-wavelength excitation, without the need for a separate UV laser system (Diaspro and Robello, 2000). Another advantage of multiphoton excitation is that fluorescent occurs "only" in the optical plane of excitation, which may enhance resolution in imaging sediments. DAPI probes, which are conventionally imaged using an excitation at $\lambda = 365$ nm, were imaged best on our multiphoton system using an excitation wavelength at 780 nm (Figure 1C and D). DAPI-labeled *Schizothrix* sp. filaments were extending from sand grains and heterotrophic bacteria were localized surrounding these filaments (Figure 1C). 2P-LSM allowed clear imaging of *Solentia* sp. within carbonate sand grains with a high resolution using DAPI and a deep penetration of laser through sediment grains (Figure 1D). This is not possible by using conventional light microscopy and scanning electron microscopy. Although DAPI staining is a conventional method to visualize bacterial cells, DAPI cannot be excited by conventional 1P laser. Therefore, the 2P-LSM has an advantage over 1P. Cell division of *Solentia* sp. and their EPS surrounding the cells can be seen in Figure 1D. DAPI-labeled microbial cells could be easily imaged through many layers of carbonate sand grains (Figure 1C). Images collected using the 2P-LSM system accurately and precisely complemented our observations using a conventional CLSM (Figure 1B). A 3D image of the top of the marine stromatolite showed that propidium iodide-labeled *Schizothrix* sp. filaments and heterotrophic bacterial cells are closely associated with $CaCO_3$ precipitates (Figure 1E). The confocal images provide a 3D optical section which has a *z*-axis thickness that can be adjusted from <0.5 to >10 μm. No significant photobleaching was observed in our study.

Confocal and two-photon imaging allows clear imaging of bacteria and their EPS in sediment systems and even inside carbonate sand grains through optical sectioning while preserving the intact 3D microspatial arrangements of their cellular and extracellular environments. Magnification of 10,000× can be achieved when a combination of the objective lens (100×) and zoom function in the Laser Sharp program are used. The method can be easily adapted for use with fluorescence in-situ hybridization (FISH) probes in the future.

Acknowledgments

This work was supported by grants from the National Science Foundation (OCE 96-17738), and Office of Naval Research (N00014-97-10024). We thank Dr. Joan Bernhard (Department of ENHS, USC) for initially suggesting the fluorescent probe approach, and the scientists of the RIBS (Research Initiative on Bahamian Stromatolites) project for helpful discussions during sample collections in the Bahamas. Mr. Don Dean (Smithsonian Institution, Washington, D.C.) is gratefully acknowledged for his technically precise thick-sectioning and mounting of our Nanoplast resin samples. We thank Dr. Susan Ellis for reviewing this manuscript. This represents RIBS (Research Initiative on Bahamian Stromatolites) Contribution #19.

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