Department of Bacteriology

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General Summary

Research projects of our department have focused on: 1) the development of a method for extracting extracellular matrixes from bacterial biofilms and its applicability, 2) high-resolution observation of bacterial biofilms with the atmospheric scanning electron microscope (ASEM), 3) biofilm formation by *Propionibacterium acnes* isolated from pacemakers, 4) high-throughput screening of antibiofilm compounds, 5) analysis of biofilm detachment factor, 6) a simple assay for measuring catalase activity: a visual approach, and 7) analysis of the viable but nonculturable (VBNC) state.

Research Activities

Development of method for extraction of extracellular matrixes from bacterial biofilms and its applicability

Biofilm-forming bacteria embedded in polymeric extracellular matrixes (ECMs) that consist of polysaccharides, proteins, and/or extracellular DNAs acquire high resistance to antimicrobial agents and host immune systems. To understand molecular mechanisms underlying the formation and maintenance of biofilms and to develop therapeutic countermeasures against chronic biofilm-associated infections, we developed an ECM extraction method using high concentrations of sodium chloride and evaluated its applicability. Our method is simple, rapid, inexpensive, and noninvasive and can be used to extract various types of ECM in biofilms formed by diverse bacteria, including Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* and Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. Furthermore, this method can be used to clarify molecular mechanisms of biofilm formation and modes of actions of antibiofilm enzymes and drugs.

High-resolution observation of bacterial biofilms with ASEM

The ASEM enables us to observe biological samples in solution at atmospheric pressure. Samples are imaged through a 35-nm-diameter film window made of silicon nitride in the base of the disposable ASEM dish. With ASEM we observed fine structures of bacterial biofilms. Biofilm matrix components (proteins, DNA, polysaccharides, and membrane vesicles) were visualized with heavy-metal labeling, positively or negatively charged nanogold labeling, and immune labeling methods optimized for biofilm samples. These methods could be used to observe biofilms formed by several bacteria, such as *S. aureus*, *S. epidermidis*, *E. coli*, and *Lactococcus lactis*. In conclusion, ASEM is a promising tool to study the fine structures of various bacterial biofilms and their matrix components in solution.

Biofilm formation by P. acnes isolated from pacemaker

P. acnes is a facultative anaerobic Gram-positive commensal bacterium of the human skin, mouth, conjunctiva, and large intestine. *P. acnes* is usually responsible for late chronic infections and rarely causes acute infections related to medical devices. This bacterium has recently been reported to cause infections associated with cardiac devices, breast implants, and prosthetic joints. In this study, colonization of bacteria on the surfaces of explanted cardiac devices (pacemaker generators) that show no signs of infection was consecutively analyzed. As a result of culture tests using agar plates followed by 16S ribosomal RNA gene sequencing, *P. acnes* was isolated from 8 of 31 devices. An *in vitro* biofilm formation assay showed that glucose is an accelerator for biofilm formation by *P. acnes*. Ultrastructural analysis of *P. acnes* biofilms with the ASEM and transmission electron microscope suggested that the efflux of extracellular substances concomitant with cell lysis contributes to biofilm formation. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology-supported Program for the Strategic Research Foundation at Private Universities, 2012-2016.

High-throughput screening of antibiofilm compounds

A potential strategy for preventing and treating biofilm-associated infections is to use small molecules that inhibit biofilm development. In collaboration with the University of Tokyo, which has a chemically diverse small-molecule library (200,000 compounds), we are now pushing ahead with high-throughput screening to identify the compounds effective against bacterial biofilm development. We have established a crystal violet staining assay of biofilm that is suitable for high-throughput screening. Additionally, we have designed a screening robot system that automates the dispensing of compounds to assay plates, cell culture handling, and activity measurement. To date, 59,600 compounds have been screened with 2 strains of *S. aureus* that form biofilms, and several promising compounds have been identified. Among the compounds, ABC-JK1 showed activity specifically against polysaccharide-dependent biofilm. Several biochemical analyses suggest that ABC-JK1 inhibits extracellular polysaccharide synthesis and thereby inhibits biofilm formation. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology-supported Program for the Strategic Research Foundation at Private Universities, 2012-2016.

Analysis of biofilm detachment factor

The bacteria within the biofilm matrix are protected from the host immune system and from antibiotic attack. Therefore, finding a biofilm-disassembling substance would prove widely useful in medical and industrial applications for preventing or eradicating biofilms. The factor responsible for the detachment effect has a molecular weight < 500 Da and is heat-stable. Culture supernatant was fractionated with gel filtration chromatography and subjected to reverse-phase column chromatography. The flowthrough was subjected to hydrophilic interaction chromatography and eluted with decreasing concentrations of acetonitrile (90% to 0%). The fraction with detachment activity was subjected to metabolome analysis. We are now attempting to identify this factor. The culture supernatant of *S. aureus* also detached the biofilms of *P. aeruginosa,* which is the

causative bacteria of biofilm infections. The molecular weight of the factor responsible for the detachment effect is greater than 10 Da and suggests that there is another detachment factor for *P. aeruginosa* biofilm.

A simple assay for measuring catalase activity: a visual approach

In this study, an assay that combines the ease and simplicity of the qualitative approach for measuring catalase activity was developed. The assay reagents comprised only hydrogen peroxide and Triton X-100. The enzyme-generated oxygen bubbles trapped by Triton X-100 were visualized as foam, whose height was estimated. A calibration plot using the defined unit of catalase activity yielded the best linear fit over a range of 20 to 300 U ($y = 0.3794x - 2.0909$; $r^2 = 0.993$). The precision and reproducibility of the assay at 100 U were 4.6% and 4.8%, respectively. The applicability of the assay for measuring the catalase activity of various samples was assessed using laboratory strains of *E. coli*, catalase-deficient isogenic mutants, clinically isolated Shiga toxin-producing *E. coli*, and human cells. The assay generated reproducible results. In conclusion, this new assay can be used to measure the catalase activity of bacterial isolates and human cells.

Analysis of the VBNC state

Some *E. coli* strains become VBNC under environmental stress conditions and escape detection by conventional culture methods. We showed that a RNA polymerase, sigma S (rpoS)-deficient mutant entered the VBNC state and that the addition of catalase or thiourea to the culture medium resulted in the resuscitation of the bacterium from the VBNC state to a culturable state. These results indicate that the VBNC phenotype is due to the death of σS-deficient stress-sensitive cells induced by oxygen-related radical generation on routine bacterial media under aerobic conditions.

After treatment to induce the VBNC state, wild-type and rpoS-deficient mutant strains were cultured in medium with or without catalase, and DNA breaks labeled with fluorescein isothiocyanate-deoxyuridine triphosphate were analyzed with fluorescence-activated cell sorting. In the wild-type strain, DNA breaks were detected after 1 hour of culture and decreased within 2 hours of culture. On the other hand, the rpoS-deficient mutant strain showed DNA breaks even after 2 hours of culture. The addition of catalase to the medium inhibited DNA breaks in both strains. These results suggest that the VBNC phenotype is due to cell death from the DNA damage induced by oxygen-related radical generation on routine bacterial media.

Publications

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