Western Blotting for *Staphylococcus aureus* AgrA

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[Abstract] *Staphylococcus aureus* has a quorum sensing system to regulate expressions of various virulence factors, which is exerted by the *agr* locus encodes *agrBDCA* and a regulatory RNA called RNAIII. AgrB, AgrD, and AgrC proteins are involved in producing and recognizing extracellular quorum sensing molecule and transduces the signal to the phosphorylation status of AgrA, which is a positive transcription factor to regulate cytolysin genes as well as the *RNAIII* gene. *RNAIII* regulates the expression of various virulence genes. Expression of the *agr* locus has been examined in depth at transcriptional level, but the investigation onto the translational expression is limited, because immunoglobulin G used to detect a specific protein highly reacts to *S. aureus* protein A. Here, we report the method to detect AgrA that is the transcription factor encoded by the *agr* regulatory system. Although this is a specific protocol for western blotting of *S. aureus* AgrA protein, this can also be used for other *S. aureus* proteins by changing the antibody.

Materials and Reagents

1. *Agr*-positive *S. aureus* strains
2. Bacto™ Tryptic Soy Broth (BD, catalog number: 211825)
3. Lysostaphin (Wako Chemicals USA, catalog number: 120-04313)
4. 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel
5. N-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) (Dojindo Molecular Technologies, catalog number: 343-08321)
6. Methanol
7. Immobilon-P (EMD Millipore, catalog number: IPVH304F0)
8. Tris(hydroxymethyl)aminometane (Nacalai Tesque, catalog number: 35434-34)
9. EDTA·2Na (Dojindo Molecular Technologies, catalog number: 345-01865)
10. Hydrochloric acid
11. Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific, catalog number: 23236)
12. Easy Blocker (GeneTex, catalog number: GTX425858)
13. Anti-AgrA IgG that was made in our laboratory (Kaito *et al*., 2013)

14. Anti-rabbit IgG conjugated with alkaline phosphatase (Promega Corporation, catalog number: S3731)
15. Nitro blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) (Roche Diagnostics, catalog number: 1681451)
16. PVDF membrane
17. TE buffer (see Recipes)
18. Lysis buffer (see Recipes)
19. Staining buffer (see Recipes)
20. 10x SDS sample buffer (see Recipes)
21. 10x TBS (see Recipes)
22. TBST (see Recipes)

Equipment

1. Sonicator (Branson, model: Sonifier 450) with double stepped microtip (3 mm) (Branson, part number: 101-063-212)
2. Plastic container
3. Centrifuge machine
4. Electrophoresis apparatus
5. Power supply
6. Wet/Tank Blotting Systems (Bio-Rad Laboratories)

Software

1. Image J (1.45 s, NIH)

Procedure

1. Pick up single colony of *S. aureus* into 5 ml of fresh tryptic soy broth (TSB) and aerobically culture it for 15 h at 37 °C with shaking at 150 rpm.
2. Inoculate 50 µl overnight culture of *S. aureus* into 5 ml (1:100 dilution) of fresh TSB and aerobically culture it for 24 h or 15 h at 37 °C with shaking at 150 rpm. A_{600} of starting and end will be approximately 0.06-0.1 and 6-10, respectively.
3. Collect 650 µl *S. aureus* cell culture by centrifugation at 10,000 *x g* for 1 min at 4 °C and discard the supernatant. Resuspend the cell pellet in 195 µl of TE buffer by vigorous vortexing. Add 5 µl of lysostaphin (1mg/ml) to the cell resuspension and incubate the resuspension at 37 °C for 30 min without agitation.

4. Sonicate the sample on ice (Branson Sonifier 450; double stepped microtip; duty cycle, constant; output control, 1; time, 15 sec), and centrifuge it at 10,000 *x g* for 10 min at 4 °C.
   Note: This step is essential to remove the most of protein A that is associated with cell wall.
5. Measure the amount of protein in the supernatant by the Bradford method using Coomassie Plus Protein Assay Reagent according to the manufacture's protocol. Equalize the protein concentration of different samples by adding TE buffer. Mix the protein samples with 3x SDS sample buffer.
6. Boil protein samples for 5 min and electrophorese the samples in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel at 100 V for 3 h.
   Note: For details of SDS-polyacrylamide gel electrophoresis, please see Kaito et al. (2013).
7. Dip the Gel in a buffer (10 mM CAPS, 20% methanol) for 10 min. Treat PVDF membrane with methanol for a few seconds and dip it in a buffer (10 mM CAPS, 20% methanol). Set the gel, membrane, and buffer (10 mM CAPS, 20% methanol) using Wet/Tank Blotting Systems and transfer the proteins from the gel to a PVDF membrane at 150 mA for 3 h at 4 °C.
8. Treat the membrane with 10 ml blocking buffer (TBST containing 5% Easy Blocker) in a plastic container at room temperature with gentle agitation for 1 h.
   Note: The use of Easy Blocker is essential to decrease the reactivity of IgG against protein A in the samples.
9. Treat the membrane with 10 ml blocking buffer containing 1:1,000 anti-AgrA IgG in a plastic container at room temperature with gentle agitation for 1 h.
   Note: For using antibodies, the appropriate concentration should be determined by serial titration.
10. After washing with 20 ml TBST for three times of 5 min agitation, treat the membrane with 10 ml blocking buffer containing 1:2,000 anti-rabbit IgG conjugated with alkaline phosphatase in a plastic container at room temperature with gentle agitation for 1 h.
11. After washing with 20 ml TBST for three times of 5 min agitation, treat the membrane with a staining buffer without agitation for 5 min.
    Note: For detection of proteins by alkaline phosphatase, please see details in Kaito et al (2013).
12. After bands appear, dip the membrane in TE buffer supplemented with 10 mM EDTA to stop overstaining and then dry the membrane on paper towel.
13. Measure the band intensity by densitometry scanning (Image J 1.45 s, NIH).

Recipes

1. TE buffer
   10 mM Tris-HCl (pH 8.0)
   1 mM EDTA (pH 8.0)
2. Staining buffer
   100 mM Tris-HCl (pH 9.5)
   100 mM NaCl
   50 mM $MgCl_2$
   2% NBT/BCIP
3. 3x SDS sample buffer
   1.5 ml 1 M Tris-HCl (pH 6.8)
   3 ml 20% SDS
   3 ml Glycerol
   500 µl 1% bromo phenol blue
   1.8 ml 2-mercaptoethanol
4. 10x TBS
   24.2 g Tris
   87.6 g NaCl
   1 L milliQ
   Adjust pH to 7.6 by adding hydrochloric acid
5. TBST
   125 ml 10x TBS
   1,168 ml milliQ
   7.5 ml 20% Tween20

Acknowledgments

This protocol was adapted from the original work (Kaito *et al.*, 2013) to provide the detailed procedures. This work was supported by Grants-in-Aid for Scientific Research (23249009, 24590519). This work was supported in part by the Naito Foundation, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and the Genome Pharmaceutical Institute.

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