Department of Bacteriology

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General Summary

Research projects of our department have focused on: 1) the analysis of *Staphylococcus aureus* biofilm formation, 2) the analysis of biofilm inhibition and destruction factors, 3) the molecular mechanisms of *S. aureus* biofilm disassembly triggered by *Staphylococcus epidermidis* Esp, 4) the effects of bacteriocins against methicillin-resistant *S. aureus* (MRSA) biofilm, 5) pathogenicity of *Escherichia coli* O157 entering the viable but nonculturable (VNC) state, and 6) the mechanism of bacterial ATP secretion.

Research Activities

Purification, crystallization, and preliminary X-ray diffraction analysis of the S. epidermidis extracellular serine protease Esp

Esp, an extracellular serine protease from *S. epidermidis*, has been shown to inhibit *S. aureus* biofilm formation and nasal colonization. The full-length 27-kDa pro-Esp was purified and digested with thermolysin to obtain mature Esp. The mature Esp containing 216 residues crystallized in space group P2(1), with unit-cell parameters a = 39.5, b = 61.2, c = 42.5 Å, β = 98.2°, and 1 molecule in the asymmetric unit, with an estimated solvent content of 42%. A diffraction data set has been collected to 1.8-Å resolution on a rotating-anode home-source facility.

Excreted molecular chaperones ClpB and DnaK stimulate bacterial biofilm formation

We have recently identified molecular chaperones ClpB and DnaK in the biofilm matrix fraction of *S. aureus*. Here, we show that extracellular ClpB and DnaK play important roles in the quality control of bacterial biofilms. Cytological analyses showed that ClpB and DnaK were attached to the cell surface of *S. aureus* embedded in biofilms during all phases of biofilm formation. Interestingly, extracellularly supplemented ClpB and DnaK stimulated *S. aureus* biofilm formation in a dose-dependent manner, whereas GroEL did not. Mutational analyses indicated that the hexamer formation and the disaggregating chaperone activity of ClpB are dispensable for biofilm promotion and, at least, the N, D1, and M domains are necessary for this extracellular function. ClpB may act as a "molecular glue" to stimulate cell-cell interaction or cell-surface interaction or both.

Spatiotemporal dynamics of the multifunctional biofilm matrix protein Eap

We have identified extracellular adherence protein (Eap), a multifunctional biofilm matrix protein related to various infectious diseases, from an *S. aureus* robust biofilm former. *In vitro* experiments revealed that purified Eap promoted biofilm development by *S. aureus*. We are now characterizing *eap*-knockout mutants to understand the

functions of Eap in biofilm development and virulence. Furthermore, we are analyzing the spatiotemporal dynamics of this protein within biofilms.

Observation of biofilms by atmospheric scanning electron microscopy
We directly observed biofilms formed by various bacteria in buffer with the newly developed atmospheric scanning electron microscope. This microscope features an open sample dish with a pressure-resistant thin film window in its base, through which the scanning electron microscopy beam scans samples in solution, from below. The locations of biofilm matrix components (proteins and polysaccharides) and fine structures of extracellular membrane vesicles and nanotubes were visualized using immunolabeling and optimized staining methods.

Effects of bacteriocins on MRSA biofilm
Control of biofilms formed by microbial pathogens is an important subject for medical researchers, because the development of biofilms on foreign-body surfaces often causes biofilm-associated infections in patients with indwelling medical devices. The present study examined the effects of different kinds of bacteriocin, which are ribosomally synthesized antimicrobial peptides produced by certain bacteria, on biofilms formed by a clinical isolate of MRSA. The activities and modes of action of 3 bacteriocins with different structures (nisin A, lacticin Q, and nukacin ISK-1) were evaluated. Vancomycin, a glycopeptide antibiotic used in the treatment of MRSA infections, showed bactericidal activity against planktonic cells but not against biofilm cells. Among the tested bacteriocins, nisin A showed the highest bactericidal activity against both planktonic cells and biofilm cells. Lacticin Q also showed bactericidal activity against both planktonic cells and biofilm cells, but its activity against biofilm cells was significantly lower than that of nisin A. Nukacin ISK-1 showed bacteriostatic activity against planktonic cells but not against biofilm cells. Mode-of-action studies indicated that pore formation leading to ATP efflux is important for the bactericidal activity against biofilm cells. Our results suggest that bacteriocins that form stable pores on biofilm cells would be highly effective for the treatment of MRSA biofilm infections.

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. We are now performing high-throughput screening (HTS) to identify compounds that inhibit bacterial biofilm development; we are collaborating with the University of Tokyo, which has a chemically diverse small-molecule library (200,000 compounds). We have established a crystal violet staining assay of biofilm that is suitable for HTS. Additionally, we have designed a screening robot system that automates the dispensing of compounds to assay plates, cell-culture handling, and activity measurement. Screening studies with several bacterial strains that can form biofilm are now in progress to assay 10,000 different conditions each month. So far, several compounds with inhibitory activity against *S. aureus* biofilm have been obtained. Hereafter, we will perform large-scale HTS to find more effective compounds, and then we will analyze in detail the composition of the

molecular interactions of these compounds with the bacteria.

Pathogenicity of E. coli O157 entering the VNC state

Some *E. coli* O157 strains become VNC under environmental stress conditions and evade detection with conventional culture methods. We showed that the addition of catalase to the culture medium resuscitated O157 from the VNC state to a culturable state and that decreased sigma factor S activity (encoded by the *rpoS* gene) caused bacteria to enter the VNC state. To investigate the pathogenicity of VNC cells, we infected germ-free mice with VNC O157 strains and found that the resuscitated VNC cells colonized the gut and induced death in all mice.

Mechanism of bacterial ATP secretion

ATP modulates immune cell functions, and ATP derived from gut commensal bacteria promotes the differentiation of T helper 17 (Th17) cells in the intestinal lamina propria. We recently reported that *Enterococcus gallinarum*, isolated from mice and humans, secretes ATP. We have since found and characterized several ATP-secreting bacteria. Of the tested enterococci, *Enterococcus mundtii* secreted the greatest amount of ATP (>2 μM/10^8 cells) after overnight culture. Glucose was essential for ATP secretion from *E. mundtii*. Analyses of energy-deprived cells demonstrated that glycolysis is the most important pathway for bacterial ATP secretion. Furthermore, exponential-phase *E. mundtii* and *Enterococcus faecalis* cells secrete ATP more efficiently than do stationary-phase cells. Other bacteria, including *Pseudomonas aeruginosa*, *E. coli*, and *S. aureus*, also secrete ATP in the exponential phase but not in the stationary phase. These results suggest that various gut bacteria, including commensals and pathogens, secrete ATP at any growth phase and modulate immune cell function.

Publications

Vengadesan K*[1]*, Macon K*[2]*, Sugimoto S, Mizunoe Y, Iwase T, Narayana SV*[2] *(*[1]*UNESCO Reg Ctr Biotechnol,* [2]*Univ Alabama). Purification, crystallization and preliminary X-ray diffraction analysis of the Staphylococcus epidermidis extracellular serine protease Esp. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2013; **69:** 49-52.

Reviews and Books

Mizunoe Y. What is biofilm? (in Japanese). *Shoni Naika.* 2012; **44:** 1198-202.

Mizunoe Y, translator. Pt.2 Chapter4. Overview of the major pathogens & introduction to anaerobic bacteria, Chapter15. Gram-positive cocci, Chapter16. Gram-negative cocci (in Japanese). In: Levinson W. Yoshikai Y, Nishiyama Y, supervisor of translation. Review of medical microbiology and immunology. 11th ed. Tokyo: Maruzen Publishing; 2012. p. 97-119.