Ultrastructural imaging and molecular modeling of live bacteria using soft x-ray contact microscopy with nanoseconds laser plasma radiation

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ABSTRACT

X ray images of the various live bacteria, such as *Staphylococcus* and *Streptococcus*, and micromolecule such as chromosomal DNA from *Escherichia coli*, and Lipopolysaccharide from *Burkholderia cepacia*, are obtained with soft x-ray contact microscopy. A compact tabletop type glass laser system is used to produce x rays from Al, Si, and Au targets. The PMMA photoresists are used to record x-ray images. An AFM (atomic force microscope) is used to reproduce the x-ray images from the developed photoresists. The performance of the 50nm spatial resolutions are achieved and images are able to be discussed on the biological view.

1. ITRODUCTION

Detection for clinical diagnosis and study of microbial cell is performed by a combination of low magnification optical microscopy and direct and indirect labeling techniques. Visual ultrastructural studies on subcellular organelles are possible with variations of electron microscopy (thin section, scanning and freeze fracture), although specimen preparation steps such as fixation, dehydration, resin embedding, ultra-thin sectioning, coating and staining are very specialized, extensive and may introduce artifacts in the original sample.

The development of high resolution x-ray microscopy is a new technique well suited to observe the intact structure of a biological specimen at high resolution without any artifacts. The advantages of x-ray microscopy are seen to be: (i) the contrast can be provided by specimen components, thereby avoiding possible artifacts caused by the staining and fixation of specimen; (ii) x-ray microscopy is capable of observing thicker specimens (up to a few μm in depth) than electron microscopy and with less damage; (iii) the actual location of the element inside a cell can be visualized when the proper x-ray wavelength is chosen; (iv) three-dimensional observation may be possible with a single exposure of x rays. X-ray contact microscopy is presently the most suitable method by which to evaluate these advantages[1].

It is generally accepted that the highest contrast in x-ray images of *in-vitro* biological specimens will be obtained with radiation of a wavelength in the so-called "water window" (2.3-4.4 nm). Both laser plasmas and synchrotrons are bright source of radiation in this region. However laser sources have the advantage that, they can be spectrally tailored to preferentially emit in specific regions, by adjustment of target and irradiation conditions and that their pulsed nature provides the capability to make single-shot

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framed images in times shorter than kinetic response times of biological organisms. Up to the present time, demonstrations of these capabilities with contact x-ray microscopy have only been made with large laser facilities, commonly used for fusion studies [2, 3]. To make this technology more condutive to clinical applications less complex systems are required.

We present soft x-ray microscopic observation of bacteria such as *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus*, *Streptococcus pneumoniae, Escherichia coli ATCC 25922 cDNA, and Burkholderia cepacia ATCC 10856 Lipopolysaccharide (LPS)*[4].

2. THE BACTERIA[4]

Staphylococcus is an aerobic organism with a typical respiratory metabolism. It is catalase positive, and this test permits its distinction from *Streptococcus* and other genera of Gram-positive cocci. The Gram-positive cocci are relatively resistant to reduced water potential, and tolerate drying and high salt fairly well. Their ability to grow in media with high salt provides a simple means for isolation. If an inoculum is spread on an agar plate with a fairly rich medium containing bically, Gram-positive cocci will often form the predominant colonies. Often, these organisms are pigmented, and this provides an additional aid in selecting Gram-positive cocci.

Staphylococci are common parasites of humans and animals, and occasionally cause serious infections. In humans, two major forms are recognized, *S. epidermidis*, a nonpigmented, nonpathogenic form that is usually found on the skin or mucous membranes, and *S. aureus*, a yellow pigmented form that is most commonly associated with pathological conditions, including boils, pimples, pneumonia, osteomyelits, meningitis, and arthritis.

The genus *Streptococcus* contains a wide variety of species with quite distinct habitats, whose activities are of considerable practical importance to humans. Some members are pathogenic to people and animals. As producers of lactic acid, certain streptococci play important roles in the production of buttermilk, silage, and other fermented products. To distinguish generally nonpathogenic streptococci from human pathogenic species, the genus *Streptococcus* has been split into three genera. The genus *Laptpcoccus* contains those streptococci of dairy significance while the genus *Enterococcus* has been created to group streptococci that are primarily of fecal origin.

Therapeutic treatment of infections caused by pathogenetic bacteria can be difficult due to the phenomenon of antibiotic resistance. Some microorganisms are resistant to some antibiotics. Antibiotic resistance can be an inherent property of a microorganism, or it can be acquired. There are several mechanisms by which a microorganism may be resistant to an antibiotic. (1) The organism may lack the structure which an antibiotic inhibits. For instance, some bacteria such as mycoplasmas, lack a typical bacterial wall and therefore are resistant to penicillins. Penicillins disrupt cell wall formation. (2) The organism may be able to alter the antibiotic to an inactive form by degradation. (3) The organism may modify the target of the antibiotic. (4) By genetic mutation, alteration may occur in a metabolic pathway in which an antimicrobial agent acts as an analog. (5) The organism may be able to pump out any antibiotic entering the cell by active efflux to reduce the effective antibiotic cencentration.

Members of the genus *Escherichia* are almost universal inhabitants of the intestinal tract of humans and warm-blooded animals, although they are by no means the dominant organisms in these habitats. *Escherichia* may play a nutritional role in the intestinal tract by synthesizing vitamins, particularly vitamin K. As a facultative aerobe, this organism probably also helps consume oxygen, thus rendering the large intestinean aerobic. Wild-type *Escherichia* strains rarely show any growth-factor requirements and are able to grow on a wide variety of carbon and energy sources such as sugars, amino acids, organic acids, and etc.

Some strains of *Escherichia* are pathogenic. The latter strains of *Escherichia* have been implicated in diarrhea in infants, occasionally occurring in epidemic proportions in children's nurseries or obstetric wards, and *Escherichia* may also cause urinary tract infections in older persons or in those whose resistance has been lowered by surgical treatment or by exposure to ionizing radiation. Enteropathogenic strains of *Escherichia coli* are becoming more frequently implicated in dysentery-like infections and in generalized fevers.

The total amount of DNA in the chromosome of a bacterium such as *Escherichia coli* is about 4700 kilobase pairs. Bacterial DNA is not surrounded by a membrane typical of the eukaryotic nucleus, although it dose tend to aggregate as a distinct structure within the cell and is visible when observed with the electron microscope.

Gram-negative bacteria, such as *Escherichia coli*, contain an outer wall layer made of lipopolysaccharide. This layer is effectively a second lipid bilayer, but is not constructed solely of phospholipid, as is the cytoplasmic membrane, but also contains polysaccharide and protein. The lipid and polysaccharide are intimately linked in the outer layer to form specific lipopolysaccharide branched chain structures. Because of the presence of lipopolysaccharide, the outer layer is frequently called the lipopolysaccharide or LPS layer.

3. CONTACT MICROSCOPY WITH PMMA PHOTORESIST

X-ray contact microscopy[5] is, in principle, a very simple method. A specimen is placed on an x-ray resist supported by a silicon base and is exposed to a parallel beam of soft x-rays, usually 1-10 nm in wavelength, roughly normal to the resist surface. Polymethylmethacrylate (PMMA) and its related polymers are widely used as a resist. The resolution of PMMA can be as high as 5nm, which is adequate for the present purpose, but its sensitivity is expected to be further improved. The intensity of x-rays passing through the specimen and reaching the surface of the resist is decreased depending on the amount of absorption by the specimen. The difference in intensities is recorded on the resist as radiation damage proportional to the number of photons received.

The exposed resist is developed by dissolving the damaged material in an appropriate solvent such as a mixture of methyl isobutyl ketone (MIBK) and isopropyl alcohol (IPA). The uneven structure which results from the development can be observed with a differential interference microscope, an electron microscope, or an atomic force microscope.

4. GENERATION OF LASER PLASMA X RAYS

The primary advantage of a laser plasma x-ray source for x-ray microscopy[6] is its compactness and flexibility. The spectral brightness of laser plasma x-ray sources can be comparable to the brightest available synchrotrons[7]. Laser plasma x-ray sources have the advantage of being compact, moveable and tolerable of modest vacuum requirements. Shown in Fig.1 is our compact tabletop glass laser system with injection seed type oscillator and multi-path amplifier. Also an SBS (Stimulated Brillouin Scattering) phase conjugation mirror is used. The x-ray emission spectrum of laser plasma has bright broad continuum emission and narrow atomic emission lines, and can easily be varied to suit specific microscopy needs. Shown in Fig.2 is the typical x-ray line emissions with Al and Si targets calculated with the RATION code[8]. The parameters used in these calculations are 300eV for electron temperature and $1x10^{21}$ $1/cm^3$ for an electron density. Each emission has strong line emission at 5.2 nm and 4.4 nm respectively. Although these lines are in fact outside of the water window, they are very suitable for thicker specimens like bacteria. Around these

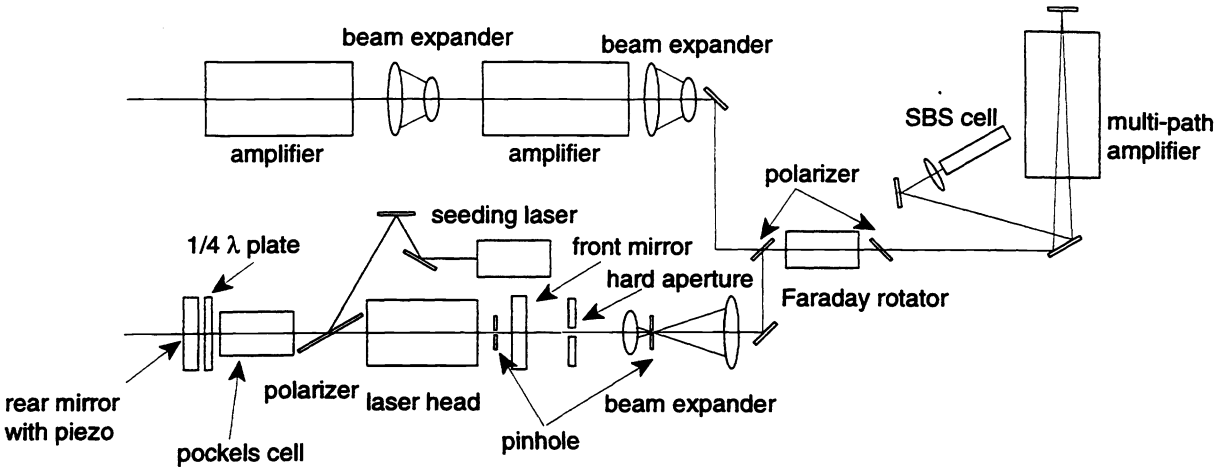

Fig.1 A compact tabletop type glass laser system with injection seeded ocillator, multi-path amplifier, and SBS phase cunjugat mirror. This laser system provides a 20J and 10ns pulse.

wavelengths 50 to 80% of absorption is expected for 1 to 2 μm thick specimens. Laser plasmas are point sources which can be highly reproducible, a requirement for precision x-ray optical systems having extremely high alignment specifications. Pulsed laser plasmas introduce the time domain element into x-ray microscopy. Whereas exposure time for microscopy with synchrotrons are measured in seconds, laser plasmas can provide x-ray emission in pulse ranging from several nanoseconds duration to less than one picosecond. This introduces the possibility of capturing kinetic, chemical, or morphological changes in biological structures in time frames on interest to understanding complex biological processes. No other source for x-ray microscopy can offer this capability.

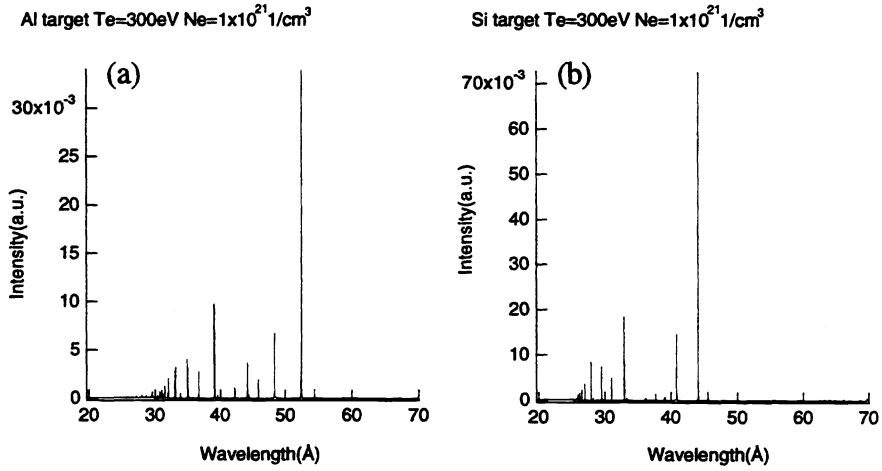

Fig.2 Typical x-ray line emissions from (a) Al and (b) Si calcurated with RATION code. Electron temperature 300eV and electron density $1x10^{21}$ $1/cm^3$ are used.

5. CONTACT X-RAY MICROSCOPY OF LIVE BACTERIA

Shown in Fig.3 is the experimental setup and a sample holder which can keep wet specimens in vacuum. The pulsed laser beam was focused onto Al or Si planar target to produce laser plasma x rays. Typical laser parameters are 1μm wavelength, 10 to 20 J energy, and 10ns pulse duration. The spot size on

the targets is about 100μm diameter. The intensity on targets is going to be 2-3x10^{13} W/cm^2. The specimen holder was placed 1 to 2 cm away from target at an angle 45 degree from target normal. Thin CH or silicon nitride (Si_3N_4) filter was placed between x-ray source and biological specimen to avoid damaging resists with plasma particles. Experiments were conducted with one single x-ray exposure (exposure time is ~10ns) for each biological specimens. The x ray photo resists were rinsed with NaOCl to remove leftover specimens from the resist and were developed with mixture of MIBK and IPA (1:1) for about 1 to 3 minutes.

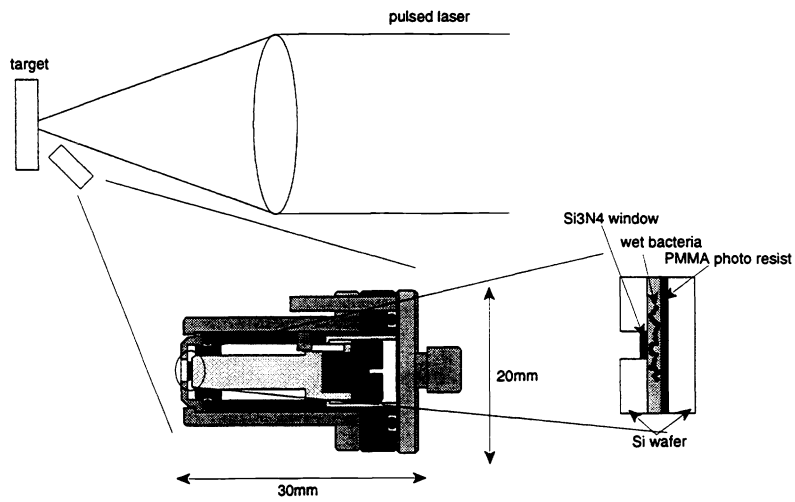

Fig.3 Experimental setup and a schematic view of a sample holder. A pulsed laser is focused to pro duce plasmas. The sample holder is placed 1cm to 2cm from target.

An Atomic Force Microscope (AFM) was used to reproduce images from developed photoresists. The AFM is a recent innovation that relies on a mechanical probe for generation of magnified images. An AFM is operable in ambient air, liquid, or vacuum to resolve features in three dimensions down to fraction of an angstrom.

The schematic view of the AFM is shown in Fig.4. The AFM is comprised of a sensing probe, piezoelectric ceramics, a feedback electronic circuit, and a computer for generating and presenting images.

The AFM measures the deflection of a cantilever. A tip is mounted on the cantilever such that, when the cantilever moves, the light beam from a small laser moves across the face of a four section photodetector. The amount of motion of the cantilever can then be calculated from the difference in light intensity on the sectors.

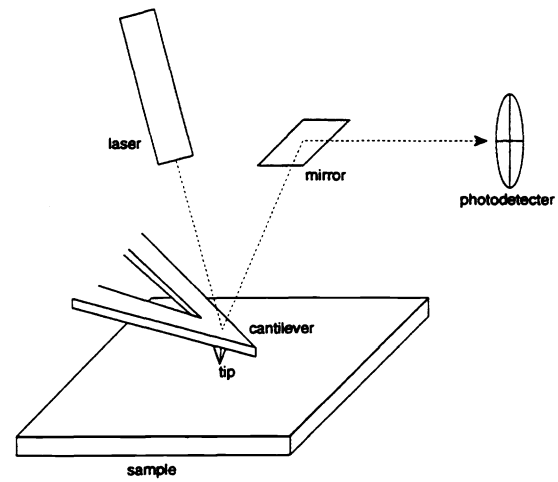

Fig.4 A schematicview of the AFM.

6. RESULTS AND DISCUSSION

Shown in Figs. 5, 6, 7, 8, and 9 are the reproduced x-ray images of *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus*, *Streptococcus pneumoniae*, DNA from *Escherichia coli*, and Lipopolysaccharide (LPS) from *Burkholderia cepacia*, respectively. Fig.5 (b) is the enlarged image of the Fig.5 (a) and Fig.6 (b) is the enlarged image of the Fig.6 (a). For *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus*, and *Escherichia coli*, Al targets and 200nm thick Si_3N_4 windows were used and laser energy was 12J. For *Streptococcus pneumoniae* and LPS, Si targets were used and laser energy was 5J. The contrast of the images with Al targets were better than the images made with Si targets, although the laser energy and filters are different. Tatter could be explained by the difference of the absorption ratios for the 1μm thick CH (typical thickness of the bacteria used for these experiment) for the Al and Si emission lines

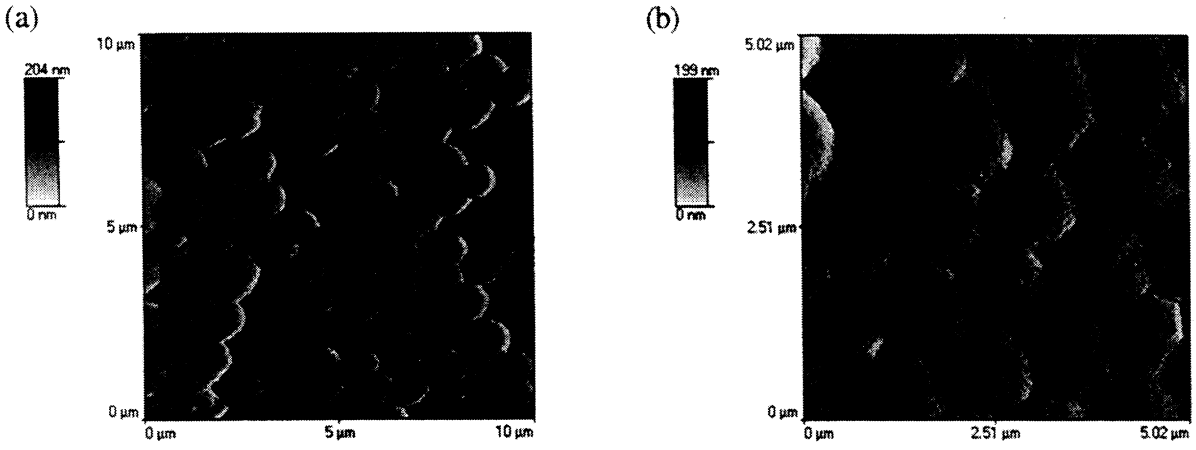

FIg.5 X-ray images of *Staphylococcus aureus*.

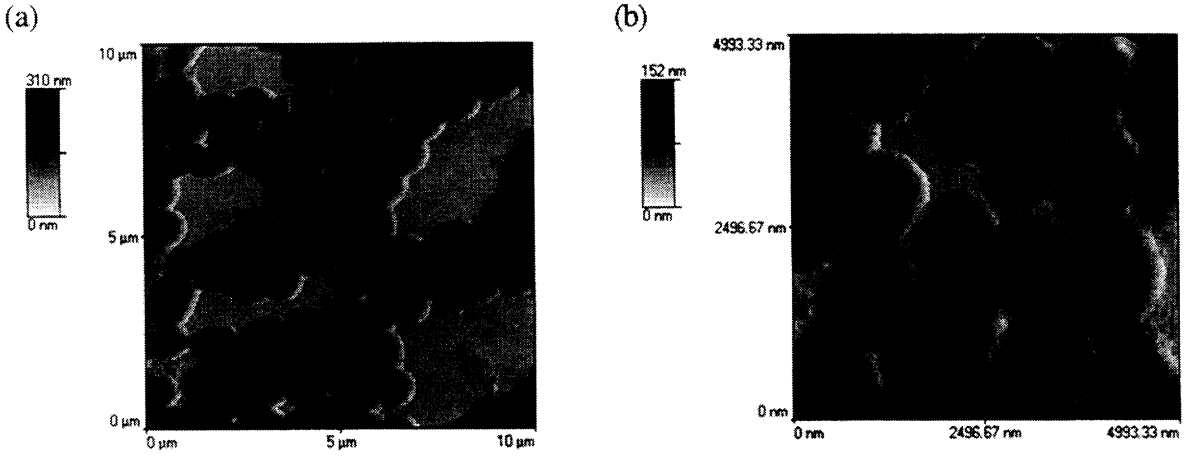

Fig.6 X-ray images of Methicillin resistant *Syaphylococcus aureus*.

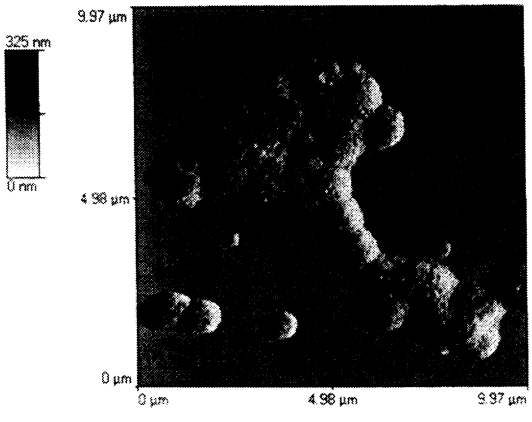

Fig.7 X-ray imege of *Streptococcus pneumoniae*.

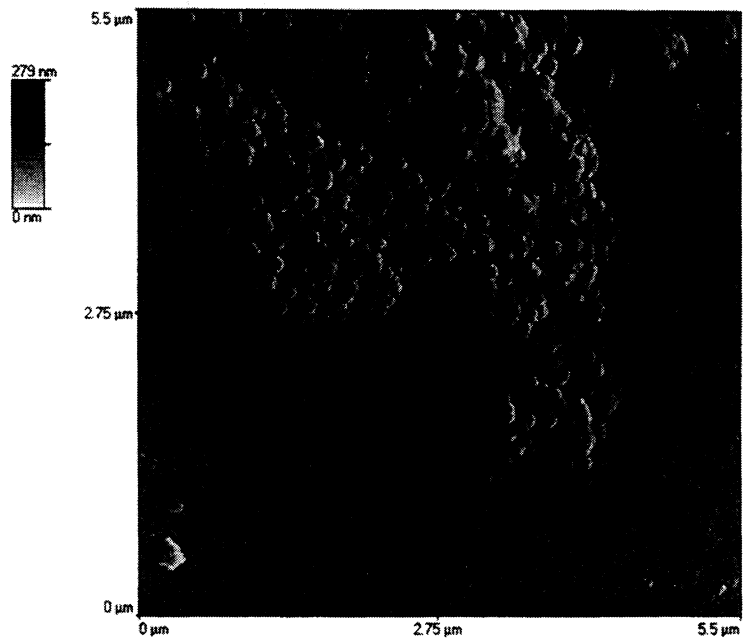

Fig.8 X-ray image of DNA from *Escherichia coli.*

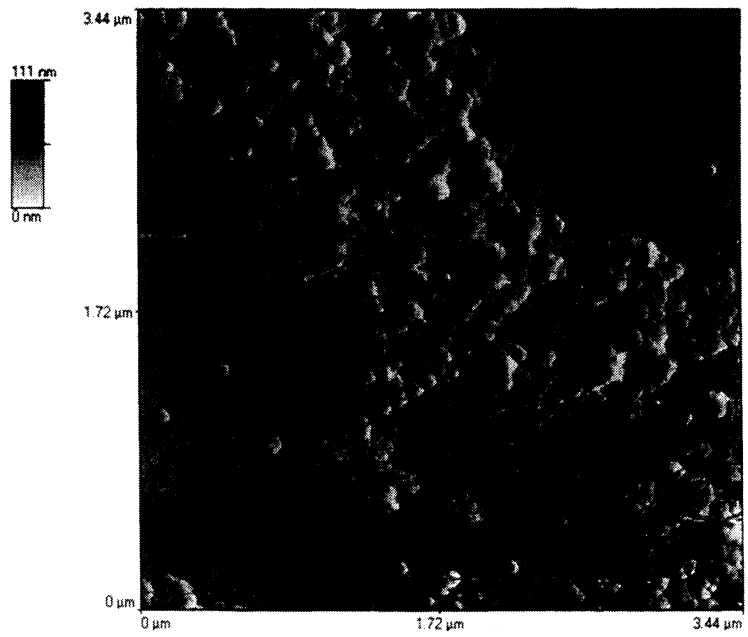

Fig.9 X-ray image of Lipopolysaccharide (LPS) from *Burkholderia capacia.*

(5.2 nm and 4.4 nm), which are 70% and 80%, respectively.

Comparing Fig.5 and Fig.6, there is no significant difference between the images of normal *Staphylococcus* and those of Methicillin resistant *Staphylococcus*.

Fig.7 shows the chain structure of *Streptococcus pneumoniae* and it is clearly different from the images of *Staphylococcus aureus* which divides in more than one plane.

The x-ray image of the DNA from *Escherichia coli* shows a particle like structure. The size of each particle was about 50nm. The x-ray image of the LPS from *Burkholderia cepacia* also shows particle structures. The size of each particle was about 50nm. Those sizes could be limited with the resolution of the AFM, while the PMMA resists have about 10nm resolution.

7. SUMMARY

Soft x-ray contact microscopy has been applied to microbiology using a compact tabletop size glass laser system. Unstained high contrast images of bacteria were obtained with high resolution. The resolution of the system was about 50nm. Particle structure of DNA and LPS were clearly imaged. Although there is no significant difference between the normal *Staphylococcus* and the Methicillin resistant *Staphylococcus*, the clear high contrast images indicate the potential of x-ray microscopy to investigate the effect of treatments on biological organism.

8. ACKNOWLEDGMENT

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