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Two Dimensional Analysis of Staphylococcal Exoproteins for the Application in Epidemiological Study

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Staphylococcus aureus strains secrete a large number of exoproteins, such as toxins and enzymes, as extracellular and cell-associated forms, many of which have been shown to contribute to pathogenic activity or to enhance virulence. These exoproteins include (i) factors involoved in invasion/tissue penetration, (ii) factors involved in evasion of host defenses and (iii) factors involved in attachment. The first group of exoproteins is α-toxin, β-hemolysin, γ-hemolysin, δ-hemolysin and phospholipase C.

The second group is toxic shock syndrome toxin-1 (TSST-1), enterotoxins, protein A, and others, and the third group contains clumping factor, fibronectin-binding proteins A and B, fibrinogen-binding protein, collagen-binding protein and coagulase. More than 30 of these exoproteins that have been significantly linked to pathogenesis have been purified to homogeneity, although no single strain produces all known exoproteins. Some of the genes that encode these exoproteins are plasmid-encoded and the others are in the chromosome and thus the repertoire of the genes encoding exoproteins varies in each *S. aureus* strain. The expression of these exoproteins are regulated by several global regulatory genes such as *agr*, *sar*, *xpr* and *sae*, and the expressions of these regulatory genes are affected by the environmental (culture) conditions. As a result, a set of exoproteins in the culture supernatant of each strain varies qualitatively and quantitatively.

Quantitative as well as qualitative evaluations of exproteins secreted from each isolate must be associated with bacterial virulence. TSST-1 and several other exoproteins in culture fluid have been measured by immunological methods, such as agglutination of latex particles coated with specific antibodies and immunoblot analysis. However, the available antisera have been limited and no other effective methods have been developed.

High-resolution two dimensional polyacrylamide-gel electrophoresis (2-DE) allows protein expression patterns of the full complements of cellular proteins from different bacterial strains to be displayed on a single gel. Then protein spots from these maps can be identified by various chemical methods, such as N-terminal microsequencer or mass spectrometry. In this study, we applied 2-DE to analysis of total exoproteins in the culture supernatant of *S. aureus* NCTC8325, a laboratory strain, and methicillin-resistant *S. aureus* (MRSA) clinical isolates and constructed a reference exoprotein map according to the genome information. In addition, we studied the effect of various culture conditions on the production of exoproteins.

NCTC8325 has been used for the genome se-

quencing project in the University of Oklahoma's Advanced Center for Genome Technology. Bacteria were cultured in LB broth if not otherwise mentioned. The culture supernatant was precipitated with trichloroacetic acid the precipitate as a total exoprotein fraction was washed with cold acetone. The precipitate was then solubilized in an appropriate buffer and applied for 2-DE analysis. After the electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane and visualized with Coomassie brilliant blue. Portein spots were excised from the membrane and the N-terminal amino acid sequences were determined with a protein sequencer. We tentatively constructed a temporary protein sequence database from NCTC8325 genome sequence data and searched the data base for homolgy with the obtained N-terminal sequences.

S. aureus secrete various exoproteins and some of these proteins are expressed growth phase-dependently. We found that the profiles of total exoproteins from NCTC8325 were dramatically changed at different stages of the growth. Many exoproteins were preferentially secreted during the stage of transition between late exponential and early stationary phases. As a 2-DE gel shows, 250-270 spots of exoproteins were observed in the culture of early stationary phase. We then determined the N-terminal amino acid sequences of 92 distinct spots by Edman degradation. These N-terminal sequences were subjected to the search for homology on the data base and more than 47 proteins were identified. Several extracellular proteins were detected as multiple spots.

The production of exoproteins was deeply affected by the conditions that bacteria were cultured. The expression of exoproteins in anaerobic condition was basically similar to that of late log phase culture in aerobic condition. In a culture at pH 5.5, the exoproteins in the region of basic and high M..W. range enhanced their expression, whereas in a culture at pH 9.5, the expression of these exoproteins was poor. When the bacteria were culture in a high NaCl concentration (12.5%), the spots of exoproteins in the region of basic range greatly decreased the number and the intensity. In the culture supplemented with 1% glucose, the expression of exoproteins was also affected. The 2-DE pattern of exoproteins in this culture was similar to that of the high NaCl culture.

In MRSA strains, we identified spots of enterotoxins A (SEA), B (SEB), H (SHE), and C_3 (SEC_3), α-and β-hemolysins, phospholipase C (PI-PLC), triacylglycerol lipase, glutamylendopeptidase, bifunctional autolysin, collagen adhesin and toxic shock syndrome toxin 1 (TSST-1). SEA, SEB and SEC_3 were detected as multiple spots. In a MRSA strain of the coagulase II group that has been prevalent in Japan, the amounts of dominant toxins, as evaluated from the gel images, were TSST-1 (1.8%), SEB (12.2%), SEC_3 (10.7%), α-hemolysin (3.6%) and β-hemolysin (5.5%) of total exoproteins. The 2-DE images of exoproteins of MRSA strains belonging to various coagulase types were compared. We found certain similarities among strains belonging to the same coagulase types. Additionally, unique expression patterns were observed in strains of coagulase type II isolated in Japan. As mentioned above, coagulase II strains secreted a somewhat larger amount of SEs than the strains of other coagulase types. TSST-1 was detected by a certain amount of total proteins from these strains.

We applied 2-DE analysis of exoproteins to MRSA strains isolated from neonatal TSS patients at the International Medical Center, Tokyo. Two strains isolated from different neonatal TSS patients in NICU had a significantly similar 2-DE gel image, suggesting that these strains were very close to, if not the same as, each other. In addition,

these gel images showed a common phase with those of coagulase type II strains. Furthermore, quantitative evaluation of the toxins showed that SEC_3 and TSST-1 were 15~16%, and 5~7%, respectively, of total exoproteins from these strains.

Several epidemiological techniques have been developed and commercialized, including phenotyping, such as coagulase typing, phage typing, antibiograms and, recently, genotypings, such as plasmid analysis, pulsed field gel electrophoresis (PFGE), polymerase chain reaction-restriction fragment length polymorphism, and ribotyping have been made available to classify strains. PFGE has been used successfully to analyze dissemination of nosocomial MRSA infections. However, these typings yield little information on the virulence of MRSA, and thus the pathogenicity of the infection could not be determined. Quantitative evaluation as well as qualitatve analysis of virulence-associated exoproteins by 2-DE of exoproteins as shown in this study, would be helpful in understanding the pathogenic character of each MRSA strain.